ADENOSINE AS AN ENVIRONMENTAL STRESSOR AFFECTING HSP27 AND CXCR4 IN EPITHELIAL CELLS

by

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Signature of Author

This thesis is dedicated to my mom.

"And then my heart with pleasure fills,
and dances with the daffodils"

- William Wordsworth

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ABSTRACT

Solid tumours are a hostile tissue environment in which the cells are exposed to many stresses including hypoxia. One consequence of hypoxic conditions is an increase in extracellular levels of the purine nucleoside adenosine, which enhances tumour cell migration. This is achieved in part through an increase in the levels of the chemokine receptor CXCR4, which along with its ligand CXCL12, is a key player in breast cancer metastasis.

The cellular response to stress is mediated by a family of proteins known as heat-shock proteins (HSPs). The small heat shock protein 27 (HSP27) has been implicated in changes in cancer cell migration. I have therefore studied the regulation of HSP27 in human breast cancer cells by conditions that normally exist in the stressful tumor environment. My project specifically aimed to establish whether changes in HSP27 are linked to hypoxia, adenosine levels and alterations in the CXCL12-CXCR4 migratory pathway.

LIST OF ABBREVIATIONS USED

ADA	adenosine deaminase
AICAR	aminoimidazole carboxamide ribonucleotide
AK	adenosine kinase
AMF	autocrine motility factor
AMP	adenosine monophosphate
APAF	apoptosis protease activating factor
ATP	adenosine triphosphate
BRCA	breast cancer susceptibility gene
BSA	bovine serum albumin
Ca^{+}	calcium
CA	carbonic anhydrase
cAMP	cyclic adenosine monophosphate
CCPA	2-chloro-N^6-cyclopentyladenosine
CNT	concentrative nucleoside transporter
CO_2	carbon dioxide
COX	cyclooxygenase
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
EMT	epithelial-mesenchymal transition
ENT	equilibrative nucleoside transporters
FBS	fetal bovine serum
Glut	glucose transporter
GPCR	G protein-coupled receptor
HIF	hypoxia-inducible factor
HPLC	high-performance liquid chromatography
HRE	hypoxia response element
HSE	heat shock element
HSP	heat shock protein
HSF	heat shock factor

HUVEC	human umbilical vein endothelial cells
IAP	inhibitor of apoptosis
IGF	insulin-like growth factor
IκB	inhibitor of κB
IKK	inhibitor of κB kinase
IL	interleukin
kDa	kilodaltons
LDH	lactate dehydrogenase
LOX	lysyl oxidase
M	moles/liter
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
MAPKAPK	mitogen-activated protein kinase-activated protein kinase
MCT	monocarboxylate transporter
MDR	multiple drug resistance
min	minute
mg	milligram
MMP	matrix metalloproteinases
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
NCS	newborn calf serum
NECA	5'-*N*-(ethylcarboxamide)adenosine
NF-κB	nuclear factor-κB
NHE	sodium/hydrogen exchanger
5'-NT	5'-nucleotidase
O_2	oxygen
ODDD	oxygen dependent degradation domains
p53	protein 53/tumour protein 53
pAb	polyclonal antibody
PBS	phosphate-buffered saline

PC	preconditioning
PDGF	platelet-derived growth factor
PDK	pyruvate dehydrogenase kinase
PI	protease inhibitor
PI3K	phosphatidylinositol 3-kinase
PKC	protein kinase C
PMSF	phenylmethylsulfonyl fluoride
pVHL	von Hippel-Lindau tumour suppressor protein
RIPA	radioimmunoprecipitation assay
ROS	reactive oxygen species
R-PIA	(R-phenylisopropyl)-adenosine
RT	room temperature
SAH	S-adenosylhomocysteine
SDF-1	stromal cell-derived factor-1
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCA	tricarboxylic acid cycle
TGF-β	transforming growth factor-beta
TNF	tumour necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
VEGF	vascular endothelial growth factor

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CHAPTER 1: INTRODUCTION

1.1 Perspective

Canadian breast cancer rates are among the highest in the world, with over 23,400 new cases expected in 2011 (Canadian Cancer Statistics 2011). Breast cancer is the most frequently diagnosed cancer in Canadian women, and the second most common cause of cancer-related mortality amongst this population. The 5-year survival rate of patients with breast cancer decreases with increasing stage, with the majority of breast cancer deaths attributed to the metastasis of the cancer to distant organs (American Cancer Society. *Cancer Facts & Figures* 2010). Despite this, the mechanisms involved in the metastatic process are not completely understood. In this project, I have examined the effects of the tumour microenvironment, including hypoxia and adenosine, on the small heat shock protein HSP27, and its possible connection to CXCR4, an important cell-surface molecule involved in breast cancer metastasis.

1.2 The Abnormal Physiology and Cell Biology of a Tumour

The tumour microenvironment is very different from that of normal tissue. In order for tumours to grow beyond 1mm in diameter, they need to incorporate existing host vasculature or create their own vessel network. Tumour neo-vasculature is abnormal in both structure and function, being unorganized and having vessel walls that are missing endothelial cells, lacking pericytes, and possessing an intermittent basement

membrane (Brown and Giaccia, 1998; Vaupel et al., 1989b). This causes irregular and slow bloodflow within leaky vessels, decreasing the amount of oxygen provided to tumour cells and ultimately resulting in a hypoxic environment.

One consequence of hypoxia is that tumour cells make a shift from aerobic to anaerobic metabolism. They switch from metabolism of glucose that relies on oxidative phosphorylation in the mitochondria to oxygen-independent glycolysis in the cytoplasm, converting pyruvate to lactic acid (Brahimi-Horn et al., 2007). The combination of an increased production of lactic acid and poor blood flow creates a more acidic tissue environment. While normal tissue has a pH around 7.0 - 7.4, the pH of solid tumours ranges to as low as 6.0 (Vaupel et al., 1989a).

While normal cells require mitogenic growth signals to proliferate, cancer cells become independent of many of these signals and proliferate in the absence of normal exogenous growth signaling (Hanahan and Weinberg, 2000). Cell-surface receptors required to transduce these signals are often abnormally expressed on cancer cells, as is seen with the receptor tyrosine kinase HER2 in breast cancer (Slamon et al., 1987; Yarden and Sliwkowski, 2001), causing greater responses to signals. Not only do cancer cells proliferate without being dependent on exogenous growth signals, they are insensitive to many anti-proliferative signals. These are the signals that cause cells to either enter the quiescent cellular state (G_o) or to permanently stop proliferating, a condition known as senescence. Many anti-proliferative signals are mediated through the retinoblastoma protein (pRb) pathway, which inhibits excessive proliferation in normal cells by preventing the G1 to S phase transition (Weinberg, 1995). In many cancers

however, pRb signaling is aberrant, allowing the proliferation of malignant cells (Donovan and Slingerland, 2000).

Tumour cells acquire other means of increasing their population size by evading cell death and having limitless replication, wherein normal cells have a finite number of potential population doublings. Unlike normal cells that are forced to enter senescence after a given number of duplications, cancer cells have essentially unlimited replicative potential (Hayflick, 2000), an ability called immortalization. Cancer cells evade apoptosis (programmed cell death) in many ways, such as activating survival pathways like the AKT/PKB pathway (Cantley and Neel, 1999). One very common mutation found in more than 50% of malignancies is the lack of the tumour suppressor p53 that normally induces apoptosis upon DNA damage (Hollstein et al., 1994).

To supply a quickly-growing population of cells with nutrients and oxygen, tumours must create a more extensive vasculature, a process called angiogenesis. Early on in tumour development cells undergo an 'angiogenic switch'. This is a change in gene transcription that increases pro-angiogenic signals such as VEGF and decreases angiogenic inhibitors like thrombospondin-1, resulting in neo-vascularization (Hanahan and Folkman, 1996).

In order for tumours to progress and to evade apoptosis, maintain angiogenesis, become immortal and be both independent of growth signals and insensitive to anti-proliferative signals, cancer cells must acquire and accumulate genetic mutations (Nowell, 1976). The number of mutations that tumours eventually acquire is inconsistent with the low mutation rate of normal human cells, suggesting that tumours must evolve an increasingly mutagenic phenotype (Loeb, 1991). The large number of mutations

gained by cancer cells is possible through the failure of 'caretaker' systems (Lengauer et al., 1998) such as the tumour suppressor protein p53. Loss of function of such tumour suppressor genes results in genomic instability generating malignant cells with an increasing tendency to develop mutations that confer selective advantages.

1.3 The Central Influence of Hypoxia in Tissue Physiology

1.3.1 The State of Hypoxia

Hypoxia is a lack of oxygen that may be acute or chronic and it is involved in many medical conditions such as rheumatoid arthritis, cancer, irritable bowel disease and atherosclerosis (Hatoum et al., 2005; Mapp et al., 1995; Savransky et al., 2007; Vaupel et al., 2004). Oxygen homeostasis is normally maintained by various genes that induce angiogenesis (VEGF), ensure sufficient blood oxygenation (EPO), permit vasodilation (NO) and allow adequate glycolysis (Glut1) (Chen et al., 2001a; Forsythe et al., 1996; Gupta and Goldwasser, 1996; Palmer et al., 1998; Pugh and Ratcliffe, 2003). These genes are primarily under the control of the transcription factor, hypoxia-inducible factor-1 (HIF-1) (Huang et al., 1998). HIF-1 is a heterodimeric complex made of two subunits, HIF-1α and HIF-1β (Wang et al., 1995a). Both HIF-1 subunits are constitutively expressed in normoxia but HIF-1α is rapidly degraded before it can bind HIF-1β (Wang et al., 1995a). The degradation of HIF-1α is mediated by the Von Hippel-Lindau protein (pVHL), which in normoxia binds HIF-1α and targets it for polyubiquitination (Ohh et al., 2000). The pVHL binds to two oxygen-dependent degradation domains (ODDDs), one in the amino-terminus of HIF-1α and one in its carboxy-terminus (Hon et al., 2002;

Huang et al., 1998). This only occurs in the presence of oxygen because in order for the interaction to occur, specific prolyl residues in one of the ODDDs must be hydroxylated, and prolyl hydroxylases require oxygen to function (Berra et al., 2003; Hon et al., 2002; Jaakkola et al., 2001; Masson et al., 2001; Yu et al., 2001). In hypoxia, hydroxylation of HIF-1α does not occur and therefore pVHL cannot bind this transcription factor and tag it for degradation, resulting in the translocation of HIF-1α to the nucleus (Kallio et al., 1998; Tian et al., 2011). Once in the nucleus, HIF-1α binds HIF-1β and the complex interacts with hypoxia-responsive elements (HREs) activating transcription of hypoxia-induced genes (Semenza, 1998). In the presence of oxygen, the transcriptional activity of HIF-1α is suppressed by factor-inhibiting HIF-1 (FIH), which hydroxylates HIF-1α on an asparganine, inhibiting HIF-1α binding of the transcriptional coactivator p300/CBP (Lando et al., 2002).

1.3.2 Hypoxia in Tumours

As stated earlier, the leaky and unorganized tumour vasculature creates a hypoxic environment. Since blood can diffuse only approximately 150μm within tissues, the further tumour cells are from a blood supply, the more hypoxic and eventually anoxic they will be, ultimately leading to local necrosis (Dewhirst et al., 1994; Thomlinson and Gray, 1955). In order to survive and grow in this hypoxic environment, tumour cells must adapt and exploit mechanisms that allow them to convert to a glycolytic phenotype, induce angiogenesis, proliferate, evade apoptosis, and have potentially limitless replication capability. HIF-1 plays an essential role in hypoxia and tumour cells in particular exploit the HIF pathway to promote survival, growth and resistance to therapy (Cummins and Taylor, 2005) (Figure 1.1).

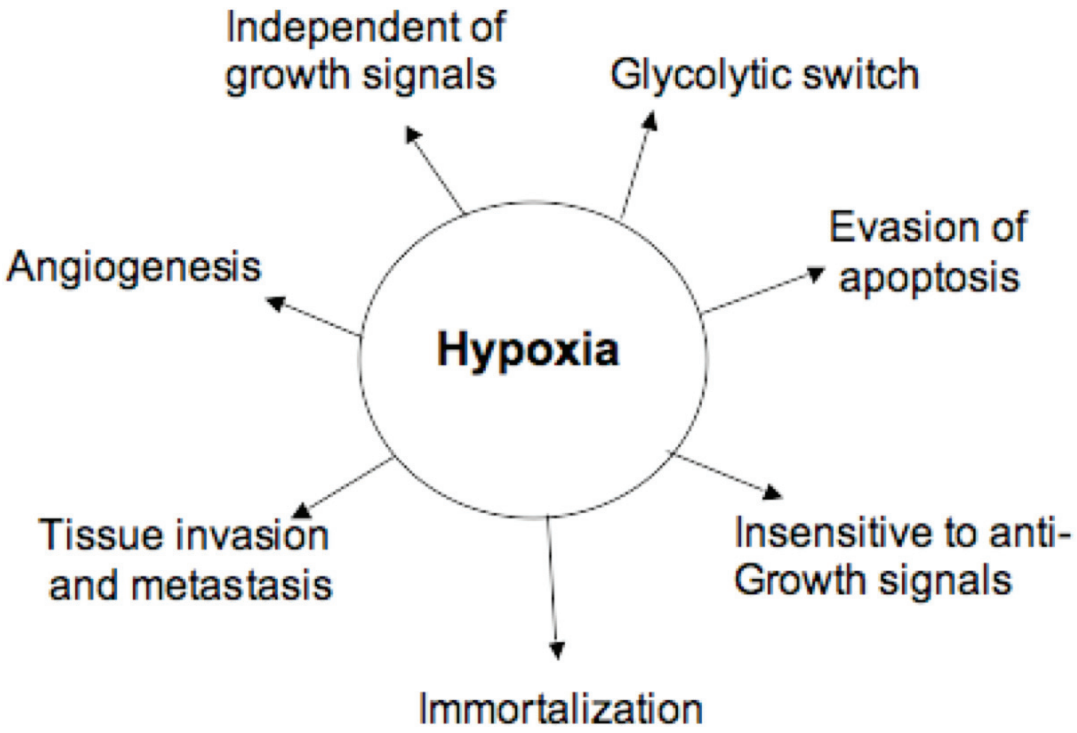


Figure 1.1 The central influence of hypoxia on different aspects of cellular and tissue behaviour in tumours
(modified from (Ruan et al., 2009))

1.3.3 Metabolism Under Hypoxic Conditions

The switch to glycolytic metabolism to adapt to hypoxia yields less ATP than aerobic metabolism. Glycolysis yields only 2 ATP for each glucose molecule utilized rather than the 38 ATP generated through aerobic metabolism (Opie, 1990). To compensate for the reduced ATP production, cells increase glucose uptake and the rate of glycolysis via HIF-1-induced expression of enzymes involved in the glycolytic pathway. Hypoxia-response elements (HREs) have been found in the genes coding for the enzymes pyruvate dehydrogenase kinase 1 (PDK1) and lactate dehydrogenase-A (LDH-A) (Semenza et al., 1996; Semenza et al., 1994) and other genes involved in glycolysis (Ebert et al., 1995; Semenza et al., 1996). LDH-A catalyzes the conversion of pyruvate to lactate and PDK1 inhibits pyruvate dehydrogenase that normally converts pyruvate into acetyl-coA beginning the TCA cycle (Holness and Sugden, 2003). Not only does this help maintain ATP levels in the cell, but it prevents the toxic creation of ROS in the mitochondria via the electron transport chain (Kim et al., 2006). Iyer and colleagues (1998) found that HIF-1α-deficient stem cells had decreased expression of several genes that encode for glycolytic enzymes and glucose transporters. HIF-1 suppresses the mTOR pathway, decreasing protein synthesis and therefore conserving energy (Pouyssegur et al., 2006). In addition to HIF-1 mediation of metabolism, the products of glycolysis, lactate and pyruvate, promote HIF-1α stabilization and expression, reflecting a tightly-controlled feedback mechanism (Lu et al., 2002).

1.3.4 Counteracting Low pH Generated by Hypoxia

To counteract the lower pH caused by the increased production of lactic acid, HIF-1 maintains the intracellular pH (pH_i) of cancer cells by mediating the up-regulation

of membrane transporters, exchangers and ecto-enzymes. Two HIF-1-inducible enzymes, carbonic anhydrases-9 (CA9) and -12 (CA12) that convert CO_2 and water to carbonic acid, are upregulated by hypoxia and downregulated by pVHL (Brahimi-Horn et al., 2007; Ivanov et al., 2001). While these membrane proteins contribute to extracellular acidification, they help to regulate the pH_i as extracellular carbonic acid is converted to bicarbonate, a weak base that can be transported into cells increasing pH_i (Chiche et al., 2009). HIF-1 regulates pH_i by mediating expression of the monocarboxylate transporters, MCT1 and MCT4, that transport lactate across the cell membrane (Perez de Heredia et al., 2010), and the Na^+/H^+ exchanger (NHE-1) (Shimoda et al., 2006).

1.3.5 Immortalization of Tumour Cells

Cancer cells become immortal by maintaining the length of telomeres at the end of chromosomes, which defeats the process of telomere-regulated senescence. Each time a normal cell divides the telomeres become a bit shorter and once they reach a certain length, senescence is triggered (Flores et al., 2005; Harley et al., 1990). Cancer cells avoid telomere-dependent senescence by increasing expression of the enzyme telomerase (Broccoli et al., 1995; Hiyama et al., 1995), which maintains telomeric length thus allowing the cells to replicate indefinitely (Bryan and Cech, 1999)**.** Hypoxia increases telomerase activity in cancer cells consequently promoting immortalization (Seimiya et al., 1999).

1.3.6 Hypoxia and Proliferation

Hypoxia can also promote cell growth. HIF-1 expression is induced by many growth factors such as platelet-derived growth factor (PDGF) (Zhang et al., 2003),

angiotensin II (Richard et al., 2000), epidermal growth factor (EGF) (Zhong et al., 2000), (transforming growth factor beta (TGF-β) (Qian et al., 2004) and insulin-like growth factor-1 and 2 (IGF-1 and IGF-2) (Feldser et al., 1999). Interestingly, hypoxia mediates the production of some of these growth factors, such as PDGF (Zhang et al., 2003), TGF-β (Falanga et al., 1991) and IGF-2 (Feldser et al., 1999), as well as VEGF (Forsythe et al., 1996), through HIF-1-dependent and -independent pathways.

1.3.7 Production of Genetic Instability by Hypoxia

Under hypoxia, cells have a diminished capacity to repair DNA (Yuan et al., 2000). Reynolds and coworkers (1996) found that the frequency of point mutations in cancer cells exposed to hypoxia was greater than in control cells, and that the frequency increased with subsequent exposures to hypoxia. Hypoxia selects for cells with p53 mutations, as those cells will preferentially survive (Graeber et al., 1996). These cells are particularly vulnerable to genetic instability (Loeb, 1991), since p53 plays a major role in DNA repair (Smith et al., 2000; Wang et al., 1995b) and cell cycle arrest (Kuerbitz et al., 1992).

1.3.8 Evasion of Hypoxia-Induced Apoptosis by Tumour Cells

Hypoxia induces apoptosis through HIF-1-dependent and -independent pathways, although tumour cells have developed mechanisms allowing them to evade hypoxia-induced cell death. Unphosphorylated HIF-1α binds to and stabilizes p53, promoting p53-dependent apoptosis, mediated by APAF-1 and caspase-9 (Soengas et al., 1999). In hypoxia however, HIF-1α is phosphorylated and binds HIF-1β preventing its interaction with p53 (Suzuki et al., 2001). Many cancer cells manage to evade hypoxia-induced

apoptosis as they have a mutated p53 gene (Graeber et al., 1996; Schmaltz et al., 1998). Hypoxia induces the expression of the anti-apoptotic genes, inhibitor of apoptosis 2 (IAP2) and apoptosis repressor with caspase recruitment domain (ARC), via a HIF-1-independent mechanism (Dong et al., 2001; Ekhterae et al., 1999). In some cell types, hypoxia promotes cell survival by increasing PI-3K/AKT activity (Chen et al., 2001b; Song et al., 2005).

1.3.9 Tumour Angiogenesis

Hypoxia promotes angiogenesis by inducing a number of angiogenic factors, such as VEGF, angiogenin, angiopoietin-2 (Ang2) and PDGF. VEGF is a receptor ligand required for the recruitment of endothelial cells to hypoxic areas and the formation of new blood vessels (Forsythe et al., 1996; Gerhardt et al., 2003). Under hypoxic conditions, VEGF mediates the up-regulation of Ang2, which is necessary for the initiation of neo-vessel sprouting (Oh et al., 1999). Ang2 is required for the destabilization and remodeling of mature vessels that are usually maintained in a dormant state (Maisonpierre et al., 1997). Ang2 is the natural antagonist for Ang1 which along with PDGF recruits pericytes to blood vessels rendering them unresponsive to VEGF (Fiedler et al., 2004; Lindblom et al., 2003; Maisonpierre et al., 1997). Not only does hypoxia up-regulate pro-angiogenic factors, it suppresses angiogenic inhibitors such as thrombospondin (Laderoute et al., 2000).

1.3.10 Hypoxia and Tumour Progression

Hypoxia induces proteomic and genomic changes in cancer cells that result in a more aggressive and malignant phenotype. The gene for glucose transporter 1 (Glut1) is

upregulated in hypoxic cancer cells and increased Glut1 expression correlates with nuclear characteristics of a high tumour grade, and the loss of estrogen and progesterone receptors that favours progression in breast cancer cells (Kang et al., 2002). In cervical cancer Glut1 is associated with malignant progression; low levels of Glut1 are present in benign cervical tissue, whereas increasing amounts are seen in dysplastic tissue and very high levels are found in malignancies and metastases (Rudlowski et al., 2003). CA9, which is highly expressed in many cancers under hypoxia, is associated with increased cancer cell invasiveness (Parkkila et al., 2000). Robertson and colleagues (2004) found that CA9-specific RNAi reduces cancer cell growth and survival under hypoxia.

HIF-1 is overexpressed in many cancers compared to normal tissues, and has been associated with tumour progression. Expression of HIF-1 correlates with poor prognosis, being associated with more aggressive and malignant phenotypes (Aebersold et al., 2001; Birner et al., 2000; Bos et al., 2003). Disruption of HIF-1-induced transcription suppresses tumour growth (Kung et al., 2000). HIF-1 expression is correlated with aberrant p53 and cell proliferation and as discussed before, hypoxia selects for cells resistant to apoptosis such as p53-negative cells. HIF-1 is more highly expressed in breast cancer metastases than in primary breast cancer tissues (Zhong et al., 1999). HIF-1 induces CA9 and Glut1 expression, which as discussed above are proteins linked to a malignant phenotype (Chen et al., 2001a; Ivanov et al., 2001).

Hypoxia upregulates interleukin-8 (IL-8), which increases tumour growth and metastatic potential in human melanoma cells (Bar-Eli, 1999) and enhances the invasiveness of murine sarcoma, carcinoma and melanoma cells (Cuvier et al., 1997).

Under hypoxic conditions, carcinoma and endothelial cells show greater invasiveness and expression of the urokinase receptor (Graham et al., 1998; Graham et al., 1999). Hypoxia promotes epithelial-mesenchymal transition (EMT), an important part of tumour progression where cancer cells develop a mesenchymal phenotype that enhances cell motility and invasion (Kalluri and Weinberg, 2009). For example, the gene Twist1 involved in the regulation of EMT is induced by hypoxia (Gort et al., 2008). One fundamental feature of EMT, the loss of E-cadherin, has now been linked to HIF-1 activation. The enzyme lysyl oxidase-like 2 (LOXL2), which stabilizes the protein Snail and results in the suppression of E-cadherin expression (Peinado et al., 2005), is highly induced by HIF-1 (Denko et al., 2003). Lysyl oxidase (LOX) and LOXL2 are overexpressed in highly invasive metastatic breast cancer cells (Kirschmann et al., 2002) and the amount of lung and liver metastasis in mice with orthotopic tumours decreases when LOX is suppressed in the MDA-MB-231 breast cancer line (Erler et al., 2006).

Hypoxia induces the expression of a few matrix metalloproteinases (MMPs) necessary for the degradation of ECM (Koong et al., 2000; Munoz-Najar et al., 2006). Tumour progression is promoted by hypoxia via the induction of proteins involved in cell migration such as CXCR4, autocrine motility factor (AMF) and the receptor tyrosine kinase c-met (Funasaka et al., 2005; Pennacchietti et al., 2003; Wang et al., 2008).

1.3.11 Resistance to Therapy as a Result of Hypoxia

Hypoxia in tumours has long been known to represent a serious problem for cancer treatment, being associated with resistance to both radio- and chemotherapy. Local areas of the tumour that have become hypoxic are resistant to radiation and cells within the hypoxic areas can survive and proliferate (Brizel et al., 1997; Wouters and Brown, 1997). Radiation causes cell death by creating oxygen free radicals that then cause DNA damage, however, in hypoxic cells the dose of radiation required to have the same effect as the equivalent dose in normoxic cells is typically 2-3 times greater (Moeller and Dewhirst, 2006; Vaupel et al., 2004). Hypoxia may cause resistance to radiotherapy through genetic changes such as those that induce HSPs, including HSP27, increasing glutathione levels (Aloy et al., 2008), or diminishing apoptotic potential as occurs in cells that have lost p53 (Graeber et al., 1996). The effectiveness of some chemotherapeutic drugs is diminished by hypoxia. Hypoxic tumour cells are less affected by alkylating agents such as cyclophosphamide, chemotherapeutic antibiotics including Actinomycin and Adriamycin, and 5-fluorouracil, and hypoxia-selective agents (misonidazole) than their non-hypoxic counterparts (Teicher et al., 1990). HIF-1 upregulates MDR1, a gene whose product contributes to tumour chemoresistance, and its expression increases up to 7–fold in hypoxia (Comerford et al., 2002). Another problem that hypoxia presents for therapy is the reduced delivery of drugs to hypoxic areas since there is a lack of well functioning vasculature (Vaupel et al., 2001).

1.3.12 Hypoxia as a Prognostic Factor

Hypoxia predicts a poor prognosis including disease-free and overall survival in head and neck and cervical cancer (Brizel et al., 1997; Hockel et al., 1996), and overexpression of HIF-1α is correlated with poor prognosis in many cancers including breast and cervical cancers (Birner et al., 2000; Bos et al., 2003). The target genes of HIF-1α are used clinically as prognostic markers. Glut1 (Kang et al., 2002; Mori et al., 2007), CA9 (Chia et al., 2001; Kon-no et al., 2006) and VEGF (Gasparini, 2000; Maeda et al., 1998) have each been correlated with poor prognosis in various cancers.

1.3.13 Role of Energy Metabolites in Hypoxia

1.3.13.1 Adenosine

The hypoxic environment of tumours increases the purine nucleoside adenosine, an essential building block for ATP and AMP (Headrick and Willis, 1989). Adenosine is found both intra- and extracellularly (Fredholm, 2007) and is produced in response to cell damage or metabolic stress (Hasko et al., 2008; Linden, 2005). Adenosine plays a role in neurotransmission (Fredholm, 2007), inflammation (Livingston et al., 2004; Spychala, 2000), angiogenesis and cardioprotection (Fredholm, 2007; Spychala, 2000).

1.3.13.2 Production and Metabolism of Adenosine

Intracellular adenosine is produced primarily by the 5'-nucleotidase (5'-NT)-dependent dephosphorylation of AMP and through hydrolysis by S-adenosyl-homocysteine (SAH). Adenosine is transported out of the cell by equilibrative nucleoside transporters (ENTs) (Fredholm, 2007) as well as concentrative nucleoside transporters (CNT2, 3) (Gray et al., 2004). Extracellular adenosine is formed through the cooperation

of two ectoenzymes; CD39 (ENTPD1; ectonucleoside triphosphate diphosphohydrolase1) and CD73 (ecto'5'-NT; ecto-5'-nucleotidase). CD39 converts ATP and ADP into AMP, which is then dephosphorylated into adenosine by CD73 (Fredholm, 2007; Hasko et al., 2008). At physiological concentrations adenosine is primarily metabolized inside the cell by adenosine kinase (AK) which phosphorylates adenosine to create AMP (Spychala, 2000). At much higher adenosine concentrations (~70μM) inside the cell however, adenosine is preferentially metabolized by the enzyme adenosine deaminase (ADA) that deaminates adenosine into inosine (Lloyd and Fredholm, 1995).

Outside the cell, adenosine breakdown is principally accomplished through ecto-ADA, which is bound to cell-surface proteins such as CD26 and certain adenosine receptors (Kameoka et al., 1993). Extracellular adenosine is present in unstressed tissue in amounts <1μM (Ballarin et al., 1991) however, during hypoxia and ischemia levels increase 10-1000 fold (Fredholm et al., 2001b). Hypoxia inhibits AK and activates the 5'NT pathway increasing the amount of extracellular adenosine (Decking et al., 1997; Headrick and Willis, 1989) (Figure 1.2).

1.3.13.3 Adenosine Receptors and Signaling

There are four known human adenosine receptor subtypes (A1R, A2aR, A2bR and A3R), which are seven-transmembrane G-protein-coupled receptors (GPCRs) each with unique signaling pathways and effects (Fredholm et al., 2001a). A1Rs have the highest affinity for adenosine followed by A2aR. These receptors are stimulated by physiological levels of adenosine, with an EC_{50} between 0.01μM and 1μM. The A2bR has lower affinity for adenosine and requires much higher, pathophysiological levels of

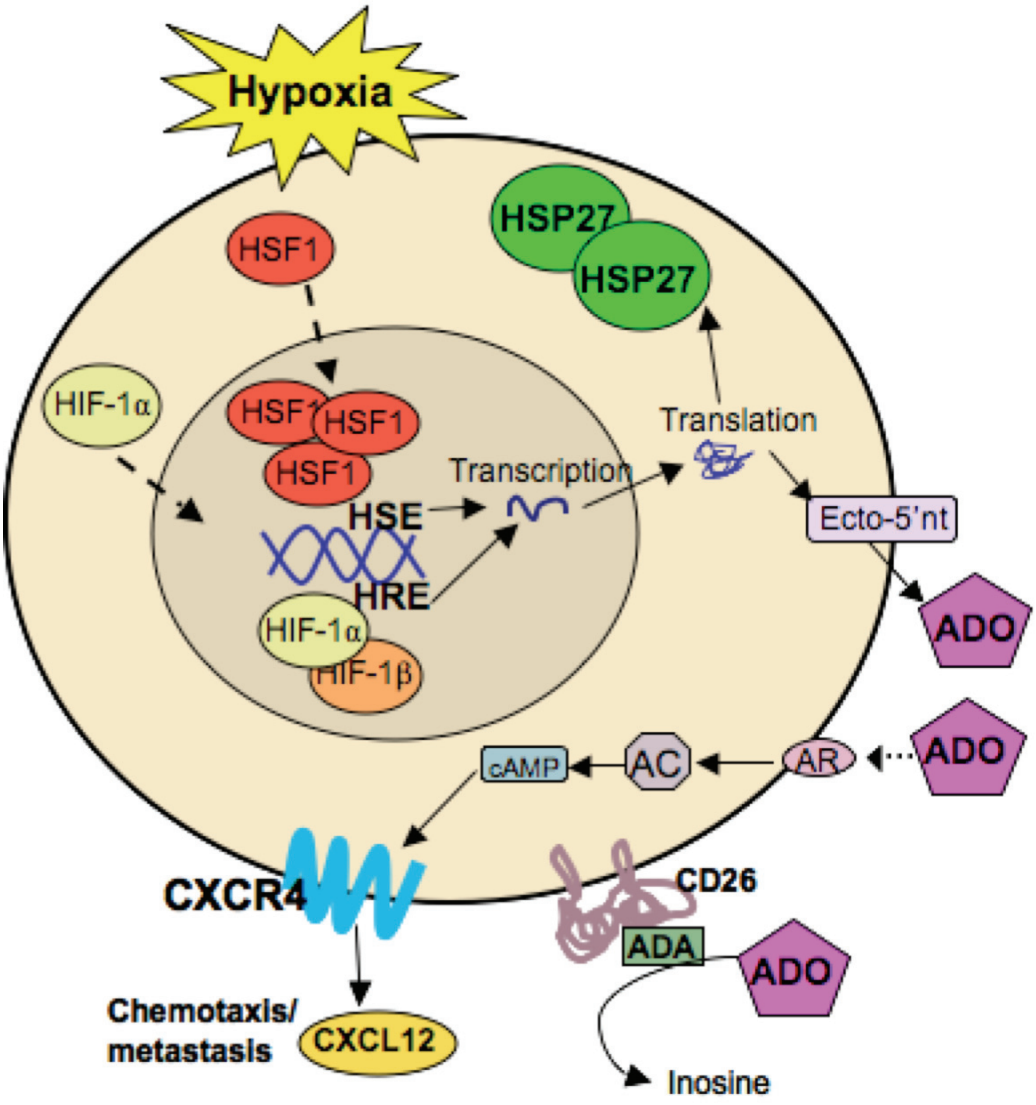


Figure 1.2 Effect of hypoxia on HSP27 and adenosine
Hypoxia induces the translocation of HSF1 to the nucleus where homotrimers bind heat shock elements (HSEs) resulting in the production of heat shock proteins including HSP27. Another consequence of hypoxia is the binding of HIF-1α to hypoxia response elements (HREs), resulting in increased production of adenosine. Adenosine then binds adenosine receptors (AR) inducing cell signaling leading to CXCR4 expression. CXCR4 binds its ligand CXCL12 leading to chemotaxis and metastasis. Adenosine is deaminated into inosine by adenosine deaminase which is bound to the cell-surface protein CD26.

adenosine (>10μM) to be activated and A3R has the lowest affinity for adenosine (Fredholm, 1995; Fredholm et al., 2001b).

