Regulation of FoxO Transcription Factors in Breast Cancer

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Statement of Originality

Unless otherwise stated in the text, this thesis is the result of my own work.
Abstract

Breast cancer is the world's most prevalent cancer. Although several drugs and chemotherapeutic strategies have been developed to tackle breast cancer, to date most patients eventually acquire resistance to these anti-cancer therapies. Therefore, identifying ways to increase the efficiency of currently used chemotherapeutic drugs and the development of new drugs for breast cancer treatment is essential. One way to achieve this goal is by identifying cellular targets which play a pivotal role in tumourigenesis and tumour progression.

Paclitaxel belongs to a class of naturally occurring anti microtubule agents used for the treatment of malignancies such as breast cancer. Previous work has shown that FoxO3a, a transcription factor downstream of the phosphotidylinsitol-3-kinase/Akt signalling pathway, mediates apoptosis and cell cycle arrest in breast cancer cells in response to paclitaxel treatment.

In order to elucidate the significance of FoxO expression and activation in response to paclitaxel treatment and oxidative stress (which is caused by paclitaxel treatment), I investigated the regulation of FoxO in endometrial and breast cancer cells. Both paclitaxel and oxidative stress were found to up-regulate FoxO expression at the protein, mRNA and gene-promoter levels. Moreover, treatment with paclitaxel and hydrogen peroxide were shown to increase FoxO3a protein stability. Paclitaxel treatment resulted in JNK mediated nuclear accumulation of FoxO3a with a corresponding reduction in Akt activity. JNK was also shown to induce FoxO3a gene-promoter activity and to phosphorylate FoxO3a at two sites. These phosphorylation events may be important in the regulation of FoxO3a stability and activity.

I also investigated the function of FoxO3a, by studying the role of BTG1, a downstream target of FoxO3a. I found that BTG1 expression was induced at the gene-promoter level by FoxO3a in MCF-7 cells. The use of a BTG1 inducible MCF-7 cell line revealed that over-expression of BTG1 results in changes in the expression levels of cell cycle regulators, reduction in cell growth and accumulation of cells in the G2/M phase of the cell cycle.
Taken together, these results show that FoxO expression and activity are upregulated following paclitaxel treatment and demonstrate that the PI3K/Akt/FoxO and JNK signalling pathways cross-talk at least at two levels. Furthermore, these results indicate that FoxO expression levels may serve as bio-marker for determining the effectiveness of paclitaxel treatment of breast cancer patients and that FoxOs may serve as a potential target for anti-cancer chemotherapeutic intervention.
To My Grandmother, Wally Ghinzburg
Acknowledgments

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Table of Contents

STATEMENT OF ORIGINALITY .................................................................................................................... 2
ABSTRACT ...................................................................................................................................................... 3
ACKNOWLEDGMENTS ...................................................................................................................................... 6
LIST OF FIGURES ......................................................................................................................................... 11
LIST OF TABLES .......................................................................................................................................... 13
ABBREVIATIONS ........................................................................................................................................... 14
PUBLICATIONS .............................................................................................................................................. 17

1 INTRODUCTION ........................................................................................................................................ 18

1.1 OVERVIEW: CANCER ............................................................................................................................. 18
  1.1.1 Genetic basis of cancer ...................................................................................................................... 19
1.2 BREAST CANCER: OVERVIEW AND TREATMENT ............................................................................... 22
  1.2.1 Taxanes in cancer treatment ............................................................................................................. 25
1.3 THE PHOSPHATIDYLINOSITOL 3-KINASE (PI3K)/AKT (PKB) SIGNALLING PATHWAY ......................... 28
  1.3.1 Overview of PI3K pathway and family members ............................................................................. 28
  1.3.2 PI3K class I: regulation and role ........................................................................................................ 30
  1.3.3 Downstream activities of class I PI3K .............................................................................................. 31
  1.3.4 Akt activation ..................................................................................................................................... 32
  1.3.5 Downstream targets of Akt ................................................................................................................ 35
  1.3.6 Deregulation of PI3K/Akt pathway in cancer ................................................................................... 38
  1.3.7 Targeting the PI3K/Akt pathway in cancer drug development ......................................................... 40
1.4 FORKHEAD TRANSCRIPTION FACTORS ............................................................................................ 41
  1.4.1 Overview: Forkhead transcription factors ......................................................................................... 41
    1.4.1.1 Forkhead chromosomal distribution, structure and regulation .................................................... 42
1.5 FOXO TRANSCRIPTION FACTORS ........................................................................................................ 43
  1.5.1 Overview: FoxO transcription factors ............................................................................................... 43
  1.5.2 Regulation of FoxO transcription factors in response to survival factors and cellular stress .......... 47
    1.5.2.1 Phosphorylation of FoxO factors which leads to inactivation of FoxO activity ......................... 49
    1.5.2.2 Phosphorylation of FoxO factors in response to cellular stress ................................................. 50
    1.5.2.3 Acetylation .................................................................................................................................... 51
    1.5.2.4 Ubiquitylation ............................................................................................................................... 53
  1.5.3 The function and downstream targets of FoxO. .............................................................................. 55
    1.5.3.1 Glucose metabolism .................................................................................................................... 55
    1.5.3.2 Apoptosis ..................................................................................................................................... 55
    1.5.3.3 DNA repair and detoxification: a role for FoxO in oxidative stress resistance and longevity ....... 56
    1.5.3.4 Cell cycle transitions .................................................................................................................... 60
    1.5.3.5 Differentiation ............................................................................................................................. 62
  1.5.4 B-Cell Translocation Gene -1 (BTG1) ............................................................................................... 64

OVERALL AIMS AND THESIS OUTLINE ....................................................................................................... 67

2 MATERIALS AND METHODS .................................................................................................................... 68

2.1 MAMMALIAN CELL CULTURE ............................................................................................................... 68
  2.1.1 Cell media .......................................................................................................................................... 68
  2.1.2 Sub-culturing of adherent cells ......................................................................................................... 68
  2.1.3 Preservation of cells ........................................................................................................................... 69
  2.1.4 Recovery of frozen cells .................................................................................................................... 69
2.1.5 Generation of a BTG1 inducible cell line ................................................................. 69
2.1.6 Sulphorhodamine B (SRB) assay ............................................................................. 70
2.1.7 DNA transfection of mammalian cells ....................................................................... 71
  2.1.7.1 Promoter assay ................................................................................................. 71
  2.1.7.2 Co-transfection reporter assay .......................................................................... 72
  2.1.7.3 Luciferase assay protocols ................................................................................ 72
2.2 FACS (FLUORESCENCE ACTIVATED CELL SORTING) ANALYSIS ................................ 74
  2.2.1 Fixing cells for FACS .......................................................................................... 74
  2.2.2 Propidium Iodide (PI) staining of fixed cells for PI FACS (cell cycle) analysis ...... 74
  2.2.3 PI FACS analysis ................................................................................................. 74
2.3 REACTIVE OXYGEN SPECIES (ROS) MEASUREMENT ............................................. 74
2.4 IMMUNOFLUORESCENCE ......................................................................................... 75
2.5 METHODS IN MOLECULAR BIOLOGY ...................................................................... 75
  2.5.1 Bacterial manipulation ......................................................................................... 75
    2.5.1.1 Bacterial strains ........................................................................................... 75
    2.5.1.2 Media and maintenance ................................................................................. 76
    2.5.1.3 Preparing chemically competent cells ........................................................... 76
    2.5.1.4 Bacterial transformations .............................................................................. 76
  2.6 DNA MANIPULATION ............................................................................................... 77
    2.6.1 DNA quantification ............................................................................................ 77
    2.6.2 Sequence analysis .............................................................................................. 77
    2.6.3 Restriction digests .............................................................................................. 77
    2.6.4 DNA ligation ........................................................................................................ 77
    2.6.5 DNA agarose gel electrophoresis ....................................................................... 77
    2.6.6 Extraction and purification of DNA ...................................................................... 78
    2.6.7 DNA precipitation ............................................................................................... 78
    2.6.8 Polymerase Chain Reaction (PCR) for DNA ....................................................... 78
    2.6.9 PCR for screening MCF-7 Tet-On/Off BTG1 cell colonies .................................. 80
    2.6.10 Plasmid constructs ............................................................................................ 80
      2.6.10.1 Plasmids used in this study ....................................................................... 80
      2.6.10.2 Plasmid construction .................................................................................. 82
    2.6.11 Screening for recombinant bacteria colonies ..................................................... 83
      2.6.11.1 Colony PCR ............................................................................................... 83
      2.6.11.2 Small scale plasmid preparation and restriction digest ................................ 83
      2.6.11.3 DNA sequencing ....................................................................................... 83
      2.6.11.4 Large scale plasmid preparation .................................................................. 84
  2.7 RNA MANIPULATION ............................................................................................... 84
    2.7.1 Total RNA extraction .......................................................................................... 84
    2.7.2 Real Time quantitative-PCR (RT-qPCR) ............................................................ 84
      2.7.2.1 First strand cDNA synthesis ....................................................................... 84
      2.7.2.2 Preparation of cDNA samples for RT-qPCR ................................................. 84
      2.7.2.3 Designing primers for RT-q PCR ................................................................. 85
      2.7.2.4 Quantification of gene expression ............................................................... 85
    2.7.3 Statistical analysis of RT-qPCR and luciferase data ............................................. 86
  2.8 PROTEIN ANALYSIS ............................................................................................... 86
    2.8.1 Preparation of total protein extracts ..................................................................... 86
    2.8.2 Determination of protein concentration .............................................................. 87
    2.8.3 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) ...... 87
    2.8.4 Western blotting of SDS-PAGE ......................................................................... 88
    2.8.5 Subcellular fractionation ..................................................................................... 90
    2.8.6 Immunoprecipitation .......................................................................................... 91
    2.8.7 Presentation of western blot data ........................................................................ 91
3 REGULATION OF FOXO GENES IN BREAST EPITHELIAL AND ENDOMETRIAL CELLS ................................................................. 93
  3.1 BACKGROUND AND OBJECTIVES ....................................................................... 93
3.2 RESULTS .......................................................................................................................... 94
  3.2.1 FoxO1 and FoxO3a expression levels........................................................................... 94
    3.2.1.1 Paclitaxel treatment induces the expression of foxO3a in MCF-7 cells ........ 94
    3.2.1.2 Paclitaxel treatment induces the expression of FoxO3a in BT-474 cells ....... 102
    3.2.1.3 The HEC-1-B endometrial cell line expresses FoxO1 ..................................... 104
    3.2.1.4 Paclitaxel treatment causes an increase in ROS (Reactive Oxygen Species) in MCF-7 cells .......................................................................................................................... 106
    3.2.1.5 H2O2 treatment of MCF-7 cells results in up-regulation of the expression of FoxO3a .......................................................................................................................... 109
  3.2.2 FoxO mRNA expression levels .................................................................................... 112
    3.2.2.1 The effect of paclitaxel and H2O2 treatment on FoxO mRNA expression levels in MCF-7 cells ................................................................................................................. 112
    3.2.2.2 FoxO3a mRNA expression levels increase in BT-474 cells following paclitaxel treatment ........................................................................................................................ 114
    3.2.2.3 FoxO1 mRNA expression levels are up-regulated in the HEC-1-B cell line following paclitaxel treatment ................................................................................................. 116
    3.2.2.4 Oxidative stress increases the stability of FoxO3a in MCF-7 cells ............... 118
  3.2.3 FoxO regulation at the DNA promoter level ................................................................ 121
    3.2.3.1 Both paclitaxel H2O2 induce FoxO promoter activity in MCF-7 cells .......... 121
    3.2.3.2 Paclitaxel treatment increases the FoxO1 promoter activity in HEC-1-B cells ................................................................................................................................. 124
    3.2.3.3 Analysis of the activity levels of FoxO3a promoter truncation constructs ... 126
    3.2.3.4 Mapping a region in the FoxO3a promoter responsive to paclitaxel treatment in MCF-7 cells .................................................................................................................... 128
    3.2.3.5 Mapping a region in the FoxO3a promoter responsive to paclitaxel treatment in BT-474 cells .................................................................................................................... 131
    3.2.3.6 The role of putative MEF2 DNA consensus binding sites in the regulation of FoxO3a promoter activity ................................................................................................. 133
    3.2.3.7 Over-expression of MEF2 increases the activity of the FoxO3a promoter in MCF-7 cells ............................................................ 136
    3.2.3.8 Co-transfection of MEF2A can further increase FoxO3a promoter activity following paclitaxel treatment ............................................................ 138
    3.2.3.9 Over-expression of MEF2A induces FoxO1 promoter activity .................... 140
  3.3 DISCUSSION .................................................................................................................... 144

4 THE ROLE OF BTG1 IN BREAST CANCER ..................................................................... 153

4.1 BACKGROUND AND OBJECTIVES .............................................................................. 153

4.2 RESULTS ......................................................................................................................... 154
    4.2.1 BTG1 is a downstream target of FoxO3a in COS-1 and MCF-7 cells ........... 154
    4.2.2 Induction in BTG1 expression levels following paclitaxel treatment in MCF-7 cells .......................................................................................................................... 157
    4.2.3 BTG1 expression levels change during the cell cycle ........................................ 159
    4.2.4 Generation of an inducible BTG1 cell line ....................................................... 161
    4.2.5 Over-expression of BTG1 decreases cell growth ........................................... 163
    4.2.6 Over-expression of BTG1 induces accumulation of cells in the G2/M phase of the cell cycle ............................................................... 165
    4.2.7 The effect of BTG1 over-expression on cell cycle regulators ....................... 167
    4.2.8 Up-regulation of BTG1 expression increases p21Cip1 and p27Kip1 expression at the promoter and mRNA levels .................................................................................. 170
    4.2.9 Over-expression of BTG1 decrease the transcription of cyclin D1 and cyclin E1 ... 172
    4.3 DISCUSSION ................................................................................................................. 174

5 REGULATION OF FOXO3A BY JNK1/2 .................................................................... 179

5.1 BACKGROUND AND OBJECTIVES .............................................................................. 179

5.2 RESULTS ......................................................................................................................... 180
5.2.1 Paclitaxel causes nuclear translocation of FoxO3a ............................................ 180
5.2.2 Inhibition of JNK1/2 by SP600125 prevents FoxO3a nuclear localisation in MCF-7 cells treated with paclitaxel ................................................................. 183
5.2.3 The effect of JNK1/2 on the sub-cellular localisation of FoxO3a is dependent on PI3K/Akt signalling .................................................................................. 185
5.2.4 JNK1 affects FoxO3a promoter activity in MCF-7 cells .................................... 187
5.2.5 JNK1 can phosphorylate FoxO3a .................................................................... 189
5.2.6 The effect of mutating FoxO3a’s serine 294 and serine 425 to alanines on the activity FoxO3a ......................................................................................... 193
  5.2.6.1 Mutating FoxO3a’s serine 294 and serine 425 to alanines reduces the ability of FoxO3a to induce the activity of the p27Kip1 promoter .................................. 193
  5.2.6.2 The Effect of mutating FoxO3a’s serine 294 and serine 425 to alanines on the ability of FoxO3a to induce BTG1 promoter activity ................................. 196
  5.2.6.3 Mutating both of FoxO3a’s JNK phosphorylation sites to alanine impairs FoxO3a’s ability to up-regulate Bim promoter activity .................................. 198
  5.2.6.4 FoxO3a cannot induce Bim (mut -164/-170) promoter activity ................. 200
5.2.7 Mutating FoxO3a’s serine 294 and serine 425 to alanines increases FoxO3a proteasomal degradation .......................................................... 202
5.2.8 Mutating FoxO3a’s serine 294 and serine 425 residues to either alanine or aspartic acid affects FoxO3a’s stability ...................................................... 205
5.2.9 The effect of mutating FoxO3a’s serine 294 and serine 425 to aspartic acids on the induction of BTG1 promoter activity ............................................ 207
5.3 DISCUSSION ....................................................................................................... 210
5.4 FUTURE WORK ............................................................................................... 215
6 FINAL DISCUSSION ........................................................................................... 217
  6.1 SUMMARY ................................................................................................. 217
  6.2 TRANSCRIPTIONAL REGULATION OF FOXO PROTEINS ......................... 218
  6.3 SENSITIVITY OF ER NEGATIVE AND ER POSITIVE CELLS TO PACLITAXEL 220
  6.4 CROSS-TALK BETWEEN JNK1/2 SIGNALLING CASCADE AND THE PI3K/AKT SIGNALLING PATHWAY ............................................................ 222
  6.5 THE ROLE OF BTG1 .................................................................................. 224
  6.6 FOXO TRANSCRIPTION FACTORS AS TUMOUR SUPPRESSORS .................. 226
  6.7 TARGETING FOXO IN CANCER THERAPY ............................................. 229
7 SUPPLEMENTARY DATA .................................................................................. 234
REFERENCES ...................................................................................................... 247
List of Figures

Figure 1-1: PI3K pathway targets AKT in mammalian cells with clear evidence of involvement in promotion of survival. ................................................................. 34
Figure 1-2: FoxO regulation, shuttling and downstream targets. ............................................................ 46
Figure 1-3: Post-translational modifications of FoxO proteins ............................................................. 48
Figure 3-1: Dose-dependent effect of 16 h paclitaxel treatment of MCF-7 cells. .............................. 98
Figure 3-2: The effect of paclitaxel treatment on the expression of FoxO, p27Kip1 and the activity of MAPK signalling in MCF-7 cells. ................................. 101
Figure 3-3: The effect of paclitaxel treatment on the expression of FoxO, p27Kip1 and the activity of JNK and p38 in BT-474 cells. ..................................................... 103
Figure 3-4: Differential expression of FoxO1 in HEC-1-B and Ishikawa cells following paclitaxel treatment. ................................................................................. 105
Figure 3-5: Treatment of MCF-7 cells with paclitaxel and hydrogen peroxide causes increase in oxidative stress. ............................................................ 108
Figure 3-6: Oxidative stress generated by H2O2 causes an increase in FoxO3a expression in MCF-7 cells ......................................................................................... 111
Figure 3-7: The effect of oxidative stress on the levels of FoxO3a and FoxO1 mRNA in MCF-7 cells. ................................................................................ 113
Figure 3-8: FoxO3a and p27Kip1 mRNA expression levels are up-regulated in BT-474 cells following paclitaxel treatment. .......................................................... 115
Figure 3-9: FoxO1 mRNA levels are induced in response to paclitaxel treatment in HEC-1-B but not Ishikawa cells. ................................................................. 117
Figure 3-10: Paclitaxel treatment increases the stability of FoxO3a in MCF-7 cells. ........................ 119
Figure 3-11: Oxidative stress increases the stability of FoxO3a in MCF-7 cells. ............................ 120
Figure 3-12: The activity levels of FoxO3a and FoxO1 promoters in response to oxidative stress in MCF-7 cells. ........................................................................ 123
Figure 3-13: The activity of the FoxO1 promoter is up-regulated in response to paclitaxel treatment in HEC-1-B but not Ishikawa cells. ............................. 125
Figure 3-14: The activity levels of FoxO3a promoter truncation constructs in MCF-7 cells. 127
Figure 3-15: The activity of the FoxO3a promoter truncation constructs in response to paclitaxel treatment in MCF-7 cells. ................................................. 130
Figure 3-16: The activity levels of the truncated FoxO3a promoter constructs in response to paclitaxel treatment in BT-474 cells. .......................................... 132
Figure 3-17: FoxO3a promoter constructs and activity levels with reference to the location of MEF2 DNA consensus binding sites ..................................... 135
Figure 3-18: The effect of MEF2 expression on the activity of the FoxO3a promoter in MCF-7 cells. ...................................................................................................... 137
Figure 3-19: Co-transfection of MEF-2 increases FoxO3a promoter activity in paclitaxel treated MCF-7 cells. .......................................................................... 139
Figure 3-20: Co-transfection of MEF-2 increases FoxO1 promoter activity in the paclitaxel treated MCF-7 cells. .......................................................................... 142
Figure 3-21: The effect of mutating MEF2 DNA consensus binding sites on the activity of the FoxO1 promoter in MCF-7 cells. ............................................. 143
Figure 3-22: Schematic representation of the changes in FoxO expression at the promoter, mRNA and protein levels in the various cell lines examined. .... 151
Figure 3-23: Schematic representation of the levels of phosphorylated JNK (P-JNK) and FoxO3a in reference to the levels of ROS in MCF-7 cells. ............................... 152
Figure 4-1: BTG1 is a downstream target of FoxO3a. ...................................................................... 156
Figure 4-2: BTG1 expression following paclitaxel treatment in MCF-7 cells. ................................. 158
Figure 4-3: BTG1 expression in synchronised MCF-7 cells. .............................................................. 160
Figure 4-4: The BTG1 MCF-7 Tet Off clone #34 expresses BTG1 in an inducible manner upon withdrawal of doxycycline from the medium. ......................... 162
Figure 4-5: Over-expression of BTG1 reduces cell growth. ............................................................ 164
Figure 4-6: Over-expression of BTG1 induces accumulation of cells in the G2/M phase of the cell cycle................................................................................................................. 166
Figure 4-7: The effect of BTG1 over-expression on cell cycle regulators.......................... 169
Figure 4-8: Induction in the expression of BTG1 affects the transcription of p21\textsuperscript{CIP1} and p27\textsuperscript{Kip1} at the promoter and mRNA levels...................................................... 171
Figure 4-9: Over-expression of BTG1 decreases the transcription of cyclin D1 and cyclin E1. ........................................................................................................................................ 173
Figure 5-1: Paclitaxel causes FoxO3a to translocate into the nucleus................................. 182
Figure 5-2: Inhibition of JNK1/2 by SP600125 prevents FoxO3a nuclear localisation in MCF-7 cells treated with paclitaxel................................................................. 184
Figure 5-3: Inhibition of JNK1/2 by SP600125 fails to prevent FoxO3a nuclear localisation in MCF-7 cells treated with inhibitors of PI3K and Akt........................................ 186
Figure 5-4: Activation of JNK1 induces the promoter activity of FoxO3a in MCF-7 cells.... 188
Figure 5-5: Akt and JNK phosphorylation sites on FoxO3a.................................................. 191
Figure 5-6: JNK1 can phosphorylate FoxO3a on Serine 294.............................................. 192
Figure 5-7: Mutating FoxO3a’s serine 294 and serine 425 to alanines impairs FoxO3a’s ability to induce p27\textsuperscript{Kip1} transcription at the promoter level............................ 195
Figure 5-8: The effect of mutating FoxO3a’s JNK phosphorylation sites to alanine on the ability of FoxO3a to up-regulate BTG1 promoter activity............................................. 197
Figure 5-9: Mutating both of FoxO3a’s JNK phosphorylation sites to alanines impairs FoxO3a’s ability to up-regulate Bim promoter activity................................................. 199
Figure 5-10: FoxO3a in unable to induce the promoter activity of a Bim promoter/reporter construct mutated at the FoxO DNA consensus binding site.................................... 201
Figure 5-11: Mutating FoxO3a’s serine 294 and serine 425 to alanines promotes proteosomal degradation of FoxO3a......................................................................................... 204
Figure 5-12: Mutating FoxO3a’s serine 294 and serine 425 to alanines promotes proteosomal degradation of FoxO3a......................................................................................... 204
Figure 5-13: FoxO3aS294D, S425D can induce Bim promoter activity................................. 209
Figure 6-1: Model summarising results obtained in this thesis coupled with predictive mechanism based on my results and available literature............................................. 233
Figure 7-1: Dose-dependent effect of 16 h paclitaxel treatment of MCF-7 cells.................. 234
Figure 7-2: The effect of paclitaxel treatment on the expression of FoxO, p27Kip1 and the activity of MAPK signalling in MCF-7 cells......................................................................................... 235
Figure 7-3: The effect of paclitaxel treatment on the expression of FoxO, p27Kip1 and the activity of JNK and p38 in BT-474 cells................................................................. 236
Figure 7-4: Differential expression of FoxO1 in HEC-1-B and Ishikawa cells following paclitaxel treatment........................................................................................................... 237
Figure 7-5: Oxidative stress generated by H\textsubscript{2}O\textsubscript{2} causes an increase in FoxO3a expression in MCF-7 cells............................................................................................................. 238
Figure 7-6: Paclitaxel treatment increases the stability of FoxO3a in MCF-7 cells. Paclitaxel treatment increases the stability of FoxO3a in MCF-7 cells............................................. 239
Figure 7-7: Oxidative stress increases the stability of FoxO3a in MCF-7 cells.................... 239
Figure 7-8: BTG1 expression following paclitaxel treatment in MCF-7 cells..................... 240
Figure 7-9: BTG1 expression in synchronised MCF-7 cells............................................... 240
Figure 7-10: The BTG1 MCF-7 Tet Off clone #34 expresses BTG1 in an inducible manner upon withdrawal of doxycycline from the medium............................................. 241
Figure 7-11: The effect of Btg1 over-expression on cell cycle regulators......................... 242
Figure 7-12: Paclitaxel causes FoxO3a to translocate into the nucleus............................... 243
Figure 7-13: Inhibition of JNK1/2 by SP600125 prevents FoxO3a nuclear localisation in MCF-7 cells treated with paclitaxel................................................................. 243
Figure 7-14: Inhibition of JNK1/2 by SP600125 fails to prevent FoxO3a nuclear localisation in MCF-7 cells treated with inhibitors of PI3K and Akt........................................ 244
Figure 7-15: JNK1 can phosphorylate FoxO3a on Serine 294........................................... 245
Figure 7-16: Mutating FoxO3a’s serine 294 and serine 425 to alanines promotes proteosomal degradation of FoxO3a................................................................. 246
Figure 7-17: Mutating FoxO3a’s serine 294 and serine 425 affects FoxO3a stability........... 246
List of Tables

Table 1: List of primers used for PCR amplification of DNA.......................................................... 80
Table 2: Plasmids used in this study ............................................................................................. 81
Table 3: PCR carried out to generate the plasmids...................................................................... 82
Table 4: Restriction digest and cloning of PCR products performed in order to generate the plasmids........................................................................................................ 83
Table 5: Primers used for RT-qPCR.............................................................................................. 86
Table 6: Proportions of the constituents of SDS-PAGE resolving and stacking gels............... 88
Table 7: Antibodies used for western blotting.............................................................................. 90
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Abbreviated Term</th>
</tr>
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<tbody>
<tr>
<td>4EBP1</td>
<td>Translation initiation factor 4E-binding protein1</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulphate</td>
</tr>
<tr>
<td>ASK1</td>
<td>Apoptosis signal-regulating kinase 1</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia telangiectasia mutated</td>
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<tr>
<td>ATR</td>
<td>Ataxia telangiectasia and Rad3 related</td>
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<td>BER</td>
<td>Base-excision repair</td>
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<td>BH3</td>
<td>Bcl-2 homology domain 3</td>
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<tr>
<td>BIM</td>
<td>Bcl-2 interacting mediator of cell death</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>CaMK</td>
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<td>Cellular Jun</td>
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<td>CKIs</td>
<td>Cyclin-dependent kinase inhibitors</td>
</tr>
<tr>
<td>CMF</td>
<td>Cyclophosphamide, methotrexate and fluorouracil</td>
</tr>
<tr>
<td>CML</td>
<td>Chronic myeloid leukaemia</td>
</tr>
<tr>
<td>CREB</td>
<td>CAMP-responsive element binding protein</td>
</tr>
<tr>
<td>CTMP</td>
<td>Carboxyl-terminal modulator protein</td>
</tr>
<tr>
<td>DAF</td>
<td>Abnormal DAuer Formation</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNA-PK</td>
<td>DNA-dependent protein kinase</td>
</tr>
<tr>
<td>DOX</td>
<td>Doxycycline</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>DYRK</td>
<td>Dual-specificity tyrosine-phosphorylated and regulated kinase</td>
</tr>
<tr>
<td>E2F4</td>
<td>E2F transcription factor 4</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ENOS</td>
<td>Endothelial nitric oxide (NO) synthase</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FASL</td>
<td>Fas Ligand</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FKH</td>
<td>Forkhead</td>
</tr>
<tr>
<td>FL</td>
<td>Full length</td>
</tr>
<tr>
<td>FOX</td>
<td>Forkhead Transcription Factors</td>
</tr>
<tr>
<td>FRE</td>
<td>FoxO-recognized element</td>
</tr>
<tr>
<td>G6Pase</td>
<td>Glucose 6 phosphatase</td>
</tr>
<tr>
<td>GADD45α</td>
<td>Growth arrest and DNA damage-inducible protein 45 alpha</td>
</tr>
<tr>
<td>GLUT4</td>
<td>Glucose transporter 4</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptors</td>
</tr>
<tr>
<td>G-Proteins</td>
<td>Guanine nucleotide–binding proteins</td>
</tr>
<tr>
<td>GSK3</td>
<td>Glycogen synthase kinase 3</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Abbreviated Term</td>
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<td>--------------</td>
<td>-------------------------------------------------------</td>
</tr>
<tr>
<td>H</td>
<td>Hours</td>
</tr>
<tr>
<td>HATs</td>
<td>Histone acetyltransferases</td>
</tr>
<tr>
<td>HAUSP/USP7</td>
<td>Herpes virus associated ubiquitin-specific protease</td>
</tr>
<tr>
<td>HDACs</td>
<td>Histone deacetylases</td>
</tr>
<tr>
<td>HIF1α or HIF2α</td>
<td>Hypoxia inducible factor α 1 or 2</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HSP27</td>
<td>Heat shock protein 27</td>
</tr>
<tr>
<td>ID1</td>
<td>Inhibitor of DNA binding 1</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor-1</td>
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<td>IGFBP1</td>
<td>IGF-binding protein-1</td>
</tr>
<tr>
<td>IKK</td>
<td>iκB kinase</td>
</tr>
<tr>
<td>ILK</td>
<td>Integrin-linked kinase</td>
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<tr>
<td>IP</td>
<td>Immuno-precipitate</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun N-terminal kinase</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>MAFbx</td>
<td>Atrogin-1</td>
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<td>MAPK</td>
<td>Mitogen Activated Protein Kinase</td>
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<tr>
<td>MBDs</td>
<td>Methyl-binding domain proteins</td>
</tr>
<tr>
<td>MDM2</td>
<td>Mouse Double Minute</td>
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<tr>
<td>MIN</td>
<td>Minutes</td>
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<tr>
<td>MMR</td>
<td>Mismatch repair</td>
</tr>
<tr>
<td>MnSOD</td>
<td>Manganese superoxide dismutase</td>
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<tr>
<td>MKKs</td>
<td>MAPK kinases</td>
</tr>
<tr>
<td>MKKKs</td>
<td>MAPK kinase kinases</td>
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<tr>
<td>MNK-1,2</td>
<td>MAPK interacting kinase-1,2</td>
</tr>
<tr>
<td>MKPs</td>
<td>MAP kinase phosphatases</td>
</tr>
<tr>
<td>MLK3</td>
<td>Mixed lineage kinase 3</td>
</tr>
<tr>
<td>MSK-1</td>
<td>Mitogen and stress activated protein kinase -1</td>
</tr>
<tr>
<td>MST1</td>
<td>Mammalian Ste20-like kinase-1</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>mTORC1</td>
<td>mTOR complex 1</td>
</tr>
<tr>
<td>Na₃VO₄</td>
<td>Sodium orthovanadate</td>
</tr>
<tr>
<td>NaF</td>
<td>Sodium fluoride</td>
</tr>
<tr>
<td>NER</td>
<td>Nucleotide-excision repair</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-κB</td>
</tr>
<tr>
<td>NLSs</td>
<td>Nuclear localization signals</td>
</tr>
<tr>
<td>NP-40</td>
<td>Nonidet P-40</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer solution</td>
</tr>
<tr>
<td>P/CAF</td>
<td>CBP-associated factor</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor receptor</td>
</tr>
<tr>
<td>PDGFR</td>
<td>Platelet-derived growth factor receptor</td>
</tr>
<tr>
<td>PDK 1</td>
<td>Phosphoinositide-dependent kinase 1</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>Peroxisome-proliferator-activated receptor γ coactivator 1 α</td>
</tr>
<tr>
<td>PH DOMAIN</td>
<td>Pleckstrin-homology domain</td>
</tr>
<tr>
<td>PEPCK</td>
<td>Phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td>PI</td>
<td>Propdium Iodide</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>p110α catalytic subunit</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>pRb</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>pRBL2 (p130)</td>
<td>Retinoblastoma-like 2</td>
</tr>
<tr>
<td>PRMT1</td>
<td>Argentine methyl transferase 1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Abbreviated Term</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PtdIns</td>
<td>Phosphatidylinositol phospholipid</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homologue deleted in chromosome 10</td>
</tr>
<tr>
<td>Rictor–TOR</td>
<td>Target of rapamycin</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Quantitative Real Time PCR</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SERMs</td>
<td>Selective estrogen receptor modulators</td>
</tr>
<tr>
<td>SGKs</td>
<td>Serum-and glucocorticoid-inducible kinases</td>
</tr>
<tr>
<td>SH2</td>
<td>Rous-sarcoma-oncogene homology-2 domain</td>
</tr>
<tr>
<td>SHIP1, SHIP2</td>
<td>Src-homology 2 (SH2)-containing phosphatases</td>
</tr>
<tr>
<td>SIR2</td>
<td>Silencing information regulator</td>
</tr>
<tr>
<td>SIRT1</td>
<td>Mammalian homologue of Silent Information Regulator-2 (SIR-2)</td>
</tr>
<tr>
<td>SAPK</td>
<td>Stress-activated protein kinase</td>
</tr>
<tr>
<td>SRB</td>
<td>Sulphorhodamine B</td>
</tr>
<tr>
<td>STE20</td>
<td>Sterile 20</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris Buffered Saline</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris Buffered Saline-Tween</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>TRADD</td>
<td>TNF receptor type 1 associated death domain</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>TRIS</td>
<td>Tris (hydroxymethyl) aminomethane</td>
</tr>
<tr>
<td>TWEEN 20</td>
<td>Polyoxyethylene-sorbitan monolaurate</td>
</tr>
<tr>
<td>TSC1/2</td>
<td>Tuberous sclerosis protein1/2</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>W/V</td>
<td>Weight to volume</td>
</tr>
<tr>
<td>W/W</td>
<td>Weight to weight ratio</td>
</tr>
<tr>
<td>YAP</td>
<td>Yes associate protein</td>
</tr>
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</table>
Publications


1 Introduction

1.1 Overview: cancer
Cancer is a genetic disease of somatic cells (Knudson, 2002). Six vital changes occur in the cell physiology, which jointly lead to malignant growth: self-sufficient growth (no growth signals required), growth in the presence of growth-inhibitory (antigrowth) signals, ability to avoid the cell’s apoptotic programme, endless proliferative potential, constant angiogenesis (growth of new blood vessels), and ability to cause metastasis (spread of malignant cells) and invade tissue. The formation of metastasis is considered the most life threatening characteristic of cancer (Hanahan and Weinberg, 2000).

According to national statistics and Cancer Research UK statistics (www.statistics.gov.uk, http://info.cancerresearchuk.org), one in three people develop cancer during their lifespan and one in four will die of cancer. The risk of getting cancer increases with age; of all cases diagnosed 74% of them are in people aged 60 and over and only 0.5% of them are in children (younger than 15 years). There are more than 200 types of cancer. The four most common types are breast, lung, bowel and prostate, together they account for over half of the new incidence. Women tend to develop cancer at an earlier age than men and this is mostly due to the high risk women have of developing breast cancer. Breast cancer is the most frequently diagnosed cancer in women aged 40-59 years in the UK and accounts for roughly half of all diagnosed cancers in this population.

Three types of genes have been shown to play a role in tumourigenesis: oncogenes, tumour-suppressor genes and stability genes. Nonetheless, cancer is not caused by a mutation in just one of these genes, but rather mutations have to occur in several genes in order for cancer to develop. Therefore, each mutation that occurs in a one of these genes increases the chances that cancer will develop (Vogelstein and Kinzler, 2004).
1.1.1 Genetic basis of cancer

Proto-oncogenes: A proto-oncogene is a gene which codes for a protein that can positively regulate cell proliferation and/or cell growth. Often proto-oncogenes encode proteins which play roles cellular signalling pathways, or are involved in transcriptional regulation. As long as an oncogene has no mutations and its function is regulated, it would be considered a proto-oncogene. However, some mutations can turn proto-oncogenes into oncogenes. These types of mutations in may result in the gene becoming either constitutively active or active under conditions different to that of the wild-type gene (Vogelstein and Kinzler, 2004). Mutations which can result in the activation of oncogenes include chromosomal translocation, gene amplifications or DNA point mutations (Vogelstein and Kinzler, 2004). An example of an oncogene is the phosphatidylinositol 3-kinase (PI3K) catalytic subunit, \textit{PIK3CA}, which will be discussed in detail later on in this introduction. Briefly, PI3Ks activity leads to an increase in cellular growth, inhibition of apoptosis and increase in cell survival. In several human cancers such as colon, breast and lung, the \textit{PIK3CA} gene has been found to be either amplified, deleted, or to harbour a somatic missense mutation that increases the activity of PI3K, leading to enhanced cellular proliferation (Karakas et al., 2006).

Tumour-suppressor genes: The proteins encoded by tumour-suppressor genes have been shown to play roles in processes such as inhibition of cell cycle, apoptosis and senescence. Mutations in tumour-suppressor genes reduce the activity of the protein encoded by the gene. Therefore, cells which express one wild type allele and one mutated allele are generally still able to prevent the development of cancer. Inactivation of tumour-suppressor genes usually results from either mutations which lead to the generation of a truncated protein, deletions, insertions or epigenetic silencing (Vogelstein and Kinzler, 2004).

\textit{p53} is the best studied example of a tumour suppressor and has been shown to play a role in several cellular processes such as inhibition of proliferation,
induction of cell cycle arrest, apoptosis and senescence. TP53, the gene which encodes p53, has been found to be mutated in most human tumours. Most TP53 germline mutations are missense mutations in the DNA binding domain of p53. Splicing mutations also occur frequently in germline, while germline deletion mutation in the TP53 gene are rare (Royds and Iacopetta, 2006).

Stability genes: Stability genes (also known as caretakers), when mutated, increase the likelihood of the development of cancer by a different mechanism to oncogenes and tumour suppressers. In healthy, non-cancerous, cells these genes play a role in the repair of subtle mistakes that occur during DNA synthesis and replication, some of which may have been induced through exposure to mutagens. Members of this class include the mismatch repair (MMR), base-excision repair (BER) and nucleotide-excision repair (NER) genes. Processes such as mitotic recombination and chromosomal segregation are controlled by other stability genes such as BRCA1, BLM and ATM (Vogelstein and Kinzler, 2004). The main role of stability genes is to keep genetic alteration to a minimum; therefore when these genes become mutated and/or inactivated, mutations accumulate at an elevated rate in replicating DNA (Friedberg, 2003). Therefore, inactivation of stability genes leads to an increase in genetic alterations in all replicating genes; however only mutations that confer a cellular growth advantage contribute to the tumourigenesis process. As with tumour suppressor genes, both the maternal and the paternal alleles of stability genes must harbour inactivation mutations in order to generate a physiological effect.

Mutations in all three classes can occur both in the germline and in single cells. Mutations in the germline cause a hereditary predisposition to cancer, while mutations in somatic cells lead to the development of sporadic tumours (Vogelstein and Kinzler, 2004). Individuals with a hereditary predisposition are more likely to develop multiple tumours at a relatively younger age compared with individuals who do not have an hereditary predisposition and
therefore must acquire all their cancer-gene mutations somatically, which statistically takes longer (Knudson, 2002).

Epigenetics: Epigenetic changes refer to changes in expression levels of genes that occur via a mechanism independent of DNA encoding, by alteration to the structure of the chromatin, the histone-DNA complex. A more condensed chromatin structure, termed heterochromatin, restricts access of transcriptional factors, while a relatively open chromatin structure, termed euchromatin, enables relatively easy access of transcription factors to the DNA. Changes in chromatin structure result from post-translational modifications that occur on histone proteins and from DNA methylation (Quina et al., 2006).

The histone proteins are spun around the DNA and are post-translationally modified, mainly by methylation, acetylation and ubiquitylation (Strahl and Allis, 2000). The term "histone code" is often used in reference to these histone modifications, and it is “read" by proteins which play a role in chromatin remodelling and transcriptional regulation. Hence, these post-translational modifications have a large impact on gene transcription (Jenuwein and Allis, 2001). Histone acetylation is the most extensively studied chromatin modification and is thought to generate a more open and transcriptionally active chromatin structure, as opposed to histone de-acetylation which condenses the chromatin, leading to a decrease in transcription. Histone acetyltransferases (HATs) covalently add an acetyl group to histones, while histone deacetylases (HDACs) catalyse the removal of acetyl groups from histone proteins; though the total level of histone acetylation is affected by the activity of both these enzymes (Struhl, 1998).

DNA methyltransferases catalyse the covalent addition of a methyl group to the DNA on a cytosine base 5’ to a guanine base, in a structure called a CpG dinucleotide. CpG are found in clusters in the DNA, which are termed CpG islands. CpG islands are generally found near or within promoters of genes. DNA methylation on CpG is highly regulated and is an important epigenetic mechanism which enables the silencing and imprinting of genes and as a
rule, methylation of the human DNA occurs on non-coding DNA (Bird, 2002). A well studied example of genomic imprinting and silencing is the methylation of CpG islands that are located on one copy of the female X chromosomes leading to the imprinting of the methylated X-chromosome (Herman and Baylin, 2003). Methylation induces gene silencing by both sterically preventing the binding of the basal transcription machinery to the DNA, and by recruiting methyl-binding domain proteins (MBDs), which repress DNA transcription also recruit other transcriptional repressors (Quina et al., 2006).

Epigenetic alterations have been implicated in tumourigenesis process. The genome of cancer cells has been shown to be DNA hypomethylated in comparison with non cancerous cells and, some gene-promoters have also been found to be hypermethylated leading to the silencing of expression of genes such as stability genes and tumour suppressors (Feinberg and Vogelstein, 1983; Jones and Baylin, 2002).

In summary, mutation in oncogenes, tumour suppressor genes and stability genes contribute to the initiation of cancer; however mutations must occur in a number of genes and not just in a single gene for cancer to occur (Vogelstein and Kinzler, 1993). Epigenetic changes may give rise to alterations in the expression levels of these three types of genes, which may increase the probability of the development of cancer.

Research conducted in this thesis focuses on breast cancer.

1.2 Breast cancer: overview and treatment  
Breast cancer is now the world’s most prevalent cancer and this rise in incidences has been attributed to changes in lifestyle (Parkin et al., 2005). The mammary gland develops from the skin epithelium and at 30 weeks from gestation the expression of Estrogen receptor (ER) alpha (ERα) gene is evident in the mammary epithelium and the expression levels ERα were found to rapidly increase after birth (Keeling et al., 2000). The majority of the mammary gland development process occurs at puberty in response to elevated levels of estrogen. Following puberty, the mammary gland is
subjected to cyclic proliferative activity, which occurs till menopause (Ali and Coombes, 2002). In addition to the ERα signalling pathway, which plays a critical role in the development of the mammary gland, other signalling pathways have also been shown to partake in this process and affect the development of the breast (Lewis et al., 1999). However, most therapies designed to treat breast cancer aim to block the activity of ERα because the ERα signalling pathway is involved in the regulation of processes such as cell proliferation (Levin, 2003).

Only some (approximately 20%) of the cells in the mammary epithelium, express the ERα and these cells were shown to not proliferate in response to estrogen, while the ERα negative cells surrounding them were found to divide in response to estrogen. However, ERα expressing cells, derived from ERα-positive breast tumour cells, were shown to divide and replicate in response to ERα (Clarke et al., 1997). In addition, over-expression of ERα in normal breast tissue has been suggested to increase the risk of developing breast cancer (Khan et al., 1998). Overall, this suggests that the presence of ERα may serve to indicate the probability of developing breast cancer and may also be indicative of sensitivity to therapy which targets the ERα signalling pathway (endocrine therapy). Pre-menopausal women with ERα-positive tumours either undergo surgery (oophorectomy) or receive endocrine therapy, which aims to either reduce the levels of estrogen in the blood or to block the ERα (Buchanan et al., 1986). Post-menopausal women with ERα-positive breast cancer usually receive aromatase inhibitors, which block the production of estrogen (Ali and Coombes, 2002).

Selective estrogen receptor modulators (SERMs) are ligands, which are used to lower ERα activity (Varshochi et al., 2005). SERMs include drugs such as ICI 182, 780, raloxifene and tamoxifen. Tamoxifen is the most commonly used SERM and in used for treatment of all stages of ERα-positive breast cancer (Varshochi et al., 2005). Tamoxifen has both agonistic and antagonistic functions, in the breast tamoxifen acts as an antagonist of estrogen while in the endometriumn, tamoxifen acts as an agonist of
estrogen. Although tamoxifen is frequently used, this therapy is not always highly effective and this because most breast cancer patients who initially benefited from the use of tamoxifen, ultimately gain resistance (Johnston et al., 1995). As mentioned above, therapeutic agents that prevent estrogen synthesis by inhibiting aromatase are also used for treatment of ERα positive breast cancer patients and can be used once resistance to tamoxifen has developed. Aromatase inhibitors include molecules such as exemestane and anastrazole and letrozole (Ali and Coombes, 2002). However, once a patient relapses on these therapies, other approaches need to be applied. Patients with ERα -positive tumours who became resistant to endocrine therapy and patients with ERα -negative tumours, which cannot benefit from endocrine therapy, are treated with inhibitors of growth factor activity. Well defined targets for breast cancer treatment are EGFR (epidermal growth factor receptor) and ERBB2/HER2/neu (a member of the EGFR family), which high levels of activity were found to associate with a bad disease prognosis (Spigel and Burstein, 2002). Several strategies have been deployed to target these growth factors signal transduction pathways, such as molecules that inhibit tyrosine kinase and monoclonal antibodies that target EGFR and HER2 (Nahta et al., 2003). The most known example is trastuzumab, a monoclonal antibody which targets the HER2 receptor and was shown to have clinical activity both as a single agent and in combination with chemotherapy in patients with tumours that over-express HER2 (Smith and Chua, 2006).

