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Role of Brain FABP and its Ligands in Malignant Glioma Cell Migration

by

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ABSTRACT

Patients diagnosed with malignant glioma tumours have median survivals of 1.6 yrs and 5 months, respectively, highlighting the deadly nature of this disease. Despite aggressive multimodal treatment, patients with malignant glioma often present with secondary brain tumours at sites distal to the primary tumour mass. These secondary tumours are a consequence of 'renegade' neoplastic cells that infiltrate the surrounding normal brain, a hallmark feature of malignant glioma. Brain fatty acid-binding protein (FABP7), which binds omega-3 docosahexaenoic acid (DHA) and omega-6 arachidonic acid (AA), is overexpressed and associated with a poor prognosis in patients with malignant glioma compared with normal brain. These data suggest that FABP7 plays an important role in gliomagenesis; however, the mechanism(s) underlying a role for FABP7 in malignant glioma has, until now, been unexplored.

Here, we demonstrate that expression of FABP7 in malignant glioma cells is accompanied by increased cell migration. Consistent with our *in vitro* results, we show that expression of FABP7 in astrocytoma tumours is associated with sites of tumour infiltration and tumour recurrence. Furthermore, we demonstrate that the fatty-acid ligands of FABP7 affect cell migration in an FABP7-dependent manner. More specifically, DHA inhibits migration, whereas AA stimulates cell migration. Finally, we reveal that uptake and incorporation of DHA and AA in the phospholipids of malignant glioma cells is enhanced by FABP7 expression, suggesting a

mechanism by which DHA and AA may affect cell migration by altering signal transduction at the cell membrane.

We propose that the inherent ability of malignant glioma cells to express the radial glial marker FABP7 underlies their infiltrative capacity, allowing tumour cells to migrate long distances from the main tumour mass. We propose a model whereby FABP7 expression and relative levels of DHA and AA determine tumour infiltrative potential. Our findings provide insight into the role of FABP7 and its fatty acid ligands in controlling the migration of malignant glioma cells and point to the potential use of DHA as a natural anti-infiltrative agent in the treatment of malignant glioma. We believe that targeting FABP7-expressing cells may make a significant impact on the treatment of high grade astrocytomas.

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LIST OF ABBREVIATIONS

abbreviation	full name
AA	arachidonic acid
ALA	alpha-linolenic acid
BCNU	bis-chloronitrosourea
BCSC	brain cancer stem cell
bp	base pair
Bmax	maximum number of binding sites
BSA	bovine serum albumin
cAMP	cyclic-AMP
CBP	CREB-binding protein
CDK	cyclin-dependent kinase
ChIP	chromatin immunoprecipitation
CNS	central nervous system
COX	cyclooxygenase
CSC	cancer stem cell
DAPI	4'-6-diamidino-2-phenylindole
DBD	DNA-binding domain
DHA	docosahexaenoic acid
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
EFA	essential fatty acid
EPA	eicosapentaenoic acid
EORTC	European Organization for Research and Treatment of Cancer
ESI/MS	electrospray ionization/mass spectrometry
FA	fatty acid
FABP	fatty acid binding protein
FCS	fetal calf serum
FRET	fluorescence resonance energy transfer
GFAP	glial fibrillary acidic protein
GBM	glioblastoma multiforme
GBMO	glioblastoma multiforme with an oligodendrial component
GDP	guanine diphosphate
GFP	green fluorescent protein
GLC	gas-liquid chromatography
GST	glutathione S-tranferase
GTP	guanine triphosphate
Gy	gray

HA	hemagglutinin
HDAC	histone deacetylase complex
HIF	hypoxia-inducible factor
HRP	horse radish peroxidase
HTH	helix-turn-helix
hu	human
IDH1	isocitrate dehydrogenase-1
kb	kilobase
Kd	dissociation constant
KO	knock-out
LA	linoleic acid
LBD	lipid binding domain
LOH	loss-of-heterozygosity
M	molar
uM	micromolar
MG	malignant glioma
MGMT	methyl-guanine methyl transferase
mM	millimolar
MRI	magnetic resonance image
NFI	nuclear factor I
NES	nuclear export signal
NLS	nuclear localization signal
NSAID	non-steroidal anti-inflammatory drug
OA	oleic acid
NCIC	National Cancer Institute of Canada
PA	palmitic acid
PC	phosphatidylcholine
PCV	procarbazine, CCNU, vincristine
PCR	polymerase chain reactin
PE	phosphatidlyethanolamine
PGE	prostaglandin-E
PI	phosphatidylinositol
PI3	phosphatidylinositol-3
PI3K	phosphatidylinositol-3-kinase
PKC	protein kinase-C
PPAR	peroxisome proliferator-activated receptor
PPRE	PPAR response elements
PS	phosphatidlyserine
PTEN	phosphatase and tensin homology
PUFA	polyunsaturated fatty acid
RT-PCR	reverse-transcription polymerase chain reaction
SA	stearic acid
SD	standard deviation
SEM	standard error of the mean
shRNA	small hairpin RNA
siRNA	small interfering RNA

SRC	sarcoma
SNP	single-nucleotide polymorphism
STAT	signal transducer and activator of transcription
SVZ	subventricular zone
RB	retinoblastoma
RT	radiation therapy
RXR	retinoid-X receptor
TMZ	temozolomide
uM	micromolar
WBRT	whole-brain radiation therapy
WHO	World Health Organization

CHAPTER 1: Introduction

1.1 Glioma: Epidemiology and classification

The vital role of the brain in controlling nearly every bodily function highlights the severity of neoplastic growths within this organ. Gliomas are the most common type of brain tumour, comprising approximately 60% of all central nervous system (CNS) malignancies (Figure 1.1). The World Health Organization (WHO) has established a system to group gliomas into three major types: astrocytomas, oligodendrogliomas, and mixed oligoastrocytomas. Recent evidence suggests the existence of a fourth group consisting of glioblastoma with an oligodendroglial component (GBMO) (He, Mokhtari et al. 2001). Categorization of these tumours into one of three types is based on histological similarities of the tumour cells to non-neoplastic mature glial cells of the CNS (i.e. astrocytes and oligodendrocytes).

In North America, the incidence of gliomas is approximately 5 – 10 per 100,000, ~75% which are astrocytomas (Louis, Ohgaki et al. 2007). While the incidence of oligodendrogliomas, oligoastrocytomas, and GBMOs is relatively lower, the numbers of confirmed cases has risen substantially over the years. Concomitantly, the incidence of astrocytoma has fallen, suggesting that some gliomas previously classified as astrocytomas are now being labeled oligodendrogliomas, mixed oligoastrocytomas, or GBMOs (Daumas-Duport, Varlet et al. 1997; Fortin, Cairncross et al. 1999; Burger 2002). Analysis of four histological criteria: nuclear atypia (variation in the appearance of the nucleus), mitotic index,

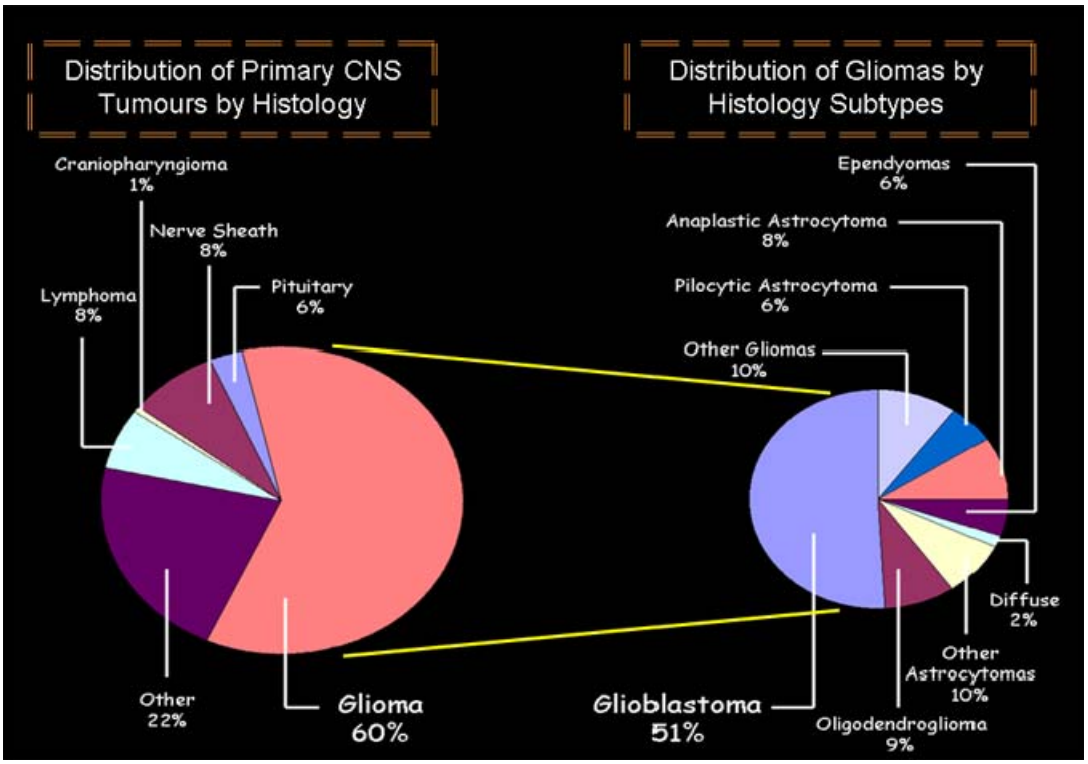

Figure 1.1 Incidence of primary CNS tumours and distribution of glioma subtypes based on histology

microvascular proliferation (indicative of angiogenesis), and necrosis (cell death), guide further subclassification of gliomas into: (i) low grade (WHO grades I and II), or (ii) high grade (WHO grades III and IV). In order of increasing malignancy, types of astrocytomas include pilocytic astrocytoma (grade I), diffuse astrocytoma (grade II), anaplastic astrocytoma (grade III), and glioblastoma multiforme (GBM; grade IV), the most aggressive and common subtype (Figure 1.2). Collectively, high-grade astrocytomas (III and IV) are referred to as malignant glioma (MG). Of note, low-grade astrocytomas possess a proclivity to undergo malignant transformation to an astrocytoma of higher grade, usually GBM. This transformational event requires that we distinguish between "secondary" MG that originate from a lower-grade astrocytoma, and "primary" MG that have arisen *de novo* with no previous evidence of disease. The lack of histopathological differences between secondary and primary GBMs has made it difficult to determine the relative frequencies at which they occur; however, distinct molecular profiles between the two groups (see Section 1.3.) indicate that primary MGs constitute >80% of reported cases (Dropcho and Soong 1996). The WHO classification for oligodendrogliomas and mixed oligoastrocytomas is simpler and includes only two grades—low grade (grade II) and anaplastic (grade III).

Proper classification of glioma is important as different types of gliomas require different treatments. While the WHO system is the most up-to-date and widely-implemented model for glioma classification, there

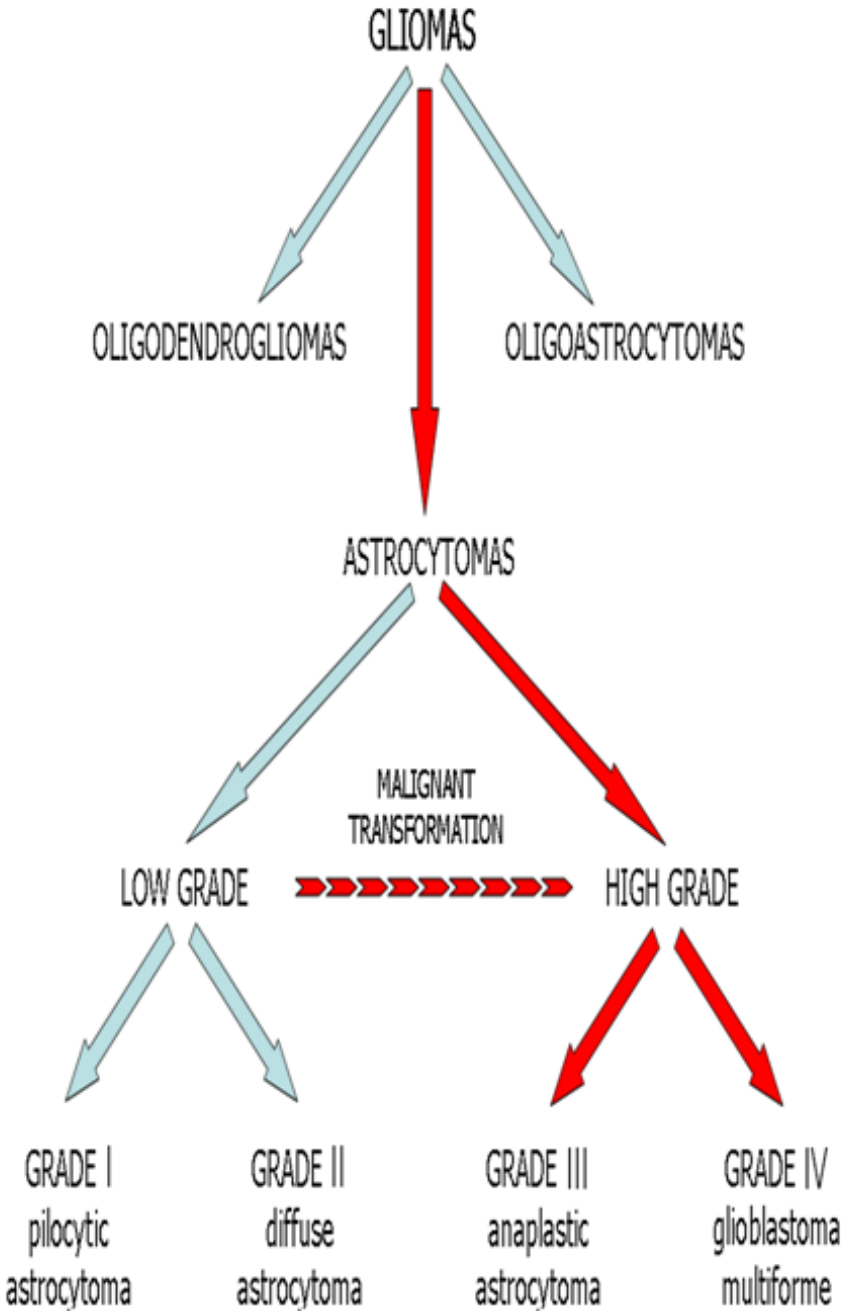

Figure 1.2 Sub-classification of astrocytomas by the World Health Organization

are limitations associated with current morphology-based guidelines. Gliomas are highly heterogeneous, with biopsies typically not representing the whole tumour, leading to possible misclassification of the tumour. In addition, agreement between medical pathologists for the classification of astrocytic versus oligodendroglial tumours, or identification of anaplastic astrocytomas, is alarmingly low (Coons, Johnson et al. 1997; Marie, Sanson et al. 2001; Sasaki, Zlatescu et al. 2002), indicating the subjective nature of morphology-based classification. Moreover, accurate identification of a glioma does not always translate into improved outcome as morphologically identical gliomas can exhibit high levels of diversity at the molecular level, resulting in differences with regards to recurrence, aggressiveness, and response to treatment. As a case in point, oligodendrogliomas characterized by loss of chromosome arms 1p and 19q (Reifenberger, Reifenberger et al. 1994), which occurs in >80% of cases, are highly sensitive to chemotherapy involving PCV (procarbazine, CCNU, vincristine); thus resulting in a more favorable clinical outcome (Cairncross, Ueki et al. 1998). In contrast, oligodendrogliomas characterized by deletions of 1p are associated with a poorer prognosis, as they do not respond well to the PCV treatment regimen (Ino, Zlatescu et al. 2000). These data suggest that in addition to morphological features, the molecular signature of a glioma is important if classification is to lead to better treatment strategies.

1.1.1 Malignant glioma: treatment and survival

Upon identification of a neoplastic lesion in the brain, surgical resection, if possible, is conducted to obtain quick relief of symptoms. Tumour tissue is used for diagnostic purposes. Following surgery, radiation therapy (RT) is administered at a total dose of 58 - 60 Gy delivered in fractions of 1.8 - 2.0 Gy to a focal field encompassing the tumour bed with a 2–3 cm margin (Walker, Alexander et al. 1978). The use of chemotherapy is recommended both during and after radiation therapy (Mason, Maestro et al. 2007). Despite this highly aggressive approach to treatment, there has been no significant increase in the survival of patients with GBM over the last 20 - 30 years (Nakada, Nakada et al. 2007). The underlying reason for the futility of current treatments lies in the fact that patients often present with secondary tumours in the brain, a hallmark of MG. These derivative tumours occur at sites proximal and distal to the primary tumour mass often without continuity between the lesions, emphasizing the infiltrative nature of these cells. In the early 1900s, when surgical methods were rather crude, hemispherectomies (removal of the affected half of the brain) failed to eradicate MG as patients still presented with secondary lesions in the remaining hemisphere! This highly infiltrative property of MG cells undermines both surgical and radiation treatments, as these procedures target the local tumour mass, leaving infiltrative neoplastic cells unscathed. In rare cases, and despite the dangers of radiation-induced cognitive dysfunction and

cell necrosis, the administration of whole-brain radiotherapy (WBRT) to target infiltrative cells has been used. In a thorough review of all the clinical trials conducted between 1990 – 2008 involving the administration of WBRT, Kalkanis *et al.* (2010) reported that although recurrence in the brain at distal sites was significantly lower in patients that received WBRT versus those that received standard local radiation treatment, there was no evidence of increased overall survival times in these patients (Kalkanis, Kondziolka et al. 2010).

Unlike surgery and radiation, chemotherapeutic agents have the ability to "seek and destroy" infiltrative malignant cells. Nitrosoureas, DNA alkylating agents with the ability to penetrate the blood brain barrier, are the most common agents used to treat MG. Several phase 3 trials have shown that the administration of nitrosoureas can improve overall patient survival, with 15 - 20% more patients alive at 18 months (Walker, Alexander et al. 1978; Walker, Green et al. 1980). However, subsequent meta-analysis of these trials has demonstrated that nitrosoureas have little to no effect on overall patient survival (Fine, Dear et al. 1993; Brandes, Tosoni et al. 2004). Such contradictions in the literature may be indicative of patient cohorts that react more or less favorably to chemotherapeutic agents based on the molecular signature of their disease.

Work by Esteller and coworkers (2000) showed that silencing of the O^6-methylguanine-DNA-methyltransferase (*MGMT*) gene in MG, by methylation of its promoter region, is a predictor of tumour resistance and

patient response to alkylating agents (Esteller, Garcia-Foncillas et al. 2000). MGMT removes DNA adducts caused by alkylating agents, thereby decreasing their ability to cause mutations. A large randomized phase 3 clinical trial coordinated by the European Organization for the Research and Treatment of Cancer (EORTC) and the National Cancer Institute of Canada (NCIC), was conducted to determine the efficacy of the alkylating agent, temozolomide (TMZ) in patients with MG. This consortium initially reported that the 2-year survival of patients receiving RT versus RT + TMZ increased from 10.9% to 27.2%, with the median survival remaining relatively unchanged at 12.1 versus 14.6 months, respectively. Segregation of these patients into two groups based on *MGMT* status revealed an increase in median survival to 23.4 months for patients whose *MGMT* gene was silenced versus 12.6 months for patients whose *MGMT* gene was active (Stupp, Hegi et al. 2009). The EORTC/NCIC consortium recently reported that the 5-year overall survival for *MGMT*-silent patients receiving RT + TMZ was 13.8% compared to 8.3% survival of *MGMT*-active patients receiving the same treatment. These data highlight the limitations of TMZ in curing patients with MG. Importantly, this clinical trial also underlines how the molecular signature of MG can define cohorts of patients that may respond favorably to specific treatments. The *MGMT* findings further support the need for a combined morphological-molecular system for classification of glioma. The emergence of such a system is

dependent on the basic understanding of the molecular signatures that define these tumours.

1.1.2 Molecular biology of MG

Like all cancers, gliomas are characterized by chromosomal abnormalities that lead to abnormal gene expression. While the altered pattern of gene expression found in gliomas is not tumour specific, their combination and accumulation have been found to be characteristic of both tumour class and grade (Figure 1.3). Technological advances in high-throughput assays such as GCH and DNA microarrays have led to the identification of several genetic alterations that regulate key pathways in the initiation and progression of MG.

Loss of heterozygosity (LOH) is the most frequent chromosomal abnormality detected in glioma. A study performed on 220 GBMs showed 75% LOH at 10p and 10q, 47% at 9p, 29% at 19q, and 19% at 1p (Houillier, Lejeune et al. 2006). Several putative tumour suppressor genes that may play a significant role in gliomagenesis have been identified on chromosome 10q, including phosphatase-tensin homology (*PTEN*) (Toledo and Wahl 2006), which regulates the phosphatidylinositol-3-kinase (PI3K) pathway by dephosphorylating phosphatidylinositol-(3,4,5)-triphosphate (PI3) (Maehama and Dixon 1998; Myers, Pass et al. 1998), an important intracellular second messenger. In doing this, PTEN acts in opposition to PI3K, which phosphorylates PI to generate PI3.

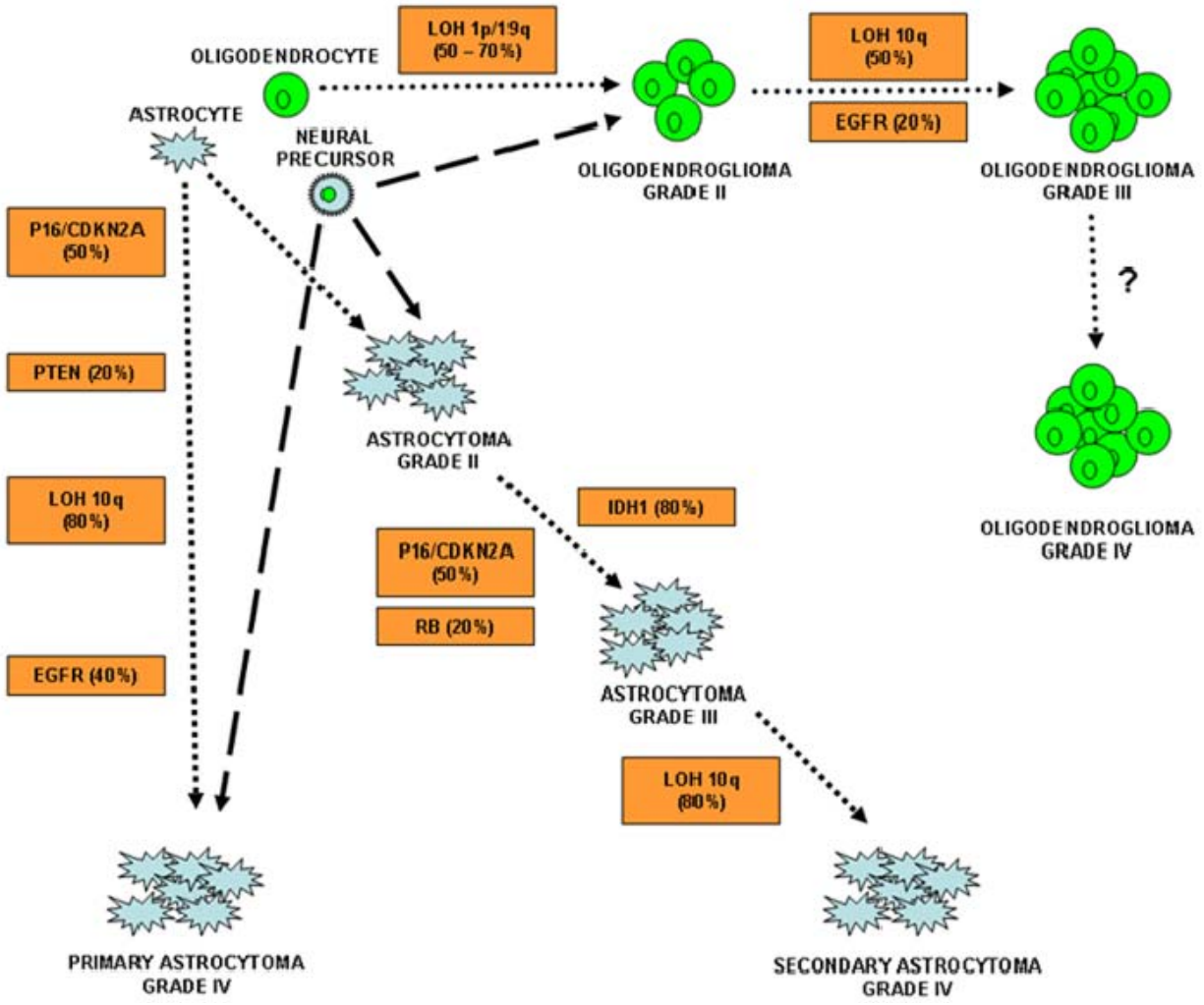

Figure 1.3 Altered patterns of gene expression found in glioma

PI3 is necessary for activation of Akt, a serine/threonine kinase involved in cell growth and survival. Induction of PTEN has been shown to cause G1-cell cycle arrest in human glioblastoma cell lines (Furnari, Huang et al. 1998; Li and Sun 1998). Interestingly, *PTEN* is mutated in 15 - 40% of primary GBM, but occurs rarely in secondary GBM (Tohma, Gratas et al. 1998; Knobbe, Merlo et al. 2002). In secondary GBM, LOH at 10p is very rare (Fujisawa, Reis et al. 2000), while partial loss of 10q, specifically 10q25p-ter, correlates with the progression of low-grade astrocytomas to the GBM phenotype (Buckner 2003). LOH at 10q has been reported in 35% - 60% of anaplastic astrocytomas (Ichimura, Schmidt et al. 1998; Sasaki, Zlatescu et al. 2001).

The most common genetic alteration in primary MG occurs at the chromosomal band 7p12, which contains the gene for epidermal growth factor receptor (*EGFR*) (Lopez-Gines, Gil-Benso et al. 2010). Amplification of *EGFR* occurs in >50% of primary GBMs, but is rare in secondary GBMs (Wong, Bigner et al. 1987; Ekstrand, Sugawa et al. 1992). Amplification of *EGFR* invariably correlates with its overexpression in primary GBM, while 70 - 90% of MG with overexpression of EGFR exhibit gene amplification (Biernat, Huang et al. 2004). In primary MG, *EGFR* is frequently rearranged, encoding the truncated (deletions of exons 2-7) and constitutively-activate tyrosine kinase receptor, EGFRvIII (Wikstrand, Hale et al. 1995). EGFRvIII continuously triggers downstream pathways that promote mitogenic effects through the activation of the PI3k/Akt pathway

(Huang, Nagane et al. 1997). Loss of PTEN in MG further enhances this effect as it functions to inhibit the activation of Akt.

TP53, encodes p53, a transcription factor that functions to induce cell-cycle arrest at G1/S and G2/M checkpoints in response to genotoxic stress (Haffner and Oren 1995). *TP53* mutation was one of the first genetic alterations described in MG, occurring in 25 - 30% of cases (Nigro, Baker et al. 1989). Further analysis revealed that *TP53* mutations are uncommon in primary GBM, occurring in <10% of cases. In contrast, >60% of secondary GBM have mutations in the *TP53* gene. Following DNA damage, p53 is activated and induces transcription of DNA repair and cell cycle related genes such as *waf1/cip1*, which encodes for p21. p21 binds and inhibits cyclin-D kinase (CDK) activity that is important for transition from G1 - S phase (Stott, Bates et al. 1998; Sherr and Roberts 1999). MDM2, a ubiquitin ligase, is also induced by p53 activation. MDM2 binds and inhibits p53 by transporting it to the cytoplasm and marking it for degradation by ubiquitination (Momand, Zambetti et al. 1992). In unstressed cells, p53 levels are kept in check by MDM2. $p14^{ARF}$ is negatively regulated by p53, and functions to bind and inhibit the function of MDM2.

Loss of p53 function can be acquired through different mechanisms. In fact, at least one of the following alterations in the TP53/MDM2/$p14^{ARF}$ pathway, *TP53* mutations, $p14^{ARF}$ homozygous deletion or promoter methylation, and *MDM2* amplification, is observed in

approximately 50% of primary and >75% of secondary GBMs (Zawlik, Kita et al. 2009). Mutations in *TP53* most often result in loss-of-function (the inability of p53 to regulate transcription). Loss-of-function mutations lead to the accumulation of inert p53 protein, as p53 can no longer self-regulate by activating *MDM2* transcription. Interestingly, the number of cells displaying p53 accumulation in MG appears to increase following subsequent biopsies, suggesting clonal expansion of these *TP53* mutant cells (Sidransky, Mikkelsen et al. 1992). In support of this hypothesis, the majority of *TP53* mutations in secondary GBM are also observed in the original lower-grade malignancy (Watanabe, Sato et al. 1997). Loss-of-function in *TP53* promotes abnormal cell division and thus a means by which anaplastic transformation of low-grade astrocytomas may be facilitated through the accumulation of genomic abnormalities. In primary MG, *TP53* mutations are rare, with evidence suggesting that *TP53* and *EGFR* mutations may be mutually exclusive events (Watanabe, Tachibana et al. 1996).

Another important pathway involved in the cell cycle that is often perturbed in MG is the p16INK4a/CDK4/retinoblastoma (pRB) pathway. The pRB pathway controls the progression of cells from G1 to the S-phase of the cell cycle. CDK4 complexes with cyclin D1 to phosphorylate pRB, thereby releasing the E2F transcription factor that activates genes involved in the G1 - S transition (Stott, Bates et al. 1998). The pRB-E2F interaction can be disrupted by loss of pRB expression or by inappropriate

pRB phosphorylation, with the latter often due to loss of the p16INK4a inhibitor of CDK4/CDK6. Homozygous deletion of *p16*^{INK4a} gene, CDK4 amplification, or loss of pRB function is found in >50% of primary GBMs and >40% of secondary GBMs (He, Allen et al. 1994; Biernat, Tohma et al. 1997). Interestingly, these alterations are, for the most part, mutually exclusive.

More recently, mutation in the isocitrate dehydrogenase 1 (*IDH1*) gene has been identified as a very early and frequent genetic alteration in secondary GBMs (Parsons, Jones et al. 2008). *IDH1* mutations in primary GBM are rare. IDH1 functions as a homodimer and catalyzes the oxidative decarboxylation of isocitrate to α-ketoglutarate, resulting in the production of NADPH which is used in the Krebs (citric acid) cycle (Smeitink 2010). *IDH1* mutations are dominant-negative, impairing the enzyme's activity by forming catalytically inactive homodimers (Zhao, Lin et al. 2009). Expression of IDH1 mutants *in vitro* results in increased levels of hypoxia-inducible factor subunit HIF-1α, a transcription factor known to facilitate tumour growth (Zhao, Lin et al. 2009).

Oligoastrocytomas are clonal tumours that are often subdivided into two groups: (i) those with *TP53* mutations, believed to be more closely related to astrocytomas, and (ii) those with 1p and 19q deletions, believed to be more closely related to oligodendrogliomas (Maintz, Fiedler et al. 1997), suggesting the existence of at least two genetic subsets of oligoastrocytomas whose response to specific therapies will likely differ.

Of note, many of the mutations/chromosomal abnormalities in MG are also found in high-grade oligodendrogliomas, including the dysregulation of the pRB pathway, amplification and overexpresion of EGFR, and LOH at chromosome 10 (Hoang-Xuan, He et al. 2001).

While the progress made in understanding the molecular biology of MG has been substantial, the importance of genetic alterations in determining patient prognosis remains controversial. For example, some studies report that EGFR overexpression correlates with a poor prognosis, whereas p53 inactivation correlates with improved prognosis in patients with GBM (Zhu, Shaeffer et al. 1996; Korkolopoulou, Christodoulou et al. 1997). However, other studies have not confirmed these results (Waha, Baumann et al. 1996; Newcomb, Cohen et al. 1998). A recent broad-spectrum investigation has concluded that the common genetic abnormalities reported in GBM have limited, if any, prognostic value (Houillier, Lejeune et al. 2006). Furthermore, clinical trials targeting the EGFR, PTEN, and PI3k/Akt pathways have shown limited success and moderate-to-high toxicity (Rich, Reardon et al. 2004; Cloughesy, Wen et al. 2006; Prados, Lamborn et al. 2006; Prados, Lamborn et al. 2006; Wrensch, Rice et al. 2006; Groves, Puduvalli et al. 2007). Nonetheless, as our knowledge of the pathways involved in gliomagenesis evolves, novel therapeutic targets that improve patient survival will undoubtedly be revealed.

1.1.3 Malignant glioma cell-of-origin

The current predominant hypothesis for the origin of cancer is the somatic mutation theory. The somatic mutation theory states that all malignant cells arise from a single cell that has acquired cell cycle-related disturbances that are mostly in the form of somatic mutations (Martin-Villalba, Okuducu et al. 2008). The origin of MG has long been a matter of debate. Traditionally, astrocytomas were believed to arise from the transformation of mature astrocytes because of their high levels of glial fibrillary acidic protein (GFAP), an astrocyte-specific marker (Dixon and Eng 1980; Gottschalk and Szymas 1987). However, GFAP expression does not always correlate with the expression of other astrocytic markers in MG (Rash, Yasumura et al. 1998; Barres 2003). Also, a number of genes expressed in neural progenitors as well as in neuronal cells have been described in MG (Kleihues and Sobin 2000; Zhou and Wei 2001; Imura, Kornblum et al. 2003; Louis, Ohgaki et al. 2007). This suggests that MG may arise from a dedifferentiated or less differentiated cell type, possibly a multipotent progenitor or stem cell. The existence of neural stem cells in the mature adult brain has been demonstrated in the hippocampus, olfactory bulb, subventricular zone (SVZ), and the dentate gyrus (DG) (Kriegstein and Alvarez-Buylla 2009). Stem cells innately display several characteristics necessary for malignancy, including self-renewal and migratory capabilities.

The cancer stem cell (CSC) theory postulates that only a small fraction of tumour cells have the capacity for self-renewal and tumour initiation. The first conclusive evidence for the existence of CSCs came in 1997 when Bonnet *et al*. isolated a subpopulation of CD34+/CD38- cells (frequency of 1/10,000) that were capable of initiating tumours in SCID mice that were histologically similar to the parent tumour (Bonnet and Dick 1997). Evidence for a stem-like cell-of-origin for MG can be traced back to experimental murine model systems demonstrating a higher rate of MG tumour formation upon treatment of fetal mice with ethylnitrosourea compared to adult mice (Druckrey, Ivankovic et al. 1966). The potential of fetal cells to generate brain tumours was further highlighted by experiments in which fetal rat brain cells transformed with both v-H-ras and v-myc gave rise to tumours of different morphology when transplanted into adult animals (Wiestler, Aguzzi et al. 1992). In CNS tumours, neural stem cells have been found to be enriched in the population expressing the marker CD133, a pentaspan transmembrane glycoprotein (Uchida, Buck et al. 2000). Recently, Singh *et al.* (2004) demonstrated that injection of as few as 100 $CD133^+$ MG cells into the brains of NOD-SCID mice could successfully initiate tumour growth, whereas 10^5 $CD133^-$ glioma cells failed to form a tumour (Singh, Hawkins et al. 2004). These data indicate the presence of brain cancer stem cells (BCSCs) in the $CD133^+$ population of glioma cells. However, separation of $CD133^+$ from $CD133^-$ populations is technically difficult (Rich and Eyler 2008), resulting in conflicting reports regarding the

notion that it is the $CD133^+$ cells that represent the BCSC population (Beier, Hau et al. 2007; Ogden, Waziri et al. 2008; Wang, Sakariassen et al. 2008). Interestingly, BCSCs differ from the majority of tumour cells in their gene expression profile and biological behavior, including proliferation, spreading from the main tumour mass, and sensitivity to chemo- and radiotherapy (Gupta, Chaffer et al. 2009). Further investigations in MG have led to the identification of a host of putative BCSC markers, including BMI-1 (Abdouh, Facchino et al. 2009), nestin (Bexell, Gunnarsson et al. 2009), Sox2 (Gangemi, Griffero et al. 2009), musashi (Johannessen, Wang et al. 2009), CD15 (Mao, Zhang et al. 2009), and activated Notch (Fan, Matsui et al. 2006). Although the identification of a distinct BCSC population in MG remains controversial, the existence of BCSCs is generally accepted.

