Interplay between Histone Deacetylases in the Modulation of Matrix Metalloproteinase-10 (MMP-10) Expression in Lung Cancer

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Abstract

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Matrix Metalloproteinase-10 (MMP-10) has been shown to be overexpressed in human non-small cell lung cancer (NSCLC), wherein it is proteolytically active, secreted predominantly by tumour cells, and implicated in retarding angiogenesis through repression of tumour vasculature development. Previous studies have indicated a role for the histone deacetylase (HDAC) enzymes, specifically HDAC7, in the regulation of MMP-10, with elevated levels of HDAC7 repressing MMP-10 expression and promoting successful blood vessel formation. The aim of this project was to evaluate the relationship between MMP-10 and HDACs and whether perturbation of the HDAC7/MMP-10 pathway maybe a viable opportunity for therapeutic exploitation in NSCLC. Using an *in vitro* model of NSCLC a central role for histone deacetylases (HDACs) in regulation of MMP-10 expression was confirmed. Although inhibition of HDAC activity increased MMP-10 expression and activity, selective inhibition of the class-II HDAC7 had no effect. However, siRNA-mediated repression of HDAC7 expression caused a significant elevation in MMP-10 expression and activity, supporting a central role for the presence but not enzymatic activity of HDAC7 in the control of MMP-10 levels within NSCLC. Despite the lack of a role for HDAC7 enzyme activity in control of MMP-10, the initial observation that pan-inhibition of HDAC activity repressed MMP-10 expression implicated a wider involvement for HDACs in regulation of MMP-10. Consequently, this study using both siRNA and pharmacological inhibitors confirmed an additional role for the presence and enzymatic activity of the class-I subfamily member HDAC3 in the regulation of MMP-10 in NSCLC. These data indicated an interplay between the class-I HDAC3 and class-II HDAC7 in the regulation of MMP-10 expression and activity, a relationship supported by the previously suggested role for HDAC7 in the nuclear shuttling and subsequent enzymatic functionality of HDAC3. This study thereby identifies and offers a novel molecular mechanism for HDAC-mediated control of MMP-10 in NSCLC. Further work is required to exploit this relationship toward improved treatment of NSCLC in the clinic.

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List of Abbreviations

B-ME: β-mercaptoethanol

Ca^{2+}: Calcium

cDNA: Complementary deoxyribo nucleic acid

$C0_2$: Carbon dioxide

DMSO: Dimethyl sulfoxide

DNA: Deoxyribo nucleic acid

ECs: Endothelial cells

ECM: Extracellular matrix

EDTA: Ethylene diamine tetracetic acid

FBS: Foetal Bovine Serum

FGF: Fibroblast growth factor

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

HAT: Histone acetyl-transferase

HBSS: Hank's Balanced Salt Solution

HDAC: Histone deacetylase

HDACi: Histone deacetylase inhibitor

HRP: Horseradish peroxidase

IC_{50}: Inhibition concentration 50%

MEF2: Myocyte enhancer factor-2

MMP: Matrix Metalloproteinase

mRNA: Messenger ribo nucleic acid

MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium Bromide; thiazolyl blue

NCOR:	Nuclear receptor co-repressor
NES:	Nuclear export signal
NLS:	Nuclear localisation signal
NSCLC:	Non-small Cell Lung Carcinoma
PBS:	Phosphate Buffered Saline
PCR:	Polymerase chain reaction
PKD:	Protein Kinase D
PTM:	Post-translational modification
RNA:	Ribo nucleic acid
RPMI:	Roswell Park Memorial Institute
RT:	Room temperature
RT-PCR:	Reverse Transcriptase polymerase chain reaction
SDS:	Sodium dodecyl sulphate
siRNA:	Small interfering RNA
SMRT:	Silencing mediator for retinoid or thyroid-hormone receptor
TBS:	Tris Buffer Saline
TF:	Transcription factor
TIMPs:	Tissue inhibitor of metalloproteinases
TME:	Tumour microenvironment
TNF-α:	Tumour necrosis factor-α
UV:	Ultraviolet
VEGF:	Vascular endothelial growth factor

Chapter I - Introduction

I.1 Cancer

Cancer is a very complex disease that can occur anywhere in the body, with a very diverse range of causes including tobacco smoke, genetic abnormalities, and infections such as the papillomavirus, amongst others (Anand *et al*., 2008; zur Hausen, 1996). It is defined as the 'uncontrolled and invasive growth of cells' with the ability to metastasise to other areas of the body, causing secondary growths (Patyar *et al*., 2010; Coussens & Werb, 2002).

There are reportedly over 200 different pathological types of human cancer affecting all tissues of the body, with approximately 350,000 new cases of adult and paediatric cancers diagnosed per year in the UK (Cancer Research UK, 2014). Furthermore, cancer is the second major cause of mortalities, responsible for the deaths of approximately 162,000 people in the UK per year. Consequently, it is now estimated that one person in three will be diagnosed with cancer during their lifetime and one in four will die of cancer. As a consequence of extended life expectancy and an increase in the global population, it is also predicted that over the next decade the prevalence of cancer would significantly increase, but due to better prediction and treatment the number of deaths from cancer should hopefully decrease (Lin *et al*., 2009; Ferlay *et al*., 2013).

I.1.1 Non-Small Cell Lung Carcinoma

Non-small cell lung carcinoma (NSCLC) accounts for approximately 80% of lung cancers and is the number one cause of cancer related mortalities (Herbst *et al*., 2008;

Bray *et al*., 2013; Perez-Soler, 2009). NSCLC is subdivided into several sub-types based on their histological appearance; adenocarcinomas, squamous cell, bronchio-alveolar and large cell carcinomas (Travis, 2002). Adenocarcinomas derive from glandular epithelia (Weinberg, 2014) and are the most frequent NSCLC sub-type reported, accounting for 40% of clinical lung cancer cases (Lu *et al*., 2010). Squamous cell carcinomas make up 25-30% of clinical lung cancer cases and develop primarily in epithelial cells that line and protect the underlying cell populations, such as the bronchial tree. Large cell carcinomas, otherwise known as undifferentiated carcinomas, account for 10-15% of cases.

Several features of NSCLC, in particular primary tumour size; tumour grade; regional lymph node involvement; presence of metastasis and tumour pathology are known to influence response to therapy and patient survival. Although treatment in the majority of NSCLC cases involves a chemotherapeutic regimen, response in many cases is often poor, with adenocarcinomas tending to have a better prognosis than large cell carcinoma or squamous cell carcinoma (Cetin *et al*., 2011). As a consequence, better understanding of the molecular pathology of NSCLC, determination of novel tumour markers and identification of targets for therapeutic exploitation are central to improving diagnosis and treatment of this cancer type. Over the past decade, a large number of newer molecular targeted treatments addressing molecular determination of NSCLC have been developed to supplement 'conventional' DNA-damaging cytotoxic drugs in the treatment of NSCLC (Hanahan & Weinberg, 2011). For instance, Bevacizumab (Avastin®) is now licensed for the treatment of NSCLC and targets the depending of tumour growth upon new blood vessel formation (Di Costanzo *et al*., 2008). Bevacizumab was found to increase the survival rate of patients

approximately 2-4 months, dependent upon the type of NSCLC it was used to target (Sandler *et al*., 2006). More recently, molecular-targeted therapeutics against NSCLC have shown symptom improvement in advanced NSCLC patients. One molecular target is the epidermal growth factor receptor (EGFR), a cell membrane receptor tyrosine kinase, which has been found to be up-regulated in certain cases of NSCLC, suggesting that the EGFR-mediated pathway might play an important role in lung carcinogenesis. High expression levels of EGFR, led to the development of tyrosine kinase inhibitors; Erlotinib (Tarceva®) and Gefitinib (Iressa®), these inhibit the tyrosine kinase activity of EGFR and have shown significantly prolonged progression-free survival and achieved higher responses than chemotherapy (Baumgart, 2015; Cascone *et al*., 2006; Gridelli *et al*., 2007; Maemondo *et al*., 2010; Zhou *et al*., 2011). However, greater knowledge of NSCLC pathophysiology is still required to better understand this disease and develop more efficacious treatment.

I.2 Molecular basis of cancer

Despite the wide range of 'causes' and 'types' of cancer, and diversity amongst individuals, cancer fundamentally derives from molecular aberrations within susceptible cells. In principle, non-lethal genetic damage causes modifications to key pathways inside the cell, which, in turn, result in lack of normal regulation of cell growth, conferring a survival advantage to the affected cells, and progressively the ability to invade neighbouring tissues and disseminate to other parts of the body.

Several types of alteration can affect the genome within these tumour cells, leading to cellular transformation and subsequently a selected growth advantage.

The types of genes affected are roughly categorised into two groups, those which promote uncontrolled cell growth (oncogenes) and those which inhibit tumour growth (tumour suppressor genes). The contrasting balance in the activity and repression of these two opposing gene categories drives the molecular evolution of cancer. However, although cancer is without doubt a genetic disease, genetic regulation involves a multitude of control factors, including epigenetic mechanisms (i.e. heritable changes in gene expression determined by factors other than alteration of the primary DNA sequence). In the same manner that DNA mutations are important, epigenetic changes can similarly control gene expression and thus cancer development (Sharma *et al*., 2010). Epigenetic regulation is largely controlled by two processes, alteration in chromatin structure to regulate the access of the transcriptional machinery to the DNA (post-translational modification of histones), and direct epigenetic modification of DNA through methylation of specific nucleotides (DNA methylation). Hypermethylation of CpG islands is common in the promoter region of many genes, preventing the recruitment of transcription factors, which is commonly involved with loss of tumour suppressor function. The post-translational modification of histones, the charged proteins around which negatively-charged DNA is packaged, effects nucleosome aggregation and compaction, dictating transcriptional activity of specific genes (Mottis *et al*., 2013). Cancer is linked to histone hypoacetylation and repression of several key tumour suppressor genes (Ropero & Estellar, 2007). Together the concerted interactions of genetic and epigenetic changes are driving forces for cancer development.

I.2.1 The hallmarks of cancer

Cancerous cells during replication do not adhere to the regulated processes involved with normal formation of eukaryotic cells, with evasion of critical checkpoints allowing the cells to carry on replicating their mutated DNA uncontrollably and gaining the capability to invade neighbouring tissues and metastasise to distant organs (Weinberg, 2014; Park & Lee, 2003). Hanahan and Weinberg originally identified six definitive hallmarks of cancer as distinctive biological capabilities of a tumour cell, defined as: limitless replicative potential; self-sufficiency in growth signals; insensitivity to anti-growth signals; evasion of cell death; development of sustained angiogenesis and, the ability to invade and metastasise (Hanahan & Weinberg, 2000). A decade later two further hallmarks were added, de-regulation of cellular energetics, and the ability to avoid destruction by the immune system (Hanahan & Weinberg, 2011). Additionally, two enabling characteristics were also identified, genome instability which generates the genetic diversity that expedites their acquisition, and tumour-promoting inflammation, which fosters multiple hallmark functions (Hanahan & Weinberg, 2011). In addition to cancer cells, tumours also exhibit another dimension of complexity: they contain a repertoire of recruited, ostensibly normal cells that contribute to the acquisition of hallmark traits by creating the "tumour microenvironment"'(Hanahan & Weinberg, 2011).

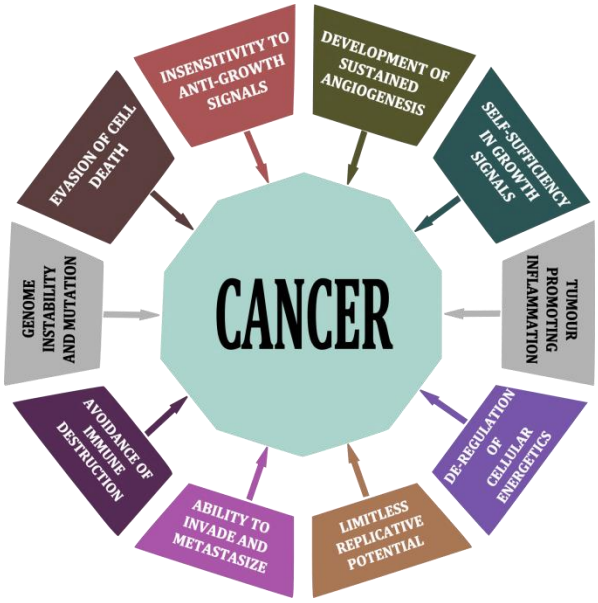


Figure I.1. Hallmarks of cancer and enabling characteristics [adapted from Hanahan and Weinberg, 2011]

I.2.1.1 Tumour cell proliferation and survival

Despite the complex nature of cancer, it is often simplistically characterised as the uncontrolled growth of cells with the consequent development of a tumour. However, uncontrolled cell growth is far from simple and results from the deregulation of a number of closely controlled features. These include the prevention of replication limiting features, the development of cellular insensitivity to anti-growth signals and the evasion of programmed cell death (Sonnenschein & Soto, 2013).

Cancer cells employ several strategies to drive proliferation and overcome insensitivities to normal growth regulation, with the ultimate aim being to move all cells into, and then through the cell cycle, culminating in production of new daughter cells. During cancer development, dysregulation of the cell cycle checkpoints,

through mutations or genetic aberrations, accelerate the accumulation of further mutations, leading to oncogene activation and tumour suppressor gene inactivation. For instance, the tumour suppressor, p53, is a key regulator of both the G1-S and G2-M checkpoints; p53 is, however, inactivated in over half of clinical cancers, allowing cells with damaged and mutated DNA to replicate and produce daughter cells (Rivlin *et al*., 2011).

Cellular proliferation is managed through the receipt of growth factors and subsequent stimulation of signalling pathways, with the tyrosine kinase class being the predominant players in this area (e.g. epidermal growth factor, EGF; Vascular endothelial growth factor, VEGF etc.). Under normal circumstances, the binding of these factors to the specific receptor on the cell surface (e.g. epidermal growth factor receptor, EGFR) provides transient receptor activation, with the signal thereafter being transduced from the cell membrane to the nucleus initiating activation of the cell cycle and cellular division (Lemmon & Schlessinger, 2010). Under normal circumstances, the vast majority of proliferation-inducing growth factors act in a paracrine fashion to stimulate proliferation of another cell type. However, a major mechanism by which cancer cells acquire self-sufficiency is through gaining the ability to both synthesize and respond to a particular growth factor, creating an autocrine loop (Brooksbank, 2001). In a second tumorigenic mechanism, some cancer cells interact with their microenvironment and 'falsely' stimulate normal cells to produce growth factors and subsequently promote cancer development. Further mechanisms associated with this cancer hallmark (self-sufficiency in growth signals) include the gain of an activating mutation (creating an oncogenic protein) in the receptor or alterna-

tively through increased expression of the receptor (gene duplication or chromosomal translocation). In these situations, cells either 'override' the normal proliferative control mechanisms (and gain independence from the external growth factor), or are rendered hyper-responsive to levels of the growth factor that would not normally trigger proliferation. A further common mechanism associated with autonomy to growth signals is through genetic alterations or epigenetic modifications of genes linking growth factor receptors to the effector mechanisms of mitogenesis in the nucleus. Such changes activate the downstream signalling pathway, effectively mimicking the growth promoting effects of growth factor pathway activation.

In the same way cells receive signals to promote proliferation and progress through the cell cycle, they also must receive messages to inhibit, retard or leave the cell cycle. Effectively these signals are provided by tumour suppressor genes, with their disruption making the cells refractory to their growth inhibitory activity and theoretically making them mimic the effects of oncogene activation (Levine *et al*., 1991). Inappropriate proliferation of cells or persistence of those with genetic mutations is normally controlled by the tightly regulated process of programmed cell death or apoptosis (Duronio & Xiong, 2013). These pathways are initiated as a response to extracellular stresses such as limitation of growth factors, oxygen or nutrients, or intracellular stresses such as DNA damage or telomere shortening (Borges *et al*., 2008). In cancer cells, defects in these pathways confer resistance to apoptosis, thereby allowing unchecked cellular replication of cells either with damaging mutations or tumorigenic phenotype (Fulda, 2009).

Another trait of cancer cells is the capacity for limitless replication and proliferative capacity (Hanahan & Weinberg, 2001). In 'non-tumorigenic' cells, the number

of cellular divisions each cell can undergo is normally restricted, regulated by specific DNA sequences at the termini of chromosomes called telomeres (O'Sullivan & Karlseder, 2010). Shortening of these telmomeric sequences causes cell cycle arrest, and senescence (O'Sullivan & Karlseder, 2010). Cancer cells demonstrate capability to renew telomeres in order to ensure their continued reproductive capacity, through upregulation of the enzyme telomerase (Jafri *et al*., 2016).

I.2.1.2 Angiogenesis

In order for cells to survive they must be within close proximity of a blood (or lymphatic) vessel, to allow maximal diffusional delivery of nutrients and oxygen or clearance of waste products (Kerbel, 2000). In agreement with normal cells, this concept is also applicable to cancer, as tumours cannot grow to a diameter larger than 1-2mm unless they have a vascular supply (Kerbel, 2000). The issue that exists with cancer is that the ever increasing tumours mass subsequently needs a larger and larger blood supply to support this rapidly expanding tumour mass (Nishida *et al*., 2006).

Angiogenesis is the formation of new blood vessels from existing vessels involved in blood vessel formation in development and disease and is one of the fundamental hallmarks of cancer (Carmeliet & Jain, 2000). In contrast to pathogenic angiogenesis, the development of blood vessels during embryo development involves recruitment of endothelial cells from immature precursor cells located within the bone marrow, a process termed vasculogenesis (Jain, 2003). Although vasculogenesis has been suggested to show an involvement in tumorigenesis, it is angiogenesis which predominates. Angiogenesis, which under normal circumstances is a highly

regulated process, is continuously triggered in the rapidly growing tumour. The ensuing hypoxia developing in the tumour, as it reaches the 'critical' size limited by diffusional restraints, prevents destruction of the oxygen-sensitive transcription factor, hypoxia-inducible-factor 1 (HIF-1α), which subsequently relocates to the nucleus and initiates expression of vascular endothelial growth factor (VEGF) (Krock *et al*., 2011). Therefore, until the tumour mass reaches this critical size associated with 'supply and demand' there is no requirement for further blood vessels. Once the tumour hits this critical size the 'Angiogenic Switch' is triggered, allowing the balance of pro-angiogenic factors to exceed anti-angiogenic factors (Baeriswyl & Christofori, 2009). The elevated production and secretion of VEGF from tumour cells and the surrounding microenvironment activates VEGF-receptors (VEGFR) on the endothelial cell surface, subsequently leading to increased permeability and vasodilation, loosening of endothelial cell contacts, and finally sprouting of endothelial cells from the existing vessels (Bates, 2010). Simultaneously, increased levels of VEGF (and other pro-angiogenic factors, such as basic fibroblast growth factor, bFGF) stimulate the release of proteolytic enzymes, including the serine protease plasmin and several zinc-dependent matrix metalloproteinases (MMPs) (Vempati *et al*., 2010) (figure I.2). The 'cleared pathway' created by these proteases permits the migration of the proliferative endothelial cells in the direction of the tumour mass, up a gradient of chemokinetic factors released from the tumour, forming 'migration columns' and primitive blood vessels (Ucuzian *et al*., 2010). Completion of the 'column' between the established vessel and the tumour mass leads to endothelial cell differentiation, involving morphological alterations and resultant adherence of these cells to form the lumen of

the vessel. Finally, the newly developed vessels are stabilised via recruitment of pericytes (smooth muscle related cells) to the external surface of endothelium. However, in tumour angiogenesis there is a decreased association of pericytes with the newly formed and immature vessels (compared to mature 'normal' blood vessels) (Bergers & Song, 2005).

Unlike 'normal' processes, the resulting vasculature initiated in the tumour, due to rapidity of tumour growth, is, however, abnormal, poorly organised, chaotic, and leaky (Nagy *et al*., 2009). Furthermore, in addition to facilitating and supporting tumour growth and expansion, the newly developed neovasculature increases the 'escape routes' for tumour cells and, by default, facilitates tumour malignancy and the spread of tumour cells to other parts of the body (metastasis) (Nishida *et al*., 2006).

Angiogenesis is a viable therapeutic target for cancer treatment because of the fact tumour progression is dependent upon its presence (Cross & Claesson-Welsh, 2001); therefore, if this stage could be halted the cancer would not be able to survive due to the lack of essential nutrients.

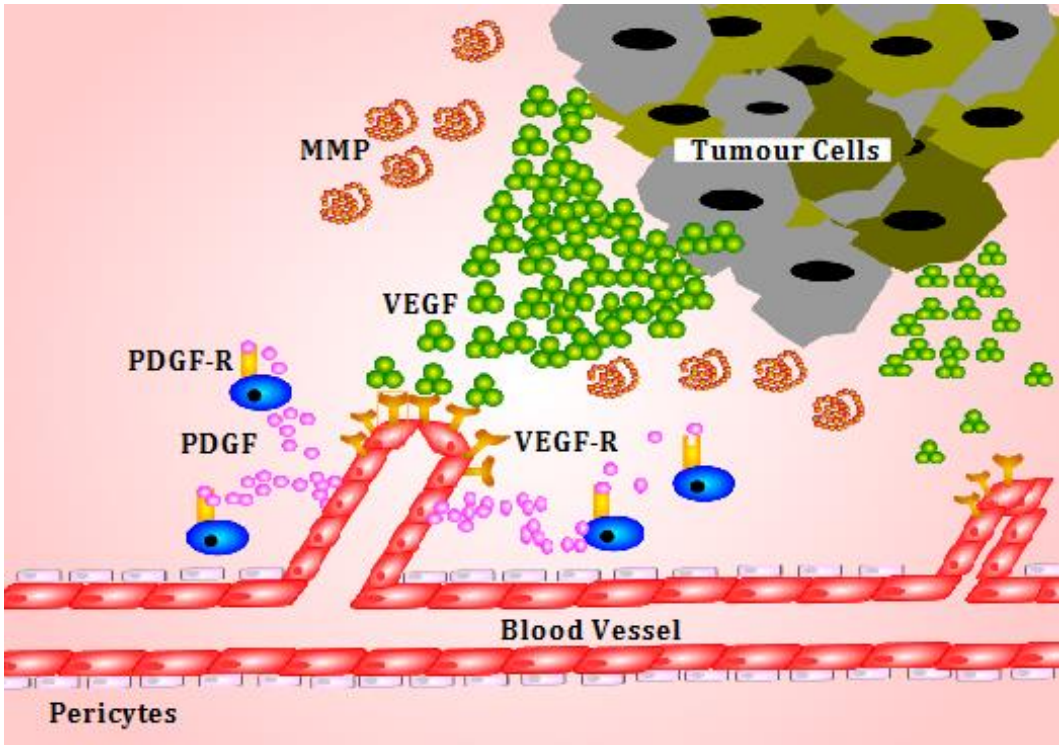


Figure I.2 Tumour Angiogenesis. Tumour cells release pro-angiogenic factors (e.g. VEGF) which bind to receptors on the endothelial cells of pre-existing blood vessels (VEGFR). This leads to secretion and activation of proteolytic enzymes, e.g. matrix metalloproteinases; MMPs, which degrade the extracellular matrix (ECM), allowing migration of endothelial cells. The existing vasculature is supported by pericytes, whereas the developing angiogenic blood vessel secretes growth factors, e.g. platelet-derived growth factor; PDGF, which attracts these supporting cells and pericytes to stabilize the new vessel.

I.2.1.3 Metastasis: Carcinoma in situ becomes an invasive carcinoma

Uncontrolled cell growth is not necessarily hazardous to the wellbeing of the patient (Chambers *et al*., 2002). Indeed, benign tumours grow without the ability to invade neighbouring tissues or relocate from their resident location to another site within the body. However, by definition, malignant cancer cells exhibit the ability to spread, show invasive potential and, ultimately, metastasise to distant regions of the body (Martin *et al*., 2013). Metastasis is the major cause of cancer mortality and, if present, dramatically worsens the prognosis for the patient; compared to if the cancer was

diagnosed prior to metastatic spread. However, it is often the case that metastasis is present by the time the primary cancer has been diagnosed (Friberg & Nystrom, 2015). It is these secondary metastases not the primary tumours that are the main attribute towards mortality and they are found to be responsible for 90% of cancer associated deaths (Yilmaz *et al.*, 2007).

The process of metastasis is a series of rate-limiting steps that must occur in order for cancer to disseminate to sites, other than the primary tumour. Tumour metastasis follows a set pattern of processes: i) The primary tumour forms an avascular mass acquiring its nutrients from the host tissue. Tumour expansion increases hypoxia within this mass, a major driver of several elements of the metastatic process (Eales *et al*., 2016), leading to activation of the angiogenic switch (Baeriswyl & Christofori, 2009). The resultant vascularisation of the tumour increases its scope for expansion and, ultimately, malignancy. ii) Next, as the tumour grows it acquires further genetic aberrations which provide it with the potential and tools to gain mobility and often trigger a conversion and transition from an epithelial to mesenchymal (EMT) phenotype. The EMT is a complex pathway during which epithelial cells lose their differentiated characteristics and acquire mesenchymal features, which include motility, invasiveness, and resistance to apoptosis (Martin *et al*., 2013). Currently, it appears that EMT-inducing transcription factors can coordinate the majority of steps in the invasion/metastatic cascade (Martin *et al*., 2013). iii) The acquisition of mobility and ability to invade through tissues, eventually results in the tumour cell(s) breaching the local basement membrane and transiting towards blood and lymphatic vessels. iv) Tumour cells can then intravasate from the 'invaded tissue' into the circulation (or lymphatic system), wherein they adhere to endothelial cells and platelets

and eventually transit through the vasculature until becoming 'trapped' or 'deposited' in capillaries of another tissue, whereby they extravasate out of the blood vessel and into the new host tissue (Gupta & Massague, 2006).

From a molecular perspective, tumour malignancy is effectively two capabilities grouped together, the initial ability of tumour cells to move and invade into neighbouring tissue (local invasion) and then secondly, the ability to move from one site and disseminate to another (metastasis). Both invasion and metastasis are dependent upon many molecular factors to allow movement and negotiation through the surrounding supportive extracellular matrix (ECM) (Egeblad & Werb, 2002). This supportive network comprises of two main classes of extracellular macromolecules, the first are the polysaccharide chains of the class called glycosaminoglycans (GAGs), which are usually found covalently linked to protein in the form of proteoglycans and secondly the fibrous proteins, including collagen and elastin which both have structural and adhesive functions (Alberts *et al*., 2002). Additional elements can be present in the ECM depending upon the cell origin, for example the ECM of the bones also contains mineral deposits (Frantz *et al*., 2010).

Following successful transit of the cells through the ECM, metastasis progresses by vascular dissemination of these cells to other sites (Valastyan & Weinberg, 2011). Although the most common route for metastatic dissemination is the haematogenous circulation (bloodstream), many tumour cells will travel to their destination via the lymphatic system (Van Zijl *et al*., 2011). Following intravasation, the tumour cells need to evade immune detection and subsequent destruction (Valastyan & Weinberg, 2011). The majority of tumour cells do not survive this challenge and are eliminated before getting to their new site (Valastyan & Weinberg, 2011). Transit of

tumour cells through the circulatory system commonly involves formation of tumour emboli, consisting of the tumour cell aggregating with platelets and other leucocyte cells, which, by default, 'cloaks' the tumour cells, and thereby avoids immune surveillance mechanisms (Hart, 2009). If they can adapt to survive within the new environment of the circulatory system, the cell must then arrive at its target organ, extravasate back out of the blood vessel and into the new host cellular environment, and then persist and eventually re-grow into a tumour, with each of these stages being inefficient (Valastyan & Weinberg, 2011).

The consequent tumour deposit (now termed a micrometastases) can remain dormant at this new site until either their growth is triggered, the host environment becomes conducive to re-grow, or the angiogenic-switch is triggered (Holmgren *et al*., 1995). The growth environment and suitability relies heavily upon the 'seed and soil' theory: the principle being that the new 'host' tissue must have a permissive environment for tumour growth, including an appropriate extracellular matrix, growth factors, and supportive cellular network. Therefore, if the new 'home' for the tumour cells is acceptable, the tumour begins to grow and form a secondary tumour (Martin *et al*., 2013). The eventual site of tumour metastasis is often consequence of the location of the primary tumour and its vascular (or lymphatic) drainage. However, in several cases this predictable route of dissemination is not straightforward and the 'rule' is disobeyed. One such example is metastasis of lung carcinoma to the brain, 'uphill' from the lungs. The reason for this 'seed and soil' theory; discussed above.

Despite metastasis being a devastating outcome of cancer, it is now accepted that, in many cases, the disseminated tumour cells may lie dormant at their new site

for many years which in some cases can actually persist but not cause clinical metastases. In several cases, it is the presence of the metastatic deposit(s) which is detected and confirms the presence of cancer in a patient, in other cases metastatic seeding may have occurred several years before diagnosis of a primary tumour. The metastatic microenvironment contributes and retards the development and regrowth of the disseminated tumour, with either progressive genetic aberrations in the tumour or perturbations in this cellular 'host' environment being the trigger for establishment of the clinically evident metastasis. Successful tumour metastasis is therefore heavily reliant upon the tumour to adapt and respond to its surroundings at each stage of the invasive and metastatic process: the primary tumour site, invasive environment, systemic circulation and the final metastatic location (Valastyan & Weinberg, 2011).

I.2.1.4 Reprogramming energy metabolism

In order to survive and adapt, the tumour must modify and respond to the surrounding environment; this includes how it derives energy and utilises the available nutrients to this effect (Zheng, 2012). The metabolic phenotype of cancer cells is comparable to that observed in other rapidly proliferating cell scenarios, with the exception that, in cancer, these changes are a consequence of genetic alterations and subsequent cell-autonomous signalling, rather than a response to the 'conventional' exogenous growth factor mediated signalling pathways (Gatenby & Gillies, 2004). In terms of metabolic changes, tumour cells demonstrate increased glucose uptake, elevated glutamine uptake and enhanced lipid and nucleotide biosynthesis. The high glucose

consumption and increased production of lactate by cancer cells, through aerobic glycolysis, was identified almost a century ago and is termed the Warburg Effect (Vander Heiden *et al*., 2009). Normally cells generate energy (in the form of adenosine triphosphate, ATP) from glucose by mitochondrial oxidative phosphorylation, producing CO_2 as an end product. However, energy production in cancer cells occurs mainly by aerobic glycolysis of glucose, even when there is sufficient oxygen, and the cells have fully functional mitochondria. Relative to oxidative phosphorylation, which produces 36 molecules of ATP per molecule of glucose, aerobic glycolysis is an inefficient means of generating ATP, with only 2 molecules of ATP produced per glucose molecule. Conversely, the rate of glucose metabolism in aerobic glycolysis is significantly greater than in oxidative phosphorylation, with lactate being produced up to 100 times faster in glycolysis. Subsequently, the two processes counterbalance one another and the amount of ATP produced is comparable between oxidative phosphorylation and aerobic glycolysis (Zheng, 2012). In light of the poor efficiency of aerobic glycosylation, and higher rate of glucose breakdown, tumour cells need to uptake a considerably high level of glucose in order to meet their energy requirements (Gatenby & Gillies, 2004). Despite the central role for the Warburg effect in cancer development, several hypotheses and molecular mechanisms have been provided for its involvement, but, as yet, no definitive function for the Warburg effect in cancer has yet been confirmed.

I.2.1.5 Evasion of immune system

The ability of tumour cells to avoid identification and removal by the immune system is now considered a fundamental hallmark of cancer (Hanahan & Weinberg, 2011). Since tumour cells are not, in effect, 'foreign', the vast majority of cancers avoid immunosurveillance and, therefore, naturally evade immune detection (Schreiber *et al*., 2011). Similarly, because tumour cells are fundamentally 'self', any autoimmune response is, generally speaking, prevented. Of the tumour cells which do raise an immune response, they generally are associated with the expression of immunogenic tumour antigens (Schreiber *et al*., 2011); however, several mechanisms have evolved to allow tumours to escape immune detection or eradication in this regard. Ultimately, the immune system is 'tricked' into failing to detect or recognise tumour cells, or to downregulate itself and drop below a level for adequate immune activation (Schreiber *et al*., 2011).

I.2.1.6 Enabling characteristics: Genome instability

The hallmarks of cancer, define the phenotypic attributes of cancer resulting from genetic mutations and epigenetic regulation of a range of oncogene and tumour suppressor genes, these are underpinned by an enabling requirement for their appearance. Despite cells throughout the human body being exposed to a panoply of putative mutagenic factors, the rate and extent of cancer development is significantly lower than that predicted by these encounters (Mertz *et al*., 2017). This is because, under normal conditions, cells are well equipped to both detect and repair DNA damage, with severe damage triggering cell removal via initiation of apoptosis (Borges *et*

al., 2008). In contrast, cancer cells are often deficient in a normal DNA repair function, with this deficiency allowing the tumour to develop genomic instability (Shen, 2011). In the presence of this deficiency, the tumour cell becomes more susceptible to disruption of tumour suppressor genes, generation of oncogenic fusion genes, and chromosomal aberrations, which subsequently progress and accelerate the tumour towards a more malignant state, thereby enabling development of subsequent cancer hallmarks (Sadikovic *et al*., 2008).

I.2.1.7 Enabling characteristics: Tumour promoting inflammation

For the success of transition of a cancer cell into a malignant neoplasm the tumour microenvironment (TME) plays an important role. The TME is the cellular surroundings in which the tumour sits. It is composed of many different types of non-neoplastic cells, such as cells of the immune system, fibroblasts and adipocytes. Tumour cells as well as immune cells can influence the TME. The TME is constantly changing as a result of tissue remodelling, metabolic alterations in the tumour and changes in the recruitment of stromal cells including a diversity of immune cells (Swartz *et al*., 2012). The TME provides the essential signals that activate the transcription factors and therefore it is the stromal or non-malignant cells that induce the requisite transcription programs allowing the necessary mesenchymal phenotypes to invade distant tissues and establish a new environment (Mbeunkui & Johann, 2009).

The presence of these tumour-associated cells drives an enabling characteristic of cancer. Although inflammation has long been associated with tumour development, the involvement and presence of this condition is now believed to be a protective

response toward the cancer, which enables malignancy. Commonly an inflammatory component is present in the microenvironment of the vast majority of solid malignancies (Grivennikov *et al*., 2010). This cancer-related inflammation involves cells of both the adaptive and innate immune system, including the infiltration of leukocytes, predominantly tumour-associated macrophages, and several inflammatory mediators, such as tumour necrosis factor-α (TNFα), interleukin-6 (IL-6), and chemokines, such as CXCR4 and CXCL12 (Grivennikov *et al*., 2010).

The presence of inflammatory cells within a tumour was initially perceived as being related to an immune reaction and an attempt by the host to 'destroy' the tumour. Although this may provide a contribution towards explaining the presence of inflammatory cells within the tumour, it is now known that growth factors, chemokines, and other cellular mediators can act to promote several tumorigenic processes, such as tumour proliferation, the invasive tumour phenotype, angiogenesis and, ultimately, tumour chemotaxis and motility (Raman *et al*., 2007).

I.2.1.8 Cancer Degradome and Malignancy

A major defining feature of malignancy is the ability to acquire an improved vasculature system, penetrate into surrounding normal tissues and disseminate to distant sites (Martin *et al*., 2013). Each one of these processes relies heavily upon the increased expression and activity of diverse extracellular endoproteases from multiple enzymatic classes, namely the metalloproteases and the serine, threonine, cysteine and aspartic proteases. These proteases constitute the cancer degradome—the repertoire of proteases that cells and tissues coordinately regulate to modulate their

local environment (Lopez-Otin & Overall, 2002; Overall & Blobel, 2007). These endoproteases are frequently upregulated within tumour tissue where they promote the development and expansion of the tumour, formation of new blood vessels to support the burgeoning energy demands of the rapidly growing tumour and facilitate the metastasis of cancer cells to distant organs (Egeblad & Werb, 2002).

There is now a considerable body of literature demonstrating elevated expression of these endoproteases in primary tumours and metastases, with the majority demonstrating an association with tumour progression or validity as prognostic indicators of clinical outcome (Gialeli *et al*., 2011; Levicar *et al*., 2003). However, it is now deemed oversimplistic to think the function of these proteases is solely to mediate tissue destruction and create a path for tumour cell motility and migration, with roles for these proteases identified in a broad range of tumorigenic processes (Lopez-Otin & Matrisian, 2007). These enzymes are known to be central mediators of growth factor activation, cellular adhesion, cellular survival and immune surveillance, to name a few (Sternlicht & Werb, 2001). Taken together their broad but essential role in tumorigenesis and their relationship to disease prognosis, these enzymes can be defined as a significant force in the phenotypic evolution of cancer (Martin *et al*., 2013).

I.3 Matrix Metalloproteinases

It has long been recognized that cancer invasiveness and dissemination to distant organ sites involves the action of many different extracellular proteases, but particularly the matrix metalloproteinases (MMPs) (Egeblad & Werb, 2002). The MMPs are

a member of the large protease metzincin superfamily, alongside the ADAMS (a disintegrin and metalloproteinase) and the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motif) (Malemud, 2006). The MMPs are themselves a family of at least 24 human proteolytic enzymes that are responsible for degrading a range of extracellular matrix proteins (Roy *et al*., 2009), contributing to the formation of a microenvironment permissive of tumour survival, growth, expansion and metastasis. They are divided into eight distinct groups; five are secreted and three are membrane-bound (membrane-type matrix metalloproteinases, MT-MMPs) based on both their substrate specificity and the arrangement of their protein domains (Roy *et al*., 2009). The majority of MMPs, with the exception of the MT-MMPs and MMP-11, -21, -23 and -28, are secreted by the cell as inactive zymogens and require activation extracellularly by cleavage of the N-terminal pro-domain resulting in a fully functional enzyme (Kessenbrock *et al*., 2010). In contrast, the MT-MMP family is not secreted from the cell but is activated intracellularly and presented on the cell surface in a proteolytically active state (Reunanen & Kahari, 2013).

Structurally the MMPs comprise of a common arrangement of four key structures; firstly, the pro-peptide which consists of 80 amino acids to sustain the MMP in the latent form (Clark *et al*., 2008). Secondly, the catalytic domain exhibiting the conserved HEXGHXXGXXH zinc-binding sequence characteristic of the metzincin superfamily of proteinases, followed by an invariant methionine which is involved in a structural feature called the Met-turn which mediates enzyme activity (Clark *et al*., 2008). Thirdly, there is the presence of the haemopexin (Hpx) domain, which provides substrate specificity to the MMP. These three central structures are all connected by a linker region of varying length (Osada *et al*., 2004). As with all protein

families, exceptions to this architecture do exist with MMPs, for instance MMP-7, MMP-23 and MMP-26 all lack the Hpx and linker domain, suggesting different proteolytic activities for these endoproteases (Nagase *et al*., 2006).