Adenosine receptor signaling is traditionally thought to be through the second messenger cAMP, either inhibiting (A1R and A3R) or stimulating (A2aR and A2bR) adenylyl cyclase. Adenosine receptors act through many other pathways with all four receptors signaling through the MAP kinase pathway (Jacobson and Gao, 2006). The A1 receptor is coupled to G_i protein and inhibits adenylyl cyclase activity, decreasing cAMP levels. Once activated, A1 receptors inhibit N-, P- and Q-type Ca^{2+} channels (Fredholm et al., 2001a; Hasko et al., 2008). A1R linkages to the PKC, PI-3K and MAPK pathways have been identified (Hasko et al., 2008; Stagg and Smyth, 2010). The A2aRs and A2bRs are coupled to G_s proteins, resulting in an increase in cAMP via the activation of adenylyl cyclase. A2aRs signal through another G protein, G_{olf}, where G_s proteins are sparse (Jacobson and Gao, 2006; Kull et al., 2000). A2aRs play an important immunogenic role (Stagg and Smyth, 2010).

1.4 Hypoxia and Heat Shock Proteins

1.4.1 Hypoxia and the Heat Shock Response

Hypoxia induces the expression of stress or heat shock proteins (HSPs), including the small heat shock protein 27 (HSP27), allowing cells exposed to hypoxia to become preconditioned to further insults. HIF-1 upregulates transcription of the heat shock factor (HSF) gene allowing for the subsequent increase in HSP synthesis (Baird et al., 2006; Sakamoto et al., 1998). Rat myoblast cells exposed to hypoxia and then re-oxygenation are resistant to hypoxia-induced cell death for up to 24h (Sakamoto et al., 1998). Resistance was accompanied by HSP27 phosphorylation and translocation of HSP27 to the nucleus and cytoskeleton. In another study HSP27 expression was upregulated by HIF-1α in ischemic preconditioning, which involves a period of ischemia followed by recovery (Whitlock et al., 2005).

1.4.2 Heat Shock Proteins

Heat shock or stress proteins are primarily molecular chaperones that assist in the proper folding of cellular proteins and are a crucial part of the cell stress response (Gusev et al., 2002). While many HSPs are constitutively expressed (Jakob et al., 1993), their expression is increased in response to a variety of stresses including heat shock (Morimoto and Santoro, 1998; Morimoto et al., 1992), hypoxia (Kacimi et al., 2000), ischemia (Wagstaff et al., 1996), extremes of pH (Rafiee et al., 2006), chemicals (Lee and Dewey, 1988; Neuhaus-Steinmetz and Rensing, 1997), or heavy metals (Morimoto et al., 1992), and they confer biological thermotolerance (Landry et al., 1982). Although the large heat shock proteins (60 - 100 kDa) have been studied more widely in mammalian

systems, the small heat shock proteins (sHSPs), which range in size from 12 to 43 kDa, play key cellular roles and are present in archaea, bacteria and eukaryotes (MacRae, 2000).

Mammals possess ten classes of sHSPs. Some sHSPs such as HSP27 and αB-crystallin are ubiquitous and some are tissue specific. α-crystallin occurs in the lens of the eye, HSPB9 in the testis and others are specific to cardiac and skeletal muscle (Taylor and Benjamin, 2005). Aberrant sHSPs have hugely damaging effects, such as the missense mutation in the αB-crystallin gene that causes desmin-related myopathy (Vicart et al., 1998) or a mutation in αA-crystallin resulting in congenital cataracts (Litt et al., 1998).

The sHSPs are characterized by a well conserved central α-crystallin domain (Berengian et al., 1999) and it is important for the oligomerization of sHSPs (Gusev et al., 2002). The N-terminal domain is less well conserved, being variable in both length and sequence, and is important for oligomerization (de Jong et al., 1998). One region in the N-terminal, the WDPF motif, is well conserved and it is important for chaperoning activity and sHSP oligomerization (Lambert et al., 1999; Theriault et al., 2004). The C-terminus is required for chaperoning activity, sHSP solubility and oligomer stability. sHSPs exist in the cell as monomers, homo-dimers and oligomers. Oligomers form between monomers of the same sHSP and between different monomers such as HSP22 and αB-crystallins or HSP27 (Bukach et al., 2009; Fontaine et al., 2005).

The sHSPs are generally ATP-independent and protect cells when they are exposed to stress by preventing the irreversible aggregation of proteins that are undergoing denaturation (Jakob et al., 1993; Sun and MacRae, 2005). During stress,

sHSP expression increases and sHSPs form oligomers that interact with substrates, which are later released and refolded when conditions return to normal (MacRae, 2000; Rogalla et al., 1999).

The transcription of HSP genes is regulated by the activation of HSFs of which there are three in humans, namely HSF1, 2 and 4 (Pirkkala et al., 2001). HSF1 plays a role in development (Xiao et al., 1999) and it is the most important HSF in mediation of the stress response (McMillan et al., 1998; Sarge et al., 1993). HSF1 is present in both stressed and unstressed cells (Baler et al., 1993). In unstressed cells, HSF1 exists as inert monomers (Baler et al., 1993) bound to chaperones and they are unable to activate transcription (Shi et al., 1998). When a cell is stressed, the increase in denaturing proteins sequesters HSPs, freeing HSF1 monomers (Santoro, 2000) which can then oligomerize into homotrimers and translocate to the nucleus (Baler et al., 1993; Sarge et al., 1993). The trimers bind heat shock elements (HSE) located in the promoter region of HSP genes, enhancing HSP gene transcription (Christians et al., 2002).

1.4.3 The Small Heat Shock Protein 27 (HSP27)

HSP27 is a ubiquitous protein although higher levels are expressed in some tissues such as breast, uterus, cervix, skin and heart (Ciocca et al., 1993). HSP27 primarily exists in cells as oligomers (Lambert et al., 1999) ranging in mass up to 800kDa but upon phosphorylation it forms smaller multimers (Lavoie et al., 1995). Homodimers are formed through the α-crystallin domain and multimers are then formed through further interactions of C- and N-terminal domains (Lambert et al., 1999).

HSP27 is constitutively expressed (Taylor and Benjamin, 2005). Like other sHSPs HSP27 confers thermotolerance on cells (Shi et al., 2011) and its expression is

induced by many stresses in addition to hyperthermia (Samali and Cotter, 1996), such as oxidation (Arrigo, 2001; Arrigo et al., 2005), acidic pH (Rafiee et al., 2006), ischemia (Wagstaff et al., 1996), hypoxia (Kacimi et al., 2000), stimulation with vasopressin (Kaida et al., 1999), and by thrombin (Hirade et al., 2002; Mehlen et al., 1995). Aside from its role as a molecular chaperone, HSP27 functions in F-actin modulation and cell movement, programmed cell death (apoptosis), and resistance to oxidative stress (Ferns et al., 2006).

Monomeric HSP27 acts as an F-actin cap-binding protein that inhibits actin polymerization (Benndorf et al., 1994; Miron et al., 1991), while oligomeric HSP27, like other sHSPs, has a chaperone function (Rogalla et al., 1999). Oligomeric HSP27 binds denaturing proteins preventing their aggregation and conserving their stability until other chaperone proteins such as HSP70 renature them (Ehrnsperger et al., 1997). Large oligomers of HSP27 protect against reactive oxygen species (ROS) (Mehlen et al., 1997). Clearly, HSP27 has several distinct roles.

1.4.3.1 HSP27 as a Molecular Chaperone

The ability of HSP27 to act as a molecular chaperone helps to prevent apoptosis in stressed cells. The build up of denatured proteins induces HSP expression (Ananthan et al., 1986) resulting in the interaction of HSP27 with denaturing proteins, that left to form large aggregates could trigger apoptosis (Soldatenkov and Dritschilo, 1997). HSP27 limits protein synthesis under cellular stress, further decreasing the number of misfolded and denatured proteins (Cuesta et al., 2000).

1.4.3.2 HSP27 as a Redox Protectant

HSP27 protects cells against oxidative damage caused by ROS, which could otherwise lead to death (Mehlen et al., 1996). Although HSP27 does not posses any endogenous ROS-detoxifying activity, large unphosphorylated oligomeric HSP27 (Mehlen et al., 1997) decreases the amount of ROS generated from TNFα stimulation by increasing the concentration of reduced glutathione (Mehlen et al., 1996; Preville et al., 1999). HSP27 also protects cells from oxidative stress by preventing the dissociation of actin filaments. Cells expressing wild-type HSP27 but not a non-phosphorylatable HSP27 mutant show increased cell survival in response to H_2O_2 (Huot et al., 1996).

1.4.3.3 HSP27 as a Modulator of Apoptosis

HSP27 modulates signaling in intrinsic and extrinsic apoptotic pathways:

Intrinsic Apoptosis Pathway

HSP27 inhibits the formation of the apoptosome consisting of cytochrome c, Apaf-1 and procaspase 9, by interacting with cytochrome c that is released from mitochondria, therefore inhibiting the caspase cascade (Bruey et al., 2000). HSP27 blocks cytochrome c release by interfering with the translocation of the pro-apoptotic protein Bid to mitochondria (Paul et al., 2002) and by maintaining mitochondrial membrane potential (Samali et al., 2001). HSP27 interacts with caspase 3 directly, therefore limiting its availability to be cleaved and activated by caspase 9 (Pandey et al., 2000).

Extrinsic Apoptosis Pathway

HSP27 inhibits extrinsic apoptosis by modulating the signaling pathways of death receptors Fas (CD95/Apo-1), TNF and TRAIL. The FAS receptor activates two apoptotic pathways, one of which involves the caspase-independent Daxx-mediated pathway.

Phosphorylated dimers of HSP27 prevent Daxx from interacting with Fas and apoptosis-signal-regulated kinase (ASK-1). HSP27 prevents Daxx translocation from the nucleus to the cytoplasm (Charette and Landry, 2000).

TNF can lead to p38-MK2 phosphorylation of HSP27, inhibiting IKK activity and therefore suppressing NF-kB (Park et al., 2003). Another group however showed that HSP27 enhances NF-kB activation by promoting the degradation of IkB (Parcellier et al., 2003). How HSP27 modulates signaling in the TNF receptor pathway is therefore not entirely clear. Less is known about the interaction of HSP27 with the signaling pathway of the death receptor ligand TRAIL, which binds to DR4 and DR5. However, in TRAIL-resistant lung cancer cells, the knockdown of HSP27 sensitized the cells to TRAIL-mediated apoptosis (Zhuang et al., 2010). HSP27 interacts with and promotes the activation of the pro-survival protein AKT/PKB (Konishi et al., 1997). AKT enhances survival in several ways including activation of NF-κB (Kane et al., 1999) and inhibition of the pro-apoptotic protein Bad (Dougherty and Morrison, 2004).

1.4.3.4 HSP27 Phosphorylation

Phosphorylation of HSP27 occurs at serines 15, 78 and 82, although unlike many other proteins, phosphorylation does not necessarily happen in a sequential order (Kostenko and Moens, 2009; Landry et al., 1992). The phosphorylation of HSP27 dissociates oligomers and decreases chaperone function (Rogalla et al., 1999). HSP27 is phosphorylated in response to a plethora of stimuli such as mitogens like VEGF (Rousseau et al., 1997), hypoxia (Kacimi et al., 2000), heat shock (Landry et al., 1991; Landry et al., 1992), cytokines including Il-1∝ and TNF-∝ (Kato et al., 1994; Mehlen et

al., 1995), ROS (Mehlen et al., 1996), H_2O_2 (Gaitanaki et al., 2003), thrombin (Nakajima et al., 2005), and vasopressin (Akamatsu et al., 2004).

The phosphorylation of HSP27 is caused indirectly by PKC, PKA, PKD, cGMP, and AKT through the p38-MAPK pathway that activates MAPKAP(MK)-2, -3 and -5 (Kostenko and Moens, 2009). While MK2, 3 and 5 (Dorion and Landry, 2002; Gerits et al., 2007; Kostenko and Moens, 2009; Ludwig et al., 1996; Stokoe et al., 1992b) all phosphorylate HSP27, MK3 and -5 do so to a much lesser degree than MK2, and it is now generally accepted that MK2 is the primary HSP27 kinase. HSP27 is dephosphorylated by protein phosphatase-2A (PP2A), a protein serine/threonine phosphatase (Berrou and Bryckaert, 2009; Cairns et al., 1994) which may affect HSP27 phosphorylation indirectly as PP2A inactivates MK2 (Stokoe et al., 1992a).

1.4.4 HSP27 - Interaction with the Cytoskeleton and its Role in Migration

1.4.4.1 Cytoskeleton Stability

Actin filament fragmentation and dissociation from the cell membrane is one of the first effects of stresses such as heat shock and oxidative stress. The induction of HSP27 expression protects cells by maintaining actin filament integrity and their contact with the cell membrane (Huot et al., 1996; Lavoie et al., 1993a; Lavoie et al., 1993b). HSP27 prevents the aggregation of unfolding actin monomers, which are later re-folded or destroyed (Pivovarova et al., 2007). Phosphorylated HSP27 protects actin since cells transfected with non-phosphorylatable HSP27 show no increase in actin stability in response to stress (Huot et al., 1996; Mounier and Arrigo, 2002). HSP27 interacts with other cytoskeletal components such as the intermediate filament proteins desmin (Blunt

et al., 2007) and vimentin (Perng et al., 1999) and microtubules (Hino et al., 2000), which may indicate a role of HSP27 in cell-cell interactions, the acquisition of a motile phenotype, and changes in the overall microtubular framework that are necessary for the cell to migrate.

1.4.4.2 Migration

HSP27 plays an important role in cell migration. HSP27 accumulates in the lamellipodia of fibroblasts (Lavoie et al., 1993b). Cells overexpressing HSP27 show enhanced migration (Hirano et al., 2004; Rousseau et al., 1997) and conversely knockdown of HSP27 inhibits cell migration (Shin et al., 2005). The phosphorylation of HSP27 is crucial for cell migration (Guo and Bhat, 2007; Hedges et al., 1999; Piotrowicz et al., 1998; Shin et al., 2005). As stated before, unphosphorylated monomers of HSP27 bind to actin filaments preventing additional actin polymerization (Benndorf et al., 1994; Miron et al., 1991). When HSP27 is phosphorylated it dissociates from actin and facilitates cell migration. HSP27 phosphorylation also plays a role in pinocytosis (Lavoie et al., 1993b) and membrane blebbing (Huot et al., 1998), and enhances cell adhesion (Di et al., 2007; Hirano et al., 2004).

1.5 Cell Migration, Invasion and Cancer Metastasis

1.5.1 Chemokines

Chemokines are a family of peptide ligands that are primarily involved in the trafficking of leukocytes. There are four groups of chemokines named on the basis of cysteine residue spacing. These ligands signal through chemokine receptors which are

named for the subclass of chemokine they recognize, followed by 'R' for receptor. Chemokine receptors are seven-transmembrane GPCRs that are linked with G_i proteins, inhibiting the activation of adenylyl cyclase (Mellado et al., 2001; Murphy et al., 2000). Eighteen chemokine receptors are identified and while there is promiscuity between the binding of receptors and chemokines, the chemokine receptor CXCR4 has a unique ligand named CXCL12 (Murphy, 2002; Murphy et al., 2000).

1.5.2 CXCR4/CXCL12 Axis

CXCR4/CXCL12 signaling is essential for embryo development and the lack of either CXCL12 or CXCR4 in mice is embryonic lethal (Nagasawa et al., 1996; Tachibana et al., 1998; Zou et al., 1998). In adult humans, CXCR4 is constitutively expressed in many cell types including lymphocytes, NK cells, monocytes, dendritic cells, endothelial cells and neurons (Balkwill, 2004)**,** and it plays an important role in tissue repair and regeneration (Ceradini et al., 2004)**.** Although CXCL12, known as stromal-derived factor-1 (SDF-1) is the only ligand for CXCR4, it binds to the receptor CXCR7 (Balabanian et al., 2005). Signaling through CXCR4 leads to the activation of several effectors including AKT, PKC (Shimizu and Hunt, 1996), Rho (Tan et al., 2006), Ca^+ (Hatse et al., 2002) and MAPK (Crespo et al., 1994). This pathway plays an important role in progression of several cancer types including breast cancer (Kato et al., 2003). CXCR4 expression is raised in more than 20 cancers (Balkwill, 2004) and it is correlated with poor prognosis, increased recurrence rate and decreased survival (Balkwill, 2004; Kim et al., 2005; Rombouts et al., 2004).

CXCR4 is undetectable in normal breast epithelial cells but is highly expressed in several breast cancer cell lines as well as primary breast cancer cells (Muller et al., 2001).

In response to CXCL12, MDA-MB-231 human breast cancer cells that express CXCR4 undergo phenotypic change characterized by actin polymerization, pseudopodia formation and directional migration and tissue invasion. Interestingly CXCL12 is more highly expressed in tissues from organs to which breast cancer preferentially metastasizes such as the lungs, lymph nodes, liver and bone marrow (Muller et al., 2001). Very low levels of CXCL12 occur in the tissues of organs to which breast cancer rarely metastasizes, including the brain, skin and muscle. Furthermore, the metastasis of MDA-MB-231 cells injected into the tail vein or orthotopically into the mammary fat pad of mice is decreased by an anti-CXCR4 antibody (Muller et al., 2001). Injection of MDA-MB-231 cells transfected with CXCR4 siRNA inhibits lung metastasis in mice (Liang et al., 2005). The increase in CXCR4 expression may occur early on in malignant transformation as it has been detected in ductal carcinoma in situ (DCIS) but not in adjacent normal tissue (Schmid et al., 2004). Apart from promoting metastasis to locations abundant in CXCL12, CXCR4 increases tumour cell proliferation and survival and induces angiogenesis (Lapteva et al., 2005; Salcedo et al., 1999; Smith et al., 2004).

1.5.3 CXCR4 and Hypoxia

Hypoxia, which is common in the tumour microenvironment, highly induces the expression of CXCR4. Staller and colleagues (2003) were the first to describe hypoxic regulation of CXCR4 expression, demonstrating that A498 renal cell cancer (RCC) cells with aberrant pVHL have strong CXCR4 expression, but when transfected with a functional pVHL, CXCR4 expression is suppressed. Staller and coworkers (2003) found that in human embryonic kidney (HEK-293) cells and primary human proximal renal tubular epithelia cells (RPTECs) with functional pVHL, CXCR4 levels increase when

exposed to hypoxia, which inactivates pVHL. HREs exist within the CXCR4 promoters of the HEK-293 cells and RPTECs (Staller et al., 2003). Hypoxia induces CXCR4 expression via HIF-1α activation in many kinds of cells including monocytes, tumour-associated macrophages, CAOV3 human ovarian cancer cells, WT2 human renal cancer cells and MCF-7 breast cancer cells, increasing their responsiveness to CXCL12 (Schioppa et al., 2003). VEGF increases CXCR4 expression, enhancing the responsiveness of cells to CXCL12 (Salcedo et al., 1999) and in some breast cancer cells estrogen upregulates CXCR4 (Sengupta et al., 2009). Additionally, CXCR4 expression is enhanced by HER2 which inhibits CXCR4 degradation (Li et al., 2004).

1.5.4 CXCR4 and Energy Metabolites

Another factor in the tumour microenvironment, adenosine, upregulates CXCR4. The adenosine receptors A2a and A2b produce a 10-fold increase in CXCR4 mRNA expression and a 3-fold upregulation of cell-surface CXCR4 expression in colorectal carcinoma cells in response to adenosine (Richard et al., 2006). The increase in CXCR4 expression enhances cell migration and proliferation in response to CXCL12 (Richard et al., 2006).

1.5.5 HER2 Involvement with CXCR4 and HSP27

The activation of the HER or *erbB* proteins (HER1-4), members of the receptor tyrosine kinase family, regulate cell growth, differentiation and survival and they are implicated in tumour growth and progression (Yarden and Sliwkowski, 2001). HER2, normally present in many cell and tissue types, is often overexpressed in cancers including breast, colon and lung (Slamon et al., 1987; Yarden and Sliwkowski, 2001). As

a result of gene amplification, HER2 is overexpressed in approximately 30% of breast cancers and it is associated with poor disease-free and overall survival (McCann et al., 1991; Slamon et al., 1987). HSP27 is highly expressed in HER2-positive tumours and ser78 phosphorylation is correlated with HER2 and lymph node positivity (Zhang et al., 2007). Kang and coworkers (2008) found that resistance to trastuzumab (Herceptin®), a monoclonal antibody to HER2, is increased by HSP27 expression and that HSP27 directly binds to and stabilizes HER2 (Kang et al., 2008). HER2 enhances tumour metastasis by upregulating CXCR4 and inhibiting its degradation (Li et al., 2004). By interacting with HER2, HSP27 may increase the amount of CXCR4 receptor at the cell-surface, enhancing cellular migration and favouring metastasis.

1.5.6 HSP27 in Cancer and Breast Cancer Cells

Increased expression of HSP27 occurs in many tumour cell lines (Morino et al., 1997) and cancers including breast, prostate, ovarian and gastric (Glaessgen et al., 2008; Kapranos et al., 2002; Langdon et al., 1995; Storm et al., 1995) and its expression is associated with malignant progression. Higher HSP27 expression is found in dysplastic gastric tissue as compared to adjacent normal gastric epithelium and expression correlates with the degree of dysplasia, the aberrant growth of cells or tissue (Kapranos et al., 2002). Expression of HSP27 correlates with more advanced stages of tumour progression, including lymph node metastasis, and it is associated with poor overall survival (Kapranos et al., 2002). Similar findings are reported for colorectal cancer (CRC), with rates of HSP27 expression increasing from 5% in normal colon mucosa to 50% in non-lymph node metastasis and 90% in lymph node metastasis (Pei et al., 2007). HSP27 is upregulated in both female and male breast cancer (Chahed et al., 2008;

Hurlimann et al., 1993) and is associated with lymph node metastasis (Storm et al., 1995).

Several studies consider the potential prognostic value of HSP27 in many kinds of cancers (Tweedle et al., 2010). For example, HSP27 expression is associated with a good prognosis in oral and esophageal cancers (Kawanishi et al., 1999; Suzuki et al., 2007) while in gastric cancer its expression is associated with a poor prognosis (Kapranos et al., 2002). No correlation exists between HSP27 expression in early ovarian cancers, but for more advanced poorly differentiated stages with metastasis, an increase in HSP27 expression is associated with poor prognosis (Arts et al., 1999). There is contradictory data on whether HSP27 is a prognostic factor for breast cancer. Têtu and coworkers (1995) found that HSP27 expression had no prognostic significance in LN+ breast cancer and similarly Love and colleagues (1994) saw no correlations with disease-free survival or overall survival in either early or advanced breast cancer. In contrast, another study shows that HSP27 expression associates with decreased disease-free survival in patients with LN+ (Thor et al., 1991).

HSP27 is implicated in increasing resistance to various chemotherapeutic drugs. For example, overexpression of HSP27 in MDA-MB-231 breast cancer cells confers resistance to the chemotherapeutic drug doxorubicin (Hansen et al., 1999). In another study using chinese hamster ovary cells, HSP27 overexpression increases resistance to doxorubicin and vincristine (Huot et al., 1991). The role of HSP27 in the chemotherapy response may be complex. The chemotherapeutic drug paclitaxel (Taxol®) suppresses HSP27 in breast cancer cells overexpressing HSP27, and this sensitizes the cells to subsequent treatment with doxorubicin (Shi et al., 2008). Fewer studies have looked at

the effect of HSP27 on radiotherapy but the results are conflicting. HSP27 overexpression confers thermo- and chemoresistance but not radioresistance in chinese hamster lung cells (Fortin et al., 2000). In contrast, down-regulation of HSP27 expression increases the sensitivity of prostate cancer cells to radiation (Teimourian et al., 2006). Another study found a decrease in the rate of radiation-induced apoptosis of prostate cells after the induction of HSP27 (Gibbons et al., 2000).

The HSP27 promotor region contains an estrogen response element (ERE) and HSP27 gene expression is induced by estrogen (Fuqua et al., 1989; Porter et al., 1996). The highest concentrations of HSP27 are found in estrogen target organs such as the cervix and uterus (Ciocca et al., 1993) and although many attempts have been made to identify a correlation between HSP27 and the estrogen receptor in cancers of these organs, no definite relationship is apparent.

The phosphorylation of HSP27 in cancer cells has not been studied as much as its synthesis, but in both normal cells (Chen et al., 2009; Rousseau et al., 1997) and cancer cells (Di et al., 2007; Guo et al., 2008; Rust et al., 1999; Shin et al., 2005) HSP27 phosphorylation promotes cell migration. When stimulated with PKC, HSP27 phosphorylation by the p38-MAPK pathway increases hepatocellular carcinoma (HCC) cell motility and invasion (Guo et al., 2008). When treated with a p38-MAPK inhibitor, phosphorylation of HSP27 is suppressed and HCC cell motility and invasion decrease (Guo et al., 2008). Blocking phosphorylation of HSP27 prevents cell migration and invasion in MDA-MB-231 cells (Shin et al., 2005) while HSP27 phosphorylation increases cell migration (Rust et al., 1999).

CHAPTER 2: HYPOTHESIS

HSP27 binds and stabilizes HER2, a protein that increases CXCR4 cell-surface expression, enhancing cellular migration. This and the fact that HSP27 is affected by many stresses led to the hypothesis of this study that:

> High levels of hypoxia and adenosine in the tumour microenvironment increase the abundance and/or phosphorylation of HSP27, which in turn upregulates the cell-surface receptor CXCR4 that is involved in directed breast cancer cell migration.

CHAPTER 3: OBJECTIVES

The overall objectives of my research are focused on how the tumour microenvironment affects HSP27 and the part that HSP27 plays in the response to hypoxia, adenosine and the CXCL12-CXCR4 migratory pathway in breast cancer cells.

Specific Objectives:

1. Determine the effects of the tumour microenvironment stressors hypoxia and adenosine on HSP27 abundance and localization in breast cancer cells;
2. Characterize HSP27 phosphorylation in response to the high levels of adenosine which are present in tumour environments;
3. Establish whether HSP27 is important for the maintenance of constitutive levels of CXCR4 and the upregulation that occurs in response to adenosine.

CHAPTER 4: METHODS

4.1 Materials

MCF-7 and T47D human breast cancer cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Media, sera, and trypsin (TripLE™ express), were from Invitrogen Canada (Burlington, ON, Canada) and culture flasks (Corning) and plates (24, 48 and 96-wells) (Nunc™) were from VWR International (Mississauga, ON, Canada). Bovine serum albumin (BSA), phenylmethylsulfonyl fluoride (PMSF) and Protease Inhibitor Cocktail Set I (PI) were from EMD Canada Inc. (Mississauga, ON, Canada). Albumin standards (BSA, 2.0mg/ml), Pierce ECL Western Blotting Substrate, SuperSignal® West Pico Chemiluminescent substrate and Restore™ Western Blot Stripping Buffer were from Thermo scientific (Ottawa, ON, Canada). Nitrocellulose and protein assay dye reagent concentrate were from BioRad Laboratories (Canada) Ltd. (Mississauga, ON, Canada). Adenosine (A9251), adenosine 5'-triphosphate (A3377), adenosine 5'-monophosphate (A2002), *N*6-(L-2-phenylisopropyl)adenosine (R-PIA), Inosine (I4125), Phosphatase Inhibitor Cocktail 3, and dimethyl sulfoxide (DMSO, 154938) were from Sigma-Aldrich (Oakville, ON, Canada). 5′ -*N*-ethylcarboxamidoadenosine (NECA) was obtained from Research Biochemicals International (Natick, MA, USA). All siRNA for HSP27, GAPDH and negative control as well as the transfection agent were purchased from Applied Biosystems (Streetsville, ON, Canada).

4.2 Antibodies

Mouse mAb against human HSP27 (clone EMD-35) was from EMD Canada Inc. (Mississauga, ON, Canada). Rabbit mAb against human HSP27 phospho (ser78) (04-447), rabbit pAb against human phospho-HSP27 (ser15) (07-388) and rabbit pAb against human phospho-HSP27 (ser82) (07-646) were from Millipore™ (Billerica, MA, USA). Mouse mAb against human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (AM4300) was from Applied Biosystems (Streetsville, ON, Canada). Rabbit pAb (HRP conjugated) against human α–tubulin (11H10) (9099S) was from Cell Signaling Technology® (Pickering, ON, Canada). Mouse IgG1 negative control (MCA928), rabbit IgG1 negative control (PRABP01), and peroxidase-conjugated AffiniPure Goat anti-rabbit IgG were from Cedarlane Laboritories Ltd. (Burlington, ON, Canada). Biotinylated goat anti-mouse IgG antibody was from R&D systems (Minneapolis, MN, USA). Alexa Fluor® 488 conjugated goat anti-mouse IgG, Alexa Fluor® 488 conjugated donkey anti-rabbit IgG and Alexa Fluor® 568 conjugated donkey anti-rabbit IgG were from Invitrogen Canada (Burlingto, ON, Canada). Mouse mAb against human CXCR4 (clone 12G5) was from BD Biosciences (San Jose, CA, USA). Secondary [^{125}I]-labeled goat anti-mouse IgG fragment (NEX159) was from PerkinElmer Life Sciences (Boston, MA, USA).

4.3 Cell Culture

MCF-7 and T47D cells were cultured in Dulbecco's modified Eagle's medium (DMEM, antibiotic free) supplemented with 10% newborn calf serum (NCS) or 5% fetal bovine serum (FBS) respectively. Cultures were maintained in 80cm^2 flasks at 37°C in a

humidified atmosphere of 90% air/10% CO_2 and were routinely sub-cultured by brief exposure to TripLE™ express. To seed cells for experiments, 0.5ml of a trypsinized cell suspension was added to 9.5ml of PBS and counted using a Coulter® counter (Beckman Multisizer^{TM} 4).

For heat shock experiments, cells were incubated at 44°C in a humidified atmosphere of 90% air/10% CO_2. During hypoxia treatments, cells were placed in a hypoxia chamber with 1% O_2 at 37°C.

4.4 Western Blots

4.4.1 Protein Collection

Cells were generally seeded into 35mm dishes and treated when 60-80% confluent. Dishes were put on ice to cool, washed 2x with 1ml cold PBS containing Ca and Mg, and then incubated for 5min in 250µl RIPA buffer (1% IGEPAL, 0.5% sodium deoxycholate, 0.1% SDS, 1mM NaF in 1x PBS) with 1x PMSF and PI. The dishes were then scraped and the cell lysate was incubated on ice for 1h prior to centrifugation (14,000xg) at 4°C for 30 min. The supernatant was collected and stored at -80°C. A modified RIPA buffer and protocol were used in later experiments where after RIPA (1% IGEPAL, 5% sodium deoxycholate, 0.1% SDS, 150mM NaCL, 50mM Tris, 1mM EDTA in dH_2O) was added, cells were scraped and lysates were incubated for 20min and centrifuged for 20min at 4°C before being stored at -80°C. For samples being collected to

look at phosphorylation, 1x phosphatase inhibitor cocktail 3 against serine/threonine protein phosphatases was added.