In addition to the identification of a CSC population in MG, evidence for a stem cell-like cell-of-origin is supported by data showing that differentiation pathways and genes identified as being important for neural stem cell maintenance are deregulated in MG (Holland 2001; Singh, Hawkins et al. 2004; Galderisi, Cipollaro et al. 2006). For example, PTEN has been shown to control proliferation and migration in neural stem cells and is often inactivated in GBM (Duerr, Rollbrocker et al. 1998; Ekdahl, Claasen et al. 2003).

EGFR and its ligands, expressed throughout brain development and into adulthood, are involved in the proliferation, migration, differentiation and survival of neural stem cells (Wong and Guillaud 2004; Nicholas, Lukas et

al. 2006). Aberrant activation of EGFR-mediated signal transduction pathways has been documented in GBM, and is believed to contribute to the induction of glial cell transformation and progression. Constitutively active EGFRvIII causes the downstream activation of the PI3K/Akt and MAPK pathways (Moscatello, Holgado-Madruga et al. 1998; Lorimer and Lavictoire 2001; Narita, Nagane et al. 2002) and can confer cell proliferation advantages and increase cell survival by inhibiting apoptosis (Nagane, Coufal et al. 1996; Sugawa, Yamamoto et al. 1998). An interesting experiment conducted by Li *et al*. (2009) demonstrated that selective expression of EGFRvIII in neural stem cells results in an increase in proliferation, centrosome amplification, colony formation in soft agar, self-renewal, and expression of CD133 (Li, Dutra et al. 2009). However, deregulation of EGFR signaling was also shown to induce GBM formation from mature astrocytes, supporting a dedifferentiation model (Bachoo, Maher et al. 2002).

Meletis *et al.* showed that p53 is expressed in the neural stem cell lineage in the adult brain (Meletis, Wirta et al. 2006). Knockdown of p53 led to increased self-renewal of neural stem cells by increasing cell proliferation and decreasing apoptotic cell death, implicating p53 as a suppressor of cancer stem cell self-renewal. Analysis of the neural stem cell transcriptome showed that p53 suppresses cell self-renewal by regulating the expression of p21, a well-known negative regulator of leukaemia stem cell self-renewal (Viale, De Franco et al. 2009). Further evidence for the role of p53 in the

suppression of stem cell differentiation was provided by Zhu *et al*. who demonstrated that p53 null mice developed astrocytomas in the SVZ, an area of the brain rich in neural stem cells (Zhu, Guignard et al. 2005).

Collectively, the existing data clearly support the notion that the cell-of-origin of MG is a less differentiated cell type rather than a mature astrocyte. Strong support for the cancer stem cell hypothesis for the origin of MG comes from the observation that similar cell signaling pathways drive gliomagenesis and neural differentiation and the identification of a subpopulation of cells in MG that can recapitulate the tumour.

1.2 Fatty Acid Binding Proteins

Fatty acid binding proteins (FABPs) are members of the lipid binding protein superfamily. In mammals, ten FABPs with overlapping cell-, tissue- and developmental-specific patterns of expression have been described (Zimmerman and Veerkamp 2002; Liu, Li et al. 2008). FABPs are named relative to the tissue in which they were first identified, i.e. brain fatty acid binding protein (B-FABP) was first identified in the brain. However, it is now known that FABPs display broad tissue distribution. To avoid confusion, a number-based nomenclature was introduced in 2000 (Hertzel and Bernlohr 2000), thus alleviating confusion over the expression profiles of this gene family.

Despite their abundance in most cells, FABP expression is tightly regulated at the transcriptional level by regulatory elements located

distally and proximally to the transcriptional start site. For example, the 0.4 kilo-base pair (kb) promoter of *FABP3* is sufficient to allow high levels of expression in muscle tissue; however, expression of FABP3 in heart requires 1.2 kb of upstream sequence (Qian, Kuo et al. 1999). Mice have been used to examine the regulation of *FABP7* in the developing CNS (Kurtz, Zimmer et al. 1994; Feng and Heintz 1995). The temporal and spatial expression of *FABP7* is dependent on an enhancer located between 0.3 and 0.8 kb upstream of the transcription start site. Down-regulation of *FABP7* during CNS development was dependent on the region between -1.2 to -1.6 kb. Analysis of the human *FABP7* promoter revealed three NFI binding sites located within 0.25 kb of the transcription start site (Bisgrove, Monckton et al. 2000). NFI is a family of transcription factors encoded by four genes. All four NFIs have been shown to regulate transcription in MG cells (Brun, Coles et al. 2009). Interestingly, the expression of FABP7 in radial glia was substantially reduced in mice lacking Notch receptors, indicating a potential role for Notch signaling in the control of FABP7 expression (Anthony, Mason et al. 2005). Not surprisingly, the regulation of FABPs by their fatty acid ligands has been observed for some members of the family (Distel, Robinson et al. 1992).

FABPs have both unique and overlapping roles in the cell and are traditionally thought to be involved in the uptake, storage, and intracellular trafficking of fatty acids, bile acids and retinoids to specific subcellular organelles, such as the mitochondria, peroxisomes, and endoplasmic

reticulum (Glatz and Storch 2001). FABPs have also been implicated in cell signaling, gene transcription, cell growth, and differentiation (Hamilton 2004). As most FABPs are present in both the nucleus and cytoplasm, it has also been postulated that an important role for FABPs is to deliver their fatty acid ligands from the cytoplasm to the nucleus. Congruent with this theory, FABPs and fatty acids have been shown to regulate the activation of transcription factors, such as the peroxisome proliferator-activated receptors (PPARs) and retinoic X receptors (RXRs), in the nucleus (Helledie, Antonius et al. 2000; Wolfrum, Borrmann et al. 2001; Helledie, Jorgensen et al. 2002; Tan, Shaw et al. 2002). FABPs also function at a systemic level, mediating effects that influence whole-body lipid and energy metabolism (Furuhashi and Hotamisligil 2008).

1.2.1 FABP structure

Members of the FABP family show moderate primary structure similarity, with amino acid sequence homology varying from 20% to 70% (Banaszak, Winter et al. 1994; Haunerland and Spener 2004). However, their tertiary structures, as determined by X-ray crystallography and nuclear magnetic resonance (NMR), are strikingly similar, consisting of a β-barrel surrounding a hydrophobic core (Marcelino, Smock et al. 2006; Oeemig, Jorgensen et al. 2009). The β-barrel itself is composed of 10 anti-parallel α-strands organized into 2 β-sheets and capped on the end by a helix-turn-helix (HTH) motif. Of particular interest, all FABPs contain a

large solvent accessible ligand binding cavity, centered at the end of the β-barrel near the HTH motif, which is thought to act as a gateway for ligand entry and exit (Sacchettini, Gordon et al. 1989; Hodsdon and Cistola 1997). The ligand binding cavity is rich in polar and hydrophobic amino acids that interact with an inward oriented fatty acid ligand and serve to stabilize the protein-fatty acid interaction (LaLonde, Levenson et al. 1994). Based on primary sequence analysis of the binding pocket, FABPs can be grouped on the basis of their ligand specificities: (i) those that are capable of binding FAs and bile salts (FABP1 and -6), (ii) those that are capable of binding FAs, retinoids, and eicosanoids (FABP3, -4, -5, -7, -8, and -9), and (iii) those which only bind FAs (FABP2) (Chmurzynska 2006). Of note, FABPs bind only a single ligand with the exception of FABP1 which can bind two FAs simultaneously (Thompson, Winter et al. 1997).

The N-terminus of FABPs is postulated to dictate the overall rate of FA transfer as it has been shown to be important for electrostatic interactions between FABPs and the cell membrane. Experiments employing fluorescence resonance energy transfer (FRET) have demonstrated that different FABPs transfer FAs at different rates using distinct ligand-transfer mechanisms. Most of the FABPs examined collect and deliver their ligands by contact/collision with a cell membrane. In contrast, FABP1 transfers its ligand(s) to and from membranes via aqueous-phase diffusion (Storch and Thumser 2000). Fatty acid transfer

by collisional mechanisms involves contact between the FABP and the cell membrane, suggesting the presence of membrane-binding domains on the FABP, and possibly specific lipid domains within the membrane itself that have a higher affinity for FABPs. In support of the former, FABP structural domains consisting of cationic residues that interact with the anionic phospholipid headgroups of membranes have been identified (Corsico, Cistola et al. 1998; LiCata and Bernlohr 1998; Wu, Corsico et al. 2001; Corsico, Franchini et al. 2005; Falomir-Lockhart, Laborde et al. 2006). Structure-function analysis using site-directed mutagenesis, chimeric FABPs in which the helical domains of diffusional and collisional FABPs have been exchanged, and a helix-less variant of FABP2, strongly support the centrality of the α-helical region of the N-terminus in determining the ligand-transfer mechanism used by FABPs (Herr, Li et al. 1999; Liou and Storch 2001; Corsico, Liou et al. 2004).

In addition to transient membrane localization for the purpose of ligand transfer, FABPs are often concentrated in the nucleus, suggesting the existence of a nuclear localization signal (NLS) within the protein. However, the primary amino acid sequence of FABPs does not harbour recognizable NLS or nuclear export signal (NES). Using site-directed mutagenesis, Ayers *et al*. (2007) reported the presence of ligand-activated non-classical NLS and NES in FABP4. Ligand-mediated nuclear localization lends itself to two models: (i) the "induced-fit" model, which states that ligand-induced conformational changes precede protein

mobilization, and (ii) the "pre-existing equilibrium" model which posits that all conformations exist within the repertoire of apoproteins, and that ligands act by stabilizing certain conformations, rather than by inducing their formation (Ayers, Nedrow et al. 2007). It was determined that the latter mechanism is employed for nuclear localization of FABP4.

1.2.2 FABPs and their ligands

Intracellular uptake and transport of amphiphilic fatty acids within the cell requires assistance from molecules such as FABPs (Veerkamp, Paulussen et al. 1990; Atshaves, Foxworth et al. 1998; Wolfrum, Buhlmann et al. 1999; Wolfrum 2007). Equilibrium binding assays and structural analysis of FABPs demonstrate that they can bind fatty acids (C16 - C22) with a high affinity and a molar stoichiometry of 1:1 (1:2 for FABP1) (Jakoby, Miller et al. 1993). In addition, biochemical evidence for a chaperone-type role for FABPs exists, with studies showing a direct interaction between fatty acid-activated nuclear receptors such as PPARs and RXRs and FABPs (Wolfrum, Borrmann et al. 2001; Tan, Shaw et al. 2002). The putative role of FABPs as transporters of fatty acids is intimately linked to their affinity for fatty acids.

Although the FABP family is highly conserved at the tertiary level, their sequence identity can be as low as 30%, allowing for differential fatty acid-binding preferences (Liu, Mita et al. 2010). There is a great deal of controversy regarding the affinities of FABPs for their ligands as a wide

range of techniques have been used to measure FABP-ligand affinities, yielding different dissociation constants for the same protein-ligand interaction. For example, the K_d values for FABP2 and palmitic acid (PA; C16:0), oleic acid (OA; C18:1) and arachidonic acid (AA; 20:4) was found to be 41, 5, and 50 nM, respectively, using the 1-sulfonate-8-anilinonaphthalene (ANS) displacement technique, a fluorescence-method. Using another fluorescence-based method, the ADI-FAB assay, K_d values of 20, 27, and 200 nM were observed for FABP2 and PA, OA, and AA, respectively. Of particular interest, human (hu)FABP7 has been reported to preferentially bind polyunsaturated fatty acids (PUFAs) such as docosahexaenoic (DHA), α-linoleic acid (ALA), linoleic acid (LA), and AA (Balendiran, Schnutgen et al. 2000). In addition to these fatty acids, rat FABP7 has been shown to bind lysophosphatidic acid (Senjo, Ishibashi et al. 1985; Xu, Sanchez et al. 1996). Although FABP7 can bind ALA, its low levels in the brain suggest that this fatty acid is not the preferred ligand of FABP7 (Marszalek, Pisklak et al. 2010). Rather, FABP7 is believed to play a role in the intracellular uptake and transport of DHA, and possibly AA, within the cell. In general, the affinity of FABPs for fatty acids is believed to increase with increasing ligand hydrophobicity, with few FABPs showing high selectivity for a specific FA ligand (Richieri, Ogata et al. 2000). The relatively high affinity of FABP7 for DHA, 4 - 40-fold greater than to any other fatty acid (Balendiran, Schnutgen et al. 2000; Richieri, Ogata et al. 2000), suggests a role for FABP7 in DHA metabolism/signaling, especially

given the high concentrations of both DHA and FABP7 in the brain. These emerging data on ligand specificity for FABP7 may be indicative of functional specificity for the different members of the FABP family and should help with the elucidation of their individual cellular roles.

1.2.3 FABP knockout mice

Despite the abundance of information regarding structure, binding properties, and *in vitro* lipid transfer mechanisms, the precise physiological roles of FABPs remain unclear. To further characterize the *in vivo* functions of FABPs, knock-out (KO) mice have been generated, and phenotypic, biochemical and gene expression differences between KO and wild-type mice have been examined (Hotamisligil, Johnson et al. 1996; Binas, Danneberg et al. 1999; Vassileva, Huwyler et al. 2000; Owada, Suzuki et al. 2002; Martin, Danneberg et al. 2003; Newberry, Xie et al. 2003; Owada, Abdelwahab et al. 2006). To date, none of the single knockout mice have shown a pronounced phenotype. This may be due to compensatory upregulation of other FABP family members, thus highlighting the importance of this gene family, as absence of FABP expression may be lethal to the cell.

FABP1 has been postulated to be involved in enterocyte lipid absorption, hepatocyte lipid transport, and lipoprotein metabolism. *fabp1-/-* mice show defects in fatty acid β-oxidation in the liver and intestine, indicating its importance in this process (Martin, Danneberg et al. 2003).

This defect is believed to represent a defect in fatty acid transport as no significant changes in the gene expression profile of these mice was observed. Furthermore, whereas wild type mice fed a diet rich in saturated fats developed fatty livers (hepatic steatosis), *fabp1-/-* mice did not (Martin, Danneberg et al. 2003). Interestingly, this protection did not occur when the mice were fed a high PUFA diet (Newberry, Xie et al. 2006), suggesting that FABP1 functions in the metabolism of saturated, but not unsaturated, fatty acids in the liver.

In addition to expressing FABP1, enterocytes of the intestine also express high levels of FABP2. Much like *fabp1-/-* null mice, *fabp2-/-* null mice exhibit hepatic steatosis in addition to other defects, including increased serum triacylglycerol levels, greater weight gain, and insulin resistance (Vassileva, Huwyler et al. 2000). These data strongly suggest a role for FABP2 in the metabolic syndrome, a combination of medical disorders that increase the risk of developing diabetes and/or cardiovascular disease (Pradhan 2007). In support of this theory, a single nucleotide polymorphism (SNP) in the human *fabp2* gene resulting in the substitution of Ala^{54} -> Thr^{54} is associated with obesity, decreased insulin sensitivity, and dislipidemia in aboriginal Canadians from an isolated community in Northern Ontario (Hegele, Harris et al. 1996). More recently, studies on individuals of non-aboriginal decent have shown that the Ala^{54} -> Thr^{54} SNP is not associated the metabolic syndrome, indicating that other genetic factors may be in play (de Luis, Gonzalez et al. 2010).

Studies show that FABP4 and FABP5 function and expression patterns are similar (Storch and Thumser 2000), suggesting that they may have overlapping roles in the cell. Adipocytes exhibit high expression of FABP4 and low expression of FABP5, whereas macrophages have high levels of both proteins. *fabp4-/-* mice have defects in controlling plasma insulin levels, suggesting a possible role for FABP4 in glucose homeostasis (Hotamisligil, Johnson et al. 1996); however, these results are confounded by the dramatic compensatory increase in FABP5 expression observed in *fabp4-/-* mice (Shaughnessy, Smith et al. 2000). *fabp4/fabp5-/-* exhibit considerably more profound defects, including protection against insulin insensitivity and hepatic steatosis (Cao, Maeda et al. 2006). *fabp5-/-*null mice display a very mild phenotype, with the major defect being lower transepidermal water loss (Owada, Takano et al. 2002). In contrast with *fabp4-/-* mice, *fabp5-/-* mice do not upregulate FABP4. Instead, upregulation of FABP3 is observed in *fabp5-/-* mice, which may explain why only minor defects have been noted. However, *fabp5/fabp3-/-* knockout mice have been generated, with no significant phenotype observed (Guthmann, Schachtrup et al. 2004).

FABP7 is highly expressed in the developing brain of animals. Unlike other members of the FABP family, *fabp7-/-* mice have not been reported to exhibit any metabolic-related defects. *fabp7-/-* mice appear to have normal brain development; however, these mice show distinct behavioral abnormalities, such as increased memory of fear and increased levels of

anxiety (Owada, Abdelwahab et al. 2006). In neurons from the amygdala, the region of the brain associated with emotional memory, N-methyl-d-aspartate receptor activity in response to DHA was decreased in *fabp7-/-* mice compared to wild-type mice. In addition, the brain of neonatal *fabp7-/-* mice had decreased brain DHA content, linking FABP7 expression in the brain to DHA uptake. No compensatory expression, as measured by PCR, of other FABP types in *fabp7-/-* mice was observed; however, protein levels were not analyzed.

1.2.4 FABPs and cancer

Although the role of FABPs in cancer is unclear, a number of reports suggest a link between FABP levels and tumour malignancy. For example, levels of FABP1, FABP2, and FABP4 decrease with the progression of liver, bladder, and colon cancer, respectively (Davidson, Ifkovits et al. 1993; Celis, Ostergaard et al. 1996; Lawrie, Dundas et al. 2004). In contrast, FABP5 is expressed at higher levels in prostate cancer compared to prostate hyperplasia (Adamson, Morgan et al. 2003). In 1998, Godbout *et al.* showed that FABP7 was expressed at high levels in MG tumour tissue (Godbout, Bisgrove et al. 1998). In addition, two independent large-scale DNA microarray studies identified FABP7 as one of the top 100 genes expressed at significantly higher levels in GBM compared to normal brain and other neoplasms of the CNS (Liang, Diehn et al. 2005; Tso, Shintaku et al. 2006). Furthermore, two independent groups showed that an

increase in FABP7 expression correlates with decreased survival in patients with GBM (Liang, Diehn et al. 2005; Kaloshi, Mokhtari et al. 2007). Mita *et al.* (2007) reported that stable clonal populations of U87MG transfected with an FABP7-expression construct showed increased migration compared with control U87 clonal populations. Conversely, stable knock-down of FABP7 in U251MG, which endogenously expresses high levels of FABP7, resulted in a decrease in cell migration (Mita, Coles et al. 2007). These data indicate an important role for FABP7 in gliomagenesis. Interestingly, FABP7 expression is associated with non-CNS malignancies. For example, high levels of FABP7 were detected in renal cell carcinoma when compared with normal kidney samples that showed no FABP7 expression at all (Domoto, Miyama et al. 2007). Zhang *et al.* (2009) identified a novel subgroup of basal-type breast cancer patients that showed a significantly better clinical outcome when FABP7 was expressed (Zhang, Rakha et al. 2009). A study comparing the gene expression pattern of primary melanoma with metastatic melanoma showed that loss of FABP7 expression correlates with the progression of this disease (Goto, Koyanagi et al. 2010). These data indicate the existence of extraneuronal functions for FABP7.

1.2.5 FABP7 and CNS development

The functions and mechanisms of action of FABP7 are not yet understood; however, a number of interesting findings are suggestive of

its physiological role in the brain. FABP7 is associated with neuronal and glial cell differentiation (Feng, Hatten et al. 1994; Kurtz, Zimmer et al. 1994) and is more abundantly expressed in fetal than in adult brain (Shimizu, Watanabe et al. 1997). During murine CNS development, FABP7 is specifically expressed in radial glial cells with levels peaking between ED12/14 and P0/5 (Feng, Hatten et al. 1994; Kurtz, Zimmer et al. 1994). Radial glia guide the migration of neurons in the brain. Once neuronal migration is over, radial glia migrate into the subventricular zone and differentiate into FABP7-negative (or low expressing) astrocytes (Schmechel and Rakic 1979). In humans, the radial glial fiber system and the migration of neuronal precursors occurs at 5-weeks gestation (Levitt 2003); however, the expression level of FABP7 at this time is unknown and can only be inferred from model systems such as the mouse. In the developing mouse brain, increased expression of FABP7 coincides with neuronal and glial cell migration, while its decreased expression coincides with the progressive differentiation of cells in the developing CNS. Addition of FABP7 antibodies to primary cell cultures prevents the extension of radial glial bipolar processes, thus inhibiting neuronal migration, suggesting that FABP7 plays an important role in the migration of cells during CNS development (Feng, Hatten et al. 1994). In further support for a role in cell migration, transient transfection of MG cells with an FABP7 expressing vector results in increased cell migration (Liang, Diehn et al. 2005).

1.3 Fatty Acids

Fatty acids are essential components of every living cell, as they play important roles in metabolism, serve as signaling molecules, affect membrane permeability and fluidity, and act as transcriptional regulators in the nucleus (Sumida, Graber et al. 1993; Foster 2004; Zechner, Strauss et al. 2005; Piomelli, Astarita et al. 2007). Structurally, they are lipophilic molecules consisting of carbon and hydrogen atoms bonded in a variable-length aliphatic chain. Fatty acids are grouped into three categories based on their level of saturation: (i) saturated - no double bonds exist between any of the carbon atoms, (ii) monounsaturated - one double-bond between two carbon atoms, and (iii) polyunsaturated - multiple double-bonds between carbons along the chain. Essential fatty acids (EFAs), required for the maintenance of optimal health, cannot be synthesized *de novo* and must be obtained from dietary sources. There are two classes of EFAs, omega(ω)-3 and ω-6 polyunsaturated fatty acids (PUFAs). The parent ω-6 fatty acid, linoleic acid (LA; 18:2) is elongated and desaturated in the body to form the longer chain fatty acid AA, whereas the parent ω-3 fatty acid α-linolenic acid (ALA; 18:3) is desaturated by the microsomal enzyme system through a series of metabolic steps to form eicosapentoaenoic acid (EPA; 20:5) and docosahexaenoic acid (DHA; 22:6) (Qui 2003; Singh 2005) (Figure 1.4). These long-chain metabolites are the precursors to respective prostaglandins, thromboxanes, leukotrienes, and prostacyclins, which affect blood flow, immune system and neurotransmitter signaling (Das 2006).

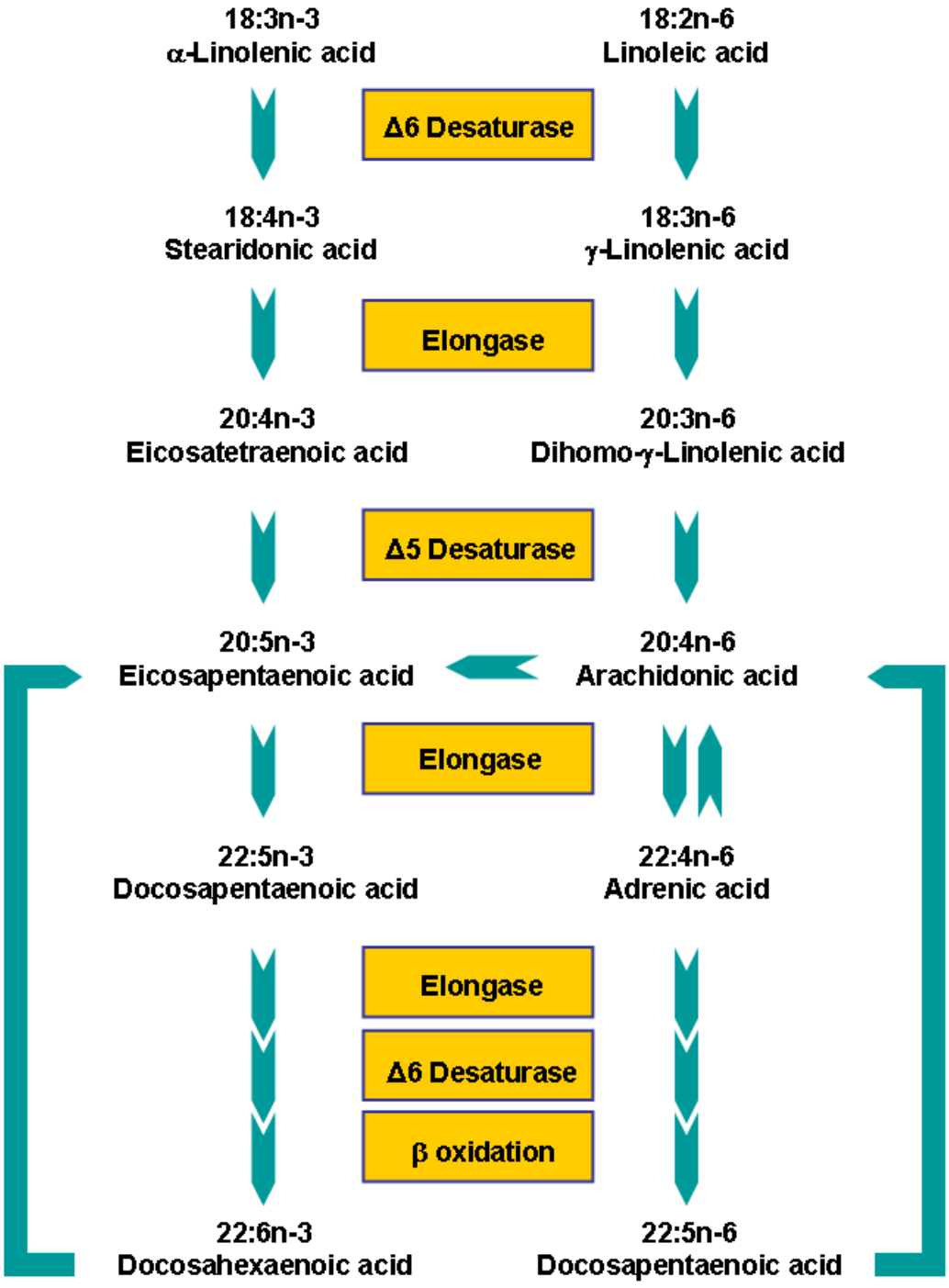

Figure 1.4 Metabolic steps to generating ω-3 and ω-6 PUFAs

In humans, fatty acids are predominately synthesized in the liver, adipose tissue, and mammary glands during lactation. Studies have shown that cells in the brain express the enzymes necessary to carry out the conversion of LA and ALA to AA and DHA, respectively (Cho, Nakamura et al. 1999; Ge, Gordon et al. 2003). These findings suggest that the brain may possess the capacity to synthesize DHA and AA from precursor molecules. However, metabolic biochemists have calculated that less than 0.5% of ALA in the brain is converted to DHA, emphasizing the limited capacity of the human body to produce DHA (Plourde and Cunnane 2007).

1.3.1 Essential fatty Acids and the human brain

The mammalian brain is rich in lipids, consisting of 10% lipids on a weight-by-weight basis, with ω-3 docosahexanoic acid (DHA; 22:6) and the ω-6 arachadonic acid (AA; 22:4) accounting for 6% of the dry matter of the cerebral cortex (Sastry 1985). DHA is the most abundant ω-3 fatty acid in the brain and retina and comprises 40% of the PUFAs in the brain (Singh 2005). The importance of lipids in the brain is highlighted by the dynamic uptake/production of fatty acids during neural development. For example, at 30-weeks gestation in humans (well beyond the 5-week cell migration period), the ω-3/ω-6 fatty acid ratio changes dramatically, with the concentration of DHA increasing quadratically and the concentration of AA decreasing linearly in the cerebellum (Martinez, Conde et al. 1974). In

contrast, the levels of saturated palmitic acid (16:0) and stearic acid (18:0) remain stable during the third trimester. This work is consistent with data demonstrating that DHA is the major lipid component of the adult brain (Singh 2005). The shift in the ω-3/ω-6 ratio is thought to be due to the release of DHA from the mother to the child through an unknown mechanism (Clandinin 1999). DHA and EPA are known to inhibit the desaturation of n-6 fatty acids, thus suggesting a negative feedback mechanism used to reduce AA synthesis (Innis 1992). Due to obvious limitations in obtaining samples, the fatty acid profile of the developing human brain is incomplete, with data prior to 24 weeks gestation unavailable; however, one can predict that the ω-3/ω-6 ratio is likely to be low in fetal brain during the first trimester based on extrapolation of data available for later developmental stages. Studies show that DHA and AA are essential for the normal development and functioning of the CNS in primates (Neuringer, Connor et al. 1986; Nakamura, Cho et al. 2001). Dietary DHA is needed for the optimum functional maturation of the retina and visual cortex in primates, with visual acuity and mental development seemingly improved by increased levels of DHA (Uauy and Dangour 2006). Reduced levels of DHA have also been correlated with impairments in cognitive and behavioral performance (Innis 2007).

Anthropological data indicate that the dietary trend of humans in the developing world has changed dramatically, shifting from an ω-3/ω-6 ratio of 1:1 10,000 years ago, to a present ratio of approximately 1:15

(Simopoulos 2006). This modification in FAs appears to have significant implications for brain function, altering learning and memory, and thermoregulation responses in mammals (Yehuda, Carraso et al. 1995; Gamoh, Hashimoto et al. 1999). In addition, ω-3/ω-6 imbalances have been associated with many neurological diseases, including multiple sclerosis, major depression, schizophrenia, and bipolar disorder (Crawford, Bazinet et al. 2009), further highlighting the importance of PUFAs for proper brain function.

1.3.2 Essential fatty acids and cancer

It is widely accepted that ω-3 PUFAs exert inhibitory effects, while ω-6 PUFAs have stimulatory effects on tumour growth and progression. The majority of cancer-related fatty acid studies have focused on breast and prostate cancer. *In vitro* studies show that ω-6 fatty acids stimulate the proliferation of both mouse (Buckman, Hubbard et al. 1991) and human (Rose and Connolly 1990) breast cancer cells by mechanisms involving the biosynthesis of lipoxygenase products.Conversely, DHA has been shown to inhibit breast cancer growth in a concentration-dependent manner (Rose and Connolly 1990); however, DHA's mechanism of action has never been elucidated completely. *In vivo* studies showed that feeding rodents a high ω-6 fatty acid diet increases the growth of transplanted rodent mammary carcinomas (Gabor and Abraham 1986), whereas a DHA-rich diet has the opposite effect (Karmali, Marsh et al. 1984). In MG

cells, AA has been associated with increased migration (McDonough, Tran et al. 1998; Giese, Hagel et al. 1999). Although the effect of DHA on MG migration has not been examined, DHA has been shown to inhibit migration in breast cancer cells (Shi, Ni et al. 1997). Interestingly, in a study of the fatty acid content of MG, Martin *et al.* (1996) showed that the AA content of MG was higher than in non-malignant brain tissue. Conversely, the DHA content was found to be much lower in MG than in non-malignant brain tissue (Martin, Robbins et al. 1996). These findings were recently confirmed by Marszalek *et al*. (2010) using nuclear magnetic resonance to analyze and compare the lipid profile of MG and normal human brain tissue (Marszalek, Pisklak et al. 2010).

1.3.3 Essential fatty acids and cell signaling

Among the oft-overlooked cell signaling components of membranes are the fatty-acid containing phospholipids. It has been proposed that the effects of PUFAs on cell signaling are mediated in part by their incorporation into the cell membrane. Cell membranes are highly complex structures composed of both lipid and protein. Coordination of cell membrane signaling is done by the segregation of its constituents into microdomains known as lipid rafts (Simons and Toomre 2000). Lipid rafts can be defined as membrane micro-domains that are rich in saturated fatty acids, sphingolipids, AA, cholesterol and several signaling proteins (Simons and Vaz 2004). Interestingly, incorporation of highly flexible PUFAs into the

cell membrane modifies the distribution of membrane-associated signaling molecules (Feller and Gawrisch 2005), which in turn changes the function of cell membranes by altering their cell signaling potential (Jones, Arai et al. 1997; Young, Gean et al. 1998; Stillwell and Wassall 2003). The presence of DHA, which is unique in its length and high level of unsaturation, causes hyperfluidization and compartmentalization of the cell membrane, expelling saturated fats from lipid rafts (Valentine and Valentine 2004; Stillwell, Shaikh et al. 2005). Electrospray ionization mass spectrometry (ESI/MS) analysis has demonstrated that lipid rafts are enriched in plasmenylethanolamines, particularly those containing AA (Pike, Han et al. 2002; Simons and Vaz 2004). Importantly, ω-3 and ω-6 fatty acids have significantly different acyl chain flexibility, allowing for the transduction of different signaling pathways (Eldho, Feller et al. 2003; Rajamoorthi, Petrache et al. 2005).

It is well-known that the activities of many cell signaling molecules depend on interactions with the cell membrane. Thus, altering the cell membrane composition may negatively or positively affect these interactions. For example, Ras proteins are recruited to the cell membrane where they cycle between inactive GDP-bound and active GTP-bound forms. The interaction of Ras with the cell membrane is facilitated by posttranslational modifications such as farnesylation and palmitoylation (Walker and Olson 2005; Bianco, Melisi et al. 2006). *In vivo* experiments using rats show that a diet rich in DHA results in a decrease in membrane

bound Ras and an increase in cytosolic Ras. This effect on Ras localization does not require farnesyl transferase activity and is independent of palmitoylation of Ras (Collett, Davidson et al. 2001), suggesting that incorporation of DHA in cell membranes is directly responsible for inhibiting Ras binding to the plasma membrane. Recent findings have demonstrated a partitioning of Ras into caveolae, a lipid microdomain rich in caveolin (Prior, Harding et al. 2001). Ma *et al*. (2004) reported decreased levels of H-Ras protein in colonic caveolae of mice fed an ω-3-rich diet compared to an ω-6-rich diet (Ma, Seo et al. 2004). Moreover, these investigations showed that ω-3 PUFAs are preferentially incorporated into caveolae phospholipids, suggesting that alterations in the caveolae lipid environment can lead to protein displacement from these membrane microdomains. Data demonstrating decreased farnesyl protein transferase activity in colonic mucosa and colonic tumours of rodents fed an ω-3–rich diet, suggests that DHA may also inhibit Ras membrane localization by interfering with the post-translational modification machinery (Singh, Hamid et al. 1997; Singh, Hamid et al. 1998).

Studies from Dr. Catherine Field's laboratory (Edmonton) indicate that DHA can be incorporated into whole membranes and lipid rafts of breast cancer cells *in vitro*, resulting in reduced EGFR levels in the rafts and increased whole cell levels of phosphorylated EGFR (Schley, Brindley et al. 2007). Increased EGFR phosphorylation was associated with p38 MAPK phosphorylation and induction of apoptosis. Another important signaling

protein, protein kinase C (PKC) has also been shown to be activated by fatty acids that exhibit large negative curvature stress such as DHA and AA (Slater, Kelly et al. 1994; Mosior, Golini et al. 1996). Giorgione *et al.* found that DHA acyl chains produced the highest level of PKC activity when incorporated into phosphatidyl ethanolamine (PE) and the activity correlated strongly with increased partitioning of PKC into the membrane (Giorgione, Epand et al. 1995). These results further support the idea that membrane microdomains are important for activation of signaling molecules and that this activation can be disrupted or enhanced via the alteration of the acyl chains present within these domains.

