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A Dynamic Transcriptome Technique for Transcriptional Profiling and Gene Regulatory Network Involving the Helicase Antigen (HAGE).

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Copyright Statement

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ACT	Adoptive T cell therapy
ADCC	Antibody-dependent cytotoxicity
AFP	Alpha-feto protein
ALL	Acute lymphoblastic leukaemia
AML	Acute myeloid leukaemia
APC	Antigen presenting cell(s)
APML	Acute promyelocytic leukaemia
APS	Ammonium persulphate
ATP	Adenosine tri-phosphate
BC	Blast crisis
BER	Base excision repair
BM	Basement membrane
BSA	Bovine serum albumin
β2M	Beta-2 microglobulin
CAGE	Cancer-associated antigen
CDK	Cyclin-dependent kinase
CEA	Carcioembryonic antigen
CHOP	Cyclophosphamide, doxorubicin, vincristine, prednisone
CMC	Complement-mediated cytotoxicity
CML	Chronic myeloid leukaemia
CMML	Chronic myelomonocytic leukaemia
CP	Chronic phase
CSC	Cancer stem cell(s)
CT	Cancer/testis
CTL	Cytotoxic T cell(s)
CTLA-4	Cytotoxic T lymphocyte antigen-4
CT-X	X-linked CT antigens
DC	Dendritic cell(s)
ddH_2O	Double-distilled H_2O
DDX	DEAD-box protein family member
DLBCL	Diffuse large B cell lymphoma
DMSO	Dimethyl sulfoxide

DPBS	Dulbecco's phosphate buffered saline
DTT	Dithioreitol
EBV	Epstein Barr virus
ECM	Extracellular matrix
EDTA	Ethyldiamine tetraacetic acid
EGF	Epidermal growth factor
EMT	Epithelial-mesenchymal transition
FACS	Flow-assisted cell sorting
FAK	Focal adhesion kinase
FAS-L	FAS-ligand
FCS	Foetal calf serum
FGF	Fibroblast growth factor
GAP	GTPase activating protein
GDP	Guanosine di-phosphate
GEF	Guanosine exchange factor
GIST	Gastrointestinal stromal tumour
GPCR	G-protein coupled receptor(s)
GSK-3β	Glycogen synthase kinase-3β
GTP	Guanosine tri-phosphate
HAGE	Helicase antigen
HCC	Hepatocellular carcinoma
HIF	Hypoxia inducible factor
HPV	Human papilloma virus
HR	Homologous recombination
HSP	Heat shock protein
HTLV-1	Human T-lymphotrophic virus type 1
H_2O_2	Hydrogen peroxide
^3H	Tritiated thymidine
IAP	Inhibitors of apoptosis
ICE	Ifosfamide, carboplatin, etoposide
IFN	Interferon
Ig	Immunoglobulin
IGF	Insulin growth factor

IGRT	Image guided radiation therapy
IL	Interleukin
IMRT	Image modulated radiation therapy
JMML	Juvenile myelomonocytic leukaemia
LOH	Loss of heterozygosity
LOI	Loss of imprinting
LSC	Leukaemia stem cell
mAb	Monoclonal antibody
MAGE	Melanoma-associated antigen
MAPK	Mitogen activated protein kinase
MDS	Myelodysplastic syndromes
MGUS	Monoclonal gammopathy of unknown significance
MHC	Major histocompatibility complex
MM	Multiple myeloma
MMP	Matrix metalloproteinase(s)
NGS	Next generation sequencing
NHL	Non-Hodgkin's lymphoma
NK	Natural killer
NOD/SCID	Non-obese/severe combined immunodeficiency
NSCLC	Non-small cell lung carcinoma
NTC	Non-template control
PARP	Poly-ADP ribose polymerase
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PH	Pleckstrin homology
PIP_2	Phosphotidylinositol-bis-phosphate
PIP_3	Phosphotidylinositol-tris-phosphate
PI3K	Phosphotidylinositol-3-kinase
PKB	Protein kinase B
PSA	Prostate-specific antigen
PTEN	Phosphatase and tensin homologue
qPCR	Quantitative PCR
Rb	Retinoblastoma
RCC	Renal cell carcinoma

RDA	Representational difference analysis
RNAi	RNA interference
ROS	Reactive oxygen species
RT	Room temperature
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription PCR
SAGE	Sarcoma-associated antigen
SCF	Stem cell factor
SCT	Stem cell transplant
SDS	Sodium dodecyl sulphate
SEREX	Serological analysis of recombinant cDNA expression
shRNA	Small-helical RNA
siRNA	Small-interfering RNA
TAA	Tumour-associated antigen
TBI	Total body irradiation
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper
TIL	Tumour infiltrating lymphocyte(s)
TIMP	Tissue inhibitor of metalloproteinase
TKI	Tyrosine kinase inhibitor
T_{Reg}	Regulatory T cell(s)
TSG	Tumour suppressor gene
VEGF	Vascular endothelial growth factor
WT	Wilm's tumour
XIAP	X-linked inhibitor of apoptosis protein

Abstract

Increased knowledge into the molecular pathways disrupted in tumours has led to the development of various therapies that can target specific mediators of these cascades. Such therapies have proven successful in patients or demonstrate significant potential for clinical use. However, this better understanding is undermined by the continued prevalence of cancer and the limitations of these drugs. Therefore, it is possible signalling networks could be influenced by as yet unknown molecules or known mediators with function that have not yet been described. As a result of this, work must continue to improve knowledge yet further to allow the design of novel therapies to aid in the treatment of cancer patients. In the present study, work was performed on the helicase antigen (HAGE), a cancer/testis (CT) antigen and DEAD-box protein found to be present in numerous types of malignancy. As with the majority of CT antigens, the role of HAGE remains unclear. In this instance, studies were carried to discover the function of HAGE in malignant cells.

Preliminary *in vitro* proliferation studies following HAGE gene knockdown or cDNA transfection strongly indicated an association between HAGE expression and increased tumour cell proliferation. This was supported by results gained from *in vivo* work performed within an immuno-compromised murine model. Expression profiling analysis of data gained from using the Genechip oligonucleotide microarray platform found significant changes to genes linked not only with proliferation but other cell processes altered during tumorigenesis. Confirmation using real-time qPCR suggested change in expression of certain genes could be recognised in other HAGE-expressing tumour cell lines. This analysis also indicated a possible interaction between HAGE and the oncogene N-RAS. Subsequent genetic and protein studies implicated HAGE acting upstream of N-RAS, markedly increasing the N-RAS level in cells. Very preliminary work has begun to demonstrate a role not just in proliferation, but in immune escape, apoptosis inhibition and metastasis, all processes potentially influenced by the RAS oncogenes. The data presented here strongly supports the hypothesis of HAGE having a significant role in malignant biology and warrant continued investigation to further confirm its role in cancer and possibly use as a target for malignancy in the future.

Chapter 1

Introduction

1.1 Tumorigenesis and the Basis of Cancer

Cancer remains one of the leading causes of both morbidity and mortality worldwide and is a disease that is especially evident in western civilisations. In 2008, there were 156,723 reported cancer deaths in the U.K., which is an increase from the 155,484 deaths recorded in 2007 (Cancer Research U.K). Disconcertingly, large numbers of people die as a result of tumours in spite of massive advancements in the diagnosis and treatment of malignancies. Progress in detection and therapy has been achieved alongside considerable investment in the education of the public regarding cancer risk factors and encouraging lifestyle changes. It is perhaps recognition of how complex a disease cancer is that it continues to represent such a prominent public health concern in the face of improved techniques and attitudes.

Cancer is a disease born out of a disruption in homeostasis, the systems of control that exist to maintain a rigorously balanced environment in cells (Bertram, 2001). Behavioural aspects of cells such as proliferation are kept in check by a highly complex series of molecular networks (Weinstein *et al*., 1997; Weinstein, 2000; Pedraza-Fariña, 2006). These consist of a vast array of molecules with highly divergent functions, which themselves are subject to strict levels of control in normal circumstances. A great deal of evidence now exists to highlight that during the development of tumours, homeostatic control systems undergo disturbance and are undermined (Weinstein, 2000; Bertram, 2001; Pedraza-Fariña, 2006). As a result, malignant cells display characteristics that almost represent a mirror image of their healthy counterparts. They can exhibit uncontrollable proliferation, resistance to apoptosis, are capable of invasion, self-sufficiency with regards to growth factors, increased resistance to the attentions of the immune system and abnormal angiogenesis. Such traits are referred to as the ‘hallmarks’ of cancerous cells (Hanahan and Weinberg, 2000).

The loss of stringent regulation and acquisition of novel function is a key stage in the development of a malignant tumour; the phenomenon known as tumorigenesis. This is an immensely intricate series of events that sees the transformation of a cell to attain a malignant phenotype, which has the potential to occur over a very long period of time. The widely held belief is that for a tumour to begin growing, at some point healthy cells must be exposed to materials/factors that are able to inflict damage to the genomic material within them, referred to as carcinogens. In the duration of their lifetime a person is subjected to countless different carcinogenic influences that are provided not only from the external environment but internally as well (Stratton *et al*., 2009). It is thought that DNA could be exposed to as many as 10,000 events per day capable of causing damage (Fortini *et al*., 2003; Sandhu *et al*., 2010). Examples of these include:

Environmental Carcinogens:

Humans readily come into contact with huge numbers of factors present in their immediate environment that have the ability to modify the physical structure of genes and are arguably the most common group of carcinogens encountered by them. Carcinogens in this category are diverse, ranging from industrial chemicals through to dietary intake. Cigarette smoke, where chemicals such as benzo[a]pyrene and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) have been associated with lung carcinoma (Shopland, 1995; Hecht, 1999), is perhaps the best known instance. Another well known example, radiation, can have profound effects on the structure of DNA, either through prolonged exposure to ultra-violet radiation (Pfeifer *et al*., 2005) or even through the use of therapeutic radiotherapy.

Viral Carcinogens:

As part of their infection cycle, viruses integrate their genome with that of the host. Numerous viruses posses genes that once unified with human genetic material induce malignant characteristics (Butel, 2000; Carillo-Infante *et al*., 2007). This comes from viral proteins being able to modify various aspects of cell behaviour. Human papilloma virus (HPV) infection is the major cause of cervical cancer and can lead to the development of head and neck carcinoma (Schiffman *et al*., 2007; Lajer and von Buchwald, 2010). The E6 and E7 gene products of HPV are able to modify different host cell molecules including p53 and retinoblastoma (Rb) along with AP-1 and E2F

transcription factors. These interactions immortalise cells and induce proliferation through increased cell cycle progression (Antinore *et al*., 1996; Hwang *et al*., 2002; Malanchi *et al*., 2002; Malanchi *et al*., 2004; Schiffman *et al*., 2007; Ghittoni *et al*., 2010).

Similar relationships can be seen with other viruses with tumorigenic properties. Epstein Bar virus (EBV) bears the EBNA and LMP gene products, which have been implicated in the generation of immortalisation, proliferation by interacting with c-MYC and affecting signalling pathways. This virus is thought to be a cause of lymphomas including Burkitt's lymphoma (Young and Murray, 2003; Thompson and Kurzrock, 2004). The TAX protein, generated from the human T lymphotrophic virus type-1 (HTLV-1) interacts with NF-κB to immortalise cells and with AP-1 transcription factors and represses the actions of cyclin-dependent kinase (CDK) inhibitors including $p21^{CIP1}$ and $p27^{KIP1}$ to promote proliferation (Grassmann et al., 2005; Boxus and Willems, 2009). The hepatitis C virus (HCV) core protein, implicated in hepatocellular carcinoma (HCC) can prevent apoptosis by affecting p53, induce cell cycle progression by interacting with Rb and CDK inhibitors and has been linked with angiogenesis (Hassan *et al*., 2009; Tsai and Chung, 2010).

Epigenetic Events:

Whereas the factors above cause changes to the coding sequence in genes, it is possible that chromatin material as a whole can be altered to bring about cancer through epigenetic modifications (Feinberg and Vogelstein, 1983; Cui *et al*., 2002). A large proportion of human gene promoter regions contain CpG islands, which may be subject to spontaneous demethylation/methylation. This can lead to the expression of genes that promote malignant behaviour or inhibit the action of those that act to inhibit transformation respectively, though the mechanisms that cause it still remains unclear (Esteller *et al*., 2001; Ehrlich, 2002; Eden *et al*., 2006). During embryogenesis, the gene alleles inherited from one parent are inactivated (imprinted) to stop their expression. In tumours, certain genes can lose this repression, called 'loss of imprinting' (LOI), resulting in the over expression of certain genes that may lend weight to transformation, as seen with insulin growth factor II (IGF-II) (Murrell, 2006). Conversely, it may be that the alleles of other genes needed to maintain cell

integrity may be randomly lost, causing 'loss of heterozygosity' (LOH), reducing the activation of that gene. If the other allele of that gene is lost via deletion or mutated, then combined LOH effectively results in the loss of that gene's expression. This is especially evident with BRCA1/2 mutations in breast carcinomas (Palacios *et al*., 2008).

If repair mechanisms cannot repair the initial damage to DNA, there is a significant risk in the primary cell acquiring yet more modifications. Cancer is a condition that develops over many years and consists of different phases: the so-called 'multi-stage' course (Vogelstein and Kinzler, 1993; Ito *et al*., 1995; Duesberg and Li, 2003). If the primary affected cell is not removed from the population by apoptosis, then it will continue to proliferate and its daughter cells will retain this initial mutation (Bertram, 2001). Cancer is considered to be a clonal disease, as all tumour masses are derived from this primary affected cell (Greenman *et al*., 2007; Stratton *et al*., 2009). This clone could be derived from a particular stem cell population in a tissue, expanded on later. Over a prolonged period of time, these cells will acquire increasing number of mutations and other modifications. This is highlighted in Figure 1.1. If modification occurs in a significant genetic region, then over time the transformed cell accumulates mutations and other subtle modifications that contribute to the development of a malignant cell (Futreal *et al*., 2001).

Until recently the number of mutations required for cells to be classified as truly malignant was unknown. Recent studies involving the use of Next Generation Sequencing (NGS) analysis, allowing the interrogation of the entire genome of cancer cells in relatively short periods of time, have started to shed light on this question. It has been proposed that a specific malignancy may involve anything from 1000 to 100,000 mutations depending on the individual and tumour type (Stratton *et al*., 2009). NGS has been employed alongside other technology to discern that specific mutational number required for tumour transformation in lung cancer and melanoma is 29,910 and 32,325, respectively (Pleasance *et al*., 2010a; Pleasance *et al*., 2010b). This work has highlighted how extensive genetic damage needs to be for a cell to be fully transformed and further emphasises the complexity of the disease.

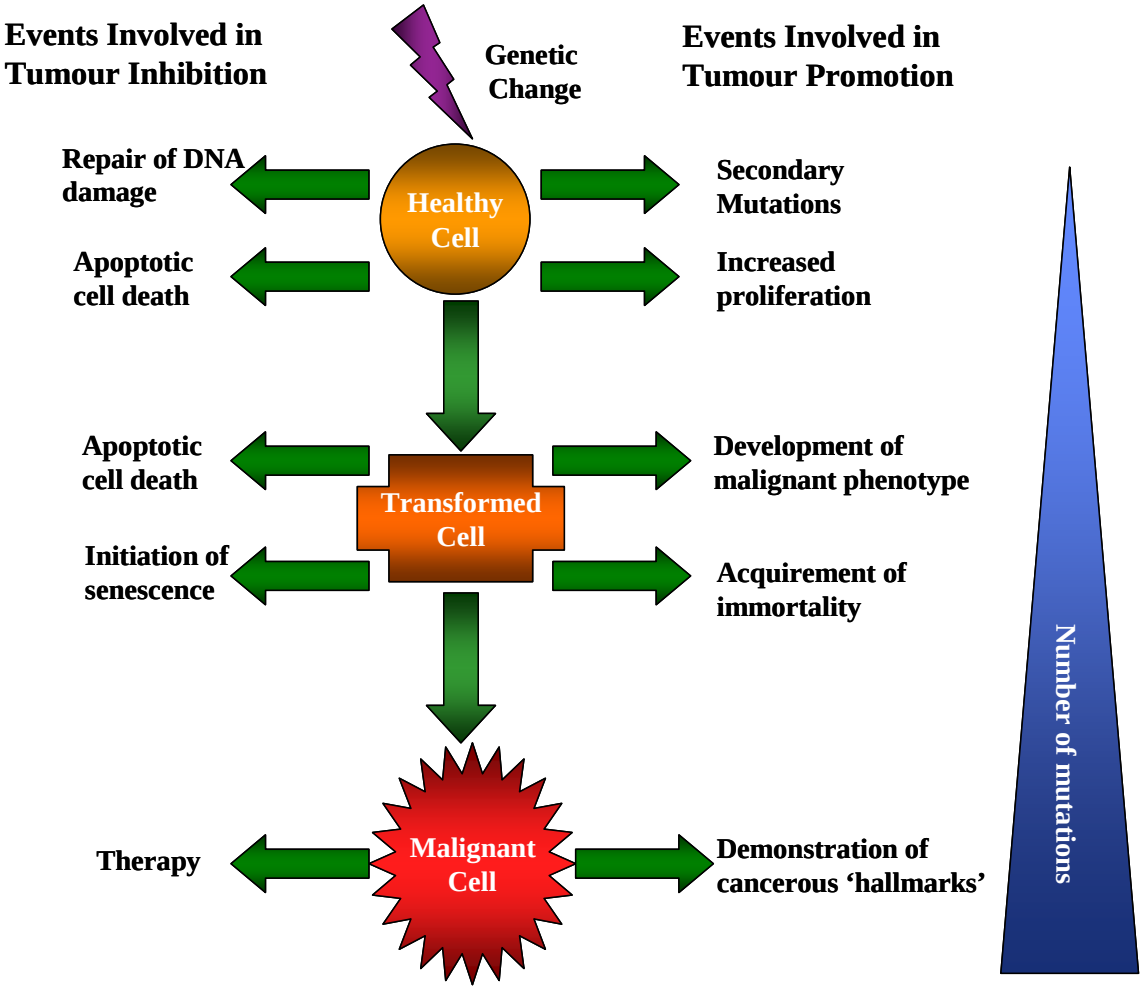


Figure 1.1: *Basic overview of events involved in the formation of a malignant cell, charting progress from initial genetic change to acquirement of full malignant phenotype. The increasing number of mutation that occur during progression are highlighted, as are steps involved to promote or prevent malignant development. Adapted from Betram, 2001.*

1.2 The Genetic Origins of Tumours

1.2.1 Concepts of Cancer Genomics

As previously alluded to, initiation of tumorigenesis is brought about genetic and epigenetic changes to genomic material. This can come in the form of somatic mutation induced by carcinogens or by the inheritance of germline mutations transmitted vertically from parent to offspring. It is a widely held belief that in the majority of instances, a cell can withstand certain levels of damage to its genome thanks to the existence of various safeguards such as damage checkpoints, DNA repair systems and the presence of tumour suppressor genes. Carcinogenic exposure has several different ways of potentially harming genes, whether it is through physical mutation or structural modifications to chromosomes. These range from point mutations (RAS, p53) affecting individual or very small number of nucleotides, or chromosomal alterations including translocations (BCR-ABL, PML-RARα), inversions, or amplification (MYC) that disrupt larger genomic regions (Futreal *et al*., 2004; Weir *et al*., 2004; Stratton *et al*., 2009).

There is an array of different proofreading and repair mechanisms within cells. These include homologous recombination (HR), a slower but very precise process that swaps damaged/incorrect bases for what is required (Whyman *et al*., 2004). However, in some instances the existence of these safeguards is still not sufficient to inhibit tumorigenesis, as shown by base excision repair (BER), which is prone to error and can actually create more chance of mutation (Fortini *et al*., 2003). The transition of a cell from healthy to malignant state is essentially dependant on the corruption of genes that can allow the development of cancerous hallmarks in addition to the inactivation of those genes whose products act to inhibit transformation: the proto-oncogenes and tumour suppressor genes respectively.

1.2.2 Proto-oncogenes and Impact of Their Activation

The very earliest studies into cancer genetics involved the examination of those viruses that can induce tumour formation as result of their infection of cells (Der, 1987). Over time, beginning with the discovery of the H-RAS and K-RAS genes

(Der *et al*., 1982), it was found that humans bear genetic regions homologous to those viral genes capable of cancer initiation, the human homologues being collectively termed proto-oncogenes. They are normal genes that are expressed to a normal level in healthy cells, their functions predominantly centred on cell signalling pathways involving proliferation, cell cycle progression, response to growth factors and angiogenesis. However, as a result of carcinogenic exposure, they become altered to form oncogenes, whereby their normal function is disrupted and utilised to induce tumorigenesis (Polsky and Cordon-Cardo, 2003). They are one of the most prevalent and influential groups of genes in cancer, not least due to their activation resulting in a significantly raised chance of tumorigenesis taking place (Der, 1987). Conversion of a proto-oncogene to its oncogenic state results in a 'gain of function' characteristic whereby their biological function, which is stringently controlled in a healthy environment, is allowed unrestrained activity, thus promoting malignant transformation (Hirota *et al*., 1998; Hirota *et al*., 2003).

Activation of oncogenes comes about through alterations to the regions of genetic code in which they reside. This can be through point mutations, affecting the protein product of the gene, increasing its activation level as in the case of B-RAF or JAK (Hingorani *et al*., 2003; Percy and McMullin, 2005; Kaushansky, 2007; Kilpivaara and Levine, 2008; Nucera *et al*., 2009), increasing its half-life or increasing its resistance to degradation species or inactivation, exemplified by RAS (Downward, 2003; Omerovic *et al*., 2007). It may also involve the physical modification of chromosomal regions that contain these genes, removing inhibitory regions via deletion thereby increasing gene expression, or transporting genes from one chromosomal region to another by way of translocation or inversion to situate a oncogene next to a highly active promoter, leading to near-constant transcription and translation of that gene (Polsky and Cordon-Cardo, 2003). This can be seen with the formation of the BCR-ABL and PML-RARα fusion genes central to chronic myeloid leukaemia (CML) and acute promyelocytic leukaemia (APML) respectively (Pandolfi, 2001; Hehlmann *et al*., 2007).

Since proto-oncogenes are actively involved in processes that increase cell populations, their mutation/conversion to an oncogenic form is considered to be an

almost forceful step in the development of malignancy in cells. In this sense, such mutations can be referred to as 'driver' mutations as such alterations compel transformation towards malignancy (Greenman *et al*., 2007; Stratton *et al*., 2009). Deregulation of pro-growth molecules provides a transformed cell with the necessary ability to uncontrollably proliferate, produce its own growth factors and potentially synthesise new vascular bundles. In this manner, it is possible for a transformed cell to achieve neoplastic expansion, however safeguards do still exist in these cells, meaning that if tumours are to develop, they must interrupt these in order to realise their full potential.

1.2.3 Tumour Suppressor Genes and Consequences of Their Loss

A cell has numerous ways in which it can protect itself from transformation. One of the most powerful is the existence of a group of genes who generate products capable of preventing cells that undergo genetic damage from furthering their progression: the so-called tumour suppressor genes (TSG). Whereas proto-oncogenes are involved in proliferation and other characteristics that can be used to the advantage of tumours, as their name would suggest, TSG function centres on those actions that aims to prevent the advance and further transmission of genetic damage (Stanbridge, 1990; Gao and Honn, 1995; Krug *et al*., 2002). These functions include cell cycle arrest, DNA repair and promotion of apoptosis. Inactivation of TSG is an arguably more significant event in tumour cell transformation, since their presence in cells still allows tumour development to be curtailed, possibly even in the face of oncogene activation.

TSG are subject to the same genetic modifications as proto-oncogenes, however whereas these events act to potentiate the latter, with TSG such events serve to restrain their activity, hence leading to a 'loss of function' situation (Wang *et al*., 2006a). Point mutations can render the protein product of the gene with shorter half life or impair its function, perhaps best shown by p53. This highly potent TSG acts as a transcription factor and can activate or inhibit specific genes in order to halt cell cycle progress in order to allow DNA repair or permit apoptosis if damage is too great. At the same time is also the most mutated TSG present in tumours. Mutations to p53 affect the function of its protein product, reducing its potency in preventing tumour formation (Farmer *et al*., 1992, Chen *et al*., 1993, Miyashita and Reed, 1995,

Levine and Oren, 2009). Alternatively, genes can be deleted, such as PTEN and with the obvious effect that no protective protein is present to prevent transformation (Martelli *et al*., 2006). In certain instances, genes that act to inhibit TSG can undergo up-regulation, leading to inactivation of their targets, as seen with the hyper-phosphorylation of Rb, promoting a pro-tumorigenic situation (Weinberg, 1995).

The initial theory into TSG inactivation in tumours stated that both alleles of the gene had to be affected, Knudson's proposed 'two-hit hypothesis' (Knudson, 1971). For example, germline mutations in genes can be inherited from parents, which primarily affects one of the alleles. If the second allele is affected by mutation, deletion or another modification, then the expression of that gene is lost. This is found with loss of BRCA expression in both breast and ovarian carcinomas (Payne and Kemp, 2005). While this premise is still thought to be true for a large number of TSG, there is increasing collections of work that indicate that for certain members of this group, inactivation of a single allele is sufficient to inhibit the action of the gene. Examples of these included NF-1 and ATM (Krug *et al*., 2002; Payne and Kemp, 2005).

1.3 Tumour Progression

1.3.1 Angiogenesis

Like healthy tissues, tumours require a vascular system in order to be provided with oxygen and nutrients that will allow them to survive while having waste products removed (Makrilla *et al*., 2009). It is generally considered such a system needs to be established once a tumour reaches a size of approximately 1-2mm^3 in order for it to survive and expand (Keyhani *et al*., 2001; Liekens *et al*., 2001). Angiogenesis is also found in haematological tumours, with strong evidence in multiple myeloma (MM) (Keyani *et al*., 2001). The generation and maintenance of new vascular bundles comes from a complex series of relationships involving various molecules that are exploited to bring about this situation (Nassenbaum and Herman, 2010). Evidence of angiogenic proteins in patients has been linked to poor disease prognosis due to increased risk of tumour spread (Makrilla *et al*., 2009). Angiogenesis is closely associated with metastasis. This stands to reason, since formation of new vessels eases the dissemination of tumour cells to other regions of the body. However, some

of the proteins involved in metastasis also play a role in neo-vascular formation (Ahmad and Hart, 1997).

The formation of new vessels is stimulated through hypoxic conditions, thus increased demand of oxygen by tissues. In their infancy, tumours are examples of such environments. However, angiogenesis comes from a fine balance between signals promoting or inhibiting the process. Therefore, there must be disruption of this balance to synthesise a novel vascular system. Vascular endothelial growth factor (VEGF), for example can be up-regulated by hypoxia-inducible factor 1 (HIF-1), a molecule generated in response to oxygen deprivation in tissues (Shweiki *et al*., 1992; Otrock *et al*., 2009; Nussenbaum and Herman, 2010). So here, a normal molecular interaction is replicated in tumour tissue in order to continue malignant development. Aside from this, VEGF mRNA can be up-regulated by growth factors including fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) and other cytokines such as transforming growth factor-β (TGF-β) (Enholm *et al*., 1997; Nussenbaum and Herman, 2010). Additionally, signalling molecules such as RAS and AKT can increase VEGF expression (Kerbel *et al*., 1998; Okada *et al*., 1998). This means that aberrant signalling, either cytokine-induced or through the action of oncogenes can also drive new vessel formation.

The importance of VEGF is also highlighted by its relationship with molecules implicated in metastasis. VEGF helps to up-regulate matrix metalloproteinases (MMP), which acts to dissolve the extracellular matrix (ECM) (Leikens *et al*., 2001; Nussenbaum and Herman, 2010). Destruction of ECM causes the release of VEGF-A, which is normally bound to the matrix, promoting neo-vascularisation (Bergers *et al*., 2000; Nussenbaum and Herman, 2010). This not only demonstrates the closeness of angiogenesis and metastasis, but also the necessity for a tumour to create a relationship with its surrounding environment in order to progress.

Though vessel formation is important, it is vital for a tumour to be able to mature and maintain them. This is mediated by the angiopoietins and their receptors, known as Tie (Klagsbrun and Moses, 1999; Karemysheva, 2008; Nassenbaum and Herman, 2010). These too are up-regulated in response to hypoxia and are found to have

distorted expression in tumours (Bach *et al*., 2007; Shim *et al*., 2007). In this manner, it is possible for cancerous lesion to not only initiate the formation of new blood vessels, but to also sustain their presence.

Due to its importance in cancer progression, angiogenesis has been the subject of focus for therapy that could potentially be used to inhibit its actions. Various inhibitors have been designed to act against those molecules involved in different stages of the process. These include Batimastat, which target most MMP members, whereas VEGF and FGF therapies have been used to limit the action of these proteins (Sledge Jr. *et al*., 1995; Goldman *et al*., 1998; Liekens *et al*., 2001). Some of these have demonstrated promise and have reached the clinic, though so far they have had very limited effect and clinical trials with Batimastat has been halted as a result of its insolubility (Liekens *et al*., 2001). The major implication of treatments against angiogenesis is that a large proportion of its effectors are in fact vital and are still required to perform their actions in regions of the body unaffected by malignancy. This makes this type of therapy very difficult to implement and as such, it means that tumour progression still remains difficult to halt.

1.3.2 Immune Escape

The human body contains a highly sophisticated system that is utilised to defend itself against pathological harm. This system is a collection of many different cells, proteins and other factors that function alongside each other to stop the actions of foreign pathogens and the effects of other influences that may threaten its survival. In its broadest sense, immunity is driven by two very different aspects that function in different ways. One aspect is innate immunity, a broad spectrum form of protection involving cells such as neutrophils and macrophages that destroy cells via physical ingestion (Phagocytosis) (Chaplin, 2003). This form of immunity also includes a subset of lymphocytes without typical lymphocytic receptors known as natural killer (NK) cells that are highly efficient at targeting both virally and malignantly transformed cells (Cooper *et al*., 2001; Moretta *et al*., 2002; Chaplin, 2003; Zamai *et al*., 2007). Whereas other lymphocytic cells interact with MHC, NK cells specifically target cells that lack MHC expression (Chávez-Galán *et al*., 2009). These cells are

assisted by the complement group of serine proteases that act to lyse the membranes of target cells (Frank and Fries, 1991; Chaplin, 2003).

The other aspect of the immunity is the more precise adaptive immunity concerning the role of the receptor-bearing lymphocytes of which there are two basic forms; B (B cells) and T (T cells), which perform humoral/immunoglobulin (Ig) and cellular-based killing, respectively (Alam and Gorska, 2003). For this to take place, the receptors found on the surface of these cells interact with immunogenic molecules presented to them referred to as antigen. More specifically, they react with peptide sequence derived from proteins that can vary in their level of immunogenicity.

In the case of B cells, their receptor is surface bound IgM antibody that can recognise vast numbers of different antigens. Upon detection, these cells undergo maturation and clonal proliferation of the cells to their mature form (Plasma cells), which secrete specific Ig molecule to coat target cells. Conversely, T cells possess a T cell receptor (TCR) complex that recognises peptides offered to them when bound to major histocompatibility complex (MHC) of cells (Hennecke and Wiley, 2001; Alam and Gorska, 2003). MHC molecules are broadly found in two forms. The first, class I, is possessed by all cells excluding erythrocytes and presents endogenously processed antigens. These interact with CD8+ T cells that activate a cytotoxic T cell (CTL) reaction, which induce apoptosis via the action of the enzyme granzyme (Chávez-Galán *et al*., 2009). The other form of MHC molecules, class II, present exogenous antigen peptides captured and processed by antigen presenting cells (APC). These antigens are recognised by CD4+ T cells that initiate a 'helper' (Th) response, which directs immunity in specific ways. MHC class II is only found on this specific group of cells, including macrophages, B cells and most potently, dendritic cells (DC) (Hennecke and Wiley, 2001; Assundani *et al*., 2007).

Both the innate and adaptive arms overlap to maximise the efficiency of the immune response. This is evident from Ig secretion by plasma cells binding to transformed cells, instigating opsonisation. Opsonisation attracts innate cells such as neutrophils and macrophages and activate mediators like complement proteins to bring about complement-mediated cell destruction. NK cells traffic to Ig-bound cells, where they can use the antibody to initiate apoptosis by antibody-dependent cytotoxicity (ADCC)

or by recruiting complement to kill targets by means of complement-mediated cytotoxicity (CMC) (Chaplin, 2003).

The immune system is highly efficient at protecting the body from damage, both foreign and endogenous. As it is possible that cells are able to counteract transformation through TSG and so forth, it is entirely feasible that during their life, a person's immune system eliminates transformed cells, preventing cancer from occurring. Knowing such information, one can therefore draw the conclusion that at some point, there must be a lapse in immune surveillance that allows malignancy to take place. Evidence collected over many years of research alludes to the fact that tumours are very capable of avoiding detection by immune cells and even able to destroy any cells that may recognise them (Whiteside, 2003).

Cancerous cells have been shown to have significantly lower expression levels of MHC molecules meaning that they do not present antigenic peptides to T cells. This effectively renders them 'invisible' to the immune system (Hicklin *et al*., 1999). Evidence from genomic studies into invasive glioma indicates that this may be due to the direct down-regulation of the genes encoding MHC proteins (Zagzag *et al*., 2005). Alternatively, decreased MHC expression could be due to mutations and abnormalities in proteins that are involved in antigen presentation, for example beta-2-microglobulin (β2M) or transport-associated protein (TAP) (Chang *et al*., 2006; Ferris *et al*., 2006; Setiadi *et al*., 2007). To compound this, other work has indicated that the majority of peptides that are presented by tumour cells are not sufficiently immunogenic to generate a substantial reaction from immune cells. Additionally these cells down-regulate the expression of co-stimulatory molecules such as B7.1 and B7.2 that act as the 'second signal' required for T cell activation. Without this essential communication, T cells enter a state of anergy and are unable to target cells, meaning that tumour cells can survive (Macián *et al*., 2004).

The affinity and avidity of T cells to the presented antigen is an important aspect of immunity. Affinity refers to the extent of interaction between the TCR to the MHC/peptide complex, while avidity is a term used to explain the potency of interaction occurring between a T cell and the antigen (McKee *et al*., 2005). Both are

vital for the optimal priming of T cells, but the concept is highly complex. It has been proposed T cells with high affinity for certain MHC/peptide complexes lead to increased T cell avidity. However this is countered by work stating affinity of T cells is not related to their reactions against an antigen (Derby *et al*, 2001; Dutoit *et al*., 2001; Dutoit *et al*., 2002; Kerry *et al*., 2003; McKee *et al*., 2005). Studies have indicated T cell clones with identical TCR have varying avidity to antigen presented by APC (Cawthon *et al*., 2001). This is made more complicated by evidence implying T cells with low avidity having strong MHC/peptide complex affinity and *vice versa* (McKee *et al*., 2005). In terms of tumour immunology, the belief is that high avidity T cells are required to eliminate malignant cells. Complications surrounding avidity comes from the concentration of antigen needed to generate a high avidity T cell clone. For example it has been shown priming with high doses of antigen results in formation of low avidity T cells. It can also lead to the death of the cells through over-exposure. Conversely, T cells primed with lower doses of antigen leads to the formation of a high avidity clone (Alexander-Miller *et al*., 1996a; Alexander-Miller *et al*., 1996b). This makes the production of CTL with high avidity for tumour antigen very complicated, especially if one considers the issues concerning malignant cells issues with antigenic peptides and MHC down-regulation.

Conversely, a cancer cell may significantly up-regulate expression of surface molecules such as FAS-ligand (FAS-L/APO-1-L/CD178) to bring about a 'counterattack' against immune cells. When ligated to its respective surface antigen FAS (CD95), FAS-L initiates pro-apoptotic signalling within the FAS-expressing cell, thus killing it (Nagata and Golstein, 1995; Minas *et al*., 2007). Since T cells express FAS as a safeguard to ensure they can be destroyed in order to avoid potential auto-immune reactions, tumours can exploit this to create immune tolerance (Daniel *et al*., 1994; Alderson *et al*., 1995; Griffith *et al*., 1996; Bennett *et al*., 1998). In this manner, as with all systems affected by cancer, there is a manipulation of installed failsafe mechanisms that a tumour uses to its advantage.

Cytokines play a vital role in immune reactions, both in their initiation and inhibition. Certain ones such as interleukin 2 and 12 (IL-2/12) have been linked with very strong immunity against cancer cells thanks to their actions in promoting the activity of CTL (Borish and Steinke, 2003). Certain sites in the body such as the brain and heart are

considered immune-privileged due to their importance in the body. To maintain this status, amongst other practices including FAS-L expression (Francavilla *et al*., 2000), immune-suppressive cytokines are secreted to reduce the activity of immune cells in these regions. Tumours have been shown to secrete high levels of specific cytokines, for example transforming growth factor β (TGF-β), IL-4 and IL-17 amongst others. Paradoxically, in the initial stages of tumorigenesis, these cytokines were found to promote anti-tumour responses, however after prolonged periods; they can aid the cancerous cells in their progression (Li *et al*., 2009a; Murugaiyan and Saha, 2009; Nagaraj and Datta, 2010). This again may link to acute-versus-chronic events that seem so pivotal in malignant transformation. Through the control of these proteins, a tumour aims to create an immuno-privileged environment. Another cytokine, IL-10, plays an important role in regulating adaptive immunity. It expressed by both CTL and Th cells as well as innate cells such DC, which act as APC for T cells, as part of a negative feedback system. Therefore, IL-10 acts to repress the immune system to help prevent inflammation and possible auto-immunity (Saraiva and O'Garra, 2010). It is possible if this is cytokine could be secreted by tumour cells, it could help prevent action by a large proportion of the adaptive immune response.

Perhaps one of the more sinister aspects of cancer comes from indications that the immune system can contribute to tumour initiation and progression. This is evident from the role of regulatory T cells (T_{Reg}), a specific subset of T lymphocyte that act as inhibitors of adaptive immunity (Groux, 2001; Ichihara *et al*., 2003; Sakaguchi *et al*., 2010). These exist naturally in the T cell pool or can be induced by the action of certain situations in the body (Sakaguchi *et al*., 2009; Sakaguchi *et al*., 2010). Their ability to inhibit immunity can be from direct contact, using granzyme or FAS-L to kill T cells (Grossman *et al*., 2004; Strauss *et al*., 2009; Sakaguchi *et al*., 2010). T_{Reg} can also inhibit immunity by employing various cytokines, including the aforementioned IL-10 (Roncarolo *et al*., 2006; Belkaid, 2007; Sakaguchi *et al*., 2010). There is strong belief that during immune reactions, the homeostatic action of T_{Reg} may act to allow tumours to form unnoticed. It is also possible that tumours may be able to use cytokines such as TGF-β and IL-17 to induce their presence, thereby inhibiting any potential anti-tumour immunity (Chen and Wahl, 2003; Chen *et al*., 2003; Beriou *et al*., 2009; Murugaiyan and Saha, 2009).

The above issues exemplify not only the extent to which the immune system must be able to monitor and react to its environment, the principle of immuno-surveillance, but also how a tumour is able to interact with and possibly alter immune reaction; so-called immuno-editing (Dunn *et al*., 2002; Dunn *et al*., 2004). This is highlighted in Figure 1.2. In spite of the array of mechanisms malignant cells might use to avoid elimination by the immune system, it is entirely possible they can be targeted and removed by immune effectors. However, at the same time there is the possibility of immuno-sculpting taking place (Dunn *et al*., 2002; Dunn *et al*., 2004; Reiman *et al*., 2007). The premise behind this concept is the possibility that certain tumour cells are indeed recognised and eliminated by CTL. However, it is thought more aggressive malignant cells existing within the tumour have the benefit of remaining unnoticed by immune cells and thus avoid deletion. This produces a problem whereby the body is able to rid itself of a certain degree of tumour mass, however it leaves behind a group of cells with a relatively more aggressive phenotype. Therefore as such immune reactions against a tumour could essentially cause an increase its aggressiveness. If this takes place, malignant cells can subsequently utilise escape mechanisms to avoid immune reactions, maintain the integrity of the tumour and progress from there (Dunn *et al*., 2002; Dunn *et al*., 2004).

The action of inflammation and phagocytic cells can involve the use of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), NAPDH oxidase and superoxide anion (O_2^-) can induce genetic changes. Production of these may not only be induced by physiological conditions like those mentioned above, they can be generated through the action of certain genes such as RAS (Heyworth *et al*., 1993; Komatsu *et al*., 2008; Pan *et al*., 2009). The action of ROS can lead to changes in the genetic structure of genes, and may contribute towards mutations in p53 (Pan *et al*., 2009), which is a significant inaction of the normal function of this TSG.

Ultimately, once a tumour gains the ability to avoid attention of the immune system, there is no host defence against its progression, hence why patients demonstrating such mechanisms may have poorer prognosis (Wang *et al*., 2006b). This renders the tumour with the opportunity to invade, metastasise and further its influence in other regions of the body.

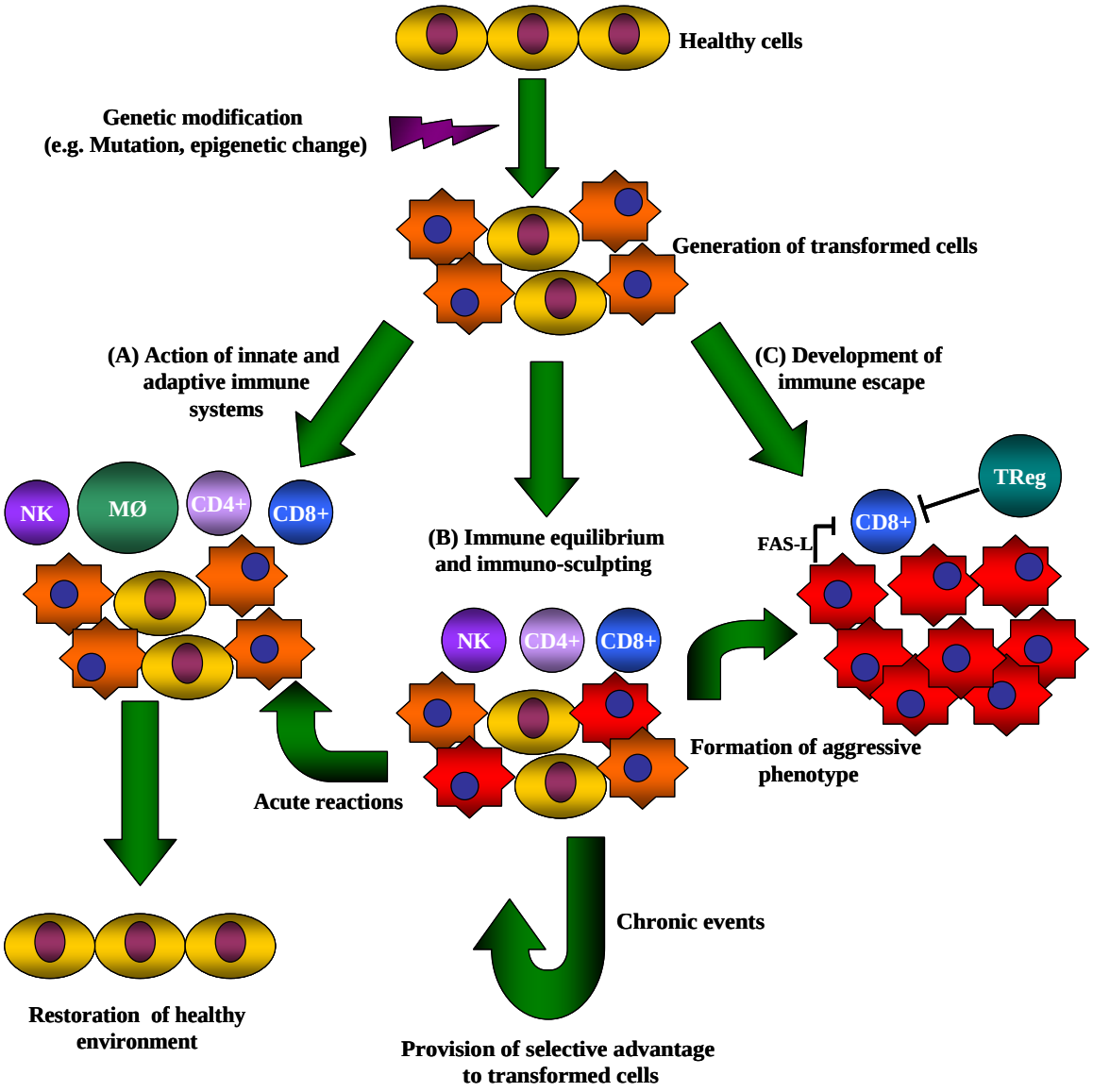


Figure 1.2: *Overview of the principles of immuno-editing in tumours. Following changes to genetic material, and formation of cell transformation various immune phenomena can occur. The combined efforts of the innate and adaptive arms of the immune response can eliminate transformed cells, returning tissue to a healthy state (A). However, it could be a balance is established (B), with acute immune reactions inducing tumour clearance or the formation of immuno-sculpting where immune cells only eliminate the least aggressive cells and promote generation of more severe phenotype. This could lead to the formation of immune-escape mechanisms, which allow a tumour to avoid elimination, possibly compounded by the actions of* T_{Reg} *cells. It is also possible for the latter to take place during their transformation (C). Adapted from Dunn et al., 2004.*

1.3.3 Metastasis

Dissemination of tumour cells from the site of origin to other distant sites in the body, referred to as metastasis, is the turning point in cancer progression and is the reason why the majority of patients succumb to their disease (Meyer and Hart, 1998; Geiger and Peeper, 2009). Until this point, tumours are confined to one distinct region that if eligible can be removed or treatment can be concentrated to that area. Following invasion of surrounding tissues, cancerous cells can enter blood or lymphatic vessel to travel to other bodily regions. If they can establish themselves in these locations, then secondary tumours may begin to form, making it difficult to not only locate them, but remove them.

In order to carry out this invasion, cancerous cells are able to initially down-regulate adherent proteins such as the cadherins and cytokeratins (Geiger and Peeper, 2009) allowing them to detach from the main mass. The action of matrix metalloproteinases (MMP), thought to play a key role in invasion (Westermarck and Kähäri, 1999), subsequently permits dissolution of the basement membrane (BM) and entry into the ECM (Shapiro, 1998). Certain members of this group are heavily linked with malignant invasion. MMP-2 and 9 (Gelatinase A and B) are highly expressed in different cancers, undergoing up-regulation to promote metastasis (Ahmad and Hart, 1997; Basset *et al*., 1997; Johnsen *et al*., 1998; Westermarck and Kähäri, 1999; Hoffman *et al*., 2005). It has been shown certain MMP can associate with one another to perform their action, as demonstrated by MMP-2 binding to MT1-MMP/MMP-14, which is found on the surface of malignant cells (Sato *et al*., 1994; Ahmad and Hart, 1997; Gilles *et al*., 1997; Hoffman *et al*., 2005). MMP are naturally inhibited by the tissue inhibitor of metalloproteinases (TIMP) family of proteins (Woessner Jr., 1991). These can undergo down-regulation during malignancy, thereby promoting MMP activity in both angiogenesis and invasion (Ahmad and Hart, 1997). However, an interesting point is that when still present, TIMP can inhibit invasion even following increased MMP expression, therefore emphasising how the balance of signals can influence this process (Ahmad and Hart, 1997).

Migration is encouraged through the up-regulation of proteins such as focal adhesion kinase (FAK) and this promotes invasion into surrounding areas. In the case of FAK,

this is due to its involvement with the organisation of adhesion molecules in cell migration and interaction with proteins such as p130Cas that can promote invasive phenotype (Cary *et al*., 1998; Tilghman *et al*., 2005; van Nimwegen and van der Water, 2007). Should malignant cells be able to reach blood or lymphatic vessels, then they have the potential to migrate to much more distant locations in the body. In order to generate new tumour lesions in these new areas, the cells must then re-expresses adhesion molecules to establish a new mass formation, again exploiting FAK and other adhesion molecules to bring this about (van Nimwegen and van der Water, 2007).

Though it represents the greatest danger to patient welfare, paradoxically it appears that metastasis is actually a very inefficient process. Studies in mice have shown that, of the cells that manage to disseminate from the original tumour mass, only a very small minority of these seem capable of settling in a new location (Luzzi *et al*., 1998). Additionally, cells that do graft in new regions seem to display dormancy. It has been suggested that this depends heavily on the capacity of single metastatic cells to proliferate and create secondary masses (Luzzi *et al*., 1998). However, one should note that this dormancy could reflect the possible situation in humans where cells may well invade surrounding tissues and organs but remain idle for long periods of time before beginning to proliferate, potentially awaiting the correct influence by signals.

Further to this, the use of multiphoton microscopy on mice injected with tumour cells is beginning to generate a better understanding of how metastasis takes place within an *in vivo* system (Alexander *et al*., 2008; Friedl, 2009). Some emerging evidence has indicated cells on the periphery of the tumour may be carrying out invasive migration (Friedl *et al*., NCRI Conference, 2009). This concept differs from the traditional view of cells in the core of tumours being more likely to metastasise due to them being considered more aggressive. This change of view as to a process central to tumour progression forces one to reassess the biological properties of cancerous cells. It begs the question as to what specific malignant cells are capable of satisfying the criteria for displaying the most aggressive phenotype.

1.4 The Cancer Stem Cell Theory

For a long period of time, the dogma central to the origins of cancer stated that tumours emanate from a single cell that is subject to genomic damage wrought by carcinogens and that all cells in the body have an even chance of being affected by this insult. It has been known for many years that certain tumours, for example CML and acute myeloid leukaemia (AML), are caused by mutations that take place in stem cells. Studies into leukaemias proposed that a specific subset of pluripotent stem cells present in the bone marrow were responsible for this group of tumours, labelled 'leukaemia stem cells' (LSC) (Warner *et al*., 2004; Bonnet, 2005; Abdel-Wahab and Levine, 2010). Bonnet and Dick (1997) then showed that such cells injected into non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice were capable of causing leukaemia, but not more mature, differentiated white cells. This data formed the basis of their argument that all tumours, solid and fluid, were derived from stem cells present in their tissues of origin. This has now been christened the 'cancer stem cell theory'.

The cancer stem cell (CSC) theory counters the traditional tumour basis model in that it states that specific cells (i.e. CSC) are present in tissues throughout a person's lifetime. In a similar fashion to how healthy cells are all derived from stem cells, the belief in that tumour cells originate from CSC (Ailles and Weissman, 2007; Bjerkvig *et al*., 2009). They are suggested to posses all the characteristics of healthy stem cells such as self-renewal and the ability to differentiate into any cell type. At some point, certain triggers will stimulate these cells and from them, malignant cells are derived to form tumours. These daughter cells then themselves proliferate to continue tumour expansion, presumably acquiring further mutations as they do so. Studies have shown that in many instances, it has been possible to isolate CSC from a variety of different tumour types including breast, prostate and head and neck carcinoma (Al-Hajj *et al*., 2003; Ponti *et al*., 2006; Signoretti and Loda; 2006; Prince *et al*., 2007). Though it is possible to recognise them, as yet it is unknown if these cells are mutated forms of normal stem cells or another variation of them (Huang *et al*., 2007). Work is now in progress to investigate these cells as fully as possible. Not only is there the potential to finally understand the possible root causes of tumours, there is massive therapeutic implications to cancer patients (Shipitsin and Polyak, 2008).

