Expression of Heat Shock Proteins on the plasma membrane of cancer cells: A potential multi-chaperone complex that mediates migration?

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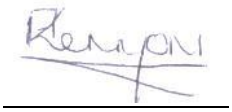
Abstract

Current dogma suggests that the Heat Shock Protein (Hsp) molecular chaperones and associated co-chaperones function primarily within the cell, although growing evidence suggests a role for these proteins on the plasma membrane of cancer cells. Hsp90 does not function independently *in vivo*, but instead functions with a variety of partner chaperones and co-chaperones, that include Hsp70 and Hsp90/Hsp70 organising protein (Hop), which are thought to regulate ATP hydrolysis and the binding of Hsp90 to its client proteins. Hsp90 on the plasma membrane appears to have distinct roles in pathways leading to cell motility, invasion and metastasis. We hypothesised that Hsp90 on the plasma membrane is present as part of a multi-chaperone complex that participates in the chaperone-assisted folding of client membrane proteins in a manner analogous to the intracellular chaperone complex.

This study characterised the membrane expression of Hsp90, Hsp70 and Hop in different cell models of different adhesive and migratory capacity, namely MDA-MB-231 (metastatic adherent breast cancer cell line), MCF-7 (non-metastatic adherent breast cancer cell line), U937 and THP1 (monocytic leukemia suspension cell lines). Membrane expression of the Hsps was analysed using a combination of subcellular fractionation, biotin-streptavidin affinity purification and immunofluorescence. This study provided evidence to suggest that Hsp90, Hsp70 and Hop are membrane associated in MDA-MB-231 and MCF-7 breast cancer cells. Hsp90, Hsp70 and Hop associated with the plasma membrane such that at least part of the protein is located extracellularly. Immunofluorescence analysis showed that Hsp90, Hsp70 and Hop at the leading edge may localize to membrane ruffles in MDA-MB-231 cells, in accordance with the published role of Hsp90 in migration. An increase in this response was seen in cells stimulated to migrate with SDF-1. By immunoprecipitation, we isolated a putative extracellular membrane associated complex containing Hsp90, Hsp70 and Hop. Using soluble Hsp90 and antibodies against membrane associated Hsp90, we suggested roles for soluble extracellular Hsp90 in mediating migration by wound healing assays and inducing actin reorganisation and vinculin-based focal adhesion formation. The effects of extracellular Hsp90 are mediated by signalling through an ERK1/2 dependent pathway. An anti-Hsp90 antibody against an N-terminal epitope in Hsp90 appeared to be able to overcome the death inducing effects of a combination of SDF-1 and AMD3100, while soluble Hsp90 could not overcome this effect. We propose that this study provides preliminary evidence that extracellular Hsp90 functions as part of a multi-chaperone complex that includes Hsp70 and Hop. The extracellular Hsp90 chaperone complex may mediate cell processes such as migration by modulating the conformation of cell surface receptors, leading to downstream signalling.

Declaration

I declare that this thesis is my own, unaided work. It is being submitted for the degree of Master of Science of Rhodes University. It has not been submitted before for any degree or examination at any other university.



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List of Abbreviations

ATP	Adenosine Triphosphate
BSA	Bovine serum albumin
BS^3	Bis[sulfosuccinimidyl]suberate
CKR	Chemokine receptor
DEN	Dengue
DMEM	Dulbecco's Modified Eagle Medium
DTSSP	3,3'-dithiobis[sulfosuccinimidyl[propionate
ECM	Extracellular matrix
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic Acid
EGTA	Ethyleneglycol-bis(beta-aminoethylether)N'N'N'N-tetraacetic acid
ER	Endoplasmic Reticulum
ERK	Extracellular signal-regulated kinase
ECD	Extracellular domain
FAK	Focal adhesion kinase
FCS	Foetal calf serum
FRET	Fluorescence Resonance Energy Transfer
GA	Geldanamyacin
GDF5	Growth Differentiation Factor 5
GRP	Glucose Regulated Protein
GPCR	G-protein-coupled receptor
Hop	Hsp90/Hsp70 organising protein

Hsp	Heat Shock Protein
HKC	human keratinocyte
HIV	Human Immunodeficiency Virus (HIV)
Ig	Immunoglobulin
LPS	Lipopolysaccharide
KSHV	Kaposi's sarcoma-associated herpes virus
MAPK	Mitogen Activated Protein Kinase
MMP2	Matrix Metalloproteinase-2
NFkB	Nuclear factor-kB
NO	Nitric oxide
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PMSF	Phenylmethanesulfonylfluoride
PrP^C	Cell surface prion protein
PS	Phosphatidylserine
RIPA	Radio-immunoprecipitation assay
RPMI	Roswell Park Memorial Institute
RT-PCR	Reverse transcriptase-polymerase chain reaction
RTK	Receptor tyrosine kinase
SDF-1	Stromal cell-derived factor-1
SDS-PAGE	Sodium-Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis
TBS	Tris-buffered Saline
TBST	Tris-buffered Saline with Tween-20
TGFα	Transforming Growth Factor α

TLR	Toll-like receptor
TPA	Tetradecanoylphorbol-13-Acetate
TPR	Tetratricopeptide repeat
VEGF	Vascular endothelial growth factor
WGA	Wheat Germ Agglutinin
αHsp90:C	Anti-Hsp90 (C-terminal epitope)
αHsp90:N	Anti-Hsp90 (N-terminal epitope)

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Chapter 1.

Literature Review

1.1 Heat Shock Proteins as Molecular Chaperones

The heat shock response was first reported in 1962 when *Drosophilia* salivary glands were subjected to heat shock resulting in an increase in the expression of certain proteins, termed heat shock proteins (Hsps), of defined molecular masses [1]. The Hsps are grouped according to their molecular masses, small Hsps (Hsp10, αA,B-crystallin, Hsp 25/27, HspB family and Hsp40), Hsp60, Hsp90 and Hsp100 [1]. It has subsequently been shown that Hsps belong to a group of conserved proteins that are both constitutively expressed and or expressed after conditions of physical or biological stresses including heavy metals, amino acid analogues, cytostatic drugs, Cox-inhibitors, actetyl salycyl acid, oxidative stress and radiation [1, 2]. The Hsps have strong cytoprotective effects and behave as molecular chaperones for other cellular proteins [3]. The term molecular chaperone is a term coined to describe the common property of assisting the assembly of other proteins (known client proteins) [1]. Co-chaperones are non client-binding partners of chaperones and may be loosely defined as proteins that facilitate in the function of other chaperones [4]. The Hsps are primarily involved in the assembly of proteins because biological stress responses result in protein unfolding. Most Hsps function to guide the conformational states of proteins and hence are critical to folding, translocation, assembly of newly synthesised proteins and the prevention of target protein aggregation [1, 5]. Under conditions of stress, inappropriate activation of signalling pathways could occur as a result of protein misfolding, aggregation or disruption of complexes. In this capacity Hsps serve to maintain homeostasis within the cell [3]. The Hsps are widely induced by cell stress and as a result they are expressed at high levels in a wide range of tumours. They are closely associated with poor prognosis and increased resistance to cancer therapy [6].

1.2 Heat Shock Protein 90 (Hsp90)

The molecular chaperone, heat shock protein (Hsp) 90, is one of the most abundant cellular proteins. Even under non-stressed conditions, Hsp90 comprises 1 % to 2 % of total cellular proteins [7]. Hsp90 is essential for protein folding, the translocation of proteins across membranes, normal protein turnover and prevention of the aggregation of non-native proteins [8]. Unlike most other Hsps, Hsp90 binds substrates that have already reached their correct folded state and exhibits specificity for its client proteins [9]. Hsp90 clientele can be extended to more than 100 proteins, many of which are mutated or overexpressed in cancers [8]. The reliance of tumour cells on Hsp90 can be further emphasized by the presence of elevated Hsp90 levels in tumour cells where Hsp90 comprises 4 % to 6 % of total proteins [10]. The Hsp90 chaperone plays a pivotal role in supporting the client proteins involved in cancer development and

spreading. Hence targeting Hsp90 could result in the disruption of multiple oncogenic signal pathways [11]. Although traditionally considered a cytoplasmic intracellular protein, the functions of Hsp90 are no longer limited to the cytoplasm [12]. Isoforms of Hsp90 include the mainly cytoplasmic Hsp90α (inducible form) isoform and Hsp90β (constitutive form) isoform that share 86 % sequence identity [13], the mitochondrial matrix homologue (TRAP1) and the endoplasmic reticulum (ER) homologue; Glucose Regulated Protein (GRP)94 [14]. The cytoplasmic isoforms, Hsp90α and Hsp90β, are also found in the nucleus [12].

1.3 The Hsp90 chaperone complex

In cancer cells, Hsp90 predominantly exists as a multi-chaperone complex, containing several accessory proteins or co-chaperones that bind to Hsp90 to mediate substrate selection and cycles of association and disassociation from the substrate [6, 10]. In normal cells Hsp90 can be found in an uncomplexed state, under non-stressed conditions [10]. An ATPase cycle is central to the chaperoning activity of Hsp90 [15]. The intracellular Hsp90 chaperone complex consists of a minimum of five proteins; an Hsp90 dimer, Hsp70, Hsp90/Hsp70 organising protein (Hop), Hsp40 and p23 as well as immunophilins [16]. The predicted model of Hsp90 chaperone activity is as follows. The newly synthesised Hsp90 client protein binds to Hsp70 in an Hsp40 dependent step to form a client protein-Hsp70-Hsp40 complex [11, 16]. The Hsp90 chaperone complex cycles between two major conformations [17]. In the 'open' state a client protein is loaded onto Hsp90 with the help of Hsp70, Hsp40 and Hop resulting in the formation of the intermediate complex. The C-terminal domain of the eukaryotic Hsp90 has a conserved pentapeptide (MEEVD) which is involved in binding of co-chaperones such as Hop that contain a tetratricopeptide repeat (TPR) domain [18]. Hop not only connects with the MEEVD motif of Hsp90 but also with Hsp70. Therefore, an important feature of Hop is the ability to link Hsp90 and Hsp70 together as is required for optimal Hsp90 chaperone complex assembly [19]. Hop binds to the MEEVD motif of Hsp90 as well as the N-terminus of Hsp90, preventing the 'closed' state. In the 'open' state of the Hsp90 dimer, the two N-termini are separated and Hsp90 can capture client proteins. In this capacity Hop binding blocks ATP binding and the ATPase activity of Hsp90 [16]. After ATP has bound to Hsp90, Hop is replaced by p23 and immunophilins, thus resulting in the formation of the mature complex [17]. This results in either the conformational maturation of the client protein or the Hsp90 complex maintains the conformation of client proteins, so that they are in an active form [17].

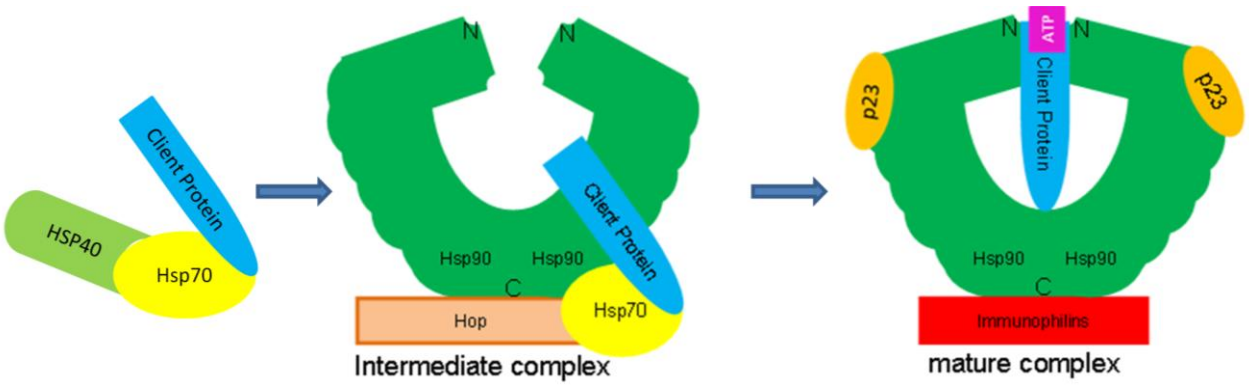

Figure 1: Intracellular Hsp90 chaperone complex

The Hsp90 chaperone complex (including Hsp90, Hsp70, Hsp40, Hop, p23 and immunophilins) cycles between two major conformations. A client protein is loaded onto Hsp90 with the help of Hsp70, Hsp40 and Hop resulting in the formation of the intermediate complex. After ATP has bound to Hsp90, Hop is replaced by p23 and immunophilins, thus resulting in the formation of the mature complex [16].

1.4 Chaperones of the Hsp90 complex

The Hsp70 family of Hsps are highly conserved and the most studied class of Hsps [3]. Hsp70 proteins function as ATP-dependent molecular chaperones by assisting the folding of newly synthesised polypeptides, the assembly of multi-protein complexes and the transport of proteins across cellular membranes [3]. Hsp70 is often overexpressed in human tumours as it is a potent anti-apoptotic protein and confers cytoprotection against a number of death inducing stimuli [20]. The Hsp70 proteins are assisted by the Hsp40 proteins, known to increase the ATPase activity of the Hsp70 proteins [1]. Human cells contain several Hsp70 homologues that include; stress inducible Hsp70, constitutively expressed Hsp70, the mitochondrial homologue (Hsp75) and the endoplasmic reticulum homologue (GRP78). Hsp70/Hsp90 organising protein (Hop) (also known as sti1, p60, stip1) is a 66 kDa protein and was first described as a co-chaperone that binds Hsp90 and Hsp70 and in this capacity regulates their activities [21]. Hop involvement in Hsp90 complexes extends to numerous cellular events that include transcription, protein folding, translocation, viral replication, signal transduction and cell division [22]. Hop is present in many cellular locations [21]. It is able to move rapidly between the cytoplasm and the nucleus [22].

1.5 Extracellular Hsp90

Hsp90 is traditionally considered an intracellular chaperone. However, the presence of Hsp90 on the plasma membrane was first published in 1986, where it was found on the surface of mouse cells as a tumour specific antigen [23]. Hsp90 has subsequently been identified on the surface of cancer cells [24-30]. Both Hsp90α and Hsp90β been found present on the surface of cells [13, 27]. Apart from Hsp90α and Hsp90β, another Hsp90 isoform, Hsp90N, encoding a protein of 75

kDa and lacking the N-terminal ATPase domain of Hsp90 was identified in a complex with a key signal transducer, the Raf kinase. It was found to target Raf to the plasma membrane for activation [31]. However, a contradictory report claimed that sequence analysis showed that there is no evidence that the Hsp90N is present in the human genome and no such homologue exists in the genomes of other organism. These authors argue that, Hsp90N should be viewed as result of chromosomal rearrangement resulting in a chimeric protein and an artefact of a single cell line [32]. The Hsp90 ER homologue gp96/GRP94 has also been shown to localise to the plasma membrane [33]. A pool of extracellular soluble Hsp90 has been found residing in the extracellular space [34].

1.6 Extracellular Chaperones of the Hsp90 Complex

Hsp70 is frequently present on the plasma membrane numerous cancer cell types [3]. The corresponding normal tissues were nearly always found to be membrane associated Hsp70 negative [3]. *In vitro* Hsp70 has been detected in the supernatants of cultured antigen presenting cells and tumour cell lines [35]. Hop has also been shown to be expressed on the surface and in the extracellular space of cells [21, 34].

Hsp90 and Hsp70, present on the surface of mammalian cells, are involved in the recognition of bacterial products that can be deadly to the mammalian host [36]. Bacterial lipopolysaccharide (LPS) form the outer-membrane of gram-negative bacteria and LPS can lead to an uncontrolled inflammatory response [37]. CD14 was one of the first membrane receptors identified that binds bacterial LPS [38]. However, since CD14 cannot transverse the cell membrane it cannot deliver a signal for activation against LPS. Using affinity chromatography and fluorescence resonance energy transfer (FRET) a complex of receptors was identified on the cell surface that include Hsp90, Hsp70, CXCR4 and growth differentiation factor 5 (GDF5) that forms after stimulation with LPS [38]. LPS is transferred from CD14 to Hsp90 and Hsp70. It has been shown that Hsp90 and Hsp70 associate with the Toll-like receptor (TLR) 4 and adaptor molecule MD2 (TLR4-MD2) following LPS stimulation. Since Hsps have an affinity for LPS they act as a transfer molecule and deliver LPS from the plasma membrane to the TLR4-MD2 afterwhich the TLR4-MD2-LPS is targeted to the Golgi apparatus [36]. It has been suggested that CXCR4 and CD55 potentially act as additional LPS transfer molecules as part of the LPS receptor cluster [36].

Hsp90 and Hsp70 were found to form part of a receptor complex required for Dengue (DEN) virus entry in neuroblastoma and monocytic cell lines, where U937 monocytic leukemia cells were used as a model. This receptor complex was similarly found on the surface of human monocyte derived macrophages [39]. Interestingly, it was shown that the entry of DEN virus into

monocytes/macrophages can be blocked by LPS Potentially because when Hsp90 and Hsp70 are clustered around CD14 preventing them from interacting with DEN [39].

The Kaposi's sarcoma-associated herpes virus (KSHV) is the cause of Kaposi's sarcoma, a cancer associated with the human immunodeficiency virus (HIV) [40]. KSHV interaction with the cell membrane triggers activation of specific intracellular signal transduction pathways which leads to virus entry into the cell [40]. Membrane associated Hsp90 facilitates KSHV gene expression primarily through the regulation of post entry events as well as serves as a co-factor for MAPK activation [40].

There is accumulating evidence to suggest that membrane associated Hsp90 and Hsp70 might be a useful tumour antigen for eliciting a host immune response [41]. As previously mentioned, Hsp90 and Hsp70 have been implicated in lipopollysaccharide (LPS) recognition which can lead to an uncontrollable inflammatory response as part of a multi-protein receptor complex [38]. Dendritic cells are key components of the innate and adaptive immune responses and it has been shown that necrotic cell death results in the secretion of Hsp70 and Hsp90 [42]. The Hsps then stimulate macrophages to secrete cytokines and induce the expression of antigen-presenting and co-stimulatory molecules on dendritic cells [42]. Extracellular Hsp70 has been found to perform other roles in the immune response. Two colon carcinoma sublines CX+ and CX- were generated and differed only in their expression of surface Hsp70. It was shown that the increase expression of Hsp70 on tumour cells correlates with increased sensitivity to lysis by natural killer (NK) cells. Antibody blocking studies of Hsp70 revealed strong inhibition of NK-mediated cell lysis [43]. The presence of Hsp70 in the serum of humans is associated with stress conditions including inflammation, bacterial and viral infections and cancer [2].

Extracellular Hop as been identified as a cell surface ligand of PrP^C [44]. The toxicity of the cell surface prion protein (PrP^C) results in prion diseases [44]. The proliferation of the glioblastoma derived cell line was stimulated by Hop in a PrP^C dependent manner, mediated by the Mitogen Activated Protein Kinase/ Extracellular signal-regulated kinase1/2 (MAPK/ Erk1/2) and PBK signalling pathways [21]. It also appears that signalling induced by Hop depends on endocytosis since inhibitors of endocytosis block Erk1/2 activity previously induced by Hop [44].

The best described role of extracellular Hsp90 is in migration. Extracellular Hsp90 is reported to play a role in the motility and thus metastasis of cancer cells [45]. Previous studies report that Hsp90 is localised on the surface of cells of the nervous tissue and contributes to cell migration processes via cytoskeletal rearrangement during normal embryonic development of the nervous tissue in a mechanism that is similar if not identical to that of tumour cell metastasis [13, 25,

46]. To specifically examine the role of extracellular Hsp90 in tumour cell motility, the small cell impermeable inhibitor, DMAG-N-oxide, significantly inhibited tumour cell migration and cytoskeletal reorganisation, a fundamental process to cell migration [41]. Hsp90 on the surface of melanoma cells correlates positively with metastatic potential [24]. Hsp90α but not Hsp90β was found in conditioned media of fibrosarcoma cells and MDA-MB-231 breast cancer cells [34]. In the extracellular space of fibrosarcoma cells Hsp90α was shown to interact with matrix metalloproteinase-2 (MMP2) and thus facilitate the maturation of MMP-2, promoting tumour invasiveness [34]. The MMP2 protease is important for digestion of major components of the extracellular matrix surrounding tumour masses and for subsequent invasion of primary tumour cells [34]. Geldanamycin-agarose, a cell impermeable Hsp90 inhibitor resulted in a reduction in cell invasiveness of HT-1080 fibrosarcoma cells [27]. Hsp90 was shown to be membrane associated on the surface of MDA-MB-453 cells by immunofluorescence and subcellular fractionation and shown to interact with the membrane receptor, HER-2 resulting in downstream signalling that leads to cell motility [26]. These researchers argue that membrane associated Hsp90 is a peripheral protein that is loosely attached to the cell membrane and thus unlikely to mediate cell membrane signalling on its own [46]. Instead, they propose that Hsp90 may interact with other proteins which through transmembrane signalling will trigger the intracellular events required for processes such as cell migration [26]. During human skin wound healing, human keratinocytes (HKCs) migrate laterally across the wound bed [47]. It has been shown that hypoxia results in the secretion of Hsp90α into the extracellular environment and extracellular soluble Hsp90α controls cell motility [48]. It was suggested that in HKCs, transforming growth factor α (TGFα) in response to hypoxia controls the release of Hsp90α from the cells via an exosomal pathway [47]. Extracellular soluble Hsp90 can then promote cell migration by interacting with the cell surface receptor CD91 which then leads to signalling events that result in cell motility [47]. Oxidative stress was shown to cause the release of Hsp90 from vascular smooth muscle cells resulting in the subsequent activation of ERK1/2 [49]. Interestingly, in endothelial cells Hsp70 was shown to be expressed on the cell surface of melanoma metastases but the corresponding skin fibroblasts were shown to be Hsp70 negative suggesting a role for Hsp70 in metastasis [50].

1.7 Membrane association and translocation of Hsps

The classical secretory membrane system allows cells to regulate the delivery of newly synthesized proteins to the cell surface [51]. This is the most recognized mechanism for membrane transport and involves the endoplasmic reticulum and Golgi apparatus [52]. Proteins are directed to the ER by a hydrophobic sequence that is recognized by a ribonucleoprotein

complex that acts as a signal recognition particle [53]. Membrane proteins that are secreted have a secretory signal sequence which differs from that of membrane associated or integral membrane proteins and is usually cleaved in the ER prior to secretion [54]. The signal sequence of type I membrane proteins is similarly cleaved where as in type II membrane proteins, the signal sequence is also the membrane anchor domain [54]. Heat shock proteins do not appear to be translocated to or across the plasma membrane by the classical secretory system as Hsp90, Hsp70 and Hop lack a signal sequence. Alternate pathways for membrane trafficking include exosomes, export via intracellular vesicles such as endosomes, direct transport across the membrane and by flip-flop mechanisms [52]. Exosomes are small membrane vesicles that are secreted by numerous cell types. Both Hsp70, Hsp90 and Hop have been found present in exosomes [2, 52]. Similarly there have been reports of an association of Hsp60, Hsp90 and Hsp70 with lipid rafts when associating with the plasma membrane. Lipid rafts are also known to be components of exosomes [52]. Hsp70 has been reported to reach the exterior of the cell via a flip-flop mechanism [2].

1.8 Cancer Development and Metastasis

Cancer was described in the earliest medical records found in the history of mankind and yet today it still remains the second most prevalent cause of death in the industrialised world [55]. The genetic instability of cancer cells and environmental stimuli leads to the acquisition of six essential alterations in cell physiology characteristic of all malignancies: sustained angiogenesis, invasion and metastasis, insensitivity to growth inhibitory signals, limitless replicative potential, self-sufficiency in growth signalling and the ability to evade apoptosis [56].

Metastasis is the movement of cancerous cells from their primary location and the development of a secondary tumour at this distal site (1). Cancer cell metastasis is the most frequent cause of death in cancer patients [57]. Secondary tumours are formed as a result of metastatic cancer cells that leave the primary tumour and travel through either blood or lymphatic vessels, to seek out new sites in the body where new tumour growth results [58]. Cancer cell metastasis consists of six steps: 1) cancers cells detach and extravasate from the primary tumour; 2) cancer cells invade the extracellular matrix and the endothelium until they reach the blood stream; 3) survive the turbulent flow of the bloodstream; 4) arrest at a distant site by adhesion to a specific endothelium; 5) extravasate across the endothelium and extracellular matrix once again and 6) stop and grow at a distant site [59]. Metastatic carcinoma cells acquire a migratory phenotype associated with increased expression of several genes involved in cell motility [60]. This allows

cancer cells to respond to cues from the microenvironment that trigger tumour migration and invasion leading to metastasis [60].

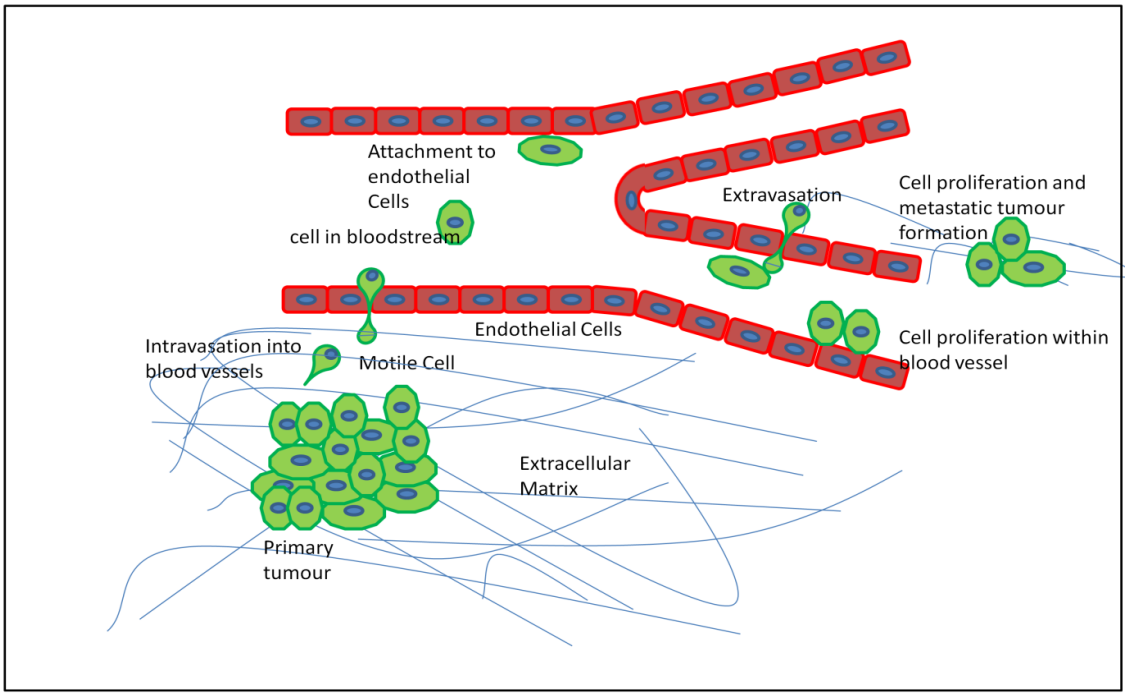

Figure 2: Cancer metastasis of cells from their primary location to a distal sight is a multi-step process.

At the primary tumour cells interact with the extracellular matrix (ECM) components such as collagen fibres. Some cells become motile (1) and invade the ECM (2). Cells can form part of blood vessels by intravasation into the blood stream (3). Cells survive the turbulent flow of the bloodstream (4) afterwhich cells attach to endothelial cells and once again extravasate across the endothelial cells and reinvade the ECM (5) migrating to sites of metastatic tumour formation (6). [Adapted from [61]].

1.9 The cell migratory machinery

Cell migration is a highly integrated multi-step process that consists of four essential steps in which intracellular and extracellular signals result in a coordinated response [62]. The initial step in cell migration is front to back polymerisation in response to extracellular cues which are often chemotactic and involves the binding of chemoattractants to cell surface receptors in the local activation of signalling proteins at the leading edge of migrating cells [62, 63]. This is followed by membrane protrusion and adhesion formation, both of which require actin [62]. Protrusive structures formed by migrating and invading cells have been termed lamellipodia, filapodia and invadapodia depending on their morphological and functional characteristics. Formation of these structures is primarily driven by actin polymerisation at the leading edge of migrating cells [63]. Lamellipodia are flat, sheet-like membrane protrusions formed at the leading edge of migrating cells. Lamellipodia attach to the underlying substrate and subsequently generate sufficient force to drag the cell forward in the direction of cell migration. Filopodia are thin, fingerlike

projections formed at the leading edge of cells. In order to migrate through the extracellular matrix (ECM), cells degrade and remodel the ECM structures by invadopodia which are ventral membrane protrusions formed by highly invasive cancer cells [64]. Adhesions are formed to the ECM through focal adhesions which include the cytoplasmic domains of clustered integrins, cytoskeletal proteins and numerous signalling molecules such as talin and vinculin [45, 61, 64]. The sites of attachment, termed focal adhesions also serve as the attachment sites for large bundles of actin filaments called stress fibres [64]. The cell is then able to migrate via the contraction of the F-actin stress fibre network which generates sufficient tension to drag the cell forward. The third step in the process of migration is the disassembly of the focal adhesions at the rear of the cell, which allows the cells to then get dragged in the direction of cell migration and resulting in the fourth and final step of rear end retraction of the cell [62, 64]

1.10 The role of integrins and chemokines in cancer metastasis

Integrins serve to regulate cell motility and other processes such as cell polarity, cell growth and survival [65]. Integrins are a family of cell adhesion molecules that mediate cellular contacts to the extracellular matrix (ECM) proteins such as fibronectin and vitronectin. Integrins comprise transmembrane αβ heterodimers. At least 18 α and 8 β subunits are known in humans, resulting in 24 heterodimers with different expression patterns and different ligand binding capabilities which are dependent largely on the α and β subunits present [66, 67]. Each integrin subunit has a large extracellular, a short transmembrane and a small intracellular domain [62]. Integrins bind to extracellular ligands via their outer domains, whereas their cytoplasmic domains are linked to both structural and signalling molecules as well as the actin cytoskeleton. Ligand binding to integrins results in integrin clustering and recruitment of actin filaments and signalling proteins to the cytoplasmic domain of integrins [65]. In this capacity integrins serve to initiate the formation of ECM attachment and signalling centres, termed focal complexes in the nascent state and focal adhesions when they have matured into larger complexes [62, 65]. The formation of focal adhesions assures substrate adhesion of the cell as well as targeted location of actin filaments and signalling components resulting in cell polarity and targeted migration [65]. The focal adhesions further serve as traction points for contractile or tensile forces through their interaction with actin. Integrins are not always in an active conformation and can switch from a low affinity to a high affinity for extracellular ligands by activation of adaptor and signalling molecules that induce changes in the conformation of the integrin which are then in a suitable form for ligand binding. Disintegration of a salt bridge (‘non-covalent clasp’) between the cytoplasmic tails of α and β subunits of integrins results in the active conformation. The association can be disrupted by treatment with chemokines which signal via G-protein coupled

receptors that result in the phosporylation of the cytoplasmic domain of the β subunit resulting in the disintegration of the non-covalent clasp [66]. Chemokines are a superfamily of small, cytokine-like proteins that induce cytoskeletal rearrangement, firm adhesion to endothelial cells and directional migration as a result of their interaction with chemokine receptors (CKRs) [55]. CKRs belong to the large family of heptahelical G protein-coupled receptors (GPCR) and share more than 20 % sequence identity. Chemokines are divided into two main subfamilies according to their sequence homology and the arrangement of the first two cysteines. For CXC chemokines, the first two cysteines are separated by one amino acid whereas CC chemokines, the first two cysteine residues are adjacent [68, 69]. A chemokine of particular importance to breast cancer metastasis is stromal-derived factor-1 (SDF-1; also called CXCL12) of which the receptors are CXCR4 and CXCR7 [70]. SDF-1 is present as two nearly identical isoforms (SDF-1α and SDF-1β) [71]. The CXCR4 activates several different intracellular events such as chemotaxis, migration and invasion all of which are important properties of cancer metastasis [72]. Amounts of CXCR4 protein is low or absent in normal breast epithelium and hence CXCR4 is generally characteristic of malignant epithelial cells of tumours [71, 73].

1.11 Problem Statement

The intracellular Hsp90 chaperone complex is a widely accepted drug target. Intracellular client proteins of Hsp90 are subject to chaperone-assisted folding. Hsp90 has recently been reported to be expressed extracellularly where it is thought to mediate biological processes such as migration, invasion and metastasis. There are increasing reports in the literature of other components of the Hsp90 chaperone complex present extracellularly, including Hsp70 and Hop. This suggests the existence of an extracellular chaperone complex and that chaperone-assisted folding may occur extracellularly. Metastatic cancer is primarily responsible for cancer mortality, understanding the mechanisms that facilitate metastatic tumour progression is of fundamental importance to our understanding of cancer biology and the design of a metastatic cancer therapy. Currently no effective cancer therapy for metastatic cancer exists as metastasis is a complex process, the mechanisms of which are not yet fully understood. Because active migration of tumour cells is a prerequisite for tumour metastasis, one such method of approach to drug design is by studying the factors regulating the migratory activity of cancer cells. By understanding how Hsp90 functions extracellularly, the viability of extracellular Hsp90 as a potential drug target may be explored.

1.12 Hypothesis

Extracellular Hsp90 is present as part of a multi-chaperone complex that participates in the chaperone assisted folding of extracellular client proteins in a manner analogous to the intracellular chaperone complex. Biological processes such as migration will be regulated by this complex.

1.13 Objectives

- Characterise the expression of members of the Hsp90 multi-chaperone complex (Hsp90, Hsp70 and Hop) on the plasma membrane of cell models of varying degrees of adhesion and migratory capacity.

- Investigate the presence of an extracellular multi-chaperone complex by examining the interactions between extracellular Hsp90 and associated chaperones and co-chaperones.

- Examine the role of extracellular soluble and membrane associated Hsp90 in SDF-1 mediated migration

Chapter 2.

