THE CHARACTERISATION OF ETS1 IN PROSTATE TUMOURS

JAMIE JOHN RODGERS
B.SC. (HONS)

THIS THESIS IS PRESENTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY OF THE UNIVERSITY OF WESTERN AUSTRALIA, AUGUST, 2014

SCHOOL OF PATHOLOGY AND LABORATORY MEDICINE
UNIVERSITY OF WESTERN AUSTRALIA
PERTH, WESTERN AUSTRALIA
Declaration

The work detailed in this thesis was performed by the candidate unless otherwise specified. This thesis is submitted for the degree of Doctor of Philosophy at the University of Western Australia and has not been submitted elsewhere for any other degree.

Candidate

Jamie John Rodgers ..........................................

Supervisors

Dr Marc Thomas .............................................

Dr Jacqueline Bentel ........................................

Winthrop Professor Jennet Harvey .......................

Dated ......................
Acknowledgements

To all who made it possible… Thanks to all the lab members who have shared/contributed to my PhD journey, Jasmine, Alison, Ebony, Abbie, Danika, Agata and Erin, I wish you all the very best in all your future endeavours. To Kimberley Roehrig for early mentorship and insightful advice. To Mike Epis, a top bloke and guru of real time PCR, thanks for your wisdom and hours of assistance, very much appreciated. Thanks also to the body that is UWA and RPH for funding me (a.k.a. food, petrol, phone, gym membership) and my project.

For Vivian, a big thank you for your companionship throughout both of our journeys, for all the memories in and out of the lab, the occult and all things creepy, being a great pair of ears to listen to my ramblings with such patience. Good luck with your PhD and beyond! Thank you and keep in touch!

For Jennet, thankyou for being my foot in the door and rock within the university. For all the times I hustled you last minute with paperwork or presentations and for your time, patience and enthusiasm. It has been invaluable amidst very busy times. Thank you!

For Mum and Dad, for the second time I have written a thesis under the support and love you both have for me. Thank you for always enquiring and looking out for me even if it doesn’t make any sense, that is unconditional love. What can I say about my mum and dad that is more important than that I love you both very much. You have given me everything I need to succeed, thankyou and thankyou in advance for your love and support in the future!

For Marc, thank you Marc for so many things, for constant mentorship regarding my growth as a scientist be it writing, presenting or lab related. Your advice has given me great insight and direction for the task at hand, and your unwavering support gave me strength when times were tough and I didn’t always understand. On a personal note, you’re a top bloke and a real inspiration as a person, scientist, husband and father. I hope to see myself following in your footsteps and if I can, I know I’ve gone in the right direction. Thankyou!

For Jacky (a.k.a ‘the boss’), thank you Jacky for countless hours of professional and personal investment/sacrifice toward my development as a scientist and as a young adult. For always being there to discuss and talk about anything and everything. Bosses aren’t always your friends, so when they are, that’s awesome. Thank you for your empathy and advice on countless occasions, for spearheading my work from day one and giving me both the opportunity and capacity to complete my PhD. I really hope Jacky that one day I can be a boss like you are, for others, for science and for the world. Thankyou!

For my fiancée Seph, it was your push that made me do my PhD (what a bad idea!!! JJKS). You always saw it in me and drove me to do my best. You have truly listened, felt and shared my entire journey and you truly embody what this thesis represents. Although you too have your own pressures, you never stopped caring or supporting me on my journey, a journey we can soon continue for a lifetime. Life never stopped during my PhD, because you are that life and love. I know this book is but another chapter of our lives, but one I am particularly proud of for both of us. I love you with all my heart. Thank you!
Awards

During my PhD I have been the recipient of the Richard Walter Gibbon Medical Research Scholarship (2011-2014)

I was the recipient of the Women and Infants award for my presentation “The Characterisation of ETS1 Overexpression in Human Prostate Tumours” at the Australian Society for Medical Research (ASMR) WA Symposium in June, 2011.

I was the recipient of the Young Investigators Day Basic Science award for my presentation “ETS1 regulates Epithelial-to-Mesenchymal Transition in Prostate Cancer Cells” at the Royal Perth Hospital Medical Research Foundation Young Investigators Day Symposium in November, 2013.
Publications (Conference Abstracts)

2014:
“ETS1 Regulates Epithelial-to-Mesenchymal Transition (EMT) in Prostate Cancer Cells”
AACR, 105th Annual Meeting, San Diego, United States of America

“ETS1 Regulates Epithelial-to-Mesenchymal Transition (EMT) in Prostate Cancer Cells”
Australian Society for Medical Research (ASMR) Medical Research Week Symposium, Perth, Australia

“ETS1 Regulates Epithelial-to-Mesenchymal Transition (EMT) in Prostate Cancer Cells”
Combined Biological Science Meeting Symposium (CBSM), Perth, Australia

2013:
“ETS1 Regulates Epithelial-to-Mesenchymal Transition (EMT) in Prostate Cancer Cells”
6th Barossa Meeting, Cell Signalling Conference, Barossa Valley, Australia

“ETS1 Regulates Epithelial-to-Mesenchymal Transition (EMT) in Prostate Cancer Cells”
**Rodgers JJ**, Harvey JM, Bentel JM & Thomas MA
Royal Perth Hospital, Medical Research Foundation (MRF) Young Investigators Day Scientific Symposium, Perth, Australia

“ETS1 Regulates Epithelial-to-Mesenchymal Transition (EMT) in Prostate Cancer Cells”
**Rodgers JJ**, Harvey JM, Bentel JM & Thomas MA
Royal Perth Hospital, Medical Research Foundation (MRF) Young Investigators Day Scientific Symposium, Perth, Australia

“ETS1 Regulates Epithelial-to-Mesenchymal Transition (EMT) in Prostate Cancer Cells”
**Rodgers JJ**, Epis MR, Roehrig K, Cohen RJ, Leedman PJ, Harvey JM, Bentel JM & Thomas MA
COMBIO Conference, Perth, Australia

“ETS1 Regulates Epithelial-to-Mesenchymal Transition (EMT) in Prostate Cancer Cells”
Combined Biological Science Meeting Symposium (CBSM), Perth, Australia
“ETS1 Regulates Epithelial-to-Mesenchymal Transition (EMT) in Prostate Cancer Cells”
Rodgers JJ, Epis MR, Roehrig K, Cohen RJ, Leedman PJ, Harvey JM, Thomas MA & Bentel JM
Australian Society for Medical Research (ASMR) Medical Research Week Symposium, Perth, Australia

2012:
“The Characterisation of ETS1 Overexpression in Human Prostate Tumours”
Rodgers JJ, Epis MR, Roehrig K, Cohen RJ, Leedman PJ, Harvey JM, Thomas MA & Bentel JM
Royal Perth Hospital, Medical Research Foundation (MRF) Young Investigators Day Scientific Symposium, Perth, Australia

“The Characterisation of ETS1 Overexpression in Human Prostate Tumours”
Rodgers JJ, Epis MR, Roehrig K, Cohen RJ, Leedman PJ, Harvey JM, Bentel JM & Thomas MA
COMBIO Conference, Adelaide, Australia

“Characterisation of ETS1 Overexpression in Human Prostate Cancers”
MA Thomas, JJ Rodgers, CJ Mitchell, DM Preece, Chai SM, Michael Epis, Ronald J Cohen, Peter Leedman, Kimberley Roehrig, JM Harvey & JM Bentel
13th Australasian Prostate Cancer Conference, Melbourne, Australia

“The Characterisation of ETS1 Overexpression in Human Prostate Tumours”
Rodgers JJ, Epis MR, Roehrig K, Cohen RJ, Leedman PJ, Harvey JM, Thomas MA & Bentel JM
Australian Society for Medical Research (ASMR) Medical Research Week Symposium, Perth, Australia

“ETS1 Overexpression in Prostate Cancer Cells”
Bentel JM, Rodgers JJ, Rouse EJ, Preece DM, Chai SM, Epis MR, Cohen RJ, Leedman PJ, Roehrig K, Harvey JM & Thomas MA
AACR Advances in Prostate Cancer Research, Orlando, United States of America

2011:
“The Characterisation of ETS1 Overexpression in Human Prostate Tumours”
Rodgers JJ, Epis MR, Roehrig K, Cohen RJ, Leedman PJ, Harvey JM, Thomas MA & Bentel JM
Royal Perth Hospital, Medical Research Foundation (MRF) Young Investigators Day Scientific Symposium, Perth, Australia
“Characterisation of ETS1 Overexpression in Human Prostate Tumours”
Rodgers JJ, Roehrig K, Epis MR, Cohen RJ, Leedman PJ, Harvey JM, Thomas MA & Bentel JM
Combined Biological Science Meeting Symposium (CBSM), Perth, Australia

“Characterisation of ETS1 Overexpression in Human Prostate Tumours”
Rodgers JJ, Roehrig K, Epis MR, Cohen RJ, Leedman PJ, Harvey JM, Thomas MA & Bentel JM
Australian Society for Medical Research (ASMR) Medical Research Week Symposium, Perth, Australia
List of Figures

Figure 1.1: Major components of the male urogenital complex including the prostate gland. 1
Figure 1.2: Gleason grading of prostatic adenocarcinomas. 3
Figure 1.3: Molecular pathway of prostate cancer initiation and progression. 7
Figure 1.4: Mechanisms of AR deregulation. 10
Figure 1.5(A): Phylogenetic tree of the ETS factor family. 14
Figure 1.5(B): The ETS factor family members. 15
Figure 1.6: ETS factor family protein domain structure. 16
Figure 1.7: ETS factor binding sub-classes. 18
Figure 1.8: ETS1 gene and mRNA isoforms. 23
Figure 1.9: Structure of ETS1 protein isoforms. 24
Figure 1.10: ETS1 expression. 26
Figure 1.11: Proposed functions of ETS1 in mammals. 30
Figure 1.12: Epithelial-to-mesenchymal transition. 33
Figure 1.13: TGF-β induced EMT. 36
Figure 4.1: ETS1 gene and mRNA isoform structure. 80
Figure 4.2: Optimisation of GAPDH qPCR. 81
Figure 4.3: Optimisation of ETS1p51 qPCR. 83
Figure 4.4: Optimisation of ETS1p42 qPCR. 85
Figure 4.5: Optimisation of ETS1p27 qPCR. 87
Figure 4.6: ETS1p51 expression in nonmalignant prostate and prostate tumour specimens. 91
Figure 4.7: ETS1p42 expression in nonmalignant prostate and prostate tumour specimens. 92
Figure 4.8: Agarose gel electrophoresis of ETS1p27 RT-qPCR products. 93
Figure 4.9: GAPDH expression in nonmalignant prostate and prostate tumour specimens. 94
Figure 4.10: ETS1-isoform copy number (log cDNA number) in nonmalignant prostate and prostate tumour specimens. 95
Figure 4.11: Normalised ETS1 isoform mRNA expression. 96
Figure 4.12: Relative ETS1 mRNA isoform expression in prostate cancer and adjacent nonmalignant prostate tissues. 97
Figure 4.13: ETS1 isoform protein expression in nonmalignant prostate and prostate tumour specimens. 100

Figure 4.14: Correlation of relative ETS1p51 mRNA and protein expression in prostate tumour and nonmalignant prostate specimens. 102

Figure 5.1: Development of an LNCaP cell model with doxycycline inducible ETS1p51 expression. 117

Figure 5.2: Ectopic expression of ETS1p51 in LNCaP cells. 120

Figure 5.3: AR and ETS1p51 mRNA expression in LNCaP cells. 122

Figure 5.4: Regulation of AR, ETS1 and NKX3.1 by DHT in LNCaP cells. 124

Figure 5.5: Gene regulation following GFP-ETS1p51 overexpression in LNCaP cells. 125

Figure 5.6: Gene regulation following DHT treatment of LNCaP cells. 126

Figure 5.7: Gene regulation following DHT treatment of GFP-ETS1p51 overexpressing LNCaP cells. 127

Figure 5.8: EMT-associated gene expression following GFP-ETS1p51 overexpression in LNCaP cells. 128

Figure 5.9: EMT-associated gene expression following DHT treatment of LNCaP cells. 129

Figure 5.10: EMT-associated gene expression following GFP-ETS1p51 overexpression and DHT treatment of LNCaP cells. 130

Figure 5.11: Clustergram analysis of gene expression following GFP-ETS1p51 overexpression and treatment with DHT. 132

Figure 5.12: Clustergram analysis of gene expression following GFP-ETS1p51 overexpression in LNCaP cells. 133

Figure 5.13: Clustergram analysis of gene expression following DHT treatment of LNCaP cells. 134

Figure 5.14: Clustergram analysis of gene expression following DHT treatment of GFP-ETS1p51 overexpressing LNCaP cells. 135

Figure 5.15: Co-regulation of genes by GFP-ETS1p51, DHT treatment or DHT treatment of GFP-ETS1p51 overexpressing LNCaP cells. 137

Figure 5.16: Pathways in cancer. 139

Figure 5.17: The TGF-β signalling pathway. 141

Figure 5.18: EMT-associated TGF-β and WNT signalling pathways. 143
Figure 5.19: Proposed interactions between ETS1 and the EMT pathway in prostate cancer cells. 144

Figure 6.1: PCR amplification of EMT-associated genes. 161

Figure 6.2: Annealing temperature optimisation of PCRs for EMT-associated genes. 162

Figure 6.3: Generation of qPCR efficiency curves. 163

Figure 6.4: Regulation of expression of EMT-associated genes by ETS1p51. 167

Figure 6.5: Regulation of EMT markers by ETS1p51. 169

Figure 6.6: Regulation of SMAD2 phosphorylation following GFP-ETS1p51 overexpression. 172

Figure 6.7: Regulation of pSMAD2 protein expression using SB431542 or TGFβ1. 174

Figure 6.8: ETS1p51- and TGF-β/SMAD-mediated LNCaP cell migration (healing assay). 176

Figure 6.9: ETS1p51- and TGF-β/SMAD-mediated LNCaP cell invasion (transwell assays). 179

Figure A3.1: pCMV-Tet3G plasmid 260

Figure A3.2: pcDNA3.1 261

Figure A3.3: pcDNA3.1/V5-His-TOPO 262

Figure A3.4: pEGFP-C2 263

Figure A3.5: pGEM-T Easy 264

Figure A3.6: pTRE3G-IRES 265

Figure A3.7: pTRE3G-Luc 266

Figure A5.1: Relative ETS1p51 protein levels between matched samples (1 - 15) 277

Figure A5.2: Relative ETS1p51 protein levels between matched samples (16 - 30) 278

Figure A5.3: Relative ETS1p51 protein levels between matched samples (31 - 45) 279

Figure A8.1: RT-qPCR amplification and melt curves 297
<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 3.1</td>
<td>PCR Conditions.</td>
<td>65</td>
</tr>
<tr>
<td>Table 3.2</td>
<td>Immunoblotting Conditions.</td>
<td>71</td>
</tr>
<tr>
<td>Table A2.1</td>
<td>Gene Expression Primers</td>
<td>257</td>
</tr>
<tr>
<td>Table A2.2</td>
<td>ETS1p51 Cloning Primers</td>
<td>258</td>
</tr>
<tr>
<td>Table A4.1</td>
<td>Prostate specimens demographics (and mRNA expression)</td>
<td>268</td>
</tr>
<tr>
<td>Table A4.2</td>
<td>Prostate specimens demographics (and protein expression)</td>
<td>272</td>
</tr>
<tr>
<td>Table A6.1</td>
<td>RT² Profiler PCR Array Human Epithelial-To-Mesenchymal Transition (EMT) (PAHS-090A)</td>
<td>281</td>
</tr>
<tr>
<td>Table A7.1</td>
<td>Array 1</td>
<td>285</td>
</tr>
<tr>
<td>Table A7.2</td>
<td>Array 2</td>
<td>291</td>
</tr>
</tbody>
</table>
**Abbreviations**

°C – Degrees celsius
ACK1 – Activated CDC42 kinase 1
ACVR2B – Activin receptor type 2B
AFS – Anterior fibromuscular stroma
AIR – Alternatively included region
AKT – Protein kinase B
AML-1 – Acute myeloid leukaemia 1
AP1 – Activator protein 1
APC – Adenomatous polyposis coli
APS – Ammonium persulphate
AR – Androgen receptor
ARE – Androgen response element
ATCC – American Type Culture Collection
ATP – Adenosine 5’ Triphosphate
β-catenin – Catenin cadherin associated protein beta 1
BAX – B-cell lymphoma-2-like protein 4
BCL-X – B-cell lymphoma-extra large
BLAST™ – Basic Local Alignment Search Tool
BMP – Bone morphogenetic protein
bp – Base pair
BPH – Benign prostatic hyperplasia
BRCA1 – Breast cancer 1, early onset
BRCA2 – Breast cancer 2, early onset
BSA – Bovine serum albumin
c-FOS – FBJ murine osteosarcoma viral oncogene homologue
CaCl₂ – Calcium chloride
CAPZB – F-actin-capping protein subunit beta
CAV2 – Caveolin 2
CBP – CREB-binding protein
CCN2 – Connective tissue growth factor
CCNG2 – Cyclin-G2
CD9 – CD9 antigen
CDH1 – E-cadherin
CDH2 – N-cadherin
CDK1 – Cyclin-dependent kinase 1
CDKN1A – Cyclin-dependent kinase inhibitor 1
CHEK2 – CHK2 checkpoint homologue
ChIP – Chromatin immunoprecipitation
CO₂ – Carbon dioxide
COL3A1 – Collagen type 3
COL5A2 – Collagen type 5
CREB – cAMP response element binding protein
CRPC – Castrate resistant prostate cancer
CSS – Charcoal stripped serum
CZ – Central zone
DAB2IP – Disabled homologue 2-interacting protein
DAVID – Database for annotation, visualisation and integrated discovery
DCS – Downstream constitutive sequence
DDX5 – DEAD box protein 5
DEPC - Diethylpyrocarbonate
DHT – 5α-dihydrotestosterone
DMSO – Dimethylsulphoxide
DNA – Deoxyribonucleic acid
dNTP - Deoxyribonucleotide
DRE – Digital rectal examination
DSC2 – Desmocollin-2
DSH – Dishevelled
DSP – Desmoplakin
DTT - Dithiothreitol
EBS – ETS binding site/s
ECL – Electrochemiluminescence
EDTA – Ethylenediaminetetraacetic acid
EGF – Epidermal growth factor
EGFR – Epidermal growth factor receptor
ELF4 – ETS-related transcription factor Elf-4
ELK – ETS like gene
EMT – Epithelial-to-mesenchymal transition
EPAC2 – Exchange protein directly activated by cAMP 2
ERBB3 – Receptor tyrosine-protein kinase erbB-3
ERG – ETS related gene
ERK – Extracellular-signal-regulated kinase
ESE – Epithelial specific ETS
EtOH – Ethanol
ETS – E26 transformation specific
ETS1 – v-ets erythroblastosis virus E26 oncogene cellular homologue 1
ETS2 – v-ets erythroblastosis virus E26 oncogene cellular homologue 2
ETV – ETS variant gene
EZH2 – Polycomb enhancer of zeste homologue 2
FCS – Foetal calf serum
FGF – Fibroblast growth factor
FGFBP1 – Fibroblast growth factor binding protein 1
FGFR – Fibroblast growth factor receptor
FLI1 – Friend leukaemia integration 1 transcription factor
FN1 – Fibronectin 1
FOXP1 – Forkhead box P1
Fz – Frizzled receptor
G418 – Geneticin
GABPα – GA-binding protein alpha chain
GAPDH – Glyceraldehyde-3-phosphate-dehydrogenase
GFI1 – Growth factor independent 1
GFP – Green fluorescent protein
GnRH – Gonadotropin releasing hormone
GSK-3 – Glycogen synthase kinase 3β
HAT – Histone acetyltransferase
HCl – Hydrochloric acid
HER2 – Human epidermal growth factor-like receptor 2
HGF/SF – Hepatocyte growth factor/scatter factor
HIF2α – Hypoxia inducible factor 2α
HNMEC – Normal human mammary epithelial cells
HNRPA2B1 – Heterogeneous nuclear ribonucleoproteins A2/B1
HSP – Heat shock protein
HUVEC – Human umbilical vein endothelial cells
ID – Inhibitory domain
IGF – Insulin-like growth factor
IL3 – Interleukin 3
IPA – Ingenuity pathway analysis
IPTG – Isopropyl β-D-1-thiogalactopyranoside
IKBKB – Inhibitor of nuclear factor kappa-B kinase subunit beta
JAG1 – Jagged 1
JNK – c-Jun N-terminal kinase
KEGG – Kyoto encyclopedia of genes and genomes
KLK2 – Kallikrein related peptidase 2
KRT – Keratin
Ku70 – X-ray repair complementing defective repair in Chinese hamster cells 6
LAR – Luciferase assay reagent
LB – Luria-Bertani
LDL – Low density lipoprotein
LOH – Loss of heterozygosity
LRP – Receptor related protein
MAPK – Mitogen activated protein kinase
MB1 – CD79a molecule, immunoglobulin-associated alpha
MEK – Mitogen-activated protein kinase kinase
MET – Mesenchymal-to-epithelial transition
MgSO₄ – Magnesium sulphate
miR – MicroRNA
MMP – Matrix metalloproteinase
MSR1 – Macrophage scavenger receptor 1
mTOR – Mechanistic target of rapamycin (serine/threonine kinase)
mwt – Molecular weight marker
NaCl – Sodium chloride
NaOH – Sodium hydroxide
NCBI – National Center for Biotechnology Information
NFκB – Nuclear factor kappa-light-chain-enhancer of activated B cells
NK – Natural killer (cell)
NLS – Nuclear localisation sequence
NODAL – Nodal growth differentiation factor
NOTCH – Notch homologue translocation-associated
ODN – Oligodeoxynucleotide decoys
OPN – Osteopontin
OST – On-sighT domain
P16INK4a – Cyclin-dependent kinase inhibitor 2A, multiple tumour suppressor 1
p21 – Cyclin-dependent kinase inhibitor 1
p53 – Tumour protein p53
p300 – E1A binding protein p300
PAGE – Polyacrylamide gel electrophoresis
PARP1 – Poly [ADP-ribose] polymerase 1
PARylation – Poly(ADP-ribosyl)ation
PAX – Paired box transcription factor
PBS – Phosphate buffered saline
PCR – Polymerase chain reaction
PDEF – SAM pointed domain-containing Ets transcription factor
PDGF – Platelet derived growth factor
PEA3 – Polyomavirus enhancer activator 3
PI3K – Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIAS – Protein inhibitor of activated STAT
PIM1 – Proto-oncogene serine/threonine-protein kinase Pim-1
PIN – Prostatic intraepithelial neoplasia
PIATES – Piperazine-N,N′-bis(2-ethanesulphonic acid)
PIT-1 – Pituitary specific positive transcription factor 1
PLB – Passive lysis buffer
PLEK2 – Pleckstrin-2
PMSF – Phenylmethanesulphonylfluoride
PNT – Pointed domain
PS – Penicillin/streptomycin
PSA – Prostate specific antigen
pSMAD2 – Phosphorylated mothers against decapentaplegic homologue 2
PTEN – Phosphatase and tensin homologue
PTHrP – Parathyroid hormone-related protein
PU.1 – Proviral integration oncogene SPI1
PZ – Peripheral zone
RAF – RAF proto-oncogene serine/threonine-protein kinase
RASE – Real-time PCR annotation splicing events software
RCC – Renal cell carcinoma
RFU – Relative fluorescence units
RhoA – Ras homologue gene family member A
RNA – Ribonucleic acid
RNASEL – Ribonuclease L (2',5'-oligoisoadenylate synthetase-dependent)
RPM – Revolutions per minute
RPMI – Roswell Park Memorial Institute
RRE – Ras responsive element
RT – Reverse transcription
RTK – Receptor tyrosine kinase
SAP – Shrimp alkaline phosphatase
SAP1 – SRF accessory protein 1
SDS – Sodium dodecyl sulphate
SEM – Standard error of the mean
SERPINE1 – Plasminogen activator inhibitor 1
shRNA – Short hairpin ribonucleic acid
SLC45A3 – Solute carrier family 45 member 3
SMAD – Mothers against decapentaplegic homologue 2
SNAIL – Zinc finger protein SNAI1
SOX10 – Transcription factor SOX-10
SP1 – Specificity protein 1
SP2 – Specificity protein 2
SPARC – Osteonectin
SPDEF – SAM pointed domain-containing Ets transcription factor
SPIB – Transcription factor Spi-B
SPP1 – Osteopontin
SRC – Steroid receptor coactivator
SUMO – Small ubiquitin-like modifier
TAD – Transactivation domain
TAE – Tris-acetate-EDTA
TBS – Tris buffered saline
TBST – Tris buffered saline/Tween 20
TCF – Ternary complex factor
TEL – Ets variant 6
TGF-α – Transforming growth factor alpha
TGF-β – Transforming growth factor beta
TIF2 – Transcriptional intermediary factor 2
TIMP – Tissue inhibitor of metalloproteinases
TMPRSS2 – Transmembrane protease serine 2
TNPO1 – Transportin-1
tPA – Tissue plasminogen activator
TRAMP – Transgenic adenocarcinoma of the mouse prostate
TRUS – Transrectal ultrasound
tSMAD2 – Total mothers against decapentaplegic homologue 2
TWIST – Twist related protein
TZ – Transition zone
UCS – Upstream constitutive sequence
UGS – Urogenital sinus
uPA – Urokinase-type plasminogen activator
UV – Ultraviolet
VCAN – Vertiscan
VDR – Vitamin D receptor
VEGF – Vascular endothelial growth factor
VEGFR – Vascular endothelial growth factor receptor
WNT – Wingless type MMTV integration site family
ZEB – Zinc finger E-box-binding homeobox 1
ZO-1 – Zona occludens 1
Thesis Summary

The transcription factor ETS1 is frequently overexpressed in prostate tumours, with its increased expression correlated with disease severity and accelerated progression to castrate resistance. Three structurally and functionally distinct ETS1 isoforms have been identified and in this study, ETS1 isoform mRNA expression was characterised in 45 human prostate tumours and adjacent non-malignant prostate tissue using isoform-specific RT-qPCR. qPCR analysis identified ETS1p51 and ETS1p42 but not ETS1p27 expression in the malignant and nonmalignant prostate specimens and significantly higher ETS1p51 expression compared to ETS1p42 in both malignant and non-malignant prostate (p<0.0001). In addition, the expression of both ETS1p51 and ETS1p42 was reduced in the majority (65-69%) of prostate tumours relative to non-malignant tissue. Western blotting of protein extracts from the same specimens detected only ETS1p51 protein, which was expressed at significantly higher levels in the majority (67%) of prostate tumours examined (p<0.01), while no association was identified between ETS1p51 mRNA and protein expression in either malignant or non-malignant prostate tissues. These findings indicated that ETS1p51 was the predominant isoform expressed in the prostate and overexpressed in prostate tumours.

The rapid growth and spread of ETS1-overexpressing prostate tumours suggested that ETS1 may promote epithelial-to-mesenchymal transition (EMT), a process associated with loss of epithelial and acquisition of mesenchymal marker expression, promotion of migration and invasion, and in tumour cells, metastasis. To examine ETS1p51-induced EMT, the expression of 84 EMT-associated genes was assessed by PCR array in the human prostate cancer cell line LNCaP that had been transiently transfected with an expression construct encoding GFP-tagged ETS1p51. ETS1p51 overexpression in LNCaP cells was found to upregulate expression of 16 EMT-associated genes including MMP3 and MMP9, which encode matrix metalloproteinases that regulate invasion, NODAL and SPARC, which encode factors that facilitate migration, and genes encoding EMT-promoting transcription factors, including ZEB1 and ZEB2. Bioinformatics analysis of PCR array results predicted significant regulation of cell movement, activation of TGF-β signalling (a well-characterised inducer of EMT), SMAD nuclear translocation (a process central to TGF-β signalling) and promotion of EMT.
Key results from the PCR arrays were validated by RT-qPCR, which confirmed ETS1p51 induced regulation of $TGF-\beta 1$, $ZEB1$, $TWIST1$ and $SNAIL1$ expression, and by western blotting, which identified increased levels of the mesenchymal marker, vimentin and the EMT-promoting transcription factors SNAIL, ZEB, SLUG and $\beta$-catenin, with progressively decreasing expression of the epithelial marker Claudin-1. The expression profiles of EMT mediators in ETS1p51-overexpressing cells typified characteristics of cells undergoing EMT and were supported by evidence of ETS1p51-mediated activation of TGF-$\beta$ signalling through phosphorylation (activation) of SMAD2, a transcriptional mediator of the TGF-$\beta$ pathway. TGF-$\beta$/SMAD signalling promotes EMT in several cancer types including prostate cancer and also regulates cell migration and invasion, which are hallmarks of cancer-associated EMT. ETS1p51 overexpression enhanced the migration of LNCaP cells in wound-healing assays and the reversal of these effects by culture of cells with the inhibitor of SMAD2 phosphorylation, SB431542 indicated that this was partially mediated by TGF-$\beta$ signalling. ETS1p51 overexpression also increased LNCaP cell invasion in Matrigel-based transwell assays, with ETS1p51 effects similarly reversed by SB431542 treatment of cells. Together, results from this thesis indicate that ETS1 overexpression in prostate tumours is largely accounted for by ETS1p51 and that high levels of ETS1p51 expression in prostate cancer cells are associated with activation of TGF-$\beta$ signalling, increased cell migration and invasion, and induction of EMT, processes that are likely to contribute to the poor prognosis of ETS1p51-overexpressing prostate tumours.
1.1 The Prostate

The prostate is an important male accessory sexual organ, producing a fluid containing phospholipids, proteins and enzymes that contribute to sperm survival and nutrition and protect the urinary tract from infection (Cunha et al. 1987; Kumar et al. 1995; Dehm et al. 2006). Located directly below bladder and surrounding the urethra, the prostate is a part of the male urogenital complex (Figure 1.1) (Timms 2008).

Figure 1.1: Major components of the male urogenital complex including the prostate gland. The anatomical zones of the prostate, the anterior fibromuscular stroma (AFS), central zone (CZ), peripheral zone (PZ) and transition zone (TZ) are indicated (Timms 2008).

In humans, the prostate originates from the urogenital sinus (UGS) typically during the 10th week of gestation and develops in response to androgens (e.g. testosterone), in particular the potent androgen 5α-dihydrotestosterone (DHT), which bind and activate the androgen receptor (AR) expressed in surrounding mesenchymal cells (Kumar et al. 1995; Timms 2008). DHT stimulation of local mesenchymal cells induces prostatic epithelial cell proliferation and differentiation, with the epithelial cells emerging from the UGS and undergoing branching morphogenesis and canalisation to form secretory ducts (Cunha et al. 1987). In the adult prostate, the epithelium lines the secretory ducts, which are composed of basal and AR-expressing luminal cells with intervening...
neuroendocrine cells, each responsible for unique roles within the prostate gland (Robinson et al. 1998; Letellier et al. 2007).

### 1.2 Prostate Cancer

#### 1.2.1 Incidence and Diagnosis

Prostate cancer is the most frequently diagnosed invasive malignancy and the second leading cause of cancer-related death of males living in western countries, accounting for almost 30% of male cancer diagnoses and more than 300,000 deaths worldwide each year (AIHW et al. 2010; Jemal et al. 2010; Siegel et al. 2013). Typical symptoms of benign or malignant growths within the prostate include difficulty or pain during urination and increased frequency of urination, none of which are specific to prostate diseases. During the initial stages of prostate cancer, men are often asymptomatic, thereby confounding early detection of disease (Miller et al. 2003).

Despite the rising incidence of prostate cancer, mortality rates have decreased in recent years, findings attributed to improvements in both early diagnosis and treatments (Jemal et al. 2010). In most countries, measurement of serum prostate specific antigen (PSA) levels and digital rectal examination (DRE) are employed to screen for, or assist in the diagnosis of prostate tumours (Grossfeld et al. 2001; Ragsdale III et al. 2014). Elevated serum PSA levels (concentrations >4ng/mL and/or reduced free-to-total ratio, variable across different laboratories) can reflect prostatic inflammation, benign prostatic hyperplasia (BPH) or prostatic malignancy, therefore abnormal findings require the use of secondary techniques to confirm a positive diagnosis of prostate cancer (Brawer 2000; Hoffman et al. 2000; Grossfeld et al. 2001; Faria et al. 2012). The use of DRE provides a cost-effective method of physical examination of the periphery of the prostate gland via the anus to identify the presence of palpable nodules, however, examination is limited to the accessible posterior region of the prostate gland and therefore potentially misses tumours located in other parts of the prostate (Coley et al. 1997; Grossfeld et al. 2001). Diagnosis of prostate cancer may be confirmed by transrectal ultrasound (TRUS)-guided needle biopsy followed by histological examination of the biopsy tissue (Grossfeld et al. 2001). Tumours are graded mainly according to gland pattern from 1 (well-differentiated, least aggressive) to 5 (poorly-differentiated, most aggressive) using the Gleason grading system, with the Gleason score (2-10) calculated as the sum of the two most common patterns within the
specimen, a system that takes into account the high incidence of heterogeneity within prostate tumours (Figure 1.2).

Figure 1.2: Gleason grading of prostatic adenocarcinomas from 1 (well differentiated) to 5 (poorly differentiated) including typical histological features (adapted from (Nguyen et al. 2012)).

### 1.2.2 Risk Factors

Risk factors for prostate cancer development include age, family history, ethnicity, diet and environmental factors (Lee et al. 1998; Cui et al. 2001; Nair-Shalliker et al. 2012; Siegel et al. 2013). Other factors such as infertility, sexual activity and specific occupations (e.g. farmers) have been investigated, however conflicting results or lack of follow-up studies have meant that the contributions of these factors to prostate carcinogenesis are not well-defined (Sharma-Wagner et al. 2000; Negri et al. 2006; Walsh et al. 2010).

Age is the best-established risk factor for prostate cancer diagnosis, with men above 40-50 years recommended to undergo routine screening, and more than two-thirds of all prostate cancer diagnoses occurring in men aged 60 and above (Howlader et al. 2010). A number of studies have identified that the risk of developing prostate cancer directly increases with the number of first-degree relatives diagnosed with the disease,
indicating genetic and/or common lifestyle risk factors (Brandt et al. 2012; Elshafei et al. 2013). The documentation of families with increased prostate cancer incidence has prompted the investigation and identification of germline mutations in a number of genes including \textit{BRCA1}, \textit{BRCA2}, \textit{EPAC2}, \textit{RNASEL}, \textit{MSR1}, \textit{CHEK2}, \textit{CAPZB} and the vitamin D receptor (VDR) which each confer increased susceptibility for prostate cancer development (Dong 2006; Damber et al. 2008; Kote-Jarai et al. 2011).

African-American males are reported to have the highest incidence of prostate cancer globally and are more likely to present at a younger age, with higher grade tumours and high mortality rates (Platz et al. 2000; Thompson et al. 2006; Glass et al. 2013). In contrast, Hispanic, Asian/Pacific Islander and Native American men exhibit lower prostate cancer rates suggestive of different underlying genetic, lifestyle and/or dietary influences (Siegel et al. 2013). Medical co-morbidities have also been implicated in enhancing the risk of prostate cancer development including cardiovascular disease, obesity, diabetes and metabolic syndrome although the mechanisms underlying these associations are not well-defined (Glass et al. 2013).

Selected studies have demonstrated that western diets which include high levels of saturated fats, calcium and dairy products are correlated with development of prostate cancer (Lee et al. 1998; Chan et al. 2001). Additional evidence includes reports that development of obesity before the age of 30 increases the risk of developing prostate cancer (Robinson et al. 2005). However, more recent studies have provided evidence that high fat diets either do not directly increase the risk of prostate cancer development or are more specifically associated with the risk of developing higher grade prostate cancer (Crowe et al. 2008; Pelser et al. 2013).

Maintaining moderate levels of regular exercise has also been suggested to lower the risk of either prostate cancer development or progression to higher-grade disease, however subsequent studies suggest that this may only be beneficial to particular ethnicities (i.e. Caucasian vs African-American), supporting interactions between genetic and lifestyle factors (Antonelli et al. 2009; Singh et al. 2013). In addition, some evidence has correlated higher rates of prostate cancer incidence among heavy smokers and those with high levels of alcohol consumption (Watters et al. 2009; Rohrmann et al. 2013; Zuccolo et al. 2013). Increased exposure to sunlight (UV) is also believed to be
linked to higher risk of prostate cancer development, however more research is required to definitively link this factor (Colli et al. 2006; Nair-Shalliker et al. 2012).

Preliminary data indicates that men with occupations which involve exposure to hazardous chemicals or extreme environments exhibit increased risk of developing prostate cancer (Sharpe et al. 2001; Zeegers et al. 2004). Male infertility and fatherhood status have also been associated with an increased risk of diagnosis of prostate cancer, however conflicting findings indicate that further study is required to clarify these as bona fide prostate cancer risk factors (Negri et al. 2006; Jorgensen et al. 2008; Walsh et al. 2010).

1.2.3 Treatment

Prostate cancer treatment can include a combination of surgery, androgen deprivation therapies, chemotherapy, radiotherapy, brachytherapy and ‘active’ surveillance (untreated), selection of which is determined by tumour stage and grade as well as the age and overall health of the patient.

For smaller, low grade, early stage tumours, intervention can be delayed and disease progression is monitored (active surveillance) by regular examination of the cancer via physical examination, PSA testing, biopsies or imaging, with the intention of initiating treatment only following evidence of cancer progression (Albertsen 2010; Yaxley et al. 2013). Due to the increased incidence of prostate cancer with age, it is common that older men diagnosed with lower grade prostate tumours will outlast the disease and ultimately die of natural or non-cancer related causes, suggesting no benefit in risking treatment.

Radical prostatectomy is a common method of surgical intervention for localised disease and involves surgical removal of the prostate. In the event that the tumour has metastasised, orchiectomy (removal of the testes) decreases the major source of androgen production upon which prostate tumours depend for survival and growth. Orchiectomy may also be performed in conjunction with radical prostatectomy and this treatment combination has been shown to increase survival (Ghavamian et al. 1999). While radical prostatectomy is the most effective method for treating early-stage prostate cancer, complications associated with the surgery include urinary incontinence and impotence (Sanda et al. 2008). Evidence shows that surgery for older men (above
70 years) or those with low-stage/low-grade tumours may risk more harm than benefit and in an effort to minimise treatment impact, patients may be monitored by active surveillance in order to detect disease progression and implement treatment (e.g. androgen deprivation therapies) as early as possible (Klotz et al. 2010).

Patients whose tumours have spread beyond the prostate or whose tumours progress following surgery are candidates for androgen deprivation therapies (e.g. gondaotropin releasing hormone (GnRH) agonists, anti-androgens) which aim to minimise the supply of androgens by inhibiting testicular or local androgen synthesis or to outcompete androgen-induced activation of the AR, respectively (Cooperberg et al. 2003; Moule et al. 2009). Although initially very successful and the frontline therapy, most prostate cancers become resistant to androgen deprivation therapies and progress to castrate-resistant prostate cancer (CRPC) in which AR-stimulated cancer cell growth is maintained despite very low levels of (circulating) androgen ligands.

A number of mechanisms have been identified by which prostate tumours may survive androgen ablation therapies including AR activation by non-androgen or alternative ligands, local (i.e. intratumoral) androgen synthesis, aberrant AR cofactor expression or activity, amplification of the AR gene, AR gene mutations and aberrant phosphorylation and activation of the AR (section 1.3) (Devlin et al. 2009). Until recently, treatment of CRPC was largely palliative, however, new chemotherapeutic agents (e.g. prednisone, docetaxel) have produced improvements in patient survival by up to 4 months (Tannock et al. 2004; Higano 2012). Despite some treatment successes, CRPC is common and due to the limited efficacy of alternative therapies, patients often succumb to the cancer (Seruga et al. 2011).

Other commonly used non-surgical interventions for prostate cancer management include external beam radiotherapy and brachytherapy (implantation of radioactive seeds into the prostate), which are employed when tumours are localised to the abdominal area. Although the lack of requirement for anaesthetic or hospital stay are an advantage of radiotherapies, radiation damage to surrounding tissues may be associated with significant morbidity, therefore assessment of risks versus benefits is an important consideration in selection of this treatment modality (Cooperberg et al. 2003; Moule et al. 2009).
1.3 Genetic Alterations in Prostate Cancer

The sequence of events that leads to the development of prostate cancer is incompletely understood, however during the initial stages of prostate carcinogenesis a number of common genomic alterations including chromosomal deletions, gene amplifications and rearrangements are evident, with specific events associated with early, mid or late stage disease (Figure 1.3).

Figure 1.3: Molecular pathway of prostate cancer initiation and progression including biological processes (red) and characterised genes/pathways (blue) (Shen et al. 2010).

The homeobox gene NKX3.1 at chromosomal locus 8p21 is disrupted by loss of heterozygosity (LOH) in approximately 80% of prostate tumours, resulting in decreased NKX3.1 expression (Vocke et al. 1996; Aslan et al. 2006). This is often an early disease event that promotes formation of the premalignant lesion, prostatic intraepithelial neoplasia (PIN), which frequently leads to the development of prostate tumours. PIN lesions are histologically characterised by a reduction in basal cells, nuclear enlargement and elevated proliferative marker expression (Shen et al. 2010). Nkx3.1 mutant mouse models and human prostate cancer cell lines expressing altered NKX3.1 levels have been used to characterise NKX3.1 functions, with Nkx3.1 mutant mice frequently developing PIN lesions. These studies have led to the hypothesis that NKX3.1 is critical for the proper maintenance of the human prostate and acts as a tumour suppressor (Abdulkadir et al. 2002; Kim et al. 2002b; Kim et al. 2002c).

Chromosomal region 8q24 is frequently amplified in prostate tumours and contains the oncogene c-MYC, which is overexpressed in the majority of prostate carcinomas.
Myc overexpression in mice results in the rapid formation of PIN and is correlated with PIN formation and prostate tumour formation in humans (Sato et al. 1999; Ellwood-Yen et al. 2003; Gurel et al. 2008). Overexpression of c-MYC in primary prostate tumours is associated with early biochemical recurrence and has been suggested to predict rapid disease progression, hallmarks of aggressive tumours (Hawksworth et al. 2010).

1.3.1 Receptor Alterations

AR alterations have been extensively investigated in prostate cancers (Figure 1.4), with amplification of the AR gene commonly occurring in ~30% of advanced prostate tumours. This is believed to result in higher levels of AR activation despite lower androgen levels, mediating increased tumour cell proliferation and therefore increased likelihood of progression toward CRPC (Visakorpi et al. 1995; Ford III et al. 2003; Haapala et al. 2007). Hundreds of somatic mutations of the AR gene have been identified in prostate tumours and these can result in hypersensitive receptors that are highly activated in the presence of low androgen levels or which may be activated by non-androgen ligands including anti-androgens (Devlin et al. 2009; Koochekpour 2010). In vitro and in vivo studies have been used to characterise specific mutant ARs including the androgen, non-androgen (oestradiol, progesterone) or anti-androgen (flutamide) responsiveness of AR (T887A) expressed in the human prostate cancer cell line, LNCaP and AR (H874T) expressed in the androgen-responsive, but androgen independent prostate cancer cell line 22Rv1 (Veldscholte et al. 1990; Grigoryev et al. 2000; Hartel et al. 2004; Linja et al. 2004; Marcias et al. 2009).

In studies using the TRAMP (transgenic adenocarcinoma of mouse prostate) mouse model, AR mutations were identified in a high proportion of tumours from castrated mice. The findings indicated that changes in the supply of androgens can promote the outgrowth of cells carrying AR mutations, which would confer a growth advantage to the prostate tumours in low androgen environments (Han et al. 2001). The AR can be phosphorylated by multiple kinases including PIM1, CDK1, ACK1 and AKT, resulting in enhanced transcriptional activity (S213, S791 phosphorylation) and increased protein levels through receptor stabilisation (S81 phosphorylation) despite low androgen conditions. The results have provided evidence that increased AR phosphorylation potentially due to increased activation of cell surface receptors and their downstream
signalling pathways, may promote disease progression, with increased AR phosphorylation detected in biochemically recurrent tumours (S213, S515) or radiotherapy-resistant CRPC (T267) (Wen et al. 2000; Chen et al. 2006b; Mahajan et al. 2012; Ha et al. 2013; Willder et al. 2013). Conversely, AR phosphorylation (S308, S791) has also been associated with enhanced patient survival or increased time to disease recurrence (McCall et al. 2013).

In addition to AR gene amplification, mutation or AR activation, prostate tumours can enhance the local supply of androgens through increased expression of genes encoding regulators of steroid biosynthesis (e.g. HMG-CoA synthase, squalene synthetase) or upregulated expression of 5α-reductase, which converts testosterone to the more biologically active androgen, DHT (Holzbeierlein et al. 2004; Nakamura et al. 2005). AR cofactors (e.g. SRC1, TIF2, SRC3) may also be aberrantly overexpressed in prostate cancers and can activate AR in an androgen-independent manner largely through modification of chromatin structures by cofactors with histone acetyltransferase (HAT) activity or by recruitment of additional transcriptional machinery. This has been shown to enhance prostate cancer cell proliferation and invasion and is most notable in tumours that arise following androgen ablation therapies (Heinlein et al. 2002; Agoulnik et al. 2005; Agoulnik et al. 2006; Chmelar et al. 2006).

