Chapter 1: Introduction

1.1 Heat Shock Proteins

The serendipitous origins of the discovery of the heat shock response date back to the early 1960s when Ferruccio Ritossa, then a student at the Genetics Institute in Pavia in Italy, was studying nucleic acid synthesis in the salivary glands of *Drosophila*. As recalled by Ritossa [1], a colleague had inadvertently increased the temperature of the incubator containing the *Drosophila* larvae, following which a transient induction of puffing in the salivary gland polytene chromosomes was observed under light microscopy [1, 2]. However, it was not until 1974 that enhanced protein synthesis of a select group of proteins in response to heat treatment was observed, following which the universality of this phenomenon as existing from bacteria to humans was recognised shortly thereafter [3, 4]. These proteins, dubbed heat shock proteins (hsps) or stress proteins, have been the subject of broad ranging biological research topics in a variety of fields and in the context of a host of human diseases.

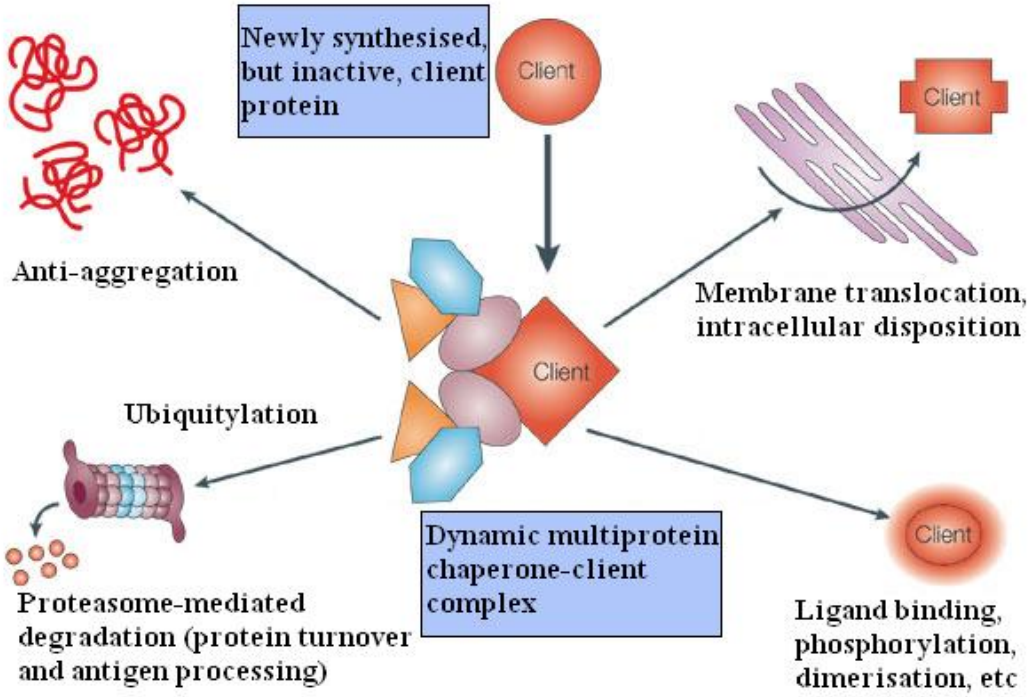
Following exposure to a range of environmental stressors, a major cellular protective mechanism is the increased expression of a select group of heat shock proteins. Hsps are an essential and highly conserved group of proteins that have been identified in every organism examined and are involved in a wide array of cellular processes [5-7]. Hsps are responsible for the folding, translocation and proteolytic turn-over of many of the fundamental regulators of cellular growth, differentiation and survival [8, 9]. As such, hsps play an essential role in the maintenance of cellular homeostasis (Fig. 1.1). Hsps perform these roles intracellularly, on the cell surface and extracellularly [8, 10-12]. Despite their name, hsps are expressed constitutively in addition to their up-regulation in response to stress [9]. They provide protection against an extensive range of cellular stressors by chaperoning and thereby mediating the activity of proteins in a non-covalent manner [9, 13]. Hsps stabilise proteins to prevent their denaturation, aggregation and/or

destruction. They also assist the re-folding of denatured proteins, prevent illicit protein interactions and promote the destruction of terminally damaged proteins [9]. This chaperoning role is typically performed as members of multi-protein chaperone complexes that consist of other molecular chaperones [6, 14]. Hsps are also involved in a variety of essential immune system functions [15].

Found in every cell of every organism, hsps are an essential and highly conserved group of proteins that are involved in a plethora of cellular pathways and processes [5-7]. Hsps primarily function as molecular chaperones that mediate the activity of other cellular proteins. In this role they are crucial for the maintenance of protein homeostasis [8-9]. Hsps facilitate the functioning of a range of fundamental cellular processes in addition to being up-regulated in response to and providing protection from a range of cellular stressors. This extensive functionality allows hsps to be involved in a diverse range of cellular processes [8, 13]. Many of these capabilities are highly sought after by malignant cells. Cancer cells are characterised by rapid and uncontrolled cellular proliferation supported by overexpressing oncoproteins. Thus cancer often manifests as a tumour mass in which the constituent cells up-regulate a range of proteins that support their survival and proliferation. These *in tumourio* conditions result in a hostile environment for the functioning of proteins, and conditions such as hypoxia, extremes of pH and other impeding factors to the functioning of proteins are common. These stressors are added to by the overexpression of multiple mutated signal transduction proteins. Thus hsps play roles in providing protection from tumour-associated stressors as well as chaperoning proteins responsible for tumour cell survival and proliferation. There is more to malignant cells than dysfunctional signalling pathways – they must possess a range of characteristics if they are to be successful. Hsps contribute to many of these fundamental characteristics and thus are intimately involved in cancer biology [8].

This thesis explores the biochemical and immunological roles of hsps with an emphasis on hsp90 in breast cancer and melanoma, two of the most common human cancers.

Figure 1.1 Functions of hsps in maintaining protein homeostasis



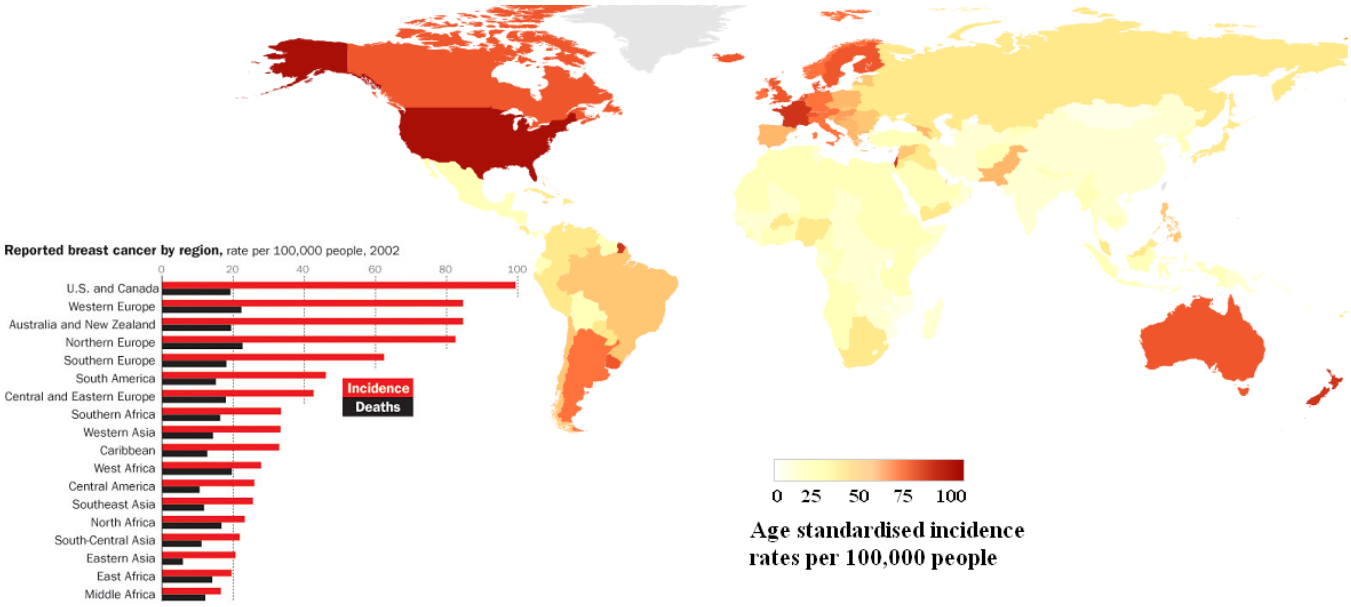
Newly synthesised and for the most part, conformationally unstable proteins are chaperoned by multi-protein chaperone complexes consisting of chaperones, co-chaperones and accessory molecules. In some instances the composition of these complexes is determined by client proteins which in turn specify the function. Dynamic chaperone-client interactions can: (a) Prevent protein aggregation and maintain these aggregated proteins in folding-competent states (b) Participate in intracellular trafficking, particularly across membranes (c) Associate with chaperone complexes and maintain many signalling proteins in meta-stable states that allows their activation by stimuli, and (d) Promote the destruction of unwanted or terminally damaged proteins through the ubiquitin proteasome pathway. Adapted from Whitesell and Lindquist [8].

1.2 Breast Cancer

Cancer is a major cause of mortality that accounts for approximately 13 % of all deaths worldwide. Among women, breast cancer is the 5th leading cause of mortality and is responsible for the majority of cancer deaths, with over 500,000 per year [16]. Breast cancer is also the most common cancer in women [17] (excluding two types of non-reportable skin cancer), and accounts for 23 % of all cancers and with over one million new cases per year. In developed countries (excluding Japan) breast cancer incidence is high (Fig. 1.2) and accounts for 2 % of all deaths, while in poor and developing countries breast cancer is responsible for 0.5 % of all deaths [18, 19]. Breast cancer incidence is increasing in most countries around the world [19], while in Australia the Age Standardised Incidence (ASI) of new cases peaked in 2001. Since then, the ASI of new cases has decreased slightly and stabilised, while the mortality rate has been falling [20]. Despite the worldwide increase in breast cancer mortality, it is decreasing in developed countries. This is attributed to improvements in disease management – namely diagnosis and treatment [17, 18].

Although developing countries have historically had low rates of breast cancer, it is these regions where breast cancer incidence is increasing at the greatest rate. This is most likely due to a combination of factors [19, 21]. In the low to middle income countries life expectancy has increased from 50 years of age in 1965 to 65 years of age in 2000. This has resulted in more women entering the 50-60+ age range in which breast cancer risk is the greatest. In addition, western lifestyle habits are a known risk factor and their increasing adoption in these countries may be a contributing factor to the increased breast cancer incidence. Specifically, factors such as high caloric diets, sedentary lifestyles and western reproductive characteristics including age of menarche, timing and number of offspring as well as other factors that influence breast cancer risk are becoming more widespread [18, 21].

Figure 1.2 Worldwide breast cancer incidence and mortality

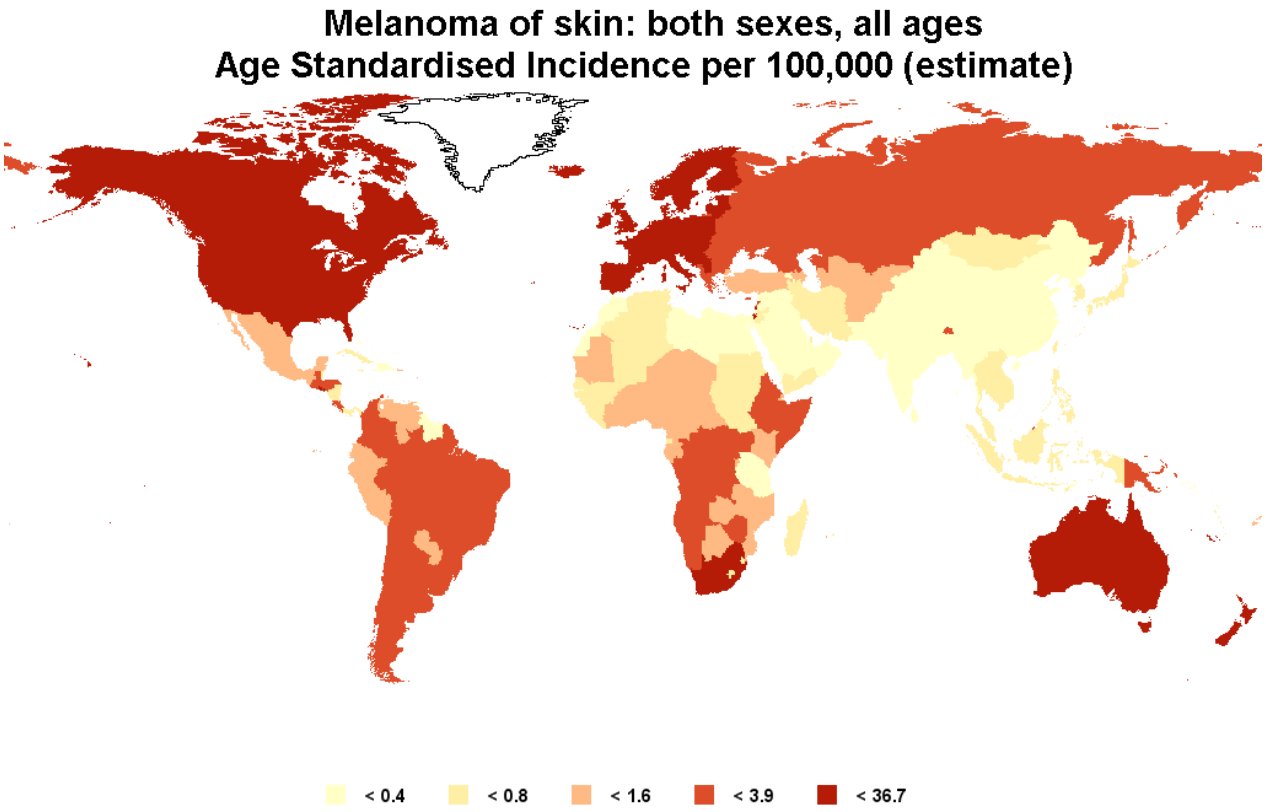


Breast cancer incidence is highest in wealthy countries, but it is here where mortality rates are showing the slowest increases or falling. In poorer regions the incidence of breast cancer is increasing faster than in wealthy countries. Figure adapted from Time Magazine [21].

1.3 Melanoma

Each year between 160,000 and 210,000 new cases of melanoma are reported throughout the world. The majority (80 %) occur in North America, Europe, Australia and New Zealand (Fig. 1.3), with incidence comparable between the sexes. Of these regions, white skinned populations in Australia are reported to have the highest incidence of melanoma in the world. Melanoma accounts for the majority of skin cancer deaths, with over 65,000 deaths in the year 2000. In many countries there is a trend of increasing incidence and mortality. The two most important risk factors are UV light exposure and the degree of skin pigmentation, with the former having a positive relationship and the latter an inverse relationship with melanoma risk. Of note is that sporadic but intense exposures are an important risk factor as well as chronic high level exposure [22, 23].

Figure 1.3 Melanoma incidence worldwide



Melanoma incidence is greatest in countries with predominately lighter skinned populations. Figure adapted from the GLOBOCAN database [24].

Survival from melanoma is greatly influenced by the degree of disease progression. If treatment is provided at or before stage 2a, the year ten relative survival is greater than 76 % [22]. This compares with 34 % for stage 4b, while less than 5 % of patients displaying systemic metastasis survive beyond two years. This is partly due to the relatively few treatment options available, although new therapies have recently become available [22, 25, 215, 216].

1.4 Hsp40

The hsp40 group of molecular chaperones are important for protein folding, refolding, unfolding, translation, degradation, aggregation suppression and translocation. This is primarily achieved through their role as intimate hsp70 co-chaperones. In this regard the predominate function of hsp40 is to regulate the ATPase activity of hsp70. This in turn regulates the activity of hsp70 by stabilising its interaction with substrates. The hsp40 proteins contain a J domain through which they bind to hsp70. Some hsp40 family members may also regulate the activity of other molecular chaperones such as hsp90. Despite their role as crucial co-chaperones, the hsp40s can bind directly to substrate proteins through zinc finger and C-terminal domains and thereby chaperone substrate proteins directly. In humans, genome wide analysis has shown that the hsp40 group consists of 41 members, while more than 20 homologues have been identified in mammals. The function of these family members depends on their localisation. In cells, they are present in multiple cellular compartments, while in multicellular organisms differential or universal tissue expression patterns may occur [26].

1.5 Hsp60

Hsp60 was long thought to be restricted to the mitochondria, but more recent studies have demonstrated that it also exists in the cytosol, both on the cell surface and extracellularly. Bacterial hsp60 (GroEL) has been well characterised as a molecular chaperone essential to protein folding [27], but human hsp60 has not been characterised as thoroughly. Hsp60 plays roles in

protein folding, trafficking and appears to be involved in a variety of cellular processes, which are yet to be fully elucidated. It is known that hsp60 associates with hsp10 to chaperone nascent polypeptides to native or functional conformations. Hsp60 also plays roles in apoptosis where it interacts with mitochondrial hsp70, survivin and p53. The mitochondrial and cytosol forms may function as both pro-survival or pro-apoptotic regulators, depending on context [28].

1.6 Hsp70

The hsp70 group of chaperone proteins play important roles in an array of folding processes including in the folding and assembly of newly synthesised proteins, solubilisation and refolding of aggregated or misfolded proteins, protein translocation, proteins disassembly as well as controlling the activity of regulatory proteins through transient interaction. Hsp70, therefore, plays important roles in the maintenance of protein homeostasis and forms part of multiple protein networks such as those involved in signal transduction and folding complexes. Many of these functions are achieved by recognition of hydrophobic regions in substrate proteins. Hsp70 consists of an N-terminal adenosine triphosphate (ATP) binding domain and a C-terminal substrate binding domain. Hsp70 performs specific functions with the assistance of other chaperone proteins such as hsp90. These co-chaperones can be divided into three groups: 1) J-domain co-chaperones (e.g. hsp40) bind to the hsp70 ATP binding domain and stimulate ATPase activity. 2) Nucleotide exchange factor co-chaperones (e.g. hsp110, hspBP1) that catalyse the release of adenosine triphosphate (ADP). These are required for completion of the hsp70 ATPase cycle. 3) Tetratrico Peptide Repeat (TPR) domain containing co-chaperones (e.g. hsp90, Hop, CHIP) are required for assembly of chaperone complexes [228]. The diversity of hsp70 function is achieved through functional mediation by co-chaperones. Many of these co-chaperones target hsp70 to specific substrates or control the release of hsp70 bound complexes. In performing this function, hsp70 co-chaperones connect hsp70 complexes to target proteins (including substrates and chaperones) by interacting with both the target and the chaperone. The ATPase cycle of hsp70

alternates between an ATP state with low affinity and fast exchange rates for substrates, and an ADP state that has high affinity and low exchange rates for substrates. This ATPase cycle is controlled by the activity of co-chaperones which can determine the lifetime of the hsp70-substrate complexes [29].

1.7 Hsp90

Hsp90 constitutes a family of chaperones that are present in eubacteria and all eukaryotes. Hsp90 is one of the most abundantly expressed and widely distributed chaperoning proteins. In eukaryotes it is ubiquitously distributed and essential for viability. Two hsp90 homologues are found in eukaryotic cellular compartments: TRAP-1/hsp75 in the mitochrondria and GRP94/GP96 in the endoplasmic reticulum (ER). Several isoforms exist, such as the α, β and N isoforms [6, 12]. Of the 17 genes that code for the hsp90 family in humans, six code for functional proteins [30]. Hsp90 is constitutively expressed at high levels and it is one of the most abundant proteins expressed under basal conditions, but expression may increase further in response to stress [31]. It resides in the cytoplasm and nucleus and its localisation may change following exposure to cellular stress [32, 33]. Hsp90 performs an essential function in the maintenance of protein homeostasis and is required by a large number of proteins including transcription factors, signal transduction and tumour suppressor proteins that control the activity of a range of physiological processes [34, 35]. This places hsp90 in a pivotal position to influence cellular survival, growth and developmental processes in addition to being involved in mitochondrial homeostasis and the propagation of RNA viruses. Hsp90 function is thus highly complex and is mediated by a select group of cofactors and co-chaperones in addition to post-translational regulation [6, 36]. Hsp90 may function in the folding of newly synthesised proteins, by stabilising folded proteins or by chaperoning unfolded proteins thereby maintaining them in a folding-competent state [6, 14]. Hsp90 associates with a large number of client proteins and the current conservative estimate exceeds 100, with this estimate continually increasing [37]. A recent review suggests hsp90 may

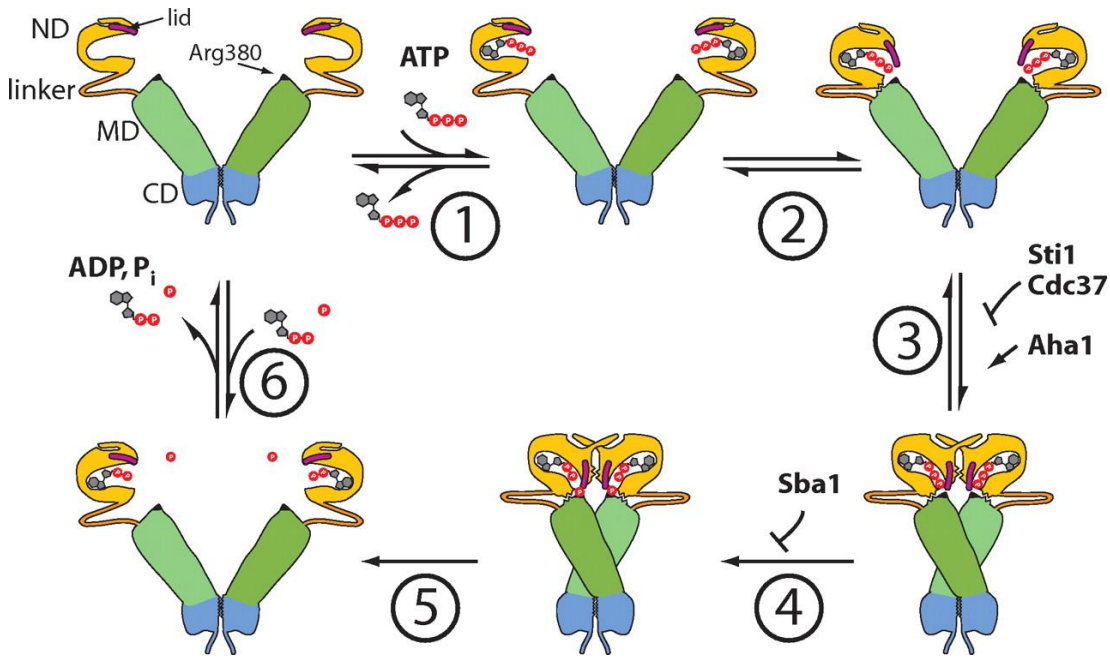
have over 200 client proteins [228]. Due to its chaperoning role, hsp90 may be able to conceal genetic mutations and variation and, as such, may play a role in potentiating evolution [38].

1.7.1 Hsp90 structure and function

Hsp90 functions primarily as a phosphorylated homodimer. Each monomer is approximately 90 kDa and consists of an N-terminal (N), Middle (M) and C-terminal (C) domains. Nucleotide binding sites exist in both the N-terminal and C-terminal domains. The N-terminal nucleotide binding domain contains a deep ATP binding pocket that binds ATP in a distinctive kinked conformation with low affinity. The N-terminal domain has high nucleotide specificity and belongs to the GHKL class of ATP-hydrolysing enzymes [35, 36, 39]. By contrast, the C-terminal nucleotide binding domain is more promiscuous and has been shown to bind both purine and pyrimidine nucleotides such as GTP and UTP. Consequently these nucleotides are C-terminal specific [39]. ATP hydrolysis is essential for hsp90 function. The process is complex, relatively slow and associated with hsp90 dimerisation. Following ATP binding, a short segment in the N-domain referred to as the –ATP lid" changes position and moves over the ATP binding pocket [35, 36]. Subsequently, another short N-terminal segment is released and binds to the other monomer, resulting in a twisted, linked and partially folded dimer (Fig. 1.4 step 3). Further conformational changes result in the re-organisation and association of the N- and M-domains. In yeast, this results in a highly conserved arginine residue in the M-domain catalytic loop to associate with the ATP γ-phosphate. This residue is the only amino acid in contact with the γ-phosphate and appears to be required for ATP hydrolysis. Crystal structure analyses have found this conformation to vary, suggesting conformational freedom exists to allow for influence by co-chaperones and client proteins. The arginine residue on the catalytic loop forms a hydrophobic network with a loop on the other monomer in this closed state, suggesting cross monomer coupling of closure and hydrolysis. ATP binding results in a change to the closed state, in which hydrophobic surfaces are less exposed [40]. Re-organisation of the N- and M-domains completes

the dimerisation process (Fig. 1.4). It is the conformational change in the nucleotide binding domains of hsp90 that regulate ATP hydrolysis. Specifically, the slow conformational changes of the lid segment within the N-domain are apparently the cause of the relatively slow rate of ATP hydrolysis [36, 41]. Despite this slow rate of hydrolysis, certain client proteins and co-chaperones may enhance it [36, 42]. This folding mechanism occurs with GP96, TRAP-1 as well as human hsp90 and therefore may be the conserved folding mechanism for all hsp90 family members [36].

Figure 1.4 Hsp90 dimerisation model



Upon ATP binding (step 1) the ATP lid segment closes over the N-terminal ATP binding pocket (step 2). Following this, the first 24 amino acids of each monomer dimerise and the first β strand and α helix associate with the N-terminal domain of the other monomer. Within each monomer the N-terminal domain contacts the Middle domain (step 3). This results in compaction of the hsp90 dimer in which the two monomers are twisted around each other [40] (step 4). Following ATP hydrolysis the N-terminal domains disassociate and both monomers separate N-terminally. The ATP lid opens which allows the release of ADP and P_i. Subsequently, hsp90 returns to the initial state (steps 5 and 6). ND, N-terminal Domain. MD, Middle Domain. CD, C-Terminal domain. Adapted from Wandinger *et al*. [36].

The two major hsp90 isoforms are hsp90α (nominally the inducible form) and hsp90β (nominally the constitutive form). Hsp90 exists predominately as homodimers (αα and ββ) and monomers (α and β), however, heterodimers (αβ) can also occur. Homologues exist in the mitochondria (TRAP-1), in the ER (GRP94/GP96) in addition to a third homologue apparently more similar to hsp90α

called hsp90N [43, 44]. Hsp90N contains a truncated N-terminal ATP binding domain, displays altered client protein association and is associated with neoplastic transformation [45]. The hsp90 isoforms are likely the product of gene duplication events and share a high degree of sequence homology [44, 46]. Although it has been reported that the α and β isoforms differ in their functionality [44], few comparative studies exist. Both isoforms have been shown to be stress inducible, but to differ in their sensitivity to stressors [47]. Differential expression of these isoforms during embryonic development has also been demonstrated [48]. Further direct comparison studies are required to elucidate the differences and similarities in the activities of the α and β isoforms.

Hsp90 is structurally flexible and functions through a three state conformation cycle. These conformations exist in equilibrium with small differences in free energy. Co-chaperones can regulate the function of hsp90 by altering conformational equilibria, kinetics of structural changes and ATP hydrolysis. ATP does not determine the conformation of hsp90, but rather shifts it between pre-existing states. Client proteins can also influence conformation - the ligand binding domain of the glucocorticoid receptor stimulates ATPase activity, implying that a conformational shift occurs towards the closed state with ATPase activity. On the other hand, Cdk4 binding results in incomplete closure and binding of yet other client proteins results in different effects on conformation. In contrast, different hsp90 conformations have been shown to affect client protein chaperoning processes. These examples highlight the ability of hsp90 to chaperone structurally diverse client proteins [40].

1.7.2 Hsp90 chaperone activity

Hsp90 functions are complex and are yet to be fully elucidated. Multiple chaperoning mechanisms exist, and these in turn are influenced by a number of factors. Hsp90 exists in different chaperoning states; as a monomer, homodimer or heterodimer, and activity is regulated on several

levels by different mechanisms, such as by co-chaperones and nucleotides as well as by post-translational regulation. Hsp90 associates with a multitude of client proteins which are capable of binding to all three domains of the protein. The outcome of which is an essential and functionally flexible chaperone that is highly complex in nature [36, 43].

The hsp90 chaperone machinery is responsible for the folding, stabilisation, degradation and translocation of a diverse group of client proteins. Hsp90 functions in association with more than a dozen co-chaperones and accessory molecules that assist in regulating chaperone activity through multiple mechanisms. Moreover, ATPase activity may be intrinsically or extrinsically modified. With the latter mediated through co-chaperone specificity for different hsp90 conformations. For example, p23/Sba1 binds to the ATP binding domain and stabilises the dimerised conformation at a late stage in the ATPase cycle. This appears to be the cause of reduced ATPase activity that is observed in the presence of p23/Sba1. Another example is Aha1, which can bind to the M-domain and stimulate ATPase activity ten fold [36]. Another set of co-factors regulate hsp90 independently of altering ATPase activity and hsp90 may be further regulated by post-translational nitrosylation, acetylation and phosphorylation [36]. Hsp90 often functions as part of multi-protein chaperone complexes. Co-chaperones provide a method of recruitment for client proteins, therefore, this is a mechanism for regulating hsp90 conformation and ATP hydrolysis and as such can influence hsp90 chaperone activity. Genomic studies in diverse organisms have shown hsp90 to have a unique repertoire of co-chaperones, suggesting different organisms tailor hsp90 chaperoning action through defining the subset of co-chaperones [40].

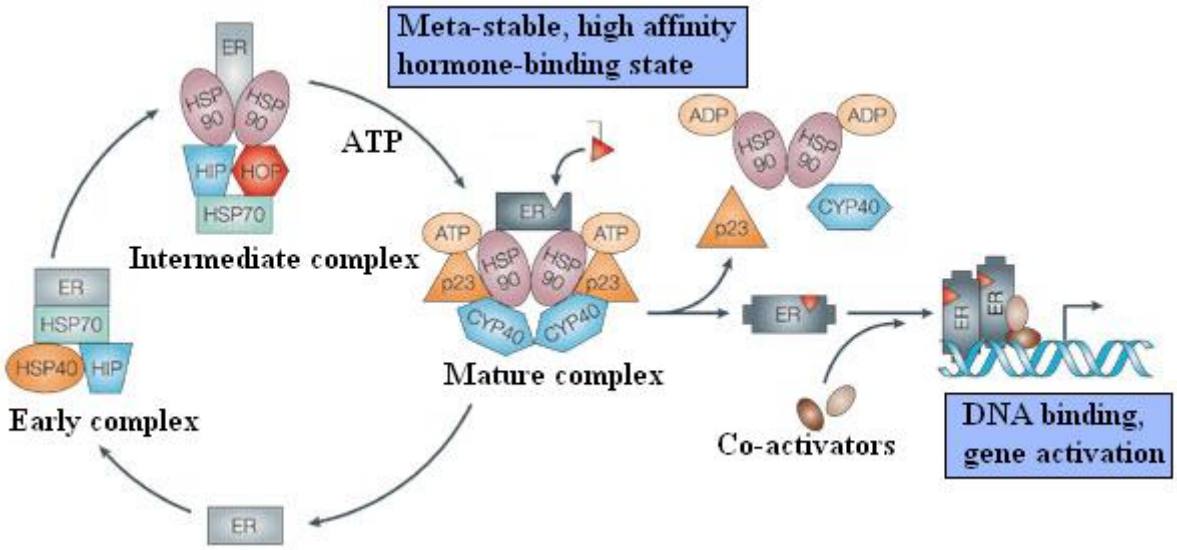
Hsp90 chaperoning action is achieved by changes in conformation throughout a functional cycle [41]. Chaperoning is an ATP driven process, however, hsp90 contains two nucleotide binding domains and may bind other nucleotides in addition to ATP. Hsp90 can also function

independently of ATP, as in the accumulation of denatured proteins [39]. ATP and ADP have been suggested to regulate the function of hsp90 and to result in different chaperoning states, and it appears as though the M-domain is responsible for directly interacting with client proteins and co-chaperones [228]. This is supported by studies showing the state of bound co-chaperones to be dependent on either the ATP or the ADP bound state of hsp90 [49]. The chaperoning action of hsp90 is commonly achieved when two monomers form a functional hsp90 homodimer which leads to ATP hydrolysis and transient enclosure of a substrate protein. This state is stabilised by p23 and subsequent ATP hydrolysis results in protein folding [34]. Dimerisation and ATP hydrolysis cause conformational changes in hsp90 that subsequently modify the conformation of the substrate protein. For example, hsp90 may induce a conformational change in the binding cleft of steroid receptors, thereby allowing a steroid to bind [50]. Hsp90 is rather promiscuous and associates with a diverse and large number of cellular proteins. Substrate specificity remains an open question but protein stability may be an important factor in the selection of hsp90 client proteins.

Hsp90 functions in association with co-chaperones that bind to functional domains on hsp90. A number of these accessory proteins bind to hsp90 via TPR domains which in turn are able to bind to other functional domains such as peptidyl-prolyl isomerase (PPIase) domains (for example on cyclophilins and immunophilins) and other TPR domains. This is the case for hsp70 - one of the major co-chaperones for hsp90 [50, 51]. Hsp90 chaperone complexes are dynamic and involve an ordered progression of co-chaperone binding and release (Fig. 1.5). This is achieved by co-chaperones differing in their binding affinity for various intermediate chaperone complexes. For example, the nascent steroid hormone receptor (SHR) is first bound by hsp70, hsp40 and Hip. This exposes the hydrophobic hormone binding domain to which hsp90 binds in association with Hop, thereby displacing hsp40. This complex is formed by hsp90 and hsp70 associating via TPR domains to Hop/Sti1. This Hop mediated hsp90-hsp70 association may be a necessary

prerequisite for hsp90 chaperone function, or at least appears to be a widely used structure in the formation of hsp90 chaperone complexes as it is found in many hsp90-client protein chaperone complexes. The SHR is transferred from hsp70 to hsp90 and is followed by the disassociation of hsp70, Hop and Hip. Driven by ATP, hsp90 then fully exposes the hormone binding domain while being stabilised by p23 that binds to the N-terminal of the hsp90 dimer. The vacant TPR domain on hsp90 becomes occupied by cyclophilin 40 (a PPIase). At this stage it seems likely that hsp90 shifts from the open to the closed conformation due to the fact that p23 associates at this point, but this has not been conclusively shown. This mature complex is meta-stable and returns to the open state following ATP hydrolysis and substrate protein release [8, 36]. This chaperoning mechanism appears to be similar for other client proteins and may represent general features of one of the hsp90 chaperoning mechanisms. In addition to folding, hsp90 may also be involved in protein translocation by connecting client proteins to protein trafficking systems. Details of these interactions are still being elucidated, but it appears as though hsp90 associates with PPIase proteins such as immunophilins which in turn link these client proteins to motor proteins such as dynein [50].

Figure 1.5 Role of heat shock proteins in chaperoning the nascent oestrogen receptor



The nascent oestrogen receptor associates with hsp70, hsp40 and the adaptor protein Hip. This results in partial exposure of the hydrophobic hormone binding domain, to which hsp90 and Hop bind, thereby displacing hsp40. The oestrogen receptor is transferred from the hsp70-Hip-Hop complex to hsp90. With the assistance of ATP, hsp90 fully exposes the hormone binding domain and is at the same time stabilised by p23 and cyclophilin 40. The binding of oestrogen leads to conformational changes that release co-chaperones and the binding to oestrogen response elements in addition to the recruitment of co-chaperones required for transcription. In the absence of ligand, the oestrogen receptor is released and undergoes further chaperoning. Adapted from Whitesell and Lindquist [8].

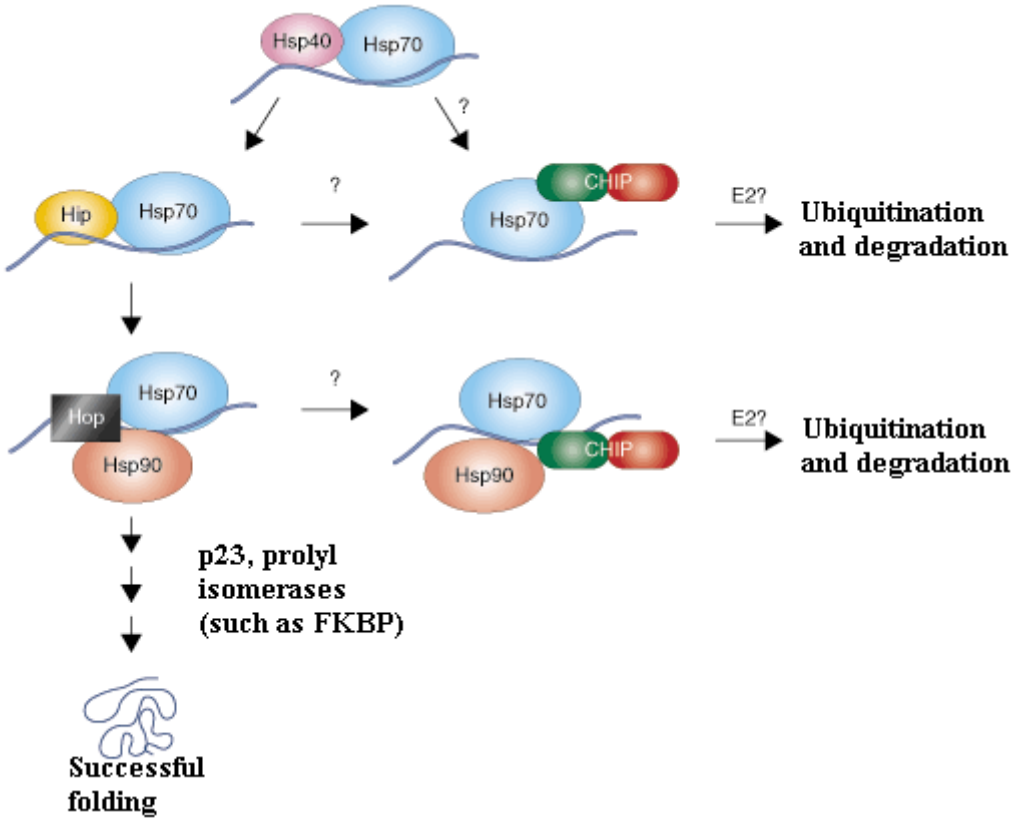
ER, Oestrogen Receptor

CYP40, Cyclophilin 40

In addition to the folding, stabilisation and translocation of proteins, hsp90 and other hsps play roles in protein degradation [52]. Maintenance of protein quality is achieved by balancing protein stabilisation and refolding with the destruction of damaged proteins. These systems have been demonstrated to be competitive, for example enhanced ubiquitin proteasome pathway (UPP) activity suppresses the refolding of denatured proteins, inhibition of the UPP results in up-regulation of hsps, while increased chaperone activity inhibits the degradation of denatured proteins [53, 54]. Despite this apparent division between these systems, there are proteins that are shared by both. One such protein is tthe C-terminus of Hsp70 Interacting Protein (CHIP), which is able to regulate the activity of hsps in addition to associating with polyubiquitinated proteins and

with the proteasome. This is possible as CHIP contains three TPR domains and a binding site that resembles the RING-finger domain characteristic of ubiquitin ligases that share a high degree of similarity with proteasome associated proteins. Consequently, CHIP has been shown to be involved in the degradation of hsp70 and hsp90 client proteins [55, 56]. The details are not known and a number of models have been proposed (Fig. 1.6) [55], however, it appears as though hsps and proteins involved in protein destruction such as CHIP associate to regulate the balance between protein folding and protein degradation to maintain protein homeostasis.

Figure 1.6 Proposed model for the role of heat shock proteins in protein degradation



Association of non-native client proteins with hsp70 and hsp40 may result in successful re-folding, but if the protein is recognised as folding incompetent, CHIP binds and re-organises the protein complex and initiates protein degradation via the ubiquitin proteasome pathway. It is not known at which stage, if any, the "decision" to abort folding is made. Adapted from McClellan and Frydman [55].

1.8 Geldanamycin

Geldanamycin is a specific hsp90 inhibitor and experimental anti-cancer drug produced by *Staphylococcus hygroscopicus*. Geldanamycin is a benzoquinone ansamycin compound that binds with greater affinity than ATP to the N-terminal binding domain of hsp90, thereby disrupting the progression of the hsp90 chaperone cycle [8]. This leads to proteasomal degradation of hsp90 client proteins and results in the inhibition of cancer cell proliferation and induction of apoptosis [35, 57, 58]. Due to its toxicity and instability, geldanamycin has not been used clinically. Instead, the development of geldanamycin derivatives such as 17-allylamino-17-demethoxygeldanamycin (17-AAG) have resulted in their evaluation in phase III clinical trials in the treatment of cancer [59].

It has been demonstrated that cancer cells display selective sensitivity to geldanamycin compared with non-malignant cells [8]. Studies have shown greater binding affinity in cancer cells for hsp90 and for hsp90 inhibitors to accumulate within tumours [60]. The mechanism has not yet been elucidated, but this may be due to hsp90 in cancer cells existing more frequently as multi-protein chaperone complexes with high ATPase activity. In addition to direct anti-tumour effects, geldanamycin has been shown to sensitise cancer cells to radiation and chemotherapeutic drugs [57]. This suggests that hsp90 inhibitors may have clinical benefits as adjuvants to existing radiotherapy and chemotherapy regimes. Despite these promising data, geldanamycin has been shown to vary in its effectiveness against cancer cell lines and has been shown to provide protection against chemotherapeutics, possibly the result of hsp70 up-regulation which is known to be caused by geldanamycin treatment [57]. These data suggest hsp90 inhibitors may be useful only in certain clinical settings.

Geldanamycin binds to the ATP binding domain of hsp90 and thus prevents conformational change. The binding of geldanamycin induces a conformation equivalent of the ADP-bound state,

but does not entirely inhibit the function of hsp90 [8, 49]. The overall result is abrogation of hsp90 cycling between the ATP and ADP conformations that are necessary to perform normal chaperone functions. Certain co-chaperones are known to preferentially associate with hsp90 in the ADP bound state locked in by geldanamycin, and the C-terminal nucleotide binding domain is likely to remain functional. This means that the ATP independent functions of hsp90 may not be inhibited by geldanamycin, instead the mechanism of action may be the result of the inability of hsp90 to change conformation.

1.9 Regulation of heat shock protein expression

Hsp expression is controlled by a family of heat shock factors (HSF) that are sensitive to cellular stressors. In mammals, at least three constitutively expressed HSFs exist. These are post-translationally activated in response to stress and this is the main mechanism of regulation, but there is a degree of regulation at the mRNA level as well. Under basal conditions, HSF1 exists in both the cytoplasm and nucleus as monomers that have no DNA binding ability. In response to stress, HSF1 accumulates in the nucleus and forms DNA-binding trimers where it binds to the Heat Shock Element (HSE) located in the promoters of most stress inducible genes. HSF1 appears to be the major regulator of hsp expression, but HSF2 may function cooperatively to assist in hsp expression. Regulation can be further modified by post-translational modifications including phosphorylation and SUMOylation. A complete understanding of HSF regulation does not currently exist, but evidence for additional mechanisms is becoming available. These include HSF regulation by HSR1 (a novel non-coding RNA) in tandem with translation elongation factor eEF1A, and others involving hsps [61, 62]. HIF (hypoxia-inducible factor) has also been shown to regulate HSF levels and consequently the expression of hsps [184].

1.10 Hsps' role in the immune system

The immune system can be divided into two diverse but complementary systems that vary in speed and specificity of response, referred to as innate and adaptive reponses. Innate immunity is relatively fast but also relatively non-specific and consists of neutrophils, monocytes, macrophages, the complement system, cytokines, acute phase proteins and physical and chemical barriers. Adaptive immunity is relatively slow and involves highly specific T lymphocyte and B lymphocyte mediated responses through the production of cytokines, cytotoxic factors and antibodies, respectively [63]. Hsps are involved in both innate and adaptive immune responses. They can activate innate immune cells, play roles in antigen presentation, maintain essential immune networks and chaperone immunogenic peptides.

Hsps are activated by innate immune system responses to fever and infection as well as cell necrosis and cytokine production. In addition, their release from cells represents a danger signal to the innate immune system, that stimulates cytokine and chemokine production and they may act as ligands for Toll-like receptors [64-67]. Hsps perform an essential chaperoning role in the maintenance of immune networks such as the interferon type I and II pathways and it has been shown that hsp90 is required for T cell activation by interferon-γ primed macrophages and the anti-viral response of interferons [68]. Hsps are also important for the function of the adaptive immune system. For example, they are involved in the assembly of major histocompatibility (MHC) molecules and chaperone peptides loaded to MHC proteins [69-71]. These peptides are produced during protein proteolysis by the proteasome. Once proteins have been degraded into peptides, they are transported across the ER membrane by the transporter associated with antigen processing (TAP) proteins. In the ER, the newly synthesised MHC proteins bind to peptide with the assistance of chaperone proteins [217].

Antigen Presenting Cells (APCs) exposed to hsps show increased migratory ability as well as increased expression of MHC proteins, co-stimulatory molecules and cytokine secretion [72]. These interactions likely occur through hsp receptors and Toll-like receptors on the surface of APCs [218] and may also be involved in hsps' role in immune recognition of extracellular antigens (cross presentation). Hsps chaperone antigenic peptides and a number of hsp receptors on APCs have been identified. Hsps have shown to mediate antigen cross presentation to APCs and for this to result in effective CD4+ and CD8+ T cell responses [73, 74]. Hsp70 has been shown to convert T cell tolerance to autoimmunity [72] and T cells recognising hsp epitopes are involved in regulating immune functions such as inflammation [67].

The role of hsps in T cell recognition of target cells is less well defined. It has been reported that hsps are essential for T cell recognition, but studies have shown that hsp90 inhibition may have the effect of abrogating or enhancing T cell recognition of tumour cells. Loss of T cell recognition is apparently due to inhibited peptide loading to MHC molecules [75-77]. Differences in the experimental systems make it difficult to discern the direct effect of hsp90 inhibition on T cell recognition, and this may be context dependent.

1.11 Hsps and cancer

Cancer is a generic term for a group of diseases that share similar characteristics, the most important of which is uncontrolled proliferation. Cancer may occur at any site in the body with over 100 distinct types currently recognised, while cancers occurring at the same site may be further broken down into molecular subtypes. Despite these complexities, cancer may be grouped into two broad categories - benign and malignant. Benign cancers are those unable to spread from the site of manifestation, while malignant cancers possess the ability to metastasise to surrounding cells, tissues and organs. The importance of this characteristic in relation to human health cannot be understated as the vast majority of cancer deaths are due to cancer metastases [16].

If cancer is to develop, multiple genetic mutations are required. These mutations typically involve proto-oncoproteins acquiring gain of function phenotypes, and tumour suppressor proteins acquiring loss of function. The result is a cell that is insensitive to death and anti-growth signals, and which simultaneously possesses self-sufficiency for endless replication. Cancer has been proposed to possess six hallmark features: self sufficiency in growth signals, insensitivity to anti-growth signals, apoptosis evasion, limitless replication potential, sustained angiogenesis, tissue invasion and metastasis. The acquisition of these features is assisted by inherent genomic instability. Despite these apparent similarities, there are multiple routes of cancer acquisition. Mutations can be caused by a variety of factors, from physical sources such as radiation to biological sources such as viruses [78]. The mutations necessary for the development of cancer represent a step by step encroachment of the multiple mutations required, generally over a long period of time [79, 80]. This idea is supported by observations that exposure to X-rays results in a higher risk of cancer, but that it does not manifest until years following exposure [81, 82]. The number of mutations required to achieve the cancer phenotype is likely to vary with different cell types.

