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The Transcriptional Regulation of Maspin

A thesis presented in partial fulfilment of the requirements for the degree
of Master of Science in Biochemistry at Massey University,
Palmerston North, New Zealand.

Andrew Hollings

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ABSTRACT

Maspin (mammary serine protease inhibitor) is a tumour suppressing member of the serpin superfamily. Maspin is expressed in normal breast and prostate cells, but reportedly down regulated during progression of cancer in these tissues. Maspin has been shown to inhibit cellular migration and invasion *in vitro*; while *in vivo,* maspin has been shown to inhibit tumour growth, metastasis, and angiogenesis. Maspin also plays a role in the sensitisation of cells to induced apoptosis. These functions of maspin are independent of serine protease inhibition; however the cellular mobility function is dependent on an intact reactive site loop. Despite this knowledge, the molecular mechanisms for all reported functions of maspin are currently unknown.

Maspin is reported to be transcriptionally regulated; to date Ets, Ap1, and p53 transcription factors have been shown to activate transcription of maspin by binding directly to the promoter. Androgen is reported to be a negative regulator through the binding of the androgen receptor to a hormone response element within the promoter. This hormone response element is also responsible for an increase in maspin expression in response to tamoxifen, an anti-oestrogen drug. Transcriptional regulation of maspin has also been reported to be activated by other molecules, including gamma linolenic acid, manganese containing super-oxide dismutase, and nitric oxide, the mechanisms of regulation by these molecules is unknown. Loss of maspin expression in cancerous cells lines has been attributed to loss of one or more of the activating factors, and aberrant methylation of cytosine residues resulting in chromatin compaction.

This study investigated the transcriptional regulation of maspin, with the aim of identifying transcriptional effectors important to the regulation of the gene. Identification of such factors may help identify a pathway in which maspin exerts its tumour suppressor functions. To this end, the maspin promoter was cloned and functional assays carried out, identifying several putative regions of the maspin promoter which may be important for the regulation of the gene. To date, the precise activator/repressor binding sites and the cognate proteins responsible for this regulation are unidentified.

ABBREVIATIONS

Amp	Ampicillin
Ap1	Activating protein-1
AR	Androgen receptor
ATP	Adenosine triphosphate
bFGF	basic fibroblast growth factor
β-gal	β-galactosidase
BLAST	Basic local alignment search tool
bp	base-pairs (DNA)
BSA	Bovine serum albumin
CM	Conditioned media
CMV	Cytomegalovirus
Contig	Contiguous sequence
CpG	Cytosine-guanine di-nucleotide
DCIS	Ductal carcinoma *in situ*
DNaseI	DeoxyribonucleaseI
dNTP	deoxy-nucleotide tri-phosphate
DTT	Dithiothreitol
E4BP4	E4 Binding Protein 4
E.coli	*Escherichia coli*
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra-acetic acid
EGF	Epidermal growth factor
EMSA	Electrophoresis mobility shift assay

eNOS	endothelial nitric oxide synthase
ER	Oestrogen receptor
Ets	E26 transformation-specific
FCS	Fetal calf serum
GCG	Genetics computer group sequence analysis software
GLA	Gamma linolenic acid
GR	Glucocorticoid receptor
HDAC	Histone deacetylase
HRE	Hormone response element
Hrs	Hours
iNOS	inducible nitric oxide synthase
kb	kilobase (DNA)
Klenow	*Klenow* fragment of *E.coli* DNA polymerase I
LB	Luria-Bertani
LMPCR	Ligation mediated PCR
MCS	Multiple cloning site
MeCP	Methyl-CpG binding protein
mMaspin	Mouse maspin
MnSOD	Manganese-containing super-oxide dismutase
mRNA	Messenger RNA
mt	Mutant
NO	Nitric oxide
ONPG	*o*-nitrophenyl-beta-D-galactopyranoside
ONP	*o*-nitrophenol
p53	53 kDa protein

PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate buffered saline
PC	Prostate cancer
PCR	Polymerase chain reaction
Pdef	Prostate derived Ets factor
Pen/Strep	Penicillin/streptomycin
PPAR	Peroxisome proliferator activated receptor
PR	Progesterone receptor
RNase	Ribonuclease
rpm	Revolutions per minute
RSL	Reactive site loop
sctPA	short chain tissue type plasminogen activator
SDS	Sodium dodecyl sulfate
SFM	Serum free medium
SNP	Single nucleotide polymorphisms
Sp1	Specificity protein 1
STS	Staurosporine
TAF	TBP-associated factor
TAg	SV-40 T-antigen
TBP	TATA binding protein
TESS	Transcriptional element search software
Tet	Tertacycline
TFIID	Transcription factor D for RNA polymerase II
tPA	tissue type plasminogen activator
TsAP	Thermostable alkaline phosphotase

TSS	Transcription start site
uPA	urokinase plasminogen activator
uPAR	urokinase plasminogen activator receptor
UTR	Un-translated region
UV	Ultra violet
V	Volts
VEGF	vascular epithelium growth factor
WAP	Whey acidic protein
wt	Wild type
YY1	Yin and Yang 1

LIST OF FIGURES

		Page Number
Figure 1.1	Maspin expression in human cell lines	2
Figure 1.2	Myoepithelial cells of the breast express maspin	3
Figure 1.3	Schematic representation of plasmin activation pathway	6
Figure 1.4	Reported regulatory elements contained in the maspin promoter	17
Figure 3.1	The human maspin promoter	48-49
Figure 3.2	2.7 kb maspin promoter PCR product and diagnostic digest	52
Figure 3.3	Schematic representation of cloning strategy	53
Figure 3.4	Gel quantification of insert and vector	54
Figure 3.5	Schematic diagram and gel of *Bgl* II digest of cloned promoter	57
Figure 3.6	Schematic diagram of sequencing primer positioning	58
Figure 3.7	Schematic diagram of deletion series creation	59
Figure 3.8	Digestion maps for the creation of deletion series	61
Figure 3.9	Example of a partial digest	62
Figure 3.10	Example of a gel extraction	63
Figure 3.11	Example of spectrophotometric DNA quantification	65
Figure 3.12	*Not* I and *Nco* I restriction map of the maspin-pGL3 constructs	65
Figure 3.13	Maspin promoter deletion series digested with *Nco* I and *Not* I	66
Figure 4.1	Schematic diagram of a transient transfection	69
Figure 4.2	Equation used to normalise transient transfection data	70
Figure 4.3	Reported elements within 500 bp of the human maspin TSS	71
Figure 4.4	Effect of increasing transfected reporter construct in HeLa cells	72
Figure 4.5	Relative activity of maspin promoter constructs in HeLa cells	73
Figure 4.6	Relative activity of the deletion constructs in MCF12a and MDA-MB-231 cell lines	75
Figure 4.7	*In silico* prediction of CpG island in the maspin promoter	77
Figure 4.8	Effect of methyltransferase inhibitor on reporter gene expression	78
Figure 4.9	Relative activity of deletion constructs in HPV-10 and PC3 cell lines	80
Figure 4.10	Schematic diagram of the -2251 bp to -1478 bp region	83
Figure 4.11	Schematic diagram of the -1078 bp to -963 bp region	85
Figure 4.12	Schematic diagram of the -660 bp to -263 bp region	86

Figure 4.13 Schematic diagram of the -134 bp to +180 bp region 87
Figure 5.1 Schematic diagram of DNaseI footprinting 92
Figure 5.2 Schematic diagram of the *in vivo* footprinting method 94
Figure 5.3 Alkaline gel of DNaseI digested DNA 95
Figure 5.4 Schematic diagram of primers used in LMPCR 96
Figure 5.5 Footprinting probe PCR gel 98
Figure 5.6 Ligation of linker to digested PCR product 100
Figure 5.7 Gel photograph of the PCR of primer extension controls 102
Figure 5.8 Photograph of footprinting PCR gel 104
Figure 5.9 The effect of increased PCR cycles on footprinting banding pattern 105
Figure 6.1 Summary of transient transfection experiments 110
Figure 6.2 Schematic diagram of an electrophoretic mobility shift assay 113
Figure 6.3 Schematic diagram of the creation of a mutated transcriptional effector binding site 114
Figure 6.4 Schematic diagram of the creation of an internal deletion 115

LIST OF TABLES

Page Number

Table 2.1 Media and additives used for growth of cells 36

Table 3.1 Results of ligated DNA transformation 55

Table 3.2 Creation of deletion series 60

Table 4.1 Change in reporter gene expression between -660 bp and -295 bp constructs 85

