The role of Hsp90/Hsp70 organising protein (Hop) in the Proliferation, Survival and Migration of Breast Cancer Cells

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Tarryn Willmer

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Abstract

Hop (the Hsp90/Hsp70 organising protein) is a co-chaperone that acts as an adapter between the major molecular chaperones Hsp90 and Hsp70 during the cellular assembly of the Hsp90 complex. The Hsp90 complex regulates the stability and conformational maturation of a range of important cellular proteins, many of which are deregulated in cancer. In this study, we hypothesised that Hop knockdown inhibits proliferation and migration of cancer cells. We characterised the expression of Hop in cell models of different cancerous status, and provided evidence that Hop was upregulated in tumour cells compared to normal cell counterparts.

Using an RNA interference approach, a 60-90% knockdown of Hop was achieved for up to 144 hours in the MDA-MB-231 and Hs578T breast cancer cell lines. Hop knockdown resulted in downregulation of the Hsp90 client proteins, Akt and Stat3, as well as a change in the expression of other Hsp90 co-chaperones, p23, Cdc37 and Aha1, while no change in the levels of Hsp90 or Hsp70 was observed. Silencing of Hop impaired cell proliferation in Hs578T cells but an increase in proliferation in MDA-MB-231, suggesting that the role of Hop in cancer cell proliferation was dependent on type of cancer cell. Hop knockdown in Hs578T and MDA-MB-231 cells did not lead to any significant changes in the half maximal inhibitory concentrations (IC_{50}) of selected small molecule inhibitors (paclitaxel, geldanamycin and novobiocin) in these cell lines after 72 hours. Hop knockdown cells were however, more sensitive than control cells to the Hsp90 inhibitors geldanamycin and novobiocin at earlier time points and in the presence of the drug transporter inhibitor, verapamil.

Hop knockdown caused a decrease in cell migration as measured by the wound healing assay in both Hs578T and MDA-MB-231 cells. Hop was present in purified pseudopodia fractions of migrating cells, and immunofluorescence analysis showed that Hop colocalised with actin at the leading edges of pseudopodia, points of adhesion and at intercellular junctions of cells that have been stimulated to migrate with the chemokine stromal derived factor-1. Hop was able to bind to actin *in vitro* using actin cosedimentation assays, and silencing of Hop dramatically reduced the capacity of Hs578T cells to form pseudopodia. These results establish a correlation between Hop and actin dynamics, pseudopodia formation and migration in the context of Hop silencing, and collectively suggest that Hop plays a role in cancer cell migration. This study presents experimental evidence for a promising alternative to targeting Hsp90 and Hsp70 chaperones, a novel drug target in cancer therapy.

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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Ms Tarryn Willmer, December 2011

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List of Abbreviations

Aha1	Activator of Heat shock protein 90 ATPase
AIF	Apoptosis-inducing factor
ATP	Adenosine Triphosphate
ATPase	Adenosine 5´-triphosphatase
BSA	Bovine serum albumin
BS3	Bis[sulfosuccinimidyl]suberate
Cdc37	Cell Division Cycle 37
Cdk 4	Cyclin-dependent kinase 4
Cns1	Cyclophilin 7 suppressor 1
Crp6	Cyclophilin 6
Crp 7	Cyclophilin 7
c- Src	Cellular-sarcoma tyrosine kinase
DMEM	Dulbecco's Modified Eagle Medium
ECL	Enhanced chemiluminescence
EEVD	glutamate-glutamate-valine-aspartate motif
EGF	Epidermal growth factor
EGFP	Enhanced Green Fluorescent Protein
EGFR	Epidermal growth factor receptor
EDTA	Ethylenediaminetetraacetic Acid
EGTA	Ethyleneglycol-bis(beta-aminoethylether)N'N'N'N-tetraacetic acid
ER	Endoplasmic Reticulum

Erbb2	See Her-2
Erk	Extracellular signal-regulated kinase
FCS	Foetal calf serum
FLT3	Fms-like tyrosine kinase receptor-3
FRET	Fluorescence Resonance Energy Transfer
GA	Geldanamyacin
Her-2	Human Epidermal growth factor Receptor 2
HIF	Hypoxia-inducible factor
HIP	HSC70-interacting protein
Hop	Hsp90/Hsp70 organising protein
Hsps	Heat shock proteins
Hsp90	Heat shock protein 90 kilodaltons
Hsp70	Heat shock protein 70 kilodaltons
Hsp40	Heat shock protein 40 kilodaltons
Ig	Immunoglobulin
KCl	Potassium chloride
MAPK	Mitogen Activated Protein Kinase
Mek	Meiosis-specific serine/threonine Kinase
MET	Hepatocyte growth factor receptor
MMP2	Matrix Metalloproteinase-2
PBS	Phosphate buffered saline
PrPC	Cell surface prion protein
Raf	Serine/threonine-specific kinase

RPMI	Roswell Park Memorial Institute
SDF-1	Stromal cell-derived factor-1
SDS	Sodium-Dodecyl-Sulphate
SDS-PAGE	Sodium-Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis
Stat3	Signal Transducer and activator of transcription 3
pStat3	Phosphorylated Signal Transducer and activator of transcription 3
TBS	Tris-buffered Saline
TBST	Tris-buffered Saline with Tween-20
TPR	Tetratricopeptide repeat
VEGF	Vascular endothelial growth factor
17-AAG	17-N-Allylamino-17-demethoxygeldanamycin

List of Symbols

α	Alpha
β	Beta
^{o}C	Degree Celsius
M	Molar
mM	Millimolar
nM	Nanomolar
µg	Micrograms
µl	Microlitres
L	Litres
g	Grams
mg	Milligrams
kDa	Kilodaltons
min	Minutes
mol	Mole
ml	Millilitre(s)
%	Percent or g/100 ml
U	Units
x *g*	Relative centrifugal force to gravity

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Chapter 1 : Literature Review

1.1 Introduction

The ability of a cell to discern whether to grow, divide, differentiate or die depends upon extracellular signals, as well as the ability to respond to these signals in an orchestrated manner (Mosser and Morimoto, 2004). Transformation of normal cells occurs through loss or mutation of tumour suppressor genes, or activation or amplification of oncogenes, leading to deregulation of signal transduction pathways that ultimately lead to a step wise progression of cells into invasive tumours (Shen and Brown, 2003). In a landmark review by Hanahan and Weinberg (2000), the multistep progression of tumour development was organised into a framework of six essential alterations in cell physiology that are responsible for the phenotype we recognise as cancer. These 'cancer hallmarks' are described as (i) self sufficiency in growth signals; (ii) evading apoptosis, (iii) limitless replicative potential; (iv) sustained angiogenesis; and (v) tissue invasion and metastasis (Hanahan and Weinberg, 2000). In addition, many tumours undergo selection through exposure to various forms of cytotoxic therapy, and develop phenotypes that are resistant to the cytotoxic therapies used (Tsuruo *et al*., 2003; Calderwood *et al*., 2006). Consequently, drugs have been developed that target a variety of factors known to influence the hallmarks of cancer (Soti *et al.,* 2005). Identification of novel molecular targets for cancer therapeutics offers the promise of great specificity, coupled with reduced systemic toxicity (Neckers, 2002). Until now, increased understanding of the roles of membrane receptors, signal transduction pathways and transcription factors in tumourigenesis has driven a mechanism-based search for chemopreventive agents (Shen and Brown, 2003). It is now widely accepted that molecular chaperones are involved not only in most stages of tumour progression, but also in the acquisition of drug resistance (Calderwood *et al*., 2006).

1.2 Molecular Chaperones and the Heat Shock Response

Molecular chaperones are fundamental components of the cellular machinery which serve to assist many signaling molecules in maintaining a stable inactive, but easily inducible state (Csermely *et al*., 1998; Pratt and Toft, 2003, Soti *et al*., 2005). Chaperone functions include the folding and maturation of nascent or newly synthesized proteins, translocation of polypeptides across intracellular membranes, solubilisation of aggregated proteins and promotion of protein degradation, as well as the assembly of macromolecular complexes (Reviewed in Chiosis, *et al*., 2004; Odunuga *et al*., 2004; Young *et al*., 2004; Huen and Chan, 2005; Soti *et al*., 2005; Cintron and Toft, 2006). Furthermore, chaperones also assist in the regulation and coordination of cellular networks that include those of signaling and the transcriptional pathway, the cytoskeleton, membrane and the organelle network (Soti *et al*., 2005).

Conditions of acute and/or chronic stress during the cell cycle include challenges such as starvation, infection, changes in physical or chemical environments, as well as genotoxic damage (Santaro, 2000). The heat shock response was first identified in Drosophilia salivary glands, where an induction in certain proteins, termed heat shock proteins (Hsps) was observed following heat shock (Rotossa and Vonborstel, 1964; Reviewed in Sorensen *et al*., 2003). These proteins are highly conserved, ubiquitous, and abundant in nearly all subcellular compartments (Fink, 1999). The major classes of Hsps are named according to their size in kilodaltons; namely, the heat shock protein 40 (Hsp40); Hsp60; Hsp70; Hsp90; Hsp100; and the small heat shock proteins (Fink, 1999; Mehta *et al*., 2005). Both prokaryote and eukaryote systems use Hsps as a protective mechanism for the preservation of protein function and homeostasis, and although

exposure of extreme stress is harmful to the cell, Hsps provide cytoprotection against stress and stress-induced molecular damage (Reviewed in Pockley, 2003).

Of the four forms of the heat shock transcription factor HSF identified, HSF1 acts as the main heat shock factor involved in the stress response (Zou *et al*., 1998). Under physiological conditions, HSF1 exists as a transcriptionally inactive, non-DNA binding monomer located within the cytoplasm, and is found in stable complexes with a number of molecular chaperones including Hsp70 and Hsp90 (Zou *et al*., 1998). Upon induction of the heat shock response, chaperones disassociate from the HSF1 complex and as a result, HSF1 assembles into active homotrimers, where they subsequently translocate to the nucleus and bind to the consensus heat shock element (HSE) that is located in the promoter region of heat shock genes (Pockley, 2003). Upon phosphorylation, HSF1 induces transcription of Hsp genes, which are subsequently translated in the cytosol to produce the functional Hsp proteins (Santoro, 2000). The regulation of the heat shock response is complex, consisting of multiple forms of redundancy and feedback control at the molecular level, and many questions regarding the mechanisms of regulation remain (Whitesell and Lindquist, 2009).

1.3 Heat Shock Protein 70 (Hsp70)

Hsp70 is found in eukaryotes, eubacteria and many archaea, and is considered one of the most prominent Hsps in the eukaryotic cytosol (Wegel *et al*., 2005). The Hsp70 family has been extensively characterised in bacteria (DnaK), yeast (Ssa and Ssb) as well as in higher eukaryotes (Johnson *et al*., 1998). In the mammalian system, four members of Hsp70 exist, namely Hsc70 (heat shock cognate), which is constitutively expressed, the inducible Hsp70, endoplasmic

reticulum resident BiP (immunoglobulin heavy-chain binding protein), and mtHsp70 (mitochondrial Hsp70) (Fink, 1999). Hsp70 proteins were first identified after their induction within the cell during times of stress, where they play an essential role in prevention of aggregation and assisting in the refolding of denatured proteins (Bukau and Horwich, 1998). However, Hsp70 is also known to function under physiological conditions, where it assists in the folding of newly synthesized polypeptides, guides translocating proteins across intracellular membranes, assists in proteolytic degradation of unstable proteins, and in certain cases, maintains cellular homeostasis by controlling the activity of regulatory proteins (Bukau and Horwich, 1998; Nollen and Morimoto, 2002; Mosser and Morimoto, 2004; Soti, *et al*., 2005; Daniel *et al*., 2007). Together with other chaperones like Hsp90, Hsp70 has also been shown to form complexes with protein kinases and transcription factors and functions in the transport of proteins to the correct cellular location, and assists in the stabilisation of conformational structure (Helmbrecht *et al*., 2000).

Hsp70 proteins consist of two functionally coupled domains. The highly conserved N-terminal ATPase domain (44 kDa) binds ATP with a high affinity in the presence of potassium or magnesium, and mediates the hydrolysis of ATP, which in turn is responsible for the regulation of substrate recognition (Rudiger *et al*., 1997). The more variable C-terminal peptide-binding domain (25 kDa) consists of two functional sub-domains, one of which recognises and binds substrate polypeptides, and the other forms a lid over the peptide-binding pocket (Rudiger *et al*., 1997). The ATP bound form of Hsp70 has a low overall affinity for substrate, while the ADP form binds substrates stably with a higher affinity (Wegel *et al*., 2005). The C-terminal domain also contains a regulatory EEVD motif, which is conserved in nearly all eukaryotic Hsp70s

(Freeman *et al*., 1995; Odunuga *et al*., 2003). Mutation of the EEVD motif effects Hsp70 ATPase activity, substrate binding, and also results in altered chaperone activity for the folding proteins refolding (Wegel *et al*., 2005; Brown *et al*., 2007).

Various proteins, known as co-chaperones, modulate the ATPase activity of Hsp70 and its affinity for substrates (King *et al*., 2001). In eukaryotes, the Hsp70 co-chaperone, Hsp40, as well as nucleotide exchange factors such as BAG1 stimulate the ATPase activity of Hsp70 (King *et al*, 2001). The Hsp70 interacting protein (Hip) has also been shown to interact with Hsc70 ATPase domain, and stimulate the assembly of the Hsp70-Hsp40- substrate complex (Rudiger, 1997). Once the Hsp70 substrate is released, it either folds into its native state or is passed on to other molecular chaperones, such as the Hsp90 chaperone or Hsp60 chaperonine machinery (Hernandez *et al*., 2002).

1.4 Heat Shock Protein 90 (Hsp90)

Hsp90 is one of the most abundant cytosolic proteins in eukaryotes, and accounts for approximately 1-2% of total protein even under unstressed conditions (Buchner, 1999; Sreedhar *et al.,* 2003; Daniel *et al.,* 2007). The Hsp90 chaperone family includes Hsp90α (inducible form), Hsp90β (constitutive form) the mitochondrial matrix homologue (TRAP1), and the endoplasmic reticulum (ER) homologue (glucose regulated protein 94 (GRP94) in humans, Hsp86 and Hsp84 in mice, Hsp83 in *Drosophilia*, Hsc82 and Hsp82 in yeast, and HtpG in bacteria) (Young *et al*., 2001). Hsp90 functions in the maintenance of major cellular proteins within the cell, and is associated with over 300 client proteins, including hormone receptors, protein kinases, and proteins controlling cell cycle and apoptosis (Zang and Burrows, 2004; Cintron and Toft, 2006).

These client proteins require Hsp90 function for stability and activation within the cell (Cintron and Toft, 2006). In addition to its role in client protein activation, Hsp90 is also involved in general protein folding; it is able to repress aggregation and maintain proteins in an inactive, but easily inducible state (Johnson *et al*., 1998). Since these client proteins are often unstable in the absence of substrate, Hsp90 serves to enhance protein stability (Buchner, 1999).

The native Hsp90 protein is an elongated dimer with multiple domains that are composed of three essential regions: an N-terminal region (24-28 kDa), middle region (38-44 kDa), and a C-terminal region (11-15 kDa) (Soti *et al*., 1998; Banerji, 2009). Different conformations of Hsp90 have been identified, indicating that this dimer is a flexible structure (Buchner, 1999). The N-terminal domain contains an ATP binding site which is responsible for the ATPase activity of Hsp90, and it is via this binding site that the N-terminal domain seems to regulate Hsp90 conformation (Buchner, 1999; Brown *et al*., 2007). The middle region of Hsp90 acts as a charged hinge region which is variable in length, and serves to bind substrates (Csermely *et al*., 1998). The Hsp90 C-terminal domain is responsible for dimerization, and contains a conserved pentapeptide sequence motif, MEEVD which is responsible for binding of proteins that contain a conserve tetratricopeptide (TPR) motif (Brown *et al*., 2007; Onuoha *et al*., 2008; Banerji, 2009). In addition, evidence has suggested that there is a second ATP-binding site located in the C-terminus of Hsp90 (Onuoha *et al*., 1998; Panaretou *et al*., 2002).

The Hsp90 ATPase activity is crucial for the functioning of Hsp90 *in vivo*, and plays a key role in the interaction with substrates and co-chaperones (Prodromou *et al*., 1997). Biochemical as well as crystallographic analysis of Hsp90 has shown that the Hsp90 ATPase activity is generally

weak, but upon binding to certain client proteins, the ATPase activity increases up to 200 times (Soti *et al*., 2002). Hsp90 interacts with a variety of co-chaperones that mediate substrate selection, as well as association and disassociation of Hsp90 from the substrate (Hernandez *et al*., 2002). These co-chaperones are described in more detail in the section, 'Hsp90 co-chaperones'.

1.5 The Hsp90 chaperone complex

Hsp90 does not function as a chaperone in isolation, but rather forms part of a multichaperone complex via association with other chaperones, in particular Hsp70, and a wide range of different co-chaperones, and client proteins in a manner that is dependent on ATP (Brown *et al*., 2007; Flom *et al*., 2007; Onuoha *et al*., 2008; Wegel *et al*., 2005; Pearl and Prodromou, 2000).

The process of Hsp90 complex mediated protein folding is best characterised in the study of steroid receptors. It is known that a minimum of five proteins, Hsp90, Hsp70, Hop, Hsp40 and p23, are essential for the assembly of the glucocorticoid receptor (GR)- Hsp90 heterocomplex (Havik and Bramham, 2006). A model for the chaperone pathway of the Hsp90 complex is outlined in Figure 1 (adapted from Wegel *et al*., 2006). The Hsp90 chaperone complex cycles between an "open" and "closed" state, with the initial interaction occurring between the client protein and Hsp40. Hsp40 subsequently delivers the client protein to Hsp70. This interaction is stabilised by the co-chaperone HIP. The client protein is loaded onto a pre-existing dimeric Hsp90-Hop complex to form an "open" state (Hernandez *et al*., 2002; Song and Masison, 2005; Havik and Bramham, 2006). Upon ATP binding and hydrolysis, the complex switches to the "closed" state. p23 is an acidic 23 kDa protein which binds to and displaces the original co-

chaperone (Hop) to assist conformational maturation of the client into an active state, leaving the TPR binding site of Hsp90 open (Prodromou *et al*., 1999; Song and Masison, 2005; Daniel *et al*., 2007; Hadden and Blagg, 2008; Havik and Bramham, 2006). High molecular weight immunophilins, namely FKBP52, FKBP51 and Cyp-40, have also been identified within the Hsp90 complex, and are similar in having peptidylprolyl isomerise activity (Pratt and Toft, 1997). Although the roles of immunophilins remain elusive, it is known that both FKBP52 and FKBP51 influence glucocorticoid receptor binding affinity, while Cyp-40 and FKBP52 may aid in steroid receptor movement (reviewed in Pratt *et al*., 2004).

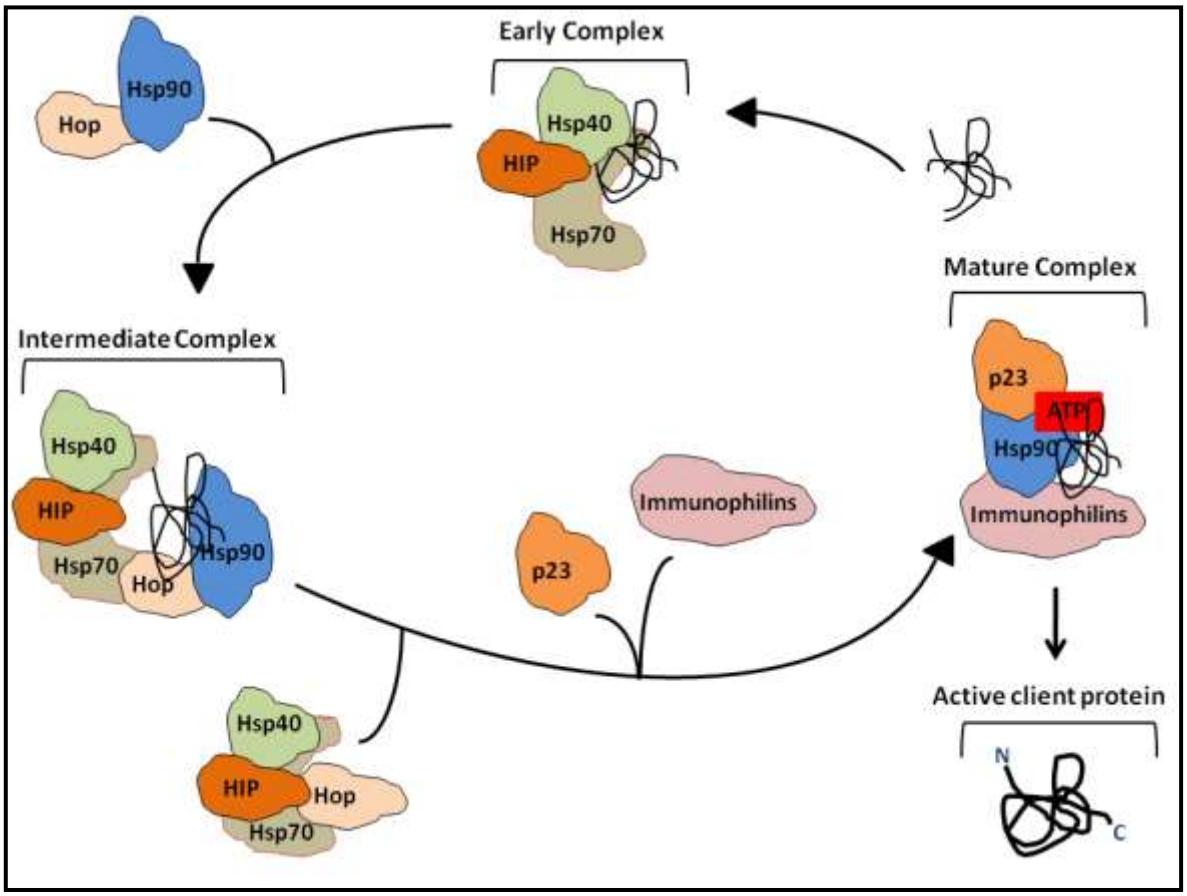


Figure 1: Model for the chaperone pathway of the Hsp70/Hsp90 complex

The Hsp90 chaperone cycle is initiated by the interaction of a protein client with Hsp40, which targets it to Hsp70 to form an 'early complex' which is stabilized by Hip. The client protein is transferred from Hsp70 to Hsp90 through the adaptor protein Hop. The binding of Hop is sufficient to stabilize the open conformation of Hsp90, resulting in the formation of the 'intermediate complex'. Hsp90 adopts the ATPase-active (closed) conformation after binding of ATP, while p23 stabilizes the closed state of Hsp90, which weakens the binding of Hop and promotes its exit from the complex. Immunophilins associate to form the 'late complex' together with Hsp90 and p23. After hydrolysis of ATP, p23 and the folded, active client is released from Hsp90 (adapted from Wegel *et al*., 2006).

1.6 Hsp90 co-chaperones

To date, a number of co-chaperones have been shown to associate dynamically with Hsp90 during the chaperone cycle (Table 1). In eukaryotes, over twenty Hsp90 co-chaperones have been identified, including Hop, cell division cycle 37 (Cdc37), activator of heat shock protein 90 ATPase (Aha1), prostaglandin-E synthase (p23), cyclophilin 7 suppressor 1 (Cns1), cyclophilin 6 (Cpr6), cyclophilin 7 (Cpr7), tetratricopeptide repeat 1 (Tpr1) and tetratricopeptide repeat 2 (Tpr 2) (Panaretou *et al*., 2002; Siligardi *et al*., 2002; Daniel *et al*., 2007). Structurally, Hsp90 associated co-chaperones such as Hop, Cpr6, Cpr7, Cns1, Tpr1 and Tpr2 bind to Hsp90 through the TPR motif. Other co-chaperones such as Cdc37, Aha1 and p23 lack TPR domains and associate with Hsp90 through unique sequences (Harst *et al.,* 2005). The co-chaperones associate with the Hsp90 complex at different stages of client activation/maturation, although the roles of most of them remain poorly defined (Panaretou *et al*., 2002; Siligardi *et al*., 2002; Daniel *et al*., 2007). It has been shown that the major immunophilin, FKBP52, binds to the free TPR acceptor site on Hsp90 after Hop has dissociated from the Hsp90 complex (Pratt and Toft, 1997). Hsp70 is usually absent in the final Hsp90 complex, or present in substoichiometric levels with respect to the substrate (Pratt and Toft, 2003).

Thus far, Hop, Cdc37, Aha1 and p23 are the most widely characterised co-chaperones in literature. The co-chaperone Hop binds and stabilizes the open conformation of Hsp90 and thus inhibits its ATPase activity (Pratt and Toft, 2003). Recent experimental results showed that Hop is a monomeric protein and the current accepted model suggests that binding of one Hop monomer to one Hsp90 dimer is sufficient to inhibit the ATPase activity of Hsp90 dimer (Carrigan *et al*., 2006). Furthermore, it has been shown that both the affinity and stoichiometry

of the association between Hsp70 and Hop are influenced by the binding of Hsp90 (Hernandez *et al.*, 2002). From these findings, it can be said that Hsp70, Hsp90 and Hop are all essential for the individual functioning of each component, and therefore work together in a highly dynamic complex.

Table 1: List of Hsp90 co-chaperones

TPR co-chaperones	
General Name	**Function**
Hop (p60, STI1)	Scaffold for Hsp90/Hsp70 interaction; involved in client protein maturation; inhibition of Hsp90 ATPase
Cns1	Nuclear transport protein; putative tumour suppressor involved in the transformation of melanocytes
Cpr6/ Cpr7	Peptidy-prolyl-isomerase; chaperone; involved in client protein maturation
TPR1	Adapter of Hsp90; involved in vesicular protein transport
TPR2	'recycling co-chaperone'; involved in regulation of glucocorticoid receptor and possibly progesterone receptor, stimulates ATP hydrolysis by Hsp70

Non-TPR co-chaperones	
General Name	**Function**
Aha1	Stimulates ATPase activity; induces conformation changes in Hsp90
Cdc37	Kinase-specific co-chaperone; inhibition of Hsp90 ATPase, chaperone
p23	Involved in client protein maturation; inhibition of Hsp90 ATPase; chaperone

(adapted from Brychzy *et al*., 2003; Lotz *et al*., 2007; Cintron Moffatt *et al*., 2008; Li *et al*., 2011)

p23 facilitates the maturation of client proteins by stabilizing the closed conformation of Hsp90 (Chen *et al*., 1998). Therefore, the ATP hydrolysis which is essential for the release of the Hsp90 client protein is inhibited in the presence of p23 (Chen *et al*., 1998). Previous studies by Li and

colleagues (2011) showed that p23 is the limiting component required for the Hsp90-client protein heterocomplex stability, and since p23 possesses chaperone activity, it may directly interact with the client protein and thus serve as the control of its conformation (Li *et al*., 2011).

Like the co-chaperone Hop, Cdc37 inhibits the ATPase activity of Hsp90 (Siligardi *et al*., 2002). Cdc37 was first identified in *S. cerevisiae* as an essential gene for cell cycle progression, while further studies on oncoprotein v-Src, revealed Cdc37 as a part of the Hsp90 – kinase complex (Siligardi *et al*., 2002). Further work by Harst and colleagues (2005) suggested that Cdc37 is a specific co-chaperone for kinases (Harst *et al*., 2005).

The most recent co-chaperone identified is Aha1, which has been reported to be involved with the maturation of both hormone receptors and protein kinases by the Hsp90 complex (Panaretou *et al*., 2002; Lotz *et al.,* 2003; Harst *et al*., 2005). Aha1 is the most prominent activator of Hsp90 ATPase activity (Li *et al*., 2011). It is proposed that during Hsp90 activation, a single Aha1 molecule is sufficient to stimulate the ATPase activity of an Hsp90 dimer (Harst *et al*., 2005), and the binding of Aha1 induces Hsp90 conformation to a 'closed state', which accelerates the progression of the ATPase cycle (Siligardi *et al*., 2002; Li *et al*, 2011).

It is speculated that the interactions of Hsp90 with these co-chaperones are tightly regulated, although the interactions between the different chaperones, Hop, Cdc37, Aha1 or p23 and Hsp90, as well as with each other, are poorly understood (Harst *et al*., 2005). Complexes of Hsp90 with one or more of these co-chaperones have been analysed in different studies, and the established dogma proposes that during hormone receptor activation, Hop and p23 bind to

Hsp90 in a defined order, while kinase activation occurs through interaction of Hsp90 in association with Cdc37 (Prodromou *et al*., 1999; Panaretou *et al*., 2002; Siligardi *et al*., 2002; Harst *et al*., 2005). Furthermore, many co-chaperones are competitive binders of each other, for example Hop competes with Aha1, p23 and Cdc37 for Hsp90 binding (Harst *et al*., 2005). It is therefore thought that modulation of Hsp90 ATPase activity by these co-chaperones may regulate progression through the chaperone cycle, controlling the efficiency Hsp90-dependent client protein maturation, and allowing Hsp90 to bind to a versatile range of client proteins (Panaretou *et al*., 2002; Daniel *et al.,* 2007). Current dogma has suggested Hop and Cdc37 may be early co-factors in the Hsp90 cycle, and upon binding, will inhibit Hsp90 ATPase activity. On the contrary, p23 and Aha1 represent late co-factors, and increase Hsp90 ATPase activity upon binding (Harst *et al*., 2005).