The phospholipid composition of a membrane can also influence the activity of ion channels including Na^+/K^+ ATPase, cAMP and cyclic nucleotides (Spector and Yorek 1985; Bourre, Dumont et al. 1991). This is of particular importance in nervous tissue where Na^+/K^+ ions are responsible for the transduction of signals between cells. Thus, a change in membrane phospholipid composition may affect neurotransmission. AA and its derivatives can modulate dopamine release and dopamine receptor activity, as well as serotonin and glutamatergic activity when incorporated into the membranes of neural cells (Skosnik and Yao 2003).

In addition to lipid rafts, plasma membranes contain phospholipid pools that function as reservoirs for the synthesis of specific lipid messengers. PUFAs are the precursors of many secondary messengers that mediate their effects by altering gene expression. It is important to note

that lipid rafts and phospholipids likely act in concert with one another. Upon stimulation, a family of proteins known as phospholipases cleave fatty acids from the cell membrane. The free fatty acids in turn participate in signaling cascades that can either promote or inhibit gene transcription. Prostaglandins, produced from AA, are synthesized as a result of cyclooxygenase activity. In the first step of the AA cascade, the short-lived precursor, prostaglandin H2, is synthesized. Additional steps in the cascade result in the synthesis of an array of prostaglandins, which participate in numerous physiological and neurological processes (Privett, Phillips et al. 1972).

The prostaglandins derived from membrane AA include prostaglandin (PG)E_1 and PGE_2. PGE_1 is important in the nervous system as it affects the release of compounds that transmit nerve impulses. PGE_1 tends to have anti-inflammatory properties and enhance immune system function. PGE_2 is a highly inflammatory substance which can cause swelling, increased pain sensitivity, and increased blood viscosity. PGE_3 tends to be mildly anti-inflammatory and immune system enhancing. PGE_3 is thought to counter the effects of the powerful inflammatory PGE_2 and prevents blood platelets from clumping and blood vessels from spasming. FAs important for PGE_3 production, such as EPA and DHA, can also reduce levels of AA in the cells. This reduces the likelihood of producing messengers from AA and represents one way in which FAs can alter the transmission of highly inflammatory messengers.

In contrast to AA, membrane DHA appears to play a role in neuroprotection. DHA is the precursor of oxygenation products now known as the docosanoids, some of which are powerful counter-proinflammatory mediators. The mediator 10, 17S-docosatriene (neuroprotectin D1, NDP1) counteracts leukocyte infiltration, NF-κB activation, and proinflammatory gene expression in brain ischemia-reperfusion and is an apoptotic mediator, potently counteracting oxidative stress-triggered apoptotic DNA damage in retinal pigment epithelial cells (Bazan 2005). At the nuclear level, PUFAs can be transported to the nucleus where they can associate with ligand-activated transcription factors such as PPARs, thus implicating them in the direct modulation of gene expression (Wolfrum 2007).

1.4 Peroxisome Proliferator Activated Receptors (PPARs)

PPARs are ligand-inducible transcription factors that belong to the nuclear hormone receptor superfamily (Kota, Huang et al. 2005). To date, three different PPAR isoforms, -α, -β/δ, and γ have been identified. Initially named for their ability to stimulate peroxisome proliferation in rodents, PPARs have since been shown to regulate a wide range of cellular and systemic functions. In line with their various developmental and physiological functions, they exhibit broad but isotype-specific tissue expression patterns, similar to the FABP family. PPARα is expressed mainly in metabolically active tissues, including the liver, heart, kidney and intestine (Auboeuf, Rieusset et al. 1997; Braissant and Wahli 1998). PPARβ

is expressed in a wide range of tissues and cells, with relatively higher levels of expression noted in the skin, adipose tissue and brain (Amri, Bonino et al. 1995). Two PPARγ isoforms, PPARγ1 and PPARγ2, have been identified in mice and humans (Zhu, Qi et al. 1995; Elbrecht, Chen et al. 1996). These two isoforms differ in that PPARγ2 has 30 additional amino acids at its N-terminus that limit its expression to adipose tissue (Fajas, Auboeuf et al. 1997), while PPARγ1 is expressed in the heart, skeletal muscle, colon, small and large intestines, kidney, pancreas and brain (Tontonoz, Hu et al. 1994).

In contrast to the steroid hormone receptor superfamily of transcription factors, which act as homodimers, PPARs form heterodimers with the RXR family (Kliewer, Umesono et al. 1992; Keller, Dreyer et al. 1993). This heterodimerization occurs without the binding of ligand; however, it is thought that upon ligand binding, a conformational change occurs in the nuclear receptors, thus stabilizing the interaction. Ligand-bound PPAR/RXR complexes modulate gene transcription by binding to a specific DNA sequence known as a PPRE (peroxisome proliferator response element) in enhancer and promoter sites of specific genes (Dreyer, Krey et al. 1992; Berger and Moller 2002). PPAR/RXR heterodimers interact with either co-activators or co–repressors to stimulate or inhibit gene expression (Dowell, Ishmael et al. 1999; Guan, Ishizuka et al. 2005; Yu, Markan et al. 2005). Several co-activators, including CBP/p300 and steroid receptor co-activator (SRC)-1, possess intrinsic

histone acetylase activity that can remodel chromatin structure (Zhu, Qi et al. 1996). Other co-activators, represented by the components of the DRIP/TRAP complex such as PPAR binding protein (PBP)/TRAP220 (Zhu, Qi et al. 1997), form a bridge between the nuclear receptor and the transcription initiation machinery.

The sequence of events underlying PPAR activation is initiated by co-activators with histone acetylase activity complexing with PPRE-bound PPAR/RXR heterodimers to disrupt nucleosomes by acetylating histones, thereby "opening up" the chromatin structure. Complexes such as DRIP/TRAP are then recruited and provide a direct link to the basal transcription machinery and initiation of transcription occurs. Interestingly, the binding of a partial agonist to PPARγ was shown to weaken PPARγ interaction with CBP or SRC-1 in contrast to a full agonist which abolished PPARγ interaction with CBP or SRC-1 (Oberfield, Collins et al. 1999). These results indicate that distinct PPAR-cofactor interactions may be critical in transmitting signals that result in unique gene regulatory activity.

Not surprisingly, PPREs have been identified in genes involved in metabolism, cell cycle, and differentiation (Gupta, Brockman et al. 2001; McClelland 2004). Interestingly, studies have shown that PPAR binding to PPREs upstream of *fabp1*, *2*, *3*, and *4* regulates the transcription of these genes (Motojima 2000; Poirier, Niot et al. 2001; Fujishiro, Fukui et al. 2002),

indicating an important role in PPAR-mediated transcriptional regulation of FABPs with the potential of a feedback mechanism.

1.4.1 PPARs and ligand binding

Similar to other members of the nuclear receptor superfamily, PPARs are characterized by three domains: the N-terminal domain (important for functional regulation), the DNA binding domain (DBD), and the ligand binding domain (LBD) located in the C-terminal region of the protein (Ehrmann, Vavrusova et al. 2002). While the N-terminal and DB-domain are highly conserved among all nuclear receptors, X-ray crystallography reveals that structural conservation of the LBD is low. The PPAR LBD consists of 12 α-helices that form a three-layer antiparallel α-helical sandwich with a small four-stranded sheet that delineates a large Y-shape hydrophobic pocket that serves as the ligand-binding cavity (Nolte, Wisely et al. 1998; Uppenberg, Svensson et al. 1998). This binding pocket is much larger in PPARs than in other nuclear receptors (Gampe, Montana et al. 2000), likely accounting for the capacity of PPARs to bind a wide range of natural and synthetic compounds.

Comparisons of the LBDs of PPARs reveals important structural differences (Xu, Lambert et al. 2001). For example, the ligand pocket of PPARβ is significantly smaller than that of either PPARα or PPARγ. Interestingly, significantly fewer ligands have been identified for PPARβ than for PPARα or -γ, suggesting that the size of the pocket directly

correlates with the ligand-binding specificity of PPARs. Furthermore, the PPARα LBD is more lipophilic than that of either PPARβ or PPARγ. This could explain why many potent PPARγ ligands do not bind PPARα, and why PPARα has such a high affinity for saturated fatty acids whereas PPARγ preferentially binds mono- and polyunsaturated fatty acids.

1.4.2 Endogenous ligands of PPARs

PPARs are believed to be involved in maintaining lipid homeostasis in the cell by sensing changes in fatty acid levels. Research has shown that fatty acids and their derivatives, including eicosanoids and prostaglandins, are endogenous activating ligands of PPARs (Xu, Lambert et al. 1999). For the most part, PPARα activators have been identified using *in vitro* approaches (Gottlicher, Widmark et al. 1992; Forman, Chen et al. 1997). PPARα is activated by many saturated and unsaturated fatty acids, including palmitic acid (PA), oleic acid (OA), LA and AA (Hostetler, Petrescu et al. 2005). Thus, natural ligands for this receptor may be generated during fatty acid catabolism and new fatty acid synthesis *in vivo* (Chakravarthy, Lodhi et al. 2009). Several enzymes such as the 8-, 12-, 15- and 5-lipoxygenases, the cyclooxygenases and cytochrome P450s utilize fatty acids as substrates to produce putative PPARα ligands (Brash 1999; Ng, Huang et al. 2007). It is unclear whether the concentrations at which binding has been noted *in vitro* are physiologically relevant; however, based on its ligands and tissue expression profiles, it seems likely that PPARα

functions primarily as a regulator of energy consumption by sensing the levels of free fatty acids in the cell (Reddy 2004).

PPARγ preferentially binds PUFAs, including the essential fatty acids LA, ALA, AA, and DHA (Krey, Braissant et al. 1997; Xu, Lambert et al. 1999). In addition, a PGD_2-derivative, 15-deoxy-112,14-prostaglandin J2, was demonstrated to be a PPARγ ligand and agonist (Forman, Tontonoz et al. 1995), although the physiological relevance of this ligand is unclear because cellular concentrations cannot be accurately determined. More recently, an oxidized alkyl phospholipid, hexadecyl azelaoyl phosphatidylcholine, was shown to bind and strongly activate PPARγ, with a similar affinity to that of the potent synthetic ligand rosiglitazone (Davies, Pontsler et al. 2001).

PPARβ interacts with both saturated and unsaturated fatty acids thus demonstrating intermediate ligand selectivity compared to PPARα and PPARγ. The PUFAs dihomo-γ-linolenic acid and AA have low affinities (in the micromolar range) for this receptor (Forman, Chen et al. 1997). PA and its metabolically stable analogue, 2-bromopalmitic acid, were also identified as PPARβ agonists (Amri, Bonino et al. 1995). In addition, a number of eicosanoids, including PGA_1 and PGD_2, have been shown to activate this PPAR isoform (Yu, Bayona et al. 1995). PPARβ is activated by binding to LA and EPA, but has very little affinity for either AA or DHA (Krey, Braissant et al. 1997). In addition, a number of synthetic drugs such as the hypolipidemic fibrates, insulin sensitizing thiazolidinediones and non-

steroidal anti-inflammatory drugs (NSAIDs) have been identified as PPAR agonists (Hess, Staubli et al. 1965; Kliewer, Lenhard et al. 1995; Lehmann, Moore et al. 1995; Berger, Bailey et al. 1996; Lehmann, Lenhard et al. 1997). Specific PPAR antagonists have also been identified (Rieusset, Touri et al. 2002).

1.4.3 PPARs and cancer

There is a growing body of evidence indicating that activation of PPARs by ligand binding can induce differentiation and inhibit proliferation of cancer (Mueller, Sarraf et al. 1998; Hisatake, Ikezoe et al. 2000; Inoue, Kawahito et al. 2001). In 1976, Reddy *et al.* described the development of hepatic tumours in mice treated with nafenopin, a potent chemical that causes an increase in peroxisome proliferation. These investigators subsequently observed that liver carcinogenesis could also be enhanced using other peroxisome proliferators, leading to the inclusion of these compounds in the list of non-genotoxic carcinogens, agents that promote cancer without causing direct DNA damage (Reddy, Azarnoff et al. 1980). The role of nuclear receptors in rat hepatocarcinomas was clearly established through experiments showing that treatment of mice with Wy-14,643, a potent activator of PPARα, increased hepatocyte proliferation and development of liver tumours. In contrast, *PPARα*-/- mice were impervious to Wy-14,643 (Peters, Cattley et al. 1997). It should be noted however, that

Wy-14,643 can activate PPARβ, and PPARγ, confounding these results (Schmidt, Endo et al. 1992; Lehmann, Lenhard et al. 1997).

Peroxisome proliferator inducing compounds causing carcinogenesis in rodents is believed to be a result of increased activity of PPARs that leads to the accumulation of high H_2O_2 levels in the cell (Reddy, Reddy et al. 1986). Observation that antioxidant treatments can inhibit PPAR-induced hepatocarcinogenesis confirms a role for oxidative stress (Rao, Lalwani et al. 1984). In humans, long term exposure of dyslipidemic patients to PPARα agonists does not result in an increase in the incidence of hepatocellular carcinomas. This may be a result of the lower expression (ten-fold) of PPARα in human liver compared to that of the mouse (Palmer, Hsu et al. 1998). Liu *et al*. (2004) were the first to report the use of TZD18, a potent PPARα ligand, to inhibit cell growth and induce apoptosis in the T98MG cell line (Liu, Zang et al. 2004).

A number of studies show that PPARγ is expressed in a variety of cancers, including gastric (Takahashi, Okumura et al. 1999), bladder (Guan, Zhang et al. 1999), pituitary (Heaney, Fernando et al. 2003), colon (Sarraf, Mueller et al. 1998) breast (Elstner, Muller et al. 1998), and malignant melanoma (Papi, Rocchi et al. 2009). PPARγ ligand-dependent activation is often associated with the suppression of tumourigenesis. Inhibition of tumour growth may be a consequence of PPARγ down-regulating the activity of a number of transcription factors, including NF-κB, STATs, and Jun/Fos (Amri, Bonino et al. 1995; Palmer, Causevic et al.

1997). Activation of PPARγ in MG using animal models can induce apoptosis, and inhibit tumour growth, migration and invasion (Zang, Wachter et al. 2003; Grommes, Landreth et al. 2006; Coras, Holsken et al. 2007). Ligands of PPARγ have also been shown to down-regulate the expression of COX-2, an enzyme widely implicated in enhancing the tumourigenicity and invasive properties of many cancers (Wu, Yiu Sung et al. 2010).

PPARγ activation by the synthetic ligands troglitazone and rosiglitazone can inhibit cell cycle progression at the G1/S checkpoint. In rat intestinal epithelial cells and MCF7 breast cancer cells, inhibition of the cell cycle was the result of cyclin D1 expression (Kitamura, Miyazaki et al. 2001; Yin, Wakino et al. 2001). On the other hand, PPARγ has been shown to be involved in the regulation of neoplastic growth. For example, the development of mammary tumours is significantly enhanced in transgenic mice expressing constitutively active PPARγ (Saez, Rosenfeld et al. 2004). In addition, PPARγ activation by thiazolidinediones appears to increase tumour formation in a genetic model of colon cancer (Lefebvre, Chen et al. 1998). Finally, PPARγ has been found to induce the production of hepatocyte growth factor, well known for its role in promoting cell growth and motility (Jiang, Johnson et al. 2001).

In comparison with PPARα and PPARγ, the role of PPARβ in cancer remains poorly elucidated. It is known that PPARβ is highly expressed in tumours of the colon, and this was originally thought to promote polyp

formation (Gupta, Tan et al. 2000; Park, Vogelstein et al. 2001). However, more recent evidence suggests that PPARβ serves to attenuate colon carcinogenesis (Harman, Nicol et al. 2004). Interesting studies on keratinocytes show that activation of PPARβ can inhibit their migration (Di-Poi, Michalik et al. 2003; Michalik, Desvergne et al. 2003).

Understanding the mechanisms of PPAR activation and gene transcription in the context of FABPs and their common fatty acid ligands may unveil new molecular targets for treating cancer.

1.5 Working Hypothesis

We propose that the inherent ability of malignant glioma cells to express the radial glial marker FABP7 underlies their infiltrative capacity, allowing tumour cells to migrate long distances from the main tumour mass. In addition, we believe that the fatty acid ligands of FABP7, DHA and AA, play a role in determining the overall effect on migration in FABP7-expressing malignant glioma cells.

1.6 Experimental Rationale

We are using U251 and U87 grade IV astrocytoma cell lines that are FABP7-positive and FABP7-negative, respectively, to investigate the role of FABP7 and its PUFA ligands, DHA and AA, in malignant glioma cell migration. U87 and U251 transfected with an FABP7 expressing construct and an FABP7 RNAi construct/siRNA, respectively, were also used in this

investigation. U87 and U251 cell lines were obtained from Jorgen Fogh, Sloane Kettering Institute, Rye, NY, USA.

1.7 Chapter Summaries

1.7.1 Chapter 2

Brain fatty acid-binding protein (B-FABP; FABP7) is normally expressed in radial glial cells where it plays a role in the establishment of the radial glial fiber network required for neuronal migration. FABP7 is also expressed in astrocytoma tumours and in some malignant glioma cell lines. To address the role of FABP7 in malignant glioma, we have studied the growth properties of clonal populations of malignant glioma cells modified for FABP7 expression. Here, we demonstrate that expression of FABP7 in FABP7(-) malignant glioma cells is accompanied by increased cell migration, morphological alterations consistent with a more differentiated phenotype and reduced ability to form colonies in soft agar. Conversely, FABP7 depletion in FABP7(+) malignant glioma cells results in extensive membrane ruffling and decreased cell migration. Consistent with *in vitro* results, expression of FABP7 in astrocytoma tumours is associated with tumour infiltration and tumour recurrence. Based on our data, we propose that the inherent ability of malignant glioma cells to express the radial glial marker FABP7 underlies their infiltrative capacity, allowing tumour cells to migrate long distances from the main tumour mass. Thus, targeting

FABP7-expressing cells may make a significant impact on the treatment of high grade astrocytomas.

1.7.2 Chapter 3

Grades III and IV astrocytomas (collectively called malignant gliomas) are highly infiltrative tumours that consistently recur within the brain despite aggressive treatment. Brain fatty acid-binding protein (FABP7), which binds ω-3 docosahexaenoic acid (DHA) and ω-6 arachidonic acid (AA), localizes to sites of tumour infiltration and is associated with a poorer prognosis in grade IV astrocytoma. Moreover, FABP7 expression in malignant glioma cells correlates with increased cell migration. Here, we show that DHA inhibits migration, whereas AA stimulates migration in a FABP7-dependent manner in malignant glioma cells. We demonstrate that nuclear localization of FABP7 is dependent on DHA binding and is necessary for FABP7-mediated inhibition of cell migration. Conversely, FABP7-induced cell migration appears to be mediated through the cyclooxygenase-2 pathway and reduction of PPARγ levels. We propose a model whereby FABP7 expression and relative levels of DHA and AA determine tumour infitrative potential. Our findings provide mechanistic insight into the role of FABP7 and its fatty acid ligands in controlling the migration of malignant glioma cells and point to the potential use of DHA in combination with COX-2 inhibitors as anti-infiltrative agents in the treatment of FABP7-expressing malignant glioma.

1.7.3 Chapter 4

Due to their highly unsaturated and flexible nature, the polyunsaturated fatty acid (PUFA) content is an important determinant of the biophysical properties of cell membranes. Changes in the proportion of PUFAs in membrane phospholipids can alter cell and tissue function. The brain is rich in PUFAs, particularly ω-3 docosahexaenoic acid (DHA) and ω-6 arachidonic acid (AA). As a result of their differing structures, acyl chain flexibility is significantly altered from ω-3 to ω-6 fatty acids, presumably effecting membrane dynamics in different ways. Due to their amphiphilic nature, intracellular uptake and transport of fatty acids requires molecular chaperones. Ligand-binding assays combined with microscopy have revealed that the family of fatty acid binding proteins (FABPs) actively binds and transports fatty acids. It has been demonstrated that FABP7 has a high affinity for both DHA and AA. Studies show that fatty acid uptake is significantly higher when cytosolic FABPs are present in the cell. Here we show that uptake of DHA and AA in MG cells in vitro is enhanced by FABP7 expression and that uptake of these fatty acids results in their incorporation into phospholipids of the cell membrane. Furthermore, we demonstrate that preferential incorporation of DHA into phosphatidylserine occurs in FABP7(+) MG only; whereas AA incorporation into phospholipids is non-specific.

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Chapter 2: FABP7-expressing radial glial cells -- the malignant glioma cell-of-origin?

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2.1 INTRODUCTION

Astrocytomas, classified as grades I to IV by the World Health Organization, are the most common CNS malignancies in humans. Grades III and IV astrocytomas, collectively referred to as malignant gliomas, are highly aggressive, with patients diagnosed with these tumours having median survivals of 1.6 yrs and 5 months, respectively (Ohgaki and Kleihues 2005). Although they have a higher survival rate, grades I and II astrocytomas frequently recur as high grade astrocytomas, making the treatment of these tumours particularly challenging.

In spite of aggressive treatment often involving a combination of surgical resection, radiation therapy and chemotherapy, patients with high grade astrocytomas usually present with secondary brain tumours at sites that are distal from the primary tumour mass. Yet, extracranial spread of disease is rare for high grade astrocytomas, occurring in only 0.4 – 2.3% of patients (Pasquier, Pasquier et al. 1980; Collignon, Holland et al. 2004). The aggressive nature of high grade astrocytomas may therefore reflect inherently migratory/infiltrative properties of the tumour cell-of-origin rather than acquired invasive properties. Astrocytomas are traditionally believed to arise from astrocytes because they express glial fibrillary acidic protein (GFAP), an astrocyte-specific marker. More recently, it has been suggested that these tumours may arise from pre-astrocytic transitional cells or from multipotent neural stem cells (Sanai, Alvarez-Buylla et al. 2005).

We have previously reported that a subset of malignant glioma cell lines established from high grade astrocytomas express brain fatty acid-binding protein (FABP7; a.k.a. FABP7, BLBP) (Godbout, Bisgrove et al. 1998). During brain development, FABP7 is expressed in radial glial cells where it plays a role in the establishment and maintenance of the radial glial fiber system that guides immature migrating neurons to their final destination (Feng, Hatten et al. 1994; Kurtz, Zimmer et al. 1994). Addition of anti-FABP7 antibodies to primary cultures of cerebellar cells blocks glial cell differentiation by preventing the extension of radial glial processes (Feng, Hatten et al. 1994). Radial glial cells give rise to GFAP-expressing astrocytes once neuronal migration is completed (Schmechel and Rakic 1979). Radial glial cells also display properties of precursor cells, generating both neurons and glial cells *in vitro*, and have been proposed to function as neural progenitor or neural stem cells (Goldman 2003).

The FABP family consists of structurally related proteins that have cell-, tissue- and developmental-specific patterns of expression. Roles for these proteins include the uptake and intracellular trafficking of fatty acids, bile acids and retinoids, as well as roles in cell signaling, gene transcription, cell growth and differentiation (Glatz and Storch 2001). Fatty acids serve as precursors for signaling molecules, metabolic substrates, membrane phospholipid constituents and are mediators of gene expression (Glatz and Storch 2001). Reports suggest a link between FABP levels and either increasing or decreasing malignancy. For

example, liver (L)-FABP and intestinal (I)-FABP levels decrease with progression of liver and colon cancers, respectively (Davidson, Ifkovits et al. 1993; Lawrie, Dundas et al. 2004). Adipocyte (A)-FABP levels are higher in low-grade bladder carcinoma compared to high grade tumours (Celis, Ostergaard et al. 1996), while epidermal (E)-FABP is expressed at higher levels in prostate cancer compared to prostatic hyperplasia (Adamson, Morgan et al. 2003). Directly relevant to our study, a recent gene profiling analysis of grade IV astrocytoma revealed an inverse correlation between expression of nuclear FABP7 and survival in patients younger than the median age (Liang, Diehn et al. 2005).

Here, we address the role of FABP7 in the growth and migration properties of malignant glioma cells. We report that stable introduction of a FABP7 expression construct into a FABP7-negative malignant glioma cell line reduces their anchorage-independence and enhances their migratory properties. Conversely, reduction of FABP7 levels in FABP7-positive cells reduces their migratory properties. Analysis of FABP7 distribution in astrocytoma tumours indicates elevated levels of FABP7 in the infiltrating regions of the tumours. Our results suggest an important role for FABP7 in controlling the migration of malignant glioma cells. Based on our *in vitro* and *in vivo* studies, we propose that FABP7 expression in astrocytoma tumours drives the infiltration of malignant cells into adjacent brain tissue.

2.2. MATERIALS AND METHODS

2.2.1 Stable transfections

U87MG and U251MG cell lines (Godbout, Bisgrove et al. 1998) were transfected by calcium phosphate-mediated DNA transfection. The FABP7 expression construct was prepared by inserting a 467 bp human FABP7 cDNA fragment containing the entire open reading frame into the pREP4 vector which carries the gene for hygromycin resistance. The pSUPER RNAi system (Oligoengine) was used to reduce levels of FABP7 in U251 cells. A 64 bp inverted repeat containing sense and antisense 19-nt gene-specific sequence (CCAACGGTAATTATCAGTC; corresponding to FABP7 nt 114 -132) was introduced into the pSUPER vector at the *Bgl*II/*Hin*dIII site. For stable transfectants, pSUPER vectors were co-transfected with pREP4 empty vector. U87 and U251 transfected cells were selected in 400 μg/ml hygromycin and individual colonies picked using cloning rings.

2.2.2 Western blot analysis

Whole cell lysates were electrophoresed in 13.5% SDS-polyacrylamide gels followed by electroblotting onto nitrocellulose. Blots were immunostained with rabbit anti-FABP7 antibody prepared by immunizing rabbits with recombinant GST-tagged human FABP7 (1:1000 dilution) (Godbout, Bisgrove et al. 1998) or goat anti-actin antibody (1:5000) (Santa Cruz). Primary antibodies were detected with horseradish

peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Biotech) using the ECL detection system (GE Healthcare).

2.2.3 Cell motility, migration and invasion

Non-directional cell motility was measured by 2-D time-lapse video microscopy. Cells were plated in triplicate (75,000 cells/35-mm tissue culture dish) and cultured for 24 h. Metamorph imaging software was used to capture a single DIC image every 30 seconds for 2 h (241 images in total) with each image containing 30 to 40 cells. A video was then constructed and the migration of single cells was tracked using the Metamorph tracking function. Directional cell migration was measured by plating 25,000 cells in DMEM in the upper chamber of Transwell culture dishes (Falcon). Cells were allowed to migrate through an 8 μm PET membrane towards a chemoattractant (DMEM + 10% FCS in bottom chamber) for 6 h. Cells were then fixed in 70% methanol and stained with 1% crystal violet. Cells were photographed and counted using Metamorph cell-counting software. Cell invasion was measured using BD Biocoat Matrigel invasion chambers (BD Biosciences) following the manufacturer's directions. Top chambers were plated with either 10,000 cells (for U87 transfectants) or 25,000 cells (for U251 transfectants) in DMEM and incubated for 22 h. Cells were fixed, stained and counted as described for the Transwell migration assay.

2.2.4 Immunofluorescence and immunohistochemical analysis

For immunofluorescence analysis, cells growing on coverslips were fixed in 1% paraformaldehyde in phosphate-buffered saline for 10 min and permeabilized in 0.5% Triton X-100 for 5 min. The cells were co-labelled with affinity-purified rabbit anti-FABP7 (1:200) followed by Alexa-488 goat-anti-rabbit (1:200) (Cedarlane Laboratories) and with Alexa-546 phalloidin (1:200) (Molecular Probes). Affinity purification of rabbit anti-FABP7 antibody (Godbout, Bisgrove et al. 1998) was performed using Hi-Trap NHS-activated Sepharose columns linked to glutathione-sepharose 4B-purified GST-FABP7. Images were collected on a Zeiss LSM 510 confocal microscope with a 40X/1.3 oil immersion lens.

For immunohistochemical analysis, grades II and IV astrocytomas embedded in paraffin were obtained from the Department of Pathology and Lab Medicine, University of Alberta, Canada or from the Brain Tumour Tissue Bank, London Health Sciences Centre, Canada. Tissues were deparaffinized in xylene, re-hydrated, microwaved for 20 min in citrate/Tween-20 epitope retrieval buffer pH 6 and incubated in affinity-purified anti-FABP7 antibody (1:800) overnight at 4°C. The slides were washed and incubated for 1 h in DakoCytomation EnvisionPlus anti-rabbit secondary antibody. Immunoreactivity was detected using DakoCytomation Liquid DAB+ Substrate Chromagen System. Slides were counterstained with hematoxylin. A low magnification picture of the entire tissue section shown in Figure 2.10 was generated by taking 28 low-

magnification pictures of the tissue and merging them using Adobe Photoshop.

2.3. RESULTS

2.3.1 Stable FABP7 transfection of U87 cells

To address the role of FABP7 in malignant glioma cells, we stably transfected the FABP7-negative U87 malignant glioma cell line with a pREP4/FABP7 expression construct. U87 cells transfected with empty vector served as the control for these experiments. Control and FABP7-transfected cells were selected in hygromycin and individual colonies picked using cloning rings.

FABP7 levels in four stable FABP7 transfectants (U87-B1, B2, B3 and B4) were examined by western blotting. As shown in Figure 2.1A, all four FABP7 transfectants expressed FABP7, with highest levels observed in U87-B4. FABP7 levels in U87-B4 were similar to the endogenous FABP7 levels found in FABP7-expressing U251 cells, with approximately 3-fold lower levels observed in U87-B1, -B2 and -B3. As expected, FABP7 was not detected in the three pREP4 control transfectants U87-(pREP4)R1, -R2 and -R3.

2.3.2 Depletion of FABP7 in U251

U251 cells express endogenous FABP7. We used RNA interference to reduce FABP7 levels in these cells. The pSUPER vector was used for these experiments because it allows long-term reduction in protein levels as the result of persistent production of shRNA in stable transfectants. Nucleotides 114-132 of the FABP7 coding region served as

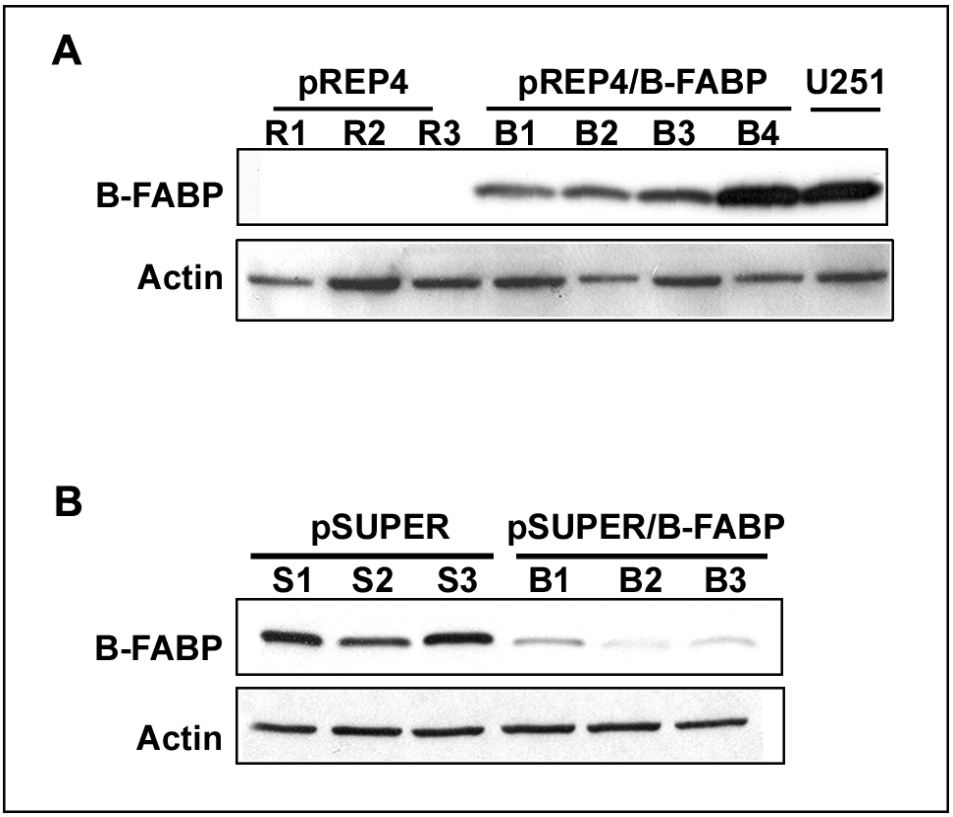


Figure 2.1 Western blot analysis of FABP7 in U87 and U251 transfected cells. (A) Whole cell extracts (50 μg per lane) were prepared from U87 cells stably transfected with empty pREP4 vector (R1, R2, R3), pREP4/FABP7 (B1, B2, B3, B4) as well as U251. (B) Whole cell extracts (50 μg per lane) were prepared from U251 stably transfected with empty pSUPER vector (S1, S2, S3) and pSUPER/FABP7 (B1, B2, B3). Proteins were separated by electrophoresis in a 13.5% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membranes and sequentially immunostained with rabbit anti-FABP7 antibody and goat anti-actin antibody, followed by HRP-conjugated secondary antibodies.

our target sequence, with empty pSUPER vector serving as the control. To select stable transfectants, pSUPER constructs were co-transfected with pREP4 and cells selected in hygromycin. These colonies were expanded and analysed for FABP7 expression after at least 10 passages of a subconfluent culture in a 100 mm plate. A 70-90% reduction in FABP7 levels was observed in three U251-pSUPER-FABP7 transfectants, labeled U251-B1, -B2 and -B3 (Figure 2.1B). All three control transfectants [S(pSUPER)1, S2 and S3] showed significantly higher levels of FABP7 compared to pSUPER/FABP7 transfectants.

2.3.3 Morphological changes in U87 and U251 transfectants

We used phase contrast microscopy to study the morphology of FABP7-negative U87, control transfectants and FABP7-expressing transfectants. The U87 parent cell line and control transfectants generally had a stellate appearance with short processes (Figure 2.2A – top panels). In contrast, FABP7-expressing U87 cells formed extended bipolar processes (indicated by arrowheads) often spanning considerable distances (as much as 250 μm) (Figure 2.2A – bottom panels). These elongated processes are similar in appearance to the radial glial processes which can span the entire width of the cortical wall during brain development (Schmid, Yokota et al. 2006).