Materials and Methods

2.1 Materials

MCF-7 (HTB-22) and MDA-MB-231 (HTB-26) breast cancer cell lines were a kind gift from Dr Sharon Prince, University of Cape Town, South Africa. U937 (CRL-1593.2) and THP1 (TIB-202) monocytic leukemia cells were from lab stocks. All general reagents were purchased from Sigma-Aldrich; USA or Saarchem, Merck; South Africa. Tissue Culture media (10 X Trypsin-EDTA, foetal calf serum, Dulbecco's Modified Eagle Medium with GlutaMAX™-I and Penicillin Streptomycin were purchased from Gibco, Invitrogen; USA and Biowhittaker; UK respectively. Tissue Culture plasticware was purchased from Corning Incorporated; USA. Subcellular Fractionation kit was from Calbiochem. Western Blotting power pack, Hybond Support Nitrocellullose and Chemidoc^{TM} EQ were purchased from Bio-Rad; UK. Anti-Hsp90α/β [F-8] (cat no.: sc-13119), anti-Hsp90α/β [N-17] (cat no.: sc-1055), anti-Hsp70/Hsc70 (cat no.: sc-24) and anti-GAPDH [FL-335] (cat no.: sc-255778) antibodies were purchased from Santa Cruz Biotechnology. Anti-actin (cat no.: A2103), anti-vinculin (cat no.: V9131) and the Streptavidin peroxidase polymer (cat no.: S2438) were purchased from Sigma. Anti-Phospho-ERK1/2 (cat no.: AF1018) and Anti-ERK1/2 (cat no.: MAB1576) were purchased from R & D Systems. Anti-CD29 [β1 integrin] (cat no.: 610468) and mouse anti-Hsp90 (cat no.: 610418) were purchased from BD Biosciences. Anti-Hop (cat no.: SRA-1500-F) was purchased from Stressgen. Wheat Germ Agglutinin (WGA)-Alexa Fluor 555 conjugate (cat no. W32464), Alexa Fluor-488 donkey anti-mouse (cat no.: A21202), Alexa Fluor-633 donkey anti-goat (cat no.: A21082), Alexa Fluor-488 chicken anti-rabbit (cat no.: A21441), Alexa Fluor-543 donkey anti-rabbit (cat no.: A10040) were purchased from Invitrogen. Anti-Hsp90α (cat no.: SM 147A/B) and Anti-Hsp90β (SMC 107) were purchased from StressMarq. The vendors of any other specialised reagents are referenced within the text.

2.2 Methods

2.2.1 Maintenance of MCF- 7 and MDA-MB-231 cancer cell lines

MCF-7 and MDA-MB-231 breast cancer cells, were maintained in modified Dulbecco's Modified Eagle Medium (DMEM) media with GlutaMAX™-I, 5 % Foetal Calf Serum (FCS), penicillin-streptomycin (100 units/mL) at 37 °C, with 9 % CO_2.Suspension and adherent U937 and THP1 monocytic leukemia cell lines, were maintained in modified Roswell Park Memorial Institute (RPMI) 1640 media with GlutaMAX™-I, 10 % FCS, penicillin-streptomycin (100 units/mL) at 37 °C, with 9 % CO_2. Adhesion of U937 and THP1 cells was carried out with 20 nM Tetradecanoylphorbol-13-Acetate (TPA; Sigma) in complete RPMI for three days.

2.2.2 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Separation of proteins by SDS-PAGE was carried out according to the Laemmli [74]. Proteins were resolved using a 12 % stacking gel (0.5 M Tris-HCl, pH 6.8) and a 12 % resolving gel (1.5 M Tris-HCl, pH 8.8) at 180 V for 45 minutes in SDS-PAGE running buffer (0.25mM Tris , 192mM glycine, and 1 % (w/v) SDS). Samples were prepared in 5 X SDS-PAGE sample buffer (0.05M Tris-HCl, 10 % glycerol, 2 % SDS, 1 % Bromophenol blue, 5 % 2-mercaptoethanol) and boiled for 5-10 minutes. Protein Marker IV (peqGOLD) and PageRuler Plus (Fermentas) were used for estimating molecular weights of proteins.

2.2.3 Western analysis with chemiluminescence detection

Western Blot analysis was performed on resolved proteins according to Towbin [75]. Transfer of proteins from the SDS-PAGE gel to the nitrocellulose membrane was carried out in transfer buffer (13 mM Tris-HCl, 100 mM glycine and 20 % methanol) for 105 minutes at 120 V with continuous stirring at 4 ˚C. Ponceau Staining (0.5 % Ponceau S, 1 % glacial acetic acid) was performed to confirm transfer of proteins from the SDS-PAGE gel to the nitrocellulose membrane. The membrane was blocked with 10 % blotto (10 % fat free milk powder in Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl, pH 7.5) for one hour and incubated with a primary antibody overnight at 4 ˚C at the manufacturer's recommended dilution. The membrane was rinsed with Tris-buffered saline-tween (TBST; (1 % Tween-20 in TBS) for one hour, replacing the TBST at 15 minute intervals. Proteins were visualised using secondary antibodies conjugated to horseradish peroxidase. After an hour of incubation with the secondary antibody in 10 % blotto, membranes were rinsed in TBST for one hour. Detection of proteins was carried out using a chemiluminescence developing kit (Enhanced Chemiluminescence [ECL], GE Healthcare; UK) in the Chemidoc^{TM} EQ system, (Biorad; UK).

2.2.4 Cell Fractionation

To sequentially isolate cytoplasmic, membrane/organelle, nuclear and cytoskeletal proteins, cell fractionation was carried out using a compartmental protein extraction kit (ProteoExtract$^{®}$ Subcellular Proteome Extraction Kit (S-PEK); CALBIOCHEM) as per the manufacturer's instructions. Cell fractionation was performed on both untreated cells and cells treated with 100 ng/mL SDF-1β (Sigma) for 5 hrs under gentle agitation. The fractions were resolved by SDS-PAGE and analysed by Western analysis with chemiluminescence detection for proteins as indicated in the figure legends. Stripping of nitrocellulose membranes was done with Restore^{TM} Western Blotting stripping buffer (Thermo Scientific, USA) for 15 minutes at 37 ºC.

2.2.5 Surface protein Biotinylation and Streptavidin-Agarose affinity purification

Confluent adherent cancer cells (MDA-MB-231, MCF-7 and adherent U937 and THP1 cells) were washed with phosphate-buffered saline (PBS [137 mM NaCl, 27 mM KCl, 4.3 mM Na_2HPO_4, 4 mM KH_2PO_4]) and incubated in 1 mL PBS (pH 8.0) containing 1 mg/mL NHS-Biotin (Sigma) at 4 ˚C for 1 hour. A second flask (negative control) was incubated with PBS (pH 8.0) alone. Both biotinylated and non-biotinylated MDA-MB-231, MCF-7 and adherent THP1 and adherent U937 cells were washed with 5 mL PBS (pH 7.2) and scraped into 1 mL of PBS (pH 7.2). Suspension (U937 and THP1) cancer cells were collected by centrifugation (2000 rpm; 2 mins, 4 ˚C), washed with PBS (pH 7.2) and resuspended in 1 mL PBS (pH 8.0) containing 1 mg/mL NHS-Biotin at 4 ˚C for 1 hour. A second flask (negative control) was incubated with PBS (pH 8.0) and subsequently both adherent and suspension cells were treated the same. Cells were harvested by centrifugation and resuspended in 600 µL of Radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM ethyleneglycol-bis(beta-aminoethylether)N'N'N'N-tetraacetic acid/ ethylenediaminetetracetic acid (EGTA/EDTA), 1 mM Na_3VO_4, 1 % NP40, 1 mM Na Deoxycholate, 1 mM phenylmethanesulfonylflouride (PMSF) and a protease inhibitor cocktail). Cell lysis was performed at 4 ˚C with gentle agitation for 30 minutes. The lysates were cleared by centrifugation at 13000 rpm in a microcentrifuge tube for 5 minutes. 100 µL of the supernatant (pre-agarose) was prepared for Western analysis. 500 µL of the supernatant was incubated with 100 µL streptavidin-agarose beads (Thermo Scientific) equilibrated with PBS. The mixture was incubated for 1 hour at 4˚ C under gentle agitation. After centrifugation the supernatant (post-agarose) was prepared for Western analysis. After washing with PBS, beads were resuspended in SDS-PAGE sample buffer. Both biotinylated and non-biotinylated fractions were resolved by SDS-PAGE and analysed by Western analysis with chemiluminescence detection.

2.2.6 Indirect Immunofluorescence Assay and Confocal Microscopy

Cells were seeded at a density of 2 X 10^4 cells/mL into 4-well plates containing coverslips and incubated (37 ˚C; 9 % CO^2) overnight before treatment and staining for immunofluorescence. Coverslips pre-treated with fibronectin (Sigma) were coated with 250 µL/mL fibronectin, incubated at 4 ºC overnight, washed with media and cells subsequently seeded as described above. Cells were treated with SDF-1β (100 ng/mL) for two hours. Cells were fixed by flash treatment (±15 seconds) with methanol (-20 ˚C) and allowed to air dry, blocked with 1 % bovine serum albumin/ Tris-buffered saline (BSA/TBS) for 30 minutes at room temperature. Cells were incubated with primary antibodies; in 1 % BSA/TBS at 4 ˚C overnight. After incubation cells were washed twice in 0.1 % BSA/TBS followed by incubation with appropriate secondary

antibodies at 25 ˚C for 1 hr in the dark. Details of individual treatments and antibody staining are described in the figure legends. Cells were washed twice with 0.1 % BSA/TBS and the nucleus stained with Hoechst-33342 Dye (1 μg/mL) before mounting on coverslips. Immunoflorescence was visualised by a Zeiss LSM 510 Meta confocal microscope and analysed using AxiovisionLE 4.7.1 (Zeiss). Imaging was performed using a 63x oil objective. Quantification of the co-localisation analysis was performed using the Zeiss LSM 5 software. Confocal figures have been saved to a compact disc for visualisation purposes.

2.2.7 Bis[sulfosuccinimidyl]suberate (BS^3) and 3,3'-dithiobis[sulfosuccinimidyl[propionate] (DTSSP) Crosslinking

Cells were harvested by centrifugation (3300 *g*, 5 mins) and washed X 3 in PBS at pH 8.0 (BS^3) or pH 7.2 (DTSSP) and incubated for one hour at 4 ºC in BS^3 (5 mM) or for 2 hours at 4 ºC DTSSP (2 mM). The crosslinking reaction was quenched by the addition of the quenching solution, Tris-HCl (pH 7.5) to a final concentration of 20 mM for 15 minutes at room temperature.

2.2.8 Immunoprecipitation

MB-MDA-231 breast cancer cells (1 X 10^7) were washed with PBS (pH 7.5). Cells were harvested by centrifugation and resuspended in 1 mL of RIPA buffer. Cell lysis was performed at 4 ˚C with gentle agitation for 30 minutes. The lysates were cleared by centrifugation at 12000 *g* for 30 minutes at 4 ˚C. 500 μL of supernatant was added to 2 μg of primary antibodies (see figure legends for antibody details) and 500 μL of supernatant served as a control (no antibody) and incubated for two hours at 4 ˚C and precipitated with 80 μL of Protein A/G agarose beads (Santa Cruz Biotechnology) overnight at 4 ˚C. Beads were washed X 4 in PBS and resuspended in 5 X SDS-PAGE sample buffer. Samples were resolved by SDS-PAGE and analysed by Western analysis with chemiluminescence detection as described in previously.

2.2.9 Crosslinking and Biotin-Streptavidin-Agarose Affinity Purification

One T75 flask of adherent MDA-MB-231 cells was treated with SDF-1 (100 ng/mL) for 2 hours at 37 ºC and one flask of cells remained untreated. Both flasks of MDA-MB-231 cells were incubated in 1 mg/mL NHS-Biotin for 1 hour, lifted with EDTA (3 mM), harvested by centrifugation (3300 rpm) and washed in PBS (pH 7.2). MDA-MB-231 cells were then crosslinked using the cell impermeable crosslinker BS^3 as described in section (2.2.7). Subsequent to the quenching of the crosslinker, the cells were washed in PBS (pH 7.2) and biotin-streptavidin agarose affinity purification carried out as described previously (2.2.5).

2.2.10 Dynabeads Immunoprecipitation

Cell surface proteins of MDA-MB-231 cells were crosslinked using the cell impermeable cleavable crosslinker DTSSP as described in section (2.2.7). After crosslinking cells were washing in PBS (pH 7.5) and then co-immunoprecipitation performed using the Dynabeads® Co-immunoprecipitation kit (Invitrogen) as per the manufacturer's instructions.

2.2.11 Biotinylation, Crosslinking and Immunoprecipitation.

Adherent MDA-MB-231 cells were incubated in 1 mg/mL NHS-Biotin for 1 hour, lifted with EDTA (3 mM), harvested by centrifugation (3300 rpm), washed in PBS (pH 7.2) and incubated for two hours at 4 ºC in DSSTP (Pierce) in PBS (pH 7.2) to a final concentration of 2 mM. The crosslinking reaction was quenched by the addition of Tris-HCl (pH 7.5) at a final concentration of 20 mM for 15 minutes at room temperature. Cells were washed in PBS (pH 7.2) and then co-immunoprecipitation performed with a mouse anti-Hsp90α/β antibody using the Dynabeads® Co-immunoprecipitation kit (Invitrogen) as per the manufacturer's instructions. The crosslinker is cleavable in sample buffer containing 5 % β-mercaptoethanol.

2.2.12 Wound Healing Assays

MDA-MB-231 cells were seeded at a density of 1 X 10^6 cells/mL into 8-well chamber slides (pre-treated with fibronectin at 4 ºC overnight) and incubated at 37 ºC until confluent. Cells were pre-treated with IgG1 (20 µg/mL), AMD3100 Octahydrochloride (AMD3100; Sigma [10 µM]),Mouse anti-Hsp90 (20 µg/mL), anti-Hsp90 α/β [N-17] (20 µg/mL), soluble Hsp90β (StressMarq [100 ng/mL]) or a combination of AMD3100 (10 µM) and anti-Hsp90 α/β [N-17] (20 ng/mL) and AMD3100 (10 µM) and soluble Hsp90β (100 ng/mL) at 4 ºC with the for 30 mins. Wounds were made by scratching the cell confluent monolayer with a p200 pipette tip followed by incubation at 37 ºC. Wound images were taken at 0 hrs and 12 hrs with a Nikon camera (Coolpix 990).

2.2.13 Protein Kinase Analysis

MDA-MB-231 cells were grown to confluency in 35 mm dishes. Cells were pre-treated with AMD3100 (10 µM) for 1 hour at 37 ºC. Cells were treated with anti-Hsp90α/β [N-17], anti-Hsp90 and soluble Hsp90β. Details of concentrations and times of treatment can be found in the figure legends. Cells were harvested by lysis in SDS-PAGE sample buffer, frozen at -20 ºC and lysates analysed by SDS-PAGE and Western analysis for pERK1/2 and ERK1/2 following stripping of the nitrocellulose membrane.

Chapter 3.

Expression of Heat Shock Proteins on the Plasma Membrane of Cancer Cells

3.1 Introduction

The plasma membrane is often thought of as a barrier between the cytosol and the extracellular space. However, the plasma membrane is a dynamic organelle in its own right as it serves to form the interface for communication between the extracellular space and the intracellular components of the cell [76]. An number of proteins, traditionally considered to be intracellular, have recently been found bound to the plasma membrane or as soluble proteins in extracellular space [77].

In the literature, extracellular is the term denoted to describe the expression of the Hsps when they are both membrane associated or present as soluble proteins in the extracellular space. This term (extracellular) will be used to refer to both membrane associated and soluble extracellular forms of Hsp90 in this thesis. Extracellular soluble proteins may associate with receptors on the plasma membrane. Many Hsps have been found on the membrane or as extracellular proteins. Hsp90 has been identified on the surface of certain cancer cell types including melanoma [24, 25], breast carcinoma [26], fibrosarcoma [27], human bladder carcinoma, [28], colorectal carcinoma [29] and lymphoma [30]. Hsp90 was present in the conditioned media of HT-1080 fibrosarcoma cells and MDA-MB-231 breast cancer cells [34]. Hsp90 was revealed to be secreted from both human keratinocytes and smooth muscle cells [47, 49]. Hsp70 is frequently present on the plasma membrane of colon, lung, pancreas, mammary, head and neck carcinomas and metastases derived thereof [3]. Bone marrow-derived leukemic blasts from patients with acute and myeloid leukemia are frequently found to have membrane associated Hsp70 [3]. *In vitro* Hsp70 has been detected in the supernatants of cultured antigen presenting cells and tumour cell lines [35]. Hop was found at the surface of a glioblastoma-derived cell line and found to be secreted from the glioblastoma-derived cell line as well as found in the conditioned media of HT-1080 fibrosarcoma cells [21, 34, 44]. Hsp25 was found on the surface of murine breast carcinoma 4T1 cells [78]. GP96, the Hsp90 ER homolog has been shown to be expressed on the plasma membrane, yet only in tumour cells [41]. Hsp47, was shown to be expressed on the surface of oral cancers [79]. Hsp60 has been found in the extracellular space of tumour cells as well as shown to associate extracellularly with the plasma membrane [80].

Extracellular membrane associated and extracellular soluble, Hsp90 appears to have distinct roles in signalling pathways leading to cell motility, invasion and hence metastasis [26, 45]. The cell impermeable Hsp90 inhibitor DMAG-N-oxide, significantly retarded tumour cell migration [28]. In the developing nervous system, Hsp90α and Hsp90β are present on the plasma membrane of neuronal cells and are involved in cell migration process such as the reorganisation

of the actin cytoskeleton [13]. It was reported that Hsp90α secreted from fibrosarcoma cells promoted the maturation of MMP-2 to promote tumour invasiveness [27]. In endothelial cells Hsp70 was shown to be expressed on the cell surface of melanoma metastases but the corresponding skin fibroblasts were shown to be Hsp70 negative suggesting a role for Hsp70 in metastasis [50].

Cell migration is largely governed by the ability of the integrin family of cell adhesion molecules to regulate adhesion to the ECM [65]. At the leading edge of migrating cells, integrins bind the ECM, recruit actin and promote actin polymerisation resulting in membrane ruffles at the leading edge (termed lamellipodia and or peripheral ruffles) [64, 65, 81]. At the rear end of the cell integrins detach from the ECM, allowing the cell to get pushed forward in the direction of cell migration [64, 65]. One method of integrin activation is via chemokine/G-coupled receptor signalling [66]. Chemokines, such as SDF-1, also induce a distinct polarised cell morphology and cell surface receptor distribution which also facilitates cell migration [82].

Given that extracellular Hsp90 has been shown to be involved in migration, the objective of this chapter was to characterise the membrane expression of Hsp90 and the chaperones and co-chaperones, Hsp70 and Hop, in different cell models of different adhesive or migratory capacity. The MCF-7 and MDA-MB-231 breast cancer cell lines are adherent cell lines whereas U937 and THP1 monocytic leukemia cells are suspension cell lines [83, 84]. MDA-MB-231 cells are metastatic whereas MCF-7 cells are non-metastatic [85, 86]. Hsp90, Hsp70 and Hop expression was studied in THP1 and U937 cells following the phorbel ester induced adhesion of the cells [87]. The effect of migration on Hsp membrane expression was studied in response to SDF-1 stimulation of migration.

3.2 Results

In order to characterise the membrane expression of Hsp90, Hsp70 and Hop in different cell models of varying degrees of adhesion and migratory capacity, a combination of three different techniques (subcellular fractionation, biotin-streptavidin affinity purification and immunofluorescence) were used.

3.2.1 Subcellular fractionation analysis of cancer cell lines to reveal membrane association of Hsps

Detergent extracted cytosolic, membrane/organelle, nuclear and cytoskeletal fractions were obtained by subcellular fractionation of MDA-MB-231 and MCF-7 breast cancer cell lines. Cells were either pre-treated with SDF-1 or left untreated. Hsp90, Hsp70 and Hop were identified in the putative cytosolic, membrane/organelle, nuclear and cytoskeletal fractions by Western analysis (Figure 3). A MDA-MB-231 whole cell lysate was used as a positive control for Western analysis.

Hsp90 was predominantly present in the cytoplasmic and membrane fractions of MDA-MB-231 and MCF-7 cells, with lower levels present in the nuclear and cytoskeletal fractions (Figure 3: Upper Panel). A strong Hsp70 signal was revealed in the cytoplasmic, membrane, nuclear and cytoskeletal fractions of both MDA-MB-231 and MCF-7 breast cancer cells (Figure 3: Upper Panel). Hop was predominantly present in the cytoplasmic fraction with lower levels present in the membrane fractions of both MDA-MB-231 and MCF-7 cells (Figure 3: Upper Panel). Following SDF-1 treatment Hsp90 was not detected in the nuclear fraction of MDA-MB-231 (Figure 2: Lower Panel). The expression pattern of Hsp90, Hsp70 and Hop did not change for MCF-7 cells after treatment with SDF-1 (Figure 3: Lover Panel). There appeared to be no change in the localisation of Hop in the fractions of either MDA-MB-231 or MCF-7 cells following SDF-1 treatment (Figure 3: Lower Panel).

Fraction markers were used to show the degree of contamination of the membrane fraction in other fractions. The fraction markers used were GAPDH (cytoplasmic protein), the β1 integrin (membrane protein) and actin (cytoplasmic and membrane protein). GAPDH was present in the cytosolic and membrane fractions of untreated MDA-MB-231 and MCF-7 breast cancer cells (Figure 3: Upper Panel). Very low levels of GAPDH were revealed in the nuclear fraction of MDA-MB-231 and MCF-7 breast cancer cells (Figure 3: Upper Panel). The β1 integrin was predominantly present in the membrane fraction of untreated MDA-MB-231 and MCF-7 cells with a small amount present in the cytoplasmic fraction (Figure 3: Upper Panel). Actin was present in the cytoplasmic, membrane, nuclear and cytoskeletal fractions of MDA-MB-231 breast cancer cells where as it was only present in the cytoplasmic and membrane fractions and in low amounts in the nuclear fraction of MCF-7 breast cancer cells (Figure 3: Upper Panel).

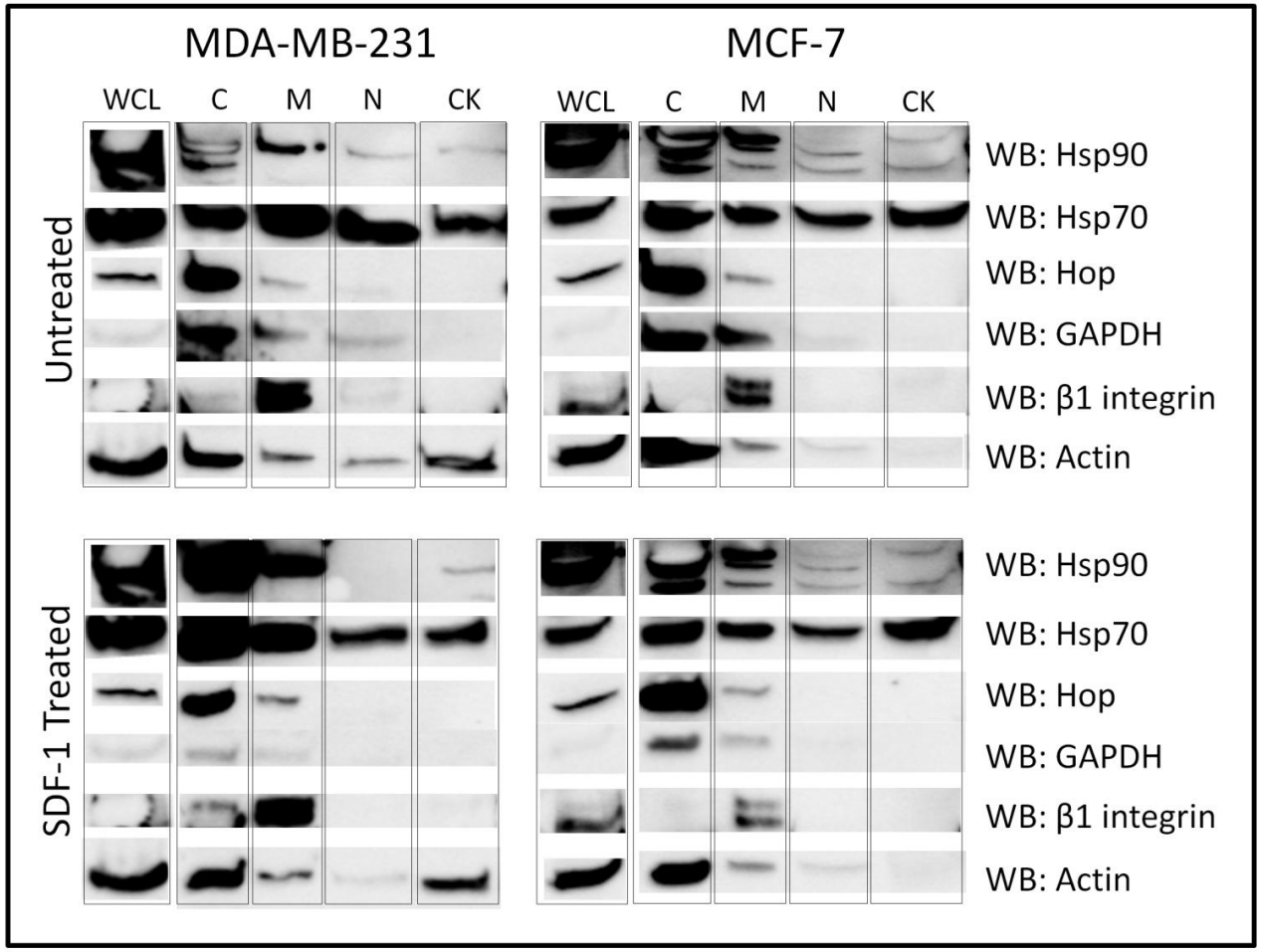

Figure 3: Hsps are membrane associated in MDA-MB-231 and MCF-7 breast cancer cells.

Subcellular fractionation into four fractions (cytoplasm [C], plasma and organelle membranes [M], nucleus [N], cytoskeleton [CK]) in untreated (upper panel) and SDF-1(100 ng/mL) treated (lower panel) MD-MBA-231 and MCF-7 breast cancer cells, followed by Western analysis to detect the localisation of Hsp90, Hsp70 and Hop in the fractions. A MDA-MB-231 whole cell lysate (WCL) was used as a positive control. GAPDH, the β1 integrin and actin were used as fraction markers to validate the specificity of the subcellular fractionation procedure.

Upon treatment with SDF-1, GAPDH was present in the cytoplasmic and membrane fractions and no longer appeared in the membrane fraction of MDA-MB-231 cells (Figure 3: Lower Panel). In MCF-7 cells GAPDH was still present in the cytoplasmic and membrane fractions (Figure 3: Lower Panel). There was no change in the localisation or a considerable change in the expression levels of the β1 integrin in the fractions following SDF-1 treatment in both MDA-MB-231 and MCF-7 cells (Figure 3: Lower Panel). Following SDF-1 treatment there was no change in the localisation of actin in either the MDA-MB-231 or MCF-7 cells although a decrease in the actin levels in the nuclear fraction of MDA-MB-231 cells occurred (Figure 3: Lower Panel).

Similarly, detergent extracted cytosolic, membrane/organelle, nuclear and cytoskeletal fractions were obtained by subcellular fractionation of U937 and THP1 monocytic leukemia cell lines with and without SDF-1 treatment. This was repeated in the leukemia cell lines after phorbel ester induced adhesion with TPA [87]. Hsp90, Hsp70 and Hop were identified in the putative cytosolic, membrane/organelle, nuclear and cytoskeletal fractions by Western analysis. An MDA-MB-231 whole cell lysate was used as a positive control for Western analysis.

Both Hsp90 and Hsp70 were present at similar levels in the cytoplasmic and membrane fractions of untreated U937 cells (Figure 4: Upper Panel). Similarly, Hsp90 was present in the cytoplasmic and membrane fractions of untreated THP1 cells with low levels revealed in the cytoskeletal fraction (Figure 4: Upper Panel). Hsp70 was present in the cytoplasmic, membrane and nuclear fractions of untreated THP1 cells (Figure 4: Upper Panel). Hop was only present in the cytoplasmic fractions of both untreated U937 and THP1 cells (Figure 4: Upper Panel). Following SDF-1 treatment Hsp90 and Hsp70 remained in the cytoplasmic and membrane fractions in U937 and THP1 cells and a decrease in the levels of Hsp70 in the nuclear fraction of THP1 cells occurred (Figure 4: Middle Panel). Hop was only present in the cytoplasmic fraction of THP1 and U937 cells, although very low levels were present in the membrane fraction of U937 cells following SDF-1 treatment (Figure 4: Middle Panel). Following adhesion there was no change in the localisation of Hsp90 and Hsp70 in U937 and THP1 cells, although low levels of Hsp70 were now present in the nuclear fraction of U937 cells and strong signals for Hsp90 and Hsp70 occurred in the cytoskeletal fractions of THP1 cells (Figure 4: Lower Panel). Hop was present in the cytoplasmic fraction of adherent U937 and THP1 cells and at low levels in the membrane fractions of both cell lines following adhesion (Figure 4: Lower Panel).

Similarly, GAPDH, the β1 integrin and actin were used as fraction markers for THP1 and U937 cells. In untreated U937 and THP1 cells, GAPDH was predominantly present in the cytoplasmic

fraction with low levels detected in the membrane fraction (Figure 4: Upper Panel). In U937 cells, the β1 integrin was present at low levels in the membrane and cytoplasmic fractions (Figure 4: Upper Panel). In THP1 cells the β1 integrin was localised to the membrane fraction with a small amount in the cytoplasmic fraction (Figure 4: Upper Panel). Actin was predominantly present in the cytoplasmic fraction with low levels in the membrane fraction of U937 cells and only present in the cytoplasmic fraction of THP1 cells (Figure 4: Upper Panel). There was no change in the localisation of actin, the β1 integrin or actin following SDF-1 treatment in U937 or THP1 cells although actin was no longer present in the membrane fraction of THP1 cells (Figure 4: Middle Panel). Following adhesion of U937 and THP1 cells GAPDH remained in the cytoplasmic and membrane fractions with low levels present in the nuclear fraction of U937 cells (Figure 4: Lower Panel). There was a considerable increase in the β1 expression levels following TPA induced adhesion and the β1 integrin was present in both the cytoplasmic and membrane fractions of U937 cells (Figure 4: Lower Panel). In the THP1 cells there appeared to be an increase in the levels of the β1 integrin, present in the cytoplasmic and membrane fractions (Figure 4: Middle Panel). Actin was present in both the cytoplasmic and membrane fractions of U937 cells and present in the cytoplasmic, membrane and cytoskeletal fractions in THP1 cells following TPA induced adhesion (Figure 4: Middle Panel).

To investigate whether a change in the proportion of Hsp90, Hsp70 and Hop in the membrane fraction upon treatment with SDF-1 occurred, density analysis was performed on the cytoplasmic and membrane fractions as obtained by subcellular fractionation. By immunofluorescence we revealed that MDA-MB-231 cells were CXCR4 positive (*data not shown)*. This is in accordance with literature as it has previously been shown that both MCF-7, MDA-MB-231, U937 and THP1 cells express CXCR4 [72, 73, 88]. In order to ensure that protein levels could be compared, the nitrocellulose membranes were stripped and reprobed. The density of the signal, representative of the proteins levels of Hsp90, Hsp70 and Hop as detected by Western analysis in the cytoplasmic and membrane fractions were normalised relative to the density of the signal obtained by Western analysis for actin and the β1 integrin respectively by dividing the value obtained for the density of the Hsp signal by that of actin and the β1 integrin in the cytoplasmic and membrane fractions respectively. Approximately similar levels of actin in the cytoplasmic fraction and the β1 integrin in the membrane fraction were observed with or without treatment with SDF-1. These markers were chosen because the subcellular localisation is well documented and the levels were not expected to change with SDF-1 treatment. Density analysis of the Hsp90, Hsp70, Hop, actin and the β1 integrin were computed using ImageJ.

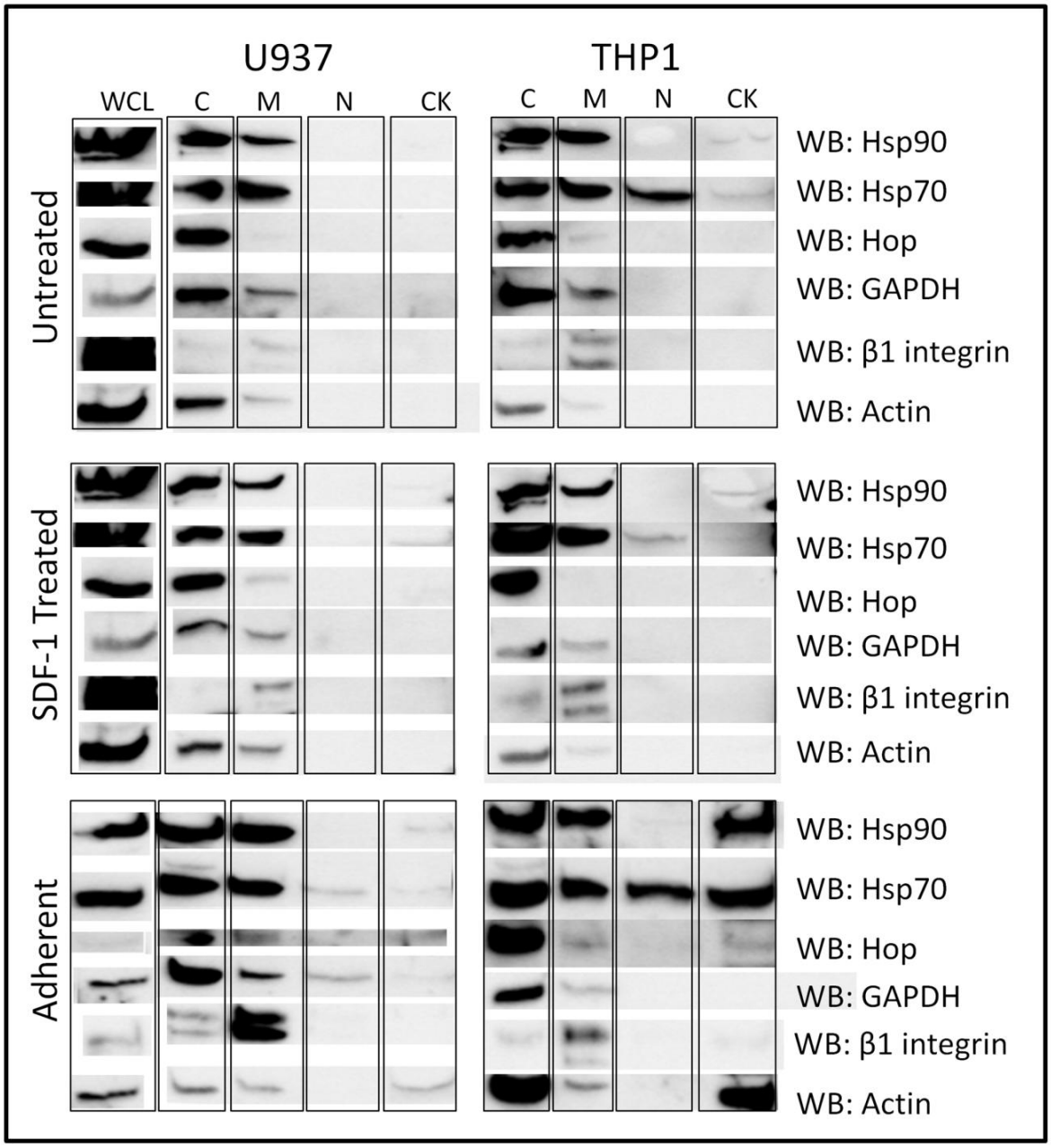

Figure 4: Selected Hsps are membrane associated in THP1 and U937 leukemia cells.