4.4.2 Bradford Assay

Protein samples were diluted 1:5 with dH_2O and 10µl of the diluted protein was put into 3 wells and 10µl of each BSA standard were pipetted into 3 wells of a 96-well plate. Protein assay dye reagent concentrate was mixed 1 part dye with 4 parts dH_2O and filtered. Using a microplate reader (PowerWave X 340, BioTek®) a standard curve created from the BSA standards was used to determine the protein concentration of the diluted samples. The average of the three wells was used as the concentration for each protein sample.

4.4.3 SDS Polyacrylamide Gel Electrophoresis

Equal amounts of protein (usually 10µg) from cell sample lysates were denatured by boiling samples for 5min with 4x loading buffer (200mM Tris-HCL pH6.8, 2% β -mercaptoethanol, 4% glycerol, 0.05% Bromophenol Blue, 8% SDS in dH_2O). Protein samples were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% gel at 20mA, 650V. The separated protein samples were then electroblotted overnight at 4°C or at RT for 1h 30min, 30V, to nitrocellulose. Blots were blocked at RT with 3% skimmed milk in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1h. Blots were probed for 1h at RT or overnight at 4°C depending on the antibody. Optimal dilutions of the primary antibodies were used: anti-HSP27 (1:7500), anti-phospho ser78 (1:5000) and anti-phospho ser82 (1:1000). Bots were washed 3 x 10min (or 5 x 5min) in TBST followed by exposure to secondary antibodies against mouse or rabbit IgG

(1:2000) for 1h at RT. Blots were washed again 3 x 10min (or 5 x 5min) with TBST and protein bands were visualized using ECL. For stripping blots were washed for 10min with TBST, exposed to 5ml of stripping buffer for 6min at RT and washed 3 x 10min with TBST before blocking and repeating the blotting procedure.

4.5 Immunofluorescence

Cells were seeded into 8-well chambered slides at 100,000 cells/well. After treatments, slides were placed on ice to cool and each well was rinsed gently with 500µl cold PBS (containing Ca and Mg, pH7.2) and then incubated in 500µl cold methanol (100%) for 10min. The methanol was tipped off and slides were allowed to air dry. Wells were incubated for 1h at RT in 120µl of primary antibody against HSP27 or phospho-HPS27, or 120µl of normal mouse or rabbit IgG1, each at a concentration of 3µg/ml. Wells were washed 3x15min with 0.5ml then 0.75ml, then 0.5ml PBS.BSA (PBS with Ca and Mg, pH7.2 with 1mg/ml BSA, passed through a 0.45µm filter). Cells were then incubated for 60min at RT in 120µl of secondary antibody at 5µg/ml. Wells were washed 3x15min at RT with 0.5ml, then 0.75ml, then 0.5ml PBS.BSA. Slides were mounted in fluorescence gel and cover slipped. Slides were observed under a Leica DM2000 upright fluorescence microscope.

4.6 siRNA

siRNA transfection of T47D cells was carried out according to the manufacture's instructions for reverse transfection using 1.5µl/well siPORT™ *Amine* Transfection

Agent (AM4503) in 24-well plates. *Silencer*® select GAPDH siRNA and *Silencer*® Select negative control #1 siRNA were used as positive and negative controls respectively. Cells were seeded at $4x10^4$ cells/well with 5nmol siRNA. Cells were shifted to 1% FBS DMEM 24h after transfection to reduce cytotoxicity.

Silencer® select pre-designed siRNA for:	Antisense sequence (5' to 3'):
HSPB2 (s6992)	AAACCAUACAUUGUGGACCat
HSPB2 (s194540)	UCUGUGUCCAAAUGUCGGCca
HPSB1 (s6991)	UUGACAUCCAGGGACACGCgc
HSPB1 (s194538)	UCUCAUCGGAUUUUGCAGCtt

4.7 Cell Based Radio-Immunobinding Assay

4.7.1 Binding Assay

All washes were done with PBS containing 0.2% BSA and antibodies were diluted in PBS with 1.0% BSA. Culture plates were placed on ice to cool and medium was aspirated from the wells. Wells were washed with 500µl of PBS.BSA and incubated on ice for 1h in 250µl of primary antibody (anti-CXCR4, 1:500 dilution) and isotype control (1:100 dilution). The antibody was aspirated and wells washed 2x with 500µl PBS.BSA. Cells were incubated for another 1h on ice in 250µl of secondary antibody (^{125}I labeled anti-mouse, 1:100 dilution). The antibody was aspirated and wells were washed 2x with 500µl PBS.BSA. After a 24h incubation in 0.5M NaOH at RT, samples

were transferred to titer tubes and read by a gamma counter (1480 Wizard™ 3, Wallac) for 300s each.

4.7.2 Cell Counting

Each well was washed 5x with 500µl PBS.BSA, cells were trypsinized with 500µl of trypsin and incubated for 15min to 1h at 37°C. The 0.5ml of trypsinized cells was then added to 9.5ml of PBS and counted using a Coulter® counter (Beckman Multisizer™ 4).

4.8 Adenosine Levels Achieved in Culture

In order to confirm that repetitive dosing with adenosine at 300µM for the siRNA experiments did not lead to accumulation to cytotoxic levels (>600µM), adenosine concentrations were measured by K Gillies, a colleague in the laboratory. Analysis by HPLC (high-performance liquid chromatography) showed that the repetitive dosing approach allowed for a concentration of adenosine (>~50µM), able to continuously activate all four receptor subtypes throughout a 36h period without adenosine reaching a cytotoxic level (Figure 4.1).

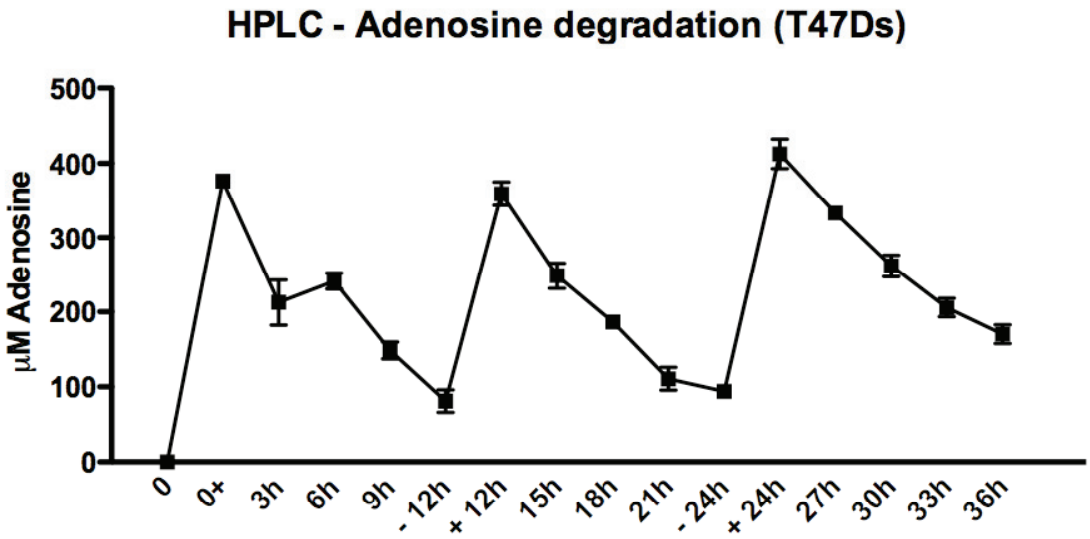


Figure 4.1 The degradation of adenosine.
T47D cells were seeded at $4x10^4$ cells/well in 48-well plates. After 24h they were downshifted to 1% FBS DMEM and after 72h (time 0h) adenosine was added to wells for an estimated final concentration of 300µM. The same amount of adenosine was added 12h and 24h later. Samples of media were collected every 3h for 36h including immediately before and after each addition of adenosine. Media samples were eventually processed with a 1200 series HPLC system (Agilent Technologies Canada Inc., Mississauga, Ontario).

4.9 Statistical Analysis

Each graph in the results section shows representative results from at least three independent experiments unless otherwise noted. Data within groups were compared using a one-way ANOVA with a Dunnett's post-test. Significance was set at $P<0.05$.

CHAPTER 5: RESULTS

5.1 Changes in HSP27 Abundance

5.1.1 Heat Shock and Hypoxia Increase HSP27

I investigated the effect of heat shock and hypoxia on the abundance of HSP27 in MCF-7 and T47D cells, to determine if the HSP27 stress-response was present. As determined by immunoprobing of western blots HSP27 protein abundance increased rapidly in T47D and MCF-7 cell lines, reaching a plateau after 1h (Figure 5.1). No clear change was seen in HSP27 in less than 0.5h of exposure to heat (data not shown). During hypoxia treatments HSP27 protein abundance in T47D cells reached a maximum at 3h and then remained steady until 48h when expression dropped to about half the peak value (Figure 5.2A). HSP27 reached maximum amounts in MCF-7 cells by 3h and then decreased, falling well below the constitutive level at 48h (Figure 5.2B). In both cell lines, an increase in HSP27 was observed after only 0.5h exposure to hypoxia (Figure 5.2 inserts).

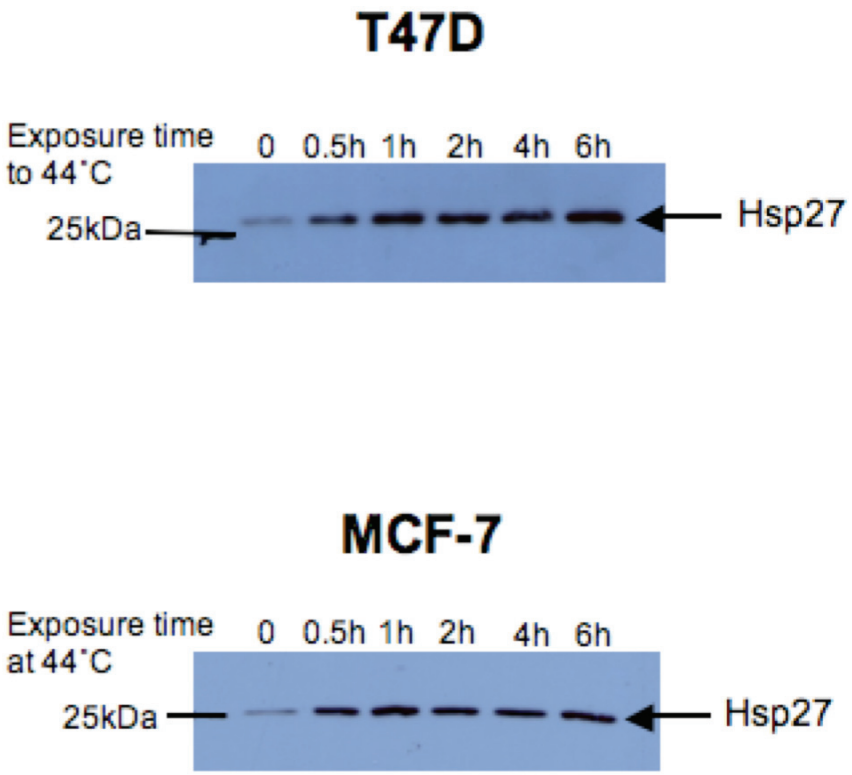


Figure 5.1 Heat shock induces an HSP27 response in breast cancer cells.
T47D and MCF-7 cells were exposed to 44˚C for varying lengths of time before protein was collected. Protein samples were resolved by separation on SDS polyacrylamide gels, blotted to nitrocellulose membranes and probed for HSP27.

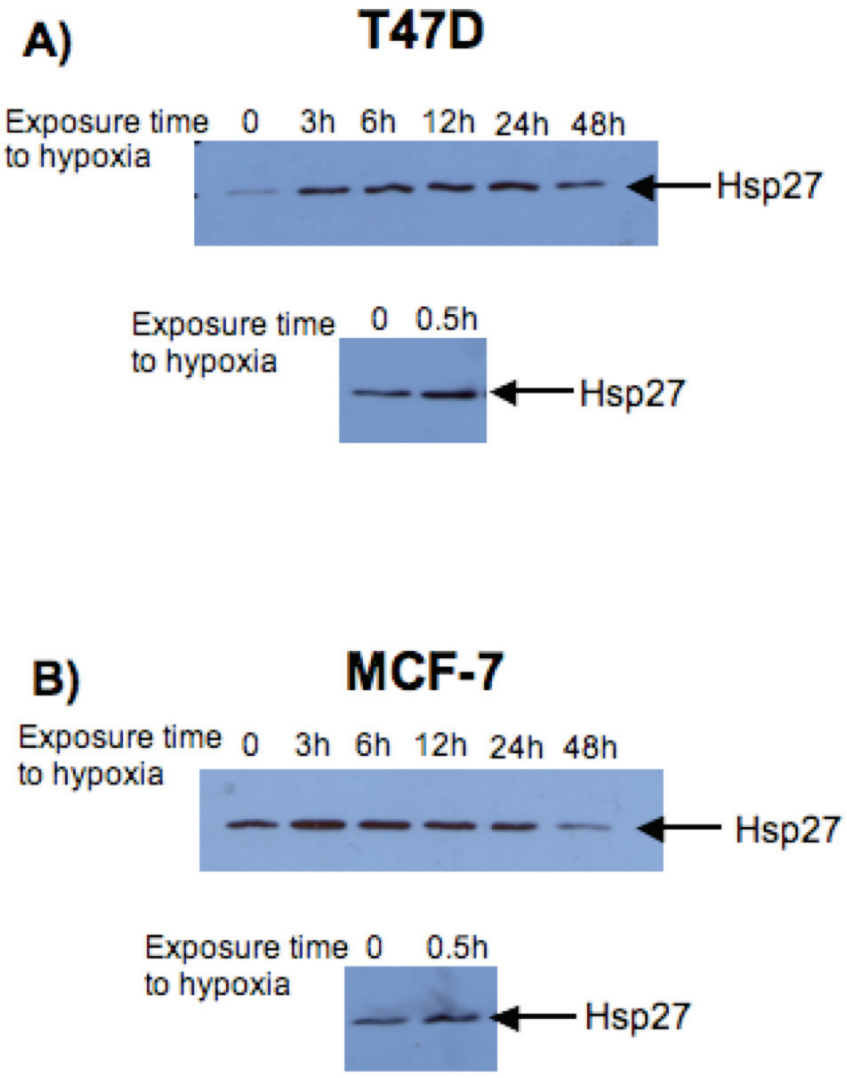


Figure 5.2 Hypoxia induces an HSP27 response in breast cancer cells.
T47D and MCF-7 cells were exposed to 1% O_2. Protein was collected at the time points indicated in the figure, resolved in SDS polyacrylamide gels, blotted to nitrocellulose and probed for HSP27.

5.1.2 Adenosine and Other Adenosine Receptor Agonists Have No Effect on HSP27 Abundance

Exposure to several concentrations of adenosine was carried out with both T47D (Figure 5.3, 5.4) and MCF-7 cells (Figure 5.5, 5.6). As determined by immuno-probing of western blots containing cell proteins resolved by SDS-PAGE no changes in HPS27 abundance were seen over 48h with doses of adenosine up to 1mM (Figure 5.3, 5.5). To reduce the effect of variation between different electrophoresis experiments samples from a dose response experiment were separated by SDS-PAGE, with samples grouped by time rather than adenosine concentration (data not shown). Under these conditions it was clear that there were no changes in HSP27 abundance due to adenosine in either cell line.

To test the possibility that the absence of an adenosine effect on HSP27 abundance was due to inactivation of the nucleoside, I treated cells with the stable adenosine analogues, NECA and R-PIA (Figure 5.7). After 48h of exposure no changes in HSP27 were evident in T47D or MCF-7 cells in response to either agonists (Figure 5.7A). In a separate pair of experiments of 2h duration, T47D and MCF-7 cells failed to exhibit changes in HSP27 abundance in response to the adenosine agonists (Figure 5.7B). The response of HSP27 to the adenosine metabolite inosine was tested. No changes in HSP27 were seen when T47D or MCF-7 cells were exposed to 300μM inosine over a 2h time frame (Figure 5.8A,C). I exposed cells to different concentrations of the adenine nucleotides, ATP or AMP, but no change in HSP27 protein expression was seen over 48h (Figure 5.8B,D). Immunostaining of T47D and MCF-7 cells treated with adenosine at 0-1000μM for 8, 24 and 48h, conditions similar to the immunoblotting studies above gave no evidence of elevation and intracellular translocation of HSP27 (data not shown).

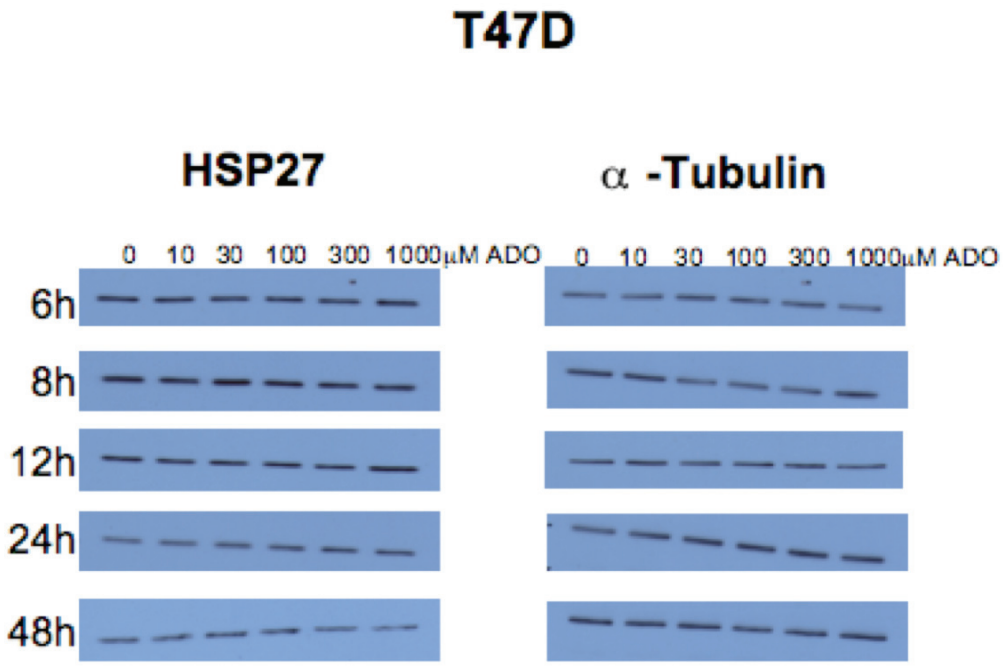


Figure 5.3 HSP27 abundance in T47D cells does not change in response to adenosine.
Dose responses (0-1000μM) with adenosine were carried out and protein was collected at the times indicated in the figure. Protein samples were separated by SDS-PAGE , blotted to nitrocellulose membranes and probed for HSP27.
Experiment n=3

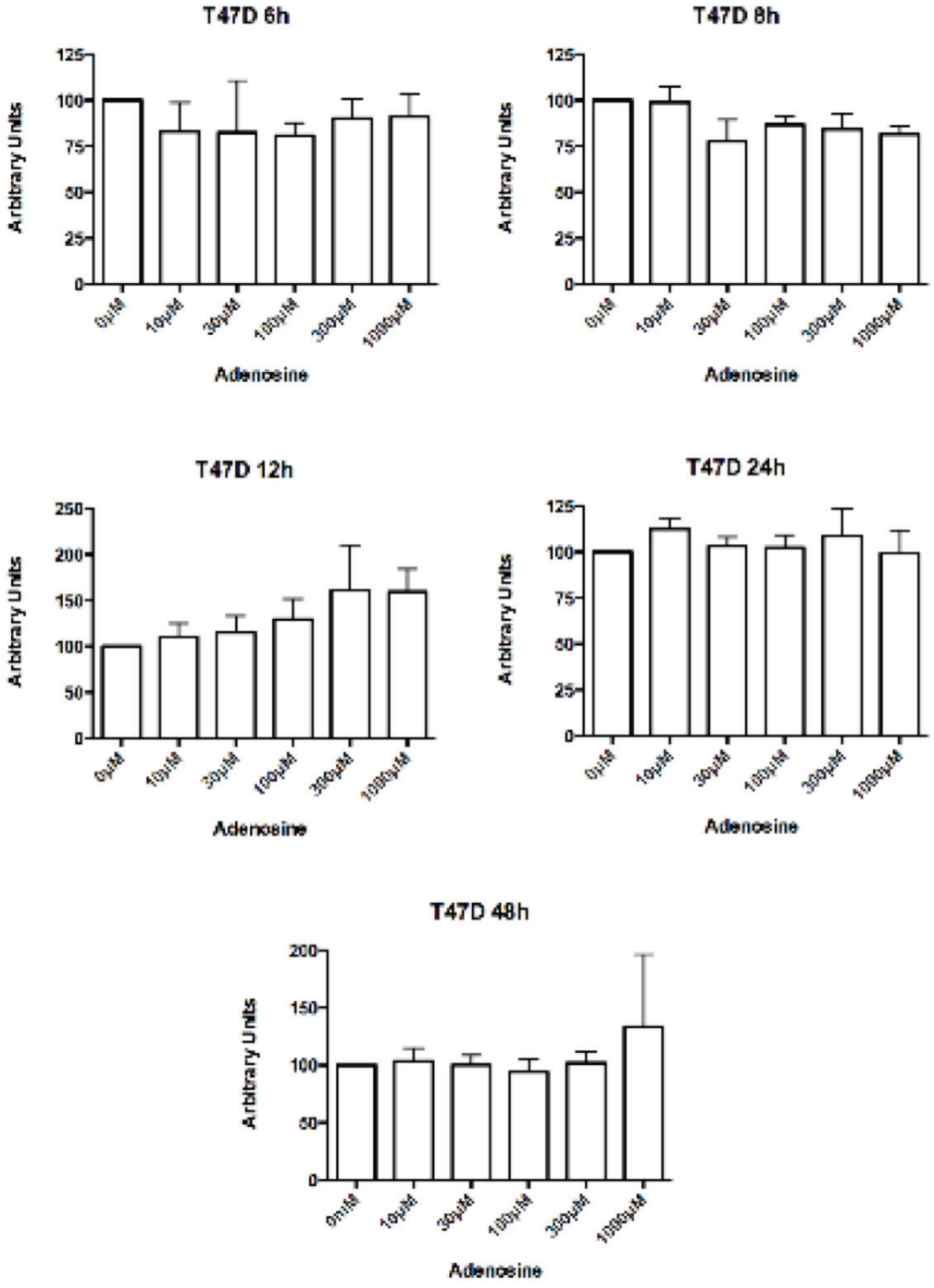


Figure 5.4 Densitometry of all three adenosine response trials with T47D cells shows no change in HSP27 abundance.
HSP27 levels of each dose response were quantified using densitometry and the results from all three trials were plotted together. The graphs are aggregated densitometry data from three independent experiments (bars show mean ± SE).

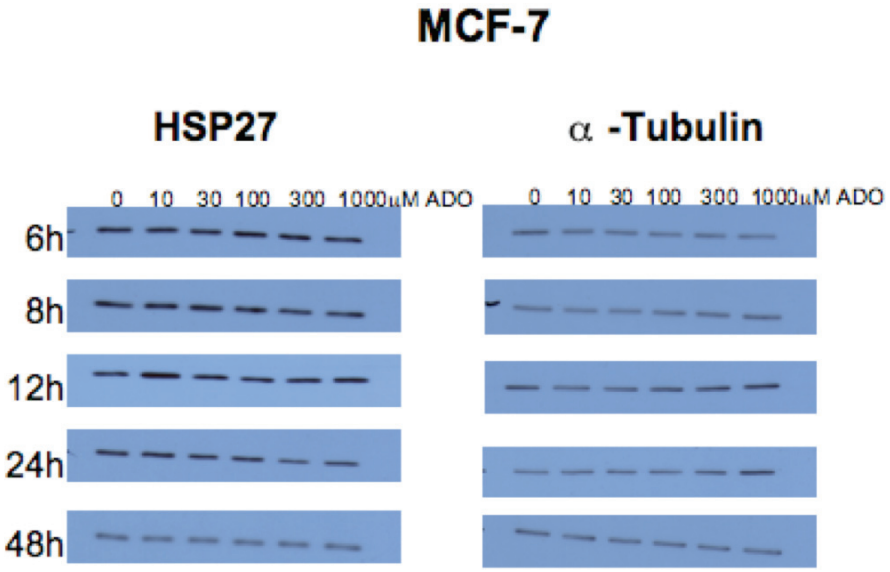


Figure 5.5 Adenosine did not increase HSP27 protein abundance in MCF-7 cells. Adenosine dose responses (0-1000μM) were carried out in MCF-7 cells and protein was collected at the times indicated in the figure. Protein samples were resolved by separation on SDS polyacrylamide gels, blotted to nitrocellulose membranes and probed for HSP27. Experiment n=3

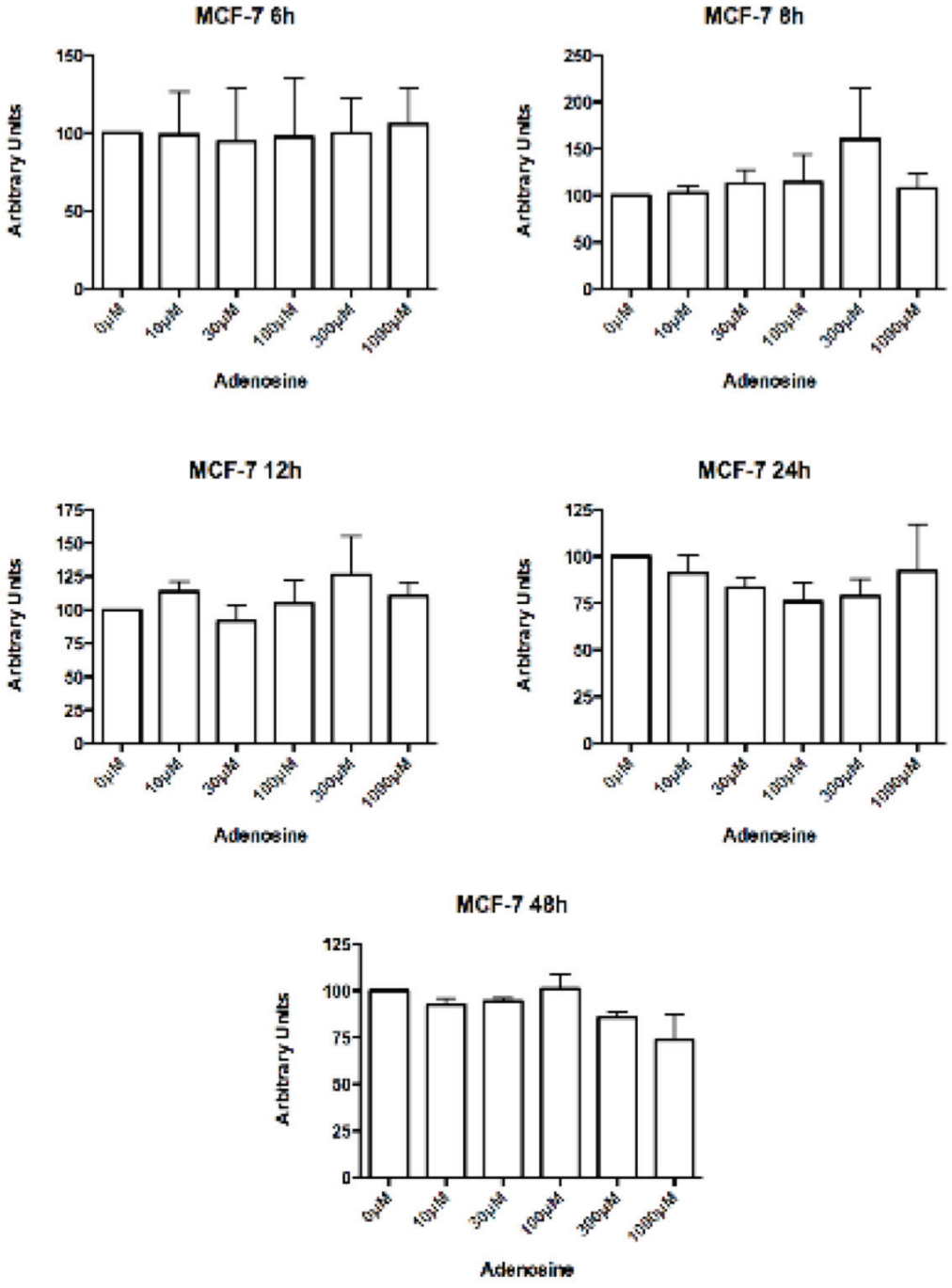


Figure 5.6 Densitometry of all three adenosine response trials with MCF-7 cells shows no change in HSP27 abundance.
HSP27 levels of each dose responses were quantified using densitometry and the results from all three trials were plotted together. The graphs are aggregated densitometry data from three independent experiments (bars show mean ± SE).

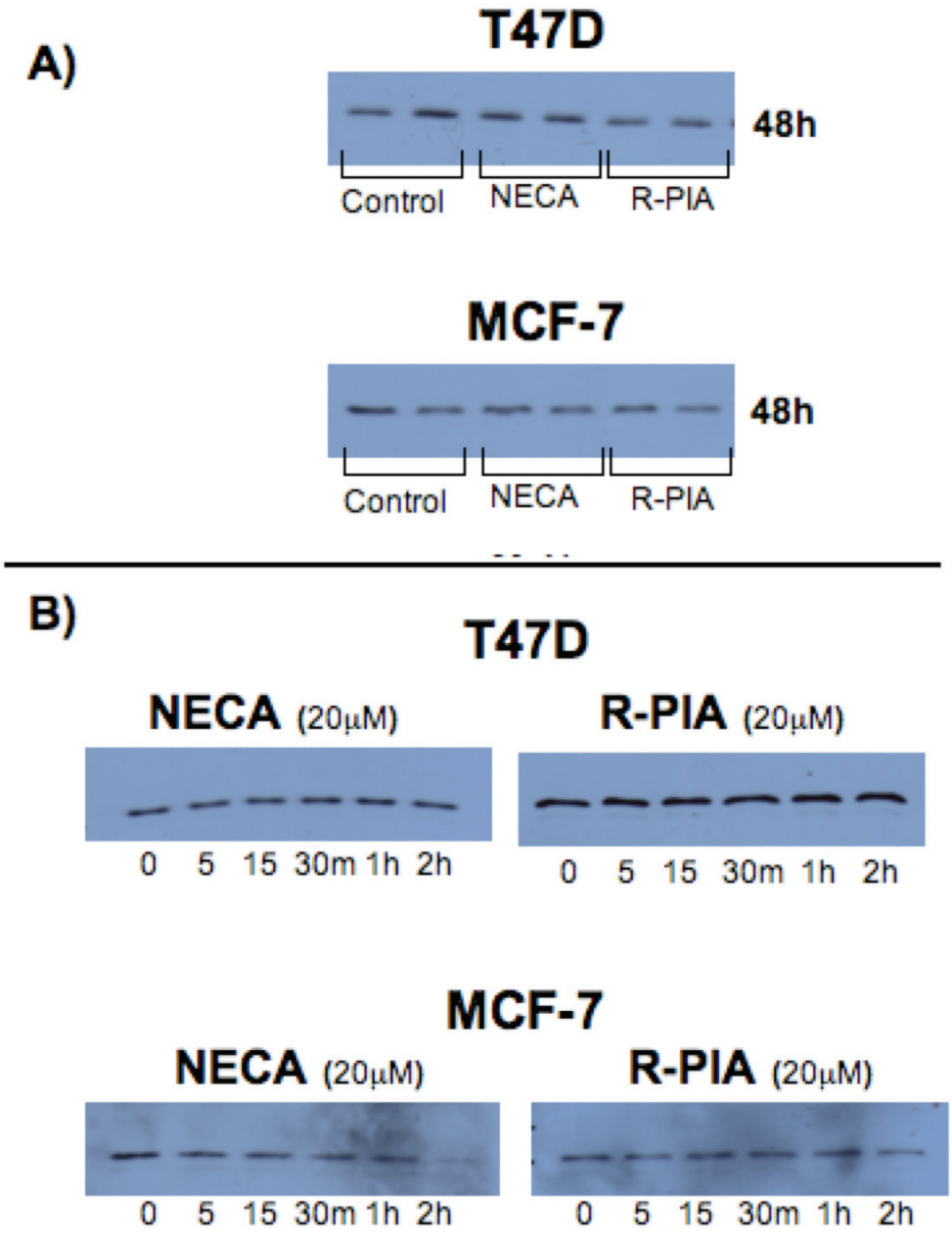


Figure 5.7 Adenosine agonists failed to elicit an HSP27 response in T47D and MCF-7 cells.
A) Cells were treated with either NECA or R-PIA at 20μM and protein was collected after 48h. An untreated control was done at the same time and all three treatments were done in duplicate. B) T47D and MCF-7 cells were treated with 20μM of either NECA or R-PIA over a 2h time course. Protein was collected and separated using SDS-PAGE, transferred to nitrocellulose membrane and were probed for HSP27.