Structural class	MMP number	Common names (s)	Notable structural feature(s)
Simple Hemopexin Domain	1	Interstitial collagenase, Collagenase-1	Standard signal peptide, propeptide domain, catalytic domain, hinge region and C-terminal hemopexin domain
	3	Stromelysin-1	
	8	Neutrophil collagenase, Collagenase-2	
	10	Stromelysin-2	
	12	Macrophage elastase	
	13	Collagenase-3	
	18	Collagenase-4	
	19	Rheumatoid arthritis-associated MMP	
	20	Enamelysin	
	22	MMP-23B	
	27		
Gelatin-Binding	2	72kDa Type IV gelatinase, Gelatinase A	Fibronectin type II repeats
	9	92kDa Type IV gelatinase, Gelatinase B	
Minimal Domain	7	Matrilysin	No C-terminal hemopexin domain; no hinge region
	26	Endometase, matrilyson-2	
Furin-Activated and Secreted	11	Stromelysin-3	Furin recognition sites
	28	Epilysin	
Vitronectin-like Insert	21	MMP-23A	
Type I Transmembrane	14	MT1-MMP	Transmembrane domain; cytoplasmic tail; MT loop
	15	MT2-MMP	
	16	MT3-MMP	
	24	MT4-MMP	

Type II Transmembrane	23	Cystein array MMP	No C-terminal hemopexin domain; no hinge region
GPI-linked	17	MT5-MMP	Transmembrane domain; cytoplasmic tail
	25	MT6-MMP	

Table I.1 Classification of the human MMPs. Adapted from Curran and Murray (2000) and Egebald and Werb (2002).

MMPs are expressed by the cell as inactive zymogens (proMMPs), this inactivation is caused by the interaction of a cysteine residue of the pro-domain with the zinc ion of the catalytic site (Egeblad & Werb, 2002; Kessenbrock *et al*., 2010). They are inactive until they are required for any one of several processes they stimulate (eg. angiogenesis, morphogenesis), wherein they are activated extracellularly via proteolytic removal (by proteolytic enzymes dependant upon the MMP; for example serine proteases and furin) of the pro-domain which opens up the active centre of the MMP molecule and allows the MMP to bind to its protein substrate(s) (Sternlicht & Werb, 2001).

I.3.1 Function of MMPs

MMPs regulate many normal physiological processes including tissue growth and embryogenesis (Brown, 1997) and they are secreted to catalyse the degradation of the ECM proteins such as collagen and gelatin as well disrupt the contact between cells (Bourboulia & Stetler-Stevenson, 2010). During malignant cell migration the

MMPs are involved in many processes, including removing sites of adhesion, cleaving cell-cell or cell-matrix receptors and releasing chemo-attractants from the ECM, thus participating in several pertinent processes associated with cancer, such as innate and adaptive immunity, inflammation, angiogenesis, bone remodelling, and neurite growth (Rundhaug, 2003; Loffek *et al*., 2011).

The controlled cleavage of malignant factors by different MMPs also is a major player in modulation of different cell signalling events, such as the ECM-tethered VEGF, which directs the specific binding pattern of VEGF to ECM components guiding the invading endothelial cells and supporting the process of neo-angiogenesis (Deryugina & Quigley, 2015). The ECM-tethered VEGF is inactive in complex with connective tissue growth factor (CTGF), but becomes active after proteolytic cleavage of CTGF by MT1-MMP, MMP-1, -3 and -13. In contrast, the cleavage of VEGF by MMP-3 and -9 generates a shorter, non-heparin binding active form of VEGF that subsequently induces irregular vessel sprouting (Lee *et al*., 2005).

I.3.2 Regulation of MMPs

Under normal physiological conditions, the activities of MMPs are precisely regulated at the level of transcription, activation of the precursor zymogens, interaction with specific ECM components, and inhibition by endogenous inhibitors (Visse & Nagase, 2003). At a local level, MMP activity is further regulated by interaction with four specific exogenous protease inhibitors, termed tissue inhibitor of metalloproteinases (TIMPs 1-4), with a broad inhibitory specificity and combined ability to inhibit all the MMPs (Nagase *et al*., 2006; Brew & Nagase, 2000; Batra *et al*., 2012). TIMPs, similarly

to MMPs, are regulated at the transcriptional level by many cytokines, growth factors and chemokines (Clark *et al*., 2008). Disequilibrium of the MMPs and TIMPs balance is associated with many diseases including cardiovascular diseases and cancers (Snoek-van Beurden & Von den Hoff, 2005; Jackson *et al*., 2017). Several studies have shown a role for the TIMPs in the vast majority of cancer hallmarks, with TIMP dysregulation evident in the tumour mass of the microenvironment and TIMP1 over-expression or TIMP3 silencing commonly related to a poor prognosis (Jackson *et al*., 2017). In NSCLC, TIMP1 and TIMP2 levels are higher in adenocarcinomas compared to squamous cell carcinomas, whereas the converse is apparent for TIMP3 (Hida & Hamada, 2012). This indicates potential different involvements of MMPs (and TIMPs) between NSCLC sub-types, with significant implications for diagnostic and therapeutic approaches.

Despite the 'conventional' function of TIMPs as endogenous inhibitors of MMPs, recent studies have now identified TIMPs also have the ability to directly influence cellular physiology as well as disease progression without an involvement of MMPs (Stetler-Stevenson & Gavil, 2014), strongly supporting TIMPs as important factors for a number of reasons (Jackson *et al*., 2017).

I.3.3 Role of MMPs in wound healing

MMPs play a role in the different stages of wound healing; they are involved in the breakdown of the damaged ECM in wounds, specifically migration of cells to close the wound and angiogenesis to facilitate cell survival and nutrition (Caley *et al*., 2015). In terms of re-vascularisation of the repairing wound, MMPs degrade the ECM

and basement membrane around the capillaries and allow endothelial cells to migrate from capillaries near the wound and establishment of new blood vessels into the wound bed through angiogenesis (Gill & Parks, 2008). Conversely, as well as degrading proteins to facilitate angiogenesis, certain MMPs (e.g. MMP-1, -3, -9 and -10) can also function to retard angiogenesis and play a role in vascular collapse, (Loffek *et al*., 2011; Chen *et al*., 2013). In this context, activated forms of these proteinases promote degradation of collagen type I, or basement membrane matrices that represent other important angiogenic substrates, supporting the hypothesis that proteolytic ECM degradation, or destruction of the angiogenic scaffold also leads to collapse of angiogenic networks as well as development of new ones (Saunders *et al*., 2005).

I.3.5 MMPs in cancer

Under normal physiological conditions MMPs activity is tightly regulated, however when unregulated or dysfunctional they contribute to a diverse range of degradative diseases such as arthritis, cardiovascular disease and cancer (Nagase *et al*., 2006; Clark *et al*., 2008). During tumorigenesis the MMPs are selectively activated and secreted into the local tumour microenvironment, with an involvement in all of the 'hallmarks of cancer' (Kessenbrock *et al*., 2010). Subsequently, because MMPs are centrally involved in cell migration, growth factor regulation, tissue remodelling, and degradation of the extracellular matrix, they are major drivers of tumour progression

if deregulated (Overall & Kleifeld, 2006). Consequently, the extracellular microenvironment is an integral and dynamic scaffold that dictates cell function and cell fate for both normal and cancer cells (Zhang *et al*., 2014).

MMPs have been found to be up-regulated in many cancer types, making them a very important target for diagnosis, prognosis and therapy (Coussens *et al*., 2002; Overall & Kleifeld, 2006; Roy *et al*., 2009). In pathological conditions such as cancer the equilibrium that is created between the MMPs and the three control states (activation of the zymogens, transcription, and inhibition of the active forms), is shifted toward increased MMP activity leading to tissue degradation (Verma & Hansch, 2007). For instance, cancers whereby MMPs have been up-regulated include; MMP-9 in breast cancer (Wu *et al*., 2008), MMP-2 in pancreatic cancer (Yokoyama *et al*., 2002) and MMP-10 and MMP-14 in lung cancer (Gill *et al*., 2004; Atkinson *et al*., 2007), amongst many others. These concerted activities subsequently provide the foundations to the many steps of remodelling and cellular rearrangements associated with tumour invasion, and ultimately metastasis.

I.3.5.1 Involvement of MMPs in establishment of pre-metastatic niche

In addition to the 'conventional' role for MMPs in modulation of the ECM and proteins within the tumour microenvironment, to permit cellular motility and invasion, they are also now known to play a role in 'establishing the premetastatic niche' (Shuman Moss *et al*., 2012). The premetastatic niche being the readying of the area in which a secondary tumour will establish and develop, a process involving many signals including VEGF, transforming growth factor β (TGFβ), tumour necrosis factor

α (TNFα) and specific chemokine proteins (Kaplan *et al*., 2006). MMPs are also activated at these distance sites, with roles involved in regulation of these cellular factors and cellular reorganisation permissive of focused and directed metastasis of the cancer cells and provision of the appropriate 'soil' for the cancer 'seed' (Shuman Moss *et al*., 2012), although the specific mechanisms need to be further explored.

I.3.5.2 Pro- and anti-tumorigenic role of MMPs

Further to the general involvement of MMPs in tumorigenesis, individual MMPs are shown to have different roles during cancer progression (Gialeli *et al*., 2011). For instance, elevated MMP-2 levels is associated with driving tumour invasion and metastasis (Kessenbrock *et al*., 2010), whereas MMP-8 over expression provides a protective effect in the metastatic process, decreasing the metastatic potential of breast cancer cells (Gialeli *et al*., 2011). Similarly, MMPs with high proteolytic homology and similar peptide selectivities have also been shown to demonstrate either comparable activities in different tumour types, or different roles in the same tumour subtype (Al-Alem & Curry, 2015). For example, MMP-10 and MMP-3 are highly homologous MMPs within the stromelysin sub-class, but show different tumour expression and activities. In breast cancer MMP-10 is downregulated, however MMP-3 showed no differential expression (Benson *et al*., 2013). Furthermore, an MMP can also have contradicting activities within the same tumour, both temporally and geographically, such as MMP-10 and MMP-3 show strong expression in NSCLC, MMP-10 over expression is localised to the tumour mass as opposed to the stroma, indicating its role in the early stages of tumour development, as opposed to MMP-3

where its strong expression in NSCLC is observed in the tumour stroma, indicating it plays a role in local growth of the tumour (Al-Alem & Curry, 2015; Gill *et al*., 2004).

The dichotomy of activities for the MMPs as being both pro- and anti-tumorigenic necessitates elucidation of the individual role of MMPs in tumour evolution, in order to identify and validate their role as diagnostic markers and whether they are targets or anti-targets for therapeutic exploitation (Overall & Kleifeld, 2006; Cathcart *et al*., 2015; Bonfil *et al*., 2008).

I.3.7 Role of MMP-10 in Tumorigenesis

One MMP for which information is limited is MMP-10, also known as stromelysin-2, which reportedly plays a role in vascular remodelling, wound healing and skeletal development (Batra *et al*., 2012). With regards to cancer, increased MMP-10 expression has been demonstrated in carcinomas of several human tissues (Tsang *et al*., 2012; Aung *et al*., 2006), including head and neck cancer (Deraz *et al*., 2011; Tsang *et al*., 2012), gastric cancer (Aung *et al*., 2006), bladder cancer (Seargent *et al*., 2005) and non-small cell lung cancer (NSCLC) (Gill *et al*., 2004). This expression, particularly in the case of NSCLC, related to altered function as exemplified by significantly higher levels of proteolytically active MMP-10 in NSCLC relative to the corresponding histologically normal lung tissue (Gill *et al*., 2004). Furthermore, MMP-10 expression in NSCLC is independent of tumour grade, stage, histological type, or lymph node status (Gill *et al*., 2004). However, elevated levels of MMP-10 are observed in recurrent NSCLC (Cho *et al*., 2004), which together suggests a lack of association of this MMP with the invasion or metastatic process (Gill *et al*., 2004). This is further supported by

the fact that MMP-10 is also not related to the invasive phenotype of skin cancer (Kerkela *et al*., 2001), or to increased pathological stage in bladder cancer (Seargent *et al*., 2005). Together these studies indicate a putative role for this MMP in the earlier stages of tumour development, rather than invasion and metastasis associated with many other MMP (Miyata *et al*., 2007). This hypothesis is supported by studies within this research group, wherein overexpression of MMP-10 in a human NSCLC tumour model did not affect cellular proliferation *in vitro*, but caused significant retardation of tumour growth *in vivo* (personal communication by Gill, Figure I.3).

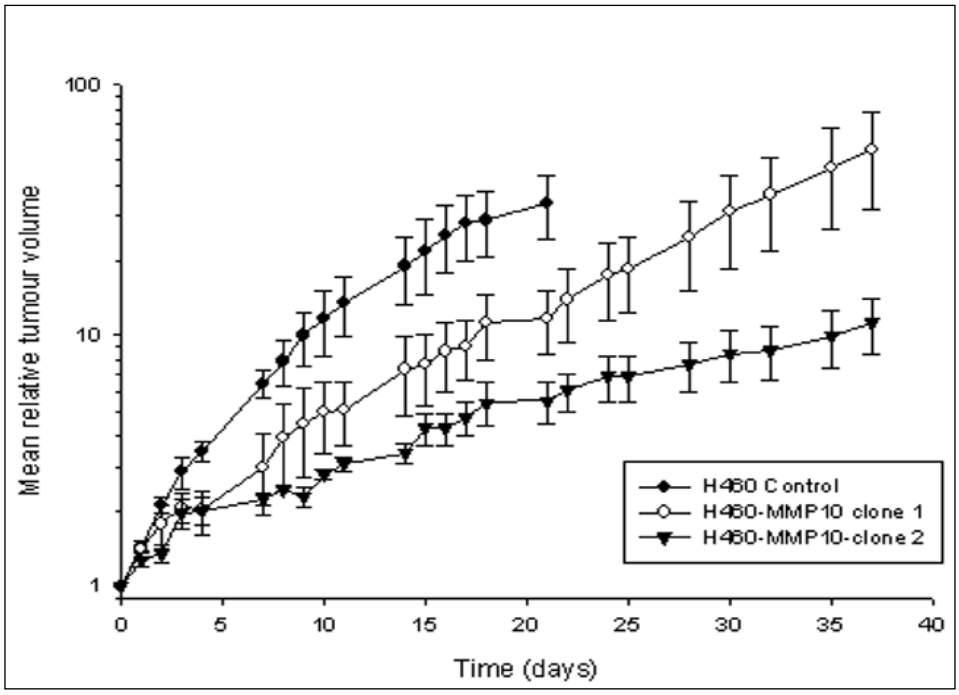


Figure I.3: Growth of MMP-10 expressing NSCLC cells *in vivo*

NSCLC H460 cells stably expressing MMP-10 were introduced sub-cutaneously into nude mice and tumour growth was determined over time.

MMP-10 expressing tumour (clone 1 and 2) growth was significantly retarded relative to mock transfected cell controls.

The degree of growth retardation is relative to level of MMP-10 selective substrate cleavage. Clones 1 and 2 demonstrate 2.5 and 4.0 fold greater MMP-10 activity than control cells, respectively

MMP-10, unlike the majority of MMPs, localises to the tumour cells rather than the supporting stromal cells within the tumour microenvironment (Gill *et al*, 2004; Seargent *et al*., 2005). The enzyme is secreted from these cells into the extracellular fluid as an inactivate 57kDa proenzyme, wherein it is converted into the 44kDa active enzyme by a cascade involving several proteases, including kallikreins, cathepsins, and other MMPs (Saunders *et al*., 2005). Once activated, MMP-10 continues the progressive proteolytic cascade through activation of pro-MMP-1, -7, -8, -9, -13, broadening the combined ability of these enzymes to restructure the tumour microenvironment (Zhang *et al*., 2014; Davis & Saunders, 2006).

The heightened concentration of MMP-10 in tumours is thought to be required for the restructuring of the endothelial cells during tumour angiogenesis (Saunders *et al*., 2005). MMP-10 has relatively broad substrate specificity (Zhang *et al*., 2007) with the ability to degrade a variety of extracellular matrix proteins, including collagen III-V and fibronectin (Batra *et al*., 2012). MMP-10 has been found to play a role in the capillary tube regression response, it causes regression of endothelial cells; they deconstruct the ECM scaffold in which the newly formed vascular network is embedded (Davis & Saunders, 2006). The MMP-10-mediated activation of MMP-1 has been found to cause consequent acceleration of capillary tube regression of ECs *in vitro*, further supporting an involvement for MMP-10 in tumour establishment rather than the invasive phenotype (Chang *et al*., 2006). Consequently, with MMP-10 being involved in vascular reconstruction, and its relationship to several cancer types, modulation of its expression and activity may function to prevent vascular stability occurring and thus interference of tumour establishment and survival.

I.3.6 Modulation of MMP activity as a therapeutic target

With MMPs being up-regulated in many cancers and several being pro-angiogenic, targeting these is hypothesised as a therapeutic target. Development of chemical inhibitors of MMP activity (MMP inhibitors; MMPi) has been a source of therapeutic activity for many years (Fisher & Mobashery, 2006). Initial MMPi's demonstrated disappointing results, due to lack of specificity and dose-limiting off-target toxicities in the clinic (Coussens *et al*., 2002; Fisher & Mobashery, 2006). Furthermore, lack of success was also attributed to poor appreciation of the pathophysiology of MMPs and inappropriate clinical trials, in which the compounds were evaluated against late stage cancers after MMP-dependent invasion and metastasis. For instance, one of the first MMPis evaluated in cancer patients was Batimastat, administered for the use of malignant effusions (Macaulay *et al*., 1999). Although highly potent against several MMPs (MMP-1, -2, -3, -7, -9 and -14) and showing clinical efficacy against malignant pleural effusion, trials were ceased due to its poor solubility and limited pharmacokinetics and the advent of MMPi with improved bioavailability such as Marimastat (Cathcart *et al.*, 2015). However, this MMPi was found to cause significant musculoskeletal pain and inflammation, with trials stopped because they did not produce significant results in terms of symptomatic progression or overall survival (Cathcart *et al.*, 2015).

Advances have been made in this area in recent years through greater understanding of these complex proteins, yielding new generations of MMPis to be designed (Overall & Lopez-Otin, 2002; Hu *et al*., 2007; Van Themsche *et al*., 2004; Cathcart *et al.*, 2015). Those with selective targeting towards specific MMPs offering a more promising outcome in reflection to the realisation that some MMPs are anti-

angiogenic, so targeting these in some cancers would have a counterproductive effect (Decock *et al*., 2011).

There are now several alternative therapeutic strategies hypothesised for modulation of MMPs, including MMP-selective antibodies (Nam *et al*., 2012). One such antibody is DX-2400, a MMP-14 specific inhibitor which blocks substrate access but does not chelate or interact with the catalytic zinc (Cathcart *et al*, 2015). Studies to date of DX-2400 indicate this antibody is selective for MMP-14 over other MMPs (Devy *et al*., 2007). In preclinical studies DX2400 prevented metastases and significantly decreased tumour mass (Devy *et al*., 2009). Similarly, the MMP-9 inhibitory antibody REGA-3G12, can differentiate between MMP-9 and its close homologue MMP-2, has shown indicative potential for therapeutic exploitation (Cathcart *et al.*, 2015). However, these antibodies have not yet been evaluated in the clinic.

Another approach currently in its infancy is the inhibition of MMP expression at the transcription or translational level, rather than targeting blockade of enzyme activity post-translationally (Cathcart *et al*., 2015). This strategy relies on an appreciation and understanding of the upstream signalling pathways and control mechanisms responsible for expression of MMPs. In this context, several pathways have now been related to MMP expression, such as NK-kappaB, p38 and Jun-N-terminal kinases (JNK), and studies are now beginning to identify and characterise these control mechanisms (Gordon *et al*., 2009). However, no studies in this area have yet been evaluated *in vivo* or clinically.

I.4 Epigenetic control of gene expression

Genetic expression in addition to being controlled by the DNA sequence, and subsequent transcription, and translation, is also regulated by epigenetic factors (Goll & Bestor, 2005; Goldberg *et al*., 2007). Epigenetics refers to heritable changes in gene expression determined by factors other than alteration of the primary DNA sequence (Goldberg *et al.*, 2007). Through modification of these non-genetic factors, gene transcription can be regulated allowing the temporal and spatial expression of genes (Goldberg *et al.*, 2007). Epigenetic regulation is largely controlled by two processes, alteration in chromatin structure to regulate 'entry' of the transcriptional machinery to the DNA (post-translational modification of histones), and direct epigenetic modification of DNA through methylation of specific nucleotides (DNA methylation).

I.4.1 Promoter methylation

In the promoter regions of genes, methylation occurs at dinucleotides comprising cytosines located prior to guanosine in the linear sequence of DNA bases. Regions of the genome with a high density of CpGs are termed CpG islands, with DNA methylation of these islands correlating with transcriptional repression and gene silencing (Robertson & Jones, 2000). This mode of silencing gene expression is essential to several 'normal' long-term processes, such as inactivation of the X-chromosome, and genomic imprinting, and is a central mechanism in the regulation of cellular differentiation and ultimately cell identify and fate (Robertson & Jones, 2000). In cancer there is global genome-wide hypomethylation, accompanied by hypermethylation of CpG islands in the promoter region of many genes commonly involved with

loss of tumour suppressor function. A central role for DNA methylation as an oncogenic factor is reinforced by the induction of growth arrest and cell death in haematological malignancies following exposure to inhibitors of DNA methyltransferases (azacitadine and decitabine) (Robertson & Jones, 2000).

I.4.2 Epigenetic modification of histones

In terms of histone modifications, DNA is wrapped around histone octamers (composed of four core histones H3, H4, H2A and H2B, organized into two dimers, H3-H4 and H2A-H2B) to form nucleosomes, these nucleosomes are the fundamental units to chromatin (Luger *et al*., 1997). The positively charged histones interact with the negatively charged DNA (due to phosphate groups) by electrostatic interactions, resulting in nucleosomal compaction (figure 1.4). Ultimately, in combination with short linker DNA sequences, these long chains of tightly compacted nucleosomes ('beads on a string') form chromatin and ultimately chromosomes (Annunziato, 2008). The degree to which the nucleosome aggregates are compacted (or relaxed) dictates transcriptional activity, causing expression or repression of specific genes. Essentially, tightly compacted nucleosomes cause "closed chromatin" (Heterochromatin) and subsequent transcriptional repression, whereas an "open" chromatin structure (Euchromatin) is characteristically transcriptionally active (Phillips & Shaw, 2008).

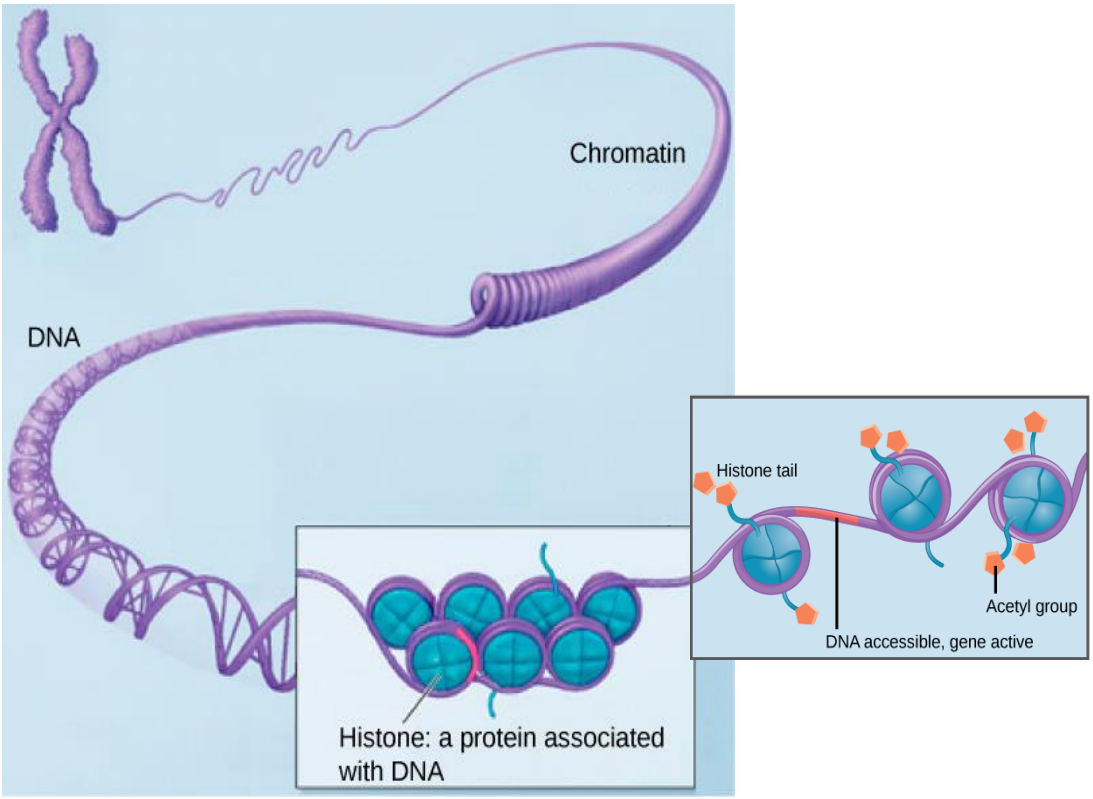


Figure I.4 Histones are proteins around which DNA can wind for compaction and gene regulation. The conformation of the histones can be altered with binding of different epigenetic factors to the histone 'tails', this opens up the DNA region where transcription machinery proteins; transcription factors and co-activators bind to turn on gene transcription. Removal of these factors from the histone tails via methylation, or deacetylation condenses DNA around histones and thus, make DNA unavailable for binding, leading to gene silencing. [Figure is adapted from National Institutes of Health 2005].

Modulation of nucleosomes, to facilitate active gene transcription, is controlled by promoting or inhibiting condensation of the DNA-histone complex, a process mediated predominantly by post-translational modifications of the N-terminal tails of the histone proteins, including methylation, acetylation, and phosphorylation, amongst others (Garcia *et al*., 2007). These changes can affect gene expression by allowing or restricting the accessibility that the transcription factors (TFs) has to the DNA (Bannister & Kouzarides, 2011). Transcriptional co-regulators play an important role in epigenetic regulation by recruiting chromatin-modifying enzymes. These co-factors bind to the TFs which are recruited to the regulatory region of genes, controlling their transcriptional activation or repression (Mottis *et al*., 2013).

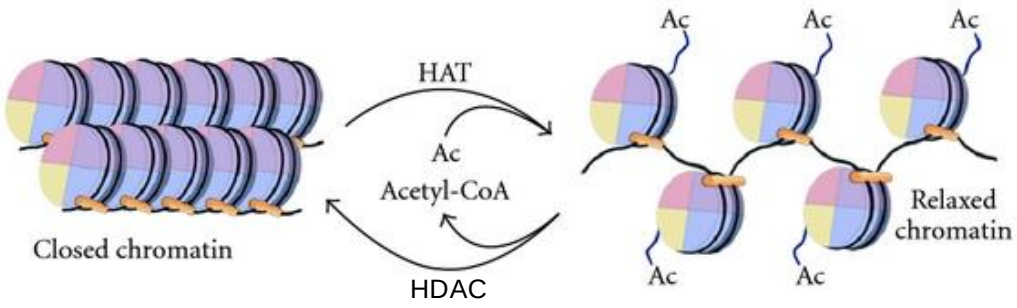


Figure I.5 The dynamic state of histone acetylation/deacetylation regulated by HAT and HDAC enzymes. Acetylation of histones alters accessibility of chromatin and allows DNA binding proteins to interact with exposed sites to activate gene transcription. Deacetylation has opposing effects to the HATs, closing off access to chromatin and repressing gene transcription [adapted from Rodd *et al*., 2012].

The epigenetic control of gene expression, particularly through modification of histone conformation, is now known to be central to the control of tumorigenesis, with many tumours exhibiting histone modifications; including NSCLC (Chervona & Costa,

2012). In terms of the hallmarks of cancer, there is growing evidence to support post-translational modifications of histones in the regulation of many of the hallmarks, including as drivers of invasion and metastasis. Furthermore, there is now compelling evidence to support a function for the enzymes responsible for histone modifications on a whole range of non-histone proteins. In many cases these proteins targeted by acetylation are the products of oncogenes or tumour suppressor genes phosphorylation and methylation, documented in several types of cancers (Singh *et al.*, 2010; Muthusamy *et al*., 2010; Kanwal & Gupta, 2012).

1.4.1 Histone deacetylase (HDAC)

The most common post-translational histone modification is acetylation (Bannister & Kouzarides, 2011; Turner, 2000). Acetylation and deacetylation of both histone and non-histone proteins is regulated by the histone acetyl-transferases (HATs) and histone deacetylases (HDACs) classes of regulatory enzymes (Petta *et al*., 2013). HATs attach acetyl groups onto the lysine amino acids of target proteins; with the HDACs having opposing effects to the HATs and removing the acetyl groups from a ε-N-acetyl lysine amino acid, particularly on histones central to epigenetic control of gene expression (Choudhary *et al*., 2009).

Class	HDAC enzymes	Cellular localisation
Class I	HDAC1	Nucleus
	HDAC2	Nucleus
	HDAC3	Nucleus and Cytoplasm
	HDAC8	Nucleus
Class IIa	HDAC4	Nucleus and Cytoplasm
	HDAC5	Nucleus and Cytoplasm
	HDAC7	Nucleus and Cytoplasm
	HDAC9	Nucleus and Cytoplasm
Class IIb	HDAC6	Cytoplasm
	HDAC10	Cytoplasm
Class IV	HDAC11	Nucleus and Cytoplasm

Table I.2 Classification of the classic zinc-dependant histone deacetylases (HDACs) [Adapted from Mohseni *et al*., 2013].

HDACs form part of the classical HDAC superfamily, constituting five groups based on their sequence similarity and function (West & Johnstone, 2014). Class I is comprised of HDAC1, 2, 3 and 8, all of which have the simplest structure; class IIa includes HDAC4, 5, 7 and 9, which have large N-terminal extensions; class IIb consists of HDAC6 and 10, with extended C-termini; and class IV includes a single member, HDAC11 (Livyatan & Meshorer, 2013; West & Johnstone, 2014). In contrast, the class III sub-family comprises the Sirtuins (SIRT1–7), which although share features with both class I and class II HDAC enzymes, are a distinct family that are NAD+ dependent rather than being zinc-mediated modulatory enzymes (de Ruijter *et al*., 2003). In this context, the sirtuins are normally not included when describing HDACs. Within the

HDAC family, differences exist between to defined classes, with class I HDACs being expressed predominantly in the nucleus, and class II HDACs shuttling between the cytoplasm and the nucleus (Chang *et al*., 2006). The differential is now believed to be associated with different roles and functions of the HDACs, with class I responsible for acetylation of histones and class II playing a role in regulation of non-nuclear HDAC targets (de Ruijter *et al*., 2003).

1.4.2 Class I Histone deacetylases

The class I HDAC family consists of HDAC1, 2, 3 and 8, and localized predominantly to the nucleus with high enzymatic activity toward histone substrates, as shown in table I.2 (de Ruijter *et al*., 2003). Class I HDACs remove the acetyl groups from a ε-N-acetyl lysine amino acid on histone tails inducing chromatin condensation, by creating a non-permissive chromatin conformation and gene transcriptional repression (Barneda-Zahonero & Parra, 2012; Glozak & Seto, 2007). This balance between acetylation and de-acetylation of histones is an important regulatory role of gene transcription in cells, with hypoacetylation resulting in a decrease in the space between the nucleosome and the DNA, diminishing accessibility for transcription factors, leading to transcriptional repression (de Ruijter *et al*., 2003).

1.4.3 Class II Histone deacetylases

The class IIa HDACs consist of HDAC4, -5, -7 and -9, as shown in table I.2. In contrast to class I, these HDACs are expressed in a tissue-specific manner (Lakshmaiah *et al*., 2014) and they contain both a nuclear localization signal (NLS) and a nuclear export signal (NES) in their structure, aiding nucleoplasmic shuttling of these enzymes (Livyatan & Meshorer, 2013). The phosphorylation of the amino termini of class II HDACs by calcium/calmodulin-dependent kinase (CaMK) and protein kinase D (PKD), creating docking sites for the 14-3-3 family of chaperone proteins, promotes shuttling of these HDACs between nucleus and cytoplasm (Chang *et al*., 2006). Furthermore, the class IIa HDACs exhibit low deacetylase activity towards histones, and preferentially target non-histone proteins, with many of these protein targets involved in regulation of gene expression, cell proliferation, migration and death pathways, including p53, E2F and MyoD (Dokmanovic *et al*., 2007; de Ruijter *et al*., 2003; Gao *et al*., 2006)

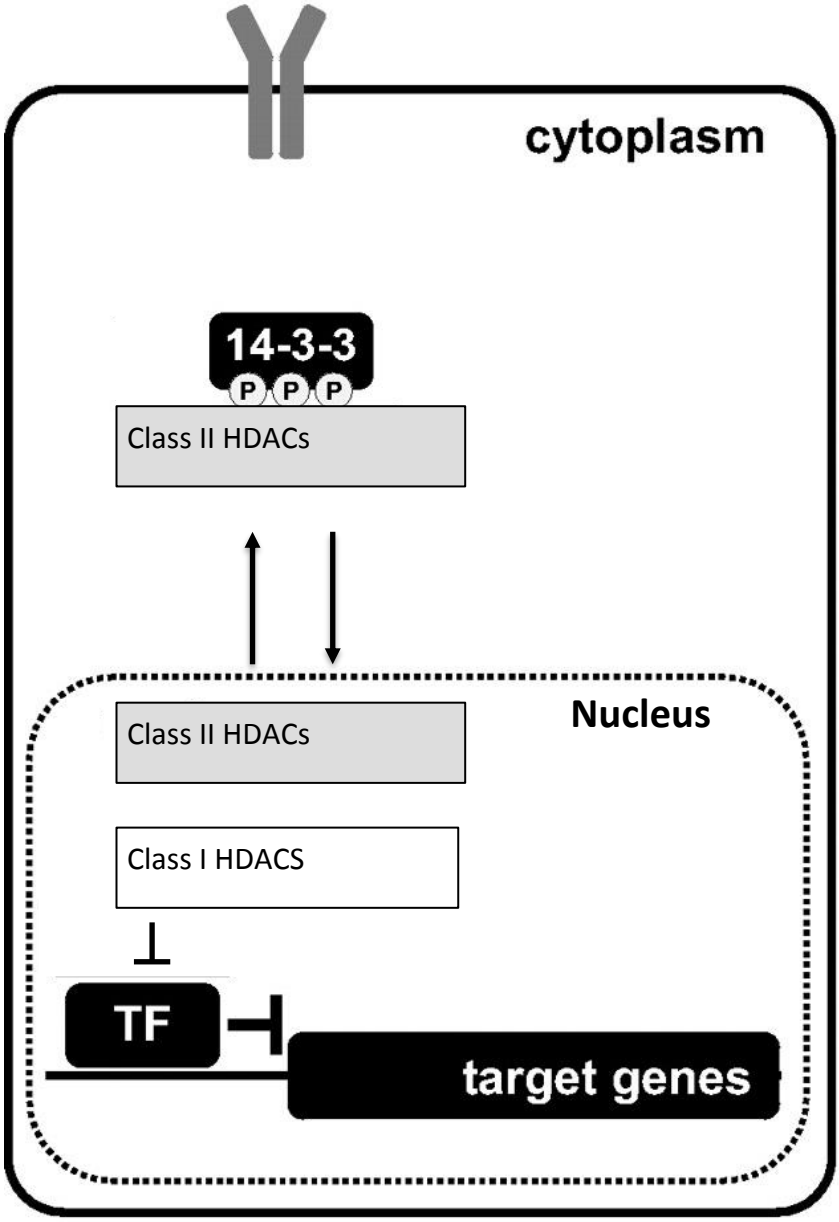


Figure I.6 Localisation of the class I HDACs versus class II HDACs. Class I HDACs are localised to the nucleus of the cell where they are known to deacetylate histones. Class II HDACs can shuttle between the nucleus and the cytoplasm of the cell, they are known to have low deacetylase activity, so are thought to work on non-histone targets [Adapted from Ha *et al*., 2008].

I.4.4 Pertubation of HDAC expression and activity in cancer

Cancer is linked to histone hypoacetylation, due largely to overexpression of class I HDACs, a factor resulting in several of these enzymes being classified as oncogenes. Abnormal expression of many HDACs is associated with numerous cancers (Barneda-Zahonero & Parra, 2012), for example HDAC1 has been found to be overexpressed in colon (Wilson *et al*., 2006), breast (Zhang *et al*., 2005), prostate (Halkidou *et al*., 2004) and gastric (Choi *et al*., 2001) cancers. Similarly, the class I HDAC; HDAC2 has been found to be overexpressed in gastric (Song *et al*., 2005) and cervical (Huang *et al*., 2005) cancers. Other HDACs, respectively class I; HDAC3 and class IIb; HDAC6 have been reported to be over expressed in colon and breast cancers (Ropero & Esteller, 2007). With regards the molecular association between HDAC overexpression and tumorigenesis, removal of acetyl groups from histones (by class I HDACs) leads to repression of several tumour suppressor genes, such as the cell cycle regulator locus CDKN2A (including $p16^{INK4A}$ and $p14^{ARF}$) and the DNA repair gene, BRCA1 (Tan *et al*., 2004). Loss of these tumour suppressor genes such as BRCA1, increases genomic instability (cancer hallmark) because of the failure of these DNA repair pathways (Zamborszky *et al*., 2017).

However, conversely to HDAC overexpression, there are now studies showing some cancers exhibit a reduction in expression of HDACs which is associated with a poor prognosis in patients (Osada *et al*., 2004; Weichert, 2009) because downregulation or deletion of HDACs may play a role in cancer progression (West & Johnstone, 2014). For instance, HDAC7 expression is decreased in lung cancer (Osada *et al*., 2004), HDAC3 has found to be decreased in cancer of the liver and HDAC4 is routinely decreased in gastric tumours (West & Johnstone, 2014). HDAC1 somatic mutations

were detected in approximately 8% of dedifferentiated human liposarcomas (Taylor *et al*., 2011), and a frame-shift mutation causing dysfunctional HDAC2 expression was observed in human epithelial cancers with microsatellite instability (Ropero *et al*., 2006). Together these data suggest a putative tumour suppressor role for some HDACs (West & Johnstone, 2014).

This association between HDACs and cancer, coupled to the potential targetability of these enzymes, led to significant efforts to develop therapeutic inhibitors with the objective of restoring the histone acetylation balance and inhibition of cancer growth (Richon, 2006; Greshock *et al*., 2008). To date, four HDAC inhibitors (Vorinostat, Romidepsin, Bellinostat, Panobinostat) have been approved for cancer treatment, specifically lymphomas and myelomas (West & Johnstone, 2014). From a molecular perspective, HDAC inhibition modulates and impinges upon many tumorigenic characteristics (hallmarks of cancer), including proliferative capacity, response to cell death signalling, angiogenesis, and immune evasion.