Human epidermal growth factor-like receptor 2 (HER2), is overexpressed in a high proportion of prostate tumours and may be involved in aberrant AR activation via activation of AKT or other signalling pathways during androgen-ablation therapy. Increased HER2 expression is correlated with loss of PTEN (phosphatase and tensin homologue) and poorer disease prognosis (Gu et al. 1996; Wen et al. 2000; Ahmad et al. 2011). HER2 expression in breast tumours is frequently associated with HER2 gene amplification, however the link between HER2 gene abnormalities and prostate cancer remains unclear as conflicting studies report either no HER2 gene amplification in prostate cancer cell lines (i.e. DU145, PC-3, LNCaP) and tumours or a high incidence (>40%) of HER2 gene amplification (Kallakury et al. 1998; Reese et al. 2001; Ullen et al. 2005). Although mechanisms of HER2 dysregulation in prostate cancers have not been well-characterised, collective evidence from studies of human prostate tumours has consistently indicated that HER2 overexpression is associated with prostate cancer progression (Kallakury et al. 1998; Reese et al. 2001; Ullen et al. 2005). An in vitro study using the prostate cancer cell line, PC-3 demonstrated that senescence induced by
PTEN knockdown could be overcome by HER2 overexpression, which enhanced ERK activation, a characteristic of metastatic prostate tumours, supporting a role of HER2 in prostate tumour progression (Ahmad et al. 2011).

**Figure 1.4:** Mechanisms of AR deregulation include AR gene amplification and mutations, increased expression of coactivators or local androgen supply, AR activation by non-androgen ligands and aberrant AR proteolysis (Devlin et al. 2009).

### 1.3.2 Signalling Pathway Alterations

The AKT/mTOR and MAPK signalling pathways are frequently dysregulated in more advanced prostate tumours, with activation of the p110β isoform of PI3K shown to alter prostate cancer cell cycle progression and rare activating mutations in AKT1 (E17K) or AKT activation of NFκB signalling reported to enhance AR expression and activity, collectively facilitating prostate tumour progression and promoting formation of CRPC (Boormans et al. 2008; Dan et al. 2008; Zhang et al. 2009a; Hill et al. 2010). Simultaneous AKT and ERK/MAPK activation has been demonstrated in advanced prostate tumours, which together promote tumour progression to CRPC in human tumours as well as in mouse prostate cancer models (Uzgare et al. 2004; Kinkade et al. 2008). In addition to these findings, loss of expression of the tumour suppressor and
Chapter 1: General Introduction

inhibitor of AKT signalling, PTEN occurs in up to 70% of prostate tumours (frequently due to allelic loss of chromosomal region 10q23) with reduced PTEN expression believed to be an early carcinogenic event which is associated with poor clinical outcome (Whang et al. 1998; Yoshimoto et al. 2007; McCall et al. 2008). Reduced PTEN expression is also proposed to be critical in the predisposition of prostate tumours towards the development of castrate resistant disease, irrespective of initial disease stage (Shen et al. 2007; Mulholland et al. 2011). Studies using the human prostate cancer cell line LNCaP have indicated that aside from AKT regulation, PTEN can interact with and modify AR function, reducing its transcriptional activity, promoting its degradation and inhibiting its nuclear translocation (Lin et al. 2004). Consistent with these findings, Pten-null mice exhibited hyperactivated AR and elevated AR expression, indicating that loss of PTEN modifies AR activity in prostate cancer cells and may enhance AR-mediated stimulation of prostate tumour growth (Lin et al. 2004).

1.3.3 Chromosomal Rearrangements

A number of chromosomal rearrangements involving the ETS family of transcription factors (e.g. ERG, ETV1, ETV4) and more than ten androgen-responsive genes including TMPRSS2, SLC45A3, FOXP1, HNRPA2B1, KLK2 and DDX5 have been detected in a high proportion (40-70%) of prostate tumours. These rearrangements frequently result in the ectopic and/or androgen-induced expression of full-length or truncated ETS factors which promotes the growth or progression of prostate tumours (Tomlins et al. 2005; Tomlins et al. 2007a; Han et al. 2008; Hermans et al. 2008; Clark et al. 2009). The most common fusion, TMPRSS2-ERG, results from intrachromosomal deletions or translocations involving the TMPRSS2 and ERG gene loci located on chromosome 21 that is proposed to occur as an early event in prostate carcinogenesis and to indicate an increased likelihood of disease progression. In prostate cancer cell lines, the TMPRSS2-ERG gene fusion has been associated with increased prostate cancer cell migration and invasion (Perner et al. 2007; Shen et al. 2010; Tian et al. 2013). The TMPRSS2-ERG fusion can result in a number of transcripts including TMPRSS2 exon 1 or 2 fused with ERG exon 4 and expression of either of these transcripts in the prostate cancer cell line VCaP in vitro or in vivo results in enhanced cell proliferation that is abrogated by shRNA-mediated silencing of ERG expression (Wang et al. 2008). In contrast, in vitro overexpression of full-length wild-type ERG in PC-3 cells does not alter proliferation, suggesting that alternatively spliced ERG transcripts can provide abnormal tumour-
specific functions (Carver et al. 2009). *TMPRSS2-ERG* rearrangements, which are significantly enriched in *PTEN*-negative, high grade PIN lesions, are able to enhance prostate cancer cell migration, and together promote progression to invasive, highly metastatic adenocarcinomas (Carver et al. 2009). A number of non-ETS fusions such as *CDKN1A-CD9* and *TNPO1-IKBKB* have been identified in *TMPRSS2-ERG* containing tumours that are likely to mediate additional tumour-associated activities and enhance aggressive characteristics of the cancers (Pflueger et al. 2010). These studies have also highlighted the high frequency of chromosomal rearrangements and gene fusions in prostate tumours.

### 1.4 ETS Factors

The E26 transformation-specific (ETS) factors form a family of 28 transcriptional regulators which are solely expressed in metazoan species and are characterised by the conserved DNA-binding ‘ETS’ domain (Figure 1.5B). The original *Ets* gene sequence was identified in 1983 as part of a *gag-myb-ets* fusion oncogene of the avian transforming retrovirus, E26 which induces leukaemias in chickens (Leprince et al. 1983). ETS factor orthologues subsequently identified in mouse (26), *Caenorhabditis elegans* (10) and *Drosophila melanogaster* (8) exhibit high sequence homology with human ETS factors, indicative of their conserved activity and thus their experimental usefulness for modelling human ETS factor functions (Wasylyk et al. 1993; Laudet et al. 1999; Hart et al. 2000; Hsu et al. 2000). ETS factors can be divided into 13 subfamilies on the basis of sequence homologies within the ETS domain and in other functional domains (Figure 1.5A) (Laudet et al. 1999).

#### 1.4.1 Protein Structure and Functional Domains

Structural and functional domains of ETS factor family members are well conserved with regards to their relative domain positions in the proteins and in regions known to be post-translationally modified (Figure 1.6) (Hollenhorst et al. 2011b). ETS proteins can be subdivided into functional regions including transactivation (TAD), ETS DNA-binding (ETS), inhibitory (ID) and pointed (PNT) domains and b-box sites (GABPα and TCF subfamily) (Oikawa et al. 2003; Hollenhorst et al. 2011b).

The highly conserved DNA-binding ‘ETS’ domain is required for direct binding of ETS factors to ETS binding sites (EBS) containing the core DNA sequence 5’-
GGA(A/T)-3’ (Kodandapani et al. 1996; Wei et al. 2010). The ETS domain (~85 amino acids) is comprised of three α-helices on a β-sheet scaffold, is typically located at the C-terminus of the protein and usually contains a nuclear localisation sequence (Donaldson et al. 1996; Kodandapani et al. 1996; Hu et al. 2005; Honsei et al. 2006).

The ETS factor PNT domain sequence (~80 residues) is conserved and present in approximately one third of ETS factors, where it functions to mediate ETS protein interactions that modify ETS factor transcriptional activity (Mackererth et al. 2004; Hollenhorst et al. 2011b). The PNT domain contains five α-helices and a 3_10-helix (helix-loop-helix) and includes amino acid residues that can be phosphorylated to enable open (active) or closed (inactive) conformations that modulate ETS-cofactor binding and ETS factor transcriptional activity (Kim et al. 2001; Nelson et al. 2010).

The TAD domain/s are variable in size, relative position and sequence compared to the well-conserved ETS and PNT domains, however mutation studies demonstrate that TAD domains are essential for ETS factor transcriptional activity (Chang et al. 1999; Oikawa et al. 2003; Bojovic et al. 2008). TAD domains are believed to mediate recruitment of the general transcriptional machinery to ETS factor-DNA complexes and are typically positioned in regions flanking the ETS and PNT domains (Oikawa et al. 2003; Verger et al. 2013).

Inhibitory domains are typically appended to either side of the ETS domain and function to auto-inhibit DNA binding or to prevent promiscuous binding activity either through blockage of the ETS domain or by promoting structural re-conformation to reduce DNA binding affinity (Greenall et al. 2001; Green et al. 2010). Cofactor interactions or phosphorylation of other ETS factor domains regulate the function of the inhibitory domain, thereby controlling the dynamics of DNA binding (Kim et al. 1999; Stinson et al. 2003).

Additional functional domains including the On-Sight (OST) and b-box region, which are specific to the ETS factors GABPα and the TCF subfamily, respectively function to mediate unique cofactor interactions (Hollenhorst et al. 2011b). The GABPα OST domain (87 residues) is a five-stranded β-sheet crossed with a distorted helix and is reported to facilitate unique cofactor recruitment independent of the PNT domain (Kang et al. 2008). Only members of the TCF subfamily (ELK1, ELK3 and ELK4) contain the b-box region, a helix-strand-helix structure that simultaneously binds DNA
and protein cofactors to enhance ETS factor-DNA binding that is independent of DNA binding via the ETS domain (Hassler et al. 2001).

Figure 1.5 (A): Phylogenetic tree of the ETS factor family based on sequence homology within the ETS domain and clustered into 13 subfamilies (bold). The branch lengths are proportional to divergence between sequences with bootstrap values (percentage) indicated (Laudet et al. 1999).
**Figure 1.5 (B):** The ETS factor family members including subfamily (group), Unigene name (original name) or alternative name, chromosomal position, size (amino acids) and locations of the ETS and Pointed domains (Findlay *et al.* 2013).
Figure 1.6: ETS factor family protein domain structure including transactivation/On-Sight domains (blue), DNA binding ETS domains (red), phosphorylation sites (orange P), pointed domains (green), inhibitory domains (yellow) and b-box (purple). Adapted from (Oikawa et al. 2003; Hollenhorst et al. 2011b).

1.4.2 Activity of ETS Factors

ETS factors can positively or negatively regulate transcription, a function that is mediated by the TAD (section 1.4.1). Given the large number of ETS factors and their diversity in structural and functional domains, this implies that transcriptional activity and specificity are regulated prior to ETS factor-DNA binding by interactions with cofactors, post-translational modification and affinities for divergent sequences flanking the core EBS (Figure 1.7) (Li et al. 2000a; Tootle et al. 2005; Wei et al. 2010).

A large number of ETS factor-cofactor interactions have been characterised to be important for transcriptional regulatory activity and selection of target DNA sequences (Li et al. 2000a). Cofactors such as paired box transcription factor 5 (PAX5) interact with multiple ETS factors (e.g. ETS1, PU.1, ELK1 and ELK3) and stabilise ETS factor-
Chapter 1: General Introduction

DNA complexes, thereby enhancing transcriptional activation of target genes, including the mb-1 gene (Fitzsimmons et al. 1996). In contrast, PIASxα is a SUMO E3 ligase that specifically interacts with the ETS family member FLI1 and represses its transcriptional activity (van den Akker et al. 2005). ESE1 is an epithelium-specific ETS factor that was identified to interact with several cofactors (e.g. Ku70, Ku86, p300, CBP), with Ku70 and Ku86 negatively regulating and p300 and CBP positively regulating ESE1 activity (Wang et al. 2004). In addition to cofactor interactions (which typically occur prior to DNA binding), post-translational modifications can independently affect ETS factor activity.

The best characterised post-translational modification of ETS factors is phosphorylation of residues typically within the TAD which regulates subcellular localisation, ETS factor-DNA complex stability, cofactor interactions and importantly, transcriptional activity (Tootle et al. 2005). NET, a member of the TCF subfamily contains four phosphorylation sensitive serine residues (S359 S365, S398 and S403) that, when phosphorylated by JNK kinase, induce nuclear export (Ducret et al. 2000). In contrast, protein kinase C-mediated phosphorylation of ELF1 promotes nuclear translocation and accumulation, enhancing transcriptional functions (Juang et al. 2002). ELK1 DNA binding activity is regulated by TAD phosphorylation, with nonphosphorylated S383 and S389 inhibiting DNA binding, but phosphorylated S383 and S389 enhancing DNA binding, thereby leading to increased transcriptional activity (Yang et al. 1999). Phosphorylation of a large number of ETS factors including ETS1/2, ELK1/3/4, GABPA, SPIB, ETV1/4/5 by mitogen-activated protein kinase (MAPK) results in their enhanced transcriptional activity. This process is particularly relevant in cancers as many tumours have aberrant MAPK signalling that can result in dysregulated ETS factor phosphorylation, resulting in increased target gene transcription (Charlot et al. 2010).

Sumoylation of ELK4 on a conserved lysine 167 residue results in increased ELK4-mediated transcriptional repression, and mutation of this residue markedly enhances ELK4 transcriptional activity, weakening its repressive activity on the c-fos target gene promoter (Kaikkonen et al. 2010). The ETS family member PEA3 is also sumoylated, however acetylation by p300 of the same residues results in enhanced transcriptional activation (Guo et al. 2011).
Figure 1.7: ETS factor binding site sub-classes (I-IV) organised by preferred DNA binding sequence including the core EBS (GGAA/T) and flanking sequences. The height of the letter is proportional to the effect of that nucleotide on binding affinity (Wei et al. 2010).

Recent evidence also indicates that ETS factor DNA binding activity is regulated by sequences flanking the EBS, and ETS factors may be divided into four classes on the basis of their preferred core/flanking sequence (Figure 1.7) (Wei et al. 2010; Hollenhorst et al. 2011b). Class I (12 members) has high affinity for the sequence ACCGGAAAGT, class II (8 members) binds with high affinity to a CCCGGAAAGT sequence, three members form class III which binds a consensus sequence element containing adenine-rich sequences 5’ to the core EBS and class four is composed of PDEF, which binds a unique EBS core (GGAT) (Wei et al. 2010). Crystallography and ChIP-seq experiments have identified that sequence preference is based on ETS factor-DNA binding and interactions with proximal non-conserved residues, altering tertiary conformation. As such, divergent sequences (Figure 1.7) confer differing DNA binding affinity to subclasses of ETS factors which modulate subsequent transcriptional specificity (Mo et al. 1998; Mo et al. 2000; Hollenhorst et al. 2009; Wei et al. 2010).
1.4.3 ETS Factor Expression and Functions

ETS factors are ubiquitously expressed in both developing and adult tissues, with the regulation of physiological processes by transcriptional control of common ETS target genes leading to redundancy of functions between ETS factor family members (Maroulakou et al. 2000; Oikawa et al. 2003; Hollenhorst et al. 2004). Individual ETS family members may also perform unique functions either by tissue-restricted expression (e.g. ESE1, PU.1), regulation of specific target genes or due to preferences (affinity) for EBS flanking sequences (Klemsz et al. 1990; Oettgen et al. 1997; Wei et al. 2010). In general, ETS factor target genes function in regulating cell proliferation, motility, invasion, angiogenesis and differentiation in both physiological and pathological contexts (Sementchenko et al. 2000). ETS factor target genes are defined as containing at least one EBS (validated by abrogation of target gene regulation following mutagenesis of the EBS) and evidence of direct regulation by at least one ETS factor, which has led to a large number (>700) of publications characterising an extensive range of ETS target genes (Sementchenko et al. 2000; Findlay et al. 2013).

Expression and function of individual ETS factors are essential for the development of a number of tissue types as evidenced by the phenotypes of ETS factor knockout mice. For example, Tel^-/- and Fli1^-/- knockout mice die in utero due to a lack of yolk sac angiogenesis and haematopoiesis, respectively, indicating that these functions cannot be compensated for by other ETS family members (Wang et al. 1997; Spyropoulos et al. 2000). In contrast, the ETS factors Spi-B and Pu.1 (SP1 sub-family) are both important for lymphocyte development however, Spi-B^-/- knockout mice display no significant phenotype whereas knockout of Pu.1 results in embryonic lethality due to aberrant lymphocyte development (Garrett-Sinha et al. 1999; Garrett-Sinha et al. 2001). Mutation or knockout of ELK4, ELF1 or ELF4 leads to impaired T cell development in adult mice and subsequent immune dysfunction (Lacorazza et al. 2002; Costello et al. 2004; Choi et al. 2011). Post-embryonic conditional knockout mice and simultaneous deletion/mutation studies of multiple ETS factors have been used to further characterise redundant and non-redundant ETS factor functions (Findlay et al. 2013). For example, targeted disruption of ETV5 (ERM) in mice results in loss of spermatogonial stem cell populations rendering male mice infertile, a unique function of ETV5 in the adult (Chen et al. 2005).
ETS fusion genes resulting from chromosomal rearrangements (section 1.3) account for a proportion of cancer-associated abnormalities of ETS factor expression, with specific ETS factor over- or under-expression that does not involve chromosomal abnormalities detected in a number of solid and non-solid tumours including prostate, breast, colon, lung, squamous carcinomas and T-cell lymphomas (Chang et al. 1997; Hida et al. 1997; Hiroumi et al. 2001; Seth et al. 2005; Watson et al. 2010; Deves et al. 2011; Lin et al. 2012). Increased expression of the ETS factors ETS1, ETS2, FLI1, ERG, ELF1 and ESE2 has been reported in human and mouse prostate tumours (Liu et al. 1997; Gavrilov et al. 2001; Alipov et al. 2005; Rostad et al. 2007). In contrast, reduced expression of the ETS factors PEA3, ELK1, PDEF and ESE3 is documented in a majority of prostate tumours (Oettgen et al. 2000; Gavrilov et al. 2001; Cangemi et al. 2008). As a consequence of the identification of widespread alterations in ETS factor expression in prostate cancer, many studies have focused on characterising ETS factor target genes and the functional consequences of aberrant ETS factor expression in prostate tumours.

ERG, an ETS factor aberrantly overexpressed in ~50% of prostate tumours is immunohistochemically detected within the nucleus of prostate cancer cells, suggesting that it is transcriptionally activating target genes such as matrix metalloproteinase 3 (MMP3) and urokinase-type plasminogen activator (uPA) which would enhance prostate cancer cell invasion (Petrovics et al. 2005; Klezovitch et al. 2008; Tomlins et al. 2008). ERG overexpression in prostate tumours that overexpress MYC and are PTEN-negative has also been correlated with shorter time to biochemical recurrence and thus represents a highly aggressive subset of prostate tumours (Paulo et al. 2012b; Leinonen et al. 2013). ELF1 and FLI1 are overexpressed in a high proportion of prostatic adenocarcinomas and co-expressed in tumours with higher levels of uPA, an ETS factor target gene believed to be dysregulated by the abnormal expression of ELF1 and FLI1 in a subset of tumours (Gavrilov et al. 2001). Similarly, in vitro overexpression of ETV5 in the benign immortalised prostate cell line RWPE results in enhanced cell invasion and expression of the ETS factor target genes, uPA and MMP9 which additionally indicates the functional redundancy and common oncogenic properties of ETS subfamily members (Helgeson et al. 2008; Hollenhorst et al. 2011a). Cumulative studies suggest that overexpression of one or more ETS factors may contribute to prostate cancer progression by regulation of common target genes.
however, as prostate tumours typically exhibit abnormal expression of multiple ETS factors, both redundant and non-redundant functions of individual ETS factors are indicated (Gavrilov et al. 2001; Watson et al. 2010; Hollenhorst et al. 2011a).

In contrast to the abovementioned ETS factors, ESE-3 expression is reported to be reduced in prostate tumours, which is proposed to alter regulation of genes controlling apoptosis and cell survival (e.g. caspase 3), with overexpression of ESE-3 inducing apoptosis of prostate cancer cells *in vitro* (Cangemi et al. 2008). PDEF is another ETS factor that is underexpressed in prostate tumours and although the function of PDEF is incompletely described, reduced PDEF is associated with increased cell migration and invasiveness, suggesting that PDEF is a suppressor of cell motility and invasion, characteristics of more aggressive, metastatic disease (Oettgen et al. 2000; Gu et al. 2007). Competitive ETS factor functions have been characterised in prostate cancer cells, supporting the co-ordinated dysregulation of expression of multiple ETS factor family members. For example, prostate tumours co-expressing high levels of ERG and low levels of ESE3 exhibit increased expression of the common target EZH2 and downregulated expression of NKX3.1 (Section 1.3), while overexpression of ESE3 and knockdown of ERG reverses this effect (Kunderfranco et al. 2010).

Chromatin immunoprecipitation (ChIP) studies have revealed a high degree of functional redundancy in ETS factor target gene binding with overlap (e.g. ETS1, ELF1, GAPBα) and co-occupancy of target gene EBS sequences (Hollenhorst et al. 2007). In addition, reciprocal regulation of ETS factor family members has been demonstrated, for example *in vitro* silencing of either ELK1 or SAP1 (TCF sub-family members) results in increased expression of the alternative factor and compensation in the regulation of expression of common target genes (Boros et al. 2009b). Redundant promoter co-occupation primarily occurs in housekeeping genes whereas specific occupancy is often associated with cell-specific functions, supporting the hypothesis that individual ETS factors possess both redundant and unique transcriptional regulatory activity, the latter of which is frequently mediated by EBS flanking sequences, post-translational modifications or cofactor interactions (Boros et al. 2009a; Hollenhorst et al. 2009; Wei et al. 2010).

ETS factor targets include microRNAs (miR) and while limited studies have been reported, both activation and repression of miRNA expression have been described.
ETS factor regulation of miRNA expression appears to be a unique activity of individual ETS factors as the effects are often disease or tissue-specific (Findlay et al. 2008; Harris et al. 2010; Findlay et al. 2013). Elevated ETV4 expression represses levels of miR-125a and miR-21 in ovarian and colorectal cancer cell lines, respectively, which results in repression of epithelial-to-mesenchymal transition (EMT) (section 1.6) (Dahl et al. 2009; Kern et al. 2012). In endothelial cells, both ETS1 and ETS2 enhance miR-126 expression which promotes angiogenesis, while in gastric cancer cells, ETS2 represses expression of miR-196b, a negative regulator of invasion and migration (Harris et al. 2010; Liao et al. 2012). ETS subfamily members may regulate common microRNAs, however this may be tissue or disease-specific, for example, ETV5 activates expression of miR-21 while ETV4 represses miR-21 expression during spermatogonial stem cell renewal and in colorectal cancers, respectively (Niu et al. 2011; Kern et al. 2012). Similar to their regulation of common target genes, ETS factor regulation of miRNAs exhibits both redundant and unique functions that are also likely to depend on tissue type in addition to post-translational modifications, cofactor interactions or co-expression of other ETS factor family members.

1.5 ETS1

ETS1 is the prototypical member of the ETS factor family and the first homologue identified in vertebrates (Watson et al. 1985). Much of the early research on the molecular structure and function of ETS factor domains and the characterisation of ETS factors as transcription factors was performed using ETS1.

1.5.1 ETS1 Gene, mRNA and Protein Isoforms

The ETS1 gene is ~60kb in length and located on chromosome 11 at 11q23.3 (NCBI Reference Sequence NG_029555.1) (Watson et al. 1985; Jorcyk et al. 1991). The ETS1 GC rich promoter has 14 documented transcription start sites, and consensus binding sequences for Sp1, Sp2, AP1 and the basal transcriptional machinery have been characterised using bioinformatics and/or functional studies (Jorcyk et al. 1991; Dittmer 2003; Pufall et al. 2005; Callaway et al. 2010). The ETS1 gene is comprised of eight exons (A, III-IX) (Figure 1.8) and is alternatively spliced to generate several mRNA transcripts, including a full-length mRNA transcript of 5242bp (NCBI Reference Sequence NM_005238.3) and two internally truncated mRNA transcripts lacking exon...
Chapter 1: General Introduction

VII (4981bp) (NCBI Reference Sequence removed) or lacking exons III to VI (4594bp) (NCBI Reference Sequence NM_001162422.1).

Three human ETS1 protein isoforms encoded by the three mRNA transcripts have been identified, a full-length isoform, c-ETS-1A or ETS1p51 (~51kDa), and the internally truncated isoforms c-ETS1-1B or ETS1p42 (lacking protein sequence encoded by exon VII, ~42kDa) and ETS1p27 (lacking protein sequence encoded by exons III to VI, ~27kDa) (Reddy et al. 1988; Laitem et al. 2009). The ETS1p51 protein (441 amino acids) contains a PNT, TAD, ETS DNA-binding (ETS) and IDs (section 1.4.1) (Figure 1.9) (Reddy et al. 1988; Dittmer 2003). The ETS1p42 isoform (355 amino acids) lacks the first of the inhibitory domains while the smallest isoform, ETS1p27 (225 amino acids) lacks both the pointed and transactivation domains, and as a result, both isoforms exhibit distinct functions (Figure 1.9) (Fisher et al. 1994; Laitem et al. 2009).

Figure 1.8: ETS1 gene and mRNA isoforms including exon size (nucleotide/bp), 5’ and 3’ untranslated regions (light blue) and coding sequence (black). Gaps in the ETS1p42 and p27 mRNA transcripts indicate spliced regions relative to the ETS1p51 sequence.

The amino-terminal of ETS1p51 and ETS1p42 contains a PNT domain (~81 amino acids) which is comprised of five α-helices. Studies using human and murine ETS1 have shown the PNT domain to function as a site of cofactor interactions and to include sites which are post-translationally modified, largely via phosphorylation which results in enhanced ETS1 transcriptional activity (Slupsky et al. 1998; Dittmer 2003; Macauley et al. 2006). Within the PNT domain is a Ras-responsive element (RRE) (threonine-38), and a number of kinases regulate phosphorylation of ETS1 including ERK2 which mediates phosphorylation of ETS1 T38, strongly enhancing ETS1 transcriptional...
activity (Yang et al. 1996; Callaway et al. 2010). Studies using the highly homologous murine Ets1 have similarly indicated that the MAPK, ERK2 binds and phosphorylates threonine-38, enhancing transcriptional activation of target genes (Seidel et al. 2002). Mutation of T38 in murine and chicken Ets1 (T38A) impedes ERK2 phosphorylation of Ets1 and reduces Ets1 transcriptional activity (Yang et al. 1996; Seidel et al. 2002; Callaway et al. 2010).

**Figure 1.9:** Structure of ETS1 protein isoforms p51, p42 and p27 including the pointed (PNT), transactivation (TAD), inhibitory (ID), DNA-binding ETS domain (ETS) and nuclear localisation sequence (NLS). The amino acid numbers of each domain and sites of phosphorylation T38, S251, S257, S282 and S285 are indicated.

The second functional domain from the N-terminus is the TAD (~108 amino acids) which is essential for the transcriptional activity of ETS1 and is present in both the ETS1p51 and ETS1p42 isoforms (Gegonne et al. 1995; Yang et al. 1998). Studies using murine Ets1 have demonstrated that deletion of the TAD significantly impairs transcriptional activity (Delannoy-Courdent et al. 1998; Yang et al. 1998). Similar to the PNT domain, the TAD mediates interactions with cofactors that cooperate with ETS1 to enhance or repress target gene transcription (Jayaraman et al. 1999; Li et al. 2000b; Dittmer 2003).

The auto-inhibitory domains (ID) of ETS1 have largely been characterised using murine Ets1 and are comprised of regions which flank the DNA-binding ETS domain and are required for auto-inhibition of Ets1-DNA binding, thereby regulating Ets1 transcriptional regulatory activity (Cowley et al. 2000; Lee et al. 2005). The ID present at the N-terminal flank of the ETS domain contains two α-helices (HI-1 and HI-2) and the ID at the C-terminus contains a single α-helix (HI-4) which interacts with HI-1 adjacent to the ETS domain, inducing a conformational change and the formation of an auto-inhibited complex that is unable to bind target DNA (Jonsen et al. 1996; Lee et al.
Chapter 1: General Introduction

2005). The ID also contains phosphorylation-sensitive serines (S251, 257, 282 and 285) which undergo calcium-responsive phosphorylation thereby increasing the stability of the autoinhibitory module and preventing transcriptional activation of target genes (Cowley et al. 2000; Liu et al. 2002).

The characteristic DNA-binding ‘ETS domain’ (~85 amino acids) is a winged helix-turn-helix structure composed of three α-helices and four β-strands arranged as H1-S1-S2-H2-H3-S3-S4 (Werner et al. 1997). The third helix (H3) directly contacts DNA within the major groove against the core GGAA/T (EBS motif) and strands S3 and S4 sit within the minor groove (Werner et al. 1997; Lamber et al. 2008). The ETS domain also contains a nuclear localisation sequence (NLS) that is required for the nuclear import of ETS1 protein (Boulukos et al. 1989). In addition to monomeric ETS1-DNA binding, homo- and hetero-dimeric ETS1-DNA complexes have been identified (Lamber et al. 2008; Babayeva et al. 2012). ETS1 homodimerisation occurs as a head-to-head complex and recognises a palindromic DNA sequence, with helices H2 and H3 bound to target DNA. As formation of this complex requires both inhibitory domains, ETS1p42 is incapable of homodimerisation (Baillat et al. 2002; Lamber et al. 2008). An example of an ETS1 heterodimer is with the transcription factor, growth factor independent-1 (GFI1) which, once formed, synergistically represses expression of target genes (e.g. Bax) by binding adjacent DNA consensus sequences (Nakazawa et al. 2007).

The truncated ETS1p42 isoform lacking exon VII that encodes part of the autoinhibitory domain is resistant to calcium-mediated phosphorylation and exhibits more stable and promiscuous DNA binding relative to ETS1p51 (Fisher et al. 1994). The smallest isoform ETS1p27 lacks both PNT and TAD regions that are responsible for transcriptional activity, but contains an intact ETS DNA-binding domain and both inhibitory domains, thereby generating an intrinsically dominant negative protein isoform (Laitem et al. 2009). Due to these properties, ETS1p27 can outcompete ETS1p51 for DNA binding and enhance ETS1p51 nuclear export, thereby repressing ETS1 target gene transcription (Laitem et al. 2009).
1.5.2 ETS1 Expression

ETS1 is expressed in human and other mammalian tissues, functioning at sites of development including the vasculature, differentiating haematopoietic and lymphoid cells, and neurons (Dittmer 2003; Hahne et al. 2008). During the implantation phase of human pregnancy, ETS1 mRNA and protein are detectable in endothelial cells of the trophoblast that invade the maternal endometrium (Luton et al. 1997; Takai et al. 2006). At 7 to 8 weeks of human embryonic development, ETS1 mRNA is detectable in the capillaries of the spinal cord and placenta (Figure 1.10) (Wernert et al. 1992). Murine Ets1 is expressed ubiquitously during development and is detected at sites of organogenesis, morphogenesis and angiogenesis, with Ets1 deletion associated with multiple disease phenotypes or embryonic lethality (Kola et al. 1993; Barton et al. 1998; Maroulakou et al. 2000; Ye et al. 2010).

ETS1 expression and function have been examined in several in vitro studies including in the human vascular endothelial cell line ECV304, which expresses high levels of ETS1 during wound-associated remodelling and re-endothelialisation (Figure 1.10) (Tanaka et al. 1998). Cultured quiescent human aortic vascular smooth muscle cells express ETS1, which is hypothesised to function in maintaining quiescence (Lo et al. 2009). Human normal mammary epithelial cells (HNMEC) express high levels of ETS1 when co-cultured in vitro with fibroblast cells (MRC-5), suggesting a physiological function in response to surrounding stroma that may be relevant during both non-cancerous mammary gland development and mammary carcinogenesis (Delannoy-Courdent et al. 1996).

Figure 1.10: ETS1 expression in (A) developing spinal cord of a 7.5 week old human embryo, (B) human vein endothelial cell line ECV304 during re-endothelialisation, (C) differentiating keratinocytes of newborn mice and (D) solid-type breast carcinoma. Adapted from (Wernert et al. 1992; Tanaka et al. 1998; Katayama et al. 2005; Nagarajan et al. 2009).
Although ETS1 expression in adult tissues was originally thought to be restricted to lymphatic cells, ETS1 is detectable in a range of tissues and cell types such as astrocytes of the human cortex, lymphocytes in the tonsils and in capillaries of skin undergoing wound healing (Amouyel et al. 1988; Wernert et al. 1992; Keehn et al. 2004). Endogenous ETS1 expression is low in the liver but is believed to be increased at tissue sites susceptible to and/or undergoing malignant transformation (Ito et al. 2000). Both newborn and adult mice maintain high levels of Ets1 expression in differentiating keratinocytes, indicating that constitutive Ets1 expression is required to regulate tissue differentiation (Figure 1.10) (Nagarajan et al. 2009). Studies of ETS1 expression in adult tissues have identified concomitant expression of ETS1 isoforms, with differences in the relative abundance of the isoforms attributed to their unique functional roles. ETS1p51 expression is relatively high in comparison to ETS1p42 in quiescent human and mouse T-cell populations and is involved in proper maintenance of the cell cycle in this population (Bhat et al. 1990; Bories et al. 1995; Grenningloh et al. 2008). In activated mouse T-cells, a time-dependent decrease of ETS1p51 levels is evident, while ETS1p42 levels remain constant, with each isoform exhibiting different transcriptional activities during an immune response (Grenningloh et al. 2008). During the menstrual cycle, ETS1p51 and ETS1p42 are expressed in both the endometrial glandular epithelium and to a lesser extent in the surrounding stroma, with both isoforms believed to contribute to menstrual cycle regulation (Kilpatrick et al. 1999). In the breast cancer cell line MDA-MB-231, ETS1p51, ETS1p42 and ETS1p27 are detectable but in different relative abundance. Overexpression of ETS1p42 was shown to reduce cell survival, with ETS1p51 DNA binding preferentially displaced by DNA bound ETS1p27, suggestive of overall repression of common target genes (Ballschmieter et al. 2003; Laitem et al. 2009).

Micro-RNAs have been more recently identified to regulate the expression of ETS1 and serve as unique repressors of ETS1 mRNA and protein, thereby modulating subsequent transcriptional functions. Micro-RNA 200b (miR-200b) is a potent repressor of EMT and using human cell lines, in vitro studies identified that miR-200b exerted translational repression of ETS1 via binding to the 3’ untranslated region (UTR) of ETS1 and was responsible for inhibiting expression of ETS1 target genes (Chan et al. 2011). In addition, miR-1 and miR-499 repressed mRNA and protein expression of ETS1 and repressed ETS1-mediated invasion and migration of the hepatocyte cell line,
Chapter 1: General Introduction

HepG2 which is used to study hepatocellular carcinoma, a disease in which ETS1 is believed to play an important role (Wei et al. 2012). In the breast cancer cell lines MCF-7 and MDA-MB-453, miR-125b directly repressed ETS1 expression in association with suppression of breast cancer cell proliferation and the induction of cell cycle arrest (Zhang et al. 2011).

In addition to its expression under normal physiological conditions, ETS1 is frequently overexpressed in carcinomas of the breast (Figure 1.10) (Katayama et al. 2005), prostate (Alipov et al. 2005), ovary (Davidson et al. 2001), colon (Ito et al. 2002), stomach (Nakayama et al. 1996), lung (Takanami et al. 2001), pancreas (Ito et al. 1998), thyroid (Nakayama et al. 1999) and other non-carcinoma (non-epithelial) malignancies (Kitange et al. 2000), where it is frequently associated with poor prognosis.

1.5.3 ETS1 Target Genes

ETS1 is widely expressed, however both general and tissue specific functions have been elucidated using in vitro and in vivo studies to characterise transcriptional regulation of target genes and the consequences of abnormal ETS1 expression, particularly in cancers. Under physiological and pathological conditions, ETS1 target genes function in the regulation of haematopoiesis, invasion, angiogenesis, proliferation, apoptosis, differentiation and organogenesis (Figure 1.11) (Dittmer 2003). ETS1 targets include MMP family members and uPA, which are required for extracellular matrix degradation and subsequent cell invasion during normal or pathological processes. In vitro and in vivo studies have identified that upregulation of MMP or uPA expression in a variety of cell types results in enhanced cell invasiveness (Watabe et al. 1998; Nakada et al. 1999; Behrens et al. 2001; Baillat et al. 2002). ETS1-mediated increases in cell invasion are important for normal tissue development but also promote cancer progression. Overexpression of ETS1 in malignant human and mouse cell lines including hepatocellular carcinoma (in vitro), mouse mammary tumour cells (in vivo) and paclitaxel-resistant PC3 prostate cancer cells (in vitro) has been linked with significantly increased migratory and invasive cell characteristics via upregulation of MMPs and uPA (Jiang et al. 2001; Furlan et al. 2008; Kato et al. 2012).

In addition to its role in enhancing cancer cell movement, ETS1 is a potent promoter of angiogenesis in the developing vasculature and under pathological conditions. Ets1−/−.
Chapter 1: General Introduction

/Ets2^{A72/A72} mice which are homozygous for Ets1 knockout and express a mutant Ets2^{A72} that affects an ERK response element, display severely impaired angiogenesis, with defects in vessel branching and vascular complexity partially due to increased apoptosis of endothelial cell populations (Wei et al. 2009). The pro-angiogenic factor fibroblast growth factor 2 (FGF2) induces expression of Ets1 in mouse yolk sac endothelial cells which subsequently transcriptionally upregulates expression of vascular endothelial growth factor receptor (VEGFR), a potent mediator of vascular development and angiogenesis (Dutta et al. 2008). Conversely, in vitro silencing of ETS1 in human pancreatic cancer cells results in reduced expression of the ETS1 targets MMP1 and uPA and is associated with an anti-angiogenic effect (Lefter et al. 2009). Correlations between ETS1 overexpression and worse prognosis of patients with ovarian, oesophageal, breast, colorectal and cervical cancers has been reported to be due to higher microvessel density and increased VEGF expression (Oettgen 2010).

ETS1 is also a regulator of proliferation and apoptosis and is required for the formation of tumours from human renal clear cell carcinoma (RCC) and glioma (U87MG) cell lines where it regulates proliferation via transcriptional upregulation of the mitogen, transforming growth factor α (TGF-α) (Holterman et al. 2010). Knockdown of ETS1 and FLI1 expression in the OCI-Ly7 human lymphoma cell line results in reduced cell proliferation and increased apoptosis, suggesting that co-operative ETS1/FLI1 overexpression and function can sustain lymphoma cell viability (Bonetti et al. 2013). Together with its cofactor p300 (section 1.5.4), ETS1 is a transcriptional activator of the cyclin-dependent kinase inhibitor, p21\textsuperscript{WAF1/Cip1}, which inhibits apoptosis of vascular smooth muscle cells (Zhang et al. 2003). In the colon carcinoma cell line DLD1, only ETS1p42 overexpression was capable of rescuing defective Fas/caspase-1 mediated apoptosis and it is therefore hypothesised to regulate unique targets (and functions) compared to other ETS1 isoforms in colorectal tumours (Li et al. 1999; Pei et al. 2005).

ETS1 is both a target and regulator of micro-RNAs, potent suppressors of target gene expression, which are often aberrantly regulated or expressed in cancers. In human umbilical vein endothelial cells (HUVEC), ETS1 was shown to transcriptionally upregulate miR-126 expression in vitro, which is reported to promote angiogenesis and vascular inflammation (Harris et al. 2010). In contrast, miR-320, a regulator of
glycolysis in response to oxidative stress, is transcriptionally repressed by ETS1 in the mouse myoblast cell line, C2C12 (Tang et al. 2012).

**Figure 1.11:** Proposed functions of ETS1 in mammals including differentiation, migration/invasion, angiogenesis, apoptosis, morphogenesis and hormone production, which is mediated by transcriptional regulation of ETS1 target genes (Dittmer 2003).

Among the better characterised functions of ETS1 is its role as a negative regulator of lymphocyte cell differentiation (Russell et al. 2010). In naïve B cells, ETS1 expression is high and is believed to suppress inappropriate activation of human and mouse B cells as evidenced by the impaired B cell differentiation and reduced B-cell numbers following loss of ETS1 expression (Eyquem et al. 2004; John et al. 2008). In resting human T cell populations, ETS1 is highly expressed and studies have illustrated defects in T cell differentiation induced by T cell receptor (TCR) signalling in Ets-1 knockout mice (Bhat et al. 1990; Eyquem et al. 2004). In addition to B and T cell regulation, ETS1 is also essential for natural killer (NK) cell development, with loss of Ets1 resulting in similarly impaired NK cell development and defects in immune functions in mice (Barton et al. 1998).

### 1.5.4 ETS1 Cofactors

Numerous factors modulate ETS1 expression, function and transcriptional activity through direct protein-protein interactions, signalling pathway crosstalk or via cooperative target gene regulation. A diverse range of ETS1 cofactors have been
characterised including transcription factors, histone deacetylases, micro-RNAs, receptor kinases, growth factors and other ETS factors (Dittmer 2003).

Transcription factors such as AML-1, HIF-2α and PIT-1 bind the human and mouse ETS1 ID and TAD resulting in repressed or enhanced ETS1 DNA binding respectively, thereby altering target gene transcription (Kim et al. 1999; Goetz et al. 2000; Augstijn 2002; Elvert et al. 2003). Interaction with the transcription factor PAX5 modifies ETS1 DNA binding domain structure via interactions with a tyrosine residue (T395), facilitating DNA binding even at low affinity binding sites and enhances ETS1-mediated transcription of target genes (Garvie et al. 2001). Interactions with factors such as CREB binding protein (CBP/p300) which possess intrinsic acetyltransferase activities, promote histone remodelling thereby exposing DNA to enhanced ETS1-mediated transcription (with transcription factors/general transcriptional machinery) (Janknecht et al. 1996; Yang et al. 1998; Li et al. 2000a).

ETS1 expression and transcriptional functions are affected by crosstalk with the transforming growth factor beta (TGF-β) signalling pathway through reciprocal regulation and interaction with the signalling intermediates SMAD2 and SMAD3 (section 1.6.1) (Koinuma et al. 2009). SMAD2 and SMAD3 directly bind the ETS1 promoter in the TGF-β stimulated human keratinocyte cell line, HaCaT in vitro and conversely, EBS’s were identified using ChIP-chip analyses in promoter regions of SMAD2 and SMAD3 (Koinuma et al. 2009). In studies using HaCaT and MDA-MB-231 breast cancer cells, silencing or overexpression of ETS1 significantly altered TGF-β/SMAD target gene expression and impaired TGF-β mediated cell growth arrest (Lindemann et al. 2001; Koinuma et al. 2009). ETS1 also complexes with SMAD2 and SMAD3 which is hypothesised to contribute to the ETS1-mediated regulation of SMAD target genes that do not contain an EBS (Koinuma et al. 2009).

AR ChIP-chip studies have identified that in the human prostate cancer cell line, LNCaP, a high proportion (~70%) of AR target gene promoters were enriched for ETS1 binding sites and able to be co-occupied by the AR and ETS1 (Massie et al. 2007). Many of the AR/ETS1 regulated target genes had been reported to be aberrantly overexpressed in primary and metastatic prostate cancers and their expression was frequently downregulated following either androgen ablation therapies or AR knockdown in vitro (Holzbeierlein et al. 2004; Velasco et al. 2004; Haag et al. 2005;
Chapter 1: General Introduction

Varambally et al. 2005; Massie et al. 2007). In addition to promoter co-occupancy, co-immunoprecipitation of the AR and ETS1 in androgen stimulated LNCaP cells suggested that the AR and ETS1 may function in the same protein complexes in prostate tumours, with ETS1 overexpression and AR activation enhancing transcriptional upregulation of target genes (Massie et al. 2007).

1.6 Epithelial to Mesenchymal Transition (EMT)

Epithelial-to-mesenchymal transition (EMT) is an evolutionarily conserved and reversible trans-differentiation process during which epithelial cells undergo a loss of cell polarity and cell-to-cell adhesion and a gain in invasive and migratory characteristics that ultimately enable migration away from the epithelial layer or tissue of origin (Kalluri et al. 2009). A number of critical events initiate and drive the EMT programme including the expression and activation of EMT promoting transcription factors, altered expression of epithelial and mesenchymal markers, and cytoskeleton reorganisation (Figure 1.12) (Kalluri et al. 2009; Sanchez-Tillo et al. 2012). The reversible and transient nature of EMT implies that when no longer under pro-EMT stimuli, cells will transition back to an epithelial-like state that is phenotypically similar to the cell of origin. This reverse process termed mesenchymal-to-epithelial transition (MET) normally occurs following completion of the movement of cells to a secondary site and is dictated by the ability of specific cell types to undergo MET, a property often referred to as plasticity (Kalluri et al. 2009).

