Characterisation of the Cellular Roles of the Non-inhibitory Serpin; Maspin

By

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Thesis submitted for the Degree of Doctor of philosophy
School of Biological Sciences
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68,000 words

Submitted September 2010

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Abstract

Maspin is a non-inhibitory member of the serine protease inhibitor (serpin) family. Maspin functions as a class II tumour suppressor, resulting from its ability to negatively influence cell migration, invasion, proliferation and angiogenesis, whilst positively regulating cell adhesion and apoptosis. The expression of maspin has been shown to correlate with an increased survival rate for patients diagnosed with several cancers, including those of the breast and the prostate. The overall aim of this work was to characterise the cellular roles of maspin focusing on its effects on cellular morphology, migration and adhesion and the underlying influence of the extracellular matrix. In addition, we aimed to provide an insight into the currently unclear molecular mechanism that maspin uses to exert its tumour suppressor effects.

Insect *Drosophila* S2 cells were used to express and produce wild type maspin protein. The biological activity of recombinant maspin was confirmed through cell migration and cell adhesion assays. We also manipulated wild type maspin gene using siRNA in mammalian cell lines and used site directed mutagenesis to generate constructs point mutated at sites potentially involved in protein-protein interaction. These mutant constructs were transfected alongside wild type maspin into maspin-null breast and prostate cancer cell lines in an attempt to identify structural motifs critical to maspin's biological function.

The addition of recombinant maspin protein or the transfection of wild type maspin gene promoted a mesenchymal-epithelial transition (MET), decreased cell motility and enhanced cell adhesion. Mutation to maspin’s α-helix G attenuated its epithelial-mesenchymal transition (EMT) and disabled its ability to reduce migration and increase adhesion. The influence of maspin on these cell behaviours was mimicked by wild type and mutant maspin peptides that spanned its α-helical G region; supporting the evidence that this helical structure is important. We also showed that maspin changed the affinity state of β1 integrins; an ability also dependent on its α-helix G.

The importance of maspin to mediate its non-metastatic effects, via its α-helix G, were consistent on collagen I, fibronectin and fibrillar matrices. However, an intact α-helical G region was not essential for maspin's ability to decrease cell migration, nor its ability to increase MET and adhesion, when plated onto laminin. Thus, in the presence of laminin, maspin must use a different mechanism of action.
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>α1-AT</td>
<td>Alpha 1-antitrypsin</td>
</tr>
<tr>
<td>ADF</td>
<td>Actin Depolymerising Factor</td>
</tr>
<tr>
<td>Arp</td>
<td>Actin Related Protein</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen Receptor</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic Acid</td>
</tr>
<tr>
<td>C1INH</td>
<td>Serpin C1 Inhibitor</td>
</tr>
<tr>
<td>CBG</td>
<td>Cortisol Binding Globulins</td>
</tr>
<tr>
<td>CCD</td>
<td>Charge-Coupled Device</td>
</tr>
<tr>
<td>cDNA</td>
<td>copy DNA</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese Hamster Ovary</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>Ct</td>
<td>Cycle threshold</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>Copper Sulphate</td>
</tr>
<tr>
<td>DAPI</td>
<td>4', 6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxyribonucleotide Triphosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>E.coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced Chemiluminesence</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>EthyleneDiamineTetra Acetic acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene Glycol Tetraacetic Acid</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-Mesenchymal Transition</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular Signal-Regulated Kinase</td>
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<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal Adhesion Kinase</td>
</tr>
<tr>
<td>FC</td>
<td>Focal Contacts</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal Calf Serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast Protein Liquid Chromatography</td>
</tr>
<tr>
<td>G</td>
<td>Relative Centrifugal Force</td>
</tr>
<tr>
<td>G-actin</td>
<td>Globular actin</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase Activating Proteins</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate Dehydrogenase</td>
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<tr>
<td>GEF</td>
<td>Guanine Exchange Factors</td>
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<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>GFOGER</td>
<td>Glycine-phenylalanine-hydroxyproline-glycine-arginine</td>
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<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine-5’-triphosphate</td>
</tr>
<tr>
<td>HCL</td>
<td>Hydrochloride Acid</td>
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<td>Histone Deacetylase 1</td>
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<tr>
<td>HEPES</td>
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<td>6xHis</td>
<td>Hexahistidine</td>
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<tr>
<td>HRE</td>
<td>Hormonal Response Element</td>
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<tr>
<td>HRP</td>
<td>Horse Radish Peroxidase</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl Thiogalactoside</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon Regulatory Factor</td>
</tr>
<tr>
<td>Kᵥ</td>
<td>Dissociation Affinity Constant</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny Broth</td>
</tr>
<tr>
<td>LRP</td>
<td>Lipoprotein Receptor-related Protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Centre for Biotechnology Information</td>
</tr>
<tr>
<td>NEAA</td>
<td>Non-Essential Amino Acids</td>
</tr>
<tr>
<td>Ni-NTA</td>
<td>Nickel Nitritetriacetic acid</td>
</tr>
<tr>
<td>MENT</td>
<td>Myeloid and Erythroid Nuclear Termination protein</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum Essential Medium</td>
</tr>
<tr>
<td>MES</td>
<td>(N-morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>MET</td>
<td>Mesenchymal-Epithelial Transition</td>
</tr>
<tr>
<td>Mg2⁺</td>
<td>Magnesium</td>
</tr>
<tr>
<td>MIDAS</td>
<td>Metal Ion-Dependent Adhesion Site</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
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<tr>
<td>MdT</td>
<td>Microtubules anti-αTubulin</td>
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<tr>
<td>NP40</td>
<td>Nonyl phenoxypolyethoxylethanol 40</td>
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<tr>
<td>PAI-</td>
<td>Plasminogen Activator Inhibitors</td>
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<tr>
<td>PAK</td>
<td>p21-Activated Kinase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PEDF</td>
<td>Pigment Epithelium-Derived Factor</td>
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<tr>
<td>pI</td>
<td>Isoelectric Point</td>
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<tr>
<td>PI3K</td>
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<td>Phosphatidylinositol 4,5-bisphosphate</td>
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<td>Piperazine-N,N'-bis(2-ethanesulfonic acid)</td>
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<tr>
<td>Q RT-PCR</td>
<td>Quantitative Real Time PCR</td>
</tr>
<tr>
<td>RCL</td>
<td>Reactive Centre Loop</td>
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<tr>
<td>RDG</td>
<td>Arginine-Glycine-Aspartate</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe Combined Immunodeficient</td>
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<tr>
<td>SdH₂O</td>
<td>Sterile distilled water</td>
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<td>SDS PAGE</td>
<td>Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis</td>
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<tr>
<td>SH3</td>
<td>Src Homology 3</td>
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<tr>
<td>siRNA</td>
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<tr>
<td>SF</td>
<td>Stress Fibers</td>
</tr>
<tr>
<td>TBG</td>
<td>Thyroxine Binding Globulins</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris Buffered Saline</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic Acid</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming Growth Factor beta</td>
</tr>
<tr>
<td>tPA</td>
<td>tissue Plasminogen Activator</td>
</tr>
<tr>
<td>tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>tween-20</td>
<td>Polyoxyethylene (20) sorbitan monolaurate</td>
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<tr>
<td>U</td>
<td>Units</td>
</tr>
<tr>
<td>uPA</td>
<td>urokinase Plasminogen Activator</td>
</tr>
<tr>
<td>UPAR</td>
<td>uPA receptor</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
</tr>
<tr>
<td>v/v</td>
<td>volume/volume</td>
</tr>
<tr>
<td>VSMC</td>
<td>Vascular Smooth Muscle Cells</td>
</tr>
<tr>
<td>WASp</td>
<td>Wiskott-Aldrich Syndrome Protein</td>
</tr>
<tr>
<td>WAVE</td>
<td>WASp Family Verprolin Homology Domain-Containing Protein</td>
</tr>
<tr>
<td>w/v</td>
<td>weight/volume</td>
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<tr>
<td>X-Gal</td>
<td>5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside</td>
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Acknowledgements

I would like to thank both my primary supervisor Dr. Rosemary Bass and my secondary supervisor Dr. Vince Ellis for giving me the opportunity to study such an interesting project, as well as their guidance and support.

Thank you to both past and present laboratory members who made my working environment a great place to be. In particular I would like to thank Dr. Laura Wagstaff for passing on her knowledge of molecular biology and cell culture techniques and who over the years has become my close friend. Also, thank you to Dr. Kelly Gray who passed on her expertise of protein purification.

I would like to thank my Mum, Dad and brother who have provided valuable moral and financial support over the years and who have often reassured my confidence when experiments were not working!

Lastly, I would like to thank the BBSRC for the funding which made my PhD possible and the UEA Gareth Roberts initiative, which provided the financial support necessary for my attendance to overseas conferences.
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1 Introduction

1.1 Introduction to the Degradome
The work of this thesis focuses on maspin, a non-inhibitory member of the serpin family of serine protease inhibitors. Maspin can be thought of as part of the Degradome; a repertoire of proteases, their natural inhibitors and the proteins with which they interact that are regulated to modulate the cellular environment. This includes proteases that reside and act in the extracellular environment, which have long been associated with tumourigenesis and metastasis (Edwards et al. 2008). The ability to degrade extracellular matrix (ECM) endows tumour cells with the ability to migrate and invade. Regulation of the ECM therefore impacts on proliferation and apoptosis of tumour cells, in addition to the control of paracrine signalling, such as the ability of the immune system to detect the diseased state. Proteases or proteinases are enzymes that cleave peptide bonds. Proteases occur naturally in all living organisms. These enzymes carry out a range of proteolytic functions and are involved in highly-regulated physiological cascades; such as the blood-clotting cascade, the complement system, apoptosis pathways, several pathways of differentiation and aiding the digestion of food. There are five main groups of protease, which are defined by the prominent functional group in their active site. These are serine, threonine, cysteine, aspartic and metalloproteases. This chapter begins as an introduction to the family of serine proteases and then provides an introduction concentrating specifically on maspin, a non-inhibitory serine protease inhibitor.

1.2 Serine Proteases
Serine proteases are a group of enzymes that catalyse the hydrolysis of covalent peptide bonds. Certain serine proteases are involved in the critical regulation of the ECM, as discussed above. The serine proteases are classified into families; based on the alignment score of their catalytic domain, and clans; which group families that show the same 3D-fold of the catalytic domain. Serine proteases are highly specific, for example chymotrypsin has a hydrophobic substrate specificity pocket, enabling it to precisely cleave peptide bonds following a hydrophobic amino acid residue.

1.2.1 Serine Protease Catalytic mechanism
There are several features critical to the catalytic activity of serine proteases. These include the catalytic triad which is located in the active site of the enzyme and the oxyanion hole which is positioned adjacent to the active site triad where it acts to stabilise substrate intermediates. Substrate recognition occurs at S1 of the serine protease; a pocket neighbouring the catalytic serine residue that interacts with the P1-P1' of the
substrate peptide scissile bond. Additional binding pockets known as protease activation domains also facilitate substrate interaction.

The best studied family of serine protease are those with chymotrypsin-like fold which share the same arrangement of catalytic residues in their active sites; histidine57, aspartic acid102 and serine195. With reference to Figure 1.1, the serine protease catalytic mechanism is as follows: The nucleophilic serine195 residue of the catalytic triad attacks the scissile bond carbonyl carbon of the protein; forming an unstable tetrahedral intermediate (A). The intermediate is stabilised by the oxyanion hole, which accepts the P1 carbonyl oxygen of the protein (N-terminal to the scissile bond to be cleaved). The free hydroxyl proton, from the serine195, is accepted by a nitrogen atom from histidine57 (B). This creates a positively charged histidine57, which is subsequently stabilised by the negative carboxyl group on aspartate102 (C). The peptide bond is now broken and a proton is transferred to the substrate which releases the N-terminal product, leaving behind the C-terminal product held in an acyl-enzyme intermediate (D). With the assistance of histidine57, water replaces the newly formed N terminus of the cleaved substrate (E). This breaks the bond forming the acyl intermediate and the C-terminus is released (F) (Figure 1.1).

![Catalytic mechanism of a chymotrypsin-like serine protease](http://en.wikibooks.org/wiki/Structural_Biochemistry/Enzyme)

**Figure 1.1** Catalytic mechanism of a chymotrypsin-like serine protease. Substrate binds into the active site of serine protease; serine195 (Ser195), histidine57 (His57) and aspartate102 (Asp102). The substrate is cleaved twice releasing product 1 and 2. Figure was adapted from [http://en.wikibooks.org/wiki/Structural_Biochemistry/Enzyme](http://en.wikibooks.org/wiki/Structural_Biochemistry/Enzyme).
Most proteolytic cascades are rapid and irreversible leading to a specific cellular response. To prevent the devastating effects of unlimited proteolysis, activation and inactivation of protease cascades has to be tightly controlled. Ways this is achieved include protease gene transcription, mRNA translation, zymogen (inactive protease precursor) activation, substrate specificity, enzyme activity and specific inhibitors.

1.2.2 Serine Protease Inhibitors
Serine proteases can be inhibited by binding to a naturally occurring serine protease inhibitor from the serpin family (Hunt and Dayoff 1980). Serpins are the largest and most diverse family of protease inhibitors and constitute one of the earliest described protein superfamilies (Rawlings et al 2004, Hunt and Dayoff 1980). Members of the serpin superfamily are present in the three major domains of life, bacteria, archaea and eukarya, as well as several eukaryotic viruses (Irving et al 2002a, Silverman et al 2004). However, serpins found in prokaryotes are sporadically distributed and most only contain one serpin gene. Over 1000 serpin sequences have been identified, including 36 human (Irving et al 2002b).

The serpin nomenclature is based on a phylogenetic analysis of 500 serpins (Silverman et al 2001). It is composed of 16 clades, termed serpin A through to serpin P. There are 29 inhibitory and 7 non-inhibitory serpin proteins, such as antithrombin and maspin respectively. Serpins are given a number that is unique to the protein within its clade. Neither alphabetic letter nor numerical designations denote order of evolution. Serpins have so far been identified from 10 different chromosomal clusters; within each cluster, all serpins belong to the same clade.

Human serpins are the most characterized group, with 36 serpins from nine clades to date being identified. The majority of human serpins are secreted and circulate in the bloodstream, where they control critical proteolytic cascades. The most studied inhibitory serpins are antithrombin and alpha 1-antitrypsin (α1-AT), for their role in coagulation/thrombosis and emphysema respectively (Table 1.1).
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Despite the majority of serpins inhibiting serine proteases, some target other proteases. For example, squamous cell carcinoma antigen-1 and -2 can complex with and thus inhibit papain-like cysteine proteases (Irving et al 2002b). Similarly, the serpin proteinase inhibitor 9 has an acidic residue present in its specificity-determining position, which is targeted by caspases resulting in cleavage and inhibition (Annand et al 1999). Furthermore, some serpins are cross class inhibitors, like viral cytokine response modifier A which shows inhibition of both cysteine and serine proteases involved in the regulation of host inflammatory and apoptotic processes.
1.3 Serpins

1.3.1 Structure of Serpin Superfamily

Serpins share a minimum of 30% sequence identity, yet these single polypeptide proteins share a precise structural architecture. Crystallography data for the archetypal serpin α1-AT and the chicken serpin ovalbumin postulated that serpins exist in a conformation whereby the region for protease binding, the reactive centre loop (RCL), must be available in the native state and unavailable once cleaved (Loebermann et al 1984, Stein et al 1990) (Figure 1.2A).

Figure 1.2 Structure of prototype homo sapiens α1-antitrypsin. A; Native serpin structure shows a lower domain (β-sheet A and 9 α-helices hA-hl), and an upper two sheeted β-barrel (β-sheets B and C). Reactive Centre Loop (RCL) is exposed from the upper β-barrel containing P1-P1’ scissile bond. B; Latent serpin structure has its RCL incorporated into β-sheet A as stand 4 (strands numbered s1-5). β-sheet C strand 1 is extracted (s1C). C; Non-covalent Michaelis complex structure has protease bound (cyan), breach and shutter regions are circled. D; covalent acyl-enzyme complex shows RCL insertion and protease transition to the lower domain. Figure adapted from Silverman et al 2001.

Serpins implement a flexible mechanism that is required for inhibitory activity and this has a highly ordered tertiary architecture; which comprises three beta-sheets (A, B and C) and eight or nine alpha-helices (A-I). All α-helices lay underneath the main 5-stranded β-sheet A. The region holding the specificity for protease binding is the RCL. The RCL is positioned on top of the main β-sheet A in an extended, solvent-exposed conformation, tethered between β-sheets A and C. The residues comprising the RCL loop are numbered relative to its P1-P1’ scissile bond (C terminal residues are denoted with prime) and consist typically 20 in length. The P1’ residue is well conserved across the serpin superfamily and mutation to P1’ affect the rate of inhibition (Olsen et al 1995). Peptide
residues P9-P15, amino terminal of the P1-P1’, are denoted the hinge region and are responsible for strand insertion into the β-sheet during inhibition (Pemberton et al 1995). Hinge region residues are involved in a hydrogen bond network between the body of the serpin; which holds the RCL in a flexible exposed position.

1.3.2 Serpin Inhibitory mechanism

Over 70 serpin structures have been determined, and these data, along with a large amount of biochemical and biophysical information, reveal that inhibitory serpins are irreversible suicide inhibitors that use a unique conformational change to inhibit proteases (Huntington et al 2000). Serpins are an exception to Anfinsen’s conjecture, for their native state is energetically unstable. This native conformation, described as metastable or ‘stressed’, can undergo intramolecular structural changes causing transformation to a more stable latent ‘relaxed’ form (Figure 1.2B). Here, the RCL inserts into β-sheet A to form a stable antiparallel five stranded sheet A where strand 1 from β-sheet C (s1C) is extracted. Latent serpins no longer hold inhibitory activity and since they are more heat stable, can only be converted back to the native state by denaturation and refolding. Upon interaction with target protease, inhibitory serpins shift to their most stable form; the cleaved form. Like the latent form, the RCL inserts into β-sheet A but s1C is not expelled. The cleaved conformation has a melting temperature of approximately 60°C more than the native state (Carrell et al 1991).

The RCL of the serpin is recognised by its target protease active site or exosite forming a reversible Michaelis complex (Ye et al 2001) (Figure 1.2C). After a major conformational change the serpin P1-P1’ is able to acetylate the serine hydroxyl of the protease. The rate of deacylation, which leads to complete proteolysis of the substrate, is slowed by 6-8 orders of magnitude to allow for a triggered re-structuring in the serpin, whilst maintaining the covalent linkage with the protease (Silverman et al 2001).

The RCL can move as much as 10 Å to interact with its protease and creates an ester bond between the backbone carbonyl of its P1 and the hydroxyl group of serine195 of the protease (Carrell et al 1991). This protease binding disrupts the peptide bond between the serpins P1-P1’ and shifts the positions of residues within the hinge region, which weakens the hydrogen bonds to the main serpin body. Thus the serpin then undergoes a dramatic conformational change whereby full RCL insertion of its amino terminal occurs as the fourth strand in β-sheet A. Like the latent serpin, the RCL loop inserts into β-sheet A, but s1C is not extracted (section 1.4). The first residue to insert is P15, followed by the hinge region to P9 (Hopkins et al 1993). The point of insertion is the breach region located at the top of β-sheet A (Whisstock et al 2000a and b). The shutter region is
situated just below the breach, and consists of strands 3 and 5 of β-sheet A. Together, the breach and shutter regions control β-sheet opening and acceptance of the hinge (Blouse et al 2003). To allow for RCL insertion, α-helix F displaces and then returns to its original place, the outer side of β-strand 3A, which in effect locks the RCL in (Gettins et al 2002b). Consequently, the protease is flipped 70 Å to the lower pole of the serpin (Huntington et al 2001). This totally distorts the alignment of the protease catalytic triad rendering the protease inactive (Huntington et al 2006) and held in an acyl-enzyme intermediate that is kinetically stable, since approximately 32kcalmol$^{-1}$ is released upon full RCL insertion (Figure 1.2D) (Im et al 2000).

1.3.3 Serpin Dimerisation and Ordered Polymerisation
The inhibitory function of serpins relies upon their ability to change conformation. Their evolutionary conserved architecture and successful mechanism of opening β-sheet A to aid RCL insertion is particularly susceptible to spontaneous conformational change. PAI-1 and antithrombin are the only serpins known to undergo slow spontaneous transition to an inactive latent state in the absence of substrate cleavage under physiological conditions (Zhou et al 2003). The latent structure of PAI-1 showed the insertion of 13 of its RCL residues, N terminal to the protease recognition site (P16-P4), into the centre of β-sheet A. Its C terminal residues P2-P10', (including protease specificity residues P2-P4') extend from β-sheet A as a rigid loop (Dupont et al 2006). The conformation of the rigid loop differs from that of the flexible RCL loop in active serpins, thus preventing protease inhibition. Akin to the structure of cleaved PAI-1, latent PAI-1 is hyperstable, owing to the many hydrogen bond interactions between its inserted strand and the already existing strands of β-sheet A. Such structural mobility can be used to an advantage by serpin co-factors. For instance, the co-factor vitronectin binds to latent PAI-1 and promotes the stabilisation of its active conformation, thus promoting the inhibition of fibrinolysis (Zhou et al 2003). Similarly, the binding of heparin to latent antithrombin causes the expulsion of the hinge region and re-orientation of the P1 side chain, thus activating antithrombin for its interaction with protease thrombin (Johnson et al 2006).

Another serpin, Myeloid and Erythroid Nuclear Termination stage-specific protein (MENT), has also adapted its structural plasticity to suit its physiological role. MENT shows no inhibitory activity but has a crucial role in organising chromatin, of which DNA binds to an exosite at its D and E α-helices. In the presence of DNA or chromatin, MENT monomers crosslink to form oligomers; the RCL of one molecule interacts with the edge β-strand of A sheet of the next molecule (McGowan et al 2006).
An alternative form of spontaneous conformational change is resultant of mutation. Notably, a mutation in almost any part of the serpin will affect the energy barrier of the incorporation of the RCL into its main β-sheet A; slowing the process or speeding it up (Huntington et al 2009). When serpins are synthesized with a point mutation that affects the critical shutter region, misfolded polymers are formed. Serpins polymerise as an unfortunate feature of their folding pathway and not because they are metastable (Huntington et al 2009). The classical mechanism for serpin polymerisation (loop-sheet A polymerisation) was based upon the Z mutation of α1-AT (Lomas et al 1992). Z mutation at P17 (glutamic acid>lysine), destabilizes β-sheet A which allows the RCL of a neighbouring α1-AT to interlock, to form a dimer. Sequential loop interlocking results in the formation of long inactive polymers (Figure 1.3). Deficiency of α1-AT which results from Z mutation can lead to chronic obstructive pulmonary disease, specifically emphysema, and liver disease (Lomas et al 2000).

![Image](image1.png)

**Figure 1.3** The molecular basis of loop-sheet A polymerisation. The reactive centre loop (RCL) (red) of α1-antitrypsin (α1-AT) is held as a β-strand that can insert into β-sheet A (blue). Mutation to its β-sheet A render α1-AT receptive to the acceptation of RCL of a neighbouring molecule of α1-AT (red arrow), thus forming a dimer. This process can extend to form polymer chains. Figure was adapted from Gooptu and Lomas 2008.

Antithrombin dimers were identified in the blood plasma of patients carrying the variant form P80S and were found to have both covalent and disulphide bond linkages. The stable antithrombin dimer was crystallized and a new mechanistic proposal for intermolecular linkage of the serpin monomer was born (Yamasaki et al 2008). They suggested a serpin folding intermediate (M*) where β-sheet A strand s5A is expelled from sheet A. M* then acts as an acceptor for a second antithrombin monomer, which donates two antiparallel β-strands (s4A and s5A) into the centre of its β-sheet A. This large, hyperstable domain swap is dependent on the unfolding of helix I, which forms an
intermolecular linker between monomers. Exposed helix I is highly hydrophobic and is suggested to mediate lateral polymer association (Figure 1.4) (Whisstock and Bottomley 2008, Huntington et al 2009).

Figure 1.4 Mechanism of serpin polymerisation by domain swapping. Partially folded intermediate (M*) serpin can either fold correctly to form native protein or, when subject to mutation, polymerise. Polymerisation involves the domain swapping of S4A and s5A β-strands from one monomer to another in a donor/acceptor manner. Figure was adapted from Whisstock and Bottomley 2008.

The inactive serpin polymer both structurally and energetically resembles the cleaved native protein. Therefore serpin polymers escape the protective pathways to clear misfolded protein from the cell. Since polymers can not be exported from the endoplasmic reticulum of secretory cells, accumulation leads to deficiency, which results in dysfunction and disease; conditions known as serpinopathies (Lomas and Carrell 2002).

1.3.4 Non-Inhibitory Serpins
A small proportion of serpins do not have any inhibitory activity. Like inhibitory serpins, non-inhibitory serpins have accessible RCLs but instead of forming stable serpin-protease complexes, the serpins themselves undergo cleavage (Pemberton et al 1995). Residues that are required for efficient stressed to relaxed re-arrangement, those that contribute to the hinge, breach and shutter regions, are conserved in inhibitory serpins, but are varied in non-inhibitory serpins (Benarafa and Remold-O'Donnell 2005).
The non-inhibitory serpin chicken protein ovalbumin (serpin B2), a storage protein from egg white, was the founding member of a subfamily of the serpin superfamily; the Ovalbumin family (Remold-O'Donnell et al 1993). Also known as the clade B serpin family, there are 13 *homo sapien* members that originate from chromosome clusters 6p25 (serpins B1, B6 and B9) and 18q21.3 (serpin B2, B3, B4, B5, B7, B8, B10, B11, B12 and B13) (Silverman et al 2001, Gettins 2002a). The human serpin members of this clade B family have 39-46% sequence identity, but also have a similar percentage identity to the larger superfamily (Barnes and Worrall 1995). Therefore, the clade B serpins are grouped together based on numerous characteristics. Relative to the prototype serpin α1-AT, they lack both N- and C-terminal extensions as well as signal peptides. The gene organisations of clade B serpins have either of two conserved structures; which are seven-exons/six-introns or eight-exons and identical six-introns. In comparison, the serpin superfamily genes have extremely variable exon intron organisation patterns (Benarafa and Remold-O'Donnell 2005). Not all members of the human clade B serpins are non-inhibitory; they do show inhibition of serine proteases and/or papain-like cysteine proteases. Human clade B family members’ that do not possess any protease-inhibitory activity include maspin (serpinB5) and epipin (serpinB11). Other non-inhibitory serpins include non-clade B members corticosteroid binding globulin (serpinA6), thyroxine binding globulin (serpinA7), angiotensinogen (serpinA8) and pigment epithelium derived factor (PEDF) (serpinF1) (Askew et al 2007). This section outlines the characteristics of non-inhibitory serpins and how they achieve their biological function. Section 1.4 describes in detail the structure and function of serpin B5; maspin.

The sequence composition at the hinge regions of non-inhibitory serpins differs from the restricted consensus sequence of inhibitory serpins (Benarafa and Remold-O'Donnell 2005) (Figure 1.5). Inhibitory serpin hinge region residues are predominantly small with strictly non-charged residues at P14 and non-proline residues at P12 and P10. This consensus creates a low activation energy that facilitates rapid RCL insertion. In comparison, the hinge region of the non-inhibitory serpin ovalbumin contains a charged arginine at P14. It was found that mutation of ovalbumin’s P14 to a non-charged threonine allowed RCL insertion into β-sheet A, but did not restore any inhibitory activity (Yamasaki et al 2002). Furthermore, the total replacement of ovalbumin’s hinge region with the inhibitory consensus sequence also did not increase the inhibitory activity of the resulting protein or decrease its stability. What did contribute to inhibitory function was the replacement of ovalbumin’s α-helix F and β-sheet strand 3A with those of inhibitory PAI-2, suggesting the serpin scaffold is responsible for non-inhibitory characteristics (McCarthy and Worrall 1997).
Figure 1.5 Alignment of serpin hinge consensus sequences. A) The consensus hinge region of inhibitory serpins. B) Alignment of P17-P9 hinge regions of non-inhibitory serpins – human maspin (serpin B5) and chicken ovalbumin, compared to those of human inhibitory serpins –PAI-2 (serpin B2), MNEI (Monocyte Neutrophil Elastase Inhibitor) and PI-6 (serpin B6).

In comparison to the inhibitory serpins non-inhibitory serpins show no conservation of RCL composition. An RCL of typically 16-17 residues is necessary for insertion into β-sheet A with enough length to span the serpin body and to compress the attached protease without sterical hindrance from surrounding loops (Schulze et al 1990). The non-inhibitory RCL of human serpin B11 was swapped with the RCL of murine serpin B11 which is known to inhibit trypsin (Askew et al 2007). This study showed that the mouse serpin scaffold containing the human RCL showed inhibitory activity, whereas human serpin scaffold containing the mouse RCL did not. They concluded that impaired serpin scaffolds and not impaired RCLs hold the reason why some serpins have lost inhibitory function.

Despite lacking inhibitory activity, some non-inhibitory serpins use the traditional stressed-to-relaxed transition as part of their function. These unique clade B serpins are thyroxine and cortisol binding globulins (TBG and CBG) (Pemberton et al 1988). TBG and CBG bind to their substrates, thyroxine or corticosteroids, at a site other than their RCL; between α-helices H and A and with strands 3–5 of the β-sheet A. The crystal structures of TBG showed that the upper half of β-sheet A was fully opened, allowing the metastability of the RCL to influence the binding and release of substrate (Zhou et al 2006). The crystal structures of cleaved CBG showed the serpin had undergone irreversible stressed-to-relaxed transition, with their cleaved RCL being fully incorporated...
into its β-sheet A. This RCL insertion disrupted substrate binding thus causing hormone release (Zhou et al 2008).

Some non-inhibitory serpins, including ovalbumin and PEDF, function independently of their RCL. PEDF has the typical serpin structure with an exposed protease-sensitive loop, yet cleavage of this region does not increase stability, promote conformational re-structuring or affect its neurite-promoting function (Becerra et al 1995). Crystallisation of PEDF and peptide studies showed α-helices C and D and their interconnecting loop to be involved in binding to a putative cell membrane receptor (Simonovic et al 2001). Later PEDF was found to bind to collagen types I and III (Kozaki et al 1998) via its α-helical G region (Meyer et al 2003).

1.3.5 Serpins in Disease
Inhibitory serpin deficiency or excess has been correlated to many diseases and cancers, since their physiological functions control numerous proteolytic cascades, which are comprehensively involved in extracellular matrix degradation and critical for tumour cell invasion, metastasis and angiogenesis. For example, antithrombin deficiency has been shown to promote thrombogenicity; the clotting of blood. Deficiency is caused by mutation to antithrombin that impairs the synthesis, secretion or function of the protein. Additionally certain mutations prevent the binding of co-factor heparin and the subsequent conversion of antithrombin from a latent to an active state. Antithrombin also regulates inflammation via a non-inhibitory mechanism, whereby it binds to heparan sulphate on endothelial and leukocyte cell surfaces. This promotes anti-angiogenic activity by the inhibition of fibroblast and vascular endothelial cell growth factors. Thus, the loss of functional antithrombin in disease states can contribute to an increased angiogenesis (Rau et al 2007). Another example is the synthesis of the serpin PAI-1, which is sensitive to many pathophysiological factors. Hyperglycaemic diabetic patients have increased synthesis of PAI-1 in vascular endothelial and smooth muscle cells, which contributes to cardiovascular disease (Rau et al 2007). There is also strong evidence that elevated PAI-1 levels are associated with gastric cancer by increasing their invasion capability (Lin et al 2008). Additionally, low levels of the serpin protein C1 inhibitor causes an unchecked activation of kinin pathways which can lead to hereditary angioedema; a swelling of the skin dermis, subcutaneous tissue, mucosa and submucosal tissues. Specifically, a deficiency of the C1 inhibitor increases levels of bradykinin in the blood, which is responsible for capillary leakage (Gompels et al 2005).

The deficiency or excess of non-inhibitory serpins can also result in pathological conditions. For example, a loss of function mutation of the non-inhibitory CBG gene
results in low plasma cortisol and has been linked to hypotension and chronic fatigue (Torpy et al. 2001). Similarly, several mutations of the non-inhibitory TBG gene have been reported to give complete or partial deficiency. Deficiency of this principal thyroid hormone transport protein results in low levels of thyroxine, culminating to hypothyroidism. On the contrary, an excess of TBG results in hyperthyroidism (Albright et al. 1955, Kopp et al. 1995). Similarly the overexpression of the plasmin inhibitor megsin, which is predominantly found in mesangial cells of the glomerulus, contributes to the proliferative glomerular disease, IgA nephropathy (Miyata et al. 1998). Furthermore, the decline in the expression of the non-inhibitory serpin maspin has been associated with tumour progression. This is described in detail in section 1.4.2.2.

1.3.6 Serpinopathies

Protein misfolding has been known as a characteristic feature of a range of disorders, termed collectively as conformational diseases. For example, β-amyloid protein and α-synuclein proteins misfold to form amyloid plaques or Lewy bodies, causing the common dementias Alzheimer’s and Parkinson’s disease respectively (Carrell et al. 1997). The misfolding of mutated serpins, which are the root of multiple disorders, are coined serpinopathies (Lomas and Carrell 2002). This section outlines the molecular and structural basis of the several serpinopathies.

For the serpin α1-AT, many dysfunctional genetic variants have been reported. The Z mutation of α1-AT causes an unstable serpin that readily forms polymers by the interlocking of one another’s RCL. Synthesised in hepatocytes, α1-AT polymers are retained and accumulate in the endoplasmic reticulum, leading to neonatal hepatitis, cirrhosis, and hepatocellular carcinoma (Eriksson 1986).

Like α1-AT, mutations in plasma serpins C1-inhibitor and anti-thrombin can lead to unstable ordered polymers. However, in comparison they are not synthesised as much as mutant α1-AT, and are able to be cleared before forming toxic accumulations. The lack of functional protein results in angioedema and thrombosis respectively (Gooptu and Lomas 2008).

Mutated serpin I1, neuroserpin, has been known to aggregate forming neuronal inclusion bodies. Familial dementia is a direct consequence to these cumulative Collin bodies. The rate and magnitude at which mutant neuroserpin is able to aggregate is correlated to the onset and severity of familial dementia (Davis et al. 2002). All mutations of neuroserpin reported to contribute to familial encephalopathies with neuroserpin inclusion bodies are found within the shutter region; found to be critical for conformational stability. Mutations
in this region increase the flexibility of β-sheet A, which favours polymeric linkage. A particular neuroserpin S49P mutant, named Siyama, forms polymers in vivo (Lomas et al 2004). Wild type shutter region residue S49 interacts with β-sheet A strands 3 and 5, to securely shut the β sheet. Mutation therefore renders the shutter very flexible, allowing easy insertion of a neighbouring RCL serpin (Davis et al 2002). Both polymer promoting mutations, S49P of neuroserpin and Z-α1-AT were found in the same shutter domain, confirming the importance of this region in the correct insertion of the RCL (Belorgey et al 2002).

Based on the structural knowledge of the serpinopathies, chemical chaperones and small loop peptides have been designed with the potential to block polymerisation and ameliorate disease. The developments of drugs that target serpinopathies have been much reliant on mechanistic knowledge of polymer formation. Thus the recently suggested mechanism of serpin polymerisation has paved way for new strategies that target the M* state or exposed 5A strand (Figure 1.4) (Yamasaki et al 2008). One group identified Z-α1-AT antagonists that reduced intracellular oligomerisation of Z-α1-AT by 70% in a cell model representing liver disease. These Z- α1-AT antagonist peptides bound to a unique hydrophobic lateral binding pocket, not present in at or a1-antichymotrypsin (Mallya et al 2007). Tiplaxtinin is a small molecule known to inhibit PAI-1 and is used therapeutically in cases where PAI-1 levels are high such as deep vein thrombosis, cancer, and type 2 diabetes (Gorlatova et al 2007).

1.4 Maspin - Serpin B5
Maspin or serpin B5 (Genbank Accession Number: U04313) was first identified from subtractive hybridisation studies using cDNAs from normal and malignant human mammary epithelial cell lines (Zou et al 1994). Maspin positioned at locus 18q21.3 encodes a 42 kDa (376 amino acids) epithelial specific, monomeric protein, with an isoelectric point of pH 5.72. Maspin can either be secreted, cytoplasmic, nuclear or cell-surface associated (section 1.4.5) (Futscher et al 2004, Pemberton et al 1997).

1.4.1 Structure of Maspin
Sequence alignment studies reported that maspin’s P1 residue is an arginine residue (Zou et al 2004). However, stability studies showed that maspin was unable to undergo the stressed-to-relaxed transition, typical of inhibitory serpins and that it could not complex with or inhibit the activity of trypsin-like, arginine specific proteases (Pemberton et al 1995). This was subsequently demonstrated that maspin had no inhibitory action (see section 1.1.4) (Bass et al 2002).
Sequence alignment studies between maspin and ovalbumin shows that the RCL of maspin was shorter by 4 residues. Maspin’s short RCL decreases the stability of the molecule and causes maspin to be highly sensitive to proteolysis by non-target proteases at physiological temperatures. Unlike target proteases which specifically cleave P1-P1’ and become inactivated by being trapped in a stable serpin:protease complex, non-target proteases cleave the serpin RCL but do not themselves become inhibited (Fitzpatrick et al. 1996). Maspin was particularly sensitive to non-target trypsin and trypsin-like proteases with limited proteolysis which caused a reduction in the molecular mass of the protein from 42 kDa to 38 kDa. As demonstrated by urea gradient gel electrophoresis, both native maspin and RCL-cleaved maspin did not undergo the stressed-to-relaxed transition and showed a reduced melting temperature compared to the prototypic serpin α1-AT (Pemberton et al. 1995). Unlike α1-AT, maspin protein was shown to be capable of spontaneous polymerisation induced by mild changes in pH. Maspin cleaved at its RCL by trypsin was also able to polymerise suggesting that maspin’s RCL remained accessible after cleavage thus supporting the finding that maspin does not adopt the stressed-to-relaxed transition (Pemberton et al. 1995). Biochemical titrimetric analyses and homology modelling showed that despite the presence of eight cysteine residues, maspin does not possess any intramolecular disulphide bonds owing to the distant positioning between residues (Fitzpatrick et al. 1996). This was later confirmed by crystallisation studies (Law et al. 2005).

Crystallisation of maspin showed that it shares the ternary structure of the native serpin; three β-sheets and nine α-helices, in which the RCL is expelled from β-sheet A making it accessible to proteolysis (Al-Ayyoubi et al. 2004). Most of maspin’s RCL is in β-strand configuration, but P4, P1 and P1’ residues are solvent-exposed as a half-turn α-helix. Maspin’s RCL lies close to the serpin body and is highly hydrophobic, contributing to the ability of maspin to form stable bonds between surface side chains of β-sheet C and cluster intermolecularly. More evidence that demonstrated maspin to be dissimilar to inhibitory serpins was its non-conserved bulky hinge region and its closed breach region; two regions necessary for inhibitory RCL insertion (Al-Ayyoubi et al. 2004).

A second study also crystallised maspin (Law et al. 2005) (Figure 1.6). The structure revealed a novel salt bridge between a glutamic acid and lysine residue on strands 1 and 2 of β-sheet A. The salt bridge distorted s1A and brought about a cluster of basic residues around D and E α-helices. Furthermore the surface of maspin centred on the α-helix D was asymmetrically charged and these areas were postulated to be involved in cofactor binding. During this particular study, two isomorphous forms of maspin were determined. These were almost identical apart from the position of α-helix G and strands
1 and 2 of β-sheet B. It was found that maspin could undergo a unique conformational switch occurring within its α-helix G, allowing the molecule to adopt both an open and closed form. In the closed configuration, the α-helix G interacted with β-sheets B and C as well as α-helix A. In the open arrangement, two glutamic acid residues E244 and E247 moved from a buried position. Upon exposure, the interfaces at either end of the α-helix G were widened which was proposed to be involved in co-factor binding/recognition (Law et al 2005).

![Ribbon diagram of maspin](image)

**Figure 1.6** Ribbon diagram of maspin. Structure is comprised of three β-sheets A, B and C surrounded by nine α-helices A-I and a reactive centre loop containing P1 residue. Diagram shows point mutations at residues R340, E244, E247, K90 and E115 (PDB entry 1XU8, Chain B). Figure was adapted from Law et al 2005.

### 1.4.2 Biological Functions of Maspin

#### 1.4.2.1 Maspin in Development

Maspin has been shown to play an essential role in development. Homozygous maspin knockout mice died at the peri-implantation stage. In particular, deletion of maspin interrupted the formation of the endodermal cell layer, disrupting the morphogenesis of the epiblast (Gao et al 2004). No other clade B serpins have been found to be embryonically lethal, apart from the loss of only one inhibitory serpin (Ishiguro et al 2000). Maspin heterozygous knockout mice were generated and these appeared morphologically normal
after birth, but the female progeny were defective in mammary duct development during puberty. Specifically, ductal elongation and side branching was reduced, both effects reliant on rapid proliferation. Defective proliferation during gland development was shown to be a consequence of a reduction of progesterone (Shi et al. 2004). This reduction in progesterone was attributed to maspin heterozygous mice having severely flawed ovarian development (Sood et al. 2002). Similarly, male maspin heterozygous mice exhibited an early developmental defect of the prostate. Between 6 and 12 months dorsal prostate tissues hyperproliferated which led to the development of hyperplastic lesions and stromal hyperplasia with increased epithelial and stromal cell proliferation seen as early as eight days (Shao et al. 2008).

In a mouse model of maspin overexpression in mammary gland alveolar cells, underdevelopment of the gland in mid-pregnancy and early lactation was observed. In particular, an excess of maspin decreased the number of alveolar cells which was suggested to be due to a disruption of alveolar cell adhesion to the ECM which in turn would decrease cell motility at a stage when invasion into the fat pad is critical. Together with an increased apoptosis, maspin lessened development and reduced milk protein production (Zhang et al. 1999).

1.4.2.2 Maspin as a Tumour Suppressor
Maspin was found to be expressed in normal mammary epithelial cell lines, but not in most mammary tumour cell lines, thus identifying maspin as a potential tumour suppressor gene. Southern blot analysis of the maspin gene in tumourous breast cells revealed no gross structural change compared to normal cells, showing that the maspin gene is not mutated or deleted in tumours, yet is down-regulated. Thus maspin was identified as a class II tumour suppressor whose down-regulation in many epithelial derived cancers is not due to mutation (Zou et al. 1994). In support of its tumour suppressor properties maspin decreases cell motility, invasion and metastasis. This was demonstrated in vitro and in vivo, whereby maspin-transfected human MDA-MB-435 cells reduced invasion through Matrigel and reduced the induction of mammary tumours and metastasis to the lung and lymph nodes of a murine model respectively (Zou et al. 1994). However, it was later found that MDA-MB-435 cell line was derivative of melanoma M14 cells and not breast carcinoma (reviewed Chambers 2009). Nonetheless, maspin has been shown to induce anti-tumourigenic properties and many subsequent studies have shown its involvement in breast cancer (section 1.4.2.2.1).

In addition to maspin’s key functions in early embryonic and gland development, maspin organises multiple cellular functions. Proteomic profiling of melanoma MDA-MB-345 cells
compared with two maspin-transfected clones showed that maspin altered both gene and protein expression of approximately 27% of the detected proteome. Many pro-apoptotic proteins were up-regulated in maspin expressing clones, whereas numerous anti-apoptotic proteins were down-regulated. This was consistent with maspin’s documented effect of increasing the rate of spontaneous cell death in cells with high metastatic propensity (Chen et al 2005).

Furthermore, maspin has been described as an inhibitor of cell migration and inducer of cell adhesion to the basement membrane and ECM resulting in inhibition of tumour invasion, metastasis and angiogenesis (reviewed Sager et al 1997). The addition of recombinant maspin protein to melanoma MDA-MB-435 cancer cells led to the conversion of a metastatic, fibroblastic phenotype to that of a benign, epithelial one. Specifically, recombinant maspin induced an increase in MDA-MB-435 cell attachment and a decrease of invasive potential through fibronectin in vitro (Seftor et al 1998). These tumour suppressive properties of maspin have been widely demonstrated in the glandular epithelial cancers of the breast and prostate. These studies have provided compelling evidence for the clinical relevance of maspin in cancer progression and metastasis.

1.4.2.2.1 Maspin in Cancer

1.4.2.2.1.1 Breast Cancer
Differential expression screening between human normal and cancerous mammary epithelial cell lines, showed maspin mRNA expression to be decreased or completely silenced in corresponding cancerous cells (Zou et al 1994). This was confirmed by RT-PCR, which revealed a reduced expression of maspin mRNA in tumour specimens of breast cancer patients. The absence of maspin expression was indicative of a high tumour aggressiveness and metastasis. Breast carcinoma specimens that had metastasized to the lymph nodes exhibited a significantly lowered expression of maspin, irrespective of the tumour stage and grade (Maas et al 2001).

A mouse model was used to study the effect of over-expressing maspin, in mammary tumour TM40D cells. The introduction of maspin significantly decreased tumour growth by 22.2%. All maspin expressing tumours were encapsulated in fibrous tissue and few showed necrosis compared to control TM40D cells which completely lacked tumour encapsulation. Mammary cancer cells TM40D showed invasion into adjacent muscle and blood vessels which permitted distant metastasis. Conversely, no invasion or metastasis was reported from maspin expressing tumours (Shi et al 2001). Another study showed that the addition of recombinant maspin or the transfection of maspin cDNA reduced the
invasion of both the highly invasive breast cancer cell line MDA-MB-231 and the slightly invasive normal mammary epithelial strain 70N through Matrigel. This was accompanied by a maspin-directed reduction in migration over a combined matrix of laminin, collagen IV and gelatin matrix (Sheng et al 1996).

The introduction of maspin gene into maspin-null melanoma MDA-MB-435 cells induced apoptosis under the influence of staurosporine (which activates the caspase-3/-8 apoptotic pathway). (Throughout this thesis, maspin-null defines cells not expressing maspin). Conversely, maspin transfected MDA-MB-435 cells in the absence of staurosporine and wild type mammary epithelial 70N cells known to express high endogenous levels of maspin, did not undergo detectable apoptosis in vitro. Therefore, endogenous maspin cannot induce apoptosis per say, yet can enhance the efficiency of an activated apoptotic cascade (Jiang et al 2002).

To determine the effect of maspin expression on in vivo angiogenesis, maspin was administered intravascularly into mammary TM40 tumours using recombinant adenovirus construct. Histological analysis of the maspin transfected mammary tumours showed disrupted neovascular and vascular leakage. The vasculature of existing vessels remained intact, which showed that maspin selectively induced apoptosis in proliferating tumour cells. In vitro experiments confirmed selective neovascular leakage of human umbilical vein endothelial cells, to be of direct consequence to an increase in apoptosis (Li et al 2005).

1.4.2.1.2 Prostate Cancer
Analysis of maspin expression during prostate cancer illustrated that a low maspin expression corresponded to an increase in higher tumour stages and histological dedifferentiation. Normal prostate basal epithelial cells had high expression of maspin, whereas low grade prostate carcinoma cells had decreased maspin expression, which decreased further in high grade carcinoma cells (Pierson et al 2002). Immunohistochemical detection of maspin in multiple tumour foci of prostate cancer specimens from 121 patients showed a significant reduction of maspin by 63% and in association to a lower Gleason score. Prostate cancer patients who received androgen deprivation therapy, showed reduced proliferation in the cancerous cells. Expression of maspin was significantly elevated in prostate tumour LNCaP cells which had been subject to androgen depletion (Zou et al 2002). Similarly the expression of maspin was found to decrease upon prostate malignancy and correlated with an increased Gleason score (Riddick et al 2005). The expression of maspin protein has been associated with a recurrence-free survival of prostate cancer patients, whereas a decreased maspin protein
expression has been correlated with increasing malignancy. Also, for patients with a low or negative maspin expression after prostatectomy, local relapse or tumour progression occurred (Machtens et al. 2001).

Between 17-45% of prostate cancers demonstrate a loss in 18q chromosome, of which the maspin gene resides. It has been shown that the re-introduction of chromosome 18q into bone-derived prostate cancer PC3 cells abrogated metastasis in vivo (Padalecki et al. 2003). The tumour suppressive activities of maspin were later found to be responsible for this phenotype, with maspin shown to reduce the metastasis of prostate cancer cells to their most common distant site; the bone. Maspin transfected PC3 cells were inoculated into athymic nude mice (absent thymus gland, immune defective mice) to create a model of bone metastasis. As a result, it was observed by radiography that the number of skeletal masses was reduced and bone degradation decreased in comparison to non-transfected PC3 clones. Furthermore, maspin transfected prostate cancer PC3 cells exhibited reduced growth in soft agar and reduced proliferation in vitro (Hall et al. 2008). This study suggested that maspin plays a role in reducing the prostate tumour cell's ability to seed to bone and/or in the inhibition of growth within the bone.

Similarly, another in vivo murine model of bone metastasis showed the expression of maspin inhibited osteolysis, tumour growth and angiogenesis. In detail, the intraosseous transfection of maspin-transfected human prostate carcinoma DU-145 cells showed tumours were uniquely encapsulated in fibrous collagen and contained original mineralised bone. Additionally, maspin expressing bone tumours had a reduced size, decreased collagen I degradation and a low growth rate, highly suggestive of an importance of maspin in ECM regulation (Cher et al. 2003). Another study analogously showed that in vitro transfection of maspin into DU-145 cells led to a significant reduction of the degradation of radiolabeled subendothelial ECM, with subsequent reduction in cell invasion (Biliran and Sheng 2001). The introduction of maspin also decreased the metastatic potential of the C2N murine prostatic adenocarcinomic cell line. This inhibited C2N cell growth in a dose-dependant manner and decreased the metastatic potential of C2N cells, by decreasing invasion and selectively increasing adhesion to a fibronectin and laminin matrix (Abraham et al. 2003). Thus these studies show that the anti-metastatic effects of maspin are mediated by its large influence on ECM regulation.

Maspin is an effective angiogenesis inhibitor. Recombinant maspin blocked in vitro endothelial cell growth, cell migration and tube formation in Matrigel. Recombinant maspin completely blocked basic fibroblast growth factor-induced corneal neovascularisation of a rat model. Additionally, maspin transfected prostate tumour
LNCaP cells when delivered into a xenograft of athymic nude mice, blocked tumour growth and caused a reduction in the density of tumour-associated microvessels (Zhang et al. 2000b). Similarly, maspin significantly reduced angiogenesis in prostate cancer cell DU-145-derived bone metastasis in vivo (Cher et al. 2003). It was found that re-expression of maspin into these DU-145 cells reduced neovessels of human origin, as opposed to both human and mouse origin in the maspin-null tumours.

1.4.2.2.1.3 Cancer of Other Organs

Experimental evidence has shown maspin to have different levels of expression, whereby in some cancers maspin expression is decreased yet in others its expression is increased. This has introduced debate regarding maspin’s well characterized tumour suppression. In the same way that maspin expression has been correlated to predicting a favourable prognosis in patients with breast or prostate cancer, maspin-positive patients with squamous cell carcinoma showed enhanced 5-year survival rates, in comparison to maspin-negative patients (Nakagawa et al. 2006). Also, a high expression of maspin in oral squamous carcinoma specimens correlated to an absence in lymph metastasis (Xia et al. 2000).