I.4.5 Role of class IIa HDAC7 in cancer

Despite significant interest in the class I HDACs and cancer, very little is known regarding class II HDACs. For instance, there is now a strong implication for an involvement of the class IIa member histone deacetylase 7 (HDAC7) in regulation of vasculature structure, and thus by default angiogenesis, a cancer hallmark (Chang *et al*., 2006). HDAC7, is expressed within the developing vascular endothelium (Chang *et al*., 2006) and plays a central role in regulating blood vessel structure and organisation (Ha *et al*., 2008). Within blood vessels, vascular integrity is regulated by tight

junctions between neighbouring endothelial cells (ECs), which form the inner lining of the blood vessels (Chang *et al*., 2006). The maintenance of blood vessel structure and integrity mediated by HDAC7 is shown to involve modulation of the expression and function of factors involved with endothelial cell morphology, cell-cell attachments, and subsequent vessel formation (Ha *et al*., 2008). HDAC7 has also been found to be involved in the initiation of tumour development with it potentially playing a role in tumour angiogenesis (Zhu *et al*., 2011). Hypoxia-inducible factor (HIF)-1∝ is a transcription factor that controls expression of genes responsive to low oxygen tension, including vascular endothelial growth factor (VEGF), which is released for blood vessel formation (Kato *et al*., 2004). The inhibitory domain of HIF-1∝ strongly interacts with the C-terminal domain of HDAC7 (Kato *et al.*, 2004). HDAC7 has also been linked to the regulation of MMP-10 in NSCLC, strongly suggested its role in tumour angiogenesis (hallmark of cancer) (Zhu *et al*., 2011).

I.4.6 HDAC7 and MMP-10

The involvement of HDAC7 in vasculature integrity is strongly linked to the activity of MMP-10. In terms of a link between HDAC7 and MMP-10, as previously discussed, MMP-10 expression has been shown to be repressed *in vivo* by the activity of HDAC7 (Chang *et al*., 2006), an association supported by the dramatic upregulation of MMP-10 expression in the developing vascular endothelium of HDAC7 mutant mouse embryos (Chang *et al*., 2006). Silencing of HDAC7 in endothelial cells resulted in alteration of their morphology, motility and led to an inability to form tube structures, an outcome mediated by proliferation or apoptosis of the endothelial cells

(Mottet *et al*., 2007), an observation in agreement with the involvement of MMP-10 (Chang *et al*., 2006; Mottet *et al*., 2007). In addition, HDAC7 angiogenic activity and modulation of MMP-10 activity has been shown to be regulated by VEGF signalling (Ha *et al*., 2008). Taken together, this strongly suggests that HDAC7 expression and activity controls MMP-10 expression and activity, offering a relationship between HDAC7 and MMP-10 in the control of tumour establishment and growth.

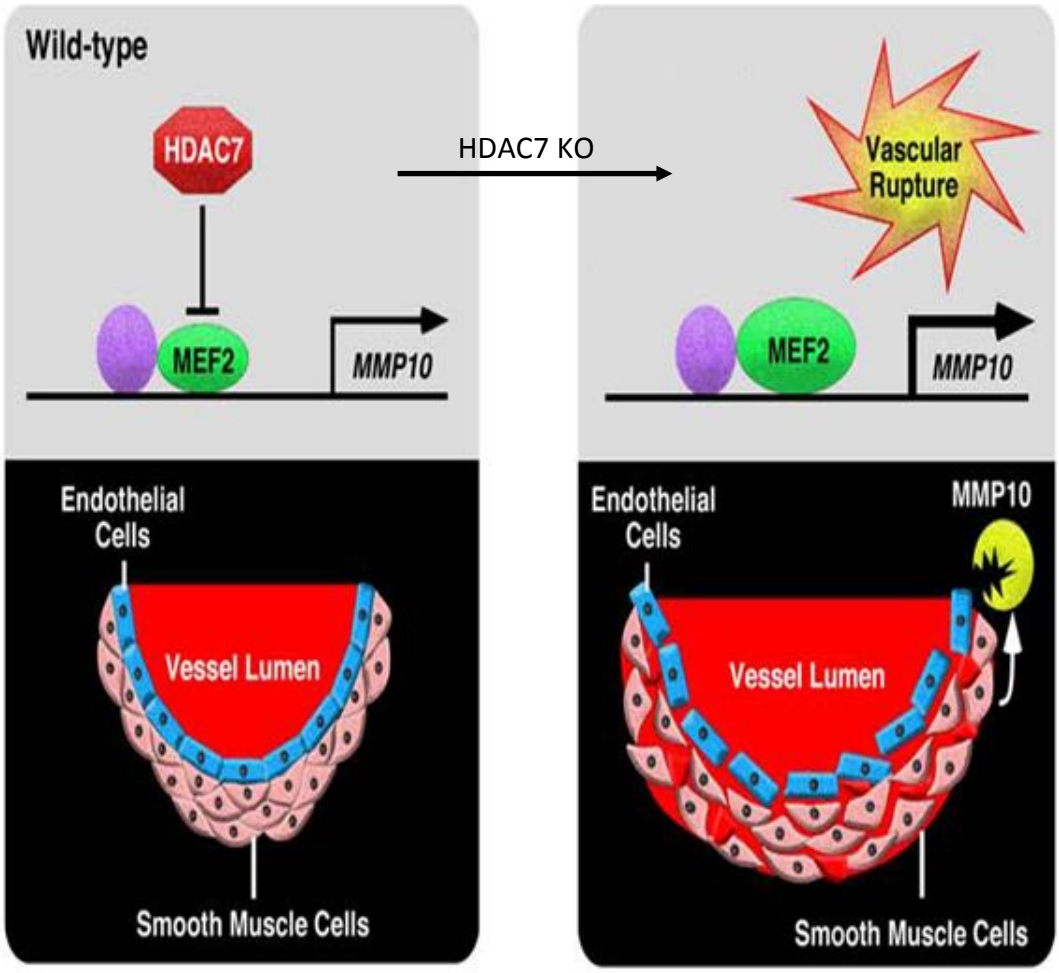


Figure I.7 A model for modulation of MMPs by HDAC7 through inhibition of MEF2 activity. MMP-10 expression is controlled by MEF2, which is antagonized by HDAC7 in ECs. In the absence of HDAC7, MMP-10 is overexpressed and vascular integrity is disrupted [Adapted from Chang *et al*., 2006].

In regards molecular pathways involved in HDAC7 activity and potentially MMP-10, HDAC7 is known to control myocyte enhancer factor-2 (MEF2), a transcription factor involved in EC differentiation, by inhibiting its transcriptional regular region; MADS (MCM1-agamous deficiens-serum response factor) region through the N-terminal of HDAC7 (Dressel *et al*., 2001). Simultaneously, it is known that MMP-10 is a direct target of MEF2, further supporting a relationship that exists between HDAC7 and MMP-10 (Chang *et al*., 2006). Mechanistically, HDAC7 undergoes nucleocytoplasmic shuttling (Fischle *et al*., 2001), allowing HDAC7 to prevent MEF2-mediated transcription (Chang *et al*., 2006). This repressive activity of HDAC7 upon MEF2 in addition to being facilitated by nucleocytoplasmic is further controlled by its phosphorylation-dependent association with the intracellular 14-3-3 proteins. Phosphorylation of conserved residues in the N-terminal regions of HDAC7 in response to cellular signals leads to interaction with 14-3-3 proteins, dissociation of the HDAC7-MEF2 complex, and a conformational change culminating in export from the nucleus (Parra *et al*., 2005). VEGF has been found to stimulate HDAC7 causing cytoplasmic accumulation of HDAC7 in the endothelial cells (ECs), by activating PKD1-mediated phosphorylation at the serine sites within its N-terminus (Ha *et al*., 2008), represented in figure 1.8.

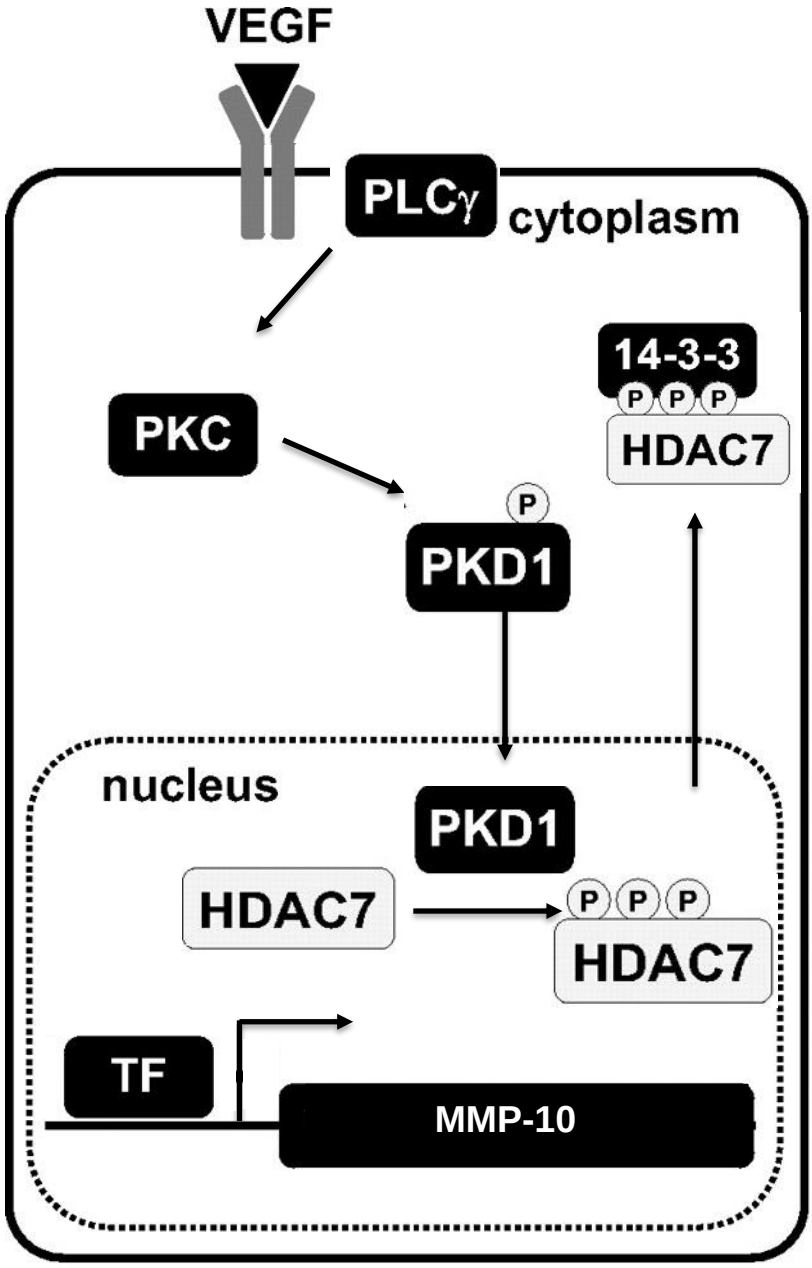


Figure I.8 Potential mechanism by which HDAC7 regulates MMP-10 gene expression. VEGF stimulated HDAC7 phosphorylation and nuclear export in endothelial cells though a VEGF receptor 2-phospholipase Cγ-protein kinase C-PKD-dependent pathway. The PKD-HDAC7 pathway mediated transcription factor; myocyte enhancer factor-2 transcriptional activation and expression of MMP-10, in response to VEGF [Adapted from Ha *et al*., 2008].

Vorinostat, an HDACi for T-cell lymphoma, was evaluated in cancers overexpressing HDAC7 with the intention of addressing the effect of inhibiting HDAC7 activity (Dokmanovic *et al*., 2007). Vorinostat treatment resulted in a reduction of HDAC7 mRNA level with no change in protein stability (Dokmanovic *et al*., 2007). Cutaneous T cell lymphoma causes lesions to appear on the skin and although there is no cure, Vorinostat has shown a benefit for a third of patients by either reducing the amount of skin affected or relieving the symptoms of pruritus (Duvic & Vu, 2007).

In comparison, HDAC7 suppression by small interfering RNA-mediated knockdown was associated with growth arrest but without detectable changes in acetylation of histones or p21 gene expression, the key biomarkers of 'conventional' histone deacetylase activity (Dokmanovic *et al*., 2007). Therefore, despite being overexpressed in cancers, this strongly supports the suggestion that HDAC7 exerts its activity not via 'conventional' HDAC nuclear activity and histone acetylation but rather by modulation some other cellular functions, which may or may not be dependent on its acetylation activity. Subsequently, in light of the weak enzymatic activity and cellular location, the repression of HDAC7 protein expression by Vorinostat also strongly suggests a regulatory involvement of HDAC activity upon expression of HDAC7 itself, implying intra-family regulation pathways.

I.5 Rationale and Aims

The objective of much current oncology research is the identification of therapeutic targets and the development of novel effective therapeutic agents. This is especially

true for those cancers which are increasing in incidence and for which current therapies are not truly effective, such as NSCLC where conventional chemotherapy has minimal efficacy. A major drawback of many current anticancer agents is their lack of tumour specificity, which has prompted a renewed focus on targeted therapy based on pathways altered during the pathogenesis of lung cancer.

This study aimed to characterise the relationship between matrix metalloproteinase-10 (MMP-10) and the histone deacetylase (HDAC) class of enzymes, particularly HDAC7. In particular, the mechanism by which HDAC7 influences expression of MMP-10 and whether this involves enzymatic activity of HDAC7, or a functionality of this enzyme independent of its deacetylase activity.

The first aim of this study was to demonstrate whether a relationship existed between HDAC7 and MMP-10. Studies have suggested an inverse correlation between HDAC7 and MMP-10 expression in clinical NSCLC; therefore, this was to be explored in an *in vitro* model of NSCLC.

The second aim of the study was to address the involvement of other HDACs in this HDAC7-MMP10 relationship.

Chapter II - General Materials and Methods

II.1 Chemicals

All general reagents were obtained from Sigma-Aldrich Company Ltd (UK) including Roswell Park Memorial Institute (RPMI) medium, OptiMEM Reduced Serum medium, Foetal Bovine Serum (FBS), L-Glutamine, Trypsin/EDTA, Hanks' Balanced Salt Solution (HBSS), Ethanol (EtOH), Phosphate buffered saline (PBS), Agarose, Ethidium Bromide, β-mercaptoethanol (β-ME), Ethylenediaminetetraacetic acid (EDTA).

II.2.1 Growth and maintenance of Non-Small Cell Lung Carcinoma (NSCLC) cell line

Human NCl-H460 (H460) cells were obtained from the America Type Culture Collection (ATCC); HTB-177. These cells are an immortalised non-small cell lung carcinoma cell line derived from the pleural effusion of patient with a large cell carcinoma of the lung.

H460 cells were grown *in vitro* and routinely maintained as monolayer cultures in RPMI medium that was supplemented with 10% FBS and 2mM L-Glutamine, (termed 'complete media'). In 75cm^2 tissue culture flasks (Corning, UK) and incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

H460 cells were routinely passaged when they reached 75-85% confluency (in exponential growth phase). The media was discarded and the cells washed twice with HBSS to remove any excess media. Cells were harvested by trypsinisation using pre-warmed 0.25% Trypsin-EDTA (Sigma, UK), centrifuged (Heraeus Megafuge 16, Thermofisher Scientific, UK) at 1000rpm (200 x g) for 5 minutes at room temperature and the resultant cell pellet resuspended in fresh complete media to neutralize activity

of trypsin, cells were then either split into new flasks or stored as a pellet at -20°C, if required for future analysis.

II.2.2 Manual counting of cell number

Cells detached using 0.25% trypsin-EDTA solution and re-suspended in fresh complete medium were counted as follows. A 10µl sample of the cell suspension was placed into a hemocytometer chamber and counts were obtained from 5 grids of the hemocytometer chamber. Cell counts were calculated as the mean of the 5 counts; cell numbers expressed as (mean cell count) x 10^4/ml medium. The value was adjusted in light of the total suspension volume to obtain total cell number per flask.

II.2.3 Cryopreservation of cells

Confluent cells were harvested and re-suspended in fresh complete media. Approximately 1x10^8 cells in a final volume of 1ml were transferred to a 2.0ml NuncTM CryoTube TM (Fisher Scientific) to which 100µl Dimethyl sulfoxide (DMSO) was added (final concentration of 10%) to protect the cells against the freezing process. Tubes were placed into a cryogenic freezing container (Mr. Frosty, Thermofisher Scientific, UK) submerged in isopropanol, and the container transferred into a -80°C freezer overnight. Cell vials were then transferred to liquid nitrogen for long term storage; the use of the cryogenic 'Mr. Frosty' freezing container permits a rate of cooling of -1°C/minute, the optimal rate for cell preservation.

II.2.4 Monitoring of cell proliferation and viability using xCELLigence real-time cell analyzer system (xCELLigence RTCA)

The xCELLigence system allows monitoring of cell proliferation and viability in real time using impedance of an electric current to non-invasively quantify cell adherence (depicted in figure II.1). Cells are seeded into E-plates (incorporating interdigital electrodes into bottom of wells), the interaction of cells with the electronic biosensors generates a cell-electrode impedance response that signifies the surface area of the respective well impeding a low current, indicating cell viability and morphology, and consequently cell number (see figure II.1).

Cells were seeded into wells of the E-plate at the required cell density (range 250-8000 cells/well). THE RTCA DP software was set up according to the manufacturer's instructions, including experimental layout (cell name, cell number, designation of well use) and experimental schedule (number of readings, interval of readings). Prior to cell addition to E-plates, 100µl of cell media was added to each well and a background reading carried out. After the background reading was performed, 100µl of cell suspension (at 2x required concentration) was added to each well in duplicate, with 100µl of media added to the control lane. The E-plate was left at room temperature for 30 minutes, to allow cell distribution and initial attachment. The E-plate was then transferred to the cradle of the RTCA DP analyser, within the incubator, and the experiment initiated. During the experiment, the data was viewed in real-time to monitor changes in cellular impedance, indicative of cell attachment, morphology change, and cell growth. Data is displayed as cell index, an arbitrary value based on changes in cellular impedance relative to cell-free background.

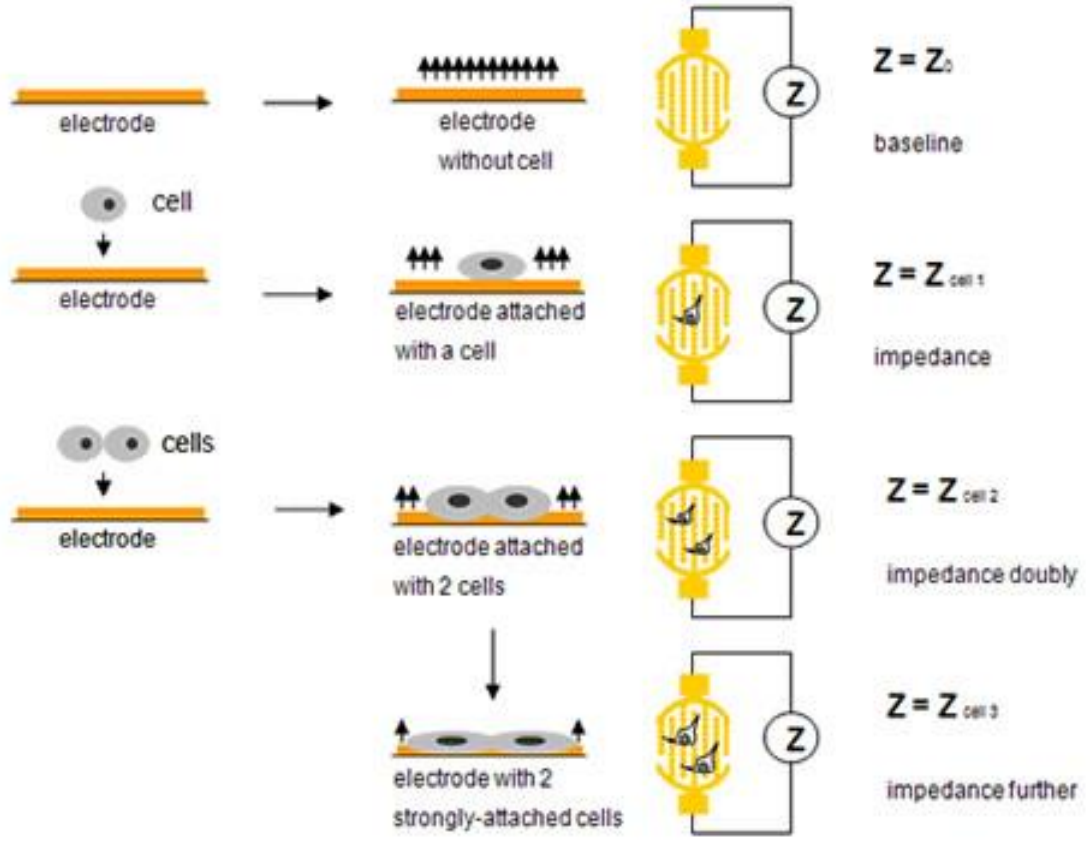


Figure II.1 Principle of impedance based cell detection. In the absence of cells, electric current flows freely through culture medium, completing the circuit between the electrodes. As cells adhere to and proliferate on the surface of the well, electrodes current flow is impeded, providing an extremely sensitive readout of cell number, cell size/morphology, and cell-substrate attachment quality [adapted from ACEA Bi-osciences, Inc, 2016].

II.2.5 Determination of cell viability using the MTT assay

The MTT assay is a cell viability analysis using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue) compound. This technique involves conversion of MTT from its soluble form (yellow in colour), to its insoluble form (purple colour). This transformation between soluble to insoluble form only occurs in living cells via the active mitochondrial hydrogenases. After treatment with a drug, its potential cytotoxic effect can be determined by the number of living or dead cells and this is quantified by the colour change (method adapted from; Mosmann, 1983). Cells were seeded on 96-well plates, and incubated for the designated time frame, the media removed and replaced with fresh media containing 1mg/ml MTT (method adapted from; Mosmann, 1983). Plates were incubated at 37°C for 4 hours, allowing mitochondrial dehydrogenase in viable cells to metabolise MTT and produce purple crystals of formazan. Media was carefully removed (so as not to disturb formazan crystals) and 150µl of DMSO added to each well to solubilise the formazan and produce a uniform colour in each well. The plate was analysed using a plate reader (Multiskan Go, Thermo Scientific) measuring absorbance at 540nm, where the absorbance reading, is directly proportional to cell number (Morgan, 1998).

II.3 Analysis of gene expression by reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR is a technique used to detect the RNA expression levels in a sample. It works by converting RNA to cDNA using the reverse transcriptase enzyme and this cDNA is used as a template for amplification in PCR.

II.3.1 RNA Extraction

Cells were grown as described in section II.2.1. Excess media was removed from cell pellets and RNA extracted using an RNeasy Mini Kit (Qiagen, UK). Briefly, the cell pellet was disturbed by the addition of lysis buffer; buffer RLT and the cell solution vortexed until the pellet was completely dispersed. The cell solution was added to a QIAshredder spin column and centrifuged for 2 minutes at 17,000 x g, at room temperature to homogenise the lysate. Solution transferred to spin column with equal volume of 70% EtOH and centrifuged at room temperature for 15 seconds at 10,000 x g, discarding the flow through. Buffer RW1 (700µl) was added to the RNeasy spin column and centrifuged at room temperature for 15 seconds at 10,000 x g, discarding the flow-through. Next, buffer RPE (500µl) was added to the spin column and the solution centrifuged at room temperature for 15 seconds at 10,000 x g. This step was repeated but centrifugation extended to 2 minutes, to dry the column and prevent ethanol carryover to RNA elution. Finally, RNase-free water (30µl) was directly added to the spin column membrane and the column centrifuged at room temperature for 1 minute at 10,000 x g to elute RNA.

Samples were analysed by measuring the relative absorbance spectrophotometrically (MultiSkan GO, Thermo Scientific, Finland) at 260nm and 280nm using a quartz cuvette. RNA purity is indicated by a 260:280 ratio of absorbance between 1.7-2.0. The concentration of RNA samples was analysed by assuming absorbance of 1.0 at 260nm is equivalent to 40µg/ml RNA (Warburg & Christian, 1942). Prior to measurement of absorbance, RNA samples were diluted into water.

II.3.2 cDNA synthesis: Reverse transcription

$dT_{23}VN$ primer (2µl) (New England Biolabs, UK) was added to 1µg of total RNA and the volume adjusted to 8µl using RNase-free water in a 0.2ml microcentrifuge tube. RNA samples were incubated at 70°C for 5 minutes, after which they were promptly put on ice to ensure primer annealing. To each RNA sample a cDNA synthesis mix was added consisting of: 10µl of 10x M-MuLV reaction mix and 2µl of 2x M-MuLV enzyme mix. This was mixed and incubated at 42°C for 1 hour, after which time the enzyme was inactivated by heating each sample to 80°C for 5 minutes. Samples were diluted with RNase-free water and vortexed to ensure proper mixing. cDNA samples were stored at -80°C. Appropriate negative controls (-RT) were constructed; RNA samples were treated as described but without the addition of M-MuLV enzyme mix in the cDNA synthesis mix, to demonstrate the level of genomic DNA contamination in subsequent PCR analyses.

II.3.3 PCR primer design

GAPDH primer design was based on findings of Daja *et al*. (2003) (Daja *et al*., 2003). MMP-10, HDAC7 and HDAC3 were designed in house (Table II.1). A BLAST search (http://www.ncbi.nlm.nih.gov/blast/index.shtml) was carried out to check homology and specificity of primers and revealed that all primers were specific to the relevant human DNA sequence. All Primers were obtained from Invitrogen Life Technologies (Glasgow, UK). Primers were made up to 10µM stock solutions in sterilised water, and stored at -20°C.

Table II.1: Sequences of primers used for RT-PCR to amplify to following human genes; HDAC7, HDAC3, MMP-10 and the housekeeping gene GAPDH**.** Supplied by Invitrogen Life Technologies

Name of gene	Orientation	Nucleotide sequences	Base pair size
MMP-10	Forward	5'-AGTTTGGCTCATGCCTACCC-3'	521
	Reverse	5'-AAAACGGTGTCCCTGCTGTT-3'	
GAPDH	Forward	5'-CCACCCATGGCAAATTCCATGGCA-3'	598
	Reverse	5'-TCTAGACGGCAGGTCAGGTCCACC-3'	
HDAC7	Forward	5'-CGCTCCCTGGGATAACTGTC-3'	538
	Reverse	5'-CTGATGAACCTGGCAGGAGG-3'	
HDAC3	Forward	5'-TCAGCCCCACCAATATGCAA-3'	511
	Reverse	5'-ACCTGGTTGATAACCGGCTG-3'	

Table II.2 RT-PCR conditions used for MMP-10, HDAC7, HDAC3 and the housekeeping gene GAPDH**.**

Primer Set	Annealing temperature (˚C)	Elongation time (seconds)	Number of cycles
MMP-10	65	60	35
GAPDH	60	90	25
HDAC7	55	60	25
HDAC3	60	90	25

II.3.4 PCR amplification of cDNA

PCR reactions were performed using the *Taq* PCR kit (New England Biolabs, UK). All reaction components were kept on ice throughout. Each cDNA sample (5µl) was placed in a 0.5µl thin-walled PCR microcentrifuge tube to which was added the following reaction mix: 10µl of Taq 2x Master Mix, 0.2µl forward primer (10µM), 0.2µl reverse primer (10µM) and 4.6µl RNase-free water (total reaction volume; 20µl). The contents of each tube were mixed well and briefly centrifuged. All samples underwent the following PCR programme, 94°C for 4 minutes followed by cycles of 94°C for 30 seconds, primer annealing for 30 seconds (Table II.2) and elongation at 72°C (Table II.2). Each program finished with 10-minute incubation at 72°C to ensure complete elongation of newly synthesised cDNA.

After cycling was complete, all PCR samples were stored at 4°C ready for analysis. The following controls underwent the PCR protocol described above; Positive controls consisted of sample DNA amplified in the presence of GAPDH primers whilst purity controls omitted cDNA. Negative (RT-) controls consisted of RT-PCR products created without the addition of M-MuLV enzyme mix (as detailed in section II.3.3).

II.3.5 Agarose gel electrophoresis to analyse PCR products

RT-PCR products were separated through a 1% v/w agarose/Tris base, acetic acid EDTA (TAE) gel containing 0.01% ethidium bromide. Prior to loading the gel, 10% loading dye (30% glycerol, 0.25% bromophenol blue) was added to each PCR reaction to allow sample visualisation. 1kb Plus DNA Ladder (2µl) (Invitrogen Life Technologies, UK) was loaded onto the gel, allowing for later verification of PCR product size.

Electrophoresis was carried out in 1 x TAE buffer (40mM Tris, 20mM acetic acid, and 1mM EDTA) at 100V for 1 hour to separate PCR products. cDNA was visualized in the gel under a UV light using the ChemiDoc MP imaging system with the image lab analysis software.

Densitometry was carried out using image J to quantify RT-PCR expression data, expressed as fold change compared to control.

II.4 Analysis of protein expression by western blotting

Western blot (also known as immunoblotting) is an important analytical technique that is used to identify a specific protein of interest from a cell sample, by separating proteins according to their molecular weight via gel electrophoresis and transferring these proteins to a PVDF membrane to probe for the protein of interest, using a specific antibody.

II.4.1 Preparation of cells for western blotting

Cells were grown to subconfluency in 6 well plates. Ice cold lysis buffer was applied directly onto the cell monolayer and a cell scraper (Sarstedt, Germany) used to collect the extract. The sample was centrifuged at 17,000 x g for 10 minutes at 4°C, the supernatant collected and the pellet discarded.

II.4.2 Protein concentration determination for western blotting

A Bradford assay is an analytical technique that is used to measure the amount of protein in a sample. Initially, 2µl of sample was removed and mixed with 38µl of di-

ionised water, vortexed. For each sample, 10µl was removed and added to the wells of a 96 well plate, several known concentration samples (standards) were created using BSA (range 0-1 µg/µl), 200µl of Bradford assay reagent (Bio-Rad, UK) was added to each of the wells containing the protein sample, and this was incubated for 5 minutes at room temperature. After 5 minutes, the plate was put into a microplate spectrophotometer (MultiSkan GO, Thermo Scientific, Finland) and read at a wave-length of 550nm. A calibration graph was constructed by plotting the absorbance of the standards against their concentrations, with this used to determine the test samples concentrations by interpolating.

II.4.3 Polyacrylamide gel electrophoresis

Proteins were separated through 10% polyacrylamide SDS gels (for composition of gel see appendix A). Each lane was loaded with a relevant amount of sample (30µg following optimisation) diluted in extraction buffer and sample buffer (Laemmli 2x concentrate) to make a final volume of 30µl. Samples were denatured at 95°C for 3 minutes and immediately transferred to ice prior to loading. Gels were loaded into running apparatus (Bio-Rad, UK) immersed in electrophoresis running buffer (25mM tris, 200mM glycine, 3.5mM SDS) and the samples loaded into the gel. In addition, one lane was loaded with Precision plus protein ladder (Bio-Rad) to allow for latter protein mass determination. Electrophoresis was performed at 200V for 45 minutes until the marker of the relevant to the size of the protein of interest was located in the centre of the gel.

II.4.4 Protein transfer to PVDF membrane

Protein was transferred from the acrylamide gel to a PVDF membrane (Ambersham Hybond P0.45, GE Healthcare Life Sciences), the gel and membrane were assembled into a sandwich (set-up shown in figure II.2), and placed in a transfer kit (VWR), immersed in transfer buffer (20mM Tris, 150mM glycine, 20% methanol). The transfer tank was placed in an ice bath and the transfer performed for 60 minutes at 300mA. After protein transfer the membrane was removed, rinsed in H_2O and then immersed in 5% w/v non-fat dry milk solution in TBS-Tween (10mM Tris, 150mM NaCl (pH8.5), 0.1% Tween 20) for 60 minutes at room temperature to block non-specific antibody binding.

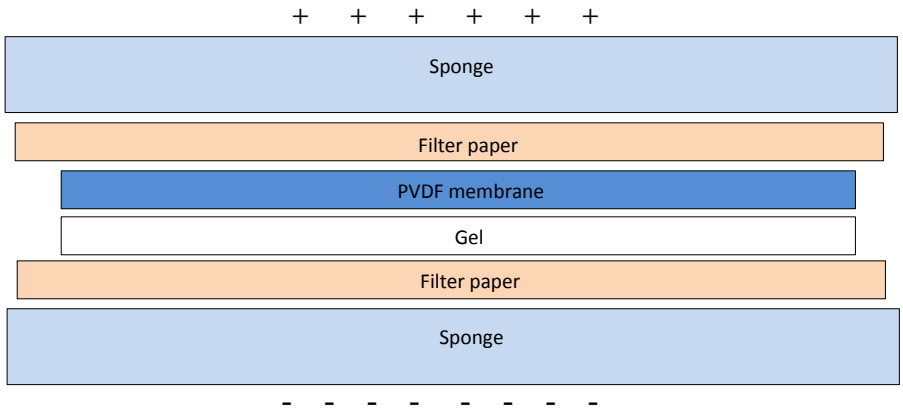


Figure II.2 Illustration of western blot transfer set-up. PVDF membrane placed closest to the cathode to allow negatively charged proteins on the acrylamide gel to be transferred to the PVDF membrane.

II.4.5 Antibody incubation

Antibodies were diluted to their final concentration (Table II.3) in 1% w/v non-fat dry milk solution in 0.1% TBS-Tween the membrane submerged in it, and this incubated overnight at 4°C with agitation. The membrane removed then excess antibody cleared by washing the blot 3 times for 5 minutes in 0.1% TBS-Tween. The blot was incubated with the appropriate horseradish peroxidase conjugated secondary antibody at a 1:2000 dilution in 1% w/v non-fat dry milk solution in TBS-Tween, for 60 minutes at room temperature. Blots were rinsed for a further 3 times for 5 minutes in 0.1% TBS-Tween to remove excess secondary antibody.

Table II.3 Western blotting conditions for MMP-10, HDAC7, HDAC3 and the house-keeping protein β-actin**.**

Primary antibody	**Type**	**Protein targeted**	**Dilution**	**Secondary antibody**	**Bands molecular weight (kDa)**
Novacastra	Mouse monoclonal	Human MMP-10	1 in 500	horse anti mouse	57 pro-MMP-10
Abcam ab32369	Rabbit monoclonal	HDAC3	1 in 1000	goat anti rabbit	49
Abcam Ab12174	Rabbit polyclonal	HDAC7	1 in 500	goat anti rabbit	105
Sigma A2228	Mouse monoclonal	β-actin	1 in 50000	horse anti mouse	42

II.4.6 Visualisation of antibody binding

Antibody binding was detected chemi-luminescently using ECL (Bio-Rad). Western blots were exposed to ECL for 3 minutes at room temperature; excess reagent blotted from the membrane, the membrane sealed in a saran wrap, and chemi-luminescence detected using the ChemiDoc MP imaging system and associated image lab analysis software (4.1), to detect proteins of interest.

Densitometry was carried out using image J to quantify protein expression data, expressed as fold change as compared to control.

II.5 Statistical analyses

All statistical analyses on MMP-10, HDAC7 and HDAC3 gene and protein expression data were performed using GraphPad Prism (version 7). Unpaired t-tests were used to analyse experiments with only one experimental treatment group versus vehicle; whereas a one-way ANOVA (analysis of variance) was used when multiple treatment groups were compared to vehicle. The ANOVA indicated equal or non-equal variance; where variance was found to be non-equal a Dunnetts test was used. For all tests, a P-value of less than 0.05 was considered statistically significant.

Chapter III: Expression of HDAC7 and MMP-10 in human NSCLC cell line *in vitro*

III.1 Introduction

Lung cancer is the leading cause of cancer deaths in both men and women, with more than 1 million deaths worldwide each year (Torre *et al*., 2015; Petty *et al*., 2004; Wang *et al*., 2016). Unfortunately, due to late prognosis and poor treatment response, this therapeutic area has shown the highest failure rate in clinical trials over the last 30 years (Constant *et al*., 2015). Consequently, greater understanding of NSCLC pathophysiology and molecular alterations therein are paramount to improving treatment of this life-threatening disease (Petty *et al*., 2004).

Next-generation sequencing has revealed that more than 50% of human cancers harbour mutations in enzymes that are involved in chromatin organization (Jones *et al*., 2016). Tumour cells are now known to employ epigenetic alterations to drive tumorigenesis and the hallmarks of cancer, and from a therapeutic perspective to support their escape from chemotherapy and host immune surveillance (Wilting & Dannenberg, 2012). Hence, a growing emphasis of recent drug discovery efforts has been on targeting the epigenome, including DNA methylation and histone modifications (Jones *et al.*, 2016). Histone modifications are post-translational modifications (PTMs) to histone proteins which can impact gene expression by altering chromatin structure or recruiting histone modifiers (Bannister & Kouzarides, 2011), including methylation, phosphorylation, acetylation, ubiquitination and sumoylation (Duan & Walther, 2015). The most common of these; histone acetylation, is the enzymatic addition of an acetyl group ($COCH_3$) from acetyl coenzyme A to the histone tails (Sterner & Berger, 2000). The process of histone acetylation is tightly involved in the

regulation of many cellular processes including chromatin dynamics and transcription, gene silencing, cell cycle progression, nuclear import (Cohen *et al*., 2011). The modifying enzymes involved in histone acetylation are called the histone acetyltransferases (HATs) (Cohen *et al*., 2011), in opposition to the HATs are the histone deacetylases (HDACs), which catalyse the hydrolytic removal of the acetyl group on the lysine tails of the histones, causing chromatin condensation and ultimately transcription repression (Legube & Trouche, 2003). Aberrant regulation of gene expression is at the basis of many human diseases and notably of many forms of cancer, including NSCLC (Gallinari *et al*., 2007; Zochbauer-Muller *et al*., 2002). Osada and colleagues found reduced expression levels of class II HDACs in clinical NSCLC, is linked to poor prognosis in patients (Osada *et al*., 2004).

Within the HDAC family the class IIa HDAC; HDAC7 has received little attention. However, a role for this HDAC has been shown in vascular development (Chang *et al*., 2006; Ha *et al*., 2008). In contrast to class I, these class II HDACs exhibit low deacetylase activity towards histones, and preferentially target non-histone proteins (de Ruijter *et al*., 2003). HDAC7 has been linked to the regulation of the secreted endoproteinase; MMP-10 (Chang *et al*., 2006). MMP-10 over expression has been demonstrated in NSCLC (Gill *et al*., 2004), with it thought to play a role in the early stages of tumour development, being key in the remodelling of vascular endothelial cells during angiogenesis, with increased expression linked to decreased tumour growth *in vivo* (personal communication by Gill, figure I.3). Therefore, there is a rationale for viewing MMP-10 and potentially HDAC7 as of molecular importance and therapeutic target in NSCLC (discussed in chapter I). The aim of this chapter is to characterise the

expression and activity of HDAC7 and MMP-10 in a human cell line (H460) to establish their putative roles as therapeutic targets in NSCLC.

III.2 Methods

III.2.1 H460 cell line growth kinetics, by manual counting

Growth kinetics of H460 cells were characterised in order to utilise exponential cell growth. Cells were trypsinised and counted as described in section II.2.2, with $1x10^4$ cells plated into each of the ten $25cm^2$ flasks in a total volume of 10mls per flask. H460 cell flasks were maintained at 37°C in conditions of 5% CO_2 (as described in section II.2.1) and cell counts performed every 24 hours, for a total of 10 days. Growth curves were repeated 3 times using different passages for the cells, with growth curves plotted as a mean of 3 growth curves.

III.2.2 H460 cell line growth analysis, by MTT metabolism

Growth curve analysis for H460 cells were conducted in 96-well plates. Varying cell densities of each cell line were seeded (25, 50, 100, 250, 500, 1000 and 2000 cells per well) and monitored for 5 days to show cell growth kinetics. On each day, the increase in cell number for each cell seeding density, was calculated using the MTT assay (method described in section II.2.5).