Hsps are associated with tumour proliferation, metastasis, differentiation and immune system recognition. They are fundamental for tumourigenesis and promote the growth of cancer cells by simultaneously providing protection from tumour associated stressors, inhibiting cell death pathways and chaperoning overexpressed oncoproteins that drive the growth of cancer cells. Oncoprotein overexpression results in a signalling imbalance that, without the protection of hsps, would result in apoptosis. In addition, hsps protect cancer cells against the hostile tumour microenvironment. Due to the proliferative nature of cancer cells and the aberrant behaviour of tumour blood vessels, tumours commonly experience stressors such as hypoxia and pH extremes that promote illicit protein interactions and lead to protein misfolding and aggregation. Hsps assist the growth and survival of cancer cells on a variety of levels and are involved in all of the

proposed hallmark features of cancer. As such, abnormal hsp expression is a feature of cancer and hsps are overexpressed in a range of human cancers. Hsp expression has thus been proposed as a biomarker for patient prognosis and response to therapy in a variety of cancer types [8, 9, 83, 84].

The following list demonstrates the involvement of hsps in the six hallmark features of cancer.

Self sufficiency in growth signals – Hsp90 maintains signalling proteins in an active conformation so they may be activated by growth signals. Client proteins are essential for the survival of cancer cells and hsp90 inhibition results in client protein degradation, growth arrest and apoptosis [58, 83].

Apoptosis evasion – Hsps 70 and 27 confer on tumour cells an ability to resist programmed cell death in response to stimuli that would lead to apoptosis in non-transformed cells, making cancer cells exceptionally resilient [83, 85, 86].

Limitless replication potential – Hsp70 decreases p53 dependent and p53 independent mechanisms of senescence. An hsp70 analogue, hsp75, is important for inhibiting replicative senescence in mitochondria and functions at least partially through p53 inhibition. Hsp90 facilitates replication by stabilising telomerase [83].

Sustained angiogenesis – Tumour cells sense hypoxia, and thereby the requirement for angiogenesis, through the induction of HIF1α. Increased expression of hsp90 and hsp70 are required for the stabilisation and accumulation of HIF1α. Further downstream, hsp90 is required for the stability and induction of proteins (such as vascular endothelial growth factor and nitric oxide synthase) that regulate the proliferation and mobility of vascular endothelial cells. In addition, hsp27 is involved in the proliferation and mobility of vascular endothelial cells [83, 87].

Tissue invasion and metastasis – Cell surface hsp90 has been suggested to be important for tissue invasion. This involves interaction with the MMP-2 protease and the HER2 oncoprotein [88]. Cell surface hsp90 has been shown to inhibit cell motility and invasion *in vitro* and cancer metastasis *in vivo* [89]. Hsps may also enhance metastasis by allowing circulating tumour cells to resist apoptosis and allowing the accumulation of genetic mutations [83].

Insensitivity to anti-growth signals – Although it has been stated that there is no evidence that increased hsp expression is important for the inactivation of tumour suppressor proteins [83], there is at least one report that demonstrates p53 may be inactivated by hsps [90].

Hsps have thus been identified as valid targets for cancer therapy. A range of hsp inhibitors have been developed and are currently under investigation in human clinical trials for a variety of cancer types [59, 91].

1.12 Hsps and breast cancer

Hsps chaperone oncoproteins essential for the growth of breast cancer cells and hsps are consequently overexpressed in breast tumours [92-95]. Hsps are associated with tumour initiation, proliferation, metastasis and apoptotic resistance [96-99]. Hsps chaperone proteins involved in these processes in breast cancer including hormone and growth factor receptors (HER2, the oestrogen receptor (OR) and the progesterone receptor (PR)), tumour suppressor proteins (p53) and signal transduction proteins (Akt, Raf/Ras, Bcr/Abl and MEK 1/2). Hsp chaperoning action confers degradation protection upon these proteins and allows the maintenance of protein networks that support the progression of breast cancer [84, 100, 101]. In addition, hsp client proteins play particularly important roles in breast cancer. The expression of HER2, OR and PR proteins are routinely screened for in breast cancer patients, as they are overexpressed in a large

proportion of breast cancer patients in addition to being routine chemotherapy and immunotherapy targets [102]. The expression of hsps is also important for patient management and predicts patient prognosis and resistance to certain therapies. Specifically, hsp70 expression is associated with poor patient prognosis, resistance to radiotherapy and chemotherapy, tumour grade, tumour proliferation, metastasis and presence of mutated p53, while high hsp90 expression correlates with reduced survival and is associated with mutated p53, cell proliferation, metastasis and ER receptor status [84, 93-95, 98, 99, 103-108]. Hsp40 has been observed to be both up- and down-regulated in breast cancer, to be associated with oestrogen receptor status and to be involved in tumour and metastasis suppression [109, 110]. These differences are likely to be associated with different hsp40 family members. Hsp105 has been shown to be increased in expression in breast tumour tissue, while hsp27 is associated with metastasis, reduced disease-free survival after chemotherapy and chemotherapy resistance but also with reduced cell proliferation [105, 108, 111-113]. Hsp60 has been reported to be associated with breast tumour lymph node metastasis, but it is not correlated with tumour size, hormone receptor or HER2 expression status [114].

1.13 Hsps and melanoma

By contrast with breast cancer, hsps have not been well established as markers of disease progression and patient prognosis in melanoma. Hsp90 has been shown to be up-regulated in primary melanoma and expression to be higher still in metastatic melanoma by comparison with melanocytic naevi. Despite this up-regulation, studies have found no association with patient survival, but expression has been associated with Breslow depth and Clark level and thus hsp90 expression is suggested to be a marker of disease progression with respect to tumour size of primary melanomas [115]. This suggests that hsp90 may have a role in disease progression and metastasis, and this is supported by studies showing hsp90 to be preferentially expressed in melanoma tissue by comparison with naevi [116, 117] and that cell surface hsp90 occurs on the

majority of metastatic melanoma cell lines [116]. This role of supporting melanoma progression may be shared by hsp70. The proportion of hsp70 expressing cells has been shown to correlate with Breslow tumour thickness in primary melanomas and presence of hsp70 with Clark level in metastatic melanomas [118, 119]. Hsp70 has been found on primary melanoma and melanoma metastasis but not on normal melanocytes, to be up-regulated in melanoma metastases compared with melanocytic naevi and to be expressed in the majority of melanoma metastases [118-120]. Hsp70 has been found in the sera of melanoma patients while hsp60 was found in the sera of melanoma patients and healthy individuals [121] (although hsp70 has been shown to be present in healthy individuals as well [229]). These data contrast with other reports showing hsp70 expression to be associated with improved survival in primary melanoma tumours and for hsp70 expression not to be associated with the Breslow depth prognostic marker in metastatic melanoma [119, 122]. This might be explained by hsp70 influencing the immunogenicity of melanomas in some patients. Still other studies have shown no correlation between prognostic factors and hsp27, hsp70 and hsp90 expression in uveal melanoma [123], but one study suggests hsp27 may have a role in predicting melanoma mortality [124]. Hsp105 has been demonstrated to be up-regulated in melanoma by comparison with normal skin and benign melanocytic naevi and to be associated with increased invasiveness, while hsp70 was observed to be expressed highly in all three of these cell types [125, 126]. Taken as a whole, it appears as though hsps play important roles in melanoma, but few clear relationships have been defined.

1.14 The role of hsps in cancer immunity and therapy

Hsps chaperone immunogenic peptides, play roles in antigen presentation and influence the immunogenicity of cells through multiple mechanisms. These roles have important implications in the context of cancer immunity and they may be exploited in the course of cancer therapy.

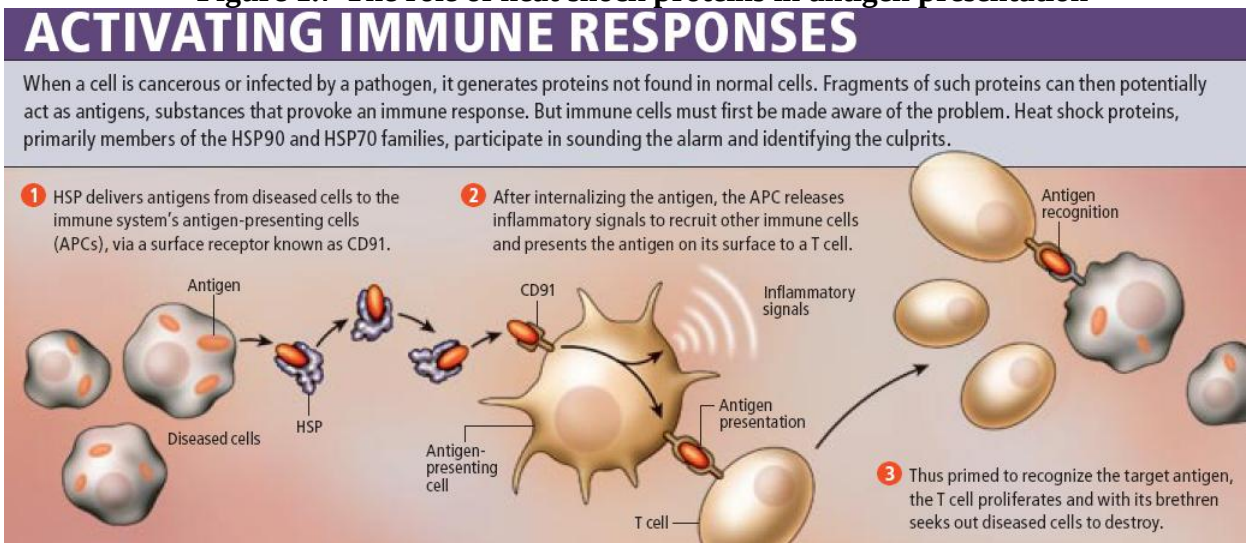
In addition to their role as protein chaperones, hsps chaperone peptides arising from protein degradation. Following degradation, peptides are produced that associate with hsps of the 90, 70 and 60 families. Malignant or infected cells synthesise and subsequently degrade unique proteins, resulting in the production of peptides specific to that cell type. These unique peptides are chaperoned by hsps and loaded onto MHC proteins on the cell surface for presentation to T cells. When hsps are exported extracellularly or isolated from cells, these peptides remain bound to hsps. Peptide-bound hsps may be taken up by receptors on APCs while simultaneously providing an APC maturation signal. The APCs process hsp chaperoned peptides in both TAP dependent and TAP independent mechanisms, resulting in their loading to MHC molecules and subsequent presentation to T cells. For some APCs, peptides have been shown to be processed through a non-acidic compartment, instead of being directly transferred to MHC I molecules. This process may result in an immune response against all cells bearing these peptides. Hsp-mediated T cell responses are independent of human leukocyte antigen (HLA) type of the cell from which the peptides are derived, and it has been suggested that hsps associate with the full antigenic repertoire [127]. These two features give this form of immune sensitisation the potential of being highly applicable in cancer vaccination [91, 128]. Hsps have been shown to activate CD4+ and CD8+ T cell responses as well as natural killer (NK) cell responses [66, 129-131]. In the course of clinical therapy, hsp-peptide complex vaccinations have been shown to result in T cell responses [130, 132]. In addition, cytosolic hsps have been identified on the cell surface and can be recognised by αβ and γδ T cells and NK cells as part of an anti-tumour response [66, 133]. As such, hsp-based immunotherapies have been identified as a valid form of therapy in the treatment of cancer. Animals immunised with hsps and their associated peptides have demonstrated resistance to a challenge of infectious cancer cells [129]. This therapy has been tested in studies with mice, rats and frogs and has undergone multiple randomised phase III human clinical trials in the treatment of cancer [64]. Despite the advantages of this type of therapy, studies to date have produced encouraging but mixed results [132, 134, 135]. This may be due to inter-patient and

inter-tumour variation in tumour immunogenicity or ineffective immune responses. Such attenuated immune responses may occur through a number of mechanisms including low expression of tumour surface antigens, down-regulation of MHC molecules, lack of differentiation from normal cells, ineffective antigen presentation, or induction of T cell tolerance. Such features may be widespread among tumours [69].

Melanoma derived hsp70 has been shown to chaperone peptides and mediate their transfer via receptors to DCs [71]. Further, hsp70 and associated peptides isolated from melanomas have been shown to stimulate anti-melanoma T cells. At higher concentrations, isolated hsp70 has also been found to stimulate anti-melanoma T cells through an MHC independent mechanism [71, 127]. Hsps have been reported to be essential for antigen presentation in cancer, but reports are conflicting [75, 76]. Hsp70 and gp96 have demonstrated an ability to bind immunogenic peptides and for this to result in T cell activation *in vitro*. This could potentially be exploited for use in cancer therapy by reconstituting hsp70 with known immunogenic peptides for use as a vaccine [129], but it should be noted that high doses of hsps have been shown to result in immune suppression. In addition to chaperoning intracellular peptides, hsps mediate the cross presentation of extracellular antigens (Fig. 1.7) [91]. Hsp-mediated cross presentation of tumour antigens may occur effectively in some cancer patients, resulting in disease stabilisation or regression. One study identified the hsp receptor CD91 to be up-regulated on monocytes (an APC) in melanoma patients displaying slow disease progression, compared to patients with typical disease progression. Moreover, there were no differences in other immune markers between these two groups, suggesting that hsp-peptide uptake may have been a factor resulting in delaying disease progression [136]. The role that hsps play in cross presentation may influence conventional chemotherapy and radiotherapy. Studies have shown necrotic cell death to result in an up-regulation of hsps by comparison with apoptotic death. This up-regulation and release of hsp and hsp-peptide complexes has been proposed to provide a stimulus to both the innate and adaptive

immune systems that may break tumour tolerance, and thus might be relevant to clinical cancer treatment and outcome [64, 69, 137]. If a cancer therapy results in the necrosis of tumour cells, this may result in the up-regulation and release of hsps and hsp-bound peptides. Hsp bound tumour-specific peptides may be taken up by APCs while simultaneously stimulating maturation of DCs and subsequent presentation to T cells [66]. This may lead to a secondary benefit of cancer therapy induced through hsp-immune stimulation.

Figure 1.7 The role of heat shock proteins in antigen presentation



Hsps chaperone immunogenic peptides from diseased cells to APCs that subsequently prime T cells against that peptide. Adapted from Scientific American [91].

In addition to the aforementioned roles, hsps can also modulate cellular immunogenicity [138]. One study observed poor T cell responses to melanoma cells with low MHC protein expression. Transfecting these cells to result in high constitutive hsp70 expression resulted in increased MHC molecule expression and improved T cell responses [142]. In contrast, hsp70 has been shown to protect tumour cells against immune system mediated mechanisms of cytotoxicity, but other studies have shown increased hsp70 expression to be associated with the breaking of heat shock induced resistance to T cells and with improved lysability to T cells [139-141]. Further, mice immunised with melanoma cells expressing high levels of hsp70 demonstrated significantly increased resistance to a challenge of live melanoma cells compared with control melanoma cells

[142]. This has also been demonstrated for the heterologous hsp65 [143]. The role of hsps in modulating tumour cell immunogenicity is supported by other studies demonstrating the ability of hsps to increase or decrease the immunogenicity of tumour cells. Another study demonstrated that a murine macrophage tumour cell line lost its ability to form tumours following transfection with hsp65. Mice immunised with the transfected cell line were resistant to subsequent challenges of the non-transfected parental cell line [144].

1.15 Thesis Aims

The aim of this doctoral thesis was to provide new insights into the roles that hsps play in human cancer. Although these roles have previously been studied in isolation, the current studies are unique in that they investigate the role of hsps in diverse cancer tissue types and tumour cell lines using a range of experimental techniques. These approaches allow for a more comprehensive evaluation of the roles of hsps in human cancer.

The specific aims of this study were:

1) To characterise the expression of hsps in human breast tissue
2) To investigate the association and identity of hsp90 client proteins in tumour and healthy breast tissue
3) To investigate differences in the expression of cell surface hsps and other proteins between primary-derived and metastasis-derived melanoma cell lines and the influence of stromal cell co-culture
4) To characterise hsp expression in a panel of melanoma cell lines cultured under low and high oxygen tension and to correlate this with cell line and patient characteristics
5) To assess hsp expression in melanoma tissue to investigate relationships with patient clinical parameters
6) To investigate the role of hsp90 on T cell recognition of melanoma cells

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Chemicals and consumables

Adefo (Dietzenbach, Germany): ECL film developer and fixer

AnalaR NORMAPUR (VWR International) (Dublin, Ireland): Methanol, xylene

Amersham GE (Life Sciences) (Munich, Germany): Hybond ECL nitrocellulose membrane, Hyperfilm ECL

Bayer (Leverkusen, Germany): GAMUNEX

BD Biosciences/Pharmingen (Heidelberg, Germany): 5 mL round bottom FACS sample tubes, Cytofix/Cytoperm, Perm/Wash, anti-human GM-CSF, anti-human GM-CSF-biotin, anti-human IFN-γ, anti-human IFN-γ-biotin, recombinant GM-CSF and IFN-γ standard proteins

BDH (VWR) (Radnor, PA, USA): Bis-acrylamide, ammonium sulphate, bromophenol blue, hydrochloric acid, mercaptoethanol, TEMED

Berlex (Bayer) (Leverkusen, Germany): Leukine (GM-CSF)

Biomol (Enzo Life Sciences) (Farmingdale, NY, USA): Geldanamycin

Bio-Rad (Gladesville, Sydney, NSW, Australia): Bio-Lyte 3/10 Ampholyte, Kaleidoscope pre-stained protein standards, mineral oil, Precast 4-15 % gradient Tris-HCl polyacrylamide mini gels, ReadyStrip IPG Strips 17 cm pH 3–10

Bio-Rad (Munich, Germany): Acrylamide solution, Tween 20

BioWhittaker (Lonza) (Cologne, Germany): X-vivo 15 medium

Boehringer Mannheim (Roche) (Dee Why, Sydney, NSW, Australia): PMSF

Cambrex (Wiesbaden, Germany): Trpysin-EDTA

Carl Roth (Karlsruhe, Germany): Glycine, Roti nanoquant

CellGenix (Freiburg, Germany): CellGro DC Medium

Chiron (Novartis) (Ratingen, Germany): Interleukin-2

Dako (Hamburg, Germany): Fluorescence mounting medium

Diploma Fonterra (North Ryde, Sydney, NSW, Australia): Skim milk powder

Endogen (Thermo Fisher Scientific) (Boston, USA): Tumour Necrosis Factor

GE Life Sciences (Rydalmere, Sydney, NSW, Australia): ECL western blotting kit, Hybond C, Hyperfilm ECL

Greiner Bio-One (Frickenhausen, Germany): Cell culture flasks (75 cm²), 24 well F-bottom and 96 well U-bottom cell culture plates

Grenzyme (Neu-Isenburg, Germany): Interleukin-7

ICN Biochemicals (MP Biomedicals) (Seven Hills, Sydney, NSW, Australia): Acrylamide, ammonium persulphate, CHAPS, Coomassie G250, glycine

ImmunoTools (Friesoythe, Germany): Interleukin-15

InvivoGen (Toulouse, France): Geldanamycin

Life Technologies (Darmstadt, Germany): RPMI 1640 medium

Merck (Kilsyth, Melbourne, Vic, Australia): Methanol

Merck (Darmstadt, Germany): Ammonium persulphate, citric acid, formaldehyde solution, hydrochloric acid, potassium chloride, potassium di-hydrogen phosphate, sodium chloride, sodium citrate, di-sodium hydrogen phosphate, 2-mercaptoethanol, urea

Miltenyi Biotec (Bergisch Gladbach, Germany): Anti-CD4-conjugated magnetic microbeads

Nunc (Langenselbold, Germany): 96 well MaxiSorp ELISA plates, cell culture flasks (175 cm²)
PAA (Pasching, Austria): Hank's Buffered Salt Solution, Dulbecco's PBS (without Ca or Mg)

Pall (Cheltenham, Melbourne, Vic, Australia): Nanosep MF GHP 0.45 µm membrane filter centrifuge tubes, Protein A coated ceramic hyperD beads

PeproTech (Hamburg, Germany): Interleukin-21

Promega (Alexandria, Sydney, NSW, Australia): DTT, glycerol

Riedel-de Haën (Sigma-Aldrich) (Seelze, Lower Saxony, Germany): Thiourea

Roche (Mannheim, Germany): DAPI

Sandoz (Holzkirchen, Germany): Interleukin-4

SAV Liquid Production (Flintsbach a. Inn, Germany): Ethanol

Serva (Heidelberg, Germany): Bovine serum albumin, sodium dodecyl sulphate

Sigma-Aldrich (Castle Hill, Sydney, NSW, Australia): Iodoacetamide, phosphoric acid, sodium dodecyl sulphate, Tris base, Tween 20, urea

Sigma-Aldrich (Munich, Germany): Avidin-HRP, ABTS, CHAPS, Bromophenol blue, donkey serum, foetal Calf Serum, hydrogen peroxide solution, PMSF, silane-Prep slides, trypan blue, TEMED, Tris base

2.1.2 Antibodies

2.1.2.1 Primary antibodies

Abgent (San Diego, CA, USA): Hsp40 rabbit polyclonal clone RB1770

Biomol (Enzo Life Sciences) Farmingdale (NY, USA): Hsp56/FKBP59 rabbit polyclonal

Cell Signaling Technology (Danvers, MA, USA): β-actin rabbit monoclonal

Dako (Hamburg, Germany): MelanA mouse monoclonal clone A103

Enzo Life Sciences (Lörrach, Germany): PE-conjugated hsp90, hsp70, hsp60, hsp40, hsp32 and unconjugated rabbit polyclonal hsp32

Santa Cruz Biotechnology (Santa Cruz, CA, USA): Hsp105: Rabbit polyclonal clone N-187, Hsp90α goat polyclonal clone N-17, hsp40 goat polyclonal clone N-19

Santa Cruz Biotechnology (Heidelberg,Germany): Hsp90α/β goat polyclonal clone N-17, hsp70 goat polyclonal clone K-20, hsp60 goat polyclonal clone N-20

Stressgen Enzo Life Sciences (Farmingdale, NY, USA): Hsp70 mouse monoclonal clone C92F3A-5, hsp60 mouse monoclonal clone LK-1

Stressmarq (Victoria, BC, Canada): Hsp90α mouse monoclonal clone Hyb-K41009, hsp70 mouse monoclonal clone C92F3A-5

2.1.2.2 Secondary antibodes

Jackson ImmunoResearch Laboratories (West Grove, PA, USA): DyLight488-conjugated donkey anti-goat IgG, DyLight488-conjugated donkey anti-rabbit IgG, Cy3-conjugated donkey anti-mouse IgG

Dako (Hamburg, Germany): HRP-conjugated polyclonal goat anti-mouse, HRP-conjugated polyclonal rabbit anti-goat, HRP-conjugated polyclonal swine anti-rabbit

Invitrogen (Life Technologies): Pacific Orange-conjugated goat anti-mouse

2.1.3 Plastic ware and general materials

Corning Incorporated (Kaiserslautern, Germany): 5, 10 and 25 mL pipette tips

Eppendorf (Hamburg, Germany): 1.5 mL centrifuge tubes, CombiTips

Greiner Bio-One (Frickenhausen, Germany): 15 mL and 50 mL centrifuge tubes

2.1.4 Peptides

Parent protein	Sequence	HLA binding specificity	Source
NY-ESO-1	LLEFYLAMPFATPMEAELARRSLAQ	HLA DR4/DP4	Dr Hubert Kalbacher Interfaculty Institute for Biochemistry, University of Tübingen
	WITQCFLPVFLAQPPSGQRR		
	SLLMWITQC	HLA A2	
	SLLMWITQV		

2.1.5 T cell clones

HLA restriction	Peptide recognised	Parent protein	Source
MHC I	ELAGIGILTV	MelanA (26-35)	Dr rer. nat. Simon Völkl Hämatologie & Internistische Onkologie, Universitätsklinikum Erlangen
MHC I	ITDQVPFSV9	gp100 (209-217)	

2.1.6 Tissue samples and cell lines

Tissue type	Source
Breast tumour tissue T47D breast tumour cell line	Dr Roger Lord, Australian Catholic University, Brisbane
Tumour and healthy breast tissue	Dr Guy Hingston, Port Macquarie Private Hospital
Tumour and healthy breast tissue	Dr Frank Sardelic, Tamworth base hospital
Melanoma tumours, squamous cell carcinomas and a lymph node from a patient with B cell chronic lymphocytic leukaemia	Dr med. Benjamin Weide, Skin Cancer Program, Sektion für dermatologische Onkologie, University of Tübingen Dermatology Clinic
Melanoma cell lines For full details see Chapters 5 and 6	European Searchable Tumour Line Database. Co-ordinated by G. Pawelec, TATI group, Sektion für Transplantationsimmunologie und Immunohämatologie, Zentrum für Medizinische Forschung, University of Tübingen

2.1.7 Software and internet resources

FACSDiva (BD Biosciences)

FlowJo (Tree Star)

Axiovision (Carl Zeiss)
Prism (GraphPad software)

European Searchable Tumour Database (ESTDAB)
http://www.ebi.ac.uk/ipd/estdab/

2.1.8 Equipment

Heidolph (Schwabach, Bavaria, Germany): Heidolph Diax900 tissue homogeniser

Branson Ultrasonics (Danbury, CT, USA): Tissue sonifier

BD Biosciences (Heidelberg, Germany): LSR II flow cytometer

Cary (Santa Clara, CA, USA): Cary Varian 50 Spectrophotometer

Bio-Rad (Gladesville, Sydney, NSW, Australia): Mini-PROTEAN Tetra Electrophoresis System

GE Life Sciences (Rydalmere, Sydney, NSW, Australia): Multiphor II Novablot semi-dry transfer unit, IPGphor II isoelectric focusing unit

Ruskinn Technology (Pencoed, UK): Concept 1000 Invivo2 hypoxic chamber

Carl Zeiss (Oberkochen, Germany): Axio Observer.Z1 confocal fluorescent microscope

2.2 Methods

2.2.1 Ethics

Ethics approval was obtained from the University of New England Human Research Ethics Committee (approval no. HE07/145). A copy is provided in appendix III.

2.2.2 Extraction and isolation of cellular proteins from breast tissue

Tumour and healthy breast tissue taken from patients during surgical procedures were cooled immediately on ice and subsequently stored at -70 °C. Samples were partially thawed and slices excised. Cellular proteins were extracted from breast tissue in a buffer containing 7 M urea, 2 M

thiourea, 4 % (w/v) CHAPS detergent and PMSF protease inhibitor. The tissues were homogenised with a bladed electric tissue homogeniser until a solution of smooth consistency was obtained. This solution was frozen at -70 °C overnight. Solutions were thawed and subjected to brief sonication and then refrozen at -70 °C. Solutions were subsequently thawed, centrifuged and the protein layer removed. Protein fractions were centrifuged twice more to ensure the final extract was free of insoluble contaminants. All steps were performed at 4 °C.

2.2.3 Protein quantification of breast tissue extracts

Protein concentration of breast tissue extracts was determined using the Bradford method [219]. Protein samples were diluted 1:500 in Bradford dye (100 mg Coomassie Blue G250, 50 mL 95 % (v/v) ethanol and 100 mL 85 % (v/v) phosphoric acid per litre). Absorbance values were read at 595 nm against a Bradford dye blank and values were converted into protein concentration with the use of a bovine serum albumin (BSA) standard curve ranging from 0-80 µg/mL.

2.2.4 Immunoprecipitation

Co-immunoprecipitation was performed under non-denaturing conditions to reveal protein associations. A total of 100 µL of protein extract was incubated with antibody to various hsps (minimum 2 µg antibody per 100 µL sample) for 1 hr at room temperature on a mechanical rocking device. This solution was subsequently incubated with 15-25 µL protein A-coated beads overnight at room temperature with gentle rocking in a 1.5 mL membrane filter centrifuge tube. Protein A beads were washed with washing solution containing 7 M urea, 2 M thiourea and 4 % (w/v) CHAPS prior to sample addition. Protein extract containing unbound protein was separated from the protein A beads by centrifugation of the membrane filter centrifuge tubes. Following centrifugation the protein A beads were washed with a minimum of 30 µL washing solution and centrifuged. The protein A beads were washed three times to ensure the removal of any unbound or weakly bound protein. The washing solution was highly stringent to ensure only tightly binding client proteins remained bound, thereby reducing the possibility of detecting non-specifically

bound proteins. Protein A beads were washed with 30 µL of washing solution following each geldanamycin treatment to remove any remaining protein and prevent sample contamination of subsequent treatments. This procedure was repeated for the two subsequent geldanamycin treatments of 25 and 50 µg/mL. Following the geldanamycin elution and washing steps, beads were treated with a denaturing solution to thoroughly disrupt any remaining protein interactions (10 % glycerol, 5 % mercaptoethanol and 2.3 % sodium dodecyl sulphate (SDS) all w/v in ddH_2O). This solution was collected as described previously and stored at 4 °C until SDS-PAGE separation. SDS-PAGE separation of eluted proteins was performed the same day as immunoprecipitation. Selected experiments were repeated to confirm the initial results. With each immunoprecipitation experiment, a set volume of total protein extract from each sample was loaded onto an SDS-PAGE gel to ensure consistent protein loading with different extracts in each experiment.

2.2.5 SDS-PAGE separation of protein extracts from breast tissue

Precast 4-15 % gradient Tris-HCl polyacrylamide mini gels were used to separate protein samples. To 15-20 µL of each sample, 3 µL of 3.75x concentrated denaturing solution (described previously) containing bromophenol blue tracking dye was added. Sample tubes were boiled in water for 3 min followed by rapid cooling on ice to mitigate against any residual protease activity. Samples were loaded and electrophoresis performed at 4 °C with running buffer (14.4 % glycine, 3.0 % tris and 1.0 % SDS, all w/v) at 30 mA per gel until the tracking dye had migrated to the bottom of the gel (typically after 1-1.5 hr). Kaleidoscope pre-stained protein standards were run on each gel.

2.2.6 Western immunoblotting of breast tissue

Following electrophoresis, proteins from SDS-PAGE gels were transferred to nitrocellulose membranes using a semi-dry transfer method by applying 120 mA for 90 min. Membranes were blocked in 5 % skim milk powder (SMP) PBS-T (8.0 g NaCl 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g

KH_2PO_4 and 1 mL Tween 20 per litre) at 4 °C overnight and washed according to the manufacturer's instructions in PBS-T before Western blotting. Primary antibody was diluted in SMP PBS-T and incubated with the membrane for 1 hr. Membranes were washed according to the manufacturer's instructions in PBS-T and appropriate HRP-conjugated (horse radish peroxidase) secondary antibody added and incubated as per primary antibody. Following incubation with secondary antibody, proteins were visualised in a dark room. Membranes were incubated with HRP substrate according to the manufacturer's instructions. Hyperfilm ECL was used to detect labelled proteins.

2.2.7 Staining of acrylamide gels

Gels were stained for a minimum of 12 hr with a highly sensitive Coomassie G250 protein stain [223] (0.12 % (w/v) dye , 10 % (w/v) ammonium sulphate, 10 % (v/v) phosphoric acid and 20 % (v/v) methanol).

2.2.8 Mass spectrometry

Liquid Chromatography-Mass Spectrometry (LC-MS) was performed on excised stained protein bands by the Bioanalytical Mass Spectrometry Facility at the University of New South Wales, Sydney. Coomassie protein stain was removed from excised protein bands before proteins were reduced, alkylated and digested with trypsin. Peptides were separated with LC and analysed with a Q-tof Ultima mass spectrometer (Micromass/Waters) and peptides corresponding in sequence to known database proteins were identified using the Mascot MS software.

2.2.9 2 Dimensional SDS-PAGE

To separate proteins according to their isoelectric point (pI) and molecular weight (kDa), a two step protein separation was performed. Proteins were first separated according to their isoelectric point using isoelectric focusing and then according to molecular weight with SDS-PAGE in the following manner. Breast tissue protein extract was diluted in IEF rehydration solution

(7 M urea, 2 M thiourea, 4 % (w/v) CHAPS, 1 % (w/v) DTT, 0.2 % (v/v) pH 3-10 ampholytes, 0.8 % (v/v) bromophenol blue solution, total volume 300 µL) and loaded onto a 2D rehydration tray. pH 3-10 Immobilised pH gradient (IPG) strips were placed on top of this solution and overlaid with mineral oil. IPG strips were allowed to rehydrate overnight. The following day, IPG strips underwent isoelectric focusing using an IPGphor II at 20 °C to a total of 45,000 kVh. Prior to second dimension separation, IPG strips were equilibrated with agitation for 15 min with IEF equilibration solution (0.375 M Tris-HCl pH 8.8, 6 M Urea, 20 % (v/v) Glycerol, 2 % (w/v) SDS with the addition of 2.5 % (w/v) DTT. This was followed by a second 15 min incubation in equilibration solution with 4.1 % (w/v) iodoacetamide and bromophenol blue in place of DTT. Isoelectrically separated proteins in IPG strips were then separated according to molecular weight using 12.0 % (w/v) acrylamide (11.6 % acrylamide and 0.4 % bis-acrylamide) SDS-PAGE gels. Gels were subsequently stained with a Coomassie G250 based protein stain (Blue Silver).

2.2.10 ELISA

A sandwich ELISA was used to quantify T cell cytokine production. Cytokine specific antibody pairs were used according to the manufacturer's instructions. To the wells of an enhanced protein binding ELISA plate, 50 µL of anti-cytokine capture antibody (1 µg/mL) diluted in binding solution (0.1 M (w/v) Na_2HPO_4, pH 9.0) was added and incubated at 4 °C overnight. The following day, ELISA plates were washed four times with approximately 200 µL of washing solution (PBS (8.0 g NaCl 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, per litre) with 0.05 % (v/v) Tween 20) and non-specific binding blocked with 200 µL blocking buffer (10 % (v/v) FCS in PBS) per well and incubated at room temperature for a minimum of 30 min. Following blocking, plates were washed three times. Samples and dilutions of standard proteins were then added and allowed to incubate overnight at 4 °C. The following day, plates were washed four times and appropriate anti-cytokine biotin-conjugated secondary antibody added (0.5 µg/mL in Blocking Buffer Tween) and incubated at room temperature for at least 1 hr. Plates were washed

six times and avidin-conjugated HRP (2.5 µg/mL) added to each well and allowed to incubate for 30 min at room temperature. Plates were washed eight times before the addition of substrate (0.03 % (w/v) ABTS in 0.1 M anhydrous citric acid, pH 4.35) including 0.9 µL 30 % hydrogen peroxide per mL, added immediately prior to use. Absorbance values were read at 405 nm and final cytokine concentrations were determined by comparison with absorbance values of standard proteins.

2.2.11 Immunofluorescence

Formalin fixed paraffin-embedded melanoma tissue sections were pre-chilled and 5 µm thick slices cut and mounted on Silane-Prep slides. Tissue sections were adhered to slides by incubation on a heating element at 37 °C overnight. These slide mounted tissue sections were used in immunofluorescence experiments. Tissue sections were de-paraffinised and rehydrated by overnight incubation at 60 °C followed by xylene treatment for 15 min. Tissue sections were subsequently rehydrated in three ethanol solutions (100 %, 96 % and 70 % ethanol) for 5 min each, then rinsed and incubated for 30 min in ddH_2O. Following deparaffinisation and rehydration, antigens were retrieved with a 2 min incubation in 10 mM citrate buffer (1.8 % (v/v) 0.1 M citric acid, 8.2 % (v/v) 0.1 M sodium citrate, pH 6) in a pressurised cooking device. Tissue sections were gradually cooled, washed for 3 min with washing buffer (8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, 0.25 mL Tween 20 and 0.5 mL 10 % BSA solution per litre) and blocked in 5 % (v/v) donkey serum (diluted in washing buffer) for 30 min. Primary antibody (diluted in washing buffer) was applied to stained sections and incubated for 60 min in a humidifying chamber. Washing buffer was applied to unstained sections during primary antibody incubation to prevent dehydration. Following primary antibody incubation, slides were rinsed and washed twice for 3 min before being incubated with secondary antibody (diluted in washing buffer) for 60 min in a dark humidifying chamber. Slides were rinsed and washed twice in washing buffer before being incubated with the DAPI (4',6-diamidino-2-phenylindole) nuclear

stain (diluted 1:2000 in washing buffer) for 15 min in a dark humidifying chamber. Slides were then rinsed and washed twice for 3 min before the addition of fluorescence mounting medium, topped with glass coverslip (0.08-0.12 mm). Slides were kept in the dark at 4 °C and visualised the following day. Stained tissue samples were analysed on the Carl Zeiss Axio Observer.Z1 confocal fluorescent microscope using Axiovision software. Exposure times for each fluorescence channel were adjusted using a tissue section stained with secondary antibodies in order to set an appropriate level of background fluorescence. This was performed for each tissue section. In order to quantify the fluorescence staining observed by eye, a small number of representative MelanA positive and MelanA negative cells were analysed using Axiovision software in a minimum of five locations in each tissue section. Secondary antibodies did not react non-specifically.

2.2.12 Extraction and isolation of cellular proteins from melanoma tissue

Melanoma samples were partially thawed and cut into fine pieces. Cellular proteins were extracted from samples in a buffer containing 9 M urea, 4 % (w/v) CHAPS detergent and 12.2 mM PMSF protease inhibitor. The tissues were homogenised with a bladed electric tissue homogeniser until a solution of smooth consistency was obtained. This solution was frozen at -70 °C overnight. The following day the solutions were thawed and subjected to sonication before being re-frozen at -70 °C. Solutions were subsequently thawed, centrifuged and the protein layer removed. Protein fractions were centrifuged until the extract was free of insoluble contaminants. All steps were performed at 4 °C.

2.2.13 Melanoma tissue protein extract quantification

A modified version of the Bradford method was used to quantify protein extracts. Roti nanoquant was used according to manufacturer's instructions. Samples were tested in triplicate.

2.2.14 Western immunoblotting of melanoma tissue

Protein extracts were denatured with 5 µL of 1.66 x sample buffer (5 x stock consisted of 50 % glycerol, 25 % 2-mercaptoethanol, 20 % 1.5 M Tris-HCl pH 6.8, 1 mL bromophenol blue solution, all v/v and 10 % (w/v) SDS) and heated at 95 °C for 5 min and immediately chilled to minimise residual protease activity. Proteins were then separated on 10 % acrylamide SDS-PAGE gels (5 mL ddH_2O, 2.5 mL acrylamide stock solution (37.5 % (w/v) acrylamide and 2.5 % (w/v) bis-acrylamide), 2.5 mL Tris-HCl 1.5 M pH 8.8, 100 µL 10 % SDS, 75 µL of 10 % ammonium persulphate solution, all w/v and 7.5 µL TEMED) by applying 200 V with migration buffer (3.0 g Tris, 14.4 g glycine, 1.0 g SDS, per litre) until the tracking dye had migrated to the bottom of the gel. Proteins were transferred to nitrocellulose membranes using a wet transfer method with 110 V for 1 hr in a buffer containing 3.0 g Tris, 14.4 g glycine and 20 % (v/v) methanol per litre (freshly prepared and chilled). Following this, membranes were stained with Ponceau Red to ensure the proteins were effectively transferred to membrane. Membranes were then blocked in 5 % SMP (diluted in TBS-Tween (2.42 g Tris, 8.0 g NaCl and 1 mL Tween 20 per litre, pH 7.6) for 90 min at room temperature before being rinsed in TBS-Tween. Primary antibody was diluted in TBS-Tween and incubated with membrane overnight at 4 °C. Membranes were rinsed and washed twice for 5 min with TBS-Tween. Appropriate HRP-conjugated secondary antibody was incubated with the membrane for 1 hr at room temperature. Following this, membranes were rinsed and washed twice for 5 min with TBS-Tween. Proteins were visualised by applying substrate for 1 min (0.022 % (w/v) luminol, 225 µM coumaric acid, 0.03 % (v/v) of 30 % hydrogen peroxide solution in 0.1 M Tris-HCl, pH 8.5) before reactive zones being recorded with Hyperfilm ECL) (visualisation performed in a dark room). All incubation and washing steps were performed with gentle rocking. In order to group samples as high or low expressors, differences in expression were group according to relative differences with respect to the level of the β-actin house keeping protein.

2.2.15 Stripping of Western blot membranes to allow for multiple probing

Subsequent to ECL detection of labelled proteins, selected membranes were stripped of antibodies and re-probed. To remove bound antibodies, membranes were incubated in stripping solution (62.5 mM Tris-HCl pH 6.7, 2 % (w/v) SDS and 100 mM 2-mercaptoethanol) (mercaptoethanol added immediately prior to use) at 50 °C for 30 min with occasional agitation. Membranes were then rinsed with water, washed for 5 min with TBS Tween before being blocked with 5 % SMP (diluted in TBS-Tween).

2.2.16 Melanoma cell line culture

Melanoma cell lines were cultured with 35 mL RPMI 1640 medium supplemented with 10 % (v/v) heat inactivated FCS in 75 cm² cell culture flasks for five days in an incubator (37 °C, 5 % CO_2, 95 % humidity) in air (20 % O_2) and in 2 % O_2 in an hypoxic chamber. Seeding cell number was adjusted according to the generation time (time for one population doubling) of each cell line in order to avoid confluence and to obtain similar cell numbers at the end of the culture period. Following the culture period cells were washed with HBSS (Hank's balanced salt solution) and detached from culture flasks by incubating with Trpysin-EDTA for approximately 3 min. Trypsin was inactivated by adding of an equal volume of RPMI medium containing 10 % (v/v) FCS.

2.2.17 Determination of cell number and viability

The trypan blue exclusion method was used to determine the number of live and dead cells. Melanoma cells were suspended in 10 mL of HBSS and mixed thoroughly. Equal volumes of a 0.4 % (w/v) trypan blue solution and the cell sample were mixed and applied to a Neubauer haemocytometer. Phase contrast microscopy was used to distinguish between stained (dead) and unstained (live) cells.

2.2.18 Flow cytometry

Following trypsinisation, cells were washed with PFEA (Dulbecco's PBS, 2 % (v/v) FCS, 2 mM EDTA, and 0.01 % (w/v) azide) and Fc receptors blocked with GAMUNEX on ice for 10 min. Cells were washed with PFEA before being permeabilised and fixed with Cytofix/Cytoperm and Perm/Wash according to the manufacturer's instructions. Antibody was diluted in Perm/Wash and incubated for 30 min on ice. If an unconjugated primary antibody was used, cells were washed in Perm/Wash and appropriate secondary antibody incubated for 20 min on ice. Following incubation cells were washed twice with Perm/Wash before being suspended in 120 µL of 1 % (v/v) formaldehyde solution in PFEA. For cell surface staining, PFEA was used in place of Cytofix/Cytoperm and Perm/Wash. Cells were immediately analysed on an LSR II flow cytometer with FACSDiva software. If multiple fluorochromes were used in the same sample, compensation was performed automatically by using single colour controls to minimise spectral overlap. Data were analysed using FlowJo software. To perform data analysis, the main population of cells was gated on a forward scatter versus side scatter dot plot according to size and granularity. For each sample a fluorescence index (FI) was calculated in order to allow the comparison of fluorescence values for each cell line. The FI is the fold increase of the mean fluorescence intensity of the main population of cells in the stained sample by comparison with the corresponding unstained sample. A minimum of 1.0×10^4 cells were counted in each experiment. FI values of less than two were considered negative. All flow cytometry expression data is intracellular unless otherwise indicated.

2.2.19 T cell culture and sensitisation

T cells were sensitised with peptide-pulsed dendritic cells in the following manner. Monocytes were isolated from PBMCs (Peripheral Blood Mononuclear Cell) by plastic adherence and matured into Dendritic Cells (DCs) with 800 IU/mL Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) and 500 IU/mL Interleukin-4 (IL-4) in Cell-Gro DC medium.

Monocytes were allowed to mature for six days with fresh cytokines added on days two and four. On day six, TNF (10 ng/mL) and LPS (0.5 μg/mL) were added. CD4+ T cells were isolated from PBMCs using antibody-conjugated magnetic microbeads according to the manufacturer's instructions. Following maturation, DCs were pulsed with peptide at a concentration of 10 μg/mL and added to CD4+ and CD4- depleted PBMC fractions with IL-2 (40 U/mL) and IL-21 (30 ng/mL). After two days in culture IL-7 (5 ng/mL) and IL-15 (5 ng/mL) were added. After seven days, T cell cultures were stimulated with autologous PBMCs (irradiated with 30 Gy), peptide (10 μg/mL) and IL-2 (40 U/mL). Subsequent re-stimulations were performed when judged microscopically to be appropriate, but at least seven days from the previous stimulation. Medium changes were performed every two to three days with 40 U/mL IL-2 or as necessary. X Vivo 15 medium was used for all T cell culture experiments.

T cell clones were stimulated with a mixture of allogeneic PBMCs (irradiated with 30 Gy) and the Laz388 B cell line (irradiated with 60 Gy). For every well in a U-bottom 96 well plate 2.5 x 10^4 T cells, 6.0 x 10^4 PBMCs and 1.5 x 10^4 Laz388 cells were added and cultured with 100 U/mL IL-2 (total volume 220 μL). Medium changes were performed twice weekly with IL-2.

The specificity of the T cells (lines or clones) was tested in co-culture assays with HLA-matched melanoma cells and with peptide-pulsed autologous PBMCs. For melanoma co-culture experiments, T cells were added to irradiated melanoma cells at a ratio of 10:1. In order to test peptide recognition, irradiated autologous PBMCs were added to T cells at a ratio of 1:1 with peptide (10 μg/mL). The co-cultures were performed in 96 well U-bottom plates for 48 hr, after which cell-free supernatants were removed and cytokine production determined with ELISA. PBMCs were irradiated with 30 Gy and melanoma cells with 80 Gy.

2.2.20 Statistical analysis

Statistical analysis was performed using Prism software. Cell line ligand adhesion data was obtained from ESTDAB. Within matching data sets, changes of less than 5.0 % were considered not to be different.

Paired two-tailed non-parametric t tests were used to assess significance for matching data points between two conditions.

Significance between two groups was assessed with two-tailed non-parametric t tests.

Correlations were tested for by using two-tailed non-parametric correlation tests.

Trends across four grouping variables were assessed with two-tailed chi-squared contingency tests.