On the contrary, maspin-positivity in non-small cell lung carcinoma patients correlated with tumour progression and displayed significantly poorer prognosis compared to maspin negative tumour patients (Hirai et al. 2005). Similarly, the expression of maspin in gastric cancer sharply contrasted the protective role of maspin (Wang et al. 2004). Unlike the breast and prostate, maspin was minimally expressed or absent in wild type gastric, pancreatic and ovarian epithelium. Using immunohistochemistry, high maspin expression was identified in 5 of 9 pancreatic cancer cell lines and in 23 of 24 pancreatic adenocarcinomas (Maas et al. 2001). Equally, maspin was up-regulated in a significant number of ovarian tumours, including that of SKOV3 cell line. Such positive correlation of maspin expression with the progression of pancreatic and ovarian carcinomas suggests maspin may act as an oncogene. However, the introduction of wild type maspin to the maspin-expressing SKOV3 tumour cell line, still reduced cell invasion; in keeping with its tumour suppressive activity (Sood et al. 2002). The exact role of maspin is not known, but its function in these cancers has been proposed to be linked to its subcellular location (section 1.4.5).

This difference in expression of maspin implies it has many versatile biological functions which can be controlled under different pathophysiological influences. Despite the questionability of maspin’s oncogenic properties, maspin has been widely accepted as a
tumour suppressor. This thesis focuses on the mechanisms that might be involved in tumour suppression using epithelial breast and prostate cell models.

**1.4.3 Maspin as a Prognostic Marker**

The anti-tumourigenic and anti-metastatic properties of maspin have prompted the investigation of its use as a possible therapeutic agent or diagnostic indicator. RT-PCR identified mRNA of maspin and mammaglobin B in the peripheral blood of patients with breast cancer, to have a negative or positive correlation respectively. The collective identification of both mRNA markers of cancer patients has thus been validated as a useful diagnostic tool (Mercatelli *et al* 2006). Maspin has also been found to be a foetal marker, being present in the maternal blood plasma during every trimester of pregnancy. Bisulfite DNA sequencing identified epigenetic differences between placental and maternal maspin gene promoters. Foetal DNA extracted from maternal plasma contained hypo-methylated maspin, whereas the maspin promoter was densely methylated in maternal blood cells. An elevated level of hypo-methylated DNA in maternal blood plasma has been associated with the pregnancy disorder pre-eclampsia (Chim *et al* 2005).

**1.4.4 Molecular Mechanism of Maspin**

The ability of maspin to inhibit cell migration and thus invasion and metastasis suggested that maspin might inhibit matrix-degrading proteases. However, it was demonstrated that maspin does not act as an inhibitor of proteases. Although no protease targets were found maspin was itself a substrate. As detected using SDS-PAGE, maspin was a substrate for chymotrypsin, trypsin, elastase, plasmin and thrombin. Proteolysis gave rise to a smaller fragment at 38 kDa, but did not form SDS-heat denaturation-resistant complexes typical of serine protease inhibition (Pemberton *et al* 1995, Zhang *et al* 1999). One study contradicted the non-inhibitory behaviour of maspin, by showing that maspin could directly bind to and form stable complex with tissue-type plasminogen activator (tPA); resulting in the inhibition of tPA and subsequently reducing levels of tPA product plasmin (Sheng *et al* 1998). However, like the initial studies (Pemberton *et al* 1995, Zhang *et al* 1999) it was confirmed that maspin does not inhibit plasminogen activators tPA or urokinase-type plasminogen activator (uPA). In comparison to the efficient homology related serpin PAI-1, maspin did not lead to any observable inhibitory effect. Such conclusive lack of inhibition is consistent with the molecular characteristics of maspin (section 1.4.7.2) (Bass *et al* 2002).

Despite maspin being a non-inhibitory serpin and its susceptibility to protease cleavage, there is evidence that suggests maspin acts through its intact RCL (Al-Ayyoubi *et al*
Maspin’s hydrophobic RCL residues (330-345) or C-terminal residues (346-375) were replaced with corresponding regions from ovalbumin. This abolished the previously seen adhesion of melanoma MDA-MB-231 cells to collagen types I and IV, fibronectin and laminin. Furthermore, a peptide consisting of maspin’s RCL residues alone aided corneal stromal cell adhesion to ECM and restrained cell invasion (Ngamkitidechakul et al 2003). It was also shown that exogenous maspin was dependent on the structural integrity of its RCL for its anti-metastatic and anti-invasive functions. This was demonstrated by the incubation of metastatic breast cancer cell lines either with a maspin RCL-specific antibody or with trypsin; both of which destroyed the biological activity of maspin (Sheng et al 1996). Additionally the RCL of maspin has been proved to be important for cell surface-associated regulation of uPA-uPAR-dependent cell detachment (section 1.4.7) (Yin et al 2006) and for its endogenous function of inducing apoptosis (Li et al 2005).

There is also evidence suggesting that maspin’s RCL is not necessary for its biological functions, where other functional region/s plays part. For example, maspin’s RCL did not function in its ability to inhibit basic fibroblast growth factor-induced angiogenesis in vivo (Zhang et al 2000b). Also, an intact RCL was not necessary for maspin to inhibit the migration of endothelial cells, whereas it was a requirement for a decreased motility of epithelial cells. This discrepancy between cell types may be due to the difference in cell surface receptors (Zhang et al 2000b). However, a lot of the mechanistic data regarding maspin’s RCL is contradictory between laboratories and thus remains a contentious issue. For example, one group have shown that an anti-maspin antibody inhibited MCF-10A cell adhesion, whereas anti-RCL did not (Cella et al 2006). This therefore suggested that maspin’s RCL was not required for its function; contradicting other studies (Ngamkitidechakul et al 2003). Similarly, mutation to maspin’s RCL, at P1 residue R340, did not perturb maspin’s ability to induce apoptosis of DU-145 cells in the presence of protein kinase C inhibitor bisindolylmaleamide (Ravenhill et al 2010). This was contradictory to another study that showed maspin’s RCL essential for the induction of apoptosis in human umbilical vein endothelial cells (Li et al 2005). However this difference in the requirement of maspin’s RCL for apoptotic promotion could be due to different cell models used, such as endothelial and epithelial respectively.

Maspin contains five tyrosine residues. Only one of these residues, T112, is accessible; positioned at the top of α-helix E. However it was suggested from the crystal structure of maspin that the orientation of residue T112 and the conformational constraint of nearby interactions with α-helices A, C and E that phosphorylation by a kinase would not be accommodated (Law et al 2005). Contradictory to this suggestion, maspin showed phosphorylation of its tyrosine residues in normal and cancerous mammary epithelial cell
lines HMEC1331/1436N1 and MDA-MB-231 respectively. Under cell-free conditions, both cell-associated and secreted maspin were phosphorylated by the protein tyrosine kinase domain of epidermal growth factor receptor which was independent of src-tyrosine kinase. It was thus suggested that phosphorylation of maspin may be important for its functions, participating as a signalling transduction molecule. Maspin tyrosine phosphorylation occurred irrespective of whether the cells were grown in suspension, on plastic or fibronectin matrix; which led to the suggestion that maspin may be involved in a signalling pathway that does not involve cell attachment (Odero-Marah et al 2002).

Like maspin, PEDF is a non-inhibitory serpin. Many studies have shown that PEDF acts as a multifunctional secreted protein with well characterised biological functions such as decreasing angiogenesis, reducing cancer cell migration and promoting apoptosis, and inducing neuronal differentiation. Comparative studies between maspin and PEDF showed both molecules prevented angiogenesis in a manner that did not require their RCL. However, the activity of PEDF was attributed to its solvent exposed C and D α-helices and surrounding area. Since maspin does not share accessibility in this region, a different mechanism for anti-angiogenesis was suggested (Simonovic et al 2001, Al-Ayyoubi et al 2004).

In conclusion, numerous efforts have been made to uncover the molecular mechanisms that maspin utilizes to exert its numerous tumour suppressive effects. However, the mechanism of action behind maspin’s tumour inhibitory activities remains unclear. The following sections provide an introduction to maspin’s localization, transcriptional regulation, protein interactions and involvement in cell signalling pathways.

1.4.5 Localisation of Maspin
Maspin is expressed in a wide variety of human tissues; mainly residing in the epithelium of several human organs, such as prostate, thymus, testis, lung, bronchus, small intestine and colon. Maspin also resides in the myoepithelium of the breast and bronchus and also stromal and oral keratocytes and endothelial cells of the cornea. Maspin gene expression is absent in normal heart, brain, placenta, liver, skeletal muscle, bone marrow, kidney and pancreas tissues (Pemberton et al 1997, Zhang et al 1997a, Futscher et al 2002, Yatabe et al 2004, Ngamkitidechakul et al 2001, Reis-filho et al 2002). This inconsistent expression of maspin between tissues and its consequential down-regulation or up-regulation upon tumour progression respectively, has questioned its role as a tumour suppressor. However many studies have established maspin a tumour suppressor (section 1.4.2.2.1.3). The localisation of maspin within a given tissue has also been shown to differ. The best example of this was seen in the stomach, in which maspin
primarily resides in epithelial cells at the base of the crypts whereas maspin is not so strongly expressed in the migratory cells further up the villi (Pemberton et al 1997). This localisation of maspin supports the many studies that have shown maspin to correlate with an anti-migratory behaviour. In addition to tissue expression, the localisation of maspin within the cell has been linked to cancer prognosis and cellular effect.

Maspin was found to have a broad localisation and resided predominantly in the cytoplasm, but also in the nucleus of cells and a small percentage was secreted. Maspin was found at the cell surface and in the endoplasmic reticulum, Golgi and secretory vesicles (Pemberton et al 1997). The secretion of maspin must occur via a non-classical mechanism, since maspin is synthesised and secreted without the presence or cleavage of a hydrophobic N-terminal signal peptide. Classically, a signal or targeting domain allows translocation into the lumen of the endoplasmic reticulum/Golgi followed by the vectorial secretion across the cell membrane. The phenomenon of protein secretion in the absence of a signal peptide, like maspin, has been denoted as non-classical or unconventional protein export. Existing data indicates various mechanistically distinct non-classical secretory routes, including extracellular heparin sulphate proteoglycan-driven membrane translocation and sequestration by secretory lysosomes or exosomes. However, the precise molecular mechanisms and machinery components that mediate these processes remain elusive (Nickel and Rabouille 2008).

Like maspin, all other members of the clade B family lack a cleavable signal peptide and reside primarily within cells, but are also often found in the nucleus and are occasionally extracellular (Silverman et al 2004). The prototypical ovalbumin, shown to be effectively secreted from chicken oviduct cells has an internal hydrophobic sequence between residues 25-45. It was proposed that this hydrophobic stretch could form one limb of a hairpin loop which could interact with cell membrane receptors (Lingappa et al 1979, Meek et al 1982). In comparison to ovalbumin, PAI-2 has a functional, yet inefficient and less hydrophobic secretion signal. PAI-2’s secretion signal was speculated to be a similar N-terminal region, with two sections H1 and H2 responsible for translocation. Fusion of PAI-2’s internal sequence, with bacterial alkaline phosphatase, caused secretion of this normally intracellular protein (Belin et al 2004). Based on hydrophobicity, the amino terminal of maspin resembles PAI-2 more than ovalbumin. Akin to PAI-2, maspin has two potential internal signal sequences at residues 26-42 and 216-278 that could direct the protein to the cell membrane (Pemberton et al 1997, Fitzpatrick et al 1996). Mutation to maspin’s N-terminal region at residues 26-42, by substitution with hydrophobic residues, showed an enhanced secretion of the chimeric protein alkaline phosphatase.
Many studies have shown maspin’s localisation to the ECM and conditioned media of maspin expressing cells (Pemberton et al 1997, Zhang et al 1997a, Ravenhill et al 2010), where it can act as an extracellular protein (Khalkhali-Ellis and Hendix 2007, Blacque and Worrall 2002). A recent observation has shown that maspin is an intracellular nucleocytoplasmic protein which cannot be secreted, with any maspin protein found extracellularly a consequence of cell damage (Teoh et al 2010). The absence of higher molecular weight forms of maspin showed that it was not glycosylated at any of its three potential cell surface asparagine residues. It was thus concluded that maspin was not able to undergo classical or unconventional secretion. Fusion of a classical signal peptide to the start of maspin cDNA directed maspin into the endoplasmic reticulum where it sequestered and was unable to move to the Golgi for export (Teoh et al 2010). This study also demonstrated using immunofluorescence that endogenous maspin was not located at the cell surface of normal mammary epithelial MCF10A cells, a finding that was previously shown by confocal microscopy (Cella et al 2006).

Maspin lacks a canonical nuclear localization signal, yet can be translocated into the nucleus. Nuclear translocation has been reported a common clade B family trait (Izuhara et al 2008). Translocation in the absence of a localisation signal can occur either by binding to a chaperone protein, diffuse passively across the nuclear pore complex or by an alternative mechanism; such as phosphorylation (Schlenstedt et al 1996, Khalkhali-Ellis et al 2006). Phosphorylation sequences of the signal-independent proteins ERK1/2, SMAD3 and MEK1 have been associated with their nuclear accumulation (Chuderland et al 2008). There is no comparable documented evidence regarding maspin, nor any conclusive evidence regarding the likely passive diffusion of maspin due to its small size of 42 kDa.

Specific subcellular localisations of maspin have been associated with different stages of tumour suppression. For example, nuclear maspin expression in invasive breast cancer is associated with a poor prognosis. Nuclear maspin correlates with the positive expression of oestrogen and progesterone receptors; markers shown to enhance the induction and growth of breast cancer. In comparison, cytoplasmic maspin correlates with a negative expression of oestrogen and progesterone receptors (Mohsin et al 2003). In contrast to the nuclear expression of maspin in breast cancer, nuclear maspin has been found to distinguish cancers with favourable clinical-pathological features. For example, the transformation of bronchial epithelia, aided by cigarette smoke concentrates, shifts a predominantly nuclear expression to one that is mainly cytoplasmic. Such decrease in nuclear maspin expression correlates with increased histological grade, proliferation and invasion of pulmonary adenocarcinoma (Lonardo et al 2005). Further evidence has
shown a nuclear partitioning of maspin in melanomas of the skin and in laryngeal carcinoma to be important for its tumour suppressive functions and association with better cancer prognoses. Specifically, nuclear maspin shows an inhibition of angiogenesis, as measured by an elevated microvessel density and tumour thickness, and an increase of cell sensitivity to apoptosis respectively (Chua et al 2009, Marioni et al 2008). Since some tissues, such as the ovary and pancreas, up-regulate maspin upon transformation, its expression has been speculated to be functionally inactive. However, the subcellular expression of maspin in these cancers were much the same as that observed for cancers that down-regulate maspin, such as bronchus and larynx discussed above. Maspin was primarily located in the cytoplasmic compartment of ovarian tumour samples and pancreatic cancer cell lines compared with benign and low malignant tumours (Sood et al 2002, Maas et al 2000). Therefore understanding the mechanism of action of maspin is critical to understanding maspin’s peripatetic expression profile.

There is evidence that shows maspin exerts different effects, depending on whether it is extracellular or intracellular. Administration of extracellular maspin to human umbilical vein endothelial cells showed no increase in cell death, yet apoptosis increased when cells were endogenously transfected with maspin (Li et al 2005). Analogously, intracellular maspin is critical for apoptotic induction in staurosporine sensitised melanoma MDA-MB-435 cells, yet extracellular maspin has no effect (Jiang et al 2002). Conversely, there are some instances where extracellular and intracellular maspin act similarly. The addition of recombinant maspin or the transfection of maspin both increase cell adhesion to the ECM, by changes to the cell integrin profile (section 1.12) (Seftor et al 1998). Likewise, maspin expressing transfectants of mammary or prostatic cancer cells decrease cell motility and invasion, as does incubation with exogenous recombinant maspin. Maspin was found primarily responsible for this anti-invasive effect as treatment with an anti-maspin antibody converted the tumour cells back to a motile, invasive state. Imaging of recombinant maspin bound to the cell surface of melanoma MDA-MB-435 cells indicates its invasion and motility activities are cell membrane associated (Sheng et al 1996).

It has been reported that extracellular maspin plays a key role in extracellular matrix remodeling, in particular its ability to up-regulate focal adhesion contacts (section 1.8.2) (Seftor et al 1998). Extracellular recombinant maspin was efficiently internalized, showing cytoplasmic fractions after 1 hour incubation. This led to the hypothesis that, initial cell surface molecular interactions and a possible inside-out signalling mechanism leads to internalized cytosolic maspin which may function similarly to endogenous maspin. Like its mechanism of export, an internalization mechanism has not been recognized (Odero-
Marah et al 2003). Also, extracellular maspin was a key contributor to high levels of paracrine anti-angiogenic activity in senescent keratinocytes. Application of maspin conditioned media from senescent keratinocyte cells was shown to up-regulate endogenous maspin, which in turn reduced endothelial cell migration in the presence of angiogenic factors (Nickoloff et al 2004).

It has been shown using an in vivo murine model of corneal wound reconstruction, that intracellular and extracellular maspin work together. Maspin expression showed a specific down-regulation in corneal keratocytes distal from the wound and as a result, differentiated into motile fibroblasts and myofibroblasts. Differentiated keratocytes migrated into the wound, where they responded to extracellular maspin secreted by neighbouring corneal epithelial cells; which increased their adhesion to the cellular matrix (Horswill et al 2008). This suggestion that maspin can act in a paracrine manner has also been reported elsewhere. Normal prostate epithelial cells can secrete maspin to the surrounding prostatic stromal cells which could act to inhibit proliferation, whereas, maspin heterozygote mice showed an increased stromal cell proliferation of the prostate (Shao et al 2008).

1.4.6 Transcriptional Regulation

1.4.6.1 Epigenetic Modulation
The expression of maspin in normal cells is regulated by epigenetic modifications in a cell-type specific manner (Futscher et al 2002). For example, the maspin promoter present in maspin-positive cells of the breast, prostate, skin and oral keratinocytes show no cytosine methylation of CpG islands and no acetylated histones, which correlates to a local accessible chromatin structure. Conversely, maspin-negative cells like gastric epithelia and skin fibroblasts, show aberrant cytosine methylation, hypoacetylated histones and an inaccessible chromatin structure. Demethylation of the maspin promoter sufficiently activated maspin expression, whereas methylation correlated with maspin gene silencing during breast cancer progression (Domman et al 2000, Futscher et al 2002). On the other hand, the transformation of differentiated maspin negative gastric epithelial cells to gastric cancer with intestinal metaplasia showed demethylation of the maspin promoter; leading to disruption of gene repression followed by an increased maspin protein. The functional significance of maspin promoter activation in cancerous cell lines remains unclear and somewhat controversial (Akiyama et al 2003). Furthermore, the epithelial specific expression of maspin between placental and normal somatic tissue was found to be controlled by methylation (section 1.4.3) (Chim et al 2005).
1.4.6.2 Activation at cis Regulatory Sites

The differential expression of tumour suppressive maspin seen between normal and carcinoma-derived epithelial cells is regulated at the transcriptional level. The maspin promoter consists of a 1.2 Kb 5’ flanking region, a non-coding exon 1, followed by a 9 Kb intron 1 sequence and a partial exon 2 sequence, that holds the major transcription start site. Within the maspin promoter there are multiple cis regulatory elements, such as Ets, AP1, AP2 and glucocorticoid response element, but not a TATA box element. Both Ets and AP1 co-operatively transactivate maspin in normal mammary epithelial cells. Loss of maspin promoter activity is seen in metastatic breast cancer cells, which correlate with endogenous mRNA levels, due to the loss of Ets/AP1 co-operation (Zhang et al 1997a). Similarly, the mechanism underlying gene regulation of maspin in normal prostate cells involves the positive transcription Ets elements, but also a negative transcriptional hormonal response element (HRE) which remains functional in carcinoma derived prostate cells. Based on sequence homology, maspin’s promoter HRE element is unique and only binds to the steroid androgen receptor (AR). AR binding suppresses maspin gene activation, possibly via sterical hindrance (Zhang et al 1997b). Patients with prostate cancer treated with androgen-ablation therapy (to reduce tumour cell proliferation and survival) followed by radical prostatectomy, showed an elevated maspin expression; clarifying maspin’s sensitivity to AR (Zou et al 2002).

Gel shift assays identified a binding consensus in the maspin promoter for the transcription factor p53. Prostate tumour cells were irradiated with ultraviolet in vitro in order to cause cellular DNA damage and subsequently induce p53 expression. The induction of p53 thus stopped cellular mitosis to allow for DNA repair and specifically induced the transcription and translation of maspin. Maspin expression was rapidly induced in breast and prostate cancer cells in a wild type p53-dependant manner (Zou et al 2000). It was shown that p53 regulated the maspin gene and subsequently its tumour suppressive effects in vivo. This was determined by the progeny of mice over-expressing maspin in mammary epithelial cells that were crossed with a repressor of p53. The progeny showed that the anti-metastatic effects of maspin decreased; specifically the apoptotic index of these cells reduced along with increased lung metastasis (Zhang et al 2000a, 2002). Correspondingly the inactivation of p53 by mutation, a trait common to the contribution of human cancers, disrupted both DNA repair and the downstream pathways of tumour suppressive genes; maspin and KAI-1 (Mashimo et al 1998). It was subsequently found that maspin expression was induced by a p53-dependent transforming growth factor-β (TGF-β) signalling pathway. TGF-β activated SMAD2/3 protein complex, which translocated to the nucleus to associate with SMAD4 and maspin.
promoter-bound p53. This interaction links the tumour suppressive function of p53/maspin and extracellular cued TGF-β signalling (Wang et al 2007).

Maspin expression in carcinoma derived lung cells was found to be under the transcriptional activation of p63 and not p53 or Ets as reported in breast and prostate cell lines. Unlike breast and prostate tumour cells, cancerous lung tissues correlate with maspin positivity. Despite this ectopic association to tumour histology, p63 bound precisely to the p53 consensus sequence which induced maspin expression and this inhibited carcinoma cell invasion. Therefore in the absence of p53, p63 modulates maspin mediated tumour restraint (Kim et al 2004).

The transcription of maspin is also activated through a stress induced protein kinase pathway. Activating transcription factor-2, phosphorylated by p38 kinase, bound to a cAMP response element in the maspin promoter. The loss of one p53 allele in heterozygous activating transcription factor-2 knockout mice drastically lowered maspin mRNA levels and increased the onset of mammary tumours. This suggested independent mechanisms for the activation of maspin (Maekawa et al 2008). Similarly, the transcription of maspin is up-regulated by the activation of the cell pathway involved in the protection from superoxide toxicity. Specifically, it was found that the over-expression of the anti-oxidant enzyme superoxide dismutase in MCF7 breast cancer cells increased maspin mRNA levels which in turn reduced the malignant phenotype and decreased tumour growth in vivo (Li et al 1998).

1.4.6.3 Hormonal Control
Since maspin’s involvement in mammary gland development was crucial, it remains credible that maspin may be under the control of the primary female sex hormone oestrogen (Zhang et al 1999). Patients with breast cancer show elevated activity of the oestrogen receptors and are commonly prescribed the anti-tumour drug Tamoxifen, an oestrogen antagonist. In addition to Tamoxifen’s antagonist effects on oestrogen responsive genes, it displays agonist effects on AP-1, Sp-1 and maspin genes. Northern blot analysis showed Tamoxifen induced maspin mRNA expression in normal mammary epithelial cell lines; specifically through the oestrogen receptor and only via its α isoform. Specifically, the LBD-AF-2 domain of Tamoxifen induced the α-oestrogen receptor, which activated the maspin promoter at a HRE independent cis element between -90 and 87 bp. Therefore, the transcriptional activation of maspin unveiled a possible mechanism of which Tamoxifen exerts its well characterised anti-metastatic effects (Liu et al 2004).
1.4.7 Binding Partners
Maspin's diverse activities, inconsistent tissue expression and varied cellular localisation have suggested the likelihood of more than one non-protease target ligand. This probability stands, with many laboratories having identified several intracellular and extracellular maspin-interacting proteins.

1.4.7.1 Intracellular
Using yeast two-hybrid screening and GSH-affinity pull-down assays, maspin was shown to interact with intracellular histone deacetylase 1 (HDAC1) (Sheng et al. 2006). Western blotting and immunoprecipitation identified the interaction of HDAC1 with maspin in both cytoplasmic and nuclear fractions of prostate cancer PC3 cells. Under physiological conditions, HDAC1 removes histone acetyl groups of its targeted genes Bax, cytokeratin-18 and p21WAF/CIP1. The transfection of maspin into maspin-null prostate cancer DU-145 cells, saw maspin bind to endogenous HDAC1 and convey an inhibitory effect; shown by an increase in acetylated histones and levels of its targeted genes. This inhibitory effect to HDAC1 was dependent on a second maspin interaction with the detoxification enzyme Glutathione S-transferase (GST) (Li et al. 2006). Additionally, it was found that the transfection of prostate cancer cells with maspin showed an increase in GST activity (Yin et al. 2005). Specifically, intracellular maspin/GST interaction was induced via the introduction of oxidative stress, using H2O2 in stably maspin-transfected DU-145 cancer cells. Interaction between maspin and GST enhanced GST activation and thus the cellular response to oxidative stress. This was shown by a reduction in oxidative stress-induced expression of vascular endothelial growth factor and the generation of reactive oxygen species. Additionally, mutation of maspin’s P1 residue significantly reduced its affinity towards GST, suggesting the involvement of maspin’s RCL in GST binding and the cellular response to stress stimuli (Yin et al. 2005).

Other intracellular targets of maspin include chaperone heat shock proteins 90 and 70 (Reviewed in Lockett et al. 2006). Maspin can regulate the cellular localization and function of active AR by competing for its nuclear chaperone protein and HDAC1 binding. Heat shock protein 90 binds to active AR, aiding AR translocation to the nucleus. Acetylated nuclear AR then binds to HDAC1 where cell proliferation and tumour survival genes are activated. This AR-dependent cell survival is negatively regulated by maspin binding to chaperone 90 or HDAC1, rendering AR prone to cytoplasmic proteasome degradation and the inhibition of tumour enhancing genes respectively (Reddy et al. 2006).

Another maspin binding protein was identified from normal human mammary epithelial cells using yeast two-hybrid screening; that of transcription factor Interferon Regulatory Factor 6 (IRF6). Maspin binding to intracellular IRF6 was confirmed through co-
immunoprecipitation of the cytosolic, but not nuclear, fraction of immortalized mammary epithelial cells. Truncation analysis of IRF6 showed contact with maspin via the interferon associated domain, its primary site of protein interaction. The corresponding maspin binding motif remains unknown (Barnes et al 2002, Bailey et al 2006). It was hypothesized that maspin/IRF6 prevented IRF6 translocation to the nucleus; in a similar way that the PAI-2/IRF3 interaction prevents the nuclear translocation of IRF. Hence, the control of transcriptional activation could perturb cellular stress from pathogenic stimuli, maintaining epithelial cell differentiation (Bailey et al 2006).

1.4.7.2 Cell Surface

Despite claims that maspin could directly inhibit tPA and uPA of the plasminogen activation system (Sheng et al 1998), it has since been shown that maspin is incapable of such inhibition (Bass et al 2002). Although maspin has no inhibitory action, another study has shown a possible indirect regulation of tPA and uPA, resulting from the observation that maspin co-localises with cell surface uPA and uPA receptor (uPAR). Antibody-targeted disruption of the uPA/uPAR complex inhibited cell surface binding and internalization of maspin thus confirming this as a cell surface ligand (Yin et al 2006). Specifically, both zymogen and active enzymes pro-tPA/tPA and pro-uPA/uPA bound to maspin independently of maspin’s RCL P1 R340 residue, which suggested the formation of an exosite-exosite complex as opposed to a connection via maspin’s protease active site. Fluorescence resonance energy transfer analysis confirmed the conclusion that maspin’s exosite does not involve its RCL, but did involve a site close-by. It was thus hypothesized that maspin:uPA/tPA complexes could bind to a cell surface receptor via maspin’s intact RCL. An intact RCL could additionally account for the properties of maspin’s-RCL in vivo (section 1.4.4) (Al-Ayyoubi et al 2007).

A novel interaction between maspin and the α2 chain of collagen subtype I, and also subtype III, but not types II, IV and V was identified (Blacque and Worrall 2002). These interactions were verified using matrix affinity agarose and immunoblot assays. Maspin-collagen I binding was shown to be covalent with a dissociation affinity constant ($K_D$) of 0.63x10$^{-6}$ M. Further yeast two-hybrid screening with N-terminal truncated maspin cDNA recognized specific collagen I interaction with maspin fragment 112-214 residues. Interestingly, the ovalbumin family member PEDF showed identical collagen binding specificity to subtypes I and III with a similar $K_D$ to maspin (Blacque and Worrall 2002). The ability of both maspin and PEDF to bind to collagen subtypes at the cell surface likely contributes to their observed increase of cell adhesion. Maspin and PEDF both have shown an inverse correlation to the progression of cancer (Ngamkitidechakul et al 2003). Like the crystal structure of maspin, PEDF held a negatively charged α-helix G and an
asymmetrically charged α-helix D (Law et al 2005, Simonovic et al 2001). The former electrostatic region has been implicated for PEDF’s collagen binding (Meyer et al 2002). However, the involvement of the latter α-helix D region would be the most probable maspin binding site for collagen I based on the identified binding fragment (Blacque and Worrall 2002). Also akin to maspin, PEDF has the ability to suppress tumour angiogenesis. Mutation to the collagen binding site of PEDF was unable to abolish tumour angiogenesis, as it was later demonstrated that neovascularization occurs through endothelial integrin engagement to collagen I. Therefore, direct binding of PEDF or maspin to collagen I could modulate integrin-ECM involvement, resulting in tumour anti-angiogenesis (Zhang et al 2006, Blacque and Worrall 2002). Correspondingly, maspin has been found to directly bind to epithelial cell integrin β1 and to integrin α5β1 (section 1.12) (Cella et al 2006, Bass et al 2009). Maspin also induced phenotypic changes to MDA-MB-435 carcinoma cells, an effect accompanied by an altered integrin expression profile (Seftor et al 1998).

Maspin has been shown to directly interact with the complement-like repeats of low density lipoprotein receptor-related protein (LRP) isoforms, using fluorescence titrations and native gel electrophoresis (Al-Ayyoubi 2007b). This interaction was sufficient to stimulate maspin internalization (Yin et al 2006). LRP typically only binds and promotes internalization of serpin-enzyme complexes; such as antithrombin-thrombin (Kounnas et al 1996). However, this specificity is not always the case; both tPA bound neuroserpin and native neuroserpin (plus the addition of un-identified co-factor) bound to and were internalized by LRP (Makarova et al 2003).

The impressive list of maspin’s direct molecular interactions reflects its central role in regulating epithelial cell homeostasis. Maspin’s pleiotropic nature is resultant of its involvement with numerous proteins which form a network of interactions. This thesis concentrates on the effects of maspin on the cell cytoskeleton and its interaction with factors involved in cell migration and adhesion. The following sections show an overview of the processes involved.

1.5 Cytoskeleton
The cellular cytoskeleton is a dynamic structure that maintains cell shape, provides protection and aids cellular motion. The cytoskeleton is composed of three distinct cytosolic filaments structured as ordered polymers; intermediate filaments, actin microfilaments and microtubules. Intermediate filaments function to organise the internal three-dimensional structure of the cell, anchoring the organelles and serving structurally for the nuclear lamina and sacromeres. Actin participates in cell-cell and cell-ECM
adhesion and signal transduction as well as providing cellular extensions necessary for directed cell movement. Microtubules form pathways for intracellular transport, form the mitotic spindle essential for mitosis, and serve structurally as cilia. Cellular morphogenesis, migration and adhesion all require the cell to extend, retract, and stabilise, all of which is aided by the cytoskeleton. Co-ordination between cytoskeletal elements is regulated by Rho GTPase signalling pathways and evidence of direct regulation explains their overlapping functions (Ridley et al 2003).

1.5.1 Actin
Actin is the most abundant protein in eukaryotic cells. Actin exists as a globular monomer; G-actin, which binds a Mg\(^{2+}\) ion and one molecule of ATP. G-actin monomers have two poles, one named the plus/barbed end which holds the ATP binding cleft, and the other termed the negative/pointed end. This section is reviewed by Pollard et al 2000 and Pollard and Borisy 2003.

1.5.1.1 Actin Nucleation
The generation of filamentous F-actin is initiated by the nucleation of three G-actin monomers. Under physiological conditions, spontaneous nucleation is slow due to their instability. However, once formed, actin filaments add to the trimer rapidly, polymerising in a head-to-tail manner to form a linear, helical chain of F-actin. After a period of growth an equilibrium phase known as treadmilling, is reached; where there is no net change in filament mass; G-actin monomers are added and disassembled at the same rate (Carlier et al 1997). Nucleation and subsequent polymerisation of existing F-actin filaments can be induced by actin related protein Arp2/3 complex. Arp2/3 anchors to free barbed ends and drives the nucleation of a new F-actin polymer at an angle of 70°. After initiating nucleation, the Arp2/3 complex becomes incorporated into the filament network.

1.5.1.2 Actin Elongation
Cells maintain a highly concentrated pool, approximately 100 µM, of unassembled Mg\(^{2+}\)-ATP bound G-actin. The concentration of monomeric actin controls the rate of polymer elongation. The ratio of dissociation: association equates to the critical concentration of Mg\(^{2+}\)-ATP-G-actin. At conditions where Mg\(^{2+}\)-ATP-G-actin is higher than the critical concentration actin will polymerise, and conversely will depolymerise when the ratios are low. The critical concentration of ADP-actin at the minus and plus ends of F-actin are the same, whereas ATP-actin has a higher critical concentration at the minus end. Thus, the rate of plus end elongation is dependent on the concentration of un-polymerised ATP-actin monomers. This varies between cell types and between cellular concentrations of actin monomer binding proteins, which subsequently alters the rate of elongation.
Capping proteins have been shown to bind at both the plus and minus ends of F-actin, where they prevent the exchange of actin monomers. Un-capping or severing of existing F-actin therefore promotes polymerisation. Capped proteins dissociate from actin filaments very slowly, thus without assistance, the actin filament rarely re-elongates. On the other hand, interaction of capping proteins with cell membrane phosphoinositides can aid uncapping and thus contributes to re-elongation; specifically towards the cell edge. Directional elongation is tightly localised and upon initiation accelerates rapidly, which is critical for cell polarity in response to stimulus and during cell movement. In addition to phosphoinositides, membrane bound GTPases and membrane lipid tyrosine kinase receptor-activated Src Homology 3 proteins, too contribute to polarisation of actin protrusion by activating localised nucleation-promoting factors.

1.5.1.3 Actin Depolymerisation
Shortly after actin monomers have been incorporated into a filament their ATP is irreversibly hydrolysed followed by the slow removal of γ-phosphate. This marks the filament for depolymerisation by actin depolymerising factor (ADF)/cofilin. ADF/cofilin binds to ADP-actin at a higher affinity than ATP-actin or ADP- γPi. Thus phosphate dissociation is rate limiting in ADF/cofilin dissociation and debranching of F-actin. The actin binding molecule tropomyosin can protect ADP-actin filaments from ADF/cofilin disassembly. Also, ADF/cofilin is inactivated via phosphorylation which is mediated by activation of GTPase Rho. This controls the rate of turnover and may coordinate with Arp2/3 pathway for regulating filament initiation.

Myosin is a motor protein that can bind to and walk along F-actin towards its plus end. Upon binding to actin, myosin-ATP hydrolyses releasing its phosphate which triggers the power stroke of myosin to move the actin filament. ATP then replaces myosin-ADP and the process can recycle. This power stroke of myosin-II generates a sliding force on the actin polymers, causing them to contract; crucial for muscle contraction and cytokinesis. Acto-myosin contraction forms bundles of actin known as stress fibres.

1.5.1.4 Actin-monomer Sequestering Proteins
Actin monomer sequestering proteins maintain a highly concentrated pool of Mg²⁺-ATP-G-actin monomers in the cell cytoplasm, which regulate the localisation and availability of monomers for polymerisation. Hence in order to prevent a massive explosion of actin polymerisation, free barbed F-actin ends are rapidly capped by capping proteins, such as the abundant gelsolin. Typically, a free plus end will elongate by >500 monomers per second, before it is stabilised by the binding of actin capping proteins; preventing further association and disassociation.
Profilin and thymosin β4 are two of the main actin-monomer binding proteins that prevent the spontaneous assembly of actin. Profilin and thymosin β4 compete for the binding of actin at a stoichiometry of 1:1. They both bind to ATP-actin 50 fold stronger than ADP-actin, of which profilin has the higher affinity (Goldschmidt-Clermont et al 1992). Thus, profilin holds ATP-actin ready for polymerisation whilst thymosin β4 maintains a reserve pool of ATP-actin that does not polymerise. Profilin shuttles ATP-actin away to barbed end filaments, and just as efficiently as free actin, promotes filament elongation. However, unlike free actin, actin-profilin does not extend the pointed ends since its binding site is hidden by the neighbouring monomer. Nevertheless, pointed ends are also capped to control polymerisation, as the affinity of actin nucleator Arp2/3 complex does not discriminate.

### 1.5.2 Microtubules

In addition to structural support, microtubules are involved in many essential cell functions; such as mitosis, vesicle/organelle motility, cell cycle progression and the control of morphogenesis. They are well adapted for these functions, since microtubules have multiple stability states and degrees of organisation which allows them to respond rapidly and effectively direct molecular transport.

Microtubules are non-covalent polymers of αβ tubulin heterodimers. Both α and β subunits bind one molecule of GTP, which is necessary for polymerisation. Heterodimers arrange in a head-to-tail manner into linear protofilaments, which vary in length from 200 nm to 25 μm. The protofilaments develop as a flat sheet which later associates laterally and closes to form 25 nm wide, robust, cylindrical hollow tubes with an intrinsic resistance to bending and compression (Nogales et al 1998). Microtubules are polar with a fast growing end and a slow growing end denoted the plus and minus ends respectively. The α-monomer is exposed at the minus end and the β-monomer orients towards the plus end. Lateral interactions between protofilaments are α-α and β-β except at the central seam region (Margolis et al 1998).

Microtubules primarily nucleate from microtubule organising centres such as centrosomes and basal bodies, but also from the Golgi complex. Specifically, γ-tubulin localises to centrosomes where it forms a ring structure which serves as the initiation site for microtubule nucleation (Wehrle-Haller and Imhof 2003). Like actin, microtubules use intrinsic nucleoside triphosphate hydrolysis. Shortly after polymer incorporation, β-tubulin hydrolyses its GTP and releases its γ-phosphate (Nogales et al 1998). GDP-tubulin is very unstable, thus the leading β-monomers remain GTP-bound or GDP-γ-phosphate-bound, which act as a protective cap from dissociation. Sometimes though, GTP
hydrolysis of the plus end microtubule leads to a catastrophe effect; characterised by rapid GDP-tubulin oligomer depolymerisation. Just as occasionally, depolymerising oligomers can change back to a polymerising state; known as rescue (Margolis et al. 1998).

The predominant mechanism governing the kinetics of microtubule polymerisation is dynamic instability. Here, a single microtubule never retains a steady state length, yet continuously switches from spouts of polymerisation and depolymerisation. Also, microtubules frequently exhibit a pause of both polymerisation and depolymerisation. Dynamic instability is measured by the rate of catastrophe/rescue. Polymerisation is dependent on the cellular free tubulin concentration, whereas depolymerisation is not. Soluble tubulin has very slow GTP hydrolysis compared to those oligomer incorporated. Many microtubule associated proteins have been identified, showing direct interaction with the tubulin lattice which stabilise and promote polymerisation. The opposite of such are microtubule destabilising factors (Watanabe et al. 2005).

1.6 Epithelial to Mesenchymal Transition

The transition between two cellular states, epithelial and mesenchymal, is crucial for vertebrate embryogenesis and organ development. During epithelial-mesenchymal transition (EMT), epithelial cells acquire fibroblast-like properties that allow the cell to invade extracellular matrix and migrate great distances. Mesenchymal cells are bipolar cells with an active front end rich in filopodia that drive motility by providing new actin cortex for the myosin endoplasm of the cell to crawl forwards on. Mesenchymal cells are elongated and extended in cell shape, which form irregular structures that are not uniform in composition or density and have weak inter-cellular adhesions. Conversely, epithelial cells form uniform arrays with regular cell-cell junctions, contributing to strong adhesive properties (Thiery et al. 2003). This EMT developmental pathway is exploited during tumour progression and malignant transformation. Here cancer cells show reduced intercellular adhesion and increased motility; a transformation from their epithelial origin. The transition of mesenchymal phenotype to an epithelial one (MET) has been shown relevant in kidney organogenesis and somitogenesis. Additionally, in a non-developmental context, MET has been hypothesised to occur in tumour cells which re-activate certain epithelial properties once they have metastasised to their secondary site (Lee et al. 2006).

The bidirectional EMT transition, which changes the adhesive and migratory properties of a cell, is mediated through an alteration to the cell cytoskeleton. Epithelial and mesenchymal cells are identified on the basis of their unique visual appearance and the
morphology of the multicellular structures they create. There are many reported signalling pathways involved in EMT with numerous transcription factors serving as markers for EMT; known to repress cell adhesion proteins and promote metastasis (Lee et al 2006).

1.7 Cell Migration
The driving force of cell movement is provided by the re-arrangement of the actin cytoskeleton and from new actin polymerisation. Cell migration coordinately alters the directed secretion of membrane components, the control of cell-ECM adhesion, changes in gene transcription and a realignment of the cell microtubular network and Golgi (Watanabe et al 2005).

1.7.1 Cell Polarisation
Migratory cells acquire a polarized morphology; with a directed, protruding leading edge and cell retraction at the rear. Cell polarisation is controlled by many signalling pathways mainly mediated by the Rho GTPases and integrins. At the leading edge of a polarized motile cell, focal contacts anchor the cell cytoskeleton to the ECM, providing a net force of forward traction. The trailing cell edge then disassembles its adhesive contacts allowing retraction to commence by actomyosin contraction (Sander et al 1999). A migrating cell has three filamentous actin structures; lamellipodia, filopodia and actin-myosin bundles, each controlled by Rho GTPase proteins and each linked to the underlying cell matrix by integrins (Ridley et al 2003).

Actin polymerisation occurs almost exclusively at the leading edge where other microfilaments are capped. Two distinct arrays of F-actin can be found at the leading edge of a migrating cell, that of lamellipodia and the lamella. The lamella is situated 3-15µm behind the leading lamellipodia separated by substrate adhesions (Figure 1.7). The lamella is comprised of long un-branched actin filaments, whereas the lamellipodia contains a thick, cross network of short-branched actin more effective in pushing the membrane forwards (Pollard and Borisy 2003). The lamellipodia has high levels of Arp2/3 and tropomyosin, poised for rapid polymerisation, whereas the lamella shows a decrease in tropomyosin with an increase in ADF/cofilin (Ponti et al 2004). Filopodia are also found at the cell edge, where they probe and form small focal complexes with the surrounding environment. By the time the protruding lamellipodia catches up to the small attachment site, it has increased in size forming a stronger focal adhesion and the filopodial extension continues to grow outwards (Ridley and Hall 1992).
Figure 1.7 Schematic of a migrating cell and the structures of its F-actin cytoskeleton. White arrow shows the direction of cell movement defined by a leading and a trailing cell edge. A. marks the lamellipodia with its dense cross-linked actin. B. labels filopodia with its parallel actin protrusions and initial focal contacts (FC). C. highlights stress fibres (SF) present in the less actin-dense lamella. R-Ras activates Rho, which promotes stress fibre formation, which in turn inhibits Rac and Rac-induced lamellipodia formation. Figure adapted from Le Clainche and Carlier 2008.

In contrast to lamellipodia, both filopodia and stress fibres are un-branched parallel bundles of actin formed by myosin II-contraction. They are nucleated by formins and involve the proteins ENA and VASP which together prevent actin capping and branching (Yamazaki et al. 2007). Stress fibres are anchored to the cell membrane and often to the outside of the cell. During cell migration stress fibres present at the rear of the cell, where their anchorage allows the generation of force from filament polymerisation and myosin-driven actin movement. This causes the fibre to contract allowing the rear of the cell to retract and reshape (Nobes and Hall 1999).

In an undifferentiated cell state, microtubules are arranged into a radial array of highly dynamic polymers. Upon differentiation, the microtubule array undergoes spatial re-organisation and rapid remodelling, so that it’s extending plus ends are primarily directed towards the direction of movement. Microtubule re-modelling occurs, whereby protofilaments are temporally captured at specific cortical binding sites in the leading lamellipodia (Watanabe et al. 2005). This interaction provides the force required to re-
arrange the microtubule organising centre and to cluster the small golgi stacks in alignment with cell movement. Differentiated cells are thus polarised with an asymmetrical distribution of signalling molecules and by the formation of a subset of unusually stable microtubules. In most cell types, both the perturbation of either actin or microtubule dynamics reduce lamellipodial activity and block membrane protrusion (Etienne-Manneville et al 2004).

1.7.2 Rho GTPase Signalling During Migration

The Rho GTPases are a small subfamily of the Ras superfamily, known for their distinct functions in actin cytoskeleton arrangement and cell migration, including Rac, Cdc42, and RhoA. Rho GTPases are active when bound to GTP and inactive when bound to GDP. They are activated by guanine exchange factors or inactivated by GTPase activating proteins (Moissoglu et al 2006).

During in vitro wound healing assay, motile cells present at the wound edge show actin rich lamellipodia and filopodial extensions from the leading edge adhered by small focal complexes. As cells from both sides of the wound edge come into close contact, cell ruffling at the leading edge quickly disappears and the cell-cell monolayer is re-formed. Using this well established cell model, the role of Rho GTPases on coordinated cell migration was studied (Nobes and Hall 1995). They found that motile cells have high levels of Rac1 and Cdc42 as opposed to low levels of RhoA (Figure 1.7). Rac1 specifically induces and regulates flat membranous lamellipodial protrusions, whilst Cdc42 regulates the formation of filopodia and actin microspikes; essential for cell movement. Specifically, Rac1, Cdc42 and PIP2 are located at the front of the cell with Rho at the rear. PIP2 localises Cdc42 to the leading edge where P13 kinase is activated, which consequentially activates downstream Rac1 and inhibits RhoA. Cdc42 along with CLIP170 and EB1 can also orientate microtubule networks towards the cell edge. Unlike Rac1 and Cdc42, RhoA controls the formation of actin-myosin filament bundles to form stress fibres and focal adhesions (Ridley and Hall 1992). RhoA activity is not essential for cell movement, but a basal RhoA is needed for cell adhesion. Constitutively active RhoA showed a decrease in cell motility, characterized by thicker, larger FAs. RhoA activation of its effectors mDia and Rho kinase/p160ROCK increased the phosphorylation of myosin light chain, and thus increased myosin-driven actin formation of stress fibres (Nobes and Hall 1999, Ridley et al 2003).

The activities of Rac and Rho have been shown antagonistic with coordinated activities crucial for effective cell migration (Nobes and Hall 1999). For example, the Rac-dependent small focal complexes of the leading lamellipodia mature into Rho-dependent
focal adhesions as the cell crawls over them; a process taking a few minutes. Hence it was proposed that cycling of the antagonistic activities of Rac and Rho at the front of the cell were coordinated by a regulatory molecule. The molecular switch between Rac and Rho was later found to be Ras-related protein R-Ras. At the start of cell migration, R-Ras activity was initially low to allow for GTP-Rac-dependent lamellipodial cell spreading. This was followed by new integrin attachment to the cell substratum which activated R-Ras causing localization along with Rac/Rho to the leading edge. R-Ras increased GTP-Rho which in turn decreased GTP-Rac to allow for strong focal adhesion attachment and cell crawling to commence (Figure 1.8) (Wozniak et al 2005). In addition to R-Ras induced GTP-Rho inhibition of GTP-Rac, active Rac has been shown to activate Rho creating a negative feedback loop (Wennerberg and Der 2004).

The continuous net addition of G-actin at un-capped filament barbed ends pushes the plasma membrane forwards. Rapid polymerisation at the leading edge must be met with a continuous supply of Arp2/3 complex. Arp2/3 complex is localized to the cell membrane via its interaction with membrane-cytoskeletal protein, vinculin. Arp2/3 must first be activated by the binding of WASp/Scar proteins, which integrate diverse signals including those of Rho family. WASp/Scar proteins are themselves activated by the binding of both membrane phospholipids and GTP-Cdc42. In vivo, there is limited cellular concentration of WASp/Scar protein compared to an abundance of Arp2/3, suggesting the former is the limiting factor in actin polymerisation. WASp proteins bind to Arp2/3 via its acidic A domain, its WASp homology domain binds actin monomers and its polyproline domain binds SH3 containing proteins, for example profilin. WASp proteins are intrinsically inactive, but are activated by the binding to GTP-Cdc42. The activation of Arp2/3 by WASp has been suggested to be via allostERIC conformational change upon WASp binding or via active recruitment of actin monomers to the site of nucleation through binding of SH3 domain to profilin. Scar protein is an effector of Rac, which also stimulates Arp2/3 nucleation and the formation of lamellipodia (Pollard et al 2003).
Figure 1.8 Schematic showing common Rho GTPase signalling pathways leading to the formation of distinct actin structures. Cdc42, Rac and Rho alternate between active-GTP bound and inactive-GDP bound by GTPase activating proteins (GAP) or guanine exchange factors (GEF). Other Rho GTPase signalling pathways including those divergent at WAVE (Wiskott-Aldrich Syndrome Protein (WASp) Family Verprolin Homology Domain-Containing Protein), PAK (p21-activated kinase) and P1(4)P5 kinase have not been included for simplicity.

1.8 Cell Adhesion
Cell adhesion provides anchorages, cues for migration and signals for growth differentiation. There are two classes of cell adhesion; cell-cell adhesion or cell-ECM adhesion.

1.8.1 Cell-Cell Adhesion
E-Cadherins are transmembrane glycoproteins which mediate epithelial cell–cell junctions, by linking to either actin filaments in adherens junctions via catenins, α-, β-, and, γ- or to intermediate filaments in desmosomes. At an adherens junction, the cytoplasmic domain of E-cadherin binds β-catenin, which in turn binds α-catenin, its binding proteins VASP and Mena and other membrane-cytoskeletal proteins necessary
for actin polymerisation of intercellular epithelial sheet formation. The extracellular domains of E-cadherin change conformation under the influence of intracellular calcium upon intercellular contact with a homotypic cell. At sites of intercellular contact, calcium-activated actin-filled filopodia extensions from both cells make antiparallel contact and incorporate into opposing cells, thus drawing the cell surfaces together.

The E-cadherin-β-catenin complex plays an essential role in the control of epithelial cell architecture and differentiation. The loss of the E-cadherin-β-catenin adhesion is a critical step in the progression of many epithelial malignancies. Down-regulation of E-cadherin promotes translocation of β-catenin to the nucleus. Under the influence of the Wnt signalling pathway, nuclear β-catenin complexes with the TCF/LEF family of transcription factors, which promote EMT. Conversely, the introduction of E-cadherin alone into fibroblast cells, impedes the role of β-catenin as a transcriptional activator, and thus increases cellular adhesion and decreases cell migration. E-cadherin is controlled via several EMT activation signalling pathways, including that of transforming growth factor beta (TGFβ), fibroblast growth factor and hepatocyte growth factor. Regulation of β-catenin by the Wnt signalling pathway results in active transcription factor Snail, which is a strong repressor of the E-cadherin gene (Nelson et al/2008).

1.8.2 Cell-Extracellular Matrix Adhesion

1.8.2.1 Integrins
Integrins play a role in cell-cell attachment, but they primarily mediate cell-ECM adhesion where they transmit bidirectional signals across the plasma membrane. Integrins are therefore regulators of many biological functions including cell differentiation, growth, migration and survival. In addition to cell adhesion, integrins regulate matrix deposition and degradation, and are important signalling platforms. Integrins are heterodimeric transmembrane proteins containing two non-covalently associated transmembrane glycoproteins; an α and β subunit (Figure 1.9). To date eight α and 18 β subunits have been identified, which through different combinations can theoretically assemble into greater than 100 possible integrin heterodimers, although only 24 unique integrins have been recognized. Integrins contain a large extracellular domain with a single spanning transmembrane domain and a smaller cytoplasmic domain. The extracellular domain binds to a range of ligands, whereas the intracellular domain anchors to cytoskeletal proteins. An integrin at resting state has a low ligand affinity. The structure of a resting integrin showed the extracellular C-terminal α and β subunits to be in a compact, bent V-shaped conformation separated only by a few Å, and stabilised by specific transmembrane and cytoplasmic interactions (Luo and Springer 2006). Ligand activated-
integrins showed a substantial conformational change. In its active state, the integrin reveals a V-shaped structure of which the ectodomain is extended. This fast and flexible structural change is critical for fast accurate cell signalling (Takagi et al 2002).