During mammalian embryogenesis, tissue development and adult tissue homeostasis, select populations of epithelial cells have been reported to undergo EMT and MET, processes also normally associated with tissue repair and inflammation in addition to other pathological processes including cancer development and progression. On the basis of the biological setting, EMT can be broadly divided into three categories, Type 1: EMT during implantation, embryogenesis and organ development; Type 2: EMT during tissue regeneration or organ fibrosis; and Type 3: EMT during cancer progression and metastasis (Kalluri et al. 2009; Zeisberg et al. 2009).

Extensive studies using vertebrate models have identified that during embryogenesis, EMT is essential for gastrulation, development of the primitive streak, neural crest delamination and for organogenesis (e.g. liver, pancreas, heart) (Shook et al. 2003; Huang et al. 2004; Thiery et al. 2009; Lim et al. 2012). In situ studies of mouse
embryos have illustrated that formation of the primitive streak, progenitor of primary germ cell layers, is an evolutionarily conserved process, occurring in the posterior epiblast and is mediated by progressive EMT accompanied by loss of basal lamina (Williams et al. 2012). Another early developmental event is EMT of the primitive endoderm to form the parietal endoderm, the primary germ cell layer which subsequently forms the interior lining of the gastrointestinal and respiratory tracts, the thyroid, thymus and epithelium of the auditory tube and bladder (Veltmaat et al. 2000). Collected evidence also indicates that during implantation in humans, trophoblasts within the chorionic villi undergo EMT to invade surrounding endometrium (Kokkinos et al. 2010).

**Figure 1.12:** Epithelial-to-mesenchymal transition including epithelial marker expression in epithelial cells (yellow), intermediate or transitioning cells (yellow-green) and mesenchymal marker expression in trans-differentiated mesenchymal-like cells (green) (Kalluri et al. 2009).

*In vitro* studies using human choriocarcinoma cells and endometrial adenocarcinoma cells lines indicate that differentiation and invasion is directed by EMT, a pivotal process for proper human embryo implantation (Uchida et al. 2012). Furthermore, *in vitro* culture of HES4 human embryonic stem cells identified that undifferentiated cells supported with embryonic fibroblasts as a feeder layer underwent limited differentiation. However, to recapitulate human development, removal of feeder cell populations resulted in EMT induction and cell differentiation, which was proposed to represent a critical process in human embryonic stem cell differentiation *in utero* (Eastham et al. 2007; Ullmann et al. 2007). Neural crest delamination is also a well-characterised EMT-mediated process in which embryonic progenitor cells undergo EMT (and subsequent MET) for proper cell movement and translocation within the embryo, prior to terminal neural crest differentiation processes (Ahlstrom et al. 2009a;
Ahlstrom et al. 2009b). Proper neural crest cell delamination is temporally regulated and disrupted following modifications in the expression of intrinsic EMT regulators (e.g. increased N-cadherin) indicative of the plasticity of the cell populations (Shoval et al. 2007). EMT-associated heart morphogenesis has been modelled in mice, quails and chickens and involves four successive cycles of EMT followed by MET to generate distinct regions of the heart including the heart primordium, endothelial cell lining and endocardial cushion, and development of epicardial-derived cells (Ladd et al. 1998; Sugi et al. 2003; Lim et al. 2012; von Gise et al. 2012).

EMT (Type 2) also occurs at sites of adult tissue regeneration or chronic fibrosis. This has been modelled in pancreatic ductal cells, a subset of which were identified using in vitro and in vivo culture to undergo EMT, representing renewal of pancreatic tissue following injury or regeneration under homeostatic conditions (Fanjul et al. 2010). Re-epithelialisation following cutaneous wounds results in basal keratinocyte cell infiltration and migration (via desmosome disruption) and induction of EMT processes for tissue repair (Arnoux et al. 2008). Culture of mouse airway epithelial cells in vitro following an artificial wound resulted in EMT-mediated cell migration at the wound edge, facilitating proper wound closure (McCormack et al. 2013). Type 2 EMT also includes non-cancer associated pathophysiological conditions which, in contrast to Type 1 EMT, occur as a response to persistent trauma and inflammation, that when ceased can result in reversal of EMT. These attributes are distinct from cell plasticity as chronic insults drive EMT and are not a result of internal cell programming via typical pro-EMT stimuli. Apparent in developed or adult tissues, Type 2 disease-based EMT characteristically occurs during events such as organ fibrosis, for which mouse models have been used to characterise organ-specific epithelia, including hepatocytes (liver) or alveolar (lung) epithelial cells that undergo EMT leading to increased deposition of extracellular matrix (ECM), contributing to the progression of liver and lung fibrosis (Kim et al. 2006; Zeisberg et al. 2007). Similar features have been identified in cells of the eye and kidney under regenerative and fibrotic conditions (Kato et al. 2007; Zheng et al. 2009). Evidence from Type 1 and 2 EMT has prompted characterisation of EMT-associated functions in cancer (Type 3), leading to the general hypothesis that EMT processes are exploited by a subset of aggressive cancers to enhance tumour cell motility and invasive characteristics to promote metastasis (Kalluri et al. 2009; Thiery et al. 2009; Nauseef et al. 2011; Grant et al. 2013).
1.6.1 EMT Signalling Pathways and Cancer Progression

The physiological and pathophysiological molecular processes of EMT are driven by a number of common signalling pathways which drive EMT in various cell types through regulation of expression of epithelial and mesenchymal associated genes (Figure 1.12). Aberrant or hyperactivated signalling events have been reported in tumours to stimulate epithelial cell invasiveness and motility, consequences of the EMT program which facilitate cancer progression and are associated with poorer disease prognosis.

One of the best characterised EMT-promoting signalling pathways is the transforming growth factor beta (TGF-β) pathway. TGF-β is a potent inducer of EMT while the TGF-β-related bone morphogenetic proteins (BMPs), which are typically inducers of MET following Type 1 EMT, have been reported to mediate physiological epithelial cell plasticity (Xu et al. 2009). Evidence of TGF-β induced EMT was first observed in mammary epithelial cells cultured in the presence of TGF-β, which underwent morphological changes from cuboidal to spindle-like cells. This occurred in conjunction with decreased expression of the epithelial marker, E-cadherin and increased expression of the mesenchymal markers, vimentin, fibronectin and N-cadherin (Miettinen et al. 1994; Lee et al. 2013). Under physiological conditions, TGF-β signalling promotes Type 1 EMT in epithelial cells of vertebrates including those of the developing mesoderm, epiblast, heart, teeth and other tissues (Lehnert et al. 1988; Sanders et al. 1991; Vaalhtokari et al. 1991; Nakajima et al. 2000).

TGF-β signal transduction occurs through binding of ligands TGF-β1, TGF-β2 and TGF-β3 to their cognate membrane receptor, TGF-β type 2 receptor (TGF-βR2) (Figure 1.13) (Lin et al. 1992; Lin et al. 2006). TGF-βR2 has serine-threonine kinase activity which, following ligand binding, phosphorylates TGF-β type 1 receptor (TGF-βR1) (Wrana et al. 1994; Kawabata et al. 1995). Subsequent to these events, SMAD molecules (SMAD2/SMAD3) are recruited and anchored to the intracellular domain of TGF-βR1, where they are activated via phosphorylation (Marcias-Silva et al. 1996; Zhang et al. 1996; Kretzschmar et al. 1997). Phosphorylated SMADs are transcriptionally active and can translocate into the nucleus (often in complexes containing SMAD4), binding to sequences containing a 5’-GTCTAGAC-3’ core element, or half sites 5’-GTCT-3’ and 5’-AGAC-3’ to regulate expression of genes
which have broad functions in cellular processes including cell proliferation, apoptosis and differentiation (Nakao et al. 1997; Labbe et al. 1998; Zawel et al. 1998; Massague et al. 2005; Koinuma et al. 2009). In the context of EMT, SMAD regulated genes include members of the SNAIL (e.g. SNAIL1, SLUG), ZEB (e.g. ZEB1/2) and TWIST (TWIST1) families that promote EMT (Xu et al. 2009). TGF-β signalling also promotes EMT via induction of non-SMAD signalling (e.g. p38/JNK MAPK, RhoA, PI3K/Akt/mTOR) and through crosstalk with other pro-EMT signalling pathways (e.g. WNT, NOTCH). Although these functions are equally significant in EMT regulation, due to their indirect regulation of common EMT processes and the complexity of signalling-pathway crosstalk, they are not as well defined (Xu et al. 2009). Disruptions in both SMAD (canonical) and non-SMAD (non-canonical) TGF-β signalling resulting in activation of EMT have been implicated in the progression of numerous cancer types (Wendt et al. 2009; Katsuno et al. 2013).

Figure 1.13: TGF-β induced EMT. TGF-β signalling induced by TGF-β ligand binding to membrane receptors (TGF-βR1/2), activation of SMAD2/3/4 signalling intermediates and regulation of SNAIL, ZEB and bHLH target gene families (yellow), decreases epithelial marker expression (green) and increases mesenchymal marker expression (pink) (Xu et al. 2009).
Chapter 1: General Introduction

In addition to TGF-β signalling, the canonical WNT (wingless orthologue)/β-catenin signalling pathway is able to induce EMT under physiological and pathophysiological conditions (Kim et al. 2002a; Clevers 2006). There are 19 human WNT proteins (with homologues in invertebrates and vertebrates), many of which bind cell surface frizzled receptor (Fz) and low density lipoprotein (LDL) receptor-related protein (LRP) complexes (Hsieh et al. 1999; Tamai et al. 2000). This results in signal transduction to a number of intracellular mediators including axin, dishevelled (DSH), glycogen synthase kinase-3β (GSK-3β), adenomatous polyposis coli (APC) and the transcriptional regulator, β-catenin (Logan et al. 2004). In the absence of WNT, phosphorylated β-catenin is degraded through the proteasome, which is directed by the GSK-3/APC/Axin complex, with loss of any complex member associated with aberrant β-catenin accumulation (Rubinfeld et al. 1996; Korinek et al. 1997; Ikeda et al. 1998). Although the exact mechanisms resulting in β-catenin accumulation are only partially characterised, this effect occurs following WNT stimulation and results in its nuclear translocation and transcriptional activity (Logan et al. 2004). β-catenin target genes include regulators of cell proliferation, migration, apoptosis and differentiation, thereby inducing both developmental and pathological EMT (Jiang et al. 2007; Kato et al. 2007; Iwai et al. 2010; Zuchini-Pascal et al. 2013). Expression of EMT-promoting factors (e.g. SLUG, TWIST and ZEB) and mediators of TGF-β signalling are regulated by β-catenin and these factors in turn have been identified to regulate β-catenin expression and activity (Conacci-Sorrell et al. 2003; Howe et al. 2003; Hirota et al. 2008; Sanchez-Tillo et al. 2011; Zhou et al. 2012).

Specific WNT ligand knockout mice have been used to characterise essential functions during embryonic development and redundancy in the WNT family, identifying roles in gastrulation, neural crest delamination, limb/organ morphogenesis and in other EMT-associated processes (Ikeya et al. 1997; Yamaguchi et al. 1999; Li et al. 2002; Barrow et al. 2003). Additional functions of the WNT/β-catenin pathway include regulation of expression of genes (e.g. cyclin D1, MMP2, MMP7, fibronectin) which function in Type 2 EMT and are associated with regeneration and chronic fibrosis of adult tissues including the kidney, lung and eye (Shtutman et al. 1999; Kato et al. 2007; Doyle et al. 2009; Zheng et al. 2009; Henderson Jr et al. 2010; Baarsama et al. 2011; Zhou et al. 2013).
Chapter 1: General Introduction

As outlined above, the process of EMT is mediated by alterations in the expression of epithelial and mesenchymal markers which are temporally controlled by the pro-EMT transcription factors SNAIL, SLUG, TWIST and ZEB. A classic hallmark of epithelial cells undergoing EMT is loss of the adherens junction protein E-cadherin via transcriptional repression or as a result of its intracellular mislocalisation. SNAIL1 can directly bind the E-cadherin gene promoter and repress its transcription, resulting in decreased E-cadherin expression and dysregulated cell-cell junction structure, thereby facilitating cell migration (Batlle et al. 2000). In addition to SNAIL1, SLUG, TWIST and ZEB1/2 have also been identified to bind the E-cadherin promoter, resulting in the same functional outcome in various cell types (Comijn et al. 2001; Grotteclaes et al. 2000; Hajra et al. 2002; Vesuna et al. 2008; Sanchez-Tillo et al. 2010). The switch from expression of epithelial to mesenchymal markers (e.g. E-cadherin to N-cadherin) and cytoskeleton proteins (e.g. cytokeratins to vimentin), and the induced expression of factors which degrade extracellular matrix (e.g. uPA, MMPs) promote the transdifferentiation and migration of cells that typifies EMT (Figure 1.12) (Jorda et al. 2005; Alexander et al. 2006; Kalluri et al. 2009; Sanchez-Tillo et al. 2012). In tumours, expression of pro-EMT transcription factors or increased mesenchymal and decreased epithelial marker expression is generally correlated with poorer prognosis due to enhanced invasiveness and metastasis (Das et al. 2014).

Both WNT induced β-catenin and TGF-β induced SMAD signalling transcriptionally enhance expression of ZEB1, which primarily represses expression of E-cadherin and is believed to be one of the first events of EMT (Shirakihara et al. 2007; Sanchez-Tillo et al. 2011). Concomitantly, factors such as TWIST1 can directly upregulate N-cadherin expression, mediating the cadherin ‘switch’ typified by cells undergoing EMT (Alexander et al. 2006). In addition to the regulation of ZEB1 expression, TGF-β and WNT signalling can cooperate or may separately induce expression of SNAIL1, SLUG and TWIST1. β-catenin transcriptionally regulates expression of the mesenchymal marker and intermediate filament, vimentin and may also indirectly regulate its expression via induction of SLUG, another mesenchymal biomarker associated with cell migration (Conacci-Sorrell et al. 2003; Gilles et al. 2003; Howe et al. 2003; Vuoriluoto et al. 2011). SMADs similarly transcriptionally activate β-catenin and vimentin expression following TGF-β stimulation, indicating the importance of signalling crosstalk during EMT that provides multiple mechanisms of regulation of common
target genes (Wu et al. 2007; Hirota et al. 2008). The transcription factors SNAIL1 and SLUG additionally repress expression of Claudin-1, and this is associated with loss of tight junctions between epithelial cells thereby facilitating epithelial cell movement (Martinez-Estrada et al. 2006). Expression of TGF-β, WNT or signalling intermediates (e.g. SMADs, β-catenin) is upregulated in many cancer types including breast, colorectal, lung, and prostate cancers, inducing EMT-associated cancer progression and metastasis in association with poor patient outcome (Wikstrom et al. 1998; Kang et al. 2000; Dimitriadis et al. 2001; Xiong et al. 2002; Huang et al. 2008; Padua et al. 2008; DiMeo et al. 2009; Thiele et al. 2011; Green et al. 2013).

Prostate tumours with elevated expression of TGF-β are reported to have higher vascular density (angiogenesis), increased metastasis and poor clinical outcome (Wikstrom et al. 1998). In contrast, expression of BMPs which function as TGF-β antagonists is reduced in prostate tumours, which are therefore unable to maintain equilibrium of TGF-β signalling (Ye et al. 2009). In the prostate cancer cell lines PC-3 and DU145, TGF-β stimulates production of uPA which promotes cell invasion (Desruisseau et al. 1996). Higher levels of WNT/β-catenin activity are also evident in more aggressive prostate cancer cell lines, and have been associated with enhancing migratory and invasive characteristics (Jiang et al. 2007). Interestingly, TGF-β1 stimulation of the LNCaP, C4-2 and C4-2B prostate cancer cell lines (in order of least to most castrate-resistant/aggressiveness) resulted in enhanced phosphorylation of SMAD2 which inhibited C4-2 and C4-2B proliferation and migration, but did not affect LNCaP migration (Miles et al. 2012). These data indicated that TGF-β/SMADs can inhibit the growth of prostate cancer cells themselves and contrast findings in primary tumours where increased TGF-β/SMAD activation is associated with metastasis and therefore higher rates of cell migration (Wikstrom et al. 1998; Miles et al. 2012). This has led to the hypothesis that the role of TGF-β in prostate cancer may be dependent on disease progression and castrate-resistance, and suggests that tumour cells may eventually reverse the inhibitory effects of TGF-β to positively regulate growth and metastasis (Barrack 1997). In prostate cancer cell lines, ZEB1 and SLUG expression is also regulated by the AR, expression of which may be high in castrate-resistant tumours. The findings indicate an alternative pathway leading to activation of pro-EMT transcription factors and its association with advanced disease (Anose et al. 2011; Wu et al. 2012).
1.7 **Statement of Aims**

The transcription factor, ETS1 is widely expressed during development and in adult tissues where it regulates cell proliferation, migration and invasion (Dittmer 2003; Hahne *et al.* 2008). ETS1 can be alternatively spliced to generate three mRNA/protein isoforms ETS1p51, ETS1p42 and ETS1p27, which are structurally and functionally distinct, although the relative expression and tissue-specific distribution of the isoforms are incompletely characterised (Fisher *et al.* 1994; Dittmer 2003; Laitem *et al.* 2009). The expression of ETS1 is elevated in up to 80% of prostate tumours and its overexpression is associated with higher Gleason score of the primary tumours, rapid progression to castrate resistance and poorer patient prognosis (Alipov *et al.* 2005; Li *et al.* 2012a; Smith *et al.* 2012a). Epithelial-to-mesenchymal transition (EMT), a reversible transdifferentiation process involved in embryogenesis, organogenesis, wound-healing and other normal physiological mechanisms is evident in rapidly progressing tumours including prostate cancers where its promotion of cell migration and invasion facilitates metastasis (Thiery *et al.* 2006; Thiery *et al.* 2009; Grant *et al.* 2013). To characterise ETS1 isoform expression in prostate cancer and the association between elevated ETS1 expression and poor disease outcomes, the specific aims of this research were:

1. To determine ETS1 mRNA and protein isoform expression in human prostate tumours and adjacent nonmalignant prostate.

2. To identify and characterise induction of EMT in prostate cancer cells following ETS1 overexpression.
Chapter 2: Materials

2.1 Reagents

2.1.1 Antibodies

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donkey Anti-Goat IgG, HRP conjugated</td>
<td>Santa Cruz Biotechnology, USA</td>
</tr>
<tr>
<td>Goat Anti-Human Polyclonal IgG, β-actin (Clone I-19)</td>
<td>Santa Cruz Biotechnology, USA</td>
</tr>
<tr>
<td>Mouse Anti-Green Fluorescent Protein (GFP) Monoclonal IgG</td>
<td>Roche Applied Science, USA</td>
</tr>
<tr>
<td>Mouse Anti-Human Monoclonal IgG, ETS1 (Clone 1G11)</td>
<td>Abcam, USA</td>
</tr>
<tr>
<td>Mouse Anti-Human Polyclonal IgG, Androgen Receptor (Clone AR441)</td>
<td>DAKO Corporation, USA</td>
</tr>
<tr>
<td>Rabbit Anti-Human Monoclonal IgG, Phospho-Smad2 (Ser465/467) (Clone 138D4)</td>
<td>Cell Signaling Technology, USA</td>
</tr>
<tr>
<td>Rabbit Anti-Human Monoclonal IgG, Smad2 (Clone EP567Y)</td>
<td>Abcam, USA</td>
</tr>
<tr>
<td>Rabbit Anti-Human Polyclonal IgG, ETS1 (Clone C-20)</td>
<td>Santa Cruz Biotechnology, USA</td>
</tr>
<tr>
<td>Sheep Anti-Mouse Ig, Affinity isolated, HRP conjugated</td>
<td>Chemicon, Australia</td>
</tr>
<tr>
<td>Sheep Anti-Rabbit Ig, Affinity isolated, HRP conjugated</td>
<td>Chemicon, Australia</td>
</tr>
</tbody>
</table>

2.1.2 Bacterial Culture and Plasmid Preparation

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>1kb Plus DNA Ladder (1.0μg/μL)</td>
<td>Life Technologies, USA</td>
</tr>
</tbody>
</table>
### Chapter 2: Materials

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar (Bacteriological grade)</td>
<td>Amresco, USA</td>
</tr>
<tr>
<td>Agarose</td>
<td>Amresco, USA</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>Amresco, USA</td>
</tr>
<tr>
<td>Bacto-Tryptone</td>
<td>Amresco, USA</td>
</tr>
<tr>
<td>Ethidium Bromide</td>
<td>ICN Biochemicals Inc, USA</td>
</tr>
<tr>
<td>Ethylenediaminotetraacetic acid (EDTA)</td>
<td>Amersco, USA</td>
</tr>
<tr>
<td>Glacial Acetic Acid</td>
<td>BDH Chemicals, Australia</td>
</tr>
<tr>
<td>Glucose</td>
<td>Sigma, USA</td>
</tr>
<tr>
<td>Glycerol</td>
<td>Sigma, USA</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>CSL Limited, Australia</td>
</tr>
<tr>
<td>Potassium Acetate</td>
<td>BDH AnalaR®, Australia</td>
</tr>
<tr>
<td>RNase H (10U/μL)</td>
<td>Promega, Australia</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>Amresco, USA</td>
</tr>
</tbody>
</table>

#### 2.1.3 Tissue Culture

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>5α-dihydrotestosterone (DHT)</td>
<td>Sigma Aldrich, USA</td>
</tr>
<tr>
<td>Charcoal Stripped Serum (CSS)</td>
<td>Serana, Australia</td>
</tr>
<tr>
<td>COS-7 Monkey Kidney Cell Line</td>
<td>American Type Culture Collection, USA</td>
</tr>
<tr>
<td>Dimethyl sulphoxide (DMSO)</td>
<td>ICN Biochemicals Inc, USA</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>Clontech Laboratories, USA</td>
</tr>
<tr>
<td>Foetal Calf Serum (FCS)</td>
<td>Serana, Australia</td>
</tr>
<tr>
<td>Geneticin (G418) (50mg/mL)</td>
<td>Life Technologies, USA</td>
</tr>
<tr>
<td>Hygromycin B (50mg/mL)</td>
<td>Life Technologies, Australia</td>
</tr>
<tr>
<td>LNCaP Prostate Cancer Cell Line</td>
<td>American Type Culture Collection, USA</td>
</tr>
<tr>
<td>MCF-7 Breast Cancer Cell Line</td>
<td>American Type Culture Collection, USA</td>
</tr>
<tr>
<td>MDA-MB-231 Breast Cancer Cell Line</td>
<td>American Type Culture Collection, USA</td>
</tr>
<tr>
<td>Metafectene Pro®</td>
<td>Biontex Laboratories GmbH, Germany</td>
</tr>
</tbody>
</table>
Chapter 2: Materials

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin/Streptomycin 1000IU/mL (1mg/mL)</td>
<td>Gibco© Life Technologies, Australia</td>
</tr>
<tr>
<td>Puromycin dichloride (10mg/mL)</td>
<td>Life Technologies, Australia</td>
</tr>
<tr>
<td>Recombinant Human TGF-beta 1 (2µg)</td>
<td>R&amp;D systems, USA</td>
</tr>
<tr>
<td>RPMI 1640 with L-Glutamine</td>
<td>Thermo Electron Corporation, Australia</td>
</tr>
<tr>
<td>SB431542 (10mg)</td>
<td>Tocris Bioscience, UK</td>
</tr>
<tr>
<td>Sodium Hydrogen Carbonate</td>
<td>Merck Pty Ltd., Germany</td>
</tr>
<tr>
<td>Trypsin/EDTA (0.25%)</td>
<td>Gibco© Life Technologies, Australia</td>
</tr>
</tbody>
</table>

2.1.4 Reverse Transcription, Polymerase Chain Reaction (PCR) and qPCR

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>dNTP mixture (100mM)</td>
<td>Promega, Australia</td>
</tr>
<tr>
<td>MgCl2 (50mM)</td>
<td>Life Technologies, Australia</td>
</tr>
<tr>
<td>Oligo dT Primer (0.5µg/µL)</td>
<td>Promega, Australia</td>
</tr>
<tr>
<td>Platinum Taq DNA Polymerase (5U/µL)</td>
<td>Life Technologies, Australia</td>
</tr>
<tr>
<td>Random Hexamers (0.5µg/µL)</td>
<td>Promega, Australia</td>
</tr>
<tr>
<td>Primers (sequences listed in Appendix 2)</td>
<td>Geneworks, Australia</td>
</tr>
<tr>
<td>RT² SYBR Green qPCR Mastermix, 2x</td>
<td>SABiosciences, Australia</td>
</tr>
</tbody>
</table>

2.1.5 Plasmids and Linear Markers

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear Hygromycin Marker (Tet-On 3G)</td>
<td>Clontech Laboratories, USA</td>
</tr>
<tr>
<td>Linear Puromycin Marker (Tet-On 3G)</td>
<td>Clontech Laboratories, USA</td>
</tr>
<tr>
<td>pCMV-Tet3G</td>
<td>Clontech Laboratories, USA</td>
</tr>
<tr>
<td>pcDNA3.1</td>
<td>Life Technologies, Australia</td>
</tr>
<tr>
<td>pcDNA3.1/V5-His-TOPO</td>
<td>Life Technologies, Australia</td>
</tr>
<tr>
<td>pEGFPC2</td>
<td>Clontech Laboratories, USA</td>
</tr>
<tr>
<td>pGEM-T Easy</td>
<td>Promega, Australia</td>
</tr>
</tbody>
</table>
Chapter 2: Materials

pTRE3G-IRES Clontech Laboratories, USA
pTRE3G-LUC Clontech Laboratories, USA

2.1.6 Cloning and Restriction Enzymes

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApaI-HF</td>
<td>New England Biolabs, UK</td>
</tr>
<tr>
<td>BamHI-HF</td>
<td>New England Biolabs, UK</td>
</tr>
<tr>
<td>Buffer 2 (10x)</td>
<td>New England Biolabs, UK</td>
</tr>
<tr>
<td>Buffer 4 (10x)</td>
<td>New England Biolabs, UK</td>
</tr>
<tr>
<td>Buffer C (10x)</td>
<td>Promega, Australia</td>
</tr>
<tr>
<td>EcoRI-HF</td>
<td>New England Biolabs, UK</td>
</tr>
<tr>
<td>Escherichia coli DH5α</td>
<td>Life Technologies, Australia</td>
</tr>
<tr>
<td>HindIII</td>
<td>New England Biolabs, UK</td>
</tr>
<tr>
<td>MluI</td>
<td>New England Biolabs, UK</td>
</tr>
<tr>
<td>SalI</td>
<td>New England Biolabs, UK</td>
</tr>
<tr>
<td>Multi Core Buffer</td>
<td>Promega, Australia</td>
</tr>
<tr>
<td>RNase A</td>
<td>Roche Diagnostics, Australia</td>
</tr>
<tr>
<td>SAP Dephosphatase Buffer</td>
<td>Roche Diagnostics, Australia</td>
</tr>
<tr>
<td>Shrimp Alkaline Phosphatase (SAP)</td>
<td>Roche Diagnostics, Australia</td>
</tr>
<tr>
<td>T4 DNA Ligase</td>
<td>New England Biolabs, UK</td>
</tr>
<tr>
<td>T4 DNA Ligase Reaction Buffer (10x)</td>
<td>New England Biolabs, UK</td>
</tr>
</tbody>
</table>

2.1.7 Western Blotting

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>40% Acrylamide/Bis-Acrylamide (37.5:1)</td>
<td>Amresco Laboratories, USA</td>
</tr>
<tr>
<td>Ammonium Persulphate (APS)</td>
<td>Bio-Rad Laboratories, USA</td>
</tr>
<tr>
<td>Bovine Serum Albumin (BSA)</td>
<td>Sigma, USA</td>
</tr>
<tr>
<td>2-Mercaptoethanol</td>
<td>BDH Chemicals, England</td>
</tr>
</tbody>
</table>
## Chapter 2: Materials

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bromophenol Blue</td>
<td>BDH Chemicals, England</td>
</tr>
<tr>
<td>Colour Plus® Prestained Protein Marker</td>
<td>New England Biolabs, England</td>
</tr>
<tr>
<td>Developer Solution A G153 (HT536)</td>
<td>AGFA-Gaevert, N.V., Belgium</td>
</tr>
<tr>
<td>Developer Solution B G153 (HT536)</td>
<td>AGFA-Gaevert, N.V., Belgium</td>
</tr>
<tr>
<td>Glycine</td>
<td>Amresco, USA</td>
</tr>
<tr>
<td>Hybond – C Extra Nitrocellulose</td>
<td>GE Healthcare, Australia</td>
</tr>
<tr>
<td>Methanol (Analytical Grade)</td>
<td>Biolab Ltd, Australia</td>
</tr>
<tr>
<td>Rapid Fixer Solution G354 (2828Q)</td>
<td>AGFA-Gaevert, N.V., Belgium</td>
</tr>
<tr>
<td>Skim Milk Powder</td>
<td>Bonlac Foods Ltd, Australia</td>
</tr>
<tr>
<td>Sucrose</td>
<td>Sigma, USA</td>
</tr>
<tr>
<td>TEMED</td>
<td>Bio-Rad Laboratories, USA</td>
</tr>
<tr>
<td>Tween 20</td>
<td>Biochemicals, Australia</td>
</tr>
</tbody>
</table>

### 2.1.8 General Reagents

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine 5' Triphosphate (ATP)</td>
<td>Sigma Aldrich, USA</td>
</tr>
<tr>
<td>Big Dye Terminator (v3.1)</td>
<td>Applied Biosystems, USA</td>
</tr>
<tr>
<td>Big Dye Terminator Sequencing Buffer</td>
<td>Applied Biosystems, USA</td>
</tr>
<tr>
<td>Boric Acid, Sodium Decahydrate (Borax)</td>
<td>Amresco, USA</td>
</tr>
<tr>
<td>Calcium Chloride</td>
<td>Ajax Chemicals, Australia</td>
</tr>
<tr>
<td>Chloroform</td>
<td>Ajax Chemicals, Australia</td>
</tr>
<tr>
<td>Co-enzyme A Lithium Salt</td>
<td>Sigma Aldrich, USA</td>
</tr>
<tr>
<td>D-Luciferin Potassium Salt</td>
<td>Life Technologies, Australia</td>
</tr>
<tr>
<td>Diethylpyrocarbonate (DEPC)</td>
<td>Sigma Aldrich, USA</td>
</tr>
<tr>
<td>Disodium Hydrogen Orthophosphate</td>
<td>BDH AnalalR®, Australia</td>
</tr>
<tr>
<td>Dithiothreitol (DTT)</td>
<td>Sigma Aldrich, USA</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Rowe Scientific, Australia</td>
</tr>
<tr>
<td>Hydrochloric Acid</td>
<td>BDH Chemicals, England</td>
</tr>
</tbody>
</table>
Chapter 2: Materials

Igepal (NP-40)     Sigma Aldrich, USA
Isopropanol     BDH Chemicals, England
Magnesium Chloride    BDH AnalaR®, Australia
Orthophosphoric Acid  Ajax Chemicals, Australia
Phenylmethanesulphonylfluoride (PMSF) Sigma Aldrich, USA
Potassium Chloride    BDH AnalaR®, Australia
Potassium Dihydrogen Orthophosphate  BDH AnalaR®, Australia
Sodium Acetate        BDH AnalaR®, Australia
Sodium Chloride  BDH AnalaR®, Australia
Sodium Dodecyl Sulphate (SDS)  Amresco, USA
Sodium Fluoride      BDH AnalaR®, Australia
Sodium Hydroxide  BDH AnalaR®, Australia
Sodium Phosphate   BDH AnalaR®, Australia
Sodium Vanadate     BDH AnalaR®, Australia
Toluidine Blue O     Amresco, USA
Tris Ultrapure Grade Amresco, USA
Triton X-100         Sigma Aldrich, USA
Water for Irrigation  Baxter Healthcare Pty Ltd, Australia
UltraspecTM RNA (200mL) Fisher Biotech, Australia
Zinc Chloride  Sigma Aldrich, USA

2.2 Equipment/Materials

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2mL Microcentrifuge Tubes</td>
<td>Bio-Rad Laboratories, USA</td>
</tr>
<tr>
<td>0.5mL and 1.5mL Microcentrifuge Tubes</td>
<td>Eppendorf, Germany</td>
</tr>
<tr>
<td>2mL Cryotubes</td>
<td>Apogent™, Denmark</td>
</tr>
<tr>
<td>5mL, 15mL and 50mL Tubes</td>
<td>Sarstedt, Germany</td>
</tr>
<tr>
<td>10, 200 and 1000μL Pipette Tips</td>
<td>Sarstedt, Germany</td>
</tr>
</tbody>
</table>
Chapter 2: Materials

Agfa CP1000 Developer  Agfa Healthcare, Belgium
Autoclave Tape  Cantech Industries Incorporated, USA
Autoradiography Cassette  Agfa Healthcare, Belgium
Baxter Medi-Vac CRD System  Baxter Healthcare Pty Ltd, Australia
Beckman Avanti™ Centrifuge J-25I  Beckman Coulter, USA
BH120 Biological Safety Cabinet Class II  Gelman Sciences Pty Ltd, Australia
C1000® Thermal Cycler  Bio-Rad Laboratories, USA
Carbon Steel Surgical Blades  Swann Morton Limited, England
Cellstar® Tissue Culture Flask 25cm² (50mL)  Greiner bio-one GmbH, Germany
and 75cm² (250mL)  Eppendorf Germany
Centrifuges 5415C, 5415R, 5804R, 5810R  Eppendorf Germany
CL-XPosure™ Blue X-ray Film (8in x 10in)  Thermo Scientific, USA
CoolSnap HQ2 Camera  Nikon, Japan
Corbett Liquid Handling Robot  Corbett Life Science, Australia
Cell Culture Inserts (0.22cm²)  IBIDI®, Germany
Dry Block Heater  Thermoline, Australia
Falcon® 96 Well Plates  Becton Dickinson, Australia
Gel Doc 2000 EQ Transilluminator  Bio-Rad Laboratories, USA
Glass Coverslips (22 x 22mm)  Bio-Rad Glaser, Denmark
Grant Waterbath  Grant Instruments Ltd, England
Horizontal Gel Electrophoresis Tank  Fischer Biotech, Australia
HP 5200 Precision Document Scanner  Hewlett-Packard Pty Ltd, Australia
IEC Magnetic Stirrer  Industrial Equipment and Control Pty Ltd, Australia
Improved Neubauer Haemocytometer  Hawksley, England
IS500 Colour CCD Camera (TCA5-N)  Tucsen, China
Laminar Flow Hood  Email-Westinghouse, Australia
Lean-Lite Light Box  Lean Pty Ltd, Australia
Chapter 2: Materials

Leica DM2500 Microscope
Leica Microsystems Pty Ltd, Australia

Microwave
Sharp, Australia

Millipore Pump with 7015 Head
Millipore, Australia

Mini PROTEAN® 3 Cell
Bio-Rad Laboratories, USA

Mini Sub™ DNA Cell
Bio-Rad Laboratories, USA

Mini Unistat Water Heater
Crown Scientific, Australia

Model 1000/500 Power Supply
Bio-Rad Laboratories, USA

Nanodrop® ND-1000 Spectrophotometer
BIOLAB Ltd, Australia

Needles 23G1 (0.63mm x 25mm)
Becton Dickinson, Australia

Nikon Coolpix 995 Digital Camera
Nikon, Japan

Nikon Eclipse Ti-E Microscope
Nikon, Japan

Olympus IX71 Microscope
Olympus, USA

Orbital Mixer Incubator
Ratek Instruments Pty Ltd, Australia

Parafilm® Laboratory Film
Pechiney Plastic Packaging, Australia

Powder Free, Latex, Examination Gloves
Ansell, Malaysia

PowerPac™ 300
Bio-Rad Laboratories, USA

PowerPac™ 3000
Bio-Rad Laboratories, USA

Protean® 2 Cell
Bio-Rad Laboratories, USA

Protean® Tetra Cell
Bio-Rad Laboratories, USA

PTC-100™ Programmable Thermal Controller
MJ Research, Australia

Red Rotor Shaker
Hoefer Scientific Instruments, USA

Sanyo CO₂ Incubator
Sanyo Electric, Japan

Tissue Culture Plate, 6 Well, Flat Bottom
Becton Dickinson, Australia

TPS pH Cube
TPS Pty Ltd, Australia

TransBlot® Turbo® Western System

Apparatus
Bio-Rad Laboratories, USA

Tuberculin Syringe, Latex Free (1ml)
Becton Dickinson, Australia

UV Sterilisation Cabinet
Starkeys, Australia
Chapter 2: Materials

Wallac VICTOR™ Luminometer (1420)  Wallac, Finland
Vortex Mixer               Raetek Instruments Pty Ltd, Australia
Whalton Plastics FC900 Fume Cabinet  Walton Plastics, Australia
Whatman® 3mm Paper       Whatman International, Australia
Zx³ Vortex              Velp Scientifica, Italy

### 2.3 Commercial Kits and Mastermixes

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD BioCoat™ Matrigel™ Invasion Chamber BD Biosciences, Australia</td>
<td></td>
</tr>
<tr>
<td>24-well BD Falcon TC Companion (354480)</td>
<td></td>
</tr>
<tr>
<td>• 24 inserts in 2 x 24-well Companion plates</td>
<td></td>
</tr>
<tr>
<td>ECL™ Prime Western Blotting Detection GE Healthcare, Australia</td>
<td></td>
</tr>
<tr>
<td>Reagent (RPN2232)</td>
<td></td>
</tr>
<tr>
<td>• Solution A: Luminol Solution (50mL)</td>
<td></td>
</tr>
<tr>
<td>• Solution B: Peroxide Solution (50mL)</td>
<td></td>
</tr>
<tr>
<td>ECL™ Select Western Blotting Detection GE Healthcare, Australia</td>
<td></td>
</tr>
<tr>
<td>Reagent (RPN2235)</td>
<td></td>
</tr>
<tr>
<td>• Solution A: Luminol Solution (50mL)</td>
<td></td>
</tr>
<tr>
<td>• Solution B: Peroxide Solution (50mL)</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 2: Materials

Epithelial-Mesenchymal Transition (EMT)    Cell Signaling Technology, USA

Antibody Sampler Kit (9782)

- Vimentin (D21H3) XP® Rabbit mAb (40μL)
- N-cadherin Rabbit mAb (40μL)
- Claudin-1 Rabbit mAb (40μL)
- β-catenin (D10A8) XP® Rabbit mAb (40μL)
- ZO-1 (D7D12) Rabbit mAb (40μL)
- Snail (C15D3) Rabbit mAb (40μL)
- Slug (C19G7) Rabbit mAb (40μL)
- TCF8/ZEB1 (D80D3) Rabbit mAb (40μL)
- E-cadherin (24E10) Rabbit mAb (40μL)
- Goat Anti-rabbit IgG, HRP-linked (100μL)

GoTaq® qPCR Master Mix (A6001)    Promega, Australia

- GoTaq® qPCR Master Mix, 2x (5 x 1mL)
- CXR Reference Dye (100μL)
- Nuclease-Free Water (2 x 13mL)

PureLink® HiPure Plasmid Midiprep Kit    Life Technologies, Australia (K2100-04)

- Resuspension Buffer R3 (100mL)
- RNase A (550μL)
- Lysis Buffer L7 (100mL)
- Precipitation Buffer N3 (100mL)
- Equilibration Buffer EQ1 (250mL)
- Wash Buffer W8 (500mL)
- Elution Buffer E4 (125mL)
- TE Buffer (15mL)
- Hi Pure Columns (25)
- Column Holders (5)
Chapter 2: Materials

QIAQuick Gel Purification Kit  
Qiagen, Australia  
(28106)
- QIAquick Spin Columns (250)
- Buffer PB (150mL)
- Buffer PE concentrate (55mL)
- Buffer EB (55mL)
- pH Indicator I (800μL)
- Collection Tubes, 2mL (250)
- Loading Dye (550μL)

QIAQuick PCR Purification Kit  
Qiagen, Australia  
(28706)
- QIAquick Spin Columns (250)
- Buffer QG (2 x 250mL)
- Buffer PE concentrate (2 x 50mL)
- Buffer EB (2 x 15mL)
- Collection Tubes, 2mL (250)
- Loading Dye (550μL)

RT² profiler: Human EMT PCR array  
SABiosciences, Australia  
(PAHS-090A)
- 384-well RT² Profiler PCR Arrays (4x)

RT² (cDNA) First Strand Kit  
SABiosciences, Australia  
(330401)
- Buffer GE (24μL)
- Buffer BC3 (48μL)
- RE3 Reverse Transcriptase Mix (24μL)
- Control P2 (12μL)
Chapter 2: Materials

- RNase-Free Water (1mL)

SuperScript™ III Reverse Transcriptase  Invitrogen, USA
(18080-093)
- SuperScript™ III
- 0.1M DTT
- 5x First-Strand Buffer

Tet-On® 3G Inducible Expression System  Clontech Laboratories, USA
(631166)
- pCMV-Tet3G – Regulator Plasmid (10μg)
- pTRE3G-IRES – Response Plasmid (10μg)
- pTRE3G-Luc – Control Response Plasmid (10μg)
- Linear Hygromycin Marker (2μg)
- Linear Puromycin Marker (2μg)
- Xfect™ Transfection Reagent (100 reactions)
- Tet System Approved FBS, US Sourced (50mL)

Renilla Luciferase® Assay System  Promega, Australia
(E2810)
- 5x Passive Lysis Buffer (30mL)
- 1x Renilla Luciferase® Assay Buffer (10mL)
- 100x Renilla Luciferase® Assay Substrate (1mL)

2.4 Computing Software

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>cellSens Standard</td>
<td>Olympus, Australia</td>
</tr>
<tr>
<td>Chromas Lite (2.1.1)</td>
<td>Technelysium, Australia</td>
</tr>
<tr>
<td>David Bioinformatics Database</td>
<td>NIH, USA</td>
</tr>
<tr>
<td>Endnote X6</td>
<td>ISI ResearchSoft, USA</td>
</tr>
</tbody>
</table>
### Chapter 2: Materials

<table>
<thead>
<tr>
<th>Software/Material</th>
<th>Provider/Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>GraphPad Prism 5</td>
<td>GraphPad Software Inc., USA</td>
</tr>
<tr>
<td>Image J</td>
<td>National Institutes of Health, USA</td>
</tr>
<tr>
<td>Ingenuity Pathway Analysis Software</td>
<td>Ingenuity Systems Inc, USA</td>
</tr>
<tr>
<td>Lightcycler® Software</td>
<td>Roche Applied Science, Australia</td>
</tr>
<tr>
<td>Microsoft Office</td>
<td>Microsoft Corporation, USA</td>
</tr>
<tr>
<td>Nanodrop 1000 Software</td>
<td>Thermo Scientific, USA</td>
</tr>
<tr>
<td>Oligo Calc</td>
<td>Northwestern University, USA</td>
</tr>
<tr>
<td>Quantity One® Imaging and Quantitation Software</td>
<td>Bio-Rad Laboratories, USA</td>
</tr>
<tr>
<td>RASE Primer Design</td>
<td>Université de Sherbrooke, Canada</td>
</tr>
<tr>
<td>RT² Profiler™ PCR array data analysis Version 3.5</td>
<td>Qiagen, Australia</td>
</tr>
</tbody>
</table>
3.1 Plasmid Preparation

3.1.1 Preparation of Competent Bacteria

To prepare competent DH5α *Escherichia coli* (E. coli), aliquots of DH5α cells were streaked onto Luria-Bertani (LB) agar plates, which were inverted and incubated at 37°C overnight. The next day, a single colony was inoculated into 10mL LB Broth and incubated overnight at 37°C and 0.56g, then 1mL of the culture was re-inoculated into 200mL LB Broth and incubated for 3 hours at 37°C and 0.56g until the OD$_{600}$ was between 0.4-0.5. The culture was centrifuged at 1700g for 10 minutes at 4°C to pellet the cells, the supernatant discarded and the bacterial cells resuspended in 5mL chilled Glycerol/PIPES buffer. The cell suspension was incubated on ice for 30 minutes, centrifuged at 1700g for 10 minutes at 4°C, the supernatant discarded and the bacterial cell pellet resuspended in 2mL chilled Glycerol/PIPES buffer, divided into 100µL aliquots and stored at -80°C.

3.1.2 Restriction Enzyme Digestion

Restriction enzyme digestion reactions containing 500ng to 1µg DNA template (section 3.1.6, 3.1.7), 2µL 10x Digestion Buffer, 2µL restriction enzyme (10U/µL) and ddH$_2$O to a final volume of 20µL were incubated at 37°C for 1-3 hours.