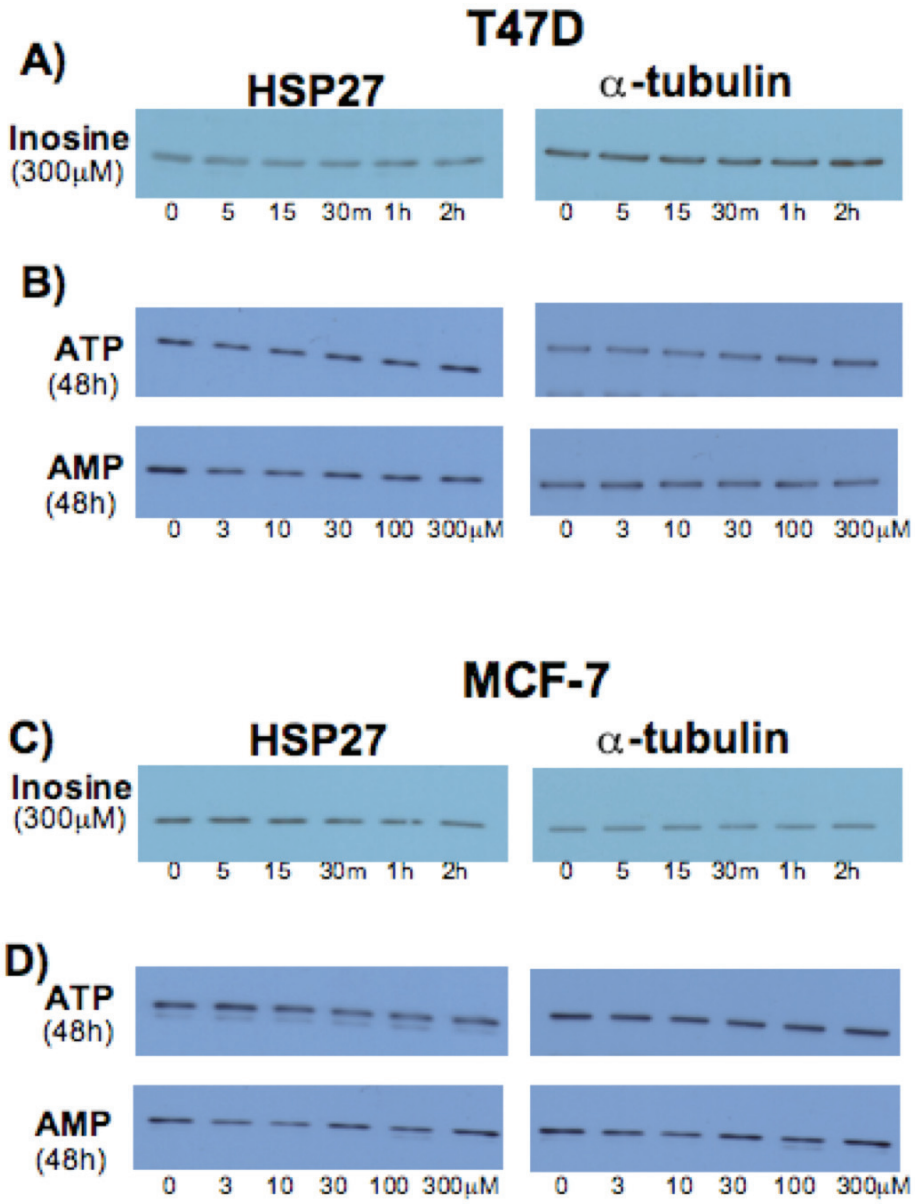


Figure 5.8 HSP27 protein abundance in T47D and MCF-7 cells does not change in response to inosine, AMP or ATP.
A/C) Cells were treated with 300μM of inosine over 2h. B/D) Cells were treated for 48h with different concentrations (0-300μM) of AMP or ATP. Protein was collected and separated by SDS-PAGE, blotted to nitrocellulose and probed for HSP27. These experiments were done once.

5.2 Handling of Cells Induced Acute Phosphorylation of HSP27 at ser78

In the experiments designed to investigate the phosphorylation of HSP27 in response to adenosine, I noticed rapid phosphorylation of HSP27 ser78 due to the handling of cells during the experiment, above which it was not possible to see an effect of adenosine.

Antibodies that specifically recognize phospho(p)-ser78, -ser82 and -ser15 of HSP27 were tested using heat shock protein samples from earlier experiments (Figure 5.1). The p-ser78 antibody gave a single strong band on western blots at the appropriate molecular weight, but the p-ser82 antibody reacted with several polypeptides indicating lack of specificity. The p-ser15 antibody did not detect any proteins on western blots. I therefore focused my attention principally on the phosphorylation on ser78 of HSP27.

I examined the effect of adenosine on HSP27 phosphorylation at ser78. In each of three independent experiments (Figure 5.9 is a representative example) where T47D cells were treated with either 300μM adenosine or vehicle for periods up to 1h, ser78 phosphorylation increased in adenosine-treated cells with maximal effect at 15-30min followed by a decline. However, the same trend occurred in cells that received only fresh culture medium. When the blots were re-probed no change was seen in the amount of HSP27 (Figure 5.9). The study showed an increase in HSP27 p-ser78, but not in HSP27, that was independent of adenosine concentration (Figure 5.10). The same experiment was done in triplicate with MCF-7 cells but no distinct patterns of ser78 phosphorylation were seen in response to any adenosine concentration (data not shown).

A similar result was obtained when I examined ser78 phosphorylation in response to the stable adenosine agonists, NECA and R-PIA. T47D and MCF-7 cells were treated

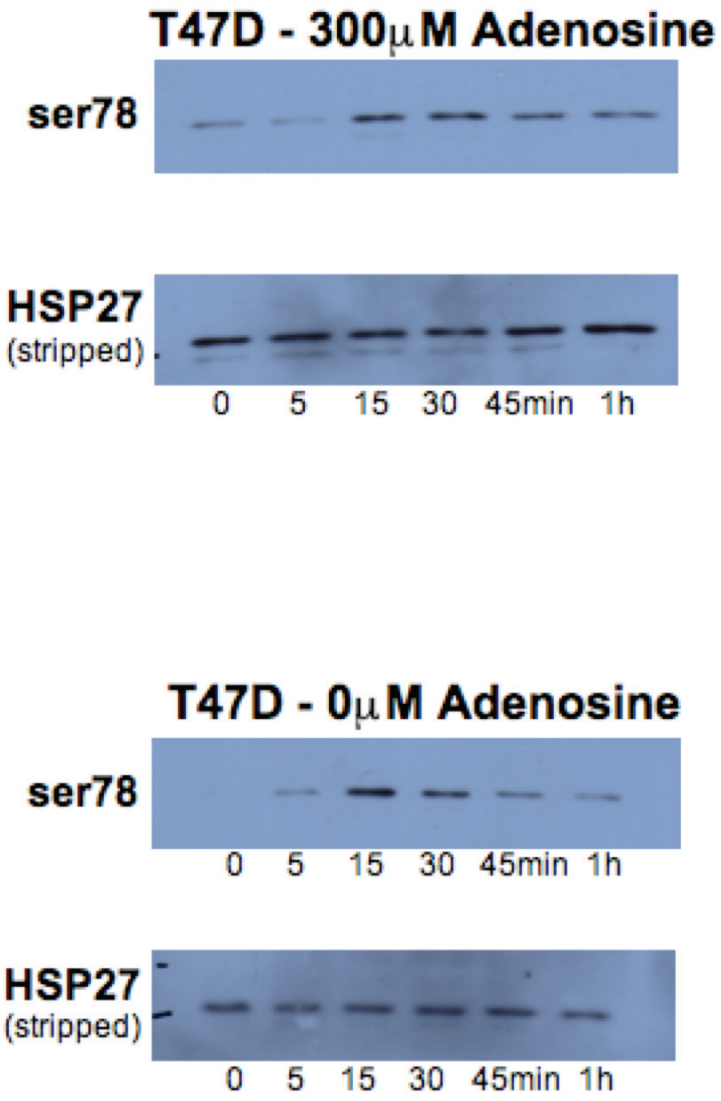


Figure 5.9 Adenosine does not increase phosphorylation of ser78 in T47D cells. Cells were treated with 0μM or 300μM adenosine over 1h. Protein was collected and resolved by separation using SDS-PAGE, transferred to nitrocellulose and probed for ser78 phosphorylation and then stripped and probed for HSP27. HSP27 served as the loading control. These blots are from a representative experiment of three independent experiments.

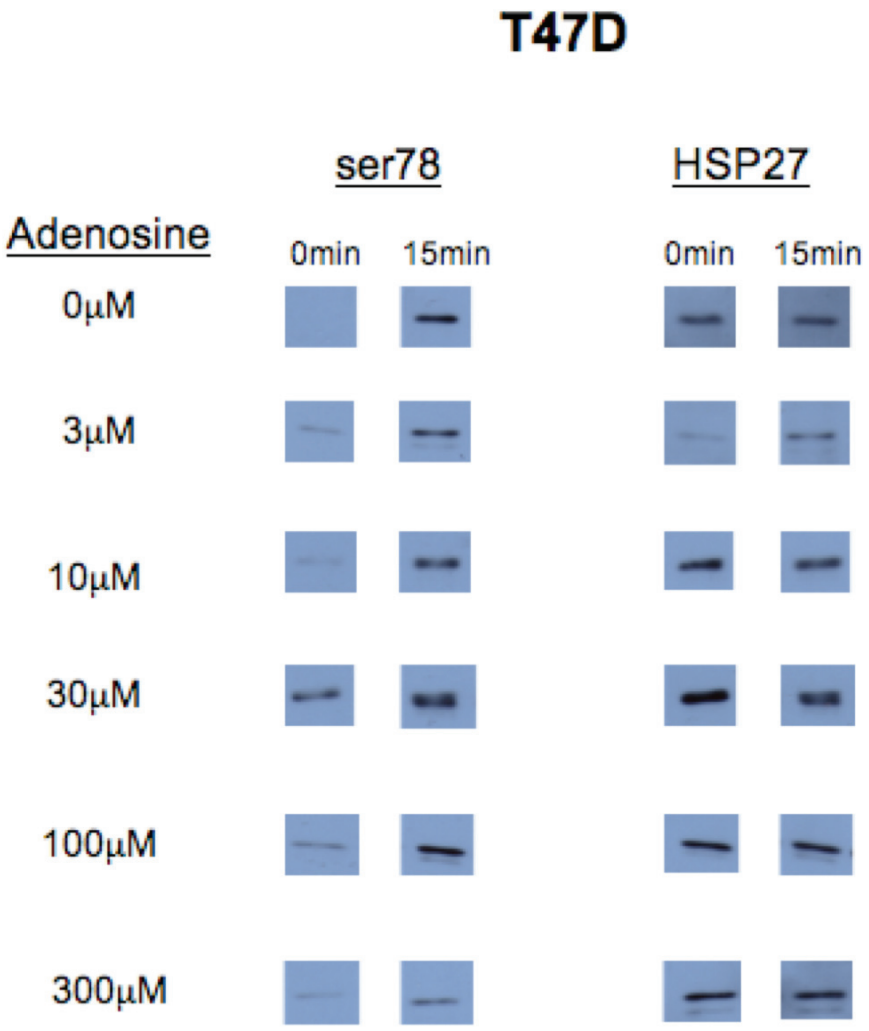


Figure 5.10 HSP27 is not phosphorylated at ser78 in T47D cells exposed to adenosine.
Different concentrations of adenosine (0-300μM) were given to cells over a 1h time course. Protein was collected and separated by SDS-PAGE, transferred to nitrocellulose and probed for ser78 and then stripped and probed for HSP27.

with 20μM of either agonist for up to 2h and ser78 phosphorylation appeared, by visual inspection, to increase in response to NECA and R-PIA with a maximal effect at 15min, a result reproduced independently 4 times. However, the same response profile was evident for the vehicle control, which included 0.02% (v/v) dimethyl sulfoxide (DMSO), the organic solvent in which NECA and R-PIA were dissolved. There was sufficient experimental variation within the 4 experiments that the results were not statistically significance (Figure 5.11). Ser78 phosphorylation of HSP27 in MCF-7 cells increased in response to NECA and the DMSO control (Figure 5.12).

I used immunostaining to determine if there was a change in the localization of phosphorylated HSP27. T47D cells were treated in duplicate with 300μM adenosine for 0, 5, 15, and 30min. At 0min phosphorylated HSP27 localized mainly to perinuclear regions. At 5 and 15min phosphorylated HSP27 in the perinuclear region became less evident, but after 30min HSP27 was again more evident in the perinuclear region (Figure 5.13). These results were not replicated but the observations suggest an adenosine-induced change in the pattern of HSP27 phosphorylation.

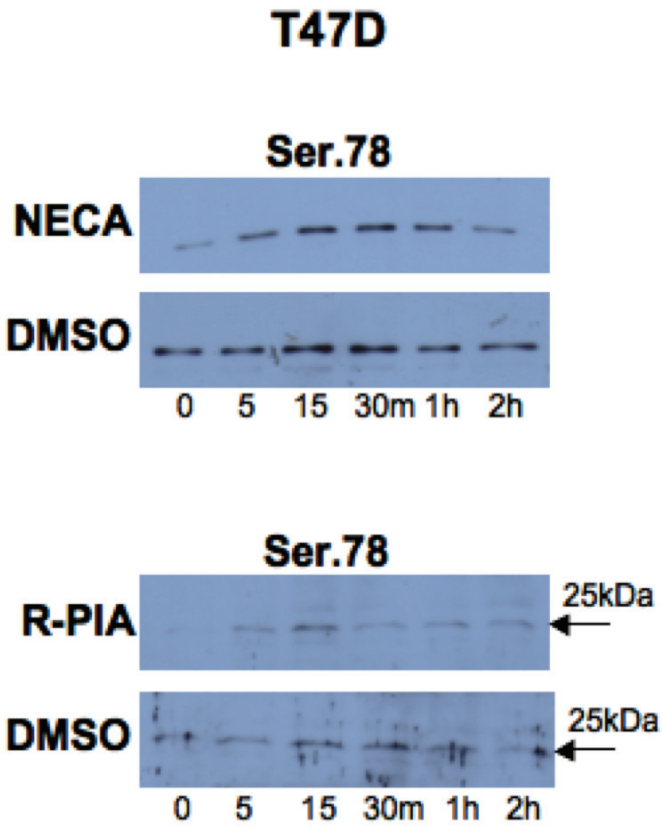


Figure 5.11 HSP27 in T47D cells is not phosphorylated at ser78 in response to adenosine agonists.
T47D cells were treated with NECA and R-PIA (20μM) or a vehicle control (DMSO) over 2h. Protein was collected and resolved by separation on SDS polyacrylamide gels, blotted to nitrocellulose and probed for ser78 and then stripped, and probed for HSP27 (refer to Figure 5.7B) which served as the loading control. The blots show representive findings.

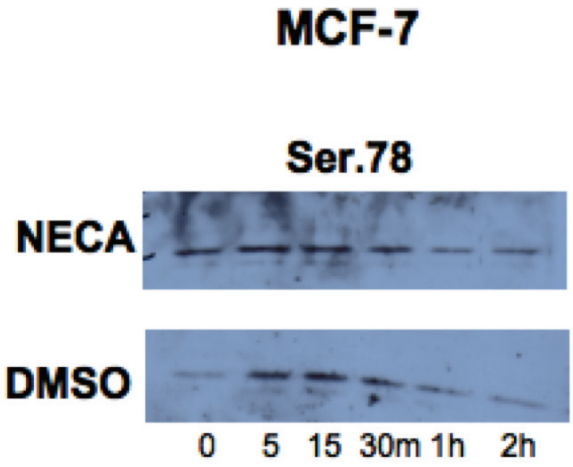
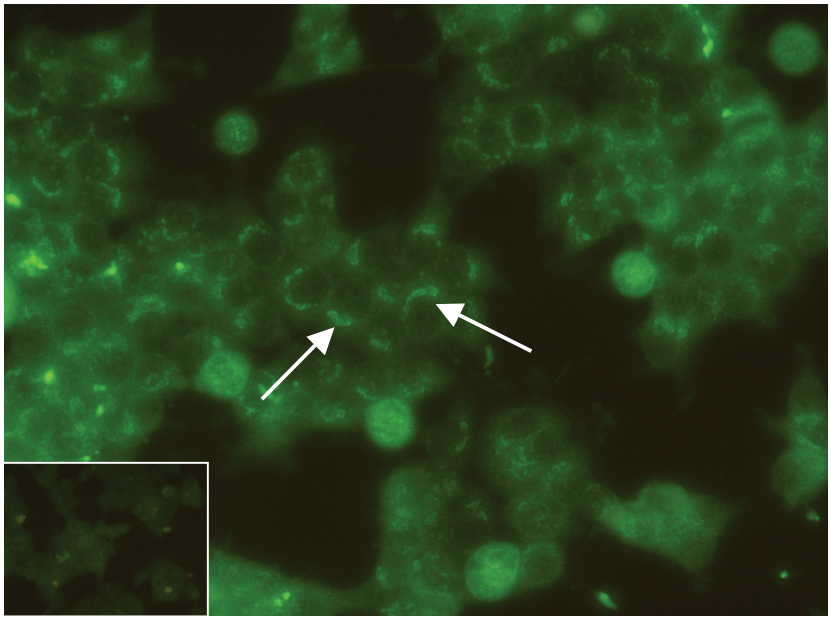
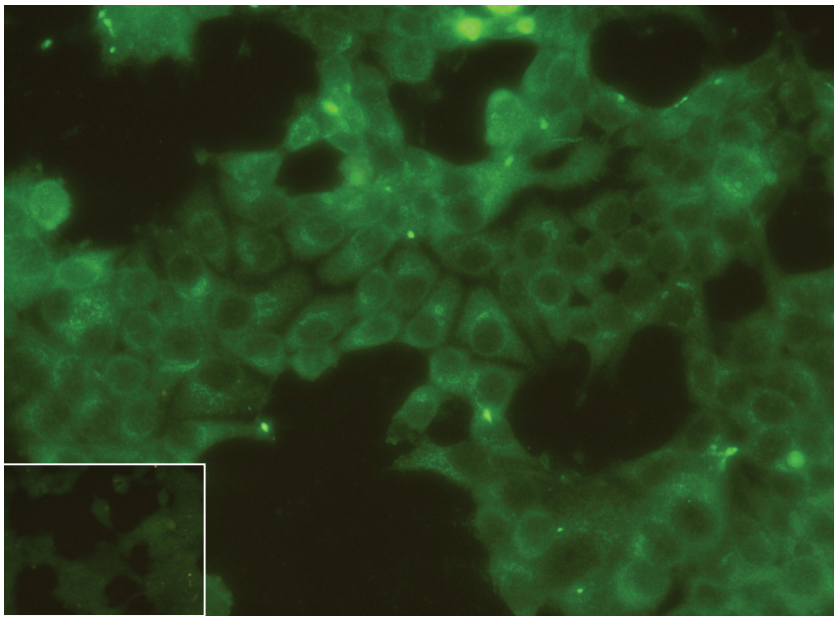


Figure 5.12 The adenosine agonist NECA does not increase HSP27 phosphorylation at ser78 in MCF-7 cells.
Cells were treated with NECA (20μM) or a vehicle control (DMSO) over 2h. Protein was collected and resolved by separation using SDS-PAGE, transferred to nitrocellulose and probed for ser78 and then stripped and probed for HSP27 (refer to Figure 5.7B) which served as the loading control. The blots show representive findings.

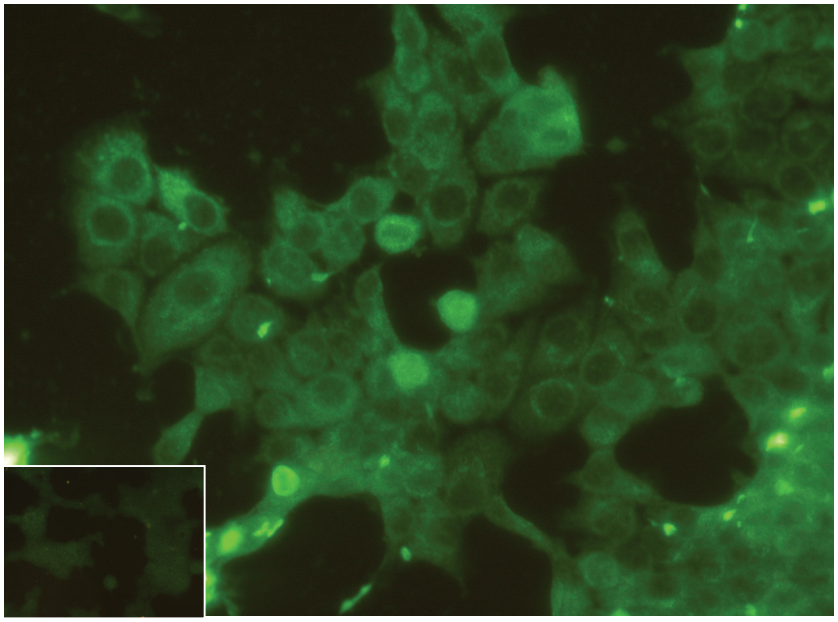
A)



B)



C)



D)

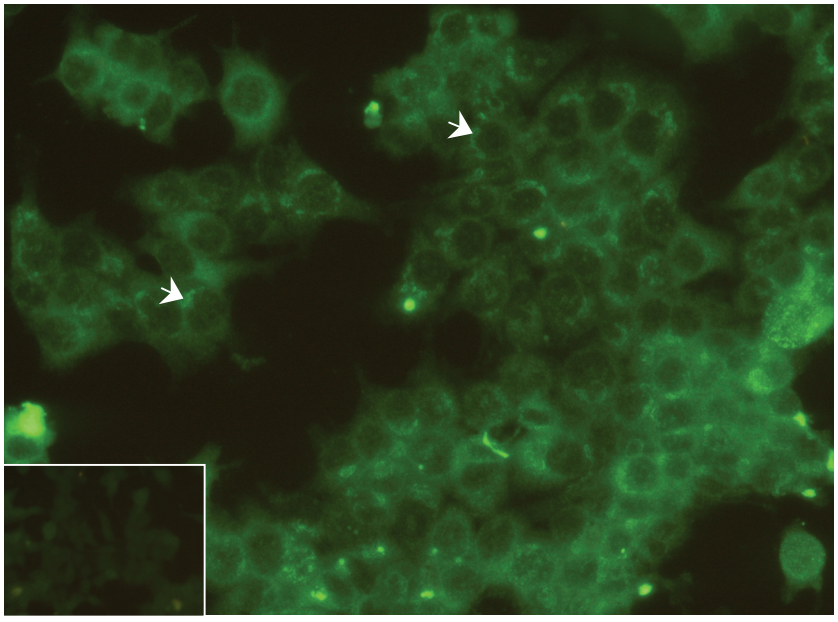


Figure 5.13 The localization of phosphorylated HSP27 in T47D cells changes in response to adenosine.
Cells grown in an 8-well chambered slide were treated with 300μM of adenosine for 5, 15 or 30min (panels B, C and D respectively). Wells were then probed with a primary antibody against phospho-HSP27 ser78 or an anti-rabbit negative control. In untreated cells (panel A) HSP27 phosphorylated at the ser78 site localized to the perinuclear region (arrows), a distribution that partially returned at the 30min timepoint (panel D, arrowheads). The boxes in the lower left hand corners of each picture are the negative controls.

5.3 Prolonged Exposure to Adenosine Increased ser78 Phosphorylation

Although there was no HSP27 phosphorylation at ser78 in response to acute adenosine treatment I found that prolonged exposures to adenosine dramatically increased HSP27 phosphorylation. These data were obtained during HSP27 siRNA knockdown experiments (section 5.4). Immunoblots of control samples for siRNA experiments, which did not have HSP27 knockdown, showed that the addition of 300μM adenosine increased ser78 phosphorylation at 24 and 48h. Ser78 phosphorylation due to adenosine increased 2.5 -fold after 24h of treatment, remained above 2-fold after 48h and declined thereafter (Figure 5.14).

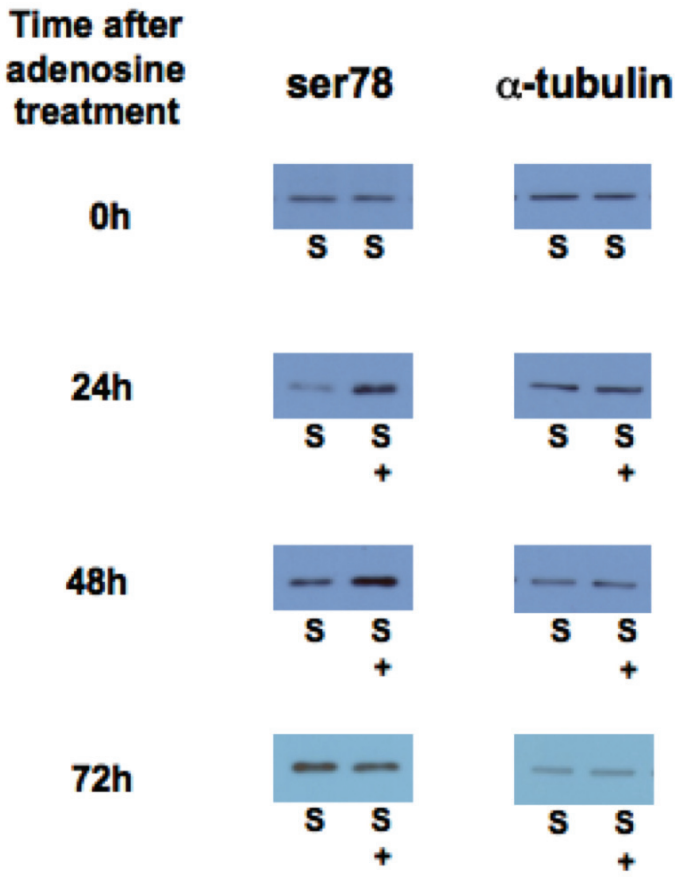


Figure 5.14 HSP27 is phosphorylated at ser78 in response to prolonged adenosine exposure.
In siRNA experiments control cells (S = scramble) were treated with adenosine at 300μM (S+) or 15μl of SF-DMEM. Protein was collected and separated by SDS-PAGE, blotted to nitrocellulose and probed for ser78 and then α-tubulin which served as a loading control. These blots are from a representative experiment of three independent experiments.

5.4 HSP27 is Not Required for Adenosine to Increase CXCR4

To test the hypothesis that adenosine increases CXCR4 and sensing of the stressful tumour microenvironment by way of HSP27 several transfection methods were used to knockdown HSP27 in MCF-7 and T47D cell lines. Using a reverse transfection method in which cells were seeded in the presence of Amine® transfection agent and siRNA simultaneously, HSP27 knockdown was obtained in T47D cells (Figure 5.15A). Both GAPDH and HSP27 were selectively knocked down with their respective siRNAs whereas neither the Amine® transfection agent nor Amine® plus the scramble siRNA affected HSP27 and GAPDH (Figure 5.15A). Attempts at HSP27 knockdown in MCF-7 cells were unsuccessful (Figure 5.16).

HSP27 knockdown in T47D cells was apparent 48h after seeding the cells and it persisted for at least 96h when 2.5μl of Amine® was used. In contrast to the manufacturers instructions indicating that knockdown would likely occur between 8-72h, the knockdown of GAPDH was not complete until 96h after transfection. α-tubulin, used as a loading control for western blots, showed no variation. However, after 72h and 96h α-tubulin increased in cells treated with siRNA against GAPDH, HSP27 and the scramble as compared to samples treated with Amine® only (Figure 5.15); this was therefore a non-specific effect of the siRNA.

Simultaneous binding assays for cell-surface CXCR4 protein were conducted to determine the effect of HSP27 knockdown on CXCR4 expression. The substantial depletion of HSP27 had no significant effect on CXCR4 at any time point (Figure 5.15B).

Although 2.5μl of Amine® was within the range suggested by the manufacturer substantial cell death occurred. Cell counts showed that 2.5μl of Amine® was cytotoxic

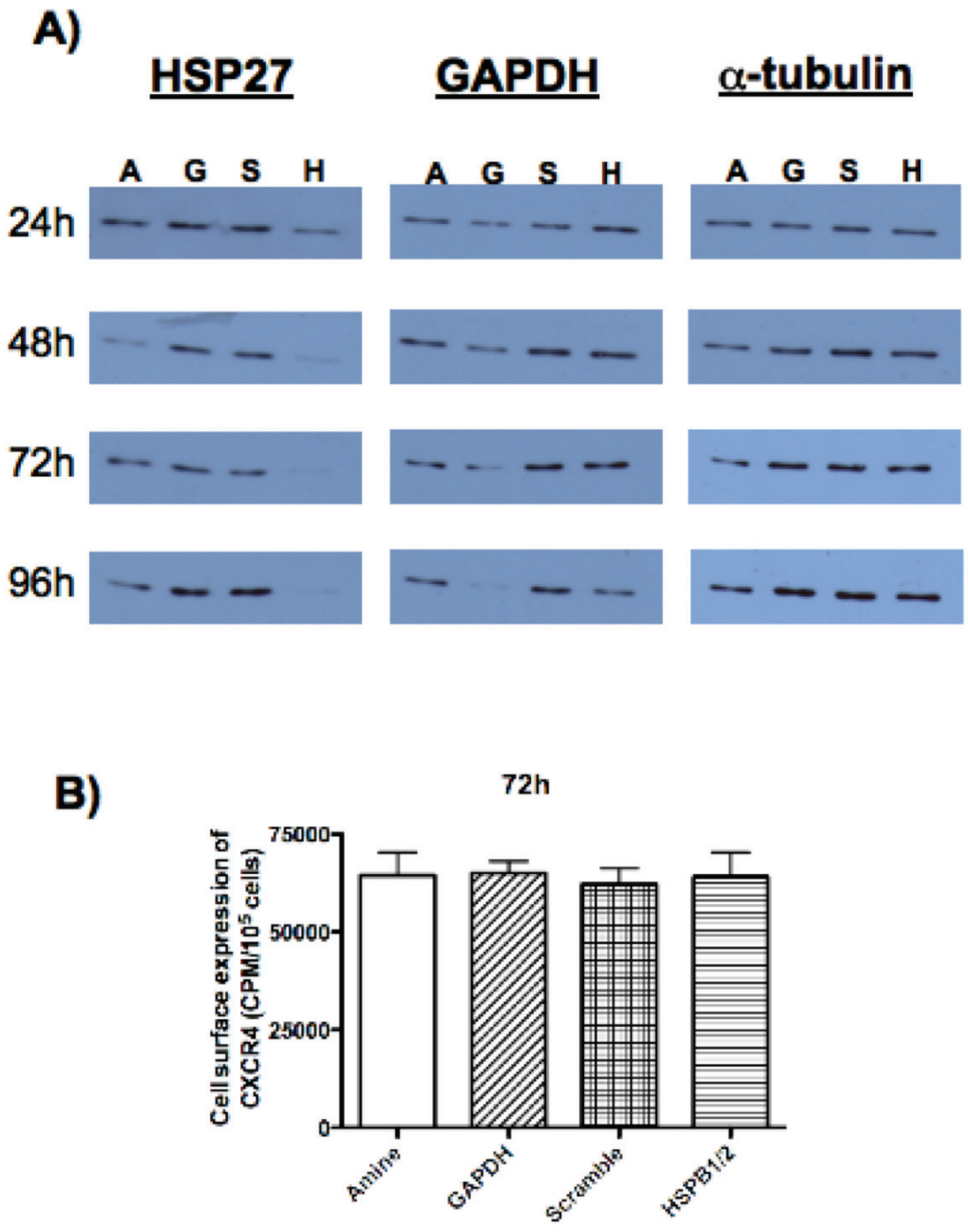


Figure 5.15 Knockdown of HSP27 in T47D cells and its effect on CXCR4 cell-surface expression.
Cells were treated individually with 1.5µl Amine only (A), siRNA against GAPDH (G), scramble siRNA (S), and a combination of four HSP27 siRNAs (H). A) Protein samples were collected 24h, 48h, 72h and 96h after transfection and were resolved by SDS-PAGE, blotted to nitrocellulose and probed for HSP27, GAPDH and α-tubulin. B) Binding assays for CXCR4 were done simultaneously (experiment n=1, in experiment n=4, bars show mean ± SE).