III.2.3 H460 cell line growth analysis, by xCELLigence RTCA

Growth curve analysis was conducted in real-time using the xCELLigence RTCA, using the method described in section II.2.4. Using a cell concentration of 80,000 cells/ml, serial dilutions were created to provide a range of cell concentrations ranging from 80,000 to 2500 cells/ml. The software was programmed as described in section II.2.4,

background readings performed, and 100µl cell suspension added to relevant wells (corresponding to 8000-250 cells/well). Cellular analysis was performed over a 5-day period, with readings taken every 30 minutes during this time (250 sweeps x 30 minutes). Cellular growth was determined by analysis of cell index over time.

III.2.4 Expression of HDAC7 and MMP-10 in human H460 NSCLC cell line *in vitro*

H460 cells were plated at a density of $4x10^5$ cells per well in 6 well plates and incubated in complete media for 24 hours. Following incubation, mRNA was extracted, converted to cDNA (as described in section II.3.2), and used as a template for reverse transcriptase with primers specific to HDAC7 and MMP-10 (see table II.1 for primer sequences and table II.2 for optimisation data). RT-PCR products were separated using agarose gel electrophoresis, visualised and analysed as described in section II.3.5.

III.2.5 Expression of HDAC7 and MMP-10 proteins in human H460 NSCLC cell line *in vitro*

H460 cells were plated at a density of $4x10^5$ cells per well in 6 well plates and incubated in complete media for 24 hours. Following incubation, protein was extracted and quantified (as described in section II.5.2), samples prepared and proteins fractionated using SDS-PAGE (see section II.5.3 for details). Proteins were transferred to a membrane and probed with antibodies to detect human MMP-10 and HDAC7, as described in section II.5.4/II.5.5 (see table II.3 for antibody details and concentrations).

III.2.6 Evaluation of MMP-10 protein activity levels in human H460 NSCLC cell line *in vitro*

H460 cells were plated at a density of $4x10^5$ cells per well in 6 well plates and incubated in complete media for 24 hours. To assess the levels of active endoprotease enzymes present in cell media, aliquots of media were removed at various time points over the duration of the time course (24-96h). The fluorometric enzyme assay was performed in a 96 well plate by incubating 10µl of media with 50µg of fluorogenic substrate M-2110 (Mca-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(Dnp)-NH_2) in 80µl of MMP activity buffer (100 mM Tris-HCl pH 7.6, 10 mM CaCl2, 100 mM NaCl, 0.16% v/v Brij-35); total reaction volume of 100µl. Fluorescence was measured (excitation = 325 nm; emission = 393 nm) over a 600-minute incubation period at 37°C. For each sample, the relative activity of the initial linear enzyme activity was calculated, and activity expressed as change in fluorescence per hour per $4x10^5$ cells. The reproducibility and reliability of the assay were determined using samples obtained from three independent experiments.

III.3 Results

III.3.1 NSCLC cell line growth kinetics, by manual counting

Growth patterns of H460 cells were determined using three methodologies to evaluate growth kinetics and characterisation of the value of each methodology. Growth curves are shown in figure III.1, from this data, the duration of the exponential phase and hence optimum time of harvesting for H460 cells was identified. The growth phases were identified as; lag phase 0-48 hours; exponential phase 48-120 hours; plateau phase >120 hours. Doubling time in exponential growth phase was 19.2 hours. For drug analysis in a T25cm^2 flask, the addition of 5-50x10^4 cells/flask would allow for drug addition during the exponential phase.

III.3.2 xCELLigence growth curve analysis

Classically determination of timings for drug addition or sample collection in *in vitro* assays is based on a time point that is convenient (24 h post seeding) rather than based on empirical or behavioural data from the cells (Kho *et al*., 2015). However, the real-time data provided by the xCELLigence RTCA reveals extra information related to the behaviour, growth and health of the cells, which can be used to guide and improve the experimental design. This information can be used to identify an appropriate time window for drug treatments and to ensure that this occurs consistently across studies or experiments. H460 cells were seeded at a high density (8000, 4000 and 2000 cells/well) and low density (1000, 500 and 250 cells/well) to determine an ideal seeding density for subsequent experiments. Low density seeding cell

numbers were found to have a long lag time and took between 61-101 hours to reach exponential phase of growth, discounting these densities for subsequent studies. The highest cell number seeded (8000 cells/well) did not exhibit lag phase of growth, discounting this cell number from subsequent studies because drug addition would have to be almost instant after seeding cells, not allowing for an incubation period. 4000 cells/well was subsequently chosen as the ideal cell number for subsequent drug treatment studies, because this would allow a 24-hour incubation period prior to drug addition (as carried out in other cellular growth studies) and the cells to be in exponential growth phase during drug incubation. The growth phases for 4000 cells/well were identified as; lag phase 0-32 hours; exponential phase 32-72 hours; plateau phase; >73 hours. Doubling time in exponential phase was 18 hours. Table III.3 compares doubling times and different phases of growth with different densities of H460 cells.

III.3.3 MTT growth curve analysis

In order to observe the growth characteristics of the H460 cell line, in which the cytotoxicity of compounds was to be assessed, growth curves of different cell densities were performed and analysed by the MTT assay (figure III.3). The three highest densities (2000, 1000 and 500 cells/well) were discounted for subsequent toxicity studies because the cell growth plateaued (2000 and 1000 cells/well) or slowed (500 cells/well) during the assay (>72 hours). The lowest cell density (100 cells/well) was also discounted for subsequent studies because it doesn't reach exponential phase of growth until 48 hours, meaning the subsequent toxicity experiments would be

carried later than other assays. Therefore, 250 cells per well was chosen as the seeding density during cytotoxicity assays because the cells are in the exponential phase of their growth cycle for the duration of the assay, which allowed drug addition of subsequent experiments after 24 hours of cell growth(figure III.3).

III.3.4 Optimisation of RT-PCR evaluation of MMP-10 and HDAC7

To assess the gene expression of the matrix metalloproteinase; MMP-10 and the class IIa HDAC; HDAC7 in NSCLC *in vitro*, primer pairs were optimised in order to identify an appropriate annealing temperature, elongation time and number of PCR cycles to yield optimum reaction products (table II.2 for optimised primer conditions). The amount of cDNA used in each PCR reaction was optimised using a range of cDNA concentrations (100-5ng) and the ideal concentration was found to be 50ng (figure III.4).

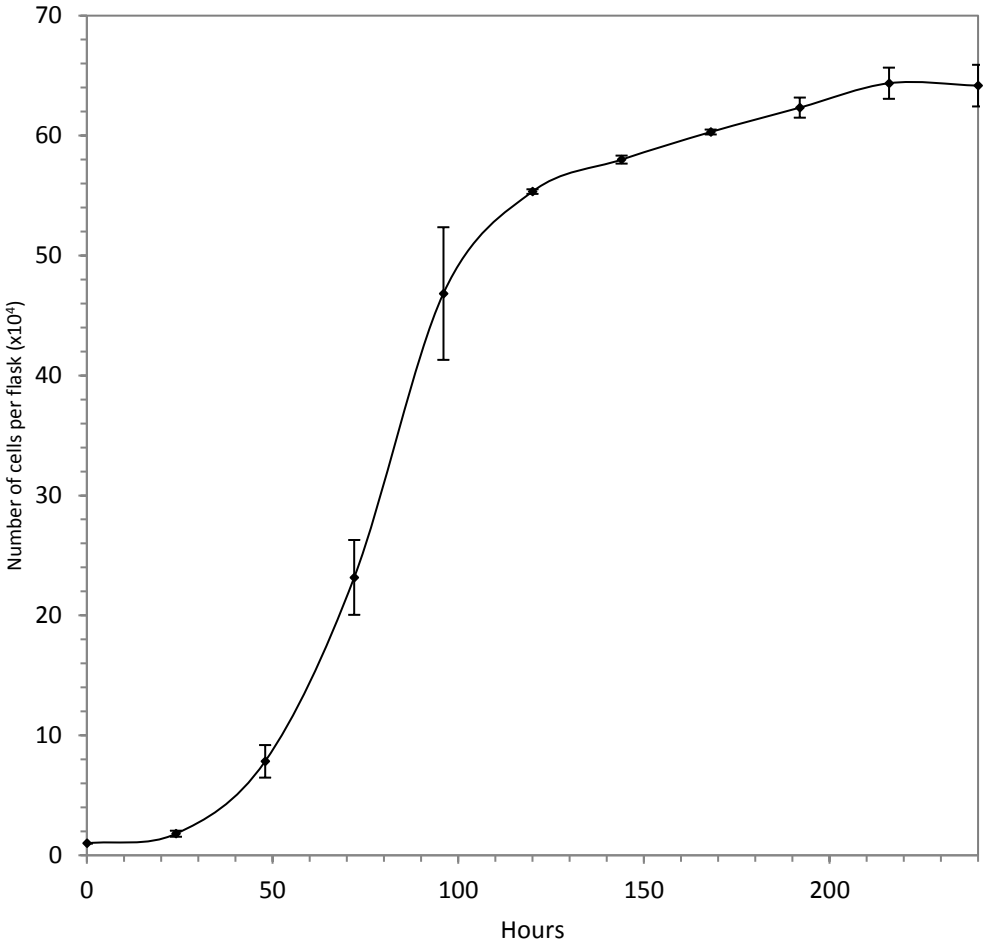


Figure III.1 Growth of H460 cells *in vitro*, determined by manual counting method. $1x10^4$ cells were plated into $10x25cm^2$ flasks, every 24 hours over the course of 10 days, cells were counted (as described in section II.2.2). Data representative of the mean ± SD of 3 independent experiments.

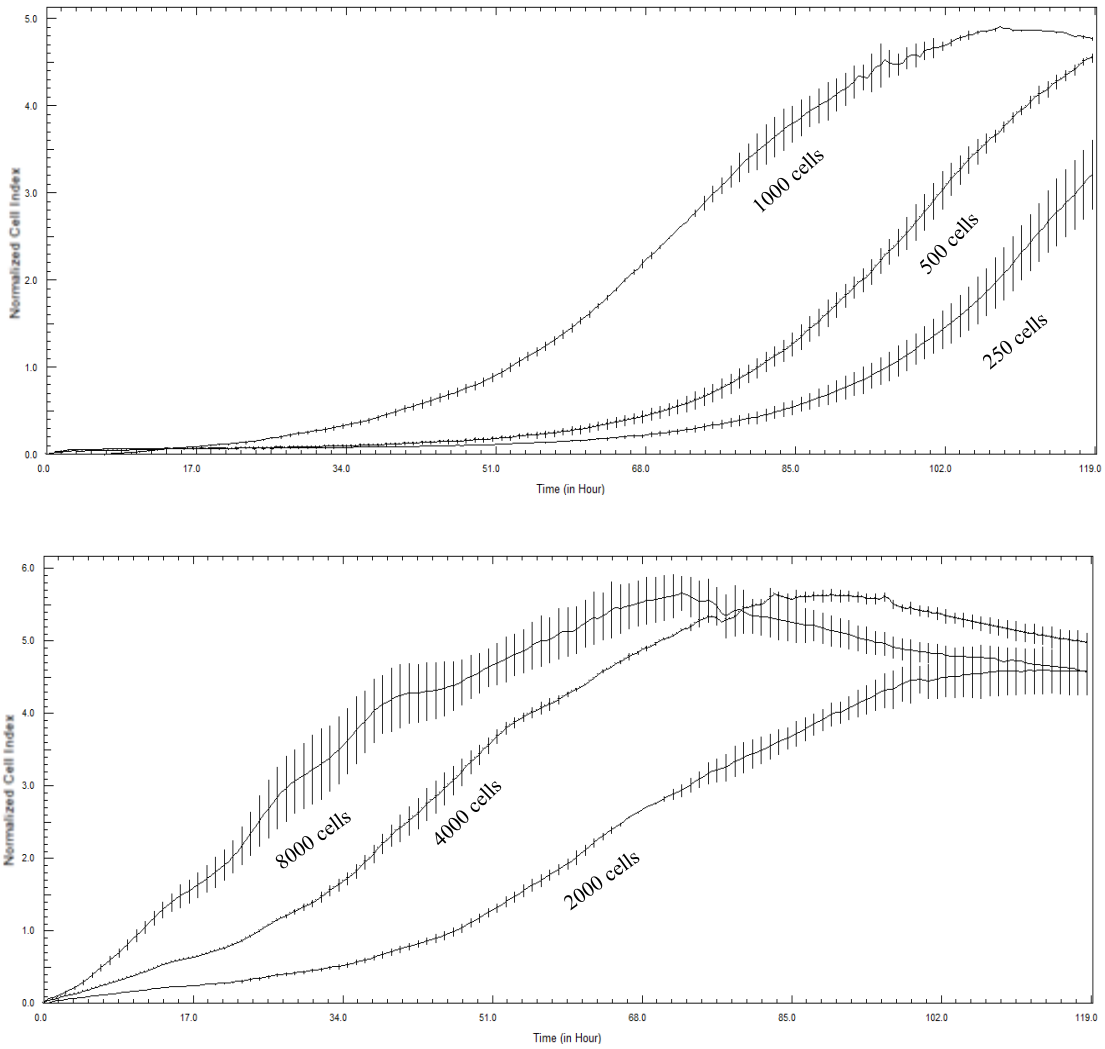


Figure III.2 Characterisation of H460 growth kinetics using the xCELLigence RTCA system**.** Cells were seeded at a range of titrations to identify the ideal seeding density and reveal time windows for drug treatment. Cells grown at A) Low density; 1000, 500, 250 cells/well and B) High density; 8000, 4000 and 2000 cells/well. Curves represent the mean Cell Index value from >2 wells ± SD.

Table III.1 Comparison of growth phases and doubling times in different seeding densities of H460 cells

Seeding density (cell number per well)	Lag phase (hours)	Exponential phase (hours)	Plateau phase (hours)	Doubling time (hours)
250	0-101	101->119	>119	17.0
500	0-85	85->119	>119	18.7
1000	0-61	61-93	>93	17.9
2000	0-51	51-95	>95	18.7
4000	0-32	32-73	>73	18.7
8000	0	0-37	>37	17.2

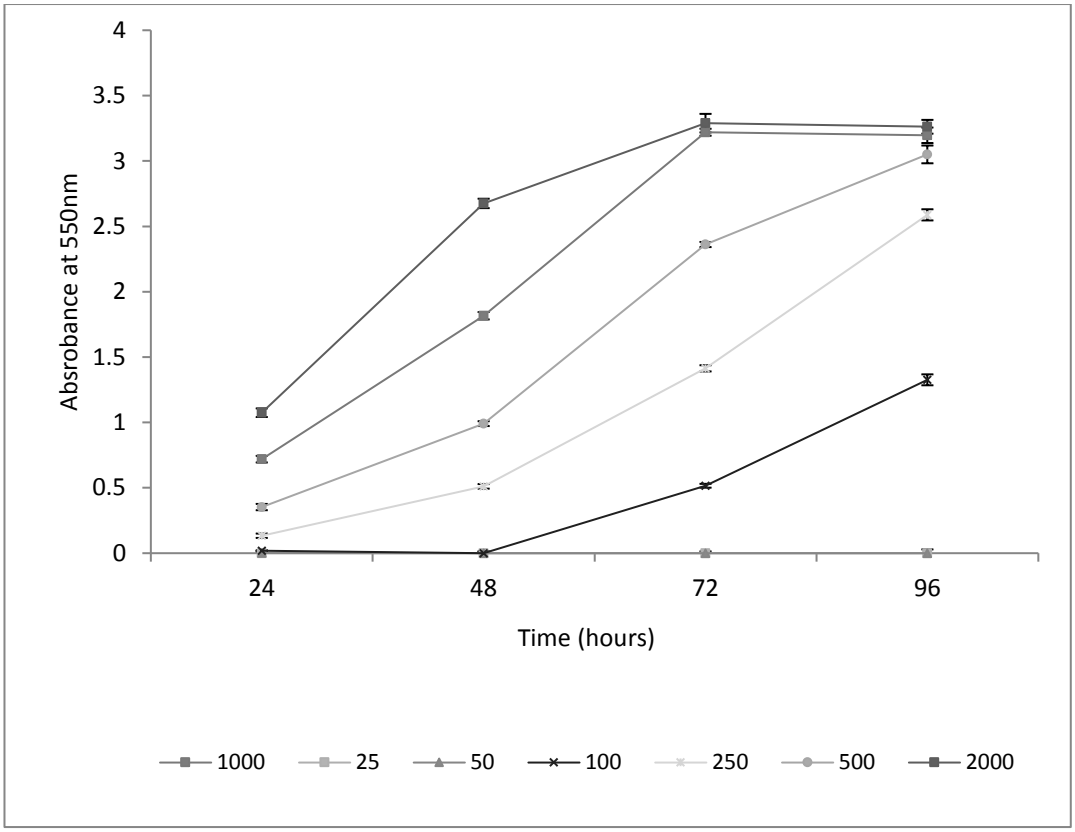


Figure III.3 Growth curve analysis of H460 cells, using the MTT assay. Different cell densities (25-2000 cells per well) were grown over a 96-hour incubation period to identify a suitable cell number to use in subsequent toxicity studies. Curves represent the mean Cell Index value from >4 wells ± SD.

Table III.2 Average doubling times during exponential growth phase of H460 cells, determined using three methodologies

Growth curve analysis method	Average doubling time (hours)
Manual	19.2 ± 1.8 hours
xCELLigence	18 ± 1 hour
MTT	20.5 ± 1.04 hours

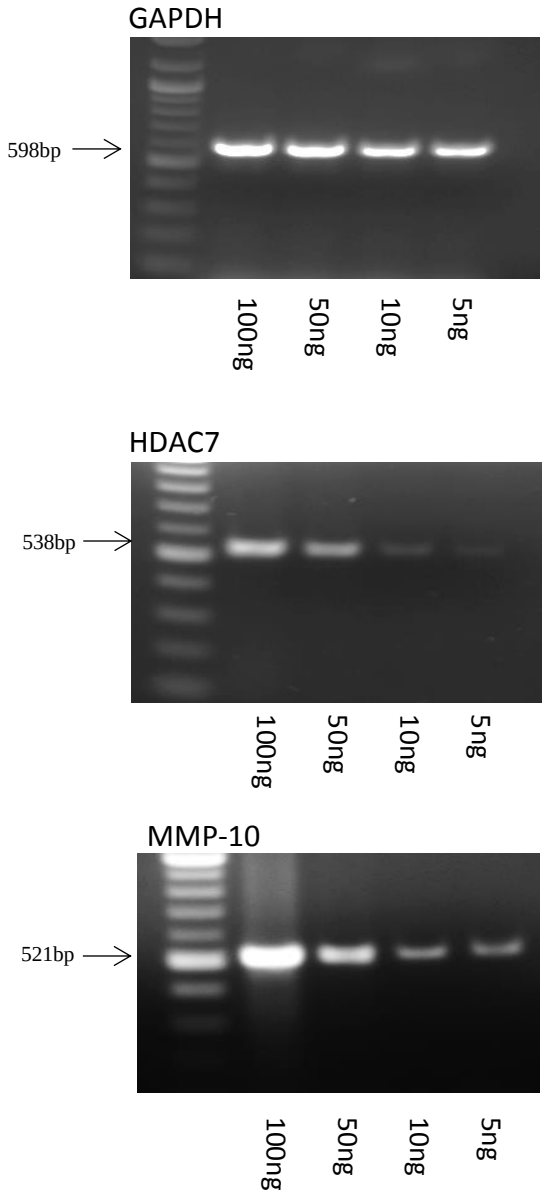


Figure III.4 Identification of optimal cDNA concentration for RT-PCR analysis. mRNA was extracted from H460 cell lysates and RT-PCR analysis was performed on different concentrations of cDNA. Optimum cDNA concentration identified as 50ng NSCLC cDNA, per reaction for GAPDH, MMP-10 and HDAC7.

III.3.5 Time-dependent expression of MMP-10 and HDAC7 in NSCLC *in vitro*

In order to use a suitable *in vitro* model of NSCLC, H460 cell lines had to be characterised for the gene expression of HDAC7 and MMP-10, this was carried out via RT-PCR. Bands of relevant sizes were present in the cells, indicative of a positive result for HDAC7 and MMP-10 expression in H460 cells (figure III.5). MMP-10 gene expression revealed stronger expression over time, showing a time-dependent increase. There was no difference in HDAC7 gene expression over time.

III.3.6 Expression of HDAC7 and MMP-10 protein in H460 NSCLC cell line *in vitro*

Western blot analysis confirmed H460 cells express both MMP-10 and HDAC7 protein, with bands of relevant sizes (figure III.6). MMP-10 can exist as both an inactive proenzyme and active enzyme, however such analyses could not be achieved due to the inability of this antibody to achieve a positive result for both the active and inactive forms of the enzyme; therefore, an assay of function was required.

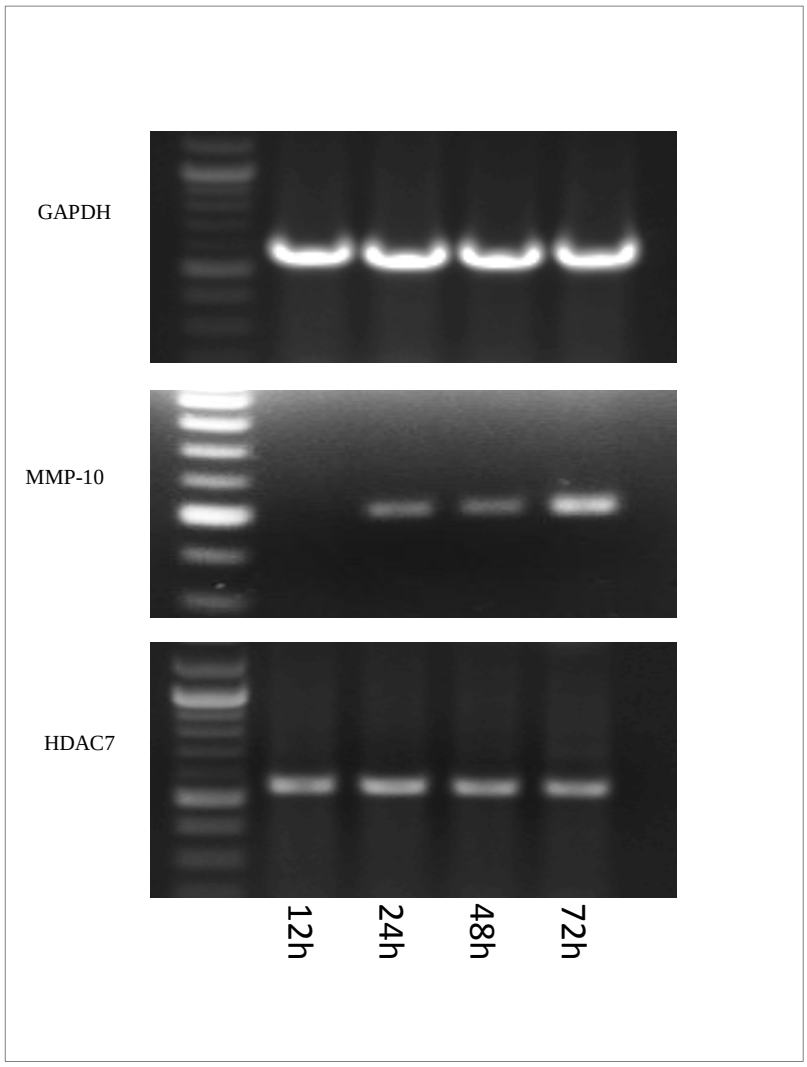


Figure III.5 RT-PCR analysis of MMP-10 and HDAC7. H460 cells were grown for 12-72 hours, mRNA was extracted from cell lysates and gene expression was evaluated by RT-PCR. Product sizes; GAPDH, 598 bp; MMP-10, 521 bp; HDAC7, 538bp. MMP-10 expression shows a time dependent increase.

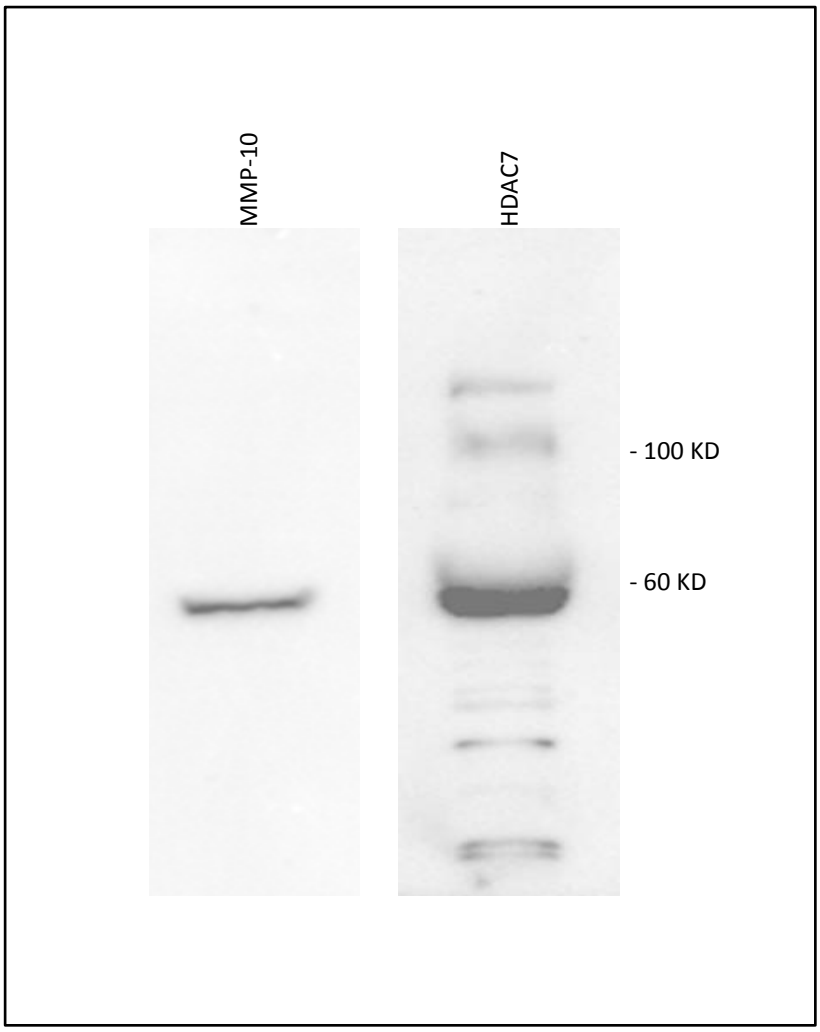


Figure III.6 Western blot analysis of MMP-10 and HDAC7 protein. H460 cells were grown for 24 hours, protein was extracted from the cell lysates and protein expression was evaluated by SDS PAGE. Both pro-MMP-10 (57kD) and HDAC7 (105kD) expression were positively identified in H460 cell line.

III.3.7 Levels of active MMP-10 in H460 cell line *in vitro*

III.3.7.1 Demonstration of MMP selectivity of M-2110 protein substrate

A continuous fluorometric assay using substrate M-2110 with different MMP recombinant proteins (-10, -9, -3 and -2) was carried out to assess the selectivity of the substrate. The substrate showed highest selectivity against active MMP-10 and MMP-3 protein, with low activation by MMP-9 and negligible activation by MMP-2 (figure III.7). This substrate could therefore be used for subsequent assay evaluations for active MMP-10 in NSCLC.

III.3.8 Secretion of enzymatically active MMP-10 by H460 NSCLC cell line in vitro

Levels of active MMP-10 were assessed in NSCLC *in vitro*, using a continuous fluorometric assay over a 24-96-hour time course, using the MMP-10 specific substrate; M-2110 (figure III.8) and normalized to cell number. Active MMP-10 was detected in media, with the levels of activity shown to increase in a time-dependent manner.

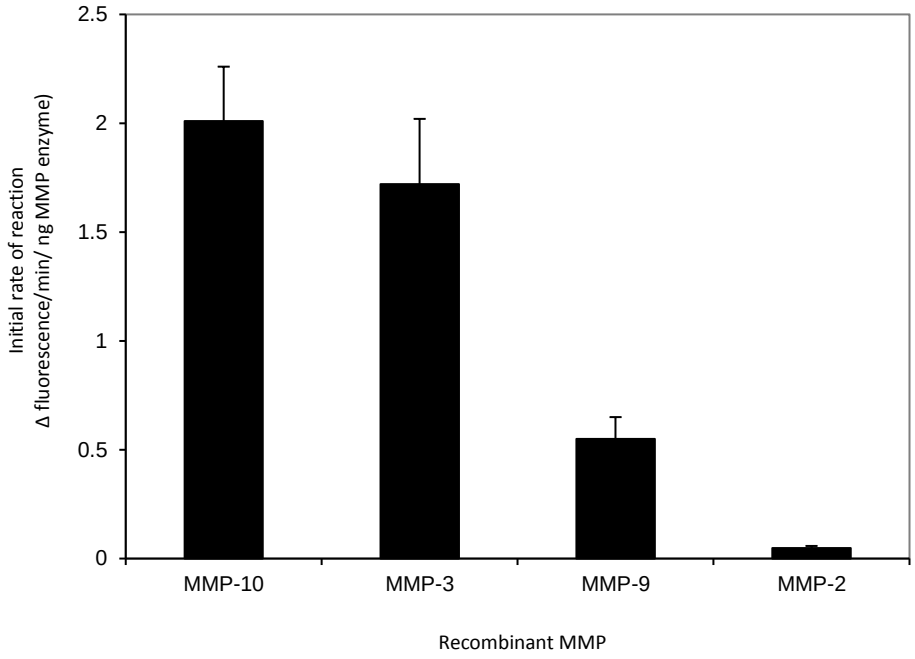


Figure III.7 Continuous fluorometric assays of MMPs with M-2110 substrate (Mca-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(Dnp)-NH_2). Assay was performed at 37°C by reacting 1µM substrate with 200ng of MMPs in 100µl solution of assay buffer. Fluorescence was read with λ_{ex}=325nm and λ_{em}=393nm. Values are represented as Δ fluorescence/min/ng enzyme.

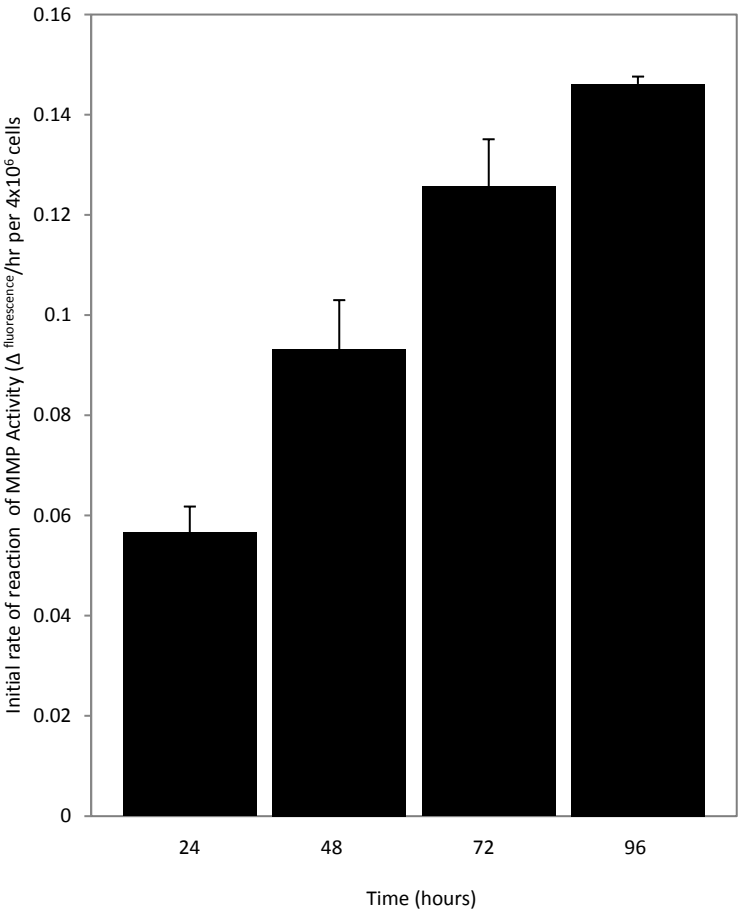
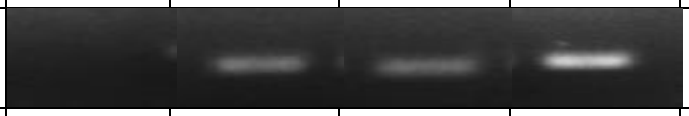


Figure III.8 Increased secretion of active MMP-10 *in vitro* NSCLC, over time continuous fluorometric assay demonstrated a time dependant increase in active MMP-10. Assay was performed at 37°C by reacting 1µM substrate with 10µl media in 100µl solution of assay buffer. Fluorescence was read with λ_{ex}=325nm and λ_{em}=393nm. Values are represented as Δ fluorescence/hr per $4x10^6$ cells.

III.3.9 Comparison of time-dependent expression of MMP-10 in H460 NSCLC cell line *in vitro*

Table III.3 Comparison of time-dependent expression of MMP-10 gene, protein and activity in H460 NSCLC cell line *in vitro*

	12h	24h	48h	72h	96h
MMP-10 gene					n.d
Relative gene expression	0	685.021	2384.891	4168.347	n.d
MMP-10 activity (ΔAbs/min/4x10^6 cells)	n.d	0.05	0.09	0.12	0.15

III.4 Discussion

This chapter serves to demonstrate the development of reproducible techniques for the detection and semi-quantification of MMP-10 expression levels in NSCLC *in vitro*. H460 cells which are of the large cell carcinoma sub-type were used as a model for *in vitro* NSCLC (Batra *et al*., 1998). H460 cells are immortalised and originally derived from a primary tumour of the lung removed via pleural effusion (Levina *et al*., 2008). Using a cell line over primary cells for *in vitro* studies is somewhat advantageous because the cells evade cellular senescence, can be grown for long periods of time, allowing studies of molecular mechanisms to be investigated. H460 cells have been shown to be a useful NSCLC model system, as they exhibit a NSCLC phenotype and demonstrate clinically relevant responses to therapeutic agents (Kellar *et al*., 2015). In this study, three techniques were utilised to characterise cell growth kinetics.

The role and significance of MMP-10 in cancer, especially NSCLC, has not yet been elucidated. However, several studies have demonstrated links between MMP-10 and tumour pathophysiology (Chang *et al*., 2006). For instance, high expression of MMP-10 in lymphoma is associated with promotion of tumour growth (Van Themsche *et al*., 2017). In contrast, elevated expression of MMP-10 did not correlate with either clinical tumour stage or grade in several cancers, including bladder, colon, and NSCLC (Seargent *et al*., 2005; Gill *et al*., 2004). However, despite lacking correlation to pathogenic classification, this MMP is linked to an invasive phenotype in many preclinical cancer studies (Frederick *et al*., 2008; Zhang *et al*., 2014). Therefore, MMP-10 appears to have a major role in cancer, but with differing roles potentially associated with tumour origin, location or surrounding microenvironment such as degree of cell-matrix interactions (Gialeli *et al*., 2011).

In NSCLC, MMP-10 expression and activity is elevated in both primary and metastatic disease (Justilien *et al*., 2012), but does not associate with tumour sub-type or pathogenesis (Gill *et al*., 2004). However, MMP-10 has been shown to play a key role in lung tumour formation via maintenance of the cancer stem-cell population and promotion of metastatic genotypes in human NSCLC (Regala *et al*., 2011; Justilien *et al*., 2012). Furthermore, the ability of MMP-10 positive NSCLC cells to form tumours in MMP-10-null mice implicates expression of MMP-10 in the tumour mass rather than the tumour microenvironment as being key to regulation of tumour growth (Justilien *et al*., 2012). However, the importance of tumour-expressed MMP-10 to tumour growth is reiterated by studies by Gill *et al*, (unpublished, Figure I.3) in which supraphysiological induced expression of MMP-10 in H460 NSCLC xenografts resulted in retardation of tumour growth *in vivo*. However, induced expression did not alter *in vitro* growth or survival (data not shown) consequently, despite being central to establishment and maintenance of the stem-cell phenotype, understanding the molecular mechanisms underlying regulation of MMP-10 has important implications for lung cancer pathogenesis and identification of therapeutic opportunities. In particular, how does elevated secretion of MMP-10 contribute to lung cancer development and how is MMP-10 expression and activity regulated? In this study, the H460 NSCLC cell line was shown to express MMP-10 at the genetic and protein level, with secretion of proteolytically active MMP-10, in an *in vitro* culture system. Therefore, the H460 cell line is a viable model to characterise regulation and function of MMP-10 in NSCLC.

Comparison of MMP-10 activity levels, as measured by a fluorometric assay and MMP-10 mRNA expression, demonstrated a correlation between the levels of active

MMP-10 and MMP-10 mRNA supporting MMP-10 gene expression as a marker for proteolytic activity. As expected, protein expression and MMP-10 proteolytic activity temporally followed gene expression. However, expression and activity of MMP-10 increased over the duration of the study between 12 and 96 hours. The reason for this apparent increase in MMP-10 expression is unclear, but maybe a consequence of deplenishment of growth factors, increasing hypoxia, or alterations in cell-matrix interactions. Such relationships have been previously identified with MMP-10, albeit not in NSCLC (Garcia-Irigoyrn *et al*., 2014; Steele *et al*., 2011).

A clear relationship has been shown between expression of HDAC7 and MMP-10, particularly in regulation of vascular integrity (Chang *et al*., 2006). MMP-10 expression has found to be overexpressed in several types of cancer as described in section I.3.5, with increased expression observed in NSCLC (Gill *et al*., 2004). HDAC7 has been linked to MMP-10 *in vitro and in vivo*; HDAC7 is thought to repress MMP-10 and has found to be under expressed in several types of cancers (examples include bladder cancer and NSCLC). Subsequently, in addition to MMP-10 facilitating maintenance of the cancer stem cell, a role for MMP-10 is postulated in the control of tumour angiogenesis and ultimately metastatic potential (Chang *et al*., 2006). Timely regulation of MMP-10 is important to endothelial motility and vascular development with unregulated activity being detrimental to successful angiogenesis, providing an explanation to retardation of NSCLC tumour growth *in vivo*.

Unexpected and specific role for HDAC7 in the maintenance of vascular integrity and have important implications for understanding the mechanisms of angiogenesis and vascular remodeling during cancer development (Chang *et al*., 2006).

The potential that HDAC7 regulates MMP-10 expression and vascular integrity has potentially important implications in the treatment of NSCLC (Chang *et al*., 2006). Inhibition of HDAC7 in tumours and consequent upregulation of MMP-10 would have antiangiogenic consequences and eventually lead to regression of tumours due to the disruption of the vascular supply (Chang *et al*., 2006).