Subcellular fractionation into four fractions (cytoplasm [C], plasma and organelle membranes [M], nucleus [N], cytoskeleton [CK]) in untreated (upper panel), SDF-1(100 ng/mL) treated (middle panel), and TPA (20 nM) induced adherent (lower panel) THP1 and U937 monocytic leukemia cells, followed by Western analysis to detect the localisation of Hsp90, Hsp70 and Hop in the fractions. A MDA-MB-231 whole cell lysate (WCL) was used as a positive control. GAPDH, the β1 integrin and actin were used as fraction makers to validate the specificity of the subcellular fractionation procedure.

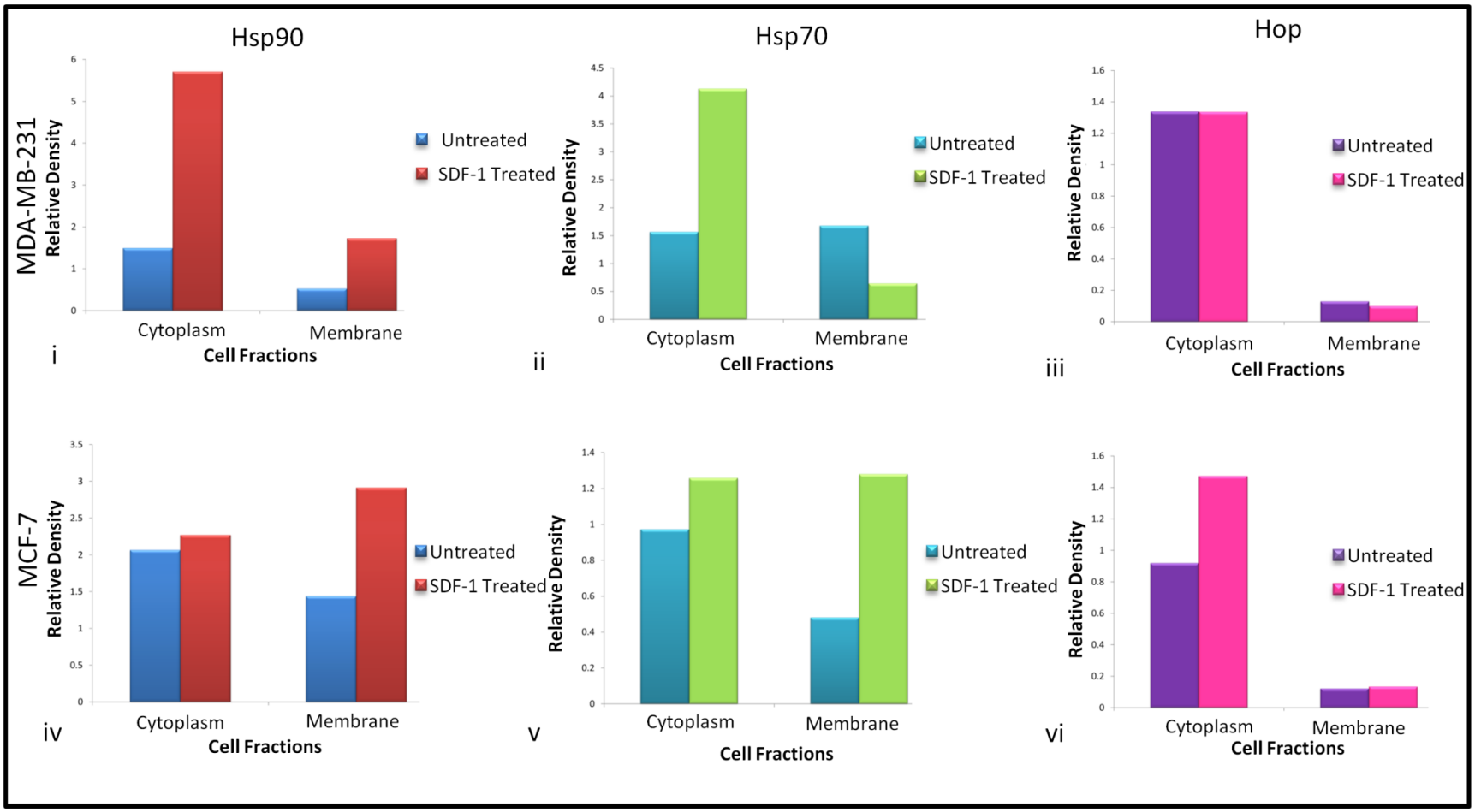

Figure 5: Density Analysis of Hsp90, Hsp70 and Hop cytoplasmic and membrane expression levels in response to SDF-1-stimulated migration.

The relative density of Hsp90, Hsp70 and Hop signal intensity as revealed by subcellular fraction and Western analysis in untreated and SDF-1 treated THP1 and U937 cells (Figure 4) was determined. The Hsp signal intensity was normalised to that of the actin (cytoplasmic fraction) and the β1 integrin (membrane fraction) signal intensity by dividing the value obtained for the density of the Hsp signal by that of actin and the β1 integrin in the cytoplasmic and membrane fractions respectively to obtain the relative density: MDA-MB-231 cells (i-iii) and MCF-7 cells (iv-vi). Density values of the Hsp and actin and the β1 integrin were computed using ImageJ.

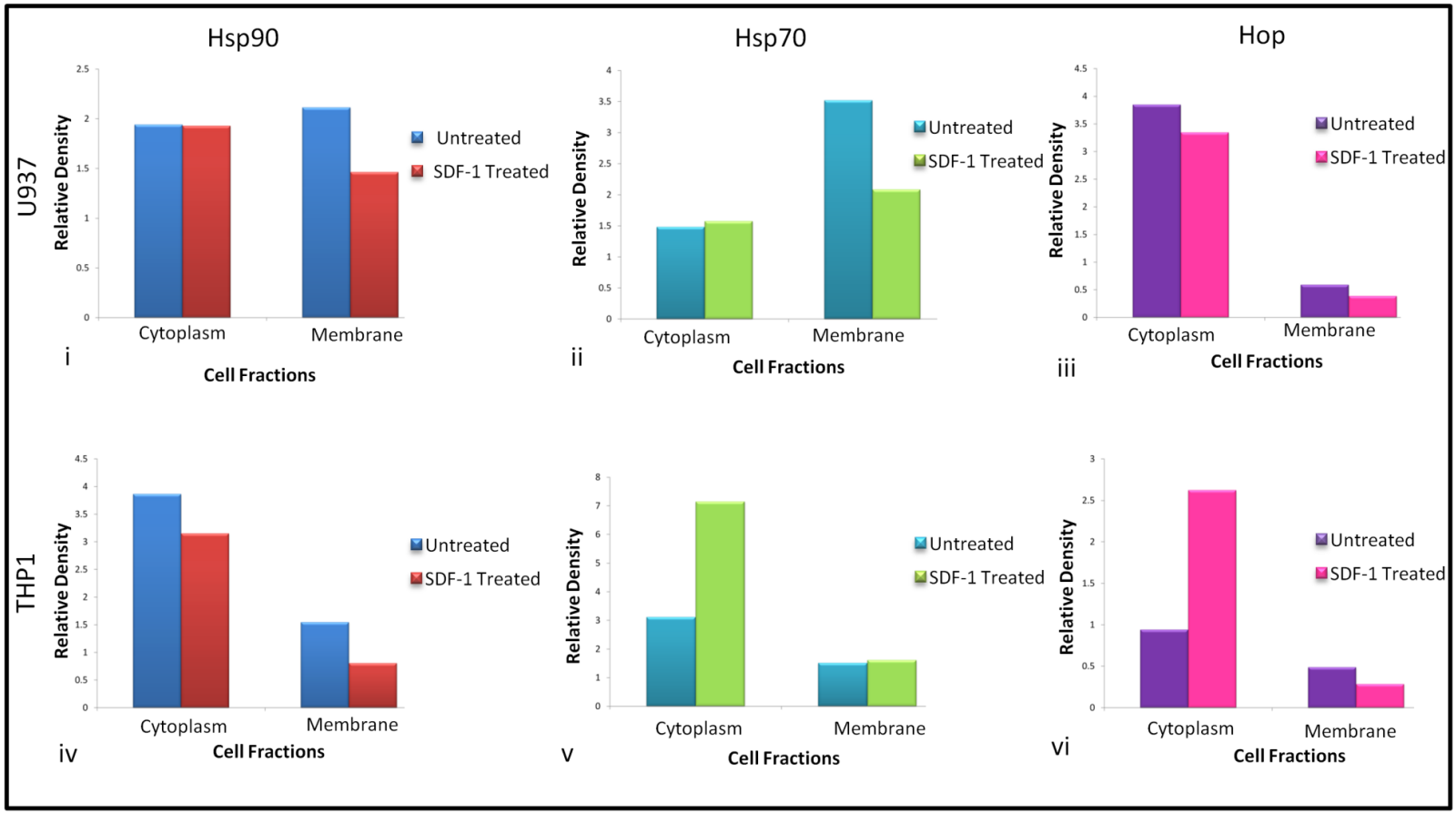

Figure 6: Density Analysis of Hsp90, Hsp70 and Hop cytoplasmic and membrane expression levels in response to SDF-1-stimulated migration

The relative density of Hsp90, Hsp70 and Hop signal intensity as revealed by subcellular fraction and Western analysis in untreated and SDF-1 treated THP1 and U937 cells (Figure 4) was determined. The Hsp signal intensity was normalised to that of the actin (cytoplasmic fraction) and the β1 integrin (membrane fraction) signal intensity by dividing the value obtained for the density of the Hsp signal by that of actin and the β1 integrin in the cytoplasmic and membrane fractions respectively to obtain the relative density: U937 cells (i-iii) and THP1 cells (iv-vi). Density values of the Hsp and actin and the β1 integrin were computed using ImageJ.

Density analysis suggested an increase in the relative density of the signal obtained for Hsp90 relative to actin of the β1 integrin in the cytoplasmic and membrane fractions of MDA-MB-231 cells respectively in response to SDF-1 (Figure 5i). An increase in the relative density of Hsp70 was revealed in the cytoplasmic fraction although there was a decrease in the relative density of Hsp70 in the membrane fraction following SDF-1 treatment (Figure 5ii). There was no change in the relative density of the Hop signal in either the cytoplasmic or membrane fractions in untreated as compared to SDF-1 treated cells (Figure 5iii). In MCF-7 cells there was a slight increase in the relative density of the Hsp90 signal in the cytoplasm and an increase in the relative density of the signal of Hsp90 in the membrane fraction in response to SDF-1 treatment (Figure 5iv). SDF-1 treatment resulted in an increase in the relative density of the signal of Hsp70 in both the cytoplasmic and membrane fractions of MCF-7 cells (Figure 5v). Although there appeared to be an increase in the relative density of the Hop signal in the cytoplasmic fraction of MCF-7 cells there was no change in the relative density revealed of the Hop signal in the membrane fraction following SDF-1 treatment (Figure 5vi).

3.2.2 Analysis of extracellular membrane association of Hsp90, Hsp70 and Hop by biotin-streptavidin affinity purification

The subcellular fractionation data showed that Hsp90, Hsp70 and Hop were identified in membrane fractions of cancer cells. However, this included membranes of intracellular organelles such as the mitochondrial membrane. Biotin-streptavidin affinity purification can be used to analyse extracellular membrane associated proteins, proteins that are present on the plasma membrane of cells and positioned such that at least part of the protein is located extracellularly or located as soluble proteins in the extracellular space and associate with the plasma membrane. Biotin-streptavidin affinity purification is based on the covalent binding of a cell-impermeable biotin moiety to lysine residues of extracellular proteins, followed by cell lysis and the selective isolation of the biotinylated surface proteins with streptavidin-agarose affinity beads (Figure 7A). The specificity of the biotinylation was determined by Western analysis with Streptavidin peroxidase. Streptavidin peroxidase specifically binds biotinylated proteins. Following biotinylation of extracellular proteins and cell lysis the cell lysate was cleared by centrifugation. Both the supernatant pre-incubation with the streptavidin-agarose beads (SPRA) and the cell pellet (P), showed high levels of extracellular biotinylated proteins (Figure 7B). High levels of biotinylated proteins were detected in the supernatant post incubation with the streptavidin-agarose beads (SPA) suggesting that not all biotinylated proteins were affinity purified (Figure 7B). Affinity purified biotinylated extracellular membrane proteins [AP(B+)] were detected by streptavidin peroxidase (Figure 7B). No biotinylated proteins were present in

the affinity purified fraction when cells weren't treated with a biotin reagent [AP(B-)] and similarly no biotinylated proteins were present in the non-biotinylated whole cell lysate [WCL(B-)] (Figure 7B). This was similarly repeated for the MCF-7, U937 and THP1 cell lines with similarly no biotinylated proteins present in the affinity purified fraction when cells weren't treated with a biotin reagent or in a non-biotinylated whole cell lysate as revealed by streptavidin peroxidase (data not shown). Subsequently the non-biotinylated whole cell lysate [WCL (B+)], affinity purified biotinylated extracellular membrane proteins [AP(B+)], affinity purified non-biotinylated extracellular membrane proteins [AP(B-)] and the supernatant post incubation with the streptavidin-agarose beads (SPA) were analysed by Western analysis to detect Hsp90, Hsp70 and Hop (Figure 8). Equal volumes of fractions from equivalent numbers of cells were loaded. The whole cell lysate served as a positive control for expression of Hsp90, Hsp70 and Hop. The affinity purification was performed in the absence of biotin and the affinity purified non-biotinylated extracellular membrane proteins [AP(B-)] analysed by Western analysis to show that Hsp90, Hsp70 and Hop did not non-specifically associate with the streptavidin-agarose beads (Figure 8). The β1 integrin was detected as a positive control for membrane associated extracellular proteins (Figure 8). Hsp90, Hsp70 and Hop were present in the affinity purified biotinylated extracellular membrane fraction of MDA-MB-231 cells and MCF-7 cells (Figure 8A and 8B). In MDA-MB-231 and MCF-7 cells the anti-Hsp90α/β antibody detected two bands of different mobilities, one at ~90 kDa and the other at ~70kDa (Figure 8A and 8B). In U937 cells neither Hsp90, Hsp70, Hop, nor, the β1 integrin were present in the affinity purified biotinylated extracellular membrane fraction (Figure 8C). Following TPA-induced adhesion low levels of Hsp90, Hsp70, Hop and the β1 integrin were present in the affinity purified biotinylated extracellular membrane associated fraction of U937 cells (Figure 8C). In THP1 cells Hsp90, Hsp70 and Hop were present in the affinity purified biotinylated extracellular membrane associated fraction, although there was no β1 integrin isolated in the extracellular membrane fraction (Figure 8D). Following TPA-induced adhesion Hsp90, Hsp70, Hop and the β1 integrin were detected at high levels in the affinity purified biotinylated extracellular membrane fraction (Figure 8D). High levels of Hsp90, Hsp70 and Hop were present in the supernatant post incubation with the streptavidin-agarose beads. This could be intracellular Hsps or indicate that the streptavidin-agarose column had been saturated (Figure 8). In order to ensure that no internalization of the cell-impermeable biotin reagent had occurred and only extracellular proteins were being isolated the affinity purified biotinylated extracellular membrane fraction should have been probed for ERK1/2 or another known intracellular protein.

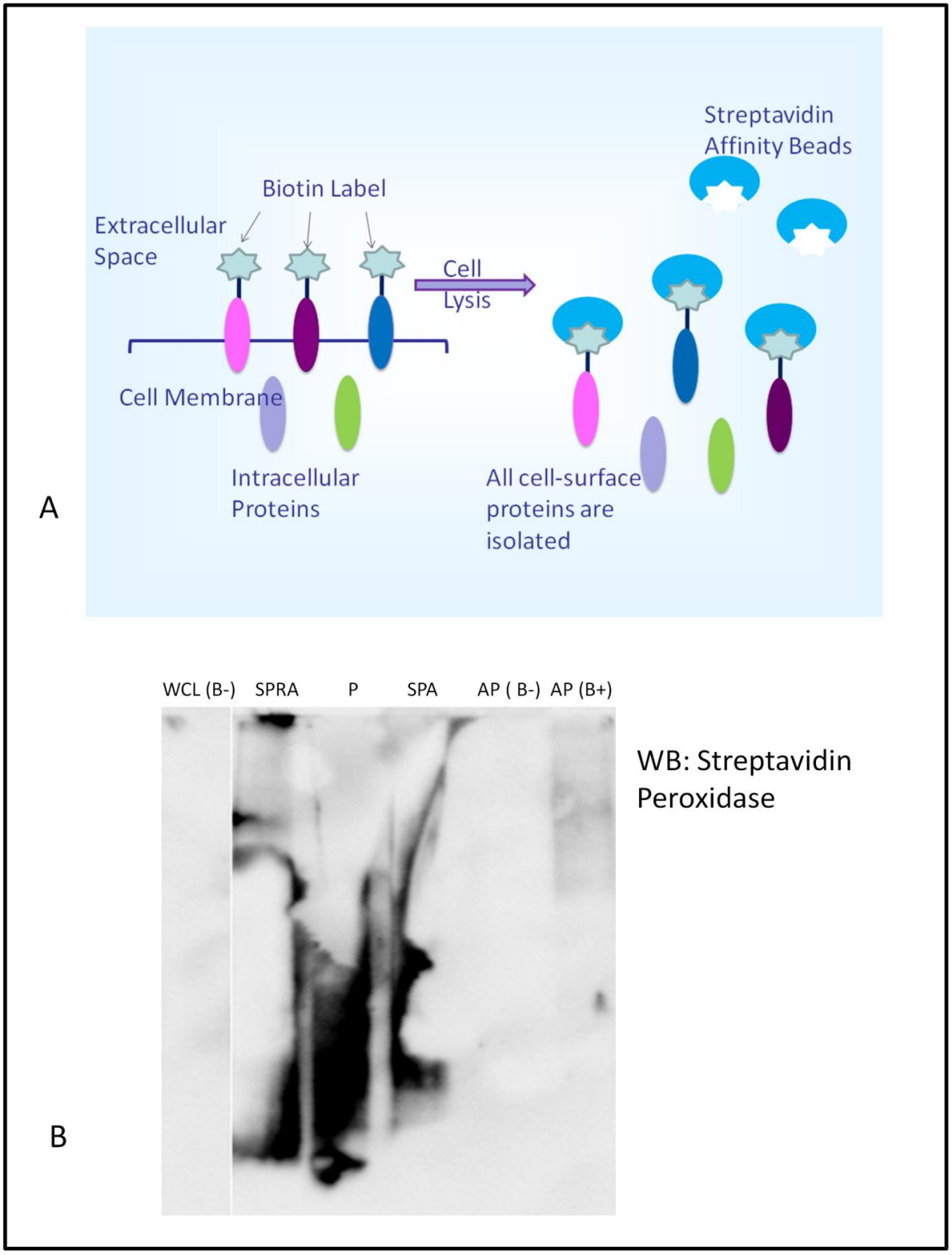

Figure 7: Determination of the specificity of extracellular biotinylation

(A) of biotin labelling of membrane associated extracellular proteins followed by isolation with streptavidin-agarose affinity beads. B) Specificity of biotinylation was determined by Western analysis using streptavidin peroxidase to detect biotinylated proteins. Supernatant pre-incubation with streptavidin-agarose (SPRA), cell pellet (P), supernatant post incubation with streptavidin-agarose (SPA), affinity purified biotinylated extracellular membrane proteins [AP(B+)], affinity purified non-biotinylated extracellular membrane proteins [AP(B-)] and a non-biotinylated whole cell lysate [WCL (B-)].

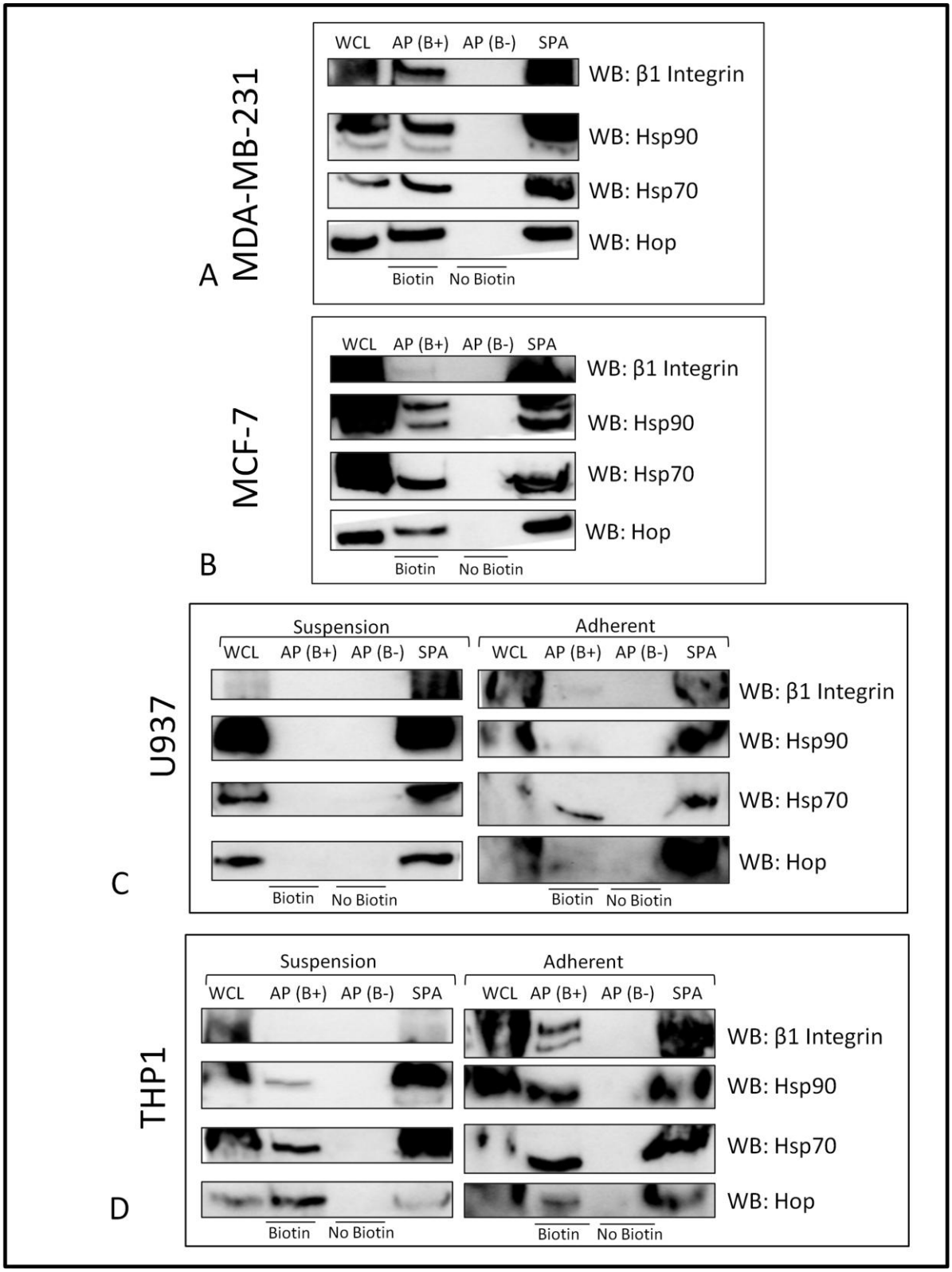

Figure 8: Selected Hsps are membrane associated and extracellular in cancer cells.

Membrane associated extracellular proteins were treated with or without NHS-Biotin followed by affinity purification using streptavidin- agarose. Eluted biotinylated and non-biotinylated (control) fractions were analysed by Western analysis to detect Hsp90, Hsp70, Hop and the β1 integrin in a biotinylated whole cell lysate (WCL), affinity purified biotinylated membrane associated extracellular proteins [AP (B+)], affinity purified non-biotinylated membrane associated extracellular proteins [AP (B-)] and supernatant post incubation with streptavidin-agarose (SPA). Equal volumes of fractions were loaded from an equivalent number of cells. The β1-integrin was detected as a positive control for membrane bound proteins.

3.2.3 Antibody specific analysis of extracellular Hsp90 isoforms

Hsp90 in the cytoplasm exists as two isoforms, Hsp90α and Hsp90β, and hence an antibody specific approach was used to determine whether Hsp90 in the membrane fractions as identified by subcellular fractionation and biotin-streptavidin affinity purification was Hsp90α, Hsp90β or both. The membrane fractions of both the untreated and SDF-1 treated MDA-MB-231, MCF-7, THP1 and U937 cells as well as the adherent U937 and THP1 cells were analysed by Western analysis for Hsp90α and Hsp90β (Figure 9).

Both Hsp90α and Hsp90β were detected in the membrane fractions obtained by subcellular fractionation, of both untreated and SDF-1 treated, MCF-7 and MDA-MB-231 cells (Figure 9 A). Hsp90β was present in the subcellular membrane fraction of untreated, SDF-1 treated and adherent THP1 and U937 cells (Figure 9A). Hsp90α was present at low levels in the membrane fraction of untreated THP1 cells, although no Hsp90α was present in the membrane fraction of SDF-1 treated THP1 cells, untreated U937 cells and SDF-1 treated U937 cells (Figure 9A). Following TPA-induced adhesion, Hsp90α was present in the membrane fractions of THP1 cells at an apparent increased level and in the membrane fraction of U937 cells (Figure 9A). Hsp90α and Hsp90β were present in the extracellular membrane affinity purified fractions as obtained by biotin-streptavidin affinity purification in MDA-MB-231, MCF-7 and THP1 cells (Figure 9B). There was no Hsp90α or Hsp90β present in the affinity purified extracellular membrane fractions of U937 cells (Figure 9B). Following adhesion, Hsp90β was present in the membrane affinity purified fraction of THP1 and U937 cells where as only Hsp90α was detected in THP1 cells (Figure 9B).

3.2.4 Immunofluorescence analysis of the membrane expression and localisation of Hsp90, Hsp70 and Hop in response to SDF-1 treatment

We previously detected Hsp90, Hp70 and Hop in membrane fractions of MDA-MB-231 cells and shown that treatment with SDF-1 resulted in a change in the Hsp expression levels on the membrane. We performed immunofluorescence to detect whether there was a change in the membrane expression and subcellular localisation of Hsp90, Hsp70 and Hop following seeding of the cells on fibronectin, the integrin ligand and extracellular matrix protein, and in response to the stimulation of migration with SDF-1. This study was carried out in the MDA-MB-231 cell line due to its metastatic potential.

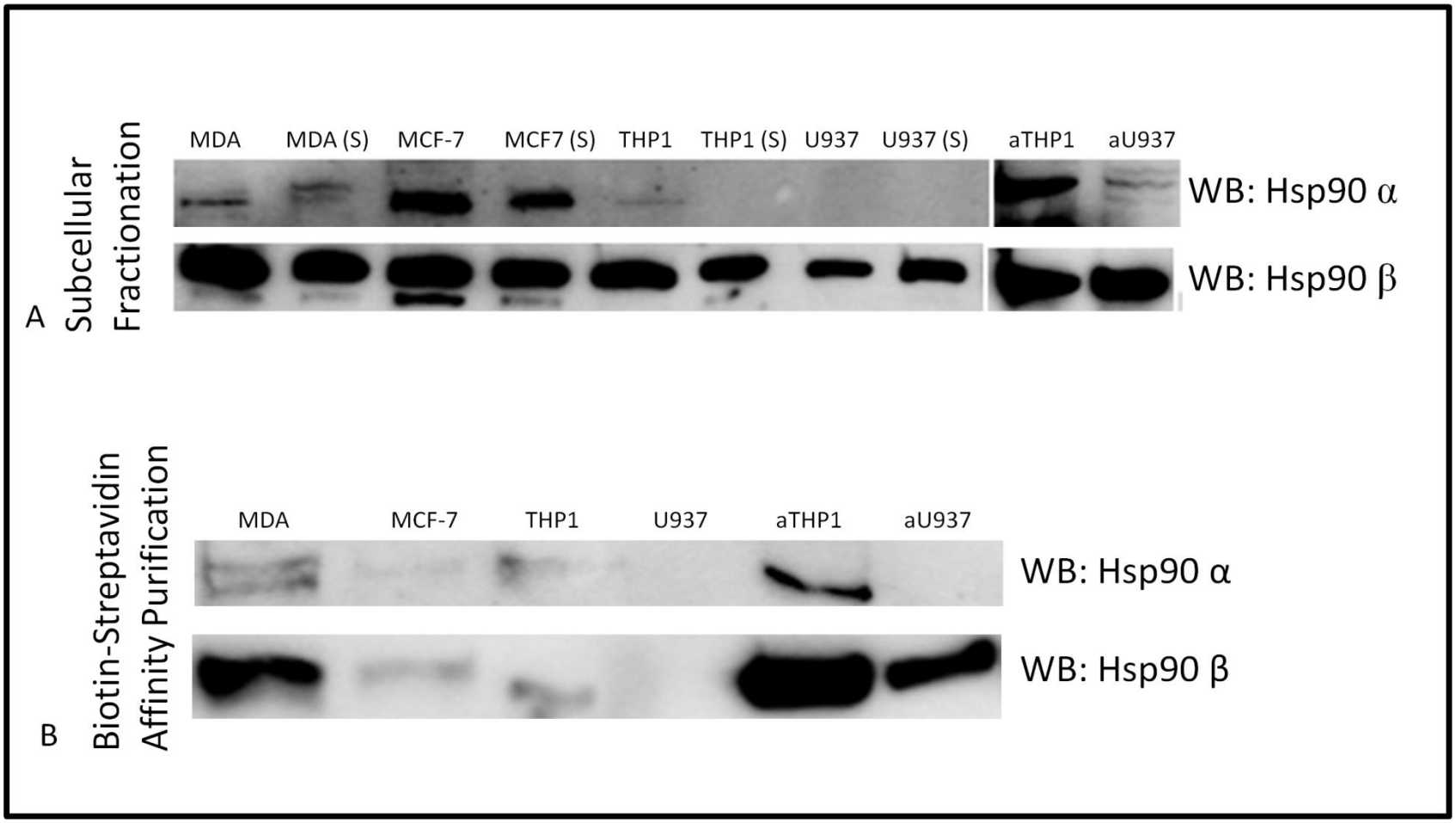

Figure 9: Detection of Hsp90α and Hsp90β in membrane fractions of cancer cells.

Untreated and SDF-1 treated membrane fractions obtained by A) subcellular fractionation (A) and biotin-streptavidin affinity purification (B) of MDA-MB-231, MCF-7, THP1, U937 and adherent THP1 and U937s (aTHP1 and aU937) were analysed by Western analysis using isoform specific antibodies for Hsp90α and Hsp90β. Details of Hsp90 membrane association as analysed by subcellular fractionation and biotin-streptavidin affinity purification are in Figures 3, 4 and 8.

Cells were stained with secondary antibodies in the absence of primary antibodies and showed negligible non-specific staining (*data not shown*). Following all treatments, cells were stained for Hsp90, Hsp70 and Hop and the membrane marker WGA-555. The immunofluorescence images are at a high magnification in order to show increased detail in the localisation of the Hsps and the morphology of the cells. Therefore, while the images may only show a few cells, the cells shown are representative of the majority of the population.

MDA-MB-231 breast cancer cells exhibit a flattened, spread out morphology. Some MDA-MB-231 cells exhibit a polarised morphology and an increase in the incidence of polarised cells occurred following SDF-1 treatment (Figure 10). The predominant staining pattern in MDA-MB-231 cells following all treatments (untreated, seeded on fibronectin, SDF-1 treatment and seeded on fibronectin followed by SDF-1 treatment) included a diffuse Hsp90 staining in the cytoplasm (Figure 10). Hsp90 staining was visible at the periphery of untreated MDA-MB-231 cells and localised to an apparent leading edge in polarised cells as indicated (white arrows) (Figure 10, untreated). Upon seeding of the cells on fibronectin, Hsp90 staining was visible as punctate staining at the periphery of the cells (Figure 10, fibronectin). Following treatment of the cells with SDF-1, Hsp90 staining was visible at the periphery of the cells where there appeared to be an increase in the levels of Hsp90 staining as compared to untreated cells (Figure 10, SDF-1). Hsp90 at the periphery of the cells treated with SDF-1 was localised to an apparent leading edge in polarised cells as indicated (white arrows) (Figure 10, SDF-1). A similar Hsp90 staining pattern to that of SDF-1 treated cells was exhibited by cells seeded on fibronectin and treated with SDF-1, with an apparent increase in the levels of Hsp90 visible at the periphery of the cells (Figure 10, fibronectin and SDF-1). Where Hsp90 staining was present at the periphery of the cells, Hsp90 staining appeared to overlap with the membrane marker WGA-555 (Figure 10). Quantification of the Hsp90 peripheral staining showed an increase in the incidence in response to SDF-1 treatment (~61 %-~94 %) (Appendix, Figure 26).