TABLE OF CONTENTS

Page Number

Acknowledgements....................i

Abstract....................ii

Abbreviations....................iii

List of Figures....................vii

List of Tables....................ix

Table of Contents....................x

Chapter 1 - Introduction....................1

1.1 Cancer....................1

1.2 Maspin....................2

1.2.1 Expression of maspin....................2

1.2.2 Maspin as a tumour suppressor....................3

1.3 Function of maspin....................5

1.3.1 Protease inhibition by maspin....................5

1.3.2 Cellular mobility....................7

1.3.3 Role of maspin in apoptosis....................9

1.3.4 Maspin and angiogenesis....................10

1.3.5 Phosphorylation of maspin....................11

1.4 Clinical studies....................12

1.4.1 Prognostic value....................12

1.4.2 Breast carcinomas....................12

1.4.3 Prostate carcinomas....................13

1.4.4 Other carcinomas....................14

1.5 Regulation of maspin expression....................15

1.5.1 Regulation of RNA polymerase II genes....................15

1.5.2 Transcriptional regulation of maspin....................16

1.5.3 Ets transcription factor....................17

1.5.4 Ap1....................18

1.5.4 p53....................19

1.5.5 Hormone response element....................20

1.5.6 Other regulatory molecules....................21

1.5.7 CpG methylation....................23

1.6 Chapter summary...24
1.7 Research aims...26
Chapter 2 - Materials and Methods ...27
2.1 Materials...27
2.1.1 DNA polymerases...27
2.1.2 Restriction endonucleases...27
2.1.3 Other enzymes...27
2.1.4 Cell culture ...27
2.1.5 Footprinting reagents...28
2.1.6 Vectors...28
2.1.7 *E.coli* strains...29
2.1.8 Other specialist materials...29
2.2 DNA modification and purification...30
2.2.1 PCR...30
2.2.2 Restriction endonuclease digests...30
2.2.3 Creation of blunt ends...30
2.2.4 Agarose gel electrophoresis...30
2.2.4 Removal of phosphate groups...31
2.2.5 Gel extraction...31
2.2.6 Column purification of DNA...31
2.2.7 Phenol chloroform extraction...32
2.2.8 Ethanol precipitation...32
2.2.9 Plasmid DNA Maxi Prep...32
2.2.10 DNA quantification...33
2.2.11 DNA sequencing...33
2.3 Transformation of *E.coli*...34
2.3.1 *E.coli* culture...34
2.3.2 'Growth on the day' competent cells...34
2.3.3 Ligation...34
2.3.4 Transformation...35
2.3.5 Rapid boil DNA preparation...35
2.3.6 Preparation of glycerol stocks...35
2.4 Mammalian cell culture...36
2.4.1 Maintenance of cell lines...36

2.4.2 Media.. 36

2.4.3 Establishing cells from frozen stocks........ 37

2.4.4 Passage of cells.............................. 37

2.4.5 Freezing cells................................ 37

2.4.6 Transient transfection........................ 38

2.4.7 Statistical analysis.......................... 39

2.5 *In vivo* DNaseI footprinting........................ 40

2.5.1 Nuclei isolation.............................. 40

2.5.2 DNaseI digestion.............................. 40

2.5.3 Alkaline gel.................................. 41

2.5.4 Primer extension.............................. 41

2.5.5 Linker preparation............................ 42

2.5.6 Ligation...................................... 42

2.5.7 Product capture............................... 42

2.5.8 PCR... 43

2.5.9 Capture of sequencing template................ 43

2.5.10 Sequencing ladder production................. 43

2.5.11 Gel electrophoresis.......................... 44

2.5.12 Transfer..................................... 44

2.5.13 Probe synthesis.............................. 45

2.5.14 Hybridisation................................ 45

2.5.15 Washes and detection......................... 45

Chapter 3 - Cloning of the Maspin Promoter...46

3.1 Identification of the human maspin promoter.............. 46

3.1.1 Analysis of maspin promoter.................. 46

3.2 Cloning of the maspin promoter............................ 50

3.2.1 Primer design................................ 50

3.2.2 Amplification of promoter.................... 51

3.2.3 Cloning...................................... 53

3.2.4 Sequencing of the maspin promoter........... 58

3.3 Creation of maspin promoter deletion series............... 59

3.3.1 Strategy..................................... 59

3.3.2 Sub cloning.................................. 63

3.3.3 Confirmation of 5' ends...................... 64

3.3.4 Construct Production........................... 64

3.4 Chapter summary... 67

Chapter 4 - Transient Transfections.. 69

4.1 Overview.. 69

4.2 HeLa cell transfections......................................72

4.2.1 Controls..72

4.2.1 Transfections....................................73

4.3 Breast cell lines... 74

4.3.1 Transfections....................................74

4.3.2 DNA methylation...............................77

4.4 Prostate cell lines.. 79

4.4.1 Transfections....................................79

4.5 Hormone response element.................................. 81

4.6 TESS interpretation of transient transfections.............. 82

4.6.1 -2551 to -2234 bp region...................... 83

4.6.2 -2234 to -1478 bp region...................... 84

4.6.3 -1078 to -963 bp region....................... 84

4.6.4 -660 to -295 bp region......................... 85

4.6.5 -295 to -263 bp region......................... 87

4.6.6 -134 bp to transcriptional start site........... 87

4.6.7 TESS summary................................. 88

4.6 Chapter summary... 89

Chapter 5 - *In vivo* DNaseI Footprinting... 91

5.1 Introduction.. 91

5.1.2 *In vivo* DNaseI footprinting technique....... 93

5.1.3 *In vivo* verses *in vitro* footprinting............ 93

5.2 *In vivo* footprinting method................................. 95

5.2.1 DNaseI digestion............................... 95

5.2.2 Amplification of footprint......................96

5.2.3 Detection of footprint.......................... 98

5.3 Controls...99

5.3.1 Ligation..99

5.3.2 Primer extension...............................101

5.3.3 Product capture................................ 102

5.3.4 PCR.....103

5.5 Chapter Summary..... 106

Chapter 6 - Discussion and Future Work.....108

6.1 Summary of the current knowledge of maspin..... 108

6.2 Summary of results..... 109

6.2.1 *In silico* studies..... 109

6.2.2 Cloning and functional analysis.....110

6.2.3 DNaseI footprinting.....111

6.3 Future work..... 112

6.3 Conclusion..... 116

References.....117

Appendix.....125

Appendix 1 - BLAST alignment of maspin mRNA with human genomic contig..... 126

Appendix 2 - TESS analysis of maspin 8 kb promoter.....130

Appendix 3 - BLAST alignments.....149

3.1 Rat and human maspin promoters.....149

3.2 Mouse and human maspin promoters..... 151

Appendix 4 - Primers.....153

4.1 Cloning primers.....153

4.2 Sequencing primers..... 153

4.3 Footprinting primers.....154

4.3.1 -2551 bp to -2251 bp region..... 154

4.3.2 -1078 bp to -963 bp region..... 154

4.3.3 -660 bp to -295 bp region..... 155

4.3.4 -134 bp to TSS region.....155

Appendix 5 - Vector maps..... 156

5.1 pGL3 Basic..... 156

5.2 pSV-β-Galactosidase.....156

5.3 pCMV-βgal..... 157

Appendix 6 - Restriction map of 2.7 kb maspin promoter fragment..... 158

Appendix 7 - Sequencing data.....161

Appendix 8 - Alignment of cloned and human genome derived maspin promoters..... 164

Appendix 9 - Transfection example data..168

Appendix 10 - Transient transfection results 169

10.1 HeLa...170

10.2 MCF12a...171

10.3 MDA-MB-231...172

10.4 PC3...173

10.5 CA-HPV-10...174

10.6 Breast cell line comparison...175

10.7 Prostate cell line comparison...175

10.8 MDA-MB-231 5-azacytidine co-transfections..................................176

CHAPTER 1 - Introduction

1.1 Cancer

Cells normally have a finite lifespan, and progress through development and differentiation to senescence in a regulated and defined manner. Cancer represents the situation where cells have lost this regulation and tend to proliferate in an uncontrolled manner. Cancer can be caused by multiple genetic and epigenetic changes, resulting in decreased expression or inactivation of tumour suppressor genes, and the increased expression or activation of oncogenes. The transformation from benign to malignant tumour is an important step in cancer progression. This step involves the gain of an invasive phenotype, i.e. the ability to invade neighbouring tissues. Malignant tumours can then become metastatic, characterised by the spreading of cancerous cells through the lymphatic or vascular system to other organs and tissues. Growth of tumours at distant sites follows, ultimately resulting in organ failure and death (DeVita et al., 2001).

In New Zealand, prostate cancer was the most common form of cancer diagnosed in 1999 at 98.2 diagnoses per 100,000 people per year, breast cancer followed with 87.6 diagnoses per 100,000 people per year. Breast and prostate cancer had the second and fourth highest mortality rates respectively in 1999 (NZHIS, 2002). Research world-wide is aimed at understanding the mechanisms of cancer development and progression, with the ultimate goal being the cure and prevention of cancer.