![Image](image.png)

**Figure 1.9** Model of integrin activation. Heterodimeric integrins show a large extracellular domain, transmembrane domain and α and β cytoplasmic legs. Individual extracellular domains are labeled. A) Bent conformer shows integrin in an inactive state with a bent extracellular domain. B) Primed conformer shows a straightening of the ectodomain and conformational change of α-helix 7 ready for acceptance of ligand. C) Active conformer shows outward swing of hybrid domain ready for acceptance of an extracellular ligand and separation of α and β cytoplasmic legs to stimulate bidirectional signalling. Figure was adapted from Luo and Springer 2006.

**1.8.2.2 Inside-out Signalling**

Integrins convey bidirectional messages across the cell membrane, from inside the cell to outside of the cell and from outside the cell to the inside. Inside-out activation of integrins results from the binding of cytoskeletal proteins to integrin cytoplasmic or transmembrane domains that are able to induce α/β separation. Many cellular proteins can bind to the α and β cytoplasmic tails, such as kindlins and the F-actin binding protein talin. Talin forms dimers with cell surface phospholipids which recruit the cytoskeletal protein vinculin; which in turn promote and stabilise integrin clustering by providing additional F-actin contacts (Gillmore and Burridge 1996).
Integrins can also be activated through intracellular force transmission. Integrins attached to the cytoskeleton, are moved in the direction corresponding to lateral force exerted by actin cytoskeleton treadmilling or contraction. Cytoskeletal force pulls apart the α and β legs creating an extended active conformation, with high ligand affinity (Zhu et al 2008).

1.8.2.3 Mechanism of Inside-out Signalling
Integrin signalling involves the equilibrium of three conformationally different states; a resting, transient and active state. In the relaxed state the two helices in the transmembrane domain are packed closely together at the exoface domain, which become separated towards the cytoplasmic end. This interhelical interaction is stabilised by two highly conserved motifs present in the cytoplasmic domain, which are involved in intracellular protein interaction. Transformation to the active state requires backbone turn-reversal of the transmembrane interhelical residues, which exposes normally hidden residues to the cytoplasmic interface. Activation collapses both transmembrane and cytoplasmic domains causing separation of α and β helices together with tilting of the β helix. In this active exposed state, integrins can interact with their extracellular ligands (Zhu et al 2009). Activated integrins form homotypic clusters by oligomerization of separated α or β transmembrane domains (Li et al 2003). The transient integrin state shows a constrained cytoplasmic/transmembrane interaction, which has been suggested for α/β priming leading to partial integrin activation/inactivation (Lau et al 2009).

1.8.2.4 Outside-in Signalling
Two possible models for outside-in signalling have been suggested. The first suggested that, upon ligand binding to the extracellular flexible β-leg subunit, the α/β transmembrane/cytoplasmic domains subsequently separate. This separation would invite cytoplasmic tail interactions with signalling complexes facilitating intracellular protein binding cascades (Luo et al 2007). The second model suggests that ligand binding encourages cis-interactions between integrin and/or other cell surface molecules (Arnaout et al 2005). It has been shown that integrin synergistic divalent cation Mn$^{2+}$/Ca$^{2+}$ metal ion binding sites, which underpin the ligand binding site, were occupied prior to ligand binding where they exerted a positive regulatory effect (Zhu et al 2008).

1.8.2.5 Integrin Ligand Binding
The most promiscuous interaction between cell matrix ligand and cell surface integrins was seen via the binding of ligands possessing an Arginine-Glycine-Aspartate (RGD) active site. RGD binds in-between α- and β-legs, with the arginine residue fitting into a β-propeller cleft on the α-leg and the aspartate coordinating to a cation in the β-leg (Xiong et al 2002). Functionally related to the RGD, ligands containing an acidic motif Lysine-
Valine-Aspartate have been postulated to bind to integrins in a similar manner to RGD ligands. Ligands such as collagen and laminin specifically bind to integrins via a glycine-phenylalanine-hydroxyproline-glycine-glutamate-arginine (GFOGER) motif. The crucial glutamate coordinates with a von Willebrand factor A bound cation in the integrin β-leg (Emsley et al 2000). Despite the importance of these RGD-integrin interactions, there is evidence to suggest that other cell surface macromolecules can promote cell adhesion in an RGD independent manner. For example, ADAM-12 and -15 disintegrin domains have shown to interact with integrin α9β1; mediating cell-cell interaction independent of an RGD sequence (Eto et al 2000). Another instance is the interaction between integrin α4β1 with fibronectin at a site other than its RGD sequence; which still promoted fibrillogenesis (Sechler et al 2000).

1.9 Focal Adhesions
Activated integrins form micro or macro clusters on the cell surface at cell periphery, together with cytoskeletal proteins such as vinculin, talin, α-actinin, fillamin, tensin and signalling molecules c-Src, focal adhesion kinase (FAK), and paxillin. These highly organized, specialized cell-ECM contact points are known as focal adhesions that transmit force and tension to maintain strong attachments sites to actin stress fibres, where numerous signalling pathways interact and emanate from. Focal complexes are smaller integrin clusters, found at the tips of filopodia or lamellipodia (Sastry and Burridge 2000).

1.9.1 Focal Adhesions during Cell Migration
In addition to cell surface-sensing entities, focal adhesions play a role in cytoskeletal organisation. Focal adhesions provide traction for a cell to migrate over a substrate, whereas large focal adhesions hinder migration due to excessive adhesion. To balance adhesive forces and migratory cues, focal adhesions switch between a motile and non-motile state. In migratory cells, focal adhesions are fixed relative to the substrate, allowing the cell body to move across them. In stationary cells, focal adhesions are motile and moved to the centre of the cell (Smilenov et al 1999).

A newly formed focal complex at the leading edge matures into a focal adhesion as the cell migrates past, which eventually finds itself at the trailing edge. Internalization of the focal adhesion via an endocytic cycle returns the integrins to the cell surface. The length of time molecules reside on the cell surface between endocytosis varies. The rate of integrin recycling has been hypothesized to contribute to the shape of a moving cell. For instance, a motile cell with a slowly circulating integrin would show uniform distribution, leading to a flattened appearance. However, a motile cell with a rapidly circulating integrin
would show a graded distribution between the leading and trailing edge of the cell, creating a ‘snail like’ appearance (Bretscher 2008).

Focal adhesions can be disassembled by the interaction with microtubules. Microtubules are guided by actin stress fibres towards focal adhesions. This has been postulated to be a direct result from binding to actin motors or indirectly via mechanosensing pathways. Such interaction is of particular importance when focal adhesions at the rear of the cell need to release their substrate during directional cell motility (Etienne-Manneville 2004).

1.9.2 Focal Adhesion Mediated RhoGTPase Signalling

Focal adhesion complex assembly and disassembly are critical for cell attachment and movement. Both the formation and maintenance of focal adhesions and stress fibres are regulated by the GTP-binding protein RhoA, whereas the smaller focal complexes are under the control of Rho proteins Rac and Cdc42 (Ridley and Hall 1995). At the start of cell attachment, RhoA levels are low as a result of activated RhoGAP’s; to allow for integrin mediated membranous extension to the ECM. As integrin mediated cell attachment/spreading increases, simultaneous increase in levels of RhoA and their respective RhoGEFs occurs (Sastry and Burridge 2000). Specifically, Rho-GTP activates Rho kinase which phosphorylates and activates myosin light chain contraction of actin filaments into stress fibres, clustering of integrins and focal adhesion formation. The activation of MLC kinase by Rac/Cdc42 which inhibits actomysosin contraction thus inhibits focal adhesion assembly. Also, the inactivation of RhoA by Ras activated RhoA GAP protein p190GAP decreases cell adhesion and increases cell polarity and migration (Ridley and Hall 1992, Qin et al 2004).

It was found that inhibitors of tyrosine kinase activity could block the formation of focal adhesions, yet integrins lacked intrinsic tyrosine kinase activity (Burridge et al 1996). The cause of this occurrence was found to be FAK; a protein tyrosine kinase which localized to sites of transmembrane integrin receptors. Upon clustering of focal adhesions, activated FAK rapidly autophosphorylates and becomes a docking site for mediators of multiple signalling events which regulate growth, survival and morphogenesis. It has been shown that FAK-null fibroblasts exhibited defects in cell migration corresponding with an elevated number of cell-substratum contact sites. FAK mediates cellular motility by increasing adhesion turnover, through the regulation of the RhoGTPases. FAK is present at the leading lamellipodia, where it enhances Rac-GTP activity and mediates Rho inhibition via Rac GEF and Rho p190GAP activation respectively. At the posterior region of migratory cells, FAK activates paxillin which inactivates the Ras protein Arf; and hence will suppress Rac activation (Chang et al 2007).
1.10 The Effect of Maspin on the Regulation of the Cytoskeleton

Previous work has shown that overexpression of maspin in invasive carcinoma cells altered ~27% of the detectable proteome, of which were a large number of proteins that regulate cytoskeletal network. These changes in protein expression predicted a less invasive/motile phenotype, which was accompanied by change in cell morphology. Maspin overexpressing cells showed prominent actin bundling in the form of stress fibres which reflected in vivo properties of reduced metastatic spread (Chen et al 2005). The non-motile phenotype of maspin expressing cells showed few lamellipod protrusions, and an increase in stress fibres and focal adhesions (Seftor et al 1998). Investigation into the signalling pathways underlying the molecular regulation of the cytoskeleton by maspin, found that maspin expressing cells inhibited Cdc42, Rac and the downstream Rac effectors PAK1 and JNK (Odero-Marah et al 2003).

Maspin has been shown to inhibit epithelial growth factor-induced epithelial-mesenchymal transition in esophageal carcinoma cells. The presence of maspin exhibited a change of cell morphology seen by an increase in cell clustering. Also, the ability of maspin to reduce cell proliferation, migration and invasion was detected (Cai et al 2008).

1.11 The Interaction of Maspin with Cell Signalling Pathways

Maspin has been reported to act via several signalling pathways, of which the common RhoGTPases play key roles in controlling cell motility and adhesion. It has been reported that the re-expression of maspin in MDA-MB-231 cells, down-regulated active Rac1 and its downstream effector PAK1. Inactive PAK1 is unable to phosphorylate myosin light chain kinase, preventing the dissolution of stress fibres and focal adhesions. Mediated via a different pathway, inhibition of PAK1 promotes actin depolymerisation, since it can no longer activate LIM kinase and its subsequent inactivation of coflin. Additionally, inactive PAK1 prevents the loss and destabilisation of paxillin in focal complexes. Maspin has also been known to stimulate P13 kinase activity and subsequent pAkt and pERK activation. This maspin-mediated P13kinase/pAkt/pERK activation was independent of Rac and was attributed to aid cell adhesion (Odero-Marah et al 2003). There has also been some evidence to suggest that maspin may hold inherent kinase activity and itself be phosphorylated (section 1.4.4) (Odero-Marah et al 2002).

Maspin has been demonstrated to control cell motility by inhibiting Cdc42/Rac1 activity. Treatment of MDA-MB-231 cells with exogenous maspin saw a decrease in both Cdc42 and Rac1 after 30 minutes or 1 hour respectively, whereas RhoA was unchanged. This wild type maspin mediated inhibition of Cdc42/Rac1 was dependent of an intact RCL and was shown to affect the JNK signalling pathway. Specifically, maspin treated MDA-MB-
231 cell extracts immunoprecipitated with JNK1. The effect of maspin on JNK1 kinase activity was measured via its downstream transcription factor c-jun; which showed a decrease in c-jun phosphorylation/activation. On the other hand maspin did not influence the activity of Rac1-activated transcription factor NFkB. It was proposed that extracellular maspin could bind to the cell surface and transmit an outside-in inhibitory signal to Cdc42/Rac1 and downstream effectors PAK1 and JNK. This RCL-dependent inhibition of Cdc42/Rac1 gave reasoning behind maspin’s attributed decrease in cell motility and invasion (Shi et al 2007). The non-motile phenotype of Rac1 inhibited cells was comparable to that of maspin expressing cells which too showed a decrease in lamellipodia and filopodia formation, with reduced focal contacts at the leading cell edge (Sheng et al 1996). This epithelial-like morphology was attributed to a non-migratory phenotype. It has been suggested that when maspin down-regulates Rac, the antagonistic activity of Rho may be up-regulated. Active Rho can stimulate MLCK, which can subsequently increase the contraction of actin resulting in an increased formation of stress fibres. The tumour suppressive pathway of p53 has been shown to reduce PI3-kinase and Rac1-GTPase activity which in turn causes an inhibition of cell migration. Since maspin can be positively regulated by p53, both molecules may converge to exert these same effects (Zou et al 2000).

1.12 The Interaction between Maspin and Integrins
There are several accounts that show maspin to interact with and/or via cell surface integrins. Recombinant maspin altered the integrin profile of MDA-MB-435 breast carcinoma cell line, which resulted in a reduced invasive phenotype. Specifically, MDA-MB-435 cell integrin profiles were determined by fluorescence-activated cell sorting analysis after 24-hour treatment with recombinant maspin. This showed an induction in cell surface levels of α5 and α3 containing integrins and a reduction in α2, α4, α6 and αV and some β1 containing integrins. Such integrin regulation resulted in an increased cell adhesion and decreased cell invasion through a fibronectin matrix, which was blocked by an antibody to the fibronectin receptor α5β1. Thus, it was concluded that maspin could act indirectly in the adhesion process through influencing cell surface integrin expression (Seftor et al 1998).

In addition to the slow effect of maspin on cell adhesion, via regulating integrin expression, it was shown that maspin could affect cell adhesion early on in the process. Incubation of recombinant maspin with maspin-expressing MCF10A normal breast epithelial cells, showed a five-fold increase in cell adhesion to the endogenous matrix after just 30 minutes. This effect was specifically due to maspin, since maspin antibody and maspin siRNA both decreased cell adhesion. Mutational analyses identified the carboxyl
residues 139-225 of maspin as responsible for maspin's adhesive properties (Cella et al 2006). They also reported on the direct extracellular association between wild type maspin and β1 integrin, as confirmed by co-immunoprecipitation and co-localisation microscopy. The physiological link between maspin and integrin β1 was highly supported by previous observations of maspin deficient mice and β1 deficient mice, which had highly comparable phenotypes (Gao et al 2004, Stephens et al 1995).

Maspin binding to β1 integrin heterodimers α3β1, α5β1, but not αVβ1 was determined. This was identified from the co-immunoprecipitation of de-differentiated vascular smooth muscle cells (VSMC) incubated with extracellular maspin. The addition of exogenous maspin to α5 stably expressing Chinese hamster ovary cells (CHO) inhibited cell migration on fibronectin and laminin matrices, but not collagen type I. On the other hand, maspin added to α5 null CHO cells did not inhibit migration over any matrix. This demonstrated that maspin could exert its inhibitory effect by binding to the non-collagen binding integrin α5β1. Maspin binding to the extracellular region of integrin α5β1 was verified by ligand blotting between maspin and α5β1 ectodomain. Immunofluorescence microscopy established that maspin inactivates cell surface integrin β1. Conformation-dependent antibody staining upon maspin binding to VSMC showed decreased expression of active β1 and increased levels of inactive β1, possibly as a direct consequence of maspin/β1 interaction (Bass et al 2009).
1.13 Hypothesis
Maspin has been widely accepted as a non-inhibitory serpin with tumour suppressive properties. However, the mechanisms behind maspin's anti-tumour activities remain unclear. Based on the crystal structure of this non-inhibitory serpin, we propose that unique structural motifs may potentially contribute to maspin's modulation of cell function. To test this hypothesis and to provide a mechanistic insight, we plan to express and generate recombinant wild type maspin protein alongside novel mutant maspin proteins, with the effect on cell migration and adhesion. Existing data shows maspin can act at the cell surface where it can indirectly influence the degradation of the ECM which may be linked to its direct binding to collagen I and III as well as cell surface LRP, tPA and uPA. Maspin's interaction with the ECM and its ability to inhibit migration and enhance adhesion suggests it can act at the cell surface and potentially directly influence integrins and cell-cell junctions with a re-organisation of the cell cytoskeleton. We propose to test this hypothesis by looking at the direct effect of maspin on cell surface associated components and by the characterisation of maspin expressing epithelial cell-phenotypes.

1.14 General Aims of Thesis

1. To generate a wild type maspin construct suitable for either insect or mammalian expression systems. To carry out site directed mutagenesis at regions postulated to have functional contributions.
2. To produce recombinant wild type maspin protein and maspin mutant proteins.
3. To characterise the effect of recombinant maspin and the re-introduction of wild type and maspin mutant constructs on the cytoskeleton of maspin-null cells.
4. To manipulate the expression of maspin and mutant maspin genes and to examine the effects on cell migration.
5. To investigate the effect of re-introducing maspin or maspin mutant constructs to the cell-cell and cell-ECM adhesion of maspin-null cells. This shall include observation on cell spreading and focal adhesion formation, as well as direct effects of maspin on integrin expression and affinity state.
6. To determine the influence of extracellular matrix on maspin’s biological function, with the aim to clarify whether maspin functions/interacts with the outside of the cell.
2 Materials and Methods

2.1 Materials and Reagents
Cell culture media's and components were all obtained from Invitrogen (Table 2.1). Chemicals used for buffers and solutions, as well as restriction enzymes, were purchased from Sigma, unless otherwise stated (Table 2.2). Primer pairs used for molecular cloning were all obtained from Sigma (Tables 2.3 and 2.4). Primary antibodies used for western blotting and immunohistochemistry were all attained from various sources (Table 2.5). Secondary antibodies used for western blotting were all purchased from DAKO. List of silencer siRNA sequences shown in Table 2.7 were procured from Applied Biosystems and Sigma. Real time polymerase chain reaction specific primers were designed using Primer Express 1.0 software and synthesized by Applied Biosystems or Primer Design. Dual labelled fluorogenic Taqman probes (Universal Probes) were purchased from Sigma. Vectors (Figures 2.1-2.6) and all other reagents were all purchased from Invitrogen, unless otherwise mentioned.
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<thead>
<tr>
<th>Homo Sapiens Cell Line</th>
<th>Description</th>
<th>Culture Medium</th>
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<tbody>
<tr>
<td>PNT1a</td>
<td>Normal prostate epithelial cell line, post-pubertal</td>
<td>RPMI 1640, 10% FCS (v/v), 1M Hepes, 1% Sodium Pyruvate (v/v)</td>
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<tr>
<td>PC3</td>
<td>Initiated from a bone metastasis of grade IV prostatic adenocarcinoma</td>
<td>RPMI 1640, 10% FCS (v/v)</td>
</tr>
<tr>
<td>LNCaP</td>
<td>Originated from a lymph node metastatic lesion of prostatic adenocarcinoma</td>
<td>RPMI 1640, 10% FCS (v/v), 1M Hepes, 1% Sodium Pyruvate (v/v)</td>
</tr>
<tr>
<td>DU-145</td>
<td>Prostate carcinoma cell line taken from brain metastasis</td>
<td>RPMI 1640, 10% FCS (v/v)</td>
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<tr>
<td>MCF7</td>
<td>Epithelial-like human caucasian breast adenocarcinoma</td>
<td>MEM 41090, 10% FCS (v/v), 1% NEAA (v/v), 1% Sodium Pyruvate (v/v)</td>
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<tr>
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<td>DMEM 21885, 10% FCS (v/v)</td>
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<td>Hek293</td>
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<th>Insect Cell Line</th>
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<td>Scheider 2 Drosophila Melanogaster cells</td>
<td>Express Five 10486, 10% L-glutamine (v/v), 10% FCS (v/v)</td>
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**Table 2.1** Cell culture conditions. FCS; heat inactivated Foetal Calf Serum, NEAA; non-essential amino acids, RPMI; Roswell Park Memorial Institute, MEM; Minimum Essential Medium and DMEM; Dulbecco's Modified Eagle Medium.
<table>
<thead>
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<th>Buffer</th>
<th>Components</th>
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<td>TE Buffer</td>
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<tr>
<td>DNA Sample Buffer</td>
<td>20% glycerol (v/v), 10% β-mercaptoethanol (v/v), 0.004% bromophenol blue (w/v), 0.125% Tris-HCL (w/v)</td>
</tr>
<tr>
<td>TAE Buffer</td>
<td>0.04M Tris, 0.001M EDTA, 0.03% glacial acetic acid (w/v)</td>
</tr>
<tr>
<td>Lysis Buffer</td>
<td>50 mM sodium phosphate, 300 mM sodium chloride, pH 8.0</td>
</tr>
<tr>
<td>Elution Buffer</td>
<td>50 mM sodium phosphate, 300 mM sodium chloride, 50 mM L-glutamic acid, 50 mM L-arginine, pH 8.0</td>
</tr>
<tr>
<td>Protein Lysis Buffer</td>
<td>50 mM Tris-HCl, 150 mM sodium chloride, 2 mM EDTA pH 8, 1% Triton X100 (v/v) and Protease Inhibitor Cocktail (Roche)</td>
</tr>
<tr>
<td>Coomassie Blue Solution</td>
<td>45% methanol (v/v), 10% glacial acetic acid (v/v), 0.25% Coomassie Brilliant Blue G-250 (w/v) (Fluka); dH₂O</td>
</tr>
<tr>
<td>Coomassie Blue De-stain</td>
<td>20% methanol (v/v), 5% glacial acetic acid (v/v), 75% dH₂O (v/v)</td>
</tr>
<tr>
<td>2 x Phemo Buffer</td>
<td>68mM PIPES, 25mM Heps, 15mM EGTA, 3mM magnesium chloride</td>
</tr>
<tr>
<td>Phemo Fix</td>
<td>0.74% paraformaldehyde/dH₂O (v/v), 1% glutaraldehyde (25% EM grade), 1% Triton X100 (v/v); 1:1 2x Phemo Buffer</td>
</tr>
<tr>
<td>Lysogeny Broth</td>
<td>1% Tryptone (w/v), 1% sodium chloride (w/v), 1.5% Yeast extracts (w/v); pH 7.5</td>
</tr>
<tr>
<td>Ecoli Buffer 1</td>
<td>100 mM Rubidium Chloride (RbCl₂), 79 mM Manganese Chloride (MnCl₂), 10.8 mM Calcium Chloride (CaCl₂), 7.5 mls (1 M pH 7.5) Potassium Acetate (KAc), 37.5 mls Glycerol</td>
</tr>
<tr>
<td>E.coli Buffer 2</td>
<td>100 mM Rubidium Chloride (RbCl₂), 100mM mM Calcium Chloride (CaCl₂), 37.5 mls Glycerol, 5mls (0.5M pH6.8) MOPS</td>
</tr>
<tr>
<td>Coating Buffer</td>
<td>5 mM sodium carbonate, 35.5 mM, sodium bicarbonate, pH 9.6</td>
</tr>
<tr>
<td>Stabilisation Buffer</td>
<td>50 mM PIPES pH 6.9, 50 mM sodium chloride, 5 mM EGTA, 5 mM magnesium chloride, 1 mM adenosine triphosphate, 1 mM Dithiothreitol, 5% glycerol (v/v), 0.1% β-mercaptoethanol (v/v); dH₂O</td>
</tr>
<tr>
<td>Extraction Buffer</td>
<td>Stabilisation Buffer supplemented with 0.5% Triton-X100 (v/v) and Protease Inhibitor Cocktail (Roche)</td>
</tr>
<tr>
<td>Protein Sample Buffer</td>
<td>4% SDS (w/v), 20% glycerol (v/v), 0.004% bromophenol blue (w/v), 10% β-mercaptoethanol (v/v), 0.125 M Tris-HCL pH 6.8; dH₂O</td>
</tr>
<tr>
<td><strong>Methylene Blue</strong></td>
<td>1:1 ethanol, 0.1 M HCL (v/v)</td>
</tr>
<tr>
<td>-------------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td><strong>Methylene Blue de-stain</strong></td>
<td>45%MeOH: 45%H2O: 10%glacial acetic acid (v/v)</td>
</tr>
<tr>
<td><strong>Hoesch Fixative</strong></td>
<td>75% acetic acid, 25% methanol (v/v)</td>
</tr>
</tbody>
</table>

**Table 2.2** Table of buffers and solutions. Tris; tris(hydroxymethyl)aminomethane, HCL; hydrochloride acid, EDTA; ethylenediaminetetraacetic acid, HEPES; β (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), PIPES; piperazine-N,N'-bis(2-ethanesulfonic acid), EGTA; ethylene glycol tetraacetic acid, SDS; sodium dodecyl sulphate.

<table>
<thead>
<tr>
<th><strong>Gateway Primers</strong></th>
<th>5' → 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>pcDNA3.2 Maspin Gateway Forward</td>
<td>G GGG ACA AGT TTG TAC AAA AAA GCA GGC</td>
</tr>
<tr>
<td></td>
<td>TTC GAA GGA GAT AGA ACA TGG ATG CCC</td>
</tr>
<tr>
<td></td>
<td>CTG C</td>
</tr>
<tr>
<td>pcDNA3.2 Maspin Gateway Reverse</td>
<td>GGG GAC CAC TTT GTA CAA GAA AGC TGG</td>
</tr>
<tr>
<td></td>
<td>GTC AGG AGA ACA G</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Restriction Site Primers</strong></th>
<th>5' → 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMTBiP Maspin Forward + EcoRI</td>
<td>G*AA TCT ATG GAT GCC CTG CAC TAG CAA</td>
</tr>
<tr>
<td></td>
<td>ATT CG</td>
</tr>
<tr>
<td>pMTBiP Maspin Reverse + NOT1</td>
<td>GC* GGC CGC TTG AGG AGA ACA GAA TTT</td>
</tr>
<tr>
<td></td>
<td>GCC AAA G</td>
</tr>
<tr>
<td>pcDNA4 Maspin Forward + Hind III</td>
<td>AAG* CTT ATG GAT GCC TGC AAC</td>
</tr>
<tr>
<td>pcDNA4 Maspin Reverse + EcoRV</td>
<td>GAT* ATC TGC AGG AGA ACA G</td>
</tr>
</tbody>
</table>

**Table 2.3** Table of primer pairs for maspin DNA amplification. Gateway primer pair for the ligation into vector pcDNA3.2 V5 has attachment sites (light blue) and maspin coding sequence residues (purple). Restriction site primer pairs for the ligation into vectors pMT BiP V5 His or pcDNA 4 V5 have ECORI/NOT1 and HindIII/EcoRV restriction sites (blue); start codons (green); stop codons mutated to glutamic acid residue (red) or mutated to alanine residue (orange); *restriction cut site.
<table>
<thead>
<tr>
<th>Site Directed Mutagenesis Primer</th>
<th>5’ → 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E244A</strong> GAT GAG TCC ACA GGC TTG <strong>GCG</strong> AAG AT GAA AAA CAA CTC</td>
<td></td>
</tr>
<tr>
<td><strong>E247A</strong> G ACA GGC TTG GAG ATT <strong>GCG</strong> AAA CAA CTC AAC TCA GAG</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.4** Primers used for site directed mutagenesis of maspin. Maspin sequence showing glutamic acid (E) residue 244 or 247 mutated to an alanine (A) (green).

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Species Antibody Raised in</th>
<th>Concentration used for western blot</th>
<th>Concentration used for immunohistochemistry</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-human maspin</td>
<td>Mouse</td>
<td>2 µg/ ml</td>
<td>25 µg/ ml</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Anti-human profilin 1</td>
<td>Rabbit</td>
<td>1 µg/ ml</td>
<td>-</td>
<td>Sigma</td>
</tr>
<tr>
<td>Anti-E-cadherin</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>1:100</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>Anti-β-Catenin</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>1:50</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>Anti-p44/42 MAPK (ERK 1/2)</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>-</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>Anti-phospho p44/42 MAPK (ERK1/2) (thr202/tyr204)</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>-</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>Anti-phospho MET (Tyr124/1235)</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>-</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>Anti-Akt 1/2/3</td>
<td>Rabbit</td>
<td>200 ng/ ml</td>
<td>-</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Anti-phospho Akt</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>-</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>Anti- human integrin β1</td>
<td>Mouse</td>
<td>10 µg/ ml</td>
<td>2.5 µg/ ml</td>
<td>*1</td>
</tr>
<tr>
<td>Anti- human integrin αV (L230)</td>
<td>Mouse</td>
<td>0.2 µg/ ml</td>
<td>10 µg/ ml</td>
<td>Chemicon</td>
</tr>
<tr>
<td>Anti- human integrin α3</td>
<td>Mouse</td>
<td>1:500</td>
<td>1:10</td>
<td>Chemicon/*2</td>
</tr>
<tr>
<td>Anti- human integrin α5</td>
<td>Mouse</td>
<td>1:500</td>
<td>1:10</td>
<td>Chemicon/*2</td>
</tr>
<tr>
<td>Antibody Description</td>
<td>Species</td>
<td>Dilution</td>
<td>Concentration</td>
<td>Supplier</td>
</tr>
<tr>
<td>------------------------------------------------------------------------------------</td>
<td>---------</td>
<td>----------</td>
<td>---------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>Anti-human integrin α6</td>
<td>Mouse</td>
<td>-</td>
<td>1:10</td>
<td>*2</td>
</tr>
<tr>
<td>Anti-human integrin β3</td>
<td>Rabbit</td>
<td>5 µg/ ml</td>
<td>10 µg/ml</td>
<td>Chemicon</td>
</tr>
<tr>
<td>Anti-human integrin α2</td>
<td>Mouse</td>
<td>-</td>
<td>1:10</td>
<td>*2</td>
</tr>
<tr>
<td>Anti-ligand occupied/active human integrin β1 (12G10)</td>
<td>Mouse</td>
<td>10 µg/ml</td>
<td>2.5 µg/ml</td>
<td>*1</td>
</tr>
<tr>
<td>Anti-inactive human integrin β1 (mAb13)</td>
<td>Mouse</td>
<td>10 µg/ml</td>
<td>-</td>
<td>*1</td>
</tr>
<tr>
<td>Anti-primed/active human integrin β1 (9EG7)</td>
<td>Rat</td>
<td>10 µg/ml</td>
<td>5 µg/ml</td>
<td>*1</td>
</tr>
<tr>
<td>Anti-human integrin α5 ligand bound integrin β1 (SNAKA51)</td>
<td>Mouse</td>
<td>10 µg/ml</td>
<td>5 µg/ml</td>
<td>*1</td>
</tr>
<tr>
<td>Microtubules anti-αTubulin (MαT)</td>
<td>Mouse</td>
<td>-</td>
<td>0.1 µg/ml</td>
<td>Sigma</td>
</tr>
<tr>
<td>Anti-Vinculin</td>
<td>Mouse</td>
<td>-</td>
<td>0.02 µg/ml</td>
<td>Upstate</td>
</tr>
<tr>
<td>Anti-actin</td>
<td>Goat</td>
<td>200 ng/ ml</td>
<td>-</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Anti-Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)</td>
<td>Rabbit</td>
<td>1:5000</td>
<td>-</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>Anti-[SV5-pk] V5 tag</td>
<td>Mouse</td>
<td>1 µg/ ml</td>
<td>-</td>
<td>Abcam</td>
</tr>
</tbody>
</table>

**Table 2.5** Primary antibodies used for western blotting and immunohistochemistry. *1; antibodies were a generous gift from Prof. Martin Humphries, University of Manchester. *2; antibodies were a kind gift from Dr. Fedor Berditchevski (University of Birmingham). Anti-human integrin β1/α6/α2 were preparations of hybridoma supernatants. 12G10/ mAb13/9EG7 (Humphries 2004), SNAKA51 (Mould et al 1995).
<table>
<thead>
<tr>
<th>Secondary Antibody</th>
<th>Fluorophore</th>
<th>Concentration</th>
<th>Absorption/ Emission</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat anti rabbit</td>
<td>Fluorescein isothiocyanate (FITC)</td>
<td>1 µg/ ml</td>
<td>495 nm, 521 nm</td>
<td>Sigma</td>
</tr>
<tr>
<td>Rabbit anti mouse</td>
<td>Alexa Fluor 488</td>
<td>2 µg/ ml</td>
<td>495 nm, 519 nm</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Rabbit anti rat</td>
<td>Alexa Fluor 488/FITC</td>
<td>1 µg/ ml</td>
<td>495 nm, 519 nm</td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stain</th>
<th>Fluorophore</th>
<th>Concentration</th>
<th>Absorption/ Emission</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phalloidin</td>
<td>Alexa Fluor 568</td>
<td>0.2 U</td>
<td>578 nm, 603 nm</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>4', 6-diamidino-2-phenylindole (DAPI)</td>
<td>-</td>
<td>300 nM</td>
<td>358 nm, 461 nm</td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>

Table 2.6 Secondary antibodies and stains used for immunohistochemistry. U; units.
<table>
<thead>
<tr>
<th>siRNA</th>
<th>Sense Sequence 5' -&gt; 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silencer® Negative control siRNA (CTRsi)</td>
<td>-</td>
</tr>
<tr>
<td>SerpinB5 siRNA target exon 5 (#3)</td>
<td>GGAUCUCACAGAUUGCCACTTG</td>
</tr>
<tr>
<td></td>
<td>UGGCCAUCUGUGAGAUCtt</td>
</tr>
<tr>
<td>SerpinB5 siRNA target exon 5 (#4)</td>
<td>GGUGGAAAAGAUUGAUUGAUTT</td>
</tr>
<tr>
<td></td>
<td>AUCAAUCAUCUUUUCACCtt</td>
</tr>
<tr>
<td>Profilin siRNA Mix: (A)</td>
<td>GAAUUUAGCAUGGAUCUUCtt</td>
</tr>
<tr>
<td></td>
<td>GAAGAUCCAUGCUAUAUUCtt</td>
</tr>
<tr>
<td>(B)</td>
<td>GCAUGGAUCUUCGUACCAAtt</td>
</tr>
<tr>
<td></td>
<td>UUGGUACGAAGAUCGAUGCtt</td>
</tr>
</tbody>
</table>

**Table 2.7** Sequence silencer RNAs (siRNAs). Maspin siRNAs #3, #4 and CTRsi were synthesised by Applied Biosystems and Profilin siRNA’s A and B were synthesised by Sigma.

**Figure 2.1** Vector map of pDONR™221. The 4.7Kb pDONR™221 vector was used in Gateway® cloning as an intermediate donor vector. Vector contains attachment sites (att), ccdB cytotoxic coding protein, and kanamycin gene resistance.
Figure 2.2 Map of mammalian vector pcDNA™3.2/V5. Plasmid pcDNA3.2 contains a V5 epitope at the 3’ end of the recombinant gene insert to allow an alternative means of detection. The vector also has Gateway® attachment sites (att) and a human cytomegalovirus (CMV) promoter.

Figure 2.3 Vector map of pGEM® T Easy. Vector was purchased from Promega. The 3.1Kb pGEM® T Easy vector was used in conventional cloning as an intermediate vector using overlapping thymidine (T) residues, forward and reverse primers T7 and SP6 and ampicillin resistance (Amp').
Figure 2.4 Map of insect expression vector pMTBiP™V5/His. Insect competent vector pMTBiP contains a methallothionein promoter; $P_{MT}$, a multiple cloning site; enclosed by brackets, a secretion signal; BiP SS, a V5 epitope and a hexa-histidine tag; 6xHis. Vector has resistance to Blasticidin and comes in three possible reading frames A, B and C.

Figure 2.5 Map of pCoBlast. Selection vector pCoBlast contains blasticidin resistance and the Drosophila promoter Pcopia.
Figure 2.6 Map of mammalian vector pcDNA™4/His. Vector pcNDA4 contains CMV promoter; pCMV, V5 epitope, hexahistidine; 6xHis Tag and SV40 origin of replication (SV40ori). Vector has ampicillin resistance and comes in three possible reading frames A, B and C.

2.2 Equipment
All lab equipment came from Eppendorf or Sigma, unless otherwise stated. All tissue culture treated plastics were Nunc branded, from Thermo Scientific.

2.3 Methods

2.3.1 Recombinant Maspin Expression constructs

2.3.1.1 Maspin Expression Constructs Cloned via Gateway Technology
Maspin-pRSETC was a kind gift from Dr. Margaret Worrall (Blacque and Worrall et al 2002) (University of Dublin). To express the maspin gene in mammalian cells, it was inserted into a mammalian competent vector pcDNA3.2 (Figure 2.1). Wild type maspin cDNA was amplified by polymerase chain reaction (PCR) (Section 2.3.3) from recombinant maspin-pRSETC using primer pairs shown in Table 2.3. PCR product was then extracted (Section 2.3.5) and recombinant maspin cloned into Gateway® vector pcDNA3.2, using Gateway® Technology (Invitrogen) (Section 2.3.2.1) to produce pcDNA3.2-maspin.

2.3.1.2 Site Directed Mutagenesis
Wild type maspin-pRSETC was subject to site directed mutagenesis to generate maspin mutants (performed with Dr. Laura Wagstaff, University of East Anglia). These were cloned into pcDNA3.2 using Gateway® Technology (Section 2.3.2.1). Site directed
mutagenesis was carried out according to manufacturer guidelines (QuickChange XL Site Directed Mutagenesis Kit, Stratagene). Wild type maspin residues lysine K90, arginine R40 and glutamic acids E115, E244 and E247 were all mutated to A; alanine. Primer sequences used for mutant pcDNA3.2-E244A and pcDNA3.2-E247A generation can be seen in Table 2.4.

2.3.1.3 Maspin Expression Constructs Cloned via Conventional Cloning
Wild type maspin and maspin mutant pcDNA3.2 constructs (Section 2.3.1.2) were amplified via PCR using restriction site primers (Table 2.3) (Section 2.3.3). PCR product attributed to the correct size in base pairs was cut from agarose gel and purified (Section 2.3.5). Purified recombinant maspin DNA was either cloned into insect or mammalian competent vector, pMTBiP/V5/His or pcDNA4 respectively, using conventional cloning (Section 2.3.2.2).

2.3.2 Molecular Biology

2.3.2.1 Gateway® Technology
2.3.2.1.1 BP Clonase Ligation
Following DNA amplification using Gateway® primers (Table 2.3), purified DNA was ligated into an intermediate, or donor vector. A BP recombination reaction was used to transfer 150 ng of purified recombinant maspin PCR product containing the gene of interest into 150 ng pDONR™221 donor vector. This was performed using BP Clonase II enzyme and TE buffer according to Invitrogen Gateway® Technology. BP recombination reaction was then transformed and plasmid DNA extracted (Sections 2.3.6, 2.3.7).

2.3.2.1.2 LR Clonase Ligation
The gene of interest contained within pDONR was transferred to pcDNA3.2 destination vector using an LR Gateway® reaction. 150 ng recombined maspin-pDONR, together with, 150 ng pcDNA 3.2, were incubated in 5x buffer containing LR clonase II enzyme at 25ºC for 2 hours. LR recombination reactions were then transformed and colonies underwent PCR screening (Section 2.3.7).

2.3.2.2 Conventional Cloning
2.3.2.2.1 Ligation into pGEM®-T Easy Vector
Amplified maspin constructs were subject to an extra 15 min PCR cycle at 70ºC with 2.5 U of DNA Taq polymerase (Roche). Constructs were then ligated into pGEM®-T Easy vector. The vector pGEM-T has higher ligation efficiency than the target vectors, and would therefore promptly allow the correct sequence alignment of the construct.
Purified restriction site-DNA containing the restriction sites and the vector pGEM were ligated at a 3:1 ratio using the Rapid Ligation Kit (Promega). Based on the equation [vector]x(Kb size of insert)/(Kb size of vector); 50 ng vector was incubated with 54.5 ng purified PCR DNA and 3 U T4 Ligase in ATP containing 2x ligation buffer at 16°C overnight.

1 μl ligation reaction was transformed with 50 μl chemically competent *Escherichia coli* (E.coli) DH5α (Section 2.3.6). Colonies were selected onto Lysogeny Broth (LB) agar containing 100 mg/ ml ampicillin, 2% X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) (v/v) and 10 mM IPTG (isopropyl thiogalactoside). Interrupted open reading frame colonies were picked and grown in 5 ml LB medium. DNA sequencing identified clones that contained maspin cDNA (Section 2.3.8).

### 2.3.2.2 Restriction Digest

Once the correct sequence of DNA plus flagging restriction sites had been verified, DNA insert was excised from 1 μg pGEM vector. 5 U restriction enzymes HindIII/EcoRV or EcoR1/NOT1 were incubated in a 10x reaction buffer at 37°C for 2 hours. Restriction digests were analysed using agarose gel electrophoresis (Section 2.3.4) and linear DNA was extracted using gel purification (Section 2.3.5). Destination vectors were cut with restriction enzymes complimentary to the DNA insert.

### 2.3.2.2.3 Ligation into Expression Vector

Cut insect and mammalian vectors, pMTBiP and pcDNA4 respectively (Figure 2.4, 2.3), were ligated with DNA construct at 1:3 ratio according to the equation [vector]x(Kb size of insert)/(Kb size of vector). 9.6 ng of mammalian vector pcDNA4 was incubated with 9.6 ng maspin DNA insert, T4 ligase and 2x ligation buffer (Promega). Likewise, 6.1 ng of insect expression vector pMTBiP was ligated with 6.9 ng maspin DNA. Ligation reactions were left overnight at 16 °C. Ligation reactions were then transformed using 100 mg/ ml ampicillin, DNA purified and sequenced (Section 2.3.5, 2.3.8).

### 2.3.3 Polymerase Chain Reaction

PCR reaction mix contained 0.5 μg plasmid DNA, 10 pmol primers, 50 mM magnesium chloride, 10 mM deoxyribonucleotide triphosphate (dNTPs) and 2.5 U of Pfu turbo DNA polymerase (Stratagene) combined in a total reaction volume of 50 μl. PCR was carried out for 1 cycle of 94°C for 2 min, 30 cycles of 94°C for 30 sec, 50°C for 30 sec and 72°C for 45 sec, and 1 cycle at 94°C for 30 sec.
2.3.4 Agarose Gel Electrophoresis
Amplified DNA was mixed with 2x DNA sample buffer and electrophoresed on a 1% agarose-TAE (w/v) gel at 100 V. DNA bands were visualised under UV light using ethidium bromide.

2.3.5 Gel Purification of DNA
Following agarose gel electrophoresis, bands corresponding to correct molecular weight were cut from the gel and purified using Qiaex II Gel Extraction Kit (Qiagen). DNA insert concentrations, eluted into sterile distilled water (SdH2O), were measured using a Nanodrop ND-100 (Labtech International).

2.3.6 Transformation
1 μl of recombined vector, was incubated with 50 μl DH5α (Section 2.3.9) for 30 min at 4 ºC. Samples were then heat shocked at 37ºC for five min before returning to 4 ºC, where 250 μl of LB medium was added. Samples were shaken horizontally at 180 rpm in a 37ºC incubator for 1 hour. 100 μl or 200 μl samples were spread onto LB agar containing antibiotic selection (50 μg/ ml Kanamycin, 100 μg/ ml Ampicillin/Chloramphenicol) and incubated at 37ºC overnight. DH5α containing recombined plasmid formed colonies.

2.3.7 Plasmid DNA PCR Screen and Isolation
Ten transformed colonies were selected and each added into 100 μl LB medium containing antibiotic. After four hours incubation at 37ºC, 0.5 μl culture was subject to PCR, with corresponding primer pairs (Table 2.3) to check the insert was present (Section 2.3.3).

Amplified colony inserts were verified using agarose gel electrophoresis (Section 2.3.4). Subsequently, cultures containing appropriate DNA insert were amplified in 3 ml LB medium plus antibiotic during 16 hours at 37ºC with aeration. Culture was centrifuged at 2600 g for five min and the DNA was purified according to Qiagen Spin Miniprep kit instructions.

2.3.8 DNA Sequencing
Plasmid DNA at 100 ng/ μl was sequenced using 1.5 pmol forward and reverse vector primers (Genome Centre, John Innes Centre). Nucleotide sequences were analysed for any mutations against matching homo-sapiens sequences, using National Centre for Biotechnology Information (NCBI), Basic Local Alignment Search Tool. Analysis for in-frame protein translation was performed using expasy.org.
2.3.9 Generating Competent *E.Coli*

Chemically competent *E.coli* strain DH5α was cultured on a LB agar plate at 37°C. One individual colony was put into 3 ml LB medium and left for 17 hours at 37°C. The 3 ml of cultured DH5α were added to 200 ml LB medium and grown to an OD$_{600}$ of 0.3-0.4. DH5α were pelleted at 1700 g, re-suspended in *E.Coli* Buffer 1 and put at 4°C for 15 min. Again, DH5α were spun at 1700 g and re-suspended in *E.Coli* Buffer 2, where they could be stored at -80°C until use.

2.3.10 Insect Cell Culture

*Drosophila Melanogaster* S2 cells were a kind gift from Professor Henrik Gårdsvoll (Finsen Laboratory, Copenhagen). They were maintained according to conditions described in Table 1.2. Cells were grown in suspension at 28°C under normal atmospheric conditions. S2 cells were propagated between a minimum density of 1x10$^6$/ ml and a maximum density of 20x10$^6$/ ml.

Wild Type S2 cells were stocked at -196°C, at 1x10$^7$/ ml density in 450 μl conditioned medium, 450 μl complete medium and 100 μl dimethyl sulfoxide. To defrost the cells, cryovials were warmed at 37°C and the thawed suspension transferred to a 4x volume of complete medium. This was then incubated at 28°C for 15 min, after which cells were centrifuged at 180 g for five min and re-suspended in fresh complete medium.

2.3.11 Transfection into Insect Cells

2.3.11.1 Transient Transfection

Up to 500 μg of pMTBiP plasmid construct was generated using Plasmid Maxi Kit (Qiagen). This high purity DNA was used for the transfection of S2 cells. Maspin expression constructs were transfected alongside the empty vector, as a negative control.

S2 cells were plated into a 6 well plate at 1x10$^6$/ 3 ml complete medium and incubated for 17 hours. Medium was replaced with 20 μg Cellfectin combined with 7 μg plasmid DNA in a total volume of 1.5 ml serum free medium for 24 hours. Following incubation, cells were returned to complete medium with 10 μM copper sulphate (CuSO$_4$), to induce gene expression for 3 to 4 days. Conditioned medium was collected and centrifuged at 180 g to separate cells from conditioned medium, ready for analysis with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) (Section 2.3.13).
2.3.11.2 Stable Cell Population

For stable transfections, S2 cells were adapted to serum free medium. S2 cells were transfected as above, with 0.4 μg of the stable selection vector pCoBlast. After 24 hour incubation, transfection medium was replaced with complete medium. After a further 24 hours, complete medium was changed with the addition of selection agent, 5 μg/ ml Blasticidin-S-HCL. Transfected cells were selected over a three week period, alongside selection of non-transfected S2 cells. During this time, selection medium was changed every four days. When the entire population became stable to Blasticidin, stable cells were then scaled up and adapted to serum free medium. Stable cell lines were maintained, stocked and defrosted in the presence of antibiotic (Section 2.3.10).

2.3.12 Purification of Maspin Protein using Nickel Agarose

2.3.12.1 Protein Extraction from S2 cells

For large scale expression of maspin, stably transfected S2 cells (Section 2.3.11.2) were adapted to serum free medium under selective conditions. When the density of stable S2 cells reached 10x10⁶/ ml, cultures were transferred from tissue culture flasks to 500 ml sterile conical flasks in 200-250 ml fresh medium. Cells were grown in a 27°C shaker at 100 rpm with aeration for 72 hours. Cell clumping in large culture volumes was prohibited by the presence of the medium constituent surfactant Pluronic-64. When densities of S2 reached 15-20x10⁵/ ml, they were induced with 0.5 mM copper sulphate for 3-4 days. At this time, conditioned medium was centrifuged at 1100 g, 28°C for five min and stored at 4°C. Cell pellets were re-suspended and added to 250 ml fresh serum free medium to grow to confluence once more, after which the cells were discarded.

Conditioned S2 medium was transferred to cellulose dialysis tubing of flat width 33 mm (Sigma). This was dialysed into 40 L 20 mM imidazole lysis buffer. Dialysis removed the surfactant Pluronic F-68; known to strip the nickel from nickel agarose during purification and retained molecular weights 12 kDa or over (Drosophila Expression System, Invitrogen).

Dialysed conditioned medium was placed in polyethylene glycol to concentrate its protein content. Concentrated medium was centrifuged for 10 min at 4°C, 2600 g. Any pellet was discarded. The resulting supernatant was de-gassed using a Schlenk flask and filter sterilised with 0.45 μm Whatman membrane filters.
2.3.12.2 First Step Purification

400 ml of conditioned medium was mixed with 1 ml nickel agarose (Ni-NTA) of 45-165 μm beads for five hours at 4°C. The Ni-NTA was moved to a disposable column and washed with 5 ml lysis buffer using gravity flow. Maspin was next eluted from the column, by 5 ml washes with elution buffer containing 20, 50, 100, 150 or 250 mM imidazole. All elution buffers contained 50 mM l-glutamic acid and 50 mM l-arginine which reduced protein aggregation and precipitation during concentration, and also protected the protein from proteolytic degradation (Golovanov et al. 2004). Reducing agent DTT was also supplemented to the elution buffers at 10 mM to prevent intermolecular disulfide bonds. All wash fractions were collected and stored at 4 ºC. Fractions were analysed for detection of maspin protein by SDS PAGE.

2.3.12.3 Second Step Purification

Maspin containing elution fractions, from first step purification, were pooled together and transferred to cellulose tubing. Dialysis in 4 L volume of 20 mM imidazole lysis buffer for 6 hours at 4ºC was performed. Re-dialysed medium was then incubated with 2 ml Ni-NTA for five hours at 4ºC. The Ni-NTA was then transferred to gravity flow disposable column, where the bound maspin was eluted with elution buffer at differing concentrations of imidazole at 20, 50, 100, 150, 250 mM. Collected elution fractions were subject to SDS PAGE followed by western blotting (2.3.13).

2.3.12.4 Final Step

Elution fractions containing maspin were transferred into cellulose dialysis tubing and were dialysed for six hours in x20 volume of lysis buffer without imidazole. Final fractions were ran on SDS PAGE and stained with Coomassie Blue and Silver (Section 2.3.13). Protein concentration was determined (Section 2.3.16).

2.3.13 SDS PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed according to the method of Laemmeli (Laemmeli 1970). Protein samples in presence of SDS were separated at 180 V per gel, alongside known molecular mass markers (BioRad) to provide approximate molecular weights of the unknown. To reduce proteins, 0.1 M DTT was added (Melford Laboratories).

2.3.14 Western Blotting

SDS PAGE gels were transferred to polyvinylidene difluoride membrane using semi-dry transfer at 15 V per gel. Transferred membrane was blocked in 5% non-fat milk (Marvel) (w/v) phosphate buffered saline (PBS)-0.02% Tween 20 (v/v) (Sigma) solution for one
hour at room temperature. Incubation of primary antibody (Table 2.5) diluted in 1% non-fat milk (w/v) PBS-0.02% Tween 20, was performed overnight at 4°C or one hour at room temperature. Blots were washed three times with PBS-0.1% Tween 20 (v/v) for 10 min each. Secondary antibody conjugated to horse radish peroxidase (HRP) (rabbit anti-mouse, goat anti-rabbit, rabbit anti-goat, goat anti-rat) was incubated at 1 μg/ ml for one hour at room temperature. Blots were washed as before. Protein detection was carried out using Enhanced Chemiluminesence (ECL) western blotting substrate (Pierce). Resulting light emission was measured using Fujifilm LAS-3000 with Image Reader LAS-3000 Software. When probing for phosphorylated proteins, PBS was replaced with tris buffered saline (TBS).