1.7 Heat shock protein 70/Heat shock protein 90 organising protein (Hop)

Hop, also known as STIP1/STI1, is a highly conserved and abundant protein, which was first identified by Nicolet and Craig during a genetic screen for proteins that were involved in the heat shock response in yeast (Nicolet and Craig, 1989). Hop belongs to the stress inducible 1 (STI1) protein family, of which numerous homologues have been identified in humans, mice, rats, plants and parasites (Daniel *et al*., 2007). This protein functions essentially as an adaptor, binding with and coordinating Hsp70 and Hsp90 during assembly of steroid receptors and other client protein complexes (Johnson *et al*., 1997; Chen and Smith, 1998; Wegele *et al*., 2006; Flom *et al*., 2007).

Structurally, Hop is comprised of three 34 amino acid, helix-turn-helix TPR motifs, namely TPR1, TPR2A and TPR2B, with a nuclear localisation signal overlapping the TPR2A domain as shown in figure 2 (Johnson *et al*., 1997; Blatch and Lässle, 1999; Young *et al*., 2001; Hernandez *et al*., 2002; Odunuga *et al*., 2003). These motifs have been shown to play a critical role in the functioning of chaperones, and trafficking of proteins during the cell cycle (Blatch and Lässle, 1999). The TPR1 and TPR2A domains of Hop have been shown to specifically interact with Hsp70 and Hsp90 independently through their conserved carboxyl terminal EEVD and MEEVD sequence, with the N-terminal TPR1 domain binding to the C-terminal of Hsp70, and the central TPR2 domain binding to the TPR receptor site on the C terminal of Hsp90 to bring the two chaperones together in an Hsp90-Hop-Hsp70 complex with an apparent stoichiometry of 2:1:1 (Johnson *et al*., 1997; Pratt and Toft, 2003; Odunuga *et al*., 2004; Longshaw *et al*., 2004; Carrigan *et al*., 2006; Americo *et al*., 2007; Flom *et al.,* 2007). More recently, studies have shown that both TPR1 and TPR2B domains bind Hsp70 with moderate affinity, where Hsp70 is transferred from the TPR1 domain to the TPR2B domain during client transfer to Hsp90 (Schmid *et al*., 2012). Studies by Odunuga and colleagues have confirmed that the specificity of Hop binding to chaperones is determined by hydrophobic contacts of the N-terminal residues of Hop, I4 and M5, with the C-termini of Hsp70 and Hsp90 respectively (Odunuga *et al*., 2003). Studies on Hop interaction with Hsp90 have revealed that a single mutation in the TPR2A domain of Hop, R341E, resulted in a disruption of Hop-Hsp90 interaction in yeast (Carrigan *et al*., 2004; Flom *et al*., 2006). In addition, deletion of the amino acid, A304, in the TPR2A domain as well as A438 in the TPR2B domain resulted in a disruption of yeast Hop-Hsp90 interaction as well as detrimental effects on yeast Hop activity *in vivo* (Flom *et al*., 2006). In a series of mutational studies by Odunuga and colleagues (2003), it was proposed that a network of

interactions existed between Hop and the Hsp90 and Hsp70 chaperones, and these interactions were not only between the charged residues in the TPR domains, but also outside the TPR domains, which allow for the functionality of Hop as a scaffolding protein (Odunuga *et al*., 2003).

Studies by Chang and colleagues (1997) proposed that Hop may not be essential for mediating the Hsp70 and Hsp90 interaction (Chang *et al*., 1997). However, this work is in contrast to that of Chen and colleagues (1996), who showed that Hsp90 and Hsp70 did not associate in a complex without the presence of Hop (Chen *et al*., 1996). Similarly, Chen and Smith (1998), as well as Johnson and colleagues (1997) proposed that Hop was indeed essential for integrating the Hsp70-Hsp90 interaction (Johnson *et al*., 1997; Chen and Smith, 1998). Interestingly, a report by Morishima and colleagues (2000) showed that Hop served to enhance the rate of Hsp90 and Hsp70 function, but was not essential for glucocorticoid receptor folding. These authors therefore proposed that an Hsp70 and Hsp90 interaction may exist independently of Hop, but that Hop was able to mediate the interaction so as to bring Hsp70 and Hsp90 into proximity of each other to promote efficient folding and transfer of substrate proteins (Morishima *et al*., 2000).

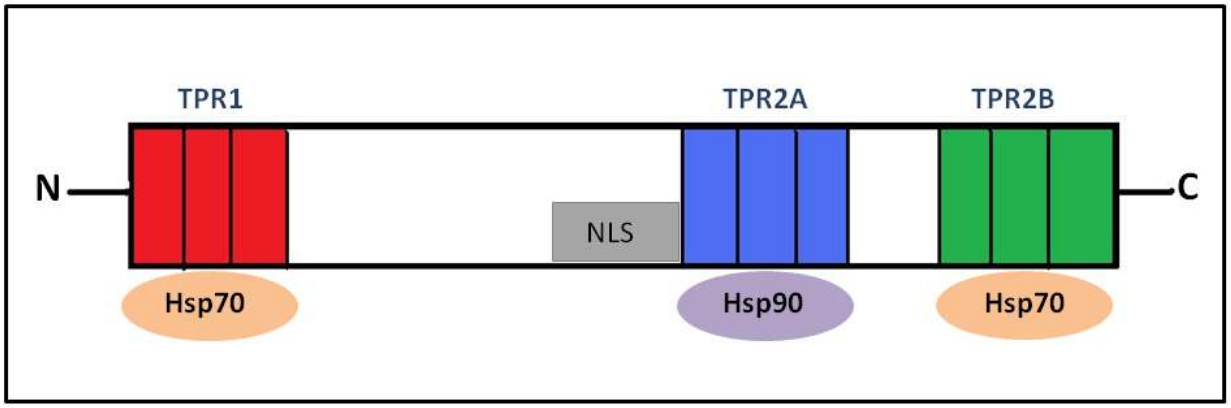


Figure 2: A schematic representation of Hop and the interaction sites with Hsp70 and Hsp90.

Hop consists of three TPR domains (TPR1; TPR2A and TPR2B) and a nuclear localization signal (NLS) (adapted from Odunuga *et al*., 2004 and Schmid *et al*., 2012)

In addition to modulating the Hsp70 and Hsp90 chaperone pathways, Hop has also shown to modulate the activity of Hsp70 and Hsp90 individually (Johnson *et al*., 1997). *In vitro*, Hop is known to modulate Hsp70 activity (Johnson *et al*., 1998; Wegel *et al*, 2003) and inhibit the ATPase activity of Hsp90 (Richter *et al.,* 2003), consistent with data suggesting that in addition to promoting the association of Hsp70 and Hsp90, Hop affects the client folding pathway through regulation of the conformations and ATPase cycles of the two chaperones (Song and Masison, 2005). Hernandez and colleagues (2002) showed that the affinity and stoichiometry of Hsp70-Hop interaction is dramatically affected by the binding of Hsp90, and although Hsp70 binds to Hop with a lower affinity than Hsp90, this affinity increased fivefold in the presence of Hsp90 (Hernandez *et al*., 2002).

Current dogma suggests that Hop is required for efficient activation of the client protein folding pathway, despite having no apparent chaperone activity of its own (Song and Masison, 2005). The impairment of the Hsp70 and Hsp90-dependent chaperone pathway has been

demonstrated by deletions of the TPR1 and TPR2 domains in yeast (Song and Masison, 2005). Studies by Morishima and colleagues (2000) also showed that the protein levels of Hop in GR-Hsp90 complexes increased in the presence of the N-terminal Hsp90 inhibitor, geldanamycin (GA), compared to GR-Hsp90 complexes without geldanamycin (Morishima *et al*., 2000). This finding may be attributed to the fact that Hsp90 inhibited by geldanamycin is in the ADP-bound conformation, which has a higher affinity for Hop when compared to the ATP-bound conformation of Hsp90 (Morishima *et al*., 2000). In addition, it was shown that GR-Hsp90 complexes incubated with geldanamycin were less stable in the absence of Hop, than GR-Hsp90 complexes in the presence of Hop. This could either be due to the retention of Hsp90 by Hop, or other possible effects of Hop on the conformation of the geldanamycin-Hsp90 complex that potentially increase its affinity for GR (Morishima *et al*., 2000).

In addition to its role as a co-chaperone, a number of reports have published interactions between Hop and other proteins that appear to be independent of Hsp70 and Hsp90. For example, Zanata and colleagues (2002) reported a role for Hop in neuroprotection, where recombinant Hop was found to bind to PrP^{c} (Cell surface prion protein), a glycosylphosphatidylinositol (GPI)-anchored cell surface sialoglycoprotein homolog, whose expression is required for the binding of prions during the propagation of neurological disease (Zanata *et al*., 2002). In addition, Gebauer and colleagues (1998) reported that Hop was able to stimulate the nucleotide exchange and protein folding activities of the cytosolic chaperonin-containing TCP-1, which is involved in the folding of actins and tubulins (Gebauer *et al*., 1998).

1.8 Chaperones as drug targets in cancer

By the early 1990s, several groups reported the observation that Hsps were over-expressed in a wide variety of cancer cells and virally transformed cells (Mosser and Morimoto, 2004; Kubota *et al*., 2010). While Kamal and colleagues (2003) have shown that Hsp90 levels do not vary greatly between tumour and normal cells (Kamal *et al*., 2003), it has been reported by others that the constitutive expression of Hsp90 is 2–10 fold higher in tumour cells compared with their normal counterparts, suggesting that it could be crucially important for the growth and/or survival of tumour cells (Neckers, 2002; McDowell *et al*., 2009). Hsp90 is required for stability of a host of proteins, including epidermal growth factor receptor (EGFR), hepatocyte growth factor receptor (MET), vascular endothelial growth factor (VEGFR), fms-like tyrosine kinase receptor-3 (FLT3), androgen and oestrogen receptors, hypoxia-inducible factor (HIF), telomerase, human epidermal growth factor receptor 2 (Her2/neu), as well as proteins downstream of Her2/neu, such as cellular-sarcoma (c-Src), and serine/threonine-specific kinase (Raf), the list of which is constantly being updated (Pick *et al*., 2007; Banerji, 2009). These proteins influence the hallmark traits of cancer, such as growth factor independence, resistance to antigrowth signals, unlimited replicative potential, tissue invasion and metastasis, avoidance of apoptosis, chemoresistance and sustained angiogenesis (Banjeri, 2009).

In addition, it has been reported that tumour Hsp90 is present almost entirely in multi-chaperone complexes with high ATPase activity, whereas Hsp90 from normal tissues is generally in a latent, uncomplexed state (Kamal *et al.*, 2003). It is proposed that the increased activity of Hsp90 in tumours can be due to the formation of multi-chaperone complexes (Kamal *et al.,* 2007). Corresponding to the elevated, complexed Hsp90 levels in tumour cells is the

overexpression of the Hsp70 (King *et al.,* 2001; Havik and Bramham, 2007). Hsp70 has been studied in various cancer cell lines, and has shown to block apoptosis at several levels, where it serves in the protection of the mitochondria-membrane integrity, promoting apoptosis-inducing factor (AIF) translocation to the nucleus, and processing caspase-3, as well as inhibiting late caspase-dependent events (Havik and Bramham, 2006). It is therefore speculated that the overexpression of the Hsp90 and Hsp70 in cancer might be indicative of the high levels of mutated oncoproteins, as well as the stress of the tumour microenvironment.

The roles played by Hsp90 and Hsp70 in tumourigenesis have made them important molecular targets in cancer, as inhibition of the Hsp90 complex has the potential to lead to inhibition of multiple signal pathways (Banerji, 2009; Neckers, 2002). Considering the complexities and redundancy in the pathways of most cancers, in addition to a range of onocogenic mutations, drugs targeting multiple signaling pathways are considered important.

Hsp90 is considered a *bona fide* drug target for anticancer treatment. More recently, other molecular chaperones, such as Hsp70 and HSF1 have been considered as drug targets. The benzoquinone ansamycin antibiotic, geldanamycin, as well as its derivative, 17-N-allylamino-17-demethoxygeldanamycin (17-AAG), represent a class of Hsp90 inhibitors that bind to the N terminus of Hsp90 and affect multiple targets in signaling pathways that are involved in tumour cell proliferation and survival (Goetz *et al*., 2003). These drugs inhibit the essential ATPase activity of Hsp90 by competing with ATP for the N-terminal nucleotide binding site (Kamal *et al*., 2004; Workman, 2004). The Hsp90 inhibitor novobiocin in contrast, binds to the carboxyl terminus of Hsp90 and disrupts Hsp90 dimerisation and the ability to bind co-chaperones and

client proteins (Neckers, 2002). A groundbreaking study by Kamal and colleagues (2003) revealed the presence of highly active Hsp90 present in 'super-chaperone' complexes in tumour cells makes the protein more susceptible to inhibition by 17-AAG compared, when compared to Hsp90 in normal cells, which is present in a latent, uncomplexed state (Kamal *et al*., 2003). These authors therefore proposed that the high binding affinity for 17-AAG to Hsp90 in cancer cells could provide an explanation for the enhanced sensitivity to the drug observed in tumour cells when compared to normal cells (Workman, 2004).

Although some Hsp90 inhibitors are currently in clinical trials and shown promise as anticancer therapeutics, these compounds are not without their problems. Studies have shown that many compounds are associated with effects unrelated to their binding to Hsp90, such as the induction of reactive oxygen species (Sreedhar and Csermely, 2004). Studies have also shown that, as with many single target treatments, tumour cells can circumvent the inhibition of one target by activating alternate pathways to support cell proliferation (Havik and Bramham, 2007). This is a result of the induction of the heat shock response (described earlier) that is triggered by Hsp90 inhibition by molecules that bind to the Hsp90 N terminus (Smith *et al.,* 2005; Powers *et al*., 2009). Consequently, Hsp90 inhibition may be followed by the upregulation of several genes with anti-apoptotic functions, such as members of the Hsp70 and Hsp27 families of chaperones (Powers *et al*., 2009). Collectively, these responses have been shown to reduce the sensitivity of cells to therapeutic strategies (Soti *et al*., 2005).

1.9 Targeting co-chaperones in cancer: a novel alternative to targeting chaperones

Consequently, targeting the Hsp90 complex through alternative strategies that do not induce the stress response, such as targeting components of the complex other than Hsp90, ,as well as using combination therapy of more than one target that may result in additive or synergistic effects are being investigated (Goetz *et al*., 2003, Tsuruo *et al*., 2003; Banerji, 2009). Examples of this include targeting the heat shock transcription factor HSF1, which regulates the expression of Hsps and has been implicated in tumourigenesis and is overexpressed in a variety of cancers (Whitesell and Lindquist, 2005; Whitesell and Lindquist, 2009). Other studies have also focused on the targeting of Hsp70 as an anticancer strategy (Evans *et al*., 2010). Since Hsp90 mediated stabilization, maturation and activation of clients all require interactions with an assortment of co-chaperones; these too have been regarded as potential drug targets. The targeting of co-chaperones such as Hop, Cdc37 and Aha1 as opposed to chaperones is a novel initiative, precipitated by a number of fundamental studies showing that many co-chaperones regulate cancer properties such as drug resistance, metastasis and invasion. Examples of this include studies by Holmes and colleagues (2008), who showed that knockdown of Aha1 in colon cancer cells led to an increase in cellular sensitivity to 17-AAG accompanying a marked decrease in Hsp90 client protein activation. Similarly, Smith and colleagues (2009) reported that silencing of Cdc37 in colorectal cancer cells induced destabilization of Hsp90 clients and sensitized cells to the Hsp90 inhibitor, 17-AAG. Studies performed in mouse embryonic fibroblasts showed that p23 in wild type cells was able to protect cells from geldanamycin, while cells carrying a disrupted p23 gene were sensitive (Forafonov *et al.,* 2008). The overexpression of Hop in cancer has been reported in range of malignant tissues, including pancreatic (Walsh *et al*., 2009; Walsh

et al., 2011), colon (Kubota *et al*., 2010), and hepatocellular carcinoma (Sun *et al*., 2007). In addition, it has been well observed that Hsp90 is found in complex with Hop in cancer when compared to the uncomplexed state of Hsp90 present in normal cells, suggesting a potential role of Hop in the regulation of cancer progression (Kamal *et al*., 2003). Since then, the role of Hop in cancer has been studied in pancreatic cancer, where knockdown led to a reduction in the metastatic and invasive potential of these cells (Walsh *et al*., 2011). Disruption the interaction of Hop with Hsp90 through the use of a TPR peptide mimic or drug in breast cancer cells resulted in a dramatic decrease in cell viability (Horibe *et al*., 2011; Pimienta *et al.,* 2011). In addition, treatment of glioblastoma cells with recombinant Hop protein led to a dramatic increase in cell proliferation compared to normal cell counterparts (Erlich *et al.,* 2007). These findings suggest that Hop may play an important role in cancer cell biology.

1.10 Problem Statement and motivation

To date, the National Cancer Registry (NCR) has listed cancer as the fourth leading cause of death in South Africa, where statistics report that one in every 6 men and one in every seven women in South Africa will develop cancer during their lifetime. Breast cancer in particular, is currently the second most common malignancy among women in South Africa (Vorobiof *et al*., 2001; Matatiele and van der Heever, 2008). Hsp90 is currently regarded as *bona fide* drug target for the treatment of cancer. However, some studies have shown that Hsp90 inhibition induces the transcriptional upregulation of Hsp70, allowing cells to circumvent the inhibition of one target by activating alternate pathways to support cell proliferation. Hop is co-chaperone that regulates chaperone folding by the Hsp90/Hsp70 heterocomplex and may have alternate functions that are independent the complex. The identification of co-chaperones as putative drug targets is the next stage in chaperone-directed drug discovery. Therefore, it is essential that the role of co-chaperones such as Hop be studied in order to describe the role in cancer cell biology and evaluate the potential use of these proteins as drug targets.

1.11 Hypothesis

The Hsp70/Hsp90 organising protein (Hop) is a potential drug target for the treatment of breast cancer. Targeting Hop may represent an indirect mechanism to target the Hsp70/Hsp90 complex in cancer cells. Therefore, Hop knockdown will lead to inhibition of cancer cell proliferation and migration.

1.12 Objectives

- Characterise the expression and localisation of Hop in different cancer cell lines;
- Establish an RNA interference (siRNA) system for the transient knockdown of Hop in breast cancer cells;
- Investigate the effect of Hop knockdown on the expression of Hsp90, Hsp70, other co-chaperones and Hsp90 client proteins;
- Investigate the effect of Hop knockdown on cancer cell proliferation and chemosensitivity;
- Investigate the effect of Hop knockdown on cancer cell migration.

Chapter 2 : Materials and Methods

2.1 Materials

MDA-MB-231 (HTB-26) and MCF-7 (HTB-22) breast cancer cell lines, purchased from American Tissue Culture Collection (ATCC), were a kind gift from Dr Sharon Prince (University of Cape Town, South Africa). The paired Hs578T (86082104) and Hs578Bst (89070101) breast cancer cell lines were purchased from ATCC and the paired SW480 and SW620 (87092801 and 87051203 respectively) colon cancer cell lines were purchased from the European Collection of Cell Cultures (ECACC). All general reagents were purchased from Sigma-Aldrich (Germany) and Saarchem (Merck; South Africa). Tissue Culture media and reagents (foetal calf serum [FCS], Dulbecco's Modified Eagle Medium [DMEM] with GlutaMAX™-I, 10 X Trypsin-EDTA, Insulin, Epidermal Growth Factor, Hydrocortisone and Penicillin Streptomycin) were from Gibco, (Invitrogen, UK) and Biowhittaker (UK). Tissue Culture plasticware was from Corning Incorporated (USA). Western Blotting power pack, Hybond Support Nitrocellullose and Chemidoc^{TM} EQ were 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), anti-Akt1/2/3 (cat no.: sc-8312) and anti-GAPDH (cat no.: sc-255778) antibodies were from Santa Cruz Biotechnology. Anti-actin (cat no.: A2103) and anti-vinculin (cat no.: V9131) were from Sigma-Aldrich. Anti-Phospho-Erk1/2 (cat no.: AF1018) and anti-Erk1/2 (cat no.: MAB1576) were purchased from R & D Systems. Mouse anti-Hsp90 (cat no.: 610418) was purchased from BD Biosciences (South Africa). Anti-Hop (cat no.: SRA-1500-F) was from Stressgen (USA), while anti-Cdc37 (cat no.: 4793) and anti-Histone 3 (cat no.: 9715) were purchased from Cell Signaling Technology (USA). Anti-Aha1 (cat no.: SMC-172C/D) and anti-p23 (cat no.: SMC-156C/D) were from StressMarq Biosciences Inc (USA). 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 from Invitrogen (UK). Pre-designed siRNA oligonucleotides were purchased from Dharmacon Technologies (UK). The siGENOME non-targeting siRNA (cat no.: D-001206-14-05) was purchased as a negative control, consisting of a scrambled oligonucleotide sequence (5'-AAGCGCGCUUUGUAGGAUUCG-3'). The siGENOME SMARTpool siRNA targeting Human Hop/STIP1 (cat no.: M-019802-01-0005) contained a pool of four siRNA oligonucleotides directed against the Hop messenger RNA sequence. Target sequences were as follows: GCAAAUAACCCUCAACUGA (cat no.: D-019802-01); CCAGAAGGCUUAUGAGGAU (cat no.: D-019802-02); GGCAAGGGCUAUUCACGAA (cat no.: D-019802-03); GAGCGUGGGUAACAUCGAU (cat no.: D-019802-04). DharmaFECT 1 (cat no.: T-2001-01) and DharmaFECT 4 (cat no.: T-2004-01) transfection reagents were purchased from Dharmacon Technologies (UK). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) cell proliferation assay kit was purchased from Rhoche (cat no.: 11 465 007 001). Pseudopodia isolation kit (cat no.: ECM 660) and Pseudopodia quantification kit (cat no.: ECM 650) were purchased from Chemicon International. Purified actin was purchased from Sigma Aldrich and recombinant human Hop protein was purchased from Stressmarq. The manufacturers/ suppliers of any other specialised reagents are referenced within the text.

2.2 Methods

2.2.1 Maintenance of cancer cell lines

MCF-7 and MDA-MB-231 breast cancer cells were maintained in DMEM media supplemented with GlutaMAX™-I, 5% (v/v) FCS, penicillin-streptomycin-amphotericin (PSA; 100 U/ml). The MCF-12A cell line was maintained in a 1:1 mixture of Ham's F12 media and DMEM with GlutaMAX™-I, 10% (v/v) FCS, PSA (100 U/ml), hydrocortisone (10 ng/ml), EGF (10 ng/ml) and

insulin (10 U/ml). Hs578T breast cancer cells were maintained in DMEM supplemented with GlutaMAX™-I, 10% (v/v) FCS and PSA (100 U/ml). Hs578Bst cells were maintained in DMEM supplemented with GlutaMAX™-I, 10% (v/v) FCS and EGF (30 ng/ml). SW480 and SW620 colon cancer cell lines were maintained in L-15 medium supplemented with GlutaMAX™-I, 5% (v/v) FCS and PSA (100 U/ml). All mammalian cancer cell lines were maintained at 37°C with 9% CO_2.

2.2.2 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Chemiluminescence-based Western detection of proteins

Cells were harvested by scraping with a 1 ml pipette tip into sample lysis buffer (50 mM Tris, pH 7.4, 5 mM EDTA, 150 mM NaCl, 1% [w/v] SDS) and protein concentrations determined using Nanodrop 2000 (Thermo Scientific). Samples were prepared for electrophoresis by boiling for 10 minutes in SDS-PAGE sample buffer (0.05M Tris pH 6.8, 10% [v/v] glycerol, 2% [w/v] SDS, 1% [w/v] Bromophenol blue, 5% [v/v] 2-mercaptoethanol). Equal amounts of proteins were separated by SDS-PAGE according to the protocol described by Laemmli (1970). Proteins were resolved using a 4% stacking gel (pH 6.8) and a 12% resolving gel (pH 8.8) at 120 V for 90 minutes in SDS-PAGE running buffer (0.25 mM Tris pH 8.3, 192 mM glycine, and 1% [w/v] SDS). Protein Marker IV (peqGOLD) and PageRuler Plus (Fermentas) were used for estimating molecular weights of proteins. For silver staining, SDS-PAGE gels were stained using the PageSilver^{TM} Silver Staining Kit (Fermentas Life Sciences) according to manufacturer's instructions. Western analysis was performed on resolved proteins from SDS-PAGE according to the method of Towbin (Towbin and Gordon, 1979). 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% [v/v] methanol) for 120 minutes at 100 V with continuous stirring at 4˚C. Protein transfer was confirmed with Ponceau staining (0.5% [w/v] Ponceau S in 1% [v/v] glacial

acetic acid). The membrane was blocked with 5% (w/v) blotto (5% fat free milk powder in Tris-buffered saline [TBS; 50 mM Tris pH 7.5, 150 mM NaCl]) for one hour and incubated with primary antibody overnight at 4˚C according to the manufacturer's recommended dilution. The membrane was washed with Tris-buffered saline containing Tween-20 (TBST; 1% [v/v] Tween-20 in TBS) twice for 20 minutes before incubation with species-matched peroxidase-conjugated secondary antibody (1:5000) for an hour at room temperature. Membranes were washed in 4 changes of TBST for a total of one hour and detection of proteins carried out using a chemiluminescence developing kit (Enhanced Chemiluminescence [ECL], GE Healthcare; UK) in the Chemidoc EQ system, (Biorad; UK).

2.2.3 Indirect Immunofluorescence staining and Confocal Microscopy

Cells were seeded at a density of 2 x 10^4 cells/ml into 4-well plates containing coverslips or 8-well chamber slides and incubated (37˚C; 9% CO2) overnight. For the Hs578Bst cell line, cells were seeded onto coverslips pre-coated overnight with 250 µg/ml fibronectin (Sigma-Aldrich) at 4^oC. For the paired SW480 and SW620 cell line, cells were seeded onto coverslips treated with poly-L-lysine (200 µg/ml, Sigma-Aldrich) for 20 minutes at room temperature. For immunofluorescence, cells were fixed by flash treatment (±5 seconds) with ice-cold methanol (-20˚C) and allowed to air dry. Cells were blocked with 1% (w/v) bovine serum albumin (BSA) in TBS (BSA/TBS) for 30 minutes at room temperature and incubated with primary antibodies in 1% (w/v) BSA/TBS at 4˚C overnight. After incubation, cells were washed twice in 0.1% (w/v) BSA/TBS for 5 minutes followed by incubation with appropriate fluorophore-conjugated secondary antibodies at room temperature for 1 hour in the dark. Cells were washed twice with 0.1% (w/v) BSA/TBS and the nucleus stained with Hoechst-33342 (1 µg/ml in distilled water) before mounting. Immunofluorescence staining was visualised using the Zeiss LSM 510 Meta

confocal microscope and analysed using AxiovisionLE 4.7.1 (Zeiss). Imaging was performed using the 63x oil objective unless specified otherwise. Quantification of co-localisation analysis was performed using the Image J software. Details of individual treatments are described in the figure legends.

2.2.4 Transient transfections of Hs578T and MDA-MB-231 cells with small interfering ribonucleotide oligomers (siRNA)

For transient knockdown experiments, Hs578T cells were seeded in 6 well plates and grown to 90% confluency, after which cells were treated with siRNA to a final concentration of 25 nM using 8 µl Dharmacon 1 transfection reagent (cat no.: T-2001-01), according to the manufacturer's instructions. MDA-MB-231 cells were seeded in 6 well plates and grown to 80% confluency, after which they were transfected with siRNA to a final concentration of 25 nM using 4 µl Dharmacon 4 transfection reagent (cat no.: T-2004-01), according the manufacturer's instructions. In separate tubes, 5 µM siRNA and DharmaFECT transfection reagent were diluted with serum-free medium to a total volume of 200 µl and incubated for 5 minutes at room temperature. The contents of each tube were mixed and incubated for 20 minutes at room temperature. Antibiotic-free, complete medium was added to the siRNA duplex to a final volume of 2 ml (final concentration of 25 nM). The culture medium was removed from the cells and the appropriate transfection medium added to each well. Cells were incubated at 37^{o}C in 9% CO_2 and harvested after 24 to 144 hours depending on the requirement for each experiment. In all experiments, cells were treated with transfection reagent alone (mock transfection) or transfected with the non-targeting siRNA and the siRNA directed against Hop.