Chapter IV - HDAC7-mediated regulation of MMP-10 expression in NSCLC

IV.1 Introduction

The regulation of the secreted endopeptidase; MMP-10 has been linked to the class IIa HDAC; HDAC7, as discussed previously. Conventionally, HDACs are important in the regulation of gene transcription because they are responsible for the removal of the acetyl groups from histone tails, inducing chromatin condensation and a non-permissive chromatin conformation, causing gene transcriptional repression (Barneda-Zahonero & Parra, 2012). This HDAC-regulated change in chromatin condensation prevents access of transcription factors to the promoter region of specific genes (Kouzarides, 2007). However, HDACs have now also been found to deacetylate non-histone proteins, with many of these non-histone targets being targets of class II HDACs (Peng & Seto, 2011). These non-histone protein targets include several transcription factors (eg HIF1α), regulatory proteins (eg MLP), tumour suppressor proteins (eg. P53), and cellular structural proteins, such as α-tubulin (Martin *et al*., 2007). The significance and diversity of these additional protein targets is only just beginning to be elucidated. However, there is strong evidence appearing to support type I HDACs, as being histone modifiers, and the type II HDACs as being largely protein deacetylases with cytoplasmic activities (Haberland *et al*., 2009), because class II HDACs exhibit low deacetylase activity against histones (de Ruijter *et al*., 2003).

HDAC7, a class IIa HDAC member, plays an important role in angiogenesis and vascular integrity (Chang *et al*., 2006; Mottet *et al*., 2007). One mechanism involved in this process is the repression of the endoprotease; MMP-10 through regulation of the transcription factor; myocyte enhancer factor 2 (MEF-2). This transcription factor is

involved in control of MMP-10 expression, is a central regulator of differentiation of the endothelial cells (Ha *et al*., 2008; Chang *et al*., 2006; Murray *et al*., 2013) and is highly expressed within the developing endothelium (Chang *et al*., 2006). A clear relationship between HDAC7 and MMP-10 is provided by mice knockout studies (Chang *et al*., 2006). In these HDAC7-modified mice, up-regulation of MMP-10 expression is observed in the perivascular region and a down-regulation of the MMP-10 antagonist TIMP1 is observed in the endothelial cells (Chang *et al*., 2006), offering a rationale to explain the loss of vascular permeability in these mice. Furthermore, Chang and colleagues also found that HDAC7 regulates vascular permeability through repressing MMP-10 expression and through inhibiting MEF2 activity (Chang *et al*., 2006). In principle, the loss of HDAC7 triggers MMP-10 expression, leading to alterations in vascular homeostasis and blood vessel defects (Clocchiatti *et al*., 2011).

In NSCLC, MMP-10 has previously been shown to be overexpressed, with high levels of proteolytically active MMP-10 detectable, and with MMP-10 localised in the tumour mass rather than the stroma (Gill *et al*., 2004). Because elevated MMP-10 is central to regulation of vascular development; it induces remodelling of vascular extracellular matrix, capillary tube regression and vascular collapse, and because HDAC7 has been linked to the regulation of HDAC7 *in vivo,* with studies indicating an inverse correlation exists between HDAC7 and MMP-10, the relationship becomes a potential area of exploration in tumour progression mechanisms.

IV. 1 .1 Aims and objectives

The main objective in this chapter is to firstly evaluate the relationship between HDAC7 and MMP-10 expression. Studies have suggested an inverse correlation exists

between HDAC7 and MMP-10 in clinical NSCLC. Using H460 cells as a model of NSCLC *in vitro*; a comparison between the effects of silencing the expression of HDAC7 upon MMP-10 expression and activity, in addition to inhibiting its activity, upon the expression of MMP-10, was explored, this is to evaluate whether any relationship between HDAC7 and MMP-10 is due to the enzymatic activity or physical presence of HDAC7.

IV.2 Methods

IV.2.1 Knockdown of HDAC7 expression in H460 NSCLC cells with siRNA

Two sets of double stranded small-interference RNA (siRNA) for HDAC7 (sequence, Table IV.1) (Ambion, UK), were reconstituted to a stock concentration of 100µM in sterile dH_2O.

H460 cells were grown in 6 well plates ($4x10^5$ cells per well) (as described in section II.2.1), media was removed from the cells, the cells washed in HBSS, and 2ml/well of serum free media added to the cells. Lipofectamine 2000 (Invitrogen, UK), a trans-fection agent, was reconstituted at a 1:35 ratio into OptiMEM (no serum or addi-tives), to a final volume of 250µl. In a parallel tube, siRNA was diluted into 250µl serum free media; OptiMEM, at a range of working concentrations (1-100nM). The Lipofectamine (250µl) and siRNA (250µl) solutions were combined and the mixture incubated for 15 minutes at room temperature, to allow the siRNA-lipid complexes to form. The siRNA-lipid complex was then added directly into the serum free media incubating the cells in the 6-well plate, and incubated for 6 hours at 37°C 5% CO_2. After the 6 hours transfection time, fresh media added. The cells were incubated for a further 12-96 hours. Cells were assayed by RT-PCR (section II.3), to assess for changes in HDAC7 and MMP-10 gene levels.

Table IV.1; HDAC7 siRNA nucleotide sequence - HDAC7 siRNA targets HDAC7 mRNA between 3040-3057 base pairs.

	HDAC7 siRNA Nucleotide sequence (5'->3')		
Target gene	**Sense**	**Antisense**	**Product size**
Human histone deacetylase 7	GGAAGAACCUAUGAAUCUCtt	GAGAUUCAUAGGUUCUUCCtc	538bp

IV.2.1.1 Detection of HDAC7 gene silencing for RT-PCR

Primers to detect the silence of the HDAC7 gene expression in H460 cells were custom designed using the primer blast tool on the National Center for Biotechnology Information (NCBI) and purchased from Invitrogen (UK). Primer sequences are shown in table II.1.

IV.2.2 Determination of MMP-10 selective enzyme activity in the presence/absence of HDAC7

H460 cells were plated at a density of $4x10^5$ cells per well in a 6 well plate and incubated in complete media for 24 hours. To compare the involvement of HDAC7 upon MMP-10 activity, cells were treated with either HDAC7 siRNA (100nM) (concentration optimised in section IV.2) or the class IIa selective inhibitor 401940 (1µM). To assess the levels of active endoprotease enzymes present in cell media, aliquots of media were removed at various time points over the duration of the time course (24-96h). The fluorometric enzyme assay was performed in a 96 well plate by incubating 10µl of media with 50µg of fluorogenic substrate M-2110(Mca-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(Dnp)-NH_2) in 80µl of MMP activity buffer (100 mM Tris-HCl pH 7.6, 10 mM CaCl2, 100 mM NaCl, 0.16% v/v Brij-35); total reaction volume of 100µl.

Fluorescence was measured (excitation = 325 nm; emission = 393 nm) over a 600 minute incubation period at 37°C. For each sample, the relative activity of the initial linear enzyme activity was calculated, and activity expressed as change in fluorescence per hour per $4x10^5$ cells. The reproducibility and reliability of the assay were determined using samples obtained from three independent experiments.

IV.2.3 Determination of cell viability upon HDAC7 inhibition, using the MTT assay

H460 cells were seeded in 96-well plates (250 cells/well, optimised in figure III.3) and incubated for 24 hours (37°C in humidified atmosphere of 5% CO_2 and 95% air), media removed and replaced with fresh media. HDAC7-selective inhibitor was prepared as a 10mM stock solution and added to the 96-well plate by serial dilution at a final concentration of 1pM-10µM and incubated for 12-96 hours (37°C in humidified atmosphere of 5% CO_2 and 95% air). After the designated incubation time, an MTT was carried out, as described in section III.2.5.

IV.2.4 Determination of cell viability upon HDAC7 inhibition, using xCELLigence assay

H460 cells were seeded into wells of the E-plate at a density of 1000 cells/well (optimised in figure III.2), the RTCA DP software was set up as described in section II.2.4, and incubated for 24 hours (37°C in humidified atmosphere of 5% CO_2 and 95% air), media removed and replaced with fresh media. HDAC7-selective inhibitor was prepared as a 10mM stock solution and added to the E-plate at a final concentration of

1µM (optimised non-toxic dose, up to 48 hours in figure IV.8) and incubated for 48 hours (37°C in humidified atmosphere of 5% CO_2 and 95% air).

IV.3 Results

IV.3.1. Lack of cytotoxicity of HDAC7 siRNA on NSCLC in vitro

Initially a cell viability assay in the form of an MTT was carried out to determine a range of appropriate non-toxic doses to use *in vitro* (figure IV.1). Cells were given a single dose of an increasing concentration of HDAC7 siRNA (between 0.39-100nM). Survival was assessed at 72 hours, with no toxicity observed at any dose over the 72 hour exposure period.

IV.3.1.2 Identification of HDAC7 siRNA concentrations required for optimal retardation of HDAC7 gene expression

To determine the optimal conditions for silencing the HDAC7 gene in NSCLC, H460 cells were treated with HDAC7 siRNA at a range of non-toxic working concentrations; between 1-100nM over a time course of 12-72 hours (figure IV.2).

A concentration and time dependant silencing of the HDAC7 gene was observed. At 12 hours, no significant silence of HDAC7 gene expression was detected at any of the four siRNA concentrations (1-100nM) evaluated (figure IV.2A). At 24 hours, there was a significant change to gene expression with all four concentrations of siRNA, the greatest effect being between 10-100nM ($p<0.01$) (figure IV.2B). At 48 hours, the highest concentration of siRNA; 100nM was the only concentration effecting a change on HDAC7 gene expression (figure IV.2C). At 72 hours, the effect of HDAC7 siRNA upon HDAC7 gene expression showed no significant difference to that observed in the absence of siRNA (figure IV.2D). No significant difference in expression

of the housekeeping gene, GAPDH, was observed at any of the time points, indicating comparability between studies. Consequently, 100nM was chosen as the optimal non-toxic dose for subsequent experiments.

IV.3.1.3 HDAC7 gene expression relates to MMP-10 gene expression in H460 NSCLC cell model in vitro

To investigate a relationship between HDAC7 gene expression and the gene expression of MMP-10 in NSCLC, H460 cells were treated with the optimised concentration (100nM) of siRNA to suppress HDAC7 gene expression in the cells. Following successful silencing of HDAC7 at 24 hours ($P<0.01$) and 48 hours ($P<0.05$) (figure IV.4A), the levels of MMP-10 gene expression were significantly increased at both 24 hours ($P<0.01$) and 48 hours ($P<0.05$) compared to control (figure IV.4B).

IV.3.1.4 Relationship between HDAC7 gene expression and regulation of MMP-10 proteolytic activity in H460 NSCLC cells model

MMP-10 can exist as both an inactive proenzyme and as an active enzyme. To evaluate whether HDAC7 expression has an effect on functional (active) levels of MMP-10, the proteolytic cleavage of the MMP-selective substrate, M2110, was determined following knockdown of HDAC7 gene expression. Increased MMP-10 activity was observed at 24 ($P<0.05$) and 96 hours ($P<0.05$) after siRNA-mediated knockdown of HDAC7 (figure IV.6), suggesting an inverse relationship between expression of HDAC7 and MMP-10 proteolytic activity.

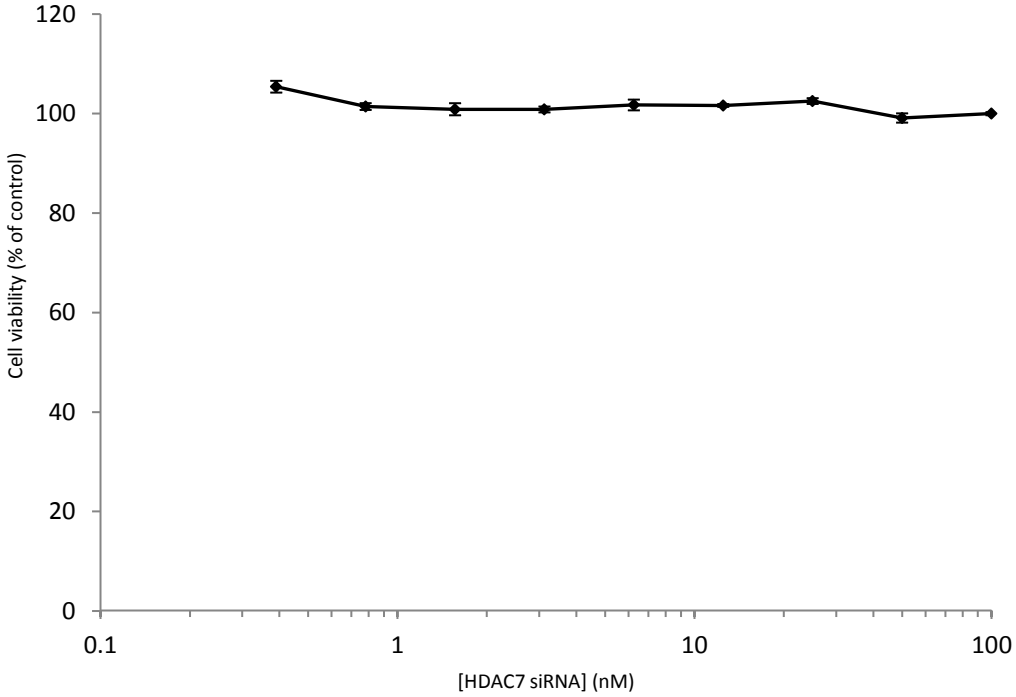


Figure IV.1 Survival curves obtained for H460 cells after 72 hours exposure to a single dose of HDAC7 siRNA, at a range of concentrations (100nM to 0.39nM). MTT assays were performed as described in II.2.5. Data representative of the mean ± S.E of 3 independent experiments.

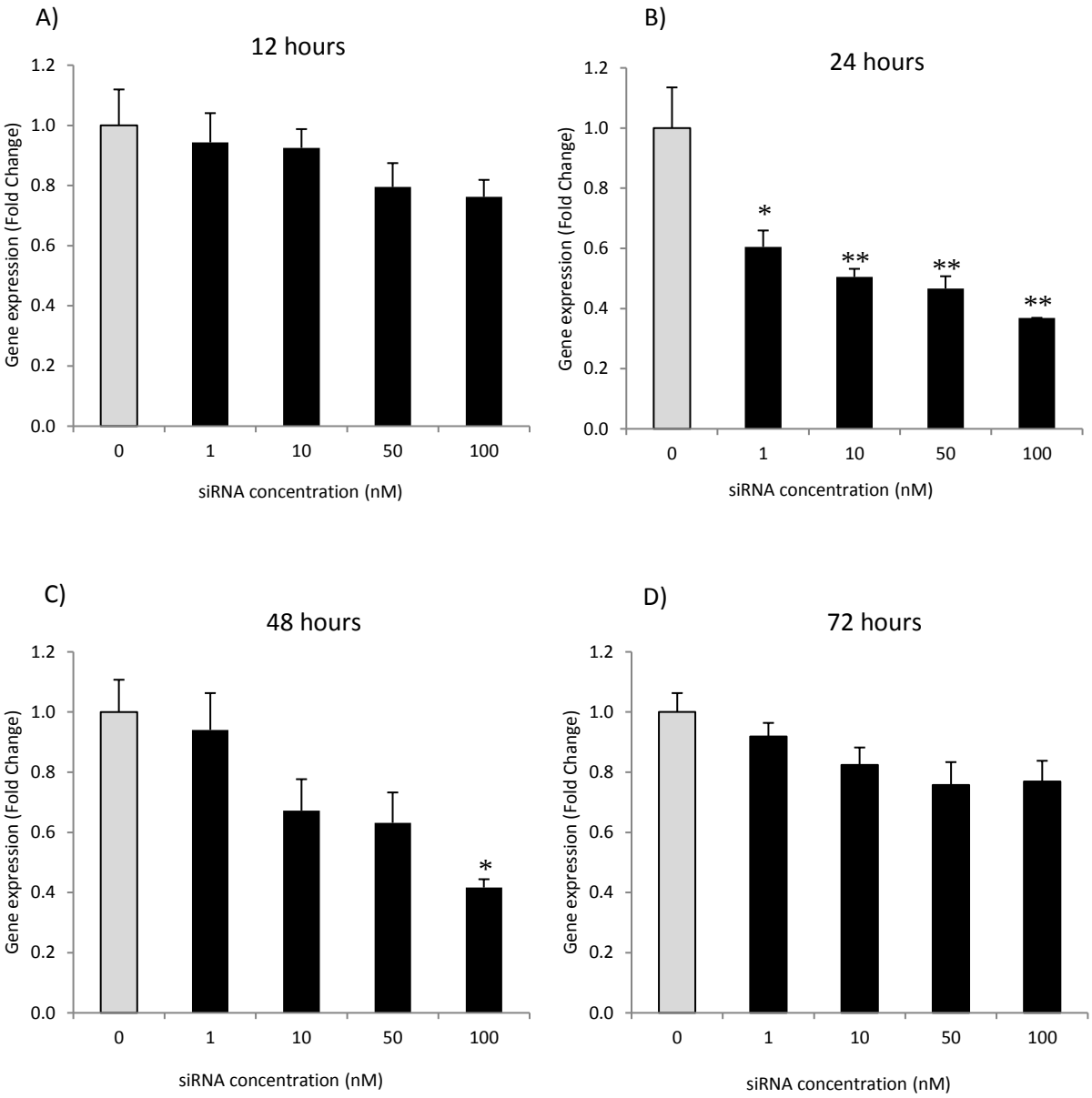


Figure IV.2 Effect of increasing concentrations of HDAC7 siRNA on the level of HDAC7 mRNA in H460 model NSCLC - H460 cells were treated with increasing concentrations (1-100nM) of HDAC7 siRNA over a time course of 12-72 hours, assessed by semi-quantitative RT-PCR. Gene expression of HDAC7 after A) 12 hours, B) 24 hours, C) 48 hours and D) 72 hours, post treatment, control is shown in grey. Signal intensity was quantified by densitometry. Values are mean ± S.E. from three independent experiments, statistical significance was calculated using a one-way ANOVA/Dunnet's post hoc test, *p<0.05, **p<0.01.

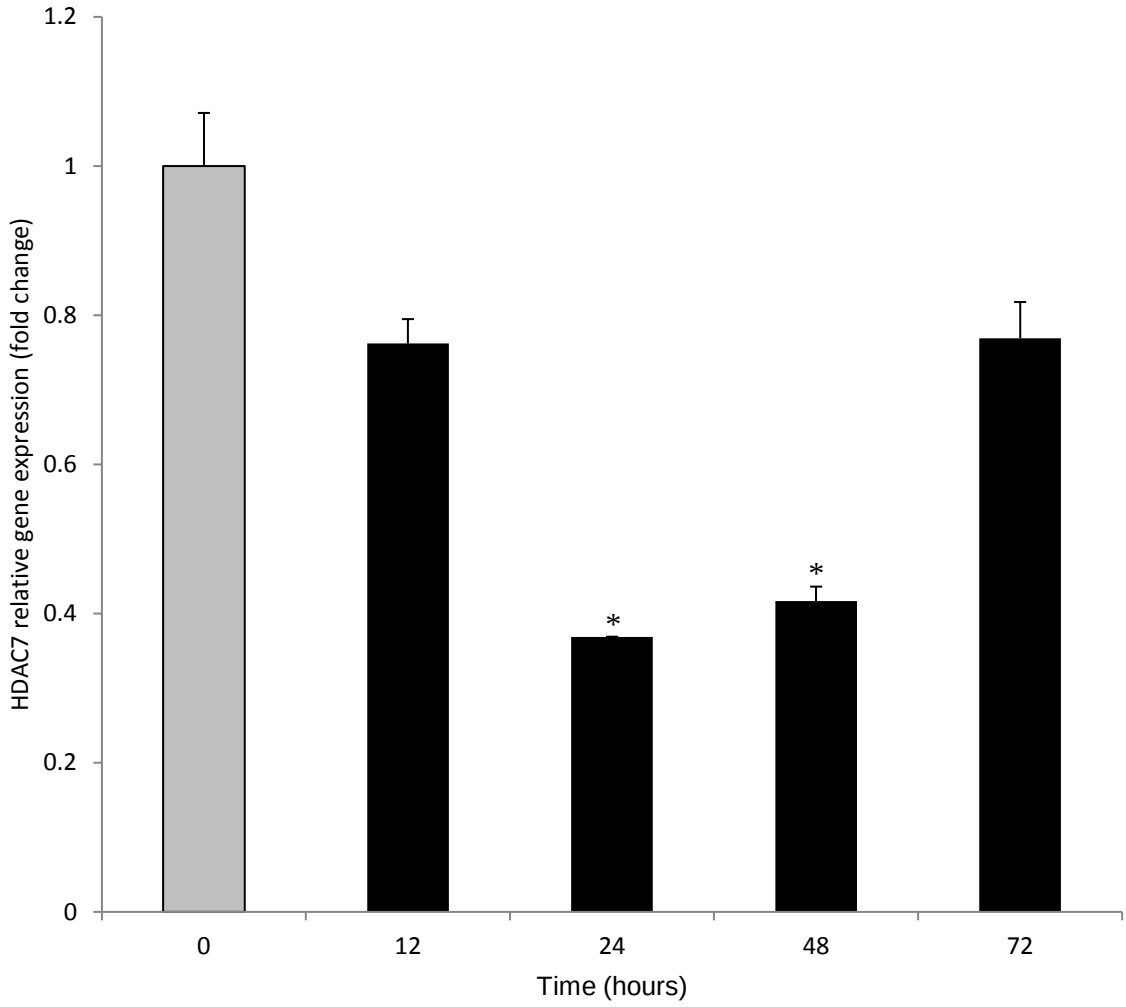


Figure IV.3 HDAC7 gene expression in the presence of 100nM HDAC7 siRNA over a time period of 12-72 hours in H460 model NSCLC**.** HDAC7 relative gene expression following exposure of H460 cells to 100nM HDAC7 siRNA. Data is summarised from figure IV.2 and illustrates 100nM siRNA as the optimal dose for silencing HDAC7 gene expression. Values are mean ± S.E. from three independent experiments, statistical significance was calculated using a one-way ANOVA/Dunnet's post hoc test, *p<0.05, **p<0.01.

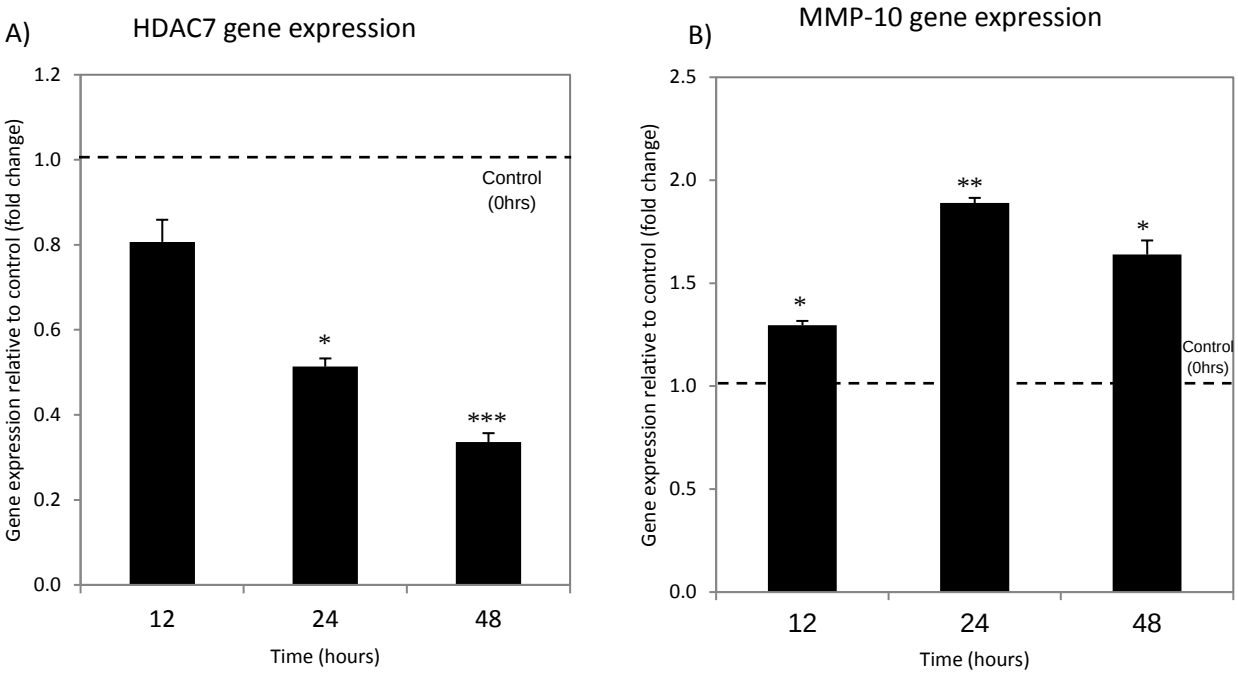


Figure IV.4 Knockdown of HDAC7 gene expression induces increased expression of MMP-10 gene - H460 cells were treated with HDAC7 siRNA (100nM) over a time course of 12-48 hours. Gene expression of A) HDAC7 and B) MMP-10, post treatment, determined by RT-PCR. Signal intensity was quantified by densitometry. Values are mean ± S.E. from three independent experiments, *p<0.05, **p<0.01, ***p<0.001, compared by unpaired t-test.

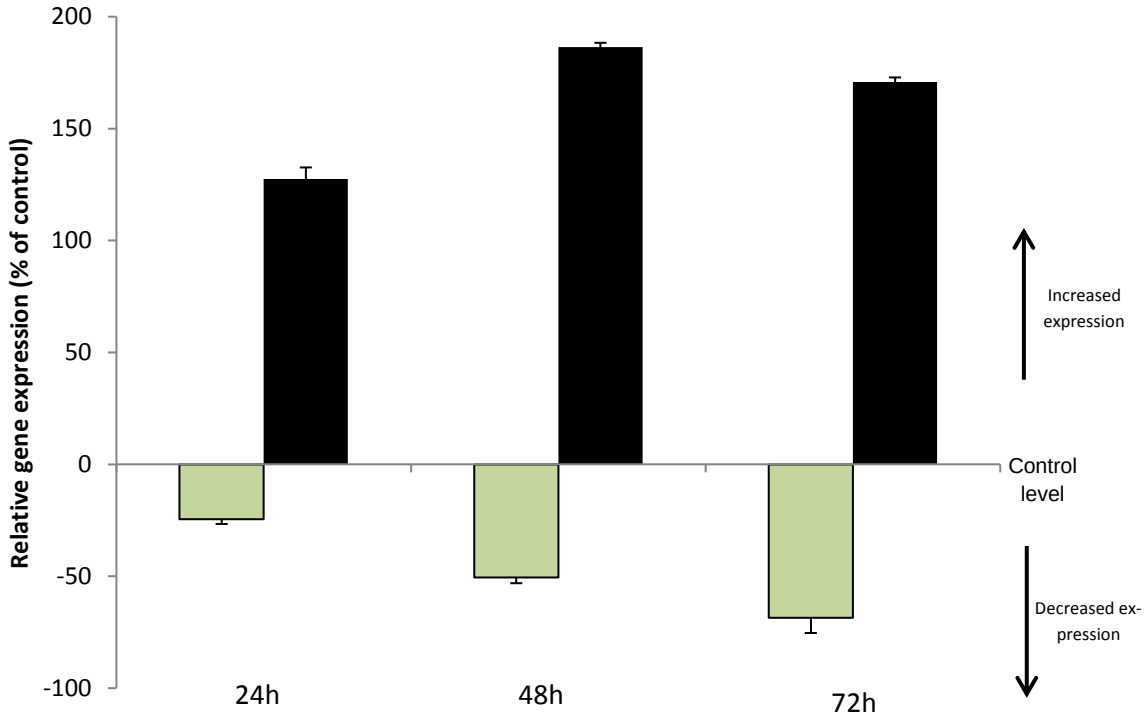


Figure IV.5 Effect of HDAC7 siRNA on the level of HDAC7 and mRNA in NSCLC - HDAC7 (green) and MMP-10 (black) relative gene expression following exposure of H460 cells to 100nM HDAC7 siRNA over 12-48h. Data is summarised from figure IV.4 and illustrates a reduction in HDAC7 gene expression following knockdown results in an increase in MMP-10 gene expression.

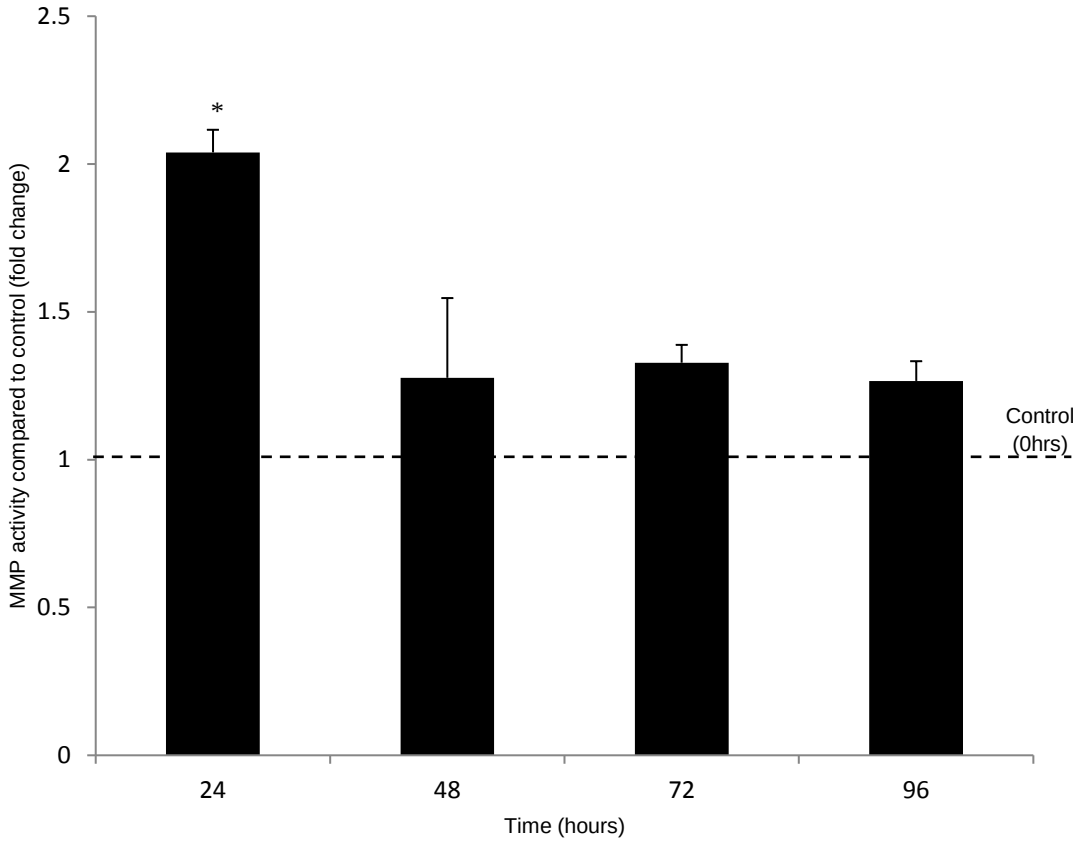


Figure IV.6 Knockdown of HDAC7 gene expression results in increased levels of active MMP-10

To assess the levels of active proteolytic enzyme, medium was removed following treatment of H460 cells with a single dose of HDAC7 siRNA (100nM) over a time course (0-96h). Activity levels were determined by measuring cleavage of the fluorogenic substrate M-2110 (Mca-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(Dnp)-NH_2) with selectivity for MMP-10. Values are mean ± S.E. from three independent experiments, *p<0.05, compared by paired t-test.

IV.3.2 Involvement of HDAC7 enzymatic activity in regulation of MMP-10

To determine whether it is the enzymatic activity of HDAC7 or the physical presence of HDAC7 which regulates the gene expression of MMP-10 in NSCLC, H460 cells were treated with the class IIa selective inhibitor; 401940.

IV.3.2.1 Evaluation of cytotoxicity of class IIa selective inhibitor against H460 NSCLC cell line in vitro

1µM of 401940 was determined as the optimal dose to use for investigating the effect of MMP-10 post treatment of the class IIa HDAC inhibitor (figure IV.7).

To confirm the lack of toxicity and mitigate any cytological response of the inhibitor and to examine the effects elicited by the class IIa selective HDAC inhibitor on the growth kinetics of the cell, the xCELLigence assay was used. The non-toxic dose; 1µM (determined by MTT assay) demonstrated no overall changes in cell index relative to control. The lack of significant modulation of cell index indicates the inhibitor does not affect attachment of the cells to the plate, induce a hyper- or hypoplastic response, or cause a detectable change in cellular morphology. Consequently, the inhibitor can inhibit HDAC7 enzymatic activity (EC_{50} HDAC7 = 30nM; Burli *et al*., 2013) but does not affect cellular characteristics at a concentration of 1µM in vitro, supporting its use to determine the role played by HDAC7 enzymatic activity upon MMP-10 expression and activity.

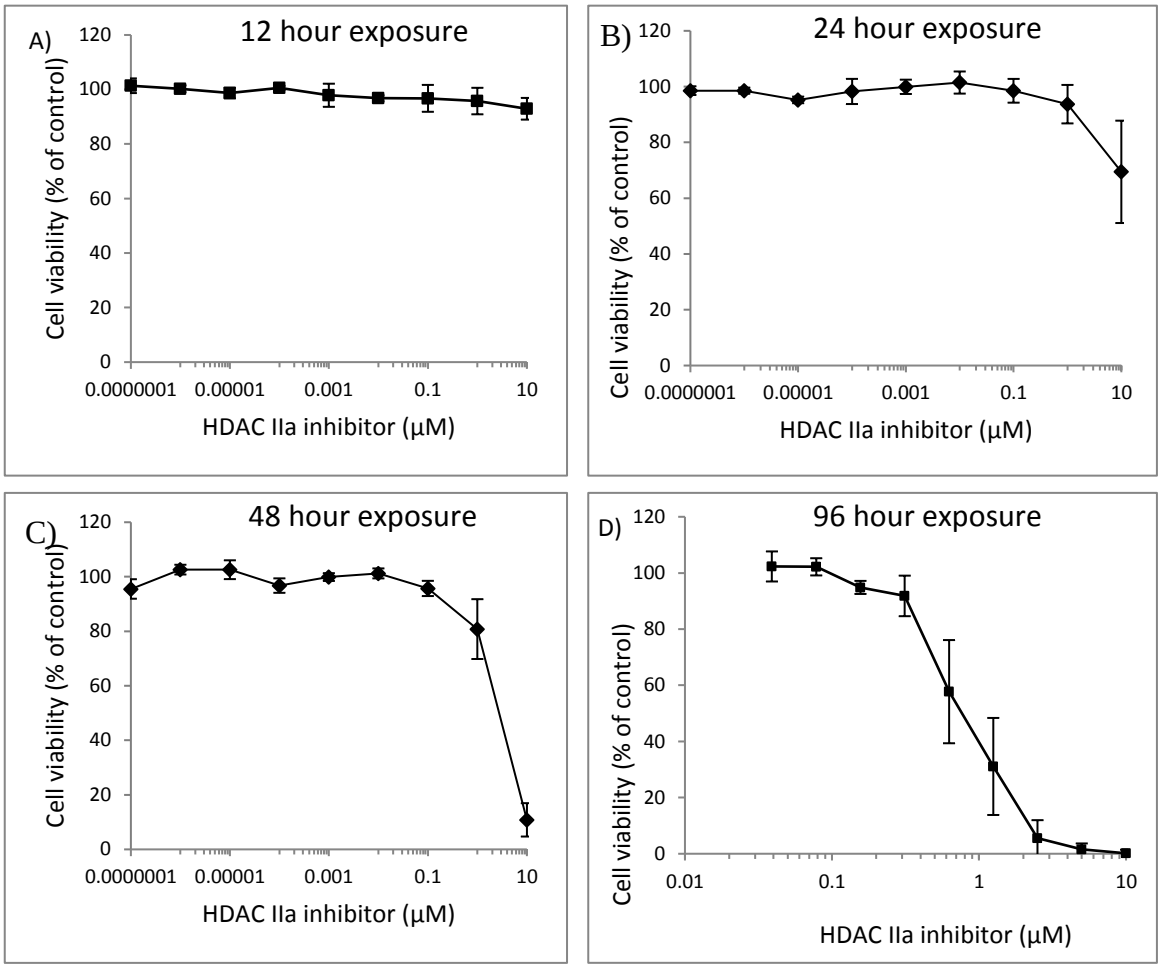


Figure IV.7 Concentration and time dependant cytotoxicity of class IIa HDAC inhibitor 401940 against H460 cell line in vitro - Survival curves obtained for H460 cells after A) 12 hours, B) 24 hours, C) 48 hours and D) 96 hours exposure to a single dose of the class IIa selective inhibitor; 401940, at a range of concentrations (10,000nM to 0.001nM). Data representative of the mean ± S.E. of 3 independent experiments.

Table IV.2; Summary of minimum toxic dose of HDAC IIa inhibition between 12-96h

Time (hours)	Minimum toxic dose	Cytotoxic IC_{50}
12	>10µM	>10µM
24	1µM	>10µM
48	1µM	5µM
96	50nM	0.75µM

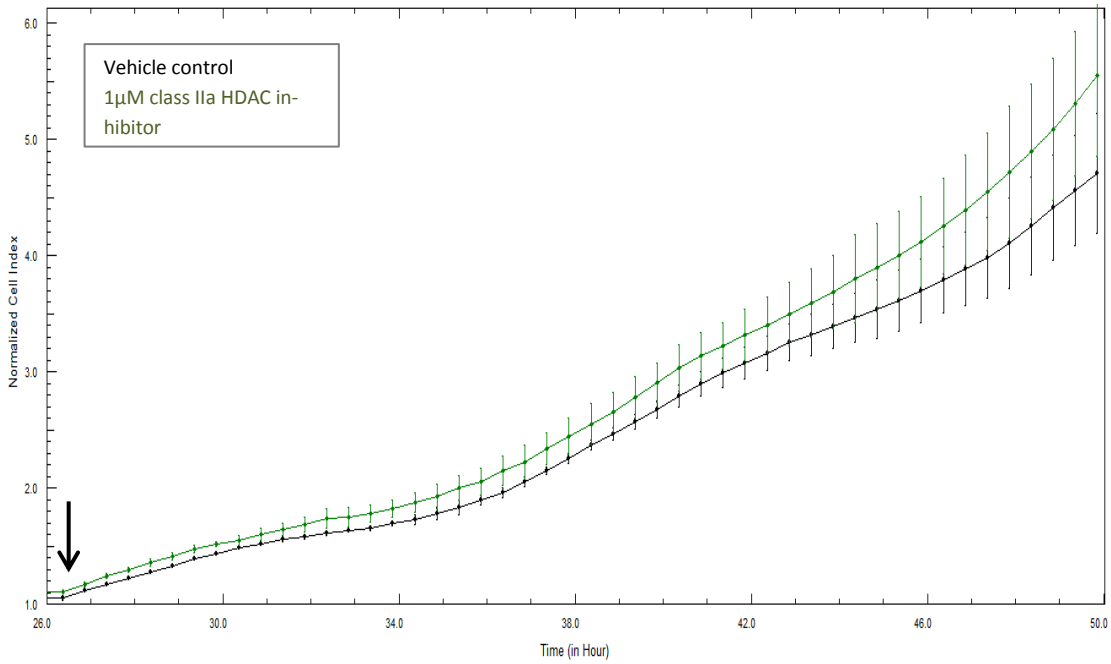


Figure IV.8 Lack of cytological effect of non-toxic concentration (1µM) of the class IIa HDAC inhibitor, 401940, obtained by xCELLigence RTCA. The black arrow indicates the time of drug addition. H460 cells plated at 4000 cells per well and observed over 24h.