U251 cells generally form longer processes compared to U87 cells, with the processes often having a bipolar appearance. The morphology of

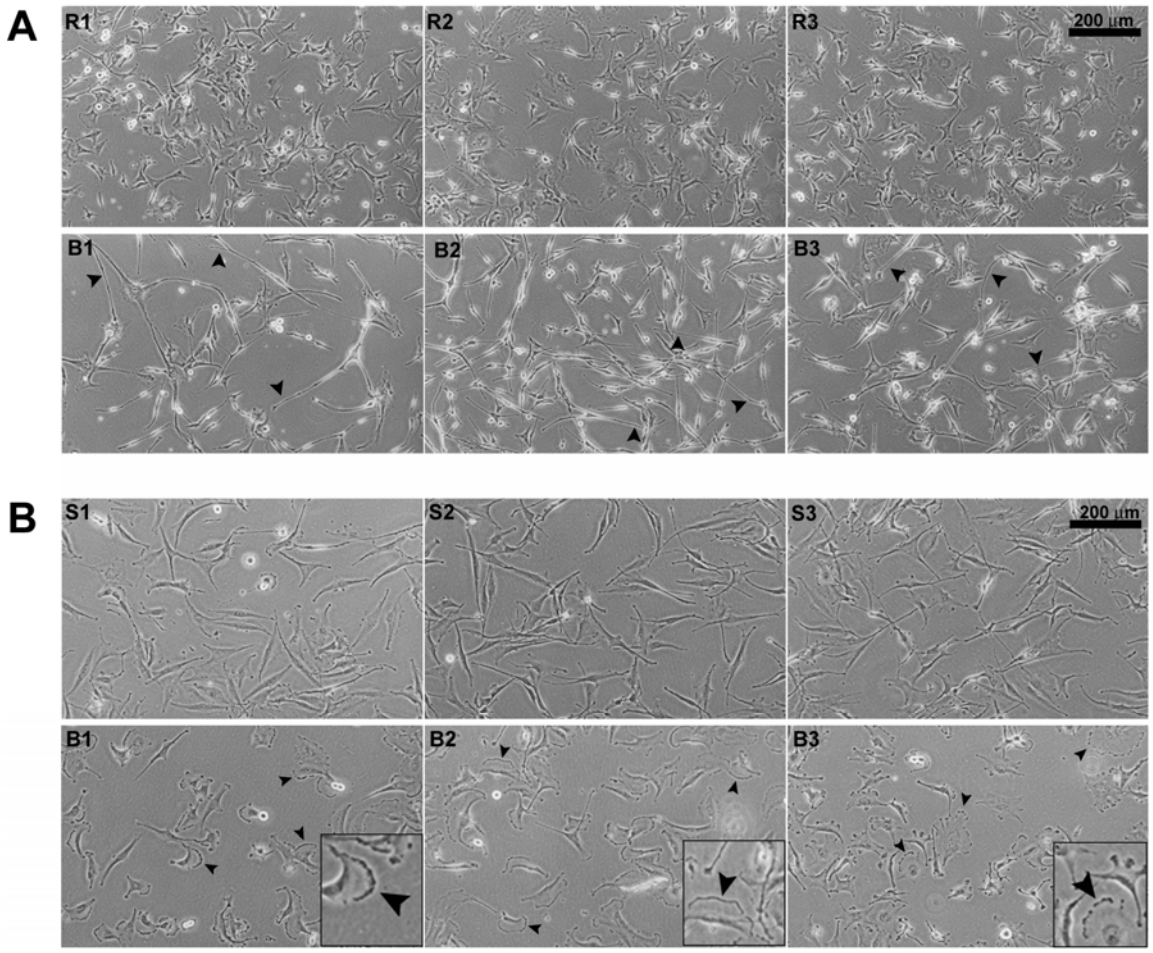


Figure 2.2 Morphological analysis of U87 and U251 transfected cells. (A) Phase contrast microscopy was used to study the morphology of U87 cells stably transfected with either pREP4 (R1, R2, R3) or the pREP4/FABP7 expression construct (B1, B2, B3). The arrowheads point to the elongated processes observed in U87 pREP4/FABP7 transfectants. (B) Phase contrast analysis of U251 cells stably co-transfected with pSUPER (S1, S2, S3) or pSUPER/FABP7 (B1, B2, B3) and pREP4. The arrowheads and insets refer to the extensive membrane ruffling observed in U251 FABP7 knock-downs.

all three U251 control transfectants was similar to that of the parent U251 cells (Figure 2.2B – top panels). Depletion of FABP7 in U251-B1, B2 and B3 resulted in loss of elongated processes and the formation of fan-like structures (indicated by arrowheads) usually associated with extensive membrane ruffling (Figure 2.2B – bottom panels). These fan-like structures were mainly observed when the cells were sub-confluent. No difference in cell piling was observed for any of the transfectants at confluence.

2.3.4 FABP7 expression and cell migration

FABP7 expression in malignant glioma cells results in the formation of elongated processes which may be associated with cell migration. We used two different approaches to study the effect of FABP7 on malignant glioma cell motility: (i) 2-D time-lapse video microscopy which measures random (or non-directed) movement and (ii) Transwell® chambers used to measure directed migration towards a chemoattractant (10% FCS).

Using 2-D time-lapse video microscopy, we followed total movement of ~100 cells per U87 clone over a period of 2 h (30-40 cells were analysed per experiment). All four U87-FABP7 transfectants had a higher motility rate than that of control transfectants, with FABP7-expressing U87 clones traveling at 75-94 μm/h compared to 48-58 μm/h for controls (Figure 2.3A). Differences in motility were found to be statistically significant using the unpaired t-test ($p<0.001$). Similar results

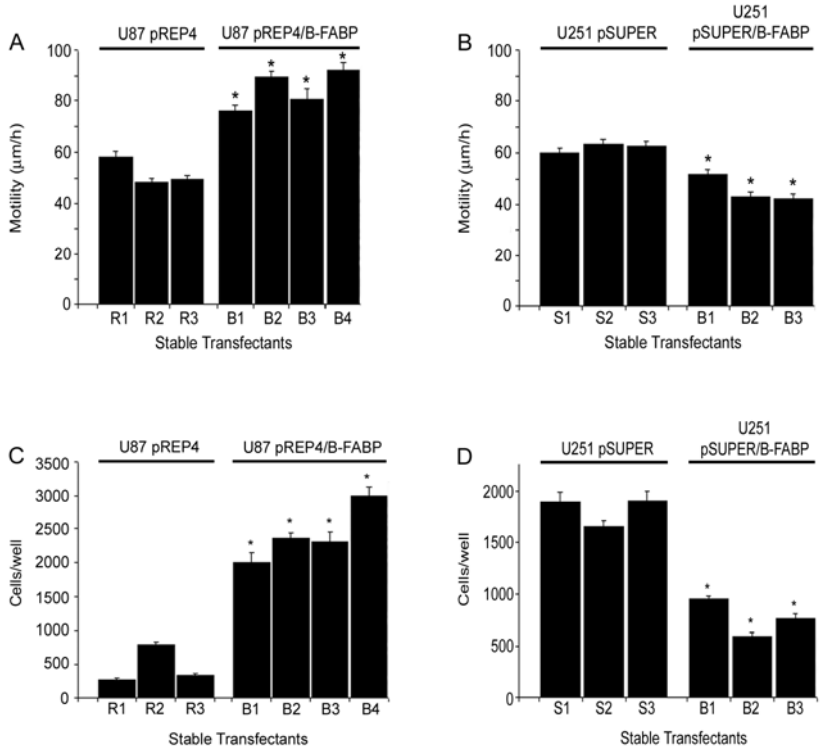


Figure 2.3 Cell motility and migration of U87 and U251 transfected cells. (A, B) Non-directional motility of U87 (A) and U251 (B) transfectants was measured using 2-D time-lapse video microscopy. Cell motility was measured over a period of 2 h with pictures taken at 30 s intervals. Distances were measured using the Metamorph tracking function. (C, D) Cell migration of U87 (C) and U251 (D) transfectants was measured using the Transwell assay (Falcon Labware). Twenty-five thousand cells were plated in triplicate, incubated for 6 h, and the cells migrating through the porous membrane fixed, stained and counted using Metamorph imaging software. Statistical significance was determined using the unpaired t-test. Error bars represent standard deviation.

were obtained with Transwell chambers, with FABP7-expressing transfectants being highly migratory compared to control transfectants. A range of 1997-2997 cells migrated through the porous filter towards the chemoattractant in the case of U87-FABP7 transfectants, in contrast to 273-816 cells in the case of U87 control transfectants (Figure 2.3C). U87-FABP7-4, with the highest levels of FABP7, had the highest number of migrating cells. These differences in migration were significant ($p<0.0001$).

Similar analyses carried out with FABP7-depleted and control U251 transfectants support a role for FABP7 in cell motility. 2-D time-lapse video microscopy revealed higher motility rates for U251 control transfectants, ranging from 60-63 μm/h, compared to FABP7-depleted U251 clones, which ranged from 43-52 μm/h ($p<0.001$) (Figure 2.3B). Results from the Transwell chambers showed decreased migration as a function of FABP7 depletion, with 1726-1890 cells/well for U251 control transfectants compared to 617-950 cells/well for FABP7-depleted U251 transfectants ($p<0.0001$) (Figure 2.3D).

2.3.5 FABP7 expression and invasion

We used the *in vitro* Matrigel® invasion assay to determine whether the increased migration rate associated with FABP7 expression in malignant glioma cells corresponded to an increase in invasive properties. The Matrigel matrix is a reconstituted basement membrane that is coated

over an 8 μm pore size filter. The basement membrane prevents non-invasive cells from migrating through the filter. This assay has been widely used to study the invasive properties of malignant glioma cells and has been shown to correlate well with a three-dimensional spheroid invasion assay and with the *in vivo* intracranial implantation assay (Kondraganti, Mohanam et al. 2000).

U87 or U251 transfected cells were plated in the upper chamber of the Matrigel chambers and incubated at 37°C for 22 h. Cells able to pass through the Matrigel matrix during this time period were stained and counted. There was a trend towards increased cell invasion as a result of FABP7 expression in U87 ($p<0.026$), with 77-136 cells/well in the control group compared to 114-236 cells/well in the FABP7-expressing group (Figure 2.4A). An approximately 2-fold reduction in the number of invading cells was observed in FABP7-depleted U251 cells compared to controls ($p<0.0001$) (Figure 2.4B).

2.3.6 FABP7 expression, cell proliferation and anchorage-independence

Doubling times of FABP7-transfected U87 and FABP7-depleted U251 were measured at 24 h intervals when cells were in the exponential growth phase. FABP7 expression in U87 cells resulted in a significant decrease in proliferation rate. Doubling times ranged from 28 to 32.5 h in control transfectants and from 49 to 77 h in FABP7 transfectants (Figure 2.5). In contrast, no significant changes in doubling times were

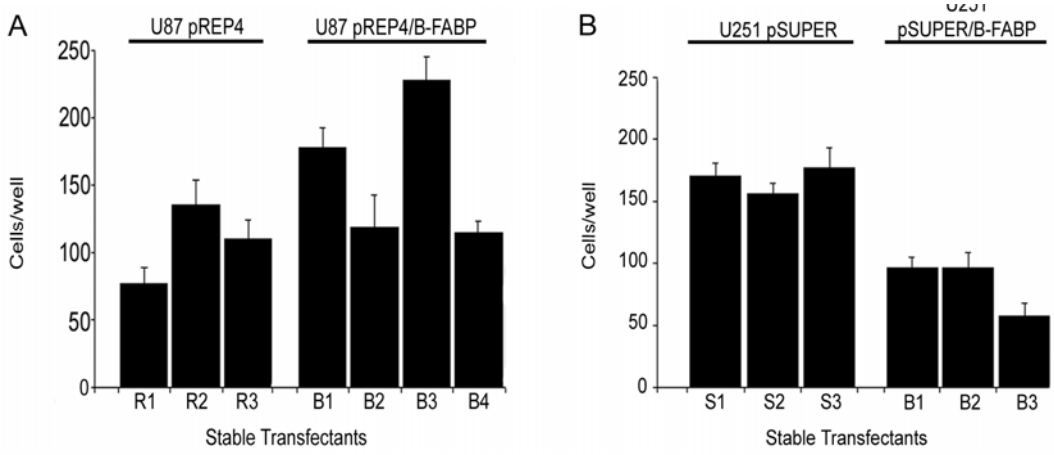
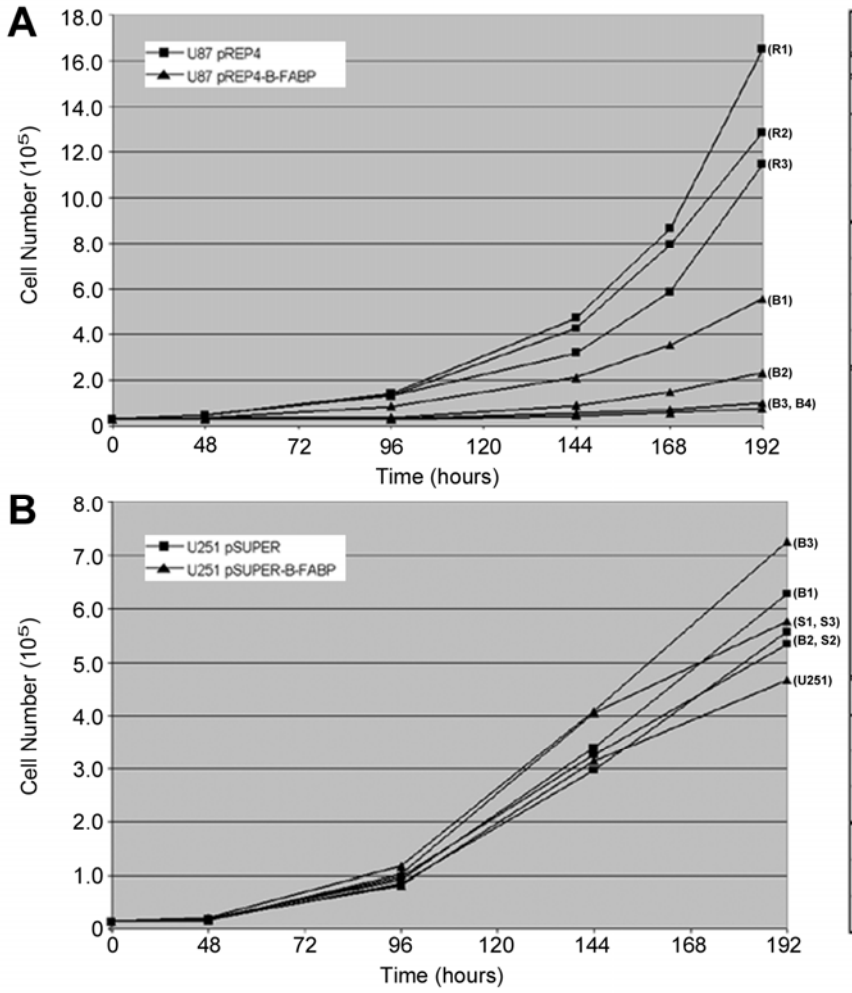


Figure 2.4 Invasion of U87 and U251 transfected cells. (A,B) Matrigel invasion of U87 (A) and U251 (B) transfected cells using Matrigel invasion chambers. For U87 transfectants, 25,000 cells were plated, incubated for 22 h and stained. For U251 transfectants, 10,000 cells were plated, incubated for 22 h and stained. Error bars indicate SEM.



Cell Line	Doubling Time
U87	21.0
pREP4-1	28.0
pREP4-2	32.5
pREP4-3	31.0
pREP4/B-FABP-1	49.0
pREP4/B-FABP-2	49.5
pREP4/B-FABP-3	77.0
pREP4/B-FABP-4	64.5
U251	26.0
pSUPER-1	23.0
pSUPER-2	24.0
pSUPER-3	23.0
pSUPER/B-FABP-1	23.0
pSUPER/B-FABP-2	24.0
pSUPER/B-FABP-3	21.0

Figure 2.5 Proliferation of U87 and U251 transfected cells. U87 control and FABP7-expressing transfectants (A) and U251 control and U251-depleted transfectants (B) were seeded at 15,000 cells per 35 mm culture dish. Cells from triplicate plates were counted using a Coulter Particle and Size Analyzer (Beckman Coulter) at 48 h, 96 h, 144 h, 168 h and 192 h after plating. Growth curves were generated using the average of the triplicate plates at each time point. Doubling times were obtained using the exponential phase of the growth curves.

observed in U251 control transfectants compared to FABP7-depleted U251 transfectants.

FABP7 has been postulated to be associated with radial glial cell differentiation and process formation (Feng, Hatten et al. 1994). FABP7 expression in malignant gliomas may therefore not only enhance migration properties but may also be a hallmark of increased cell differentiation and decreased cell transformation. Anchorage-independent growth is an *in vitro* transformation assay that correlates well with tumour formation in nude mice (Bigner, Bullard et al. 1981). U87 and U251 transfectants were plated in soft agar and colonies >50 cells were counted after 4 weeks in culture. FABP7 expression in U87 cells resulted in a significant decrease in soft agar growth, with an average of 12-118 colonies per plate for U87-FABP7 transfectants, compared to an average of 255-333 colonies per plate for control transfectants ($p<0.0001$) (Figure 2.6). A difference, albeit of smaller magnitude, was also noted between U251 control and FABP7-depleted transfectants, with an average of 290-387 colonies/plate observed in FABP7-depleted transfectants compared to an average of 225-230 colonies/plate in control transfectants ($p<0.008$).

2.3.7 Subcellular localization of FABP7 in malignant glioma cell lines

Cell migration involves protrusion and adhesion at the front of the cell, contraction of the cell body and detachment at the rear (Lauffenburger andHorwitz 1996). These processes require actin

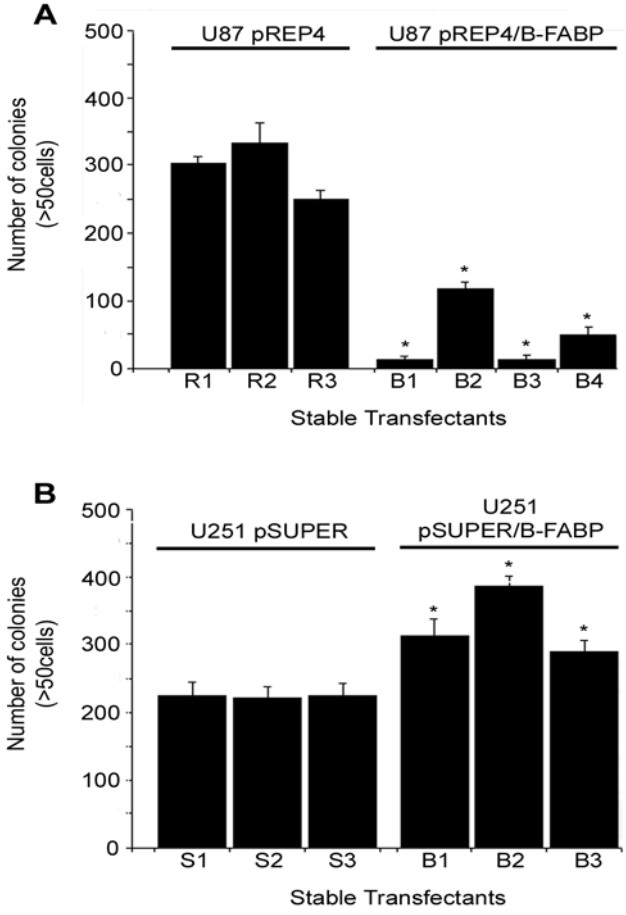


Figure 2.6 Growth of U87 and U251 transfectants in soft agar. (A) U87 pREP4 control and pREP4/FABP7 transfectants (A) and U251 pSUPER control and pSUPER/FABP7 (B) transfectants were plated in quadruplicate at 10^3 and 10^4 cells per dish. Colonies (>50 cells) were counted after 4 wks incubation using a Nikon Diaphot 300 light microscope with a 4X objective. Error bars indicate SEM.

cytoskeleton remodeling involving actin polymerization/depolymerization and are regulated by the small GTPases (Fukata, Nakagawa et al. 2003; Li, Guan et al. 2005). We used immunofluorescence microscopy to study the subcellular distribution of FABP7 in relation to filamentous actin. As shown in Figure 2.7A, FABP7 is enriched in the nuclei of U251 control transfectants and is concentrated at the leading edge of the cells (arrow). There is extensive staining of F-actin at the leading edge of U251 cells and actin stress fibers are abundant in these cells (Figure 2.7B). Interestingly, there is a significant amount of co-localization between FABP7 and F-actin in U251 cells (Figure 2.7C), implying a possible association between FABP7 and F-actin. FABP7-depleted U251 cells demonstrate a reduction in F-actin at the plasma membrane, as well as a reduced number of stress fibers (Figure 2.7D,E). There is little co-localization of FABP7 and F-actin in these cells (Figure 2.7F). FABP7-expressing U87 cells (U87-FABP73) have a similar staining pattern to that observed in U251 control transfectants (Figure 2.7G-I).

2.3.8 Distribution of FABP7 in astrocytoma tumours

Immunohistochemical analysis of a grade II astrocytoma indicates FABP7 expression in gemistocytic astrocytes (Figure 2.8). Although gemistocytes have a low proliferative index, their presence in astrocytomas is considered a sign of poor prognosis, with gemistocyte-rich tumours rapidly progressing to high-grade astrocytomas (Watanabe,

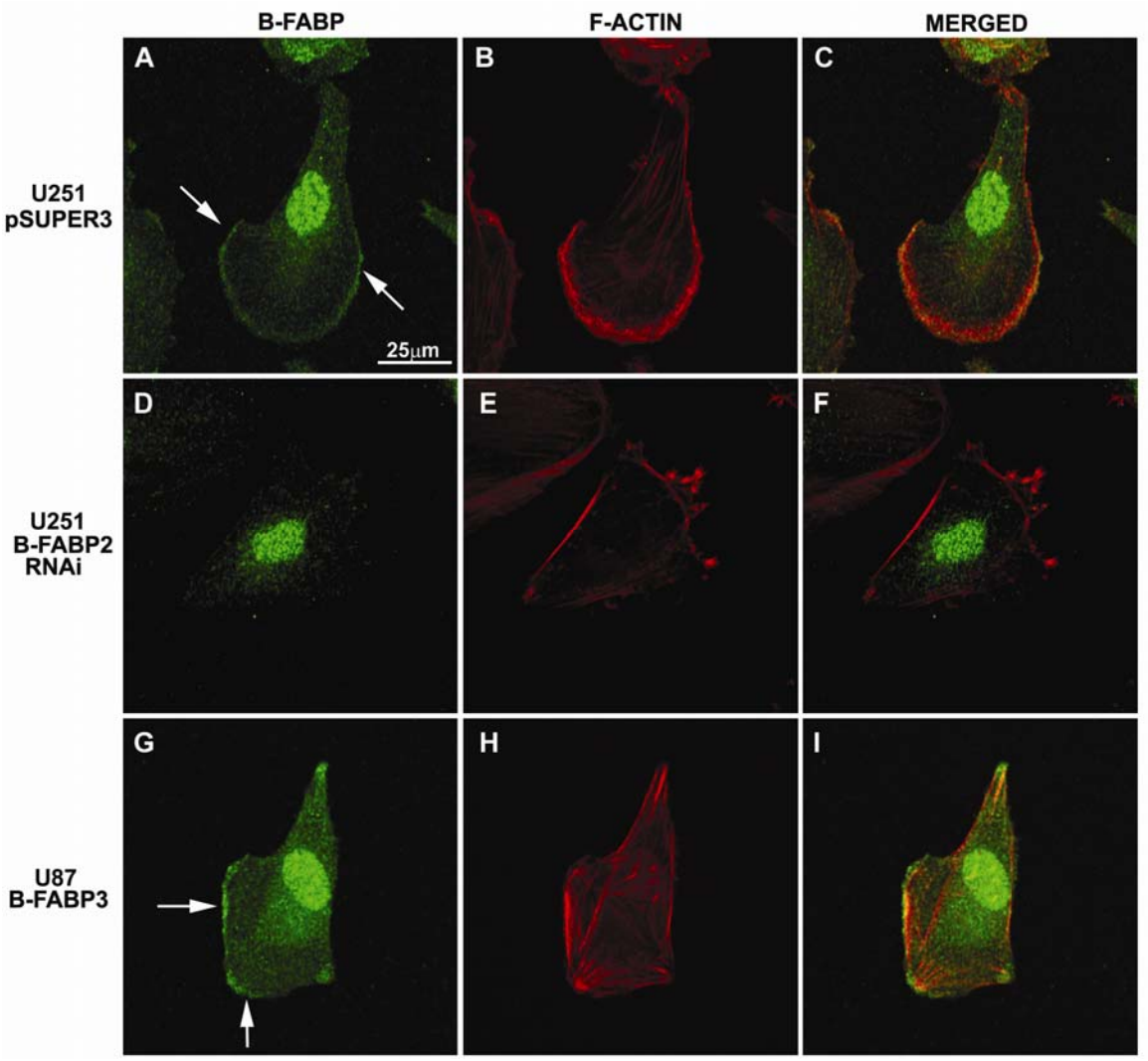


Figure 2.7 Subcellular localization of FABP7 in U87 and U251 transfectants. The subcellular location of FABP7 in U251-pSUPER3 control (A-C), U251-pSUPER/FABP72 (D-F) and U87 FABP73 (G-I) was analysed by immunofluorescence using: (i) anti-FABP7 primary antibody followed by Alexa-488-conjugated secondary antibody (A, D, G) and (ii) Alexa-546 phalloidin which stains F-actin (B, E, H). FABP7 and F-actin signals were merged in C, F. I.

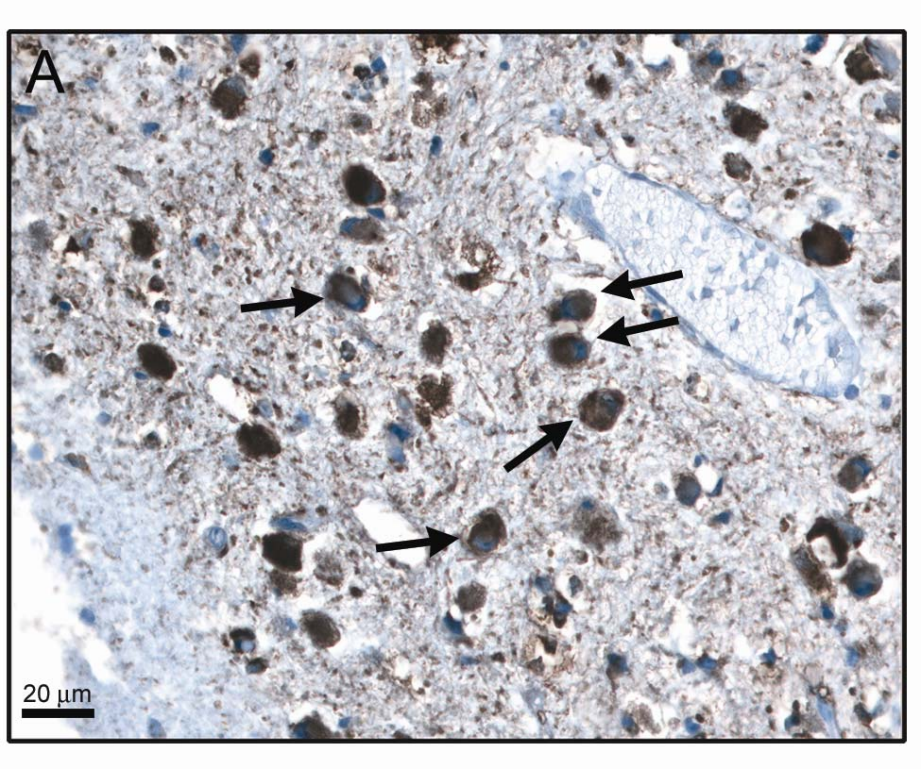


Figure 2.8 Immunohistochemical analysis of FABP7 in grade II astrocytoma. The tissue section was immunostained with affinity-purified anti-FABP7 antibody and counterstained with hematoxylin. The arrows point to FABP7-positive gemistocytic astrocytes.

Tachibana et al. 1997; Yang, Kim et al. 2003). Immunohistochemical analysis of grade IV astrocytomas revealed considerable variation in FABP7 expression, as previously reported (Liang, Bollen et al. 2006; Kaloshi, Mokhtari et al. 2007). As we were particularly interested in the role of FABP7 in migration and infiltration, we selected tumours for analysis that contained regions of high infiltration based on pathology reports. In Figure 2.9, we show a grade IV astrocytoma specimen (patient 670) that contains the following tissue grades: no visible tumour cells (1), scant tumour cells (2), tumour center (3), and heavy tumour infiltrate (4). The top panel shows a low-magnification view of tissue immunostained with anti-FABP7 antibody and counterstained with hematoxylin. The cortical tissue on the left is characterized by low cellularity, with weak FABP7 staining that is mostly cytoplasmic (panel 1). FABP7 staining is stronger in panel 2, but remains primarily cytoplasmic although some FABP7-positive nuclei are observed. The tumour center (panel 3) is characterized by high cellularity, with ~25% of nuclei staining positive for FABP7. Regions of the tumour classified as heavy tumour infiltrate (panel 4) show high cellularity, numerous blood vessels and abundant nuclear and cytoplasmic FABP7 staining especially in cells located in the vicinity of blood vessels. The surrounding of blood vessels by FABP7-positive cells was commonly observed in grade IV astrocytomas (e.g. patient 1022; Figure 2.10A). Accumulation of FABP7-expressing cells in the sub-pial

region of the brain was also observed in grade IV astrocytoma (e.g. patient 1032; Figure 2.10B).

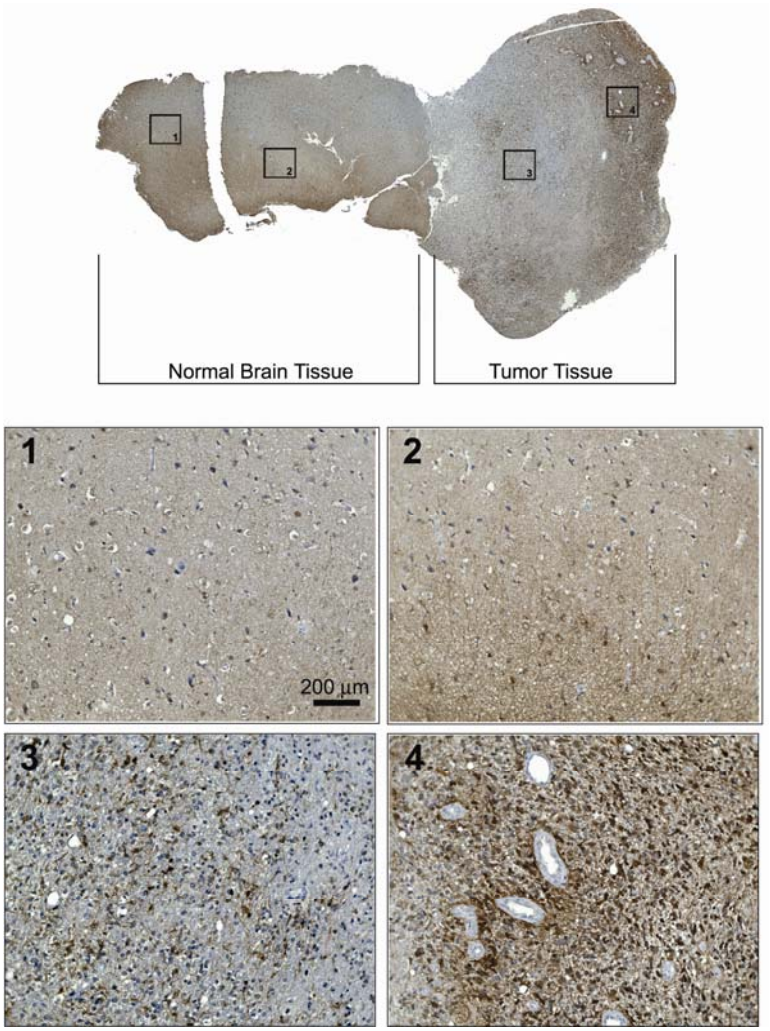


Figure 2.9 Immunohistochemical analysis of FABP7 in grade IV astrocytoma. Tissue section was immunostained with affinity-purified anti-FABP7 antibody and counterstained with hematoxylin. The top diagram is a low magnification view of the entire tissue section consisting of cortical tissue and tumour tissue. The areas labeled 1 (no visible tumour cells), 2 (scant tumour cells), 3 (tumour center) and 4 (heavy tumour infiltration of the CNS parenchyma) are enlarged in the bottom panels.

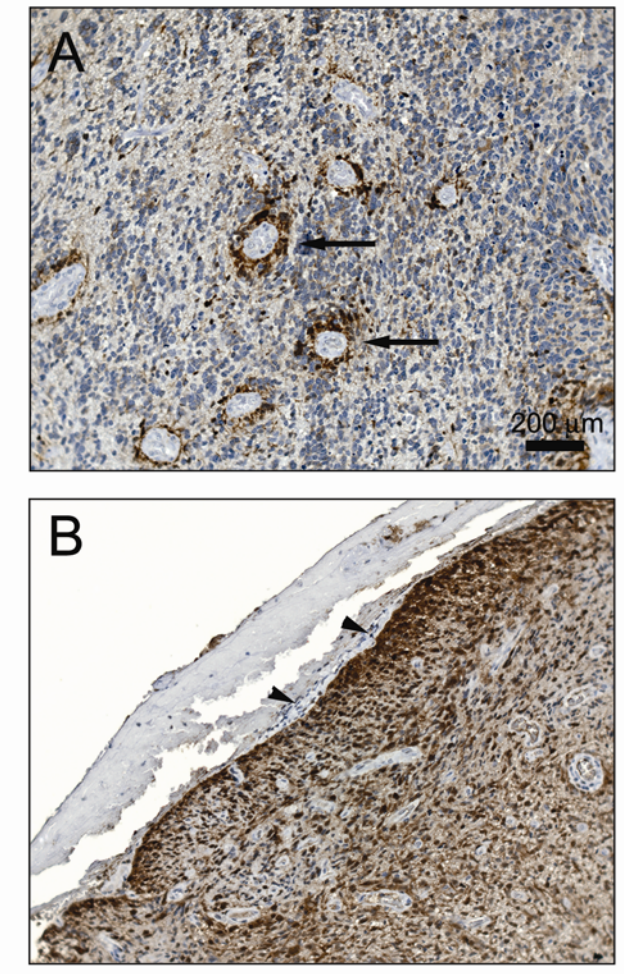


Figure 2.10 FABP7 localizes to blood vessels and pia surface. Tissue sections from patient 1022 (A) and patient 1032 (B) were immunostained with affinity-purified anti-FABP7 antibody and counterstained with hematoxylin. The arrows point to blood vessels surrounded by FABP7-staining cells. The arrowheads point to the pia mater.

2.4. DISCUSSION

Despite recent advances in the treatment of high grade astrocytomas, the prognosis for these patients remains dismal. Current therapies are effective at destroying cells within or near the primary tumour mass; however, we still have no way of identifying and targeting the malignant cells that have migrated away from the main tumour. As a result, patients with high grade astrocytomas consistently relapse, presenting with secondary tumours at sites that are distal from the primary tumour. We need to find ways of targeting malignant cells that infiltrate the brain if we are to make an impact on the treatment of these tumours.

We have previously shown that the radial glial marker FABP7 is expressed in astrocytoma tumours and malignant glioma cell lines (Godbout, Bisgrove et al. 1998). In addition to serving as a scaffold for neuronal migration, radial glial cells can transform into GFAP-expressing astrocytes and can also function as neuronal cell progenitors (Schmechel and Rakic 1979; Anthony, Klein et al. 2004). Radial glial cells proliferate throughout neurogenesis (Hartfuss, Galli et al. 2001) and demonstrate neural stem cell-like properties in embryonic and adult mice brain (Merkle, Tramontin et al. 2004). Two large-scale DNA microarray studies have shown an association between FABP7 expression and astrocytomas. In one study, FABP7 was identified as one of the top 100 genes expressed at significantly higher levels in primary grade IV astrocytomas compared to normal brain tissue (Tso, Shintaku et al. 2006). In the second study,

nuclear FABP7 expression was shown to correlate with a worse prognosis in astrocytoma grade IV patients below the median age (Liang, Diehn et al. 2005). Comparison of FABP7 levels in grade I versus grades II, III, IV astrocytomas, and analysis of cytoplasmic versus nuclear FABP7 levels in grade IV astrocytomas as a function of survival, also support a link between nuclear FABP7 expression and invasion/infiltration (Liang, Bollen et al. 2006; Kaloshi, Mokhtari et al. 2007).