The CSC theory is highly credible and entirely possible for most cancer types. It is believed that they are the aggressive cells present in poor prognosis cases. This is feasible as stem cells are more resistant to drug treatments as well as apoptosis (Ailles and Weissman, 2007). The expression of telomerase, a protein that maintains chromatid length following cell division is expressed by all stem cells (Shay and Wright, 2010), and accounts for the 'immortal' nature of aggressive tumour cells. Likewise, being able to 'self renew' following division necessitates them being continuously present in tumour tissue. The theory also means that certain cancers, for example teratocarcinomas, may harbour cells with the potential to differentiate into any cell type and would go a significant way to explain this tumour (Andrews, 2002; Chambers and Smith, 2004). Additionally, lack of MHC expression by stem cells would explain the ability of tumours cells to escape the immune system (Shufaro and Reubinoff, 2004). Indeed, this has been shown on suspected CSC isolated from patients suffering from glioblastoma (Di Tomaso *et al*., 2010).

In spite of this, there are certain aspects of this theory that can be challenged. It states that all initiators only affect CSC to bring about all forms of cancer. However, it is very notable that this theory cannot explain the aetiology of those tumours induced by viruses. Specifically, it cannot argue a case as to why viruses should only enter CSC. For example, EBV enters B cells to cause lymphoma by binding to CD21 (Young and Murray, 2003; Thompson and Kurzrock, 2004). This marker is only expressed by these cells at the early-B cell stage of differentiation, long after the primitive stages of a cell's development. An obvious counter argument is maybe CSC may aberrantly express cell surface markers. The theory also states that CSC must exist due to their ability to self-renew, effectively making them immortal and a hallmark of cancer cells. This, as previously mentioned is due to the action of telomerase. However, it has been shown that telomerase is frequently mutated in cancer cells (Deng and Chang, 2007), thus permitting them immortality, therefore bringing the existence of CSC into question. Moreover, the notion of CSC means that there are cells present that already have multiple mutations/abnormalities in genes such as RAS, p53, and PTEN amongst others, yet are able to somehow keep very strong pro-proliferative signals in check until a specific trigger is encountered (Bjerkvig *et al*., 2009).

Ultimately, controversy alongside argument and counter-argument in this field has led to a divergence in the CSC theory. It is now stated that cells develop stem cell-like abilities during the course of their transformation and progression (Passegué *et al*., 2003; Bjerkvig *et al*., 2009). In many ways, this acts to marry the more traditional beliefs with the more modern way of interpreting cancer biology. While many parts of the theory are still very unclear, work continues and is shedding light on many aspects of the disease.

1.5 Cancer Therapeutic Strategies

That cancer remains a public health concern is testament to the notion that for the most part, it continues to exist as a disease that cannot be prevented. In response, concentration has been placed on therapeutic protocols in order to combat tumours. Due to this focus, massive advancement has been made in all aspects of cancer treatment, from detection to palliative care. In combination with research using genomic, proteomic and immunological techniques, this has gone further to the point where the first prophylactic vaccines are now in use and the prospect of personalised therapeutic regimens is a possibility. However, while all treatments currently in use have many advantages, a number of drawbacks exist with each regimen and if one is to use these therapies effectively, one must fully understand and respect these disadvantages.

1.5.1 Surgery

Of all the treatments used to combat cancer, surgery remains the preferential first line option and is the one that arguably poses least risk to a patient's wellbeing. If a tumour is accessible and still present in its place of origin, then the best option is to remove it using surgical intervention. In this manner, the removal of malignant tissue *en masse* prevents prolonged treatment, meaning a reduction in the time taken from diagnosis to remission (Tsim *et al*., 2010). If different surgical techniques can be used for a specific malignancy, then algorithms are employed to determine which procedure would best suit a specific patient (Tsim *et al*., 2010). The same principle is used to consider if a patient is suitable for surgery in the first instance.

Like all cancer therapies, there are complications to surgery. In certain circumstances, for example the nature of the organ where the tumour resides, a large number of considerations must be made to ensure surgery is successful as possible but not cause harm to the patient. This is found with colorectal carcinoma (Yoshida *et al*., 2010). Additionally, post-operative complications including haemorrhage can occur, which can be life-threatening and must therefore be taken into account (Yoshida *et al*., 2010). Furthermore, it is possible not all of a tumour is removed by surgery (Tsim *et al*., 2010), leading to relapse.

Additionally, surgery has limitations in so much that in following healthcare guidelines for surgery, it is possible that patients with less aggressive tumours that could be treated topically, undergo removal of bodily areas which is entirely unnecessary, a practice referred to as 'over-diagnosis'. This has been seen in breast cancer, where women have undergone mastectomy since they satisfy surgical criteria, only to find that it was not needed. This can be highly traumatic to the patients concerned. A similar situation is seen with prostate cancer patients. Ultimately, a balance is required to further investigate a person's malignancy to fully assess if surgery is the true option for them (Etzioni *et al*., 2002; Draisma *et al*., 2003; Day, 2005: Moss, 2005).

1.5.2 Radiotherapy

The use of radiation to combat tumours is one of the oldest forms of cancer therapy. It involves the administration of radiation to a patient in a variety of potential ways. In certain instances radioactive isotopes have been conjugated to antibodies to maximise the efficacy of therapy (Juweid, 2002). Radiotherapy works due to knowledge that upon exposure to radiation, DNA undergoes fragmentation and is severely damaged thereby resulting in apoptosis of the affected cell. The hope is that by exposing tumour tissues to this environment, the resulting DNA damage will lead to their destruction. This is further supported in the knowledge that radiation damage preferentially affects rapidly dividing cells of which tumour cells are a good example of.

Radiotherapy remains a heavily utilised form of cancer therapy and is front-line treatment for certain malignancies for example head and neck carcinomas, where it has demonstrated a high success rate (Mittal *et al*., 1983; Bailet *et al*., 1992; Ikushima, 2010). Furthermore, it is used as part of standard therapy regimen for non-small lung cell carcinoma, oesophageal, cervical and prostate carcinomas along with lymphoma (Ikushima, 2010; Katz *et al*., 2010; Nakano *et al*., 2010).

The majority of radiotherapy is performed by external beam technology, employing a focused beam of radiation that is fired from an external source into the area where the tumour resides (Nakano *et al*., 2005; Nakano *et al*., 2010). Initially, this principle relied on the use of X-rays and CT scans to 'guide' a clinician on the location of the tumour and where to direct the beam of radiation. There are obvious drawbacks with this such as missing the tumour entirely, so radiotherapy has undergone rapid sophistication and is now a very precise practice. Currently used methods include stereotactic irradiation, which uses multiple beams that can attack tumour masses from various angles and image-modulated radiation therapy (IMRT) (Ikushima, 2010). These advancements themselves have evolved to overcome shortfalls, evident from the use of the highly sophisticated Cyberknife® system (Gibbs, 2006, Coppa *et al*., 2009; Vahdat *et al*., 2010) and development of image-guided radiation therapy (IGRT) (Ikushima, 2010).

Like chemotherapy, detailed below, the reasons why radiotherapy is so successful are also the reasons why it has so many disadvantages. While radiation can be focused onto a particular region or group of cells, especially with the assistance of advanced tumour imaging, in essence there is still a risk of radio beams affecting healthy tissues. Perhaps the most sinister aspect of radiotherapy comes from the notion that while it is very effective, radiation is one of the most powerful of the known carcinogens. For example, it has been shown that the use of ionising radiation on glioblastoma cell lines can in fact activate the highly potent AKT signalling pathway (Li *et al*., 2009b). This finding entails that in a clinical setting the use of radiotherapy has the potential to contribute to increasing resistance to the very therapy being used to treat malignant cells. While the benefits of radiotherapy far outweigh the risks, there is still a chance that this type of treatment can actually initiate the development of further tumours, effectively causing the very disease it is supposed to treat.

1.5.3 Chemotherapy

Chemotherapy remains the most heavily employed frontline form of treatment for cancer. Even though anti-malignant drugs have evolved to a point where they are extremely targeted and precise, the use of chemical poisons to destroy tumour cells remains popular. A vast array of different drugs can be administered orally, intravenously or intra-spinally to combat virtually every known form of cancer.

This form of treatment acts in a similar manner to radiotherapy in that chemotherapy acts to damage the DNA of tumour cells. Cytarabine, for example, is a purine analogue, so-called due to its ability to substitute purine bases in target cell DNA, thereby disrupting the cell's genome, ultimately leading to apoptosis of the cell (Galmarini *et al*., 2002). Other drugs may attack tumour cells by inhibiting the actions of proteins central to DNA metabolism, for instance shown by the action of etoposide on topoisomerase II (Montecucco and Biamonti, 2007). Alkylating agents such as cisplatin are first-line therapy for a wide array of different cancers and function not only to disrupt the DNA pattern of cells, but also attacks the cytoskeleton, which destroys cellular integrity (Siddik, 2003).

Chemotherapy is also as well known for its drawbacks as it is for its effectiveness. It is most effective against rapidly dividing cells, such as tumour cells. This is because it is during cell division where DNA is most vulnerable to nucleotide substitution and attack against proteins used to maintain genomic integrity. However, chemotherapy is completely indiscriminate and as a result, it kills other cells in the body capable of rapid cell division. These include keratinocytes in the hair, leucocytes and gastric cells. The death of these cells results in the stereotypical side-effects seen in oncology/haematology patients such as hair loss, neutropaenia and nausea (Antonarakis and Hain, 2004; Caggio *et al*., 2005; Mols *et al*., 2009). The former can be distressing to patients undergoing chemotherapy, while the latter two require particular attention, as it renders a patient susceptible to infection or dramatic weight loss thanks to loss of appetite respectively. In addition to this, chemotherapeutic drugs can cause nephron toxicity, resulting in renal damage (Hanigan and Devarajan, 2003). All of these side effects mean that the use of drugs requires very strict, careful

monitoring and guidelines towards their use and management of supportive therapy (Aapro *et al*., 2006).

Another drawback of chemotherapy comes in the form of the resistance that can be demonstrated by tumours towards these drugs. Even though they are extremely potent, it is known that tumours are able to withstand their effects. This is mainly a characteristic of highly aggressive or late stage tumours, but nonetheless it remains a concern that cells are able to form resistance against even the most harmful of poisons.

The mechanisms that underlie this response are to date very unclear. Resistance to platinum-based drug compounds such as cisplatin and carboplatin has been well documented (Siddik, 2003; Stewart, 2007) and could represent a good model to examine chemotherapy resistance. Cancerous cells could reduce their uptake of the compounds or use efflux pumps to withstand effects of chemotherapy (Siddik, 2003; Stewart, 2007). Alternatively, DNA repair mechanisms could be utilised by malignant cells to reduce the impact of damage induced by drugs (Martin *et al*., 2008). Expression of specific molecules could additionally aid resistance, including AKT or up-regulation of inhibitors of apoptosis (IAP) (LaCasse *et al*., 1998; Gagnon *et al*., 2004). To overcome this, combinational chemotherapy regimens may be employed, where certain drugs are used in association with each other. A well known example is the use of ICE (Ifosfamide, carboplatin, etoposide) and CHOP (Cyclophoshamide, doxorubicin, vincristine, prednisone) therapy used to treat haematological tumours such as lymphoma (Zelenetz *et al*., 2003; Hertzberg *et al*., 2006; Pfreundshuh *et al*., 2006). Not only does this reduce the risk of a malignancy becoming resistant to any one of the drugs used, but it can maximise their effects, thereby giving more chance of success for the patient.

1.5.4 Specific Therapies and the Evolution of the Single Agent Treatment

Knowing that both radio- and chemotherapy, while effective, are non-selective and in themselves can represent a threat to patient wellbeing, there was obvious scope to design drugs that could have the same impact as the older forms, but reduce patient risk. Increased understanding of cancer molecular biology has made it possible to

now explicitly recognise the precise genes and proteins that are responsible for certain cancer types. Concurrent improved pharmacological/pharmokinetic techniques has resulted in better drug design. This has led to the development of a new generation of cancer drugs that now seek to specifically inhibit target molecules that act to promote tumorigenesis or have a key role within a cancer cell.

1.5.4.1 Oncogene Addiction and Synthetic Lethality

The evolution of NGS analysis has now made it possible to begin examining the genome of cancer cells in far greater detail and in much shorter time than has been imaginable in the past (Stratton *et al*., 2009; Pleasance *et al*., 2010a; Pleasance *et al*., 2010b). This work has already been able to highlight the huge number of mutations that are present in certain tumours. Cancer is a disease characterised not just by mutation, but also by the aberrant up- and down-regulation of a whole host of different genes that may contribute to the malignant phenotype. Nevertheless, speculation exists as to the number and identity of the genes whose expression are fundamental to the development of tumour cells. Over-expression of certain genes may confer the majority of characteristics required for the malignant phenotype. Alternatively, due to the loss of both alleles of certain genes (e.g. TSG) cancer cells depend on particular genes (Kaelin Jr., 2009). As such, expression of these genes is vital for tumour cells, creating a situation dubbed 'oncogene addiction' (Sharma and Settleman, 2007; Weinstein and Joe, 2008).

This theory was initially proposed by Weinstein, citing evidence from cell line culture experiments, murine models and patient studies as the foundation for this argument. Induction of the c-MYC gene in haematopoietic progenitor cells in mice has been shown to bring about myeloid and T lymphocyte leukaemias. Interestingly, inhibition of this gene subsequently resulted in these cells exhibiting healthy characteristics (Felsher and Bishop, 1999; Weinstein and Joe, 2008). One can recognise addictive genes in specific cancers, for example RAS, B-RAF, BCR-ABL and HER2 amongst others (Weinstein and Joe, 2008).

It is thought that if the products of these genes can be inhibited or the genes themselves inactivated, then tumour cells will die. The premise behind this is that

since a malignant cell could be so dependent on a gene in order to survive, then targeting this molecule would leave the cell unable to cope and undergo apoptotic death as a result of 'oncogenic shock'. While this theory goes a long way to account for the action and dependence of oncogenes in cancerous cells, it does not explain why their inhibition leads to differentiation in certain instances or the possible existence of CSC (Weinstein and Joe, 2008). A counter argument to Weinstein's oncogene addiction theory is that of 'oncogene amnesia' proposed by Felsher. This proposes the existence of almost induced ignorance of malignant events within and outside cells, which brings about the same phenomena as oncogene addiction but also attempts to answer the other issues (Felsher, 2008; Weinstein and Joe, 2008).

Due to this apparent dependence, gene addiction is closely linked to synthetic lethality. The principle behind this comes from the idea that certain mutations/events lead to loss of specific gene expression and because of this, cells become dependent on other genes in order to survive and these could be targeted to induce death. This notion is being employed with great promise with the PARP inhibitor Olaparib. BRCA- deficient breast and ovarian carcinomas depend on BER DNA repair in order to survive. Work by Ashworth and colleagues was able to identify the molecule, poly-ADP ribose polymerase-1 (PARP-1), as being central to BER in BRCA-deficient cells, hence PARP-1 is addictive. The drug has been highly effective and recently completed a Phase II clinical trial (Ashworth *et al*., NCRI Conference, 2009). Olaparib specifically targets PARP-1 in both healthy and malignant cells. However, healthy cells still possess HR DNA repair and will therefore survive (Fong *et al*., 2009; Fong *et al*., 2010; Sandhu *et al*., 2010). Other PARP-1 inhibitors exist and are part of clinical trials for numerous tumour types (Sandhu *et al*., 2010).

1.5.4.2 Monoclonal Antibody Therapy

One of the more popular methods of targeted cancer treatment can be found in the form of monoclonal antibody (mAb) therapy. The use of Ig molecules for use against specific tumour antigens has shown significant success with various different tumour types. mAb treatment is a specific variation of immunotherapy in that its goal is to try and induce immune reactivity against tumour cells that express particular antigens.

Ig molecules utilised in mAb therapy can be found in different forms, depending on the method utilised to generate them.

The administration of mAb aims to mimic the action of the natural order of Ig reactions within the body, yet modified to the extent that only malignant cells are targeted. Ig binding to specific antigens present on tumour cells can attract NK cells to the region and promote the actions of both ADCC and other mechanisms to destroy these cells. Since NK cells are potent producers of various cytokines including interferon-γ (IFN-γ) (Cooper *et al*., 2001; Moretta *et al*., 2002; Zamai *et al*., 2007), there is the potential for them to provoke an adaptive reaction from CTL.

It stands to reason that in order for these specific uses of mAb to be of any use to patients, the target molecules must be either unique or significantly over-expressed by cancer cells. Likewise, the patient must actually express the molecule on their tumours in order to be considered for the use of this therapy. There are well known and successful mAb therapies that have been employed for different malignancies. Some began their use as immunosuppressive drugs for ablative therapy prior to transplants. This can be seen with alemtuzumab (CAMPATH 1-H®), an anti-CD52 mAb used for stem cell transplant (SCT) which has now been re-employed for patients with haematological tumours or tumours where myeloid cells are implicated in specific aspects of tumour phenotype (Tibes *et al*., 2006; Alinari *et al.,* 2007; Pulaski *et al*., 2009).

Perhaps the best publicised is the drug trastuzumab (Herceptin®) which is used to target the HER-2 molecule over-expressed in breast carcinomas and acts to inhibit the action of signalling pathways that can be initiated by EGF members. This has shown impressive results in clinical trials, achieving therapy response rates of up to 35% when used alone and has shown to slow the rate of disease progression and increase patient survival when combined with chemotherapy agents such as paclitaxel and docetaxel (Slamon *et al*., 2001; Esteva *et al*., 2002; Nahta *et al*., 2009; Redana *et al*., 2010).

Another drug, rituximab has been highly successful in the treatment of B cell non-Hodgkin's lymphoma (NHL) and is considered to be one of the most accomplished forms of tumour immunotherapy (van Meerten and Hagenbeek, 2009). This is a chimeric antibody raised against the CD20 antigen present on B cells, which due to its potency is incorporated into first-line chemotherapy for patients with mantle zone, follicular and diffuse large B cell (DLBCL) lymphomas (Coiffier *et al*., 2002; van Meertenn and Hagenbeek, 2009). Retrospective studies into the use of rituximab have shown that it can considerably improve the survival of DLBCL patients (Rodenburg *et al*., 2009). Upon recognising its effectiveness, there has been development of next generation anti-CD20 mAb therapies that have either been approved for patient use or are undergoing clinical trial (Lim *et al*., 2010).

However, like all cancer therapies to date, there are issues with mAb treatments. While initial impressions of trastuzumab are impressive, there are some quite fundamental flaws with its use as a single agent. The first comes from the recognition that only 25-30% of patients with metastatic breast carcinoma over-express the HER-2 oncogene (Nahta *et al*., 2009). Additionally, it has been shown that a large number of patients whose tumours express the antigen do not respond. Likewise, the same studies that show the benefits of combined trastuzumab/chemotherapy regimens have highlighted following single treatment, time taken to develop resistance and go on to progress is made shorter. Such disadvantages have now led to the development and use of other drugs such as lapatinib for HER-2 positive tumours (Nahta *et al*., 2009).

For rituximab, issues include the development of resistance to the antibody following prolonged periods of application. Though the reasons for this refraction still remain unclear, there are suggestions that it may be a result of tumour resistance mechanisms. These include antigen (CD20) down-regulation, promotion of anti-complement mechanisms and activation of survival mechanisms (Meerten and Hagenbeek, 2009; Borgerding *et al*., 2010). It has been shown that its effectiveness as part of salvage therapy for relapsed DLBCL patients is quite limited, hinting at its use being confined to newly diagnosed patients (Borgerding *et al*., 2010). This limitation is compounded by the recognition that rituximab is incorporated alongside combinational chemotherapy regimens such as CHOP (R-CHOP) and ICE (R-ICE) (Pfreundshuh *et al*., 2006; Griffen *et al*., 2009). Although rituximab is very

successful in this setting, such employment brings its use as single therapy into question, much like trastuzumab.

Therefore, mAb therapy regimens, though extremely promising and in certain cases highly effective, still suffer from the same problems as the more traditional methods of treatment. It has to be said that as a therapy, the use of mAb has made significant improvements to the treatment of cancer patients. It has been noted though that this improvement has been relatively slow and as yet requires even more improvements and modifications in order to optimise it fully (Yan *et al*., 2009). As a result, it is necessary to find other types of treatment applications.

1.5.4.3 Tyrosine Kinase Inhibitors

Cancer exerts its effects in cells through the manipulation of a large number of different cell signalling cascades. By doing this, it can bring about the prominent hallmarks that are representative of the malignant phenotype. While many effector molecules such as RAS, PTEN and AKT are corrupted in order to cause this, the receptor molecules that make use of these effectors can also be modified in cancer, with perhaps the most recognisable being the receptor tyrosine kinases (RTK). This group of surface receptors span the membrane of cells to transmit stimuli brought about by ligand binding, which they then propagate through the cell. More specifically, they communicate the action of growth factors including epidermal growth factor (EGF), FGF, IGF and PDGF amongst others.

Receptors to these mitogens may be over-expressed or rather subject to increased binding caused by autocrine signalling that is common in tumour cells; in either case, the result is similar. Since RTK are receptors for growth factor ligands, then they bring about pro-proliferative, anti-apoptotic signalling in cells, thereby increasing cellular populations. This is of obvious use to malignant cells and can allow them to grow efficiently. Well known examples of RTK subverted in cancer include HER-2, a member of the EGF receptor (EGFR) family and BCR-ABL in CML (Parsons and Parsons, 2004; Arighi et al., 2005). What is noticeable about some of the molecules that bind to these receptors is that aside from proliferation, they act in processes such as angiogenesis and metastasis. Therefore, there is obvious scope to inhibit such

molecules and in more recent years, therapies have been developed, referred to collectively as tyrosine kinase inhibitors (TKI).

One of the best examples of this type of therapy comes in the form of Imatinib (Gleevec), a TKI that was designed to inhibit BCR-ABL in CML. As already mentioned, this new genetic sequence is due to a reciprocal translocation that takes place between chromosomes 9 and 22 within haemopoietic stem cells. This translocation causes the formation of a smaller version of chromosome 22 known as the Philadelphia (Ph) chromosome with all cells harbouring this being referred to as Ph-positive. The result of this modification is a fusion gene that combines the ABL-1 proto-oncogene and placed next to the active BCR promoter region (Deininger *et al*., 2000; Kabarowski and Witte, 2000). This translocation is responsible for approximately 95% of all cases of CML and is present in the cells of 20% of adult acute lymphoblastic leukaemia (ALL) patients (Druker *et al*., 2001b). The protein product of this gene is a highly active RTK that impels the malignant characteristics exhibited by the cell clone derived from the stem cell affected by this translocation. The BCR-ABL protein itself can be found in different molecular weight forms, either 190, 210 and 230 kDa (p190, p210 and p230 respectively). The p210 form is most common in CML, while p190 predominates in Ph-positive ALL (Chopra *et al*., 1999; Deninger *et al*., 2000a).

Imatinib, the drug designed to combat BCR-ABL, was the original TKI to be accepted for clinical use and arguably remains the most effective example of it. It works by acting as a competitive inhibitor of the ATP binding site within the BCR-ABL protein. The attachment of Imatinib to this region stops the function of the protein and therefore signalling from it cannot proceed and the cell dies through apoptosis due to the attenuation of a pathway upon which it depends (Deininger *et al*., 2000b).

The use/discovery of Imatinib revolutionised treatment for CML (Druker *et al*., 2001a; Druker *et al*., 2001b). Until its acceptance, standard therapy was through the use of interferon-alpha (IFN-α), which was the only way to induce lost lasting remission along with allogenic SCT (Goldman, 2009). However, since its inception,

the use of Imatinib has meant that 70-90% of patients in chronic phase (CP) can achieve a complete cytogenic response, whereby their bone marrow does not contain detectable Ph-positive cells. In blast-crisis (BC) patients, this reaction is found in up to 60% (Palandri *et al*., 2007; Hehlmann *et al*., 2007). Further to this, 84% of patients have been able to remain disease-free for five years. Patients who relapse following SCT are also treated with this drug (Druker *et al*., 2006; Hehlmann *et al*., 2007).

Though its effectiveness cannot be doubted, Imatinib, as with all cancer therapy, suffers from flaws. In this case, it comes in the form of resistance, rendering the treatment ineffective against malignant cells. It is thought that up to 30% of patients may form resistance against Imatinib (Quintás-Cardama *et al*., 2009). For the former, resistance is thought to be due to specific mutant variants of the BCR-ABL gene created during the Ph translocation. Conversely for BC patients, the consideration is that the amalgamation of mutations gained during their disease course produces resistance (Bixby and Talpaz, 2009; Quintás-Cardama *et al*., 2009). Formation of Imatinib resistance led to the production of second generation TKI against BCR-ABL. These are Nilotinib, Dasatinib and Borotinib, all of which have displayed significant effectiveness for use in treatment (Kantarjian *et al*., 2006; Fava *et al*., 2008; Aguilera and Tsimberidou, 2009; Ramchandren and Schiffer, 2009; Swords *et al*., 2009).

One of the more recent members to be part of TKI therapy is Masatinib, a drug that is capable of inhibiting the action of c-KIT (Stem cell factor (SCF)/CD117), a receptor involved in cell production (such as erythropoiesis and gametogenesis) (Roskoski Jr., 2005). This is a proto-oncogene that due to modification achieves gain of function constitutive activation in various tumour types including the acute leukaemias, melanoma and gastrointestinal stromal tumours (GIST) (Lennartsson *et al*., 2005; Dubreuil *et al*., 2009). Masatinib was designed to combat the actions of this receptor and was initially shown to be successful in pre-clinical models against both the murine and human forms of c-KIT as well as specific gain of function mutant of the protein. It can also inhibit the de-granulation of mast cells thereby reducing inflammatory episodes, for which c-KIT is a potent driver (Dubreuil *et al*., 2009). Combined with the chemotherapeutic agent gemcitibine, Masatinib is effective against human pancreatic cancer cell lines *in vitro* and within a murine host model,

which shadowed a Phase II clinical trial involving the same regimen on patients with pancreatic carcinoma (Humbert *et al*., 2010; Mitry *et al*., 2010). Therefore, the effectiveness of this drug appears to show the potential of TKI in therapy and how this follows on from the promise made by Imatinib.

Though they are highly effective at inducing remission and reducing disease burden, TKI remain unable to cure their target tumours. SCT still remains the only true way of curing haematological tumours such as CML and ALL, while it remains unclear if Masatinib will be an effective single. Even though problems remain, the creation of such drugs signals progress towards finally identifying a regimen that can target and potentially cure specific cancers. Having said that, there remain aspects of therapy that need to be explored in order to achieve this goal.

1.5.4.4 Immunotherapy

For many years, the goal of both cancer researchers and clinicians has been to create a therapy that can generate long lasting effects against tumours within patients. The perception that the body's immune repertoire can react towards malignant cells allowed the establishment of immunotherapy and it was believed that this could address the ultimate goal of cancer therapy. By manipulating a patient's own immunity to limit the growth of their tumour or even to destroy it entails that it may be possible to avoid the harsh side effects that blight both radio- and chemotherapy. Seminal work was performed by Baldwin and co-workers, using various immune-based therapies in studies. This concept has been built and expanded upon to promote such treatment and has made the principle of immunotherapy a realistic prospect for combating tumours. (Baldwin and Pimm, 1973; Rees *et al*., 1975; Shah *et al*., 1976; Greager and Baldwin, 1978; Robins *et al*., 1979).

Since its inception, virtually every facet of the immune system has been manipulated in an attempt to induce anti-tumour immunity (Borghaei *et al*., 2009). As previously described, the use of mAb such as rituximab exemplifies the use of immunotherapy as front-line cancer treatment for various cancers. In this setting, immunotherapy has been successful, however it is flawed. One of the aims of immunotherapy is to try and create immune memory to ensure long lasting resistance against a tumour. One

of the issues with mAb treatment is it does not induce immunological memory, so there is always the possibility of patient relapse.

Aside from this, many other concepts have been used to activate the immune system, with increasing emphasis being placed on the use of vaccines. Immune activation can be achieved in either non-specific or specific methods. Non-specific immune stimulation includes the use of cytokines. Due to the fact that a large number of these proteins are highly capable of activating immune cells in acute situations, they represent an inviting proposition for this type of treatment. IL-2 bolus injection has been used as part of trial of a large number of patients with metastatic melanoma, in which 16% of patients responded, 6% displaying complete remission (Atkins *et al*., 1999). This trial was able to significantly prolong patient survival for more than 30 months (Atkins *et al*., 2000). Other cytokines, such as IFN have been shown to activate NK cells that can go on to inhibit the proliferation of cells transformed by a carcinogenic virus (Fresa and Murasko, 1986). Furthermore IFN-α combined with the chemotherapy drug vinblastine was shown to prolong the survival of renal cell carcinoma patients better than vinblastine alone (Pyrhönen *et al*., 1999).

The alternative to the broad-spectrum approach of using cytokines can be found through the employment of more specific forms of immunotherapy, which involves the more targeted utilisation of immune components. Specific immunotherapy can be targeted due to the fact that tumour cells possess antigens that can be recognised by the immune system, known as tumour associated antigen (TAA). Such antigens are varied in their expression patterns and composition (Boon and van der Bruggen, 1996; Kawakami and Rosenberg, 1997; Novellino *et al*., 2005); therefore meaning that one should use caution when designing therapy against them. A list of well known TAA is shown in Table 1.1 and they are discussed briefly below.

<u>Over-expressed Antigens:</u>

These antigens are derived from molecules expressed by a wide range of tissues. Members include the HER-2 antigen found in breast carcinoma (Peoples *et al*., 1995), MUC-1 in breast and ovarian carcinoma (Brossart *et al*., 1999) and Wilm's tumour-1

(WT-1) in a variety of tumours such as gastric, lung, ovarian, uterine and hepatic carcinomas as well as leukaemias (Ohminami *et al*., 2000; Azuma *et al*., 2002). Expression of these TAA is higher in malignant tissue compared to healthy cells; however expression does not seem to be higher in any one particular cancer (Boon and van der Bruggen, 1996; Kawakami and Rosenberg, 1997; Novellino *et al*., 2005).

Differentiation/ Lineage Specific Antigens

This group of antigens are tissue specific, being found in both the healthy and malignant cells of that particular tissue. The majority of differentiation antigens isolated to date are derived from melanoma, including gp100, Melan-A/MART-1, tyrosinase and gp75 (Brichard *et al*., 1993; Bakker *et al*., 1994; Kawakami *et al*., 1994; Wang *et al*., 1995; Boon and van der Bruggen, 1997). Other than melanoma-based antigens, this group contains prostate-specific antigen (PSA) and mammaglobin-A in prostate and breast carcinoma respectively (Corman *et al*., 1998; Jaramillo *et al*., 2002). Some of these antigens have been used as part of clinical trials in an attempt to utilise them as part of immunotherapy, as seen with gp100 and Melan-A/MART-1 for melanoma patients (Di Pucchio *et al*., 2006; Yuan *et al*., 2009).

Virally-derived Antigens

Those proteins that are responsible for the neoplastic transformation of cells following viral infection are well characterised. Viral proteins such as E6 and E7 products of HPV, EBNA from EBV and TAX produced by HTLV infection are responsible for the production of malignant phenotype in affected cells. Due to the importance of these proteins in tumorigenic transformation, there is the belief these proteins are necessary for maintenance of this situation and thus act as highly inviting targets for therapy (Hung *et al*., 2008). Such promise has led to various work investigating the use of possible immunotherapy against such antigens (Taylor *et al*., 2004; Demachi-Okamura *et al*., 2008; Hung *et al*., 2008; Uozumi, 2010). Investigations into targeting viral TAA led to the development of prophylactic vaccines for immunisation against HPV (Harper, 2008)

Onco-foetal Antigens

Some genes which are normally inactivated following birth can become re-expressed in tumours. For example, proteins that promote proliferation and reduce apoptosis, a situation required during foetal development, should become redundant in neo-natal life. However, as shown by carcioembryonic antigen (CEA) in colorectal carcinoma (Novellino *et al*., 2005) and alpha-foetal protein (AFP) in HCC, these genes become expressed again and result in malignant changes in adult cells (Ribas *et al*., 2003). A similar situation can be found with the cancer/testis antigens, discussed in greater detail later on.

Table 1.1: *A list of the major classes of tumour associated antigen (TAA) found in cancer, along with common examples and types of malignancy in which they can be found. References detailing these TAA are found in the main test.*

Antigen Type	Antigen Name	Malignancy
Over-expressed	HER-2	Breast
	MUC-1	Breast, ovarian
	WT-1	Gastric, lung, HCC, leukaemias
Differentiation/Lineage specific	gp100	Melanoma
	Melan-A/MART-1	Melanoma
	Tyrosinase	Melanoma
	PSA	Prostate
Viral	E6/E7	Cervical, head and neck
	EBNA	Lymhomas
	TAX	T cell leukaemia
	HCV Core	HCC
Onco-foetal	AFP	HCC
	CEA	Colorectal

One of the original methods of specific immunotherapy which is still being used as part of clinical trial is adoptive T cell therapy (ACT). ACT exploits the observation that a patient's tumour contains lymphocytes that have trafficked to the lesion: purported tumour infiltrating lymphocytes (TIL). The notion that these cells can

migrate into malignant tissue suggests some form of immune reactivity. The principle behind ACT is to isolate TIL from tumour lesions, which are treated *in vitro* with a cytokine cocktail including IL-2 that allows the clonal expansion of these cells. During this time, patients undergo conditioning therapy using drugs such as cytarabine and fludarabine to ablate lymphocytes, total body irradiation (TBI) to bring about general marrow ablation and cytokine therapy to remove all other immune cells. Once the patient is completely immuno-suppressed, then the modified TIL are infused back into their peripheral blood, allowing them to re-traffic to the tumour and attack cells within it (Dudley *et al*., 2003; Rosenberg and Dudley, 2009).

ACT therapy has been employed by Rosenberg and colleagues for a number of years against late-stage melanoma patients in several clinical trials. It has been able to achieve highly significant results in these studies, to the point where extremely large lesions showed complete regression (Dudley *et al*., 2008; Rosenberg *et al*., 2008; Rosenberg and Dudley, 2009). Furthermore some of these TIL were shown to be able to recognise specific antigens prevalent in melanoma (Panelli *et al*., 2000; Seiter *et al*., 2002). ACT does seem to highlight the extent of immune down-regulation taking place in tumours, due to the vigorous conditioning therapy required for TIL to help in eliminating lesions. The necessity of traditional therapy also implies that immunotherapy may be less potent as a single agent. Other issues one must take into account include the principle of T cell affinity and avidity discussed earlier (McKee *et al*., 2005). Extent of T cell priming and the strength of interaction between TIL and antigen presented to them would all impact on the success of ACT.

However, while ACT has been successful for melanoma, there has been limited impact on other tumours. Indeed, retrieving TIL from tumour types other than melanoma has proved problematic, though evidence exists for their extraction from bladder and lung carcinoma (Weynants *et al*., 1999; Heidecker *et al*., 2000). Difficulty in extracting TIL from lesions other than melanoma may be due, at least in part, to the relative ease of access to tumours. Nonetheless, the use of specific immunotherapy in the form of ACT has provided hope that immune based medicine other than mAb can be used successfully against tumours.

One of the aspects of specific immunotherapy that has become more focused upon in recent years is the development of therapeutic vaccines that could potentially be used to initiate immune reactivity against malignant cells (Jäger *et al*., 2003). This concentration has come about thanks to the discovery of the cancer/testis antigens that are discussed in detail further below (van der Bruggen *et al*., 1991; Rosenberg, 1999). The formulation of vaccines that could lead to the elimination of tumour spread would satisfy the goal of a therapy that could be used to target cancer and spare healthy tissue. Following the formation of this concept, nearly every single conceivable form of vaccine has been utilised against a whole host of different TAA ranging from irradiated autologous tumour cells to gene therapy involving viruses (Ribas *et al*., 2003). One of the most widely used approaches includes the use of DNA vaccines, whereby plasmids containing targeted genome material are administered using gene gun or direct injection. This has been utilised with DNA for both TAA and immune ligands (Ribas et al., 2003; Zhou *et al*., 2005) with the intention of increasing recognition of tumour targets or stimulating immunity respectively. Likewise, dendritic cells (DC) have been at the centre of research into vaccination against tumours due to their pivotal role in immunity (Melief, 2008). Their use in immunotherapy has involved either the *ex vivo* treatment of autologous patient DC using peptides and other material or the design of modified immune ligands that could stimulate *in situ* DC within the patient to act against tumours (Hanks *et al*., 2005) . The aim is to reintroduce these DC into patients and allow them to traffic to lymphatic tissue where they can interact with T cells and activate immunity against malignant cells.

So far tumour vaccination has had extremely limited success, certainly in a therapeutic setting, where only a minority of patients have exhibited actual tumour regression (Rosenberg *et al*., 2004). However, there is more promise with vaccines in the realm of prophylactic prevention of cancer. This can be seen with Cervarix, a DNA vaccine used via needle injection that has undergone large scale trials for immunisation of women to reduce the risk of cervical cancer induced by HPV infection (Harper, 2008). Very recently initial evidence has emerged of a prophylactic therapy against breast carcinoma that has shown great promise in murine studies (Jaini *et al*., 2010). For vaccination to work as a therapy against established

tumours however, it may be that our knowledge of the relationship that exists between tumours and the immune system remains insufficient.

1.6 Cell Signalling in Tumour Cells

1.6.1 Significance of Cellular Signalling

All actions performed by cells are controlled by extremely complex molecular communication networks. Everything from proliferation, DNA repair, trafficking through to apoptosis are subject to the machinations of signalling cascades found in the intracellular environment. These are initiated and inhibited by the actions of both external influences such as ligand-receptor interactions and internal factors including genomic damage. The actions of these stimuli are mediated by vast numbers of genes and proteins that interact with each other in order to bring about a phenotypical action. Signalling cascades are subject to intense levels of control present to maintain strict levels of homeostasis. This entails that in a healthy environment, cells respond to initiation and inhibitory stimuli when required, thus maintaining a stable population of cells.

1.6.2 Consequences of Abnormal Cellular Signalling

While signalling within cells is subject to stringent levels of control, it is very possible that this can be corrupted with significant consequences. Cancer is a disease occurring when signalling homeostasis is undermined. Cellular signalling is a very finely balanced process that involves both pro- and anti-communication systems. Every molecule shown to undergo mutation or modification in malignancy more often than not is central or at least has a key role in signal transduction. This tips the balance of a particular cell process in favour of tumorigenic behaviour and results in the formation of the hallmarks exhibited by tumour cells.

1.6.3 Major Pathways Subverted in Tumours

1.6.3.1 The Magnitude of the RAS Proto-oncogene

Perhaps one of the most recognisable molecules to be affected in cancer is that of RAS. This is a group of human genes initially discovered as homologues of viral genes capable of initiating tumours in mouse cell lines, the first members to be discovered being Harvey rat sarcoma viral gene homologue (H-RAS) and the Kirsten rat sarcoma homologue (K-RAS) (Der *et al*., 1982). These two alongside N-RAS, the third member to be discovered in neuroblastoma (Shimizu *et al*., 1983), represent the three major species of this gene that are present in human cells (Downward, 2003). To understand the importance of RAS in signalling and more pertinently to what impact this has in cancer, then one must appreciate the function of the molecule and the diversity of other molecules it effects.

RAS belongs to a family of small-GTP binding proteins present in the cytoplasm of cells, with a molecular weight of 21kDa ($p21^{RAS}$) (Wennerberg *et al*., 2005). It acts to communicate pro-proliferative signals from receptors on the cell surface to a diverse number of different cascades in the cytoplasm. It can be activated by a number of different receptors including RTK and G-protein coupled receptors (GPCR) (Mitin *et al*., 2005). Following receptor-ligand binding, RAS is recruited to the membrane where it too can be made active. The RAS protein is constantly bound to guanine phosphate and it is the extent of this molecule's phosphorylation that determines its state of activation. When bound to GDP, RAS is inactive and in order to undergo activation, this is swapped for GTP in a reaction catalysed by guanine exchange factors (GEF), which include the son-of-sevenless (SOS) proteins. RAS itself possesses an intrinsic GTPase ability that breaks down GTP as a form of self-regulation towards ensuring it can be made inactive. This however, is relatively slow and inactivation is vastly potentiated by proteins that convert the GTP to GDP, referred to as GTPase activating proteins (GAP) such as p120RASGAP (Downward, 2003; Mitin *et al*., 2005; Schubbert *et al*., 2007). An overview of this is shown in Figure 1.3.

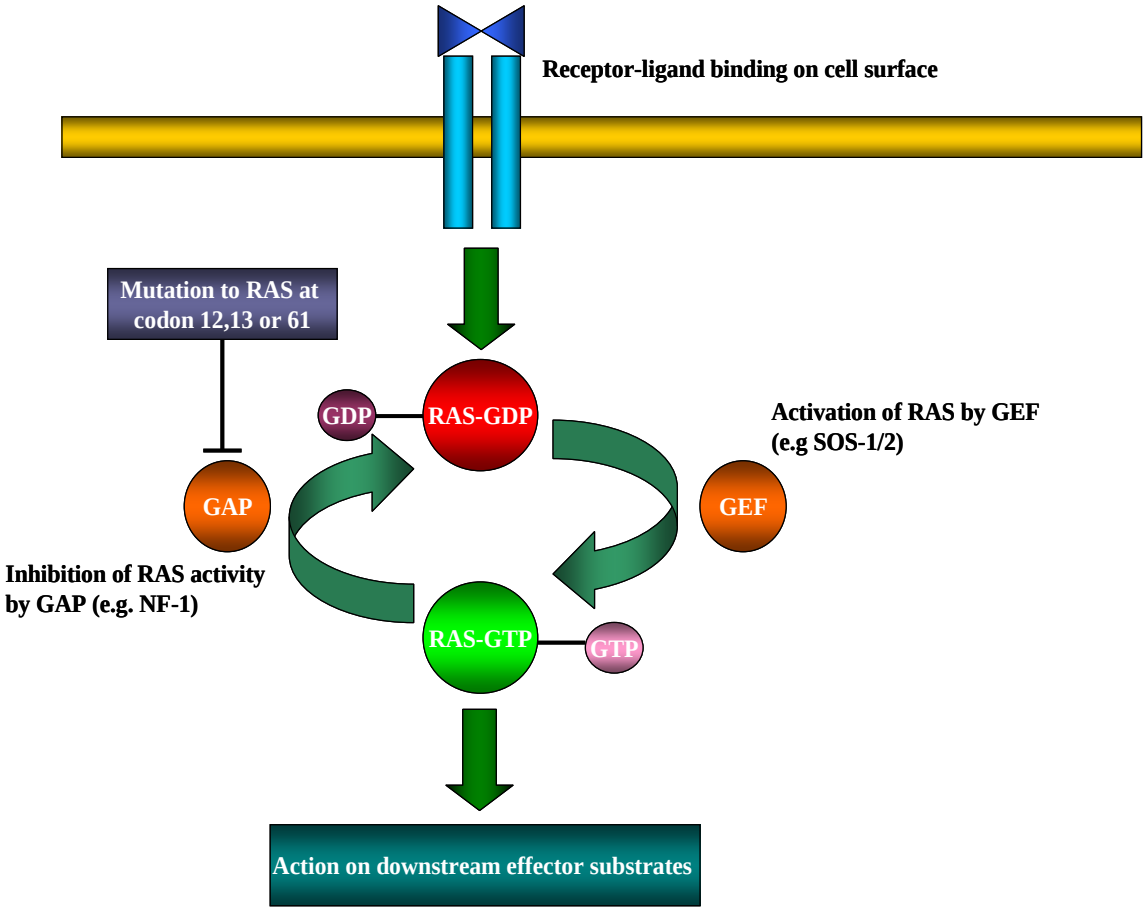


Figure 1.3: *Basic overview of the steps involved in activation of RAS. Stimuli to cell receptors leads to guanine exchange factors (GEF) transferring GDP to GTP on RAS, thereby activating it. RAS activity is stopped by GTP-ase activating proteins (GAP) catalysing the hydrolisis of GTP to GDP. RAS frequently undergoes mutations occurring at codons 12, 13 or 61, leading to resistance to the actions of GAP, thus making it constitutively active in affected tumour cells. Adapted from Cox and Der, 2002.*

When present in a GTP-bound form, RAS is extremely efficient in its role as a mediator of signal transduction. Its actions lead to a large number of molecular changes in the cell to induce a wide range of different behaviour by cells (Campbell *et al*., 1998; Shields *et al*., 2000). The reason for having roles with such a diverse range of cell functions comes from its position in signal pathways and recognising the sheer number of molecules that are influenced by RAS as a result. It is because of this that RAS represents one of the most important molecules in cancer.

The major reason for its importance comes from the recognition that RAS is subject to point mutations that most commonly occur at codons 12, 13 and 61 of its genetic sequence (Bos, 1989; Downward, 2003). The effect of these mutations is a change to the amino acid sequence of the protein product, which results in RAS becoming resistant to the GTPase action of GAP members (Bos, 1989). Lack of inhibition entails the constitutive action of a highly potent promoter of proliferation and inhibitor of apoptosis. RAS mutations are thought to be present in up to 30% of all human cancers, underlining its importance and effect in cells (Bos, 1988). The extent of this is made clearer when one examines the array of different molecules that make up the signalling cascades controlled by RAS. Due to the fact that RAS controls molecules that can induce proliferation, cell survival, production of ROS and angiogenesis along with metastasis, then its constant activation is a significant event in tumour cells.

1.6.3.2 The PI3-Kinase Pathway and the Importance of AKT and PTEN

Some signalling pathways present in cells can be highly beneficial tumours once they become corrupted. One of the more notable involves phophotidylinositol-3-kinases (PI3K), a series of heterodimeric lipid kinases situated within the cytoplasm close to the cellular membrane. PI3K proteins consist of a regulatory and catalytic subunit (p85 and p110 respectively in the case of class I PI3K) and may be activated by different types of cell surface receptors (Cantley, 2002; Vara *et al*., 2004). For example, the class I subtype IA is activated by RTK while IB responds to signals from GPCR (Vara *et al*., 2004; Calhoub and Baker, 2009). Activation can take place following direct interaction with the phosphorylated SH2 regions of activated receptors or via the activation of upstream regulators that include RAS. The action of

PI3K is to initiate signal relay from growth factor receptors following mitogen-receptor binding by phosphorylating the D3/3'OH region of inositol lipids to bring about pro-survival signalling in cells (Vara *et al*., 2004). Activation of these lipids in this manner leads to increased production of phosphotidylinositol-tri-phosphate (PIP_3), a compound that specifically binds to pleckstrin homology (PH) domains of target proteins (Calhoub and Baker, 2009).

One such example of these targets is AKT (Protein kinase B/PKB), the human homologue of the v-AKT viral oncogene (Staal, 1987; Nicholson and Anderson, 2002). The importance of this molecule in tumour cells is evident following the understanding the sheer number of signalling events that are controlled by it. AKT is used to promote proliferation, survival, angiogenesis and motility amongst other actions (Datta *et al*., 1997; Vara *et al*., 2004). To do this, it is recruited to the membrane following the stimulation of upstream molecules, where it becomes activated either by phosphorylation of its PH region or via residue phosphorylation by PDK or mTOR (Nicholson and Anderson, 2002; Calhoub and Baker, 2009). A brief overview of PI3K/AKT signalling is shown in Figure 1.4.

PI3K/AKT signalling is significantly subverted in cancer. PIK3CA, the gene that encodes the p110α catalytic subunit of PI3K undergoes amplification in a wide range of tumours including head and neck, lung, ovarian and breast carcinoma (Isakoff *et al*., 2005; Calhoub and Baker, 2009). This gene is subject to point mutations, as seen in a wide range of different tumours, most commonly in prostate carcinoma, but also in breast, endometrial and colonic carcinoma (Calhoub and Baker, 2009). The effect of both of these alterations is the constitutive activation of PI3K which in turn leads to the continuous activation of AKT. Knowing that PI3K is stimulated by RAS (Pacold *et al*., 2000; Shields *et al*., 2000), then obviously over-expression/amplification and mutations of these respectively can result in increased signalling from this pathway. Mutations may additionally take place in the AKT genes (Staal, 1987; Calhoub and Baker, 2009).

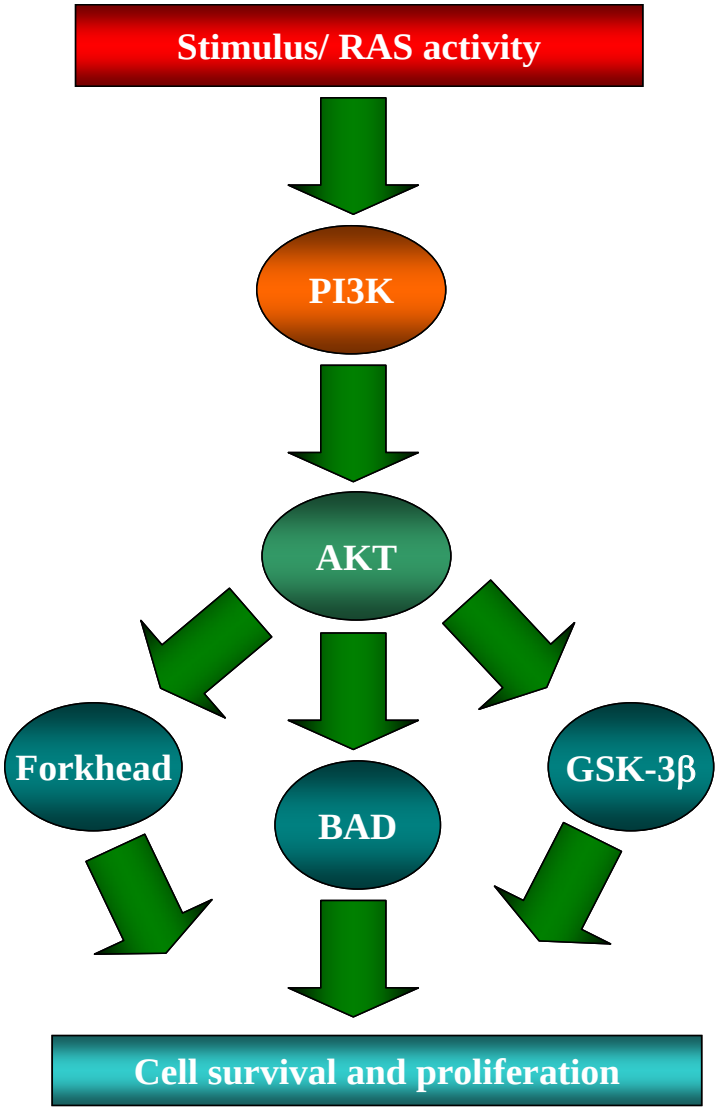


Figure 1.4: *Basic overview of the PI3K/AKT signal cascade. Signalling stimuli from receptor-ligand binding or the actions of RAS leads to activation of PI3K. Action of PI3K can result in AKT recruitment and activation resulting in numerous downstream effects. These include direct interactions with proteins or acting on transcription factors to promote survival of a cell and induce proliferation. Adapted from Downward, 2003.*

The consequence of excessive AKT activity from theses changes is the development of an ideal setting for tumorigenesis. AKT can create such an environment by acting on transcription factors that may promote apoptosis for example the Forkhead proteins that are used to transcribe BAD, BIM and Pro-caspase-9 (Brunet *et al*., 1999; Dijkers *et al*., 2000; Nicholson and Anderson, 2002). It can additionally directly phosphorylate target proteins to induce the same effect. In this manner, AKT inhibits the action of BAD and through that promotes survival (Datta *et al*., 1997; Calhoub and Baker, 2009). Likewise, it renders GSK-3β inactive, thus promoting survival, cell cycle progression and β-catenin proliferative signalling (Calhoub and Baker, 2009). The interaction with GSK-3β is also thought to promote metastatic development through the increased maintenance of SNAIL (Qiao *et al*., 2008)

Since it is implicated in such a range of different aspects of pro-tumorigenic biology, increased AKT activation or mutation is considered to be a poor prognostic factor in cancer. Evidence suggests that its activity can lead to increased radiotherapeutic resistance in head and neck carcinoma patients (Bussink *et al*., 2008). Moreover, as previously mentioned, there are indications that AKT may be induced by exposure to radiotherapy (Li *et al*., 2009), therefore implying a potential risk of using this form of treatment for cancer.