1.2 Maspin

1.2.1 Expression of maspin

Maspin (mammary serine protease inhibitor) was initially discovered by subtractive hybridisation between normal and cancerous breast cell lines, with maspin being expressed in normal breast cell lines, whilst down-regulated in breast cancer cell lines (Zou et al., 1994). Expression of maspin has since been found in cells of epithelial origin; in the mouth, airway, skin, breast, and prostate and also in testis, thymus, and small intestine (Zhang et al., 1997a; Futscher et al., 2002). In addition, expression of maspin has been reported to be progressively down-regulated as cancers of the breast and prostate progress (Zou et al., 1994; Hojo et al., 2001; Maass et al., 2001a; Maass et al., 2001c). Supporting this, the normal breast cell line MCF12a expresses maspin, whilst cancerous breast cell lines MDA-MB-231, MCF7 and T47D do not (figure 1.1) Interestingly, the prostate cancer cell lines PC3 and CA-HPV-10 both express maspin, as does the cervical cancer cell line HeLa (see figure 1.1). Hepatocyte carcinoma HepG2, lymphoblast K562, and colon cancer CaCO-2 cell lines do not express maspin (Bradbury, 2004).

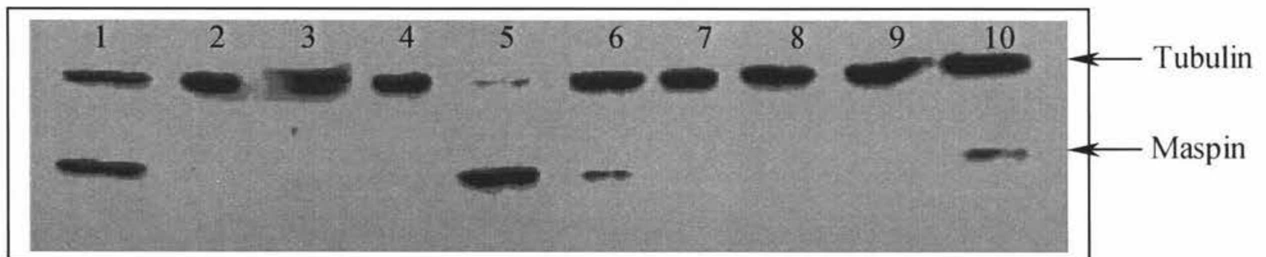

Figure 1.1. Maspin expression in human cell lines. Cells were grown to ~80% confluence and whole cell protein extracted. Protein extracts were then subjected to electrophoresis on an acrylamide gel in presence of SDS. Proteins were then transferred to nylon membrane and probed for maspin and tubulin expression. 1 = MCF12a, 2 = MCF7, 3 = MDA-MB-231, 4 = T-47D, 5 = CA-HPV-10, 6 = PC-3, 7 = HepG2, 8 = K562, 9 = CaCO-2, 10 = HeLa. Western blot courtesy of Dr Penny Bradbury (Bradbury, 2004)

1.2.2 Maspin as a tumour suppressor

Maspin has been reported to be a tumour suppressor gene, whose function is to inhibit cellular mobility, angiogenesis, and increase stress-induced apoptosis *in vitro* and *in vivo* (Zou et al., 1994; Pemberton et al., 1995; Sheng et al., 1996; Sternlicht et al., 1997; Seftor et al., 1998; Zhang et al., 1999; Zhang et al., 2000b; Zou et al., 2000; Jiang et al., 2002; Shi et al., 2002). Inhibition of cellular mobility can be affected by the addition of recombinant maspin to cultured cells, while apoptosis requires endogenously expressed maspin (Sheng et al., 1994; Zou et al., 1994; Sheng et al., 1996; Jiang et al., 2002).

Myoepithelial cells of the breast are considered to be a protective cell layer (Sternlicht et al., 1997; Reis-Filho et al., 2002). These cells surround the ductal cell layer (figure 1.2) and secrete high levels of many serpins, including maspin, which may act as paracrine inhibitors of tumour growth (Sternlicht et al., 1997).

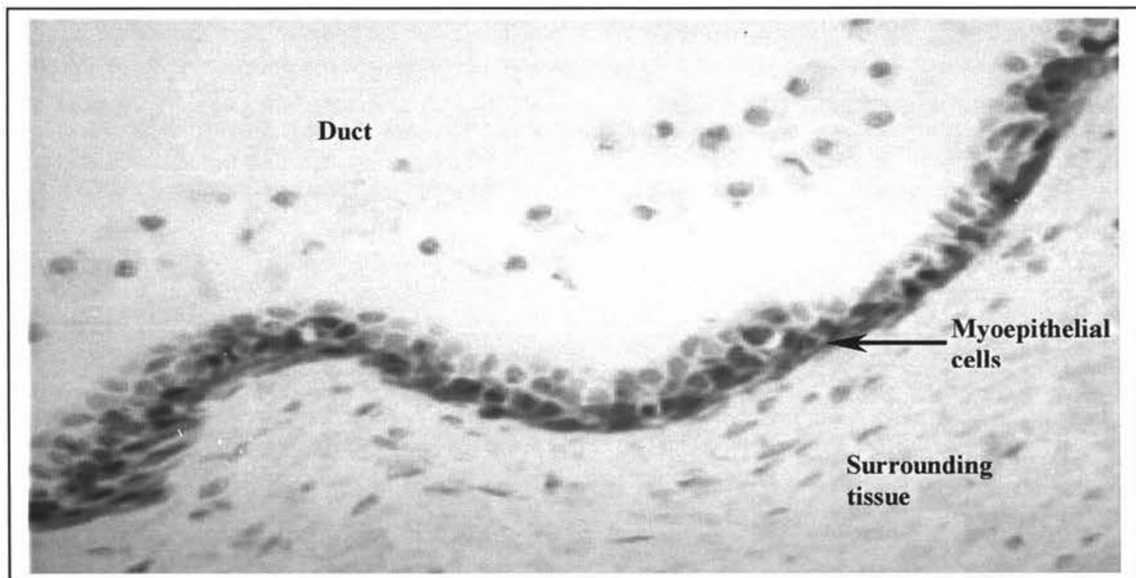

Figure 1.2. Myoepithelial cells of the breast express maspin. The myoepithelial cell layer expresses high levels of maspin as shown by brown staining with anti-maspin antibody. The myoepithelial cell layer separates the ducts of the breast from the surrounding tissue. Figure courtesy of Dr Penny Bradbury (Bradbury, 2004).

Maspin is mainly present in the cytosol, but also contains an N-terminal signal sequence which causes a small amount to be packaged into microsomes and secreted, where it acts to inhibit migration and invasion (Zou et al., 1994; Sheng et al., 1996; Pemberton et al., 1997; Sternlicht et al., 1997). Although controversial, nuclear localisation of maspin has also been demonstrated using immunohistochemistry and sub-cellular fractionation. Interestingly some tumour cells showed only nuclear localisation of maspin (Maass et al., 2002b; Odero-Marah et al., 2002; Reis-Filho et al., 2002; Sood et al., 2002; Khalkhali-Ellis and Hendrix, 2003; Kim et al., 2003; Mohsin et al., 2003; Khalkhali-Ellis et al., 2004). To date, the function of nuclear maspin is unknown.

Mouse and rat homologues of human maspin have been isolated, and at the amino acid level show 89% and 88% identity respectively to human maspin (Umekita et al., 1997; Zhang et al., 1997c). Mouse, rat, and human maspin also show similar expression patterns and are down-regulated in cancerous cells (Umekita et al., 1997; Zhang et al., 1997c). Mouse maspin (mMaspin) was shown to exhibit similar characteristics as human maspin: reactive site loop-dependent inhibition of cellular migration and invasion *in vitro* (discussed in 1.3.2) (Zhang et al., 1997c). Rat maspin, however did not show tumour suppressive properties *in vivo* (Umekita et al., 1997).

1.3 Function of maspin

1.3.1 Protease inhibition by maspin

As suggested by its name, maspin shares a high level of identity to the serine protease inhibitor (serpin) super-family (Hopkins and Whisstock, 1994; Zou et al., 1994). Serpins act by presenting the highly conserved reactive site loop (RSL) to the target protease, which recognises and cleaves the RSL. This cleavage results in the protease and serpin becoming trapped in a 1:1 heat and detergent resistant, covalently bound complex (Bass et al., 2002). The P_1 residue of the RSL determines specificity of the serpin for the type of serine protease. Maspin contains an arginine at this residue indicating that it is an inhibitor of trypsin-like proteases (Zou et al., 1994).

It has been suggested that maspin is not an inhibitory serpin i.e. does not inhibit serine proteases, as the hinge region of the RSL is highly divergent from known inhibitory serpins. The RSL also contains a deletion, leaving maspin with the shortest RSL of all serpins (Hopkins and Whisstock, 1994; Pemberton et al., 1995; Fitzpatrick et al., 1996; Bass et al., 2002). Maspin was shown to be unable to go through the stressed to relaxed transition, which is characteristic of inhibitory serpins, also indicating that maspin is a non-inhibitory serpin (Pemberton et al., 1995). Finally, maspin does not inhibit a range of trypsin-like proteases, in fact maspin acts as a substrate for these proteases (Pemberton et al., 1995; Bass et al., 2002).