2.2.5 Cell proliferation and viability assays

Twenty-four hours after siRNA transfection, cells were seeded into 96 well plates at a density of 1 x 10^4 cells/ml (total volume of 100 µl) and allowed to adhere overnight. Cell viability was assessed after 24, 48 and 72 hours respectively using the MTT cell proliferation assay kit. At the appropriate time intervals, 10 µl MTT reagent was added to each well to a final concentration of 0.5 mg/ml and cells were incubated at 37^{o}C in 9% CO_2 for 4 hours. To each well, 100 µl of solubilization solution was added and the cells incubated overnight, after which the absorbance measured at 570 nm. Cell viability curves were constructed of the cell viability as absorbance measured for each siRNA transfection treatment over time.

2.2.6 Cell cytotoxicity assays

Twenty-four hours after siRNA transfection, cells were seeded into 96 well plates at a density of 1.2 x 10^5 cells/ml (total volume of 50 µl) and allowed to adhere overnight. The following morning, cells were incubated with varying concentrations of paclitaxel, geldanamycin or novobiocin as indicated in figure legends and cell viability assessed after 72 hours using the MTT assay as previously described. Cells were treated with an equivalent amount DMSO to that present in the highest concentration of drug used. The IC_{50} values were calculated as the drug concentration that led to a 50% reduction in cell viability compared to DMSO treated cells. A time course cytotoxicity study was also performed, where cells were treated with single concentrations of paclitaxel, geldanamycin or novobiocin as indicated in figure legends, harvested at set time intervals of 24, 48 and 72 hours and cell viability assessed using the MTT

assay. Cell viability curves were constructed as the absorbance measured for each siRNA transfection treatment over time.

2.2.7 Wound healing assays

Following transfection with siRNA, Hs578T and MDA-MB-231 cells were seeded into 96 well plates (pre-treated with fibronectin at 4^{o}C overnight) at a density of 2 x 10^{5} cells/ml in a total volume of 100 µl and allowed to adhere overnight. Wounds were made by scratching the confluent cell monolayer with a sterile toothpick followed by incubation at 37^{o}C. Images of the wound were taken at 0 hrs, 6 hrs and 12 hrs (to exclude a contribution of cell proliferation to results) with a Nikon camera (Coolpix 990) attached to a standard light microscope.

2.2.8 Pseudopodia isolation

Pseudopodia were isolated using the pseudopodia isolation assay kit. The polycarbonate porous membranes of the chamber inserts were coated with fibronectin (5 µg/ml in PBS) by addition of 2 ml of fibronectin solution to the well containing each insert (lower chamber) and 1 ml inside of each insert (upper chamber) and incubation overnight at 4^{o}C. Serum-starved Hs578T cells were trypsinized and resuspended in migration media (Hs578T media without serum supplemented with 0.2% [w/v] BSA) to a total of 1 x 10^{6} cells/ml. Cells were allowed to attach and spread on the upper surface of the membrane for 2 hours, after which cells were stimulated with the chemokine stromal derived factor 1 (SDF-1/CXCL12) at a concentration of 250 ng/ml in Hs578T media containing 10% (w/v) FCS. The SDF-1 containing medium was placed in the lower chamber to establish a chemoattractant gradient. Cells were allowed to extend pseudopodia through the pores toward the direction of the gradient for 90 minutes, after which

the chambers were fixed with ice cold methanol for 30 minutes. The cell body on the upper surface was manually removed with a cell scraper and the total pseudopodia protein on the lower surface of the insert harvested according to the manufacturer's instructions. Alternatively, the cell body was harvested by first removing the pseudopodia from the lower surface of the insert using a cotton swab before collecting the cell body fractions from the upper surface of the insert. Protein concentration of each fraction was quantified using the Nanodrop 2000 (Thermo Scientific), and equal protein concentrations (40 ug/ml) resolved by SDS-PAGE and the presence of Hop, Hsp90, actin, vinculin and histone 3 in the pseudopodia and cell body fractions determined by Western analysis. Whole cell lysates prepared from untreated cells were used as a control.

2.2.9 Pseudopodia quantification

Pseudopodia were quantified in Hs578T cells transfected with either siRNA targeting Hop or with non-targeting siRNA, using the pseudopodia quantification assay kit. The polycarbonate porous membranes of the chamber inserts were coated with fibronectin (5 µg/mL in PBS) by adding 600 µL of the fibronectin solution to the bottom of the well containing each insert (lower chamber) and 100 µL inside of each insert (upper chamber) overnight at 4^{o}C. Serum-starved siRNA-transfected Hs578T cells were trypsinized and resuspended in migration media (Hs578T media without serum supplemented with 0.2% [w/v] BSA), and a total of 7.5 $x10^{4}$ cells placed into the upper chamber of each insert. Cells were allowed to attach and spread on the upper surface of the membrane for 2 hours, after which cells were stimulated with SDF-1 (250 ng/ml in Hs578T media containing 10% [w/v] FCS), which was placed in the lower chamber to establish a chemoattractant gradient. Cells were allowed to extend pseudopodia through the pores

toward the direction of the gradient for 90 minutes. Insert filters were fixed with ice cold methanol and the upper and lower surfaces of the membrane stained for the presence of actin (rabbit anti-actin antibody followed by donkey anti-rabbit conjugated to Alexa Fluor 488), and nuclei (labelled with Hoescht 33342). Z stack images encompassing both the upper and lower surfaces of the membrane were captured using the Zeiss LSM 510 Meta confocal microscope and analysed using AxiovisionLE 4.7.1 (Zeiss).

2.2.10 Actin and Hop cosedimentation assay

The actin cosedimentation assay was adapted from methods described by Srivastava (2008). Purified actin protein (Sigma Aldrich) was resuspended to a concentration of 10 mg/ml in buffer G (5 mM Tris , pH 8, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM DTT) and centrifuged at 16 000*g* for 10 minutes at 4^{o}C. The supernatant containing monomeric actin was polymerised by dilution to 2 mg/ml with polymerization buffer F (50 mM KCl, 1 mM ATP, 2 mM $MgCl_2$) and incubated at room temperature for one hour. Recombinant human Hop protein (1 mg/ml, Stressmarq) or BSA was incubated with polymerised F-actin in Interaction Buffer (10 mM Tris HCl pH 7, 1 mM ATP, 0.2 mM DTT, 1 mM EGTA, 0.1 mM $CaCl_2$ and 2 mM $MgCl_2$) in a total volume of 200 µl for 1 hour at room temperature. Sedimentation of F-actin was performed by centrifugation at 100,000*g* for 30 minutes at 22°C in a Beckman ultracentrifuge. Supernatants were carefully removed by pipetting into an eppendorf tube, and pellets resuspended in 1 ml 5 x SDS PAGE buffer. The supernatant was concentrated using Amicon centrifugal spin columns (Millipore) to 1ml total volume per sample. Equivalent amounts of supernatant and pellet fractions were resolved and analyzed by SDS-PAGE, silver staining and Western blot analysis for Hop and actin in the different fractions.

Chapter 3 : Hop as a co-chaperone in cancer

3.1 Introduction

The overexpression of Hop in cancer has been reported in a range of malignant tissue (McDowell *et al.,* 2009), including pancreatic (Walsh *et al*., 2011), colon (Kubota *et al*., 2010), and hepatocellular carcinoma (Sun *et al*., 2007). In addition, Hsp90, a *bona fide* anticancer drug target, is found in complex with Hop in cancer cells, in contrast to the uncomplexed state of Hsp90 present in normal cells (Kamal *et al*., 2003). Previous studies utilizing RNA interference (RNAi) techniques have explored the roles of Hop and other Hsp90 co-chaperones in tumourigenesis. Examples of this include the knockdown of Hop, Cdc37 and Aha1 in murine embryonic stem cells, colon and pancreatic cancer cells respectively (Holmes *et al.,* 2008; Longshaw *et al.,* 2008; Smith *et al.,* 2009; Kubota *et al.,* 2010; Welsh *et al.,* 2011). From these studies, it was shown that co-chaperones played key roles in cancer progression, and that knockdown led to either decreased cell proliferation, increased sensitivity to anti-cancer drugs, a decrease in migratory ability, or a combination of these effects. Importantly, it has been shown that the knockdown of these co-chaperones induce changes in major signaling pathways involved in oncogenesis by downregulation of key oncogenic proteins such as Akt and Erk kinases, hormone receptors, as well as various transcription factors such as Stat3, which are dependent on Hsp90 mediated folding for stabilization and activity (Whitesell and Lindquist, 2005). Recently, similar effects have been shown in studies employing alternative methods to siRNA silencing, by means of disrupting the interaction of Hop with Hsp90 through the introduction of a TPR peptide or drug in order to investigate the role of Hop in breast cancer (Horibe *et al*., 2011; Pimienta *et al.,* 2011).

In this chapter, we characterised the expression and localisation of Hop in a range of different cancer cell lines, as well as investigating the effects of siRNA mediated silencing of Hop on the expression of Hsp90, Hsp70, selected clients proteins and co-chaperones of Hsp90 in breast cancer.

3.2 Results

3.2.1 Analysis of the constitutive expression of Hop in human cell lines

The constitutive expression of Hop was examined in a panel of seven cell lines of differing malignancy by Western blot analysis (Figure 3A). Hop expression in the transformed, non-malignant human mammary epithelial cell line (MCF-12A) was compared to the non invasive breast cancer cell line (MCF-7), the highly invasive and metastatic breast cancer cell line (MDA-MB-231) (Arteaga *et al*., 1987), and MCF-7 mammosphere-derived cells (enriched in putative stem-like cancer cells) (Mani *et al*., 2008). Hop expression was also assessed using two paired cell line models, namely the paired colon cancer cell line model incorporating metastatic (SW620) and non-metastatic (SW480) cell lines (Futschik *et al.,* 2002); and the paired breast cell model that included the breast carcinoma (Hs578T) and the autologous normal fibroblast breast cell line derived from tissue adjacent to the tumour (Hs578Bst) (Eckert *et al*., 2004).

Western analysis of whole cell lysates for Hop showed a single band at the expected molecular weight of approximately 66 kDa (Figure 3A); although, there were minor differences in the mobility of the Hop protein between the different cell lines. GAPDH was used as a loading control and the density of the Hop signal in the cell lines was normalized against the density of the GAPDH signal in the same cell line using the program ImageJ (Figure 3B). Results showed a

modest but noticeable reduction in Hop expression levels in the MCF-12A cell line when compared to the MDA-MB-231 and MCF-7 cell lines (Figure 3B). Higher expression of Hop was observed in MCF-7 cells compared to MCF-7 mammosphere cells. The paired metastatic (SW620) and non-metastatic (SW480) colon carcinoma cell lines showed no obvious difference in Hop protein expression level. In contrast, when comparing the paired Hs578T (breast carcinoma) and Hs578Bst (normal breast fibroblast) cell lines, Hop protein levels were markedly higher in the Hs578T cell line compared to the Hs578Bst cell line.

3.2.2 Analysis of Hop localisation in a panel of human cell lines

The localisation of Hop in each cell line described in the panel above was evaluated by immunofluorescence and confocal microscopy. Cells were seeded onto glass coverslips and allowed to adhere overnight, after which they were fixed, permeabilized, and stained for Hop and nuclear DNA (Figure 4). Images showed cytoplasmic, and punctuate, perinuclear staining of Hop in MDA-MB-231 cells, as well as a strong detection of Hop on the periphery of the cell, proposed to correspond to the plasma membrane and at apparent points of adhesion (Figure 4; white arrows). MCF-12A cells showed a more diffuse cytoplasmic staining, and interestingly, also displayed Hop localisation at defined points of adhesion. MCF-7 cells showed a diffuse cytoplasmic and perinuclear staining of Hop, as well as defined Hop staining between intercellular junctions and on the periphery of cells. The paired metastatic and non-metastatic cell lines, SW620 and SW480 respectively, were smaller in size, and displayed nuclei of larger proportion to the cytoplasm. Hop localisation was diffuse in the cytoplasm, with enriched regions of staining localised at points of adhesion on apparent leading edges of polarized cells (Figure 4; white arrows). In the Hs578Bst cell line, Hop localised predominantly to the nuclear

and perinuclear regions, with no apparent membrane location. This was in contrast to the other cell line of the pair (Hs578T cell line), which showed cytoplasmic and perinuclear staining, along with prominent Hop staining at the periphery of cells adhering to the surface.

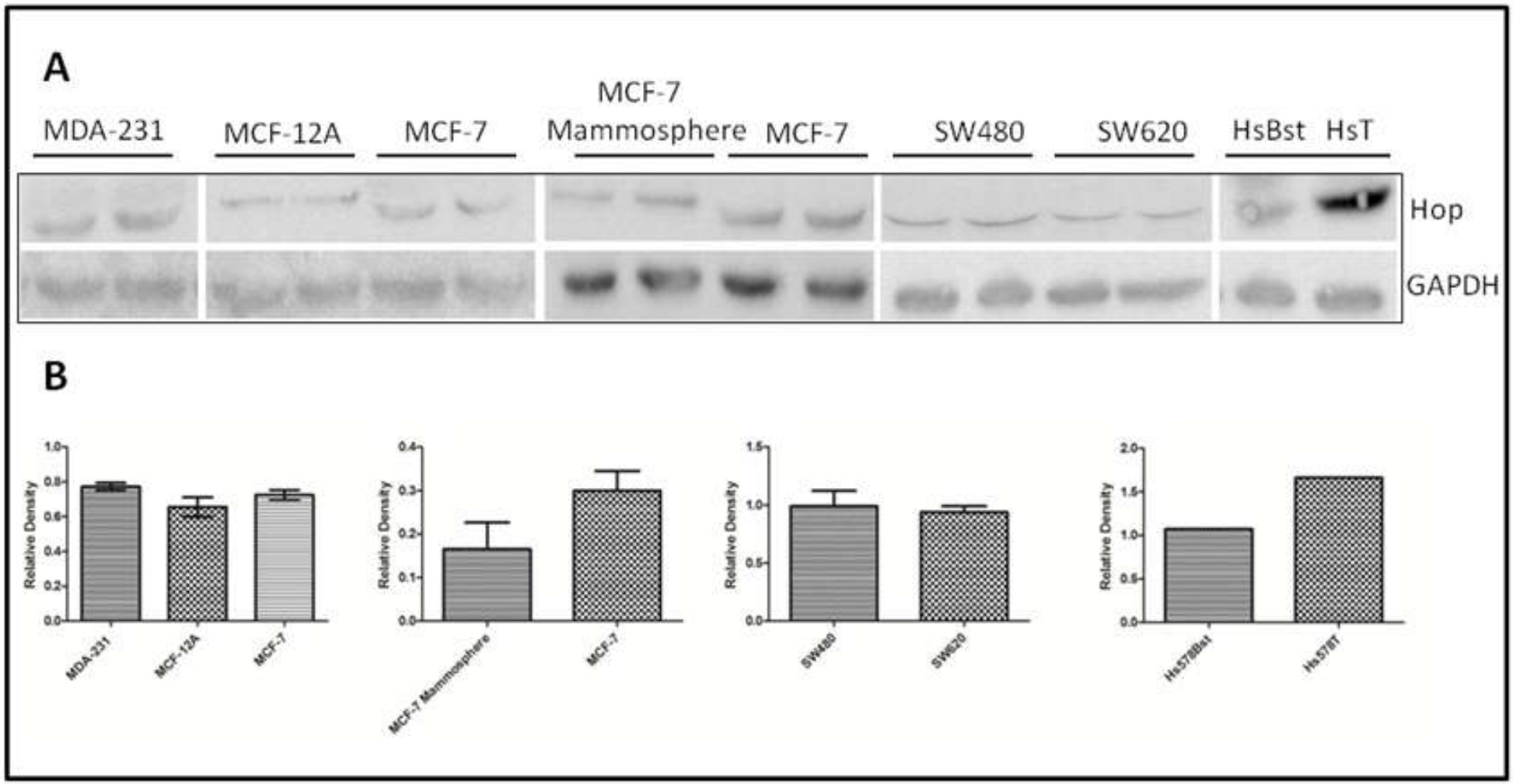


Figure 3: Constitutive expression of Hop in a range of human cancer cell lines

Western blot analysis of constitutive Hop protein expression levels in a panel of seven different human cancer cell lines (A). Hop expression in duplicate samples of cell lysates was determined by SDS-PAGE and Western analysis. GAPDH was included as a loading control. (B). The intensity of the Hop signal was normalized to that of GAPDH (representing total protein) by dividing the value obtained for the density of the Hop signal by that of the GAPDH to obtain the relative density. All densitometry was determined using ImageJ. Results are representative of two independent experiments, performed on separate Western blot membranes, with the exception of the Hs578Bst and Hs578T cell lines. Error bars represented ranges in density.

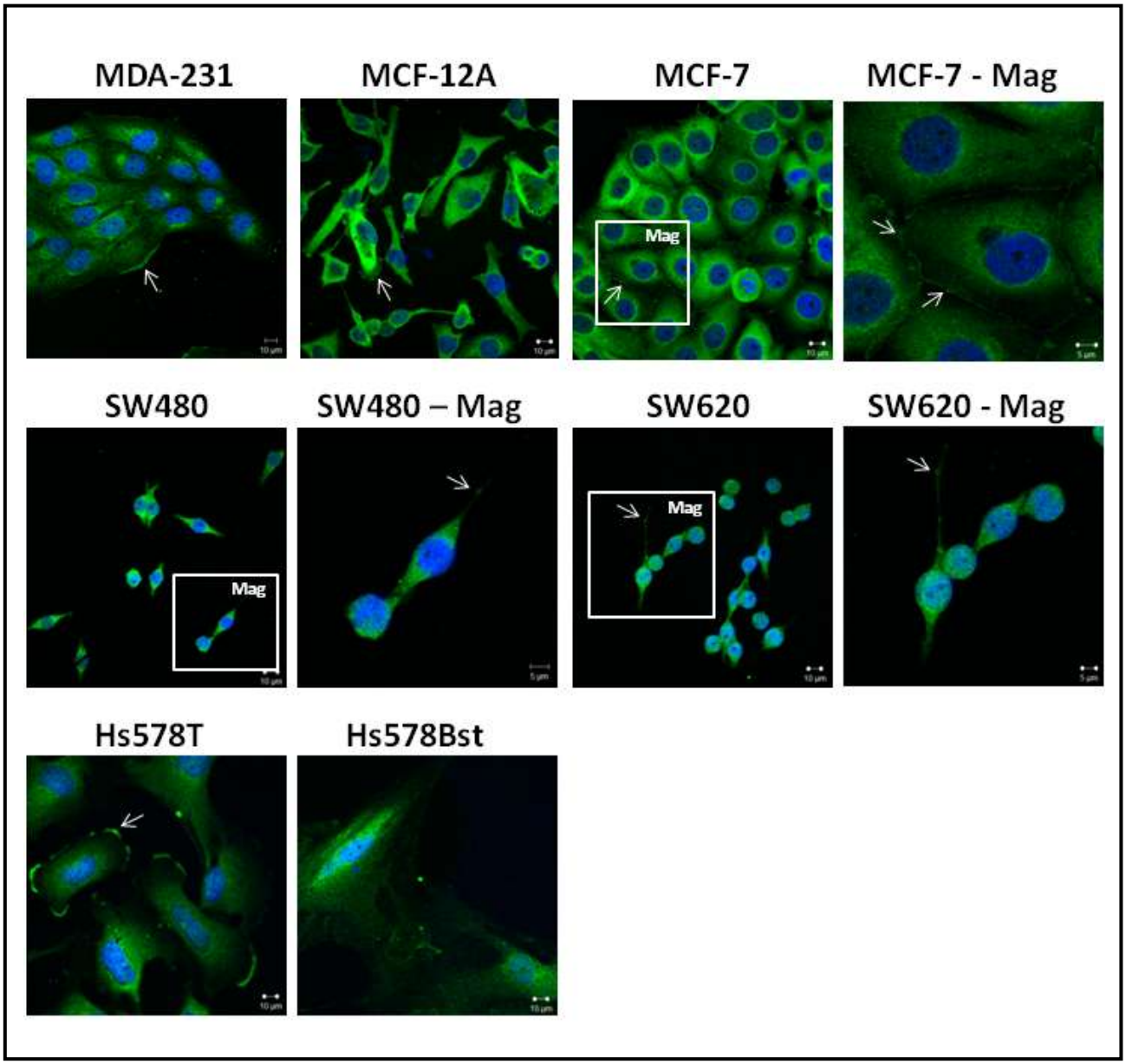


Figure 4: Constitutive localisation of Hop in a panel of human cancer cell lines.

The subcellular localisation of Hop in a panel of seven human cancer cell lines was assessed by indirect immunofluorescence staining and confocal microscopy. Cells were seeded overnight on uncoated glass coverslips, with the exception of SW480 and SW620 cell lines which were seeded onto poly-L-lysine (200 µg/mL) coated coverslips. Cells were incubated with mouse anti-Hop primary antibody followed by donkey anti-mouse-488 secondary antibody (green). The nucleus was stained with Hoechst-33342 (blue). White arrows indicate Hop staining at points of adhesion at the periphery of the cell as well as intercellular junctions. Images were captured using the x63 objective on the Zeiss LSM 510 Meta confocal microscope and analysed using AxiovisionLE 4.7.1 (Zeiss). Scale bars represent 10 µm or 5 µm as indicated. Images shown are examples from triplicate randomly selected fields and are representative of the dominant morphology observed in all cells. Mag: magnification.

3.2.3 siRNA mediated silencing of Hop and effects on chaperones and co-chaperones in Hs578T and MDA-MB-231 cells

In order to assess the role of Hop as a co-chaperone in breast cancer cell biology, a siRNA knockdown approach was used to silence Hop in Hs578T and MDA-MB-231 cells, both of which are triple negative breast carcinoma cell lines, which are characterised by a lack of expression of oestrogen, progesterone, and ERBB2 (Her-2) receptors. In addition, these cell lines were both determined to have relatively high constitutive levels of Hop protein expression. Cells were transfected with pre-designed Dharmacon SMARTpool siRNA, which contained a mixture of four siRNA oligonucleotides targeting Hop at different sequences along the mRNA. A mock control was included where cells were treated with transfection reagent only, as well as a non-targeting siRNA negative control, which contained a non-targeting RNA sequence. A time course study was performed over a period of 144 hours, where cells were treated with siRNA oligonucleotides against Hop, negative siRNA oligonucleotides or mock transfected. Cells were harvested every 24 hours during the 144 hour experiment and whole cell lysates containing equal amount of protein assessed for levels of Hop by Western analysis. In the mock transfected Hs578T cells, a band at approximately 66 kDa was detected with the anti-Hop antibody (Figure 5A). The levels of Hop were reduced after treatment of cells for 48 hours with siRNA against Hop, indicating that knockdown of Hop was successful. The reduction in the levels of Hop protein was maintained for 144 hours after treatment with Hop siRNA. In contrast, there was no change in the level of Hop in Hs578T cells treated with non-targeting negative siRNA at any of the time intervals analysed (Figure 5B). Knockdown of Hop was quantified by densitometry, where the intensity of the Hop signal for cells treated with siRNA against Hop and non-targeting siRNA were normalised to the GAPDH loading control and in each case are presented as a ratio of the mock transfection control (Figure 6).

The effect of Hop knockdown on the levels of chaperones (Hsp90 and Hsp70) and co-chaperones (p23, Aha1 and Cdc37) was determined by Western analysis and densitometry (Figure 5 and 6). Results showed that in Hs578T cells treated with siRNA against Hop, Hsp90 levels remained relatively constant, indicating that Hsp90 expression was not affected by a decrease in the levels of Hop protein. Similarly, knockdown of Hop did not induce any considerable effects on the levels of Hsp70 protein. On the contrary, analysis of the co-chaperone expression levels after Hop knockdown showed a time-dependent increase in the levels of both Aha1 and Cdc37, while a decrease in p23 levels was observed compared to cells treated with negative non- targeting siRNA. In all cases, treatment of cells with negative non-targeting siRNA did not result in any noticeable time dependent change in the expression level of all proteins tested (Figure 5B and 6). Similarly, treatment of MDA-MB-231 cells with siRNA against Hop showed an obvious decrease in Hop protein levels after 48 hours (Figure 7A). This reduction was maintained for 144 hours after treatment, while no change of Hop protein was observed in MDA-MB-231 cells treated with non-targeting negative siRNA (Figure 7B). These observations were confirmed by densitometry analysis (Figure 8). A similar trend in chaperone and co-chaperone expression was observed in MDA-MB-231 cells after treatment with siRNA targeting Hop (Figure 7A). Densitometry analysis (Figure 8) revealed no change in Hsp90 or Hsp70 levels after 144 hours post siRNA transfection when compared expression levels of cells transfected with non-targeting siRNA (Figure 8). Similar to the trend in Hs578T cells, a gradual increase in Aha1 and Cdc37 expression levels was observed, while p23 levels decreased over time. In all cases, co-chaperone expression levels in cells treated with negative non-targeting siRNA did not change (Figure 7B and 8).

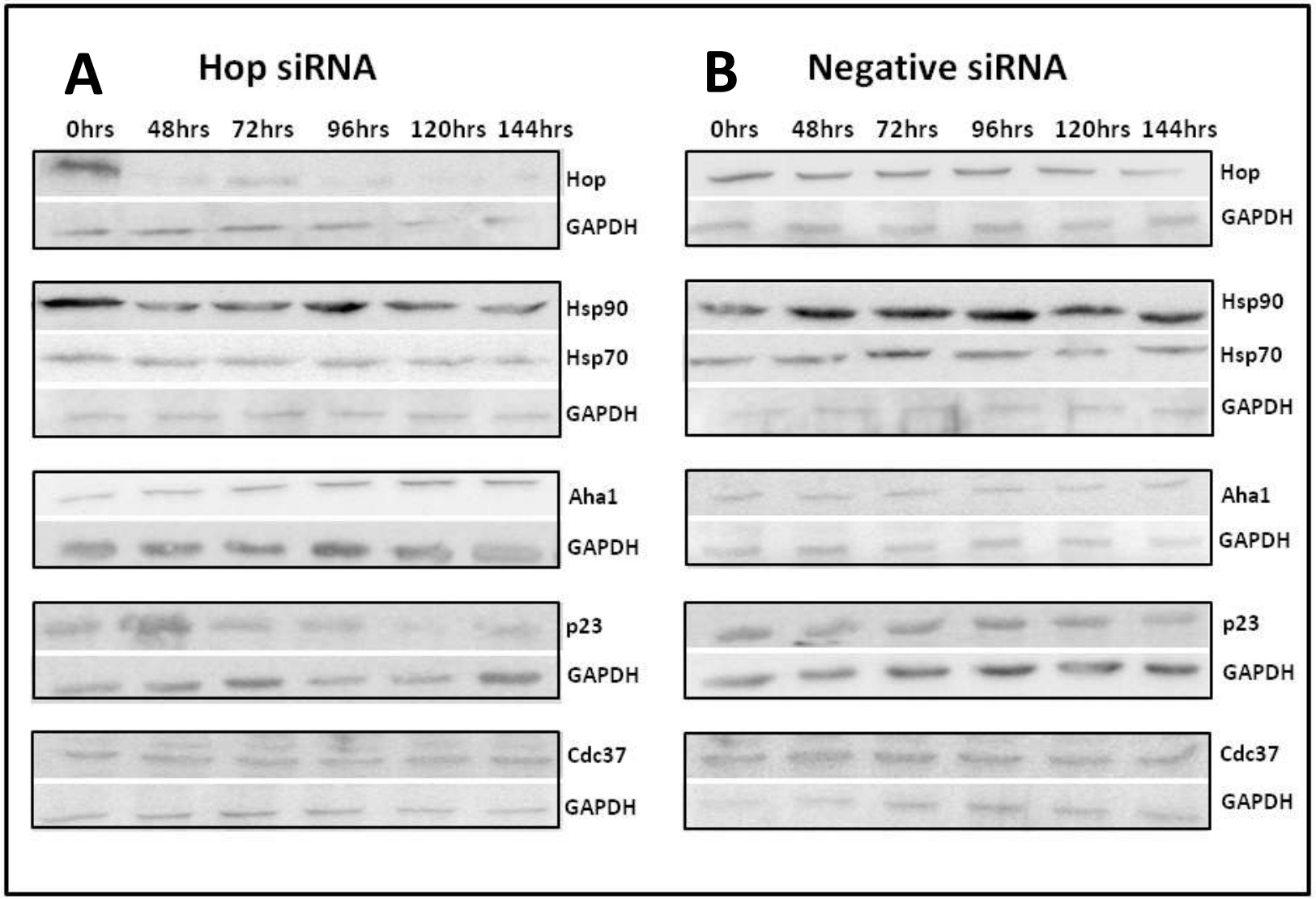


Figure 5: Effect of Hop knockdown on expression of chaperones and co-chaperones in Hs578T cancer cells.