The predominant Hsp70 staining pattern was similarly localised to the cytoplasm and visible at the periphery of untreated cells, cells seeded on fibronectin, cells treated with SDF-1 and cells seeded on fibronectin and treated with SDF-1 (Figure 11). Hsp70 staining was visible at the periphery of untreated MDA-MB-231 cells and localised to the apparent leading edge in polarised cells as indicated (white arrows) (Figure 11). Upon seeding of the cells on fibronectin, Hsp70 staining was visible at the periphery of the cells although there were sites of punctate Hsp70 staining along the periphery of the cells (Figure 11, fibronectin). Following treatment of the cells with SDF-1, Hsp70 staining was visible at the periphery of the cells where unlike for the Hsp90 staining there was no apparent increase in the levels of Hsp70 staining in those cells

treated with SDF-1 (Figure 11, SDF-1). Hsp70 at the periphery of the cells treated with SDF-1 was localised to an apparent leading edge in polarised cells as indicated (white arrows). A similar Hsp70 staining pattern to that of SDF-1 treated cells was exhibited by cells seeded on fibronectin and treated with SDF-1 (Figure 11, fibronectin and SDF-1). Where Hsp70 was seen at the periphery of the cells there was an apparent overlap with the membrane marker WGA-555 (Figure 11). However, there was not as a considerable increase in the incidence of cells with Hsp70 peripheral staining in response to SDF-1 treatment as compared to the incidence of Hsp90 staining, although the constitutive membrane expression of Hsp70 appeared to be higher (~85 %-87 %) (Appendix, Figure 26).

The predominant Hop staining in MDA-MB-231 cells was a diffuse staining in the cytoplasm of cells. In some cells Hop staining was revealed to overlap with the Hoechst staining for the nucleus. Similarly, Hop staining was localised to the periphery of some of the cells in untreated cells, cells seeded on fibronectin, cells treated with SDF-1 and cells seeded on fibronectin and SDF-1 (Figure 12). Following seeding of the cells fibronectin, Hop staining was similarly present at the periphery of the cells and areas of increased punctate staining were visible (Figure 12, fibronectin). Following treatment with SDF-1 or a combination of seeding of cells on fibronectin and treatment with SDF-1, Hop was visible at the periphery of the cells although unlike as was shown for the Hsp90 staining there did not appear to be an increase in the expression levels of Hop at the periphery of MDA-MB-231 cells (Figure 12, SDF1, fibronectin and SDF-1). As revealed by quantification of the peripheral Hop staining, there did not appear to be a considerable in the incidence of cells with Hop staining at the periphery of the cells in response to SDF-1 treatment (~55 %-~47 %) (Appendix, Figure 26).

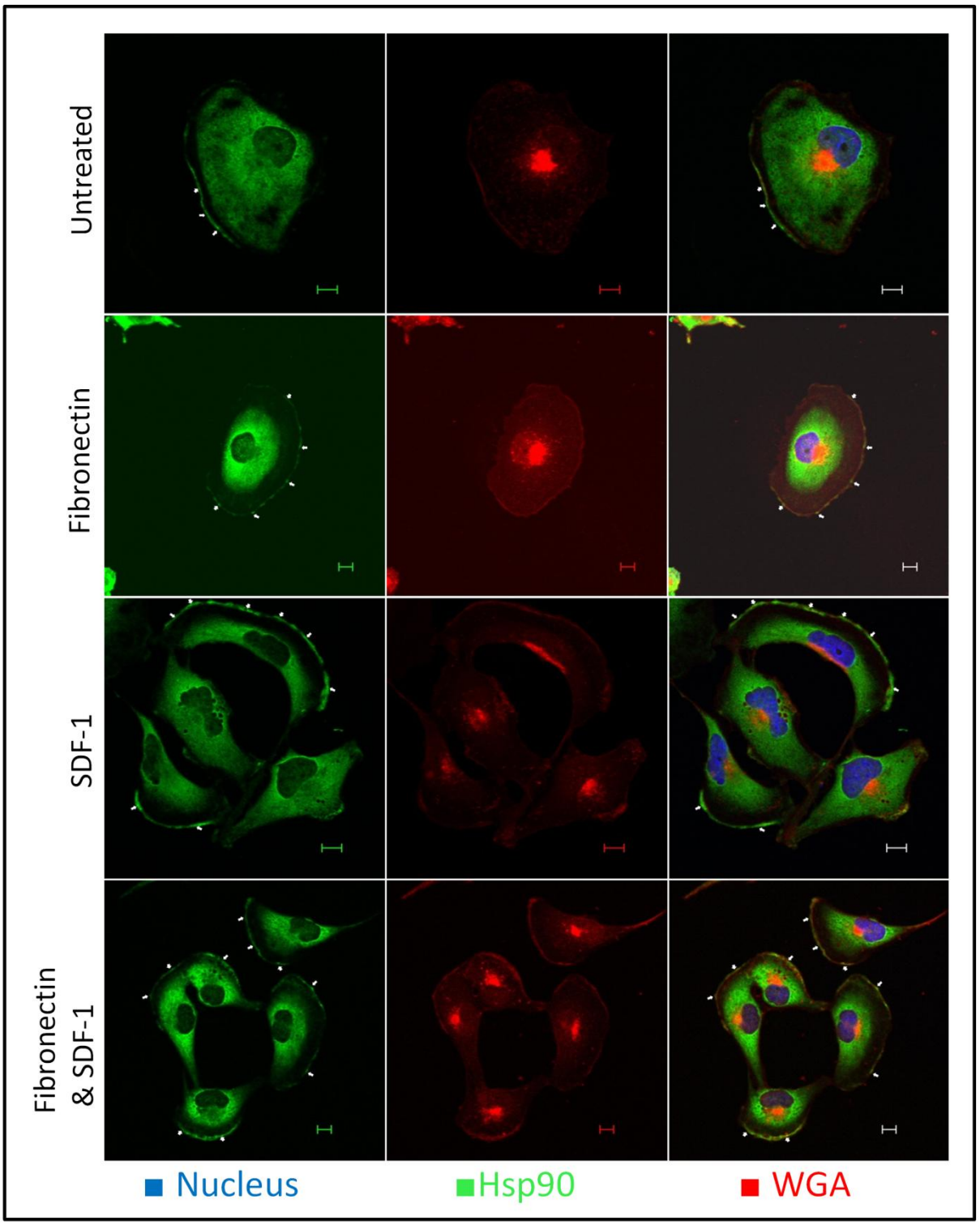

Figure 10: Immunofluorescence revealed Hsp90 localisation in SDF-1-stimulated and unstimulated MDA-MB-231 breast cancer cells.

Cells were seeded with or without fibronectin (250 µg/mL) overnight and treated with or without SDF-1 (100 ng/mL) for two hours. Cells were fixed and incubated with a mouse anti-Hsp90α/β primary antibody followed by a donkey anti-mouse-488 secondary antibody (green) and with the membrane marker WGA-555 (red). White arrows indicate Hsp90 staining at the periphery (reminiscent of the leading edge). The nucleus was stained with Hoechst-33342 (blue). Images were captured using the Zeiss LSM 510 Meta confocal microscope and analysed using AxiovisionLE 4.7.1 (Zeiss). Scale bars represent 10 µm. Experiment was carried out in duplicate.

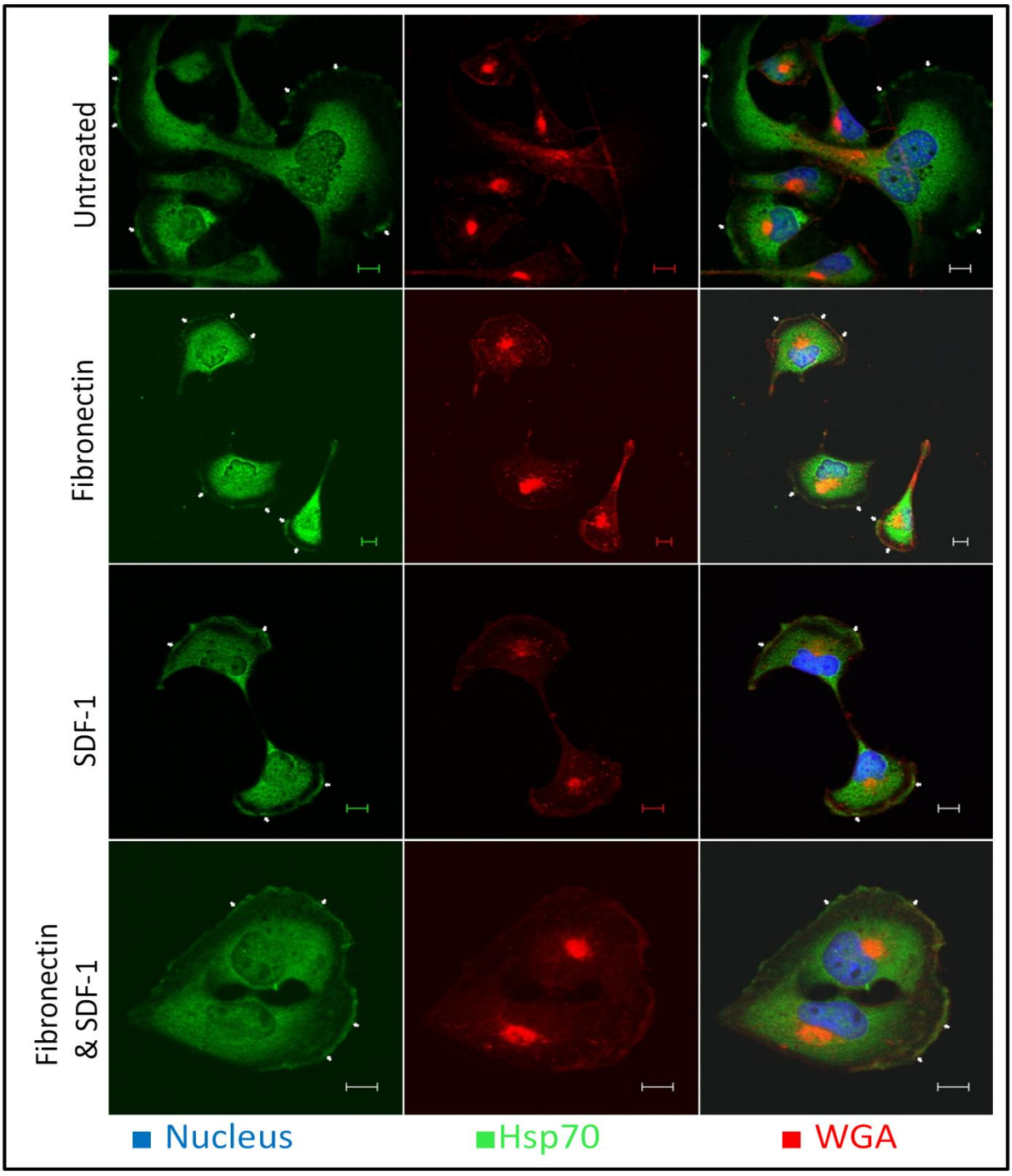

Figure 11: Immunofluorescence revealed Hsp70 localisation in SDF-1-stimulated and unstimulated MDA-MB-231 breast cancer cells.

Cells were seeded with or without fibronectin (250 µg/mL) overnight and treated with or without SDF-1 (100 ng/mL) for two hours. Cells were fixed and incubated with a mouse anti-Hsp70 primary antibody followed by a donkey anti-mouse-488 secondary antibody (green) and with the membrane marker WGA-555 (red). White arrows indicate Hsp70 staining at the periphery (reminiscent of the leading edge). The nucleus was stained with Hoechst-33342 (blue). Images were captured using the Zeiss LSM 510 Meta confocal microscope and analysed using AxiovisionLE 4.7.1 (Zeiss). Scale bars represent 10 µm. Experiment was carried out in duplicate.

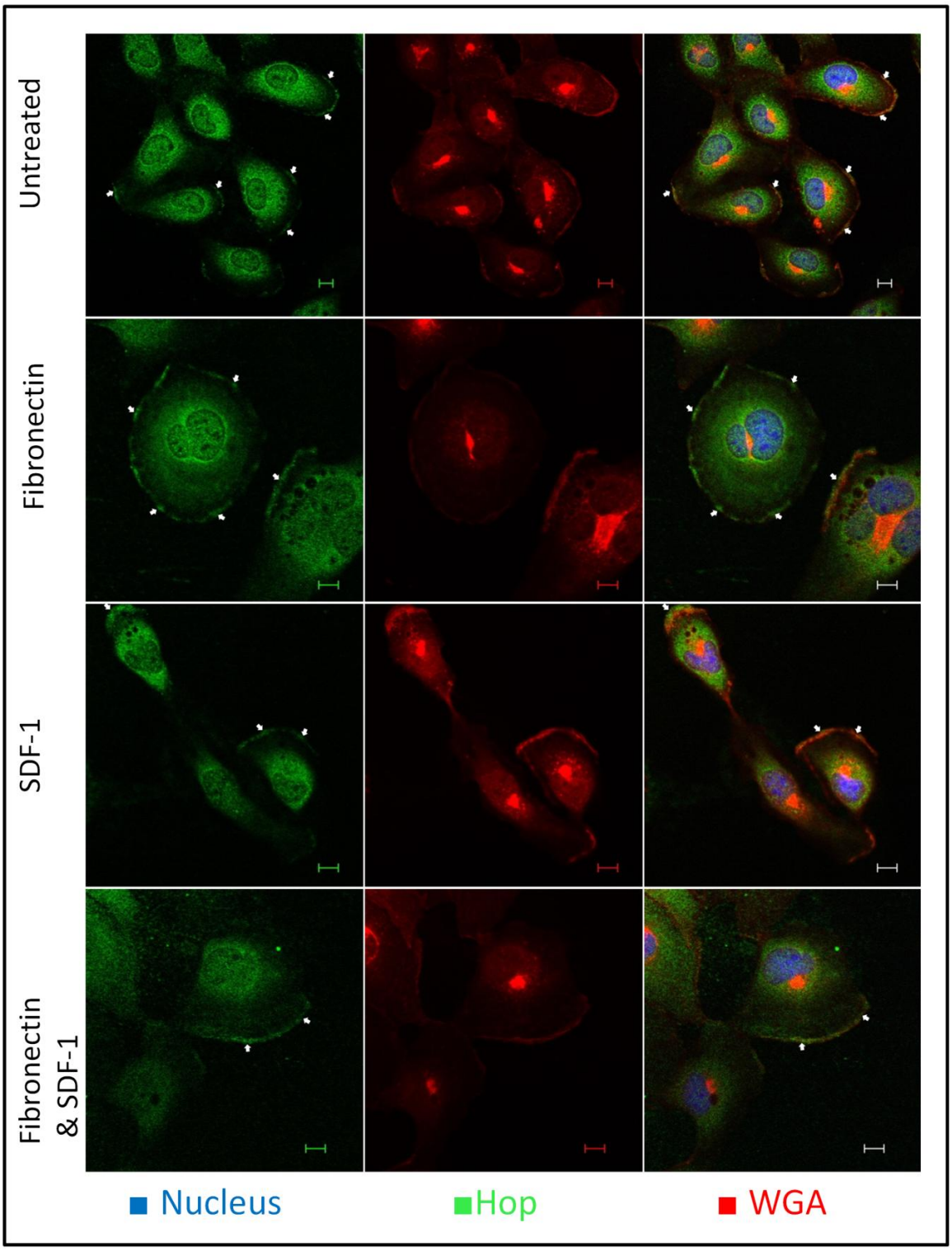

Figure 12: Immunofluorescence revealed Hop localisation in SDF-1-stimulated and unstimulated MDA-MB-231 breast cancer cells.

Cells were seeded with or without fibronectin (250 µg/mL) overnight and treated with or without SDF-1 (100 ng/mL) for two hours. Cells were fixed and incubated with a mouse anti-Hop primary antibody followed by a donkey anti-mouse-488 secondary antibody (green) and with the membrane marker WGA-555 (red). White arrows indicate Hop staining at the periphery (reminiscent of the leading edge). The nucleus was stained with Hoechst-33342 (blue). Images were captured using the Zeiss LSM 510 Meta confocal microscope and analysed using AxiovisionLE 4.7.1 (Zeiss). Scale bars represent 10 µm. Experiment was carried out in duplicate.

3.2.5 Validation of membrane localisation in MDA-MB-231 cells by intensity profiling of and GAPDH and Hsp90 staining.

To verify that immunofluorescence was a suitable technique for membrane localisation of proteins and to observe that the response of the Hsps to SDF-1 stimulation, the same treatments (untreated, fibronectin, SDF-1, fibronectin and SDF-1) were performed on MDA-MB-231 cells, and stained for GAPDH and WGA-555. GAPDH is a predominantly cytoplasmic protein that is not known to be involved in migration, the localisation of which is not expected to change with SDF-1 treatment. Subsequently, intensity profiles were carried out on cells stained for GAPDH or Hsp90 and WGA-555 in order to compare the staining intensity patterns at the periphery of the cells. Intensity profiles can be used to show the levels of protein staining along a section of a cell. The intensity profiles are representative of the fluorescent intensity along the dotted white line drawn through the cell (Figure 13). WGA-555 is a membrane marker and as a result, the distance from the nucleus after which the WGA-555 signal intensity becomes negligible is the membrane of the cell and usually revealed by a sharp increase in the staining intensity of WGA-555.

Immunofluorescence showed a predominantly cytoplasmic staining for GAPDH (Figure 13). In some cells, GAPDH staining could be seen in the nucleus as was similarly observed in the subcellular fractionation analysis (Figure 3). Immunofluorescence revealed little or no GAPDH staining at the periphery of the cells overlapping with the staining for the membrane marker WGA-555 (Figure 13). The predominant Hsp90 staining was localised to the cytoplasm, with Hsp90 staining visible at the periphery of cells and localised to the leading edge in polarised cells where it overlapped with WGA-555 (Figure 13). Intensity profiles of untreated cells stained for Hsp90 showed an overlap in the signal intensity of Hsp90 at a distance that correlated with an increase in the signal intensity of the membrane marker WGA-555 (Figure 13A). In cells stained with GAPDH there was no increase in signal intensity for GAPDH at a distance from the nucleus correlating with the increase in the signal intensity of the membrane marker WGA-555 (Figure 13A). An overlap in the signal intensity of the punctate Hsp90 staining and WGA-555 at an equivalent distance from the nucleus occurred in cells seeded on fibronectin as was revealed by an intensity profile (Figure 13B). No similar overlap in signal intensity at a distance from the nucleus correlating with an increase in signal intensity of the membrane marker WGA-555 was seen in cells seeded on fibronectin and stained for GAPDH (Figure 13B).

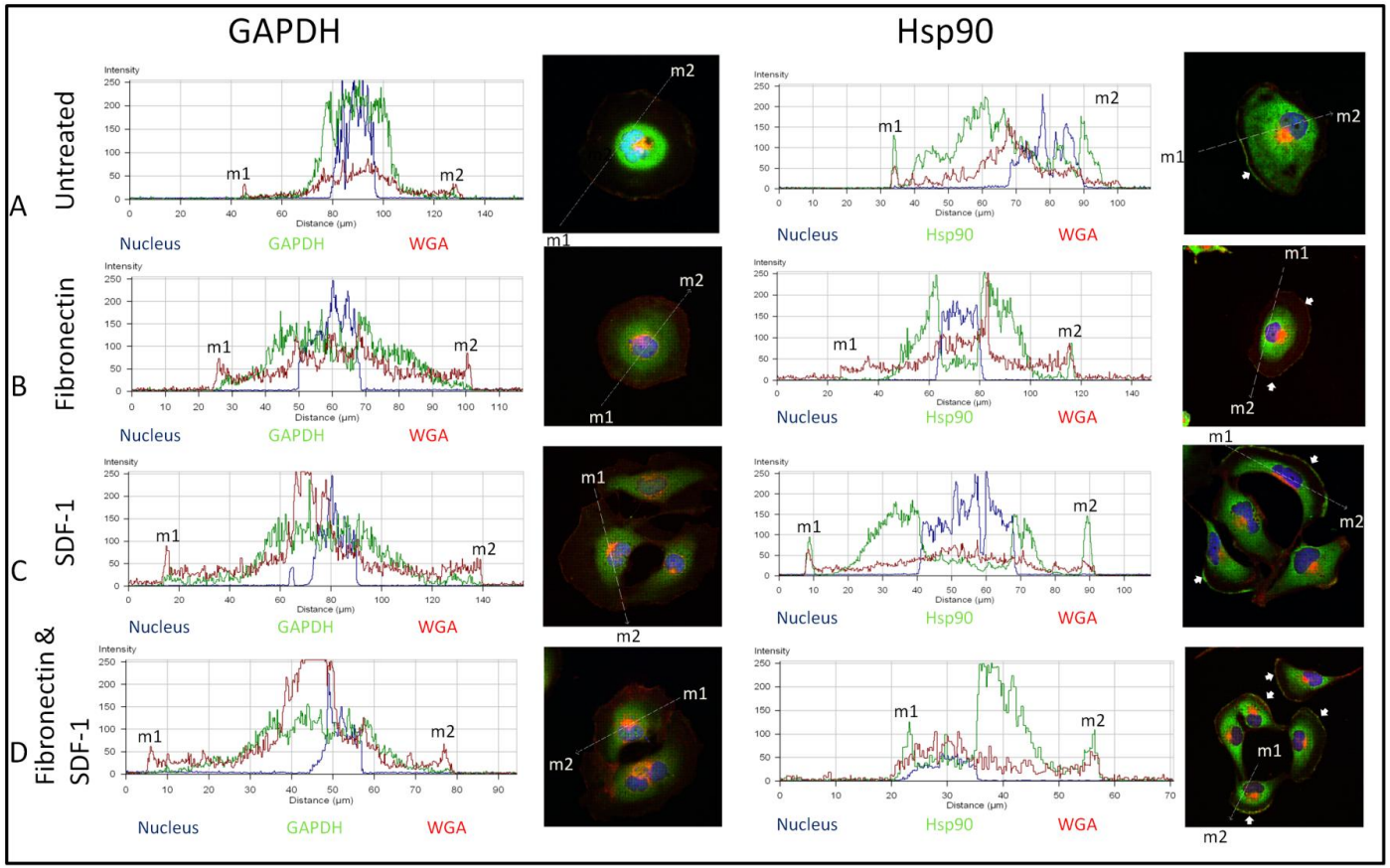

Figure 13: Validation of Hsp response to the simulation of migration in the MDA-MB-231 cell line by a comparison of GAPDH and Hsp90 staining.

Cells were seeded with or without fibronectin (250 µg/mL) overnight and with or without SDF-1 (100 ng/mL) for two hours at 37 ºC. Cells were fixed and incubated with a rabbit anti-GAPDH primary antibody or a mouse anti-Hsp90α/β primary antibody followed by chicken anti-rabbit-488 secondary antibody (green) and donkey anti-mouse-488 secondary (green) antibodies respectively along with the membrane marker WGA-555 (red). The nucleus was stained with Hoechst-33342 (blue). Images were captured using the Zeiss LSM 510 Meta confocal microscope and analysed using AxiovisionLE 4.7.1 (Zeiss). Intensity profiles show fluorescence intensities of the dotted line drawn through the cell. White arrows indicate staining at the periphery of cells (reminiscent of the leading edge). The images shown are a single confocal z-slice through the cells.

In MDA-MB-231 cells treated with SDF-1 and cells seeded on fibronectin and treated with SDF-1, there was an increase in the signal intensity of Hsp90 at a distance from the nucleus corresponding to an increase in the WGA-555 signal intensity (Figure 13C and 13D). Although there is an increase in the signal intensity of WGA-555 in the cells stained for GAPDH at a distance from the nucleus assumed to be the membrane, there is no similar increase in the signal intensity for GAPDH in cells treated with SDF-1 and cells seeded on fibronectin and treated with SDF-1 (Figure 13C and Figure 13D).

3.2.6 Investigating the membrane localisation of Hsp90, Hsp70 and Hop by intensity profiles

A considerable number of MDA-MB-231 breast cancer cells exhibited a polarised morphology with Hsp90, Hsp70 and Hop peripheral staining localised to what was predicted to be the leading edge. In order to investigate the membrane localisation of Hsp90, Hsp70 and Hop and in particular the association with membrane ruffles at the leading edge, intensity profiles of the staining of actin, vinculin, Hsp90, Hsp70 and Hop were generated and compared to the membrane marker WGA-555. The intensity profiles are representative of the fluorescent intensity at set distances along the dotted white line drawn through the cell (Figure 14).

The intensity profile of actin shows a sharp increase in staining intensity of actin at a distance from the nucleus that is equivalent to the distance from the nucleus of the signal corresponding to the membrane marker WGA-555 (Figure 14A). Vinculin is a protein present in focal adhesions in the cytoplasm just before the plasma membrane. Its serves to link the actin cytoskeleton to talin which interacts with the cytoplasmic domain of integrins [89]. An increase in the vinculin signal intensity occurred at a distance from the nucleus roughly correlating with an increase in the signal intensity of the membrane marker WGA-555 (Figure 14B). Hsp90, Hsp70 and Hop were present at a distance correlating with that of the membrane marker WGA-555 from the nucleus as is shown by an increase in the staining intensity of Hsp90, Hsp70 and Hop coinciding with an increase in the staining intensity of WGA-555 (Figure 14C, 14D and 14E).

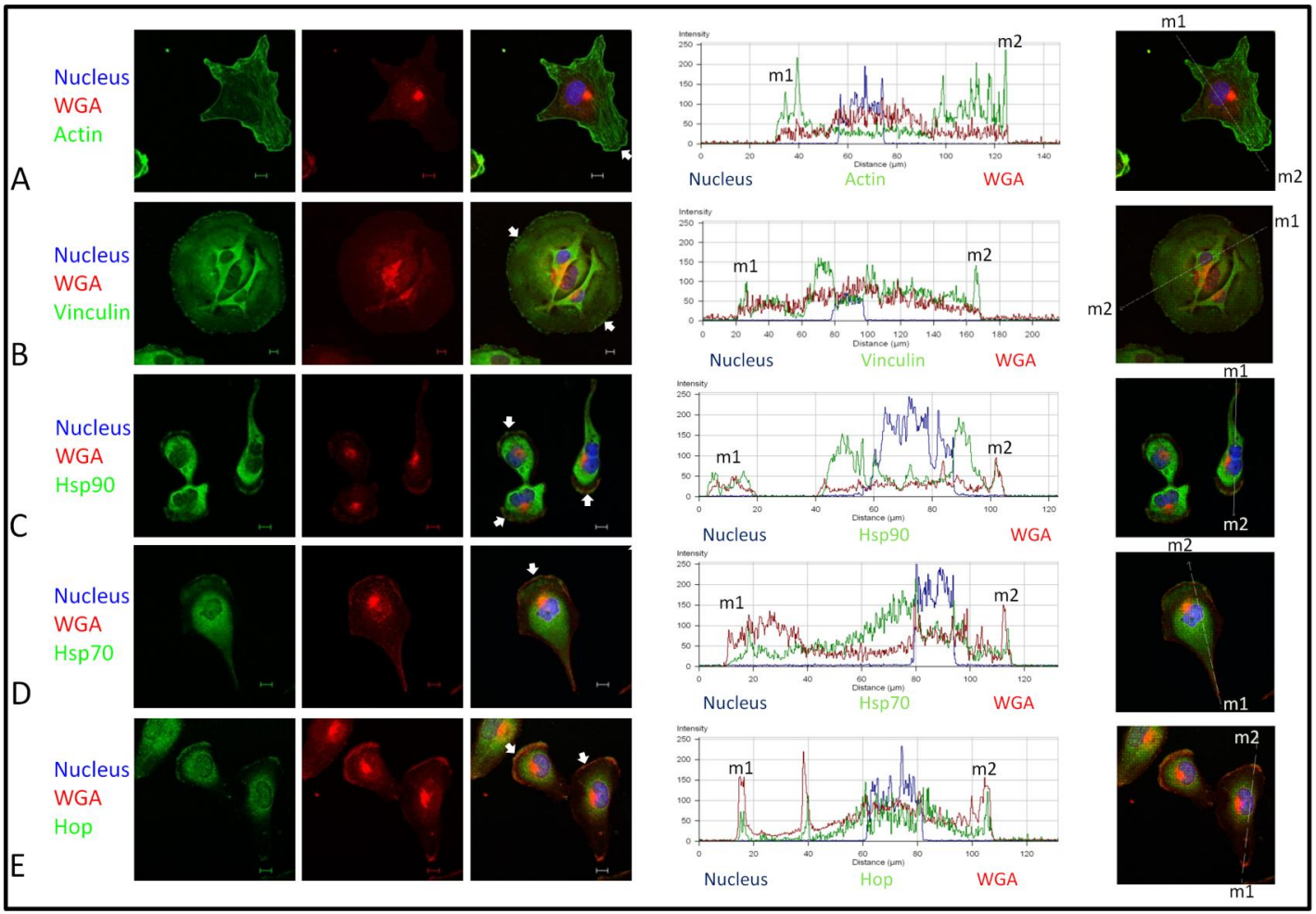

Figure 14: Investigation of Hsps association with membrane ruffles in the MDA-MB-231 cell line.

Staining intensity profiles were carried out on untreated MDA-MB-231 cells and show fluorescence intensities of the dotted line drawn through the cell. The images shown are a single confocal z-slices through the cells. All scale bars measure 10µm. Blue: nucleus, Red: WGA-555, Green: Actin (A), Vinculin (B), Hsp90 (C), Hsp70 (D), Hop (E). White arrows indicate the leading edge of polarised cells. m1 and m2 represent the periphery of the cell. Intensity Profiles were generated using Zeiss LSM 510 software.

3.3 Discussion

3.3.1 Hsp90, Hsp70 and Hop are membrane-associated in breast cancer cells

Hsp90, Hsp70 and Hop were detected in the membrane fractions as obtained by subcellular fractionation from the adherent MCF-7 and MDA-MB-231 breast cancer cell lines. In order to identify any contamination of the membrane fraction with other fraction, fraction markers of known localisation were used. Actin was used as a marker for the subcellular fractionation to show that if proteins are localised to more than one cellular compartment, subcellular fractionation would not result in the proteins only being detected in the first fraction but in every appropriate fraction. Actin was present in the cytoplasmic, membrane, nuclear and cytoskeletal fractions of MDA-MB-231 cells and in the cytoplasmic, membrane and nuclear fractions of MCF-7 cells. The actin antibody used recognised all isoforms of actin (α, β and γ). The presence of actin in both the cytoplasm, membrane (β-actin) and cytoskeleton is well documented [90]. The functions of actin have recently been expanded to the nucleus where it is implicated in processes such as gene expression, transcription factor regulation and intranuclear motility [91]. Therefore depending on its function within a specific cell type one would expect actin to be observed in all fractions.

GAPDH was used as a fraction marker for the cytoplasmic fraction. GAPDH was present in the cytosolic and membrane fractions with very low levels of GAPDH detected in the nuclear fraction of MDA-MB-231 and MCF-7 breast cancer cells. The presence of GAPDH in the membrane fraction may either be partial contamination with other fractions or may be GAPDH localised to intracellular membranes. Although GAPDH is traditionally considered a cytoplasmic signalling molecule that is involved in glycolysis, there have been reports of its expression on the plasma membrane and in the nucleus of cancer cells. Diverse roles of the mammalian GAPDH protein include, membrane transport and in membrane fusion, microtubule assembly, nuclear RNA export, protein phosphotransferase/ kinase reactions, the translational control of gene expression, DNA replication and DNA repair [92]. GAPDH was found localised in pseudopodial protrusions which are distinct plasma membrane domains involved in the directed migration of cells [93]. GAPDH has been shown to play critical role in apoptosis of several cell types and was shown to translocate to the nucleus [94].

The β1 integrin was used as a fraction marker for the membrane. The β1 integrin was predominantly present in the membrane fractions and visible at low levels in the cytoplasmic fraction of MDA-MD-231 and MCF-7 breast cancer cells. Both normal cells and those derived from tumours can switch between different modes of migration and specific signalling pathways

activated downstream of integrins. This is possibly as a result of the recycling of integrins [95]. Receptor signalling pathways act to transport specific integrins forward during cell migration. Therefore, the small band that appears in the cytoplasmic fraction of these cancer cells can potentially be accounted for by integrin switching [95].The fraction markers of the subcellular fractionation procedure suggested that although the membrane fractions for MDA-MB-231 and MCF-7 cells may not have been a completely 'pure' fractions, there was little cross contamination of proteins between the cell fractions.

Therefore, having investigated the degree of contamination of the fractions obtained by subcellular fractionation, using marker proteins of known localisation, we concluded that Hsp90, Hsp70 and Hop were potentially membrane associated in adherent MCF-7 and MDA-MB-231 breast cancer cell lines.

3.3.2 Hsp90, Hsp70 and Hop are extracellular and associated with the plasma membrane in MDA-MB-231 and MCF-7 breast cancer cells.

The membrane fraction isolated using the subcellular fractionation technique included proteins resident in both the plasma membrane and intracellular membranes. We identified Hsp90, Hsp70 and Hop from proteins isolated using cell surface biotinylation followed by streptavidin purification. Surface biotinylation followed by streptavidin affinity purification can be used to isolate both membrane associated and extracellular soluble proteins that associate with the plasma membrane. These data are in accordance with reports in the literature that demonstrated the presence of these chaperones in the plasma membrane of numerous cancer cell types as well as in the extracellular space [2, 21, 34, 45].

Taken together with the subcellular fractionation data, we proposed that Hsp90, Hsp70 and Hop were associated with the plasma membrane and at least parts of these proteins are located extracellularly in the adherent breast cancer cells.

3.3.3 Hsp90α and Hsp90β isoforms were detected in the plasma membrane

Both Hsp90α and Hsp90β were detected in the membrane extracellular fractions of MCF-7 and MDA-MB-231 cells. In MDA-MB-231 and MCF-7 cells the anti-Hsp90α/β antibody detected to bands of different mobility, one at ~90 kDa and the other at ~70kDa following biotin-streptavidin affinity purification.