Adjuvant chemotherapy is mostly used for treatment of younger women with moderate to high risk breast cancer. Chemotherapy usually involves treatment with a combination of three different drugs such as cyclophosphamide, methotrexate and fluorouracil (CMF). CMF is often combined with anthracyclines (inhibitors of DNA and RNA synthesis), such as doxorubicin or epirubicin, as this was shown to further increase survival. For patients with node positive cancer (when cancer cells from the tumour in the
breast can be found in the lymph nodes) it has been demonstrated that adding taxanes (paclitaxel and docetaxel) to anthracyclines further improves disease free survival in these women (Smith and Chua, 2006). Moreover, treatment with trastuzumab in combination with one the taxanes has been shown ti increase survival in patients with metastatic breast cancer (Luck and Roche, 2002).

1.2.1 Taxanes in cancer treatment

Taxanes, paclitaxel and docetaxel, are a type of chemotherapy used for treatment of cancers, such as breast and non-small cell lung cancer, (Gelmon, 1994). Paclitaxel, a complex diterpene, originates from the bark of the Pacific yew tree, *Taxus brevifolia* (Wani et al., 1971). Researchers examined the bark of the Pacific yew tree following positive results obtained from a previous screening process aimed at finding new drugs for treatment of cancer (Altmann and Gertsch, 2007). In 1992 paclitaxel was launched for treatment of stage 4 ovarian cancer and is now also used for treatment of other cancers, as mentioned above. Since the production of paclitaxel proved to be an expensive process, mainly due to the lack of availability of barks of Pacific yew trees, docetaxel, a semi-synthetic paclitaxel analogue, was developed using the bark of the European yew tree and is now used almost interchangeably with paclitaxel.

The anti-proliferative activity of paclitaxel has been attributed to the ability of paclitaxel to bind to polymerised αβ-tubulin, thereby stabilising cellular microtubules and causing mitotic arrest (Schiff et al., 1979). Paclitaxel was principally shown to inhibit the formation of mitotic spindles, which prevents chromosomes from segregating (Long and Fairchild, 1994). In addition, at therapeutic concentrations paclitaxel was shown to induce two forms of cell cycle arrest and each one was shown to lead to the induction of a different apoptotic pathway. Arrest at M phase was shown to induce cell death by a p53 independent pathway, while arrest at G1 was shown to induce apoptosis via a p53 dependent pathway (Woods et al., 1995). Nonetheless, paclitaxel was also shown to affect the activity of cellular signalling pathways.
Paclitaxel has been shown to activate the Jun N-terminal Kinase (JNK)/Stress Activated Protein Kinase (SAPK) signalling pathway in cancer cells by activating either the apoptosis signal-regulating kinase (ASK1) or the Ras GTP binding protein (Wang et al., 1998). JNK may induce apoptosis by phosphorylation of the B-cell lymphoma 2 (Bcl-2) protein (Yamamoto et al., 1999). Paclitaxel also activates p38 MAPK, another member of the SAPK family, and inhibition of this kinase was surprisingly found to protect cells from paclitaxel induced cell death (Seidman et al., 2001). Paclitaxel treatment has been shown to activate the mitogen-activated protein kinases (MAPK) extracellular signal-regulated kinase 1/2 (ERK1/2) and induce apoptosis (Bacus et al., 2001; Seidman et al., 2001). However, another report (MacKeigan et al., 2000), showed enhanced apoptosis in cells in response to paclitaxel treatment following inhibition of ERK1/2. Moreover, elevated levels of activation of the phosphatidylinositol 3-kinase (PI3K)/Akt (PKB) signalling pathway has been shown to protect cells from paclitaxel induced cell death (Page et al., 2000; VanderWeele et al., 2004).

Recently, it has been demonstrated that increase in oxidative stress is one of the first events that occurs following paclitaxel treatment and is essential for the induction of cell death (Alexandre et al., 2006). This may be due to the suggested role reactive oxygen species (ROS) play in tumourigenesis process (Szatrowski and Nathan, 1991). Furthermore, while paclitaxel treatment was found to increase the levels of H\textsubscript{2}O\textsubscript{2} in the cells; it was shown that agents which decrease the levels of free radicals diminish paclitaxel induced cell cycle arrest and apoptosis (Ramanathan et al., 2005). It has been shown that enhanced ROS production leads to an increase in the activity of JNK and p38 MAPK and that oncogenically transformed cells, which are more sensitive to anticancer agents, have higher levels of ROS and JNK activity (Benhar et al., 2001). Taken together, this indicates that control of ROS levels and regulation MAPK signalling may affect cellular response and sensitivity to anticancer drugs, such as taxanes.
Taxanes have proven to be important chemotherapeutic agents in treatment of breast cancer. Taxanes do not target the ERα signalling pathway and therefore can be used for treatment of patients with ERα negative breast cancer tumours and for the treatment of patients which are resistant to endocrine therapy. Furthermore, it has been observed that paclitaxel treatment is more effective for treatment of patients with ERα negative breast tumours than for treatment of those with ERα positive tumours (Razandi et al., 2000; Stearns et al., 2003). A recent study has shown that expression of ERα in the ERα negative cell line, BCap37 reduces the ability of paclitaxel to induce apoptosis, but does not prevent paclitaxel induced mitotic arrest (Sui et al., 2007). The same study also showed that the addition of the anti-estrogen ICI 182,780, overturned the resistance of ERα -positive BCap37 cells to paclitaxel and increased the sensitivity of the MCF-7 and T47D cell lines to paclitaxel. Overall, these observations suggest a link between resistance to cancer therapy and expression of ERα, as it was shown that and imply that ERα paclitaxel can be used to which further confirms the correlation between ERα and drug resistance in ERα positive tumour cells. These findings suggest that ERα expression and activity facilitate resistance to paclitaxel-induced apoptosis in breast cancer cells.

In summary, while ERα signalling is important for the development of healthy breast tissue it also plays a significant role in breast cancer tumourigenesis. To date, the most successful way to treat breast cancer has been by either blocking ERα activity or by reducing estrogen concentrations. However, these treatments are not beneficial against ERα negative tumours. Furthermore, most patients with ERα positive breast cancer tumours eventually develop resistance to endocrine therapy. Chemotherapy is mostly used for treatment of younger women with moderate to high risk breast cancer, and the most commonly used regimens are combination therapies with drugs such as CMF together with anthracyclines and/or taxanes. Taxanes, paclitaxel and docetaxel, stabilise cellular microtubule and induce a number of cellular
signalling events. The exact mechanism taxanes exploit to induce cell death has not been fully elucidated; but has been linked to the increase observed in the level of ROS following treatment with paclitaxel. Elevated levels of ROS have been shown to stimulate the activity of MAPK signalling pathways such as JNK. The efficacy of treatment of breast cancer patients with paclitaxel has also been shown to be influenced by the activity of the PI3K/Akt signalling pathways, as elevated levels of PI3K/Akt activation were shown to protect cells from the apoptotic effect of paclitaxel.

The PI3K signalling pathway regulates cellular processes, such as cell cycle entry, cell survival, proliferation, growth, metabolism and cell migration. Elevated levels of PI3K/Akt activity have been shown to contribute to the tumourigenesis process and to the protect cells from cytotoxic effects of chemotherapy, with agents such as paclitaxel (Page et al., 2000; VanderWeele et al., 2004). Hence, understanding the role and regulation of this signal transduction pathway in healthy and cancerous cells may significantly contribute to cancer therapy development.

1.3 The phosphatidylinositol 3-kinase (PI3K)/ Akt (PKB) signalling pathway

1.3.1 Overview of PI3K pathway and family members
The PI3Ks constitute a large family of lipid and serine/threonine kinases which include a number of phosphatidylinositol kinases, such as ataxia telangiectasia and Rad3 related (ATR) kinase and the related DNA-dependent protein, ataxia telangiectasia mutated (ATM) kinase (Fruman et al., 1998; Hennessy et al., 2005). This family of proteins initiate and take part in the signal transduction.

The PI3K family of proteins are activated by a receptor that could be a G-protein coupled receptor or a trans-membrane tyrosine kinase receptor. The receptors bind to their ligands (growth factor or hormone molecule) on the outer-side of the membrane, and then activate the signal transduction through the PI3K family of proteins. This process translates a
chemical/hormonal extracellular signal into an intra-cellular phosphorylation cascade, initiated by the PI3K proteins.

The PI3K family of proteins have been divided into three classes (I II and III) (Fruman et al., 1998; Vanhaesebroeck et al., 1997). PI3K class I are defined by a heterodimeric form, and consist of an associated regulatory subunit and a catalytic subunit. Class I has been further divided into sub-classes; class 1A PI3K enzymes are heterodimers of one of the p110α, p110β or p110δ catalytic subunits and one of the p50-55/p85 regulatory subunits. The Class IB PI3K enzymes are heterodimers of the p110γ catalytic subunit bound to either p101 or p84 regulatory subunits. Class 1A and class 1B PI3Ks are initially activated by membrane bound tyrosine kinases and G-protein-coupled receptors, respectively. Following their activation, class I PI3Ks phosphorylate phosphatidylinositol phospholipid (PtdIns) and PtdIns(4,5)P2 giving rise to PtdIns(3,4,5)P3. PtdIns(3,4,5)P3, are also membrane bound, serve as an anchor for a subgroup of pleckstrin-homology (PH) domain proteins (Hawkins et al., 2006; Wymann and Marone, 2005).

Class II of PI3Ks are large proteins (170–210 kDa) that consist the phosphoinositide kinases conserved “PIK domain” and a catalytic kinase domain which is only 45–50% similar to class I of PI3Ks (Fruman et al., 1998). Class II PI3Ks seem to preferentially phosphorylate PtdIns and PtdIns(4)P in vitro (Fruman et al., 1998). Three class II isoforms have been identified in mammals; PI3K-C2α isoform and the less ubiquitously expressed isoforms, PI3K-C2β, and liver-specific PI3K-C2γb (Hennessy et al., 2005).

Class III of PI3Ks are heterodimers that are made of a catalytic (Vps34, 100 kDa) subunit and an adaptor (p150) subunit. Class III PI3Ks phosphorylate only PtdIns to give rise to PtdIns(3)P. Class III PI3Ks are associated with endocytosis, receptor internalisation, vesicle trafficking, and have been implicated in regulation of autophagy, a process which enables survival under stress conditions (Lemmon, 2003; Wymann and Marone, 2005).
Of all three classes of PI3Ks, class I is the best characterised. PI3K class I are activated by a large number of cell surface receptors, which include the majority of growth factors, many inflammatory stimuli, hormones, neurotransmitters and antigens (Hawkins et al., 2006). Furthermore, mutations in the PI3Kα, a PI3K class IA member, whose catalytic domain is encoded by *PIK3CA*, has been found to be associated with several tumour types and have been suggested to have oncogenic potential (Samuels and Ericson, 2006).

1.3.2 **PI3K class I: regulation and role**

PI3K class IA are activated by growth factor receptor following binding to their ligands. The receptors are tyrosine kinase receptors may autophosphorylate or phosphorylate their substrates. The phosphorylated tyrosines on the receptors intracellular domains or on their substrate molecules are recognised by the SH2 domains (Rous-sarcoma-oncogene homology-2 domain), and the p85 subunit directly interact with the activated receptor tyrosine kinase. Consequently, this interaction relieves an inhibitory effect on the p110 subunit which can now catalyse the phosphorylation of its substrates, the PtdIns, at their 3' position (Samuels and Velculescu, 2004; Cantley, 2002). The regulatory p85 subunit interacts with the transmembrane tyrosine kinase-linked receptors, but tyrosine kinase receptors are only part of its activators, as it also binds to and incorporates signals from a range of intracellular proteins such as Rac, Rho, hormonal receptors, protein kinase C (PKC), mutated Ras and Src (Hennessy et al., 2005). It has been suggested that the main role of class IA PI3Ks is to direct energy into cell growth and proliferation (Wymann and Marone, 2005). The p110 and p85 are the only class IA PI3Ks isoforms associated with tumours (Samuels and Velculescu, 2004). p110 and p85 have isoform specific processes, but share some overlap in their function as reviewed by Henessy *et al* (Hennessy et al., 2005).

PI3Ky, the only class IB member (locus PIK3cy), heterodimerises with either a p101 or p87 regulatory subunits (Wymann and Marone, 2005). The PI3Ky
heterodimer is activated, at the membrane, by the guanine nucleotide-binding proteins (G proteins) on a G-protein coupled receptor. PI3Kγ is highly expressed in lymphocytes, and is involved in controls processes of inflammation and allergy (Deane and Fruman, 2004; Wymann et al., 2003). PI3Kγ is also associated with the control of vascular tone and heart contractility (Crackower et al., 2002; Oudit et al., 2004; Patrucco et al., 2004; Vecchione et al., 2005).

1.3.3 Downstream activities of class I PI3K
The product of active PI3Ks are the PtdIns(3,4,5)P3 (PIP3) generated by phosphorylations of phosphatidylinositol-4,5-bisphosphate PtdIns(4,5)P2 (Hawkins et al., 2006; Wymann and Marone, 2005). PIP3 are secondary messengers, and recruit downstream target substrates for phosphorylation by binding to pleckstrin-homology (PH), Phox (PX) and other lipid-binding domains in these substrates. Genetic screens in model organisms have identified the serine/threonine kinase Akt (Protein Kinase B), a member of the AGC family of serine/threonine kinases, as the primary downstream mediator of the effects of PI3K. Another target protein activated by PIP3 is the phosphoinositide-dependent kinase 1 (PDK 1) molecule (Vivanco and Sawyers, 2002).

The PI3K signalling is stopped by segregation of the P(3,4,5)P3. There are two known phosphatases that remove the phosphate group of P(3,4,5)P3. the phosphatase and tensin homologue (PTEN) phosphatase and the Src-homology 2 (SH2)-containing phosphatases (SHIP1 and SHIP2) (Cantley, 2002).

SHIP1 and SHIP2 dephosphorylate the 5' position of the inositol ring leading to the production of P(3,4)P2, while PTEN dephosphorylates the 3' position of P(3,4,5)P3 to produce P(4,5)P2. deficiency in SHIP2 leads to insulin hypersensitivity, suggesting that this phosphatase is a crucial regulator of the PI3K signalling pathway downstream of insulin (Clement et al., 2001).
PTEN deficiency and mutations are associated with advanced malignancies in human, suggesting that uncontrolled, constitutively active PI3K pathway is highly tumourigenic (Maehama and Dixon, 1999).

The main downstream target of PI3K pathway is the Akt (PKB) protein. Akt phosphorylates several downstream target proteins and is involved in processes of cell growth, cell cycle and survival.

1.3.4 Akt activation

Akt (PKB) is an important signalling intermediate for controlling metabolic activity, growth and proliferation. There are 3 AKT isoforms which share homology with the viral v-Akt and with the AGC kinase family. The three Akt isoforms are derived from distinct genes (Akt1/PKBα, Akt2/PKBβ and Akt3/PKBγ) (Manning and Cantley, 2007).

Cytoplasmic Akt is recruited to the plasma membrane by the PIP3 molecules which bind the N-terminal PH domain on Akt. The PIP3 interaction results in conformational change that exposes Akt to phosphorylation on two separate residues (threonine 308 and serine 473 in Akt1). Threonine 308 is phosphorylated by PDK1, a constitutively active kinase that is recruited by the PH domain as well. Serine 473, located within the hydrophobic C-terminal domain, is phosphorylated by PDK2 (an unknown molecule yet). All this takes place in a complex that is anchored to the plasma membrane by PIP3 molecules. Phosphorylation of Akt on threonine 308 stabilises Akt’s activation loop, while phosphorylation of serine 473, activates Akt (refers to Akt1, only) (Alessi et al., 1996; Blume-Jensen and Hunter, 2001).

Several reports have suggest that cyclic adenosine monophosphate (cAMP)-elevating agents activate AKT in a independently of PI3K, indicating that Akt is a possible phosphorylation target of protein kinas A (PKA). Neither the PH domain of Akt, nor phosphorylation of Serine 473, is required for PKA induced activation of Akt However, phosphorylation of Threonine 308 is essential. The mechanism by which PKA activates Akt remains to be elucidated as well (Filippa et al., 1999; Sable et al., 1997).
Another site of phosphorylation that is probably meaningful to the regulation and activation of Akt is tyrosine 474. It is phosphorylated in response to insulin. This phosphorylation has been implied to be involved in the activation of Akt (Conus et al., 2002). Since tyrosine 474 is widely conserved in the AGC family, it is possible that this mechanism of regulation may be evolutionarily conserved, however, more data is required to determine the importance of this phosphorylation (Conus et al., 2002).

More proteins that regulate Akt’s activity have been suggested as alternatives to PI3K activation. The Carboxyl-terminal modulator protein (CTMP) was found to bind to Akt resulting in a reduction in phosphorylation on Threonine 308 and Serine 473, thereby inhibiting the activity of Akt (Maira et al., 2001).

Positive regulators of Akt have also been identified. For example; the heat shock protein 27 (Hsp27) that was first suggested to activate Akt (Konishi et al., 1997). The function of Hsp27 has been further investigated by Rane et al, who have demonstrated that the interaction between Hsp27 and Akt up-regulates Akt activity and inhibits apoptosis in neutrophils (Rane et al., 2003).

Akt plays an important role in increasing cell survival, cell growth and to inhibit apoptosis and cell cycle arrest. A schematic overview of Akt activation and downstream cellular targets is presented in figure 1.1.
Figure 1-1: PI3K pathway targets AKT in mammalian cells with clear evidence of involvement in promotion of survival. Adapted from Song et al., 2005 and Downward 2004.
1.3.5 Downstream targets of Akt

The downstream targets of Akt play a role in enhancing cell survival, cellular metabolism, cell growth and proliferation. Some targets are directly regulated by Akt, while other cellular signalling pathways, such as the NF-κB, SAPK and p53 signalling pathways may be affected by Akt activation in a manner that increases pro-survival signalling.

Akt plays a role in increasing cell survival in more than one way. The best established mechanism include inactivation through phosphorylation of pro-apoptotic proteins such as BAD, a Bcl-2 homology domain 3 (BH3)-only protein (Datta et al., 1997; del Peso et al., 1997). Furthermore, it has been reported that activated Akt can phosphorylate procaspase-9, resulting in a decrease in the protease activity of caspase-9 activity (Cardone et al., 1998).

Another mean by which Akt promotes cell survival is by phosphorylation of glycogen synthase kinase 3 (GSK3), which results in inhibition of its activity and an increase in storage of glucose as glycogen (Cross et al., 1995). Although it has been demonstrated that inhibition of GSK3 protects against apoptosis in many conditions, the molecular basis for this is not fully understood but has been linked to the role of Akt in cell metabolism (Pap and Cooper, 1998).

Akt downstream effects are mostly associated with the promotion of cell growth. This predominantly occurs by activation of mammalian target of rapamycin (mTOR) complex 1 (known as either mTORC1 or mTOR-raptor complex) (Manning et al., 2002). mTORC1 is controlled by cell signalling that is stimulated by growth factors and nutrients, and it plays a central role in translation initiation complexes and ribosome biogenesis (Wullschleger et al., 2006). Akt indirectly activates mTORC1 by phosphorylating and by inhibiting the mTORC1 negative regulator, tuberous sclerosis protein-2 (TSC2) (Manning et al., 2002). mTORC1 inhibitors, such as rapamycin have been shown to have an inhibitory effect over proliferation of cancer cells in vitro and in tumours in mouse models that were shown to posses oncogenic
activity due to increased PI3K/Akt pathway, suggesting that mTORC1 is an essential downstream target of Akt (Sabatini, 2006).

More AKT important targets are the cyclin-dependent kinase inhibitors $p27^{\text{Kip1}}$ and $p21^{\text{Cip1}}$. Akt has been found to stimulate cell proliferation by blocking the $p27^{\text{Kip1}}$ and $p21^{\text{Cip1}}$ CDK inhibitory activity by phosphorylating of $p27^{\text{Kip1}}$ and $p21^{\text{Cip1}}$ (Liang et al., 2002; Zhou et al., 2001a), resulting detention of $p27^{\text{Kip1}}$ and $p21^{\text{Cip1}}$ from the nucleus, therefore, leading to an attenuation in their ability to inhibit the cell cycle (Sekimoto et al., 2004; Zhou et al., 2001b).

The GSK3 and TSC2 proteins are targets for Akt dependent phosphorylation. The phosphorylated GSK3 mediates the downstream phosphorylation of cyclins and transcription factors which play a role in the transition from the G1 phase to the S phase of the cell cycle, the phosphorylation by GSK mark the substrate proteins for an E3 ligase that ubiquitylates these proteins for proteasome degradation. Therefore, the phosphorylations of GSK3 by Akt inhibits its activity and stabilises cyclins and transcription factors that promote G1-S transition, and induce cell cycle progression and proliferation (Diehl et al., 1998; Wei et al., 2005; Welcker et al., 2003; Yeh et al., 2004).

Furthermore, Akt phosphorylation of TSC2 and subsequent activation of mTORC1 leads to a complementary increase in translation of proteins that are important for cell cycle progression such as CDKs (Mamane et al., 2004).

As mentioned above, Akt signalling pathway cross-talks with other cellular signal transduction pathways, resulting in an overall positive response on cell survival, proliferation and growth. Akt can phosphorylate kinases upstream of SAPK, such as Apoptosis signal-regulating kinase 1 (ASK1), a MAP kinase kinase kinase (MKKK), which results in the inhibition of ASK1 induced apoptosis (Kim et al., 2001). Akt has also been shown to phosphorylate the mixed lineage kinase 3 (MLK3), thereby, inactivating MLK3 and promoting of cell survival (Barthwal et al., 2003).

Akt also affects the p53 pathway by regulating the main p53 controller protein, the mouse double minute (MDM2 or HDM2 in humans), an E3 ubiquitin ligase that marks p53 for proteasome degradation. Akt
phosphorylates MDM2, which translocates MDM2 into the nucleus, leading to MDM2 interaction with p53 and subsequent degradation of p53 (Mayo and Donner, 2001; Zhou et al., 2001b). Reduction of the amount of p53 in the cell, allows quicker cell proliferation. p53 is a transcription factor that transactivates the expression of genes that are associated in induction of apoptosis, such as the BH3-only proteins Puma and Noxa (Villunger et al., 2003).

Akt downstream phosphorylation also reduces the activity of other p53 family members like p73. p73 induces apoptosis by up-regulating the expression of similar target genes as p53 (Strano et al., 2001). Akt phosphorylates the Yes Associated Protein (YAP), a transcriptional co-activator which complexes with p73 to induce its transcription factor activity. The phosphorylated YAP is exported to the cytoplasm, therefore, inhibiting p73 activity (Basu et al., 2003).

Positive cross-talk between the Akt and NF-κB pathways results in the expression of pro-survival genes, such as Bcl-xL and caspase inhibitors (Barkett and Gilmore, 1999). Akt has been reported to phosphorylate cAMP response element binding protein (CREB) transcription factor, up-regulating the transcriptional activity of CREB (Du and Montminy, 1998).

The best studied Akt downstream targets involved in transcriptional control of cell survival are three members of the FoxO subfamily of Forkhead transcription factors, FoxO1, FoxO3a, FoxO4 (previously known as FKHR, FKHRL1 and AFX) (Biggs et al., 1999; Brunet et al., 1999; Kops et al., 1999). Several downstream targets of FoxO are important in apoptosis, for instance, Fas ligand (Brunet et al., 1999), TRAIL (TNF-related apoptosis-inducing ligand) (Modur et al., 2002), TRADD (TNF receptor type 1 associated death domain) and Bim (Bcl-2 interacting mediator of cell death) (Burgering and Medema, 2003). Other targets of FoxO have been implicated in inhibiting cell cycle progression, such as p27Kip1 and cyclin G2 (Martinez-Gac et al., 2004; Medema et al., 2000; Nakamura et al., 2000).
The activity of the PI3K/Akt pathway increases following growth factor stimulation. However, uncontrolled activation of the PI3K/Akt pathway leads to an increase in cell growth and survival, which ultimately, results in a selective growth advantage, metastatic competence and occasionally therapy resistance. Furthermore, deregulation of the PI3K/Akt pathway is a frequent cause in cancer and in neoplastic transformation.

1.3.6 Deregulation of PI3K/Akt pathway in cancer
Angiogenesis: The vascular endothelial growth factor (VEGF) activates the PI3/Akt pathway in endothelial cells (Olsson et al., 2006). In turn, the Akt phosphorylates and activates endothelial nitric oxide (NO) synthase (eNOS) (Dimmeler et al., 1999; Fulton et al., 1999) and the NO that is released, induces angiogenesis (generation of new blood vessels) (Morbidelli et al., 2003). Moreover, the active Akt pathway, increases the levels of the transcription factors hypoxia inducible factor α (HIF1α and HIF2α) which induce the expression and secretion of VEGF and other angiogenic factors, thus, inducing angiogenesis both in an autocrine and paracrine fashion (Gordan and Simon, 2007).

Another manner, in which deregulation of the PI3K/Akt pathway may occur and contribute to tumourigenesis, is via mutations in the p110α catalytic domain of PI3K. Functional analyses of mutations in the gene coding for PIK3CA (the gene which encodes the p110α subunit) revealed that mutations that increase p110α enzymatic activity, can lead to constant Akt activation and result in growth-factor independent cell growth and increased cell invasion and metastasis.

PIK3CA mutations in human cancers are somatic, cancer-specific and heterozygous. Most of the mutations are missense mutations and to date, no truncations or nonsense mutations were detected (Samuels and Ericson, 2006; Campbell et al., 2004; Lee et al., 2005b; Samuels and Velculescu, 2004). The PIK3CA cancer specific mutations are recurrent in patient samples, suggesting that these mutations provide a proliferative advantage. PIK3CA mutations map to three separate sites in p110α. Mutations that occur
any of the three sites, result in increased enzymatic activity (Kang et al., 2005; Samuels and Velculescu, 2004).

Mutations in p110α and other mechanisms that activate PI3K signalling seem to be mutually exclusive (Bader et al., 2005). For example, mutations in the p85α subunit and chromosomal translocations are generally rare, but have been detected in some human cancers. A human lymphoma cell line was shown to express a C-terminal truncated form of p85 (Jucker et al., 2002). Mutant p85 subunits render the PI3K/Akt signalling pathway constitutively active (Borlado et al., 2000; Jimenez et al., 1998; Philp et al., 2001). Nonetheless, in breast cancer, the most common genetic alterations are somatic missense mutations in the PIK3CA gene and these mutations are mostly found in HER2-amplified and ERα-positive breast cancers (Campbell et al., 2004).

Other targets of genetic aberrations, in this pathway, are PTEN and Akt. Akt2 is frequently amplified, at the genome level, in pancreatic, breast and ovarian cancers and Akt3 is frequently over-expressed, in hormone-insensitive breast and prostate tumours (Testa and Bellacosa, 2001). These observations are in agreement with findings that indicate that the different isoforms of Akt have specific tumourigenic functions, Akt2 in motility and invasion and Akt3 in hormone independence (Arboleda et al., 2003).

The PI3K/Akt pathway may be desregulated by aberrations in molecules and signalling pathways that are related to the PI3K/Akt, but are not part of it, for example, in pancreatic cancers which frequently harbour Ras mutations that activate PI3K, or mutations in EGFR (epidermal growth factor receptor) which mediates an oncogenic effect through the PI3K/Akt pathway and increases PI3K activity. (Almoguera et al., 1988; Nelson et al., 1996; Rodriguez-Viciana et al., 1997; Yanez et al., 1987).

However, unlike tumour-suppressors related cancers, such as p53, p73 and Rb, which become oncogenic due to loss of function, signal transduction pathways, such as the PI3K pathway are highly active in cancer, making it a convenient target for therapeutic intervention, because inhibition of its
activation is easier than replacing a loss of tumour suppressor function. In addition, mutated kinases are ideal therapeutic targets, in particular when mutated forms of these proteins are solely expressed in cancerous cells (Bader et al., 2005; Hennessy et al., 2005).

Taken together, these observations suggest that the PI3K/Akt signalling pathway may serve as an ideal target for the development of novel cancer therapeutics.

### 1.3.7 Targeting the PI3K/Akt pathway in cancer drug development

To date, no drugs developed, which exclusively target the PI3K/Akt pathway, have entered cancer clinical trials. Nonetheless, some drugs in clinical use or preclinical evaluation which were developed for other purposes but have been shown to target the PI3K/Akt signalling pathway in either a direct or indirect manner.

These compounds include mTOR inhibitors (Sabatini, 2006), which appear to exclusively target the PI3K/Akt pathway and the small protein kinase inhibitor imatinib, which is used to treat chronic myeloid leukaemia (CML) patients by inhibiting the effects of BCR-ABL which constitutively activate the PI3K pathway (Burchert et al., 2005). However, imatinib (Glivec) has only partial success in treatment and it has been suggested that imatinib also activates mTOR, and therefore, a combination of mTOR inhibitors might improve treatment of CML patients.

New therapies have been suggested to work in combination with the tyrosine kinase inhibitor imatinib, such as the use of specific monoclonal antibodies which target the receptor on the outer membrane of the cell (Adachi et al., 2004).

The P110α mutations are tumour-specific and cause gain-of-function, suggesting that they contribute to the malignant phenotype of the cell. Small molecule inhibitors that target the mutant kinase could be developed into a novel anti-cancer drug. These drugs may be found to demonstrate sufficient anti-tumour activity alone, or may require combination approaches with other
signal transduction inhibitors, chemotherapy and radiation therapy to reach optimal efficacy for treatment of patients

As mentioned above, one of the best studied downstream targets of Akt are FoxO transcription factors. These transcription factors have been implicated in cellular events such as apoptosis, cell cycle arrest, oxidative stress resistance, differentiation and longevity. Hence, these proteins may play a significant role in determining sensitivity to chemotherapeutic agents, such as paclitaxel. FoxOs are members of the large family of transcription factors named Forkhead transcription factors.

1.4 Forkhead transcription factors

1.4.1 Overview: Forkhead transcription factors
All members of the forkhead family of transcription factors possess a highly conserved DNA-binding domain dubbed the 'Forkhead domain'. The family gained their name from the initial observation that in Drosophila, mutations in the first gene identified as a member of this family, result in homoeotic transformation of certain gut structures, which leads to the substitution of the fore- and hind- gut by ectopic protruding head structures (Weigel et al., 1989). The 110 amino acid DNA binding domain is highly conserved in over 100 forkhead genes identified in species ranging from yeast to human. The number of forkhead genes varies from species to species, there are 4 known members in Saccharomyces cerevisiae and over 45 forkhead family members in mouse and human (Wijchers et al., 2006). Several years ago, a unified nomenclature was introduced, which based on phylogenetic analysis of the forkhead domains led to the assignment of most of these genes into 19 subclasses named FoxA to FoxS (Kaestner et al., 2000). Briefly, the word Fox indicates the presence of a forkhead box and the following letter represents the subfamily and the number represents the member of the subfamily. Upper case or lower case letters represent the species which the gene is derived from; uppercase letters for human (e.g. FOXO1), only a
capital F for mouse (e.g. Foxo1) and the first and subclass letters appear in uppercase for all other chordate species (e.g. FoxO1) (Kaestner et al., 2000; Wotton and Shimeld, 2006).

1.4.1.1 Forkhead chromosomal distribution, structure and regulation
Forkhead genes are distributed throughout the genomes and only the human subclasses FOXC, FOXF, FOXL1 and FOXQ1 were found to form clusters that map to two areas of the human genome; the genes FOXL1, FOXC1, FOXF2 are mapped to chromosome 16 and the genes FOXC2, FOXF1 and FOXQ1 are mapped to chromosome 6 (Mazet et al., 2006). Studies suggest, on the basis of the observation that amphioxus orthologues of these Forkhead genes are found clustered in one region of the amphioxus genome, that block duplication of this region occurred in vertebrates during the evolutionary development of chromosomes 6 and 16 loci (Mazet et al., 2006).

Based on its similarity to the shape of butterflies, the term “winged helix” is often used to describe the 3D structure of a forkhead DNA binding domain (Clark et al., 1993). The forkhead domain is comprised of α-helices, β-sheets and loops. The number of α-helices β-sheets and loops varies (Liu et al., 2002; Stroud et al., 2006; van Dongen et al., 2000; Weigelt et al., 2001).

Most forkhead transcription factors, except from some members of the FoxP subfamily, bind target DNA sequences as monomers (Li et al., 2004). The initial binding of Forkhead to the DNA occurs through the third α-helix, known as the recognition helix and it binds the target DNA in the major groove. The binding of the second loop leads to the interaction between the forkhead domain and the minor groove of the DNA. This affects the stability and specificity of the binding (Clark et al., 1993; Marsden et al., 1998). Forkhead factors bind to a core DNA consensus binding sequence, A/C-A-A-C/T-A, which is essential but not sufficient to enable this interaction (Pierrou et al., 1994). The forkhead domain of most family members also contains nuclear localisation signals (NLSs). Aside from these similarities, within the conserved forkhead domain, forkhead proteins largely differ in other regions.
and only members of the same subclass possess additional conserved domains or motifs (Barthel et al., 2005). Forkhead transcription factors can act as both positive or negative transcriptional regulators, depending on the type of complex they associated with; whether an activator or a repressor complex (Wijchers et al., 2006). Forkhead activity is predominantly regulated by post-translational modifications, whilst phosphorylation of FoxO proteins by Akt following stimulation with insulin and growth factors is the most studied post-translational modification. Phosphorylation of FoxOs by Akt results in nuclear exclusion and localisation of FoxO proteins in the cytosol, which inhibits the activity of FoxO (Brunet et al., 1999; Rena et al., 1999). FoxA2 is also regulated by Akt in response to insulin, this also results in cytosolic localisation of FoxA2 (Wolfrum et al., 2003).

Forkhead factors are involved in diverse biological process, such as cell cycle regulation (FoxM1, FoxO, FoxA1, FoxG1, FoxK1), survival (FoxO), metabolism (FoxA, FoxC, FoxO), immunoregulation (FoxP3, FoxJ1, FoxN1, FoxO, FoxD2, FoxP1) and embryonic development (all except FoxB, FoxG, FoxK, FoxO, FoxQ, FoxS). Therefore, deregulation of the expression of FOX gene through mechanisms such as gene amplification, chromosomal translocation, or impaired transcriptional regulation can lead to the development of physiological disorders and cancer (Lehmann et al., 2003).

1.5 FoxO transcription factors

1.5.1 Overview: FoxO transcription factors
Mammalian FoxO transcription factors have conserved Akt phosphorylation sites located either near or with their forkhead DNA binding domains. These family of proteins are the mammalian orthologues of the soil-dwelling nematode worm, Caenorhabditis elegans DAF-16 (abnormal DAuer Formation-16 (Lin et al., 1997; Ogg et al., 1997). Research conducted in C.elegans has proved there is a link between the insulin-like signal transduction pathway and longevity, which is mediated by the activity of FoxO
transcription factors. Mutations resulting in the inactivation DAF-2, the
*C. elegans* insulin receptor and upstream regulator of DAF-16, were shown to
increase the lifespan of *C. elegans* and require functional DAF-16 (Lin et al.,
1997; Ogg et al., 1997)

There are 4 members in human family of FOXO factors: FOXO1, FOXO3a,
FOXO4 and FOXO6. FOXO1, FOXO3a, and FOXO4 were first identified in
chromosomal translocations in human tumours, suggesting they may play a
role in the development of cancer. FOXO1 was found in childhood tumour
alveolar rhabdomyosarcoma (Davis et al., 1994; Shapiro et al., 1993) and
FOXO3a and FOXO4 in acute myeloid leukaemia (Parry et al., 1994; So and
Cleary, 2003). These chromosomal translocations result in a fusion protein,
composed of the C-terminal region of FOXO fused to the N-terminal region of
transcription factors, such as Pax3 or Pax7 (del Peso et al., 1999). FOXO1,
FOXO3a, and FOXO4 mRNAs are expressed in most human tissues but in
distinct and diverse levels (Anderson et al., 1998), while FOXO6 mRNA is
chiefly expressed in the developing brain (Biggs et al., 2001; Jacobs et al.,
2003).

Based on the results of genetic studies, it appears that FoxO factors have
distinct but overlapping functions. A study conducted using FoxO1, FoxO3a
and FoxO4 null mice (Hosaka et al., 2004) showed that FoxO4 and FoxO3a
null mice are generally indistinguishable from wild-type mice; however,
female FoxO3a-null mice have an age dependent infertility phenotype
combined with abnormal ovarian follicular development. FoxO1-null mice
were found to have the most prominent phenotype, these mice were found to
be embryonic lethal at day 10.5, due to defects in vascular development
(Hosaka et al., 2004). In addition, results from another study (Nakae et al.,
2002), in insulin resistant mice has shown that haploinsufficiency of the
FoxO1 gene can rescue a diabetic phenotype. However, the same study also
showed that a gain of function mutation in the FoxO1 gene in the liver and
pancreatic beta-cells of mice leads to the development of diabetes, indicating
the important role FoxO1 plays in insulin downstream signalling. Taken together, these studies suggest the functions of FoxO factors are diverse, but that a degree of redundancy also exists. Furthermore, the observed differences in the phenotypes of FoxO-null mice may be caused by variations in the levels of expression of each isoform in the specific tissues.

Since FoxO proteins are transcription factors, they can only function when in the nucleus. Monomers of FoxO bind to a consensus DNA sequence (G/C)(T/A)AA(C/T)AA, named the FoxO-recognised element (FRE) (Biggs et al., 1999; Furuyama et al., 2000; Gilley et al., 2003). All the FoxO factors appear to be able to regulate the expression of the same target genes. This further strengthens the hypothesis that these proteins function with a certain degree of redundancy.

A schematic overview of the function and regulation can be found in figure 1.2.
Figure 1-2: FoxO regulation, shuttling and downstream targets.
Most of the known post-translational modification of FoxO protein, which affect FoxO shuttling and/or FoxO activity, are summarized in this figure. Some of the modifications presented have, to date, been shown to be isoform specific. β-catenin binding to FoxO enhances transcriptional activity. The role of acetylation of FoxO proteins is currently unresolved, however the general consensus is that SIRT1 deacetylation of FoxO induces transcription of genes important for stress resistance and longevity. * JNK phosphorylation of FoxO4 has been shown to induce transcription of genes involved in oxidative stress detoxification. FoxO proteins are believed to be able to transcribe genes when phosphorylated by both Akt and JNK under oxidative stress conditions in the presence of growth factors. Adapted from Huang and Tindall 2007.
1.5.2 Regulation of FoxO transcription factors in response to survival factors and cellular stress

FoxO transcription factors are expressed in most adult tissues in a specific yet overlapping manner (Anderson et al., 1998; Biggs et al., 2001). All FoxO proteins appear to bind the same consensus DNA binding site (Biggs et al., 1999; Furuyama et al., 2000). Taken together, these observations suggest that regulation on FoxO activity is not predominantly achieved by isoform specific expression or binding to the DNA. In fact, the regulation of FoxO transcription factors seems to occur mainly at post-translational level. The factors which drive the activity of post-translational modifiers are mostly extracellular signals. Post-translational modifications of FoxO factors include phosphorylation, ubiquitylation and acetylation. A schematic summary of the various post-translational modifications FoxO1, FoxO3a and FoxO4 are subjected to, can be found in figure 1.3. The effects of these post-translational modifications on FoxO activity are illustrated in figure 1.2.
Figure 1-3: Post-translational modifications of FoxO proteins. Schematic representation of post-translational modifications of FoxO. The enzymes responsible for the modification are noted where known. Akt and SGK phosphorylate the same sites. FKH-forkhead domain, NLS-nuclear localisation signal, NES-nuclear export sequence, TA-transactivation domain. Adapted from van der Horst et al., 2007.
1.5.2.1 Phosphorylation of FoxO factors which leads to inactivation of FoxO activity

Several kinases have been found to phosphorylate FoxO transcription factors leading to their inactivation, which is generally achieved by nuclear exclusion of these proteins. Phosphorylation of FoxO factors following stimulation with insulin was the first identified and best characterised post-translational modification of FoxO. As mentioned above, the first indication that FoxO factors are downstream targets of the PI3K/Akt signalling pathway came from studies conducted in *C. elegans* which showed that the DAF-16 is phosphorylated and hence, negatively regulated by DAF-2 (Lin et al., 1997; Ogg et al., 1997). This mechanism of regulation was found to also occur in mammalian cells. Stimulation by growth factors, such as insulin and insulin like growth factor (IGF-1), results in the activation of PI3K and subsequent Akt phosphorylation. Phosphorylation of Akt results in the activation of Akt and thereby phosphorylation of FoxO1, FoxO3a and FoxO3a at three evolutionary conserved residues, leading to the inactivation of FoxO transcription factors (Biggs et al., 1999; Brunet et al., 1999; Kops et al., 1999; Rena et al., 1999; Tang et al., 1999). The mechanism underlying the inactivation of FoxO following phosphorylation by Akt, appear to be the resulting nuclear exclusion of these factors. Phosphorylation of FoxO factors by Akt leads to the binding of 14-3-3 proteins to FoxO and this interaction appears to occur in the nucleus. The 14-3-3 FoxO complex is then exported from the nucleus to the cytoplasm. The interaction of 14-3-3 proteins with FoxO proteins seems to mask the FoxO NLS and therefore, may be the mechanism which causes FoxO proteins to translocate to the cytoplasm and not re-enter the nucleus (Brunet et al., 2002; Zhao et al., 2004). FoxO6 lacks the third Akt regulatory site, and although phosphorylated by Akt, at two sites, leading to its inactivation, it does not translocate to the cytoplasm (van der Heide et al., 2005) and is therefore, not further discussed.

Serum and glucocorticoid inducible kinases (SGKs) are serine/threonine kinases, which were found to phosphorylate FoxO3a, at the same residues as Akt (Brunet et al., 2001). Like Akt, SGKs are downstream targets of the
PI3K pathway and localise in the nucleus following exposure to survival factors. Hence, the same extracellular stimuli leads to both SGK and Akt activation, both which results in the phosphorylation of FoxO3a at the same three residues leading to the nuclear export and inactivation of FoxO3a (Brunet et al., 2001). Similarly, FoxO1 was also found to be phosphorylated by a member of the SGK family leading to its inactivation (Liu et al., 2000). Several additional mechanisms of regulation by phosphorylation, resulting in FoxO inactivation, have been identified for specific FoxO isoforms. In addition the residues subjected to this phosphorylation are generally not conserved from C.elegans to mammals and have been only shown to occur in mammalian cells. FoxO1 has been shown to be phosphorylated by the dual-specificity tyrosine-phosphorylated and regulated kinase (DYRK) at serine 329, resulting in a decrease in FoxO1 transcriptional activity (Woods et al., 2001). The cyclin-dependent kinase 2 (CDK2) was shown to phosphorylate FoxO1 at serine 249, leading to FoxO1 inactivation and re-localisation in the cytoplasmic compartment (Huang et al., 2006).

IKK (IkB kinase) was found to phosphorylate FoxO3a on serine 644 leading to the inhibition of FoxO3a activity and its cytoplasmic localisation (Hu et al., 2004). This mechanism of regulation was found to be independent of Akt activity and therefore, IKK may be regarded as an Akt-independent inhibitor of FoxO3a activity (Hu et al., 2004).

Following stimulation with growth factors and/or insulin, FoxO factors translocate from the nucleus to the cytoplasm. Nonetheless, following exposure to oxidative and genotoxic stress inducing agents, FoxO proteins localise to the nucleus, despite induction with growth factors and insulin (Brunet et al., 2004; Essers et al., 2004; Huang et al., 2006). This implies that other factors affect the cellular distribution of FoxO.

1.5.2.2 Phosphorylation of FoxO factors in response to cellular stress

Oxidative stress affects the activity of cellular transduction pathways such as the JNK signalling pathway (Davis, 2000). FoxO has been shown to be
regulated by JNK in several organisms. In *Drosophila*, JNK was found to induce the nuclear translocation and activation of *Drosophila* FoxO (Wang et al., 2005). In *C. elegans*, it was shown that following heat stress the JNK orthologue can bind to and phosphorylates DAF-16 and this is assumed to lead to nuclear localisation of DAF-16 (Oh et al., 2005). In mammalian cells, following the induction of oxidative stress, JNK was also found to be able to phosphorylate a member of the FoxO family, FoxO4, at two sites, threonine 447 and threonine 451. This was found to be dependent on the activation of the small GTPase Ral and result in the accumulation of FoxO4 in the nucleus, leading to the transcription of downstream targets (Essers et al., 2004). JNK has also been shown to also induce the nuclear translocation of FoxO indirectly, by the phosphorylation of 14-3-3. This leads to the dissociation of FoxO from 14-3-3, which enables FoxO to re-localise to the nucleus (Sunayama et al., 2005). Similarly, the mammalian Ste20-like kinase-1 (MST1), a MAPKKKK, upstream of JNK was found to phosphorylate FoxO3a at a conserved site, located in forkhead domain (serine 207). This has been shown to affect the interaction between FoxO3a and 14-3-3 resulting in the release of FoxO3a from the complex with 14-3-3, thereby promoting the ability of FoxO3a to translocate to the nucleus (Lehtinen et al., 2006).

Taken together, following exposure to oxidative stress and oxidative stress inducing agents, such as paclitaxel (Sunters et al., 2003), FoxO factors predominately localise to the nucleus. One of the mechanisms underling FoxO nuclear translocation/retention appears to be mediated, at least partially, by the activity of JNK and other members of the JNK signal transduction pathway.

1.5.2.3 Acetylation

In addition to phosphorylation, FoxO proteins are subjected to post-translational regulation by acetylation. FoxO proteins have been found to form complexes with an abundance of gene expression regulators; co-
activators and co-repressors, some of which affect the acetylation status of FoxO proteins. The acetylation status of FoxO has been suggested to tip the balance between positive and negative regulation of the cell fate. As a result, the set of FoxO downstream genes transcribed may change (Greer and Brunet, 2005).

