TUMOUR-SUPPRESSIVE ACTIVITY OF THE GROWTH ARREST-SPECIFIC GENE, GAS1

A thesis submitted to the University of Adelaide as the requirement for the degree of Doctor of Philosophy

by

Andreas Evdokiou B.Sc.

Department of Physiology, The University of Adelaide

and

Department of Surgery, The University of Adelaide ,

The Queen Elizabeth Hospital

June 1997

TABLE OF CONTENTS

	Page No
Table of Contents	ii
Summary	x
Declaration	xiv
Acknowledgments	xv
Publications Arising	xvi
Conference Presentations	xvii
List of Abbreviations	xviii

Chapter 1

Literature Review

1.1	General Introduction	2
1.2	The Mammalian Cell Cycle	3
1.3	Identification of Gene Products Associated with Growth Arrest	8
1.3.1	Antiproliferative mRNAs	8
1.3.2	Interferons	9
1.3.3	Transforming Growth Factor β (TGF-β)	10
1.3.4	Prohibitin	12
1.3.5	Growth Arrest and DNA Damage-inducible (GADD) Genes	14
1.3.6	Cyclins, Cyclin-Dependent Kinases (CDK), and their inhibitors	
1.4	Tumour Suppressor Genes	21
1.4.1	Cell Hybrids	21
1.4.2	Familial Cancers	22
1.4.3	Loss of Heterozygosity (LOH)	25

1.4.3.1 p53 26
1.4.3.2 Deleted in Colorectal Carcinoma (DCC) 31

1.5 Non-Coding RNAS as Tumour Suppressors 33

1.6 Growth Arrest-Specific (GAS) Genes 34

1.6.1 Growth Arrest-Specific Gene 1 (GAS1) 35
1.6.2 Growth Arrest-Specific Gene 2 (GAS2) 39
1.6.3 Growth Arrest-Specific Gene 3 (GAS3) 40
1.6.4 Growth Arrest-Specific Gene 5 (GAS5) 42
1.6.5 Growth Arrest-Specific Gene 6 (GAS6) 42

1.7 Aims of the Thesis 44

Chapter 2

General Materials and Methods

2.1 Materials 47

2.1.1 Chemicals and reagents 47
2.1.2 Enzymes 48
2.1.3 Radiochemicals 49
2.1.4 Kits 49
2.1.5 Buffers and solutions 50
2.1.6 Bacterial media 51
2.1.7 Antibiotics 51
2.1.8 Bacterial strains 52
2.1.9 Plasmid vectors 52
2.1.10 Cloned DNA sequences 52
2.1.11 Synthetic oligonucleotides 53
2.1.12 Tissue culture solutions 54
2.1.13 Cell lines 55

2.1.14 Miscellaneous 56

2.2 General Methods 56

2.2.1 Plasmid DNA preparation 57
2.2.2 Restriction enzyme digestions 57
2.2.3 Preparation of DNA restriction fragments 58
2.2.4 Ligation of cloning vectors 58
2.2.5 Ligation of DNA fragments into plasmid vectors 59
2.2.6 Preparation of competent bacteria 59
2.2.7 Transformation of *E. coli* 60
2.2.8 Southern transfer 60
2.2.9 Labelling of DNA with ^{32}P 61
2.2.10 Prehybridisation, hybridisation and washing of filters 62
2.2.11 Re-use of filters 62
2.2.12 RNA preparation 63
2.2.13 Northern blot analysis 64
2.2.14 Purification of oligonucleotides 64
2.2.15 Polymerase chain reaction (PCR) 65
2.2.16 Reverse transcriptase polymerase chain reaction (RT-PCR) 66
2.2.17 Removal of contaminating DNA prior to reverse transcription 66
2.2.18 Preparation of protein from cell extracts 67
2.2.19 SDS-polyacrylamide gel electrophoresis and Western blot analysis 67
2.2.20 Maintenance of cultured cell lines 69
2.2.21 Transfection of adherent cells with expression vectors 69
2.2.21 Sequencing reactions 70
2.2.22 Sequencing gels 71
2.2.23 Single strand conformational polymorphism (SSCP) 71
2.2.24 Computer programmes 72