Under normal physiological conditions, the action of AKT is repressed by the phosphatase and tensin homologue deleted on chromosome 10 (PTEN) protein (Li *et al*.,. 1997; Steck *et al*., 1997; Dahia, 2000). PTEN does this by converting PIP_3 to its bis-phosphate form (PIP_2) (Maehama and Dixon, 1998; Chow and Baker, 2006), thereby reducing stimulation of AKT by PI3K as it cannot migrate to the membrane to be acted upon (Vara *et al*., 2004). In this sense, PTEN is an example of a highly potent TSG and is therefore in turn highly important in preventing tumorigenic transformation. It is also of note that evidence has shown that PTEN is also required to maintain the integrity of chromosomes (Puc *et al*., 2005; Shen *et al*., 2007; Calhoub and Baker, 2009) and may play an active role in the HR DNA repair (Mendes-Pereira *et al*., 2009). Ironically, in spite of its great potency, PTEN is frequently affected, causing loss of PTEN function, whether that is due to deletion, mutation or reduction in protein activity (Li *et al*., 1997; Steck *et al*., 1997; Dahia,

2000; Chow and Baker, 2006; Calhoub and Baker, 2009). Without such a barrier in place, then PI3K signalling through AKT is unrestricted, thus promoting tumour development and progression (Vara *et al*., 2004; Chow and Baker, 2006; Calhoub and Baker, 2009). The PI3K/AKT/PTEN pathway is an excellent example of a cellular network involving the oncogenic activation of specific molecules that takes place alongside the inhibition of TSG.

1.6.3.3 The MAPK Pathway

The mitogen activated protein kinase (MAPK) signalling network represents another cascade that has been shown to be vastly modified in tumour cells. Much like the PI3K pathway, MAPK is involved in a series of cell behaviour ranging from proliferation through to differentiation, apoptosis, migration and cell transformation (Huang *et al*., 2004; Kim and Choi, 2010). Whereas PI3K is a single line of communication, MAPK consists of a series of different pathways that interact with one another to bring about their action.

Broadly, they are organised into a three-level system that leads from stimulus brought about by ligand-receptor binding and is carried through to a response by a series of intermediary kinases (Schaeffer and Weber, 1999; Kim and Choi, 2010). These kinase effectors act upon each other to bring about activation by phosphorylation and, tracing upstream to downstream, are usually organised as a MAPK kinase-kinase (MAPKKK) that acts on a MAPK kinase (MAPKK) before finally initiating the action of a MAPK (Schaeffer and Weber, 1999). From this point, MAPK can act on a series of genes or proteins that bring about the effect of this pathway, as shown by Figure 1.5. MAPK signalling abnormalities have been shown to be present in a variety of different human pathological conditions. This is not simply restricted to cancer and includes neurodegenerative lesions such as Parkinson's and Alzheimer's diseases along with amyotrophic lateral sclerosis (Kim and Choi, 2010).

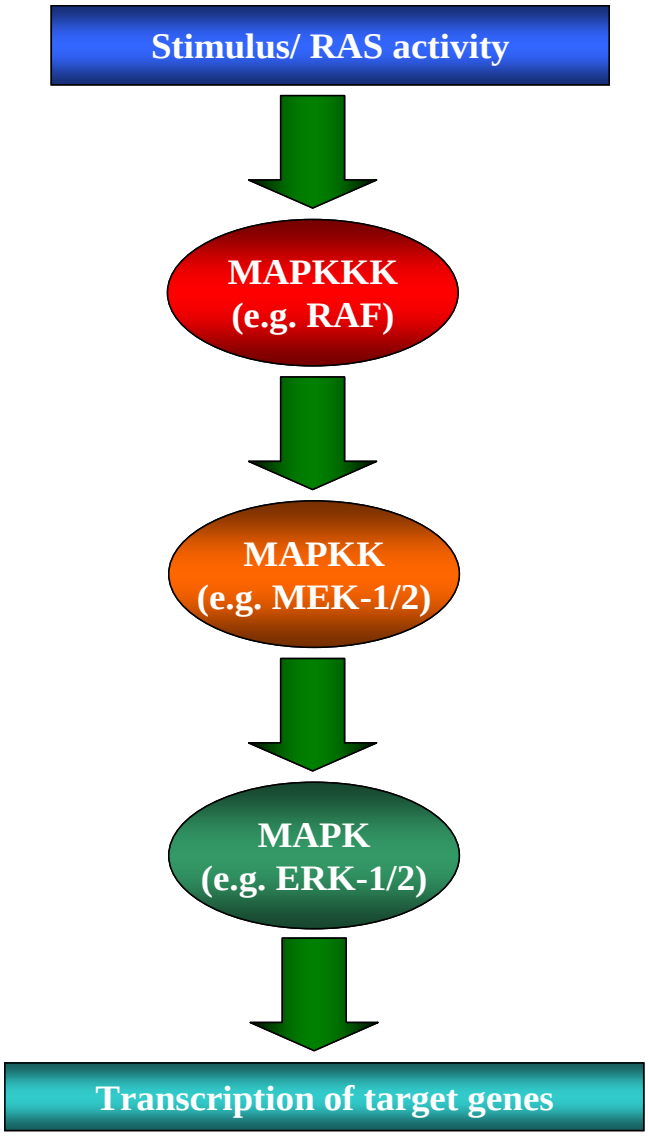


Figure 1.5: *Representation of the MAPK signalling network. This typically involves the action of a MAPKKK, which activates a MAPKK that in turn serves to promote MAPK function. MAPK subsequently target their downstream targets to induce effect. The pathway shown above details the nature of the RAF proliferation pathway, but the can involve JNK or p38 mediated cascades. Adapted from Kim and Choi, 2010.*

As can be assumed thanks to the range of different MAPK networks, different pathways respond to different stimuli. For example, the B-RAF/ERK pathway acts in response to growth factors to stimulate proliferation, as opposed to the p38 and JNK cascades, which act due to stress to stimulate inflammation, differentiation and apoptosis (Schaeffer and Weber, 1999; Weston and Davis, 2007; Feng *et al*., 2009). All of these pathways are capable of inducing cell migration (Huang *et al*., 2004). Since MAPK signalling involves a series of different communication pathways, there

are a wide variety of different molecules that act in specific capacities in each of the specific tiers of the cascades. Aberrations in MAPK signalling has been implicated in numerous different tumour types, including breast carcinoma where alterations to the ERK-1/2 (Growth factor) cascade has been connected to disease progression (Whyte *et al*., 2009), and is also modified in AML (Zebisch *et al*., 2006).

In cancer, the MAPK network is subject to various influences that can disrupt its normal way of functioning. This can come from changes to external stimuli such as up-regulation of receptors or autocrine signalling caused by transformation, particularly with the ERK-1/2 path (Kim and Choi, 2010). Stress and inflammation, environments that are common in tumour tissue, may bring changes to the JNK and p38 networks (Davis, 2000; Feng *et al*., 2009). The role of these latter mediators is unusual and open to controversy in tumours. JNK has been said to be involved in tumorigenesis, using evidence from cell lines to promote this idea (Ip and Davis, 1998; Kennedy and Davis 2003; Weston and Davis, 2007). There was also belief JNK was required for transformation mediated by RAS, however other evidence points to possibility of JNK actually suppressing RAS activity (Kennedy and Davis, 2003). On the other hand, certain p38 isoforms (α and β) can induce proliferation in cells and prevent apoptosis, while the other members (γ and δ) can promote cell death (Feng *et al*., 2009). This conflicting data and variation in isoform-specific activity means the exact signalling properties of JNK and p38 in cancer remain unclear and may depend entirely on the balance of various signalling molecules.

While all of these are changes taking place with molecules that control these pathways, significantly molecules that actually participate within them undergo manipulation, perhaps most recognisably with RAF. This is a MAPKKK of three isoform members (A-RAF, B-RAF and C-RAF) mediating pro-proliferative signalling with the ultimate activation of the MAPK ERK-1/2 via the MAPKK MEK (Robinson and Cobb, 1997). Much like RAS, a molecule that has control over RAF activation, RAF is also significantly mutated in tumours. Though numerous mutations to RAF have been found, the most common occurs at position 600 of the amino acid chain of B-RAF, where valine is replaced with glutamic acid (RAF^{V600E}) (Garnett and Marais, 2004; Nucera *et al*., 2009). This mutated protein has a 500

times greater activity rate, with obvious downstream effects to the RAF-MEK-ERK cascade (Wan *et al*., 2004). Mutations to B and C-RAF have been shown in melanoma, lung, hepatic and colorectal carcinomas (Davies *et al*., 2002; Yuen *et al*., 2002; Garnett and Marais, 2004).

Attempts to tackle mutant RAF have shown limits, much like with RAS. The kinase inhibitor Sorafenib has been used as part of clinical trials and has demonstrated promising results both *in vitro* and in animals (Tuveson *et al*., 2003; Sharma *et al*., 2005). However, it has yet to have a significant impact in patients, especially in melanoma, where B-RAF is the most commonly occurring mutation and is only licensed for use against renal cell (RCC) and hepatocellular carcinoma (HCC). More advances have been seen using anti-MEK therapy, but this is only effective in the presence of particular RAF mutants.

The knowledge that cancer cell phenotype is inextricably linked to cell communication networks and the interactions of the molecules involved means that other genes and proteins central to tumours can be investigated with the intention of developing therapy against them. The success of Imatinib and the considerable promise of Olaparib also give hope that better understanding of certain cancer-related molecules could potentially result in the formulation of successful therapy against certain types of malignancy.

1.7 The Cancer/Testis Antigens

Whereas other classes of TAA discussed previously are over-expressed, lineage specific or mutated forms of ubiquitous cellular components (van den Eynde and van der Bruggen, 1997), their use in cancer therapy, especially immunotherapy, is limited because they are expressed by normal cells. The risk of their inclusion in therapy is the destruction of healthy tissue as well as malignant and in the case of immune-based treatment, the potential threat of generating auto-immunity. Conversely, another TAA group exists that has the promise of by-passing these issues. The cancer/testis (CT) antigens are unique amongst TAA due to their expression being confined to cancerous cells, with very little or no expression found in healthy cells, with the exception of normal testis and placenta tissue (Scanlan *et al*., 2004; Simpson *et al*.,

2005). Since these latter tissues lack expression of MHC, they are considered immune-privileged sites and cannot be targeted by the body's immune system (Haas *et al*., 1988; Ribas *et al*., 2003). This means that they have a great deal of promise for cancer therapy.

1.7.1 Discovery

The existence of CT antigens was initially recognised by van der Bruggen, who along with colleagues at the Ludwig Institute (Belgium), used a genetic technique of transfecting cDNA from tumour cells into cells that express MHC prior to interrogation by patient autologous TIL. It was this work that led to the advent discovery of the melanoma antigen (MAGE) (van der Bruggen *et al*., 1991). Since many cancers do not readily produce TIL, another technique, serological analysis of recombinant cDNA expression libraries (SEREX), came into being (Sahin *et al*., 1995; Li *et al*., 2004). This requires the existence of humoral immunity against TAA within patients. Antibodies taken from cancer patients can be used to screen cDNA libraries to identify particular antigens. This has been used to successfully identify the CT antigen SYCP-1 along with a number of TAA in a range of tumours and has even been shown to discover antigens that can be recognised by CD8+ T cells (Old and Chen, 1998; Türeci et al., 1998; Kawakami *et al*., 2004; Miles *et al*., 2007). Another method, representational difference analysis (RDA), which involves the comparison of simpler synthetic forms of genomes extracted from cancerous and normal tissue panels (Hubanks and Schatz, 1994), has led to the discovery of CT antigens including MAGE-C and MAGE-D (Lucas *et al*., 1998, Lucas *et al*., 1999).

Collectively, the use of the above techniques has allowed the discovery of over 100 antigens that are part of 44 separate families. The vast majority of CT antigens are encoded by genes found on the X-chromosome (CT-X antigens), however an increasing number of these molecules are being found to be coded for by genes on other chromosomes (non-X-linked CT antigens) (Scanlan *et al*., 2004; Simpson *et al*., 2005; Linley *et al*., 2009). A list of CT antigens is shown in Table 1.2. Though a large number of these antigens have been shown to exist, very little is known as to what function they perform.

Table 1.2: *Examples of members of the cancer/testis (CT) family of TAA. Antigens shown are listed according to their class (X-linked or non-X-linked), identifier, chromosomal location and the technique used to recognise them in malignancies.*

Class	Antigen	CT Identifier	Chromosome Location	Discovery Method
X-linked	**MAGE-A**	CT1	**Xq28**	CTL cloning
	GAGE	CT4	**Xp11.23**	CTL cloning
	NY-ESO-1	CT6	**Xq28**	SEREX
	MAGE-C	CT7	**Xq26**	RDA
	CAGE	CT26	**Xp22.11**	SEREX
Non-X-linked	**BAGE**	CT2	**21p11.1**	CTL cloning
	SYCP-1	CT8	**1p13-p12**	SEREX
	HAGE	CT13	**6q12-q13**	RDA
	SP17	CT22	**11q24.2**	Genetic screen
	OY-TES-1	CT23	**12p13.31**	Genetic screen

1.7.2 Involvement of CT Antigens in Cell Biology

All of the molecules described so far have a known function in both a healthy environment as well as a malignant one. CT antigens differ from this in that following their discovery, it has yet to be shown what role they have in tumour cells. Interestingly, certain non-X-linked antigens have had their function described in healthy cells. For example, Sperm Protein 17 (Sp17) has been proposed to be involved in cell migration and adhesion that involves heparin and heparan sulphate and may act as a kinase in the binding of sperm with zona pellucida (Frayne and Hall, 2002, Linley *et al*., 2010). Likewise, CDCA-1 along with three other subunits form the Ndc80 complex that associates with the kinetochore of chromosomes and may stabilise microtubule attachment (Ciferri *et al*., 2005; Hayama *et al*., 2006; Linley *et al*., 2010). Conversely, it is unknown what these antigens do in cancerous cells, whether they have the same role as in normal tissue and if so, what purpose they serve within a tumour cell. Due to their expression pattern being confined to normal

testis and placenta, it has previously been proposed these antigens may be involved in embryogenesis (Lucas *et al*., 1998).

1.7.3 Progress in CT Antigen Therapy

The discovery of MAGE by van der Bruggen and colleagues led to a resurgence in interest in cancer immunotherapy due to promise that CT antigens have with regards to this type of treatment. Thanks to this, large numbers of different members of this class of TAA were found and treatments were designed against them, with emphasis placed firmly in the realm of tumour vaccination. Virtually every known type of vaccination strategy has been employed against a wide variety of CT antigens (Ribas *et al*., 2003). It could be argued that the development of these vaccines has significantly increased our knowledge as to how immunotherapy functions in the body.

Certain CT antigens have been shown to be highly immunogenic and some of the therapies designed against them are highly effective against tumour cells expressing them both *in vitro* and within murine models. This has been seen most prominently with the CT-X antigens MAGE and NY-ESO-1 (Marchand *et al*., 1999; Jäger *et al*., 2000) and is becoming evident with the non-X-linked antigen Sp17 (Chiriva-Internati *et al*., 2002a; Chiriva-Internati *et al*., 2002b; Chiriva-Internati *et al*., 2003; Linley *et al*., 2009). For this promise, some antigens have proceeded to clinical trial or, in the case of Sp17, are part of pre-clinical testing (Dadabeyev *et al*., 2005; www.clinicaltrials.gov).

Specifically, Thierry Boon and colleagues from the Ludwig Institute (Belgium) have placed a large amount of focus attempting to use immunotherapy to target the immunogenic antigen MAGE in various tumours. They employed peptide injection, peptide-pulsed DC, adjuvant therapy or viral vectors that code for the target antigen (Marchand *et al*., 1999; Marchand *et al*., 2003; van Baren *et al*., 2005; Carrasco *et al*., 2008). These vaccines have been able to induce tumour regression and this was strongly linked with anti-tumour immunity. However, while tumour shrinkage was possible, the extent of complete or partial regression in the cohorts tested was low.

This perhaps reflects on the issues with other immunotherapeutic techniques and concepts of immunity discussed earlier.

To date however, clinical trials have displayed very limited impact within patients or are at such an early stage, it is difficult to estimate if they will be successful. This means that despite having such a significant level of potential, using CT antigens as a target for therapy has not fulfilled this. In part, this could be due to the fact that cancer is such a multifaceted disease and the immune system so complex, that the use of CT antigens for therapy remains a distant prospect.

1.8 The Helicase Antigen (HAGE) and the Rationale for this Project

1.8.1 HAGE

As has been described so far, following the identification of MAGE, large numbers of CT antigens were isolated using a variety of techniques. One such example of this is the helicase antigen (HAGE). This was initially identified by Martelange and co-workers along with the CT-X antigen SAGE (Sarcoma antigen) using RDA of a rhabdomyosarcoma cell line (Martelange *et al*., 2000). HAGE, itself, is a non-X-linked CT antigen (CT13) encoded by a gene located at chromosome 6q12-q13, which produces a putative protein of 648 amino acids. This contains the sequence motif D (Asp), E (Glu), A (Ala), D (Glu) that is homologous to the DEAD-box family of ATP-dependant RNA helicases where HAGE is denoted DDX43 (Linder, 2006). The structure of HAGE is shown in Figure 1.6.

DEAD-box proteins belong to a super-family of RNA helicases, which include the DEHD-box and Ski families, collectively termed the DExD/H-box family. The function of RNA helicases centres around the unwinding of double stranded RNA, which can occur in cells in particular circumstances (Du *et al*., 2002; Linder, 2006; Marsden *et al*., 2006). They are believed to have a variety of roles in the metabolism of RNA, ranging from the splicing of mRNA, nuclear export, ribosome biogenesis, initiation of translation, RNA decay and interference (Silerman *et al*., 2003; Rocak and Linder, 2004; Cordin *et al*., 2006; Fuller-Pace, 2006; Linder, 2006). Though different members of the DEAD-box family have had some of these functions

assigned to them, it is unknown if a single member may have multiple different functions. However, due to the nature of these functions and examples of this family, including translational initiators such as eIF4a/b, it could be speculated that they have some role in cellular proliferation (Linder, 2006).

Different DEAD-box proteins have been implicated in cancer. Notable examples include p68 (DDX5), which is over-expressed in both colorectal and prostate carcinomas (Causevic *et al*., 2001; Clark *et al*., 2008). Since this has been linked to splicing of H-RAS, there is indeed a potential link with tumours (Liu, 2002; Guil *et al*., 2003; Camats *et al*., 2008). Other examples include p54/RCK, which like p68 has been associated with colorectal carcinoma, where there may be some link with c-myc (Nakagawa *et al*., 1999; Hashimoto *et al*., 2001), but has been additionally found in a B-cell lymphoma cell line (Akao *et al*., 1995). The cancer-associated antigen (CAGE/DDX53) is also a CT antigen that has been found in different tumour types (Cho *et al*., 2002; Iwata *et al*., 2005; Shim *et al*., 2006).

Work into HAGE has predominantly concentrated on its mRNA expression in different cancers and a large amount of focus has been placed on expression in haematological tumours. HAGE is expressed in both CML and AML cell lines and patient material (Adams *et al*., 2002; Roman-Gomez *et al*., 2007). Specifically for CML, mRNA expression of HAGE has been found in more than 50% of CP-patients and over 70% of BC-patients. Work performed by Roman-Gomez *et al*. (2007) indicated that as patients progress from chronic phase to blast crisis, there is significant hypomethylation of the HAGE gene promoter, meaning that its expression increases dramatically. This notion would seem to agree with the work of Adams *et al*. (2002), suggesting that HAGE expression could be associated with more aggressive disease. Such an idea has been previously alluded to in a review demonstrating a relationship between BCR-ABL and HAGE expression in patients who failed to respond to Imatinib (Riley *et al*., 2009). Microarray analysis has found HAGE to be expressed in cells of patients with both MM and monoclonal gammopathy of unknown significance (MGUS) (Condomines *et al*., 2007). This indicates that the gene is expressed by malignant lymphocytes as well as myeloid cells, though it is yet to be shown if HAGE is present in both chronic lymphocytic leukaemia (CLL) and ALL. Aside from blood-borne cancers, it has been shown that

HAGE gene expression is evident within both malignant and benign tumours of the salivary glands (Nagel *et al*., 2003).

Though some work has been carried out into detecting HAGE expression, it has only focused on its mRNA expression. This laboratory was the first to describe evidence that HAGE is actually translated within tumour cells. It has been possible to demonstrate HAGE protein expression in both cell lines and patient material taken from an assortment of different tumour types (Mathieu *et al*., 2010). Other work has identified that HAGE can act as a target for immunotherapy. In the first instance immunogenic class I and II peptides derived from the protein were identified, which have been shown to be processed by immune cells and could therefore presented to T cells (Mathieu *et al*., 2007). Secondly, the use of HAGE cDNA as part of therapeutic gene gun vaccination approach was able to bring about significant reduction in growth of a lymphoma cell line within an *in vivo* murine tumour model, thus indicating that HAGE has the potential to be utilised in this setting (Riley *et al*., 2009).

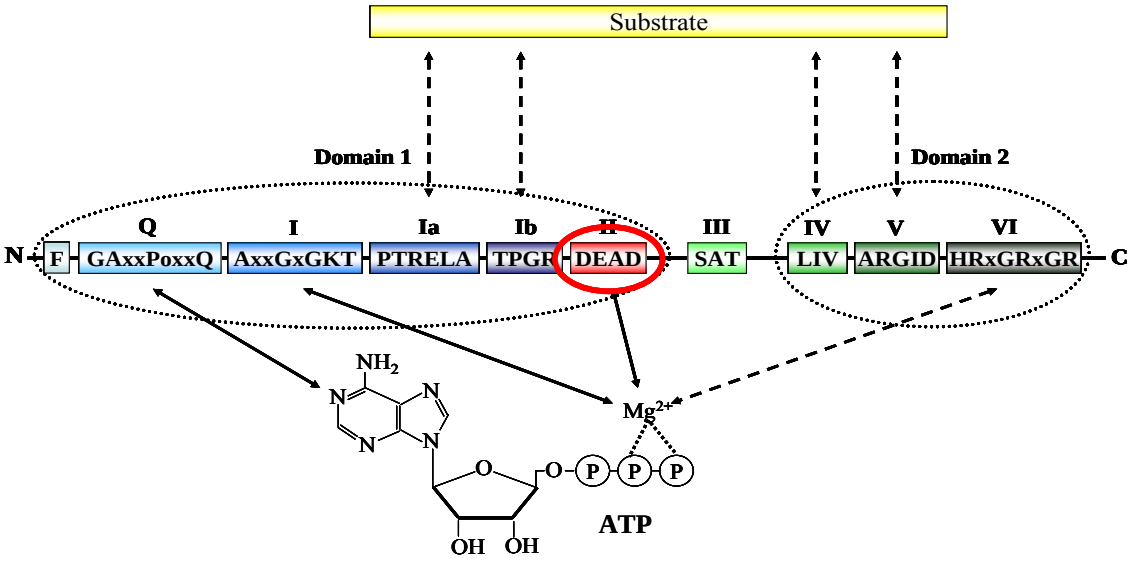


Figure 1.6: *Representation of the protein structure of the CT antigen and DEAD-box protein HAGE. Each of the sequence motifs found in members of the DEAD-box family are indicated, with the homologous DEAD region highlighted (Red ring). Regions which interact with ATP, needed for protein function and substrate (Double stranded RNA) are additionally noted. Adapted from Rocak and Linder, 2004.*

1.8.2 Rationale of this Study

One of the major criticisms that could be levelled at the use of immunotherapy against CT antigens, is that relatively little progress has been made, especially if one considers the significant amount of investment placed in its research in terms of time, cost and effort. This may well be as a result of the lack of knowledge into the exact role these molecules might have within tumour cells and the inability of current assays to predict patient response. A comparison could be drawn with Olaparaib, which as a result of the in-depth knowledge of PARP-1 action in DNA repair, has made fast progress from basic research into Phase II clinical trial. On the other hand, since discovery in 1991, no FDA approved drug against CT antigens is in use. One may argue that better understanding of a specific molecule's involvement in cancer may improve the design of therapy targeted against it.

The establishment that HAGE exists as a protein and is both a CT antigen and a DEAD-box protein implies that it has a potential role in cancerous cells, possibly associated with cell proliferation. If it were possible to discern what function this antigen has in cells, or identifying the processes it might be involved in, there is the potential to design and formulate a drug for use against HAGE-expressing cancers. It could even be possible to redeploy currently existing therapy if this knowledge were to be attained. Therefore, this study aims to use molecular screening and functional techniques to determine the possible role of HAGE in malignancy.

Chapter 2

Materials and Methods

2.1 Laboratory Consumables and Equipment

2.1.1 Reagents used and list of producers

All reagents were stored according to the manufacturer's instructions and used within their expiry date.

Culture Media	**Supplier**
RPMI 1640	Lonza
Reduced serum Opti-MEM (With L-glutamine and HEPES)	Invitrogen

Culture Media Supplements	**Supplier**
Foetal calf serum (FCS)	Bio Whittaker Europe
L-glutamine	Lonza
Geneticin (G418)	Promega

Other Cell Culture Materials	**Supplier**
Dimethyl sulfoxide (DMSO)	Sigma
Dulbecco's phosphate buffered saline (DPBS)	Lonza
Trypan blue stain	Sigma
Trypsin/Versene	Lonza

Chemical Reagents	**Supplier**
Acrylamide	Geneflow
Ammonium persulphate (APS)	Geneflow
Bovine serum albumin (BSA)	Sigma
Chloroform	Sigma
Deoxycholate	Sigma
$DilC_{12}(3)$ fluorescent dye	SLS
Dithiothreitol (DTT)	Sigma
dNTP	Bioline

Ethanol	BDH
Ethyldiamine tetraacetic acid (EDTA)	Sigma
Glycerol	Sigma
Hydrochloric acid (HCl)	Fisher Scientific
INTERFERin transfection reagent	Polyplus Transfection
Isopropanol	Fisher Scientific
Lipofectamine 2000	Invitrogen
Marvel milk powder	Premier Brands
Methanol	Fisher Scientific
Microscint-O scintillation media	Perkin-Elmer
PBS tablets	Sigma
Phenol/chloroform/isomyl alcohol	Sigma
Potassium acetate (KOAc)	Sigma
RapidStep ECL Reagent	Calbiochem
RNA-STAT-60	AMS Biotechnology
Sodium azide (NaN_3)	Sigma
Sodium chloride (NaCl)	Fisher Scientific/ Sigma
Sodium dodecyl sulphate (SDS)	Sigma
Sodium fluoride	Sigma
Sodium orthovanadate	Sigma
Stacking gel buffer for SDS gels	Geneflow
SYBR Green Supermix	BioRad
TEMED	Geneflow
Tris-base	Melford/Sigma
Tween-20	Sigma
Western-C Precision Plus molecular weight marker	BioRad

Immunochemical Reagents	**Supplier**
Mouse anti-human DDX43 (HAGE) monoclonal antibody	Santa Cruz/Sigma
Mouse anti-human FAK monoclonal antibody	Santa Cruz
Mouse anti-human FAS-ligand (CD178) antibody	Sigma
Mouse anti-human MMP-9 monoclonal antibody	Santa Cruz
Mouse anti-human N-RAS monoclonal antibody	Sigma

Mouse anti-human $p21^{CIP1}$ monoclonal antibody	AbCam
Mouse anti-human TIMP-4 monoclonal antibody	Santa Cruz
Rabbit anti-human polyclonal HAGE antibody	Pacific Immunology
Rabbit anti-human phospho-AKT (Ser 473) antibody	Cell Signalling Technology
Rabbit anti-human total AKT antibody	Cell Signalling Technology
Rabbit anti-human β-actin antibody	Cell Signalling Technology
Rabbit anti-human phospho-GSK-3β (Ser9) antibody	Cell Signalling Technology
Rabbit anti-human total GSK-3β antibody	Cell Signalling Technology
Mouse IgG isotype negative control antibody	ABD Serotec
Rabbit IgG isotype negative control antibody	Pierce Biotechnology
Alexa-Fluor 448 fluorescent secondary antibody	Santa Cruz
Alexa-Fluor 558 fluorescent secondary antibody	Santa Cruz
Goat anti-mouse-HRP secondary antibody	Dako
Steptavidin-HRP secondary antibody	BioRad
Swine anti-rabbit-HRP secondary antibody	Dako

Plasmids	**Supplier**
pBudCE 4.1, pcDNA3.1	Invitrogen
HAGE shRNA and negative control shRNA plasmids	S.A. Biosciences

Small Inhibitory RNA (siRNA) Molecules	**Supplier**
HAGE, H-RAS, K-RAS, N-RAS, negative control	EuroGentec

Kits	**Supplier**
Reverse Transcription:	
M-MLV reverse transcription kit	Promega
Total Protein Assay:	
Total protein assay kit	BioRad
Phospho-protein evaluation:	
Proteome Profiler MAPK array kit	R&D Systems
Active Ras (Ras-GTP) detection:	
Active Ras Pull Down and Detection kit	Thermo
RNA Extraction:	
RNA extraction kit	Ambion
Caspase detection:	
Caspase-Glo 3/7 Assay	Promega
Cell Invasion:	
Biocoat Matrigel Invasion System plate (8μm pore)	BD
Falcon FluoroBlok 24-Multiwell Insert System (8μm pore)	BD
Apoptosis:	
Annexin/7-AAD staining kit for apoptosis detection	Beckman-Coulter

2.1.2 Equipment

Glassware

Pyrex glassware was used. All items were washed in dilute presept/ teepol and then rinsed twice in distilled water and autoclaved prior to use.

Disposable equipment and plasticware	**Supplier**
Bijou tubes (7ml)	Sterilin
Coverslips	SLS
Cryovials (1.5ml)	TPP
Eppendorf tubes (0.5ml/1.5ml)	Sarstedt
FACS tubes	Elkay
Flat-bottom culture dishes (6 well/ 24 well)	Sarstedt
Filter tips (20µl/200µl/1000µl)	Sarstedt/ Deuchter Scientific
Hybond-P PVDF western blot membrane	G.E. Healthcare
Pasteur pipettes	Sarstedt
96-well round-bottom culture plates	Sarstedt
Real-time qPCR tubes	Qiagen
Scalpels	Swann Morton
Scrapers	TPP
Screw top tubes	Sarstedt
Serological pipettes	Sarstedt
Tips (20µl/200µl/1000µl)	Sarstedt
T25/T75/T175cm^3 culture flasks	Sarstedt
Unifilter 96-well filter plates	Perkin-Elmer
20ml Universal tubes	Sterilin
Western blot filter paper	Schleicher-Schwell
0.2µm filters	Sartorious

Equipment	**Supplier**
2100 Bioanalyser	Agilent Technologies
Biofuge Pico 4^{o}C micro-centrifuge	Heraeus
CCD Camera	Fuji Systems
4^{o}C refrigerated centrifuge	Eppendorf
Class II safety cabinet	Walker
Confocal microscope	Leica
Cryostat	Leica

Cryostore	Forma Scientific
Drying cabinet	SLS
Electrophoresis gel tanks	Geneflow
-80^{o}C Freezer	Thermo Scientific
Combined 4^{o}C fridge/-20^{o}C freezer	Premier
Gallios flow cytometer	Beckman-Coulter
PALM Laser-capture microdissector (LCM)	Carl Zeiss
Microscope	Nikon
Microwave	Matsui
Mini-orbital shaker	Stuart
Nanodrop 8000 spectrophotometer	Thermo Scientific
pH meter	Mettler Toledo
Plate reader	BioRad and Tecan
Plate rocker	VWR
96-well plate harvester	Packard
PCR workstation cabinet	Grant-Bio
Power packs	BioRad
Real-time qPCR thermal cycler	Qiagen
Sealing film	Bando Chemicals
Transfer apparatus	Geneflow
37^{o}C/5%CO_2 incubators	Forma Scientific
Top count scintillation counter	Packard
UNO-thermoblock	Biometra
Vortex mixer	Scientific Industries
Water baths	Grant

2.1.3 Buffers

Buffers used for cell culture**:**

Trypan Blue for cell counts:

0.1% (v/v) solution Trypan Blue in DPBS.

Buffers used for western blot analysis**:**

1X 10% Resolving SDS gel:

1900µl ddH_2O
1700µl Acrylamide
1300µl 1.5M Tris-HCl (pH 8.8)
50µl 10% (w/v) SDS
50µl 10% (w/v) APS
2µl TEMED

1X 15% Resolving SDS gel:

1100µl ddH_2O
2500µl Acrylamide
1300µl 1.5M Tris-HCl (pH 8.8)
50µl 10% (w/v) SDS
50µl 10% (w/v) APS
2µl TEMED

1X 5% Stacking SDS gel:

1400µl ddH_2O
330µl Acrylamide
250µl 1M Tris-HCL (pH 6.8)
20µl 10% (w/v) SDS
ddH2O to final volume

Reducing sample buffer:

0.5M Tris-HCl (pH 6.8)
2% (w/v) SDS
10% (v/v) Glycerol
1% DTT

Tris buffered saline+ Tween-20 (TBST):

1.21g Tris base
22.33g NaCl
pH made to 7.5 with HCl
100mM sodium orthovanadate
100mM sodium fluoride
0.05% Tween-20

TBST+ Marvel blocking buffer:

100ml TBST
10% (w/v) Marvel milk powder

5X SDS gel running buffer:

15.1g Tris-base
94g Glycine
50ml 10% (w/v) SDS
1L ddH_2O
Made to 1x for each use using ddH_2O

SDS gel transfer buffer:

5.8g Tris-base
2.9g Glycine
0.37g SDS
200ml Methanol
800ml ddH_2O

Buffers used for flow cytometry (FACS) analysis:

Fixation solution:

1% (v/v) paraformaldehyde in DPBS

Permeablisation solution:

70% (v/v) Ethanol in DPBS

FACS buffer:

0.1% (w/v) BSA
0.02% (w/v) NaN_3
1xDPBS

Buffers used for immunohistochemistry:

Primary antibody:

100µg/ml rabbit anti-HAGE antibody
5% (v/v) goat serum
Made to final volume using PBS

Secondary antibody:

10µg/ml goat anti-rabbit IgG-biotin
1.5% (v/v) goat serum
Made to final volume using PBS

ABC Reagent:

2.5ml PBS
1 drop Reagent A
1 drop Reagent B

DAB Reagent:

2.5ml DDH_2O
1 drop buffer
2 drops DAB
1 drop H_2O_2

Buffers for Immunofluorescence

PBST

1L ddH_2O

Buffered to pH 7.2 using PBS tablets

0.1% (v/v) Tween-20

Blocking solution

1X PBST

10% (w/v) BSA

Buffers for LCM section staining

Ethanol solutions

Diluted to strength using molecular grade water.

Cresyl Violet

1% (w/v) solution in 50% (v/v) ethanol

Filtered before use

2.1.4 Cell Lines and Growth Conditions

All cells were grown cultured in incubators supplying a 37^{o}C/5%CO_2 atmosphere

Table 2.1: *List of tumour cell lines used in this study, describing their tumour histology, growth conditions as well as their source supplier*

Cell Line	Description	Media	Source
FM-3	Human melanoma	RPMI 1640+10% (v/v) FCS+2mM L-glutamine	Prof. D Schadendorff (Tuebingen University)
FM-6	Human melanoma	RPMI 1640+10% (v/v) FCS+2mM L-glutamine	Prof. D Schadendorff (Tuebingen University)
FM-82	Human melanoma	RPMI 1640+10% (v/v) FCS+2mM L-glutamine	Prof. D Schadendorff (Tuebingen University)
FM-3/-ve	Human melanoma transfected with pcDNA3.1/-ve plasmid	RPMI 1640+10% (v/v) FCS+2mM L-glutamine+500μg/ml G418	Nottingham Trent University
FM-3/HAGE	Human melanoma transfected with pcDNA3.1/HAGE plasmid	RPMI 1640+10% (v/v) FCS+2mM L-glutamine+500μg/ml G418	Nottingham Trent University
FM-82/Ctrl	Human melanoma transfected with control shRNA plasmid	RPMI 1640+10% (v/v) FCS+2mM L-glutamine+500μg/ml G418	Nottingham Trent University
FM-82/ shRNA	Human melanoma transfected with HAGE shRNA plasmid	RPMI 1640+10% (v/v) FCS+2mM L-glutamine+500μg/ml G418	Nottingham Trent University
Jurkat	Human T cell lymphoma	RPMI 1640+10% (v/v) FCS+2mM L-glutamine	ATCC
PCI-13	Human head and neck carcinoma	RPMI 1640+10% (v/v) FCS+2mM L-glutamine	Prof. E Tatour (Hôpital Européen George Pompidou)

2.2 Methods and Procedures

2.2.1 Examination of HAGE in Tumour Proliferation

2.2.1.1 Transient HAGE cDNA Transfection to Induce HAGE Gene Expression

Induction of HAGE gene expression was done transiently by transfecting the HAGE-negative human melanoma cell line FM-3 with the pBud CE4.1/HAGE cDNA plasmid or control plasmid using Lipofectamine 2000 reagent. Briefly, cells were plated in the wells of an 8 well chamber slide (for immune-staining) or a T25 flask (for RNA extraction and real-time qPCR) and allowed to grow normally overnight. The following day, cells underwent transfection. In one tube, Opti-MEM media was combined with the plasmid. In a separate tube, the same amount of Opti-MEM media was used to dilute Lipofectamine 2000 transfectant reagent. These tubes were agitated using a vortex mixer and allowed to incubate for five minutes at room temperature (RT). Following this, the contents of both tubes were combined, mixed using a vortex and allowed to stand at RT for 20 minutes. During this time, media was removed from the cells and replaced with RMPI 1640 media. Following incubation, transfection mixtures were applied to all relevant cells. To ensure even coverage of all cells, after incubation, plates/ flasks were agitated using a rocking platform while all materials were applied and plates were allowed to rock for five minutes post application.

2.2.1.2 Transfection of Small Inhibitory RNA (siRNA) to Bring about Transient RNA Interference

Transient silencing of HAGE and RAS members via RNA interference (RNAi) was brought about using a siRNA molecule that was specific for each gene, the sequences of which are shown in Table 2.2. To transfect this material into the cells, INTERFERin transfection material (Polyplus) was used. Briefly, cells were grown overnight in either 24-well plates or T25cm^3 flasks. Reagent mixtures were then made in Opti-MEM media. 40μM of siRNA or a negative control siRNA was diluted into this media and placed briefly on a vortex mixer. INTERFERin reagent was then added in a certain volume and vortex mixed again. For *in vitro* proliferation studies,

some cells were treated with Opti-MEM media laced with INTERFERin only or simply Opti-MEM media alone, to create further negative controls. In all cases, these latter two control measures were prepared at the same time as those involving siRNA, only omitting inclusion of said molecule. All reagents were added to appropriate cells while vessels were agitated on a rocking platform to ensure transfection and plates were allowed to rock for a further five minutes.

Table 2.2: *Sequences of sense and anti-sense strands of siRNA molecules used to knockdown expression of genes in specific cell lines.*

Gene	siRNA Strand	Sequence
HAGE	Sense	5′ AUUAGAGAGGAAGGUUUGA 3′
	Anti-sense	5′ UCAAACCUUUCCUCUCUAAU 3′
H-RAS	Sense	5′ CGUGAGAUCCGGCAGCACATT 3′
	Anti-sense	5′ UGUGCUGCCGGAUCUCACGTT 3′
K-RAS	Sense	5′ UCAAAGACAAAGUGUGUAATT 3′
	Anti-sense	5′ UUACACACUUUGUCUUUGATT 3′
N-RAS	Sense	5′ CAGCAGUGAUGAUGGGACUTT 3′
	Anti-sense	5′ AGUCCCAUCAUCACUGCUGTT 3′

2.2.1.3 Generation of Human Melanoma Cells Stably Transfected with HAGE cDNA or shRNA

To investigate the effect of induced expression/silencing of the HAGE gene over prolonged periods of time, it was necessary to create stable transfectant cell lines. For stable cDNA transfection, the cell line FM-3, normally negative for HAGE expression was used, while the FM-82 cell line, which demonstrates high HAGE gene expression was utilised to induce stable knockdown. Both cell lines were grown overnight in eight wells each of two 24 well plates. The following day, the cells of one plate were transfected using the gene-specific plasmid, with the other plate being treated with the control. For the generation of a stable cell line with induced HAGE expression, the pcDNA.3.1/HAGE plasmid was employed, while the other was treated with the pcDNA.3.1/-ve plasmid, thus producing a negative control cell line. To create a cell line with stable knockdown of HAGE, one plate of FM-82 cells was

treated with a HAGE-specific shRNA, while the other underwent treatment with the negative control plasmid.

In both cases, cells underwent transfection using Lipofectamine 2000 reagent in the same manner as with the transient transfection of the pBud CE4.1 plasmid described above. In this instance, the amount of media used was enough to ensure that there was 100µl/well and enough plasmid for 1.2µg/well. In the second tube, the same amount of Opti-MEM media was used to dilute Lipofectamine 2000 transfectant reagent for 2µl/well. During plasmid/Lipofectamine incubation, plated cells had their growth media removed and this was replaced with 500µl/well of fresh complete RPMI 1640 media.

48 hours following transfection, media was removed from all wells and cells were washed using DPBS before undergoing trypsinisation to remove them from the plate wells. All of the cells were pooled into a tube containing complete RPMI media, therefore providing two tubes, which were then centrifuged to wash each cell line. Supernatant was discarded before cells were resuspended in complete RPMI media containing 500µg/ml G418 selective antibiotic and transferred to flasks and allowed to grow. Since all the plasmids used contain an antibiotic resistance gene, addition of G418 would potentially allow all cells that expressed the plasmid to continue growing, while eliminating all those that did not. This way it was possible to maximise the number of transfected cells. Cells were cultured for four passages prior to RNA extraction and real-time qPCR confirmation that HAGE was expressed by cells transfected with the HAGE containing plasmid and not those transfected with the empty vector. This process of passaging and examination of HAGE gene expression was continued until 10 passages had been completed since initial transfection.

2.2.1.4 Tritiated thymidine (^{3}H) Incorporation Proliferation Assay

To examine effect of HAGE expression on cell proliferation, FM-3, FM-6 and FM-82 were grown and treated in 24-well plates as described above. Stable transfectants were grown in 24-well plates as per normal growth conditions. Proliferation readings

were taken at three and seven days post plating/treatment for all cells except FM-3/-ve and FM-3/HAGE, which due to extremely fast growth had to have the measurement times optimised to four and six days post plating. In all cases, the day prior to measurement, cells had their media removed and replaced with media that had been laced with ^{3}H. Each well was treated with 200µl of cell culture media containing 2µl ^{3}H and plates were incubated overnight at 37^{o}C/5% CO_2. The day following ^{3}H treatment, plates were removed from incubation and the media was extracted from all wells using a pipette. Each well of the 24-well plate was then washed with 200µl of ddH_20 to allow cell lysis and release the incorporated ^{3}H before this water was transferred to the well of a 96-well round bottom culture plate. This process was repeated for all wells to be tested. The contents of the plate were subsequently harvested onto a Unifilter 96-well plate using a Filtermate Harvester, which was allowed to dry using a drying cabinet. Each test well was filled with 40µl of Microscint-O media that would allow the ^{3}H to be read. Plate reading was carried out using a Top-Count NXT microplate scintillation counter.

2.2.1.5 Examination into the *in vivo* Growth of HAGE stable transfectants

To check extent of growth of HAGE-positive tumours *in vivo*, a malignant growth model was established. Non obese diabetic/severe combined immunodeficiency (NOD/SCID) mice were acquired (Harlan Laboratories, U.K) and kept in accordance with U.K Home Office regulations. Mice were kept in IVC cages supplied with laminar air flow along with autoclaved food and water taken *ad libitum*. All work with these mice was performed in sterile conditions within a laminar flow hood. Two sets of studies were carried out, the first comparing FM-3/-ve with FM-3/HAGE while the second involved a comparison between FM-82/Ctrl and FM-82/shRNA. In both studies, two groups of mice were utilised, each being injected with one of the specific cell lines. In all cases, mice were injected sub-cutaneously (s.c) into the right flank. $1x10^{6}$ cells/100µl were injected per mouse. Tumour development was observed twice weekly using calliper measurement. When required, mice were euthanised prior to tumours being excised, placed on cork boards, coated in OCT mounting media and snap frozen in liquid nitrogen-cooled isopentane and stored at-80^{o}C.

Tumours were sectioned using a cryostat to produce 8μm thick sections that were placed on sialinised microscope slides. These were permeabilised using 4% (w/v) paraformaldehyde at RT for 10 minutes, washed in PBS, then fixed in 70% (v/v) ethanol for 10 minutes at RT, before a final PBS wash. Slides were then placed in storage at -80^{o}C.

2.2.1.6 Detection of HAGE Protein in Human Melanoma Cell Lines Using Immunofluorescence Dual Staining.

For examination of protein expression in cell lines, methanol washed coverslips were placed in to the wells of a flat-bottomed 24-well plate. Cells were counted and diluted accordingly before cells were plated into wells at 5x10^{4} cells/well and allowed to grow for 48 hours. For staining, media was removed and cells were washed in PBST for 10 minutes. Following washes, 4% paraformaldehyde was applied for one hour at RT to fix cells, after which cells were washed using PBST. PBST was removed and replaced with 200μl of blocking solution applied for two hours at RT while the plate was moving on a rocking platform. Once the blocking period had ended, primary antibodies from different host species for proteins of interest or isotype controls were added to the blocking solution in relevant wells. The plate was placed on a rocking platform and staining took place overnight at 4^{o}C.

The following day, blocking solution was removed and cells underwent three separate washes with PBST. A cocktail of Alexa-Fluor 468 (Green) and 568 (Red) fluorescent secondary antibodies were diluted in blocking media and once the final wash had been completed, 200μl of this was added to all relevant wells. The plate was covered in foil to retain signal and placed on a rocking platform for one hour at RT after which cells were washed as above. To individual microscope slides, a small amount of Vectashield containing DAPI stain was added. The coverslips bearing the stained cells were then removed, inverted and placed coverslip face up on the slides. These were viewed using a fluorescence microscope.

2.2.1.7 Immunofluorescent Staining of Murine Tumours.

Slides were removed from -80^{o}C storage and treated with a 1-3% (v/v) H_2O_2 solution for 15 minutes at RT. Slides were rinsed in PBS and placed in a PBS bath for five

minutes to fully remove the prior reagents. Dual immune staining was then performed. In brief; a solution of 5% (w/v) human serum in PBS was applied to slides to act for 30 minutes at RT and washed in PBS. Primary antibodies were diluted in a solution of PBS with 10% BSA and 0.1% (v/v) Tween-20. The primary antibodies used were raised in two separate species (i.e. Rabbit and mouse) to ensure distinct separate staining. This mixture was placed onto slides and allowed to act at RT for two hours. Slides were rinsed with PBS before being placed in a PBS/0.1% (v/v) Tween-20 bath for five minutes. This was repeated to ensure complete washing. Secondary antibodies specific to the primaries, were prepared in the same manner as the primary, applied to slides for 30 minutes at RT. Slides were rinsed and washed as above ahead of slides being mounted using Vectashield+DAPI and a coverslip. Slides were incubated at 4^{o}C overnight and the following day, were examined using a fluorescence microscope.

2.2.1.8 Immunohistochemistry of Murine Tumours

Slides were removed from -80^{o}C storage and 0.03% (v/v) H_2O_2 diluted in 1X PBS was added to the tissue sections for five minutes before being washed with PBS. Sections were then blocked for 10 minutes with 10% (v/v) serum made from the species of the secondary antibody. In the case of HAGE, goat serum was used. Following incubation, the serum was removed and 100μg/ml of the primary antibody, a mono-specific polyclonal antibody was added for overnight incubation at 4°C. Slides were then washed in 1X PBS and 10μg/ml of the secondary antibody (biotin-conjugated goat anti-rabbit IgG) was added. Slides were incubated for 30 minutes at RT and washed thoroughly with 1X PBS. The ABC reagent was laid onto the slides, left to react with secondary antibodies for 30 minutes at RT and washed off with PBS. The DAB reagent was added to the sections in order to react with the ABC reagent for 10 minutes at RT and washed off with ddH_2O. Finally, frozen and paraffin-embedded sections were counterstained in Gill's and Harris' staining, respectively, and fixed consecutively in graded ethanol (70%, 100%, 100% (v/v)) and in xylene. Slides were mounted, air-dried and observed under the microscope.

2.2.1.9 PALM LCM and RNA Extraction of NOD/SCID Tumours

PALM membrane slides were exposed to U.V. light in a sterile hood for 15 minutes to improve adherence of tissue. NOD/SCID tumours were sectioned using a cryostat in the same manner as sections used for IHC. Slides were placed on dry ice and stored at -80^{o}C. For fixation and staining, slides were treated in molecular grade solvents. They were placed in 95% (v/v) ethanol for 40 seconds, 75% (v/v) ethanol for 30 seconds and 50% (v/v) ethanol for 30 seconds while agitating the slide on the final wash to remove OCT media. 300µl filtered cresyl violet stain was added to each slide to inhibit the action of any RNase molecules present. Slides were drained, placed in 50% (v/v) ethanol for 30 seconds, 75% (v/v) ethanol for 30 seconds, 95% (v/v) ethanol for 40 seconds before two separate washes in 100% (v/v) ethanol for 40 seconds. Slides were then rinsed xylene and then bathed in this solvent for five minutes.

After fixation and staining was complete, LCM was performed immediately to maintain specimen quality. Section was done using a PALM LCM instrument, using onboard software to isolate approximately 500 cells. Cut sections were captured into the caps of LCM tubes and immediately 100µl RNA lysis buffer was placed into the tube. Tubes were sealed, vortex mixed and placed in storage at -20^{o}C.
RNA extraction of these sections was done using an RNAqueous Micro extraction kit as according to the manufacturer's protocol, specifically the protocol for extraction of tissue taken by LCM. The manufacturer's advice for DNAse treatment was also adhered to.

2.2.1.10 Cell Cycle Analysis

Cells were starved overnight in serum free media to bring about synchronisation of their cell cycle. The following day, cells were harvested and washed in FACS buffer. They were subsequently fixed using ice cold 70% (v/v) ethanol mixed using a vortex and incubated at 4^{o}C for 30 minutes. After this cells were pelleted by centrifugation at 2,000 rpm for five minutes ahead of two washes using FACS buffer. 50µl of a 100µg/ml RNAase solution was applied to degrade RNA in the cells, followed by

50µl of a 25µl/ml 7-AAD solution to stain DNA. Cells were analysed using a Gallios flow cytometer, collecting 25,000 events for each sample tested. The percentage of cells in each phase of the cell cycle was determined using Kaluza® software (Beckman- Coulter).

2.2.2 Examination of the Genetic Interactions of HAGE

2.2.2.1 Extraction of RNA Using RNA-STAT-60

Cells were grown as already stated and had their culture media removed before being carefully washed using DPBS. Following the removal of DPBS, RNA-STAT- 60 was applied in either 500µl (For 24-well plates and T25cm^3 flasks) or 1ml (For T75cm^3 flasks) quantities. The extraction of material was carried out as according to the manufacturer's protocol. RNA pellets retrieved from 24-well plates and T25cm^3 flasks were re-suspended in 10µl ddH_2O or in 20µl ddH_2O if the sample pellets came from cells grown in a T75cm^3 flask.

Quantity of RNA extracted was tested using a Nanodrop 8000 U.V. spectrophotometer after the instruments had been calibrated using a ddH_2O blank. This allowed readings to be taken at 260nm and 280nm wavelengths, permitting a ratio to be given that could give a general reading of RNA quality. Measurements with Nanodrop 8000 involved pipetting 1.5µl of extracted RNA onto the measurement wells. The concentration of RNA in µg/µl was calculated before each sample's quantity was adjusted to 1µg/µl using ddH_2O. RNA samples were stored at -80^{o}C for future use.