Mammalian FoxOs are acetylated by HATs, such as, p300, core binding protein (CBP) and CBP-associated factor P/CAF. Deacetylation of FoxOs is carried out by histone deacetylases (HDAC); the best studied being sirtuin (SIRT1), which is the mammalian orthologue of SIR-2 (silent information regulator-2), which remove acetyl groups from both histones and other proteins (Brunet et al., 2004; Daitoku et al., 2004; Fukuoka et al., 2003; Kobayashi et al., 2005; Mahmud et al., 2002; Motta et al., 2004; Perrot and Rechler, 2005; van der Horst et al., 2004; Yang et al., 2005b). Most probably, SIRT1 is not the only HDAC that regulates FoxO activity, as inhibition of other HDACs results in increased FoxO acetylation (Brunet et al., 2004; Frescas et al., 2005; Matsuzaki et al., 2005; Motta et al., 2004; van der Horst et al., 2004).

In C. elegans, the over-expression SIR-2 gene of significantly increased the lifespan of the worms by a mechanism which was shown to dependent on the activation of DAF-16 (Tissenbaum and Guarente, 2001). This implying that DAF-16 is regulated, to some extent, by acetylation and deacetylation (Tissenbaum and Guarente, 2001). In addition, SIR-2 dependent increase in lifespan seems to be mediated, at least partially, by 14-3-3 proteins (Berdichevsky et al., 2006; Wang et al., 2006). DAF-16 was shown to interact with 14-3-3; therefore, as suggested by van der Horst et al, the connection between SIR-2 and DAF-16 may be conducted through the interaction of both proteins with 14-3-3 (van der Horst and Burgering, 2007).

The regulatory significance of FoxO acetylation and deacetylation (by SIRT1) is currently inconclusive. Some studies suggest that acetylation inhibits FoxO activity, as there is evidence that SIRT1 activates FoxO (Daitoku et al., 2004; Frescas et al., 2005; Gan et al., 2005; Kobayashi et al., 2005; van der Horst et al., 2004; van der Horst et al., 2004).
et al., 2004). Another approach, as suggested by Motta et al., is that the acetylation activates FoxO (Motta et al., 2004). Brunet and colleagues, proposed a context-specific regulation mechanism, in which SIRT1 stimulates the expression of FoxO-induced target genes which play important roles in cell cycle regulation and stress resistance, while de-acetylation by SIRT1 inhibits the expression of FoxO induced pro-apoptotic target genes (Brunet et al., 2004). This model is based on three observations; following exposure to stress, SIRT1 intertracts with and deacetylates FoxO factors in the nucleus (Brunet et al., 2004; Kitamura et al., 2005), SIRT1 expression up-regulates FoxO3a induced cell cycle arrest by upregulation of p27Kip1 expression (Brunet et al., 2004), and the inhibition of SIRT1 increases the expression of FoxO3a downstream apoptotic target genes, such as Bim, (Brunet et al., 2004). Therefore, SIRT1 interaction was suggested to tip the activity of FoxO3a from apoptosis and cell death towards stress resistance and cell survival (Greer and Brunet, 2005).

FoxO family of proteins are subjected to another type of post-translational modification which is ubiquitylation, the addition of ubiquitin groups.

1.5.2.4 Ubiquitylation
There are two known types of protein ubiquitylation; polyubiquitylation and monoubiquitylation. Polyubiquitylation is irreversible and tags the protein for degradation by the proteasome, while monoubiquitylation is reversible and does not culminate in the degradation of the protein.

The amounts of FoxO1 and FoxO3a proteins in mammalian cells have been shown to be regulated by PI3K/Akt pathway dependent polyubiquitylation of these proteins and proteasome degradation (Matsuzaki et al., 2003; Plas and Thompson, 2003). The phosphorylation of FoxO1 by Akt on serine 256 leads to the recruitment of Skp2, the substrate recognition component of the E3 ligase complex the SCF (SKP1-CUL1-F-box protein) (Huang et al., 2005). The interaction results in the polyubiquitylation of FoxO1 and subsequent degradation by the proteasome (Huang et al., 2005). Similarly, the IKK
mediated phosphorylation of FoxO3a at serine 644 (discussed later on in the introduction) also results in ubiquitylation and subsequent degradation by proteasome. The E3 ligase responsible has not yet been identified (Hu et al., 2004). To date, only FoxO1 and FoxO3a have been shown to be regulated post-translationally by polyubiquitylation. However, FoxO4 was found to be monoubiquitylated, at the conserved residues, lysine 199 and lysine 211. This post-translational modification occurs after exposure of cells to oxidative stress and leads to FoxO4 nuclear localisation and to upregulation in FoxO4 dependent transcription of target genes (van der Horst et al., 2006). FoxO4 was shown to interact with the herpesvirus associated ubiquitin-specific protease (HAUSP/USP7), resulting in the inhibition of FoxO4 activity. Therefore, it is plausible that USP7 interaction with FoxO4 leads to the removal of monoubiquitin from FoxO4 (van der Horst et al., 2006). The E3 ligase responsible for the addition of the ubiquitin group is unknown, yet. Taken together, monoubiquitylation of FoxO4 may be a form of post-translational regulation following oxidative stress. Hence, monoubiquitylation of FoxO4 is probably a mechanism which enables the upregulation of FoxO4 transcriptional activity.

In summary, the main determinates that regulate the sub-cellular distribution and activities of FoxO transcription factors are growth factors, insulin and oxidative stress. Abundance in growth factors and insulin result in the phosphorylation of FoxO by Akt, which leads to cytoplasmic localisation and inactivation of FoxO factors. By contrast, oxidative stress results in nuclear retention and activation of FoxO proteins. The mechanism underlying regulation of FoxO proteins following exposure to oxidative stress is complex. Several post-translational modifications, such as phosphorylation by JNK and MST1 were found to retain FoxO in the nucleus, even after treatment with insulin, whilst deacetylation of FoxO is presumed to lead to transcription of FoxO target genes involved in detoxification of ROS and cell cycle arrest. Monoubiquitylation of FoxO has also been implicated in oxidative stress regulation of FoxO, leading to FoxO re-localisation and retention in the
nucleus. The next section discusses the end result of FoxO regulation, the function of FoxO transcription factors.

1.5.3 The function and downstream targets of FoxO

FoxO downstream targets have been implicated in several cellular activities, such as glucose metabolism, cell cycle arrest, apoptosis, DNA repair, detoxification of oxidative stress and differentiation. As mentioned above, phosphorylation of FoxO proteins by Akt results in inhibition of FoxO activity, therefore, taken together, this may result in a pro-survival anti-apoptotic advantage to the cells, which could lead to uncontrolled proliferation and reduced sensitivity to chemotherapeutic agents. FoxO transcription factors influence the following physiological activities: glucose metabolism, apoptosis, DNA repair and detoxification, cell cycle and differentiation.

1.5.3.1 Glucose metabolism

FoxO transcription factors transactivate the expression of genes that are involved in gluconeogenesis. It has been found that FoxO transcription factors can upregulate the expression of the metabolic enzyme, glucose 6 phosphatase (G6Pase), which converts glucose 6 phosphate to glucose and the expression of phosphoenolpyruvate carboxykinase (PEPCK), an enzyme that converts oxaloacetate to phosphoenolpyruvate (Nakae et al., 2001; Puigserver et al., 2003; Schmoll et al., 2000; Yeagley et al., 2001). As FoxO activity is controlled by insulin signalling, it can be suggested that insulin affects glucose metabolism by repressing FoxO activity, thereby, decreasing gluconeogenesis.

1.5.3.2 Apoptosis

FoxO factors induce apoptosis via both the mitochondria dependent and independent pathways. In the mitochondria independent pathway, FoxO factors were found to up-regulate the expression of the death receptors ligand genes, Fas-Ligand (FasL) and TRAIL. The FasL gene, which encodes a protein that activates the Fas death receptor, was found to have three consensus FRE sequences, to which the binding of FoxO3a was shown to
result in \textit{de novo} transcription of FasL (Brunet et al., 1999). TRAIL was identified in a study, conducted using prostate cancer cells, to be regulated by FoxO3a (Modur et al., 2002).

In the mitochondrial dependent (intrinsic) pathway, FoxO proteins were shown to induce apoptosis by up-regulating the transcription of Bim. The \textit{Bim} gene-promoter was found to posses two functional FRE sites (Gilley et al., 2003) and FoxO factors were shown to induce the transcription of \textit{Bim} in haematopoietic cells deprived of growth factors (Dijkers et al., 2000a; Stahl et al., 2002). In addition, the transcriptional repressor Bcl-6, which expression results in the down-regulation of the expression of Bcl-XL, a pro-survival Bcl-2 family member, was found to be induced by FoxO4 (Tang et al., 2002) and bNIP3, another proapoptotic member of the Bcl-2 family, was also identified as a FoxO target gene (Tran et al., 2003).

Taken together, the significance of the role of FoxO transcription factors play in the induction of apoptosis is illuminated but the fact that FoxO factors can up-regulate cell death by both mitochondria dependent and independent pathways.

\textbf{1.5.3.3 DNA repair and detoxification: a role for FoxO in oxidative stress resistance and longevity}

\textit{The role of FoxO in the detoxification of oxidative stress}: FoxO transcription factors have been implicated in oxidative stress detoxification based on the identification of FoxO downstream transcriptional targets and the role FoxO factors play in haematopoietic stem cell resistance to physiologic oxidative stress. The \textit{manganese superoxide dismutase} (MnSOD) and catalase genes, both which encode antioxidant scavenger proteins, were found to be positively regulated at the transcriptional level by FoxO factors (Kops et al., 2002a; Nemoto and Finkel, 2002). In a conditional knock out of FoxO1, FoxO3a and FoxO4 in adult mouse models, the importance of FoxO proteins was shown in the haematopoietic system. The haematopoietic stem cells have lost their longevity (and self renewal) under conditions of elevated oxidative stress, in comparison with wild-type haematopoietic stem cells; a
phenotype which can be reversed following treatment with an antioxidant agent. These observations indicate that FoXO play a role in the maintenance and longevity of the hematopoietic stem cells and resistance to oxidative stress (Tothova et al., 2007). The maintenance of the hematopoietic stem cells has implications on lifespan. This is in accord with the increase in lifespan observed in *C. elegans* which has been linked to the activity of FoxO and JNK orthologues as detailed below.

**Oxidative stress results in JNK-dependent activation of FoxO leading to an increase in lifespan:** Cross talk between the JNK signal transduction pathway and PI3K/Akt/FoxO pathway has been suggested based on the results of several studies (De Ruiter et al., 2001; Essers et al., 2004; Lehtinen et al., 2006; Oh et al., 2005; Sunayama et al., 2005). It is a well established fact that the JNK signal transduction pathway becomes active following exposure to oxidative stress (Davis, 2000). JNK activity has also been shown to decrease the levels of ROS and extend lifespan in *Drosophila* (Wang et al., 2005) However, more recently, it has been shown that in *Drosophila*, JNK activation induces FoxO to localise in the nucleus and to transcribe genes involved in growth regulation and stress defence (Wang et al., 2005). Similarly, heat stress was also found to extend lifespan in *C. elegans* in a manner dependent on both DAF-16 and JNK-1 (Oh et al., 2005). In mammalian cells FoxO factors accumulate in the nucleus, even following treatment with growth factors (Brunet et al., 2004). In mouse cells, it was shown that following treatment with H$_2$O$_2$ FoxO4 becomes phosphorylated at threonine 447 and threonine 451 in a JNK dependent manner, resulting in the accumulation of FoxO4 in the nucleus (Essers et al., 2004). JNK was also found to phosphorylate 14-3-3, which leads to the dissociation of 14-3-3 proteins from FoxO, which most likely enables FoxO factors to re-enter the nucleus and antagonise PI3K/Akt activity (Sunayama et al., 2005). In addition, in primary mammalian neuron cells, an upstream regulator of JNK, MST1, a MAPKKKK, was shown to phosphorylate FoxO3a at the conserved serine 207, which is located in the DNA binding domain site. This
phosphorylation event causes the FoxO3a-14-3-3 complex to dissociate, resulting in re-localisation of FoxO3a in the nucleus (Lehtinen et al., 2006), Taken together, activation of MST1/JNK following oxidative stress neutralises the activity of the PI3K/Akt pathway, by inducing FoxO nuclear localisation and transcription of target genes involved in cellular responses to stress. 

**FoxO deacetylation following oxidative stress tips FoxO activity towards cell cycle arrest and oxidative stress resistance:** FoxO transcription factors have been implicated in various cellular functions and have been shown to have diverse cellular roles which often may seem to be contradicting. However, the results of research conducted on the acetylation and de-acetylation by SIRT1 of FoxO factors, has helped to shed some light on this topic (Brunet et al., 2004; Daitoku et al., 2004; Fukuoka et al., 2003; Kobayashi et al., 2005; Mahmud et al., 2002; Motta et al., 2004; Perrot and Rechler, 2005; van der Horst et al., 2004; Yang et al., 2005b). In yeast, the increase in lifespan following caloric-restriction was found to be dependent on the *SIR-2* gene, which encodes the silencing protein Sir2p (Lin et al., 2000). While in *C.elegans*, it was shown by the use of strains which contain a chromosomal duplication of the region encompassing the *SIR2.1* gene, a gene highly homologous to the yeast *SIR-2* gene, that SIR-2 plays a role in the prolonging lifespan and that this function is dependent on the downstream DAF-16 protein (Tissenbaum and Guarente, 2001). In mammalian cells, it has been demonstrated that following exposure to oxidative stress stimuli, such as \( \text{H}_2\text{O}_2 \), SIRT1, one of the human orthologues of SIR-2 forms a complex with FoxO3a and deacetylates FoxO3a. This interaction between FoxO3a and SIRT1 and the subsequent deacetylation of FoxO3a have been shown to induce the ability of FoxO3a to upregulate resistance to oxidative stress and induce cell cycle arrest, but to impair the ability of FoxO3a to upregulate apoptosis. Taken together, oxidative stress leads to deacetytelation of FoxO3a by SIRT1, thereby, shifting the activity of FoxO3a from cell death to detoxification of oxidative stress and longevity (Brunet et al., 2004).
**FoxO4 monoubiquitylation following oxidative stress results in the nuclear retention of FoxO4, and transcriptional activation of FoxO4**: In mammalian cells, monoubiquitylation of FoxO4 at lysine 199 and lysine 211 occurs following exposure to H₂O₂ and was shown to result in FoxO4 nuclear accumulation and upregulation of target gene transcription (van der Horst et al., 2006). Interestingly, lysine 199 and lysine 211 were also shown to be acetylated by CBP/p300 following exposure to oxidative stress (van der Horst et al., 2004); however, the kinetics of these reactions appears to be different. While monoubiquitylation appears to occur almost instantly after treatment with H₂O₂ (within 5 minutes), acetylation occurs at a later time (after longer than 60 minutes). This suggests that oxidative stress results, initially in FoxO4 monoubiquitylation and activation and that this is later followed by FoxO4 deubiquitylation and acetylation, resulting in FoxO4 inactivation (van der Horst et al., 2006).

**β-catenin induces FoxO activity in detoxification of oxidative stress**: Experiments conducted in both *C.elegans* and mammalian cells have suggested that β-catenin, a multifunctional protein which plays a role in Wnt signalling, can bind to FoxO following treatment with H₂O₂. This was shown to result in an increase in FoxO transcription of target genes (Essers et al., 2005). In mammalian cells, exposure to oxidative stress stimuli was shown to result in the binding of β-catenin to FoxO4 and an increased transcription the FoxO downstream transcriptional target, p27^Kip1^. In *C.elegans*, it was demonstrated that the expression of the DAF-16 target gene, *superoxide dismutase 3 (sod-3)*, the MnSOD homologue, following exposure to an oxidative-stress inducing agent is dependent on BAR-1, the β-catenin orthologue (Essers et al., 2005). Taken together, it appears that β-catenin interaction with FoxO4 is induced following oxidative stress, leading to an increase in FoxO4 dependent transcription of target genes, such as p27^Kip1^, a gene important in cell cycle regulation.

**FoxO factors may also play a role in reducing genotoxic stress**: CDK2, a kinase which activity is reduced following DNA damage, has been shown to
phosphorylate FoxO1; an event which leads to a decrease in FoxO1 transcriptional activity (Huang et al., 2006). Based on this observation, it is possible that genotoxic stress may be another stress factor which induces the activity of FoxO transcription factors.

Overall, oxidative stress results in changes in the regulation and activity of multiple cellular targets, some of which have been found to affect the activity of FoxO factors. This is predominantly achieved by altering FoxO post-translational modifications, which mainly affect the sub-cellular distribution of FoxO, but also may have an impact on the array of genes transcribed by FoxO.

1.5.3.4 Cell cycle transitions
The cell cycle process is a tightly regulated process that is promoted by a family of proteins called cyclins and cyclin dependent kinases (CDK) and is inhibited by cyclin kinases inhibitors (CKI), such as, p21^{Cip1} and p27^{Kip1}. Cyclins and their kinases form complexes. The balance between cyclin-CDK complexes and CKI promotes or inhibits the progression of the cell cycle (Hunter and Pines, 1994; Sherr, 1994; Sherr and Roberts, 1995, 1999).
The cell cycle responds to growth factor stimulation, as quiescent cells (G0) move into the G1 phase of the cell cycle. The transition increases the transcriptional activity in the cell, include degradation of p27^{Kip1} protein and up-regulation of cyclin D expression, this activates cyclin D–CDK4 and cyclin E–CDK2 complexes and results in progression of the cells into S phase.
The growth factor signal is carried through the cell by signal transduction pathways. The signal molecule interact with a receptor on the outer side of the cell membrane, which translates the hormonal/growth factor signal into a phosphorylation signal which cascades down until it concludes in consequent gene expression and cell growth. One of the main signal transduction pathways is the PI3K/Akt pathway. Activation of the PI3K/Akt signalling pathway leads to the entry of quiescent cells (G0) into G1-phase.
FoxO proteins have a role in cell cycle regulation. Growth factor stimulation of cells results in activation of the PI3K/Akt signalling pathway and in activation of FoxO. In the presence of a growth signal by exposure of the cells to the transforming growth factor β (TGFβ), FoxO proteins were found to bind the promoter of $p21^{Cip1}$ gene, a cell cycle inhibitor, leading to an induction in cell cycle arrest (Seoane, 2004). Therefore, in the absence of growth factors, FoxO activity is sustained resulting in up-regulation of $p27^{Kip1}$, p130 and cyclin G2 expression, as well as, inhibition of cyclin D1 and D2 expression, thereby insuring maintenance of a quiescent state.

Over-expression of FoxO1, FoxO3a and FoxO4 induce G1 arrest via $p27^{Kip1}$ expression (De Ruiter et al., 2001; Medema et al., 2000; Nakamura et al., 2000; Stahl et al., 2002; Tanaka et al., 2001). FoxO proteins reduce the expression of cyclin D1 and cyclin D2 and may induce cell cycle arrest independently of $p27^{Kip1}$ (Schmidt et al., 2002; Ramaswamy et al., 2002). The FoxO transcription factors have been shown to regulate the transcription of $p27^{Kip1}$ and cyclin D by binding the promoters of these genes. On the promoter of $p27^{Kip1}$ there is a canonical FRE sequence to which FoxO proteins can directly attach (Medema et al., 2000; Nakamura et al., 2000). However, binding of FoxO to the cyclin D promoter is probably indirect, via interaction with other proteins, or by binding to non-canonical sequence, as cyclin D promoter lacks the FRE sequence (Ramaswamy et al., 2002; Schmidt et al., 2002).

FoxO has been suggested to be directly involved with the regulation of cell quiescence (G0) in cell lines (Kops et al., 2002b) and in vivo in the regulation of the quiescent haematopoietic stem cells (Tothova et al., 2007). Possible pathways in which FoxO may induce cell quiescence, may involve the retinoblastoma (Rb), by transactivation of the p130 gene (pRBL2, retinoblastoma-like 2), in a FRE dependent manner, resulting in the accumulation of p130 protein (Kops et al., 2002b). p130, is expressed at low levels in dividing cells, but is highly expressed during quiescence (Grana et al., 1998). Moreover, p130 is hypo-phosphorylated and bound to the
transcription factor E2F-4 that is associated with activation of cell-cycle promoting genes. In complex with p130 E2F-4 is inhibited and re-entry to the cell cycle is blocked, thereby maintaining the quiescent state (Stahl et al., 2002).

Therefore, it is possible to summarise that the PI3K/Akt signalling pathway controls the abundance of p130 during the cell cycle via FoxO regulation and hence, controls the cell cycle and quiescent status of the cell.

Another pathway in which FoxO proteins are involved in cell quiescent is through the by increasing *cyclin G2* gene transcription (Martinez-Gac et al., 2004). Cyclin G2 is an unconventional cyclin that is highly expressed in quiescent cells, but is down-regulated in a manner dependent on activation of the PI3K/Akt signalling pathway as cells enter the cell cycle (Bennin et al., 2002; Horne et al., 1997).

In addition to G0 and G1 arrest, FoxO was also found to be able to induce G2 delay and arrest (Furukawa-Hibi et al., 2002; Tran et al., 2002). DNA microarray analysis has implicated the GADD45α (growth arrest and DNA damage-inducible protein 45 alpha) as a potential mediator of G2 arrest induced by FoxO activation (Furukawa-Hibi et al., 2002; Tran et al., 2002). GADD45α induces the dissociation of the cyclin B–CDK1 complex which is required for G2 transition (Zhan et al., 1999). FoxO directly binds to and activates the promoter of the GADD45α gene, thereby increasing the amount of GADD45α protein (Furukawa-Hibi et al., 2002; Tran et al., 2002).

### 1.5.3.5 Differentiation

As the function of FoxO is linked to cell cycle, it has also been found to be involved in a special type of cell division that is differentiation. Cellular differentiation is a polar cell division which gives rise to two different cells. One daughter cell resembles the cell they derived from and the other has a different phenotype.

The role of FoxO factors in differentiation may be linked to their function in tumourigenesis. FoxO factors have been implicated in both blocking and promoting differentiation, depending on the FoxO isoform and the cell type.
Therefore, it is plausible that impaired FoxO activity in differentiation may result in a differentiation block, the accumulated cells will still be capable of proliferation, though the might have an increasing tumourigenic potential (Arden, 2006).

The most studied differentiation system is the hematopoietic system. It is a highly regulated system (Lapidot et al., 1993). Deregulation of haematopoiesis results in leukaemia. For example, the disruption of PI3K/Akt pathway in haematopoietic cells as a result of the expression of the fusion gene BCR/ABL, results in leukaemia (Era et al., 2002). FoxO3a and FoxO4 are fusion partners of MLL and cause acute myeloid leukaemia (Parry et al., 1994).

A recent publication has demonstrated that FoxOs have a significant role in maintaining the haematopoietic stem cell (HSC) compartment. The HSCs are a population of quiescent cells, with enhanced survival, which maintain for very long time (the life time of a human) (Tothova et al., 2007). Knock out mice of the FoxO1, FoxO3a and FoxO4 resulted in severe defects to the HSC compartment, as a result, with decreased ability of the cells to react to ROS (Tothova et al., 2007). FoxO3a appears to induce erythroid differentiation. This has been shown to be potentially linked to the ability of FoxO3a to bind to the promoter of the Id1 (inhibitor of differentiation1), ultimately resulting in reduced transcription of Id1 activity (Birkenkamp et al., 2007). Another mean by which FoxO3a has been implicated in differentiation is the induction of erythroid differentiation is by transactivation of the B-cell translocation gene 1 (BTG1). The activated BTG1 causes, in an unknown manner, methylation of arginine residues on substrate proteins. This methylation activity results in increased erythroid differentiation (Bakker et al., 2004).

In other tissues such as adipocytes and myoblasts, expression of a constitutively active FoxO1 inhibits differentiation in vitro (Nakae et al., 2003; Hribal et al., 2003). In adipocytes, differentiation block has been linked to FoxO1 ability to up-regulate p21Cip1 expression (Nakae et al., 2003). FoxO4 has been implicated in the repression of differentiation of smooth muscle cells
by interacting with myocardin resulting in a disruption of the transcriptional activity (Liu et al., 2005b).

In chapter 4 of the thesis, I investigate the function of FoxO3a by examining the role and activity of BTG1, a novel downstream target gene of FoxO3a, initially identified in B-cells. BTG-1 was first identified as a downstream target of FoxO3a in erythroid progenitors and has been implicated in differentiation. Despite this fact, the exact function of BTG1 is currently unknown. Examination of the activity of this downstream target of FoxO3a was performed regardless to its suggested role in differentiation, the sole purpose being characterisation of BTG1 activity in breast cancer and not in differentiation.

### 1.5.4 B-Cell Translocation Gene -1 (BTG1)

The BTG/Tob family of proteins consists of six known members in vertebrates, BTG1, BTG2/TIS21/PC3, BTG3/ANA, PC3B, Tob and Tob2 (Matsuda et al., 2001). All members of this family posses a highly conserved 110-amino-acid N-terminal domain, named the BTG/Tob homology domain. BTG proteins have been shown to play a role in restriction of cell growth (antiproliferative activity) and differentiation (Guardavaccaro et al., 2000; Guehennieux et al., 1997; Raburn et al., 1995; Rodier et al., 1999). The BTG/Tob homology domain consists of two highly homologous regions, named box A and box B. BTG1 and BTG2 bear homology to each other in another region called box C, which associates with protein arginine methyl transferase 1 (PRMT1).

Research conducted on the function and role of BTG2 has revealed that BTG2 is a p53 downstream target gene; BTG2 is up-regulated as a response to exposure to DNA damaging agents. BTG2 also plays a role in down-regulation of cyclin D1 transcription and has been shown to induce the inhibition of cell cycle progression at G1 by decreasing the levels of phosphorylated Rb (Rouault et al., 1996).
BTG has a specific role in the normal mammary gland development and in initiation and progression of breast cancer. In the rat mammary gland model, BTG2 was found to be expressed in epithelial cells and its levels were shown to decrease in pregnant and lactating animals. However, BTG2 levels increased during involution. Estrogen and progestin were found to suppress BTG2 expression, implying that these steroids, which stimulate proliferation of mammary cell, also down-regulate BTG2. Furthermore, the expression levels of BTG2 in human breast cancer cell lines are lower than in non-tumourigenic mammary cells and BTG2 was found to be stimulated in breast cancer cells by activation of NF-κB (Kawakubo et al., 2004). Loss of BTG2 was shown to occur in the early stages of the tumourigenesis process. Taken together, this suggests that down-regulation of BTG2 may be play a significant role in the development of mammary tumours (Kawakubo et al., 2006).

To date, little is known about the function and regulation of BTG1, however over-expression of BTG1 was found to inhibit proliferation of NIH3T3 (Bogdan et al., 1998) cells and in QM7 quail myoblasts cells BTG1 localises in the nucleus of non-dividing cells and to the cytoplasm of proliferating cells (Rodier et al., 1999). DNA microarray identified the BTG1 gene as a FoxO3a target in erythroid progenitors and FoxO3a was also found to regulate the transcription of BTG1 (Bakker et al., 2004). The BTG1 induced proliferation inhibition is abolished by deletion of box C in BTG1, the PRMT1 binding domain (Bakker et al., 2004). Furthermore, BTG1 expression is induced in response to DNA damage (Cortes et al., 2000), this appears to be at least partially mediated by the activity of stress stimuli induced JNK signal transduction pathway (Devary et al., 1991; Kawasaki et al., 2000; van Dam et al., 1995). The CRE (cAMP-responsive element) site in the BTG1 promoter has been shown to recruit c-Jun and/or Atf-2. c-Jun and Atf2 are positively regulated by JNK, binding of Creb/Atf1 to the CRE site in the BTG1 promoter induces FoxO3a-mediated expression late in differentiation, while binding of c-Jun/Atf2 following JNK activation is presumed to increase FoxO-mediated
expression in response to oxidative or genotoxic stress (Bakker et al., 2007). BTG1 lacks enzymatic activity but contains several protein interaction domains, which suggests that BTG1 may be an adaptor/scaffold protein or a regulatory co-factor (Bakker et al., 2004).
Overall aims and thesis outline
FoxO transcription factors target genes have been implicated in cell cycle arrest, apoptosis, oxidative stress detoxification and DNA repair. Therefore, I hypothesised that FoxO transcription factors play a vital role in prevention of cancer. To date, most of the research conducted regarding the regulation of FoxO transcription factors focused on post-translational regulation of these transcription factors, however not much is known about the regulation of FoxOs at the transcriptional level. Therefore, I decided to examine whether FoxO transcription factors are regulated at the transcriptional level, in particular in response to treatment with the drug paclitaxel, which is used to treat cancer patients, including those suffering from breast and endometrial cancer. This drug has been shown to increase the expression of FoxO3a at the protein level. Hence, my hypothesis was that in addition to the regulation of FoxO at the post-translational level, these proteins are also regulated at the transcriptional level and that treatment with chemotherapeutic drugs increase FoxO transcription. The results of these experiments are presented and discussed in chapter 3.

In chapter 4, I focused on the function of FoxO3a. My aim was to investigate whether BTG1, a novel downstream target of FoxO3a identified in erythroid progenitors, also played a role in epithelial cells. To this end, I examined the effects of BTG1 over-expression in the MCF-7 breast cancer cell line.

In addition to FoxO3a, JNK1/2 activity has also been shown to be up-regulated following paclitaxel treatment. Therefore, my aim was to examine whether the JNK1/2 signalling pathway plays a positive role in the regulation of FoxO3a in response to paclitaxel treatment. To this end, I examined the effect of the JNK1/2 signalling pathway on FoxO3a sub-cellular distribution, on the stability of FoxO3a protein expression and the activity of FoxO3a. The results of these experiments are summarised and discussed in chapter 5.
2 Materials and Methods

2.1 Mammalian cell culture

2.1.1 Cell media
Human breast cancer cell lines (MCF-7, BT-474) and COS-1 (Green monkey kidney epithelial cells) were obtained from ATCC and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% foetal calf serum (FCS), 2mM glutamine, and 100U/ml of penicillin/streptomycin. (Invitrogen, Paisley, UK) MCF-7 Tet-Off BTG1 cells were selected and maintained in the presence of 100μg/ml of G418 (InvivoGen) and 200μg/ml hygromycin (InvivoGen), in a humidified incubator in an atmosphere of 10% CO2 at 37°C. Human endometrial cancer cells (HEC-1-B and Ishikawa cells) were maintained in Dulbecco’s modified Eagle’s medium/F12 (GIBCO Industries Inc., Carlsland, CA, USA) supplemented with 10% foetal bovine serum (Hyclone, Logan, UT, USA), 2mM L-Glutamine and 100 U/ml penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA), in a humidified incubator in an atmosphere of 5% CO2 at 37°C.

Inhibitors and drugs which were added to cell media: LY294002, SP600125 and Triciribine were obtained from Calbiochem and dissolved in dimethyl sulphoxide (DMSO). Paclitaxel was obtained from Sigma-Aldrich (Poole, UK) and dissolved in DMSO. MG-132 was obtained from Merck Biosciences Ltd. (Nottingham, UK) and dissolved in DMSO. 30% w/w H2O2 in H2O was obtained from Sigma-Aldrich (Poole, UK).

2.1.2 Sub-culturing of adherent cells
Cells were grown until a sub-confluent (80% confluence) state was reached. The media was then removed and the monolayer of cells was washed once with PBS. The monolayer was then detached using 1x trypsin-EDTA (ethylenediaminetetraacetic acid), 2ml were used for 100mm dish, for 3 min at 37°C) and the trypsin-EDTA was then inactivated by adding 7ml of complete media. The cells were then either plated at a defined ratio (1 in 5 of...
the total cells were re-plated), or were counted using a haemocytometer and plated at the required density, and were then maintained at 37°C in either 10% or 5% CO2 (according to cell line type).

2.1.3 Preservation of cells
Adherent cells from a sub-confluent (~80% confluence) dish were trypsinized, washed once in complete media and spun down at 700 xg for 5 min to remove any traces of trypsin, and re-suspended at 10⁶ cells/ml in FCS with 10% DMSO (BDH). 1ml aliquots were wrapped in several layers of tissue paper, transferred to cyrotubes and frozen at -80°C. The tubes were then transferred to liquid nitrogen after 48 h.

2.1.4 Recovery of frozen cells
Cells were removed from liquid nitrogen storage and thawed rapidly at 37°C. 1ml of the cell suspension was added to 9ml of complete media in a 15ml falcon tube and then spun at 700 xg for 5 min to remove the DMSO-containing media. The pellet was then re-suspended in 6ml of complete media and transferred to a 25cm² flask and incubated at 37°C incubator (either 5% or 10% CO2 depending on cell line type) until sub-confluence was reached, at which point the cells were sub-cultured.

2.1.5 Generation of a BTG1 inducible cell line
In order to determine the function of the BTG1 gene in breast cancer a stably transfected breast cancer cell lines which is able to express BTG1 in an inducible manner was generated. Initially both the ‘Tet-On’ and ‘Tet-Off’ systems were used. These systems enable expression of a gene under specifically regulated conditions. In the Tet-Off system, gene expression is up-regulated when doxycycline (Dox) or tetracycline (Tet) is removed from the medium, while expression is up-regulated in the Tet-On system by the addition of either Dox or Tet to the medium. Both systems regulate gene expression in response to Tet and Dox concentration (Clontech manual for Tet-On/Off systems).
The Tet system is made up of two components, the first one being the regulatory protein, which in the Tet-Off system is the tetracycline-controlled transactivator (tTA) and in the Tet-On system it is a "reverse" Tet repressor (rTetR). Both of the plasmids these plasmids have a neomycin-resistance gene (Clontech manual for Tet-On/Off systems). The second component is the response plasmid, which expresses the gene of interest under the regulation of the tetracycline-response element (TRE). When cells contain both the regulatory (pTet-Off or pTet-On) and the response (e.g. pTREhyg-gene B) vectors, gene B is expressed only when either the tTA or rtTA proteins bind to the TRE. In the Tet-Off system, tTA binds the TRE and activates transcription in the absence of Tet and Dox. In the Tet-On system, rtTA binds to the TRE and activates transcription in the presence of Tet and Dox. In both systems, transcription is turned either on or off in response to either Tet or Dox in a dose-dependent fashion (Clontech manual for Tet-On/Off systems). Hence, the coding region of BTG1 was amplified by PCR and cloned into the pTRE2hyg vector. MCF7 Tet-Off and MCF7 Tet-On cells were transfected with these constructs and the empty pTRE2hyg vector as a control. The transfected cells were grown in selective DMEM media that contained 100μg/ml of G418 (InvivoGen) and 200μg/ml hygromycin (InvivoGen). The media of the transfected MCF-7 Tet-Off cells also contained Dox at the concentration of 1μg/ml in order to abstain from BTG1 expression in case it may have a toxic effect on the cells. Single cell cloning was performed on the transfected cell populations. The clones isolated were screened for the incorporation of pTRE-BTG1 using PCR as detailed in section 2.5.8.

2.1.6 Sulphorhodamine B (SRB) assay
Cells were either untreated or treated and then plated, in quadruplicate, in a 96 well tissue culture plate at 3000 cells/well. After 24 h cells were fixed by adding 100μl of 40% ice cold TCA (Trichloroacetic acid) to each well and incubating at 4°C for 1 h. The plate was then washed five times with water. In order to stain the fixed cells, 100μl of 0.4% SRB in 1% acetic acid were
added to each well and the plate was incubated at RT (room temperature) for 1 h. The plate was then washed five times with 1% acetic acid. The plate was left to dry for 1 h at RT. In order to determine the amount of total protein in each well, 100µl of 10mM Tris were added to each well and the plate was left to shake for 30 min at RT and optical density (OD) was measured at 492nm.

2.1.7 DNA transfection of mammalian cells

2.1.7.1 Promoter assay
In preparation for transfection of MCF-7, COS-1, Ishikawa and BT-474 cells; cells were cultured in 96-well dishes in 100µL of complete media at approximately 7000cell/well. The following day the cells were transfected using the Fugene 6 transfection reagent (Roche, UK) according to manufacture’s instructions. The transfections were performed in quadruplicates. 20ng of pGL3 firefly-luciferase reporter plasmid (with the appropriate promoter sequence cloned in the MCS of this vector) and 5ng of pRLTK Renilla plasmid (Promega Southampton, UK) were added to 0.075µl of Fugene diluted in 5µl of DMEM without serum per well. The mixture incubated at RT for 15 min prior to adding it to the cells.

HEC-1-B cells were transfected using lipofectamine 2000 (invitrogen). Similarly to transfection carried out with Fugene 6, cells were cultured in 96-well dishes in 100µl of complete media at approximately 7000cell/well. The following day the cells were transfected using lipofectamine 2000 according to manufacture’s instructions. The transfections were performed in triplicates. 160ng of pGL3-FoxO1 and 40ng of pRLTK Renilla plasmid were diluted in 25µl of serum free DMEM media; 0.5µl of lipofectamine 2000 were also diluted in 25µl of serum free DMEM media. After 5 min incubation at RT of lipofectamine 2000 with the serum free media, the two tubes were combined and incubated for a further 10 min at RT. The mixture was then added to the cells.

For both types of transfections, the cells were left to recover for 16 h before treatment with drugs or inhibitors begun. At the appropriate time-points cells
were harvested for renilla and luciferase activity assay (described in 2.1.7.3) and kept at -20°C till all samples were ready to be read.

2.1.7.2 Co-transfection reporter assay
In preparation for co-transfection MCF-7, COS-1 and were cultured in 96-well dishes at approximately 7000 cell/well in a total volume of 100μL of complete DMEM per well. The transfections were performed in triplicates. The cells were transfected with a constant total amount of 85ng DNA/well which consisted of 20ng of the various pGL3 promoter constructs, 5ng of pRLTK Renilla plasmid (Promega Southampton, UK) and of 0-50ng of a protein expression constructs. In order to maintain an equal amount of total DNA transfected per well, 10-60ng of the empty expression vector backbone were added to the mix. The DNA mixture was then added to 0.255μl of Fugene diluted in 5μl DMEM without serum. The DNA-Fugene mix was incubated at RT for 15 min and then added to the cells. After 24 h incubation at 37°C, cells were harvested for firefly/renilla luciferase assay.

2.1.7.3 Luciferase assay protocols
Luciferase assay was performed either with Dual-Luciferase Reporter Assay System (Promega) according to manufacturer's instruction or LucLite Luminescence Reporter Gene Assay System (Perkin ElmerTM Life Sciences, Monza (Milano), Italy) coupled with native coelenterazine (n-CTZ, Lux Biotechnology, Edinburgh, UK).

When using the Dual-Luciferase Reporter Assay System, cells were lysed in 100μl 1x of the provided passive lysis buffer for 15 min at RT. Then 10μl were transferred to a 96 well microplate (OptiPlate-96, Perkin ElmerTM Life Sciences) and 50μl of Luciferase Assay Reagent II were added and firefly luciferase activity was measured. The luminescence was read by Packard TopCount Luminometer (Perkin–Elmer Life Sciences), two wells are read simultaneously for duration of one second, and counts per second are determined. Afterwards 50μl Stop & Glo Reagent was added and Renilla activity was measured, using the same machine and programme used for
reading luciferase activity. In LucLite Luminescence Reporter Gene Assay System, cells were lysed in 1xLucLite Reagent for 15 min at RT. 100μl of lysate were transferred to OptiPlate-96 (Perkin ElmerTM Life Sciences) and firefly luciferase activity was read with TopCount Luminometer (Perkin ElmerTM Life Sciences), as described above. Afterwards, 25μl of RenLite reagent [Coelenterazine (50μg/ml) in Renilla Buffer (0.5M HEPES, pH7.8, 40mM EDTA)] were added and the mixture was incubated for 20 min in the dark at RT before measurement of renilla activity, using the same programme and machine as mentioned above. The relative luciferase activity levels were determined by dividing experimental luciferase values by renilla luminescence values. As a negative control, renilla and luciferase values were measured in two types of negative controls, one type was blank wells, which contained only the lysis buffer, luciferase buffer and renilla buffer and the second type was wells which contained non-transected non-transfected cells and all of the three buffers. This was performed in order to give an indication on the efficiency of the co-transfection and to determine if the levels of luciferase and renilla activity were higher than the background reading.

Below is an example of raw data readings for luciferase and renilla obtained from three independent experiments performed in triplicates:

<table>
<thead>
<tr>
<th>Luciferase</th>
<th>Renilla</th>
<th>Replicas</th>
</tr>
</thead>
<tbody>
<tr>
<td>1278576</td>
<td>16144</td>
<td>Experiment A</td>
</tr>
<tr>
<td>1197968</td>
<td>14016</td>
<td></td>
</tr>
<tr>
<td>1181304</td>
<td>17312</td>
<td></td>
</tr>
<tr>
<td>1266032</td>
<td>20056</td>
<td></td>
</tr>
<tr>
<td>1186976</td>
<td>17760</td>
<td>Experiment B</td>
</tr>
<tr>
<td>1190646</td>
<td>17456</td>
<td></td>
</tr>
<tr>
<td>1108456</td>
<td>13960</td>
<td>Experiment C</td>
</tr>
<tr>
<td>1000328</td>
<td>12968</td>
<td></td>
</tr>
<tr>
<td>1077628</td>
<td>14328</td>
<td></td>
</tr>
</tbody>
</table>
2.2 FACS (Fluorescence Activated Cell Sorting) analysis

2.2.1 Fixing cells for FACS
Cells were trypsinized as usual and pelleted at 700 xg for 5 min, washed twice with 1 X PBS. Subsequently cells were fixed by re-suspending them in 90% ethanol in PBS, transferred in to a round bottomed polypropylene tube (Falcon) and incubated at 4ºC for at least 30 min until ready for FACS analysis.

2.2.2 Propidium Iodide (PI) staining of fixed cells for PI FACS (cell cycle) analysis
Fixed cells were pelleted at 700 xg for 5 min and were washed in 1ml of PBS prior to staining. Cells were then re-suspended in a staining solution of PBS, containing 10μg/ml propidium iodide (Sigma, Poole, UK) and 5ug/ml RNase A (Sigma, Poole, UK) to ensure that RNA is not stained. The cell suspension was subsequently passed through a 0.6mm bore needle in order to generate a single cell suspension. Tubes were wrapped in foil and incubated at 37ºC for 30 min before fluorescence-activated cell sorting analysis was performed using a FACSCalibur (Becton Dickinson, Cowley, UK).

2.2.3 PI FACS analysis
All FACS analysis was carried out using FACSCalibur (Beckton Dickinson, Cowley, UK) and the Cell Quest software (Becton Dickinson, Cowley, UK). Further analysis was carried out using FlowJo (Tree Star, Oregon, USA).

2.3 Reactive Oxygen Species (ROS) measurement
Cells were grown in 6-well tissue culture plates to 80% confluence. Shortly before performing the experiments, the ROS indicator (a mixture of reduced fluorescein and calcein) (C6827, Molecular Probes) was made up to a concentrated stock solution (50μM in DMSO). The cells were then removed from growth media via scarping and centrifugation at 700 xg. Cells were re-suspended in 1ml of pre-warmed PBS containing the ROS indicator probe diluted to the final working concentration of 5μM and incubated 37ºC for 15-20 min. After which, cells were centrifuged and re-suspended in pre-warmed
PBS. Cells that were not treated with oxidative stress inducing reagents and were not incubated with the probe were used to determine the level of autofluorescence in healthy cells and to calibrate the forward and side scatter of the flow cytometer. Negative controls, cells which were treated only with the probe, were used to assess the levels of ROS in healthy cells. Positive controls were generated by treating cells with 100µM H₂O₂ for 15 min before incubation with probe. The levels of fluorescence of experimental samples were compared to those of the negative and positive controls.

2.4 Immunofluorescence
MCF-7 cells were grown on sterile 13mm-diameter coverslips and fixed in 4% formaldehyde before being permeabilized in 0.01% v/v Triton X-100 for 10 min. Coverslips were blocked for an hour in PBS containing 3% bovine serum albumin (BSA) and antibody recognizing FoxO3a (06-951, Upstate) was added at 80µg/mL. Rabbit IgG at the same concentration was used as negative control. After washing three times for 5 min with PBS-Tween (0.05%), samples were incubated in the dark at RT for 30 min with a 1:1000 dilution of a secondary antibody conjugated to Alexa 488 anti-rabbit (Invitrogen, Paisley, UK). The cells were then washed three times for 5 min with PBS-Tween (0.05%), and were then mounted with Vector Shield anti-fade with DAPI (Vector Laboratories, Peterborough, UK). Analysis was performed using a Zesis confocal microscope with LSM meta 510 software.

2.5 Methods in molecular biology
2.5.1 Bacterial manipulation
2.5.1.1 Bacterial strains
In most cases, the XL1-Blue Subcloning-Grade competent E. coli cells (Stratagene) were used for plasmid preparation (standard transformation). The XL1-Gold Ultra competent cells (Stratagene) were used when cloning was performed (i.e. preparation of FoxO3a and BTG1 constructs).
2.5.1.2 Media and maintenance
Competent cells were grown in either Luria-Bertani (LB) broth or LB Agar. LB broth was made up by mixing 20g/L of LB broth powder (Sigma-Aldrich) with deionised water and autoclaving for 20 min at 120°C. LB Agar was made by mixing 37g/L of LB agar pellets (Miller) with deionised water and autoclaving as above. Where necessary, ampicillin (Sigma-Aldrich) was added to a final concentration of 100μg/ml.

2.5.1.3 Preparing chemically competent cells
XL-10 Gold *E.coli* (originally provided by Stratagene) were streaked on to a LB agar plate and incubated overnight at 37°C. A single colony was picked from the plate and grown overnight in 2ml of LB at 37°C in a shaking incubator. 1ml of the overnight culture was used to inoculate 100ml LB (a 1:100 dilution) in a 1000ml flask. The bacteria were grown until they reached an Optical Density (OD) of 0.4 at 600nm. The bacteria were then spun down at 8000 xg for 10 min at 4°C. The pellet was then re-suspended in 100 ml ice cold MgCl₂ (100mM) and incubated on ice for 30 min. The bacteria were then pelleted as before and the pellet was re-suspended in 100 ml ice cold CaCl₂ (100mM) and incubated on ice for another 30 min. The cells were pelleted again at 8000 xg and then re-suspended in 10ml freezing medium (30mM CaCl₂, 15% (v/v) Glycerol). The competent cells were snap-frozen in 100μl aliquots on dry ice and then stored till use at -80°C.