Hs578T cells were transfected with either siRNA oligonucleotides targeted against Hop (A) or non-targeting siRNA control oligonucleotides (B). Cells were harvested at regular intervals over a total of 144 hours with mock transfected cells (0) included as a control. Hop, Hsp90, Hsp70, Aha1, p23 and Cdc37 levels were detected by SDS-PAGE and Western analysis as indicated in the methods. GAPDH was used as a loading control to normalise for the levels of total protein.

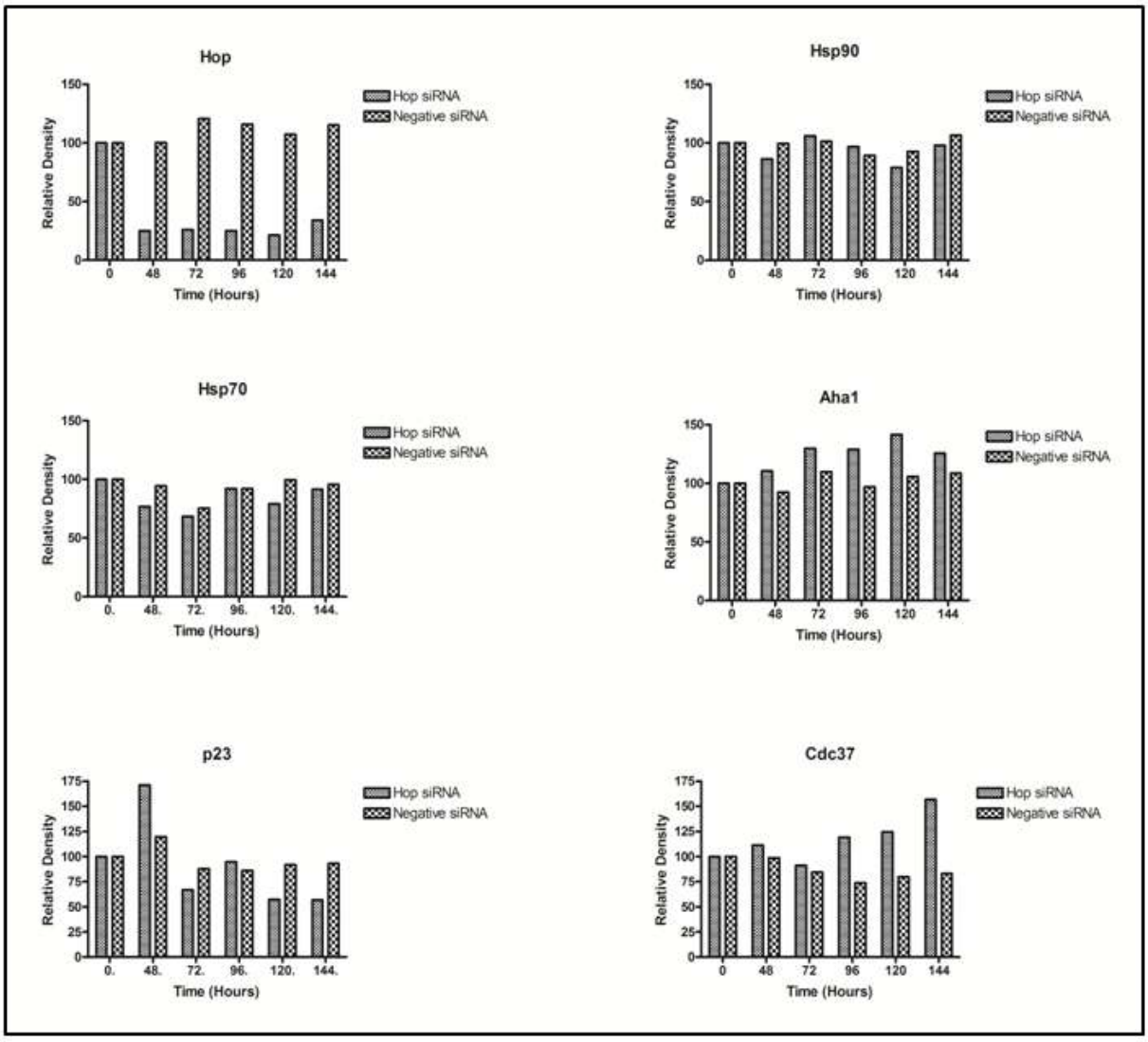


Figure 6: Changes in the expression of client proteins or chaperones in Hs578T cells as determined by densitometry.

Semi-quantitative analysis of the expression levels of proteins shown in Fig 5 by densitometry. All values were normalised to the GAPDH loading control and in each case are presented as a ratio of the no transfection control. The intensity of the Hop signal was normalized to that of GAPDH (representing total protein) by dividing the value obtained for the density of the Hop signal by that of the GAPDH to obtain the relative density. All densitometry was determined using ImageJ. Each experiment was performed once.

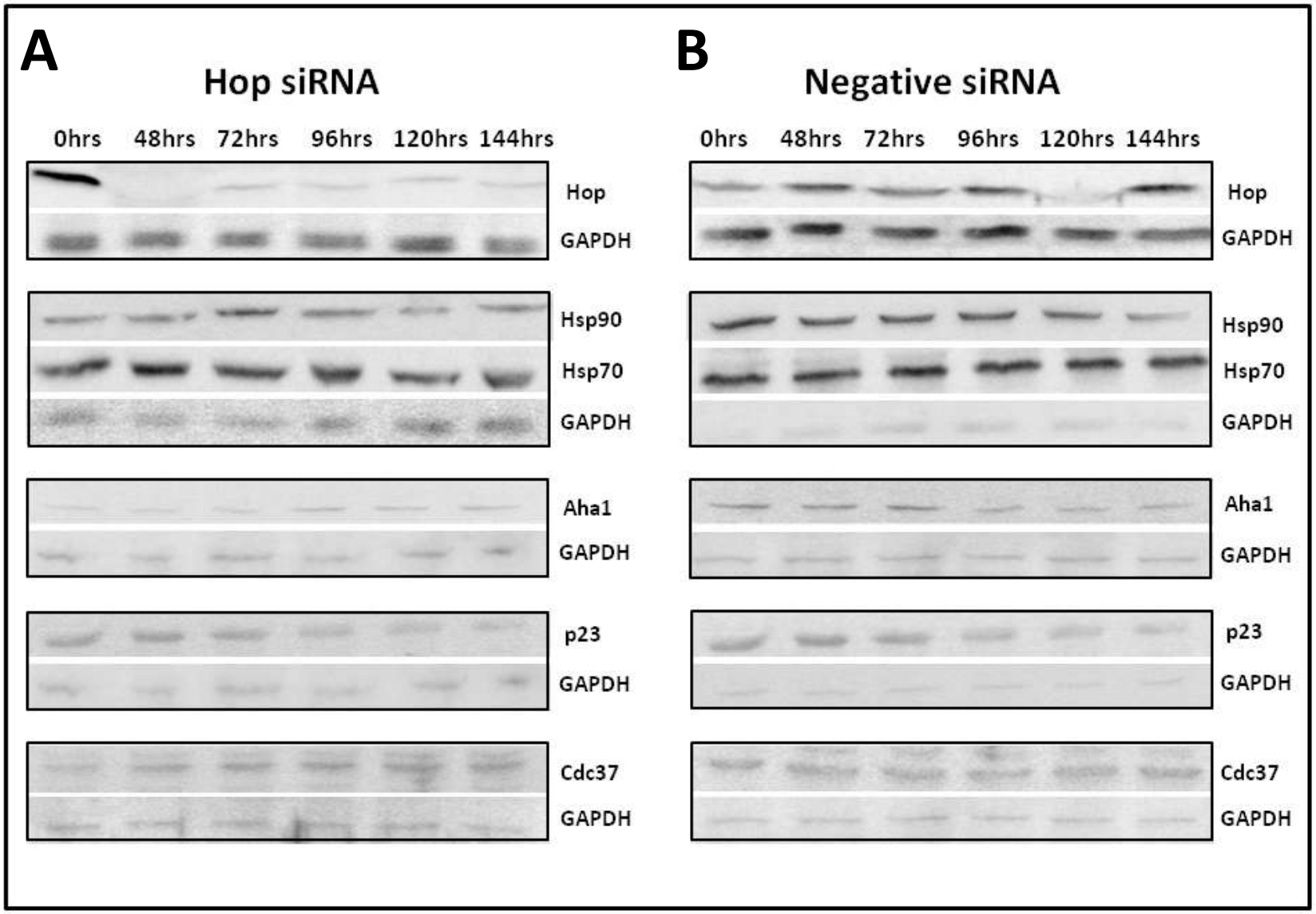


Figure 7: Effect of Hop knockdown on Hsp90, Hsp90 and other co-chaperones in MDA-MB-231 cancer cells

MDA-MB-231 cells were transfected with either siRNA oligonucleotide against Hop (A) or scrambled siRNA control oligonucleotide (B). Cells were harvested at regular intervals over a total of 144 hours with mock transfected cells (0) included as a control. Hop, Hsp90, Hsp70, Aha1, p23 and Cdc37 levels were detected by SDS PAGE and Western analysis as indicated in methods. GAPDH was included as a loading control.

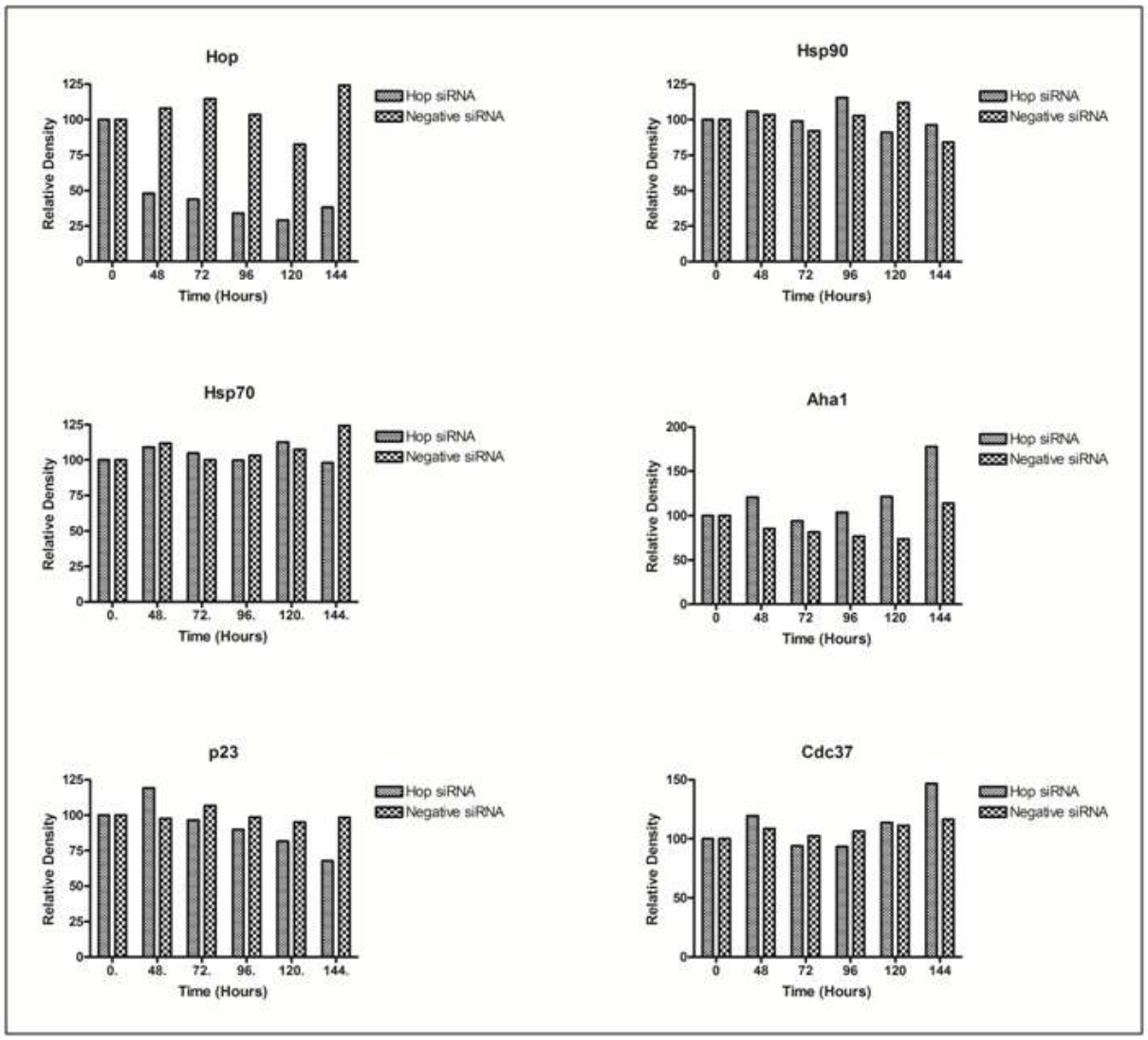


Figure 8: Changes in the expression of client proteins or chaperones in MDA-MB-231 cells as determined by densitometry.

Semi-quantitative analysis of the expression levels of proteins shown in Fig 7 by densitometry. All values were normalised to the GAPDH loading control and in each case are presented as a ratio of the no transfection control. The intensity of the Hop signal was normalized to that of GAPDH (representing total protein) by dividing the value obtained for the density of the Hop signal by that of the GAPDH to obtain the relative density. All densitometry was determined using ImageJ. Each experiment was performed once.

3.2.4 Effect of Hop silencing on Hsp90 client expression in Hs578T and MDA-MB-231 cells

The expression of Hsp90 clients in cell lines treated with siRNA against Hop or non-targeting control siRNA for 144 hours was tested. The Hs578T cell line treated with siRNA against Hop showed a reduction in Akt protein levels, along with that of phosphorylated signal transducer and activator of transcription 3 (pStat3). There was no obvious change in the levels of Stat3, while a decrease in the pStat3/Stat3 ratio was observed, indicating that a decrease in the proportion of phosphorylated Stat3 to total Stat3 levels occurred (Figure 9A and 10). There was fluctuation in the expression levels of phosphorylated extracellular regulated kinase (pErk) and total Erk occurred over the time course of 144 hours post transfection. However, after 144 hours, total Erk levels appeared to be lower than those observed in cells treated with negative non-targeting siRNA after 144 hours, while no particular trend was observed for pErk levels. However, an increase in the pErk to total Erk ratio was observed at 120 hours and 144 hours post transfection with siRNA targeting Hop compared to cells transfected with negative, non-targeting siRNA. It is important to note that although minor changes were observed in the levels of some proteins in the Hs578T cell line treated with the negative non-targeting siRNA over the 144 hour time interval (Figure 9B), all noteworthy changes in expression observed in cells treated with siRNA against Hop were directly compared with the corresponding negative siRNA transfected cells by densitometry analysis at each specific time point (Figure 10).

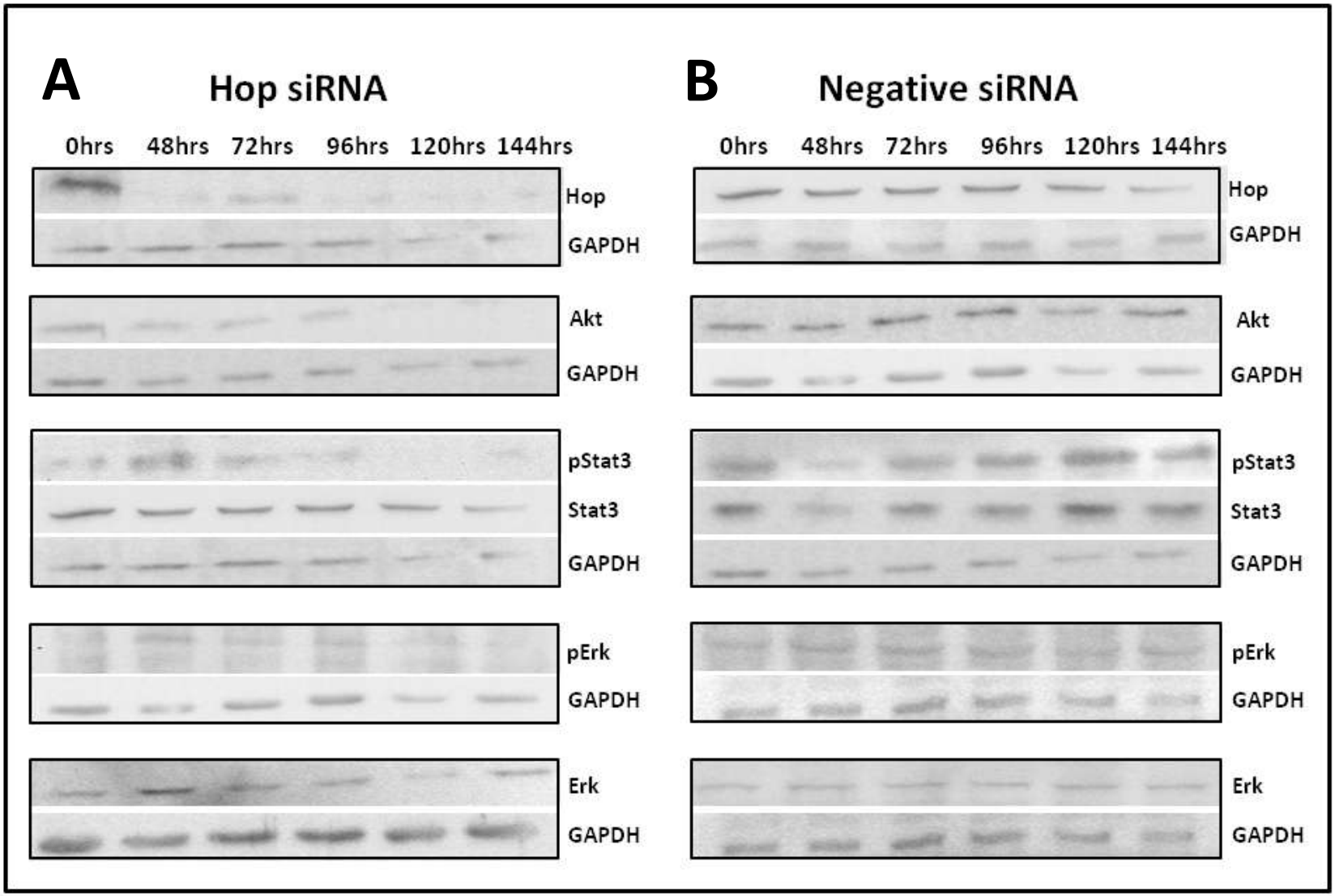


Figure 9: Effect of Hop knockdown on Hsp90 clients in Hs578T cancer cells

Hs578T cells were transfected with either siRNA oligonucleotide against Hop (A) or scrambled siRNA control oligonucleotide (B). Cells were harvested at regular intervals over a total of 144 hours with mock transfected cells (0) included as a control. Hop, Akt, pStat3, Stat3, pErk, and Erk levels were detected by SDS PAGE and Western blot analysis as indicated in methods. GAPDH was included as a loading control.

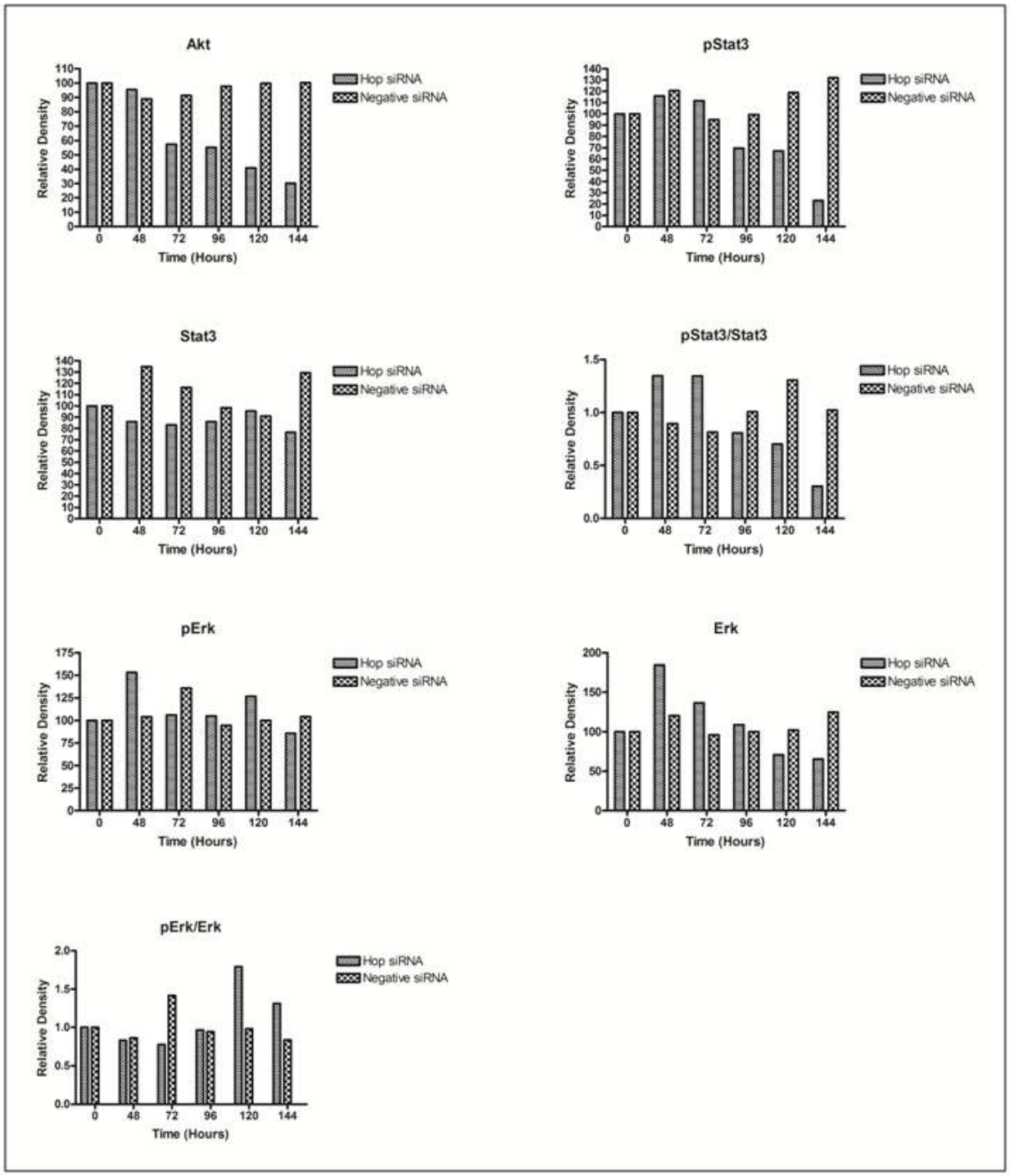


Figure 10: Changes in the expression of client proteins or chaperones in Hs578T cells as determined by densitometry

Semi-quantitative analysis of the expression levels of proteins shown in Fig 9 by densitometry. All values were normalised to the GAPDH loading control and in each case are presented as a ratio of the no transfection control. The intensity of the Hop signal was normalized to that of GAPDH (representing total protein) by dividing the value obtained for the density of the Hop signal by that of the GAPDH to obtain the relative density. All densitometry was determined using ImageJ. Each experiment was performed once.

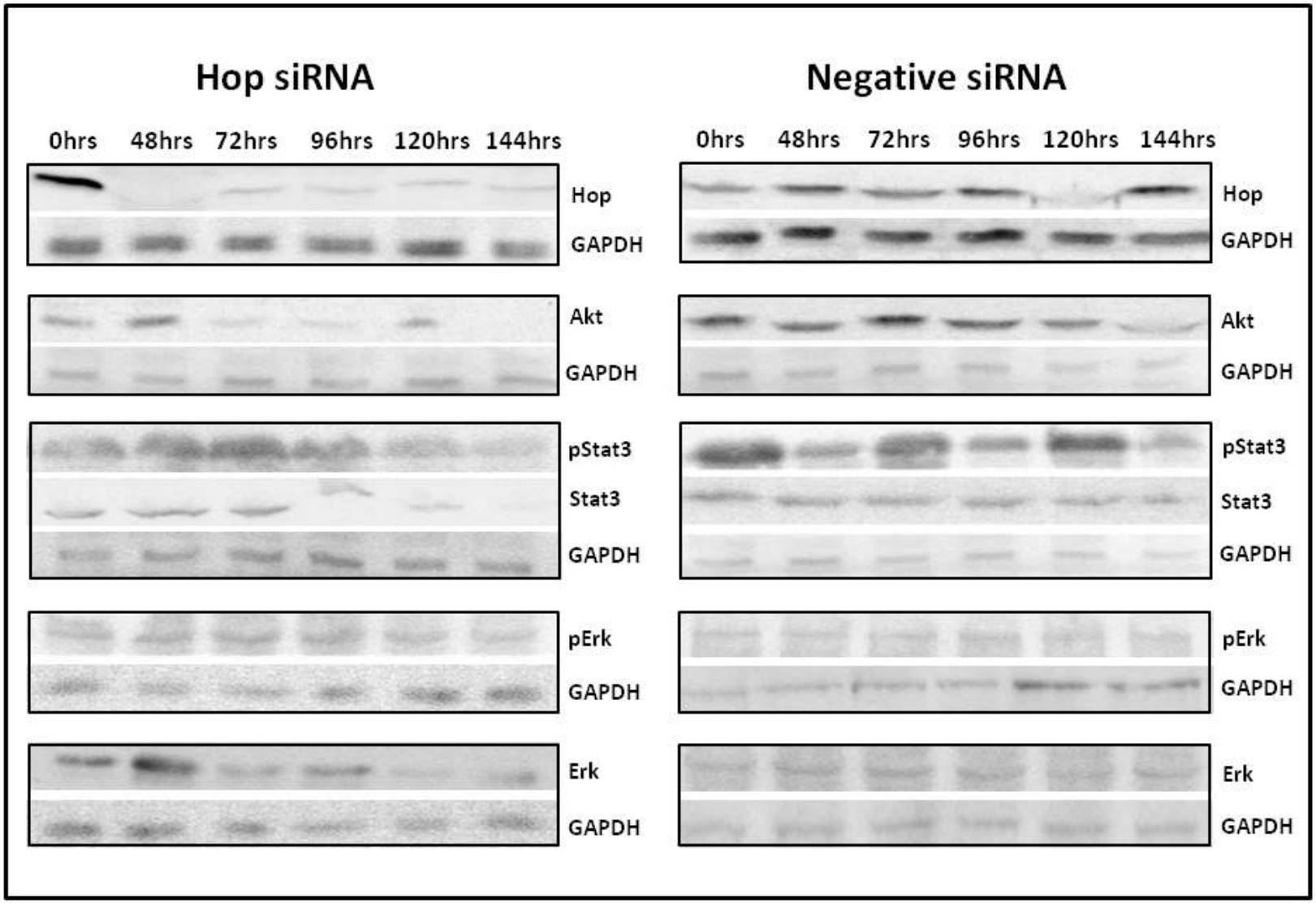


Figure 11: Effect of Hop knockdown on Hsp90 clients in MDA-MB-231 cancer cells

MDA-MB-231 cells were transfected with either siRNA oligonucleotide against Hop (A) or scrambled siRNA control oligonucleotide (B). Cells were harvested at regular intervals over a total of 144 hours with mock transfected cells (0) included as a control. Hop, Akt, pStat3, Stat3, pErK, and ErK levels were detected by SDS PAGE and Western blot analysis as indicated in methods. GAPDH was included as a loading control.