Chapter 3: Heat shock protein expression in tumour and healthy breast tissue

3.1 Introduction

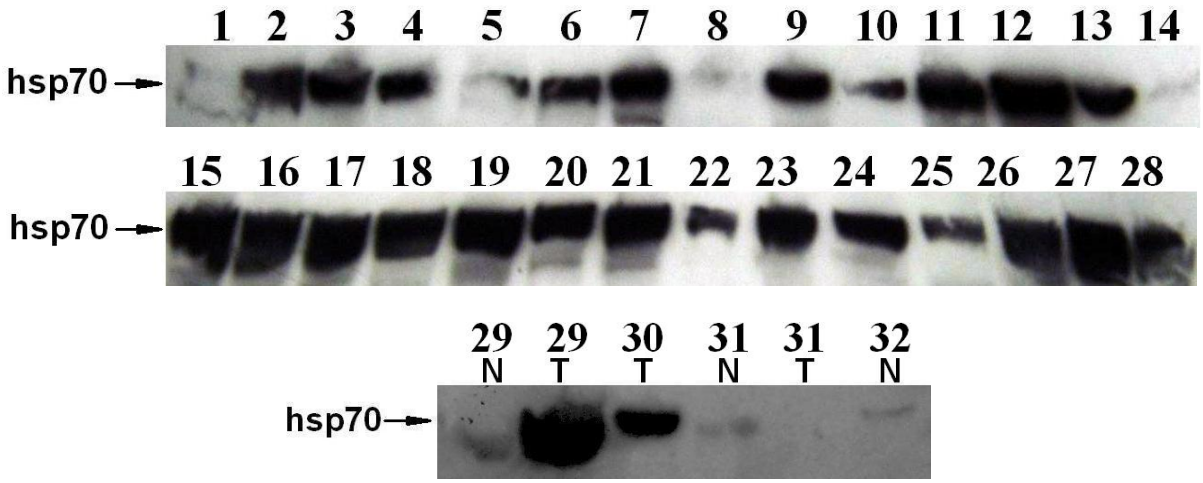
The hsp chaperone system is essential for the maintenance of the cancer phenotype. Hsps chaperone a range of signal transduction and pro-survival proteins that are required for the growth and progression of cancer [51, 145, 146]. Consequently, abnormal hsp expression has been reported in a range of human cancers and has been shown in some cases to correlate with therapeutic response and patient prognosis. In breast cancer, overexpression of hsp90 and hsp70 predicts poor patient prognosis and up-regulation of hsp70 is associated with chemotherapeutic resistance [84, 104]. The expression of oncoproteins such as HER2, OR and PR are important for the therapeutic management and prognosis of breast cancer patients and are chaperoned by hsps [104, 147]. Accordingly, hsps have been identified as targets in cancer therapy and a number of hsp inhibitors are currently under evaluation in human clinical trials in the treatment of cancer [148]. Since hsps play important roles in breast cancer and associate with other proteins essential for breast cancer progression, the expression of hsps and other proteins was investigated in tumour and healthy breast tissue samples obtained from breast cancer patients. The sample cohort consisted of 30 breast tumours, two with corresponding healthy breast tissue and one healthy breast tissue sample from a breast cancer patient. Western immunoblotting and 2D SDS-PAGE were employed to assess differences in protein expression.

3.2 Results

Using Western immunoblotting, hsp70 was observed to be expressed in the majority of breast tumour samples (Fig. 3.1). Comparing the tumour and healthy tissue obtained from the same individual showed that hsp70 was up-regulated in the tumour tissue by comparison with the healthy tissue (Fig. 3.1, Patient 29). Despite this observation, hsp70 was not universally expressed

in the breast tumour tissue samples, and was not significantly expressed in any of the three healthy breast tissue samples (Fig. 3.1, healthy tissue from Patients 29, 31 and 32 and tumour tissue from Patients 1, 8 and 14 did not show expression of hsp70)

Figure 3.1 Hsp70 expression in tumour and healthy breast tissue

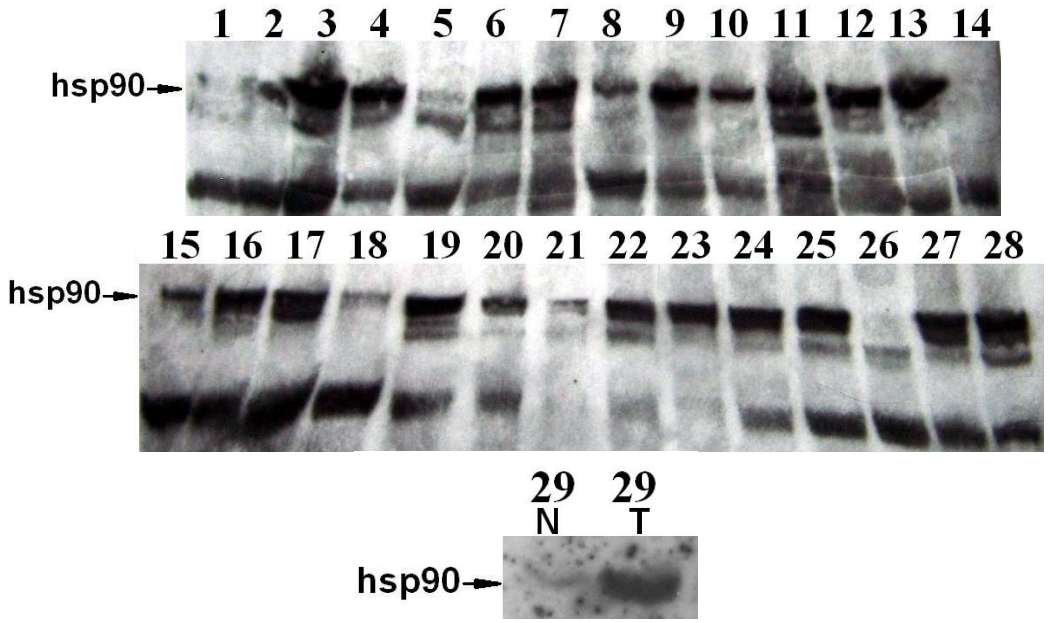


Thirty two breast cancer patients were screened for hsp70 expression using Western immunoblotting. The results demonstrate that hsp70 is expressed in the majority of these breast tumours.

Number, Patient number
T, Tumour sample
N, Normal sample

Hsp90 was also observed to be highly expressed in the majority of breast tumour samples, but was not expressed in all tumours examined here (for example, Patients 1, 5 and 26 in Fig. 3.2). The matched samples from Patient 29 showed that the tumour tissue up-regulated hsp90 by comparison with the healthy tissue. Some tumours that expressed low relative levels of hsp70 also expressed low relative levels of hsp90, but this trend was not seen in all samples (Patients 1 and 14, Figs. 3.1 and 3.2)

Figure 3.2 Hsp90 expression in tumour and healthy breast tissue



Twenty nine breast cancer patients were screened for hsp90 expression using Western immunoblotting. The results demonstrate that hsp90 is highly expressed in the majority of these breast cancer patients.

Number, Patient number
T, Tumour sample
N, Normal sample

Histological analysis of HER2, PR and OR expression performed during routine testing of surgically removed breast tumours was available for 22 of these breast tumour samples. Statistical analysis demonstrated a relationship between the expression of PR and OR ($P < 0.0005$) and a borderline significant relationship between OR and HER2 ($P = 0.058$), but no association between PR and HER2 ($P > 0.4$) (Table 3.1, PR and HER2 correlation data not shown). These results suggest the expression of PR and OR are very closely associated, while a weaker but noteworthy relationship between the expression of HER2 and the OR may exist.

Table 3.1 Relationship between oestrogen receptor, progesterone receptor and HER2 expression in breast tumour tissue

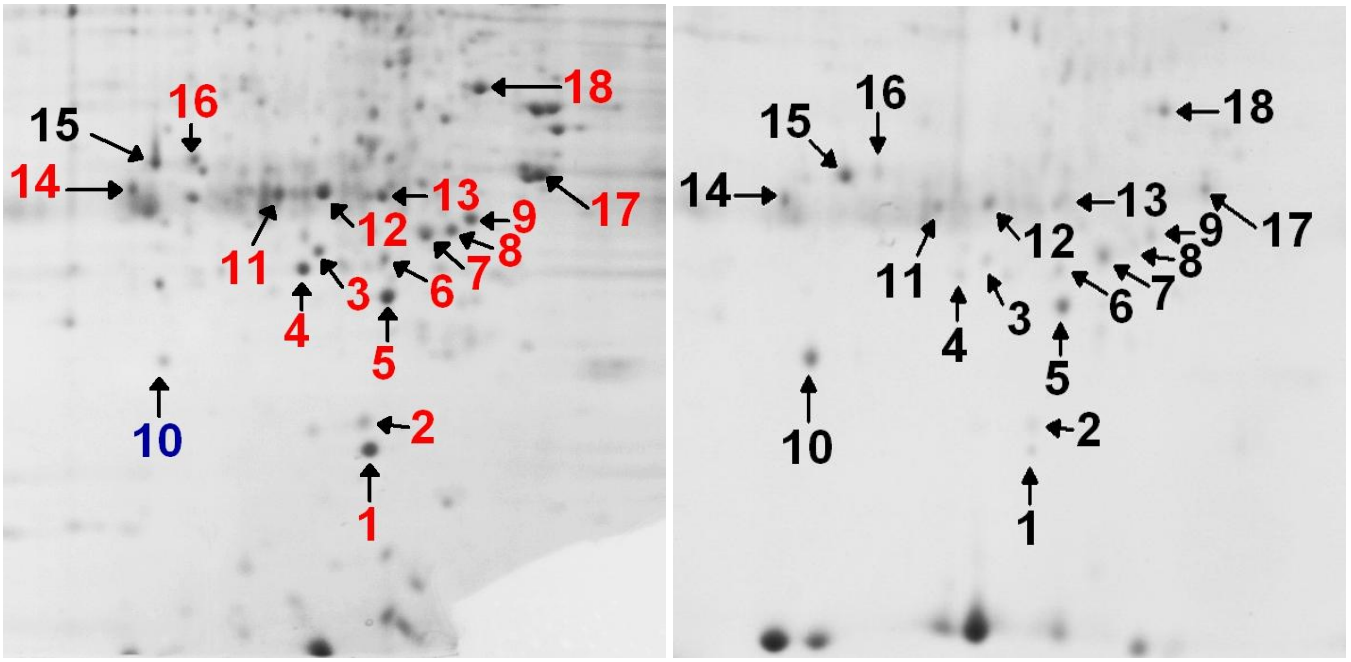
	PR Negative	PR Positive	P value
OR Negative	7	1	< 0.0005
OR Positive	1	12	

	HER2 Negative	HER2 Positive	P value
OR Negative	0	7	> 0.05
OR Positive	5	8	

The observation of differential hsp expression between the tumour and healthy breast tissue samples from Patient 29 was investigated more thoroughly using 2D SDS-PAGE. This technique separates proteins according to their isoelectric point (pI) and size (kDa) and results in a far more comprehensive protein separation when compared to 1D SDS-PAGE. Comparing the relative protein presence between tumour and healthy tissues obtained from the same individual serves as an ideal experimental control to evaluate proteins that may be differentially expressed in breast tumour tissue by comparison with healthy breast tissue. This approach allows for the identification of differentially expressed proteins that may be associated with breast cancer carcinogenesis, free of inter-patient variability.

Eighteen distinct matching protein spots were identified in a cognate area of the 2D separation between the tumour and healthy breast tissue samples from Patient 29 (Fig. 3.3). Seventeen of these were observed to be differentially expressed in the breast tumour tissue by comparison healthy breast tissue: Sixteen of these were up-regulated in the tumour sample, while one showed no change (Fig. 3.3, protein number 15) and one was observed to be down-regulated (Fig. 3.3, protein number 10). These results therefore suggest differential protein expression may be associated with acquisition of the breast cancer phenotype.

Figure 3.3 2D SDS-PAGE separation of proteins breast tumour and healthy tissue from the same individual



Tumour and healthy breast tissue protein extracts from the same patient were separated using 2D SDS-PAGE. The results suggest that proteins are differentially expressed in breast tumour tissue by comparison with healthy breast tissue. Representative results of replicate experiments are shown.

Tumour tissue labelling:
Red number indicates protein is up-regulated in the tumour sample
Blue number indicates protein is down-regulated in the tumour sample
Black number indicates protein displayed no difference in expression between samples

3.3 Discussion

Notwithstanding the limitations of the present study, differential protein expression in breast tumour tissue by comparison with healthy breast tissue was indicated in this study. Given that these tissues were derived from a single patient, differences in protein expression cannot be due to variation across individuals. As such these differentially expressed proteins may be associated with breast cancer carcinogenesis and play important roles in breast cancer. Of note is the limited number of matched tumour and healthy patient samples included in this study. These results therefore should be regarded as very preliminary. These observations prompt questions as to the identity of these proteins, their chaperone complex association, the mechanism of differential expression and their role in breast cancer. Since the vast majority of these proteins were observed to be increased in expression, up-regulation of these proteins suggests their function is under greater demand in transformed cells. This in turn implies that these proteins may be important for the survival of breast cancer cells, and as such their expression may be relevant to patient prognosis or therapeutic management and might consequently have use as biomarkers or as therapeutic targets in breast cancer patients. This has already been demonstrated for a number of proteins that are up-regulated in breast cancer, for example hsp70 expression is associated with tumour grade and lymph node status, and high hsp90 expression is associated with reduced survival in breast cancer patients [98, 104]. In line with previous reports, the widespread expression of hsp90 and hsp70 was observed in the majority of breast cancer tissue samples in this study. In addition, the expression of hormone receptors and HER2 were shown to correlate. This suggests the function of these proteins is related and that they assist one another in the growth and development of breast cancer. Alternatively, these proteins may support the growth of breast cancer through separate mechanisms. Since these proteins are chaperoned by hsps and the expression of all these proteins has implications for patient prognosis and therapeutic management, it is therefore possible that these proteins participate in a network or networks that are integral to the growth and progression of breast cancer. This hypothesis is supported by

previous studies showing the expression of hsp90 to correlate with HER2 and OR, while other studies have shown a relationship between the expression of hsp70 and OR [98, 104, 149]. Co-immunoprecipitation directed at these proteins followed by Western immunoblotting could be used to investigate the hypothesis that they associate with one another, therefore supporting the hypothesis that they participate in the same protein network.

Using this approach, further studies are required to identify proteins important for breast cancer growth, some of which may be relevant to patient outcome. To the best of the author's knowledge few, if any, studies have assessed protein expression using matched tumour and healthy breast tissue with 2D SDS-PAGE such as performed here. To expand on the current study, a larger number of matched tumour and healthy breast tissue samples could be screened for differentially expressed proteins using 2D electrophoresis. Proteins observed to be differentially expressed throughout the sample cohort could then be identified using approaches such as mass spectrometry. Larger scale follow-up studies would confirm or deny the importance of these differentially expressed proteins as breast cancer biomarkers. To better understand the role that these proteins play, their association and interaction with other proteins could be investigated in breast tissue and cell lines. By doing so, new protein networks that facilitate cancer progression may be uncovered. These approaches used in parallel are likely to add significantly to understanding the biology of breast cancer.

Chapter 4: Characterisation of heat shock protein 90 client proteins in tumour and healthy breast tissue

4.1 Introduction

The hsp90 molecular chaperone is responsible for the stabilisation of a multitude of cellular pathways and processes [145, 150]. Hsp90 is essential for the survival of eukaryotic cells and it functions by mediating and stabilising the activity of other cellular proteins [6, 150]. Hsp90 is an attractive therapeutic target in the treatment of cancer as many of its client proteins are involved in signal transduction and hsp90 appears to be unique in that its inhibition results in the destabilisation of multiple signalling pathways. Specifically, hsp90 is essential for the function of multiple growth and survival pathways that are required for the maintenance of the cancer phenotype [51, 145, 146]. Reflecting perhaps the fundamental role that hsp90 plays in the maintenance and progression of cancer, hsp90 is abnormally expressed in a variety of human cancer types. In breast cancer, hsp90 has been shown to be up-regulated and this correlates with poor patient prognosis [104]. Multiple hsp90 inhibitors of the ansamycin type, such as geldanamycin derivatives with more suitable pharmacological profiles, have been evaluated for the treatment of cancer in human clinical trials, including a phase III clinical trial [59] .

Despite the clinical use of hsp90 inhibitors, knowledge of the effect(s) of hsp90 inhibition is limited. It is known that hsp90 interacts with a large number of client proteins, however a thorough knowledge of specific interactions in different biological contexts does not currently exist [6, 150]. This study presents a preliminary investigation of hsp90 client proteins sensitive to the hsp90 inhibitor geldanamycin in human breast tissue. This study included the use of tumour and healthy (non-cancerous) breast tissue from breast cancer patients and healthy breast tissue from a cancer-free individual. In addition to client proteins that bind to hsp90, hsp90 client proteins that associate with hsp40, hsp56, hsp60, hsp70 and hsp105 were examined. Client

proteins of these hsps sensitive to geldanamycin were investigated in an effort to increase the current body of knowledge regarding hsp and hsp client protein associations in cancer and to elucidate the role of hsps in the assembly of chaperone complexes in health and disease.

4.2 Results

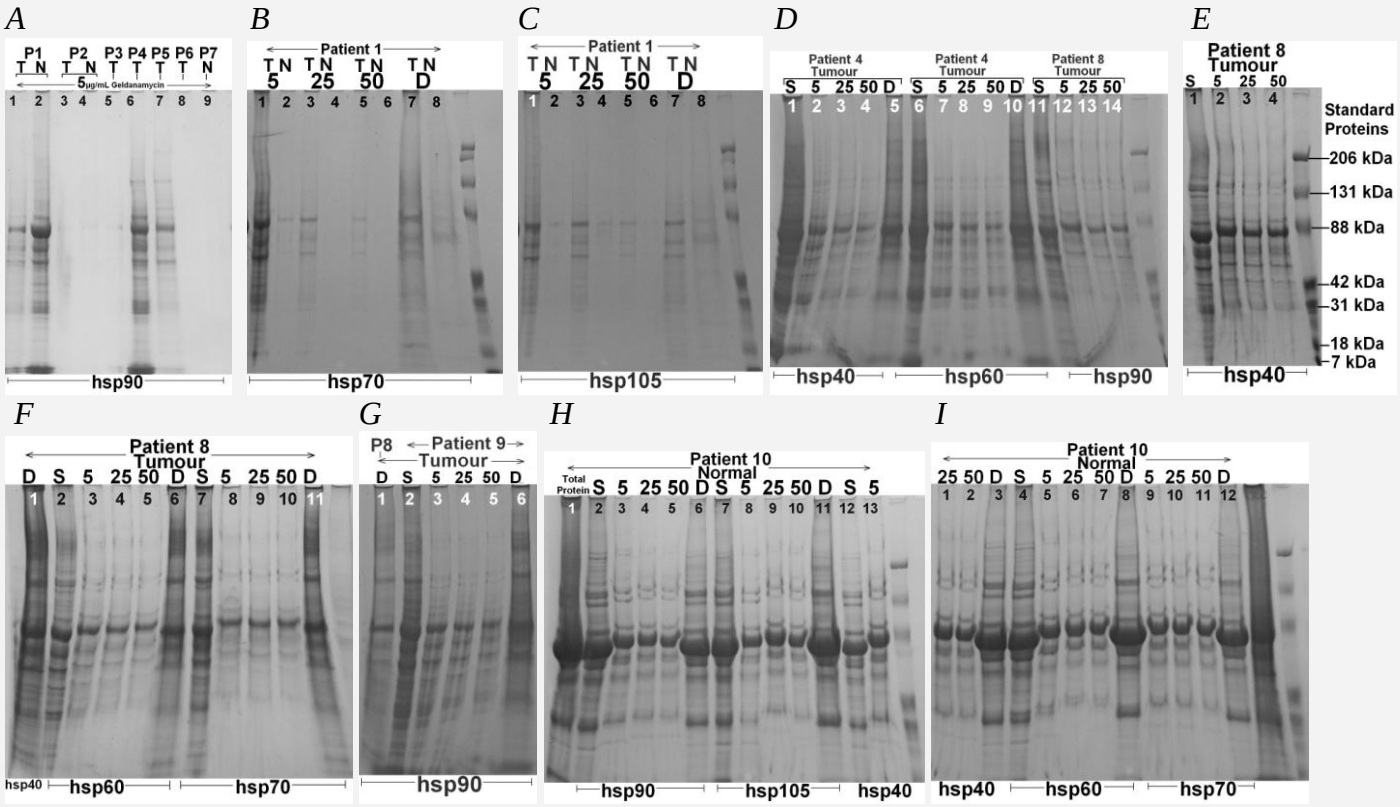
Hsp90 client proteins in breast cancer patients

Protein extracts of tumour and healthy breast tissue from ten breast cancer patients (three matched for tumour and healthy tissue) and one cancer-free individual were screened for hsp90 client proteins using co-immunoprecipitation followed by geldanamycin treatment. Since geldanamycin is a specific hsp90 inhibitor, proteins eluted from the immunoprecipitated protein complexes, following treatment with geldanamycin, were taken as representing a select subset of hsp90 client proteins. Hsp90α client proteins eluted by geldanamycin were identified in seven of 11 patients. Among the seven patients in which hsp90 client proteins were identified (Fig. 4.1A lanes 1-2 and 6-7, Fig. 4.1D lanes 11-14, Fig. 4.1G, Fig. 4.1H lanes 2-6; one patient data not shown), two were matched for tumour and healthy tissue and no differences in the client proteins between tissue types were observed for both patients. Hsp90 client proteins sensitive to geldanamycin were not identified in four of the 11 patients (Fig. 4.1A lanes 3-5 and 8-9). Treatment of these four patient samples with denaturant did not result in any observable protein, suggesting that these samples either contained an undetectable level or an absence of hsp90α client proteins (denaturation data not shown).

Among the seven patients in which hsp90α client proteins were identified, the majority (5/7) displayed the same protein group consisting of approximately 20 proteins, most of which migrated to a position corresponding to 90kDa or less. One of these seven patients (patient 10, Fig. 4.1H lanes 2-6) was unique in displaying a different group of proteins compared to the other patients. It is noteworthy that this was the only sample of healthy tissue obtained from a cancer-free

individual. Despite this, at least three protein bands in common to all patients were identified, as shown in Fig. 4.3. All client proteins were sensitive to geldanamycin at a minimum concentration of 5 µg/mL

Figure 4.1 Geldanamycin-sensitive hsp90 client proteins in tumour and normal breast tissue extracts



Tumour and healthy breast tissue extracts were immunoprecipitated with hsp antibodies and hsp90 client proteins eluted with geldanamycin, separated using SDS-PAGE and stained with Coomassie G250.

P, patient number
T, tumour breast tissue sample
N, healthy breast tissue sample
S, supernatant (unbound protein after immunoprecipitation)
D, denaturation applied to protein A beads
5/25/50, geldanamycin concentration (µg/mL)
hsp, immunoprecipitation performed with the noted hsp antibody

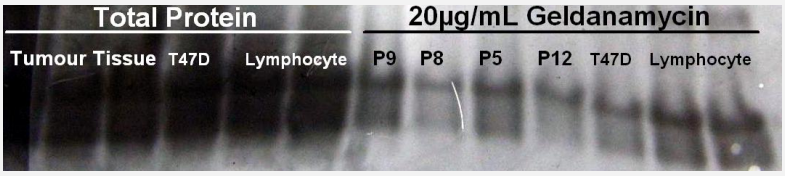
Hsp90 associates with hsps 40, 56, 60, 70 and 105 in human breast cancer

To investigate the role of other hsps associating with hsp90 in breast cancer, the original immunoprecipitation experiments were repeated using hsp 40, 60, 70 and 105 antibodies in place of anti-hsp90 and followed by elution with geldanamycin, as before. Client proteins sensitive to geldanamycin (inferred as hsp90 client proteins since they were eluted from the immunoprecipitates by the hsp90 inhibitor geldanamycin) were observed in association with all hsp antibodies tested in samples from all five patients (Fig. 4.1B, C lanes 1-8, Fig. 4.1D lanes 1-10, Fig. 4.1E lanes 1-4, Fig. 4.1F lanes 1-11, Fig. 4.1H lanes 7-13 and Fig. 4.1I lanes 1-12). In four of the five patients, these proteins were the same as those identified in experiments with hsp90 antibodies. Therefore, these experiments suggested that these hsps associate with hsp90 in human breast cancer and that they assemble in a complex with the same proteins sensitive to geldanamycin identified using hsp90 antibodies as the target for immunoprecipitation. All eluted proteins were sensitive to geldanamycin at a minimum concentration of 5 μg/mL.

One of the five patients (patient 1, Fig. 4.1A lanes 1-2 and Fig. 4.1B, C lanes 1-8) showed differences in the association of hsp90 client proteins between the tumour and normal tissue samples. While the tumour sample displayed the same group of client proteins sensitive to geldanamycin for each hsp, the normal sample only displayed this group of proteins in experiments with hsp90 antibodies. By contrast, in experiments using hsp40 (data not shown), hsp60 (data not shown), hsp70 and hsp105 antibodies, a single protein band was detected in the healthy tissue. These experiments implied that in this patient there was a selective association of hsp90 client proteins with hsps 40, 60, 70 and 105 in the breast tumour tissue, while this association was absent in the healthy breast tissue.

Patient 10 (healthy tissue sample from a cancer-free individual, Fig. 4.1H, I) showed a different group of client proteins by comparison with the other patients. This result was consistent with the experiments performed with hsp90 antibodies.

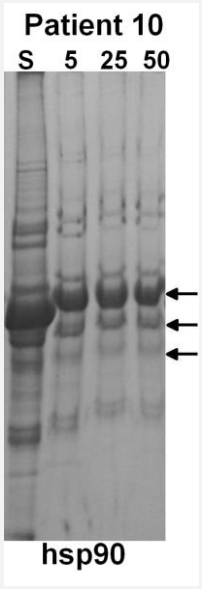
Figure 4.2 Hsp56 Western immunoblot of immunoprecipitated hsp90 client proteins in breast tumour tissue extracts



Western blotting inferred hsp56 as an hsp90 client protein. Breast tumour tissue extracts from four breast cancer patients and the T47D breast tumour cell line were immunoprecipitated with hsp90 antibodies and eluted with geldanamycin. Western blotting with anti-hsp56 was subsequently performed on the eluted proteins
P, patient number.

Immunoprecipitation with hsp56 antibodies was performed with protein extracts from Patients 1 and 9 (data not shown). Client proteins sensitive to geldanamycin were observed in both patients. To confirm this result, hsp90-bound geldanamycin-sensitive proteins from four breast cancer patient samples and the T47D tumour cell line were transferred to membranes and probed with hsp56 and hsp90 antibodies using Western immunoblotting (Figs. 4.2, 4.4). The presence of hsp90 and hsp56 was observed in all samples. Taken together, these data provided two independent sources of evidence for an association between hsp56 and hsp90 in breast cancer. These data also implied that hsp56 is a member of the geldanamycin-sensitive protein group.

Figure 4.3 Hsp90 client proteins analysed with LC-MS



Geldanamycin-sensitive hsp90 client proteins common to all patients and both tissue types were sequenced by LC-MS. Arrows indicate bands analysed. Top arrow indicates lumican.

S, supernatant (unbound protein after immunoprecipitation) 5/25/50, geldanamycin concentration (µg/mL)

Lumican is an hsp90 client protein in human breast tissue

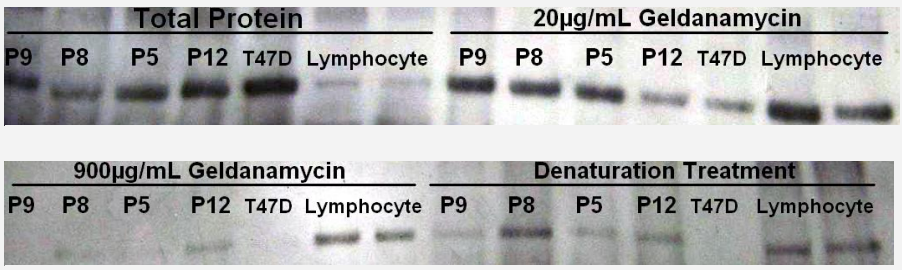
A select number of hsp90 client proteins eluted with geldanamycin after immunoprecipitation with anti-hsp90 were also observed to be present in immunoprecipitation using antibodies against hsps 40, 60, 70 and 105. Three of these common proteins were excised (Fig. 4.3, Patient 10) and sequenced. The same three protein bands were evident in all samples (both tumour and healthy) where hsp90 client proteins were observed. Lumican, a protein that plays an important role in breast stromal tissue, was identified as one of these common hsp90 client proteins and, thus, as a member of the geldanamycin-sensitive group of proteins.

A subset of hsp90 displays resistance to geldanamycin in breast cancer tissue

In immunoprecipitation experiments, bound protein complexes were treated with geldanamycin solutions of increasing concentration (5, 25 and 50 µg/mL). This was followed by application of a denaturing solution to ensure all protein associations had been disrupted, thereby resulting in the removal of all client proteins. In the example shown in Fig. 4.1B the healthy tissue sample eluted protein only by the 5 µg/mL geldanamycin treatment condition (Fig. 4.1B, lane 2). The higher concentration solutions failed to elute protein from the immunoprecipitated protein complexes (Fig. 4.1B, lanes 4 and 6). This suggests all hsp90 and hsp90 client proteins had been eluted from the immunoprecipitated protein complexes.

However, treatment of this sample with a denaturant resulted in the elution of the same protein band (Fig. 4.1B, lane 8). This was a feature of all immunoprecipitation experiments and was further investigated using Western immunoblotting. In the Western blots shown in Fig. 4.4, an hsp90 immunoprecipitation was performed with four breast cancer patient (tumour) samples and the T47D tumour cell line. The immunoprecipitated protein complexes were treated with solutions containing 20 and 900 µg/mL geldanamycin before being treated with a denaturant.

Figure 4.4 Geldanamycin resistant hsp90 in breast tumour tissue extracts



Hsp90 displays resistance to geldanamycin in human breast tissue. Four breast tumour tissue samples and the T47D breast tumour cell line were immunoprecipitated with hsp90 antibodies and eluted with geldanamycin. Western blotting with hsp90 antibodies was subsequently performed on the eluted proteins.
P, patient number

The samples were transferred to nitrocellulose membranes and probed with an hsp90 antibody using Western immunoblotting. The geldanamycin solution (20 µg/mL) eluted a single band in all breast cancer samples. Increasing the concentration to 900 µg/mL did not result in further elution of this band in Patients 5 and 9. However, when these samples were treated with a denaturant, a reappearance of this same protein band was seen. These data suggest that hsp90 is at least partially resistant to geldanamycin. It is not known whether this was due to reduced sensitivity or resistance to geldanamycin or whether two distinct pools of hsp90 exist, one sensitive to geldanamycin and the other insensitive.

4.3 Discussion

Hsp90 client proteins in breast cancer patients

Hsp90 and its client proteins are involved in the growth of cancer cells and, hence, are used as therapeutic targets [145, 146]. As such, the degree to which hsp90 and hsp90 client proteins are expressed may predict patient response to hsp90 inhibitors. Furthermore, it is not known whether expression of hsp90 client proteins correlates with expression of hsp90. Client proteins were identified in seven of the 11 patients screened in this study. Subsets of breast cancer patients with reduced expression of hsp90 or hsp90 client proteins thus may not respond to hsp90-targeted drugs as favourably if at all. To date, studies have focused on the expression of hsp90 or a small number of well-characterised client proteins. No studies have investigated the spectrum of hsp90 client proteins in cancer tissue as performed here and no clinical studies have investigated the expression of hsp90 or of hsp90 client proteins in relation to response to hsp90 inhibitors. In addition, it remains to be determined whether the observed absence of client proteins was due to down-regulation or an absence of these proteins. These aspects of the hsp90 chaperone machinery should be investigated in future studies.

Hsp90 associates with hsps 40, 56, 60, 70 and 105 in human breast cancer

This study is the first to report the association of hsp90 with these heat shock proteins in human breast tumour tissue. The specific effect(s) of hsp90 inhibition remains a pressing question. It is known that hsp90 functions as a member of a multi-protein chaperone complex with other hsps [6, 8]. These chaperone complexes are delicately balanced and transiently dynamic in their function [6, 36]. A change in the activity of one protein within the complex is likely to result in a cascade of repercussions for the functionality of the complex as a whole. As an example of the intimate and co-dependent role these hsps possess, hsp90 inhibition by geldanamycin has been shown to induce hsp70 expression [151]. As a consequence, the chaperoning of client proteins is likely to be altered and this may be a major mechanism of action of hsp90 inhibitors *in vivo*.

Hsp56, otherwise known as FKBP52, is an important hsp90 co-chaperone that possesses peptidyl-prolyl isomerase activity [152]. This is the first report of an association between FKBP52 and hsp90 in breast cancer, however FKBP52 has previously been reported to be up-regulated in breast tumour tissue [153]. Given that hsp90 is up-regulated in breast cancer [104, 154], increased expression and association of these two proteins may be a supporting mechanism for the growth of breast tumour cells. This study has provided evidence for the inherent complexity of the hsp-chaperone system in breast cancer and for the co-chaperones and client proteins whose activity may be altered by hsp90 inhibitors in breast cancer.

One patient displayed a select group of hsp90 client proteins that was only present in immunoprecipitation experiments using antibodies to hsps 40, 60, 70 and 105 in the tumour tissue, while the healthy tissue from the same patient did not contain these proteins. These data provided evidence of a cancer-specific group of proteins that may be used as a therapeutic target to specifically deliver a drug to cancerous breast cells. However, to qualify as a clinically useful drug, this cancer-specific group needs to be present in a substantial proportion of the breast cancer patient population which is known to be highly diverse in both causative mechanisms and prognostic outcomes.

The hsp-client protein interactions observed in this study were eluted from chaperone complexes following their extraction from tissues using mechanical disruption, sound energy, sub-zero temperatures in a urea-detergent based extraction solution. Of note is that it is not known what effect(s) these treatments may have on the association of chaperone complexes. These results therefore may present a distorted or incomplete view of the chaperone complexes found *in vivo*.

Molecular chaperones function throughout a dynamic cycle and are involved in a host of biochemical processes such as protein folding, translocation and destruction [8, 9].

Therefore it seems plausible that the following inferences can be made about hsps in their role as molecular chaperones: 1) Hsp molecular chaperones associate with different chaperone complexes that vary in composition in order to perform different roles. 2) One particular species of hsp may simultaneously participate with a number of chaperone complexes that vary in composition in order to chaperone a large number of client proteins. From this perspective, a complex view of proteome-wide chaperone activity begins to emerge, and it is clear that the data presented in these experiments neglect these aspects. It is therefore possible that samples which showed the same set of proteins, in fact, differed in the association of these proteins, but that it was not observed in these experiments. In order to discern the chaperone complex associations, non-denaturing native PAGE could be employed. Patient 10 was observed to display a separate set of geldanamycin-sensitive proteins compared with the other patients, this therefore suggests a more substantial difference in the chaperone complexes than that observed in the remainder of the patients who all displayed a similar set of proteins. Although Patient 10 was the only sample of healthy breast tissue from a cancer-free individual, it may be speculated that this set of proteins is the norm for healthy individuals, whereas in the other samples, the chaperone complexes may have been altered due to the carcinogenic state. Alternatively, this set of chaperone complexes may be associated with a predisposition for breast cancer in these individuals. Larger scale follow-up studies will assist in characterising the chaperone complex association in healthy individuals and breast cancer patients and may reveal if, as of yet, unidentified sets of chaperone complexes are present, as a degree of variation in the chaperone complexes across individuals may exist beyond that observed in this study. Native PAGE will assist in characterising the association of chaperone complexes within the spectrum of proteins observed using SDS-PAGE.

Lumican is an hsp90 client protein in human breast tissue

Lumican mRNA has previously been demonstrated to be up-regulated in breast cancer patients [155]. But this study is the first to report the association between lumican and hsp90. As this protein band was identified in hsp90 immunoprecipitates and eluted by geldanamycin in breast tumour and healthy tissue extracts, it can be inferred that lumican associates with hsp90 in breast tissue. Leygue *et al.* [155] reported that increased expression of lumican is associated with higher tumour grade and increased hsp90 expression in breast cancer patients is predictive of a poor prognosis. These findings taken together with the present study reporting that lumican associates with hsp90 in breast tissue, suggest that these proteins may function in tandem to facilitate the progression of breast cancer. Given the results of the immunoprecipitation experiments using antibodies to other hsps, the association of lumican with hsps 40, 60, 70 and 105 can be inferred and these proteins are likely to support the progression of breast cancer through their chaperoning action. Indeed, increased hsp70 expression has been reported to be associated with decreased survival in breast cancer patients [107]. By further mapping hsp90 client proteins whose expression is associated with the progression of breast cancer, a range of biomarkers may become available and this may allow a more accurate prediction of a patient's prognosis and, as such, may have consequences for the therapeutic management of breast cancer patients as decided by oncologists on a patient-by-patient basis. Therefore, this work contributes at a preliminary preclinical level to the growing trend of personalised cancer care. In addition, this avenue of research will give greater insight to the progression of breast cancer at the biochemical level, which may, in turn, lead to the development of more effective therapies by revealing new families of hsp-associated biochemical targets.

In contrast to the study by Leygue *et al.* [155], low levels of lumican expression in breast cancer patients have been shown to correlate with large tumour size, shorter time to progression and poorer survival [235]. These data suggest that lumican may play a role as a tumour-suppressor

protein. Similarly, studies that have investigated the role of lumican in other cancer types do not present a clear role for this protein. For example, presence of lumican in the stromal tissues of pancreatic cancer patients correlated with shorter survival times compared with patients with lumican negative stromal tissues [230]. Of note is that lumican expression in cancer cells of these patients did not correlate with clinical parameters, yet female gender, advanced stage, retroperitoneal and duodenal invasion and residual tumour correlated with lumican expression in stromal tissue. In lung adenocarcinoma, lumican expression in cancer cells correlated with pleural invasion and larger tumour size, but lumican in stromal tissues did not correlate with clinical parameters [231]. This study also noted that lumican was localised either in the cytoplasm of lung cancer cells and/or in stromal tissues adjacent to cancer cells. In colorectal cancer, survival was significantly shorter in patients with tumours that expressed lumican compared with patients that showed an absence of lumican expression [232]. These studies suggest that lumican expression in both tumour and stromal tissue may be relevant to unfavourable patient clinical parameters. Studies examining the role of lumican in cell lines have shown that its over-expression may increase or decrease markers of aggressiveness and for this to be dependent on the cell line. Yet further experiments by the same experimenters showed that lumican over-expression *in vivo* resulted in uniformly smaller tumours, likely through an anti-angiogenic mechanism [233]. Other studies have suggested that lumican polymorphisms may be associated with breast cancer risk [234]. These studies hint at the complex role for lumican in cancer and that these roles may vary depending on the tissue type in which it is expressed (stromal or tumour) or on the type of cancer. Given that in this study lumican was inferred to occur in association with hsp90 in both tumour and healthy breast tissue, it may play a role as a tumour-suppressor or tumour-promoter protein that may be mediated through tumour-stromal interactions or directly within tumours. It is evident that further studies are required in order to obtain a better understanding for the role of lumican in cancer.

A subset of hsp90 displays resistance to geldanamycin in breast cancer tissue

A subset of hsp90 was observed to be resistant to the effects of geldanamycin, even at a high physiological concentration of 0.9 mg/mL. This observation may be important to the clinical application of hsp90 inhibitors. The ability of some hsp90 to resist inhibition may be a limitation to these drugs. In cancer patients treated with hsp90 inhibitors, the proportion of hsp90 that is resistant to geldanamycin may increase in an attempt by the cancer to continue to grow. Consequently, the proportion of cells resistant to hsp90 inhibitors may increase in response to treatment, a potential mechanism for the development of resistance. The basis for geldanamycin resistance is not known, but several possibilities exist. There may be an as of yet unidentified hsp90 isoform which is less sensitive to geldanamycin inhibition, possibly due to alteration of the ATP binding domain. The hsp90N isoform which contains a truncated ATP binding domain is a potential candidate. This isoform has a substantially lower molecular weight of approximately 75kDa [45] and therefore would not be expected to migrate to a similar position as full length hsp90 in SDS-PAGE experiments. Given that similar migration positions were observed, it is unlikely that the hsp90N isoform is the cause of the geldanamycin resistance observed here. Alternatively, geldanamycin resistance may be due to post-translation modification such as acetylation. Hsp90 acetylation has been reported to hinder the binding of ATP [36], and since geldanamycin and ATP bind to the same site, this may similarly affect the binding of geldanamycin.

Chapter 5: Identification of differential protein expression in primary-derived versus metastasis-derived melanoma cell lines and the effect of stromal cell interactions

5.1 Introduction

Cancer metastasis results from the spread of cancer cells from the primary originating tumour to distant organs. This process accounts for the majority of deaths among cancer patients [16] and despite its obvious clinical importance, is currently not well understood. It has been observed that if cancers are treated before metastasis occurs, patient prognosis is significantly enhanced. However, if treatment is delayed and metastasis is allowed to develop, treatments are less effective. Moreover, even after apparent successful treatment of the primary cancer, metastases can develop years later. For the metastatic process to occur effectively, a series of steps additional to those involved in primary tumour formation must be completed. Accordingly, metastatic tumours are associated with genetic mutations and altered protein expression similar to but distinct from the primary cancer from which they develop [156-158]. Melanoma tumours typically possess a high propensity to metastasise (personal communication, Dr med. Benjamin Weide) and it has been suggested that proteins expressed on the cell surface assist in this process. For example, cell surface hsp70 has been shown to occur more frequently on metastatic melanoma tissue than on primary melanoma tissue [120] and cell surface hsp90 has been shown to correlate with melanoma progression, while its inhibition results in the disruption of cell motility and metastasis [89, 159, 160]. Adhesion molecules play crucial roles in the progression and development of cancer generally, while in melanoma the expression of cell surface adhesion molecules has been demonstrated to be important for progression and invasion [161, 162]. Altered MHC protein expression is a mechanism associated with tumour immune escape, and has been shown to be a feature of cancer and its metastasis [163].

Given the need to better understand the metastatic process, this study was designed to investigate the expression of cell surface proteins between primary-derived and metastasis-derived melanoma cell lines in addition to investigating the influence of stromal cell co-culture. The aim of this study was to investigate if the expression of cell surface hsps, adhesion molecules, apoptosis receptors and MHC molecules show differential expression in primary- versus metastasis-derived melanoma cell lines. The proteins chosen as representing members of these distinct functional groups were hsp90, hsp70, hsp60, hsp40, hsp32, CD44, CD54, CD95, CD155 and MHC I. As an additional aspect to this study, the effect of fibroblast co-culture was investigated in an attempt to facilitate the development of an *in vivo* culture model. Fibroblasts are one of the major cell types that compose the skin and their presence may provide a culture environment more similar to *in vivo* growth conditions. It was therefore of interest to assess the interactions, if any, between melanoma cells and fibroblasts, thereby revealing potential cross-talk between tumour cells and normal stromal cells.

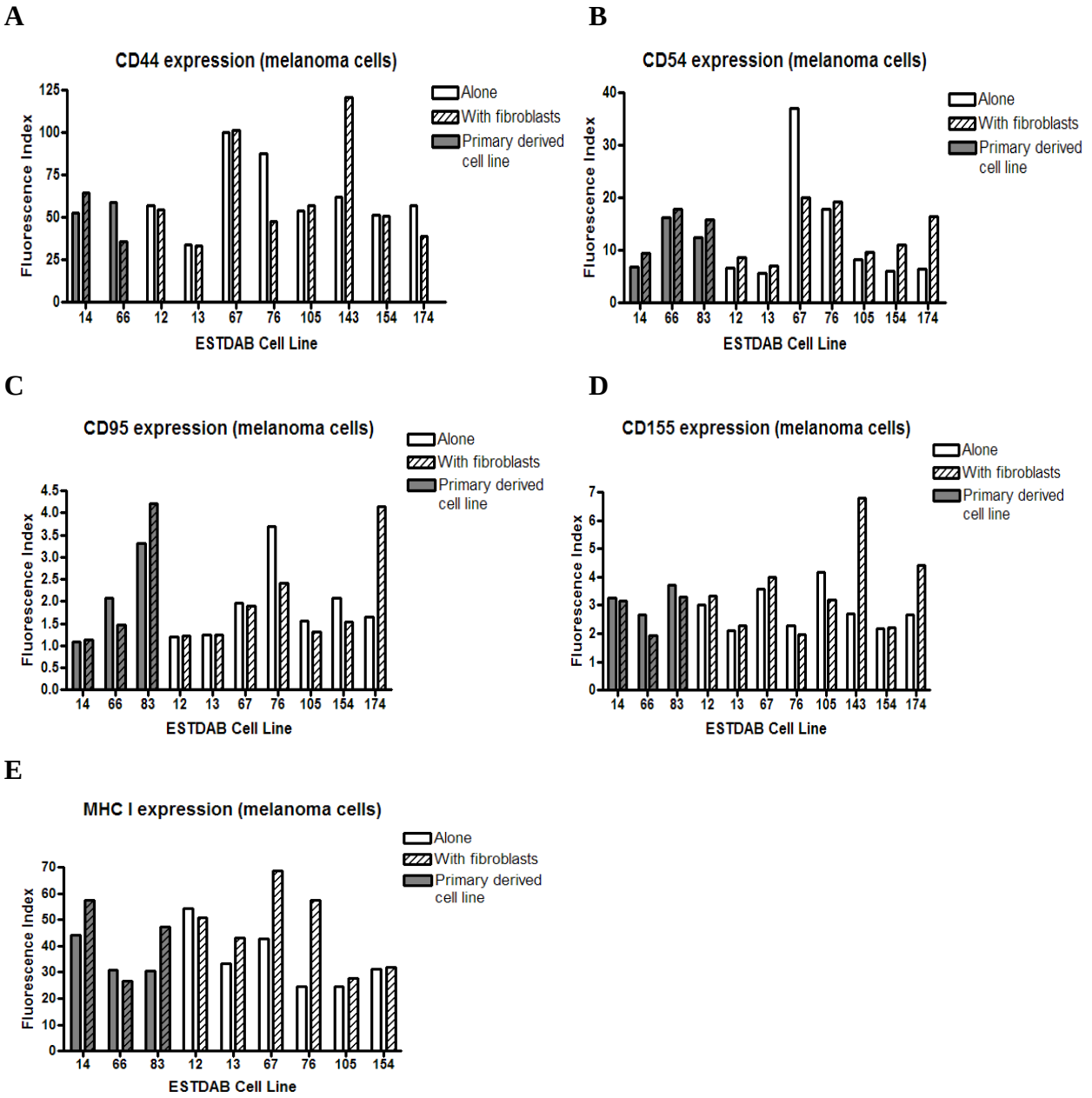
To this end, 11 melanoma cell lines consisting of three matched pairs of primary- and metastasis-derived cell lines from the same patient and four non-matched metastasis-derived cell lines were cultured with or without fibroblasts and screened for the expression of the proteins of interest. The following cell lines were used: 1) Matched cell lines EST66 (primary-derived) and EST76 (metastasis-derived), 2) Matched cell lines EST14 (primary-derived), EST12 (metastasis-derived) and EST13 (metastasis-derived), 3) Matched cell lines EST83 (primary-derived) and EST67 (metastasis generated in mouse) and four metastatic cell lines EST154, EST143, EST105, EST174. Cell cultures were performed in 175 cm^2 cell culture flasks for a period of nine days under identical conditions with or without fibroblasts. To determine changes in protein expression on fibroblasts co-cultured with melanoma cells, fibroblasts were additionally cultured alone as controls. Following the culture period, cells were harvested with trypsinisation, stained with antibodies and protein expression assessed with flow cytometry. All antibodies were unconjugated

except PE-conjugated hsp antibodies and FITC-CD90. Multicolour flow cytometry was employed where unconjugated antibodies were first applied, followed by binding to a PO-conjugated secondary antibody. To prevent binding of directly conjugated mouse antibodies to the PO-conjugated secondary antibody, blocking with 10 % (v/v) mouse serum before incubation with conjugated antibodies was performed. The fibroblast specific marker CD90 [164] was used to differentiate between melanoma cells and fibroblasts. All cultures were set with 3.3×10^5 melanoma cells and fibroblasts (where applicable) each with 45 mL RPMI 1640 medium containing 10 % (v/v) heat inactivated FCS. To analyse these data, the main population of cells in each sample was gated based on forward scatter versus side scatter dot plot and the mean fluorescence intensity of each fluorochrome in this population obtained. The fold increase in fluorescence of the stained sample by comparison with the unstained sample was used to quantify protein expression, and is subsequently referred to as the Fluorescence Index (FI). For co-culture experiments, melanoma cells and fibroblasts were analysed separately by gating the CD90 positive (fibroblast) and CD90 negative (melanoma) cells.