Using two different assays for cell motility, one non-directional (time-lapse video microscopy), the other directional (Transwell chambers), we have observed striking differences in the migration of FABP7-expressing versus FABP7-negative/depleted clonal cell populations, with the former showing much higher motility. These results are consistent with a previous report demonstrating an increase in migration upon transiently transfecting SF767 malignant glioma cells (which express low levels of FABP7) with a FABP7 expression construct (Liang, Diehn et al. 2005). While we also observe increases in cell invasion in FABP7-expressing clones compared to FABP7-negative/depleted clones, this could be due to increased cell motility. Thus, a major consequence of FABP7 expression in malignant glioma cells is enhancement of their motility.

Radial glial cells are traditionally believed to be static with neuronal progeny migrating along radial glial fibers. However, recent evidence suggests that radial glial cells are much more than migratory guides,

generating radial glial daughter cells that migrate away from the parent cells, form extended processes and become neurons (Kriegstein 2005). Furthermore, migration of radial glial cells by somal translocation has been documented in mouse, rat and monkey brain (Schmechel and Rakic 1979; Kakita 2001; Smith, Ohkubo et al. 2006). In keeping with a role for FABP7 in cell migration, FABP7-expressing U87 cells have longer processes than U87 controls. FABP7 expression is accompanied by a distinctive migratory behavior resembling translocation (i.e., extension of the leading process followed by snapping forward of the cell body) as opposed to locomotion (i.e., movement of the entire cell) (Nadarajah, Brunstrom et al. 2001). In contrast, reduction of FABP7 levels in U251 results in fan-like membrane ruffling with almost no extended protrusions. Although membrane ruffling is associated with increased cell motility, it is the specific concentration of membrane ruffling at the ends of lamellipodia-filopodia extensions that has been linked to this process (Rinnerthaler, Geiger et al. 1988). Fan-like structures such as those observed in FABP7-depleted U251 cells do not appear to be associated with cell migration as inhibition of these fan-like structures does not affect cell motility (Heaysman, Pegrum et al. 1982). The reduction in cell migration observed upon FABP7 depletion is in agreement with FABP7 playing a central role in cell migration.

In vitro ligand binding studies show that docosahexanoic acid (DHA) (22:n-6) is the preferred ligand of FABP7 (Xu, Sanchez et al. 1996;

Balendiran, Schnutgen et al. 2000). DHA is the longest and most highly unsaturated fatty acid found in membranes. Highly unsaturated long chain fatty acids can alter the structure and function of membranes, increasing their fluidity, elasticity and permeability, and potentially affecting signal transduction and gene expression (Hashimoto, Hossain et al. 1999; Rojas, Martinez et al. 2003; Stillwell and Wassall 2003). Recent data support a role for DHA in the hyperfluidization of membranes (Valentine and Valentine 2004). Preferential localization of FABP7 at the leading edges of cells may result in increased DHA content at these sites. Increased fluidity may directly promote cell motility or may activate cell signaling events associated with cell motility. Future work will involve characterizing the effect of fatty acid ligands on the growth properties of FABP7-expressing malignant glioma cells.

FABP7 is primarily located in the nucleus of U251 and FABP7-transfected U87 cells, and can be found in both the cytoplasm and nucleus of astrocytoma tumour cells, in keeping with a role in the regulation of gene expression. It has been postulated that FABP-bound fatty acids may serve as ligands for PPARs which then activate the transcription of genes containing PPAR response elements (Wolfrum, Borrmann et al. 2001). PPARs are expressed in malignant glioma cell lines although their relationship to FABP7 and its fatty acid ligands is not known (Kato, Nagaya et al. 2002). A link to PPARs would suggest a direct

role for FABP7 and/or its ligands in the regulation of genes involved in cell migration.

In addition to the nucleus, FABP7 localizes to structures associated with actin cytoskeleton remodeling and cell migration, such as stress fibers and sites of focal adhesions (e.g. leading edges) (Maidment 1997). Proteins involved in cell migration include cadherins, integrins, actin and actin binding proteins, the Rho family of small GTPases, growth factors and receptors (rev. in Christofori, 2006). To identify pathways altered as a consequence of FABP7 expression, we compared genes expressed in U87 control and FABP7-transfected cells. A number of differentially expressed transcripts encoding proteins involved in cell migration and adhesion were identified by cDNA microarray analysis, including cadherins, laminin, tensin and integrin β4 (our unpublished data). Based on these preliminary results, we postulate that the mechanism underlying FABP7-enhanced cell migration will likely be through the modulation of cell adhesion properties.

High grade astrocytomas are highly invasive within the brain itself; however, metastasis outside the brain is rare. This has been attributed to the short life expectancy of the patient and/or the blood/brain barrier. However, the blood/brain barrier is disrupted in nearly all patients with grade IV astrocytomas (Schneider, Ludwig et al. 2004). Moreover, the majority of high grade astrocytoma patients undergo surgery followed by radiation treatment, both risk factors for metastasis (Hou, Veeravagu et al.

2006). Astrocytoma tumour cells have been shown to migrate along white matter tracks, encircle neurons and blood vessels and pile up at the sub-pial surface of the brain (Peiffer and Kleihues 1999; Holland 2000). The FABP7-positive cells observed in highly infiltrative regions of grade IV astrocytomas mimic the previously reported migratory behavior of astrocytoma cells, with FABP7-positive cells encircling blood vessels and accumulating near the pia mater. Of note, these features (i.e. high motility, association with blood vessels and white matter tracts) are also properties of neural stem cells and progenitor cells (Holland 2001).

Migrating FABP7-positive tumour cells may not be particularly tumourigenic, as suggested by the decreased proliferation rate and reduced anchorage-independent growth of FABP7-expressing malignant glioma cells. This is consistent with reports indicating that the expression of FABPs such as I-FABP, L-FABP and A-FABP is reduced with increasing malignancy, and that FABP expression is generally associated with increased differentiation (Zimmerman and Veerkamp 2002). The enhanced motility associated with FABP7-positive tumour cells may thus represent a property associated with the cell-of-origin of the tumour rather than a consequence of the malignant process. An intriguing observation is the presence of FABP7 in gemistocytic astrocytes in grade II astrocytomas. Although gemistocytes have a low proliferative index, their presence in low-grade astrocytomas is an indication of poor prognosis as these tumours rapidly progress to high-grade astrocytomas (Watanabe,

Tachibana et al. 1997; Yang, Kim et al. 2003). FABP7-expressing gemistocytes may therefore represent pre-cancerous cells that have the potential of developing into more aggressive cancer cells. Thus, FABP7-positive gemistocytic astrocytes in low-grade astrocytomas, FABP7-positive infiltrating tumour cells in high-grade astrocytomas, and FABP7-negative proliferating cells in the tumour centre, may represent cells at different stages in the tumourigenic process, each carrying progressively more mutations. Loss of FABP7 expression may enhance the tumourigenic phenotype by decreasing cell migration, allowing tumour cells to accumulate at one site.

In conclusion, we have used complementary approaches to demonstrate that FABP7 expression in malignant glioma cells increases migratory activity. Changes in migratory activity are accompanied by morphological alterations: longer processes in FABP7-expressing U87 cells and extensive membrane ruffling in the case of FABP7-depleted U251 cells, suggesting a role for FABP7 in altering membrane properties. Based on transfection experiments and immunohistochemical analysis of astrocytoma tumours, we propose that the spread of astrocytoma cells within the brain is through migration of FABP7-expressing tumour cells that originate from radial glial cells. Manipulation of FABP7 levels in malignant glioma cells has a dramatic effect on their migratory and growth properties. A better understanding of FABP7-expressing cells may lead to novel approaches for the treatment of both low- and high-grade

astrocytomas, perhaps through manipulation of FABP7 or its ligand DHA. FABP7 may also serve as a useful prognostic marker for astrocytomas, particularly as related to the prediction of tumour spread and recurrence within the brain.

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Chapter 3: Brain Lipid Binding Protein: Mediating the Effects of PUFAs on Malignant Glioma Cell Migration

3.1 INTRODUCTION

Anaplastic astrocytoma (grade III astrocytoma) and glioblastoma multiforme (GBM; grade IV astrocytoma), collectively called malignant gliomas (MG), are the most common cancers of the central nervous system (CNS). The prognosis for these cancers is dismal, with median survival times of 30 months for grade III astrocytoma (Lin, Lieu et al. 2003) and 20.4-40.9 weeks for grade IV astrocytoma (Ohgaki and Kleihues 2005; Deorah, Lynch et al. 2006). Standard treatment for high grade astrocytomas includes surgical resection, radiation therapy and adjuvant chemotherapy involving nitrosourea-based compounds and temozolamide (Mason, Maestro et al. 2007). As a consequence of their highly infiltrative nature, these tumours recur in the brain at sites that can be either proximal or distal to the original tumour mass (Giese, Bjerkvig et al. 2003). Thus, improving patient survival may depend on developing new therapies that target the infiltrative cells while minimizing damage to normal brain tissue.

We have shown that brain fatty acid binding protein (FABP7; B-FABP; BLBP) is expressed in astrocytoma tumour biopsies as well as in a subset of MG cell lines (Godbout, Bisgrove et al. 1998). Furthermore, FABP7 levels are up-regulated in tumour tissue compared to normal adult brain (Liang, Diehn et al. 2005; Tso, Freije et al. 2006). Immunohistochemical analysis of grade IV astrocytomas reveals FABP7 expression in regions of the tumour typically associated with infiltration;

i.e., in the subpial region of the brain, along white matter tracts and surrounding blood vessels (Mita, Coles et al. 2007). Furthermore, introduction of FABP7 into FABP7-negative MG cells confers a pro-migratory/pro-invasive phenotype to the cells, whereas knock-down of FABP7 in MG cells that naturally express FABP7 results in reduced migration/invasion (Liang, Diehn et al. 2005; Mita, Coles et al. 2007). These results, combined with studies demonstrating decreased survival times in FABP7-positive grade IV astrocytoma patients (Liang, Diehn et al. 2005; Kaloshi, Mokhtari et al. 2007), suggest that FABP7 may enhance the infiltrative properties of tumour cells. Importantly, FABP7 has also been associated with the progression of malignant melanoma, basal-type breast cancer and renal cell carcinoma (Seliger, Lichtenfels et al. 2005; Slipicevic, Jorgensen et al. 2008; Goto, Koyanagi et al. 2009; Zhang, Rakha et al. 2009).

FABP7 is a member of the FABP family involved in the uptake and intracellular trafficking of fatty acids. *In vitro* ligand binding studies have shown that the polyunsaturated fatty acid (PUFA), ω-3 docosahexaenoic acid (DHA; 22:6) is the preferred ligand of FABP7. FABP7 also binds ω-6 arachidonic acid (AA; 20:4) with a ~4-fold lower affinity (Xu, Sanchez et al. 1996; Balendiran, Schnutgen et al. 2000; Zimmerman, van Moerkerk et al. 2001). ω-3 and ω-6 fatty acids typically exhibit opposite effects on tumour growth, with ω-3 fatty acids inhibiting (Larsson, Kumlin et al. 2004) and ω-6 stimulating (McDonough, Tran et al. 1998) growth. Growth inhibition by

DHA has been shown to be mediated by blocking STAT1 signaling (Altenburg and Siddiqui 2009) and inducing β-catenin degradation (Lim, Han et al. 2009). In contrast, AA stimulates growth when it is converted to eicosanoids, by cyclooxygenases and lipoxygenases (Ford-Hutchinson 1994; Smith, Garavito et al. 1996; Funk 2001; Turini and DuBois 2002; Soberman and Christmas 2003). Eicosanoids are lipid mediators that stimulate cell proliferation and promote cell survival and migration by activating factors such as VEGF and EGFR (Romano, Catalano et al. 2001; Akbulut, Regner et al. 2009).

Comparison of the fatty acid composition of MG tumour tissue versus normal brain indicates significantly higher levels of AA and its precursor linoleic acid (LA), and lower levels of DHA, in tumour tissue (Ledwozyw and Lutnicki 1992; Martin, Robbins et al. 1996; Kokoglu, Tuter et al. 1998). While these data suggest that fatty acids are metabolized differently in cancer cells compared to normal cells, the molecular mechanisms underlying these differences and a possible role for FABP7 in these processes have not been investigated.

There is indirect evidence supporting a role for FABPs and their fatty acid ligands in the regulation of gene expression. First, most FABPs are found in both the nucleus and cytoplasm. Second, FABPs are known to activate peroxisome proliferator-activated receptors (PPARs), nuclear receptors which function as transcription factors. Specifically, liver FABP and adipocyte FABP can bind and activate PPARα and PPARγ,

respectively, (Helledie, Antonius et al. 2000; Wolfrum, Borrmann et al. 2001; Helledie, Jorgensen et al. 2002; Tan, Shaw et al. 2002). Thus, it has been postulated that nuclear FABPs can deliver their fatty acid ligands to PPARs, thereby regulating PPAR transcriptional activity. Increased expression of PPARα is associated with a worse prognosis in MG (Benedetti, Galzio et al. 2010), whereas PPARγ is generally associated with growth arrest and apoptosis in these tumours (Chearwae and Bright 2008; Papi, Tatenhorst et al. 2009)

Although links have been described between specific fatty acids and FABPs, FABP7 and MG, fatty acids and MG, PPARs and MG, there has been no concerted effort to investigate the interrelationships between fatty acids, FABPs and PPARs in MG. Here, we examine the effect of DHA and AA on FABP7-mediated cell migration in MG. We describe an inhibitory role for DHA in FABP7-mediated cell migration and a permissive role for AA in FABP7-mediated cell migration. Intriguingly, DHA-mediated inhibition of cell migration requires nuclear localization of FABP7 whereas stimulation of cell migration by FABP7 appears to depend on AA-mediated activation of cyclooxygenase 2 (COX-2) and PGE_2 production. We propose a model whereby relative levels of DHA and AA, and the subcellular distribution of FABP7, determine the migratory potential of MG cells. The importance of our findings resonates 2-fold as they provide a molecular mechanism for FABP7-induced MG cell migration and point to

the potential use of DHA as an anti-infiltrative therapeutic agent in the treatment of MG.

3.2 MATERIALS AND METHODS

3.2.1 Cell lines, culture conditions, generation of stable and transient transfectants

The human MG cell line U87 was obtained from Dr. Jorgen Fogh, and has been previously characterized for FABP7 expression (Godbout, Bisgrove et al. 1998). Unless otherwise stated, cells were grown in Dulbecco's Modified Essential Medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS), 100 μg/ml streptomycin and 100 U/ml penicillin. Generation of U87 clonal populations stably transfected with empty vector [FABP7(-)] or FABP7 expression construct [FABP7(+)] have been previously described (Mita, Coles et al. 2007). Transient transfection of U87 cells was by calcium phosphate-DNA precipitation, with 40-50% transfection efficiency. Cells were examined 48 h after removal of the DNA.

3.2.2 Western blot analysis

Whole cell lysates were prepared by incubating cells on ice for 20 min in 0.5 M Tris-HCl pH 7.5, 0.15 M NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mM NaF, 0.1% SDS, 1X protease inhibitor cocktail (Roche), 1 mM PMSF, 2 mM DTT. Lysates were centrifuged at 14,000 x g for 15 min, and the supernatant collected. Protein concentration was measured using the Bradford assay. Nuclear cell extracts were prepared by lysing cells at 4^{o}C in 10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM

EDTA, 0.4% NP40, 1 mM DTT, 0.5 mM PMSF. Nuclei were collected by centrifugation (15,000 X g at 4ºC for 3 min) and lysed by vigorous shaking in 20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 10% glycerol, 1 mM DTT, 0.5 mM PMSF. Nuclear lysates were centrifuged at 14,000 X g for 5 min at 4^{o}C and the supernatants collected. Lysates were separated by electrophoresis in a 13.5% SDS-polyacrylamide gel followed by electroblotting to nitrocellulose. Membranes were immunostained with rabbit anti-FABP7 antibody (Godbout et al., 1998), mouse anti-HA (Santa Cruz), goat anti-COX-2 (sc-1747; Santa Cruz), mouse anti-PPARα (Chemicon), mouse anti-PPARβ (Chemicon), mouse anti-PPARγ (Chemicon) antibodies. Primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Biotech) using the ECL detection system (GE Healthcare). The signal was quantitated using Adobe Photoshop CS2.

3.2.3 Preparation of fatty acids and COX-2 inhibitor NS398

Fatty acids (Sigma) were dissolved in ethanol, then complexed to fatty acid-free BSA (Roche) over a steady stream of nitrogen gas. The final concentration of ethanol in our experiments was 0.1%. NS398 (Sigma) was dissolved in DMSO (Sigma) to generate a 200 mM stock solution. The final concentration of DMSO in the medium was 0.1%.

3.2.4 Cell migration

Twenty thousand cells were plated in DMEM (without FCS) in the top chamber of HTS multiwell inserts with a porous 8 μM PET membrane separating the top and bottom chambers (Transwell culture dishes; BD Biosciences). Cells were incubated for 6 h in a CO_2 incubator to allow cell migration to the bottom chamber containing chemoattractant (DMEM plus 10% FCS). Cells that migrated to the bottom chamber were fixed with ice-cold 100% methanol for 2 min and stained with 2% crystal violet in 20% methanol for 5 min. The cells were photographed using a 2.5X lens and four frames combined to reconstruct the entire well. Cells were then counted using Metamorph version 7.7 imaging software.

3.2.5 Immunofluorescence

Cells were fixed with 1% paraformaldehyde for 10 min then permeabilized in PBS + 0.5% Triton X-100 for 5 min. Affinity-purified rabbit anti-FABP7 antibody was used at a 1:200 dilution, followed by secondary Alexa 488-conjugated goat anti-rabbit secondary antibody (Cedarlane Laboratories). All images were collected on a Zeiss LSM 510 confocal microscope with a 40X/1.3 oil immersion lens.

3.2.6 Site-directed mutagenesis

The 400 bp human FABP7 cDNA encoding the entire open reading frame was inserted into the pcDNA3.1 vector at the EcoRV site. To generate the nuclear localization signal (NLS) mutant, site directed

mutagenesis of FABP7 at K21A (AAG -> GCG), R30A (AGG -> GCG), and Q31A (CAG -> GCG) was carried out by sequential PCR (Cormack and Castano 2002) using partially complementary primers carrying the appropriate point mutations. These primers were used in conjunction with pcDNA3.1 vector primers to generate DNA fragments corresponding to full length FABP7. To generate the non-fatty acid binding (FAB) mutant, the same technique was applied, using primers carrying the following point mutations: F104A (TTT -> AGA), R126A (CGC -> GCC), and Y128A (TAT -> GCT). These primers, which contained an Xho1 recognition site were used along with primers to the pcDNA3.1 vector sequence spanning the BamH1 site to allow directional cloning into pcDNA3.1. The FABP7 cDNA inserts were sequenced to ensure that they were error-free. Expression of mutant FABP7 was confirmed by transfection into U87MG and western blot analysis.

3.2.7 Delipidation of FABP7 and fatty-acid binding assay

Lipidex® 1000 (Sigma) was used to delipidate recombinant FABP7 and carry out the fatty acid binding assays. Lipidex 1000 effectively removes unbound and/or protein-bound fatty acids from aqueous solutions in a temperature-dependent manner. For delipidation, recombinant GST-tagged FABP7 was incubated in 1.0-ml amber glass vials along with 50% w/v Lipidex in 10 mM potassium phosphate buffer (pH 7.4) for 10 min at 37^{o}C. The Lipidex/protein mixture was then spun down for 2 min at 4,000

x g, and the incubation repeated twice using fresh Lipidex-conjugated beads.

For fatty acid binding assays, delipidated FABP7 was incubated in 10 mM potassium phosphate buffer (pH 7.4) with varying concentrations of [1-^{14}C]-DHA (Perkin Elmer) and incubated at 37^{o}C for 10 min. Unbound fatty acids were removed from the solution by adding 50 µl of an ice-cold Lipidex-buffer suspension (1:1, w/v) followed by 10 min incubation at 4^{o}C. The mixture was then centrifuged for 5 min at 4,000 x g and fatty acid binding calculated from the amount of radioactivity present in the supernatant as measured by a scintillation counter. Graphs, K_d, and B_{max} values were generated using Graphpad Prism software version 5. B_{max} value, provided by Graphpad in counts per minute (cpm) was converted to pmol/µg assuming 100% efficiency (1 cpm = 1 dpm) and using Equation 1, where $B_{max'}$ is the Graphpad provided reading, *M* is the dpm per µCi constant (2.22 x 10^6 dpm / µCi), *P* is the amount of µCi per mmol ^{14}C-DHA (5.6 x 10^4 µCi / mmol; Moravek Biochemicals) and N is the amount of protein used for assay (5 µg).

$$Bmax = Bmax' M^{-1}P^{-1} / N \qquad \text{(Eq. 1)}$$

The free energy change of binding was calculated as shown by Equation 2, where *R* is the gas constant (8.31 J/K mol) and *T* is absolute temperatue (310.15 K).

$$\Delta G = RT \ln K_d^{-1} \qquad \text{(Eq. 2)}$$

3.2.8 Knockdown of PPARγ and PPARβ by siRNA

Cells were transfected with 10 nM Stealth siRNAs (Invitrogen) using Lipofectamine™ RNAiMAX (Invitrogen). The following siRNAs were used: to PPARγ [5'-CCAGUGGUUGCAGAUUACAAGUAUG-3' (NM_13872_stealth_425) and 5'-AGGGAGUUUCUAAAGAGCCUGCGAA-3' (NM_13772_stealth_1295)]; to PPARβ [5'-CCACUACGGUGUUCAUGCAUGUGAG-3' (NM_006238_stealth_561) and 5'-UCAGUGAUAUCAUUGAGCCUAAGUU-3' (NM_006238_stealth_1280)]; and scrambled siRNA (Stealth™ RNAi Negative Control Medium GC Duplex). The Transwell migration assay was carried out 72 h post-transfection using 20,000 cells/well. The remaining cells were used for the preparation of cell lysates and used for western blot analysis to verify knockdown.

3.2.9 Enzyme-linked immunosorbent assay (ELISA)

PGE_2 levels were measured by ELISA using the protocol supplied by the manufacturer (Amersham Prostaglandin E_2 Biotrak Enzymeimmunoassay System, GE Healthcare). Cells were seeded in triplicate on 96-well plates at 50,000 cells/well and cultured for 24 hours. Cells were then lysed and PGE_2 was quantified using manufacturer's protocol 8.4. One hundred µl of 1 M H_2SO_4 was added to each well

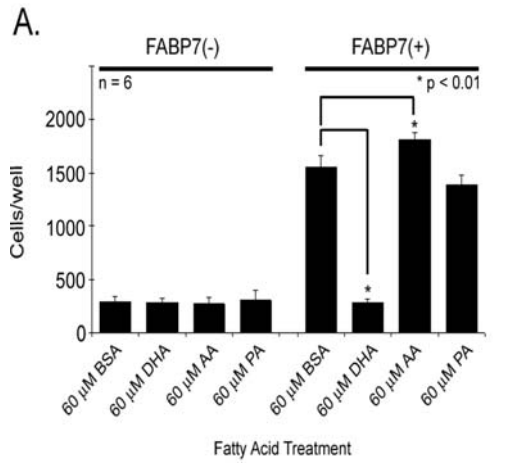
following substrate incubation and the plates were read at 450 nm using a microplate reader (FLUOstar OPTIMA, BMG Labtechnologies).

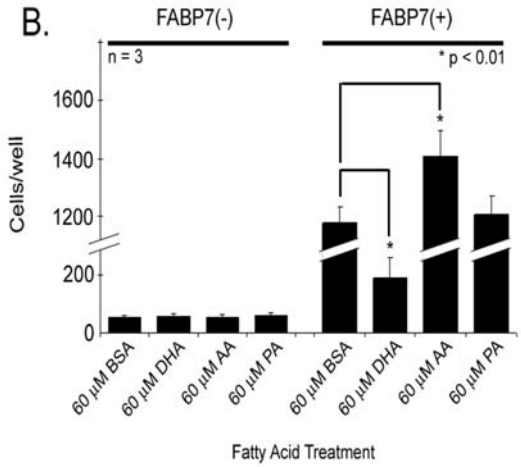
3.3. RESULTS

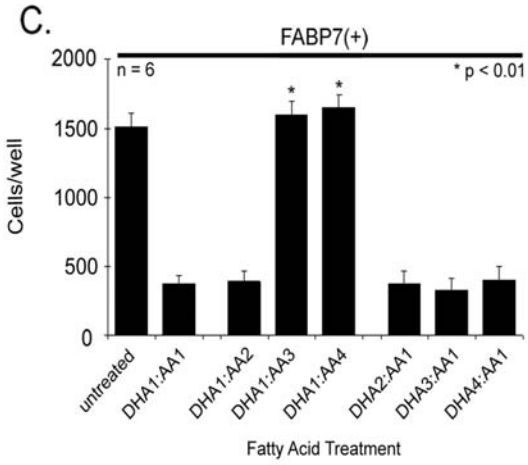
3.3.1 FABP7-induced malignant glioma cell migration is fatty acid ligand-dependent

Previous work has demonstrated that expression of FABP7 in U87 MG cells increases their motility and their ability to migrate towards a chemoattractant (Mita, Coles et al. 2007). *In vitro* ligand binding studies indicate that FABP7 binds both ω-3 DHA and ω-6 AA fatty acids, with equilibrium dissociation constants (K_d) of 53.4 nM and 207 nM, respectively (Balendiran, Schnutgen et al. 2000). We therefore used the Transwell assay to examine the effect of DHA and AA on the migration of FABP7(-) and FABP7(+) U87 cells. Growth of FABP7(-) U87 cells in the presence of 60 μM BSA-complexed DHA, AA, palmitic acid (PA; 16:0), or BSA alone for 24 hours had no effect on cell migration, with an average of ~250 cells migrating through the upper chamber of Transwell inserts (Figure 3.1A). As expected, FABP7-expressing U87 cells cultured in 10% FCS supplemented with BSA showed increased migration compared to FABP7(-) U87 cells (1553 versus 294 cells/well). Growth in PA had no effect on cell migration; however, DHA resulted in a 5-fold decrease in cell migration, with an average of 279 cells migrating to the bottom chamber compared to 1553 cells in the BSA control ($p < 0.01$) (Figure 3.1A). In contrast, cells cultured in AA showed a small but significant increase in cell migration, with 1812 cells/well migrating to the bottom chamber

Figure 3.1 Cell migration of U87-FABP7(-) and U87-FABP7(+) cells in the presence of FAs. (A) Cells cultured in DMEM supplemented with 10% FCS were treated with 60 μM BSA, 60 μM DHA, 60 μM AA or 60 μM palmitic acid (PA) for 24 h. (B) Cells were serum-starved for 24 h then treated with either BSA or the indicated fatty acids for 24 h. (C) Cells were cultured in DMEM supplemented with 10% FCS, and treated with the different ratios of DHA and AA: 1 = 30 μM, 2 = 60 μM, 3 = 90 μM, 4 = 120 μM). (A, B, C) 20,000 cells (in DMEM) were plated in the upper chambers of Transwell inserts. The bottom chambers contained DMEM plus 10% FCS. After 6 h, the cells migrating through the porous membrane were fixed, stained and counted using Metamorph imaging software. Statistical significance was determined using the unpaired t-test. Error bars represent standard deviation.


A.
FABP7(-)
FABP7(+)
n = 6
* p < 0.01
Cells/well
2000
1500
1000
500
0
60 µM BSA
60 µM DHA
60 µM AA
60 µM PA
60 µM BSA
60 µM DHA
60 µM AA
60 µM PA
Fatty Acid Treatment


B.
FABP7(-)
FABP7(+)
n = 3
* p < 0.01
Cells/well
1600
1400
1200
200
0
60 µM BSA
60 µM DHA
60 µM AA
60 µM PA
60 µM BSA
60 µM DHA
60 µM AA
60 µM PA
Fatty Acid Treatment


C.
FABP7(+)
n = 6
* p < 0.01
Cells/well
2000
1500
1000
500
0
untreated
DHA1:AA1
DHA1:AA2
DHA1:AA3
DHA1:AA4
DHA2:AA1
DHA3:AA1
DHA4:AA1
Fatty Acid Treatment

compared to 1553 in the BSA control ($p < 0.01$). These results indicate a strong link between FABP7-mediated cell migration and specific PUFAs.

Fetal calf serum (FCS) contains >20 different fatty acids including DHA and AA at concentrations of 4 μM and 11.5 μM, respectively (Dr. Catherine Field, personal communication). To study the effects of DHA and AA on migration in the absence of FCS, U87-FABP7(-) and U87-FABP7(+) MG cells were cultured in DMEM without FCS for 24 h, then treated with DHA, AA, PA or BSA in the absence of FCS for 24 hours (i.e. 48 hours total serum starvation). Although there was a 30% decrease in the number of serum-starved BSA-treated U87-FABP7(+) cells that migrated to the bottom chamber compared to cells cultured in 10% FCS (Figure 3.1B), the overall trend was similar to that observed for cells grown in the presence of FCS, with DHA inhibiting and AA stimulating cell migration. There was an average of 1202 cells/well for the BSA control compared to 199 cells/well for DHA-treated cells and 1446 cells/well for AA-treated cells. Again, PA treatment did not affect cell migration and none of the fatty acids affected the migration of U87-FABP7(-) cells. These results indicate that PUFAs DHA and AA can regulate FABP7-mediated U87 cell migration.

3.3.2 The ratio of DHA:AA determines the migration of FABP7(+) U87 MG cells

A number of studies have shown that ω-3 fatty acids have anti-tumourigenic properties, inhibiting tumour growth and preventing vascularizaton and cell migration. In contrast, ω-6 fatty acids have pro-tumourigenic properties. It has been postulated that the ratio of ω-3 to ω-6 fatty acids is key to the pathogenesis of many diseases, including cancer (Simopoulos 2009). To address whether the ratio of DHA:AA, as opposed to individual concentrations of DHA or AA, is driving the effects observed in Figures 3.1A and 3.1B, we treated U87-FABP7(+) cells with varying concentrations of both ω-3 DHA and ω-6 AA, and measured their migration using the Transwell assay (Figure 3.1C).

The migration of U87-FABP7(+) cells cultured in DMEM plus 10% FCS supplemented with 60 μM each of DHA and AA was inhibited by 75%, with an average of 371 cells/well migrating to the bottom chamber compared to 1513 cells/well in the untreated control. Cell migration was also inhibited by 74% when the concentration of DHA was halved (30 μM DHA; 60 μM AA). However, when levels of AA exceeded those of DHA by 3-fold or more, the inhibitory effects of DHA on cell migration were negated, with 1596 cells/well migrating to the bottom chamber when the DHA:AA ratio was 1:3 (30 μM DHA; 90 μM AA), and 1646 cells/well migrating to the bottom chamber with a DHA:AA ratio of 1:4 (30 μM DHA; 120 μM AA). These results are in agreement with *in vitro* data indicating that DHA has a 4X greater binding affinity for FABP7 than AA (Balendiran, Schnutgen et al. 2000). Increasing the concentration of DHA, from 60 to

90 to 120 μM, in the presence of 30 μM AA, did not result in further inhibition of cell migration (Figure 3.1C). PA at concentrations ranging from 30 μM to 120 μM had no effect on cell migration (Figure 3.2). Based on cellular morphology and growth rate, high concentrations of fatty acids had no adverse effect on the survival of FABP7(+) U87 cells. These combined data suggest that different pathways are activated when FABP7 is bound to DHA or AA, with DHA inhibiting and AA stimulating cell migration.

3.3.3 Subcellular localization of FABP7 requires binding to PUFAs

FABPs play an important role in the transport of their fatty acid ligands to various subcellular compartments, including cell membranes (for incorporation into the phospholipids of plasma membranes and endoplasmic reticulum), mitochondria (for energy production), and nucleus (for use as secondary messengers). FABP7 is found in both the cytoplasm and nucleus of MG cells, with some tumours preferentially expressing FABP7 in either the nucleus or cytoplasm (Liang, Diehn et al. 2005; Kaloshi, Mokhtari et al. 2007). To investigate whether the subcellular localization of FABP7 might be dependent on availability of specific fatty acid ligands, we examined the subcellular distribution of FABP7 in U87-FABP7(+) and U251, a MG cell line that endogenously expresses FABP7, in the presence and absence of DHA and AA. When cells were cultured in medium depleted of fatty acids (i.e., serum-starved for 24 h), FABP7 was

primarily found in the cytoplasm (Figures 3.3A and 3.3D). Addition of 60 μm DHA to DMEM resulted in FABP7 localization to the nucleus (Figures 3B and 3E), suggesting that DHA-bound FABP7 is sequestered to the nucleus. In contrast, a relatively uniform distribution of FABP7 was observed throughout the cell in the presence of 60 μm AA (Figures 3.3C and 3.3F). Alterations in the subcellular distribution of FABP7 were not accompanied by any changes in the levels of FABP7 protein (Figure 3.4). The dynamic shuttling of FABP7 from the cytoplasm to the nucleus in the presence of DHA (and to a lesser extent AA) suggests a role for FABP7 in delivering its fatty acid ligands to the nucleus.