3.1.3 Shrimp Alkaline Phosphatase Digestion

To prevent the re-circularisation of restriction enzyme digested plasmid DNA (section 3.1.2), phosphate groups at the 5’ termini were removed by shrimp alkaline phosphatase (SAP) digestion. Reactions containing 3µg of digested plasmid (section 3.1.2), made up to a volume of 40µL by the addition of 4µL 10x SAP Dephosphorylation Buffer, 1µL SAP(1U/µL) and ddH$_2$O. SAP reactions were incubated for 30 minutes at 37°C before the addition of an extra 1µL of SAP and incubation at 37°C for 30 minutes. The SAP enzyme was inactivated by incubation at 65°C for 15 minutes.

*: superscript numbers refers to Buffers/Solutions number (Appendix 1)
3.1.4 Ligation Reactions

To clone PCR amplified DNA into the pGEM-T Easy Vector (Appendix 3) using PCR generated 3’-A overhangs, amplified PCR products were purified (section 3.3) and quantitated spectrophotometrically (section 3.7.2). Ligation reactions contained 5μL 2x Promega Rapid Ligation Buffer, 1μL pGEM-T Easy vector (50ng), 1μL T4 DNA ligase (3U/μL), the appropriate amount of purified DNA insert as calculated below for a 1:1 molar ratio of insert:vector and ddH₂O to a final volume of 10μL.

\[
x(\text{ng})_{\text{insert}} = \frac{50\text{ng vector}}{x(\text{kb})_{\text{insert}}} \times y(\text{kb})_{\text{vector}}
\]

Ligation reactions were incubated overnight at 4°C and stored at -20°C.

For ligation into the pTRE3G-IRES vector (Appendix 3), reactions contained 30ng vector and the appropriate amount of insert (1:1 insert:vector ratio), 2μL 10x T4 DNA Ligase Reaction Buffer, 1μL T4 DNA Ligase (10U/μL) and ddH₂O to a final volume of 20μL. Reactions were incubated at 16°C overnight and stored at -20°C.

3.1.5 Transformation of Competent Bacteria

Aliquots of 200μL competent DH5α E. coli cells (section 3.1.1) were thawed on wet ice and entire ligation reactions (section 3.1.4) or 50-100ng plasmid (section 3.1.6, 3.1.7) added, the solution briefly mixed by stirring then incubated on ice for 30 minutes. Bacteria were heated at 42°C for 90 seconds, tubes were placed on ice for 2 minutes, 800μL LB Broth\(^2\) was added to each tube and the tubes were incubated at 37°C and 0.56g for 1 hour. Aliquots of 50μL, 100μL and 150μL of the transformed cells were spread onto LB agar/ampicillin\(^2\) or LB agar/kanamycin\(^2\) plates either alone or supplemented with IPTG and X-Gal\(^2\), which were incubated inverted at 37°C overnight then stored inverted at 4°C.
3.1.6 Small Scale Plasmid Preparation

To isolate plasmids from transformed *E. coli* cells (section 3.1.5), individual colonies were picked from culture plates, inoculated into 5mL LB broth/ampicillin\textsuperscript{29} or LB broth/kanamycin\textsuperscript{31} and the cultures incubated overnight at 37°C and 0.56g. For plasmid preparation, 1.5mL of each culture was centrifuged at 16000g for 30 seconds in a microcentrifuge tube, LB broth was aspirated and the bacterial pellet was resuspended in 100µL cold Solution I\textsuperscript{54}. 200µL Solution II\textsuperscript{55} was added to each tube, the tubes were inverted to mix, 150µL cold Solution III\textsuperscript{56} was added to each tube and the tubes were immediately inverted and vortexed briefly then incubated on ice for 5 minutes. Tubes were centrifuged at 16000g for 5 minutes, supernatants were collected and placed into fresh 1.5mL microcentrifuge tubes, 900µL 100% EtOH was added to each tube and the tubes were immediately vortexed, incubated at room temperature for 2 minutes then centrifuged at 11752g for 5 minutes. Supernatants were discarded, 600µL 70% EtOH\textsuperscript{17} was added to each tube, the tubes centrifuged at 11752g for 5 minutes, supernatants discarded and the pellets air dried for 10-15 minutes. DNA was dissolved in 35µL ddH\textsubscript{2}O, 1µL (20U) RNase A\textsuperscript{41} was added to each tube, the tubes incubated at 37°C for 1 hour then stored at -20°C.

3.1.7 Large Scale Plasmid Preparation

To purify plasmids from frozen bacterial stocks (section 3.1.8), a pipette tip was used to scrape the frozen stock then placed into 5mL LB broth/ampicillin\textsuperscript{29} or LB broth/kanamycin\textsuperscript{31} and incubated for ~8 hours at 37°C and 0.56g. The 5mL culture was added to 95mL LB broth/ampicillin\textsuperscript{29} or LB broth/kanamycin\textsuperscript{31} and incubated overnight at 37°C and 0.56g. The following day, cultures were centrifuged at 2655g for 10 minutes at room temperature, the supernatants discarded, bacterial pellets resuspended in 4mL QIAGEN Resuspension Buffer/RNase A (R3), 4mL QIAGEN Lysis Buffer (L7) added to each tube and the mixtures inverted 6 times then incubated for 5 minutes at room temperature. Four mL pre-chilled QIAGEN Precipitation Buffer (N3) was added to each preparation, the solutions immediately mixed by inversion until homogeneous, transferred to 50mL ultracentrifuge tubes, centrifuged at 2655g for 10 minutes at 4°C and the supernatants transferred to fresh tubes. QIAGEN-tip 100 (columns) were
Chapter 3: Methods

equilibrated with 10mL of QIAGEN Equilibration Buffer (E1), which was passed through the columns by gravity flow, the lysed bacterial supernatants were added and allowed to pass through the columns by gravity flow, the columns were washed twice with 10mL QIAGEN Wash Buffer (W8), then the DNA was eluted using 5mL QIAGEN Elution Buffer (E4). Eluted plasmid DNA samples were transferred to fresh 50mL ultracentrifuge tubes, 3.5mL 100% isopropanol was added to precipitate the DNA and the tubes were centrifuged at 20000g for 40 minutes at 4°C. Supernatants were discarded and the plasmid DNA pellets were washed with 1.5mL 70% EtOH\(^\text{17}\) then centrifuged at 20000g for 40 minutes at 4°C. The supernatants were again discarded, the DNA pellets air dried, redissolved in 100 - 150µL TE buffer and stored at -20°C.

3.1.8 Preparation of Bacterial Glycerol Stocks

To prepare bacterial glycerol stocks, 5mL cultures in LB broth/ampicillin\(^\text{29}\) or LB broth/kanamycin\(^\text{31}\) that had been incubated overnight at 37°C and 0.56g were centrifuged at 2655g for 3 minutes (4°C) and the supernatants discarded. 1mL pre-chilled LB broth/10% glycerol\(^\text{30}\) was added to each bacterial cell pellet, cells were evenly resuspended, transferred to labelled 2mL cryotubes and stored at -80°C.

3.2 Agarose Gel Electrophoresis

For agarose gel electrophoresis, 1% or 2% agarose gels\(^2\) were placed into electrophoresis tanks and immersed in 1x TAE Buffer\(^\text{59}\). The appropriate amount of 6x Loading Dye\(^\text{32}\) was added to each sample, the samples (5 - 20μL) were loaded into the wells and a well of each gel was loaded with 5μL 1kb plus DNA ladder. Gels were electrophoresed at 100V for 20 – 40 minutes, viewed under UV transillumination and imaged using Quantity One\textsuperscript{TM} software.

3.3 DNA Purification

To purify DNA using QIAquick PCR Purification Kits, 5 volumes of Buffer PBI was added to each sample, the samples mixed and added to QIAquick spin columns placed in 2mL collection tubes, and the columns were centrifuged for 60 seconds at 11752g.
Chapter 3: Methods

The flow throughs were discarded, the columns replaced in the 2mL tubes, 0.75mL Buffer PE was added to each column and the columns were centrifuged for 60 seconds at 11752g. The flow throughs were again discarded and the columns replaced into the 2mL tubes then centrifuged for 60 seconds at 11752g. The QIAquick columns were placed into fresh 1.5mL microcentrifuge tubes and to elute the DNA, 50μL Buffer EB was added directly to the centre of the QIAquick column membrane, the columns were incubated at room temperature for 60 seconds, centrifuged for 60 seconds at 11752g and the eluted DNA stored at -20°C.

To gel purify DNA, DNA samples that had been electrophoresed in agarose gels (section 3.2) were viewed under low intensity UV transillumination, the bands of interest excised using a scalpel blade, placed into 1.5mL microcentrifuge tubes and the gel fragment weights calculated. 3 volumes (300μL) Buffer QG was added per 100mg sample, the tubes incubated at 50°C for 10 minutes to melt the agarose and each mixture was added to a QIAquick spin column placed in a 2mL collection tube. Column assemblies were centrifuged for 60 seconds at 11752g, the flow throughs discarded, the columns replaced into the 2mL tubes, 0.75mL Buffer PE was added to each column and the columns were centrifuged for 60 seconds at 11752g. The flow throughs were again discarded, the columns replaced into the 2mL tubes, centrifuged for 60 seconds at 11752g and each column was placed into a fresh 1.5mL microcentrifuge tube. To elute the DNA, 50μL Buffer EB was added directly to the centre of each QIAquick column membrane, the columns were incubated at room temperature for 60 seconds, centrifuged for 60 seconds at 11752g and the eluted DNA was stored at -20°C.

3.4 DNA Sequencing

DNA sequencing was performed by Sanger sequencing. Reactions contained 8μL 2.5x Big Dye™ Terminator sequencing buffer, 3pmol primer (Appendix 2), 250-500ng of plasmid DNA, 0.5μL Big Dye™ Terminator (v3.1) and ddH2O to 20μL. Reactions were performed in PTC-100™ thermal cyclers with 25 cycles of 96°C for 15 seconds, 50°C for 10 seconds and 60°C for 4 minutes. To precipitate the DNA, 2μL 3M sodium acetate (pH4.6) and 50μL of 95% ethanol were added to each tube, the tubes were vortexed, incubated on ice for 10 minutes then centrifuged at 13200g for 30 minutes at
4°C. Supernatants were removed by pipette, 250μL 70% ethanol\textsuperscript{17} was added to each tube, the tubes were centrifuged at 13200g for 5 minutes at 4°C, the supernatants removed by pipette and the DNA air dried for 15 minutes at room temperature. Reactions were run on a ABI Prism 3730 capillary sequencer at the Lotterywest State Biomedical Facility (Royal Perth Hospital). Sequencing chromatograms were visualised using Chromas Lite (2.1.1) and the DNA sequences compared to human genomic and mRNA sequences using BLAST® (NCBI).

3.5 Tissue Culture

3.5.1 Routine Maintenance of Cell Lines

The LNCaP, MDA-MB-231 and COS-7 cell lines obtained from the American Type Culture Collection (ATCC, USA) were used in this study. The human prostate cancer cell line LNCaP was originally isolated from the lymph node metastasis of a human prostate cancer (Horoszewicz, Leong et al. 1983), the human breast cancer cell line MDA-MB-231 was originally isolated from the pleural effusion of a human breast adenocarcinoma (Cailleau, Young et al. 1974) and the immortalised monkey kidney cell line COS-7 was originally derived from the SV40 transformed CV-1 cell line (from the kidney of the African Green Monkey, Cerocopithecus aethiops) (Gluzman 1981).

Cells were routinely cultured in stock medium\textsuperscript{46} composed of RPMI-1640\textsuperscript{43} supplemented with 10% (v/v) foetal calf serum (FCS) and 100U/mL penicillin/100mg/mL streptomycin (PS) (RPMI/10%FCS/PS\textsuperscript{46}) in humidified incubators at 37°C and 5% CO\textsubscript{2}. Stock medium\textsuperscript{46} was replaced every 2-3 days and cells were passaged every 4-5 days when ~80% confluent. To passage cells, medium was aspirated, the cells were rinsed with 4mL phosphate buffered saline (PBS\textsuperscript{37}) then incubated for 2-4 minutes at 37°C with ~1mL per 75cm\textsuperscript{2} flask trypsin/EDTA until cells had detached from the culture surface. 4mL stock medium\textsuperscript{46} was added to each flask to inactivate the trypsin, cells were dispersed by pipetting and aliquotted into new 75cm\textsuperscript{2} flasks or plates/dishes as required (section 3.5, 3.6, 3.7.1, 3.8.1, 3.9, 3.10). For routine culture, LNCaP cells were typically passaged at a 1:5 dilution, MDA-MB-231 cells were passaged at a 1:6 dilution and COS-7 cells were passaged at a 1:15 dilution.
3.5.2 DHT Treatment

To treat LNCaP cells with $10^{-8}$M 5α-dihydrotestosterone (DHT), cells were trypsinised (section 3.5.1), diluted with RPMI-1640 medium$^{42}$ containing 5% (v/v) CSS (RPMI/5% CSS/PS$^{45}$), seeded at 1:3 - 1:5 dilution (10cm petri dishes), 4 x $10^5$ - 8 x $10^5$ cells/well (6-well plates), or 2 x $10^5$ - 4.5 x $10^5$ cells/well (24 well plates) then cultured overnight at 37°C and 5% CO₂. The following day, the medium was replaced with RPMI/5%CSS/PS$^{42}$ supplemented with $10^{-8}$M DHT$^{10}$ (or 0.1% v/v ethanol vehicle) and the cells cultured for a further 8 hours to 5 days prior to harvesting for RNA extraction (section 3.7.1) or western blotting (section 3.8.1). Medium was replaced every 2-3 days as required.

3.5.3 Cryopreservation and Thawing of Cell Lines

To prepare frozen stocks of cultured cells, 75cm$^2$ flasks of cells were trypsinised and diluted with stock medium$^{46}$ (section 3.5.1), the cell suspensions were centrifuged at 152g and 20°C for 5 minutes, the medium aspirated, the cell pellets resuspended in 3mL cold RPMI/10%FCS/10%DMSO/PS$^{47}$ and divided into 1mL aliquots in pre-chilled 2mL cryotubes. Tubes were frozen at -80°C overnight then placed in long term storage in liquid nitrogen.

To thaw cryopreserved cells, 10mL stock medium$^{46}$ was added to 75cm$^2$ flasks and the flasks were incubated at 37°C and 5% CO₂ for >1 hour. Cryotubes containing the cells were removed from liquid nitrogen storage, thawed rapidly in a 37°C water bath and the cell suspension added dropwise to the prepared flasks. Flasks were incubated at 37°C and 5% CO₂ overnight, the stock medium$^{46}$ was replaced the following day and cells were routinely maintained as described in section 3.5.1.
3.5.4 Transient Transfection of Cell Lines

For transient transfection, LNCaP cells were trypsinised (section 3.5.1) and $7 \times 10^5$ cells seeded into each well of 6-well plates or diluted 1:3 from confluent 75cm$^2$ flasks into 10cm petri dishes to achieve 70-80% confluency the following day. After overnight incubation at 37°C and 5% CO$_2$, transfection reagents were prepared as follows. For each transfection, a tube containing RPMI-1640$^{43}$ (700μL per 10cm$^2$ dish, 100μL per well of 6-well plates, 30μL per well of 24-well plates) and Metafectene Pro$^{TM}$ (42μL, 6μL, 2μL, respectively), and a second tube containing RPMI-1640$^{43}$ (700μL, 100μL, 30μL, respectively) and plasmid (42μg, 6μg, 2μg, respectively) (section 3.1.7) were prepared. The plasmid DNA mixture was added to the RPMI.Metafectene Pro$^{TM}$ mixture and incubated for 20 minutes at room temperature, during which time the culture medium in each well was replaced. Plasmid.Metafectene Pro$^{TM}$ mixtures were added dropwise to each well and the plates were incubated at 37°C and 5% CO$_2$.

3.5.5 Stable Transfection of Cell Lines

For stable transfection, LNCaP or pCMVTet3G-LNCaP cells cultured in 75cm$^2$ flasks were trypsinised (section 3.5.1), seeded into 10cm petri dishes at a 1:3 dilution to achieve 70-80% confluency the following day, then incubated overnight at 37°C and 5% CO$_2$. To stably transfect LNCaP cells with the pCMV-Tet3G plasmid (Appendix 3), two tubes per petri dish were prepared, with one containing 4μL (4μg) pCMV-Tet3G plasmid and 96μL Xfect Reaction Buffer and the second tube containing 1.5μL Xfect Polymer and 98.5μL Xfect Reaction Buffer. The tubes were vortexed for 10 seconds, the contents of the second tube added directly to the first tube, the mixture vortexed for 10 seconds then incubated at room temperature for 10 minutes. Following incubation, the transfection mixture was added dropwise to the cells, the petri dishes were incubated at 37°C and 5% CO$_2$ for 6 hours, the stock medium$^{46}$ was replaced and the transfected cells were incubated at 37°C and 5% CO$_2$. Cultures reached confluency approximately 72 hours post-transfection and were trypsinised (section 3.5.1), passaged at a 1:2 dilution into 2 petri dishes, incubated for a further 48 hours at 37°C and 5% CO$_2$ before the medium was replaced with RPMI/10%FCS/PS$^{46}$ containing 400μg/mL G418. To select transfected cells, cultures were maintained in this medium for ~1 month with the
medium replaced every 1-3 days as required, then the concentration of G418 was reduced to 200µg/mL. G418 resistant cells were initially passaged into 24 well plates and subsequently into larger culture flasks.

3.5.6 Optimisation of Antibiotic Selection Concentration

To optimise the antibiotic concentration for selection of stably transfected cells, cultures were trypsinised (section 3.5.1) and seeded at 1 x 10^6 cells/well into 6-well plates. The following day, the culture medium was replaced with fresh medium supplemented with 0, 100, 150, 200, 250 or 300µg/mL Hygromycin B and cultures were incubated at 37°C and 5% CO₂. Cells were cultured for 7 days with the medium replaced every 2 days. For pCMVTet3G-LNCaP cells, all stock medium was supplemented with 200µg/mL G418.

3.6 Luciferase Reporter Assays

For luciferase assays, cells were trypsinised (section 3.5.1) and seeded into 24-well plates at 4 x 10^5 cells/well in stock medium (LNCaP cells) or stock medium supplemented with 200µg/mL G418 (pCMVTet3G-LNCaP cells). The following day, cells were transiently transfected with 500ng plasmid per well then cultured in medium that was further supplemented with 0.5 or 1µg/mL doxycycline (section 3.5.4). At 24 hours post-transfection, medium from each well was replaced with 50µL Passive Lysis Buffer (PLB) and the plates were incubated on a rocking platform for 15 minutes at room temperature. To assay firefly luciferase activity, 160µL Luciferase Assay Reagent (LARIItm) per well was added, 180µL of the mixture was transferred to individual wells of a 96-well plate and luminescence was assayed using a VICTOR™ Light (1420) luminometer.
3.7 **RNA Extraction, Reverse Transcription and PCR**

3.7.1 **RNA Extraction**

For RNA extraction, cells cultured in 10cm petri dishes were trypsinised, the cell suspensions diluted with culture medium (section 3.5.1), centrifuged at 2655g for 5 minutes at 20°C, the supernatants aspirated and the cell pellets stored at -80°C until use. To extract RNA, cell pellets were thawed in 1mL Ultraspec RNA™ and pipetted until homogeneous. The cell lysates were transferred to 1.5mL microcentrifuge tubes, incubated on ice for 5 minutes, 0.2 volumes of chloroform was added to each lysate and the samples were shaken vigorously for 30 seconds then incubated on ice for 5 minutes. Tubes were centrifuged at 13200g and 4°C for 15 minutes, the upper (aqueous) phase was transferred to fresh 1.5mL microcentrifuge tubes, an equal volume of isopropanol was added to each sample and tubes were inverted 5-6 times then incubated on ice for 10 minutes. The tubes were centrifuged at 13200g for 10 minutes at 4°C to pellet the RNA, supernatants were removed and the RNA pellets were washed twice with 1mL 75% ethanol with centrifugation at 5156g for 5 minutes at 4°C. RNA pellets were dried at 60°C for ~1 minute then dissolved in ~100µL DEPC-treated ddH₂O and stored at -80°C.

3.7.2 **Spectrophotometric Quantitation of DNA/RNA**

RNA and DNA concentrations were determined spectrophotometrically using a NanoDrop® ND-1000 spectrophotometer against the appropriate vehicle solution (TE-Buffer/ddH₂O). An $A_{260}$ of 1 for DNA was estimated to be 50µg/mL and for RNA was estimated to be 40µg/mL. Measurements at 260nm and 280nm were used to evaluate the purity of samples, with a 260/280 ratio of 1.8 to 2 indicating a relatively pure sample.

3.7.3 **Reverse Transcription – SuperScript III**

For reverse transcription reactions using Superscript III, a mixture of 1µg RNA (section 3.7.1), 1µL (0.5µg) Oligo(dT) (or 1µL (0.5µg) Random Hexamers), 1µL 10mM
dNTP\textsuperscript{12} and ddH\textsubscript{2}O to a volume of 12µL was incubated at 65°C for 5 minutes then placed on ice for 2-3 minutes. 4µL 5x First Strand Buffer, 2µL 0.1M DTT and 1µL Superscript III Reverse Transcriptase were added, the solution was incubated at room temperature for 5 minutes then heated at 50°C for 60 minutes and at 70°C for 15 minutes. cDNA was stored at -20°C.

3.7.4 Reverse Transcription – RT\textsuperscript{2} First Strand Kit

For reverse transcription reactions using the RT\textsuperscript{2} First Strand Kit, 2 µL Buffer GE and 1µg RNA (section 3.7.1) were combined with RNase free ddH\textsubscript{2}O to a final volume of 10µL, the mixture was incubated at 42°C for 5 minutes then placed on ice for 1 minute. A mixture of 4µL 5x Buffer BC3, 1µL Control P2, 2µL RE3 Reverse Transcriptase Mix and 3µL RNase-free ddH\textsubscript{2}O was added and tubes were incubated at 42°C for 15 minutes then at 95°C for 5 minutes. 91µL RNase-free ddH\textsubscript{2}O was added to each reaction and the cDNA samples were stored at -20°C.

3.7.5 Polymerase Chain Reaction (PCR)

PCRs were composed of 1-2µL cDNA, 5µL 5x PCR Buffer\textsuperscript{35} (with 25mM dNTP\textsuperscript{12}), 1µL (15pmol) each of forward and reverse primers (Appendix 2), 2-3mM MgCl\textsubscript{2}, 0.1µL (0.5U) Platinum Taq DNA Polymerase and ddH\textsubscript{2}O to a final volume of 25µL. Reactions were performed in BioRad C1000 thermal cyclers with an initial denaturation step of 5 minutes at 95°C, 35 PCR cycles as listed in Table 3.1 and a final extension of 5 minutes at 72°C. Amplified PCR products were electrophoresed in 1-2% agarose gels (section 3.2) or stored at -20°C.
### Table 3.1 – PCR Conditions (Primer sequences are listed in Appendix 2).

<table>
<thead>
<tr>
<th>Gene</th>
<th>MgCl₂ (mM)</th>
<th>Denaturation Temperature/Duration (°C/s)</th>
<th>Annealing Temperature/Duration (°C/s)</th>
<th>Extension Temperature/Duration (°C/s)</th>
<th>Cycles</th>
<th>Amplicon Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR</td>
<td>2</td>
<td>95/15</td>
<td>65/60</td>
<td>-</td>
<td>40</td>
<td>168</td>
</tr>
<tr>
<td>β-actin</td>
<td>2</td>
<td>95/15</td>
<td>60/60</td>
<td>-</td>
<td>40</td>
<td>319</td>
</tr>
<tr>
<td>ETS1p51</td>
<td>3</td>
<td>94/30</td>
<td>58/30</td>
<td>72/60</td>
<td>35</td>
<td>144</td>
</tr>
<tr>
<td>ETS1p42</td>
<td>2</td>
<td>94/30</td>
<td>58/30</td>
<td>72/60</td>
<td>35</td>
<td>192</td>
</tr>
<tr>
<td>ETS1p27</td>
<td>2</td>
<td>94/30</td>
<td>60/30</td>
<td>72/30</td>
<td>40</td>
<td>282</td>
</tr>
<tr>
<td>GAPDH</td>
<td>3</td>
<td>95/15</td>
<td>60/60</td>
<td>-</td>
<td>40</td>
<td>112</td>
</tr>
<tr>
<td>NODAL</td>
<td>2</td>
<td>94/30</td>
<td>58/30</td>
<td>72/30</td>
<td>40</td>
<td>93</td>
</tr>
<tr>
<td>SLUG</td>
<td>2</td>
<td>94/30</td>
<td>60/30</td>
<td>72/30</td>
<td>40</td>
<td>118</td>
</tr>
<tr>
<td>SMAD2</td>
<td>2</td>
<td>94/30</td>
<td>60/30</td>
<td>72/30</td>
<td>40</td>
<td>128</td>
</tr>
<tr>
<td>SNAIL1</td>
<td>2</td>
<td>94/30</td>
<td>63/30</td>
<td>72/30</td>
<td>40</td>
<td>140</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>2</td>
<td>94/30</td>
<td>60/30</td>
<td>72/30</td>
<td>40</td>
<td>136</td>
</tr>
<tr>
<td>TWIST1</td>
<td>2</td>
<td>94/30</td>
<td>63/30</td>
<td>72/30</td>
<td>40</td>
<td>77</td>
</tr>
<tr>
<td>ZEB1</td>
<td>2</td>
<td>94/30</td>
<td>60/30</td>
<td>72/30</td>
<td>40</td>
<td>72</td>
</tr>
</tbody>
</table>

### 3.7.6 Quantitative PCR (qPCR)

qPCR was performed using SYBR-green and contained (per tube) 5µL GoTaq® (containing Taq DNA polymerase, dNTP mix and 2mM MgCl₂), 1-2µL cDNA (section 3.7.3), 2-3mM MgCl₂ (final concentration) (Table 3.1), 1µL (10pmol) each forward and reverse primers and ddH₂O to a final volume of 10µL. Reactions were performed in duplicate or triplicate in each run and were loaded into 384-well plates, sealed with adhesive film, centrifuged (1 minute at 1000g and room temperature) then loaded into a Roche LightCycler 480. Cycling conditions for each primer set are detailed in Table 3.1 and melt curve analysis was performed following each PCR (section 3.7.7) with raw data exported as Notepad and jpeg images using the LightCycler® software. Data was imported into Microsoft Excel for analysis and unless otherwise stated, the Pfaffl Method of relative quantification was used to determine relative gene expression using the following formula (Pfaffl 2001):
In this equation, $E_{\text{target}}$ and $E_{\text{ref}}$ are the efficiencies of the target and reference genes, respectively (section 3.7.8) and $\Delta Ct_{\text{target}}$ and $\Delta Ct_{\text{ref}}$ are the differences in $C_t$ values between the treated (experimental) and the corresponding control samples for the target and reference gene, respectively. The resulting ratio indicates higher (>1) or lower (<1) expression of the gene of interest in the treated (experimental) sample compared to the control sample. For all samples, the standard deviation between replicates was used to calculate the standard error of the mean (SEM).

Tissue specimens were additionally analysed by absolute quantitation to determine the copy number (log cDNA relative to efficiency curve) using the following formula:

$$\text{Quantity} = 10^{((C_t - b)/m)}$$

where $C_t$ is the average cycle recorded between sample replicate PCRs, $b$ is the y-axis intercept determined by the efficiency curve for that PCR (section 3.7.8) and $m$ is the slope value of the respective efficiency curve. Data analysed using Excel was exported to GraphPad Prism 5 to graph results.

Tissue specimens were also analysed using the $\Delta Ct$ method using a reference gene (GAPDH). For this method, the target gene was normalised against the reference gene using the following formula

$$\text{Ratio (reference/target)} = 2^{(C_t(\text{reference}) - C_t(\text{target}))}$$

where $C_t$ values were calculated for the target and reference gene in both samples (control and test) and the difference between test $C_t$ values (reference – target) was
plotted over control Ct values (reference – target). The difference of Ct values (ΔCt method) is a variation of the Livak method (Livak and Schmittgen 2001).

3.7.7 Melt Curve Analysis

For melt curve analysis using the Roche LightCycler 480, plates were incubated for 1 minute at 95°C followed by 1 minute at 40°C, with progressive increases in temperature from 58°C to 95°C at a ramp rate of 0.02°C/second. Melt curves were produced by plotting the derivative of the change of relative fluorescence units (RFUs) over the change in time, against the change in temperature (°C): - ((d(RFU)/dT)). The identification of a single peak (product) in the melt curve profile corresponding to the melting temperature (T_m) of the PCR product is indicative of specific amplification of the target DNA sequence. Additional peaks (not of the corresponding T_m) or the presence of peaks with multiple heads are indicative of amplification of non-specific products, primer dimers or partial amplification of product. As the presence of multiple peaks produces signal that may interfere with accurate quantitation of the target PCR product, PCRs which exhibited non-specific peaks were excluded from subsequent analyses.

3.7.8 Generation of Efficiency Curves

To generate qPCR efficiency curves, cDNA samples were diluted at 1:10, 1:100, 1:1000 and 1:10000 in ddH2O and qPCR performed according to optimised cycling conditions (section 3.7.5). Data were imported into Excel and the log amount of the starting cDNA plotted against average C_T (y-axis). Linear trendlines were drawn using Excel, R² values were determined with the slope of the trendline used in the following equation to estimate the PCR efficiency.

\[ E = 10^{-1/(\text{slope})}, \text{Efficiency \%} = (E-1) \times 100 \]

Acceptable results for these calculations included R² > 0.98 and reproducible PCR efficiencies between 90-110%. Efficiency curves using undiluted, 1:10 and 1:100 cDNA dilutions were typically included in each qPCR as an internal control.
3.7.9 Construction of ETS1 Isoform Specific Primers

To generate qPCR primer pairs that would specifically amplify the ETS1 p51, p42 or p27 isoforms, the full-length ETS1 mRNA sequence (accession NM_005238.3) was copied into the real-time PCR annotation splicing events software (RASE) at (http://designs.lgfus.ca). The software identified the splice sites for the p42 and p27 isoforms and regions upstream and downstream of the splice sites to generate boundary-spanning and splice-site encompassing primer pairs for each ETS1 isoform. The specificity and compatibility of putative primers was validated using BLAST® and primer BLAST® tools (with and without reference sequence), and the primers were screened for potential self-annealing, hairpin formation or dimerisation using the www.basic.northwestern.edu/biotools/oliocalc.html software. Additional parameters for acceptance of primer pairs included oligomer length (18 – 24bp), melting temperature (55 – 60°C), GC% (45 – 65%) and amplicon size (90 – 200bp).

3.7.10 PCR Array

For PCR array, 102µL cDNA that had been reverse transcribed using the RT² First Strand Kit (section 3.7.4) was mixed with 650µL 2x RT² SYBR Green Mastermix and 1300µL RNase-free ddH₂O and 10µL of this mastermix was added to wells of the 384-well PCR array (Human EMT, PAHS-090A) using a Corbett liquid handling robot according to manufacturer’s instructions. Plates were sealed with adhesive film, centrifuged for 1 minute at 1000g and room temperature and cycled in a Roche LightCycler 480 at 1 cycle of 10 minutes at 95°C (to activate the polymerase) then 45 cycles of 15 seconds at 95°C (denaturation) and 1 minute at 60°C (annealing/extension). Fluorescence data was collected after each cycle and a melt curve (section 3.7.7) was performed following qPCR to examine the specificity of each PCR. Results were exported into Notepad then Excel, then imported into the SABiosciences PCR Array Data Analysis Template (for Excel) and uploaded onto the RT² Profiler™ PCR array data analysis programme at pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php. Analysed data was annotated using online graphing tools and Excel. Relative quantitation of gene expression was performed using the 2⁻ΔΔCt or Livak Method and the following formulae (Livak and
Schmittgen 2001). Initially, the $C_T$ of the target gene (gene of interest) was normalised to that of the reference gene for the experimental sample (test) and the control sample (calibrator).

\[ \Delta C_T^{(test)} = C_T^{(target, test)} - C_T^{(ref, test)} \]

\[ \Delta C_T^{(calibrator)} = C_T^{(target, calibrator)} - C_T^{(ref, calibrator)} \]

Secondly, the $\Delta C_T$ of the test was normalised against the $\Delta C_T$ of the calibrator:

\[ \Delta \Delta C_T = \Delta C_T^{(test)} - \Delta C_T^{(calibrator)} \]

Finally, the expression ratio was calculated as:

\[ 2^{-\Delta \Delta C_T} = \text{Normalised expression ratio} \]

The resulting ratio is a fold increase or decrease of the gene of interest in the test sample relative to the calibrator sample (normalised to the reference gene).

### 3.8 Western Blotting

#### 3.8.1 Preparation of Whole Cell Lysates

To prepare whole cell lysates for western blotting, medium was aspirated from cells growing in 6 well plates then scraped with a rubber spatula and collected in 250µL Whole Cell Lysis Buffer\(^73\). Alternatively, cell pellets were lysed in 250µL Whole Cell Lysis Buffer\(^73\) by pipetting. Lysates were transferred into 1.5mL tubes and repeatedly passed through a 23G needle into a 1mL syringe to reduce viscosity, then stored at -20°C.
3.8.2 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed using a BioRad Protean 3 Cell electrophoresis system which was assembled and run according to the manufacturer’s instructions. Polyacrylamide separating gel (12%)\textsuperscript{38} was prepared and cast between glass plates, overlayed with ddH$_2$O and allowed to polymerise at room temperature for $\sim$45 minutes. The ddH$_2$O was replaced with polyacrylamide stacking gel (4%)\textsuperscript{39}, the well combs inserted and the gel allowed to polymerise at room temperature for $>$30 minutes. Protein samples (18µL) were prepared in 0.5mL tubes containing 2µL 10x Western Loading Dye\textsuperscript{72}, samples were denatured by heating to 95°C for $\sim$5 minutes and cooled to room temperature prior to loading of 15µL aliquots into the wells. Gels were electrophoresed in 1x Running Buffer\textsuperscript{48} for 50-55 minutes at 200V. Each gel included a well containing 5µL ColourPlus\textsuperscript{80} Prestained Protein Marker.

3.8.3 Western Transfer

Following SDS-PAGE (section 3.8.2), the apparatus was disassembled and gels were placed in chilled Transfer Buffer\textsuperscript{62}. Transfer cassettes were assembled from negative to positive with Scotchbrite pads, two layers of filter paper, the polyacrylamide gel, Hybond C-Extra nitrocellulose membrane, two layers of filter paper and Scotchbrite pads that had each been pre-soaked in chilled Transfer Buffer\textsuperscript{62}. Cassettes were closed then loaded into a Mini-Protean III transfer apparatus containing chilled Transfer Buffer\textsuperscript{62}, a magnetic stirrer and an ice block. Proteins were transferred overnight at 30V with stirring. The following day, the nitrocellulose filters were either immunoblotted (section 3.8.4) or stored at 4°C wrapped in filter paper and aluminium foil.

3.8.4 Immunoblotting

For immunoblotting, nitrocellulose filters (section 3.8.3) were trimmed as required and incubated in Blocking Solution (Table 3.2) for 90 minutes, then primary antibody (Table 3.2) for 90 minutes (*or overnight at 4°C, Table 3.2). Filters were washed in TBST\textsuperscript{66} for 3 x 10 minutes, incubated with secondary antibody (Table 3.2) for 90 minutes, washed in TBST\textsuperscript{66} for 3 x 10 minutes, incubated in ECL\textsuperscript{16} for 3-5 minutes.
(Table 3.2), wrapped in plastic wrap, exposed to X-ray film for 10 seconds to 30 minutes as required and the films developed using an AGFA CP1000 developer. Films were scanned using a HP Photosmart 2710 tabletop scanner and quantitated using BioRad Quantity One® software.

Table 3.2 – Immunoblotting Conditions (*Primary antibody: overnight at 4°C)

<table>
<thead>
<tr>
<th>Antigen (Antibody species)</th>
<th>Blocking Solution</th>
<th>Antibody Diluent</th>
<th>Primary Antibody Dilution</th>
<th>Secondary Antibody Dilution</th>
<th>ECL</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR (Mouse)</td>
<td>TBS/3% Blotto&lt;sup&gt;65&lt;/sup&gt;</td>
<td>TBST/1% Blotto&lt;sup&gt;67&lt;/sup&gt;</td>
<td>1:1500</td>
<td>1:15000</td>
<td>Prime</td>
</tr>
<tr>
<td>β-actin (Goat)</td>
<td>TBS/3% Blotto&lt;sup&gt;65&lt;/sup&gt;</td>
<td>TBST/1% Blotto&lt;sup&gt;67&lt;/sup&gt;</td>
<td>1:3000</td>
<td>1:30000</td>
<td>Prime</td>
</tr>
<tr>
<td>β-catenin* (Rabbit)</td>
<td>TBST/5% Blotto&lt;sup&gt;69&lt;/sup&gt;</td>
<td>TBST/5% Blotto&lt;sup&gt;69&lt;/sup&gt;</td>
<td>1:1000</td>
<td>1:10000</td>
<td>Prime</td>
</tr>
<tr>
<td>Claudin-1* (Rabbit)</td>
<td>TBST/5% Blotto&lt;sup&gt;69&lt;/sup&gt;</td>
<td>TBST/5% Blotto&lt;sup&gt;69&lt;/sup&gt;</td>
<td>1:1000</td>
<td>1:10000</td>
<td>Select</td>
</tr>
<tr>
<td>E-cadherin* (Rabbit)</td>
<td>TBST/5% Blotto&lt;sup&gt;69&lt;/sup&gt;</td>
<td>TBST/5% BSA&lt;sup&gt;70&lt;/sup&gt;</td>
<td>1:1000</td>
<td>1:10000</td>
<td>Prime</td>
</tr>
<tr>
<td>ETS1 (Rabbit)</td>
<td>TBS/3% Blotto&lt;sup&gt;65&lt;/sup&gt;</td>
<td>TBST/1% Blotto&lt;sup&gt;67&lt;/sup&gt;</td>
<td>1:1000</td>
<td>1:10000</td>
<td>Prime</td>
</tr>
<tr>
<td>GFP (Mouse)</td>
<td>TBS/3% Blotto&lt;sup&gt;65&lt;/sup&gt;</td>
<td>TBST/1% Blotto&lt;sup&gt;67&lt;/sup&gt;</td>
<td>1:1500</td>
<td>1:15000</td>
<td>Prime</td>
</tr>
<tr>
<td>N-cadherin* (Rabbit)</td>
<td>TBST/5% Blotto&lt;sup&gt;69&lt;/sup&gt;</td>
<td>TBST/5% BSA&lt;sup&gt;70&lt;/sup&gt;</td>
<td>1:1000</td>
<td>1:10000</td>
<td>Select</td>
</tr>
<tr>
<td>SLUG* (Rabbit)</td>
<td>TBST/5% Blotto&lt;sup&gt;69&lt;/sup&gt;</td>
<td>TBST/5% BSA&lt;sup&gt;70&lt;/sup&gt;</td>
<td>1:1000</td>
<td>1:10000</td>
<td>Select</td>
</tr>
<tr>
<td>pSMAD2* (Rabbit)</td>
<td>TBST/5% Blotto&lt;sup&gt;69&lt;/sup&gt;</td>
<td>TBST/5% BSA&lt;sup&gt;70&lt;/sup&gt;</td>
<td>1:1000</td>
<td>1:10000</td>
<td>Select</td>
</tr>
<tr>
<td>SMAD2 (Rabbit)</td>
<td>TBST/5% Blotto&lt;sup&gt;69&lt;/sup&gt;</td>
<td>TBST/1% Blotto&lt;sup&gt;67&lt;/sup&gt;</td>
<td>1:1000</td>
<td>1:10000</td>
<td>Prime</td>
</tr>
<tr>
<td>SNAIL1* (Rabbit)</td>
<td>TBST/5% Blotto&lt;sup&gt;69&lt;/sup&gt;</td>
<td>TBST/5% BSA&lt;sup&gt;70&lt;/sup&gt;</td>
<td>1:1000</td>
<td>1:10000</td>
<td>Prime</td>
</tr>
<tr>
<td>Vimentin* (Rabbit)</td>
<td>TBST/5% Blotto&lt;sup&gt;69&lt;/sup&gt;</td>
<td>TBST/5% BSA&lt;sup&gt;70&lt;/sup&gt;</td>
<td>1:1000</td>
<td>1:10000</td>
<td>Select</td>
</tr>
<tr>
<td>ZEB1* (Rabbit)</td>
<td>TBST/5% Blotto&lt;sup&gt;69&lt;/sup&gt;</td>
<td>TBST/5% BSA&lt;sup&gt;70&lt;/sup&gt;</td>
<td>1:1000</td>
<td>1:10000</td>
<td>Select</td>
</tr>
<tr>
<td>Zona Occludens1* (Rabbit)</td>
<td>TBST/5% Blotto&lt;sup&gt;69&lt;/sup&gt;</td>
<td>TBST/5% Blotto&lt;sup&gt;69&lt;/sup&gt;</td>
<td>1:1000</td>
<td>1:10000</td>
<td>Prime</td>
</tr>
</tbody>
</table>
3.9 Migration Assays

LNCaP cell migration was assessed in wound-healing assays performed using IBIDI® silicone spacers in 24-well plates. For the assays, LNCaP cells were trypsinised (section 3.5.1), collected in RPMI/5%CSS/PS, diluted to 1.35x10^6 cells/mL and transiently transfected with either 2μg pcDNA3.1 (Appendix 3) or 2μg pEGFP-ETS1p51 (Appendix 3) (section 3.5.4). The transfected cells were seeded at 5.4x10^4 cells/40μL per side of IBIDI® silicone spacers, incubated for 24 hours at 37°C/5% CO₂ and the following day when cells were ~80-90% confluent, the silicone inserts were removed using sterile forceps and the RPMI/5% CSS/PS supplemented with either 10μM SB431542, 10ng/mL TGF-β1 or 0.1% DMSO (vehicle control). Medium was replaced every 2 days and to assess cell migration, the leading edge of the cells was imaged using a TUCSEN camera (IS500, TCA5-N) under 100x phase contrast microscopy every 24 hours, with the images imported into Image J where the colour threshold was adjusted and all cells were coloured red. The 500μm space at 0hr was determined initially, with the dimensions of the space (~500μm) overlaid onto subsequent images to calculate the percentage of red (cell covered) vs the non-coloured (empty) space to determine wound closure (%). Replicate percentage values were analysed using a (non-parametric) Mann Whitney U test, with significance determined at p<0.05. Non-parametric statistical tests were conducted as a Shapiro-Wilk analysis revealed a significant violation of normality.

3.10 Invasion Assays

To determine LNCaP cell invasion through Matrigel™, LNCaP cells were trypsinised (section 3.5.1), resuspended in RPMI/10% FCS, transiently transfected with either 2μg pcDNA3.1 (Appendix 3) or 2μg pEGFP-ETS1p51 (Appendix 3) and 8x10^5 cells/well added to 6-well plates (section 3.5.4). Plates were incubated at 37°C/5% CO₂ for 24 hours and the following day, the required number of transwell inserts containing Matrigel™ were warmed to room temperature for ~15 minutes to allow the Matrigel™ to gel, immersed in 0.5mL RPMI/5% CSS (lower and upper chambers) in 24 well plates and incubated for 2 hours at 37°C/5% CO₂. Transfected cells were trypsinised and suspended at 2x10^5 cells/mL in RPMI/5% CSS containing
either 10μM SB431542\textsuperscript{49}, 10ng/mL TGF-β1\textsuperscript{60} or 0.1% DMSO (vehicle control). Medium was aspirated from the upper chamber of the transwell inserts, the inserts were transferred to fresh 24-well plates containing RPMI/10% FCS\textsuperscript{46} (lower chamber) and 0.5mL of the cell suspension was added to the upper chamber of each transwell. Plates were incubated for 48 hours at 37°C/5% CO\textsubscript{2} and to prepare the inserts for counting, the contents of the upper chambers (medium, cells, Matrigel\textsuperscript{TM}) were aspirated, the upper sides of the filters were cleaned with cotton buds and the inserts were removed from the plates and immersed in 100% methanol for 2 minutes, 1% Toluidine Blue\textsuperscript{61} for 10 minutes followed by 2x2 minutes in ddH\textsubscript{2}O. Inserts were inverted, air dried for ~10 minutes, the insert membranes excised using scalpel blades and placed lower side down onto a drop of immersion oil on glass microscope slides. A drop of immersion oil was placed on top of each insert, a coverslip placed over the insert and the edges of the coverslips sealed using nail polish. Inserts were viewed at 100x magnification using a Leica DM 2500 camera, imaged using the Olympus software Cell Sens Standard (Exposure: 27.65ms, Sensitivity: ISO200, Contrast: vivid) and a grid used to standardise cell counting in 10 fields per slide, which was performed manually using Image J. Cell numbers were calculated as percentages of control vector transfected (untreated) cells to determined the invasion index (%). Replicate percentage values were analysed using a (non-parametric) Mann Whitney U test, with significance determined at p<0.05. Non-parametric statistical tests were conducted as a Shapiro-Wilk analysis revealed a significant violation of normality.
Chapter 4: Characterisation of ETS1 Isoform Expression in Human Prostate Tumours

4.1 Introduction

Alternative gene splicing is a normal physiological process that is important for maintaining diversity of protein functions (Leoni et al. 2011). Specific mechanisms that promote alternative splicing are not well-characterised, however aberrant expression and function of splice variants have been detected in cancers and are implicated in the abnormal behaviour of cancer cells (Venables 2004; Ghigna et al. 2008). In prostate cancer, alternative splicing of genes including the androgen receptor, KLF6, Cyclin D1, BCL-X and the ETS factor gene fusion, TMPRSS2-ERG have been reported and are proposed to contribute to disease progression by their aberrant functions (Sette 2013). Alternative splicing of the TMPRSS2-ERG gene fusion in prostate cancer cells results in expression of multiple mRNA and protein variants, altered ratios or differences in relative abundance of which are associated with less favourable disease outcomes (Hu et al. 2008). In vitro and in vivo studies of TMPRSS2-ERG splice variants have been used to demonstrate differential regulation of prostate cancer cell proliferation, invasiveness and motility (Wang et al. 2008), establishing significant roles of alternative splice variants during the carcinogenic process and in disease progression.