Despite the weight of evidence that maspin is a non-inhibitory serpin, controversy still remains. Maspin was demonstrated to inhibit urokinase-plasminogen activator (uPA) and tissue-type plasminogen activator (tPA). uPA and tPA convert plasminogen into active plasmin, which in turn degrades the extracellular matrix (ECM) (figure 1.3). Thus uPA and tPA cause an increase in cellular mobility (Sheng et al., 1998; Biliran and Sheng, 2001). Interestingly, maspin activated the activity of tPA when a co-factor (fibrinogen or poly-lysine) was not bound however, maspin acted as a competitive inhibitor when a co-factor was bound to tPA (Sheng et al., 1998).

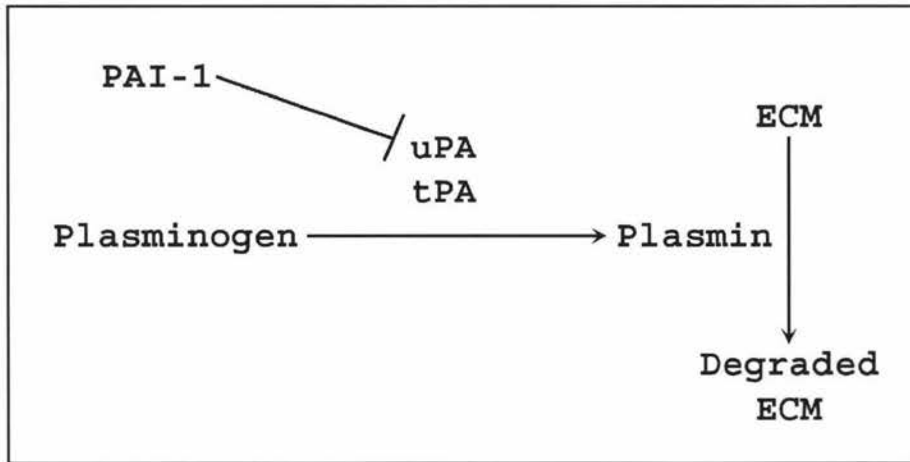

Figure 1.3. Schematic representation of plasmin activation pathway. Plasminogen is cleaved into active plasmin by uPA and tPA, which are inhibited by the serpin PAI-1. Active plasmin causes the degradation of the extracellular matrix, and thus an increase in cellular mobility.

uPA was also shown to be inhibited only when associated with the cell surface. The levels of uPA and uPA Receptor (uPAR) were shown to be decreased in cells expressing maspin. The authors suggested that this decrease was due to increased endocytosis and degradation of a maspin-uPA-uPAR complex (Sheng et al., 1998; Biliran and Sheng, 2001). The inhibition of uPA and tPA dependent proteolysis of plasminogen by maspin may decrease the amount of active plasmin, thus resulting in a decrease in ECM degradation and cellular migration (Sheng et al., 1998; Biliran and Sheng, 2001). uPAR is also known to modify the integrin profile of a cell, which may also play a role in inhibition of cellular mobility (discussed in 1.3.2) (Biliran and Sheng, 2001).

The inhibition of uPA and tPA, and the decrease in uPA and uPAR may explain how maspin inhibits cellular migration and invasion, however several publications dispute these findings (Zhang et al., 1999; Bass et al., 2002). Bass, et al., (2002) demonstrated that maspin does not inhibit cellular mobility by inhibiting proteolysis, and that maspin is a non-inhibitory serpin. Under a wide range of conditions, in which plasminogen activator inhibitor-1 (PAI-1) (figure 1.3), a serpin with a high level of identity to maspin, is an effective inhibitor, maspin showed no detectable inhibition of proteolysis (Bass et al., 2002). PAI-1 inhibits both cell-surface bound and free uPA with similar kinetics, indicating that the cell-surface is not required for the inhibitory activity of this serpin. Maspin then, would also be expected to show little difference between bound

and non-bound inhibition (Bass et al., 2002). Also, the competitive inhibition shown by Sheng, et al., (1998) is not a recognised mechanism of serpin action (Bass et al., 2002)

Taken together the data indicate that maspin is not an inhibitory serpin, and that the inhibition of migration and invasion is not dependent on protease inhibitory activity.

1.3.2 Cellular mobility

For a cell to gain the ability to invade, it must modify cell-cell and cell-ECM adhesions (DeVita et al., 2001). Proteolysis of the ECM and basement membrane is important for the invasion of tumour cells into surrounding tissues. This is carried out by many different proteases including metalloproteases and serine proteases (Seftor et al., 1998; DeVita et al., 2001; Liotta and Kohn, 2001). Modification of the integrin profile of a cell, which mediates the interactions with the ECM, changes the ability of the cell to interact with and perceive its environment; thus affecting signal transduction, and can lead to the acquisition of an invasive phenotype (Seftor et al., 1998; DeVita et al., 2001).

Maspin has been shown to inhibit the migration of cancerous cells across a membrane, and invasion through a membrane *in vitro* (Sheng et al., 1994; Zou et al., 1994; Sheng et al., 1996). Both endogenously expressed, and purified recombinant maspin have been shown to inhibit cellular mobility. Maspin exerts this effect at the plasma membrane, and cellular mobility can be restored to normal levels by addition of maspin monoclonal antibody (Sheng et al., 1994; Zou et al., 1994; Sheng et al., 1996). An antibody to the RSL of maspin prevents maspins inhibition of migration and invasion only when pre-incubated with, or added to the migration assay concurrently with recombinant maspin. This indicates that the maspin RSL is buried, and necessary for migration inhibition (Sheng et al., 1996). Cleavage at the P_1 position of the maspin RSL (thereby removing RSL) abrogates the migration inhibitory activity of maspin (Sheng et al., 1994; Pemberton et al., 1995; Sheng et al., 1996; Zhang et al., 2000b). Thus the RSL is necessary for inhibition of cellular mobility.

Myoepithelial cell lines have been shown to inhibit invasion and migration of cancerous cells. Myoepithelial cell conditioned media (CM) or co-culture has been shown to

reduce invasion and migration of cancerous cell lines *in vitro*. Immunoprecipitation of maspin from CM abrogates cancer cell mobility inhibition (Sternlicht et al., 1997; Shao et al., 2000). This suggests that myoepithelial cells secrete maspin, which inhibits the invasion of cancerous cells in a paracrine manner.

Inhibition of cellular migration and invasion has also been shown *in vivo* (Zou et al., 1994; Zhang et al., 1999; Zhang et al., 2000a; Zhang et al., 2000b; Shi et al., 2002). Maspin expressing cancerous cells implanted into nude mice, showed reduced tumour growth, and number of metastatic sites. This effect was also seen if maspin was delivered as a recombinant protein, or as an expression vector in liposome mediated gene therapy (Zou et al., 1994; Zhang et al., 2000b; Shi et al., 2002).

Transgenic mice containing maspin under the influence of the whey acidic protein (WAP) promoter, which is expressed exclusively in mammary epithelial cells during mid-pregnancy and lactation, showed disrupted mammary gland development (Zhang et al., 1999). This was due to the inability of the cells to migrate, and increased apoptosis (Zhang et al., 1999). Mice containing the SV-40 T-antigen under the control of the WAP promoter (WAP-TAg) produce mammary tumours with 100% efficiency. These mice were crossed with WAP-maspin mice (Zhang et al., 2000a). When compared to WAP-TAg mice, WAP-TAg:WAP-maspin bi-transgenic mice showed a reduction in tumour growth, and number of lung metastases, however maspin did not affect tumour initiation (Zhang et al., 2000a). Thus endogenously expressed maspin inhibits tumour growth and migration to metastatic sites *in vivo*.

Addition of recombinant maspin to non-expressing cancerous cells has been shown to change the integrin profile of these cells (Seftor et al., 1998). Cells expressing maspin had increased levels of α_3 and α_5 containing integrins, whilst decreased levels of other integrin monomers was observed. This addition of maspin inhibited cellular migration, which was reversed by the addition of $\alpha_5\beta_1$ integrin antibody (Seftor et al., 1998). Maspin has also been demonstrated to interact with collagen type I and III (Blacque and Worrall, 2002). Binding to collagen was within a region of the maspin protein which shares homology to the collagen binding protein colligin, and was independent of the RSL (Blacque and Worrall, 2002). These reports may help explain how maspin inhibits cellular migration and invasion; however the mechanism of action is unknown.

Taken together these data suggest that maspin can inhibit cellular migration and invasion *in vitro* and *in vivo*; and can act in an autocrine and paracrine manner. The molecular mechanisms responsible for the inhibition of cellular mobility are as yet unknown; however they may involve binding to collagen and/or modification of the cellular integrin profile. Inhibition of cellular mobility by maspin is dependent on an intact RSL, however does not involve inhibition of serine proteases (discussed in 1.3.1).