2.5.1.4 Bacterial transformations
A single 100μl aliquot of frozen competent bacteria was thawed on ice. 1μl of plasmid DNA (0.5-1μg/μl) was mixed with the thawed cells and left on ice for 30 min. Cells were then subjected to heat shock by immersing them in a 42°C water bath for 45 seconds and subsequently, kept on ice for a further 2 min. 1ml of LB medium (without ampicillin) was then added and the transformed bacteria were incubated at 37°C in a bacterial shaker for 1h. Transformed bacteria were then plated onto Agar plates containing ampicillin and incubated overnight at 37°C.
2.6 DNA manipulation

2.6.1 DNA quantification
The concentrations of DNA preparations were determined by measuring the optical density of the samples at 260nm (OD\textsubscript{260}) using a BioRad spectrophotometer (Bio-rad Smart Spec\textsuperscript{TM}3000 Spectrophotometer). DNA concentration was calculated using the relationship:

\[
1 \text{ OD unit at 260nm} = 50\mu\text{g/ml DNA}
\]

2.6.2 Sequence analysis
DNA sequences and PCR products were compared to the wild type DNA sequence using BLAST and BLAST two sequences (http://www.ncbi.nlm.nih.gov/blast). Localising of promoter regions and start transcription sites were performed using UCSC Genome Bioinformatics (http://www.genome.ucsc.edu) and Ensembl (www.ensembl.org). Mapping of transcription factors consensus binding sites in the various promoter-constructs was performed using TESS (http://www.cbil.upenn.edu/tess).

2.6.3 Restriction digests
Restriction digestions of DNA plasmids were performed using restriction endonucleases (New England Biolabs, Hitchin. Hertfordshire, UK). Briefly, the 10X buffer provided for each enzyme was diluted to 1 X in the final reaction mixture. Incubation time and temperature were adjusted according to the conditions specified by the manufacturer.

2.6.4 DNA ligation
DNA fragments with compatible over-hang ends were covalently joined using Rapid DNA Ligation Kit (Roche Applied Science) according to manufacture’s protocol.

2.6.5 DNA agarose gel electrophoresis
DNA fragments mixed in a final 1x Orange G loading dye (6x, Promega Southampton, UK) were separated by electrophoresis in 1-2% (w/v) agarose
gel (incorporated with 0.5μg/ml ethidium bromide) in 1% TAE (40mM Tris-acetate and 2mM EDTA) buffer. The gel image was recorded with a UVIpro Gel Documentation system (Uvitec, Cambridge, UK). DNA fragments which were to be extracted from the gel were exposure to only 70% UV light in order to reduce DNA damage through generation of thymine dimers (Grundemann and Schomig, 1996).

2.6.6 Extraction and purification of DNA
DNA was purified from the gel using either QIAquick Gel Extraction Kit or MinElute Gel Extraction Kit (Qiagen, Crawley, West Sussex, UK) according to manufacture’s instructions.

2.6.7 DNA precipitation
DNA precipitation was performed when either the concentration of DNA in the sample was lower than desired, or when farther purification of the sample was required, i.e. purifying the DNA from restriction enzymes. Ethanol precipitation was carried out as follows: 1 volume of DNA was mixed with 0.1 volumes of 3M sodium acetate pH 5.2 and 2.5 volumes of 100% ethanol. The mixture was stored at -20ºC for at least 2 h and then centrifuged using a bench top centrifuge at 13,000 rpm for 15 min at 4ºC. The pellet was washed using 70% ethanol and re-centrifuged at 13,000 rpm for 15 min at 4ºC. The supernatant was then discarded and the DNA pellet was left to air-dry for 15 min. The DNA was re-suspended in the appropriate volume of 10mM Tris or sterile water.

2.6.8 Polymerase Chain Reaction (PCR) for DNA
For PCR amplification of genomic or plasmid DNA reactions were carried out using the Qiagen Taq DNA polymerase kit. PCR reactions were set up in a total volume of up to 50μl consisting of 1x PCR buffer (Qiagen), 1x Q solution (Qiagen), 2mM dNTP (each) (Qiagen), 0.5μM of each forward and reverse primers, 0.1μl/10μl of Taq polymerase (Qiagen) and sterile water. The PCR was carried out using a Gene Amp® PCR system 9700 (Applied Biosystems) using the the following PCR programme:
1) 95°C 5 min
2) 95°C 30 seconds
3) Tm 30 seconds
4) 72°C 1 min/Kb

Repeat steps 2-4 30 times

5) 95°C 30 seconds
6) Tm 30 seconds
7) 72°C 10 min/Kb
8) 4°C hold

(Tm- melting temperature)

A List of the primers used for PCR can be found in Table 1

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5'-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>262FHBTG</td>
<td>TCCGCAGCATTTCGAAGATC</td>
</tr>
<tr>
<td>1210RHBTG</td>
<td>GCGTTATATCTGCTTTTCAGTAACTGG</td>
</tr>
<tr>
<td>BTG1HF</td>
<td>AAAAAGCTTCGCCATGCATCCCCCTTCTACAG</td>
</tr>
<tr>
<td>BTG1HR</td>
<td>AAAATCGATGCCCCACCCAAAGCAGAAAAA</td>
</tr>
<tr>
<td>BTG2HF</td>
<td>AAAAAGCTTACTTCCCCGCGAGACCTGCT</td>
</tr>
<tr>
<td>BTGHR</td>
<td>AAAATCGATTACGGCGAGCATGAGCAGCG</td>
</tr>
<tr>
<td>F-1514PFOXO3aNHE</td>
<td>CTAGCTAGCTCTCTCTCTTTCAGATTGTACTCG</td>
</tr>
<tr>
<td>R-2968HPFOXO3aXHO</td>
<td>CGGCCTCGAGAGCAGCACAAGTATAGACACACG</td>
</tr>
<tr>
<td>mMEF2F3F</td>
<td>GTCTTTTGCGAAAGTCAAAATGCCACG</td>
</tr>
<tr>
<td>mMEF2F3R</td>
<td>TTGACTTTTGCGCAAGACGTATCTTTTA</td>
</tr>
<tr>
<td>mef2FO3a-2F</td>
<td>GGATTTTCGAAATTTATGAACTAG</td>
</tr>
<tr>
<td>mef2FO3a-2R</td>
<td>AAATATTTTCCGAATCTCTTGTAGTCAC</td>
</tr>
<tr>
<td>hFO17FKPN</td>
<td>GCCGGTGACCCTATTTTTTCCTTTTTCCCTTC</td>
</tr>
<tr>
<td>HFO11845RH3</td>
<td>CCGGAAGCTTGGAGTGAAGCGCGAGCC</td>
</tr>
<tr>
<td>mef2FO1-1F</td>
<td>GGCAAGAAGATCGAAACACCCTACTTGATCAT</td>
</tr>
<tr>
<td>mef2FO1-1R</td>
<td>GGTGTTTCGTATCTTGTCAAAAAGGACAG</td>
</tr>
<tr>
<td>mef2FO1-2F</td>
<td>TATTTTTCCCTGCGAAAGCAAAAGCATAAACA</td>
</tr>
<tr>
<td>mef2FO1-3R</td>
<td>AACTTTTGCGAAATGCTAAGAAATGAGGAAC</td>
</tr>
<tr>
<td>GL2</td>
<td>CTTTATGTTTTGGCGCTTTCCA</td>
</tr>
<tr>
<td>Primer</td>
<td>Sequence 5’-3’</td>
</tr>
<tr>
<td>------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>RV3</td>
<td>CTAGCAAAATAGGCTGTCCC</td>
</tr>
<tr>
<td>M13F</td>
<td>GTAAACGACGGCCAGT</td>
</tr>
<tr>
<td>M13R</td>
<td>GGAAACAGCTATGACCATG</td>
</tr>
<tr>
<td>BTG1ex2-R61</td>
<td>TACAACGTAACCCCGATA</td>
</tr>
<tr>
<td>Rev-Hy-F2200</td>
<td>AGGTCGCCAACATCTTCTTC</td>
</tr>
</tbody>
</table>

Restriction sites are indicated in bold.
Mutations in primer sequence for site-directed mutagenesis are underlined.

Table 1: List of primers used for PCR amplification of DNA

### 2.6.9 PCR for screening MCF-7 Tet-On/Off BTG1 cell colonies

In order to screen MCF-7 Tet On/Off colonies for incorporation of the pTRE-BTG1 plasmid a PCR was performed using the Advantage 2 Polymerase mix (Clontech, California, USA). The template for the PCR was prepared as follows: Cell pellets were boiled in 10µl of 5mM Tris pH 7.5 and 0.5mM EDTA for 6 min and then centrifuged using a bench top centrifuged at 6000 rpm for 2 min at RT. 3µl of the supernatant were used as template for the PCR. The PCR was set out in a total volume of 50 µl consisting of 5µl buffer Ad2 PCR, 1µl dNTPs (10mM each), 10µM Rev-Hy-F2200 primer, 10µM BTG1ex2-R61 primer, 1µl Ad2 polymerase mix and 38µl sterile water. The empty vector (pTRE-hyg) was used as template for the negative control and the pTRE-hBTG1 plasmid was as template for the positive control. The PCR was carried out using the following programme : 95ºC for 3 min, 40 cycles of : 95ºC for 20 seconds, 60ºC for 30 seconds, 68ºC for 90 seconds and a final cycle: 95ºC for 30 seconds, 60ºC for 3 min, 68 ºC for 3 min.

### 2.6.10 Plasmid constructs

#### 2.6.10.1 Plasmids used in this study

Plasmids used to carry out the work presented in this thesis are listed in Table 2:
<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pLPC-FoxO3a(wt)</td>
<td>(Brunet et al., 1999; Fernandez de Mattos et al., 2004)</td>
</tr>
<tr>
<td>pLPC-FoxO3a(A3)</td>
<td>(Brunet et al., 1999; Fernandez de Mattos et al., 2004)</td>
</tr>
<tr>
<td>pCDNA3-FOXO3a</td>
<td>(Kwon et al., 2004)</td>
</tr>
<tr>
<td>pCMV-FOXO3a(wt)</td>
<td>Dr. Simon Arthur, MRC Unit, Dundee</td>
</tr>
<tr>
<td>pCMV-FOXO3aS294A</td>
<td>Dr. Simon Arthur, MRC Unit, Dundee</td>
</tr>
<tr>
<td>pCMV-FOXO3aS425A</td>
<td>Dr. Simon Arthur, MRC Unit, Dundee</td>
</tr>
<tr>
<td>pCMV-FOXO3a S294A, S425A</td>
<td>Dr. Ho Ka Kei, CRUK Labs, Imperial College</td>
</tr>
<tr>
<td>pCMV-FOXO3aS294D, S425D</td>
<td>Dr. Ho Ka Kei, CRUK Labs, Imperial College</td>
</tr>
<tr>
<td>pGL3-FOXO3a(-1480/-25)(full-length-FL)</td>
<td>This study</td>
</tr>
<tr>
<td>pGL3-FOXO3a(-1349/-25)</td>
<td>Dr. Ines Soeiro, CRUK Labs, Imperial College</td>
</tr>
<tr>
<td>pGL3-FOXO3a(-1155/-25)</td>
<td>Dr. Ines Soeiro, CRUK Labs, Imperial College</td>
</tr>
<tr>
<td>pGL3-FOXO3a(-984/-25)</td>
<td>Dr. Ines Soeiro, CRUK Labs, Imperial College</td>
</tr>
<tr>
<td>pGL3-FOXO3a(-478/-25)</td>
<td>Dr. Ines Soeiro, CRUK Labs, Imperial College</td>
</tr>
<tr>
<td>pGL3-FOXO3a(FL)mut1(-921/-918)</td>
<td>This study</td>
</tr>
<tr>
<td>pGL3-FOXO3a(FL)mut2(-1072/-1069)</td>
<td>This study</td>
</tr>
<tr>
<td>pGL3-FOXO3a(FL)mut1+2</td>
<td>This study</td>
</tr>
<tr>
<td>(-1072/-1069, -921/-918)</td>
<td>This study</td>
</tr>
<tr>
<td>pGL3-FOXO1(-1609/+230)(FL)</td>
<td>This study</td>
</tr>
<tr>
<td>pGL3-FOXO1(FL)mut1(-1444/-1441)</td>
<td>This study</td>
</tr>
<tr>
<td>pGL3-FOXO1(FL)mut2(-1368/-1365)</td>
<td>This study</td>
</tr>
<tr>
<td>pGL3-FOXO1(FL)mut3(-836/-833)</td>
<td>This study</td>
</tr>
<tr>
<td>pGL3-p27\textsuperscript{kip1}(-679/+1)</td>
<td>(Lynch et al., 2005)</td>
</tr>
<tr>
<td>pGL3-p21\textsuperscript{cip1}(-2325/+8)</td>
<td>(Koutsodontis et al., 2002; Lam et al., 1992)</td>
</tr>
<tr>
<td>pGL3-cyclin D1(-1179/+162)</td>
<td>(Leung et al., 2001)</td>
</tr>
<tr>
<td>pGL3-Bim(-689/+1)(wt)</td>
<td>(Essafi et al., 2005)</td>
</tr>
<tr>
<td>pGL3-Bim(-689/+1)(mut -164/-170)</td>
<td>(Essafi et al., 2005)</td>
</tr>
<tr>
<td>pCDNA3-HA-JNK2-JNK1</td>
<td>Dr. Axel Behrens (Zheng et al., 1999)</td>
</tr>
<tr>
<td>pGL3-BTG1(-871/+47)</td>
<td>This study</td>
</tr>
<tr>
<td>pTRE-BTG1</td>
<td>This study</td>
</tr>
<tr>
<td>pBABE-BTG1</td>
<td>(Bakker et al., 2004)</td>
</tr>
</tbody>
</table>

**Table 2:** Plasmids used in this study
2.6.10.2 **Plasmid construction**  

Plasmids constructed for this study using Polymerase Chain Reaction (PCR) are listed in Table 3 and Table 4:

<table>
<thead>
<tr>
<th>Resulting plasmid</th>
<th>Template used for PCR</th>
<th>Primers used for PCR</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGL3-BTG1(-871/+47)</td>
<td>human genomic DNA</td>
<td>262FHBTG, 1210RHBTG</td>
<td>948bp</td>
</tr>
<tr>
<td>pTRE-BTG1</td>
<td>human cDNA library</td>
<td>BTG1HF, BTG1HR</td>
<td>583bp</td>
</tr>
<tr>
<td>pGL3-FOXO3a(-1480/-25)</td>
<td>human genomic DNA</td>
<td>F1514PFoxO3aNHE, R2968HPoxO3aXHO</td>
<td>1455bp</td>
</tr>
<tr>
<td>pGL3-FOXO3a(FL)mut1(-921/-918)</td>
<td>pGL3-FOXO3a(FL)</td>
<td>mMEF2F3F, GL2</td>
<td>580bp</td>
</tr>
<tr>
<td>pGL3-FOXO3a(FL)mut2(-1072/-1069)</td>
<td>FOXO3a(FL)</td>
<td>mMEF2F3R, RV3</td>
<td>950bp</td>
</tr>
<tr>
<td>pGL3-FOXO3a(FL)mut1+2(-1072/-1069, -921/-918)</td>
<td>FOXO3a(FL)mut1</td>
<td>mEF2FO3a-2F, GL2</td>
<td>430bp</td>
</tr>
<tr>
<td>pGL3-FOXO1(-1609/+230)(FL)</td>
<td>human genomic DNA</td>
<td>hFO17FKPN, HFO11845RH3</td>
<td>1839bp</td>
</tr>
<tr>
<td>pGL3-FOXO1(FL)mut1(-1444/-1441)</td>
<td>pGL3-FOXO1(FL)</td>
<td>mEF2FO1-1F, GL2</td>
<td>180bp</td>
</tr>
<tr>
<td>pGL3-FOXO1(FL)mut2(-1368/-1365)</td>
<td>pGL3-FOXO1(FL)</td>
<td>mEF2FO1-2F, GL2</td>
<td>260bp</td>
</tr>
<tr>
<td>pGL3-FOXO1(FL)mut3(-836/-833)</td>
<td>pGL3-FOXO1(FL)</td>
<td>mEF2FO1-3F, GL2</td>
<td>900bp</td>
</tr>
</tbody>
</table>

**Table 3**: PCR carried out to generate the plasmids

<table>
<thead>
<tr>
<th>Resulting plasmid</th>
<th>Vector</th>
<th>Enzymes used for digestion and cloning</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGL3-BTG1(-871/+47)</td>
<td>pGL3</td>
<td>Bgl I, BamHI</td>
</tr>
<tr>
<td>pTRE-BTG1</td>
<td>pTRE2hyg</td>
<td>Cla I, Hind III</td>
</tr>
<tr>
<td>pGL3-FOXO3a (-1480/-25)</td>
<td>pGL3</td>
<td>Nhe I, Xho I</td>
</tr>
<tr>
<td>pGL3-FOXO3a(FL)mut1(-921/-918)</td>
<td>pGL3</td>
<td>Nhe I, Xho I</td>
</tr>
<tr>
<td>Resulting plasmid</td>
<td>Vector</td>
<td>Enzymes used for digestion and cloning</td>
</tr>
<tr>
<td>-------------------------------------------</td>
<td>--------</td>
<td>----------------------------------------</td>
</tr>
<tr>
<td>pGL3-FOXO3a(FL)mut2(-1072/-1069)</td>
<td>pGL3</td>
<td>Nhe I, Xho I</td>
</tr>
<tr>
<td>pGL3-FOXO3a(FL)mut1+2(-1072/-1069, -921/-918)</td>
<td>pGL3</td>
<td>Nhe I, Xho I</td>
</tr>
<tr>
<td>pGL3-FOXO1(-1609/+230)</td>
<td>pGL3</td>
<td>Kpn I, Hind III</td>
</tr>
<tr>
<td>pGL3-FOXO1(FL)mut1(-1444/-1441)</td>
<td>pGL3</td>
<td>Kpn I, Hind III</td>
</tr>
<tr>
<td>pGL3-FOXO1(FL)mut2(-1368/-1365)</td>
<td>pGL3</td>
<td>Kpn I, Hind III</td>
</tr>
<tr>
<td>pGL3-FOXO1(FL)mut3(-836/-833)</td>
<td>pGL3</td>
<td>Kpn I, Hind III</td>
</tr>
</tbody>
</table>

**Table 4:** Restriction digest and cloning of PCR products performed in order to generate the plasmids

### 2.6.11 Screening for recombinant bacteria colonies

#### 2.6.11.1 Colony PCR

A colony was picked from the agar plate using a 10μl pipette tip and placed in an eppendorf containing 10μl of sterile water. The samples were boiled for 10 min and then spun down. 1μl of supernatant was used as template in a 20μl PCR reaction using vector specific forward and reverse sequencing primer to determine if an insert was present in the colony.

#### 2.6.11.2 Small scale plasmid preparation and restriction digest

DNA was purified from the overnight culture using Miniprep Plasmid Purification Kit (Qiagen) according to manufacture’s instructions. Restriction digests were designed to determine whether the plasmid contained the correct insert in the right orientation.

#### 2.6.11.3 DNA sequencing

The DNA sequence of all plasmids was verified by DNA sequencing on a PerkinElmer 3700 automated DNA analyser in the DNA core laboratory using terminator cycle sequencing ready reaction kit (Applied Biosystems, Warrington, UK). 10μl of sequencing sample was prepared with 3.2 pmol sequencing primer and 200-500ng/3kb plasmid DNA or 30-90ng/1kb PCR product. The obtained chromatogram was analysed using either DNA strider (http://cellbiol.com/DNAStrider1_1_sit.bin) or Chromas 1.45 software (http://www.technelysium.com.au/chromas.html).
2.6.11.4 *Large scale plasmid preparation*
Once DNA sequence was verified large scale preparation of the DNA sample was performed. Larger amount of DNA was isolated from 250 ml of overnight bacteria culture using Maxiprep Plasmid Purification Kit (Qiagen) according to the manufacture's instructions.

2.7 RNA manipulation

2.7.1 *Total RNA extraction*
Total RNA was isolated using the RNeasy Mini kit (Qiagen) according to manufacture's instructions without DNase treatment as the silica-membrane spin column removes DNA. The concentrations of RNA preparations were determined by measuring the optical density of the samples at 260nm (OD$_{260}$) using a BioRad spectrophotometer (Bio-rad Smart Spec™3000 Spectrophotometer).
RNA concentration was calculated using the relationship:

\[ 1 \text{ OD unit at } 260\text{nm} = 40 \mu g/ml \text{ RNA} \]

2.7.2 *Real Time quantitative-PCR (RT-qPCR)*

2.7.2.1 *First strand cDNA synthesis*
Two-step reverse transcription PCR (RT-PCR) was used to generate cDNA from RNA samples. Depending on the RNA yield, 1-5 µg of total RNA was reverse transcribed to first strand cDNA using the Superscript III reverse transcriptase and oligo T primers (Invitrogen).

2.7.2.2 *Preparation of cDNA samples for RT-qPCR*
The resulting cDNA samples were used as template for RT-qPCR. In order to generate a standard, cDNA samples were pooled to a final concentration of 1µg/µl and four 1:5 serial dilutions were performed. For experimental cDNA the samples were further diluted to a final concentration of 10ng/µl. 5µl of each cDNA (standard and experimental) was used as template for a 25µl RT-qPCR.
2.7.2.3 Designing primers for RT-q PCR

Gene-specific primer pairs were designed using the ABI Primer Express software (Applied Biosystems). Primers with amplicon size 50-150bp spanning intron/exon boundary were selected whenever possible to avoid potential amplification of genomic DNA in cases were cDNA samples may be contaminated. Specificity of each primer was determined using NCBI BLAST module. Primer optimisation was performed as follows: Each primer set was optimised using the highest concentration of standard cDNA (final concentration of cDNA in the reaction was 40ng/μl) in order to determine the minimum concentration required for maximum yield (ΔRn) and minimum non-specific amplification. Dilutions of 50-900nM of forward and reverse primers in different combinations were tested. The concentration that gave the lowest Ct value (10 -35) with standard DNA and highest Ct value (40) with water was selected for use in further experiments. Specificity of PCR product was confirmed by dissociation curve analysis.

Primers used for Real Time PCR are listed in table 5

2.7.2.4 Quantification of gene expression

Real time PCR was performed using and Power SYBR® Green PCR Master Mix (Applied Biosystems Brackley, UK) and an ABI PRISM® 7700 Sequence Detection System (Applied Biosystems). All measurements were performed in triplicates. The PCR programme used was as follows: 50°C for 2 min, 95°C for 10 min and 40 cycles of: 95°C for 15 seconds followed by 60°C for 1 minute. L19, a ribosomal housekeeping gene, was used as an internal control to normalize input RNA. The quantification of gene expression was analysed with Sequence detection software v1.7 using the relative standard curve method. Standard deviation of the normalised value was calculated using the following formula:

\[ SD = \frac{m1}{m2} \times \left[ \frac{(s1/m1)^2 + (s2/m2)^2}{2} \right]^{0.5} \]

\( m = \text{mean}, \ s = \text{standard deviation}, \ 1 = \text{sample}, \ 2 = \text{endogenous gene L19 control} \)

Efficiency of PCR was determined from the slope of standard curve:

\[ \text{Eff} = 10^{(-1/slope)} - 1 \]
Ideal slope values are between -3.1 to -3.6.

The list of primers use for RT-qPCR can be found in Table 5.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>human FOXO1-sense</td>
<td>TGGACATGCTCAGCAGACATC</td>
</tr>
<tr>
<td>human FOXO1-antisense</td>
<td>TCTACGAGTGATGGTGCGTT</td>
</tr>
<tr>
<td>human FOXO3A-sense</td>
<td>CGACTATGCACTGACAGGTTGTG</td>
</tr>
<tr>
<td>human FOXO3A-antisense</td>
<td>GCAGCCGCGCGCAAA</td>
</tr>
<tr>
<td>human L19-sense</td>
<td>GAGCCTCTGGATGGTGCAAT</td>
</tr>
<tr>
<td>human L19-antisense</td>
<td>CTGGCCATGAACTACCTG</td>
</tr>
<tr>
<td>human cyclin E1-sense</td>
<td>GCGGAAGGGTGACAGCCAAAT</td>
</tr>
<tr>
<td>human cyclin E1-antisense</td>
<td>GTGGACGGGTTTACCCAAA</td>
</tr>
<tr>
<td>human cyclin D1-sense</td>
<td>GCAGCCGCGCGCAAA</td>
</tr>
<tr>
<td>human cyclin D1-antisense</td>
<td>GCAGCCGCGCGCAAA</td>
</tr>
<tr>
<td>human p27-sense</td>
<td>ACGGGAGCCCTAGCCTGGAGC</td>
</tr>
<tr>
<td>human p27-antisense</td>
<td>TGCCCTTCTCCACCTCTTGCC</td>
</tr>
<tr>
<td>human p21-antisense</td>
<td>AGGAACCTCTCATTAACCGGC</td>
</tr>
<tr>
<td>human BTG1-sense</td>
<td>AGACCTTCAGCCAGACGCC</td>
</tr>
<tr>
<td>human BTG1-antisense</td>
<td>GCGAATAAACGGTAAACCC</td>
</tr>
</tbody>
</table>

Table 5: Primers used for RT-qPCR

2.7.3 Statistical analysis of RT-qPCR and luciferase data
A two-tailed unpaired homoscedastic student’s t-test was performed in order to statistically analyse the results of luciferase and RT-qPCR experiments. This test enables the comparison of two arrays of data to each other in order to determine whether the values in the arrays are statistically different. p<0.05 and p<0.01, indicate that arrays are statistically different. While p<0.001 indicates that the arrays are different and that this is of higher statistical significance.

2.8 Protein analysis

2.8.1 Preparation of total protein extracts
For the lysis of adherent mammalian cells, cells were washed twice with ice cold 1 X PBS, scraped into 1ml of ice cold 1 X PBS and subsequently
transferred to a eppendorf tube. Alternatively, cells were washed once with 1 X PBS and then trypsanized. The cells were then centrifuged at 700 xg at 4°C for 5 min and the pellets were then immediately frozen at -80°C until lysis was performed. Frozen pellets were lysed on ice for 15 min in 2 volumes of NP40 lysis buffer (1% (v/v) Nonidet P-40, 100mM NaCl₂, 20mM Tris-HCl (pH 7.4), 10mM NaF, 1mM Sodium orthovanadate, 30mM β-glycerophosphate and protease inhibitors (‘Complete’ protease inhibitor mixture, as instructed by the manufacturer, Roche Applied Science). Cellular debris was spun down using a bench top centrifuge at 13000 rpm for 15 min at 4°C. The lysate was transferred to a fresh tube and its protein concentration was assayed.

2.8.2 Determination of protein concentration
Protein concentrations were determined using the Bio-Rad Dc protein assay (Bio-Rad Laboratories), a protein assay based on the Lowry assay (Lowry et al., 1951). Working reagent A was made by adding 20µl of reagent S (Bio-Rad) to each ml of reagent A (Bio-Rad) as instructed by the manufacturer. A standard curve was established by assaying 5 dilutions of a protein standard within the range of 0.2mg/ml to 1.5mg/ml protein. 100µl of reagent A+S was added to 2µl of each dilution and subsequently mixed with 800µl of reagent B (Bio-Rad). Absorbance was read at 750nm after 15 min and a standard curve was established. Absorbance at 750nm was then measured for each of the experimental samples and their concentrations were determined by multiplying the absorbance of the sample by the standard curve’s regression coefficient.

2.8.3 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)
Gels for resolution of proteins were made by using a 30% (w/v) Acrylamide/Bis stock solution (containing a ratio of 29 acrylamide: 1 NN’-methylenebisacrylamide; Bio-Rad). As a polymerisation catalyst, 25% (w/v) ammonium persulphate (APS) and TEMED were used. For all percentages of resolving gels, a 5% (w/v) stacking gel was used. SDS-PAGE gels were
topped with 2cm of stacking gel (see Table 6).

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Resolving gel</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7%</td>
<td>10%</td>
</tr>
<tr>
<td>30% Acrylamide</td>
<td>7.0 ml</td>
<td>10.0 ml</td>
</tr>
<tr>
<td>1M Tris*</td>
<td>11.2 ml</td>
<td>11.2 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.3 ml</td>
<td>0.3 ml</td>
</tr>
<tr>
<td>25% APS</td>
<td>100 μl</td>
<td>100 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>40 μl</td>
<td>40 μl</td>
</tr>
<tr>
<td>H₂O</td>
<td>11.5 ml</td>
<td>8 ml</td>
</tr>
</tbody>
</table>

* The resolving gels were made using 1M Tris.HCl pH 8.8, whereas the stacking gels were made using 1M Tris.HCl pH 6.8

Table 6: Proportions of the constituents of SDS-PAGE resolving and stacking gels

For separation of the proteins, 20μg of each cell lysate was mixed with 1 volume of 2 X SDS loading buffer (4% (w/v) SDS, 62.5mM Tris-HCl (pH 6.8), 1% (v/v) Glycerol, 0.01% (w/v) Bromophenol blue, 100mg dithiothreitol (DTT)) and boiled at 100ºC for 5 min. Samples were then loaded in to the stacking gel and fractionated by electrophoresis on SDS-PAGE gels (7%, 10% or 12.5%) for 2 h in running buffer (25mM Tris-base, 190mM Glycine, 0.1% (w/v) SDS) using a Mini-Protean III apparatus (Bio-Rad Laboratories, Hemel Hempstead, UK). Proteins were separated alongside broad-range pre-stained SDS-PAGE standards (Full-Range Rainbow Molecular Weight Markers, Amersham Biosciences).

2.8.4 Western blotting of SDS-PAGE
Following separation via SDS-PAGE, proteins were transferred to a 0.45μm Protran nitrocellulose membrane (Schleicher and Schuell) by electrophoretic transfer in a wet tank blotting system (Mini Trans-Blot Electrophoretic Transfer Cell, Bio-Rad Laboratories). The transfer was carried out in transfer buffer (25mM Tris, 190mM glycine and 20% (v/v) methanol) for 1-2 h at a constant current of 0.4A at RT. The nitrocellulose membrane was then blocked by incubating the membrane for 1 h at RT in either 5% (w/v)
skimmed milk powder (Marvel, Premier Brands) dissolved in Tris Buffered Saline-Tween (TBS-T) (20mM Tris-base, 136mM NaCl, adjusted to pH 7.6 supplemented with 0.05% Tween) or in 5% (w/v) bovine serum albumin (BSA) in Tris buffered solution with 0.05% Tween-20 (TBST, pH 7.5). The filter was then incubated for 2 h at room temperature (or overnight at 4ºC) with the primary antibody (see table 7) diluted (according to manufacture’s recommendations) in 5% BSA-TBS-T. The filter was then washed five times (10 min each at room temperature) in TBS-T prior to a 30 min incubation with horseradish peroxidase (HRP) conjugated secondary anti-mouse, anti-rabbit or anti-goat conjugates as appropriate (DAKO UK Ltd., Ely, UK). The membrane was then washed 5 more times, in TBS-T and visualized by enhanced chemiluminescence (Amersham Biosciences, UK). The membrane was then exposed to an auto-radiographic film (Amersham Biosciences, UK) for times varying from 10 seconds to 1 h.

<table>
<thead>
<tr>
<th>Primary Antibody</th>
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<tr>
<td>Akt (9272)</td>
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<tr>
<td>Phospho-Akt (Ser(^{473})) (9271S)</td>
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<td>CDK4 (C-22)</td>
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<tr>
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<td>Dr. Simon Arthur, MRC Unit, Dundee</td>
</tr>
<tr>
<td>Phospho-JNK(Ser^{425})</td>
<td>Dr. Simon Arthur, MRC Unit, Dundee</td>
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<td>Phospho-pRB Thr^{821} 44-582G</td>
<td>Biosource</td>
</tr>
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**Table 7:** Antibodies used for western blotting

### 2.8.5 Subcellular fractionation

Cells were pelleted and re-suspended in 100μl of cytosolic extraction buffer (10mM HEPES, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 2mM DTT, and complete protease inhibitor cocktail) for 15 min on ice. NP-40 was added to final concentration of 1% (v/v) and vortexed for 10 seconds in order to lyse the cells. The lysate was centrifuged at 13,000 rpm using a bench top centrifuge at 4°C. The supernatant fraction, which contains the cytosolic proteins, was snap frozen on dry ice. The pellet (containing nucleic extract), was then washed in 50μl of cytosolic buffer in order to reduce contamination of the nucleic fraction with cytosolic proteins and re-suspended and vortexed in 30μl of high salt nuclear extraction buffer (10mM HEPES, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 2mM DTT, 400mM NaCl, 1% NP40, and protease inhibitors). Afterwards, the lysate was rotated in the cold room for 15 min and centrifuged at 13,000 rpm for 5 min. The supernatant obtained was the nuclear extract. The protein concentration was determined as described in section 2.8.2. After calculating the yield of the protein extraction a ratio of 3:1 cytosolic to nucleic was determined. Therefore, 6μg of nuclear extract and 20μg of cytosolic extract were loaded on to a SDS-PAGE gel.
2.8.6 Immunoprecipitation

Cells were grown in 60mm plates to 60-70% confluency in the appropriate medium and growth conditions. Cells were washed twice with ice cold PBS and then scraped in a total volume of 50-100μl of NP40 lysis buffer (1% NP40, 150 mM NaCl, 20 mM Tris pH7.4, 10 mM sodium molibdate, 1 mM sodium orthovanadate, 1 mM sodium fluoride and Complete Mini protease inhibitors (Roche)) and incubated on ice for 15 min. The lysate was then centrifuged at 13,000 rpm, using a bench-top centrifuge, at 4°C for 15 min and the supernatant was transferred to a fresh centrifuge tube. 100μl of 50% slurry of protein A or G beads (Sigma) in PBS was then added to the cell lysate and incubated at 4°C for 1 h on an orbital shaker in order to pre-clear the lysate. The beads were then removed by centrifugation and the pre-cleared lysate was assayed for protein concentration. The cell lysate was diluted to approximately 1mg/ml in PBS and the recommended volume of immunoprecipitating antibody was added to 1ml of lysate. 15μl of the 50% protein A or G sepharose slurry was added to the cell lysate/antibody mix and the samples were incubated at 4°C for 3-4 h on an orbital shaker. Following incubation, the beads were collected by pulse centrifugations (5 seconds at 13,000 rpm) and washed 5 times with 500μl of lysis buffer. The beads were then re-suspended in 20μl of loading buffer and boiled for 5 min to dissociate the immuno-complexes from the beads. The beads were separated from the loading buffer by centrifugation and the supernatant was either frozen or loaded onto SDS-PAGE gels for protein fractionation.

2.8.7 Presentation of western blot data

For all western blot experiments presented in this thesis, I obtained results demonstrating a similar trend at least twice. Furthermore, results for some western blotting experiments are further confirmed by immunofluorescence experiments (chapter 5, figures 5.1, 5.2) or by sets of similar experiments performed in different cell lines (chapter 3), or experiments based on the results attained in the previous experiment (time-course following dose-
response- chapter 3, figures 3.1, 3.2). Experiments showing similar trends to
the blots presented in the result chapters (chapters 3-5) can be found in the
supplementary data (chapter 7).
3 Regulation of FoxO Genes in Breast Epithelial and Endometrial Cells

3.1 Background and objectives
Breast cancer is one of the most common cancers affecting women in the Western world and occurs following the accumulation of mutations which either increase the rate of cellular proliferation or reduce the levels of apoptosis. Mutations often arise following exposure to extracellular stimuli. Signal transduction pathways are responsible for transmitting extracellular signals to transcription factors, leading to changes in the expression of downstream target genes. These changes can often lead to the initiation of processes such as cell growth, differentiation and apoptosis. The PI3K pathway signal transduction pathway is highly conserved in eukaryotes and is activated following stimulation with growth factors, such as insulin and IGF-1. Activation of the PI3K signalling pathway results in activation and phosphorylation of Akt (Vanhaesebroeck et al., 1999; Vanhaesebroeck et al., 1997). A key downstream target of Akt is the FoxO family of transcription factors (Engstrom et al., 2003; Guo et al., 1999). Following activation of the PI3K pathway, FoxO is phosphorylated by Akt, leading to the export of FoxO from the nucleus, thereby inhibiting FoxO dependent transcription of target genes (Biggs et al., 1999). Two important target genes of FoxO are the cyclin-dependent kinase inhibitor (CKI) p27Kip1, which inhibits cell cycle progression and Bim, a BH3-only protein implicated in the induction of apoptosis. Therefore, activation of the PI3K pathway leads to the inactivation of FoxO and thereby FoxO induced growth arrest and apoptosis (Dijkers et al., 2000a; Dijkers et al., 2000b; Gilley et al., 2003; Medema et al., 2000).

Generally, breast cancer is treated with surgery and endocrine therapy. Endocrine therapy targets the ERα, by two different mechanisms, one by blocking the binding of estrogen to ERα by the use of drugs such as tamoxifen the second by blocking the estrogen synthesis process by the use of drugs such as aromatase inhibitors which reduce the concentration of
estrogen (Ali and Coombes, 2002). Although endocrine therapy is usually effective in treating estrogen dependent tumours, some tumours are resistant or develop resistance to these therapies and therefore other therapies such as taxanes are used (Ali and Coombes, 2002; Blagosklonny and Fojo, 1999; Symmans, 2001). Although taxanes primarily target the microtubules, understanding of the signal transduction pathways involved in taxanes-induction apoptosis in breast cancer is incomplete. However, it has recently been shown that accumulation of H$_2$O$_2$ is an required for paclitaxel-induced cell death (Alexandre et al., 2006). Sunters and colleagues (Sunters et al., 2003) were able to demonstrate an induction in FoxO3a expression following paclitaxel treatment, which resulted in an increase in cell death following an increase in the expression of Bim. The molecular basis for the up-regulation in FoxO3a protein expression in response to paclitaxel has not yet been elucidated. In addition the role of oxidative stress in the regulation of FoxO transcription and protein expression has not been determined. Therefore, I decided to examine the regulation of FoxO1 and FoxO3a transcription in cancer cell lines, in particular in response to paclitaxel treatment and oxidative stress.

3.2 Results

3.2.1 FoxO1 and FoxO3a expression levels

3.2.1.1 Paclitaxel treatment induces the expression of FoxO3a in MCF-7 cells

Previous research conducted in Prof. Lam’s lab (Sunters et al., 2003), has shown that paclitaxel treatment can up-regulate FoxO3a expression in MCF-7 cells. Initially, I decided to confirm these results and to determine the minimum concentration of paclitaxel required for up-regulation of FoxO3a expression in MCF-7 cells. This would enable me to determine at which concentrations I should treat the cells in order to examine the ability of paclitaxel treatment to induce transcription of FoxO genes. Hence, I conducted a dose response experiment, in which MCF-7 cells were treated...
with increasing concentration of paclitaxel (0-0.5µM) for 16 h and examined the level of FoxO1 and FoxO3a expression by western blot (Fig 3.1).

Western blot analysis (Fig 3.1) revealed that a basal level of FoxO3a expression is detectable in MCF-7 cells, as cells treated with the DMSO vehicle (0nM paclitaxel) expressed FoxO3a. An induction in FoxO3a expression was noticeable at the low concentrations of paclitaxel (e.g. 1nM paclitaxel). However, a dramatic increase and peak in FoxO3a expression levels were observed at the higher concentration of 10nM paclitaxel. FoxO3a expression remained high at 20nM paclitaxel, but an evident decrease in the level of FoxO3a expression was noted at 50nM paclitaxel. When cells were treated with higher doses of paclitaxel (200 and 500nM) the level of FoxO3a expression was low and comparable to non-treated cells, indicating that at these high doses, paclitaxel is ineffective in inducing the expression of FoxO3a. Examination of FoxO1 expression levels in MCF-7 cells revealed that the level of FoxO1 expression in MCF-7 cells was below western blot detection level.

I also decided to examine the activity of the up-stream regulator of FoxO3a, Akt, by monitoring the phosphorylation level of this protein at Ser-473, a site implicated in the activation of Akt (Alessi et al., 1996). In MCF-7 cells that were not treated with paclitaxel, I expected to see high levels of phospho-Akt expression, as these cells were grown at their optimum conditions in medium supplement with 10% foetal calf serum. However, although the level of expression of phosphorylated Akt was detectable, it appeared that only a small proportion of the total Akt expressed in the cells was phosphorylated at this site. Nonetheless, this still indicates that the Akt signalling pathway was activated in the non-treated MCF-7 cells and that therefore FoxO3a should be inactive and cytoplasmic. Interestingly, although the levels of total Akt expression remained constant throughout the dose response experiment, changes in presence of phospho-Akt were detected; at lower concentrations of paclitaxel a gradual increase in phospho-Akt was observed. The up-regulation in phospho-Akt reached its peak at the concentration of 50nM.
paclitaxel, which is a higher dose than that required for FoxO3a expression to peak. In fact, at the concentration of 50nM paclitaxel there was a clear decrease in the expression of FoxO3a in comparison to the lower doses. A decrease in the level of phosphorylated Akt was observed at 100nM and 200nM paclitaxel, with a significant decrease in the presence of phospho-Akt at 500nM paclitaxel, the highest dose of paclitaxel examined.

Apart from the PI3K/Akt signalling pathway, other signalling pathways may also affect paclitaxel sensitivity. The mitogen-activated protein kinases (MAPK) extracellular signal-regulated kinase 1/2 (ERK1/2) has been shown to be activated following paclitaxel treatment (Bacus et al., 2001) and an increase in paclitaxel related cellular toxicity has also been noted following ERK1/2 inhibition (MacKeigian et al., 2000). In addition, p38 MAPK and JNK signalling pathways have also been shown to be activated following paclitaxel treatment (Amato et al., 1998; Lee et al., 1998; Seidman et al., 2001). Therefore, I decided to examine the activity of these signalling pathways in response to the different concentrations of paclitaxel. The expression levels of total JNK were not affected by the use of increasing concentrations of paclitaxel; although a small stable increase in the expression of JNK1 (faster migrating band) was observed at 1-100nM paclitaxel, which decreased when cells were treated with higher concentrations of paclitaxel (e.g. 200nM and 500nM). However, significant changes in the phosphorylation levels of JNK at Thr183/Tyr185 were detected. A prompt increase in phospho-JNK was observed at 1nM paclitaxel, the lowest concentration of paclitaxel examined. Phosphorylation levels remained high at the concentrations of 10nM and 20nM paclitaxel. A slight decrease in phospho-JNK was observed at 50nM paclitaxel, but a rapid decrease in the expression levels of phospho-JNK was observed at 100nM paclitaxel, with virtually undetectable levels of phosphorylated JNK at the higher concentrations of paclitaxel (e.g. 200nM and 500nM).

Comparable levels of p38 expression were observed when cells were treated with 1-200nM paclitaxel. The level of p38 expression at these concentrations
was higher than that observed for non-treated MCF-7 cells. Nonetheless, dramatic changes in the phosphorylation status of p38 (Thr180/Tyr182) were observed. While in untreated cells phospho-p38 levels were undetectable, an increase in phosphorylated p38 was observed at 1nM paclitaxel and the phospho-p38 levels continued to gradually increase, peaking at 50nM paclitaxel. At concentrations of 100-500nM, a decrease in phosphorylated p38 was observed with no detectable phospho-p38 at 500nM paclitaxel.

The expression levels of total ERK1/2 remained predominantly the same throughout the doses of paclitaxel tested, although an increase in phosphorylated ERK1/2 (Thr202/Tyr204) was observed at 1nM paclitaxel. The levels of phospho-ERK1/2 then remained stable and a decrease was only observed at the higher concentrations of 200nM and 500nM paclitaxel.

Analysis of the data obtained in this experiment led to the decision to treat the MCF-7 breast cancer cell line with 10nM paclitaxel. Cells treated at this concentration for a period of 16 h showed a high level of FoxO3a expression that correlated with activation of the JNK, p38 and ERK signalling pathways, which have all been implicated in cellular response to paclitaxel.
Figure 3-1: Dose-dependent effect of 16 h paclitaxel treatment of MCF-7 cells.
MCF-7 cells were treated with 0-500nM paclitaxel for 16 h. Following treatment cell lysates were prepared, separated on SDS-polyacrylamide gels and immunoblotted with specific antibodies. The expression levels of FoxO3a were analysed using a total FoxO3a antibody. The activity of FoxO3a upstream regulator, Akt and the activity of stress induced signalling pathways, JNK, p38 and ERK were analysed using specific phospho (indicated by a P in-front of the protein name) and total antibodies. β-actin was used as a loading control. An experiment showing the same trend can be found in the supplementary data, figure 7.1.
I next decided to monitor, by western blot, the effect of treatment of MCF-7 cells with 10nM paclitaxel on FoxO3a expression during the course of 72 h. As shown in figure 3.2, treatment of MCF-7 cells with 10nM paclitaxel caused an up-regulation in total FoxO3a expression. A noticeable increase in FoxO3a expression occurred at 8 h, with the expression peaking 48 h after treatment begun. At 72 h a decrease in FoxO3a expression was observed, although the level of expression of FoxO3a remained elevated in comparison to the non-treated cells (0 h). Examination of the phosphorylation status of FoxO3a on the Akt phosphorylation site, Thr32 revealed an increase in phosphorylation of FoxO3a commencing at 8 h post treatment and ending 48 h post treatment. Interestingly, this pattern of expression was similar to that of total FoxO3a, which may suggest that the increase observed in total FoxO3a consisted also of Akt phosphorylated FoxO3a, which is inactive. Therefore, I decided to also examine the levels of total Akt and active Akt (phosphorylated on Ser-473), in order to determine if indeed an increase in Akt activity occurred following paclitaxel treatment. Western blot analysis revealed that levels of total Akt remained stable throughout the experiment. A decrease in phosphorylated Akt levels was observed at the beginning of the time-course and phospho-Akt expression was at its lowest at 4 h post treatment. An increase in phospho-Akt levels was observed at 8 h and phospho-Akt expression levels continued to rise up to 48 h post treatment, while a decrease in phospho-Akt levels was observed at 72 h. Hence, the expression patterns of phospho-Akt and phospho-FoxO3a were similar, indicating that the induction in Akt activity which occurred during the course of paclitaxel treatment resulted in the phosphorylation of FoxO3a on Thr32. However, it is important to note that the levels of phospho-FoxO3a only measure inactive FoxO3a and therefore have no bearing on active FoxO3a. As regards to the changes observed in the expression pattern of phospho-Akt, the decrease in phosphorylated Akt was expected as paclitaxel induces apoptosis, therefore inhibiting the pro-survival PI3K/Akt signalling pathway. However, the increase in phosphorylated Akt, observed at later time-points, was less expected. This
increase could be due to prolonged exposure of the cells to oxidative stress caused by paclitaxel treatment, as it has recently been shown that Akt can also be activated by oxidative stress through a PI3K dependent pathway (Lahair et al., 2006; Nishino et al., 2002). In order to determine if the increase observed in total FoxO3a expression consisted of transcriptionally active FoxO3a, I also examined the expression pattern of p27Kip1, a downstream target of FoxO3a. Up-regulation of p27Kip1 expression was observed, with expression levels peaking at 24 and 48 h after treatment. This correlated with the increase in the expression of total FoxO3a suggesting that the increase in p27Kip1 expression is due to activation of FoxO3a.