Chapter 3

Chromosomal Localisation of the Mouse and Human GAS1 Genes

3.1 Introduction 74

3.2 Materials and Methods 76

3.2.1 DNA probes for *in situ* hybridisation 76

3.2.2 Labelling of cDNA probes with ^{3}H-dNTPs by nick translation 77

3.2.3 Chromosome preparation from mouse splenic lymphocytes 78

3.2.4 Chromosome preparation from human peripheral lymphocytes 79

3.2.5 *In situ* ybridisation 79

3.2.5.1 Treatment of slides with RNase 79

3.2.5.2 Acetylation 80

3.2.5.3 Denaturation and hybridisation 80

3.2.5.4 Autoradiography and staining 81

3.2.6 Somatic cell hybrid DNA panel 82

3.2.7 Polymerase chain reaction 82

3.3 Results 83

3.3.1 Localisation of *Gas-1* to mouse Chromosome 13 83

3.3.2 The human GAS1 gene does not map to human chromosome 5 85

3.3.3 Localisation of the human GAS1 gene to human chromosome 9q21.3-q22 86

3.3.4 Confirmation of the location of GAS1 to human chromosome 9 87

3.3.5 *GAS1* maps to a region frequently deleted in myeloid malignancies 88

3.4 Discussion 89

Chapter 4

Analysis of Gas-1 in Normal and Transformed Mouse Cells

4.1 Introduction 92

4.2 Materials and Methods 93

4.2.1 Demethylation of *Gas-1* by treatment with azacytidine 93

4.3 Results 94

4.3.1 Induction of Gas-1 mRNA in growth arrested cells 94

4.3.2 *Gas-1* is abnormally methylated in mouse tumour cell lines 95

4.3.3 The methylation state of the Gas-1 gene does not change in serum stimulated cells 96

4.3.4 Mouse tumour cell lines do not express Gas-1 mRNA 97

4.3.5 Demethylation by 5-azacytidine does not effect the expression of Gas-1 in Lewis lung carcinoma cells 98

4.4 Discussion 100

Chapter 5

Expression of GAS1 in Human Cells and Growth-Suppressive Activity in NIH3T3 Fibroblasts

5.1 Introduction 105

5.2 Materials and Methods 106

5.2.1 Plasmid clones 106

5.2.2 Detection of GAS1 mRNA in IMR90 cells by DNase treatment and RT-PCR 108

5.2.3 Transfections 109
5.2.4 Analysis of growth rate 110
5.2.5 Soft agar cloning assay 110

5.3 Results 110

5.3.1 Expression of GAS1 mRNA in human fibroblasts 110
5.3.2 Detection of GAS1 mRNA in IMR90 cells by RT-PCR 111
5.3.3 Constitutive expression of GAS1 mRNA in NIH3T3 cells is antiproliferative 112
5.3.4 Dexamethasone-inducible expression of GAS1 in NIH3T3 fibroblasts leads to growth arrest 114
5.3.5 Expression of antisense GAS1 mRNA in NIH3T3 fibroblasts 116

5.4 Discussion 117

Chapter 6

Tumour-Suppressive Activity of GAS1 in Human Cell Lines

6.1 Introduction 125

6.2 Materials and Methods 127

6.2.1 Cell lines used for transfections 127
6.2.3 Transfections 128
6.2.4 Tumorigenicity in nude mice 128
6.2.5 Analysis of tumours 129

6.3 Results 129

6.3.1 Constitutive expression of GAS1 in A549 cells 129
6.3.2 Dexamethasone-inducible expression of GAS1 mRNA 131
6.3.3 Growth of A459-GAS1 transfectant colonies in semisolid agar 133

6.3.4 Suppression of tumorigenicity of A549 GAS1-transfesfectant cells *in vivo* 134
6.3.5 Transfection of GAS1 into HTD114 fibrosarcoma cells 136
6.3.6 P53 status of HTD114 cells 137
6.3.7 Expression of MDM2 in HTD114 cells 138
6.3.8 Transfection of GAS1 in HT1080 cells with wild-type or mutant p53 and low levels of MDM2 140

6.4 Discussion 142

Chapter 7

Identification and Characterisation of an Alternatively-Spliced Variant of the MDM2 Oncogene Product

7.1 Introduction 148

7.2 Materials and Methods 149

7.2.1 Cloning of the antisense MDM2 fragment into the pReCMVneo constitutive expression vector 149
7.2.2 Transfection of HTD114 cells 150
7.2.3 Nested RT-PCR for the detection of the MDM2 alternatively-spliced variant 150
7.2.4 Sequencing of the MDM2 alternatively spiced variant 152

7.3 Results 153

7.3.1 Transfection with pReCMV-GAS1: Colony numbers and expression of GAS1 mRNA 153
7.3.2 Transfection with pReCMV-MDM2-antisense: Colony numbers and inhibition of expression of the endogenous MDM2 mRNA 154

7.3.3 Co-transfection of HTD114 cells with pRcCMV-GAS1 and pRcCMV-MDM2-antisense induces the expression of a variant MDM2 transcript 155

7.3.4 The variant transcript is a novel alternatively-spliced variant of MDM2 156

7.3.5 The MDM2 alternatively-spliced variant contains most of the p53 binding domain but is devoid of DNA binding motifs 157

7.4 Discussion 158

Chapter 8

General Discussion

8.1 Discussion 162

8.2 Future Directions 168

Bibliography 170

Appendix I 198

Sequencing data of exons 5, 6, 7 and 8 of the p53 gene in HTD114 cells

Appendix II 199

Sequencing data of the MDM2 [AE-V] alternatively spliced variant

SUMMARY

The growth arrest-specific gene, GAS1, codes for a membrane-associated protein which accumulates at the cell surface following growth arrest mediated by either serum starvation or density-dependent inhibition. Expression of both GAS1 mRNA and protein is rapidly down-regulated when cells are activated to enter S phase. Overexpression of the mouse gas-1 gene in normal and oncogene-transformed NIH3T3 cells blocks proliferation of actively-growing cells and inhibits entry by quiescent cells into S phase when activated by serum. The growth suppressing activity of the mouse gas-1 gene is mediated by a p53-dependent signalling pathway, but the transcriptional activation function of p53 is not required for gas-1-induced growth arrest. Since the expression of the GAS1 gene is tightly confined to the quiescent stage of the cell cycle and GAS1 has growth-inhibitory activity *in-vitro,* its expression either reflects requirements to sustain growth arrest, or may be involved in the control of differentiation.