1.3.3 Role of maspin in apoptosis

Cells expressing maspin have been shown to be more sensitive to stress-induced apoptosis (Jiang et al., 2002; Khalkhali-Ellis and Hendrix, 2003). In breast cancer cells, increased levels of nitric oxide (NO) induced maspin expression and an increase in apoptosis. This increase in apoptosis was partially reduced by the addition of maspin antibody (Khalkhali-Ellis and Hendrix, 2003). NO pro-apoptotic effects are believed to be due to an increase in p53, activation of the caspase cascade, and DNA fragmentation (Khalkhali-Ellis and Hendrix, 2003). Supporting these suggestions Jiang, et al., (2002) demonstrated that maspin expressing cells were more sensitive to staurosporine (STS)-induced apoptosis, due to increased caspase activation. (Jiang et al., 2002). Protein kinase C prevents caspase-dependent apoptosis, STS induces apoptosis by inhibition of this enzyme. Maspin was shown to increase levels of caspase 3 and caspase 8 activity upon STS treatment; this was associated with increased DNA fragmentation and apoptosis. Increased apoptosis was not seen in untreated maspin expressing cells, indicating that maspin did not increase apoptosis directly, but sensitised cells to apoptotic inducers (Jiang et al., 2002).

PAI-1, a close relative of maspin, is an inhibitory serpin which inhibits the plasminogen activator-dependent proteolysis of plasminogen into active plasmin. This activation of plasmin results in the increased degradation of the ECM. Thus PAI-1 is an inhibitory serpin which inhibits cellular migration and invasion. Recombinant maspin, conditioned media, and N- and C-terminal maspin:PAI-1 fusion proteins did not sensitise cells to induced apoptosis, indicating that this is a function of intracellular maspin, and dependent on an intact protein (Jiang et al., 2002). Both N- and C-terminal maspin:PAI-1 fusion proteins inhibited cellular mobility, indicating that the fusion proteins were correctly folded and functional.

An increase in apoptosis in tumour cells when expressing maspin was observed in the WAP-TAg/WAP-maspin, bi-transgenic mouse model (see Chapter 1.3.2), when compared to the WAP-TAg single transgenic mouse (Zhang et al., 2000a). Also the WAP-maspin single transgenic mice were shown to have disrupted mammary gland development during pregnancy. This was due to the inappropriate expression of maspin causing a decreased rate of migration and increased apoptosis (Zhang et al., 1999). The cause of this increased rate of apoptosis in WAP-maspin mice is unknown, however it may be due to inappropriate integrin-ECM contacts/signalling due to inhibition of migration (Zhang et al., 1999). Maspin expressing cells may be more sensitive to apoptosis caused by such inappropriate integrin interactions.

Taken together, maspin sensitises cells to stress-induced apoptosis *in vitro* and *in vivo*. Maspin increases the activation of caspase 3 and 8, resulting in increased DNA fragmentation, and cell death. This function seems to be dependent on intact intracellular maspin. The molecular mechanism of the sensitisation of cells to induced apoptosis is currently unknown.

1.3.4 Maspin and angiogenesis

Angiogenesis is the development of new blood vessels from existing capillaries (Beecken et al., 2000). Tumour growth is angiogenesis-dependent, as cells in the centre of a tumour become hypoxic due to lack of nutrients and oxygen. This hypoxia causes proliferation and apoptosis of tumour cells to be in balance (Beecken et al., 2000). Metastasis is also angiogenesis-dependent as tumours require access to vessels to be able to spread to other organs (Beecken et al., 2000).

Maspin, independently of the RSL, inhibited the ability of prostate cancer cells to migrate toward vascular epithelium growth factor (VEGF), a stimulator of angiogenesis *in vitro* (Zhang et al., 2000b). Prostate cancer cells, grafted into nude mice, were supplied with exogenous maspin and the effect on tumour growth and vascularization measured. Maspin reduced the size of tumours and the density of vascularization compared to controls (Zhang et al., 2000b). In corneal neovascularization experiments, pellets containing basic fibroblast growth factor (bFGF), an angiogenesis stimulator, and maspin (mutant and normal) were implanted into rat corneas. Maspin, and maspin

lacking the RSL inhibited the formation of vessels, whilst maspin lacking the N-terminus had no effect (Zhang et al., 2000b). Thus as bFGF acts as a paracrine angiogenesis stimulator, maspin with an intact N-terminus, independent of the RSL, acts as a paracrine angiogenesis inhibitor. This report is supported by WAP-TAg/WAP-maspin bi-transgenic mice which showed a decrease in tumour vascular density (Zhang et al., 2000a). In addition, a clinical study carried out by Hojo, et at., (2001) demonstrated that vascularization was significantly inhibited in breast tumours expressing maspin.

These reports indicate that maspin acts as an inhibitor of angiogenesis *in vivo*. Maspin can exert this effect in a paracrine manner, and is independent of the RSL. Blacque and Worrall, (2002) showed that maspin could bind collagen I and III independently of the RSL. This binding may play a role in the inhibition of angiogenesis; however its significance is yet to be demonstrated.

1.3.5 Phosphorylation of maspin

Maspin has also been shown to be phosphorylated on tyrosine residues (Odero-Marah et al., 2002). Phosphorylation of tyrosine residues can be important for enzyme activity and/or signal transduction. Maspin was shown to be phosphorylated in normal and cancerous cell lines, and was phosphorylated by the tyrosine kinase domain of the epidermal growth factor receptor (Odero-Marah et al., 2002). The potential change of function upon phosphorylation of maspin was not studied. The phosphorylation of maspin may be involved in activation or inhibition of the protein; or may be part of a signal transduction pathway.

1.4 Clinical studies

1.4.1 Prognostic value

Maspin has been reported to be present in normal breast and prostate tissue but down-regulated, or lost in cancers of these tissues (Zou et al., 1994; Hojo et al., 2001; Maass et al., 2001a; Maass et al., 2001c). This, along with the reported tumour suppressor functions of maspin suggest that the loss of maspin may contribute to the progression of cancer. Initial studies indicated that major mutations/rearrangements of the maspin gene did not contribute to the loss of maspin, indicating that maspin was down-regulated rather than mutated (Zou et al., 1994). Many clinical studies have been carried out testing the prognostic value of maspin in several different cancer types. If maspin is not mutated, and loss of maspin is important for the progression of cancer, then maspin expression in cancers may be a prognostic marker for a less aggressive cancer.

1.4.2 Breast carcinomas

Many studies have been carried out on maspin expression in breast cancers. Several studies have shown that maspin is down-regulated during breast cancer progression (Zou et al., 1994; Maass et al., 2001c). Maass, et al., (2001c) showed a stepwise decrease in maspin expression as cancers progressed from ductal carcinoma *in situ* (DCIS) to invasive carcinoma to lymph node metastases; however a subset of invasive tumours were shown to express high levels of maspin. Another study by Maass, et al., (2001a) indicated that loss of maspin correlated with a higher risk of distant metastasis and shorter disease free survival. These results were supported by Hojo, et al., (2001).

In other reports, maspin expression has been correlated with poor prognosis (Umekita et al., 2002; Umekita and Yoshida, 2003). A step wise increase from DCIS to invasive cancer was seen (Umekita and Yoshida, 2003). Maspin expression correlated with larger tumour size, higher histological grade, and shorter disease free survival, and may have been related to an aggressive phenotype (Umekita et al., 2002; Umekita and Yoshida, 2003)

Mohsin, et al., (2003) studied nuclear and cytoplasmic maspin staining, and breast cancer progression; 96% of cancers stained for maspin showed nuclear staining, whilst 35% showed cytoplasmic staining. Nuclear staining was associated with good prognosis, and presence of oestrogen and progesterone receptor (ER and PR respectively). Cytoplasmic staining was correlated with the absence of ER and PR; and poor prognosis (Mohsin et al., 2003).

Another study showed that maspin was frequently associated with a higher histological grade, similar to the subset of invasive tumours found by Maass, et al., (2001c) which showed strong maspin staining (Kim et al., 2003). The histological grade of a tumour is influenced by the percent of tubular structures; size and shape of cells (uniformity); and rate of growth as determined by number of cells undergoing mitosis. Tumours are graded from 1 to 3, a tumour with a higher grade is more aggressive, faster growing, with poorly differentiated cells (www.imaginis.com). Kim et al., (2003) investigated 192 specimens, with 13% of tumours stained having high expression of maspin. Nuclear and cytoplasmic staining was noted. Maspin staining was not of prognostic value, and differed between types of breast cancer (Kim et al., 2003).

Maspin staining in breast cancers is controversial. Different methods of staining, quantification of staining, nuclear and cytoplasmic staining, and statistical analyses may be the cause of some of these reported differences. The contradictory nature of these results suggests that more large studies need to be carried out to determine if maspin expression in breast cancers is of any prognostic value.