The ETS1 gene is alternatively spliced, resulting in the generation of three mRNA transcripts, each of which produces a structurally and functionally distinct ETS1 protein (i.e. ETS1p51, ETS1p42, ETS1p27) (section 1.5.1) (Dittmer 2003; Laitem et al. 2009). Studies using human foetal and adult tissues and cancer cell lines have identified co-expression of ETS1 isoforms in the majority of human tissue types, with the relative abundance and levels of expression of ETS1 isoforms infrequently equal within individual tissues or cell lines (Laitem et al. 2009; Lulli et al. 2010; Shaikhibrahim et al. 2012). In addition, individual tissues can show exclusive expression or lack of expression of a specific ETS1 isoform, which is hypothesised to represent tissue-specific or isoform-specific ETS1 functions (Laitem et al. 2009; Lulli et al. 2010). For example, ETS1 isoform mRNA expression in foetal and adult tissues can be exclusive (e.g. ETS1p51 is the only isoform detectable in peripheral blood leukocytes), ETS1 isoform expression may change during development (e.g. only ETS1p27 is detectable in foetal lung and only ETS1p51 is detectable in adult lung) or ETS1 isoforms may be co-expressed in characteristic ratios (e.g. ETS1p51 is more highly expressed compared with ETS1p27 in foetal kidney tissue) (Laitem et al. 2009).
Chapter 4: Characterisation of ETS1 Isoform Expression in Human Prostate Tumours

Studies of human solid tumours or cancer cell lines have demonstrated diverse levels of ETS1 mRNA and protein isoform expression, however total ETS1 levels are typically elevated in the majority of cancer types investigated (Davidson et al. 2001; Buggy et al. 2004; Alipov et al. 2005; Laitem et al. 2009). PCR products corresponding to ETS1p51, ETS1p42 and ETS1p27 mRNA are reported to be expressed in the prostate cancer cell lines LNCaP, VCaP, DU145 and PC-3 and although the methods were not quantitative, levels of the ETS1 PCR products differed between the cell lines (Shaikhhibrahim et al. 2012). In a study using prostate cancer cell lines with features that correlate with disease progression (LNCaP, C4, C4-2 and C4-2B), ETS1p51 mRNA expression was not found to be associated with disease progression however, ETS1p51 protein levels were progressively increased in the cell lines exhibiting more aggressive growth characteristics (Smith et al. 2012a). ETS1 mRNA and protein is expressed in the human prostate, with lower levels detected in normal prostate or BPH tissue compared with prostate tumours, and with progressively increasing ETS1 expression in higher grade tumours (Alipov et al. 2005; Li et al. 2012a; Smith et al. 2012a). Elevated ETS1 expression is also correlated with tumour aggressiveness and the more rapid development of castrate resistant prostate cancer (Li et al. 2012a). Immunohistochemical studies that identified higher ETS1 levels in prostate tumours have additionally reported aberrant localisation of ETS1 in the cytoplasm of tumour cells, while in the breast cancer cell line MDA-MB-231, which expresses both ETS1p51 and ETS1p27, higher levels of ETS1p27 were shown to enhance the nuclear export and cytoplasmic accumulation of ETS1p51 (Alipov et al. 2005; Laitem et al. 2009). Based on these findings, the co-expression of ETS1p51 and ETS1p27 has been hypothesised to contribute to the detection of cytoplasmic ETS1, suggesting that isoform co-expression can influence the intracellular distribution of ETS1 and therefore its transcriptional activity (Laitem et al. 2009).

ETS1 expression and function are regulated post-transcriptionally through modulation of mRNA/protein stability and turnover however, these events and isoform-specific differences in the post-transcriptional regulation of ETS1 levels are not well-characterised. Experimental evidence that Ets1 protein turnover is likely to affect its overall expression has been reported in studies using murine full-length Ets1 in COS-7 cells. In these studies, Ets1 was shown to be degraded via ubiquitination-induced proteolysis and the stability of Ets1 protein was enhanced by the upregulated expression
of PIASγ, a cofactor that reduced Ets1 degradation or turnover via its interaction with the Ets1 TAD (Nishida et al. 2007). As the Ets1 TAD is absent in the ETS1p27 isoform but present in the ETS1p51 and ETS1p42 isoforms, the findings suggest differential regulation of ETS1 isoform stability and changes in the relative expression of ETS1 isoforms in association with PIASγ levels. In the prostate cancer cell line LNCaP and LNCaP subcultures that exhibit features of disease progression, phosphorylation of ETS1p51 at T38 was found to be increased in the more aggressive sublines (Smith et al. 2012a). Phosphorylation of T38, which is absent in ETS1p27, potentially alters protein stability by enhancing ETS1 transcriptional activity, promoting nuclear localisation and thus reducing available (free cytoplasmic) ETS1 for degradation (Smith et al. 2012a). ETS1p42 has been shown to form more stable DNA complexes compared to ETS1p51, decreasing its availability for degradation and thereby altering the relative expression of ETS1 isoforms (Fisher et al. 1994). In addition to post-translational mechanisms, androgen stimulation of LNCaP prostate cancer cells has been reported to enhance ETS1p51 protein levels, while androgen depletion reduced ETS1p51 expression (Preece 2009; Smith et al. 2012a). In contrast, androgen-mediated increases in ETS1p42 and ETS1p27 levels have not been reported and specific modulation of expression of individual ETS1 isoforms by the large number of transcription factors and miRNAs identified to regulate ETS1 expression has not been documented (Chan et al. 2011; Zhang et al. 2011; He et al. 2013; Williams et al. 2013).

Common and unique functions of each ETS1 isoform have been identified, with a number of studies reporting tissue-specific roles of individual isoforms. For example, studies using the colorectal cancer cell line DLD1 identified that only ETS1p42 can induce apoptosis, a finding that was attributed to the more stable DNA-binding kinetics of ETS1p42 compared with ETS1p51 which resulted in sustained (versus transient) transactivation of the caspase-1 gene (Suzuki et al. 1995; Li et al. 1999b). Differences in ETS1p51 and ETS1p42 function were also demonstrated using the mouse fibroblast cell line NIH3T3, with microarray analysis used to show the differential regulation of target genes by ETS1p51 and ETS1p42 (Hahne et al. 2011). For example, ETS1p42 overexpression enhanced TIMP4 expression while overexpression of ETS1p51 repressed TIMP4 expression, and overexpression of ETS1p51 promoted while ETS1p42 repressed NIH3T3 cell migration and invasion relative to parental cells (Hahne et al. 2011). Unique activities of ETS1 isoforms were also characterised, with regulation of
MMP3 mRNA levels only detected in NIH3T3 cells that overexpressed ETS1p42, findings that may partially account for differences in cell behaviour (e.g. invasion, apoptosis) associated with overexpression of specific ETS1 isoforms (Hahne et al. 2011). Tissue-specific functions of ETS1 isoforms are also evident, with studies using the HEK293 human embryonic kidney cell line identifying only ETS1p51 regulation of MMP3 expression, which was facilitated by an intact ID (ETS1p42 lacks one ID), while in the MDA-MB-231 breast cancer cell line, ETS1p51 and ETS1p27 compete with each other to induce transcriptional activation or repression, respectively of the target genes, MMP3, uPA, p53 and p21 (Laitem et al. 2009; Leprivier et al. 2009). Overexpression of ETS1p27 in MDA-MB-231 cells repressed proliferation, migration, invasion, anchorage-independent growth and induced cell cycle arrest, all functions that were promoted by overexpression of ETS1p51 (Laitem et al. 2009). In contrast to the tumour growth promoting activities of ETS1p51, overexpression of ETS1p27 repressed the growth of subcutaneous MDA-MB-231 xenografts in mice (Laitem et al. 2009).

The elevated expression of ETS1 reported in human prostate tumours has been characterised using mRNA detection methods (in-situ hybridisation, RT-qPCR) or immunohistochemical analyses that detect all ETS1 isoforms (Alipov et al. 2005; Li et al. 2012a; Smith et al. 2012a). As such, the expression and co-expression of ETS1 isoforms in either nonmalignant or malignant prostate and the isoform or isoforms that constitute the increased ETS1 expression detected in prostate tumours are unknown. In order to determine expression and co-expression of the ETS1 mRNA and protein isoforms ETS1p51, ETS1p42 and ETS1p27 in prostate tumours, mRNA and proteins extracted from 45 human prostate tumours and the adjacent nonmalignant prostate of the 44 patients were analysed using RT-qPCR and western blotting in this study.
Chapter 4: Characterisation of ETS1 Isoform Expression in Human Prostate Tumours

4.2 Results

For analysis of ETS1 isoform mRNA and protein isoforms, mRNA and proteins extracted from 45 prostate cancers by M Epis (Hormone-Dependent Cancers Laboratory, Harry Perkins Institute of Medical Research) were obtained. The prostate tumour and adjacent nonmalignant prostate tissues had been selected by a pathologist (R Cohen, Uropath, West Leederville) from 44 radical prostatectomy specimens. Limited clinical information was available in association with the specimens, however Gleason scores for each tumour were listed (Appendix 4).

4.2.1 Construction of ETS1 Isoform-Specific qPCR Primers

To determine expression of the ETS1 mRNA isoforms ETS1p51, ETS1p42 and ETS1p27 in prostate tumours, isoform-specific qPCR primers were designed according to RASE primer design online software (http://designs.lgfus.ca) (Brosseau et al. 2010) (Figure 4.1). The ETS1p51 (NM_005238.3) and ETS1p42 (NM no longer available) NCBI reference sequences were entered into RASE by dividing the sequence into the upstream constitutive sequence (UCS), alternatively included region (AIR, spliced region) and downstream constitutive sequence (DCS). Parameters selected for algorithm-derived primer pairs included minimum amplicon length of 90bp and maximum amplicon length of 200bp. Additional primer restrictions included oligonucleotide length ~18-24bp, Tm ~55-60° and GC% ~45-65%. To confirm their specificity, primer sequences were entered into Primer BLAST™ and to determine primer penalties (incompatibility) or potential self-complementarity, primers were analysed using the online OligoCalc Calculator (www.basic.northwestern.edu/tiotools/oligocalc.html) that examined potential self annealing, GC% content and hairpin or dimer formation. Primers were accepted following completion of all tests (Appendix 2). Primer sequences for ETS1p27 (Laitem et al. 2009) and GAPDH (Rogojina et al. 2003) were designed according to published data.

4.2.2 Optimisation of RT-qPCR primers

To optimise the qPCR conditions for ETS1p51, ETS1p42, ETS1p27 and the reference gene GAPDH, RNA was extracted from MDA-MB-231 and LNCaP cells using Ultraspec™ RNA (section 3.7.1), 1μg RNA was reverse transcribed using SuperScript
Chapter 4: Characterisation of ETS1 Isoform Expression in Human Prostate Tumours

III (section 3.7.3) and PCRs were performed (section 3.7.5) using a range of MgCl₂ concentrations and annealing temperatures. For GAPDH, PCRs were performed using 3mM MgCl₂ (Rogojina et al. 2003), annealing temperatures of 55°C to 65°C and with 1μL MDA-MB-231 cDNA (section 3.7.5). Following PCR, 15μL of each reaction was electrophoresed in a 2% agarose gel and the gels were visualised under UV transillumination (Figure 4.2A) (section 3.2). The single PCR product of ~112bp in each reaction corresponded to the predicted size of GAPDH thereby supporting primer specificity. The strongest bands that indicated more optimal PCR conditions were detected in PCRs performed with annealing temperatures of 59°C to 63.3°C. Existing qPCR protocols with an annealing temperature of 60°C were selected as this was within the optimum range (section 3.7.6). Using the same approach as for the GAPDH primer set, ETS1-isofrm specific primer pairs were optimised using MDA-MB-231 cDNA to the following conditions: ETS1p51 (MgCl₂: 3mM, Tm: 58°C, Amplicon: 144bp, Figure 4.3) ETS1p42 (MgCl₂: 2mM, Tm: 58°C, Amplicon: 192bp, Figure 4.4) and ETS1p27 (MgCl₂: 2mM, Tm: 60°C, Amplicon: 282bp, Figure 4.5).

4.2.3 Generation of RT-qPCR Efficiency Curves

PCR efficiency curves were generated ≥3 times for each primer pair using at least duplicate 10μL reactions (section 3.7.5) containing 2.5, 5, 10, 20 or 40ng of purified MDA-MB-231 cDNA (GAPDH, ETS1p51) (section 3.7.8), serial dilutions of pEGFP-ETS1p42 (a plasmid encoding ETS1p42) or pGEM-ETS1p27 (a plasmid encoding ETS1p27). PCRs were performed in a Roche Lightcycler 480, with Ct values calculated from amplification curves used to generate the efficiency curves and melt curve analysis used to confirm amplification of a single product (GAPDH (Figure 4.2), ETS1p51 (Figure 4.3), ETS1p42 (Figure 4.4) and ETS1p27 (Figure 4.5)). To assemble efficiency curves, Ct value replicate averages were plotted against the log of cDNA (ng) and graphed as dot plots (± SEM) with a line of best fit. R² values were each >0.98, with GAPDH: R²=0.99937, ETS1p51: R²=0.98649, ETS1p42: R²=0.98903 and ETS1p27: R²=0.99957. Amplification efficiencies were also acceptable, with GAPDH: 102%, ETS1p51: 105%, ETS1p42: 91% and ETS1p27: 81% (section 3.7.8). (ETS1p27 PCR efficiency was low, however this isoform was not able to be detected in the tissue samples (section 4.2.4)).
Figure 4.1: *ETS1* gene and mRNA isoform structure. Included are nucleotide positions and size (bp) of exons (red), untranslated regions (green) and coding sequence (black). ETS1 mRNA isoforms *ETS1p51*, *ETS1p42* and *ETS1p27* (black lines) with gaps representing spliced regions relative to *ETS1p51*. Blue arrows indicate locations of ETS1 isoform-specific primers generated using RASE. The *ETS1p42* reverse and *ETS1p27* forward primers were designed to encompass splice junctions.
Chapter 4: Characterisation of ETS1 Isoform Expression in Human Prostate Tumours

(A)  

1. 55°C  6. 63.3°C  
2. 55.7°C  7. 64.5°C  
3. 57°C  8. 65°C  
4. 59°C  9. 59°C (-ve)  
5. 61.4°C

(B)  

Efficiency  102%  
R² (correlation)  0.99937  
Curve equation  \( y = -3.271x + 19.935 \)
Figure 4.2: Optimisation of GAPDH qPCR. (A) GAPDH PCRs were prepared using 1μL MDA-MB-231 cDNA and performed with annealing temperatures of 55°C to 65°C. 15μL of each reaction was electrophoresed in a 2% agarose gel, with a single band at the expected size of ~112bp amplified in each PCR. GAPDH PCR efficiency was evaluated in a Roche Lightcycler 480 using reactions containing 2.5, 5, 10, 20 and 40ng of MDA-MB-231 cDNA. (B) Fluorescence intensity and Ct values were calculated for each reaction, (C) melt curve analysis was performed post-PCR to confirm amplification of a single product and (D) average Ct values for each cDNA concentration were plotted against the log of the starting amount of cDNA. Using the optimised qPCR conditions, GAPDH was amplified with 102% efficiency ($R^2 = 0.999$).
Chapter 4: Characterisation of ETS1 Isoform Expression in Human Prostate Tumours

(A) Efficiency

1. 1mM MgCl₂
2. 1.5mM MgCl₂
3. 2mM MgCl₂
4. 2.5mM MgCl₂
5. 3mM MgCl₂
6. -ve (2mM MgCl₂)

(B) Curves

1. 55°C
2. 55.7°C
3. 57°C
4. 59°C
5. 61.4°C
6. 63.3°C
7. 64.5°C
8. 65°C
9. -ve (59°C)

(C) Graph 1

(D) Graph 2

(E) Efficiency Curve

Efficiency 105%
R² (correlation) 0.98649
Curve equation y=-3.1979x + 26.933
Figure 4.3: Optimisation of ETS1p51 qPCR. (A) ETS1p51 PCRs were prepared containing 1-3mM MgCl₂, amplified with an annealing temperature of 58°C and 15μL of each reaction was electrophoresed in a 2% agarose gel. (B) To optimise annealing temperature, PCRs were prepared using MDA-MB-231 cDNA and 2mM MgCl₂, amplified with annealing temperatures of 55-65°C and 15μL of each PCR product was electrophoresed in a 2% agarose gel. ETS1p51 PCR efficiency was evaluated in reactions containing 2.5, 5, 10, 20 and 40ng of MDA-MB-231 cDNA. (C) Fluorescence intensity and Ct values were calculated for each reaction, (D) melt curve analysis was performed post-PCR to confirm amplification of a single product and (E) average Ct values for each cDNA concentration were plotted against the log of the starting amount of cDNA. Using the optimised qPCR conditions, ETS1p51 was amplified with 105% efficiency ($R^2 = 0.986$).
Chapter 4: Characterisation of ETS1 Isoform Expression in Human Prostate Tumours

(A) 1. pGFP-ETS1p42 (+ve control) (2mM MgCl₂)
2. 1mM MgCl₂
3. 1.5mM MgCl₂
4. 2mM MgCl₂
5. 2.5mM MgCl₂
6. 3mM MgCl₂
7. -ve (2mM MgCl₂)

(B) 1. 55°C  6. 63.3°C
2. 55.7°C  7. 64.5°C
3. 57°C  8. 65°C
4. 59°C  9. -ve (59°C)
5. 61.4°C

(C) 192bp

(D) 192bp

(E) Efficiency 91%
R² (correlation) 0.98903
Curve equation y = -3.56x + 24.961
Figure 4.4: Optimisation of ETS1p42 qPCR. (A) ETS1p42 PCRs were prepared containing 1-3mM MgCl₂, amplified with an annealing temperature of 58°C and 15μL of each reaction was electrophoresed in a 2% agarose gel. (B) To optimise annealing temperature, PCRs were prepared using MDA-MB-231 cDNA and 2mM MgCl₂, amplified with annealing temperatures of 55-65°C and 15μL of each PCR product was electrophoresed in a 2% agarose gel. ETS1p42 PCR efficiency was evaluated in reactions containing serial dilutions (1:2) of a pGFP-ETS1p42 plasmid. (C) Fluorescence intensity and Ct values were calculated for each reaction, (D) melt curve analysis was performed post-PCR to confirm amplification of a single product and (E) average Ct values for each cDNA concentration were plotted against the log of the starting amount of cDNA. Using the optimised qPCR conditions, ETS1p42 was amplified with 91% efficiency (R²=0.98903).
Chapter 4: Characterisation of ETS1 Isoform Expression in Human Prostate Tumours

(A) 1. 1mM MgCl₂  
2. 1.5mM MgCl₂  
3. 2mM MgCl₂  
4. 2.5mM MgCl₂  
5. 3mM MgCl₂  
6. –ve (2mM MgCl₂)

(B) 1. 55°C  
2. 55.7°C  
3. 57°C  
4. 59°C  
5. 61.4°C  
6. 63.3°C  
7. 64.5°C  
8. 65°C  
9. –ve (59°C)

(E) Efficiency 81%  
R² (correlation) 0.99957  
Curve equation y=−3.8656x + 25.935
Chapter 4: Characterisation of ETS1 Isoform Expression in Human Prostate Tumours

Figure 4.5: Optimisation of ETS1p27 qPCR. (A) ETS1p27 PCRs were prepared containing 1-3mM MgCl₂, amplified with an annealing temperature of 58°C and 15μL of each reaction was electrophoresed in a 2% agarose gel. (B) To optimise annealing temperature, PCRs were prepared using MDA-MB-231 cDNA and 2mM MgCl₂, amplified with annealing temperatures of 55-65°C and 15μL of each PCR product was electrophoresed in a 2% agarose gel. ETS1p27 PCR efficiency was evaluated in reactions containing serial dilutions (1:2) of a pGEM-ETS1p27 plasmid. (C) Fluorescence intensity and Ct values were calculated for each reaction, (D) melt curve analysis was performed post-PCR to confirm amplification of a single product and (E) average Ct values for each cDNA concentration were plotted against the log of the starting amount of cDNA. Using the optimised qPCR conditions, ETS1p27 was amplified with 81% efficiency (R² = 0.99957).
4.2.4 Determination of ETS1-Isoform mRNA Expression in Nonmalignant Prostate and Prostate Tumour Tissues

To determine ETS1 isoform mRNA expression in human nonmalignant prostate and prostate tumour tissues, RNA concentration in each of the mRNA samples extracted from 45 prostate tumour samples and 44 adjacent nonmalignant prostate tissues (section 4.2) was determined (section 3.7.2, results not shown), cDNA was prepared by reverse transcription of 1μg RNA (section 3.7.3) and a minimum of duplicate qPCRs were prepared per cDNA sample (section 3.7.6). ETS1p51 qPCR and melt curve analysis identified ETS1p51 mRNA expression in all nonmalignant prostate and prostate tumour samples (89/89, 100%) as evidenced by amplification of a single product using melt curve analysis (section 3.7.7, Figure 4.6, Appendix 4). ETS1p42 RT-qPCR of the clinical RNA specimens identified ETS1p42 mRNA in 84% (37/44) non-malignant prostate and in 75% (34/45) prostate tumour specimens, accounting for 80% (71/89) of samples (Figure 4.7, Appendix 4). RT-qPCR for ETS1p27 failed to amplify specific products in any of the RNA samples (data not shown), with agarose gel electrophoresis of 15μL of each of the amplified products confirming these findings (Figure 4.8). ETS1 mRNA expression in the specimens was normalised using RT-qPCR for GAPDH (Figure 4.9) and all qPCR runs included internal efficiency curve controls for each gene analysed (neat, 1:10 and 1:100 dilutions of positive control cDNA/plasmid (section 4.2.3)) and negative controls (ddH2O).

In order to determine ETS1p51 and ETS1p42 cDNA (mRNA) copy number and to compare ETS1p51 and ETS1p42 expression in nonmalignant prostate and prostate tumour specimens, absolute quantitation of ETS1p51 and ETS1p42 mRNA expression was performed using the means of replicate qPCR Ct values (section 3.7.6). Log cDNA values for ETS1p51 and ETS1p42 in nonmalignant prostate and prostate tumour tissues were plotted and from these analyses, mean ETS1p51 mRNA levels in both nonmalignant prostate and prostate tumour tissues were found to be ~1000-fold higher than mean ETS1p42 mRNA levels in these tissues (p<0.0001) (Figure 4.10A). Statistical comparison of ETS1p51 mRNA expression between nonmalignant prostate and prostate tumour tissue identified no statistically significant differences (p=0.0940). Similarly, nonmalignant prostate and prostate tumour tissue ETS1p42 mRNA expression also identified no significant differences (p=0.1014). ETS1p51 and ETS1p42
mRNA expression was also sorted by the Gleason score of the tumours to identify correlations between $ETS1$ isoform expression and tumour grade however, no associations between Gleason score and $ETS1$ isoform mRNA expression were apparent (Figure 4.10B,C). Raw data and calculations are listed in Appendix 4.

To determine normalised $ETS1p51$ and $ETS1p42$ mRNA expression in nonmalignant and malignant specimens, prostate tumour samples were re-matched with adjacent nonmalignant prostate tissue samples for each specimen and Ct values were normalised against respective $GAPDH$ Ct values using the $\Delta$Ct method (section 3.7.6) (Figure 4.11A,B). These analyses identified that normalised $ETS1p51$ mRNA expression levels were significantly higher in nonmalignant prostate relative to prostate tumour samples ($p<0.0005$) (Figure 4.11A). In contrast, comparison of $ETS1p42$ mRNA expression between nonmalignant prostate and prostate tumour tissues did not identify significant differences ($p=0.2022$) (Figure 4.11B).

Relative $ETS1p51$ and $ETS1p42$ mRNA expression in prostate tumours versus adjacent nonmalignant prostate was also calculated using Ct values and the Pfaffl method of relative quantification (section 3.7.6). Presentation of results as a waterfall plot identified that $ETS1p51$ mRNA expression was lower in 31/45 (69%) of prostate tumour specimens compared to adjacent nonmalignant prostatic tissue (Figure 4.12A). Similarly, $ETS1p42$ mRNA expression was lower in 20/31 (65%) of prostate tumours compared to adjacent nonmalignant prostatic tissue (Figure 4.12B).

### 4.2.5 Optimisation of Western Blotting for Detection of ETS1 Protein Isoforms

To detect ETS1 protein isoforms, western blotting was optimised using lysates from the COS-7, DU145 and LNCaP cell lines, each of which is reported to express at least one ETS1 protein isoform (Sugimoto et al. 2003; Itoh et al. 2012; Smith et al. 2012a). For these analyses, cells that had been routinely cultured were immunoblotted for ETS1 and $\beta$-actin using conditions optimised previously in this laboratory for detection of ETS1p51 (Preece 2009; section 3.7.4). In COS-7 cell lysates, strongly immunoreactive bands at $\sim 51kDa$ and $\sim 27kDa$ were detected using the ETS1 antibody, with a faint band evident at $\sim 42kDa$ (Figure 4.13A). DU145 cells also expressed high levels of ETS1p51, with weaker ETS1 immunoreactive bands at $\sim 42kDa$ and $\sim 27kDa$, while LNCaP cells expressed lower levels of ETS1p51, with a very faint ETS1 immunoreactive band at $\sim 27kDa$ (Figure 4.13A). Longer exposures of ETS1 western blots using LNCaP cell
Figure 4.6: *ETS1p51* expression in nonmalignant prostate and prostate tumour specimens. (A) *ETS1p51* RT-qPCR of (i) the first 16 samples pairs and (ii) the remaining 29 sample pairs resulted in Ct values between 22 and 30 (Appendix 4). (B) Melt curve analysis of the amplified PCR products from (i) the first 16 sample pairs and (ii) the remaining 29 sample pairs identified single products in all cases. Positive amplification (red), unsure/late amplification (green) and negative amplification (blue) are indicated. mRNA samples were reverse transcribed twice with qPCR performed in at least duplicate for each cDNA and mean results used for subsequent analyses. Representative results are depicted.
Figure 4.7: *ETS1p42* expression in nonmalignant prostate and prostate tumour specimens. (A) *ETS1p42* RT-qPCR of (i) the first 16 sample pairs and (ii) the remaining 29 sample pairs resulted in Ct values between 24 and 32 (Appendix 4). (B) Melt curve analysis of the amplified PCR products from (i) the first 16 sample pairs and (ii) the remaining 29 sample pairs identified single products in all cases. Positive amplification (red), unsure/late amplification (green) and negative amplification (blue) are indicated. mRNA samples were reverse transcribed twice with qPCR performed in at least duplicate for each cDNA and mean results used for subsequent analyses. Representative results are depicted.
Figure 4.8: Agarose gel electrophoresis of ETS1p27 RT-qPCR products. ETS1p27 qPCR of the prostate specimens (Appendix 4) followed by melt curve analysis (not shown) did not identify amplification of a specific product in any of the samples. Electrophoresis of samples in 2% agarose gels supported the lack of amplification of ETS1p27 from the tissue specimens. ETS1p27 (~282bp) was detected in RNA/cDNA derived from MBA-MB-231 cells and a pGEM-ETS1p27 plasmid.
Figure 4.9: GAPDH expression in nonmalignant prostate and prostate tumour specimens. (A) GAPDH RT-qPCR of (i) the first 16 samples pairs and (ii) the remaining 29 sample pairs resulted in Ct values between 15 and 25 (Appendix 4). (B) Melt curve analysis of the amplified PCR products from (i) the first 16 sample pairs and (ii) the remaining 29 sample pairs identified single products in all cases. Positive amplification (red), unsure/late amplification (green) and negative amplification (blue) are indicated. mRNA samples were reverse transcribed twice with qPCR performed in at least duplicate for each cDNA and mean results used for subsequent analyses. Representative results are depicted.
Figure 4.10: *ETS1* isoform copy number (log cDNA number) in nonmalignant prostate and prostate tumour specimens. *ETS1* isoform mRNA levels in nonmalignant prostate and prostate tumour tissues were determined by RT-qPCR using the respective efficiency curve equations. (A) *ETS1* isoform expression (log cDNA number) in nonmalignant prostate samples (blue) and prostate tumour samples (red). (B) *ETS1p51* expression (log cDNA copy number) sorted by Gleason score. (C) *ETS1p42* expression (log cDNA copy number) sorted by Gleason score. Experiments were performed twice with representative data shown. Significance was determined using Wilcoxon matched signed ranks testing.
Figure 4.11: Normalised ETS1 isoform mRNA expression. ETS1 isoform mRNA levels in nonmalignant prostate (blue) and prostate tumour (red) tissues were determined by RT-qPCR and quantitated using the ΔCt method. (A) ETS1p51 isoform expression normalised to GAPDH and (B) ETS1p42 isoform expression normalised to GAPDH. Experiments were performed twice with representative data shown. Significance was determined using Wilcoxon matched signed ranks testing.
Chapter 4: Characterisation of ETS1 Isoform Expression in Human Prostate Tumours

Figure 4.12: Relative ETS1 mRNA isoform expression in prostate cancer and adjacent nonmalignant prostate tissues. ETS1p51, ETS1p42 and GAPDH Ct values from each of the samples were used to determine (A) relative ETS1p51 and (B) relative ETS1p42 expression in matched prostate tumour (T) and adjacent nonmalignant prostate (N) specimens using the Pfaffl equation (incorporating respective PCR efficiencies). Samples were ordered from highest to lowest relative ETS1 isoform mRNA expression. Dashed orange line represents average relative ETS1 expression.
lysates produced more distinct immunoreactive bands at ~51kDa, however these conditions resulted in overexposed images for ETS1 western blots of other cell lines (not shown). ETS1p51 expression was also detected in additional cell lines including the MDA-MB-231 and MCF-7 breast cancer cell lines, and an alternative ETS1 antibody (Abcam) was also tested, which only detected the full-length (~51kDa) isoform (not shown).

4.2.6 Determination of ETS1 Protein Isoform Expression in Nonmalignant Prostate and Prostate Tumour Specimens

To examine ETS1 protein isoform expression, western blotting was performed using 15μL aliquots of proteins that had been extracted from the same cohort of nonmalignant prostate and prostate tumour specimens that were used to examine ETS1 mRNA isoform expression (sections 3.8.2, 3.8.3, 3.8.4, 4.2.4). ETS1 immunoreactivity was detected at ~51kDa in the tissue specimens, indicating that although ETS1p51 and ETS1p42 mRNA were expressed, only ETS1p51 protein levels were within detectable limits of the antibody used (Figure 4.13B). A wide range of ETS1p51 expression was evident in both the nonmalignant prostate and prostate tumour specimens, with ETS1p51 protein detected in 38/44 (86%) of nonmalignant prostate and 43/45 (95%) of prostate tumour specimens. ETS1p51 protein levels were normalised using β-actin immunoblots for each sample, indicating that ETS1 protein levels were overall significantly lower (p<0.0016) in nonmalignant prostate compared with prostate tumours (Figure 4.13C). The data also indicated a broad range of ETS1 protein expression in nonmalignant prostate (range: 0.01 - 1.84) and more noticeably in prostate tumours (range: 0.006 - 3.47) (Figure 4.13C). Relative ETS1p51 levels in prostate tumours compared to adjacent nonmalignant prostate tissue were also determined and identified that ETS1p51 was more highly expressed in 30/45 (67%) prostate tumours (mean=2.2 fold, median=1.44 fold) (Figure 4.13D) (Appendix 5). Higher ETS1p51 levels did not correlate with increasing Gleason score (results not shown).

4.2.7 Comparison of ETS1 mRNA and Protein Expression in Prostate Specimens

To identify correlations between ETS1 mRNA and protein isoform expression, relative ETS1p51 mRNA expression (section 4.2.4) was matched with relative ETS1p51 protein expression (section 4.2.6) for each specimen (Figure 4.14A), however no associations were evident within the cohort. These findings were further supported in
scatter plots of normalised $ETS1p51$ mRNA and ETS1p51 protein levels in either nonmalignant prostate (Figure 4.14Bi) or prostate tumour tissues (Figure 4.14Bii), with $R^2$ values of 0.005 and 0.0007 obtained, respectively (Figure 4.14B,C). Additionally, no correlations were identified when mRNA and protein expression data were stratified according to Gleason score (results not shown).
Chapter 4: Characterisation of ETS1 Isoform Expression in Human Prostate Tumours

(A)  
![Image of immunoblot showing ETS1 and β-actin bands with molecular weights indicated: 51kDa, 42kDa, and 27kDa for ETS1, and 42kDa for β-actin. Lanes labeled 1, 2, 3 correspond to COS-7, DU145, and LNCaP cells, respectively.]

(B)  
![Image of immunoblot showing ETS1p51 and β-actin bands with molecular weights indicated: 51kDa and 42kDa. Lanes labeled with sample numbers N1 to T12 representing different samples.]

(C)  
![Graph showing normalised ETS1p51 protein levels with p-value 0.0016.]

(D)  
![Graph showing relative ETS1p51 levels across sample pair numbers.]
Figure 4.13: ETS1 isoform protein expression in nonmalignant prostate and prostate tumour specimens. (A) ETS1 western blotting of prostate cancer (DU145, LNCaP) and COS-7 cell lysates identified bands of ~51, ~42 and ~27kDa consistent with the molecular sizes of ETS1p51, ETS1p42 and ETS1p27. (B) ETS1 and β-actin western blotting of matched nonmalignant prostate (N) and prostate tumour tissues (T). Western blotting was performed twice for all samples with representative blots shown. (C) Western blotting results were quantitated by densitometry and normalised using respective β-actin blots for nonmalignant prostate (blue) and prostate tumour specimens (red). Bars indicate mean normalised ETS1p51 levels. Normalised ETS1p51 protein levels were compared using the Wilcoxon matched signed ranked test. (D) Waterfall plot of relative ETS1p51 protein levels (T/N) sorted from highest to lowest with sample pair number indicated. Dashed orange line represents average relative ETS1 expression, which was ~2.2 fold higher in tumour samples.
Figure 4.14: Correlation of relative ETS1p51 mRNA and protein expression in prostate tumour and nonmalignant prostate specimens. (A) Relative ETS1p51 mRNA and ETS1p51 protein levels in prostate tumour (T) and nonmalignant prostate (N) specimens sorted in order of relative ETS1p51 mRNA expression (highest to lowest). (B) Comparison of ETS1p51 mRNA and ETS1p51 protein levels in (i) nonmalignant prostate and (ii) prostate tumour specimens. Correlation was determined by (linear) line of best fit.
4.3 Discussion

Altered expression of members of the ETS transcription factor family is common in prostate tumours and may arise due to diverse mechanisms including genomic translocations resulting in the formation of gene fusions (e.g. *TMPRSS2-ERG*) (Tomlins *et al.* 2005) or mutations (e.g. *ETV6*) (Kibel *et al.* 2002), while processes leading to aberrant expression of other ETS factors are unresolved (e.g. ETS1, ETS2, FLI1) (Watson *et al.* 2010). In addition to their abnormal expression, alternative splicing of ETS factor genes may produce variant transcripts and proteins with distinct functions that promote cancer-associated cell behaviours including proliferation and migration (Giovane *et al.* 1997; Hu *et al.* 2008; Mao *et al.* 2008; Xue *et al.* 2008; Laitem *et al.* 2009). While ETS1 protein overexpression in human prostate tumours has been confirmed in several immunohistochemical studies (Alipov *et al.* 2005; Li *et al.* 2012a; Smith *et al.* 2012a), ETS1 mRNA levels are less well defined (Alipov *et al.* 2005; Smith *et al.* 2012a) and the expression or co-expression of ETS1 isoforms in prostate cancers has not been reported. Due to the distinct and potentially opposing functions of ETS1 isoforms (Fisher *et al.* 1994; Dittmer 2003; Laitem *et al.* 2009), the functional consequences of ETS1 overexpression in human prostate tumours will be dependent on the relative expression of ETS1 isoforms in the tumour cells.

The characterisation of ETS1 isoform expression in prostate tumours and in adjacent nonmalignant prostate was determined in this study by examining RNA and protein extracts from the prostate tissues of 45 patients who had undergone radical prostatectomy as treatment for prostate cancer. While all specimens were pre-screened by a pathologist (R. Cohen, Uropath) to select nonmalignant and malignant areas, prostate tissues are heterogeneous and contain different proportions of nonmalignant/malignant epithelial cells and other cell types including stromal, endothelial and inflammatory cells, each of which are known to express ETS1. For example, high levels of ETS1 have been detected in multiple immune cell types (e.g. NK, T and B cells) as well as in stromal cells supporting neoplasms (Behrens *et al.* 2001; Behrens *et al.* 2003; Garrett-Sinha 2013). Therefore, while the proportions of additional cell populations in the samples were likely to be minor due to careful selection of tissue areas, ETS1 mRNA and protein expression analyses performed in
this study represent averages from the multiple cell types present in the tissue specimens. In addition, prostate tumours are known to be heterogeneous and may contain foci of tumour cells with different levels of expression of subsets of marker mRNA and protein isoforms including ETS1, which would contribute to the average ETS1 mRNA and protein levels detected in this study (Barry et al. 2007; Mehra et al. 2007; Furusato et al. 2008).

A strong feature of this cohort was that both mRNA and proteins were able to be extracted from nonmalignant and malignant tissues from the same patient specimens, which permitted direct comparison of ETS1 expression between individual prostate cancers and the adjacent nonmalignant prostate, as well as comparison of ETS1 mRNA and protein expression in the individual tissue types. However, no information regarding patient treatments prior to or concurrent with radical prostatectomy was available and it is unknown whether these may alter expression of ETS1 in the tissues examined. Previous studies have demonstrated that chemotherapeutic agents can affect expression of ETS family members (e.g. FLI-1) in other tumour types including Ewing’s sarcoma and leukaemias, indicating that ETS1 expression may have been altered if patients had received neoadjuvant treatments (Li et al. 2012b; Li et al. 2014b).

Expression of multiple ETS1 isoforms has been documented in a variety of nonmalignant and diseased tissues, with ETS1p27 isoform expression only recently identified in multiple cancer cell lines and nonmalignant human tissues (Laitem et al. 2009). This is consistent with expression of isoforms of other ETS factors in prostate cancer cell lines and human prostate tumour specimens where, for example, co-expression of the ESE3 splice variants ESE3a and ESE3b has been reported, with relatively higher levels of ESE3b found in both sample types (Cangemi et al. 2008). Based on the limited number of specific investigations of ETS1 isoform expression, it is also feasible that additional uncharacterised ETS1 isoforms are expressed in the nonmalignant prostate and in prostate tumours, however use of isoform-specific RT-qPCR primers in this and previous studies would limit the ability to detect novel ETS1 variants. Future studies to identify novel isoforms including proposed additional ETS1 isoforms (Shaikhibrahim et al. 2012) may involve multiple techniques including tandem mass spectrometry, transcriptomics/proteomics, transcript isoform sequencing (TIF-
Use of RT-qPCR resulted in detection of *ETS1p51* and *ETS1p42* mRNA in nonmalignant prostate and prostate tumour tissues. This result is consistent with a previous report of *ETS1p51* mRNA expression in the nonmalignant prostate, prostate tumour specimens and the prostate cancer cell lines, LNCaP, C4, C4-2 and C4-2B (Smith *et al.* 2012a). In contrast, an earlier study failed to detect *ETS1* in the nonmalignant prostate using in situ hybridisation, but only reported its expression in prostate tumours, suggesting that *ETS1* mRNA levels were higher in prostate tumours compared with nonmalignant prostate where *ETS1* expression, if present, was at levels too low to detect using that method (Alipov *et al.* 2005). In the only published RT-qPCR study (Smith *et al.* 2012a), qPCR was performed specifically against *ETS1p51*, while the prior in situ hybridisation method did not discriminate between *ETS1* isoforms (Alipov *et al.* 2005), therefore until the present study, individual and relative *ETS1* isoform expression in the nonmalignant prostate and in human prostate tumours was unknown.

*ETS1p27* mRNA was not detected in nonmalignant prostate, prostate tumours or the LNCaP cell line, contrasting a previous study which reported *ETS1p27* mRNA expression in the prostate cancer cell lines LNCaP, VCaP, DU145 and PC-3 (Shaikhhibrahim *et al.* 2012). Although it is feasible that RT-qPCR methods used in this thesis were less sensitive, or that different isolates of LNCaP cells express distinct subsets of *ETS1* isoforms, the larger reported size of amplified PCR products in comparison to the primers used to amplify *ETS1p27* in the study of Shaikhhibrahim *et al* indicate that sequencing analysis of the amplified PCR products would be required to confirm *ETS1p27* expression in the cell line isolates (Shaikhhibrahim *et al.* 2012). Consistent with the original report of *ETS1p27* mRNA expression, *ETS1p27* was detected in MDA-MB-231 cells which, taken with findings from this study suggest that if present, *ETS1p27* expression in prostatic tissues is at near undetectable levels for RT-qPCR methods (Laitem *et al.* 2009). Taking into account the technical limitations of methods employed for these investigations, the undetectable expression of *ETS1p27*...
indicates its limited contribution to ETS1 function in the adult prostate or in prostate cancer.

In this study, ETS1p51 and ETS1p42 were found to be co-expressed in the majority of nonmalignant and malignant samples but with levels of ETS1p51 expression an order of magnitude above that of ETS1p42 in every specimen. Multiple ETS1 isoforms are expressed in various foetal and adult tissues, yet PCR studies highlight that levels are rarely equal and this is likely to indicate non-redundant isoform functions (Laitem et al. 2009). Similarly, higher expression of the ESE3 isoform, ESEb relative to ESEa and undetectable levels of the isoform ESEj in dendritic cells have been proposed to reflect more critical functions of ESEb and to a lesser extent, ESEa with no determined role of ESEj in this cell type (Sprater et al. 2012). Co-expression of the ETS1p51 and ETS1p42 isoforms has been reported in human breast and colon cancer, melanoma cell lines and mouse fibroblast cell lines in which each isoform was proposed to perform distinct functions (Huang et al. 1997; Behrens et al. 2001; Rothhammer et al. 2004; Hahne et al. 2011). In addition, evidence from Ets1p51 and Ets1p42 knockout mouse models indicates a high degree of non-overlapping functions of each Ets1 isoform during the development of multiple lymphoid cell populations (Higuchi et al. 2007).

Characterisation of mechanisms that promote alternative splicing of the ETS1 gene resulting in differential expression of ETS1 isoforms is also limited, however the similar relative levels of ETS1p51 and ETS1p42 between malignant and nonmalignant tissues suggest that the preferential splicing and transcription of ETS1 may be conserved in nonmalignant and malignant prostatic tissues. In the MDA-MB-231 breast cancer cell line which expresses all three ETS1 isoforms but with reduced ETS1p42 levels, overexpression of ETS1p42 impairs cell survival in vitro, suggesting that repression of ETS1p42 expression in breast cancer cells specifically enhances cell survival (Ballschmieder et al. 2003). In prostate tumours harbouring the TMPRSS2-ERG fusion, differential expression of alternative ERG transcripts has been associated with outcome, and in vitro these transcripts differentially regulate prostate cancer cell proliferation, invasion and motility (Hu et al. 2008; Wang et al. 2008). Similarly, knockout of the ETS factor GABP isoforms GABPβ1L or GABPβ1S results in normal development compared to embryonic lethality, respectively (Xue et al. 2008).
Comparison of ETS1 mRNA isoform expression in nonmalignant and malignant prostate tissues indicated that ETS1 transcripts were expressed at significantly lower levels in prostate tumours compared to nonmalignant prostate. Expression of ETS2, another member of the same ETS factor subfamily is also downregulated in prostate tumours compared to nonmalignant prostate (Ernst et al. 2002) and similarly, levels of ERG mRNA are lowest in higher grade prostate tumours (Petrovics et al. 2005). Analysis of large transcriptome and genome studies of human prostate tumour cohorts using Oncomine™ has identified that ETS1 mRNA expression is reduced in the majority of prostate tumours screened (50-80%), supporting the findings of this study (Rhodes et al. 2007; Tomlins et al. 2007b; Barwick et al. 2010; Grasso et al. 2012). No ETS1 deletions or LOH are included in Oncomine™, indicating that these are not primary mechanisms which contribute to reduced ETS1 mRNA expression in prostate tumours. While no ETS1 mutations were detected in the large number of prostate tumours included in the database, hypermethylation of DNA was identified in chromosomal regions encompassing the FLI1 and ETS1 genes, which are located adjacent to each other on chromosome 11 in a head-to-head gene orientation (not shown). Therefore it is feasible that DNA hypermethylation potentially contributes to the reduced ETS1 mRNA expression detected in prostate tumour specimens.