2.2.2.2 Genechip® Oligonucleotide Microarray Analysis

FM-82 cells were grown in T25cm^3 flasks and treated with either control or HAGE-specific siRNA. RNA from test and control flasks was extracted using RNA-STAT-60 as mentioned above. All experiments were performed in triplicate, therefore providing six samples. Since high quality RNA is required for microarray analysis, in the first instance, quality was checked using Nanodrop spectrophotometry as

mentioned above. Once this was satisfactory, RNA was supplied to NASC Affymetrix Service based at University of Nottingham, where microarray testing was carried out. Prior to testing, quality was assessed again through the use of Agilent Bioanalysis. Microarray analysis was performed using the Affymetrix Genechip® microarray platform with Human U133 Plus 2.0 arrays. Data provided from these experiments was subsequently supplied back to this laboratory.

2.2.2.3 Data Analysis of Genechip® data

Genechip® report data was checked using Genespring software (Version 9.0) (Agilent Technologies) at University of Nottingham by Dr Neil Graham. This checked the noise level, extent of RNA hybridisation and housekeeping gene expression for every Genechip® tested. All of this data was satisfactory for analysis to continue. Analysis was performed in association with Dr Morgan Mathieu and Dr Graham Ball using differential and regression analysis of the data. For both experiments, the averaged values for those cells that expressed HAGE were compared to the averaged values of samples taken from control cells from the same experiment. This provided ratios of relative expression for all genes and generated a list of genes that were up/down-regulated when HAGE was silenced.

2.2.2.4 Literature Searches

From the list of genes generated from microarray testing, those with a change of expression ratio of 2.5 fold or greater were isolated for further study. The function of these genes was researched using both Entrez Nucleotide and Online Mendelian Inheritance in Man (OMIM) online database searches (www.ncbi.nlm.nih.gov). Both of these databases allowed the definition of each gene's possible role in tumour-modified events and could allow the possibility of having further work carried out on them.

2.2.3 Gene Expression Analysis

2.2.3.1 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

cDNA was synthesised using the RNA extracted from the cells used in different experiments. cDNA was generated using reagents obtained from Promega and used according to the manufacturer's protocol. Briefly, 2µg sample RNA was mixed in an Eppendorf tube with 1µl oligo-dT primers and diluted to 15µl with ddH_2O. This was repeated for all samples to be tested. The primers were allowed to anneal to the RNA by heating all tubes to 70°C for five minutes using a UNO-Thermoblock. During incubation, a reverse transcriptase mix was produced by combining Muloney murine leukaemia virus (M-MLV) 5X buffer, dNTPs, RNasin RNase inhibitor, M-MLV-reverse transcriptase and ddH_2O. This mix was made to allow 10µl to be added to each sample, thus bringing the total volume of each sample to 25µl. Following the addition of RT mix to all samples, tubes were placed in a water-bath pre-heated to 39.2°C for 80 minutes to allow cDNA synthesis to take place. Once this was complete, tubes were removed from water-bath incubation and were again placed in a UNO-Thermoblock and heated to 95°C for five minutes to stop the reaction. Finally, all tubes were placed in a -20°C freezer for future use.

2.2.3.2 Real Time qPCR

cDNA generated as described above was used to act as a template in real-time RT-PCR with the reagents shown below:

- 6.25µl iQ Sybr Green
- 0.5µl Gene specific sense primer
- 0.5µl Gene specific anti-sense primer
- 4.75µl ddH_2O
- 0.5µl cDNA template

These reagent mixtures were added to all tubes for a specific gene. All samples were tested in duplicate alongside negative controls that contained the same reagent mixture, omitting sample cDNA, to act as a non-template control (NTC). Real-time

qPCR was carried out using the Rotogene 6000 real-time qPCR analyser (Qiagen) to test the expression of genes of interest. The expression of the housekeeping genes GAPDH, TBP-1 and HPRT-1 was used to calculate relative gene expression using 2ΔCT calculations. Real-time qPCR was carried out using the following protocols:

Stage	Step	Temperature	Time	Cycles
Hold	N/A	95ºC	5 minutes	1
Cycling	1 (Denaturation)	95ºC	30 seconds	40
	2 (Annealing)	x oC	30 seconds	
	3 (Extension)	72ºC	*y* seconds*	
Melt	N/A	x *-95ºC	5 minutes	1

χ refers to the annealing temperature used to allow the annealing of gene specific primers to template cDNA, shown in Table 2.3 while *y* refers to extension times shown in Table 2.3.

Table 2.3: *List of primers used in real-time qPCR experiments to examine expression of genes of interest following HAGE expression modification in various cell lines. Sequences, annealing temperatures and extension times are detailed for each set of primers.*

Gene	Primer	Annealing Temperature (ºC)	Extension Time (Seconds)
HAGE	F: 5' GGAGATCGGCCATTGATAGA 3'	64.5	30
	R: 5' GGATTGGGGATAGGTCGTTT 3'		
GAPDH	F: 5' ACCACCCCCTGCTTAGCACC 3'	58	10
	R: 5' CCATCCACAGTCTTCTGGGT 3'		
HPRT-1	F: 5' TGACACTGGCAAAACAATGCA 3'	55	10
	R: 5'GGTCCTTTTCACCAGCAAGCT 3'		
TBP-1	F: 5' TGCACAGGAGCCAAGAGTGAA 3'	56	10
	R: 5' CACATCACAGCTCCCCACCA 3'		
CCNE2	F: 5' CCCCAAGAAGCCCAGATAAT 3'	58	10
	R: 5' AATCAGGCAAAGGTGAAGGA 3'		
CIDE-A	F: 5' CTTAACGTGAAGGCCACCAT 3'	60	10
	R: 5' AGAAACTGTCCCGTCACCTG 3'		
CXCL-6	F: 5' GTCCTGTCTCTGCTGTGCTG 3'	61	10
	R: 5' AACTTGCTTCCCGTTCTTCA 3'		
E2F2	F: 5' GGCCAAGAACAACATCCAGT 3'	60	10
	R: 5' TGTCCTCAGTCAGGTTGCTTG 3'		
FAS-L	F: 5' GGAAAGTGGCCCATTTAACA 3'	60	10
	R: 5' CAAGATTGACCCCGGAAGTA 3'		
HSP-27	F: 5' CGAGTACGAATTTGCCAACC 3'	60	10
	R: 5' GTCACCTCGTCTGGGGTAAAA 3'		
HSP-70	F: 5' CGGTTTGACCTGACTGGAAT 3'	58	10
	R: 5' TGCAGCAATTTTCTCCCTCT 3'		
IL-15	F: 5' ATTTTGGGCTGTTTCAGTGC 3'	58	10
	R: 5' ACTTTGCAACTGGGGTGAAC 3'		
JUN-D	F: 5' GCCCTGGAGGATTTACACAA 3'	60	10
	R: 5' CTCAGGTTCGCGTAGACAGG 3'		
PKC-β1	F: 5' TGAAGGGGAGGATGAAGATG 3'	60	10
	R: 5' TAAGGGGGCTGGATCTCTTT 3'		
PTEN	F: 5' ACCAGGACCAGAGGAAACCT 3'	61	10
	R: 5' GCTAGCCTCTGGATTTGACG 3'		
OAS-1	F: 5' CAAGCTCAAGAGCCTCATCC 3'	59	10
	R: 5' TGGGCTGTGTTGAAATGTGT 3'		
HRAS	F: 5' TGCCATCAACAACACCAAGT 3'	58	10
	R: 5' ATCTCACGCACCAACGTGTA 3'		
KRAS	F: 5' TACAGTGCAATGAGGGACCA 3'	60	10
	R: 5' TCCTGAGCCTGTTTTGTGTCT 3'		
NRAS	F: 5' AAACCTCAGCCAAGACCAGA 3'	60	10
	R: 5' CCCTGAGTCCCATCATCACT 3'		
SFRP-1	F: 5' ATCTCTGTGCCAGCGAGTTT 3'	60	10
	R: 5' GAAGTGGTGGCTGAGGTTGT 3'		
STAT-1	F: 5' AAATTCCTGGAGCAGCTTCA 3'	59	10
	R: 5' TGGCCCCAGTCACTTAATC 3'		
WNT-1	F: 5' TCCTGCTCAGAAGGTTCCTA 3'	60	10
	R: 5' GCCTCGTTGTTGTGAAGGGTT 3'		

2.2.4 HAGE in Tumour Modified Events

2.2.4.1 Investigation into HAGE Influence over Cell Signalling

Mitogen activated protein kinase (MAPK) Proteome Profiler kits were obtained from R&D systems. These were used in conjunction with HAGE-stable shRNA transfectant lines. These kits were used as according to the manufacturer's protocol. Prior to testing, protein concentration of cells was assessed under manufacturer's advice and carried out using the total protein assay protocol described below. Membranes were developed using the same technique as for immune blots also described below. Spot densitometry to test intensity was carried out using Aida software.

2.2.4.2 Total Protein Assay

For the calculation of cell lysate protein concentration, total protein assay were carried out using BioRad Dc protein assay reagents according to the manufacturer's protocol. Standards were produced from a stock BSA solution (10mg/ml) to create a series of standard dilutions (2, 1.5, 1.0, 0.8, 0.5, 0.4, 0.2 and 0mg/ml). Standards were tested in duplicate, while protein extracts were tested in triplicate. All assays were carried out in 96-well round bottom plates. 25µl of reagent A was applied to all samples followed by 200µl reagent B. Plates were covered in foil and left to incubate for one hour at RT. Absorbance measurements for plates were read using a plate reader set to a 750nm wavelength. Using these absorbance values, protein fraction concentrations were calculated.

2.2.4.3 Development of Proteome Profiler Arrays

After secondary antibody application, arrays were washed as stated in the protocol. ECL reagents were used to lightly wash each membrane. Following this, membranes were placed into a CCD camera chamber cooled to -25^{o}C for development prior to the image being captured.

2.2.4.4 Western Blot Analysis of Protein

Following culture, cells were counted and diluted to a $1x10^6$ cells/ml concentration. $1x10^6$ cells were taken from this dilution, placed into a tube and pelleted by centrifugation at 1,500rpm for three minutes. This pellet was resuspended in 1ml DPBS, transferred to a 1.5ml Eppendorf tube and centrifuged at 2,000rpm for one minute to remove residual media. DPBS was removed and cells were resuspended in 300µl sample reducing buffer, breaking down disulphide bonds in the protein structure and transferred to a 500µl Eppendorf tube. These were placed into a thermoblock set to 95^oC for 10 minutes to allow protein denaturation and either separated using SDS electrophoresis or stored at -20^oC for future use.

40µl of sample was loaded into the wells of a SDS gel immersed in a tank containing 1X tris-glycine-SDS running buffer. All samples were run alongside a molecular weight marker. When loaded, a 70V current was applied to allow proteins to pass through the 5% stacking gel and once this was achieved, the current was increased to 90V to permit proteins to be separated in 10% resolving gel. PVDF transfer membranes were prepared by washing with 100% methanol for five seconds, ddH_2O for five minutes and transfer buffer for 10 minutes. When proteins had run through the gel they were transferred to the PVDF membrane using 'wet transfer'. Briefly, a gel frame was immersed in cold transfer buffer. To this, a buffer sponge soaked in transfer buffer was placed into the frame. This was followed by one piece of filter card, then by the gel, PVDF membrane, another piece of filter card and finally a second buffer sponge soaked in transfer buffer. The frame was sealed and placed into an ice-cooled transfer tank filled with cold transfer buffer. A 100V current was applied for one hour to allow the transfer of the proteins.

When complete, membranes were cut accordingly so they could be treated with different antibodies, placed in vessels and washed in a 10% (w/v) Marvel milk solution for one hour at RT to block non-specific binding sites. The blocking solution was decanted; protein-specific antibody was diluted in 10% (w/v) Marvel solution and applied to the necessary membranes, which were agitated on a plate rocker

overnight at 4°C. The following day, membranes underwent three separate 10-minute washes using TBST, while vessels were agitated on a plate orbital shaker at RT.

Once washing was completed, secondary antibody specific to the host species of the primary was diluted in 10% (w/v) Marvel and applied to the required membranes for one hour while being agitated at RT on an orbital shaker. A streptavidin-HRP secondary antibody was also added to each membrane in order to stain the molecular weight marker present on each membrane.

Following staining with secondary antibodies, washes were carried out as above and membranes were developed. This was done by removing the membrane from its wash vessel and placing it in a clean tray, lightly washing it with ECL reagent and exposing it for a certain period of time using a CCD camera.

2.2.4.5 Detection of Activated RAS Protein

To determine if HAGE expression led to an increase in the levels of activated RAS (RAS-GTP) in tumour cells, a pull-down/detection kit was acquired (Thermo Scientific). This employs an antibody specific for the RAS-binding domain (RBP) of the RAF protein which is specific for activated RAS protein to isolate the active protein. Once extracted, this protein can be detected using western blotting with an anti-RAS antibody supplied with the kit. This was carried out on shRNA stable transfectants and performed according to the manufacturer's protocol, using 500μg of cell lysate proteins. In all cases, a positive control was created using GTPγS. All recommendations for modifications to in-house immune blot techniques were followed. Membranes were developed using a CCD camera.

2.2.4.6 Detection of Caspase 3/7 Following Apoptotic Drug Treatment

The levels of caspase 3/7 in HAGE stable cDNA and shRNA transfectant cell lines were measured using a luminometer screening method (Promega). Briefly, cells were cultured in six-well plates and treated with different concentrations of staurosporine and cisplatin to optimise the amount of drug required to induce cell death. For

screening, cells were plated at $1x10^4$ cells/well into a 96-well plate and grown overnight. The following day, media was removed from cells and replaced with 100µl media containing 0.3µg/ml of staurosporine or cisplatin. To determine background, media/drug mixture was added to wells containing no cells. The plate was incubated at $37^oC/5\%CO_2$ for four hours prior to the addition of 100µl caspase 3/7 detection reagent and incubated at RT for one hour. The plate was read using a luminometer and caspase levels were determined by normalising test values by subtracting background reading.

2.2.4.7Analysis of Apoptosis in Response to Cisplatin Treatment

To investigate if reduction of HAGE expression could leave tumour cells more susceptible to apoptosis induced by cisplatin, FACS analysis using Annexin-V-FITC/7-AAD staining was carried out according to the manufacturer's protocol. In brief, shRNA stable transfectants and Jurkat cells (positive control) were grown in $T25cm^3$ flasks in their normal culture conditions. For stable knockdown cells, the day prior to testing, media was decanted and replaced containing 0.3µg/ml cisplatin. For Jurkat cells, 100ng/ml of a CD95 agonist antibody was added to the media to initiate FAS induced apoptosis. For each cell line, a flask that underwent no treatment was used to act as a comparison. Cells were cultured for 24 hours. On the day of testing, cells were divided into tubes and either stained using Annexin-V-FITC/7-AAD or left unstained. All cells underwent FACS testing using a Gallios flow cytometer and data gained was analysed using Kaluza® software.

2.2.4.8 Detection of Surface Expression of FAS-Ligand

In order to confirm up-regulation of FAS-ligand (FAS-L) in HAGE-expressing cells, FACS analysis was used to determine surface expression of the antigen. cDNA and shRNA stable transfectants were cultured as normal before being divided into tubes. Following two washes in FACS buffer, cells were treated either with an antibody specific for FAS-L (CD178) or an isotype-specific negative control and incubated on ice for 30 minutes. Two washes with FACS buffer were carried out prior to the addition of a species-specific FITC-conjugated secondary antibody. Cells were

incubated on ice in darkness for 30 minutes, washed twice in FACS buffer and finally resuspended in isoton. FAS-L expression was screened by FACS testing using a Gallios flow cytometer and data was analysed used Kaluza® software.

2.2.4.9 Screening of Invasive Capability of HAGE-Expressing Cells

Invasive capability of HAGE-expressing cells was tested using a Matrigel-Invasion System and used in accordance with the manufacturer's guidelines to achieve real-time observation of cell migration. Briefly, FM-82/Ctrl and FM-82/shRNA were grown overnight in their normal conditions. On the say of testing, media was decanted and replaced with media containing 10μg/ml $DilC_{12}(3)$ fluorescent dye and cells were incubated for one hour at $37^oC/5\%CO_2$. Following incubation, cells were counted and diluted in serum-free media to $1x10^5$ cells/ml. Cells were seeded at $5x10^4$ cells/well of either a test plate with wells containing Matrigel or a negative control plate containing no barrier material prior to the addition of a chemo-attractant (Media containing 5% FCS) under the migration pore and were incubated at $37^oC/5\%CO_2$. Detection of stained cells passing through the pore was done using a fluorescent plate reader over a 25-hour period. Percentage invasion at each time point was calculated by dividing reading for the test plate by the reading for the control plate and multiplying by 100.

2.2.5 Statistical Analysis

For various experiments performed in this study, statistical analysis was carried out to highlight their significance. In the first instance, on studies where biological repeats were performed, the difference between repeats was determined using standard deviation, calculated from average values gained for the control or test measures used in each experiment. In certain cases, the significance between values gained was examined. On these occasions, the p value was calculated using a student's t test, which would allow the recognition of differences that were significant ('*'/p=<0.05), very significant ('**'/p=<0.01) or highly significant ('***'/p=<0.001).

Chapter 3

The Effect of HAGE Expression on Tumour Cell Proliferation

3.1 Introduction

The heightened ability of cancerous cells to proliferate is perhaps the best known characteristic of tumours (Hanahan and Weinberg, 2000). Uncontrollable proliferation is the reason why a tumour is capable of rapid expansion in its site of origin. Angiogenesis may also be initiated thanks to proliferation due to tumours reaching a certain size and requiring oxygen for survival. Furthermore, this ability is essential for these cells that have metastasised to distant locales in order to generate additional malignant lesions.

This high proliferative capability is due to the expression of molecules that enable proliferation and progression through the cell cycle and mitosis, while inhibiting those that act to prevent these actions and promote apoptosis. To achieve this cancer cells either alter their communication with other cells or modify that affect their signalling pathways.

Interestingly, under normal conditions cells themselves have a finite ability to proliferate. This was first demonstrated in culture cells by Hayflick (Hayflick, 1997). Following every round of replication, the telomeres of chromosomes are 'shortened' by a loss of approximately 50 to 100 base pairs (Hanahan and Weinberg, 2000). Progressive deficit of genomic material ultimately leads to a cell undergoing apoptosis because it is unable to support further mitotic division. Thus, cell division and proliferation are self-limiting processes that can regulate the extent that a cell is able to expand the population from itself. However, in both established cell lines and malignant cells in culture and *in vivo*, there appears to be an infinite ability to proliferate with the acquisition of immortality, made possible by repair of telomeres after each mitotic division. Tumour cells can continuously divide without ever being in danger of entering apoptosis. In the vast majority of instances, they achieve this by the abnormal over-expression of telomerase, the enzyme that acts to maintain

telomeres in stem cells (Shay and Bacchetti, 1997; Bryan and Cech, 1999). Alternatively, the alternative lengthening of telomeres (ALT) mechanism, a form of genomic recombination is employed to accomplish the same outcome (Bryan *et al*., 1995; Henson and Reddel, 2010). In addition to this other genetic abnormalities affecting external and internal signalling will eventually lead to uncontrollable proliferation.

The former can be provided by tumour cells developing autocrine and paracrine secretion of growth factors such as EGF, FGF and PDGF, thus making them self-sufficient (Zandi *et al*., 2007). A similar condition can be achieved via abnormal over-expression of receptors for these growth factors on the surface of malignant cells. Once ligand-receptor binding has taken place, initiation of pro-growth cascades occurs, involving molecules such as RAS, PI3K and AKT (Cox and Der, 2002; Vara *et al*., 2004; Yoeli-Lerner and Toker, 2006). These activate the expression of various molecules that encourage cell cycle progression at the same time as inhibiting the molecules that inhibit growth. A good example of this can be seen with the PI3K pathway (Vara *et al*., 2004). In this setting, the molecules that communicate the signal are not mutated themselves, instead they are subverted and their actions manipulated as a result of increased stimulation to surface receptors.

As opposed to cell to cell communication, modifications can take place within a cell itself, granting it heightened potential to proliferate. This can come in the form of gain of function modifications occurring with the molecules implicated in encouraging proliferation or loss of action of those that act to prevent this behaviour. This is evident with the former with regards to the generation of oncogenes, for example the point mutations that occur in B-RAF that predominates in melanoma (Tuveson *et al*., 2003) and RAS species in numerous tumours (Downward, 2003). These are supplemented by translocations, for example BCR-ABL in CML and ALL (Druker *et al*., 2001b), and amplifications as found with MYC (Al-Kuraya *et al*., 2007; Rodriguez-Pinilla *et al*., 2007).

The level to which malignant cells can proliferate is important. Increases in tumour mass can only be achieved if apoptosis is limited, therefore limiting the rate of cell

turnover (Hanahan and Weinberg, 2000). Loss of pro-apoptotic functionality is a common event in a malignant cell. In some instances, this takes place as a result of increased pro-proliferative signalling. In cells where there is amplified AKT signalling, for example from the loss of PTEN or mutations to RAS, there is a natural inhibition of certain genes or proteins that act to cause cell death. Initiation of AKT function leads to inactivation of the pro-apoptotic proteins BAD and BIM along with the anti-proliferative GSK-3β, thus allowing a cell to increase the rate at which it enters mitotic cell division (Kandel and Hay, 1999). Events like these are compounded further by the inactivation of pro-apoptotic molecules through deletion, mutation or insensitivity to stimulation, for example as seen with p53 (Levine and Oren, 2009). Once a cell can achieve this, then there is a raised chance to which it can proliferate.

It is essential to emphasise the importance of TSG in the evolution of tumour proliferation. Some of the best examples of these genes and their products are central to proliferation signalling or progression through the cell cycle, the two processes required for cells to increase their population. This can be seen with PTEN, which undergoes deletion in many tumour types subsequently allowing continual signalling via AKT, a highly potent signalling mediator (Chalhoub and Baker, 2009). A similar situation can be seen with the retinoblastoma (Rb) protein. Rb undergoes inactivation by mutation, increased phosphorylation by cyclin-CDK complexes or the action of the E7 gene product of HPV (Dyson *et al*., 1989; Zuo *et al*., 1996; Hanahan and Weinberg, 2000). The normal function of Rb is to regulate activity of E2F transcription factor family members by complexing with them. Loss of Rb activity means that E2F are free to act to propel the cell from G1 to S phase of the cell cycle and from there to mitosis (Weinberg, 1995). Alternatively, other molecules that serve to stimulate TSG activity may be inhibited in the same fashion or TSG become insensitive to them. In the case of Rb, insensitivity to or loss of function of TGF-β and $p15^{INK}$ means that it cannot be activated and in turn, this has a comparable effect to loss of this TSG itself (Fynan and Reiss, 1993; Hanahan and Weinberg, 2000). Collectively, these mechanisms guide a malignant cell to increase the size of the nascent tumour mass. Despite knowing so much regarding this process, it still

remains one of the most difficult to curtail, implying that other factors must exist within cell communication networks that cause this phenotype.

Work previously performed by this laboratory has indicated that the expression of HAGE in a tumour cell line may promote proliferation (Unpublished data). Expression of the HAGE protein in tumour cell lines (Mathieu *et al*., 2010) means that it potentially has a viable role in cells. Since other DEAD-box proteins and CT antigens have been linked to proliferation (Por *et al*., 2010), it is possible that HAGE could also share this involvement. To that end, the initial stages of this study aimed to investigate if this antigen contributes to tumour proliferation.

3.2 Results

3.2.1 Effect of Transient Transfection on HAGE Expression in Cells

In order to understand what role HAGE has in tumours, it was initially important to discern what impact its expression has in malignant cells. Any study into functionality requires either inducing target gene expression in negative cell line or reducing expression in a cell line that is naturally positive. For this study, both techniques were utilised, either in a transient capacity or in a stable manner.

In the case of transient modification to expression, a human melanoma cell line naturally negative for HAGE expression (FM-3) was transfected with a HAGE bearing plasmid (pBudCE4.1/HAGE). At the same time, a human melanoma cell line that naturally expresses the HAGE gene (FM-82) was treated with a gene-specific siRNA molecule. To ensure that both transfections had their desired effects at the genetic level, RNA was extracted from treated cells and used to synthesise cDNA using RT-PCR. This was carried out a number of days post-transfection (3, 5 and 7 days) to find out how long the effects of each treatment lasted. Screening of this DNA involving real-time qPCR with the use of HAGE-specific primers was able to show that transfection of cDNA and siRNA was successful at inducing and silencing HAGE expression respectively. Figure 3.1 indicates that the gene was detected using PCR, therefore showing that cDNA had been able to enter and be utilised by the cells to create HAGE gene expression in a negative cell line. Relative expression, calculated using CT values of both HAGE and housekeeping genes, point to the greatest level of expression being shown at 3 days post-transfection, with expression decreasing as time progressed. In the case of gene knockdown, Figure 3.2 demonstrates that it was possible to decrease the level of HAGE in FM-82 cells. Relative expression in this graph highlights that it was possible to achieve an 85% decrease in gene expression 72 hours following siRNA treatment which decreased to approximately 45% one week after transfection had been carried out. Real-time PCR screening of the genes OAS-1 and STAT-1 was previously used to confirm that transfection of HAGE siRNA does not trigger an IFN-γ mediated cell death as can happen when using siRNA.

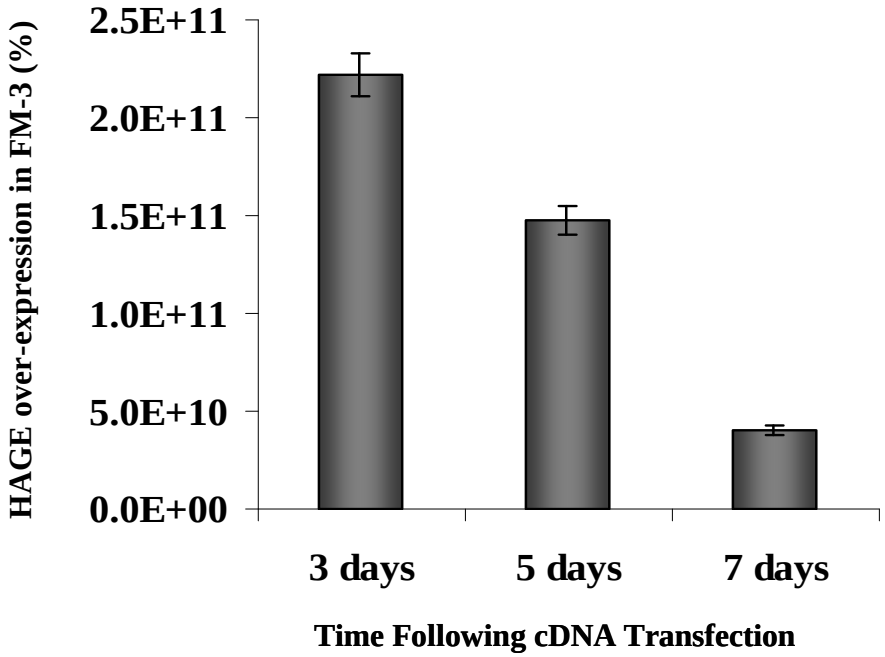


Figure 3.1*: Relative HAGE gene expression in FM-3 human melanoma cells transiently transfected with the pBud CE4.1/HAGE cDNA plasmid (n=4). Expression determined using 2ΔCT calculations of real-time qPCR data of RNA taken 3, 5 and 7 days post-transfection.*

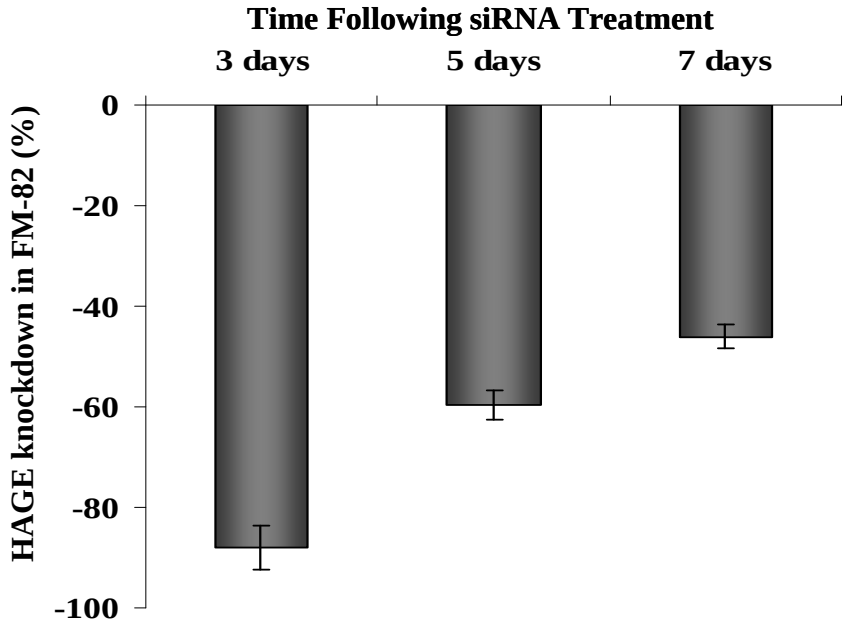


Figure 3.2: *Relative gene expression of HAGE in FM-82 human melanoma cells following treatment with a specific siRNA molecule (n=4). Expression determined from 2ΔCT calculations derived from results of real-time qPCR of RNA extracted from these cells 3, 5 and 7 days post treatment.*

The findings of genetic changes were mirrored by the alterations in protein expression in these cells. Figure 3.3 and 3.4 illustrates both cDNA transfected FM-3 cells and FM-82 cells treated with siRNA in the same manner as with the genetic experiments. These underwent immune staining using a mono-specific polyclonal antibody specific for the HAGE protein. In terms of cDNA transfection, Figure 3.3 shows that HAGE protein expression was greatest after three days, but decreased as time progressed. It could be seen that three days following siRNA treatment, HAGE was still expressed as a protein in these cells, evident by the level of FITC (Green) staining present and that HAGE appears to be confined to the cytoplasm. This could be compared to the same cells stained with an isotype control antibody, where only the nucleus was visible. However, five days after treatment, there was a decrease in staining, implying a reduction in the protein level, indicating that the siRNA had successfully managed to silence HAGE in these cells not only at the gene level, but also at the protein level. By seven days, protein expression appeared to increase again for FM-82 cells, while FM-3 cells demonstrated very little protein expression.

3.2.2 Creation of HAGE Stable Expression in Tumour Cells

While it is important to examine the effects of a temporary change in HAGE genetic expression, it is necessary to investigate what effects long term modifications could have. To do this, cells were stably transfected in order to bring about long lasting changes to HAGE expression. To induce gene expression, FM-3 cells were subject to transfection using the pcDNA3.1/HAGE plasmid or empty plasmid vector. For gene silencing, FM-82 underwent treatment with a HAGE-specific shRNA plasmid or negative control shRNA vector. All four of these new cell lines were grown in media supplemented with G418 selective antibiotic. Figures 3.5 and 3.6 show that the real-time qPCR of cDNA generated from RNA extracted from these cells was able to confirm the change in HAGE expression that was hoped for. For the cDNA transfectants, Figure 3.5 shows that with the test cell line (FM-3/HAGE), HAGE expression was nearly 200,000 times greater than the FM-3/-Ve control line ($p=<0.001$). This obviously stands to reason since FM-3/-Ve was treated with a plasmid that bore no HAGE cDNA, however it demonstrates the extent to which HAGE had become over-expressed in FM-3/HAGE.

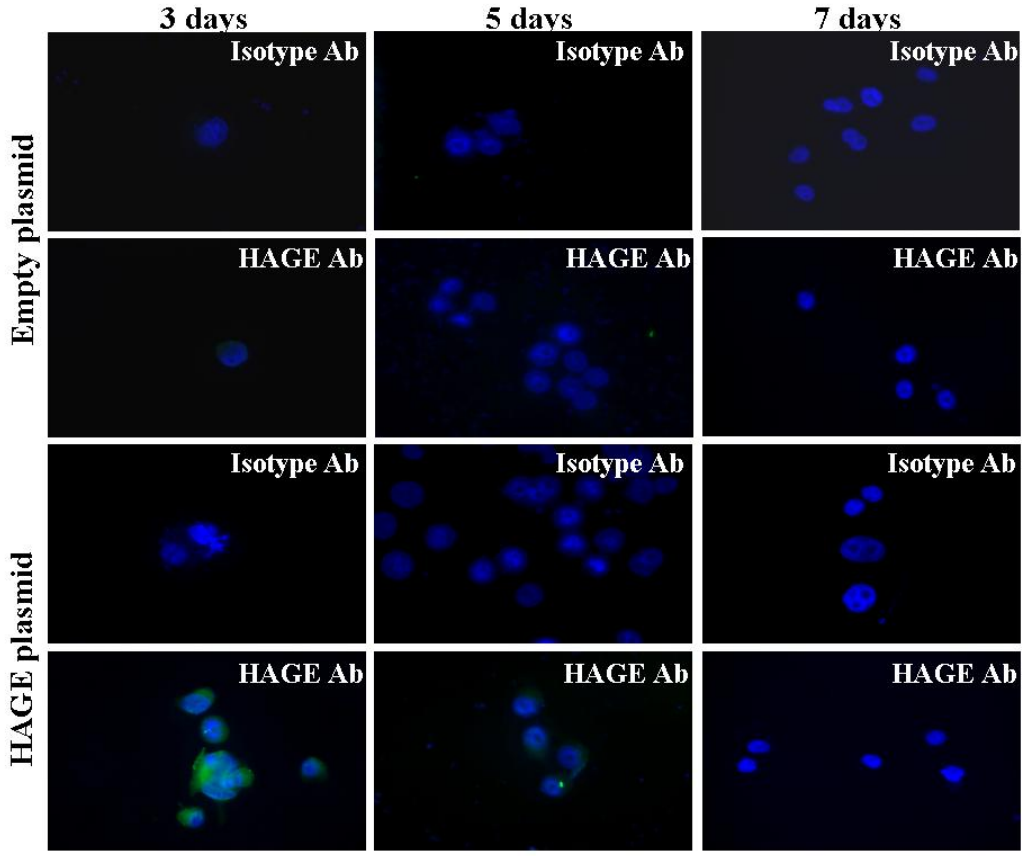


Figure 3.3: *Immunofluorescent staining of HAGE protein in FM-3 cells transiently transfected with the pBud CE4.1/-Ve empty plasmid or pBud CE4.1/HAGE cDNA plasmid (n=2). Staining involved the use of a mono-specific polyclonal antibody against HAGE and counterstained with FITC -conjugated secondary antibody. DAPI was used to stain the nucleus of cells. Image magnification at x40.*

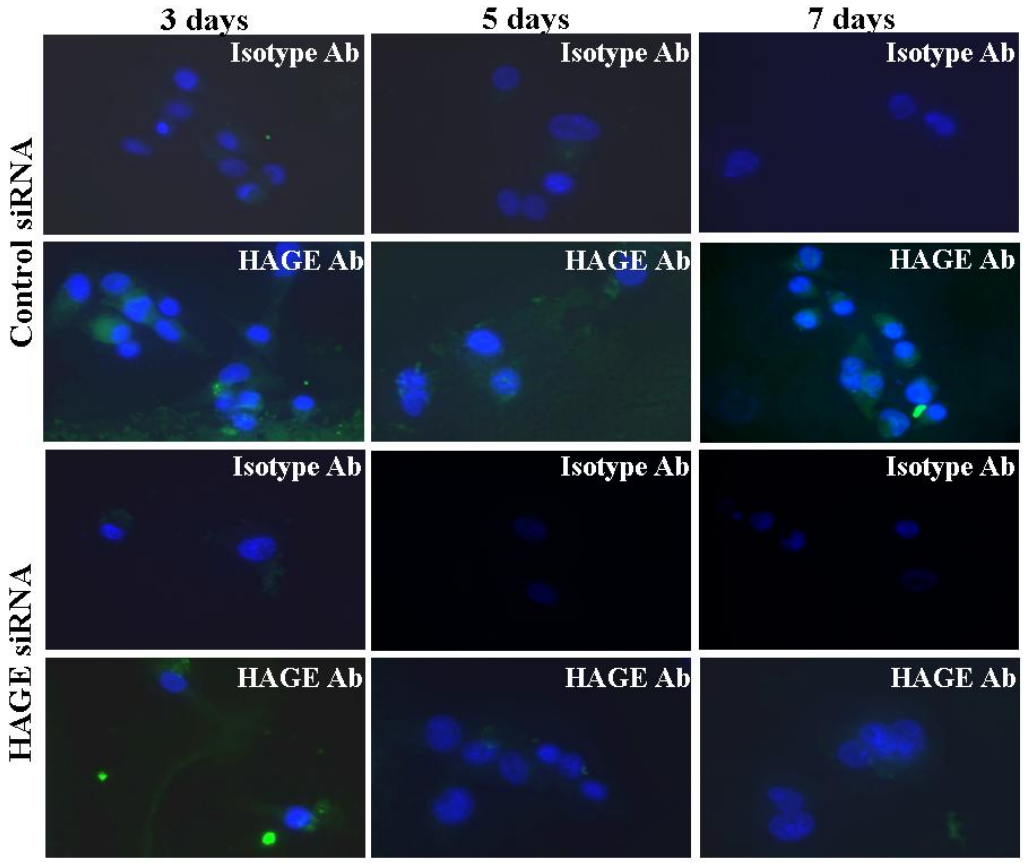


Figure 3.4: *Immunofluorescent staining of HAGE protein in FM-82 cells transiently transfected with either control or HAGE-specific siRNA (n=2). Cells were stained in the same manner as cDNA plasmid transfected cells. Image magnification at x40.*

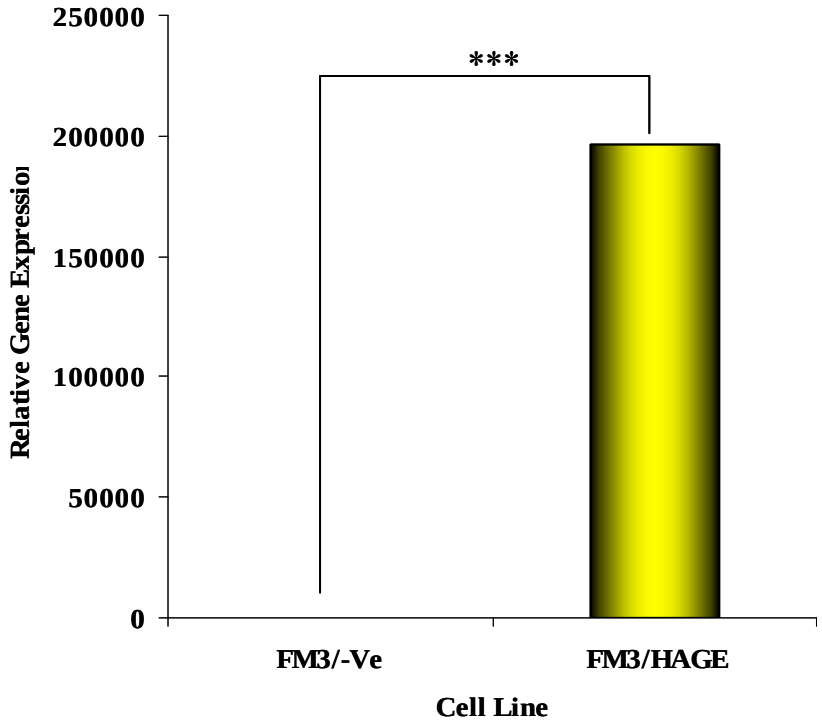


Figure 3.5: *Relative gene expression of HAGE in FM-3 human melanoma cells gained from 2ΔCT calculations of real-time qPCR data (n=4). Cells were stably trasnfected either with the pcDNA3.1/HAGE DNA vector to create the FM-3/HAGE stable line, or the pcDNA3.1 empty vector to form the FM-3/-Ve control cell line (p=<0.001).*

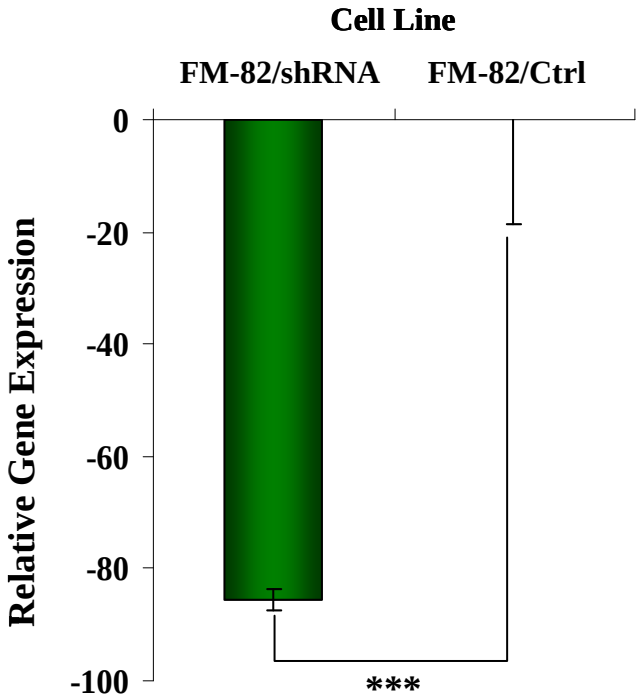


Figure 3.6*: Extent of gene knockdown in FM-82 human melanoma cells stably transfected with shRNA (n=4). Cells were treated with either a HAGE specific shRNA plasmid (FM-82/shRNA) or a negative control plasmid (FM-82/Ctrl). Expression was determined using 2ΔCT calculations of real-time qPCR data (p=<0.001)*

On the other hand, Figure 3.6 shows the decrease in expression in the stably knocked-down cell line. The FM-82/shRNA test cell line demonstrated approximately 80% less expression of the HAGE gene than the control cell line FM-82/Ctrl.

As with the transient treatments, the effect of transfection seen at the genetic level was confirmed additionally at the protein level. Figure 3.7 (A) shows the difference in HAGE expression between FM-3/-Ve and FM-3/HAGE. No protein was found to be expressed in FM-3/-Ve, demonstrated by only the DAPI staining of the nucleus of these cells being present. Conversely, examination of FM-3/HAGE cells shows that not only the molecule, but the level of staining visible indicated the extent to which the antigen was expressed within these cells.

For the shRNA cell lines, Figure 3.7 (B) displays how the incorporation of the HAGE-specific molecule was able to significantly reduce the level of protein generated in FM-82/shRNA. Interestingly, it is notable that HAGE is still expressed by the test cell line, however to a much lesser extent than FM-82/Ctrl. This is reassuring, since shRNA treatment is an RNAi technique and does not involve gene knockout. As a result, it means that HAGE was still expressed by FM-82/shRNA, but to a much lower concentration. This would appear to agree with the relative gene expression calculated from real-time qPCR described earlier.

Importantly, it must be noted that for FM-3/HAGE, expression of the protein was confined to the cytoplasm. This matches what was found with the transient cDNA and also agrees with the localisation of HAGE in FM-82 which naturally expresses the protein. This indicated that further work on these cells could potentially match what may be taking place in a naturally occurring cell line.

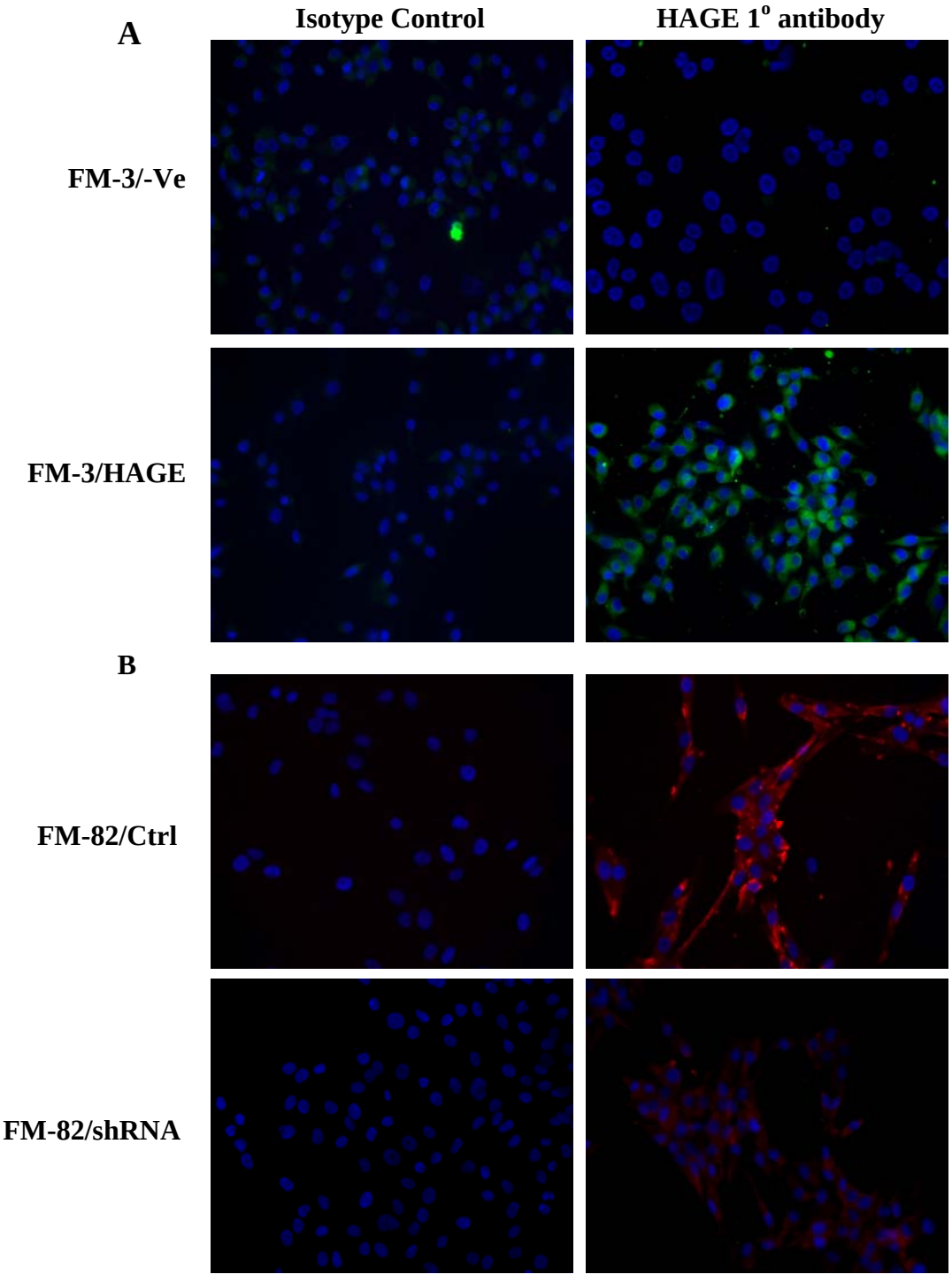


Figure 3.7: *Immunofluorescent staining of HAGE protein in stable transfectant cell lines (n=2). (A) cDNA stable transfectants stained with rabbit anti-HAGE mono-specific polyclonal primary antibody and with Alexa-468 (Green) secondary antibody staining. (B) shRNA stable transfectants stained with mouse anti-HAGE monoclonal antibody and Alexa-568 (Red) secondary antibody. Cell nuclei stanied with DAPI.*

3.2.3 Effect of HAGE Expression on Tumour Cell Proliferation *in vitro*

Work performed by this laboratory in the past has shown that the transient transfection of HAGE cDNA into the FM-3 results in a significant increase in the proliferation level of these cells *in vitro* (Unpublished data). To confirm these findings, a reciprocal experiment was performed to discern if reduction of HAGE expression leads to reduced proliferative capacity. This involved the use of FM-82 cells treated with HAGE-specific siRNA in ^{3}H incorporation proliferation assays.

Figure 3.8(A) shows that three days following siRNA treatment, there was a significant decrease in the ability of those treated with HAGE-specific siRNA to proliferate in comparison to cells treated with control siRNA, transfection material alone or when growing naturally ($p=<0.05$). Conversely, Figure 3.8(A) also shows that seven days following treatment, the pattern is not repeated and there is no reduction in proliferation of HAGE-siRNA treated FM-82 cells compared to control treatments.

To investigate if the same finding could be repeated in another HAGE-expressing cell line, the human melanoma cell line FM-6 underwent the same technique, shown in Figure 3.8(B). As can be seen in this instance, cells demonstrated the same reaction in response to HAGE knockdown as FM-82. Three days after transfection, there was a reduction in the number of cells treated with HAGE siRNA compared to control cells. This could be supported by the result gained for cells treated with INTERFERin reagent alone, where the number of cells was considerably lower than cells grown in their natural state.

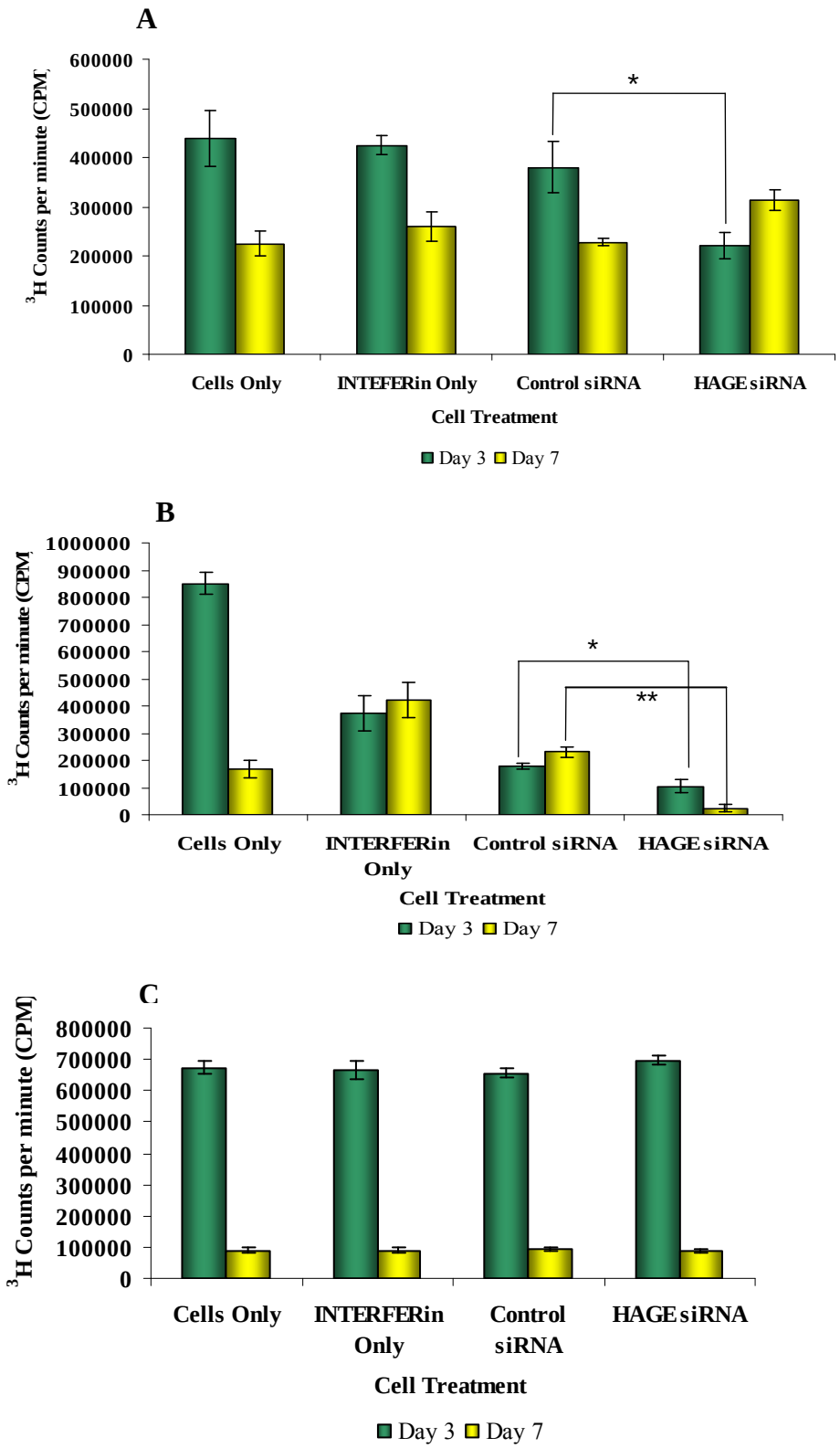


Figure 3.8: *Readings from in vitro ^{3}H incorporation proliferation assays to assess the effect of HAGE siRNA treatment on three human melanoma cell lines; (A) FM-82, (B) FM-6 and (C) FM-3 (n=3). Measurements were taken 3 days (Green bars) and 7 days (Yellow bars) post siRNA transfection (*=p=<0.05; **=p=<0.01).*

In order to ensure that these findings were due to a reduction in HAGE expression and not a consequence of the transfection procedure used, HAGE-negative FM-3 cells were subject to the same experimental conditions. This was done to act as a true negative control, as FM-3 does not express HAGE, specific siRNA should have no effect on this cell line. Figure 3.8(C) shows that there was no significant difference between the treatments used at both times of measurement.