IV.3.2.3 Inhibition of HDAC7 enzyme activity does not affect MMP-10 gene expression in NSCLC in vitro

To investigate whether HDAC7s enzymatic activity is required to regulate the gene expression of MMP-10 in NSCLC, H460 cells were treated with the optimal non-toxic dose (1μM) of the class IIa selective inhibitor; 401940 over a time course (12-48 hours). After exposure to the inhibitor, there was no significant differential MMP-10 expression relative to control (figure IV.9B), implying HDAC7-mediated regulation of MMP-10 is independent of HDAC7 activity.

IV.3.2.4 Inhibition of HDAC7 enzymatic activity does not affect MMP-10 proteolytic activity

Because MMP-10 can exist as both an inactive proenzyme and as an active enzyme, the effect of inhibition of HDAC7 enzyme activity upon proteolytic activity of MMP-10 was assessed in NSCLC cells *in vitro*.

The inhibition of HDAC7 enzymatic activity using the class IIa selective inhibitor, 401940, did not retard the proteolytic activity of MMP-10 in the H460 NSCLC cell line model *in vitro*. This further supports the lack of involvement of HDAC7 enzymatic activity in the regulation of MMP-10 in NSCLC.

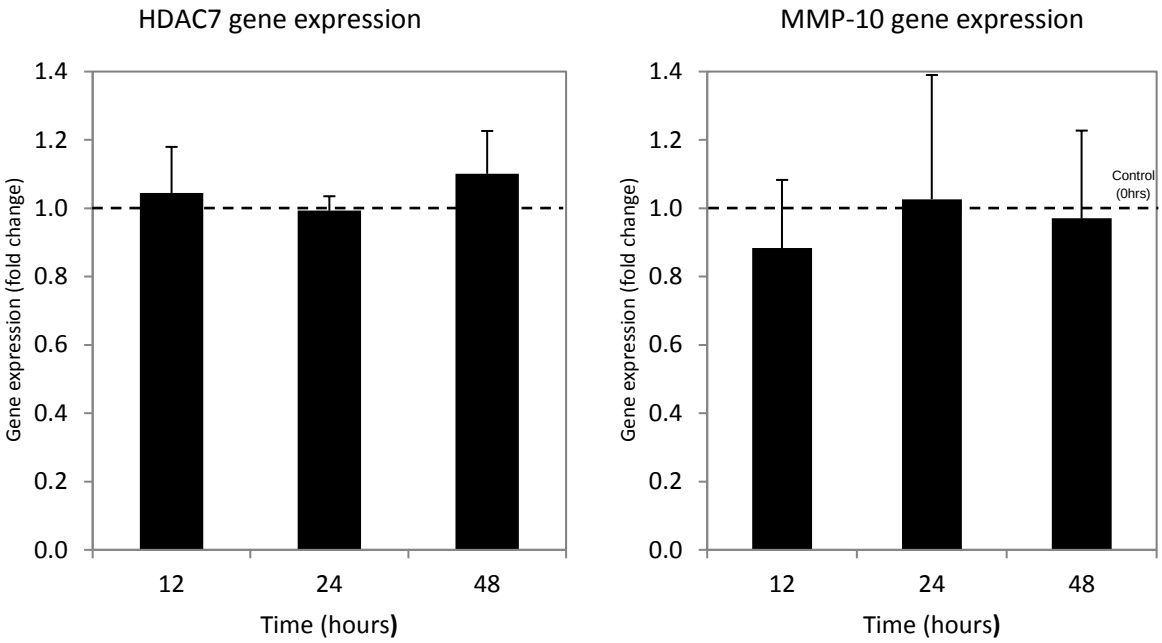


Figure IV.9 The selective class IIa inhibitor; 401940, does not affect levels of HDAC7 and MMP-10 mRNA in NSCLC. H460 cells were treated with 1µM of the class IIa selective inhibitor; 401940 over a time course of 12-48 hours. Gene expression of A) HDAC7 and B) MMP-10, after exposure to the selective inhibitor, determined by RT-PCR. Signal intensity was quantified by densitometry. Values are mean ± S.E. from three independent experiments.

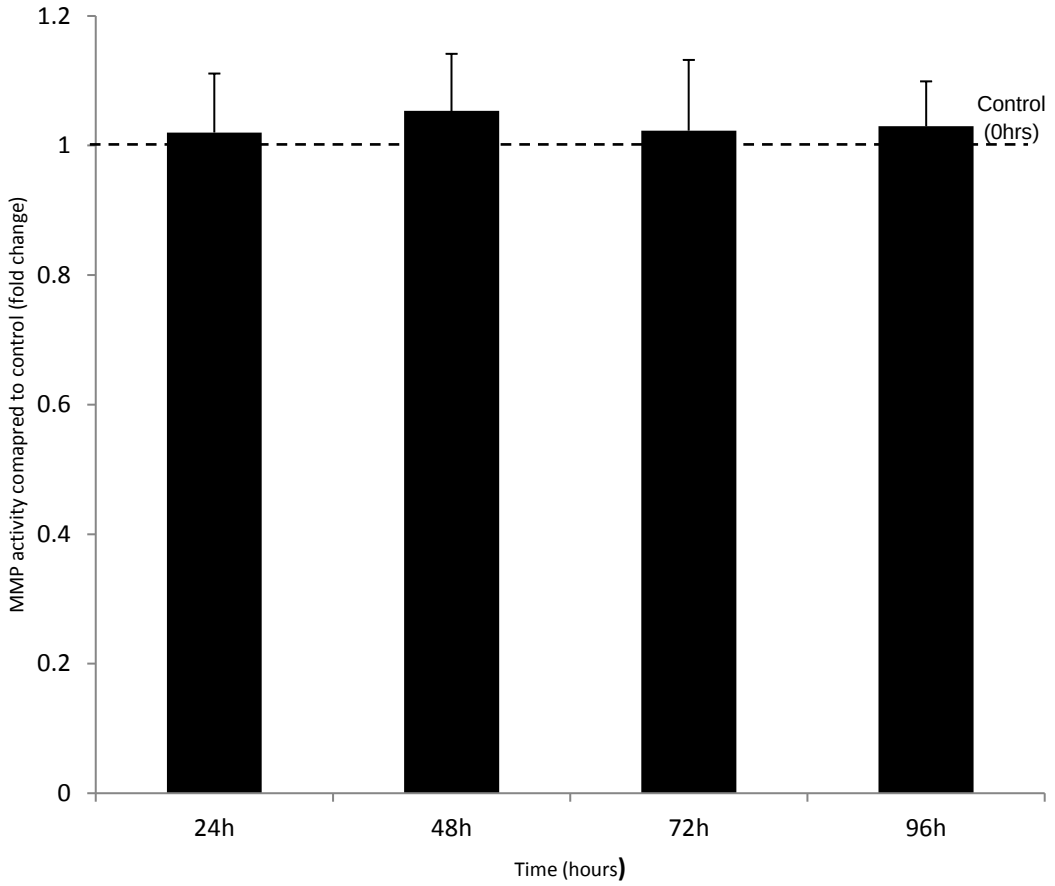


Figure IV.10 Inhibition of HDAC7 enzymatic activity does not affect MMP-10 activity in H460 NSCLC cells *in vitro*

The levels of active MMP-10 proteolytic enzyme, in the presence of the class IIa selective inhibitor; 401940 (1µM), was determined over a time course (24-96h). Activity levels were determined by measuring cleavage of the fluorogenic substrate M-2110 (Mca-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(Dnp)-NH_2) with selectivity for MMP-10. Values are mean ± S.E. from three independent experiments.

IV.3.3 Discussion

The secreted endopeptidase; MMP-10, is involved in degradation of the ECM and is thought to play a central role in the regulation of vascular development (Chang *et al*., 2006). Elevated MMP-10 levels have shown to induce remodelling of the vascular extracellular matrix, capillary tube regression and vascular collapse *in vivo* (Chang *et al*., 2006). MMP-10 expression has been shown to be repressed *in vivo* by HDAC7 in the developing vasculature to maintain vascular integrity (Chang *et al*., 2006). High levels of proteolytically active MMP-10 have been observed in human primary NSCLC, and recurrent NSCLC, regardless of the histologic type (Cho *et al*., 2004; Gill *et al*., 2004). Recently MMP-10 has been linked to cancer stem cells, Justilien and colleagues reported MMP-10 is highly expressed in lung cancer stem cells (CSC) (Justilien *et al*., 2012). Furthermore, MMP-10 expression is also postulated to be elevated due to its involvement during endothelial restructuring required for angiogenesis. In this context, MMP-10 is involved with vessel formation and stabilization rather than contributing to the invasive nature of tumours, an action supported by published studies (Heo *et al*., 2010). Preliminary data carried out by Gill *et al.,* (chapter 1, figure I.3) showed that overexpression of MMP-10 in a human NSCLC tumour model did not affect cellular proliferation *in vitro*, but caused significant retardation of tumour growth *in vivo*, further supporting this role for MMP-10 in NSCLC. The aim of the work in this chapter was to determine whether the expression and activity of HDAC7 is involved in the regulation of MMP-10 in NSCLC.

The silencing of HDAC7 in H460 cells using siRNA resulted in a time-dependent increase in MMP-10 gene expression. The increase in MMP-10 gene expression in re-

sponse to inhibition of HDAC7 gene expression, indicating an inverse relationship between these factors, was also accompanied by an increase in proteolytic activity of MMP-10. This elevation of MMP-10 activity occurred post-transcriptionally, supporting regulation at gene level. This result suggested that the proteolytic activity of MMP-10 is significantly increased along with its expression upon HDAC7 repression, reinforcing the involvement of HDAC7 in the regulation of MMP-10 (Herbst *et al*., 2008). To determine whether the existence of HDAC7 or its enzymatic activity is responsible for the effects upon MMP-10, inhibition of HDAC7 activity was evaluated. However, the inhibition of HDAC7s enzymatic activity using the selective IIa inhibitor did not cause a change in MMP-10 gene expression, compared to control. In addition, inhibition of HDAC7 enzymatic activity did not alter levels of proteolytically active MMP-10. This suggests that although HDAC7 is central to MMP-10 expression the activity of HDAC7 has no influence on the regulation of MMP-10 in *vitro* unlike the class I HDACs which directly enzymatically regulate gene expression. The class IIa HDACs; -4, -5, -7 and -9, are reported to have a low deacetylase activity against acetylated histones (Haberland *et al*., 2009). Furthermore, HDAC7 has been reported to recruit class I HDACs through its C-terminal HDAC domain, with its enzymatic activity dependent upon being bound to the class I HDAC; HDAC3 (Fischle *et al*., 2001), in agreement with other class IIa HDACs, although predominantly localised in the cellular nucleus HDAC7 can also locate in the cytoplasm, suggesting nucleoplasmic shuttling of this enzyme occurs (Kao *et al*., 2001). In the cytoplasm, HDAC7 is not bound to HDAC3 and is therefore enzymatically inactive with the converse apparent in the nucleus (Fischle *et al*., 2001). The interaction between HDAC7 and HDAC3 is in itself

indirect with these two HDACs being co-recruited by the transcriptional co-repressors SMRT and N-CoR, which serve as crucial mediators of HDAC7 activity by simultaneously binding HDAC7 and HDAC3 via two repressor domains (Fischle *et al*., 2001).

Therefore, although HDAC7 is required for MMP-10 activity, as shown in this study and those investigating vascular development, it is not as straightforward as first postulated and is neither a direct acetylation effect nor a 'conventional' HDAC pathway. It is well established that HDAC7 is regulated post-translationally. The class IIa HDACs possess a conserved C-terminal catalytic HDAC domain and interact with myocyte enhancer factor 2 (MEF2) transcription factors through an N-terminal 17-amino acid motif. This interaction leads to the recruitment of class IIa HDACs to select promoters, where MEF2 is bound, resulting in the repression of its transcriptional activity. The repressive activity of class IIa HDAC; HDAC7 is tightly regulated by nucleoplasmic shuttling and by their phosphorylation dependant association with the intracellular 14-3-3 proteins. Phosphorylation of conserved residues in the N-terminal regions of HDAC7 in response to cellular signals leads to interaction with 14-3-3 proteins, dissociation of the class IIa HDAC-MEF2 complexes and a conformational change culminating in export from the nucleus (Parra *et al*., 2004). However, this study implies another potential mechanism for HDAC7 in the regulation of MMP-10, HDAC7 could act as transporter, shuttling a class I HDAC, potentially HDAC3 into the nucleus where it acts upon the control of MMP-10 expression and activity, indicative in the importance of HDAC7 presence but not activity in the regulation of MMP-10. In this mechanistic model, although HDAC7 presence is important for regulation of MMP-10 expression and activity its role would be to 'mobilise' gene regulators, specifically

HDAC3, with these latter proteins being the drivers of MMP-10 expression. In terms of NSCLC and the involvement of MMP-10 therein, a much larger and more complex pathway than HDAC7-directed regulation of MMP-10 is now evident.

Chapter V – Class I HDAC-mediated regulation of MMP-10 expression in NSCLC

V.1 Introduction

Class I HDACs, which include; HDAC1, -2, -3 and -8 are ubiquitously expressed in tissues and are the most thoroughly investigated with respect to function (Fischle *et al*., 2001; Hayashi *et al*., 2010). They are known to have a high deacetylase activity and control the deacetylation state of lysine sidechains in histone tails, promoting chromatin condensation and consequent transcriptional repression (Chang *et al*., 2006; Watson *et al*., 2016). The class I HDACs regulate a number of genes by directly interacting with transcription factors; including Ef2, Stat3, p53 and NF-Kb (Delcuve *et al*., 2012). Deletion of these enzymes individually leads to deregulation of a limited set of genes, further supporting their involvement in transcription regulation, with a role in cell proliferation and survival (Reichert *et al*., 2012; Delcuve *et al*., 2012).

Members of the class I HDACs (all except HDAC8) have been found to form several large multiplexes with other proteins, each with particular biological functions, offering a further way they regulate gene expression (Fischle *et al*., 2001). The formation of these complexes are targeted to specific genomic regions by interactions with DNA binding factors that include transcription factors, nuclear receptors, and other epigenetic modifier genes, such as methyl-binding proteins (MBDs), DNA methyl transferases (DNMTs) and histone methyl transferases (HMTs) (Fischle *et al*., 2001; Ropero & Esteller, 2007).

HDAC1 and -2 are close homologues and exclusively nuclear, they are known to form homo- and heterodimers between each other, which presumably allows them to act together or separately from each other, and it is thought that the dimer is required

for HDAC activity (Delcuve 2012). They are found together in repressive complexes such as sin3, NuRD, CoREST and PRC2 complexes, which are recruited to chromatin regulatory regions by transcription factors and have diverse, often cell-specific roles (Haberland *et al*., 2011; Gallinari *et al*., 2007; Delcuve *et al*., 2012). These complexes are recruited to chromatin through interaction with repressive transcription factors or other silencing co-factors and their enzymatic activity shows significant enhancement when incorporated into their cognate co-repressor complexes (Watson *et al*, 2016). There is extensive evidence to support the activity of these nuclear-restricted HDACs as being exclusively toward histone modulation and gene expression (Gallinari *et al*., 2007).

HDAC3, another class I HDAC, unlike HDAC1 and -2, has been found to be present in both the nucleus and cytoplasm, suggesting nucleoplasmic shuttling of this enzyme. Like HDAC1 and -2 it to is found in distinct complexes, such as the two highly related complexes nuclear receptor corepressor (NCoR or NCOR1) and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) (Haberland *et al*., 2011). These nuclear corepressors are regulated by different kinase pathways and play different roles in development. It is noteworthy that repression by NCoR/SMRT is an integral phase of the cyclical process that is the transcriptional activation of genes controlled by liganded receptors (Delcuve *et al*., 2012). NCoR/SMRT repression is necessary to prime chromatin for subsequent transcription initiation. Besides its role in transcriptional control, the HDAC3-NCoR-SMRT axis is critical to the maintenance of heterochromatin content and genomic stability (Delcuve *et al*., 2012). Deregulated functions of NCor and SMRT have been observed in many types of cancers; including breast and prostate (Wong *et al*., 2014).

In addition to their central role in HDAC3 activity and gene expression, NCoR and SMRT also interact with class IIa HDACs, which have minimal deacetylase activity, but are believed to recruit NCoR/SMRT HDAC3 activity to distinct promoters, such as myocyte enhancer factor 2 (MEF2) (Martin *et al*., 2007). The class IIa HDACs are signal transducers characterised by the presence in their regulatory N-terminal domains of two or three conserved serine residues subject to reversible phosphorylation (Delcuve *et al*., 2012). Phosphorylation leads to the binding of the 14-3-3 proteins, the nuclear export of HDACs and the derepression of their target genes. A range of kinases and phosphatases acting downstream of diverse biological pathways have been shown to regulate the nucleocytoplasmic trafficking of class IIa HDACs (Delcuve *et al*., 2012).

For instance, HDAC7 is phosphorylated by PKD1 and this is shown to facilitate cytoplasmic retention of this HDAC and subsequently its functional translocation activity (Parra *et al*., 2005). In the context of vascular development and particularly angiogenesis, VEGF the driving factor for this process directly regulates HDAC7 as demonstrated in chapter IV, although HDAC7 is important for MMP-10 expression and activity, this involves a mechanism dependent of HDAC7 enzymatic activity but dependent upon HDAC7 presence.

One viable explanation is that the class IIa HDAC; HDAC7 may be responsible for recruiting class I HDACs, which allows them to elicit their repressive activity against MMP-10 in NSCLC. Such a collaborative role between HDAC7 and HDAC3 has already been shown in several studies, indicating a close relationship between HDAC7 and HDAC3 in the control of biological functions (Fischle et al., 2002; Fischle et al., 2001). For instance, in cancer cells HDAC3 and HDAC7 collaboratively suppress expression

of autotaxin, a secreted glycoprotein responsible for production of lysophosphatidic acid (LPA) involved in cancer progression, including NSCLC (Li et al., 2014). Whereas down-regulation of HDAC3 facilitates histone acetylation in the ATx promotor, concomitant down-regulation of HDAC7 was required to induce ATx expression (Li et al., 2014). These data suggest HDAC3-mediated acetylation of ATx is sufficient and the presence of HDAC7 is needed to 'modulate' HDAC3 activity (Fischle *et al*., 2002; Fishel *et al*., 2001). Therefore, it is highly plausible that such a function could exist in the case of MMP-10, with HDAC3 facilitated by HDAC7 being the actual regulatory process.

V.1.1 Aims and objectives

The aim of this chapter is to build upon the confirmation of the involvement of HDAC7 in the regulation of MMP-10 expression and activity in NSCLC *in vitro* and further define the mechanistic basis of this process. In particular this chapter will address the existence of a dynamic relationship between HDAC7 and the class I family member HDAC3 in the regulation of MMP-10. In order to evaluate such a mechanism, the effect of regulating activity and presence of HDACs and more specifically HDAC sub-families and individual HDACs upon MMP-10 expression and activity in the *in vitro* NSCLC model system will be evaluated.

V.2 Methods

V.2.1 Determination of cell viability upon HDAC inhibition, using the MTT assay

H460 cells were seeded in 96-well plates (250 cells/well, optimised in figure III.3) and incubated for 24 hours (37°C in humidified atmosphere of 5% CO_2 and 95% air), media removed and replaced with fresh media. The range of HDAC inhibitors were prepared as a 10mM stock solution and added to the 96-well plate by serial dilution at a final concentration of 1pM-10µM and incubated for 12-96 hours (37°C in humidified atmosphere of 5% CO_2 and 95% air). After the designated incubation time, an MTT was carried out, as described in section III.2.5.

V.2.2 Determination of cell viability upon HDAC inhibition, using xCELLigence assay

H460 cells were seeded into wells of the E-plate at a density of 1000 cells/well (optimised in figure III.2), the RTCA DP software was set up as described in section II.2.4, and incubated for 24 hours (37°C in humidified atmosphere of 5% CO_2 and 95% air), media removed and replaced with fresh media. The range of HDAC inhibitors were prepared as a 10mM stock solution and added to the E-plate at varying final concentrations (previously optimized by MTT assay), and incubated for 48 hours (37°C in humidified atmosphere of 5% CO_2 and 95% air).

V.2.3 Knockdown of HDAC3 expression in H460 NSCLC cells with siRNA

Two sets of double stranded small-interference RNA (siRNA) for HDAC3 (sequence, Table V.1) (Ambion, UK), were reconstituted to a stock concentration of 100µM in sterile dH_2O. For *in vitro* cell studies, siRNA was further diluted into 250µl serum free media; OptiMEM, at a range of working concentrations (50-200nM).

H460 cells were grown in 6 well plates ($4x10^5$ cells per well) (as described in section II.2), media was removed from the cells, the cells washed in HBSS, and serum free media added to the cells. The transfection agent Lipofectamine 2000 (Invitrogen, UK), was reconstituted at a 1:35 ratio into OptiMEM, to a final volume of 250µl. The Lipofectamine (250µl) and siRNA (250µl) solutions were combined and the mixture incubated for 15 minutes at room temperature, to allow the siRNA-lipid complexes to form. The siRNA-lipid complex was added directly into the serum free media and incubated for 6 hours at 37°C 5% CO_2. After the 6 hours transfection time, fresh media was added and the cells were incubated for a further 12-96 hours. Cells were assayed for RT-PCR, see section II.3 for details.

Table V.1; HDAC3 siRNA nucleotide sequence - HDAC3 siRNA targets HDAC3 mRNA between 605-619bp

	HDAC3 Nucleotide sequence (5'->3')		
Target gene	**Sense**	**Antisense**	**Product size**
Human histone deacetylase 3	GCUUUCUACCUCACUGACCtt	GGUCAGUGAGGUAGAAAGCtt	511bp

V.2.3.1 Detection of HDAC3 gene silencing for RT-PCR

Primers to detect the silence of the HDAC3 gene expression in H460 cells were custom designed using the primer blast tool on the National Center for Biotechnology Information (NCBI) and purchased from Invitrogen (UK). Primer sequences are shown in table II.1.

V.2.4 Detection of HDAC3 gene silencing on HDAC3 protein expression

H460 cells were plated at a density of $4X10^6$ cells per well in a 6 well plate, and incubated in complete media for 24 hours (37°C in humidified atmosphere of 5% CO_2 and 95% air). HDAC3 gene expression was silenced using HDAC3 siRNA, as previously described and optimised in section V.2.3. Following knockdown, protein was extracted and quantified (as described in section II.5.2), samples prepared and proteins fractionated using SDS-PAGE (see section II.5.3 for details). Proteins were transferred to a membrane and probed with antibodies to detect human HDAC3, as described in section II.5.4/II.5.5 (see table II.3 for antibody details and concentrations).

V.2.5 Determination of MMP-10 selective enzyme activity in the presence/absence of HDAC7

H460 cells were plated at a density of $4x10^5$ cells per well in a 6 well plate and incubated in complete media for 24 hours. To compare the effect of expression of activity upon MMP-10 activity, cells were treated with either HDAC3 siRNA (50nM) or a class

I HDAC inhibitor (MS275, 1µM). To assess the levels of active endoprotease enzymes present in cell media, aliquots of media were removed at various time points over the duration of the time course (24-96h). The fluorometric enzyme assay was performed in a 96 well plate by incubating 10µl of media with 50µg of fluorogenic substrate M-2110(Mca-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(Dnp)-NH_2) in 80µl of MMP activity buffer (100 mM Tris-HCl pH 7.6, 10 mM CaCl2, 100 mM NaCl, 0.16% v/v Brij-35); total reaction volume of 100µl. Fluorescence was measured (excitation = 325 nm; emission = 393 nm) over a 600-minute incubation period at 37°C. For each sample, the relative activity of the initial linear enzyme activity was calculated, and activity expressed as change in fluorescence per hour per $4x10^5$ cells. The reproducibility and reliability of the assay were determined using samples obtained from three independent experiments.

V.3 Results

V.3.1.1 Cytotoxicity of the pan inhibitor Trichostatin A on NSCLC *in vitro*

For up to 24 hours; 100nM of TSA was determined as the optimal dose to use for investigating the effect of MMP-10 post treatment of the HDAC pan inhibitor (figure V.1), with an IC_{50} value of >10µM (table V.2).

To confirm lack of toxicity and mitigate against any drug induced cytological effects and to examine the effects elicited by TSA, at a non-toxic concentration for 24-hour drug exposure on the growth kinetics of the cell, the xCELLigence assay was used. The non-toxic dose; 100nM (determined by MTT assay) demonstrated no overall changes in cell index relative to control (figure V.2). This indicates at this non-toxic concentration that pan-inhibition of class I and II HDACs does not affect cellular via-bility.

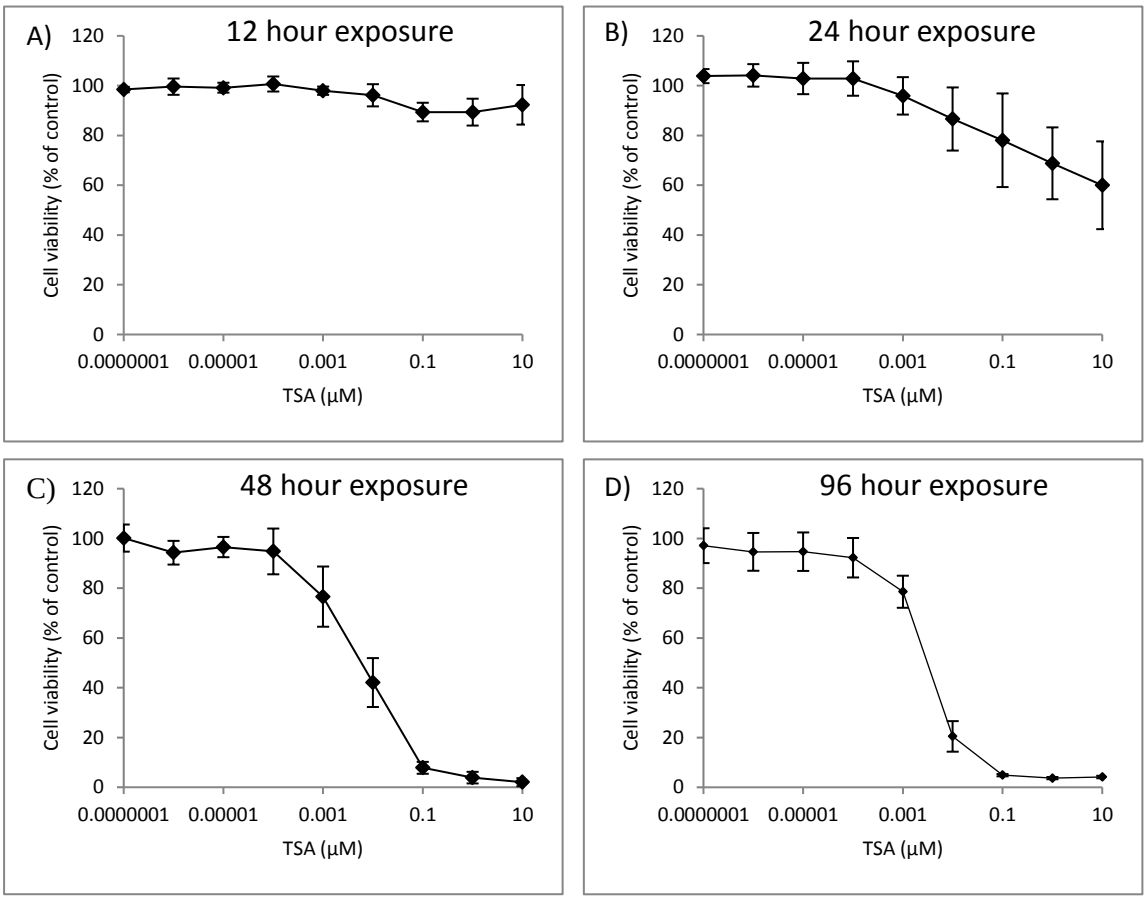


Figure V.1 The pan-HDAC inhibitor induces dose and time-dependant cytotoxicity against H460 NSCLC in vitro. Survival curves obtained for H460 cells after A) 12 hours, B) 24 hours, C) 48 hours and D) 96 hours exposure of the HDAC pan inhibitor; TSA, at a range of concentrations (1000nM to 0.001nM). Data representative of the mean ± SD of 3 independent experiments.

Table V.2; Summary of minimum toxic dose of TSA for 12-96h

Time (hours)	Minimum toxic dose	Cytotoxic IC_{50}
12	>10μM	>10μM
24	1μM	>10μM
48	1nM	5nM
96	1nM	2nM

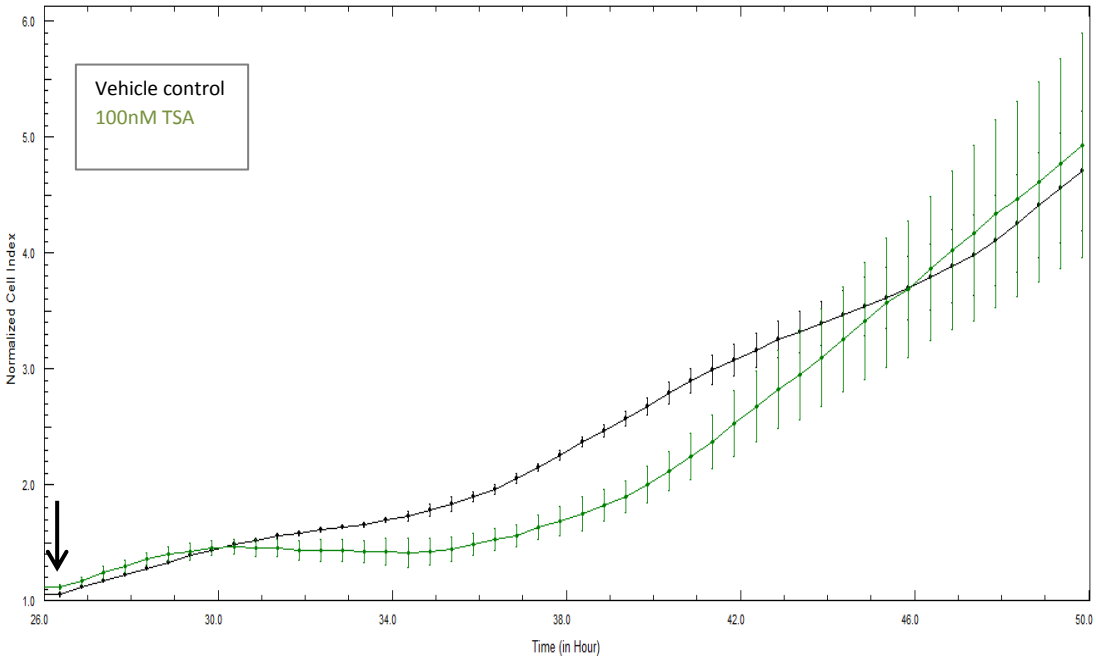


Figure V.2 Lack of significant cytological effect of 100nM TSA against H460 NSCLC in vitro, obtained by xCELLigence RTCA. The black arrow indicates the time of drug addition. H460 cells plated at 4000 cells per well and observed over 24h.

V.3.1.3 Inhibition of class I and II HDACs activity induces expression of MMP-10 gene in NSCLC

To investigate the effect on MMP-10 expression following inhibition of all the HDACs in NSCLC, H460 cells were treated with the optimised concentration (100nM) of the pan inhibitor; TSA over a time course (12 and 24 hours). After exposure to the inhibitor, MMP-10 expression significantly increased at both 12 hours ($p<0.05$) and 24 hours ($p<0.01$), compared to control (figure V.3B).

V.3.1.4 Inhibition of HDACs induces MMP-10 proteolytic activity in H460 NSCLC cells model

Because MMP-10 can exist as both an inactive proenzyme and as an active enzyme, MMP activity following treatment upon pan inhibition was assessed. Using the MMP-selective substrate, M2110, activity relating to MMP-10 activity can be evaluated. Increased MMP-10 activity was observed at 48 hours ($P<0.001$), suggesting a relationship between HDAC inhibition and MMP-10 activity (figure V.4).

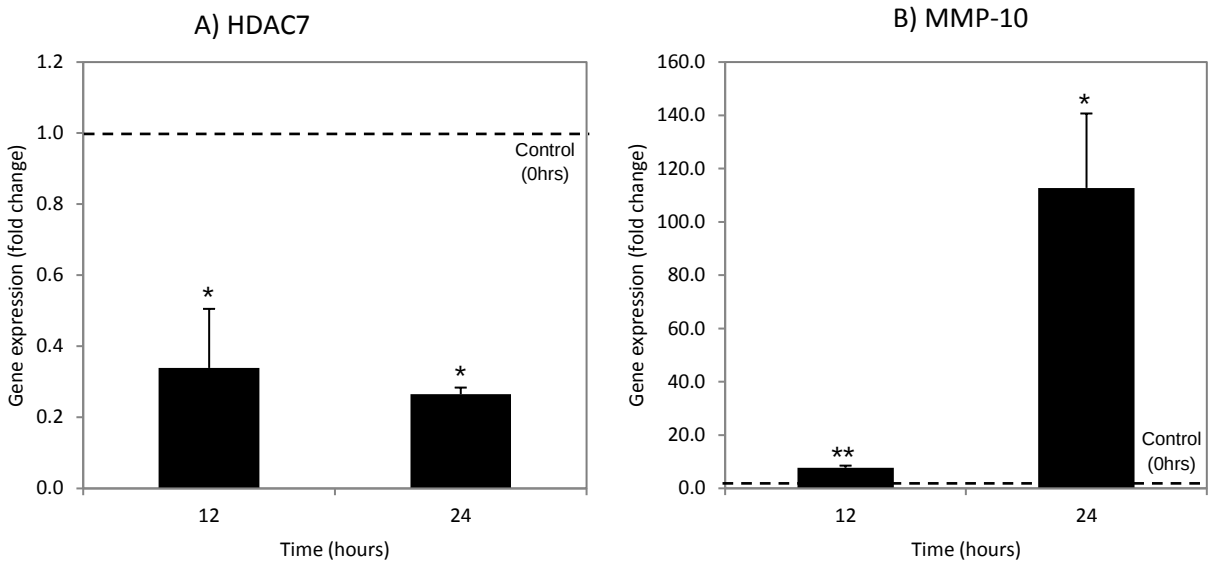


Figure V.3 The HDAC pan inhibitor; TSA, inhibits expression of HDAC7 mRNA and induces MMP-10 mRNA expression in NSCLC. H460 cells were treated with 100nM of the HDAC pan inhibitor; TSA over a time course of 12 and 24 hours. Gene expression of A) HDAC7 and B) MMP-10, post treatment, determined by RT-PCR. Signal intensity was quantified by densitometry. Values are mean ± S.E. from three independent experiments, *p<0.05, **p<0.01, compared by unpaired t-test.

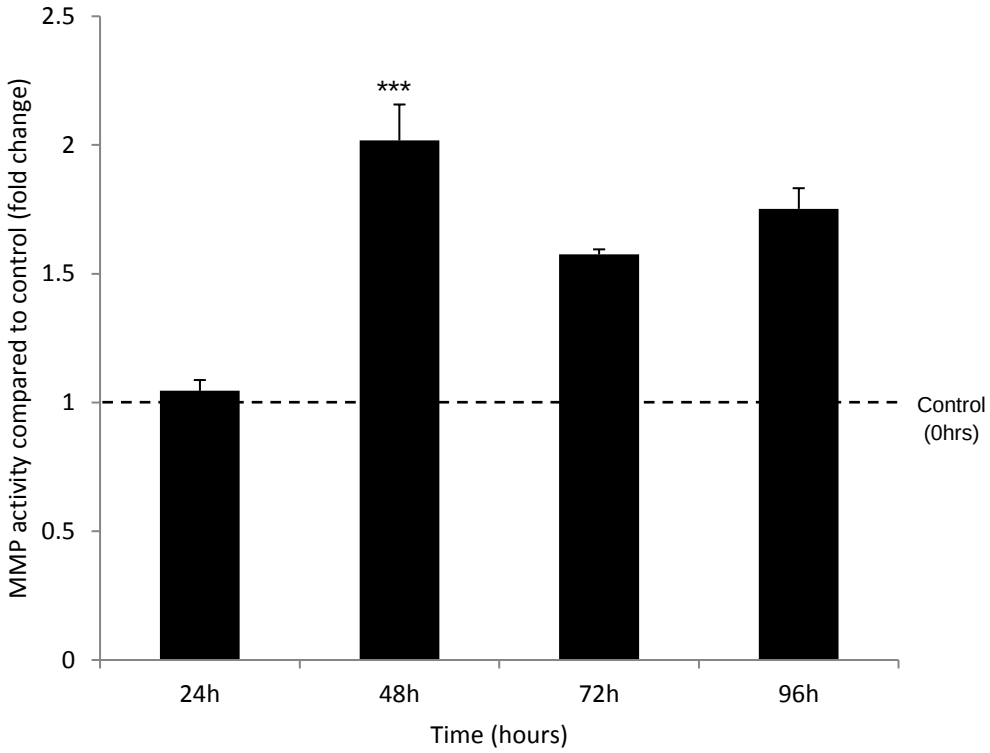


Figure V.4 The induction of MMP-10 activity by inhibition of HDAC enzymes in H460 NSCLC model

To assess the levels of active proteolytic enzyme, medium was removed following treatment of H460 cells with a single dose of TSA (100nM) over a time course (0-96h). Activity levels were determined by measuring cleavage of the fluorogenic substrate M-2110 (Mca-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(Dnp)-NH_2) with selectivity for MMP-10. Values are mean ± S.E. from three independent experiments, $p<0.001$ compared by unpaired t-test.

V.3.2 Cytotoxicity of class I HDAC inhibitor on NSCLC in vitro

1µM of MS275 was determined as the optimal dose to use, for investigating the effect of MMP-10, post treatment of the class I HDAC inhibitor (figure V.5).

To confirm lack of toxicity and identify any time-dependent drug-induced cytological effects and to examine the effects elicited by MS275 on the growth kinetics of the cell, the xCELLigence assay was utilised. The non-toxic dose; 1µM (determined by MTT assay) demonstrated no overall changes in cell index relative to control (figure V.6). This indicates that inhibition of class I HDACs does not alter cell proliferation, adherence or morphology of cells.