Examination of the activation status of MAPKs signalling pathways demonstrated a biphasic expression pattern for phosphorylated JNK. An increase in phospho-JNK was observed as early as 2 h post treatment with levels remaining elevated 4 h post treatment. The second peak in phospho-JNK expression was observed at 24-48 h after treatment began. No significant changes in the phosphorylation status of p38 were observed. A decrease in phospho-ERK1/2 was observed 8 h after treatment commenced, with an increase in phospho-ERK1/2 at 16 h and 24 h. Despite this increase in phosphorylation of ERK1/2, levels of phospho-ERK1/2 remained similar to those observed for the non-treated MCF-7 cells.
Figure 3-2: The effect of paclitaxel treatment on the expression of FoxO, p27Kip1 and the activity of MAPK signalling in MCF-7 cells.

Whole cell lysates were prepared at indicated time-points following treatment with 10nM paclitaxel, and then separated on SDS-polyacrylamide gels and immunoblotted. The levels of total FoxO1, total FoxO3a, Akt-phosphorylated (Thr32) FoxO3a, Akt, phospho-Akt (Ser473), p27Kip1, as well as the levels and activity of the, JNK, p38 and ERK signalling pathways were analysed by western blot using specific phospho (indicated by a P in-front of the protein name) and total antibodies. β-actin was used as a loading control. The arrow indicates the location of the FoxO3a-specific band. An experiment showing the same trend can be found in the supplementary data, figure7.2.
3.2.1.2 Paclitaxel treatment induces the expression of FoxO3a in BT-474 cells

In order to confirm that the results obtained for MCF-7 cells were not cell-type specific and in an effort to find a breast cancer cell line that expressed detectable levels of FoxO1, I also decided to test the response of another ER positive breast cancer cell line, BT-474, to treatment with 10nM paclitaxel during the course of 48 h. I decided to treat BT-474 cells with paclitaxel for 48 h because the results obtained for the MCF-7 cell line showed that treatment with paclitaxel for longer than 48 h did not generate a further increase in FoxO3a expression. This could be attributed to paclitaxel induced cell death occurring at later time-points.

Western blot analysis (Fig 3.3) of BT-474 cells treated with 10nM paclitaxel showed no detectable expression of FoxO1; however, an increase in the expression of FoxO3a occurred. Expression levels of FoxO3a were at their highest 16 to 24 h after treatment begun. An increase in phospho (Thr32)-FoxO3a was also detected at the same time-points, interestingly, no significant changes in the levels of phospho(Ser473)-Akt were noted, Therefore, in order to confirm that FoxO3a activity was up-regulated in the cells, I examined the expression pattern of the downstream transcriptional target, p27Kip1. The expression of p27Kip1 was induced 24-48 h after treatment with paclitaxel. This induction occurred after the initial increase in FoxO3a expression, suggesting that FoxO3a is responsible for the induction observed in p27Kip1 and that FoxO3a was transcriptionally active in BT-474 cells following paclitaxel treatment.

When examining the expression pattern of phospho(Thr183/Tyr185)-JNK, a biphasic expression pattern emerged, similar to the one noted for MCF-7 cells. A peak in phospho-JNK expression was observed at 8 h followed by a decrease in expression at 16 h and another peak in expression at 24-48 h post treatment. A small increase in phospho(Thr180/Tyr182)-p38 levels were observed at 8 h and the levels remained elevated for up to 24 h after treatment.
Figure 3-3: The effect of paclitaxel treatment on the expression of FoxO, p27^{Kip1} and the activity of JNK and p38 in BT-474 cells.

Whole cell lysates were prepared at indicated time-points following treatment with 10nM paclitaxel, and then separated on SDS-polyacrylamide gels and immunoblotted. The levels of total FoxO3a, Akt-phosphorylated (Thr32) FoxO3a, Akt, phospho-Akt (Ser473), p27^{Kip1} and the levels and activity of stress induced signalling pathways, JNK and p38 were analysed by western blot using specific phospho (indicated by a P in-front of the protein name) and total antibodies. β-actin was used as a loading control. An experiment showing the same trend can be found in the supplementary data, figure 7.3.
3.2.1.3 The HEC-1-B endometrial cell line expresses FoxO1

In attempt to find a cell line that expressed FoxO1, I decided to examine the response of the endometrial cell lines, HEC-1-B and Ishikawa, to paclitaxel treatment. Endometrioid endometrial cancer cells are estrogen-regulated and account for 80% of all endometrial carcinomas (Kong et al., 1997). FoxO1 expression in endometrium increases during the secretory phase of the cycle, in contrast FoxO3a expression is repressed in differentiating endometrium (Labied et al., 2006). It has also been demonstrated that FoxO1 plays a role in endometrial homeostasis by regulating cyclic differentiation and apoptosis of stromal cells in response to changes in ovarian progesterone levels (Kajihara et al., 2006). FoxO1 is also abundantly expressed in endometrial epithelial cells, however its function in this cellular compartment remains to be confirmed. Hence, these cell lines were good candidates for expression of FoxO1. Indeed as seen in figure 3.4, HEC-1-B cells expressed FoxO1. In addition, these cells showed up-regulation in FoxO1 expression following 10nM paclitaxel treatment. An increase in FoxO1 expression was observed 8 h post treatment, which remained stable throughout the 48 h time-course. The expression levels of FoxO1 in the Ishikawa endometrial cell line were detectable but low; however no induction in the expression of FoxO1 following paclitaxel treatment was observed.
Figure 3-4: Differential expression of FoxO1 in HEC-1-B and Ishikawa cells following paclitaxel treatment.

Whole cell lysates were prepared at indicated time-points following treatment with 10nM paclitaxel, and then separated on SDS-polyacrylamide gels and immunoblotted. The expression of total FoxO1 was analysed using a specific total FoxO1 antibody. β-actin was used as a loading control. An independent experiment showing the same trend can be found in the supplementary data, figure 7.4.
3.2.1.4 Paclitaxel treatment causes an increase in ROS (Reactive Oxygen Species) in MCF-7 cells

In order to test the hypothesis that oxidative stress may play a role in FoxO3a up-regulation in response to paclitaxel treatment, I decided to initially validate the observation that paclitaxel treatment can cause oxidative stress (Alexandre et al., 2006). Oxidative stress is exerted by the intracellular accumulation of ROS. Therefore, I conducted an assay, with the help of Dr. Stephen Myatt (CRUK Labs, Imperial College, London) to monitor the levels of ROS produced by MCF-7 cells following paclitaxel treatment. MCF-7 cells were treated with paclitaxel for 24 and 48 h and the level of oxidative stress was compared to that of MCF-7 cells treated with pure H2O2. Levels of oxidative stress were measured by monitoring oxidation of probes, which generate an increase in fluorescence and was analysed by flow cytometry. The results of the flow cytometer analysis are presented in figure 3.5A.

MCF-7 cells were treated with 10nM paclitaxel for 24 and 48 h. The levels of fluorescence at each time-point were compared to that of the negative control, non-treated MCF-7 cells and to that of a positive control, MCF-7 cells treated with 200µM H2O2 for 15 min, which is supposed to cause an increase in the generation of ROS in the cells. As shown in figure 3.5A, cells treated with H2O2 for 15 min generated more fluorescence than non-treated MCF-7 cells, the fluorescence mean was larger and the median value was greater for the H2O2 treated cells in comparison to the non-treated MCF-7 cells. The values obtained for MCF-7 cells treated with paclitaxel for 24 h and 48 h were similar. The fluorescence median values for paclitaxel treated cells resided between that of non-treated cells to that of H2O2 treated cells. This indicates that cells treated with paclitaxel for 24 or 48 h generate ROS, but to a lesser extent than cells treated with pure H2O2 for 15 min. I next set out to examine whether the prolonged treatment was the cause of the lower levels of ROS observed. Therefore, I decided to treat MCF-7 cells with H2O2 for 24-48 h. As can be seen in figure 3.5B, cells treated with H2O2 for 24 and 48 h did generate ROS, but to a lesser extent than cells treated for only 15 min, in fact
the levels obtained were similar to those acquired with paclitaxel treatment. This suggests that paclitaxel treatment causes oxidative stress in a similar manner to \( \text{H}_2\text{O}_2 \); however examination of the levels of ROS following 15 min of paclitaxel treatment is required in order to validate this statement.
Figure 3-5: Treatment of MCF-7 cells with paclitaxel and hydrogen peroxide causes increase in oxidative stress.

5µM of reduced fluorescein and calcein in DMSO were added to cells treated with paclitaxel (A) or with H₂O₂ (B) 1 h before measuring oxidation of these probes, which was detected by monitoring the increase in fluorescence with a flow cytometer. Mean and median measurements of fluorescence were taken. Cells treated with H₂O₂ for 15 min served as positive control and untreated cells served as negative control (A, B).
3.2.1.5 H₂O₂ treatment of MCF-7 cells results in up-regulation of the expression of FoxO3a

Since the previous experiment had demonstrated that paclitaxel treatment does indeed cause oxidative stress, I next decided to examine whether treatment with H₂O₂ during the course of 48 h rendered the same effect as paclitaxel treatment. To that end, MCF-7 cells were treated with 200µM H₂O₂ for 48 h and western blot analysis was performed (Fig 3.6).

Western blot analysis revealed an increase in FoxO3a expression that was prominent at 24 h post treatment. Levels of phosphorylated FoxO3a were high in the non-treated cells (0 h), which is probably because these MCF-7 cells were not exposed to oxidative stress and therefore should be proliferating and growing, due to the abundance of growth factors in the medium. An anticipated decrease in Akt-phosphorylation of FoxO3a was observed at 4 h post H₂O₂ treatment. An increase in phospho-FoxO3a was noted at later time-points, which correlated with the up-regulation in total FoxO3a expression. Hence, I also examined the expression pattern of active Akt (phospho-Akt). Interestingly, elevated levels of phospho-Akt expression were observed almost throughout the entire time-course. This could be due to oxidative stress because, as mentioned above, Akt can also be activated by oxidative stress (Lahair et al., 2006; Nishino et al., 2002). Therefore, it is plausible that the increase in phosphorylation of FoxO3a could be attributed to the up-regulation in Akt activity. Thus, in order to find out if FoxO3a is activated in the cells, I examined the expression pattern of the FoxO3a transcriptional downstream target, p27Kip1. A clear increase in total p27Kip1 was observed, indicating that FoxO3a activity was up-regulated in cells treated with H₂O₂.

Examination of the activation status of the MAPKs signalling pathways ERK1/2, JNK and p38, which have been implicated in cellular response to paclitaxel treatment (Amato et al., 1998; Lei and Davis, 2003; MacKeigan et al., 2000; Seidman et al., 2001) revealed that the levels of activated ERK (phospho-ERK) remained stable, while a slight decrease in phospho-p38
levels was detected 16 h after treatment. Similar to the results obtained with paclitaxel treatment, a biphasic expression pattern was detected for phospho-JNK. The first peak in expression was observed at 4-8 h after paclitaxel treatment and the second increase was observed at 24-48 h post treatment. Hence, both paclitaxel and H₂O₂ treatment induce the expression of FoxO3a and activate the JNK signal transduction pathway in a similar manner.
Figure 3-6: Oxidative stress generated by H$_2$O$_2$ causes an increase in FoxO3a expression in MCF-7 cells.

Whole cell lysates were prepared at indicated time-points following treatment with 200µM H$_2$O$_2$, and then separated on SDS-polyacrylamide gels and immunoblotted. The levels of total FoxO3a, Akt- phosphorylated (Thr32) FoxO3a, Akt, phosho-Akt (Ser473), p27Kip1 and the levels and activity of the signalling pathways, JNK, p38 and ERK were analysed by western blot using specific phospho (indicated by a P in-front of the protein name) and total antibodies. β-actin was used as a loading control. The arrow indicates the location on the FoxO3a-specific band. An experiment showing the same trend can be found in the supplementary data, figure 7.5.
3.2.2 FoxO mRNA expression levels

3.2.2.1 The effect of paclitaxel and H$_2$O$_2$ treatment on FoxO mRNA expression levels in MCF-7 cells

Since it was established that both paclitaxel treatment and H$_2$O$_2$ treatment can induce FoxO expression at protein level, I examined if this up-regulation could also be detected at the transcriptional, mRNA level. To this end, I treated MCF-7 cells with either 10nM paclitaxel or 200µM H$_2$O$_2$ for 48 h and determined the mRNA levels of FoxO1 and FoxO3a using Real Time quantitative PCR (RT-qPCR). The results were normalised with ribosomal protein L19 mRNA. When examining the levels of FoxO1 mRNA expression (Fig 3.7A, B) following either paclitaxel or H$_2$O$_2$ treatment, no significant changes in normalised FoxO1 mRNA levels were observed. This could be due to the fact that FoxO1 expression levels in MCF-7 cells are low, as indicated by western blotting experiment (figure 3.1). Results obtained for FoxO3a mRNA expression following paclitaxel treatment (Fig 3.7C) demonstrated a relatively stable level of mRNA expression with an increase in expression at 16 h. When monitoring FoxO3a mRNA expression following H$_2$O$_2$ treatment (Fig 3.7D), an increase in FoxO3a mRNA expression was noticeable at 8 h and at 48 h, with expression levels remaining relatively stable at other time-points examined. The increase at 16 h in FoxO3a mRNA expression following paclitaxel treatment is not likely to solely account for the up-regulation observed in FoxO3a protein expression, as levels of protein expression increased at 8 h and remained high for up to 48 h post treatment. When comparing FoxO3a protein expression to FoxO3a mRNA expression after H$_2$O$_2$ treatment, an increase in both protein and mRNA expression were observed 8 h after treatment commenced, however while protein expression levels peaked at 24 h, mRNA levels returned to a level similar to that of the non-treated MCF-7 cells (with the exception of elevated mRNA levels at 48 h). Hence, although the increase in FoxO3a mRNA expression may have contributed to the up-regulation in FoxO3a protein expression levels, it seems
unlikely that it explains the high levels of expression observed at later time-points, unless FoxO3a has a very long half-life.

Figure 3-7: The effect of oxidative stress on the levels of FoxO3a and FoxO1 mRNA in MCF-7 cells.
MCF-7 cells were treated with either 10nM paclitaxel (A and C) or 200µM H2O2 (B and D) for indicated times. Total RNA was extracted and analysed for the expression levels of either FoxO1 mRNA (A and B) or FoxO3a mRNA (C and D) using RT-qPCR and normalised to the level of L19 mRNA expression. All data shown represent the average of three independent experiments, and the error bars show the standard deviation, * p<0.05 in comparison to 0 h.
3.2.2.2 FoxO3a mRNA expression levels increase in BT-474 cells following paclitaxel treatment

Due to the fact that the RT-qPCR results obtained using MCF-7 did not match the protein expression pattern observed in these cells; I decided to also examine the levels of FoxO3a mRNA expression in BT-474 cells, as these cells also showed an increase in FoxO3a protein expression following paclitaxel treatment. BT-474 cells were treated for duration of 48 h with 10nM paclitaxel and levels of FoxO3a mRNA expression were assessed. As seen in figure 3.8A, an increase in FoxO3a mRNA was clearly observed, from as early as 8 h post treatment with expression levels peaking at 16 h. This expression pattern is similar to that of the FoxO3a protein (Fig 3.3) with the exception that FoxO3a protein levels remained elevated at 24 and 48 h, while mRNA levels returned to levels similar to those observed for non-treated cells. Hence, the increase in FoxO3a mRNA expression detected following paclitaxel treatment could account for the increase observed in FoxO3a protein expression.

In light of these results, I decided to examine the levels of p27Kip1 mRNA expression in BT-474 cells after paclitaxel treatment (Fig 3.8B). A gradual increase in p27Kip1 mRNA was observed 8-24 h after paclitaxel treatment, with mRNA expression levels peaking 24 h post treatment and then plummeting sharply at 48 h. This decrease could be due to the cell “switching-off” transcription of the p27Kip1 target gene. The peak observed in p27Kip1 protein expression occurred 24-48 h following paclitaxel treatment (Fig 3.3). Therefore, it is possible that the up-regulation in p27Kip1 protein expression occurred due to the increase in p27Kip1 transcription. Since the increase in p27Kip1 mRNA and protein expression occurred after the increase in FoxO3a mRNA and protein expression, it is likely that the up-regulation in FoxO3a was responsible for the increase in p27Kip1 expression in BT-474 cells.
Figure 3-8: FoxO3a and p27<sup>Kip1</sup> mRNA expression levels of are up-regulated in BT-474 cells following paclitaxel treatment.

BT-474 cells were treated with 10nM paclitaxel (A and B) for indicated times. Total RNA was extracted and analysed for the expression levels of either FoxO3a mRNA (A) or p27<sup>Kip1</sup> mRNA (B) using RT-qPCR and normalised to the level of L19 mRNA expression. All data shown represent the average of three independent experiments, and the error bars show the standard deviation, *p<0.05 in comparison to 0 h (non-treated cells).
3.2.2.3 FoxO1 mRNA expression levels are up-regulated in the HEC-1-B cell line following paclitaxel treatment

Since the levels of FoxO1 mRNA expression did not change following paclitaxel treatment in MCF-7 cells and previously, when using the HEC-1-B cell line, an increase in FoxO1 protein expression was observed (Fig 3.4); I decided to treat the endometrial cancer cell lines, HEC-1-B and Ishikawa with paclitaxel and monitor the levels of FoxO1 mRNA. RT-qPCR analysis of the expression pattern of FoxO1 mRNA in the Ishikawa cell line (Fig 3.9A) showed no significant up-regulation in FoxO1 mRNA expression following paclitaxel treatment. The levels of FoxO1 mRNA remained constant throughout the course of treatment. When examining the expression levels of FoxO1 mRNA in HEC-1-B cells following paclitaxel treatment (Fig 3.9B), an increase in FoxO1 mRNA expression was evident. The levels of FoxO1 mRNA expression peaked 48 h post treatment. These results are in agreement with the data obtained for FoxO1 protein expression in the Ishikawa and HEC-1-B cell lines (Fig 3.4). Thus, following paclitaxel treatment, up-regulation in the transcription and protein expression of FoxO1 occurs in the HEC-1-B but not the Ishikawa cell line.
Figure 3-9: FoxO1 mRNA levels are induced in response to paclitaxel treatment in HEC-1-B but not Ishikawa cells. Total RNA was extracted from Ishikawa cells (A) or HEC-1-B cells (B) and analysed for the expression level of FoxO1 mRNA using RT-qPCR and normalised to the level of L19 mRNA expression. All data shown represent the average of three independent experiments, and the error bars show the standard deviation. *p<0.05 **p<0.001 when compared to non-treated cells (0 h).
3.2.2.4 Oxidative stress increases the stability of FoxO3a in MCF-7 cells

In previous experiments, I was able to detect an increase in FoxO3a protein expression in MCF-7 cells treated with the oxidative stress inducing agents, paclitaxel and H2O2 (figures 3.1, 3.2, 3.6). However, I was unable to detect an increase in FoxO3a mRNA expression levels (figure 3.7C, D) of a magnitude that could account for the accumulation in FoxO3a protein observed in these cells. Hence, I decided to examine the stability of the FoxO3a protein in MCF-7 cells and determine if exposure of the cells to oxidative stress increased FoxO3a protein stability.

To that end, I pre-treated MCF-7 cells with either paclitaxel or H2O2 for 16 h and then conducted an 8 h time-course with cycloheximide, an inhibitor of protein biosynthesis. I then compared the rate of FoxO3a protein degradation in the pre-treated cells to that of MCF-7 cells that were not pre-treated with oxidative stress inducing agents. In order to confirm that the cycloheximide was indeed inhibiting the activity of the 60S ribosome, I also monitored the rate of degradation of the Id1 protein, which is known to have a short half-life (Bounpheng et al., 1999; Trausch-Azar et al., 2004). β-actin was used as an equal loading control, as this protein is relatively stable. The results obtained (figures 3.10, 3.11) demonstrate that both paclitaxel (Fig 3.10) and H2O2 (Fig 3.11) treatment affect the stability of FoxO3a. While a decrease in FoxO3a expression was noted for the non-pre-treated MCF-7 cells (Fig 3.10, 3.11 right panel), no prominent changes in FoxO3a levels were observed for cells pre-treated with either paclitaxel or H2O2 (Fig 3.10, 3.11 respectively, left panel). Hence, it is possible that exposure of MCF-7 cells to oxidative stress increases FoxO3a protein stability. The mechanism behind the increase in protein stability remains to be determined.
Figure 3-10: Paclitaxel treatment increases the stability of FoxO3a in MCF-7 cells. Cells were either pre-treated with 10nM paclitaxel for 16 h (left panel), or not pre-treated (right panel). After which cells were treated with 10μg/ml cycloheximide for up to 8 h. Whole cell lysates were prepared at indicated time-points, then separated on SDS-polyacrylamide gels and immunoblotted. The expression levels of FoxO3a and Id1, a protein with a short half-life were analysed by western blot. β-actin, a protein with a longer half life, was used as a loading control. An independent experiment showing the same trend can be found in the supplementary data, figure 7.6.
Figure 3-11: Oxidative stress increases the stability of FoxO3a in MCF-7 cells. Cells were either pre-treated with 200µM H$_2$O$_2$ (left panel) or not pre-treated (right panel) for 16 h. Following which, cells were treated with 10µg/ml cycloheximide for up to 8 h. Whole cell lysates were prepared at indicated time-points, and then separated on SDS-polyacrylamide gels and immunoblotted. The levels of FoxO3a and Id1, a protein with a short half-life were analysed by western blot. β-actin, a protein with a longer half life, was used as a loading control. An independent experiment showing the same trend can be found in the supplementary data, figure 7.7.
3.2.3 FoxO regulation at the DNA promoter level

3.2.3.1 Both paclitaxel H₂O₂ induce FoxO promoter activity in MCF-7 cells

In an attempt to further understand how FoxO genes are transcriptionally regulated, I decided to clone the regulatory 5' region of the human FoxO1 (-1609/+230) and FoxO3a (-1480/-25) genes into the luciferase reporter vector, pGL3 (Fig 3.12 top panel). The 5' regulatory regions were identified using http://genome.ucsc.edu/ and http://www.ensembl.org/index.html. Briefly, the 5' up-stream region of the genes was found by identifying the genomic coordinates of the genes and requesting the DNA sequence 5' the transcription start site of the genes. In order to examine the activity level of these promoters in response to oxidative stress, I transiently transfected MCF-7 cells with either the FoxO1 or FoxO3a promoter/luciferase constructs and pRLTK Renilla (serves to normalise transfection activity/efficiency) after which, I treated the transfected cells with either paclitaxel or H₂O₂.

FoxO1 promoter activity in MCF-7 cells (figure 3.12A, B) was up-regulated following both paclitaxel (Fig 3.12A) and H₂O₂ treatment (Fig 3.12B). Relative luciferase activity values obtained for paclitaxel treatment were higher (approximately double) than those obtained using H₂O₂. Both treatments induced the promoter activity 16 h after treatment. Paclitaxel treatment generated a gradual effect on FoxO1 promoter activity, with values peaking at 48 h post treatment. H₂O₂ treatment caused an increase in FoxO1 promoter activity at 16 h and its levels remained constant and high throughout the 48 h time-course.

FoxO3a promoter activity levels following paclitaxel (Fig 3.12C) and H₂O₂ treatment (Fig 3.12D) yielded similar results to those obtained with cells transiently transfected with the FoxO1 promoter construct. Both treatments caused an up-regulation in the promoter activity of FoxO3a that was noted 16 h post treatment. Like the FoxO1 promoter, FoxO3a promoter activity increased gradually following paclitaxel, but not H₂O₂ treatment, and reached levels twice as high as those observed for H₂O₂ treatment. Thus, paclitaxel
treatment is more effective than H$_2$O$_2$ in inducing $FoxO$ promoter activity, suggesting that the mechanism by which paclitaxel induces $FoxO$ promoter activity may be due to additional factors and not just oxidative stress.
Figure 3-12: The activity levels of FoxO3a and FoxO1 promoters in response to oxidative stress in MCF-7 cells.
Top panel, schematic representation of the human FoxO1 and FoxO3a promoters cloned into the pGL3 luciferase reporter construct. MCF7 cells were transiently transfected with 20ng of either the FoxO1 promoter construct (C and D) or the FoxO3a promoter construct (A and B) together with either 10nM paclitaxel (A and C) or 200µM H2O2 (B and D) for times indicated. The time-course was performed in a manner that enabled all samples to be harvested together, 72 h after transfection. Samples were assayed for luciferase activity. Values are corrected for co-transfected renilla activity. All data shown represent the average of three independent experiments, and the error bars show the standard deviation. *p<0.001 when compared to non-treated cells (0 h).
3.2.3.2 Paclitaxel treatment increases the FoxO1 promoter activity in HEC-1-B cells

Since an increase in both FoxO1 protein and mRNA expression was noted in the HEC-1-B cell line following paclitaxel treatment, I decided to examine FoxO1 promoter activity in this cell line in response to paclitaxel treatment. To this end, I transiently transfected HEC-1-B and Ishikawa cells with the FoxO1 promoter construct and then treated the cells with paclitaxel (figure 3.13). The basal level of FoxO1 promoter activity was lower in the Ishikawa cells in comparison to the HEC-1-B cells. While an increase in FoxO1 promoter activity was observed in the HEC-1-B cell line, no significant change was detected for the Ishikawa cell line. The promoter activity level was at its highest 24-48 h post-paclitaxel-treatment. Therefore, FoxO1 is expressed in the HEC-1-B cell line in a detectable manner at protein (Fig 3.4) mRNA (Fig 3.8) and promoter (Fig 3.13) levels. This expression can be induced at transcriptional (promoter, mRNA) and translational (protein) levels following paclitaxel treatment.
Figure 3-13: The activity of the FoxO1 promoter is up-regulated in response to paclitaxel treatment in HEC-1-B but not Ishikawa cells.

16 h after HEC-1-B and Ishikawa cells were transiently transfected with 20ng of the human FoxO1 promoter construct, cells were treated with 10nM paclitaxel for indicated times. Time course was performed in a manner that enabled all samples to be harvested together, 72 h after transfection and samples were assayed for luciferase activity. Values are corrected for co-transfected renilla activity. All data shown represent the average of three independent experiments, and the error bars show the standard deviation. *p<0.001 in comparison to non-treated HEC-1-B cells (0 h).
3.2.3.3 Analysis of the activity levels of FoxO3a promoter truncation constructs

In order to map region/s in the FoxO3a promoter important for up-regulation of FoxO3a transcription, truncated forms of the original FoxO3a promoter construct were generated (by Dr. Ines Soerio, CRUK Labs, Imperial College, London). These constructs were transiently transfected into cells and their activity levels were compared to that of the full-length construct. The FoxO3a promoter construct and the truncated forms are illustrated in figure 3.14A.

The FoxO3a full-length and truncated promoter constructs were transiently transfected into MCF-7 cells and their activity levels were normalised to that of co-transfected renilla (Fig 3.14B). The full-length FoxO3a promoter construct (-1480/-25) yielded the highest activity level, while the second largest construct (-1349/-25) was less active. Interestingly, both the -1155/-25 and the -984/-25 FoxO3a promoter constructs exhibited very low basal activity levels, while an even shorter form of the FoxO3a promoter (-478/-25) was more active. These results indicate that regulation of FoxO3a at the gene level is complex. The low levels of activity observed for the -1155/-25 and -984/-25 constructs could be due to the binding of a transcriptional repressor to a region between -478 to -1155bp upstream to the FoxO3a transcription start site. This effect was masked when using the longer construct (-1349/-25) owing to binding of a transcriptional activator to a region between -1155 to -1349bp upstream to the FoxO3a transcription start site. This hypothesis remains to be validated.
Figure 3-14: The activity levels of FoxO3a promoter truncation constructs in MCF-7 cells.
(A), Schematic representation of the human full length FoxO3a promoter construct and the derived truncated constructs cloned into the pGL3 luciferase reporter construct. (B), MCF-7 cells were transiently transfected with 20ng of either of the FoxO3a promoter constructs. Cells were harvested 24 h after transfection and assayed for luciferase activity. Values are corrected for co-transfected renilla activity. All data shown represent the average of three independent experiments, and the error bars show the standard deviation.
3.2.3.4 Mapping a region in the FoxO3a promoter responsive to paclitaxel treatment in MCF-7 cells

Previously, I was able to demonstrate that paclitaxel treatment up-regulates FoxO3a transcription at promoter level (Fig 3.12C). Examination of the basal activity levels of the truncated FoxO3a promoter constructs suggested the existence of regions in the FoxO3a promoter that negatively regulate transcription, while other regions generally have a positive effect on FoxO3a transcription (Fig 3.14B). Therefore, I next examined whether the up-regulation in FoxO3a transcription following paclitaxel treatment could be attributed to a specific region or regions within the FoxO3a promoter.

To this end, I transiently transfected MCF-7 cells with either the full length or one of the truncated FoxO3a promoter constructs (illustrated in Fig 3.14A) and treated the transfected cells with paclitaxel for 24 h. Normalised luciferase activity values are presented in figure 3.15A. As previously noted (Fig 3.12C), the full-length FoxO3a promoter construct (-1480/-25) was responsive to paclitaxel treatment and its activity levels increased following this treatment. The truncated FoxO3a promoter construct (-1349/-25) showed no change in FoxO3a promoter activity following paclitaxel treatment. This suggests that the region between -1349bp to -1480bp upstream to FoxO3a transcription start site is responsible for the increase in FoxO3a promoter activity observed for the full-length promoter construct. As observed before (3.14B), the basal promoter activity levels of the -1155/-25 and -984/-25 FoxO3a promoter constructs was very low and although these constructs showed an increase in activity levels following paclitaxel treatment, the values obtained were still very low (practically indetectable in comparision to the other constructs) and might be due to an experimental error rather than true up-regulation in activity following paclitaxel treatment. The activity level of the shortest truncated FoxO3a promoter construct tested (-478/-25) increased following paclitaxel treatment, indicating that the region between -478bp to -25bp upstream to the transcription start site, is positively regulated by paclitaxel treatment. A schematic representation of the results obtained can
be found in figure 3.15B. Areas within the promoter coloured in black represent regions in the promoter which are positively regulated by paclitaxel treatment.
Figure 3-15: The activity of the FoxO3a promoter truncation constructs in response to paclitaxel treatment in MCF-7 cells.

(A), MCF-7 cells were transiently transfected with 20ng of either of the FoxO3a promoter construct. Cells were treated with 10nM paclitaxel 16 h after transfection and were harvested 24 h following initiation of treatment and assayed for luciferase activity. Values are corrected for co-transfected renilla activity. All data shown represent the average of three independent experiments, and the error bars show the standard deviation. (B), Schematic representation of the full-length FoxO3a promoter construct, regions responsive to paclitaxel are indicated in black. *p<0.001 in comparison to cells transfected with the same construct but not treated with paclitaxel.
3.2.3.5 Mapping a region in the FoxO3a promoter responsive to paclitaxel treatment in BT-474 cells

In order to confirm the results obtained in the previous experiment, which indicated that two regions in the FoxO3a promoter construct are responsive to paclitaxel treatment (Fig 3.15), I decided to conduct a similar experiment using the BT-474 breast cancer cell line. Other experiments using this cell line showed that the FoxO3a protein expression is up-regulated following paclitaxel treatment (Fig 3.3) and that this up-regulation can also be observed at the mRNA level (Fig 3.8A). Hence, BT-474 cells were transiently transfected with either the full-length or the truncated forms of the FoxO3a promoter construct (illustrated in Fig 3.16A) and then treated with paclitaxel for up to 48 h. The results obtained for all five constructs are presented in Figure 3.16C, while the activity levels for untreated transfected cells are presented in figure 3.16B.

When examining the normalised promoter activity level of these constructs (0 h, Fig 3.16B), a similar pattern to that obtained using MCF-7 cells emerged. Using these cells, the -1155/-25 and -984/-25 constructs exhibited low levels of FoxO3a promoter activity. Furthermore, similar to the results acquired with the transfected MCF-7 cells, the activity levels of the full-length construct (-1480/-25) and the -478/-25 construct were up-regulated following paclitaxel treatment, while the -1349/-25 promoter construct was not responsive to paclitaxel treatment. A schematic representation of the regions responsive to paclitaxel treatment can be found in figure 3.16D.

In conclusion, the results attained from transfecting the BT-474 cell line with the FoxO3a promoter constructs supports the data acquired from the experiment conducted using MCF-7 cells. Taken together, these experiments suggest that the FoxO3a promoter contains two separate regions that are responsive to paclitaxel treatment and up-regulate the promoter activity following paclitaxel treatment.
Figure 3-16: The activity levels of the truncated FoxO3a promoter constructs in response to paclitaxel treatment in BT-474 cells. 
(A), schematic representation of the human full length FoxO3a promoter construct and the derived truncated constructs cloned into the pGL3 luciferase reporter construct. (B, C), BT-474 cells were transiently transfected with 20ng of either of the FoxO3a promoter construct. (C) Cells were treated with 10nM paclitaxel 16 h after transfection and were harvested at time-points indicated, up to 48 h following initiation of treatment and assayed for luciferase activity. Values are corrected for co-transfected renilla activity. All data shown represent the average of three independent experiments, and the error bars show the standard deviation. *p<0.001 in comparison to cells transfected with the same construct but not treated with paclitaxel (0 h). (D), Schematic representation of the human full-length FoxO3a promoter construct, regions responsive to paclitaxel are indicated in black.
3.2.3.6 The role of putative MEF2 DNA consensus binding sites in the regulation of FoxO3a promote activity

In order to further investigate the regulation of FoxO3a transcription at promoter level, I decided to map the transcription factor DNA consensus binding sites located in the promoter, using the Transcription Element Search System (TESS) (www.cbil.upenn.edu/tess) website.

Analysis of the FoxO3a promoter sequence revealed the presence of two putative myocyte enhancer factor 2 (MEF2) DNA consensus binding sites (-1072 and -922 upstream to FoxO3a transcription start site). Studies have shown that the MEF2 family of transcription factors play an important role in linking calcium-dependent signalling pathways to genes which play key roles in cell division, differentiation and death (McKinsey et al., 2002). Four vertebrate MEF2 genes have been identified, MEF2A, -B, -C and -D, which are expressed in distinct, but overlapping, patterns during embryogenesis, and in adult tissues. MEF2 proteins have a MADS domain in their nearly identical N-terminal. This domain mediates dimerization and binding to the DNA sequence CTA(A/T)₄TAG/A. An adjacent MEF2-specific domain influences DNA-binding affinity and interactions with cofactors. The C-terminal regions of MEF2 proteins are necessary for activation of transcription. Like other MADS domain proteins, MEF2 protein interact with an array of transcriptional cofactors to control specific sets of downstream target genes. The p38 and ERK pathways have been shown to increase MEF2 activity. The PI3K signalling pathway has also been implicated in the regulation of MEF2 (Tamir and Bengal, 2000; Xu and Wu, 2000). Activated forms of PI3K and its downstream effector, Akt, have been shown to increase MEF2-dependent transcription (Xu and Wu, 2000). Therefore, both MEF2 and FoxO are regulated by the PI3K/Akt signalling pathway. The p38 MAPK signalling pathway has been implicated in cellular response to paclitaxel treatment (Seidman et al., 2001). Taken together these data may indicate a possible link between MEF2 and FoxO, in particular in response to paclitaxel treatment.
A schematic illustration of the FoxO3a promoter constructs and the relative location of the MEF2 DNA consensus binding can be found in figure 3.17A. Results presented in figure 3.17B, show the relative basal activity levels of the truncated FoxO3a promoter constructs in reference to the location of MEF2 DNA consensus binding sites. This data was previously presented in figure 3.14B, with the exception of the locations of the MEF2 DNA consensus binding sites in each of the constructs. Analysis of the data presented in figure 3.17B divulged that the MEF2 DNA consensus binding sites are located in a region of the FoxO3a promoter which displayed low levels of basal promoter activity (the -1155/-25 and the -984/-25 truncated constructs). Therefore, it is possible that MEF2 binding represses FoxO3a promoter activity. However, in the context of the longer promoter constructs (-1349/-25 and -1480/-25) an activator of FoxO3a transcription binds to the promoter, therefore masking the negative effect of MEF2 on FoxO3a transcription. In order to further examine the role of MEF2 in the regulation of FoxO3a transcription experiments were conducted to determine whether exogenous expression of MEF2 affects FoxO3a promoter activity levels and if exogenous expression of MEF2 increases FoxO3a promoter activity in response to paclitaxel treatment.
Figure 3-17: FoxO3a promoter constructs and activity levels with reference to the location of MEF2 DNA consensus binding sites.

(A), Schematic representation of the location of MEF2 DNA consensus binding sites in the full-length FoxO3a promoter construct and the derived truncated constructs cloned into the pGL3 luciferase reporter construct (as before). (B), Relative activity of the truncated FoxO3a promoter/reporter constructs in reference to the location of MEF2 DNA consensus binding sites. All data shown represent the average of three independent experiments, and the error bars show the standard deviation. The grey oval shape represents MEF2 DNA consensus binding site.
3.2.3.7 Over-expression of MEF2 increases the activity of the FoxO3a promoter in MCF-7 cells

At first, I set out to examine the effect of MEF2 over-expression on the activity of the wt FoxO3a promoter construct. To that end, I cloned the MEF2A and the MEF2B cDNA (acquired from RZPD Deutsches Ressourcenzentrum für Genomforschung GmbH) into the pCDNA3 expression vector. MCF-7 cells were transiently co-transfected with the FoxO3a (wt) promoter construct and increasing amounts of either the MEF2A or the MEF2B expression plasmid (method explained in 2.1.6.2) and the activity levels of the FoxO3a promoter were monitored (Fig 3.18).

Both MEF2A and MEF2B were found to be able to induce FoxO3a promoter activity. This induction in FoxO3a promoter activity occurred even when relatively low amounts of MEF2 were co-transfected, indicating that the FoxO3a promoter may be highly sensitive to changes in MEF2 cellular concentrations.
Figure 3-18: The effect of MEF2 expression on the activity of the FoxO3a promoter in MCF-7 cells.

MCF-7 cells were transiently co-transfected with 20 ng of the FoxO3a promoter construct and increasing amounts of MEF2A or MEF2B. Cells were harvested 24 h after transfection and assayed for luciferase activity. Values are corrected for co-transfected renilla activity. All data shown represent the average of three independent experiments, and the error bars show the standard deviation. p<0.01 in comparison to cells transfected with 0 ng MEF2.
3.2.3.8 Co-transfection of MEF2A can further increase FoxO3a promoter activity following paclitaxel treatment

In order to further analyse the role of MEF2 in the regulation of FoxO3a transcription, the FoxO3a promoter construct was either transiently transfected or transiently co-transfected with 10ng MEF2A into MCF-7 cells (Fig 3.19) following the transfection some of the cells were also treated with 10nM paclitaxel. As previously observed (Fig 3.18), co-transfection of MEF2A resulted in an increase in FoxO3a promoter activity. An increase in FoxO3a promoter activity was also observed for cells which were transfected with the FoxO3a promoter construct alone, and then treated with paclitaxel. Nonetheless, a further increase in FoxO3a promoter activity was noted for cells which were co-transfected with MEF2A and then treated with paclitaxel, indicating that exogenous expression of MEF2 can augment the activity of the FoxO3a promoter in response to paclitaxel. Hence, it seems that over-expression of MEF2 may serve as a mean to induce the activity of the FoxO3a promoter, in particular in response to paclitaxel, which suggests that the MEF2 consensus binding sites identified in the FoxO3a promoter may facilitate the up-regulation observed. Analysis of FoxO3a promoter activity in cell in which MEF2 has been knockdown, with and without paclitaxel treatment, may help clarify the role of MEF2 in the regulation of FoxO3a promoter activity.
Figure 3-19: Co-transfection of MEF-2 increases FoxO3a promoter activity in paclitaxel treated MCF-7 cells.

MCF-7 cells were either transiently transfected with 20ng of the FoxO3a promoter construct or transiently co-transfected with 20ng of the FoxO3a promoter construct and 10ng MEF2A. 16 h post-transfection the cells were either not-treated or treated with 10nM paclitaxel 16 h for duration of 24 h. Cells were then harvested and assayed for luciferase activity. Values are corrected for co-transfected renilla activity. All data shown represent the average of three independent experiments, and the error bars show the standard deviation. *p<0.01 when compared to non treated (NT) cells transfected with just the FoxO3a promoter construct.
3.2.3.9 Over-expression of MEF2A induces FoxO1 promoter activity

In light of the results obtained from studying the role of MEF2 in regulating FoxO3a promoter (Fig 3.17-Fig 3.19), I analysed the FoxO1 promoter sequence using TESS (www.cbil.upenn.edu/tess) and found three putative MEF2 DNA consensus binding sites within the region cloned into the pGL3 vector (-1690/+230). In order to examine the role of MEF2 in the regulation of FoxO1 promoter activity, I conducted a similar experiment to the one performed using the FoxO3a promoter construct (figure 3.19). The FoxO1 promoter construct was either transiently transfected or transiently co-transfected with 10ng MEF2A into MCF-7 cells. Following the transfection some of the cells were also treated with 10nM paclitaxel. As shown in figure 3.20 co-transfection of MEF2A resulted in an increase in FoxO1 promoter activity. As expected, an increase in FoxO1 promoter activity also occurred in cells which were transfected with the FoxO1 promoter and treated with paclitaxel. Furthermore, an additional increase in FoxO1 promoter activity was noted for cells which were co-transfected with MEF2A and then treated with paclitaxel, indicating that exogenous expression of MEF2 can further enhance the activity of the FoxO1 promoter in response to paclitaxel. Therefore, it seems that over-expression of MEF2 may serve as a mechanism to induce FoxO1 promoter, in particular in response to paclitaxel treatment. In order to further study the role of the MEF2A DNA consensus binding sites found in the FoxO1 promoter sequence, I mutated each one of the MEF2 DNA consensus binding sites within the FoxO1 promoter construct. A schematic representation of the constructs generated can be found in figure 3.21A. Following which, MCF-7 cells were transiently co-transfected with either the wild-type or one of the mutated FoxO1 promoter constructs (Fig 3.21 B).

As shown in figure 3.21B, there were differences in the basal promoter activity level of the four promoter constructs. The wild-type FoxO1 promoter construct exhibited high levels of basal promoter activity which were comparable to that of the FoxO1mut3 construct. The FoxO1mut1 and
FoxO1mut2 constructs exhibited lower levels of promoter activity. These results suggest that mutating two of the three MEF2 DNA consensus binding sites (-1447 and -1370) identified in the FoxO1 promoter sequence may lead to a decrease in FoxO1 promoter activity. Therefore, these sites might be involved in positively regulating FoxO1 transcription. In order to confirm that these sites positively regulate FoxO1 transcription, by the binding of MEF2 to the promoter, a chromatin immunoprecipitation experiment need to be carried out. Analysis of FoxO1 promoter activity, with and without paclitaxel treatment, in cells in which MEF2 is knocked down, will help determine whether MEF2 plays a role in up-regulating FoxO1 promoter activity in response to paclitaxel.
Figure 3-20: Co-transfection of MEF-2 increases FoxO1 promoter activity in the in paclitaxel treated MCF-7 cells.
MCF-7 cells were either transiently transfected with 20ng of the FoxO1 promoter construct or transiently co-transfected with 20ng of the FoxO1 promoter construct and 10ng MEF2A. 16 h post-transfection the cells were either not-treated or treated with 10nM paclitaxel 16 h for duration of 24 h. Cells were then harvested and assayed for luciferase activity. Values are corrected for co-transfected renilla activity. All data shown represent the average of three independent experiments, and the error bars show the standard deviation. *p<0.01 when compared to non treated (NT) cells transfected with just the FoxO1 promoter construct.
Figure 3-21: The effect of mutating MEF2 DNA consensus binding sites on the activity of the FoxO1 promoter in MCF-7 cells.
(A), schematic representation of the human full-length wtFoxO1 promoter construct (top) and the derived constructs which are mutated in one of the three MEF2 DNA consensus binding sites. The grey oval shape represents MEF2 DNA consensus binding sites, the X symbol within the grey oval shape indicates a point mutation in the MEF2 DNA consensus binding site. (B) MCF-7 cells were transiently transfected with 20ng of the FoxO1 promoter construct shown in (A) and were harvested and assayed for luciferase activity 36 h post-transfection. Values are corrected for co-transfected renilla activity. All data shown represent the average of three independent experiments, and the error bars show the standard deviation. *p<0.05 when compared to cells transfected with the wtFoxO1 promoter construct (-1690/+230).
3.3 Discussion
The FoxO family of transcription factors have been shown to have anti-proliferative and pro-apoptotic functions (Brunet et al., 1999; Dijkers et al., 2000a; Dijkers et al., 2000b; Medema et al., 2000; Nakamura et al., 2000; Stahl et al., 2002; Tran et al., 2002) and their activity is negatively regulated by the PI3K/Akt signalling pathway (Biggs et al., 1999; Brunet et al., 1999; Kops et al., 1999; Rena et al., 1999; Tang et al., 1999). FoxO transcription factors have been suggested to play an important role in preventing tumourigenesis (Yang et al., 2005a). Hence, the FoxO family of transcription factors has been the focus of many recent studies in hope to better understand their function and regulation. Although progress has been made as regards to the post-translational regulation of FoxO proteins, little is known about the transcriptional regulation of these proteins.