All of the above had been performed on cells that had undergone transient transfection. To confirm that long term modified HAGE expression has the same effect on cell proliferation, both sets of stable transfectant cell lines were used for ^{3}H proliferation assays. Figure 3.9(A) shows that following stable induction of HAGE gene expression, there was significant increase in the proliferation four days after they had been plated however there was no difference at day 6. Findings for Day 4 with this cell line further agrees with previous proliferation assays in that once HAGE had begun to be expressed by these cells, then their level of proliferation was made more potent compared to the control cell line. To corroborate the above, stable knockdown of HAGE in FM-82, shown in Figure 3.9(B), matched the pattern of results. This was evident from reduction of HAGE gene expression by specific shRNA leading to a significant loss of proliferation as opposed to the cells transfected with control shRNA. This effect was even more pronounced at Day 7, possibly implying that prolonged reduction in this gene's expression resulted in an even greater reduction in cell proliferation.

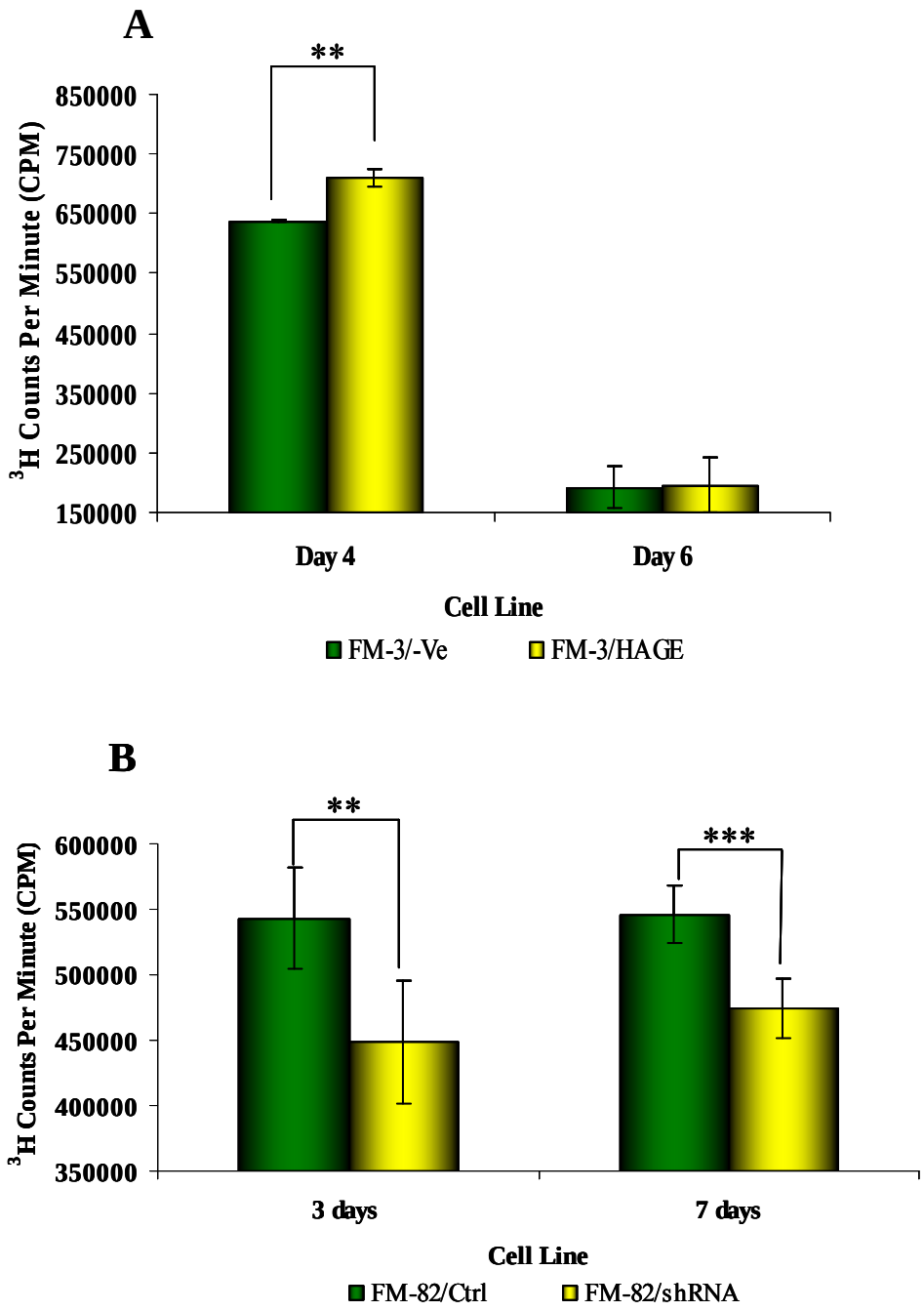


Figure 3.9: *In vitro ^{3}H incorporation proliferation assay readings (n=3) gained for human melanoma cells stably transfected with (A) HAGE cDNA or (B) HAGE shRNA compared to control cell lines. Readings were taken at 3 or 4 days (Green Bars) or 6 or 7 days (Yellow bars) (**=p=<0.01; ***=p=<0.001).*

3.2.4 Effect of HAGE Expression in Tumour Cell Proliferation *in vivo*

While it was of importance to discern the effect that HAGE had on *in vitro* proliferation, if this molecule is to be considered a realistic target for cancer therapy, then the same findings must be recognised *in vivo*. To this end, FM-3/-Ve and FM-3/HAGE cells were injected into two groups of NOD/SCID mice to investigate if HAGE expression could lead to increased tumour growth in an *in vivo* setting. This strain of mice was used since they lack adaptive immunity as well as NK cell activity and as such, represents a suitable model to allow tumour growth to be monitored with the smallest risk of interference from the immune system of the host.

As Figure 3.10(A) highlights, from approximately Day 12 onwards, mice that had been injected with FM-3/HAGE bore tumours that were greater in size on average when compared to the group of mice that had been inoculated with FM-3/-Ve. Moreover readings taken after Day 28 found that HAGE-positive tumours were significantly larger than the control tumours. Subsequently, this would appear to point towards HAGE being associated with increased proliferation of malignant cells. The significant difference between the two groups ($p=0.05$; $p=<0.01$), of mice suggests a possibility that HAGE could have more of an impact on cell biology over a protracted length of time.

To ratify these findings, the experimental conditions were reciprocated using FM-82/Ctrl and FM-82/shRNA. On this occasion, as shown by Figure 3.10(B), the mice injected with FM-82/shRNA developed tumours at a significantly slower rate than the control group ($p=<0.05$), complementing the findings of the previous experiment. As expression of this gene was the only variable for these two experiments, it could be suggested that HAGE is able to promote increased proliferation in an *in vivo* environment. This furthers the evidence of HAGE and proliferation and goes further to support that this antigen not only has a role *in vitro* but within an *in vivo* setting as well.

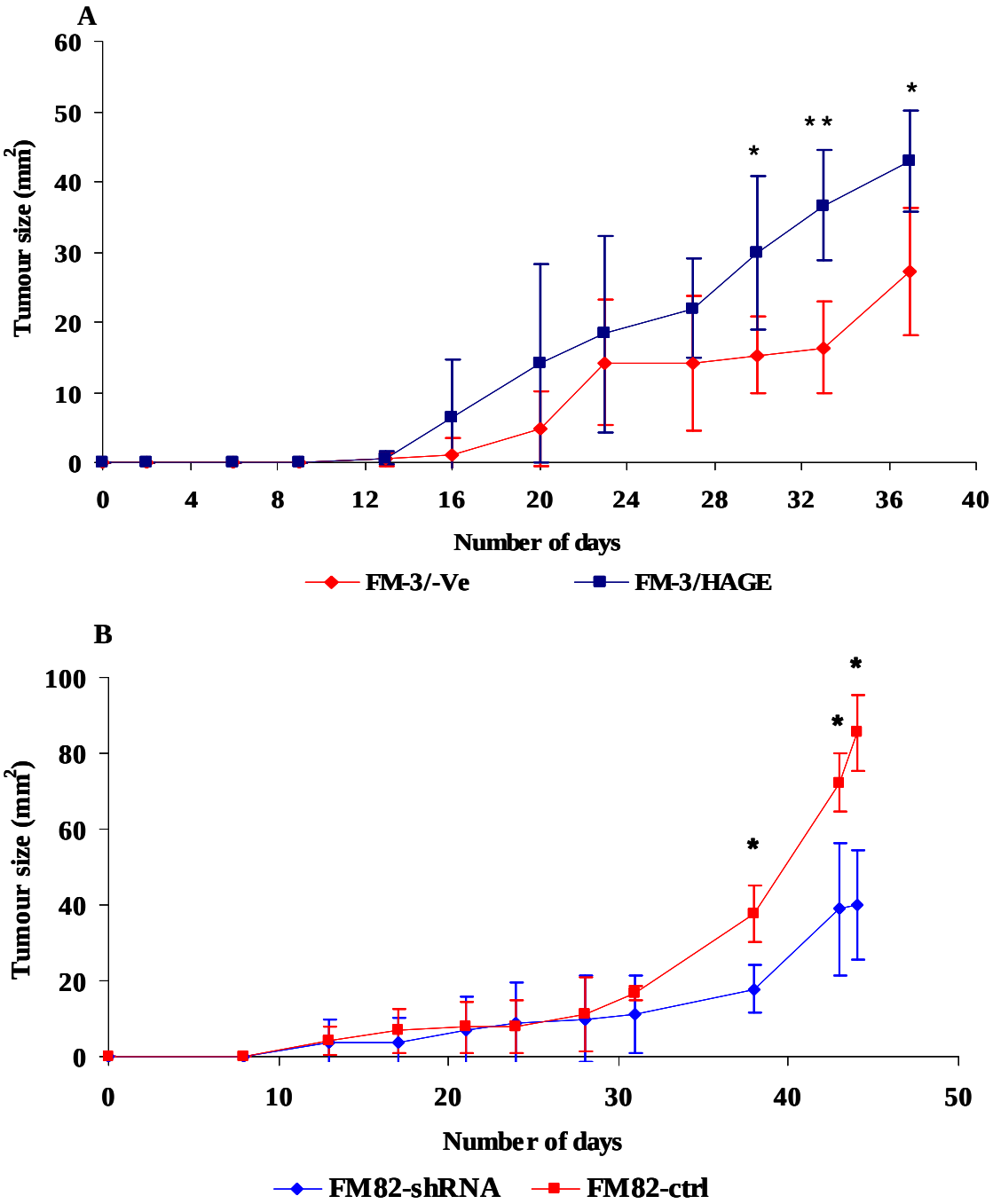


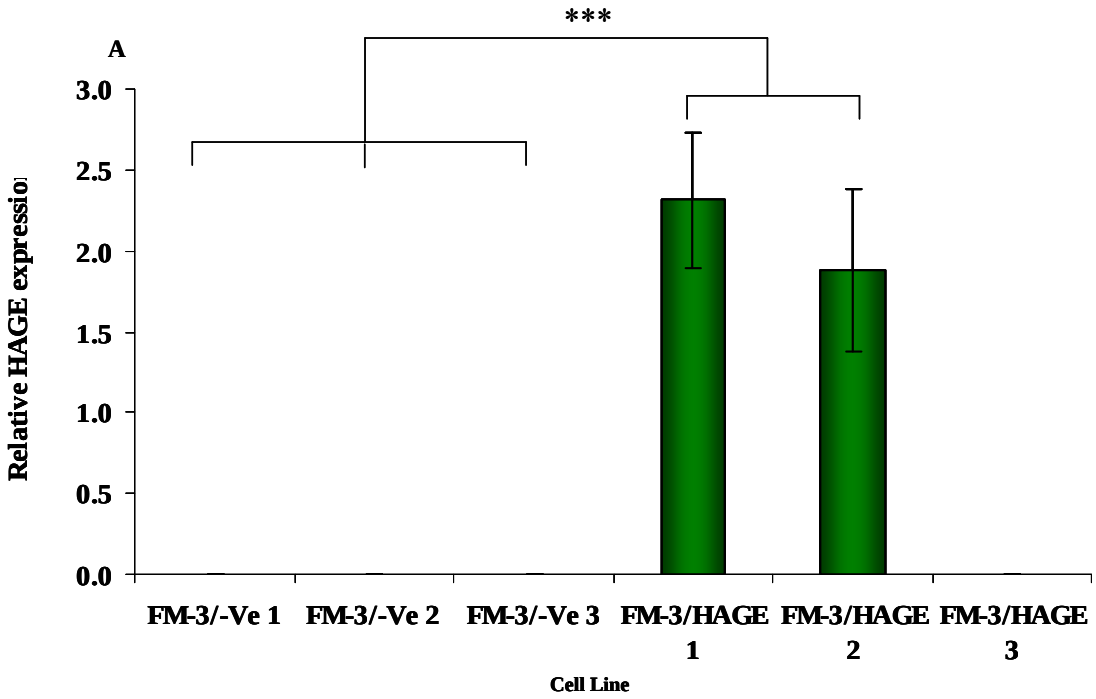
Figure 3.10: *Size of tumours that developed in NOD/SCID mice following the injection of either (A) HAGE cDNA stable transfectant and control cell lines or (B) HAGE shRNA and control cell lines (n=1). Readings were taken bi-weekly using calliper measurement (*=p=<0.05; **=p=<0.01).*

3.2.5 Verification of HAGE Expression in NOD/SCID Tumours

By way of performing complete ratification of HAGE expression in these tumours at all levels, extra sections were taken, stained using cresyl violet and underwent laser-capture microdissection (LCM). The cell islets removed from these sections underwent RNA extraction and this was used to generate cDNA for analysis of HAGE gene expression using real-time qPCR. The levels of relative expression are displayed in Figure 3.11. Here, it could be recognised that three out of three FM-3/-Ve cell islets tested demonstrated no HAGE mRNA, as could be expected. For FM-3/HAGE cells, two out of three islets expressed the mRNA to a relatively high degree. Through the use of LCM, it had been possible to identify HAGE gene expression in these tumours, even from a very small number of cells and in turn, this was able to confirm the presence of the antigen in these cells and connect its presence with the increase proliferation in these tumours.

In order to confirm increase tumour growth *in vivo* could be due to the expression HAGE, work was performed on these tumours to ratify the molecular expression of HAGE. Mice were euthanized and their tumours removed and snap frozen in liquid nitrogen-cooled isopentane. Sections taken from these tumours were used for immunohistochemical staining of HAGE and viewed using a microscope, the images taken from this illustrated in Figure 3.12. No positive staining was evident in FM-3/-Ve tumours, however widespread brown HRP was noticed in FM-3/HAGE based masses displaying a relatively high concentration of the HAGE protein in these cells. As can been seen, no brown from HRP staining was gained using the isotype control primary antibody.

To further validate the presence of increased proliferation within these tumours, immune staining was performed on them to detect both HAGE and Ki-67, a protein marker that is produced by cells going through proliferation. As can be seen in Figure 3.13, when stained for both proteins, FM-3/HAGE was found to express both, as did FM-82/Ctrl. On the other hand, in both FM-3/-Ve and FM-82-shRNA there was none or decreased HAGE expression respectively and a reduced level of staining for Ki-67. This hints that HAGE-bearing tumours were actively proliferating as a result of HAGE protein expression in cells.



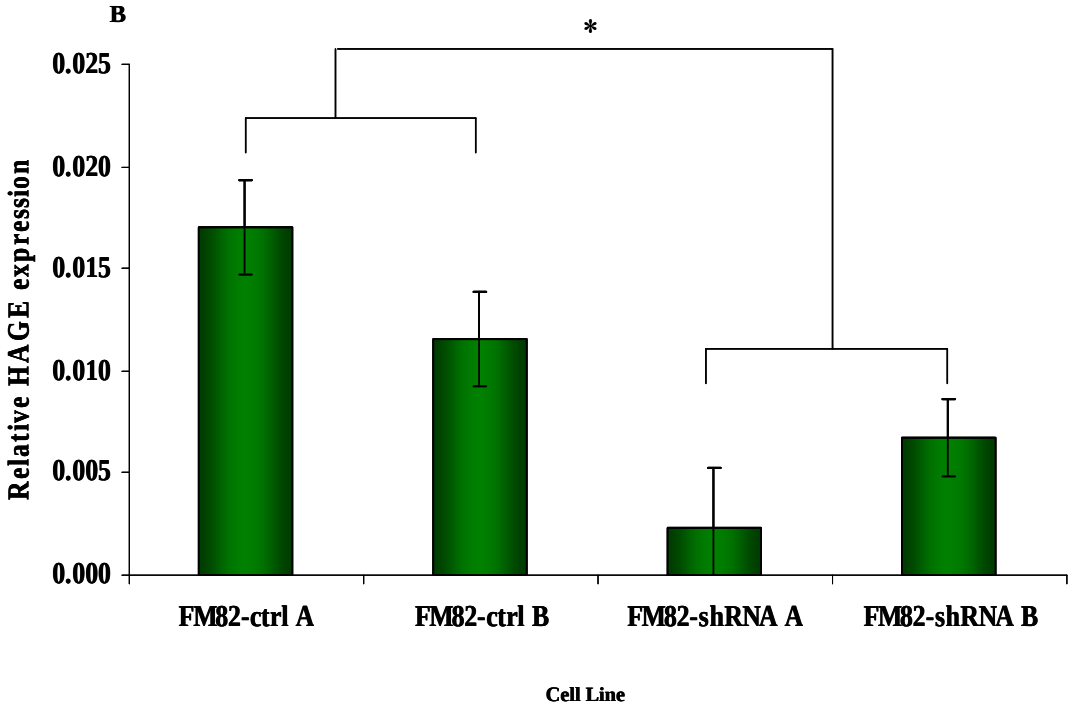


Figure 3.11: *Relative expression of HAGE mRNA expression in NOD/SCID HAGE cDNA tumours isolated by LCM. Extracted tumours were grown from either (A) cDNA stable transfectants (p=<0.001) or (B) shRNA stable transfectants (p=<0.05). Expression was determined using 2ΔCT calculations of CT values derived from real-time qPCR (n=4).*

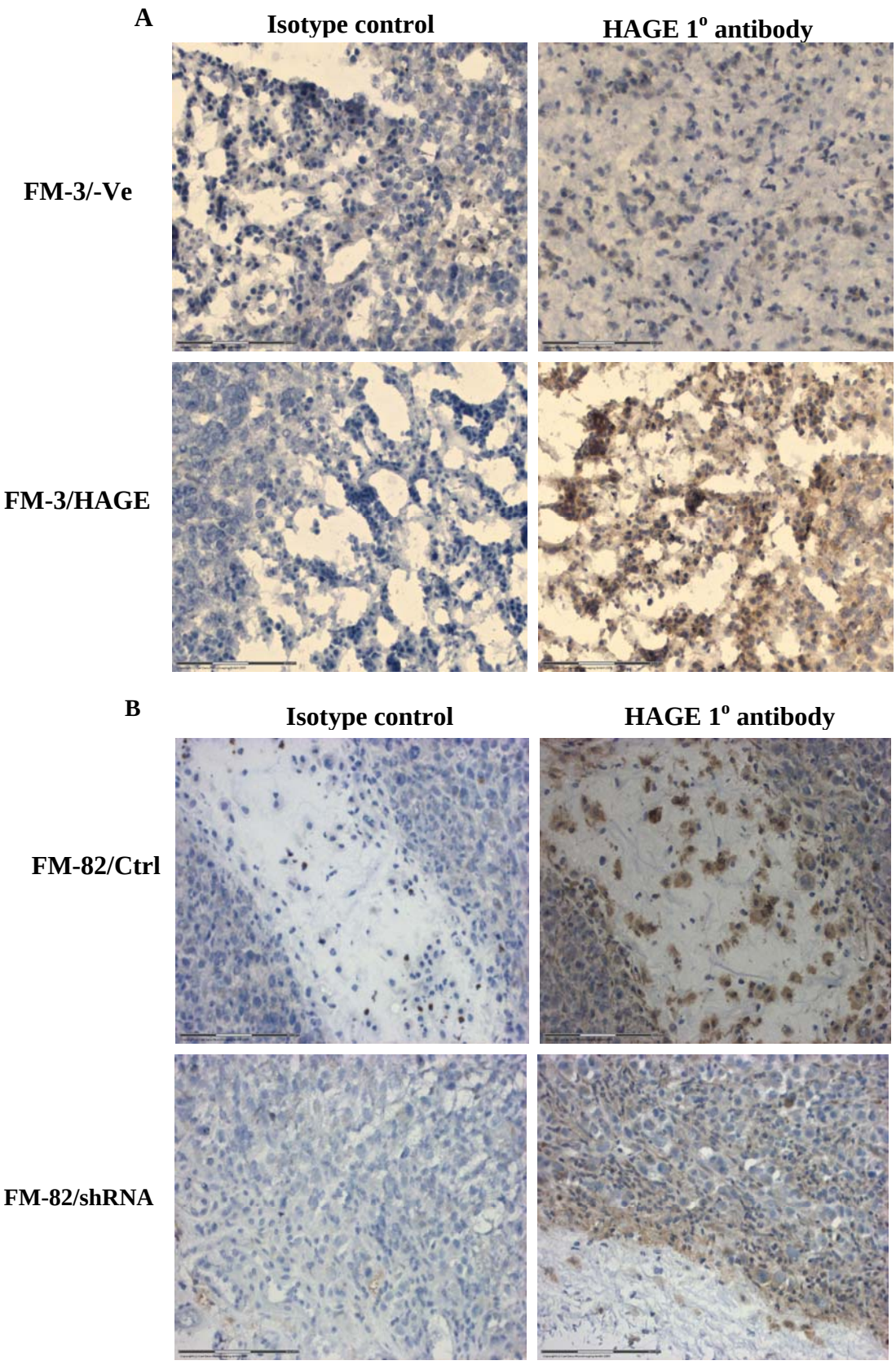


Figure 3.12: *Images taken from LCM viewing of tumours grown in NOD/SCID mice following sectioning and immunohistochemistry staining of HAGE (n=2). Tumours were either (A) HAGE cDNA stable transfectants or (B) HAGE shRNA transfectants. Images taken when viewed at x20 magnification.*

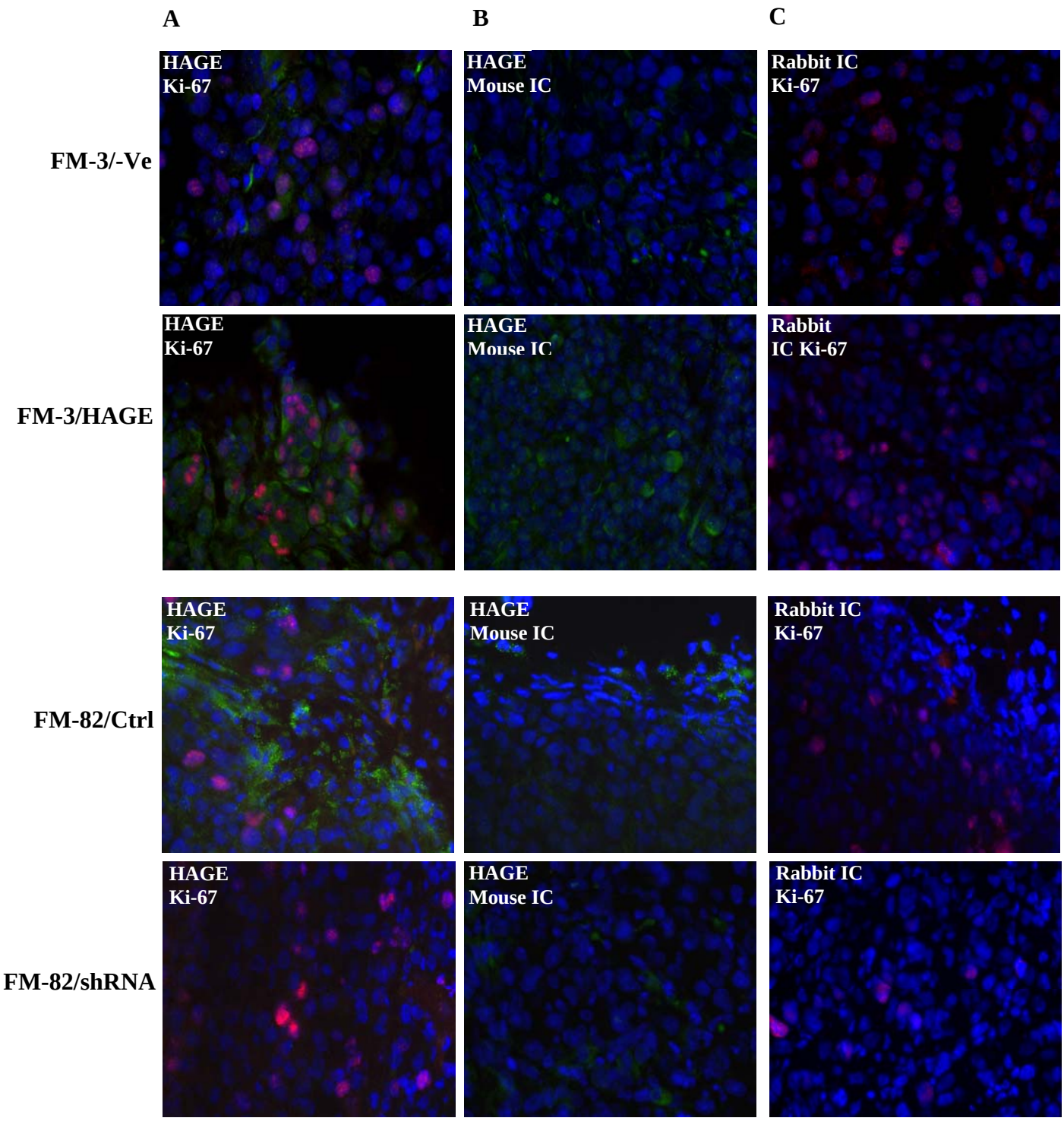


Figure 3.13: *Dual immunofluorescent staining of HAGE (Green) and the Ki-67 cell proliferation marker (Red) in NOD/SCID tumours (n=2). Cells were stained with either (A) Primary antibodies against both proteins; (B) HAGE primary antibody and the isotype control against the primary for Ki-67 or (C) Ki-67 primary antibody and the isotype control against the primary for HAGE. Images taken when viewed at X40 magnification.*

3.2.6 HAGE and Progression through the Cell Cycle

If HAGE-positive cells have higher levels of proliferation, then it is possible that they have undergone modifications to permit increased progression through the cell cycle and through this, enter mitosis quicker. Both cDNA and shRNA stable transfectants underwent serum starvation in order to synchronise cells to the same stage of the cell cycle. Once completed, serum was replaced in the media and subsequently cells were analysed using FACS following staining using the 7-AAD genetic stain. Data retrieved from this testing was analysed using the Kaluza® software package (Beckman Coulter). This suggested that expression of HAGE permitted cells to advance through the cell cycle at a faster rate (Figure 3.14). Examination of peaks gained from FACS analysis showed that there appeared to be more HAGE-positive cells in S phase once serum was replaced when compared to cells negative for the antigen. For example, for FM-3/HAGE, 35% of cells were found to be in S phase as opposed to 13% with FM-3/-Ve, a very significant difference. This was complemented with data gained for FM-82/Ctrl cells, where 8% cells were at S phase, whereas 4% of FM-82/shRNA cells were at this stage. While this latter difference was not as significant as seen with the cDNA transfectants, it was still considered to be significant.

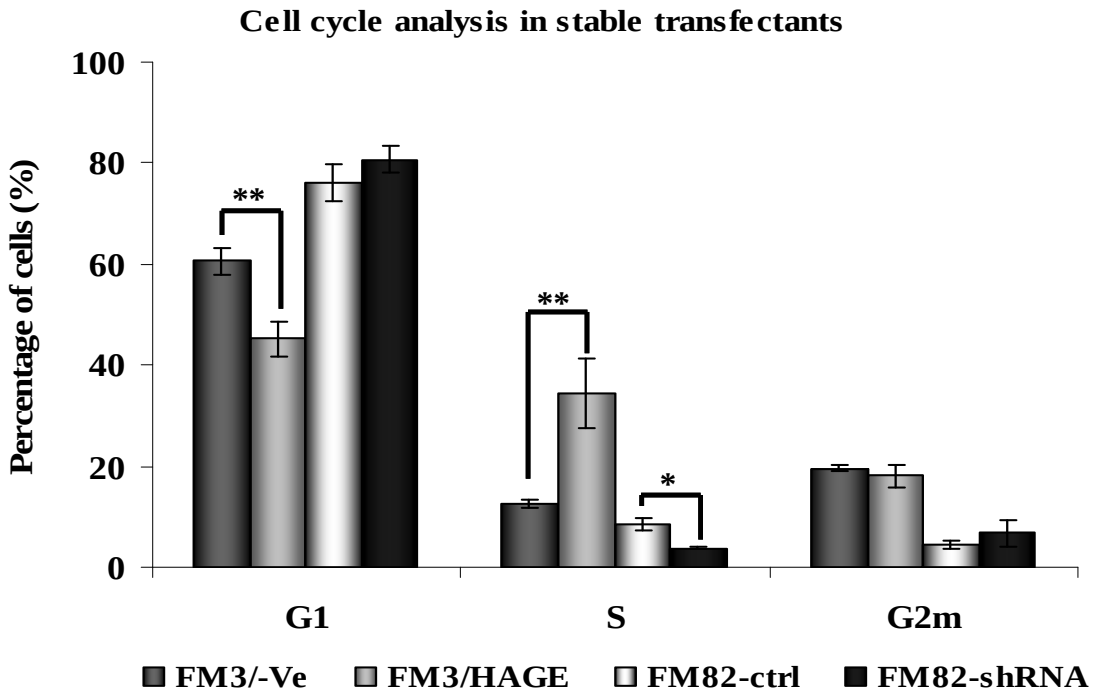


Figure 3.14: *Proportion of cDNA or shRNA stable transfectant cells in different stages of the cell cycle (n=2). This was determined once cells had been synchronised by serum starvation and subsequent replenishment of serum in media. Proportion of cells was calculated using FACS data analysed using Kaluza® software (*=p=<0.05; **=p=<0.01).*

3.3 Discussion

The initial aim of this study was to investigate the hypothesis that HAGE could have an association with tumour cell proliferation. Genetic manipulation of HAGE, either in the form of cDNA, siRNA or shRNA transfection was employed to activate or reduce genetic expression. It was initially possible to show that HAGE expression could be manipulated as required in a successful manner. It was interesting to note that in both sets of experiments, optimal changes to expression were seen 72 hours following introduction of cDNA and siRNA. Likewise, in both cases, after this point, there was a steady reversal back to each cell line's natural genotypic pattern for HAGE. In the case of both cDNA transfection and siRNA treatment, following the supplementation of cell media with these molecules and transfection lipids, they would be taken up by cells, and incorporated into the genome for DNA, while siRNA would initiate RNAi activity in the cells. Data shown here implies that both these events require approximately the same amount of time to exert their effects on the gene.

The reversal in gene expression over time can be explained in two ways. For cDNA, not all cells in culture will take up the plasmid. This means that unless one treats media with selective antibiotics to kill non-transfected cells as with stable transfection, then it means that there is a relatively more heterogeneous population in culture. Non-affected cells will continue to proliferate, thereby diluting the level of target gene expression. In the case of siRNA, the same applies. Therefore when the cell divides by mitosis, the daughter cells do not contain a template for the DICER complex to carry out RNAi, thus these new cells will express genes in their natural fashion (Pushparaj *et al*., 2008). Hence over time, target gene expression returns to its natural state.

siRNA treatment was shown to optimally affect gene expression by 72 hours, but five days were needed before the protein level changed. This suggests that until this point, translation of HAGE was still being carried out by the cells and by Day 5, this was not possible due to the degradation of HAGE mRNA being carried out as part of RNAi. It appeared that protein expression was returning to its natural level after seven days, with a certain amount of FITC staining being visible. Such a result

would appear to suggest that once gene silencing has begun to diminish, then there could be a rapid re-initiation of the translation of target mRNA, quite possibly as part of a compensation mechanism in the cell after a certain time of losing a certain gene's expression.

Inhibition of HAGE expression resulted in a significant reduction in the proliferative capacity of the tumour cells. In the case of FM-82, a significant reduction ($p=<0.05$) was seen three days following siRNA treatment, but not at Day 7. Therefore proliferation seemed to be affected at the same time siRNA had the greatest impact on HAGE gene and protein expression. At Day 7 however, there was a resurgence in HAGE expression at both RNA and protein levels. It could be due to the loss in effect of the siRNA and cells had regained the ability to proliferate at the capacity prior to transfection. The increase in proliferation would seem to match the increase in HAGE molecular expression and loss of siRNA activity. Transfection of siRNA duplexes into cells can induce cell death mediated by cytokines such as IFN-γ (Robbins *et al*., 2009). Such a reaction could bring proliferation results into dispute as it would mean reduction in cell growth would be due to cytokine activated cell death and not inactivation of the gene of interest. Activity of this pathway can be monitored by screening genes involved in this reaction, such as OAS-1 and STAT-1. This has been previously performed for HAGE siRNA and it was shown that these were not significantly increased therefore IFN-γ was not activated and responsible for the effect observed (Unpublished data).

Similarly, the above effect was also observed in FM-6 cells, where at Day 3, there was a significant decrease ($p=<0.05$) in tumour cell proliferation once HAGE knockdown had taken place. Interestingly, in contrast to FM-82, this effect was significant at Day 7 as well ($p=<0.01$). It is difficult to assess why this was the case. Since HAGE is expressed as mRNA by FM-6 to a very high level (Data not shown), the effect of gene-specific silencing may have a more detrimental effect on this cell line. Alternatively, it could be that FM-6 cells were more sensitive to the transfection procedure and by Day 7, cells had begun to die. This notion could be supported by the evident difference in proliferation of cells grown naturally and treated with INTERFERin alone. There was a large decrease in cells treated with the transfection

reagent, hinting that this cell line was more sensitive to the action of transfection as compared to FM-82. The findings gained for FM-3 cells treated in the same manner, where there appeared to be no decrease in cell proliferation after siRNA treatment, seemed to ratify the notion that HAGE may be linked to proliferation. FM-3 cells are naturally negative for HAGE gene expression and treatment with a HAGE specific siRNA had no impact on the number of cells in culture. This was in contrast to the other cell lines, which are HAGE positive and following knockdown of this gene, experienced significant decreases in cell number.

The results derived from siRNA studies were repeated using cells that had undergone stable transfection. Once HAGE was stably expressed by FM-3, their level of growth was significantly increased four days after plating compared to cells transfected with an empty plasmid vector ($p=<0.01$), suggesting there was a connection between the introduction of HAGE expression and the ability of a cell line to proliferate at a greater rate. This antigen may bring about the same phenotypic reaction when expressed for a relatively longer period of time. However by Day 6, there appeared to be little difference between the two cell lines. As mentioned above, FM-3 cells naturally proliferate at a fast rate. The introduction of HAGE seemed to increase this yet further and thus, it is quite possible that by this point, cells were beginning to die through being over-confluent, resulting in the observed outcome. In contrast, cells that had undergone stable knockdown using shRNA displayed significant reduction in proliferation compared to the control cell line. This reduction was found to be very significant at Day 3 ($p=<0.01$) and became highly significant by Day 7 ($p=<0.001$). The stable knockdown of HAGE in this cell line complemented the findings gained from transient knockdown. It again promoted the idea that HAGE could play a role in the proliferation of the cells.

In vivo studies of stable transfectant cell lines were able to demonstrate that HAGE expression led to larger tumour masses over the same time period. Genetic and protein studies of the tumour tissues grown in this manner confirmed the expression of HAGE. Immune staining of the protein showed that HAGE was confined to the cytoplasm as was observed in *in vitro* cultures. This was encouraging as it showed that it was possible to detect HAGE in the same cellular region in cells grown either *in vitro* or *in vivo*. In terms of *in vivo* work, this was only carried out once for both

sets of stable transfectants, meaning repetition of these studies is required to confirm the initial findings. This implies that the expression of the antigen in these cells was able to contribute to the development of larger tumours. In turn, this could mean that HAGE has a role within the *in vivo* environment.

Dual staining for HAGE and the proliferation marker Ki-67 in these NOD/SCID tumours demonstrated some interesting findings. It was shown that there was strong co-expression of both proteins in HAGE-expressing tumours, implying a possible link between HAGE and proliferation *in vivo*. However in tumours derived from cells with reduced or no HAGE expression, staining of Ki-67 was still evident. It could well be HAGE does not directly influence Ki-67 and both of these proteins are expressed at different stages of the proliferative cycle of cells. If HAGE does act to promote proliferation, then it is possible it acts in a specific manner before Ki-67 becomes expressed by other means. Nonetheless, data gained from them is highly encouraging as it implies that HAGE could have a role in patient disease and is not an *in vitro* phenomenon.

The final interesting point concerning HAGE and proliferation comes from the apparent links to cell cycle progression. FACS analysis of synchronised cells indicated that a greater percentage of HAGE-expressing cells were in S phase than those with negative expression. This way highly significant for cDNA transfectants ($p=<0.001$) and very significant for shRNA cell lines ($p=<0.01$). This was complemented by the finding that more HAGE-negative cells were found to be at the G1 stage of the cycle. Tumour cells are notorious for manipulation of the cell cycle to promote cell proliferation. This can be seen with the manipulation of the E2F family, Rb and other TSG like $p21^{CIP1}$ and $p27^{KIP1}$ (Weinstein, 1995; Payton and Coats, 2002; Cam and Dynlacht, 2003; Coqueret, 2003). Therefore, data from these analyses would propose that HAGE-expressing cells were passing through the cell cycle at a faster rate than their counterparts. This could account for the other findings that are detailed above.

Collectively, this means that a large volume of data now exists to associate HAGE with the ability to which tumour cells are able to proliferate. This is the first time that such a link has been made with this molecule and would appear to confirm the

original theory into what process it is associated with. One of the first proposals for the possible role of CT antigens was that they could be involved in embryogenesis due to the pattern of their expression (Lucas *et al*., 1998). The nature of embryogenesis would require cells to proliferate at a very fast rate and therefore would appear to link the antigen family to this required phenotype. It has been implied that both MAGE-A1 and NY-ESO-1 are involved in the proliferation of both male and female germ cells (Gjerstorff *et al*., 2007). This would seem to agree with the localisation of these antigens and go some way to begin confirming the initial notion put forward by Lucas and colleagues (1998). Likewise, due to their numerous potential roles, there is an argued role for members of the DEAD-box family in cell proliferation. Evidence for this has been shown with the protein p68 (DDX5) and its associate member p72 (DDX17) promoting proliferation and inhibiting apoptosis. The over-expression of these antigens in cancer has been suggested to promote these actions in malignant cells (Janknecht, 2010). CT antigens are expressed in healthy tissues, being confined to testis and placenta (Simpson *et al*., 2005) where it would seem they contribute to proliferation. Once they become re-expressed in tumour cells, it would seem appropriate that they reprise this role and increase the extent to which these cells can proliferate.

Data is beginning to emerge confirming these notions. The CT antigen CAGE (Cancer-associated antigen) is also a member of the DEAD-box family (DDX53). It has been found to increase the proliferation of tumour cells by influencing cell cycle progression through the up-regulation of cyclin D1 (Por *et al*., 2010). Due to its classification as a CT antigen and DEAD-box protein, CAGE serves as an interesting comparison to HAGE. The initial data from this study would appear to have similarity to the evidence gained for CAGE. Therefore, together there seems to be more confirmation of the role of CT antigens and DEAD-box proteins in this role in malignant cells.

Proliferation comes about through complex interactions that take place between vast numbers of different molecules. In cancer, these relationships are severely disrupted, either through the modification of key mediators or by the expression of certain molecules (Weinstein, 2000). With the expression of HAGE being restricted to malignant cells and its classification as a DEAD-box protein, results suggest that this

antigen may be involved in both. Since HAGE is an RNA helicase and acts to unwind RNA (Linder, 2006), it is possible that it could have interactions with specific molecules that lead to this kind of activity in cells. It would be important to investigate if HAGE has relationships with molecules that promote specific areas of the proliferative response, whether it is those that act at the highest part of signalling cascades or the cell cycle. Likewise, it would be necessary to find out if HAGE leads to these molecules up or down regulation and whether this effect is via direct contact with HAGE or through a series of different mediators.

Chapter 4

Tumour Cell Gene Expression in Response to HAGE

4.1 Introduction

As previously described, perhaps one of the most unusual aspects of CT antigens is the apparent lack of understanding into their role in malignant cells. Large collections of work have been carried out describing their expression, immunogenicity and response to *in vitro* and murine drug models. Clinical trials involving the most immunogenic or promising CT antigens have had limited success in patients and this has led to the current situation that no FDA-approved drug exists towards these molecules. Increasingly, there seems to be more evidence to promote these molecules as biomarkers for disease progression for a number of different cancers.

A feasible argument, connecting the lack of progress observed in CT antigen drug development and the lack of knowledge surrounding them, could be raised. This is perhaps best shown by CT-X antigens such as MAGE, GAGE and NY-ESO-1 amongst others. Members of this group were some of the original antigens of this class to be discovered and are amongst the most immunogenic, especially MAGE-A3 and NY-ESO-1 (Marchand *et al*., 1999; Jäger *et al*., 2000). At the same time, very little is known of their biological function. There is however mounting evidence that certain CT-X antigens are employed in spermatogenesis, as MAGE and NY-ESO are expressed by both spermatogonia and primary spermatocytes (Simpson *et al*., 2005; Gjerstorff *et al*., 2007). Further to this, genetic and protein data have been collected to propose that MAGE-A and GAGE may have a role in the differentiation of the earliest forms of somatic cells (Gjerstorff *et al*., 2008). For malignant cell biology, MAGE-A3 and MAGE-C1 have been shown to promote survival of tumour cells in MM (Atanackovic *et al*., 2010). This means that more is being addressed to identify what processes CT-X antigens are involved in, not just in terms of malignant cells but also in healthy situations.

What is noticeable is that those CT antigens to which most is known as far as function is concerned appear to be non-X-linked antigens. Sperm protein 9 and 17 (Sp9/Sp17), OY-TES-1 amongst others have had their proposed mode of action described in healthy cells, for example Sp17 involvement in binding of sperm to zona pellucida (Frayne and Hall, 2002). In part, this could be due to some of these antigens being recognised as testis proteins initially, before being found to be re-expressed in tumour tissue, as with Sp17 (Frayne and Hall, 2002). While this is the case, it is difficult to appreciate what purpose some of these functions such as conjugation to zona pellucida and heparin-heparan sulphate binding would play in a malignant environment. It is possible that these antigens could be aberrantly re-expressed thanks to random promoter demethylation, possibly on account of modifications to other genes or proteins. It would seem unlikely however that a tumour would express genes that it has no use for, particularly when functional redundancy is taken into account.

Progress has been made more recently with the antigen CAGE, a CT antigen that like HAGE is a member of the DEAD-box family (DDX53). It was originally discovered by SEREX technology to be expressed by a variety of different tumours (Cho *et al*., 2002). Unlike other members of this group of antigens however, work has been carried out to find out what purpose CAGE has and the impact its expression has on malignant cell biology. It was initially suggested that the molecule had an impact on both proliferation and cell motility (Kim and Jeoung, 2008). Evidence has since emerged that CAGE promotes metastasis by up-regulating both MMP-2 and FAK (Shim *et al*., 2006; Kim and Jeoung, 2009) and proliferation by influencing the expression of cyclin D and E (Por *et al*., 2010). By investigating CAGE in this way, it has been possible to isolate the processes it is involved in. This, in turn has allowed initial recognition of other molecules that could interact with the antigen to cause these effects. Ultimately, it may be possible to pinpoint a particular part of these interactions with therapeutic intervention; therefore working to discern CT antigen function could improve the design of drugs against them.

Cancer in general is a disease caused by alterations taking place in molecular pathways (Weinstein *et al*., 1997). Changes can be found in physical modifications to the mediators of these pathways, loss of expression of them or the expression of other

molecules that can influence pathways in terms of promotion or inhibition. In tumours, CT antigens become re-expressed by random demethylation, yet the series of events leading to this remain unclear. It stands to reason that the presence of these molecules in tissues where they are not normally expressed could have a significant impact on cell biology. This is evident as seen for CAGE and also with the data presented in the previous chapter with HAGE. It is important to examine what effect HAGE expression has on the expression pattern of other genes in tumour cells.

Attempting to determine which genes are affected in response to tumorigenic changes has been common practice for a number of years. The evolution of oligonucleotide microarray analysis has allowed gene expression profiling to perform on a much larger scale. This is especially apparent with the Affymetrix Genechip® system analysis that allows the transcriptomic analysis of the entire human genome (Kim *et al*., 2004; Eszlinger *et al*., 2007). Thus, it is possible to investigate the impact of a target across all known genes and gain as much information as possible. Microarray has been used widely in cancer research for a number of years for different tumours to investigate all aspects of cancer such a drug response and methylation (Kim *et al*., 2004; Eszlinger *et al*., 2007). Combined with real-time PCR, it is a very useful tool to screen gene expression.

More recently, this type of technology has evolved yet further with the arrival of Next Generation Sequencing (NGS). This system screens the genetic sequence of a cell at such a rate that it is possible to screen the entire human genome in a relatively short period of time. It is also possible to recognise mutated, duplicated and deleted gene regions, amongst other factors, that may be taking place in a cancerous cell. The power of this technology has been recently utilised alongside other analysis systems to find the number of mutations present in both a lung carcinoma and melanoma patient (Pleasance *et al*., 2010a; Pleasance *et al*., 2010b). Though in both cases this information was gained from the cells of an individual patient, it demonstrates the huge potential of NGS and the extent of data that can be discerned at the current time.

The previous chapter detailed the effect that HAGE expression has on the proliferation of tumour cells. There is an implication that there are various genetic interactions that are altered to induce this phenotype. To that end, gene expression

profiling was performed in an attempt to discover what genes are influenced in tumour cells as a result of HAGE gene expression.

4.2 Results

4.2.1 Genome wide Expression Analysis

To examine the possible changes that occur to cancer cell genome expression in response to the presence of HAGE, expression profiling oligonucleotide microarray was performed. FM-82 cells were treated with either control or HAGE-specific siRNA to recreate the environment used in the initial *in vitro* proliferation studies. This was carried out in triplicate to generate a suitable number of repeats to examine the effect of gene expression change. RNA from these cells was extracted and supplied to the NASC service to undergo analysis. Microarray testing requires the use of very high quality RNA and the material supplied was suitable for this purpose as determined by spectrophotometry and Agilent Bioanalysis. RNA was hybridised to the Human U133 Plus 2.0 Genechip arrays which have transcripts for approximately 56,000 genes including the entire human genetic sequence, housekeeping genes and negative control genes. These were used to investigate the impact of HAGE on the whole human genome.

Once the report data had been validated using Genespring software, differential analysis of the gene data took place. Figure 4.1 shows the volcano plot derived from Genespring showing all the genes analysed, their change in signal intensity and fold change in expression following HAGE knockdown. Differential analysis involved initially averaging the signal data gained for all the genes from the chip from the triplicate samples. Averaged signals from the test samples were then divided by the averaged signals from the control samples to gain a ratio of test versus control. This gave a basic indication as to the change in expression of all tested genes in response to the knockdown of HAGE. To ensure that this analysis had been successful, the data was searched for the ratio of HAGE. It was found that the ratio of the gene was 0.18, meaning that there had been an 82% decrease in expression after siRNA treatment. This meant that a suitable level of HAGE knockdown had been achieved and implied that other results were reliable.

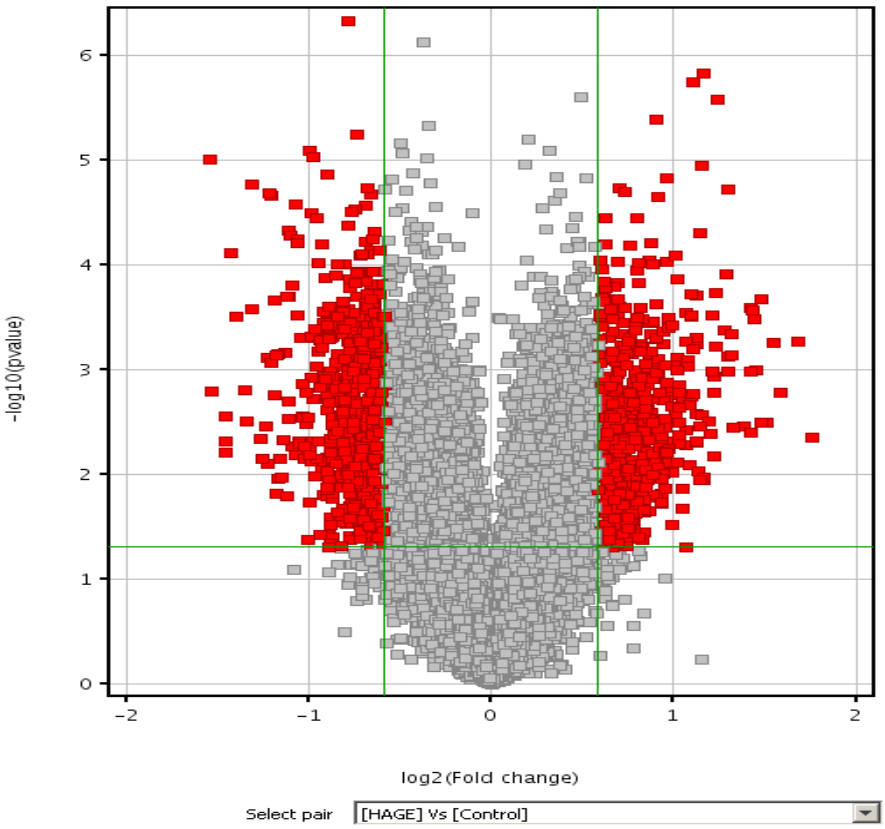


Figure 4.1: *Volcano plot derived from analysis of Genechip microarray data using Genespring software. Points on the plot represent all genes screened using the arrays of choice. The X-axis shows the fold change in expression of these genes, while the Y-axis shows the significance (p value) of change. Points coloured in red denote genes deemed to be the most significantly changed.*

Differential analysis generated a considerable collection of genes in which to search. To refine this, in the first instance a smaller list was created by isolating all genes that showed a 2.5 fold change in expression, either up or down-regulation. From this list, identities and functions were assigned to each gene using Entrez Nucleotide and OMIM database searches. When completed, a collection of genes was chosen based on the extent of their expression change and published role in processes that undergo modification in cancer cells, shown in Table 4.1. Some of these genes are involved in events that a tumour would promote such as proliferation and cell cycle progression (E2F2, CCNE2, WNT-1), immune escape (FAS-L), and cell survival (HSP-27, HSP-70). There were also genes that a tumour would inhibit in order to progress such as immune reactivity (CXCL-6, IL-15), apoptosis (PKC-β1, SFRP-1, CIDE-A) and inhibition of angiogenesis (JUN-D).