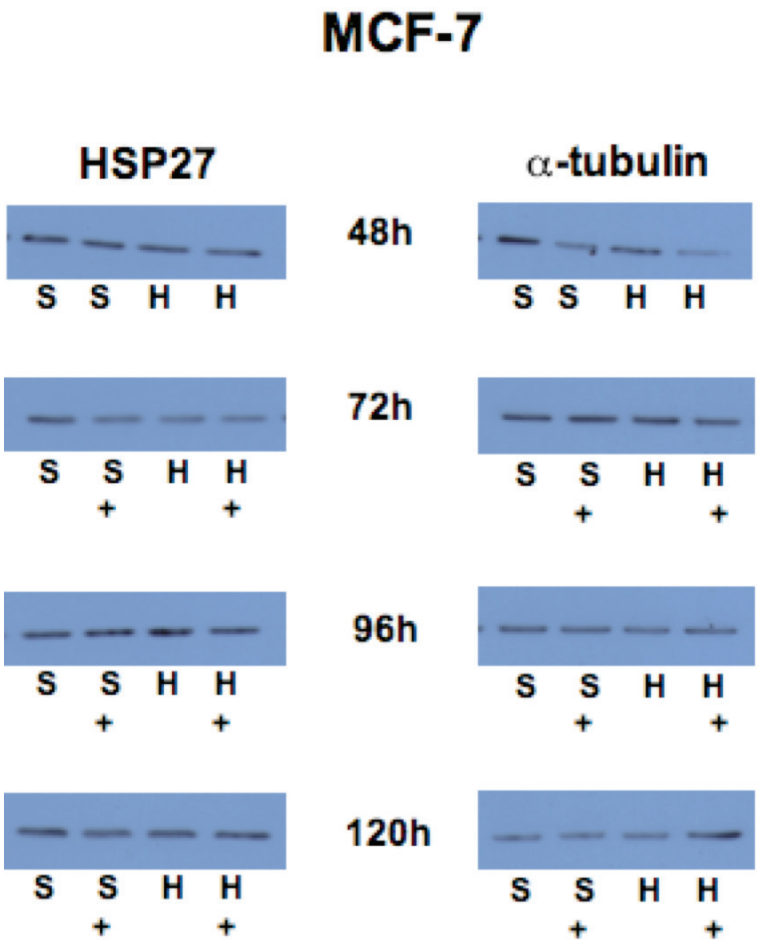


Figure 5.16 siRNA knockdown of HSP27 was not achieved in MCF-7 cells.
MCF-7 cells were transfected with a combination of siRNA against HSP27 using 1.5µl of Amine. Cells were downshifted to medium with a lower (1%) NCS concentration after 24h and at 48h were treated with 300µM adenosine 3 times at 12h intervals over a 72h period. Protein samples collected over the entire 120h time course were separated by SDS-PAGE, transferred to nitrocellulose and probed for HSP27 and then α-tubulin.

24h after seeding and that these effects were still evident 48h post-transfection after changing the cells to fresh medium (Figure 5.17A). Furthermore, probing of western blots showed that 2μl and 2.5μl of Amine® increased HSP27, likely due to chemical stress (Figure 5.17B). To maintain a balance between minimal cytotoxicity and effective knockdown I chose 1.5μl of Amine® for subsequent siRNA experiments. This amount of Amine® neither increased HSP27 nor significantly depressed cell counts compared to untreated controls (Figure 5.17).

Finding no change in CXCR4 levels in response to HSP27 knockdown, I nevertheless wanted to determine if HSP27 knockdown affected the increase of CXCR4 that occurs in response to adenosine. An adenosine effect on CXCR4 was achieved by successively treating cells three times at 12h intervals with 300μM of adenosine, with the first exposure 48h after seeding. In three separate experiments (combined data shown in (Figure 5.18) the elimination of HSP27 by knockdown had no effect on the response of CXCR4 to adenosine. In these same experiments the knockdown of HSP27 was confirmed by western blotting (Figure 5.19) and was typically ~95% after 96h (four independent experiments).

From these blots I determined that adenosine did not increase HSP27 abundance at any time after treatment, as shown earlier (Figure 5.3). However, as mentioned in section 5.3, when these blots were stripped and probed with the antibody to p-ser78 phosphorylation occurred in response to adenosine. In HSP27 knockdowns there was too little HSP27 present to determine if adenosine increased phosphorylation (Figure 5.20).

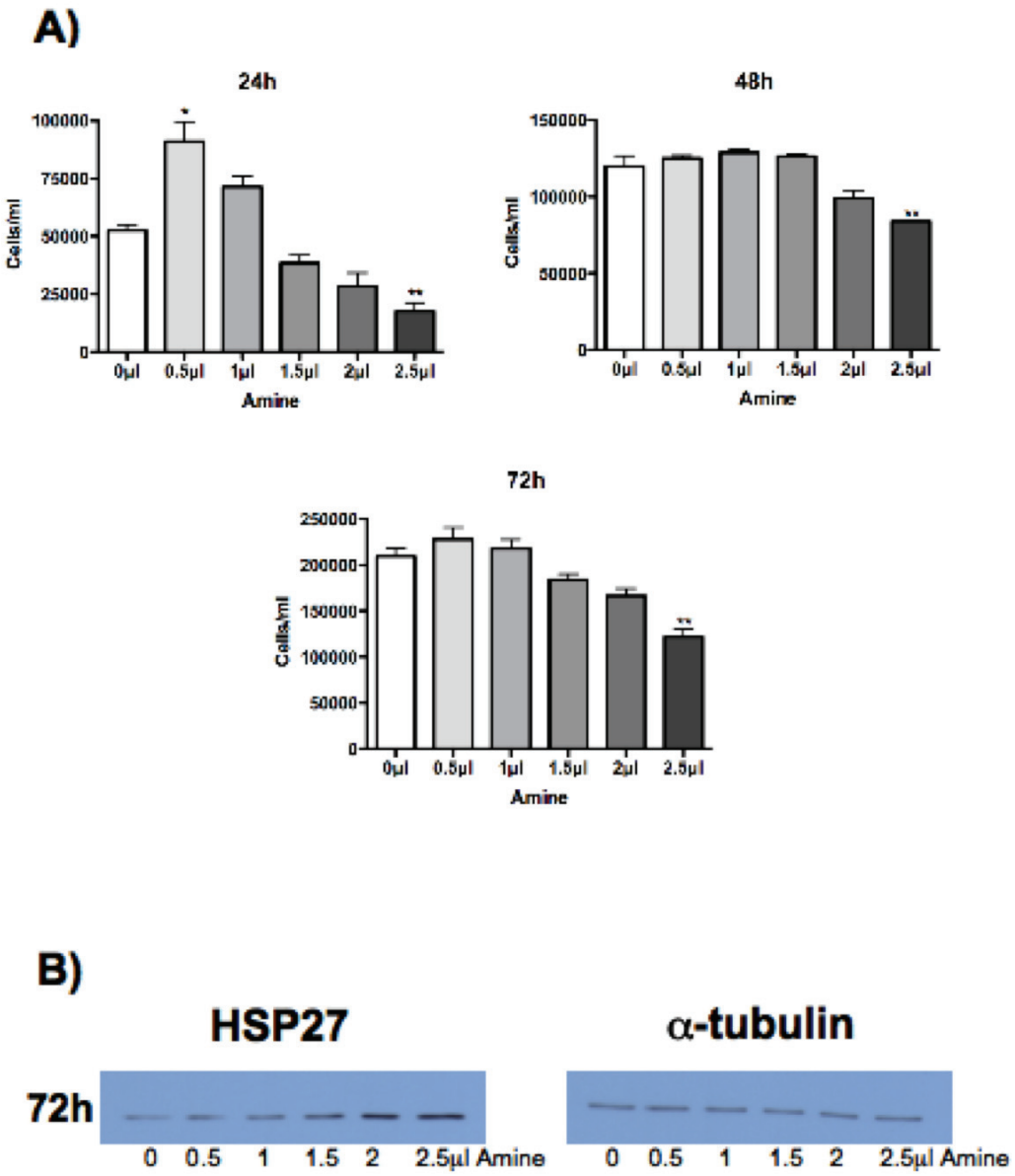


Figure 5.17 High amounts of Amine reagent were cytotoxic and elicited an HSP27 stress response.
T47D cells were treated with different amounts of Amine for 24, 48 and 72h. A) Cells were trypsonized and counted at each time point. B) Protein samples were collected and resolved by separation on SDS polyacrylamide gels, blotted to nitrocellulose and probed for HSP27 (experiment n=1, in experiment n=3, bars show mean ± SE). Differences to control are $p<0.05$ (*) and $p<0.01$ (**).

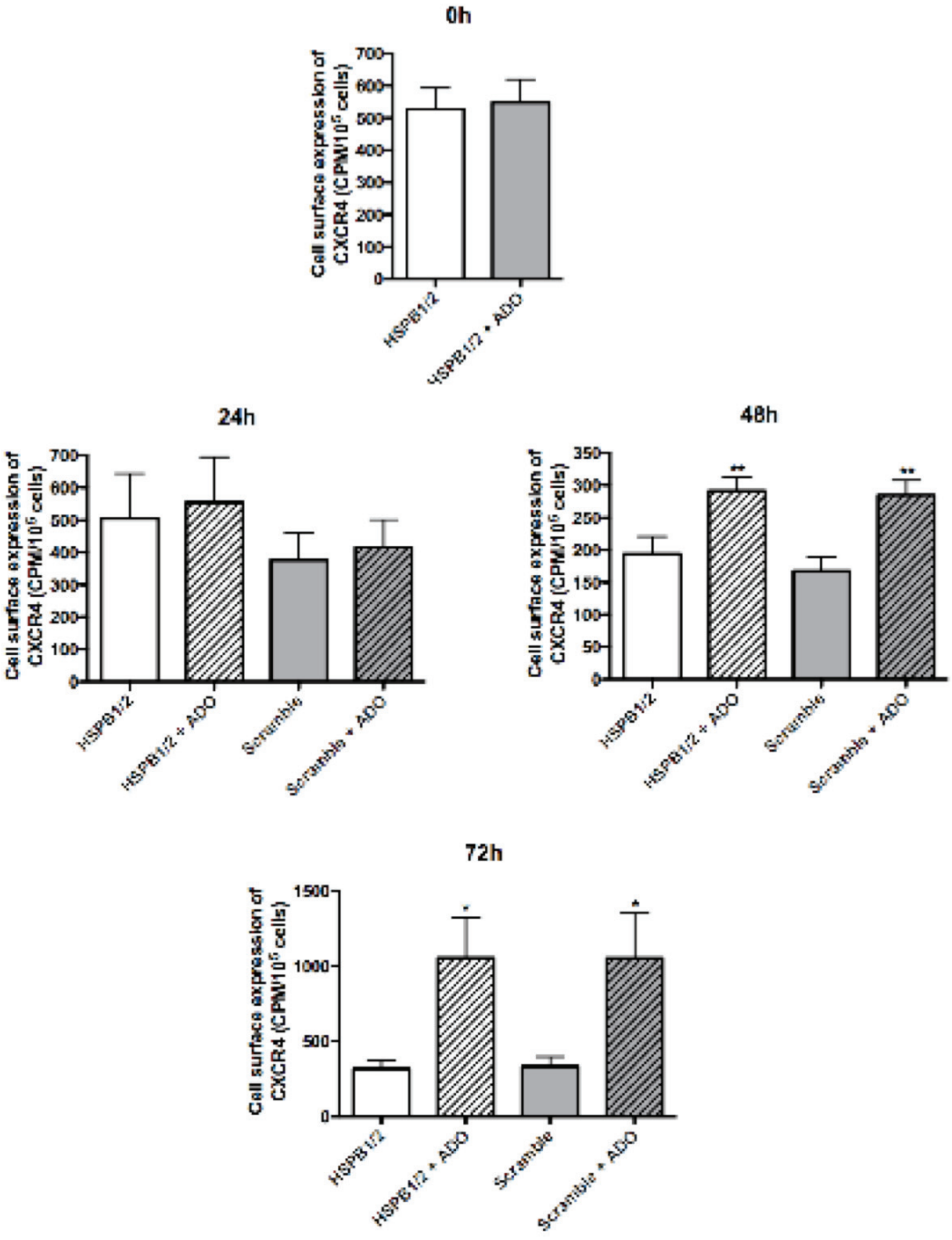


Figure 5.18 HSP27 is not involved in the upregulation of CXCR4 cell-surface expression in response to adenosine in T47D cells.
Using 1.5µl of Amine transfection agent, cells were reverse transfected with siRNA against HSP27 (HSPB1/2) or a negative control (Scramble) and 48h after seeding (time 0h), were treated with 0µM or 300µM of adenosine 3 times at 12h intervals. Binding assays for CXCR4 were done before treatment (0h), and 24-72h after treatment (in each experiment, n=4, bars show mean ± SE). Differences to control (HSP1/2 or Scramble) are $p<0.05$ (*) and $p<0.01$ (**)

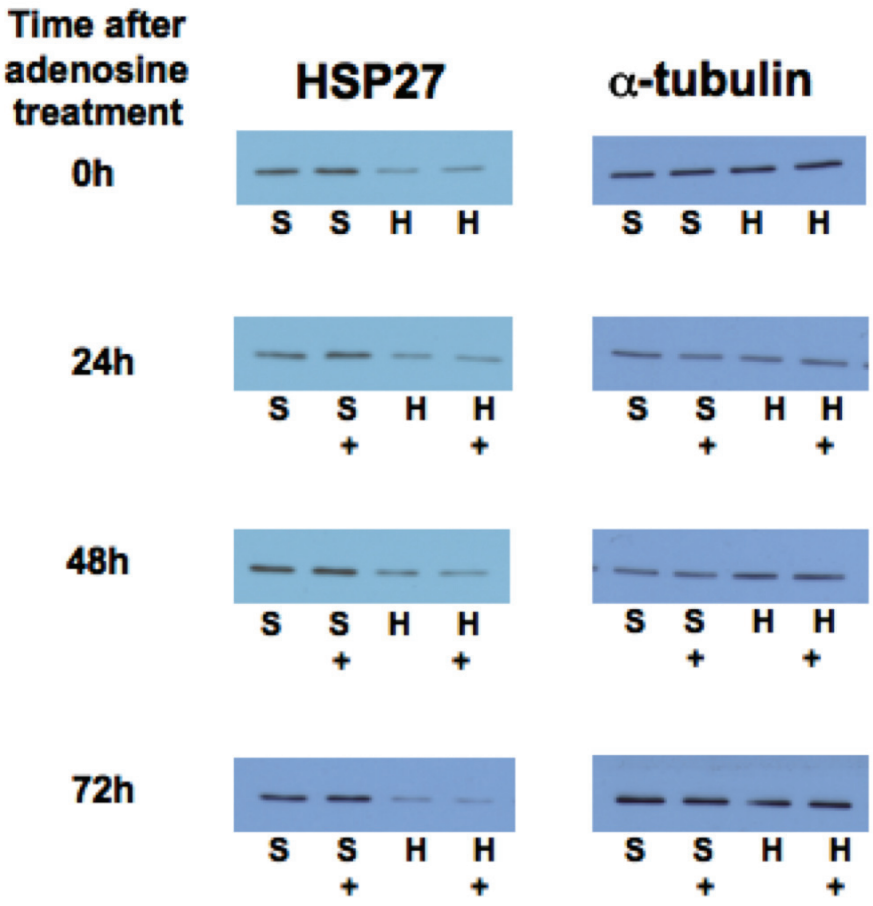


Figure 5.19 HSP27 knockdown in T47D cells.
Cells were reverse transfected with siRNA against HSP27 (H) or a negative control (S) and 48h after seeding (time 0h), were treated with 0μM or 300μM (+) of adenosine 3 times at 12h intervals. Protein samples were collected over 120h and separated by SDS-PAGE, blotted to nitrocellulose and probed for HSP27. These blots are from a representative experiment of three independent experiments.

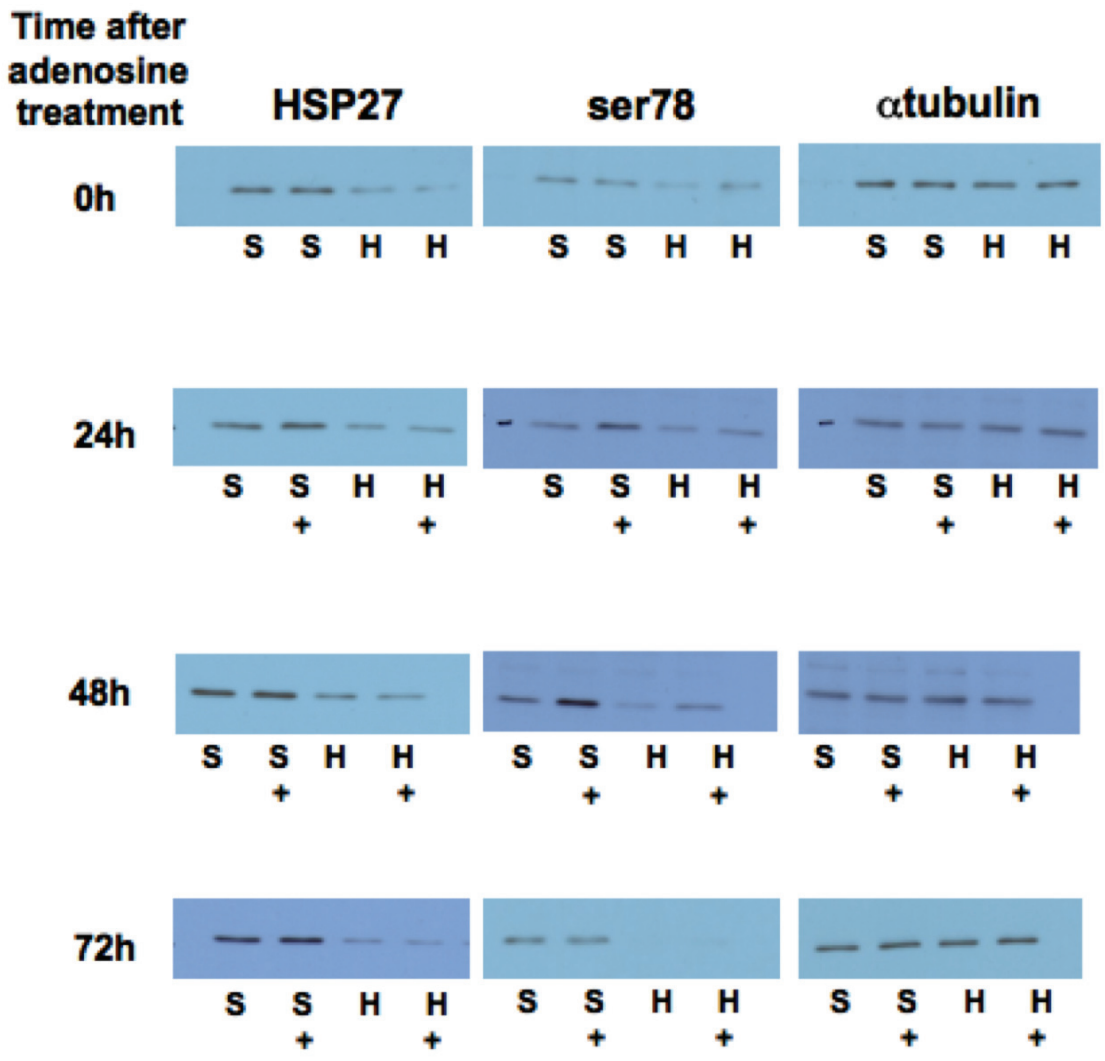


Figure 5.20 Phosphorylation of ser78 increased upon prolonged exposure to adenosine.
T47D cells were reverse transfected with siRNA against HSP27 (H) or a negative control (S) and 48h after seeding (time 0h), were treated with 0μM or 300μM (+) of adenosine 3 times at 12h intervals Protein samples were collected over 120h and by SDS-PAGE, transferred to nitrocellulose and probed for ser78. These blots are from a representative experiment of three independent experiments.

5.5 Phosphorylations of HSP27 at ser78 and 82 are Similar

Although the clearest results were obtained for HSP27 ser78 phosphorylation, the same pattern of phosphorylation occurred at ser82 in acute experiments with adenosine, NECA and R-PIA (Figure 5.21). However as with ser78, phosphorylation of ser82 occurred in control samples in response to handling masking any phosphorylation that occurred in response to adenosine and its analogues. I saw late phosphorylation of ser82 in the knockdown experiments in response to adenosine (Figure 5.22). The results were however compromised by the poor quality of the antibody (Millipore™ polyclonal IgG Cat# 07-646).

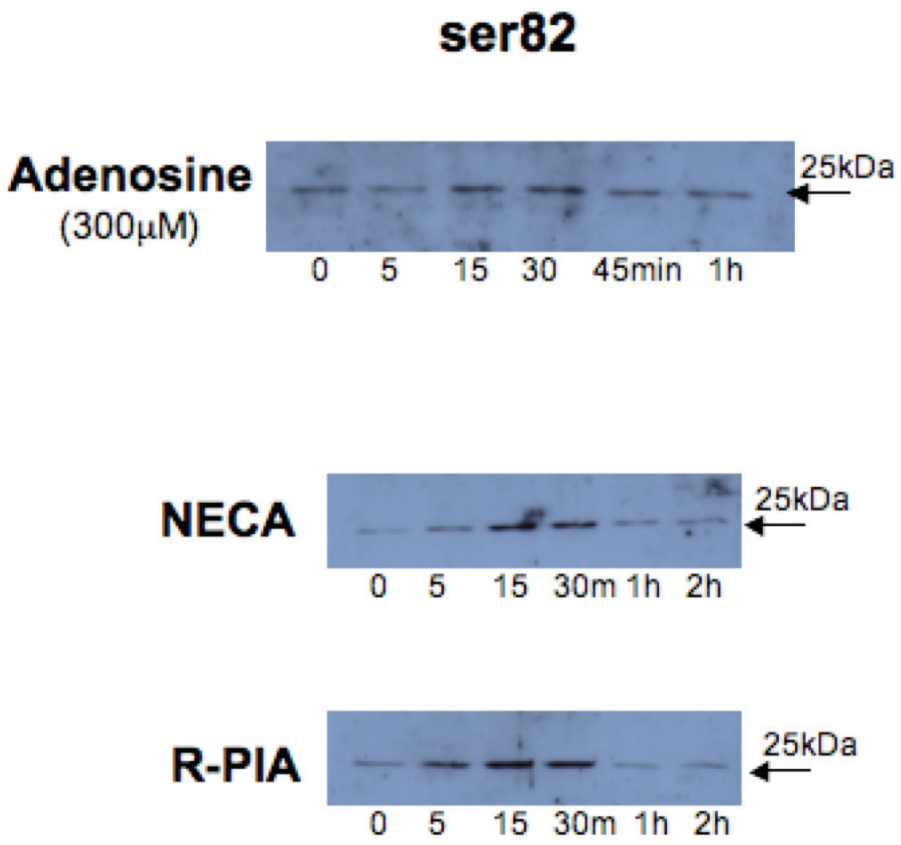


Figure 5.21 Ser82 of HSP27 is phosphorylated in a similar manner to ser78 in T47D cells.
Cells were treated with adenosine (300μM) over 1h, and NECA and R-PIA (20μM) over 2h. Protein samples were collected and resolved by separation on SDS polyacrylamide gels, blotted to nitrocellulose and probed for HSP27 ser78, stripped, and then re-probed for ser82.

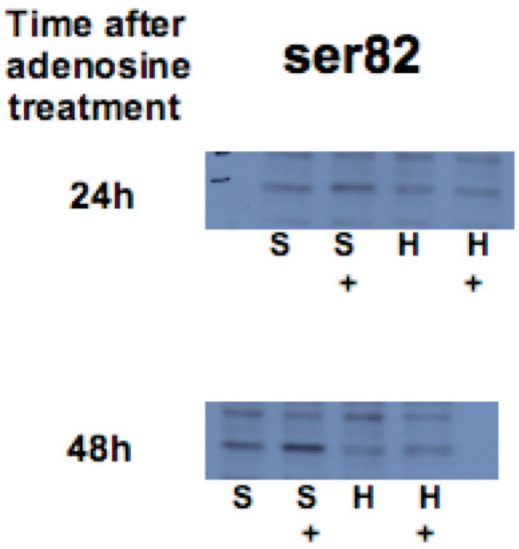


Figure 5.22 Phosphorylation of ser82 after prolonged exposure to adenosine.
T47D cells were reverse transfected with siRNA against HSP27 (H) or a negative control (S) and 48h after seeding (time 0h), were treated with 0μM or 300μM (+) of adenosine 3 times at 12h intervals Protein samples were collected over 120h and separated by SDS-PAGE, blotted to nitrocellulose and probed for ser82.

CHAPTER 6: DISCUSSION

6.1 HSP27 Increase in Response to Heat Shock and Hypoxia

MCF-7 and T47D cells responded to stress by increasing HSP27. To test HSP27 induction by heat shock in MCF-7 and T47D, cells were initially exposed to 44°C for up to 6h. This is consistent with the usual practice reported in the literature where most heat shock experiments are conducted between 43° and 45°C (Arrigo et al., 1988; Landry et al., 1982; Lavoie et al., 1993a; Lee et al., 1997; Li and Werb, 1982). Most heat shock studies relate to the acquisition of thermotolerance where cells are first briefly exposed to heat for 20 – 60 min, allowed to recover at 37°C for 2-12h, and then heated again to see if they survive. Landry and colleagues (1982) showed that after 0.5h at 43°C many HSPs such as HSP27, 65, and 70 were induced and persisted at high levels for several hours after cessation of heat shock. Cells became more thermoresistant with a longer post-incubation time at 37°C. Thermotolerance was fully induced after 8h and lasted 2-3 days. (Arrigo et al., 1988; Landry et al., 1982; Lavoie et al., 1993a; Lee et al., 1997; Li and Werb, 1982). HSP27 is a key molecule in thermotolerance and it increases several-fold after heat shock (Landry et al., 1989; Landry et al., 1991).

In the tumour microenvironment cells are constantly under stress, so my experiments were designed to mimic chronic stress by using up to 48h of heat shock, rather than acute thermal stress. In both T47D and MCF-7 cells there was an increase of HSP27 with a maximal effect reached by 1h, but no change seen before 0.5h, in response to heat. This is consistent with reports in the literature (Landry et al., 1989; Landry et al.,

1991). Chinese hamster lung fibroblast cells, O23, were exposed to 20min of heat shock (44°C) which increased HSP27 beginning 1h after treatment and lasting several hours (Landry et al., 1989; Landry et al., 1991).

Hypoxia increases HSP27 abundance (Baird et al., 2006; Headrick and Willis, 1989; Whitlock et al., 2005). One of my main objectives was to determine if some of the effects of hypoxia on HSP27 are attributable to adenosine, which is elevated in response to hypoxia (Headrick and Willis, 1989). I first explored what effect hypoxia had on HSP27 in the MCF-7 and T47D cells. For hypoxia treatments cells were exposed to 1% O_2 in keeping with most experiments that use 1-2% O_2 (8-16mmHg) (Graham et al., 1998; Palmer et al., 1998; Pichiule et al., 2004). The increase in HSP27 when oxygen was reduced to 1% from the normal of ~20% was consistent with the cellular stress response seen in the heat shock experiments in both cell lines. Constantly exposing cells to 1% O_2 mimics chronic hypoxia which occurs in the tumour microenvironment as a result of poor tumour vasculature (Brown and Giaccia, 1998; Vaupel et al., 1989b). This approach is appropriate for investigations of other hypoxia-regulated proteins and it was used by Pichiule and coworkers (2004) where the effect of hypoxia on Ang2 was investigated by exposing HUVECs to hypoxic conditions for 3-24h (Pichiule et al., 2004).

6.2 Adenosine Has No Effect on HSP27 Abundance

Adenosine is found at high levels in the tumour microenvironment due to hypoxic conditions (Blay et al., 1997) and it plays an important role in the protective response of tissue against ischemia. Adenosine 'preconditions' myocardial tissue so that cells are more tolerant of subsequent ischemic insults (Baxter et al., 1994; Liu et al., 1991). This

result is observed in the well-studied ischemic preconditioning (PC) model where short sublethal exposure of cells to ischemia confers tolerance to later ischemic insults. *In vivo* studies by Liu and colleagues (1991) on rabbit hearts showed that 5min intracoronary infusion of adenosine or a stable adenosine analogue (R-PIA) was as protective as 5min of ischemic preconditioning. Adenosine pretreatment reduced the size of infarcts that resulted from subsequent ischemia. These researchers found that inhibition of adenosine receptors by antagonists prevented the protective effects of ischemic PC. The molecular mechanisms by which adenosine mediates tolerance are however not completely known, so perhaps another protein such as HSP27, which is involved in preconditioning, could play a role.

It is well established that HPS27 expression is induced by a plethora of stresses and that this provides cytoprotection. Overexpression of HSP27 produces cellular resistance to heat shock (Landry et al., 1989), chemicals (Lavoie et al., 1993a), and oxidative stress (Mehlen et al., 1995). As indicated above, the induction of HSP27 expression confers tolerance to subsequent stress (Landry et al., 1982; Landry et al., 1989; Lee and Dewey, 1988; Li and Werb, 1982) including ischemia (Whitlock et al., 2005). The involvement of HSP27 in ischemia is of particular interest not only in the study of myocardial infarcts but in retinal degenerative diseases (Dana et al., 2000; Whitlock et al., 2005). Li's research group (2003) found that 24-72h after retinal ischemic PC HSP27 expression increased 200% before dropping back to basal levels 120h after PC. In contrast, two other prominent HSPs, HSP70 and 90, showed no consistent increase. An increase in HSP27 protects against subsequent ischemic insults with the most protection afforded 24-72h after pretreatment with $CoCl_2$ which mimics

hypoxia (Whitlock et al., 2005). Cells rendered thermotolerant from brief exposure to heat shock are more resistant to anti-cancer drugs (Ciocca et al., 1992; Jaattela, 1999), for example cells exposed to 2h of non-lethal heat followed by a rest phase of 4h at 37°C were more resistant to a 1h treatment with doxorubicin (Ciocca et al., 1992).

I found no increase in HSP27 abundance at any time up to 48h in response to adenosine concentrations ranging from 0.01 to 1mM. Two adenosine analogues, NECA and R-PIA, failed to induce HSP27 expression which is consistent with a study where an adenosine receptor agonist, 2-chloro-N^6-cyclopentyladenosine (CCPA), was unable to induce the expression of HSP27 in rabbit tissue after 24h (Dana et al., 2000). Lee and coworkers (2007) however, saw an increase in HSP27 expression in immortalized porcine renal tubule cells (LLC-PKI) after 6-8h of CCPA exposure (Lee et al., 2007).