There is much controversy in the literature surrounding the isoforms of membrane associated Hsp90. Cases have been reported of both membrane associated Hsp90α and membrane associated Hsp90β expression [13]. Hsp90α and Hsp90β were reported to be membrane associated on the surface of neuronal cells [13]. Both isoforms were shown to play a role in migration as migrating Schwann cells cultured in the presence of antibodies of both isoforms prevented actin reorganisation and lamellipodia formation [13]. We provide evidence to show that both Hsp90α and Hsp90β were present on the surface of breast cancer cells.

Western analysis of the subcellular fractions using Hsp90 specific isoforms revealed that Hsp90β was membrane associated in untreated, SDF-1 treated and adherent THP1 and U937 cells whereas Hsp90α was present at very low levels in untreated THP1 cells and high levels adherent THP1 cells. Hsp90α was not present in the membrane fraction of U937 cells when they were in suspension but following adhesion Hsp90α was present in the membrane fraction of U937 cells. We further showed that treatment with SDF-1 resulted in an increase in the expression levels of Hsp90α/β associating with cell membranes as compared to the β1 integrin.

In the intracellular environment, Hsp90α is the stress-inducible isoform where as Hsp90β is the constitutive isoform [96]. Inhibition of Hsp90 with 17-AAG in MDA-MB-231 breast cancer cells showed a decrease in the translocation of Hsp90α to the surface, suggesting that Hsp90α may be inducible on the plasma membrane similar to the intracellular environment [97].

The absence of Hsp90α in untreated cells as compared to the consistent expression of Hsp90β until following adhesion when both isoforms are present suggests that similarly to intracellular Hsp90α and Hsp90β, Hsp90α may be induced where as Hsp90β may constitutively expressed. However, in order to confirm that Hsp90α was inducible in the membrane we need to quantify Hsp90α membrane expression levels using flow cytometry in response to a dose treatment with SDF1.

Hsp90N, the 75 kDa homologue of Hsp90 is thought to associate with the plasma membrane and lacks the N-terminus or ATPase domain of the protein [9]. The 70 kDa band detected in this

study by biotin-streptavidin affinity purification may possibly be Hsp90N. In order to validate that the band of lower mobility as revealed by biotin-streptavidin affinity purification was Hsp90N, immunoprecipitation using an antibody that would specifically target the amino acid sequence at the N-terminus of Hsp90N that differs from that of Hsp90α and Hsp90β could be performed. Alternately, since the sequence identity of the first 509 amino acids of Hsp90N is identical to that of Hsp90α, immunoprecipitation could be performed using an anti-Hsp90α antibody that specifically binds to the C terminus and then mass spectrometry done on any band visible at ~70 kDa. Hsp90, Hsp70 and Hop membrane expression correlates with adhesion in THP1 and U937 cells

In U937 and THP1 cells, Hsp90 and Hsp70 were present in membrane fractions of subcellular fractionation. Hop was present only in the cytoplasmic fraction of untreated U937 and THP1 cells. Following adhesion, Hsp90, Hsp70 and Hop were present in the membrane fraction of U937 and THP1 cells. Neither Hsp90, Hsp70, Hop, nor the β1 integrin were affinity purified by biotin-streptavidin affinity purification before adhesion of U937 cells, whereas after adhesion, low levels of Hsp90, Hsp70, Hop and the β integrin were affinity purified. In THP1 cells Hsp90, Hsp70 and Hop were affinity purified by biotin-streptavidin affinity purification and following adhesion the β1 integrin was also isolated.

The degree of contamination of the fractions obtained by subcellular fractionation of the THP1 and U937 cell line was similarly carried out as for the breast cancer cell lines using actin, GAPDH and the β1 integrin as fraction markers. However, when the U937 cell line was grown in suspension, virtually all of the proteins were localised in the cytoplasmic and membrane fractions upon subcellular fractionation. This suggested that subcellular fractionation process in the suspension U937 cell line was not specific and that proteins from all fractions were isolated in the first two fractionation stages (cytoplasm and nucleus). Suspension cell lines are small rounded cells with a small cytoplasm and predominant nucleus and hence this might have affected the accuracy of the fractionation procedure.

The increase in expression of the adhesion protein, the β1 integrin visible in both the subcellular fractionation and the biotin-streptavidin affinity purification validated TPA as a method to induce adhesion in THP1 and U937 cells. A similar response to that of the β1 integrin, was revealed for the Hsps in that, following adhesion, there was Hop membrane associated in THP1 cells as detected by subcellular fractionation; and Hsp90, Hsp70 and Hop were shown to associate with the membrane such that at least part of the protein was located extracellularly in U937 cells. This suggested a putative role for the Hsps in adhesion.

3.3.4 SDF-1 treatment resulted in a change in the levels and localisation of Hsp90, Hsp70 and Hop

SDF-1 stimulation of MDA-MB-231 breast cancer cells resulted in an increase in the levels of Hsp90 on the plasma membrane as compared to the membrane expression levels of the β1-integrin. A similar trend was observed by immunofluorescence, where SDF-1 treatment resulted in an apparent increase in the signal intensity of Hsp90 at the leading edge. An increase in both Hsp90 and Hsp70 membrane expression levels as compared to the membrane expression levels of the β1 integrin occurred on the surface of MCF-7 cells. This suggested that SDF-1 stimulation of migration may have resulted in a recruitment of Hsps to the plasma membrane.

We observed that in response to SDF-1 stimulation there was an increase in the number of MDA-MB-231 cells exhibiting a polarised morphology. Hsp90, Hsp70 and Hop were localised to the leading edge of polarised cells and showed a similar localisation at the leading edge to actin. Staining of MDA-MB-231 cells with GAPDH revealed no GAPDH localised to the leading edge in polarised cells. Intensity profiles have previously been used as a technique to show leading edge localisation and membrane ruffle association comparing the localisation of membrane proteins with the localisation of actin in membrane ruffles [98]. Environmental signals induce the formation of membrane ruffles (termed lamellipodia or peripheral ruffles), sites of actin polymerisation at the leading edge of cells [64, 81]. In a similar study, intensity profiling of inducible nitric oxide synthetase (iNOS) was carried out and compared to actin at the leading edge of trophoblasts [98]. Nitric oxide has been shown to have an effect on cell migration and invasion in various cell types [98]. They demonstrated that the distribution of iNOS showed considerable overlap with the region of actin staining at the leading edge of cells and later showed that iNOS was responsible for site specific *S*-nitrosylation at the leading edge when localised to membrane ruffles [98]. The potential localisation of the Hsp90, Hsp70 and Hop to membrane ruffles at the leading edge of metastatic breast cancer cells may be indicative of a role in mediating migration as this phenotype is characteristic of proteins involved in migration. In mouse embryonic fibroblasts, Hsp90 along with HDAC6 was shown to localise to membrane ruffles following stimulation with the growth factor PDGF [81]. Our results suggested that Hsp90, Hsp70 and Hop localise to membrane ruffles in MDA-MB-231 cells which correlates with the published role of Hsp90 in migration [45]. We show that the localisation of the Hsps to the leading edge is a specific response as GAPDH did not show the same distribution following the stimulation of migration.

3.3.5 Conclusions

This chapter provided evidence that Hsp90, Hsp70 and Hop are plasma membrane associated and positioned such that at least part of the Hsps is extracellular in certain cell lines. SDF-1-stimulated migration resulted in the localisation of Hsp90, Hsp70 and Hop to the leading edge of migrating metastatic breast cancer cells suggesting a collective role at the leading edge. Intracellular Hsp90 is known to function as part of a multi-chaperone complex [6] and these data suggested that similarly membrane associated Hsps may be functioning as part of a multi-chaperone complex.

Chapter 4.

Evidence for an Extracellular Multi-chaperone complex

4.1 Introduction

In previous studies Hsp90 [24-26, 34, 45, 47, 49], Hsp70 [2, 3] and Hop [21, 34, 44] have been reported to be extracellular membrane associated, or extracellular soluble proteins found in the extracellular space.

Previous studies have shown extracellular Hsps in multi-protein complexes. Hsp60 was shown to associate with the $\alpha_3\beta_1$ integrin on the surface of breast carcinoma cells [80]. The $\alpha_3\beta_1$ integrin plays a role in both tumourigenesis and metastasis of breast cancer. Surface labelling of the membrane proteins with a biotin moiety followed by immunoprecipitation using an anti-β1 antibody revealed a 55 kDa protein by Western analysis with streptavidin peroxidase which was later confirmed by mass spectrometry to be Hsp60 [80]. The motility of cells is associated with integrin activation. One mode of integrin activation is through the association of or disassociation of other molecules with integrins that results in conformation changes which induce activation [66]. These studies showed that the $\alpha_3\beta_1$ integrin in its active conformation preferentially associates with Hsp60 on the cell surface.

A protein with which extracellular Hsp90 interacts is the receptor tyrosine kinase HER-2 (or ErB-2). Hsp90 was specifically shown to interact with the extracellular domain (ECD) of Her2 using a GST pull down assay with the ECD domain of HER2 coupled to GST [26]. Interruption of the Hsp90-HER-2 interaction leads to receptor destabilisation and consequently proteosomal degradation of HER-2 [46]. Using the monoclonal antibody (mAb) 4C5 which binds to extracellular Hsp90 disruption of the extracellular interaction of Hsp90 with HER-2 was observed. This resulted in the inhibition of heregulin (HRG)-induced HER-2/ErbB-3 heterodimer formation and impaired downstream kinase signalling required for cell motility [26].

Recent studies have revealed membrane complexes containing multiple Hsps. A complex of receptors was identified on the cell surface of monocytes that included Hsp90, Hsp70, CXCR4 and growth differentiation factor 5 (GDF5) that forms after stimulation with LPS using affinity chromatography and peptide mass fingerprinting and later confirmation with FRET analysis and immunofluorescence [38]. Similarly Hsp90 and Hsp70 were found to form part of a receptor complex required for Dengue virus entry in neuroblastoma and monocytic cell lines by affinity purification and the membrane association of Hsp90 and Hsp70 confirmed by biotin-streptavidin affinity purification [39]. This receptor complex was also found on the surface of human monocyte derived macrophages [39].

Current dogma suggests that intracellular Hsp90 does not function independently *in vivo*, but instead associates with a variety of partner chaperones and co-chaperones which are thought to be essential for the binding of Hsp90 to its client proteins and ATP hydrolysis [10]. We propose that instead of functioning independently extracellularly, as most groups suggest, that extracellular Hsp90 functions as part of a multi-chaperone complex. The objective of this chapter was to investigate whether extracellular membrane associated or soluble Hsp90, Hsp70 and Hop form a multi-chaperone complex similar to that observed in the cytoplasm.

4.2 Results

This chapter investigated the presence of an extracellular multi-chaperone primarily by means of immunoprecipitation. This chapter focused on the metastatic breast cancer cell line MDA-MB-231 as not only was there consistent expression of Hsp90, Hsp70 and Hop observed on the plasma membrane in this cell line (Chapter 3) but membrane associated Hsp90 is thought to play a role in metastasis [45].

4.2.1 Chemical crosslinking analysis of extracellular Hsp90 complexes

In order to test whether extracellular Hsp90 was present as part of a multi-protein complex, cells were treated with the cell impermeable, non-cleavable chemical crosslinker BS^3 which crosslinks all proteins within 11.4 Å of each other. BS^3 crosslinking is based on the covalent binding of amine residues in the crosslinker to lysine residues on the proteins [99]. The crosslinked whole cell lysate ($+BS^3$) and untreated whole cell lysate ($-BS^3$) were analysed by Western analysis using an antibody against Hsp90 (Figure 15A). In the whole cell lysate the Hsp90 band was detected at approximately 90 kDa (Figure 15A, lane $-BS^3$). Following chemical crosslinking using the cell impermeable BS^3, higher molecular weight species containing Hsp90 were revealed as is shown by prominent bands above 170 kDa (Figure 15A, lane $+BS^3$). Hsp90 that has not been crosslinked is still present as shown by the less prominent 90 kDa band (Figure 15A, lane $+BS^3$). Although the higher molecular weight Hsp90 containing species are insuffuciently resolved, more than one band is visible, suggesting the presence of more than one putative complex containing Hsp90 (Figure 15A, lane $+BS^3$).

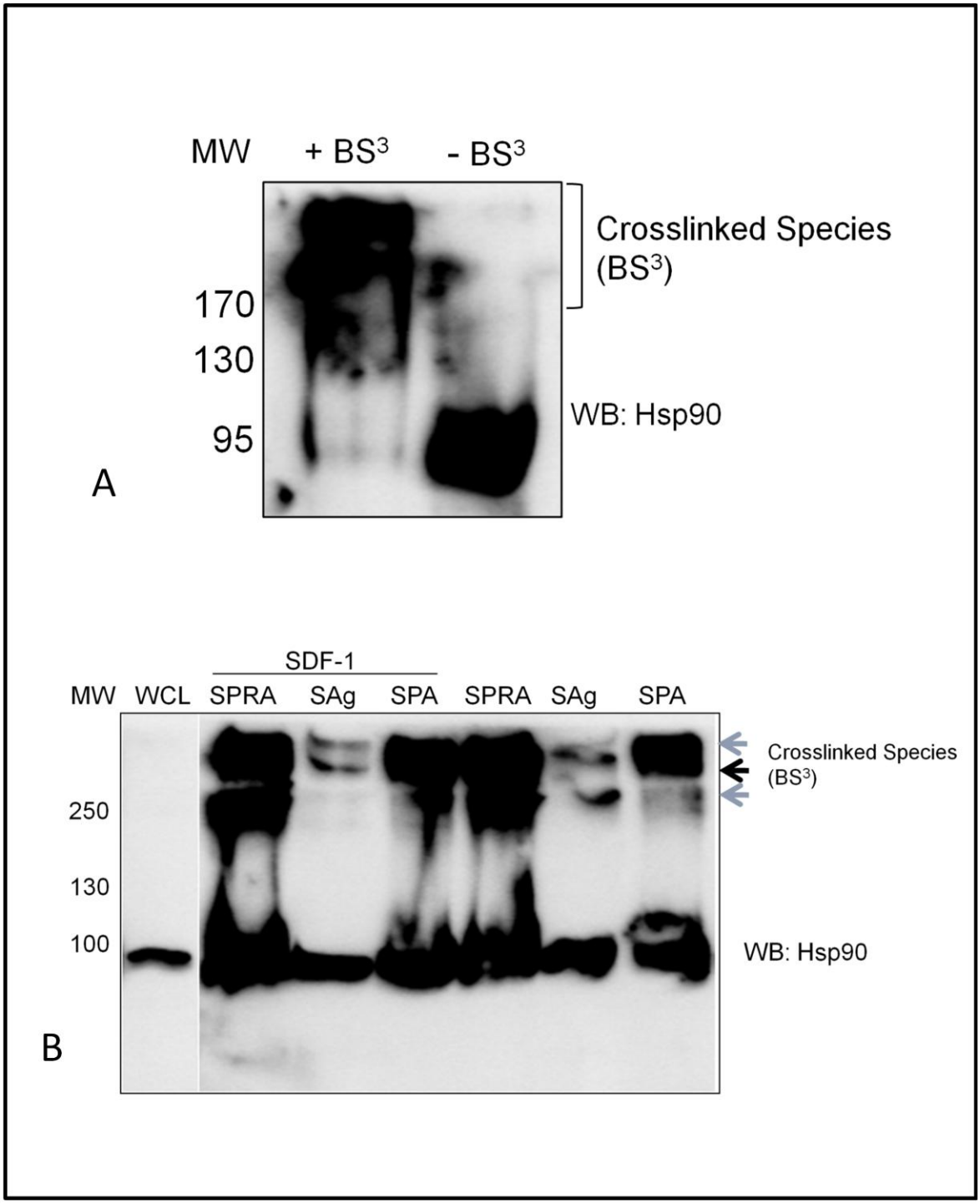

Figure 15: Hsp90 is present in a putative multi-protein complex on the plasma membrane.

(A) Extracellular proteins of MDA-MB-231 cells were chemically crosslinked with the cell impermeable BS^3 reagent (5 mM) for 1 hour at 4 ºC followed by quenching of the crosslinker with 20 mM Tris-HCl (pH 7.5). The BS^3 crosslinked whole cell lysate (+BS^3) and whole cell lysate (-BS^3) were analysed by Western analysis for Hsp90α/β. Experiment was carried out in triplicate. (B) Equal cell numbers were either untreated or treated with SDF-1β (100 ng/mL) for 2 hours, after which extracellular proteins of MDA-MB-231 cells were biotinylated, chemically crosslinked with the BS^3 cell-impermeable reagent (5 mM) and the biotinylated membrane proteins affinity purified with streptavidin-agarose beads. The whole cell lysate (WCL), supernatant pre-incubation with streptavidin-agarose (SPRA), streptavidin-agarose affinity purified fraction (SAg), supernatant post incubation with streptavidin-agarose (SPRA), for both untreated and SDF-1 treated cells were analysed by Western analysis for Hsp90α/β. Experiment was carried out in duplicate.

We investigated the response of the potential Hsp90 containing extracellular complexes to SDF-1 treatment. A combination of chemical crosslinking and biotin-streptavidin affinity purification was performed in the presence and absence of SDF-1. Equal cell numbers were either untreated or treated with SDF-1. Untreated and SDF-1-treated cells were biotinylated with a cell impermeable biotin-reagent, followed by crosslinking with BS^3 and biotin-streptavidin affinity purification (Figure 15B). A whole cell lysate (WCL), the supernatant pre-incubation with streptavidin-agarose (SPRA), streptavidin-agarose affinity purified fraction (SAg) and supernatant post incubation with streptavidin-agarose (SPA) were analysed by Western analysis for Hsp90 (Figure 15B). The whole cell lysate served as a positive control for Western analysis and to indicate the mobility of non-crosslinked Hsp90 (Figure 15B, lane WCL). The supernatant pre-incubation with the streptavidin-agarose beads showed high molecular weight crosslinked Hsp90 containing species, as indicated by the presence of two thick, high molecular weight bands by Western analysis in both untreated and SDF-1 treated cells (Figure 15B, lane SPRA). Non-crosslinked Hsp90 was also present as shown by the 90 kDa band (Figure 15B, lane SPRA). Biotin-streptavidin affinity purification of the extracellular Hsp90, isolated higher molecular weight, crosslinked Hsp90 containing species in both the untreated cells and SDF-1 treated cells. In the untreated and the SDF-1 treated cells, non-crosslinked Hsp90 was isolated as is revealed by the bands at 90 kDa (Figure 15B, lane SAg). In the untreated cells, two distinct Hsp90 containing high molecular weight bands can be seen as indicated by the grey arrows (Figure 15 B, lane SAg). In the SDF-1 treated cells, at least two Hsp90 containing higher molecular weight bands can be seen as indicated by the top two arrows (Figure 15B, lane SDF-1-SAg). In both untreated and SDF-1 treated cells, isolated crosslinked high molecular mass bands that contain Hsp90 were detected above 170 kDa (Figure 15B). In both the untreated and SDF-1 treated cells a decrease in the intensity of the higher molecular weight bands representative of the crosslinked higher molecular weight Hsp90 containing species is shown in the supernatant post incubation with the streptavidin-agarose beads (Figure 15B, lane SPA).

4.2.2 Optimisation of the isolation of higher molecular weight Hsp90 containing complexes by immunoprecipitation.

The immunoprecipation protocol was optimised with the main objective to isolate extracellular Hsp90 in a complex.

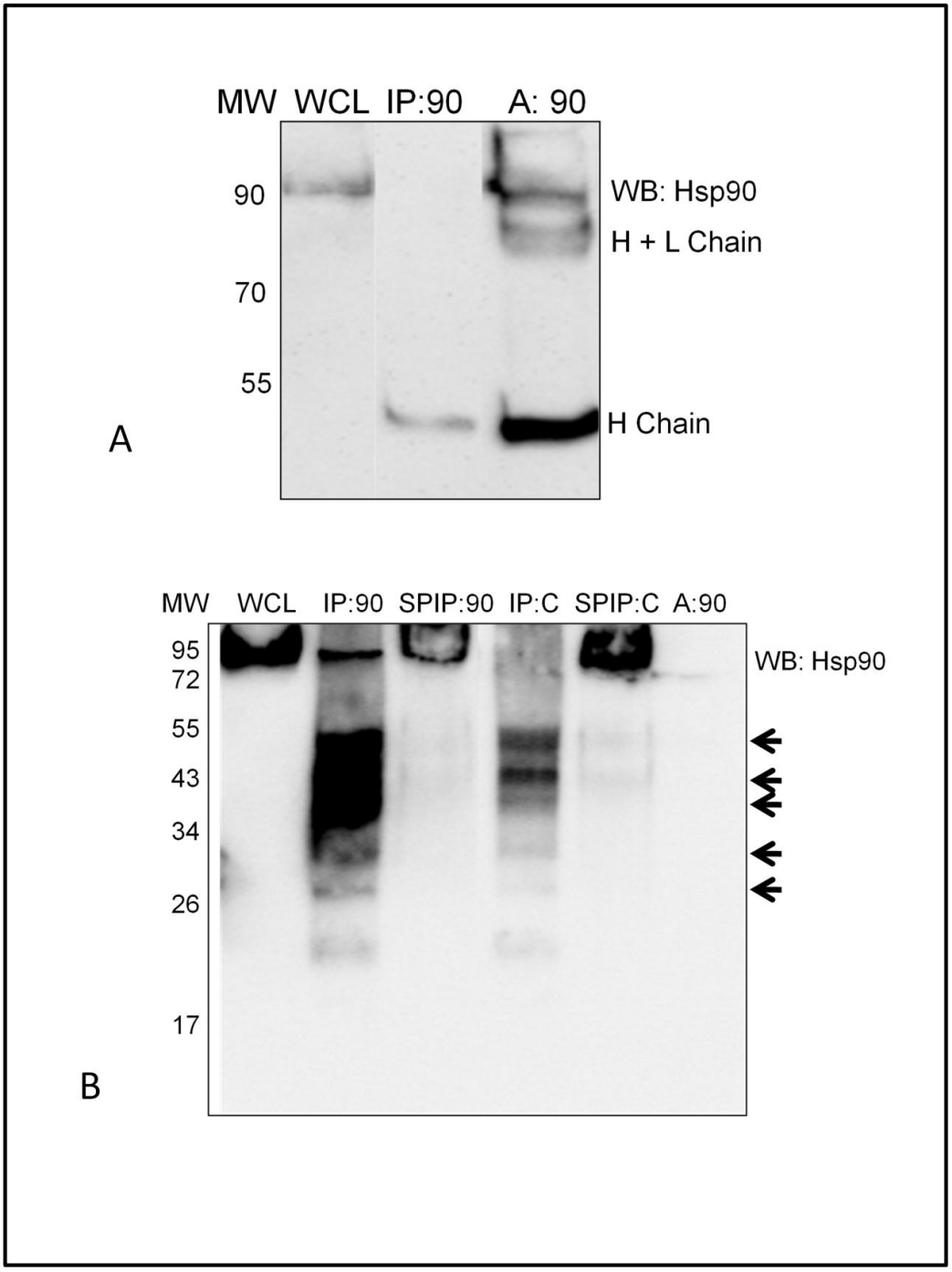

Figure 16: Optimisation of Immunoprecipitation protocol for isolation of Hsp90 membrane complexes.

A). Immunoprecipitation was performed following cell lysis of untreated MDA-MB-231 cells using a mouse anti-Hsp90α/β antibody coupled to Protein A/G agarose, followed by Western analysis with mouse anti-Hsp90α/β: whole cell lysate (WCL), immunoprecipitated proteins as isolated by mouse anti-Hsp90α/β (IP:90), mouse anti-Hsp90α/β antibody (A:90). B) immunoprecipitation was performed on equal volumes of lysate using a goat anti-Hsp90α/β antibody coupled to Protein A/G agarose beads and in the presence of no antibody (control), followed by Western analysis using mouse anti-Hsp90α/β: whole cell lysate (WCL), immunoprecipitated proteins as isolated by goat anti-Hsp90α/β (IP:90), supernatant post immunoprecipitation with anti-Hsp90α/β (SPIP:90), immunoprecipitated proteins as isolated by Protein A/G agarose with no antibody (IP:C), supernatant post immunoprecipitation control (SPIP:C), goat anti-Hsp90α/β antibody (A:90).

In order to optimise the immunoprecipitation procedure, immunoprecipiation was performed without crosslinking with the initial aim of isolating non-crosslinked Hsp90. Immunoprecipitation was carried out using a mouse anti-Hsp90α/β antibody and Protein A/G agarose beads. Western analysis was performed on a whole cell lysate (WCL), immunoprecipitated proteins as isolated by mouse anti-Hsp90α/β (IP:90) and the mouse anti-Hsp90α/β antibody control (A:90) (Figure 16A). The whole cell lysate served as a positive control for Western analysis (Figure 16A, lane WCL). The anti-Hsp90α/β antibody allowed identification of the presence and mobility of heavy and light chains of the antibody, as both the immunoprecipitation and the Western analysis were performed using an anti-mouse secondary antibody (Figure 16A, lane A:90). It was evident that no Hsp90 was pulled down with the mouse anti-Hsp90α/β antibody as Western analysis revealed no 90 kDa band in the immunoprecipitation sample, although the heavy chain of the antibody was present (Figure 16A, lane IP:90).

Next, immunoprecipitation using a goat anti-Hsp90α/β antibody coupled to Protein A/G agarose beads was performed. Western analysis was performed on the whole cell lysate (WCL), immunoprecipitated proteins as isolated by goat anti-Hsp90α/β (IP:90), supernatant post immunoprecipitation with anti-Hsp90α/β (SPIP:90), immunoprecipitated proteins as isolated with only the Protein A/G beads and no antibody (IP:C), supernatant post immunoprecipitation control (SPIP:C) and the goat anti-Hsp90α/β antibody (A:90). The whole cell lysate served as a positive control for Western analysis (Figure 16B, lane WCL). The immunoprecipitation procedure with only Protein A/G agarose beads and no antibody served as a negative control for the immunoprecipitation procedure to show that Hsp90 did not bind non-specifically to the Protein A/G agarose beads. Western analysis was performed using the mouse anti-Hsp90α/β antibody to probe for Hsp90. A band corresponding to Hsp90 as indicated by a band detected at 90 kDa was observed in the immunoprecipitation sample (Figure 16B, lane IP:90). The supernatant post immunoprecipitation with the goat anti-Hsp90 antibody shows less protein than the supernatant post control (Figure 16B, lane SPIP:90). A number of lower molecular weight bands were detected in the lane corresponding to Hsp90 immunoprecipitates as indicated by the black arrows (Figure 16B, lane IP:90). These bands could not be attributed to the goat antibody used in the IP as the secondary antibody used in this case would only detect mouse antigen. This conclusion was supported by the fact that the heavy and light chains of the antibody are not visible on the Western membrane when the goat anti-Hsp90 antibody was loaded alone (Figure 16B, lane A:90). It was concluded that the low molecular weight bands were most likely from loss of Protein A/G from the agarose column (Figure 16B, lane IP:90). No Hsp90 was present

when the Protein A/G beads without antibody were used for the IP although the lower molecular weight bands that were present in the (IP:90) lane are still visible, supporting the conclusion that this was Protein A/G that was being detected by the anti-mouse secondary antibody (Figure 16B, lane IP:C).

As the goat anti-Hsp90α/β antibody was successful in immunoprecipitation of non-crosslinked Hsp90, it was used to isolate the crosslinked membrane associated Hsp90. Extracellular proteins were crosslinked with the cell impermeable crosslinker BS^3 and then immunoprecipitation performed with the goat anti-Hsp90 α/β antibody. The whole cell lysate (WCL), crosslinked whole cell lysate (CLWCL), immunoprecipitated non-crosslinked proteins as isolated with anti-Hsp90α/β (IP:90), supernatant post immunoprecipitation with anti-Hsp90α/β (SPIP:90), immunoprecipitated proteins as isolated with anti-Hsp90α/β after crosslinking (IP:C90), supernatant post immunoprecipitation with anti-Hsp90α/β after crosslinking (SPIP:C90), immunoprecipitated proteins as isolated by Protein A/G agarose with no antibody (IP:C) and supernatant post immunoprecipitation control (SPIP:C) were analysed by Western analysis (Figure 17A). The whole cell lysate served as a positive control for Western analysis (Figure 17A, lane WCL). The crosslinked whole cell lysate showed that extracellular proteins were crosslinked as indicated by the high molecular weight Hsp90-containing bands (Figure 17A, lane CWCL). A 90 kD band of Hsp90 was detected in the absence of crosslinking (Figure 17A, lane IP:90). Hsp90 present at 90 kDa was either intracellular Hsp90 or membrane associated Hsp90 that had not been crosslinked. No higher molecular weight bands containing Hsp90 were detected in the immunoprecipitate following crosslinking (Figure 17A, lane IP:C90). There was detection of lower molecular weight bands in the lane, which was observed in previous experiments and was suggested to be Protein A/G agarose that has leached off the column. The supernatant post immunoprecipitation with antiHsp90α/β after crosslinking showed high levels of crosslinked Hsp90 (Figure 17A, lane SPIP:C90). The immunoprecipitation in the presence of no antibody but only Protein A/G agarose showed no Hsp90 indicating that Hsp90 did not bind non-specifically to the beads (Figure 17A, lane IP:C). Accordingly, both crosslinked and non-crosslinked Hsp90 species were detected in the supernatant post incubation with the Protein A/G agarose (Figure 17A, lane SPIP:C). The conclusion was reached that immunoprecipitation of Hsp90 containing crosslinked complexes using Protein A/G agarose would not be possible.

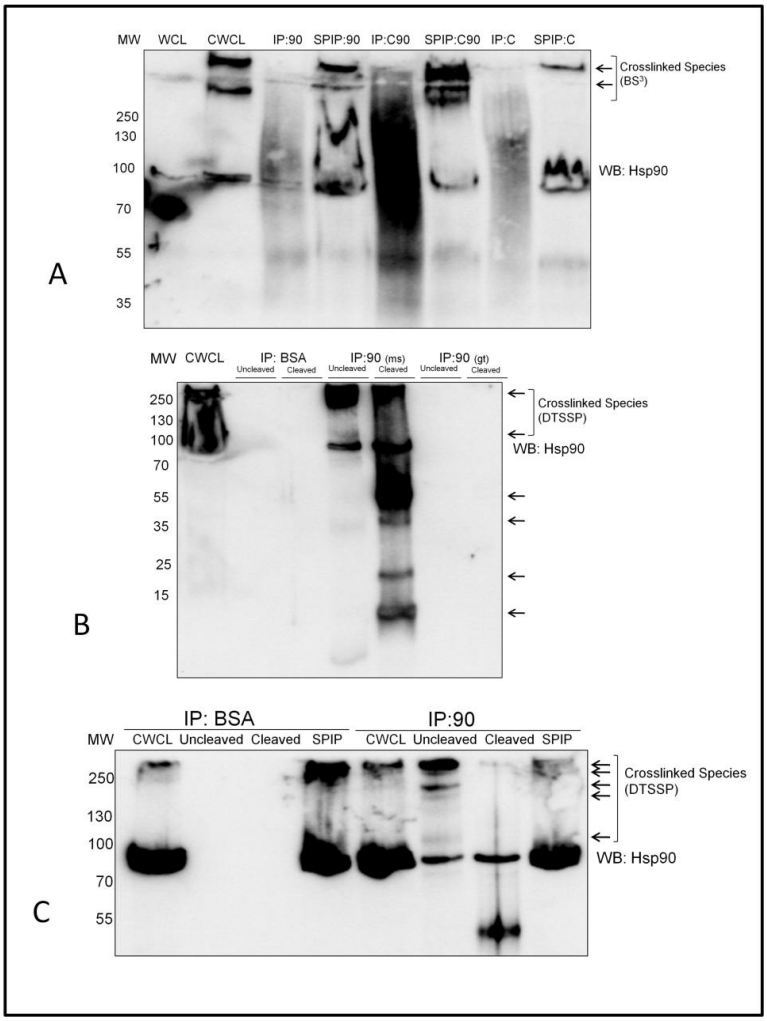

Figure 17: Optimisation of immunoprecipitation protocol following chemical crosslinking.

(A) Extracellular proteins of MDA-MB-231 cells were chemically crosslinked with the cell impermeable BS3 reagent (5 mM) for 1 hour at 4 ºC followed by quenching of the crosslinker with 20 mM Tris-HCl (pH 7.5). Immunoprecipitation was performed on equal volumes of whole cell lysate and crosslinked whole cell lysate with a goat anti-Hsp90α/β antibody coupled to Protein A/G agarose beads and a crosslinked whole cell lysate in the presence of Protein A/G agarose beads with no antibody (control), followed by Western analysis using mouse anti-Hsp90α/β. Whole cell lysate (WCL), crosslinked whole cell lysate (CLWCL), immunoprecipitated non-crosslinked proteins as isolated by goat antiHsp90α/β (IP:90), supernatant post immunoprecipitation with anti-Hsp90α/β (SPIP:90), immunoprecipitated proteins as isolated with goat anti-Hsp90α/β after crosslinking (IP:C90), supernatant post immunoprecipiation with anti-Hsp90α/β after crosslinking (SPIP:C90), immunoprecipitated proteins as isolated with Protein A/G agarose with no antibody (IP:C), supernatant post immunoprecipitation control (SPIP:C). (B) Cell surface proteins were crosslinked with the cleavable, cell impermeable DTSSP reagent (2 mM) for two hours at 4 ºC followed by quenching of the crosslinker with 20 mM Tris-HCl (pH 7.5). Cell lysates were immunoprecipitated with two different anti-Hsp90α/β antibodies using the Dynabeads® Co-immunoprecipitation kit. Mouse anti-Hsp90α/β and goat anti-Hsp90α/β were coupled to magnetic beads. BSA coupled to the beads served as a negative control followed by Western analysis with mouse anti-Hsp90α/β. Crosslinked whole cell lysate (CWCL), immunoprecipitated proteins as isolated with BSA, where the crosslinker was left uncleaved or cleaved (IP:BSA-uncleaved and cleaved), immunoprecipitated proteins as isolated with mouse anti-Hsp90α/β, where the crosslinker was left uncleaved or cleaved (IP:90 (ms)-uncleaved and cleaved), immunoprecipitated proteins as isolated by goat anti-Hsp90α/β where the crosslinker was left uncleaved or cleaved (IP:90 (gt)-uncleaved and cleaved) (C) The Dynabeads immunoprecipitation was repeated followed by Western analysis using mouse anti-Hsp90α/β. Crosslinked whole cell lysate (CWCL), immunoprecipitated proteins as isolated with BSA, where the crosslinker was left uncleaved or cleaved (IP:BSA-uncleaved and cleaved), immunoprecipitated proteins as isolated with mouse anti-Hsp90α/β, where the crosslinker was left uncleaved or cleaved (IP:90 (ms)-uncleaved and cleaved), supernatant post immunoprecipitation (SPIP). The Experiment was carried out in triplicate.