3.3.4 FAB7-induced cell migration is dependent on a direct FABP7-fatty acid ligand interaction

Based on X-ray crystallography, Balendiran *et al.* (2002) predicted that the amino acid F104 is essential for the formation of π-π interactions between FABP7 and DHA, whereas the guanidinium group of R126 and the hydroxyl group of Y128 were predicted to bind directly to the carboxylate moiety of DHA (Balendiran, Schnutgen et al. 2000). We therefore mutated the FABP7 F104/R126/Y128 residues to A104/A126/A128 ($FABP7_{FAB}$) in order to gain further insight into the importance of fatty acid binding to FABP7-mediated migration in MG cells. $FABP7_{FAB}$ was cloned into the pcDNA3.1 expression vector and transfected into U87-FABP7(-) MG cells. In keeping with a need for fatty

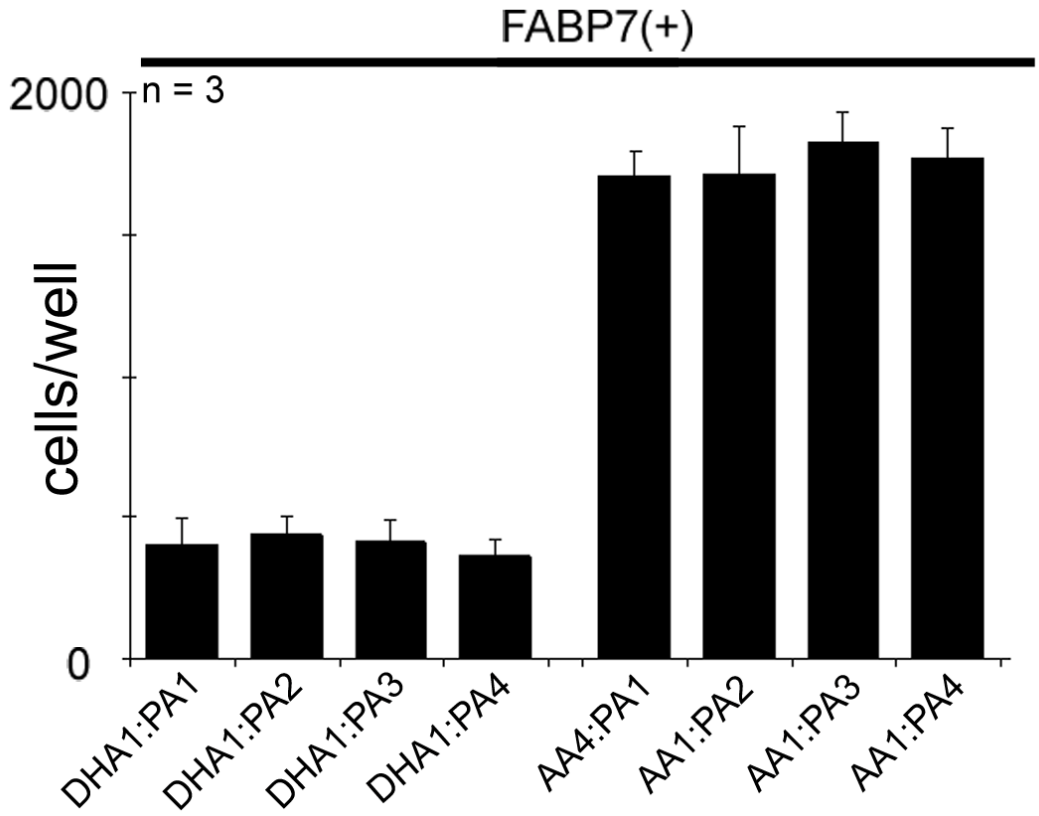
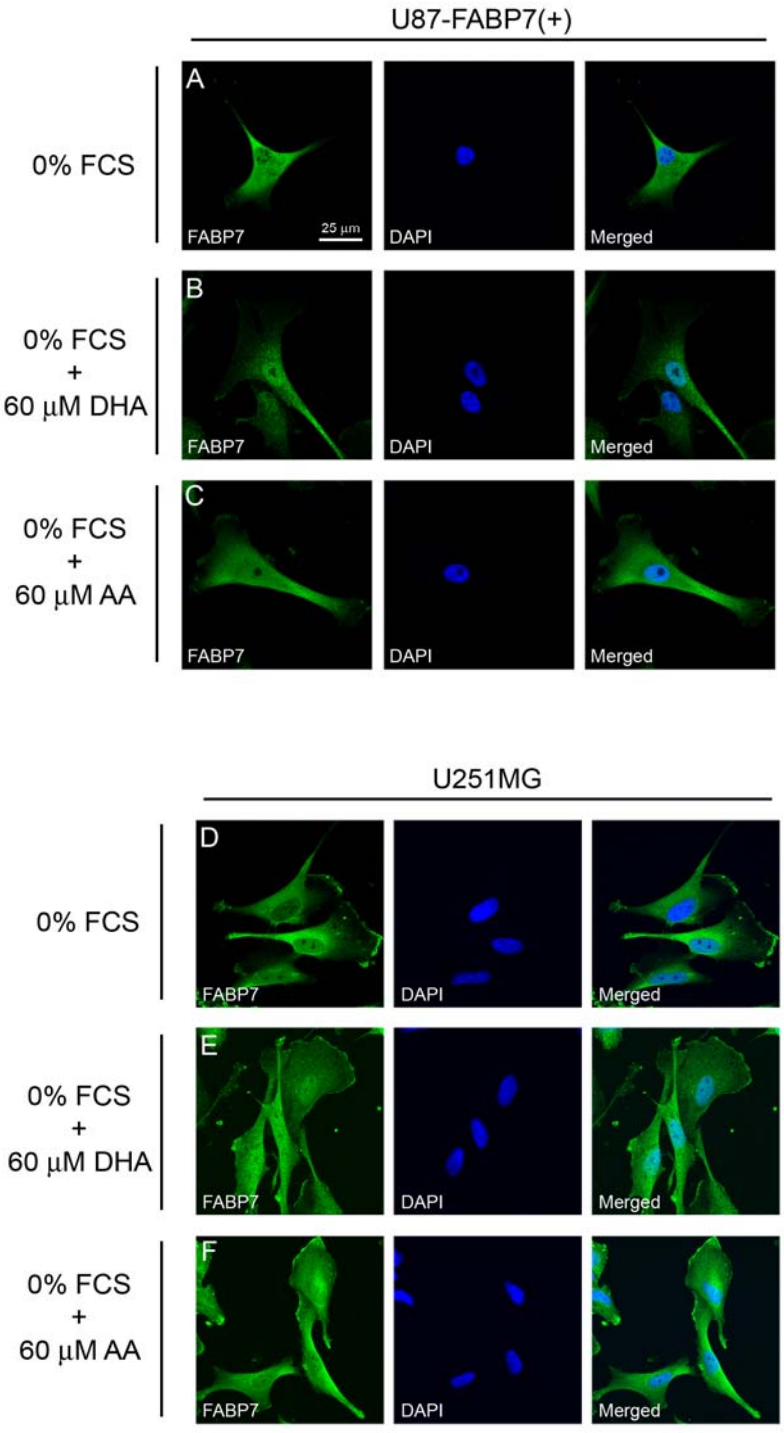


Figure 3.2 Cell migration of U87-FABP7(+) cells treated with varying FA ratios. U87-FABP7(+) cells were grown in DMEM plus 10% FCS and treated with varying fatty acid ratios for 24 h: 1 = 30 μM, 2 = 60 μM, 3 = 90 μM, 4 = 120 μM). Cell migration was measured using the Transwell assay. Twenty thousand cells/well were plated, incubated for 6 h, and the cells migrating through the porous membrane were fixed, stained and counted using Metamorph imaging software. Statistical significance was determined using the unpaired t-test (*$p < 0.01$). Error bars represent standard deviation.

Figure 3.3 PUFA-dependent subcellular localization of FABP7 in U87-FABP7(+) and U251MG cell lines. U87-FABP7(+) and U251MG cells expressing endogenous FABP7 were serum-starved for 24 h (A, D), then were either treated with 60 μM DHA in the absence of FCS for an additional 24 h (B, E), or treated with 60 μM AA in the absence of FCS for 24 h (C, F). The subcellular localization of FABP7 in U87-FABP7(+) and U251MG was analyzed by immunofluorescence using α-FABP7 antibody followed by Alexa-488-conjugated secondary antibody. The DNA was counterstained with DAPI.


U87-FABP7(+)
0% FCS
A
FABP7
25 μm
DAPI
Merged
0% FCS
+
60 μM DHA
B
FABP7
DAPI
Merged
0% FCS
+
60 μM AA
C
FABP7
DAPI
Merged
U251MG
0% FCS
D
FABP7
DAPI
Merged
0% FCS
+
60 μM DHA
E
FABP7
DAPI
Merged
0% FCS
+
60 μM AA
F
FABP7
DAPI
Merged

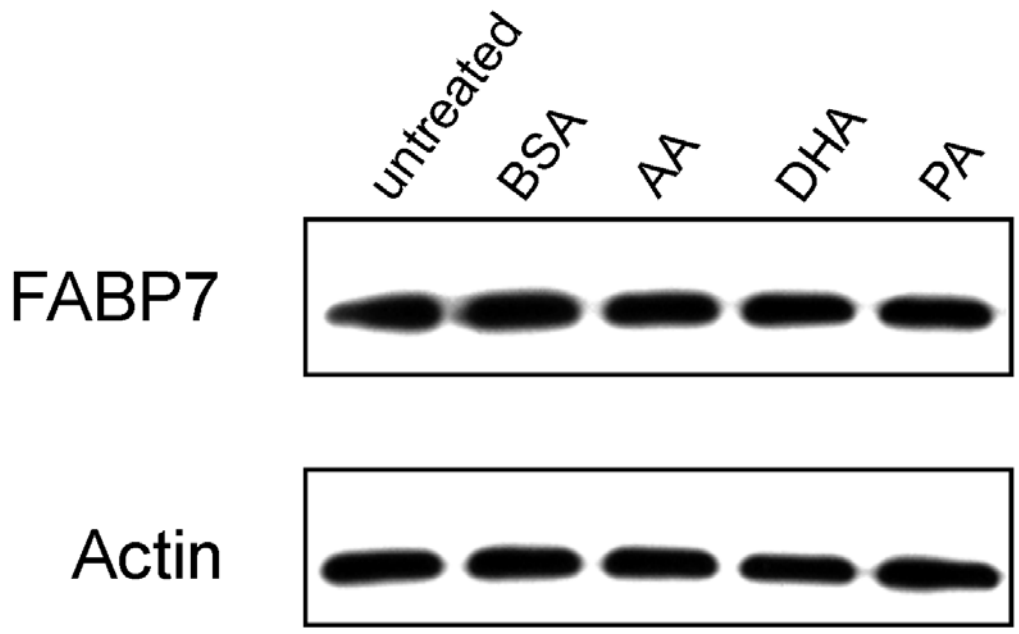


Figure 3.4 Western blot analysis of FABP7 in fatty acid-treated U87-FABP7(+). Whole cell lysates (25 μg/lane) were prepared from U87-FABP7(+) treated with 60μM BSA, AA, DHA, or PA and electrophoresed in a 13.5% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membranes and sequentially immunostained with rabbit anti-FABP7 antibody and mouse anti-actin antibody, followed by HRP-conjugated secondary antibodies.

acid ligands for nuclear FABP7 localization, western blot analysis demonstrated abundant $FABP7_{FAB}$ in the cytoplasm but little mutant protein in the nucleus (Figure 3.5). The abundance of $FABP7_{FAB}$ in the cytoplasm indicates that any structural changes resulting from the site-directed mutagenesis had little or no effect on protein stability. Subcellular localization experiments verified that $FABP7_{FAB}$ mostly resides in the cytoplasm, in contrast to wild-type FABP7 ($FABP7_{WT}$) which is abundant in the nucleus (Figures 3.6A and 3.6B). These findings are in keeping with our previous results indicating that serum (and fatty acid) starvation prevents FABP7 from going to the nucleus.

Next, we assessed the binding of fatty acids to recombinant $FABP7_{FAB}$ using Lipidex® and radiolabeled DHA (Glatz, Baerwaldt et al. 1984). As shown in Figure 3.7, the K_d for binding of $FABP7_{WT}$ to [1-^{14}C]DHA was 35.5 nM, similar to that previously reported (Balendiran, Schnutgen et al. 2000). There was no detectable binding of $FABP7_{FAB}$ to [1-^{14}C]DHA. These results confirm the predictions made from the three-dimensional model of the FABP7/ligand complex and indicate that the three mutated residues are essential for fatty acid binding to FABP7.

To directly test the effect of fatty acid binding on FABP7-mediated MG cell migration, U87 cells were transiently transfected with $FABP7_{WT}$ or $FABP7_{FAB}$ expression constructs and migration assays were conducted 48 h post-transfection. As shown in Figure 3.6C, transient transfection of $FABP7_{WT}$ resulted in a significant increase in migration compared to

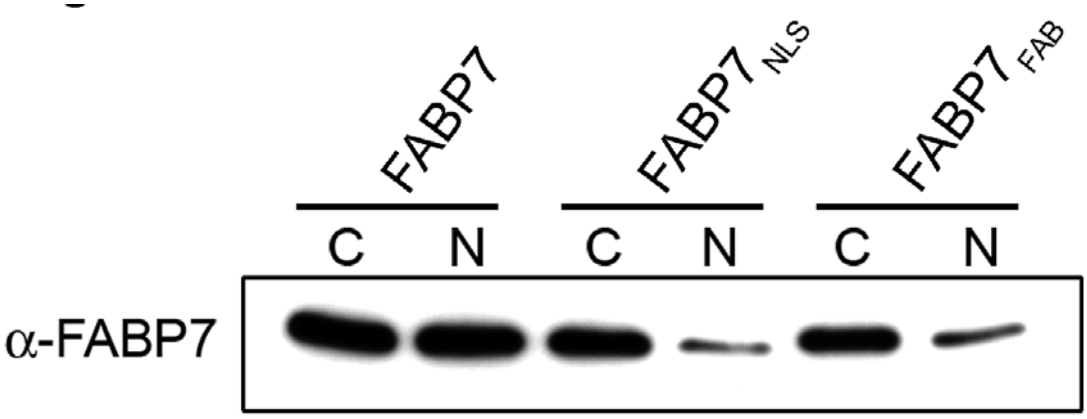


Figure 3.5 FABP7 levels in U87MG cells transfected with wild-type and mutant FABP7 expression constructs. U87MG cells were transfected with pcDNA3.1-FABP7$_{WT,}$ -FABP7$_{NLS}$, and -FABP7$_{FAB}$ expression constructs. Cytoplasmic and nuclear fractions were prepared and 25 µg protein were loaded in each lane. Proteins were electrophoresed in a 13.5% SDS-polyacrylamide gel, transferred to nitrocellulose membranes and immunostained with rabbit α-FABP7 antibody followed by horseradish peroxidase-conjugated secondary antibodies. The signal was detected using ECL reagent (GE Healthcare).

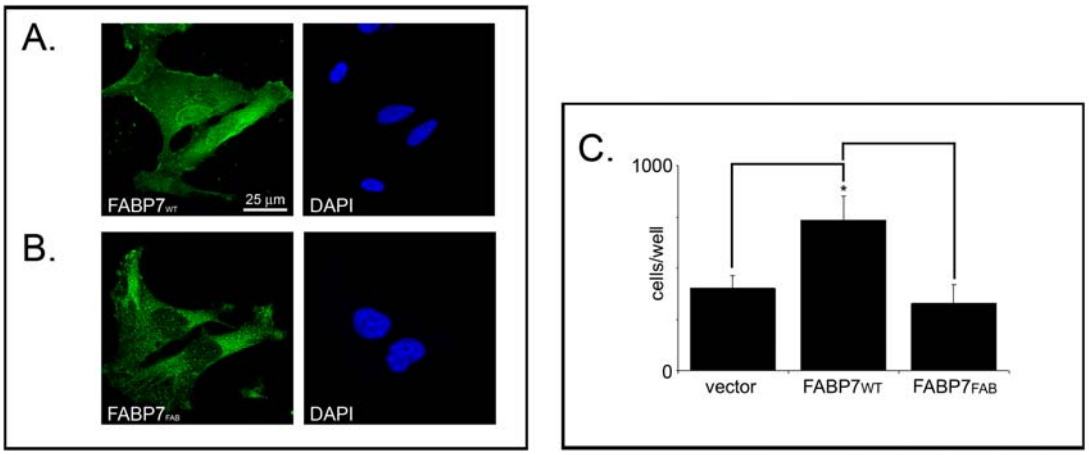
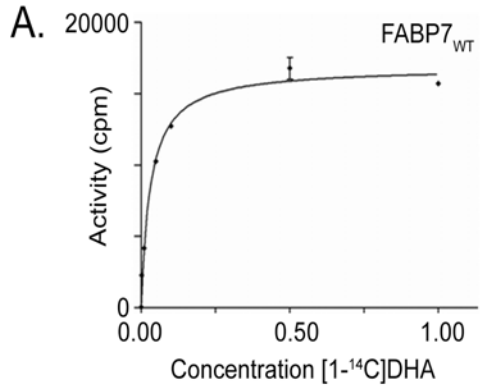
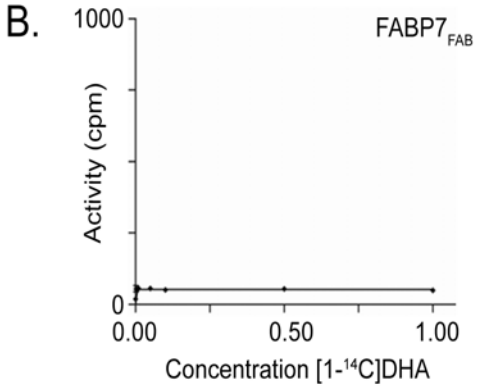
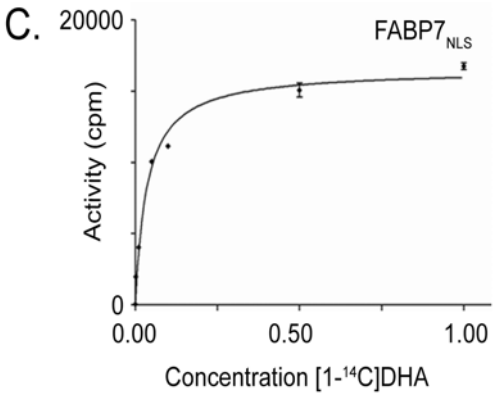


Figure 3.6 Fatty acid binding is required for FABP7-dependent cell migration. Subcellular localization of FABP7 in U87MG cells transiently transfected with pcDNA3.1-FABP7$_{WT}$ (A) or pcDNA3.1-FABP7$_{FAB}$ (B) expression constructs. Transfection efficiency was ~40%. Immunofluorescence was carried out 48 h after transfection using anti-FABP7 antibody followed by Alexa 488-conjugated secondary antibody. DNA was counterstained with DAPI. (C) The migration of U87MG cells transfected with empty vector, pcDNA3.1-FABP7$_{WT}$ and pcDNA3.1-FABP7$_{FAB}$ was measured using the Transwell assay. Twenty thousand cells were plated in the upper chamber and incubated for 6 h. Cells migrating towards the bottom chamber (which contained DMEM plus 10% FCS) were fixed, stained and counted using Metamorph imaging software version 7.7. Statistical significance was determined using the unpaired t-test (n = 3; *p < 0.01). Error bars represent standard deviation.

Figure 3.7 Binding of DHA to $FABP7_{WT}$, $FABP7_{FAB}$, and $FABP7_{NLS}$ proteins. Reactions were carried out in the presence of different concentrations of ^{14}C-labeled DHA ranging from 0.001-5.0 μM (0.001, 0.01, 0.05, 0.1, 0.5, 1, and 5 μM) using 5 μg of recombinant $FABP7_{WT}$ (A), $FABP7_{FAB}$ (B) and $FABP7_{NLS}$ (C). Data points are the mean ± the standard deviation of three experiments.


A.
20000
FABP7WT
Activity (cpm)
0
0.00
0.50
1.00
Concentration [1-14C]DHA


B.
1000
FABP7FAB
Activity (cpm)
0
0.00
0.50
1.00
Concentration [1-14C]DHA


C.
20000
FABP7NLS
Activity (cpm)
0
0.00
0.50
1.00
Concentration [1-14C]DHA

control transfectants, with an average of 736 cells and 400 cells migrating to the bottom chamber, respectively ($p < 0.01$). Cells transiently transfected with the $FAB7_{FAB}$ expression construct showed no increase in cell migration compared to control cells with an average of 329 cells migrating to the lower chamber. These results indicate that FABP7-mediated migration is dependent on FABP7 binding to its fatty acid ligands.

3.3.5 FABP7-induced migration is independent of nuclear localization

In light of the inhibitory effects on migration observed in the presence of DHA, and the nuclear localization of FABP7 in the presence of DHA, we reasoned that the increased migration observed in FABP7-expressing MG cells might be mediated through AA and cytoplasmic FABP7. To address this hypothesis, we generated a mutant FABP7 that could no longer localize to the nucleus. The nuclear localization signals (NLS) of CRABP-II and FABP4 consist of three basic residues situated in the N-terminus helix-loop-helix region (Sessler and Noy 2005; Ayers, Nedrow et al. 2007). The sequence similarity and structural homology of CRABP-II and FABP4 to FABP7 raises the possibility that nuclear import of the latter may also be mediated through an N-terminal NLS, consisting of conserved residues, K21, R30, and K31. A FABP7 mutant with all three residues mutated to alanines ($FABP7_{NLS}$) was generated and cloned into pcDNA3.1. $FABP7_{NLS}$ and $FABP7_{WT}$ were transfected into U87 cells and

protein distribution patterns examined. As shown in Figure 3.5, overall levels of $FABP7_{NLS}$ and $FABP_{WT}$ were similar in transfected cells, although very little $FABP7_{NLS}$ was detected in the nucleus. $FABP7_{NLS}$ was clearly excluded from the nucleus based on immunofluorescence analysis (Figure 3.8A).

As the subcellular localization pattern of the NLS mutant was similar to that of the $FABP7_{FAB}$ mutant, we examined whether the ability of $FABP7_{NLS}$ to bind to fatty acid ligands was compromised. K_d values of 35.5 nM and 30.6 nM were obtained for binding of [1-^{14}C]-DHA to $FABP7_{WT}$ and $FABP7_{NLS}$, respectively (Figure 3.7). The ligand-binding affinity of the NLS mutant is thus similar to that of the wild-type protein, indicating that the mutations did not significantly alter the folding of the protein or hinder its capacity to bind to its fatty acid ligands.

We next examined the effect of the $FABP7_{NLS}$ mutant on cell migration. U87 cells were transiently transfected with $FABP7_{WT}$ or $FABP7_{NLS}$ expression constructs, and cultured in the presence of 10% FCS supplemented with 60 μM BSA (control), DHA or AA. Expression of either wild-type or NLS mutant construct resulted in a significant increase in migration (average of 736 or 687 cells/well) compared to U87 cells transfected with empty vector (average of 400 cells/well). Whereas DHA reduced the migration of $FABP7_{WT}$-transfected cells to that observed in control cells, DHA had no significant effect on the migration of $FABP7_{NLS}$-transfected cells. A small but significant effect was seen on migration

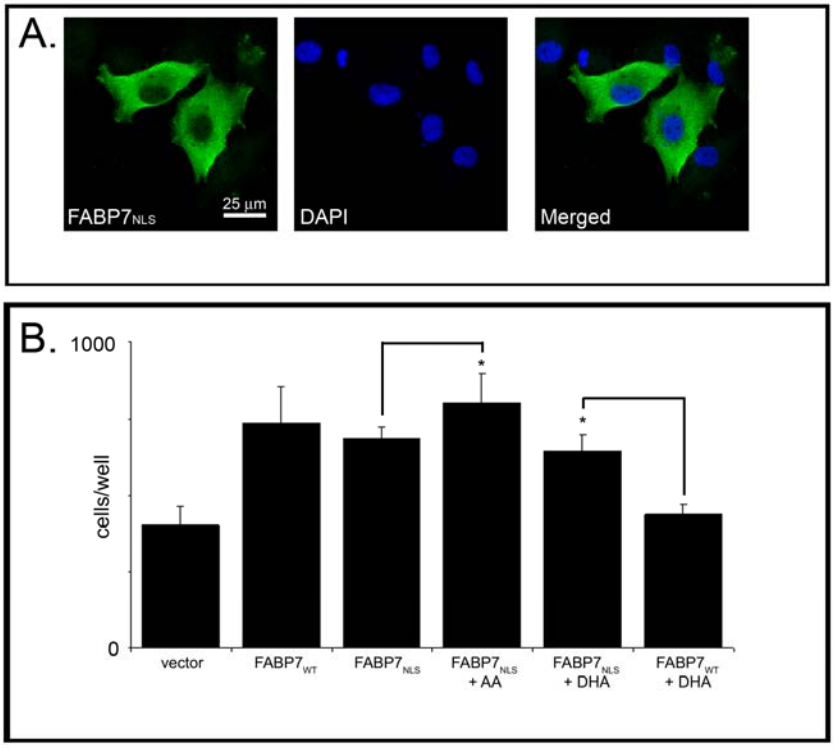


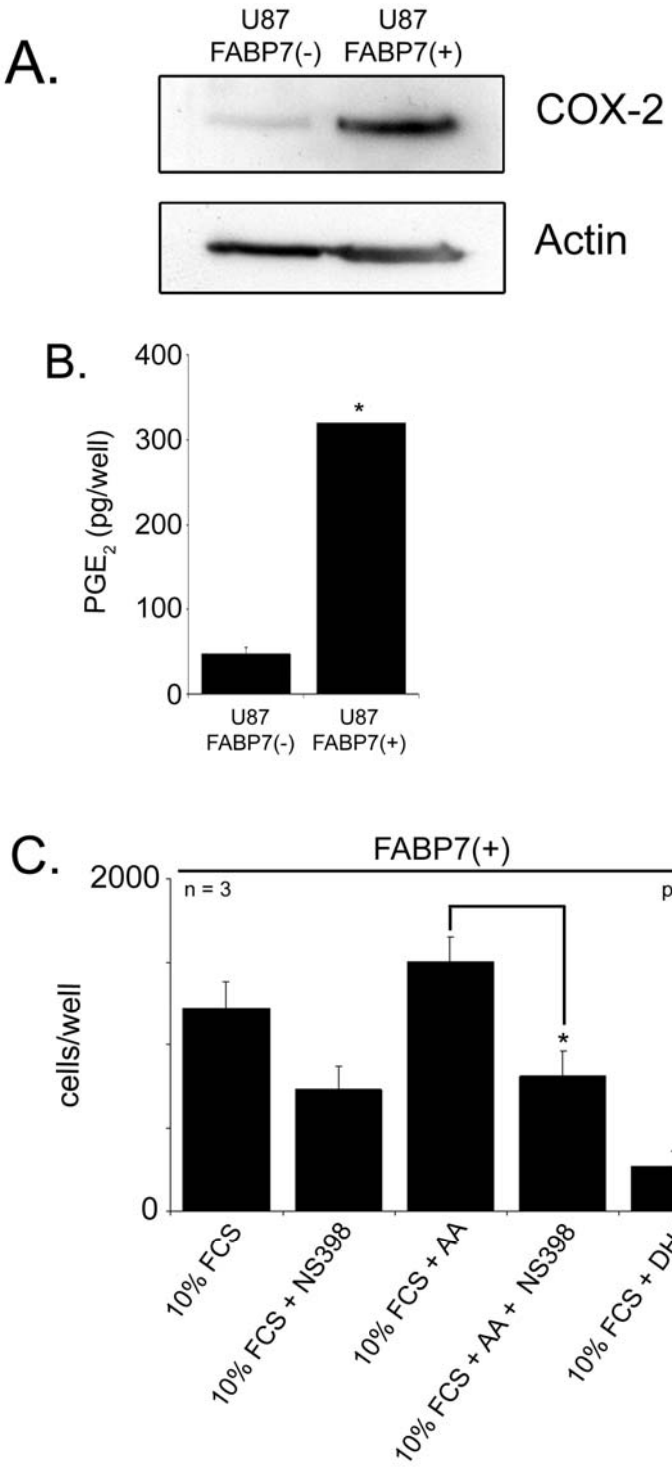
Figure 3.8 FABP7-induced cell migration is independent of nuclear localization. (A) U87 cells were transfected with a pcDNA3.1-$FABP7_{NLS}$ expression construct. $FABP7_{NLS}$ localization was analyzed 48 h post-transfection by immunofluorescence using anti-FABP7 antibody followed by Alexa-488-conjugated secondary antibody. (B) Cell migration of U87-$FABP7_{WT}$ and U87-$FABP7_{NLS}$ cells treated with BSA, 60 µM AA or 60 µM DHA was measured by Transwell®. Twenty thousand cells were plated in the top chamber. Following 6 h incubation, cells migrating through the porous membrane were fixed, stained and counted using Metamorph software. Statistical significance was determined using the unpaired t-test ($n = 6$; $^*p < 0.05$). Error bars represent SD.

when $FABP7_{NLS}$-transfected cells were cultured in the presence of AA, with an average of 802 cells/well ($p < 0.01$). These results suggest that FABP7 localization to the nucleus is not required for FABP7-mediated cell migration, whereas inhibition of migration by DHA is dependent on localization of FABP7 to the nucleus.

3.3.6 Inhibition of COX-2 blocks FABP7-induced cell migration

COX-2 is commonly up-regulated in a variety of cancers, including malignant gliomas (Perdiki, Korkolopoulou et al. 2007). Importantly, the conversion of AA to prostaglandins by COX-2 has been linked to an increase in cell migration (Giese, Hagel et al. 1999). PGE_2 is a primary metabolite of AA and has been shown to function as a mediator of cell migration (Buchanan, Wang et al. 2003). COX-2 protein levels are increased ~5-fold in U87-FABP7(+) compared to U87-FABP7(-) cells (Figure 3.9A). This increase in COX-2 levels is accompanied by a concomitant >6X increase in the prostaglandin E_2 (PGE_2) (Figure 3.9B). To determine whether COX-2 might be involved in the increase in cell migration observed in FABP7-expressing U87 cells, cells cultured in 60 μM AA were treated with the COX-2 inhibitor NS398, and tested for cell migration using the Transwell assay. A significant decrease (46%; $p < 0.01$) in the number of cells migrating to the bottom chamber was observed in the presence of the COX-2 inhibitor (Figure 3.9C). In comparison, there were 5-fold fewer migrating cells when the medium was

Figure 3.9 COX-2 involvement in FABP7-induced cell migration. (A) Western blot analysis of whole cell lysates (25 µg/lane) prepared from U87 cells stably transfected with FABP7 or empty vector. Nitrocellulose membranes were sequentially immunostained with goat anti-COX-2 antibody and mouse anti-actin antibody, followed by horseradish peroxidase-conjugated secondary antibodies. (B) ELISA was used to measure PGE_2 levels. The data were obtained from two independent experiments measured in triplicate. No error bars are shown for U87-FABP7(+) as all the wells were saturated for PGE_2. (C) U87-FABP7(+) stable transfectants were treated 60 µM AA, 60 µM DHA and/or 200µM NS398, as indicated. Cell migration was measured using the Transwell assay. Statistical significance was determined using the unpaired t-test (*$p < 0.01$). Error bars represent standard deviation (SD).


A.
U87 FABP7(-)
U87 FABP7(+)
COX-2
Actin
B.
400
300
200
100
0
PGE2 (pg/well)
*
U87 FABP7(-)
U87 FABP7(+)
C.
FABP7(+)
2000
n = 3
p < 0.01
cells/well
0
*
10% FCS
10% FCS + NS398
10% FCS + AA
10% FCS + AA + NS398
10% FCS + DHA

supplemented with DHA. These results suggest opposing pathways governing cell migration in FABP7-expressing MG cells, with AA/COX-2 increasing migration through cytoplasmic FABP7, and DHA decreasing migration through nuclear FABP7.

3.3.7 Nuclear receptors PPARβ and PPARγ play a role in FABP7-induced cell migration

FABPs have been shown to interact and activate members of the PPAR family in a fatty acid-dependent manner. Using western blotting, we examined the expression of PPARs in U87-FABP7(-) and U87-FABP7(+) cultures. An increase in PPARβ levels were observed upon FABP7 expression (Figure 3.10A). In contrast, a decrease in PPARγ levels accompanied FABP7 expression in U87 cells. No change was observed in PPARα levels. To determine if PPARβ and/or PPARγ might be involved in FABP7-induced cell migration, we used siRNAs to knock-down their levels in U87-FABP7(+) cells. As shown in Figure 3.10B, siRNA transfection resulted in significant decreases in PPARβ and PPARγ levels. All three populations of transfected cells (scrambled siRNA control, PPARβ siRNA and PPARγ siRNA) were cultured in DMEM plus 10% FCS supplemented with BSA, AA or DHA for 24 h, and cell migration examined using the Transwell assay. A significant decrease in migration was observed in cells transfected with PPARβ siRNA compared to cells transfected with scrambled siRNA (1712 versus 1065 cells/well for BSA control; 365

versus 206 cells/well for DHA-treated cells; 2035 versus 843 for AA-treated cells) (Figure 3.10C). No such effect was seen in cells transfected with PPARγ siRNA (1712 versus 1729 cells/well, respectively for BSA control). Intriguingly, there was a partial reversal of DHA inhibition upon PPARγ knock-down (791 cells/well for DHA-treated PPARγ knock-down compared to 365 cells/well in DHA-treated control). Knock-down of PPARγ in the presence of AA had little effect on cell migration (2317 cells/well in control cells treated with AA compared to 2035 cells/well in PPARγ knock-down cells treated with AA). These combined data suggest that the effect of AA on cell migration is not mediated through either PPARβ or PPARγ, but that the inhibitory effect of DHA on cell migration is mediated through PPARγ.

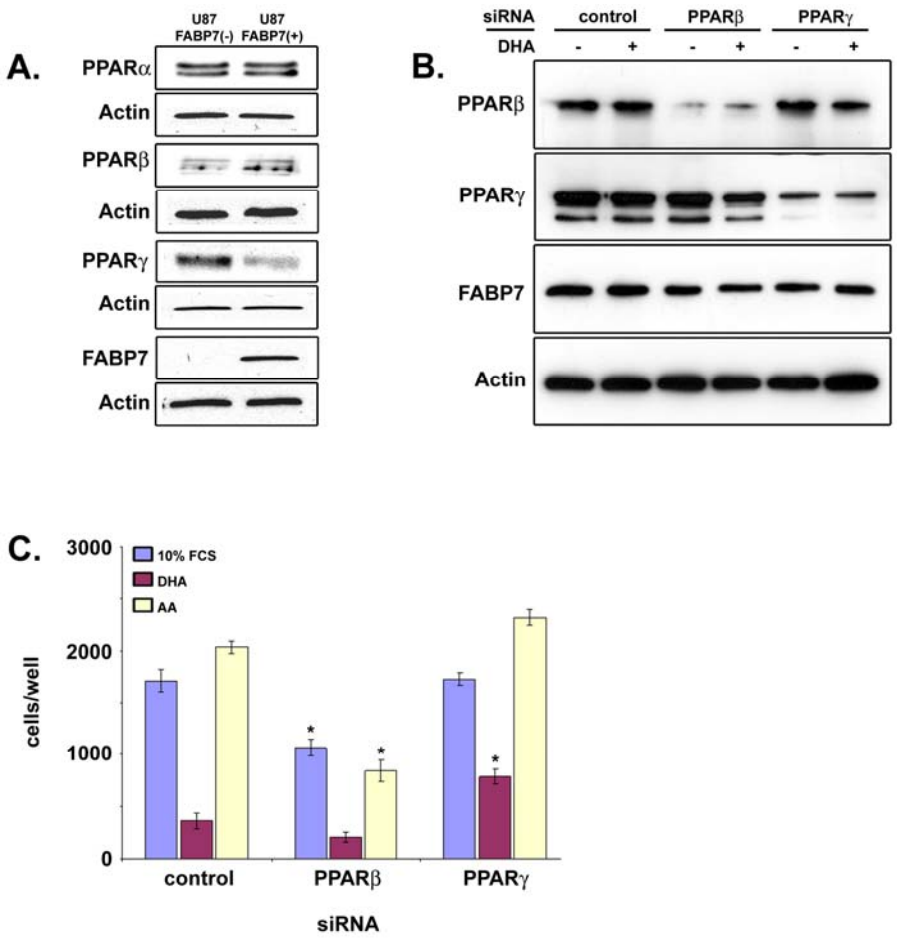


Figure 3.10 Role of PPARs in FABP7/FA-mediated cell migration. (A) Western blot of PPARα, PPARβ, and PPARγ whole cell lysates (25 µg/lane). Lysates were separated by electrophoresis in a 13.5% SDS-polyacrylamide gel. (B) Western blot of U87-FABP7(+) cells transfected with scrambled siRNA (control), or siRNAs targeting PPARβ or PPARγ. (C) Twenty-four h post-transfectioṇ cells cultured in DMEM plus 10% FCS were treated with 60 µM BSA, 60 µM DHA or 60 µM AA for an additional 24 h, and subjected to the Transwell assay. The data were obtained from two independent experiments carried out in triplicate. * = $p < 0.01$.

3.4 DISCUSSION

The hallmark of MG is their ability to infiltrate surrounding brain tissue, thus escaping therapies aimed at eradicating the main tumour mass. As a result, MG has a dismal prognosis, with most patients dying within two years of diagnosis. Importantly, MG is rarely metastatic, suggesting that controlling local disease may be key to the successful treatment of these tumours. FABP7, a radial glia/neural progenitor cell marker, is often expressed in MG where it localizes to regions associated with tumour infiltration such as the subpial surface and surrounding blood vessels (Mita, Coles et al. 2007). FABP7 expression is associated with decreased patient survival in grade IV astrocytomas (Liang, Bollen et al. 2006; Kaloshi, Mokhtari et al. 2007). Manipulation of FABP7 levels in MG cell lines demonstrates a direct link between FABP7 expression and cell migration (Mita, Coles et al. 2007).