5.2 Results

The expressions of CD44, CD54, CD95, CD155 and MHC I proteins in the melanoma cell lines were not associated with primary or metastatic tumour origin, nor were trends observed within matched pairs of primary- and metastasis-derived cell lines (Fig. 5.1). This was true with or without fibroblast co-culture, although these proteins displayed altered expression in the presence of fibroblasts. Of note was that the matched cell lines EST14 (primary), EST12 and EST13 (both metastatic) showed relatively low expression of CD54 and CD95 with or without fibroblast co-culture (Figs. 5.1B and C). In summary, the changes in protein expression were variable for each cell line and each protein.

Figure 5.1 CD44, CD54, CD95, CD155 and MHC I protein expression on primary- and metastasis-derived melanoma cell lines with or without fibroblast co-culture

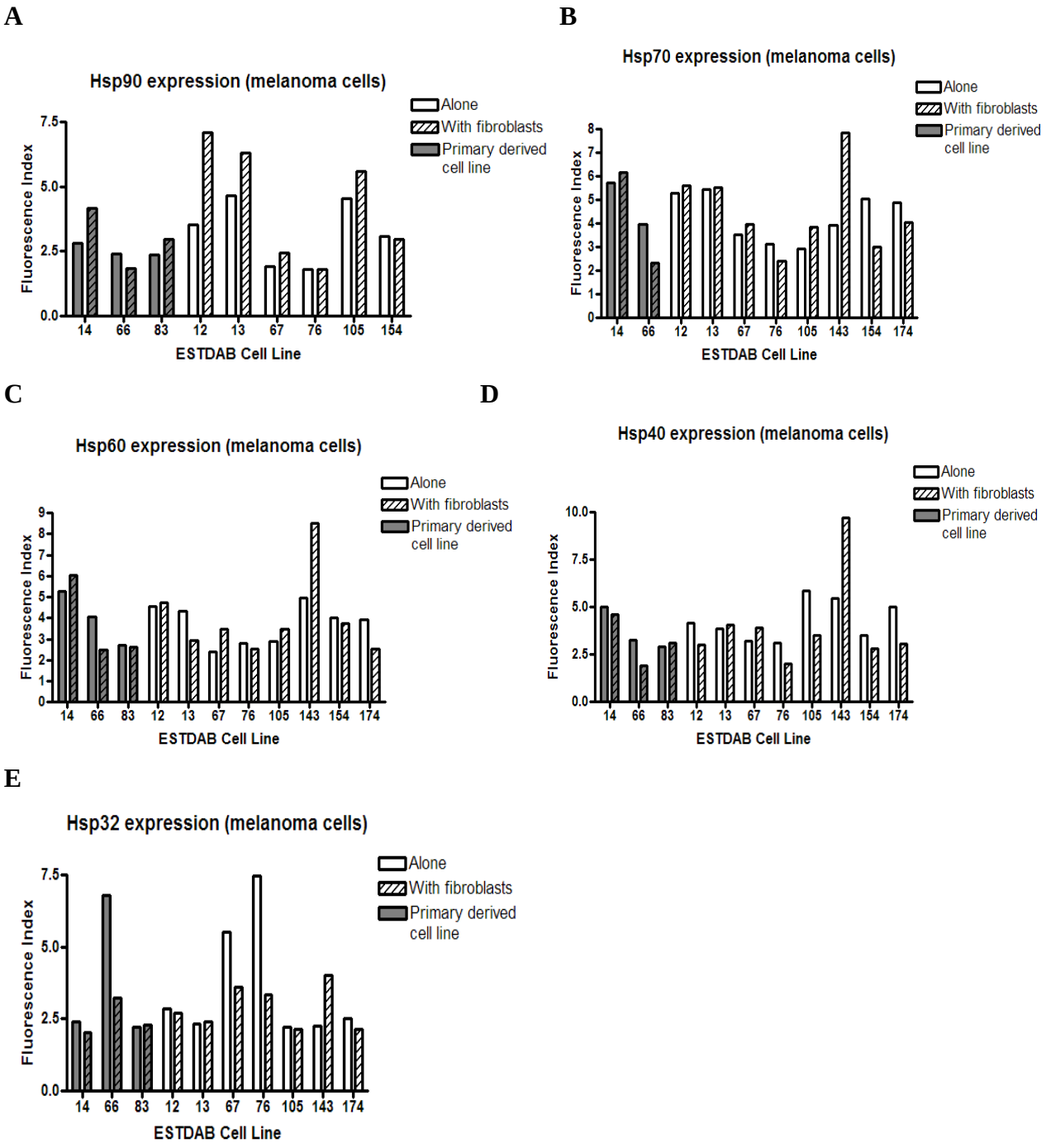


Eleven melanoma cell lines were cultured alone or with fibroblasts for nine days. Following the culture period the cell lines were harvested, stained with fluorochrome-labelled antibodies to CD44, CD54, CD95, CD155, MHC I and protein expression assessed by flow cytometry.

Following this, hsp surface expression was assessed on these cell lines. Hsp expression was not found to be associated with primary- or metastasis-derived cell line type, however, trends were observed within matched primary- and metastasis-derived cell lines. One set of matched cell lines showed an equivalent or increased level of hsp90 expression when cultured with fibroblasts by

comparison with the expression seen when cultured alone (Fig. 5.2A matched cell lines EST14, EST12 and EST13). Within this set of matched cell lines, a slight increase in hsp90 expression with or without fibroblast co-culture in the metastasis-derived cell lines by comparison with the primary-derived cell line was observed. For hsp70, the change in expression in response to fibroblast co-culture in the primary derived cell lines was observed to be similar to the response in the matched metastasis-derived lines (Fig. 5.2B matched cell lines EST66 and EST76 both showed slight decreases with fibroblast co-culture relative to culture alone, while matched lines EST14, EST12 and EST13 all showed equivalent expression with or without fibroblast co-culture). For hsp32, the matched cell lines EST66 and EST76 displayed relatively high expression without fibroblast co-culture, while this was substantially reduced when these cell lines were cultured with fibroblasts (Fig. 5.2E). For hsps 70, 60, 40 and 32 all cell lines, apart from EST143, showed equivalent or reduced expression with fibroblast co-culture (Figs. 5.2B, C, D and E). In the co-culture condition, EST143 increased the expression of all hsps tested (Fig. 5.2). Cell lines EST66, EST76 (matched primary- and metastasis-derived), EST67, EST83, EST105, EST154 and EST174 showed equivalent or reduced expression of all hsps tested in the co-culture condition relative to culture alone (Fig. 5.2). The remainder of the hsps showed variable expression that was not consistent with primary or metastatic cell line origin.

Figure 5.2. Heat shock protein expression on primary- and metastasis-derived melanoma cell lines with or without fibroblast co-culture

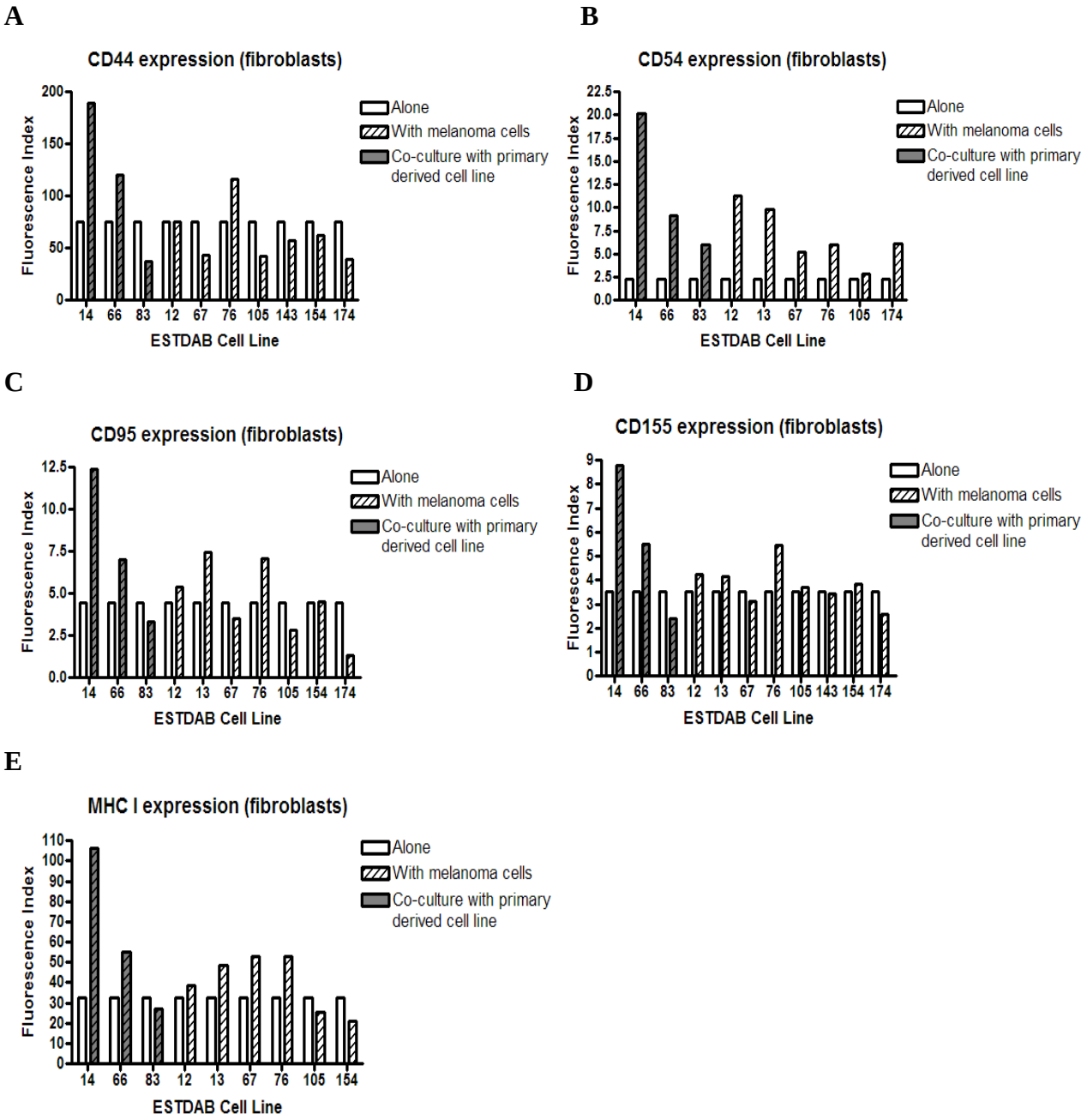


Eleven melanoma cell lines were cultured alone or with fibroblasts for nine days. Following the culture period the cell lines were harvested, stained with fluorochrome-labelled antibodies to hsp90, hsp70, hsp60, hsp40, hsp32 and protein expression assessed by flow cytometry.

Additionally, the expression of cell surface proteins was assessed on fibroblasts cultured alone or with melanoma cells, since it was of interest to explore the influence that melanoma cells may exert on neighbouring non-malignant cells.

As with the melanoma cells, fibroblasts displayed variable expression of CD44, CD54, CD95, CD155 and MHC I proteins when co-cultured with melanoma cells. Co-culture with melanoma cells resulted in substantially higher expression of CD54 in every cell line, with the exception of EST105 that showed equivalent expression (Fig. 5.3B). Within the matched cell lines the level of CD54 expression on fibroblasts cultured with primary-derived cell lines was higher or equivalent compared to fibroblasts cultured with the corresponding metastasis-derived cell line, as illustrated in Fig. 5.3B co-culture with matched cell lines EST14 (primary) resulted in higher expression than with corresponding metastatic lines EST12, EST13. A similar but weaker trend was observed with matched cell lines EST66 (primary) and EST76 (metastatic), and EST83 (primary) and EST67 (metastatic). Fibroblasts cultured with the matched cell lines EST66 (primary) and EST76 (metastatic) showed relatively high expression of CD44 and CD155 (Figs. 5.3A and D). Fibroblasts cultured with matched cell lines EST14 (primary-derived) and EST12 (metastasis-derived) show higher or equivalent expression of CD44 than fibroblasts cultured alone and than many of the other co-cultured fibroblasts (Fig. 5.3A). Relative to culture alone, fibroblasts cultured with matched pairs of primary- and metastasis-derived cell lines EST14 (primary), EST12, EST13 (metastatic) and EST66 (primary), EST76 (metastatic) all showed equivalent or increased expression of all markers tested (Fig. 5.3). In contrast, matching cell lines EST83 (primary-derived) and EST67 (metastasis-derived) showed equivalent or decreased expression for all proteins with the exception of CD54 and MHC I for EST67 (Fig. 5.3).

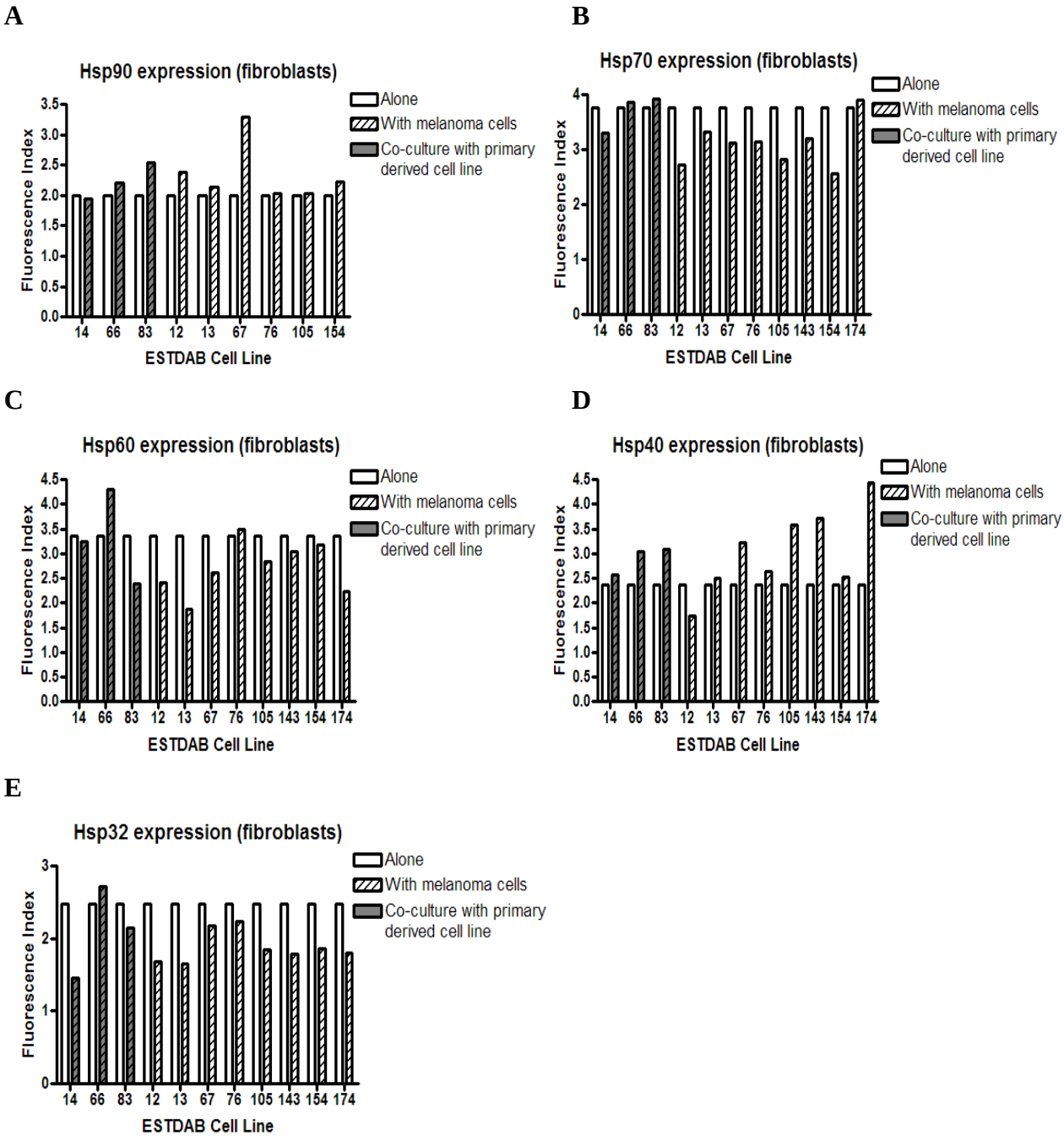
Figure 5.3 CD44, CD54, CD95, CD155 and MHC I protein expression on fibroblasts cultured with primary- and metastasis-derived melanoma cell lines



Fibroblasts were cultured alone or with melanoma cell lines for nine days. Following the culture period, cells were harvested, stained with fluorochrome-labelled antibodies to CD44, CD54, CD95, CD155, MHC I and protein expression assessed by flow cytometry.

The expression of hsps on fibroblasts cultured with primary- and metastasis-derived cell lines was not exclusively associated with primary or metastatic cell line type. Within the matched pairs of cell lines, fibroblasts cultured with primary-derived cell lines showed similar changes in the expression of hsp60 on fibroblasts cultured with the matched metastasis-derived cell lines relative to fibroblasts cultured alone as illustrated in Fig. 5.4C, the matched pairs EST14, EST12 and EST13 showed equivalent or decreased expression, EST66 and EST76 showed similar or increased expression, and EST67 and EST83 showed decreased expression relative to fibroblasts cultured alone. For hsp90, all co-cultured fibroblasts showed equivalent or increased expression relative to fibroblasts cultured without melanoma cells, this was also the case for hsp40 with the exception of co-culture with EST12. In contrast, hsp70 showed equivalent or reduced expression following fibroblast co-culture with all of the melanoma cell lines tested. This was also the general trend for hsp60 and hsp32 expression with the notable exception of co-culture with EST66. Hsp expression on many of the remaining fibroblasts cultured with cell lines did not show remarkable expression differences relative to fibroblasts cultured alone.

Figure 5.4 Heat shock protein expression on fibroblasts cultured with primary- and metastasis-derived melanoma cell lines



Fibroblasts were cultured alone or with melanoma cell lines for nine days. Following the culture period, cells were harvested, stained with fluorochrome-labelled antibodies to hsp90, hsp70, hsp60, hsp40, hsp32 and protein expression assessed by flow cytometry.

5.3 Discussion

This study was performed in an attempt to identify cell surface proteins that may be differentially expressed in melanoma metastases by comparison with melanoma primary tumours and to investigate tumour-stromal cell interactions. None of the proteins investigated were observed to be differentially expressed in the primary-derived or the metastasis-derived melanoma cell lines, however, trends were observed within the matched pairs of cell lines.

Some of the matched melanoma cell line sets used in this study were observed to express similar levels of surface proteins. These similarities may be due to genetic relatedness that is known to exist between a primary cancer and its metastases [156, 165]. However despite these similarities, differences in the expression of surface proteins were also observed.

Of note was the finding that hsp70 was not differentially expressed on primary- or metastasis-derived cell lines. This result contrasts with previous studies (using flow cytometry and immunohistochemistry) performed with tissue-derived melanoma cells in short-term culture and melanoma tissues that showed increased hsp70 expression to be associated with melanoma metastases [118, 120]. Furthermore, this study demonstrated that fibroblasts express hsp70, whereas in a previous study hsp70 was reported to be absent on tissue-derived fibroblasts in short-term culture using flow cytometry [120]. Hsp90 expression was not associated with primary-derived or metastasis-derived cell line type in two of the three matched sets of cell lines, but did show modest increased expression on metastasis-derived cell lines by comparison with the matching primary-derived cell line with or without co-culture (EST14, EST12 and EST13 in Fig. 5.2A). These results lend some support to the numerous previous studies that have shown hsp90 to be involved in melanoma metastasis [89, 159, 160].

The expression of all proteins was influenced on both melanoma cells and fibroblasts in co-culture experiments, providing evidence of cross-talk between these two cell types in culture. This was particularly evident for CD54 which showed increased expression on fibroblasts cultured with all the melanoma cell lines examined (Fig. 5.3B). Primary-derived cell lines were observed to influence the expression of fibroblast protein expression differently to metastatic-derived cell lines. In particular, CD54 showed higher expression on fibroblasts cultured with primary cell lines compared with the corresponding matched metastasis-derived lines (Fig. 5.3B). This suggests primary melanoma cells may exert different effects on stromal cells compared to metastatic melanoma cells and this may be due to genetic differentiation associated with acquisition of the metastatic phenotype.

Heterogeneity in the expression of all proteins in these cell lines both with or without co-culture was observed. This may be due to genetic differences as these cell lines originated from melanomas in separate individuals, while cell lines sourced from the same individual were observed to be more similar. Another possibility is that the culture environment resulted in artificial modification of the expression of these proteins and the findings are not representative of *in vivo* expression. Despite the potential for cell culture-induced artefacts, it may have still been possible that these cell lines differentially expressed a subset of cell surface proteins. Given that this was not observed, the proteins investigated in this study may therefore not be associated with melanoma metastasis. Furthermore, it remains possible that differential protein expression in melanoma is not a feature of all tumours or that this is only observable in melanoma tissue, and/or that this may only exist within patient matched primary and metastatic tumours.

As demonstrated by the changes in protein expression induced by fibroblast co-culture, melanoma cells appear to be highly sensitive to their culture environment. In order to control for the potential effects of cell culture induced artefacts, a comparison of protein expression should be made

between a freshly removed melanoma tumour and after this tumour has been established as a cell line. Using flow cytometry, Farkas *et al*. [120] indicated that freshly prepared melanoma tumour samples show similar expression of hsp70, Mel-1, Mel-2 and Mel-5 on the cell surface after the same tumours had been established as a cell line. This suggests that it was not the use of cell lines, but rather the panel of proteins chosen that may account for the observation that protein expression was not observed to be associated with primary or metastatic cell line type. Although it should be noted that other studies have shown that growth conditions can influence the attributes of other cancer cell line types [202, 204] and to differ compared to the tumour tissues from which they were derived [203].

The methodology and results described in this study must be regarded as preliminary and did not provide a clear picture of proteins that may be differentially expressed in primary or metastatic melanoma. Nonetheless, this study demonstrated the feasibility of exploring the issues of differential protein expression in primary and metastatic melanomas. Examination of a wide range of cell surface protein markers ideally with a larger number of matched primary and metastatic cell lines should be explored. This immediately raises the issue as to the selection of appropriate marker proteins, an issue that currently remains unresolved. Within the matched sets of primary- and metastasis-derived cell lines used in this study, genes that are differentially expressed in the metastatic cell lines have previously been proposed (personal communication of unpublished data, Dr Evelyna Derhovanessian). These genes included Beta-Pix/Cool-1, DOCK1, DOCK4, MUSK, WAS, MADD and THRAP1. In addition, it has been proposed by Farkas *et al*. [120] that metastatic melanomas preferentially express hsp70, but not Mel-5 on the cell surface, while another study identified the chemokine receptor CXCR4 to be associated with melanoma metastasis [225]. Other proteins worthy of investigation may be those known to be associated with metastasis in other cancer types, such as metastasis-associated protein 1 [226].

These results suggest that further investigation of differential protein expression in melanoma metastases is warranted, in addition to proposing candidate protein markers that might be included in future studies.

A major challenge for such an undertaking and one that should not be underestimated, is the very limited availability of matched primary- and metastasis-derived melanoma cell lines. The cell lines used in this study were selected from ESTDAB, which is the largest existing collection of melanoma cell lines that contains over 230 lines. Of these, only three sets of matched primary- and metastasis-derived cell lines exist, all of which were included in the present study.

Chapter 6: Heat shock protein expression in melanoma cell lines: effect of culture under low oxygen tension, relationship with cell line characteristics and patient clinical parameters

6.1 Introduction

Hsps play crucial roles in the maintenance of protein homeostasis and protection from cellular stressors. Malignant tumours are characterised by oncoprotein overexpression, a high rate of cellular proliferation and a hostile internal environment. Given their intrinsic molecular and cellular roles, hsps are consequently essential for the growth and progression of cancer. A common and important feature of the tumour microenvironment is hypoxia [166-168]. Hypoxia is the result of the combined effects of rapid proliferation of malignant cells and the abnormal behaviour of the tumour microvasculature, resulting in insufficient blood supply to the tumour mass [166-169]. Hypoxia is widespread among human tumours and contributes to tumour biology through multiple mechanisms including the promotion of genetic instability, facilitating immune evasion and assisting in the selection of cells more resistant to apoptosis and the tumour microenvironment [167, 170-174]. Furthermore, hypoxia has been associated with resistance to therapy, more aggressive tumours, tumour invasion, poor prognosis and patient death [170, 173, 175, 176]. Despite typically being a relatively small tumour frequently found on the skin, hypoxia is nevertheless a feature of human melanoma [166, 177, 178]. In melanoma, hypoxia is associated with tumour metastasis and may serve to enhance metastatic spread [179-182].

The major mechanism by which cells respond to hypoxic stress is by rapid modulation of the expression of the HIF transcription factor [183]. HIF directly regulates the expression of hsps and hsp90 has been shown to assist in the stabilisation of HIF under hypoxic conditions in melanoma cells [184, 185]. Other studies suggest hsp90 is involved in HIF expression and transactivation

under hypoxia [186]. Many roles of hsps contribute to the survival of tumour cells; accordingly, hsps have been shown to be abnormally expressed in a range of human cancers [9].
They promote the growth of cancer cells through multiple mechanisms such as inhibiting apoptosis, enhancing angiogenesis and providing protection against tumour-associated stressors such as hypoxia [9, 83, 86, 87, 187]. Hsps perform these roles in addition to chaperoning a range of proteins essential for the maintenance of the cancer phenotype including overexpressed oncoproteins that drive the proliferation of tumour cells [9, 145, 188]. In addition, hsps facilitate cancer differentiation and metastasis. Perhaps due to these roles, hsps are overexpressed in a range of human cancers and their expression correlates with prognosis and resistance to therapies. Hsps have been identified as valid targets in the treatment of cancer and are currently being evaluated in clinical trials in a number of cancer types, including melanoma [8, 9, 83, 84, 145, 148, 189].

The relevance of hypoxia and hsps to cancer is well documented, but has rarely been studied in the context of human cancer cell cultures. Despite relatively few studies having been performed, at least one previous study suggests that altered oxygen tension can result in biologically relevant changes in tumour cell cultures *in vitro* [224]. Laboratory cell culture is routinely performed under hyperoxic conditions (i.e. 20 % O_2 in air) and for this reason may be a limitation of this model for the study of human cancer cells which frequently experience hypoxia *in vivo*. Hsps have been investigated in melanoma tumour tissue and play multiple roles important for cancer growth, but have not been extensively studied in melanoma cell lines. Hence this study sought to assess hsp expression in relation to melanoma cell line characteristics, patient clinical parameters and low and high oxygen culture conditions in a large panel of melanoma cell lines. Since melanoma cells experience hypoxia *in vivo*, it was hypothesised that 2 % O_2 may be more similar to *in vivo* growth conditions. To this end, 42 melanoma cell lines were selected from the European Searchable Tumour Line Database (ESTDAB) and screened for hsp expression using flow cytometry. These cell lines were generated in air and for this reason 2 % O_2 was considered to

model hypoxic stress. Cell lines were cultured in 75cm² cell culture flasks for five days in a humidified incubator (37 °C 5 % CO_2, 95 % humidity) in air (20 % O_2) with 35 mL medium (RPMI 1640 with 10 % (v/v) heat inactivated FCS). To investigate the effect of hypoxia, a number of cell lines were simultaneously cultured in 2 % O_2 under identical conditions. Seeding cell number was adjusted according to the generation time of each cell line in order to obtain similar cell numbers at the end of the culture period. This was performed for consistency and to avoid 100 % confluence, a state that could stress the cells thereby inducing the stress response and result in altered hsp expression. Cells were harvested by trypsination and 3.0 x 10^5 cells were stained with each antibody. In order to better differentiate between differences in protein expression, antibodies conjugated to phycoerythrin (PE) were used, which is one of the brightest fluorochromes commercially available [190, 191]. Brighter fluorochromes allow for greater sensitivity in measuring differences in protein expression compared with duller fluorochromes. In addition, the use of a single fluorochrome for all antibodies allowed for a more direct comparison between the expression of each protein of interest across the sample cohort, while this is not possible when different fluorochromes are used for each protein of interest.

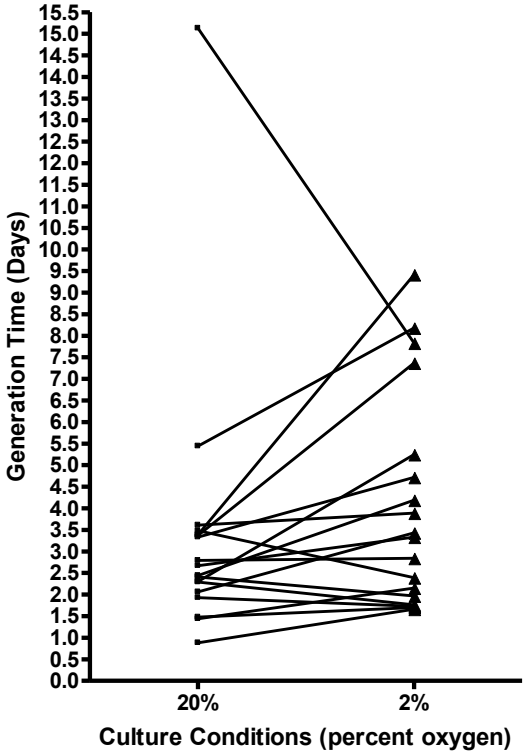
6.2 Results

Heat shock protein expression, growth rate and response to low oxygen tension in melanoma cell lines

Eighteen melanoma cell lines were selected for culture in 2 % O_2 (Fig. 6.1). Twelve of these 18 cell lines showed a mean increase of 70 % in generation time when cultured in 2 % O_2 compared with the 20 % O_2 condition ($P = 0.054$). Five of the remaining six cell lines showed reduced generation times (mean decrease 26 %), while the remaining cell line did not change. The mean generation time for cell lines grown in 20 % O_2 was 3.4 days with a range of 0.9 - 15.1 days. When these cell lines were grown in 2 % O_2 the mean generation time increased slightly to 4.1

days with a range of 1.7 to 9.4 days. As indicated by a reduced range in the cell line generation times, there was less variation in the generation times when these cells were cultured in 2 % O_2. Within matching data sets, changes of less than 5.0 % were considered not to be different.

Figure 6.1 Effect of low oxygen tension on melanoma cell line generation time

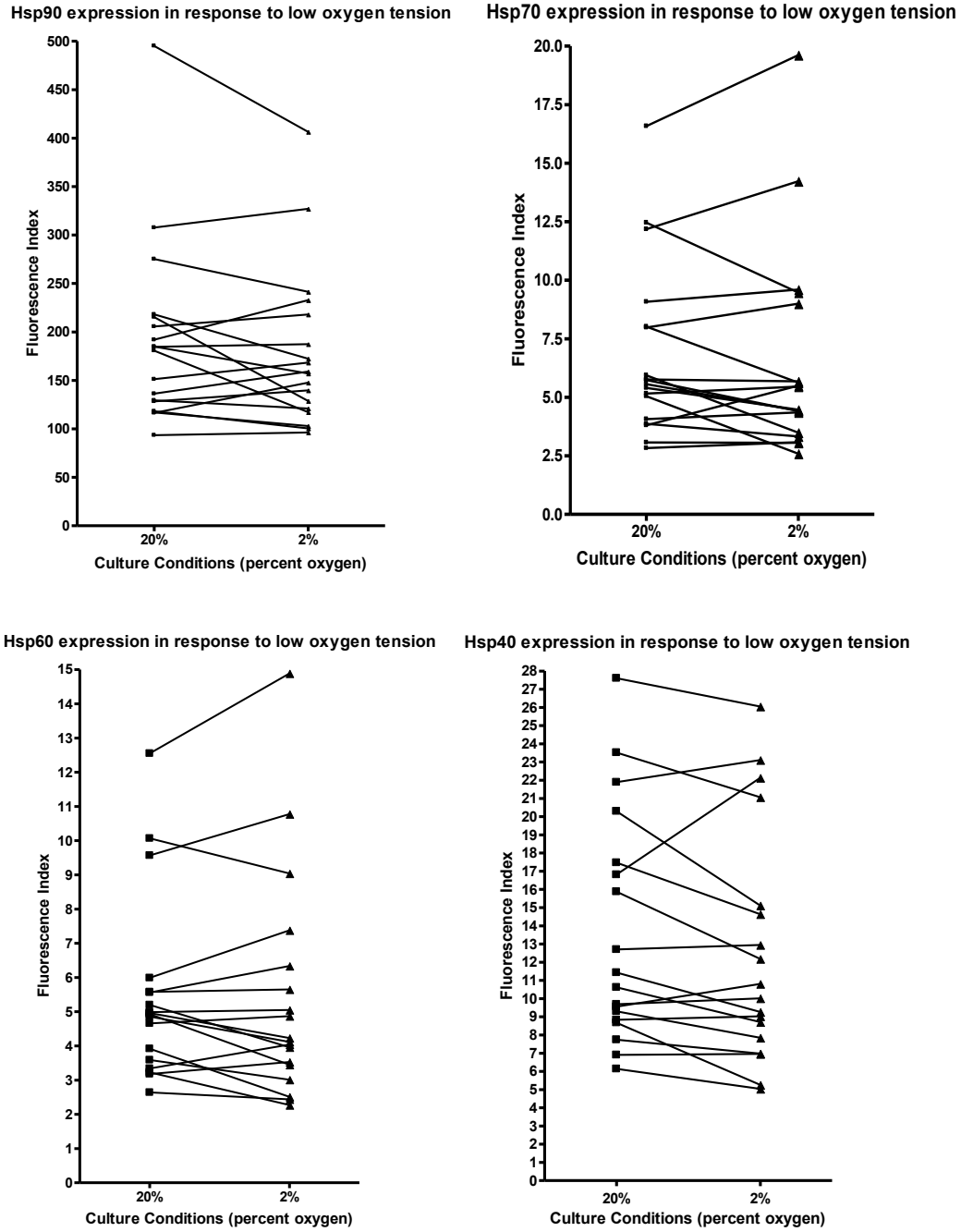


Low oxygen tension reduces melanoma cell line growth rate. Eighteen melanoma cell lines were cultured under identical conditions in 2 % or 20 % O_2 for five days. Following the culture period, the cell lines were harvested and counted. Total number of live cells was used to calculate generation time.

Given that these cell lines displayed altered growth rates in response to low oxygen tension, changes in hsp expression associated with low oxygen tension were investigated. Comparison of the change in expression of hsps within each cell line showed that five of 18 cell lines decreased the expression of hsps 90, 70, 60 and 40 in 2 % O_2 relative to 20 % O_2. Another set of three cell lines increased the expression of hsps 90, 70 and 60 but not hsp40. The change for the remainder of the cell lines was variable. Low oxygen tension resulted in both up- and down-regulation of individual hsps (Fig. 6.2). For each of the four hsps examined, approximately half of the cell lines showed decreased expression in 2 % O_2, while a smaller proportion increased (Table 6.1).

When investigating the relationship between change in hsp expression and generation time in response to low oxygen tension, modest relationships emerged. For hsp90, 8/8 cell lines that decreased expression of hsp90 in 2 % O_2 showed an increased generation time and 5/7 cell lines that showed increased hsp90 expression in 2 % O_2 showed reduced generation times. For hsp40, 7/9 cell lines that decreased expression in 2 % O_2 showed increased generation times. Data for the other hsps examined were either negative or inconclusive. Of note was that cell line EST207 showed a substantially reduced generation time when in the 2 % O_2 culture condition. Within matching data sets, changes of less than 5.0 % were considered not to be different.

Figure 6.2 Effect of low oxygen tension on heat shock protein expression



Low oxygen tension results in the up- or down-regulation of hsp expression. Eighteen melanoma cell lines were cultured under identical conditions in 2 % or 20 % O_2. After five days the cell lines were harvested, stained with PE conjugated hsp antibodies and hsp expression assessed with flow cytometry. A Fluorescence Index was calculated (fold increase in mean fluorescence of the stained cells compared with the unstained cells) and was used as a comparative measure of protein expression across the cell lines.

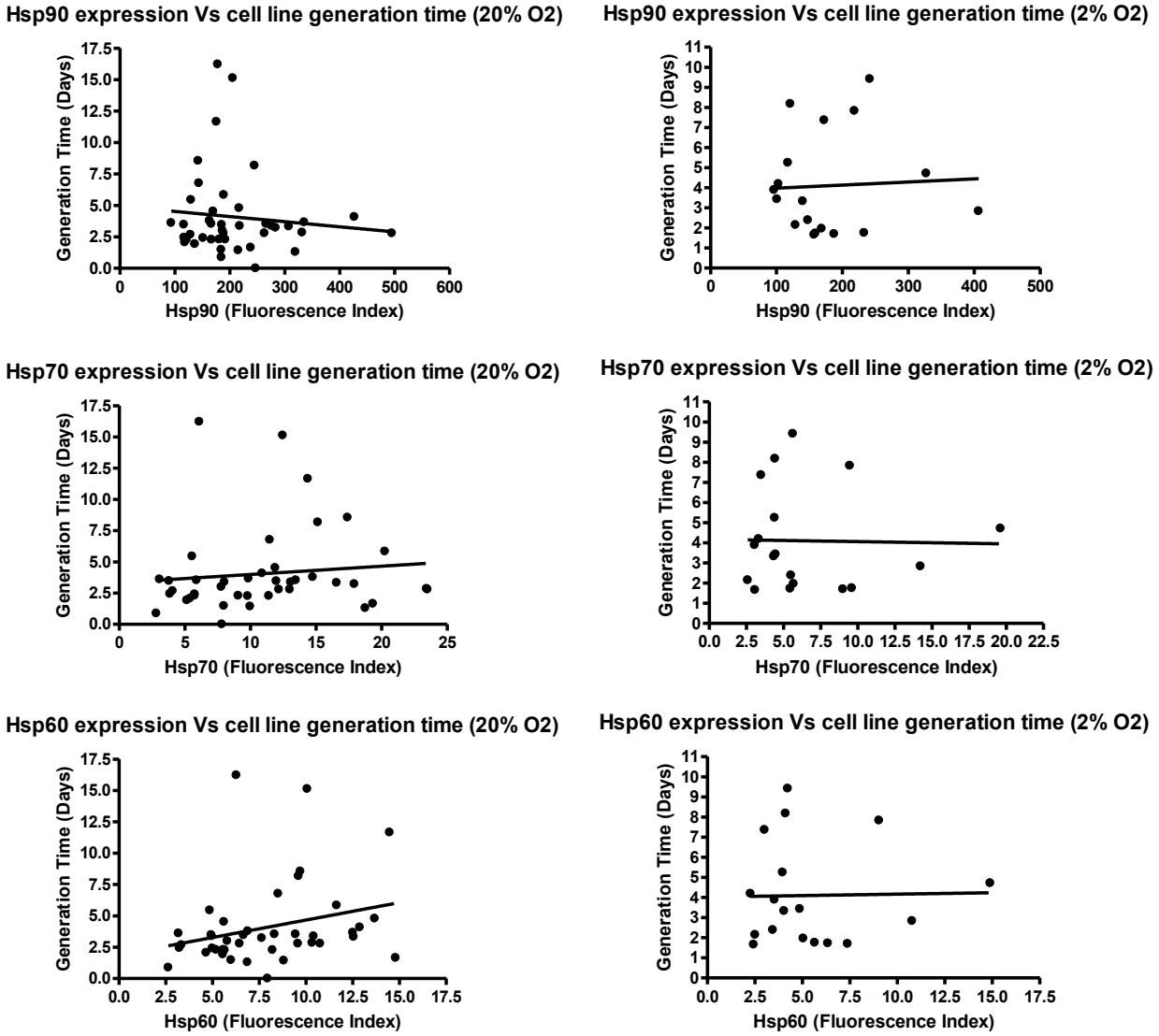
Table 6.1 Change in hsp expression in response to low oxygen tension

Hsp	Decrease of hsp expression in 2% O_2 (percent of cell lines)	Mean decrease (%)	Increase of hsp expression in 2% O_2 (percent of cell lines)	Mean increase (%)
90	**50.0**	**19.5**	**38.9**	**13.9**
70	**44.4**	**27.3**	**44.4**	**15.0**
60	**50.0**	**20.4**	**33.3**	**16.8**
40	**61.1**	**18.3**	**16.7**	**16.8**

Low oxygen tension results in the up- or down-regulation of hsp expression. Eighteen melanoma cell lines were cultured under identical conditions in 2 % or 20 % O_2. After five days the cell lines were harvested, stained with PE conjugated hsp antibodies and protein expression assessed by flow cytometry. A Fluorescence Index was calculated (fold increase in mean fluorescence of the stained cells compared with the unstained cells) and was used as a comparative measure of protein expression.

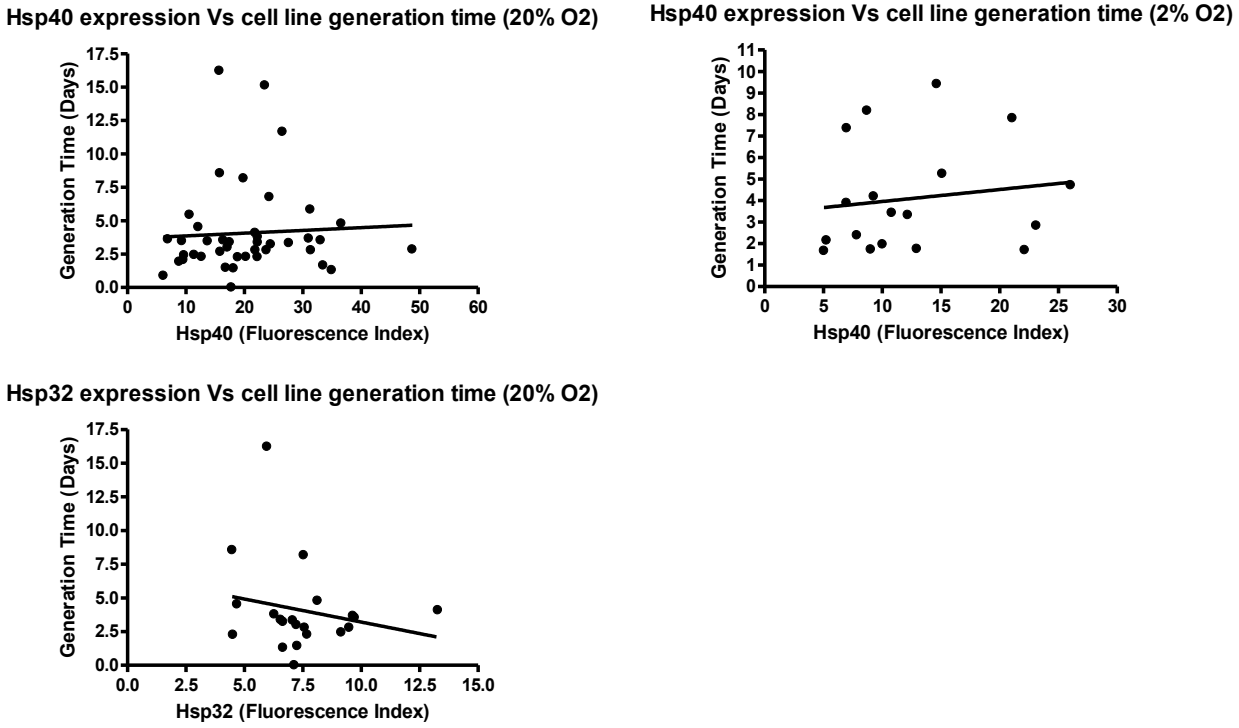
The relationship between the total expression levels of hsps and the growth rate of these melanoma cell lines was then investigated. With the exception of hsp60 in 20 % O_2 ($P < 0.03$) there was no association between hsp expression and cell line generation time for either 2 % or 20 % O_2 ($P > 0.13$) (Fig. 6.3). This suggests the change in hsp expression in response to low oxygen tension, but not the overall level of expression, is relevant to melanoma cell line growth rate under low oxygen tension.

Figure 6.3 Relationship between heat shock protein expression and melanoma cell line generation time in 2 % or 20 % O_2 culture conditions



$P < 0.03$

Figure 6.3 continued

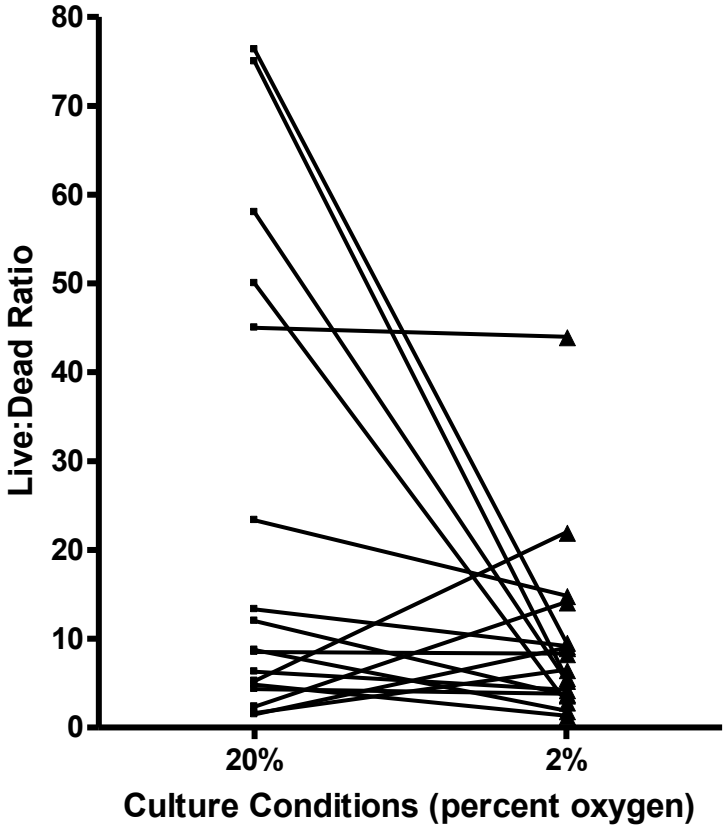


Forty-two melanoma cell lines were cultured in 20 % O_2 and 18 of these additionally in 2 % under identical conditions for five days. Following the culture period, the cell lines were harvested and live and dead cells enumerated microscopically using trypan blue. Total number of living cells was used to calculate generation time.