1.4.3 Prostate carcinomas

Several clinical studies on prostate cancers (PC) have been carried out. Machtens, et al., (2001) showed that a decrease in maspin expression correlated with the loss of cellular differentiation, increased tumour grade, and shorter disease free survival. Zou, et al., (2002) also showed that a decrease in maspin expression correlates with dedifferentiation of the tumour.

Pierson et al., (2002) demonstrated that prostate epithelial cells express maspin; however the secretory cells from which PC arises did not. Interestingly the pre-

malignant prostate tumours, and surrounding cells, were shown to express maspin (Pierson et al., 2002). Thus maspin is up-regulated in the pre-cancerous stage of prostate cancer development. The pre-malignant stage can preceed PC by up to 10 years, and Pierson, et al., (2002) suggested that this up-regulation of maspin may prolong the pre-malignant stage, and may act as a marker for potential PC development. This report also demonstrated that once past the pre-malignant stage, maspin expression inversely correlated with prostate cancer tumour grade, and was down-regulated step-wise as cancer progresses (Pierson et al., 2002).

Maspin expression in prostate cancers is less controversial than in breast cancers, with all reports to date indicating that maspin expression correlates with higher grade PC. This may be due to fewer reports on maspin expression in prostate cancers. The up-regulation of maspin in pre-malignant prostate cancers may be a cellular response to tumourigenic changes, however whether maspin up-regulation in the pre-malignant to PC transition is a direct or indirect cellular response needs further investigation.

1.4.4 Other carcinomas

Studies in cancers of other tissues have also been conducted. In oral squamous cell carcinomas, maspin expression correlated with higher survival rates, and absence of lymph node metastases (Xia et al., 2000). This report also suggested that maspin expression may be a favourable prognostic marker in this type of cancer.

High levels of maspin expression have been shown in most pancreatic cancers, whilst normal pancreatic cells do not express maspin (Maass et al., 2001b; Ohike et al., 2003; Sato et al., 2003). In tumour specimens studies by Maass, et al., (2001b), 23 out of 24 expressed maspin; while 51 out of 57 tumours studied by Ohike et al., (2003) expressed maspin. Maspin expression was correlated with low grade (grade1) pancreatic cancers (Ohike et al., 2003). Maass, et al., (2001b) showed that maspin expression increased with the malignancy grade of the tumour.

A similar trend in maspin expression was seen in ovarian cancers (Sood et al., 2002). Maspin expression was not found in normal ovary tissue, whilst maspin was present in 71% of ovarian invasive cancers, and was correlated with a higher tumour grade.

Interestingly cancers with greater than 50% nuclear staining were associated with improved patient survival (Sood et al., 2002). Three mutations in maspin were reported in two ovarian cancerous cell lines. One of these mt-maspin cell lines was transfected with wt-maspin, resulting in inhibited cellular migration, which was abrogated by addition of maspin antibody (Sood et al., 2002). This suggests that the mt-maspin expressed in this cell line is not functional in cellular mobility inhibition. This raises the possibility that maspin may be mutated in ovarian cancers. Maspin mutations have also been reported in prostate cancer cell lines (Umekita et al., 1997)

The tumour suppressive function of maspin seems to be supported by its down-regulation in prostate and oral carcinomas, while the case for maspin down-regulation is not so clear cut in breast cancers. Up-regulation of maspin in ovarian and pancreatic cancers however, does not support maspin as a tumour suppressor. Pancreatic and ovarian cells may not be responsive to maspin i.e. interacting partners may not be present in these tissues; however maspin is up-regulated in a large proportion of pancreatic and ovarian cancers, which indicates expression of maspin is an advantage to these cancers. It is possible that maspin expressed in these cancers is mutated. This mutant form may carry out a function facilitating cancer progression. Few maspin mutations have been reported to date, and so little is known about the frequency of such mutations in cancers and their effect on cancer cells.

1.5 Regulation of maspin expression

1.5.1 Regulation of RNA polymerase II genes

Eukaryotes contain three RNA polymerase enzymes, of which only RNA polymerase II transcribes hn-RNA (heterogeneous-nuclear RNA), the precursor to mRNA. RNA polymerase II cannot directly recognise the promoter of target genes, and thus requires additional factors for the recognition of a promoter and subsequent transcription of the gene. Transcription factor II D (TFIID) is essential for transcription of RNA polymerase II genes. TFIID is a multi-subunit protein, made up of TATA box-binding protein (TBP) and TBP-associated factors (TAFs). TFIID is responsible for the recognition of

the TATA box element of a promoter. The TATA box is an important element in many promoters which binds TFIID resulting in the positioning of the polymerase over the transcription start site and activation of transcription. TFIID recognises and binds to the TATA box through the TBP subunit. Other essential factors including TFIIA and TFIIB are then recruited by the DNA bound TFIID. RNA polymerase then binds; this assembled complex, which is capable of transcriptional activation, is called the basal apparatus. The binding of RNA polymerase II to the assembled transcription factor complex positions the polymerase, allowing transcription to occur, with additional factors binding to help the polymerase clear the promoter.

Despite the importance of the TATA box, many promoters do not contain this element; such promoters are termed TATA-less promoters. In TATA-less promoters, the TBP sub-unit of TFIID cannot directly bind to the DNA. In these cases TFIID (or TFIIB) is recruited by transcriptional activators (e.g. Sp1) which are bound to the promoter at their corresponding element. Protein-protein interactions between activation domains of the transcription factor, or co-activator, and TAF sub-units anchor TFIID to the promoter, allowing the basal apparatus to assemble, and leading to the transcription of the gene. Thus, the transcription of TATA-less promoters relies upon TFIID sub-unit interactions with activating transcription factors (Weaver, 1999). The maspin promoter is a TATA-less promoter (Zhang et al., 1997a).

1.5.2 Transcriptional regulation of maspin

Maspin is regulated transcriptionally, and is classed as a class II tumour suppressor gene, because it is down-regulated rather than mutated (Lee et al., 1991; Zou et al., 1994). Several different factors have been shown to bind to the maspin promoter and activate/repress maspin transcription (figure 1.3). All regulatory factor sites reported to date are located within 500 base pairs (bp) of the transcription start site (TSS). Ets, Ap1 and p53 sites have been shown to be important for the activation of transcription, whilst androgen receptor (AR) binding to the hormone response element (HRE) represses transcription (Zhang et al., 1997a; Zhang et al., 1997b; Zou et al., 2000). Methylation of CpG di-nucleotides in this region of the promoter has also been shown to inhibit trans-activation of maspin (Domann et al., 2000; Futscher et al., 2002; Maass et al., 2002a; Oshiro et al., 2003; Sato et al., 2003).

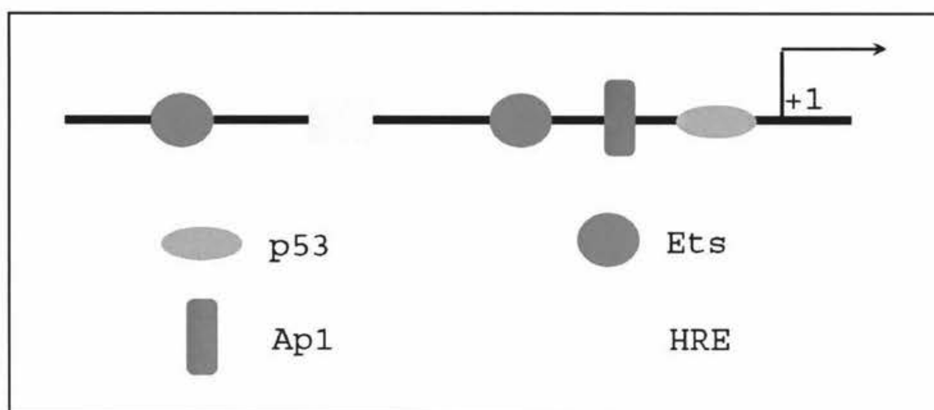

Figure 1.3. Reported regulatory elements contained in the maspin promoter. All sites are contained within 500 bp of the transcription start site. Thick black line indicates DNA, bent arrow indicates TSS (Zhang et al., 1997a; Zhang et al., 1997b; Zou et al., 2000).

1.5.3 Ets transcription factor

Two binding sites for the Ets family of transcription factors have been reported within 500 bp of the TSS (Zhang et al., 1997a; Zhang et al., 1997b) (figure 1.3). The proximal Ets site was sufficient for transcription of the reporter gene-maspin promoter construct in normal breast and prostate cell lines (Zhang et al., 1997a; Zhang et al., 1997b). Transient transfections in cancerous breast cell lines showed decreased trans-activation by the Ets site. This suggests that the decrease in maspin expression in breast cancer cells may be due in part to decreased transactivation by the Ets factors.