In addition to promoter methylation, altered levels of ETS1 in prostate cancers may result from the increased or decreased expression of regulators of ETS1 expression. ETS1 mRNA expression is regulated by microRNAs, which target the 3’UTR, and multiple microRNAs including miR-1, miR-125b, miR-200b, miR-145 and miR-499 have been shown to repress expression of ETS1 (Chan et al. 2011; Zhang et al. 2011; Wei et al. 2012; Fleming et al. 2013; Zheng et al. 2013). In prostate tumours, numerous microRNAs are over- or under-expressed including miR-1, miR-200b and miR-499 (Porkka et al. 2007; Walter et al. 2013). miR-125b and miR-221/222, which repress expression of ETS1 in human breast cancer and embryonal kidney cell lines, respectively (Mattia et al. 2011; Zhang et al. 2011) are overexpressed in prostate cancers and in more aggressive prostate cancer cell lines, potentially contributing to the reduced ETS1 mRNA expression detected in prostate tumours (Shi et al. 2007; Sun et al. 2009; Pang et al. 2010). Future correlations between miRNA and ETS1 levels in
human prostate tumour specimens will indicate involvement of specific miRNAs or other transcriptional regulators in the regulation of ETS1 expression.

Although patient numbers were very low in this study, ETS1 mRNA isoform expression was not found to correlate with increasing disease severity (Gleason score), an observation that is consistent with ETS1p51 mRNA expression in the LNCaP cell line and its more aggressive sub-lines, C4, C4-2 and C4-2B (Smith et al. 2012a). In contrast, the same report identified that ETS1p51 mRNA expression was significantly correlated with increasing Gleason score (4-9) in 32 individuals with prostate cancer (Smith et al. 2012a). As specimen numbers in the present study and in the previous report of Smith et al were low, larger studies will be required to confirm results and in particular to identify whether ETS1 mRNA or protein levels are the better predictors of disease severity.

ETS1p51 was the only protein isoform detected in the prostate specimens and was overexpressed in the majority of prostate tumours in comparison to nonmalignant prostate tissues. This finding is consistent with previous immunohistochemistry studies that have reported higher levels of ETS1 protein in prostate tumours relative to nonmalignant or BPH tissues (Alipov et al. 2005; Li et al. 2012a; Smith et al. 2012a). Immunohistochemical methods used in previous studies were not able to differentiate the relative abundance and expression of individual ETS1 isoforms as the antibody used in those as well as the present study is raised against an epitope within the ETS1 ETS domain (Santa Cruz, C-20) and therefore will recognise ETS1p51, ETS1p42 and ETS1p27, which each contain the ETS domain. The identification of ETS1p51, ETS1p42 and ETS1p27 by western blotting in this thesis is comparable to previous reports in which the three isoforms were detected in the VCaP, LNCaP, PC-3 and DU145 prostate cancer cell lines (Shaikhibrahim et al. 2012; Smith et al. 2012a). Supporting the findings of the present study, ETS1 protein expression was verified in a subset of specimens using an alternative ETS1 antibody (Abcam, 1G11), which confirmed expression of ETS1p51 but not other ETS1 isoforms in both nonmalignant and malignant prostate tissue specimens (results not shown).
Chapter 4: Characterisation of ETS1 Isoform Expression in Human Prostate Tumours

Western blotting analysis of proteins extracted from nonmalignant and malignant prostate tissues indicated that the quality of samples was not equivalent to protein extracts from cell lines which exhibited sharper immunoreactive bands suggesting less protein degradation. The lower quality of protein extracts isolated from human prostate and prostate tumours was not unexpected due to delays in tissue processing and the possible presence of areas of ischaemia or necrosis in the tissue specimens. Although the quality of tissue protein extracts may have reduced the sensitivity of ETS1 protein detection, in combination with the significantly lower levels of \textit{ETS1p42} mRNA in the prostate specimens, results of the present study indicate that either ETS1p42 protein levels were too low to be detected using this western blotting procedure, or that \textit{ETS1p42} mRNA is not being translated into protein. The results are consistent with a previous study in which \textit{ETS1p51} and \textit{ETSp42} transcripts were expressed in multiple leukaemia cell lines however only ETS1p51 protein was detected (Lulli \textit{et al.} 2010).

In addition to the large differences in \textit{ETS1p51} and \textit{ETS1p42} mRNA levels, post-transcriptional mechanisms may differentially regulate the translation and/or half-life of ETS1p51 and ETS1p42, leading to the detection of ETS1p51 protein only in the present study. Differences in the relative abundance of ETS1 protein isoforms has been observed in breast cancers (Buggy \textit{et al.} 2004) as well as in other tissue and cell types (Laitem \textit{et al.} 2009), suggesting that mechanisms regulating ETS1 isoform expression are likely to be tissue or disease specific. Due to the isoform-specific functions of ETS1, the consequences of ETS1 overexpression will be dependent on the ETS1 isoforms that are overexpressed. For example, ETS1p51 and not ETS1p42 is responsible for the transcriptional activation of \textit{MMP3} (a regulator of extracellular matrix remodelling), a distinct function of ETS1p51 which is likely similar for other target genes in non-malignant and malignant prostate tissue (Leprivier \textit{et al.} 2009). It is also possible that ETS1p42 is translated but that the protein is less stable than ETS1p51. Accelerated degradation of ETS factors has been proposed previously, with studies of ETV1 expression in LNCaP cells identifying that despite expression of \textit{ETV1} mRNA, no ETV1 protein was detectable and this was attributed to the instability or high turnover of ETV1 protein (Vitari \textit{et al.} 2011). Similarly, \textit{PDEF} mRNA expression is elevated in a majority of breast tumours, however protein expression is undetectable, a finding proposed to result from uncharacterised post-transcriptional or post-translational
mechanisms (Feldman et al. 2003). Post-translational modifications may alter the stability or turnover of ETS1 proteins, for example, polyubiquitination of ETS1 in HEK293T cells results in increased ETS1 degradation via the 26S proteasome pathway (Ji et al. 2007). In addition, inhibition of ETS1 PARylation (poly(ADP-ribosyl)ation), leads to the accumulation of ETS1 and is proposed to participate in proteasome-mediated degradation of ETS1p51, but not ETS1p27 which lacks the target ubiquitination sites, indicating the likelihood of additional isoform-specific regulatory mechanisms (Legrand et al. 2013).

No correlations between ETS1 mRNA and protein expression were identified in either nonmalignant prostate tissue or in prostate tumours. Furthermore, while ETS1 transcripts were expressed at lower levels in prostate tumours compared with non-malignant prostate, the majority of the same specimens exhibited elevated levels of ETS1p51 protein in prostate tumours relative to the adjacent non-malignant prostate. This disparity is comparable to findings in human breast specimens, in which ETS1 mRNA expression was found to be similar in nonmalignant and malignant breast specimens, however in the majority of breast tumours, ETS1p51 levels were increased (Buggy et al. 2004). Although ETS1p51 protein expression was not correlated with Gleason score in the present and a previously published study (Li et al. 2012a), higher expression of ETS1 has been correlated with more rapid disease progression and a poorer prognosis of prostate cancer patients (Li et al. 2012a). Due to the increased incidence of biochemical recurrence in patients with ETS1-overexpressing prostate tumours (Li et al. 2012a), it is important that future studies further characterise associations between ETS1 expression and disease severity, biochemical recurrence or patient responses to existing therapies. As patient numbers are small in all reported studies, the potential use of reduced ETS1 mRNA and/or elevated ETS1 protein as a disease biomarker will require further investigations using larger cohorts to identify definitive associations between ETS1 mRNA or protein expression with prognosis or progression of disease. Major findings of this initial study therefore indicate elevated ETS1 protein expression in human prostate tumours that entirely or almost entirely consists of ETS1p51. Based on these findings, subsequent studies have evaluated the effects of elevated ETS1p51 expression in prostate cancer cells.
Chapter 5: ETS1p51 Effects on the Expression of EMT-Associated Genes

5.1 Introduction

Epithelial-to-mesenchymal transition (EMT) is a reversible physiological process that is essential for the development and homeostasis of foetal and adult tissues (Kalluri et al. 2009; Zeisberg et al. 2009). The three primary types of EMT occur during embryogenesis and development (Type I), tissue regeneration and fibrosis (Type II) and EMT-associated cancer progression and metastasis (Type III) (Kalluri et al. 2009; Zeisberg et al. 2009) (section 1.6). Classification of type III EMT has stemmed from findings that aggressive cancers can exploit EMT-associated processes, in particular cell migration and invasion to extravasate and form metastases (Thiery et al. 2009). Typically, cancer-associated EMT processes are modulated by the aberrant activation of EMT promoting signalling pathways including the TGF-β, WNT and NOTCH pathways and tyrosine kinase receptor pathways (e.g. EGFR, FGFR) (section 1.6.1). Induction of EMT in cancer cells involves reduced epithelial and increased mesenchymal marker expression with concomitantly enhanced migratory, invasive and proliferative characteristics, and has been associated with increased cell survival and the promotion of therapy-resistance (Thiery et al. 2009; Nauseef et al. 2011).

Progression of prostate cancer can occur in conjunction with the activation of EMT-related signalling pathways and EMT-like characteristics (e.g. elevated expression of EMT-promoting transcription factors and mesenchymal markers), suggesting that EMT is a bona fide mechanism underpinning the aggressive tumour growth, metastasis and poorer patient outcome associated with advanced disease (Wikstrom et al. 1998; Nauseef et al. 2011; Sethi et al. 2011; Behnsawy et al. 2013). Cadherin type ‘switching’ typifies EMT and multiple studies have documented loss of E-cadherin and the gain of N-cadherin expression in prostate cancer cells undergoing EMT (Tomita et al. 2000; Gravdal et al. 2007). Consistent with this finding, the EMT promoter, SNAIL1 is overexpressed in the majority of prostate tumours where it may transcriptionally regulate expression of genes which enhance cell migration and invasion (Smith et al. 2012b; Fawzy et al. 2013; Deep et al. 2014). The SNAIL family member, SLUG is also a promoter of EMT which has been shown to enhance prostate cancer cell migration and invasion while potently repressing expression of E-cadherin (Bolton et al. 2007; Uygur et al. 2011; Wu et al. 2012). Despite conflicting results of studies investigating
the expression and role of β-catenin in prostate tumours, higher expression and nuclear accumulation of β-catenin is associated with reduced E-cadherin expression and destabilisation of adherens junctions, and is responsible for enhancing the EMT-associated invasive characteristics of prostate cancer cell lines (Jaggi et al. 2005; Whitaker et al. 2008; Vlad-Fiegen et al. 2012; Wu et al. 2013). The EMT-promoting transcription factor TWIST1 is also highly expressed in aggressive prostate tumours, where it functions as a predictive marker of metastatic potential. In vitro, TWIST1 promotes mesenchymal marker expression and cell invasion while repressing apoptosis in prostate cancer cell lines (Kwok et al. 2005; Yuen et al. 2007; Pellecchia et al. 2012; Eide et al. 2013). Studies using prostate cancer cell lines have characterised ZEB1-mediated repression of E-cadherin, activation of mesenchymal marker expression and cell migration (Drake et al. 2009). In addition, higher levels of ZEB1 are found in androgen-deprived LNCaP prostate cancer cells and are associated with the acquisition of EMT characteristics, increased tolerance to the chemotherapeutic agent, docetaxel and progression toward CRPC, which is mediated in part via AR-signalling crosstalk (Sun et al. 2012).

Recent studies have begun to characterise crosstalk between established prostatic signalling pathways (e.g. AR signalling) and EMT-related genes/pathways (e.g. EMT-promoting transcription factors, TGF-β and WNT signalling), suggesting that their interaction mediates critical functions during prostate tumorigenesis, prostate cancer cell survival, metastasis and subsequent responses to therapies (Nauseef et al. 2011; Grant et al. 2013). In particular, AR signalling has been linked to the induction of expression of EMT-promoting transcription factors including SNAIL1, SLUG and β-catenin, cadherin type-switching, and enhanced prostate cancer cell migratory and invasive characteristics (in vitro) which are each proposed to be important during EMT as well as the development of CRPC (Bolton et al. 2007; Zhu et al. 2010; Vlad-Fiegen et al. 2012; Wu et al. 2012). In addition, AR interaction with and the induction of β-catenin is implicated in the disruption of cell-to-cell adhesion, a critical event of EMT evident in prostate cancer progression (Mulholland et al. 2002; Vlad-Fiegen et al. 2012; Grant et al. 2013). Activation of EMT is also believed to enhance the development of therapy resistance and cell survival, with AR-mediated activation of EMT and associated genes/pathways proposed to promote therapy resistance during the
development of CRPC (Thiery et al. 2009; Nauseef et al. 2011). Based on the findings of these experimental studies, characterisation of individual EMT mediators and signalling pathways will further the understanding of prostate tumour progression by elucidating mechanisms that facilitate the development of CRPC, thereby identifying candidate factors or pathways suitable for therapeutic targeting.

In prostate tumours, AR-mediated signalling can synergise with aberrantly co-expressed molecules such as EZH2 (polycomb) and the ETS factor fusion protein, TMPRSS2-ERG to collaboratively enhance EMT-like traits in prostate cancer cells through regulation of genes including DAB2IP, which represses E-cadherin expression and enhances vimentin expression (Xie et al. 2010; Yu et al. 2010). In the nonmalignant prostate cell line RWPE, overexpression of the ETS factor ETV4 results in enhanced mesenchymal marker and MMP expression and reduced epithelial marker expression and is associated with a mesenchymal-like state with elevated TWIST1 and ZEB1 expression. These data suggest that ETS factor overexpression may be important during prostate cancer progression potentially via induction of EMT processes (Pellecchia et al. 2012). The ETS factor ERG is also overexpressed a high proportion of prostate tumours and is associated with activation of the WNT/β-catenin signalling pathway, driving EMT-mediated prostate cancer cell invasiveness (Wu et al. 2013). Additional studies have reported ETS factor-mediated suppression of EMT-like characteristics including PDEF-mediated downregulation of EMT-related gene expression (i.e. SLUG and SLUG target genes) in breast cancer cells (Findlay et al. 2011). Interestingly, PDEF expression is reduced in both breast and prostate tumour cells in comparison to non-malignant breast and prostate, respectively (Watson et al. 2010).

The overexpression of ETS1 in breast cancer cell lines promotes cell invasiveness and EMT-like characteristics including elevated uPA, MMP1, MMP3 and vimentin expression, while simultaneously downregulating E-cadherin expression (Gilles et al. 1997). Several studies report that the overexpression of ETS1 in prostate tumours is associated with disease severity and biochemical recurrence (Alipov et al. 2005; Li et al. 2012a), with more recent functional studies indicating that ETS1 promotes aggressive prostate tumour characteristics through the upregulation of cell motility and invasive properties (Shaikhibrahim et al. 2011; Smith et al. 2012a).
Evidence of ETS1-mediated increases in cell motility and invasion has been documented in cancer cell lines and occurs in conjunction with regulation of the target genes uPA and MMPs, expression of which is elevated in prostate tumours and known to function during EMT (Gilles et al. 1997; Taki et al. 2006; Massie et al. 2007; Ghosh et al. 2012b; Behnsawy et al. 2013). In addition, previous studies have initiated characterisation of ETS1/TGF-β signalling cross-talk, with the TGF-β target gene ZEB1 also demonstrated to be a direct target of ETS1 and a potent repressor of E-cadherin expression, thereby repressing epithelial-like cell characteristics (Shirakihara et al. 2007; Dave et al. 2011). ETS1 transcriptionally upregulates expression of TGF-β receptor 2 (TGF-βR2) in embryonal carcinoma cell lines, enhancing the potential activation of TGF-β signalling (Kopp et al. 2004). Other studies have identified a high degree of overlap in ETS1/SMAD target gene binding sites, promoter co-occupancy and co-regulation of target genes (Koinuma et al. 2009) (section 1.5.4).

Given the recent association of ETS factor-mediated regulation of EMT in multiple cancer types and the pro-migratory and pro-invasive functions of ETS1 in prostate tumour cells, it was feasible that ETS1 was able to induce an EMT programme in prostate cancer cells. Therefore, ETS1-mediated regulation of EMT processes was investigated, with initial studies examining differential expression of EMT-associated genes following overexpression of ETS1p51. Due to the previously reported interaction between ETS1 and the AR in prostate cancer cells (Massie et al. 2007), the effects of DHT treatment on the expression of EMT-associated genes was similarly determined in LNCaP cells that expressed low or high ETS1p51 levels.
5.2 Results

To investigate the effects of ETS1 overexpression on EMT, a cell culture model of ETS1p51 overexpression was required, which would allow comparison of gene expression between prostate cancer cells with low or high ETS1p51 levels. An appropriate model would be use of stably transfected cells with doxycycline inducible ETS1p51 expression. Several experimental systems for inducible gene expression have been developed including the Tet-On system (Clontech) in which cells are stably transfected to express a doxycycline-inducible protein (Tet-On 3G) encoded by the pCMVTet-3G doxycycline responsive plasmid, with Tet-On 3G transactivating expression from a second construct containing the coding sequence of the gene of interest (ETS1p51) under control of a Tet-On 3G responsive promoter (pTRE3G) (Gossen et al. 1995). Therefore, to generate doxycycline-inducible expression of ETS1p51 in the androgen-responsive prostate cancer cell line, LNCaP which expresses low levels of endogenous ETS1p51, LNCaP cells would be stably transfected with pCMVTet-3G. Following ligation of the ETS1p51 coding sequence into pTRE3G, the pTRE3G-ETS1p51 plasmid would be stably transfected into LNCaP pCMV-Tet3G cells to generate cells in which doxycycline treatment induces high levels of ETS1p51 expression.

5.2.1 Stable transfection of LNCaP cells with pCMVTet-3G

In the first stage of development of LNCaP cells with doxycycline-inducible ETS1p51 expression, LNCaP cells were stably transfected with the pCMV-Tet3G plasmid (section 3.5.5, Appendix 3). Transfected cells were selected following culture with the antibiotic G418 (400µg/mL) and maintained in medium containing 200µg/mL G418 (section 3.5.5; results not shown). To assess the doxycycline responsiveness of the resulting (uncloned) LNCaP pCMVTet-3G cell line, cells were transiently transfected with the pTRE3G-LUC luciferase reporter construct which expresses firefly luciferase under control of a Tet-inducible promoter (section 3.5.4, Appendix 3). Following transfection, cultures were incubated with 0-1µg/mL doxycycline for 24 hours prior to measurement of firefly luciferase activity (section 3.6). LNCaP pCMVTet-3G cells transiently transfected with the pTRE3G-LUC construct and
cultured in the absence of doxycycline exhibited no firefly luciferase activity, while cells cultured with 0.5-1µg/mL doxycycline exhibited a dose-dependent increase in firefly luciferase activity (Figure 5.1A). These results indicated that the stable transfection was successful as the resulting LNCaP pCMVTet-3G cells exhibited no expression of pTRE3G in the absence of doxycycline, while expression was induced in the presence of doxycycline.

### 5.2.2 Construction of the pTRE3G-ETS1p51 Plasmid

To enable doxycycline-induced ETS1p51 expression, LNCaP pCMVTet-3G cells were stably transfected with a pTRE3G-IRES ETS1p51 construct containing the ETS1p51 coding sequence under control of the Tet-inducible promoter (Appendix 3). In order to generate the pTRE3G-IRES-ETS1p51 expression construct, the ETS1p51 coding sequence was PCR-amplified from an ETS1p51-V5 expression construct (Appendix 3) using primers containing MLU1 restriction enzyme sites (section 3.7.5; not shown). Following gel extraction, the ~1.3kb ETS1p51 PCR product was ligated into the pGEM-T cloning vector (section 3.1.4; not shown), transformed into competent DH5α cells (section 3.1.5) and plasmids were isolated from 8 of the resulting colonies (section 3.1.6). Agarose gel electrophoresis of the MLU1-digested plasmids identified the ~1.3kb ETS1p51 product in clones 1, 5, 7 and 8 (Figure 5.1B). Clones 1 and 5 were sequenced (section 3.4) to confirm the absence of mutations in the ETS1p51 coding sequence (Appendix 9), following which ETS1p51 coding sequence obtained by MLU1 digestion of clone 5 was ligated into MLU1-digested, SAP-treated pTRE3G-IRES expression construct (section 3.1.3, 3.1.4). Ligation products were transformed into competent DH5α cells (section 3.1.5), and plasmids were extracted from 3 of the resulting colonies (section 3.1.6), digested with MLU1, electrophoresed in agarose gels (section 3.1.2) and visualised under UV transillumination (Figure 5.1C). A ~1.3kb ETS1p51 insert was identified in all plasmids and clones 2 and 3 were sequenced (section 3.4), confirming the presence of ETS1p51 with no mutations (Appendix 9). Glycerol stocks of clones 2 and 3 containing pTRE3G-ETS1p51 were prepared (section 3.1.8) and subsequently used to purify plasmids from large scale cultures for use in transfection experiments (section 3.1.7).
Chapter 5: ETS1p51 Effects on the Expression of EMT-Associated Genes

Figure 5.1: Development of an LNCaP cell model with doxycycline-inducible ETS1p51 expression. (A) Following stable transfection of LNCaP cells with pCMVTet-3G (LNCaP pCMVTet-3G), the doxycycline inducibility of Tet-3G expression was determined by transient transfection of the cells with the pTRE3G-LUC luciferase reporter construct. No luciferase activity was detected when cells were cultured without doxycycline, while luciferase activity was progressively increased when cells were cultured with 0.5 or 1μg/mL doxycycline. Results are mean ± SEM firefly luciferase activity. Experiments were performed three times with representative results shown. (B) To generate the pTRE3G-ETS1p51 expression construct, the ETS1p51 coding sequence was PCR-amplified from pcDNA3.1-ETS1p51-V5 using primers containing 5’ MLU1 restriction enzyme sites and ligated into the pGEM-T easy cloning vector. MLU1 digestion of pGEM-ETS1p51 isolated from 8 clones identified ~1.3kb ETS1p51 inserts in clones 1, 5, 7 and 8. (C) Following subcloning of ETS1p51 from pGEM-ETS1p51 into pTRE3G, MLU1 digestion of pTRE3G-ETS1p51 plasmids isolated from 3 of the clones identified ~1.3kb ETS1p51 inserts in each of the plasmids (Appendix 9).
5.2.3 Transfection of LNCaP cells with pTRE3G-ETS1p51

To test the doxycycline-induced expression of ETS1p51, LNCaP cells were transiently transfected with pTRE3G-IRES or pTRE3G-ETS1p51 (section 3.5.4), or a combination of pCMVTet3G and pTRE3G-ETS1p51 and cultured in the absence or presence of 1µg/mL doxycycline for 24 hours. Proteins extracted from the cultures were separated by SDS-PAGE (section 3.8.2), and immunoblotting for ETS1 and β-actin resulted in the identification of ~51kDa bands corresponding to ETS1p51 in each sample (section 3.8.4, Figure 5.2A), with highly elevated ETS1p51 levels in doxycycline-treated LNCaP cells that had been transfected with pCMVTet3G and pTRE3G-MLU1-ETS1p51. Similar experiments performed using LNCaP pCMVTet3G cells (section 5.2.1; not shown) produced equivalent results, indicating that these vectors would be suitable to use for preparation of stably transfected LNCaP cells with doxycycline inducible ETS1p51 expression.

To generate LNCaP cells containing both pCMVTet3G (section 5.2.1) and pTRE3G-MLU1-ETS1p51, LNCaP pCMVTet3G cells were co-transfected with the pTRE3G-ETS1p51 construct (section 5.2.2, Appendix 3) and either Hygromycin or Puromycin linear resistance markers (section 3.5.5). Alternatively, parental LNCaP cells were co-transfected with pCMVTet3G, pTRE3G-ETS1p51 and Hygromycin or Puromycin linear markers (section 3.5.5). Following transfection, cells were selected using culture medium supplemented with 200µg/mL G418 and either 50-200µg/mL Hygromycin B or 0.5µg/mL Puromycin at 24, 48 or 72 hours post-transfection. Outgrowth of colonies was observed during several weeks of culture of transfected cells under all experimental conditions and cloning rings were used to isolate individual colonies, with clonal selection performed by limiting dilution in 96-well plates. Parental LNCaP cells were also added into cultures to serve as a ‘feeder’ population (Bennett et al. 1997), however under all conditions tested, stably transfected colonies were not able to be maintained. For these reasons, alternative methods were required in order to investigate the effects of ETS1p51 overexpression on expression of EMT regulators.
5.2.4 Transient overexpression of GFP-ETS1p51 in LNCaP Cells

To develop experimental protocols for transient overexpression of ETS1p51 in LNCaP cells as an alternative to doxycycline-induced ETS1p51 overexpression, (sections 5.2.1 – 5.2.3), LNCaP cultures were transiently transfected with pEGFP-C2 that encoded GFP-tagged ETS1p51 (pEGFP-C2-ETS1p51) or a control vector (pcDNA3.1) (section 3.5.4, Appendix 3). Cells were harvested 1-5 days following transfection and immunoblotting of protein extracts for GFP identified immunoreactive bands at ~80kDa corresponding to the size of GFP-tagged ETS1p51 in extracts of LNCaP cells that had been transfected with pEGFP-C2-ETS1p51 but not in extracts from pcDNA3.1 transfected cultures (Figure 5.2B). Immunoblotting for ETS1 confirmed the presence of GFP-tagged ETS1p51, with similar levels of immunoreactivity at ~80kDa detected (Figure 5.2B). Using this protocol, ETS1p51 was overexpressed between 1 to 5 days post-transfection, with peak GFP-ETS1p51 levels at day 3 (Figure 5.2B). Endogenous ETS1 immunoreactivity was not detected in either pEGFP-C2-ETS1p51 or pcDNA3.1 transfected LNCaP cells, which indicated the presence of low to undetectable endogenous ETS1 levels in comparison to GFP-ETS1p51. Due to the technical difficulties associated with stable transfection of LNCaP cells (section 5.2.3) and evidence of robust and reproducible GFP-ETS1p51 overexpression following transient transfection of LNCaP cells, subsequent analysis of regulation of expression of EMT-associated genes was performed using transient transfection methods.

5.2.5 Characterisation of GFP-ETS1p51 Overexpressing LNCaP Cells

Prior to studies investigating the effects of ETS1 on expression of EMT-associated genes, expression of AR, ETS1 and AR-responsive genes was determined in the LNCaP cell culture model. In initial experiments, AR and ETS1p51 mRNA levels were quantitated in LNCaP cells following culture in the presence of 10⁻⁸M DHT (or 0.01% EtOH (vehicle)) for 2 to 24 hours (section 3.5.2). RT-qPCR for AR, ETS1p51 and GAPDH was performed and relative mRNA expression was determined using the Pfaffl method (section 3.7.6). Under these culture conditions, AR and ETS1p51 mRNA expression was reduced by ≥50% following DHT treatment, with a nadir at 2 – 12 hours
Chapter 5: ETS1p51 Effects on the Expression of EMT-Associated Genes

Figure 5.2: Ectopic expression of ETS1p51 in LNCaP cells. (A) ETS1p51 expression was determined by immunoblotting in LNCaP cells that had been transiently transfected with 4 μg pTRE3G, 4 μg pTRE3G-ETS1p51 or co-transfected with 1 μg pCMV-Tet3G and 3 μg pTRE3G-ETS1p51, and cultured in the absence or presence of 1 μg/mL doxycycline for 24 hours. (B) LNCaP cells were transiently transfected with 2 μg pcDNA3.1 (control) or 2 μg pEGFP-C2-ETS1p51 then cultured for 1 to 5 days. ETS1p51 and GFP-ETS1p51 levels were determined by immunoblotting for GFP (GFP-ETS1p51) and ETS1 (ETS1p51, GFP-ETS1p51), with β-actin used as a loading control. Experiments were performed three times with representative western blots shown.
and with expression increasing by 24 hours but not returning to baseline (Figure 5.3A). Similar experiments were performed to determine AR and ETS1p51 mRNA levels following transient transfection of LNCaP cells with pEGFP-C2-ETS1p51 (or control pcDNA3.1) vectors (sections 3.5.4, 3.7.6). Consistent with the previous experiments, AR (not shown) and endogenous ETS1p51 (Figure 5.3B) levels were reduced following DHT treatment of pcDNA3.1 transfected cells. AR mRNA levels were not markedly altered following overexpression of GFP-ETS1p51 (not shown), however in pEGFP-C2-ETS1p51 transfected LNCaP cells, ETS1p51 mRNA expression was increased >6000 fold compared to pcDNA3.1 transfected cells (Figure 5.3C) and was further increased to ~11000 fold following DHT treatment of pEGFP-C2-ETS1p51 transfected LNCaP cells (Figure 5.3C).

To characterise expression of AR, ETS1 and NKX3.1 (an AR and ETS1 responsive gene/protein) (Preece et al. 2011), western blotting of proteins extracted from LNCaP cells cultured for 1-8 days in the presence of 10^{-8}M DHT or (0.01% EtOH (vehicle)) was performed (sections 3.5.2, 3.8). When AR, ETS1 and NKX3.1 protein levels were normalised using β-actin western blots, it was identified that AR protein levels were elevated between 1 to 4 days then progressively decreased between 5 to 8 days of DHT treatment (Figure 5.4A). The findings indicate that despite the reduction in AR mRNA expression, AR protein levels are increased following culture of LNCaP cells with DHT, results that have been reported previously (Yeap et al. 1999). Levels of ETS1p51 protein were also elevated in DHT-treated cultures, with progressively increased levels of 3-9 fold above controls between 1 to 6 days, reducing to 7-fold above controls by day 8 of DHT treatment (Figure 5.4B). Expression of NKX3.1 was similarly increased 6-10 fold above controls during 8 days of DHT treatment of LNCaP cells, a finding that has been documented previously (He et al. 1997; Prescott et al. 1998) (Figure 5.4C). Based on these initial experiments, the effects of ETS1p51 overexpression and DHT treatment on expression of EMT-associated genes was investigated using LNCaP cells harvested at 24 hours following transfection with pEGFP-C2-ETS1p51 (or pcDNA3.1 (control)) and cultured in the presence of 10^{-8}M DHT (or 0.01% EtOH (vehicle)).
Figure 5.3: *AR* and *ETS1p51* mRNA expression in LNCaP cells. Following treatment of LNCaP cells with 10⁻⁸M DHT for 2-24 hours (A) *AR* and (B) *ETS1p51* mRNA expression was determined by RT-qPCR (C) *ETS1p51* mRNA expression was determined by RT-qPCR in LNCaP cells transiently transfected with either pcDNA3.1 or pEGFP-C2-ETS1p51 and cultured with 10⁻⁸M DHT or 0.01% EtOH (vehicle control) for 24 hours. Gene expression was normalised using *GAPDH* RT-qPCR and relative expression was determined using the Pfaffl method (Pfaffl et al. 2001). PCRs were performed in duplicate and the experiment was performed twice with representative results shown. Error bars indicate standard error of the mean (±SEM).
Chapter 5: ETS1p51 Effects on the Expression of EMT-Associated Genes

5.2.6 EMT PCR Array

For PCR array analysis (RT² profiler: Human EMT PCR array, SABiosciences) of expression of 84 EMT-associated genes, LNCaP cells cultured in 10cm petri dishes were transiently transfected with pEGFP-C2-ETS1p51 or pcDNA3.1 then cultured for 24 hours post-transfection in medium containing 10⁻⁸M DHT or 0.01% EtOH prior to RNA extraction (sections 3.5.4, 3.5.2, 3.7.1). Before use in the PCR array, levels of AR and ETS1p51 mRNA were evaluated using methods described in section 5.2.5 to confirm that transfection and treatment of cells had produced the expected effects on expression of these genes (not shown) (sections 3.7.6). For the PCR array, cDNA was prepared using an RT² First Strand Kit (section 3.7.4) and added to RT² SYBR green mastermix prior to PCR array (section 3.7.10). The EMT PCR array was conducted twice using RNA extracted from independent experiments (Appendix 6, 7, 8) and data was imported into the RT² Profiler™ PCR array data analysis (version 3.5) programme using an Excel™ spreadsheet template (pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php). Online analysis tools included quality controls such as amplification of negative and positive controls, reverse transcription controls and housekeeping gene expression (also used for gene normalisation), with each requirement satisfactory for these experiments (not shown). Regulation of gene expression was visualised using heatmaps for LNCaP cells overexpressing GFP-ETS1p51 (Figure 5.5), treated with 10⁻⁸M DHT (Figure 5.6) or overexpressing GFP-ETS1p51 and treated with 10⁻⁸M DHT (Figure 5.7) for 24 hours. Genes regulated ≥ 1.5 fold relative to vehicle (EtOH) treated controls were chosen as candidates for further investigation (Figures 5.8, 5.9, 5.10). Following pEGFP-C2-ETS1p51 overexpression in LNCaP cells, 21/84 (25%) genes were regulated with the majority, (16/21) up-regulated and 5/21 genes down-regulated (Figure 5.8). In DHT-treated cultures, 39/84 (46%) genes were regulated, with 10/39 genes up-regulated while the majority, (29/39) were down-regulated (Figure 5.9). When pEGFP-C2-ETS1p51 overexpressing cells were treated with DHT, expression of 50/84 (59%) genes was altered, with 19/50 genes up-regulated and, 31/50 genes down-regulated (Figure 5.10).
Figure 5.4: Regulation of AR, ETS1 and NKX3.1 by DHT in LNCaP cells. LNCaP cells were cultured with 10⁻⁸M DHT or 0.01% EtOH (vehicle control) for 0-8 days prior to immunoblotting for (A) AR, (B) ETS1 and (C) NKX3.1, with protein levels normalised using β-actin western blots and quantitated relative to vehicle control. The experiment was performed twice with representative blots shown.
Figure 5.5: Gene regulation following GFP-ETS1p51 overexpression in LNCaP cells. Heatmap of gene expression (fold change) in LNCaP cells at 24 hours following transient transfection with pEGFP-C2-ETS1p51, indicating reduced (green), unchanged (black) or increased (red) expression relative to gene expression in pcDNA3.1 (control) transfected cells. Genes highlighted in yellow text indicate ≥1.5 fold regulation of expression. Fold change was calculated using RT² Profiler™ PCR array data analysis software (Version 3.5).
Figure 5.6: Gene regulation following DHT treatment of LNCaP cells. Heatmap of gene expression (fold change) in LNCaP cells at 24 hours of $10^{-8}$M DHT treatment indicating reduced (green), unchanged (black) or increased (red) expression relative to gene expression in 0.01% EtOH (vehicle control) treated cells. Genes highlighted in yellow text indicate $\geq 1.5$ fold regulation of expression. Fold change was calculated using RT² Profiler™ PCR array data analysis software (Version 3.5).
Chapter 5: ETS1p51 Effects on the Expression of EMT-Associated Genes

Figure 5.7: Gene regulation following DHT treatment of GFP-ETS1p51 overexpressing LNCaP cells. Heatmap of gene expression (fold change) in LNCaP cells at 24 hours following transient transfection with pEGFP-C2-ETS1p51 and 10^-8 M DHT treatment is included, indicating reduced (green), unchanged (black) or increased (red) expression relative to gene expression in pcDNA3.1 (control) transfected and 0.01% EtOH (vehicle control) treated cells. Genes highlighted in yellow text indicate ≥1.5 fold regulation of expression. Fold change was calculated using RT² Profiler™ PCR array data analysis software (Version 3.5).
Table 5.1: EMT-associated gene expression following GFP-ETS1p51 overexpression in LNCaP cells.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Fold regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPARC</td>
<td>6.774</td>
</tr>
<tr>
<td>WNT5B</td>
<td>6.6807</td>
</tr>
<tr>
<td>TGFB2</td>
<td>5.7358</td>
</tr>
<tr>
<td>VCAN</td>
<td>4.6589</td>
</tr>
<tr>
<td>TGFB1</td>
<td>2.9282</td>
</tr>
<tr>
<td>ZEB1</td>
<td>2.9282</td>
</tr>
<tr>
<td>FGFBP1</td>
<td>2.4284</td>
</tr>
<tr>
<td>MMP9</td>
<td>2.2658</td>
</tr>
<tr>
<td>NODAL</td>
<td>2.2346</td>
</tr>
<tr>
<td>SPP1</td>
<td>1.8921</td>
</tr>
<tr>
<td>TIMP1</td>
<td>1.8921</td>
</tr>
<tr>
<td>MMP2</td>
<td>1.8532</td>
</tr>
<tr>
<td>SOX10</td>
<td>1.8277</td>
</tr>
<tr>
<td>ZEB2</td>
<td>1.8025</td>
</tr>
<tr>
<td>KRT7</td>
<td>1.7171</td>
</tr>
<tr>
<td>TGFB3</td>
<td>1.5052</td>
</tr>
<tr>
<td>RGS2</td>
<td>-1.5263</td>
</tr>
<tr>
<td>WNT5A</td>
<td>-1.5692</td>
</tr>
<tr>
<td>CTNNB1</td>
<td>-1.6358</td>
</tr>
<tr>
<td>COL3A1</td>
<td>-1.7901</td>
</tr>
<tr>
<td>MMP3</td>
<td>-3.4822</td>
</tr>
</tbody>
</table>

**Figure 5.8:** EMT-associated gene expression following GFP-ETS1p51 overexpression in LNCaP cells. Gene expression data from the EMT PCR array were normalised and fold regulation determined using the RT2 Profiler™ PCR array data analysis (version 3.5) programme. Representative values from duplicate PCR array results are shown.
### Chapter 5: ETS1p51 Effects on the Expression of EMT-Associated Genes

#### Figure 5.9: EMT-associated gene expression following DHT treatment of LNCaP cells.

Gene expression data from the EMT PCR array were normalised and fold regulation determined using RT² Profiler™ PCR array data analysis (version 3.5) programme. Representative values from duplicate PCR array results are shown.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Fold regulation</th>
<th>Gene symbol</th>
<th>Fold regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNAI2</td>
<td>28.6408</td>
<td>ESR1</td>
<td>-2.2815</td>
</tr>
<tr>
<td>KRT14</td>
<td>5.0281</td>
<td>BMP1</td>
<td>-2.4116</td>
</tr>
<tr>
<td>TWIST1</td>
<td>5.0281</td>
<td>MST1R</td>
<td>-2.4794</td>
</tr>
<tr>
<td>KRT19</td>
<td>4.2281</td>
<td>TGFB3</td>
<td>-2.639</td>
</tr>
<tr>
<td>WNT5B</td>
<td>3.0314</td>
<td>ZEB1</td>
<td>-2.8481</td>
</tr>
<tr>
<td>JAG1</td>
<td>2.7321</td>
<td>NODAL</td>
<td>-3.0738</td>
</tr>
<tr>
<td>SPARC</td>
<td>2.1886</td>
<td>OCLN</td>
<td>-3.0738</td>
</tr>
<tr>
<td>TGFB2</td>
<td>1.9588</td>
<td>CAV2</td>
<td>-3.1821</td>
</tr>
<tr>
<td>AHNAK</td>
<td>1.6245</td>
<td>FN1</td>
<td>-3.249</td>
</tr>
<tr>
<td>PTK2</td>
<td>-1.5052</td>
<td>FGFBP1</td>
<td>-3.3173</td>
</tr>
<tr>
<td>VPS13A</td>
<td>-1.5583</td>
<td>TGFB1</td>
<td>-3.4822</td>
</tr>
<tr>
<td>WNT11</td>
<td>-1.5583</td>
<td>TCF4</td>
<td>-3.5554</td>
</tr>
<tr>
<td>FZD7</td>
<td>-1.5801</td>
<td>SNAI1</td>
<td>-4.4076</td>
</tr>
<tr>
<td>ITGA5</td>
<td>-1.6472</td>
<td>RGS2</td>
<td>-5.4264</td>
</tr>
<tr>
<td>MMP3</td>
<td>-1.7777</td>
<td>BMP7</td>
<td>-7.1602</td>
</tr>
<tr>
<td>SIP1</td>
<td>-1.8277</td>
<td>COL5A2</td>
<td>-8.5742</td>
</tr>
<tr>
<td>TCF3</td>
<td>-1.9053</td>
<td>IL1RN</td>
<td>-8.8766</td>
</tr>
<tr>
<td>TMEFF1</td>
<td>-1.9588</td>
<td>COL3A1</td>
<td>-10.411</td>
</tr>
<tr>
<td>PLEK2</td>
<td>-2.1287</td>
<td>CAMK2N1</td>
<td>-69.071</td>
</tr>
<tr>
<td>GSC</td>
<td>-2.1435</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Chapter 5: ETS1p51 Effects on the Expression of EMT-Associated Genes

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Fold regulation</th>
<th>Gene symbol</th>
<th>Fold regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNAI2</td>
<td>26.5382</td>
<td>SNAI3</td>
<td>-1.7532</td>
</tr>
<tr>
<td>SPARC</td>
<td>9.4479</td>
<td>TGFB3</td>
<td>-1.7777</td>
</tr>
<tr>
<td>WNT5B</td>
<td>9.2535</td>
<td>TMEFF1</td>
<td>-1.8025</td>
</tr>
<tr>
<td>TGFB1</td>
<td>6.3203</td>
<td>NODAL</td>
<td>-1.815</td>
</tr>
<tr>
<td>KRT14</td>
<td>4.5315</td>
<td>CDH1</td>
<td>-1.8404</td>
</tr>
<tr>
<td>TWIST1</td>
<td>4.0558</td>
<td>SIP1</td>
<td>-1.8532</td>
</tr>
<tr>
<td>KRT7</td>
<td>3.5064</td>
<td>WNT5A</td>
<td>-1.8532</td>
</tr>
<tr>
<td>KRT19</td>
<td>2.6759</td>
<td>DSP</td>
<td>-1.9588</td>
</tr>
<tr>
<td>ESR1</td>
<td>2.4116</td>
<td>MMP3</td>
<td>-2.0279</td>
</tr>
<tr>
<td>TGFB2</td>
<td>2.3295</td>
<td>NOTCH1</td>
<td>-2.042</td>
</tr>
<tr>
<td>SERPINE1</td>
<td>2.2501</td>
<td>TCF3</td>
<td>-2.0562</td>
</tr>
<tr>
<td>TIMP1</td>
<td>2.2501</td>
<td>FZD7</td>
<td>-2.1735</td>
</tr>
<tr>
<td>CDH2</td>
<td>2.1886</td>
<td>CTNNB1</td>
<td>-2.2974</td>
</tr>
<tr>
<td>JAG1</td>
<td>2.114</td>
<td>TMEM132A</td>
<td>-2.2974</td>
</tr>
<tr>
<td>FGFBP1</td>
<td>1.9725</td>
<td>SNAI1</td>
<td>-2.5847</td>
</tr>
<tr>
<td>ZEB1</td>
<td>1.8921</td>
<td>FN1</td>
<td>-2.8879</td>
</tr>
<tr>
<td>MMP9</td>
<td>1.7777</td>
<td>OCLN</td>
<td>-3.0314</td>
</tr>
<tr>
<td>ZEB2</td>
<td>1.5801</td>
<td>TCF4</td>
<td>-3.0951</td>
</tr>
<tr>
<td>SPP1</td>
<td>1.5583</td>
<td>GSC</td>
<td>-3.249</td>
</tr>
<tr>
<td>AKT1</td>
<td>-1.5476</td>
<td>BMP7</td>
<td>-5.2416</td>
</tr>
<tr>
<td>ERBB3</td>
<td>-1.6245</td>
<td>COL3A1</td>
<td>-5.3889</td>
</tr>
<tr>
<td>PTK2</td>
<td>-1.6245</td>
<td>RGS2</td>
<td>-6.9644</td>
</tr>
<tr>
<td>VPS13A</td>
<td>-1.6702</td>
<td>COL5A2</td>
<td>-7.21</td>
</tr>
<tr>
<td>BMP1</td>
<td>-1.6935</td>
<td>IL1RN</td>
<td>-10.928</td>
</tr>
<tr>
<td>DSC2</td>
<td>-1.7532</td>
<td>CAMK2N1</td>
<td>-59.302</td>
</tr>
</tbody>
</table>

**Figure 5.10: EMT-associated gene expression following GFP-ETS1p51 overexpression and DHT treatment of LNCaP cells.** Gene expression data from the EMT PCR array were normalised and fold regulation determined using RT² Profiler™ PCR array data analysis (version 3.5) programme. Representative values from duplicate PCR array results are shown.
To determine the predicted impact of genes regulated in response to DHT and/or ETS1p51 overexpression on cellular functions, gene expression data were stratified according to their associated functions using categories on the QIAGEN/SABiosciences website (http://www.sabiosciences.com/rt_pcr_product/HTML/PAHS-090A.html) (Figure 5.11). Overexpression of GFP-ETS1p51 largely upregulated expression of genes involved in invasion (e.g. MMP2, MMP9), migration (e.g. NODAL, SPARC), adhesion (e.g. SPP1, TIMP1, VCAN) and differentiation (e.g. SOX10, TGF-β2, TGF-β3, WNT5B), with the majority of these genes also functioning as components of EMT-related signalling pathways (e.g. TGF-β and WNT pathways) (Figure 5.12). Functionally, the overexpression of ETS1p51 appeared to up-regulate the expression of mediators of TGF-β and WNT signalling, which are well characterised initiators and regulators of EMT in multiple epithelial cell types, promoting mesenchymal-like characteristics (Thiery et al. 2006).