Many studies use adenosine agonists due to the short half life ($t_{1/2}$ ~1s) of adenosine, particularly in whole animal models (Moser et al., 1989). In the cell monolayer model used in these experiments however, the degradation of adenosine is much slower (Figure 4.1, (Mujoomdar et al., 2003). Experimentally, it is better to use adenosine rather than its synthetic analogues, which sometimes act differently than adenosine (Colquhoun and Newsholme, 1997). While adenosine stimulates all four adenosine receptor subtypes at appropriate concentrations, its analogues may be selective for just one or two receptor subtypes (Merighi et al., 2001). Similarly, problems may be encountered when endogenous adenosine levels are modulated by the addition of ADA inhibitors (Sandberg, 1983) as ADA signals through binding to A1R and A2bR adenosine receptors (Ciruela et al., 1996; Herrera et al., 2001; Saura et al., 1996). Employing adenosine at 300μM avoided the use of ADA inhibitors in my experiments,

and this concentration does not lead to cytotoxicity in most cancer cell lines (Tan et al., 2004).

Extracellular levels of the adenosine metabolite inosine increase in response to hypoxia and ischemia as a result of adenosine breakdown (Bell et al., 1998; Wang et al., 1994) and inosine induces various cellular responses by binding adenosine receptors (Hasko et al., 2000; Jin et al., 1997). Inosine however did not have any affect on HSP27 in my experiments. This is consistent with other findings such as the increase of CXCR4 or the downregulation of CD26 in colorectal carcinoma cells (Richard et al., 2006; Tan et al., 2004) and they show that the effects of adenosine are not the result of its breakdown to inosine. Some effects of adenosine are produced through its metabolites ATP, ADP and AMP. ATP and AMP stimulate DNA synthesis, which was partially repressed by a 5'NT inhibitor (Mujoomdar et al., 2003). This suggests that while adenine nucleotides evoke cellular responses through conversion to adenosine, they may do so without being dephosphorylated, for example by ATP binding to P2-purinergic receptors (Hopfner 1998).

6.3 Adenosine Does Not Induce Acute Phosphorylation of HSP27

Phosphorylation of HSP27 mediates cell functions such as migration and cytoprotection, and is induced by multiple stimuli (Hedges et al., 1999; Huot et al., 1996; Landry et al., 1991; Mounier and Arrigo, 2002; Rousseau et al., 1997). After determining that adenosine did not increase HSP27 abundance, I tested if adenosine signaling leads to the phosphorylation of HSP27. HSP27 is phosphorylated primarily by MK2 which is activated by p38 MAPK (Kostenko and Moens, 2009). Adenosine receptors are upstream

stimulators of MAPKs, and several studies confirm that adenosine induces the phosphorylation of p38 MAPK (Carini et al., 2001; Dana et al., 2000; Feoktistov et al., 1999).

A 1h time course was initially chosen for the phosphorylation studies, this based on the extensive literature that shows very quick phosphorylation of HSP27, sometimes in less than 5min, and then decrease to basal levels within 1h (Akamatsu et al., 2004; Nakajima et al., 2005). This time course is typical of protein phosphorylation events in signaling pathways of mammalian cells. The pattern of ser78 phosphorylation that I first saw, namely an increase in phosphorylation starting at 5min, reaching a maximal at 15-30min and then decreasing, was consistent with results in the literature. Phosphorylation of HSP27 typically increases dramatically in the first 20min of stress exposure (Landry et al., 1989; Landry et al., 1991). The pattern of phosphorylation in my experiments was very similar to the finding that vasopressin increased the phosphorylation of rat HSP27 as early as 2min, with a maximal effect at 20-45min, and a decrease by 60min in aortic smooth muscle cells (Akamatsu et al., 2004). Similar phosphorylation patterns of rat HSP27 were seen in response to thrombin (Nakajima et al., 2005).

However, the increase in HSP27 ser78 phosphorylation in control cells, as well as in cultures with adenosine or its analogues, indicated that HSP27 was stimulated by something other than the drug treatments. We hypothesized that during the 24-48h growth period of the cultures prior to treatment, a gradient of nutrients and other factors is created in the unstirred medium above the cell monolayer. For instance, in close proximity to the monolayer of metabolically-active cells, glucose decreases while lactate levels and adenosine increase. When fresh medium is added during treatment, cells are

suddenly exposed to a rush of glucose, the lactate levels decrease and the medium pH returns to normal from ~7.0 to ~ 7.4. This may result in cellular stress and stimulate the substantial increase in 'background' HSP27 phosphorylation observed. Perhaps a similar effect occurred in other studies with HSP27 phosphorylation due to the manipulation of cell cultures rather than the stressors themselves. For example, cells were left in SF-DMEM for 48h before treatment and in these papers there is no mention of a simultaneous control (Akamatsu et al., 2004; Nakajima et al., 2005).

6.4 Adenosine Alters the Localization of Phosphorylated HSP27

In the immunohistochemical work I saw a rapid change in the localization of phosphorylated HSP27 from the perinuclear region to the cytoplasm in response to adenosine, even though there was no demonstrable change in HSP27 phosphorylation. The data on localization of HSP27 in unstressed and stressed cells varies among different studies, but localization to the perinuclear region in unstressed cells was demonstrated in HeLa cells (Arrigo et al., 1988). In contrast, Mehlen and coworkers (1994) found that unphosphorylated HSP27 is dispersed in the cytoplasm of HeLa cells but upon serum induction of phosphorylation it redistributes towards the perinuclear region (Mehlen and Arrigo, 1994). Most studies have shown, however, that under normal conditions phosphorylated HSP27 is randomly distributed in the cytoplasm (Lavoie et al., 1993b; Sakamoto et al., 1998). Heat shock usually shifts HSP27 to the perinuclear region and after heat shock of HeLa cells HSP27 localizes within the nucleus (Arrigo et al., 1988). Phosphorylated HSP27 translocates to the nucleus and cytoskeleton after heat shock and

phosphorylated HSP25 in rat cells moves into the nucleus (Geum et al., 2002; Sakamoto et al., 1998).

6.5 Prolonged Exposure to Adenosine Phosphorylates HSP27 on ser78 and ser82

The phosphorylation of HSP27 was examined extensively for an acute response to various stimuli, with no convincing result. While adenosine failed to elicit the immediate phosphorylation of HSP27 I saw a substantial increase in phosphorylation after 24h of adenosine exposure. Phosphorylation of HSP27 occurred 24h after pretreatment of rabbit tissue with an A1R-selective agonist linking the late phosphorylation of HSP27 to the protection of tissue against subsequent ischemia, with the cytoprotection attributed to cytoskeletal stabilization (Dana et al., 2000). A single experiment quantifying the expression of mRNA encoding adenosine receptors using qPCR in T47D cells showed no A1R expression but did show significant amounts of each subtype of A2R. Adenosine signaling through A2aR and A2bR increases CXCR4, a response linked to cell migration (Richard et al., 2006). Adenosine does not appear to cause HSP27 phosphorylation via A1R signaling, but it is possible that the late phosphorylation of HSP27 occurs through A2Rs. For example, pretreating hepatocytes with an A2aR agonist activated p38 MAPK which ultimately leads to HSP27 phosphorylation (Carini et al., 2001).

In my experiments that looked at the long-term effects of adenosine on HSP27 phosphorylation, adenosine levels were maintained by 3 additions of the nucleoside to the cultures, so that there was a constant adenosine exposure acting as a stressor. As indicated in the *Materials and Methods*, this provided a persistent adenosine

concentration greater than 10μM, without reaching cytotoxic concentrations. The maximum concentration of adenosine reached during experiments was no greater than about 400μM.

Prolonged exposure of more than 24h to elevated adenosine levels caused HSP27 phosphorylation on ser78 and ser82, potentially causing small phosphorylated HSP27 oligomers to bind actin filaments (Mounier and Arrigo, 2002), strengthening the cell structure which increases resistance to subsequent stressors. One of the immediate consequences of multiple stresses is the fragmentation of actin filaments. The overexpression of HSP27 in chinese hamster cells protected against stress-induced F-actin fragmentation but a non-phosphorylatable form of HSP27 did not (Huot et al., 1996). Similar observations were made for chinese hamster cells in response to heat shock where overexpression of HSP27, but not a phosphomutant form in which individual or multiple serine residues were replaced with glycines, increased resistance against cytochalasin D, an actin reactive drug (Lavoie et al., 1995). In the tumour microenvironment where adenosine is in high concentrations this could be used by cancer cells to protect against stressors like hypoxia and chemotherapeutic agents.

The late time course of adenosine-induced HSP27 phosphorylation indicates linkage to a late cellular response to this purine nucleoside. One possibility is that HSP27 phosphorylation in response to adenosine is part of the cell migration response. Phosphorylation of HSP27 is crucial for cell migration in many cell types (Kwon et al., 2011; Rousseau et al., 1997) including cancer cells (Guo et al., 2008; Hedges et al., 1999; Piotrowicz et al., 1998; Rust et al., 1999; Shin et al., 2005). The expression of a non-phosphorylatable form of HSP27 slows endothelial cell motility by 40% and

phosphorylated HSP27 promotes the polymerization of microfilaments in lamellipodia (Piotrowicz et al., 1998). In smooth muscle cells migration in response to PDGF, TGF-β, and IL-1β, all known to mediate HSP27 phosphorylation via MK2, is blocked by a p38 inhibitor (SB203580), a p38-MAPK dominant-negative mutant and a HSP27 phosphorylation mutant (Hedges et al., 1999). In metastatic MDA-MB-231 breast cancer cells, blocking HSP27 phosphorylation inhibits migration in a Boyden chamber assay using serum as a chemoattractant (Shin et al., 2005).

Phosphorylated HSP27 dissociates from the ends of actin allowing for actin polymerization (Benndorf et al., 1994; Miron et al., 1991; Mounier and Arrigo, 2002), which may facilitate migration. Adenosine induces migration in melanoma and colorectal carcinoma cells (Richard et al., 2006; Woodhouse et al., 1998). The delayed phosphorylation of HSP27 in response to an adenosine agonist is mediated through PKC/p38-MAPK, the same pathway shown to increase HCC cell motility and invasion (Dana et al., 2000; Guo et al., 2008).

6.6 HSP27 Knockdown Does Not Alter CXCR4 Expression

One way adenosine affects cell migration is through regulation of the chemokine receptor CXCR4 (Richard et al., 2006). However the siRNA experiments I conducted clearly demonstrated that HSP27 does not play a role in maintaining the steady-state cell-surface expression of CXCR4 and is not involved in the adenosine-mediated increase of CXCR4. Although I had shown that adenosine did not increase HSP27 abundance or its acute phosphorylation there were other reasons for investigating a possible link between HSP27 and CXCR4. As mentioned before, HSP27 stabilizes HER2 (Kang et al., 2008), a

protein which upregulates CXCR4 (Li et al., 2004). Since the binding of HSP27 to HER2 stabilizes the receptor, it was thought that perhaps HSP27 does the same with CXCR4.

Adenosine upregulates CXCR4 as shown herein and by Richard and coworkers (2006) but the mechanism is not fully understood. In the siRNA experiments described in section 5.4, T47D cells treated with adenosine exhibited a 3-fold increase in CXCR4 cell-surface expression (Figure 5.18), the same as when human colorectal carcinoma cells (HT-29) are treated with 300μM of adenosine (Richard et al., 2006). The adenosine effect is mediated through A2aR and A2bR. This leads to the hypothesis that an increase in CXCR4 expression is mediated through cAMP leading to PKA activation (Fredholm et al., 2000). However, the inhibition of PKA only partially blocks the increase in CXCR4 abundance in response to adenosine (Richard et al., 2006) indicating that other coupled G-proteins or receptors are involved.

CXCR4 is a GPCR able to increase MAPK, including p38, activity (Crespo et al., 1994; Holland et al., 2006). An increase in CXCR4 expression in response to adenosine may increase p38 activity leading to the phosphorylation of HSP27 and allowing actin polymerization and migration. That is, the direction of causality may be from elevation of CXCR4 to HSP27 phosphorylation. In support of this proposal, inhibiting p38 activation or using siRNA against HSP27 blocked HeLa cell migration towards the CXCR4 ligand CXCL12 (Rousseau et al., 2006).

CHAPTER 7: CONCLUSIONS

7.1 Summary

I have shown that adenosine and its stable analogues (NECA, R-PIA) do not affect HSP27 expression in T47D or MCF-7 human breast carcinoma cells. I found no evidence that inosine or the adenosine metabolites AMP and ATP change HSP27 abundance. Limited immunostaining results suggests that adenosine did not elevate HSP27 expression in either cell line.

T47D cells treated with adenosine, NECA or R-PIA showed phosphorylation over short time courses due to manipulation of the cell cultures rather than adenosine exposure. There was however a change in the localization of phosphorylated HSP27 between the cytoplasm and the perinuclear region. Although there was no acute increase in HSP27 phosphorylation in response to adenosine, phosphorylation occurs after 24h of adenosine exposure. This effect lasted until 48h and disappeared after 72h adenosine treatment.

Using HSP27 siRNA knockdowns I established that HSP27 does not affect the steady-state cell-surface expression of CXCR4. The adenosine response of CXCR4 is unchanged by the abolition of HSP27. Any link between HSP27 and CXCR4 is therefore likely to be downstream of CXCR4 signaling.

7.2 Significance of Findings

Adenosine does not induce HSP27 expression or immediate phosphorylation of HSP27 and HSP27 does not affect the cell-surface expression of CXCR4. However, late phosphorylation of HSP27 in response to adenosine is very interesting and may be relevant to the tumour situation where cells are bathed continuously in high levels of adenosine. Late HPS27 phosphorylation by adenosine may play a role in ischemic preconditioning, where pretreatment with adenosine protects cells against subsequent ischemic assaults (Baxter et al., 1994; Liu et al., 1991). In the heart this effect protects against infarction (Liu et al., 1991). In tum3ours where there is a high concentration of adenosine, the phosphorylation of HSP27 may provide cancer cells with persistent resistance against elements of the harsh tumour microenvironment such as hypoxia. If this is the case then adenosine-induced phosphorylation of HSP27 may play an important role in tumour resistance against chemotherapeutic agents. As phosphorylation of HSP27 leads to migration, it could be a necessary step for CXCL12-induced migration and metastasis. The ability of chronic exposure to adenosine to facilitate this mechanism might be one way in which metastasis becomes favoured as tumours develop.

7.3 Future Directions

To determine the pathways leading to late HSP27 phosphorylation, siRNA against candidate kinases such as p38 and MK2 could be used. It would be interesting to see if adenosine is preconditioning cells via a delayed phosphorylation of HSP27 thereby protecting tumour cells from other stresses in the tumour microenvironment and from

chemotherapeutic agents. This possibility could be evaluated by treating cells with adenosine and 24h later exposing cells to hypoxia or anti-cancer drugs and assessing the resistance of the cells using, for example, colony-forming assays. Further experiments could then address the putative causal link to HSP27 phosphorylation.

Migration assays with HSP27 knockdown cells could be used to see if HSP27 is involved in cell migration towards CXCL12. Cells transfected with non-phosphorylatable HSP27 could be used in similar migration assays to establish whether the phosphorylation of HSP27 is needed for CXCL12 induced migration.

REFERENCES

American Cancer Society. *Cancer Facts & Figures 2010*. Atlanta: American Cancer Society; 2010.

Canadian Cancer Society's Steering Committee: Canadian Cancer Statistics 2011. Toronto: Canadian Cancer Society, 2011."

Aebersold, D.M., P. Burri, K.T. Beer, J. Laissue, V. Djonov, R.H. Greiner, and G.L. Semenza. 2001. Expression of hypoxia-inducible factor-1alpha: a novel predictive and prognostic parameter in the radiotherapy of oropharyngeal cancer. *Cancer research*. 61:2911-2916.

Akamatsu, S., K. Nakajima, A. Ishisaki, H. Matsuno, K. Tanabe, M. Takei, M. Takenaka, K. Hirade, N. Yoshimi, H. Suga, Y. Oiso, K. Kato, and O. Kozawa. 2004. Vasopressin phosphorylates HSP27 in aortic smooth muscle cells. *Journal of cellular biochemistry*. 92:1203-1211.

Aloy, M.T., E. Hadchity, C. Bionda, C. Diaz-Latoud, L. Claude, R. Rousson, A.P. Arrigo, and C. Rodriguez-Lafrasse. 2008. Protective role of Hsp27 protein against gamma radiation-induced apoptosis and radiosensitization effects of Hsp27 gene silencing in different human tumor cells. *International journal of radiation oncology, biology, physics*. 70:543-553.

Ananthan, J., A.L. Goldberg, and R. Voellmy. 1986. Abnormal proteins serve as eukaryotic stress signals and trigger the activation of heat shock genes. *Science (New York, N.Y.* 232:522-524.

Arrigo, A.P. 2001. Hsp27: novel regulator of intracellular redox state. *IUBMB life*. 52:303-307.

Arrigo, A.P., J.P. Suhan, and W.J. Welch. 1988. Dynamic changes in the structure and intracellular locale of the mammalian low-molecular-weight heat shock protein. *Molecular and cellular biology*. 8:5059-5071.

Arrigo, A.P., S. Virot, S. Chaufour, W. Firdaus, C. Kretz-Remy, and C. Diaz-Latoud. 2005. Hsp27 consolidates intracellular redox homeostasis by upholding glutathione in its reduced form and by decreasing iron intracellular levels. *Antioxidants & redox signaling*. 7:414-422.

Arts, H.J., H. Hollema, W. Lemstra, P.H. Willemse, E.G. De Vries, H.H. Kampinga, and A.G. Van der Zee. 1999. Heat-shock-protein-27 (hsp27) expression in ovarian carcinoma: relation in response to chemotherapy and prognosis. *International journal of cancer*. 84:234-238.

Baird, N.A., D.W. Turnbull, and E.A. Johnson. 2006. Induction of the heat shock pathway during hypoxia requires regulation of heat shock factor by hypoxia-inducible factor-1. *The Journal of biological chemistry*. 281:38675-38681.

Balabanian, K., B. Lagane, S. Infantino, K.Y. Chow, J. Harriague, B. Moepps, F. Arenzana-Seisdedos, M. Thelen, and F. Bachelerie. 2005. The chemokine SDF-1/CXCL12 binds to and signals through the orphan receptor RDC1 in T lymphocytes. *The Journal of biological chemistry*. 280:35760-35766.

Baler, R., G. Dahl, and R. Voellmy. 1993. Activation of human heat shock genes is accompanied by oligomerization, modification, and rapid translocation of heat shock transcription factor HSF1. *Molecular and cellular biology*. 13:2486-2496.

Balkwill, F. 2004. The significance of cancer cell expression of the chemokine receptor CXCR4. *Seminars in cancer biology*. 14:171-179.

Ballarin, M., B.B. Fredholm, S. Ambrosio, and N. Mahy. 1991. Extracellular levels of adenosine and its metabolites in the striatum of awake rats: inhibition of uptake and metabolism. *Acta physiologica Scandinavica*. 142:97-103.

Bar-Eli, M. 1999. Role of interleukin-8 in tumor growth and metastasis of human melanoma. *Pathobiology*. 67:12-18.

Baxter, G.F., M.S. Marber, V.C. Patel, and D.M. Yellon. 1994. Adenosine receptor involvement in a delayed phase of myocardial protection 24 hours after ischemic preconditioning. *Circulation*. 90:2993-3000.

Bell, M.J., P.M. Kochanek, J.A. Carcillo, Z. Mi, J.K. Schiding, S.R. Wisniewski, R.S. Clark, C.E. Dixon, D.W. Marion, and E. Jackson. 1998. Interstitial adenosine, inosine, and hypoxanthine are increased after experimental traumatic brain injury in the rat. *Journal of neurotrauma*. 15:163-170.

Benndorf, R., K. Hayess, S. Ryazantsev, M. Wieske, J. Behlke, and G. Lutsch. 1994. Phosphorylation and supramolecular organization of murine small heat shock protein HSP25 abolish its actin polymerization-inhibiting activity. *The Journal of biological chemistry*. 269:20780-20784.

Berengian, A.R., M. Parfenova, and H.S. McHaourab. 1999. Site-directed spin labeling study of subunit interactions in the alpha-crystallin domain of small heat-shock proteins. Comparison of the oligomer symmetry in alphaA-crystallin, HSP 27, and HSP 16.3. *The Journal of biological chemistry*. 274:6305-6314.

Berra, E., E. Benizri, A. Ginouves, V. Volmat, D. Roux, and J. Pouyssegur. 2003. HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1alpha in normoxia. *The EMBO journal*. 22:4082-4090.

Berrou, E., and M. Bryckaert. 2009. Recruitment of protein phosphatase 2A to dorsal ruffles by platelet-derived growth factor in smooth muscle cells: dephosphorylation of Hsp27. *Experimental cell research*. 315:836-848.

Birner, P., M. Schindl, A. Obermair, C. Plank, G. Breitenecker, and G. Oberhuber. 2000. Overexpression of hypoxia-inducible factor 1alpha is a marker for an unfavorable prognosis in early-stage invasive cervical cancer. *Cancer research*. 60:4693-4696.

Blay, J., T.D. White, and D.W. Hoskin. 1997. The extracellular fluid of solid carcinomas contains immunosuppressive concentrations of adenosine. *Cancer research*. 57:2602-2605.

Blunt, B.C., A.T. Creek, D.C. Henderson, and P.A. Hofmann. 2007. H2O2 activation of HSP25/27 protects desmin from calpain proteolysis in rat ventricular myocytes. *Am J Physiol Heart Circ Physiol*. 293:H1518-1525.

Bos, R., P. van der Groep, A.E. Greijer, A. Shvarts, S. Meijer, H.M. Pinedo, G.L. Semenza, P.J. van Diest, and E. van der Wall. 2003. Levels of hypoxia-inducible factor-1alpha independently predict prognosis in patients with lymph node negative breast carcinoma. *Cancer*. 97:1573-1581.

Brahimi-Horn, M.C., J. Chiche, and J. Pouyssegur. 2007. Hypoxia and cancer. *Journal of molecular medicine (Berlin, Germany)*. 85:1301-1307.

Brizel, D.M., G.S. Sibley, L.R. Prosnitz, R.L. Scher, and M.W. Dewhirst. 1997. Tumor hypoxia adversely affects the prognosis of carcinoma of the head and neck. *International journal of radiation oncology, biology, physics*. 38:285-289.

Broccoli, D., J.W. Young, and T. de Lange. 1995. Telomerase activity in normal and malignant hematopoietic cells. *Proceedings of the National Academy of Sciences of the United States of America*. 92:9082-9086.

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

Bruey, J.M., C. Ducasse, P. Bonniaud, L. Ravagnan, S.A. Susin, C. Diaz-Latoud, S. Gurbuxani, A.P. Arrigo, G. Kroemer, E. Solary, and C. Garrido. 2000. Hsp27 negatively regulates cell death by interacting with cytochrome c. *Nature cell biology*. 2:645-652.

Bryan, T.M., and T.R. Cech. 1999. Telomerase and the maintenance of chromosome ends. *Current opinion in cell biology*. 11:318-324.

Bukach, O.V., A.E. Glukhova, A.S. Seit-Nebi, and N.B. Gusev. 2009. Heterooligomeric complexes formed by human small heat shock proteins HspB1 (Hsp27) and HspB6 (Hsp20). *Biochimica et biophysica acta*. 1794:486-495.

Cairns, J., S. Qin, R. Philp, Y.H. Tan, and G.R. Guy. 1994. Dephosphorylation of the small heat shock protein Hsp27 in vivo by protein phosphatase 2A. *The Journal of biological chemistry*. 269:9176-9183.

Cantley, L.C., and B.G. Neel. 1999. New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. *Proceedings of the National Academy of Sciences of the United States of America*. 96:4240-4245.

Carini, R., M.G. De Cesaris, R. Splendore, D. Vay, C. Domenicotti, M.P. Nitti, D. Paola, M.A. Pronzato, and E. Albano. 2001. Signal pathway involved in the development of hypoxic preconditioning in rat hepatocytes. *Hepatology (Baltimore, Md*. 33:131-139.

Ceradini, D.J., A.R. Kulkarni, M.J. Callaghan, O.M. Tepper, N. Bastidas, M.E. Kleinman, J.M. Capla, R.D. Galiano, J.P. Levine, and G.C. Gurtner. 2004. Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. *Nature medicine*. 10:858-864.

Chahed, K., M. Kabbage, B. Hamrita, C.L. Guillier, M. Trimeche, S. Remadi, L. Ehret-Sabatier, and L. Chouchane. 2008. Detection of protein alterations in male breast cancer using two dimensional gel electrophoresis and mass spectrometry: the involvement of several pathways in tumorigenesis. *Clinica chimica acta; international journal of clinical chemistry*. 388:106-114.

Charette, S.J., and J. Landry. 2000. The interaction of HSP27 with Daxx identifies a potential regulatory role of HSP27 in Fas-induced apoptosis. *Annals of the New York Academy of Sciences*. 926:126-131.

Chen, C., N. Pore, A. Behrooz, F. Ismail-Beigi, and A. Maity. 2001a. Regulation of glut1 mRNA by hypoxia-inducible factor-1. Interaction between H-ras and hypoxia. *The Journal of biological chemistry*. 276:9519-9525.

Chen, E.Y., N.M. Mazure, J.A. Cooper, and A.J. Giaccia. 2001b. Hypoxia activates a platelet-derived growth factor receptor/phosphatidylinositol 3-kinase/Akt pathway that results in glycogen synthase kinase-3 inactivation. *Cancer research*. 61:2429-2433.

Chen, H.F., L.D. Xie, and C.S. Xu. 2009. Role of heat shock protein 27 phosphorylation in migration of vascular smooth muscle cells. *Molecular and cellular biochemistry*. 327:1-6.

Chia, S.K., C.C. Wykoff, P.H. Watson, C. Han, R.D. Leek, J. Pastorek, K.C. Gatter, P. Ratcliffe, and A.L. Harris. 2001. Prognostic significance of a novel hypoxia-regulated marker, carbonic anhydrase IX, in invasive breast carcinoma. *J Clin Oncol*. 19:3660-3668.

Chiche, J., K. Ilc, J. Laferriere, E. Trottier, F. Dayan, N.M. Mazure, M.C. Brahimi-Horn, and J. Pouyssegur. 2009. Hypoxia-inducible carbonic anhydrase IX and XII promote tumor cell growth by counteracting acidosis through the regulation of the intracellular pH. *Cancer research*. 69:358-368.

Christians, E.S., L.J. Yan, and I.J. Benjamin. 2002. Heat shock factor 1 and heat shock proteins: Critical partners in protection against acute cell injury. *Critical care medicine*. 30:S43-S50.

Ciocca, D.R., S.A. Fuqua, S. Lock-Lim, D.O. Toft, W.J. Welch, and W.L. McGuire. 1992. Response of human breast cancer cells to heat shock and chemotherapeutic drugs. *Cancer research*. 52:3648-3654.

Ciocca, D.R., S. Oesterreich, G.C. Chamness, W.L. McGuire, and S.A. Fuqua. 1993. Biological and clinical implications of heat shock protein 27,000 (Hsp27): a review. *Journal of the National Cancer Institute*. 85:1558-1570.

Ciruela, F., C. Saura, E.I. Canela, J. Mallol, C. Lluis, and R. Franco. 1996. Adenosine deaminase affects ligand-induced signalling by interacting with cell surface adenosine receptors. *FEBS letters*. 380:219-223.

Colquhoun, A., and E.A. Newsholme. 1997. Inhibition of human tumour cell proliferation by analogues of adenosine. *Cell biochemistry and function*. 15:135-139.

Comerford, K.M., T.J. Wallace, J. Karhausen, N.A. Louis, M.C. Montalto, and S.P. Colgan. 2002. Hypoxia-inducible factor-1-dependent regulation of the multidrug resistance (MDR1) gene. *Cancer research*. 62:3387-3394.

Crespo, P., N. Xu, W.F. Simonds, and J.S. Gutkind. 1994. Ras-dependent activation of MAP kinase pathway mediated by G-protein beta gamma subunits. *Nature*. 369:418-420.

Cuesta, R., G. Laroia, and R.J. Schneider. 2000. Chaperone hsp27 inhibits translation during heat shock by binding eIF4G and facilitating dissociation of cap-initiation complexes. *Genes & development*. 14:1460-1470.

Cummins, E.P., and C.T. Taylor. 2005. Hypoxia-responsive transcription factors. *Pflugers Arch*. 450:363-371.

Cuvier, C., A. Jang, and R.P. Hill. 1997. Exposure to hypoxia, glucose starvation and acidosis: effect on invasive capacity of murine tumor cells and correlation with cathepsin (L + B) secretion. *Clinical & experimental metastasis*. 15:19-25.

Dana, A., M. Skarli, J. Papakrivopoulou, and D.M. Yellon. 2000. Adenosine A(1) receptor induced delayed preconditioning in rabbits: induction of p38 mitogen-activated protein kinase activation and Hsp27 phosphorylation via a tyrosine kinase- and protein kinase C-dependent mechanism. *Circulation research*. 86:989-997.

de Jong, W.W., G.J. Caspers, and J.A. Leunissen. 1998. Genealogy of the alpha-crystallin--small heat-shock protein superfamily. *International journal of biological macromolecules*. 22:151-162.

Decking, U.K., G. Schlieper, K. Kroll, and J. Schrader. 1997. Hypoxia-induced inhibition of adenosine kinase potentiates cardiac adenosine release. *Circulation research*. 81:154-164.

Denko, N.C., L.A. Fontana, K.M. Hudson, P.D. Sutphin, S. Raychaudhuri, R. Altman, and A.J. Giaccia. 2003. Investigating hypoxic tumor physiology through gene expression patterns. *Oncogene*. 22:5907-5914.

Dewhirst, M.W., T.W. Secomb, E.T. Ong, R. Hsu, and J.F. Gross. 1994. Determination of local oxygen consumption rates in tumors. *Cancer research*. 54:3333-3336.

Di, K., Y.C. Wong, and X. Wang. 2007. Id-1 promotes TGF-beta1-induced cell motility through HSP27 activation and disassembly of adherens junction in prostate epithelial cells. *Experimental cell research*. 313:3983-3999.

Dong, Z., M.A. Venkatachalam, J. Wang, Y. Patel, P. Saikumar, G.L. Semenza, T. Force, and J. Nishiyama. 2001. Up-regulation of apoptosis inhibitory protein IAP-2 by hypoxia. Hif-1-independent mechanisms. *The Journal of biological chemistry*. 276:18702-18709.

Donovan, J., and J. Slingerland. 2000. Transforming growth factor-beta and breast cancer: Cell cycle arrest by transforming growth factor-beta and its disruption in cancer. *Breast Cancer Res*. 2:116-124.

Dorion, S., and J. Landry. 2002. Activation of the mitogen-activated protein kinase pathways by heat shock. *Cell stress & chaperones*. 7:200-206.

Dougherty, M.K., and D.K. Morrison. 2004. Unlocking the code of 14-3-3. *Journal of cell science*. 117:1875-1884.

Ebert, B.L., J.D. Firth, and P.J. Ratcliffe. 1995. Hypoxia and mitochondrial inhibitors regulate expression of glucose transporter-1 via distinct Cis-acting sequences. *The Journal of biological chemistry*. 270:29083-29089.

Ehrnsperger, M., S. Graber, M. Gaestel, and J. Buchner. 1997. Binding of non-native protein to Hsp25 during heat shock creates a reservoir of folding intermediates for reactivation. *The EMBO journal*. 16:221-229.

Ekhterae, D., Z. Lin, M.S. Lundberg, M.T. Crow, F.C. Brosius, 3rd, and G. Nunez. 1999. ARC inhibits cytochrome c release from mitochondria and protects against hypoxia-induced apoptosis in heart-derived H9c2 cells. *Circulation research*. 85:e70-77.

Erler, J.T., K.L. Bennewith, M. Nicolau, N. Dornhofer, C. Kong, Q.T. Le, J.T. Chi, S.S. Jeffrey, and A.J. Giaccia. 2006. Lysyl oxidase is essential for hypoxia-induced metastasis. *Nature*. 440:1222-1226.