Since the main objective of the analysis was to purify Hsp90 in a complex, the method of immunoprecipitation was changed. Subsequent to crosslinking with the thiol-cleavable cell impermeable crosslinker DTSSP, immunoprecipitation was performed with the Dynabeads® Co-immunoprecipitation kit. Instead of using Protein A/G agarose beads, this technique is based on the covalent coupling of the antibody to magnetic beads. Following the coupling of antibody to the beads, the magnetic beads are resuspended in a cell lysate. Subsequent to binding of the protein to the beads, the supernatant is separated from the beads by means of a magnet. This technique therefore prevents the breaking up of complexes as occurs during centrifugation in other immunoprecipitation techniques. Immunoprecipitation using the Dynabeads® Co-immunoprecipitation kit, was performed using both the goat anti-Hsp90α/β antibody and the mouse anti-Hsp90α/β antibody (Figure 17B). Control beads that were covalently linked to BSA were used to account for any non-specific binding of proteins to the beads. The thiol-cleavable crosslinker DTSSP can be cleaved with 5 % β-mercaptoethanol in SDS-PAGE sample buffer.

The crosslinked whole cell lysate (CWCL), immunoprecipitated proteins as isolated with BSA-conjugated magnetic beads, without (uncleaved) or treated with (cleaved) 5 % β-mercaptoethanol (IP:BSA-uncleaved and cleaved), immunoprecipitated proteins as isolated with mouse anti-Hsp90α/β (IP:90 (ms)-uncleaved and cleaved), immunoprecipitated proteins as isolated with goat anti-Hsp90α/β (IP:90 (gt)-uncleaved and cleaved), were analysed by Western analysis (Figure 17B). Extracellular proteins were crosslinked as was shown by the higher molecular weight Hsp90 containing bands in the crosslinked whole cell lysate (Figure 17B, lane CWCL). BSA coupled to the magnetic beads served as a negative control and no Hsp90 was shown to associate non-specifically with the BSA-conjugated beads as no Hsp90 was detected by Western analysis (Figure 17B, lane IP:BSA-uncleaved and cleaved). Both Hsp90 and crosslinked higher molecular mass Hsp90 containing species were isolated using the mouse anti-Hsp90α/β antibody-conjugated beads as shown by the presence of both a 90 kDa band and higher molecular weight Hsp90 containing bands indicated by the black arrows (Figure 17B, lane IP:90(ms)-uncleaved). Upon cleavage of the DTSSP crosslinker a reduction in the proportion of higher molecular weight species to 90 kDa molecular weight species occurred, although some higher molecular mass species remained (Figure 17B, lane IP:90(ms)-cleaved). This suggested insufficient cleavage of the crosslinker. Upon cleavage of the crosslinker, lower molecular mass bands detected by the Hsp90 antibody were also detected as indicated by the black arrows. Neither Hsp90 nor Hsp90 containing higher molecular weight species were isolated with the goat anti-Hsp90α/β antibody as no Hsp90 was revealed by Western analysis (Figure 17B, lane IP:90(gt)-uncleaved and cleaved). This reaction serves as an additional antibody control that

confirmed that Hsp90 present in the samples was not due to non-specific binding. The immunoprecipitation was repeated and the crosslinked whole cell lysate (CWCL), immunoprecipitated proteins using BSA-conjugated magnetic beads, without (uncleaved) or treated with (cleaved) 5 % β-mercaptoethanol (IP:BSA-uncleaved and cleaved), immunoprecipitated proteins as isolated with mouse anti-Hsp90α/β (IP:90 (ms)-uncleaved and cleaved) analysed by Western analysis. The proteins were allowed to migrate further on the SDS-PAGE gel leading to better resolving of the proteins. Five distinct bands of putative higher molecular weight isolated Hsp90 species can be seen as indicated by the black arrows (Figure 17C, lane IP:90-uncleaved).

4.2.3 Detection of Hsp70 and Hop in Hsp90 containing membrane complexes

To detect whether the immunoprecipitated complexes containing Hsp90 also contained extracellular Hsp70 and Hop, the extracellular proteins were biotinylated with a cell impermeable biotin reagent and crosslinked with a cell-impermeable cleavable crosslinker (DTSSP). Crosslinked, biotinylated Hsp90 containing complexes were immunoprecipitated using the anti-Hsp90α/β antibody coupled to the magnetic beads of the Dynabeads co-immunoprecipitation kit. Proteins immunoprecipitated with the anti-Hsp90α/β antibody-coupled beads were identified by Western analysis using streptavidin peroxidase (to detect the presence of the biotin label) and antibodies against Hsp90, Hsp70 and Hop, where the crosslinker was left uncleaved or cleaved using 5% β-mercaptoethanol (Figure 18). When the crosslinker was left uncleaved streptavidin peroxidase revealed the presence of high molecular mass biotinylated species of above 170 kDa as well as lower molecular weight biotinylated species of approximately 65-70 kDa, 55 kDa and 42 kDa molecular weights [Figure 18, lane SA(U)]. Upon cleavage of the crosslinker, the higher molecular mass biotinylated species disappeared [Figure 18, lane SA(C)]. BSA coupled to the beads served as a negative control and no biotinylated proteins were visible as shown by Western analysis (Figure 18, lane CNTRL). Three distinct bands of higher molecular mass Hsp90 containing species were visible in the uncleaved lane probed with anti-Hsp90 antibody as indicated by the black stars [Figure 18, lane 90(U)]. Upon cleavage of the crosslinker the higher molecular mass Hsp90 containing bands disappeared and an increase in the intensity of the 90 kDa non-crosslinked Hsp90 band occurred [Figure 18, lane 90(C)].

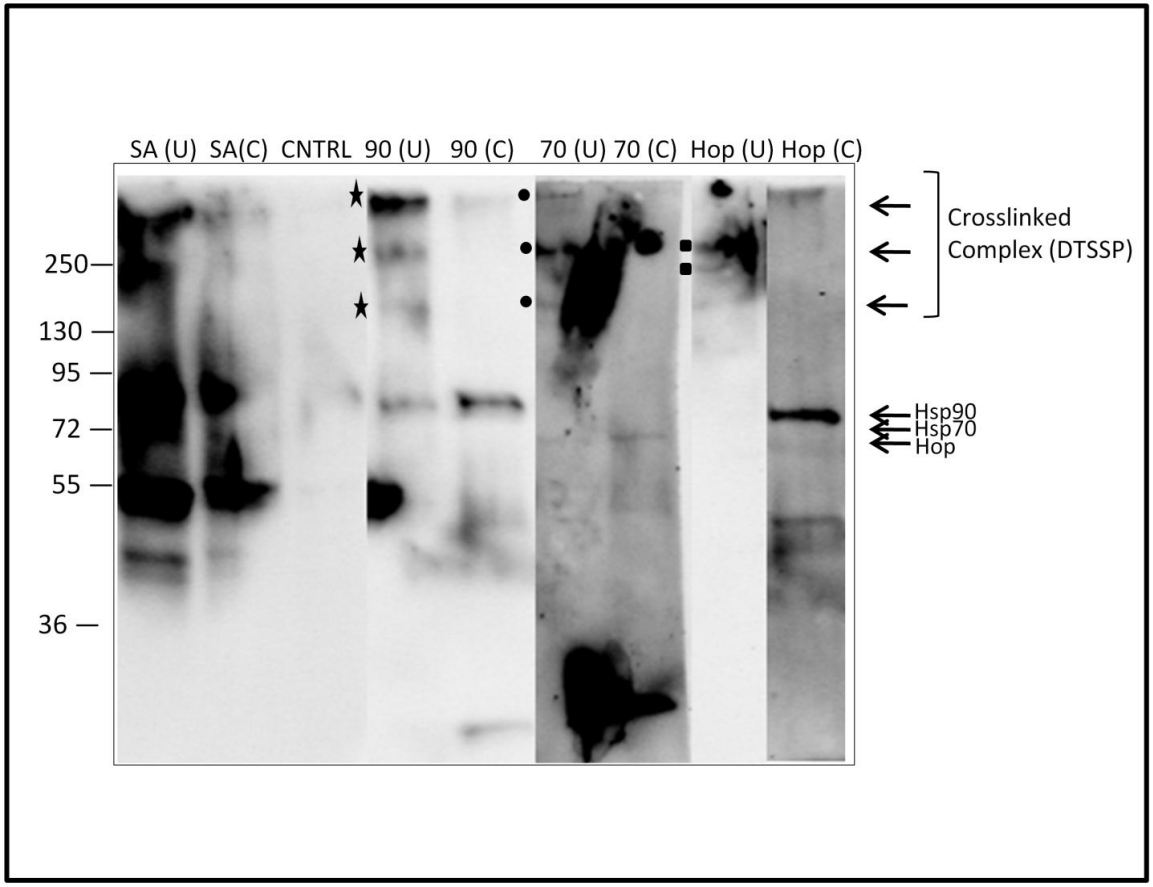

Figure 18: Hsp90, Hsp70 and Hop may be present as part of an extracellular multi-chaperone complex in MD-MBA-231 breast cancer cells.

Following cell-surface biotinylation, cell surface proteins were crosslinked with the cleavable cell impermeable DTSSP reagent (2 mM) for two hours at 4 ºC. Cell lysates were immunoprecipitated with an anti-Hsp90α/β antibody using the Dynabeads® Co-immunoprecipitation kit. Mouse anti-Hsp90α/β was coupled to magnetic beads. BSA coupled to the beads served as a negative control. Eluted proteins where the crosslinker was cleaved or left uncleaved were analysed by Western analysis with streptavidin peroxidase, antiHsp90α/β, anti-Hsp70 and anti-Hop. SA (U): streptavidin peroxidase (Uncleaved), SA (C): streptavidin peroxidase (Cleaved), 90 (U): Hsp90 (Uncleaved), 90 (C): Hsp90 (Cleaved), 70 (U): Hsp70 (Uncleaved), 70 (C): Hsp70 (Cleaved), Hop (U): Hop (Uncleaved), Hop (C): Hop (Cleaved).

Similarly, three distinct bands of higher molecular mass of different intensities were detected using an antibody against Hsp70 as indicated by the black circles [Figure 18, lane 70(U)]. Upon cleavage of the crosslinker, these higher molecular mass Hsp70 containing bands disappeared and an increase in the intensity of the 70 kDa non-crosslinked Hsp70 bands occurred [Figure 18, lane 70(C)]. Hop was detected in higher molecular mass complexes as indicated by the black squares, although in the uncleaved form no non-complexed Hop (66 kDa) was observed [Figure 18, lane Hop (U)]. Upon cleavage a faint band corresponding to non-complexed Hop is present at 66 kDa [Figure 18, lane Hop (C)]. The other band identified in the Hop blot is Hsp90 and is due to the fact that the membrane was stripped and reprobed for Hop following cleavage of the crosslinker as was necessitated by lack of sample. The presence of Hsp90 following stripping may be attributed to the fact that not all of the bound Hsp90 primary antibody was removed and since the Hsp90 and Hop antibodies were both mouse in species, Hsp90 was detected following binding of the mouse secondary antibody to detect Hop.

When probing for Hsp90, Hsp70 and Hop when the crosslinker has been cleaved, lower molecular mass bands are detected. To ensure that the higher molecular mass species detected were Hsp90 containing complexes and not antibody coupled into the complex, we probed an uncleaved sample with just the mouse secondary antibody and no primary antibody. The mouse antibody should detect mouse antigen and therefore detect if any antibody had been complexed into the higher molecular mass species as the Hsp90, Hsp70 and Hop antibodies were all mouse species. No bands were detected by Western analysis (*data not shown*) suggesting that these lower molecular weight species are not derived from the antibody being released from the magnetic beads.

4.2.4 Co-localisation analysis of Hsp90, Hsp70 and Hop membrane staining by confocal microscopy

Immunofluorescence analysis was carried out on the MDA-MB-231 cell line in an attempt to identify a co-localisation between the three proteins that could corroborate the biochemical data that suggested the presence of a putative extracellular chaperone complex. MDA-MB-231 cells seeded with or without fibronectin and with or without SDF-1 were stained for Hsp90, Hsp70 and Hop in order to examine the co-localisation of the Hsps in response to the stimulation of migration. In untreated cells, specific populations of Hsp90, Hsp70 and Hop were observed at the periphery of the cells, in addition to cytoplasmic staining. The staining signal of Hsp90, Hsp70 and Hop overlapped at the periphery of the cells as indicated by arrows in the merged images (Figure 19). In the cells seeded on fibronectin, Hsp90, Hsp70 and Hop were all visible as punctate staining at distinct points at the periphery of the cells.

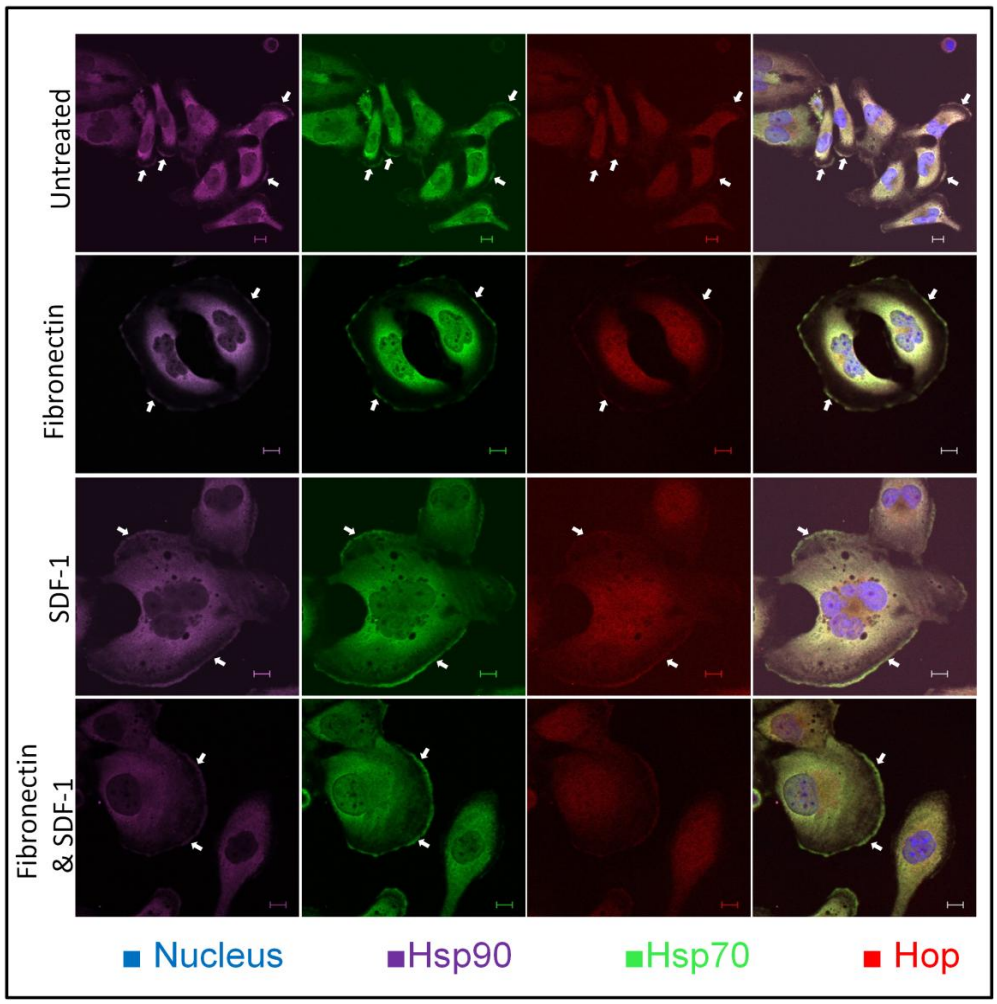

Figure 19: Potential co-localisation between Hsp90, Hsp70 and Hop at the plasma membrane of MDA-MB-231 breast cancer cells.

Cells were seeded with or without fibronectin (250µg/mL) overnight treated with or without SDF-1 (100 ng/mL) for two hours. Cells were fixed and incubated with a goat anti-Hsp90α/β primary antibody, mouse anti-Hsp70 primary antibody and a rabbit anti-Hop primary antibody followed by donkey anti-goat-633 (purple), donkey anti-mouse-488 secondary (green) and donkey anti-rabbit-546 (red) secondary antibodies. The nucleus was stained with Hoechst-33342 (blue). Membrane staining is indicated by the white arrows. Images were captured using the Zeiss LSM 510 Meta confocal microscope and analysed using AxiovisionLE 4.7.1 (Zeiss). Scale bars represent 10 µm. Experiment was carried out in duplicate.

Treatment	Channels	Pearson's Coefficient (Rr)	Mander's Overlap (R)
Fibronectin	Hsp90 and Hsp70	0.846	0.924
	Hsp70 and Hop	0.31	0.593
	Hsp90 and Hop	0.366	0.63
Fibronectin & SDF-1	Hsp90 and Hsp70	0.837	0.952
	Hsp70 and Hop	0.277	0.682
	Hsp90 and Hop	0.315	0.702

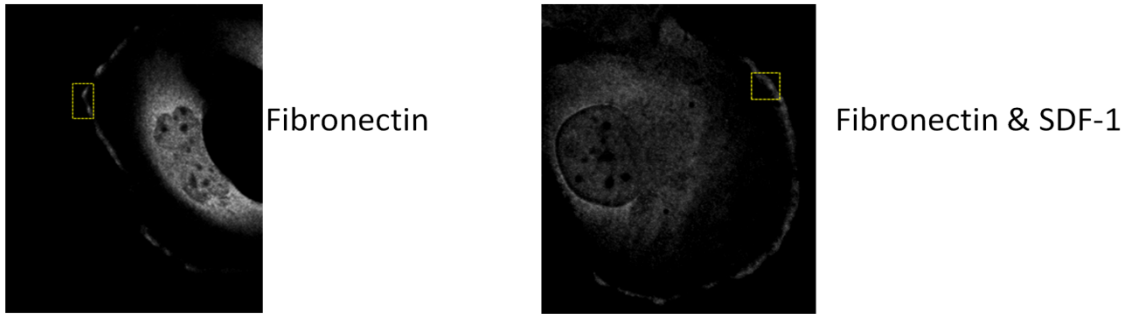

Figure 20: Co-localisation Analysis of Hsp90, Hsp70 and Hop Immunofluorescence using ImageJ

The Pearson's correlation coefficient (Rr) and Mander's Overlap coefficient (R) were computed using ImageJ Co-localisation analysis software for Hsp90 and Hsp70, Hsp70 and Hop and Hsp90 and Hop when cells were seeded on fibronectin or when cells were seeded on fibronectin and treated with SDF-1. Yellow blocks show the region for which the coefficients were calculated.

Similarly the signal at the distinct points on the periphery of the cells overlaps as indicated with arrows in the merged image (Figure 19). Following SDF-1 treatment, an increase in staining of Hsp90, Hsp70 and Hop was seen on the periphery of the cells. The fluorescence signal of the Hsps overlapped at the periphery of the cells as indicated by arrows in the merged image (Figure 19). Similarly in cells seeded on fibronectin and treated with SDF-1 there is a general increase in Hsp90, Hsp70 and Hop staining at the periphery of the cells and the Hsps appeared to localise to membrane ruffles (Figure 19).

To quantify the co-localisation visualised in Figure 19, the Pearson's correlation coefficient (Rr) and the Mander's Overlap coefficient were computed using ImageJ. Selected regions of MDA-MB-231 cells seeded on fibronectin and cells seeded on fibronectin and treated with SDF-1 were tested. For the Pearson's correlation coefficient, a value of 1 represents perfect co-localisation whereas a value of -1 represents perfect exclusion and zero represents random localisation [100]. The Mander's Overlap coefficient ranges between 0 and 1, with 1 being high co-localisation and 0 being low co-localisation [100]. The staining intensity of the Hsp70 was greater than that of the Hsp90 and the staining intensity of Hop was lower than that of Hsp90 and Hsp70 (Figure 19). The cells seeded on fibronectin and cells seeded on fibronectin and treated with SDF-1 had Rr values for Hsp90 and Hsp70 co-localisation close to 1 (0.846 and 0.837 respectively) (Figure 20). Similarly, the cells seeded on fibronectin and cells seeded on fibronectin and treated with SDF-1 have R values for Hsp90 and Hsp70 co-localisation close to 1 (0.924 and 0.952 respectively) (Figure 20). However, cells seeded on fibronectin and cells seeded on fibronectin and treated with SDF-1 have Rr values for Hsp70 and Hop co-localisation of 0.31 and 0.277 respectively, and R values of 0.593 and 0.682 respectively (Figure 20). Similarly, cells seeded on fibronectin and cells seeded on fibronectin and treated with SDF-1 have Rr values for Hsp90 and Hop co-localisation of 0.366 and 0.315 respectively, and R values of 0.63 and 0.702 respectively (Figure 20).

4.3 Discussion

4.3.1 Identification of a putative Hsp90 chaperone complex in the plasma membrane

This chapter provided putative evidence for the presence of an extracellular Hsp90 complex, either membrane associated or extracellular soluble associating with the plasma membrane. Chemical crosslinking of MDA-MB-231 cells revealed crosslinked higher molecular mass species containing Hsp90 with a mobility of more than 170 kDa. Biotin-streptavidin affinity purification revealed higher molecular mass species that contained Hsp90 ranging from ~170

kDa to above 250 kDa. Similarly, immunoprecipitation revealed higher molecular mass species containing Hsp90 of mobility between ~150 kDa to above 250 kDa. An additional higher molecular mass species was present at ~105 kDa following immunoprecipitation. Hsp90 is a dimer (~180 kDa) and therefore, together with Hsp70 and Hop (~136 kDa in total), an other associated proteins, an extracellular Hsp90 containing complex could easily be expected to be more than 250 kDa.

Biotinylation followed by chemical crosslinking and immunoprecipitation resulted in the isolation of biotinylated crosslinked species above 170 kDa that contained Hsp90 as detected by streptavidin peroxidase. Three crosslinked species containing Hsp90 with a mobility of just more than 130 kDa, just more than 250 kDa and one just after entering the stacking gel were subsequently identified using an anti-Hsp90α/β antibody. Similarly three crosslinked higher molecular mass species containing Hsp70 were detected and resolved at a similar mobility to the bands of Hsp90. At least two higher molecular mass bands containing Hop were identified although the most prominent band had a mobility of ~250 kDa. These data provided putative evidence for an extracellular multi-chaperone complex that may contain Hsp90, Hsp70 and Hop. It may alternately be that the multiple higher molecular mass bands are not all representative of different complexes but rather complexes of different sizes that were present as a result of the heterogenous degrees of crosslinking.

In order to specifically and accurately isolate these membrane associated protein complexes, the techniques used relied on the fact that both biotinylation and chemical crosslinking occurred prior to cell lysis and that both reagents used were cell impermeable. To account for no internalisation of the crosslinker or biotin reagent, immunoprecipitation could have been performed on a known intracellular protein followed by mass spectrometry or streptavidin peroxidase analysis. This would have revealed whether the intracellular protein carried either a biotin label or was crosslinked, which would be indicative of internalisation of the reagents. Although BSA was used as a negative control to show that there was no non-specific binding of the proteins to the magnetic beads, an IgG control could similarly have been used. However, it was shown that the goat Hsp90 antibody that binds the N-terminus of the antibody did not pull down any Hsp90 which indicated that the complexes that occurred when using the Hsp90 antibody with a C-terminal epitope were *bona fide* Hsp90 containing complexes.

A similar technique was used to identify the extracellular proteins with which membrane associated Pro-AMADTS9 interacts. In order to identify cellular proteins that complex with the metalloprotease Pro-AMADTS9, proteins were crosslinked using a cell permeable crosslinker

followed by mass spectrometry analysis. It was found that Pro-AMADTS9 associated with gp96 (the ER homologue of Hsp90) and BiP (GRP78; the ER Homolgue of Hsp70) and ERdj3 (an Hsp40 homologue). Since Pro-AMADTS9 is known to localise to the cells surface, cell surface biotinylation using a cell-impermeable inhibitor, western blotting of streptavidin-agarose purified membrane proteins revealed that gp96, BiP and Pro-AMAMTS9 were biotinylated and hence extracellular membrane associated [33]. With evidence of a membrane associated proteins, cell surface proteins were crosslinked with DSTTP or BS^3 which are cell impermeable and immunoprecipitation performed with an antibody against Pro-AMAMTS9 to reveal crosslinked high molecular weight complexes of Pro-AMAMTS9, gp96, and BiP.

Following chemical crosslinking, the anti-Hsp90 antibody that recognised the C-terminus resulted in the successful isolation of the higher molecular weight complexes containing Hsp90 by immunoprecipitation. This could suggest that the C-terminal epitope was accessible to the antibody after cell lysis because the N-terminus of Hsp90 was masked by crosslinking. It remains to be determined how membrane-associated Hsp90 is orientated within the membrane. Using a flow cytometry based approach, which allows for the binding of the antibody cell surface proteins without cell lysis, antibodies recognising a C-terminal epitope for Hsp90 failed to identify Hsp90 on the plasma membrane of Hela Cells. By contrast, antibodies recognising an N-terminal epitope detected membrane associated Hsp90 [40]. Similarly using a flow cytometry approach, an antibody recognising a C-terminal epitope of Hsp90 identified Hsp90 on the surface of melanoma cells [24]. Therefore there are contradicting reports in literature pertaining to the orientation of Hsp90 within the membrane and our results are in agreement with only some of the published literature.

We cannot discount an alternative interpretation that the biotin-streptavidin affinity purification and chemical crosslinking resulted in the biotinylation or crosslinking of proteins that are extracellular soluble but are associating with the plasma membrane by binding to other proteins on the cell surface. Therefore, although the isolated complexes contain extracellular Hsp90, it remains to be determined whether this is extracellular membrane associated Hsp90 or extracellular soluble Hsp90 that is associating with the membrane. Previous studies have shown that Hsp90α, Hop and p23 were all secreted by HT-1080 fibrosarcoma cells, although there was no evidence if these proteins were in a chaperone complex [34]. In separate studies PrP^C was shown to be released from THP1 cells via exosomes and Hsp70 was shown to interact with PrP^C not only in cell lysates but in secreted exosomes, suggesting the association of extracellular forms of chaperones with client proteins [101]. Hop was similarly shown to be a cell surface ligand of PrP^C on the surface of the glioblastoma-derived cell line A172 [21]. A complex of

receptors was identified on the cell surface of monocytes that included Hsp90, Hsp70 that forms after stimulation with LPS [38].

4.3.2 SDF-1 treatment altered the predominant Hsp90 complexes identified

Crosslinking and biotin-streptavidin affinity purification resulted in the presence of additional bands and a change in the prominent Hsp90 containing higher molecular weight species in response to SDF-1 treatment.

In Hsp receptor complexes which recognise bacterial LPS it was found that CD14, Hsp70 and Hsp90 were associated in a membrane complex prior to LPS stimulation, but TLR4, CXCR4 and GDF5 were not and were only present after LPS stimulation [38]. It has been suggested that transforming growth factor α (TGFα) controls the release of Hsp90α in response to hypoxia [47]. Extracellular soluble Hsp90 can then promote cell migration by binding through the cell surface receptor CD91 which then leads to signalling events that result in cell motility [47].

Taken together our results suggested that following SDF-1-stimulation of migration, additional members of the multi-protein machinery, be they chaperones or client proteins were recruited to a membrane associated Hsp90 chaperone complex. It may alternately be that following SDF-1 stimulation, there was an increase in binding of the extracellular soluble members of the extracellular Hsp90 complex to a receptor on the cell surface.

4.3.3 Co-localisation of chaperones at the leading edge in SDF-1 treated MDA-MB-231 cells

Immunofluorescence analysis revealed an overlap in the staining of Hsp90, Hsp70 and Hop at specific points on the periphery of the cells. Quantification of the overlap showed Pearson's and Mander's Overlap coefficients that were close to the value 1 for the overlap between Hsp90 and Hsp70, suggesting a co-localisation of the two proteins.

The Pearson's and Mander's Overlap Coefficients representative of the co-localisation between Hsp70 and Hop, and Hsp90 and Hop, showed poor correlation and values that were not close to 1. This suggests that there was no co-localisation between Hsp70 and Hop and Hsp90 and Hop on the cell membrane. However, there is a considerable difference in the signal intensity between Hsp90 and Hsp70 and Hop which potentially resulted in the poor correlation indicated by the co-localisation coefficients and challenges the accuracy of these calculations.

4.3.4 Conclusions

This chapter provided putative evidence for the presence of an extracellular multi-chaperone complex in the metastatic breast cancer cell line, with additional evidence provided for a membrane associated Hsp90 chaperone complex. The different higher molecular weight complexes that contain Hsp90 have been sent for mass spectrometry analysis in order to confirm the Hsp90 chaperone complex. The potential presence of Hsp90, Hsp70 and Hop in an extracellular complex supported our proposal that extracellular Hsp90 may function as a molecular chaperone assisted by Hsp70 and co-chaperones. Although there is growing evidence for the presence of extracellular membrane-associated or soluble forms of multiple Hsps, this research is the first report to suggest that extracellular Hsp90 formed a putative chaperone complex containing Hsp70 and Hop. Although extracellular Hsp90 has been thought to play a role in migration [13, 45], it remains to be determined whether it is the membrane associated pool of Hsp90 that is mediating migration or the extracellular soluble pool of Hsp90 and the mechanisms by which extracellular Hsp90 mediated migration.

Chapter 5.

Investigation of the role of extracellular Hsp90 in SDF-1 mediated migration

5.1 Introduction

Previous studies have revealed a role for intracellular Hsp90 in migration. Hsp90 is known to bind actin directly in the cytoplasm [97, 102]. Following inhibition of Hsp90 with 17AAG, immunoprecipitation resulted in an increase in the pull down of actin with Hsp90. Furthermore, a decrease in the numbers of filapodia and lamellipodia were observed following Hsp90 inhibition [97]. The reorganisation of the actin cytoskeleton involves equilibrium processes between globular (G-actin) and filamentous (F-actin) actin [97]. Therefore, it is thought that Hsp90 interacts with G-actin following inhibition, not allowing the polymerisation of G-actin monomers into F-actin which may in turn contribute to the observed decrease in numbers of lamellipodia and filapodia [97].

Membrane associated and extracellular Hsp90 has been shown to play a role in cancer metastasis. Previous studies have shown that membrane associated Hsp90 is involved in the mediation of cell migration [13, 27, 45]. In the extracellular space of fibrosarcoma cells, Hsp90α was shown to interact with matrix metalloproteinase-2 (MMP2) and thus facilitate the maturation of MMP-2, promoting tumour invasiveness [34]. In the literature, the term extracellular is used interchangeably to describe both membrane associated and extracellular soluble forms of Hsp90. It remains to be determined which extracellular pool of Hsp90 is mediating migration, if not both.

Cell migration is stimulated by environmental signals such as growth factors and extracellular matrix molecules [45, 97]. Following binding to cell surface receptors, the environmental signals stimulate intracellular signalling pathways that regulate numerous signal transduction networks that control processes such as the reorganisation of the actin cytoskeleton and ERK1/2 signalling [60, 103]. For example, in integrin dependent migration, integrins bind extracellular ligands via their outer domains whereas their cytoplasmic domains directly bind to cytoskeletal proteins such as talin. Talin in turn binds actin directly or via intermediates such as vinculin which then directly binds to actin [62]. Actin dynamics play a crucial role in cell motility, primarily by producing forces to push forward the leading edge of motile cells [104]. Environmental signals induce reorganisation of the actin cytoskeleton as well as the formation of membrane ruffles at the leading edge (termed lamellipodia and or peripheral ruffles) [64, 81]. ERK1/2 activation results in the transduction of environmental signals from the cell membrane to the nucleus [49]. ERK1/2 signalling results in immediate gene transcription leading to several cell functions including cell growth, transformation, differentiation, metastasis as well as survival after stress [49, 105, 106].

In a previous study, it was shown that treatment of tumour cells stimulated in suspension with SDF-1 resulted in intense F-actin staining in the periphery of cells and a redistribution of actin towards the leading edge, a prerequisite for the formation of pseudopodia necessary for migration. Furthermore, SDF-1 induced directional migration of breast cancer cells through a reconstituted basement membrane where breast cancer cells were forced to migrate towards SDF-1 [107]. Blocking the SDF-1/CXCR4 interactions with neutralising anti-CXCR4 or anti-SDF-1 antibodies significantly reduced these migratory responses [107].

This study previously showed a change in the expression of membrane associated Hsp90 and Hsp70 in response to SDF-1 stimulated migration as compared to the β1 integrin in MDA-MB-231 cells. Hsp90, Hsp70 and Hop were shown to localise to the leading edge of metastatic breast cancer cells suggesting a role for the Hsp90 complex in migration. Following treatment with SDF-1 a change in the putative extracellular Hsp90 containing species occurred. Based on these observations, the objective of this chapter was to investigate the role of Hsp90 during SDF-1 mediated cell migration with particular reference to the role of membrane associated Hsp90 and extracellular soluble Hsp90.

5.2 Results

This chapter explored the role of extracellular Hsp90 mediated migration by means of wound healing assays, analysis of actin reorganisation and focal adhesion formation in the presence of Hsp90 antibodies that target either the N or C terminus of the protein, as well as in the presence of soluble Hsp90. We further explored the effect of antibodies against Hsp90, and soluble Hsp90 on ERK signalling. This analysis focused on the metastatic breast cancer cell line MDA-MB-231.