Clustergram analysis of gene regulation following DHT treatment indicated that processes affected included downregulation of factors associated with adhesion (e.g. COL5A2, BMP1, MMP3), cytoskeleton (e.g. CAV2, PLEK2) and migration (e.g. FN1, NODAL, TGFβ1) (Figure 5.13). The majority of genes regulated encoded factors previously characterised to interact with, or to function as components of the EMT-related signalling pathways, the integrin, WNT and TGF-β pathways (Figure 5.13) (Thiery et al. 2006). Potential effects due to this pattern of gene regulation includes reduction and disruption of the integrin pathway which is required for maintenance of cell-cell junctions and an epithelial-like phenotype (Martin et al. 2009), while downregulation of WNT and TGF-β pathway components would not likely contribute to initiation of EMT. DHT treatment of GFP-ETS1p51 overexpressing cells regulated levels of a subset of genes responsive to either DHT treatment or ETS1p51 overexpression alone (e.g. NODAL, SPARC), along with some unique genes including SERPINE1 (upregulated), and SNAI3 and ERBB3 (downregulated). Clustergram analysis of regulated genes indicated their roles in adhesion, differentiation, invasion and migration, with downregulation of the majority of affected genes and associated processes, including downregulation of components of EMT-related signalling pathways (e.g. integrin, WNT, TGF-β pathways) (Figure 5.14). Based on these results,
Figure 5.11: Clustergram analysis of gene expression following GFP-ETS1p51 overexpression and treatment with DHT. Clustergram analysis indicates downregulated (green) or upregulated (red) gene expression in control (EtOH) LNCaP cells, in LNCaP cells treated with $10^{-8}$M DHT (DHT), in LNCaP cells overexpressing GFP-ETS1p51 (ETS1 + EtOH) and in $10^{-8}$M DHT treated LNCaP cells that overexpress GFP-ETS1p51 (ETS1 + DHT). Changes in gene expression were calculated using RT² Profiler™ PCR array data analysis software (Version 3.5).
Chapter 5: ETS1p51 Effects on the Expression of EMT-Associated Genes

Figure 5.12: Clustergram analysis of gene expression following GFP-ETS1p51 overexpression in LNCaP cells. (A) Genes regulated following GFP-ETS1p51 overexpression (ETS1 + EtOH) sorted by associated ontology (genes may fall into multiple categories or regulate multiple processes). (B) Components of EMT promoting signalling pathways. Downregulated (green) or upregulated (red) genes relative to control (pcDNA3.1) transfected and vehicle (0.01% EtOH) treated cells (EtOH). Changes in gene expression were calculated using RT² Profiler™ PCR array data analysis software (Version 3.5).
Figure 5.13: Clustergram analysis of gene expression following DHT treatment of LNCaP cells. (A) Genes regulated following 10^{-8}M DHT treatment (DHT) sorted by associated ontology (genes may fall into multiple categories or regulate multiple processes). (B) Components of EMT promoting signalling pathways. Downregulated (green) or upregulated (red) genes relative to control (pcDNA3.1) transfected and vehicle (0.01% EtOH) treated cells (EtOH). Changes in gene expression were calculated using RT² Profiler™ PCR array data analysis software (Version 3.5).
Figure 5.14: Clustergram analysis of gene expression following DHT treatment of GFP-ETS1p51 overexpressing LNCaP cells. (A) Genes regulated following 10^{-8}M DHT treatment of GFP-ETS1p51 overexpressing cells (ETS1 + DHT) sorted by associated ontology (genes may fall into multiple categories or regulate multiple processes). (B) Components of EMT promoting signalling pathways. Downregulated (green) or upregulated (red) genes relative to control (pcDNA3.1) transfected and vehicle (0.01% EtOH) treated cells (EtOH). Changes in gene expression were calculated using RT² Profiler™ PCR array data analysis software (Version 3.5).
DHT treatment alone appears likely to minimally contribute to the induction of EMT in prostate cancer cells even following the overexpression of ETS1p51.

Comparison of gene regulation between the treatment groups identified that of the 39 DHT-regulated and 21 GFP-ETS1p51 regulated genes, 11 of the genes were regulated by both of the treatments, 7 of the genes were similarly up or downregulated, while 5 of the genes, *NODAL, FGFBP1, TGFβ1, TGFβ3* and *ZEB1* were upregulated following ETS1p51 overexpression (or DHT treatment of ETS1p51 overexpressing cells), but down-regulated following DHT treatment of cells (Figure 5.15). The largest overlap of regulated genes occurred between DHT-treated cells and DHT-treated, ETS1p51-overexpressing cells, with 22 commonly regulated genes of which 16/22 were downregulated, 5/22 upregulated and only 1/22 genes (ESR1) differentially regulated (Figure 5.15). In comparison, 7 genes were commonly regulated in ETS1p51-overexpressing and DHT-treated, ETS1p51-overexpressing cells, with expression of 5/7 genes upregulated and 2/7 genes downregulated (Figure 5.15). Eleven genes were regulated in all treatment groups, 6 genes were uniquely regulated by DHT treatment, while 3 genes were solely regulated in GFP-ETS1p51 overexpressing cells and 10 genes were only regulated following DHT treatment of GFP-ETS1p51 overexpressing cells (Figure 5.15).

### 5.2.7 Bioinformatics Analysis of ETS1p51 regulation of EMT

To further characterise ETS1p51-induced regulation of EMT, bioinformatics analysis was used to interrogate and interpret PCR array findings (section 5.2.6) following the overexpression of GFP-ETS1p51. For these analyses, PCR array data (expression values) were exported into both the DAVID (Database for Annotation, Visualisation and Integrated Discovery) Bioinformatics Resource version 6.7 (Huang *et al.* 2009b; Huang *et al.* 2009a) and the QIAGEN Ingenuity® Pathway Analysis Programme (www.qiagen.com/ingenuity). The 21 genes regulated ≥1.5-fold following GFP-ETS1p51 overexpression were entered into the functional annotation analysis from DAVID, which interrogates a number of databases to predict functions and pathways regulated. Following analysis, the functional annotation chart indicated that the most affected processes relevant to this study were cell proliferation (p=3.3x10⁻⁶), the TGF-
Chapter 5: ETS1p51 Effects on the Expression of EMT-Associated Genes

Figure 5.15: Co-regulation of genes by GFP-ETS1p51, DHT treatment or DHT treatment of GFP-ETS1p51 overexpressing LNCaP cells. EMT-associated genes regulated ≥ 1.5 fold in LNCaP cells overexpressing GFP-ETS1p51, in cells treated with $10^{-8}$M DHT or in DHT-treated LNCaP cells that overexpressed GFP-ETS1p51 were presented using a Venn diagram to indicate numbers of genes uniquely regulated by each of the treatments or coregulated by 2 or all 3 of the treatments.
Chapter 5: ETS1p51 Effects on the Expression of EMT-Associated Genes

βR signalling pathway ($p=6.5 \times 10^{-5}$), SMAD protein nuclear translocation ($p=4.7 \times 10^{-5}$), mesenchymal cell development and differentiation ($p=4 \times 10^{-5}$) and (positive regulation of) EMT ($p=3.5 \times 10^{-5}$) (significance calculated using modified Fisher’s exact test). Functional annotation using the BIOCARTA (Nishimura 2001), KEGG (Kanehisa et al. 2000; Kanehisa et al. 2014), PANTHER (Mi et al. 2013) and REACTOME (Milacic et al. 2012; Croft et al. 2014) pathway databases also determined that several pathways were enriched for the genes regulated by GFP-ETS1p51 overexpression, including pathways in cancer ($p=1.2 \times 10^{-5}$) (Figure 5.16) and the TGF-β signalling pathway ($p=5.3 \times 10^{-4}$) (Figure 5.17) (output from the KEGG pathway database). Results from bioinformatics analysis using DAVID were consistent with predicted functions determined by clustergram analysis (section 5.2.6) and indicated that GFP-ETS1p51 overexpression was significantly associated with upregulation of the TGF-β signalling pathway, which induces EMT in prostate cancer cells (Grant et al. 2013).

ETS1p51 regulation of EMT-associated processes was also analysed using QIAGEN Ingenuity® Pathway Analysis by examination of EMT-associated molecules, pathways and functions. Ingenuity® contains 217 core molecules associated with EMT, which are subdivided into 75 effectors, 59 repressors and 115 activators. Core analysis of this data categorised molecules associated with EMT as participants of EMT-associated signalling pathways and inducers of EMT-associated functions (e.g. cellular movement). The canonical EMT pathways were functionally and molecularly subdivided into 4 major signalling pathways, TGF-β, WNT, NOTCH and a combined Receptor Tyrosine Kinase pathway (including EGF, HGF, IGF, PDGF and FGF growth factor-mediated signalling). Overlay of expression data from the PCR arrays with no cut-offs assigned (section 5.2.6) onto the core EMT molecules indicated that following the overexpression of GFP-ETS1p51, 28 molecules present on the array and components of EMT signalling were significantly enriched ($p<0.01$). Further analysis of these components indicated that the top cellular functions regulated include cell movement ($p=3.82 \times 10^{-31} – 5.49 \times 10^{-5}$), cell development ($p=9.37 \times 10^{-25} – 5.15 \times 10^{-5}$) and cell growth and proliferation ($p=1.94 \times 10^{-22} – 4.78 \times 10^{-5}$) (predicted range of significance, one tailed Fisher’s exact test).
Chapter 5: ETS1p51 Effects on the Expression of EMT-Associated Genes
Figure 5.16: Pathways in cancer. Major signalling pathways involved in cancer determined bioinformatically via DAVID and the KEGG pathway databases. Included are core signalling components (black boxes) with specific genes (green), pathway-associated functions (bold black boxes) and genes identified by PCR Array to be responsive to GFP-ETS1p51 overexpression highlighted in association with pathways in cancer (red stars). Also included are direct relationships (solid lines and arrows) or indirect relationships (dotted lines and arrows) with direction of regulation.
Figure 5.17: The TGF-β signalling pathway. The major components (green) and associated functions of the TGF-β signalling pathway determined bioinformatically using the DAVID and KEGG pathway databases. Included are core signalling components (black boxes) with specific genes (green), pathway-associated functions (no boxes) and genes identified to be responsive to GFP-ETS1p51 overexpression in LNCaP cells (red stars). Also included are direct relationships (solid lines and arrows) or indirect relationships (dotted lines and arrows) with direction of regulation.
Data were also analysed with reference to EMT-associated signalling pathways, identifying significant regulation of the EMT pathway \( (p=5.74 \times 10^{-35}) \) and TGF-\( \beta \) signalling \( (p=6.09 \times 10^{-16}) \) and were depicted in relation to EMT-associated TGF-\( \beta \) and WNT signalling (Figure 5.18). Overlay of PCR expression data onto the EMT-related TGF-\( \beta \) and WNT signalling pathways indicated primarily upstream activation of both pathways through core ligands (e.g. TGF-\( \beta 1/2/3 \) and WNT, respectively) and downstream via upregulation of EMT-associated transcription factors (e.g. ZEB1). Evidence from Ingenuity\textsuperscript{®} was consistent with original clustergram analysis (section 5.2.6) and DAVID analysis, providing stronger evidence of GFP-ETS1p51-mediated regulation of EMT, principally through activation of EMT-associated signalling pathways. GFP-ETS1p51-regulated EMT components were further connected using the build-pathway tool and expanded to include direct/indirect components potentially required for ETS1p51-mediated EMT, thereby creating a larger list that was subsequently filtered to 16 molecules (including ETS1) by examining factors regulated by \( \geq 1.5\)-fold from the PCR array (section 5.2.6). The resulting list was specific to genes assayed on the PCR array that were regulated \( \geq 1.5\)-fold following GFP-ETS1p51 overexpression and significantly associated with EMT processes, highlighting these as the most suitable candidates for subsequent analysis. These genes comprised the core ETS1p51-regulated and EMT pathway components (Figure 5.19) and Ingenuity\textsuperscript{®} core analysis was used to confirm the processes and pathways regulated following GFP-ETS1p51 overexpression in LNCaP cells. The core analysis re-confirmed previous bioinformatics data suggesting significant regulation \( (p<0.01, \text{right tailed Fisher’s exact test}) \) of cell movement and cell growth as well as regulation of EMT pathways including TGF-\( \beta \) and WNT signalling. Overall, bioinformatics data strongly indicated that ETS1p51 overexpression in prostate cancer cells initiated EMT-associated programmes at least in part through the up-regulation of TGF-\( \beta \) and WNT signalling components.
Chapter 5: ETS1p51 Effects on the Expression of EMT-Associated Genes

**Figure 5.18: EMT-associated TGF-β and WNT signalling pathways.** Included are the canonical pathways related to EMT with core components (black circles) and genes identified to be either up-regulated (pink/red) or down-regulated (green) by GFP-ETS1p51 overexpression in the EMT PCR Array. Direct relationships (solid lines and arrows) or indirect relationships (dotted lines and arrows) with direction of regulation are indicated. This pathway was overlayed with expression data derived from bioinformatics analysis of all genes regulated on the EMT PCR array following ETS1p51 overexpression using Ingenuity Pathway Analysis Software, QIAGEN.
Figure 5.19: Proposed interactions between ETS1 and the EMT pathway in prostate cancer cells. Genes predicted to mediate/participate in ETS1-induced EMT including up-regulated (pink/red) or down-regulated (green) genes, direct (solid line) and indirect (dashed line) relationships and direction of regulation shown by arrows. Data and pathway were constructed using Ingenuity Pathway Analysis by importing gene expression data following GFP-ETS1p51 overexpression derived from the EMT PCR array.
5.3 Discussion

The overexpression of ETS1 in prostate tumours is associated with increased severity and rapid progression of disease (Li et al. 2012a; Smith et al. 2012a). Several in vitro studies have demonstrated that higher ETS1 levels increase prostate cancer cell line migration, invasion and anchorage-independent growth (Shaikhibrahim et al. 2011; Smith et al. 2012a), characteristics consistent with aggressive and metastatic prostate tumours. The process of EMT results in higher cell motility and invasiveness, facilitating the development of metastases (Grant et al. 2013), attributes that correlate with both the clinical features of ETS1-overexpressing prostate tumours and ETS1-overexpressing prostate cancer cell lines. In this thesis, ETSp51 was determined to be the predominant ETS1-isoform expressed in the human prostate and overexpressed in the majority of prostate tumours (Chapter 4). On the basis of these findings, characterisation of ETS1 regulation of EMT was investigated initially by determining ETSp51-induced regulation of expression of EMT-associated genes.

The human prostate cancer cell line LNCaP was used to conduct in vitro studies as it is a well-characterised, androgen-responsive prostate cancer cell line with low levels of ETS1 expression (Horoszewicz et al. 1983; Preece et al. 2011). LNCaP cells have been used previously to model stable overexpression of genes using Tet-On and doxycycline-responsive systems (Jin et al. 2008; Das et al. 2010; Jeter et al. 2011), while ETSp51 has been cloned into doxycycline or tetracycline inducible vectors for use in other cell lines and in transgenic mice (Yamamoto et al. 2000; Nagarajan et al. 2009). The inability to generate LNCaP cells with inducible ETSp51 expression in this thesis was largely due to the failure of transfected cells to sustain growth in the selection media. Reasons for this were unresolved but may have included poor expression of antibiotic resistance genes. A number of publications have documented difficulties in the clonal expansion of LNCaP cells and outlined troubleshooting techniques to facilitate the isolation of clonal populations of stably transfected cell lines, including limiting dilution methods (Iguchi et al. 2007), use of feeder cell populations (Bennett et al. 1997) and clonal expansion using cloning rings (Kim et al. 2010). Although each of these methods was tested during this study, and despite the success of initial stable transfection of LNCaP cells with the pCMVTet3G vector which encoded G418 resistance, either the
efficiency of expression from linear resistance markers for hygromycin B or puromycin or the inability of LNCaP cells to be stably transfected with multiple plasmids resulted in the failure to generate LNCaP cells with inducible ETS1p51 expression. Transient transfection with the pTRE3G-ETS1p51 construct did however result in doxycycline-induced ETS1p51 expression and therefore the plasmids generated in this study may be used in future experiments to develop prostate cancer cell lines or other experimental models with inducible ETS1p51 expression. ETS1p51 overexpression has been achieved by transient transfection in a number of cancer cell lines including HEK293 (Baillat et al. 2002) and LNCaP cells (Massie et al. 2007), and results from this thesis confirmed that transient transfection of LNCaP cells to overexpress GFP-tagged ETS1p51 resulted in high levels of ETS1p51 expression for up to 5 days post-transfection, which was suitable for subsequent in vitro experiments in place of stably transfected cell lines.

PCR array analysis of ETS1p51 overexpressing LNCaP cells identified altered expression of a subset of EMT-associated genes. Although ETS1-mediated regulation of EMT is not characterised, the promotion of an EMT programme would be consistent with reports of the increased migration and invasion of prostate cancer cells in which ETS1p51 was overexpressed in vitro (Shaikhibrahim et al. 2011; Smith et al. 2012a). Other ETS factors have been reported to regulate the expression of EMT-associated genes including PDEF, which suppresses SLUG and SLUG target gene expression in multiple breast cancer cell lines in association with downregulation of EMT processes (Findlay et al. 2011). In the nonmalignant prostate epithelial cell line RPWE-1, constitutive overexpression of ETV4 enhanced expression of the extracellular matrix modifiers MMP2 and MMP3 as well as the EMT promoting transcription factors ZEB1 and TWIST1 and the mesenchymal markers vimentin and N-cadherin, while reducing expression of the epithelial markers E-cadherin and Zona Occludens 1 (Pellecchia et al. 2012). These reports support findings of the present study and indicate that dysregulated ETS factor expression and/or activity may be correlated with modified expression of EMT genes.

Elevated expression of ETS1p51 in breast cancer cell lines has been correlated with upregulation of uPA, MMP1, MMP3 and vimentin expression, while simultaneously...
downregulating E-cadherin expression, typical of a mesenchymal-like phenotype (Gilles et al. 1997). In this study, expression of the majority of EMT-associated genes regulated following ETS1p51 overexpression was enhanced (16/22) (e.g. *MMP2*, *MMP9*, *VCAN*, *SPARC*, *NODAL*, *TIMP1*, *WNT5B*). Among these genes was *ZEB1*, which encodes an EMT promoter that is a transcriptional repressor of expression of the epithelial marker, E-cadherin and is a direct target of ETS1p51 in mouse mammary, pancreatic and colorectal carcinoma cell lines (Shirakihara et al 2007, Dave et al 2011). Other genes such as *MMP2* and *MMP9*, encoded products of which promote invasiveness, have also been identified as ETS1 target genes in multiple cell types including prostate cancer cells (Behrens et al. 2001; Deryugina et al. 2006; Taki et al. 2006; Roy et al. 2009; Ghosh et al. 2012b; Behnsawy et al. 2013). Protein products of *NODAL* and *SPARC* regulate cell invasiveness (Bradshaw et al. 2001; DeRosa et al. 2012; Quail et al. 2014), suggesting that increased expression and/or function of NODAL and SPARC may contribute to the increased invasiveness of prostate cancer cells. Induction of mRNA’s encoding VCAN and TIMP1 which alter adhesion and motility of prostate cancer cells and prostate cancer associated fibroblasts *in vivo* similarly facilitate cell movement, invasiveness and proliferation of prostate cancer cells (Gao et al. 2012; Gong et al. 2013).

ETS1p51 overexpression regulated expression of components of EMT signalling pathways includes *WNT5B*, which encodes a ligand of the WNT pathway, with WNT signalling proposed to contribute to prostate cancer progression through the upregulation of EMT (Jiang et al. 2007; Bisson et al. 2009; Li et al. 2014a). Although ETS1 regulation of WNT signalling has not been characterised previously, higher levels of ERG have been correlated with increased expression of WNT1, WNT2, WNT3A and WNT11, and ERG-mediated activation of WNT signalling enhanced expression of the critical signalling intermediate, β-catenin which regulates WNT signalling target gene expression and promotes invasive characteristics of prostate cancer cells (Wu et al. 2013). Elevated WNT5B expression is detected in highly aggressive breast tumours and is associated with enhanced metastasis and decreased disease-free survival (Yang et al. 2014). WNT5B is also reported to function as a differentiation marker that can be secreted by mesenchyme-like cells that have undergone EMT to enhance surrounding epithelial tumour cell EMT and disease progression (Kato et al. 2014). Gene expression
data from this study also indicated that ETS1p51 overexpression in LNCaP cells enhanced expression of a number of TGF-β signalling pathway components (i.e. TGF-β1, TGF-β2, TGF-β3, NODAL and ZEB1). Although the mRNA expression of TGF-β ligands in prostate tumours is incompletely characterised, it is proposed that TGF-β signalling is a promoter of EMT in prostate cancer cells and prostate tumours, and likely augments the progression of disease (Wikstrom et al. 1998; Zhang et al. 2009b; Vo et al. 2013). Upregulation of mRNA’s encoding both TGF-β signalling ligands and the downstream effector ZEB1 suggest activation of TGF-β signalling in ETS1p51 overexpressing LNCaP cells. Together with the enhanced expression of genes that encode EMT-promoting factors and effectors of an invasive, mesenchymal phenotype, gene expression data support promotion of EMT processes by ETS1p51 overexpression.

PCR arrays also identified that expression of a smaller proportion of EMT-associated factors (6/22) was downregulated following ETS1p51-overexpression, COL3A1, CTNNB1, MMP3, RGS2, SNAI1 and WNT5A. At the 24-hour timepoint tested, expression of COL3A1 which encodes a collagen type 3 protein that associates with extracellular matrix was reduced, which contrasts findings from studies of colorectal (Kahlert et al. 2011) and ovarian cancers (Lili et al. 2013), where EMT was associated with elevated COL3A1 expression. In this thesis, ETS1p51-overexpression downregulated SNAI1 (SNAI1) which is reported to promote EMT and increase motility of prostate cancer cells and prostate tumours (Smith et al. 2012b), although additional ETS factors (e.g. PEA3) are characterised to promote SNAIL expression in other cell types in association with EMT-induction (Yuen et al. 2011). SNAIL1 (encoded by SNAI1) and ZEB1 (encoded by ZEB1) both function as transcriptional repressors of E-cadherin in multiple cell types (Batlle et al. 2000; Shirakihara et al. 2007) and in this thesis, ETS1p51-overexpression downregulated SNAI1 expression at 24 hours while simultaneously upregulating ZEB1 expression, with the repression of E-cadherin (CDH1) expression not reaching significance.

DHT treatment of LNCaP cells upregulated expression of 10 EMT-associated genes in the PCR array including SLUG, TWIST1, WNT5B, TGFβ2 and SPARC, and downregulated the expression of 29 EMT-associated genes including SNAI1, TGFβ1, ZEB1, NOTCH1, MMP3 and WNT11. Androgen-induced AR signalling is required for
the maintenance of differentiation and proliferation of prostatic epithelial cells (Cunha et al. 1987; Cunha et al. 1992; Berger et al. 2004) and AR signalling is reported to enhance expression of EMT-associated genes and promote EMT processes in prostate tumours (Nauseef et al. 2011; Grant et al. 2013). In this study, markers of the epithelial phenotype, KRT14 and KRT19 were upregulated following DHT treatment of LNCaP cells. Keratin 14 (KRT14) and Keratin 19 (KRT19) are markers of basal cells, while Keratin 19 is expressed in a proportion of luminal cells in nonmalignant prostate and both KRT14 and KRT19 are highly expressed in proliferating cells (Hudson et al. 2001; van Leenders et al. 2001). As expression of keratin mRNA including that of KRT14 and KRT19 is downregulated in prostate tumours (Pascal et al. 2009), the DHT-induced increases in KRT14 and KRT19 expression are consistent with androgen/AR-induced cell proliferation and (epithelial) differentiation of LNCaP cells. JAG1 is a ligand for the NOTCH receptor and an AR target gene, upregulated following DHT treatment in this study and shown previously to be expressed at high levels in androgen-treated LNCaP cells (Martin et al. 2004) and to enhance the proliferation of prostate cancer cell lines in vitro (Yu et al. 2014).

The EMT-promoting transcription factors SLUG and TWIST1, are characterised AR target genes found to be upregulated in this study following DHT treatment of LNCaP cells (Bolton et al. 2007; Wu et al. 2012; Eide et al. 2013; Grant et al. 2013). SPARC (osteonectin), was also among the genes upregulated by AR signalling and encodes a multifunctional protein that in normal tissues binds to extracellular matrix (ECM) proteins, enhances the expression of MMPs, acts as a counter-adhesive factor and modifies growth factor activity by preventing growth factor – receptor interactions (McClung et al. 2007; Said et al. 2007). SPARC is involved in cell migration, proliferation and differentiation and its effects on cell adhesion and migration can contribute to EMT, which is supported by the correlation between elevated SPARC mRNA expression in primary prostate cancer and metastasis (Bradshaw et al. 2001; DeRosa et al. 2012). SPARC is also a regulator of cell shape and therefore may contribute to the cytoskeletal reorganisation induced by AR signalling (Zhu et al. 2010).

Although previous studies have identified that DHT-mediated activation of the AR in LNCaP and AR-transfected PC-3 prostate cancer cells is associated with induction of
EMT via increased expression of SNAIL1 and N-cadherin, and decreased expression of E-cadherin (Zhu et al. 2010), results from this study identified that SNAIL1 expression was reduced following DHT-treatment of LNCaP cells. Studies of AR signalling in breast and colon cancer cell lines have also identified repression of E-cadherin expression via SLUG and other EMT-associated transcription factors and this was correlated with the increased expression of mesenchymal markers including vimentin, an indicator of cell motility (Liu et al. 2008). In this study however, expression of E-cadherin was not significantly reduced despite increased levels of SLUG and genes encoding other repressors of E-cadherin including TWIST1 and ZEB1 (Sanchez-Tillo et al. 2010). This result contrasts previous findings of DHT-induced expression of ZEB1 in AR-transfected PC-3 cells (Anose et al. 2011). Among other genes repressed by DHT-induced AR signalling was MMP3, which encodes the matrix metalloproteinase, MMP3 that promotes cell invasion through degradation of ECM (Deryugina et al. 2006). MMP3 in addition to family members MMP1 and MMP7, has been reported previously to be downregulated in DHT-treated LNCaP cells (Schneikert et al. 1996). Although DHT-induced regulation of MMP (protein) expression and function have not been characterised in prostate cancer cells, it is feasible that MMP activity and the alteration of ECM surrounding prostate malignancies, which are both increased during progression of disease and during development of castrate-resistance (Brehmer et al. 2003; Trudel et al. 2003; Deryugina et al. 2006; McCall et al. 2012), are repressed in androgen abundant conditions. FN1 which encodes the high molecular weight glycoprotein fibronectin that binds integrins is a previously reported androgen-responsive gene and AR target that is repressed in androgen-stimulated LNCaP cells as was found in this study (Nelson et al. 2002; Bolton et al. 2007) and characterised to be upregulated in cells undergoing EMT (Anastassiou et al. 2011).

Due to the potent EMT-promoting role of the TGF-β signalling pathway, it is interesting to note that expression of TGFβ1 and TGFβ3 was downregulated, while TGFβ2 was upregulated in DHT-treated LNCaP cells. siRNA-mediated knockdown of AR-signalling reduces TGFβ2 expression in patient-derived prostate cancer associated fibroblasts, supporting a hypothesis that TGFβ2 is an AR target gene (Yu et al. 2013). While further investigations are required, it is relevant that expression of all TGFβ
ligands was low in both untreated and DHT-treated LNCaP cells, suggesting that AR activation does not induce marked upregulation of ligand-induced TGF-β signalling.

In contrast to evidence of androgen/AR induced promotion of EMT, androgen deprivation may also induce EMT in prostate cancer cells and prostate tumours (Zhu et al. 2010; Sun et al. 2012; Li et al. 2014a). For example, in mouse prostate and in xenografts of the human prostate cancer cell line LuCaP35 grown in mice, cells were phenotypically more mesenchymal-like following androgen deprivation and expressed higher mRNA and protein levels of mesenchymal markers including ZEB1, TWIST1, N-cadherin and vimentin (Sun et al. 2012). Additionally, low AR levels in shRNA treated LNCaP cells were found to sensitise the cells to subsequent androgen-induced cell invasion and EMT marker expression (Zhu et al. 2010). In this study, gene ontology analysis following PCR arrays indicated that DHT treatment downregulated expression of genes encoding regulators and effectors of adhesion, cytoskeletal reorganisation and migration, opposing EMT promoting events. As higher AR expression is reported to suppress androgen-induced EMT while low AR expression sensitises prostate cancer cells to androgen-induced EMT, the inverse relationship between AR expression and EMT induction may account for the gene expression profiles observed in the DHT-treated LNCaP cells in this study which do not support promotion of EMT (Zhu et al. 2010).

Interaction between the AR and ETS1p51 has been reported in a previous study, which identified common ETS factor binding sites in the promoters of AR target genes, DHT-induced binding of ETS1p51 with the AR in LNCaP cells and ETS1p51-mediated potentiation of androgen/AR induced regulation of target genes (Massie et al. 2007). In addition, previous studies in the laboratory and in this thesis have characterised reciprocal regulation of ETS1p51 and AR levels, which is likely to enhance their transcriptional activities (Preece 2009) (section 6.2.2.3). Consistent with these findings, DHT treatment of ETS1p51-overexpressing cells resulted in the regulation of a larger number of genes in comparison to either treatment alone in addition to enhancing the (up- or down-) regulation of subsets of target genes. In contrast to PCR array results from ETS1p51-overexpressing or DHT-treated LNCaP cells where regulation of CDH1 or CDH2 was either not detected or did not reach significance, DHT treatment of
Chapter 5: ETS1p51 Effects on the Expression of EMT-Associated Genes

ETS1p51 overexpressing cells downregulated \textit{CDH1} and upregulated \textit{CDH2} levels. Repression of E-cadherin (\textit{CDH1}) and upregulation of N-cadherin (\textit{CDH2}) expression are typically related to cadherin type ‘switching’, a distinctive characteristic of epithelial cells undergoing EMT, including prostate cancer cells (Grant \textit{et al.} 2013). It is feasible therefore that the previously characterised interactions between ETS1p51 and the AR increase the magnitude of transcriptional regulation of target genes or accelerate transcriptional regulatory effects, resulting in their detection at earlier timepoints.

Consistent with an interaction between ETS1p51 and the AR, enhanced regulation of several EMT-associated factors was detected in DHT-treated ETS1p51-overexpressing cells. This included enhanced downregulation of \textit{DSC2} and \textit{DSP}, which encode desmocollin-2 and desmoplakin, respectively, proteins that form core components of desmosome (cell-to-cell) adherens. Reduced DSC2 and DSP are associated with disrupted desmosome structure, an early event in tumour progression that reduces cell-cell adhesion and facilitates motility of epithelial cells including prostate cancer cells (Thiery \textit{et al.} 2006; Thomson \textit{et al.} 2011; Celia-Terrassa \textit{et al.} 2012). While DSC2 and DSP are not characterised as direct ETS1 targets, ZEB1, which is upregulated in ETS1p51 overexpressing cells represses the expression of desmosome proteins including DSP in colorectal and squamous epidermoid carcinoma cell lines (Vandewalle \textit{et al.} 2005), and potentially mediates similar functions in prostate cancer cells. Comparable to ETS1, the AR has not been reported to directly regulate expression of desmosome proteins, but is known to interact with and modulate \(\beta\)-catenin nuclear localisation and transcriptional functions (Mulholland \textit{et al.} 2002), which includes regulation of ZEB1 expression and activity (Sanchez-Tillo \textit{et al.} 2011).

Additional examination of genes regulated following DHT treatment of ETS1p51-overexpressing LNCaP cells identified increased expression of \textit{SPARC} that would be associated with promotion of EMT. However, the regulation of a number of genes including \textit{SERPINE1}, \textit{SNAIL3}, \textit{KRT7}, \textit{AKT1} and \textit{NOTCH1} would not support activation of EMT but are more consistent with gene expression profiles detected in DHT-treated cells. For example, increased mRNA levels of \textit{SERPINE1} which encodes plasminogen activator inhibitor 1, a repressor of the proteases tPA and uPA (Ghosh \textit{et al.} 2012a) indicates downregulation of invasive characteristics mediated by protease activity and
therefore does not support induction of EMT. Reduced expression of NOTCH1 and AKT1, components of the NOTCH and the PI3K/AKT signalling pathways, respectively, support the inactivation or reduced activity of these pathways which are both considered promoters of EMT (Grille et al. 2003; Bao et al. 2011). Following DHT treatment of ETS1p51-overexpressing LNCaP cells, the downregulation of SNAIL1 and upregulation of SNAI2 expression were enhanced in comparison to either treatment alone and in addition, SNAI3 expression was decreased. SNAI3 is not as well characterised as its family members, but has been identified as a poor inducer of EMT (Gras et al. 2014) and may only be expressed in select tissue types (Katoh et al. 2005), therefore its downregulation is unlikely to contribute to induction of EMT. Although further studies are required, overall the profile of gene regulation in DHT-treated ETS1p51-overexpressing LNCaP cells appears most similar to that in DHT-treated cells and does not support promotion of EMT.

Clustergram and bioinformatics analysis of gene expression data from ETS1p51-overexpressing cells was used to predict the significance of gene regulation on cellular processes including EMT. Due to the role of ETS1 during development and in nonmalignant cells where it regulates adhesion, differentiation, migration and invasion (Dittmer 2003), it was unsurprising that overexpression of ETS1p51 in prostate cancer cells was predicted to enhance these cellular processes. Gene expression analysis predicted that ETS1p51 overexpression altered cell adhesion, which was consistent with the increased expression of TIMP1 and VCAN detected in ETS1p51-overexpressing LNCaP cells. The collapse of cell-cell boundaries and disrupted adhesion are typical characteristics of the early stages of EMT and facilitate cell motility (Tran et al. 1999; Tran et al. 2002; Yilmaz et al. 2009; Grant et al. 2013). The disruption of cell-cell contact is mediated by increased expression and activity of MMPs (e.g. MMP2, MMP9), induction of pro-EMT signalling pathways (e.g. TGF-β) (Zheng et al. 2009) and activation of EMT transcription factors (e.g. ZEB1, ZEB2) (Comijn et al. 2001; Sanchez-Tillo et al. 2010), which were detected in ETS1p51-overexpressing LNCaP cells. Factors such as TIMP1 interact with and mediate the activation of integrins and additional cell surface receptors (Li et al. 1999a; Liu et al. 2003; Jung et al. 2006; Jung et al. 2012), which engage ECM and can promote cell movement (Hynes 2002; Yilmaz et al. 2009). Simultaneously, upregulation of factors including ZEB1 will downregulate
expression of genes which encode proteins that maintain the tight junction, adherens junctions, gap junctions and desmosomes, structures required for the maintenance of adhesion of epithelial cells (Vandewalle et al. 2005).

Results of bioinformatics data predicted significantly increased prostate cancer cell motility following ETS1p51 overexpression, supported by the upregulation of genes such as NODAL and SPARC. Following disruption of cell-cell adhesion, the progression of EMT involves upregulation of factors such as NODAL which contribute to cell migration (Quail et al. 2014) or SPARC, a counter-adhesive protein that also enhances the expression and activity of MMPs, promoting the movement of cells within local tissue (Bradshaw et al. 2001; McClung et al. 2007; Said et al. 2007). During this phase of EMT, the upregulation of EMT transcription factors including ZEB1 can indirectly promote cell motility by repressing the expression of epithelial-specific adhesion molecules (Aigner et al. 2007) or directly downregulating expression of migration-inhibitory miRNAs (e.g. miR-34a) (Ahn et al. 2012) and E-cadherin (Sanchez-Tillo et al. 2010). Increased expression of EMT transcription factors and mesenchymal motility markers can occur through several mechanisms including elevated expression of ETS1 (Shirakihara et al. 2007; Dave et al. 2011) or the activation of TGF-β signalling (Wu et al. 2007). Upregulation of factors such as MMP2, MMP9 and SPARC, are also likely to contribute to the induction of cell invasion, which was predicted by bioinformatics analyses performed in this study to be significantly upregulated following ETS1p51 overexpression. The production and secretion of MMPs via factors including ETS1 or SPARC (McClung et al. 2007; Said et al. 2007) are recognised mediators of cell invasion. During EMT, MMPs degrade ECM and promote invasive characteristics in conjunction with disruption of cell adhesion and induction of mesenchymal-like cell movement to infiltrate local tissue or vasculature and contribute to the formation of metastases (Deryugina et al. 2006).

Stratification of gene ontology data and bioinformatics analysis to further delineate ETS1p51-mediated regulation of EMT processes revealed a significant likelihood of activation of TGF-β signalling, which is known to contribute to the regulation of EMT in multiple cancer types, including prostate cancer (Nauseef et al. 2011; Grant et al. 2013; Katsuno et al. 2013). In addition, significant SMAD nuclear translocation was
also predicted, which occurs following the activation of TGF-β signalling. In ETS1p51-overexpressing LNCaP cells, high expression of TGF-β1, TGF-β2 and TGF-β3, the primary ligands of TGF-β signalling were detected. TGF-β ligands are required to bind the extracellular domain of the primary TGF-β signalling receptor, TGF-β receptor type II (Lin et al. 1992; Lin et al. 2006), which mediates the activation of TGF-β signalling. In addition to this, ETS1p51-overexpressing cells expressed high levels of NODAL. NODAL, an alternative ligand for TGF-β signalling can bind the TGF-β receptor type II, ACVR2B (Reissmann et al. 2001) also resulting in the activation of TGF-β signalling. Irrespective of the ligand/s responsible for activation of TGF-β receptor type II, this subsequently activates cognate type I receptors (Wrana et al. 1994; Kawabata et al. 1995), which in turn directly phosphorylate and activate SMADs (Marcias-Silva et al. 1996; Zhang et al. 1996; Kretzschmar et al. 1997), thereby intrinsically linking the activation of TGF-β signalling and increased nuclear translocation of SMADs, both processes predicted to occur in ETS1p51-overexpressing LNCaP cells. Additional genes such as SPARC which were upregulated in ETS1p51 overexpressing cells, can similarly induce the expression of TGF-β1 (Francki et al. 1999) and enhance SMAD phosphorylation and nuclear translocation (Francki et al. 2004). Similarly, activation of TGF-β signalling will result in increased expression of target genes including ZEB1, an ETS1 target gene also upregulated in ETS1p51-overexpressing cells (Shirakihara et al. 2007; Dave et al. 2011).

The EMT programme encompassing alterations in cell adhesion, motility, invasion, migration and the increased expression of well-characterised transcription factors was predicted by bioinformatics analyses to be significantly upregulated in ETS1p51 overexpressing cells. The finding of significant activation of TGF-β signalling including SMAD nuclear translocation in these cells suggested that the TGF-β signalling pathway was mediating or contributing to ETS1p51-induced EMT, and these predictions were investigated in subsequent studies that were performed to validate PCR array results.
6.1 Introduction

The progression of prostate cancer and development of metastatic disease are associated with the accumulation of multiple genetic and epigenetic abnormalities, dysregulated activity of signalling pathways and acquisition of castrate-resistant growth characteristics, which are frequently accompanied by the activation of EMT (Shen et al. 2010; Grant et al. 2013). EMT characteristics have been identified in multiple epithelial tumour types including prostate cancer and are typified by specific processes including loss of epithelial and gain of mesenchymal marker expression, reduced cell polarity and adhesion and acquisition of migratory and invasive properties that define EMT-associated events and the EMT process (Grant et al. 2013) (section 1.6). EMT that occurs in association with normal tissue physiology has been extensively investigated, establishing the critical functions of signalling pathways which promote the expression of EMT effectors and which also contribute to the induction of EMT in various epithelial cancers, including prostate cancer (Thiery et al. 2009). TGF-β, WNT, NOTCH and a number of growth factor receptor regulated tyrosine kinase signalling pathways promote EMT, and crosstalk between the pathways enhances the expression of common EMT-promoting transcription factors including ZEB1, SNAIL and TWIST (section 1.6) (Thiery et al. 2009). The EMT-promoting TGF-β and WNT signalling pathways facilitate the induction of EMT in prostate cancers, promoting cell motility and invasiveness, which are also characteristics of aggressive and metastatic prostate tumours (Nauseef et al. 2011; Grant et al. 2013).

During prostate development, EMT-inducing paracrine signalling, which is believed to be derived from mesenchymal cells within the urogenital sinus facilitates the migration and survival of local epithelial cells that subsequently form the prostate gland (Grant et al. 2013). TGF-β ligands and receptors are expressed in the prostate and in vitro, TGF-β signalling inhibits the proliferation of nonmalignant prostate epithelial cells (Sutkowski et al. 1992; Guo et al. 1997; Gerdes et al. 1998). In BPH tissues, high levels of TGF-β signalling components and pro-EMT transcription factors including phosphorylated SMADs, SNAIL1 and SLUG are associated with mesenchymal-like morphological characteristics of the epithelial cell layer and with increased infiltration of macrophages (inflammation) (Alonso-Magdalena et al. 2009; Lu et al. 2012b).
TGF-β signalling has also been shown to inhibit the proliferation and migration and induce apoptosis of prostate cancer cell lines in vitro and in vivo (Desruisseau et al. 1996; Barrack 1997; Landstrom et al. 2000). Hyperactivation of TGF-β signalling is proposed to be insufficient for EMT-mediated cancer progression (Type III EMT), but likely simulates persistent or chronic EMT-mediated induction of ‘wound healing’ (Type II EMT) and local tissue remodelling within the prostate tumour microenvironment, facilitating tumour cell motility (Verona et al. 2007). In advanced cancers, tumour cells may become insensitive to the growth suppressor activity of TGF-β signalling, which coincides with the acquisition of mesenchymal traits and is also therefore proposed to contribute to prostate cancer progression through the promotion of EMT (Nauseef et al. 2011; Grant et al. 2013). TGF-β signalling promotes EMT characteristics (e.g. increased vimentin expression) in prostate cancer cells and in prostate tumours, higher expression of mediators of TGF-β signalling has been correlated with increased invasiveness, disease severity and was predictive of biochemical recurrence (Zhang et al. 2009b).

Pathways that augment TGF-β signalling including the AR, WNT and other EMT-associated signalling pathways exhibit high levels of signalling crosstalk and contribute to the regulation of EMT in prostate cancer cells by inducing expression of EMT promoting factors (e.g. TWIST, SLUG, ZEB1) (section 5.2.7, 5.2.8) (Conacci-Sorrell et al. 2003; Howe et al. 2003; Nauseef et al. 2011; Sanchez-Tillo et al. 2011; Grant et al. 2013). The roles of EMT-associated WNT signalling in prostate cancer are not as well-defined as TGF-β signalling, but studies using prostate cancer cell lines have demonstrated that WNT/β-catenin signalling also mediates the induction of EMT and is associated with increased cell invasiveness and plasticity (Jiang et al. 2007; Bisson et al. 2009; Li et al. 2014a). In addition, β-catenin expression is elevated in the majority of prostate tumours and is associated with invasive characteristics (Morita et al. 1999; Chesire et al. 2000; Jaggi et al. 2005; Whitaker et al. 2008; Francis et al. 2013). In prostate cancer cell lines, silencing of β-catenin resulted in increased epithelial and reduced mesenchymal marker expression, while higher β-catenin levels were positively correlated with more mesenchymal features, providing evidence that β-catenin is able to promote EMT in prostate cancer cells (Jiang et al. 2007; Zhao et al. 2011). Studies using human cell lines and mouse models have been used to demonstrate the formation
Chapter 6: Regulation of EMT by ETS1p51 Overexpression

of transcriptional regulatory complexes that contain both the TGF-β transcriptional intermediates, SMAD2/3 and the WNT transcriptional intermediate β-catenin, and that directly activate expression of target genes including EMT promoting factors (Conacci-Sorrell et al. 2003; Lei et al. 2004; Kim et al. 2009; Brandl et al. 2010). Activation of either TGF-β or WNT signalling can also result in co-activation of other pathways via signalling crosstalk and due to overlapping activities of SMAD2/3 and β-catenin, resulting in the regulation of common target genes, for example SLUG and vimentin (Conacci-Sorrell et al. 2003; Gilles et al. 2003; Jian et al. 2006; Wu et al. 2007; Hirota et al. 2008; Medici et al. 2008; Guo et al. 2009; Brandl et al. 2010; Zhou et al. 2012; Kim et al. 2013).