Ets (E26 transformation-specific) is a family of proteins which are known to play a role in apoptosis, cell proliferation, and differentiation. About 30 members of the Ets family have been identified to date, all containing a highly conserved, approximately 85 amino acid, Ets DNA binding domain (Verger and Duterque-Coquillaud, 2002; Oikawa and Yamada, 2003). The Ets domain confers specific binding to a sequence containing the core GGA(A/T) sequence, with the surrounding sequence and binding partners defining the specificity of the Ets protein for a particular site (Verger and Duterque-Coquillaud, 2002; Oikawa and Yamada, 2003). Some Ets proteins are ubiquitously expressed, while expression of others is developmentally regulated, or tissue-specific (Oikawa and Yamada, 2003). Some Ets transcription factors are targets of the Ras/MAP kinase signalling cascade, and have been implicated in breast cancer progression (Feldman et al., 2003; Oikawa and Yamada, 2003).

Pdef (prostate derived Ets factor) was shown to activate transcription of the maspin promoter in HeLa cell line transient transfections (Feldman et al., 2003). Pdef was found to be present in prostate, colon and breast tissue, and seemed to be down-regulated in invasive breast cancer cell lines. Pdef was not present in tissues of non-epithelial origin. The activation induced by Pdef, was reduced by co-transfection with Ets-1 (Feldman et al., 2003). This demonstrates that Ets factors are competing for binding sites, and so up-regulation of an Ets factor which does not activate maspin may cause a decrease in maspin expression by competing for the Ets binding site. The mouse Pdef (mPse) has also been shown to activate expression of the human maspin promoter in transient transfections using human kidney 293T cell line (Yamada et al., 2000). This indicates Ets factors have conserved recognition sequences, and possibly function.

1.5.4 Ap1

In breast cell lines, the transcription factor Ap1 (Activating protein-1) was shown to co-operate with Ets to activate maspin-reporter expression in transient transfections (Zhang et al., 1997a). Ap1 was not sufficient to activate transcription of the reporter gene, however with a functional Ets site a large increase in expression was seen compared to the Ets site alone (Zhang et al., 1997a).

Ap1 is a family of proteins that homo or heterodimerise to activate transcription of target genes (Shaulian and Karin, 2001). Ap1 proteins play a role in cell proliferation, neoplastic transformation, and apoptosis. Formation of active dimers by differential regulation of Ap1 monomers and phosphorylation of Ap1 contribute to the regulation of target genes (Shaulian and Karin, 2001). Ap1 proteins, like Ets transcription factors, are under control of the Ras/MAP kinase signalling cascade. Ap1 proteins can also be activated by UV, growth hormones, and alkylating agents (Shaulian and Karin, 2001). The identification of the Ap1 dimer which along with Ets is responsible for co-operative activation may help identify the stimulus which results in maspin expression.

1.5.4 p53

Another positive regulator of maspin expression, present within 500 bp of TSS, is the p53 element (Zou et al., 2000). Elevated levels of maspin expression were seen after exposure of cells containing wt-p53, to DNA-damaging agents. This effect was not seen in mt-p53 cells (Zou et al., 2000). p53 bound to the maspin promoter in electrophoretic mobility shift assays (EMSA), and p53 could not activate transcription when the p53 site was mutated (Zou et al., 2000).

Oshiro, et al., (2003) showed that transfection of MDA-MB-231 breast cancer cell lines with wt-p53 increased maspin transcription by modification of histones present at the promoter. The maspin promoter in MDA-MB-231 cell lines is reported to be methylated, and in a compact chromatin structure (see 'CpG methylation' below) (Maass et al., 2002a). Transfection of p53 expression vector did not effect the methylation status of the promoter, but allowed transcription by recruiting histone acetylases and opening up chromatin structure (Oshiro et al., 2003).

These studies were supported by a report by Umekita, et al., (2002), which showed that maspin expression positively correlated with p53 expression in breast cancer patients. Interestingly, a clinical study carried out on prostate cancer patients indicated that expression of p53 was correlated with lower maspin expression (Machtens et al., 2001). This result may be partially explained by a recent report which showed that missense p53 mutants could repress expression of target genes in a dominant negative manner (Willis et al., 2004). Missense mutations are the most common type of mutation in p53 (Gasco et al., 2002), and may cause repression of maspin transcription. This may be responsible for the correlation between p53 expression and the absence of maspin in prostate cancers. Interestingly only 20% of breast cancers express a mutant p53 allele, possibly explaining why p53 correlated with maspin expression in breast cancers (Gasco et al., 2002).

p53 is known to be involved in DNA repair, apoptosis and cell cycle arrest; the regulation of maspin by p53 may also indicate that p53 is involved in angiogenesis and inhibition of metastasis (Gasco et al., 2002).

1.5.5 Hormone response element

The presence of a hormone response element (HRE) in the maspin promoter has also been shown (Zhang et al., 1997b). This element was responsive to the androgen receptor (AR) in prostate cell lines, and caused transcriptional repression of a reporter gene in transient transfections. Interestingly this repression was mediated independently of the androgen ligand (Zhang et al., 1997b). This androgen-independence may be due to hormone analogues contained within the media, as charcoal stripped foetal calf serum (FCS) and phenol red free media were not used in these experiments. Supporting this, maspin-reporter gene expression was increased, in androgen responsive prostate cancer cell lines, when cultured in charcoal stripped FCS (Zou et al., 2002). This reporter gene expression was reduced upon growth with supplemented androgen. Attempts to reproduce the EMSA experiments performed by Zhang, et al., (1997b) showed weak non-specific binding of AR to the maspin HRE (van Dijk, 2003; Bradbury, 2004).

The role of androgen in the regulation of maspin in prostate cancers was supported by androgen ablation studies. Patients who underwent androgen ablation treatment before surgery showed significantly increased maspin expression (Zou et al., 2002). Prostate cancer cells showed greater maspin expression when implanted into castrated mice compared to non-castrated (Zou et al., 2002). It has also been shown that anti-androgen treatment of breast cancer cell lines (which over-express AR compared to normal breast cells) caused an increase in maspin expression (Khalkhali-Ellis et al., 2004). Interestingly no difference in prostate, kidney, or intestinal maspin expression was seen in normal and castrated rats, indicating rat maspin is not responsive to androgen (Umekita et al., 1997).

Androgen receptor plays a role in the development and differentiation of male sex organs, and also plays a role in prostate carcinogenesis (Cato and Peterziel, 1998; Bonaccorsi et al., 2000). The unliganded androgen receptor is normally present in the cytoplasm and upon binding to the androgen hormone, the receptor-ligand complex is translocated to the nucleus where it binds to DNA as a homodimer activating or repressing transcription (Cato and Peterziel, 1998).

Tamoxifen is an anti-oestrogen drug, used in cancer treatment, which competes with oestrogens for the oestrogen receptor (ER) (Khalkhali-Ellis et al., 2004). Treatment of breast cancer cell lines expressing ER with tamoxifen decreases invasion and migration, and increases apoptosis (Khalkhali-Ellis et al., 2004). Oestrogen, progesterone, and dihydrotestosterone had no effect on maspin expression, however treatment with tamoxifen increased expression in breast cancer cells, and in myoepithelial cells secretion of maspin was increased without increasing mRNA levels (Shao et al., 2000; Khalkhali-Ellis et al., 2004). Tamoxifen was shown to activate ERβ, but not ERα, and mutation of the maspin HRE abrogated the effect of tamoxifen on maspin expression (Shao et al., 2000; Khalkhali-Ellis et al., 2004). Increased maspin expression was not seen in MCF12a normal breast cells when treated with tamoxifen; however secreted maspin was not measured (Bradbury, 2004).

These data indicate that maspin is repressed by the androgen receptor, in response to androgen, in human and mice, but not rats. This suggests that maspin may have a role in development of male sex organs in mice and humans. The activation of maspin by anti-oestrogen suggests that other hormones may also play a role in regulation of maspin.

1.5.6 Other regulatory molecules

Maspin has been shown to be activated by several other mechanisms, however as yet the mechanism of action of these activators is not known. Manganese containing super-oxide dismutase (MnSOD) is a protein which converts oxygen radicals, a by-product of metabolism, into peroxide which is then converted to water by catalase. Oxygen radicals can react with proteins, lipids, and DNA potentially causing cellular and DNA damage (Li et al., 1998; Cullen et al., 2003). Super-oxide radicals may promote cellular proliferation, differentiation, and apoptosis by changing the redox potential of the cell, thus regulating redox-sensitive signal transduction pathways (Li et al., 1998; Cullen et al., 2003).

MnSOD is lost, or down-regulated in many cancer cell lines. Re-expression of MnSOD in MCF-7 breast cancer cell line, reduced cellular migration and invasion, concomitant with maspin re-expression (Li et al., 1998). Maspin re-expression was MnSOD dose dependent (Li et al., 1998). This indicates that maspin is activated by MnSOD, however

MnSOD is localised in mitochondria (Cullen et al., 2003), and so maspin induction due to MnSOD is likely to be indirect. Thus the re-expression of MnSOD in cancer cells may restore redox potential, thus restoring redox-sensitive signalling, increasing maspin expression.