2.3.15 Staining of SDS PAGE Gels
SDS PAGE gels were stained in Coomassie Brilliant Blue G-250 solution for one hour at room temperature. Gels were then rinsed and left overnight in Coomassie De-stain. Staining with Coomassie allowed protein detection as low as 50 ng (Neuhoff et al 1988).

One dimensional SDS PAGE gels were stained with Silver Stain, according to Pierce guidelines. Silver staining gave protein detection to levels as low as 0.1 ng. Stained gels were imaged under white light using a trans-illuminator with Quantity One 4.4.6 Image Software (BioRad).

2.3.16 BCA Protein Assay
This assay was used to evaluate protein concentration. The bichinchoninic acid (BCA) assay was carried out to company guidelines (Pierce). The BCA assay combines the well-known reduction of Cu²⁺ to Cu⁺ by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu⁺) by bichinchoninic acid.

2.3.17 Mammalian Cell Culture
Mammalian cell lines were cultured according to the conditions defined in Table 2.1, at 37°C, with 5% CO₂. All cells came from American Type Culture Collection unless otherwise stated (Manassas, VA).

The generation of DU-145 stable cell lines expressing maspin constructs was unfortunately not achieved. However MCF7 stable cell lines were generated by Dr. Laura Wagstaff (University of East Anglia), to express vector only pcDNA3.2, pcDNA3.2-maspin, and pcDNA3.2-E244A. Two isolates of pcDNA3.2-maspin cells were produced. Both isolates showed similar levels of maspin protein expression, therefore one clone was
chosen at random for subsequent passaging and cell-banking. Only one isolate was identified for pcDNA3.2-E244A and pcDNA3.2 stable cells. Stable cells were maintained under wild type cell line conditions with Geneticin G418 selection at 500 μg/ml. Stable cells were frozen containing antibiotic.

Mammalian cells were stored at -196°C, at 1x10^6/ml density in freezing medium (800 μl complete medium, 100 μl FCS and 100 μl dimethyl sulfoxide). To defrost the cells, cryovials were warmed at 37°C for three min. Cell suspension was added to 50 ml complete medium pre-warmed to 37°C and centrifuged at 180 g for five min. Cells were then re-suspended in fresh complete medium.

2.3.18 Transfection into Mammalian Cells

Cells were plated at 2x10^5/ml and incubated for 17 hours in complete medium. According to Fugene 6 manufacture instructions (Roche), Fugene 6 transfection reagent was incubated with 1 μg plasmid DNA. Transfection mixture was added to cells in serum free medium and placed at 37°C. After 24 hours conditioned serum free medium was removed and replaced. 48 hours after the transfection, conditioned medium was collected and cells were lysed (Section 2.3.20). SDS PAGE was carried out to detect protein of the expressed plasmid in both cell medium and lysate (Section 2.3.13).

2.3.19 Collection of Maspin Protein from Mammalian Cells

2.3.19.1 Transfection of COS-7 cells

COS-7 cells were transfected with pcDNA4 constructs (Figure 2.3), where under CMV expression the gene of interest was expressed at high output. COS-7 cells were plated at 3x10^5/5 ml complete medium in a 9 cm dish. After 17 hours under normal conditions, cells were washed twice in 5 ml sterile PBS and replaced with 4 ml serum free medium. Transfection mix containing 7.5 μl Fugene 6 and 290 μl serum free medium was incubated for five min at room temperature. Next, 2.5 μg plasmid DNA was added to the mix and incubated for 15 min. After incubation, transfection mix was added to the dish of COS-7 cells and swirled gently to mix. Fresh serum free medium replaced transfection medium at 24 hours. Cells were left to proliferate over 4 to 5 days, when all CMV-gene of interest transfected cells had died.

2.3.19.2 Extraction of Maspin Conditioned Medium

Conditioned medium from COS-7 transfected cells was collected and centrifuged free of large cell debris at 180 g for five min. Pooled volumes of 200 ml conditioned medium was transferred to 33 mm dialysis tubing and concentrated in PEG for six hours. Expression of
maspin constructs was qualitatively quantified using SDS PAGE and western blot (Section 2.3.13, 2.3.14).

2.3.20 Preparation of Cell Lysates
Cells were washed and isolated in PBS. After centrifugation at 2000 g for five min, cell supernatant was re-suspended in protein lysis buffer. If cell proteins to be analysed were phosphorylated proteins, cells were washed in TBS and protein lysis buffer was supplemented with phosphatase inhibitors; 1 mM sodium orthovanadate and 20 mM sodium fluoride.

2.3.21 TCA Precipitation
To concentrate protein, DNA or RNA macromolecules, trichloroacetic acid precipitation was performed. Conditioned cell medium samples were mixed at 1:3 ratios with 10% TCA/dH₂O (v/v) and incubated at 4°C for five min. Samples were centrifuged at 10 000 g, 4°C, for five min and the pellet washed twice in acetone chilled to 4°C. Supernatant was discarded, pellets air dried and re-suspended in protein sample buffer ready for SDS PAGE (Section 2.3.13).

2.3.22 Protein Glycosylation
To detect protein glycosylation, cell samples were incubated with 1 U N-glycosidase F (Roche) for two hours at 37°C. Protein samples were then subject to SDS PAGE and western blotting (Section 2.3.13, 2.3.14).

2.3.23 Immunohistochemistry
Circular coverslips were pre-treated overnight at 4°C with 5 µg/ml matrix components; Collagen type 1 (Sigma), Fibronectin or Laminin (Sigma). Cells were plated onto coverslips at 0.05x10⁶/ml. After 17 hours incubation, coverslips were washed twice in PBS at 37°C prior to fixation.

2.3.23.1 Paraformaldehyde Fixation for Cellular Proteins
Cells were washed in PBS and fixed with 4% paraformaldehyde/PBS (w/v) at room temperature for 10 min. Fixed cells were washed three times for ten min in 1% goat serum/PBS (v/v). Cells were then blocked in 10% goat serum/PBS (v/v) for 30 min, followed by incubation with primary antibody (Table 2.5) diluted in 1% goat serum/PBS. Cells were washed in 1% goat serum/PBS for one hour. Secondary antibody (Table 2.6) diluted in 1% goat serum/PBS was incubated for one hour, in the dark to prevent bleaching. Cells were then washed for two 15 min intervals and slides were mounted in Hydromount plus 0.2 M 1, 4 diazabicyclooctane DABCO (National Diagnostics).
2.3.23.2 Paraformaldehyde Fixation for Actin
4% paraformaldehyde/PBS (w/v) fixed cells were washed twice in PBS for ten min. Actin within the cells was stained with 0.2 U phalloidin in PBS for 40 min. Two 15 min PBS washes rinsed the cells before they were mounted in Hydromount.

2.3.23.3 MeOH/Mes Fixation
Cells were fixed at -20°C in 9:1 methanol/N-morpholino-ethanesulfonic acid (MeOH/Mes) (v/v) buffer for five min. Samples were rinsed in 1% goat serum/PBS (v/v) and then cells were permeabilised in 1% nonyl phenoxylpolyethoxyethanol 40/dH2O (v/v) for 5 min. Cells were blocked in 10% goat serum/PBS (v/v) followed by the addition of primary antibody (Table 2.5) in 1% goat serum/PBS for one hour. Samples were then washed for 30 min in 1% goat serum/PBS, before incubation with secondary antibody (Table 2.6) in the dark for 30 min. Remaining out of direct light, cells were washed twice, each for 15 min and mounted in Hydromount.

2.3.23.4 Phemo-Fixation for Actin and Microtubules
Cells were fixed in Phemo-Fix for ten min at 37°C. Next, two 10 min washes in 1x Phemo Buffer at 37°C, and two 5 min washes in PBS at room temperature were performed. The cells were permeabilised in 50 mM ammonium chloride/PBS for 10 min and washed three times for 5 min in PBS. Cells were blocked in 10% goat serum/PBS (v/v) for 30 min and incubated in primary mouse anti-α tubulin in 1% goat serum/PBS (v/v) (Table 2.5). Unbound antibody was removed with two 30 min washes in 1% goat serum/PBS. Following this, the incubation of secondary antibody (Table 2.6) in 1% goat serum/PBS and 0.2 U phalloidin/PBS was performed for 30 min in the dark. After two 15 min washes the cells were mounted with Hydromount.

2.3.24 Microscopy
Prepared slides were visualised under a charge-coupled device (CCD) Upright microscope or a LSM Confocal Microscope (Carl Zeiss Ltd, Hertfordshire, UK). Images were captured with Axiovision 4.7.1 software and Zeiss LSM Examiner 4.0. Fluorescence intensity, and thus protein localisation, was measured using Andor iQ 1.9.1, as a measure of fluorescence intensity distribution per µm². Fluorescence intensity was normalised to non-specific background staining.

2.3.25 Actin Fractionation
In this section the method for fractionating actin is described (Zou et al 2007). Cells were plated onto substrate at 2x10⁵/ml serum free medium and incubated for 17 hours. After 24 hours cells were washed twice in Stabilisation Buffer. Extraction Buffer was added for
10 min at 37°C and soluble proteins removed. The culture plate was then washed in Extraction Buffer and insoluble proteins scraped into protein sample buffer warmed to 37°C.

2.3.26 siRNA Transfection
Cells were put in 6 well plates at a density of 2x10^5/ ml complete medium and incubated for 17 hours at 37°C. Transfection solutions were prepared as follows, 1) 100 nM/ 200 nM siRNA plus 184 μl optimem 2) oligofectamine plus 10 μl optimem and incubated individually at room temperature for five min, before combining for a further 15 min. Wells were washed in Opti-MEM® I Reduced Serum Medium and replaced with 800 μl medium plus the transfection solutions. After four hours, 500 μl complete medium was supplemented. Over a specific time course, cell lysates were collected (Section 2.3.20).

2.3.27 Time-Lapse Video Microscopy
Migration assays were adapted from Entschladen et al 2005. Cells were plated onto 24 well plates at a density of 7000 /ml complete medium. All treatments were performed in triplicate. Adhered cells were incubated in serum free medium for six hours. Cells were again changed to fresh serum free medium containing treatment. Plates were immediately placed in a humidified chamber (37°C/ 5% CO_2) and frames were positioned for recording every 15 min over a 13 hour time course using the CCD Inverted Microscope (Zeiss). Using Axiovision 4.7.1 software, ten individual cells per frame were selected at random and their movement tracked in μm. Migration speed was calculated using the equation: Distance travelled/ Time and expressed as μm/ hr.

2.3.28 Apoptosis Assays
A DNA binding dye was used to visualise apoptotic changes in cells based on nuclei morphology. This method was adapted from Allen et al 2001. Cells were seeded at 0.05 x 10^6 cells/ well in a 24 well plate and incubated in serum free medium for 15 hours. Cells were then treated with 20 μM bisindolylmaleimide III or phorbol 12-myristate 13-acetate (PMA) (Sigma, MO, USA) in serum free medium for 24 hours at 37°C. Cells were rinsed twice in PBS, once in 1:1 diluted Hoescht Fixative/PBS and once in Hoescht fixative. Fixative was left on the cells for 10 min, after which cells were washed twice in dH_2O. Cells were stained in Hoescht for 10 min, followed by two more washes in dH_2O. Cells were visualised and captured with a CCD inverted microscope and images analysed using Axiovision 4.5.
2.3.29 RNA Extraction and Reverse Transcription
To extract RNA from cells, subconfluent monolayers of cells were washed twice in 10 ml PBS and isolated according to SV RNA Isolation guidelines set by Promega.

Extracted RNA was quantified using a Nanodrop NR-1000 spectrometer (Labtech International). 1 µg RNA was reverse transcribed to cDNA by incubation with 2 µg random hexamers for 10 min at 70°C. This was followed by incubation for 1 hour at 42°C, with 10 U/µl Superscript II reverse transcriptase, 0.1 M DTT, 5x first strand buffer, 10 mM dNTP mix and 40 U/µl RNase Inhibitor. Superscript was deactivated at 65°C for 15 min and stored at -80°C.

2.3.30 Quantitative Real-Time PCR
Quantitative Real-Time PCR (qRT-PCR) is used to quantify mRNA. Specific primers and corresponding Universal Probes were identified for gene to be analysed at National Centre for Biotechnology Information (NCBI Blast).

qRT-PCR was performed using 7500 Fast System SDS Software (Roche). Final reaction volume of 20 µl, contained 5 ng cDNA, 50% 2 x Fast mastermix (v/v), 200 nM primer and 100 nM of Universal Probe. 1 ng cDNA were used for 18S reactions. The qRT-PCR run was programmed to: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 sec at 95°C and 1 min at 60°C. For each gene, standard curves were devised using dilutions of placental cDNA at 20, 10, 5, 1 and 0 ng.

Results were calculated using the comparative cycle threshold (Ct) method of relative quantization, normalized to the internal control of 18S or according to Genorm (Primer Design) (Kevorkian et al 2004).

2.3.31 SYBR Green Quantitative Real Time PCR
SYBR Green analysis was carried out as in Section 2.3.29 with minor changes. Fast mastermix was replaced with 2x Sybr Green mastermix and probes were omitted. In addition to the qRT-PCR cycle, there was an extension phase comprising of eight 15 sec cycles at 95°C, followed by 2 min at 60°C and 15 sec at 95°C. Dissociation curves were analysed for non-specific amplification.
2.3.32 Cell Derived Matrix

This method of capturing ECM laid down by cells has been adapted from Cella et al 2006. Cells at density 3.5x10⁴/100 μl serum free medium were plated in 96 well plates for 24 hours. Cells were next washed twice in PBS and treated with 20 mM ammonium hydroxide for five min at 37°C to remove cells. Cell derived matrices were neutralised with washing in SdH₂O, followed by washing in PBS.

2.3.33 Adhesion Assays

The following method describes cell adhesion assays which have been adapted from Methylene Blue Assay; Oliver et al 1989. When not using ECM laid down by cells (Section 2.3.31), tissue culture treated 96 well plates were coated with adhesion substrates; 5 μg/ml matrix components diluted in coating buffer and left overnight at 4ºC respectively. Matrix coated wells were then washed twice with PBS and blocked in 1% bovine serum albumin/PBS (w/v) for 30 min at 37°C. The optimal number of cells per 100 μl well (previously determined) were plated and incubated for 30 min at 37°C. The plate was inverted to remove any non-adherent cells and adhered cells were fixed in 4% paraformaldehyde/PBS (w/v). Fixed wells were stained with Methylene Blue for 30 min. Plates were rinsed clear in dH₂O and replaced with 100 μl Methylene Blue de-stain. At 10 min, absorbance of stained adhered cells was determined at 630 nm in a ThermoMAX microplate reader (Molecular Device).

2.3.34 Aggregation Assay

Cell aggregation was measured using a light microscope; a method adapted from Tan et al 1999. Sub-confluent cell cultures were serum starved for 24 hours. Single cell suspensions at 5x10⁵/ml were plated in complete medium containing 0.5% bovine serum albumin/PBS (w/v) and incubated for two hours at 37°C, with aeration at 30 rpm. A control plate was duplicated at 4°C. Cells were fixed in 2% Grade 1 Glutaraldehyde solution (v/v) (Sigma) and imaged immediately using a Nikon D50 SR digital camera.

2.3.35 Cell Spreading

To analyse the individual spread of cells, cells were first seeded on coverslips at 0.025x10⁵/ml complete medium. Adhered cells were changed to serum free medium for four hours, when they were stained with Haemotoxin and Eosin. This was adapted from the method described in Humphries et al 2007.
2.3.36 Haemotoxin and Eosin Stain
Cells adherent to coverslips were washed twice in PBS and fixed in methanol for 10 min. Eosin Solution (Sigma) was added to cover the coverslip and left for five min. After removal of Eosin, coverslips were stained with Mayers Haemotoxin Solution for five min (Sigma). Coverslips were rinsed clear in PBS and mounted in Hydromount.

2.3.37 Proliferation Assay
This is a colorimetric method used for determining the number of viable cells in a proliferation assay (Cell Titer96 Aqueous One Solution Cell Proliferation Assay, Promega). MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) is an electron coupling reagent that is reduced by dehydrogenase enzymes found in metabolically active cells. Cells into soluble formazan product that can be measured at 490 nm. The quantity of formazan product as measured by the amount of 490 nm absorbance is directly proportional to the number of living cells in culture. 4000 cells/150 µl complete medium were plated onto 96 well plates, and left to adhere for 24 hours at 37 °C. Methanethiosulfonate reagent was added and left for two hours after which the absorbance at 490 nm was measured. To relate optical density to cell number, a series of increasing number of cells were plated per 150 µl media and incubated immediately with 15 µl Methanethiosulfonate reagent for two hours and measured at 490 nm.
3 Expression and Purification of Maspin

3.1 Introduction
This chapter presents the molecular and protein tools used to express and purify wild type and mutant maspin proteins. Five mutant maspin constructs were produced using site directed mutagenesis (Figure 1.6). Constructs were point mutated at various residues postulated to be key in the tertiary structure of human maspin in an attempt to identify their functional contribution. All mutated residues were substituted with non charged alanine residues. Mutated residues were lysine 90, glutamic acid 115, arginine 340 and glutamic acid residues 244 and 247. K90 and E115 form a novel salt bridge between strands 1 and 2 of β-sheet A, which maintains a basic amino acid cluster around α-helices D and E, postulated to aid co-factor binding. Residue R340 is positioned at P1 of maspin’s RCL which has no inhibitory function, yet evidence regarding its contribution to non-inhibitory functions remain conflicted. E244 and E247 are both key amino acids in the α-helix G of maspin. Two conformational variants of maspin, ‘closed’ and ‘open’ were identified from the crystal structure (Law et al 2005). The rotation angle of α-helix G that hinges around residue 249 was calculated to be ~5°. In the open conformation, glutamic acid residues 244 and 247 are exposed, so that a continuous band of negative charge spans the helix. These exposed residues have been proposed to mediate interactions with protein or ligand (Law et al 2005). The five constructs expressing mutant maspin proteins were named K90A, R340A, E115A, E244A and E247A. Wild type and maspin mutants were cloned conventionally into the mammalian expression vector pcDNA3.2. Transient transfection of maspin constructs into mammalian cells was used to study over-expression and the effect of each mutation on the function of maspin. The cellular effects of cells transfected with mutant maspin protein were compared to those expressing wild type maspin protein as well as endogenous expression.

Using Gateway ® and conventional cloning, wild type maspin and maspin mutant cDNA constructs were cloned into the inducible insect competent vector pMTBiP-6xHis and the mammalian competent vector pcDNA4-6xHis. These expression vectors contain a C terminal encoded hexahistidine tag (6xHis) which will be utilised for its interaction with nickel ions in the process of affinity chromatography. Constructs were stably or transiently transfected into insect or mammalian cells respectively. Polyclonal stable insect cell lines were created that expressed protein at a high level. Collected protein was purified via the binding affinity between 6xHis and nickel cations. As an alternative to insect expression, maspin proteins were constitutively expressed using a mammalian expression system with a strong enhancer region, which underwent episomal replication. Purified maspin proteins were quantified and used to evaluate the cellular effects of exogenous protein.
3.1.1 S2 Drosophila Expression System

To produce recombinant wild type and mutant maspin proteins, for use in functional studies *in vitro*, a desirable expression system was essential. Recombinant proteins can be produced on a large scale by using either bacterium, yeast, mammalian or insect cells. For small scale protein expression, a cell free system can be used involving a coupled transcription and translation reaction *in vitro*. All expression systems differ, having advantages and disadvantages to each other. Insect cell expression systems hold advantages over both mammalian, bacterial and yeast systems. Insect cells are a higher eukaryotic system than yeast and are able to carry out more complex post-translational modifications. Like mammalian cells, insect cells can produce post-translational modifications and active proteins. Producing a high yield, quickly growing insect cells are easy to transfect and are grown in suspension on a large scale.

In this study we have used the *Drosophila* expression system, with *Drosophila melanogaster* Schneider line 2 (S2) cells. S2 cells have previously been used to express mammalian proteins in our laboratory, such as pro-urokinase-type plasminogen activator (Kilpatrick et al 2006) and pro and mature neurotrophins; brain-derived neurotrophic factor and nerve growth factor (Gray and Ellis 2005). The *Drosophila* expression system offers ease of propagation of S2 cells. Cells are grown at room temperature and grow exponentially to high density, producing high levels of expression. Proteins for purification can be over-expressed in *Drosophila* cells and allow for efficient protein folding, disulphide bond formation, post-translational modification and secretion; thus turning out a high quality product. The insect cells do not require serum or CO₂ to thrive which reduces the risk of contamination. These non-lytic, stable cell lines can be maintained indefinitely owing to their immortality; which is particularly desirable for continuous protein production (Muerhoff and Dawson *et al* 2004).

3.1.2 Purification of Recombinant Maspin Protein

Recombinant maspin has previously been expressed in insect *Spodoptera frugiperda* cells and purified by process of anion exchange and heparin affinity chromatography (Sheng *et al* 1994). Also, wild type maspin and maspin fused to glutathione S-transferase have previously been expressed in *E.coli* and purified using anion exchange followed by metal affinity chromatography and by glutathione affinity chromatography respectively (Blacque and Worrall 2002, Sheng *et al* 1994). Similarly, another laboratory expressed maspin cDNA in *E.coli* and purified maspin protein using nickel-NTA chelate and Q-Sepharose column chromatography. However all of maspin’s eight cysteine residues were mutated to a serine or alanine to increase the solubility of its purified protein (Al-Ayyoubi *et al*...
Additionally, recombinant maspin has been expressed in *Saccharomyces cerevisiae* and purified by anion-exchange chromatography (Pemberton et al. 1995). Despite maspin having not previously been purified using a *Drosophila* cell expression system, insect S2 cells were chosen in this study to express maspin protein with purification via metal affinity chromatography. This decision was largely influenced by laboratory expertise (Gray and Ellis 2008).

### 3.2 Chapter Aims

1. To establish suitable tools for the expression of wild type and mutant forms of maspin protein. Maspin constructs shall be cloned into insect or mammalian competent vectors utilising both Gateway® and conventional cloning.

2. To purify recombinant proteins using metal-affinity chromatography for subsequent use in experiments.
3.3 Results

3.3.1 Recombinant Maspin Mammalian Expression Constructs
Successful clones containing wild type human maspin or mutated constructs, K90A, R340A, E115A, E244A and E247A were identified by DNA sequencing. These were subcloned into mammalian destination vector pcDNA3.2 using Gateway® Technology. Sequencing verified the correct order of amino acids for maspin's coding sequence which was in alignment with the vector V5 epitope. The wild type stop codon was removed by PCR primer amplification to allow for translation of the V5 epitope. The sequence did not contain any spontaneous mutations (Figure 3.1). Expression of V5 would not occur in the empty vector since there would not be a start codon to initiate translation.

Figure 3.1 Sequence of human maspin in pcDNA3.2 vector. Figure shows in-frame orientation with the V5 epitope (orange) of the pcDNA3.2 vector, downstream of a mutated stop codon (red). Mutated maspin constructs pcDNA3.2-K90A (pink), -E115A (light blue), -E244A (dark blue) and -E247A (green), -R340A (white).

3.3.2 Endogenous Maspin Expression in Mammalian Cell Lines
To check our mammalian maspin constructs were capable of protein expression, a maspin-null cell line was required. The presence or absence of endogenous maspin was determined by western blotting, for three prostatic adenocarcinoma cell lines; LNCaP, PC3 and DU-145, and one normal epithelial cell line PNT1a. Endogenous maspin protein was detected in LNCaP, PC3 and PNT1a cells. This was seen as a doublet that corresponded approximately to the 35 and 37 kDa markers (Figure 3.2). Quantification of endogenous maspin protein was carried out using densitometry analysis of western blot.
and mRNA via qRT-PCR (Figure 3.3). Protein levels were related to total cell protein. PC3 cells showed highest expression of maspin, at 2.5 ng/μg total cell protein, followed by LNCaP at 1 ng/μg and PNT1a at 0.75 ng/μg. No detectable maspin protein or mRNA was seen for DU-145 cells. Hence this cell line was most suitable for checking the transfection of wild type and mutant maspin proteins.

Figure 3.2 Western blot detection of endogenous maspin expression in normal and cancerous prostate cell lines. Lane 1. LNCaP, 2. DU-145, 3. PC3, 4. PNT1a. 10 μg soluble cell lysate was probed for A) human maspin and B) GAPDH.

Figure 3.3 Quantification of endogenous maspin expression in normal and cancerous prostate cell lines. A) Western blot showing probed with anti-maspin; showing 5 ng/1 ng/0.5 ng recombinant maspin (lanes 1/2/3), and 10 μg cell lysate DU-145/LNCaP/PC3/PNT1a (lanes 4/5/6/7). B) Representative graph showing densitometry analysis of western blot A (pink bars). Relative maspin mRNA levels are also plotted for each cell line (blue bars) as detected by RT-PCR. Experimental data provided by Dr. Rosemary Bass.
3.3.3 Transient Transfection of Mammalian Cells
DU-145 cells were transfected with wild type maspin and mutant maspin constructs and also with empty control vector, pcDNA.3.2. Maspin expression was detected in cells transfected with each construct, but was absent from vector only treated cells (Figure 3.4A). Expression of maspin was detected for four days post transfection using a monoclonal antibody against the V5 epitope encoded by the vector (Figure 3.4B). Transfected maspin constructs were seen as a doublet and ran at approximately 39 and 41 kDa, a higher molecular weight than observed for endogenous maspin, owing to their additional V5 tag.

![Figure 3.4](image)

**Figure 3.4** Western blots showing transiently transfected maspin protein expression. A) Transiently transfected DU-145 cell lysate or conditioned medium were probed with anti-maspin. Lane 1. empty vector pcDNA3.2, 2. pcDNA3.2-maspin, 3. pcDNA3.2-E244A, 4. pcDNA3.2-E247A, 5. pcDNA3.2-EK90A, 6. pcDNA3.2-E115A, 7. pcDNA3.2-R340A. B) DU-145 cells were transiently transfected over four days. 10 µg cell lysate from each day was probed with anti-V5. Lane 1-4. pcDNA3.2-maspin - day one to four, 5. pcDNA3.2.

3.3.4 Recombinant Maspin Insect Expression Constructs
It was shown that mutant maspin constructs E244A and E247A reduced maspin’s ability to decrease cell motility (Chapter 5). Thus to study these effects further, wild type maspin and mutant constructs E244A and E247A were conventionally cloned into insect expression vector pMTBiP for the expression and purification from insect cells. Sequencing analysis of wild type human maspin and E244A/E247A mutant constructs revealed no spontaneous amino acid changes and were verified to be in-frame with the C terminal V5 epitope and 6xHis tag, and the N terminal secretion signal BiP (Figure 3.5). In-frame orientation of maspin gene and BiP and 6xHis tag, allow maspin to be efficiently secreted out of the insect cell and for purification purposes using metal ion affinity chromatography respectively. Plasmids are referred to as pMTBiP-maspin, pMTBiP-E244A and pMTBiP-E247A respectively.
Figure 3.5 Sequence of human maspin in pMTBiP vector. Figure shows in-frame orientation with C-terminal V5 epitope (orange) and 6xHis tag (light blue) of the pMTBiP vector. Mutated stop codon (red) and maspin residues E244A (blue) and E247A (green).

3.3.5 Transient Transfection of S2 Cells

S2 cells were transfected with wild type recombinant maspin construct; pMTBiP-maspin. Western blotting showed that the transfection of S2 cells was successful as secretion of protein could be detected after the activation of the metallothionen promoter with copper ions; with moderate expression on day one and high expression on day two (lanes 3 and 4 Figure 3.6A). Maspin protein was detected via western blotting as a single band at approximately 45 kDa; a higher molecular weight than endogenous maspin protein expressed in mammalian cells, detected as a doublet at 35 and 37 kDa. This difference was due to the presence of the fused C-terminal V5 and 6xHis tags and S2-cell specific glycosylation. Incubation of N-glycosidase F enzyme with conditioned medium from S2 cells expressing wild type maspin, showed a cleaved product a few kDa smaller at approximately 43 kDa (Figure 3.7A). In contrast, endogenous maspin from mammalian PC3 cell lysate, matrix or conditioned medium was not glycosylated (Figure 3.7B). Expression of wild type maspin in S2 cells also produced higher molecular weight bands at approximately 90-100 kDa and at the very top of the acrylamide gel loading lane (Figure 3.6A). These higher bands were speculated to be aggregates of maspin protein that decreased in the presence of reducing agent DTT. Unlike maspin endogenously expressed in mammalian cells (Figure 3.2A), insect cell expressed wild type maspin did not show a double protein band separated by a few kDa (Figure 3.6A).
Figure 3.6 Western blotting of transiently transfected S2 cells with recombinant maspin constructs. A) Wild type pMTBiP-maspin transfection. Protein was probed with anti-maspin. Lane 1. 30 µl cell lysate, 2. 40 µl conditioned medium from non-induced cells, 3. 40 µl conditioned medium from induced cells (day one), 4. 40 µl conditioned medium from induced cells (day two), 5. 40 µl conditioned medium from induced cells (day two) reduced with DTT. B) Transfection of pMTBiP-E244A. Protein was probed with anti-maspin. Lane 1-3. 30 µg transfected cell lysate from day one, three or six, Lanes 4-6. 40 µl induced conditioned medium from day one, three or six.

Figure 3.7 Western blots probed with anti-maspin showing protein glycosylation. A) 20 µl PC3 cell fractions; matrix, lysate or conditioned medium (CM) were incubated with (+) or without (-) N-glycosidase F. B) 20 µl conditioned medium from S2 cells transfected with pMTBiP-maspin, plus or minus N-glycosidase F.

S2 cells were transfected with mutant maspin pMTBiP constructs and expression was detected by method of western blot. In comparison to wild type maspin protein, there was no detectable expression of E247A protein from transfected S2 cell lysate or conditioned medium, over a period of six days after CuSO₄ induction. Mutant E247A maspin protein was not detected with either maspin or V5 antibodies. In comparison to E247A protein, a weak expression of E244A protein from S2 cell lysate was observed at day six of
activation of the promoter with CuSO$_4$, yet was absent from conditioned medium. The
detection of E244A protein at day six was only observed with anti-maspin and not anti-V5
antibody. This suggested that mutation to maspin’s residues E244 or E247 had perturbed
correct protein folding (Figure 3.6B). In an attempt to optimise transfection, the
transfection conditions were altered. The amounts of plasmid DNA was increased (3-9
µg) with corresponding Cellfectin amounts (9-25 µl). However, mutant protein constructs
remained un-expressed.

Since the expression and secretion of correctly folded mutant maspin proteins was
unsuccessful, it was decided to use a mammalian expression system for the purification of
E244A and E247A proteins (section 3.39). The use of a mammalian expression system
was supported by the previous observation that both wild type and mutant maspin
proteins were expressed in, and secreted from mammalian cells (Figure 3.4).

### 3.3.6 Stable Transfection of S2 Cells
Following successful transient transfection of pMTBiP-maspin, stably expressing S2
clones were generated by selection with blasticidin. Stable protein expression under the
induction of copper sulphate for 24 hours was detected via western blot with maspin and
V5 antibodies (Figure 3.8). Stable clones were maintained in serum free medium under
Blasticidin selection. For the large scale production of maspin protein, cultures were
expanded to a maximum of 250 ml where they were then incubated with CuSO$_4$.

![Figure 3.8](image)

**Figure 3.8** Conditioned medium from S2 cells stably transfected with pMTBiP-maspin. 25
µl samples were subject to SDS PAGE and western blotting. Blots were probed with A)
anti-maspin or B) anti-V5.
3.3.7 Insect Expressed Protein Purification of Wild type Maspin

Wild type maspin protein was purified from the conditioned medium of stably transfected S2 cells, by affinity chromatography that utilised the interaction between maspin’s C terminal fusion histidine tag and nickel.

3.3.7.1 First Step Purification using Ni-NTA

2 L of conditioned medium containing recombinant maspin protein was dialysed in lysis buffer plus 20 mM imidazole and passed over a Ni-NTA gravity flow column. To reduce problems with the column filter becoming blocked with air bubbles, dialysed conditioned media was concentrated with PEG and de-gassed before adding to Ni-NTA. To elute maspin bound-Ni-NTA, a step-wise succession of elution buffer containing the nickel ion chelator imidazole was used.

Figure 3.9A shows initial purification fractions analysed by western blotting. A high proportion of maspin in the conditioned medium (lane 1) did not bind to the Ni-NTA, as there was still a large amount in the flow through fraction and wash steps (lanes 2 and 3). The elution of maspin protein from the column can be seen in fractions of 50 mM and 100 mM imidazole (lanes 4 and 5). This suggested that additional steps at 50 mM were required for specific elution. A strongly expressed protein was seen at approximately 45 kDa by Coomassie Blue stain (Figure 3.9B). This band corresponded to maspin protein and was present in 20 mM wash and 50/100 mM elution fractions. A second strongly expressed band was identified at approximately 100 kDa. This was attributed to maspin aggregates, like that previously observed (Figure 3.6) as the higher bands were decreased in the presence of reducing agent DTT. Additional elution steps at 50 mM decreased contaminants, such as those seen in fractions with higher imidazole concentration (lanes 5 and 6).
Figure 3.9 First Step purification of wild type maspin using Ni-NTA. A) 25 µl samples from 5ml collected fractions were analysed by SDS-PAGE followed by western blotting using anti-V5. Lane 1. conditioned medium, 2. flow through, 3. 20 mM wash fraction, 4. 50 mM, 5. 100 mM, 6. 150 mM, 7. 250 mM elution fractions. B) 45 µl samples from 1ml collected fractions were analysed by SDS-PAGE followed by Coomassie Blue staining. Lane 1. conditioned media, 2. 20 mM wash fraction, 3. 50 mM, 4. 100 mM, 5. 150 mM, 6. 250 mM elution fractions. Samples were not reduced. Arrow indicates maspin protein at ~45 kDa.

3.3.7.2 Second Step Purification using Ni-NTA

Maspin containing fractions from the first step purification were pooled and re-dialysed into lysis buffer plus 20 mM imidazole. Pooled fractions were then incubated with fresh Ni-NTA. To achieve purer protein preparations, 5 x 2 ml fractions were collected using an increasing imidazole gradient. Intermolecular protein aggregation was decreased by the presence of DTT in the elution buffers.

Maspin specifically eluted at 50 mM (Figure 3.10). This low concentration of nickel ion competitor confirms our earlier observation of a low affinity between maspin-6xHis and Ni-NTA (Figure 3.9). The presence of higher affinity contaminants could be due to endogenous S2 proteins containing histidine repeats. The band corresponding to maspin, at approximately 45 kDa, is of similar size to a S2 contaminating protein of approximately 55 kDa (Figure 3.11). This has been observed previously by other laboratory members using the same expression system.
Figure 3.10 Second step Ni-NTA purification of wild type maspin. Pooled maspin fractions were passed over a Ni-NTA column and collected as 2 ml fractions. Maspin western blot of 10 µl column samples. Lane 1. conditioned media, 2-4. 20 mM imidazole wash, 5-9. 50 mM imidazole elution, 10-14. 100 mM imidazole elution, 15-19. 150 mM imidazole elution, 20-24. 250 mM imidazole elution.

Figure 3.11 Second step Ni-NTA purification of wild type maspin. Coomassie Blue stain of 45 µl column samples. Lane 1. conditioned media, 2-3. 20 mM imidazole wash, 4-6. 50 mM imidazole elution, 7-9. 100 mM imidazole elution, 10-12. 150 mM imidazole elution, 13-14. 250 mM imidazole elution. Arrow indicates maspin protein at ~45 kDa.

3.3.7.3 Final Step

Final fractions containing maspin were pooled and dialysed against control buffer; lysis buffer without imidazole. The purity of the final pooled fraction was shown by Coomassie Blue and Silver staining (Figure 3.12). A strong contaminating band can be seen at approximately 55 kDa, with two lesser impurities at approximately 49 and 39 kDa.
3.3.7.4 Concentration of Purified Wild type Maspin Protein

The concentration of the final maspin protein preparation was assessed by BCA assay. The yield was calculated as 16 mg/L S2 cell-conditioned medium and the concentration of the maspin preparation was 0.8 mg/ml, which was used for all further calculations.

3.3.8 Biological Activity of Wild type Maspin Protein

Since there is no standard functional test for maspin, we evaluated its biological activity through well established cell based assays, determining its effect on in vitro cell migration and cell adhesion. The activity of the final wild type maspin protein preparation was first investigated. In the subsequent sections and chapters, the maspin preparation is referred to as recombinant maspin.

Although the final maspin protein preparation was only partially pure, the biological activity of maspin was determined by measuring the migration rate of DU-145 cells using time-lapse video microscopy. DU-145 cells were incubated with increasing maspin concentration or a corresponding volume of control buffer and compared to non-treated cells (Figure 3.13A). This showed that recombinant maspin significantly reduced DU-145 cell migration in comparison to its carrier control at concentrations greater than 121 µg/ml. Using this effective concentration of recombinant maspin, of 121 µg/ml, its effect on DU-145 cell migration was repeated in triplicate (Figure 3.13B). It was found that the addition of recombinant maspin significantly reduced DU-145 cell motility by 30% in comparison to equivalent control buffer or non-treated cells.
Figure 3.13 The effect of recombinant maspin on DU-145 cell migration. A) Cells were treated with recombinant maspin of increasing concentration, or control buffer of equivalent volume. Cells only, were not treated. 100% migration was defined as DU-145 cells in the presence of control buffer. B) % migration of cells treated with 121 µg/ml recombinant maspin was compared to equivalent volume of control buffer or cells only. 100% migration is defined as DU-145 cells only. Experiments were performed in triplicate. Statistical significance was measured by Students t-test (*p<0.05).

The functional activity of maspin was also assessed using a cell adhesion assay that measures the absorbance of adherent cells stained with Methylene Blue. Maspin null DU-145 cells were either treated with S2 conditioned medium expressing maspin or 161 µg/ml recombinant maspin, compared to S2 conditioned medium expressing empty vector or control buffer respectively (Figure 3.14). The addition of maspin expressing conditioned media or recombinant maspin both significantly increased cell adhesion to a fibrillar matrix laid down by HT29 cells, by 71% or 51% in comparison to their respective negative controls. Both cell assays used to assess the activity of the final maspin protein preparation showed maspin to have corresponding cell function with previous published findings (Chapters 5 and 6).
3.3.9 Expression and Extraction of Recombinant Maspin from Mammalian COS-7 Cells

Following unsuccessful expression and/or secretion of mutant maspin in insect cells, E244A, E247A and wild type maspin DNA constructs were cloned conventionally into vector pcDNA4. Sequencing analysis verified the correct order of amino acids and the in-frame alignment with C terminal V5 epitope and a 6xHis tag necessary for purification. Vector pcDNA4 was chosen for it has a cytomegalovirus (CMV) promoter that induces high levels of replication of the recombinant gene and vector encoded SV40 origin of replication. In turn the SV40 origin of replication can be activated by large T antigen, to allow efficient constitutive expression of the recombinant gene.
These mutant maspin and wild type maspin constructs were transiently transfected into COS-7 cells; a cell line known to express large T antigen. In comparison to transfection of empty vector pcDNA4, each protein was detected 48 hours post transfection using an anti-maspin antibody (Figure 3.15A). A strong band was detected at ~40 kDa and a faint, non-specific band of 28 kDa was also detected. The introduction of SV40 origin of replication into large T antigen-containing COS-7 cells induces episomal replication. It was found that after four to five days of episomal replication levels of the episomal plasmid had become toxic and 90% of the COS-7 cells had died.

At the end of episomal replication, conditioned medium of transiently transfected COS-7 cells was collected. This contained a high level of expressed maspin protein. Due to time constraints, E244A and E247A proteins were used as conditioned medium. Multiple transient transfections of COS-7 cells grown in 9cm dishes enabled the collection of conditioned media for all three constructs and empty vector. Each pooled volume of conditioned medium was dialysed into control buffer and concentrated using PEG. Maspin protein was detected in the conditioned medium of each construct by western blot which showed a relatively equal protein expression, however lane 2 appeared to have less protein (Figure 3.15B). These were subsequently used in cell based experiments (Chapter 5).

![Figure 3.15](image)

**Figure 3.15** Western blots showing expression of pcDNA4-maspin constructs in COS-7 cells. A) 10 µg cell lysate was collected 48 hours after transient transfection. Blots were probed with anti-maspin (top) or anti-GAPDH (bottom). B) 40 µl transiently transfected conditioned media samples were pooled and concentrated. Lane 1. pcDNA4, 2. pcDNA4-maspin, 3. pcDNA4-E244A, 4. pcDNA4-E247A. Blot was probed with anti-maspin.
3.4 Discussion

3.4.1 Mammalian Expression of Maspin Constructs

The successful generation of pcDNA3.2-maspin and various mutant maspin constructs enabled us to determine which expressed proteins, if any, were detrimental to the function of maspin. As discussed in later chapters, mutant maspin protein mutated at residues E244 and E247 showed a detriment to the function of wild type maspin. Thus it was decided to purify wild type maspin and these particular mutant maspin proteins.

The expression of wild type and mutant maspin proteins in mammalian cells were detected as a doublet on western blot. This doublet was not the result of V5 tag cleavage since this doublet band was also detected on western blotting using anti-V5 antibody. There was a difference in the molecular weight of wild type maspin doublets detected from either endogenous or transfected protein, of either 35/37 kDa (Figure 3.2A) or 39/41 kDa (Figure 3.4A) respectively. This additional size was attributed to be a consequence of the V5 epitope fusion.

Although maspin has no known conventional secretion signal; the possibility that the doublet resulted from the cleavage of a novel secretion signal was addressed. This was found to be unlikely since an endogenous maspin doublet was identified in the conditioned medium and matrix of cells (Figure 3.7A). This maspin doublet has previously been identified from endogenous cell lysate treated with trypsin (Al-Ayyoubi et al 2007). This study stated that the doublet was the result of cleavage of its RCL releasing a fragment of 30 base pairs. In our hands this seems unlikely as the transfection of pcDNA3.2-R340A still showed the maspin doublet. This construct was mutated at its arginine-scissile bond P1 residue, which would prohibit the interaction with trypsin and thus prevent cleavage (Appendix Figure A2). In contrast to mammalian cells, the expression of wild type maspin in S2 cells was not detected as a doublet in either cell lysate or conditioned medium.

The Drosophila expression system was chosen for its ability to correctly fold and post-translationally modify proteins. However the endogenous insect cell N-glycosylation processing machinery does not produce complex, terminally sialylated N-glycans such as those found in mammalian cells. The N-linked high mannose oligosaccharides of insect cell produced proteins are not trimmed or modified by the addition of other types of high-mannose N-linked glycosylation and thus have a high mannose content. Since maspin has five potential N-glycosylation sites (Pemberton et al 1997) wild type maspin from either S2 cells or PC3 cells was incubated with N-glycosidase F enzyme (Figure 3.7). The incubation of maspin form S2 cell conditioned medium showed a smaller de-glycosylated
product, whereas endogenous human maspin from lysate, matrix or conditioned medium of PC3 cells was not glycosylated. This non-complex N-glycosylation of maspin protein produced in S2 cells could provide an explanation for the detection of maspin at approximately 45 kDa; larger than the size of endogenous maspin at 39 kDa. Additionally, incubation with N-glycosidase F did not modify the protein doublet of mammalian produced maspin. Another possible reason behind the maspin doublet could be phosphorylation, but this was not investigated. Phosphorylation at tyrosine residues has been previously observed for both intracellular and extracellular maspin (Odero-Marah et al 2002). However, they did not report on potential un-phosphorylated maspin forms, nor did they mention the appearance of a doublet from their stably transfected maspin-MDA-MB-231 cell lines.

3.4.2 Insect Cell Expression System

The Drosophila expression system was effective in producing wild type maspin, but failed to express E247A and did not secrete E244A. The expression of E244A could be seen at day six after CuSO₄ induction. In comparison to the high expression of wild type maspin at just day one post induction, E244A expression was much lower. The inability of S2 cells to secrete E244A protein suggests that mutation of maspin's α-helix G may prevent correct protein folding and/or deter its entrance to secretory vesicles. However, effective expression and secretion of E244A and E247A in mammalian cells implied the correct folding of such proteins.

3.4.3 Purification of Wild Type Maspin

Conditioned medium from S2 cells transfected with pMTBiP-maspin showed relatively high expression levels of wild type maspin protein. Maspin was identified by western blot at a band of approximately 45 kDa, as well as a higher molecular weight band at approximately 90 kDa. Both bands were visible when the conditioned medium was probed with antibodies to both maspin and V5. Under reducing conditions, the larger 90 kDa band disappeared. This did not affect the lower 45 kDa band since maspin does not form intramolecular disulphide bonds, despite its eight cysteine residues (Fitzpatrick et al 1996). It could be plausible that the maspin preparation under non-reducing conditions would all remain at 90 kDa. Since the protein sample buffer used for protein detection by western blotting contains SDS and β-mercaptoethanol, this could only be determined in the absence of these reducing reagents. It may be possible that maspin exists as an intracellular dimer, a characteristic of serpins. However serpin dimers are not usually bound by disulphide linkage, as discussed in section 1.4.1. It also remains possible that this observed intermolecular aggregation may be an artefact resulting from SDS-PAGE. The presence of SDS opens up the structure of proteins exposing cysteine residues. This
may allow intermolecular disulphide bond formation, creating protein aggregates that
would not likely occur otherwise. For purification purposes DTT was added to elution
buffers to prevent intermolecular aggregation.

The purification of wild type maspin using nickel affinity chromatography proved
problematic. Maspin-6xHis tag fusion protein had a low affinity for Ni-NTA, shown by the
presence of maspin protein in flow through fractions and wash fractions. This low binding
efficiency is unlikely to be due to a lack of Ni-NTA binding capacity, because 1 ml of Ni-
NTA can bind between 5-10 mg of His-tagged protein. It may result from the tertiary
folding of maspin, shielding the 6xHis tag from maximum availability for Ni-NTA binding.
The presence of S2 proteins containing histidine repeats could have competed with
maspin 6xHis tag for binding to Ni-NTA. Additionally, binding to Ni-NTA could be
perturbed by the presence of copper ions or surfactant Pluronic-64 from serum free
media. However, these should have been removed during dialysis.

To determine whether the gravity flow apparatus of Ni-NTA affinity chromatography was
affecting the binding between maspin and the nickel matrix, an alternative technique of
Fast Protein Liquid Chromatography (FPLC) was tried. From the resulting chromatogram,
masin protein eluted in the presence of 50 mM imidazole, which was confirmed via
western blot (Appendix Figure A3), much like that observed from gravity flow (Figure 3.9).
However, by method of FPLC the amount of detected maspin protein was low in
comparison to protein detected in the flow through and washes fractions. This technique
was not continued with, due to the low yield. A possible reason for the low yield of maspin
could be from the unusually large peak in electrical conductance (Appendix Figure A3).
This suggested that the flow across the column somehow became interrupted, implying
that the protein sample could have flowed straight to the wash fraction.

Fractions eluted from the Ni-NTA gravity column that contained maspin also contained
multiple contaminating proteins, making their removal difficult without losing maspin
protein. Most contaminants smaller than 12 kDa were removed as determined by the
molecular weight cut-off of the dialysis membrane. The reduction of impurities above 12
kDa could have been enhanced by increasing the wash steps. However, this was decided
against, for large quantities of maspin protein would have also been eluted, due its low
affinity for the nickel cations.

Throughout purification the yield of maspin dropped. Although the pcDNA3.2 vector,
which encodes the V5 epitope, does not contain an internal protease cleavage site,
cellular proteases have the propensity to cleave exposed V5 and His tags, and hence
reduce the final yield. However, cleavage of the V5 tag by contaminating proteases was not observed in the presence or absence of protease inhibitors, since maspin protein was detected using an antibody to V5. Although this suggests that the V5 tag may be partially hidden, a broad range protease inhibitor cocktail was supplemented to elution buffers to prevent cleavage of the His tag. The protease inhibitor cocktail did not contain EDTA, as EDTA has been shown to bind to Ni-NTA.

Dialysis of the pooled maspin fractions into a final buffer proved difficult. Dialysis into PBS showed a large net loss of protein, undetectable by BCA assay or by Coomassie Blue staining. This suggested that the protein had adhered to the dialysis cassette membrane. To prevent this from happening, final dialysis was carried out with the fractions contained in dialysis tubing and not a cassette. The final buffer was changed from PBS to purification buffer minus imidazole, pH 8.0.

To yield a purer final sample of maspin protein, further purification techniques could be employed. Gel filtration would separate maspin from impurity proteins based on size exclusion, although the separation of maspin from its three major contaminants would prove difficult as their molecular weights are very similar. Ion exchange chromatography utilises the pI of a protein and its interaction with the oppositely charged matrix. Maspin has a slightly acidic pI of 5.6, and thus would bind to a positively charged matrix, such as a matrix with the functional group diethylaminoethyl or quaternary ammonium cations, possibly increasing its purity.

3.4.4 Biological Activity of Recombinant Wild Type Maspin
Since there is no functional test for the biological activity of maspin, the activity of recombinant wild type maspin protein was assessed qualitatively. The function of partially purified recombinant wild type maspin on in vitro cell migration and cell adhesion was assessed. These cell assays were chosen because maspin’s tumour suppressive properties are well characterised in relation to these functions within the cell. Although a completely pure preparation of wild type maspin was not achieved, the biological function of wild type maspin was successfully assessed in relation to the appropriate controls. The preparation of partially purified maspin showed a significant decrease in DU-145 cell migration and a significant increase in cell adhesion in contrast to non-treated DU-145 cells or those treated with control buffer. Additionally, S2 cell conditioned medium expressing wild type maspin significantly enhanced cell adhesion in relation to conditioned medium expressing empty vector. This suggested that the contaminant proteins in the final preparation of wild type recombinant maspin were not contributing to any biological effect or interfering with the function of recombinant maspin.
3.4.5 Mammalian Expression of Maspin Mutant Constructs
Following the ineffective expression of maspin mutant constructs in S2 cells, E244A and E247A were expressed in a mammalian system. COS-7 cells were chosen for their ability to express E244A and E247A, as well as the ability to undergo episomal replication of transfected plasmids and hence a high levels of protein production.

Due to time constraints, conditioned medium from COS-7 cells transfected with wild type maspin, mutant maspin E244A and E247A, or empty vector pcDNA4 was produced on large scale and quantified without purification.

3.5 Future Perspectives
The purification of maspin using S2 cell expression system turned out to be a lengthy process; fraught with difficulties. Nonetheless, wild type maspin protein was purified. The use of both purified wild type and mutant maspin constructs in cellular based systems would be ideal for comparing their exogenous effects on cell behaviour. The purification of mutant and wild type maspin protein from mammalian cells seems encouraging for purification conditions specific to maspin have now been established. Mammalian cells offer the highest level of correct post-translational modifications with the higher probability of producing fully functional proteins. However, mammalian cells in comparison to insect cells can be hard to transfet and produce a lower protein yield.

3.6 Conclusions
Wild type and mutant maspin constructs were transiently expressed and detected in mammalian cells. The expression of wild type maspin, but not mutated maspin, was detected in insect cells. The development of an ‘in house’ expression and purification system was essential, as the high purchasing cost of commercial recombinant protein was not feasible and mutant protein forms were not available. Functionally active wild type maspin protein was produced, that yielded a partially pure preparation.
4 The Effect of Maspin on the Cell Cytoskeleton

4.1 Introduction
This chapter concentrates on the effect of transfecting wild type or mutant E244A maspin on the cell cytoskeleton of DU-145 and MFC7 cells. Specifically, the morphology of maspin expressing cells will be characterised with emphasis on the actin architecture. Additionally maspin’s interaction with profilin-1, a protein involved in cytoskeletal architecture, will be investigated. This investigation stemmed from the potential importance of maspin in the influence with cytoskeletal protein expression (Chen et al 2005).