Gene products with growth-inhibitory properties frequently act as tumour suppressor genes and the studies described in this thesis seek to establish a possible role for GAS1 as a candidate tumour suppressor gene whose function is to inhibit uncontrolled proliferation and to prevent the development of the malignant phenotype.

The first part of this thesis reports on the chromosomal localisation by *in-situ* hybridisation of the mouse and human GAS1 genes. The mapping of GAS1 to mouse Chromosome 13 at bands B3-C2 and, more particularly, to human chromosome 9 at bands q21.3-q22, places the human gene in a region of the genome which is highly significant as a site of a number of potential tumour suppressor genes. Genetic

alterations in this region of chromosome 9q have previously been implicated as early events in the progression of myeloid malignancy and bladder carcinoma.

The human GAS1 gene, when overexpressed in NIH3T3 cells, is able to block proliferation of actively growing cells. Cells overexpressing exogenous GAS1 display remarkable changes in phenotype that resemble growth arrested cells or cells in crisis. These results clearly demonstrate that the human GAS1 gene product is directly involved in mediating growth suppression in a similar manner to that previously observed with the mouse homologue.

To investigate whether GAS1 is able to suppress the growth of human tumours, GAS1-negative tumour cells were transfected with GAS1-expressing plasmids and the growth characteristics of stable transfectants were analysed. The A549 lung adenocarcinoma cell line contains wild type p53 and normal levels of the MDM2 oncogene product. When a constitutively-expressing GAS1 plasmid was transfected into A549 lung adenocarcinoma cells, no stable clones expressing GAS1 were isolated. When A549 cells were transfected with a dexamethasone-inducible GAS1 plasmid, expression of GAS1 inhibited growth *in vitro* and produced fewer slow-growing tumours in nude mice. GAS1 also inhibited proliferation of an HT1080 fibrosarcoma subline with wild-type (wt) p53 and normal levels of MDM2. In contrast, when the HT1080 subline, HTD114, was transfected with the constitutive GAS1 plasmid, there was no reduction in colony number. GAS1-transfectant clones had unaltered growth *in vitro*, and showed no difference in their ability to form tumours in nude mice. Although HTD114 cells contain wt p53, levels of MDM2 were elevated by 10-15 fold. The product of the MDM2 gene physically associates with wild type p53 protein and inhibits its transactivation function. Overexpression of MDM2 therefore provides an alternate mechanism by which p53 function is inhibited. The HT10806TGc5 subline with mutant

p53 and normal levels of MDM2 was also refractory to GAS1. These results indicate that GAS1 suppresses the growth and tumorigenicity of tumour cells in a p53 dependent manner and that p53 mutation and/or overexpression of MDM2 abrogates the GAS1-mediated growth-suppressing pathway.

To further define the role of MDM2 in the inhibition of GAS1-mediated growth suppression, the endogenous levels of MDM2 in the non-responsive HTD114 cells were downregulated using an antisense strategy. HTD114 cells were co-transfected with MDM2-antisense and GAS1 plasmid constructs. If overexpression of MDM2 in HTD114 cells inhibits the suppression of growth mediated by GAS1, then downregulation of the endogenous levels of MDM2 in these cells using the antisense construct, should increase the sensitivity of the co-transfectants to exogenous GAS1. Although there was a reduction in the levels of endogenous MDM2 mRNA, these cells remained refractory to overexpression of GAS1. However, these cells now expressed a previously unrecognised and novel MDM2 splice variant, which, by sequence analysis, was shown to contain most of the p53 binding domain and was devoid of the putative DNA binding motifs present in the carboxy end of the MDM2 gene. Based on these observations, the following model is proposed. The net effects of antisense MDM2 changes the balance in favour of free and functional p53 protein within the cell and that these cells now overcome the GAS1-mediated growth suppression by expressing the alternate splice variant of MDM2, thus maintaining the cells in a transformed state.

Taken together, the results presented in this thesis have established the growth-suppressive activity of the human GAS1 gene and provided the first direct evidence that GAS1 can inhibit the growth of tumours. The antiproliferative effects of GAS1 are mediated by a p53 dependent pathway. Functional inactivation of p53 by either p53 mutation and/or overexpression of the MDM2 oncogene product abrogates GAS1-

mediated growth-suppression. This study has therefore identified another downstream element, MDM2, that is involved in the cascade of events that lead to growth arrest mediated by GAS1. Further characterisation of the previously unrecognised alternate splice variant of MDM2 identified in HTD114 cells presents a challenge of defining its role and oncogenic potential.