Nitric oxide (NO) has also been shown to activate maspin expression (Shao et al., 2000; Khalkhali-Ellis and Hendrix, 2003). Increasing NO in media, or expression of endothelial nitric oxide synthase (eNOS) in MCF7 breast cancer cells caused re-expression of maspin (Khalkhali-Ellis and Hendrix, 2003). Increased levels of maspin correlated with decreased migration and invasion, and increased apoptosis (Khalkhali-Ellis and Hendrix, 2003).

Interestingly, increased NO levels have been shown to cause p53 phosphorylation and acetylation in a DNA damage-like response. This corresponded to increased levels of p53 target gene trans-activation (Hofseth et al., 2003). The MCF7 cell line used in the above experiments has been shown to express wt-p53 (Vojtesek and Lane, 1993). This may indicate NO treatment results in increased maspin expression by increasing p53 trans-activation; however this remains to be proven.

Gamma linolenic acid (GLA) has also been shown to induce maspin expression (Jiang et al., 1997; Jiang et al., 2000). The action of GLA was shown to be mediated through Perixosome proliferator activated receptor γ, which was translocated into the nucleus upon GLA treatment (Jiang et al., 2000). GLA is believed to be an anti-cancer fatty acid, with animals on a pure GLA diet showing a decrease in the occurrence of cancer (Jiang et al., 2000), however GLA treatments used by Jiang, et al., (1997 & 2000) in cell culture experiments seemed to be outside physiological levels (50 – 75 μM).

These reports show that other mechanisms can up-regulate the expression of maspin, however they seem to be indirect. The exact method of maspin regulation by these modulators is yet to be demonstrated.

1.5.7 CpG methylation

Methylation on the cytosine residue of CpG dinucleotides in the promoter region of genes has been demonstrated to prevent transcriptional activation of genes (Brown and Strathdee, 2002). Addition of a methyl group to the cytosine residue can prevent the binding of some transcription factors; however this is not believed to be the main mechanism by which methylation prevents trans-activation. Methylation leads to the binding of methyl-CpG binding proteins (MeCP), which recruit histone deacetylases, therefore causing compaction of chromatin in the methylated area (Brown and Strathdee, 2002). This compaction of chromatin prevents transcription factors gaining access to their respective binding sites, resulting in loss of trans-activation.

Methylation of the maspin promoter has been shown to prevent expression of maspin in some cancerous cells, and also in cells from non-expressing tissues (Domann et al., 2000; Futscher et al., 2002; Maass et al., 2002a; Oshiro et al., 2003; Sato et al., 2003). The maspin promoter has been reported to contain two CpG islands (Domann et al., 2000). A CpG island is defined as a region of DNA that has an unusually high cytosine (C), guanine (G), and CpG di-nucleotide content (Costello and Vertino, 2002). The proximal CpG island of the maspin promoter overlaps the transcription start site and positive effector sites reported to date. This island was shown to be highly methylated in cancerous cell lines, and tissues which do not normally express maspin, whilst maspin expressing cells had little methylation in this region. The distal CpG island is located approximately 120 bp upstream of the proximal island and was shown to be methylated in both expressing and non-expressing cells (Domann et al., 2000; Futscher et al., 2002; Maass et al., 2002a; Oshiro et al., 2003; Sato et al., 2003).

In cell lines which contained methylation of the proximal island, maspin could be re-expressed by addition of a histone deacetylase inhibitor, and/or methyltransferase inhibitors (Domann et al., 2000; Costello and Vertino, 2002; Maass et al., 2002a; Oshiro et al., 2003; Sato et al., 2003). Pancreatic tissue does not express maspin, however many pancreatic cancers do. Treatment of normal pancreatic cells with methyltransferase, and histone deacetylase inhibitor caused expression of maspin in these cells. Cancerous pancreatic cells were hypomethylated, whilst normal cells were found to be hypermethyalated (Maass et al., 2001b; Ohike et al., 2003; Sato et al., 2003). p53 was

able to induce maspin expression in cells with methylated promoters by recruiting histone acetylases, which in turn caused the opening of chromatin structure, thus allowing transcription factors to bind (Oshiro et al., 2003).

These findings indicate that at least some factors required for maspin transcription are present in many, if not all, tissues and cancers. This suggests that a major loss of maspin transcription may be due to methylation of the promoter, and concomitant histone compaction. The loss of trans-activation of maspin in methylated cell lines may be controlled by the balance of MeCP induced histone acetylation and p53 induced deacetylation. Thus the status and expression levels of p53 may be important.

1.6 Chapter summary

Maspin is a tumour suppressor gene whose expression has been observed in many tissues of epithelial origin, including prostate and breast. The tumour suppressive function of maspin is related to its ability to inhibit cellular mobility and invasion, apoptosis, and angiogenesis *in vitro* and *in vivo*. Whilst some interaction partners have been identified, no mechanism is known for the reported functions of maspin. Although maspin is a non-inhibitory serpin, inhibition of cellular migration and invasion is dependent on the RSL, however angiogenesis inhibition is reliant upon the N-terminus. This indicates that maspin contains at least two functional sites.

Maspin was initially reported to be down-regulated in breast cancers. Several reports support this hypothesis, however other reports disagree. Maspin down-regulation in prostate cancer however, is less controversial. The differing results seen in clinical studies may be due to the lack of standardised methods. The sub-cellular localisation of maspin also needs further investigation, as well as the role of maspin in these compartments. The up-regulation of maspin in pancreatic and ovarian cancers seems to oppose its reported tumour suppressive function; however these cells may be non-responsive to maspin, or may have high levels of mutation. The expression of mutant maspin may be an advantage to cancers of these tissues.

Expression of maspin is positively regulated by Ets, Ap1, and p53 transcription factors. The binding sites for these factors and the negative HRE element are contained within 500 bp of the transcription start site. Ets, Ap1 and p53 all play roles in cell proliferation and apoptosis. Ets and Ap1 also regulate cell differentiation. Androgen influences cellular differentiation and development. The development of tissues involves cellular migration, apoptosis, differentiation, and proliferation. The regulation of maspin by these factors suggests that maspin may be important for the development and differentiation of tissues. This hypothesis is supported by abnormal expression of maspin during pregnancy in WAP-maspin mice causing disrupted mammary gland development. These factors also support the functional evidence that maspin plays a role in apoptosis, as all activators of maspin transcription are involved in apoptosis regulation. Ets, Ap1 and AR play roles in cellular differentiation, and in prostate cancers the loss of maspin correlated with the loss of differentiation (higher tumour grade), however the role of maspin in cellular differentiation has not been studied.

Repression of maspin transcription by methylation of the promoter may be important for tissue-specific regulation. This silencing mechanism may also be used by cells to silence maspin as cancer progresses. The methylation of the proximal CpG island in the maspin promoter results in histone deacetylation and chromatin compaction in this area. This then prevents transcription factors from activating transcription, causing the loss of the tumour suppressor functions of maspin, and thus allowing cancer progression.

The mechanisms by which MnSOD, NO, and GLA stimulate maspin expression are yet to be deciphered, however NO may activate p53, and p53 in turn activate maspin expression.

1.7 Research aims

The elements that are involved in transcriptional regulation of maspin suggest that maspin is involved in apoptosis, cellular migration, and proliferation; hypotheses which are supported by functional studies. To date however, the molecular mechanisms of the function of maspin are unknown. The identification of further transcriptional elements which are involved in the transcriptional regulation of maspin may provide insights in the search for a molecular function of maspin. For example, if a transcriptional effector, responsible for activating transcription of genes which act in a particular apoptosis pathway, is identified as activating maspin transcription, then maspin may play a role in this pathway. This hypothesis can then be tested further.

The regulatory elements that have been functionally identified to date are all contained within 500 bp of the transcriptional start site. Transient transfection of deletion series has shown that transcriptional elements may be present in regions further upstream from the TSS (Zhang et al., 1997a; Zhang et al., 1997b), however these regions have not been investigated. The reported activation of maspin expression by MnSOD, GLA, and NO also suggest that other transcriptional regulators may play a role in maspin expression.

The aim of this study was to identify putative regulatory factors which are important for the regulation of the gene, by assessing the regulation of maspin transcription in cancerous and normal cell lines. To this end, the maspin gene was identified from the human genome sequence, and a fragment representing the promoter cloned and analysed for putative transcriptional elements. Transient transfection of cell lines with a series of maspin promoter deletion constructs was the first step in functional analysis. This was followed by footprinting of regions identified as being important for the regulation of the maspin gene. Footprinting has the potential to specifically identify protein binding motifs at the nucleotide level, enabling putative protein binding DNA elements to be identified. The DNA elements can then be used to predict the transcription factor which binds to this region.