Falanga, V., S.W. Qian, D. Danielpour, M.H. Katz, A.B. Roberts, and M.B. Sporn. 1991. Hypoxia upregulates the synthesis of TGF-beta 1 by human dermal fibroblasts. *The Journal of investigative dermatology*. 97:634-637.

Feldser, D., F. Agani, N.V. Iyer, B. Pak, G. Ferreira, and G.L. Semenza. 1999. Reciprocal positive regulation of hypoxia-inducible factor 1alpha and insulin-like growth factor 2. *Cancer research*. 59:3915-3918.

Feoktistov, I., A.E. Goldstein, and I. Biaggioni. 1999. Role of p38 mitogen-activated protein kinase and extracellular signal-regulated protein kinase kinase in adenosine A2B receptor-mediated interleukin-8 production in human mast cells. *Molecular pharmacology*. 55:726-734.

Ferns, G., S. Shams, and S. Shafi. 2006. Heat shock protein 27: its potential role in vascular disease. *International journal of experimental pathology*. 87:253-274.

Fiedler, U., M. Scharpfenecker, S. Koidl, A. Hegen, V. Grunow, J.M. Schmidt, W. Kriz, G. Thurston, and H.G. Augustin. 2004. The Tie-2 ligand angiopoietin-2 is stored in and rapidly released upon stimulation from endothelial cell Weibel-Palade bodies. *Blood*. 103:4150-4156.

Flores, I., M.L. Cayuela, and M.A. Blasco. 2005. Effects of telomerase and telomere length on epidermal stem cell behavior. *Science (New York, N.Y.* 309:1253-1256.

Fontaine, J.M., X. Sun, R. Benndorf, and M.J. Welsh. 2005. Interactions of HSP22 (HSPB8) with HSP20, alphaB-crystallin, and HSPB3. *Biochemical and biophysical research communications*. 337:1006-1011.

Forsythe, J.A., B.H. Jiang, N.V. Iyer, F. Agani, S.W. Leung, R.D. Koos, and G.L. Semenza. 1996. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Molecular and cellular biology*. 16:4604-4613.

Fortin, A., H. Raybaud-Diogene, B. Tetu, R. Deschenes, J. Huot, and J. Landry. 2000. Overexpression of the 27 KDa heat shock protein is associated with thermoresistance and chemoresistance but not with radioresistance. *International journal of radiation oncology, biology, physics*. 46:1259-1266.

Fredholm, B.B. 1995. Astra Award Lecture. Adenosine, adenosine receptors and the actions of caffeine. *Pharmacology & toxicology*. 76:93-101.

Fredholm, B.B. 2007. Adenosine, an endogenous distress signal, modulates tissue damage and repair. *Cell death and differentiation*. 14:1315-1323.

Fredholm, B.B., I.J. AP, K.A. Jacobson, K.N. Klotz, and J. Linden. 2001a. International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacological reviews*. 53:527-552.

Fredholm, B.B., G. Arslan, L. Halldner, B. Kull, G. Schulte, and W. Wasserman. 2000. Structure and function of adenosine receptors and their genes. *Naunyn-Schmiedeberg's archives of pharmacology*. 362:364-374.

Fredholm, B.B., E. Irenius, B. Kull, and G. Schulte. 2001b. Comparison of the potency of adenosine as an agonist at human adenosine receptors expressed in Chinese hamster ovary cells. *Biochemical pharmacology*. 61:443-448.

Funasaka, T., T. Yanagawa, V. Hogan, and A. Raz. 2005. Regulation of phosphoglucose isomerase/autocrine motility factor expression by hypoxia. *Faseb J.* 19:1422-1430.

Fuqua, S.A., M. Blum-Salingaros, and W.L. McGuire. 1989. Induction of the estrogen-regulated "24K" protein by heat shock. *Cancer research*. 49:4126-4129.

Gaitanaki, C., S. Konstantina, S. Chrysa, and I. Beis. 2003. Oxidative stress stimulates multiple MAPK signalling pathways and phosphorylation of the small HSP27 in the perfused amphibian heart. *The Journal of experimental biology*. 206:2759-2769.

Gasparini, G. 2000. Prognostic value of vascular endothelial growth factor in breast cancer. *The oncologist*. 5 Suppl 1:37-44.

Gerhardt, H., M. Golding, M. Fruttiger, C. Ruhrberg, A. Lundkvist, A. Abramsson, M. Jeltsch, C. Mitchell, K. Alitalo, D. Shima, and C. Betsholtz. 2003. VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *The Journal of cell biology*. 161:1163-1177.

Gerits, N., T. Mikalsen, S. Kostenko, A. Shiryaev, M. Johannessen, and U. Moens. 2007. Modulation of F-actin rearrangement by the cyclic AMP/cAMP-dependent protein kinase (PKA) pathway is mediated by MAPK-activated protein kinase 5 and requires PKA-induced nuclear export of MK5. *The Journal of biological chemistry*. 282:37232-37243.

Geum, D., G.H. Son, and K. Kim. 2002. Phosphorylation-dependent cellular localization and thermoprotective role of heat shock protein 25 in hippocampal progenitor cells. *The Journal of biological chemistry*. 277:19913-19921.

Gibbons, N.B., R.W. Watson, R.N. Coffey, H.P. Brady, and J.M. Fitzpatrick. 2000. Heat-shock proteins inhibit induction of prostate cancer cell apoptosis. *The Prostate*. 45:58-65.

Glaessgen, A., S. Jonmarker, A. Lindberg, B. Nilsson, R. Lewensohn, P. Ekman, A. Valdman, and L. Egevad. 2008. Heat shock proteins 27, 60 and 70 as prognostic markers of prostate cancer. *Apmis*. 116:888-895.

Gort, E.H., G. van Haaften, I. Verlaan, A.J. Groot, R.H. Plasterk, A. Shvarts, K.P. Suijkerbuijk, T. van Laar, E. van der Wall, V. Raman, P.J. van Diest, M. Tijsterman, and M. Vooijs. 2008. The TWIST1 oncogene is a direct target of hypoxia-inducible factor-2alpha. *Oncogene*. 27:1501-1510.

Graeber, T.G., C. Osmanian, T. Jacks, D.E. Housman, C.J. Koch, S.W. Lowe, and A.J. Giaccia. 1996. Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. *Nature*. 379:88-91.

Graham, C.H., T.E. Fitzpatrick, and K.R. McCrae. 1998. Hypoxia stimulates urokinase receptor expression through a heme protein-dependent pathway. *Blood*. 91:3300-3307.

Graham, C.H., J. Forsdike, C.J. Fitzgerald, and S. Macdonald-Goodfellow. 1999. Hypoxia-mediated stimulation of carcinoma cell invasiveness via upregulation of urokinase receptor expression. *International journal of cancer*. 80:617-623.

Gray, J.H., R.P. Owen, and K.M. Giacomini. 2004. The concentrative nucleoside transporter family, SLC28. *Pflugers Arch*. 447:728-734.

Guo, G., and N.R. Bhat. 2007. p38alpha MAP kinase mediates hypoxia-induced motor neuron cell death: a potential target of minocycline's neuroprotective action. *Neurochemical research*. 32:2160-2166.

Guo, K., Y. Liu, H. Zhou, Z. Dai, J. Zhang, R. Sun, J. Chen, Q. Sun, W. Lu, X. Kang, and P. Chen. 2008. Involvement of protein kinase C beta-extracellular signal-regulating kinase 1/2/p38 mitogen-activated protein kinase-heat shock protein 27 activation in hepatocellular carcinoma cell motility and invasion. *Cancer science*. 99:486-496.

Gupta, M., and E. Goldwasser. 1996. The role of the near upstream sequence in hypoxia-induced expression of the erythropoietin gene. *Nucleic acids research*. 24:4768-4774.

Gusev, N.B., N.V. Bogatcheva, and S.B. Marston. 2002. Structure and properties of small heat shock proteins (sHsp) and their interaction with cytoskeleton proteins. *Biochemistry*. 67:511-519.

Hanahan, D., and J. Folkman. 1996. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell*. 86:353-364.

Hanahan, D., and R.A. Weinberg. 2000. The hallmarks of cancer. *Cell*. 100:57-70.

Hansen, R.K., I. Parra, P. Lemieux, S. Oesterreich, S.G. Hilsenbeck, and S.A. Fuqua. 1999. Hsp27 overexpression inhibits doxorubicin-induced apoptosis in human breast cancer cells. *Breast cancer research and treatment*. 56:187-196.

Harley, C.B., A.B. Futcher, and C.W. Greider. 1990. Telomeres shorten during ageing of human fibroblasts. *Nature*. 345:458-460.

Hasko, G., D.G. Kuhel, Z.H. Nemeth, J.G. Mabley, R.F. Stachlewitz, L. Virag, Z. Lohinai, G.J. Southan, A.L. Salzman, and C. Szabo. 2000. Inosine inhibits inflammatory cytokine production by a posttranscriptional mechanism and protects against endotoxin-induced shock. *J Immunol*. 164:1013-1019.

Hasko, G., J. Linden, B. Cronstein, and P. Pacher. 2008. Adenosine receptors: therapeutic aspects for inflammatory and immune diseases. *Nat Rev Drug Discov*. 7:759-770.

Hatoum, O.A., D.G. Binion, and D.D. Gutterman. 2005. Paradox of simultaneous intestinal ischaemia and hyperaemia in inflammatory bowel disease. *European journal of clinical investigation*. 35:599-609.

Hatse, S., K. Princen, G. Bridger, E. De Clercq, and D. Schols. 2002. Chemokine receptor inhibition by AMD3100 is strictly confined to CXCR4. *FEBS letters*. 527:255-262.

Hayflick, L. 2000. The illusion of cell immortality. *British journal of cancer*. 83:841-846.

Headrick, J.P., and R.J. Willis. 1989. 5'-Nucleotidase activity and adenosine formation in stimulated, hypoxic and underperfused rat heart. *The Biochemical journal*. 261:541-550.

Hedges, J.C., M.A. Dechert, I.A. Yamboliev, J.L. Martin, E. Hickey, L.A. Weber, and W.T. Gerthoffer. 1999. A role for p38(MAPK)/HSP27 pathway in smooth muscle cell migration. *The Journal of biological chemistry*. 274:24211-24219.

Herrera, C., V. Casado, F. Ciruela, P. Schofield, J. Mallol, C. Lluis, and R. Franco. 2001. Adenosine A2B receptors behave as an alternative anchoring protein for cell surface adenosine deaminase in lymphocytes and cultured cells. *Molecular pharmacology*. 59:127-134.

Hino, M., K. Kurogi, M.A. Okubo, M. Murata-Hori, and H. Hosoya. 2000. Small heat shock protein 27 (HSP27) associates with tubulin/microtubules in HeLa cells. *Biochemical and biophysical research communications*. 271:164-169.

Hirade, K., O. Kozawa, K. Tanabe, M. Niwa, H. Matsuno, Y. Oiso, S. Akamatsu, H. Ito, K. Kato, Y. Katagiri, and T. Uematsu. 2002. Thrombin stimulates dissociation and induction of HSP27 via p38 MAPK in vascular smooth muscle cells. *Am J Physiol Heart Circ Physiol*. 283:H941-948.

Hirano, S., E.A. Shelden, and R.R. Gilmont. 2004. HSP27 regulates fibroblast adhesion, motility, and matrix contraction. *Cell stress & chaperones*. 9:29-37.

Hiyama, K., Y. Hirai, S. Kyoizumi, M. Akiyama, E. Hiyama, M.A. Piatyszek, J.W. Shay, S. Ishioka, and M. Yamakido. 1995. Activation of telomerase in human lymphocytes and hematopoietic progenitor cells. *J Immunol*. 155:3711-3715.

Hockel, M., K. Schlenger, B. Aral, M. Mitze, U. Schaffer, and P. Vaupel. 1996. Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. *Cancer research*. 56:4509-4515.

Holland, J.D., M. Kochetkova, C. Akekawatchai, M. Dottore, A. Lopez, and S.R. McColl. 2006. Differential functional activation of chemokine receptor CXCR4 is mediated by G proteins in breast cancer cells. *Cancer research*. 66:4117-4124.

Hollstein, M., K. Rice, M.S. Greenblatt, T. Soussi, R. Fuchs, T. Sorlie, E. Hovig, B. Smith-Sorensen, R. Montesano, and C.C. Harris. 1994. Database of p53 gene somatic mutations in human tumors and cell lines. *Nucleic acids research*. 22:3551-3555.

Holness, M.J., and M.C. Sugden. 2003. Regulation of pyruvate dehydrogenase complex activity by reversible phosphorylation. *Biochemical Society transactions*. 31:1143-1151.

Hon, W.C., M.I. Wilson, K. Harlos, T.D. Claridge, C.J. Schofield, C.W. Pugh, P.H. Maxwell, P.J. Ratcliffe, D.I. Stuart, and E.Y. Jones. 2002. Structural basis for the recognition of hydroxyproline in HIF-1 alpha by pVHL. *Nature*. 417:975-978.

Huang, L.E., J. Gu, M. Schau, and H.F. Bunn. 1998. Regulation of hypoxia-inducible factor 1alpha is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway. *Proceedings of the National Academy of Sciences of the United States of America*. 95:7987-7992.

Huot, J., F. Houle, S. Rousseau, R.G. Deschesnes, G.M. Shah, and J. Landry. 1998. SAPK2/p38-dependent F-actin reorganization regulates early membrane blebbing during stress-induced apoptosis. *The Journal of cell biology*. 143:1361-1373.

Huot, J., F. Houle, D.R. Spitz, and J. Landry. 1996. HSP27 phosphorylation-mediated resistance against actin fragmentation and cell death induced by oxidative stress. *Cancer research*. 56:273-279.

Huot, J., G. Roy, H. Lambert, P. Chretien, and J. Landry. 1991. Increased survival after treatments with anticancer agents of Chinese hamster cells expressing the human Mr 27,000 heat shock protein. *Cancer research*. 51:5245-5252.

Hurlimann, J., S. Gebhard, and F. Gomez. 1993. Oestrogen receptor, progesterone receptor, pS2, ERD5, HSP27 and cathepsin D in invasive ductal breast carcinomas. *Histopathology*. 23:239-248.

Ivanov, S., S.Y. Liao, A. Ivanova, A. Danilkovitch-Miagkova, N. Tarasova, G. Weirich, M.J. Merrill, M.A. Proescholdt, E.H. Oldfield, J. Lee, J. Zavada, A. Waheed, W. Sly, M.I. Lerman, and E.J. Stanbridge. 2001. Expression of hypoxia-inducible cell-surface transmembrane carbonic anhydrases in human cancer. *The American journal of pathology*. 158:905-919.

Jaakkola, P., D.R. Mole, Y.M. Tian, M.I. Wilson, J. Gielbert, S.J. Gaskell, A. Kriegsheim, H.F. Hebestreit, M. Mukherji, C.J. Schofield, P.H. Maxwell, C.W. Pugh, and P.J. Ratcliffe. 2001. Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. *Science (New York, N.Y.* 292:468-472.

Jaattela, M. 1999. Escaping cell death: survival proteins in cancer. *Experimental cell research*. 248:30-43.

Jacobson, K.A., and Z.G. Gao. 2006. Adenosine receptors as therapeutic targets. *Nat Rev Drug Discov*. 5:247-264.

Jakob, U., M. Gaestel, K. Engel, and J. Buchner. 1993. Small heat shock proteins are molecular chaperones. *The Journal of biological chemistry*. 268:1517-1520.

Jin, X., R.K. Shepherd, B.R. Duling, and J. Linden. 1997. Inosine binds to A3 adenosine receptors and stimulates mast cell degranulation. *The Journal of clinical investigation*. 100:2849-2857.

Kacimi, R., J. Chentoufi, N. Honbo, C.S. Long, and J.S. Karliner. 2000. Hypoxia differentially regulates stress proteins in cultured cardiomyocytes: role of the p38 stress-activated kinase signaling cascade, and relation to cytoprotection. *Cardiovascular research*. 46:139-150.

Kaida, T., O. Kozawa, T. Ito, K. Tanabe, H. Ito, H. Matsuno, M. Niwa, H. Miyata, T. Uematsu, and K. Kato. 1999. Vasopressin stimulates the induction of heat shock protein 27 and alphaB-crystallin via protein kinase C activation in vascular smooth muscle cells. *Experimental cell research*. 246:327-337.

Kallio, P.J., K. Okamoto, S. O'Brien, P. Carrero, Y. Makino, H. Tanaka, and L. Poellinger. 1998. Signal transduction in hypoxic cells: inducible nuclear translocation and recruitment of the CBP/p300 coactivator by the hypoxia-inducible factor-1alpha. *The EMBO journal*. 17:6573-6586.

Kalluri, R., and R.A. Weinberg. 2009. The basics of epithelial-mesenchymal transition. *The Journal of clinical investigation*. 119:1420-1428.

Kameoka, J., T. Tanaka, Y. Nojima, S.F. Schlossman, and C. Morimoto. 1993. Direct association of adenosine deaminase with a T cell activation antigen, CD26. *Science (New York, N.Y.*. 261:466-469.

Kane, L.P., V.S. Shapiro, D. Stokoe, and A. Weiss. 1999. Induction of NF-kappaB by the Akt/PKB kinase. *Curr Biol*. 9:601-604.

Kang, S.H., K.W. Kang, K.H. Kim, B. Kwon, S.K. Kim, H.Y. Lee, S.Y. Kong, E.S. Lee, S.G. Jang, and B.C. Yoo. 2008. Upregulated HSP27 in human breast cancer cells reduces Herceptin susceptibility by increasing Her2 protein stability. *BMC cancer*. 8:286.

Kang, S.S., Y.K. Chun, M.H. Hur, H.K. Lee, Y.J. Kim, S.R. Hong, J.H. Lee, S.G. Lee, and Y.K. Park. 2002. Clinical significance of glucose transporter 1 (GLUT1) expression in human breast carcinoma. *Jpn J Cancer Res*. 93:1123-1128.

Kapranos, N., A. Kominea, P.A. Konstantinopoulos, S. Savva, S. Artelaris, G. Vandoros, G. Sotiropoulou-Bonikou, and A.G. Papavassiliou. 2002. Expression of the 27-kDa heat shock protein (HSP27) in gastric carcinomas and adjacent normal, metaplastic, and dysplastic gastric mucosa, and its prognostic significance. *Journal of cancer research and clinical oncology*. 128:426-432.

Kato, K., K. Hasegawa, S. Goto, and Y. Inaguma. 1994. Dissociation as a result of phosphorylation of an aggregated form of the small stress protein, hsp27. *The Journal of biological chemistry*. 269:11274-11278.

Kato, M., J. Kitayama, S. Kazama, and H. Nagawa. 2003. Expression pattern of CXC chemokine receptor-4 is correlated with lymph node metastasis in human invasive ductal carcinoma. *Breast Cancer Res*. 5:R144-150.

Kawanishi, K., H. Shiozaki, Y. Doki, I. Sakita, M. Inoue, M. Yano, T. Tsujinaka, A. Shamma, and M. Monden. 1999. Prognostic significance of heat shock proteins 27 and 70 in patients with squamous cell carcinoma of the esophagus. *Cancer*. 85:1649-1657.

Kim, J., H. Takeuchi, S.T. Lam, R.R. Turner, H.J. Wang, C. Kuo, L. Foshag, A.J. Bilchik, and D.S. Hoon. 2005. Chemokine receptor CXCR4 expression in colorectal cancer patients increases the risk for recurrence and for poor survival. *J Clin Oncol*. 23:2744-2753.

Kim, J.W., I. Tchernyshyov, G.L. Semenza, and C.V. Dang. 2006. HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. *Cell metabolism*. 3:177-185.

Kirschmann, D.A., E.A. Seftor, S.F. Fong, D.R. Nieva, C.M. Sullivan, E.M. Edwards, P. Sommer, K. Csiszar, and M.J. Hendrix. 2002. A molecular role for lysyl oxidase in breast cancer invasion. *Cancer research*. 62:4478-4483.

Kon-no, H., G. Ishii, K. Nagai, J. Yoshida, M. Nishimura, M. Nara, T. Fujii, Y. Murata, H. Miyamoto, and A. Ochiai. 2006. Carbonic anhydrase IX expression is associated with tumor progression and a poor prognosis of lung adenocarcinoma. *Lung cancer (Amsterdam, Netherlands)*. 54:409-418.

Konishi, H., H. Matsuzaki, M. Tanaka, Y. Takemura, S. Kuroda, Y. Ono, and U. Kikkawa. 1997. Activation of protein kinase B (Akt/RAC-protein kinase) by cellular stress and its association with heat shock protein Hsp27. *FEBS letters*. 410:493-498.

Koong, A.C., N.C. Denko, K.M. Hudson, C. Schindler, L. Swiersz, C. Koch, S. Evans, H. Ibrahim, Q.T. Le, D.J. Terris, and A.J. Giaccia. 2000. Candidate genes for the hypoxic tumor phenotype. *Cancer research*. 60:883-887.

Kostenko, S., and U. Moens. 2009. Heat shock protein 27 phosphorylation: kinases, phosphatases, functions and pathology. *Cell Mol Life Sci*. 66:3289-3307.

Kuerbitz, S.J., B.S. Plunkett, W.V. Walsh, and M.B. Kastan. 1992. Wild-type p53 is a cell cycle checkpoint determinant following irradiation. *Proceedings of the National Academy of Sciences of the United States of America*. 89:7491-7495.

Kull, B., P. Svenningsson, and B.B. Fredholm. 2000. Adenosine A(2A) receptors are colocalized with and activate g(olf) in rat striatum. *Molecular pharmacology*. 58:771-777.

Kung, A.L., S. Wang, J.M. Klco, W.G. Kaelin, and D.M. Livingston. 2000. Suppression of tumor growth through disruption of hypoxia-inducible transcription. *Nature medicine*. 6:1335-1340.

Kwon, S.M., S.A. Kim, S. Fujii, H. Maeda, S.G. Ahn, and J.H. Yoon. 2011. Transforming growth factor beta1 promotes migration of human periodontal ligament cells through heat shock protein 27 phosphorylation. *Biological & pharmaceutical bulletin*. 34:486-489.

Laderoute, K.R., R.M. Alarcon, M.D. Brody, J.M. Calaoagan, E.Y. Chen, A.M. Knapp, Z. Yun, N.C. Denko, and A.J. Giaccia. 2000. Opposing effects of hypoxia on expression of the angiogenic inhibitor thrombospondin 1 and the angiogenic inducer vascular endothelial growth factor. *Clin Cancer Res*. 6:2941-2950.

Lambert, H., S.J. Charette, A.F. Bernier, A. Guimond, and J. Landry. 1999. HSP27 multimerization mediated by phosphorylation-sensitive intermolecular interactions at the amino terminus. *The Journal of biological chemistry*. 274:9378-9385.

Lando, D., D.J. Peet, J.J. Gorman, D.A. Whelan, M.L. Whitelaw, and R.K. Bruick. 2002. FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. *Genes & development*. 16:1466-1471.

Landry, J., D. Bernier, P. Chretien, L.M. Nicole, R.M. Tanguay, and N. Marceau. 1982. Synthesis and degradation of heat shock proteins during development and decay of thermotolerance. *Cancer research*. 42:2457-2461.

Landry, J., P. Chretien, H. Lambert, E. Hickey, and L.A. Weber. 1989. Heat shock resistance conferred by expression of the human HSP27 gene in rodent cells. *The Journal of cell biology*. 109:7-15.

Landry, J., P. Chretien, A. Laszlo, and H. Lambert. 1991. Phosphorylation of HSP27 during development and decay of thermotolerance in Chinese hamster cells. *Journal of cellular physiology*. 147:93-101.

Landry, J., H. Lambert, M. Zhou, J.N. Lavoie, E. Hickey, L.A. Weber, and C.W. Anderson. 1992. Human HSP27 is phosphorylated at serines 78 and 82 by heat shock and mitogen-activated kinases that recognize the same amino acid motif as S6 kinase II. *The Journal of biological chemistry*. 267:794-803.

Langdon, S.P., G.J. Rabiasz, G.L. Hirst, R.J. King, R.A. Hawkins, J.F. Smyth, and W.R. Miller. 1995. Expression of the heat shock protein HSP27 in human ovarian cancer. *Clin Cancer Res*. 1:1603-1609.

Lapteva, N., A.G. Yang, D.E. Sanders, R.W. Strube, and S.Y. Chen. 2005. CXCR4 knockdown by small interfering RNA abrogates breast tumor growth in vivo. *Cancer gene therapy*. 12:84-89.

Lavoie, J.N., G. Gingras-Breton, R.M. Tanguay, and J. Landry. 1993a. Induction of Chinese hamster HSP27 gene expression in mouse cells confers resistance to heat shock. HSP27 stabilization of the microfilament organization. *The Journal of biological chemistry*. 268:3420-3429.

Lavoie, J.N., E. Hickey, L.A. Weber, and J. Landry. 1993b. Modulation of actin microfilament dynamics and fluid phase pinocytosis by phosphorylation of heat shock protein 27. *The Journal of biological chemistry*. 268:24210-24214.

Lavoie, J.N., H. Lambert, E. Hickey, L.A. Weber, and J. Landry. 1995. Modulation of cellular thermoresistance and actin filament stability accompanies phosphorylation-induced changes in the oligomeric structure of heat shock protein 27. *Molecular and cellular biology*. 15:505-516.

Lee, G.J., A.M. Roseman, H.R. Saibil, and E. Vierling. 1997. A small heat shock protein stably binds heat-denatured model substrates and can maintain a substrate in a folding-competent state. *The EMBO journal*. 16:659-671.

Lee, H.T., M. Kim, M. Jan, R.B. Penn, and C.W. Emala. 2007. Renal tubule necrosis and apoptosis modulation by A1 adenosine receptor expression. *Kidney international*. 71:1249-1261.

Lee, Y.J., and W.C. Dewey. 1988. Thermotolerance induced by heat, sodium arsenite, or puromycin: its inhibition and differences between 43 degrees C and 45 degrees C. *Journal of cellular physiology*. 135:397-406.

Lengauer, C., K.W. Kinzler, and B. Vogelstein. 1998. Genetic instabilities in human cancers. *Nature*. 396:643-649.

Li, G.C., and Z. Werb. 1982. Correlation between synthesis of heat shock proteins and development of thermotolerance in Chinese hamster fibroblasts. *Proceedings of the National Academy of Sciences of the United States of America*. 79:3218-3222.

Li, Y.M., Y. Pan, Y. Wei, X. Cheng, B.P. Zhou, M. Tan, X. Zhou, W. Xia, G.N. Hortobagyi, D. Yu, and M.C. Hung. 2004. Upregulation of CXCR4 is essential for HER2-mediated tumor metastasis. *Cancer cell*. 6:459-469.

Liang, Z., Y. Yoon, J. Votaw, M.M. Goodman, L. Williams, and H. Shim. 2005. Silencing of CXCR4 blocks breast cancer metastasis. *Cancer research*. 65:967-971.

Lindblom, P., H. Gerhardt, S. Liebner, A. Abramsson, M. Enge, M. Hellstrom, G. Backstrom, S. Fredriksson, U. Landegren, H.C. Nystrom, G. Bergstrom, E. Dejana, A. Ostman, P. Lindahl, and C. Betsholtz. 2003. Endothelial PDGF-B retention is required for proper investment of pericytes in the microvessel wall. *Genes & development*. 17:1835-1840.

Linden, J. 2005. Adenosine in tissue protection and tissue regeneration. *Molecular pharmacology*. 67:1385-1387.

Litt, M., P. Kramer, D.M. LaMorticella, W. Murphey, E.W. Lovrien, and R.G. Weleber. 1998. Autosomal dominant congenital cataract associated with a missense mutation in the human alpha crystallin gene CRYAA. *Human molecular genetics*. 7:471-474.

Liu, G.S., J. Thornton, D.M. Van Winkle, A.W. Stanley, R.A. Olsson, and J.M. Downey. 1991. Protection against infarction afforded by preconditioning is mediated by A1 adenosine receptors in rabbit heart. *Circulation*. 84:350-356.

Livingston, M., L.G. Heaney, and M. Ennis. 2004. Adenosine, inflammation and asthma--a review. *Inflamm Res*. 53:171-178.

Lloyd, H.G., and B.B. Fredholm. 1995. Involvement of adenosine deaminase and adenosine kinase in regulating extracellular adenosine concentration in rat hippocampal slices. *Neurochemistry international*. 26:387-395.

Loeb, L.A. 1991. Mutator phenotype may be required for multistage carcinogenesis. *Cancer research*. 51:3075-3079.

Lu, H., R.A. Forbes, and A. Verma. 2002. Hypoxia-inducible factor 1 activation by aerobic glycolysis implicates the Warburg effect in carcinogenesis. *The Journal of biological chemistry*. 277:23111-23115.

Ludwig, S., K. Engel, A. Hoffmeyer, G. Sithanandam, B. Neufeld, D. Palm, M. Gaestel, and U.R. Rapp. 1996. 3pK, a novel mitogen-activated protein (MAP) kinase-activated protein kinase, is targeted by three MAP kinase pathways. *Molecular and cellular biology*. 16:6687-6697.

MacRae, T.H. 2000. Structure and function of small heat shock/alpha-crystallin proteins: established concepts and emerging ideas. *Cell Mol Life Sci*. 57:899-913.

Maeda, T., S. Matsumura, H. Hiranuma, A. Jikko, S. Furukawa, T. Ishida, and H. Fuchihata. 1998. Expression of vascular endothelial growth factor in human oral squamous cell carcinoma: its association with tumour progression and p53 gene status. *Journal of clinical pathology*. 51:771-775.

Maisonpierre, P.C., C. Suri, P.F. Jones, S. Bartunkova, S.J. Wiegand, C. Radziejewski, D. Compton, J. McClain, T.H. Aldrich, N. Papadopoulos, T.J. Daly, S. Davis, T.N. Sato, and G.D. Yancopoulos. 1997. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science (New York, N.Y.* 277:55-60.

Mapp, P.I., M.C. Grootveld, and D.R. Blake. 1995. Hypoxia, oxidative stress and rheumatoid arthritis. *British medical bulletin*. 51:419-436.

Masson, N., C. Willam, P.H. Maxwell, C.W. Pugh, and P.J. Ratcliffe. 2001. Independent function of two destruction domains in hypoxia-inducible factor-alpha chains activated by prolyl hydroxylation. *The EMBO journal*. 20:5197-5206.

McCann, A.H., P.A. Dervan, M. O'Regan, M.B. Codd, W.J. Gullick, B.M. Tobin, and D.N. Carney. 1991. Prognostic significance of c-erbB-2 and estrogen receptor status in human breast cancer. *Cancer research*. 51:3296-3303.

McMillan, D.R., X. Xiao, L. Shao, K. Graves, and I.J. Benjamin. 1998. Targeted disruption of heat shock transcription factor 1 abolishes thermotolerance and protection against heat-inducible apoptosis. *The Journal of biological chemistry*. 273:7523-7528.

Mehlen, P., and A.P. Arrigo. 1994. The serum-induced phosphorylation of mammalian hsp27 correlates with changes in its intracellular localization and levels of oligomerization. *European journal of biochemistry / FEBS*. 221:327-334.

Mehlen, P., E. Hickey, L.A. Weber, and A.P. Arrigo. 1997. Large unphosphorylated aggregates as the active form of hsp27 which controls intracellular reactive oxygen species and glutathione levels and generates a protection against TNFalpha in NIH-3T3-ras cells. *Biochemical and biophysical research communications*. 241:187-192.

Mehlen, P., C. Kretz-Remy, X. Preville, and A.P. Arrigo. 1996. Human hsp27, Drosophila hsp27 and human alphaB-crystallin expression-mediated increase in glutathione is essential for the protective activity of these proteins against TNFalpha-induced cell death. *The EMBO journal*. 15:2695-2706.

Mehlen, P., X. Preville, P. Chareyron, J. Briolay, R. Klemenz, and A.P. Arrigo. 1995. Constitutive expression of human hsp27, Drosophila hsp27, or human alpha B-crystallin confers resistance to TNF- and oxidative stress-induced cytotoxicity in stably transfected murine L929 fibroblasts. *J Immunol*. 154:363-374.