4.1.1 Localisation of Maspin
Maspin has been shown predominantly to reside in the cytoplasm, but also the nucleus, endoplasmic reticulum, Golgi, secretory vesicles and cell surface. A small percentage has also been identified outside of the cell, by an un-identified non-classical secretion pathway (Pemberton et al 1997, Zhang et al 1997a). The roles of maspin has been characterised as dependent on both its intracellular and extracellular activities. However the presence of extracellular maspin secreted from cells remains a contentious issue.

4.1.2 Cell Cytoskeleton
The actin cytoskeleton mediates a variety of essential biological functions in all eukaryotic cells. In addition to providing a structural framework around which cell shape and polarity are defined, its dynamic properties are important for motility, cytokinesis and endocytosis. The cytoskeleton is made of three components; intermediate filaments, actin and microtubules, all of which are controlled by Rho GTPases.

Epithelial-mesenchymal transition (EMT) is defined by the loss of epithelial characteristics and polarity, and the acquisition of a mesenchymal phenotype with an increased migratory and decreased adhesive behaviour (Lee et al 2006). Epithelial or mesenchymal-like cells can be identified microscopically based on their unique visual appearance, due to cytoskeletal rearrangement and the morphology of the multicellular structures they create. Mesenchymal-like cells can be identified by the acquisition of spindle-like morphology with cytoskeleton re-organisation, showing numerous filopod and lamellipod membrane extensions and few mature focal adhesions, whereas epithelial cells can be identified by their uniform shape and size with regular cell-cell junctions and focal adhesions; presented as an array of cortical actin. The transition of epithelial cells to mesenchymal-like cells is also characterised by a reduction of epithelial markers, such as E-cadherin and occludins, and the increase of mesenchymal markers, such as vimentin and smooth
muscle actin. Additionally, differentiated epithelial cells are maintained by a well organized polarity with apical basal and lateral domains. In contrast mesenchymal-like cells lose this differentiation and become bipolar; characteristic of cells undergoing directional migration (Figure 1.7). The establishment and maintenance of cell polarity and cell shape are complex processes, involving the coordination of numerous overlapping signalling cascades which highly regulate the spatial control of the actin cytoskeleton. Dysregulation of cell polarity and subsequent tissue disorganization is a hallmark of cancer.

Rapid changes in the levels of polymeric F-actin and monomeric G-actin are involved in the morphological changes associated with cell-cell interaction, motility, differentiation and adhesion. Cellular F-actin levels have been used as a marker for transformation. F-actin levels were shown to increase in dividing cells. However, undifferentiated cells which divide at a higher rate show an overall lower level of F-actin compared to differentiated cells (Rao et al 1990). A low F-actin concentration and a high G-actin concentration correlated with cellular abnormality and risk of bladder cancer (Rao et al 1991). Cells showing a reduced migration have shown a redistribution of G-actin, increase in thymosin β4 and a reduced F-actin in the cell interior, with an increase at the cell cortex (McCormack et al 1999). An epithelial phenotype, typical of a differentiated non-motile cell will typically show an increase in actin associated focal adhesions, actin bundling in the form of stress fibres and a thick actin cortex. Conversely, highly motile mesenchymal cells will show an overall reduction in F-actin focal adhesions and stress fibres, yet show an increase in thin membrane protrusions and polarised lamellipodia.

Polarised microtubule re-orientation involves the rearrangement of pre-existing microtubules and the local concentration of regulation factors guiding microtubule dynamics. Microtubules typically emanate from the centrosome and their plus end associated proteins search for a cortical binding site. These sites then capture the microtubules, providing the force required to move the microtubule organising centre. This 'search and capture' mechanism is necessary for directed protein trafficking; where the Golgi aligns with cell movement and the microtubules deliver a constant supply of proteins and membrane components to the projecting leading edge (Etienne-Manneville 2004). A polarised migrating cell has three distinctive actin structures; stress fibres, lamellipodia and filopodia. In brief, filopodia and lamellipodia are positioned at the leading edge of a cell where the former extends into the surrounding matrix where it makes a weak attachment; focal complex. The thick actin array of the lamellipodia undergoes polymerisation which pushes the leading cell membrane forwards. Stress fibres enable the cell to make stable connections to the underlying substrate. These are positioned at
the trailing edge of a polarised cell, whereby their contraction provides traction for the cell to move (Walcott and Son 2010).

4.1.3 Profilin

Profilin-1 is a 15 kDa globular actin monomer binding protein found in all eukaryotes. Three profilin genes have been identified; profilin -1, -2 and -3. Profilin-1 is the most ubiquitously expressed isoform which is highly expressed throughout and post development in most tissues. Profilin -2 and -3 isoforms are neuronal or testes specific, respectively (NCBI). Profilin has a high affinity for ATP-actin ($K_d$ of 0.1 µM) and a lower affinity for ADP-actin ($K_d$ of 0.5 µM). When bound to ATP-actin, profilin inhibits spontaneous nucleation and promotes nucleotide exchange. In the absence of un-capped filament barbed ends it acts as a sequestering protein, and in its presence it promotes assembly (reviewed in Pollard et al 2000).

Profilin competes with ADF/cofilin for the binding of depolymerised ADP-actin and subsequently re-charges the monomer with ATP. The excess of ADP in the cell cytoplasm favours profilin binding and the exchange of ADP for ATP. Thus, monomeric actin returns to the ATP-actin pool ready for elongation and ADF/cofilin is poised for severing and depolymerisation, thus completing the actin cycle (Blanchoin et al 2000). The availability of profilin has been postulated to be under the control of interaction with WASp and formins and also via the metabolism of phospholipids. The actin-binding domain of profilin partially overlaps with its site for phosphatidylinositol 4, 5-biphosphate, which could disrupt actin polymerisation (Pollard et al 2000).

The exact role of profilin in vivo is not clear. Overexpression of profilin did not affect the total actin concentration, but caused accumulation and increased actin polymerisation specifically at lamellipodia, and a reduced density of cytoplasmic stress fibres. In cells where a concentration of profilin was high, ATP was in excess of ADP, which increased the concentration of F-actin. Profilin over-expressing cells resembled those of highly motile cells responding to growth factors or Ras (Finkel et al 1994). While this suggested a positive role of profilin in cell movement, other studies have provided contradictory evidence. Differential display screening between several tumourigenic breast cancer cell lines with non-tumourigenic cell lines showed that expression of human profilin-1 gene was reduced in the former. Hence, it was suggested that profilin suppressed the tumourigenic phenotype, by a decrease in cell growth, cell spreading and cell motility and an increase in cell clustering (Janke et al 2000). The role of profilin in mammalian cell migration is complex and may be cell-type dependent.
4.1.4 Maspin and the Cell Cytoskeleton

When MDA-MB-435 cells were transfected with maspin gene, ~27% of the detectable proteome was altered. In particular maspin altered the regulation of proteins involved in cytoskeletal architecture, such as α-actin, plectin 1, actinin-4, caldesmon 1, and profilin 1 (Chen et al 2005). It is likely that maspin’s influence on the cytoskeleton is due to an indirect mechanism, since it has been shown that maspin did not associate with the insoluble actin cytoskeleton of MCF10 cells (Teoh et al 2010).

Maspin, known for its anti-tumour properties, has been shown to inhibit the EMT of cells, in particular showing an up-regulation of stress fibres and focal adhesions and a down-regulation of membrane protrusive structures (Seftor et al 1998). In keeping with this, maspin was found to inhibit the lamellipod and filopod regulators, Rac1 and Cdc42 and their downstream effectors PAK1 and JNK, and as a result specifically decreased cell motility (Odero-Marah et al 2003, Shi et al 2007). Through regulating the balance between activated Rac and Rho proteins, maspin can affect cell morphology (Burridge and Wennerberg 2004).

4.2 Chapter Aims

1. To investigate the effect of wild type maspin and its mutant protein E244A on the organisation of the cellular cytoskeleton.

2. To characterize the cellular morphology of cells transfected with wild type or mutant maspin, maintained on various matrices.

3. To study the effect of maspin on cytoskeletal proteins.
4.3 Results

4.3.1 Localisation of Cellular Maspin Protein

Maspin null DU-145 cells were transiently transfected with pcDNA32-maspin or empty vector pcDNA3.2. Maspin expression was detected in both the lysate and conditioned medium of cells by western blotting (Figure 4.1A). Immunofluorescence microscopy of non-permeabilised pcDNA3.2-maspin transfected cells, fixed in PFA, revealed specific maspin expression throughout the cell with a punctate appearance at the cell surface (Figure 4.1B). Maspin-null MCF7 cells were stably transfected with pcDNA3.2-maspin, pcDNA3.2-E244A or vector only pcDNA3.2. Protein expression was confirmed using western blotting and immunofluorescence microscopy with anti-maspin (Figure 4.2). The expression of E244A protein was approximately 50% that of wild type maspin. Both wild type and mutant maspin proteins showed punctate staining throughout the cells. Non-specific background staining was visible in vector only expressing DU-145 and MCF7 cells, however this was not punctate.

To determine where endogenous maspin protein resides within the cell, PC3 cells were subject to cellular fractionation (Figure 4.3). PC3 cells were chosen since they express a high level of endogenous maspin protein (Figure 3.2). The sequential addition of different extraction buffers to collected PC3 cells, coupled with centrifugation, gave the selective isolation of proteins from different cellular compartments. As detected by western blot, maspin protein was found in all four subcellular compartments; cytosol, plasma membrane, nuclear and cytoskeleton. To check the integrity of the fractionation, the samples were also probed for nuclear-specific protein histone 2B and the cytoplasmic-specific protein GAPDH. These proteins were detected only in their specific compartments, which indicated that the isolation of subcellular proteins was successful. Our observation that maspin is associated with the cell cytoskeleton, led us to investigate whether this location of maspin has any affect on the cytoskeletal re-arrangement.
Figure 4.1 Wild type maspin protein expression of transiently transfected DU-145 cells. A) Western blot analysis of 10 µg soluble cell lysate or 1 ml TCA precipitated conditioned medium. Protein was detected using anti-maspin (upper panel) and equivalent loading was confirmed using anti-GAPDH (lower panel). B) Immunofluorescence labelling of transfected cells with mouse anti-maspin and Alexa Fluor 488-conjugated anti-mouse (left panel). Phase contrast (right panel). Images were taken using CCD upright at x63 magnification. Graph shows relative fluorescence intensity per µm². Statistical significance measured by Students t-test (p*<0.05).
Figure 4.2 Stable expression of maspin constructs in MCF7 cells. A) Western blot analysis of 10 µg cell lysate probed with anti-maspin (upper panel) and anti-GAPDH (lower panel). 1 ml conditioned medium prepared by TCA precipitation was probed with anti-maspin. B) Immunofluorescence showing maspin expression labelled with Alexa Fluar 488 and nuclear staining shown by DAPI. Corresponding graph showing relative measure of fluorescence intensity per µm². Statistical significance measured by Students t-test (p*<0.05) in relation to cells transfected with vector only pcDNA3.2.
Figure 4.3 Fractionation showing localisation of endogenous maspin protein in PC3 cells. Western blots showing 10 µg protein from cell compartments; Lane1. cytoplasmic, 2. cell membrane, 3. nuclear, 4. cytoskeletal. Fractionation was carried out with Qproteome kit, Qiagen. Experimental data was prepared with the assistance of Dr. Laura Wagstaff (UEA).

4.3.2 The Effect of Maspin on Cell Cytoskeleton

4.3.2.1 The Effect of Maspin on Actin Architecture
The cytoskeletal organisation of F-actin in transfected DU-145 cells was stained using phalloidin and visualised using confocal microscopy (Figure 4.4A). DU-145 cells transfected with empty vector showed numerous long, thin, filopodia extending from the basal cell surface into the surrounding environment. Filopodia extended from the entire cell periphery and formed thin intercellular bridges with adjacent cells. In comparison DU-145 cells transfected with wild type maspin also showed intercellular bridges, yet the frequency and length of filopodia were reduced; specifically at the periphery where neighbouring cells were not present. Maspin expressing cells showed a replacement of thin protrusions with thick focal adhesion like structures at the cell edge.

It was noted during the culturing of MCF7 stable cell lines (expressing wild type maspin, mutated E244A or vector only pcDNA3.2), that the morphology of these cells differed (Figure 4.5). MCF7 cells expressing empty vector appeared small with an elongated, often front-to-back polarised morphology. Comparatively, cells expressing wild type maspin appeared larger and rounded, with a uniform shape, which grew predominantly in tightly packed colonies. MCF7 cells expressing E244A displayed a mixed phenotype; similar to both cells expressing empty vector or wild type maspin. These cells were often, small and irregular in shape, yet some showed a rounded and regular shape respectively.
MCF7 cells stably expressing maspin, E244A or vector only, were stained for F-actin with phalloidin and visualised via confocal microscopy (Figure 4.4B). Control MCF7 cells expressing empty vector, showed a mesenchymal-like morphology with typical polarised arrangement with thin lamellipodia; characteristic with a leading edge of a motile cell. In comparison, the expression of wild type maspin altered the mesenchymal-like irregular cell shape seen in control cells to a more regular epithelial-like one. Specifically, wild type maspin stable cells showed a decrease in thin membranous extensions; similar to that seen with maspin transfected DU145 cells. Maspin MCF7 cells also showed thick, un-polarised lamellipodial actin networks which could be seen around the entire cell periphery. In contrast, the expression of mutant maspin E244A did not undergo this transformation, with a large proportion of cells remaining predominantly mesenchymal-like. Similar to maspin expressing cells, mutant E244A cells often showed thick peripheral actin-rich lamellipodia that could be seen around the cell circumference in a non-polarised manner. However these were often much thinner in diameter and showed increased ruffling with frequent membrane protrusions, similar to mesenchymal characteristics of the control cells. Therefore it appears that expression of wild type maspin protein can influence the re-organisation of actin cytoskeleton to produce an epithelial-like morphology. Such influence on cell morphology is interrupted by mutation to its α-helix G.
Figure 4.4 Confocal images showing filamentous actin labelled with Alexa Fluor 568-labelled phalloidin. A) Transiently transfected DU-145 cells as indicated. Intercellular bridges are shown by white arrows and filopodia with grey arrows. B) Stably transfected MCF7 cells as indicated. Lamellipodia are shown by white arrows and white arrowhead shows retracting trailing edge.
Figure 4.5 Phase contrast images of MCF7 cells stably expressing maspin. Cells were plated at sub-confluent densities and left to adhere overnight in serum containing medium on plastic dishes. Three independent images are shown per condition.

In addition to cells plated on plastic, this maspin-dependent re-organisation of the actin cytoskeleton, was also seen in cells grown on ECM components collagen I, laminin and fibronectin (Figure 4.6). The cellular structure of maspin expressing MCF7 cells grown on collagen I resembled that of the cells cultured on plastic. Wild type maspin MCF7 cells grown on collagen I retained their non-polarised epithelial-like morphology showing thick peripheral lamellipodia extending in all directions, with few filopodia. Both E244A and control expressing MCF7 cells were generally smaller in size, irregular in shape and frequently polarised. Similarly, wild type maspin MCF7 cells grown on laminin showed a dense meshwork of actin around the cell edge; however, cells exhibited a more irregular shape. In comparison, mutant E244A cells grown on laminin were similar in both size and shape and also showed a similar actin meshwork encircling the cells. However, control treated cells grown on laminin showed an increased membrane ruffling and cytoplasmic extensions with an irregular cell shape. The phenotype of each wild type, mutant and
control cells grown on either laminin or fibronectin all increased in mesenchymal-like attributes. In each case, cells were more elongated and polar, suggestive of an increase in motility on this substrate. The motility of these cells on ECM components was later studied (Chapter 5).

Figure 4.6 Confocal images of F-actin stained with Alexa Fluor 568-labelled phalloidin and nuclear staining with DAPI. MCF7 cells stably expressing pcDNA3.2, pcDNA3.2-maspin or pcDNA3.2-E244A were grown on plastic or matrix components for 24 hours.
4.3.2.2 The Effect of Maspin on Cell Morphology

A scoring system for morphometric analysis was developed (Figure 4.7). Five cellular morphologies were denoted a score; 1, 3, 5, 7 or 9, representative of increasing mesenchymal-like characteristics. Cells with a score of 1 were rounded in shape with a thick membranous periphery with few filopodial protrusions. Cells with a similar spherical morphology but with thinner, ruffled lamellipodia were scored 3. Cells which were not so uniform in shape and often showed a polar morphology with frequent filopodia were scored 5. Score 7 and 9 represented cells which displayed numerous peripheral extensions or interactive polar lamellipodia with an increasing irregularity in shape. This scoring system was applied to MCF7 cells stably expressing wild type maspin, E244A or empty vector that were cultured onto plastic or matrix components. Cells were stained with phalloidin and DAPI and their cellular morphology was scored upon visual inspection (Figure 4.8). Each stable MCF7 cell line showed the greatest mesenchymal phenotype on a laminin matrix; laminin > fibronectin > collagen I > plastic. On each substrate, control cells gave the highest score of mesenchymal-like phenotype. In comparison, cells expressing wild type maspin had a low score of mesenchymal-like attributes, and were thus more epithelial-like. E244A stable cells gave a lower score of mesenchymal-like phenotype than control cells. However the score of E244A cells was higher than maspin cells on plastic, collagen I and fibronectin, whereas on laminin E244A cells shared a similar morphological score to maspin cells.
Figure 4.7 Classification phenotype-scoring system. Five panels showing cells of similar morphology stained with Alexa Fluor 568-conjugated phalloidin. Respective scores 1, 3, 5, 7 and 9 indicate an increase in mesenchymal-like phenotype. Images were taken from non-transfected MCF7 cells.

Figure 4.8 Assessment of MCF7 stable cell morphology using phenotype-scoring system. MCF7 cells stably expressing pcDNA3.2, pcDNA3.2-maspin or pcDNA3.2-E244A were cultured on matrix components or tissue culture-treated plastic and stained with phalloidin. The cellular morphology of 68 cells for each cell type was given a score of mesenchymal-like phenotype and averaged scores were plotted.
4.3.3 Maspin α-helix G Peptide Mimics the Effect of Wild type Maspin on F-actin Rearrangement

In addition to the observation that mutation to maspin at E244A perturbs its influence on the re-arrangement of the cell cytoskeleton, it was shown that mutant maspin constructs E244A and E247A both abolished maspin’s ability to decrease cell motility (Chapter 5). Therefore, since both residues E244 and E247 reside on maspin’s α-helix G, we set out to determine whether a peptide representing this region in isolation could have similar effects. Four 15-amino acid peptides were designed that span the sequence of maspin’s α-helix G with two additional residues at either end. Peptides included a wild type peptide (G-helix), two peptides with mutated residues (E244A and E247A) and a control peptide (Control) (Figure 4.9). The control peptide has the same amino acids as the wild type G-helix peptide but is in a re-arranged sequence, and thus has the same charge. The maspin peptides were synthesised with an amide on their C-terminus and a biotin tag on their N-terminus. The peptides are predicted to be unstructured in aqueous solution (ExPASy.org), but could attain a helical structure when bound to their cell surface target, in the same way that uPAR inhibitory peptides only have secondary structure in complex with uPAR (Llinas et al 2005).

![Figure 4.9](image)

**Figure 4.9** Table showing peptide sequences spanning the α-helix G region. Peptide sequences of wild type G-helix, E244A, E247A and control peptide. Wild type α-helix G is highlighted and glutamic acid residues E244 and E247 are substituted with alanine (blue and green respectively).

Each peptide was added to maspin-null DU-145 cells plated on tissue culture treated plastic, at a concentration of 5 μM. This concentration was found to be the optimal, for 5 μM G-helix reduced cell migration akin to wild type maspin protein (Chapter 5). Incubation of DU-145 cells with peptides for three hours did not lead to any effect on the actin cytoskeleton (data not shown). However after 20 hours incubation, we observed that the wild type G-helix peptide mimicked the effect of wild type maspin protein as it also re-organised the actin cytoskeleton (Figure 4.10). DU-145 cells incubated with G-helix showed a rounded phenotype with an increased actin cortex often exhibiting lamellipodia.
structures around the whole of the cell, like that seen with wild type maspin in Figure 4.4B. G-helix treated cells showed a marked decrease in filopodia like that exerted by wild type maspin in Figure 4.4A. Additionally, cells subject to G-helix showed stress fibres arranged parallel to the cell edge. Comparatively, cells treated with either mutant α-helix G peptides E244A or E247A, control peptide or peptide carrier, DMSO, showed longer central stress fibres and multiple membrane protrusions often forming directed lamellipodia. Unlike wild type G-helix peptide, DU-145 cells treated with mutant peptides E244A or E247A did not show F-actin re-arrangement to an epithelial-like phenotype. Rather, E244A or E247A treated cells resembled those treated with control peptide; displaying a mesenchymal-like morphology. Therefore the effect of wild type maspin protein on the re-organisation of the actin cytoskeleton was mimicked by a peptide spanning its wild type α-helix G. Similarly, the inability of mutant maspin protein E244A to promote epithelial-like characteristics was mimicked by peptide E244A and peptide E247A. Thus this helical region of maspin is important for the re-arrangement of the cell cytoskeleton and thus promotes the transition from a mesenchymal-like to epithelial-like phenotype.
Figure 4.10 Confocal images of DU145 cell F-actin. Cells were stained with Alexa Fluor 568-labelled phallloidin and nuclear stained DAPI. DU-145 wild type cells were plated onto plastic. Adhered cells were incubated with 10 µM peptides; G-helix, E244A, E247A, or control, or peptide carrier control DMSO for 24 hours in serum free medium. White arrows indicate stress fibres.
4.3.4 Maspin Alters Cellular Concentrations of G and F actin

Following from the observation that maspin expressing cells showed a thick cortical rim of actin we determined whether maspin influenced cellular levels of globular and filamentous actin. Fractionation was used to separate soluble G-actin and insoluble F-actin from MCF7 cells stably expressing wild type maspin, mutant E244A maspin or empty vector, that were plated onto either plastic, collagen I, laminin or fibronectin (Figure 4.11). It was shown using western blotting, that cells expressing wild type maspin grown on plastic, collagen I and laminin showed an increase in G-actin content compared to both mutant maspin and control stable cells. Conversely, each stable cell type fractionated from a fibronectin matrix showed equal expression of G-actin. Compared to the levels of G-actin, the difference in levels of F-actin between stable cell lines was greater. The wild type maspin MCF7 cell line had increased levels of F-actin in relation to cell lines expressing E244A maspin or control vector. The greatest increase in F-actin between maspin and E244A/control treated cells was seen on plastic and collagen I. This corresponded with a reduced mesenchymal phenotype of maspin expressing cells, characterised by an increase in peripheral F-actin (Figure 4.6). The levels of F-actin were reduced for cells expressing E244A maspin compared to those expressing wild type. This was seen for cells plated on plastic and each matrix component; collagen I, laminin and fibronectin. In conclusion, cells transfected with wild type maspin showed a slight increase in the content of both G-actin and F-actin which corresponds with its phenotype of a thick actin periphery and large flattened phenotype. As with the influence on cytoskeletal re-arrangement, the mutation of maspin at residue E244 showed a reduced level of both G- and F- actin akin to that of control cells. This further implicates the importance of maspin’s α-helix G for its ability to influence EMT.
Figure 4.11 Fractionation of MCF7 stable cells expressing maspin constructs. Cells were cultured on A) plastic, B) collagen I, C) laminin or D) fibronectin. Western blots show fractionation fractions: 10 µg soluble G-actin fraction or 30 µl insoluble F-actin fractions. Blots were probed with anti-actin (upper panel) or anti-GAPDH (lower panel). Western blotting was performed in triplicate.

4.3.5 The Effect of Maspin on the Actin Monomer Binding Protein, Profilin

Following the observation that maspin alters the cellular levels of both G- and F-actin, we investigated whether maspin had an affect on the level of profilin; an actin monomer binding protein that enhances actin polymerisation. Lysate of MCF7 cells stably expressing wild type or E244A mutant maspin proteins were analysed by western blotting for the expression of profilin-1. Profilin-1 was identified at approximately 14 kDa (Figure 4.12A) and showed a small down-regulation in MCF7 cells expressing wild type maspin, in comparison to the vector only control. E244A expressing cells showed a level of profilin-1 that was intermediate between vector only cells and maspin cells. To determine whether
this maspin-directed down-regulation of profilin was due to a change in transcription of the profilin-1 gene, three mRNA samples of each MCF7 cells expressing wild type, E244A or empty vector pcDNA3.2 were collected and subject to qRT-PCR. Amplification of profilin-1 gene showed no difference in mRNA levels between cell lines (Figure 4.12B); suggesting maspin alters the levels of profilin-1 post-translationally.

**Figure 4.12** Expression of profilin-1 in MCF7 stable cells expressing maspin constructs cultured on plastic. A) Western blot showing 10 µg cell lysate probed with anti-profilin-1 (upper panel) or anti-GAPDH (lower panel). B) Quantitative densitometry data of profilin-1 protein expression corresponding to western blot A. C) The effect of maspin expression on the gene expression of profilin-1. Samples were conducted in triplicate and subject to qRT-PCR. These were standardised to 18S.

**4.3.5.1 Profilin-1 Knockdown Reduces Maspin Expression**

To determine whether the expression of profilin directly influences the expression of maspin, profilin-1 siRNA was used to see if levels of maspin protein simultaneously changed. MCF7 vector only cells were treated with two siRNAs targeted to profilin-1, namely A and B, compared to a scrambled control siRNA (CTRsi). Optimal profilin-1 protein silencing occurred at 72 hours post transfection for both siRNA A and B (Figure 4.13).
Figure 4.13 MCF7 control cells treated with siRNA to profilin-1; A or B compared to CTRsi. 10 µg MCF7 cell lysate expressing empty vector pcDNA3.2 were treated with 200 nM siRNA. Cell Lysate was collected at 24, 48 and 72 hours. Blots were probed with anti-profilin-1 (upper panel) and anti-GAPDH (lower panel).

The effect of profilin knockdown on maspin expression was then investigated. MCF7 cells stably expressing either wild type or E244A maspin were treated with profilin-1 siRNA, A and B for 72 hours. Cell lysate was then separated via SDS PAGE followed by western blotting to anti-profilin-1 and anti-maspin (Figure 4.14A). Profilin-1 knockdown was verified in both stable cell lines (upper panel). Treatment of both MCF7 cells expressing wild type maspin or E244A with siRNA A or B, showed a decrease in maspin expression, compared to cells treated with CTRsi (lower panel). This reduction in maspin protein was confirmed by immunohistochemistry and fluorescence quantification (Figure 4.14B). Therefore it appears that profilin-1 protein may be upstream of maspin protein.
Figure 4.14 The effect of profilin-1 gene knockdown on maspin expression. MCF7 stable cells expressing pcDNA3.2-maspin or pcDNA3.2-E244A were treated with 200 nM profilin-1 siRNA A or B or CTRsi. A) Western blots show 10 µg cell lysate collected after 72 hours treatment and probed with anti-profilin-1 (upper panel), anti-GAPDH (middle panel) and anti-maspin (lower panel). B) Immunofluorescence shows maspin expression labelled with Alexa Fluor 488 and nuclear staining shown by DAPI. Each row shows images from three independent knockdown experiments. Corresponding graphs show measurement of fluorescence intensity per µm². Statistical significance measured by Students t-test (p<0.05) in relation to cells treated with CTRsi.
4.3.5.2 Maspin Knockdown Does Not Effect Profilin Expression

To see whether the expression of profilin is influenced by the expression of maspin, MCF7 cells stably expressing wild type or mutant maspin were treated with maspin siRNA, namely #3 and #4 in comparison to CTRsi. Efficient knockdown of maspin protein was found using 200 nM siRNA 72 hours after transfection of MCF7 cells expressing wild type or mutant maspin protein (Figure 4.15 upper panel). Knockdown of maspin did not affect levels of profilin-1, relative to the treatment of cells with CTRsi (lower panel). This therefore supports the previous experiment that showed maspin protein is downstream of profilin-1.

**Figure 4.15** The effect of maspin gene knockdown on profilin-1 expression. MCF7 stable cells expressing pcDNA3.2-maspin or pcDNA3.2-E244A were treated with 200 nM maspin siRNA #3 or #4 and CTRsi. Western blots show 10 µg cell lysate collected after 72 hours treatment, probed with anti-maspin (upper panel), anti-GAPDH (middle panel) and anti-profilin-1 (lower panel).
4.4 Discussion

4.4.1 Localisation of Maspin
Maspin null DU-145 cells were transfected with wild type maspin or control vector. Maspin protein expression was effectively detected by western blot and immunohistochemistry which showed punctuate staining of maspin protein at the surface of non-permeabilised cells. The compartmentalisation of maspin within the cell was studied by cell fractionation using centrifugation. Endogenous maspin protein from PC3 cells was identified in the nuclear, membrane, cytoplasmic and cytoskeletal fractions. Fractionation was successful, judged by the compartment specific localisation of histone 2B and GAPDH. Although GAPDH is primarily a cytoplasmic protein, evidence has been provided that it can shuttle to and from the nucleus, which may explain why we observed GAPDH in both of these compartments. The observation of maspin in the cytoskeletal compartment suggested that maspin may have a role as a cytoskeletal regulator. These results also matched early observations that maspin resides in the nucleus, cytoplasm and at the cell membrane, supporting its diverse cellular roles (Pemberton et al 1997). Recently, maspin protein expression in MCF10A epithelial breast cells was shown to be intracellularly restricted, with any extracellular expression the result of cell damage or necrosis (Teoh et al 2010).

In an attempt to further study the cellular localisation and trafficking of maspin, maspin-GFP fusion proteins were constructed. However, we failed to see expression of either wild type or E244A maspin-GFP constructs in MCF7 or DU-145 cells, despite the sequence being in-frame with the GFP tag and the successful transfection and observation of GFP from positive control (data not shown). We chose to tag maspin with GFP at its C-terminus, as prior V5 and His tagging in this region did not hinder its protein expression. However, the GFP protein is itself large, at approximately 28 kDa, which could easily interfere with protein folding. Nonetheless, there have been previous reports of a successful GFP-tagged maspin protein, although this was adjoined to maspin’s N-terminus (Zhang et al 2005, Bailey et al 2005, Shi et al 2001).

4.4.2 The Effect of Maspin on Cytoskeletal Components

4.4.2.1 Maspin Directed Changes in Cell Cytoskeleton
When maspin or empty vector transfected DU-145 cells were stained with phalloidin, a noticeable difference in their actin cytoskeleton was observed. Parental DU-145 cells are deficient in gap junctions, yet communicate with adjacent cells through intercellular bridges composed of filopodia (Vidulescu et al 2004). Vector only treated DU-145 cells
showed an extensive filopodial network. In comparison, cells expressing wild type maspin showed a reduction in filopodial formation, particularly at the periphery where there was not a neighbouring cell. The expression of maspin altered the mesenchymal-like cell phenotype of DU-145 cells to an epithelial-like one. The maspin-dependent reduction of filopodia implicates that cells would have reduced transport of ligand receptors to the filopodial tips and an accompanied reduction in sensitivity towards external stimuli such as guidance cues. Thus, a reduction in peripheral filopodial filaments suggests that maspin could use this mechanism to perturb directed cell locomotion. Maspin expressing cells did not show a reduced number of intercellular bridges. This implies that maspin does not perturb cell-cell adhesion and may influence filopodial localisation which is normally controlled by active Rac1.

Monoclonal MCF7 stable cells expressing wild type maspin, E244A maspin or empty vector were stained with phalloidin and classified according to their phenotype. Analysis of the actin cytoskeleton of MCF7 cells expressing wild type maspin showed an increase of epithelial-like features, such as smooth discoid cell shape and marginal F-actin bundles arranged along the whole cell periphery. This was suggestive of a non-motile, adhesive phenotype. In comparison, control treated stable cells showed the mesenchymal characteristics expected of migratory tumour cells; an elongated fibroblast-like morphology with a leading lamellipodia and trailing edge. This difference was reflected by the morphometric scoring system, of cells plated onto plastic, collagen I, laminin and fibronectin. Such an increase in epithelial-like characteristics induced by wild type maspin was consistent with an overall reduction in cell migration (Chapter 5, Figure 5.13). The relationship between the cellular morphology of maspin and E244A expressing cells and cell migration is addressed in detail in Chapter 5. The differentiated mammary epithelial MCF7 cell line is known to grow in clusters and often form domes. Upon two dimensional cell culturing of our MCF7 stable cell lines, it was noted that cells expressing wild type maspin formed large, tightly grouped clusters compared to cells containing either E244A maspin or empty vector. This morphological difference was studied further by analysis of cell-cell adhesion (Chapter 6).

When cultured onto plastic, collagen I or fibronectin, stable cells expressing mutant maspin protein showed an intermediate phenotype, scoring a reduced epithelial phenotype with mesenchymal-like attributes. However, when grown on a laminin substrate, cells expressing mutant maspin exhibited very similar epithelial-like characteristics to that of wild type maspin expressing cells. This laminin specific effect suggests that maspin may be acting extracellularly, perhaps by the interaction with a laminin specific cell surface receptor, or maybe with laminin itself. The mechanism that
maspin utilises in the presence of laminin is thus independent of its α-helix G. The observation of mesenchymal-like characteristics was highest for all three cell lines when cultured onto laminin. It has previously been shown that MCF7 cells have a high binding affinity for laminin, which promotes cell attachment and spreading and subsequent promotion of metastasis (Terranova et al 1983).

Morphometric analysis suggests that wild type maspin protein causes a mesenchymal to epithelial transition and that maspin mutated at its α-helix G, is unable to cause MET on matrices other than laminin. E244A expressing cells were metastable; a term introduced to represent the appearance of epithelial-like and mesenchymal-like characteristics within the same cell population (Lee et al 2006). E244 mutation could possibly hinder maspin’s interaction with proteins controlling directed actin formation and elongation. However, it remains possible that the increased fibroblastic nature of E244A cells compared to wild type maspin transfected cells was due to differential expression. Western blot analysis showed that MCF7 stable cells expressed wild type maspin protein at a level twice that of the mutant protein. This reduction of expression implicates that mutant maspin presents difficulties in folding of the protein, and could be a result of elimination of a misfolded folding intermediate (Chapter 3). Despite differential levels of expression, fluorescence staining of both wild type and E244A proteins displayed similar punctate patterning.

Results indicate that maspin protein has a pivotal role in the reorganisation of the actin cytoskeleton and the ability to promote an epithelial-like phenotype. MET involves the cytoplasmic arrangement of both actin and microtubules. The observed actin-rich cell periphery of wild type maspin expressing MCF7 cells corresponded with our observations of microtubule organisation (Appendix Figure A4). Although there was no observed change in microtubule organisation between MCF7 cells stably transfected with wild type maspin or control vector it was noted that the microtubule plus ends were not as dense at the cell periphery of maspin treated MCF7 cells. This was characteristic of microtubule plus ends of a polarised cell; which rarely enter a protruding lamellipodium. However, maspin treated cells were not polarised and microtubule filaments kept a distance from a majority of the cell periphery. Such arrangement suggested that maspin expressing cells contained an actin-rich cell boundary, preventing microtubule diffusion.

Parallel and thick actin filaments have been identified previously from maspin-expressing MDA-MB-435 cells (Chen et al 2005). Maspin expressing cells decreased filopod protrusions. In support of this, filopodia are controlled by Cdc42, of which maspin has been shown to decrease its GTP active form (Odero-Marah et al 2003). Additionally, wild type maspin increased lamellipodia which formed a thick marginal apron. This would
suggest that levels of Rac1, responsible for lamellipodial formation, were up-regulated. However, it has been shown that in response to exogenous or stably transfected maspin, levels of active Rac1 and the Rac1 effector PAK1 decreased; resulting in a regulation of cell motility and adhesion (Odero-Marah et al. 2003), cell behaviours that are addressed in Chapters 5 and 6.

Unlike monoclonal MCF7 cells stably expressing maspin, DU-145 cells transiently transfected with wild type maspin were not scored according to their phenotype because transfection efficiency could not be controlled. To overcome this problem, transiently transfected cells were co-stained with both maspin and F-actin Alexa Fluor antibodies. Subsequent imaging using an upright microscope, which fixed the exposure between cell types, ensured the imaging of only maspin transfected cells. However, in order to retrieve sharper images of the actin cytoskeleton a confocal microscope was required, yet this was not performed.

4.4.2.2 Maspin’s α-helix G is Critical for the Rearrangement of Actin Cytoskeleton

Wild type maspin caused the rearrangement of the actin cytoskeleton to form an epithelial-like morphology. This was mimicked by the addition of a peptide composed of maspin’s α-helix G residues. Alike the expression of wild type maspin, G-helix peptide induced an increase in actin rich periphery and reduced both the number and thickness of filopodia extensions. Although maspin has been shown to induce stress fibre formation in MDA-MB-435 cells, we did not observe an increased amount of stress fibres in DU-145 or MCF7 cells from either the addition of G-helix or the transfection of wild type maspin (Seftor et al. 1998). Additionally we did not observe a promotion of stress fibre formation in the MCF7 stable cell lines; a common property of cells grown in vitro due to the inability of a cell to contract because of its rigid adhesion to tissue culture plate. Instead MCF7 stable maspin cell lines showed flocculent actin and few stress fibres. However, it was noticed that the addition of G-helix peptide to DU-145 cells showed a stress fibres parallel to the cell edge as opposed to longer central stress fibres, seen in cells treated with E244A/E247A/control peptides or DMSO. This observed peripheral arrangement of stress fibres suggests that they are stabilised along the cell periphery by an up-regulation of focal adhesions. This was investigated in Chapter 6 (Figure 6.14).

Unlike the addition of G-helix, the addition of either E244A or E247A peptide did not show any rearrangement of actin different from cells treated with control peptide or carrier control. The morphology of cells expressing mutated E244A maspin protein was metastable, showing a phenotype of mixed epithelial-like and mesenchymal-like character (Figure 4.10). This metastable appearance questions whether mutation to maspin at
E244A renders it unable to promote the formation of epithelial-like characteristics, or whether it can not due to its lower intrinsic expression compared to wild type maspin protein. The results shown by E244A peptide suggest that the former is more likely; since the peptides E244A or E247A failed to show any cytoskeletal re-organisation. Thus maspin’s α-helix G plays a critical role in its ability to promote epithelial-like phenotype.

In comparison to wild type maspin protein, the maspin peptides would not form the tertiary structure of an α-helix, and thus may not interact with its cellular targets in the same way. However, the observation that maspin’s G-helix peptide has the same effect on the actin cytoskeleton as maspin protein, supports the importance of this region to promote a non-motile phenotype. How maspin’s G-helix peptide exerts its effects is of great interest for future investigation; for example whether the negatively charged G-helix peptide can bind to a cell surface receptor, or whether the peptide can diffuse through the membrane or become internalised and act at an intracellular target. We used immunohistochemistry to try to identify the biotin-labelled maspin peptides, but unfortunately we could not detect a specific signal for the peptides in relation to their carrier control. Fluorescent labelled peptides may overcome this problem. Although we have shown that maspin transfected DU-145 and MCF7 cells express maspin protein in the cell lysate and conditioned medium (Figure 4.1A, 3.4A), it remains undetermined whether this maspin-dependent change in the cytoskeleton is an effect caused by maspin located either intracellularly or extracellularly, or both.

4.4.3 The Effect of Maspin on Cellular Actin
MCF7 cells stably expressing wild type maspin formed thick peripheral bands of F-actin, which was accompanied by an increase in F-actin content. Maspin induced a relative increase in F-actin content of cells cultured on substrates plastic, collagen I, laminin and fibronectin, which corresponded to a reduction of mesenchymal-like character. This is supported by evidence that a decrease in F-actin content has previously been used as a marker for transformation in relation to cell differentiation (Rao et al 1990). The maspin-directed increase in F-actin did not form structures typical to a motile cell, like filopodia or directed lamella; suggesting the direct result of a down-regulation of active Rac and active Cdc42 (Shi et al 2007). However, maspin expressing cells showed depolarised lamellipodia around the entire cell periphery, suggesting the requirement of active Rac. MCF7 cells expressing mutant maspin E244A protein showed lower levels of F-actin on all substrates compared to cells expressing wild type protein. A lower content of F-actin complied with the scoring of E244A cells that showed mesenchymal-like attributes like control cells, and a reduced cortical actin in comparison to wild type maspin.
The G-actin content of wild type maspin MCF7 cells was also increased compared to mutant maspin or empty vector cells. An increase in G-actin suggested an increased cell motility aided by the promotion of F-actin polymerisation. However this is most likely when the G-actin: F-actin ratio is high, which was not the case of maspin expressing cells. Nonetheless, cells expressing maspin did show an increase in actin-rich lamellipodia like structures at the cell periphery, which would require actin polymerisation. Thus, maspin may regulate the polymerisation of F-actin, by influencing the levels of G-actin. For example, an increase in G-actin could result from an increase in ADF/cofilin expression/activity. An increase in ADF/cofilin activity could also contribute to the formation of long stable actin filaments; which are seen at the cell edge of maspin expressing cells.

4.4.4 Relationship between Maspin and Profilin Expression

The expression of the G-actin binding protein profilin-1 in MCF7 maspin stable cells cultured on plastic was determined using western blotting. Unfortunately antibody detection via fluorescence staining was not viable. MCF7 cells producing wild type maspin showed a reduced expression of profilin-1, compared to control cells or those expressing E244A which showed a higher level of profilin-1 expression. Maspin’s reduction in profilin-1 protein was not reciprocated at the mRNA level; as equal amplification of profilin-1 gene was detected for each stable cell line. Previous analysis of the carcinoma proteome transfected with wild type maspin, showed a change in expression of proteins that regulate cytoskeletal architecture, including a 2-fold down-regulation of profilin-1 at both protein and mRNA level (Chen et al 2005). Nonetheless it has been reported that maspin has a high influence on post-transcriptional regulation, which supports this finding. Additionally maspin was shown to reduce expression of F-actin capping protein and actin related protein, which supports maspin’s involvement with actin nucleation and polymerisation. In our cell model we propose that the expression of profilin-1 was not acting as a tumour suppressor and rather acting as a positive regulator of cell motility (Section 4.1.3). Thus the reduced levels of profilin-1 in maspin expressing cells would reduce actin polymerisation at leading edge lamellipodia, creating a non-motile phenotype like that observed.

Cells expressing wild type maspin, exhibited a post-transcriptional decrease in profilin-1, yet did not show a decrease in its high affinity ligand G-actin. Thus it could be suggested that maspin up-regulates other actin binding proteins in place of profilin. A good candidate for this would be thymosin β4, which would prevent spontaneous or induced actin polymerisation. Simple down-regulation of profilin-1 would increase spontaneous nucleation of actin filaments. This may play part in maspin-directed formation of F-actin
structures under the influence of Rho, such as actin bundling to form thick cortical boundaries. Conversely, control cells have a greater profilin:G-actin ratio, suggestive of a more efficient polymerisation (reviewed in Pollard and Borisy 2003). This suggests that cells lacking maspin have an increased rate of nucleotide exchange characteristic of a migratory cell; which corresponds with the morphological character of these cells. A high concentration of profilin has previously been associated with reduced density of parallel actin bundles (Finkel et al 1994), of which we also observed in cells deficient in maspin.

The knockdown of profilin-1 simultaneously reduced the expression of wild type or mutant maspin protein, as detected by western blotting and immunofluorescence. Concurrent knockdown of maspin suggests that profilin-1 was upstream of maspin and could thus influence maspin’s expression. If the reduction of maspin was due to a direct protein interaction with profilin, mutation to the α-helix G of maspin would not function since mutation to this region did not perturb the reduction of protein. However, it also remains likely that maspin could be upstream of profilin-1, since in cells over-expressing maspin, profilin-1 levels were lowered. Where profilin-1 expression was lowered considerably, as a result of targeted siRNA, a negative effect on the levels of maspin protein occurred. If maspin was acting as an upstream negative regulator of profilin-1, it would be expected that a decrease in maspin expression would increase expression of profilin-1. However, this was not observed when cells were treated with maspin siRNA; profilin-1 expression did not alter.

The suppressive affect of maspin on profilin-1 may result from the upstream inhibition of profilin-1 activating proteins. Profilin-1 is under the control of actin nucleators WASp/formins. WASp and its relative formins are both effectors of Rac and Cdc42. As discussed earlier, maspin has been shown to reduce levels of active Rac and its effector PAK1 (Odero-Marah et al 2003). It was shown that through a down-regulation of the Rac effector PAK1, maspin induced an increase in focal adhesions and stress fibres which led to an increase in cell adhesion. This cytoskeletal re-organisation has been mimicked in our MCF7 cells over-expressing wild type maspin. Thus maspin-dependent reduction of active Rac could also decrease its effector formins and respective profilin-1 protein; resulting in a reduction of directed actin nucleation typical to non-motile cells. Additionally maspin could also potentially down-regulate the expression of the Rac effector phosphatidylinositol 4-phosphate 5-kinase (PI5K), which controls the expression of phospholipid phosphatidylinositol 4, 5-biphosphate (PIP2). Profilin can specifically bind to PIP2, forming a profilin dominant protruding cell edge. Thus maspin may regulate the localisation of profilin, reverting the cellular phenotype away from a polarised one (Figure 4.16). It is interesting to note that signalling proteins WASp/formins, along with related
Scar/WAVE components, normally concentrate in filopodia; of which were significantly reduced in cells over-expressing wild type maspin. Furthermore, it has previously been reported that maspin down-regulated the expression of actin-related protein, which is also under the control of WASp (Chen et al 2005).

![Figure 4.16](image)

**Figure 4.16** Hypothetical schematic showing the regulation of the cellular actin cytoskeleton by maspin. Maspin reduces levels of active Rac1 and Cdc42 (Odero-Marah et al 2003). Subsequent reductions of downstream effectors Formins and P15K reduce profilin-1 and PIP2. PIP2 can regulate localisation and concentration of profilin-1. Reduction in actin nucleator profilin-1 results in decreased actin polymerisation and contributes to a mesenchymal-epithelial transition. Low levels of profilin-1 can negatively feedback to maspin.

### 4.5 Future Perspectives

The introduction of wild type maspin showed a dramatic change in the actin cytoskeleton, whereas the change in microtubular array was subtle. Since actin and microtubule rearrangement are coupled, the effect of maspin on microtubule associated proteins could be investigated. The maspin-dependent increase in the epithelial-like features could be reflected in the cytokeratin expression profile of intermediate filaments; of which changes within epithelium as it undergoes malignant transformation.

Our results suggest that maspin regulates the F-actin assembly, possibly through the control of cellular actin levels. In the future we would like to determine whether the amount of F-actin occurs in a maspin dose-dependent manner. The levels of profilin-1 was only determined for cells cultured onto plastic, therefore the effect of cells cultured on matrix components would also be of interest. These results lead us to the hypothesis that
maspin can control profilin-1 via a down-regulation of Rac/Cdc42 and its effectors. Investigation into the levels of formins and P15K would be of promising interest. Furthermore, the effect of maspin on profilin competitor thymosin β4 and ADF/cofilin could too provide additional insight into the way maspin co-ordinates assembly of the cytoskeleton.

The maspin-dependent induction of MET was mimicked by a peptide consisting of wild type sequence of maspin’s α-helix G region. This effect was noted on cells cultured onto plastic. It would be interesting to study the effect of maspin peptides on cells cultured on matrix components and if the peptides mimic the morphology scoring seen by cells expressing wild type or mutant proteins. Additionally, the identification of the site of action of the maspin peptides could provide valuable mechanistic insight. Furthermore, maspin may influence markers of MET transition, such as snail and slug which are regulated by Ras/MAPK pathways, or Twist which has been implicated in the regulation of metastasis.

4.6 Conclusions
Our findings are consistent with other reports that maspin restores the epithelial-like phenotype of cells. Specifically, the expression of wild type maspin exhibited a phenotypic change in the cytoplasmic arrangement of actin stress fibres. The transfection of wild type maspin into breast cancer cells resulted in a transition to a non-motile, adhesive epithelial phenotype. In comparison, the non-transfected cells showed a transformed morphology typical of a locomotive metastatic phenotype; with polarized actin architecture with extending filopodia and irregularity of cell shape. Cells expressing mutant maspin E244A showed an equal reversion to an epithelial-like phenotype when cultured on a laminin matrix. However, E244A cells grown on plastic, collagen I or fibronectin did not show epithelial-like character. Both wild type and mutant maspin showed reduced cellular levels of profilin-1, a major controller of actin polymerisation at the cell edge and thus regulator of motility. Our data points towards a new hypothesis of the molecular mechanism of maspin in the regulation of tumour metastasis. The tumour suppressive effect of maspin could be mediated via its interaction with the F-actin network, through the control of cellular levels of monomeric actin and via the regulation of actin monomer binding protein profilin-1. The resulting cytoskeletal re-arrangements are likely to be governed by maspin's regulation of the family of Rho GTPases.
5 The Effect of Maspin on Cell Migration

5.1 Introduction
The work in this chapter investigates the effect of manipulating maspin expression on cell migration. This included transfection of wild type maspin and the over-expression or the knockdown of wild type maspin on cell migration. Experiments were performed on a range of cell lines including, DU-145, MCF7, LNCaP, PC3 and PNT1a. The transfection of various mutant maspin constructs and their effect on cell migration was also studied. The effect of adding recombinant maspin or maspin peptides to the migration of maspin-null cells was also explored.

5.1.2 Cell Migration
Directional cell migration is important in both physiological processes such as embryonic development and wound repair, but also in pathological processes including tumour invasion and metastasis. Cell migration is a complex process involving the spatio and temporal integration of cell membrane protrusions and adhesions at the leading edge, with an establishment of an anterioposterior cell axis (Figure 1.7). Conceptually, the typical migrating cell has rapidly changing activities that are spatially segregated, particularly at the cell front and rear. Such polarised morphology shows a directed, protruding leading edge and cell retraction at the rear. This re-arrangement of the actin cytoskeleton accompanied by new actin polymerization provides the driving force for cell movement. Cell migration co-coordinately alters the directed secretion of membrane components, the control of cell-extracellular adhesion, changes in gene transcription and a re-alignment of the cell microtubular network and Golgi. At the leading edge focal adhesions anchor the cell cytoskeleton to the ECM providing a net force of forward traction. The trailing cell edge then retracts by actomyosin contraction and adhesion disassembly. A migrating cell has three filamentous structures; lamellipodia, filopodia and actin-myosin bundles, each controlled by Rho GTPase proteins and each linked to the underlying cell matrix by integrins (Chapter 6). Factors that can influence directional migration include the topography of the ECM, cell polarity and cell adhesion.

5.1.3 Maspin Dependent Inhibition of Cell Migration
The tumour suppressive activity of maspin via an anti-migratory mechanism has been demonstrated. Maspin has been shown to inhibit the migration of either parental or tumour-derived endothelial or epithelial cell lines. Furthermore, it has been demonstrated that these effects arose via either transfection of maspin cDNA or from the addition of recombinant maspin to the extracellular medium. For example, recombinant maspin inhibited the migration of both differentiated and non-differentiated VSMC (Bass et al.
Human epithelial cells, fibroblasts, keratinocytes and mammary carcinoma cells also showed a decreased motility in the presence of exogenous maspin (Zhang et al 2000b). Similarly, maspin expressing transfectants of mammary and prostatic cancer cells decreased cell motility (Sheng et al 1996). The reduction in cell migration by the addition of recombinant maspin to the extracellular milieu suggests an extracellular mechanism of action. This suggestion was found to be in agreement with the observation that recombinant maspin bound to the cell surface of tumour MDA-MB-435 cells and to VSMC (Sheng et al 1996, Bass et al 2009).