4.2.2 Validation of Microarray Data

To examine if the data from microarray analysis was correct, specific primer molecules for the genes listed in Table 4.1 were acquired. The annealing temperatures for these primers were optimised by synthesising cDNA using RT-PCR and using gel electrophoresis to examine if the generated product was of the correct size (Data not shown). When done, these primers were used in real-time qPCR to ratify microarray data analysis. In the first instance, this involved RNA extracted from FM-82 cells treated with control or HAGE siRNA thus recreating the setting of the microarray testing. Samples were run in duplicate and for each gene analysed a non-template control was run to act as a negative control. Housekeeping genes GAPDH, TBP-1 and HPRT-1 were also analysed and the data from these was used to normalise test data. Following 2ΔCT calculations, relative expression of these genes was calculated by comparing the results of test to control. These are shown in Figure 4.2.

Table 4.1: *List of genes isolated for further study following differential data analysis of microarray data and literature searches. Selection was based around the extent of expression change following HAGE knockdown and the published roles of the gene in a process known to be altered in cancerous cells.*

Genes up-regulated when HAGE is expressed	
CCNE2	Cell signalling/cell cycle/ cell proliferation
E2F2	
NRAS	
WNT-1	
FAK	Cell invasion/metastasis
MMP9	
FASL	Lymphocyte apoptosis
HSP27	Tumour Cell Survival
HSP70	
Genes down-regulated when HAGE is expressed	
JUN-D	Inhibition of angiogenesis
PKCb1	Apoptosis
CIDEA	
SFRP1	
CXCL6	Memory T cell development
IL-15	
$p21^{CIP1}$	Tumour suppression
Rb	
TIMP4	Inhibition of MMPs

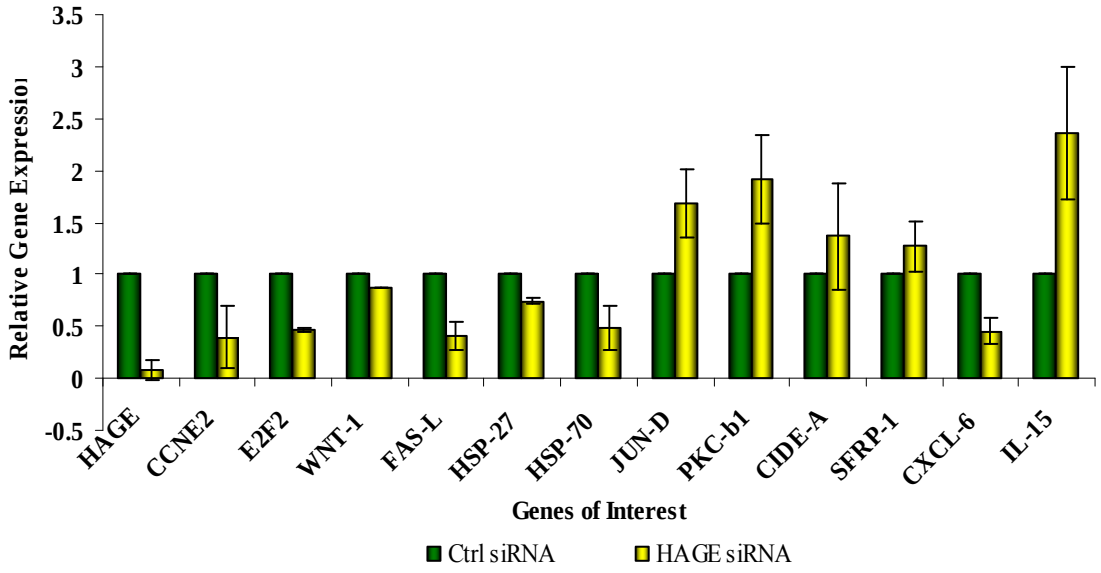


Figure 4.2: *Relative expression of genes of interest in FM-82 cells treated with either control siRNA (Green bars) or HAGE-specific siRNA (Yellow bars) as determined by real-time qPCR (n=4). CT readings were normalised using the housekeeping genes GAPDH, TBP-1 and HPRT-1 and used to calculate 2ΔCT values.*

One can notice that HAGE gene expression decreased by 92%, therefore this followed the same pattern as the microarray analysis. As can be seen, those genes which would be advantageous for a tumour to promote were decreased following HAGE knockdown, with CCNE2, E2F2 and FAS-L displaying the greatest change. This entails that when HAGE is expressed, expression of these genes increases. Similarly, the majority of genes that are involved in anti-tumour activities increased their expression once HAGE underwent interference, with JUN-D, PKC-β1 and IL-15 all showing large changes in expression. This would go to suggest that in tumour cells, HAGE is acting to decrease their expression. The only exception to this was CXCL-6, which demonstrated a decrease in expression. This meant in total, out of 13 genes tested including HAGE, 12 (92%) shared the same change in expression as was found with microarray testing.

Since it was important to determine if these results were due to HAGE knockdown and not simply cell line-specific, the above was repeated using RNA extracted from the HAGE-positive FM-6 cell line treated in the same way as FM-82. As displayed by Figure 4.3, the relative expression of the gene panel demonstrated a very similar pattern in this cell line compared to that of FM-82. On this occasion, the genes suspected of being promoted by HAGE again all decreased in expression, going yet further to consolidate the proposed role of HAGE in cancer. With the role of genes that are involved in anti-tumour processes, the majority matched the expression change seen with microarray, with PKC-β1 and IL-15 demonstrating large increases in their expression. CXCL-6 showed an increase expression after HAGE knockdown and matched the expression profiling but was in contrast to the expression found in FM-82. Conversely, with FM-6 both CIDE-A and SFRP-1 actually decreased their expression in response to HAGE knockdown, seeming to contradict the other data. Despite this, 11/13 genes (85%) shared the pattern of expression discovered using microarray analysis.

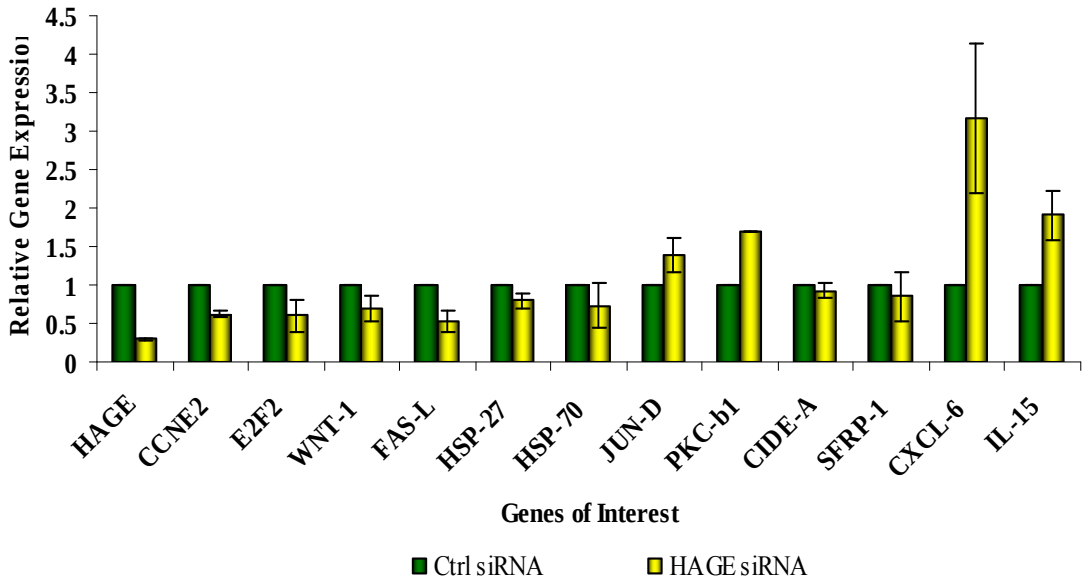


Figure 4.3: *Relative expression of genes of interest in FM-6 cells treated with either control siRNA (Green bars) or HAGE-specific siRNA (Yellow bars) as determined by real-time qPCR (n=4). Relative expression was determine in the same manner used for FM-82.*

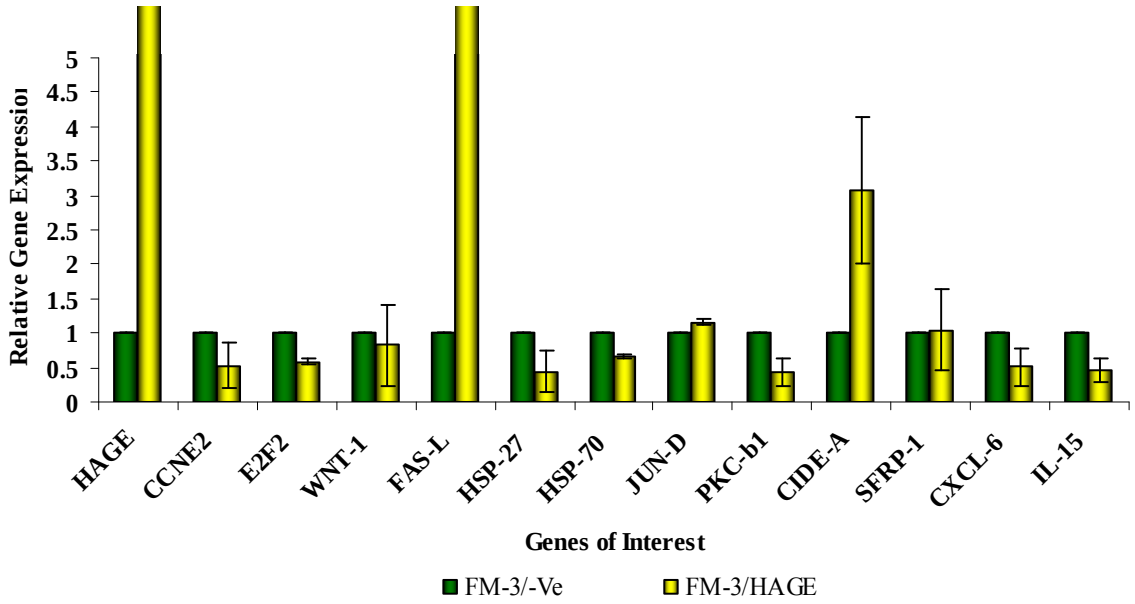


Figure 4.4: *Relative expression of genes of interest in HAGE cDNA stable transfectants determined by real-time qPCR (n=4). Expression in cells transfected with the empty plasmid vector (Green bars) was compared to that in cells treated with pcDNA3.1/HAGE cDNA plasmid (Yellow bars). Relative expression was determined in the same manner used for previously described results.*

Confirmation had been attained using HAGE-positive cell lines that had undergone transient RNAi. To establish if similar results could be gained from cells with long term modifications to HAGE expression, the same experiment was carried out with cDNA generated from the RNA extracted from both sets of stable transfectants. Figures 4.4 and 4.5 highlight that considerable differences to expression changes could be seen in these cells compared to that observed in transient knockdown. In terms of the stable cDNA transfection (Figure 4.4), it was found that HAGE had indeed been successfully introduced to a high degree, with a relative expression increase of approximately 196,000-fold compared to the control cell line. However, the majority of genes tested did not share the expression changes seen previously. Only FAS-L increased its expression in response to stable induction of HAGE, with a 221-fold rise in expression. Other genes expected to increase with stable HAGE expression such as CCNE2, E2F2 amongst others, actually decreased. With the genes previously shown to decrease with HAGE expression, there were more promising findings. PKC-β1, CXCL-6 and IL-15, all showing reductions in their expression, thereby giving the reciprocate picture as would be hoped for.

A similar series was found with the stable knockdown cells (Figure 4.5). A considerable decrease in HAGE expression was achieved, being around 82%, matching that attained with siRNA for microarray analysis. In spite of this, like the cDNA transfectants, changes to target gene expression varied to that seen with transient knockdown. Only HSP-27 and 70 demonstrated a large decrease, while the remainder showed increases or a very small decrease in WNT-1. Comparatively, the pro-apoptotic genes PKC-β1, CIDE-A and SFRP-1 all increased, matching previous results but JUN-D, CXCL-6 and IL-15 all decreased. Therefore there seems to be some discrepancies between the effects of transient and stable transfection, hinting towards possible effects of prolonged changes to gene expression.

In order to confirm that the effects in gene expression were not lineage-specific, cDNA was synthesised from the RNA extracted from a HAGE-positive head and neck carcinoma cell line PCI-13. This had been treated transiently with HAGE-specific siRNA in the same manner utilised for FM-82 and FM-6. As shown by Figure 4.6, in this instance, it was possible to reduce HAGE expression by

approximately 66%. However, not all genes matched the pattern exhibited by the melanoma cell lines. This cell line did not appear to express either PKC-β1 or SFRP-1 as it was not possible to gain any signal from real-time qPCR. For the remaining genes, FAS-L and the two HSP members demonstrated decreases to go alongside HAGE, while JUN-D and CXCL-6 increased, thereby supporting earlier impressions that HAGE seems to inhibit their expression. So, it was possible to show that some genes are influenced by HAGE across malignancies.

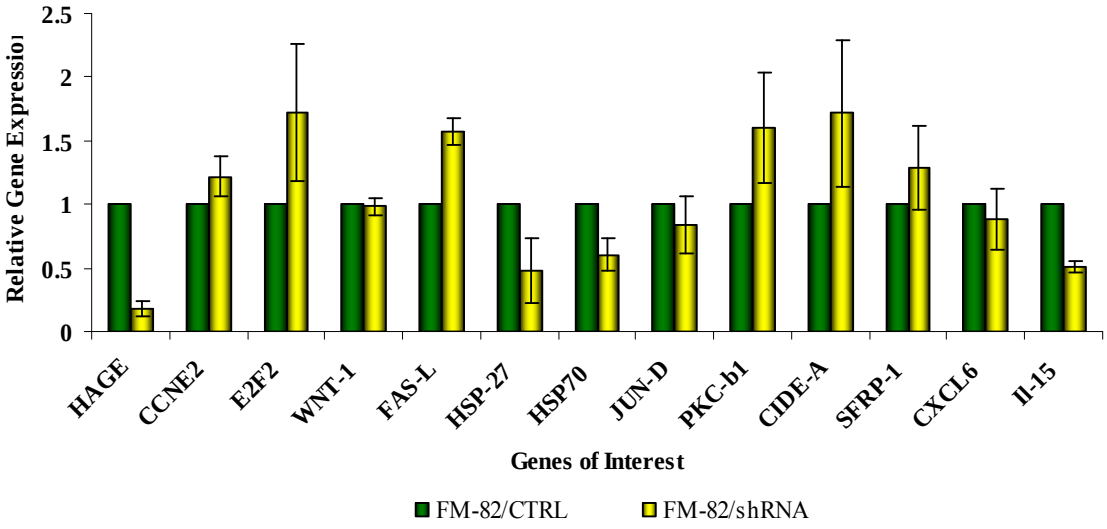


Figure 4.5: *Relative expression of genes of interest in HAGE shRNA stable transfectants as determined by real-time qPCR (n=4). Expression in cells transfected with the control shRNA (Green bars) was compared to that in cells treated with a HAGE specific shRNA (Yellow bars). Relative expression was determined in the same manner used for previously described results.*

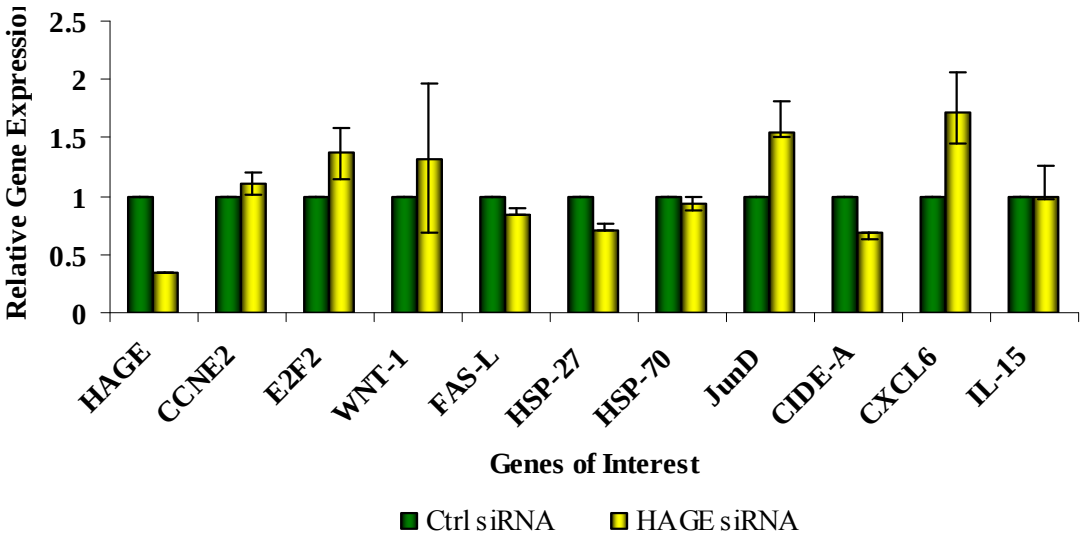


Figure 4.6: *Relative expression of genes of interest in the PCI-13 cell line following siRNA treatment, determined by real-time qPCR (n=4). Comparison was made between those cells treated with control siRNA (Green bars) or HAGE specific siRNA (Yellow bars) Relative expression was determined in the same manner used for experiments involving melanoma cell lines.*

Table 4.2: *Summary of expression change seen in genes of interest following modification to HAGE gene expression in melanoma or head and neck carcinoma cell lines using siRNA, shRNA or cDNA. Changes displayed are those noted using expression analysis involving real-time qPCR.*

	Cell Lines and Treatment				
Gene	**FM-82/ siRNA**	**FM-6/ siRNA**	**FM-3/ HAGE**	**FM-82/ shRNA**	**PCI-13/ siRNA**
HAGE	Down-reg	Down-reg	Up-reg	Down-reg	Down-reg
CCNE2	Down-reg	Down-reg	Down-reg	Up-reg	Up-reg
E2F2	Down-reg	Down-reg	Down-reg	Up-reg	Up-reg
WNT-1	Down-reg	Down-reg	Down-reg	Down-reg	Up-reg
FAS-L	Down-reg	Down-reg	Up-reg	Up-reg	Down-reg
HSP-27	Down-reg	Down-reg	Down-reg	Down-reg	Down-reg
HSP-70	Down-reg	Down-reg	Down-reg	Down-reg	Down-reg
JUN-D	Up-reg	Up-reg	Up-reg	Down-reg	Up-reg
PKC-β1	Up-reg	Up-reg	Down-reg	Up-reg	N/A
CIDE-A	Up-reg	Down-reg	Up-reg	Up-reg	Down-reg
SFRP-1	Up-reg	Down-reg	Up-reg	Up-reg	N/A
CXCL-6	Down-reg	Up-reg	Down-reg	Down-reg	Up-reg
IL-15	Up-reg	Up-reg	Down-reg	Down-reg	No change

4.3 Discussion

It has been shown on many occasions that the expression of particular genes in cancer leads to changes in behaviour of a transformed cell, whether it is just one particular aspect or several. Changes to biology come about thanks to alterations in the molecular interactions that take place within the cell. Previously, it was discussed how initial experiments found evidence to support the involvement of HAGE in increasing the ability of tumour cells to proliferate. In order to establish what possible molecular relationships may exist to provide this phenotype, gene expression profiling in the form of oligonucleotide microarray analysis was performed. Specifically, this was carried out on a human melanoma cell line that had undergone transient HAGE knockdown using siRNA to discover what occurs to a cancer cell transcriptome following this event. Since the entire transcriptome of these cells was screened using microarray, a large number of different genes could have been analysed. Selection of those chosen for further study was based on the extent of expression change, which was chosen to be a 2.5 fold up/down-regulation, and genes generating a product with published involvement in a cell process modified in malignancy

What was noticeable about the confirmation of the microarray data using qPCR was the similarity in the change in expression in target genes in both FM-82 and FM-6. Both showed very close correlation between the genes that were both up and down regulated in response to the knockdown in HAGE expression. On the other hand, this was not shared by both sets of stable transfectants. While some genes did match overall, it was relatively fewer than might have been first thought of, especially in light of the change of HAGE expression that was gained in each set. For the cDNA transfectants, this may result from the stable incorporation of the plasmid into the cell's genome. Since this was not a promoter-specific induction of gene expression, it is difficult to know what impact the plasmid might be having on the genetic stability of the cell. Evidence gained from proliferation assays showed that these cells matched the behaviour of cells that naturally express HAGE, but this phenotype might be brought about through different genetic changes. This is made all the more possible when one considers that this was originally a HAGE-negative line in which a significant change to a particular gene has taken place.

The shRNA lines were more of a concern as they in essence followed the same biological pattern as cells treated with siRNA. However, it is difficult to say what effect long-term knockdown of a specific gene has on a cell's genomic make-up. It may well be that prolonged exposure to RNAi caused by integration of shRNA specific to HAGE could cause genetic changes that were seen in this experiment.

It would appear that HAGE does have genetic effects shared across different tumour types. This could be seen with the expression of genes seen in the PCI-13 cell line. On the other hand, like the stable transfectants this was not universal across the entire gene panel. This series of experiments was carried out to investigate if any similarity existed. In that respect, it could be called successful, since certain genes do alter their expression in the same way. It would be highly unusual if two completely different tumours shared the exact same genetic pattern. This can be argued further when one considered that naturally, these cell lines would naturally have very different levels of expression of all of the genes tested. Therefore silencing of HAGE allowed the recognition of genes that could be implicated across different forms of cancer. However, this was only performed in one head and neck carcinoma line compared to the two natural and two modified melanoma cell lines. Therefore, to confirm the hypothesis further, the same studies need to be to be repeated in other cell lines derived from this malignancy. In the future, it would be interesting to perform this type of expression analysis using a panel of cells taken from a variety of HAGE-expressing tumour types to further establish the extent of gene expression change involving this antigen.

Primary indications from the analysed microarray data provided evidence that HAGE gene expression affects genes involved in proliferation and cell cycle progression. This was shown with decreases expression of CCNE2, E2F2 and WNT-1 once HAGE was silenced, therefore implying that their expression would be up-regulated in the presence of HAGE. WNT-1 belongs to a series of secreted signalling molecules used in epithelial homeostasis that act on frizzled receptors on the cell surface and activate proliferation through the β-catenin pathway (Fodde and Brabletz, 2007; Suzuki *et al*., 2008). WNT itself is classed as being an oncogene and is implicated in tumorigenesis

(Behrens and Lustig, 2004). It has been shown that inhibition of WNT signalling in colorectal carcinoma can result in apoptosis of cells (He *et al*., 2005). Aberrant WNT can be found in numerous different cancers, especially breast cancer, where there is evidence of autocrine signalling (Schlange *et al*., 2007; Oloumi *et al*., 2010). Of note is the data that has been published indicating that WNT signalling is inhibited by SFRP-1 (Matsuda *et al*., 2009), a gene that according to this study's data was found to be down-regulated by HAGE. Increased expression of WNT by HAGE would seem to suggest that the cells are capable of autocrine production of a potent signalling protein, given previous work showing its secretion from cells. As this is initial data based on gene expression, this would obviously require confirmation at the protein level. If however, this is the case, then increase signalling by this molecule could provide an answer for how HAGE expressing cells are able to proliferate quicker. This is made all the more interesting by the proposed role of SFRP-1 in WNT blockade, suggesting some sort of molecular interaction involving HAGE.

CCNE2 (Cyclin E2) and E2F2 are both involved in the cell cycle process. The latter is a member of the E2F family of transcription factors and promotes the transition from G1 to S phase, therefore promoting proliferation of a cell. In particular E2F1-3 have been implicated in this role. Normally, the action of E2F members is inhibited by the TSG retinoblastoma (Rb), which binds to them and prevents them from inducing target gene transcription (DeGregori, 2002). In a healthy environment, E2F2 alongside its associate member E2F1 can promote either apoptosis or cell cycle progression (Cam and Dynlacht, 2003). In the absence/inactivation of Rb, a situation found in tumours, this balance is tipped towards the latter and hence proliferation (Cam and Dynlacht, 2003). E2F2 over-expression has been shown to activate hyper-proliferation in a transgenic murine model (Scheijen *et al*., 2004). Furthermore, there is evidence indicating that E2F2 is required by MYC, a gene amplified in cancer, to promote cell cycle progression and that its ablation stops this process (Leone *et al*., 2001).

Other work has suggested E2F2 might inhibit tumorigeneis, thereby contradicting previous work into the molecule (Zhu *et al*., 2001; *Li et al*., 2003; Scheijen *et al*., 2004). It is possible that certain members of the E2F family have both pro and anti-tumorigenic properties, but which depends on the state of other molecules in the cell.

It is possible that in HAGE expressing cells, E2F2 acts to promote proliferation and increased mRNA expression could result in increased protein that contributes to the apparent raised cell turnover. As with WNT-1, evaluation of the protein level of E2F2 in HAGE expressing cells would be needed to corroborate this theory further.

Interestingly, further evidence of increased cell cycle progression came in the form of increased CCNE2 mRNA expression in HAGE-positive cells. Increased expression of this gene has been shown in tumours and over-expression of CCNE2 is connected with increased cell cycle progression and increases the number of cells in S phase (Payton and Coats, 2002). It can also be induced by oncogenic proteins by viruses (Zariwala *et al*., 1998). This immediately suggests that HAGE positive cells could be capable of increased progression due to the enhanced expression of CCNE2. It is able to bring about passage into S phase by complexing with CDK-2 (Gudas *et al*, 1999). The ability of CCNE2 to reduce the time taken for the cell cycle to occur comes from it acting on the TSG Rb, repressing its activity (Ohtsubo *et al*., 1995; Sherr and Roberts, 1999; Payton and Coats, 2002). This is intriguing, since it is known that Rb acts to inhibit the E2F family. As such, a hypothesis could be stated that in HAGE expressing cells, inhibition of Rb by CCNE2 and evidently increased E2F2 expression could lead to an enhancement of proliferation. In order to confirm this, activation of Rb would need to be checked, as would possible coupling of CCNE2 protein to CDK2. As with other regulatory molecules in tumours, there is a paradox as to the relationship that CCNE2 has with proteins that regulate it. Both $p21^{CIP1}$ and $p27^{KIP1}$ are known to inhibit CCNE2-CDK complexes (Harper and Elledge, 1996; Payton and Coats, 2002). However other work also states that the former can interact with $p27^{KIP1}$ to inhibit its function (Sheaff *et al*., 1997), meaning that there is a need to study further some of the regulators and effectors of CCNE2.

Examination of the differential analysis results revealed that it was not just proliferation that seemed to be affected by HAGE expression, but other cell processes involved in cancer too. Collectively, these data indicate that HAGE promotes survival mechanisms in cells that expressed it. This came from the recognition that genes reported to promote apoptosis, PKC-β1, CIDE-A and SFRP-1, increased their expression when HAGE was silenced. At the same time, there was a decrease in the

expression of both HSP-27 and 70. This implies that HAGE may be able to reduce and up-regulate these two groups of genes respectively.

In terms of the former group, PKC-β1 is one of two splice variants of the PKC-β gene (Martiny-Baron and Fabbro, 2007). It has been shown that this specific variant can contribute towards activating apoptosis. This occurs during oxidative stress through the influence of ROS, where it contributes to the expression of p66. This proteins acts to disrupt the mitochondrial membrane and through that activate apoptosis in response to oxygen related injury (Pinton *et al*., 2007). It is possible then that HAGE expression leads to a reduction in pro-apoptotic molecules. On the other hand, PKC-β2 has been shown to be involved in driving various tumorigenic events (Gökeman-Polar *et al*., 2001; Martiny-Baron and Fabbro, 2007). Elevation in levels has been linked to the development of colorectal carcinoma and expression. The expression of PKC-β in general is associated with uncontrollable proliferation and associated with poor prognosis in DLBCL (Martiny-Baron and Fabbro, 2007). However there is evidence of this loss in expression in melanoma (Martiny-Baron and Fabbro, 2007). This is thought to be part of the differentiation process and could explain what was seen in the result gained. On the other hand, this does not explain why reduction in HAGE results in reduced expression of PKC-β1, hinting to a possible link with apoptosis.

The gene CIDE-A (Cell death- inducing DNA fragmentation factor-a-like effector A) was first recognised in mice as being related to DNA fragmentation factor (DFF) family. These are responsible for the break-up of DNA to cause apoptosis (Inohara *et al*., 1998). To date, this molecule is predominantly linked to a role in adipocytes and loss of expression has been associated with obesity (Lin and Li, 2004; Nordström *et al*., 2005). There have been two converse links with CIDE-A and cancer. On the one hand, there has been an association with over expression of CIDE-A and cachexia, the weight loss that takes place in later-stage cancer patients (Laurencikiene *et al*., 2008). On the other hand other work has shown that the gene undergoes methylation and thus silencing (Hill *et al*., 2010; Huang *et al*., 2010), possibly to stop its potential role in apoptosis. Results from this study indicated the latter and would appear to fit with the hypothesis that HAGE is involved in increasing tumour cell proliferation.

One of the most interesting molecules that HAGE seems to inhibit is SFRP-1 (Secreted frizzled-related protein-1). This belongs to a group of five glycoproteins that have been proposed to act as inhibitors of WNT (Jones and Jomary, 2002; Sogabe *et al*., 2008). Work performed on cervical, colorectal and hepatocellular carcinoma (HCC) has shown that expression of SFRP-1 antagonises WNT signalling, while its loss has the opposite effect (Suzuki *et al*., 2004; Shih *et al*., 2007; Chung *et al*., 2009). Additionally, loss or silencing of the gene has been found in bladder, breast, prostate and non-small cell lung carcinoma (NSCLC) (Stoehr *et al*., 2004; Fukui *et al*., 2005; Lodygin *et al*., 2005; Lo *et al*., 2006). Both microarray and PCR data hinted at the decreased expression of this gene when HAGE was present in cells. As described above, WNT-1 was up-regulated in the same situation. Therefore, there seemed to be some sort of agreement with previously published findings as to the relationship between WNT and SFRP-1 with the results displayed here. The change in these genes' expression following HAGE knockdown also insinuates that the demonstration of HAGE in cells could influence their expression. Moreover, these findings act as another example of how HAGE expression has an effect on the relationship of genes that have involvement in cancer.

Aside from affecting genes that could induce apoptosis and repress signalling, the data collected showed that genes promoting survival such as HSP-27 and 70 were also affected. The heat shock proteins (HSP) are a group of chaperone molecules which serve to protect cells from death during stress events (Garrido *et al*., 2006). An example of this is raised levels of both HSP-27 and 70 following the reoxygenation of cells, stopping cell death by oxidative injury (Kabakov *et al*., 2003). HSP-27 and 70 act by inhibiting the action of various prominent apoptotic mediators. HSP-70 can inhibit the actions of BID and BAX, the JNK signal cascade and can prevent the actions of caspase 3, 7 and 9. It is also capable of reversing apoptosis, even in its latter stages (Jolly and Morimoto, 2000; Garrido *et al*., 2006). The necessity of HSP-70 is exemplified by its down-regulation resulting in apoptotic cell death (Nylansted *et al*., 2000). HSP-27, like 70 also inhibits caspases; in this case, it directly inhibits the actions of caspase 3. It is also capable of inhibiting the activation of caspase 9 by preventing the action of cytochrome *c* (Garrido *et al*., 1999; Paul *et al*., 2002). Caspase independent cell death may also be stopped by HSP-27 through an apparent

inhibition of the pro-apoptotic molecule DAXX (Charette *et al*., 2000; Garrido *et al*., 2006; Schmitt *et al*., 2007). Of particular note, is the ability of HSP-27 to phosphorylate and therefore activate AKT (Schmitt *et al*., 2007). This not only helps to halt apoptosis through the inactivation of BAD, but can also lead to proliferation. The TSG cell cycle inhibitor $p27^{KIP1}$ is ubiquinylated and therefore degraded as a result of HSP-27 and through that promoting cell cycle progression (Garrido *et al*., 2006; Parcellier *et al*., 2006).

HSP molecules are known to be up-regulated in cancers (Soti and Csermely, 1998; Jolly and Morimoto, 2000). Indeed, HSP-27 is linked to drug resistance in tumours (Garrido *et al*., 1997; Choi *et al*., 2007). This would again link HAGE to proliferation and prevention of apoptosis, particularly the possible relationship between HSP-27 and the pro-proliferative AKT molecule. More to the point, this acts as evidence that HAGE may cause tumour cells to survive. Up-regulation of HSP-27, a molecule known to create chemo-resistance, suggests that HAGE may be promoting a more aggressive phenotype in tumours. On top of this is the connection that HSP-27 has with the inhibition of $p27^{KIP1}$. This latter protein inactivates CCNE2-CDK2 complexes, meaning that there is yet more possibility that HAGE is influencing various cellular interactions.

A rather paradoxical change in expression came in the form of JUN-D. This belongs to the JUN family of genes that have been associated with cancer in the past (Hirai *et al*., 1989; Ryder *et al*., 1989). This member, like its other counterparts, has been classified as being an oncogene. It has been described that expression of JUN-D promotes cell survival against oxidative stress (Gerald *et al*., 2004). So, there appears to be a paradox in the results obtained, showing that JUN-D would be down-regulated when HAGE is expressed by malignant cells. However, the same work that showed JUN-D protects against ROS described that angiogenesis is inhibited by this gene through this antagonism of the oncogene RAS (Gerald *et al*., 2004). One could therefore argue that in a HAGE expressing cell, while JUN-D does protect a cell, it does so at the cost of a less aggressive phenotype. Since it was possible to show evidence of other survival genes being up-regulated by HAGE, there is a theory that JUN-D is inhibited in order to promote RAS activation of angiogenesis and through that encourage a more aggressive disease state.

Aside from the hallmarks of cancer detailed above that HAGE seems to have an effect on, gene data collected in this study indicated that HAGE expression could aid in tumour immune escape. This came from the recognition that once HAGE expression appeared to lead to a decrease in the expression of both CXCL-6 and IL-15. The former is a chemokine connected with IL-8 and acts as ligand for innate immune cells. It has been shown to both bring about innate immune reactions while also being increased during hypoxic conditions (Zhu *et al*., 2006; Mittal *et al*., 2008). So in this instance, it is possible that HAGE could allow tumour cells to protect themselves from attack from innate immune cells. The finding that CXCL-6 is greater in hypoxia seems to give another link between HAGE and stress that reoccurred through the gene data gained.

IL-15 on the other hand is an important cytokine used in the homeostasis of T cells. It stimulates continued proliferation of activated T cells (Waldmann, 2003), and contributes to both NK cell survival and maturation (Carson *et al*., 1997; Cooper *et al*., 2002; Cho and Campana, 2009), a factor has been demonstrated *in vivo* (Cooper *et al*., 2002). Perhaps the most important aspect of IL-15 is its role in the generation and survival of memory T cells (Schluns *et al*., 2002; Surh and Sprent, 2008), which has been demonstrated *in vivo* (Zhang *et al*., 1998). These are of great importance in immunity, especially in terms of tumour immunity as T cells capable of 'remembering' tumour antigens are a powerful tool against malignancies. This is one of the reasons why IL-15 is considered a potentially valuable tool in cancer immunotherapy (Cho and Campana, 2009). This implies that a HAGE expressing tumour could have the potential to inhibit the actions to cause T cell memory and in turn grant itself a better chance of survival against immune reactivity. Furthermore, the possible maturation of NK cells could be of threat to a tumour, due to the anti-malignant activity of these cells. Collectively, evidence for change in expression of this and CXCL-6 allows the impression that both aspects of immunity could potentially be altered by the expression of HAGE.

Ultimately, it was shown that a number of different genes are affected by the expression of HAGE in tumour cells. Aside from the initial confirmation that genes involved in cell proliferation and the cell cycle appeared to be affected as was hoped,

there seemed to other processes that could be manipulated by HAGE. While these findings were very promising, it must be stressed that this was genetic data and therefore must be confirmed at the protein level before any further conclusions could be made. It did however appear to validate the use of the combined use of microarray and qPCR as an effective tool for achieving more insight into the possible role of a gene in cancer.

Chapter 5

Expression of HAGE and its Impact on Cell Signalling Pathways in Cancer

5.1 Introduction

Cancer is a disease which comes about through a disruption in the signalling networks that control cell behaviour. Every behavioural facet of a cell is governed by communication cascades that induce them to perform a given action at all stages of their life. This is brought about through the action of different proteins that either interact and influence other proteins, or act as transcription factors to provoke expression of specific genes. Cellular signalling is such a highly complex subject that it cannot be covered comprehensively here, but the basic principles will be outlined.

There are numerous different networks within cells, involving a huge number of different molecules. They may consist of a single cascade or multiple pathways that connect together but bear the potential to act autonomously. Furthermore, a cascade may well be controlled by its most upstream molecule but can also be influenced by other mediators that lie even further upstream than this, adding yet more complexity to the subject. Good examples of this can be found with the PI3K pathways, which can act alone, reacting to ligand-receptor binding, or be activated through the actions of RAS members, which lies further upstream (Rodriguez-Viciana *et al*., 1994; Krasilnikov, 2000; Pacold *et al*., 2000). Due to the extremely complicated nature of signalling, it is often portrayed as a situation where one pathway/network is active at a particular moment. This is especially true in the case of explaining proliferation versus apoptosis signalling. It would be naïve to believe that this is the case *in vivo*. A cell may well be constantly exposed to various simultaneous stimuli, that both encourage a cell expand its population or to inhibit it, senesce or die. The key is the balance of these signals, which comes from homeostasis, which promotes specific cascades.

In cancer, this balance is tipped in favour of promoting the acquisition of hallmarks described by Hanahan and Weinberg (2000). For this to take place, there must be a situation where molecules, which can cause this phenotype, are promoted and others with the potential to inhibit are lost or down-regulated. It is at this point that the modifications that occur to the proto-oncogenes and TSG become more apparent and their significance made more noticeable. These alterations can take place not just at the genetic level, but also affect post-translational modification of protein products. Different alterations can affect molecules in different ways. Point mutations in RAS and B-RAF significantly increase their activity and can cause increased proliferation of cells through either the PI3K or MAPK pathway (Bos, 1989; Davies *et al*., 2002). Conversely, the same change to p53 causes abnormal function, reducing the capability of affected cells to cause cell cycle arrest and apoptosis, contributing to proliferation (Levine and Oren, 2009). Chromosomal translocation can also lead to increased proliferation of cells, seen following the formation of BCR-ABL in CML and ALL (Druker *et al*., 2001b). At the same time, loss of PTEN through deletion allows continual signalling through the PI3K pathway (Blanco-Aparicio *et al*., 2007). Post-translational modifications that affect protein activity are most frequently seen in terms of phosphorylation. AKT can experience this as a result of PTEN loss, which leads to more production of PIP_3 that modifies AKT (Maehama and Dixon, 1998; Chow and Baker, 2006). In this state, the molecule is more active and can cause increased proliferation by causing the transcription of various genes and inhibits apoptosis by inhibiting other proteins like BAD (Datta *et al*., 1997).

Phosphorylation is just one of the numerous different ways that proteins can be modified following translation. Others include glycosylation, sumolysation (PML) (Dohmen, 2004), farnesylation and palmitoylation (both RAS) (Roy *et al*., 2005; Wright and Philips, 2006). This diversity of modifications adds to the complexity of tumour signalling and targeting molecules involved. This is especially true with RAS. Upon translation of RAS isoforms, it was originally believed that they must be subject to farnesylation by farnesyl transferases in order to truly active and be capable of trafficking to the cell membrane (Downward, 2003; Omerovic *et al*., 2007). This led to the development of various different inhibitors against farnesyl transferases as anti-RAS cancer therapy, which have had very limited effect to date (Downward,

2003). In part, this is due to the discovery that both K-RAS isoforms and N-RAS can still undergo prenylation by the geranylgeranyl transferase enzyme (Whyte *et al*., 1997). The other issue is that farnesyl transferase inhibitors are most effective against H-RAS, mutations of which occur in less than 1% of human tumours with oncogenic RAS (Bos, 1989; Downward, 2003; Omerovic *et al*., 2007).

Cell signalling disruption is integral to the concept of Weinstein's "oncogene addiction" theory and Felsher's "oncogene amnesia" hypothesis (Felsher, 2008; Weinstein and Joe, 2008). Both suggest evident reliance of tumour cells on certain gene products for their development. This would seem to come about two ways. The first could be from loss of genes leading to dependence on specific genes to maintain particular signalling pathways to maintain survival. This can be seen with the reliance on PARP-1 for DNA repair signalling following the loss of BRCA (McCabe *et al*., 2006). Alternatively, it could be that signalling involving specific oncogenes are so profitable to tumour cells, they become increasingly reliant upon that pathway. Moreover, it could be argued that a tumour cell moves to down-regulate other cascades that are not as efficient as the transformed ones so only this latter pathway is active. Evidence for this can be found in the success of Imatinib in CML. Use of this TKI causes signal attenuation via the BCR-ABL protein, leading to the death of Ph-positive malignant white cells through apoptosis (Deininger *et al*., 2000b). If other proliferative pathways were readily available to those cells, one would assume it would be possible to use them. However, it would seem that loss of BCR-ABL signalling changes the signal environment and in the time taken for other cascades to be re-established, signalling homeostasis is tipped in favour of apoptosis and death occurs. Therefore, oncogene addiction/amnesia and synthetic lethality are closely bound to signalling within tumour cells.

Knowledge about tumour cell signalling has come from extensive research investigating how normal signals become corrupted. This has involved combinations of genetic, proteomic and bioinformatic approaches over the years to piece together pathways and networks and changes to them. Understanding of malignant cell signalling in general is currently highly extensive, with knowledge of certain pathways and the molecules involved being in-depth. Paradoxically, in spite of this vast level of awareness, efforts to curtail signalling in malignant cells have been very

limited. Molecules that lie the furthest upstream are fewer in numbers and have proven very difficult to treat, as with RAS. On the other hand, trying to target further downstream is arguably more testing due to the sheer number of effectors present. This is compounded by the possibility that signalling could be diverted by the tumour cell, rendering treatment useless. Limitation in curtailing malignant signalling networks implies that there are molecules present in these pathways that currently remain unclear. This would certainly account for difficulties being present in the face of apparently well developed understanding. Therefore, other entities must exist that have influence over signalling that are yet to be discovered or are known molecules with extra abilities that are currently unclear.

The data gained previously highlights a possible involvement of HAGE in tumour proliferation. These also indicate that various genes concerned with various cell processes undergo expression change in response to HAGE being present in cells. Therefore work was carried out to investigate if this antigen has some form of influence over signalling networks in cancerous cells to cause the observed phenotype.

5.2 Results

5.2.1 HAGE Expression and Alterations to PI3K Signal Molecule Phosphorylation

To determine if signalling pathways, known to be altered in cancer, were affected by HAGE expression, the phosphorylation status of proteins involved in some of these paths were examined. This was done using MAPK proteome arrays (R & D Systems) specific for the MAPK and PI3K pathways. These can be used to determine the relative levels of phosphorylated forms of specific signalling proteins involved in these cascades. Arrays were used in conjunction with protein lysates extracted from the shRNA stable transfectant cell lines. Cells transfected in this manner represented the ideal way of further investigating HAGE as they represented the effect of long-term knockdown in a line naturally positive for the antigen. While the cDNA transfected cell line appeared to cause the same phenotype, it could be that this may be brought about through a different pathway. To this end, the stable shRNA cell lines were used in preference.

A representative image of arrays used with these lysates is shown in Figure 5.1(A). Every two spots seen in the image represents a specific signalling protein and its repeat. Both the positive and negative controls for each array are highlighted. Once images were gained for control and test lysates, densitometry was carried out to compare the levels of phospho-proteins following each treatment. The average of these readings is shown in Figure 5.1(B). As highlighted here, there appeared to be a difference in the levels of certain phospho-proteins known to be altered in tumours. Specifically, it seemed that once HAGE was reduced, there was a decrease in the levels of both AKT-1 and GSK-3β, proteins involved in the PI3K signal pathway. Conversely, it could be noted that there was an increase in the level of phosphorylated ERK-2 in the same conditions. For the former two proteins, this would appear to suggest that if HAGE is expressed by tumours, then there would be an increase in the level of their phosphorylation, suggesting possible post-translational modification resulting from HAGE expression.

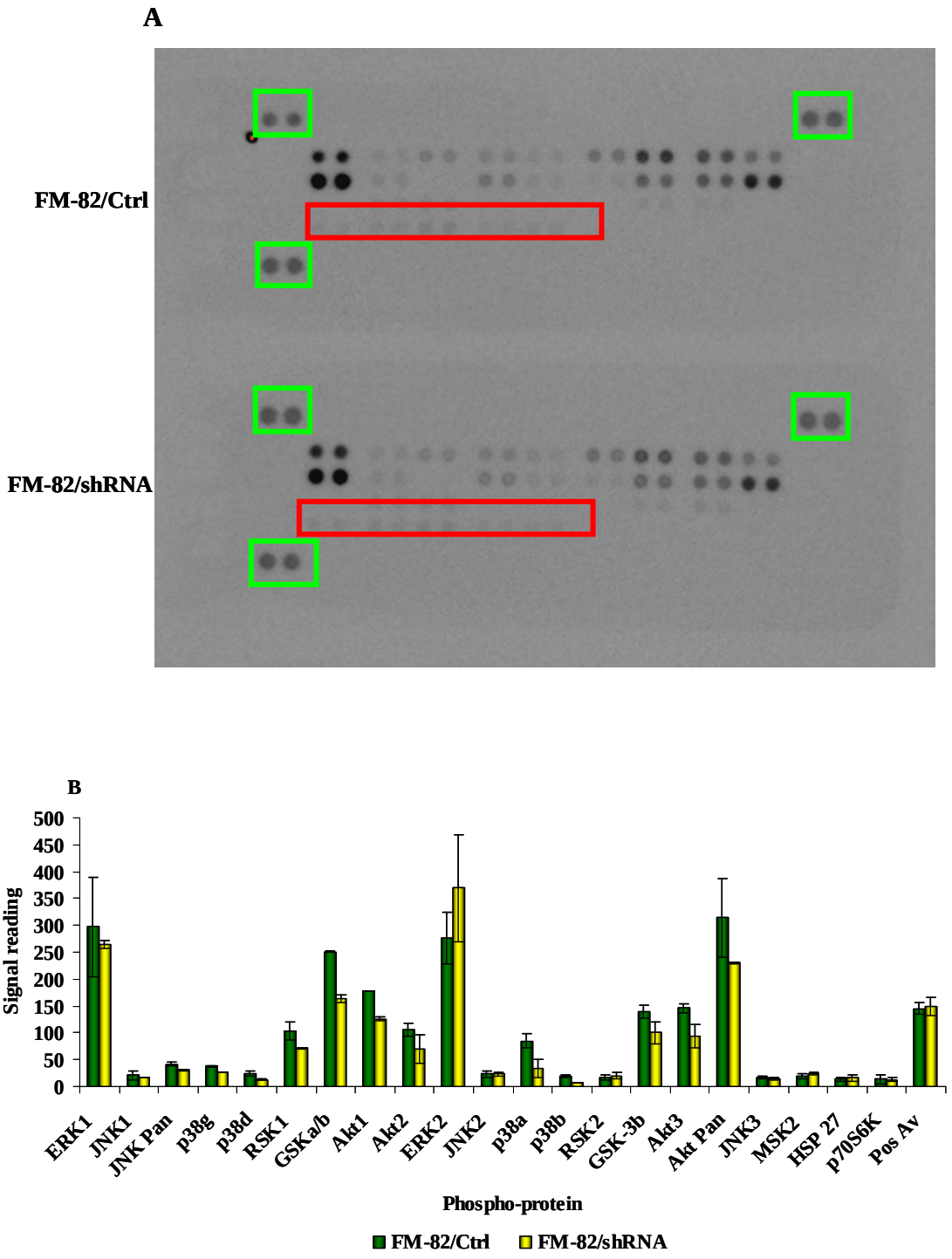


Figure 5.2: *MAPK Proteome Profiler array data obtained from lysates taken from shRNA stable knockdown cells (n=2). An image of the arrays used for densitometry is shown by (A). Each two spots represents the capture region for a specific signalling protein and its repeat. Positive controls are highlighted in green and negative controls in red. Densitometry readings of these arrays are shown in (B).*

5.2.2 Confirmation of Changes to Signal Protein Phosphorylation

In order to confirm changes in phosphorylation status of AKT and GSK-3β hinted to by the proteome arrays, antibodies were obtained against these proteins for western blotting. These would detect the phosphorylation at the same residues as detected by the MAPK arrays. Antibodies against the total form of both these proteins were also acquired to assess whether changes observed are due to changes in the overall concentration of the protein. Western blotting involved the initial separation of protein lysates derived from both FM-82/Ctrl and FM-82/shRNA. These were then exposed to overnight staining for using these antibodies along with those against the housekeeping protein β-actin. Detected protein bands following membrane development are shown in Figure 5.2. Bands detected by western blotting shared the same pattern that was noted with the Proteome Profiler arrays. It was found that both AKT and GSK-3β total proteins were increased when HAGE was silenced yet with the phosphorylated forms of both proteins, it was found when HAGE was stably knocked-down using shRNA; there was marked decrease in their level.

Densitometry was performed on these bands in order to calculate the difference in ratio of phosphorylated AKT and GSK-3β between FM-82/Ctrl and FM-82/shRNA. Readings for the test bands were normalised using the readings gained for β-actin bands. The ratio was then calculated by dividing the normalised reading for FM-82/shRNA by the reading for the control cell line. This was used to determine the proportion of phosphorylated protein in relation to total protein in each group of cells. For p-AKT (Ser473), the ratio gained for FM-82/Ctrl was 1.82 compared to 0.49 for FM-82/shRNA. Though the visual difference between the bands was stark and the bars shown for this protein showed a difference, this was not considered to be statistically significant, implying more biological significance. There was a similar pattern with p-GSK-3β. On this occasion, the ratio in FM-82/Ctrl was 0.69, which was greater than the 0.36 calculated for FM-82/shRNA. Once again, though there was a recognisable difference between the levels of p-GSK-3β in each cell line, this was not seen as being statistically significant, suggesting biological significance.

Thus, there seemed to be a connection between HAGE expression and the phosphorylation status of these two proteins. In turn, this suggests HAGE could have some influence over molecules involved the PI3K signalling pathway. If these modifications were a result of changes to gene expression or possible post-translational modifications, the data from microarray analysis was referred back to. This showed that there was no significant change in the mRNA levels of both AKT and GSK-3β following HAGE gene knock down (Data not shown). This implied that changes occurring to these proteins were a result of post-translational modification.