The relationship between hsp expression and viability of these cell lines was investigated. Eleven of 17 cell lines showed reduced viability in 2 % O_2, but this did not reach statistical significance ($P = 0.096$), while four showed improved viability and two did not change (Fig. 6.4) (no data for one cell line). Four of the five cell lines that showed reduced length of generation time in the 2 % O_2 condition also displayed improved viability in 2 % O_2. Mean live to dead ratio for 20 % O_2 was 23.3:1, while this was reduced to 9.8:1 when the cells were cultured in 2 % O_2. Since it was clear that the 2 % O_2 condition exerted biological effects on these cell lines, the relationship between hsp expression and cell viability was investigated. There was no association between hsp expression and cell viability when the cells were cultured in 20 % O_2, however, significant ($P < 0.05$) relationships were found between hsp90, hsp70 and hsp60 when the cell lines were cultured in 2 % O_2 (Fig. 6.5). Examining the change in hsp expression showed that 7/8 cell lines that

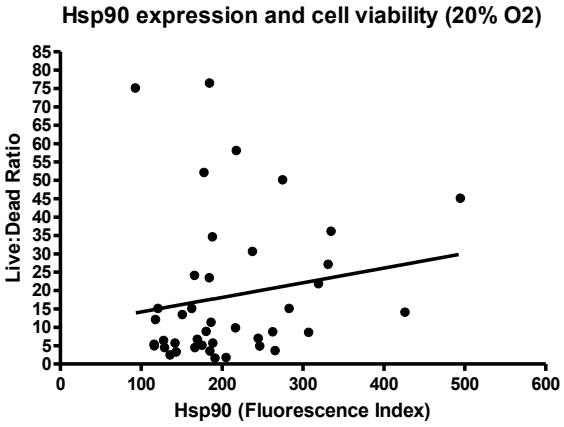
reduced expression of hsp90 in 2 % O_2 also displayed reduced viability in 2 % O_2, while 4/7 cell lines that increased expression of hsp90 in 2 % O_2 displayed improved cell viability compared with the 20 % O_2 condition.

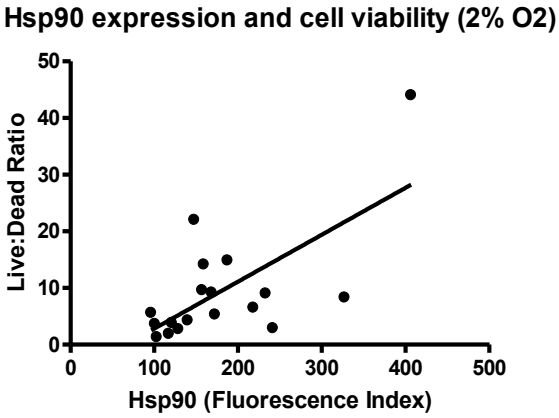
Figure 6.4 Effect of low oxygen tension on melanoma cell line viability



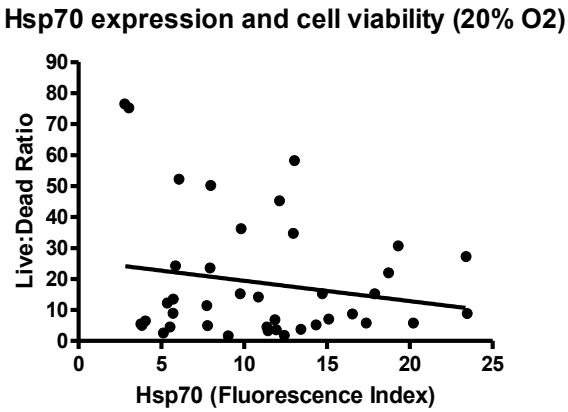
Low oxygen tension reduces melanoma cell line viability. Eighteen melanoma cell lines were cultured under identical conditions in 2 % or 20 % O_2 for five days. Following the culture period, the cell lines were harvested and live and dead cells enumerated microscopically using trypan blue. Ratio of live to dead cells was used as a marker of cell viability.

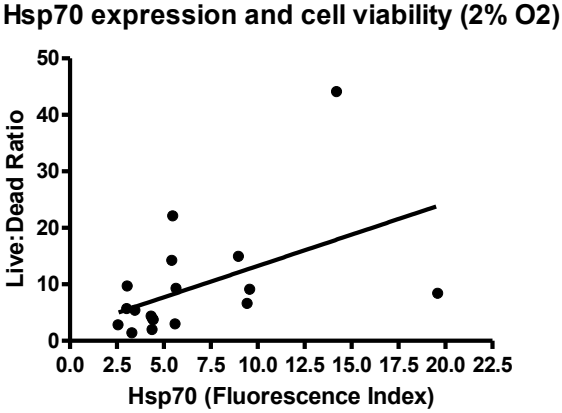
Figure 6.5 Relationship between heat shock protein expression and melanoma cell line viability in 2 % or 20 % O_2 culture conditions



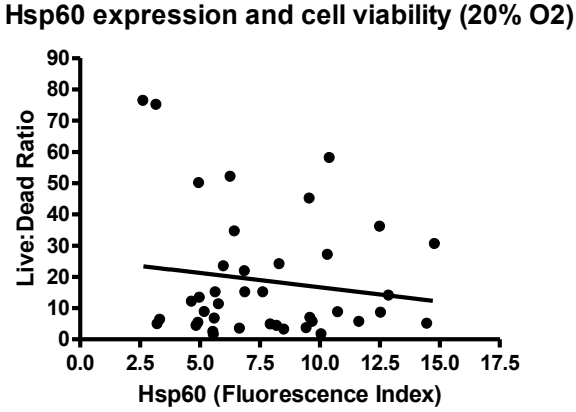


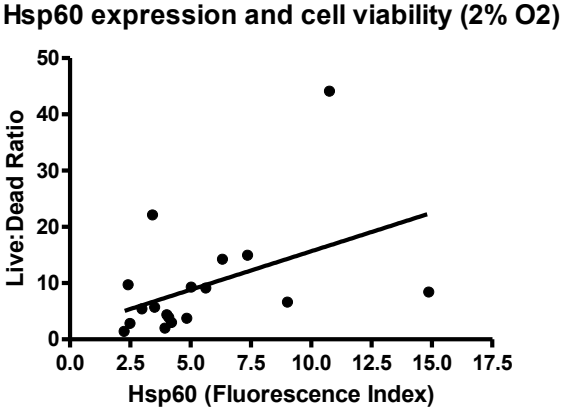
Spearman r = 0.529 P = 0.024





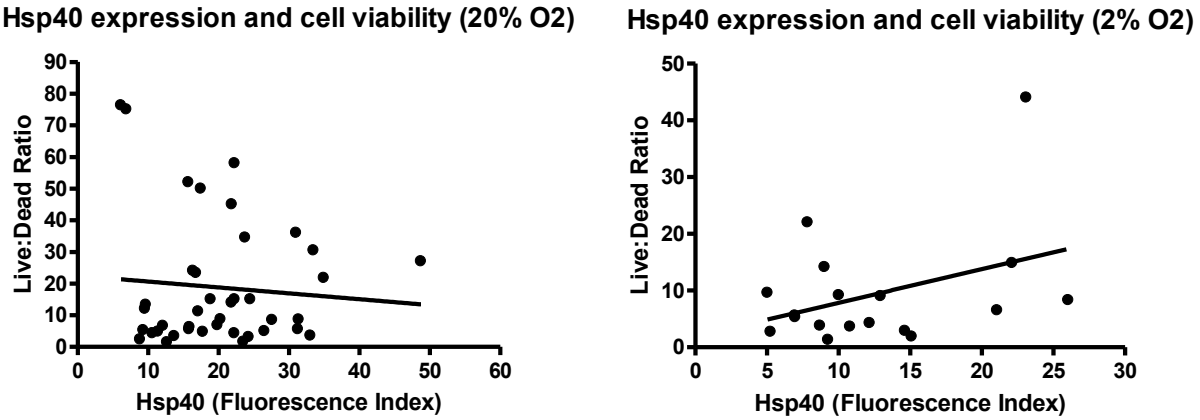
Spearman r = 0.527 P = 0.025





Spearman r = 0.474 P = 0.047

Figure 6.5 continued

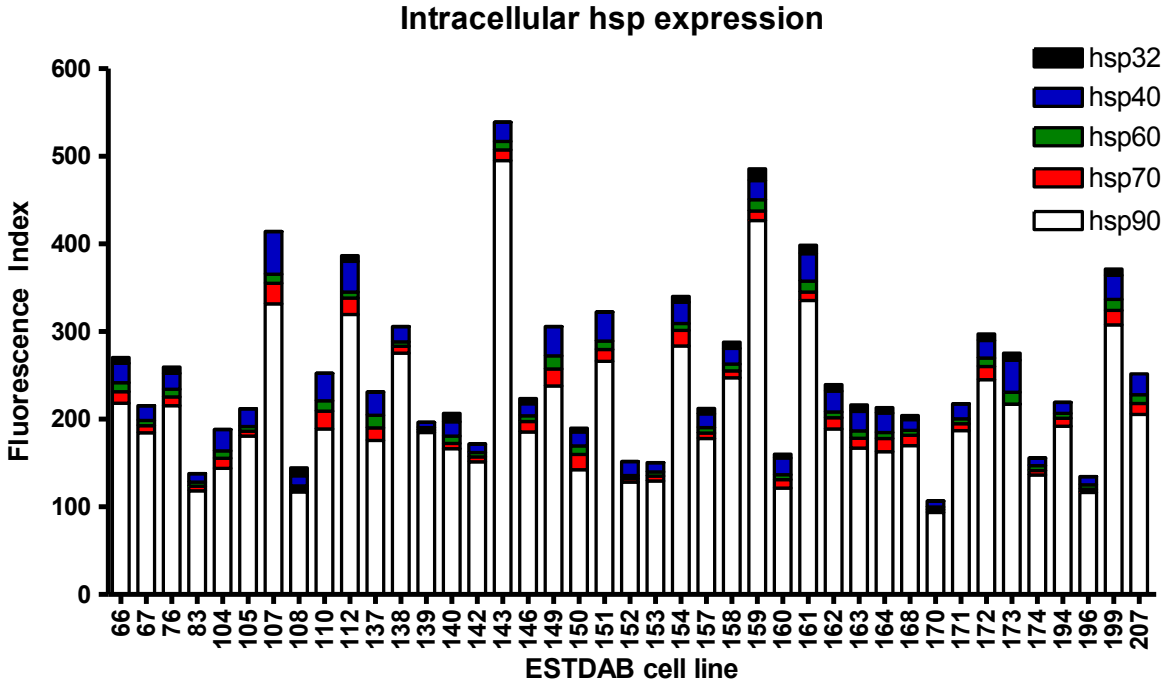


Hsp expression is associated with melanoma cell line viability in 2 % O_2. Forty two melanoma cell lines were cultured in 20 % O_2 and 18 of these cell lines additionally in 2 % O_2 for five days. Following the culture period, the cell lines were harvested and the number of live and dead cells determined microscopically using trypan blue. The ratio of live to dead cells was used as a marker of cell viability.

Hsp expression in melanoma cell lines

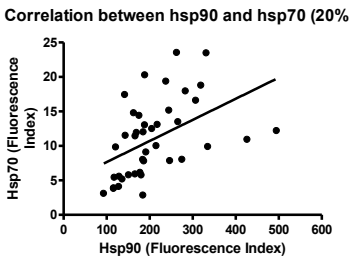
Hsp expression was compared throughout the cohort of melanoma cell lines cultured in 20 % O_2 (Fig. 6.6). Hsp90 was observed to account for the vast majority of total hsp expression in all cell lines, followed by hsp40 and hsp70, while hsps 60 and 32 were similarly expressed at relatively low levels. Furthermore, it was observed that the relative expression of each hsp was observed to be consistent across the cohort of cell lines. Although the cell lines showed some variation in the expression of hsps, overall the majority expressed similar levels. A small number of cell lines displayed a relatively high (for example EST 143, 159) or low (EST 170, 83) level of hsp expression. Given that the relative expression of hsps was observed to be consistent, relationships between the expression of these hsps were investigated. The expression of hsp90, hsp70, hsp60 and hsp40 but not hsp32 were found to correlate in 2 % ($P = 0.015$) and 20 % O_2 ($P = 0.0001$) culture conditions (Figs. 6.7 and 5.8, hsp32 not tested in 2 % O_2).

Figure 6.6 Heat shock protein expression across melanoma cell lines

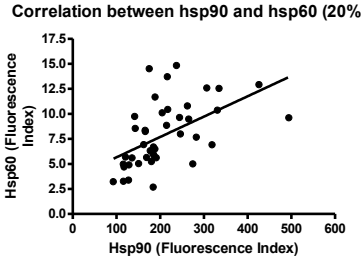


Forty-two melanoma cell lines were cultured in 20 % O_2 for five days. Following the culture period cell lines were harvested, stained with PE conjugated antibodies to hsps 90, 70, 60, 40 and 32 and protein expression assessed by flow cytometry. A Fluorescence Index was calculated (fold increase in mean fluorescence of the stained cells compared with the unstained cells) and was used as a comparative measure of protein expression across the cell lines.

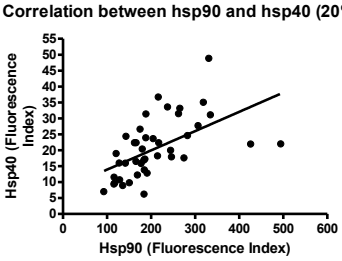
Figure 6.7 Relationship between the expression of different heat shock proteins in melanoma cell lines cultured under 20 % O_2



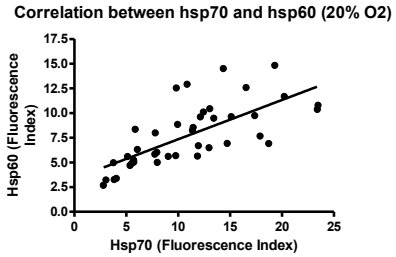
Spearman r = 0.605



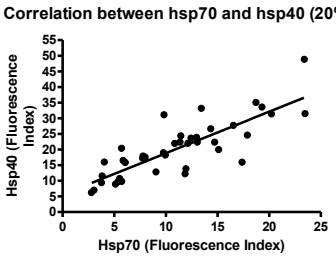
Spearman r = 0.629



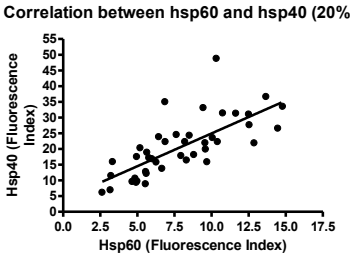
Spearman r = 0.671



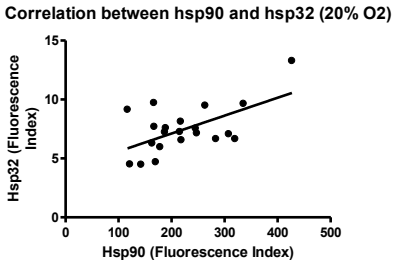
Spearman r = 0.797

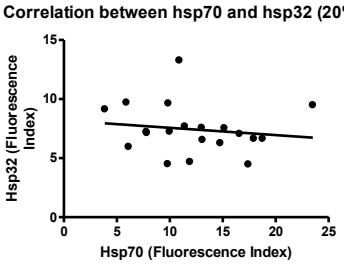


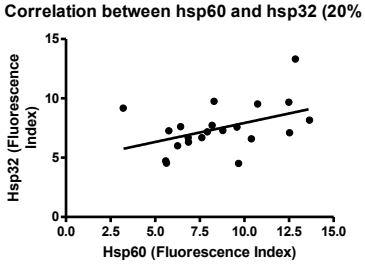
Spearman r = 0.829

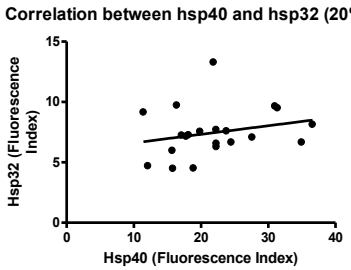


Spearman r = 0.799



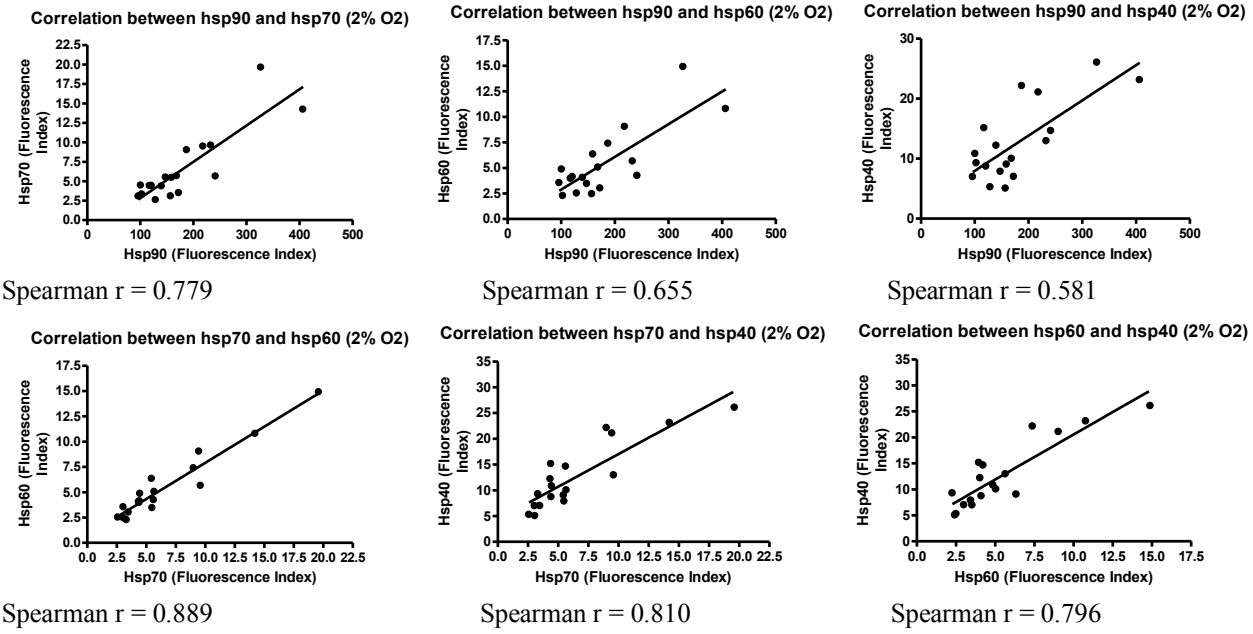






The expression of hsps 90, 70, 60 and 40 correlated with one another, but not with hsp32. Forty-two melanoma cell lines were cultured in 20 % O_2 for five days. Following the culture period cell lines were harvested, stained with PE conjugated antibodies to hsps 90, 70, 60, 40 and 32 and protein expression assessed by flow cytometry. Fluorescence indexes for each hsp were calculated and underwent significance testing using two-tailed nonparametric correlation tests.

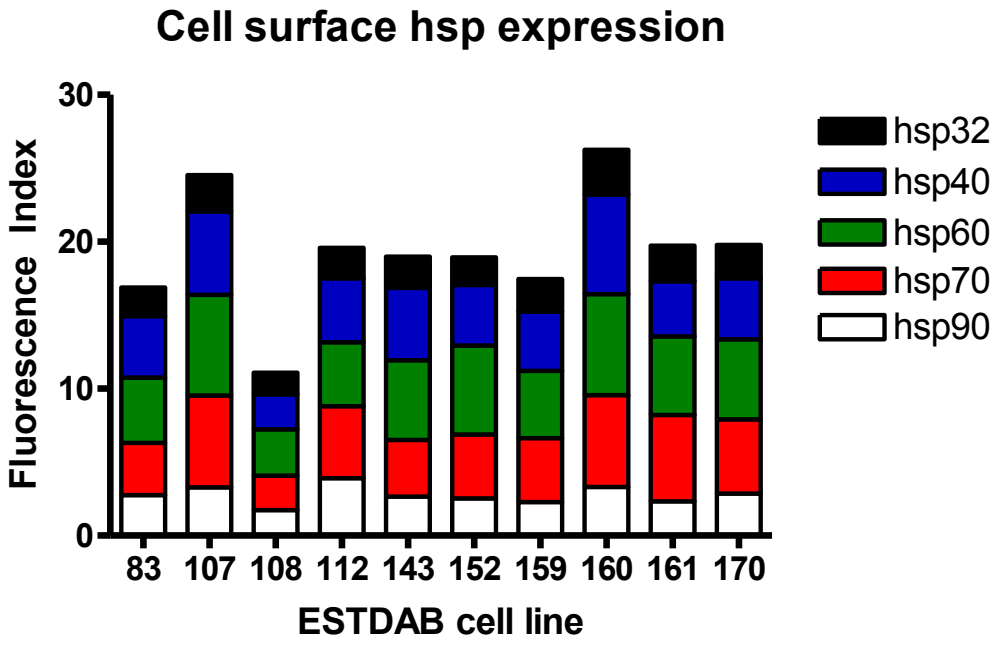
Figure 6.8 Relationship between the expression level of different heat shock proteins in melanoma cell lines cultured under 2 % O_2



The expression of hsps 90, 70, 60 and 40 correlated with one another. Eighteen melanoma cell lines were cultured in 2 % O_2 for five days. Following the culture period cell lines were harvested, stained with PE conjugated antibodies to hsps 90, 70, 60 and 40 and protein expression assessed by flow cytometry. Fluorescence indexes for each hsp were calculated and underwent significance testing using two-tailed nonparametric correlation tests.

Cancer cells have previously been reported to express hsps on the cell surface, which may be important for the invasion and progression of cancer [89]. For this reason ten melanoma cell lines were screened for expression of cell surface hsps (Fig. 6.9). Hsps 90, 70, 60, 40 and 32 were observed to be expressed on all ten cell lines with the exception of three cell lines being negative for hsp32, while one was negative for hsp90. In comparison to intracellular expression, cell surface hsp expression was low. Of note is that hsp90 was not the most highly expressed hsp on the cell surface, in contrast to intracellular hsp expression level. Although trends were present, there were no statistically significant correlations between cell surface and intracellular expression of the same hsp (data not shown). Similarly, the expression of cell surface hsps did not correlate with cell line generation time (data not shown).

Figure 6.9 Expression of cell surface heat shock proteins in melanoma cell lines



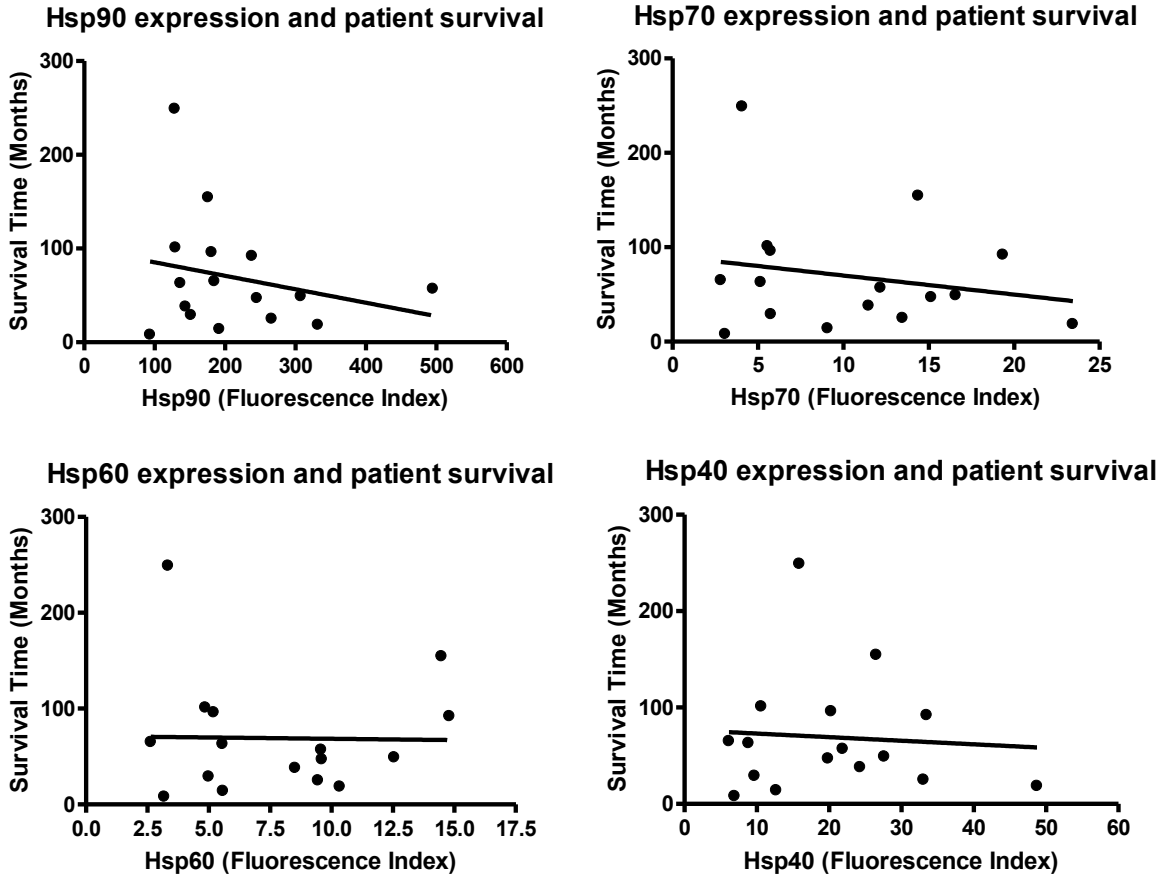
Ten melanoma cell lines were cultured in 20 % O_2 for five days. Following the culture period cell lines were harvested, stained for cell surface hsp90, hsp70, hsp60, hsp40 and hsp32 and protein expression assessed by flow cytometry. A Fluorescence Index was calculated (fold increase in mean fluorescence of the stained cells compared with the unstained cells) and was used as a comparative measure of protein expression.

Correlation of hsp expression with clinical parameters

The expression of hsps in melanoma tissue has been shown to correlate with clinical parameters, but few, if any, studies have been performed on hsp expression in melanoma cell lines in this context. Patient survival time, Clark level and Breslow depth of the primary tumour tissue were known for a limited number of the metastasis-derived cell lines in the Biobank used here, and were tested for a correlation, if any, with the expression of hsps

No significant relationships were observed between hsp expression and patient survival time, but, hints of a possible inverse relationship were present for hsp90 (Spearman r = -0.25, P > 0.35) (Fig. 6.10). In order to reduce patient variation, only those diagnosed at stages I and II (i.e. absence of metastasis) were included in this analysis. Of note was that one patient survived substantially longer than the others and also showed low relative hsp expression.

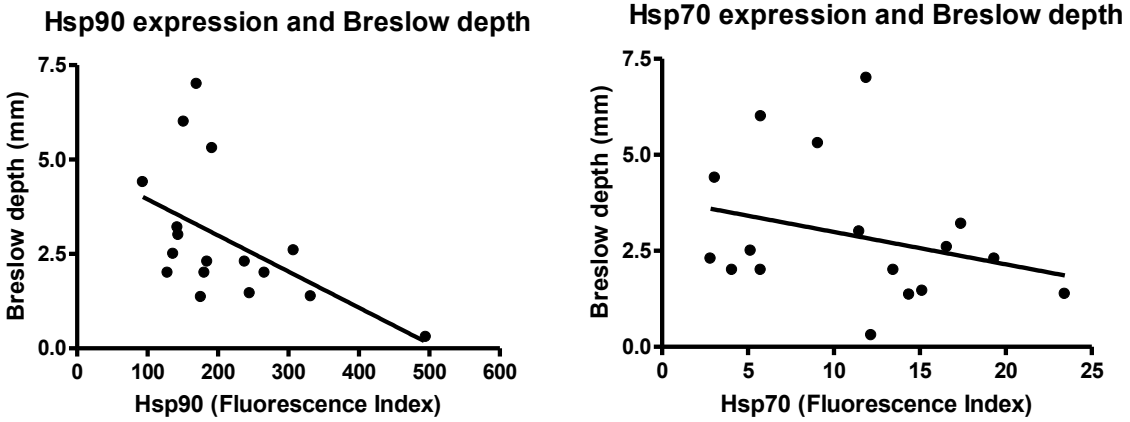
Figure 6.10 Relationship between heat shock protein expression and patient survival in melanoma cell lines



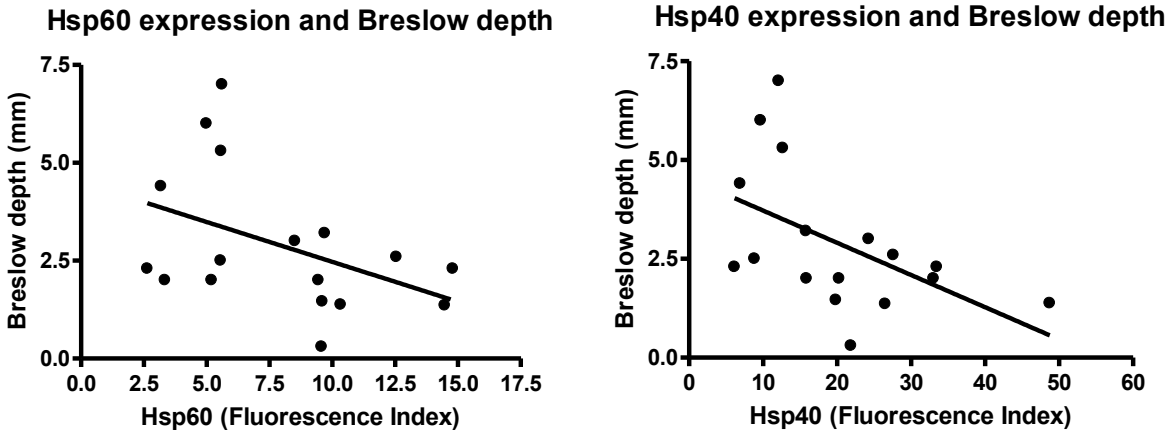
The expression of hsps in melanoma cell lines was correlated with survival time of the patients from which they were established using nonparametric two-tailed correlation tests.

Examining the association with other clinical parameters showed that the expression of hsp90 and hsp40 was inversely correlated with Breslow depth, but no relationships were observed with Clark level (Fig. 6.11, for Clark level data see appendix IV).

Figure 6.11 Relationship between heat shock protein expression and Breslow depth in melanoma cell lines



Spearman r = -0.507 P = 0.038



Spearman r = -0.552 P = 0.022

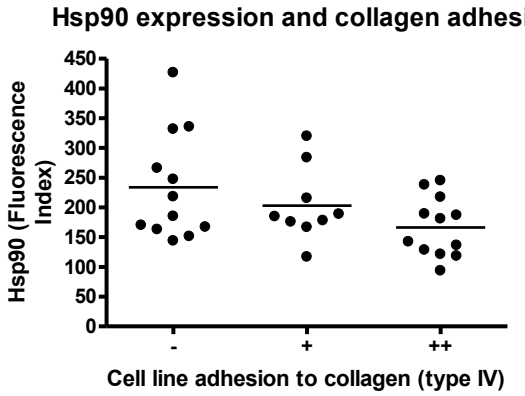
Hsp90 and hsp40 expression correlated with Breslow depth. The expression of hsps in melanoma cell lines was correlated with Breslow depth of primary tumours using nonparametric two-tailed correlation tests.

Relationship between hsp expression and cell surface proteins

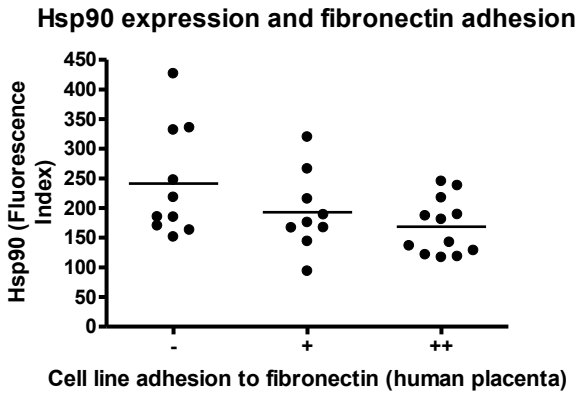
Hsps are essential for the maintenance of protein homeostasis under basal conditions and play crucial roles in protein folding, translocation, degradation and are associated with cell adhesion [192]. As such hsp expression may be relevant to the expression of other cellular proteins. The hsp expression data previously obtained for 42 melanoma cell lines was correlated with the expression of melanoma surface proteins and melanoma cell line adhesion to various ligands. These data were obtained from ESTDAB. Each protein was placed into one of four groups based on the degree of expression or adhesion reported in ESTDAB (absent, present, high and very high

which correspond to "-" "+" "++" and "+++", respectively) and this was matched with the fluorescence index for each hsp of the corresponding cell lines and placed into the appropriate category. To assess significance, two-tailed non-parametric t tests were used. The following proteins were investigated: CD11b, CD40, CD44, CD49d, CD54, CD56, CD57, CD58, CD95, CD95L, CD112, CD155, ULBP1, ULBP2, ULBP3, gp100, B7-H1, TRAIL-R1, TRAIL-R2, TRAIL-R4, Bcl-2, FUT1, MICA/B and cell adhesion to the following ligands: Collagen type IV, fibronectin (from human placenta) and laminin (from EHS mouse sarcoma). Significant correlations were found between hsp expression and surface protein expression and adhesion to the following ligands (Fig. 6.12): hsp90 with collagen, laminin and fibronectin (borderline significant) and hsp40 with TRAIL-R4. A strong trend was observed between hsp90 with CD155 and CD54 and hsp40 with CD155. These correlations are shown in Fig. 6.12. For a full list of results see appendix V.

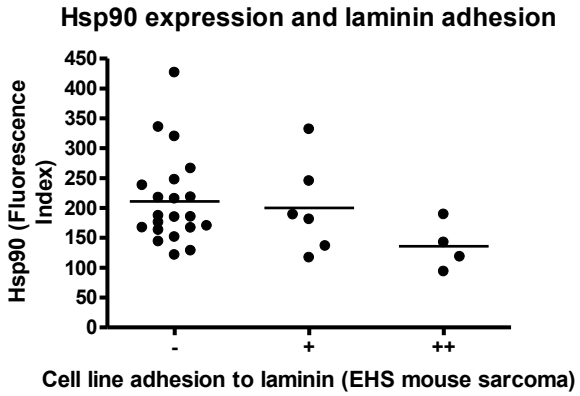
Figure 6.12 Relationship between heat shock protein expression, ligand adhesion and expression of cell surface proteins in melanoma cell lines



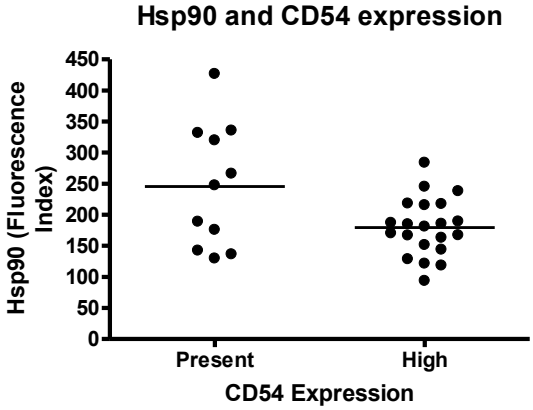
P value between "-" and "++" = 0.046



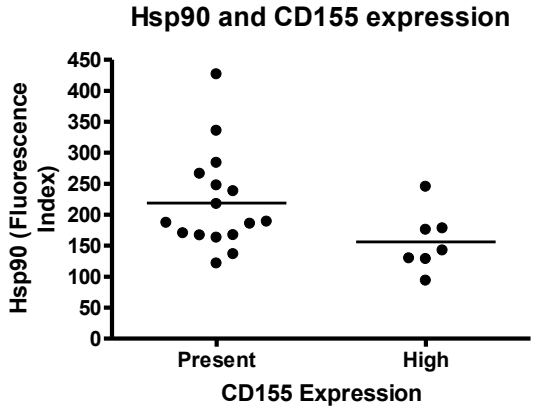
P value between "-" and "++" = 0.052



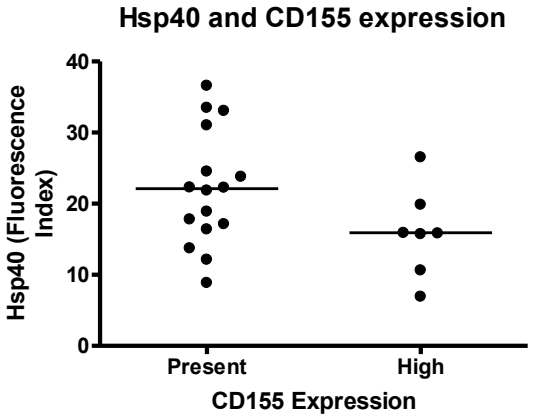
P value between ~~–~~" and ~~+~~+" = 0.042



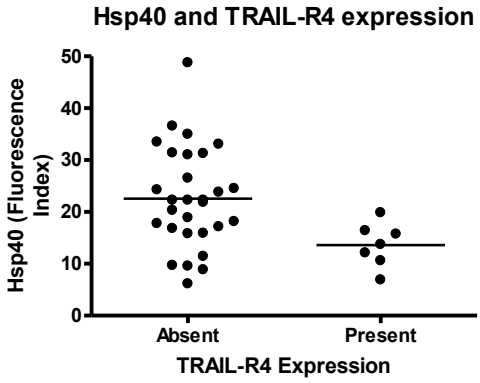
P value between ~~–~~present" and ~~–~~high" = 0.081



P value between ~~–~~present" and ~~–~~high" = 0.057



P value between ~~–~~present" and ~~–~~high" = 0.077



P value between ~~–~~absent" and ~~–~~present" = 0.013

The expression of hsps correlated with cell line ligand adhesion and TRAIL-R4 expression. Hsp expression was tested for correlation with melanoma cell surface protein expression and ligand adhesion using two-tailed nonparametric t tests.

To more thoroughly investigate the relationship between hsps and cell surface proteins in these melanoma cell lines, the effect of hsp90 inhibition on the expression of these proteins was investigated. Three melanoma cell lines were treated for 12 hr with 20 μM of the hsp90 inhibitor geldanamycin or left untreated. The cell lines were subsequently harvested and stained with antibodies to CD44, CD54, CD95, CD155, MHC I and MHC II. Protein expression was assessed by flow cytometry. Table 6.2 summarises the results.

Table 6.2 Changes in expression of surface marker proteins in response to hsp90 inhibition

Surface protein	EST02	EST27	EST160
CD44	19.8 % Decrease	17.9 % Decrease	29.8 % Increase
CD54	NC	10.7 % Decrease	10.1 % Decrease
CD95	10.8 % Decrease	15.4 % Increase	ND
CD155	6.9 % Increase	NC	NC
MHC I	19.8 % Decrease	9.7 % Decrease	17.4 % Decrease
MHC II	17.5 % Decrease	NC	23.6 % Decrease

Hsp90 inhibition results in differential protein expression. Three melanoma cell lines were set in duplicate cultures and treated with 20 μM geldanamycin for 12 hr or left untreated and the expression of cell surface proteins assessed with flow cytometry. Values indicate change in protein expression in the geldanamycin treated cell line relative to the matching untreated cell line.

NC, No Change
ND, No Data

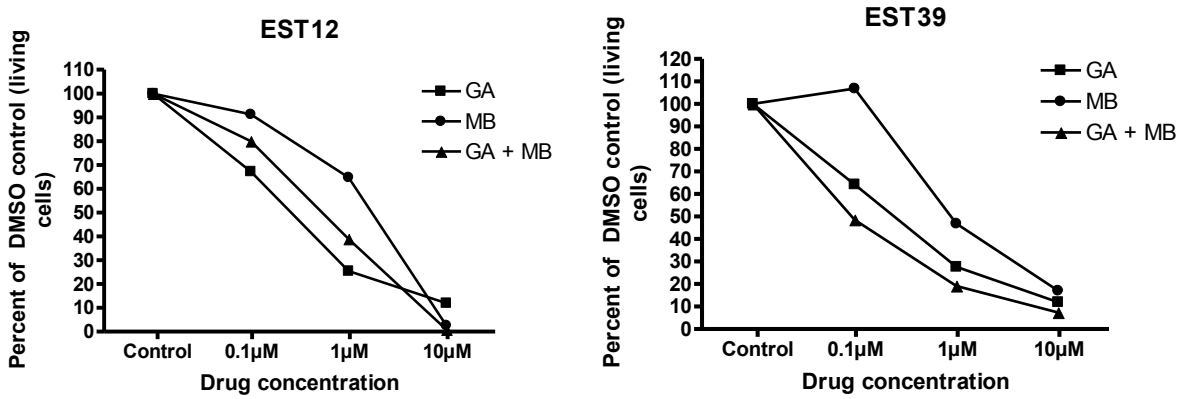
Changes in the expression of all proteins were observed to be induced by hsp90 inhibition, but these were largely inconsistent. MHC I proteins however showed a consistent but modest reduction in expression in all three cell lines.

Effect of hsp inhibition on melanoma cell line growth

Since hsp inhibitors are under clinical evaluation in the treatment of cancer [148], the effect of hsp inhibition on the growth of melanoma cells was investigated in two melanoma cell lines. The well-studied hsp90 inhibitor geldanamycin and the newly identified hsp70 inhibitor methylene blue were tested alone and in combination for their ability to inhibit the growth of two melanoma cell lines [193, 194]. Both drugs inhibited proliferation, with higher concentrations resulting in more effective growth inhibition. Methylene blue was observed to result in minor growth

inhibition compared to geldanamycin at low concentrations (0.1 μM), and only in one cell line. When treated with these inhibitors in combination, cell line EST39 but not cell line EST12 displayed an additive effect on growth inhibition.

Figure 6.13 Effect of geldanamycin and methylene blue on melanoma cell line growth



Geldanamycin and methylene blue inhibit melanoma cell line growth. Identical cultures of the melanoma cell lines EST12 and EST39 were treated with geldanamycin (GA) or methylene blue (MB) alone and in combination at concentrations of 0.1 μM, 1 μM and 10 μM or left untreated (control cultures contained an equivalent or greater concentration of DMSO). After 48 hr cells were harvested and live and dead cell numbers determined with trypan blue.

6.3 Discussion

Heat shock protein expression, growth rate and response to low oxygen tension in melanoma cell lines

Cancer cell lines are widely used models in cancer studies, but standard practice dictates culture under hyperoxic conditions (i.e. 20 % O_2 in air). Since hypoxia is a common and important feature of cancer, culture of these cells under high oxygen conditions may produce misleading results. For this reason viability, generation time and hsp expression was investigated in order to assess melanoma cell line response to low oxygen tension. One feature of the data was that for many of the characteristics, a large degree of variability was observed. Perhaps this is because each melanoma cell line is the result of independent mutation events accumulated in separate individuals. Consistencies in the data, for example that of increased generation time and reduced

viability in response to low oxygen tension, are presumably due to general physiological and biochemical properties shared by all cells and may not truly reflect the cancer phenotype.

Although hypoxia is a feature of human melanoma, the majority of the cells lines tested here displayed retarded growth and reduced viability under low oxygen conditions. This may be due to the fact that they have been generated and cultured under hyperoxic conditions which have prevented the selection of hypoxia-resistant cells that would normally occur [195]. Since these cell lines were cultured for a relatively short period of time that allowed only a small number of population doublings to occur, extending the culture period under low oxygen tension may have enabled the selection of cells more resistant to these conditions.

Hypoxia is a known inducer of the hsp response [196], but to the best of the author's knowledge, no studies have examined the effect of low oxygen on hsp expression in melanoma or indeed in any other cancer cell lines in the manner performed here. Unexpectedly, a widespread induction of hsp expression did not occur in response to low oxygen tension. Despite this, changes in hsp expression were associated with tolerance to low oxygen as measured by generation time and viability. These data suggest that improved hypoxic tolerance is associated with the induction of hsps in melanoma cells. Further, higher total hsp expression was associated with improved viability in low but not high oxygen conditions. This suggests that low oxygen tension culture is more relevant with respect to the role of hsps in these melanoma cell lines and simultaneously suggests that oxygen tension is a valid experimental variable in studies with other cancer types, especially given the relative lack of investigation in this area.

A relationship between higher expression of hsp90, hsp70 and hsp60 and greater cell viability in 2 % but not 20 % O_2 was observed. The greater demand placed on the hsp chaperone system under low oxygen stress where the level of hsp expression is more likely to fall below the threshold

required for cellular survival may account for the observation that hsp expression was not associated with viability under high oxygen levels. Since under hypoxic stress the requirement for chaperoning proteins is likely to be increased, cells that express higher levels of hsps may have a survival advantage compared with cells that express a lower level of hsps. Considering that hsp90 is essential for cellular viability and hsps provide protection against apoptosis, under stress conditions when the requirement for chaperones is increased, cell lines that express lower levels of hsp90 may suffer a loss in viability as the level of hsps falls below the threshold required for survival [6, 86]. Supporting this hypothesis is the observation that an increase in hsp90 expression in 2 % O_2 relative to 20 % O_2 was associated with increased cell viability in 2 % O_2 relative to 20 % O_2, while a decrease in hsp90 expression in 2 % O_2 relative to 20 % O_2 was related to decreased cell viability. Furthermore, changes in the expression of hsp90 and hsp40 in 2 % O_2 relative to 20 % O_2 were associated with growth rates in low oxygen tension, suggesting that up-regulation of these proteins is associated with improved growth under hypoxic stress, while down-regulation results in retarded growth. These data suggest that under stress but not basal conditions, both the degree of total hsp expression and the change in hsp expression in response to low oxygen tension are associated with the ability of cells to tolerate hypoxic stress. These data are consistent with reports showing hsps to have a protective function under hypoxic conditions and perhaps reflect the anti-apoptotic role that hsps play in cancer [85, 86, 187, 197]. If this observation is at all reflective of *in vivo* conditions then it follows that anti-neoplastic hsp inhibiting drugs may be relatively more effective in hypoxic tumours in which a range of standard therapies are known to be less effective [170]. Indeed, melanoma cells are sensitive to hsp70 and hsp90 inhibiting drugs, confirming previous reports that they require these proteins in order to proliferate [198, 199]. Thus, *in vivo* under hypoxic conditions their effectiveness may be enhanced.

Relative hsp expression within each cell line was observed to be consistent across the entire cohort of cell lines and levels of hsp 90, 70, 60 and 40 expression were shown to correlate with one another but not with hsp32. Hsp90 is essential for eukaryotic cell viability and it chaperones a large number of overexpressed client proteins in cancer. This may explain why hsp90 was expressed at a level many fold greater than the other hsps examined [6, 145]. However hsps most often function in cooperation with other molecular chaperones [6, 8]. The other hsps examined in this study are major hsp90 co-chaperones, consistent with the notion that hsps cooperate closely in their role as molecular chaperones [6]. The intimacy of their association may explain the observation that their expression correlated with one another but not with hsp32, because this protein is not a molecular chaperone and its function as a haem oxygenase is less related to that of the other hsps [200]. Future studies could employ isoform-specific antibodies in order to identify differences that may exist in hsp isoforms. Furthermore, potential differences in the binding affinity of these antibodies make it difficult to draw conclusions on the relative level of hsp expression.

Cell surface expression of hsps in melanoma has not been widely investigated. Among existing studies conflicting findings have been reported [120, 201]. In this study all five hsps investigated were observed to be present on the cell surface, but their function and biological relevance is unknown. Immune recognition is a potential function for these cell surface hsps, as membrane located hsps have been shown to act as targets for T cells and NK cells [131, 133]. These hsps may also possess a chaperone function, as cell surface hsps have been shown to be involved in tissue invasion and metastasis [89].