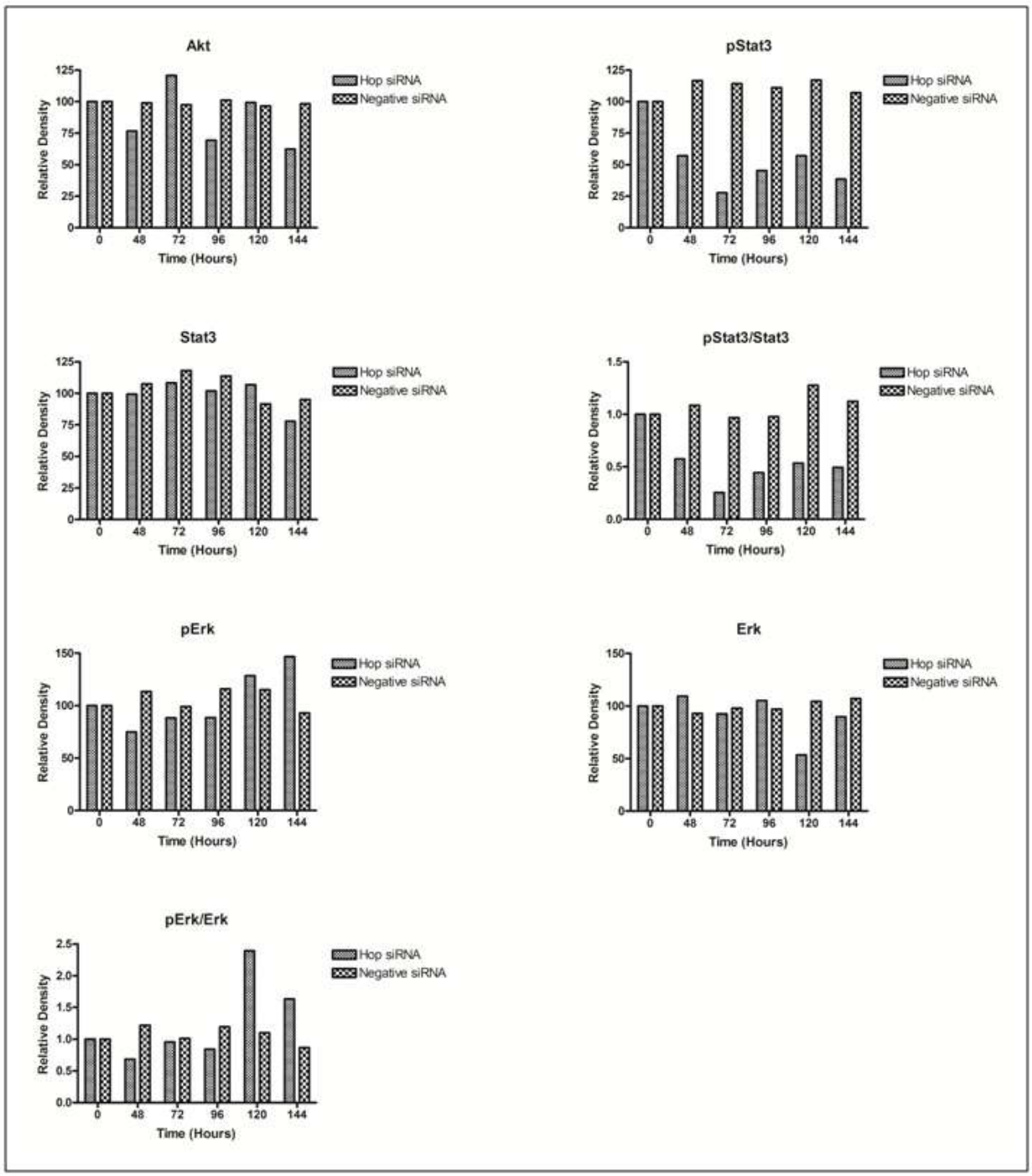


Figure 12: Changes in the expression of client proteins or chaperones in MDA-MB-231 cells as determined by densitometry

Semi-quantitative analysis of the expression levels of proteins shown in Fig 11 by densitometry. All values were normalised to the GAPDH loading control and in each case are presented as a ratio of the no transfection control. The intensity of the Hop signal was normalized to that of GAPDH (representing total protein) by dividing the value obtained for the density of the Hop signal by that of the GAPDH to obtain the relative density. All densitometry was determined using ImageJ. Each experiment was performed once.

Silencing of Hop in the MDA-MB-231 cell line (Figure 11A) resulted in no consistent pattern of Akt expression compared to cells transfected with negative non-targeting siRNA (Figure 11B). However, similar effects on pStat3 expression to the Hs578T cell line were observed, where protein levels reduced after 144 hours compared to cells transfected with negative non-targeting siRNA (Figure 11B). In addition, although the levels of total Stat3 did not differ greatly, a time dependent reduction in the pStat3 to total Stat3 ratio was observed. There was a general increase in pErk levels while total Erk remained relatively constant in cells transfected with Hop siRNA compared to cells treated with non-targeting siRNA, and these levels did not follow any particular trend. Therefore, the ratio of pErk to total Erk increased at the later time points post transfection (Figure 12). Like the Hs578T cells, slight changes in the levels of some proteins in negative non-targeting siRNA treated cells at various time intervals was observed (Figure 11B), however, all noteworthy changes in expression observed in cells treated with siRNA against Hop were directly compared with the corresponding negative siRNA transfected cells by densitometry analysis at each specific time point (Figure 12).

3.3 Summary and Conclusions

Hop expression and subcellular localisation was evaluated in seven different cell lines, with all cell lines tested expressing Hop to varying levels. Analysis of the subcellular localisation of Hop in the same panel of cell lines showed that the Hop was commonly located within the cytoplasm, but was also detected in the nucleus and on the periphery of cells. The RNA interference system for transient silencing of Hop in the Hs578T and MDA-MB-231 cell lines was successfully achieved; wherein Hop protein expression was reduced over a period of 144 hours by between 60 - 90% of the control for both cell lines, while the non-targeting siRNA oligonucleotides did not result any noticeable in Hop levels. The Hs578T and MDA-MB-231 cell lines were selected for RNA interference studies to study the role of Hop in cancer cell biology as these cell lines are characterized as triple negative tumours, a breast cancer subtype which is known to be highly aggressive (Arteaga *et al*., 1987). In addition, Hs578T had the highest level of Hop expression, and strong Hop staining was detected at the cell membrane of both Hs578T and MDA-MB-231 cells. In both cell lines, Hop knockdown did not alter Hsp90 or Hsp70 levels, while there was an increase in the levels of the co-chaperones Cdc37 and Aha1, and a decrease in p23 levels. Furthermore, Hop knockdown resulted in a reduction in the client protein Akt as well as the proportion of pStat3 to total Stat3, while the proportion of pErk to total Erk increased. The fact that Hop could be silenced for at least 6 days in both of these cell lines validated the use of this transient RNAi system for subsequent cell biological analyses. To the best of our knowledge, this is the first report of siRNA-mediated Hop silencing in breast cancer cell lines.

Chapter 4 : The role of Hop in cancer cell proliferation and chemosensitivity

4.1 Introduction

Hsp90 chaperone function is important for the self sufficiency in growth signals observed in a wide range of cancers, where key oncogenic protein kinases and hormone receptors are dependent on Hsp90 mediated folding and stabilization for activity (Whitesell and Lindquist, 2005). Recently, the role of Hsp90 in cancer cell proliferation has extended to studies on other accessory proteins involved in the chaperone cycle, and it has been shown that Hsp90 co-chaperones such as Hop, Aha1, Cdc37 and p23 play a role in cell proliferation as well as sensitivity to anti-cancer drugs (Erlich *et al*., 2007; Forafonov *et al.,* 2008; Holmes *et al.,* 2008; Smith *et al.,* 2009; Horibe *et al*., 2011). To date, studies on the role of Hop in cancer cell proliferation have shown that treatment of glioma cells with recombinant Hop led to increased cell proliferation (Erlich *et al.,* 2007), while other reports have shown that targeting the interaction between Hsp90 and Hop resulted in a reduction in proliferation of numerous cell lines, including breast, human lung, kidney as well as embryonic kidney cells (Horibe *et al*., 2011; Pimienta *et al*., 2011). However, the effect of Hop knockdown on breast cancer cell lines has not been reported. Therefore, using our validated RNAi knockdown system for Hop, we investigated the effect of Hop knockdown on the proliferation and chemosensitivity of the Hs578T and MDA-MB-231 cell lines.

4.2 Results

4.2.1 Effects of Hop silencing on Hs578T and MDA-MB-231 cell proliferation

Using the RNA interference approach described previously, cell growth curves were determined using the MTT assay. Hs578T and MDA-MB-231 cells were treated with siRNA against Hop, non-targeting negative siRNA or transfection reagent alone (mock) and the cell viability measured at regular intervals over a 72 hour period (Figure 13). The growth curve for Hs578T treated with the transfection reagent alone (mock) or with the negative siRNA were similar. However, treatment of Hs578T cells with Hop siRNA resulted in a significant reduction in cell growth (Figure 13A). Mock and negative siRNA treated MDA-MB231 cells showed a similar growth curve. In contrast to the Hs578T data, Hop silencing in MDA-MB-231 cells resulted in a significant increase in proliferation of the cells when compared to untreated and control siRNA groups (Figure 13B). The knockdown of Hop was verified for both the Hs578T (Figure 13C) and MDA-MB-231 (Figure 13D) cell lines by Western analysis at the end of the experiment (72 hours).

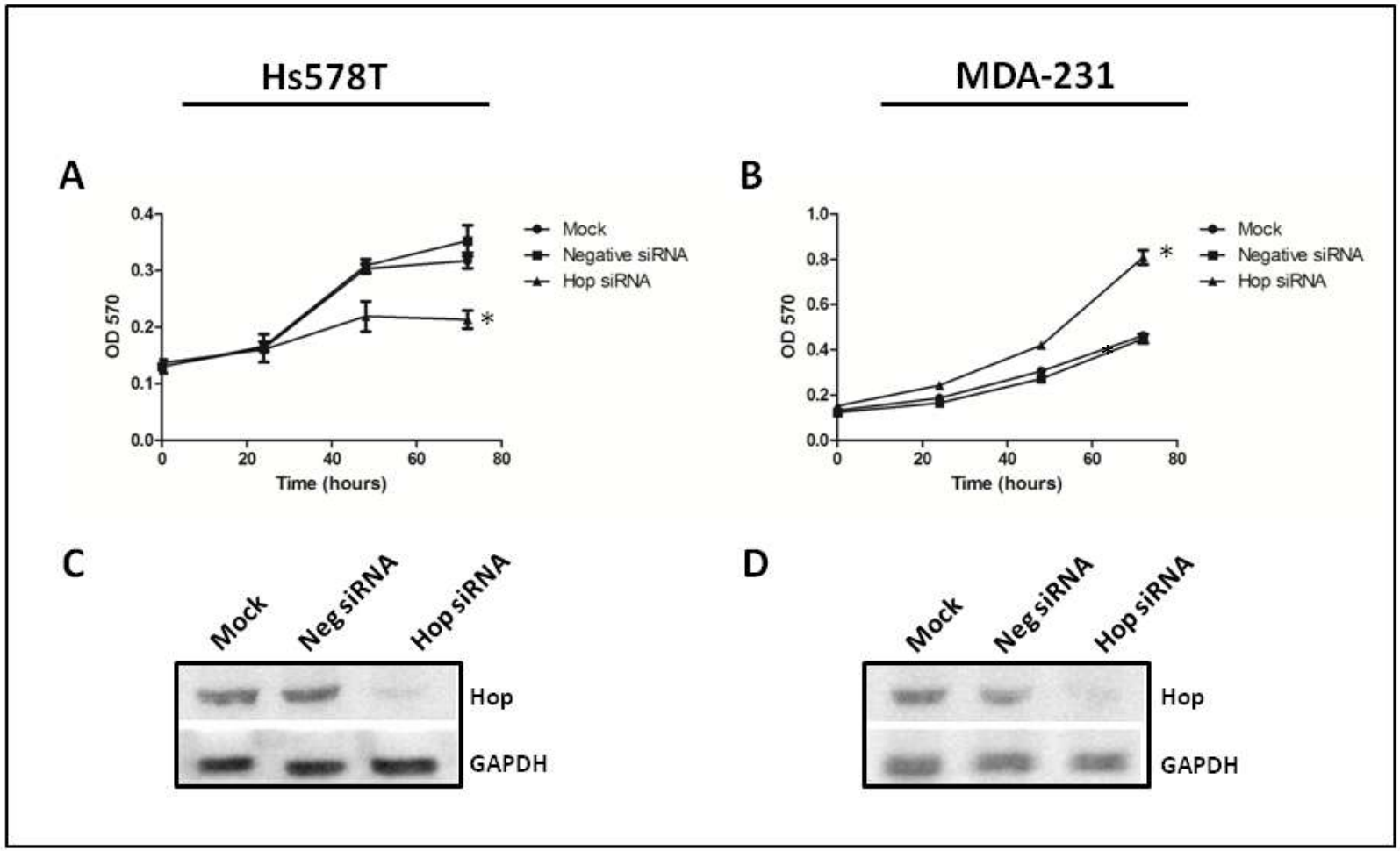


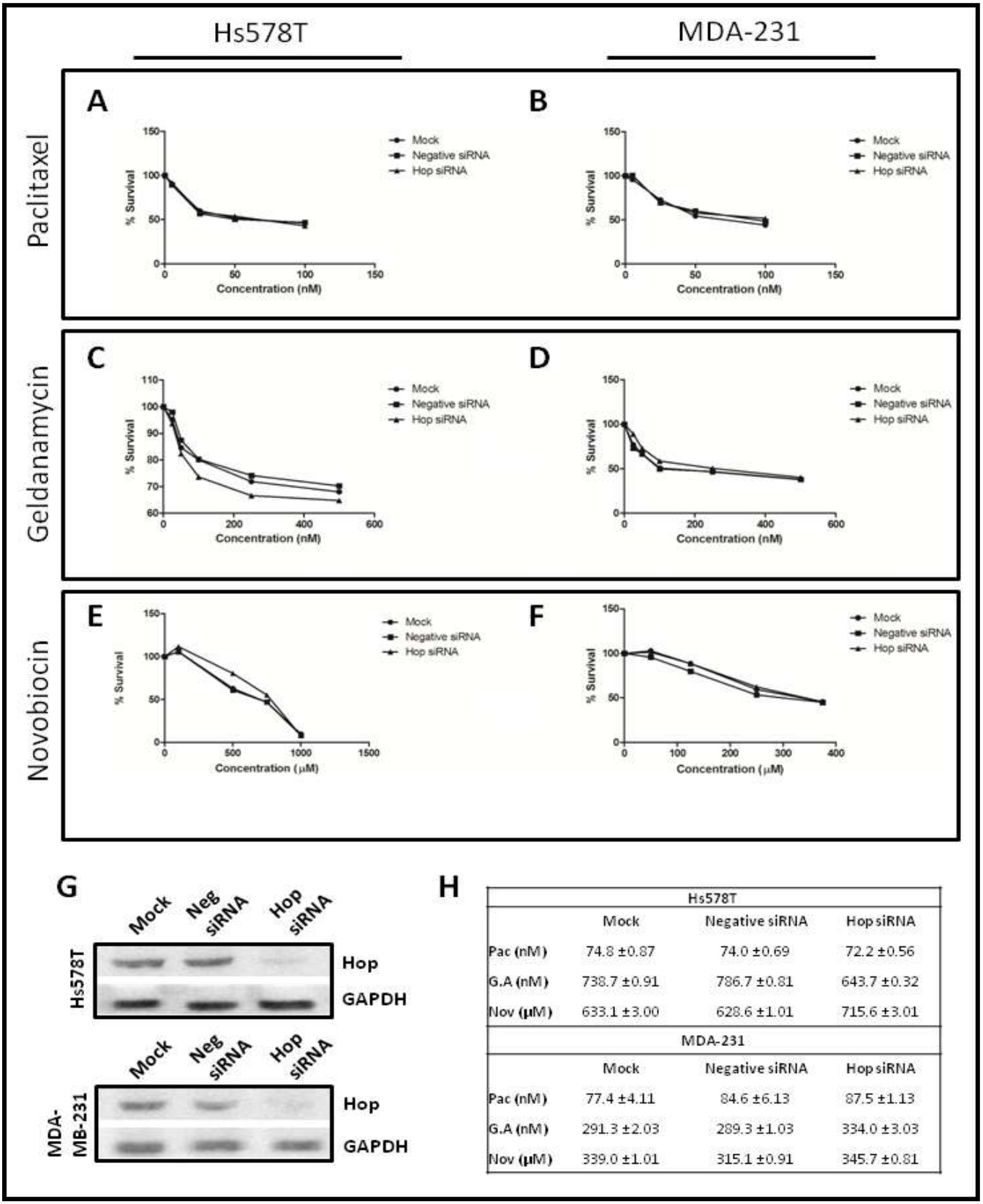
Figure 13: Effect of siRNA knockdown of Hop on cell proliferation of Hs578T and MDA-231 cancer cells

Proliferation of Hs578T (A) and MDA-231 (B) cells was assessed by the MTT assay at the time points indicated after either mock transfection, transfection with siRNA oligonucleotides against Hop, or transfection with scrambled siRNA negative control oligonucleotides. Knockdown was confirmed by Western analysis for Hs578T cells (C) and MDA-231 cells (D) at the end of each experiment (72 hours). Each curve represents the mean and standard deviation of nine replicates, and is representative of three independent experiments. Statistical significance was analyzed by the two-way ANOVA test (*= $p < 0.05$).

4.2.2 Effects of Hop silencing on chemosensitivity in Hs578T and MDA-MB-231 cells

Next, the effect of silencing of Hop on the chemosensitivity of cells to selected pharmacological inhibitors was tested. In this study, it was investigated whether silencing of Hop would sensitize Hs578T and MDA-MB-231 cancer cells to the anticancer compound, paclitaxel, as well as two Hsp90 inhibitors, geldanamycin and novobiocin.

Hs578T and MDA-MB-231 cells were treated with increasing concentrations of paclitaxel, geldanamycin or novobiocin over a period of 72 hours after mock transfection, transfection with siRNA oligonucleotides against Hop, or transfection with scrambled siRNA control oligonucleotides. Cell viability was assessed by the MTT assay, and the IC_{50} values for each drug were determined as the drug concentration that inhibited cell growth by 50% (Figure 14). The growth curves for Hs578T and MDA-MB-231 cells treated with paclitaxel after mock transfection or transfection with siRNA targeting Hop or with the negative siRNA showed a similar trend, whereby cell viability decrease with increasing paclitaxel concentration (Figure 14A and 14B respectively). Calculation of the corresponding IC_{50} values showed no significant difference between each treatment (Figure 14H), where Hs578T cells treated with either transfection reagent alone or negative non-targeting siRNA had IC_{50} values of approximately 75 nM and 74 nM respectively, and cells treated with siRNA targeting Hop had an IC_{50} value of approximately 72 nM, while MDA-MB-231 cells treated with transfection reagent alone, negative non-targeting siRNA or siRNA targeting Hop had similar IC_{50} values of approximately 77 nM, 85 nM and 88 nM respectively.



Hs578T	Mock	Negative siRNA	Hop siRNA
Pac (nM)	74.8 ±0.87	74.0 ±0.69	72.2 ±0.56
G.A (nM)	738.7 ±0.91	786.7 ±0.81	643.7 ±0.32
Nov (μM)	633.1 ±3.00	628.6 ±1.01	715.6 ±3.01
MDA-231			
	Mock	Negative siRNA	Hop siRNA
Pac (nM)	77.4 ±4.11	84.6 ±6.13	87.5 ±1.13
G.A (nM)	291.3 ±2.03	289.3 ±1.03	334.0 ±3.03
Nov (μM)	339.0 ±1.01	315.1 ±0.91	345.7 ±0.81

Figure 14: Effects of siRNA silencing of Hop on chemosensitivity to paclitaxel, geldanamycin and novobiocin.

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Figure 14: Effects of siRNA silencing of Hop on chemosensitivity to paclitaxel, geldanamycin and novobiocin

The sensitivity of Hs578T cells (left panel) and MDA-231 cells (right panel) to paclitaxel (A and B), geldanamycin (C and D) and novobiocin (E and F) at the concentrations indicated was assessed by the MTT assay over 72 hours, following either mock transfection, transfection with siRNA oligonucleotides against Hop, or transfection with scrambled siRNA control oligonucleotides. (G) Knockdown was confirmed by Western analysis for Hs578T cells and MDA-231 cells at the end of each experiment. Each assay represents the mean ±SD of three replicates, and is a representative of three independent experiments. Statistical significance was analyzed by the two-way ANOVA test (*= $p < 0.05$). (H) IC_{50} values were calculated as the drug concentrating inhibiting cell viability by 50%, and are summarized in the table.

A similar trend was observed for MDA-MB-231 cells treated with geldanamycin (Figure 14D respectively), where mock transfected cells, or cells treated with negative non-targeting siRNA or siRNA targeting Hop had IC_{50} values (Figure 14H) that were not significantly different. The IC_{50} values for mock transfected cells, or cells treated with negative non-targeting siRNA or siRNA targeting Hop were approximately 291 nM, 289 nM and 334 nM respectively for the MDA-MB-231 cell line. In contrast, in the Hs578T in which Hop was silenced there was an apparent dose dependent increase in sensitivity to geldanamycin when compared to mock or negative siRNA treated cells, with IC_{50} values of approximately 784 nM, 787 nM and 644 nM for mock, negative and Hop siRNA transfections respectively.

The growth curves of Hs578T cells treated with increasing concentrations of novobiocin together with either transfection reagent alone or negative non-targeting siRNA showed similar dose responses compared to cells treated with siRNA targeting Hop (Figure 14E). The calculated IC_{50} values for each treatment did not differ significantly (Figure 14H; approximately 633 µM, 629 µM and 716 µM for mock transfected cells, cells treated with negative siRNA or siRNA targeting Hop respectively). In addition, the growth curves for MDA-MB-231 cells treated with

increasing concentrations of novobiocin and either transfection reagent alone, negative non-targeting siRNA or siRNA targeting Hop showed similar dose response curves (Figure 14D) and the IC_{50} too did not differ significantly (Figure 14H; approximately 339 µM, 315 µM and 345 µM for mock transfected cells, cells treated with negative siRNA or siRNA targeting Hop respectively). After each experiment the knockdown of Hop was confirmed by Western blot analysis (Figure 14G).

We had previously shown that Hop knockdown had opposing effects on cell growth in the Hs578T and MDA-MB-231 cell lines. In order to account for the differences in growth rate, a kinetic study was performed in which cells were treated with fixed drug concentrations and the effect on cell viability determined at 24 hour intervals over a 72 hours period. In addition, in order to assess whether the difference in dose response trends between cells in which Hop was silenced in comparison to control treated cells were a result of acquired chemoresistance, the kinetic study was repeated with the addition of the drug transport inhibitor, verapamil.

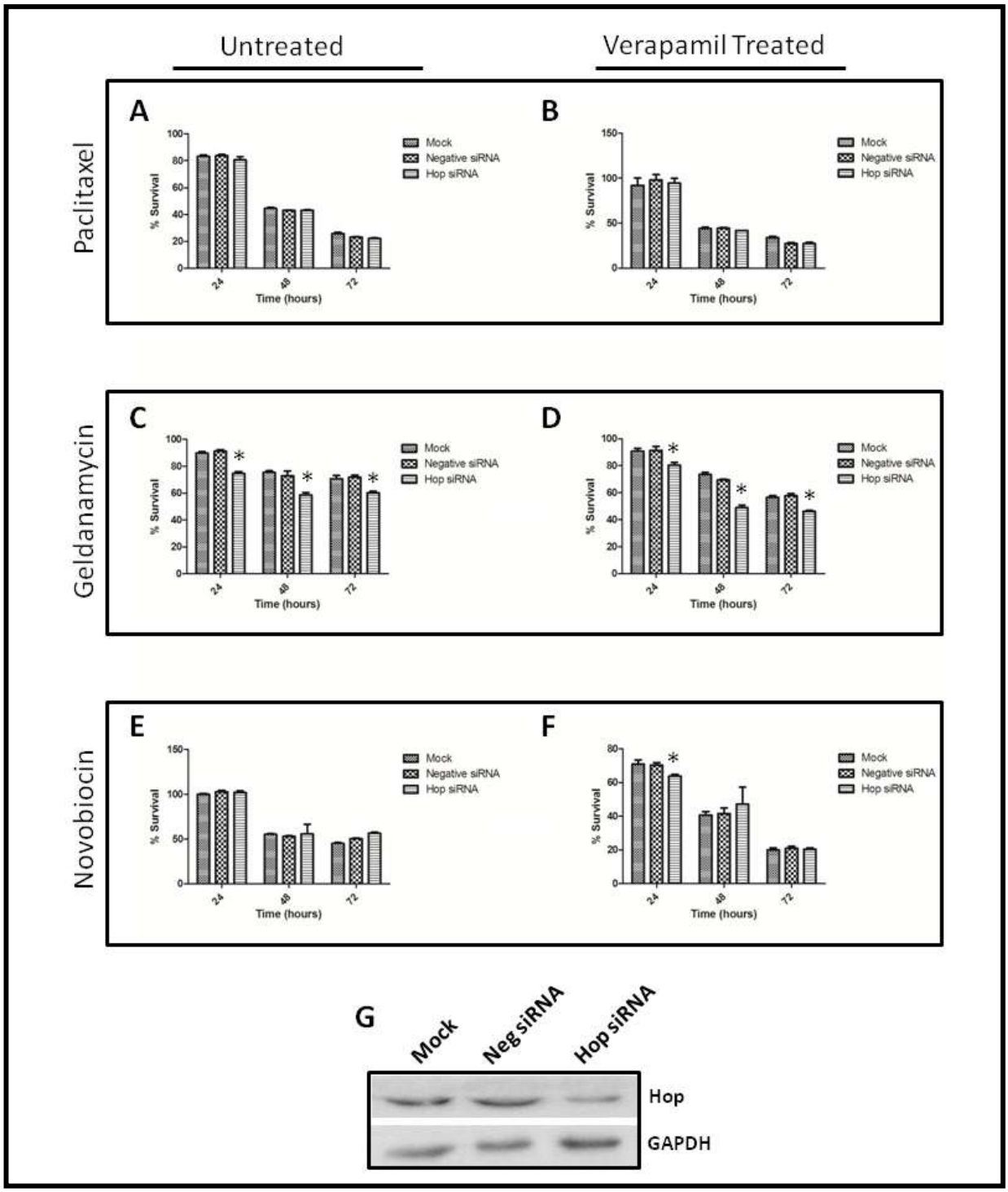


Figure 15: Effect of siRNA silencing of Hop and verapamil treatment on chemosensitivity of Hs578T cells to paclitaxel, geldanamycin and novobiocin

Effect of siRNA silencing of Hop in Hs578T cells treated without (left panel) or with verapamil (50 µg/ml) (Right panel) on the chemosensitivity of cells to the calculated IC_{50} values of paclitaxel (100 nM; A and B), geldanamycin (750 nM; C and D) and novobiocin (750 µM; E and F). Percentage survival was assessed by the MTT assay at 24 hour intervals over a time course of 72 hours after treatment following either mock transfection, transfection with siRNA oligonucleotides against Hop, or transfection with scrambled siRNA control oligonucleotides. All values were normalized to cells treated with DMSO and verapamil alone. Knockdown was confirmed by Western blot analysis at the end of each experiment (G). Each chart represents the mean ±SD of four replicates, and is a representative of two independent experiments. Statistical significance was analyzed by the two-way ANOVA test (* = $p < 0.05$).

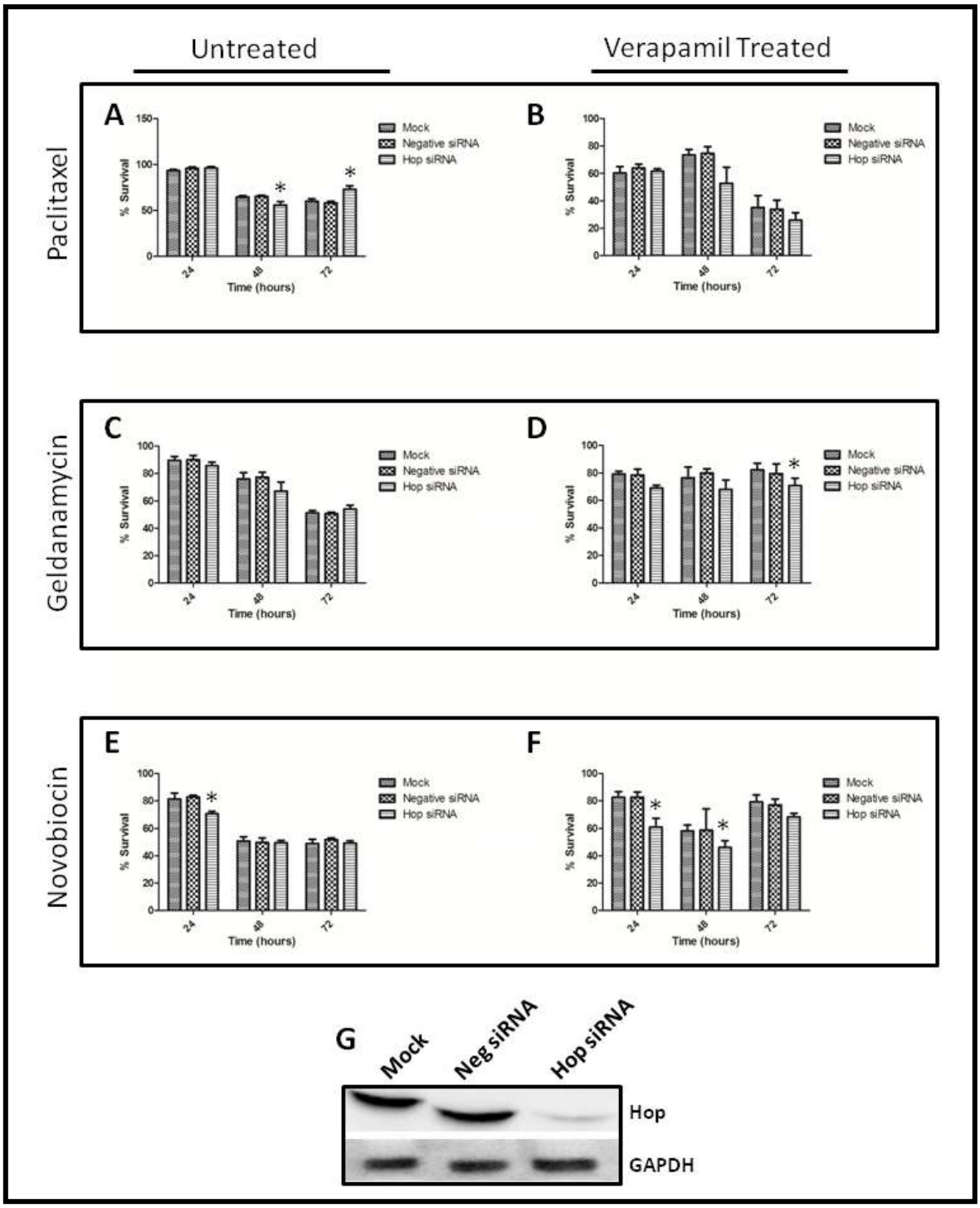


Figure 16: Effects of siRNA silencing of Hop and verapamil treatment on chemosensitivity of MDA-231 cells to paclitaxel, geldanamycin and novobiocin

Effect of siRNA-mediated silencing of Hop in MDA-MB-231 cells treated without (left panel) or with verapamil (50 µg/ml) (right panel) to the corresponding IC_{50} values of paclitaxel (100 nM; A and B), geldanamycin (300 nM; C and D) and novobiocin (300 µM; E and F). Percentage survival was assessed by the MTT assay at 24 hour intervals over a time course of 72 hours after treatment following either mock transfection, transfection with siRNA oligonucleotide against Hop, or transfection with scrambled siRNA control oligonucleotide. All values were normalized to cells treated with DMSO. Knockdown was confirmed by Western blot analysis at the end of each experiment (G). Each assay represents the mean ±SD of four replicates, and is a representative of two independent experiments. Statistical significance was analyzed by the two-way ANOVA test (* = $p < 0.05$).