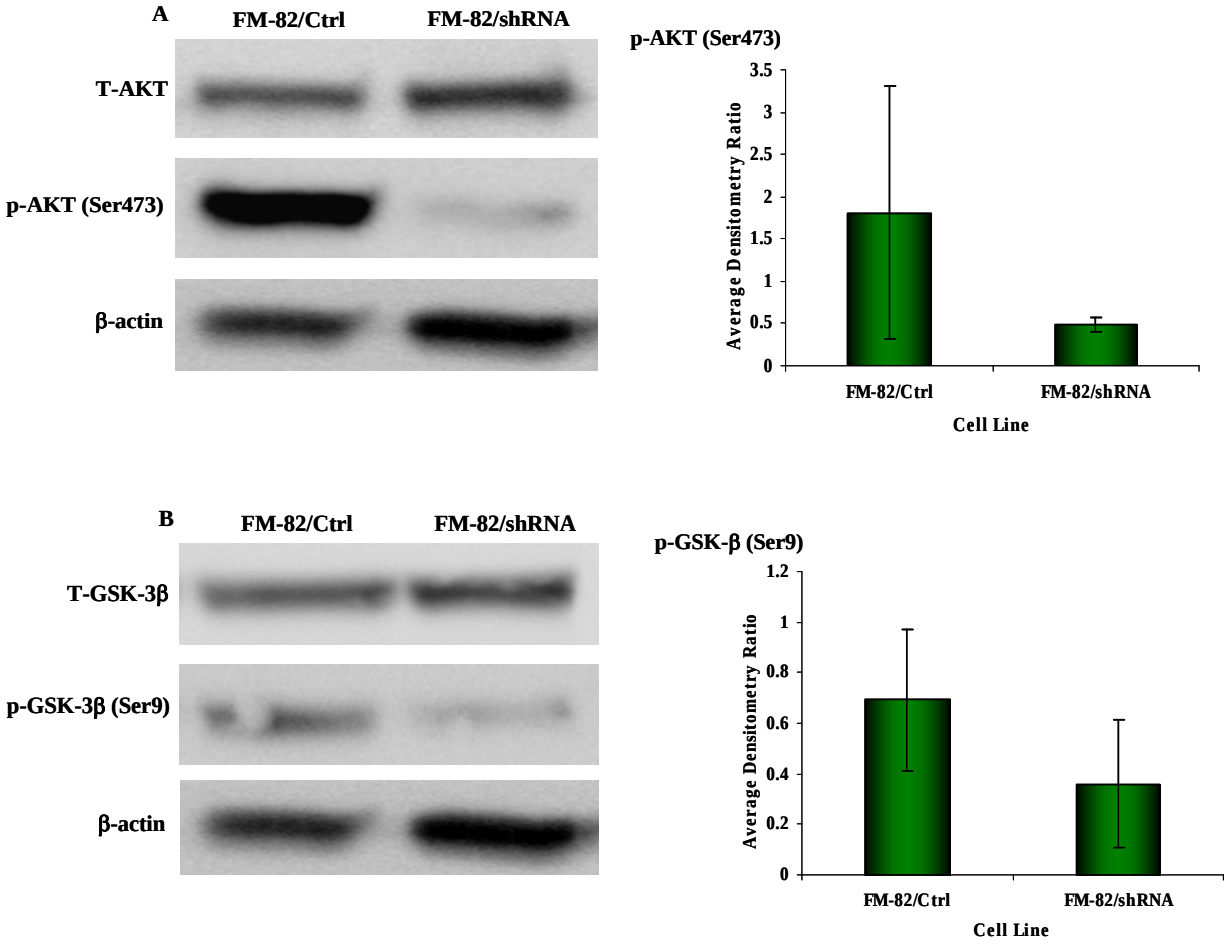


Figure 5.2: *Confirmation of (A) AKT and (B) GSK-3β expression in HAGE stable knockdown cell lines. Protein bands gained from western blotting (n=2) for total and phosphorylated proteins are shown along with averaged densitometry ratios used to determine the difference of p-AKT and p-GSK-3β in each cell line. Proteins were stained overnight with antibodies optimised to 1/500 for T-AKT, T-GSK-3β, p-GSK-3β and β-actin. Antibody for p-AKT was optimised to 1/250. Ratios were calculated by normalising readings for test proteins using β-actin readings and dividing the modified readings for the phosphorylated protein by that of the total, to represent the proportion of phospho-protein in that cell line.*

5.2.3 Effect of HAGE Expression on Molecules Associated with Cell Cycle Progression

Increased phosphorylation of the PI3K effectors AKT and GSK-3β has links to increased proliferation of cells. Therefore it was necessary to check the impact of HAGE expression on proteins involved in cell cycle progression. To investigate this, an antibody against the CDK inhibitor $p21^{CIP1}$, a downstream target of AKT, were obtained and used in western blotting using protein lysates from shRNA stable transfectants. An image of these can be seen in Figure 5.3. It was found that in FM-82/shRNA cells, visually there appeared to be more $p21^{CIP1}$ present than in control cell line. This implied that in HAGE expressing cells, there would be a lower concentration of this CDK inhibitor. Densitometry of the western blotting bands was performed to compare the level of the protein in each cell line, also shown in Figure 5.3. This was calculated by dividing the reading for the test band by its respective reading for β-actin. The averages of these were taken for each cell line and compared to one another. The slight increased visually recognised in the protein bands was reflected in the ratios between the two lines, however this was deemed not to be statistically significant, suggesting that the difference was more biological.

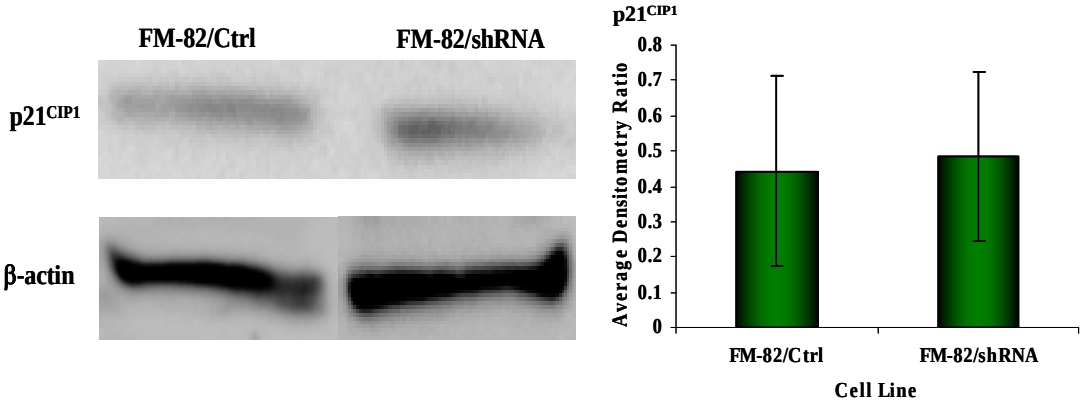


Figure 5.3: *Expression of the CDK inhibitor and AKT target substrate $p21^{CIP1}$ in HAGE stable knockdown cells (n=2). Proteins bands detected using a specific antibody are shown along with densitometry analysis of bands. Proteins were separated using a 10% acrylamide SDS gel and stained overnight with antibody optimised to a 1/250 concentration. β-actin antibody was optimised to 1/500. Densitometry ratios were calculated by dividing readings for test band by reading for respective β-actin, then averaging these readings.*

5.2.4 Impact of HAGE on the Expression of the N-RAS Oncogene

Due to the apparent changes to the phosphorylated forms of both AKT and GSK-3β, it was important to determine if the expression of HAGE had any impact on other aspects of signalling pathways. In the first instance, a retrospective study of the microarray data analysis was performed to see if there were changes to molecules upstream of AKT and GSK-3β or more commonly modified downstream molecules. This review found that the oncogene N-RAS, which exists upstream of AKT and controls the PI3K cascade demonstrated a 70% decrease in expression following HAGE knockdown. This implied HAGE would act to up-regulate this gene in tumour cells.

Since RAS members are highly homologous, it was necessary to check if changes took place to K-RAS and H-RAS. Examination of microarray analysis data indicated that there was no significant change in these latter two molecules, hinting that the effect of HAGE was confined to N-RAS. To confirm this, primers specific for all three RAS members were obtained and used in real-time qPCR. This initially involved cDNA generated from RNA extracted from FM-82 cells treated with siRNA to ratify the microarray findings. Subsequently, cDNA synthesised from RNA extracted from shRNA stable transfected cells was used. The relative expression of the three RAS genes in both instances is shown in Figure 5.4. Figure 5.4(A) shows that following transient HAGE knockdown, there was a marked decrease in the mRNA expression of N-RAS. Conversely, this change was not shown by either K-RAS or H-RAS mRNA. So in this instance, real-time qPCR was able to confirm the findings of microarray expression analysis. When the same experiment was performed using stable knockdown RNA the pattern was reversed, highlighted in Figure 5.4(B). Here, it could be seen that in response to prolonged reduction in HAGE expression, there was no change to the expression of N-RAS but a reduction to both K-and H-RAS gene mRNA expression.

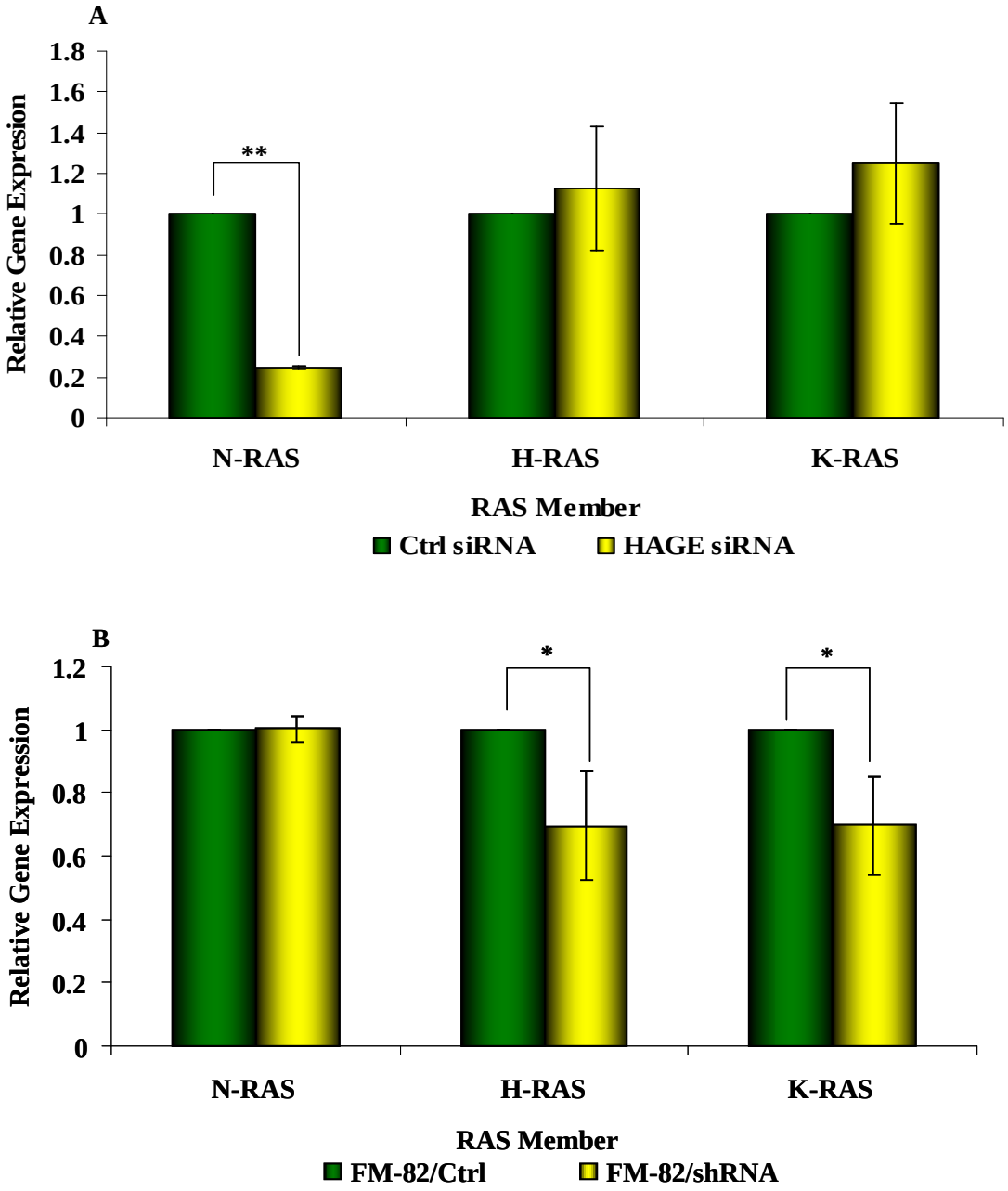


Figure 5.4: *Relative expression of RAS member expression determined by real-time qPCR in response to HAGE silencing (n=4). cDNA used for PCR was generated from RNA taken from either (A) FM-82 cells transfected transiently with siRNA (p=<0.01) or (B) FM-82 shRNA stable trasnfectants (p=<0.05). Relative expression was calculated as previously described.*

Though there seemed to be discrepancy between changes to N-RAS expression depending on the nature of HAGE knockdown, the implication was the oncogene's mRNA expression was modified due to the presence of HAGE. To gain more insight into this possible relationship, a siRNA duplex specific to N-RAS was obtained. This was used to silence the FM-82 cell line, which is naturally positive for HAGE, to investigate if reduction in N-RAS expression resulted in a decrease in HAGE mRNA expression. RAS gene expression was knocked down in the same manner used for HAGE. Expression was detected using real-time qPCR and gene expression calculated using 2ΔCT of PCR CT values normalised using the housekeeping genes GAPDH, TBP-1 and HPRT-1. Due to the aforementioned homology that exists between RAS members, siRNA molecules unique for K-RAS and H-RAS were obtained and used in the same way as above. In all cases, 'BLAST' nucleotide searches were performed on the designed siRNA sequence to ensure that they were unique to their target gene. Following the knockdown of these genes, expression of OAS-1 and STAT-1 was checked using real-time qPCR to ensure that addition of siRNA would not lead to apoptosis mediated by the IFN-γ pathway, as done with HAGE in the past. It was found for all siRNA molecules used that there was no significant increase in the expression of OAS-1 and STAT-1 (Data not shown), indicating that RAS siRNA did not induce the IFN-γ pathway.

The relative expression for RAS and HAGE are shown in Figure 5.5. As shown, transfection of siRNA into FM-82 cells was able to bring about reduced mRNA expression of specific RAS members. Additionally, it was found that decreasing the expression of each RAS member did not lead to a simultaneous reduction in the expression of HAGE mRNA. If one focuses on N-RAS, the specific RAS member of interest, Figure 5.5(A) shows that it was possible to achieve a marked reduction in the expression of the gene, reaching approximately 80%. Conversely, though there was a decrease in HAGE, it was not as dramatic. This begins to imply that HAGE may act upstream of N-RAS.

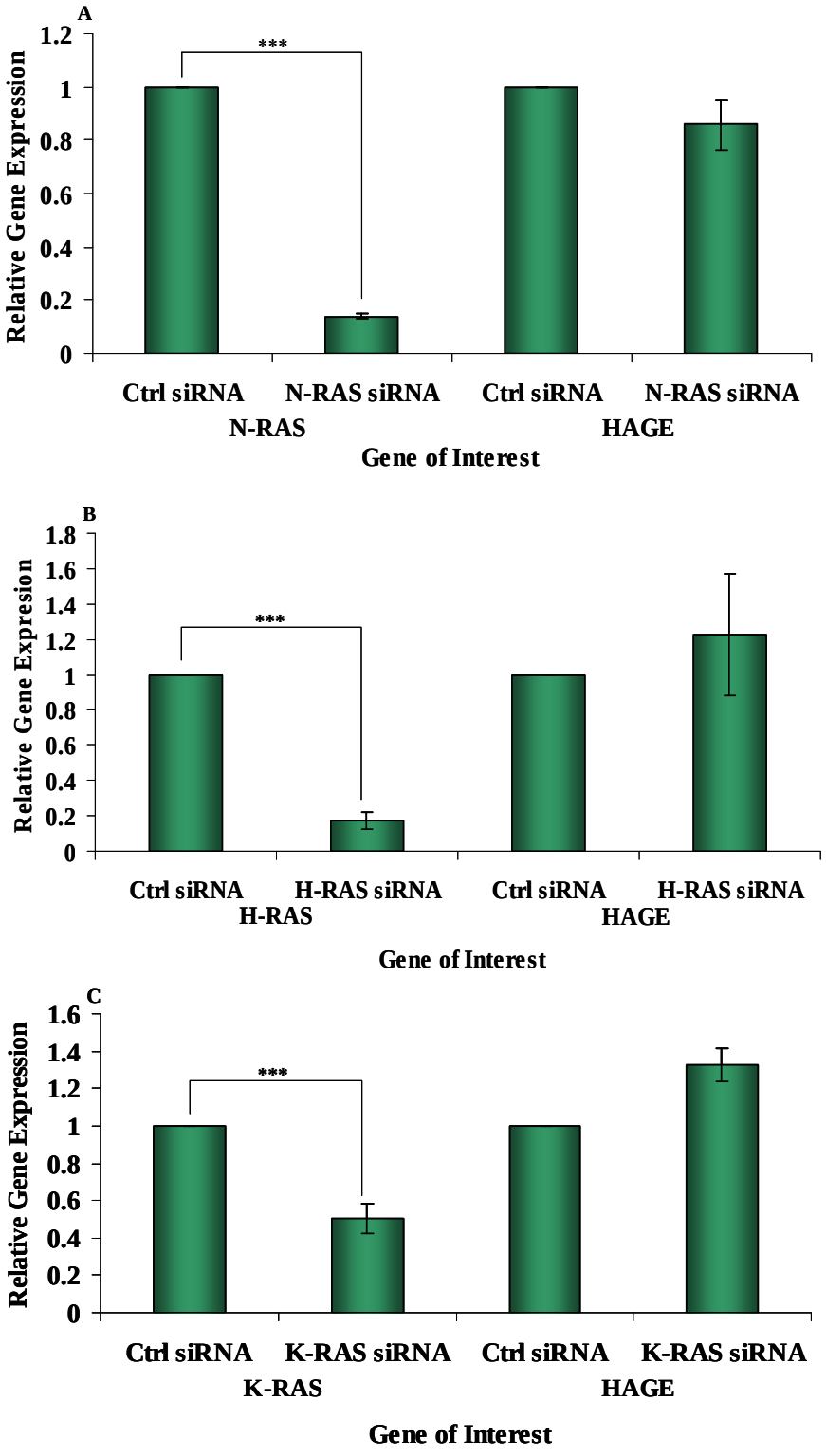


Figure 5.5: *Relative gene expression of RAS members and HAGE in FM-82 following specific RAS siRNA transfection (n=4). Expression was determined by real-time qPCR analysis of cDNA synthesised from RNA extracted from cells treated with (A) N-RAS siRNA, (B) H-RAS siRNA or (C) K-RAS siRNA. Relative expression was calculated from 2ΔCT equations of PCR CT values following normalisation using GAPDH, TBP-1 and HPRT-1 housekeeping genes (p=<0.001).*

If HAGE does act to promote N-RAS, it was important to examine if this up-regulation was noticeable at the protein level or confined to mRNA. An antibody specific for N-RAS was obtained and used for western blotting of protein extracted from both FM-82/Ctrl and FM-82/shRNA. An image of the detected bands along with β-actin can be seen in Figure 5.6(A). It could be noticed that following stable knockdown of HAGE expression in cells, there was a distinct decrease in the expression of the N-RAS protein. This was in contrast to the mRNA of N-RAS in this situation, where there was no change following stable HAGE knockdown. The decrease in protein was reflected in the densitometry ratio shown in Figure 5.6(A). From this, it could be argued that HAGE appears to promote N-RAS expression at the protein level.

Since there seemed to be an increase in the protein level in HAGE expressing cells, there was the potential for an increase in the level of RAS-GTP, the active form of RAS proteins. Should this be the case, there would be the possibility of HAGE interacting with GEF and/or GAP molecules that activate and inactivate RAS proteins respectively. To that end, a pull-down method was used to isolate active RAS from FM-82/Ctrl and FM-82/shRNA and detected using western blotting. For each cell line, part of the extracted lysate was supplemented with GTPγS, thereby creating a positive control lysate to compare each test lysate. The image taken for this is shown in Figure 5.6(B). Here, it could be seen that there was no evident decrease in RAS activity following the stable knockdown of HAGE. This meant that it did not seem HAGE had any influence over the direct activation or inactivation of RAS in cells.

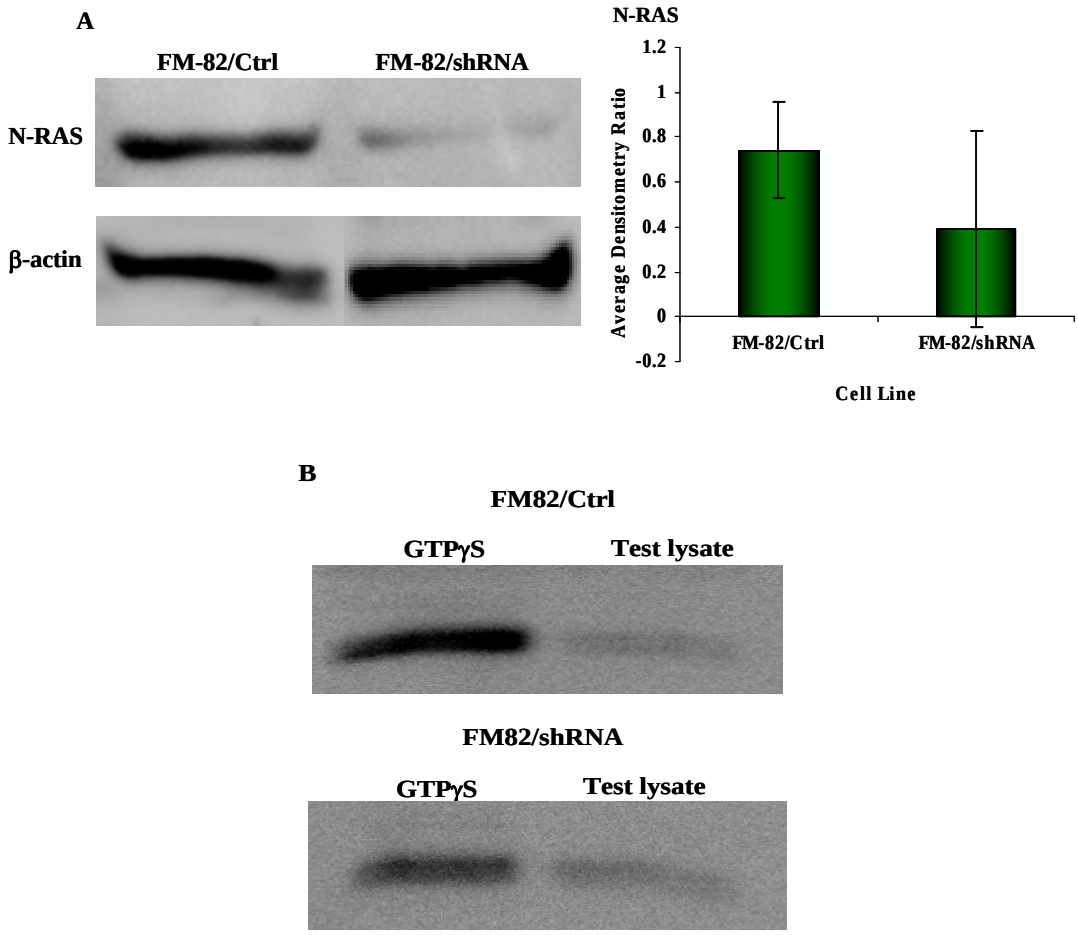


Figure 5.6: *Western blotting images of N-RAS protein expression in HAGE shRNA stable transfectants (n=2). (A) shows bands detected following SDS electrophoresis using a 15% acrylamide gel ahead of transfer to membrane and staining with an anti-human antibody optimised to a 1/250 concentration. β-actin antibody was used at 1/500. Densitometry is also shown, calculated in the same manner for $p21^{CIP1}$. (B) shows western blotting of lysates isolated using a RAS-GTP pull down kit to isolate active RAS and run alongside lysate treated with GTPγS acting as a positive control (n=1).*

5.3 Discussion

Signalling pathways that reside within cells are remarkably difficult to target. Therefore, it therefore stands to reason there are aspects to these pathways that remain unknown. Additionally, it means the possible existence of factors, as yet undiscovered, that are gained or lost as a result of malignant transformation that bear the facility to modify the actions of effectors of signalling pathways. HAGE may be one of these factors.

Preliminary work into this involved a basic screening of the MAPK and PI3K pathways, both of which are commonly corrupted in tumours. The arrays utilised as part of this work were capable of identifying levels of the phosphorylated forms of major effectors proteins that exist within these cascades. When used to screen protein lysates extracted from stable knockdown cell lines, there seemed to be a change in the level of certain phospho-proteins. In particular, there were particularly noticeable changes to the PI3K effectors, AKT and GSK-3β. It was found once HAGE expression had been decreased using shRNA the phosphorylated forms of these proteins experienced clear reductions according to densitometry analysis. These were matched by reductions to both the total AKT pool (AKT pan) and both GSK isoforms (GSK-3α/β), providing further evidence that there were changes to these proteins. Aside from this, there were additional reductions to the p38 isoforms, components of the MAPK signalling network.

The MAPK arrays were validated using western blotting and antibodies against the total and specific phosphorylated forms of AKT and GSK-3β. Examination of these bands using densitometry software allowed the determination of changes in protein following normalisation using readings for the control band β-actin. In terms of the ratio of p-AKT (Ser473) to total AKT in each cell line, densitometry analysis indicated that there was a higher ratio of the phospho-protein in FM-82/Ctrl compared to FM-82/shRNA. While the results were not found to be statistically significant, the stark decrease in protein level visualised between the control and test cell line implies a strong biological significance. The same could be said of the ratio of p-GSK-3β to total GSK-3β in each cell line. The large error bars seen with densitometry can be

explained by the difficulties in gaining protein bands of exactly the same intensity when carrying out western blotting, despite identical experimental conditions. Though the same pattern was found when performing these experiments, there was variation in the darkness of the protein bands, hence the extent of the standard deviation noted. Interestingly, examination of gene microarray data showed that there was no significant change to the gene expression of both these molecules. This means that there was potentially no more protein available that could be phosphorylated. An argument could therefore be put forward that HAGE expression leads to some form of alteration to post-translational modification of these proteins.

Both AKT and GSK-3β play significant roles within PI3K signalling. AKT, also called protein kinase B (PKB), is made active in cells through phosphorylation of specific amino acid residues. This can either be threonine at position 308 (Thr308) caused by PDK-1 or serine at position 473 (Ser473), currently thought to be mediated by the mTORC-2 protein (Mora *et al*., 2004; Sarbassov *et al*., 2005). Indeed, there have been attempts to target this latter relationship for tumour therapy. Data presented here focused on the latter, perhaps showing increased activity of mTORC-2 encouraging this increase in activity, though this would require confirmation. AKT perhaps represents the major effector of the PI3K pathway, inducing both cell survival and proliferation in cells. It can do this by the direct interaction with target substrates, instigating the phosphorylation and subsequent inhibition of proteins that include BAD and pro-caspase 9, both of which promote apoptosis (Datta *et al*., 1997; Vara *et al*., 2004). Evidence shows an interaction between HSP-27 and AKT, which can interact with each other to promote survival (Arya *et al*., 2007; Schmitt *et al*., 2007; Havasi *et al*., 2008). AKT can additionally act indirectly via the inhibition of transcription factors like FOXO, which stops the production of mRNA for pro-apoptotic FAS-L, BIM and the cell cycle repressor $p27^{KIP1}$ (Brunet *et al*., 1999; Liang and Slingerland, 2003; Vara *et al*., 2004; Calhoub and Baker, 2009).

Therefore, the stark increase in p-AKT seen from western blotting could help to explain the phenotype shown by HAGE-expressing cells. It could be that expression of HAGE is somehow increasing AKT phosphorylation, in turn causing cells to survive and proliferate to increase their population. This would appear to correlate

with the proliferation data presented in Chapter 3. Heightened expression of HSP-27 mRNA shown in Chapter 4 found by expression profiling could be seen as interesting, indeed if this mRNA undergoes translation, there could be more target material for AKT to act on and promote survival. An unusual point is the knowledge that AKT activity reduced production of FAS-L through FOXO inhibition, which seemingly contradicts the increased mRNA expression of FAS-L recognised in Chapter 4. FAS-L can also protect tumour cells and this point is discussed in greater detail in Chapter 6.

One of the major downstream substrates for p-AKT is the molecule glycogen synthase kinase-β (GSK-3β) one of two isoforms of GSK. GSK-3β is thought to be an important molecule in the metabolism of glucose and is linked with various pathological conditions other than cancer including diabetes and Alzheimer's disease (Kaytor and Orr, 2002; Lee and Kim, 2007). Its implication in malignancy comes from its function to inhibit the action of β-catenin and through that inhibit canonical signalling through WNT. Phosphorylation of the GSK-3β isoform at serine 9 (Ser9) is thought to inhibit the action of the protein and causes accumulation of β-catenin (Stambolic and Woodgett, 1994; Kandel and Hay, 1999; Rayasam *et al*., 2009). From this, WNT signalling can take place, increasing cell proliferation. Hence, GSK-3β inhibition is intrinsically connected to abnormal WNT communication, a mechanism shown in tumours (Luo *et al*., 2007). Phospho-AKT acts as a kinase to GSK-3β and can bring about the phosphorylation that is required to stop its action. So in HAGE-expressing cells, as well as higher levels of p-AKT (Ser473), MAPK arrays and western blotting were able to show increased levels of GSK-3β (Ser9). This would suggest that in these cells, there is an environment where AKT activation is promoted and through that, GSK-3β activity is restrained. This situation could lead to increased WNT signalling through the abundance of free β-catenin. Ultimately, these data strongly suggest that HAGE presence in tumour cells could lead to the manipulation of PI3K signalling to promote cell survival and proliferation.

Cell cycle progression that could allow increased proliferation seemed to be confirmed by the recognition that the CDK inhibitor and downstream effector of AKT, $p21^{CIP1}$, was affected. HAGE shRNA transfected cells demonstrated higher

levels of this protein, indicating a reduction when the gene is expressed, a phenomenon reported for AKT activity in tumours (Zhou *et al*., 2001). Since this CDK inhibitor acts to stop cyclin E activity (Kandel and Hay, 1999), this data could have some relevance and connection to the gene data for CCNE2 described in Chapter 4. This would entail that HAGE expressing cells have a lower presence of a potent inhibitor of the cell cycle, furthering its link to proliferation. As seen with AKT and GSK-3β, though the visual changes were matched by the densitometry readings, they were not significant at the statistical level; however, the difference in protein level could lead one to argue the biological importance of this change. Again the large degree of error seen could be explained due to the issues of band intensity between western blots described for AKT and GSK-3β. These data would seem to be connected and it is very interesting that changes appeared to be associated with expression of HAGE. It means that possible future work could include further examination of other cell cycle inhibitors affected by AKT such as $p27^{KIP1}$.

Since there was possible deregulation of post translational modification to AKT and GSK-3β, this required further investigation. Increased GSK-3β phosphorylation could be explained by raised AKT function, but changes to the latter come about through actions of upstream mediators. Aside from autocrine growth factor signalling, increases to AKT activity can come from events such as mutation to the catalytic region of PI3K (p110α), loss of the TSG PTEN or modifications that occur to RAS (Calhoub and Baker, 2009). Due to the prolific nature of RAS in human cancer, the microarray data analysis was referred back to examine if any expression changes took place with RAS isoforms. There was initial evidence to suggest that HAGE expression could be associated with up-regulation of mRNA for the N-RAS oncogene isoform.

Microarray data showed a decrease of approximately 63% in N-RAS gene expression following transient knockdown of HAGE in FM-82 cells. Real-time qPCR of cells using specific N-RAS primers against cells undergoing the same treatment seemed to confirm this notion with N-RAS being shown to have around an 80% reduction in relative expression ($p=<0.01$). RAS genes are highly homologous, sharing approximately 85% of their amino acid sequence (Downward, 2003). In view of this

homology, expression change to H- and K-RAS was also checked. Microarray data analysis of FM-82 cells treated with siRNA hinted at no significant change in the other RAS members upon HAGE knockdown. This was confirmed further using real-time qPCR, which suggested that in terms of a temporary reduction in expression, HAGE was specifically linked to N-RAS.

Paradoxically, the above data did not seem to be confirmed with shRNA stable transfectant cells. Here, there was no change in N-RAS expression after HAGE was stably knocked down, whereas there was a significant decrease in both H- and K-RAS ($p=<0.05$). This indicated a possible difference between transient and stable RNAi techniques. It could be that short term exposure to siRNA caused a sharp decrease in N-RAS expression in response to the transfection. The apparent lack in change in expression following prolonged RNAi points to either possible cellular compensation or the potential that HAGE may not act on N-RAS mRNA specifically. The difference in expression changes that took place with the other RAS isoforms will require further investigation.

A decrease in N-RAS expression following HAGE knockdown would thus hint towards a situation in tumours where expression of HAGE could lead to increased levels of a known oncogene being produced. Knockdown of N-RAS in the HAGE-positive cell line FM-82 using a specific siRNA was capable of reducing the RAS member's relative expression by around 80%. However, the mRNA expression of HAGE was not reduced and thus one could argue that HAGE acts upstream of N-RAS. It was also noted that using the same technique with siRNA specific for H- and K-RAS resulted in no change in HAGE expression, meaning that reduction in any of the RAS members has no impact on the expression of HAGE mRNA.

Evidence gained using western blotting would suggest the notion that HAGE could act on N-RAS protein and not mRNA might occur. It was found that FM-82/shRNA had a marked reduction in N-RAS protein levels compared to the FM-82/Ctrl cells. As such, the HAGE expressing line therefore possessed a raised level of N-RAS protein. Once again, the difference in N-RAS protein level was not considered statistically important, but similarly to p-AKT, such a notable decrease recognised with the protein bands could lead to the argument of this reduction being biologically

significant. Thus, it may well be possible that HAGE expression does not result in an increase in N-RAS mRNA, but instead leads to accumulation of protein. This entails that HAGE could have a potential role in the translation of N-RAS. DEAD-box members have potential roles in the translation of proteins in cells (Linder, 2006) and therefore it is possible HAGE could act in this manner. No results to date have associated a CT antigen with this function, meaning this could represent a highly novel finding for this family of molecules. Extraction of active RAS (RAS-GTP) from shRNA stable transfectants revealed that there was no discernable difference between the test and control cell lines. The lack of change seen implied no interaction between HAGE and the GEF and GAP molecules that regulate RAS function such as SOS-1/2 and NF-1 (Downward, 2003). It must be noted that the technique used to extract RAS-GTP from cells did not discriminate between RAS isoforms since no kit is available to do this. Subsequently, all active forms of RAS were examined, so it is impossible to say for certain at this point in time if there is increased N-RAS protein activity in HAGE expressing cells.

RAS genes represent one of the most frequently mutated molecules in cancer. Approximately 30% of all human tumours bear a mutation to one the members of this group (Bos, 1988). Genetic alteration comes in the form of point mutation that occurs most commonly at codons 12, 13 or 61 (Bos, 1989). Modification causes insensitivity to GTP hydrolysis by GAP molecules, meaning that mutant RAS is constitutively active in affected cells. Mutations to RAS are considered to be amongst the primary genetic events in tumours, thanks to recognition of them in pre-malignant lesions (Kranenburg *et al*., 2004). K-RAS signifies the most commonly mutated isoform, with 85% of mutations occurring to the gene. This is followed by N-RAS with around 15% and lastly by H-RAS, making up less than 1% (Downward, 2003). N-RAS is the most common isoform mutant in melanoma, AML and myelodysplastic syndromes (MDS) (Downward, 2003). So there is an interesting overlap between the most commonly altered RAS member in melanoma, of which the FM-82 cell line is derived from and HAGE.

The significance of RAS mutation in tumours can be recognised from the sheer scale of its influence in cells. RAS proteins act as molecular switches, relaying signals initiated from receptors located at the surface of cells to induce the required

phenotype (Downward, 2003). They control a multitude of different pathways and mutation to them has been implicated in proliferation, survival, angiogenesis and metastasis (Shields *et al*., 2000; Campbell and Der, 2004; Kranenburg *et al*., 2004). Therefore, the above data would seem to suggest that HAGE expression in tumours could lead to increased production of a protein that is associated with a wide range of tumorigenic behaviour.

RAS mediates the actions of two major pathways. One is the MAPK network while the other is the PI3K cascade. This is intriguing because it could be possible to connect the findings that have been seen with N-RAS to those that were presented above with AKT and GSK-3β. It is especially interesting that subsequent steps in this pathway appear to be affected by HAGE expression. In the first instance this would apparently further the idea of HAGE involvement with proliferation and survival. Furthermore, it potentially links HAGE expression with other, more aggressive cancer hallmarks. Ultimately, strong evidence now exists to suggest a novel interaction that occurs between a CT antigen and DEAD-box protein with a notorious oncogene in N-RAS. Due to the number of malignancies involving N-RAS mutation, this work gives potential for the possibility of targeting HAGE. More work is needed to establish if HAGE is intrinsically linked to N-RAS or if there is involvement with H- and K-RAS. Moreover, it needs to be established if HAGE can induce similar effects in tumours without RAS mutation. At this point in time though, these data are promising.

Chapter 6

HAGE Expression and Aggressive Tumour Phenotype

6.1 Introduction

The rapid expansion of tumour mass is driven by malignant traits that include growth factor self-sufficiency, hyper-proliferation and de-regulation of apoptosis. However, the most aggressive tumours bear other phenotypic traits that further their progression even more, ultimately allowing them to grow, evade and survive the body's defences, and disseminate to other regions of the body (Hanahan and Weinberg, 2000). Should a tumour gain the capability to do this, then a patient's malignancy becomes more aggressive and significantly raises the threat it poses to them.

6.1.1 Apoptosis

Programmed cell death (Apoptosis) is a vital process in cellular homeostasis (Schultz and Harrington Jr., 2006; Elmore, 2007). It is used to efficiently eliminate older or damaged cells from the population in order to be replaced by new daughter cells generated by mitotic division. Apoptosis is one of the primary mechanisms affected within cancerous cells. Loss of apoptosis coupled with rapid proliferation is required for expansion of the tumour mass and the eventual progression towards angiogenesis and metastasis. It is also central to progression in terms of resistance to therapy and survival against anti-neoplastic agents.

Broadly, apoptosis can be brought about through two separate pathways. The extrinsic pathway is induced by the actions of certain ligands binding to death receptors on the surface of cells. These include members of the TNF receptor (TNFR) superfamily, which includes TNF-related apoptosis-inducing ligand (TRAIL) receptors and the aforementioned FAS (Ashkenazi and Dixit, 1998; Rath and Aggarwal, 1999; Schultz and Harrington Jr., 2003). In the case of FAS, once FAS-L is bound, the adaptor protein FAS-associated death domain (FADD) becomes bound. With other TNF receptors, once ligation takes place, TNF receptor-associated death

domain (TRADD) is bound, recruiting FADD and RIP (Hsu *et al*., 1995; Wajant, 2002; Elmore, 2007). FADD can subsequently bind to pro-caspase 8, leading to the formation of the death-inducing signalling complex (DISC), activating caspase 8 (Kischkel *et al*., 1995), which brings about the execution phase and cell death (Schultz and Harrington Jr. 2003; Elmore, 2007).

Comparatively, the intrinsic apoptosis pathway involves signals originating from a cell's internal environment. Effects that are derogatory to a cell, for example loss of growth factors, radiation, action of chemicals and viral infection, can result in activation of apoptosis. In this case, events such as these can act on the mitochondrial membrane, releasing pro-apoptotic mediators such as cytochrome *c* and Smac/DIABLO. The latter represses IAP, while secreted cytochrome *c* causes the activation of both pro-caspase 9 and APAF-1, creating a complex referred to as the apoptosome (Hill *et al*., 2004, Schultz and Harrington Jr., 2003, Elmore, 2007), which induces the execution phase. Intrinsic apoptosis is controlled by members of the BCL-2 family, including BID, BAD and BAX (Cory and Adams, 2002; Schultz and Harrington Jr., 2003; Elmore, 2007). This pathway can also be activated through the action of caspase 8 (Schultz and Harrington Jr., 2003), therefore showing overlap between the extrinsic and intrinsic cascades.

Malignant changes to apoptotic machinery can occur in numerous different ways. Molecules that serve to promote apoptosis can be limited or altered. This is perhaps most noticeable with loss of function of p53. Amongst the consequences of this includes the loss of transcription of BAX, for which p53 is involved in, limiting apoptosis (Miyashita and Reed, 1995). Oncogenic expression of AKT and aberrant expression of HSP can also lead to increased survival of cells and reduced apoptotic activity (Garrido *et al*., 2003). Caspase enzymes, the effectors of cell death signalling, can be affected, as seen with major members of this groups including caspases 3, 7, 8 and 9; affecting both the intrinsic and extrinsic pathways (Schultz and Harrinton Jr., 2003; Elmore, 2007). Therefore, a tumour cell can manipulate both promoting influences and mediators of cell death. Inhibitors of apoptosis (IAP) molecules, which include survivin, play a large role in preventing apoptosis and so in turn, could provide pivotal influence in tumour survival to promote malignant development (LaCasse *et al*., 1998; Sah *et al*., 2006). The combination of IAP and

other survival molecules may permit cells to avoid anoikis and contribute to the metastatic spread of a tumour.

Loss of apoptosis is core to problems experienced with lack of cancer drug effectiveness. Central to both radio- and chemotherapy is the principle of inducing widespread genomic damage, such that it is beyond repair, resulting in the death of the cell. Drug resistance in tumours is a well-known phenomenon and is associated with poor patient prognosis. Development of resistance hints towards expansion of apoptosis inhibition to convey protection against therapeutic agents. For example, PI3K/AKT expression has been linked to radio-/chemo-resistance in head and neck carcinoma and therefore poorer prognosis in these patients (Bussink *et al*., 2008). IAP have been implicated in protecting cultured cells against a wide range of different agents that trigger apoptosis, such as X-linked IAP (XIAP) in protecting MCF-7 cells against cisplatin (Duckett *et al*., 1998; LaCasse *et al*., 1998). Hence, loss of apoptosis is a process closely associated with tumour progression. It is especially important when designing drugs for late-stage patients, as it represents one of the biggest issues with therapy.

6.1.2 Immune Escape

6.1.2.1 Down-Regulation of the Immune System in a Healthy Environment

The collected components of the immune system are highly effective at preventing pathological harm coming to the body. However, it is important that this system can be controlled, as if immune reactions, especially those originating from the adaptive system, were not mediated, there would be a considerable risk of auto-immunity. Various mechanisms are in place to prevent the possibility of auto-immune reactions. Once T cells become activated by MHC-bound peptide, they become increasingly sensitive to apoptosis and up-regulate various death receptors such as TRAIL-R and FAS. In this manner, surface bound or secreted ligands to these receptors can bind to them and cause immune cell apoptosis via a process referred to activation induced cell death (AICD) (Shi *et al*., 1989; Hildeman *et al*., 2002; Lu and Finn, 2008). This brings about apoptosis through the extrinsic pathway described above and acts as a feedback system to prevent prolonged T cell activity. Closely related to this is the

action of cytotoxic T lymphocyte antigen-4 (CTLA-4), an antigen found on the T cell surface. Once T cells become activated, expression of this marker increases and has affinity for the co-stimulatory molecule B7.1 and when bound to it, prevents its signal being transmitted (Oosterwesgel *et al*., 1999). Since T cell activation is dependent on the second signal provided by antigens such as B7.1, the action of CTLA-4 acts as another form of negative feedback. Other mechanisms include the secretion of inhibitory cytokines such as TGF-β, IL-10 and IL-17, which act to suppress the action of CTL and create immune homeostasis (Murugaiyan and Saha, 2009; Saraiva and O'Garra, 2010). One of the most effective suppression methods involves T_{Reg}. Though these cells can be found naturally within the population of T cells, following T cell activation by antigenic peptide, their formation can be induced (Sakaguchi *et al*., 2009; Sakaguchi *et al*., 2010). These then repress CTL activity by using either FAS-L-FAS interaction or the perforin/granzyme pathway to bring about apoptosis in CTL and control their actions (Grossman *et al*., 2004; Strauss *et al*., 2009; Sakaguchi *et al*., 2010).

6.1.2.2 Manipulation of Immune Inhibition by Malignant Cells

Though homeostatic mechanisms are in place to curtail the immune system, tumours possess various methods to escape the attentions of the immune system. These involve either directly affecting key components involved in immunity or deregulating processes vital to it for the advantage of the tumour (Whiteside, 2003). Malignant cells can affect MHC processing to prevent the presentation of antigenic peptide to T cells and thus avoiding immune detection (Hicklin *et al*., 1999). This can be achieved by affecting MHC expression itself by down-regulation of molecules associated with it such as β2M or TAP (Zagzag *et al*., 2005; Chang *et al*., 2006; Ferris *et al*., 2006; Setiadi *et al*., 2007). Similarly, cancerous cells have been shown to reduce their expression of co-stimulatory molecules. As with CTLA-4 action, loss of secondary signalling results in T cell anergy and renders them unresponsive to transformed cells (Macián *et al*., 2004). Alternatively, tumours can either down-regulate their expression of FAS or up-regulate FAS-L expression, to avoid T cell mediated apoptosis or to cause apoptosis of T cells respectively (O'Connell *et al*., 1999; Peli *et al*., 1999). Since tumours have the ability to avoid detection and

clearance by the immune system, better understanding of this and finding molecules that are able to mediate this phenomenon is vital in helping restrict tumour progression.

6.1.3 Metastasis

By its very nature, metastasis heralds a major step in tumour progression (Ahmad and Hart, 1997; Meyer and Hart, 1998; Geiger and Peeper, 2009). If a malignant mass can be held in its original site, then efforts to treat it are relatively easier. Therefore dissemination of a tumour from its primary site to other regions poses the greatest threat to patient health and the biggest challenge to clinicians. Like most aspects of cancer biology, metastasis comes from a fine balance of communication and expression of key molecules. For example, the matrix metalloproteinase (MMP) enzymes are central to solid tumour metastasis for their ability to degrade basement membrane (BM) (Westermarck and Kähäri, 1999; Hofmann *et al*., 2005). Their expression can up-regulated by the action of certain oncogenes such as RAS, hence associating such tumorigenic molecules with malignant progression (Campbell and Der, 2004). MMP can be inhibited naturally by the action of the tissue inhibitor of metalloproteinase (TIMP) group of proteins (Woessner Jr., 1991). They do this by binding in an irreversible manner to MMP and inhibiting MMP-substrate binding (Jezierska and Motyl, 2009). There is a belief that even in the face of raised MMP function, TIMP proteins have the ability to stop them. This means that increase in MMP expression should coincide with reduced activity of the inhibitory molecules, thereby indicating a balance of molecular expression required to promote metastasis (Ahmad and Hart, 1997).

Both groups of molecules are complicit in angiogenesis, with MMP required for neo-vascular formation and TIMP being able to stop it (Ahmad and Hart, 1997). Since these two processes are closely linked together, it stands to reason that targeting angiogenesis aids in combating metastasis. In spite of this, no completely curative regimen exists for cancer as yet, and it is therefore important to design new therapies against both of these phenomena in an attempt to limit tumour progression. The identification and characterisation of more molecules that can influence either pro- or anti- angiogenic/metastatic molecules before targeting them is a pre-requisite.

So far HAGE has been linked with increased proliferation in tumours. However, emerging data in Chapters 4 and 5 suggest further roles for this antigen. Preliminary microarray analysis highlighted genes involved in processes aside from tumour growth HAGE could be involved in. Meanwhile, retrospective examination of this data was able to find a possible relationship with N-RAS, an oncogenic molecule also linked to other aspects of malignant biology including metastasis. For that reason, preliminary work was performed to investigate the potential contribution HAGE makes in various activities connected to malignant progression.

6.2 Results

6.2.1 HAGE and Protection against Drug-Induced Apoptosis

Microarray analysis showed that HAGE expression led to down-regulation of genes linked with apoptosis, insinuating that other than proliferation, antigen expression could confer protection against induced cell death. In order to examine this, HAGE shRNA stable transfectants were exposed to two different drugs capable of initiating apoptosis; staurosporine and cisplatin. Initially, both sets of cells were exposed to different concentrations of both drugs. Light microscopy of these cells over 48 hours observing morphological changes was used to determine the concentration to be used in future work, specifically whereby 50% of the observed cells were dead.

FM-82/Ctrl and FM-82/shRNA were subsequently grown overnight in a 96-well plate. The following day, they were treated with either 0.3μg staurosporine or cisplatin for four hours prior to detection. Cells were run alongside a blank, consisting of media alone laced with the drug, or a negative control, where cells were not treated. As shown in Figure 6.1(B), when treated with cisplatin, the FM-82/shRNA cell line demonstrated a higher level of caspase 3/7. However, when staurosporine was added (Figure 6.1 (A)), the knockdown line appeared to have a lower amount of caspase compared to the control line. The suggestion from this was that HAGE expressing cells were able to have a lower amount of active caspase 3/7 upon exposure to cisplatin, but expression was not able to lead to the same effect against staurosporine.

Since it seemed that HAGE expression was leading to greater protection towards the actions of cisplatin, this was verified further using preliminary FACS analysis. Cells were again treated with the drug for four hours, stained using Annexin/7-AAD, and measured. After Kaluza® analysis of FACS data, it was found there that was a greater number of FM-82/shRNA cells undergoing apoptosis than FM-82/Ctrl. Figure 6.2 shows images of Kaluza® data with the 'C+-' quadrant used to detect cells undergoing early apoptosis. 0.92% FM-82/Ctrl compared to 3.82% of FM-82/shRNA

cells experienced this, suggesting that loss of HAGE expression led to reduced resistance against cisplatin.