Correlation of hsp expression with clinical parameters

Expression of some hsps was observed to correlate with Breslow depth but not Clark level or patient survival. Studies using primary melanoma tissue have shown increased hsp90 expression

to be associated with greater Breslow depth and Clark level [115], but in the present study with metastatic melanoma cell lines an inverse relationship was observed. These data contrast with studies using melanoma tissue that show increased expression of hsp70 to be associated with improved survival and higher Clark level but not Breslow depth. A further study identified hsp70 expression to be associated with Breslow depth [115, 118, 119, 122]. The data presented here suggest that hsp90 and hsp40 expression in cell lines may thus be a marker for Breslow depth. In the case of hsp90 this appears to be a surrogate marker, since in contrast to studies performed with melanoma tissue an inverse relationship was observed. Similar reports exist in other contexts [202]. For example, Walter *et al*. [203] demonstrated MMP-2 mRNA levels in renal cancer cell lines to inversely correlate with patient survival. A potential disadvantage of using cultured cells as models for *in vivo* processes is that it is not known how reflective the parameters measured under these conditions are of *in vivo* conditions. The data presented here coupled with at least one previous report suggests that cancer cell lines maintain relevance to patient clinical parameters and thus may be of use as models for cancer, even if this relevance is maintained only indirectly [203]. Since in these cell lines hsp expression was relevant to viability in 2 % but not 20 % O_2 culture conditions, low oxygen tension culture conditions may be more relevant to *in vivo* growth conditions, but more studies are needed to confirm this.

A trend between increased hsp90 expression and reduced survival time in patients from which the cell lines were generated was observed. If as in other cancer types, increased hsp90 expression is associated with decreased patient survival, then it is possible that hsp90 was more highly expressed in these patients' tumours *in vivo* and this feature has been maintained under *in vitro* cell culture conditions. However, it may also be that due to the artificial cell culture environment, the level of hsp expression has been altered and therefore the expression levels observed are not reflective of *in vivo* expression, especially since a study conducted with melanoma tissue showed no association between hsp90 expression and survival in primary melanoma tissue [115]. A

comparison between the hsp expression of freshly isolated melanoma tumour tissue and that of the same tumour established as a cell line in culture is required to answer this question. One previous study that examined hsp gene expression in prostate cancer cells growing as monolayers *in vitro* and as tumour xenografts *in vivo* demonstrated a reduction in hsp gene expression when the cell lines were grown as tumours *in vivo* [204], suggesting culture conditions influence hsp expression. Furthermore, to establish a correlation between hsp expression in melanoma cell lines and patient survival, a larger number of cell lines with accompanying patient data will need to become available. The results from this study should be compared to those investigating the relationship between hsp expression and patient survival in melanoma tissue, such as that performed in Chapter 7 of this thesis. The results given in this chapter suggest that there is an association between higher hsp90 expression and reduced survival following metastasis in metastatic melanoma patients. Given that an inverse trend was observed between hsp90 expression and patient survival in melanoma cell lines derived from metastatic melanomas, this supports the notion that these melanoma cell lines are perhaps in some way reflective of their *in vivo* properties, perhaps if only indirectly.

The aforementioned experiments will further assist in determining if *in vitro* cultured cell lines maintain *in vivo* relevance with respect to hsp expression. A previous study has shown tumour derived cell lines to vary in MMP-2 mRNA expression, whereas expression of MMP-2 protein in tumour tissue from which the cell lines were derived was uniformly high [203], however in contrast another study showed that freshly prepared melanoma tumour samples show similar expression of hsp70, Mel-1, Mel-2 and Mel-5 on the cell surface after the same tumours had been established as a cell line [224]. The data presented here combined with the small number of studies undertaken on the topic of cell line *in vivo* relevance is clearly open to interpretation, but it appears as though *in vitro* cell culture may influence the properties of cell lines, but that they may still be reflective of *in vivo* conditions. Given that in this study the cell lines were observed not to

be uniform in any of the measured attributes, it appears as though *in vitro* cell culture conditions did not exert an overwhelming influence on the cell cultures. Instead, it is possible that the influence of the cell culture environment interplays with the intrinsic properties of each cell line, thereby allowing the possibility that they are in some way reflective of their *in vivo* state.

If the level of hsp expression observed in this study is reflective of *in vivo* expression, then previous studies using melanoma tissue that have shown higher hsp expression to be associated with greater Breslow depth contradict the observations here that higher hsp expression in cell lines correlates with reduced Breslow depth. This contradiction may be due to hsps influencing the immunogenicity of these cells. This might be explained by greater hsp expression resulting in increased immunogenicity, producing a more effective immune response and a smaller resultant tumour [137, 138, 142-144, 205]. Supporting this is a study in which expression of hsp70 was found to correlate with improved survival in melanoma patients [122].

Relationship between hsp expression and cell surface proteins

Significant inverse correlations between the expression of hsp90 and adhesion ability of the cell lines to collagen and laminin ligands were observed. The expression of cell adhesion molecules influences the metastatic potential of cancer cells and the observation of a relationship between hsp90 expression and cell adhesion may relate to the role of hsp90 in metastasis [162]. Hsp expression was also found to correlate with expression of melanoma cell surface proteins. These correlations do not necessarily imply that a direct relationship exists. In order to investigate this, hsp90 function was inhibited and the change in expression of a number of melanoma surface proteins was assessed. In response to hsp90 inhibition, the expression of all proteins examined was altered, but this was not observed in all cell lines and the changes were largely inconsistent. Considering the observed variability in the degree of expression change, it is unlikely that any of the proteins examined are hsp90 client proteins. Instead, the altered patterns of protein expression

may be due to the loss of general protein chaperoning (~~h~~ouse keeping") that hsp90 is known to be involved in, thereby producing a cascade of indirect effects propagated through altered activity of co-chaperone and client proteins, and disruption of protein homeostasis. Since these are indirect effects, this may explain the observation that proteins showed increased expression in some cell lines, while in other cell lines decreased expression was observed. MHC I was the only protein to display a consistent response and as such, there may be a more direct relationship between the hsp90 and MHC I proteins. To date, there are mixed reports on the effect of hsp90 inhibition on the expression of MHC I proteins. Studies have shown hsp90 inhibitors to have no effect on MHC I protein expression, but for this to result in reduced levels of folded MHC I proteins, while others have reported an increase in expression [75-77].

Effect of hsp inhibition on melanoma cell line growth

Hsp inhibitors are currently being evaluated in clinical trials for the treatment of melanoma. Therefore the assessment of melanoma growth under *in vitro* conditions is of clinical importance. The hsp inhibitors used in this study were observed to result in the inhibition of melanoma cell line proliferation. This observation supports the notion that hsps are essential proteins required for rapid cellular proliferation such as that in cancer.

These data suggest hsps associate closely with one another in melanoma cells and that their expression is associated with tolerance to low oxygen tension and patient clinical parameters. The *in vivo* importance of hypoxia coupled with the finding that hsp expression is relevant to melanoma cell viability in low but not high oxygen tension suggests cancer cell cultures should more frequently be subjected to oxygen tension that more closely resemble *in vivo* levels in order to cast more light on the largely unknown influence that this condition has on the myriad of facets investigated in modern cancer research.

Chapter 7: Heat shock protein expression in melanoma tumour tissue and relationship with patient clinical parameters

7.1 Introduction

Hsps are fundamental for tumourigenesis and contribute to the biology of cancer through multiple diverse mechanisms [83]. Hsps are abnormally expressed in a range of cancer types and their expression has been shown to predict patient prognosis and therapeutic response [83, 104, 107]. The importance of hsps in human cancers is well established, for example hsp inhibitors are currently under clinical evaluation for the treatment of a variety of cancer types [148]. Hsp expression has been thoroughly characterised for a number of cancer types, although reports are lacking, limited and have provided conflicting data [104, 115]. Inferring the role of hsps based on previous studies is thus problematic as their role and clinical relevance varies depending on the cancer type [84]. The significance of hsp expression with respect to cancer is undefined in many contexts and remains enigmatic to date.

It may well be that differences in the clinical relevance of abnormal hsp expression are products of the diverse roles that hsps perform. Hsps were originally thought to provide protection from tumour-associated stressors and to chaperone oncoproteins responsible for tumour cell proliferation, but they are now recognised to contribute to many of the characteristic features of cancer including sustained angiogenesis and apoptosis evasion [83]. These well recognised roles are shadowed by a second, less well characterised role of hsps as immunomodulators. Modifying hsp expression results in biologically relevant alteration of the immunogenicity of cells [138, 142, 143]. Furthermore, cell surface hsps act as targets for NK cells and T cells, and hsps are involved in antigen presentation whereby they can chaperone tumour antigens to APCs [71, 131, 133]. Moreover, the interplay between these two diverse roles may vary across cancer types or within tumours of the same type.

Melanoma is one such cancer type for which conflicting reports on the role of hsps exist. Melanoma is an invasive cancer with poor survival rates once metastasis occurs [22]. Melanoma incidence is increasing in many countries, consequently it constitutes a significant burden on human health [22, 23]. The validation of hsps as therapeutic targets in melanoma is of particular importance because hsp inhibitors are currently being evaluated in the treatment of melanoma patients [206]. Studies to date have demonstrated that hsps play important roles in melanoma, but few clear relationships have been defined and conflicting data have been reported [115, 122, 123, 236, 237]. It was the aim of this study to assess the expression of hsps in melanoma tumour tissue and to assess correlations with patient clinical parameters.

7.2 Results

The expression of hsp90, hsp70, hsp60, hsp40 and hsp32 was investigated with Western immunoblotting in 32 stage III/IV metastatic melanomas, one metastatic squamous cell carcinoma (sample 17) and a lymph node from a patient with B cell chronic lymphocytic leukaemia (sample 28). The vast majority of patient samples were observed to express all hsps examined (Fig. 7.1). Samples 11 and 32 are lymph node and ureter metastases from the same patient, respectively, and it was noteworthy that these tumours were observed to express similar levels of all hsps examined. Sample 17, metastasis of a squamous cell carcinoma, consistently displayed relatively reduced expression of hsps as compared to many of the melanoma tumours. By contrast sample 28, a lymph node from a patient with B cell chronic lymphocytic leukaemia, showed levels of hsp expression comparable with many of the melanoma tumours. All expression levels were normalised with respect to the level of the β-actin house keeping protein.

Among the melanoma tumours, a number of samples showed relative higher or lower hsp expression. These differences in expression were investigated for association with a range of patient clinical parameters. The results of this analysis demonstrated that higher relative hsp90 (P

< 0.02) and hsp40 ($P < 0.03$, Appendix VI) expression correlated with advanced tumour stage (stage III to stage IV) and higher hsp90 expression with reduced patient follow-up time ($P < 0.04$) (survival time since removal of the metastasis that was herein examined), both prognostic markers in melanoma (Table 7.1). Note that only patients with a follow-up period of at least two months were included in the analysis. In contrast, the expression of the other hsps was not associated with patient clinical parameters (Appendix VI). Additionally, t tests were used to investigate differences between selected prognostic factors but no significant relationships were identified (Appendix VI). Samples number 1 and 26 contained very low levels of protein according to the β-actin loading control, and with the exception of sample 26 probed for hsp32, these samples showed a corresponding low or absent level of hsp expression. All experimentation and data analysis were performed blinded, that is, without prior knowledge of patient clinical parameters.

Figure 7.1 Heat shock protein expression in melanoma, a squamous cell carcinoma and a lymph node from a patient with B cell chronic lymphocytic leukaemia

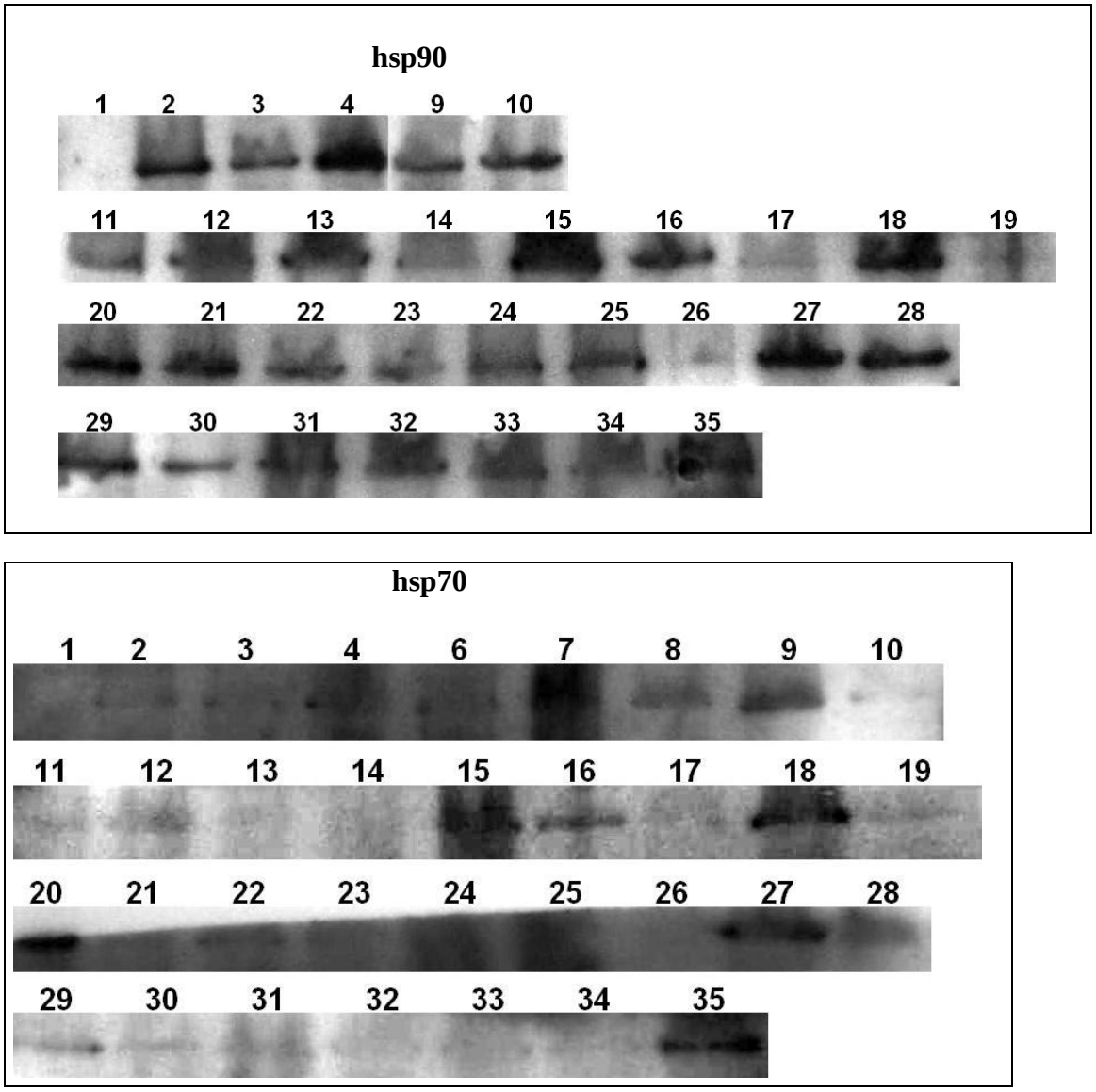
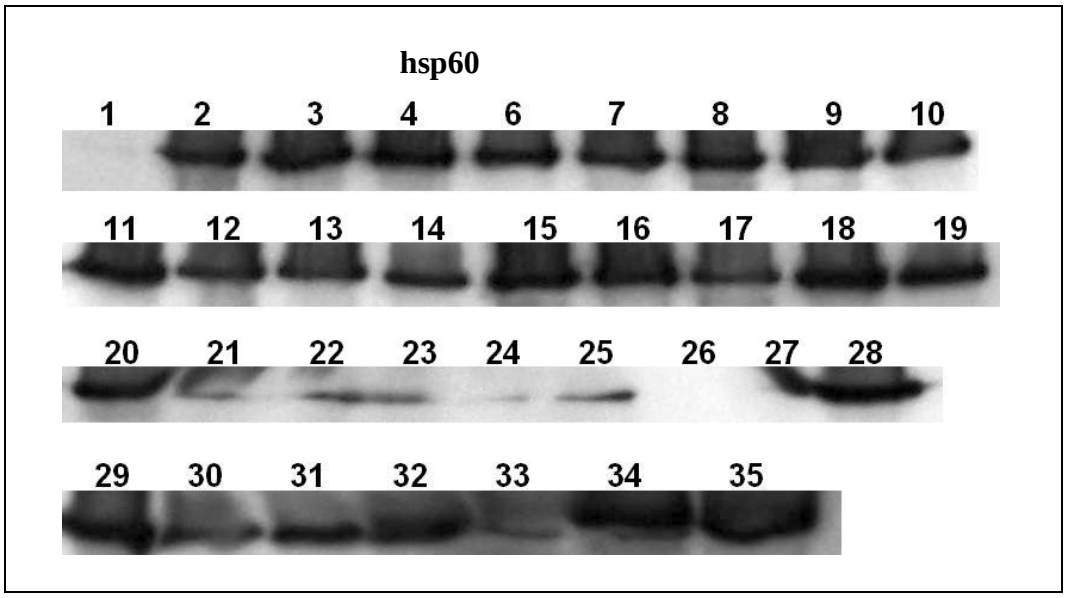


Figure 7.1 continued



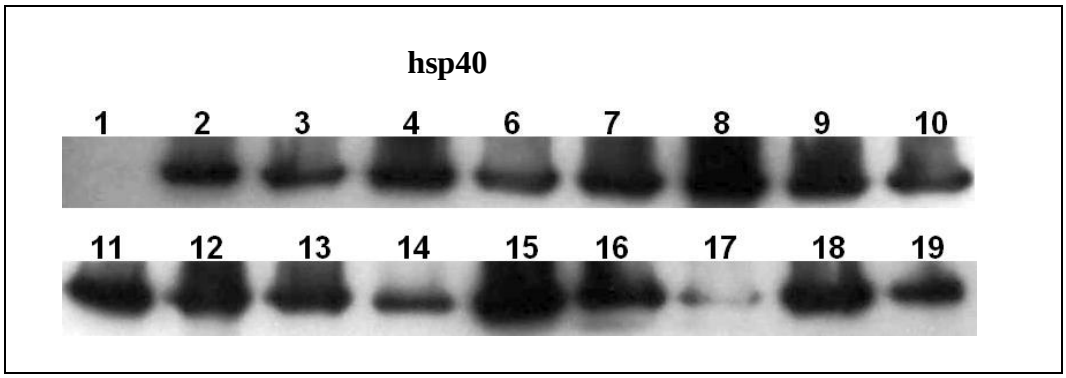
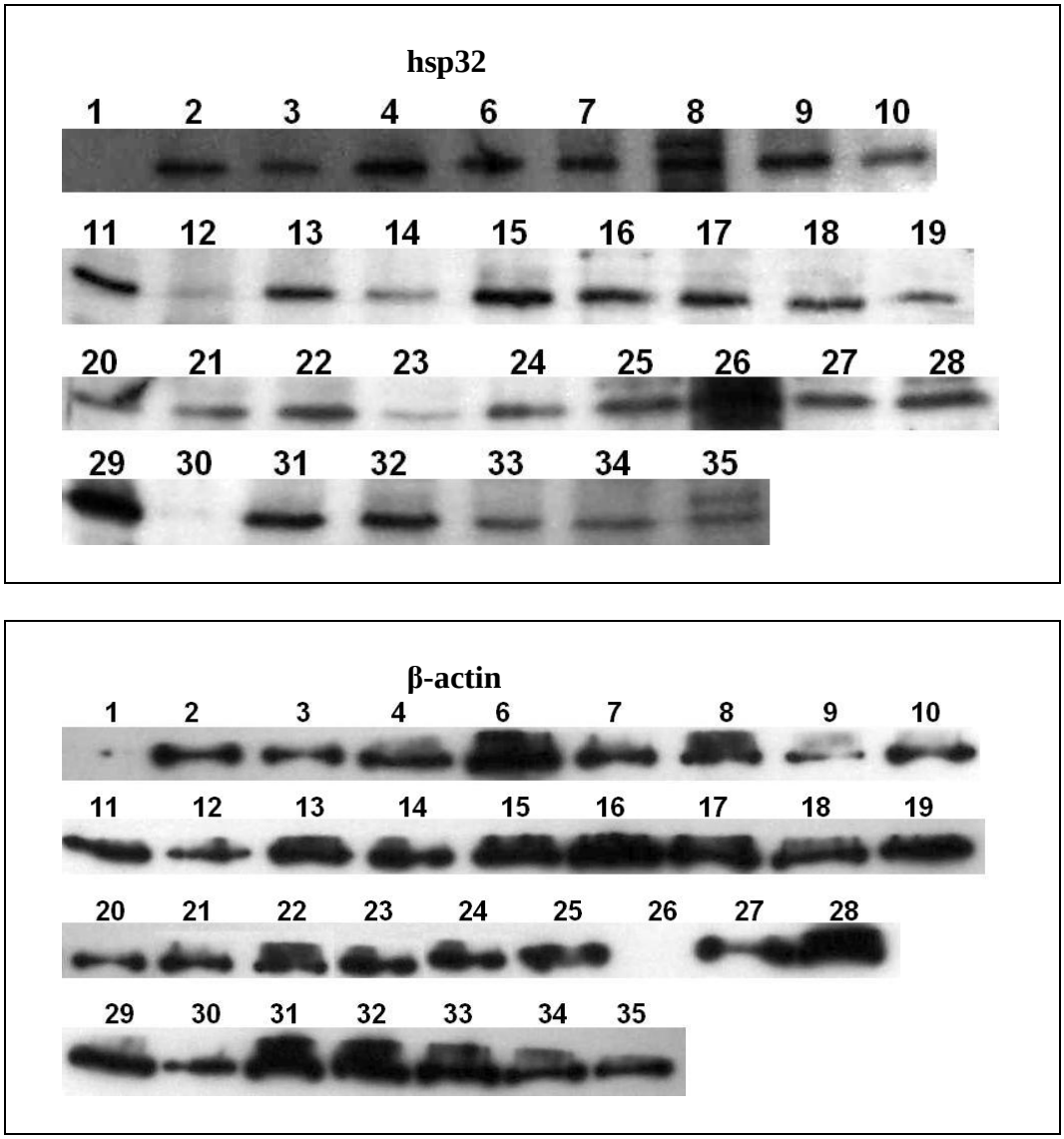


Figure 7.1 continued



Expression levels of hsps 90, 70, 60, 40 and 32 were assessed in 32 stage III/IV metastatic melanomas, one metastatic basal cell carcinoma and a lymph node from a patient with B cell chronic lymphocytic leukaemia using Western immunoblotting. Numbers indicate patient sample number. Samples were grouped accordingly: hsp90 high: 2, 4, 15, 18, 20, 21, 27, 29, 30, 3, 9, 10. hsp90 low: 13, 14, 16, 19, 22, 23, 24, 31, 32, 33. hsp70 high: 9, 15, 18, 27, 35. hsp70 low: 2, 3, 4, 6, 8, 10, 11, 12, 13, 14, 16, 19, 22, 23, 29, 30, 31, 32 , 33, 34. hsp60 high: 2, 3, 4, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 18, 19, 20, 29 30, 31, 32, 34, 35. hsp60 low: 21, 22, 23, 24, 25, 33. hsp40 high: 2, 4, 7, 8, 9, 11, 12, 15, 18. hsp40 low: 3, 6, 10, 13, 14, 16, 19. hsp32 high: 2, 3, 4, 6, 7, 8, 9, 10, 11, 13, 15, 16, 18, 20, 21, 22, 24, 25, 26, 27, 29, 31, 32. hsp32 low: 12, 14, 19, 23, 30, 33, 34, 35

Table 7.1 Association between hsp90 expression and patient clinical parameters

Clinical parameter		Low hsp90 tumours	High hsp90 tumours	P value
Sex (n = 22)	Male	9	9	> 0.35
	Female	1	3	
Ulceration (n = 11)	Positive	2	4	> 0.12
	Negative	4	1	
Breslow depth (n = 13)	0-2 mm	4	3	> 0.35
	>2-4 mm	2	0	
	>4 mm	1	3	
Stage (n = 22)	III	4	0	< 0.02
	IV	6	12	
Clark Level (n = 9)	III	0	1	> 0.15
	IV	4	3	
	V	1	0	
Lymph node metastasis (n = 22)	Positive	5	4	> 0.40
	Negative	5	8	
Skin metastasis (n = 22)	Positive	3	7	> 0.15
	Negative	7	5	
Primary tumour localised to lower extremity (n = 17)	Positive	3	3	> 0.85
	Negative	5	6	

Table 7.1 continued

Clinical parameter		Low hsp90 tumours	High hsp90 tumours	P value
Superficial spreading histological sub-type (n = 12)	Positive	5	2	> 0.25
	Negative	2	3	
Follow-up time* (n = 17)	≤6 months	5	8	< 0.04
	>6 months	4	0	
Survival since diagnosis (n = 22)	≤33 months	6	8	> 0.70
	>33 months	4	4	
Stage III to IV progression time (n = 9)	<23 months	3	3	> 0.60
	≥23 months	2	1	
Time to first metastasis (n = 12)	≤29 months	4	2	> 0.20
	>29 months	2	4	
Age (n = 22)	≤56	8	6	> 0.12
	>56	2	6	

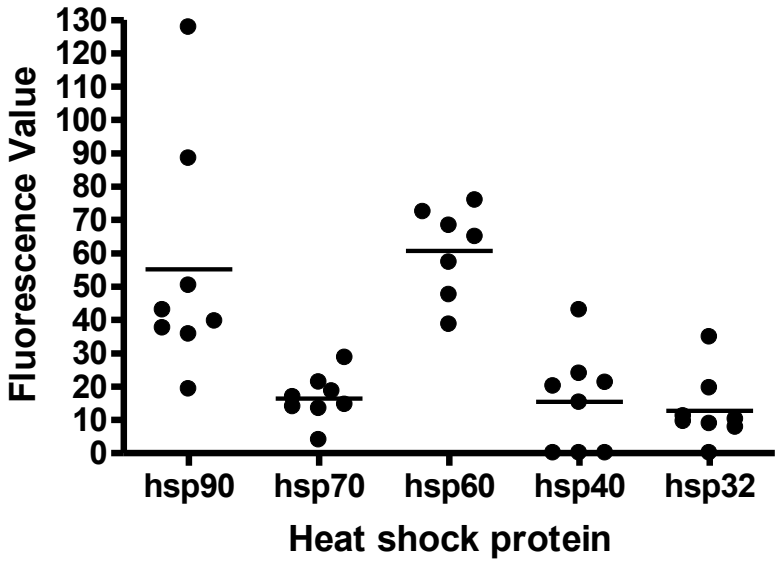
* Only patients with a follow-up period of at least two months were included in the analysis

Higher hsp90 expression is associated with advanced melanoma tumour stage and reduced follow-up time. Patient clinical parameters were correlated with hsp expression using chi-squared contingency tests. Data for hsps 70, 60, 40 and 32 appear in Appendix VI.

Fluorescence microscopy was used to assess hsp expression in melanoma tissue samples in order to visualise the distribution of hsp expression among melanoma cells within tumour tissue. Melanoma tissue sections from eight stage III skin-localised metastatic melanomas were screened for the expression of hsp90, hsp70, hsp60 (n = 7), hsp40, hsp32 and the diagnostic melanoma marker, MelanA [222]. In all patient tumours, positive staining for hsp90, hsp70 and hsp60 was

observed, while three patients were negative for hsp40 and one negative for hsp32. Of these hsps, hsp90 and hsp60 were observed to be the most highly expressed, while hsp70, hsp40 and hsp32 were expressed at relatively lower levels. Among positive samples, the majority of melanoma cells were observed to express hsps. In order to quantify these observations, average fluorescence values were obtained and compared (Fig. 7.2). It was noteworthy that hsp40 was expressed in the cell nucleus, whereas the other hsps showed either weak or no nuclear staining.

Figure 7.2 Heat shock protein expression level in melanoma tumour tissue

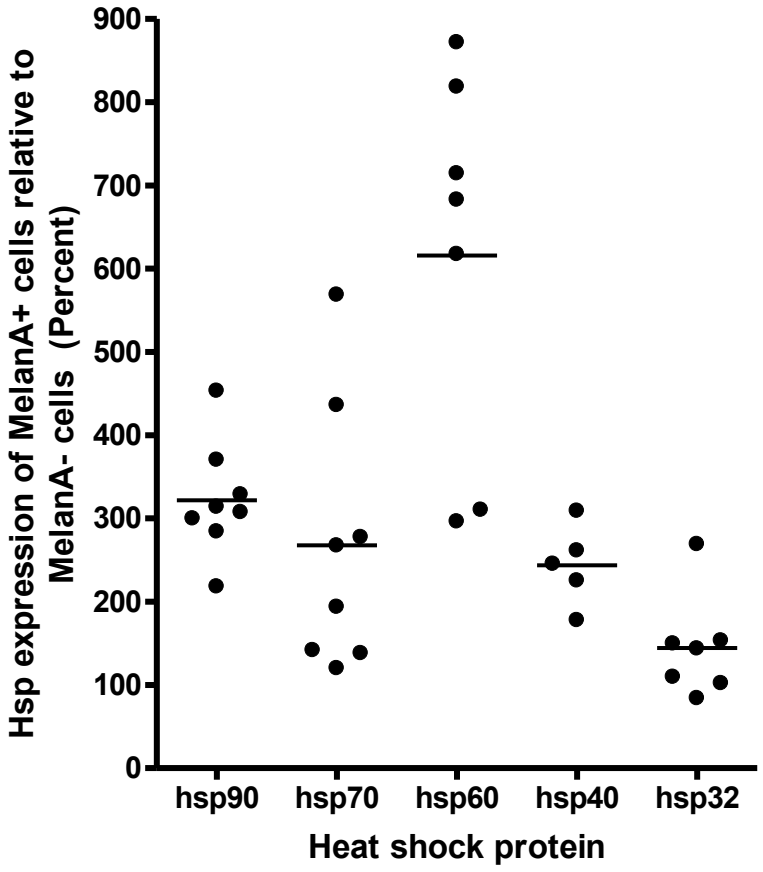


Formalin fixed paraffin-embedded melanoma tissue sections were stained for hsp expression using fluorescent antibodies and analysed using fluorescence microscopy. Differences in protein expression were assessed with appropriate software quantification by obtaining the mean fluorescence value of a small number of representative cells in a minimum of five regions within the tissue section. Bars indicate mean value for each group.

Hsp expression was visually observed to be higher in the MelanA+ cells compared with neighbouring MelanA- cells in each individual tissue section. To quantify this difference, the mean fluorescence of each hsp for a small number of representative MelanA+ and MelanA- cells within the same tissue region were compared (Fig. 7.3).

Hsps 90 and 60 showed the highest expression in MelanA+ cells relative to MelanA- cells with between a 200 – 450 % and 300 – 900 % increase, respectively. Hsp40 showed modest increased expression in MelanA+ cells of between 150 – 300 %. In the case of hsp70 and hsp32, protein expression in MelanA+ cells compared with MelanA- cells was observed to be increased by between 100 – 600 % and 100 – 300 %, respectively. It should be noted that a few tumours did not show relative increased expression of hsp70 or hsp32. Representative images for each hsp are shown in Fig. 7.4.

Figure 7.3 Fluorescence microscopy of heat shock protein expression in melanoma and non-melanoma cells within melanoma tumour tissue



Formalin fixed paraffin-embedded melanoma tissue sections were stained for hsp expression with fluorescent antibodies and analysed using fluorescence microscopy. Differences in hsp expression between MelanA+ and MelanA- cells were quantified with appropriate software and the percentage increase in the MelanA+ cells by comparison with the MelanA- cells calculated. Bars indicate mean value for each group.

Figure 7.4 Fluorescence microscopy of heat shock protein expression in melanoma tumour tissue

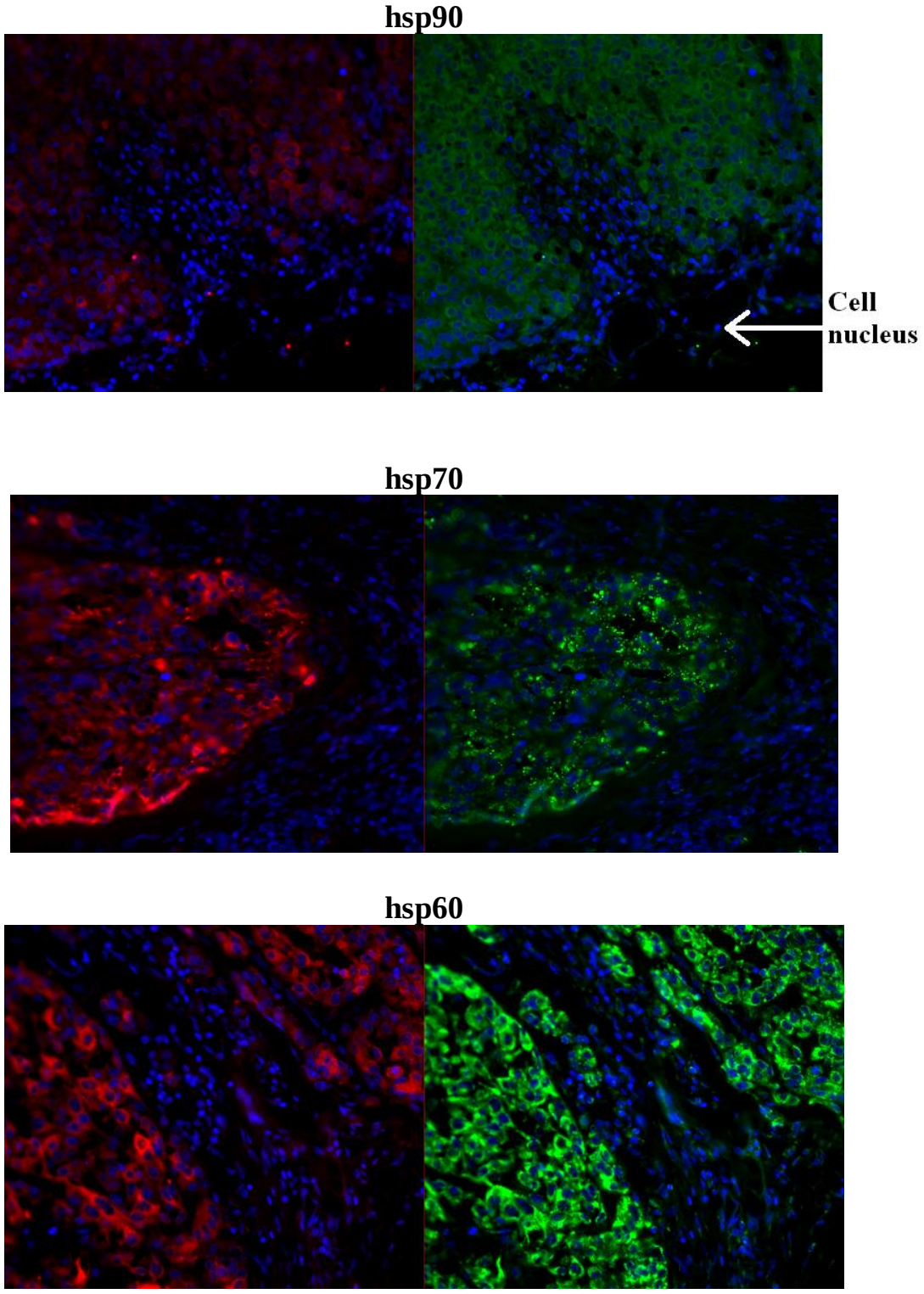
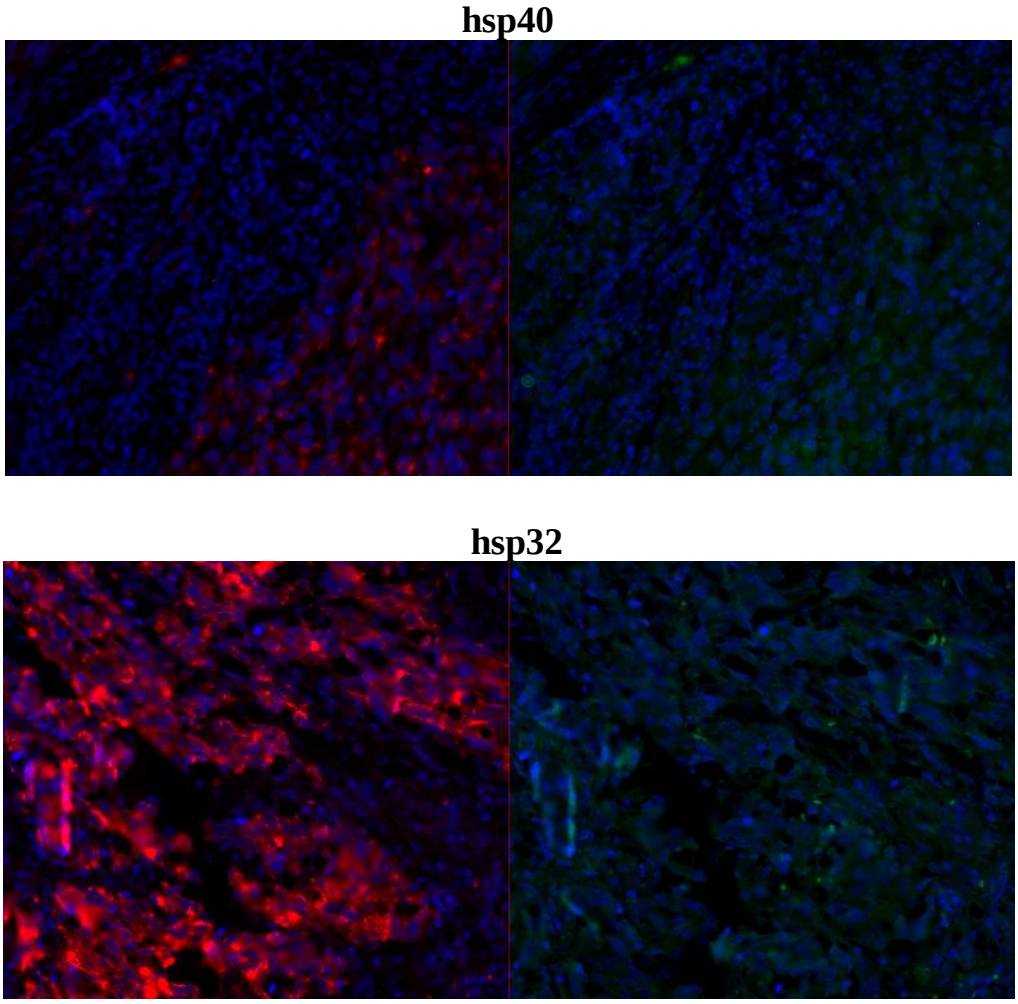


Figure 7.4 continued



Hsps 90, 70, 60, 40 and 32 were increased in expression in MelanA+ cells relative to MelanA- cells. Formalin fixed paraffin-embedded melanoma tissue sections were stained for hsp expression with fluorescent antibodies and analysed using fluorescence microscopy. Representative images shown.

Red, MelanA (marker for melanoma cells)
Green, hsp (marker for hsp expression)
Blue, DAPI DNA stain (marker for cell nucleus)
20x magnification

7.3 Discussion

This study demonstrated that hsps are widely expressed in melanoma tumours and that their expression correlated with patient clinical parameters (for example, stage III progression to stage IV, Table 7.1). Using immunofluorescence to examine hsp expression within tumour tissue showed that hsps were expressed at higher levels in melanoma cells relative to neighbouring non-melanoma cells (Fig. 7.3). These observations have a number of important implications.

This study presents preliminary findings, albeit not previously reported, that hsp90 and hsp40 expression levels may be connected with advanced tumour stage and hsp90 with reduced survival following metastasis in melanoma patients (Table 7.1). Interestingly, an earlier study using primary melanoma tissue reported an association between increased hsp90 expression with greater Breslow depth and higher Clark level, but not patient survival [115]. These data may not be entirely contradictory with the present study in that it may be proposed that the importance of hsp90 may not be fully evident until the primary tumour progresses to a more advanced metastatic stage. The higher hsp90 expression and connection with advanced tumour stage and reduced survival time following metastasis suggests that hsp90 may play a role in the progression of metastatic melanoma and thus may be a potential biomarker of poor patient prognosis. This may be achieved through the role that hsp90 plays in stabilising multiple signalling pathways exploited by cancer cells in order to proliferate [145]. Higher hsp40 expression was also found to correlate with advanced tumour stage (stage III Vs stage IV) and this may be achieved through the shared chaperoning role that these two proteins have [8]. If hsp90 is involved in activating the immune system against melanoma tumours, higher hsp90 expression would not be expected to correlate with markers of disease progression as observed in this study. Therefore these data additionally suggest that hsp90 is not involved in activating the immune system against melanoma tumours, on the contrary increased expression may even protect against tumour directed immune system attack

[140]. These findings contribute to the proposal of hsp90 as a valid therapeutic target in the treatment of melanoma and to the understanding of melanoma tumours.

A number of relationships bordering on statistical significance with other patient parameters were observed and, in contrast to previous studies [119, 122], hsp70 expression was not associated with Clark level or patient survival, but did correlate with the sex of the patient (Appendix VI). On the other hand, in accordance with previous studies, hsp90 expression was not associated with tumour ulceration, patient age or sex, and hsp70 expression did not relate to Breslow depth [115, 119]. Discrepancies between previous studies and the results presented in this thesis are most likely related to differences in the patient sample groups or due to differences between primary and metastatic melanomas. Although hsp60 and hsp32 were widely expressed in melanoma tumours, there was no obvious correlation with disease progression or patient parameters. The negative associations and apparent contradictions to previous studies may be due to the small sample sizes of this and of previous studies. These inconsistencies and potential relationships require more comprehensive larger scale follow-up studies in order to conclusively define the role of hsps in melanoma.

In agreement with the results presented in Chapter 6 of this thesis which investigated hsp expression in melanoma cell lines, hsp expression in metastatic melanomas was not connected with Clark level or Breslow depth (hsp70, hsp60) nor was expression of hsps 70, 60 and 40 connected with patient survival, although hsp90 expression was connected with patient follow-up time following metastasis in melanoma tissue. On the other hand, the expression of hsp90 and hsp40 did not correlate with Breslow depth as it did in cell lines. This is most likely due to clinical differences between the patient groups, for example stage of diagnosis. In the melanoma cell lines examined in Chapter 6, hsp90 was by far the most highly expressed hsp, followed by relatively low and equivalent levels of the other hsps (Fig. 6.6). With the notable exception of hsp60, a

similar trend was observed in the melanoma tissue with immunofluorescence experiments (Fig. 7.2), but not with Western immunoblotting experiments (Fig. 7.1) where hsps 60, 40 and 32 were the most highly expressed. Nonetheless, differences in experimental settings such as antibody concentrations, incubation times, differences between the antibodies themselves and other factors make it difficult to draw conclusions, especially as antibodies may differ in their binding affinity, making comparisons between the relative level of expression difficult. Nevertheless these experiments suggest that hsp90 and hsp60 are relatively highly expressed in melanoma. The results of future studies will refute or support the observations reported here. In immunofluorescence experiments, hsp90 and hsp60 were observed to be the most highly expressed hsps, while hsps 70, 40 and 32 showed relatively weak staining. In contrast, hsp90 was one of the least highly expressed hsps by Western immunoblotting, while hsps 60, 40 and 32 were observed to be the most highly expressed. Identical antibody panels were employed for both techniques, thus observational differences are most likely attributed to differences in experimental procedures: Western immunoblotting involves protein extraction, heat denaturation, separation by gel electrophoresis, transfer to nitrocellulose membrane, primary and secondary antibody blotting involving HRP-conjugated antibodies and signal detection onto X-ray film, whereas immunofluorescence involves the use of formalin-fixed paraffin embedded tissues in which the proteins have been denatured by heat and acid. Following which primary and fluorochrome-conjugated secondary antibodies are applied and the signal detected by light microscopy, thus throughout this procedure the proteins remain embedded within the whole tissue matrix. Thus these two methods are not absolutely comparable with respect to the relative levels of hsp expression.

Tumours are a dynamic, evolving tissue mass [212]. This is an important characteristic when considering hsp expression determined experimentally in biopsy tissue samples. The degree of hsp expression observed in such tissue samples is representative of the tumour at the time of

excision only, and does not take into account the variation of hsp expression that may occur throughout *in vivo* tumour development. Ideally, freshly excised tumour and non-tumour biopsies should be taken, maintained in short-term tissue culture, subjected to stressors such as heat or free radicals and analysed for hsp expression. Following this, hsp expression levels could be quantified and compared with the levels observed under basal conditions. Of interest are potential differences between the basal and induced level of hsp expression within and between these tissue types, as malignant tissues and healthy tissues may respond differently to stress and possess an altered ability for the induction of hsp expression. Additionally, multiple biopsies from a tumour growing *in vivo* could be taken and analysed for hsp expression throughout its development in order to document the variation, if any, in hsp expression. Although technically difficult, these experiments would assist in determining the degree of tumour hsp expression more accurately, if hsp expression does indeed vary throughout stages of tumour development and if this is significant to patient clinical parameters or outcome.

It is known that hsps are regulated at the post-translational level [36], thus the experimental quantification of hsp levels may not be indicative of their biological importance across different experimental samples due to the potential for variation in the activity and functionality induced post-translationally. To account for this, antibodies that recognise post-translational specific forms of hsps (such as phosphorylation) could be employed and considered along with the level of total hsp expression. Additionally, isoform-specific antibodies could be employed to distinguish between differences that may exist in hsp isoforms.

Hsp90 inhibiting drugs are under clinical assessment for the treatment of cancer, noteworthy is that this study indicated that melanoma cells express higher levels of hsp90 relative to non-melanoma cells. These observations therefore support the clinical use of hsp90 inhibitors in the treatment of melanoma. Given that hsps 70, 60 and 40 were also observed to be increased in

expression in melanoma cells, these hsps may make useful therapeutic targets in the treatment of melanoma as well, although it should be noted that the expression of hsp70 and hsp60 was not associated with prognostically relevant patient clinical parameters. These data further suggest that the chaperoning activity of these hsps is under greater demand in these cells. This is likely due to the many roles that these proteins play that support cancer progression such as providing protection from tumour-associated stressors and chaperoning overexpressed oncoproteins. Indeed, the mutated B-Raf oncoprotein V600E is dependent on hsp90 for stability [207] and hsp90 inhibitors have been shown to reduce expression of multiple members of the MAP kinase pathway in the clinical treatment of melanoma [220]. However, in immunofluorescence experiments, a number of samples were observed to be negative for hsps, suggesting that not all tumours exploit the hsp molecular chaperone system. The identity of the MelanA-negative cells is unknown, but they may be tumour-associated fibroblasts that support the growth of the tumour [212, 227]. Despite the widespread apparent increased hsp expression in MelanA+ cells with respect to neighbouring MelanA- cells, considerable variation was observed both between individuals and between hsps in any individual. Therefore melanoma patient sub-groups that do not exhibit up-regulated hsp expression or that have reduced or a lack of hsp expression within their tumours should be carefully screened as they may be unsuitable for treatment with hsp inhibiting drugs due to the likelihood of low efficacy and the selection of hsp negative melanoma cells.