For the Hs578T cell line, no significant difference in cell viability was observed between mock transfected cells, or cells treated with negative siRNA or siRNA targeting Hop after treatment with paclitaxel (100 nM) for any of the time intervals assessed (Figure 15A). Furthermore, the addition of verapamil caused a 20% increase in cell viability compared to cells treated with paclitaxel alone, although this effect was independent of siRNA treatment (Figure 15B). Interestingly, for Hs578T cells treated with geldanamycin (750 nM), a significant decrease in cell viability was observed in cells treated with siRNA targeting Hop compared to mock transfected cells or cells treated with negative siRNA for all time intervals (Figure 15C). The addition of verapamil did not change the trend and there was still a statistically significant sensitisation of Hop knockdown cells to geldanamycin compared to mock and negative siRNA transfected Hs578T cells (Figure 15D). Finally, no significant difference between mock transfected cells and cells treated with negative siRNA or siRNA targeting Hop was observed after treatment with novobiocin (750 μM) for any of the time points assessed (Figure 15E). However, the addition of verapamil caused a significant decrease in cell viability in response to novobiocin of cells treated with Hop siRNA in comparison to mock transfected cells or cells treated with negative siRNA (Figure 15E). After each experiment the knockdown of Hop was confirmed by Western analysis (Figure 15G).

The results from the kinetic study in the MDA-MB-231 cell line showed a significant decrease in the viability of cells treated with siRNA targeting Hop compared to mock transfected cells, or cells treated with negative siRNA at 48 after treatment with paclitaxel (100 nM), although no decrease in viability occurred after 24 hours (Figure 16A). However, at 72 hours post drug treatment, the cells treated with siRNA targeting Hop showed significantly higher cell viability in

comparison to mock transfected cells or cells treated with negative siRNA. Addition of verapamil was able to overcome the resistance of Hop knockdown cells at 72 hours and lead to a reduction in cell viability that was determined to be not significantly different to the mock or negative control transfected cells treated with paclitaxel (Figure 16B). MDA-MB-231 cells treated with geldanamycin (300 nM) showed no significant difference in cell viability irrespective of the different transfections for all time intervals (Figure 16C). However, the addition of verapamil resulted in a significant decrease in the viability of cells treated with siRNA targeting Hop when compared to mock transfected or cells treated with negative siRNA at 72 hours post drug treatment (Figure 16D). Finally, it was observed that MDA-MB-231 cells treated with siRNA targeting Hop had a statistically significant reduction in cell viability after 24 hours of treatment with novobiocin (300 μM) in comparison to mock transfected cells or cells treated with negative siRNA, but this effect was not observed after 48 or 72 hours of treatment (Figure 16E). Interestingly, the addition of verapamil induced sensitivity of MDA-MB-231 cells treated with siRNA targeting Hop at 48 hours post novobiocin treatment compared to mock transfected cells or cells treated with negative siRNA, although this effect was not observed after 72 hours (Figure 16F). After each experiment the knockdown of Hop was confirmed by Western analysis (Figure 16G).

4.3 Summary and Conclusions

In this chapter, we investigated the effects of Hop silencing on cell proliferation and sensitivity to anti-cancer drugs. We observed that Hop had apposing effects in cell proliferation that was dependent on cellular context, where knockdown of Hop decreased Hs578T cell proliferation but increased MDA-MB-231 cell proliferation. We next investigated the effects of Hop silencing on chemosensitivity of Hs578T and MDA-MB-231 cells to an anticancer compound, paclitaxel, as well as two Hsp90 inhibitors, geldanamycin and novobiocin. Results from the dose dependent studies showed that silencing of Hop did not cause any significant sensitization to paclitaxel, geldanamycin or novobiocin in Hs578T or MDA-MB-231 cell lines, as determined by the relative IC_{50} values in comparison to mock and negative siRNA transfected cells. In the time-dependent study Hop knockdown did in fact induce some sensitization to geldanamycin in Hs578T cells, and paclitaxel and novobiocin in MDA-MB-231 cells at earlier time points. However, in some cases, cells in which Hop was silenced were able to circumvent the sensitivity observed after 72 hours treatment, and showed an apparent increase in drug resistance compared to control cells. These effects seemed to be dependent on cell type, as well as time and each individual drug treatment. Interestingly, it was observed that in cases where cells became chemoresistant upon silencing of Hop, sensitization was partially restored by addition of verapamil, an inhibitor of the P-gp drug transporter (Takara *et al*., 2002).

The association of Hop with the cell membrane as well as the extracellular matrix has been reported in several studies (Zanata *et al*., 2002; Erlich *et al.,* 2007 Walsh *et al*., 2011). Hop has also been linked to migration and invasion in pancreatic cells (Walsh *et al*., 2011). As a result, we next investigated the role of Hop in the migration of breast cancer cells.

Chapter 5 : The role of Hop in cell migration

5.1 Introduction

The presence of Hop in the extracellular matrix has been described in glioma cells (Erlich *et al*., 2007), pancreatic cancer cells (Walsh *et al.,* 2011), and fibrosarcoma cells (Eustace *et al*., 2004). In studies by Walsh and colleagues (2011), the presence of Hop in the extracellular matrix was linked to the invasive properties of these cells by association with matrix metalloproteinase-2 (MMP-2) (Walsh *et al.,* 2011). In the extracellular space of fibrosarcoma cells, Hsp90α in complex with Hop and Hsp70 was shown to interact with matrix metalloproteinase-2 (MMP2) and thus facilitate the maturation of MMP-2, promoting tumour invasiveness (Eustace *et al.,* 2004). MMPs are known to play a role in cancer metastasis by promoting degradation of the extracellular matrix (Xie *et al.,* 2004). Cell migration is dependent on the reorganization of the actin cytoskeleton, which involves an equilibrium between globular (G-actin) and filamentous (F-actin) actin, and results in the protrusion of the lamellipodium at the leading edge of the cell and the retraction of the cell rear (Taiyab *et al*., 2011). The protrusion of the lamellipodium is provided by continuous growth of actin filaments toward the leading edge of the lamellipodium, while the retraction of the rear is regulated by the release of adhesive contacts from extracellular matrix proteins (Lauffenburger and Horwitz, 1996). Hsp90 has been linked to cancer cell migration through a direct interaction with actin filaments and inhibition of Hsp90 led to a decrease in lamellipodia and filopodia formation (Taiyab *et al*., 2011). It is thought that upon inhibition, Hsp90 interacts with G-actin and prevents polymerisation of G-actin monomers into F-actin, thus resulting in reduced lamellipodia and filopodia formation (Taiyab *et al*., 2011).

We previously observed the localisation of Hop at points of adhesion and the leading edge of polarized cells by confocal microscopy (Chapter 3). As this staining was the most prominent in

the Hs578T cell line, the effect of siRNA mediated knockdown of Hop on cancer cell migration was investigated.

5.2 Results

5.2.1 Investigation of the effect of Hop silencing on migration of Hs578T and MDA-MB-231 cells

In order to investigate the effect of Hop silencing on the migratory capacity of the Hs578T and MDA-MB-231 cell lines, wound healing assays were employed (Figures 17 and 18 respectively). Cells were transfected with either siRNA targeting Hop, or non-targeting siRNA, and cell migration monitored over a period of 12 hours in order to exclude a contribution of cell proliferation. The size of each wound was recorded by at the start of the experiment (0 hours) and after 6 and 12 hours using a digital camera attached to a standard light microscope. The wound area was measured at the different time points using ImageJ. The area of the wound at the start of the experiment was taken to be 100% and the size of the wound after 6 hours or 12 hours was represented as a percentage of the original wound size. Therefore, no cell migration would be recorded as little to no change in the wound size (i.e. a percentage that is close to 100%), while an increase in migration would lead to a decrease in the wound area. All wound healing assays were performed with a total of 9 replicates, and images shown are representative of three independent experiments. Statistical analysis of the average area of each wound for all replicates was calculated using the Student's t-test, where $p = <0.05$ (Figure 17B and 18B) was considered statistically significant. After each experiment, knockdown of Hop in Hs578T and MDA-MB-231 cells was confirmed by Western analysis (Figure 17C and 18C).

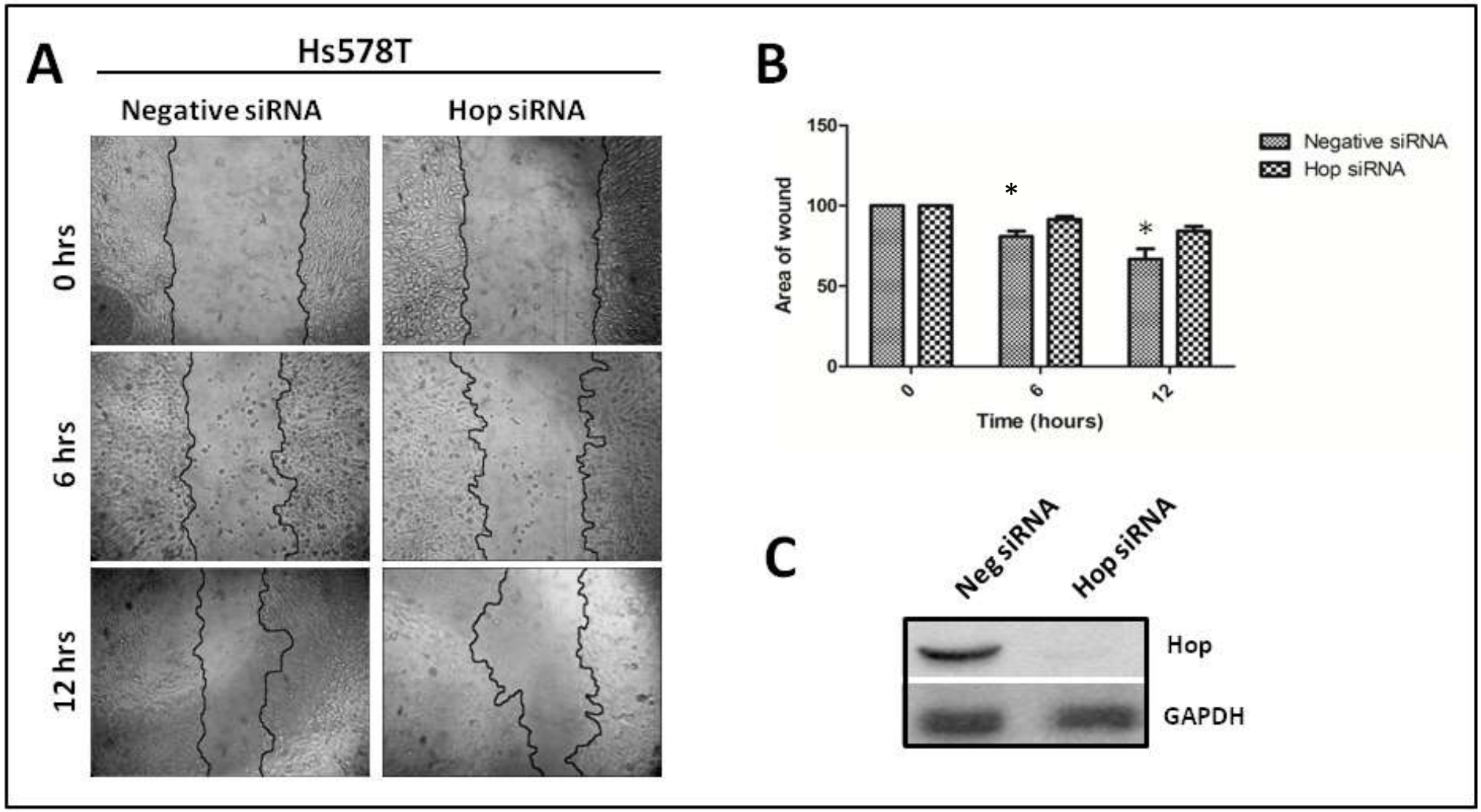


Figure 17: Analysis of the effect of Hop knockdown in migration of Hs578T cells in the wound healing assay

Hs578T cells were transfected with either non-targeting siRNA (A, left panel) or siRNA against Hop (A, right panel), and seeded on fibronectin coated 96-well plates 24 hours post transfection. Cells were allowed to adhere overnight and wounds were made by scraping the cell monolayer with a sterile toothpick. Images were taken at 0 hrs and after 6 hrs and 12 hrs of incubation. Images shown in (A) are representative of three independent experiments consisting of nine replicates, while (B) shows the average change in wound size for all experiments. Statistical significance was analyzed by the paired t-test (*= $p < 0.05$). The levels of Hop in the transfected lysates was confirmed at the end of each experiment by Western analysis (C).

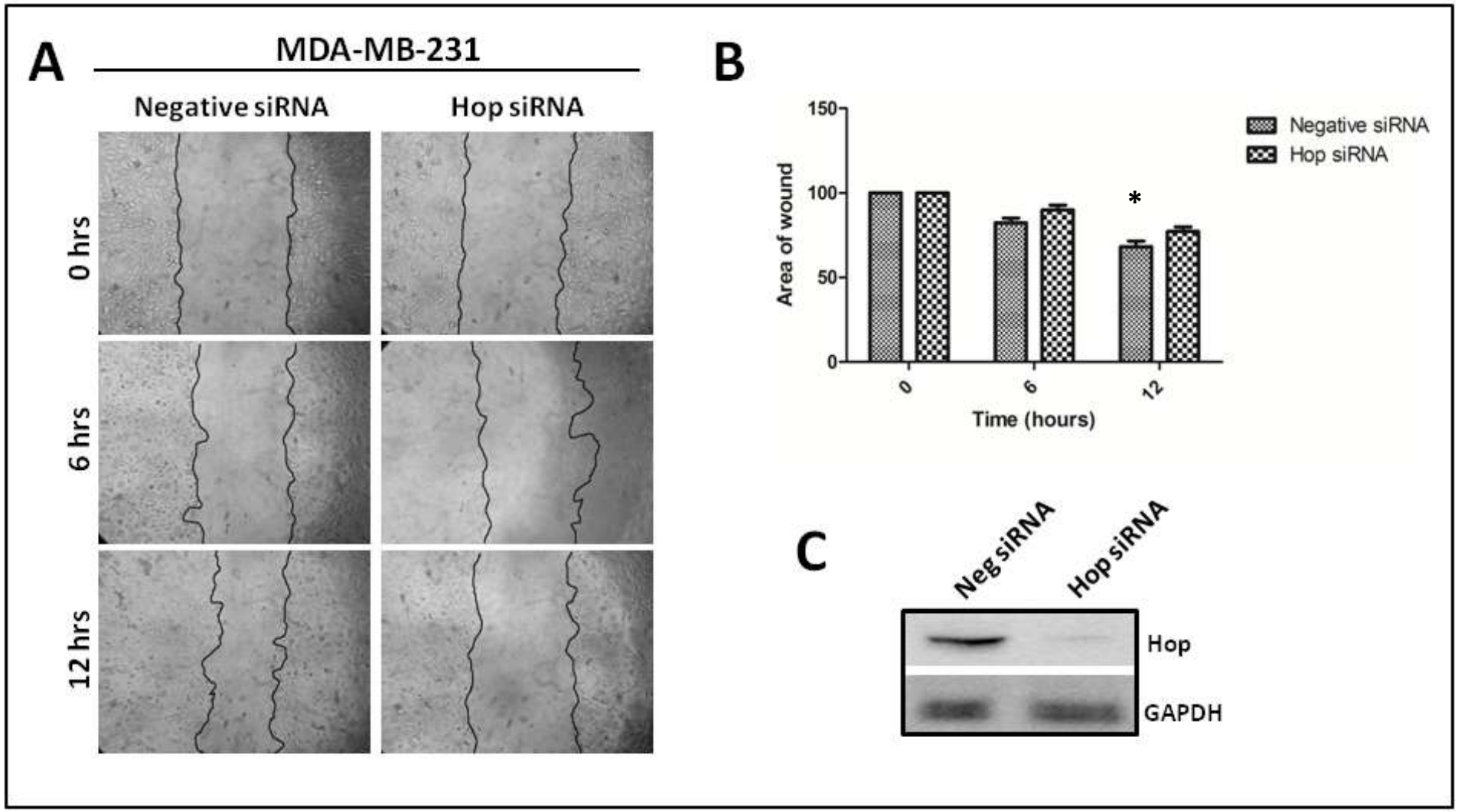


Figure 18: Analysis of the effect of Hop knockdown in migration of MDA-MB-231 cells in the wound healing assay

MDA-MB-231 cells were transfected with either non-targeting siRNA (A, left panel) or siRNA against Hop (A, right panel), and seeded on fibronectin coated 96-well plates 24 hours post transfection. Cells were allowed to adhere overnight and wounds were made by scraping the cell monolayer with a sterile toothpick. Images were taken at time 0 hrs and after 6 hrs and 12 hrs of incubation. Images shown in (A) are representative of three independent experiments consisting of nine replicates, while (B) shows the average change in wound size for all experiments. Statistical significance was analyzed by the paired t-test (*= $p < 0.05$). The levels of Hop in the transfected lysates was confirmed at the end of each experiment by Western analysis (C).

Results in Hs578T cells showed that knockdown of Hop led to a larger wound area after 6 and 12 hours of migration, compared to the area of the wound in the Hs578T cells treated with non-targeting siRNA. The reduction in cell migration was deemed to be significant after 12 hours (Figure 17A, right panel; Figure 17B) when compared to the migration of negative siRNA transfected control cells (Figure 17A, left panel; Figure 17B). In addition, the cells in which Hop was silenced migrated in a non-synchronized manner compared to non-targeting siRNA transfected cells. This led to an irregular wound border in the Hop knockdown cells that was observed after 12 hours (Figure 17A).

Similar to the results observed in Hs578T cells, knockdown of Hop in MDA-MB-231 cells showed a statistically significant decrease in migration after 12 hours (Figure 18A, right panel; Figure 18B) in comparison to cells transfected with non-targeting siRNA (Figure 18A, left panel). Taken together, these results suggested that Hop silencing led to a reduction in cell motility in Hs578T cells and to a lesser effect, MDA-MB-231 cells.

5.2.2 Identification of Hop in the pseudopodia of Hs578T cells

Hop knockdown caused a statistically significant decrease in cell motility in Hs578T cell line and therefore this cell line was selected for further analyses of the mechanism by which cell migration was reduced. Since directional cell migration is dependent on the formation of pseudopodial protrusions, we sought to investigate the role of Hop in pseudopodia formation.

The presence of Hop was assessed in purified pseudopodia fractions isolated from Hs578T cells that had been treated with the chemokine SDF-1. The expression of the SDF-1 receptor, CXCR4,

in Hs578T cells was confirmed by immunofluorescence confocal microscopy (*data not shown*) and is in accordance with previous reports of CXCR4 expression in Hs578T cells (Sauvé *et al.,* 2009). Cells were seeded on 3-µm transwell chambers and pseudopodia formation stimulated using an SDF-1 chemoattractant gradient, after which pseudopodia and cell body fractions were harvested. Proteins were resolved by SDS-PAGE and the presence of selected protein (including Hop) in the pseudopodia and cell body fractions determined by Western analysis (Figure 19).

The purity of the pseudopodia fractions was assessed by probing for proteins known to be either enriched in the pseudopodia (actin and vinculin) or absent from the pseudopodia (nuclear protein, histone 3). Whole cell lysates of untreated cells were used as a control for the Western analysis (Figure 19A). Western analysis of equal amounts of protein in all fractions revealed the presence of actin and vinculin but not histone 3 in the pseudopodial fraction. Actin was enriched in the pseudopodial fractions, compared to the cell body. Histone 3 was only detected in the cell body and whole cell lysate fractions. These data suggested that the purified pseudopodia fractions were largely uncontaminated with proteins from the cell body. Hop expression was detected in both the cell body and the pseudopodial fractions isolated from Hs578T. Hsp90, which is known to be in the cytoplasm, was detected in the cell body, with extremely low levels in the pseudopodial fraction. The presence of Hop in the pseudopodia was confirmed in three independent pseudopodial isolations (Figure 19B).

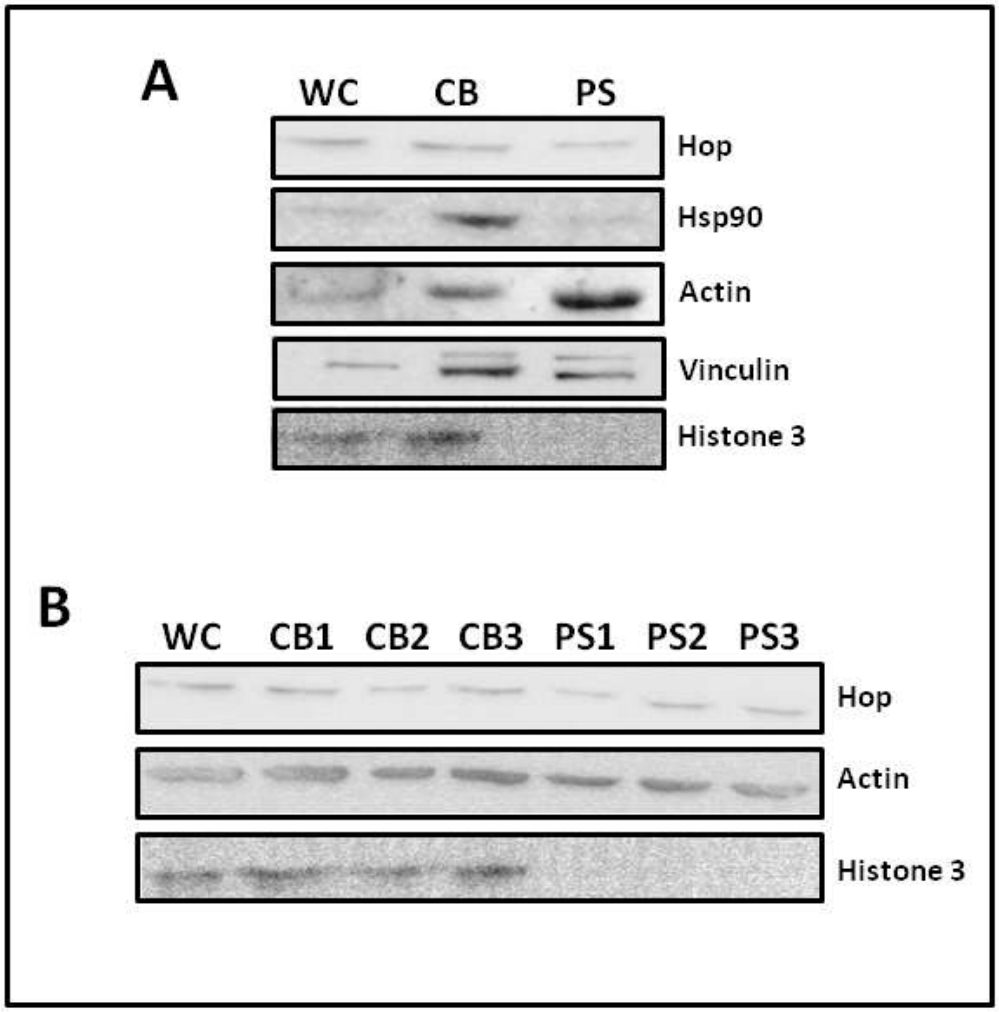


Figure 19: Identification of Hop in the pseudopodia isolated of Hs578T cells

Pseudopodia were isolated from serum starved cells that had been treated with 250 ng/ml SDF-1 for 90 minutes using the Chemicon pseudopodia isolation kit. Pseudopodia (PS) and Cell Body fractions (CB) were harvested and equal concentrations of protein (40 ug/ml) were resolved by SDS-PAGE and the presence of Hop, Hsp90, actin, vinculin and/or Histone 3 determined by Western analysis. (A) Confirmation of the isolation of pseudopodial fractions using pseudopodial markers. (B) Detection of the relative levels of Hop in replicate PS and CB fractions. Whole cell lysates of untreated cells were used as a control for the Western analysis (WC). Data are representative of three independent pseudopodial isolations.

5.2.3 Colocalisation analysis of Hop and actin in SDF-1 treated Hs578T cells

In order to assess the presence of Hop in pseudopodia within the cell, immunofluorescence confocal microscopy was performed in Hs578T cells. Cells were treated with SDF-1 and stained with antibodies against Hop and actin, followed by the relevant secondary antibodies. Figures 20 and 21 show a representative image of the staining patterns observed (Figure 20 and 22), where Hop staining is shown in green and actin staining in red.

The presence of Hop in the pseudopodia of Hs578T cells was confirmed using indirect immunofluorescence and confocal microscopy (Figure 20 and 22; green staining). Hop staining was observed throughout the cytoplasm of Hs578T cells as well as at distinct points at the end of long extensions of the cytoplasm that were indicative of pseudopodia (Figure 20). The presence of pseudopodia was confirmed by co-staining with actin (Figure 20; red staining) and by differential interference contrast (DIC) images (Figure 21). Hop staining was also observed in cytoplasmic extensions between cells that also showed positive staining for actin (Figure 22). The Hop staining appeared to colocalise with the actin at specific points in the extreme end of the pseudopodia and the intercellular junctions.

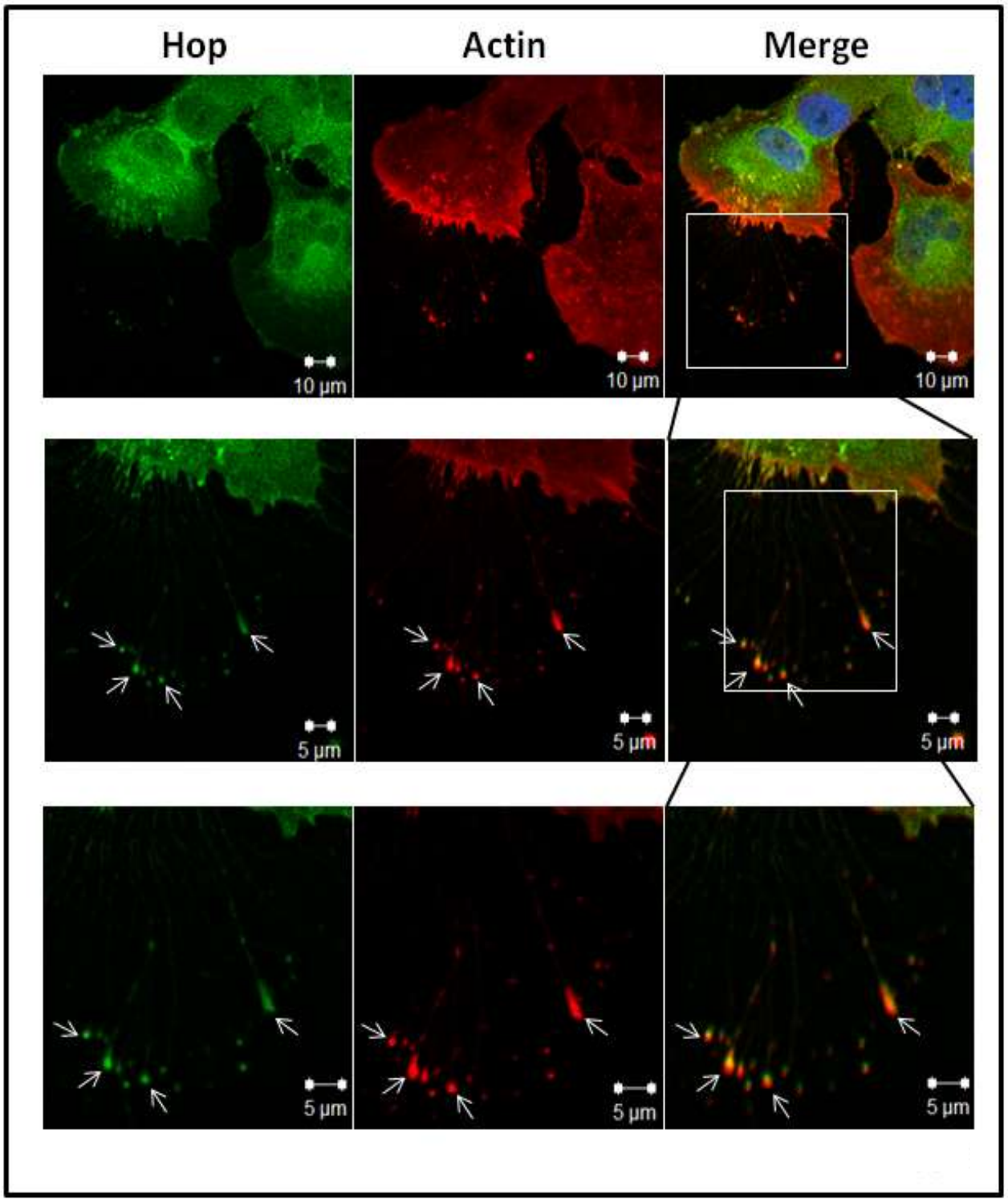


Figure 20: Localisation of Hop and actin in the pseudopodia of SDF-1 treated Hs578T cells.