5.2.1 Investigation of the role of extracellular Hsp90 in migration by wound healing assays

In order to investigate the putative roles of membrane associated and extracellular soluble Hsp90 in migration, we performed wound healing assays in the presence of Hsp90 antibodies and soluble Hsp90 and monitored cell migration over 12 hrs. Wound healing assays were done in the presence of antibodies of Hsp90 that bind either the N- or C-terminus of the protein respectively (henceforth referred to as αHsp90:N and αHsp90:C, respectively) (Figure 21). The antibodies were expected to bind to Hsp90 on the plasma membrane and in doing so inhibit the mechanism by which Hsp90 mediates migration. By performing wound healing assays in the presence of soluble Hsp90, we proposed to determine whether the soluble form of the protein had an effect on migration, which would indicate a role for extracellular soluble Hsp90 in mediating

migration. IgG was used as the isotype control for the potential effect of binding of Fc receptors on cell migration. The expression of CXCR4 in MDA-MB-231 cells was confirmed by immunofluorescence (data not shown) and is in accordance with previous reports of CXCR4 expression in MDA-MB-231 cells in literature [73]. AMD3100 was used as a negative control for migration since it inhibits the SDF-1 receptor CXCR4 mediated signalling. Wound healing assays were carried out in the presence of a combination of αHsp90:N and AMD3100, soluble Hsp90 and a combination of soluble Hsp90 and AMD3100. Cells were treated for 30 minutes at 4 ºC to allow for binding of the antibodies and to prevent the internalisation of the antibodies. Wound healing assays were done in the absence and presence of SDF-1, although over 12 hrs there was no considerable difference in migration with and without the additional stimulant of migration (data not shown). We subsequently showed that ERK1/2 was constitutively active in MDA-MB-231 cells and that a dose response study of MDA-MB-231 cells treated with increasing concentrations of the CXCR4 inhibitor AMD3100 showed a decrease in ERK1/2 phosphorylation (See Appendix: Figure 27). This indicated that there may be constitutive activation of CXCR4 in MDA-MB-231 cells and therefore subsequent wound healing assays were done in the absence of SDF-1. Cells were grown to confluency on 8-well chamber slides pre-treated with fibronectin. A linear wound was made by scraping the cell monolayer with a sterile pipette tip and pictures taken along the entire wound at time zero. Following 12 hours of incubation a second set of images of the wounds were taken. To quantify the changes in wound distance, eight distances were measured along the wounds and the change from 0 hours to 12 hours in the average decrease in the wound size calculated. Wound healing assays were performed in triplicate and the decrease in wound quantified (Figure 22).

Wound healing assays showed an increase in distance migrated after 12 hours following treatment with IgG1, as compared to the distance migrated in untreated MDA-MB-231 cells after 12 hours (15.4 mm and 19.2 mm respectively) (Figure 21A, Untreated and 21B, IgG1). When treated with αHsp90:N a further increase in distance migrated relative to the IgG1 control occurred (20.7 mm) (Figure 21C, αHsp90:N). However treatment with αHsp90:C did not result in a decrease in the distance migrated cells (11.9 mm) as compared to untreated cells (Figure 21D, αHsp90:C). Treatment with the inhibitor of CXCR4, AMD3100, was roughly equivalent to the distance migrated of untreated cells (15.4 mm) (Figure 21E, AMD3100).

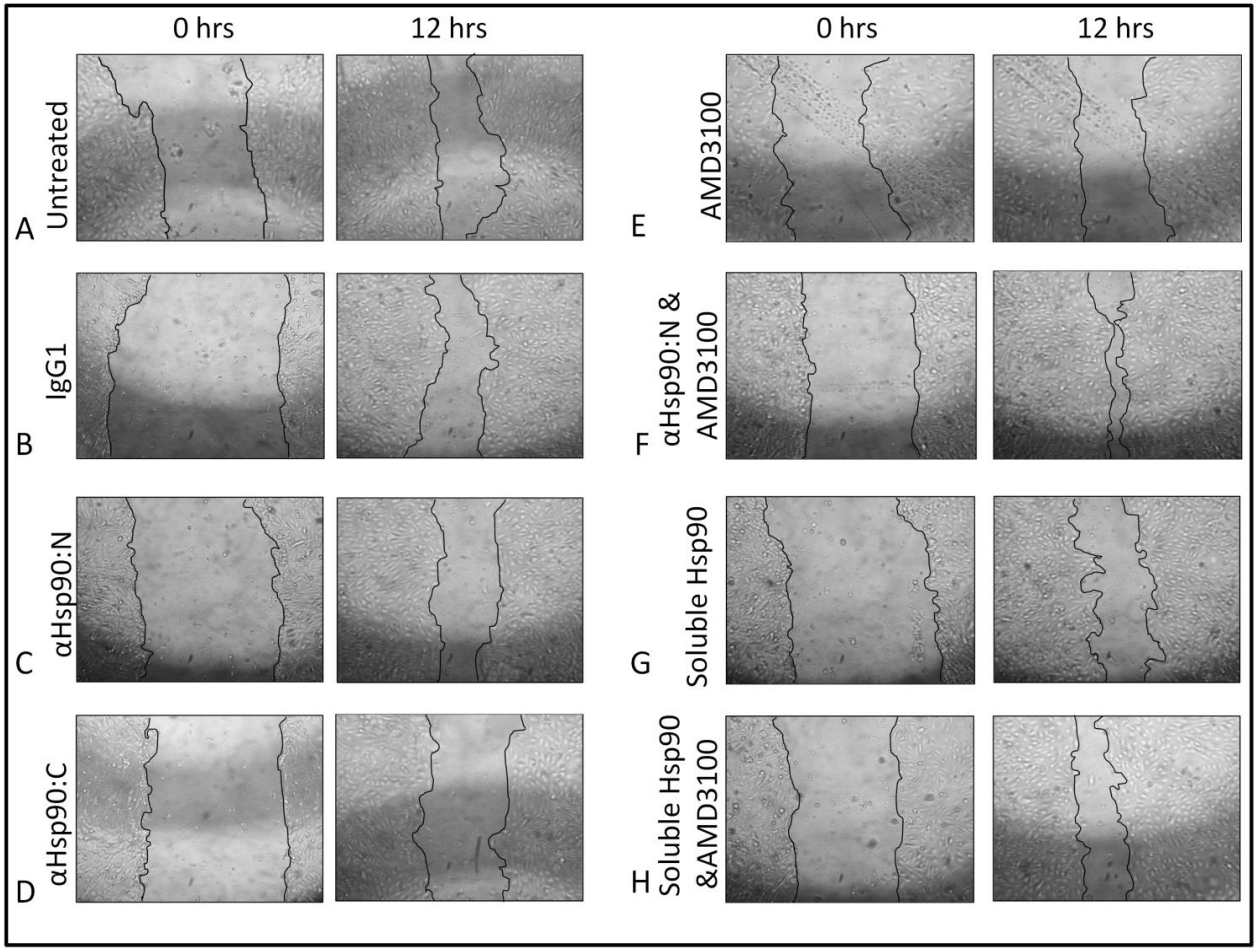

Figure 21: Role of Extracellular Hsp90 in migration of MDA-MB-231 breast cancer cells

MDA-MB-231 breast cancer cells were grown to confluency on fibronectin (0.7 µg/mm^2) coated 8-well chamber slides, after which cells were left untreated [A] or treated with IgG (20 µg/mL) [B], αHsp90:N (20 µg/mL)[C], αHsp90:C (20 µg/mL) [D], AMD3100 (10 µM) [E], αHsp90: N (20 µg/mL) and AMD3100 (10 µM) [F], Soluble Hsp90 (10ng/mL) [G] and Soluble Hsp90 (100 ng/mL and AMD3100 [H] for 30 minutes at 4 ºC after which wounds were made by scraping the cell monolayer with a sterile pipette tip. Images were taken at time 0 hrs and after 12 hrs of incubation.

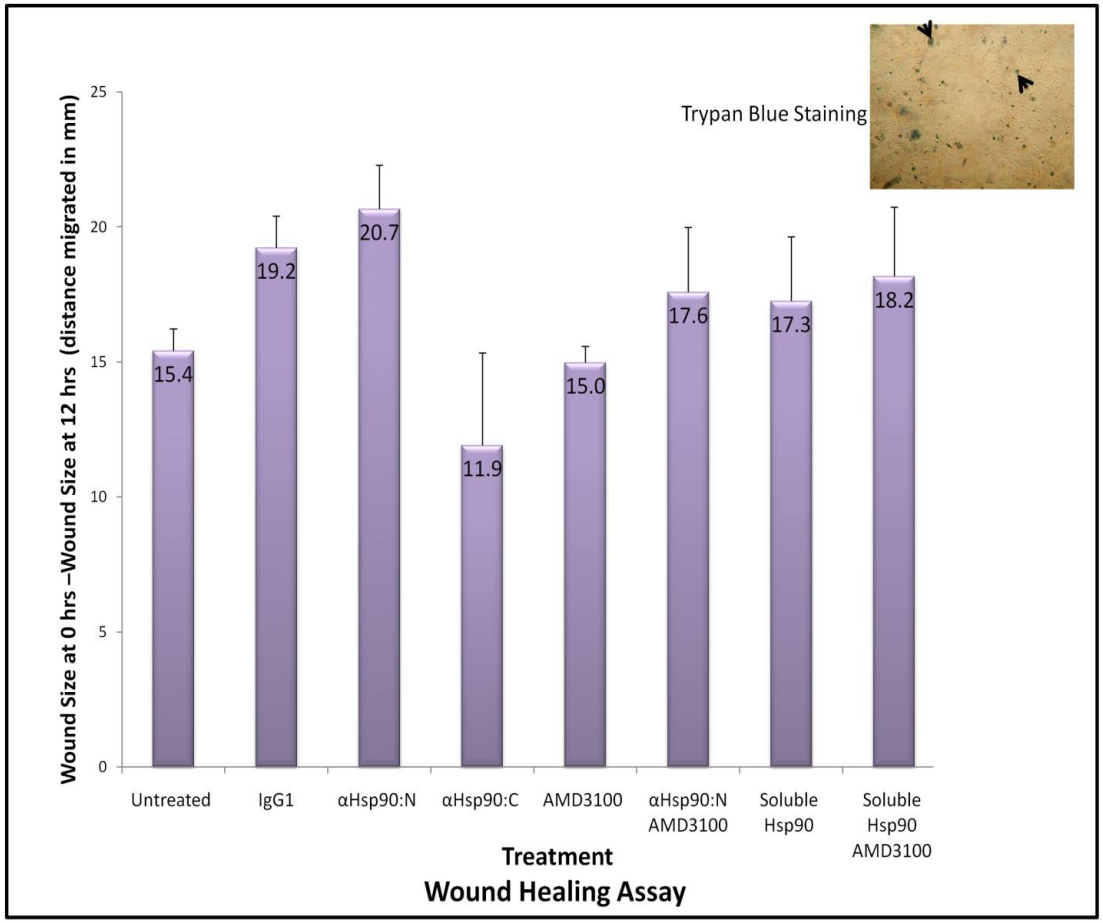

Figure 22: Wound Healing Assays suggested a role for extracellular Hsp90 in migration.

Quantification of distance migrated by cells during the wound healing assay. Eight measurements were taken along each wound and the average wound size calculated for untreated cells (Fig 21A) or cells treated with IgG (Fig 21B), αHsp90:N (Fig 21C), αHsp90:C (Fig 21D), AMD3100 (Fig 21E), αHsp90: N and AMD3100 (Fig 21F), Soluble Hsp90 (Fig 21G) and Soluble Hsp90 and AMD3100 (Fig 21H). Values represent the mean of the change in the wound size after 12 hours ±SD (n=3). (Insert) Trypan blue staining indicating viable cells. Image shown is a section of the wound when cells were treated with soluble Hsp90.

When treated with a combination of αHsp90:N and AMD3100 however, there was a considerable increase in the distance migrated as compared to untreated cells (17.6 mm) (Figure 21F, αHsp90:N and AMD3100). Soluble Hsp90 resulted in an increase in the distance migrated similar to that seen when a combination of soluble Hsp90 and AMD3100 was used as compared to untreated cells (17.3 mm and 18.2 mm respectively) (Figure 21G, Soluble Hsp90 and Figure 21H, Soluble Hsp90 & AMD3100 respectively). In order to confirm that cells were not dead following wound healing, the cells were stained with Trypan blue which is a cell viability stain. Trypan blue can only traverse the membrane of dead cells and hence dead cells appear blue under a microscope (Figure 22: insert). The trypan blue staining showed a few dead cells as indicated by the black arrows, but not a sufficient amount to affect the results of the wound healing assay.

5.2.2 Investigation of effect of extracellular Hsp90 on actin and vinculin staining by immunofluorescence

Based on the results from the wound healing assay, we examined the reorganisation of the actin cytoskeleton and focal adhesion formation in the metastatic breast cancer cell line, MDA-MB-231, in response to the same treatments as used in the wound healing assay. MDA-MB-231 cells were left untreated or treated with IgG1, αHsp90:N, αHsp90:C, AMD3100, αHsp90:N and AMD3100, soluble Hsp90, and soluble Hsp90 and AMD3100. Cells were seeded on fibronectin in the absence and presence of SDF-1. Cells were pre-incubated for 30 minutes at 4 ºC, followed by the addition of SDF-1, after which cells were cultured at 37 ºC for 2 hours. Following fixation with methanol, cells were stained for Hsp90, actin and vinculin, with the exception of cells treated with αHsp90:C which were not stained for vinculin as the antibodies were of the same species. The images shown in this section are indicative of the typical response obtained (See Appendix, Figure 28 for additional images).

The predominant phenotype of untreated MDA-MB-231 cells was a flattened, spread out morphology. Vinculin staining was observed near the periphery of the untreated cells and within the cells as punctate points of staining. A diffuse cytoplasmic vinculin staining was also visible (Figure 23, untreated). Actin staining was observed in the cytoplasm as well as on the periphery of the cells. F-actin filaments were visible in the cytoplasm as long thin disordered strands. Following treatment with SDF-1, the staining intensity and incidence of the actin filaments increased. The vinculin appeared to localise to the tip of the actin filaments although no overlap in the staining of Hsp90 and vinculin is visible (Figure 24, untreated).

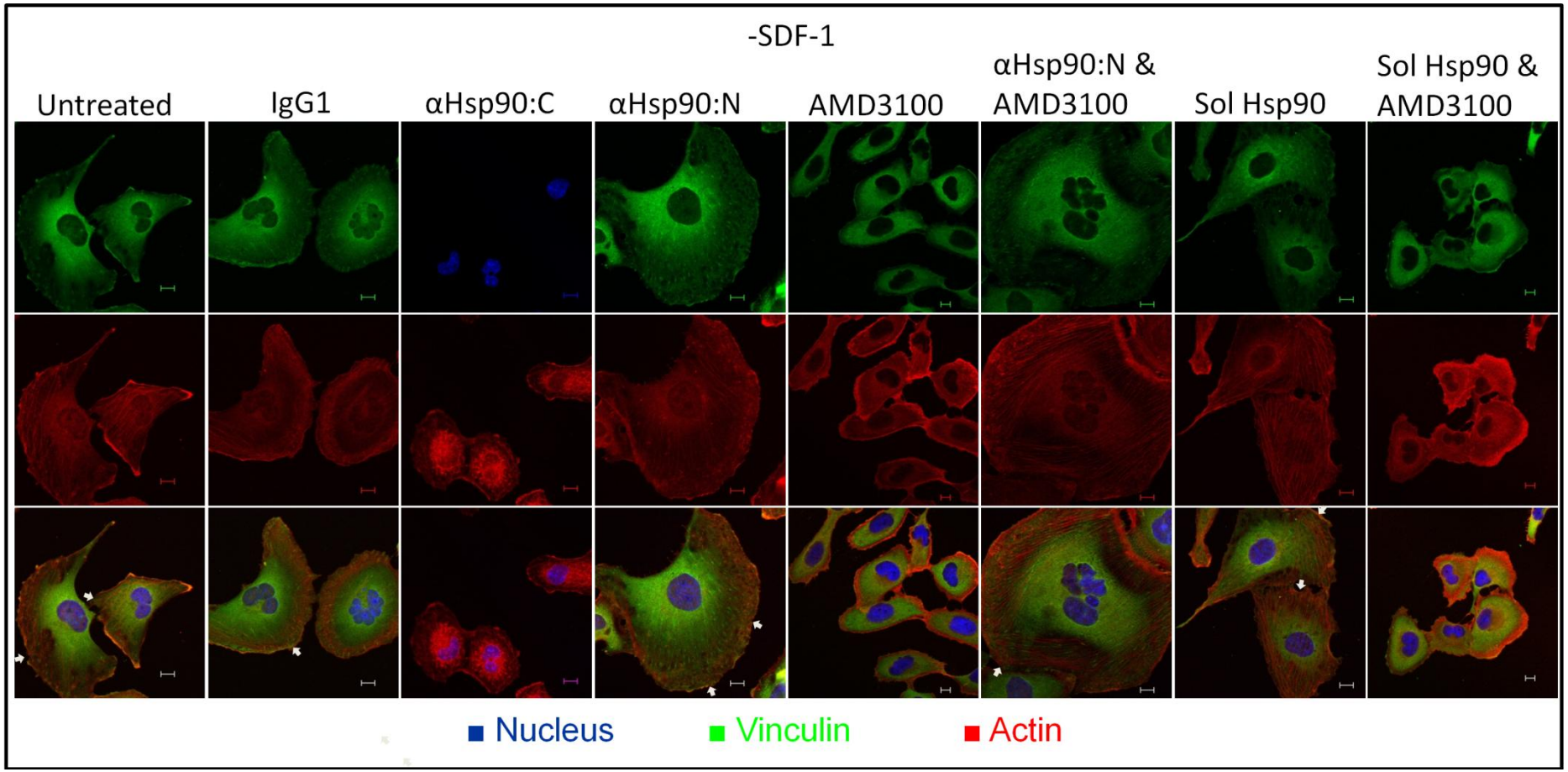

Figure 23: Effect of extracellular Hsp90 on cytoskeleton reorganisation in the absence of SDF-1.

MDA-MB-231 cells were seeded on fibronectin (250 µg/mL).Cells were left untreated or treated with IgG (20 µg/mL) , αHsp90:C (20 µg/mL), αHsp90:N (20 µg/mL), AMD3100 (10 µM), αHsp90:N (20 µg/mL) and AMD3100 (10 µM), Soluble Hsp90 (10 ng/mL) and Soluble Hsp90 (100 ng/mL) and AMD3100 (10 µM) for 30 minutes at 4 ºC followed by incubation for 2 hrs at 37 ºC. Cells were fixed and incubated with a mouse anti-vinculin antibody, rabbit anti-actin antibody followed by donkey anti-mouse-488 (green), donkey anti-rabbit 546 (red) secondary antibodies. Cells treated with αHsp90:C were not stained for vinculin. The nucleus was stained with Hoechst-33342 (blue). Images were captured using the Zeiss LSM 510 Meta confocal microscope and analysed using AxiovisionLE 4.7.1 (Zeiss). Scale bars represent 10 µm. Experiment was carried out in duplicate.

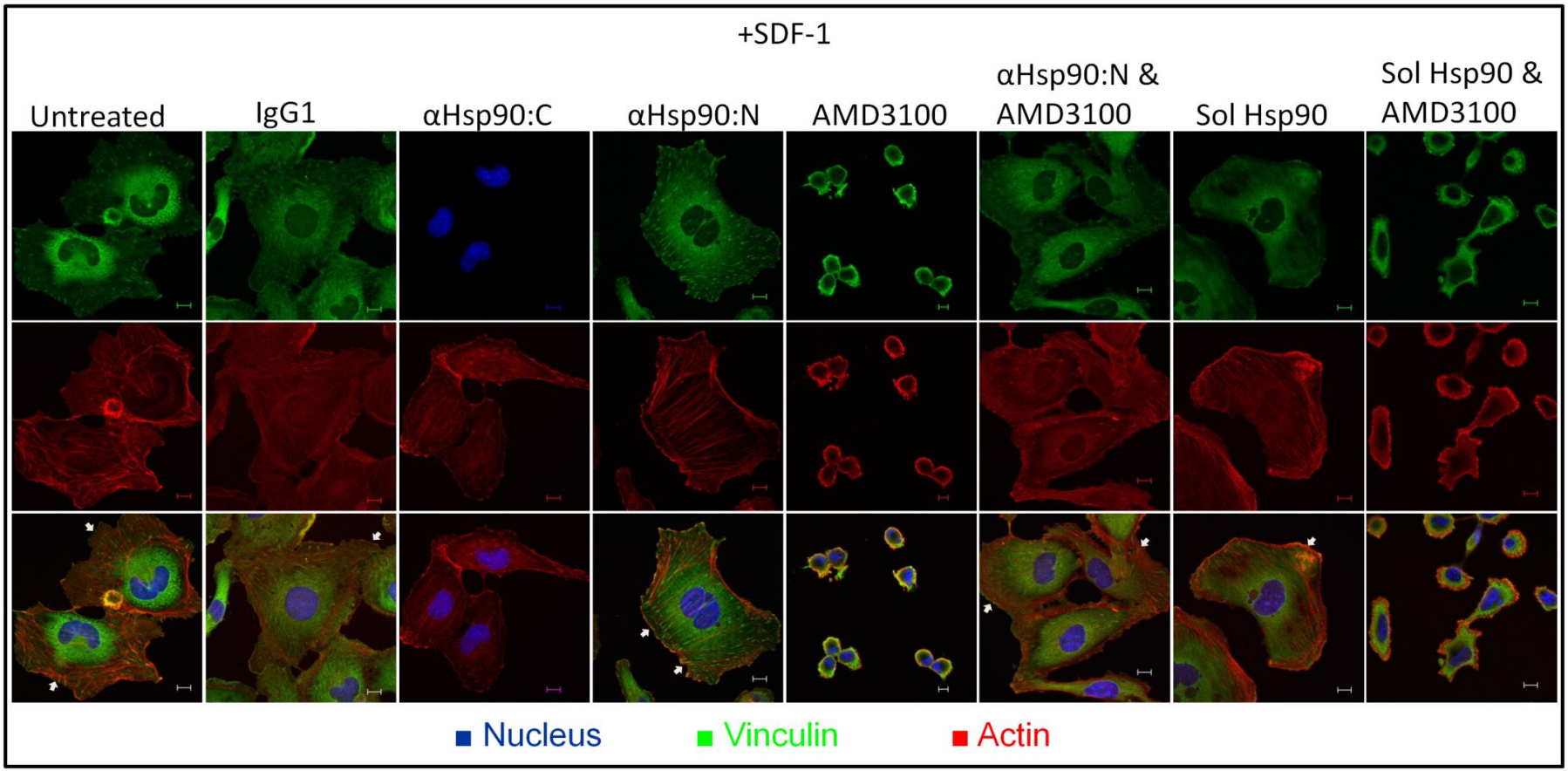

Figure 24: Effect of extracellular Hsp90 on cytoskeleton reorganisation in the presence of SDF-1

MDA-MB-231 cells were seeded on fibronectin (250 µg/mL).Cells were left untreated or treated with IgG (20 µg/mL) , αHsp90:C (20 µg/mL), αHsp90:N (20 µg/mL), AMD3100 (10 µM), αHsp90:N (20 µg/mL) and AMD3100 (10 µM), Soluble Hsp90 (10 ng/mL) and Soluble Hsp90 (100 ng/mL) and AMD3100 (10 µM) for 30 minutes at 4 ºC followed by the addition of SDF-1 (100 ng/mL) to selected cells and incubation for 2 hrs at 37 ºC. Cells were fixed and incubated with a mouse anti-vinculin antibody, rabbit anti-actin antibody followed by donkey anti-mouse-488 (green), donkey anti-rabbit 546 (red) secondary antibodies. Cells treated with αHsp90:C were not stained for vinculin. The nucleus was stained with Hoechst-33342 (blue). Images were captured using the Zeiss LSM 510 Meta confocal microscope and analysed using AxiovisionLE 4.7.1 (Zeiss). Scale bars represent 10 µm. Experiment was carried out in duplicate.

A similar phenotype was noted in cells treated with IgG1 (Figure 23, IgG). Following treatment with SDF-1, there was no considerable change in the morphology of the cells, although the intensity of the actin and vinculin staining appears to increase (Figure 24, IgG).

In cells treated with αHsp90:C, the cells appeared smaller and less spread out. Although actin staining was still observed in the cytoplasm and on the periphery of the cells, there was a considerable decrease in the prevalence and intensity of the F-actin filaments (Figure 23, αHsp90:C). There was no considerable difference between the phenotypes observed for cells treated with anti-Hsp90C and SDF-1 and cells treated with anti-Hsp90C without SDF-1 (Figure 24, αHsp90:C). Upon treatment with αHsp90:N, MDA-MB-231 cells had a more flattened, polarised and more spread out morphology as compared to untreated cells, with a prominent punctuate vinculin staining pattern throughout the cell. A diffuse cytoplasmic vinculin staining was also visible. Prominent F-actin filaments were visible. Furthermore, the actin filaments became more ordered, compared to the actin filaments observed in untreated cells, and appeared orientated towards the leading edge of the polarised cells as indicated by the white arrows (Figure 23: αHsp90:N). Following SDF-1 treatment, although there was no distinct change in morphology of the cells, there was an increase in the intensity and incidence of the F-actin filaments and vinculin staining (Figure 24, αHsp90:N).

When cells were treated with AMD3100, there was a considerable reduction in the size and loss of polarity of the cells (Figure 23, AMD3100). Vinculin was present at the periphery and as diffuse cytoplasmic staining but no punctate vinculin staining could be seen (Figure 23, AMD3100). Although actin staining was present in the cytoplasm and at the periphery of the cells, no F-actin filaments were visible (Figure 23, AMD3100). When treated with AMD3100 and SDF-1, the cells were considerably small and assumed a rounded morphology indicative of dead cells (Figure 24, AMD3100).

In cells treated with a combination of αHsp90:N and AMD3100 the cells had a flattened, spread out, polarised morphology, with punctuate vinculin staining and visible actin stress fibres. The actin filaments were ordered, and appeared orientated towards the leading edge of the polarised cells (Figure 23, αHsp90:N and AMD3100). There was no distinct difference in the staining pattern and morphologies of cells after SDF-1 treatment (Figure 24, αHsp90:N and AMD3100). This suggested that treatment with anti-Hsp90N was able to overcome the deleterious effects of AMD3100, both in the presence and absence of SDF-1 (Figure 24, αHsp90:N and AMD3100).

In cells treated with soluble Hsp90, the cells appeared flattened, spread out and some cells appeared polarised. Punctate vinculin staining was visualised near the periphery of the cells. Within the cytoplasm vinculin staining was diffuse with punctate vinculin staining visible at certain points. Actin staining is present in the cytoplasm as well as on the periphery of the cells. Actin fibres were visible in the cytoplasm and in some cells the actin filaments were ordered such that they were orientated towards the leading edge of the cells (Figure 23, soluble Hsp90). No considerable changes in staining or morphology were apparent in those cells further treated with SDF-1 (Figure 24, soluble Hsp90). However, when treated with a combination of AMD3100 and soluble Hsp90, there was a decrease in the size of the cells and the actin filaments disappeared. Similarly to AMD3100 on its own, upon the addition of SDF-1, cells treated with soluble Hsp90 and AMD3100, reduced further in size and appeared to be dead (Figure 24, Soluble Hsp90). This suggested that soluble Hsp90 was not able to overcome the effects of AMD3100 on MDA-MB-231 cells.

5.2.3 Analysis of ERK phosphorylation by extracellular Hsp90

In order to determine whether the effects of αHsp90: N, αHsp90: C and soluble Hsp90 were mediated through an ERK1/2 dependent pathway, time course and dose dependent studies of the effects of the antibodies and soluble Hsp90 on ERK1/2 phosphorylation were studied. Cells were pre-treated with AMD3100 in order to knock down the constitutive expression of ERK1/2 in the MDA-MB-231 cell line. For dose dependant studies cells were treated with increasing concentrations of antibodies and soluble Hsp90 for one hour before Western analysis. For time course studies, cells were treated with a fixed concentration of antibody and soluble Hsp90 and samples taken after 5 minutes, 10 minutes and 20 minutes.

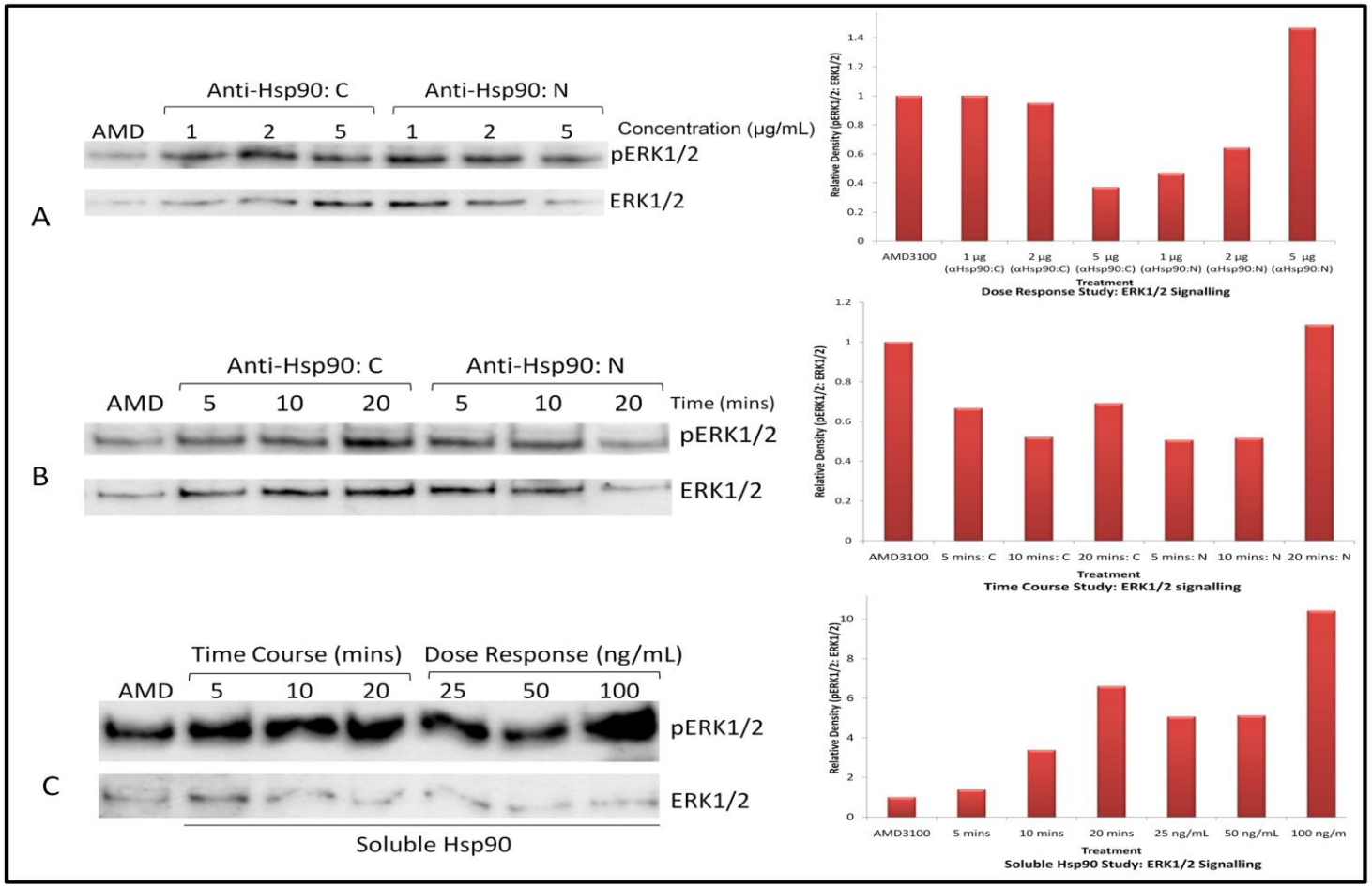

Figure 25: Dose and Time dependent study of ERK1/2 signalling.

Confluent cultures of MDA-MB-231 cells were treated with 10 µM AMD3100 for 1 hour (AMD) followed by dose dependent and time course analyses at the indicated concentrations and times. Cell lysates were analysed for pERK1/2 by Western analysis, the nitrocellulose membrane stripped and reprobed and analysed for ERK1/2. The results were quantified by densitometry using ImageJ and the pERK1/2 density normalised relative to the ERK1/2 values. (A) Dose Dependent study: αHsp90: C (anti-Hsp90 antibody with a C-terminal epitope) and αHsp90: N (anti-Hsp90 antibody with an N-terminal epitope), (B) Time Course Study: αHsp90: C and αHsp90:N, (C) Dose Dependent and Time Course Studies: Soluble Hsp90. Experiment was carried out in duplicate.

Samples were analysed by Western analysis for pERK1/2 followed by stripping of the nitrocellulose membranes which were reprobed for ERK1/2. Density analysis of the pERK1/2 signal and ERK1/2 signals were computed using ImageJ. The pERK1/2 signal intensity was normalised relative to that of the ERK1/2 signal intensity by dividing the pERK1/2 density value obtained from ImageJ analysis by the ERK1/2 density value and the relative signal intensities in response to the treatments compared. All analyses were done in duplicate. The dose dependent study of the effect of αHsp90: C on ERK1/2 signalling showed a decrease in the proportion of phosphorylated ERK1/2 relative to total ERK1/2 following treatment with αHsp90: C (Figure 25A, αHsp90:C). The time course study revealed a decrease in the pERK1/2: ERK1/2 ratio intensity after 5 minutes and 10 minutes relative to the pERK1/2: ERK1/2 signal intensity taken after the knockdown with AMD3100 in response to αHsp90: C. After 20 minutes an increase in the pERK1/2: ERK1/2 signal intensity was observed, although it was still lower than the relative pERK1/2: ERK1/2 signal intensity after knockdown with AMD3100 (Figure 25B, αHsp90:C). An initial decrease in the pERK1/2: ERK1/2 ratio following treatment with αHsp90: N was seen, followed by a dose dependent increase in pERK1/2: ERK1/2 ratio intensity with increasing antibody concentration (Figure 25A, αHsp90:N). At a concentration of 5 μg/mL of αHsp90: N there was a considerable increase in the pERK: ERK signal intensity (Figure 25A, αHsp90:N) compared to the AMD3100 treated cells. Treatment with anti-Hsp90N antibody induced an initial decrease in the relative pERK1/2: ERK1/2 signal intensity as compared to the pERK1/2: ERK1/2 ratio signal intensity after AMD3100 knockdown (Figure 25B: αHsp90:N). After 20 minutes treatment with anti-Hsp90N an increase in the relative ERK1/2 signal was seen (Figure 25B, αHsp90:N). The effect of soluble Hsp90 on ERK1/2 signalling showed an increase in the relative pERK1/2: ERK1/2 signal intensity over time relative to that of AMD3100 knockdown, as well as a more considerable increase in the relative pERK1/2: ERK1/2 signal intensity when treated with increasing concentrations of soluble Hsp90 over an hour (Figure 25C). For all analyses, duplicate studies showed similar trends.