Similar studies using melanoma tissue have reported hsp90 and hsp70 to be expressed in a minority of tumour cells and for hsp90 to show nuclear localisation [117, 123], but the results from this study are not in accordance with these previous reports. Although a number of studies have shown the expression of hsp70 and hsp90 to be up-regulated in melanoma tissue relative to melanocytic naevi or melanocytes [115, 116, 118, 120], to the best of the author's knowledge, this is the first report that hsp90, hsp70, hsp60 and hsp40 are increased in expression in melanoma cells relative to neighbouring non-transformed cells. Hsp32, the inducible form of the haem

oxygenase enzyme, was observed to be widely expressed in melanoma tissue as analysed by Western blotting but in the majority of samples did not show significantly higher expression in MelanA+ cells by comparison with MelanA- cells using immunofluorescence. This contrasts with a previous study using immunohistochemistry which indicated hsp32 to be up-regulated in prostate cancer tissue [209], but since it was observed to be widely expressed it may act as a pro-survival protein as it may in mastocytosis [208]. The roles that these hsps play in cancer may thus vary depending on the biological context, as has been previously demonstrated for other cancer types [84].

In agreement with previous studies, the current data demonstrated that hsps play important roles in melanoma. It is evident that larger scale follow-up studies are required to confirm these results and to provide definitive evidence of the significance of hsp expression with respect to patient clinical parameters. Furthermore, the results presented in this study are indicative of a key role for hsps in melanoma tumours as they were observed to be widely expressed and were related to patient clinical parameters and point to the putative clinical application of hsp inhibitors for the treatment of cancer. Furthermore, these results indicate that hsp inhibitors may not be an efficacious form of therapy in all patients and that their use should be accompanied with prior assessment of intra-tumoural levels of hsp expression.

Chapter 8: The role of hsp90 in T cell recognition of tumour cells

8.1 Introduction

The hsp90 molecular chaperone is multi-faceted. Although originally identified as a stress inducible protein, it was later recognised to play ~~"h~~ouse keeping" roles in a range of diverse cellular processes [36]. This is achieved by the ability of hsp90 to chaperone a large number of client proteins, with current estimates of over 100 client proteins [37]. Consequently, hsp90 is essential for multiple cellular pathways and processes including proliferation, differentiation, protein folding, degradation and translocation [35]. In addition, hsp90 is required for immune system functions such as interferon signalling and antigen presentation [68, 75].

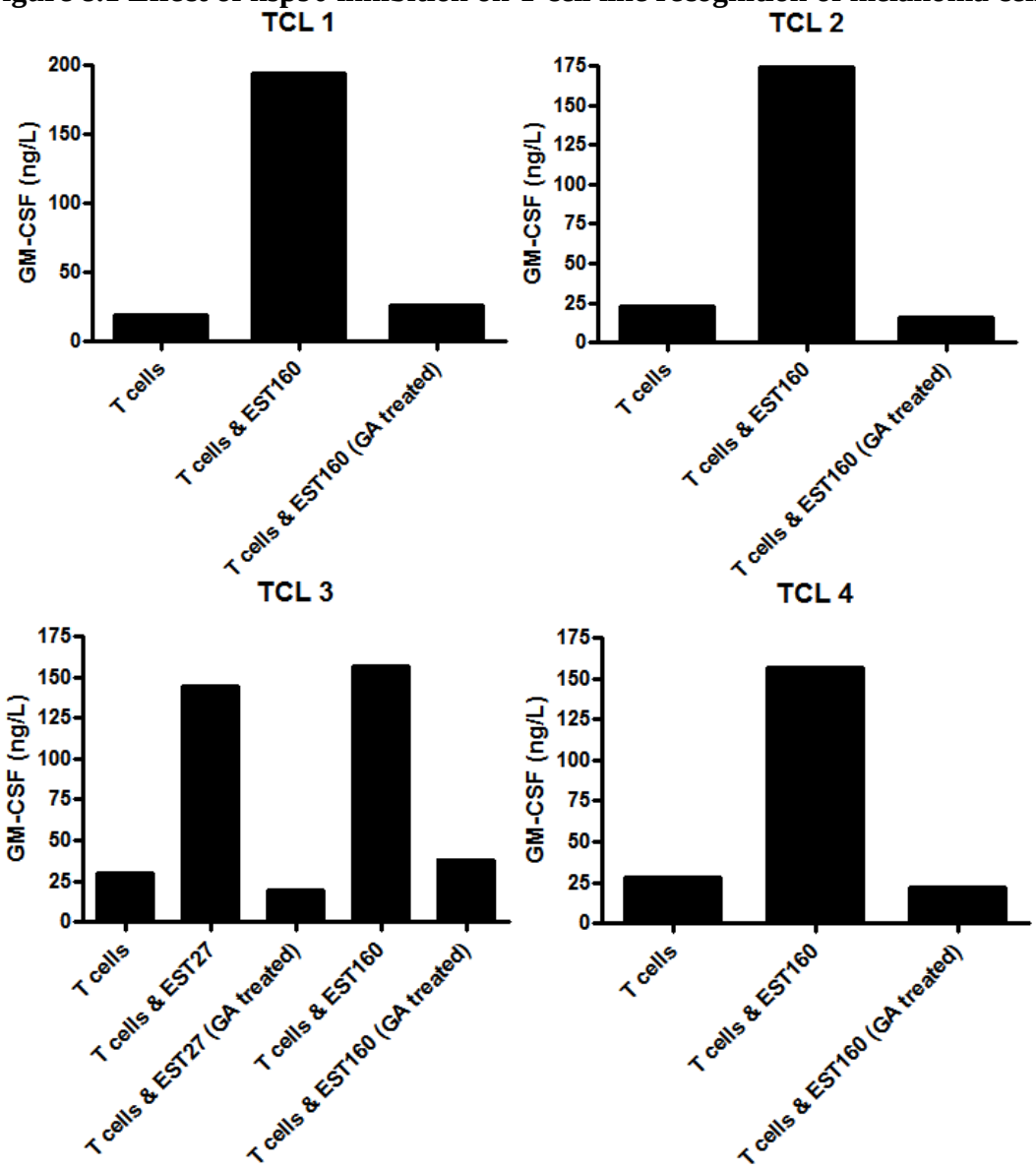
A number of studies have examined the role of hsp90 in direct antigen presentation, but the results from these studies are conflicting. For example, inhibition of hsp90 function in target cells has been shown to both abrogate and enhance T cell recognition [75, 76]. It was the aim of this study to examine the role of hsp90 in direct antigen presentation using melanoma cell lines.

8.2 Results

In order to investigate the role of hsp90 in T cell recognition, T cell lines were generated that recognise melanoma tumour cell lines. Four T cell lines were tested in co-culture recognition experiments with melanoma cells. Two T cell lines (TCL1 and TCL2) were derived from CD4+ T cells isolated from PBMCs, and two (TCL3 and TCL4) from this CD4-depleted fraction. In these experiments, melanoma cell lines (EST27, EST160) were either pre-treated with the hsp90 inhibitor geldanamycin or left untreated. All four T cell lines produced significant amounts of the cytokine GM-CSF when cultured with melanoma cells in the absence of geldanamycin, indicating that the T cells had recognised the melanoma cells (EST27 only tested with TCL3).

In contrast, these T cell lines did not produce significant levels of GM-CSF when cultured with melanoma cells pre-treated with geldanamycin (Fig. 8.1).

Figure 8.1 Effect of hsp90 inhibition on T cell line recognition of melanoma cells

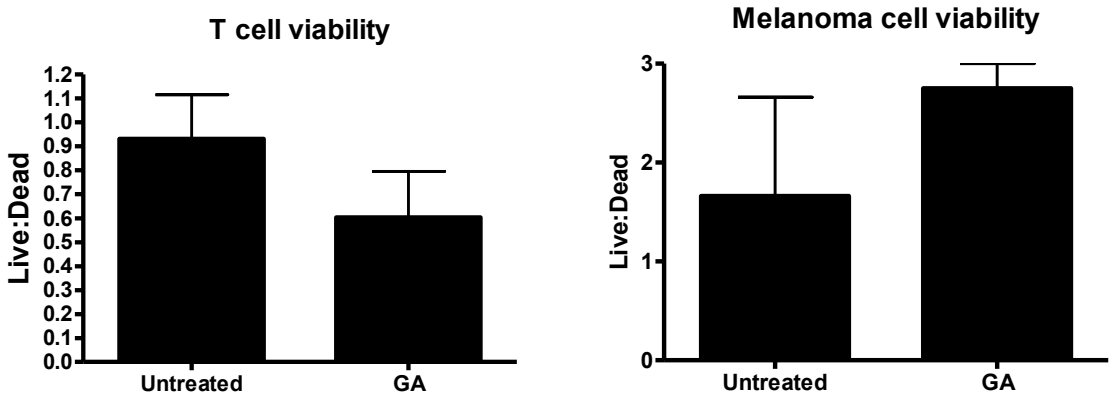


Geldanamycin inhibits recognition of melanoma cells by T cells. T cell lines were generated by incubating CD4+ (TCL1 and TCL2) and CD4-depleted PBMCs with peptides derived from the NY-ESO antigen. After two rounds of stimulation with the peptide, they were tested for recognition of melanoma cell lines in 48 hr co-culture recognition experiments at a target to effector cell ratio of 10:1. Melanoma cell lines underwent 12 hr pre-treatment with 20 µM geldanamycin or were left untreated. After 48 hr, cell-free supernatant was removed and tested for GM-CSF using ELISA. Repeated experiments produced comparable results.

TCL, T cell line
EST, Melanoma cell line
GA, Geldanamycin

Since pre-treating melanoma cells with geldanamycin was observed to abrogate T cell recognition, geldanamycin cytotoxicity may have reduced cell viability and may explain these observations. To investigate this, T cells and melanoma cells were assessed with the trypan blue exclusion method following 48 hr co-culture and the ratio of live to dead cells in the geldanamycin and the untreated conditions were compared. Live to dead cell ratios were not significantly different between conditions for T cells or melanoma cells (T cells in the geldanamycin culture condition showed a live to dead ratio of approximately 0.6:1, and 0.9:1 without geldanamycin ($P > 0.2$), while melanoma cells showed viability ratios of approximately 1.8:1 (untreated) and 2.9:1 (geldanamycin) (Fig. 8.2).

Figure 8.2 Effect of pre-treating melanoma cells with geldanamycin on T cell and melanoma cell viability in co-culture experiments

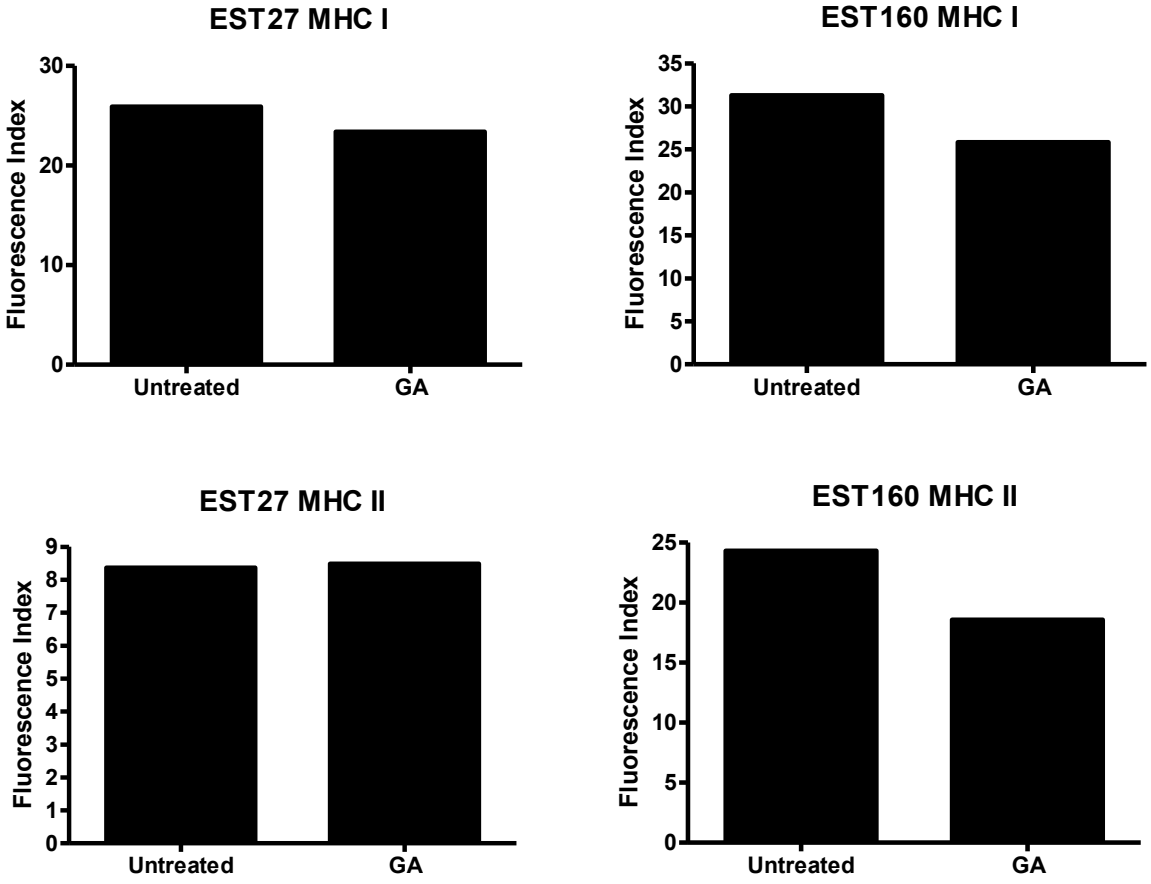


Following 48 hr of T cell line and melanoma cell co-culture, the ratio of live to dead cells was determined using the trypan blue exclusion method. Representative results shown.
GA, Geldanamycin

Given that reduced viability was not the cause of the loss of T cell recognition, further experiments were designed in order to elucidate the mechanism of abrogation of T cell recognition. In order for a target cell to be effectively recognised by a T cell, it must present a relevant MHC-bound peptide on its cell surface. Since hsps are known to interact with MHC proteins [143], and considering the data presented in this thesis from three other melanoma cell lines (Table 6.2), it was hypothesised that geldanamycin resulted in reduced expression of MHC proteins on melanoma cells. To test this, flow cytometry was used to evaluate MHC protein

expression following co-culture experiments. Geldanamycin treatment was observed to result in modest or no down-regulation (EST27, MHC II) of MHC I and II proteins in both cell lines EST27 and EST160 (Fig. 8.3). Similar results were obtained after 48 hr subsequent to geldanamycin treatment, suggesting this effect did not diminish for the duration of the co-culture experiments (data not shown).

Figure 8.3 Effect of geldanamycin on melanoma cell line MHC expression



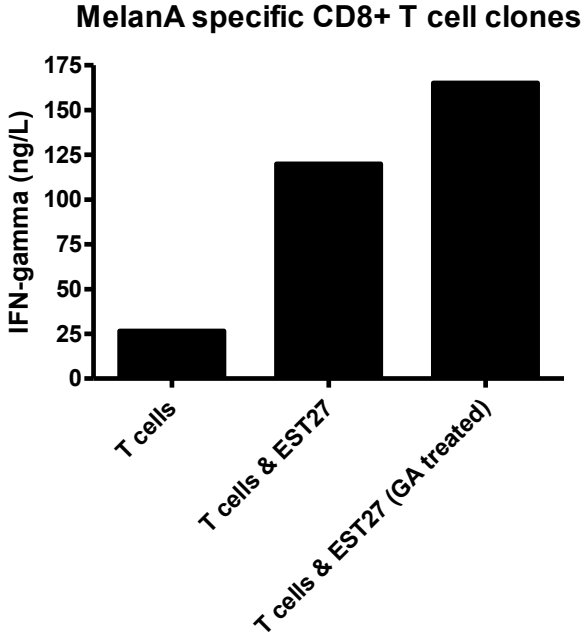
Melanoma cells were pre-treated for 12 hr with 20 μM geldanamycin or were left untreated. Cells were harvested and stained for MHC I and II protein expression with fluorescently-labelled antibodies and analysed by flow cytometry.

GA, Geldanamycin
EST, Melanoma cell line

The effect of hsp90 inhibition was then investigated using interferon-γ-producing CD8+ T cell clones that recognise a peptide derived from MelanA [221], a melanoma differentiation antigen recognised by T cells [222]. Repeating the original co-culture experiments showed that in contrast

to the T cell lines, pre-treating melanoma cell lines with geldanamycin did not result in abrogation of recognition (Fig. 8.4). This result also confirmed the observation that pre-treating melanoma cells with geldanamycin did not reduce melanoma or T cell viability in co-culture experiments.

Figure 8.4 Effect of hsp90 inhibition on recognition of melanoma cells by a MelanA-specific CD8+ T cell clone



Geldanamycin does not inhibit recognition of melanoma cells by MelanA-specific T cell clones. Interferon-γ-producing MelanA-specific CD8+ T cell clones were tested in 48 hr co-culture experiments at a target to effector cell ratio of 10:1. Melanoma cell lines underwent 12 hr pre-treatment with 20 μM geldanamycin or were left untreated. After 48 hr, cell-free supernatant was removed and tested for IFN-γ using an ELISA.

8.3 Discussion

Hsp90 has been widely studied in a variety of biological contexts, yet much remains to be learnt about this protein. With respect to the role of hsp90 in antigen presentation, two opposing roles have been demonstrated; that hsp90 inhibition in target cells results in abrogation of T cell recognition, or that hsp90 inhibition may result in improved T cell recognition [75-77]. Despite these conflicting reports, both of these functions may be genuine observations; these studies may serve to hint at the complexity that this protein plays in antigen presentation. The data presented in this study confirms the complexity of hsp90 function in antigen presentation.

Using T cell lines as effector cells, inhibiting hsp90 with geldanamycin in target melanoma cells abrogated T cell recognition, yet using T cell clones as effector cells, inhibiting hsp90 in the same melanoma cells did not perturb T cell recognition. This is the first report of the duality of the role of hsp90 in T cell recognition within the same study. Since these T cell lines are derived from isolated fractions, they are most likely predominately CD4+ and CD8+ cells. This is the first suggestion that hsp90 inhibition results in abrogation of both CD4+ and CD8+ T cell recognition in melanoma. Geldanamycin, the hsp90 inhibitor used in this study, was seen to result in a modest reduction of MHC proteins on melanoma cells, and therefore it seems unlikely that this was the cause of the abrogation of T cell recognition. Since MHC molecules are only present on the cell surface as folded, peptide-bound molecules, this suggests that inhibiting hsp90 resulted in reduced levels of peptide presented on the cell surface. Hsp90 may thus be involved in peptide processing and the loss of T cell recognition observed in this study may have been due to the absence of peptide on the cell surface. Indeed, this has been shown to be the mechanism of loss of T cell recognition caused by hsp90 inhibiting drugs [75]. This is supported by studies that suggest hsps are involved in the transport of antigenic peptides to MHC molecules [210].

Yet this hypothesis does not adequately explain the observations in this study since hsp90 inhibition did not have an effect on the ability of MelanA+ T cell clones to recognise target cells. Since only a modest reduction in MHC molecule expression was caused by hsp90 inhibition, it may be that hsp90 chaperones a subset of MHC-bound peptides, and that it is the MHC loading of only these peptides that are inhibited by geldanamycin. These results therefore suggest the peptide(s) that the T cell lines recognise are chaperoned by hsp90, whereas the peptide that the MelanA-specific T cell clones recognise are not chaperoned by hsp90.

A study by other investigators has shown hsp90 inhibition to result in improved T cell recognition, and that this is due to degradation of the parent protein from which the peptide that the T cells recognise is derived [76]. In that study, hsp90 inhibition was demonstrated to cause a loss of chaperoning and to induce degradation of the client protein. This in turn probably resulted in greater peptide presence on the cell surface. Therefore any protein degraded by hsp90 inhibition (the majority of which will be hsp90 client proteins) is likely to result in improved recognition by T cells that recognise peptides derived from this protein. It is not known if MelanA is an hsp90 client protein, and this remains to be confirmed or otherwise before any definitive conclusions can be drawn from the present observations. For the remaining peptides presented on the cell surface, the effect of hsp90 inhibition is likely to be dependent on the role of hsp90 in loading the peptide to the MHC, since hsp90 may only chaperone a subset of peptides to MHC molecules. However, it remains a possibility that all peptides associate with hsp90 in their loading to MHC molecules [75, 211]. If this is the case, hsp90 inhibition will prevent the normal loading of a range of peptides to MHC molecules, but peptides that increase in frequency after hsp90 inhibition (i.e. derived from hsp90 client proteins) may, due to their increased abundance, increase in frequency on the cell surface. The specific effects of hsp90 on antigen presentation are not as yet understood, therefore further studies should be focused on more clearly defining the

relationship between hsp90 and the loading of the spectrum of peptides to MHC molecules and the consequences for T cell recognition.

Defining the role of hsp90 in T cell recognition has important consequences for clinical use of hsp90 inhibitors. These drugs are used to disrupt proliferating cancer cells by inhibiting the chaperone function of hsp90. If patients treated with these inhibitors possess T cells with cytotoxic activity against peptides that derive from hsp90 client proteins present in their tumours, a secondary therapeutic benefit may result through improved immune response against these tumours. On the other hand, if there are T cells that recognise peptides derived from non-hsp90 client proteins on their tumour, their activity may be impaired. These factors should be considered as part of the clinical evaluation of hsp90 inhibiting drugs.

Chapter 9: Conclusions and Future Directions

Cancer is characterised by a number of features that result in an aberrant biological condition. In order for this inherent abnormality to exist, distinct genetic, biochemical and physiological perturbations are required. In the year 2000 Hanahan and Weinberg published a landmark paper that considered the body of cancer research to date [80]. In this paper the authors proposed six essential "hallmark" features of cancer: self sufficiency in growth signals, apoptosis evasion, limitless replication potential, sustained angiogenesis, tissue invasion and metastasis and insensitivity to anti-growth signals. It has long been known that hsps play important roles in cancer, and as the investigation of molecular chaperones in this context became more extensive, they were identified as participating in all of the hallmark features proposed by Hanahan and Weinberg. Improved understanding of cancer biology in the ensuing years resulted in the publication in 2011 of a substantially updated review by Hanahan and Weinberg [212] in which two additional emerging hallmark features were proposed: evading immune destruction and reprogramming energy metabolism. This revised perspective encompassing these eight features is proposed to occur within an enabling environment of genetic instability and inflammation.

Hsps also contribute to these more recently proposed hallmark features of cancer. For example, hsps can mask deleterious genetic mutations and this may result in buffering the negative effects of genetic instability while allowing potentially advantageous mutations to arise [38, 83]. Hsps also play roles in maintaining pro-inflammatory networks, chaperone proteins involved in energy metabolism and provide resistance to immune system attack [68, 140, 213, 214]. The body of molecular chaperone and cancer research to date has shown hsps and cancer to be intimately linked, implicating this group of proteins as an essential and intimately associated feature of cancer biology. The diverse mechanisms by which hsps contribute to cancer are primarily achieved through their ability to chaperone a diverse set of client proteins. These roles are

complemented by other functions of hsps such as providing protection against tumour-associated stressors, buffering negative genetic mutations, assisting the function of mutated and overexpressed proteins that support cancer growth, amongst others. This thesis contributes to our understanding of molecular chaperone cancer research by taking a broad and multidisciplinary approach in the context of two of the most common human cancers.

In a preliminary study, breast cancer tissues (n = 30) were demonstrated by Western immunoblotting to widely express hsps 90 and 70. Two-dimensional gel electrophoresis indicated that a number of proteins were differentially expressed in tumour and healthy breast tissue from the same patient. These results suggest that a number of possibly unidentified proteins may play important roles in breast cancer and thus have use as therapeutic targets or biomarkers. The role of hsp90 and associated client proteins in breast cancer was further investigated by non-denaturing immunoprecipitation followed by elution with geldanamycin, a specific inhibitor of hsp90. Geldanamycin-sensitive hsp90 client proteins were observed in seven of 11 protein extracts from breast cancer patients and one healthy individual. Immunoprecipitation, Western immunoblotting and LC-MS identified hsps 40, 56/FKBP52, 60, 70, 105 and lumican as potential hsp90 client proteins. These proteins may thus assist breast cancer progression alongside hsp90. In one patient sample, a cancer-specific group of proteins was identified, while in all experiments geldanamycin resistance was observed. The results of this study may have relevance for the future of breast cancer research and clinical treatment. To expand on the present study, comparisons could be made between the identity of hsp90 client proteins in breast tissue and those found in other cancerous tissues and corresponding normal tissues. Non-denaturing SDS-PAGE could be used to characterise the associations of chaperone complexes within this group of proteins. Ideally, this should be accompanied with assessment of the effects of hsp inhibitors alone and in combination with existing chemotherapeutic drugs *in vivo*. Assessment of differences in the expression and

association of chaperone complex group members would assist in revealing the mechanism of hsp90 function and in turn may lead to the development of improved hsp inhibitors.

Cell surface proteins involved in cell adhesion, apoptosis, antigen presentation as well as hsps were investigated for their role in melanoma metastasis. In addition, the influence of co-culture with stromal cells was investigated as a potential model system for *in vivo* growth conditions. Matched sets of primary-derived versus metastasis-derived melanoma cell lines were cultured and screened for the expression of CD44, CD54, CD95, CD155, MHC 1, hsps 90, 70, 60, 40 and 32. In some instances, differential protein expression was observed in metastasis-derived cell lines as compared to lines derived from primary tumours and, although a number of consistent changes in protein expression were observed within the cell lines, these were not exclusively associated with primary or metastatic tumour origin. To expand on this preliminary study, advances could be made to the development of an *in vivo* cell culture model. The inclusion of additional cell types such as stromal and immune cells cultured under physiological oxygen levels might be considered. Using such a model system, these original experiments may be repeated and cell lines screened for the intracellular and cell surface expression of proteins that may be important for the metastatic process such as those involved in cell adhesion, cell migration, signal transduction as well as chemokine, mitogen and apoptosis receptors. The modification of biological or physical growth conditions is likely to be important in an attempt to re-create *in vivo* growth conditions, but this may not be possible with existing cell culture methods since these conditions may vary temporally and regionally within a tumour [212]. To control for the influences that the cell culture environment may induce on cancer cell cultures, comparisons of hsp expression between freshly isolated tumour cells and those cells established as a cell line could be made. Manipulation of the culture environment in the manner previously described would enable the determination of the conditions that most accurately reflect those *in vivo* with respect to hsp expression.

Hypoxia is a well established characteristic of cancer cells. Paradoxically, *in vitro* studies on cancer cell lines are routinely performed under hyperoxic conditions. In this study, melanoma cell lines were cultured under high (20 % O_2, n = 42) and low (2 % O_2, n = 18) oxygen tension and monitored for the expression of hsps 90, 70, 60, 40 and 32. Total higher expression of hsps 90, 70 and 60 correlated with improved viability in low ($P < 0.05$) but not high oxygen tension. Relative hsp expression was consistent across the cohort of cell lines and the expression levels of hsps 90, 70, 60 and 40 correlated with one another ($P = 0.0001$), but not with hsp32. Expression of hsp90 was associated with cell line adhesion to collagen type IV and laminin ($P < 0.05$). Expression of hsp90 and hsp40 correlated with Breslow depth of the primary tumour from which these metastatic tumour cell lines were derived ($P < 0.04$), however, hsp expression was not correlated with other clinical parameters including Clark level or patient survival. Interestingly, all hsps were identified on the cell surface and these proteins may thus play roles in tissue invasion, metastasis and immunological recognition. In future studies, culturing of melanoma and other tumour cell lines under a wider range of oxygen concentrations ranging from hypoxic to hyperoxic could be accomplished. Cell lines could then be monitored for the expression of cell surface and intracellular hsps and correlated with cell line characteristics as performed here. Additionally, the functional consequences of these hsps could be examined by their inhibition by siRNA or overexpression by transfection.

Using Western immunoblotting to examine the expression of hsp90, hsp70, hsp60, hsp40 and hsp32 in uncultured metastatic melanomas (n = 32) demonstrated that these proteins are widely expressed in melanoma tumour tissue. Correlating the expression of these hsps with patient clinical parameters showed that increased hsp90 ($P < 0.02$) and hsp40 ($P < 0.03$) expression was correlated with progression to advanced tumour stage (stage III to stage IV), higher hsp90 expression correlated with reduced patient follow-up time ($P < 0.04$) (survival since removal of the metastatic tumour that was therein examined) and hsp70 expression was associated with sex of

the patient ($P < 0.05$). On the other hand, expression of the other hsps was not associated with any recorded patient clinical parameters. Fluorescence microscopy of whole melanoma tissues using the MelanA antigen as a specific marker for melanoma cells demonstrated increased expression of hsps 90, 70, 60, 40 and 32 in MelanA-positive cells compared to adjacent MelanA-negative (non-melanoma) cells. These data contribute to the proposal that hsps are valid therapeutic targets in the treatment of melanoma. Further experiments should be concerned with the identification of these MelanA-negative cells since they may be a non-transformed cell type such as tumour-associated fibroblasts that support tumour growth [212, 227]. In order to more accurately determine the relevance of hsps to cancer patient parameters and outcome, Western immunoblotting experiments could be expanded with antibodies specific to post-translationally modified states of the hsps. Given that functional activity of hsps is known to be altered by post-translational modifications (e.g. phosphorylation), there may be a discrepancy between the functional state of the hsps independent from the level of hsp expression.

The role of hsp90 in the immunological recognition of cancer cells was investigated by treating target melanoma cells with the hsp90 inhibitor geldanamycin and using T cell lines and T cell clones as effector cells. Geldanamycin treatment was observed to abrogate the recognition of melanoma cells by T cell lines. In contrast, under identical conditions, T cell clones were able to recognise geldanamycin-treated melanoma cells. These data allude to the complexity of the hsp90 molecular chaperone and may have consequences for the rarely considered immunological aspects of hsp90 inhibitors used clinically. This study, and others, have shown that hsp90 inhibition may abrogate or improve T cell recognition of target cancer cells, however the mechanism of this duality has not been resolved. Similarly, many of the details of the effect of hsp90 inhibition on T cell recognition are unknown. Future studies could be directed towards *in vitro* characterisation of the effect of hsp90 inhibition and its consequences for the recognition of different CD4+ and CD8+ T cells specific to a number of peptide sequences derived from the same and different

parent proteins should be performed. Subsequently, tumour-directed T cell responses in cancer patients treated with hsp90 inhibitors could be monitored in order to assess the immunological consequences of hsp90 inhibitors *in vivo*. These experiments should be accompanied with an investigation into the mechanistic effect of hsp90 inhibition on antigen presentation, for example, identification of hsp90 client proteins at each stage of peptide processing until its eventual presentation on the cell surface, in a model system with and without hsp90 inhibition. Differences in client protein association could then be investigated for their potential role in peptide processing.

One of the most consistent observations throughout this course of research was the heterogeneity in the results, reflective perhaps of the multi-dimensional and complex nature of cancer. This study showed that hsps are widely expressed in tumour tissues, but that they are expressed in non-malignant tissues and cells as well. The expression of hsps was demonstrated to correlate with patient clinical parameters, but was dependent on whether tissue or cell lines were examined, even within the same cancer type. Despite these differences, similarities in the relative proportion of hsp expression were found between these tissues and cell lines of the same cancer type. Further complexities were revealed in other investigations. Characterising hsp90 client proteins in tumour and normal breast tissue showed no differences between these tissue types, and yet one patient sample was observed to show a different set of hsp90 client proteins, and yet another sample showed hsp-dependent differences in the association of client proteins, and other patient samples showed no client proteins at all. This complex theme continued when probing the role of hsp90 in T cell recognition of target melanoma cells. Depending on the T cell type used, hsp90 inhibition in target cells was observed to either have no effect or to abrogate T cell recognition. These findings, taken place in different experimental settings but part of a single study, further revealed the complexity of the molecular chaperone system.

The present studies employed a diverse range of experimental approaches to examine the role of hsps, hsp-client proteins and cell surface proteins in a wide range of human cancer cells, including breast cancer, melanomas and related cell lines. A number of novel experimental approaches were developed which included screening of hsp expression in matched primary and metastatic tumour cell lines, the adoption of cancer cell line culture under conditions of low oxygen tension, immunofluorescence to identify hsp expression in melanoma and adjacent non-melanoma cells *in situ* and attempts to relate hsp expression to patient clinical parameters. The overall outcomes of these studies have clearly demonstrated a critical role for hsps in cancer pathogenesis and in the immune recognition of cancer cells. Moreover, these studies have provided fresh evidence that hsps provide a valid therapeutic target in the treatment of cancers and may have consequences for the development of hsp inhibiting drugs and for the management of patients treated with these drugs. Nevertheless, many of the present results were equivocal and contradicted some previous studies. These observations simply confirm our limited understanding of the complexities of cancer pathogenesis and the hsp molecular chaperone system.

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Appendix III

1.

Research Development & Integrity
Research Services
Armidale, NSW 2351, Australia
Telephone: 02 6773 3449
Facsimile: 02 6773 3543
http://www.une.edu.au/research-services\ethics
E-mail: jo-ann.sozou@une.edu.au

HUMAN RESEARCH ETHICS COMMITTEE

MEMORANDUM TO: A/Prof G Lloyd Jones & Mr C Shipp
School of Science & Technology

This is to advise you that the Human Research Ethics Committee has approved the following:

PROJECT TITLE: Protein interactions in human breast cancer.

COMMENCEMENT DATE: 03/09/2007

COMMITTEE APPROVAL No.: HE07/145

APPROVAL VALID TO: 31/12/2010

COMMENTS: Nil. Conditions met in full.

The Human Research Ethics Committee may grant approval for up to a maximum of three years. For approval periods greater than 12 months, researchers are required to submit an application for renewal at each twelve-month period. All researchers are required to submit a Final Report at the completion of their project. The Progress/Final Report Form is available at the following web address: http://www.une.edu.au/research-services/ethics/human-ethics/hrecforms.php

The *NHMRC National Statement on Ethical Conduct in Research Involving Humans* requires that researchers must report immediately to the Human Research Ethics Committee anything that might affect ethical acceptance of the protocol. This includes adverse reactions of participants, proposed changes in the protocol, and any other unforeseen events that might affect the continued ethical acceptability of the project.

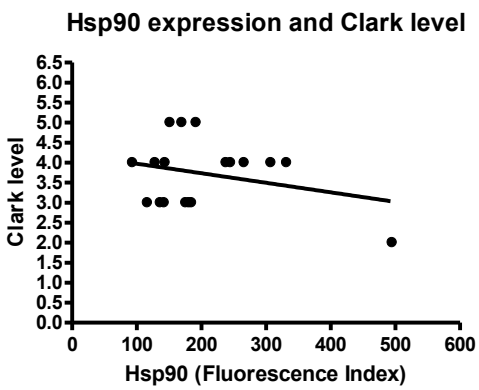
In issuing this approval number, it is required that all data and consent forms are stored in a secure location for a minimum period of five years. These documents may be required for compliance audit processes during that time. If the location at which data and documentation are retained is changed within that five year period, the Research Ethics Officer should be advised of the new location.

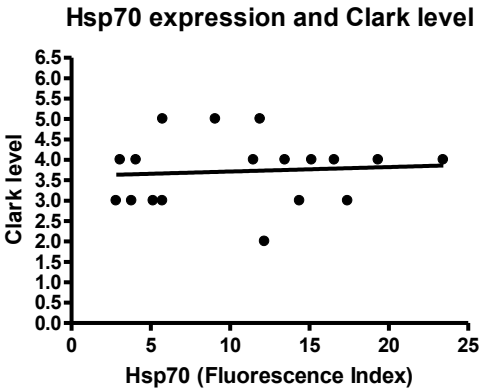
[redacted]

Jo-Ann Sozou
Secretary

30/10/2008

Appendix IV


Hsp90 expression and Clark level
Clark level
Hsp90 (Fluorescence Index)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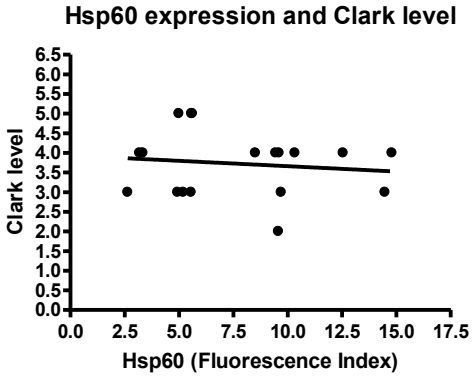
Clark level
Hsp70 (Fluorescence Index)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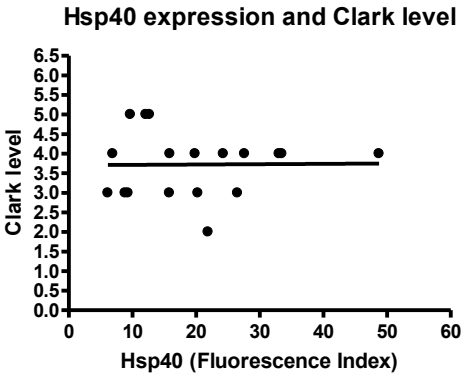
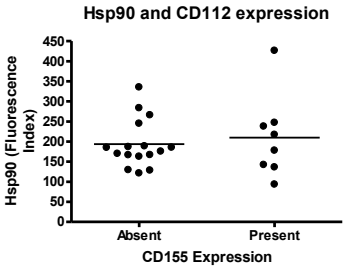
Clark level
Hsp60 (Fluorescence Index)


Hsp40 expression and Clark level
Clark level
Hsp40 (Fluorescence Index)

Appendix V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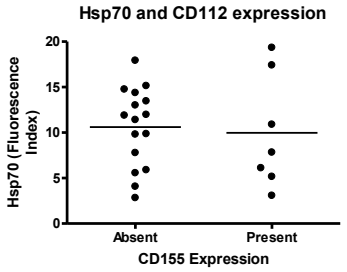
Hsp90 (Fluorescence Index)
Absent
Present
CD155 Expression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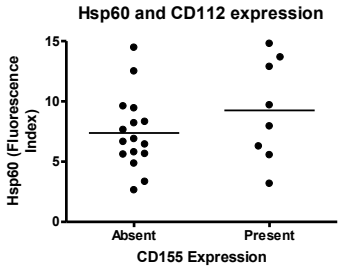
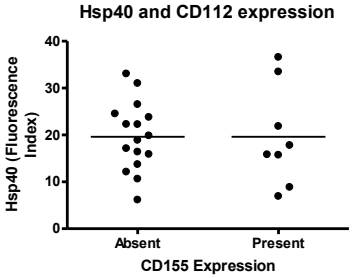
Hsp70 (Fluorescence Index)
Absent
Present
CD155 Expression


Hsp60 and CD112 expression
Hsp60 (Fluorescence Index)
Absent
Present
CD155 Expression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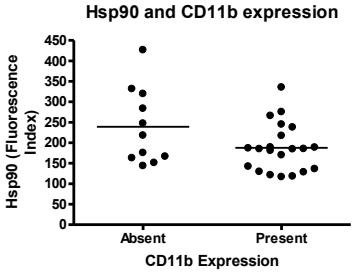
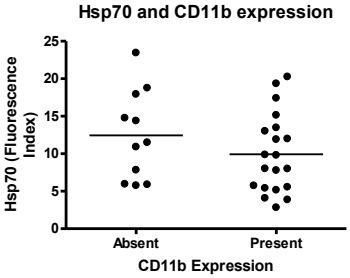
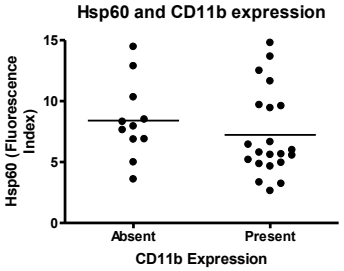
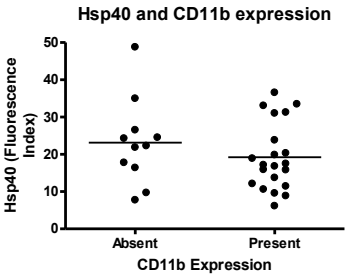
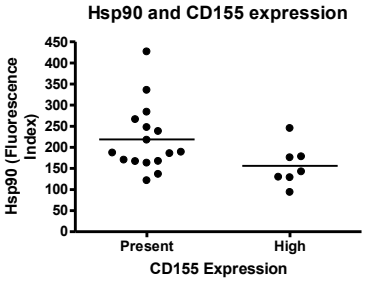
Hsp40 (Fluorescence Index)
Absent
Present
CD155 Expression


Hsp90 and CD11b expression
Hsp90 (Fluorescence Index)
Absent
Present
CD11b Expression


Hsp70 and CD11b expression
Hsp70 (Fluorescence Index)
Absent
Present
CD11b Expression


Hsp60 and CD11b expression
Hsp60 (Fluorescence Index)
Absent
Present
CD11b Expression


Hsp40 and CD11b expression
Hsp40 (Fluorescence Index)
Absent
Present
CD11b Expression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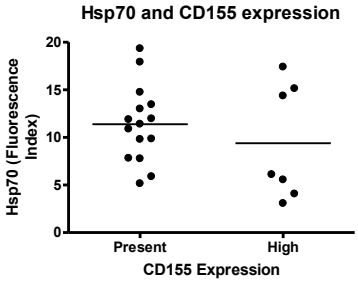
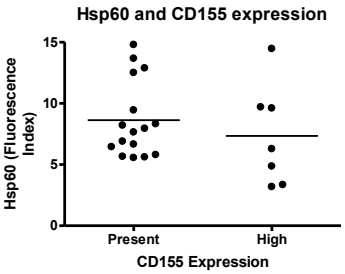
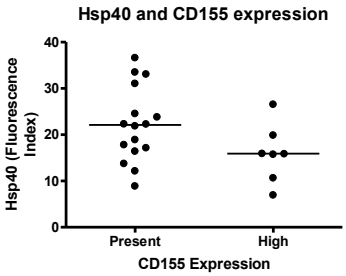
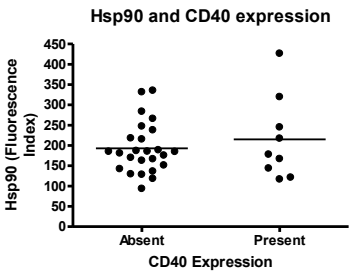
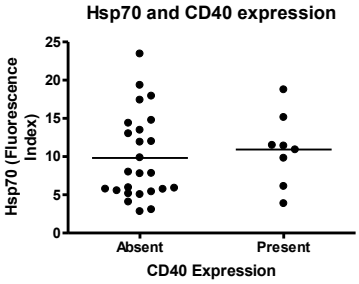
Hsp90 (Fluorescence Index)
Present
High
CD155 Expression

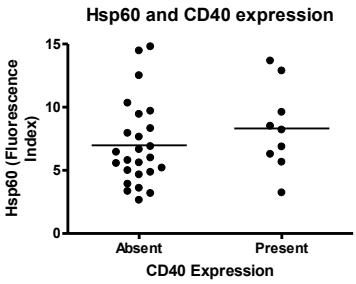

Hsp70 and CD155 expression
Hsp70 (Fluorescence Index)
Present
High
CD155 Expression

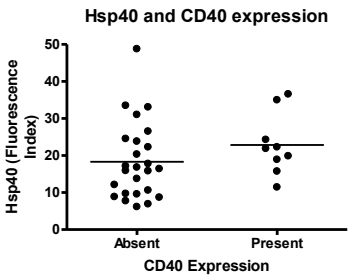

Hsp60 and CD155 expression
Hsp60 (Fluorescence Index)
Present
High
CD155 Expression

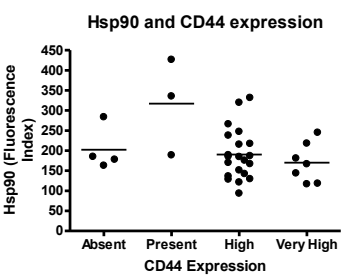

Hsp40 and CD155 expression
Hsp40 (Fluorescence Index)
Present
High
CD155 Expression

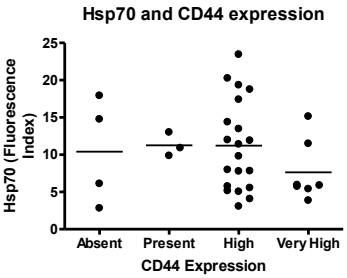

Hsp90 and CD40 expression
Hsp90 (Fluorescence Index)
Absent
Present
CD40 Expression

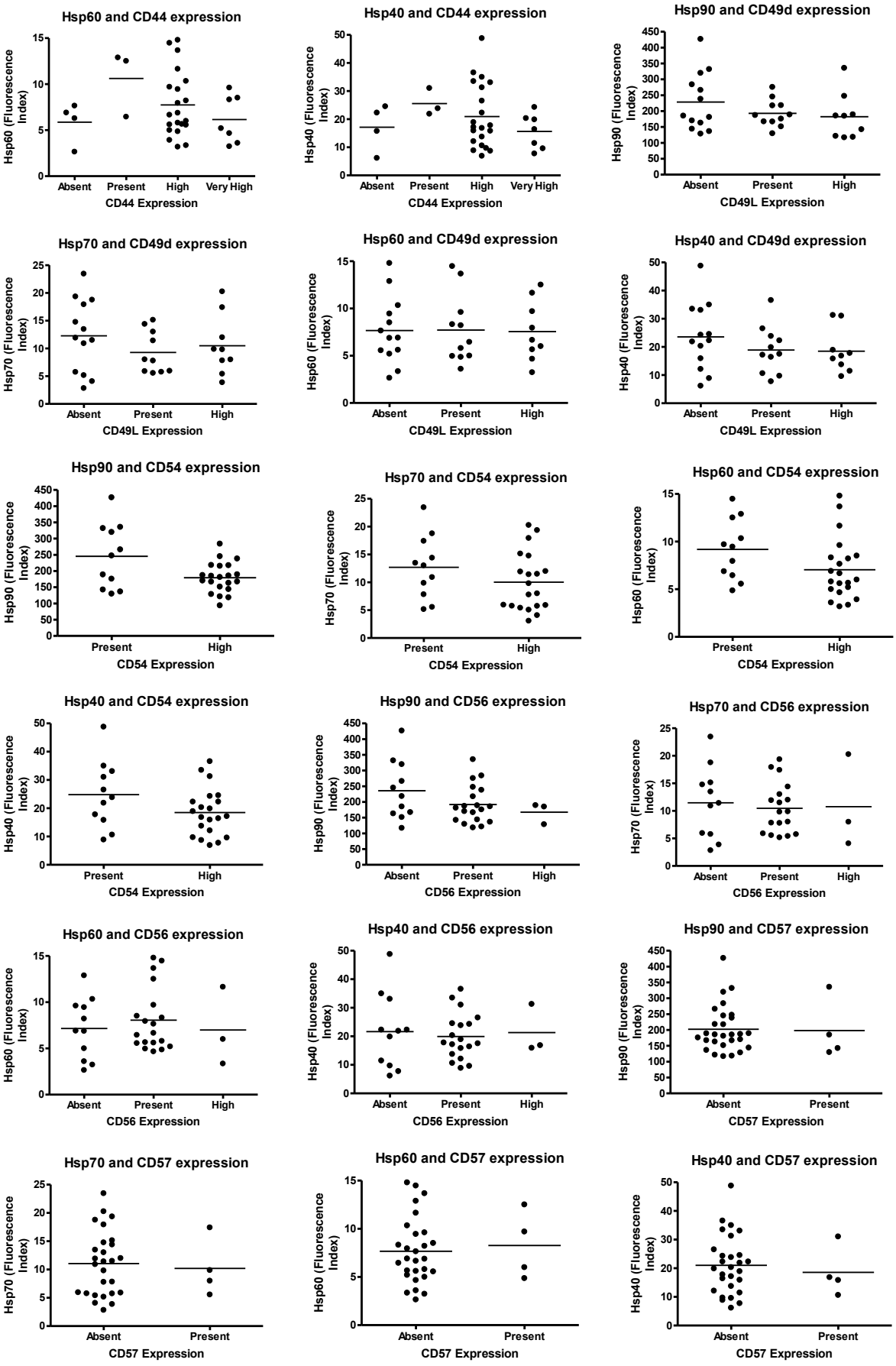

Hsp70 and CD40 expression
Hsp70 (Fluorescence Index)
Absent
Present
CD40 Expression