The exact mechanism that maspin uses to exert its inhibitory effect on cell migration is not known. However there have been several experimental insights. For instance, the tumour suppressive effect of maspin on cell migration occurs in the absence of any protease-inhibition activity. Studies have shown that in comparison to the efficient protease inhibitor PAI-1, maspin does not inhibit uPA bound to its cell surface receptor, nor tPA bound to fibrin (Bass et al 2002). Additionally, it was shown that the anti-invasive properties of maspin were resultant from the blocking of cell migration. Specifically these anti-migratory activities were disrupted in the presence of anti-RCL, thus suggesting a mechanism that depends on an intact RCL (Sheng et al 1996). In correspondence, it was shown that a mutated RCL abolished the ability to block migration in human epithelial cells (Zhang et al 2000). Furthermore a peptide corresponding to the maspin’s RCL competed with wild type maspin for cell surface binding, suggesting the importance of maspin’s RCL in a possible extracellular mechanism responsible for the inhibition of cell motility (Ngamkitidechakul et al 2003).

5.2 Chapter Aims

1. To investigate the effect of wild type maspin on cell migration using transient or stable transfection to introduce or over-express maspin, or siRNA to knock down endogenous maspin expression.
2. To study the effect of cell migration upon the transfection of maspin constructs point mutated at structurally important elements.
3. To investigate the effect of adding maspin peptides on cell migration.
4. To determine whether the effect of maspin on cell migration is influenced by the ECM.
5. To study the influence of exogenous wild type and mutant maspin proteins on cell migration.
5.3 Results

5.3.1 The Effect of Knocking Down Maspin Expression on Cell Migration

Following from the observations that wild type maspin exhibits an anti-migratory phenotype (Chapter 4), we investigated the effect of cell migration when endogenous maspin expression was altered. The optimisation of maspin siRNA was first performed. Prostate cancer cell lines LNCaP and PC3, and normal prostate epithelial cell line PNT1a all express endogenous maspin (Figure 3.2A). These cell lines were treated with two siRNAs targeted to maspin, named #3 and #4, and a scrambled control siRNA, CTRsi. Maspin silencing was verified by western blotting (Figure 5.1), whereby an optimal concentration of siRNA was determined; 100 nM or 200 nM siRNA #3 or #4 respectively. Optimal knockdown of maspin was found between 48 and 72 hours post transfection for both siRNAs. The combined treatment of cell lines with both #3 and #4 siRNA gave no further knockdown than cells treated with #3 or #4 siRNA alone (data not shown). Maspin silencing was also verified for cells fixed in PFA using immunofluorescence against anti-maspin (Figure 5.2).

The migration of prostate cell lines LNCaP, PC3 and PNT1a were analysed using time-lapse video microscopy (Figure 5.3A). In comparison to each other, PC3 cells migrated at a higher rate than LNCaP cells, which in turn were higher than PNT1a cells. The migration of these cell lines treated with maspin siRNA was next analysed, also using time-lapse video microscopy (Figure 5.3B) that commenced at 48 hours post transfection. For each cell line, treatment with maspin siRNA #3 or #4 significantly increased migration in comparison to cells treated with CTRsi. This confirmed that the expression of maspin inhibits cell migration. The addition of either maspin siRNA #3 or #4 had an almost identical effect on cell migration when added to PC3 or PNT1a cells, showing an increase in cell migration by approximately 50% and 95% respectively. However, siRNA #4 only increased the cell migration of LNCaP cells by 31%, compared to siRNA #3 which caused a 94% increase. This was not an effect due to an increased concentration as the migration of LNCaP cells treated with either 100 nM or 200 nM CTRsi showed a similar rate of migration (µm/ hr) (data not shown). Also it was unlikely that this difference in effect was due to a difference in protein knockdown between the two maspin siRNA’s since both showed a similar level of effective knockdown (Figure 5.1). Thus the difference between #3 and #4 siRNA on LNCaP cell migration could have been due to analytical error. For example, since transfection of the cell population with siRNA was a transient process, there was no guarantee that the cells analysed had indeed been transfected. This difference in knockdown could be due to off-target effects of the siRNA, for example, if the siRNA holds a homology to mRNA transcripts other than that of maspin.
Figure 5.1 Knockdown of maspin expression using targeted siRNA. Western blots show 10 µg soluble cell lysate from A) LNCaP, B) PC3 and C) PNT1a cells subject to maspin siRNA #3 or #4 and CTRsi over a period of 72 hours. Maspin siRNA #3 or #4 were added to cells at 100 nM or 200 nM respectively, with corresponding concentration of CTRsi. Blots were probed with anti-maspin (upper panels) or anti-GAPDH to confirm equal loading (lower panels).
Figure 5.2 Maspin knockdown was verified by immunohistochemistry. Maspin siRNA #3 or #4 were added alongside CTRsi, at either 100 nM or 200 nM respectively, to A) LNCaP, B) PC3 or C) PNT1a cells. Cells were fixed 72 hours post transfection, stained with anti-maspin and DAPI and imaged using CCD upright microscope. Panel B shows cells imaged with DIC III in the absence of DAPI. Fluorescence intensity was measured per µm².
Figure 5.3 The effect of maspin siRNA on cell migration. A) Graph showing rate of migration (µm/hr) of LNCaP, PC3 and PNT1a cells. B) LNCaP, PC3 and PNT1a cells were subject to 100 nM #3 siRNA or 200 nM #4 siRNA (grey) versus the same concentrations of CTRsi (blue). Inset shows verification of protein knockdown. 100% migration indicates cells treated with CTRsi. Time-lapse video microscopy experiments were performed in triplicate and repeated three times. Statistical significance was measured by Students t-test (*p<0.05).

5.3.2 The Effect of Wild Type or Mutant Maspin Constructs on Cell Migration
Following from the observation that knocking down maspin expression increases cell migration, the effect of introducing maspin expression on cell migration was determined. As assessed by time-lapse video microscopy, maspin-null DU-145 cells transfected with wild type maspin reduced cell migration by 30%, compared to DU-145 cells transfected with empty vector (Figure 5.4A). To determine whether mutation of maspin at structurally important elements would affect its anti-migratory behaviour, DU-145 cells were transiently transfected with maspin mutant constructs; K90A, R340A, E115A, E244A and E247A (Section 3.1). Time-lapse video microscopy was performed alongside DU-145 cells transfected with wild type maspin and empty vector control (Figure 5.4A).
Verification of transfection was performed using western blotting, which detected wild type and mutant maspin proteins in the cell lysate and conditioned medium (Figure 3.4A).

Transfection with either maspin mutant constructs K90A, E115A or R340A retained the functionality of wild type maspin; shown by a similar reduction in cell migration. However, the transfection of DU-145 cells with either E244A or E247A mutant maspin constructs showed a migration similar to cells transfected with vector only. Therefore mutation to maspin residue E244 or E247 significantly eradicated the inhibitory effect of wild type maspin on cell migration. The effect of wild type maspin or maspin mutant proteins E244A or E247A was replicated in MCF7 and PC3 cells, which had been manipulated to express or over-express the maspin constructs via transient or stable transfection respectively (Figure 5.4B). The over-expression of wild type maspin protein reduced cell migration by 30% in PC3 cells and by 62% in MCF7 cells, whereas the expression of proteins E244A or E247A did not change the migration of these cells. The level of E244A protein expression in MCF7 stable cells was approximately half that of wild type maspin, which could indicate the reason for the perturbed effect of E244A in cell migration. However, our results shown by the equally expressing transient transfectants indicate that this is highly unlikely. In summary, the overexpression of wild type maspin reduces cell migration, by a mechanism dependent on an intact α-helix G of which its residues E244 and E247 reside.

To determine the effect of maspin on apoptosis, DU-145 cells transiently transfected with wild type maspin and mutant constructs K90A, R340A, E115A, E244A and E247A were treated with protein kinase C inhibitor or activator, bisindoylmaleimide III or PMA respectively (Figure 5.5). In the presence of PMA, wild type and mutant maspin constructs did not induce apoptosis in relation to DU-145 cells expressing empty vector. However, cells expressing wild type maspin in the presence of bisindoylmaleimide III increased the sensitivity of cells to challenge with the protein kinase C inhibitor, and thus stimulated apoptosis. Cells expressing mutant maspin constructs all retained the ability of the wild-type molecule to stimulate apoptosis. Since mutation to maspin's α-helix G did not perturb its ability to increase cellular sensitivity to apoptosis, the effects of maspin on cell migration can be uncoupled from its action on apoptosis.
Figure 5.4 Influence of wild type and mutant forms of maspin on cell migration. Data is presented as % of cell migration for A) transiently transfected DU-145 cells and B) transiently transfected PC3 and stably transfected MCF7 cells; 1; pcDNA3.2, 2; pcDNA3.2-maspin, 3; pcDNA3.2-E244A, 4; pcDNA3.2-E247A. Corresponding western blots show 10 µg cell lysate, detected by anti-V5. 100% migration indicates cells transfected with pcDNA3.2 (DU-145 cells have an average speed of 2.28 µm/hr, PC3 cells 16.1 µm/hr and MCF7 cells 4.6 µm/hr). Statistical significance was measured by Students t-test (*p<0.05). Time-lapse video microscopy experiments were performed by Dr. Laura Wagstaff (University of East Anglia).
Figure 5.5 The effect of wild type and mutant maspin proteins on cellular apoptosis. DU-145 cells were transiently transfected with empty vector-pcDNA3.2, wild type or mutant maspin constructs as indicated. Cells were treated with either phorbol 12-myristate 13-acetate (white bars) or protein kinase C inhibitor; bisindolylmaleimide (blue bars) to decrease or increase the sensitivity of cells respectively. Time-lapse video microscopy experiments were performed on three separate occasions, with four replicates for each condition and five fields analysed for each sample. Normal and apoptotic cells were counted and data is presented as % apoptotic cells and statistical significance determined by Students t-test (*p=<0.05). Experiments were performed by Dr. Laura Wagstaff (University of East Anglia).

5.3.3 Maspin Peptide Mimics the Effect of Wild type Maspin on Cell Migration
Following from the observation that maspin’s α-helix G is critical for maspin’s inhibitory influence on cell migration, we set out to determine whether the α-helix G in isolation could have similar effects. Four maspin peptides were designed to span the sequence of the α-helix G, including a wild type sequence, mutant peptides E244A and E247A, and a control peptide with a re-arranged sequence (Figure 4.9). The migration of maspin-null DU-145 cells whilst incubated with increasing concentration of wild type G-helix peptide was analysed using time-lapse video microscopy (Figure 5.6A). A significant reduction in cell migration was observed of cells treated with a minimum concentration of 5 µM G-helix peptide and a maximum 29 µM; of which beyond this concentration the peptide carrier DMSO became toxic to the cells. Thus to reduce potential toxicity, peptides were used at 5 µM. DU-145 cells were next treated with 5 µM of each maspin peptide to see their effects on cell migration in comparison to cells treated with peptide carrier control DMSO (Figure 5.6B). Wild type G-helix peptide significantly reduced cell migration by 45% compared to cells treated with mutant peptides E244A or E247A, or DMSO treated cells. The effect of control peptide on cell migration showed a rate of migration similar to that of cells treated with carrier control (Ravenhill et al 2010).
Figure 5.6 The effect of maspin peptides on DU-145 cell migration. Analysis was performed using time-lapse video microscopy. A) DU-145 cells incubated with G-helix peptide at increasing concentration. B) DU-145 cells were treated with either 5 µM peptides G-helix, E244A, E247A or carrier control DMSO. 100% migration indicates cells treated with DMSO, at an average speed of 2.3 µm/hr. Statistical significance was measured with Students t-test (*p<0.05).

The effect of adding maspin peptides to the migration of cells already expressing wild type maspin was next determined. Peptides were incubated with MCF7 cells stably expressing wild type maspin or empty vector pcDNA3.2 and analysed using time-lapse video microscopy (Figure 5.7). MCF7 cells expressing wild type maspin showed a reduced migration in comparison to control cells by 63%. However, the addition of wild type G-helix peptide did not elicit any further reduction in cell migration. Conversely, MCF7 cells expressing empty vector showed a 52% reduction in cell migration when G-helix peptide was added. Mutant peptides E244A and E247A were unable to reduce cell migration to either maspin expressing or control MCF7 cells. Thus, these results suggest that the wild type sequence of maspin's α-helix G in isolation holds responsibility for maspin's ability to reduce cell motility.
Figure 5.7 The effect of maspin peptides on MCF7 cell migration. Time-lapse video microscopy traced the migration of MCF7 stable cells expressing vector only pcDNA3.2 or wild type maspin, in the presence of either 5 µM G-helix, E244A, E247A or carrier control DMSO. 100% migration indicates vector only cells treated with DMSO at an average speed of 4.6 µm/hr. Three independent, triplicate experiments were performed. Students t-test denotes statistical significance (*p<0.05).

To determine whether the mutant maspin peptides could compete with the wild type G-helix peptide, the migration of VSMC was analysed in the presence of combined peptides (Figure 5.8) (Ravenhill et al 2010). The wild type G-helix peptide was able to influence VSMC migration in the presence of equimolar mutant peptide, suggesting that the mutant peptides were not able to compete with the wild type peptide. The presence of G-helix peptide significantly reduced VSMC migration by approximately 60%.

Figure 5.8 The effect of maspin peptides on VSMC cell migration. Cell migration was determined by time-lapse video microscopy of VSMC incubated with 10µM+10µM peptide or DMSO. 100% migration indicates cells treated with DMSO+DMSO at an average speed of 5 µm/hr. Three independent, triplicate experiments were performed. Students t-test denotes statistical significance (*p<0.05). Experiments were performed by Dr. Rosemary Bass (University of East Anglia).
5.3.4 Maspin G-helix Peptide Exerts a Greater Effect on the Migration of Cells Pre-treated with Maspin siRNA

The effect of adding maspin peptides to the migration of cells that had been pre-treated with maspin siRNA to silence the expression of endogenous maspin was next determined. PC3 cells were treated with maspin siRNA #3 versus CTRsi (Figure 5.1). At 48 hours post siRNA treatment, cells were treated with G-helix peptide and subject to time-lapse video microscopy, to find an optimal concentration that could produce a significant reduction of cell migration (Figure 5.9). The optimal concentration of G-helix was found to be 10 µM, which showed a 50% decrease in cell motility in comparison to cells treated with peptide carrier control DMSO.

Using this optimal concentration, the effect of maspin peptides on the migration of LNCaP, PC3 and PNT1a cells treated with maspin siRNA #3 or #4 was determined (Figures 5.10-5.12). For each experiment, maspin knockdown was verified (data not shown). All cell lines pre-treated with #3 or #4 maspin siRNA, in the absence of peptide, significantly increased cell migration compared to cells treated with CTRsi. This agreed with previous findings that knockdown of maspin increased cell motility (Figure 5.3). The addition of wild type G-helix peptide to LNCaP cells treated with siRNA #3 or #4 resulted in a significant decrease in cell migration in relation to cells treated with CTRsi, by 56% and 51% respectively. Similarly, G-helix Peptide significantly reduced the migration of PC3 and PNT1a cells pre-treated with #3 or #4 siRNA; by 40% and 60%, and 50% and 131% respectively. In comparison, G-helix peptide only decreased the migration of PC3 cells pre-treated with 100 nM CTRsi, by 21%. This was not reciprocated for LNCaP and PNT1a cells, or for cells treated with 200 nM CTRsi. Thus, the addition of G-helix peptide only induced a decrease in migration in cells where endogenous maspin expression had not been knocked down.

The addition of peptides E244A or E247A, in comparison to DMSO, did not reduce the migration of either LNCaP or PC3 cells pre-treated with maspin siRNA or CTRsi. However, it was observed that the presence of either mutant peptide to PNT1a cells treated with siRNA #4 significantly reduced cell migration. This was the only condition that showed this result, as the mutant peptides showed no effect on the migration of PNT1a cells treated with #3 or 100/200 nM CTRsi. In fact, PNT1a cells treated with 100 nM siRNA #3 or CTRsi showed a significant increase in cell migration in the presence of E244A or E247A. Since the addition of E244A or E247A peptides had no inhibitory effect of their own, the difference between cells treated with maspin siRNA or CTRsi remained. However, for LNCaP cells treated with E247A, the difference between maspin siRNA and
CTRsi was not significant. Nonetheless the trend of increased migration with a reduced maspin expression remained. This re-enforces the importance of an intact α-helix G.

**Figure 5.9** The effect of wild type G-helix peptide on PC3 cell migration. Time-lapse video microscopy analysed the migration of PC3 cells that were incubated with G-helix peptide at increasing concentration as indicated. 100% migration shows PC3 cells treated with CTRsi and DMSO; an average speed of 8.6 μm/hr. Statistical significance was measures by Students t-test (*p>0.05).
Figure 5.10 The migration of LNCaP cells treated with maspin peptides. Cells were treated with 10 µM G-helix, E244A, E247A peptides or carrier control DMSO. Cells were pre-treated with 100 or 200 nM maspin siRNA #3 or #4 respectively, alongside CTRsi, and analysed using time-lapse video microscopy. 100% migration indicates cells treated with CTRsi and DMSO; an average speed of 4.8 μm/hr. Statistical significance measured by Students t-test (*p<0.05, **p<0.005, ***p<0.0005...e.t.c).
Figure 5.11 The migration of PC3 cells treated with maspin peptides. Cells were treated with 10 µM G-helix, E244A, E247A peptides or carrier control DMSO. Cells were pre-treated with 100 or 200 nM maspin siRNA #3 or #4 respectively, alongside CTRsi, and analysed using time-lapse video microscopy. 100% migration indicates cells treated with CTRsi and DMSO; an average speed of 6.2 µm/hr. Statistical significance measured by Students t-test (*p<0.05, **p<0.005, ***p<0.0005…e.t.c).
Figure 5.12 The migration of PC3 cells treated with maspin peptides. Cells were treated with 10 µM G-helix, E244A, E247A peptides or carrier control DMSO. Cells were pre-treated with 100 or 200 nM maspin siRNA #3 or #4 respectively, alongside CTRsi. 100% migration indicates cells treated with CTRsi and DMSO; an average speed of 3.3 μm/hr. Analysis was performed using time-lapse video microscopy. Statistical significance measured by Students t-test (*p<0.05, **p<0.005, ***p<0.0005…e.t.c).

5.3.5 The Effect of Wild type and Mutant Maspin on Cell Migration over Extracellular Matrix Components and Fibrillar Matrix

The migration of MCF7 cells stably expressing wild type or mutant maspin, over ECM components or a fibrillar matrix was performed using time-lapse video microscopy (Figure 5.13). Human colon adenocarcinoma HT29 cell line was chosen for the production of fibrillar matrix since they produce large amounts of ECM. MCF7 cells expressing wild type maspin significantly reduced cell migration over each matrix component and fibrillar matrix compared to cells expressing vector only. The reductive effect of cell migration by wild type maspin was greatest over collagen I which showed a decrease in cell migration of 74%. This was followed by plastic of 62%, fibronectin at 51%, fibrillar matrix at 42% and laminin at 33%. This result re-iterates the importance of the role wild type maspin plays in the process of cell migration.
Figure 5.13 The migration of MFC7 cells over matrix components and a fibrillar matrix. MCF7 cells stably expressing pcDNA3.2, pcDNA3.2-maspin or pcDNA3.2-E244A were plated onto plastic, collagen I, fibronectin, laminin or a fibrillar matrix deposited by HT29 cells. 100% migration represents cells expressing empty vector pcDNA3.2; with average speeds of 4.6, 3.7, 3.5, 5.7, 3.1 μm/hr for plastic, collagen I, fibronectin, laminin and HT29 respectively. Time-lapse video microscopy experiments were each performed in triplicate and statistical significance was measured by Students t-test (*p<0.05).

When plated onto either, a plastic, fibronectin or fibrillar HT29 cell matrix, MCF7 cells expressing mutant E244A protein behaved similar to control cells expressing vector only. Here cells transfected with mutant maspin E244A did not inhibit cell migration, unlike wild type maspin. However, when MCF7 stable cell lines were plated onto laminin, both cells expressing wild type or mutant E244A maspin inhibited cell migration by 33% and 41% respectively. Thus, mutation to maspin’s α-helix G does not perturb its anti-migratory behaviour when on laminin, but does when on other components and fibrillar matrix.

5.3.6 The Binding of Exogenous Maspin to Cell Surface Proteins
Following from the observations that G-helix peptide dynamically inhibited cell migration, we hypothesised that this effect was due to the binding of peptide to cell surface proteins involved in cell-ECM interactions. In line with this hypothesis was the previous demonstration that low concentrations of recombinant maspin, over a short time scale, could inhibit the migration of VSMC (Bass et al 2002). Additionally, recombinant maspin protein was found to bind to the surface of maspin-null VSMC which displayed a punctate staining in a concentration dependent manner (Bass et al 2009). This latter result was replicated from the binding of wild type maspin, obtained from the conditioned medium of pMTBiP-maspin transfected S2 cells (Section 3.3.5); to maspin-negative DU-145 cells.
Immunofluorescence showed a punctate cell surface staining of maspin protein after one hour incubation with 15% maspin conditioned medium: 85 % serum free medium. Maspin-DU-145 cell surface binding was seen in comparison to DU-145 cells incubated with medium from empty vector transfected cells.

It was next determined whether the interaction of maspin with cell surface proteins was dependent on an intact α-helix G; akin to its regulation of cell migration. Conditioned medium was collected from COS-7 cells which expressed wild type maspin, or mutant maspin proteins E244A or E247A. Media was pooled together and dialysed into control buffer (Section 3.3.9). Maspin-null DU-145 cells were analysed for anti-maspin protein by immunofluorescence after the incubation of 15% conditioned medium: 85% serum free medium. Incubation of wild type and mutant maspin conditioned media’s were performed alongside vector only conditioned medium and control buffer for five hours (Figure 5.14B). DU-145 cells incubated with medium containing wild type maspin showed a weak punctate staining at the cell surface. Comparatively, incubation with medium containing E244A protein showed slightly less staining than wild type maspin, and cells incubated with E247A protein did not show any punctate staining at the cell surface. Also, cells treated with conditioned medium from empty vector expressing cells, or control buffer, did not show any punctate staining. However, under these maspin-negative conditions, cells showed relatively strong background staining with maspin antibody, thus indicating that the actual binding of wild type maspin and E244A proteins from conditioned medium was weak. Incubation of cells with maspin conditioned medium’s at volumes greater than 15% had a toxic affect on DU-145 cells and was thus not viable for experiments. The incubation of conditioned medium for 24 hours did not show an increase in cell surface binding of either maspin protein (data not shown). Therefore in summary, wild type exogenous maspin bound to the cell surface whereas E244A showed a much weaker fluorescence suggesting that mutation to E244A perturbs this putative binding. Furthermore, mutation to maspin’s E247A results in a protein which may be unable to bind to this putative site at the cell surface.
Figure 5.14 Immunofluorescence showing cell surface binding of exogenous maspin to DU-145 cells. Cells were incubated with A) conditioned medium from cells transfected with vector only pMTBiP or pMTBiP-maspin for one hour, or B) conditioned medium collected from cells expressing pcDNA4, -maspin, -E244A or -E247A, or serum free medium (SFM); for five hours. DU-145 cells were fixed in 4% paraformaldehyde, stained with anti-maspin and DAPI, and imaged using CCD upright microscope at x63 magnification.
5.3.6.1 The Effect of Exogenous Maspin on Cell Migration

The effect of exogenous wild type maspin and maspin mutants on cell migration was studied using time-lapse video microscopy (Figure 5.15). Conditioned medium containing wild type maspin or maspin mutant proteins E244A or E247A, were added to DU-145 cells at a ratio of 15%: 75% serum free medium. Cell migration was analysed in comparison to cells incubated with conditioned medium lacking maspin protein and 100% serum free medium. The addition of conditioned medium containing wild type maspin to DU-145 cells showed a 37.3% reduction in cell migration, compared to the cells treated with vector only medium or serum free medium. Such maspin dependent reduction in cell migration was also significant in relation to cells treated with mutant maspin E244A and E247A conditioned medium. Thus like cells transfected with mutant maspin E244A or E247A, the addition of exogenous mutant proteins no longer held the ability to decrease cell motility. This experiment was performed alongside DU-145 cells treated with 121 µg/ ml recombinant maspin versus control buffer (Section 3.3.7.4). Similar to the effect of maspin conditioned medium, the addition of recombinant maspin significantly reduced cell migration by 33.2% compared to cells treated with control buffer. Therefore maspin can reduce cell migration by an extracellular mechanism that is dependent on an intact α-helix G region.

![Figure 5.15](image)

Figure 5.15 The effect of exogenous wild type and mutant maspin proteins on cell migration. DU-145 cells were subject to time-lapse video microscopy immediately after the addition of exogenous maspin proteins (light blue), control buffer or 121 µg/ ml recombinant maspin (pale blue). Conditioned medium was collected from COS-7 cells transfected with pcDNA4 vector, -maspin, -E244A and –E247A. 100% migration is represented by DU-145 cells treated with 100 % serum free medium (SFM) (dark blue), with an average speed of 2.7 µm/hr.
5.4 Discussion

5.4.1 Knocking Down Maspin Expression Increases Cell Migration

Maspin siRNAs #3 and #4 both effectively reduced the endogenous maspin expression in LNCaP, PC3 and PNT1a cells in comparison to negative CTRsi (Figure 5.1 and 5.2). In each cell line, optimal maspin knockdown was achieved between 48 and 72 hours; during which cell migration assays were carried out. The endogenous silencing of maspin significantly increased cell migration, supporting evidence that maspin is involved in the negative regulation of this cell process. Maspin siRNA #3 or #4 targeted maspin exons 4 or 7 respectively. Both siRNAs achieved similar rates of cell migration in PC3 and PNT1a cells (Figure 5.3B). However, the migration of LNCaP cells treated with #4 siRNA was lower than LNCaP cells treated with #3 siRNA. This effect could have been due the efficiency of siRNA in this cell line, whereby siRNA #3 reduced maspin expression more so than siRNA #4 (Figure 5.1). Nonetheless the knockdown of maspin using either #3 or #4 still caused a significant increase in LNCaP cell migration compared to those treated with CTRsi.

The migration of prostate cell lines non-treated with siRNA, PC3 > LNCaP > PNT1a (Figure 5.3A) inversely correlated with their levels of endogenous maspin (Figure 3.2). This suggests that the presence of endogenous maspin was not having an anti-migratory effect on such cells. However the silencing of endogenous maspin enhanced cell migration, thus suggesting otherwise. This observation that levels of endogenous maspin do not correlate with cell motility, has also been seen previously (Sheng et al 1996). Thus this may be explained by the localisation of maspin within these cells, whether the protein predominantly resides in a particular area, either influencing cell migration or not. A shift in the nuclear/cytoplasmic ratio of maspin from normal to cancerous cells has previously been used to indicate cancer prognosis. For example, the nuclear partitioning of maspin in cancerous breast tissue or prostatic tumour samples was found important for its tumour suppressive functions; correlating positively with the good prognostic markers oestrogen and progesterone receptors (Mohsin et al 2003, Zou et al 2002). Thus, in keeping with this correlation, perhaps the expression of endogenous maspin in PC3 cells is predominantly cytoplasmic, and could not inhibit cell migration, in relation to PNT1a cells which may show a predominance of nuclear maspin. Indeed, immunofluorescence of endogenous maspin showed a slight increase in nuclear localisation of PNT1a cells compared to LNCaP and PC3 (Appendix Figure A6). However, the lack of maspin in the nuclear region of these cells could have been the result of a bleaching effect from nuclear DAPI staining. Additionally, the mechanism that maspin exerts to inhibit cell migration is likely to be via an extracellular/cell surface mechanism, since the addition of exogenous
maspin significantly reduced cell migration (Figure 5.15). Thus perhaps the motility of these cell lines is influential on the presence of extracellular maspin, either resultant from non-classical secretion or from the necrosis of neighbouring cells (Pemberton et al. 1997, Teoh et al. 2010, Section 4.1.1).

5.4.2 Maspin’s α-helix G is Critical for the Modulation of Cell Migration

The introduction of wild type maspin gene reduced cell migration, further supporting maspin's negative influence on cell motility, which has been previously recognised (Seftor et al. 1998, Zhang et al. 2000). Transient transfection of wild type maspin into DU-145 cells reduced cell migration by 30%, and overexpression into PC3 cells showed a similar reduction of 25% (Figure 5.4). Comparatively, the stable transfection of wild type maspin into MCF7 cells reduced migration further by 62%. This difference in effectiveness of wild type maspin can be attributed to the fact that stably expressing cells express twice the level of maspin protein compared to transiently transfected cells.

The effect of introducing wild type maspin gene on cell migration was analysed in relation to numerous point mutated forms of maspin. Mutations to wild type maspin were chosen within structurally important areas. These included the arginine P1 residue of maspin’s RCL (R340A), lysine and glutamic acid residues comprising a salt bridge between maspin's β-sheet A strands 1 and 2 (K90A and E115A) and glutamic acid residues resident within maspin’s α-helix G (E244A and E247A) (Law et al. 2005) (Figure 1.6). Mutation to maspin’s RCL did not change the properties of wild type maspin on cell migration. This result showing that an intact RCL is not necessary for maspin to reduce cell migration correlated with previous studies (Cella et al. 2006, Cher et al. 2003, Zhang et al. 2000b), but contrasted with others (Sheng et al. 1994, Ngamkitidechakul et al. 2003). Similarly, cells expressing maspin mutated at its salt bridge showed an inhibitory effect on cell migration akin to wild type maspin. This shows that the region around D and E helices suggested to be influenced by K90 and E115 salt bridge is not required for the mechanism by which maspin inhibits cell migration.

Mutation to maspin’s α-helix G, at either residue E244A or E247A abolished its influence on cell migration in both transiently transfected prostate cancer cell lines and stably transfected breast carcinoma MCF7 cells. The stable expression of mutant E244A protein was half that of wild type maspin in MCF7 cells. This lower level of expression could attribute to the non-inhibitory effect of mutant E244A protein on cell migration. However, the level of mutant E244A and E247A proteins in transiently transfected cell lines were comparable to the wild type and showed the same migratory results as stable MCF7 cells. Therefore data suggests that an intact α-helix G was required for maspin’s interaction with
the cellular effectors that lead to a reduction in cell migration. Maspin’s mechanism of action that is dependent on an intact α-helix G, which mediates the inhibition of cell migration, was also found in common to maspin’s pro-adhesive effects (Chapter 6). However, such mechanism was different from that exerted by maspin to stimulate cell apoptosis. Here, mutation to maspin’s α-helix G did not interfere with its induction of apoptosis (Figure 5.5). This particularly shows that the inhibitory effect on migration was not due to an up-regulation in cell death, which would have also been noticed during the analysis of time-lapse video microscopy. Similarly, mutation to maspin’s RCL or salt bridge did not affect its pro-apoptotic behaviour of its wild type form. It was expected to see that mutation to maspin’s RCL would perturb its function on cellular apoptosis, because previous evidence had described that the RCL of endogenous maspin critical for this function (Li et al 2005). However the cell model used in this previous study was endothelial and not epithelial like that shown in this Chapter. Thus this suggests maspin’s mode of action concerning apoptosis is controversial between cell types. In summary, maspin is a multi-functional protein that can exert its effects via different molecular mechanisms. Of which we have demonstrated a novel importance of maspin’s α-helix G in the process of cell migration.

5.4.3 G-helix Peptide Mimics the Effect of Wild type Maspin on Cell Migration

Treatment of maspin-null DU-145 cells with G-helix peptide significantly reduced cell migration in comparison to treatment with peptides E244A or E247A, or peptide carrier control DMSO (Figure 5.6A). The addition of either wild type G-helix peptide or mutant peptide E244A or E247A thus mimicked the effects of wild type and mutant maspin proteins. Therefore, our results suggest that an intact α-helix G is not only essential, but also sufficient for mediating the effects of maspin on cell migration. Mutation to either residue E244 or E247 could be disrupting maspin’s potential interaction with either a protein or ligand which in turn would have the potential to mediate an inhibition of migration. It was the specific sequence of residues within the wild type G-helix peptide which mediated the inhibition of cell migration, because control peptide consisting of a rearranged sequence of the same α-helix G residues of matching charge; showed no such inhibitory effect. Similarly, the two mutant peptides and the DMSO carrier control, the control peptide did not reduce cell migration (Ravenhill et al 2010).

The inhibitory effects of wild type G-helix peptide on cell migration were replicated using other cell lines including MCF7, LNCaP, PC3 and PNT1a. The concentration required for G-helix peptide to exert a significant inhibitory action on the migration of DU-145 and MCF7 cells was 5 µM (Figure 5.6), compared to that of 10 µM for the other prostatic cell lines (Figure 5.9). This difference in concentration could be due to the dissimilar levels of
endogenous maspin between cell lines. For example DU-145 and MCF7 are absent of endogenous maspin, whereas PC3, LNCaP and PNT1a each show endogenous maspin expression (Figure 3.2, Figure 4.2A). Therefore, the G-helix peptide may be able to exert a more effective anti-migratory effect at a lower concentration in those cells absent of endogenous maspin; because maspin’s potential binding sites/interactions would be unoccupied. In correlation to this, wild type G-helix peptide significantly reduced the migration of MCF7 cells expressing empty vector, but did not exhibit any further reduction to the migration of MCF7 cells stably expressing wild type maspin (Figure 5.7). This suggests that a further reduction in cell migration would not be plausible as the rate of migration of cells expressing high levels of maspin would already be low, and that the G-helix peptide could not access its putative binding site as it was already occupied. In conjunction with the observation that wild type G-helix peptide caused a greater reduction in the migration of cells lacking endogenous maspin, the response to G-helix peptide was enhanced in cells where the basal level of maspin expression was reduced by siRNA (Figures 5.10-5.12).

Similarly, the addition of G-helix peptide to VSMC at a concentration of 5 µM significantly reduced the migratory capacity of this cell line void of endogenous maspin (Ravenhill et al 2010). We have previously shown that 50 nM recombinant maspin added to VSMC reduced cell migration (Bass et al 2002). Together this data suggests that wild type recombinant maspin has a much greater effect on VSMC migration than the peptide fragment alone. This indicates that the tertiary structure of maspin surrounding the important structural α-helix G could also play an important role in establishing an inhibitory effect on cell motility, or that it may enhance the interaction of this exposed region in some way. However, for the case of DU-145 cells similar concentrations of G-helix peptide or recombinant maspin, at 5 µM or 121 µg/ ml (2.88 µM), showed a similar reduction of cell migration (Figure 5.6 and 5.15). This variation between the concentrations of recombinant maspin sufficient to cause a significant inhibitory effect could be because two different preparations of purified maspin were used.

It has been previously shown that the time course of migration in the presence of maspin was linear, suggesting that maspin exerts an immediate inhibitory effect which continued to take effect during the course of the assay (Bass et al 2002). In the same way, the addition of maspin peptide G-helix showed an immediate reduction on the motility of cells, which continued over the 13 hour time period studied. This short term and long term effect of maspin protein/ G-helix peptide on cell migration correlates with the behaviour of maspin previously observed during its pro-adhesive activities. For example, maspin increased cell adhesion after 30 minutes (Cella et al 2006), and showed an altered
integrin profile at 24 hours respectively (Seftor et al 1998). Furthermore, maspin G-helix peptide showed a long term effect on the re-arrangement of the cell cytoskeleton, akin to the effect exerted by wild type maspin protein (Figure 4.10). Thus it can be hypothesised that the involvement of maspin’s intact α-helix G may play a role in the mechanism maspin uses to increase cell adhesion. This was investigated in Chapter 6.

5.4.4 The Extracellular Environment affects Maspin's Inhibition of Migration via its α-helix G

The effect of MCF7 cell migration stably transfected with either wild type maspin, α-helix G mutant maspin or vector only was analysed over matrix components collagen I, fibronectin, laminin, a fibrillar matrix and plastic (Figure 5.13). On each substrate the expression of wild type maspin protein significantly reduced cell migration in comparison to cells expressing vector only. The greatest reduction, was seen on a collagen I substrate, followed by plastic. This haptotactic-driven cell movement correlated with our observation that the morphology of MCF7 cells expressing wild type maspin on a collagen I and plastic substrate had a phenotype typical of non-motile cells, lacking in directional cues (Figure 4.6).

Cells which expressed mutant maspin protein E244A, that were plated onto plastic, collagen I, fibronectin or HT29 matrix, abolished the inhibitory effect of wild type maspin; showing a migration similar to the empty vector control. On the same substrates, the morphological analysis of MCF7 cells showed that expression of E244A produced a medial phenotype between that of mesenchymal-like cells expressing empty vector and epithelial-like cells expressing wild type maspin (Figure 4.8). The morphology of cells plated onto fibrillar HT29 matrix was not studied. Therefore the inability of E244A protein to promote a complete epithelial-like phenotype is reflected in its inability to reduce cell migration. This confirms the critical role of maspin’s α-helix G and implicates its importance in the regulation of cell actin architecture; the mechanism through which maspin exerts a decreased cell motility.

Conversely, when MCF7 cells expressing mutant E244A maspin were plated onto a laminin substrate, they maintained the ability to reduce cell migration, akin to wild type maspin. This also correlates with the morphological studies which showed that when on a laminin substrate, cells expressing E244A displayed an epithelial-like phenotype equivalent to that of cells expressing wild type maspin and were dissimilar to the mesenchymal-like score of control cells. Therefore the presence of a laminin matrix prevented the discrimination between wild type maspin and maspin mutated at its α-helix
G, suggesting an alternative mode of action to inhibit migration; one that is no longer dependent on its α-helix G.

The influence of laminin on the inhibitory behaviour of maspin, leads us to postulate a direct extracellular interaction that does not involve maspin’s α-helix G. For instance, maspin could bind to activated integrin receptors which hold specificity to laminin, such as heterodimers β4 with α3, α6, and α7. This could in turn enhance the cells attachment to the laminin matrix and/or trigger outside-in signalling pathways that result in the rearrangement of the actin cytoskeleton and negatively influence migration. However, β4-laminin receptors do not culminate in the linking to actin, but that of intermediate filaments. Therefore investigation into the effect of maspin on the keratin profiling of cells could be important here. Furthermore it could be possible that maspin present at the cell surface (bound to perhaps an integrin) could interact directly with laminin at a site other than its α-helix G. A direct interaction of maspin with matrix components collagen I and III have previously been identified (Blacque and Worrall 2002). The most probable binding site for collagen I was proposed at maspin’s α-helix D and not its α-helix G. Furthermore the G-helix peptide does not appear to directly bind to collagen I (data not shown). Therefore maspin could bind to laminin at a similar region independent of its helical G region.

Initial interaction between a cell and its ECM is not only required for adhesive contacts and the subsequent transduction of bi-directional signalling, but also the physical organisation of the ECM. ECM contact promotes the reorganization of the cellular cytoskeleton, specifically the insertion of orientated actin stress fibres into focal adhesion sites. This provides the cell with the tension required to remodel the surrounding ECM. Matrix remodelling is an important process during both gastrulation and postnatal life, and processes such as wound healing; however under diseased states the highly organized ECM is disrupted. For example, mammary epithelial cells are tightly surrounded by a collagen matrix which orientates along the axis of the gland. In contrast, invasive tumour cells reorientate the extracellular collagen fibres perpendicular to the gland which through physical cues can guide filopodia; necessary for migration and the initiation of metastasis. Thus it can be postulated that wild type maspin could contribute to the prevention of directional alignment of fibronectin fibrils, collagen and laminin fibres, resulting in a reduction of cell migration. This theory can be backed up by the morphological analysis of wild type maspin expressing MCF7 cells; which showed a polygonal array of actin filament bundles as opposed to actin bundles organised parallel to margins (Figure 4.6). Furthermore, the translocation of supramolecular matrix complexes is dependent on the intracellular focal adhesion-associated molecule; focal adhesion kinase (FAK). Thus it
may be possible for wild type maspin to stabilise FAK interactions, and thus influence matrix remodelling.

In addition to the orientation of extracellular fibrils, the in vivo environment contains many other molecules that have the potential to affect directional migration. These include growth factors bound to the ECM and proteoglycans. Consequently, in vivo cell migration tends to differ from that studied in vitro; probably reflective of different signalling mechanisms or cellular mechanics. To overcome this problem, we analysed the migration of cells over a fibrillar matrix laid down by HT29 cells (Figure 5.13). Results showed that, wild type maspin could also significantly inhibit the movement of cells over a fibrillar matrix, supporting its dynamic importance in cell migration.

5.4.5 Exogenous Maspin Reduced Cell Migration via an Extracellular Mechanism

The addition of recombinant maspin to DU-145 cells significantly reduced cell migration (Figure 5.15) to a rate comparable to that exerted by the addition of G-helix peptide (Figure 5.6B). Similarly the addition of conditioned medium expressing wild type maspin significantly decreased the rate of cell migration in comparison to medium absent of maspin or that containing mutant maspin proteins E244A or E247A. Thus, this data further complies with the necessity of maspin's intact α-helix G in its mode of inhibition of motility. Furthermore, our results suggest that maspin can act via an extracellular mechanism and as consequence can apply its anti-migratory behaviour on a small time scale. This extracellular mechanism can also be applied to cells transfected with maspin, since both wild type and mutant forms of maspin protein were present in the ECM and conditioned medium (Figure 3.4).

In support of maspin's extracellular action is the observation that wild type maspin interacts with the surface of maspin-null DU-145 cells; producing a punctate staining as shown by immunohistochemistry (Figure 5.14). The binding of wild type maspin to this cell surface site was observed after five hours of incubation with conditioned medium from pMTBiP-maspin transfected cells. It would have been interesting to see whether the G-helix peptide competed with the same cell surface binding site. However, as discussed in section 4.4.2.2, immunofluorescence of the biotin-labelled peptides was not successful. The strong cell surface interaction with maspin protein from pMTBiP-maspin transfected cells was weaker when conditioned medium from cells transfected with pcDNA4-maspin was added. This could be due to the greater expression of maspin produced from cells transfected with pMTBiP vector constructs compared to pcDNA4 (Section 3.4.5). Ideally, this experiment would have been performed with purified mutant maspin proteins.
alongside purified wild type maspin, but this was not achieved during the time scale of this project.

The observation that mutation to maspin’s α-helix G perturbs its anti-migratory function, suggests that mutant E244A maspin may be unable to bind to this cell surface binding site, or that it can bind to, but cannot promote subsequent signalling/ECM interactions. Immunofluorescence of DU-145 cells incubated with mutant maspin protein E244A showed very weak punctate staining at the cell surface, thus suggesting that it can bind to a cell surface receptor, but with weak affinity. Additionally, the presence of E247A protein did not show any cell surface binding, suggesting that mutation to maspin’s α-helix G critically perturbs this interaction.

The transfection of wild type maspin gene, the addition of exogenous maspin or G-helix peptide all reduced the rate of cell migration, which correlated with the effect observed on cell morphology; an un-polarised, non-motile phenotype (Section 4.4.2.2). Specifically the stable transfection of wild type maspin into MCF7 cells formed lamellipodial structures around the entire cell periphery in comparison to the directed lamellipodia seen at the leading edge of control cells (Figure 4.4B). Thus it can be hypothesised that the maspin-directed formation of lamellipodia extensions in all directions are causing a net negative effect on cell movement. Investigation into the mode of migration could be explored further using chemotactic-driven motility studies, or wound-induced migration studies over a solid phase.

5.5 Future Perspectives
The potent anti-migratory behaviour of maspin’s G-helix peptide has the prospective to serve as a therapeutic agent, particularly in tumour cells where endogenous maspin is down-regulated as they become more metastatic; such as breast and prostate cancers. Since the wild type G-helix peptide had the greatest impact on cells with little or no maspin expression, we suggest that the G-helix peptide would principally target maspin null tumour cells. Thus G-helix peptide has the potential for anti-cancer therapies in a similar way that the peptide alphasatin is used to target angiogenesis (Staton et al 2004). So that maspin G-helix peptide can be exploited for therapeutic applications, its mechanism of action needs to be fully characterised. The potential of the wild type maspin peptide competing with endogenous maspin protein for the putative interaction with cellular effectors is an important mechanistic item that needs to be considered in more depth. Additionally, further studies are warranted to ascertain whether the anti-migratory effects of maspin G-helix peptide seen in vitro play an anti-invasive/metastatic role in tumour-
beating mice. Additionally, the effect of prolonged exposure of G-helix peptide need be established.

Future analysis could optimally include the development of three-dimensional systems that would allow imaging of cellular and molecular dynamics. This is also important, as the migration of cells in vivo tends to be more directed than that of cells migrating in vitro. Furthermore we would like to look at cell invasion in the presence of wild type or mutant forms of maspin. This would help our understanding of the mechanism underlying maspin’s mediated inhibition of migration.

It was demonstrated that maspin bound specifically to the surface of DU-145 cells. Although not shown in this thesis, it has been shown that maspin directly binds to α3β1 and α5β1 on the surface of VSMC and HT1080 fibrosarcoma cells (Bass et al. 2009). This was demonstrated to be at least partially responsible for maspin’s anti-migratory properties. The requirement of an intact α-helix G for such binding remains to be determined. However, the influence of maspin on the direct expression of cellular integrins was studied and is shown in Chapter 6.

Furthermore, the results presented in this Chapter implicate a unique mechanism of action required for the inhibition of cell migration over a laminin matrix component; one that does not involve its α-helix G. To support this finding, it would be of interest to determine whether like the expression of E244A protein, the addition of mutant peptides E244A/E247A also retain the ability to reduce cell migration over laminin. Additional investigations into whether maspin can potentially up-regulate or down-regulate proteins that restrict or promote migration; such as α-actinin-4 tropomyosin-1, caldesmon-1, coronin and desmuslin, or actin-related protein, α-tubulin and leupaxin respectively, would help understand maspin’s mechanism of action (Chen et al. 2005). Furthermore, to investigate the hypothesis that maspin plays a role in matrix remodelling, future work could include the staining of ECM components and to investigate possible interactions with proteins influential to matrix organisation/degradation; such as matrix metalloproteases, and ECM remodelling related proteins biglycan and neutrophil gelatinase-associated lipocalin (Sousa et al. 2005). The deposition of ECM by cells expressing maspin was investigated via the influence on cell adhesion in Chapter 6.
5.6 Conclusions
The findings presented in this Chapter are consistent with other reports that maspin can significantly reduce the migratory capacity of epithelial cells. In support of this, it was also shown that the reduction of maspin using siRNA significantly increased the rate of cell migration. Maspin mutated at its α-helix G attenuated the wild type inhibition of cell migration. In contrast, mutations to maspin’s internal salt bridge or P1 position of the RCL all retained the ability of the wild type molecule to inhibit motility. An intact α-helix G was necessary for the inhibition of cell migration over a collagen I, fibronectin and endogenous fibrillar matrix. The necessity of maspin’s α-helix G was reflected by its dependence to promote mesenchymal to epithelial transition on these substrates. A unique mechanism of action was suggested for cells migrating over laminin. The anti-migratory influence of maspin expressing cells over laminin did not require an intact α-helix G. It is shown for the first time that a 15-amino acid peptide derived from the intact α-helix G of maspin has potent anti-migratory properties that are interchangeable with wild type protein. Both wild type maspin and G-helix peptide showed a greater anti-migratory response to those cells absent or showing reduced levels of maspin. This was demonstrated on multiple cell lines (and beyond those reported here); suggesting a fundamental mechanism to how maspin works. Thus, the potential that G-helix peptide can be used as a therapeutic agent to inhibit tumour cell migration via an extracellular mechanism could be very promising.
6 The Impact of Maspin on Cellular Adhesion

6.1 Introduction
This chapter investigates the effect of maspin on cell adhesion. We specifically look at maspin's influence on both cell-extracellular matrix and cell-cell adhesion; focusing on direct changes to cell surface integrin expression and changes to junctional proteins respectively. Throughout this chapter we explore whether or not any effect on cell adhesion was dependent on an integral α-helix G. For this we compared MCF7 cells stably transfected with wild type maspin or mutant E244A maspin.

6.1.2 Cell Adhesion
Cell adhesion plays a fundamental role in cell communication and regulation, which is essential during development and the maintenance of tissues. Cell adhesion stimulates signals that regulate cell differentiation, the cell cycle, and cell survival. Both cell-cell and cell-ECM adhesion are responsible for a wide range of normal and aberrant cellular activity; including directional migration of immune cells and invasion and metastasis of tumour cells respectively.

6.1.2.1 Cell-Extracellular Matrix Adhesion
The ECM is a complex structural and functional network of proteins including those of the collagen family, elastic fibres, glycosoamino- and proteo- glycans and adhesive glycoproteins, which can interact simultaneously with multiple cell surface receptors. The structural and functional composition of the ECM is important in maintaining normal tissue architecture, in development, and in tissue specific function. Growth factors, hormones, vitamins, cytokines and cell-cell contacts regulate ECM production. In turn, the ECM regulates the biological response of the cell by influencing gene expression; a process which is mediated by interactions with two families of cell surface receptor; integrins and syndecans. Dysfunctional matrix components and abnormalities in ECM biosynthesis and catabolism are associated with numerous acquired pathological responses, thus implicating the importance of the ECM.

Integrins are the major class of cell transmembrane proteins that link the cell cytoskeleton to ECM proteins, leading to transduction of bidirectional signalling pathways between the intracellular and extracellular compartments, which regulate cell survival, proliferation, migration and differentiation. Functional integrins are heterodimeric molecules, of which different combinations of their α and β subunit counterparts give rise to 24 distinct integrins that can either have specific or broad recognition for ECM ligands (Figure 6.1) (Hynes 2002). A subset of integrins have an inserted domain on their extracellular α-
subunit; termed the αA domain or the α-I domain. Integrins carrying this α-I domain, such as α1β1, and α2β1 can recognise and bind to collagens. Integrins that do not contain an α-I domain, have an RGD ligand binding site positioned on their extracellular β subunit termed the βA domain, such as integrins αV, α8 and α5 (Figure 1.9). There is also evidence that suggests that integrins can adhere to the ECM and other cells in an RGD-independent manner, such as the interaction between integrin α4β1 and fibronectin (Sechler et al 2000). The intracellular domain of integrins binds to actin linker proteins such as vinculin and talin. Clustering of activated integrins at the cell surface is known as focal adhesions. In addition to emanating numerous cell signalling pathways, focal adhesions are strong sites of adhesion maintained by the force and tension transmitted from the attached actin stress fibres (Luo and Springer 2006).

Figure 6.1 The integrin receptor family. Diagram shows mammalian integrin subunits with αβ associations; eight β subunits associate with 18 α subunits to form 24 unique heterodimers. α subunits with specificity for laminins are shown in purple and those specific for fibronectin are coloured blue. Those subunits that are restricted to chordates are shown in green and those integrins that have restricted expression on white blood cells are shown in yellow box. Figure was adapted from Hynes 2002.

Integrins have three highly controlled activation states depending on the accessibility of their intra and extracellular domains to respective ligands. Integrins configured with bent extracellular α and β domains represent a low ligand affinity and avidity state (Figure 1.9A). In this state the intracellular integrin legs are close together which is also suggestive of an inactive state. In the primed state, cation binding to the integrin MIDAS
region causes a conformational change in α-helix 7 of α-I domain; displacement and re-orientation. The primed integrin has an extended ectodomain (Figure 1.9B). Activated integrins bound to ligand, undergo outward conformational change of the hybrid region and a subsequent swinging apart of its α and β cytoplasmic legs (Figure 1.9C) (Arnaout et al 2005, Humphries 2004). Both integrin clustering and conformational changes can be triggered either from the inside or outside of the cell.