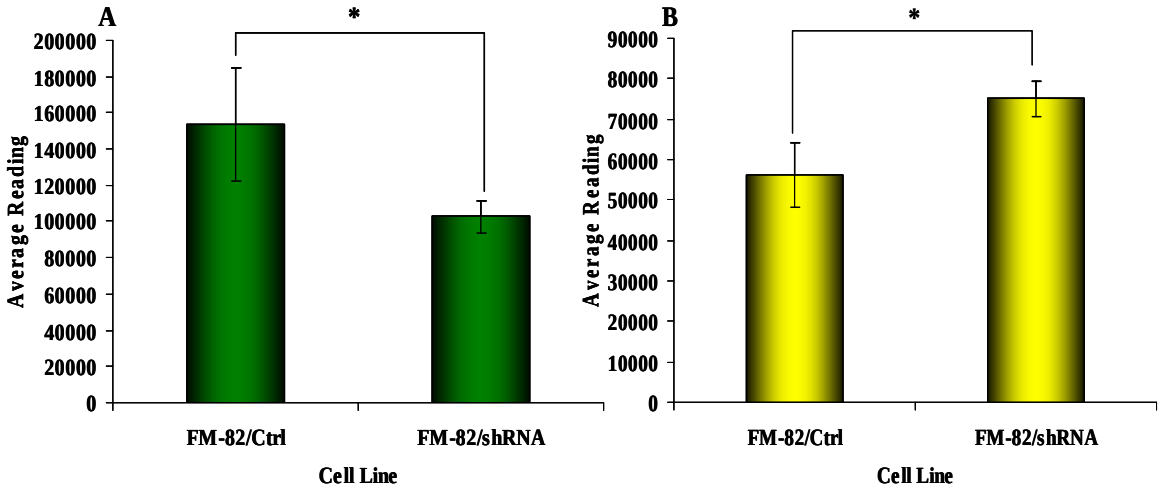


Figure 6.1: *Average readings to determine levels of caspase 3/7. Either staurosporine (Green bars (A)) or cisplatin (Yellow bars (B)) were used to treat stable knockdown cells. Cells were treated for four hours prior to addiction of detection reagent and scanning using a luminometer. Readings were normalised using values gained for blank control wells.*

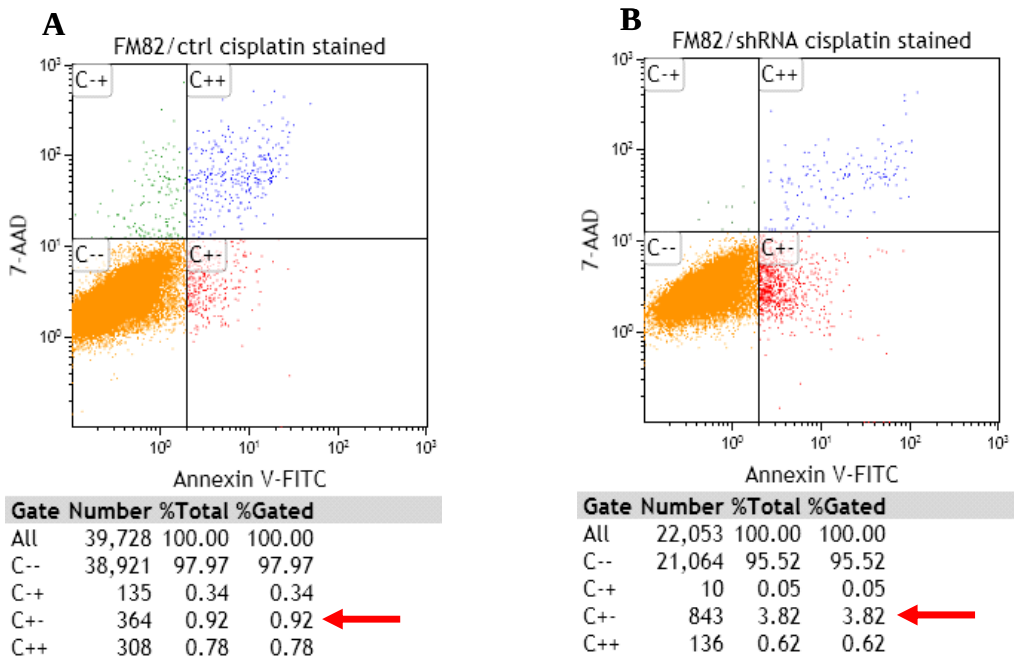


Figure 6.2: *Images taken from Kaluza® software analysis of FACS examination of drug induced apoptosis in stable knockdown cells (n=1). Either (A) FM-82/Ctrl or (B) FM-82/shRNA were treated for four hours with cisplatin prior to staining with Annexin/7-AAD and FACS analysis. The C+- quadrant (Red arrow) contained cells undergoing early apoptosis and used to measure the effects of cisplatin.*

6.2.2 HAGE and Tumour Immune Escape

In Chapter 4, it was noted that there was a reduction in FAS-L expression following HAGE siRNA treatment. The implication of this is expression of HAGE could lead to a raised level of FAS-L on tumour cells. Expression of this latter antigen on the surface of tumour cells could potentially cause CTL death via FAS-L-FAS interaction to avoid immune reactivity.

To examine this, an antibody specific against FAS-L (CD178) was obtained and used for FACS screening of cell lines. Data gained from FACS was analysed using Kaluza® software to determine expression of the antigen, detected using specific antibody compared to cells stained using an isotype control and cells alone. This is shown in Table 6.1.

Table 6.1 shows a significantly greater level of FAS-L surface expression on cells that had significantly higher HAGE expression (p=<0.05). With cDNA transfectants, it could be seen that stable expression of HAGE in FM-3 led to over a 4% increase in FAS-L. A similar situation was noted with stable knockdown. On this occasion, there was over 6% decrease once FM-82 cells had been treated with HAGE-specific shRNA. Hence, HAGE expression would appear to lead to increased surface FAS-L and correlated with the increased mRNA expression seen in Chapter 4.

Table 6.1: *Expression of FAS-ligand (FAS-L) on the surface of both sets of stable transfectant cell lines determined by FACS analysis involving a FAS-L specific antibody (n=2). Values were gained following interpretation of FACS data using Kaluza® software. (*=p<0.05)*

Cell Line	FAS-L Expression
FM3/-Ve	10.14% (+/- 0.61)
FM3/HAGE	14.68% (+/- 1.20)*
FM82-ctrl	16.42% (+/- 2.1)
FM82-shRNA	10..37% (+/- 0.85) *

Secondly, cells were co-cultured with CTL clones to see if HAGE-expressing cells were able to cause a greater level of CD8+ cell death compared to HAGE-negative cells. This latter experiment was done in collaboration with Prof. Francine Jotereau and Dr. Nathalie Labarrière (Université de Nantes, France). For this experiment, only HAGE cDNA stably transfected cells were tested, as creation of shRNA stable transfection had not been performed. The CTL clones used for co-culture were specific for the Melan-A/MART-1 melanoma antigen. Real-time qPCR confirmed high expression of this antigen in the cell lines (Data not shown) prior to the cells being sent to collaborators. Cells were cultured for 48 hours alongside either the 10C10 CTL clone (Apoptosis resistant; negative control) or 24B7 clone (Apoptosis sensitive; test clone). FACS analysis was performed with Annexin/PI staining to examine cell death in CD8+ cells and TNF-α detection to measure the extent of apoptotic cytokine released by CD8+ T cells. Data gained from these experiments is summarised in Tables 6.2 and 6.3.

Table 6.2: *Extent of apoptosis found in CD8+ cells using FACS analysis following co-culture of HAGE cDNA stable transfectants with apoptosis resistant (10C10) and susceptible (24B7) CTL clones (n=1). Cell death was determined using Annexin/PI staining and analysis was performed on gated CD8+ cells.*

CD8+ T cell clone	24h co-culture		48h co-culture	
	FM3/-Ve	FM3/HAGE	FM3/-Ve	FM3/HAGE
10C10	14.45%	14.06%	3.32%	3.40%
24B7	7.37%	8.07%	14.68%	18.19%

Table 6.3: *Degree of TNF-α release from CD8+ cells after co-culture of HAGE cDNA stable transfectants with apoptosis resistant and susceptible CTL clones (n=1). TNF-α was measured using FACS analysis and gating of CD8+ gated cells.*

CD8+ T cell clone	FM3/-Ve	FM3/HAGE
10C10	59.61%	59.90%
24B7	63.41%	68.57%

There was little difference after 24 hours of co-culture between FM-3/-Ve and FM-3/HAGE in the amount of CD8+ T cell death detected using Annexin/PI staining. After 48 hours however, there was nearly a 4% increase in the amount of Melan-A/MART-1 specific 24B7 CTL grown with FM-3/HAGE undergoing apoptosis as opposed to the same clone grown with FM-3/-Ve (Table 6.2). Screening for TNF-α release from the above clones seemed to further such a notion, with a five-point increase of TNF-α producing 24B7 CTL clone cultured with FM-3/HAGE compared to the same clone grown with FM-3/-Ve. In both experiments, there was little difference in readings for the 10C10 CTL clone, which is apoptosis resistant (Table 6.2 and 6.3). Increased amounts of apoptosis taking place in CTL were therefore obtained when confronted with HAGE expressing cells, possibly linked to the expression of FAS-L. Significance of these results could not be determined for co-culture experiment as they were only performed once and require repetition

6.2.3 HAGE and Tumour Invasion

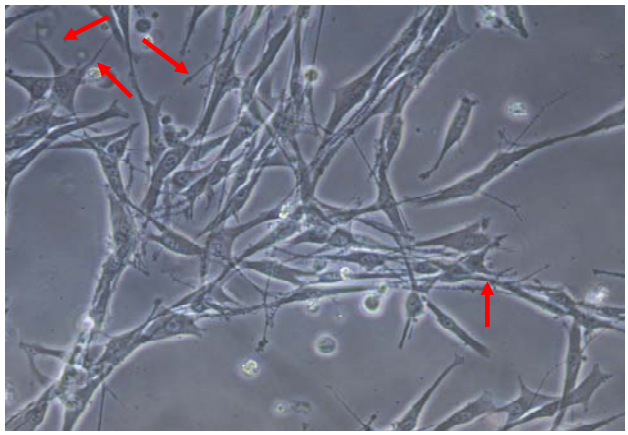
Data gained here have begun to indicate a possible relationship between HAGE expression and aggressive tumour phenotype. Retrospective analysis of microarray data indicated the down-regulation of the pro-invasion genes, FAK and MMP-9, when HAGE was knocked down, implying the antigen up-regulates them when expressed. Conversely, expression of the MMP inhibitor TIMP-4 was up-regulated following a reduction in HAGE expression, suggesting inhibition of this molecule by HAGE. During the creation of the stable knockdown cells, it was noted that once HAGE specific shRNA was stably transfected, there seemed to be a reduction in the number of dendrites normally shown by FM-82 cells (Figure 6.3). Loss of dendrites upon HAGE knockdown gave rise to a possible notion linking HAGE to cell migration and from that metastasis. To see if cell migration was affected by expression of the antigen, an *in vitro* invasion assay was carried out using HAGE stable knockdown cells. Migration of cells was monitored in Matrigel containing test plates and compared to passage of cells through control membrane plates. Readings from both allowed the calculation of percentage invasion (Test plate reading/control plate reading x 100) and allowed the comparison of migration between FM-82/Ctrl

and FM-82/shRNA. The plot showing the percentage invasion for each cell line is shown in Figure 6.4.

It was found that a greater number of FM-82/Ctrl cells had passed through the Matrigel at each points during the experiment compared to cells where HAGE expression had been reduced. At certain time points, the difference in invasion between the two cell lines was statistically significant ($p=<0.05$; $p=<0.01$). This implied that expression of the antigen was leading to cells being more capable of migration across a barrier.

As previously described, there was a change in the gene expression of certain molecules involved in metastasis. To investigate if these changes were also evident at the protein level, antibodies specific to FAK, MMP-9 and TIMP-4 were obtained and used in western blotting. Images from blots and respective densitometry are shown in Figure 6.5.

There was an evident change in the levels of both FAK and TIMP-4. Levels of FAK markedly decreased after HAGE knockdown while TIMP-4 showed the opposite effect. These findings were reflected in the densitometry ratios gained for both these proteins. From this, it could be seen that HAGE expression could lead to an up/down-regulation of a proteins known in promoting and inhibiting metastasis in FAK and TIMP-4 respectively. With MMP-9, there seemed to be more protein visually present in FM-82/shRNA cells. Ratios calculated from densitometry indicated that there was an increase of the protein in cells where HAGE expression had been reduced.



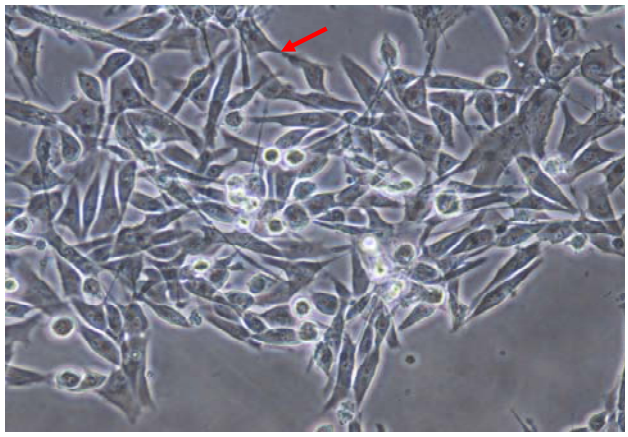


Figure 6.3: *Light microscopy images (Magnification X20) of HAGE stable knockdown cell lines used to recognise the reduction in evident dendrites (Exemplified by red arrows), following stable transfection of HAGE shRNA.*

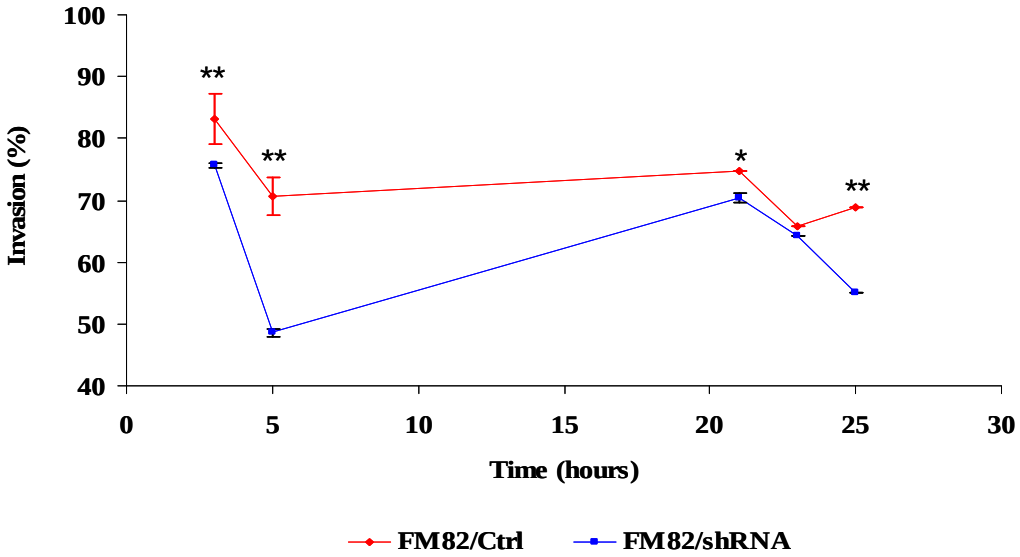


Figure 6.4: *In vitro invasion assay readings taken for HAGE stable knockdown cells (n=1). Readings were taken over a 25 hour period after cells had been stained with $DilC_{12}(3)$ to allow luminometer measurements to be collected in real-time (*=p=<0.05; **=p=<0.01).*

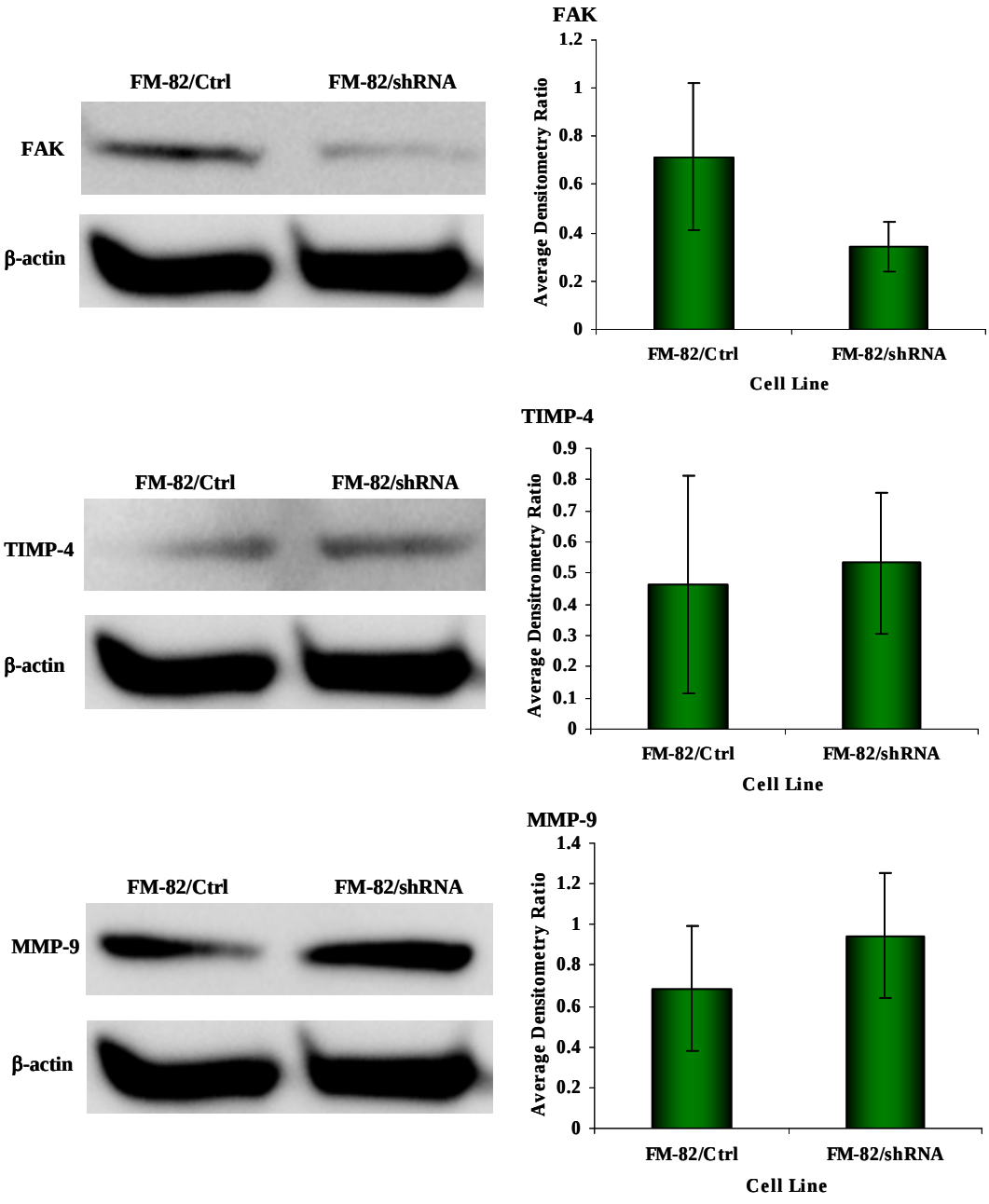


Figure 6.5: *Images of bands for proteins with known involvement in metastasis detected by western blotting (n=2). Proteins were separated using 10% acrylamide SDS gels except TIMP-4, which was performed using a 15% gel. Membranes were stained overnight specific with anti-human antibodies diluted in 10% Marvel milk. Antibodies were optimised to a 1/250 concentration apart from β-actin, optimised to 1/500. Densitometry ratios calculated as for $p21^{CIP1}$ and N-RAS.*

6.3 Discussion

Gene expression analysis suggested the possibility of HAGE being involved in other malignant processes. If evidence could be found to support this then it could mean that the expression of the antigen in tumours results in a more aggressive phenotype. Initial analysis had suggested HAGE involvement in apoptosis and immune escape while further study implicated a possible role in invasion.

Suppression of apoptosis is a malignant phenomenon not only in terms of proliferation but also in respect to cell survival, which adds to the complication of drug resistance. Preliminary work involved treatment of stable knockdown cells with either staurosporine or cisplatin, two drugs known to cause apoptosis in cells. These cells were used as part of a basic screen to specifically detect relative levels of the effector caspases 3 and 7. There was a significant increase in the amount of caspase 3/7 detected in FM-82/shRNA treated with cisplatin ($p=<0.05$), implying HAGE expression could result in a reduction in the levels of these two caspase enzymes. Interestingly, when the same cell lines were treated with staurosporine, the level of caspase 3/7 showed the opposite effect ($p=<0.05$). This could mean that HAGE expression offered more protection against cisplatin than staurosporine.

Further confirmation of this was achieved using FACS analysis, where results showed an increase in the number of FM-82/shRNA cells entering apoptosis compared to the control cell line. Taken together these data seemed to show that a reduction in HAGE expression was making cells more susceptible to drug-induced apoptosis. In other words, the presence of this molecule in tumours could confer protection against certain drugs.

Resistance to platinum compounds such as cisplatin and carboplatin can be caused by a variety of different mechanisms in tumour cells (Stewart, 2007). These can include alterations to drug uptake and drug efflux mechanisms in malignant cells (Siddik, 2003; Stewart, 2007). IAP could also play an important role in resistance. For example, up-regulation of survivin has been linked with the ability to withstand effects of cisplatin (Nomura *et al*., 2005; Wang *et al*., 2005). Furthermore, the up-regulation of XIAP can have the same effect (Nomura *et al*., 2005). Interestingly,

evidence has shown a potential interaction between XIAP and the PI3K pathway to mediate resistance (Cheng *et al*., 2002). XIAP appears to increase resistance to cisplatin and inhibit the action of caspase 3 and therefore apoptosis in different cell lines (Amantana *et al*., 2004; Yang *et al*., 2005). It may be that in HAGE expressing cells, such a situation is taking place. Evidence gained from this study has shown the possible effects HAGE expression has on this signal network, again providing a link to reduced caspase 3 in cells treated with cisplatin. PI3K also acts to inhibit pro-caspase 9 (Vara *et al*., 2004), stopping its cleavage to an active form and from that initiating effector caspases. Future work would involve examining the levels of this caspase in HAGE positive cells to see if there is more data to connect HAGE, PI3K and apoptosis repression.

Other evidence collected in this study could be associated with this. Chapter 4 details the apparent increase in HSP-27 and 70 mRNA in HAGE positive cells, while Chapter 5 details RAS expression. All of these chaperones have a role in suppressing apoptosis by either acting on caspase 3 (HSP) or inhibiting caspases through unknown mechanisms (RAS). If work could match protein expression with the observed mRNA results, it may further the link between HAGE and the results seen with response to cisplatin and apoptosis.

Caspases 3 and 7 are important in apoptosis as they, along with caspase 6, make up the effector members that bring about cell death (Cohen, 1997; Elmore, 2007). All three of these share some of the same targets such as PARP and NuMA (Slee *et al*., 2001; Elmore, 2007). Caspase 3 and 7 bear protein homology to each other and can recognise the same DXXD sequence motif of target proteins. However, caspase 3 is considered to be the most important of the effectors, as it can be activated by any of the initiating caspases (Caspases 8, 9 and 10). Additionally, it exclusively acts on ICAD to release CAD, leading to cell death (Sakahira *et al*., 1998; Elmore, 2007). Therefore, repression of caspase 3 in HAGE expressing cells, possibly mediated by the PI3K network could explain cisplatin resistance.

Aside from protecting itself from apoptosis, data collected here would seem to indicate that HAGE expressing cells could induce the reaction in immune cells. Gene expression analysis detailed in Chapter 4 found increased FAS-L mRNA in HAGE

expressing cells. Expression of FAS-L has the potential to bind to FAS/CD95 expressed by activated T cells and survive anti-tumour immunity. Using FACS examination of stable knockdown cell lines, it was possible to show HAGE expressing cells had significantly more of the FAS-L protein on their surface ($p=<0.05$). Increased protein expression in this instance would seem to have confirmed the genetic data gained previously. In turn, it is possible that this could confer protection against CTL. Data collected from collaborative co-culture experiments appeared to begin corroborating this theory. With this experiment, annexin staining and TNF-α detection found a greater level of apoptosis in CD8+ positive cells grown alongside HAGE positive compared to control cells. Confirmation of this would be needed; however there may be an association between FAS-L expression of HAGE-positive cells and increased CD8+ cell death.

FAS-L (CD178/APO1-L) is a death inducing ligand, which upon binding to the antigen FAS (CD95), initiates apoptosis in the cell that bears FAS (Nagata and Golstein, 1995; Minas *et al*., 2007). FAS-L-FAS interaction results in activation of the extrinsic apoptotic signalling pathway, initiating caspase 8 to induce caspase 3 (Schultz and Harrington Jr., 2006; Elmore, 2007). There is a belief that in certain cases, tumours are able to express FAS-L on their surface to offer protection against possible action by CTL: the so-called counter-attack mechanism (O'Connell *et al*., 1999). Anti-CTL killing is a process close to transplant immunity and the provision of immune tolerance (Bellgrau *et al*., 1995; Griffith *et al*., 1996). This suggests tumours can avoid immune reactions by helping to generate a tolerogenic environment. Expression has been detected in gastric, colorectal and ovarian carcinomas (O'Connell *et al*., 1996; Bennett *et al*., 1999; Houston *et al*., 2003; Minas *et al*., 2007). It is also found in haematological tumours, as FAS-L killing has been found in mycosis fungoides (Ni *et al*., 2001). Furthermore, this phenomenon has been shown *in vivo* (Bennett *et al*., 1998). Indeed, since FAS induced killing of CTL is linked to progression (Kim *et al*., 2004), there could be a link with prognosis of patients (Okada *et al*., 2000). In this instance, data collected for HAGE could agree with the above and are potentially promising in terms of a factor that could increase this situation.

As with a lot of malignant biology, there is controversy surrounding FAS counterattack (Maher *et al*., 2002). It has been argued FAS counterattack is not responsible for the generation of immune tolerance (Favre-Felix *et al*., 2000; Maher *et al*., 2002). In part this is due to possible issues surrounding the techniques used to detect tumour FAS-L expression (Maher *et al*., 2002). There seems to be a large degree of contradiction involved with FAS-L expression. A good example of this can be seen with Chappell *et al*. (1999), who found that no human melanoma tested expressed FAS-L mRNA. This is thought to be contrary to other work performed by Hahne and co-workers (Hahne *et al*., 1996; Maher *et al*., 2002). Evidence presented here contradicts the work of Chappell *et al*. Furthermore, it seems interesting that HAGE expression led to increased FAS-L expression, despite increased AKT activity, a molecule known to suppress FAS-L transcription by inhibiting FOXO (Calhoub and Baker, 2009). Therefore, HAGE expression could lead to the increased expression of FAS-L through another mechanism or pathway as yet unrecognised. Further work would be needed to investigate this phenomenon.

There is emerging data to support HAGE expression being connected to tumour invasion. Preliminary data from an *in vitro* invasion assay indicated reduction of HAGE expression in tumour cells seemingly impairing their migration through Matrigel. Although this came from an initial experiment and would require repeating, early impressions of phenotype would look as if HAGE positive cells were more competent at invasion. Western blotting of protein extracted from the same cell lines seemed to support the above. Expression of the pro-metastatic protein FAK appeared to be increased, both in terms of visual recognition and ratios calculated from densitometry. On the other hand, there was a decrease in the expression of the inhibitory protein TIMP-4. Initially, it seemed some molecular basis to initially associate HAGE expression and increased invasive ability of malignant cells, though some of the data could be considered paradoxical. Densitometry analysis of these bands seemed to agree with the visualisation of the proteins. Much like previous densitometry, the calculated changes were not considered statistically significant. However, some of the changes could be considered biologically significant, especially if one refers to the change seen in FAK protein level noted by western blotting. Again the extent of error seen with this analysis could be explained due to the variability in band intensity between western blots, as mentioned in Chapter 5.

FAK is a well described molecule in tumour biology with its expression is heavily linked to malignant cell invasion and other tumorigenic behaviour (Luo and Guan, 2010). It is a highly important factor in the formation of invasion in cells (Hsia *et al*., 2003) and increased expression could be connected with cells gaining independent invasive phenotype (Luo and Guan, 2010). Indeed, loss of FAK expression can lead to reduction in invasiveness of tumour cells (Hauck *et al*., 2002). There is also evidence that FAK has a controlling role in the epithelial-mesenchymal transition (EMT), an important stage in tumour invasion (Avizienyte and Frame, 2005; Luo and Guan, 2010). The evident increase of FAK noted in cells with greater HAGE expression could therefore be associated with raised metastatic potential and increased *in vitro* invasive ability.

The reduction in TIMP-4 protein expression seen in western blotting could further this. TIMP proteins are known inhibitor of the MMP series of proteases prevalent in tumour metastasis, acting to prevent MMP interacting with substrates (Jezierska and Motyl, 2009). Whereas other members of the TIMP family and their links to metastasis inhibition are better known, there is relatively less evidence surrounding TIMP-4 (Melendez-Zajgla *et al*., 2008). In spite of this, studies have shown the expression of TIMP-4 is decreased in progressive tumours (Riddick *et al*., 2005) and the presence of the molecule can curb malignant growth and invasion by interacting with molecules such as MMP-9 (Wang *et al*., 1997; Groft *et al*., 2001; Zhao *et al*., 2004; Riddick *et al*., 2005). Interestingly, other work has found that expression of TIMP-4 can actually result in development of tumourigenic behaviour (Jiang *et al*., 2001; Tunuguntla *et al*., 2003). Commentary of all these studies point out possible difference in TIMP-4 induction causing changes in cellular function (Riddick *et al*., 2005). Moreover, opposing roles of the same molecule may come down to a balance existing between that molecule, its promoters and effectors and the produced phenotype.

This latter point regarding balance could be used to explain findings for MMP-9. Here, seemingly higher protein expression was found in FM-82/shRNA cells, implying increased production following HAGE expression knockdown. Such a result would appear strange through the apparent increased invasion capability of

HAGE positive cells, but having less MMP-9 compared to the knockdown cells. MMP-9 (Gelatinase B) belongs to a large group of metalloproteinases involved in metastasis (Ahmad and Hart, 1997; Hoffman *et al*., 2005). Along with MMP-2 (Gelatinase A), it has been found to be highly expressed in tumours (Johnsen *et al*., 1998; Westermarck and Kähäri, 1999). So, findings presented here would appear to be contradictory to previous work, especially if one considers that both RAS and FAK, were found to be up-regulated in this group of work, are capable of initiating MMP-9 (Hsia *et al*., 2003; Campbell and Der, 2004).

However, various concepts still need to be taken into account. The activation status of MMP-9 was not evaluated in this study. Therefore, while HAGE expressing cells may have had relatively less protein compared to cells with less antigen expression, it has yet to be seen if HAGE bearing cells have more active MMP-9 within them. It may also be that increased protein seen in FM-82/shRNA is due to abundance of inactive MMP-9. As yet it is impossible to tell and future work investigating HAGE and metastasis would need to examine this along with the status of other MMP and TIMP members. As has been seen in Chapter 5 with AKT and GSK-3β, activation could be considered very important. Foremost though, balance of promoters and inhibitors is perhaps the most important factor here. Though cells with higher HAGE expression have lower MMP-9 levels, this may not detract from metastatic potential thanks to the decreased level of TIMP-4 seen. If there is a decrease in MMP inhibitors, then it may be that changes to MMP may have little impact in stopping metastasis. Coupled with the increased level of N-RAS, this molecular pattern could be sufficient to drive tumour cell invasion. Ultimately, work presented here has begun to indicate that HAGE expression might result in a more aggressive phenotype and further work need to be performed in order to establish if this is the case.

Chapter 7

Discussion

7.1 Discussion

The prevalence of cancer as a public health concern necessitates continued work to refine and discover therapeutic options. Such research has led to the detection of a large number of molecules that can be used to target a wide range of tumours. Therapy is now at a stage where agents can be designed to selectively act against specific targets and is highly promising. In part, this has come about through better understanding of target molecules and the signalling networks in which they are involved. Despite this knowledge, efforts to therapeutically inhibit significant pathways, for example proliferation or angiogenesis, have had limited impact. Therefore, it is possible these signal pathways could be influenced by other means, either due to as yet unknown means or known molecules having abilities or functions not yet described.

Overall, the role of cancer/testis (CT) antigens remains elusive. Certain members have a known function but this is restricted to normal cell homeostasis, while their role in malignant biology is poorly understood. If CT antigens are to be considered realistic targets for tumour therapy, then their position in the cancer environment should be made clear. For the most part, previous work performed on HAGE has matched its CT antigen counterparts, focusing on gene expression in different tumours (Martelange *et al*., 2000; Adams *et al*., 2002; Condomines *et al*., 2007; Roman-Gomez *et al*., 2007). Work performed in this laboratory was the first to demonstrate the protein form of the molecule and investigate its possible use for immunotherapy (Mathieu *et al*., 2007; Riley *et al*., 2009; Mathieu *et al*., 2010).

Preliminary studies by this group also began to focus on the potential cell processes HAGE could be involved in. Here, transient cDNA transfection into a HAGE-negative cell line resulted in the significant increase of cell proliferation (Unpublished data). Classification of this antigen as a DEAD-box RNA helicase implies its

possible role in proliferation as seen with other members (Nakagawa *et al*., 1999; Janknecht, 2010; Por *et al*., 2010). So in the first instance, the preliminary work seemed to match the possible action of the antigen. The data provided by this study attempted to define what processes HAGE is involved in following cell transfection and whether its role as a potential target for therapy could be confirmed.

Initial evidence appeared to corroborate the hypothesis that HAGE could be involved in tumour proliferation, shown in Chapter 3. Reduction of antigen genetic expression, either in a transient or longer lasting manner led to a significant decrease in the ability of human melanoma cell lines to increase their number. This phenomenon was not restricted to the *in vitro* environment as *in vivo* studies using NOD/SCID mice complemented the findings. Additionally, murine investigations using a cell line stably transfected with HAGE cDNA acted as a reciprocal set of data.

Together, these data supplied a strong argument to suggest that HAGE had some involvement in promoting tumour proliferation. Other published evidence has shown the involvement of the CT antigen CAGE in the same process (Por *et al*., 2010), this is interesting as this molecule is also a member of the DEAD-box family (DDX53) and shares 55% homology with HAGE (Linder, 2006). Results for HAGE therefore seem to complement these findings. Additionally, *in vivo* data gained here was similar to those found with its counterpart.

Cancerous cells are able to proliferate due to the modification of many molecules and subversion of numerous signalling pathways. This means they would be more than able to proliferate without expressing HAGE. Results gained with the FM-3/-Ve cell line *in vivo* exemplify such a notion. By their very nature, tumours undergo genetic diversity and are highly heterogeneous (Yegnasubramanian *et al*., 2008), meaning not all cells within a mass would express HAGE and there would be variations between those that did. Immune staining was able to highlight this. However, results presented here could suggest expression of HAGE leads to tumour cells having an increased capacity to proliferate. Therefore as such, expression of this antigen could serve to greatly exaggerate this aspect of malignant cell biology.

Heightened proliferation in HAGE-expressing cells appears to be brought about through changes to a variety of different molecules. Chapter 4 details how gene expression profiling using the Affymetrix Genechip® platform made it possible to examine the genome of a human melanoma cell line to investigate the impact of HAGE. Genes such as CCNE2, E2F2 and WNT-1, involved in cell cycle progression and proliferation, seem to be up-regulated by HAGE and could help explain heightened proliferation shown by HAGE-expressing cells. Genetic examination alone is not sufficient to draw conclusions and so protein expression analysis was performed to further examine HAGE molecular interactions leading to increased proliferation. This involved looking at molecules with strong influence over signalling pathways that could provide this phenotype, shown in Chapter 5. The results showed here strongly support the hypothesis linking HAGE expression with modification to cellular signalling.

In the first instance, this came from the observed changes to AKT and GSK-3β phosphorylation. Proteome Profiler arrays and western blotting were able to indicate increased amounts of both p-AKT (Ser473) and p-GSK-3β (Ser9) in cells with higher HAGE expression. Though as previously described in Chapter 5, GSK-3β has a prominent role in glucose metabolism and has been implicated in neurodegenerative disorders; it could also play a highly important one in cancer. GSK-3β acts to restrict the activity of β-catenin, phosphorylating it and maintaining it in a complex along with APC, and axin, promoting catenin degradation (Rayasam *et al*., 2009). β-catenin represents one of the major components of WNT signalling and since WNT/β-catenin signalling has been shown to be altered in different tumours (Luo *et al*., 2007; Rayasam *et al*., 2009), this means as such, GSK-3β could inhibit tumour growth.

As also mentioned previously, AKT has been shown to inactivate GSK-3β through phosphorylation, which in turn permits β-catenin signalling (Nicholson and Anderson, 2002) and WNT can also act to inactivate GSK-3β to cause the same effect (Rayasam *et al*., 2009). Microarray data implied an increase in the mRNA expression of WNT-1 in cells expressing HAGE, although no protein investigations were performed to confirm this was the case. However, demonstrating an increase in

the protein levels for WNT would suggest an interesting connection between HAGE and the expression of these molecules. This is made even more evident through the knowledge that this signalling pathway is found in embryogenesis (Logan and Nusse, 2004) and it has been suggested that CT antigens could be involved in this process (Lucas *et al*., 1998). Cancer/testis antigen expression in healthy tissue would appear to agree with this idea. Indeed, expression of the CT antigens, MAGE-A3 and NY-ESO-1, has been correlated to proliferation taking place at this stage of development (Gjerstorff *et al*., 2007). The notion that HAGE expression could up-regulate signalling involved in this way could agree with its potential function. Other than its involvement with β-catenin, GSK-3β has been implicated in the separation of chromosomes (Tighe *et al*., 2007), meaning that loss of function could lead to genetic abnormality.

The effect of β-catenin activity requires the interaction with the transcription factor lymphoid enhancer factor (LEF), whose substrates include cyclin D and c-MYC, both of which are involved in cell cycle progression (Nicholson and Anderson, 2002; Jope and Johnson, 2004; Patel *et al*., 2004; Rayasam *et al*., 2009). So from this data, there is the possibility that HAGE expression causes an accumulation of β-catenin as a result of increased AKT activity and reduced action of GSK-3β. Increased cell cycle progression brought on by this series of interactions may explain the increased proliferation seen in HAGE-expressing cells. Data shown in Chapter 3 indicated there was greater cell cycle progression in HAGE-expressing cells, again associating the antigen with this phenomenon. Results gained here echoed those found with CAGE, where it was shown that CT expression led to progression in the cycle involving cyclins D1, E and the E2F transcription factors (Por *et al*., 2010). In this study, microarray data indicated both CCNE2 and E2F mRNA could be increased by HAGE expression. Though this would require confirmation at the protein level, there is interesting similarity between the works involving these antigens.

In contrast to the work of Por and colleagues (2010), it was possible to show an increase in the level of the cyclin inhibitor $p21^{CIP1}$ in HAGE knockdown cells. This means that within HAGE expressing cells, there is a reduction in the level of $p21^{CIP1}$. Por's study showed little change in this protein's expression in cells over-expressing

the CAGE antigen (Por *et al*., 2010). Since $p21^{CIP1}$ acts to repress cell cycle progression, its down-regulation at the protein level would seem to further the impression of HAGE-expressing cells being able to proliferate faster. Furthermore, this protein is a target substrate for AKT (Vara *et al*., 2004) providing even more evidence to support this hypothesis.

Though identification of key effector molecules in signalling is important, two problems persist. While molecules at the highest points of cascades are notoriously affected in malignancy, it has been difficult to find one that could be easily targeted for therapy. To compound this further, downstream pathway events become more diverse, making target selection far more complex. The advantage of employing genome expression profiling analysis using techniques such as oligonucleotide microarray and NGS is the ability to refer back to the data to expand a specific area of work. This method was used to identify the change in expression to the N-RAS oncogene.

Initial evidence could be found in PCR experiments used to confirm expression profiling results, shown in Chapter 5. Here it was shown once HAGE expression was reduced, N-RAS expression did also. This was not matched at the mRNA level using shRNA-treated cells; however there was a marked reduction in N-RAS protein when HAGE expression had been knocked down. Reciprocal silencing of N-RAS using specific siRNA did not lead to reduction in HAGE mRNA expression, implying HAGE existing upstream of the RAS molecule. Taken collectively, there is now a strong argument to promote the possibility of HAGE acting to promote N-RAS protein expression in tumour cells. If this is the case, then it would explain the change in expression seen in molecules described earlier, such as AKT, GSK-3β and $p21^{CIP1}$. This is due to the knowledge that RAS can influence PI3K to promote both survival and proliferation (Pacold *et al*., 2000; Sheilds *et al*., 2000).

DEAD-box involvement with RAS has been shown in the past, with work describing the splicing of H-RAS by p68/DDX5 (Guil *et al*., 2003; Camats *et al*., 2008). So far, it is impossible to determine the exact effect HAGE may have on N-RAS. Evidence gained here using stable knockdown cell lines would suggest possible effect on the

protein. DEAD-box proteins have been connected to translation (Linder, 2006), meaning that HAGE could promote the translation of N-RAS mRNA into protein. To date, no published data has been able to associate DEAD-box proteins or CT antigens with N-RAS expression, meaning in this instance, this work represents the first to do so. Speculation exists to say RAS mutation could be one of the primary genetic events to occur in tumours (Kranenburg *et al*., 2004). Possible up-regulation of N-RAS by HAGE could therefore come about through a feedback loop following up-regulation of one of the substrates of RAS. Since CT antigen re-expression in malignant cells is poorly understood, this needs further investigation.

The group of proteins HAGE belongs to acts by unwinding double-stranded RNA (Fuller-Pace, 2006; Linder, 2006). Studies into DEAD-box helicase function in other species have been able to demonstrate the action of some members of this family (Marsden *et al*., 2006). Therefore in the future, work will be carried out to examine if the HAGE protein is able to exclusively and directly target N-RAS RNA to allow better understanding towards HAGE promotion of N-RAS expression.

RAS is one of the most frequently mutated molecules in malignancy. Approximately 30% of all human tumours demonstrate mutation to one of the three major members (Bos, 1988). K-RAS is the most frequently mutated isoform with around 85% of RAS mutations affecting it. This is followed by N-RAS with approximately 15% and finally H-RAS with less than 1% of mutations (Downward, 2003). High frequency of K-RAS compared to the other family members was thought to be a result of its apparent necessity for cell development. Indeed, in mice, germline mutation of K-RAS is fatal, whereas this is not the case for H- or N-RAS (Umanoff *et al*., 1995; Johnson *et al*., 1997; Koera *et al*., 1997; Esteban *et al*., 2001). This has led to the assumption of K-RAS being vital for healthy development as loss of the other RAS members does not have such dramatic consequences. This could be due to the prevalence of K-RAS involvement in organelle localisation and trafficking in the cytoplasm, which seems greater than N- or H-RAS (Omerovic *et al*., 2007).

However, while N-RAS mutants are not as frequent as K-RAS, it represents the most common isoform mutant in myeloid leukaemias including AML, chronic myelomonocyctic leukaemia (CMML), and juvenile myelomonocytic leukaemia

(JMML). It is also common in MDS, melanoma and hepatocellular carcinoma and can bring about haematological tumours in mice (Downward, 2003; Parikh *et al*., 2006; Koike and Matsuda, 2008). Theoretically therefore, there is the possibility of HAGE exerting some influence in these tumours. If HAGE is able to control the expression of an oncogene with the potency of RAS, therapy against it could be designed in order to inhibit the effects of RAS. This, therefore, makes HAGE a very inviting novel target for therapy, though as already stated, more work is required to confirm the relationship between the two molecules. As mentioned above, N-RAS mutations appear to have more prevalence in haematological malignancies, a group of tumours frequently associated with HAGE expression (Adams *et al*., 2002; Condomines *et al*., 2007; Roman-Gomez *et al*., 2007). However, work is still required to confirm the co-existence of N-RAS and HAGE in cancers.

Analysis of relative expression for K-RAS and H-RAS in FM-82/Ctrl and FM-82/shRNA showed a decrease in these isoforms upon HAGE silencing. This requires further investigation and future work will involve examination of protein levels of the other RAS members to determine if they conform to the same pattern of expression as N-RAS. If this were to be the case, HAGE could recognise an mRNA sequence common to all isoforms. This would be understandable since RAS members are thought to be 85% homologous (Downward, 2003). Other work could involve examining the helicase activity of HAGE on K- and H-RAS RNA.

The possible up-regulation of RAS by HAGE could go some way to explain the phenotype displayed by cells with higher HAGE expression. This is evident in terms of proliferation, where RAS is able to increase the activity of AKT by acting upon the PI3K signalling pathway (Cox and Der, 2002). If more data could be gained to confirm the HAGE-NRAS or HAGE-RAS interaction, this would act as significant evidence as to assigning a role to the CT antigen. Apart from this, up-regulation of the protein expression of a RAS isoform and through this, AKT, could also go some way to explain the results of other phenotypic aspects of HAGE-expressing cells.

The serine/threonine kinase AKT can inhibit apoptosis and has links to therapy resistance. It can down-regulate pro-caspase 9 (Vara *et al*., 2004), which can stop initiation of caspase 3 thus preventing cell death (Schultz and Harrington, 2003).

FM-82/Ctrl cells treated with cisplatin showed lower levels of caspase 3/7 compared to the FM-82/shRNA line, perhaps linking increased p-AKT (active) with reduced effector caspase levels. PI3K signalling involving AKT can also prevent apoptosis through interactions with IAP (Cheng *et al*., 2002), inferring a potential mechanism for HAGE to prevent cell death. If this is the case, then expression of the antigen could act as a biomarker for aggressive disease or disease progression. It also re-emphasises the potential usefulness of HAGE as a target for therapy to at least limit disease progression.

Yet another association for RAS, HAGE and disease progression was the initial data connecting expression of the CT antigen with increased invasiveness. RAS expression has association with angiogenesis and metastasis, two very closely related processes in tumours (Campbell and Der, 2002; Kranenburg *et al*., 2004). In terms of metastasis specifically, HAGE expression seemed to lead to an increase of the pro-metastatic FAK. The importance of this molecule in cell motility, such as leading edge formation in migrating cells, and invasion is well known and underscored by the demonstration that loss of FAK can result in loss of invasion in certain cases (Hauck *et al*., 2002; Hsia *et al*., 2003; Tilghman *et al*., 2005). One could argue that HAGE expression contributes towards metastatic potential. Yet again, RAS and PI3K involvement can be brought into play, since it has been shown in breast cancer that FAK is required to support tumorigenesis and progression involving these two onco-proteins (Pylayeva *et al*., 2009). The only area of contradiction came with MMP-9 expression. RAS has been shown to induce expression of this metalloprotease, as has FAK (Kranenburg *et al*., 2004). Yet there was no increase of MMP-9 in HAGE-expressing cells. However as already mentioned, there is a great deal of focus placed on the balance between pro- and anti-invasion mediators in metastasis (Ahmad and Hart, 1997). Therefore, the reduction of TIMP-4 seen in FM-82/Ctrl could play a part in promoting metastasis in HAGE expressing cells. Future work should focus on developing a more in-depth picture of HAGE and tumour invasion and in particular the changes in expression of TIMP and MMP relative to cell invasion.

Tumour-driven neo-vascularisation is necessary for malignant survival and a pre-requisite for dissemination to other parts of the body (Kranenburg *et al*., 2004). This is why many proteins, aberrantly expressed to cause new vessel development, are

additionally implicated in metastasis. RAS is not only important for angiogenic behaviour in malignant cells, but also affects healthy cells in the surrounding environment, such as stroma to cooperate in promoting the process (Rak *et al*., 1995; Kranenburg *et al*., 2004). Furthermore, AKT has been shown to promote angiogenesis, hinting at another way in which RAS can act to bring this about (Jiang and Liu, 2008). Though angiogenesis was not specifically looked at in this work, one of the genes found to be down-regulated by microarray analysis was JUN-D. This has been shown to down-regulate angiogenesis induced by processes mediated by RAS to prevent oxidative stress (Gerald *et al*., 2004). This preliminary finding necessitates further work investigating the role of HAGE in angiogenesis.

The role of HAGE in immune escape is a more complex relationship, requiring further investigation. Seemingly raised level of FAS-L surface expression on tumour cells positive for HAGE along with increased T cell death caused by the co-culture with HAGE expressing cells would point towards evidence of immune escape. Certain organs of the body bear immune privilege and for obvious reasons, this includes the brain, eye, testis and placenta. The testis have been shown to have FAS-L expression (Francavilla *et al*., 2000), which could confer immune privilege by helping to deter T cell activity. Since HAGE is a CT antigen and therefore normally expressed by healthy testis, there is the possibility this molecule is repeating its function in healthy cells upon re-expression in malignant cells. In terms of the possible HAGE-RAS relationship, RAS has been shown to down-regulate FAS expression (Peli *et al*., 1999), allowing tumour cells to avoid apoptosis possibly induced by FAS-L expressing CTL. Expression of the latter, along with FLIP, would need to be analysed to gain confirmation of RAS-mediated immune survival.

Avoidance of immune reactivity by the up-regulation of FAS-L is contentious. Work focusing on melanoma has indicated that FAS-L is not expressed, while others claim the opposite (Hahne *et al*., 1996; Chappell *et al*., 1999; Ugurel *et al*., 1999). As stated previously, the results obtained in this thesis would appear to agree with the latter. There has been speculation on the role of RAS and increased expression of FAS-L by tumours (Urquhart *et al*., 2002). A study by Urquhart and co-workers (2002) examined a possible link between N-RAS and FAS-L in a melanoma cell line. Their data suggested FAS-L was not expressed, which runs contrary to data obtained

in the present study. However, this was analysis based on one cell line, so there is a need to further examine this concept using different cell lines of different tissue origin. It would seem the data presented here adds to the confusion in terms of FAS-L expression on tumour cells.

7.2 Conclusion and Future Work

Cancer/testis antigens represent one of the most promising targets for cancer therapy. Very little is known as to their role in cancer cells, though hypotheses have been proposed. The pattern of their expression, being predominantly confined to tumours and healthy testis and placenta, would imply that they become re-expressed during transformation to carry out a role in malignant cells. One way of improving the development of therapy targeting CT antigens could be to increase knowledge into the function they possess in cancer. Since HAGE is also classified as a DEAD-box protein, there was the initial hypothesis that it could be involved in proliferation.

From the data collected and presented here, there is now a strong argument to implement HAGE not just in proliferation, but other aspects of malignant cell biology. Gene silencing and phenotypic studies have shown the possible involvement of this antigen in different processes disrupted in cancer. HAGE action seems to be most prominent in proliferation, not just *in vitro*, but *in vivo* as well. Microarray analysis and western blotting demonstrated the expression changes of different molecules within HAGE-expressing cells that could promote cell cycle progression and proliferation. Change in expression of N-RAS, AKT, GSK-3β and $p21^{CIP1}$ has been shown using protein extracted from cell lines. Since increased proliferation has been shown *in vivo*, protein will be extracted from tumours grown in NOD/SCID mice and used for western blotting to confirm expression pattern of these mediators at this level. To increase understanding for the role of HAGE in proliferation, NGS analysis will be used in the first instance to validate the expression of genes shown to be altered by HAGE such as CCNE2, E2F2 and WNT-1. Following this, western blotting will be carried out to discover if changes to these molecules also takes place at the protein level.

Proliferation, along with a large proportion of other aspects of the malignant phenotype can be controlled by RAS members. Due to the position of RAS within cell signalling, its level of influence in cells is substantial, hence its importance in cancer in response to its mutation. The discovery of the possible relationship between HAGE and the N-RAS oncogene acts as a novel finding potentially linking CT antigen expression and the phenotype described here. Due to the prevalence of RAS in human cancer, the discovery of a tumour-specific molecule with possible control over it represents a significant finding. While N-RAS expression change was indicated at the gene and protein level, this was carried out in melanoma. In the first instance, silencing of HAGE will be reduced in other tumour types that express the antigen to confirm if this relationship could be applied other malignancies. Gene studies, in the form of real-time qPCR and protein studies involving western blotting will be employed to confirm expression. In addition to this, studies using the above techniques will be used to investigate if this interaction is specific for N-RAS or also applies to both H- and K-RAS. Further to N-RAS, mechanistic unwinding studies will be performed and a helicase assay will be developed to examine if HAGE can specifically and directly target N-RAS mRNA in order to confirm the hypothesis that it could promote RAS protein translation. HAGE protein will also be extracted from cells using immuno-precipitation, which combined with either mass spectrometry or ChIP-Prot sequencing could help determine what molecules HAGE binds to.

For tumours to pose the biggest threat to a patient, they must be as aggressive as possible. Data obtained in this study have begun to indicate that HAGE could help to promote this, such as invasion. In order to gain a better understanding of this, a study will be performed in HAGE-expressing cell lines of different tumours to examine the levels of metastatic promoters and inhibitors such as MMP and TIMP to examine the balance of these molecules and how this could influence tumour invasion. Additionally, an *in vivo* metastasis model will be established in NOD/SCID mice using the stable shRNA cell line to investigate if HAGE-expressing tumours have greater potential to invade compared to cells where its expression is reduced. Validation of key mediators in this process such as MMP, TIMP, as well as RAS signalling substrates will be looked into, either by extracting RNA from these tumours for use with PCR or proteins for use in conjunction with western blotting.

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Communications resulting from this study

Publications

Original Article

Mathieu, M.G, **Linley, A.J**, Reeder, S.P, Badoual, C, Tartour, E, Rees, R.C and McArdle, S.E, (2010), HAGE, a cancer/testis antigen expressed at the protein level in a variety of cancers, *Cancer Immunity*, **10:2**.

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HAGE Expression and Head and Neck Cancer. A Potential Target for Immunotherapeutic Inetervention?

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Presentations

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