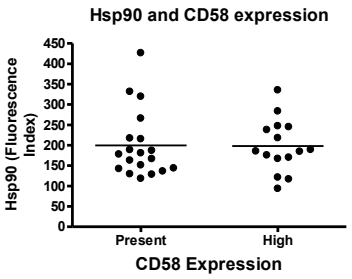

Hsp60 and CD40 expression
Hsp60 (Fluorescence Index)
Absent
Present
CD40 Expression

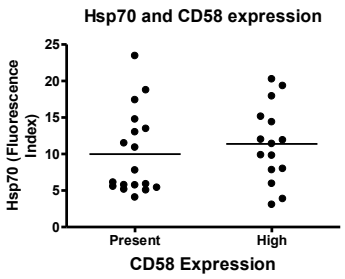

Hsp40 and CD40 expression
Hsp40 (Fluorescence Index)
Absent
Present
CD40 Expression

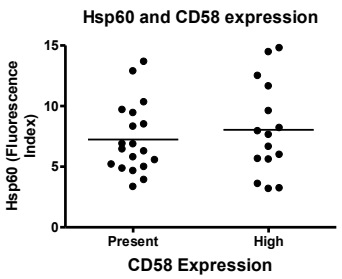

Hsp90 and CD44 expression
Hsp90 (Fluorescence Index)
Absent
Present
High
Very High
CD44 Expression

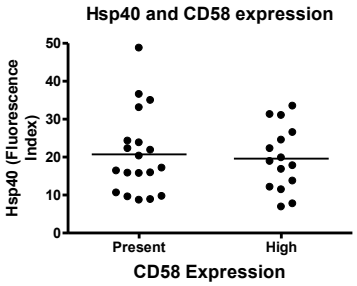

Hsp70 and CD44 expression
Hsp70 (Fluorescence Index)
Absent
Present
High
Very High
CD44 Expression

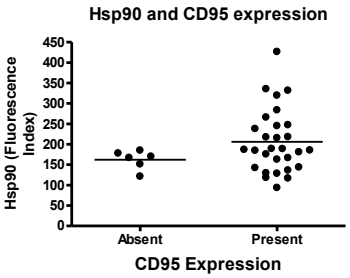

Hsp60 and CD44 expression
Hsp60 (Fluorescence Index)
Absent
Present
High
Very High
CD44 Expression
Hsp40 and CD44 expression
Hsp40 (Fluorescence Index)
Absent
Present
High
Very High
CD44 Expression
Hsp90 and CD49d expression
Hsp90 (Fluorescence Index)
Absent
Present
High
CD49L Expression
Hsp70 and CD49d expression
Hsp70 (Fluorescence Index)
Absent
Present
High
CD49L Expression
Hsp60 and CD49d expression
Hsp60 (Fluorescence Index)
Absent
Present
High
CD49L Expression
Hsp40 and CD49d expression
Hsp40 (Fluorescence Index)
Absent
Present
High
CD49L Expression
Hsp90 and CD54 expression
Hsp90 (Fluorescence Index)
Present
High
CD54 Expression
Hsp70 and CD54 expression
Hsp70 (Fluorescence Index)
Present
High
CD54 Expression
Hsp60 and CD54 expression
Hsp60 (Fluorescence Index)
Present
High
CD54 Expression
Hsp40 and CD54 expression
Hsp40 (Fluorescence Index)
Present
High
CD54 Expression
Hsp90 and CD56 expression
Hsp90 (Fluorescence Index)
Absent
Present
High
CD56 Expression
Hsp70 and CD56 expression
Hsp70 (Fluorescence Index)
Absent
Present
High
CD56 Expression
Hsp60 and CD56 expression
Hsp60 (Fluorescence Index)
Absent
Present
High
CD56 Expression
Hsp40 and CD56 expression
Hsp40 (Fluorescence Index)
Absent
Present
High
CD56 Expression
Hsp90 and CD57 expression
Hsp90 (Fluorescence Index)
Absent
Present
CD57 Expression
Hsp70 and CD57 expression
Hsp70 (Fluorescence Index)
Absent
Present
CD57 Expression
Hsp60 and CD57 expression
Hsp60 (Fluorescence Index)
Absent
Present
CD57 Expression
Hsp40 and CD57 expression
Hsp40 (Fluorescence Index)
Absent
Present
CD57 Expression

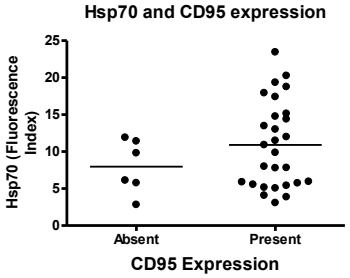

Hsp90 and CD58 expression
Hsp90 (Fluorescence Index)
Present
High
CD58 Expression

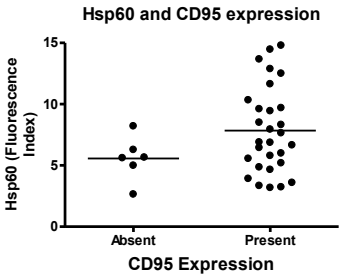

Hsp70 and CD58 expression
Hsp70 (Fluorescence Index)
Present
High
CD58 Expression

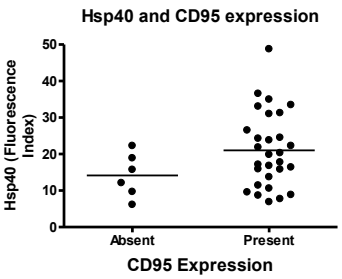

Hsp60 and CD58 expression
Hsp60 (Fluorescence Index)
Present
High
CD58 Expression

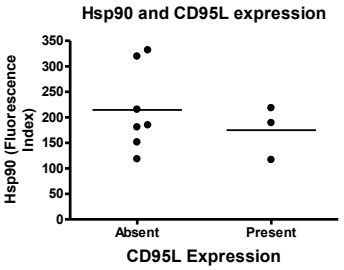

Hsp40 and CD58 expression
Hsp40 (Fluorescence Index)
Present
High
CD58 Expression

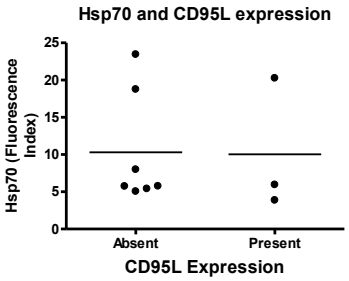

Hsp90 and CD95 expression
Hsp90 (Fluorescence Index)
Absent
Present
CD95 Expression

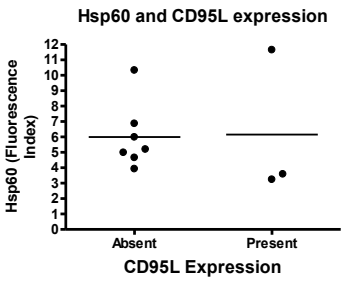

Hsp70 and CD95 expression
Hsp70 (Fluorescence Index)
Absent
Present
CD95 Expression

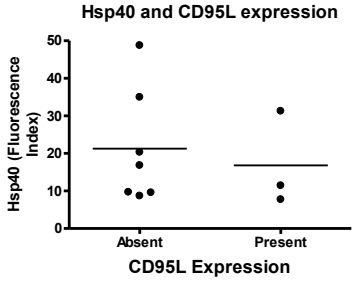

Hsp60 and CD95 expression
Hsp60 (Fluorescence Index)
Absent
Present
CD95 Expression

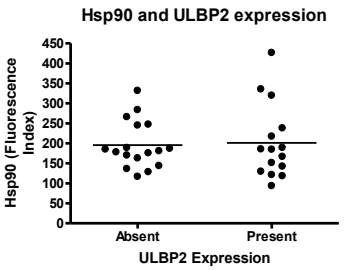

Hsp40 and CD95 expression
Hsp40 (Fluorescence Index)
Absent
Present
CD95 Expression

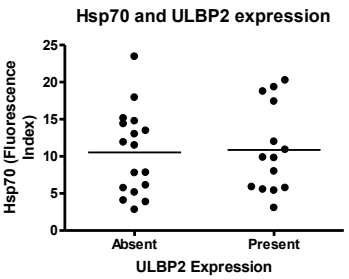

Hsp90 and CD95L expression
Hsp90 (Fluorescence Index)
Absent
Present
CD95L Expression

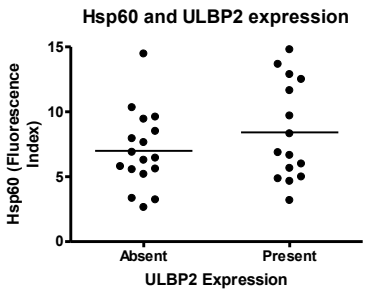

Hsp70 and CD95L expression
Hsp70 (Fluorescence Index)
Absent
Present
CD95L Expression

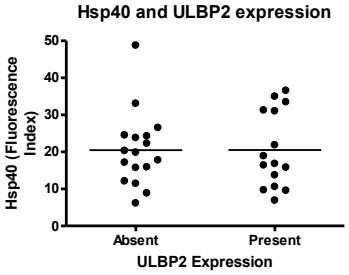

Hsp60 and CD95L expression
Hsp60 (Fluorescence Index)
Absent
Present
CD95L Expression

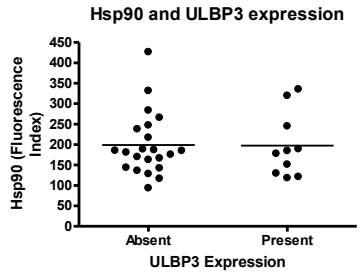

Hsp40 and CD95L expression
Hsp40 (Fluorescence Index)
Absent
Present
CD95L Expression

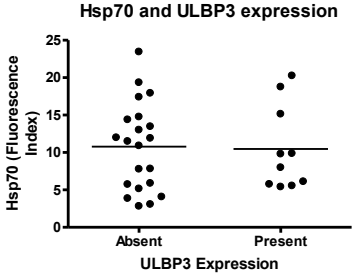

Hsp90 and ULBP2 expression
Hsp90 (Fluorescence Index)
Absent
Present
ULBP2 Expression

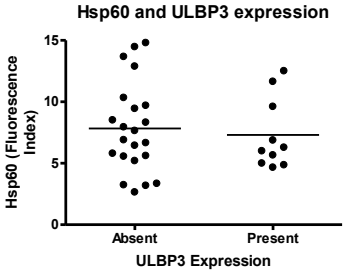

Hsp70 and ULBP2 expression
Hsp70 (Fluorescence Index)
Absent
Present
ULBP2 Expression

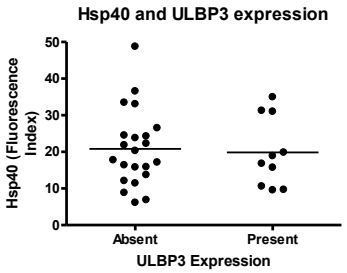

Hsp60 and ULBP2 expression
Hsp60 (Fluorescence Index)
Absent
Present
ULBP2 Expression

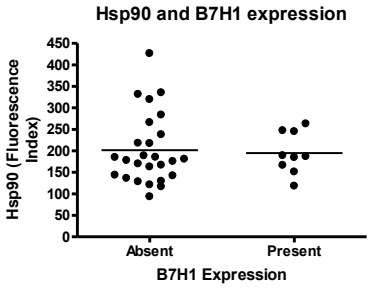

Hsp40 and ULBP2 expression
Hsp40 (Fluorescence Index)
Absent
Present
ULBP2 Expression

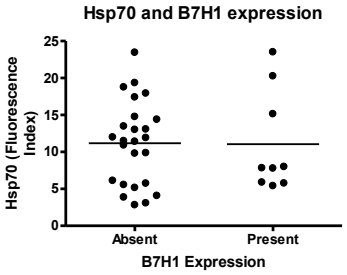

Hsp90 and ULBP3 expression
Hsp90 (Fluorescence Index)
Absent
Present
ULBP3 Expression

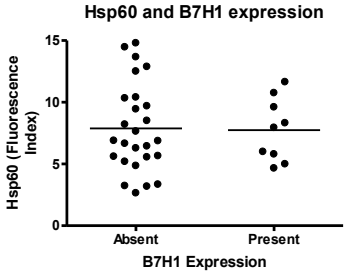

Hsp70 and ULBP3 expression
Hsp70 (Fluorescence Index)
Absent
Present
ULBP3 Expression

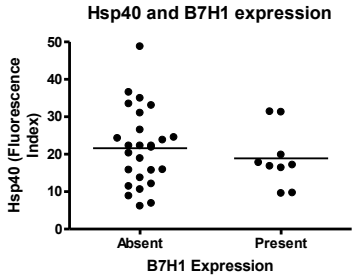

Hsp60 and ULBP3 expression
Hsp60 (Fluorescence Index)
Absent
Present
ULBP3 Expression

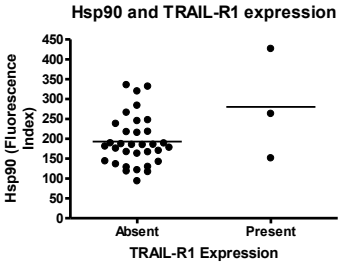

Hsp40 and ULBP3 expression
Hsp40 (Fluorescence Index)
Absent
Present
ULBP3 Expression

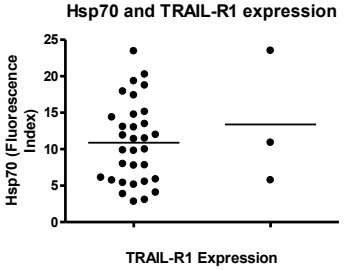

Hsp90 and B7H1 expression
Hsp90 (Fluorescence Index)
Absent
Present
B7H1 Expression

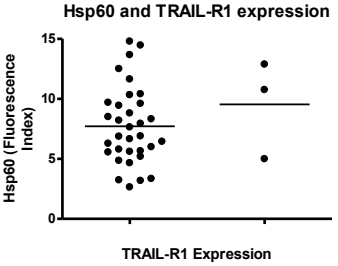

Hsp70 and B7H1 expression
Hsp70 (Fluorescence Index)
Absent
Present
B7H1 Expression

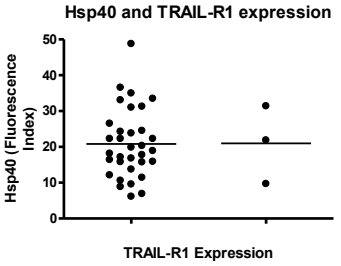

Hsp60 and B7H1 expression
Hsp60 (Fluorescence Index)
Absent
Present
B7H1 Expression

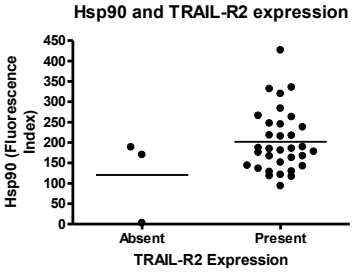

Hsp40 and B7H1 expression
Hsp40 (Fluorescence Index)
Absent
Present
B7H1 Expression

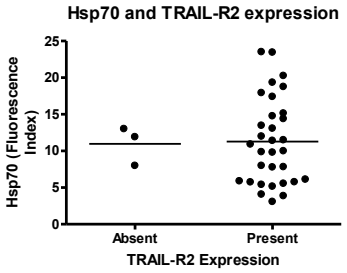

Hsp90 and TRAIL-R1 expression
Hsp90 (Fluorescence Index)
Absent
Present
TRAIL-R1 Expression

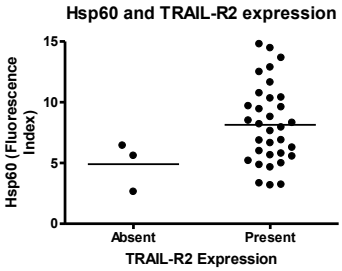

Hsp70 and TRAIL-R1 expression
Hsp70 (Fluorescence Index)
Absent
Present
TRAIL-R1 Expression

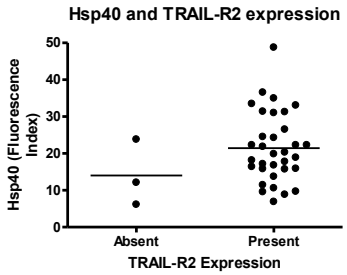

Hsp60 and TRAIL-R1 expression
Hsp60 (Fluorescence Index)
Absent
Present
TRAIL-R1 Expression


Hsp40 and TRAIL-R1 expression
Hsp40 (Fluorescence Index)
Absent
Present
TRAIL-R1 Expression

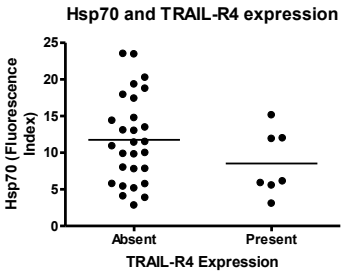

Hsp90 and TRAIL-R2 expression
Hsp90 (Fluorescence Index)
Absent
Present
TRAIL-R2 Expression

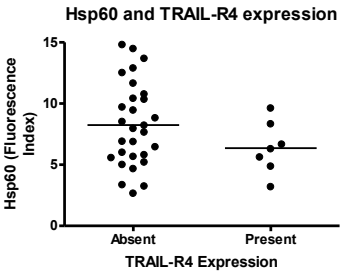

Hsp70 and TRAIL-R2 expression
Hsp70 (Fluorescence Index)
Absent
Present
TRAIL-R2 Expression

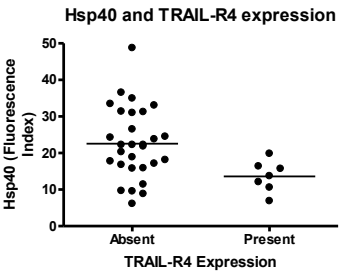

Hsp60 and TRAIL-R2 expression
Hsp60 (Fluorescence Index)
Absent
Present
TRAIL-R2 Expression

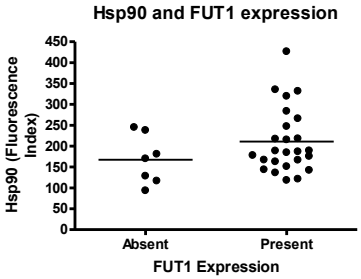

Hsp40 and TRAIL-R2 expression
Hsp40 (Fluorescence Index)
Absent
Present
TRAIL-R2 Expression

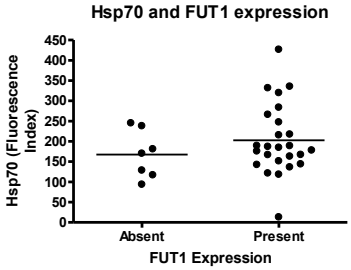

Hsp90 and TRAIL-R4 expression
Hsp90 (Fluorescence Index)
Absent
Present
TRAIL-R4 Expression

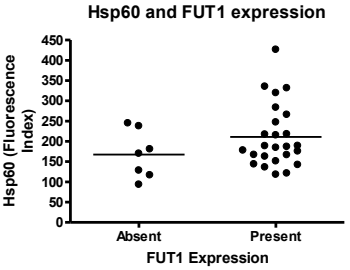

Hsp70 and TRAIL-R4 expression
Hsp70 (Fluorescence Index)
Absent
Present
TRAIL-R4 Expression

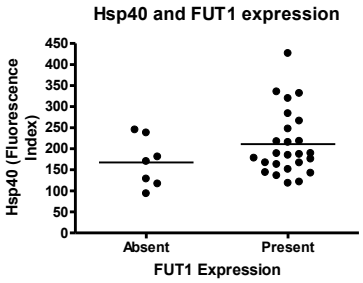

Hsp60 and TRAIL-R4 expression
Hsp60 (Fluorescence Index)
Absent
Present
TRAIL-R4 Expression

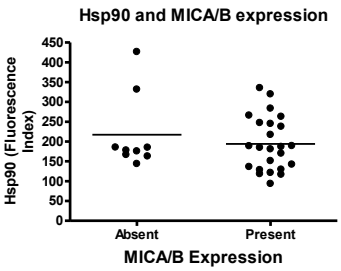

Hsp40 and TRAIL-R4 expression
Hsp40 (Fluorescence Index)
Absent
Present
TRAIL-R4 Expression

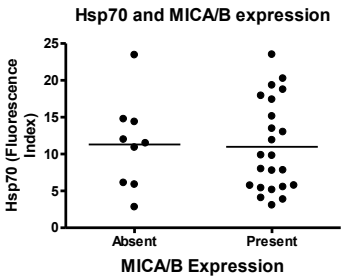

Hsp90 and FUT1 expression
Hsp90 (Fluorescence Index)
Absent
Present
FUT1 Expression

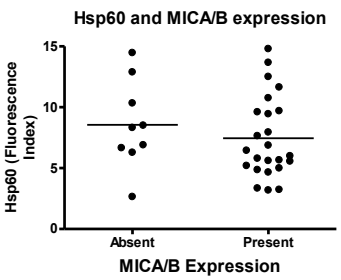

Hsp70 and FUT1 expression
Hsp70 (Fluorescence Index)
Absent
Present
FUT1 Expression

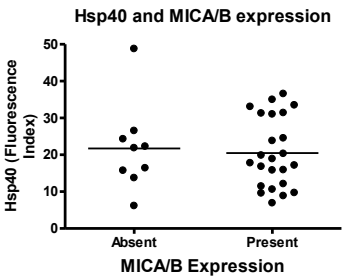

Hsp60 and FUT1 expression
Hsp60 (Fluorescence Index)
Absent
Present
FUT1 Expression

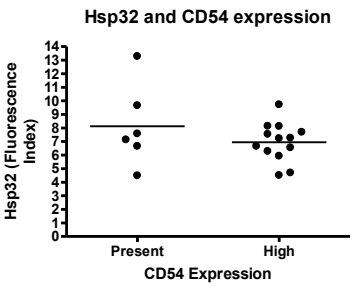

Hsp40 and FUT1 expression
Hsp40 (Fluorescence Index)
Absent
Present
FUT1 Expression

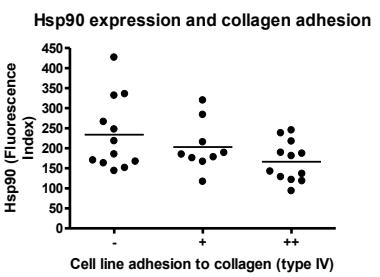

Hsp90 and MICA/B expression
Hsp90 (Fluorescence Index)
Absent
Present
MICA/B Expression

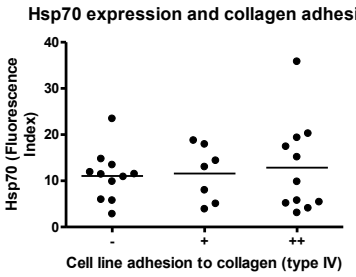

Hsp70 and MICA/B expression
Hsp70 (Fluorescence Index)
Absent
Present
MICA/B Expression

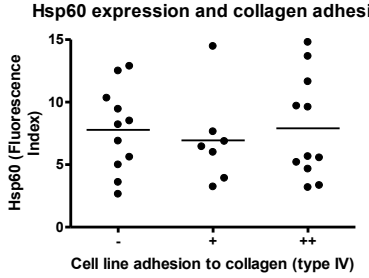

Hsp60 and MICA/B expression
Hsp60 (Fluorescence Index)
Absent
Present
MICA/B Expression

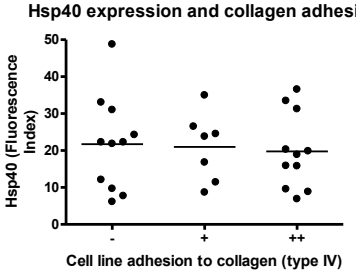

Hsp40 and MICA/B expression
Hsp40 (Fluorescence Index)
Absent
Present
MICA/B Expression

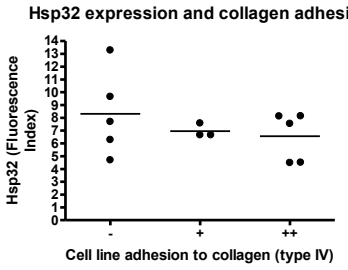

Hsp32 and CD54 expression
Hsp32 (Fluorescence Index)
Present
High
CD54 Expression

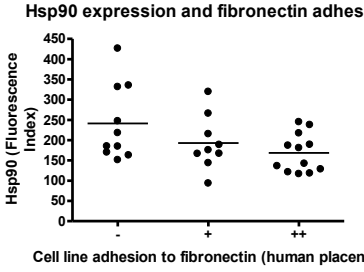

Hsp90 expression and collagen adhesion
Hsp90 (Fluorescence Index)
-
+
++
Cell line adhesion to collagen (type IV)

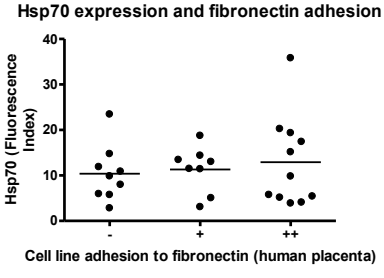

Hsp70 expression and collagen adhesion
Hsp70 (Fluorescence Index)
-
+
++
Cell line adhesion to collagen (type IV)

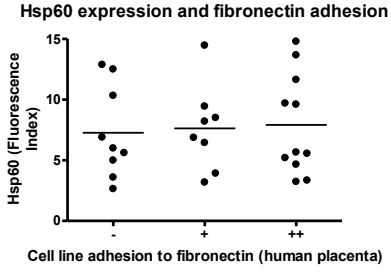

Hsp60 expression and collagen adhesion
Hsp60 (Fluorescence Index)
-
+
++
Cell line adhesion to collagen (type IV)

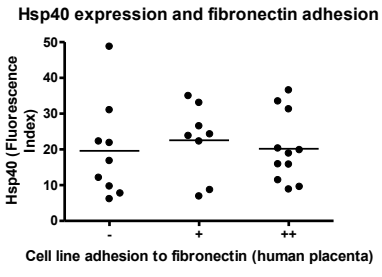

Hsp40 expression and collagen adhesion
Hsp40 (Fluorescence Index)
-
+
++
Cell line adhesion to collagen (type IV)

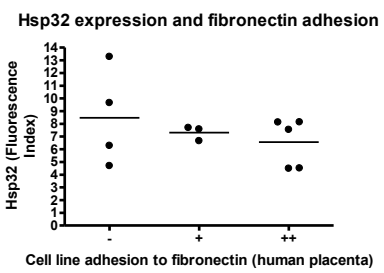

Hsp32 expression and collagen adhesion
Hsp32 (Fluorescence Index)
-
+
++
Cell line adhesion to collagen (type IV)

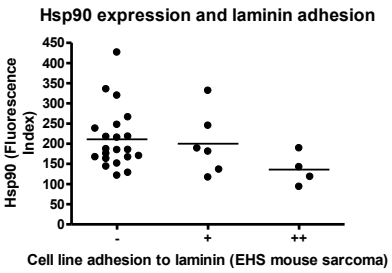

Hsp90 expression and fibronectin adhesion
Hsp90 (Fluorescence Index)
-
+
++
Cell line adhesion to fibronectin (human placenta)

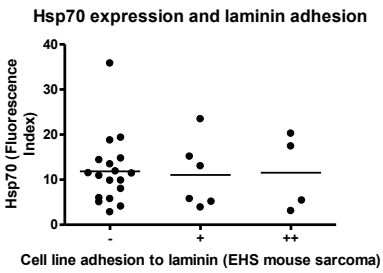

Hsp70 expression and fibronectin adhesion
Hsp70 (Fluorescence Index)
-
+
++
Cell line adhesion to fibronectin (human placenta)

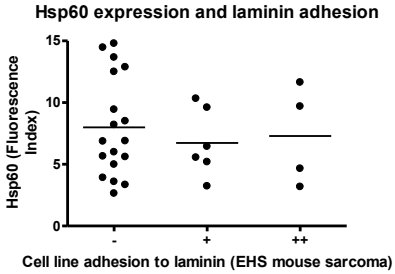

Hsp60 expression and fibronectin adhesion
Hsp60 (Fluorescence Index)
-
+
++
Cell line adhesion to fibronectin (human placenta)

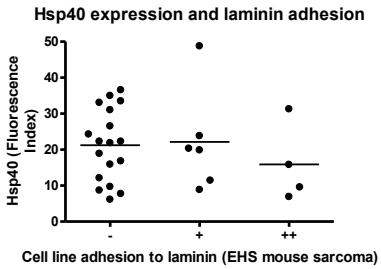

Hsp40 expression and fibronectin adhesion
Hsp40 (Fluorescence Index)
-
+
++
Cell line adhesion to fibronectin (human placenta)

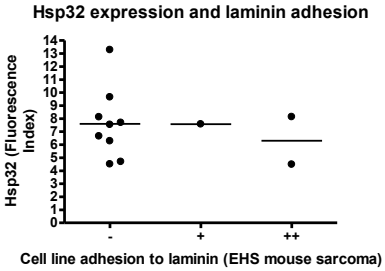

Hsp32 expression and fibronectin adhesion
Hsp32 (Fluorescence Index)
Cell line adhesion to fibronectin (human placenta)
-
+
++


Hsp90 expression and laminin adhesion
Hsp90 (Fluorescence Index)
Cell line adhesion to laminin (EHS mouse sarcoma)
-
+
++


Hsp70 expression and laminin adhesion
Hsp70 (Fluorescence Index)
Cell line adhesion to laminin (EHS mouse sarcoma)
-
+
++


Hsp60 expression and laminin adhesion
Hsp60 (Fluorescence Index)
Cell line adhesion to laminin (EHS mouse sarcoma)
-
+
++


Hsp40 expression and laminin adhesion
Hsp40 (Fluorescence Index)
Cell line adhesion to laminin (EHS mouse sarcoma)
-
+
++


Hsp32 expression and laminin adhesion
Hsp32 (Fluorescence Index)
Cell line adhesion to laminin (EHS mouse sarcoma)
-
+
++

Appendix VI

Clinical parameter		Low hsp70 tumours	High hsp70 tumours	P value
Sex (n = 24)	Male	16	2	< 0.05
	Female	3	3	
Stage (n = 24)	III	5	0	> 0.15
	IV	14	5	
Skin metastasis (n = 25)	Positive	7	3	> 0.30
	Negative	13	2	
Survival since diagnosis (n = 24)	≤33 months	12	2	> 0.30
	>33 months	7	3	
Time to first metastasis (n = 15)	≤29 months	7	1	> 0.15
	>29 months	4	3	
Age (n = 24)	≤56	12	2	> 0.30
	>56	7	3	

Clinical parameter		Low hsp60 tumours	High hsp60 tumours	P value
Sex (n = 28)	Male	5	17	> 0.70
	Female	1	5	
Ulceration (n = 15)	Positive	3	4	> 0.45
	Negative	2	6	
Breslow depth (n = 18)	0-2 mm	3	6	> 0.55
	>2-4 mm	1	3	
	>4 mm	1	4	
Stage (n = 28)	III	3	4	> 0.10
	IV	3	18	
Clark Level (n = 12)	III	0	1	> 0.10
	IV	2	8	
	V	1	0	
Lymph node metastasis (n = 29)	Positive	4	9	> 0.20
	Negative	2	14	
Skin metastasis (n = 29)	Positive	2	10	> 0.65
	Negative	4	13	
Primary tumour localised to lower extremity (n = 24)	Positive	4	6	> 0.15
	Negative	2	12	

Clinical parameter		Low hsp60 tumours	High hsp60 tumours	P value
Superficial spreading histological sub-type (n = 19)	Positive	3	6	> 0.85
	Negative	3	7	
Follow-up time* (n = 23)	≤6 months	4	11	> 0.40
	>6 months	1	7	
Survival since diagnosis (n = 28)	≤33 months	4	11	> 0.45
	>33 months	2	11	
Stage III to IV progression time (n = 12)	<23 months	2	5	> 0.70
	≥23 months	1	4	
Time to first metastasis (n = 18)	≤29 months	2	7	= 1
	>29 months	2	7	
Age (n = 28)	≤56	2	13	> 0.25
	>56	4	9	

* Only patients with a follow-up period of at least two months were included in the analysis

Clinical parameter		Low hsp40 tumours	High hsp40 tumours	P value
Sex (n = 16)	Male	6	8	> 0.80
	Female	1	1	
Ulceration (n = 9)	Positive	0	3	> 0.12
	Negative	3	3	
Breslow depth (n = 10)	0-2 mm	3	2	> 0.10
	>2-4 mm	1	1	
	>4 mm	0	3	
Stage (n = 16)	III	3	0	< 0.03
	IV	4	9	
Clark Level (n = 7)	III	1	0	> 0.20
	IV	2	4	
Lymph node metastasis (n = 16)	Positive	2	2	> 0.75
	Negative	5	7	
Skin metastasis (n = 16)	Positive	4	5	> 0.90
	Negative	3	4	

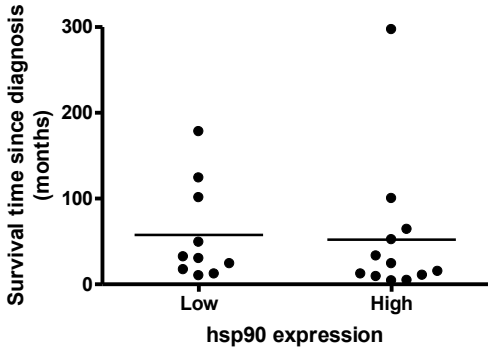
Clinical parameter		Low hsp40 tumours	High hsp40 tumours	P value
Superficial spreading histological sub-type (n = 9)	Positive	1	3	> 0.60
	Negative	2	3	
Follow-up time* (n = 12)	≤6 months	5	4	> 0.50
	>6 months	1	2	
Survival since diagnosis (n = 16)	≤33 months	4	4	> 0.60
	>33 months	3	5	
Stage III to IV progression time (n = 5)	<23 months	1	1	> 0.70
	≥23 months	1	2	
Time to first metastasis (n = 11)	≤29 months	3	3	> 0.70
	>29 months	2	3	
Age (n = 16)	≤56	5	6	> 0.80
	>56	2	3	

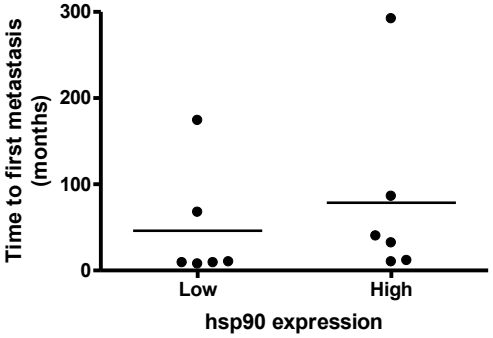
* Only patients with a follow-up period of at least two months were included in the analysis

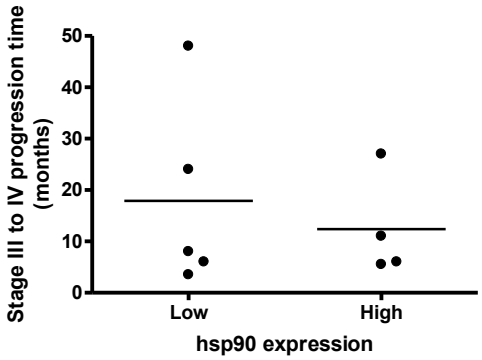
Clinical parameter		Low hsp32 tumours	High hsp32 tumours	P value
Sex (n = 30)	Male	4	18	> 0.08
	Female	4	4	
Ulceration (n = 16)	Positive	3	5	> 0.55
	Negative	2	6	
Breslow depth (n = 18)	0-2 mm	2	7	> 0.40
	>2-4 mm	2	2	
	>4 mm	2	3	
Stage (n = 30)	III	3	5	> 0.40
	IV	5	17	
Clark Level (n = 12)	III	0	1	> 0.13
	IV	3	7	
	V	1	0	
Lymph node metastasis (n = 31)	Positive	5	8	> 0.15
	Negative	3	15	
Skin metastasis (n = 31)	Positive	2	12	> 0.15
	Negative	6	11	
Primary tumour localised to lower extremity (n = 25)	Positive	5	6	> 0.08
	Negative	2	12	

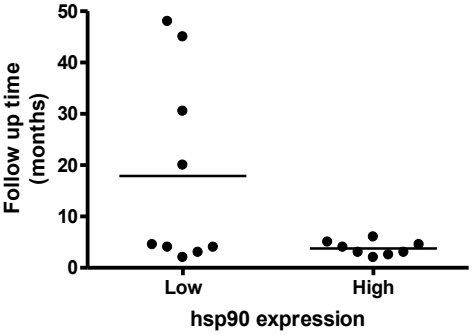
Clinical parameter		Low hsp32 tumours	High hsp32 tumours	P value
Superficial spreading histological sub-type (n = 19)	Positive	4	5	> 0.50
	Negative	3	7	
Acral lentiginous histological sub-type (n = 19)	Positive	2	3	> 0.85
	Negative	5	9	
Follow-up time* (n = 25)	≤6 months	3	14	> 0.09
	>6 months	4	4	
Survival since diagnosis (n = 30)	≤33 months	6	11	> 0.22
	>33 months	2	11	
Stage III to IV progression time (n = 12)	<23 months	3	4	> 0.90
	≥23 months	2	3	
Time to first metastasis (n = 18)	≤29 months	4	5	> 0.10
	>29 months	1	8	
Age (n = 30)	≤56	4	12	> 0.80
	>56	4	10	

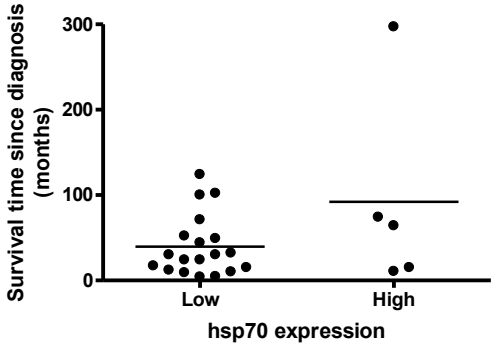
* Only patients with a follow-up period of at least two months were included in the analysis

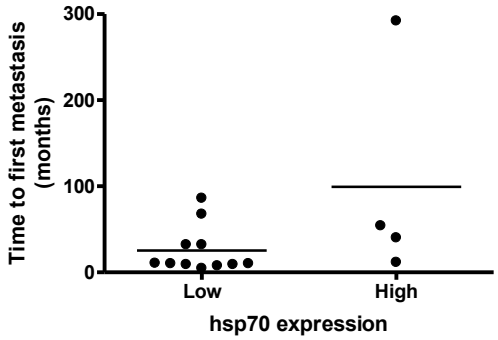

Survival time since diagnosis (months)
300
200
100
0
Low
High
hsp90 expression

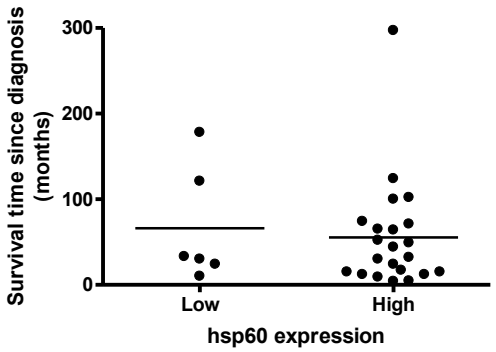

Time to first metastasis (months)
300
200
100
0
Low
High
hsp90 expression

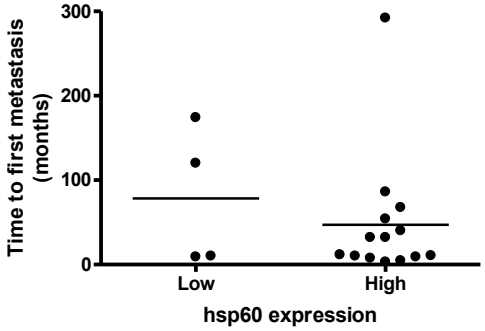

Stage III to IV progression time (months)
50
40
30
20
10
0
Low
High
hsp90 expression

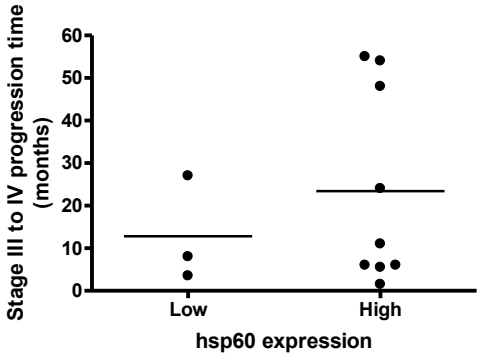

Follow up time (months)
50
40
30
20
10
0
Low
High
hsp90 expression

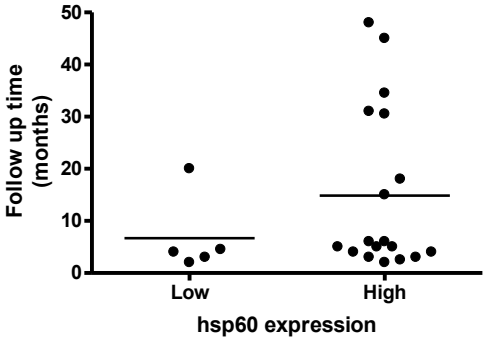

Survival time since diagnosis (months)
300
200
100
0
Low
High
hsp70 expression

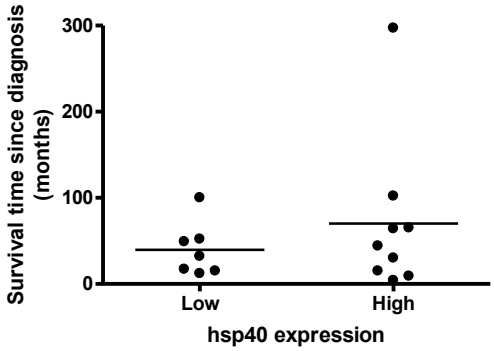

Time to first metastasis (months)
300
200
100
0
Low
High
hsp70 expression

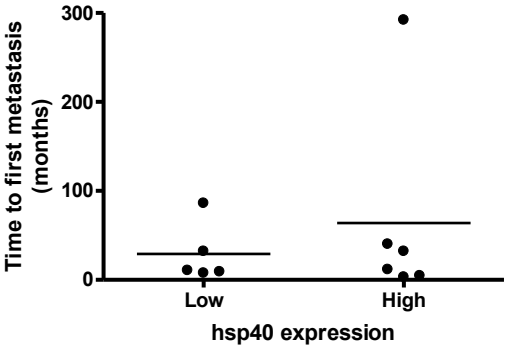

Survival time since diagnosis (months)
300
200
100
0
Low
High
hsp60 expression

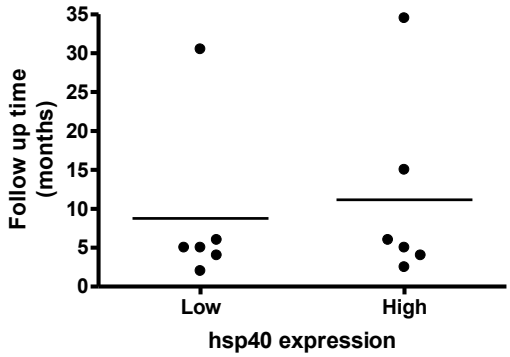

Time to first metastasis (months)
300
200
100
0
Low
High
hsp60 expression

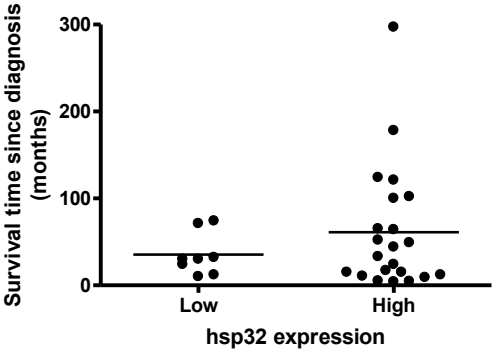

Stage III to IV progression time (months)
60
50
40
30
20
10
0
Low
High
hsp60 expression

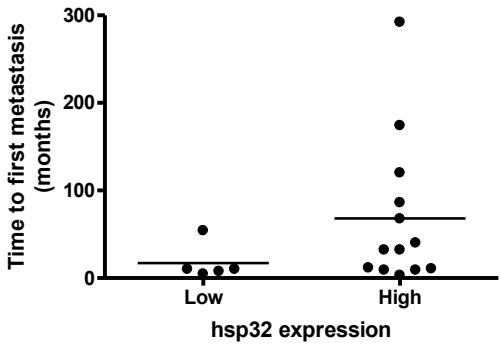

Follow up time (months)
50
40
30
20
10
0
Low
High
hsp60 expression

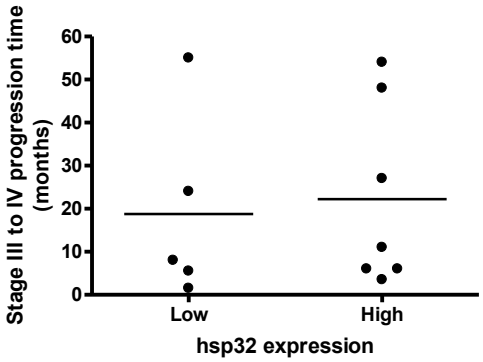

Survival time since diagnosis (months)
300
200
100
0
Low
High
hsp40 expression

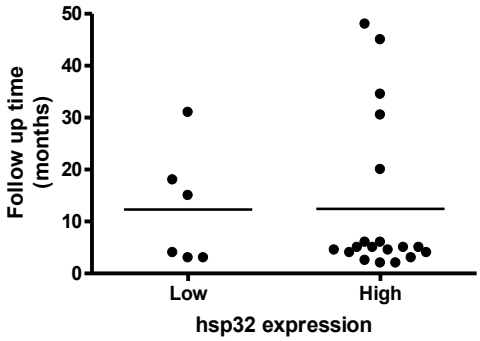

Time to first metastasis (months)
300
200
100
0
Low
High
hsp40 expression


Follow up time (months)
35
30
25
20
15
10
5
0
Low
High
hsp40 expression


Survival time since diagnosis (months)
300
200
100
0
Low
High
hsp32 expression


Time to first metastasis (months)
300
200
100
0
Low
High
hsp32 expression


Stage III to IV progression time (months)
60
50
40
30
20
10
0
Low
High
hsp32 expression


Follow up time (months)
50
40
30
20
10
0
Low
High
hsp32 expression