Hs578T cells were seeded on fibronectin (1 µg/ml) and treated with 250 ng/ml SDF-1 for 5 hours at 37^{o}C. Cells were fixed and incubated with mouse anti-Hop and rabbit anti-actin antibody followed by donkey anti-mouse-488 (green) and donkey anti-rabbit 555 (red) secondary antibodies. The nucleus was stained with Hoescht-33342 (blue). Images were captured using the Zeiss LSM 510 Meta confocal microscope and analysed using AxiovisionLE 4.7.1 (Zeiss).

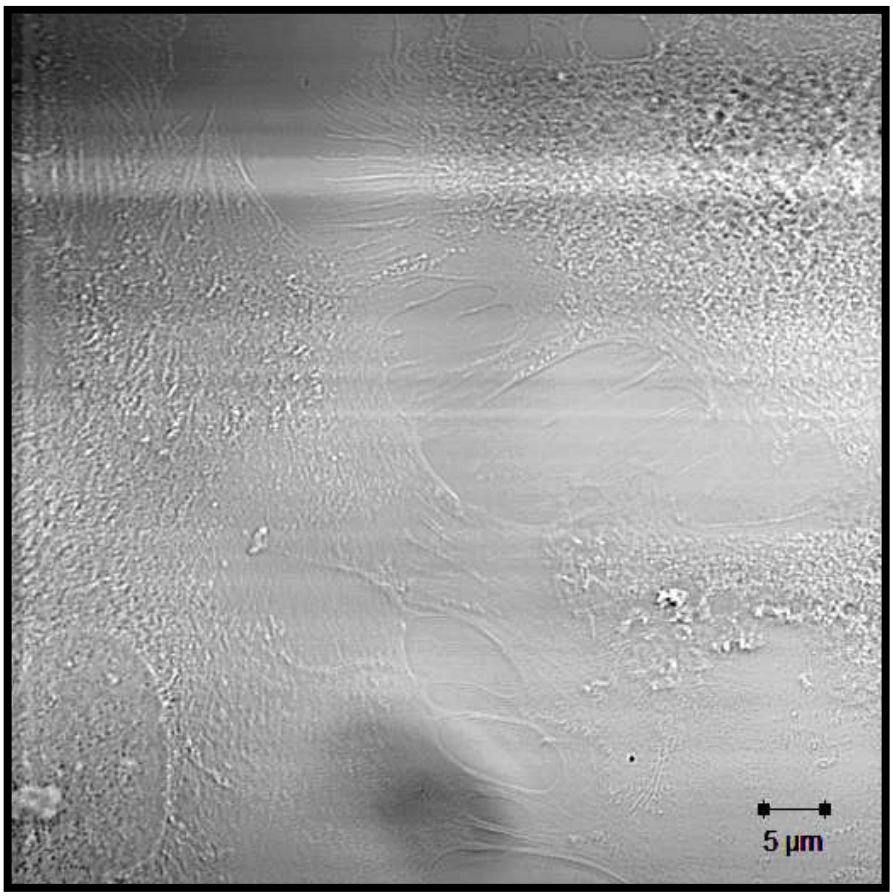


Figure 21: Differential Contrast (DIC) Image of Pseudopodial extensions in SDF-1 treated Hs578T cells

Hs578T cells were seeded on fibronectin (1 µg/mL) and treated with 250 ng/mL SDF-1 for 5 hours at 37^{o}C. Pseudopodia extensions are shown by white arrows. Images were captured using the Zeiss LSM 510 Meta confocal microscope and analysed using AxiovisionLE 4.7.1 (Zeiss).

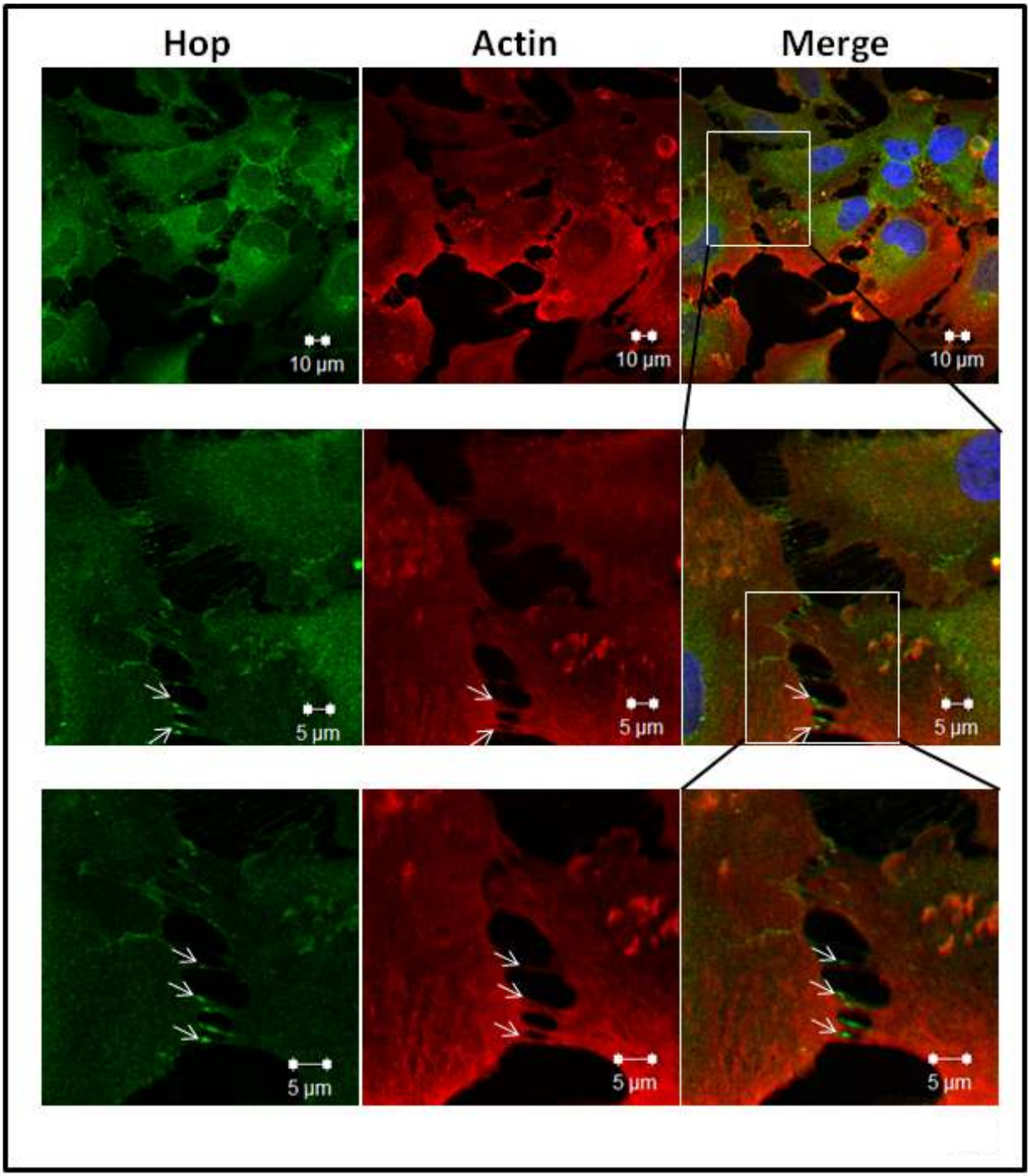
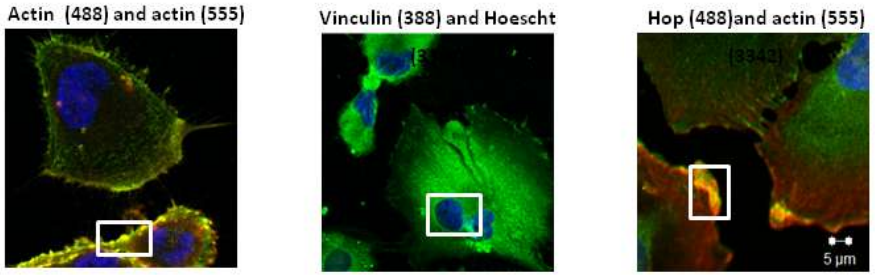


Figure 22: Localisation of Hop and actin in intercellular junctions in SDF-1 treated Hs578T cells

Hs578T cells were seeded on fibronectin (1 µg/ml) and treated with 250 ng/ml SDF-1 for 5 hours at 37°C. Cells were fixed and incubated with mouse anti-Hop and rabbit anti-actin antibody followed by donkey anti-mouse-488 (green) and donkey anti-rabbit 555 (red) secondary antibodies. The nucleus was stained with Hoescht-33342 (blue). Images were captured using the Zeiss LSM 510 Meta confocal microscope and analysed using AxiovisionLE 4.7.1 (Zeiss).

A



B

	Channel		
	Actin (488) and actin (555)	Vinculin (388) and Hoescht (3342)	Hop (488)and actin (555)
Pearson's Coefficient (Rr)	0.933	-0.45	0.859
Mander's Overlap (R)	0.97	0.589	0.92
Intensity Correlation Quotient	0.408	-0.093	0.241

C

n	Pearson's Coefficient (Rr)	Mander's Overlap (R)	Intensity Correlation Quotient
1	0.762	0.852	0.4
2	0.79	0.818	0.403
3	0.788	0.895	0.253

Figure 23: Co-localisation analysis of Hop and actin immunofluorescence using ImageJ

The Pearson's correlation coefficient (Rr) and Mander's Overlap coefficient (R) and Intensity Correlation Quotient (ICQ) were computed using ImageJ co-localisation analysis software. Coefficients were calculated for the positive control that included the same actin primary antibody detected with two different secondary antibodies linked to different fluorophores (Alexa Fluor 488; green and Alexa fluor 555; red); the negative control that included colocalisation analysis of the vinculin (Alexa Fluor 488; green) and the nuclear staining (Hoechst 3342); and the test sample in which the colocalisation of Hop (Alexa Fluor 488; green) and actin (Alexa fluor 555; red) were included. (A), and summarized in (B). White blocks indicate the region of interest (ROI) for which the coefficients were calculated. Hop and actin colocalisation coefficients were quantified at least three times in independent experiments (C).

The potential colocalisation between Hop and actin in the pseudopodia of Hs578T were analysed using the quantitative colocalisation software on ImageJ to determine the Pearson's coefficient (Rr) and Mander's overlap coeffiecent (R). Pearson's correlation coefficient defines the correlation between two channels as a value between -1 and 1, where a value of 1 represents perfect co-localisation whereas a value of -1 represents perfect exclusion and zero represents random localisation (Adler and Parmryd, 2010). If the ratio between the two channels (i.e. Ch1:Ch2 ratio) is equal to 1, colocalisation can be quantified using Mander's overlap coefficient. For the Mander's Overlap coefficient, values range between 0 and 1, where a value of 1 represents high co-localisation and 0 represents low co-localisation (Adler and Parmryd, 2010). Additional analysis of the synchrony between the staining intensities for the channels for each co-localisation analysis was performed using the Intensity Correlation Quotient (ICQ) computed in Image J. ICQ values range between -0.5 and 0.5, where a value of 0 indicated random staining, values between -0.5 and 0 indicated segregated staining and values between 0 and 0.5 indicated dependent staining. The results of the quantitative co-localisation analysis are shown in Figure 23.

For colocalisation analysis, a positive control was included where cells were treated with SDF-1 and stained with an anti-rabbit antibody against actin followed by detection with both donkey anti-rabbit-488 (green) and donkey anti-rabbit 555 (red) secondary antibodies. A negative control where the colocalisation between two signals that did not overlap, namely vinculin (in the cytoplasm) and the cell nucleus stained with Hoechst 3342 was quantified (Figure 23A). The positive control showed strong overlap between the two secondary antibodies specific for actin, where Pearson's and Mander's Overlap coefficients were close to values of 1 (0.933 and 0.97

respectively) and the ICQ value was 0.408 which suggested dependent staining between the two signals (Figure 23 A and B). Quantification of the overlap between Hoechst and vinculin staining showed poor co-localisation, where Pearson's and Mander's Overlap coefficients were not close to 1 (-0.45 and 0.589 respectively) and the ICQ value of -0.093 indicated independent staining (Figure 23 A and B). Results for Hop and actin staining showed that Pearson's and Mander's Overlap coefficients were close to the value of 1 (0.859 and 0.92 respectively) and the ICQ value was 0.241 was in the range of 0 to 0.5 that suggested dependent staining (Figure 23A and B). All colocalisation quantifications for Hop and actin were performed for at least three independent experiments (Figure 23C). Results showed that for all Hop and actin colocalisation tests, the ICQ values were between the values of 0 and 0.5, indicating that each signal represented dependent staining. In all cases, the Hop and actin colocalisation results were more similar to the results obtained with the positive control than the negative control. This suggested that there was co-localisation between Hop and actin.

5.2.4 Analysis of the interaction between Hop and actin *in vitro*.

Having shown that Hop colocalised with actin to pseudopodial protrusions in Hs578T cells, we tested whether Hop protein could bind to actin filaments *in vitro* using cosedimentation assays. Hop protein was incubated with F-actin under a medium salt conditions (50 mM KCl), and subjected to high-speed ultracentrifugation to separate the polymerised actin filaments from soluble globular actin. Controls included incubation of actin alone, Hop alone, BSA (as a non-specific protein) alone, and actin with BSA. Following centrifugation, supernatants containing soluble, unbound protein as well as pellet fractions containing polymerized actin filaments with bound protein were resolved by SDS PAGE and Western analysis (Figure 24).

Figure 24A shows the control samples in which the cosedimentation assay was conducted with actin alone and actin incubated with a non-specific protein, BSA. After centrifugation, actin was detected in both the supernatant and pellet fractions. This suggested that the *in vitro* polymerization of actin was successful. BSA when incubated alone was detected mainly in the soluble fraction, with low to undetectable levels in the pellet fraction (Figure 24A). Importantly, there was no change in the proportions of BSA present within the supernatant or pellet fractions when actin and BSA were incubated together, compared to when actin and BSA were incubated alone. This suggested that there was no binding between actin and BSA.

The cosedimentation assay for Hop and actin was assessed by incubation of actin with Hop at two different concentrations, namely 0.05 mg/ml and 0.1 mg/ml (Figure 24B). Following centrifugation and Western analysis, it was observed that although Hop and actin were present in both supernatant and pellet fractions when incubated alone, the majority of actin was present within the pellet fraction, while the Hop was present in both the supernatant and pellet fractions (Figure 24B). However, when actin and Hop were incubated together, a significant shift of Hop from the supernatant fraction to the pellet fraction was observed. This suggested that Hop was able to bind to actin filaments *in vitro*.

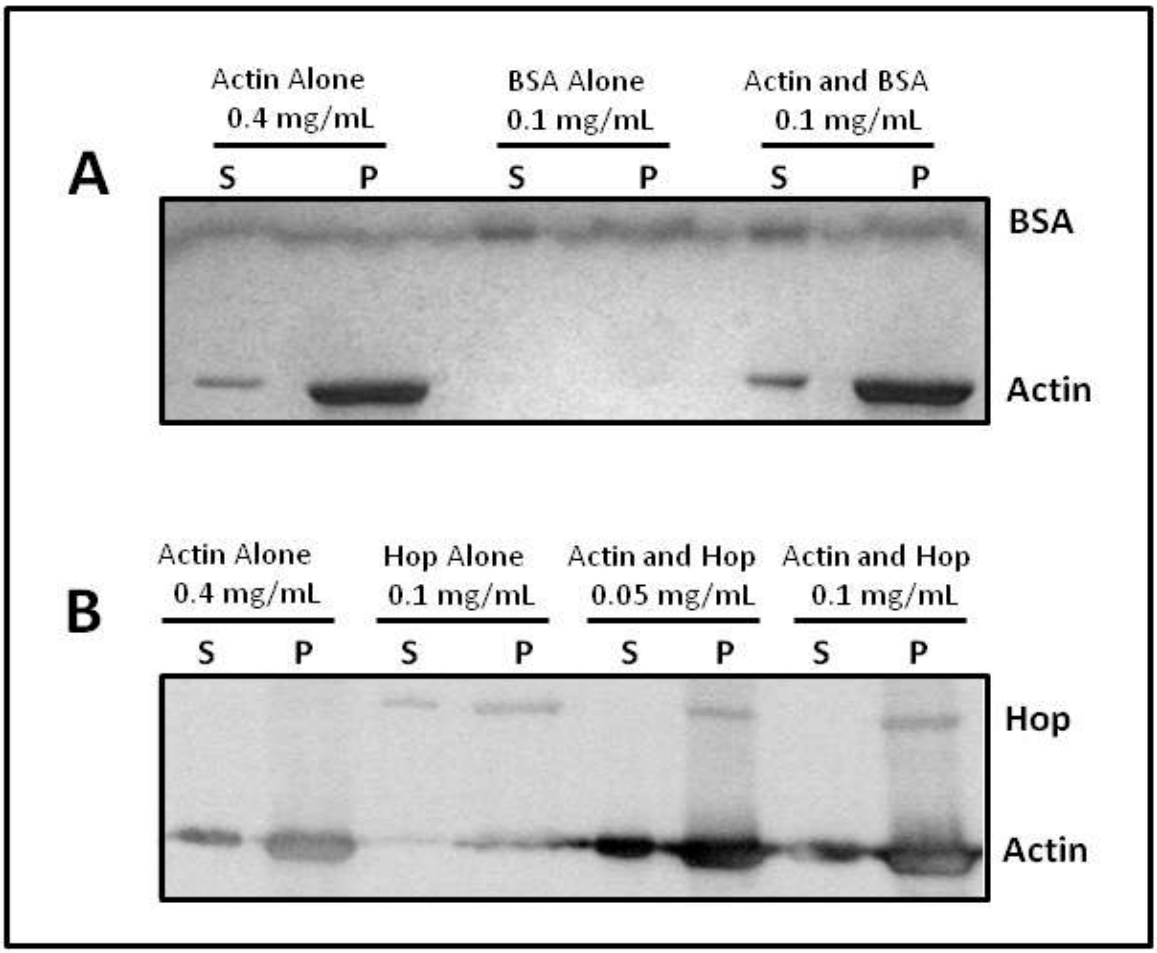


Figure 24: Analysis of the interaction between Hop and actin by in vitro cosedimentation assay

The ability of a Hop to bind F-actin was examined *in vitro* by an actin cosedimentation assay. BSA was used as a control reaction, which was incubated in the presence or absence of F-actin, followed by centrifugation at 100,000*g*, and the pellet (P) and supernatant (S) fractions resolved by SDS-PAGE and silver staining (A). Similarly, Hop protein was incubated in the presence of F-actin, as well as in the absence of F-actin, and samples centrifuged at 100,000*g* after which supernatant and pellet fractions were resolved by SDS-PAGE and Western blot analysis (B).

5.2.5 Analysis of Hop knockdown on pseudopodia formation

Having shown that Hop colocalised with actin in pseudopodia and could bind to actin *in vitro*, we investigated whether Hop knockdown would reduce the formation of pseudopodia in Hs578T cells. The siRNA mediated knockdown of Hop was performed in Hs578T cells, after which cells were seeded onto 3-µm transwell filters and pseudopodia formation stimulated using an SDF-1 chemoattractant gradient. The transwell filters were removed from the inserts and stained for actin and nuclei prior to fixation onto glass coverslips for confocal microscopy. A series of Z-stack images across the membrane were obtained and the three dimensional reconstruction of the pseudopodia and cell body across the membrane insert were generated (Figure 25).

The density of actin and Hoechst (nuclear) staining in cells treated with negative siRNA or siRNA targeting Hop at the upper and lower membrane surfaces were compared (Figure 25A and B respectively). Similarly, single Z-slice images focusing on the cell bodies stained for nuclei at the top and the actin rich protrusions at bottom sides of the filter were taken. In each case the actin and Hoechst signals were collected at the equivalent depth above or below the filter, and the actin staining in cells in which Hop had been silenced was quantified and compared to actin staining in negative control cells (Figure 25C and D). At the end of each experiment, knockdown of Hop was confirmed by Western blot analysis (Figure 25E). The images shown in Figure 25 are representative of three independent experiments with images captured in triplicate.

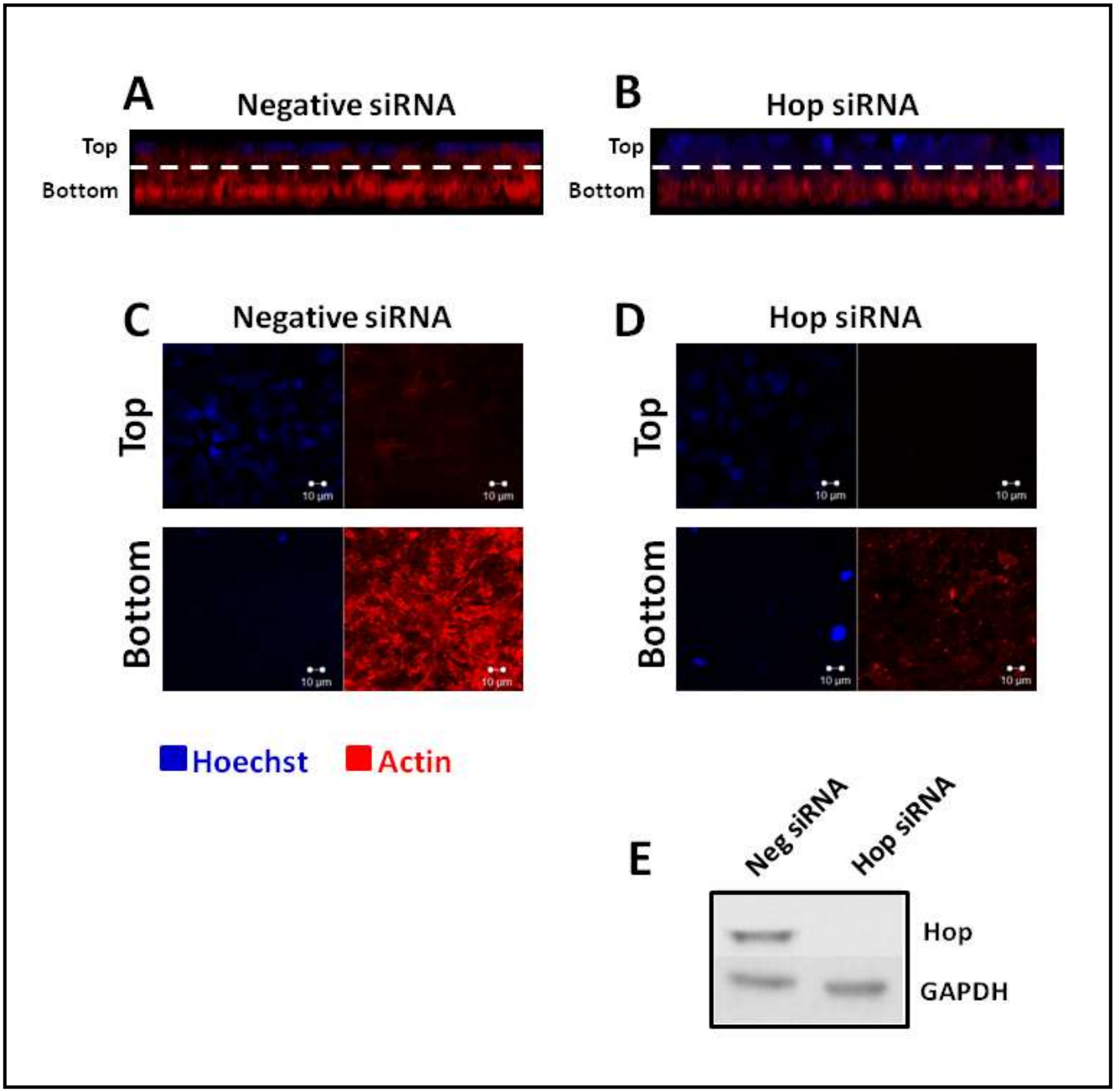


Figure 25: Hop knockdown reduced pseudopodia formation in Hs578T cells

Hs578T cells were transfected with either negative siRNA (A) or siRNA targeting Hop (B) and plated onto 3 µm pore filters. Pseudopodia formation was stimulated with 250 ng/mL SDF-1 (90 minutes at 37^{o}C) after which filters were fixed with ice cold methanol and actin labelled with rabbit anti-actin antibody followed by donkey anti-mouse-555 (red), and nuclei labelled with Hoescht 33342 (blue). Images were captured using the Zeiss LSM 510 Meta confocal microscope and analysed using AxiovisionLE 4.7.1 (Zeiss). (A) and (B) show three dimensional reconstructions of Z stack images across the membrane (which is represented on the image by the dashed white line). The size of the Z stack is approximately 16 µm in both images.(C) and (D) show single Z slices taken from the top (showing the cell body stained with Hoechst) and bottom (pseudopodia stained with actin) of the filters. Knockdown of Hop was confirmed after each experiment by Western blot analysis (E). Data are representative of three independent pseudopodial quantifications.

The resulting Z-stack images showed a noticeable decrease in actin staining at the bottom of transwell filter slides in cells in which Hop had been silenced, as compared to cells transfected with non-targeting siRNA (Figure 25A and B). Similarly, the single Z-slice images focused on the bottom of each filter showed that cells in which Hop had been silenced had considerably lower quantity of actin filaments, compared to cells treated with non-targeting siRNA (Figure 25C and D). Collectively these results suggested that knockdown of Hop led to a reduction in the number and size of pseudopodia formed.

5.3 Summary and Conclusions

The results of this chapter suggested a role for Hop in cell migration. Here we show that siRNA knockdown of Hop in both Hs578T and MDA-231 cancer cells led to a reduction in cell motility as measured by the wound healing assay. The concentration of Hop along the cell membrane at intercellular junctions, sites of adhesion, as well as at the leading edge of migrating cells led to the hypothesis that Hop may mediate migration through interaction with actin during the reorganization of the actin cytoskeleton, and serve to modulate the dynamics of pseudopodia formation. We showed by immunofluorescence microscopy that Hop colocalised with actin in pseudopodial extensions and migrating cells, and that Hop directly binds to actin *in vitro.* Furthermore, we provided evidence of the presence of Hop in purified pseudopodia of migrating cancer cells, and that knockdown of Hop dramatically reduced the capacity of Hs578T cells to form pseudopodia. To our knowledge, this is the first report implicating Hop as a direct actin binding protein, as well as the potential involvement of Hop in pseudopodial formation.

Chapter 6 :Discussion and conclusion

6.1 Introduction

The Hsp90/Hsp70 organising protein (Hop) is an important co-chaperone of the Hsp90 complex, and serves as an adapter protein, coordinating the interactions of Hsp90 and Hsp70 (Csermely *et al*., 1998). Hop influences the functional activities of Hsp90 and Hsp70, which in turn is required for the regulation of protein folding pathways (Daniel *et al.*, 2007). The Hsp90 complex is required for the maintenance of many signaling molecules involved in oncogenesis (Csermely *et al*., 1998; Sıti *et al*., 2005). Where mutation, overexpression or over-activation would normally render these signaling molecules prone to aggregation, the Hsp90 complex, consisting of chaperones, together with an assortment of co-chaperones and co-factors, serves to maintain the unstable states of these proteins in an active form that permits the development of cancer. For this reason, an extensive range of functional studies have been carried out on the putative roles of these components in tumourigenesis, as inhibition would lead to combined inhibition of multiple signal pathways (Neckers, 2002; Banerji, 2009).