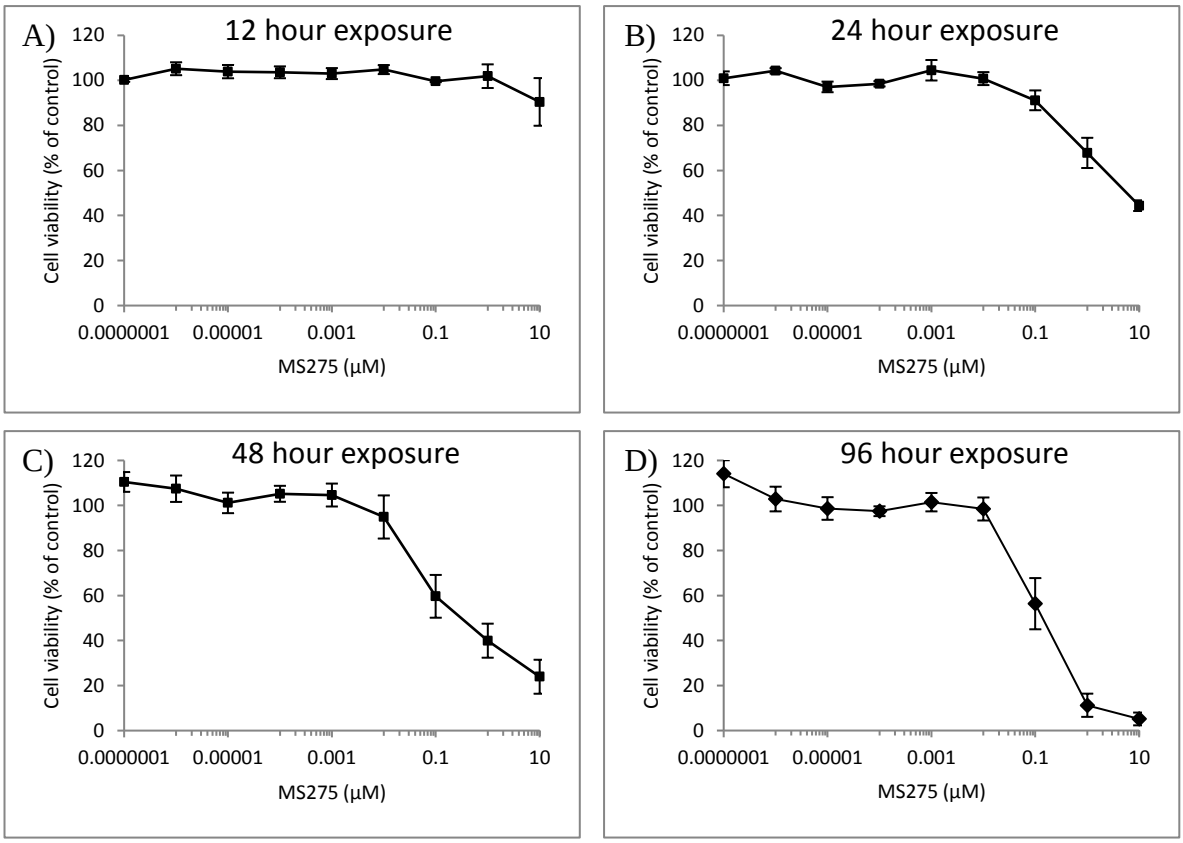


Figure V.5 The class I HDACi induces concentration of time-dependent cytotoxicity. Survival curves obtained for H460 cells after A) 12 hours, B) 24 hours, C) 48 hours and D) 96 hours exposure, to a single dose of the class I HDAC inhibitor; MS275, at a range of concentrations (1000nM to 0.001nM). Data representative of the mean ± SD of 3 independent experiments.

Table V.3; Summary of minimum toxic dose of MS275 between 12-96h

Time (hours)	Minimum toxic dose	Cytotoxic IC_{50}
12	>10µM	>10µM
24	1µM	5µM
48	10nM	50nM
96	10nM	10nM

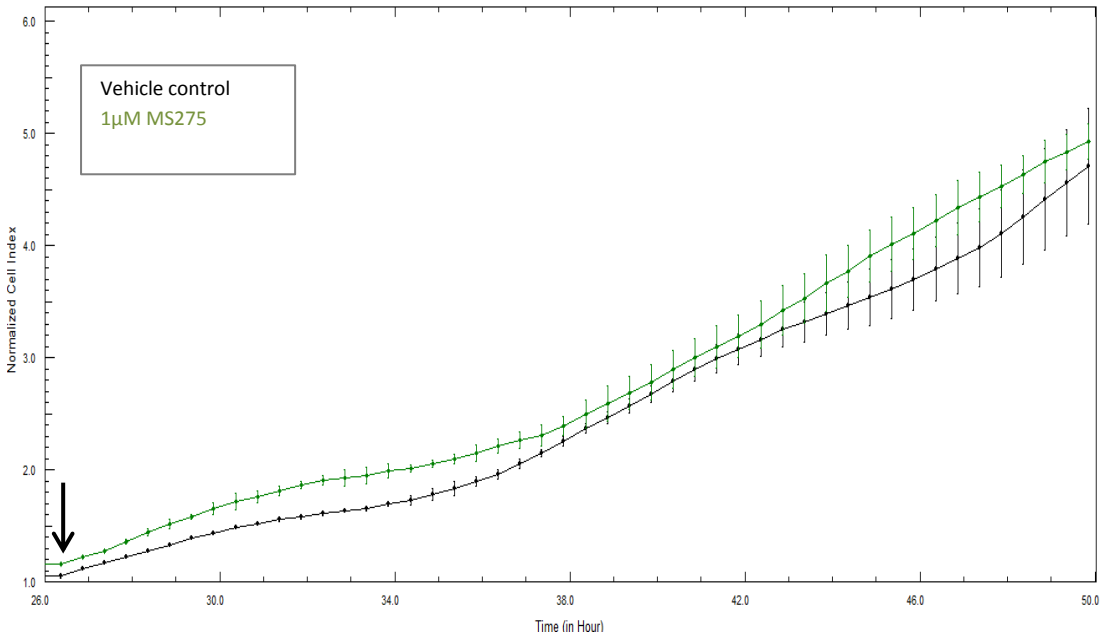


Figure V.6 Lack of significant cytological effect of 1µM class I HDACi; MS275 against H460 in vitro, obtained by xCELLigence RTCA. The black arrow indicates the time of drug addition. H460 cells plated at 4000 cells per well and observed over 24h.

V.3.2.2 Inhibition of class I HDAC activity reduces HDAC7 expression and induces MMP-10 gene expression in H460 NSCLC cells.

To investigate the mechanism of which HDAC is responsible for regulating the gene expression of MMP-10 in NSCLC, H460 cells were treated with the optimal non-toxic dose (1µM) of the class I selective inhibitor; MS275. MS275 was used to inhibit the enzymatic activity of class I HDACs, over a time course of 12 and 24 hours. HDAC7 gene expression significantly decreased after 12 (p<0.05) and although recovering toward control levels was still significantly reduced after 24 hours (p<0.001), post exposure to MS275 (figure V.7A). Conversely, MMP-10 gene expression was elevated 5-fold 12 hours after MS275 treatment (p<0.05) (figure V.7B) and 2.5-fold after 24 hours (figure V.7B).

V.3.2.3 Exposure to the class I HDAC inhibitor MS275 does not significantly alter MMP-10 proteolytic activity in H460 NSCLC cells model

Because MMP-10 can exist as both an inactive proenzyme and as an active enzyme, MMP activity following treatment of class I HDAC inhibition was assessed. Using the MMP-selective substrate, M2110, MMP-10 activity can be evaluated. Although MMP-10 proteolytic activity was elevated at 24 and 48 hours post-exposure to MS275 this was not shown to be significantly significant (figure V.8). Overall, no significant difference in MMP-10 activity was expressed between control and treated cells over the time course (24-96h) (figure V.8).

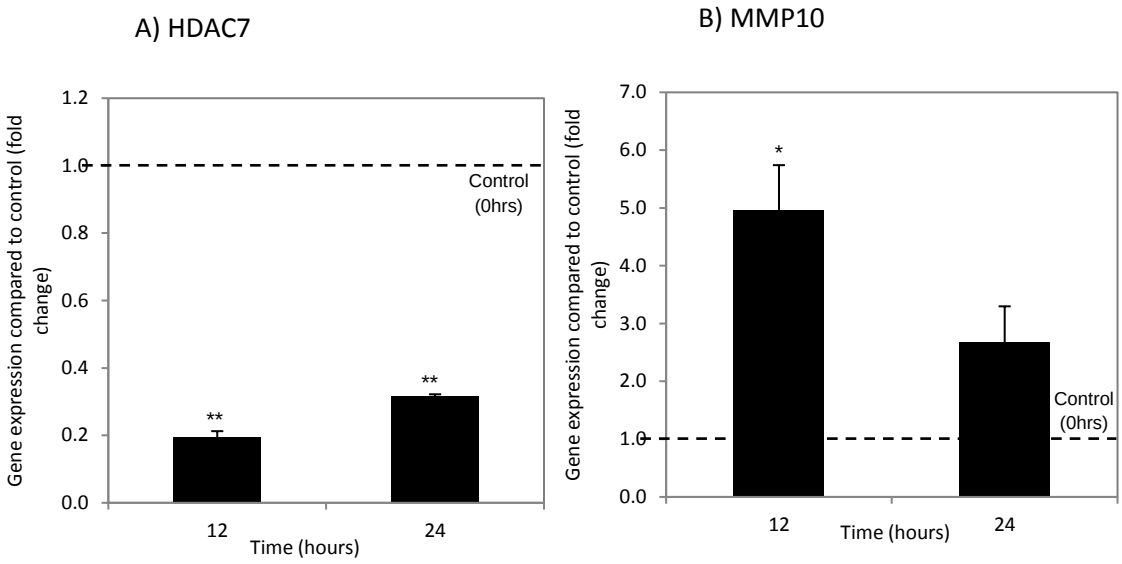


Figure V.7 The selective class I HDAC inhibitor; MS275, reduces HDAC7 mRNA and increases MMP-10 mRNA in NSCLC. H460 cells were treated with 1µM of the class I selective HDAC inhibitor; MS275 over a time course of 12 and 24 hours. Gene expression of A) HDAC7 and B) MMP-10, after exposure to the selective inhibitor, determined by RT-PCR. Signal intensity was quantified by densitometry. Values are mean ± S.E. from three independent experiments, *p<0.05, **p<0.01, compared by unpaired t-test.

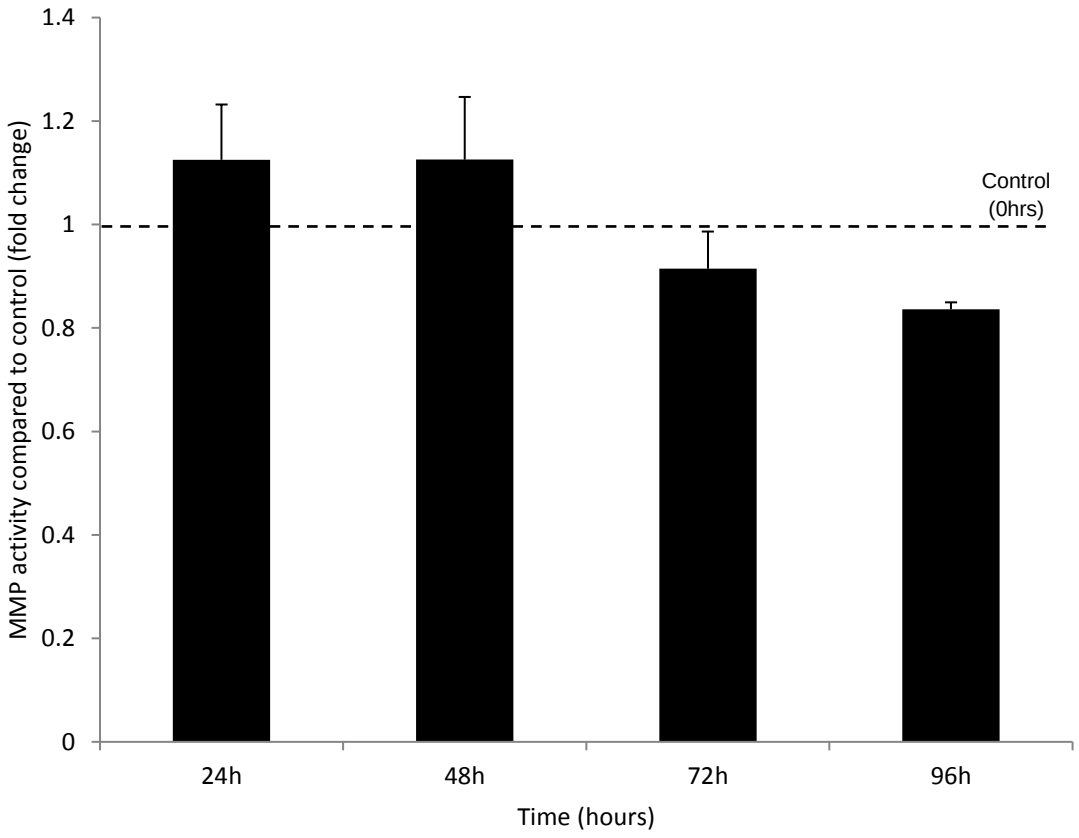


Figure V.8 Lack of significant effect of inhibiting class I HDAC activity on MMP-10 enzymatic activity in H460 NSCLC model

To assess the levels of active proteolytic enzyme, medium was removed following treatment of H460 cells with a single dose of MS275 (1µM) over a time course (0-96h). Activity levels were determined by measuring cleavage of the fluorogenic substrate M-2110 (Mca-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(Dnp)-NH_2) with selectivity for MMP-10. Values are mean ± S.E. from three independent experiments.

V.3.3 Evaluation of cytotoxicity of HDAC3 selective inhibitor on NSCLC in vitro

1µM of MI192 was determined as the optimal dose to use for investigating the effect of MMP-10, post treatment of the HDAC3-selective inhibitor.

To confirm lack of toxicity and identify any drug-induced modulation of cell proliferation, attachment or morphology and to examine the effects elicited by MI192 on the growth kinetics of the cell the xCELLigence assay was used. The non-toxic dose; 1µM (determined by MTT assay) again showed no overall changes in cell index, indicative of no effect upon morphology, number and adherence (figure V.9).

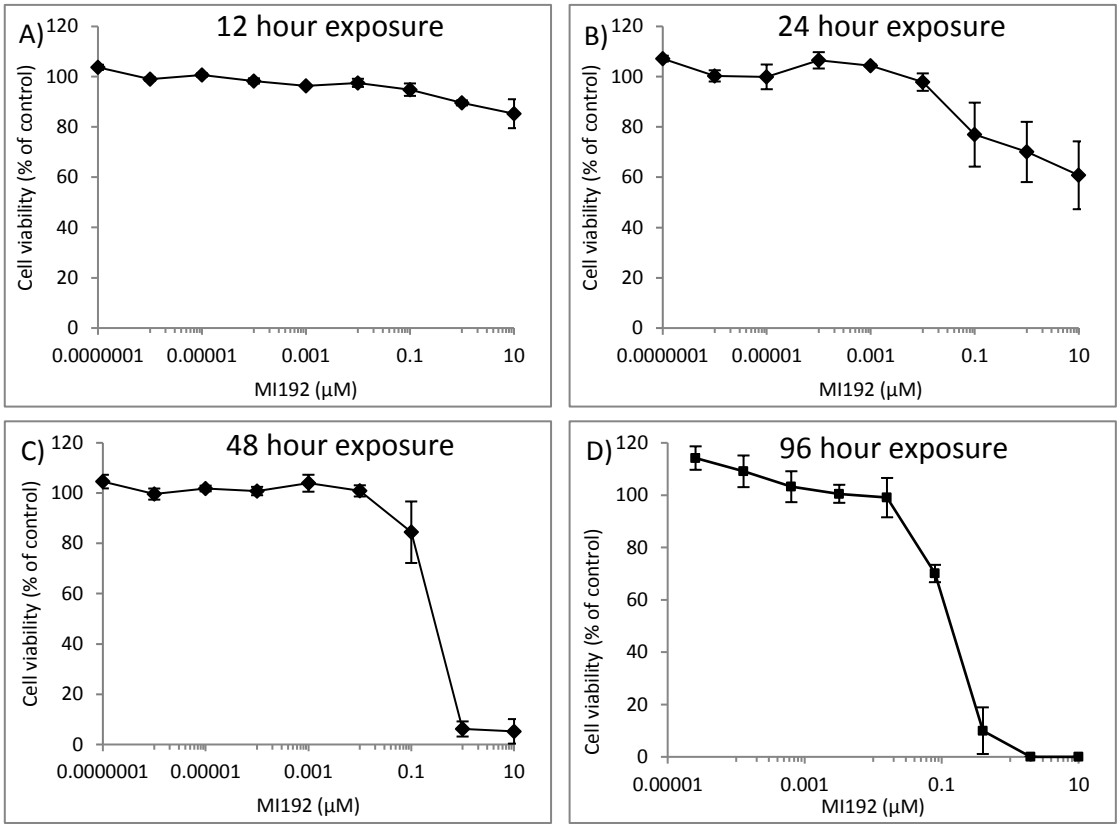


Figure V.9 The HDAC3-selecitve inhibitor induces concentration and time dependent cytotoxicity. Survival curves obtained for H460 cells after A) 12 hours, B) 24 hours, C) 48 hours and D) 96 hours exposure to a single dose of the class I selective HDAC3 inhibitor; MI192, at a range of concentrations (1000nM to 0.001nM). MTT assays were performed as described in II.2.5. Data representative of the mean ± SD of 3 independent experiments.

Table V.4; Summary of minimum toxic dose of MI192 between 12-96h

Time (hours)	Minimum toxic dose	Cytotoxic IC_{50}
12	>10µM	>10µM
24	1µM	>10µM
48	100nM	0.5µM
96	80nM	0.2µM

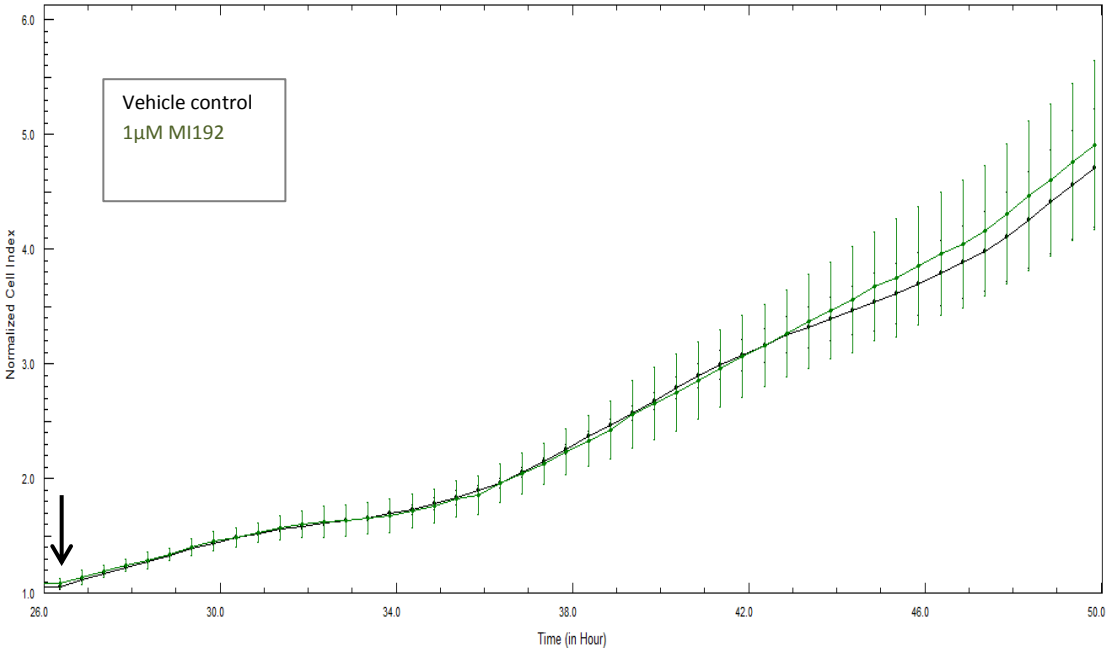


Figure V.10 Lack of significant effect of 1µM HDAC3-selective inhibitor on H460 cell survival, attachment or morphology obtained by xCELLigence RTCA. The black arrow indicates the time of drug addition. H460 cells plated at 4000 cells per well and observed over 24h.

IV.3.3.2 Inhibition of HDAC3 enzyme activity induces expression of MMP-10 but does not modulate HDAC7 expression in H460 NSCLC cells in vitro

To investigate the mechanism of which HDAC is responsible for regulating the gene expression of MMP-10 in NSCLC, H460 cells were treated with the optimal non-toxic dose (1μM) of the HDAC3-selective inhibitor; MI192. Effects upon the expression of HDAC7 and MMP-10 in the cell were then evaluated. MMP-10 gene expression significantly increased after 12 (p<0.001), 24 (p<0.001) and 48 hours (p<0.001) (figure V.11). Conversely, HDAC7 gene expression didn't significantly change, post exposure to MI192 (figure V.11).

V.3.3.3 Inhibition of HDAC3 activity induces MMP-10 proteolytic activity in H460 NSCLC cells model

Because MMP-10 can exist as both an inactive proenzyme and as an active enzyme, MMP activity following treatment of class I HDAC inhibition was assessed. Using the MMP-selective substrate, M2110, activity relating to MMP-10 activity can be evaluated. MMP-10 proteolytic activity increased in a time dependant manner, MMP-10 showed a significant increase (p<0.05) at 96 hours, compared to control (figure V.12).

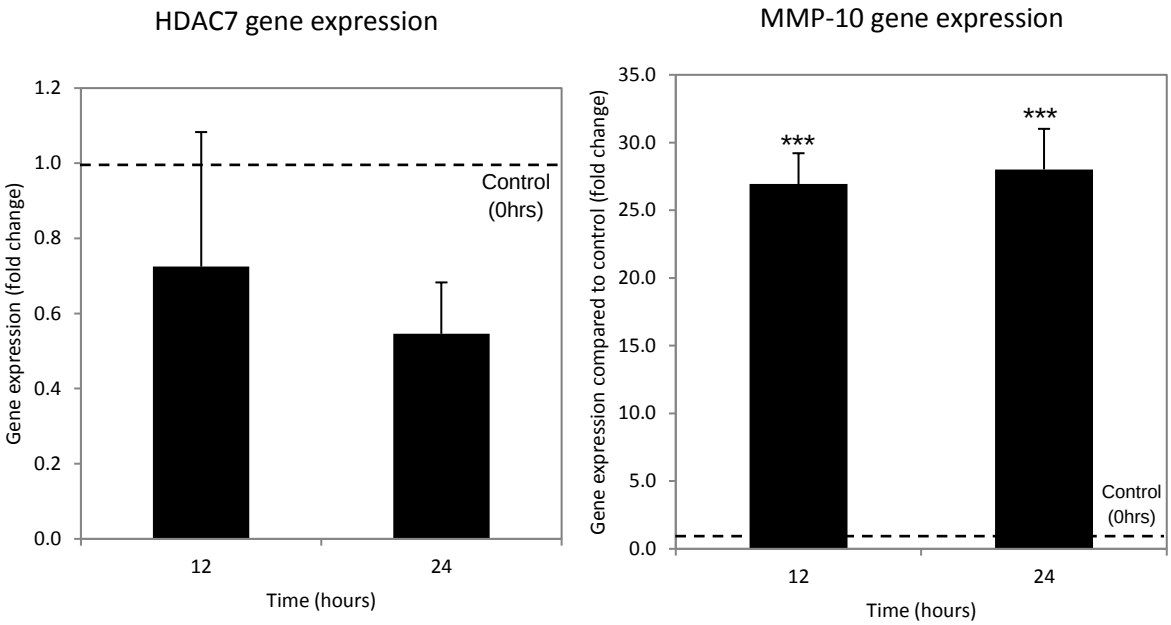


Figure V.11 The HDAC3-selective inhibitor MI192 does not significantly change HDAC7 expression but induces MMP-10 mRNA in NSCLC. H460 cells were treated with 1µM of the selective HDAC3 inhibitor; MI192 over a time course of 12-48 hours. Gene expression of A) HDAC7 and B) MMP-10 after exposure to the selective inhibitor, determined by RT-PCR. Signal intensity was quantified by densitometry. Values are mean ± S.E. from three independent experiments, ***p<0.001, compared by unpaired t-test.

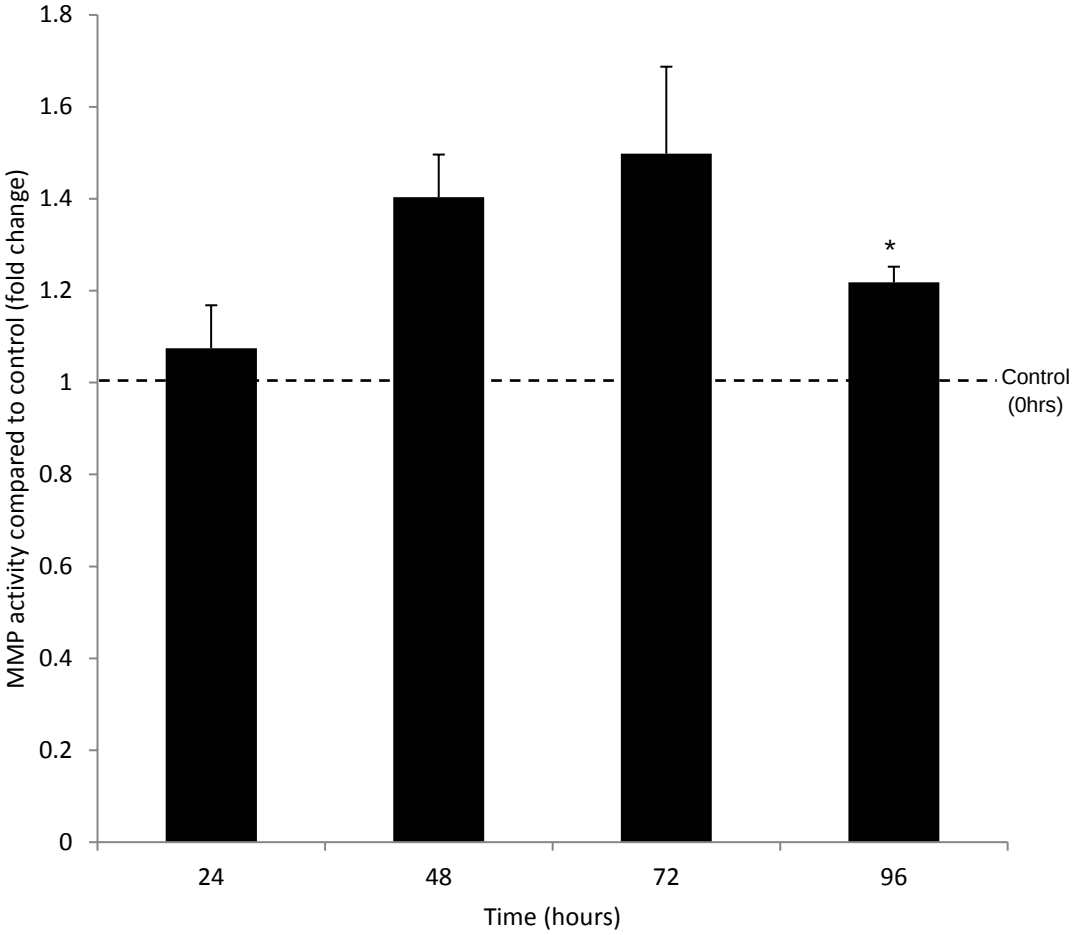


Figure V.12 Inhibition of HDAC3 activity induces MMP-10 enzymatic activity in H460 NSCLC model

To assess the levels of active proteolytic enzyme, medium was removed following treatment of H460 cells with a single dose of MI192 (1µM) over a time course (0-96h). Activity levels were determined by measuring cleavage of the fluorogenic substrate M-2110 (Mca-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(Dnp)-NH_2) with selectivity for MMP-10. Values are mean ± S.E. from three independent experiments, $p<0.05$, compared by unpaired t-test.

V.3.4 Effect of HDAC3 presence upon HDAC7 expression and MMP-10 expression and activity

IV.3.4.1 Lack of cytotoxicity of HDAC3 siRNA on NSCLC *in vitro*

Initially a cell viability assay in the form of an MTT was carried out to determine a range of appropriate non-toxic doses of HDAC3 siRNA to use *in vitro* (figure V.13). Cells were given a single dose of an increasing concentration of HDAC3 siRNA (between 0.8-200nM). Cell survival was assessed at 72 hours. No cytotoxicity was observed at any concentration of HDAC3 siRNA over the 72-hour exposure period.

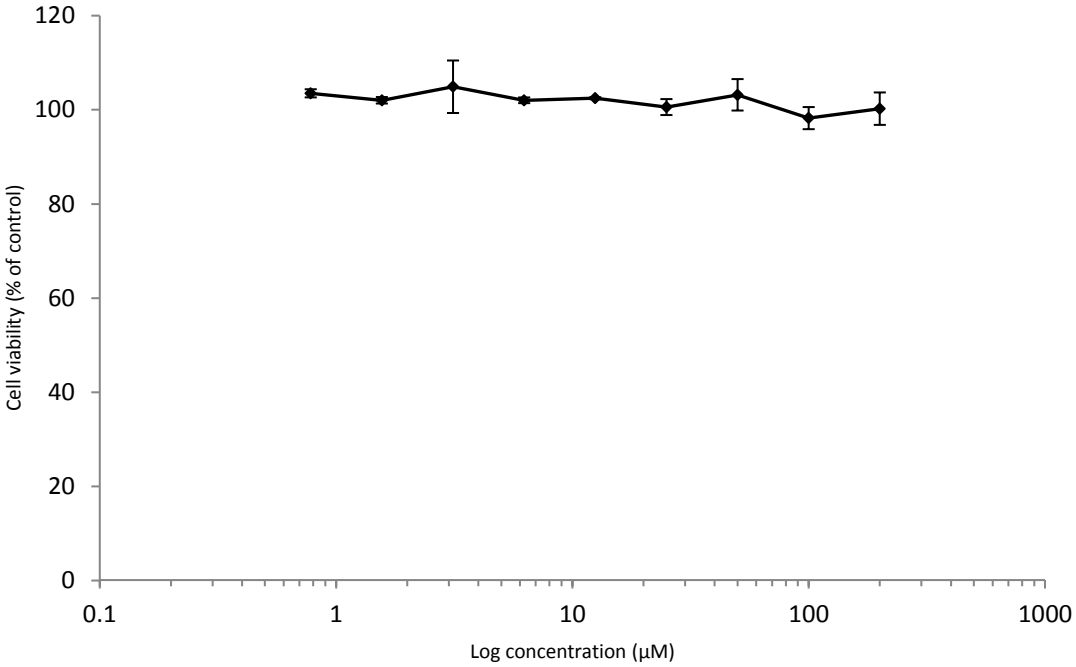


Figure V.13 Survival curves obtained for H460 cells after 96 hours exposure to a single dose of HDAC3 siRNA, at a range of concentrations (200nM to 80pM). MTT assays were performed as described in II.2.5. Data representative of the mean ± SD of 3 independent experiments.

V.3.4.2 Identification of HDAC3 siRNA concentrations required for optimal retardation of HDAC3 expression

To determine the optimal conditions for silencing the HDAC3 gene in NSCLC, H460 cells were treated with HDAC3 siRNA at a range of non-toxic working concentrations; between 50-200nM over a time course of 12-72 hours (figure V.14).

A concentration and time dependant silencing of the HDAC3 gene was observed. At 12 hours no significant silence of HDAC3 gene expression was detected at any of the four siRNA concentrations (1-100nM) evaluated (figure V.14A). At 24 hours, there was a significant change to gene expression with all four concentrations of siRNA, with a similar effect for each (p<0.0001) (figure V.14B). At 48 hours, the two highest concentrations of siRNA (150 and 200nM) showed the largest significant change to gene expression (p<0.001) (figure V.14C). At 72 hours, the effect of HDAC3 siRNA upon HDAC3 gene expression showed no significant difference to that observed in the absence of siRNA (figure V.14D). No significant difference in expression of the housekeeping gene, GAPDH, was observed at any of the time points, indicating comparability between studies. Consequently, 50nM was chosen at the optimal non-toxic does for subsequent experiments.

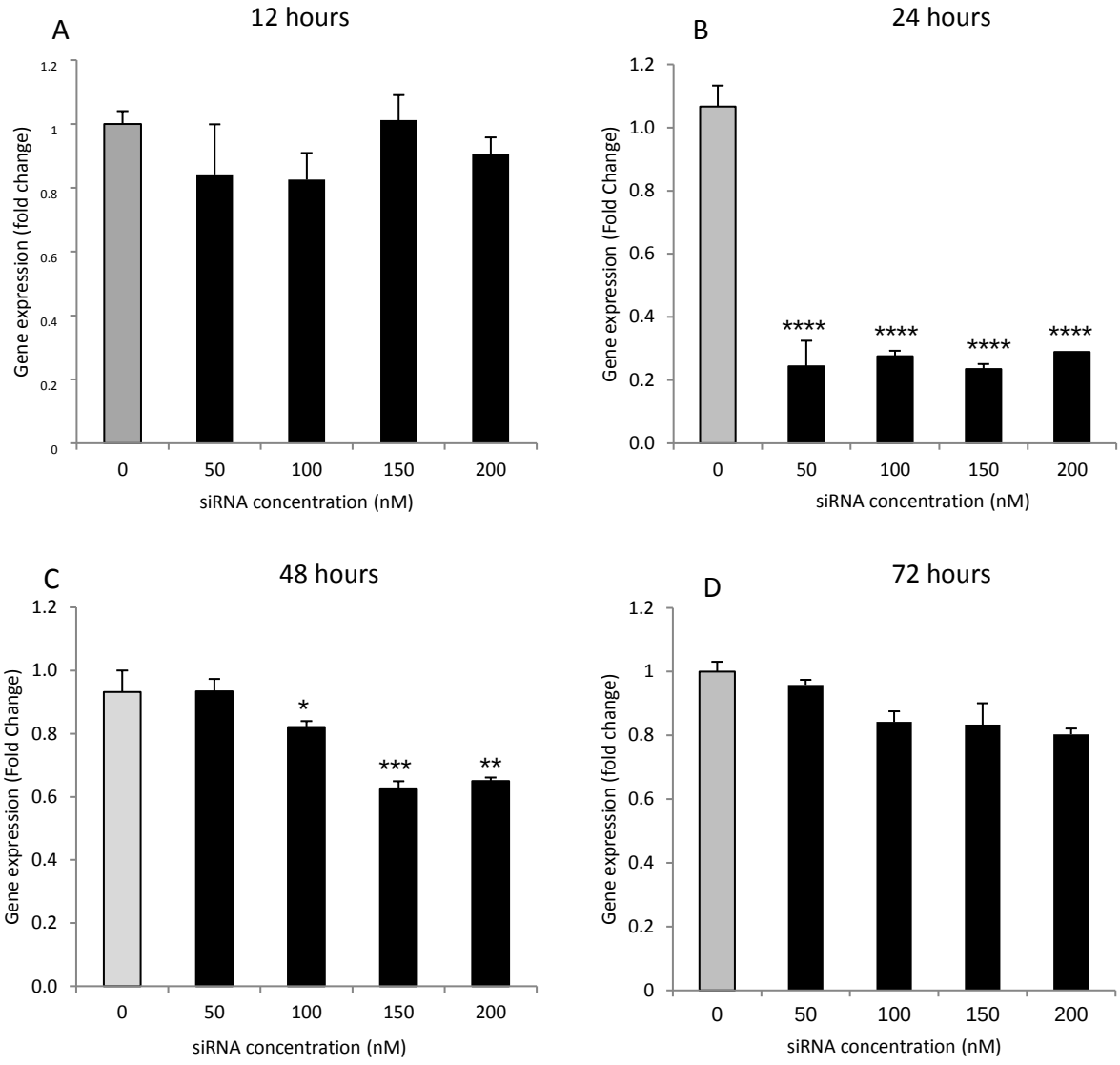


Figure V.14 Effect of increasing concentrations of HDAC3 siRNA on the level of HDAC3 mRNA messenger in NSCLC - H460 cells were treated with increasing concentrations (50-200nM) of HDAC3 siRNA over a time course of 12-72 hours. Gene expression of HDAC3 after A) 12 hours, B) 24 hours, C) 48 hours and D) 72 hours, post treatment, control is shown in grey, determined by RT-PCR. Signal intensity was quantified by densitometry. Values are mean ± S.E. from three independent experiments, *p<0.05, **p<0.01, ***p<0.001****p<0.0001 determined by one-way ANOVA/Dunnet's post hoc test.

V.3.2.3 The effect of HDAC3 silence upon the level of HDAC3 protein in NSCLC

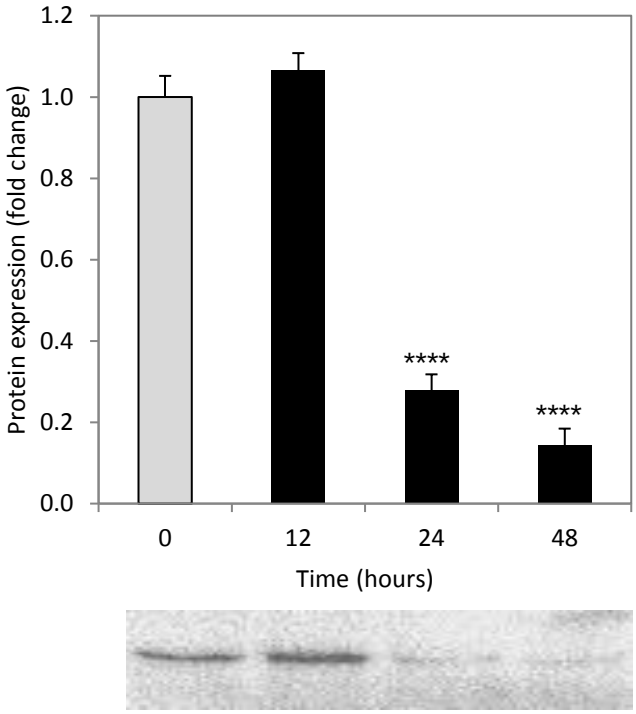


Figure V.15 Effect of HDAC3 siRNA on the level of HDAC3 protein in NSCLC**.** H460 cells were treated with the optimal non-toxic dose of siRNA (50nM) over a time course of 12, 24 and 48 hours. HDAC3 protein expression was determined by western blot analysis. A representative gel from each experiment is included below each graph. Signal intensity was quantified by densitometry. Values are mean ± S.E. from three independent experiments, ****p<0.0001 determined by one-way ANOVA/Dunnet's post hoc test.

V.3.4.3 Silencing of HDAC3 expression results in inhibition of HDAC7 expression and induction of MMP-10 expression in NSCLC

To investigate the mechanism by which HDAC3 regulates the gene expression of MMP-10 in NSCLC, H460 cells were treated with the optimal non-toxic dose (50nM) of siRNA to suppress the HDAC3 expression in the cells. siRNA knockdown of HDAC3 gene and protein was significantly achieved at 24 hours ($P<0.05$), post treatment (figures V.15 and V.16A). MMP-10 gene expression in the presence of siRNA at 24 hours ($p<0.05$) showed a significant increase, as compared to vehicle treated cells only (figure V.16B). In addition, HDAC7 gene expression showed a significant decrease at 24 ($p<0.05$) and 48 hours ($p<0.05$) post exposure to HDAC3 siRNA (figure V.16C). Therefore, knockdown of HDAC3 expression regulates HDAC7 and MMP-10 expression.