Anti-integrin monoclonal antibodies, that recognise specific conformation-dependent epitopes, have provided important information regarding the mechanisms of integrin conformational change that underlies priming and activation. Using integrins α5β1 and αIIbβ3, stimulatory monoclonal antibodies have been shown to increase the binding affinity of these integrins by decreasing the ligand off-rate. Stimulatory integrin antibodies can recognise primed or ligand occupied conformers which create a conformational equilibrium in favour of these forms and away from inactive and unoccupied conformers. It has previously been found that different stimulatory monoclonal antibodies induced different conformational changes, suggesting integrins could convey unique responses to individual ligands. Inhibitory monoclonal antibodies bind allosterically near to the ligand binding site, at the hybrid / βA domain; where they prevent the conformational change necessary for activation (Figure 1.9). Thus in the presence of ligand activated integrin, the binding of ligand-attenuated binding site monoclonal antibodies is lowered (Humphries 2004).

When an anchorage-dependent cell first comes into contact with its substratum, it spreads because of actin polymerisation and attaches via integrins. For the membrane to form stable sites of cell-substratum contact, integrins and focal adhesion proteins are recruited from adjacent regions to form concentrated sites called focal adhesions. Focal adhesion proteins such as talin and vinculin form the link between integrins and the actin microfilaments. Focal adhesion contacts are formed and disassembled in a dynamic, cyclical manner as cells translocate through sequential recruitment and loss of cytoskeletal and signalling molecules (Burridge et al 1996, Humphries et al 2007). Activation of FAK elicits intracellular signal transduction pathways that promote the turnover of focal adhesions; a process which leads to the promotion of cell migration.

6.1.2.2 Cell-Cell Adhesion

Like cell-ECM adhesion, cell-cell adhesion plays a major role both in development and cancer. In the developing embryo, individual cells and sheets of epithelial cells migrate to specific areas; a movement dependent on regulated changes in cellular adhesiveness. Similarly, primary tumours grow until single cells separate away where they invade other
regions of body via crawling and transport by the bloodstream. Upon development of a secondary site tumour, adhesion of cancer cells to normal cells occurs.

E-cadherin and β-catenin are two major proteins that contribute to adherens junctions; playing an essential role in the control of epithelial cell architecture and differentiation. E-cadherin is a type I transmembrane protein whose extracellular region is formed by variable numbers of repeated cadherin modules with a calcium cation binding site in-between each repeat. Upon the binding of calcium cations, homophilic adhesion to the E-cadherin of a close-contacting neighbouring cell is formed creating a rigid link. The intracellular domain of E-cadherin binds to the proteins p120-catenin and β-catenin; which creates the link between the cytoplasmic face and the actin cytoskeleton (Nelson et al 2008). β-catenin is a multifunctional protein that in addition to its role at the plasma membrane can be translocated from the cytoplasm to the nucleus where it acts as a transcriptional activator. E-cadherin has a potent ability to recruit β-catenin to the cell membrane and prevent its nuclear localization and transactivation. The loss of the E-cadherin-β-catenin adhesion complex is a critical step in the progression of many epithelial malignancies. The loss of E-cadherin and thus the consequential increase in the nuclear translocation of β-catenin has been associated with epithelial cell migration and EMT in various physiological and pathological processes (Orsulic et al 1999).

6.1.3 The Role of Maspin in Cell Adhesion

In the previous chapters, it has been shown that maspin inhibits cell migration, which is accompanied by active cytoskeletal changes; both of which are accomplished by a constant interaction between cells and the substratum. In addition it was shown that wild type maspin protein binds to the surface of cells, suggesting an extracellular mechanism and the possible role in adhesion. The importance of maspin in cell adhesion has been highlighted in vivo using mouse models. Homozygous knockout of maspin prevented the developing epiblast from adhering to the endodermal layer, which prevented embryonic development. Equally, the expression of maspin directly increased endodermal adhesion to a laminin matrix (Gao et al 2004). Heterozygous maspin knockout mice developed prostate hyperplastic lesions with a related increase in levels of the proliferation marker Ki-67. This developmental defect was associated with a reduction in cell adhesion (Shao et al 2008). Subsequently the transfection of maspin in vitro of many cell lines has demonstrated a significant increase of cell adhesion. It was shown that the re-introduction of maspin to C2N prostate epithelial cells, isolated from transgenic adenocarcinoma of the mouse prostate, resulted in consistently more adhesion to a laminin or fibronectin matrix. Concomitantly, maspin expressing C2N tumour cells demonstrated a reduction in cell migration and invasion (Abraham et al 2003). Similarly the treatment of maspin-null
corneal stroma cells with exogenous maspin, significantly increased adhesion to fibronectin, collagen I, IV and laminin (Ngamkitidechakul et al 2001).

These pro-adhesive effects of maspin have been attributed to the ability of maspin to influence cellular integrin expressions. Specifically, it has been found that the treatment of MDA-MB-435 cells with recombinant maspin for 24 hours significantly increased the relative expressions of α3, α4 and α5 containing integrins and decreased the integrin subunits α2, α6, αV and β1, as assessed by fluorescence-activated cell sorting. This correlated with a maspin-dependent increase of cell attachment and reduction of invasion into a fibronectin matrix by 30% and 43% respectively (Seftor et al 1998). Following this, another study showed that the addition of maspin to normal mammary epithelial MCF10A cells increased cell adhesion by changing the integrin cell profile. By method of co-immunoprecipitation and double immunofluorescence microscopy, a direct association of maspin with β1 integrin was demonstrated. Thus this suggested a functional change in cell adhesion was likely to be due to this direct integrin interaction (Cella et al 2006). Another study similarly showed a specific binding of maspin to the β1 integrin heterodimers α3β1, α5β1 by co-immunoprecipitation and affinity chromatography (Bass et al 2009). Specifically, it was shown that maspin interacted with the extracellular part of recombinant α5β1; suggesting a means to maspin’s ability to bind to the cell surface. The direct binding of exogenous maspin at the cell surface inhibited the migration of Chinese hamster ovary cells over-expressing integrin subunit α5, on fibronectin and laminin matrices. By contrast, exogenous maspin did not bind to α5-null cells and had no inhibitory effect on the migration of such cells. Therefore this data demonstrated that maspin specifically binds to α5β1 and that this interaction is sufficient to inhibit cell migration. In addition it was found that recombinant maspin could redistribute the balance of active and inactive conformations of β1 integrin, and it was suggested that this could be a consequence to its direct binding to β1/α3β1 and/or β1/α5β1. Specifically, maspin lead to an overall reduction in β1 activity, as assessed using conformation-dependent antibodies (Bass et al 2009). In addition to maspin’s ability to directly influence integrin activation, indirectly change integrin expression levels and increase adhesion, the addition of recombinant maspin altered downstream signalling pathways involved in motility and invasion. Maspin’s ability to increase cell adhesion was attributed to a reduction in active Rac1 and its downstream effector, PAK and increased activities of P13K and ERK 1/2 (Odero-Marah et al 2003).

When anchorage-dependent cells detach from each other and from the ECM, they undergo programmed cell death, a process known as anoikis. Survival and proliferation of normal adherent cells are only possible in the presence of sustained signalling by both
soluble factors and those emanating from the ECM. However, metastatic tumour cells can escape anchorage-dependent anoikis and thus survive without attachment to the ECM or cell-cell contact. This characteristic of tumour cells is a key aspect of their metastatic potential. The over-expression of maspin in PC3 cells was found to reduce tumour growth and inhibit the growth of cells independent of anchorage. Consequently, PC3 cells over-expressing maspin showed a reduced ability to seed to bone; a common metastatic site of prostate cancer cells (Hall et al 2008). Another study showed the transfection of maspin into C2N epithelial derived prostatic tumour cells showed a reduced rate of cell cycle progression. Specifically, maspin transfected cells showed a reduced activity of cyclin-dependent kinase 2 and increased expressions of its cell cycle inhibitors p27 and p21. Since increased cell-cell and cell-ECM adhesion negatively influence cell cycle progression, it was postulated that maspin’s directed increase of cell adhesion leads to its observed decrease in cell proliferation (Shao et al 2008).

Changes to the ECM of both malignant cells and their targeted site of metastasis, are necessary for their successful translocation. In general, during neoplastic transformation cells promote the degradation of the ECM as well as perturb its production and deposition, in order to facilitate migration and invasion. Such changes to the ECM rely on the dynamic and reciprocal communication between epithelial cells and the underlying stromal cells, which include fibroblasts, endothelial cells, smooth muscle cells, adipocytes, inflammatory cells and nerve cells. In the case of the breast, basal and luminal epithelial cells form a complex alveolo-tubular network that is embedded in a specialized underlying stroma. This thus implicates the importance of such communication and its significance on tumour cell behaviour. It has been shown that although both tumour and normal mammary tissue are composed of the same collagen types I and II, the supramolecular collagen architecture is significantly disturbed in malignant breast lesions. The presence of extracellular maspin has been shown to maintain the matrix integrity of breast cancer cells. Specifically, MDA-MB-231 cells plated onto a collagen matrix that had incorporated maspin protein; were less susceptible to cathepsin D-mediated degradation (Khalkhali-Ellis and Hendrix 2007). It was suggested that the ability of maspin to deter the incorporation of cathepsin D into a collagen matrix, could be due to competitive binding. Consistent with this, it had previously been shown that maspin directly interacts with collagen types I and III (Blacque and Worrall 2001). A similar role of maspin in matrix remodelling was provided by an alternative explanation for the early developmental defect of the prostate caused by a heterozygous loss of maspin; which showed a changed pattern of matrix composition (Shao et al 2008). The partial loss of maspin resulted in an increased deposition of laminin surrounding prostatic epithelial cells, which showed intrusion by stromal cells. In comparison wild type prostate showed a tightly packed
basement membrane effectively separating epithelial and stromal cells. The improper formation of the ECM by loss of maspin resulted in a reduced adherence of epithelial cells to the basement membrane (Shao et al. 2008).

6.2 Chapter Aims

1. To determine the effect of wild type maspin on cell-extracellular matrix adhesion and the importance of an intact α-helix G, looking at cell spreading and expression of focal adhesion proteins.

2. To investigate the influence of maspin on the integrin expression profile of the human mammary carcinoma MCF7 cells at the mRNA and protein levels.

3. To look at the effect that maspin expression has on the activation status of integrin β1, by using anti-β1 conformation-dependent monoclonal antibodies.

4. To determine the effect of cell-cell adhesion of cells transfected with maspin or maspin targeted siRNA.

5. To study the effect that wild type maspin and mutant E244A maspin have on the expression of cell-cell adhesion molecules.
6.3 Results

6.3.1 Maspin Expression Enhances Cell-Extracellular Matrix Adhesion

6.3.1.1 Importance of Maspin’s α-helix G in Cell-Extracellular Matrix Adhesion

Chapter 3 has presented data confirming previous reports that the addition of recombinant maspin to maspin-null cells significantly enhances cell adhesion by 51 ± 23% (Figure 3.14). Here we examined the effect on cell adhesion when cells were transfected with wild type pcDNA3.2-maspin. Using an in vitro assay to measure cell adhesion, it was found that wild type maspin enhanced MCF7 cell adhesion to various ECM components (Figure 6.2). Specifically, MCF7 cells stably expressing wild type maspin significantly increased cell adhesion by 113 ± 5% on a laminin matrix and by 45 ± 6% on both collagen I or fibronectin matrices, in comparison to cells expressing vector only. On a collagen I or fibronectin matrix, cells expressing mutant E244A maspin showed a significant reduction in cell adhesion, akin to control cells and in contrast to cells expressing wild type maspin protein. However, on a laminin matrix, mutation at E244A had no effect on maspin’s ability to increase adhesion and showed a significant increase in adhesion in contrast to vector only cells. This suggested that the mechanism maspin uses to exert its pro-adhesive effect is dependent on an intact α-helix G, but a separate mechanism could be activated in the presence of laminin; one that does not involved this helical region.

![Figure 6.2](image-url) Adhesion of MCF7 stable cells on matrix components. MCF7 cells stably transfected with pcDNA3.2, pcDNA3.2-maspin, or pcDNA3.2-E244A were plated onto 5 µg/ml matrix components. Adhered cells were stained with methylene Blue and absorbance was measured at 630 nm. 100% adhesion is defined by pcDNA3.2 cells; an average OD630 of 0.7, 0.25, 0.7 for collagen I, laminin or fibronectin respectively. Three independent experiments were performed in triplicate. Statistical significance was compared to cells transfected with pcDNA3.2 and measured by Students t-test (p*<0.05).
For a more representative model of an in vivo ECM, we studied the adhesion of MCF7 stable cells to various pre-laid fibrillar matrices. Fibrillar matrices used included those laid down by maspin-expressing HT29, LNCaP, and PC3 cells, as well as maspin-null DU-145 and MCF7 cells (Figure 6.3A). MCF7 cells expressing wild type maspin showed a significant increase in adhesion to each fibrillar matrix compared to control cells. MCF7 cells expressing wild type maspin promoted a similar increase in adhesion on either a fibrillar matrix or ECM components, between 47-92% and 55-113% respectively (Figure 6.3B). The expression of wild type maspin enhanced cell adhesion regardless of whether the ECM had bound maspin protein or not. When plated onto fibrillar matrices, MCF7 cells expressing wild type maspin showed a significant increase in adhesion compared to control cells.

It was also noted that the overall cell adhesion, for each stable cell line, was lower to matrices not containing maspin; DU-145 and MCF7, and enhanced to a maspin-containing matrix; HT29, LNCaP, PC3 (data not shown). Following from this observation the effect of cell adhesion to a matrix containing wild type or mutant maspin proteins was investigated. Parental MCF7 cells were plated onto a fibrillar matrix previously laid down by MCF7 stable cells (Figure 6.4). Cell adhesion to matrix containing wild type maspin, was significantly enhanced by 89 ± 7 %, in comparison to a matrix deposited by cells expressing empty vector. In contrast, no increase in cell adhesion was seen on a matrix laid down by cells expressing mutant maspin E244A. This result supports the previous implication that maspin acts extracellularly to increase cell adhesion (Figure 3.13). Furthermore, this evidence suggests that there may be a preferred role for matrix-bound maspin. It also supports the necessity of an intact α-helix G for maspin’s ability to promote cell adhesion.
Figure 6.3 Adhesion of MCF7 stable cells on cell-derived fibrillar matrix. A) Western blots showing the presence of maspin in respective cell matrix fractions. Protein was probed with anti-maspin. B) Stable MCF7 cells transfected with pcDNA3.2, pcDNA3.2-maspin or pcDNA3.2-E244A were plated onto fibrillar matrix; laid down by HT29, LNCaP, PC3, DU-145 and MCF7 cells. The absorbance of adherent cells stained with methylene Blue, were measured at 630 nm. 100% adhesion is defined by pcDNA3.2 expressing cells; with an average OD630 of 0.7, 0.5, 0.9, 0.9, 0.2 or 0.2 per matrix component respectively. Three independent experiments were performed in triplicate. Statistical significance was measured by Students t-test (p*<0.05).

Figure 6.4 Adhesion of parental MCF7 wild type (WT) cells to a fibrillar matrix deposited by MCF7 cells stably transfected with empty vector pcDNA3.2-, pcDNA3.2-maspin or pcDNA3.2-E244A. The absorbance of adherent cells stained with methylene Blue, were measured at 630 nm. 100% adhesion is defined by vector only expressing cells and an average OD630 of 0.4. Three independent experiments were performed in triplicate. Statistical significance was measured by Students t-test (p*<0.05).
6.3.1.2 Expression of Maspin Alters Integrin Profiling of MCF7 Cells

6.3.1.2.1 Integrin Protein Expression

Following the evidence that maspin directly affects adhesion to the ECM, and the existing evidence that suggests this may be mediated by interactions with integrins (Bass et al 2009), the integrin expressions of maspin expressing cells were characterised using immunohistochemistry. However, it needs to be considered that any effects maspin has on integrin protein expression are effects achieved over a longer time period than the 30 minute course of the adhesion assays presented earlier. This could include integrin redistribution from the cellular cytoplasmic pool.

MCF7 cells stably expressing either wild type maspin, E244A or empty vector that had been cultured on plastic, collagen I, laminin or fibronectin for 17 hours, were labelled with integrin antibodies and analysed by immunofluorescence (Figure 6.5-6.11). The distribution of fluorescence intensity was quantified per unit area using Andor iQ computer software. A summary of the results presented in this section can be seen in Table 6.1. On conclusion it can be said that wild type maspin influenced the expression of all integrin subunits that were studied. Although no clear trend was observed, more often than not cells that contained wild type maspin caused a relative increase in integrin proteins than cells that did not. However, the largest difference in expression between cells containing or absent of wild type maspin was a down-regulation of integrin subunit β1 respectively. It was also observed more often than not, that integrin expression differed between cells expressing wild type maspin and those with mutant maspin E244A. Although the presence of different matrix components influenced individual integrin subunit expressions, maspin also influenced these changes, thus re-enforcing the possibility that this is achieved by direct interaction, as reported by others (Cella et al 2006, Bass et al 2009), and provides a mechanism of which maspin can influence long-term cell adhesion. Therefore, the data presented in this section complies with the work of others (Seftor et al 1998), that maspin alters the expression profile of integrins in breast carcinoma cells. The expression of most integrins is increased, which implicates a reason behind maspin's ability to strongly increase adhesion to the ECM. It needs to be considered that any change in integrin expression for cells expressing mutant maspin E244A may be the result of a lower endogenous expression level compared to levels of expressed wild type maspin.

In addition to investigating the expression of integrins at the protein level, qRT-PCR was used to examine integrin expression at the mRNA level (data not shown). This indicated that the differences in the adhesive properties of the MCF7 cell lines used here were not
due to changes in integrin gene expression, as the profile of integrin mRNA was virtually identical between MCF7 stably expressing empty vector, maspin or E244A. However, a trend was seen whereby maspin expressing cells showed increased mRNA of integrin αV and a decrease of integrin β1, in comparison to cells expressing empty vector. Additionally, integrin subunits that were not studied by immunofluorescence such as β4 and β5 also showed an increased mRNA for cells expressing maspin, than those cells that did not (Appendix Figure A6). However none of the mRNA data was significant as assessed by Students t-test ($p^*<0.05$), because only two independent RNA samples were collected for each condition.

6.3.1.2.1.1 Integrin α2
The protein expression of integrin α2 was significantly up-regulated in MCF7 cells expressing wild type maspin compared to those expressing mutant maspin E244A or vector only (Figure 6.5). This maspin induced up-regulation of integrin α2 was seen on each plastic, laminin and fibronectin. When plated onto collagen I the expression of integrin α2 was relatively similar for each stable MCF7 cell line. The relative level of integrin α2 present in cells absent of maspin was similar between matrix components.

6.3.1.2.1.2 Integrin α3
Similar to the up-regulation of α2 on plastic or fibronectin, cells expressing wild type maspin showed significantly increased levels of integrin α3 protein, compared to cells absent of maspin or those expressing mutant maspin E244A (Figure 6.6). However, when plated onto laminin, cells expressing either wild type or mutant maspin showed a significant decrease of α2 protein expression compared to control cells. Each MCF7 stable cell line plated onto collagen I showed similar levels of integrin α3 protein. The overall expression of integrin α3 for cells absent of maspin expression was greater on plastic or fibronectin than when on collagen I or laminin.

6.3.1.2.1.3 Integrin α5
The expression of integrin α5 was the same for each stable MCF7 cell line when grown on plastic substrate (Figure 6.7). This result was replicated using western blotting (Appendix Figure A7). In contrast, cells expressing wild type maspin plated onto either laminin or fibronectin showed a significant increase of integrin α5, which was not observed for cells expressing mutant maspin E244A. When plated onto collagen I, cells expressing wild type maspin showed no increase or decrease of integrin α5 whereas cells expressing E244A maspin showed a significant reduction of α5. The ability of maspin to up-regulate α5 on laminin or fibronectin may be due to the overall expression of α5 on these
substrates; which was lower than plastic and collagen I respectively, and thus possibly more likely to generate an increase in expression.

6.3.1.2.1.4 Integrin α6
Consistent up-regulation of integrin α6 by cells expressing wild type maspin was observed on all matrices in comparison to cells absent of maspin (Figure 6.8). This significant up-regulation was also seen for cells expressing mutant maspin when on a collagen I or laminin substrate. However, E244A cells showed levels of α6 similar to those absent of maspin, when plated onto plastic or fibronectin. The relative expression of integrin α6 for cells absent of maspin was greatest when on plastic or collagen I, rather than laminin or fibronectin. However, this had no relation to the consistent ability of wild type maspin to increase these integrin levels further.

6.3.1.2.1.5 Integrin αV
MCF7 cells expressing wild type maspin significantly increased relative levels of integrin αV when on collagen I or laminin matrix in comparison to cells absent of maspin. This increase was also seen for wild type maspin expressing cells on a fibronectin matrix, yet this was not significant (Figure 6.9). Cells expressing E244A did not show this increase in αV protein on collagen I or fibronectin, but did when plated onto laminin. Unlike that observed on matrix component, cells expressing wild type maspin did not show increased levels of αV protein on a plastic substrate. The expression pattern of integrin αV on plastic was replicated on western blot (Appendix Figure A7). Similar relative levels of αV were observed for control cells on each substrate.

6.3.1.2.1.6 Integrin β1
MCF7 cells expressing wild type maspin showed a large reduction in integrin β1, which was significant in comparison to cells absent of maspin when plated onto plastic, collagen I or fibronectin (Figure 6.10). This significant reduction was also seen for cells expressing mutant maspin E244A when plated onto plastic, collagen I or fibronectin. Similar to the immunofluorescence data, western blotting of soluble protein obtained from cells grown on a plastic substrate showed a down-regulation of β1 for those expressing wild type maspin, in relation to those expressing empty vector, but did not show a down-regulation of β1 for those expressing E244A (Appendix Figure A7). In contrast to this relatively consistent down-regulation of β1, maspin expressing cells plated onto laminin showed an increase in β1 protein levels compared to control or E244A cells.
6.3.1.2.1.7 Integrin β3

Cells expressing wild type maspin showed a significant increase of β3 protein expression in comparison to cells lines absent of maspin when plated onto plastic, collagen I or laminin (Figure 6.11). This increase of β3 was not replicated by cells expressing mutant maspin E244A. Matching results were shown by western blotting of cell lysate cultured on plastic (Appendix Figure A7). In contrast to the other substrates, cells expressing either wild type or mutant maspin plated onto fibronectin, showed a significant decrease in relative β3 protein levels in comparison to control cells.
Figure 6.5 Protein expression of integrin α2 in MCF7 stable cell lines transfected with pcDNA3.2-maspin, pcDNA3.2-E244A or pcDNA3.2. Integrin α2 was detected by Alexa Flour 488 fluorescence and nuclei were counterstained with DAPI. Three images were taken from randomly chosen fields, from three independent experiments, using CCD upright at x63 magnification. Graph shows relative fluorescence intensity per unit area µm². Statistical significance was measured by Students t-test (p*<0.05, p**<0.005).
Figure 6.6 Protein expression of integrin α3 in MCF7 stable cell lines. Integrin α3 was detected by Alexa Flour 488 fluorescence and nuclei were counterstained with DAPI. Three images were taken from randomly chosen fields, from three independent experiments, using CCD upright at x63 magnification. Graph shows relative fluorescence intensity per unit area µm². Statistical significance was measured by Students t-test (p*<0.05, p**<0.005).
Figure 6.7 Protein expression of integrin α5 in MCF7 stable cell lines. Integrin α5 was detected by Alexa Flour 488 fluorescence and nuclei were counterstained with DAPI. Three images were taken from randomly chosen fields, from three independent experiments, using CCD upright at x63 magnification. Graph shows relative fluorescence intensity per unit area µm². Statistical significance was measured by Students t-test (p*<0.05).
Figure 6.8 Protein expression of integrin α6 in MCF7 stable cell lines. Integrin α6 was detected by Alexa Flour 488 fluorescence and nuclei were counterstained with DAPI. Three images were taken from randomly chosen fields, from three independent experiments, using CCD upright at x63 magnification. Graph shows relative fluorescence intensity per unit area µm². Statistical significance was measured by Students t-test (p*<0.05).
Figure 6.9 Protein expression of integrin αV in MCF7 stable cell lines. Integrin αV was detected by Alexa Flour 488 fluorescence and nuclei were counterstained with DAPI. Three images were taken from randomly chosen fields, from three independent experiments, using CCD upright at x63 magnification. Graph shows relative fluorescence intensity per unit area µm². Statistical significance was measured by Students t-test ($p^*<0.05$, $p^{**}<0.005$).
Figure 6.10 Protein expression of integrin β1 in MCF7 stable cell lines. Integrin β1 was detected by Alexa Flour 488 fluorescence and nuclei were counterstained with DAPI. Three images were taken from randomly chosen fields, from three independent experiments, using CCD upright at x63 magnification. Graph shows relative fluorescence intensity per unit area µm². Statistical significance was measured by Students t-test (p*<0.05, p**<0.005, p***<0.0005).
Figure 6.11 Protein expression of integrin β3 in MCF7 stable cell lines. Integrin β3 was detected by Alexa Flour 488 fluorescence and nuclei were counterstained with DAPI. Three images were taken from randomly chosen fields, from three independent experiments, using CCD upright at x63 magnification. Graph shows relative fluorescence intensity per unit area µm². Statistical significance was measured by Students t-test (p*<0.05, p**<0.005).
The following table summarises the changes in integrin expression between MCF7 stable cell lines either expressing wild type or E244A maspin compared to cells expressing empty vector (Table 6.1).

<table>
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<th>Plastic</th>
<th>Collagen I</th>
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<th>Fibronectin</th>
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<th>α3 heterodimers</th>
<th>α5 heterodimers</th>
<th>α6 heterodimers</th>
<th>αV heterodimers</th>
<th>β1 heterodimers</th>
<th>β3 heterodimers</th>
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<tr>
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<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
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<td>pcDNA3.2-maspin</td>
<td>↑3</td>
<td>0</td>
<td>↓2</td>
<td>↑3</td>
<td>α3β1, α3β4</td>
<td></td>
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<td></td>
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<tr>
<td></td>
<td>pcDNA3.2-E244A</td>
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<td>0</td>
<td>↓2</td>
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<tr>
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<td>↑2</td>
<td>↑1.5</td>
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<tr>
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Table 6.1 Changes in integrin protein expression by comparison of fluorescence intensity. The change increase ↑ or decrease ↓ in fluorescence intensity of MCF7 cells stably transfected with pcDNA3.2-maspin or pcDNA3.2-E244A is shown in comparison to cells transfected with vector only pcDNA3.2, which were given a score of 0. An increase or decrease was recorded when a difference of 1.5 arbitrary units (fluorescence intensity per µm²) or more was observed. Far right column shows possible integrin heterodimers. Heterodimers highlighted green primarily bind to collagen, blue to laminin and purple to fibronectin.
6.3.1.3 Maspin Expression alters β1 Integrin Activation Status

Following the observation that maspin altered the expression profile of integrins in breast carcinoma cells, investigation into a possible mechanism for this was carried out. This investigation stemmed from the idea that the dynamic effects of maspin could not be accounted for just by changes in expression of cell surface integrins. It was investigated whether maspin could influence the activation status of β1 integrins as performed using three distinct monoclonal conformation-dependent antibodies in immunofluorescence microscopy. Any effect observed would be suggested to be a direct effect since recombinant maspin bound directly to the cell surface (Figure 6.14) and in particular integrin β1 (Bass et al. 2009). The conformation-dependent antibodies used were SNAKA51, mAb13 and 9EG7. SNAKA51 specifically recognises calf-1/calf-2 region of ligand bound integrin α5. mAb13 recognises an epitope of inactive β1. 9EG7 is an allosteric activator of β1 which recognises the unbent active conformer of β1 (Mould et al. 1995, Mould et al. 1996, Humphries et al. 2006).

MCF7 stable cells expressing wild type maspin, E244A or empty vector, were plated onto plastic and labelled with the β1 conformation-dependent antibodies. Detection of α5β1, by SNAKA51, was found equal between each MCF7 stable cell line (Figure 6.12). In contrast to the detection of α5β1, clear differences in staining intensities of inactive/active β1 were observed for cells expressing wild type maspin in comparison to those absent of maspin or those with mutant maspin. A significantly increased binding of mAb13, which recognises inactive β1, was observed for MCF7 cells expressing wild type maspin in relation to cells expressing vector only or E244A. The staining intensity of active β1 conformation detected by 9EG7 was significantly reduced for cells expressing wild type maspin compared to cells absent of maspin or those with E244A. In summary, wild type maspin altered the activation state of β1 integrin; favouring that of an inactive one. An increase in β1 inactive conformation, shown by 3 units, was accompanied by a decrease in β1 inactive conformation, of 6 units. Additionally, this is achieved by a mechanism involving its intact α-helix G since mutation to this region prevented the change in activation state.

The ability of maspin to redistribute the balance between active and inactive β1 conformation fits with the earlier experiment shown in this chapter; that expression of wild type maspin consistently reduces β1 integrin expression at the protein and mRNA level on plastic, collagen I and fibronectin substrates (Figure 6.10). However, a decrease of integrin β1 does not correlate with the ability of maspin to increase adhesion to the ECM. Nonetheless, maspin increased cell surface expression of integrins α2, α3, α5, α6, αV and β3, and so it remains possible that maspin may influence the activation of these in the
favour of an active conformer and in favour of ECM adhesion. The ability of maspin to influence the equilibrium of integrin conformation fits with its quick effects on cell adhesion and its reported direct interaction with integrins.

**Figure 6.12** Protein detection of β1 integrin activation status in MCF7 stable cell lines. β1 integrin states were detected using Alexa Flour 488 fluorescence and nuclei were counterstained with DAPI. Images were taken from randomly chosen fields, from three independent experiments, using CCD upright at x63 magnification. Graph shows relative fluorescence intensity per unit area \( \mu \text{m}^2 \). Statistical significance was measured by Students t-test \( (p^*<0.05, p^{**}<0.005) \).
6.3.1.4 Maspin Promotes Cell Spreading and Focal Adhesion Protein Expression

Following from the observation that wild type maspin alters integrin protein expression levels; maspin’s influence on cell spreading was investigated. Cell spreading is a process dependent on integrin co-localisation, concentration and attachment to the substratum. The area (µm²) of MCF7 cells expressing wild type maspin, mutant maspin E244A or empty vector was quantified as a measure of cell spreading. Cells were plated at a low density to minimize any non-specific electrostatic and repulsive steric forces between cells which may influence spreading. MCF7 cells stably expressing wild type maspin showed a significantly increased cell area on plastic, collagen I and laminin, in comparison to vector only cells (Figure 6.13). However no difference was observed on a fibronectin substrate. The largest difference between the spreading of MCF7 cells expressing wild type maspin and those with vector only was seen on a collagen I substrate. On comparison of cell spreading between matrix components, it was observed that cells expressing maspin were less spread on a laminin and fibronectin substrate than on plastic or collagen I. In contrast, the expression of mutant E244A protein blocked the ability of maspin to increase cell spreading onto each substrate, as shown by a significant reduction in cell area. The spread of E244A cells on plastic, collagen I and laminin were equal to that of vector only cells. However, when plated onto a fibronectin substrate, E244A cells were significantly smaller in area than vector only cells and also to those expressing wild type maspin.

In summary, the observed maspin-dependent increase in cell spreading on plastic or collagen I correlate with its increase in cell adhesion, whereby both processes are dependent on its α-helix G. Similarly, the expression of wild type maspin increased cell spreading on laminin, but this was also dependent on its α-helix G; a region shown to be independent for the adhesion to this specific substrate. When on fibronectin, either cells expressing or not expressing maspin, were equally spread, implicating a similar effect on cell adhesion to this substrate. However, this was not the case (Figure 6.2).
Figure 6.13 Spreading analysis of MCF7 stable cells on matrix components in serum containing conditions. A) MCF7 cells stably transfected with pcDNA3.2-, -maspin, or -E244A were plated onto plastic or 5 µg/ ml matrix components. Cells were stained with Hoescht and Eosin and imaged using DIC III at x 63 magnification of CCD upright. B) Mean cell area was measured in µm² using Velocity. Error bars indicate standard deviations of three replicate experiments, analysing a total of 90 cells. Statistical significance was measured by Students t-test (p*<0.05, p**<0.005 etc).
In keeping with maspin’s pro-adhesive nature the relative expression levels of the focal adhesion protein vinculin was determined. Using an anti-vinculin antibody, immunofluorescence showed frequent focal adhesive structures around the periphery of maspin expressing MCF7 cells (Figure 6.14). Comparatively, the focal adhesions of control MCF7 cells were not as thick or frequent and did not occur around the entire cell boundary. Similar to maspin expressing cells, cells expressing mutant E244A maspin protein showed frequent focal adhesion sites. However, these were smaller. Therefore, this indicates that maspin expressing cells have an increased number of established focal adhesions as a consequence of their ability to increase cell attachment to the ECM. Furthermore the presence of focal adhesion sites around the entire periphery of maspin expressing cells could contribute to the thick cortical actin previously seen in the morphology of these cells (Figure 4.4B). The inability of E244A expressing cells to create as many, or as established, focal adhesion complexes suggests a possible reason why these show deficient adhesion in comparison to its wild type protein.

![Figure 6.14](image)

**Figure 6.14** Immunofluorescence showing staining for the focal contact protein vinculin. Upper and lower panels show MCF7 stable cells transfected with pcDNA3.2, pcDNA3.2-maspin or pcDNA3.2-E244A were stained for vinculin expression, with Alexa Fluor 488, and nuclear proteins with DAPI. Images were acquired using CCD upright at x63 magnification.
Interestingly, under serum free conditions we observed cell attachment to a non-tissue culture treated plastic in the presence of wild type maspin (Figure 6.15). Comparatively, MCF7 cells stably expressing either mutant maspin E244A or vector only did not adhere to the plastic and remained as non-adherent cells. Thus in the absence of cell attachment and ECM proteins and also in the absence of a carbon source, cells expressing maspin were able to uniquely adhere to the plastic substrate. This re-enforces the strong ability of maspin to increase cell adhesion and may indicate the ability of maspin to influence matrix deposition.

![image]

**Figure 6.15** Spreading of MCF7 stable cells in serum free conditions. MCF7 stable cells were plated onto sterile non-tissue culture treated plastic dishes. Images were taken after 20 hour incubation at 37°C with Nikon D50 SR digital camera at x10 magnification.

### 6.3.2 Maspin Expression Enhances Cell-Cell Adhesion

#### 6.3.2.1 Increased Cell Aggregation is Dependent on Maspin’s α-helix G

The influence of wild type maspin and mutant maspin on cell-cell adhesion was investigated by analysing the frequency and number of aggregated cells. To look at cell-cell adhesion and not cell-ECM adhesion, single cell suspensions were rotated for two hours, before being fixed and imaged. The stable transfection of wild type maspin in MCF7 cells showed a significant increase in both the number of cell aggregates and the number of cells per each aggregate, compared to that of MCF7 cells absent of maspin protein (Figure 6.16). The expression of mutant maspin E244A, showed a significantly lower frequency of MCF7 cell aggregation and lower aggregation size in relation to MCF7 cells expressing the wild type form. In fact, the expression of E244A significantly impaired the frequency of aggregation, in relation to control cells, yet the number of cells per
aggregate was similar. Therefore the expression of wild type maspin increases the adhesion between adjacent cells; by a process reliant on its wild type α-helix G.

Figure 6.16 Aggregation of MCF7 cells stably transfected as indicated. A) Phase contrast images of fixed cells after two hours rotation. Images were taken with Nikon D50 SR digital camera at x10 magnification. B) Data is presented as number of aggregates (blue bars) and number of cells per aggregate (purple bars). Data represents mean of three independent experiments. Statistical significance was determined by Students t-test (p*<0.05, p**<0.005, p****<0.00005).

To further investigate the importance of maspin in promoting cell-cell interactions, the aggregation of MCF7 stable cells that had been treated with maspin siRNA was analysed. For each experiment, the knockdown of maspin protein in MCF7 cells expressing wild type or E244A maspin, using either siRNA #3 or #4 was verified by western blotting. An example is shown in Figure 4.15. To check that the cells analysed in this assay were behaving as we had seen in Figure 6.16, the aggregation of cells expressing maspin were compared to control cells, which had each been treated with CTRsi. It was found that indeed both the frequency and number of cells per aggregate were significantly greater when maspin was present and that no difference in cell-cell aggregation was observed for cells expressing E244A compared to control cells (Figure 6.17). The knockdown of wild
type maspin with both targeting siRNAs showed a significant reduction in the mean number of aggregates compared to cells treated with CTRsi. However, only the treatment with siRNA #3 gave a significant reduction in the number of cells per aggregate. This supported the importance of wild type maspin with its ability to promote cell aggregation. The knockdown of E244A expression with either siRNA #3 or #4 showed no significant difference to either the number of aggregates or the number of cells per aggregate in comparison to those treated with CTRsi. Thus, the expression of mutant maspin E244A has no involvement with the promotion of cell-cell contact, as its reduced protein levels showed no further adverse effect.

Figure 6.17 Aggregation of MCF7 stable cells treated with maspin siRNA. Cells were treated with 200 nM maspin siRNA #3 or #4 or CTRsi for 48 hours before subject to aggregation assay. Data is presented as number of aggregates (blue bars) and number of cells per aggregate (purple bars). Data represents mean of three independent experiments. Statistical significance was determined by Students t-test (p*<0.05).

Following from the observation that maspin expression in MCF7 cells increased cell-cell interactions in suspension, we studied the effect of maspin on colony formation. MCF7 stable cell lines transfected with pcDNA3.2-maspin, pcDNA3.2-E244A or pcDNA3.2 were maintained in serum free medium on a non-adherent plastic for 15 hours. This caused the MCF7 cells to form suspended colonies, which were then transferred to a serum containing medium on an adherent plastic. Here, colonies made cell-substrate contact whilst maintaining their cell-cell contacts formed in suspension (Figure 6.18A). MCF7 cells expressing wild type maspin showed a significant increase in the area occupied by its cell colonies, in comparison to those cells expressing either mutant maspin E244A or empty vector (Figure 6.18B). This increase in colony size was attributed to an increased
number of cells per colony, thus further indicating the ability of maspin to promote numerous cell-cell contacts.

Figure 6.18 Colony area of MCF7 stably transfected cells. A) Images taken using DIC III with a CCD inverted microscope at x20 magnification. B) Graph shows mean colony area measured in \( \mu m^2 \) (light blue bars) as determined by Velocity and number of cells per colony (dark blue bars). 50 cell colonies were randomly imaged over nine independent wells of a 6-well plate. Statistical significance was determined by Students t-test \((p^*<0.05)\).

6.3.2.2 Maspin Alters the Expression of Cell-Cell Adhesion Molecules

Following the demonstration that maspin significantly promotes cell-cell interaction, its influence on the expression of the cell adhesion molecules E-cadherin and \(\beta\)-catenin was studied. Western blot analysis was used to detect protein levels of E-cadherin in MCF7 cells stably expressing pcDNA3.2-maspin, pcDNA3.2-E244A or pcDNA3.2 cells (Figure 6.19A). An up-regulation of E-cadherin was observed for wild type maspin expressing MCF7 cells in comparison to those expressing E244A or empty vector. This observation was also seen by immunofluorescence analysis (Figure 6.19B). Cells stably transfected with wild type maspin showed a strong, specific E-cadherin staining between adjacent cell membranes. In comparison, cells stably transfected with mutant maspin E244A or empty vector showed a reduced staining at cell-cell junctions. MCF7 cells expressing wild type maspin formed larger cell-cell interacting colonies, like those observed in Figure 6.18. Despite this increase in cell-cell contact, there is also an overall increased fluorescence of
individual cells representing mature adherens or desmosomal junctions, compared to cells expressing mutant maspin or control vector; as measured by fluorescence intensity. Fluorescence intensity is measured per \( \mu \text{m}^2 \), which ensures that the increased intensity of E-cadherin is not due to a difference in cell number.

Western blot analysis was also used to detect protein levels of the E-cadherin binding partner \( \beta \)-catenin (Figure 6.20A). MCF7 cells expressing maspin up-regulated \( \beta \)-catenin in comparison to cells expressing empty vector. Unlike the regulation of E-cadherin, cells expressing mutant maspin E244A showed an increase of \( \beta \)-catenin similar to cells expressing wild type maspin. This was replicated using immunofluorescence, which showed an increased \( \beta \)-catenin expression at cell-cell contacts of MCF7 cells expressing either wild type or mutant E244A maspin (Figure 6.20B). Although maspin expressing cells have an increased surface area and therefore more cell-cell contact in relation to control cells, the \( \beta \)-catenin staining was weaker at the junctions of control cells, as well as less frequent. In summary, maspin appears to promote the expression of E-cadherin and \( \beta \)-catenin. However, the apparent increase of E-cadherin protein requires the involvement of maspin's intact \( \alpha \)-helix G, whereas its mechanism used to increase levels of \( \beta \)-catenin appears to be independent of this region.
Figure 6.19 E-cadherin expression of stably transfected MCF7 cells as indicated. A) Western blot shows detection of 10 µg stable cell lysate with E-cadherin (left) or GAPDH (right). B) Three sets of immunofluorescence images showing labelling of cells with rabbit anti-E-cadherin conjugated to Alexa Fluor 488 and cell nuclei that were stained with DAPI. Images were taken using CCD upright at x63 magnification. Graph shows relative fluorescence intensity per unit area µm². Statistical significance was measured by Students t-test in relation to cells transfected with pcDNA3.2 (p*<0.05).
Figure 6.20 β-catenin expression of stably transfected MCF7 cells as indicated. A) Western blot shows detection of 10 µg stable cell lysate with β-catenin (left) or GAPDH (right). B) Three sets of immunofluorescence images showing labelling of cells with rabbit anti-β-catenin conjugated to Alexa Fluor 488 and cell nuclei that were stained with DAPI. Images were taken using CCD upright at x63 magnification. Graph shows relative fluorescence intensity per unit area µm². Statistical significance was measured by Students t-test in relation to cells transfected with pcDNA3.2 (p*<0.05).
6.4 Discussion

6.4.1 Summary
The findings presented in this chapter are consistent with other reports that maspin can significantly increase the adhesion capacity of epithelial cells. Cells expressing wild type maspin showed strong adhesion to the ECM, which was accompanied by an increased cell spreading; giving more surface area of the contact region with the matrix and other cells. The transfection of wild type maspin also increased cell-cell adhesive strength by inducing the formation of intercellular junctions by an up-regulation of E-cadherin and β-catenin. The expression of wild type maspin in MCF7 breast carcinoma cells significantly increased cell adhesion to the ECM, which correlated with its ability to increase the formation of stable focal adhesions. The presence of exogenous recombinant maspin or maspin trapped in a fibrillar cell matrix increased the adhesion of maspin null parental MCF7 cells. Thus, it can be suggested that the mechanism of action that maspin utilises to enhance adhesion acts predominantly extracellularly. However the possibility of an intracellular mechanism that works in cooperation with an extracellular mechanism must be considered. The mechanism/s that maspin uses to enhance cell adhesion and cell aggregation were at least in part dependent on its intact α-helix G. However, maspin was able to increase cell adhesion to extracellular laminin via a mechanism independent of an intact α-helix G. Following MCF7 cell adhesion to components of the ECM, expression of wild type maspin changed integrin expression forming a reduced invasive phenotype. Maspin increased the distribution of laminin specific receptors β4 and α6, the former of which was up-regulated in an α-helix G independent manner, which we postulate to contribute to maspin's adhesion to laminin in a manner also independent of its α-helix G. Wild type maspin also increased the distribution of integrin α2, α3 and α5 and we saw a consistent decrease in levels of integrin β1. Wild type maspin prevented the transition of inactive integrin β1 to a primed or active conformer, probably via direct interaction. Overall maspin has the powerful ability to increase cell adhesion, contributing to its epithelial-like character.

6.4.2 Maspin Increases Cell Adhesion via its α-helix G
Throughout this chapter, the effect of MCF7 cell adhesion stably transfected with wild type maspin or maspin mutant E244A was studied. The focus of maspin mutant E244A was studied because previous observations showed that its α-helix G region was critical for maspin's influence on MET and critical for its inhibitory effect on cell migration (Chapters 4 and 5). Stable transfected cells were used because the method used to measure cell adhesion gave unreliable results when transiently transfected cells were used, because of the inconsistent efficiency of gene delivery (data not shown). The measurement of cell
adhesion was highly reproducible when stable cells were used, and this also ensured that any effect observed was due to the presence/absence of maspin because of permanent integration of protein-coding constructs.

The addition of either recombinant maspin or maspin containing conditioned medium, to maspin-null DU-145 cells significantly enhanced cell adhesion (Figure 3.14). This complied with the work of other laboratories (Seftor et al. 1998, Cella et al. 2006, Ngamkitidechakul et al. 2001). This increase in adhesion was greater for cells treated with maspin conditioned medium than cells treated with recombinant maspin. It was also demonstrated that the stable transfection of wild type maspin into MCF7 cells significantly enhanced cell adhesion to matrix components; collagen I, laminin and fibronectin (Figure 6.2). This too complied with the work of other laboratories (Abraham et al. 2003, Odero-Marah et al. 2003). The greatest increase in cell adhesion of wild type maspin cells compared to control cells was seen on a laminin matrix. This could be due to the fact that overall MCF7 stable cell adhesion to a laminin substrate was much lower than that seen on a collagen I and fibronectin substrate. Thus, MCF7 stable cells plated onto a laminin substrate had the greatest potential to increase adhesion, whereas those on collagen I or fibronectin which were already highly adherent had the least.

For a more representative ECM, cell adhesion to a fibrillar matrix was determined (Figure 6.3). For each fibrillar matrix laid down by several cell lines, a consistent enhancement in adhesion of MCF7 cells transfected with wild type maspin was observed compared to control cells. The presence of maspin in the fibrillar matrix did not further enhance the adhesion of maspin expressing cells from that of a fibrillar matrix absent of bound maspin protein. This suggested that maspin present in the cellular matrix was redundant, leaving the other determining factors for the observed increased adhesion being intracellular maspin and/or maspin secreted into the medium. However, the overall adhesion of MCF7 stable cells to a fibrillar matrix containing maspin protein was greater than the adhesion to a matrix void of maspin protein. This not only indicates that maspin incorporated into an ECM is in fact not redundant and can enhance cell adhesion, but that maspin acts extracellularly bound to the matrix. Additionally, it was demonstrated that a fibrillar matrix containing maspin protein could enhance the adhesion of maspin-null cells in relation to a fibrillar matrix absent of maspin (Figure 6.4). This supports a functional role of maspin protein incorporated in the matrix. Here maspin may bind to a cell surface receptor and directly or indirectly facilitate cell adhesion. Furthermore, the binding of matrix-bound maspin to the cell surface may not be as effective when in the presence of cells endogenously expressing maspin protein, where this site could potentially already be occupied. Another possible mechanism of matrix-bound maspin could be to play a role in
paracrine signalling; where its incorporation into the cell-derived matrix may enhance its scope of effectiveness on enhancing cell adhesion. Similarly, the paracrine signalling of maspin trapped in the fibrillar matrix to enhance nearby cell adhesion, may not be as effective when in the presence of cells endogenously expressing maspin protein. In support of this latter hypothesis, maspin has previously been reported to act in a paracrine manner. For example, maspin secreted from corneal epithelial cells increased the adhesion of motile fibroblasts to the ECM in vivo (Horswill et al 2008). Also, maspin secreted from prostate epithelial cells in vivo inhibited the proliferation of surrounding prostatic stromal cells (Shao et al 2008).

Unlike the expression of wild type maspin, the expression of mutant maspin E244A did not increase cell adhesion in comparison to control cells, when plated onto either plastic, collagen I or fibronectin matrices (Figure 6.2). This suggested a role for an intact α-helix G in maspin’s ability to increase cell-ECM adhesion. However, when plated onto a laminin matrix cells expressing E244A showed a significant increase in adhesion on par with the effect seen by its wild type form, suggesting that on this specific matrix subtype mutation to this region does not diminish maspin’s pro-adhesive action. To determine whether maspin’s wild type α-helix G mimicked the enhanced adhesion of wild type maspin protein, the effect of maspin peptides (Figure 4.9) on cell adhesion was studied. However, wild type α-helix G peptide was not sufficient to induce adhesion in isolation, as the G-helix peptide did not mimic the increased cell adhesion as seen by exogenous or endogenous maspin.

Further indication that maspin’s α-helix G is crucial to its adhesive effect was observed by the inability of cells expressing mutant maspin E244A to enhance adhesion to fibrillar matrices (Figure 6.3). Also showing an intact α-helix G is crucial to maspin’s adhesive effect, was the inability of a matrix containing E244A to enhance cell adhesion, whereas a matrix containing wild type maspin did (Figure 6.4). Although this could suggest that E244A bound to the matrix was unable to bind to the cell surface and hence promote cell adhesion, it could also imply that cells expressing mutant maspin produce less ECM than cells with its wild type form. In support of this suggestion, cells expressing E244A or empty vector did not adhere to a non-adherent plastic in the absence of glucose, whereas cells expressing wild type maspin were able to (Figure 6.15). Therefore implying that cells expressing wild type maspin are able to either promote the synthesis of, or influence ECM deposition, and that mutation to its α-helix G prevents this from occurring. Such hypothesis, that wild type maspin can influence cell matrix deposition, is supported by the observation that breast tumour cells treated with the oestrogen antagonist Tamoxifen showed cell quiescence and regulation of ECM deposition (Hattar et al 2009). It is likely
that Tamoxifen exerts its effects via maspin since Tamoxifen has been shown to increase expression of maspin in breast tumour cells (Liu et al 2004), and maspin has been shown to induce the effects of Tamoxifen; a reduced ECM proteolysis and decreased activity of pro-enzyme metalloprotease-2 (Khalkhali-Ellis and Hendrix 2007, Seftor et al 2002).

Specifically, mammary tissue from Tamoxifen-treated rats had decreased fibronectin and increased collagen I levels, with a reduction in ECM turnover as detected by reductions in fibronectin, laminin 1, laminin 5 and collagen I cleavage fragments, and a decrease in expression and activity of metalloprotease-2 (Hattar et al 2009). Therefore maspin most probably also plays a part in regulating matrix deposition, contributing to its tumour suppressive properties.

The inability of cells expressing mutant maspin E244A to produce an increase of cell adhesion on matrix components and fibrillar matrices could be due to differential protein expression levels. For example, western blotting of MCF7 stable cell lysates showed that expression of mutant maspin E244A was slightly lower than that of wild type maspin (Figure 4.2A). However, the specific effect of cells expressing E244A on a laminin substrate suggests otherwise, especially as this was mirrored in the effect of cell migration (Chapter 5). Also, we have shown by transient transfection, that when the expressions of wild type and mutant E244A maspin were equal, the same effect on cell migration was seen as with stably expressing cells.

The specific ability of maspin to increase cell adhesion on a laminin substrate in a manner independent of its α-helix G was particularly interesting as it had been observed that the migration of maspin expressing cells specifically over a laminin matrix was also not influenced by mutation at residue E244 (Figure 5.13). Furthermore, this specific independence of maspin’s helical G region on its effect on cell adhesion fits well with the previous morphological studies. It was shown that cells expressing E244A on a laminin substrate had an epithelial-like phenotype equivalent to that of cells expressing wild type maspin, which were dissimilar to the mesenchymal-like score of control cells (Figure 4.6). This phenotype was also specific to laminin. Such consistent discrimination between matrix subtypes, leads to the proposal that maspin plays a key role in communicating with the extracellular environment. As discussed in section 5.4.4, it could be probable that maspin interacts with, or promotes the interaction of laminin specific integrin receptors, such as the heterodimers β4/β1 with α3, α6, and α7. In support of this is the demonstration that maspin directly associates with heterodimer α3β1 (Bass et al 2009). Such laminin specific interaction would thus be independent of its α-helix G, whereas interactions with collagen I or fibronectin integrin specific receptors would not. Indeed, as discussed in the following section, integrins α3, α6 and αV were increased in a manner
independent of its intact α-helix G when specifically on a laminin substrate. Furthermore, perhaps secreted maspin protein can interact directly with laminin at a site other than that involving its α-helix G.