Paclitaxel is used to treat breast cancer but the means by which paclitaxel promotes apoptosis has not been fully determined (Gelmon, 1994; Luck and Roche, 2002). Previously, it has been shown that in MCF-7 cells the levels of FoxO3a protein expression and its downstream pro-apoptotic transcriptional target, Bim, increase treatment with paclitaxel (Sunters et al., 2003). In this study, I was able to show that indeed paclitaxel treatment caused up-regulation in the expression of FoxO3a and downstream target p27Kip1, in two ER positive breast cancer cell lines, MCF-7 and BT-474 (Fig 3.2, 3.3); strengthening the hypothesis that FoxO3a mediates paclitaxel induced cell death. Others have shown that over-expression of Akt can result in increased resistance to paclitaxel further emphasising the role of the PI3K/Akt/FoxO pathway in determining sensitivity to this drug (Page et al., 2000; VanderWeele et al., 2004).

Since the ERK1/2, p38 and JNK signal transduction pathways may also affect paclitaxel sensitivity (Amato et al., 1998; Bacus et al., 2001; Lee et al., 1998; Lei and Davis, 2003; MacKeigan et al., 2000; Seidman et al., 2001), I also examined cellular levels and activation status of these kinases (Fig 3.1, 3.2, 3.3) following paclitaxel treatment. In a dose-response experiment an
increase in levels of activated JNK, p38 and ERK1/2 were observed at concentrations ranging from 1-100nM paclitaxel 16 h post treatment (Fig 3.1). Since the largest induction in FoxO3a expression was observed at 10nM paclitaxel, MCF-7 cells were treated with 10nM paclitaxel for duration of 72 h (Fig 3.2). At this concentration of paclitaxel, prominent biphasic JNK activation was detected, while no significant induction in p38 activity was observed. Levels of activated ERK decreased during the course of treatment and then returned to levels similar to that of untreated cells, suggesting that this signalling pathway was not activated following treatment with 10nM paclitaxel. Results obtained for treatment of BT-474 cells with 10nM paclitaxel (Fig 3.3) also showed a biphasic increase in JNK activity, but no prominent change in p38 activation status was detected. Taken together, these results suggest that out of the three signalling pathways, the JNK signalling pathway is the predominant signal transduction pathway to become activated in response to treatment with 10nM paclitaxel in both the MCF-7 and BT-474 cell lines.

Since I was unable to detect FoxO1 expression in the MCF-7 and BT-474 cell lines, I examined the expression levels of FoxO1 in the endometrial cells lines, Ishikawa and HEC-1-B. Both cell lines expressed FoxO1, however the HEC-1-B cell line expressed FoxO1 at higher levels and expression was further induced following 10nM paclitaxel treatment (Fig 3.4). This result indicates that not only FoxO3a expression, but also FoxO1 expression can be induced following paclitaxel treatment and that this regulation depends on which isoform of FoxO is the predominantly expressed in the cell line.

The molecular basis for the up-regulation in FoxO proteins in response to paclitaxel is unknown. I found that both paclitaxel and H2O2 treatment increased the levels of ROS in the cells and a previous report demonstrated that accumulation of H2O2 is required in order for paclitaxel to induce apoptosis (Alexandre et al., 2006). Oxidative stress results from an imbalance between the production and removal of ROS. One of the target genes of FoxO is catalase, an enzyme responsible for breakdown of peroxide (Nemoto
et al., 2004). Therefore, oxidative stress could play a key role in inducing FoxO expression. Treatment of MCF-7 cells with H$_2$O$_2$ induced the levels of FoxO3a expression and activated the JNK signalling pathway (Fig 3.6). Therefore, both paclitaxel and H$_2$O$_2$ treatment increase the levels of FoxO3a expressed in the cells and activate the JNK signalling pathway (schematically represented in figure 3.23). The role of the JNK signalling pathway in the regulation of FoxO3a will be discussed in detail in chapter 5.

In order to determine the mechanism of induction in FoxO protein expression following exposure to oxidative stress, I examined the effect of paclitaxel and H$_2$O$_2$ treatment of transcription of FoxO genes. BT-474 cells and HEC-1-B cells showed an increase in FoxO3a and FoxO1 mRNA and promoter activity, respectively. Hence, paclitaxel treatment generates an increase in FoxO protein expression by up-regulation of FoxO transcription in BT-474 and HEC-1-B cells (schematically represented in figure 3.22B and C, respectively). However, the mechanism by which paclitaxel and H$_2$O$_2$ treatments induced the expression of FoxO3a in MCF-7 cells differs from that observed in the HEC-1-B and BT-474 cell lines. Although both treatments caused up-regulation in FoxO3a promoter activity, the increase observed in FoxO3a mRNA levels was not sufficient to account for the accumulation in the FoxO3a protein (schematically represented in figure 3.22A). Examination of FoxO3a protein stability in MCF-7 cells showed an increase in FoxO3a half-life in MCF-7 cells that were exposed to oxidative stress (Fig 3.10, 3.11). This increase in the stability of FoxO3a could cause accumulation of the protein, augmenting protein expression levels without up-regulation in protein synthesis.

An increase in protein stability could arise from either preventing or decreasing the rate of protein degradation. FoxO1 and FoxO3a have both been shown to be regulated by the ubiquitin–proteasome pathway (Aoki et al., 2004; Hu et al., 2004; Huang et al., 2005; Matsuzaki et al., 2003; Plas and Thompson, 2003). FoxO1 and FoxO3a ubiquitin dependent degradation occurs following phosphorylation of these proteins at the Akt regulatory sites.
(Aoki et al., 2004; Matsuzaki et al., 2003). The Skp2 ubiquitin ligase was shown to be required for FoxO1 proteolysis (Huang et al., 2005), however the ubiquitin ligase responsible for the degradation FoxO3a is unknown. In addition, changes in the phosphorylation status of proteins have been shown to affect protein stability (Yaglom et al., 1995). Phosphorylation of certain protein residues can serve to mask motifs that ubiquitin proteasome pathway recognises (Khanday et al., 2006). Therefore, it is possible that in MCF-7 cells oxidative stress stabilises FoxO3a expression by causing FoxO3a to become phosphorylated at a site/s which masks motif/s recognised by ubiquitin-26S proteasome, therefore preventing FoxO3a degradation. Maintaining constant levels of FoxO3a synthesis and increasing FoxO3a protein half-life, could generate an increase in total levels of FoxO3a expressed in the cells.

Analysis of the regulation of FoxO at the promoter level demonstrated that both FoxO1 and FoxO3a can be regulated by oxidative stress inducing reagents (Fig 3.12, 3.13, 3.15, 3.16). FoxO1 promoter activity was up-regulated in the MCF-7 cell line, despite the fact that no increase in FoxO1 mRNA expression was observed and FoxO1 protein expression in this cell line was undetectable. This differs from the result obtained for the HEC-1-B cell line which showed up-regulation at the protein, mRNA and promoter levels of FoxO1 expression following paclitaxel treatment (schematically represented in figure 3.22C). Therefore, it seems that MCF-7 cells express the transcription factors required for up-regulation of FoxO1 activity at the promoter level, but under endogenous conditions these cells negatively regulate the expression of FoxO1. Previously it has been demonstrated that all FoxO proteins can regulate the expression of the same target genes. Hence, it is likely that since MCF-7 cells express FoxO3a at high and inducible levels that down regulation of FoxO1 expression occurred in this cell line and transcriptional regulation of FoxO targets genes occurs via FoxO3a. One way of negatively regulating gene expression is via methylation
of CpG islands on the gene promoter. Thus, negative regulation of FoxO1 expression in MCF-7 cells could be achieved by methylation of the FoxO1 promoter CpG islands; this waits to be determined. However, it has recently been shown that low levels of FoxO1 expression in Ishikawa cells were the result of an increase in mRNA turnover and promoter methylation or activity, (Goto et al., 2007). Therefore, increased mRNA turnover this is another mechanism cells can manipulate to decrease the expression levels of genes, and may also account for the low levels of FoxO1 expression in MCF-7 cells.

Analysis of the activity levels of truncated FoxO3a promoter constructs in MCF-7 and BT-474 cells in response to paclitaxel treatment revealed 2 regions in the promoter sequence which are responsive to paclitaxel treatment (Fig 3.15, 3.16). The first region located at -478 to -25bp and the second region located at -1480 to -1349bp upstream to the FoxO3a transcription start site. Since the first site is located at close proximity to the transcription start site it is possible that up-regulation in activity in this region, in response to paclitaxel treatment, is not exclusively related to paclitaxel treatment but to the binding of the general transcription machinery to the core promoter (Sadhale et al., 2007). The core promoter is about 50 bases upstream to the transcription initiation site of genes and usually contains binding sites for the basal transcription complex and RNA polymerase II (Thomas and Chiang, 2006). The second region responsive to paclitaxel treatment is located further upstream. The up-regulation in activity observed for this region may be related to binding of transcription factors which activity is up-regulated following paclitaxel treatment. Binding of these transcription factors to the promoter increases FoxO3a promoter activity in response to paclitaxel treatment. It is probable that these transcription factors are downstream targets of a signalling transduction pathway which is activated following oxidative stress, such as the JNK signalling pathway (Davis, 2000). Downstream targets of JNK include the AP-1 and ATF-1 transcription factors (Ahmed et al., 2003; Botteron and Dobbelaaere, 1998; Caelles et al., 1997; Leppa et al., 2001; Zoumpouriis et al., 2000). Although the FoxO3a promoter
sequence contains several consensus binding sites for these transcription factors, none of them are located solely in this region of the promoter. Hence, further experiments need to be conducted in order to determine which transcription factor/s up-regulate FoxO3a mRNA expression in response to paclitaxel treatment. One possibility is to perform cDNA microarray analysis. Comparison of cDNA microarray of cells exposed to paclitaxel to those untreated will reveal which transcription factors’ expression is induced following treatment. If the transcription factors identified will be shown to bind the FoxO3a promoter at the -1480 to -1349bp region, responsive to paclitaxel treatment, this may imply that these transcription factors play a role in the up-regulation of FoxO3a transcription following paclitaxel treatment.

The MEF2A transcription factor was found to regulate transcription of both FoxO1 and FoxO3a in MCF-7 cells (Fig 3.18, 3.19, 3.20, 3.21). Over-expression of MEF2A induced both FoxO1 and FoxO3a promoter activity in MCF-7 cells. Mutating two of the three MEF2 DNA consensus binding sites in the FoxO1 promoter led to a decrease in FoxO1 promoter activity (Fig 3.21B). Therefore, it seems that MEF2 may up-regulate FoxO promoter activity, by binding to MEF2 DNA consensus binding sites located in the promoter sequence. Since transcription factors are bound to the DNA in complexes it is possible that the nature of regulation depends on the presence or absence of other transcription factors in the complex which, in concert, determine whether the net effect on transcription will be positive or negative. It is also possible that regulation of FoxO genes by MEF2 could be cell type specific or isoform specific. Regulation of transcription by MEF2 may also be influenced by cross-talk between signalling pathways affecting the expression of other transcription factors. In order to further understand the role of MEF2 is the regulation of FoxO genes, an additional experiment in which MEF2 expression is knocked down (siRNA) should be performed. Furthermore, it was shown that exogenous expression of MEF2A can result in an additional increase in FoxO promoter activity in response to paclitaxel (Fig 3.19, 3.20). Therefore, it is possible that paclitaxel treatment activates
signal transduction pathways upstream to MEF2, such as the p38 signalling pathway (Seidman et al., 2001) and this results in MEF2 activation and a further increase in FoxO transcription. In addition, paclitaxel treatment could affect signal transduction pathways involved in the regulation of transcription factors which bind with MEF2 to the DNA and activation or repression of these factors could result in an increase in FoxO transcription. Furthermore, since I was able to demonstrate, as previously observed (Alexandre et al., 2006), that paclitaxel treatment generates ROS and since it has also been shown that oxidative stress can induce the activity of MEF2 (Al-Khalili et al., 2004), it would be interesting to determine whether up-regulation of MEF2 activity following oxidative stress and ROS production could lead to MEF2-dependent increase in FoxO transcription.

The work presented in this chapter demonstrates that paclitaxel and oxidative stress can induce the levels and activity of FoxO transcription factors, indicting that FoxOs may act as downstream effectors of chemotherapeutic drugs, such as paclitaxel. Up-regulation of FoxO activity can result in FoxO-dependent increase in the transcription of target genes involved in apoptosis (Brunet et al., 1999; Dijkers et al., 2000a; Modur et al., 2002; Stahl et al., 2002) and therefore, may be important in determining sensitivity to chemotherapy. However, since FoxOs can also up-regulate the transcription of genes involved in detoxification of ROS, such as catalase and MnSOD (Balaban et al., 2005; Kops et al., 2002a; Nemoto and Finkel, 2002), it is possible that FoxOs may also play a role in resistance to chemotherapy. Hence, better understanding of the regulation of FoxO transcription factors is required in order to prevent FoxOs from facilitating resistance to chemotherapy.
Figure 3-22: Schematic representation of the changes in FoxO expression at the promoter, mRNA and protein levels in the various cell lines examined. (A) Changes in FoxO3a expression levels in MCF-7 cells (based on figures 3.2, 3.7C and 3.12C). (B) Changes in FoxO3a expression levels in BT-474 cells (based on figures 3.3, 3.8A and 3.16C). (C) Changes in FoxO1 expression levels in HEC-1-B cells (based on figures 3.4, 3.9BA and 3.13).
Figure 3-23: Schematic representation of the levels of phosphorylated JNK (P-JNK) and FoxO3a in reference to the levels of ROS in MCF-7 cells. Levels of ROS produced were attained based on figure 3.5, levels of P-JNK and FoxO3a are based on figures 3.2 and 3.6.
4 The Role of BTG1 in Breast Cancer

4.1 Background and objectives
In recent years many efforts have been made to better understand the function of FoxO transcription factors. This has been predominately performed by analysing the role and regulation of FoxO downstream target genes. FoxO proteins have been implicated in cell cycle arrest and apoptosis by inducing transcription of downstream targets such as p27\textsuperscript{Kip1}, Bim, FasL (Brunet et al., 1999; Dijkers et al., 2000a; Essafi et al., 2005; Stahl et al., 2002). However, some studies also point to a role for FoxO in regulation of differentiation. BTG1 was identified in a DNA microarray as a target gene of FoxO3a in erythroid progenitors and was shown to directly regulated BTG1 transcription (Bakker et al., 2004).

FoxO3a is suggested to induce erythroid differentiation by a mechanism dependent on BTG1 and protein arginine methyl transferase 1 (PRMT1), activity (Bakker et al., 2004). PRMT1 was also shown to play an important role in anti-proliferative functions in cells such as T-lymphocytes and fibroblasts (Berthet et al., 2002; Matsuda et al., 2001 2001; Prevot et al., 2001; Sasajima et al., 2002). BTG1 is a member of the Tob/BTG anti-proliferative protein family. The human family of Tob/BTG proteins has six members: BTG1, BTG2, PC3B, Tob, Tob2 and ANA/BTG3 (Matsuda et al., 2001). One hundred and ten amino acids located in the N-terminal region of these proteins bear homology and termed the Tob/BTG homology domain. Within this domain there are two highly conserved regions, box A and box B. BTG1 and BTG2 have another homologous region called box C which associates with PRMT1. The box B and the C-terminal domain of BTG1 are responsible for its nuclear localisation. Deletion of box C, the PRMT1 binding domain in BTG1, has been found to stop BTG1 induced proliferation inhibition. The BTG1 protein lacks enzymatic activity but possesses protein interaction domains, implying that BTG1 may be an adaptor protein or a cofactor (Bakker et al., 2004). BTG1 has also been shown to interact with
p/CAF1, a protein which may be involved in transcriptional regulation and is deleted in many human tumours (Prevot et al., 2001). PRMT1 and p/CAF were shown to play a role in ER-dependent signalling in breast epithelial cells. In addition, it has also been suggested that down-regulation of BTG2, which shares 66% homology with BTG1, may be involved in the development of mammary tumours (Kawakubo et al., 2004). Therefore, in order to further understand role of FoxO3a in breast cells, I decided to examine whether \textit{BTG1} was also a target gene of FoxO3a in the breast cancer cell line, MCF-7 and to determine its function of BTG1 in breast cells.

\section*{4.2 Results}

\subsection*{4.2.1 \textit{BTG1} is a downstream target of FoxO3a in COS-1 and MCF-7 cells}

In order to examine whether \textit{BTG1} is a downstream target of FoxO3a in breast epithelial cells, I amplified by PCR 871bp of the human \textit{BTG1} upstream regulatory region and cloned it into the pGL3 luciferase vector (Fig 4.1A). The region cloned into pGL3 includes a FoxO DNA consensus binding site located -211bp upstream to the \textit{BTG1} transcription start site. The construct was transiently co-transfected with increasing amounts of either wild-type FoxO3a or constitutively active FoxO3a (FoxO3aA3) into COS-1 and MCF-7 cells (Fig 4.1B, C).

As seen in figure 4.1B, both the wild-type FoxO3a and the constitutively active FoxO3a increased \textit{BTG1} promoter activity in COS-1 cells. The up-regulation in activity observed when the constitutively active FoxO3a was co-transfected was greater than that observed for the wild-type FoxO3a. Both constructs generated a dose dependent increase in the promoter activity which reached saturation at 40ng of co-transfected FoxO3a. This indicates that \textit{BTG1} is a transcriptional downstream target of FoxO3a. In order to determine if BTG1 was also a downstream target of FoxO3a in breast carcinoma cells, I co-transfected MCF-7 cells with the \textit{BTG1} promoter/reporter construct and increasing amounts of either wild-type or constitutively active FoxO3a (Fig 4.1C). Co-transfection of both wild-type
FoxO3a and constitutively active FoxO3a increased BTG1 promoter activity, however only the increase observed with the wild-type FoxO3a was dose-dependent. The increase in activity observed with the constitutively active FoxO3a was larger than that noted with the wild type FoxO3a and activity levels peaked at 10ng of co-transfected FoxO3aA3. Hence, BTG1 transcription can be up-regulated by FoxO3a in COS-1 cells and in MCF-7 breast cancer cells.
**Figure 4-1: BTG1 is a downstream target of FoxO3a.**

(A). Schematic representation of the BTG1 promoter/reporter construct generated. The human BTG1 upstream regulatory region was amplified by PCR and cloned into the pGL3 luciferase vector. COS-1 cells (B), or MCF-7 cells (C), were transiently co-transfected with 20ng of the BTG1 promoter construct and increasing amount of either FoxO3a or constitutively active FoxO3a (FoxO3a3A). Values are corrected for co-transfected renilla activity. All data shown represent the average of three independent experiments, and the error bars show the standard deviation, for all conditions it was found that p<0.05 in comparison to cells transfected with 0ng FoxO3a/FoxO3a3A.
4.2.2 Induction in BTG1 expression levels following paclitaxel treatment in MCF-7 cells

In chapter 3, I was able to show that treatment of MCF-7 cells with paclitaxel induced the expression of FoxO3a and downstream target p27Kip1 (Fig 3.2, 3.3, 3.8B). Therefore, I decided to examine whether BTG1 was also a target gene of FoxO3a following paclitaxel treatment. MCF-7 cells were treated with paclitaxel and the levels of BTG1 mRNA and protein expression were determined. As seen in figure 4.2A, BTG1 mRNA expression levels remained relatively stable throughout the course of treatment with paclitaxel, except at 48 h when an increase in BTG1 mRNA expression was detected. Generally, BTG1 protein expression levels also remained constant throughout the course of treatment; although a slight increase in protein expression levels was detected 48 h after treatment commenced (Fig 4.2B). Hence, paclitaxel treatment caused up-regulation in BTG1 expression at both the protein and mRNA levels in MCF-7 cells. However, the increase was subtle and occurred at a late time-point.
Figure 4-2: BTG1 expression following paclitaxel treatment in MCF-7 cells. MCF-7 cells were treated with 10nM paclitaxel for indicated times. (A), Total RNA was extracted and analysed for the expression levels of BTG1 mRNA using RT-qPCR and normalised to the level of L19 mRNA expression. All data shown represent the average of three independent experiments and the error bars show the standard deviation. *p<0.05 in comparison to 0 h. (B), Whole cell lysates were prepared, then separated on SDS-polyacrylamide gels and immunoblotted. The levels of total FoxO3a and BTG1 were analysed by western blot using specific total antibodies. β-actin was used as a loading control. The arrow indicates the location of the FoxO3a specific band. An experiment showing the same trend as 4.2B can be found in the supplementary data, figure 7.8.
4.2.3 BTG1 expression levels change during the cell cycle

The FoxO transcription factors play a role in the regulation of cell cycle progression and cell cycle arrest, by regulating the expression of genes such as p27^Kip1, p21^Cip1 cyclin D1 and D2, cyclin G2 and p130 (Kops et al., 1999; Martinez-Gac et al., 2004; Medema et al., 2000; Nakamura et al., 2000; Ramaswamy et al., 2002; Schmidt et al., 2002; Seoane, 2004). BTG2, which shares several homologous domains with BTG1, was shown to inhibit cyclin D1 expression (Kawakubo et al., 2006). Therefore, to determine whether BTG1 expression changes throughout the cell cycle, I decided to examine the expression pattern of FoxO3a transcriptional target, BTG1 in cell cycle synchronised MCF-7 cells.

As shown in figure 4.3, BTG1 expression increased 16 h after cells re-entered the cell cycle. 24 h after release from G0, an increase in the expression of polo like kinase (Plk) was evident. Plk expression is up-regulated during the M phase of the cell cycle (Lee et al., 1995), therefore the increase in BTG1 expression occurred prior to entry of the cells to the G2/M phase. An increase in the expression of FoxO3a was observed at 8 h, during the G1 phase of the cell cycle. It is notable that a corresponding decrease in the mobility of FoxO3a also occurred; this is probably due to an increase in FoxO3a’s phosphorylation during S phase. Taken together, BTG1 expression is cell cycle regulated and peaks at the early stages of the cell cycle, following an induction in FoxO3a expression.
Figure 4-3: BTG1 expression in synchronised MCF-7 cells.
MCF-7 cells were synchronised at G0 by decreasing the concentration of foetal calf serum (FCS) from 10% to 0.5% in the growth medium for 36 h; after which growth medium was replaced by fresh media containing 10% FCS. Whole cell lysates were prepared, and then separated on SDS-polyacrylamide gels and immunoblotted. The levels of total FoxO3a, BTG1 and Plk were analysed by western blot using specific total antibodies. β-tubulin was used as a loading control. An experiment showing the same trend can be found in the supplementary data, figure 7.9.
4.2.4 Generation of an inducible BTG1 cell line

Since previous experiments demonstrated that BTG1 is a downstream target of FoxO3a and that BTG1 expression changes through the course of the cell cycle, I next sought to examine the role of BTG1 in breast epithelial cells. In order to determine the function of the BTG1 in breast cancer cells, I decided to generate a stably transfected MCF-7 breast cancer cell line which expressed BTG1 in an inducible manner. To that end, I chose to use the ‘Tet-On’ and ‘Tet-Off’ systems which enable expression of a gene under specifically regulated conditions. The process of cloning and selecting of BTG1 MCF-7 Tet On and Tet Off clones is explained in detail in chapter 2 sections 2.1.5 and 2.5.8. One clone, BTG1 MCF-7 Tet Off clone #34 (hereby known as BTG1 Tet-Off), was found to express BTG1 at the highest inducible levels and was chosen for further analysis of the role of BTG1 in breast epithelial cells. As seen in figure 4.4A, BTG1 mRNA expression was up-regulated upon withdrawal of doxycycline (Dox) from the medium. BTG1 mRNA levels increased rapidly and peaked 8 h after removal of Dox to levels more than 3.5 times higher than those observed for non-induced cells (0 h). Levels of BTG1 mRNA remained elevated, in comparison to non induced cells, for the duration of the experiment. In order to examine whether up-regulation of BTG1 mRNA expression generates an increase in BTG1 protein expression, western blot analysis was performed. As shown in figure 4.4B, the expression of BTG1 increased upon removal of Dox from the medium. The up-regulation in BTG1 expression was observed 8 h after Dox was removed from the media and remained elevated for the rest of the time-course. At 24 h a slight decrease in the expression of BTG1 was observed. Results obtained in the previous experiment (Fig 4.3) indicate that BTG1 expression is cell cycle regulated and peaks prior to M phase. Therefore, it is possible that up-regulation of BTG1 expression affects cell cycle progression by inducing a transient G1 cell cycle arrest, which results in the synchronisation of the cells at G1. Upon release from this transient cell cycle arrest, BTG1 expression levels decrease.
Figure 4-4: The BTG1 MCF-7 Tet Off clone #34 expresses BTG1 in an inducible manner upon withdrawal of doxycycline from the medium.
MCF-7 Tet off cells, which were stably transfected with pTRE2Hyg-BTG1, were cultured in the presence of 100μg/ml of G418, 200μg/ml and 1μg/ml doxycyclin. At the beginning of the time-course (0 h) all antibiotics were removed from the media. (A) Total RNA was extracted and analysed for the expression levels of BTG1 mRNA using RT-qPCR and normalised to the level of L19 mRNA expression. All data shown represent the average of three independent experiments, and the error bars show the standard deviation. *p<0.01 and was determined comparing the levels of expression to 0 h. (B) Whole cell lysates were prepared, then separated on SDS-polyacrylamide gels and immunoblotted. The levels of BTG1 was analysed by western blot using specific total antibodies. β-tubulin was used as a loading control. An experiment showing the same trend as 4.4B can be found in the supplementary data, figure7.10
4.2.5 Over-expression of BTG1 decreases cell growth

BTG1 was initially identified as target gene of FoxO3a in erythroid progenitors and appears to play a role in erythroid differentiation (Bakker et al., 2004). In an effort to discover the function of BTG1 in breast cancer cells, I decided to examine whether BTG1 played a role in regulation of cell growth by performing a Sulphorhodamine B (SRB) assay. To this end, BTG1 Tet-Off cells were induced to express BTG1 and cellular protein content was compared to that of cells harbouring the empty vector (MCF-7 Tet-Off pTRE2hyg, hereby known as pTRE Tet-Off). As seen in figure 4.5, cells expressing BTG1 had lower cellular protein content than cells harbouring the empty vector. A decrease in cellular protein content was also observed for BTG1Tet-Off cells, which were not induced to express BTG1 (grown in the presence of doxycycline), indicating that the inducible BTG1 expression system may be leaky. However, the decrease in cellular protein content for cells induced to express BTG1 (grown without doxycycline) was statistically significant in comparison to the cells which were not induced. Therefore, it seems that over-expression of BTG1 in MCF-7 cells reduces cell growth, suggesting that BTG1 may either negatively regulate cell cycle progression or play a role in inducing cell death.
Figure 4-5: Over-expression of BTG1 reduces cell growth.

BTG1 MCF-7 Tet-Off Clone #34 cells (BTG1) and MCF-7 Tet Off pTRE2hyg (EV) cells were grown in 96 well plates for 48 h in DMEM medium without Dox and cellular protein content was determined every 24 h by performing SRB assay and measuring OD at 492nm. OD measurements were normalised using initial (0 h) OD measurement as the value 1. All data shown represent the average of three independent experiments, and the error bars show the standard deviation. *p<0.001 when comparing relative protein content of Tet-Off Clone #34 cells (induce and not induced) to each other and to that of MCF-7 Tet Off pTRE2hyg at 48 h.
4.2.6 Over-expression of BTG1 induces accumulation of cells in the G2/M phase of the cell cycle

In the previous experiment, I was able to demonstrate that over-expression of BTG1 reduced cell growth, indicating that BTG1 plays a role in either cell cycle arrest or induction of apoptosis. In addition, I was also able to show that BTG1 expression is cell cycle regulated (Fig 4.3). Therefore, I next sought to examine the effect of BTG1 over-expression on the cell cycle profile by performing PI staining followed by FACS analysis.

As seen in figure 4.6, BTG1 Tet-Off cells that were not induced to express BTG1 were found predominantly in the G1 phase of the cell cycle. Over-expression of BTG1 induced accumulation of cells at G2/M phase of the cell cycle and a slight increase in sub-G1 population was also observed. Hence, previously I was able to demonstrate that expression of BTG1 peaks prior to M phase; in this experiment I was able to show that over-expression of BTG1 induces G2/M cell cycle arrest and that BTG1 over-expression may also increase cell death. Therefore, it is possible that BTG1 expression is induced at G1 or S phase of the cell cycle but causes cell cycle arrest and apoptotic cell death at the later G2/M phase of the cell cycle.
Figure 4-6: Over-expression of BTG1 induces accumulation of cells in the G2/M phase of the cell cycle.
PI-FACS analysis was performed on BTG1 Tet-Off cells. Cells were induced to express BTG1 by removal of Dox from the medium 24 h before FACS analysis was performed.
4.2.7 The effect of BTG1 over-expression on cell cycle regulators

In the last experiment, I was able to demonstrate that over-expression of BTG1 induced accumulation of the cells in the G2/M phase of the cell cycle. In attempt to find out how BTG1 inflicts this G2/M accumulation, I next examined the effect of BTG1 over-expression on the following cell cycle regulators: cyclin D1, cyclin E1, cyclin A, cyclin B cyclin-dependent kinase 4 (CDK4), CDK2, retinoblastoma protein (pRb), CDK kinase inhibitors (CKI’s): p27Kip and p21Cip. In order to understand the results obtained in this experiment it is important to first clarify the role of each of these proteins in cell cycle regulation. Briefly, the literature shows that during the transition from quiescence into the cell cycle, levels of cyclin D1 increase in response to extracellular mitogens (Aktas et al., 1997; Cheng et al., 1998; Lavoie et al., 1996) and cyclin D1, in combination with CDK4 and CDK6, can phosphorylate, pRb. The phosphorylation of pRb is leads to the release of E2F transcription factors, which then activate genes that are required for cell cycle progression. Cyclin E synthesis is mediated by E2F following pRb phosphorylation by CDK4/6–cyclin D complex (Harbour et al., 1999; Malumbres and Barbacid, 2001; Zhang et al., 1999) and that CDK2 is activated through binding to cyclin E., The CDK2-cyclin E complex phosphorylate target proteins, thereby initiating S phase. One important target of CDK2–cyclin E is pRb. In addition to promoting entry to S phase by phosphorylating pRb (on threonine 821) CDK2-cyclin E also phosphorylate a number of proteins which play important roles in DNA replication (Caldon et al., 2006; Hwang and Clurman, 2005). CKIs form heterotrimeric complexes with the G1/S CDKs. However, in stoichiometric amounts, they only kinase activity of CDK2–cyclin E complex is inhibited (Malumbres and Barbacid, 2001). Cyclin A expression is required for progression through S and G2 phases of the cell cycle and cyclin B1 is required for M phase progression (Clute and Pines, 1999; Lukas et al., 1999).
Hence, BTG1 Tet-Off cells were induced to express BTG1 for 16 h and the expression pattern of BTG1 and cell cycle regulators was analysed by western blot (Fig 4.7). As seen in figure 4.7, following the removal of Dox from the medium an increase in BTG1 expression was observed. I also examined the levels of FoxO3a expression, in order to determine if over-expression of the FoxO3a downstream target, BTG1 caused a feedback effect; the result obtained showed no significant change in FoxO3a expression. However, a decrease in cyclin D1 expression was observed, while no significant change in the expression of CDK4 or phosphorylation of the tumour suppressor, pRb on Ser 801/807 was noted. A decrease in cyclin E1 expression was also observed, however no change in CDK2 expression and phospho-Thr821pRb was noted. Nonetheless, an increase in the expression of the p27^Kip and p21^Cip was observed. A slight decrease in cyclin A expression was observed. No change in the expression levels of cyclin B1, was detected (Clute and Pines, 1999; Lukas et al., 1999). The levels of total pRb and β-tubulin did not change, indicating equal loading, Taken together, over-expression of BTG1 correlates with a decrease in the expression levels of cyclin D1 and cyclin E1 which are required for G1 phase initiation and progression, respectively. Following the induction in BTG1 expression up-regulation in the expression of p21^Cip and p27^Kip, which inhibit the activity of the CDK2-cyclin E complex, was also noted.

Overall, I was able to show that induction in BTG1 expression results in accumulation of the cells at the G2/M phase of the cell cycle (Fig 4.6) and that BTG1 over-expression may have a negative effect on cell cycle progression at the G1 phase of the cell cycle, as it was shown that over-expression of BTG1 results in increased expression of p21^Cip and p27^Kip which repress the activity of the CDK2-cyclin E complex (Fig 4.7).
Figure 4-7: The effect of BTG1 over-expression on cell cycle regulators.
BTG1 Tet-Off cells were induced to express BTG1, by removal of Dox from the cell growth medium for a period of 24 h. After which cell lysates were prepared, separated on SDS-polyacrylamide gels and immunoblotted with specific antibodies. The levels of BTG1, FoxO3a and cell cycle regulators was analysed using specific phospho and total antibodies. β-tubulin was used as a loading control. An experiment demonstrating the same trend an be found in the supplementary data, figure 7.11
4.2.8 Up-regulation of BTG1 expression increases p21\textsuperscript{Cip1} and p27\textsuperscript{Kip1} expression at the promoter and mRNA levels

In the previous experiment, I was able to show an induction in both p21\textsuperscript{Cip1} and p27\textsuperscript{Kip1} protein levels following an induction in BTG1 expression in BTG1 Tet-Off cells. Therefore, I next set out to examine whether BTG1 could up-regulate p21\textsuperscript{Cip1} and p27\textsuperscript{Kip1} expression at the transcriptional level. To that end, MCF-7 cells were co-transfected with either the p21\textsuperscript{Cip1} or p27\textsuperscript{Kip1} promoter/reporter construct together with increasing amounts of BTG1 (Fig 4.8 A, B).

As shown in figure 4.8A, B, the activity levels of both the p21\textsuperscript{Cip1} and the p27\textsuperscript{Kip1} promoters were up-regulated following co-transfection with BTG1 in MCF-7 cells. The increase in the p21\textsuperscript{Cip1} promoter activity occurred at lower concentrations of BTG1 than the increase observed for the p27\textsuperscript{Kip1} promoter. The p27\textsuperscript{Kip1} promoter also exhibited a dose dependent increase in activity in response to BTG1. An increase in both p21\textsuperscript{Cip1} and p27\textsuperscript{Kip1} mRNA levels was observed 16 h after BTG1 expression was induced in BTG1 Tet-Off cells (Fig 4.8 C,D). Therefore, over-expression of BTG1 seems to positively regulate transcription of p21\textsuperscript{Cip1} and p27\textsuperscript{Kip1} at both the promoter level and the mRNA levels.
Figure 4-8: Induction in the expression of BTG1 affects the transcription of p21Cip1 and p27Kip1 at the promoter and mRNA levels.

MCF-7 cells were transiently co-transfected with increasing amounts of BTG1 (p-BABE-BTG1) and 20ng of either the p21Cip1 promoter construct (A) or the p27Kip1 promoter construct (B) samples were harvested 24 h after transfection and were assayed for luciferase activity. Values are corrected for co-transfected renilla activity. All data shown represent the average of three independent experiments, and the error bars show the standard deviation. *p<0.05 when compared to cell transfected with 0ng. Total RNA was extracted from BTG1 Tet-Off cells (C, D) and the expression levels of p21Cip1 (C) and p27Kip1 (D) mRNA were analysed using RT-qPCR and normalised to the level of L19 mRNA expression. All data shown represent the average of three independent experiments, and the error bars show the standard deviation. *p<0.05 when compared to levels at 0 h.
4.2.9 Over-expression of BTG1 decrease the transcription of cyclin D1 and cyclin E1

Since a decrease in both cyclin D1 and cyclin E1 expression was observed following an induction in BTG1 expression in the BTG1 Tet-Off cells (Fig 4.7), I decided to examine the effect of BTG1 over-expression on transcription of cyclin D1 and cyclin E1. To that end, I examined the levels of *cyclin D1* and *cyclin E1* mRNA following an induction in BTG1 expression in the BTG1 Tet-Off cells (Fig 4.9A, C). A decrease in *cyclin D1* mRNA expression was evident as early as 8 h after induction of BTG1 expression with a further decrease in *cyclin D1* mRNA expression levels occurring 24 h after BTG1 expression was induced (Fig 4.9A). A decrease in the levels of *cyclin E1* mRNA expression were only noticeable 24 h after BTG1 expression was induced (Fig 4.9C). This does not correlate with cyclin E1 protein expression levels which were found to decrease rapidly 16 h after induction in BTG1 expression (Fig 4.7). A possible explanation may be that the cyclin E1 protein underwent rapid degradation, with not significant changes in the transcription levels, until 24 h post induction in BTG1 expression, by which time transcription of cyclin D1 was also down-regulated.

In order to examine if the decrease observed in cyclin D1 expression at protein and mRNA expression levels also occurred at the promoter level, I co-transfected the cyclin D1 promoter/reporter construct with increasing amounts of BTG1 into MCF-7 cells. A decrease in *cyclin D1* promoter activity was observed when 30ng of BTG1 were co-transfected with the *cyclin D1* promoter. Therefore, over-expression of BTG1 can lead to a decrease in *cyclin D1* mRNA expression and promoter activity levels. BTG1 over-expression also appears to down-regulate *cyclin E1* mRNA levels, but at a later time-point than observed at the protein level.
Figure 4-9: Over-expression of BTG1 decreases the transcription of cyclin D1 and cyclin E1.

Total RNA was extracted from BTG1 Tet-Off cells (A,C) and the expression levels of cyclin D1 (A) and cyclin E1 (C) mRNA was analysed using RT-qPCR and normalised to the level of L19 mRNA expression. All data shown represent the average of three independent experiments, and the error bars show the standard deviation. *p<0.05 in comparison to 0 h.

MCF-7 cells were transiently co-transfected with increasing amounts of BTG1 expression vector (p-BABE-BTG1) and 20ng of the cyclin D1 promoter construct (B). Samples were harvested 24 h after transfection and were assayed for luciferase activity. Values are corrected for co-transfected renilla activity. All data shown represent the average of three independent experiments, and the error bars show the standard deviation. *p<0.05 when compared to cells transfected with 0ng.
4.3 Discussion

In chapter 3, I focused on the regulation of FoxO at the transcriptional level; in this chapter, I attempted to understand the function of FoxO3a in breast cancer by studying the role of FoxO3a downstream target, BTG1.

As previously mentioned, BTG1 was initially identified as a downstream target of FoxO3a in erythroid development and differentiation (Bakker et al., 2004). In this chapter, I examined whether BTG1 is a downstream target of FoxO3a in breast carcinoma cells and the role BTG1 plays in these cells. I was able to demonstrate that BTG1 is a transcriptional target of FoxO3a in MCF-7 cells (Fig 4.1C). However, the nature by which FoxO3a regulates and induces BTG1 expression in these cells is unclear, since induction of FoxO3a expression following paclitaxel treatment seemed to only slightly increase BTG1 protein and mRNA expression (Fig 4.2). Hence, it may be possible that up-regulation of FoxO expression following paclitaxel treatment does not lead to an increase in BTG1 expression, as it was previously mentioned that FoxO factors may differentially induce the transcription of target genes dependent on the form of cellular stress (Greer and Brunet, 2005). Analysis of BTG1 expression levels following paclitaxel treatment in BT-474 cells, which have been shown to up-regulate FoxO3a expression following paclitaxel treatment (Fig 3.3), will help determine whether treatment of breast carcinoma cells with paclitaxel results in FoxO3a-dependent up-regulation of BTG1 expression. Furthermore, analysis of the cell cycle profile and survival status of cells over-expressing BTG1 and cells in which BTG1 expression has been knocked down, in the presence and absence of paclitaxel, will help determine whether BTG1 expression is important in mediating FoxO3a-dependent effects of paclitaxel treatment in breast cancer cells. However, up-regulation of FoxO3a expression alone was not sufficient in inducing BTG1 expression in MCF-7 cells.

The BTG1 mRNA contains a long 3’ AU-rich untranslated region, typical of unstable mRNAs, like those found in cell cycle control genes (Kozak, 1991). This also implies that BTG1 may be a target for microRNA (Jing et al., 2005).
Furthermore, the BTG1 protein sequence contains PEST residues (Proline, Glutamine, Serine and Threonine), typical of highly unstable proteins (Rogers et al., 1986) including cyclins (Bai et al., 1994; Salama et al., 1994) and transcription factors (Bogdan et al., 1998; Ernst et al., 1995). Previous studies showed that BTG1 is expressed predominantly in quiescent cells, at the G0/G1 phase transition, with levels declining as cells enter S phase (Rouault et al., 1992). Indeed, I was able to show that in MCF-7 cells BTG1 expression peaks at G1/S prior to entry of the cells to G2/M (Fig 4.3). Furthermore, I also demonstrated that FoxO3a expression levels appear to be cell cycle regulated.

Prior to this study, BTG1 was shown to play a role in cell cycle regulation, differentiation and apoptosis. Experiments conducted by other groups demonstrated that exogenous expression of BTG1 resulted in a reduction in proliferation, G1 arrest and/or apoptosis in several cell types (Corjay et al., 1998; Lee et al., 2003a; Rodier et al., 1999; Rouault et al., 1992). Recently, BTG1 was also shown to upregulate apoptosis, by enhancing antisense Bcl-2 related cytotoxic effects in breast cancer cells (Nahta et al., 2006). BTG1 has been suggested to play a role in cellular differentiation based on experiments that showed that BTG1 expression induces myoblast differentiation (Rodier et al., 1999) and that in leukemic cells following treatment with chemicals that induce differentiation, the expression of BTG1 is increased (Cho et al., 2004). However, it is unclear how BTG1 exerts its effect. Therefore, in order to learn more about the function of BTG1, in particular in breast epithelial cells, I generated a cell line that was able to express BTG1 in a controlled and inducible manner. The conditional MCF-7 cell line was designed to up-regulate BTG1 expression upon removal of doxycycline from the medium (Fig 4.4).

Using these cells, I was able to show that over-expression of BTG1 reduced cell growth (Fig 4.5) and that cells accumulated at the G2/M phase of the cell cycle with a higher percentage of cells undergoing apoptosis (Fig 4.6). This result contradicts previous observations indicating that exogenous expression
of BTG1 causes G1 arrest (Rouault et al., 1992), but agrees with studies showing an increase in cell apoptosis (Corjay et al., 1998; Lee et al., 2003a). However, my results were attained using a stably transfected cell line. Hence, the region in the genome and the numbers of copies of the gene integrated into the genome may affect the outcome of the experiments (Murnane et al., 1990; Wurm, 2004). Thus, it is possible that the results of the experiments were affected by the use of a stably transfected cell line and not just the over-expression of BTG1 and therefore may account for the discrepancy in the outcome of my experiments in comparison to those previously published.

Examination of the expression patterns of some cell cycle regulators following an induction in BTG1 expression revealed that BTG1 predominately affects the expression levels of G1 and G1/S cell cycle regulators (Fig 4.7). A decrease in cyclin D1 and cyclin E1 expression was observed while an increase in p21^{Cip1} and p27^{Kip1} expression was noted. Hence, BTG1 induced the expression of negative regulators of cell cycle progression while decreased the expression of positive cell cycle progression regulators. A recent study, showed that in ER positive breast tumours an increase in tumour grade and size, and over-expression of cyclin D1 correlated with loss of nuclear expression of BTG2 (Kawakubo et al., 2006). Therefore, it seems that BTG1 and BTG2 may have overlapping effects on G1 phase cell cycle regulators.

BTG1 over-expression increased transcription of p21^{Cip1} and p27^{Kip1} at the promoter and mRNA levels (Fig 4.8) and generated a decrease in both cyclin D1 promoter activity and mRNA expression levels. (Fig 4.9A, B) A decrease in cyclin E1 transcription was also noted (Fig 4.9C). Hence, it appears that BTG1 can influence gene transcription in both a positive and a negative way. However, the net effect of BTG1 regulation, in these experiments, resulted in the same outcome, generation of an expression pattern of G1 phase regulators which inhibits cell cycle progression. Since BTG1 has not been shown to bind to DNA and since it was previously suggested that BTG1 may act as an adaptor protein (Bakker et al., 2004), it seems likely that these
effects are mediated by transcription factor/s that bind to BTG1. Identifying which transcription factor/s bind to BTG1 will enable us to determine which promoters contain a DNA consensus binding site for these transcription factors, and therefore may be affected by the levels of BTG1 expression. Although I was able to demonstrate that BTG1 over-expression inhibits the expression of cyclin D1 and cyclin E1 and increases the expression of p21Cip1 and p27Kip1, which agrees with previous observations that BTG1 can induce G1 cell cycle arrest (Rouault et al., 1992), these results disagree with the cell cycle analysis data which showed accumulation of the cells at the G2/M phase of the cell cycle (Fig 4.6). Since regulation of cell cycle progression depends on concerted activity of multiple regulators, over-expression of one may not on its own bring about changes in cell cycle status, as abundance of BTG1 binding partners may be a limiting factor. Therefore, it would be interesting to perform a complementary experiment, in which BTG1 expression is knocked down via siRNA, and examining the effect on cell cycle progression. Identifying BTG1 binding partners may also help in further understanding the functional mechanistic role of BTG1 in cell cycle regulation. Moreover, I previously mentioned (section 4.2.4) that it may be possible that over-expression of BTG1 causes a transient G1 arrest, a hypothesis which is further strengthened by the observation that BTG1 expression appears to have an overall negative effect on G1 phase cell cycle regulators that induce cell cycle progression. Therefore, taken together, theses results indicate that the accumulation of cells in G2/M, 24 h after an induction in BTG1 expression, may not be the direct effect of BTG1 expression but may reflect synchronised cells coming out of G1 following the transient G1 arrest. Hence, in order to determine if BTG1 expression can indeed induce G1 arrest, synchronising the cells in a reversible manner, for instance at M phase, using a drug such as colcemid, may be beneficial. This is due to the fact that the G1 is the longest phase of the cell cycle and therefore the majority of unsynchronised cells reside in this phase, making it difficult to detect a G1 arrest using PI-FACS analysis. Analysis of the cell
cycle profile of non-induced BTG1 Tet-Off cells released from M phase synchronisation, followed by an induction in BTG1 expression for up to 24 h (approximant length of the cell cycle), will enable the detection of a G1 arrest. This is due to the fact that if BTG1 expression does indeed induces a G1 arrest, cells will remain in this phase of the cell cycle and not be able to re-enter G2/M.