5.3 Discussion

5.3.1 Effect of anti-Hsp90 antibodies and soluble Hsp90 on MDA-MB-231 cell migration

Treatment with an antibody directed against the N terminus of Hsp90 (αHsp90:N) resulted in a decrease in wound gap size after 12 hours whereas treatment with the antibody that binds to the C-terminus of Hsp90 (αHsp90:C) did not result a considerable decrease in wound size. Treatment with the αHsp90:N antibody induced an increase in the incidence of F-actin filaments

and focal adhesion formation in MDA-MB-231 cells as measured by an increase in punctate vinculin staining. In contrast, treatment with αHsp90:C led to a decrease in F-actin filaments and focal adhesion formation as measured by a loss of punctate vinculin staining. Soluble Hsp90 had a pro-motility effect as was shown by a decrease the wound gap size after 12 hours and an increase in the incidence of F-actin filaments and focal adhesion formation. We provided evidence that antibodies targeted to the N-terminus of Hsp90 increased cell migration while an antibody against the C-terminus of Hsp90 decreased cell motility, as measured by the wound healing assays.

IgG1 was used as a control for the effect of antibodies binding to Fc receptors in both the wound healing assays and the immunofluorescence phenotype studies. In the wound healing assays, IgG1 resulted in a decrease in the wound gap suggesting an increase in migration, although it did not appear to have an effect on actin and vinculin. The different effects of the antibodies to the N and the C terminus of Hsp90 are validated by the effects that they have on the fundamental cell motility mechanism of actin reorganisation. The loss of actin stress fibres that was seen in response to treatment with the antibody that targets the C-terminus of Hsp90, as compared to the increase and reorganisation of the actin filaments that was seen in response to the antibody that targets the N-terminus. This suggests that the different effects of the antibodies on cell motility are *bone fide* rather than a non-specific effect of any antibodies.

Previous studies have similarly used an antibody based approach to target extracellular Hsp90. We showed that targeting the C-terminus of Hsp90 inhibits migration. Similarly, an Hsp90 antibody that targets the C-terminus was shown to inhibit migration in T24 bladder carcinoma cells using a wound healing assay [45]. In migrating Schwann cells, lamellipodia at the leading edge were less developed and spread out in cells treated with anti-Hsp90α and anti-Hsp90β as compared to untreated Schwann cells [13]. Similarly, cells treated with an antibody that binds a C-terminal epitope, followed by phalloidin (a toxin that interacts specifically with F-actin bundles) staining showed a marked reduction in phalloidin staining [45]. To the best of my knowledge, this is the first report that used an antibody that binds to an N-terminal epitope of Hsp90 and showed an increase in migration.

5.3.2 Effect of anti-Hsp90 antibodies and soluble Hsp90 on MDA-MB-231 on ERK1/2 phosphorylation

Treatment with the αHsp90:N antibody resulted in a dose and time dependent increase in the relative pERK1/2: ERK1/2 intensity, while in contrast treatment with the αHsp90:C antibody

resulted in a dose and time dependent decrease in the relative pERK1/2: ERK1/2 signal intensity. Soluble Hsp90 resulted in a dose and time dependent increase in relative pERK1/2: ERK1/2 signal intensity.

Using the cell impermeable Hsp90 inhibitor DMAG-N-oxide, dose dependent inhibition of basal ERK1/2 phosphorylation was shown in Hela cells, similarly suggesting ERK1/2 activation as a potential mechanism by which membrane associated Hsp90 mediates migration [40]. Previous studies have similarly shown an activation in ERK1/2 signalling by soluble Hsp90. Oxidative stress was shown to cause the release of Hsp90α from vascular smooth muscle cells resulting in the subsequent activation of ERK1/2 [49]. However, ERK1/2 signalling controls several cell functions including cell growth, transformation, differentiation, metastasis as well as survival after stress [49, 105, 106]. Therefore, the activation of ERK1/2 signalling in response to extracellular Hsp90 might regulate additional downstream process other than cell motility.

Immunofluorescence revealed that a combination of AMD3100 and SDF-1 resulted in a cell morphology that suggested that the cells were dead. However, in the presence of AMD3100, αHsp90:N and SDF-1, the cell phenotypes observed were that of healthy live motile cells. Treatment with the αHsp90:C antibody or soluble Hsp90 in the presence of AMD3100 and SDF-1 resulted in a cell morphology reminiscent of dead cells. This suggested that soluble Hsp90 or an antibody against the C-terminus of Hsp90 was not able to overcome the effect of AMD3100 and SDF-1 on MDA-MB-231 cells. In contrast, αHsp90:N was able to overcome the negative effects of AMD3100 and SDF-1. This suggests that the activation of ERK1/2 signalling in response to extracellular Hsp90 may be in cell survival.

Taken together, these results suggested that extracellular Hsp90 may mediate cell migration and possible other processes in an ERK1/2 dependent manner. It is however necessary to test whether the effects of the antibodies and soluble Hsp90 on ERK1/2 signalling was in response to cell migration and not another cell function mediated by extracellular Hsp90 and ERK1/2 signalling. Wound healing assays and immunofluorescence studies of actin reorganisation and focal adhesion formation could have been done in the presence of αHsp90:N, αHsp90:C and soluble Hsp90 with U0126, a known inhibitor of the ERK1/2 signalling pathway in mammalian cells.

5.3.3 Mechanism of action of anti-Hsp90 antibodies

Both soluble Hsp90 and αHsp90:N antibody had a pro-migratory effect. While soluble Hsp90 was not able to overcome the inhibitory effects of AMD3100 and SDF-1, treatment with αHsp90:N overcame the negative effects of AMD3100 and SDF-1. The mechanism by which

this antibody induced the effect remains to be determined. There are two possible mechanisms by which the antibody could have induced its effect. The first is that the antibody was binding to a membrane-associated form of Hsp90 and thereby inducing the response. The alternate hypothesis was for the antibody to bind to soluble Hsp90 in solution and prevent it from inducing its response.

Our data showed that soluble Hsp90 did not rescue the deleterious effects of treatment of MDA-MB-231 cells with SDF-1 and ADM3100. This was despite the fact that soluble Hsp90 induced phosphorylation of ERK1/2 in MDA-MB-231 cells and had a positive effect on cell migration. Therefore, if the antibody against the N-terminus inhibited soluble Hsp90, it would be more likely that it would inhibit ERK1/2 phosphorylation and not overcome the negative effects of AMD3100 and SDF-1. Taken together these results suggest that the effect of the αHsp90:N was more likely due to binding membrane-associated Hsp90 as opposed to soluble forms of Hsp90. To confirm this proposal it would be necessary to conduct the experiment to examine the effect of co-stimulation with soluble Hsp90 in the presence of the αHsp90:N or αHsp90:C antibodies.

We could not detect Hsp90 in the conditioned media of MDA-MB-231 cells using an antibody targeted at the C-terminus *(data not shown)*. These data are corroborated by published reports that indicated that Hsp90 secreted into the conditioned media of tumour cells could not be detected using an antibody to the C-terminus. Soluble Hsp90 however, could be detected using an antibody to the N-terminus, suggesting that secreted Hsp90 may be cleaved somewhere at the C-terminus [108]. Our data suggests that this might be the same for MDA-MB-231 cells. Although we do not know the exact epitope at which the αHsp90:C antibody bound, its inhibitory effects together with the fact that we could not detect extracellular soluble Hsp90 suggested that αHsp90:C was not targeting soluble Hsp90.

The chemokine, SDF-1, binds to and signals both CXCR4 and CXCR7 receptors in breast cancer cells [71, 109]. In T-lympocytes it has been suggested that there is some crosstalk between CXCR7 and CXCR4. When using a monoclonal antibody to inhibit CXCR7, the ability of membrane associated CXCR4 to activate lymphocyte integrin adhesiveness was severely impaired [70]. A recent study showed that the CXCR4 inhibitor AMD3100 is also a ligand for CXCR7. AMD3100 positively regulated SDF-1 effects and binding to CXCR7 [110]. Therefore if AMD3100 was inhibiting SDF-1 by binding CXCR4, and membrane associated Hsp90 was able to overcome that inhibition, this may suggest that there was a role for CXCR4 and another receptor in Hsp90 signalling. If there was similarly crosstalk between CXCR4 and CXCR7 in

breast cancer cells, one might speculate that αHsp90:N prevented cell death through a CXCR7 mediated pathway.

These data do not conclusively show that the αHsp90:N antibody bound to membrane associated Hsp90, as opposed to soluble forms of Hsp90. However, based on the fact that soluble Hsp90 could not prevent cell death induced by SDF-1 and AMD3100 but the αHsp90:N antibody could, we interpreted our results to suggest that the antibody targeted a membrane-associated Hsp90 species. This provided putative evidence for an alternative function for membrane associated Hsp90 in response in cell death.

5.3.4 Conclusions

Our data suggested a role for both membrane associated Hsp90 and extracellular Hsp90 in cell migration. We propose that the mechanism by which membrane associated Hsp90 mediates migration in MDA-MB-231 cells is SDF-1 dependent and that in mediating migration both membrane associated Hsp90 and extracellular Hsp90 play a role in the reorganisation of the actin cytoskeleton and focal adhesion formation. We provided some evidence that extracellular Hsp90 mediated migration at least in part through an ERK1/2 dependent pathway and described putative evidence for a role for membrane associated Hsp90 in cell survival.

Chapter 6.

Discussion and Conclusions

6.1 Objectives and Summary of Results

The broad objectives of this study were to characterise the expression of the members of the Hsp90 chaperone complex on the surface of cancer cells of varying degrees of adhesion and migratory capacity, investigate a possible interaction of extracellular Hsp90 with associated chaperones and examine the role of both extracellular membrane associated and extracellular soluble Hsp90 in migration.

This study provided evidence to suggest that Hsp90, Hsp70 and Hop were membrane associated in MDA-MB-231 and MCF-7 breast cancer cells. We showed a potential membrane association of Hsp90 and Hsp70 in THP1 cells and that following adhesion, Hop was similarly membrane associated. We showed that Hsp90, Hsp70 and Hop in MDA-MB-231, MCF-7 and THP1 cells possibly associated with the plasma membrane such that at least part of the protein is located extracellularly. In U937 cells, we provided putative evidence to suggest that Hsp90, Hsp70 and Hop are membrane associated such that part of the proteins were located extracellularly following adhesion. We revealed that Hsp90, Hsp70 and Hop at the leading edge of polarised MDA-MB-231 cells may localise to membrane ruffles. Henceforth we provided putative evidence for an extracellular membrane associated complex containing Hsp90, Hsp70 and Hop. We showed that when stimulated with SDF-1, there was a change in the multi-protein complexes that contained extracellular Hsp90. We proposed that membrane associated and extracellular soluble Hsp90 may mediate migration by controlling actin reorganisation and focal adhesion formation and that extracellular Hsp90 may mediate signalling through an ERK1/2 dependent pathway. We further showed that whereas as αHsp90:N appeared to be able to overcome the death inducing effects of a combination of SDF-1 and AMD3100, soluble Hsp90 could not. These data suggest a role for an extracellular Hsp90 complex that mediates migration and other biological functions.

6.2 The plasma membrane as a chaperone protein folding compartment

Cellular chaperones are typically defined by their ability to assist the folding and assembly of proteins. Previous studies suggested that protein folding occurs within the cell and proteins in their active conformation are subsequently transported to the plasma membrane or secreted from the cell where they function extracellularly [111]. We hypothesise that the plasma membrane, as with the cytoplasm or intracellular organelles, is a dynamic organelle in its own right and proteins are subject to chaperone-assisted folding when associating with the plasma membrane. As with the intracellular Hsp90 chaperone machinery, an extracellular Hsp90 chaperone

complex would require chaperones, associated co-chaperones and ATP in order to modulate the conformation of client proteins. There are many unanswered questions as to how the extracellular chaperone complex may function.

6.3 Extracellular and membrane-associated chaperones and co-chaperones

Although we provide evidence to show that in certain cancer cell models Hsp90, Hsp70 and Hop are membrane associated and associated with the plasma membrane such that at least part of the protein is located extracellularly, it remains to be determined how Hsp90 reaches the plasma membrane or extracellular space. The known isoforms of Hp90 do not have the signal sequences that would target the protein to the membrane or a secretory pathway [112]. Similarly, neither Hsp70 nor Hop carry the necessary signal sequences [2, 21]. The amino acid sequence of Hsp90 on the plasma membrane has never been fully elucidated and it may be that extracellular Hsp90 is an alternately spliced variant [112]. Without knowing the signal sequence it also becomes impossible to predict the orientation of the protein and although we provided putative evidence to suggest that the N-terminus was exposed to the extracellular space, there is contradicting evidence in the literature to support this [24]. A possibility is that Hsp90, Hsp70 and Hop associate with lipid components in the plasma membrane. It has been shown that Hsp70 associates with phosphatidylserine (PS) in tumour cells and it is hypothesised that following binding of Hsp70 to PS, a flip flop mechanism may result in the transport of Hsp70 from the cytoplasm to the outer leaflet of the cell membrane [35]. Interestingly it was shown that both Hsp90 and Hsp70 associate with lipid rafts in the plasma membrane in response to LPS [38]. Hsp70 has been shown to be secreted via an exosomal pathway [2]. Similarly, Hsp90 has been reported to be present in exosomes, suggesting exosomal export as a possible mechanism for Hsp90 release form the cell and hence account for the presence of Hsp90 in the extracellular space [113].

6.4 Extracellular and membrane-associated client proteins of Hsp90

The intracellular Hsp90 complex is a multi-faceted machinery complex interacting with over 100 client proteins. Membrane associated Hsp90 binding proteins include, TLR4 and HER2 whereas extracellular soluble Hsp90 has been shown to act with CD91 and MMP2. This suggested that similar to intracellular Hsp90, the extracellular pool of Hsp90 may govern the stabilisation and activity of an array of client proteins, extending its extracellular function beyond that of migration and the immune response. We provided some evidence to show that upon stimulation with SDF-1 there is a change in the isolated extracellular complexes that contain Hsp90. This

may suggest a possible recruitment of a client protein to a potential Hsp90 chaperone complex. This study focused in particular of the role of extracellular Hsp90 in mediating migration. If an extracellular chaperone complex is mediating migration, one might propose that the client proteins of the extracellular chaperone complex, will be membrane receptors of the cell migratory machinery such as cadherins, integrins or chemokine receptors.

The integrin family of adhesion molecules mediates cellular contacts to the ECM, and in this way regulates cell motility. We revealed that inhibiting Hsp90 prevents reorganisation of the actin cytoskeleton and focal adhesion formation. Since Hsp90, Hsp70 and Hop were all only present on THP1 and U937 cells following adhesion this might suggest that the role of the Hsp90 chaperone complex in migration is linked to a role in adhesion. Integrins initiate focal adhesion formation following binding to extracellular ligands, ensuring adhesion to an underlying substrate as well as the targeted location of the actin filaments generating cell polarity [65]. Integrins switch from a low affinity for extracellular ligands to a high affinity, and the high affinity conformation is necessary for ligand binding and subsequent downstream signalling process leading to cell motility. Hsp90 binds substrates that have already reached their correct folded state [9]. Therefore, the extracellular Hsp90 chaperone complex may serve to maintain the active conformation of integrin membrane receptors and in this capacity mediate cell migration. One mechanism of integrin activation is through the binding of extracellular chemokines such as SDF-1 to G-protein coupled receptors which in turn lead to integrin activation. Previous studies have shown that an interaction of Hsp60 with $\alpha_3\beta_1$ is sufficient to activate the $\alpha_3\beta_1$ integrin [80]. The endoplasmic reticulum chaperone, calreticulin, has been identified on the cell surface where it was thought to stimulate cell spreading of melanoma cells [114]. Calreticulin is thus crucial for motility and has been shown to associate with the $\alpha_2\beta_1$ integrin on platelet cell surfaces where it was shown to modulate the conformation of the integrin for interaction with extracellular ligands [115]. Hsp90 has been shown to interact with the intracellular domain of $\alpha_v\beta_3$ following VEGF stimulation signalling. Hence, not only would an integrin as a possible membrane associated Hsp90 clients account for the SDF-1 dependent manner in which membrane associated Hsp90 appears to control migration, but it would also account for the dependence on focal adhesion formation and actin cytoskeleton reorganisation.

This study showed that following SDF-1 stimulated migration, Hsp90, Hsp70 and Hop localise to membrane ruffles and that SDF-1 stimulation resulted in a change in extracellular complexes that contain Hsp90. This suggests a role for Hsp90 in SDF-1 mediated migration. Another potential client protein of the extracellular membrane associated chaperone complex may be CXCR4. Membrane associated CXCR4 modulates migration in response to SDF-1 by

controlling the conformation of integrins on the cell surface subsequently leading to focal adhesion formation and actin reorganisation [65, 66]. Previous studies have revealed membrane complexes with Hsps that include CXCR4 [38]. Interestingly we show that one of the biotinylated cell surface proteins interacting with Hsp90 as revealed by immunoprecipitation was resolved at 42 kDa, the molecular mass of CXCR4.

Although we propose that membrane associated Hsp90 mediates adhesion and migration in an SDF-1 dependent manner, this is not to say that extracellular soluble Hsp90 doesn't play a role in migration. There is precedence in the literature for proteins that are functional in both membrane associated and soluble forms, eg CD23 [116]. It may be that soluble Hsp90 associates with the plasma membrane and is able to bind a receptor on the cell surface as was shown by the binding of extracellular soluble Hsp90α to CD91 in promoting both dermal and epidermal cell migration [47]. Using biotin-streptavidin affinity purification we showed that Hsp90, Hsp70 and Hop associate with the plasma membrane such that at least part of the proteins are located extracellular and may include extracellular soluble proteins associating with receptors on the plasma membrane. Alternatively, soluble Hsp90 may execute its pro-motility effect in response to its ability to mediate invasion. Hsp90α was shown to be important for tumour invasiveness in HT-1080 cells. Hsp90 was shown to interact with MMP2 by immunoprecipitation. The MMP2 protease is important for digestion of many components of the ECM and for subsequent invasion of primary tumour cells. It was further shown that Geldanamycin-agarose inhibits the pro-invasive nature of Hsp90α [112].

6.5 ATP and ATPase activity in the membrane and extracellular environment

The intracellular Hsp90 chaperone complex modulates the tertiary structure and conformation of client proteins in an ATP dependent manner. Extracellular Hsp90, be it soluble or membrane associated, would similarly be thought to require ATP hydrolysis. Yet the levels of ATP outside the cell are relatively low [34]. Hsp90N, the 75 kDa homologue of Hsp90 is known to associate with the plasma membrane and lacks the N-terminus or ATPase domain of the protein. Although we provided some evidence for a lower molecular weight Hsp90 species, presence of a potential chaperone complex on the cell membrane suggested that it is the full length Hsp90 that is mediating migration. Furthermore, there is evidence in the literature to suggest that extracellular Hsp90 functions in an ATP dependent manner. Geldanamycin, which binds the ATPase domain of Hsp90, was shown to inhibit the invasive nature of extracellular soluble Hsp90 in fibrosarcoma cells suggesting that ATP is essential for extracellular Hsp90 to function [34].

Similarly, DMAG-N-oxide which is cell impermeable and binds to the ATPase domain of Hsp90 was shown to inhibit both motility and invasion [45]. Similarly, using an antibody targeted to the N-terminus of Hsp90 we revealed an increase in motility suggesting that the ATPase domain is involved in for migration. This would suggest that Hsp90 on the cell surface that mediates migration is not Hsp90N as this homologue lacks that ATPase domain of the full length protein. It may be that membrane associated Hsp90 is orientated within the plasma membrane such that the ATPase domain is orientated towards to cytoplasm. Therefore, the exact mechanism of ATPase hydrolysis essential for the chaperone function of Hsp90 remains to be elucidated for the extracellular pool of the protein.

6.6 Future work

Although we provide evidence for the expression of an Hsp90 chaperone complex on the plasma membrane, this should be validated by chemical crosslinking followed by immunoprecipitation with an Hsp90 antibody and mass spectrometry performed on any molecular weight bands that are resolved higher than 90 kDa. Since we have showed that immunoprecipitation of extracellular Hsp90 results in numerous higher molecular mass species containing Hsp90, mass spectrometry may reveal more than one extracellular Hsp90 complex that may include both extracellular soluble or membrane associated Hsp90. This may provide evidence to confirm an extracellular Hsp90 chaperone complex and further extend the members of the extracellular chaperone complex to other chaperones and co-chaperones of the Hsp90 chaperone complex such as p23 and Hsp40. Mass spectrometry could also reveal the membrane associated client protein of the Hsp90 chaperone complex. In light of our results it may be necessary to stimulate the cells with SDF-1 to reveal the client protein of membrane associated Hsp90 involved in the mediation of migration and provide valuable insight into the mechanism of Hsp90 mediated migration. Mass spectrometry may be used to sequence proteins. Similarly, mass spectrometry could be used to reveal whether a specific homologue of Hsp90 is present in the Hsp90 complex by analysis of the amino acid sequence obtained.

The results of immunoprecipitation and mass spectrometry analysis could be further validated using fluorescence resonance energy transfer (FRET). FRET is a non-invasive imaging technique used to measure molecular proximity [37]. FRET occurs when donor and acceptor labelled fluorophores have sufficiently large spectral overlap and a proximity of 1-10 nm resulting in an overall increase in emission [117]. Using a FRET based approach it would be possible to directly measure the interactions between Hsp90 in a multi-protein receptor complex and a client protein. FRET analysis could also be used to show receptor complex formation. Not

only did we show that SDF-1 treatment resulted in an upregulation of Hsp90 on the surface of breast cancer cells, but also that SDF-1 treatment resulted in a change in the expression patterns of extracellular Hsp90 containing complexes. This suggested that the membrane associated Hsp90 complex formation may be a multi-step process. Using FRET it would be possible to monitor the distance of proteins previously identified to be part of the multi-protein Hsp90 complex with and without SDF-1 as well as quantify the interaction with and without SDF-1 as it may be that there is some basal interaction occurring. Furthermore, since the Hsp90 multi-protein complex may not be limited to the Hsp90 chaperone complex and a single client protein but rather a sequence of signalling events including multiple proteins, using a FRET live imaging based approach it would be possible to monitor the client protein Hsp90 interaction as well as the proximity of the membrane associated proteins in signal transduction pathways that follow highlighting the signalling events [118].

6.7 Conclusion

This study is the first of its kind to provide evidence for an extracellular multi-chaperone complex. We challenge previous dogma as we suggest that proteins are not in fact transported to the plasma membrane in an active conformation but rather that chaperone assisted folding and conformational regulation of client proteins can occur on the plasma membrane. The presence of a putative extracellular multi-chaperone complex provides a novel and attractive target for the treatment of metastatic cancer using cell-impermeable inhibitors of Hsp90. Intracellular Hsp90 inhibitors are an attractive anti-cancer therapy as although they are directed towards a specific molecular target, they simultaneously inhibit multiple oncogenic pathways. There is growing evidence to suggest that Hsp90 on the cell surface may interact with a number of receptors and in doing so mediate a number of cell functions and therefore targeting extracellular Hsp90 may similarly disrupt multiple oncogenic pathways. Not only is Hsp90 known to be upregulated in tumour cells but Hsp surface expression appears to be a feature of tumour cells. Although Hsp70 is found on the plasma membrane of various carcinoma cells, the corresponding normal tissues were nearly always found to be membrane associated Hsp70 negative [1]. Similarly, the incidence of membrane associated Hsp90 expressing cells is upregulated in malignant melanomas as compared to benign melanocytic lesions [24]. Our studies do not extend to the corresponding normal tissues and this remains to be done in the future. Targeting the extracellular pool of Hsp90 would not have the negative effect that other inhibitors have on healthy tissue. An understanding of how Hsp90 and its chaperone machinery functions extracellularly as well as the elucidation of the chaperone complex client proteins is key information for the design of a drug to target extracellular Hsp90.

Chapter 7.

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Chapter 8.

Appendix

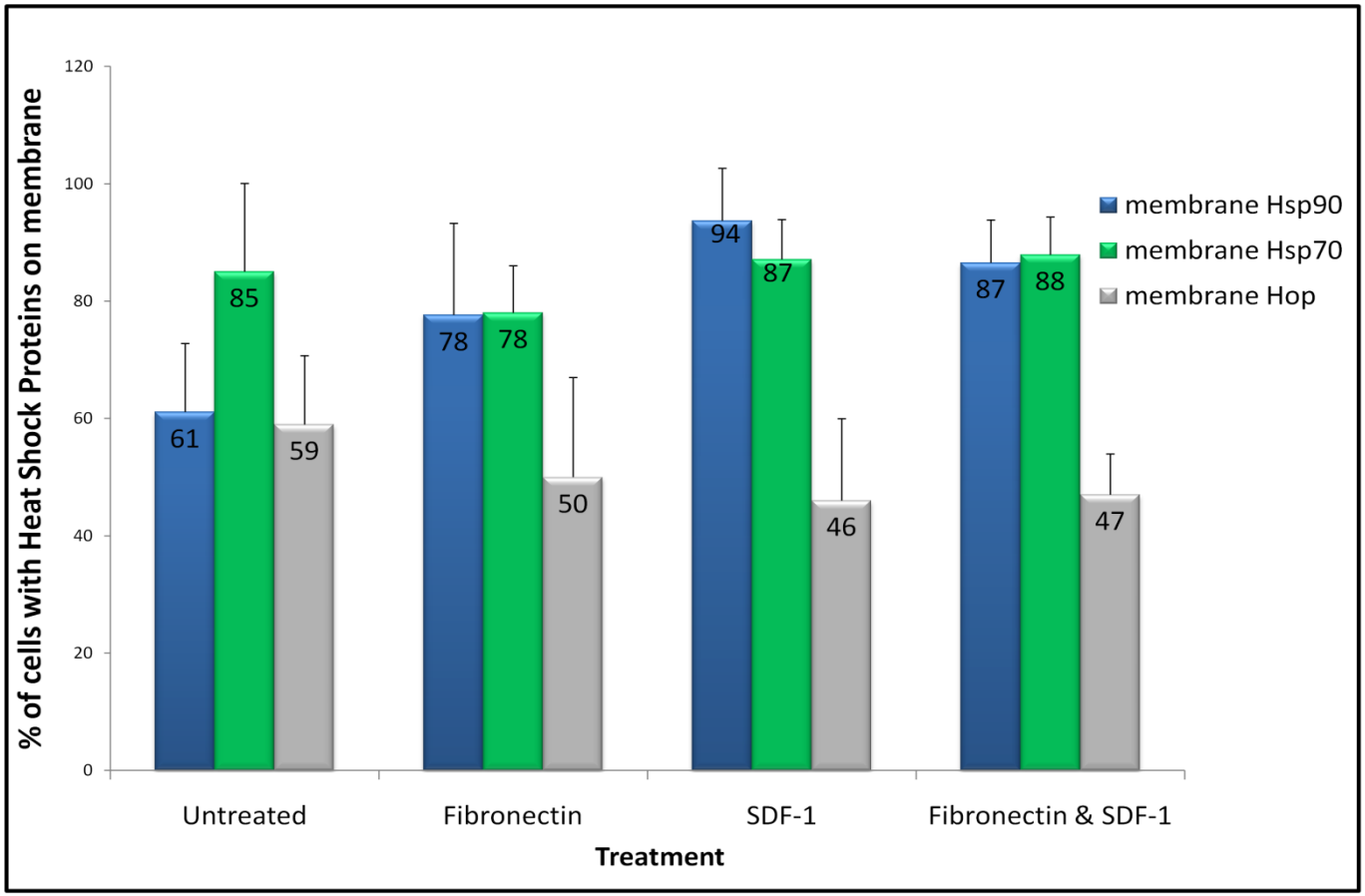

Figure 26: Quantification of Hsp membrane expression in response to SDF-1 stimulation in MDA-MB-231 cells as detected by immunofluorescence.

Hsp90, Hsp70 and Hop membrane expression as shown in Figures 10A-C was counted in 5 different fields of view at 20x magnification and the percentage of Hsps localized to the membrane determined for untreated cells, cells seeded on fibronectin, cells treated with SDF-1 and cells seeded on fibronectin and treated with SDF-1.

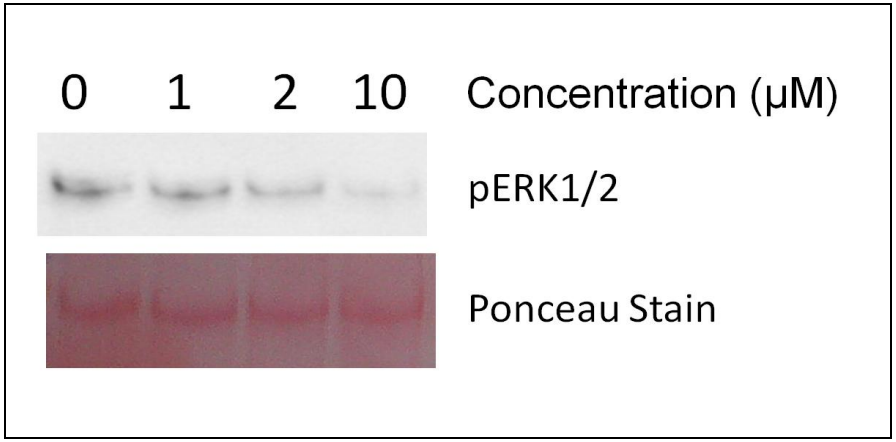

Figure 27: Constitutive activation of CXCR4 in MDA-MB-231 cells.

Dose response study of cells treated with the CXCR4 inhibitor AMD3100. Cell lysates were analysed for pERK1/2 by Western analysis. The Ponceau stain serves as a control for the levels of pERK1/2.

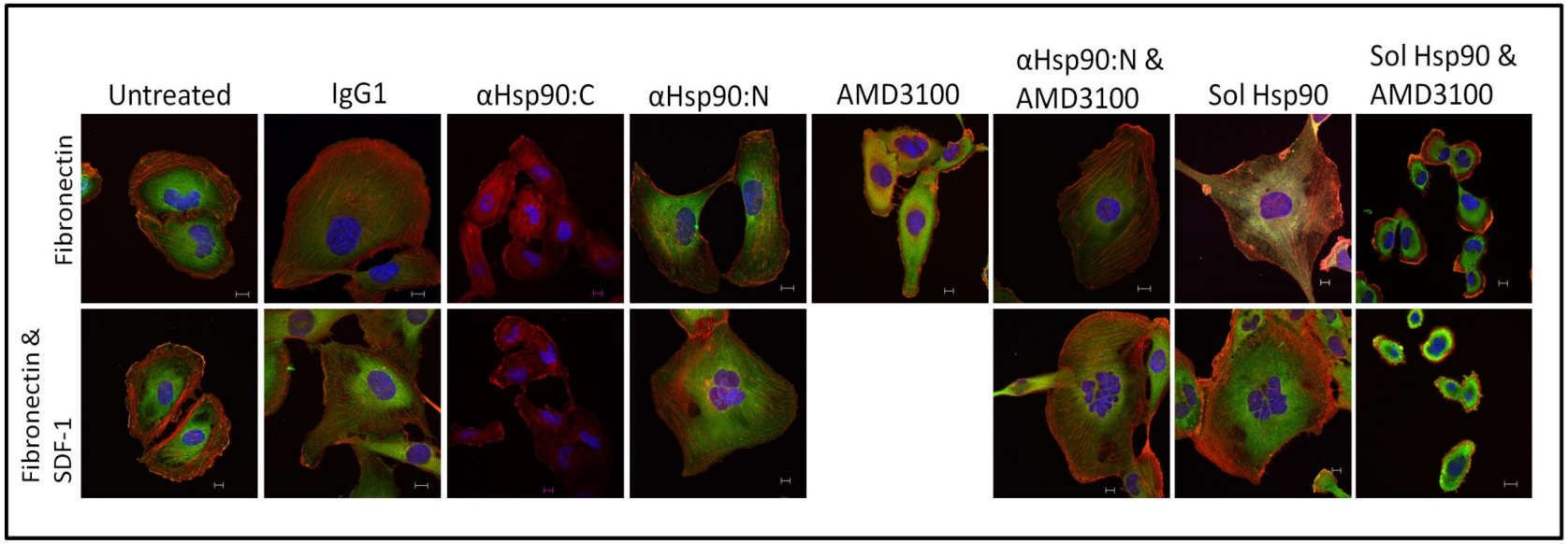

Figure 28: Effect of extracellular Hsp90 on cytoskeleton reorganisation in the absence and presence of SDF-1.

MDA-MB-231 cells were seeded on fibronectin (250 µg/mL) and treated with or without SDF1 (100 ng/mL).Cells were left untreated or treated with IgG (20 µg/mL) , αHsp90:C (20 µg/mL), αHsp90:N (20 µg/mL), AMD3100 (10 µM), αHsp90:N (20 µg/mL) and AMD3100 (10 µM), Soluble Hsp90 (10 ng/mL) and Soluble Hsp90 (100 ng/mL) and AMD3100 (10 µM) for 30 minutes at 4 ºC followed by the addition of SDF-1 (100 ng/mL) to selected cells and incubation for 2 hrs at 37 ºC. Cells were fixed and incubated with a mouse anti-vinculin antibody, rabbit anti-actin antibody followed by donkey anti-mouse-488 (green), donkey anti-rabbit 546 (red) secondary antibodies. Cells treated with αHsp90:C were not stained for vinculin. The nucleus was stained with Hoechst-33342 (blue). Images were captured using the Zeiss LSM 510 Meta confocal microscope and analysed using AxiovisionLE 4.7.1 (Zeiss). Scale bars represent 10 µm.