Mellado, M., J.M. Rodriguez-Frade, S. Manes, and A.C. Martinez. 2001. Chemokine signaling and functional responses: the role of receptor dimerization and TK pathway activation. *Annual review of immunology*. 19:397-421.

Merighi, S., K. Varani, S. Gessi, E. Cattabriga, V. Iannotta, C. Ulouglu, E. Leung, and P.A. Borea. 2001. Pharmacological and biochemical characterization of adenosine receptors in the human malignant melanoma A375 cell line. *British journal of pharmacology*. 134:1215-1226.

Miron, T., K. Vancompernolle, J. Vandekerckhove, M. Wilchek, and B. Geiger. 1991. A 25-kD inhibitor of actin polymerization is a low molecular mass heat shock protein. *The Journal of cell biology*. 114:255-261.

Moeller, B.J., and M.W. Dewhirst. 2006. HIF-1 and tumour radiosensitivity. *British journal of cancer*. 95:1-5.

Mori, Y., K. Tsukinoki, M. Yasuda, M. Miyazawa, A. Kaneko, and Y. Watanabe. 2007. Glucose transporter type 1 expression are associated with poor prognosis in patients with salivary gland tumors. *Oral oncology*. 43:563-569.

Morimoto, R.I., and M.G. Santoro. 1998. Stress-inducible responses and heat shock proteins: new pharmacologic targets for cytoprotection. *Nature biotechnology*. 16:833-838.

Morimoto, R.I., K.D. Sarge, and K. Abravaya. 1992. Transcriptional regulation of heat shock genes. A paradigm for inducible genomic responses. *The Journal of biological chemistry*. 267:21987-21990.

Morino, M., T. Tsuzuki, Y. Ishikawa, T. Shirakami, M. Yoshimura, Y. Kiyosuke, K. Matsunaga, C. Yoshikumi, and N. Saijo. 1997. Specific expression of HSP27 in human tumor cell lines in vitro. *In vivo (Athens, Greece)*. 11:179-184.

Moser, G.H., J. Schrader, and A. Deussen. 1989. Turnover of adenosine in plasma of human and dog blood. *The American journal of physiology*. 256:C799-806.

Mounier, N., and A.P. Arrigo. 2002. Actin cytoskeleton and small heat shock proteins: how do they interact? *Cell stress & chaperones*. 7:167-176.

Mujoomdar, M., D. Hoskin, and J. Blay. 2003. Adenosine stimulation of the proliferation of colorectal carcinoma cell lines. Roles of cell density and adenosine metabolism. *Biochemical pharmacology*. 66:1737-1747.

Muller, A., B. Homey, H. Soto, N. Ge, D. Catron, M.E. Buchanan, T. McClanahan, E. Murphy, W. Yuan, S.N. Wagner, J.L. Barrera, A. Mohar, E. Verastegui, and A. Zlotnik. 2001. Involvement of chemokine receptors in breast cancer metastasis. *Nature*. 410:50-56.

Munoz-Najar, U.M., K.M. Neurath, F. Vumbaca, and K.P. Claffey. 2006. Hypoxia stimulates breast carcinoma cell invasion through MT1-MMP and MMP-2 activation. *Oncogene*. 25:2379-2392.

Murphy, P.M. 2002. International Union of Pharmacology. XXX. Update on chemokine receptor nomenclature. *Pharmacological reviews*. 54:227-229.

Murphy, P.M., M. Baggiolini, I.F. Charo, C.A. Hebert, R. Horuk, K. Matsushima, L.H. Miller, J.J. Oppenheim, and C.A. Power. 2000. International union of pharmacology. XXII. Nomenclature for chemokine receptors. *Pharmacological reviews*. 52:145-176.

Nagasawa, T., S. Hirota, K. Tachibana, N. Takakura, S. Nishikawa, Y. Kitamura, N. Yoshida, H. Kikutani, and T. Kishimoto. 1996. Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature*. 382:635-638.

Nakajima, K., K. Hirade, A. Ishisaki, H. Matsuno, H. Suga, Y. Kanno, E. Shu, Y. Kitajima, Y. Katagiri, and O. Kozawa. 2005. Akt regulates thrombin-induced HSP27 phosphorylation in aortic smooth muscle cells: function at a point downstream from p38 MAP kinase. *Life sciences*. 77:96-107.

Neuhaus-Steinmetz, U., and L. Rensing. 1997. Heat shock protein induction by certain chemical stressors is correlated with their cytotoxicity, lipophilicity and protein-denaturing capacity. *Toxicology*. 123:185-195.

Nowell, P.C. 1976. The clonal evolution of tumor cell populations. *Science (New York, N.Y.* 194:23-28.

Oh, H., H. Takagi, K. Suzuma, A. Otani, M. Matsumura, and Y. Honda. 1999. Hypoxia and vascular endothelial growth factor selectively up-regulate angiopoietin-2 in bovine microvascular endothelial cells. *The Journal of biological chemistry*. 274:15732-15739.

Ohh, M., C.W. Park, M. Ivan, M.A. Hoffman, T.Y. Kim, L.E. Huang, N. Pavletich, V. Chau, and W.G. Kaelin. 2000. Ubiquitination of hypoxia-inducible factor requires direct binding to the beta-domain of the von Hippel-Lindau protein. *Nature cell biology*. 2:423-427.

Opie, L.H. 1990. Myocardial ischemia--metabolic pathways and implications of increased glycolysis. *Cardiovascular drugs and therapy / sponsored by the International Society of Cardiovascular Pharmacotherapy*. 4 Suppl 4:777-790.

Palmer, L.A., G.L. Semenza, M.H. Stoler, and R.A. Johns. 1998. Hypoxia induces type II NOS gene expression in pulmonary artery endothelial cells via HIF-1. *The American journal of physiology*. 274:L212-219.

Pandey, P., R. Farber, A. Nakazawa, S. Kumar, A. Bharti, C. Nalin, R. Weichselbaum, D. Kufe, and S. Kharbanda. 2000. Hsp27 functions as a negative regulator of cytochrome c-dependent activation of procaspase-3. *Oncogene*. 19:1975-1981.

Parcellier, A., E. Schmitt, S. Gurbuxani, D. Seigneurin-Berny, A. Pance, A. Chantome, S. Plenchette, S. Khochbin, E. Solary, and C. Garrido. 2003. HSP27 is a ubiquitin-binding protein involved in I-kappaBalpha proteasomal degradation. *Molecular and cellular biology*. 23:5790-5802.

Park, K.J., R.B. Gaynor, and Y.T. Kwak. 2003. Heat shock protein 27 association with the I kappa B kinase complex regulates tumor necrosis factor alpha-induced NF-kappa B activation. *The Journal of biological chemistry*. 278:35272-35278.

Parkkila, S., H. Rajaniemi, A.K. Parkkila, J. Kivela, A. Waheed, S. Pastorekova, J. Pastorek, and W.S. Sly. 2000. Carbonic anhydrase inhibitor suppresses invasion of renal cancer cells in vitro. *Proceedings of the National Academy of Sciences of the United States of America*. 97:2220-2224.

Paul, C., F. Manero, S. Gonin, C. Kretz-Remy, S. Virot, and A.P. Arrigo. 2002. Hsp27 as a negative regulator of cytochrome C release. *Molecular and cellular biology*. 22:816-834.

Pei, H., H. Zhu, S. Zeng, Y. Li, H. Yang, L. Shen, J. Chen, L. Zeng, J. Fan, X. Li, Y. Gong, and H. Shen. 2007. Proteome analysis and tissue microarray for profiling protein markers associated with lymph node metastasis in colorectal cancer. *Journal of proteome research*. 6:2495-2501.

Peinado, H., M. Del Carmen Iglesias-de la Cruz, D. Olmeda, K. Csiszar, K.S. Fong, S. Vega, M.A. Nieto, A. Cano, and F. Portillo. 2005. A molecular role for lysyl oxidase-like 2 enzyme in snail regulation and tumor progression. *The EMBO journal*. 24:3446-3458.

Pennacchietti, S., P. Michieli, M. Galluzzo, M. Mazzone, S. Giordano, and P.M. Comoglio. 2003. Hypoxia promotes invasive growth by transcriptional activation of the met protooncogene. *Cancer cell*. 3:347-361.

Perez de Heredia, F., I.S. Wood, and P. Trayhurn. 2010. Hypoxia stimulates lactate release and modulates monocarboxylate transporter (MCT1, MCT2, and MCT4) expression in human adipocytes. *Pflugers Arch*. 459:509-518.

Perng, M.D., L. Cairns, I.P. van den, A. Prescott, A.M. Hutcheson, and R.A. Quinlan. 1999. Intermediate filament interactions can be altered by HSP27 and alphaB-crystallin. *Journal of cell science*. 112 (Pt 13):2099-2112.

Pichiule, P., J.C. Chavez, and J.C. LaManna. 2004. Hypoxic regulation of angiopoietin-2 expression in endothelial cells. *The Journal of biological chemistry*. 279:12171-12180.

Piotrowicz, R.S., E. Hickey, and E.G. Levin. 1998. Heat shock protein 27 kDa expression and phosphorylation regulates endothelial cell migration. *Faseb J*. 12:1481-1490.

Pirkkala, L., P. Nykanen, and L. Sistonen. 2001. Roles of the heat shock transcription factors in regulation of the heat shock response and beyond. *Faseb J*. 15:1118-1131.

Pivovarova, A.V., N.A. Chebotareva, I.S. Chernik, N.B. Gusev, and D.I. Levitsky. 2007. Small heat shock protein Hsp27 prevents heat-induced aggregation of F-actin by forming soluble complexes with denatured actin. *The FEBS journal*. 274:5937-5948.

Porter, W., F. Wang, W. Wang, R. Duan, and S. Safe. 1996. Role of estrogen receptor/Sp1 complexes in estrogen-induced heat shock protein 27 gene expression. *Molecular endocrinology (Baltimore, Md*. 10:1371-1378.

Pouyssegur, J., F. Dayan, and N.M. Mazure. 2006. Hypoxia signalling in cancer and approaches to enforce tumour regression. *Nature*. 441:437-443.

Preville, X., F. Salvemini, S. Giraud, S. Chaufour, C. Paul, G. Stepien, M.V. Ursini, and A.P. Arrigo. 1999. Mammalian small stress proteins protect against oxidative stress through their ability to increase glucose-6-phosphate dehydrogenase activity and by maintaining optimal cellular detoxifying machinery. *Experimental cell research*. 247:61-78.

Pugh, C.W., and P.J. Ratcliffe. 2003. Regulation of angiogenesis by hypoxia: role of the HIF system. *Nature medicine*. 9:677-684.

Qian, D., H.Y. Lin, H.M. Wang, X. Zhang, D.L. Liu, Q.L. Li, and C. Zhu. 2004. Involvement of ERK1/2 pathway in TGF-beta1-induced VEGF secretion in normal human cytotrophoblast cells. *Molecular reproduction and development*. 68:198-204.

Rafiee, P., M.E. Theriot, V.M. Nelson, J. Heidemann, Y. Kanaa, S.A. Horowitz, A. Rogaczewski, C.P. Johnson, I. Ali, R. Shaker, and D.G. Binion. 2006. Human esophageal microvascular endothelial cells respond to acidic pH stress by PI3K/AKT and p38 MAPK-regulated induction of Hsp70 and Hsp27. *Am J Physiol Cell Physiol*. 291:C931-945.

Richard, C.L., E.Y. Tan, and J. Blay. 2006. Adenosine upregulates CXCR4 and enhances the proliferative and migratory responses of human carcinoma cells to CXCL12/SDF-1alpha. *International journal of cancer*. 119:2044-2053.

Richard, D.E., E. Berra, and J. Pouyssegur. 2000. Nonhypoxic pathway mediates the induction of hypoxia-inducible factor 1alpha in vascular smooth muscle cells. *The Journal of biological chemistry*. 275:26765-26771.

Rogalla, T., M. Ehrnsperger, X. Preville, A. Kotlyarov, G. Lutsch, C. Ducasse, C. Paul, M. Wieske, A.P. Arrigo, J. Buchner, and M. Gaestel. 1999. Regulation of Hsp27 oligomerization, chaperone function, and protective activity against oxidative stress/tumor necrosis factor alpha by phosphorylation. *The Journal of biological chemistry*. 274:18947-18956.

Rombouts, E.J., B. Pavic, B. Lowenberg, and R.E. Ploemacher. 2004. Relation between CXCR-4 expression, Flt3 mutations, and unfavorable prognosis of adult acute myeloid leukemia. *Blood*. 104:550-557.

Rousseau, S., I. Dolado, V. Beardmore, N. Shpiro, R. Marquez, A.R. Nebreda, J.S. Arthur, L.M. Case, M. Tessier-Lavigne, M. Gaestel, A. Cuenda, and P. Cohen. 2006. CXCL12 and C5a trigger cell migration via a PAK1/2-p38alpha MAPK-MAPKAP-K2-HSP27 pathway. *Cellular signalling*. 18:1897-1905.

Rousseau, S., F. Houle, J. Landry, and J. Huot. 1997. p38 MAP kinase activation by vascular endothelial growth factor mediates actin reorganization and cell migration in human endothelial cells. *Oncogene*. 15:2169-2177.

Ruan, K., G. Song, and G. Ouyang. 2009. Role of hypoxia in the hallmarks of human cancer. *Journal of cellular biochemistry*. 107:1053-1062.

Rudlowski, C., A.J. Becker, W. Schroder, W. Rath, R. Buttner, and M. Moser. 2003. GLUT1 messenger RNA and protein induction relates to the malignant transformation of cervical cancer. *American journal of clinical pathology*. 120:691-698.

Rust, W., K. Kingsley, T. Petnicki, S. Padmanabhan, S.W. Carper, and G.E. Plopper. 1999. Heat shock protein 27 plays two distinct roles in controlling human breast cancer cell migration on laminin-5. *Mol Cell Biol Res Commun*. 1:196-202.

Sakamoto, K., T. Urushidani, and T. Nagao. 1998. Translocation of HSP27 to cytoskeleton by repetitive hypoxia-reoxygenation in the rat myoblast cell line, H9c2. *Biochemical and biophysical research communications*. 251:576-579.

Salcedo, R., K. Wasserman, H.A. Young, M.C. Grimm, O.M. Howard, M.R. Anver, H.K. Kleinman, W.J. Murphy, and J.J. Oppenheim. 1999. Vascular endothelial growth factor and basic fibroblast growth factor induce expression of CXCR4 on human endothelial cells: In vivo neovascularization induced by stromal-derived factor-1alpha. *The American journal of pathology*. 154:1125-1135.

Samali, A., and T.G. Cotter. 1996. Heat shock proteins increase resistance to apoptosis. *Experimental cell research*. 223:163-170.

Samali, A., J.D. Robertson, E. Peterson, F. Manero, L. van Zeijl, C. Paul, I.A. Cotgreave, A.P. Arrigo, and S. Orrenius. 2001. Hsp27 protects mitochondria of thermotolerant cells against apoptotic stimuli. *Cell stress & chaperones*. 6:49-58.

Sandberg, G. 1983. Regulation of thymocyte proliferation by endogenous adenosine and adenosine deaminase. *International journal of immunopharmacology*. 5:259-265.

Santoro, M.G. 2000. Heat shock factors and the control of the stress response. *Biochemical pharmacology*. 59:55-63.

Sarge, K.D., S.P. Murphy, and R.I. Morimoto. 1993. Activation of heat shock gene transcription by heat shock factor 1 involves oligomerization, acquisition of DNA-binding activity, and nuclear localization and can occur in the absence of stress. *Molecular and cellular biology*. 13:1392-1407.

Saura, C., F. Ciruela, V. Casado, E.I. Canela, J. Mallol, C. Lluis, and R. Franco. 1996. Adenosine deaminase interacts with A1 adenosine receptors in pig brain cortical membranes. *Journal of neurochemistry*. 66:1675-1682.

Savransky, V., A. Nanayakkara, J. Li, S. Bevans, P.L. Smith, A. Rodriguez, and V.Y. Polotsky. 2007. Chronic intermittent hypoxia induces atherosclerosis. *American journal of respiratory and critical care medicine*. 175:1290-1297.

Schioppa, T., B. Uranchimeg, A. Saccani, S.K. Biswas, A. Doni, A. Rapisarda, S. Bernasconi, S. Saccani, M. Nebuloni, L. Vago, A. Mantovani, G. Melillo, and A. Sica. 2003. Regulation of the chemokine receptor CXCR4 by hypoxia. *The Journal of experimental medicine*. 198:1391-1402.

Schmaltz, C., P.H. Hardenbergh, A. Wells, and D.E. Fisher. 1998. Regulation of proliferation-survival decisions during tumor cell hypoxia. *Molecular and cellular biology*. 18:2845-2854.

Schmid, B.C., M. Rudas, G.A. Rezniczek, S. Leodolter, and R. Zeillinger. 2004. CXCR4 is expressed in ductal carcinoma in situ of the breast and in atypical ductal hyperplasia. *Breast cancer research and treatment*. 84:247-250.

Seimiya, H., M. Tanji, T. Oh-hara, A. Tomida, I. Naasani, and T. Tsuruo. 1999. Hypoxia up-regulates telomerase activity via mitogen-activated protein kinase signaling in human solid tumor cells. *Biochemical and biophysical research communications*. 260:365-370.

Semenza, G.L. 1998. Hypoxia-inducible factor 1: master regulator of O2 homeostasis. *Current opinion in genetics & development*. 8:588-594.

Semenza, G.L., B.H. Jiang, S.W. Leung, R. Passantino, J.P. Concordet, P. Maire, and A. Giallongo. 1996. Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. *The Journal of biological chemistry*. 271:32529-32537.

Semenza, G.L., P.H. Roth, H.M. Fang, and G.L. Wang. 1994. Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. *The Journal of biological chemistry*. 269:23757-23763.

Sengupta, S., R. Schiff, and B.S. Katzenellenbogen. 2009. Post-transcriptional regulation of chemokine receptor CXCR4 by estrogen in HER2 overexpressing, estrogen receptor-positive breast cancer cells. *Breast cancer research and treatment*. 117:243-251.

Shi, P., M.M. Wang, L.Y. Jiang, H.T. Liu, and J.Z. Sun. 2008. Paclitaxel-doxorubicin sequence is more effective in breast cancer cells with heat shock protein 27 overexpression. *Chinese medical journal*. 121:1975-1979.

Shi, Y., D.D. Mosser, and R.I. Morimoto. 1998. Molecular chaperones as HSF1-specific transcriptional repressors. *Genes & development*. 12:654-666.

Shi, Y., K. Nishida, D. Campigli Di Giammartino, and J.L. Manley. 2011. Heat shock-induced SRSF10 dephosphorylation displays thermotolerance mediated by Hsp27. *Molecular and cellular biology*. 31:458-465.

Shimizu, Y., and S.W. Hunt, 3rd. 1996. Regulating integrin-mediated adhesion: one more function for PI 3-kinase? *Immunology today*. 17:565-573.

Shimoda, L.A., M. Fallon, S. Pisarcik, J. Wang, and G.L. Semenza. 2006. HIF-1 regulates hypoxic induction of NHE1 expression and alkalinization of intracellular pH in pulmonary arterial myocytes. *American journal of physiology*. 291:L941-949.

Shin, K.D., M.Y. Lee, D.S. Shin, S. Lee, K.H. Son, S. Koh, Y.K. Paik, B.M. Kwon, and D.C. Han. 2005. Blocking tumor cell migration and invasion with biphenyl isoxazole derivative KRIBB3, a synthetic molecule that inhibits Hsp27 phosphorylation. *The Journal of biological chemistry*. 280:41439-41448.

Slamon, D.J., G.M. Clark, S.G. Wong, W.J. Levin, A. Ullrich, and W.L. McGuire. 1987. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science (New York, N.Y.* 235:177-182.

Smith, M.C., K.E. Luker, J.R. Garbow, J.L. Prior, E. Jackson, D. Piwnica-Worms, and G.D. Luker. 2004. CXCR4 regulates growth of both primary and metastatic breast cancer. *Cancer research*. 64:8604-8612.

Smith, M.L., J.M. Ford, M.C. Hollander, R.A. Bortnick, S.A. Amundson, Y.R. Seo, C.X. Deng, P.C. Hanawalt, and A.J. Fornace, Jr. 2000. p53-mediated DNA repair responses to UV radiation: studies of mouse cells lacking p53, p21, and/or gadd45 genes. *Molecular and cellular biology*. 20:3705-3714.

Soengas, M.S., R.M. Alarcon, H. Yoshida, A.J. Giaccia, R. Hakem, T.W. Mak, and S.W. Lowe. 1999. Apaf-1 and caspase-9 in p53-dependent apoptosis and tumor inhibition. *Science (New York, N.Y.* 284:156-159.

Soldatenkov, V.A., and A. Dritschilo. 1997. Apoptosis of Ewing's sarcoma cells is accompanied by accumulation of ubiquitinated proteins. *Cancer research*. 57:3881-3885.

Song, G., G. Ouyang, and S. Bao. 2005. The activation of Akt/PKB signaling pathway and cell survival. *Journal of cellular and molecular medicine*. 9:59-71.

Spychala, J. 2000. Tumor-promoting functions of adenosine. *Pharmacology & therapeutics*. 87:161-173.

Stagg, J., and M.J. Smyth. 2010. Extracellular adenosine triphosphate and adenosine in cancer. *Oncogene*. 29:5346-5358.

Staller, P., J. Sulitkova, J. Lisztwan, H. Moch, E.J. Oakeley, and W. Krek. 2003. Chemokine receptor CXCR4 downregulated by von Hippel-Lindau tumour suppressor pVHL. *Nature*. 425:307-311.

Stokoe, D., D.G. Campbell, S. Nakielny, H. Hidaka, S.J. Leevers, C. Marshall, and P. Cohen. 1992a. MAPKAP kinase-2; a novel protein kinase activated by mitogen-activated protein kinase. *The EMBO journal*. 11:3985-3994.

Stokoe, D., K. Engel, D.G. Campbell, P. Cohen, and M. Gaestel. 1992b. Identification of MAPKAP kinase 2 as a major enzyme responsible for the phosphorylation of the small mammalian heat shock proteins. *FEBS letters*. 313:307-313.

Storm, F.K., K.W. Gilchrist, T.F. Warner, and D.M. Mahvi. 1995. Distribution of Hsp-27 and HER-2/neu in in situ and invasive ductal breast carcinomas. *Annals of surgical oncology*. 2:43-48.

Sun, Y., and T.H. MacRae. 2005. Small heat shock proteins: molecular structure and chaperone function. *Cell Mol Life Sci*. 62:2460-2476.

Suzuki, H., H. Sugimura, and K. Hashimoto. 2007. Overexpression of heat shock protein 27 is associated with good prognosis in the patient with oral squamous cell carcinoma. *The British journal of oral & maxillofacial surgery*. 45:123-129.

Suzuki, H., A. Tomida, and T. Tsuruo. 2001. Dephosphorylated hypoxia-inducible factor 1alpha as a mediator of p53-dependent apoptosis during hypoxia. *Oncogene*. 20:5779-5788.

Tachibana, K., S. Hirota, H. Iizasa, H. Yoshida, K. Kawabata, Y. Kataoka, Y. Kitamura, K. Matsushima, N. Yoshida, S. Nishikawa, T. Kishimoto, and T. Nagasawa. 1998. The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. *Nature*. 393:591-594.

Tan, E.Y., M. Mujoomdar, and J. Blay. 2004. Adenosine down-regulates the surface expression of dipeptidyl peptidase IV on HT-29 human colorectal carcinoma cells: implications for cancer cell behavior. *The American journal of pathology*. 165:319-330.

Tan, W., D. Martin, and J.S. Gutkind. 2006. The Galpha13-Rho signaling axis is required for SDF-1-induced migration through CXCR4. *The Journal of biological chemistry*. 281:39542-39549.

Taylor, R.P., and I.J. Benjamin. 2005. Small heat shock proteins: a new classification scheme in mammals. *Journal of molecular and cellular cardiology*. 38:433-444.

Teicher, B.A., S.A. Holden, A. al-Achi, and T.S. Herman. 1990. Classification of antineoplastic treatments by their differential toxicity toward putative oxygenated and hypoxic tumor subpopulations in vivo in the FSaIIC murine fibrosarcoma. *Cancer research*. 50:3339-3344.

Teimourian, S., R. Jalal, M. Sohrabpour, and B. Goliaei. 2006. Down-regulation of Hsp27 radiosensitizes human prostate cancer cells. *Int J Urol*. 13:1221-1225.

Theriault, J.R., H. Lambert, A.T. Chavez-Zobel, G. Charest, P. Lavigne, and J. Landry. 2004. Essential role of the NH2-terminal WD/EPF motif in the phosphorylation-activated protective function of mammalian Hsp27. *The Journal of biological chemistry*. 279:23463-23471.

Thomlinson, R.H., and L.H. Gray. 1955. The histological structure of some human lung cancers and the possible implications for radiotherapy. *British journal of cancer*. 9:539-549.

Thor, A., C. Benz, D. Moore, 2nd, E. Goldman, S. Edgerton, J. Landry, L. Schwartz, B. Mayall, E. Hickey, and L.A. Weber. 1991. Stress response protein (srp-27) determination in primary human breast carcinomas: clinical, histologic, and prognostic correlations. *Journal of the National Cancer Institute*. 83:170-178.

Tian, Y.M., K.K. Yeoh, M.K. Lee, T. Eriksson, B.M. Kessler, H.B. Kramer, M.J. Edelmann, C. Willam, C.W. Pugh, C.J. Schofield, and P.J. Ratcliffe. 2011. Differential sensitivity of hypoxia inducible factor hydroxylation sites to hypoxia and hydroxylase inhibitors. *The Journal of biological chemistry*. 286:13041-13051.

Tweedle, E.M., I. Khattak, C.W. Ang, T. Nedjadi, R. Jenkins, B.K. Park, H. Kalirai, A. Dodson, B. Azadeh, M. Terlizzo, H. Grabsch, W. Mueller, S. Myint, P. Clark, H. Wong, W. Greenhalf, J.P. Neoptolemos, P.S. Rooney, and E. Costello. 2010. Low molecular weight heat shock protein HSP27 is a prognostic indicator in rectal cancer but not colon cancer. *Gut*. 59:1501-1510.

Vaupel, P., F. Kallinowski, and P. Okunieff. 1989a. Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review. *Cancer research*. 49:6449-6465.

Vaupel, P., A. Mayer, and M. Hockel. 2004. Tumor hypoxia and malignant progression. *Methods in enzymology*. 381:335-354.

Vaupel, P., P. Okunieff, and L.J. Neuringer. 1989b. Blood flow, tissue oxygenation, pH distribution, and energy metabolism of murine mammary adenocarcinomas during growth. *Advances in experimental medicine and biology*. 248:835-845.

Vaupel, P., O. Thews, and M. Hoeckel. 2001. Treatment resistance of solid tumors: role of hypoxia and anemia. *Medical oncology (Northwood, London, England)*. 18:243-259.

Vicart, P., A. Caron, P. Guicheney, Z. Li, M.C. Prevost, A. Faure, D. Chateau, F. Chapon, F. Tome, J.M. Dupret, D. Paulin, and M. Fardeau. 1998. A missense mutation in the alphaB-crystallin chaperone gene causes a desmin-related myopathy. *Nature genetics*. 20:92-95.

Wagstaff, M.J., Y. Collaco-Moraes, B.S. Aspey, R.S. Coffin, M.J. Harrison, D.S. Latchman, and J.S. de Belleroche. 1996. Focal cerebral ischaemia increases the levels of several classes of heat shock proteins and their corresponding mRNAs. *Brain research*. 42:236-244.

Wang, G.L., B.H. Jiang, E.A. Rue, and G.L. Semenza. 1995a. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. *Proceedings of the National Academy of Sciences of the United States of America*. 92:5510-5514.

Wang, T., J. Sodhi, R.M. Mentzer, Jr., and D.G. Van Wylen. 1994. Changes in interstitial adenosine during hypoxia: relationship to oxygen supply:demand imbalance, and effects of adenosine deaminase. *Cardiovascular research*. 28:1320-1325.

Wang, X., C. Li, Y. Chen, Y. Hao, W. Zhou, C. Chen, and Z. Yu. 2008. Hypoxia enhances CXCR4 expression favoring microglia migration via HIF-1alpha activation. *Biochemical and biophysical research communications*. 371:283-288.

Wang, X.W., H. Yeh, L. Schaeffer, R. Roy, V. Moncollin, J.M. Egly, Z. Wang, E.C. Freidberg, M.K. Evans, B.G. Taffe, and et al. 1995b. p53 modulation of TFIIH-associated nucleotide excision repair activity. *Nature genetics*. 10:188-195.

Weinberg, R.A. 1995. The retinoblastoma protein and cell cycle control. *Cell*. 81:323-330.

Whitlock, N.A., N. Agarwal, J.X. Ma, and C.E. Crosson. 2005. Hsp27 upregulation by HIF-1 signaling offers protection against retinal ischemia in rats. *Investigative ophthalmology & visual science*. 46:1092-1098.

Woodhouse, E.C., D.F. Amanatullah, J.A. Schetz, L.A. Liotta, M.L. Stracke, and T. Clair. 1998. Adenosine receptor mediates motility in human melanoma cells. *Biochemical and biophysical research communications*. 246:888-894.

Wouters, B.G., and J.M. Brown. 1997. Cells at intermediate oxygen levels can be more important than the "hypoxic fraction" in determining tumor response to fractionated radiotherapy. *Radiation research*. 147:541-550.

Xiao, X., X. Zuo, A.A. Davis, D.R. McMillan, B.B. Curry, J.A. Richardson, and I.J. Benjamin. 1999. HSF1 is required for extra-embryonic development, postnatal growth and protection during inflammatory responses in mice. *The EMBO journal*. 18:5943-5952.

Yarden, Y., and M.X. Sliwkowski. 2001. Untangling the ErbB signalling network. *Nature reviews*. 2:127-137.

Yu, F., S.B. White, Q. Zhao, and F.S. Lee. 2001. HIF-1alpha binding to VHL is regulated by stimulus-sensitive proline hydroxylation. *Proceedings of the National Academy of Sciences of the United States of America*. 98:9630-9635.

Yuan, J., L. Narayanan, S. Rockwell, and P.M. Glazer. 2000. Diminished DNA repair and elevated mutagenesis in mammalian cells exposed to hypoxia and low pH. *Cancer research*. 60:4372-4376.

Zhang, D., L.L. Wong, and E.S. Koay. 2007. Phosphorylation of Ser78 of Hsp27 correlated with HER-2/neu status and lymph node positivity in breast cancer. *Molecular cancer*. 6:52.

Zhang, S.X., D. Gozal, L.R. Sachleben, Jr., M. Rane, J.B. Klein, and E. Gozal. 2003. Hypoxia induces an autocrine-paracrine survival pathway via platelet-derived growth factor (PDGF)-B/PDGF-beta receptor/phosphatidylinositol 3-kinase/Akt signaling in RN46A neuronal cells. *Faseb J*. 17:1709-1711.

Zhong, H., K. Chiles, D. Feldser, E. Laughner, C. Hanrahan, M.M. Georgescu, J.W. Simons, and G.L. Semenza. 2000. Modulation of hypoxia-inducible factor 1alpha expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. *Cancer research*. 60:1541-1545.

Zhong, H., A.M. De Marzo, E. Laughner, M. Lim, D.A. Hilton, D. Zagzag, P. Buechler, W.B. Isaacs, G.L. Semenza, and J.W. Simons. 1999. Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. *Cancer research*. 59:5830-5835.

Zhuang, H., W. Jiang, W. Cheng, K. Qian, W. Dong, L. Cao, Q. Huang, S. Li, F. Dou, J.F. Chiu, X.X. Fang, M. Lu, and Z.C. Hua. 2010. Down-regulation of HSP27 sensitizes TRAIL-resistant tumor cell to TRAIL-induced apoptosis. *Lung cancer (Amsterdam, Netherlands)*. 68:27-38.

Zou, Y.R., A.H. Kottmann, M. Kuroda, I. Taniuchi, and D.R. Littman. 1998. Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. *Nature*. 393:595-599.