I also examined whether over-expression of BTG1 affected the expression levels of FoxO3a. However, no feedback mechanism appears to exists, as over-expression of BTG1 did not affect the levels of FoxO3a expression (Fig 4.7) in MCF-7 cells.

In summary, my data demonstrates that BTG1 is a downstream target of FoxO3a in breast cancer cells. BTG1 expression appears to generate accumulation of cells in the G2/M phase of the cell cycle as well as cause a slight increase in the levels of apoptosis. Hence, it is possible that BTG1 may play a role in mediating some of the effects of FoxO3a on cell cycle and cell death.
5 Regulation of FoxO3a by JNK1/2

5.1 Background and objectives
In chapter 3, I demonstrated that treatment of breast cancer cells with paclitaxel caused up-regulation in FoxO3a expression levels, which led to an increase in the transcription of the downstream target, p27\textsuperscript{Kip}(Fig 3.2, 3.3, 3.6, 3.8B), a gene implicated in the induction of cell cycle arrest (Dijkers et al., 2000b). Other studies have shown that elevated levels of Akt activity can lead to resistance to paclitaxel treatment (Page et al., 2000; VanderWeele et al., 2004), further implicating the PI3K/Akt/FoxO pathway in determining drug sensitivity (Sunters et al., 2006).

In chapter 3, I was also able to show that the JNK signalling pathway may play a role in the response of breast cancer cells to treatment with paclitaxel and H\textsubscript{2}O\textsubscript{2}. Following treatment of breast cancer cells with paclitaxel a biphasic expression pattern of phosphorylated JNK (Thr183/Tyr185) was observed with the first peak in expression occurring prior to the increase in FoxO3a expression (Fig 3.2, 3.3, 3.6). The JNK signalling cascade has previously been shown to be activated in response to cell stresses such as UV irradiation (Tournier et al., 2000) and has been implicated in proliferation, apoptosis, metabolism and DNA repair (Hayakawa et al., 2004; Lee et al., 2005a; Sakurai et al., 2006; Davis, 2000; Weston and Davis, 2002; Aguirre et al., 2000; Lee et al., 2003b). Deregulation of JNK signalling is believed to occur in many diseases such as chronic inflammation, neurodegeneration and cancer (Hunot et al., 2004; Kennedy and Davis, 2003; Su et al., 1998; Sumara et al., 2005).

Recent studies have also demonstrated that JNK signalling is activated following paclitaxel treatment (Amato et al., 1998; Lee et al., 1998; Lei and Davis, 2003) and that inhibition of JNK reduces apoptosis following paclitaxel treatment (Lee et al., 1998). Taken together, my data and previous observations suggest possible cross-talk between the PI3K/Akt/FoxO and JNK signalling pathways. Therefore, I next sought to examine whether the
JNK signalling pathway plays a role in the regulation of FoxO3a in breast cancer, in particular in response to treatment with paclitaxel.

5.2 Results

5.2.1 Paclitaxel causes nuclear translocation of FoxO3a

Previously, I was able to demonstrate that paclitaxel treatment can cause an increase in FoxO3a expression in the MCF-7 and BT-474 breast cancer cell lines. In addition, prior research conducted in Prof. Lam’s lab (Sunters et al., 2003) showed that in MCF-7 cells treatment with paclitaxel upregulates apoptosis and this was found to be mediated by an increase in expression and transcriptional activity of FoxO3a, which resulted in increased transcription of Bim, a pro-apoptotic gene. However, in order to activate the apoptotic machinery, FoxO3a has to reside in the nucleus, suggesting that treatment with paclitaxel results in changes in the sub-cellular localisation of FoxO3a. To test this notion, I examined the sub-cellular localisation of FoxO3a in MCF-7 following 10nM paclitaxel treatment using confocal microscopy, with the help of Dr. Andrew Sunters, (CRUK labs, Imperial College, London). MCF-7 cells were either treated with DMSO vector alone (0nM) or treated with 10nM paclitaxel for 16 h.

As shown in Figure 5.1A, FoxO3a resided almost exclusively in the cytosol of untreated MCF-7 cells. However, following 10nM paclitaxel treatment FoxO3a resided predominantly in the nuclei of MCF-7 cells. In order to further validate these results, I also conducted a nuclear and cytoplasmic fractionation experiment and analysed the relative amounts of FoxO3a in the nucleus and cytoplasm following 8 h of 10nM paclitaxel treatment. The results obtained are presented in figure 5.1B. These results show that the majority of FoxO3a in control cells (untreated with paclitaxel) was located in the cytoplasm, and that following treatment with paclitaxel, FoxO3a was predominantly located in the nucleus. In agreement with the data obtained from the immunocytochemical staining, treatment of MCF-7 cells with paclitaxel induced nuclear translocation of FoxO3a. These experiments were performed
at 8 and 16 h after paclitaxel treatment; in chapter 3, I was able to show that the predominate increase in p27\(^{\text{Kip1}}\) expression occurred 24 h after initiation of treatment and Sunters and colleagues (Sunters et al., 2003), demonstrated an increase in the pro-apoptotic target, Bim 48 h after treatment. Therefore, these data support the hypothesis that nuclear translocation of FoxO3a is required for the activation of anti-proliferative and pro-apoptotic genes, such as \(p27^{\text{Kip1}}\) and \(\text{Bim}\), in response to paclitaxel.
Figure 5-1: Paclitaxel causes FoxO3a to translocate into the nucleus.

(A), MCF-7 cells were cultured on sterile coverslips and treated for 16 h with paclitaxel (10 nM), before being fixed in 4% formaldehyde. FoxO3a was using a rabbit polyclonal antibody and subsequent addition of the ALEX488 (green) labelled anti-rabbit antisera, TRITC-labelled phalloidin (red) and DAPI (blue) were also applied to visualize the cytoplasm and nuclei, respectively. (B), Western blot using cytoplasmic and nuclear extracts isolated from MCF-7 cells that were either treated with 10nM paclitaxel or untreated (control) for 8 h The levels of total FoxO3a and Akt-phosphorylated FoxO3a (P-FoxO3a) was determined in both cellular components. β-actin was used as a loading control. An experiment showing the same trend as 5.1B can be found in supplementary data, figure7.12.
**5.2.2 Inhibition of JNK1/2 by SP600125 prevents FoxO3a nuclear localisation in MCF-7 cells treated with paclitaxel**

In chapter 3, I was able to show that JNK1/2 became activated (phosphorylated) following paclitaxel treatment (Fig 3.1, 3.2, 3.3). Research conducted in parallel in Prof Lam’s lab (Sunters et al., 2006), indicated that treatment of MCF-7 cells with SP600125, an inhibitor of JNK1/2, in combination with paclitaxel, attenuated the decrease in phospho-Thr32FoxO3a (cytoplasmic FoxO3a) observed for cells treated with paclitaxel alone. This suggested that JNK1/2 may act upstream to FoxO3a and might affect FoxO3a sub-cellular localisation following paclitaxel treatment.

To determine whether inhibition of JNK1/2 by SP600125 prevented nuclear translocation of FoxO3a, I performed, with the help of Dr. Andrew Sunters (CRUK Labs, Imperial College, London), confocal microscopy on MCF-7 cells treated with paclitaxel, SP600125, or the combination for 16 h. As shown in figure 5.2A, the majority of the endogenous FoxO3a remained in the cytoplasmic compartment in the untreated control cells and in the cells treated with SP600125. Moreover, treatment with SP600125 almost completely prevented nuclear accumulation of FoxO3a in response to paclitaxel treatment. In order to confirm these results, I performed nuclear and cytoplasmic fractionation experiments and analysed the relative amounts of FoxO3a in the nucleus and cytoplasm. The results are presented in figure 5.2B and show that the majority of FoxO3a in control (untreated) and SP600125-treated cells was located in the cytoplasm and after paclitaxel treatment FoxO3a was predominantly localised in the nucleus. As observed using confocal microscopy, SP600125 prevented FoxO3a nuclear accumulation following paclitaxel treatment. The observation that treatment of MCF-7 cells with a SP600125 blocked the paclitaxel-induced nuclear translocation of FoxO3a, indicates that JNK1/2 may play a role in nuclear translocation of FoxO3a following paclitaxel treatment.
Figure 5-2: Inhibition of JNK1/2 by SP600125 prevents FoxO3a nuclear localisation in MCF-7 cells treated with paclitaxel.

(A) MCF-7 cells were grown on coverslips and then treated with paclitaxel (10 nM), SP600125 (20 μM), or a combination of paclitaxel and SP600125. Cells were fixed 16 h after treatment in 4% formaldehyde and FoxO3a was visualized with a rabbit polyclonal antibody and subsequent addition of the ALEX488 (green) labelled anti-rabbit antisera. TRITC-labelled phalloidin (red) and DAPI (blue) were also applied to visualize the cytoplasm and nuclei, respectively.

(B), Western blotting of cytoplasmic and nuclear extracts isolated from MCF-7 cells which were untreated, treated with paclitaxel (10 µM), SP600125 (20 µM), or SP600125 and paclitaxel for 8 h. Protein levels were analysed using a specific phospho- FoxO3a (P-FoxO3) antibody and specific total antibodies. β-actin was used as a loading control. * Non-specific marking on the autorad. The mark left to the nuclear control sample may be part of the protein size marker which ran in that lane (Molecular weight of about 100kDa). An experiment showing a similar trend as 5.2B can be found in the supplementary data, figure 7.13.
5.2.3 The effect of JNK1/2 on the sub-cellular localisation of FoxO3a is dependent on PI3K/Akt signalling

The previous experiment showed that inhibition of JNK1/2 prevented nuclear translocation of FoxO3a following paclitaxel treatment and work conducted in Prof. Lam’s lab by Dr. Andrew Sunters and colleagues (Sunters et al., 2006) demonstrated that inhibition of JNK1/2 activity diminished the decrease in cytoplasmic, Akt phosphorylated (transcriptionally inactive) FoxO3a following paclitaxel treatment. Therefore, I next decided to examine whether the effect of JNK on the sub-cellular localisation of FoxO3a was dependent on PI3K/Akt signalling. To investigate this, MCF-7 cells were treated with either Triciribine (Akt inhibitor) or LY294002 (PI3K inhibitor) alone or in combination with SP600125, and the sub-cellular localisation of FoxO3a was examined by performing nuclear and cytoplasmic fractionation experiments (Fig 5.3). As seen in figure 5.3, the majority of FoxO3a in control (untreated cells) was located in the cytoplasm. Following treatment with SP600125 a decrease in FoxO3a expression in both the cytoplasmic and nuclear compartments was observed. Furthermore, FoxO3a was predominantly located in the nucleus of MCF-7 cells following treatment with LY294002 or Triciribine. However, treatment of cells with SP600125 failed to prevent the translocation of FoxO3a to the nucleus which is triggered by the inhibition of Akt activity through LY294002 or Triciribine. Together, these data suggest a role for JNK1/2 in the regulation of FoxO3a sub-cellular localisation. Moreover, these results also suggest that the main determinant of the sub-cellular localisation of FoxO3a is PI3K-acticated Akt activity, and that the effect of JNK1/2 on the sub-cellular distribution of FoxO3a occurs predominantly via modulating the PI3K/Akt pathway and not by acting directly on FoxO3a.
Figure 5-3: Inhibition of JNK1/2 by SP600125 fails to prevent FoxO3a nuclear localisation in MCF-7 cells treated with inhibitors of PI3K and Akt.

Western blotting of cytoplasmic and nuclear extracts isolated from MCF-7 cells treated with inhibitors of PI3K and Akt. Cells were treated with either LY294002 (30 µM), Triciribine (30 µM), or SP600125 (20 µM), or a combination of either LY294002 and SP600125 or Triciribine and SP600125 for 8 h. Protein levels were analysed using specific phospho (indicated by a P in-front of the protein name) and total antibodies. β-actin was used as a loading control. An experiment showing a similar trend can be found in the supplementary data, figure 7.14.
5.2.4 JNK1 affects FoxO3a promoter activity in MCF-7 cells

In order to determine whether the JNK signalling pathway may also have a direct affect on FoxO3a, I decided to examine the effects of JNK activation on FoxO3a promoter activity in MCF-7 cells. Hence, MCF-7 cells were transiently co-transfected with the FoxO3a promoter/reporter construct and a plasmid which expresses constitutively active JNK1 (Fig 5.4A). This expression plasmid was a generous gift from Dr. Axel Berhrens, (CRUK Labs, Lincoln’s Inn Fields, London) and harbours a JNKK2-JNK1 fusion protein. Fusion of JNK1 to its upstream activator JNKK2 results in constitutive JNK1 phosphorylation and activation. The JNKK2-JNK1 fusion protein is highly specific for the JNK pathway and does not activate either p38 or ERK (Zheng et al., 1999).

As shown in figure 5.4A, cells transiently transfected with constitutively active JNK1 showed an increase in FoxO3a promoter activity. Moreover, when MCF-7 cells were transiently transfected with the FoxO3a promoter/reporter construct and then treated with the JNK inhibitor, SP600125 for 8 h a decrease in FoxO3a promoter activity was observed (Fig 5.4B). Taken together, these data imply that the JNK signalling pathway, in addition to playing a role in the nuclear/cytosolic shuttling of FoxO3a, may also affect FoxO3a transcription at the gene promoter level.
Figure 5-4: Activation of JNK1 induces the promoter activity of FoxO3a in MCF-7 cells. Top panel, a schematic representation of the FoxO3a promoter/reporter construct. (A), MCF-7 cells were transiently co-transfected with 20ng of pGL3-FoxO3a and either 30ng of pCDNA3 (EV) or 30ng of pCDNA3-JNKK2-JNK1. (B), Cells were transiently transfected with the FoxO3a promoter/reporter construct and 16 h post transfection cells were either untreated or treated with SP600125 (20 µM) for 8 h. Cells were harvested 24 h after transfection and assayed for luciferase activity. Values are corrected for co-transfected renilla activity. All data shown represent the average of three independent experiments, and the error bars show the standard deviation, *p<0.01.
5.2.5 JNK1 can phosphorylate FoxO3a

JNK has been found to phosphorylate FoxO4 at threonine 447 and threonine 451, which are located within the transactivation domain of FoxO4. This occurs following oxidative stress induced activation of the small GTPase Ral (Essers et al., 2004). Research conducted in Prof. Lam’s lab by Dr. Muriel Aubert revealed that JNK and FoxO3a interact and mass spectrometry performed in collaboration with Dr. Simon Arthur (MRC Unit, Dundee) identified two potential FoxO3a phosphorylation sites by JNK, serine 294 and serine 425. The relative location of these sites with reference to the Akt phosphorylation sites can be found in figure 5.5. In order to examine if these sites are indeed phosphorylated by JNK in MCF-7 cells, I decided to transiently transfect MCF-7 cells with a plasmid expressing constitutively active JNK1 (the JNKK2-JNK1 fusion protein as explained in 5.2.4) and monitor the phosphorylation status of FoxO3a at serine 294 and serine 425 residues with specific phospho-antibodies generated against these sites. These antibodies were also kindly provided by Dr. Simon Arthur (MRC Unit, Dundee) and works best on precipitated protein rather than whole cell lysate. Therefore, I decided to immunoprecipitate (IP) cells transfected with the JNKK2-JNK1 fusion protein. Since I was experiencing some problems performing IP using the FoxO3a total antibody, I also co-transfected the MCF-7 cells with the pCMV-FoxO3a construct (a generous gift from Dr. Simon Arthur MRC Unit, Dundee) which enabled me to IP transfected cells using the Flag antibody which targets the Flag sequence fused to FoxO3a in this construct. The results are presented in figure 5.6.

As seen in figure 5.6, transfection of the JNKK2-JNK1 fusion protein increased the levels of active JNK as indicated by the increase in phosphorylated JNK (Thr183/Tyr185) in total cell lysate, while the levels of total JNK remained the same in both cells transfected with the empty vector and cells transfected with the JNKK2-JNK1 expression plasmid. Immunoblotting with the total FoxO3a antibody revealed two bands and it is possible that the faster migrating band represents the endogenous FoxO3a, while the
top band represents transfected FoxO3a which is fused to Flag. In cells transfected with JNKK2-JNK1 fusion protein, an increase in the intensity of the lower band was observed. This may indicate an increase in the expression of endogenous FoxO3a. It is likely that transfection of active JNK1 also generates an increase in the expression of exogenous FoxO3a, but that the increase in endogenous FoxO3a is more prominent because this band in the western blot is under-exposed. This implies that phosphorylated/active JNK1 can increase the level of FoxO3a expression. Western blotting of Immunoprecipitated flag-tagged FoxO3a transfected cells, using the phospho-specific antibodies, revealed an increase in FoxO3a phosphorylated at the serine 294 residue, but not at the serine 425 residue. Hence, these results demonstrate that JNK1 can phosphorylate FoxO3a at serine 294. However, it is also possible that JNK1 can phosphorylate FoxO3a at the serine 425 site but the antibody used was unable to detect this phosphorylation under these experimental conditions. In addition, it is also plausible that phosphorylation at the serine 425 site occurs at a lower level than phosphorylation at serine 294.
Figure 5-5: Akt and JNK phosphorylation sites on FoxO3a.
Schematic representation of FoxO3a and the relative location of the Akt and JNK phosphorylation sites (FKH-forkhead domain, NLS-nuclear localisation signal, NES-nuclear export sequence, TA-transactivation domain).
Figure 5-6: JNK1 can phosphorylate FoxO3a on Serine 294. MCF-7 cells were co-transfected with either the combination of pCMV-FoxO3a and pCDNA3- JNKK2-JNK1, or with PCMV-FoxO3a and the empty vector, pCDNA3. Cell lysates were prepared 36 h after transfection was performed and 20µg were used for western blot and the remaining 60µg of lysate was Immunoprecipitated (IP) with an antibody against Flag. The levels of total JNK and phospho-JNK (Thr183/Tyr185) and total FoxO3a in transfected cells was determined by western blot analysis of total cell lysate. The precipitated anti-Flag complexes were resolved on SDS-polyacrylamide gels and immunoblotted with a Flag antibody (Immunoprecipitated protein, indicating equal amounts of precipitated protein) and antibodies against phosphorylated FoxO3a (predicated JNK phosphorylation sites). An experiment demonstrating a similar trend can be found in the supplementary data, figure 7.15.
5.2.6 The effect of mutating FoxO3a's serine 294 and serine 425 to alanines on the activity FoxO3a

As mentioned in section 5.2.5, JNK has been found to phosphorylate FoxO4 following oxidative stress. This phosphorylation has been suggested to increase the interaction between FoxO4 and the basal transcription machinery (Essers et al., 2004). In addition, Akt phosphorylation of FoxO proteins induces the binding of 14-3-3 proteins to FoxO leading to the export of FoxO from the nucleus; whereas JNK has been shown to phosphorylate 14-3-3, thereby leading to the release of FoxO from the complex with 14-3-3 (Sunayama et al., 2005; Tsuruta et al., 2004).

Previously, I was able to demonstrate that activation of the JNK signalling cascade was important for nuclear translocation of FoxO3a following paclitaxel treatment (Fig 5.2). Given that I was also able to show that JNK1 can phosphorylate FoxO3a at serine 294 (Fig 5.6), I next examined the effect of mutating FoxO3a JNK phosphorylation sites, serine 294 and serine 425 to alanine, on the activity of FoxO3a. Mutating these serine residues to alanine residues prevents these sites from being phosphorylated; though these proteins mimic constitutively un-phosphorylated FoxO3a proteins at the JNK phosphorylation sites. To this end, I used pCMV5-Flag vectors (all of which were a generous gift from Dr. Simon Arthur) that harboured one of the following: FoxO3a (wt), FoxO3aS294A, FoxO3aS425A or FoxO3aS294A, S425A.

5.2.6.1 Mutating FoxO3a's serine 294 and serine 425 to alanines reduces the ability of FoxO3a to induce the activity of the p27Kip1 promoter

MCF-7 cells were transiently co-transfected with the p27Kip1 promoter/reporter construct and with either wild-type FoxO3a or FoxO3a mutated at one or both of JNK phosphorylation sites. As seen in figure 5.7A, co-transfection of the wild-type FoxO3a resulted in an increase in p27Kip1 promoter activity, while co-transfection of either of the single mutants or the double mutant did not affect p27Kip1 promoter activity. I repeated the same transfection, but this time, in addition, treated the transfected cells with 30µM LY294002. This was
performed in order to inactivate the PI3K/Akt pathway which in turn should induce FoxO3a nuclear translocation and activity. As shown in figure 5.7B, treatment of the transfected cells with LY294002 for 8 h did not significantly alter the results previously attained; an increase in p27Kip1 promoter activity was only observed for cells co-transfected with the wild-type FoxO3a.
Figure 5-7: Mutating FoxO3a’s serine 294 and serine 425 to alanines impairs FoxO3a’s ability to induce p27Kip1 transcription at the promoter level.
Top panel, schematic representation of the p27Kip1 promoter/reporter construct, the location of the FRE-FoxO-recognised element is indicated. (A), MCF-7 cells were transiently co-transfected with 20ng of pGL3-p27Kip1 and 10ng of either pCMV5-FoxO3a (wt), pCMV5-FoxO3aS294A (S294A), pCMV5-FoxO3aS425A (S425A) or pCMV5-FoxO3aS294A,S425A (S294A,S425A). (B), Transfected cells were treated with LY294002 (30 µM) for 8 h. All cells were harvested 24 h after transfection and assayed for luciferase activity. Values are corrected for co-transfected renilla activity. All data shown represent the average of three independent experiments, and the error bars show the standard deviation.*p<0.05 when compared to cells transfected with 0ng.
5.2.6.2 The Effect of mutating FoxO3a’s serine 294 and serine 425 to alanines on the ability of FoxO3a to induce BTG1 promoter activity

MCF-7 cells were transiently co-transfected with the BTG1 promoter/reporter construct and with either wild-type FoxO3a or FoxO3a mutated at one or both of JNK phosphorylation sites. As shown in figure 5.8A, an increase in BTG1 promoter activity was observed only when wild-type, and not mutated FoxO3a proteins, were co-transfected with the promoter/reporter construct. As shown in figure 5.8B, treatment of transfected cells with 30µM LY294002 for 8 h induced an increase in BTG1 promoter activity, when wild-type FoxO3a, the single mutants (FoxO3aS294A and FoxO3aS425A), but not the double mutant (FoxO3aS294A, S425A) were co-transfected. These results imply that inhibition of PI3K/Akt pathway, which leads to FoxO3a nuclear translocation, is a prerequisite for induction of BTG1 promoter activity by the single site JNK phosphorylation mutants, albeit to levels lower than observed for wild-type FoxO3a. Nonetheless, inhibition of PI3K/Akt pathway did not result in an increase BTG1 promoter activity when FoxO3a mutated in both JNK phosphorylation sites was co-transfected, suggesting that at least one intact JNK phosphorylation site is required for FoxO3a to up-regulate BTG1 promoter activity.
Figure 5-8: The effect of mutating FoxO3a’s JNK phosphorylation sites to alanine on the ability of FoxO3a to up-regulate BTG1 promoter activity. 

Top panel, a schematic representation of the BTG1 promoter/reporter construct, the location of the FRE-FoxO-recognised element is indicated. (A), MCF-7 cells were transiently co-transfected with 20ng of pGL3-BTG1 and 10ng of either pCMV-FoxO3a (wt), pCMV5-FoxO3aS294A (S294A), pCMV5-FoxO3aS425A (S425A), pCMV5-FoxO3aS294A,S425A (S294A,S425A). (B), Transfected cells were treated with LY294002 (30 µM) for 8 h. All cells were harvested 24 h after transfection and assayed for luciferase activity. Values are corrected for co-transfected renilla activity. All data shown represent the average of three independent experiments, and the error bars show the standard deviation; *p<0.01, when compared to 0ng of the same construct ^ p<0.05 when compared to cells transfected with the wild-type construct and treated with LY294002.
5.2.6.3 Mutating both of FoxO3a’s JNK phosphorylation sites to alanine impairs FoxO3a’s ability to up-regulate Bim promoter activity

MCF-7 cells were transiently co-transfected with the *Bim* promoter/reporter construct and with either wild-type FoxO3a or FoxO3a mutated at one or both of the JNK phosphorylation sites. As seen in figure 5.9A, an increase in *Bim* promoter activity was observed when both wild-type FoxO3a and FoxO3a harbouring one of the mutations (S294A or S425A), but not both of the mutations, was co-transfected into MCF-7 cells. Treatment of transfected cells with 30µM LY294002 for 8 h, figure 5.9B, also resulted in an increase in *Bim* promoter activity, for MCF-7 transiently co-transfected with either FoxO3a (wt), or one of the single-site mutants (FoxO3aS294A, FoxO3aS425A). Yet again, no increase in *Bim* promoter activity was observed for cell co-transfected with the double mutant. It appears that the FoxO3a S294A mutant can induce the activity of the *Bim* promoter to levels similar to that of the wild-type, while FoxO3a S425A induces *Bim* promoter activity to levels which are statistically lower than those of obtained for the wild-type. Taken together, these results indicate that the double FoxO3a mutant is unable to up-regulate, under the circumstances tested, the activity of the *Bim* promoter. In addition, inhibition of PI3K/Akt pathway, obtained by treatment of the transfected cells with LY294002, was not required in order to generate an increase in *Bim* promoter activity following transient transfection of either FoxO3a (wt) or one of single-site mutants.
Figure 5-9: Mutating both of FoxO3a’s JNK phosphorylation sites to alanine impairs FoxO3a’s ability to up-regulate Bim promoter activity.

Top panel, a schematic representation of the Bim promoter/reporter construct the location of the FRE-FoxO-recognised element, is indicated. (A), MCF-7 cells were transiently co-transfected with 20ng of pGL3-Bim and 10ng of either pCMV5-FoxO3a (wt), pCMV5-FoxO3aS294A (S294A), pCMV5-FoxO3aS425A (S425A) or pCMV5-FoxO3aS294A,S425A (S294A,S425A). (B), Transfected cells were treated with LY294002 (30 µmol/L) for 8 h. All cells were harvested 24 h after transfection and assayed for luciferase activity. Values are corrected for co-transfected renilla activity. All data shown represent the average of three independent experiments, and the error bars show the standard deviation. *p<0.01, when compared to cells transfected with 0ng.
5.2.6.4 FoxO3a cannot induce Bim (mut -164/-170) promoter activity

To determine whether the increase observed in Bim promoter activity when co-transfected with FoxO3a (wt), FoxO3aS294A and FoxO3aS425A was mediated through the FoxO DNA consensus binding site, located in the Bim promoter; I decided to perform the same co-transfection experiment, but instead of transfecting pGL3-Bim (wt), I transfected the pGL3-Bim (mut-164/170) construct (mutated in the FoxO DNA binding site).

MCF-7 cells were co-transfected with pGL3-Bim (mut-164/170), together with wild-type FoxO3a or FoxO3a mutated at one or both of the JNK phosphorylation sites. Half of the transfected cells were also treated with LY294002 in order to inhibit the activity of the PI3K/Akt signalling pathway, which should induce FoxO3a activation and translocation to the nucleus. As seen in figure 5.10A, no increase in the mutated Bim promoter activity was observed when cells were co-transfected with wild-type or mutated FoxO3a proteins. Treatment of the transfected cells with LY294002 (Fig 5.10B) also yielded the same result. Taken together, this experiment shows that the increase observed in Bim (wt) promoter activity, when co-transfected with either FoxO3a (wt), FoxO3aS294A or FoxO3aS425A (Fig 5.9A, B), was due to the presence of the FoxO DNA consensus binding site in the Bim promoter and that the increase in activity was FoxO specific.
Figure 5-10: FoxO3a is unable to induce the promoter activity of a Bim promoter/reporter construct mutated at the FoxO DNA consensus binding site. Top panel, schematic representation of the Bim promoter/reporter construct mutated in the FoxO DNA consensus binding site. FRE—FoxO-recognised element, black rectangular indicates mutation. (A), MCF-7 cells were transiently co-transfected with 20 ng of pGL3-Bim (mut) and 10 ng of either pCMV5-FoxO3a (wt), pCMV5-FoxO3aS294A (S294A), pCMV5-FoxO3aS425A (S425A) or pCMV5-FoxO3aS294A,S425A (S294A,S425A). (B), Transfected cells were treated with LY294002 (30 µM) for 8 h. All cells were harvested 24 h after transfection and assayed for luciferase activity. Values are corrected for co-transfected renilla activity. All data shown represent the average of three independent experiments, and the error bars show the standard deviation.
5.2.7 Mutating FoxO3a’s serine 294 and serine 425 to alanines increases FoxO3a proteasomal degradation

Previous experiments demonstrated that FoxO3aS294A, S425A (mutated at both of the potential JNK phosphorylation sites) was unable to up-regulate promoter activity of FoxO3a downstream targets in MCF-7 cells (Fig 5.7, 5.8, 5.9). Treatment with LY294002 did not increase promoter activity levels, indicating that inducing nuclear translocation of this protein could not rescue this phenotype. Since induction of transcription by FoxOs has been shown to be affected by FoxO stability (Plas and Thompson, 2003), it is plausible that JNK phosphorylation of FoxO3a might play a role in modulating FoxO3a stability. Therefore, I next sought to examine the stability of wild-type FoxO3a in comparison to single mutants (FoxO3aS294A, FoxO3aS25A) and the double mutant (FoxO3aS294A, S425A). To that end, I transiently transfected MCF-7 cells with pCMV5-Flag vectors harbouring one of the following: FoxO3a (wt), FoxO3aS294A, FoxO3aS425A or FoxO3aS294A, S425A. After which cells were either untreated (NT) or treated with the proteasomal degradation inhibitor, MG132 for 4 h. Whole cell lysates were prepared and western blotting using an anti-flag antibody was performed.

As shown in figure 5.11A, MG132 treatment of MCF-7 cells transfected with FoxO3a (wt) caused an increase in levels of FoxO3a, indicating that FoxO3a, as previously observed, is degraded at least partially, via the proteasomal pathway (Plas and Thompson, 2003). Both untreated FoxO3a single amino-acid mutants (FoxO3aS294A, FoxO3aS425A) showed lower levels of protein expression (Fig 5.11A), although slightly higher levels of expression were observed for FoxO3aS294A in comparison to the FoxO3aS425A. This could be due to variations in transfection efficiency, or stability of the transfected proteins. However, this is not related to plasmid degradation as indicated by the results of the DNA gel electrophoresis of the plasmids, which is presented in Figure 5.11B.

Treatment with MG132 was able to increase the levels of FoxO3aS294A expression, which appear similar to those observed for wild-type FoxO3a (Fig
5.11A). No increase in FoxO3aS425A expression was observed following treatment with MG132, indicating that this mutant form of FoxO3a was not degraded via the proteasome. However, it is possible that this result was caused by poor transfection efficiency of the cells as indicated by the very low levels of expression of the transfected protein. Untreated cells transfected with FoxO3a double amino-acid mutant (FoxO3aS294A, S425A) also demonstrated very low levels of protein expression, in comparison to cells transfected with wild-type FoxO3a (Fig 5.11A). Again, this could be due to either differences in transfection efficiency or stability of the transfected protein. Though, it is worth considering the possibility that other proteins might also modulate FoxO3a post-translationally and that this may affect FoxO3a’s stability and degradation via the ubiquitin-proteasome pathway. Nonetheless, treatment with MG132 caused a noticeable increase in FoxO3aS294A, S425A expression which appears to be even higher than observed for FoxO3aS425A and possibly than observed for wild-type FoxO3a. Taken together, these results suggest that the FoxO3a double-mutant is prone to degradation via the proteasomal pathway, most probably more than the wild-type FoxO3a.
Figure 5-11: Mutating FoxO3a’s serine 294 and serine 425 to alanines promotes proteosomal degradation of FoxO3a.

MCF-7 cells were transfected with either pCMV-FoxO3a (wt), pCMV-FoxO3aS294A (S294A), pCMV-FoxO3aS425A (S425A) or pCMV-FoxO3aS294A, S425A (S294A, S425A). 24 h post-transfection cells were treated with proteasome inhibitor MG132 (50 µmol/L) for 4 h or not treated (NT). (A), Whole cell lysates were prepared and separated on SDS-polyacrylamide gels and immunoblotted. Flag protein levels were analysed by western blot. β-tubulin was used as a loading control. An experiment showing a similar trend can be found in the supplementary data, figure 7.16 (B). DNA agarose gel electrophoresis of the four constructs transfected in (A). 1µg of plasmid DNA construct was run on a gel, 1- pCMV-FoxO3a, 2- pCMV-FoxO3aS294A, 3- pCMV-FoxO3aS425A, 4- pCMV-FoxO3aS294A, S425A.
5.2.8 Mutating FoxO3a’s serine 294 and serine 425 residues to either alanine or aspartic acid affects FoxO3a’s stability

In order to further understand the role of JNK post-translational regulation of FoxO3a, I decided to monitor the stability of wild-type FoxO3a in comparison to two different FoxO3a mutants, one which cannot be phosphorylated by JNK (FoxO3aS294A, S425A) and one which mimics a constitutively JNK-phosphorylated FoxO3a (FoxO3aS294D, S425D). To that end, MCF-7 were transiently transfected with one of the following constructs: pCMV5-Flag-FoxO3a (wt), pCMV5-Flag-FoxO3a (S294A, S425A) or pCMV5-Flag-FoxO3a (S294D, S425D), (which was generated by Dr. Ho Ka Kei, CRUK Labs, Imperial College, London), and then treated with 10µg/ml cycloheximide for up to 6 h. Western blot analysis was performed using the anti-Flag antibody.

As shown in figure 5.12, a decrease in the expression levels of wild-type FoxO3a and FoxO3a S294A, S425A was noticeable 6 h after treatment with cycloheximide commenced. However, there appeared to be no noticeable decrease in the expression levels of FoxO3aS294D, S425D through the time-course. Take together, it seems that FoxO3aS294D, S425D is less susceptible to degradation than wild-type FoxO3a and FoxO3a S294A, S425A. Therefore, it is possible that phosphorylation of FoxO3a by JNK, at serine 294 and serine 425, increases the stability of FoxO3a by reducing its degradation rate.
Figure 5-12: Mutating FoxO3α’s serine 294 and serine 425 affects FoxO3α stability.

MCF-7 cells were transfected with either pCMV5-FoxO3α (wt), pCMV5-FoxO3αS294A, S425A (S294A,S425A) or pCMV5-FoxO3αS294D, S425D (S294D, S425D). 24 h post-transfection cells were treated with 10μg/ml cycloheximide for up to 6 h. whole cell lysates were prepared at indicated time-points, and then separated on SDS-polyacrylamide gels and immunoblotted. The levels of Flag were analysed by western blot. β-tubulin was used as a loading control. An experiment demonstrating a similar trend can be found in the supplementary data, figure7.17.
5.2.9 The effect of mutating FoxO3a’s serine 294 and serine 425 to aspartic acids on the induction of BTG1 promoter activity

In order to examine whether phosphorylation of FoxO3a by JNK affects FoxO3a activity, I decided to transiently co-transfect MCF-7 cells with the BTG1 promoter/reporter construct and FoxO3a S294D, S425D, which mimics a constitutively JNK-phosphorylated FoxO3a, and compare the levels of BTG1 promoter activity to those attained co-transfecting either wild-type FoxO3a or FoxO3aS294A, S425A.

As seen in figure 5.13A, co-transfection of both FoxO3a (wt) and FoxO3aS294D, S425D increased BTG1 promoter activity in a similar magnitude. However, as previously observed (Fig 5.8), FoxO3aS294A, S425A was unable to up-regulate BTG1 promoter activity. I next decided to conduct the same co-transfection experiment, but this time, in order to inhibit JNK, I also treated the transfected cells with SP600125 for 8 h. This was performed because previously, I was able to demonstrate that JNK is active in normal cycling MCF-7 (Fig 5.4B) and therefore the effect of JNK phosphorylation of FoxO3a may not be prominent in cells that were not treated with a JNK inhibitor. As shown in figure 5.13B, inhibiting JNK activity caused a decrease in the ability of FoxO3a (wt) to up-regulate BTG1 promoter activity (p<0.01, when compared to 10ng FoxO3a (wt) non-treated in figure 5.13A). As observed for untreated cells, no change in BTG1 promoter activity levels were observed when FoxO3aS294A, S425A was co-transfected into MCF-7 cells. Nonetheless, a similar pattern of increase in BTG1 promoter activity was observed for FoxO3aS294D, S425D, as to that observed for untreated cells, suggesting that inhibition of the JNK signalling pathway does not render the ability of this mutant to increase BTG1 promoter activity. Taken together, these data imply that inhibition of the JNK signalling pathway reduces the ability of FoxO3a to up-regulate BTG1 transcription at the promoter level. Furthermore, this result strengthens the notion that JNK phosphorylates FoxO3a at serine 294 and serine 425 as the ability of
FoxO3aS294D, S425D to induce *BTG1* promoter activity was not affected by treatment with SP600125.
Figure 5-13: FoxO3aS294D, S425D can induce BTG1 promoter activity. 
Top panel, a schematic representation of the BTG1 promoter/reporter construct, the location of the FRE (FoxO recognising element) is indicated. (A), MCF-7 cells were transiently co-transfected with 20ng of pGL3-BTG1 and 10ng of either pCMV5-FoxO3a (wt), pCMV5-FoxO3aS294A, S425A (S294A,S425A) or pCMV-5FoxO3aS294D,S425D (S294D,S425D). (B), transfected cells were treated with SP600125 (20 µM) for 8 h. All cells were harvested 24 h after transfection and assayed for luciferase activity. Values are corrected for co-transfected renilla activity. All data shown represent the average of three independent experiments, and the error bars show the standard deviation. *p<0.01 when compared to cells transfected with 0ng.
5.3 Discussion
In chapter 3, I was able to show that in the MCF-7 breast cancer cell line paclitaxel treatment lead to up-regulation in FoxO3a expression, followed by an increase in the expression of FoxO3a downstream target p27^Kip1. I also observed a raise in JNK1/2 phosphorylation after treatment with paclitaxel (Fig 3.1, 3.2, 3.3). Other work conducted in our lab (Sunters et al., 2006), showed that paclitaxel treatment of MCF-7 cells caused an increase in FoxO3a expression and downstream pro-apoptotic target Bim, resulting in apoptosis of the cells. In this chapter, I was able to show that paclitaxel treatment of MCF-7 cells led to an increase in nuclear FoxO3a (Fig 5.1) and that treatment of MCF-7 cells with SP600125, a JNK1/2 inhibitor, inhibited nuclear accumulation of FoxO3a (Fig 5.2), which is a essential for FoxO3a transcriptional activation of downstream proapoptotic and anti-proliferative target genes. However, the use of specific inhibitors of PI3K and Akt resulted in nuclear accumulation of FoxO3a, which could not be blocked by SP600125 (Fig 5.3), indicating that the effect of JNK1/2 on FoxO3a nuclear localisation, in response to paclitaxel treatment, was dependent on PI3K/Akt signalling pathway. Hence, these results suggest that the main determinant of FoxO3a sub-cellular distribution is the PI3K/Akt signalling pathway and that this pathway may be subject to regulation by JNK1/2.

In addition to the role of JNK1/2 in the regulation of FoxO3a sub-cellular localisation, this pathway also seems to be important for regulation of FoxO3a transcription. Activation of the JNK1 signalling cascade induced FoxO3a promoter activity in MCF-7 cells and treatment of MCF-7 cells with SP600125 appeared to reduce FoxO3a promoter activity (Fig 5.4). This effect remains to be tested at the mRNA level. However, it is plausible that a transcription factor downstream of JNK1 can up-regulate FoxO3a transcription. Indeed, examination of FoxO3a 5' upstream regulatory region revealed the presence of several DNA consensus binding sites of transcription factors which are downstream targets of the JNK signalling pathway, such as c-Jun (Derijard et al., 1994; Murray et al., 2004), Elk-1.
(Whitmarsh et al., 1995; Yang et al., 1998) and c-Myc (Alarcon-Vargas and Ronai, 2004). Conducting experiments such as chromatin immunoprecipitation (ChIP) could help determine whether these transcription factors do indeed bind to the FoxO3a promoter following activation of the JNK signalling pathway.

Previously it was shown that JNK can phosphorylate FoxO4 in response to H$_2$O$_2$ treatment, leading to the translocation of FoxO4 from the cytoplasm to the nucleus (Essers et al., 2004). In the nuclear FoxO4 was found to be able to up-regulate the transcription of MnSOD, resulting in the detoxification of ROS and by doing so FoxO activity may promote longevity (Essers et al., 2004). I was able to show that activation of the JNK1 signalling pathway induced phosphorylation of FoxO3a on serine 294 (Fig 5.6). Other experiments conducted in Prof. Lam’s lab by Dr. Muriel Aubert and in collaboration with Dr. Simon Arthur (MRC Unit, Dundee) demonstrated that FoxO3a interacted with JNK both in vitro and in vivo and identified FoxO3a’s serine 425 and serine 294, as JNK phosphorylation sites. Phosphorylation of FoxO3a by JNK seems to be important for regulation of transcription by FoxO3a as mutating serine 294 and serine 425 to alanine residues inhibited FoxO3a induced up-regulation of $p27^{kip}$, BTG1 and Bim promoter activity (Fig 5.7, 5.8, 5.9). Interestingly, mutating each site individually showed a specific pattern of regulation unique to each of the promoters tested. $p27^{kip}$ promoter activity was not induced by either FoxO3aS294A or FoxO3S425A. BTG1 promoter activity was induced by FoxO3aS425A and FoxO3aS294A, in the presence of LY24002, a PI3K inhibitor; whereas Bim promoter activity was induced by both FoxO3aS294A and FoxO3S425A in the presence and absence of LY294002. Therefore, phosphorylation of FoxO3a by JNK might be involved in tightly regulating the specificity of FoxO3a downstream target genes transcribed. These results suggest that phosphorylation of FoxO3a by JNK at both serine 294 and serine 425 preferentially induces transcription of genes important for inhibition of cell cycle progression, while phosphorylation of FoxO3a at just one of the
two serine residues, seems to be important for induction of BTG1, a gene implicated in differentiation of B-cells and the induction of transcription of genes involved in apoptosis, such as Bim. However, results obtained monitoring BTG1 promoter activity; indicate that inhibition of the PI3K pathway may reduce the effect of fine-tuning by JNK phosphorylation, as FoxO3aS425A and FoxO3aS294A were able to induce BTG1 only in the presence of LY294002. It is important to note that the induction in the activity of all of the promoter constructs tested by wild-type FoxO3a is statistically significant but moderate. This could be due to the fact that it has recently been shown that the main role FoxO3a play in induction of transcription appears to be chromatin remodelling (Hatta and Cirillo, 2007). FoxO proteins induce transcription by opening and remodelling the chromatin structure and since transfected DNA have little chromatin structure, the effect of FoxO remodelling of DNA is lost.

Previously, it has been shown that FoxO3a is degraded via the proteasomal pathway (Plas and Thompson, 2003). Indeed, I was able to show that treatment of MCF-7 cells with the proteasome inhibitor, MG132 caused an increase in the expression levels of FoxO3a, indicating that this protein is degraded via the proteasome (Fig 5.11A). Interestingly, mutating both of the FoxO3a-JNK-phosphorylation-sites to alanines appeared to increase FoxO3a degradation rate, as suggested by what appears to be higher levels of protein expression after treatment with MG132 for FoxO3a S294A, S425A in comparison to wild-type FoxO3a. Interestingly, it seems as though mutating each site individually does not cause this effect, indicating the complexity of this regulation. Hence, it seems plausible that phosphorylation of FoxO3a by JNK at both of these sites inhibits degradation of FoxO3a. Examination whether FoxO3a phosphorylated by JNK at both sites is protected, at least partially, from proteasomal degradation could help validate this statement.

I also examined the rate of degradation of wild-type FoxO3a, FoxO3a unphosphorylated by JNK (FoxO3aS294A, S425A) and FoxO3a mimicking a constitutively JNK-phosphorylated FoxO3a (FoxO3aS294D, S425D) (Fig
The results of this experiment indicate that phosphorylation of FoxO3a by JNK increases FoxO3a stability, as it appears that FoxO3aS294D, S425D may have a longer half-life than FoxO3a S294A, S425A following treatment with cycloheximide. Results presented in figure 5.12 do not clearly indicate whether FoxO3a S294D, S425D also has a longer half-life than wild-type FoxO3a, however, it is possible that the effect of JNK phosphorylation on FoxO3a’s stability is greater than observed in this experiment. This is because I was able to show that the activity level of JNK in normal cycling MCF-7 cells, grown in 10% FCS, is high (Fig 5.4B). Hence, it is possible that a percentage of the wild-type FoxO3a-Flag, transfected into the cells, got phosphorylated by JNK and therefore exhibited increased stability. Nonetheless, the results of this experiment strengthen the hypothesis that JNK phosphorylation of FoxO3a increases FoxO3a half-life by protecting FoxO3a from proteasomal degradation. It would be interesting to examine the half-life of FoxO3a harbouring a serine to aspartic acid mutation at each one of the JNK phosphorylation sites separately. This will help determine whether phosphorylation by JNK at both sites causes a cumulative increase in FoxO3a’s half-life, or if phosphorylation at only one of these sites is sufficient to cause an increase in the stability of FoxO3a.