Fatty acids are highly versatile molecules that serve as: (i) building blocks for the production of membranes, (ii) fuel for energy generation, and (iii) direct or indirect regulators of cell signaling and gene regulation. The fatty acid composition of the phospholipids in the plasma membrane determines membrane fluidity and permeability, with changes in membrane fluidity/permeability in turn influencing the formation of lipid microdomains and altering cell signaling and enzymatic activity. As FABPs can directly affect fatty acid uptake, expression and subcellular localization of FABPs can significantly alter cellular properties and function. Here, we

provide evidence of a central role for FABP7 in regulating the growth properties of MG cells, particularly as related to migration.

FABP7 binds the long chain PUFAs DHA (K_d 53 μM) and AA (K_d 207 μM) (Balendiran, Schnutgen et al. 2000). Treatment of cells with DHA and AA generally leads to opposite outcomes, with DHA having anti-tumourigenic properties and AA having pro-tumourigenic properties (Larsson, Kumlin et al. 2004). Our results support a role for FABP7 at the apex of two competing fatty acid-dependent signaling pathways, one pro-migratory, the other anti-migratory. In particular, our data indicate that: (i) FABP7-mediated cell migration is dependent on availability of AA and activation of COX-2 and PGE_2 production in the cytoplasm, and (ii) FABP7 bound to DHA has anti-migratory effects, through activation of the nuclear receptor PPARγ. Importantly, these pro- and anti-migratory effects are governed by the ratio of DHA to AA rather than empirical levels of DHA and AA.

Transfection of FABP7 into U87MG, a cell line that normally does not express FABP7, is accompanied by increased cell migration in regular growth medium (3:1 ratio of AA:DHA), as well as increased COX-2 expression. A significant proportion of FABP7 is expected to be bound to AA under these conditions, suggesting a functional link between FABP7 bound to AA and COX-2. In support of this idea, cyclooxygenases, along with lipooxygenases, convert AA into eicosanoids, biologically active signaling molecules that include prostaglandin PGE_2. Thus, one can

imagine a feedback loop whereby increased FABP7/AA gives rise to increased PGE_2 production (through increased COX-2 expression) which in turn maintains elevated levels of COX-2, and drives the pro-migratory phenotype (Figure 3.11).

COX-2 is overexpressed in many different types of cancers, including breast, prostate, pancreas, skin, lung, and is found at higher levels in high grade astrocytomas compared to low grade astrocytomas and normal brain (Perdiki, Korkolopoulou et al. 2007). It is noteworthy that a study examining the effect of the COX-2 inhibitor, NS398, on the growth properties of U251 (FABP7+) and U87 (FABP7-), showed that NS398 was a potent inhibitor of cell migration in these two cell lines (Joki, Heese et al. 2000). However, the effect on U87 was determined to be due to an increase in apoptosis and a decrease in cellular proliferation, in support of FABP7 being required to induce cell migration via the COX-2 pathway. It may therefore be critical to assess FABP7 expression in order to predict the effect of AA and COX-2 on MG growth properties.

It is well established that DHA suppresses tumourigenesis *in vivo* (Gonzalez, Schemmel et al. 1993; Rose, Connolly et al. 1996; Noguchi, Minami et al. 1997) and cancer cell proliferation *in vitro* (Chajes, Sattler et al. 1995; Connolly, Gilhooly et al. 1999); however, the mechanism by which DHA exerts its tumour suppressing activity remains controversial, with competing theories proposing that DHA can indirectly or directly modulate gene expression (Biondo, Brindley et al. 2008). Indirect effects

appear to be mediated through modification of the intracellular lipid second messenger profile, a consequence of DHA competing with AA for modification by COX, leading to the production of metabolically inactive compounds such as Δ^{17}-6-keto-PGF and TXB_3 (Rose and Connolly 1999). However, the role of COX proteins in the conversion of DHA into secondary compounds remains controversial. In contrast, direct effects of DHA have been postulated to be a consequence of binding and activating transcription factors (e.g. PPARs and RXRs) in the nucleus (Crawford, Golfetto et al. 2003).

Our results show that DHA is a strong inhibitor of AA/COX-2/PGE_2-mediated cell migration, and that this anti-migratory effect is dependent on: (i) the presence of FABP7 and (ii) localization of FABP7 to the nucleus. Furthermore, our data demonstrate that binding of DHA to FABP7 sequesters FABP7 to the nucleus, indicating that DHA binding may alter the configuration of FABP7 and promotes its translocation to the nucleus. Gillilan *et al.* (2007) (Gillilan, Ayers et al. 2007) used x-ray crystallography to compare structural features of FABP4 bound to ligands that induce its translocation to the nucleus versus apoFABP4 and FABP4 bound to ligands that do not induce nuclear translocation, These authors reported that binding of FABP4 to nuclear-homing ligands results in the stabilization of structures that expose a nuclear localization signal. Binding of DHA to FABP7 may have a similar effect.

Protein interaction studies using laser-scanning microscopy and co-immunoprecipitation indicate that FABPs and fatty acids can co-operate in the activation of transcription factors in the nucleus (Helledie, Antonius et al. 2000; Wolfrum, Borrmann et al. 2001; Helledie, Jorgensen et al. 2002; Tan, Shaw et al. 2002). There is a growing body of evidence indicating that ligand-dependent activation of PPARs can inhibit tumour growth (Mueller, Sarraf et al. 1998; Hisatake, Ikezoe et al. 2000; Inoue, Kawahito et al. 2001). For example, activation of PPARγ in MG using synthetic ligands can induce apoptosis, and inhibit growth, migration and invasion in animal models (Zang, Wachter et al. 2003; Grommes, Landreth et al. 2006; Coras, Holsken et al. 2007; Spagnolo, Glick et al. 2007; Spagnolo, Grant et al. 2007). DHA has been reported to bind PPARγ but not PPARα or PPARβ (Itoh, Fairall et al. 2008; Banga, Unal et al. 2009). In addition, FABP7 has been shown to interact with PPARγ *in vitro* (Adida, Friedrich 2006). Using siRNAs targeting PPARβ or PPARγ, we demonstrate that PPARγ is important for DHA-dependent inhibition of migration in U87-FABP7(+) cells. We propose that FABP7 activates PPARγ in the nucleus by DHA-ligand transfer, thereby inhibiting cell migration through alteration in the transcription of PPARγ target genes (Figure 3.11). A possible target of PPARγ is COX-2, as studies have shown that activated PPARγ can inhibit COX-2 expression (Bren-Mattison, Meyer et al. 2008; Takano, Kubota et al. 2008). In keeping with our observation that PPARγ levels are decreased and COX-2 levels are increased in U87-FABP7(+) versus U87-

FABP7(-) cells, others have reported that PPARγ is downregulated in response to prostaglandins produced by COX-2 (Yan, Kermouni et al. 2003). These data suggest complex feedback loops regulating the FABP7/PUFA/COX-2/PPARγ pathways. To add an additional level of complexity, PPARs are directly involved in the transcription of at least four FABPs (FABP1, 2, 3 and 4) (Motojima 2000; Poirier, Niot et al. 2001; Fujishiro, Fukui et al. 2002). We also show that knock-down of PPARβ results in a general decrease in the migration of U87-FABP7(+) cells, suggesting an interaction between FABP7 and PPARβ. The increase in PPARβ observed upon expression of FABP7 in U87MG cells is compatible with a relationship between these two proteins.

Our experiments indicate that the migratory response of FABP7-expressing MG cells depends on the ratio of DHA:AA, suggesting cross-talk between the DHA- and AA-related signaling/gene regulation pathways through FABP7. Such molecular cross-talk is not surprising as FABP7-expressing cells are unlikely to be exposed to only AA or DHA in their natural environment. During brain development, when cell migration is important and FABP7-expressing radial glial cells are abundant, levels of AA exceed that of DHA. The DHA:AA ratio gradually increases upon brain maturation, along with a reduced need for cell migration and down-regulation of FABP7 (Liu, Li et al. 2008). Two independent reports indicate that the DHA:AA ratio is considerably lower in MG than in normal adult brain (Martin, Robbins et al. 1996; Marszalek, Pisklak et al. 2010).

Combined with the elevated levels of FABP7 in a subset of MG and the importance of deregulation in the promotion of MG cell migration, these data underline the importance of FABP7 in controlling the growth properties of MG tumours.

Despite major advances in neurosurgical/radiological procedures, and the discovery of chemotherapeutic agents that can penetrate the blood-brain barrier, the survival rate for patients with MG has not improved significantly over the past 20 - 30 years (Mason, Maestro et al. 2007; Nakada, Nakada et al. 2007). To compound an abysmal prognosis, treatment of MG patients is often associated with significant local and systemic side effects. Many small phase-II MG clinical trials have been carried out over the past years. Some of these trials, including one involving COX-2 inhibitors and PPARγ agonists (Hau, Kunz-Schughart et al. 2007), have shown moderate effects suggesting that the treatment regimen might be suited to only a subset of patients. We show that DHA/AA, COX-2 and PPARs are linked through FABP7, and that FABP7 may be key to controlling which signaling pathways are activated or repressed in MG. We propose that early use of DHA as an adjuvant therapy in those tumours that express FABP7 may alter the migration potential and infiltrative properties of MG cells.

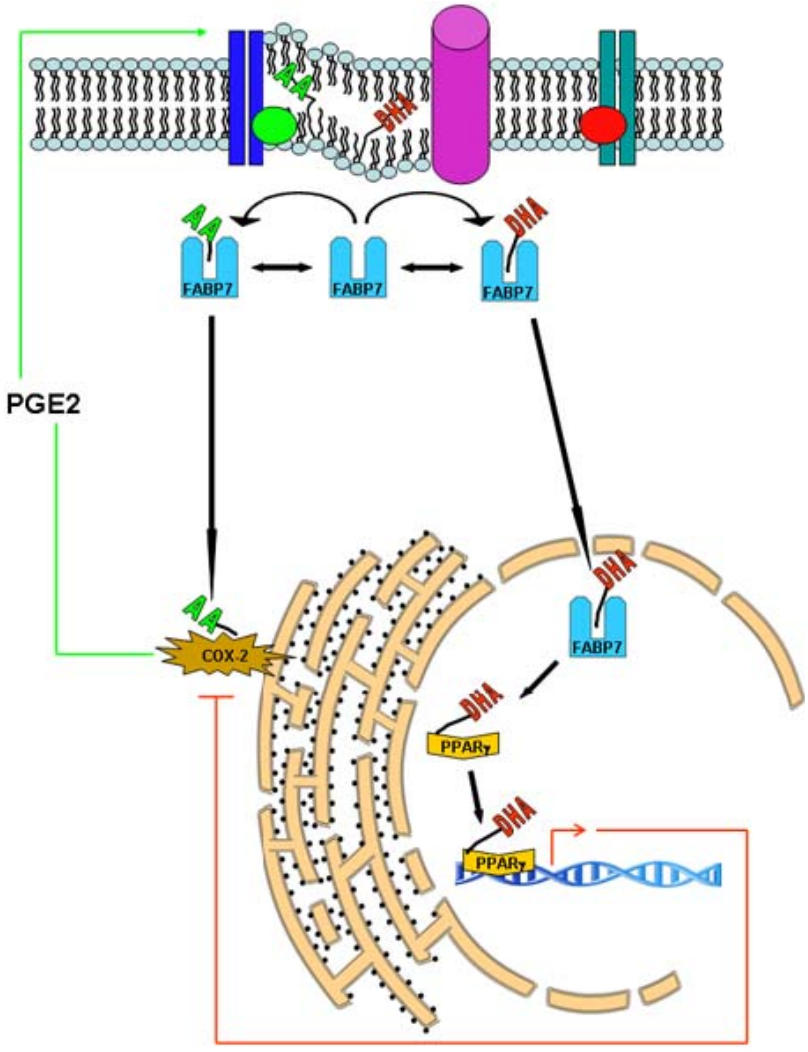


Figure 3.11 Model of cytoplasmic and nuclear roles for FABP7/fatty acids in cell migration. Cytosolic FABP7 can bind to either DHA or AA depending on the relative abundance and availability of these two fatty acids. Binding of FABP7 to AA induces cell migration through transfer of AA to the COX-2 pathway, whereby AA is metabolized into pro-migratory signaling molecules such as PGE_2. Binding of FABP7 to DHA results in translocation of FABP7 to the nucleus where DHA is transferred to PPARγ, resulting in down-regulation of pro-migratory genes.

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Chapter 4: FABP7 Increases the Uptake and Incorporation of PUFAs in Malignant Glioma Cell Membranes

4.1 INTRODUCTION

Biological membranes are poorly understood structures consisting primarily of phospholipids interspersed with proteins and carbohydrates. Although highly structured, membranes are dynamic, with constant positioning and re-positioning of proteins and lipids in relation to one another. As a consequence, membranes can affect a multitude of extracellular and intracellular processes, including ligand-receptor binding, antigen presentation, lipid raft formation, and cell signaling (Shaikh, Edidin et al. 2006).

The fatty acid composition of phospholipids, particularly as related to length and saturation of the carbon chain, can have a major impact on the biophysical properties of cell membranes (Gawrisch, Soubias et al. 2008). Importantly, there is a general increase in membrane fluidity and permeability as the content of the highly flexible polyunsaturated fatty acids (PUFA) increases in phospholipids (Salem, Litman et al. 2001). Phospholipids have been shown to play an important role in mediating the distribution and/or activation of G-protein coupled receptors (Soubias, Teague et al. 2006), receptor tyrosine kinases (Schley, Brindley et al. 2007), and other intracellular signaling molecules (Jude, Martel et al. 2007),

The long chain PUFAs, ω-3 docosahexaenoic acid (DHA) and ω-6 arachidonic acid (AA), are essential fatty acids implicated in numerous physiological and pathological processes, including lipoprotein synthesis,

atherosclerosis, inflammation, and cancer (Simopoulos 1991). AA and DHA can be synthesized in the body from linoleic acid (LA; 18-2 ω-6) and α-linolenic acid (ALA; 18:3 ω-3), respectively, or directly ingested. In the lipid-rich brain, there is a dynamic increase in the levels of DHA relative to AA during perinatal development, with DHA and AA each comprising ~40% of total PUFAs in the mature brain (Farquharson, Cockburn et al. 1992; Chen, Green et al. 2008; Liu, Mita et al. 2010). DHA, like AA, is selectively incorporated into the (sn)-2 position of phospholipids, suggesting competition between DHA and AA for incorporation into the plasma membrane (Chen, Green et al. 2008).

The effects of AA deficiency remain relatively unexplored as conversion of LA to AA is highly efficient, reducing the likelihood of AA deficiency (Makrides, Neumann et al. 1994). Animals fed DHA-deficient diets show deficits in cognitive ability, photoreceptor responsiveness, and visual acuity (Makrides, Simmer et al. 1995; Chang, Contreras et al. 2001; Crawford, Bazinet et al. 2009; Simopoulos 2009). DHA deficiency in humans during development has been linked to reduced intelligence quotients, and aberrant cortical and neural development (Farquharson, Cockburn et al. 1992). In human adults, low tissue levels and intake of DHA have been linked to major depression, bipolar disorder, age-related maculopathy, Alzheimer's disease, Huntington's disease, and schizophrenia (rev. in (Crawford, Bazinet et al. 2009)). Interestingly, the brain lipid profile of patients with bipolar disorder (Chang, Contreras et al.

2001), major depression (Rao, Ertley et al. 2006), and schizophrenia, reveal higher levels of AA compared to normal brain. Of particular relevance to our study, levels of DHA are significantly lower in malignant glioma tissue compared to normal brain, suggesting major changes in the lipid metabolism of brain tumours (Martin, Robbins et al. 1996; Marszalek, Pisklak et al. 2010). As the acyl chain flexibility of phospholipids containing DHA is significantly different from that containing AA, DHA and AA may affect cell membrane dynamics in different ways.

Intracellular uptake and transport of hydrophobic fatty acids requires molecular chaperones. The family of fatty acid-binding proteins (FABPs) has been shown to effectively bind and transport fatty acids within the cell. One member of this family, brain fatty acid binding protein (FABP7, B-FABP, BLBP), effectively binds DHA and AA *in vitro* with K_d's of 30 nM and 207 nM, respectively (Balendiran, Schnutgen et al. 2000). FABP7 is first expressed in radial glial cells during brain development, and is down-regulated upon maturation of radial glial cells (Feng, Hatten et al. 1994). FABP7 is significantly overexpressed in malignant glioma tissue compared to normal tissue, suggesting a role for FABP7 in tumour formation, progression and/or maintenance. In addition, FABP7 expression in malignant glioma cells is associated with increased migration and FABP7 localizes to areas of tumour infiltration, suggesting a role for FABP7 in the control of tumour growth properties (Mita, Coles et al. 2007). Furthermore, we have shown that FABP7-induced MG cell

migration is AA-dependent and can be inhibited by DHA. In this study, we investigate how FABP7 affects the uptake and incorporation of DHA and AA into membrane phospholipids of human malignant glioma cells.

4.2 MATERIALS AND METHODS

4.2.1 Cell culture

U87FABP7(-) and U87FABP7(+) cell lines were generated as previously described. Unless otherwise stated, cells were cultured in Dulbecco's modified essential medium (DMEM; Gibco) supplemented with 10% (v/v) fetal calf serum (FCS) and antibiotics (100 μg/ml streptomycin and 100 U/ml penicillin). Cells were maintained at 37^{o}C in a humidified 5% CO_2 atmosphere. For analysis of fatty acid incorporation into cells, 1 X 10^6 cells were plated on a 100 cm dish. After 2 days, the medium was replaced with medium containing 60 μM BSA, DHA or AA. At 2, 4, 8, 24, 32, and 48 h, cells were collected and processed for lipid analysis. Each set of experiment was carried out using three independent cell cultures.

4.2.2 Preparation of fatty acids

Fatty acids (Sigma) were dissolved in ethanol, then complexed to fatty acid-free BSA (Roche) over a steady stream of nitrogen gas. Final concentration of ethanol in our experiments was 0.1%.

4.2.3 Isolation and analysis of lipids

Lipids were extracted from cells by a modified Folch procedure (Folch, Lees et al. 1957; Field, Ryan et al. 1988). Individual phospholipids were separated from the lower phase on thin layer chromatography plates (HPK silica gel 60 nm 10 X 10 cm, Whatman, Clifton, NJ) using a solvent

system consisting of chloroform : methanol : 2-propanol : 0.25% w/v KCl : triethylamine (30:9:25:6:18 by volume) as previously described (Bradova, Smid et al. 1990). Separated phospholipids were visualized with 8-anilino-1-naphthalene-sulfonic acid (Sigma) and identified under ultraviolet light using appropriate standards as references (Supelco). Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS) fatty acid methyl esters were prepared from the scraped silica bands using 14% w/v BF3/methanol reagent (Sigma). Fatty acid methyl esters were separated by automated gas liquid chromatography (Varian CP-3800) on an SGE BP20 column (60 m x 0.25 mm internal diameter). The analytical conditions were designed to separate all saturated, mono-, di-, and polyunsaturated fatty acids from 14 to 24 carbons in chain length.

4.3 RESULTS

4.3.1 Incorporation of DHA and AA into total lipids increases as a function of FABP7 expression

A number of studies have shown that fatty acid uptake increases when cytosolic FABPs are present in the cell. Conversely, decreasing levels of cytosolic FABPs using siRNA or reducing the binding affinity of specific FABPs by site-directed mutagenesis of residues involved in fatty acid binding, results in reduced fatty acid uptake. Fatty acid profiling of MG tissue has revealed lower levels of DHA and higher levels of AA compared with normal brain tissue (Marszalek, Pisklak et al. 2010). Combined with the fact that FABP7 is overexpressed in MG, this suggests a possible involvement of FABP7 in altering the fatty acid profile of MG membrane phospholipids. FABP7-negative U87 cells were transfected with empty vector (pREP4) and a FABP7 expression construct. Stable U87-FABP7(-) and U87-FABP7(+) clonal populations were selected in hygromycin, and analyzed for FABP7 expression as previously described (Mita, Coles et al. 2007).

Determining the fatty acid composition of U87-FABP7(-) and U87-FABP7(+) cells cultured in 10% FCS was conducted using the total lipid fraction of these cells. Similar to previous reports on the fatty acid composition of brain tissue (Martin, Robbins et al. 1996; Marszalek, Pisklak et al. 2010), the most abundant fatty acids present in neural tissue were palmitic acid (PA, 16:0), stearic acid (SA, 18:0), and oleic acid (OA,

18:1) (Table 4.1). However, contrary to previous reports, which indicated that 9 – 10% of human brain tissue is DHA and AA, our MG cell cultures contained very little of these fatty acids. Our results show that DHA constitutes only a small fraction of the fatty acids in total lipids, consisting of 0.5% in U87-FABP7(-) and 0.7% in U87-FABP7(+) (Figure 4.1A; Table 4.1). In comparison, AA makes up 1.8% and 4.4% of the fatty acids in total lipids extracted from U87-FABP7(-) and U87-FABP7(+), respectively (Figure 4.1B; Table 4.1). The relatively higher levels of AA in U87-FABP7(+) compared to U87-FABP7(-) (resulting in DHA:AA ratios of 0.16 versus 0.28) suggests that the presence of FABP7 allows preferential accumulation of AA in cells that are cultured in 10% FCS, which contains 4 μM and 11 μM, DHA and AA, respectively. The low levels of DHA and AA observed in our cells compared to brain tissue can be explained by the relatively low levels of these fatty acids in our culture medium.

Next, we tested whether FABP7 could potentiate DHA and AA uptake in MG cells cultured in DMEM plus 10% FCS supplemented with either 60 μM DHA or 60 μM AA. As shown in Figure 4.1, increased levels of either DHA or AA in the culture medium resulted in increased levels of DHA and AA in total lipids extracted from both U87-FABP7(-) and U87-FABP7(+) lipids, with peak levels of DHA and AA observed 24 h post-treatment. Importantly, the percentage of DHA or AA in lipids extracted from U87-FABP7(+) was higher than that observed in U87-FABP7(-),

Table 4.1. Lipid profile of U87-FABP7-expressing and -nonexpressing cells grown in DMEM plus 10% FCS[a,b]

	% of total lipid fraction	
	U87-FABP7(-)	U87-FABP7(+)
Palmitic	26.8 ± 1.2	27.9 ± 1.4
Stearic	30.2 ± 0.9	33.2 ± 1.9
Oleic	22.3 ± 1.2	25.3 ± 1.7
Linoleic	7.7 ± 2.1	6.9 ± 1.3
α-linolenic	ND	ND
Docosahexaenoic	0.5 ± 0.5	0.7 ± 0.2
Arachidonic	1.8 ± 0.8	4.4 ± 0.5*
DHA:AA	0.28	0.16*
	% of phospholipid fraction	
	U87-FABP7(-)	U87-FABP7(+)
Palmitic	21.7 ± 3.4	28.2 ± 1.8
Stearic	32.1 ± 2.9	31.3 ± 1.6
Oleic	19.5 ± 1.0	22.3 ± 1.7
Linoleic	8.7 ± 1.5	6.1 ± 4.6
α-linolenic	ND	ND
Docosahexaenoic	0.4 ± 0.4	0.6 ± 0.2
Arachidonic	2.1 ± 0.6	2.1 ± 0.4
DHA:AA	0.19	0.29

a: values are means ± SD, n = 3
b: within each row, statistical significane is represented by * $p < 0.01$

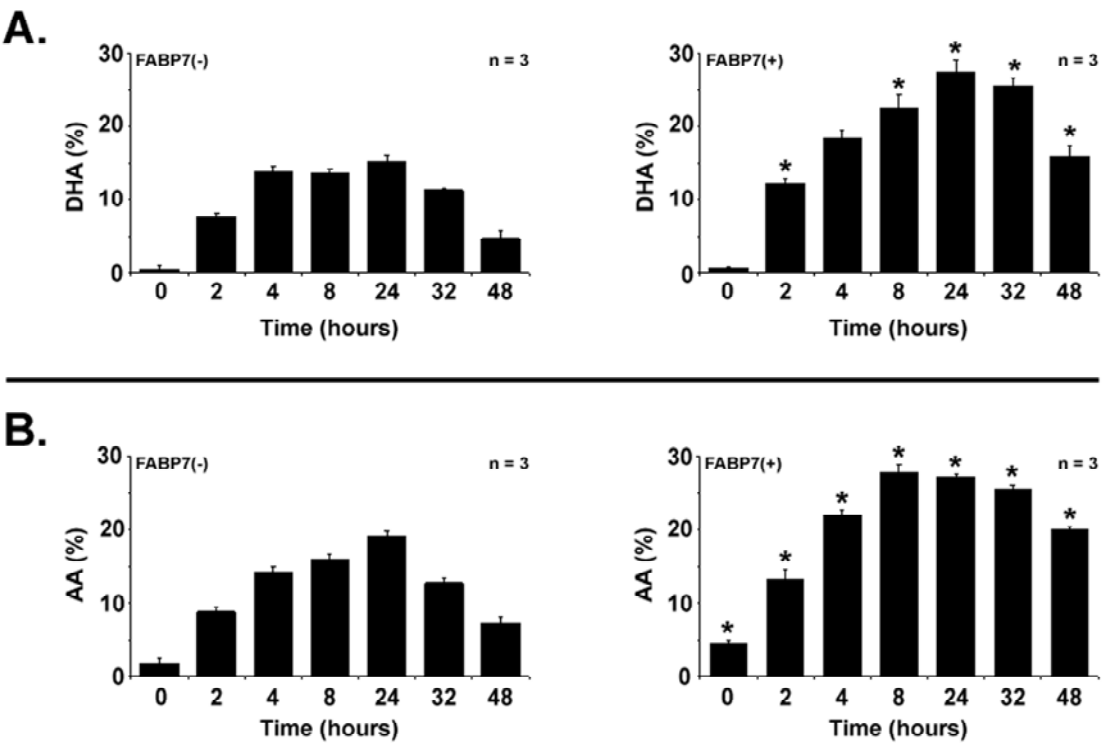


Figure 4.1 Uptake of DHA and AA into the total lipid fraction of MG cells. U87-FABP7(-) and (+) cells were treated with (A) 60 μM DHA or (B) 60 μM AA and cells were harvested at the indicated time points of 2, 4, 8, 24, 32, 48 h. Values represent the means of three independent experiments ± standard deviation. Control represents cells grown in 10% FCS without extra fatty acids. * represents statistical significance ($p < 0.05$) between the two cell populations.

reaching 27.3% (DHA) and 27.2% (AA) of total fatty acids at 24 h (Figure 4.1; Table 4.2).

The ratio of DHA to AA in BSA-treated U87-FABP7(-) and U87-FABP7(+) was 0.15 and 0.14, respectively (Table 4.2). Addition of 60 μM DHA to the culture medium increased the DHA:AA ratio to 12.58 for U87-FABP7(-) and 28.14 for U87-FABP7(+). Of note, the DHA:AA ratio in lipids extracted from cells cultured in 60 μM AA was 0.02, regardless of FABP7 status (Table 4.2). These combined data suggest that the presence of FABP7 enhances both DHA and AA uptake; however, whereas increased uptake of DHA results in a concomitant decrease in AA uptake, no such effect on DHA uptake is observed upon increased AA uptake.

4.3.2 Incorporation of DHA and AA into phospholipids is enhanced by FABP7

Lipids are present in the cell in a variety of physical forms, of which the phospholipids in the cell membrane are most abundant. The fatty acid content of the phospholipid bilayer affects the distribution of other lipids such as cholesterol as well as protein receptors and signaling molecules (Wassall and Stillwell, 2009). To determine whether DHA and AA were also increased in phospholipids upon supplementation with DHA and AA, we extracted phospholipids from U87-FABP7(-) and U87-FABP7(+) cells cultured in medium supplemented with 60 μM BSA, 60 μM DHA or 60 μM AA.

Table 4.2: DHA and AA profile of U87-FABP7-expressing and -nonexpressing cells grown in 10% FCS supplemented with BSA, DHA, or AA at 24 hours[a,b]

	U87FABP7 (-)			U87FABP7 (+)		
	BSA	DHA	AA	BSA	DHA	AA
	Ctrl	60 uM	60 uM	Ctrl	60 uM	60 uM
Total Lipid						
AA	2.80 ± 0.7	1.20 ± 0.3	19.00 ± 0.8	5.30 ± 1.4	0.97 ± 0.5	27.20 ± 0.5
DHA	0.43 ± 0.3	15.10 ± 1.0	0.35 ± 0.2	0.76 ± 0.4	27.30 ± 1.7	0.61 ± 0.4
DHA:AA	0.15	12.58	0.02	0.14	28.14	0.02
Total Phospholipid						
AA	2.80 ± 1.0	1.70 ± 0.7	10.30 ± 0.5	3.10 ± 1.2	1.10 ± 0.5	15.70 ± 0.6
DHA	0.14 ± 0.1	7.50 ± 0.8	0.12 ± 0.1	0.35 ± 0.2	15.30 ± 0.8	0.29 ± 0.2
DHA:AA	0.05	4.41	0.01	0.11	13.91	0.02
Phospholipid Species						
			PC			
AA	0.68 ± 0.3	0.58 ± 0.3	2.73 ± 0.4	0.67 ± 0.3	0.56 ± 0.2	3.40 ± 0.3
DHA	0.25 ± 0.2	1.45 ± 0.3	0.17 ± 0.1	0.23 ± 0.2	2.18 ± 0.5	0.19 ± 0.1
			PE			
AA	3.69 ± 0.8	2.10 ± 0.5	8.96 ± 0.5	4.20 ± 0.7	1.00 ± 0.4	10.83 ± 1.0
DHA	0.43 ± 0.2	7.20 ± 0.5	0.31 ± 1.0	0.98 ± 0.3	8.30 ± 0.6	0.67 ± 0.1
			PI			
AA	2.93 ± 0.4	2.71 ± 0.6	9.52 ± 1.2	3.17 ± 1.0	2.82 ± 0.7	11.07 ± 0.4
DHA	0.2 ± 0.1	1.05 ± 0.4	0.21 ± 0.2	0.08 ± 0.1	1.30 ± 0.5	ND
			PS			
AA	0.47 ± 0.3	0.41 ± 0.2	0.82 ± 0.2	0.60 ± 0.2	0.46 ± 0.2	1.30 ± 0.4
DHA	0.05 ± 0.1	0.25 ± 0.2	ND[c]	0.18 ± 0.1	4.78 ± 0.6	0.17 ± 0.1

a: values are means ± SD, n = 3

b: within each row, statistical significane is represented by * $p < 0.01$

c: ND = non detectable

Our fatty acid profile of cellular phospholipids revealed that the overwhelming majority of fatty acids in the cell membrane are found in phospholipids form (Table 4.1). DHA constituted 0.4% and 0.6% of all fatty acids found in phospholipids extracted from U87-FABP7(-) and U87-FABP7(+) cells, respectively, in DMEM plus 10% FCS and 60 μM BSA (Table 4.1). Significant increases in DHA incorporation were observed for both U87-FABP7(-) and U87-FABP7(+) cells when 60 μM DHA was added to the culture medium, with maximum incorporation of DHA occurring at or near 24 h (Figure 4.2A). DHA incorporation into phospholipids was generally higher in U87-FABP7(+) compared to U87-FABP7(-) cells, reaching a peak of 15.3% [compared to a peak of 7.5% for U87-FABP7(-) cells] 24 h post-DHA-treatment. In comparison, BSA-treated U87-FABP7(-) and U87-FABP7(+) cells showed 2.8% and 3.1% DHA incorporated into phospholipids, respectively.

The percentage of AA in the phospholipids of cells cultured in regular growth medium (supplemented with 60 μM BSA) was 2.1% for both U87-FABP7(-) and U87-FABP7(+) cells (Figure 4.2B; Table 4.1). Supplementation with 60 μM AA resulted in significant increases in AA incorporation for both cell cultures, with peak incorporation observed at 24 h. As with DHA, AA incorporation was enhanced by FABP7 expression [with AA making up 10.3% and 15.7% of all fatty acids in U87-FABP7(-) and U87-FABP7(+) cells, respectively]. In contrast to DHA-supplemented cells which showed an overall decrease in AA incorporation compared to

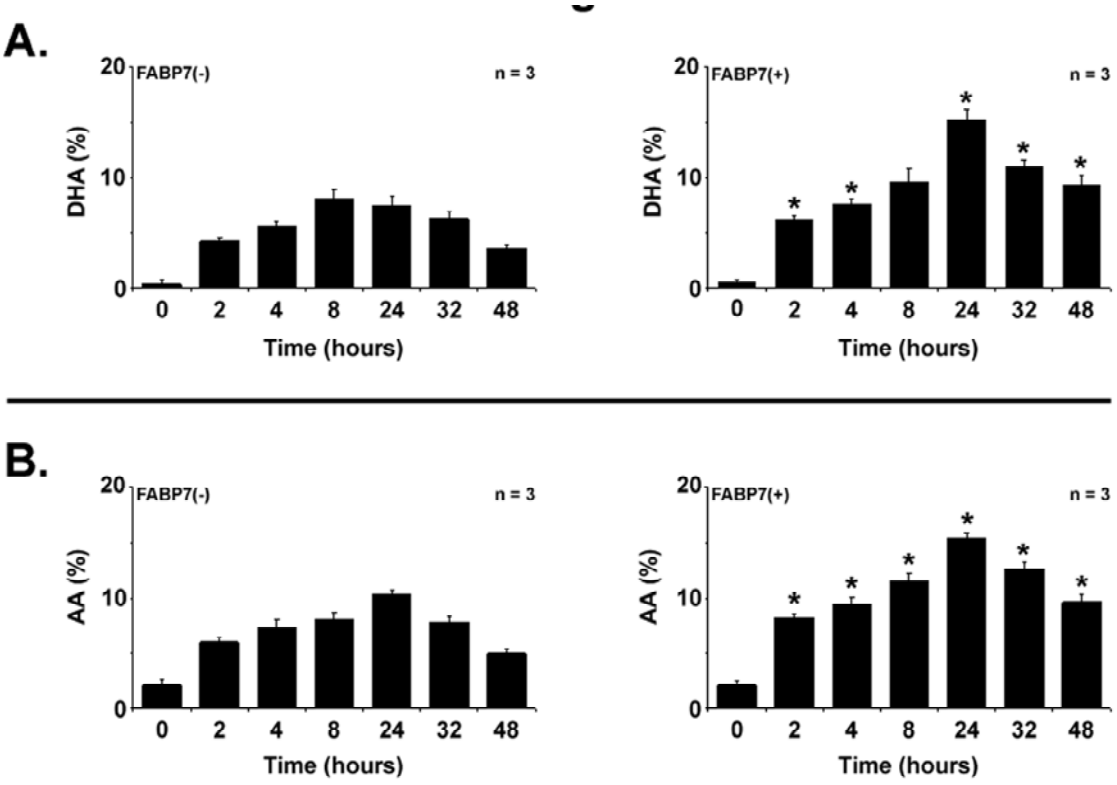


Figure 4.2 Incorporation of DHA and AA into phospholipids of MG cells.

U87-FABP7(-) and (+) cells were treated with (A) 60 μM DHA or (B) 60 μM AA and harvested at the indicated time points of 2, 4, 8, 24, 32, 48 h. Values represent the means of three independent experiments ± SD. Control represents cells grown in 10% FCS without extra fatty acids. * represents statistical significance ($p < 0.05$) between two cell populations.