IV.3.4.4 Silencing of HDAC3 induces proteolytic activity of MMP-10 in NSCLC

Because MMP-10 can exist as both an inactive proenzyme and as an active enzyme, MMP activity following treatment of HDAC3 siRNA was assessed. Using the MMP-selective substrate, M2110, activity relating to MMP-10 activity can be evaluated. Increased MMP-10 activity was observed at 24 hours ($P<0.05$) (figure V.17), suggesting a relationship between HDAC3 and MMP-10.

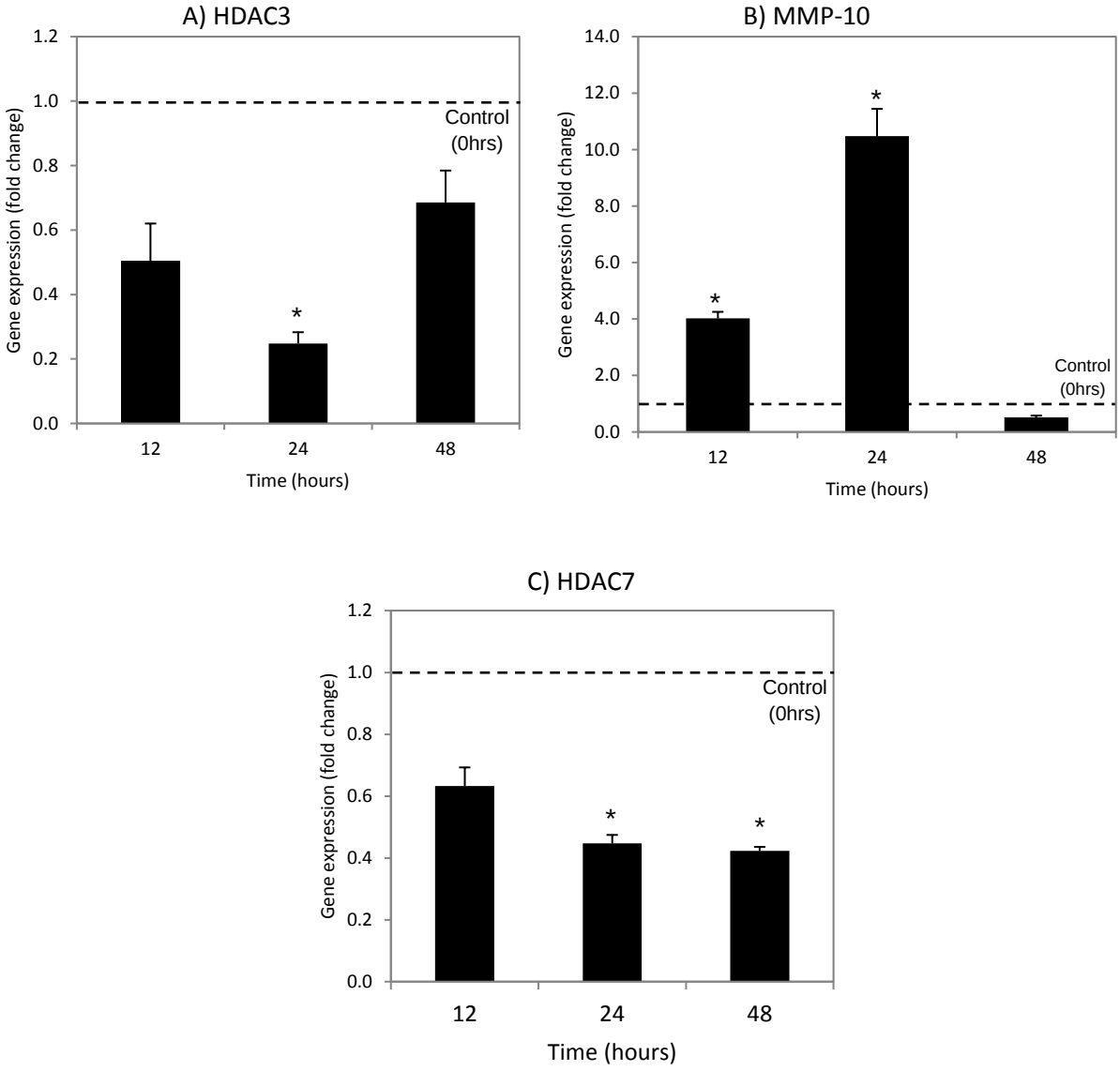


Figure V.16 Silencing of HDAC3 expression retards HDAC7 and induces MMP-10 expression in NSCLC. H460 cells were treated with the optimal non-toxic dose of siRNA (50nM) over a time course of 12-48 hours. Gene expression of A) HDAC3, B) MMP-10 and C) HDAC7, post treatment, determined by RT-PCR. Signal intensity was quantified by densitometry. Values are mean ± S.E. from three independent experiments, *p<0.05, compared by unpaired t-test.

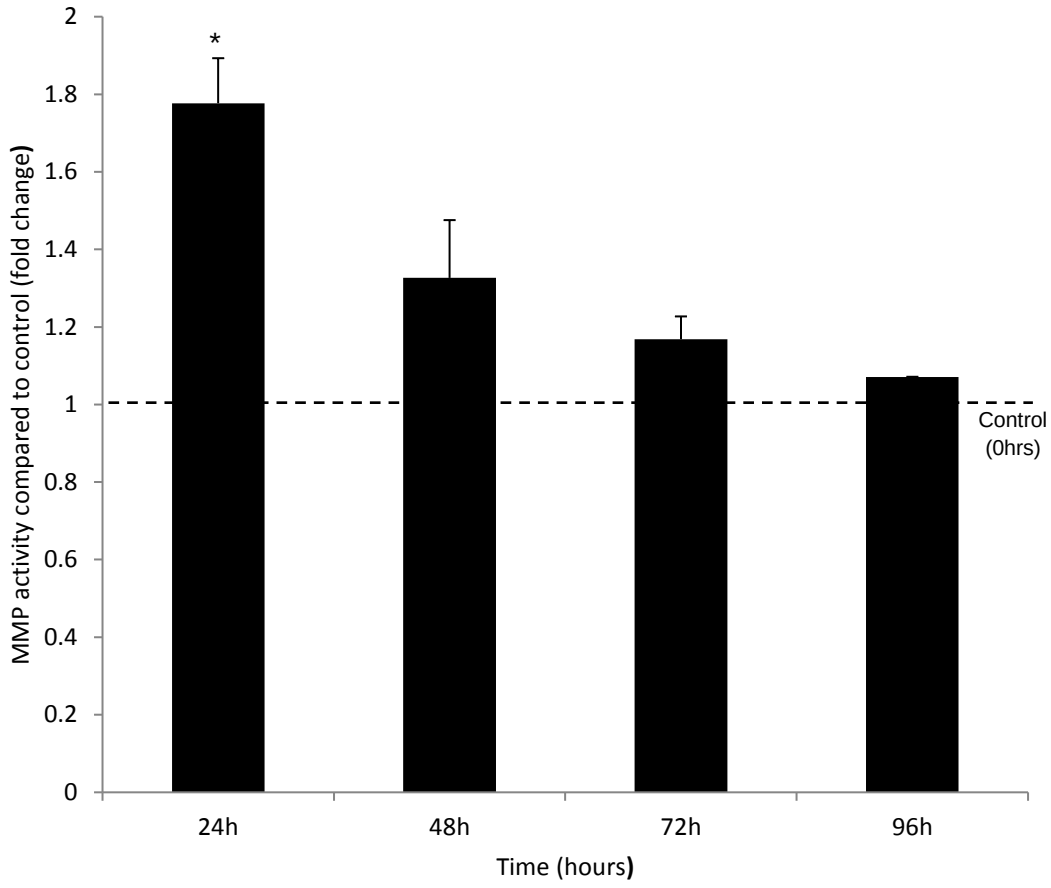


Figure V.17 Knockdown of HDAC3 results in MMP-10 enzymatic activity in H460 NSCLC model - To assess the levels of active proteolytic enzyme, medium was removed following treatment of H460 cells with a single dose of HDAC3 siRNA (50nM) over a time course (0-96h). Activity levels were determined by measuring cleavage of the fluorogenic substrate M-2110 (Mca-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(Dnp)-NH_2) with selectivity for MMP-10. Values are mean ± S.E. from three independent experiments, *p<0.05, compared by paired t-test.

V.4 Discussion

Histone acetylation modulates chromatin structure regulating DNA replication and gene transcription (Delcuve *et al*., 2012), through regulating access of transcription factors to chromatin structures (Delcuve *et al*., 2012; Ropero & Esteller, 2007). Additionally, HDACs form multiprotein corepressor complexes with nuclear receptors and transcription factors (eg HDAC3, -5 and -6 independently form complexes with the SMRT-NCoR complex) offering an alternative mechanism for gene regulation (Jayne *et al*., 2006). However, it is also now known HDACs are involved in the deacetylation not only of chromatin proteins but also deacetylation of non-histone proteins, which regulate important functions that, in turn, regulate cellular homeostasis (cell-cycle progression, differentiation, and apoptosis), non-histone targets include several transcription factors (eg HIF1α), regulatory proteins (eg MLP), tumour suppressor proteins (eg p53), and cellular structural proteins, such as α-tubulin (Martin *et al*., 2007). Strong evidence appears to support deacetylation of non-histone proteins to be the action of the class II HDACs because they are known to have low deacetylase activity towards histones and class I HDACs being histone modifiers (Haberland *et al*., 2009).

Expression of the proteolytic enzyme; MMP-10 is known to play a key role in NSCLC, MMP-10 expression has previously been linked to the action of the class IIa HDAC; HDAC7 with roles suggested in tumour expansion and invasion, studies described in chapter IV suggested the expression but not the activity of HDAC7 was important in the regulation of MMP-10 in NSCLC *in vitro*, this suggests that the class IIa HDACs repressive activity may be independent of the catalytic domain of the deacetylase,

instead they have been reported to recruit class I HDACs through assembly into cognate co-repressor complexes, for example interaction with the SMRT-N-CoR co-repressors and they regulate transcription by bridging the enzymatically active complexes and selective transcription factors independent of any intrinsic HDAC activity (Hudson *et al*., 2015; Fischle *et al*., 2002). The formation of these complexes between HDACs potentially offers rationale for the regulation of MMP-10, with HDAC7 being involved in formation of this complex and regulation of a class I HDAC and subsequently expression of MMP-10.

Inhibition of all the family of HDACs in H460 cells using the pan inhibitor TSA, inhibiting both class I and II HDACs, caused a significant increase in MMP-10 expression and activity, suggesting the enzymatic activity of one or more HDACs is centrally involved in the regulation of MMP-10 alongside an involvement for presence of HDAC7. The previous study results had confirmed class II HDAC activity was not required for MMP-10 expression, implying an additional involvement for class I HDACs. Inhibition of class I HDACs using the sub-family inhibitor MS275 resulted in an induction in gene expression of MMP-10 by up to 5-fold. However, no significant comparative increase in MMP-10 proteolytic activity was detectable. This confirmed an involvement for class I HDACs alongside HDAC7 in the control of MMP-10 expression in NSCLC. The lack of modulation of MMP-10 proteolytic activity with inhibition of class I HDACs is likely to be due to the level of MMP-10 expression in the cells, with MS275-induced expression being 5-fold compared to 120-fold with the pan-HDACi Trichostatin A, with the latter causing a significant increase in MMP-10 proteolytic activity. The observation that MMP-10 expression and activity increased after inhibition with the selective HDAC3 inhibitor; MI192, in contrast to the general class I

HDACi MS275, further supports a role for HDAC3 expression and subsequently HDAC3 activity in the regulation of MMP-10 expression and activity, over other class I HDACs. This role is further reinforced by silencing of HDAC3 in H460 cells which also resulted in an increase in MMP-10 expression and activity of MMP-10 with the results demonstrating an increase in active MMP-10 following successful silencing of HDAC3, reinforcing the involvement of HDAC3 in the regulation of MMP-10.

Several studies have indicated a close relationship between HDAC7 and HDAC3 in the control of biological functions (Fischle *et al*., 2002; Fishcle *et al*., 2001). For instance, in cancer cells HDAC3 and HDAC7 collaboratively suppress expression of autotaxin, a secreted glycoprotein responsible for production of lysophosphatidic acid (LPA) involved in cancer progression, including NSCLC (Li *et al*., 2014). Whereas down-regulation of HDAC3 facilitates histone acetylation in the ATx promoter, concomitant down-regulation of HDAC7 was required to induce ATx expression (Li *et al*., 2014). These data suggest HDAC3-mediated acetylation of ATx is insufficient and the presence of HDAC7 is needed to 'modulate' HDAC3 activity, a scenario in agreement with the control of MMP-10 expression and activity in this current study.

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The effects of HDAC7 are mediated through a mechanism independent of deacetylase activity, with the deacetylase domain of HDAC7 dispensable for repression of genes required for osteoclast differentiation (Pham *et al*., 2011). This deacetylase-independent role for HDAC7 supports the observations in this study in which HDAC7-inhibition did not affect MMP-10 expression or activity, whereas down-regulation of HDAC7 expression resulted in upregulation of MMP-10 function. Consequently, the function of class IIa HDACs is now believed to regulate 'transportation' of class I HDACs (and other proteins, including transcription factors) between the cytoplasm and their functional workplace in the nucleus (Fischle *et al*., 2001). This is evidenced by the observation by Fischle and colleagues demonstrating that *in vivo* HDAC7 is associated with enzymatic activity when located in the cell nucleus but not when located in the cytoplasm (Fischle *et al*., 2001). The lack of the mechanistically important Tyr306 and its replacement with a histidine in the active site of HDAC7 (and other class IIa HDACs) creating a largely non-functional deacetylase enzyme activity additionally supports a function for HDAC7 as an acetyl-lysine reader similar to bromodomains (Arrowsmith *et al*., 2012). In this case, HDAC7 is essential for modulating expression of key genes with the deacetylation activity being contributed by class I HDACs, especially HDAC3, and both enzymes being associated with specific corepressors (Fischle *et al*., 2001). Such a relationship is strongly supported by this study, with HDAC3 enzymatic activity and HDAC7 presence being central to regulation of MMP-10 and its ultimate proteolytic activity.

As described previously, HDAC7 and HDAC3 constitute members of large transcriptional repressor complexes containing SMRT and N-CoR corepressors (Fischle *et al*., 2001), and in the case of MMP-10 regulation the transcription factor myocyte enhancer factor 2 (MEF2) (Chang *et al*., 2006). In this complex, the carboxy-terminus of HDAC7 interacts with HDAC3, whereas SMRT (also known as N-CoR2) interacts with HDAC3 through its deacetylase activating domain (DAD) and to HDAC7 via interaction with the second zinc ion located on the surface of the catalytic site of the enzyme (Codina *et al*., 2005; Bottomley *et al*., 2004). Thus the catalytically inactive HDAC7 is recruited into the SMRT repressor complex whose deacetylase activity is conferred by adjacently bound HDAC3 (Fischle *et al*., 2001; Desravines *et al*., 2017), with the interaction resulting in presentation of enzymatically active HDAC3 and locationally-regulation through conformational and post-translational modulation of HDAC7.

As discussed by Falkenberg & Johnstone (Falkenberg & Johnstone, 2014), the physical or enzymatic effect of one HDAC on activity of another HDAC or HDACs as part of a multiprotein complex is often overlooked and subsequently underappreciated. For instance, the findings from this study have shown both HDAC7 and HDAC3, as part of a larger complex, are required for regulation of MMP-10 expression and activity in NSCLC. This co-dependence has also been demonstrated by the class IIa HDAC4, which also interacts with HDAC3 in a NCoR-SMRT complex, and is essential for repression of genes involved in other cellular systems (Fischle *et al*., 2001) and regulation of calmodulin dependent protein kinase II (CaMKII) through association with the class IIa enzyme, HDAC5 (Backs *et al*., 2008).

This HDAC7-NCoR-SMRT-HDAC3 complex is central to regulation of MMP-10, providing an explanation as to why inhibition of HDAC7 enzymatic activity did not affect

MMP-10 expression but inhibition of HDAC3 activity and presence of HDAC3/HDAC7 did modulate MMP-10 expression and activity in NSCLC.

VI Discussion

Lung cancer is the leading cause of cancer-related deaths worldwide, accounting for more than 1 million fatalities per year; more than breast, colon and prostate cancers combined. The majority of lung cancer cases are diagnosed after the lung cancer has spread to other areas of the body (~60 percent), making it more difficult to treat. Despite therapeutic advances, the overall 5-year survival still remains at only 15% (Zappa & Mousa, 2016), therefore novel treatment strategies are needed to improve patient outcomes. One promising treatment strategy involves the further subdivision of NSCLC into clinically relevant molecular subtypes based on specific driver mutations, rather than the histological consideration as previously done. These specific driver mutations occur in genes that encode signalling proteins crucial for cellular proliferation and survival (Pao & Girard, 2011). One particular gene of interest is the secreted endoproteinase; MMP-10, because it has been found to be overexpressed in many cancers, including NSCLC (Gill *et al*., 2004). Although the role of this protease in cancer has not yet been elucidated, several studies have demonstrated links between MMP-10 and tumour pathophysiology (Chang *et al*., 2006). In NSCLC, MMP-10 expression and activity is elevated in both primary and metastatic disease (Justilien *et al*., 2012), but does not associate with tumour sub-type or pathogenesis (Gill *et al*., 2004). Elevated MMP-10 is central to regulation of vascular development; it induces remodelling of vascular extracellular matrix, capillary tube regression and vascular collapse (Chang *et al*., 2006). The ability of MMP-10 positive NSCLC cells to form tumours in MMP-10-null mice implicates expression of MMP-10 in the tumour mass rather than the tumour microenvironment as being key to regulation of tumour

growth (Justilien *et al*., 2012). The importance of tumour-expressed MMP-10 to tumour growth is reiterated by studies by Gill *et al,* in which supraphysiological induced expression of MMP-10 in H460 NSCLC xenografts resulted in retardation of tumour growth *in vivo*. However, induced expression did not alter *in vitro* growth or survival consequently, despite being central to establishment and maintenance of the stem-cell phenotype. Therefore, understanding the molecular mechanisms underlying regulation of MMP-10 has important implications for lung cancer pathogenesis and identification of therapeutic opportunities.

MMP-10 regulation has been linked to the histone deacetylase; HDAC7, particularly in regulation of vascular integrity (Chang *et al*., 2006). HDAC7 is thought to repress MMP-10, and has found to be under expressed in several types of cancers, including NSCLC. Class IIa HDACs possess a conserved C-terminal catalytic HDAC domain and interact with myocyte enhancer factor 2 (MEF2) transcription factors through an N-terminal 17-amino acid motif. This interaction leads to the recruitment of class IIa HDACs to select promoters, where MEF2 is bound, resulting in the repression of its transcriptional activity. The repressive activity of class IIa HDAC; HDAC7 is tightly regulated by nucleoplasmic shuttling and by their phosphorylation dependant association with the intracellular 14-3-3 proteins. Phosphorylation of conserved residues in the N-terminal regions of HDAC7 in response to cellular signals leads to interaction with 14-3-3 proteins, dissociation of the class IIa HDAC-MEF2 complexes and a conformational change culminating in export from the nucleus (Parra *et al*., 2004). Subsequently, in addition to MMP-10 facilitating maintenance of the cancer stem cell, a role for MMP-10 is postulated in the control of tumour angiogenesis and ultimately

metastatic potential (Chang *et al*., 2006). Timely regulation of MMP-10 is important to endothelial motility and vascular development with unregulated activity being detrimental to successful angiogenesis, providing an explanation to retardation of NSCLC tumour growth *in vivo*. Unexpected and specific role for HDAC7 in the maintenance of vascular integrity have important implications for understanding the mechanisms of angiogenesis and vascular remodeling during cancer development (Chang *et al*., 2006).

In this study, the silencing of HDAC7 in H460 cells using siRNA resulted in a time-dependant increase in MMP-10 gene expression. The increase in MMP-10 gene expression in response to inhibition of HDAC7 gene expression, indicating an inverse relationship between these factors, was also accompanied by an increase in proteolytic activity of MMP-10. This elevation of MMP-10 activity occurred post-transcriptionally, supporting regulation at gene level. This result suggested that the proteolytic activity of MMP-10 is significantly increased along with its expression upon HDAC7 repression, reinforcing the involvement of HDAC7 in the regulation of MMP-10 (Herbst *et al*., 2008). To determine whether the existence of HDAC7 or its enzymatic activity is responsible for the effects upon MMP-10, inhibition of HDAC7 activity was evaluated. However, the inhibition of HDAC7s enzymatic activity using the selective IIa inhibitor did not cause a change in MMP-10 gene expression, compared to control. In addition, inhibition of HDAC7 enzymatic activity did not alter levels of proteolytically active MMP-10. This suggests that although HDAC7 is central to MMP-10 expression, the activity of HDAC7 has no influence on the regulation of MMP-10 *in vitro*. Therefore, although HDAC7 is required for MMP-10 activity, as shown in this

study and those investigating vascular development, it is not as straightforward as first postulated and is neither a direct acetylation effect nor a 'conventional' HDAC pathway. However, over the past decade, it has become increasingly apparent that histones are not the only targets of acetylation and deacetylation, an abundance of non-histone proteins are now known to also be regulated by the actions of HATs and HDACs. Acetylation and deacetylation of non-histone proteins have pleiotropic effects on protein function, including modulation of protein–protein interactions, protein stability and subcellular localization (Glozak, 2007). Non-histone targets include several transcription factors (eg HIF1α), regulatory proteins (eg MLP), and cellular structural proteins, such as α-tubulin (Martin *et al*., 2007). They have also been found to be products of oncogenes or tumour-suppressor genes and are directly involved in tumorigenesis, tumour progression and metastasis, with aberrant activity of HDACs has been documented in several types of cancers (Singh *et al*., 2010).

In the past few years, much has been learned about class IIa HDACs, they contain a N-terminal extension of ~600 residues which provides a platform for several protein-protein interactions and post-translational modifications, which enables them to recruit class I HDACs, which are known to be the main drivers of histone deacetylation, via transcription co-repressors, which are targeted to specific promoters or subcellular localization. Examples of these multi protein complexes are the transcriptional co-repressors; nuclear receptor co-repressor (N-CoR) and silencing mediator for retinoid and thyroid hormone receptor (SMRT) (Singh *et al*., 2010; Fischle *et al*., 2001). The transcriptional corepressors SMRT and NCoR serve as critical mediators of HDAC7 activity by binding class IIa HDACs and HDAC3 by two distinct repressor domains,

suggesting an importance of another HDAC in this regulation of MMP-10 (Fischle *et al*., 2001).

Inhibition of all the family of HDACs in H460 cells using the pan inhibitor TSA, inhibiting both class I and II HDACs, caused a significant increase in MMP-10 expression and activity, suggesting the enzymatic activity of one or more HDACs is centrally involved in the regulation of MMP-10 alongside an involvement for presence of HDAC7. The previous study results had confirmed class II HDAC activity was not required for MMP-10 expression, implying an additional involvement for class I HDACs. Inhibition of class I HDACs using the sub-family inhibitor MS275 resulted in an induction in gene expression of MMP-10 by up to 5-fold. However, no significant comparative increase in MMP-10 proteolytic activity was detectable. This confirmed an involvement for class I HDACs alongside HDAC7 in the control of MMP-10 expression in NSCLC. The lack of modulation of MMP-10 proteolytic activity with inhibition of class I HDACs is likely to be due to the level of MMP-10 expression in the cells, with MS275-induced expression being 5-fold compared to 120-fold with the pan-HDACi Trichostatin A, with the latter causing a significant increase in MMP-10 proteolytic activity. The observation that MMP-10 expression and activity increased after inhibition with the selective HDAC3 inhibitor; MI192, in contrast to the general class I HDACi MS275, further supports a role for HDAC3 expression and subsequently HDAC3 activity in the regulation of MMP-10 expression and activity, over other class I HDACs. This role is further reinforced by silencing of HDAC3 in H460 cells which also resulted in an increase in MMP-10 expression and activity of MMP-10 with the results demonstrating an increase in active MMP-10 following successful silencing of HDAC3,

reinforcing the involvement of HDAC3 in the regulation of MMP-10. HDAC3, which is a class I HDAC, is a nuclear protein that plays a role in regulating multiple genomic activities including transcriptional repression (Hudson *et al*., 2015). Several studies have indicated a close relationship between HDAC7 and HDAC3 in the control of biological functions (Fischle *et al*., 2002; Fishcle *et al*., 2001). For instance, in cancer cells HDAC3 and HDAC7 collaboratively suppress expression of autotaxin, a secreted glycoprotein responsible for production of lysophosphatidic acid (LPA) involved in cancer progression, including NSCLC (Li *et al*., 2014). Whereas down-regulation of HDAC3 facilitates histone acetylation in the ATx promoter, concomitant down-regulation of HDAC7 was required to induce ATx expression (Li et al., 2014). These data suggest HDAC3-mediated acetylation of ATx is insufficient and the presence of HDAC7 is needed to 'modulate' HDAC3 activity, a scenario in agreement with the control of MMP-10 expression and activity in this current study.

The observation that HDAC7 and HDAC3 have opposing effects on osteoclast differentiation is also supportive of this study regarding regulation of MMP-10 in NSCLC (Pham *et al*., 2011). In osteoclasts although HDAC3 activity modulates gene expression, surprisingly, inhibition of class I HDAC with MS275 resulted in a decreased expression of HDAC7. This suggests HDAC7 expression is under control of class I HDACs, with potentially controlling expression directly at the gene level. Several studies have reported a direct relationship between the class II HDAC; HDAC7, and the class I HDAC; HDAC3 (Fischle *et al*., 2002; Fischle *et al*., 2001). In this perspective, HDAC7 is postulated to regulate nuclear-cytoplasmic translocation of HDAC3 and thus its enzymatic activity because HDAC3 can only exhibit its deacetylase activity when located

within the nucleus of the cell. The effects of HDAC7 are mediated through a mechanism independent of deacetylase activity, with the deacetylase domain of HDAC7 dispensable for repression of genes required for osteoclast differentiation (Pham *et al*., 2011). This deacetylase independent role for HDAC7 supports the observations in this study in which HDAC7- inhibition did not affect MMP-10 expression or activity, whereas down-regulation of HDAC7 expression resulted in upregulation of MMP-10 function. Consequently, the function of class IIa HDACs is now believed to regulate 'transportation' of class I HDACs (and other proteins, including transcription factors) between the cytoplasm and their functional workplace in the nucleus (Fischle *et al*., 2001). This is evidenced by the observation by Fischle and colleagues demonstrating that *in vivo* HDAC7 is associated with enzymatic activity when located in the cell nucleus but not when located in the cytoplasm (Fischle *et al*., 2001). The lack of the mechanistically important Tyr306 and its replacement with a histidine in the active site of HDAC7 (and other class IIa HDACs) creating a largely non-functional deacetylase enzyme activity additionally supports a function for HDAC7 as an acetyl-lysine reader similar to bromo-domains (Arrowsmith *et al*., 2012). In this case, HDAC7 is essential for modulating expression of key genes with the deacetylation activity being contributed by class I HDACs, especially HDAC3, and both enzymes being associated with specific corepressors (Fischle *et al*., 2001). Such a relationship is strongly supported by this study, with HDAC3 enzymatic activity and HDAC7 presence being central to regulation of MMP-10 and its ultimate proteolytic activity. As described previously, HDAC7 and HDAC3 constitute members of large transcriptional repressor complexes containing SMRT and N-CoR corepressors (Fischle *et al*., 2001), and in the case of MMP-10 regulation the transcription factor myocyte enhancer factor 2

(MEF2) (Chang *et al*., 2006). In this complex, the carboxy-terminus of HDAC7 interacts with HDAC3, whereas SMRT (also known as N-CoR2) interacts with HDAC3 through its deacetylase activating domain (DAD) and to HDAC7 via interaction with the second zinc ion located on the surface of the catalytic site of the enzyme (Codina *et al*., 2005; Bottomley *et al*., 2004). Thus, the catalytically inactive HDAC7 is recruited into the SMRT repressor complex whose deacetylase activity is conferred by adjacently bound HDAC3 (Fischle *et al*., 2001; Desravines *et al*., 2017), with the interaction resulting in presentation of enzymatically active HDAC3 and locationally- regulation through conformational and post-translational modulation of HDAC7.

As discussed by Falkenberg & Johnstone (Falkenberg & Johnstone, 2014), the physical or enzymatic effect of one HDAC on activity of another HDAC or HDACs as part of a multiprotein complex is often overlooked and subsequently underappreciated. For instance, the findings from this study have shown both HDAC7 and HDAC3, as part of a larger complex, are required for regulation of MMP-10 expression and activity in NSCLC. This co-dependence has also been demonstrated by the class IIa HDAC4, which also interacts with HDAC3 in a NCoR-SMRT complex, and is essential for repression of genes involved in other cellular systems (Fischle *et al*., 2001) and regulation of calmodulin dependent protein kinase II (CaMKII) through association with the class IIa enzyme, HDAC5 (Backs *et al*., 2008). HDAC7, which is thought to have lost its deacetylase activity during evolution, could potentially shuttle components of the HDAC3-SMRT-NCoR complex, (because it has been found to express both NLS and NES) in and out of the nucleus and therefore function as a corepressor, and HDAC3 alone or in conjunction with other factors would be the sole enzymatic activity of the

HDA7-HDAC3 complex, functioning within the nucleus by deacetylating the histones, and repressing gene transcription of MMP-10 *in vitro*. Because the class I HDACs, unlike the class IIa HDACs, show a significant enhancement of lysine deacetylase activity when bound to the corepressor. However, this is a relationship that needs to be further explored, to find out by which means this complex exists, which could help in understanding the pathogenesis of NSCLC in the future.

VII Conclusion

The secreted endoproteinase; MMP-10, has been shown to be overexpressed in human non-small cell lung cancer (NSCLC), in both the primary and metastatic disease, wherein it is proteolytically active and secreted predominantly by tumour cells (Gill *et al*., 2004). MMP-10 over expression has been linked to tumour pathophysiology (Chang *et al*., 2006), its role has been implicated in retarding angiogenesis through repression of tumour vasculature development. Elevated levels have shown to induce remodelling of the vascular extracellular matrix, capillary tube regression and vascular collapse *in vivo* (Chang *et al*., 2006). MMP-10 is involved with vessel formation and stabilization rather than contributing to the invasive nature of tumours, an action supported by published studies (Heo *et al*., 2010). Preliminary data carried out by Gill *et al*., (chapter 1, figure I.3) showed that overexpression of MMP-10 in a human NSCLC tumour model did not affect cellular proliferation *in vitro*, but caused significant retardation of tumour growth *in vivo*, further supporting this role for MMP-10 in NSCLC.

Previous studies have indicated a role for the histone deacetylase (HDAC) enzymes, specifically HDAC7, in the regulation of MMP-10, with elevated levels of HDAC7 repressing MMP-10 expression and promoting successful blood vessel formation (Chang *et al*., 2006). The potential that HDAC7 regulates MMP-10 expression and vascular integrity has potentially important implications in the treatment of NSCLC (Chang *et al*., 2006). Inhibition of HDAC7 in tumours and consequent up regulation of MMP-10 would have antiangiogenic consequences and eventually lead to regression of tumours due to disruption of the vascular supply (Chang *et al*., 2006). The aim of this project was to evaluate the relationship between MMP-10 and HDACs and

whether perturbation of the HDAC7/MMP-10 pathway maybe a viable opportunity for therapeutic exploitation in NSCLC. Using an *in vitro* model of NSCLC, a central role for histone deacetylases (HDACs) in regulation of MMP-10 expression was confirmed. Although inhibition of HDAC activity increased MMP-10 expression and activity, selective inhibition of the class-II HDAC7 had no effect. However, siRNA-mediated repression of HDAC7 expression caused a significant elevation in MMP-10 expression and activity, supporting a central role for the presence but not enzymatic activity of HDAC7 in the control of MMP-10 levels within NSCLC. Despite the lack of a role for HDAC7 enzyme activity in control of MMP-10, the initial observation that pan-inhibition of HDAC activity repressed MMP-10 expression implicated a wider involvement for HDACs in regulation of MMP-10. Consequently, this study using both siRNA and pharmacological inhibitors confirmed an additional role for the presence and enzymatic activity of the class-I subfamily member HDAC3 in the regulation of MMP-10 in NSCLC.

These data indicated an interplay between the class-I HDAC3 and class-II HDAC7 in the regulation of MMP-10 expression and activity. HDACs are known to form multiprotein corepressor complexes with nuclear receptors and transcription factors (eg HDAC3, -5 and -6 independently form complexes with the SMRT-NCoR complex) offering an alternative mechanism for gene regulation (Jayne *et al*., 2006). Several studies have indicated a close relationship between HDAC7 and HDAC3 in the control of biological functions (Fischle *et al*., 2002; Fishcle *et al*., 2001). For instance, in cancer cells HDAC3 and HDAC7 collaboratively suppress expression of autotaxin, a secreted glycoprotein responsible for production of lysophosphatidic acid (LPA) involved in cancer progression, including NSCLC (Li *et al*., 2014). Whereas down-regulation of

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This study thereby identifies and offers a novel molecular mechanism for HDAC-mediated control of MMP-10 in NSCLC, however further work is required to exploit this relationship toward improved treatment of NSCLC in the clinic.

VIII Overall summary

Much effort has been made in this project to investigate the regulation of MMP-10 expression in NSCLC by histone deacetylases, primarily the class IIa member HDAC7 and subsequently the class I member HDAC3. The major findings from the study were i) the importance of HDAC7 physical presence, but not enzymatic activity, in the regulation of MMP-10 expression and function, and ii) the requirement for HDAC3 enzymatic activity to facilitate the HDAC7-MMP10 signalling axis. It is thereby postulated that HDAC7 and HDAC3 demonstrate a physical relationship to regulate MMP-10 activity, potentially through regulation of nucleoplasmic shuttling, post-translational regulation of non-histone proteins, and differential interaction with epigenetic and genetic regulatory factors. In this context, further studies are proposed to both strengthen and confirm the promising findings presented herein and further elucidate the molecular mechanisms underpinning these regulatory effects:

1) Improved definition of the relationship between the HDAC3:SMRT:N-CoR complex.

· To further elucidate if nuceloplasmic shuttling of HDAC7 and HDAC3 enzymes occur in situ, using co-locational fluorescence microscopy methodologies

· Create truncated and modified HDAC7 and HDAC3 proteins to identify sites of protein interaction, responsible for physical interaction, co-binding, or co-factor relational positioning

2) Involvement of other HDACs in the regulation of MMP-10 activity

· Evaluate whether any of the other class IIa HDACs (-4, -5, -9) play a role in the regulation (positive and negative) of MMP-10 expression and function.

3) Selectivity of HDAC7-HDAC3 signalling axis toward MMP-10

· Investigate whether HDAC7:HDAC3 is a generic regulatory mechanism for MMP expression and activity, or a specific control process for tight control of MMP-10. Expansion of inhibitor and siRNA studies, presented in this thesis, will permit such evaluations.

4) Confirmation of perturbation of the HDAC3:HDAC7:MMP-10 axis as a clinically validated approach for putative clinical management of NSCLC.

· Examine the genetic and protein expression of HDAC3, HDAC7 and MMP-10 in specimens of NSCLC and correlations to clinical and pathological parameters, addressing potential subcellular co-localisation of these proteins

· Evaluate therapeutic consequences of selective HDAC3 and HDAC7 inhibition and genetic knockdown using preclinical in vivo tumour models, determining effects on tumour vascularity, growth and functional MMP-10 levels

Appendix A - Solutions for western blotting

MRC Lysis Buffer	
Chemical	**Concentration**
Tris HCl pH 7.5	50mM
EGTA	1mM
EDTA	1mM
Sodium ortho vandate	1mM
β-glycerol phosphate	10mM
Sodium fluoride	50mM
Sodium pyrophosphate	5mM
Sucrose	0.27M
Triton x100	1% (v/v)
β-mercaptoethanol	0.1%
Mini protease inhibitor tablet (Roche, Germany)	1 tablet per 50ml of lysis buffer

4x Sample Buffer	
Chemical	**Concentration**
Tris HCl pH 6.8	240mM
SDS	8% (w/v)
Glycerol	40% (v/v)
Bromophenol blue	0.04% (v/v)

10% resolving gel	
Chemical	**Concentration**
Bis/acrylamide	10% (v/v)
Tris HCl pH 8.8	380mM
SDS	0.1%
Ammonium persulfate	0.1%
TEMED	0.4µl/ml

5% stacking gel	
Chemical	**Concentration**
Bis/acrylamide	5% (v/v)
Tris HCl pH 8.8	750mM
SDS	0.1%
Ammonium persulfate	0.1%
TEMED	1µl/ml

Running buffer	
Chemical	**Concentration**
Trizma Base	250mM
Glycine	190mM
SDS	0.1% (v/v)

Transfer buffer	
Chemical	**Concentration**
Trizma Base	20mM
Glycine	150mM
Methanol	20% (v/v)

TBS-T	
Chemical	**Concentration**
Trizma Base	20mM
NaCl	150mM
Tween-20	0.1% (v/v)

Conference Abstract

Interplay Between Histone Deacetylases in the Modulation of MMP-10 Expression in Lung Cancer

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Cancer development is highly dependent upon the Matrix Metalloproteinase (MMP) family of proteolytic enzymes. Matrix metalloproteinase-10 (MMP-10) is overexpressed in many human carcinomas including non-small cell lung cancer (NSCLC), wherein it is expressed, proteolytically active, and secreted predominantly by tumour cells. During mammalian development, MMP-10 is central to regulation of vascular development; elevated MMP-10 induces remodelling of vascular extracellular matrix, capillary tube regression and vascular collapse. Such a role implicates an involvement for MMP-10 in tumour angiogenesis, offering a viable target for therapeutic exploitation in NSCLC. Previous studies have shown a role for the class-IIa Histone Deacetylase-7 (HDAC7) in regulation of MMP-10 expression, with elevated levels of HDAC7 repressing MMP-10 expression and promoting successful blood vessel formation. In this study, we evaluate whether perturbation of the HDAC7/MMP-10 pathway maybe a viable opportunity for therapeutic exploitation in NSCLC. Using an *in vitro* model of NSCLC, we confirm a central role for histone deacetylases (HDACs) in regulation of MMP-10 expression. Pharmacological inhibition of HDAC7 activity had no effect on genetic expression of MMP-10, whereas siRNA-mediated knockdown of HDAC7 expression caused an elevation of MMP-10 mRNA expression. Together this suggests HDAC7 plays a non-enzymatic role in MMP-10 regulation. Surprisingly, pharmacological inhibition of other HDACs, particularly HDAC3 (a member of the class-I HDAC sub-family) repressed MMP-10 expression. These data suggest interplay between HDAC7 and HDAC3 in the regulation of MMP-10 expression in NSCLC. Previous reports have supported a role for HDAC7 in the nuclear shuttling of HDAC3, offering a novel molecular mechanism for HDAC-mediated control of MMP-10 and a potential therapeutic strategy for investigation in NSCLC.

Poster Presentation;
Metalloproteinases and their inhibitors: beginning, past and future,
Oxford, UK.

Publications

Cross, J.M., Blower, T.R., Gallagher, N., Gill, J.H., Rockley, K.L. & Walton, J.W. (2016). Anticancer RuII and RhIII Piano-Stool Complexes that are Histone Deacetylase Inhibitors. ChemPlusChem 81(12): 1276-1280.

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