Phosphorylation of FoxO3a by JNK also seems to play a role in determining the ability of FoxO3a to induce transcription of target genes at the promoter level (Fig 5.13). The FoxO3a S294D, S425D mutant was able to induce the activity of the *BTG1* promoter, while FoxO3aS294A, S425A was unable to do so. Treatment of cells with the JNK inhibitor, SP600125, did not affect FoxO3a S294D, S425D ability to up-regulate *BTG1* activity, but did cause a decrease in ability of wild-type FoxO3a to do so. Therefore, it is plausible that phosphorylation of FoxO3a by JNK positively affects FoxO3a’s ability to up-regulate transcription of target genes. However, this hypothesis needs to be tested at the mRNA and protein expression levels. In addition, this effect may also be target gene specific and therefore should be examined on other transcriptional targets of FoxO3a.
Taken together, the data presented here demonstrates that FoxO3a can be regulated by JNK at various levels. At the transcriptional (gene promoter) level, I found that JNK1 was able to induce FoxO3a promoter activity, while inhibition of this pathway had a negative effect on up-regulation of FoxO3a. At the post-translational level, I was able to show that JNK1/2 can regulate FoxO3a sub-cellular localisation following treatment with paclitaxel. Treatment of MCF-7 cells with paclitaxel induces FoxO3a translocation to the nucleus. This event seems to be predominantly regulated by the PI3K/Akt pathway. However, the activation of JNK1/2 and suppression of Akt following treatment with paclitaxel suggest that there may be cross-talk between these two signal transduction pathways. This assumption is further strengthened by work other published data. It has also been shown that DAF-16 (the homologue of mammalian FoxO) is directly phosphorylated by *C. elegans* JNK after heat shock, resulting in an increase in stress resistance and longevity by a mechanism dependent on nuclear translocation of DAF-16 (Oh et al., 2006). Moreover, binding of FoxO to 14-3-3 following Akt activation results in the translocation of FoxO from the nucleus to the cytoplasm. JNK has been shown to phosphorylate 14-3-3 proteins, which terminates the interaction between 14-3-3 and FoxO (Tsuruta et al., 2004).

Data obtained in our lab, which was partly presented in this chapter, demonstrates that JNK can post-translationally regulate FoxO3a by facilitating phosphorylation at the serine 294 and serine 425 sites, and that this phosphorylation can affect the stability and perhaps even the activity of FoxO3a. It appears that phosphorylation of FoxO3a by JNK at both sites may increase FoxO3a’s stability by reducing FoxO3a proteasomal degradation. This phosphorylation may also increase FoxO3a’s ability to induce the activity of downstream target genes; however, the results obtained in this experiment were inconclusive. Furthermore, this effect may be target-gene specific, related to the activation status of the PI3k/Akt pathway and may not necessarily require phosphorylation of FoxO3a by JNK at both sites identified. Nonetheless, a recent report using a *C. elegans jnk-1* deletion
mutant revealed that JNK-1 plays a role in inducing the nuclear localisation of DAF-16 and subsequent DAF-16 dependent transcription of the target gene sod-3 (Wolf et al., 2007).

In summary, I was able to show that paclitaxel treatment of MCF-7 cells induces FoxO3a translocation to the nucleus and that this post-translational regulation involves both the PI3K/Akt and JNK signalling pathways, indicating that these two pathways cross-talk. Further analysis on the role of JNK in the regulation of FoxO3a revealed that at the gene promoter level, activation of JNK1 appears to induce the transcription of FoxO3a and that at the post-translational level, JNK1 can phosphorylate FoxO3a at two different sites and this leads to an increase in FoxO3a’s stability, which is probably partially mediated by the ubiquitin-proteasome pathway. Moreover, mutating both of the JNK phosphorylation sites in the FoxO3a protein decreased FoxO3a’s ability to induce the promoter activity of downstream targets.

5.4 Future work
It would be interesting to explore whether treatment with paclitaxel induces JNK phosphorylation of FoxO3a and if inhibiting these phosphorylation events would decrease FoxO3a’s ability to induce cell death in response to paclitaxel. Furthermore, since treatment with paclitaxel can also up-regulate the activity of other signalling pathways, such as p38 (Bacus et al., 2001; Seidman et al., 2001), it would be beneficial to conduct a similar experiment in which only the activity JNK is up-regulated, such as treating MCF-7 cells with TNF-α, which activates JNK but not p38. In addition, an experiment using MEFs derived from wild-type mice, jnk1 and jnk2 double knockout mice and p38 knockout mice treated with paclitaxel; would help confirm the role of JNK in the nuclear translocation of FoxO3a and also indicate if this role is reserved to the JNK signal transduction pathway or whether p38 can also influence the sub-cellular localisation of FoxO3a in Jnk1/2 knockout cells. ΔMEKK3:ER cells, which express phospho-JNK1/2 in an inducible manner following treatment with 4-hydroxytamoxifen (and are available in Prof. Lam’s...
lab), could be used in order to determine whether JNK can, in addition to inducing *FoxO3a* promoter activity, also up-regulate the expression of *FoxO3a* at the mRNA level.
6 Final Discussion

6.1 Summary
The FoxO family of transcription factors are negatively regulated by the PI3K signalling pathway. This pathway has been found to be deregulated and mutated in several cancers and has been implicated in tumourigenesis and tumour progression (Campbell et al., 2004; Ma et al., 2000; Mizoguchi et al., 2004; Woenckhaus et al., 2002).

The work presented in this thesis focused on the role and regulation of FoxO transcription factors. I initially studied the regulation of FoxOs at the transcriptional level, using breast epithelial and endometrial cells as model systems. Next, I examined the function of FoxO3a in cell proliferation through investigating the role of BTG1, a novel downstream target of FoxO3a (Bakker et al., 2004), in breast cancer. Finally, I studied the cross-talk between the PI3K signalling pathway and the JNK signalling cascade in the regulation of FoxO. Through my work, I was able to demonstrate that FoxO proteins are targets of the chemotherapeutic drug, paclitaxel. I was also able to show that FoxO3a integrates signals from both the PI3K signalling pathway and the JNK signalling cascade, to mediate an increase in the transcription of downstream targets, in response to paclitaxel and oxidative stress. FoxO proteins regulate the expression of a spectrum of genes which are important for both mediating cell death and for inducing longevity, such as genes involved in cell cycle arrest (De Ruiter et al., 2001; Medema et al., 2000; Nakamura et al., 2000; Seoane, 2004; Stahl et al., 2002; Tanaka et al., 2001), apoptosis (Brunet et al., 1999; Modur et al., 2002), DNA repair (Furukawa-Hibi et al., 2002; Tran et al., 2002) and ROS detoxification (Balaban et al., 2005; Kops et al., 2002a; Nemoto and Finkel, 2002). BTG1 was shown to up-regulate the expression and activity of some of FoxO3a’s target genes and to inhibit cell cycle progression.

Taken together, these data validate the initial hypothesis that FoxO transcription factors are downstream targets of chemotherapeutic drugs, such
as paclitaxel and are important in cancer development and progression. Hence, by defining and exploiting a mechanism that would enable us to shift FoxO activity from stress resistance and longevity towards induction of cell death, we might be able to influence cancer cell fate through inducing apoptosis, restoring cell cycle arrest and senescence, and counteracting longevity in these cells.

6.2 Transcriptional regulation of FoxO proteins

The study detailed in chapter 3 examined whether FoxO transcription factors are regulated at the transcriptional level by paclitaxel, in cancer cells. I was able to demonstrate that indeed paclitaxel treatment caused an increase in FoxO3a protein expression, mRNA levels and promoter activity in the breast cancer cell lines. Consistent with this, work conducted on the endometrial cell line HEC-1-B, also indicated that FoxO1 expression is induced by paclitaxel at the protein, mRNA and promoter levels. However, in the MCF-7 breast cancer cell line, despite the increase observed in FoxO3a expression and promoter activity, FoxO3a mRNA levels remained unaffected by paclitaxel. The reason for this is unknown as previous work conducted in our lab by Dr. Sunters and colleagues (Sunters et al., 2003) showed an increase in FoxO3a mRNA levels following paclitaxel treatment. Nonetheless, further experiments led to the possibility that paclitaxel can regulate FoxO3a post-translationally by reducing FoxO3a protein turnover, which increases protein stability. In the literature it has been shown that oxidative stress can increase protein stability (Nguyen et al., 2003). In line with this, my results and previously published data (Alexandre et al., 2006), demonstrate that paclitaxel treatment can increase the levels of oxidative stress in the cells. Furthermore, similar to the results obtained with paclitaxel, I was also able to show that FoxO3a protein stability increases following H₂O₂ treatment in MCF-7 cells. However, the mechanism enabling the increase in protein stability remains to be elucidated.

Another aspect of the work presented in chapter 3, is the regulation of FoxO transcription factors at the gene-promoter level. Generation of truncated
FoxO3a promoter constructs revealed that FoxO3a is most probably regulated at the transcriptional level by both negative and positive transcription regulators. These studies also enabled mapping a region of 131bp in the FoxO3a 5’ regulatory region which is responsive to paclitaxel treatment. FoxO1 and FoxO3a were shown to be partially regulated at the promoter level by the MEF2 transcription factor. Over-expression of MEF2 was shown to increase FoxO promoter activity, while mutating the MEF2 DNA consensus binding sites located within the FoxO1 promoter, yielded mixed results. While I was able to show that mutating one of the three sites decreased FoxO1 basal promoter activity, no statistically significant change in FoxO1 promoter activity were observed when mutating the other two sites. However, co-transfection of MEF2 was found to augment both FoxO1 and FoxO3a promoter activity following paclitaxel treatment. This additive effect indicates that paclitaxel and MEF2 regulate FoxO promoter activity by two separate mechanisms. Therefore, targeting MEF2 may serve as a therapeutic strategy for treatment of cancer in synergy with classical chemotherapeutic treatments, such as paclitaxel. These results suggest that increasing the expression of MEF2 in the cells may increase the response of cells to paclitaxel treatment by further inducing the levels of FoxO expression, which could lead to an induction in the expression levels of downstream pro-apoptotic target genes.

These finding are important because this is the first time that FoxO transcription factors were shown to be regulated at the transcriptional level. Prior to this study, the regulation of FoxO transcription factors focused on the study of post-translational modifications. FoxO proteins were shown to be phosphorylated by Akt, which decreases the ability of FoxO transcription factors to bind to the DNA and leads to the translocation of FoxO from the nucleus to the cytosol and inhibition of FoxO’s activity (Biggs et al., 1999; Brunet et al., 1999; Kops et al., 1999; Rena et al., 1999; Tang et al., 1999).
Recently, it has also been shown that acetylation of FoxO proteins plays a role in the post-translational regulation of FoxO transcription factors (Brunet et al., 2004; Daitoku et al., 2004; Fukuoka et al., 2003; Kobayashi et al., 2005; Mahmud et al., 2002; Motta et al., 2004; Perrot and Rechler, 2005; van der Horst et al., 2004; Yang et al., 2005b); however the significance of this only became known once I already started working on this dissertation.

The significance of the regulation of FoxO at the transcriptional level is emphasised by the work of Dr. Sunters and colleagues (Sunters et al., 2003), which demonstrated that FoxO3a expression levels differ between breast cancer cell lines and that these differences are indicative of cell sensitivity to paclitaxel treatment. This work showed that paclitaxel treatment induced apoptosis in cell lines that expressed FoxO3a at elevated levels while G2/M arrest was induced in cells that expressed FoxO3a at lower levels.

Overall, FoxO can be important bio-markers for monitoring the effectiveness of chemotherapeutic drugs, such as paclitaxel. Furthermore, treatment of cancer patients with paclitaxel in conjunction with a substance which would generate an increase in the number of FoxO transcripts in the cells may prove as an avenue worth exploring for the development of cancer therapy strategies.

6.3 Sensitivity of ER negative and ER positive cells to paclitaxel
A connection between FoxO activity and ER expression has been previously suggested in the literature (Goto et al., 2007; Schuur et al., 2001; Sui et al., 2007; Sunters et al., 2003; Zhao et al., 2001; Zou et al., 2008). Activation of the ER signalling pathway by estrogen has been shown to increase cell proliferation and contribute to the tumourigenic process, while FoxO activation can result in inhibition of cell proliferation and induction of apoptosis. As one study (Schuur et al., 2001) suggested that ER interacts with FoxO and represses FoxO-mediated transactivation of target genes, and thereby ER is inhibiting cell cycle arrest. Others studies suggest that FoxO
and ER interact, but that this interaction leads to inhibition of ER induced transactivation of target genes (Zhao et al., 2001; Zou et al., 2008). Further discrepancies can be found in the literature regarding the sensitivity of ER negative and ER positive cells to treatment with paclitaxel. Whilst some reports suggest that ER negative cells are more sensitive to paclitaxel (Razandi et al., 2000; Sui et al., 2007), other reports suggest the opposite (Sunters et al., 2003; Zou et al., 2008). Sunters and colleagues found that ER negative breast cancer cells (MDA-MB-231) were less sensitive to paclitaxel and did not undergo apoptosis following treatment with paclitaxel, while the ER positive breast cancer cells (MCF-7) were sensitive to paclitaxel and the apoptotic programme was initiated in the cells following treatment with paclitaxel (Sunters et al., 2003). My data shows that FoxO3a expression is up-regulated, following paclitaxel treatment, in two ER positive breast cancer cell lines (MCF-7 and BT-474). This up-regulation resulted in nuclear localisation of FoxO3a in MCF-7 cells and for both cell lines, an increase in the expression of a FoxO3a downstream target (p27) was observed. Taken together, some of the data presented in the literature suggest that ER positive breast cancer cells may be less sensitive to paclitaxel-induced cell death and that ER expression may hinder FoxO induced transcription of target genes. My results and others’ (Zou et al., 2008) show that treatment of ER positive breast cancer cells with paclitaxel causes up-regulation in FoxO expression and nuclear translocation, signifying that treatment of ER positive breast cancer with paclitaxel may be beneficial. This could be dependent on whether the cells express FoxO at high and inducible levels (Sunters et al., 2003) and may also be affected by the cross-talk with other cellular signaling pathways. Elevated levels of HER2 and EGFR expression have been linked to resistance to treatment with SERMs (Sengupta and Jordan, 2008). Furthermore, clinical data suggest that treatment of breast cancer patients with trastuzumab (inhibits the HER2 signaling pathway) in combination with paclitaxel is more efficacious than treatment with paclitaxel alone (Nicolini et al., 2006; Smith and Chua, 2006). Therefore, the levels of activity of other
signaling pathways, such as HER2, may affect the activity of the ER signaling pathway and lead to phenotypes, such as reduced sensitivity to paclitaxel.

6.4 Cross-talk between JNK1/2 signalling cascade and the PI3K/Akt signalling pathway

Experiments conducted in chapter 5 demonstrated that treatment of MCF-7 cells with paclitaxel led to an increase in nuclear FoxO3a and that this increase correlated with a paclitaxel induced reduction in active Akt. This suggested that paclitaxel-induced inhibition of Akt leads to a decrease in the levels of phosphorylated FoxO3a, which results in the nuclear localisation of FoxO3a. Furthermore, blocking the JNK signalling pathway in MCF-7 cells treated with paclitaxel prevented nuclear accumulation of FoxO3a, which is essential for FoxO-induced transcriptional activation of target genes (Sunters et al., 2006). The use of specific inhibitors of the PI3K/Akt pathway demonstrated that the effect of JNK on FoxO3a nuclear localisation, in response to paclitaxel, was dependent on PI3K/Akt signalling pathway. In fact, it has previously been shown that over-activation of Akt or expression of a constitutively active form of Akt protects cells from the cytotoxic effects of paclitaxel (MacKeigan et al., 2002; Page et al., 2000; Sunters et al., 2006; VanderWeele et al., 2004).

The observation that following paclitaxel treatment Akt is inactivated and JNK1/2 is activated imply that these two signal transduction pathways cross-talk. This notion is further strengthened by the observation that activation of JNK in an inducible MEKK3-expressing cell line resulted in reduced activity of Akt and that MEF cells derived from JNK null mice had elevated levels of active Akt (Sunters et al., 2006). Hence, these data suggest that stress-induced JNK1/2 activity regulates FoxO3a by inhibiting Akt which results in the nuclear accumulation of FoxO3a.

Further work conducted in chapter 5 (and data obtained from ongoing research in Prof. Lam’s lab) demonstrated that FoxO3a and JNK interact and that JNK can phosphorylate FoxO3a. This phosphorylation was shown to
increase FoxO3a’s stability, while preventing JNK-mediated phosphorylation was shown to cause a decrease in the stability of FoxO3a. It has previously been shown that phosphorylation of the p53 transcription factor reduces the affinity of p53 to the E3-ligase MDM2, resulting in an increase in the stability of p53 (Kubbutat et al., 1997). Taken together, it possible that phosphorylation of FoxO3a by JNK increases the stability of FoxO3a by preventing the binding of ubiquitin ligase to FoxO3a. JNK has been found to phosphorylate FoxO4 following exposure to ROS (Essers et al., 2004). Hence, it is possible that a link exists between the results obtained in chapter 3, which demonstrated that treatment with paclitaxel and H$_2$O$_2$ increased FoxO3a stability, to the results attained in chapter 5, which showed that phosphorylation of FoxO3a by JNK also rendered the same effect. Taken together, this suggests that oxidative stress may cause JNK to phosphorylate FoxO3a which results in an increase in the stability of FoxO3a.

This is not the first time that JNK signalling was shown to affect the activity of members of the FoxO family of transcription factors and affect lifespan. Previous research conducted in *C. elegans*, *Drosophila* and mammalian cells have shown that JNK regulates FoxO activity. In *C. elegans* DAF-16, the orthologue of mammalian FoxO, was found to be phosphorylated by JNK-1 following heat shock, leading to the nuclear localisation of DAF-16 (Oh et al., 2005). Furthermore, it has been suggested that JNK-1 induces DAF-16 nuclear accumulation leading to the transcription of the target gene sod-3 (Wolf et al., 2007). In *Drosophila* it was shown that JNK can extend the lifespan of *Drosophila* and that this was dependent on *Drosophila* FoxO, which was shown to translocate to the nucleus following JNK activation and induce transcription of target genes (Wang et al., 2005). In mammalian cells, it has been shown, in response to oxidative stress JNK becomes activated and phosphorylates FoxO4 at two residues, threonine 447 and threonine 451. This leads to the nuclear accumulation of FoxO4 and induction of the transcription of the downstream target MnSOD (Essers et al., 2004).
Therefore, another aspect of the work related to the regulation of FoxO3a by JNK focused on the function of FoxO3a following phosphorylation by JNK, however results obtained are insufficient to determine the effect of JNK phosphorylation on the transcription of FoxO target genes.

The mechanism by which FoxO factors regulate diverse cellular fates such as cell death versus longevity is not entirely clear, but has been suggested to be related to environmental stimuli and other types of post-translational modification of FoxO factors, such as acetylation and de-acetylation (by SIRT1) (Brunet et al., 2004; Motta et al., 2004). Taken together, these data imply that FoxO transcription factors are able to specifically control the expression of downstream targets according to cellular conditions. This is achieved by differential post-translational modification of FoxO proteins, which enables FoxO transcription factors to participate in such varied and opposing processes, like longevity and apoptosis. One possibility is that under oxidative stress conditions, in the presence of growth factors, FoxO proteins are phosphorylated by JNK and translocate into the nucleus. In the nucleus, acetylated-JNK-phosphorylated-FoxO proteins induce cell death and apoptosis. However, if FoxO proteins are deacetylated by SIRT1 in the nucleus, a shift towards up-regulation of FoxO target genes involved in cell cycle arrest and detoxification of ROS occurs.

6.5 The role of BTG1
FoxO transcription factors have been implicated in differentiation (Bakker et al., 2004; Birkenkamp et al., 2007; Hribal et al., 2003; Nakae et al., 2003). BTG1 was shown to be a downstream target of FoxO3a in erythroid progenitor cells; and expression of BTG1 appears to potentiate erythroid differentiation (Bakker et al., 2004). Recently, it has also been shown that FoxO3a induces differentiation of BCR-ABL transformed cells by down-regulating the transcription of Id1 (inhibitor of DNA binding 1) (Birkenkamp et al., 2007). However, some reports have demonstrated that in adipocytes and
myoblasts expression of FoxO1 inhibits differentiation *in vitro* (Hribal et al., 2003; Nakae et al., 2003).

Research conducted in chapter 4 revealed that BTG1 was a downstream target of FoxO3a in breast epithelial cells. Although, I was unable to determine to the precise function of BTG1 in MCF-7 cells, I was able to demonstrate that over-expression of BTG1 resulted in a decrease in cell growth and accumulation of cells at the G2/M phase of the cell cycle. Furthermore, over-expression of BTG1 resulted in an increase in the expression of \( p21^{\text{Cip1}} \) and \( p27^{\text{Kip1}} \) at the protein, mRNA and promoter levels, while a decrease in the levels of cyclin D1 at the protein, mRNA and gene-promoter levels was observed. A decrease in cyclin E1 expression was also noted at the protein and mRNA levels. PRMT1 and p/CAF are cofactors involved in ER-dependent signalling in breast cells and it was found that interaction of BTG1 with p/CAF can have either a negative or positive effect on the regulation of the function ER (Prevot et al., 2001). Hence, this observation further strengthens the possibility that BTG1 may be involved in both positive and negative regulation of transcription. Furthermore, it was shown that the rat homologue of BTG2 inhibits the expression of cyclin D1, which induces ER transactivation (Prevot et al., 2001). Therefore, it is possible that BTG1 may be a key factor in treatment of ER positive breast cancer patients. Moreover, results presented in chapter 4 imply that induction of BTG1 expression in breast cancer cells can lead to cell cycle arrest. Over-expression of BTG1 was not shown to induce apoptosis; however, a slight increase in BTG1 expression was observed in MCF-7 cells following paclitaxel treatment. Therefore, it is possible that BTG1 may play a role in the induction of cell death following paclitaxel treatment in MCF-7 cells and that due to the fact that I was using an inducible gene expression system; I was unable to detect an increase in cell apoptosis. The inducible system enables up-regulation of only *BTG1* mRNA and therefore if BTG1 requires the presence of other proteins or binding partners, such as transcription factors, to exert a pro-apoptotic effect, the abundance of these co-factors will...
determine the ability of BTG1 to induce cell death. Nonetheless, the results obtained in chapter 4 emphasise the role of FoxO3a in breast cancer as BTG1, a downstream target of FoxO3a in MCF-7 cells, is involved in the regulation of the cell cycle in breast cancer cells. Previously it has been shown that FoxO transcription factors require co-factors, in order to bind to the promoters of target genes and regulate their transcription specific target genes. In gastric cancer cells FoxO3a was shown to interact with the RUNX3 transcription factor on the \textit{Bim} promoter, leading to an induction in the expression of Bim (Yamamura et al., 2006). Smad and FoxO were found to interact following treatment with TGF-β (transforming growth factor β) resulting in an increase in the expression of the cyclin-dependent kinase inhibitor p21\textsuperscript{Cip1} (Gomis et al., 2006; Seoane et al., 2004). It is possible that FoxO3a up-regulation of \textit{BTG1} expression requires the presence of other transcriptional co-factors. Determining which transcription factors have DNA consensus binding sites adjacent to that of FoxO's on the \textit{BTG1} promoter, may help reveal possible candidates for co-regulation of \textit{BTG1} expression. One such candidate may be c-Jun, a target of the JNK signalling pathway, as its DNA consensus binding site is adjacent to that of FoxO’s. Mutating this DNA consensus binding site (or those of other candidates) and examining the ability of transfected FoxO3a to up-regulate \textit{BTG1} expression, and/or performing ChIP (chromatin immunoprecipitation), may help determine if indeed FoxO3a requires the presence and binding of other transcriptional factors in order to induce \textit{BTG1} expression in breast cancer cells.

6.6 FoxO transcription factors as tumour suppressors
The hypothesis that FoxO transcription factors may act as tumour suppressors is mainly based on their role in induction of cell cycle arrest and apoptosis (Essafi et al., 2005; Fernandez de Mattos et al., 2004; Hu et al., 2004; Liu et al., 2005a; Park et al., 2005; Seoane, 2004; Sunters et al., 2006). However, since the formal definition of a tumour suppressor gene, is a gene which exhibits a loss of function mutation in cancer (Christophorou et al.,
research has also focused on the implications of mutating FoxO genes and their regulators on the development of cancer. In human breast cancer, it has been suggested that the localisation of FoxO predominantly in that cytoplasm (inactive FoxO) is associated with a poor prognosis for patients (Hu et al., 2004). Recently, FoxO factors in mice were found to have what appears as tissue specific tumour suppressors activities (Paik et al., 2007). The C. elegans defective in germ line development (gld-1) gene is considered to be a tumour suppressor (Francis et al., 1995). Mutating both the gld-1 gene and the DAF-2 gene has been shown to increase the lifespan of C. elegans by a mechanism that appears to be dependent on DAF-16 and p53 activity (Pinkston et al., 2006). Extensive screening using C. elegans mutated in both the gld-1 gene and the DAF-2 gene identified 29 DAF-16 target genes which were shown to have an affect on either tumour cell proliferation or apoptosis, some of which are orthologues of human oncogene and tumour suppressor genes. Of the 29 genes identified, half have also been shown to play a role in normal aging which implies that there is a link between the aging process and tumourigenesis (Pinkston-Gosse and Kenyon, 2007). Taken together, it appears that FoxO transcription factors act as tumour suppressors and that this feature is evolutionary conserved. However, these results also imply that this activity of FoxO may be tissue specific.

Another link between FoxO activity and cancer development, is based on the fact the FoxO proteins are downstream targets of the PI3K/PTEN/AKT signalling pathway, which has been shown to be highly mutated in many different types of cancer (Bachman et al., 2004; Broderick et al., 2004; Samuels et al., 2005; Samuels et al., 2004; Altomare and Testa, 2005). FoxO activity was found to oppose the effect of mutations in this signalling pathway (Kau et al., 2003; Trotman et al., 2006; Samuels et al., 2005).
The similarities between FoxO activity and the activity of p53, also suggest that FoxO may be a tumour repressor. The activity of both these proteins is regulated by environmental stimuli. FoxO proteins have been shown to be activated following exposure to oxidative stress leading to the transcription of target genes such as MnSOD and catalase (Kops et al., 2002a; Nemoto and Finkel, 2002) which play an important role in detoxification of oxidative stress; while p53 has been shown to become activated following genotoxic stress, leading to the transcription of GADD45 (Kastan et al., 1992). In addition both these transcription factors upregulate the transcription of genes involved in the regulation of the cell cycle and even share the same target gene. FoxO3a has been shown to induce the transcription of p27\(^{kip1}\) (Medema et al., 2000) and p21\(^{cip}\) (Seoane, 2004), while p53 has been shown to upregulate the transcription of p21 (el-Deiry et al., 1993). Furthermore, both FoxO and p53 can induce apoptosis. FoxO3a has been found to increase the transcription of the pro-apoptotic genes Bim (Dijkers et al., 2000a) and FasL (Brunet et al., 1999) and p53 has been shown to upregulate the transcription of PUMA (Nakano and Vousden, 2001).

Furthermore, both FoxO and p53 are regulated post-translationally, and in some cases by the same enzymes, SIRT1, JNK and USP7 have been shown to deacetylate, phosphorylate and deubiquitylate FoxO and p53, respectively (Adler et al., 1997; Essers et al., 2004; Brunet et al., 2004; Luo et al., 2001; Motta et al., 2004; van der Horst et al., 2004; Vaziri et al., 2001; Li et al., 2002; van der Horst et al., 2006). Deacetylation of FoxO3a by SIRT1 has been shown to affect the activity of FoxO; however, there is no clear agreement as to how this influences FoxO activity. Nonetheless, it has been suggested that deacetylation of FoxO by SIRT1 causes FoxO to selectively transcribe genes involved in increasing lifespan (Greer and Brunet, 2005). Deacetylation of p53 by SIRT1 results in the inhibition of the activity of p53 (Luo et al., 2001). JNK has a positive effect on both FoxO and p53 activity. JNK has been shown to phosphorylate and activate FoxO4 and induce nuclear localisation of FoxO3a (Essers et al., 2004; Sunters et al.,
The phosphorylation of p53 by JNK has been shown to increase the stability and activity of p53 (Buschmann et al., 2001). Deubiquitylation of FoxO and p53 by USP7 does not affect these proteins in the same way. USP7 deubiquitylation of FoxO results in the inhibition of FoxO activity, while USP7 deubiquitylation of p53 lead to an increase in the stability and thereby activity of p53 (Li et al., 2002; van der Horst et al., 2006). Overall, FoxO and p53 have been show to be regulated post-translationally by similar mechanisms, though not always leading to the same effect on their activity. In addition, FoxO and p53 have been found to be able to regulate the expression of target genes that participate in the same or similar cellular processes and even share some of the same transcriptional downstream targets. All this, is suggesting that FoxO, like p53, may also be a tumour repressor.

### 6.7 Targeting FoxO in cancer therapy

The necessity to increase the efficiency of cancer therapy is a longstanding goal. Therefore identifying cellular targets which may serve for chemotherapy is of utmost importance. As discussed above, FoxO transcription is up-regulated following paclitaxel treatment. FoxO target genes have been implicated in a broad spectrum of activities, with induction of apoptosis at one extreme of the spectrum and induction of longevity at the other. To date, it has been shown that breast cancer cells that express FoxO3a at high levels are more susceptible to paclitaxel treatment in comparison with breast cancer cells that express FoxO3a at low and non-inducible levels (Sunters et al., 2003). This has been mainly attributed to the up-regulation in the expression of FoxO3a’s pro-apoptotic target gene, Bim. Hence, expression and activation of FoxO3a in cancer cells may lead to the activation of the apoptotic pathway. In addition, it has also recently been shown that FoxO induced apoptosis occurs in tumour cells and not normal cells (Pinkston-Gosse and Kenyon, 2007).

Furthermore, the PI3K/Akt pathway, which regulates vital processes, such as cell survival, proliferation, growth, metabolism and cell migration (see
introduction); has as mentioned above, been found to be deregulated or mutated in many cancers. FoxO factors are downstream effectors of this signalling pathway and although data suggests that there is a decrease in the function of FoxO in some cancers, no FoxO gene promoter methylation or mutations in the genes have been identified in human cancers. Nonetheless, chromosome translocations (Davis et al., 1994; Parry et al., 1994; Shapiro et al., 1993; So and Cleary, 2003) and deletions (Dong et al., 2006) have been identified. Therefore, taken together, these data imply that FoxOs may prove to be an attractive target for cancer therapy.

However, targeting FoxO genes for cancer therapy may prove to be a complicated task as FoxO genes play vital roles at both the organismal and the cellular levels. FoxO1-null mice were found to be embryonic lethal (Hosaka et al., 2004), and conditional knock-out of three isoforms of FoxO in mice (FoxO1, Foxo3a, FoxO4) has also been shown to lead to the development of certain malignancies (Paik et al., 2007). Furthermore, these proteins also play a vital role in up-regulating the expression of genes that control glucose metabolism; hence, their expression is essential in normal non-cancerous cells (Nakae et al., 2001; Puigserver et al., 2003; Schmoll et al., 2000; Yeagley et al., 2001). In addition, FoxO factors exhibit functional redundancy, as they are able to bind the same target DNA sequence, regulate the transcription of the same target genes and knock-out of all three isoforms is required in order for some forms of tumours to develop (Castrillon et al., 2003; Hosaka et al., 2004; Paik et al., 2007). Therefore, targeting FoxO genes for cancer therapy must be done in a manner that would not hinder FoxO expression in cells but would modulate their activity in cancer cells.

In this thesis, I was able to show that JNK and PI3K/Akt pathway cross-talk. This was found to affect the sub-cellular distribution of FoxO3a. Furthermore, it was also appears that JNK and FoxO interact and that JNK can phosphorylate FoxO (Essers et al., 2004). Under stress conditions FoxO does not translocate to from the nucleus to the cytoplasm, regardless of
growth factors in the media (Brunet et al., 2004). This could be due to the activation of JNK (Brunet et al., 2004; Essers et al., 2004; Huang et al., 2006; Lehtinen et al., 2006; Sunayama et al., 2005). Therefore, it is plausible that exposure to oxidative stress generating agents leads to the activation of JNK, which in turn phosphorylates FoxO, thereby preventing FoxO from translocating from the nucleus into the cytosol (Essers et al., 2004). Hence, activation of JNK may be therapeutically desirable, as co-administration of agents that activate JNK may synergise with paclitaxel to promote tumour cell death. Though, since FoxOs can upregulate, following exposure to oxidative stress, the expression of a broad range of genes, some of which are involved in inducing cell cycle arrest (De Ruiter et al., 2001; Medema et al., 2000; Nakamura et al., 2000; Seoane, 2004; Stahl et al., 2002; Tanaka et al., 2001) and apoptosis (Brunet et al., 1999; Modur et al., 2002), and some of which are involved in detoxification of ROS (Balaban et al., 2005; Kops et al., 2002a; Nemoto and Finkel, 2002) and DNA repair (Furukawa-Hibi et al., 2002; Tran et al., 2002); inducing FoxO activity in cancer cells would only be beneficial if it was possible to selectively up-regulate the expression of target genes involved in induction of cell death.

SIRT1 was found to be able to deacetylate FoxO transcription factors (Brunet et al., 2004; Daitoku et al., 2004; Fukuoka et al., 2003; Kobayashi et al., 2005; Mahmud et al., 2002; Motta et al., 2004; Perrot and Rechler, 2005; van der Horst et al., 2004; Yang et al., 2005b). Although the role of acetylation and SIRT1 in FoxO post-translational regulation is not fully understood, it appears that SIRT1 expression alters FoxO activity (Brunet et al., 2004; Furukawa-Hibi et al., 2002; Motta et al., 2004; Tran et al., 2002). The presence of SIRT1 was found to increase FoxO3a-induced cell cycle arrest, while FoxO3a-induced expression of apoptotic genes such as Bim was further upregulated following inhibition of SIRT1 activity (Brunet et al., 2004). Thus, it appears that SIRT1 may shift FoxO function from cell death to survival. Therefore, it is logical that the best way to target FoxO in cancer therapy is by inducing FoxO activity in cancer cells, for instance by activating
JNK, and co-administration of a drug, such as Sirtinol (Ota et al., 2006), which would prevent the activity of SIRT1 in the cells, thereby restricting FoxO function to the up-regulation of genes involved in induction of apoptosis, such as Bim. Research into or understanding of the role and regulation of FoxO transcription factors in normal and cancer cells might help to open up new avenues in cancer therapy. We are currently only at the beginning of the process of learning how these transcription factors work and how to exploit this knowledge in cancer prevention and treatment.
Briefly (Regulation of FoxO), paclitaxel treatment was shown to induce oxidative stress in MCF-7 cells. This treatment results in an increase in FoxO transcription and protein expression in BT4-74 cells (FoxO3a) and HEC-1-B cells (FoxO1). In MCF-7 cells this treatment resulted in an increase in FoxO3a promoter activity and protein expression, which appeared to result from an increase in protein stability. JNK is activated following treatment with paclitaxel and phosphorylates FoxO3a, this phosphorylation may account for the increase observed in FoxO3a stability. The JNK and PI3K/Akt pathway cross-talk Inactivation of the PI3K/Akt pathway leads to FoxO3a nuclear localisation. Speculative, combined with inhibition of SIRT1 activity this will lead to an increase in transcription of target genes involved in promoting cell death. (Function of FoxO3a), BTG-1 is a downstream target of FoxO3a. Up-regulation of BTG1 leads to a decrease in cyclin D1 and cyclin E1 expression and an increase in p21 and p27 expression. In addition, cells over-expressing BTG1 were shown to accumulate at the G2/M phase of the cell cycle. * only shown at protein and mRNA levels, rectangular ended arrow, only shown in MCF-7 cells.
Figure 7-1: Dose-dependent effect of 16 h paclitaxel treatment of MCF-7 cells. MCF-7 cells were treated with 0-200nM paclitaxel for 16 h. Following treatment cell lysates were prepared, separated on SDS-polyacrylamide gels and immunoblotted with specific antibodies. The expression levels of FoxO3a were analysed using a total FoxO3a antibody. The activity of FoxO3a upstream regulator, Akt and the levels and activity of stress induced signalling pathways, JNK and p38, were analysed using specific phospho (indicated by a P in-front of the protein name) and total antibodies. β-actin was used as a loading control.
Figure 7-2: The effect of paclitaxel treatment on the expression of FoxO, p27Kip1 and the activity of MAPK signalling in MCF-7 cells.

Whole cell lysates were prepared at indicated time-points following treatment with 10nM paclitaxel, and then separated on SDS-polyacrylamide gels and immunoblotted. The levels of total FoxO3a, Akt-phosphorylated (Thr32) FoxO3a, Akt, phospho-Akt (Ser473), p27Kip1, and the levels and activity of the signalling pathways, JNK, p38 and ERK were analysed, using specific phospho (indicated by a P in-front of the protein name) and total antibodies. β-actin was used as a loading control.
Figure 7-3 The effect of paclitaxel treatment on the expression of FoxO, p27Kip1 and the activity of JNK and p38 in BT-474 cells.
Whole cell lysates were prepared at indicated time-points following treatment with 10nM paclitaxel, and then separated on SDS-polyacrylamide gels and immunoblotted. The levels of total FoxO3a, Akt-phosphorylated (Thr32) FoxO3a, Akt, phospho-Akt (Ser473), p27Kip and the levels and activity of stress induced signalling pathways, JNK and p38 were analysed by western blot using specific phospho (indicated by a P in-front of the protein name) and total antibodies. β-actin was used as a loading control.
Figure 7-4: Differential expression of FoxO1 in HEC-1-B and Ishikawa cells following paclitaxel treatment.

Whole cell lysates were prepared at indicated time-points following treatment with 10nM paclitaxel, and then separated on SDS-polyacrylamide gels and immunoblotted. The expression of total FoxO1 was analysed using a specific total FoxO1 antibody. β-actin was used as a loading control.
Figure 7-5: Oxidative stress generated by H$_2$O$_2$ causes an increase in FoxO3a expression in MCF-7 cells.

Whole cell lysates were prepared at indicated time-points following treatment with 200µM H$_2$O$_2$, and then separated on SDS-polyacrylamide gels and immunoblotted. The levels of total FoxO3a, Akt- phosphorylated (Thr32) FoxO3a, Akt, phosho-Akt (Ser473), p27^Kip1 and the levels and activity of the signalling pathways, JNK, p38 and ERK were analysed by western blot using specific phospho (indicated by al P in-front of the protein name) and total antibodies. β-actin was used as a loading control. The arrow indicates the location on the FoxO3a-specific band.
Figure 7-6: Paclitaxel treatment increases the stability of FoxO3a in MCF-7 cells. Paclitaxel treatment increases the stability of FoxO3a in MCF-7 cells.
Cells were either pre-treated with 10nM paclitaxel for 16 h (left panel), or not pre-treated (right panel). After which cells were treated with 10\(\mu\)g/ml cycloheximide for up to 8 h. whole cell lysates were prepared at indicated time-points, then separated on SDS-polyacrylamide gels and immunoblotted. The levels of FoxO3a and Id1, a protein with a short half-life were analysed by western blot. \(\beta\)-actin, a protein with a longer half life, was used as a loading control.

![Western Blot](image)

Figure 7-7: Oxidative stress increases the stability of FoxO3a in MCF-7 cells. Oxidative stress increases the stability of FoxO3a in MCF-7 cells.
Cells were either pre-treated with 200\(\mu\)M \(H_2O_2\) (left panel) or not pre-treated (right panel) for 16 h. Following which, cells were treated with 10\(\mu\)g/ml cycloheximide for up to 8 h. whole cell lysates were prepared at indicated time-points, and then separated on SDS-polyacrylamide gels and immunoblotted, the levels of FoxO3a and \(\beta\)-actin were analysed.

![Western Blot](image)
Figure 7-8: BTG1 expression following paclitaxel treatment in MCF-7 cells.
MCF-7 cells were treated with 10nM paclitaxel for indicated times. Whole cell lysates were prepared at indicated times and then separated on SDS-polyacrylamide gels and immunoblotted. The levels of total FoxO3a and BTG1 were analysed by western blot using specific total antibodies. β-actin was used as a loading control. The arrow indicates the location of the FoxO3a specific band.

Figure 7-9: BTG1 expression in synchronised MCF-7 cells.
MCF-7 cells were synchronised at G0 by decreasing the concentration of foetal calf serum (FCS) from 10% to 0.5% in the growth medium for 36 h; after which growth medium was replaced by fresh media containing 10% FCS. Whole cell lysates were prepared, and then separated on SDS-polyacrylamide gels and immunoblotted. The expression levels of total FoxO3a, BTG1 and Plk were analysed by western blot using specific total antibodies. β-tubulin was used as a loading control.
Figure 7-10: The BTG1 MCF-7 Tet Off clone #34 expresses BTG1 in an inducible manner upon withdrawal of doxycycline from the medium. MCF-7 Tet off cells, which were stably transfected with pTRE2Hyg-BTG1, were cultured in the presence of 100μg/ml of G418, 200μg/ml and 1μg/ml doxycycline. At the beginning of the time-course (0 h) all antibiotics were removed from the media. Whole cell lysates were prepared, and then separated on SDS-polyacrylamide gels and immunoblotted. The levels of BTG1 were analysed by western blot using specific total antibodies. β-tubulin was used as a loading control.
Figure 7-11: The effect of BTG1 over-expression on cell cycle regulators.
BTG1 Tet-Off cells were induced to express BTG1, by removal of Dox from the cell growth medium for a period of 24 h. After which cell lysates were prepared, separated on SDS-polyacrylamide gels and immunoblotted with specific antibodies. The levels of BTG1, FoxO3a and cell cycle regulators was analysed using specific phospho and total antibodies. β-tubulin was used as a loading control.
Figure 7-12: Paclitaxel causes FoxO3a to translocate into the nucleus.
Western blotting of cytoplasmic and nuclear extracts isolated from MCF-7 cells that were either treated with 10nM paclitaxel or untreated (control) for 8 h. The levels of total FoxO3a and Akt-phosphorylated FoxO3a (P-FoxO3a) was determined in both cellular components. β-actin was used as a loading control.

Figure 7-13: Inhibition of JNK1/2 by SP600125 prevents FoxO3a nuclear localisation in MCF-7 cells treated with paclitaxel.
Western blotting of cytoplasmic and nuclear extracts isolated from MCF-7 cells which were untreated, treated with paclitaxel (10 µM), SP600125 (20 µM), or SP600125 and paclitaxel for 8 h. Protein levels were analysed using a specific phospho-FoxO3a (P-FoxO3) antibody and specific total antibodies. β-actin was used as a loading control.
Figure 7-14: Inhibition of JNK1/2 by SP600125 fails to prevent FoxO3a nuclear localisation in MCF-7 cells treated with inhibitors of PI3K and Akt.

Western blotting of cytoplasmic and nuclear extracts isolated from MCF-7 cells treated with inhibitors of PI3K and Akt. Cells were treated with either LY294002 (30 µM), Triciribine (30 µM), or SP600125 (20 µM), or a combination of Triciribine and SP600125 for 8 h. Protein levels were analysed using specific phospho (indicated by a P in-front of the protein name) and total antibodies. β-actin was used as a loading control.
Figure 7-15: JNK1 can phosphorylate FoxO3a on Serine 294. MCF-7 cells were co-transfected with either the combination of pCMV-FoxO3a and pCDNA3-JNKK2-JNK1, or with PCM-V-FoxO3a and the empty vector, pCDNA3. Cell lysates were prepared 36 h after transfection was performed and 20µg were used for western blot and the remaining 60µg of lysate was Immunoprecipitated (IP) with an antibody against Flag. The levels of total JNK and phospho-JNK (Thr183/Tyr185) in transfected cells was determined by western blot analysis of total cell lysate. The precipitated anti-Flag complexes were resolved on SDS-polyacrylamide gels and immunoblotted with a Flag antibody (Immunoprecipitated protein, indicating equal amounts of precipitated protein) and antibodies against phosphorylated FoxO3a (predicated JNK phosphorylation sites).
Figure 7-16: Mutating FoxO3a’s serine 294 and serine 425 to alanines promotes proteasomal degradation of FoxO3a.

MCF-7 cells were transfected with either pCMV-FoxO3a (wt), pCMV-FoxO3aS294A (S294A), pCMV-FoxO3aS425A (S425A) or pCMV-FoxO3aS294A, S425A (S294A, S425A). 24 h post-transfection cells were treated with proteasome inhibitor MG132 (50 µmol/L) for 4 h or not treated (NT). Whole cell lysates were prepared and separated on SDS-polyacrylamide gels and immunoblotted. Flag protein levels were analysed by western blot. β-tubulin was used as a loading control.

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Figure 7-17: Mutating FoxO3a’s serine 294 and serine 425 affects FoxO3a stability.

MCF-7 cells were transfected with either pCMV5-FoxO3a (wt), pCMV5-FoxO3aS294A, S425A (S294A, S425A) or pCMV5-FoxO3aS294D, S425D (S294D, S425D). 24 h post-transfection cells were treated with 10µg/ml cycloheximide for up to 8 h. whole cell lysates were prepared at indicated time-points, and then separated on SDS-polyacrylamide gels and immunoblotted. The levels of Flag were analysed by western blot. β-tubulin was used as a loading control.

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