6.4.3 Maspin Alters the Cellular Integrin Profile

Changes in integrin expressions are characteristic of cells undergoing differentiation, migration and transformation, whereby they undergo altered adhesion and cytoskeletal organization. Since we know that maspin promotes an epithelial-like phenotype accompanied by a biologically significant increased cell adhesion and decreased cell motility of mammary carcinoma cells, we can therefore assume that changes to the integrin profile contribute to such characteristics.

It has previously been shown by fluorescence activated cell sorting, that wild type maspin expressing cells increased levels of both α3 and α5 integrins (Seftor et al. 1998). Indeed, integrin α3 and α5 proteins were increased on plastic or fibronectin and on laminin or fibronectin respectively (Figure 6.6 and 6.7). However, since β1 integrin was down-regulated in maspin expressing cells, on plastic, collagen I and fibronectin (Figure 6.10), the heterodimer receptors α3β1 and α5β1 may not be preferentially formed on these substrates. Conversely, when maspin expressing cells are plated onto laminin, integrin β1 increased, suggesting the preference for the heterodimers α3β1 and α5β1. The greatest decrease of β1 was seen on a fibronectin matrix; however a simultaneous decrease of β1 fibronectin specific heterodimer integrins α5 and αV was not seen. Moreover, fibronectin specific integrin β3 was also down-regulated on fibronectin in the presence of maspin, possibly suggesting a preference for αVβ6 heterodimers on this substrate.

The expression of wild type maspin increased protein levels of integrin α2 and α6, irrespective of matrix component (Figure 6.5 and 6.8). Both α2 and α6 can bind to β1 to form collagen I or laminin specific heterodimers respectively. Since α2 only dimerises with β1, and our results showed that maspin decreased levels of β1 on a collagen I matrix (Figure 6.10), the up-regulation of this integrin may not serve a purpose in the enhanced adhesion to this matrix. On the other hand, maspin induced an up-regulation of integrin β1 unique to a laminin matrix. When surrounded by laminin, maspin expressing cells may up-regulate α6β1, a common laminin receptor, to enhance its adhesion to this matrix. Similarly, α6 can also dimerise with integrin β4, which was shown to have an elevated mRNA level upon the transfection of maspin (data not shown). Thus, maspin may also specifically up-regulate the laminin specific receptor; α6β4 to create strong integrin mediated adhesions to this ECM protein. In support of an up-regulation of integrin β4 by
maspin, is the correlation between low levels of β4 and the progression of mammary carcinoma (Pontier and Muller 2009); an effect negatively correlated to levels of maspin.

In comparison to cells expressing wild type maspin, cells expressing mutant maspin E244A showed a mixed profile of integrin expression. For example, unlike cells expressing wild type maspin, cells expressing E244A showed different levels of integrin α2, α5, αV and β3. This suggested an importance for maspin’s intact α-helix G region. However, like the expression of integrins between maspin expressing cells, the dependence of maspin’s intact α-helix G was dependent on the matrix subtype. For example, when on plastic or fibronectin, maspin increased levels of integrin α6 in a manner dependent of this helical region, yet on collagen I or laminin, maspin increased integrin α6 independent of this region. In addition to maspin’s up-regulation of α6, levels of α3 and αV were increased in a manner independent of its intact α-helix G when specifically on a laminin substrate. This correlates with the previous observation that the maspin directed increase in adhesion on a laminin matrix was independent of its α-helix G (Figure 6.2). Thus maspin may regulate these integrins specific to laminin and contribute to the increased cell adhesion via a mechanism not reliant on its helical G region. Furthermore, maspin may influence non-integrin laminin receptors, such as syndecans and 37/67 kDa laminin receptor, in a manner independent of its α-helix G, and which may contribute towards the previously observed cellular effects on laminin matrix.

In general cells expressing maspin mostly showed increased levels of integrin protein, suggesting that an up-regulation of integrin interaction with the surrounding ECM would enhance cell adhesion. In deed this fits with the results seen at the beginning of this chapter. The effect of maspin on the protein levels of integrins was dependent on the surrounding ECM subtype. This suggests that maspin may be altering the integrin profile by its involvement with the ECM itself, such as its specific binding to collagen I and III (Blacque and Worrall 2002). Additionally, maspin may be able to bind directly to the extracellular region of integrins, like that shown for integrins α3β1/α5β1 (Bass et al 2002), and influence integrin expression. The independence/dependence of maspin’s α-helix G on integrin expression may arise from its involvement with either matrix component binding or integrin binding. It also remains possible that intracellular maspin may be promoting integrin expression via down-stream signal transduction pathways, which may be affected by the ECM.
6.4.4 Maspin Inactivates Integrin β1
The investigation into the effect of maspin on β1 integrin activation status stemmed from the observation that a consistent down-regulation of β1 expression was identified using immunofluorescence in MCF7 cells and VSMC transfected with maspin (Bass et al 2009). Although the expression of wild type maspin mostly increased post-translational integrin expression, its specific down-regulation of β1 was also attributed to be influential to maspin’s pro-adhesive effect. Furthermore, although the expression of β1 has been shown to be essential for normal mammalian development, the expression of β1 and its downstream FAK have been found to be important for breast tumour progression (Pontier and Muller 2009); an effect opposite to that of the tumour suppression activities of maspin.

Immunofluorescence of MCF7 cells stably transfected with control vector showed diffuse staining of inactive β1 across the cell and a distribution of active β1 across the cell surface with localization to adhesion complexes (Figure 6.12). Comparatively, transfection of cells with wild type maspin showed a decrease of active β1 which was also specific to the cell edge. The reduction in binding of the 9EG7 antibody could possibly be due to simple steric hindrance of antibody binding by maspin, rather than any change in integrin conformation. However a conformational inactivation of the integrin is strongly supported by the observed concomitant increase in mAb13 binding to inactive β1 which was localised to the cell periphery. Therefore the expression of wild type maspin was able to hinder the conformational change of integrin β1 to a primed or active conformer; holding β1 in its inactive conformation at cell surface focal adhesions. This result was reproducible on VSMC, where binding of exogenous maspin to the cell surface showed a decreased expression of active β1 and increased levels of inactive β1, as shown by conformation-dependent antibody staining (Bass et al 2009). This redistribution of balance between active and inactive conformation was proposed to be a direct consequence of maspin/β1 interaction. Also the dependence of this interaction was shown for the ability of maspin to decrease cell migration (Bass et al 2009). Although how maspin interacts with β1 integrins has yet to be determined, maspin binding to the extracellular region of integrin β1 prevents the separation of the flexible α/β-leg subunits and subsequently interruption of this fast and flexible structural change could alter ECM binding and fast accurate cell signalling.

In contrast to cells expressing wild type maspin, those expressing E244A showed a low expression of inactive β1 and a higher expression of active β1, akin to controls cells (Figure 6.12). Therefore, we propose a mechanism involving interactions between β1 integrin on the cell surface and maspin through its α-helix G, resulting in an inactivation of β1 and concomitant downstream effects. The effects of maspin on migration and
adhesion could represent separate consequences of the interaction of maspin through its α-helix G to the same cell surface receptor, although they could both involve independent mechanisms. We suggest it is probable that cross-talk between maspin and β1 integrins is involved in both mechanisms of cell adhesion and migration.

Immunofluorescence detection of α5β1 by conformation-dependent antibody SNAKA51 showed punctate staining. This antibody specifically labelled ligand-bound or ligand-competent β1 integrin translocated to fibrillar adhesive complexes (Figure 6.12). Equal expression of SNAKA51 was observed between stable cell lines, indicating that the presence of maspin or mutant maspin did not alter the amount of fibrillar adhesions. Since wild type maspin reduced levels of the active β1 conformer and a direct binding of maspin to α5β1 has been demonstrated (Bass et al 2009), decreased levels of α5β1 were expected. Despite there being an equal detection of α5β1 fibrillar adhesions, it remains to be determined whether the maspin-dependent increase of both fibronectin specific αV and β3 (Table 6.1), function to enhance the binding to fibronectin. Therefore, on conclusion although wild type maspin reduces the levels of active, high ligand-binding affinity conformation, the binding of α5β1 to ligand fibronectin was not reduced. Since integrin β1 is expressed at high levels in mammary epithelial cells, it could be that inactivation of a proportion of this integrin did not perturb its ligand binding capacity, but yet may cause effect on a downstream pathway of β1, to influence its adhesive and migratory properties.

6.4.5 Maspin Increases Cell Spreading and Focal Adhesion Formation

The expression of maspin promoted the spreading of MCF7 cells, which correlated with its increase in adhesion to substrate. Specifically wild type maspin increased MCF7 cell spreading over a plastic, collagen I or laminin substrate in comparison to MCF7 control cells (Figure 6.13B). The greatest spreading of maspin expressing cells was seen on a plastic and collagen I substrate. This correlated with the highest score of epithelial-like character seen on these substrates (Figure 4.6); indicative of a highly adherent nature. When plated onto a fibronectin substrate, cells expressing wild type maspin were equally spread to those expressing empty vector. This suggests that both maspin expressing cells and control cells have the same adhesive capacity on fibronectin; however this was not the case, since expression of maspin had significantly greater adhesion to fibronectin than control cells (Figure 6.2). Moreover, the microscope images of MCF7 control cells on fibronectin showed an elongated, irregular mesenchymal-like phenotype, with numerous membrane protrusions; characteristic of motile, non-adhesive cells (Figure 6.13A and 4.6). The lack of effect on fibronectin may be related to our previous observation that both cell lines showed equal expression of ligand bound α5β1 (Figure 6.12). Cells expressing wild type maspin showed a consistent large cell area, increasing the strength of adhesion onto
the substrate but also between cells. In support of this, we have previously shown that cells transfected with wild type maspin showed an increased F-actin content (Figure 4.11). An increase of F-actin has been shown to aid cell spreading (McLeod et al. 2003).

The expression of maspin mutant E244A did not affect MCF7 cell spreading on plastic, collagen I, laminin or fibronectin (Figure 6.13B). This correlated with the inability of cells expressing E244A to increase adhesion to the ECM (Figure 6.2), whereas cells expressing wild type maspin increased cell spreading with a concomitant increase in cell adhesion. The observation that mutation to maspin’s α-helix G did not increase cell spreading, suggests that this region is critical in the ability of maspin to influence integrin-ECM interactions and subsequently initiate the complex cascade of signalling events that trigger cellular morphological changes resulting in cell spread. It had been expected that E244A cells would show an increased surface area on laminin since these cells adhered very well to this ECM component, yet this was not seen. This therefore suggests that when on laminin another mechanism independent of maspin’s α-helix G can sufficiently enhance adhesion onto laminin substrate that does not involve the influence of integrin-ECM interactions; such as the possible influence on non-integrin laminin receptors like syndecans.

The early stage of cell spreading is where cellular integrins first contact the ECM, flatten and deform extensively. This is induced by active Rac and Cdc42, which aid cell spreading by putting out membranous extensions to contact the surface and form focal contacts. In comparison, the later stage of cell spreading is induced by active RhoA which involves myosin driven tension to exert the force required to induce outward movement, along with the formation of strong focal adhesions. In turn, RhoA expression negatively regulates levels of active Rac. Since both assays measuring cell adhesion and cell spreading to the ECM in this chapter concern late cell spreading, it can be suggested that the ability of wild type maspin to increase late cell spreading increases levels of active RhoA. This corresponds with data showing maspin to decrease the RhoA antagonist GTP-Rac1 (Odero-Marah et al. 2003). In following to this implication, the expression of vinculin was looked at; a focal adhesive protein controlled by RhoA.

Immunofluorescence analysis with anti-vinculin showed that MCF7 control cells had few focal adhesions around the cell periphery which were often situated at polar ends (Figure 6.14). Comparatively, MCF7 cells expressing wild type maspin showed thicker, larger focal adhesions which were situated around the entire cell periphery. This showed that the expression of maspin created strong adhesive attachments in an un-directional manner which can explain the non-motile character of the cells in which maspin is
expressed. This can therefore suggest that maspin may enhance levels of active RhoA or one of its effectors, such as mDia or Rho kinase (Hall and Nobes 2000, Riento and Ridley 2003). Alternatively maspin may up-regulate the R-Ras driven localisation of Rho-GTP to the membrane in order to create new integrin clustering and strong focal adhesions to the cell substratum (Wozniak et al 2005).

Unlike the well established focal adhesions seen by wild type maspin expressing cells, the focal adhesions of cells expressing mutant E244A protein were not as thick or as large, which comparatively can help explain the lesser cell spread and increased motility phenotype. However, these smaller focal adhesions were situated around the majority of the cell cortex, actually suggesting an adhesive nature. Since cells expressing E244A are not as adhesive as cells expressing wild type maspin, it can be postulated that these cells have a high turnover of focal adhesions, a process controlled by FAK. A high turnover would explain why the focal adhesions of E244A expressing cells were not as established and showed a non-adhesive stance.

The ability of maspin to up-regulate focal adhesions may be supported by its direct interaction with integrin β1 and ability to alter the activation of β1, to an inactive conformer. Since integrin extension, from a bent to an extended conformation, takes place at focal adhesions, it is likely that in maspin expressing cells active β1 is reduced at these sites. However, integrin β1 is expressed at very high levels in epithelial cells and therefore, maspin is not likely to cause an overall inactivation of β1 subunits, but its reduction of β1 extension at focal adhesions could in turn reduce the activation of FAK. A reduction in FAK would subsequently decrease signalling via Ras/Raf/MEK pathway, culminating in a decreased level of active ERK. In support of this, maspin expressing cells showed a reduced level of phosphorylated, active ERK, as shown by western blotting (Appendix Figure A8). Furthermore, when FAK was blocked, breast cancer cells became less metastatic due to an increased number of focal adhesions and decreased mobility (Ren et al 2000); both of which we have shown to be characteristic to the introduction of wild type maspin (Figure 6.14 and 5.4).

6.4.6 Maspin Increases Cell-Cell Adhesion via its α-helix G

In addition to the observation that the expression of wild type maspin increased cell spreading and cell-ECM adhesion, it was shown that maspin also enhanced cell-cell contact. Specifically, maspin increased the frequency of cell-cell aggregation and also the number of cells in each aggregate (Figure 6.16B). The importance of the expression of wild type maspin for the enhancement of cell-cell contact was demonstrated using targeted siRNA (Figure 6.17). Results showed that the reduction of wild type maspin
accordingly reduced cell aggregation, whereas the reduction of mutant maspin protein did not. The ability of wild type maspin to increase cell-cell adhesion was reiterated when we observed an increased MCF7 colony area in the presence of maspin (Figure 6.18). To show that indeed this increased area was due to an increase in cell-cell contacts and not due to an increased cell spreading to the substratum, a mean number of cells per colony were counted. Thus on average wild type maspin expressing MCF7 cells formed significantly larger colony areas composed of a greater number of cell-cell interactions. This maspin directed effect on cell aggregation was dependent on an intact α-helix G. Correspondingly, mutation to maspin’s α-helix G hindered its ability to form colonies of a high quantity of cell-cell associations. It was additionally noted that cell-cell contacts within MCF7 colonies expressing E244A or empty vector were frequently adherent by long finger-like projections. In comparison, cells expressing wild type maspin were adjoined to one another by large areas of the cell surface. This not only suggests that the tight packing of colonies expressing wild type maspin was dependent on its α-helix G, but that the overall strength of the cell-cell adhesions was far greater.

Epithelial cells require cell-cell contact for optimal survival and growth and when required to migrate they move as sheets of interacting cells, whereas fibroblast-like cells migrate individually at low densities only requiring a low substrate attachment (Boyer et al 2000). The promotion of intercellular contact by wild type maspin thus coincides with its increased epithelial-like morphology. For example, maspin expressing cells were large and rounded with a uniform shape, which grew predominantly in tightly packed colonies (Figure 4.5). Furthermore, it has been shown that a loss of intercellular junctional communication correlates with metastatic potential, a factoring role in the carcinogenesis of mammary adenocarcinoma cells (Nicholson et al 1988). Thus a loss of cell-cell adhesion in tumour cells, correlates with a simultaneous loss in the expression of maspin; contributing to a reduced strength of cell-cell contacts.

6.4.7 Maspin Up-regulates Cell-Cell Adhesion Molecules

Succeeding the finding that maspin enhanced intercellular adhesion; the effect of maspin on the adherens junction proteins E-cadherin and β-catenin was investigated. Like maspin, E-cadherin is a tumour suppressor, whereby immunohistological studies have shown that the expression of E-cadherin is partially or totally lost in various human epithelial tumour types (Birchmeier and Behrens 1994). Like maspin, a reduced expression of E-cadherin in breast cancer is associated with invasiveness and loss of differentiation characteristics (Oka et al 1993). In accordance to this data, breast carcinoma MCF7 cells transfected with wild type maspin showed increased levels of E-cadherin protein specifically located at adjacent cell contacts, as detected by
immunofluorescence (Figure 6.19). In contrast cells expressing maspin mutant E244A or empty vector showed weaker staining of E-cadherin and western blotting showed that mutation to maspin’s α-helix G disabled this ability to increase homophilic E-cadherin-E-cadherin interactions. Respectively, this correlates with our earlier finding that cell-cell adhesion was dependent on maspin’s α-helix G. The positive correlation between wild type maspin and E-cadherin was in association with other laboratories. For instance, the heterozygous loss of maspin showed a decreased localisation of E-cadherin staining between basal-lateral junctions (Shao et al 2008). Additionally, a correlation can be linked with the effective restoration of E-cadherin seen by MCF7 cells treated with oestrogen receptor antagonist Tamoxifen (Bracke et al 1994), which Tamoxifen specifically increases the transcriptional activation of maspin gene (Liu et al 2004). Thus maspin may be the key mediator between the effects at the oestrogen receptor to the regulation of E-cadherin mediated adhesion.

MCF7 cells stably transfected with maspin showed an increased staining of β-catenin at regions of cell-cell contact (Figure 6.20). This fitted well with the ability of maspin to increase E-cadherin, and the subsequent potent ability of E-cadherin to bind to and recruit β-catenin to the adherens junctions. However, unlike the increased expression of E-cadherin which was dependent on maspin’s intact α-helix G, the increased expression of β-catenin was not. Therefore, the results suggest that β-catenin was recruited to the membrane of E244A expressing cells by a mechanism other than via E-cadherin; and one that is influenced by maspin at a site other than its α-helix G. This alternative mechanism could be via the phosphorylation of β-catenin, a modification which prevents its nuclear translocation and promotes its involvement at the cell membrane. Phosphorylation of β-catenin by maspin independent of its α-helix G was backed by the observation of a second β-catenin band of increased molecular weight by approximately two kDa, detected by western blot (Figure 6.20A). Furthermore, the nuclear translocation of β-catenin has been shown to be influenced by active GTP-Rac1 under Wnt signalling (Orsulic et al 1999); a GTPase that maspin has been shown to down-regulate (Odero-Marah et al 2003), which may enhance β-catenin phosphorylation. However, if according to this premise that maspin influences β-catenin phosphorylation; this higher band on the western blotting should have been seen from wild type maspin-cell lysate also. Moreover, cells expressing mutant maspin with higher levels of β-catenin than E-cadherin would not necessarily indicate the formation of strong cell-cell interactions. On the other hand, cells expressing wild type maspin were able to up-regulate E-cadherin/β-catenin complex and consequently form strong cell-cell junctions.
6.5 Future Perspectives

It was shown that maspin had the ability to powerfully increase cell adhesion, an action in which its α-helix G was crucial, unless in the presence of laminin; where maspin increased adhesion to this substrate independent of this region. Throughout this chapter stable cell lines were used to assess cell adhesion and cell aggregation, since transiently transfected cells did not give consistent results. Therefore, to re-enforce the necessity of maspin’s α-helix G in the enhancement of adhesion, we could generate MCF7 stable cells expressing mutant E247A protein. It would be likely that cells expressing E247A would behave similarly to those expressing E244A, since both residues reside on the same α-helical region. Similarly, stable cell generation with other mutagenic constructs (Figure 3.4A) could provide further interest to the pro-adhesive mechanism. For example, investigation into the effect of maspin’s RCL on adhesion could help make sense of conflicting studies which show either its P1-P1’ scissile region of the RCL essential (Ngamkitidechakul et al 2003, Locket et al 2006) or not essential for cell adhesion (Cella et al 2006). One such avenue of investigation would be to produce a peptide consisting of maspins wild type or mutated RCL and apply this to a cell adhesion assay.

As we have shown maspin clearly alters cellular integrin expression levels, a future perspective would be to analyse the levels of integrin proteins using fluorescence activated cell sorting, a specialized type of flow cytometry. This may allow us to study those integrin proteins that we did not look at, such as laminin specific α7, fibronectin specific α8 and collagen specific integrin α1, α10 and α11. Additional experiments to develop our understanding of maspin’s ability to increase cell adhesion via cell surface integrins, would be to analyse functional assays in the presence of integrin blocking antibodies. For example, we could look at cell adhesion to laminin or collagen I in the presence of β4 or αV blocking antibodies respectively.

The wild type G-helix peptide did not mimic the effect of wild type maspin protein on cell adhesion. However, we have shown a re-organisation of the actin cytoskeleton by wild type G-helix peptide (Figure 4.10), which showed the critical involvement of this region to aiding ECM re-arrangement, an effect most likely mediated via integrin engagement. Therefore, we could explore the preliminary effects of maspin G-helix peptides on integrin expression. This would indicate whether the re-organisation of the integrin profile by wild type or mutant maspin protein can be mimicked with wild type G-helix or mutant 244 peptides respectively. The involvement of maspin’s α-helix G in cell adhesion was consistent on all fibrillar matrices and matrix components with the exception of laminin (Figure 6.2 and 6.3). The possibility that maspin may interact with laminin directly, or regulate the binding of laminin receptors in a way independent of its helical G domain is an exciting concept, one that should be looked into further.
The observation that maspin increases levels of inactive β1 integrin, correlates with maspin's direct engagement with β1 (Cella et al 2006, Bass et al 2009). It would have been interesting to see whether maspin prevented the active conformer of β1 on matrix components, other than the studied plastic substrate. In correspondence with other evidences, it was also shown that maspin binds to the cell surface (Sheng et al 1996, Bass et al 2009) where it can elicit its anti-migratory and pro-adhesive effects. Despite this evidence suggesting maspin functions extracellularly at the cell surface, a recent publication disagreed with this (Teoh et al 2010). Their report presented maspin as a non-secretory protein which did not play a role at the cell surface and showed no co-localisation with integrin β1. They suggest that maspin indirectly affects cell-matrix interaction via an intracellular mechanism. This conflicts with the results shown in this thesis; that recombinant maspin added to the outside of cells can increase their adhesive capacity. However, the possibility that this may be internalised and indeed function intracellularly has not been ruled out, yet the observation that maspin trapped in the ECM was sufficient to increase cell adhesion suggests otherwise.

Further investigation into whether maspin influences the synthesis or secretion of matrix components would be of interest. For example we could asses the quantity of secreted matrix components using immunofluorescence. Furthermore we could look at the interaction of maspin with molecules involved in cell matrix production such as transforming growth factor-β; which has been shown to regulate proteolytic enzyme and ECM production. One particular avenue of future investigation could be to determine whether maspin affects fibronectin matrix synthesis. This process requires the presence of α5β1 integrin, an integrin recently shown to directly interact with maspin (Bass et al 2009). Furthermore, maspin expressing cells showed a particularly high level of adhesion to fibronectin and collagen I. Collagen type I contains a unique fibronectin binding region (Guidry et al 1990) and a maspin binding region (Blacque and Worral 2002). An increase in ECM deposition occurs during wound healing. This is important because it provides guidance cues for cells to migrate into the wound, as well as increasing the strength of the wound. Once epithelial sheets of cells migrate into the wound, they stop migrating under cues known as contact inhibition (Boyer et al 2000). On the other hand, an increase in ECM deposition can be detrimental. For example, an enhanced matrix deposition has been shown to be a characteristic feature of lung fibrosis and accumulation of proteoglycans in the walls of atherosclerotic vessels contributing to the pathogenesis of cardiovascular disease (Eickelberg et al 1999). Therefore, if maspin does indeed aid the secretion of matrix components, it would not serve to aid migration like that seen in these mesenchymal-like processes; since maspin clearly inhibits cell migration (Chapter 5) and enhances cell adhesion.
It can be hypothesised that the maspin mediated up-regulation of β-catenin (Figure 6.20) is predominantly located at the cell membrane, since nuclear accumulation of un-phosphorylated β-catenin has been associated with an enhanced cell migration and EMT (Lin et al 2000A). Cellular fractionation experiments would help clarify this. Also, it could be determined whether Wnt signalling, which influences the nuclear accumulation of β-catenin, is perturbed in maspin expressing cells. Another useful avenue of investigation would be to determine whether wild type maspin can influence the phosphorylation of β-catenin, which would have high clinical significance.

The experiments performed in this chapter to assess cell adhesion were all performed in two dimensional cultures. In comparison, a three dimensional in vitro system to assess the expressions of intra-and extracellular proteins has certain advantages. A 3D environment incorporates both cell-ECM and cell-cell interactions, creating an in vivo-like morphology with a more realistic biology and function. Therefore, it would be ideal to determine the effect of wild type maspin and mutant maspin E244A on the expression of vinculin, cell surface integrins and cell-cell proteins E-cadherin and β-catenin in a 3D matrix designed to mimic the tumour microenvironment. Research into maspin’s affect on cell adhesion could also be broadened to examine its biological response on communicative gap junctions and occluding tight junctions, in addition to the anchoring junctions studied here. Furthermore, it was found that expression of maspin increased protein levels of integrin α6 and mRNA levels of β4; a heterodimer (α6β4) involved in the formation of hemidesmosome structures that link cytosolic keratins to extracellular laminin. This could also be explored.

6.6 Conclusions
On conclusion, the expression of wild type maspin or the addition of recombinant maspin significantly increases cell adhesion to matrix components and fibrillar matrices. In correlation with this increased adhesion, cells expressing wild type maspin are more spread, thus providing a larger surface area for attachments to the ECM to occur. Correspondingly cells expressing maspin showed an enhanced formation of focal adhesions, which correlated with a general increase in integrin expression. Cells expressing wild type maspin also showed a decrease in the activation status of integrin β1, yet this did not show a decrease in the α5β1 ligand binding to fibronectin. Therefore, it is likely that maspin may, through direct extracellular interaction with β1 have an effect on downstream signalling pathways, potentially leading to an increased cell adhesion and decreased cell migration. The expression of wild type maspin also increased cell-cell adhesion which was accompanied by an increase in junctional proteins. It was found that mutation to maspin’s α-helix G at residue E244A did not increase adhesion to either
fibrillar matrices or adjacent cells. This conferred the importance of such region for these adhesion processes. However, a unique mechanism of action was suggested for cells adhering to laminin, since the pro-adhesive influence of maspin expressing cells over laminin did not require an intact α-helix G. Such unique mechanism is suggested to overlap with the mechanism used for maspin's anti-migratory behaviour over laminin (Chapter 5); also independent of this helical region.
7 General Discussions

7.1 Thesis Summary
Over a decade of research on maspin has solidified its function as a non inhibitory serpin and tumour suppressor (Bass et al 2002, Zou et al 1994, Sager et al 1997). This prompted our investigations into how maspin achieves its pleiotropic effects. There have been conflicting interpretations of how maspin carries out its functions. Opinions differ on maspin's site of action whether it functions intra- or extracellularly, or both. For example, intracellular maspin has been shown to be essential for sensitizing cells to apoptosis (Jiang et al 2002), whereas maspin located either extracellularly or intracellularly has been shown to promote cell adhesion, and decrease migration and invasion (Seftor et al 1998, Sheng et al 1996). There are also studies that question whether maspin has oncogenic properties in certain cancers, where maspin’s expression has been found to positively correlate with the progression and metastasis of ovarian and pancreatic carcinomas (Sood et al 2002, Maas et al 2001). In comparison, the majority of studies have focused on demonstrating maspin’s tumour suppressive properties. The negative correlation of maspin with increasing grade of cancer has been largely demonstrated in the breast and prostate (Zou et al 1994, Pierson et al 2002), and also in squamous cell carcinoma (Nakagawa et al 2006).

The initial objective of this thesis was to characterise the biological effects of maspin expression using breast and prostate cancer cell models. It was shown that maspin is present within the nuclear, cell membrane, cytoplasmic and cytoskeletal compartments as well as in the conditioned medium of cells. Also, previous observations were confirmed that maspin decreases cell migration and proliferation, whilst increases cell adhesion and apoptosis. Site directed mutagenesis was performed to mutate residues that resided within key tertiary structures of maspin, with the prospective of providing an insight into maspin's mechanism of action. It was demonstrated that mutation to maspin’s α-helix G at either residue E244 or E247 perturbed maspin’s inhibition of migration. In comparison to the requirement of maspin’s intact α-helix G, an intact RCL was not necessary for its ability to decrease cell motility (Ravenhill et al 2010). This happens to disagree with other studies that demonstrate the importance of maspin's RCL in its anti-migratory behaviour (Sheng et al 1996), specifically in epithelial cells (Zhang et al 2000b).

Following this novel mechanistic insight into maspin’s critical α-helix G, wild type maspin protein was expressed using an insect system and purified. Also maspin variant proteins E244A and E247A were expressed using a mammalian CMV system and concentrated proteins were collected from the conditioned cell medium. The biological activity of
recombinant maspin and mutant maspin proteins were clarified using a well established migration assay. Using recombinant wild type maspin and mutant maspin proteins in conditioned medium, it was confirmed that maspin can act extracellularly to inhibit epithelial cell migration, and that this inhibition was dependent on its intact α-helix G. Similarly, it was demonstrated that the addition of exogenous maspin, or the transfection of maspin, increased cell-ECM and cell-cell adhesion; both effects critical to maspin’s integral α-helix G. Specifically, maspin’s enhanced adhesion to the ECM was found to correlate with its ability to increase expression of E-cadherin/β-catenin complex and focal adhesions. Additionally wild type maspin showed a direct regulation of cell surface integrin expressions, and also promoted the inactivation of β1 integrin; by promoting its inactive, bent conformer. The requirement of maspin’s α-helix G, was specific to its anti-migratory and pro-adhesive effects, since it was not essential for maspin’s pro-apoptotic properties.

The extracellular function of maspin was likely to be the outcome of its binding to a cell surface ligand/s, since the addition of recombinant maspin showed punctate cell surface staining whereas exogenous mutant proteins did not. Corresponding to the effects of extracellular maspin, the transfection of wild type maspin decreased epithelial cell migration and increased cell adhesion. It was not determined whether these effects were due to the secretion of maspin or due to the release of maspin as a consequence to cell necrosis, however the large amount of maspin present in the conditioned medium indicates maspin’s action extracellularly was due to secretion.

Maspin’s non-motile and pro-adhesive nature was reflected in the morphology of maspin expressing cells. Maspin directed MET through alteration of the actin cytoskeleton, a process that was perturbed when maspin was mutated at its α-helix G. It was demonstrated that maspin directly increased the content of F-actin and G-actin with a decrease in the expression of actin filament elongating protein profilin. Cells expressing maspin exhibited a lower profilin:G-actin ratio, which suggested a lower tendency towards directional polymerisation, correlating with its anti-migratory, epithelial-like stance.

Interestingly it was discovered that a wild type peptide consisting of residues from maspin’s α-helix G exerted identical anti-migratory, pro-adhesive and MET effects as wild type maspin protein. Furthermore, this G-helix peptide was shown to compete with endogenously expressed maspin protein in order to exert its cellular effects, implicating competitive binding for a cell surface/extracellular binding site.
Overall, it was clarified that maspin is a multifunctional protein whose anti-metastatic activity is mediated via an extracellular mechanism, likely to interact with cell surface integrins and ECM components (Bass et al 2009, Blacque and Worrall 2002). Such interaction in turn would catalyse the re-organisation of the cellular cytoskeleton, influencing cell migration, adhesion and proliferation.

7.2 Conceptual Model

Based upon the observations presented in this thesis, Figure 7.1 represents a proposed model for the regulatory action of maspin on cell adhesion, migration and epithelial residence. Although additional levels of complexity exist, this diagram has highlighted those relevant to this project. This model does not exclude or undermine the existing intracellular roles/binding partners of maspin; instead this study most strongly indicates an extracellular/cell surface role for its effects of adhesion, migration and actin reorganisation.

The demonstration that maspin re-arranged the cellular profile of integrins was akin to other studies (Seftor et al 1998, Cella et al 2006). Maspin was found to up-regulate laminin and fibronectin specific receptors α6β4 and αVβ5 respectively. In the presence of collagen I, it was found that maspin up-regulated the fibronectin specific receptor αVβ3. We hypothesise that via αVβ3 maspin encourages the interaction between collagen and fibronectin. This effect could also be possibly due to maspin's direct interaction with collagen subtypes I and III (Blacque and Worrall 2002). The heterodimer αVβ3 is also a receptor for vitronectin, and its up-regulation by maspin may be to aid adhesion to this secreted matrix protein. Interestingly, it was found that maspin’s α-helix G was not essential for its effect on either cell migration or adhesion over a laminin matrix. In relation to this finding, integrins α6β1, αV and α3 were up-regulated in a manner independent of maspin’s α-helix G, and could therefore contribute to maspin’s unique behaviour on this substrate. Furthermore, it can be proposed that maspin interacts with non-integrin laminin receptors in a manner independent of an intact α-helix G, such as syndecans, to aid this unique effect.

These maspin-directed integrin re-arrangements encouraged integrin clustering and the formation of stable focal adhesions. This re-arrangement could potentially be mediated by the small GTPase Rap2, which has been shown to promote F-actin assembly and cell spreading (McLeod et al 2003); two characteristics corresponding to the effect of maspin. Therefore, maspin can increase adhesion and decrease migration via integrin clustering and its interactions with the ECM.
Figure 7.1 Schematic representation of the extracellular influence of maspin on cell behaviour. The black arrows represent known pathways and the red arrows indicate novel pathways implicated from the results found from this thesis. The down-regulation/inhibition of a process is marked by ⊗. Reference to pathways/interactions - 1; Al-Ayyoubi et al 2007, 2; Blacque and Worrall 2001, 3; Khalkhali-Ellis and Hendrix 2007, 4; Seftor et al 1998, 5; Cella et al 2006, 6; Bass et al 2009, 7; Odero-Marah et al 2003, 8; Shao et al 2008. EGFR Epidermal growth factor receptor, uPAR urokinase plasminogen activator receptor, PAI1 Plasminogen Activator Inhibitors, PIP2 Phosphatidylinositol 4,5-bisphosphate, PI5K Phosphatidylinositol 5-Kinase, MMP Matrix Metalloproteinase, ERK Extracellular Signal-Regulated Kinase.
It was shown that the introduction of wild type maspin influenced a decrease in the activation status of β1 integrin, as did the addition of exogenous maspin (Bass et al. 2002). This action could be mediated by the ability of maspin to directly interact with integrin β1 (Seftor et al. 1998, Cella et al. 2006), and the ectodomain of α5β1 or α3β1 (Bass et al. 2009). In addition, this direct interaction and regulation of integrin active state could mediate the re-organisation of cell surface integrin expressions. It is hypothesised that maspin’s influence on the inactivation of integrin β1 would cause a subsequent down-regulation in FAK and the following Ras/Raf/MEK pathway, culminating in a decreased level of ERK activation and the previously reported inhibition of the JNK mediated c-jun phosphorylation (Shi et al. 2007). The activation of FAK by tyrosine phosphorylation is essential for G1 phase cell cycle progression mediated by its downstream activation of extracellular signal-regulated kinase (ERK) (Aplin et al. 2001). Therefore, as a result of FAK down-regulation, oncogenic transcription would be suppressed and cell cycle progression halted; fitting with maspin’s tumour suppressive, anti-proliferative nature.

The decrease of active integrin β1 by wild type maspin may perturb the interaction between β1 and uPAR; an interaction shown previously at the surface of MDA-MB-231 breast cancer cells which increases β1-containing focal adhesions and redistributes them to the leading and rear edges of the cell (Sturge et al. 2001). The interaction with β1 and uPAR was shown to be essential for fast cancer cell chemotaxis towards an uPA gradient and the activation of N-WASP with its subsequent up-regulation of pro-migratory microspikes and lamellipodia; during invasion and metastasis. Also, the expression of uPAR correlated with an increased progression in cancers of the breast and prostate (reviewed in Ellis 2003). Since, unlike uPAR, the expression of maspin negatively correlates with the progression of breast and prostate cancers (Zou et al. 1994, Pierson et al. 2002), maspin may directly decrease active β1 conformer and consequently perturb the β1/uPAR interaction and the dissemination of tumour cells in malignant disease. Subsequently, an interruption between β1 and uPAR can inactivate epidermal growth factor receptor (EGFR) in an EGF independent manner (Xue et al. 1997). The anti-adhesive and pro-migratory effects of uPAR, mediated by Cdc42 and Rac1 would thus be down-regulated. A decreased Rac1 would likely increase levels of its antagonistic GTPase Rho, which in turn would increase stress fiber and focal adhesion formation and reduce microspikes. In support of this is the previous report that Cdc42 and Rac1 are down-regulated as a downstream effect to maspin expression (Odero-Marah et al. 2003), and the observations seen in this thesis that the expression of maspin increased focal adhesions, reduced membranous protrusions and subsequently increased adhesion and decreased cell motility. Further interpretation of maspin with uPAR is supported by an exosite interaction between maspin and uPA (Al-Ayyoubi et al. 2007). Although maspin
holds no inhibitory action towards uPA and plasminogen activation (Bass et al 2002), its interaction with uPA may contribute to a down-regulation of cancer cell chemotaxis, as could perturbation of β1/uPAR association.

It was also demonstrated in this thesis, that maspin negatively regulated the expression of profilin, a protein that binds ADP-actin to promote directional polymerisation. Profilin is associated with high motility and often in the response to growth factors. Therefore maspin could potentially influence profilin via profilin’s upstream regulators WASp/formins or via the metabolism of phospholipids.

Furthermore, it was shown that maspin increased the expression of E-cadherin/β-catenin complex, which elucidated its tumour suppressive attribute of increased cell-cell clustering. Therefore it can be speculated that the positive effect of maspin on the E-cadherin/β-catenin complex, would inhibit the nuclear translocation of β-catenin and the subsequent transcription of anti-apoptotic genes; a feature akin to the pro-apoptotic nature of maspin.

Maspin has previously been shown to bind to both active and zymogen forms of the extracellular enzymes tPA and uPA. This occurs via an exosite: exosite interaction which does not involve maspin’s RCL and hence does not use any inhibitory action (Al-Ayyoubi et al 2007, Bass et al 2002). Thus it is possible that the binding of maspin to PAI-1-bound tPA could enhance the inhibitory action of PAI-1; the inhibited conversion of plasminogen to plasmin and subsequent matrix degradation. Similarly, the direct interaction of maspin with collagen I matrix (Blacque and Worrall 2001) can prevent the binding of cathepsin D (Khalkhali-Ellis and Hendrix 2007) and its matrix degrading action which would otherwise promote cell migration.

7.3 Relevance of Findings

In this study, we focused on characterising maspin’s function in breast and prostate cells, since the expression of maspin has been shown to inversely correlate with pathophysiological progression in these tissues. However, one of the major problems with using a single isolated tumour cell type is that interactions with stroma cells and the systemic environment involving complex interactions and stimuli are removed. Therefore, all observations need to be considered within this context. Furthermore, we are using one subset of tumour cell, when in vivo numerous epithelial cancer subtypes derive from a variety of distinct normal epithelial cells. Therefore it would be ideal to test a culture system formed from different cell subtypes from the same epithelium. Also it must be
considered that when our results are extrapolated to a complex *in vivo* model of breast cancer, our findings may not be as functionally important.

The data in this thesis indicates that wild type G-helix maspin peptide has the potential to be a novel cancer therapy. We show that G-helix peptide has effective anti-cancer properties, which specifically inhibited epithelial derived tumour cell migration, promoted cell attachment and produced an epithelial-like phenotype. Wild type G-helix peptide has a high prospective for being used as a treatment for breast and prostate cancers, where maspin expression is down-regulated and sometimes silenced. Since we have also shown that maspin’s α-helical G structure does not influence apoptosis, we propose that cancer treatment with G-helix peptide would not induce cell death to neighbouring non-transformed cells, yet potently decrease the metastasis of cancer cells where maspin expression is lowest.

Investigation into the exogenous role of maspin was hindered in these studies by the unsuccessful production of mutant maspin proteins by an insect expression system. However, maspin mutant proteins were expressed using a mammalian expression system, but these were not purified due to time limitations within the project. Nonetheless, conditioned medium from cells expressing maspin mutated at its α-helical G residue E244 or E247 were able to mimic the effects of cells transfected with mutant E244 or E247 maspin constructs; an elimination of maspin’s native tumour suppressive actions. Unlike the data using recombinant maspin, conditioned medium proteins were used in experiments at qualitative volumes and so this needs to be considered when looking at this data. We also must consider that adding exogenous maspin to cells may be beyond realistic physiological concentrations, especially if maspin is not secreted from the cell but is only released upon necrosis; as recently suggested (Teoh *et al* 2010). Nonetheless we showed that outside of the cell, maspin retained functionality and is most likely to exert its effects via direct interaction with cell surface ligand/s and by direct matrix interactions.

7.4 Future Perspectives

Throughout this thesis the cellular role of maspin in breast and prostate epithelial cells was characterised. A novel mechanism of action whereby many of maspin’s biological effects hold a lot of responsibility to its α-helix G was also identified. This has opened numerous avenues of future investigation in both breast/prostate cells but also in other cancer models where maspin expression has been reported to behave as a tumour suppressor; such as squamous cell carcinoma. This section provides a general overview of the directions for future work.
A key finding to this project was the ability of wild type α-helix G peptide to mimic the anti-migratory and pro-adhesive effects of native maspin protein. These tumour suppressive effects of G-helix peptide, possibly mediated via the re-arrangement of the actin cytoskeleton, need to be assessed under native conditions such as using an in vivo model of epithelial cell derived cancers. In addition, the functional consequences of long term exposure to G-helix peptide would need to be examined, in order to support the therapeutic use of the peptide. As well as an in vivo model, the effect of G-helix peptide on migration/invasion could also be studied using a three dimensional model, such as Matrigel.

Maspin is a unique tumour suppressor, most akin to its family member PEDF which is also an anti-tumourigenic protein. Both maspin and PEDF correlate inversely with the progression of cancer (Ngamkitidechakul et al 2001, Ngamkitidechakul et al 2003), bind to collagen subtypes I and III (Blacque and Worrall 2002), and share a unique α-helical G region (Law et al 2005, Simonovic et al 2001). Although the α-helical G region of PEDF has been implicated in its binding to collagen (Meyer et al 2003), the binding of maspin to collagen involves its α-helical D region. It would be interesting to determine whether PEDF’s α-helix G can mediate an anti-migratory, adhesive effect like maspin.

It would be ideal to effectively isolate and purify mutant maspin proteins to provide additional insight to whether the native extracellular biological function of maspin is affected. Also, protein structure studies of maspin mutant proteins, such as nuclear magnetic resonance spectroscopy, could determine whether mutation caused the partial folding of maspin; something that was suggested since proteins E244A and E247A were not secreted from S2 cells. Additionally, protein structure studies could be used to determine the structure of maspin before and after its direct interaction with integrin β1 or the heterodimers α3β1 or α5β1 (Bass et al 2009). Although the effect of mutant maspin E244A and E247A on cell migration and adhesion was determined using collated conditioned medium, an isolated protein would provide less margin of error. Additionally, isolated maspin protein could be used in the identification of protein:protein interactions via co-immunoprecipitation and ligand blotting techniques. For instance, whether mutant protein E244A can bind to the ectodomain of integrin α5β1, like native maspin (Bass et al 2009), would clarify our observation that the expression of E244A is no longer able to promote the inactivate conformer of β1. Isolated maspin protein may also help us to determine the discriminatory mechanism of action that maspin uses when on a laminin matrix, whereby the α-helix G is no longer necessary for its anti-migratory role. This ability of maspin to exert its tumour suppressive effects on laminin in a manner independent of its α-helix G was perplexing; one which needs to be further addressed. One route of
further investigation could be the influence of maspin on matrix deposition. We hypothesise that maspin can influence matrix deposition to aid strong cell-ECM adhesion. In keeping with this, it has been reported that the deposition of laminin and fibronectin were lost from heterozygous knockout-maspin murine models (Shao et al 2008). However, this may be a secondary effect to maspin’s ability to inhibit cathepsin D mediated matrix deposition (Khalkhali-Ellis and Hendrix 2007). Additionally, the purification of maspin proteins mutated at other critical sites, such as the salt bridge or α-helices A-I could identify unique protein interactions, and may be necessary for other cellular processes such as maspin’s ability to decrease angiogenesis.

The ability of maspin to re-arrange cell surface integrin expression is critical for its perturbation of cell movement and promotion of an epithelial-like morphology. Further techniques into the identification of integrin expression, such as fluorescence activated cell sorting analysis need to be considered. Also further investigation should be given to the levels of active FAK and whether indeed maspin’s ability to down-regulate active ERK was down-regulated in response to FAK regulation by integrin clustering. In addition we could determine whether nuclear ERK substrates were subsequently less active. An experiment could have been performed to determine whether maspin stabilised focal adhesions by elevating the expression of vinculin on matrix components and fibrillar substrates; like that observed on plastic. Also, the morphology of the cytoskeleton of MCF7 cells in the presence of an ERK inhibitor could be investigated to see if the phenotype reverted to an epithelial-like one; more akin to those re-introduced to maspin.

Another avenue of investigation could extend from the observation that maspin down-regulated the expression of profilin; regulators of actin polymerisation at the plasma membrane. Since maspin expressing cells did not show a decrease in the profilin substrate G-actin, it can be assumed that maspin may up-regulate profilin competitors such as the G-actin sequestering protein thymosin β4 and/or the actin depolymerisation family ADF/cofilin. In addition to actin, profilin binds to a variety of signalling molecules implicated in cell motility, such as Arp2/3 complex, Mena, VASP, N-WASP, dynamin I. Thus a look into the expression of binding partners and upstream regulators of profilin could provide a closer insight into this maspin-mediated down-regulation. Other opportunities for investigation would be to completely characterise maspin’s influence on MET markers; in addition to E-cadherin, such as Desmoplakin and Cytokeratin, and EMT markers; such as Snail, Slug and Twist.

The non-motile phenotype of maspin expressing cells consisting of a dense lamellipodial network around the whole cell periphery is akin to a non-motile phenotype induced by
fungus Strongylophorine-26, which showed increased levels of active Rho (McHardy et al 2005). This particular study proposed that active Rho would act via the effector mDia and not via Rho kinase. As a result Rho would reduce central stress fibres and induce actin polymerisation required for lamellipodia extension, as opposed to the promotion of stress fibre formation, respectively. Thus it remains possible, that maspin could be signalling via Rho and mDia. Future studies would investigate the effect of maspin with lamellipodial or filopodial specific proteins, such as Scar/WAVE or Mena/VASP.

Investigation into the hypothesised influence of maspin on Rap2 could provide a mechanistic insight into its elevated cell spreading and integrin-mediated cell adhesion. This could be particularly interesting seeing that the process by which Rap-GTP promotes integrin activation is also not currently understood. On the contrary, the activation of the GTPase Rap1 was associated with the spreading and membrane extension of cells which have a polarised morphology and has been associated with an increased cell migration (McLeod et al 2004). Thus it could also be determined whether the potential down-regulation of Rap1 is influenced by maspin.

Another avenue of future investigation could include the relationship between maspin and oestrogen. This stems from the similar morphology of control and E244A expressing MCF7 cells with that documented for MCF7 cells treated with oestrogen (Sapino et al 1986). The documented study reported that hormone-responsive MCF7 cells supplemented with oestrogen show cytoplasmic protrusions rich in F-actin, which were often associated with peripheral lamellipodia. This resemblance can be attributed to the fact that our MCF7 cells were cultured in MEM medium which contained phenol red; a chemical reported to act as a weak oestrogen. Conversely, it was reported that MCF7 cells grown in oestrogen deprived medium showed a flattened phenotype, with strong F-actin ruffling around the entire cell periphery with multiple focal adhesions. Additionally MCF7 cells treated in the presence of oestrogen antagonist Tamoxifen, showed strong F-actin staining at the cell periphery with the formation of multiple stress fibres (Sapino et al 1986). This latter, oestrogen deprived, phenotype is much alike our MCF7 cells expressing wild type maspin. Therefore, it can be suggested that wild type maspin may itself be causing an antagonistic effect on the oestrogen receptors of these cells. Furthermore, mutation at maspin’s α-helix G, which perturbs maspin’s ability to re-distribute the actin cytoskeleton, may occur via an inadequate interaction with oestrogen receptors. In keeping with this hypothesis, is the comparable reduction in cell proliferation shown by either blocking oestrogen or the transfection of wild type maspin (Hall et al 2008) (Appendix Figure A5).
Appendix

Figure A1 Expression of maspin-mutated RCL construct in DU-145 cells. 10 µg cell lysate transiently transfected with pcDNA3.2-maspin, cDNA3.2-R340A or pcDNA3.2, were detected by A) anti-maspin or B) GAPDH.
Figure A2 Fast Protein Liquid Chromatography using maspin-pMTBiP conditioned media from stably expressing S2 cells. A) Chromatogram shows arbitrary units (mAU) representing measurement of UV wavelength (complete line) and electrical conductance (dotted line). Concentration gradient of imidazole buffer (dashed line). Step increase in concentration of imidazole (triangle); 20 mM wash fractions A1-A15, 50 mM elution fractions B10-B1, 100 mM elution fractions C1-C10, 150 mM elution fractions C11-C15/D15-D12 and 250 mM elution fractions D11-D6/D5-D1/E1-E10. Protein eluted from the column at fraction B6 and B8 (circle), large peak in electrical conductance (star). B) Chromatogram fractions identified by western blot using anti-maspin. Samples were not reduced.
Figure A3 Immunofluorescence showing microtubule organisation of stably transfected MCF7 cells. Cells were stained with anti-β tubulin. Slides were visualised using confocal microscope LSM Zeiss.
Figure A4  Graph showing proliferation of maspin expressing cells. DU-145 cells transiently transfected with pcDNA3.2-maspin or pcDNA3.2 were subject to Methanethiosulfonate assay. Methanethiosulfonate is reduced into a soluble product which is measured at 490nm. The formation of product is proportional to the number of viable cells. Statistical significance was measured by Students t-test (p*<0.05).

Figure A5  Immunostaining showing maspin expression. Cancerous prostatic cell lines LNCaP and PC3, and normal prostate epithelial cell line PNT1a were labelled with anti-maspin Alexa Fluor 488 (green) and cells were imaged with DIC III and DAPI (blue) overlay.
Arbitrary expression of integrin β4 or β5 mRNA for MCF7 stable cell lines as indicated was measured by RT-PCR. Two repeat experiments were performed in triplicate. Gene expression was standardised to the expression of housekeeping gene 18S.

Figure A6

Western blots showing integrin expression. 10 µg soluble cell lysate was collected from MCF7 stable cells expressing pcDNA3.2-maspin, pcDNA3.2-E244A or pcDNA3.2 cultured onto plastic. Blots were probed with A) anti-integrin α5 B) anti-integrin αV C) anti-integrin β1 D) anti-integrin β3 E) anti-GAPDH.

Figure A7
Figure A8 Western blots showing expression of ERK in MCF7 stable cells. 10 µg MCF7 soluble lysate was probed with anti-ERK (total) and anti-pERK (phosphorylated) and anti-GAPDH to show equal loading.
B Publications

B1  Bass R, Wagstaff L, Ravenhill L, Ellis V.  

References