The broad objective of this study was to investigate the role of the Hsp70-Hsp90 organising protein (Hop) co-chaperone in cancer. We aimed to characterise expression and localisation of Hop in different cancers, as well as explore the effects of Hop knockdown on expression of chaperones, Hsp90 client proteins and co-chaperones, and ultimately cell biological effects such as cancer proliferation and migration.

6.2 Hop as a co-chaperone in cancer

6.2.1 Hop in overexpressed in certain cancer cell lines

The expression of Hop was examined in a panel of seven human cell lines. The cell lines chosen represented a wide spectrum of cell types of different origin and biological status, ranging from those that were non-tumourigenic to those with varying degrees of invasive and metastatic potential (Sommers *et al.,* 1990; Zeillinger *et al.,* 1996; Charafe-Jauffret *et al.,* 2006; Chatterjee *et al.,* 2006; Gelmann *et al.,* 2006; Moser *et al*., 2007; Hattar *et al.,* 2009; Subik *et al.,* 2010; Arteaga *et al.,* 2011). Hop was detected by Western analysis as a single band at the expected molecular weight of approximately 66 kDa, although minor differences in the mobility of the Hop protein was observed between the different cell lines. Various acidic isoforms of Hop have been identified, and it was proposed that these isoforms were directly related to post-translational phosphorylation (Daniel et al., 2008). Therefore, the phosphorylation status of Hop may account for the varying mobilities of the Hop protein observed across different cell lines, but the factors influencing potential phosphorylation status in relation to cell context remains to be elucidated. Our results showed that the expression of Hop correlated with invasive status, where it was observed that Hop expression noticeably increased in an order from non-invasive (MCF-12A) cells to cells of higher invasive potential (MCF-7 and MDA-MB-231) in the panel of breast cancer cells examined. Previously, Walsh and colleagues (2011) showed that a defined correlation existed between increased Hop expression and invasive potential in a panel of seven human pancreatic cancers, which is consistent with our data in breast cancer cells (Walsh *et al.,* 2011). We next examined whether Hop expression was related to cancer stem cell characteristics, where we compared the breast cancer cell line, MCF-7, with the MCF-7 cells grown under mammosphere conditions that enrich for the presence of stem-like cancer cells

(Mani *et al.,* 2008). Results showed a noticeably higher level of Hop expression in the MCF-7 cell line when compared to mammosphere cells. Although no reports of chaperone expression in mammosphere development have been established, these findings suggest that Hop may not be required, or is required to a lesser extent, for the maintenance of stem like characteristics in MCF-7 cells. A paired primary and metastatic cell line, derived from different stages of colon carcinoma in the same patient (Futschik *et al.,* 2002) was used in this study to illuminate any correlation between Hop expression and metastatic characteristics. No noticeable difference in Hop expression levels were observed, indicating that Hop may not necessarily contribute to the metastatic property of this specific cell line. Our results showed a strong correlation between Hop expression and tumourigenic status in the paired Hs578T/Hs578Bst cell line, which is in accordance with work by Kubota and colleagues (2010), who showed that the levels of Hop were frequently increased in colonic carcinoma tissue samples compared to non-tumour tissues obtained from the same colonic carcinoma patients (Kubota *et al*., 2010). Previous experiments showed an increase in Hop mRNA and protein expression upon transformation of Rat1 fibroblast cells with the Ras oncogene (Van der Spuy, PhD thesis 2000). It is interesting to note that the tumourigenic Hs578T cell line, in which Hop protein levels were elevated, has been shown to express activated forms of the Ras oncogene when compared to the normal equivalent, Hs578Bst (Eckert *et al*., 2004). Collectively, these results suggest that Hop expression levels may be indicative of invasive and tumourigenic status in breast cancer cells, although, cell context may be important in determining these effects.

6.2.2 The subcellular localization of Hop does not necessarily correlate with tumourigenicity

We did not detect any obvious relationship between the subcellular localization of Hop and malignant status in this study. Immunofluorescence confocal microscopy revealed that the predominant localisation of Hop in the cell lines examined was within the cytoplasm, which is in accordance with work proposed by Lässle and colleagues in NIH3T3 cells (Lässle *et al*., 1997). A small proportion of Hop was identified in the nucleus, this occurrence being more prevalent in some cells than others. For example, in the paired Hs578T and Hs578Bst cell line, it was observed that Hop staining in the Hs578Bst cell line was predominantly within and around the nucleus as opposed to a predominant cytoplasmic location with minimal nuclear Hop staining in the tumourigenic counterpart, Hs578T. The presence of Hop in the nucleus has been described by Longshaw and colleagues (2004) in murine fibroblast cells, where the ability of murine Hop to shuttle between the nucleus and the cytosol after phosphorylation by casein kinase II and cell division cycle-2 (cdc2) kinase at sites upstream of a putative nuclear localisation signal (Longshaw *et al.,* 2000, Longshaw *et al.,* 2004). It has been postulated that the phosphorylation status of Hop might serve as regulatory mechanism mediating its location within the cell (Longshaw *et al*., 2004; Daniel *et al*., 2008). As previously mentioned, the Hs578T cell line exhibits elevated levels of Ras, a trait that is not shared with the normal cell counterpart, Hs578Bst. Interestingly, work by Gomez showed that mSTI1 staining in rat fibroblast cells shifted from nuclear to cytoplasmic upon transformation with the Ras oncogene (Gomez, Honours thesis 2002).

Six out of the seven cell lines showed Hop staining at or near the cellular membrane. Prominent Hop staining was observed on the periphery of cells, as well as at distinct points of adhesion

along leading edges of polarized cells. Furthermore, Hop staining was detected on what appeared to be membrane ruffles on the periphery of some cells and was also present within intercellular junctions between adjacent MCF-7 cells. The association of Hop with the cell membrane has been reported in several other studies, where Hop was identified by Zanata and colleagues (2002) as a prion ligand (PrP^C) binding protein localised at the cell surface. In addition, membrane staining of Hop was detected in invasive human pancreatic tumours (Walsh *et al*., 2011). Interestingly, despite our findings and other published reports of Hop association with the membrane, no transmembrane or signal peptide is contained within the Hop sequence (Lässle *et al*., 1997), and whether association with the membrane is through non-classical routes or through interactions with other proteins such as prions remains to be elucidated (Zanata *et al*., 2002, Walsh *et al*., 2011).

6.2.3 Hop knockdown does not affect Hsp90 expression or induce transcriptional upregulation of Hsp70

Hop plays a central role in the delivery of client proteins from Hsp70 to Hsp90 and can therefore be seen as a regulator of Hsp90 and Hsp70 activity (Hernandez *et al.,* 2002; Goetz *et al.,* 2003; Carrigan *et al.,* 2006). Since the Hsp90-Hop-Hsp70 complex is important for the maintenance of client proteins, many of which are important oncoproteins, we investigated the effects of siRNA mediated knockdown of Hop on Hsp90 and Hsp70 expression, as well as the expression of Hsp90 and Hsp70 client proteins. The Hs578T and MDA-MB-231 cell lines were chosen as cell models for the development of an RNAi-based system to knockdown Hop as both are aggressive, triple negative cancers (Arteaga *et al.,* 2011). The Hs578T cell line in particular represented a promising candidate for knockdown studies, as it was previously shown that Hop was highly upregulated in these cells in comparison to the non-tumourigenic counterpart,

Hs578Bst. In addition, studies have shown that Hsp90 in both MDA-MB-231 (Horibe *et al*., 2011) and Hs578T cells (Kamal *et al*., 2003) was present almost entirely in complex with Hop. This was compared to normal cells, where Hsp90 remained in an uncomplexed, latent state (Kamal *et al.,* 2003).

In this study, we were able to successfully develop a system for transient knockdown of Hop for up to 144 hours in the breast cancer cell lines chosen. This time frame was sufficient to allow the evaluation of the biological effects of Hop knockdown, including cell proliferation and migration. Hop protein expression was knocked down in Hs578T and MDA-MB-231 cells over a time course of 144 hours, and whole cell lysates analysed for the levels of the major chaperones with which Hop interacts, namely Hsp90 and Hsp70, as well as other co-chaperones known to mediate Hsp90 interaction with client proteins. Results revealed that Hop silencing had no noticeable effect on the levels of Hsp90 or Hsp70 in both Hs578T and MDA-MB-231 cell lines. In a recent study, it was shown that treatment of MDA-MB-231 cells with a drug that prevented the interaction of Hop and Hsp90, led to a decrease in Hsp70 mRNA and protein levels (Pimienta *et al.*, 2011). The fact that Hop silencing or disruption of the Hsp90 and Hop interaction did not upregulate Hsp70 levels shows a promising advantage over conventional N-terminal Hsp90 ATPase inhibitors such as geldanamycin, which have been shown to induce a compensatory upregulation of Hsp70 by heat shock factor-1 (HSF1). This induction of Hsp70 expression is thought to contribute, at least in part, to the development of resistance to N-terminal Hsp90 inhibitors (Song *et al*., 2008).

6.2.4 Hop knockdown affects expression and phosphorylation of certain Hsp90 clients

The effect of Hop downregulation on selected Hsp90 client proteins (signaling intermediates Akt, Erk and Stat3) was investigated by Western analysis over a time course of 144 hours. Silencing of Hop dramatically reduced the levels of Akt in both Hs578T and MDA-231 cells. These results are in accordance with other studies showing that the disruption of Hop association with Hsp90 in T47D breast cancer cells (Horibe *et al*., 2011), or downregulation of Hop in colon cancer cells (Kubota *et al*., 2011), led to a loss of multiple cancer associated Hsp90 clients, including protein kinases such as Akt, Bcr-Abl, v-Src and Cdk4. In contrast to these findings however, studies by Pimienta and colleagues (2011) showed that Akt was not affected upon the disruption of Hop interaction with Hsp90 in MDA-MB-231 cells (Pimienta *et al.*, 2011). Although the response of Akt to Hsp90 inhibition is known to be dependent on cell context (Theodoraki *et al*., 2007), our findings and the contradictory reports by Pimienta and colleagues were observed in the same cell line. This suggested that siRNA mediated silencing of Hop may follow a different mechanism to drug mediated disruption of Hop interaction with Hsp90 in cancer. In light of this, it is interesting that Hop silencing was able to downregulate a kinase such as Akt that is thought to preferentially associate with the Hsp90 co-chaperone, Cdc37, for stabilization (Gray *et al*., 2007) and despite the fact that Cdc37 levels were shown to increase upon Hop knockdown.

In addition to the reduction in Akt levels, Hop silencing also induced a marked reduction in the ratio of pStat3 to total Stat3 levels in both cell lines. This data is consistent with work previously described by Longshaw and colleagues (2009) in murine embryonic stem cells where Hop knockdown led to a reduction in pStat3 (Longshaw *et al*., 2009). No consistent trend was

observed in the expression of Erk levels in both Hs578T and MDA-MB-231 cell lines, as the levels fluctuated over the 144 hour time course, and this is consistent with work by Pimienta and colleagues (2011). Although Pimienta and colleagues did not evaluate the levels of phosphorylated Erk in their study, we observed an increase in the proportion of pErk to Erk levels in both cell lines. These observations were unexpected may be explained by the possibility that depletion of Hop within the cell caused compensatory interactions between signaling pathways. In particular, the loss of Akt may have induced activation of the Erk pathway, as links between these two pathways in breast cancer cells has been described (Moelling *et al*., 2002).

6.2.5 Hop knockdown alters the expression of other Hsp90 co-chaperones

Hsp90 function is mediated not only by Hop, but by an assortment of other co-chaperones that are involved in client stabilization and activation. The composition of the Hsp90 complex is limited by sterical overlap and competition for binding sites, and in addition, also selected by the client protein involved (Harst *et al*., 2005). In order to assess whether the downregulation of Hop had any effect on the expression of other Hsp90 co-chaperones, Western analysis was performed to detect protein expression levels of Aha1, Cdc37 and p23 after Hop knockdown in Hs578T and MDA-MB-231 cells. Results showed a time dependent increase in Aha1 in both cell lines, while a decrease in p23 levels was observed. In addition, Cdc37 protein levels were upregulated in both Hs578T and MDA-MB-231 cells, a finding that is in contrast to that of Pimienta and colleagues (2011), who showed that disruption of Hop interaction with Hsp90 led to a decrease in Cdc37 levels (Pimienta *et al.,* 2011). In this case, it is interesting to note that we observed a reduction in Akt levels after Hop silencing, even though levels of Cdc37 were

increased, while Pimienta and colleagues observed no changes in Akt levels despite a reduction in Cdc37. These findings challenge the dogma that client proteins interact with only one type of co-chaperone. Our data, along with the findings of Pimienta et al (2011), suggest that Cdc37 may not be the only co-chaperone for the client protein Akt and that other co-chaperones such as Hop may be involved in the chaperoning of Akt. Although the effects of Hop knockdown on Hsp90 client stability have been studied in both pancreatic and colonic cancer (Kubota *et al*., 2010; Walsh *et al*., 2011), this is the first study in which the changes in the levels of other Hsp90 co-chaperones when Hop is silenced have been taken into consideration.

The involvement of co-chaperones in cancer has been reported in numerous studies (Oxelmark *et al.,* 2006; Smith *et al.,* 2009; Holmes *et al.,* 2008). Work by Smith and colleagues (2009) showed that downregulation of Cdc37 caused a significant decrease in the expression of protein kinases such as Cdk4, Akt, c-Raf and Brbb2, but did not alter the expression of Hop, Aha1 or p23 (Smith *et al.*, 2009) while studies carried out by Holmes and colleagues (2008) showed that siRNA mediated knockdown of Aha1 in human colon cancer had no effect on the levels of the Hsp90 kinase clients, C-Raf, Cdk4, Erbb2, Mek1/2, Erk1/2 or Akt, but resulted in a decrease in phosphorylation of C-Raf, Mek1/2, Erk1/2 and Akt. These studies suggested that Aha1 was responsible for the activation status of Hsp90 client kinases, while Cdc37 was required for stability. Downregulation of Hsp90 clients was still observed upon Hop silencing, despite the fact that upregulation of Aha1 and Cdc37 occurred, which suggested that Aha1 and Cdc37 were not able to compensate for the reduction in Hop levels. In contrast to the findings on Cdc37 and Aha1 expression, a significant depletion of p23 was observed after Hop silencing in both Hs578T and MDA-MB-231 cells. Although knockdown studies on the effects of p23 on Hsp90 clients in

cancer have not been published, it is hypothesized that effects would mimic those observed upon knockdown of Aha1, since both are considered late cofactors that are thought to regulate activation of Hsp90 clients rather than stabilization (Harst *et al*., 2005). Although Hop silencing induced depletion of phosphorylated Hsp90 clients such as Stat3, it is difficult to ascertain whether the effects on activation of these clients were due to downregulation of Hop, p23, or both.

Collectively, these findings show that the study of co-chaperones in oncogenesis may require a collective view of these components as a whole, instead of separate entities within the Hsp90 complex. As a result, additional studies on the effects of knockdown of individual as well as a combination of co-chaperones on the Hsp90 complex are required. It is speculated that there is redundancy between the different co-chaperones, whereby the knockdown of one isoform could lead to the binding of an alternative co-chaperone to the Hsp90 complex. We suggest that knockdown experiments on individual co-chaperones followed by immunoprecipitation of the Hsp90 complex from Hop knockdown cells and detection for the presence of other co-chaperones in that complex could be performed. The specific associations of other Hsp90 co-chaperones with the complex may reveal additional insight into the competitive nature that is thought to occur between these co-chaperones for Hsp90 binding.

6.3 The role of Hop in cell proliferation and chemosensitivity

6.3.1 The effects of Hop knockdown on cell proliferation are cell line specific

We previously reported that Hop silencing resulted in a downregulation of Akt, as well as phosphorylated and total levels of Stat3 Hs578T and MDA-MB-231 cells. Since these proteins are signaling intermediates in pathways that control cell growth (Moelling *et al*., 2002), it was hypothesized that Hop silencing affects cell proliferation. Results showed that Hop silencing in Hs578T cells induced a significant anti-proliferative effect after 72 hours. These findings are supported by reports in glioblastoma cells, where treatment of cells with recombinant Hop caused a significant increase in cell proliferation, mediated via the Erk and Akt signaling pathways (Erlich *et al*., 2007). Furthermore, it has been shown that treatment of human lung and kidney cells with an anti-TPR peptide disrupting the Hsp90–Hop interaction caused a marked decrease in cell proliferation, as well as a depletion of Akt levels after 48 hours (Horibe *et al.,* 2011). In contrast to the results observed in Hs578T cells, studies on the effect of Hop silencing in MDA-MB-231 cells showed a significant increase in cell proliferation. This was unexpected, since earlier studies on Hsp90 client expression in both cell lines showed a clear reduction in both total Akt and Erk levels after 72 hours of Hop silencing. This observation suggested that the effect of Hop silencing in the two cell lines was associated with differential signaling pathways in mediating cell proliferation. As the role of Hop in cancer cell proliferation has not been widely studied, to date there is no established mechanism to explain any dependency on cell context and therefore this warrants further study.

6.3.2 Hop knockdown led to differential affects on sensitivity of cells to anti-cancer drugs

We characterized the effect of Hop silencing on the sensitivity of breast cancer cells to anti-cancer drugs, including inhibitors of both the N-terminus (geldanamycin) and C-terminus (novobiocin) of Hsp90 (Powers *et al*., 2009). A dose dependent study was performed where Hs578T and MDA-MB-231 cells were subjected to increasing doses of paclitaxel, geldanamycin and novobiocin for a period of 72 hours. There was no significant relationship between Hop silencing and cancer cell sensitivity to each of the three drugs tested as determined by the corresponding IC_{50} values. Subsequently, a kinetic study was performed, whereby cells were exposed to a fixed drug concentration, and viability assessed every 24 hours over a 72 hour period. There was a statistically significant increase in the sensitivity of Hs578T cells to geldanamycin in which Hop was silenced, compared to untreated and mock treated cells. There was no change in the chemosensitivity of MDA-MB-231 cells in which Hop was knocked down compared to the control transfections for any of the drugs.

Earlier reports by Kamal and colleagues (2003) showed that the apparent affinity of Hsp90 for 17-AAG was increased dramatically from an IC_{50} value of 600 nM for native Hsp90 alone, to 12 nM when Hsp90 was in complex with Hop, Hsp70 and p23 (Kamal *et al*., 2003). Therefore, it might be expected that knockdown of Hop would lead to a loss of chemosensitivity in cancer cells by disrupting the Hsp90 complex. Interestingly, studies have shown that treatment of cells with geldanamycin affects the composition of the Hsp90 complex. An and colleagues (2000), showed that treatment of K562 cells with geldanamycin caused a shift in the composition of co-chaperones within the Hsp90 complex (An *et al*., 2000). Geldanamycin binds to the N-terminal ATP binding domain of Hsp90, and upon treatment, competitively displaces the co-chaperones

p23 and Cdc37, which also bind to the Hsp90 N-terminus. In turn, binding of geldanamycin induces a change in Hsp90 conformation to an open, ADP bound state, which has a high affinity for Hop (Tsuruo *et al.,* 2003). In this way, it is hypothesized that blockade of one or more co-chaperones potentially influenced a transition from one status to another (An *et al*., 2000). Morishima and colleagues (2000) showed that the levels of Hop protein were increased in geldanamycin blocked GR-complexes of Hsp90 compared to Hsp90 complexes without geldanamycin (Morishima *et al*., 2000). Collectively these observations suggest that depletion of Hop within the cell increased the affinity of other co-chaperones for Hsp90. It is therefore possible that the knockdown of Hop shifted the Hsp90 complex to one favourable for Aha1 and Cdc37 binding. The increased level of Aha1 and Cdc37 may also have contributed to the lack of sensitivity, as knockdown of these proteins has been shown to induce sensitivity to geldanamycin (Holmes *et al*., 2008; Smith *et al*., 2009).

Amongst the obstacles faced with the undesirable effects of Hsp90 inhibitors, other forms of resistance to anticancer drugs exist. The heritable ability of cells to gain resistance to drug treatment is not an old phenomenon, and was first reported as early as the 1970s (Juliano *et al.,* 1967). Interestingly, chemosensitivity studies for the both the Hs578T and MDA-MB-231 cell lines, results showed that while Hop knockdown cells were more sensitive to the controls at the earlier time points, these cells appeared able to gain resistance, after 72 hours, when the viability of Hop knockdown cells was not significantly different from those of the control treated cells. In the case of MDA-MB-231 cell lines, there was a statistically significant increase in chemoresistance to paclitaxel in the Hop knockdown cells after 48 and 72 hours. The addition of verapamil, an inhibitor of the major P-gp class of drug transporters, was able to overcome the

resistance of the Hop knockdown cells at 72 hours. This suggested that the chemoresistance in Hop knockdown cells was related to increased drug transporter activity in these cells. The expression ATP-dependent plasma membrane glycoprotein (P-gp) drug transporters (Lavie *et al.,* 1996) is known to induce resistance due to the ability to reduce intracellular concentrations of a variety of drugs, including etoposide and taxol (Krishna and Mayer, 2000), and several strategies have developed against multidrug-resistance based on inhibition or modulation of P-gp (Takara *et al*., 2002). P-gp is known to be a client protein of the Hsp90 complex. Studies described by Bertram and colleagues (1996) in human colon carcinoma and the murine cell line, S180, showed that Hsp90β associated with P-gp, and that upon knockdown of Hsp90β, resistance to doxurubin was significantly reduced (Bertram *et al.,* 1996). Therefore, inhibition of Hop may have had an effect on the interaction between Hsp90β and P-gp leading to a change in activity of the drug transporter. Whether the effects were due to an increased expression of the transporter itself or an increase in the transporter activity remains to be determined and future work includes studies such as Western analysis detecting the levels of P-gp post Hop silencing and treatment with anti-cancer drugs.

6.4 The role of Hop in cancer cell migration

6.4.1 Hop knockdown reduced cell migration

Hop knockdown in both Hs578T and MDA-MB-231 cells resulted in reduced migration of cells in wound healing assays, although the effect was less pronounced in the MDA-MB-231 cell line. Earlier reports by Walsh and colleagues (2011) suggested a role for Hop in promoting the migration of pancreatic cancer cells. The siRNA mediated knockdown of Hop in pancreatic cancer cells reduced invasion by reducing the expression levels of MMP-2 (Walsh *et al.,* 2011).

The presence of Hop in the extracellular matrix has also been described in different experimental models, namely glioma cells (Erlich *et al.,* 2007), as well as fibrosarcoma cells (Eustace *et al.,* 2004). In the study by Eustace and colleagues (2004), the presence of Hop in complex with Hsp90α and Hsp70 in the extracellular matrix was linked to the invasive properties of these cells by association with MMP-2. MMP are secreted into the extracellular environment in a latent, unstable form and require maturation for proteolytic activity to facilitate invasion and metastasis as result of the degradation of the extracellular matrix (Xie *et al.,* 2004). Although the interaction of Hop and MMP-2 was not explored in this study, our data that showed Hop knockdown led to a decrease in cell migration are supported by those of Eustace and colleagues (2004), Walsh and colleagues (2011) and Xie and colleagues (2004). We proposed that Hop knockdown led to a decrease in cell migration as a result of a reduction in pseudopodia formation.

6.5 Hop knockdown decreased pseudopodia formation

As pseudopodia formation is critical for cell migration (Brahmbhatt and Klemke, 2003), we investigated whether Hop knockdown would be sufficient to attenuate pseudopodia formation in cells when stimulated with a chemoattractant gradient. Hs578T cells were transfected with either siRNA targeting Hop or non-targeting negative control siRNA and pseudopodia quantified after stimulation with the chemokine SDF-1. Our hypothesis that Hop was involved in pseudopodia formation was confirmed by the significant reduction in the number of actin-rich pseudopodia that protruded through the transwell membrane pores when Hop levels were reduced. Since migration is initiated by the protrusion of lamellipodia and filopodia at the cell

front (Lauffenburger and Horwitz; 1996), the observed loss of these structures upon Hop silencing suggested an alternative mechanism by which Hop may regulate cancer cell migration.

Since the protrusion of pseudopodia at the leading edge of the cell is controlled by the reorganization of the actin cytoskeleton (Lauffenburger and Horwitz, 1996), we hypothesised that Hop interacted with actin during pseudopodia formation. Indeed, Taiyab and colleagues (2011) showed a direct interaction between Hsp90 and soluble actin, where it was hypothesized that Hsp90 was involved in the process of actin polymerization (Taiyab and Rao, 2010). The putative interaction between Hop and actin was assessed using an *in vitro* actin cosedimentation assay. Our results suggested that Hop was able to bind to F-actin *in vitro*. To the best of our knowledge this is the first report of Hop in a direct association with actin. Whether Hop is independently required for pseudopodia dynamics or whether it is involved in conjunction with other components of the Hsp90 complex remains to be elucidated. However, it was interesting to note that only low levels of Hsp90, relative to the levels of Hop, were detected in the isolated pseudopodia fractions. Together, these results suggested that Hop controlled cell migration by regulating pseudopodia formation, which may be as a result of the ability of Hop to bind directly to actin. The actin cosedimentation assay could be further verified by incorporating a positive control experiment such as α-actinin, a known actin-binding protein (Coghill *et al.,* 2003). Futhermore, the *in vitro* interaction between Hop and actin would need to be validated *in vivo* using a technique such as fluorescent resonance energy transfer (FRET) analysis. FRET is defined as the transfer of energy from a donor fluorophore to an acceptor fluorophore upon excitation of the donor, requires the donor emission curve to overlap the excitation spectrum of the acceptor, and for them to be in close proximity to each other (about

5–10 nm) (Wallrabe *et al*., 2003). This study could easily be performed using the fluorophore pairs used in the colocalisation analysis in this study and would provide *in vivo* evidence that Hop and actin are able to interact directly.

Our results, together with those from others showing the involvement of Hop in migration, as well as our data suggesting a role for Hop in pseudopodia formation indicated that Hop effects migration by multiple mechanisms. As a result, Hop could potentially serve as a candidate drug target for treatment of metastasis.

6.6 Hop as a drug target for cancer

A growing understanding of the aspects contributing to molecular, genetic and biochemical changes that occur in cancer progression has resulted in a shift of focus from empirical drug development, to therapeutics that act on specific molecular targets that are responsible for each cancerous phenotype (Goetz *et al*., 2003). Current studies have therefore emphasized drug development focused on aspects such as regulators of signal transduction, cell survival, oncogenic proteins, cell cycle regulating proteins as well as proteins involved in angiogenesis (Goetz *et al*., 2003). Bakheet and colleagues (2009) described key features that should be considered during the identification and validation of a good drug target: a role in major pathways, including that of signaling and communication; a signal peptide motif; a long half-life as well as membrane location (Bakheet *et al*., 2009).

Hop and its associated Hsp90 heteroprotein complexes are critical to the stabilization of a large number of oncoproteins that are involved in the six different hallmarks of cancer. In this study

alone, we showed that silencing of Hop in two different breast cancer cell lines was sufficient in promoting downregulation of Hsp90 associated clients involved in tumourigenesis. The roles played by Hop in pseudopodia formation, actin dynamics and hence cancer cell migration are indicative that Hop is a multifactoral mediator of cellular pathways, and is consistent with work by others (Walsh *et al*., 2011). Indeed, other studies have contributed to the understanding of the role of Hop in cancer and show proof of concept that small molecules inhibiting the TPR domain association of Hop with Hsp90 are successful in reversing numerous cancer phenotypes such as cell proliferation, drug resistance and migration (Horibe *et al*., 2011; Pimienta *et al*., 2011).

In contrast, however, given the opposing effects of Hop knockdown on the proliferation of two different cell lines observed in our study, the targeting of Hop may not be without limitations. In this case, the risk is that targeting Hop may induce growth in some tumours but inhibit growth in others. In addition, our data suggest that Hop may be involved in resistance of cancer cells to drug treatment possibly through the upregulation or activation of drug transporters, and therefore loss of Hop could potentially contribute to an increase in drug resistance. These findings collectively question the feasibility and efficacy of Hop as a potential anticancer drug target, and this approach requires further investigation.

This study in addition raised a possibility of cross-talk between other Hsp90 co-chaperones, and that the targeting of Hop may result in undesirable effects in the equilibrium of other Hsp90 co-chaperones within the cell. Additional work is thus required to provide a more detailed insight into possible mechanisms mediating the changes in abundance and composition of the Hsp90 complex. If this can be achieved, the validation of other co-chaperones such as Aha1, Cdc37 and

p23 as potential drug targets may result in additive or synergic cell killing when either targeted alone or in combination with other Hsp90 inhibitors.

Collectively, the work presented in this study, together with work by others provide emerging evidence for a role of Hop in cancer. Further understanding of the distinct, as well as cooperative roles of Hop in tumourigenesis will provide important information for the rationale design of therapeutic drugs to target Hop for cancer treatment in the future.

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