BSA control, there was not a significant decrease in DHA incorporation in AA-supplemented cells (Table 4.2).

The DHA:AA ratio in phospholipids at 24 h in BSA-treated cells was 0.05 and 0.11 for U87-FABP7(-) and U87-FABP7(+) cells, respectively. In AA- supplemented cells, the respective DHA:AA ratios were 0.01 and 0.02. In comparison, DHA-treated U87-FABP7(-) and U87-FABP7(+) cells had significantly different ($p < 0.01$) DHA:AA ratios of 4.4 and 13.9, respectively at 24 h. These data indicate that FABP7 expression is particularly effective at increasing DHA levels in phospholipids.

4.3.3 DHA and AA profiling of the four major species of phospholipids in MG cells

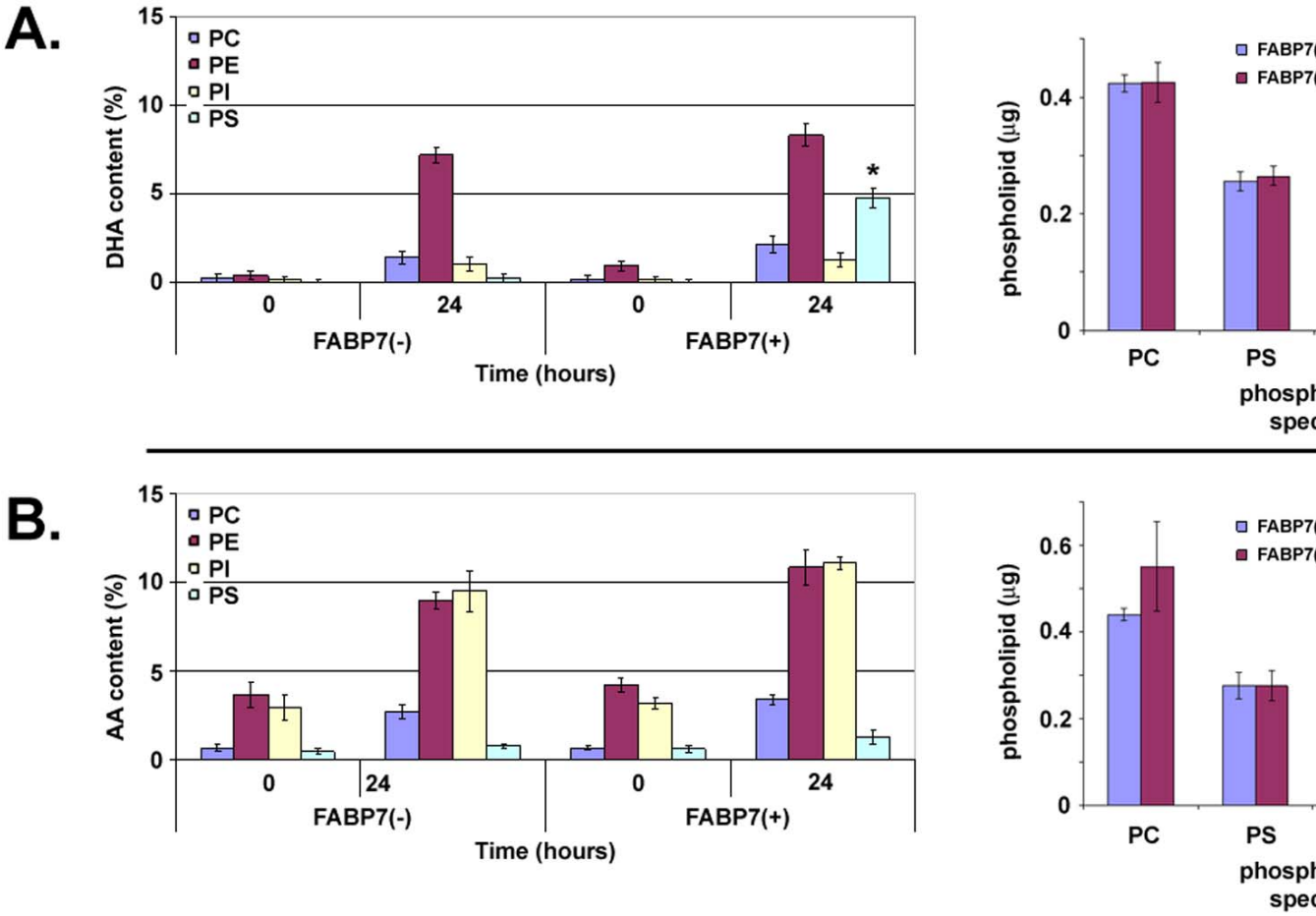
Plasma membranes are made up of five major species of phospholipids: phosphotidylcholine (PC), phosphotidylethanolamine (PE), phosphotidylserine (PS), phosphotidylinositol (PI), and sphingomyelin (SM). As different phospholipids have been implicated in different cellular functions, and PUFA content of phospholipids affects membrane fluidity, we examined the DHA and AA content of PC, PE, PS and PI in U87-FABP7(-) versus U87-FABP7(+) cells. These experiments were carried out using cells cultured for 24 h in growth medium supplemented with 60 μM BSA, 60 μM DHA or 60 μM AA.

DHA-supplemented U87-FABP7(-) cells showed significant increases in the DHA content of all four species of phospholipids, with

highest levels of DHA observed in PE, followed by PC, PS and PI (Figure 4.3A). A similar trend was observed with U87-FABP7(+) cells, with one exception. Whereas proportional increases in DHA were observed in PC, PE and PI, the DHA content in PS surpassed that of PC, comprising 5% of the total fatty acids in PS. In comparison, the DHA content of PE was ~8% whereas that of PC was ~2%. Next, we measured the amount of PC, PE, PI and PS in U87-FABP7(-) and U87-FABP7(+) cells treated with DHA for 24 h. Based on the phosphorus assay, there was no significant differences in PC, PE, PI and PS content in these cell populations (Figure 4.3, right panel). These results indicate that DHA is preferentially accumulated in PS in FABP7-expressing cells cultured in DHA-supplemented medium for 24 h.

There was a significant increase in the AA content of PC, PE, PI, and PS in cells cultured for 24 hrs in growth medium supplemented with 60 μM AA for both U87-FABP7(-) and U87-FABP7(+) cells. Expression of FABP7 resulted in ~15-25% higher levels of AA in the phospholipids of U87-FABP7(+) compared to U87-FABP7(-) cells (Figure 3B). There was no change in the amount of PC, PE, PI and PS at the 24 hr timepoint between the two cell populations; however in both FABP7(-) and (+), incorporation of AA was significantly higher compared with incorporation of AA in untreated control cells (Figure 3B, left panel). There was no difference in the phospholipid profiles of U87-FABP(-) versus U87-FABP7(+) cells (Figure 3B; right panel).

Figure 4.3 Incorporation of DHA and AA into specific phospholipid species of MG cells. U87-FABP7(-) and (+) cell populations were treated with (A) 60 μM DHA or (B) 60 μM AA (B) and allowed to grow for 24 h. Values represent the means of three independent experiments ± SD. Control represents cells grown in 10% FCS without extra fatty acids. * represents statistical significance ($p < 0.01$) across cell populations. There was a significant increase in the AA content of PC, PE, PI, and PS in cells cultured for 24 h in growth medium supplemented with 60 μM AA for both U87-FABP7(-) and U87-FABP7(+) cells. Expression of FABP7 resulted in ~15-25% higher levels of AA in the phospholipids of U87-FABP7(+) compared to U87-FABP7(-) cells (Figure 4.3B). There was no change in the amount of PC, PE, PI and PS at the 24 h timepoint between the two cell populations; however in both FABP7(-) and (+), incorporation of AA was significantly higher compared with incorporation of AA in untreated control cells (Figure 4.3B, left panel). There was no difference in the phospholipid profiles of U87-FABP(-) versus U87-FABP7(+) cells (Figure 4.3B; right panel).


A.
DHA content (%)
PC
PE
PI
PS
0
5
10
15
0
24
0
24
FABP7(-)
FABP7(+)
Time (hours)
*
phospholipid (μg)
0
0.2
0.4
PC
PS
B.
AA content (%)
PC
PE
PI
PS
0
5
10
15
0
24
0
24
FABP7(-)
FABP7(+)
Time (hours)
phospholipid (μg)
0
0.2
0.4
0.6
PC
PS

4.4 DISCUSSION

A role for FABPs in mediating intracellular fatty acid uptake and transport is intimately linked to their binding affinities for fatty acids (Storch and Thumser 2000). In addition, studies show that cellular fatty acid uptake increases when cytosolic FABP expression is increased by transfection or induction (Renaud, Foliot et al. 1978; Murphy, Prows et al. 1996; Atshaves, Foxworth et al. 1998; Murphy 1998; Burczynski, Fandrey et al. 1999; Darimont, Gradoux et al. 2000). Conversely, decreasing levels of cytosolic FABPs using siRNA or reducing the binding affinity of specific FABPs by site-directed mutagenesis of residues involved in fatty acid binding results in a reduction in fatty acid uptake of cells (Ockner and Manning 1976; Wu-Rideout, Elson et al. 1976; Memon, Bass et al. 1999; Wolfrum, Buhlmann et al. 1999). It is well established that the PUFA content of phospholipids can be readily altered both *in vitro* and *in vivo* by addition of PUFAs to cell culture and by increasing PUFA intake in the diet (Jurkowski and Cave 1985; Grammatikos, Subbaiah et al. 1994; Robinson, Clandinin et al. 2002). However, there is no clear evidence linking FABP expression to this phenomenon.

Here, we demonstrate that uptake of both DHA and AA is higher in U87MG cells that have been stably transfected with an FABP7-expression construct compared with U87MG cells that have been transfected with control vector. Furthermore, our results show that uptake of DHA and AA by MG cells results in the incorporation of these fatty acids into

phospholipids, specifically PC, PE, PI, and PS. In addition, we show that FABP7 expression results in a higher incorporation of DHA into PS. These data provide a clear association between FABP expression and fatty acid uptake and incorporation into the plasma membrane.

The relative ratios of ω-3 and ω-6 fatty acids in the cell have been recently implicated as having an important impact in human health and disease (Simopoulos 2009). For example, lipid profiling of MG tissue reveals higher levels of AA and lower levels of DHA compared with normal brain (Martin, Robbins et al. 1996; Marszalek, Pisklak et al. 2010). Combined with our data showing that FABP7 can increase the uptake of DHA and AA in MG cells, we suggest a model by which FABP7 expression can alter the lipid profile of MG cells. The higher affinity of FABP7 for DHA seems to contradict the possibility that increasing FABP7 expression would lead to higher AA than DHA uptake in MG cells; however, this relationship may be dependent on availability of DHA and/or AA for binding. For the most part, DHA and AA levels in the human body are affected by dietary intake. Anthropological data indicate that the dietary trend of humans in the developing world has changed dramatically, shifting from an ω-3/ω-6 ratio of 1:1 ten thousand years ago, to a present ratio of approximately 1:15 (Simopoulos 2006). These data suggest that there is significantly more AA than DHA readily available for binding by FABP7 in normal brain today compared the brains of our ancestors.

An alternative possibility for increased AA uptake at the expense of DHA may lie in the existence of single nucleotide polymorphsims (SNPs) in FABP7 that either alter its binding affinity to favor AA or altogether inhibit its binding to fatty acids. In support of this theory, an SNP in the human *fabp1* gene resulting in Ala^{54} -> Thr^{54} substitution is associated with obesity, decreased insulin sensitivity, and dislipidemia in aboriginal Canadians from an isolated community in Northern Ontario and is thought to decrease the ability of FABP1 to bind its fatty acid ligands. Moreover, complete inhibition of fatty acid binding by FABP7 would most certainly decrease the levels of DHA in cells as no other FABP can bind DHA with such high affinity; whereas other FABPs can bind AA with affinities that are similar to that of FABP7.

The preferential incorporation of DHA into phosphatidylserine (PS) by FABP7 is an interesting phenomenon. While best characterized for its role as a macrophage recognition site during apoptosis, several proteins and signaling pathways unrelated to apoptosis have been shown to require PS for optimal activity. Moreover, the presence of DHA in PS, rather than PS itself, has been associated with alterations in cell signaling properties. For example, PS is known to act as a specific activator of the family of protein kinase C (PKC) through interactions at the plasma membrane (Nishizuka 1986). In a recent study, Jude *et al.* (2007) showed that the incorporation of ω-3 PUFAs in cardiac membrane phospholipids of dogs led to a differential translocation (less membrane localization) of

PKC-δ and PKC-ε (Jude, Martel et al. 2007) away from the cell membrane. These results may be explained by the production of structurally different lipids that may have varying affinities for PKC isoforms. Differences in structure may be a direct reflection of fatty acid composition.

In addition to PKC enzymes, PS promotes the translocation of cytosolic Raf-1 (cRaf-1) protein kinase to the plasma membrane (Nagai, Aoki et al. 1999). Using DHA-treated Neuro2A cells, Kim *et al.* (2003) showed that the *in vitro* interaction between Raf-1 and the cell membrane was inhibited by the fatty acyl composition in PS (Kim, Akbar et al. 2003), suggesting that depletion/repletion of DHA from neuronal tissues may have an effect on Raf-1 translocation thereby affecting downstream signaling pathways. The constitutive activation of Raf-1 induces glioma formation in mice (Lyustikman, Momota et al. 2008), implicating it as a key regulator of tumour development and progression. Given the importance of elevated Raf-1 signaling in tumour growth promotion, specific inhibitors of Raf-1 are being developed and tested singly or in combination with other anticancer therapies (Kloog and Cox 2000; Walker and Olson 2005; Basso, Kirschmeier et al. 2006). Taken together, these studies suggest that DHA may affect Raf-1 signaling and could enhance the effects of anti-Raf-1 therapies in cells that express FABP7.

It was apparent to us that the majority of arachidonic acid found to be incorporated into the phospholipids was much higher than the amount of arachidonic acid found in the four phospholipid species analyzed. This

difference could possibly be accounted for in the sphingolipid, lysophosphatidic acid, and cardiolipin fractions that were not analyzed, as studies show that arachidonic acid can be readily incorporated into these lipid species (Lee, Bazinet 2006). These lipid species can incorporate AA and DHA at the sn-2 position, thus our results indicate that in the absence of FABP7, there remains a basal level of DHA and AA uptake that could be attributed to the expression of other FABPs in the brain. Brain tissue is known to express four FABP types: FABP3, FABP5, FABP7 and FABP8. *In vitro* binding assays reveal that these four FABPs have overlapping ligand specificity. FABP3 and FABP7 have similar affinities for arachidonic acid, with Kds of 370, and 210 nM, respectively (Maatman, Degano et al. 1994; Balendiran, Schnutgen et al. 2000); however, the affinity of FABP3 for DHA is only 410 nM compared to 30 nM for FABP7 (Crawford, Bazinet et al. 2009). FABP5 binds AA and DHA with Kds of 170 and 420 nM, respectively; whereas the binding affinities of FABP8 for AA and DHA is similar to that of FABP7 (Richieri, Ogata et al. 2000). These studies suggest that FABPs of nervous tissue have overlapping binding properties that potentiate PUFA enrichment in the lipids of this tissue. While the variable expression of multiple FABPs in the brain has been well-described, only FABP7 has been observed to be highly expressed in malignant glioma tissue (Godbout, Bisgrove et al. 1998; Liang, Bollen et al. 2006; Kaloshi, Mokhtari et al. 2007; Mita, Coles et al. 2007), suggesting that it is likely the key FABP involved in the uptake of these fatty acids.

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Chapter 5: Discussion

5.1 Discussion

5.1.1 Treating malignant glioma: Missing the target

Malignant gliomas are the most common and deadly brain tumours in adults. Despite an aggressive multimodal treatment regimen, the life expectancy for patients with GBM is approximately 1 year. In recent years, technologies for treating patients with malignant giloma have improved substantially. For example, new radiotherapy technology now allows the clinician to focus the beam and tailor it to the irregular contours of brain tumours; thereby minimizing the dose to nearby critical structures (Oermann, Collins et al. 2010). In addition, new surgical techniques are improving the short-term prognosis of patients. A recent phase 3 clinical trial in Germany showed that the use of 5-aminolevulinic acid to visualize the tumour during neurosurgery, resulted in a significantly higher rate of complete resection as measured by magnetic resonance imaging (MRI) (Stummer, Pichlmeier et al. 2006). It is well accepted that maximal safe resection improves survival (Pichlmeier, Bink et al. 2008). While optimism for the improvement of patient prognosis lies in these new technologies, the inherent capacity of malignant glioma cells to infiltrate normal brain tissue enables the malignant cells to escape surgical and radiological therapies that focus on eradicating the main tumour mass. This poses a serious clinical challenge, as these infiltrative cells are widely believed to be responsible for tumour recurrence which leads to patient death.

To combat these infiltrative malignant glioma cells, the use of chemotherapeutics, which have wide-spread targeting abilities, has become a staple of malignant glioma treatment. The use of carmustine-impregnated biodegradable wafers post- surgical resection of GBM increases the time to disease progression and overall survival in selected patients (Westphal, Hilt et al. 2003; Ewend, Brem et al. 2007). Administration of TMZ concurrently and post-radiotherapy has improved overall survival (Mason, Maestro et al. 2007). While the value in extending patient survival using the above-mentioned agents is irrefutable, the side-effects of these compounds combined with limits to their efficacy cannot be ignored. For example, implantation of carmustine-impregnated biodegradeable wafers is associated with more frequent episodes of brain seizures, oedema, and intracranial infections in patients receiving this intervention (Brem, Piantadosi et al. 1995; Valtonen, Timonen et al. 1997). With regards to TMZ, it is now recognized that its efficacy is directly related to the silencing of the *MGMT* gene promoter by methylation, limiting its usefulness to a subset of patients (Stupp, Hegi et al. 2009).

The failure of current therapies in successfully curing patients with malignant glioma is not surprising. It is obvious that without the direct targeting of 'renegade' malignant cells that have infiltrated the normal brain paranchyma, a cure will not be possible. To this end, we feel that our work on FABP7 and its role in malignant glioma cell migration may lead to novel clinical applications that may result in a cure.

5.1.2 An important role for FABP7 in MG

Here, using U87MG and U251MG cells lines, the former FABP7(-) and the latter FABP7(+), we established a unique malignant glioma cell model system to delineate a functional role for FABP7 in MG. By expressing FABP7 in U87MG, and knocking down FABP7 levels in U251 using RNAi, we generated clonal populations of cells that differ in FABP7 levels compared with their parent cell line. U87-FABP7(-), U87-FABP7(+), U251-FABP7(+), and U251-FABP7(reduced) clonal populations were used to address the role of FABP7 in MG. We report that FABP7 expression results in an increase in cell migration. Conversely, reducing FABP7 expression results in a decrease in cell migration. In support of a role for FABP7 in cell migration, we also show that FABP7 is expressed at sites typically associated with malignant glioma cell infiltration: i.e. surrounding blood vessels and at the sub-pial surface (Bellail, Hunter et al. 2004). These data suggest that the spread of astrocytoma cells within the brain is through migration of FABP7-expressing tumour cells. In addition to migration, expression of FABP7 also results in reduced rates of proliferation, a decrease in anchorage independence, and altered cell morphology.

Furthermore, we show that the cell migration effects exerted by FABP7 are inextricably related to its role as a fatty acid binding protein. Inhibition of fatty acid binding to FABP7 by site-directed mutagenesis of residues crucial for this activity demonstrates that FABP7-dependent cell

migration is dependent on binding to arachidonic acid. In addition, binding of FABP7 to DHA can override the pro-migratory effects of FABP7-AA, thus inhibiting the migratory process. It is generally accepted that ω-3 fatty acids have anti-tumourigenic properties, whereas the effects of ω-6 fatty acids are often reported to be pro-tumourigenic (Gleissman, Johnsen et al. 2010). Our data are consistent with these observations; however, the importance of fatty acid binding proteins in mediating the effects of fatty acids is a novel finding. Analysis of downstream effectors of FABP7/AA suggests that FABP7 transfers AA to the COX-2 pathway, thus increasing the production of PGE_2, a well-known pro-migratory signalling molecule (Greenhough, Smartt et al. 2009). DHA inhibition of cell migration is dependent on PPARγ and may function by competing with AA for binding to FABP7.

Lastly, we demonstrate that expression of FABP7 can alter the lipid profile of malignant glioma cells This is an important observation in light of reports indicating that malignant glioma tissue have increased levels of AA and decreased levels of DHA compared with normal brain. Cellular uptake of DHA and AA and the incorporation of these fatty acids into the plasma membranes of malignant glioma cells are significantly higher in FABP7-expressing compared to non-expressing malignant glioma cells. Our data implicate FABP7 in altering the lipid profile of MG. We showed that the incorporation of AA into the phospholipid pool is widespread and non-specific to the specific phospholipids analyzed; whereas DHA is

preferentially incorporated in the phosphatidylserine (PS) species of phospholipids in FABP7 cells. While we do not fully understand the significance of DHA incorporation into PS in malignant glioma cells, others have shown that DHA-rich PS may serve to inhibit pro-migratory signaling events at the level of the cell membrane (Nishizuka 1986; Walker and Olson 2005).

5.1.3 A developmental parallel for FABP7-induced cell migration

Besides revealing new pathways that may be targeted for the treatment of patients with malignant glioma, our results also serve to further the notion that malignant glioma is a disease with developmental origins. As previously discussed, there are similarities in the signaling pathways and protein expression patterns associated with neurogenesis and gliomagenesis. Not surprisingly, the expression pattern of FABP7, the uptake and incorporation of DHA and AA, and the expression of PPARs are no exception to this developmental-like trend of glioma expression.

FABP7 is normally expressed specifically in radial glial cells during CNS development, with levels peaking between ED12/14 and P0/5 (Feng, Hatten et al. 1994; Kurtz, Zimmer et al. 1994). Interestingly, peak levels of FABP7 in the developing murine brain coincides with neuronal and glial cell migration, with subsequent decreases in expression coinciding with the progressive differentiation of cells in the developing CNS. Addition of anti-FABP7 antibodies to primary cell cultures prevents the extension of radial

glial bipolar processes, thus inhibiting neuronal migration, suggesting that FABP7 plays an important role in the migration of cells during CNS development (Feng, Hatten et al. 1994). Following neuronal cell migration, radial glial cells migrate into the subventricular zone and differentiate into FABP7-low/negative expressing astrocytes (Schmechel and Rakic 1979). In humans, the radial glial fiber system and the migration of neuronal precursors occurs at 5-weeks gestation (Levitt 2003). However, the levels of FABP7 at this time have not been analyzed and can only be inferred from model systems such as the mouse. Recent reports have shown that radial glia demonstrate properties of neural stem cells as they can give rise to different types of cells in the brain, including migratory neuronal precursors and astrocytes (Malatesta, Hartfuss et al. 2000; Noctor, Flint et al. 2001; Parnavelas and Nadarajah 2001; Campbell and Gotz 2002; Bonfanti and Peretto 2007). These data indicate that FABP7 is specifically found in less differentiated neural cell types that may be the cell-of-origin of malignant glioma.

Although the fatty acid profile of the developing human brain is incomplete, research by Martinez *et al.* (1989) provides some insight during the last trimester of pregnancy. At 30-weeks gestation in humans (far beyond the 5-week cell migration period), the concentration of AA in the brain is greater than that of DHA. At this time, the levels of DHA begin to increase quadratically while the levels of AA decrease linearily (Martinez 1993). These data indicate a low DHA:AA ratio during early

neural development when FABP7 expression is high and cell migration is common, in contrast to the higher DHA:AA ratio during later stages of neural development when cell migration is less common and FABP7 levels are decreasing. The lower DHA:AA ratio observed during early CNS development parallels the fatty acid profile of MG. When combined with data showing elevated levels of FABP7 in both malignant glioma and the developing brain, the importance of deregulated developmental signaling in the promotion of malignant glioma cell migration becomes apparent.

All three PPAR isotypes (PPARα, PPARβ, and PPARγ) are co-expressed in the rat nervous system during late embryogenesis, with levels of all three PPARs peaking between day 13.5 and 18.5 of gestation. However, whereas PPARβ/δ remains highly expressed in the post-natal brain, levels of PPARα and PPARγ show significant decreases postnatally (Braissant, Foufelle et al. 1996; Braissant and Wahli 1998; Cullingford, Bhakoo et al. 1998). Much like the late stages of rat brain development, malignant glioma express all three PPAR isotypes (Benedetti, Galzio et al. 2010). These data suggest that insight into malignant glioma development and progression may be gained by studying the mechanisms underlying neural development.

5.1.4 Future directions

The inherent capacity of malignant glioma cells to penetrate normal brain tissue presents a serious clinical challenge because these infiltrative

cells are widely believed to be responsible for tumour recurrence following surgery, radiation, and chemotherapy. Cell migration in general, and malignant glioma cell migration in particular, is a complex and dynamic process that involves many biological features. Malignant glioma tumour cell invasion in the brain shares characteristics with stromal infiltration by non-neural carcinomas, namely: (1) cell detachment from the primary tumour mass and receptor-mediated adhesion to components of the ECM (Giese, Rief et al. 1994), (2) activation of processes related to cell motility such as actin reorganization through modifications in integrin-extracellular matrix (ECM) interactions (Palecek, Loftus et al. 1997), and (3) degradation of ECM by the secretion of proteolytic enzymes (Belien, Paganetti et al. 1999).

To identify pathways altered as a consequence of FABP7 expression, we need to identify differentially expressed cell migration/cell adhesion-related genes in FABP7(-) and FABP7(+) cell populations. As a first step, our lab has already carried out cDNA microarray analysis of U87-FABP7(-) versus U87-FABP7(+) cell lines. A number of differentially expressed transcripts encoding proteins involved in cell migration and adhesion have been identified in this manner, including tensin1, integrin β4, and Rac3. Validation of differential expression followed by manipulation of the expression of these genes is a logical next step to understanding the role of FABP7 in malignant glioma. Furthermore, cDNA microarray analysis of U87-FABP7(-) and U87-FABP7(+) cell lines treated

with DHA and AA has also been carried out and will hopefully lead to the identification of differentially expressed target genes that can then be analyzed in order to gain further insight into the mechanisms underlying FABP7-induced malignant glioma cell migration.

To validate the differential expression of genes of interest *in vivo*, we will carry out double immunohistochemical staining of malignant glioma tissue samples and look for overlapping FABP7 and target protein expression. It has previously been demonstrated that RNAs isolated from single cells of paraffin-embedded and immunohistochemically stained tissue sections are sufficient to serve as a template for RT-PCR (Becker, Becker et al. 1996; Roehrl, Becker et al. 1997; Suarez-Quian, Goldstein et al. 1999). Given that only a subset of cells in malignant glioma tissue express FABP7 and specific antibodies for proteins of interest are not always readily available, this technique would be of value in analyzing the intratumoural expression of our genes of interest in FABP7(-) and FABP7(+) cells.

Proliferation and migration have been proposed to be mutually exclusive cell properties (Giese, Loo et al. 1996). That is, if a cell is proliferating, it does not migrate, and if a cell is migrating, it does not proliferate. Interestingly, FABP7 expression slows proliferation of U87MG and U251MG at the expense of increasing cell migration. It has been postulated that a component of the switch between proliferation and migration is controlled by the Crk-associated substrate (CAS)/Crk pathway

(Klemke, Leng et al. 1998). Recently, Nomura *et al.* (2007) showed that PKC induced cell migration in glioblastoma cell line A172 was dependent on activation of the Cas/Crk signaling pathway (Nomura, Nomura et al. 2007). As we have shown that FABP7 expression results in the preferential incorporation of DHA into phosphatidylserine, an event that can inhibit the activation of PKC, investigation into whether Cas/Crk signaling activates cell migration in our system warrants further investigation.

PPARs are known to bind PPRE DNA elements which are usually found upstream of transcription start sites of genes. Our data suggest that PPARγ and, to a lesser extent, PPARβ, may be downstream effectors of FABP7/fatty acid-mediated cell migration. Chromatin immunoprecipitation (ChIP) can be used to identify PPAR targets that are dependent on interactions with FABP7 and its fatty acid ligands. Experiments will focus on differences and similarities in PPAR-DNA binding in U87-FABP7(-) versus U87-FABP7(+) cell populations treated with DHA or AA. Genes of interest would include those involved in cell migration as well as genes involved in PUFA synthesis. These experiments are important because they will provide insight into the role of FABP7 in the nucleus. Furthermore, identification of genes regulated by FABP7/DHA/AA/PPAR interactions will shed light on how these factors act in concert to control specific pathways. The understanding of these pathways may lead to the development of novel agents for treating MG.

In addition to activating PPARs to control gene expression, we hypothesize that FABP7 itself may act as transcription factor. The basis for this theory lies in the following facts: (i) FABP7 forms homodimers when bound to DHA, a common trait of transcription factors, (ii) upon binding to DHA, FABP7 translocates to the nucleus indicating it may be activated upon binding, and (iii) the structure of FABPs consists of a helix-turn helix motif, a tertiary structure that has long been known to be a DNA-binding motif (Brennan and Matthews 1989). Thus, another avenue of exploration would be to use ChIP to test whether FABP7 binds directly to DNA. This has never been shown before with any other member of the FABP family, and would be a significant discovery.

Our results indicate that FABP7 expression results in significant alterations in the fatty acid composition of the cell membrane. To further explore how changes in fatty acid composition might affect membrane-associated signal transduction, we could examine the incorporation of DHA and AA into membrane lipid rafts. Lipid rafts are specialized membrane microdomains enriched in specific lipids, cholesterol and proteins. Many functions have been attributed to lipid rafts, including cholesterol and protein trafficking, regulation of neurotransmission, and assembly of signaling molecules (Pike 2009). Small invaginated membrane structures called “caveolae” are the best characterized lipid rafts. Caveolins are widely expressed in the nervous system, including oligodendritic and astrocytic cells (Korade and Kenworthy 2008). Caveolae are known to be enriched in

arachidonic acid (Pike, Han et al. 2002), suggesting that arachidonic acid may play an important role in signal transduction through lipid rafts.

In addition, lipid rafts appear to play a role in signaling through both the insulin and insulin-like growth factor receptors (Bickel 2002; Vainio, Heino et al. 2002). A number of studies suggest that ω-3 fatty acids provided in the diet and in culture can modulate aspects of insulin signaling in various cell types (Yorek, Leeney et al. 1989; Murata, Kaji et al. 2001; Taouis, Dagou et al. 2002). In addition, insulin signaling has been shown to influence cell migration in malignant glioma cell lines (Schlenska-Lange, Knupfer et al. 2008; Mendes, Wang et al. 2010). Furthermore, FABPs have been linked to insulin resistance in animal mouse models (Boord, Fazio et al. 2002). Therefore, an examination of the effects of ω-3 and ω-6 fatty acids on raft modulation of insulin receptor and insulin-like growth factor receptor-associated signaling is warranted.

In addition to altering cell signaling pathways, PUFAs also alter the blood–brain barrier by increasing the permeability of cells (Hussain and Roots 1994). Membrane permeability can regulate the influx/efflux of hydrophobic drugs that pass through the membrane by diffusion (Das, Madhavi et al. 1998). In support of this, enrichment of ω-3 fatty acids in cell membranes correlates with an increase in the intracellular accumulation and/or retention of chemotherapeutic drugs (doxorubicin, vincristine, mitoxantrone) and enhanced drug cytotoxicity in a variety of tumour cell types (Burns and North 1986; Ikushima, Fujiwara et al. 1991;

Abulrob, Mason et al. 2000; Vibet, Maheo et al. 2007). Increased uptake of hydrophilic drugs may also occur as ω-3 PUFAs can affect transport proteins interspersed within the membrane. For example, EPA and DHA treatment increased the rate of purine uptake by a nucleoside transport protein in lymphoblastic leukemia cells *in vitro* (Martin and Meckling-Gill 1996). It would be interesting to explore how FABP7 expression and different fatty acids affect uptake and/or retention of common drugs used to treat patients with malignant glioma such as temozolamide or BCNU.

The blockade of PGE_2 production using COX inhibitors such as NSAIDs or specific COX-2 inhibitors such as celecoxib, rofecoxib and NS-398 have shown potential to inhibit both tumour growth and metastasis in experimental animal models (Dandekar and Lokeshwar 2004; Patel, Subbaramaiah et al. 2005). Encouraging data from these model systems have led to clinical trials designed to assess the efficacy of these compounds in the clinic. As evidenced by both the VIGOR and APC clinical trials, COX-2 inhibitors showed moderate to no effect on tumour growth, but did increase the risk of adverse cardiovascular events (Krotz, Schiele et al. 2005); (Luo, He et al. 2005). Unlike the synthetic COX-2 inhibitors, ω-3's are widely believed to be associated with a reduced risk of cardiovascular disease and sudden cardiac death (Marchioli, Barzi et al. 2002; Holub and Holub 2004). In addition, studies have shown that doses of up to 4 g/day of DHA are well tolerated (Harrison and Abhyankar 2005). Therefore, the use of DHA as a natural adjuvant therapy for patients with

malignant glioma may prove to be a potent COX-2 inhibitor that functions to block PGE_2 production, thereby inhibiting malignant glioma cell migration while sparing the surrounding healthy tissue.

A natural next step to test the clinical implications of our findings is the generation of an *in vivo* murine model system. To study the effect of FABP7 and PUFAs on tumour cell migration *in vivo*, U87-FABP7(-) and U87-FABP7(+) cell lines could be transfected with a Green Fluorescent Protein (GFP) expression construct. The resulting cells would then be intracranially injected in athymic nude mice according to previously described protocols (Strojnik, Kavalar et al. 2006). Tumour cells are allowed to grow for seven days at which point MRI imaging is performed weekly to assess tumour growth. Once the tumour has formed, 50 µmol/L of fatty acid (DHA or AA), prepared as per Huber *et al.* (2006), is injected through the tail vein of the mouse once every 8 hours for 24 hours (Huber, Kampf et al. 2006). Once the tumours have reached an appropriate volume, the tumour can be resected, frozen, and subjected to GLC to determine fatty acid composition.

To investigate migration of U87 cells, the brain (minus resected tumour) can be embedded in paraffin and sectioned coronally through the injection site as previously described (Bradley, Kataoka et al. 1999). Immunofluorescence can then be used to locate FABP7(+) cells in normal brain. FABP7(+) tumour cells originating from the main tumour mass will be differentiated from normal FABP7-expressing cells by their co-

expression of GFP. The number of FABP7(+)/GFP(+) and FABP7(-)/GFP(+) control cells migrating away from the main tumour and their distance of migration can then be quantified. The results of this experiment will indicate whether the relationship between FABP7 and its fatty acids *in vitro* holds true *in vivo*.

5.1.5 Conclusion

Excluding this publication, studies on FABPs and cancer, including FABP7, have been observational/correlational, with no data on functionality being published. Here, we show the existence of an intimate relationship between protein (FABP7), ligand (DHA and AA), and subcellular localization (nuclear vs. cytoplasmic) in modulating malignant glioma cell migration. Our study links FABP7-expression and the availability of fatty acid ligands to the spread of neoplastic cells in the brain, revealing potential targets for malignant glioma therapy. The possibility of replacing current chemotherapeutic agents with natural and safe products such as ω-3 PUFAs is a novel and manipulatable approach to malignant glioma intervention and possibly prevention. The potential application of FABP7 as a biomarker to predict tumour-suppressive response to ω-3 PUFAs warrants further investigation. Finally, our results lend credence to the paradigm integrating signaling pathways in neural development with those present during gliomagenesis.

5.2 References

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