Preliminary findings from this study indicate that ETS1 promotes EMT and that this potentially occurs through regulation of signalling pathways including TGF-β and WNT. In breast cancer cell lines, elevated ETS1 expression is associated with a more mesenchymal phenotype, with higher vimentin and lower E-cadherin expression and increased cell invasiveness (Gilles et al. 1997). The downstream target of TGF-β signalling and an EMT-promoting transcription factor, ZEB1 is also a direct target of ETS1 in multiple human and mouse cell lines (Shirakihara et al. 2007; Dave et al. 2011). Interestingly, ETS1-mediated regulation of ZEB1 is hypothesised to be the primary mechanism of TGF-β associated induction of ZEB1 expression, with TGF-β signalling inducing high levels of ETS1 expression that subsequently regulates the expression of target genes such as ZEB1 and CCN2 (Dave et al. 2011; Geisinger et al. 2012). Several target genes are co-regulated by ETS1 and EMT transcription factors, for example TWIST1 and SNAIL1 cotransactivate or individually promote ZEB1 expression by engaging respective consensus sites located upstream of the EBS (Dave et al. 2011). In the same study, knockdown of SNAIL1 resulted in reduced levels of ETS1 and ZEB1, suggesting that SNAIL1 may regulate ETS1 expression, while induction of SNAIL1 resulted in elevated ETS1 protein levels, enhanced ETS1 nuclear translocation and increased ZEB1 expression (Dave et al. 2011). ETS1 may also participate in EMT signalling processes due to the formation of complexes with SMAD2, which bind to target gene promoters, including promoters that do not contain EBS sequences, representing an indirect mechanism of ETS1 activation of TGF-β target genes (Koinuma et al. 2009). For example, ETS1 expression and activity are required.
for the regulation of expression of Parathyroid hormone-related protein (PTHrP), a SMAD3/ETS1 and TGF-β signalling target gene (Lindemann et al. 2001). In liver, breast and colorectal cancers, high levels of ETS1 correlate with increased β-catenin expression, and transcriptional complexes containing ETS1 and β-catenin interact with EBS sequences located in the promoters of ETS1/β-catenin target genes including osteopontin, MMP7 and ZEB1 (Brabletz et al. 1999; El-Tanani et al. 2004; Mole et al. 2011; Sanchez-Tillo et al. 2011). While PCR array analysis of ETS1p51 overexpressing LNCaP cells indicated upregulation of EMT-promoting factors including TGF-β signalling, results of gene screening methods require validation to confirm and characterise regulation of expression of the candidate genes and their encoded proteins. Promotion of EMT is associated with increased cell migration and invasion and ETS1p51 regulation of these processes as well as the involvement of TGF-β signalling in the ETS1p51-induced regulation of migration and invasion was investigated in this thesis.
6.2 Results

6.2.1 Optimisation of RT-qPCR for EMT-Associated Markers

To validate PCR array results (section 5.2.7, 5.2.8) and confirm ETS1p51-mediated regulation of EMT-associated gene expression, mRNA levels of 7 genes whose expression was regulated according to the PCR array (section 5.2.7) were evaluated by RT-qPCR (section 3.7.6, 3.7.7). For these studies, primers were designed or selected from published studies to amplify: NODAL (Vo et al. 2011), SLUG (Lefever et al. 2009), SMAD2 (Yu et al. 2009), SNAIL1 (Lefever et al. 2009), TGF\(\beta\)1 (Zaravinos et al. 2008), TWIST1 (self-designed) and ZEB1 (Drake et al. 2009) (Appendix 2). PCR conditions were optimised using RNA extracted from LNCaP, COS-7 or MCF-7 cells (section 3.7.1) and agarose gel electrophoresis (section 3.2) of amplified products indicated amplification of single products of the expected size for SLUG, SMAD2, SNAIL1 and ZEB1, while TGF\(\beta\)1 and TWIST1 PCRs each amplified 2 bands and no specific products were amplified in the NODAL PCR (Figure 6.1) (section 3.7.6, 3.7.7). Alternative TGF\(\beta\)1 and TWIST1 primers were subsequently obtained from published studies or databases (Reinhold et al. 2006; Lefever et al. 2009) and products of the expected sizes were successfully PCR-amplified (results not shown). NODAL PCRs were performed using RNA/cDNA from LNCaP, MDA-MB-231, MCF-7 and COS-7 cells (section 3.7.1, 3.7.3), with modification of PCR parameters including MgCl\(_2\) concentration, annealing temperature and cycling times, however due to low expression in all available cell lines and due to the lack of a positive control, NODAL qPCR was not successfully optimised and was excluded from quantitative analysis (results not shown). PCR annealing temperatures were optimised in reactions using annealing temperatures between 58°C to 66°C (section 3.2) (Figure 6.2), while melt curve analysis was used to verify amplification of single products in each PCR (section 3.7.6, 3.7.7) (results not shown). In addition, MgCl\(_2\) concentration was optimised for each gene in PCRs using a range of 2-4mM MgCl\(_2\) with melt curve analysis (section 3.7.6, 3.7.7) (results not shown). Using the optimised PCR conditions for each gene, efficiency curves were generated and each was suitable for subsequent experiments: SMAD2 = 99%, SLUG = 109%, SNAIL1 = 101%, TWIST1 = 118%, ZEB1 = 101% and TGF\(\beta\)1 = 100% (Figure 6.3) (section 3.7.8).
Figure 6.1: PCR amplification of EMT-associated genes. PCRs for NODAL, SLUG, SMAD2, SNAIL1, TGFβ1, TWIST1 and ZEB1 were performed using LNCaP cDNA and an annealing temperature of 60°C, with 15μL of each reaction electrophoresed in a 2% agarose gel. Expected size of PCR-amplified products (bp) are indicated for each gene.
1. 66°C  6. 59.6°C
2. 65.6°C  7. 58.5°C
3. 64.7°C  8. 58°C
4. 63.1°C  9. -ve
5. 61.2°C

**Figure 6.2: Annealing temperature optimisation of PCRs for EMT-associated genes.** PCRs were performed using 2μL LNCaP cDNA for (A) *SLUG* (~118bp), (B) *SMAD2* (~128bp), (C) *TWIST1* (~250bp), using 2μL COS-7 cDNA for (D) *SNAIL1* (~140bp), (E) *ZEB1* (~72bp) and using 2μL MCF-7 cDNA for (F) *TGFβ1* (~276bp). Annealing temperatures of between 58°C and 66°C were tested, and 10μL of each product was electrophoresed in a 2% agarose gel. Aliquots of PCRs were separately analysed by melt curve analysis to confirm the presence of single products (not shown).
Chapter 6: Regulation of EMT by ETS1p51 Overexpression

(A)  
\[ y = -3.122x + 38.755 \]
\[ R^2 = 0.99893 \]
\[ E = 109\% \]

(B)  
\[ y = -3.3437x + 37.398 \]
\[ R^2 = 0.99785 \]
\[ E = 99\% \]

(C)  
\[ y = -2.9467x + 38.296 \]
\[ R^2 = 0.98477 \]
\[ E = 118\% \]

(D)  
\[ y = -3.287x + 36.026 \]
\[ R^2 = 0.99279 \]
\[ E = 101\% \]
Figure 6.3: Generation of qPCR efficiency curves. PCR efficiency was evaluated in reactions containing neat, 1:10, 1:100, 1:1000 and 1:10000 dilutions of LNCaP cDNA for (A) SLUG, (B) SMAD2, (C) TWIST1, COS-7 cDNA for (D) SNAIL1, (E) ZEB1 and MCF-7 cDNA for (F) TGFβ1. Fluorescence intensity and Ct values were calculated for each reaction with melt curve analysis performed post-PCR to confirm amplification of single products. Average Ct values for each cDNA concentration were plotted against the log of the starting amount of cDNA. Using the optimised qPCR conditions, each gene was amplified with the specified efficiency and correlations of $R^2 > 0.98$. 

\[
y = -3.2833x + 37.602 \\
R^2 = 0.99635 \\
E = 101\%
\]

\[
y = -3.304x + 36.464 \\
R^2 = 0.99385 \\
E = 100\%
\]
6.2.2 Regulation of Expression of EMT-Associated Genes by ETS1p51 and DHT

ETS1p51- and DHT-induced regulation of expression of EMT-associated genes detected in the PCR array was further validated in LNCaP cells that had been transfected with pEGFP-C2-ETS1p51 and/or treated with $10^8$M DHT (section 3.5.2, 3.5.4). Cells were harvested and RNA extracted from the cultures at 1-5 days following transfection or initiation of treatment and evaluated by RT-qPCR using the optimised PCR conditions (sections 3.7.1, 3.7.6, 6.2.1). *ETS1p51* mRNA expression in pEGFP-C2-ETS1p51 transfected and/or DHT-treated LNCaP cells was $>30000$ fold higher than that in control (pcDNA3.1) transfected cells for the 5 days following transfection (Figure 6.4A). Levels of *SLUG* mRNA were increased $\sim1.2$ - $\sim60$ fold above controls in DHT-treated LNCaP cells, which was consistent with PCR array data, with peak levels after 3 days (section 5.2.7) (Figure 6.4B). *SLUG* mRNA levels were not regulated by GFP-ETS1p51-overexpression, however *SLUG* mRNA expression was most highly sustained in DHT-treated GFP-ETS1p51 overexpressing cells (Figure 6.4B). Levels of *SNAIL1* were strongly upregulated by up to 24-fold on day 3 in DHT-treated GFP-ETS1p51 overexpressing cells, although its expression was not markedly regulated by either GFP-ETS1p51 overexpression or DHT treatment alone (Figure 6.4C). These results contrasted findings from the EMT PCR arrays, in which DHT treatment repressed *SNAIL1* expression, an effect that was enhanced by GFP-ETS1p51 overexpression (section 5.2.7).

Expression of *TGFβ1* mRNA was upregulated up to 10-fold in LNCaP cells which overexpressed GFP-ETS1p51, with further increases to 60-fold detected in GFP-ETS1p51 overexpressing cells cultured with $10^8$M DHT, findings that were consistent with PCR array data (section 5.2.7) (Figure 6.4D). When treated with DHT, LNCaP cells that overexpressed GFP-ETS1p51 maintained very high levels of *TGFβ1* over 5 days of culture (Figure 6.4D). *SMAD2* mRNA expression was not markedly altered in any of the treatment groups, however levels were generally reduced on day 1 following transfection and/or initiation of DHT treatment and increased to peak levels of $<2$ fold on day 3 in GFP-ETS1p51 overexpressing and/or DHT treated cells (Figure 6.4E). Levels of *ZEB1* mRNA were increased by up to 4.5-fold in LNCaP cells that overexpressed GFP-ETS1p51 and further enhanced to $>60$-fold above controls in GFP-
ETS1p51 overexpressing cells cultured in the presence of DHT, findings that were consistent with PCR array data (section 5.2.7) (Figure 6.4F). Although GFP-ETS1p51 overexpression did not alter TWIST1 levels, expression of TWIST1 mRNA was enhanced up to 30-fold in control (pcDNA3.1) transfected LNCaP cells treated with DHT, with highest TWIST1 expression detected in LNCaP cells that also overexpressed GFP-ETS1p51 (Figure 6.4G). In addition, the overexpression of ETS1p51 in DHT-treated LNCaP cells appeared to sustain elevated TWIST1 expression during 5 days of culture (Figure 6.4G). Overall, RT-qPCR results confirmed the general pattern of GFP-ETS1p51- and DHT-induced regulation of gene expression initially identified using PCR arrays (section 5.2.7) and supported initial findings of ETS1p51- and DHT-mediated upregulation of expression of EMT-associated genes.

6.2.3 Regulation of Expression of EMT Markers by ETS1p51

The effects of GFP-ETS1p51 overexpression on protein levels of EMT transcription factors, mesenchymal or epithelial markers was evaluated by western blotting during 1-5 days following transfection of LNCaP cells with pEGFP-C2-ETS1p51 or pcDNA3.1 (control) (section 3.5.4, 3.8). In pEGFP-C2-ETS1p51 transfected cells, levels of ETS1p51 (GFP-ETS1p51) were increased by >100-fold compared to pcDNA3.1 (control) transfected cells during the 5 days of the assay (Figure 6.5A). Immunoblotting for the EMT-associated markers identified immunoreactivity against the EMT transcription factors SNAIL (~29kDa), SLUG (~30kDa), ZEB1 (~200kDa) and β-catenin (~92kDa) in all samples tested (Figure 6.5) (section 3.8.4). Similarly, expression of the mesenchymal markers vimentin (~57kDa), N-cadherin (~140kDa, not shown) and the epithelial markers Claudin-1 (~20kDa), E-cadherin (~135kDa, not shown) and Zona Occludens 1 (~220kDa, not shown) was also detected in all samples tested (Figure 6.5). Following normalisation of cellular protein levels using β-actin western blots for each of the samples and quantitation relative to pcDNA3.1 (control) transfected samples, levels of each of the EMT-associated transcription factors were identified to be increased in ETS1p51-overexpressing samples (Figure 6.5). The maximum levels of the transcription factors were detected on days 2-5 following transfection, with SNAIL1 (~2.5 fold, day 4), SLUG (~4.5 fold, day 5), ZEB1 (~2.5 fold, day 2) and β-catenin (~1.5 fold, day 5) (Figure 6.5B, C, D, E). Expression of SLUG and β-catenin gradually
Chapter 6: Regulation of EMT by ETS1p51 Overexpression

(A) ETS1p51

(B) SLUG

(C) SNAIL1

(D) TGFβ1

(E) SMAD2

(F) ZEB1

(G) TWIST1
Figure 6.4: Regulation of expression of EMT-associated genes by ETS1p51. LNCaP cells were transfected with pEGFP-C2-ETS1p51 or pcDNA3.1 (control) and treated with $10^8\text{M}$ DHT or 0.01% EtOH (vehicle control) for 1, 3 or 5 days prior to RNA extraction and RT-qPCR for (A) $ETS1p51$, (B) $SLUG$, (C) $SNAIL1$, (D) $TGF\beta1$, (E) $SMAD2$, (F) $ZEB1$, (G) $TWIST1$ and $GAPDH$ (reference gene). Relative expression of each of the genes was calculated using the Pfaffl method relative to untransfected/vehicle treated controls (EtOH). qPCRs were performed in duplicate per condition with averages quantitated ($\pm$SEM). The experiment was performed three times with representative results shown.
Chapter 6: Regulation of EMT by ETS1p51 Overexpression

(A) FTS1

(B) SNAIL1

(C) SLUG

(D) ZEB1

(E) β-catenin

(F) Vimentin

(G) Claudin-1

Day
0 1 2 3 4 5

Fold Increase

ETS1
β-actin

51kDa
44kDa

SNAIL1
β-actin
29kDa
44kDa

SLUG
β-actin
30kDa
44kDa

ZEB1
β-actin
200kDa
44kDa

β-catenin
92kDa
44kDa

Vimentin
β-actin
57kDa
44kDa

Claudin-1
β-actin
20kDa
44kDa
Figure 6.5: Regulation of EMT markers by ETS1p51. LNCaP cells were transiently transfected with a pEGFP-C2-ETS1p51 expression construct or pcDNA3.1 for 1-5 days. Immunoblotting was performed for (A) ETS1, (B) SNAIL1, (C) SLUG, (D) ZEB1, (E) β-catenin, (F) vimentin and (G) Claudin-1 and protein levels were normalised against β-actin for each sample and relative to the pcDNA3.1 transfected cells (-) (day 5). Experiments were performed three times with representative blots depicted.
increased during the 5 days, while peak levels of SNAIL1 and ZEB1 were detected prior to day 5 and had begun to decline by the end of the experiment (Figure 6.5). Levels of the mesenchymal marker, vimentin were increased to a maximum of ~2.5 fold by day 3, with small decreases thereafter (Figure 6.5F), whereas N-cadherin expression was not markedly altered during 5 days of GFP-ETS1p51 overexpression (not shown). In contrast to vimentin, levels of the epithelial marker, Claudin-1 were progressively reduced in GFP-ETS1p51 overexpressing LNCaP cells to 20% of initial levels on day 5 (Figure 6.5G), while expression of E-cadherin or Zona Occludens 1 was not regulated (not shown). These findings demonstrated that in addition to ETS1p51-mediated regulation of the expression of EMT-associated genes, protein levels of EMT mediators were also altered following GFP-ETS1p51 overexpression in LNCaP cells.

6.2.4 Regulation of TGF-β/SMAD Signalling in LNCaP Cells

To determine whether ETS1p51 overexpression resulted in activation of TGF-β/SMAD signalling, relative levels of phosphorylated SMAD2 (pSMAD2) compared to total cellular SMAD2 (tSMAD2) were estimated using western blotting (section 3.8). Following transfection of LNCaP cells, western blotting demonstrated that ETS1p51 (GFP-ETS1p51) levels were >100-fold higher relative to pcDNA3.1 (control) transfected cells during the 5 days following transfection (Figure 6.6). Immunoblotting detected pSMAD2 and tSMAD2 in all samples (Figure 6.6), including parental (untransfected/untreated) LNCaP cells (not shown). In LNCaP cells which overexpressed GFP-ETS1p51, levels of pSMAD2 were increased relative to pcDNA3.1 (control) transfected cells by up to ~7 fold at 24 hours following pEGFP-C2-ETS1p51 transfection, gradually decreasing to baseline levels by days 4-5 (Figure 6.6). In other experiments, the elevated levels of pSMAD2 were sustained for the (5 day) duration of GFP-ETS1p51 overexpression (results not shown). In these cells, no marked differences were detected in tSMAD2 levels (~1 to ~1.4-fold), suggesting that only phosphorylation of SMAD2 was altered following GFP-ETS1p51 overexpression (Figure 6.6). Calculation of pSMAD2/tSMAD2 ratios indicated that in comparison to pcDNA3.1 (control) transfected cells, SMAD2 phosphorylation was up to 9-fold higher in pEGFP-C2-ETS1p51 overexpressing cells.
Chapter 6: Regulation of EMT by ETS1p51 Overexpression

(A) pcDNA3.1 pEGFP-C2-ETS1p51

<table>
<thead>
<tr>
<th>Day</th>
<th>5</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP-ETS1p51</td>
<td>~80 kDa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pSMAD2</td>
<td>~60 kDa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tSMAD2</td>
<td>~58 kDa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>~44 kDa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(B) GEPF-ETS1p51 (Fold Increase)

0 50 100 150 200 250 300

pcDNA3.1 1 2 3 4 5 pEGFP-C2-ETS1p51 (Days)

(C) pSMAD2/SMAD2 (Fold increase)

0 1 2 3 4 5 6 7 8 9 10

pcDNA3.1 1 2 3 4 5 pEGFP-C2-ETS1p51 (Days)
Figure 6.6: Regulation of SMAD2 phosphorylation following GFP-ETS1p51 overexpression. LNCaP cells were transiently transfected with a pEGFP-C2-ETS1p51 expression construct (or pcDNA3.1 control vector) and harvested at 1-5 days following transfection. **(A)** Immunoblotting was performed for GFP (GFP-ETS1p51), pSMAD2 and tSMAD2, with protein levels normalised against β-actin for each sample and levels calculated relative to pcDNA3.1 (control) transfected samples. **(B)** GFP immunoblotting was used to monitor GFP-ETS1p51 overexpression and **(C)** normalised pSMAD2/tSMAD2 levels were used to determine GFP-ETS1p51-induced changes in SMAD2 phosphorylation. Experiments were performed three times with representative blots depicted.
Figure 6.7: Regulation of SMAD2 phosphorylation by SB431542 or TGFβ1. LNCaP cells were treated with either (A) 10ng/mL TGF-β1, (B) 10μM SB431542 or (C) 10μM SB431542 + 10ng/mL TGF-β1 or vehicle control (0.01% v/v DMSO) for up to 5 days. Immunoblotting was performed against pSMAD2 and tSMAD2 to evaluate SMAD2 phosphorylation levels. Experiments were performed twice with representative blots depicted.
Chapter 6: Regulation of EMT by ETS1p51 Overexpression

For subsequent studies investigating the activation of TGF-β/SMAD signalling in GFP-ETS1p51 overexpressing cells, the inhibitor of SMAD2 phosphorylation, SB431542 was used. In LNCaP cells cultured with 10μM SB431542, pSMAD2 was undetectable by ~1 hour (not shown) and for up to 5 days of SB431542 treatment, with no marked changes in tSMAD2 expression (Figure 6.7B). As expected, culture of LNCaP cells with 10ng/mL TGF-β1 increased pSMAD2/tSMAD2 levels (Figure 6.7A) while co-treatment of cells with 10ng/mL TGF-β1 and 10μM SB431542 reduced pSMAD2 to undetectable levels during 5 days of culture (Figure 6.7C). Together, these results indicated that GFP-ETS1p51 overexpression enhanced pSMAD2/tSMAD2 levels, providing evidence of activated TGF-β signalling, and that the SB431542 inhibitor specifically repressed SMAD2 phosphorylation induced by either GFP-ETS1p51 overexpression or TGF-β treatment of cultures.

6.2.5 Regulation of Cell Migration by ETS1p51

To investigate the roles of ETS1p51 overexpression on LNCaP cell migration, wound-healing assays were performed using IBIDI® silicone spacers (section 3.9). At the completion of the 72 hour assay, the migration of GFP-ETS1p51 overexpressing LNCaP cells was significantly enhanced (13.42% wound closure, p<0.05) relative to pcDNA3.1 (control) transfected cells that did not overexpress GFP-ETS1p51 (7.224% wound closure) (Figure 6.8). Incubation of cells with 10μM SB431542 attenuated the migration of control transfected LNCaP cells from 7.224% to 4.989% (p>0.05), while treatment of GFP-ETS1p51 overexpressing LNCaP cells with 10μM SB431542 significantly reduced cell migration from 13.42% to 3.796% (p<0.05) (Figure 6.8). TGF-β1 (10ng/mL) treatment of GFP-ETS1p51 overexpressing LNCaP cells increased migration from 13.42% to 16.728% and increased migration of control transfected LNCaP cells from 7.224% to 9.915%, however these differences did not reach significance (Figure 6.8). Migration of TGF-β1 treated LNCaP cells which overexpressed GFP-ETS1p51 (16.728%) was significantly greater than that of TGF-β1 control transfected LNCaP cells which did not overexpress GFP-ETS1p51 (9.915%) (p<0.05) (Figure 6.8). These results indicated that GFP-ETS1p51 overexpression significantly enhanced LNCaP cell migration, which was in part mediated by activation of TGF-β signalling.
Chapter 6: Regulation of EMT by ETS1p51 Overexpression

(A) pEGFP-C2-ETS1p51               pEGFP-C2-ETS1p51 + SB431542 (10μM)               pEGFP-C2-ETS1p51 + TGFβ1 (10ng/mL)

0hr

24hr

48hr

72hr

(B) pcDNA3.1                       pcDNA3.1 + SB431542 (10μM)                       pcDNA3.1 + TGFβ1 (10ng/mL)

0hr

24hr

48hr

72hr
Chapter 6: Regulation of EMT by ETS1p51 Overexpression

Figure 6.8: Regulation of LNCaP cell migration by ETS1p51. LNCaP cell migration was assayed during 3 days in cultures transiently transfected with (A) pEGFP-C2-ETS1p51 or (B) pcDNA3.1 (control) and treated with 10μM SB431542, an inhibitor of SMAD2 phosphorylation, 10ng/mL TGF-β ligand (TGF-β1) or vehicle (0.01% v/v DMSO). The leading edge of migration is highlighted (red line), with (C) cell migration quantitated by calculating the occupancy of cells (%) in the ‘wound’ relative to 0hr. Imaging was conducted every 24 hours and analysed using ImageJ software. Each treatment was performed in duplicate and the experiment was performed three times with averages graphed and representative images displayed. (D) Significant differences following the completion of the assay were calculated using a one-tailed Mann Whitney U test (p<0.05). Graphs represent mean ± SEM.
6.2.6 Regulation of LNCaP Cell Invasion by ETS1p51

To investigate ETS1p51-mediated regulation of cell invasion, LNCaP cell invasion was determined using Matrigel™ transwell assays (section 3.10). After 48 hours, the average number of LNCaP cells that had invaded through the Matrigel™ inserts ranged between ~650 to ~1500. In comparison to pcDNA3.1 (control) transfected cells treated with 0.01% DMSO (vehicle), 10μM SB431542 treatment did not alter cell invasion, while 10ng/mL TGF-β1 treatment elevated cell invasion by ~50%, (p<0.05) (Figure 6.9). Cell invasion was increased to ~160% (p<0.05) in GFP-ETS1p51 overexpressing cells with this increased reversed by co-treatment of the cultures with SB431542 (p<0.05). These findings suggest that increased invasiveness was in part mediated by activation of TGF-β signalling in the GFP-ETS1p51 overexpressing cells. TGF-β1 treatment of GFP-ETS1p51 overexpressing cells further enhanced cell invasion (Figure 6.9) in line with the known functions of TGF-β signalling in the regulation of cell invasion (Katsuno et al. 2013).
Figure 6.9: Regulation of LNCaP cell invasion by ETS1p51. LNCaP cell invasion was assessed following transient transfection with a GFP-ETS1p51 expression construct or pcDNA3.1 (control) vector and treated with 10μM SB431542, an inhibitor of SMAD2 phosphorylation, 10ng/mL TGF-β ligand (TGF-β1) or 0.01% (v/v) DMSO vehicle control. (A) At 48 hours, cells that had invaded through the Matrigel were imaged using phase contrast microscopy and (B) 10 fields (100x magnification) were counted to determine cell invasion in comparison to control-transfected, vehicle-treated cultures. Each treatment was performed in duplicate, the experiment was performed four times with average invasion index (%) graphed (±SEM) relative to pcDNA3.1 control (vehicle-treated) cultures, and representative images shown. Significant differences following the completion of the assay were calculated using a one-tailed Mann Whitney U test (p<0.05).
6.3 Discussion

The overexpression of ETS factor family members in prostate cancer has been linked to more aggressive growth characteristics which are typically associated with enhanced cell proliferation, migration, invasion and tumour-associated angiogenesis (Watson et al. 2010). In prostate tumours, the consequences of aberrant ETS factor expression and/or activity on the abnormal activation (or repression) of target gene expression have been partially characterised and include genes encoding ECM-degrading enzymes (MMPs, uPA), cyclins (cyclin D1), growth factors (VEGF, FGF) and more recently, EMT promoting factors (SLUG, β-catenin) (Sementchenko et al. 2000; Oikawa et al. 2003; Hsu et al. 2004; Gupta et al. 2010; Findlay et al. 2013). In this study ETS1p51-mediated regulation of expression of EMT-associated genes was screened by PCR array (section 5.2.6), following which validation of results was required to confirm gene regulation using independent RT-qPCR methods (Rajeevan et al. 2001). As the PCR array studies had investigated differential gene expression following 24 hours of ETS1p51 overexpression and/or DHT treatment of LNCaP cells, determination of gene expression during a 5 day period permitted more extensive characterisation of the regulation of mRNA levels in addition to validation of the PCR array results. Genes confirmed to be regulated by ETS1p51 overexpression and/or DHT treatment included members of the TGF-β signalling pathway TGF-β1 and ZEB1 as well as EMT-associated transcription factors, SLUG, SNAIL and TWIST1.

TGF-β1 mRNA expression was elevated following ETS1p51 overexpression in either the absence or presence of DHT treatment. Although ETS1 or ETS factor regulation of TGF-β1 expression has not been reported previously, TGF-β1 mRNA levels are reduced in the majority of human prostate tumours (Soulitzis et al. 2006), most of which would overexpress one or more ETS factors including ETS1. DHT-induced AR signalling downregulates the expression of TGF-β1 in LNCaP cells (Chipuk et al. 2002) as was observed in this study, however DHT treatment augmented the ETS1p51-induced upregulation of TGF-β1 expression, potentially due to DHT-mediated increases in ETS1 levels (Preece 2009). ETS1p51 overexpression or DHT treatment of LNCaP cells did not markedly alter expression of the TGF-β signalling intermediate SMAD2, which is reported to be similar in nonmalignant and malignant...
prostatic tissues (Latil \textit{et al.} 1999). A putative EBS has been identified in the \textit{SMAD2} promoter (Koinuma \textit{et al.} 2009), however its function has not been characterised and binding of ETS1 or other ETS factors to the sequence has not been determined. In contrast to the lack of DHT regulation of \textit{SMAD2} in prostate cancer cells, testosterone-induced AR-signalling has been reported to upregulate expression of \textit{SMAD2} in the TM4 Sertoli cell line (Itman \textit{et al.} 2011).

Levels of \textit{ZEB1} mRNA were higher in ETS1p51-overexpressing LNCaP cells and further increased following DHT treatment of these cells. \textit{ZEB1} is a target of ETS1 that has been previously characterised using mouse and human cell lines (Shirakihara \textit{et al.} 2007; Dave \textit{et al.} 2011), with both \textit{ZEB1} (Graham \textit{et al.} 2008) and \textit{ETS1} (Smith \textit{et al.} 2012a) mRNA levels reported to be increased in prostate cancer cell lines that are representative of more aggressive prostate tumours. The finding in this study of DHT-induced upregulation of \textit{ZEB1} mRNA expression was also similar to the AR-mediated regulation of \textit{ZEB1}, which contains two AREs (Anose \textit{et al.} 2011), supporting the likelihood of collaborative regulation of \textit{ZEB1} by ETS1 and AR signalling.

In these studies, ETS1p51 overexpression did not markedly alter expression of the EMT transcription factor, \textit{SLUG}, however \textit{SLUG} mRNA expression was enhanced following DHT treatment, which was consistent with results from the PCR arrays (section 5.2.6). \textit{SLUG} is an androgen responsive gene and its elevated expression in DHT-treated LNCaP cells has been reported previously (Chen \textit{et al.} 2006a; Bolton \textit{et al.} 2007; Baygi \textit{et al.} 2010; Wu \textit{et al.} 2012). Although \textit{SLUG} expression was not markedly altered following ETS1p51 overexpression, it is regulated by other ETS factors in nonmalignant prostate or prostate cancer cell lines and its expression has been correlated with that of ERG and ETV4 (Gupta \textit{et al.} 2010; Pellecchia \textit{et al.} 2012). Conversely, PDEF has been shown to negatively regulate \textit{SLUG} expression in breast cancer cell lines (Findlay \textit{et al.} 2011). \textit{SNAIL1} mRNA levels were not regulated by ETS1p51 overexpression or DHT treatment of LNCaP cells but were elevated following DHT treatment of ETS1p51 overexpressing LNCaP cells. This contrasts previous findings of elevated \textit{SNAIL1} mRNA expression in DHT treated LNCaP cells (Zhu \textit{et al.} 2010) and results from the PCR arrays which identified reduced \textit{SNAIL1} mRNA levels in DHT-treated or ETS1p51-overexpressing LNCaP cells (section 5.2.6). Direct AR
transactivation of SNAIL1 or the presence of an ARE in the SNAIL1 gene have not been reported and it is feasible that androgen-induced activation of SNAIL1 may be indirect or requires the function of additional coactivators, potentially including ETS1. Comparable to its family member SLUG, SNAIL1 expression is reported to be regulated by other ETS factors including PEA3/ETV4, which directly regulates SNAIL expression in breast cancer cell lines (Yuen et al. 2011), and ERG, overexpression of which in RPWE cells has been associated with upregulated SNAIL1 levels (Gupta et al. 2010).

TWIST1 expression was not altered following ETS1p51 overexpression but was enhanced by DHT treatment of LNCaP cells, which was consistent with a previous report of androgen-induced upregulation of TWIST1 mRNA expression in LNCaP cells (Eide et al. 2013). Although ETS1-mediated regulation of TWIST1 was not identified in the present study, in vitro silencing of ETS1 results in reduced expression of TWIST1 (Schroeder et al. 2011), while PEA3 directly binds EBS sequences in the TWIST1 promoter and upregulates TWIST1 expression in mouse non-small cell lung cancer cell lines (Howe et al. 2003; Qin et al. 2009). The TWIST1 promoter contains numerous putative EBS sequences (Howe et al. 2003; Qin et al. 2009) and although ETS1p51 overexpression did not regulate TWIST1 mRNA levels in LNCaP cells under the culture conditions employed in this study, it is feasible that ETS1 regulates TWIST1 expression in other cell types or under different growth conditions.

The functional consequences of ETS1p51-induced changes in the expression of mRNA’s encoding EMT-associated factors is largely directed by altered levels of expression of the protein products encoded by these genes. In this study, changes in expression of a selection of EMT-associated factors in ETS1p51-transfected cells was determined by western blotting. These experiments identified upregulation of expression of the EMT transcription factors SNAIL1, SLUG, ZEB1 and β-catenin in ETS1p51 overexpressing cells, which collectively represent the spectrum of EMT-associated transcription factors expressed during EMT (Nauseef et al. 2011; Grant et al. 2013).

In particular, SNAIL1 a repressor of E-cadherin in epithelial tumour cells (Batlle et al. 2000) is reported to be overexpressed in prostate tumours (Fawzy et al. 2013) and
was identified to be upregulated following ETS1p51 overexpression. This contrasts RT-qPCR data which identified that ETS1p51 overexpression had no effect on \textit{SNAIL1} mRNA levels, but enhanced \textit{SNAIL1} expression following DHT treatment. Relative expression of ETS1 and SNAIL1 in prostate tumours or prostate cancer cell lines has not been reported and SNAIL1 is not a previously characterised ETS1 target however, in breast cancer cell lines transfected to overexpress PEA3, increased SNAIL1 levels were identified (Yuen \textit{et al.} 2011). Similar to SNAIL1, increased SLUG protein levels were detected in ETS1p51-overexpressing LNCaP cells, contrasting previous RT-qPCR results, which did not identify increases in \textit{SLUG} mRNA expression following ETS1p51 overexpression. Correlations between ETS factor or ETS1p51 and SLUG protein expression have not been characterised in prostate cancer cells however, as the \textit{SLUG} promoter contains EBS sequences and \textit{SLUG} is negatively regulated by PDEF (Findlay \textit{et al.} 2011) it is feasible that other ETS factors are able to regulate \textit{SLUG} expression. In the absence of evidence of ETS1p51 regulation of \textit{SLUG} mRNA expression in this study, ETS1p51 overexpression on its own or in association with other factors expressed in LNCaP cells may contribute to post-transcriptional or post-translational mechanisms which mediate the observed increases in SLUG protein levels.

\textit{ZEB1} has been previously characterised as an ETS1 target gene in mouse mammary epithelial and human colorectal cancer cell lines (Shirakihara \textit{et al.} 2007; Dave \textit{et al.} 2011), and results from this study indicate that this regulation is conserved in prostate cancer cells. In this study ETS1p51 overexpression enhanced both \textit{ZEB1} mRNA and protein levels, which are similarly overexpressed in a majority of prostate tumours and bone metastases (Graham \textit{et al.} 2008; Sethi \textit{et al.} 2011). \textit{ZEB1} is also a target gene of ERG in prostate cancer cells and overexpression of ERG directly upregulates both mRNA and protein levels of \textit{ZEB1}, suggesting that multiple ETS factors can enhance \textit{ZEB1} expression (Leshem \textit{et al.} 2011).

Divergent regulation of β-catenin by ETS1p51 was identified in this study, with reduced β-catenin mRNA in PCR arrays (section 5.2.6), and increased protein levels identified by western blotting. A number of studies have investigated β-catenin expression in prostate cancer and interestingly report that β-catenin expression is reduced in the majority of tumours (Morita \textit{et al.} 1999; Chesire \textit{et al.} 2000; Jaggi \textit{et al.}}
However, several studies have identified elevated nuclear β-catenin levels in higher stage disease, suggesting that measurement of levels of activated β-catenin may be more relevant in the investigation of its function in prostate cancer.

Of the epithelial and mesenchymal markers investigated, only differences in Claudin-1 (epithelial) and vimentin (mesenchymal) expression were observed in ETS1p51-overexpressing cells. Although changes in the expression of other epithelial markers, E-cadherin or Zona Occludens-1 or the mesenchymal marker, N-cadherin were not detected in ETS1p51 overexpressing cells, their intracellular localisation was not investigated and future studies using microscopy techniques may further characterise their regulation, if any, by ETS1p51. Progressively reduced expression of the epithelial tight junction molecule Claudin-1 was identified in ETS1p51 overexpressing LNCaP cells, which is functionally associated with the reduction of tight junction structures, enabling cell motility (Ikenouchi et al. 2003; Martin et al. 2009). Claudin-1 has not been characterised as a direct ETS1 target, however studies in endothelial and mouse epithelial cells have demonstrated ERG- and ETV5-mediated regulation, respectively of the Claudin family member, Claudin-5 (Morrow et al. 2009; Yuan et al. 2012). Of the mesenchymal markers investigated in this study, vimentin was increased in ETS1p51 overexpressing LNCaP cells, although vimentin mRNA expression was not significantly altered in the PCR arrays (section 5.2.6). Vimentin expression is reported to be elevated in prostate tumours and is significantly associated with other established prognostic factors (e.g. biochemical recurrence) (Sethi et al. 2011; Behnsawy et al. 2013). Previous studies using breast cancer cell lines also report that ETS1 and vimentin are abundantly co-expressed in the more invasive cell lines (Gilles et al. 1997), and PEA3 and ETS1 have been stated to transactivate vimentin expression indirectly via hepatocyte growth factor signalling in tumour cells (Hsu et al. 2004), suggesting that the altered levels of vimentin observed in this study were an indirect effect of ETS1p51 overexpression.

ETS1p51 overexpression in LNCaP cells was associated with increased phosphorylation of SMAD2, which is consistent with bioinformatics analyses that predicted ETS1p51-induced activation of TGF-β signalling (section 5.2.7). SMAD2 is a
transcription factor that mediates TGF-β signalling-induced gene expression in association with other SMADs, transcription factors and co-regulatory molecules (Shi et al. 2003). SMAD2 activation results in the transactivation of other EMT promoting transcription factors including SNAIL1 and SLUG (Brandl et al. 2010), β-catenin (Hirota et al. 2008), and mesenchymal markers such as vimentin (Wu et al. 2007), with expression of each increased at the mRNA and/or protein level in ETS1p51 overexpressing LNCaP cells in this study. Although not directly characteristic of EMT processes, elevated expression of EMT transcription factors are considered the master regulators of EMT in various cancers including prostate cancer (Thiery et al. 2009; Grant et al. 2013) potentially implicating ETS1 as a promoter of EMT in prostate cancer.

Induction of cancer-associated EMT characteristically results in the increased migration and invasion of tumour cells, both of which were observed in this study. The ETS1p51-induced increases in LNCaP cell migration detected in this thesis were consistent with previous in vitro studies which similarly reported enhanced migration of ETS1p51 overexpressing LNCaP cells (Shaikhbrahim et al. 2011; Smith et al. 2012a). ETS1 directly regulates expression of genes that encode mediators of cell migration including MMPs and uPA, and proteases which degrade ECM and facilitate cell movement (Watabe et al. 1998; Baillat et al. 2002; Dittmer 2003; Rothhammer et al. 2004). In addition, ETS1 is reported to increase expression of the EMT-associated transcription factors SNAIL1, SLUG, ZEB1 and β-catenin as well as the mesenchymal marker vimentin, each of which have been identified to promote cell migration (Lang et al. 2002; Graham et al. 2008; Odero-Marah et al. 2008; Uygur et al. 2011; Smith et al. 2012b).

The overexpression of ETS1p51 was also found to induce activation of TGF-β signalling, as evidenced by enhanced SMAD2 phosphorylation and predicted from bioinformatics analysis of PCR array results (Chapter 5). TGF-β is a potent inducer of cancer cell motility (Katsuno et al. 2013) and findings from this thesis indicate that it both increased the migration of LNCaP cells and in part mediated the effects of ETS1p51 overexpression on LNCaP cell migration. Divergent effects of TGF-β on the migration of prostate cancer cells have been reported previously, with reduced
migration on fibronectin of LNCaP cells and the metastatic/castrate-resistant LNCaP derivatives C4-2 and C4-2B4 (Miles et al. 2012) but increased migration of DU145 cells on collagen (Vo et al. 2013) following TGF-β treatment. Conversely, treatment of cells with the inhibitor of SMAD2 phosphorylation, SB431542 reduced LNCaP cell migration and TGF-β1 induced cell migration in this study, and reversed the inhibition of migration on fibronectin of LNCaP, C4-2 and C4-2B4 cells cultured with TGF-β1 (Miles et al. 2012). As cells were cultured on different substrates in the present and in previous studies, results are not directly comparable, in particular as ECM components can directly regulate cell migration (Lu et al. 2012a). However, further characterisation of ETS1p51-induced cell migration in future studies may involve the use of different ECM substrates, which will clarify interactions between ETS1p51-overexpressing prostate cancer cells and their microenvironment in the regulation of cell migration.

LNCaP cell invasion displayed similar trends to the cell migration assays following ETS1p51 overexpression. These findings are comparable to a previous study that evaluated transient ETS1p51 overexpression in LNCaP cells (Smith et al. 2012a), but contrast an additional report which found that ETS1 overexpression did not affect prostate cancer cell invasion (Shaikhibrahim et al. 2011). ETS1 overexpression has been shown to upregulate the expression of mediators of cell invasion including MMPs, uPA, HGF/SF, PTHrP, vimentin and integrin-β2 (Gilles et al. 1997; Watabe et al. 1998; Baillat et al. 2002; Cataisson et al. 2002; Rothhammer et al. 2004; Hahne et al. 2005; Furlan et al. 2008) and was associated in this study with increased expression of the EMT transcription factors SNAIL1, SLUG, ZEB1 and β-catenin in addition to the mesenchymal marker vimentin, factors which promote cell invasion (Aigner et al. 2007; Iwai et al. 2010; Uygur et al. 2011; Neal et al. 2012).

The processes of migration and invasion are frequently linked in cancer cells, and ETS1p51-induced increases in LNCaP cell invasion was shown to be associated with induction of TGF-β signalling in the present study as was observed for ETS1p51-induced LNCaP cell migration. TGF-β enhances PC-3 cell invasion (Vo et al. 2013) but is also reported to inhibit of LNCaP and C4-2 cell invasion (Miles et al. 2012). Comparable to findings of this study, treatment of LNCaP cells with the inhibitor of SMAD2 phosphorylation SB431542, inhibited LNCaP cell invasion (Miles et al. 2012).
and while TGF-β treatment of LNCaP cells enhanced invasion in this study, this contrasted TGF-β-mediated inhibition of LNCaP cell invasion of the published study. The role of TGF-β signalling in the regulation of LNCaP cell invasion is supported by the reversal of its effects when cells in the present and previous studies were co-treated with SB431542 (Miles et al. 2012). Major findings from this study therefore indicate that ETS1p51 overexpression in prostate cancer cells is associated with increased expression of EMT mediators and transcription factors, activation of TGF-β/SMAD signalling via enhanced SMAD2 phosphorylation and enhanced cell migration and invasion that is in part mediated by ETS1p51-induced TGF-β signalling. Collectively, these gene and protein expression profiles, signalling activity and cellular processes constitute an EMT programme. Based on the known roles of EMT on cancer invasion and metastasis, these processes are likely to contribute to disease progression and the poorer prognosis of ETS1 overexpressing prostate tumours.
7.1 General Discussion

Members of the E26 Transformation Specific (ETS) transcription factor family are ubiquitously expressed in developing and adult tissues, with prevalent roles in a variety of cellular processes (Dittmer et al. 1998; Maroulakou et al. 2000; Hollenhorst et al. 2004). ETS factor over- or under-expression due to gene amplification or loss, point mutations and chromosomal rearrangements has been investigated in many types of cancer including leukaemias, Ewing’s sarcoma, breast and prostate tumours (Tenen et al. 1997; Seth et al. 2005; Watson et al. 2010). Expression of the prototypical ETS factor family member, ETS1 is aberrantly increased in up to ~70% of prostate tumours (Alipov et al. 2005; Li et al. 2012a; Smith et al. 2012a) and studies performed in this thesis have characterised prostate tumour ETS1 isoform expression in comparison to the adjacent nonmalignant prostate, identifying elevated ETS1p51 expression in prostate cancers that induces EMT in prostate cancer cells. In conjunction with previously published reports of the functions of other ETS factor family members in prostate tumours, this study provides strong evidence of the importance of the ETS family in the formation and progression of prostate cancer.

Of the 28 ETS factor family members, 25 are detectable at the mRNA level in the nonmalignant prostate (Hollenhorst et al. 2004) and at least 16 have been identified to be aberrantly expressed in prostate cancer (Seth et al. 2005; Watson et al. 2010). The majority of ETS factors abnormally expressed in prostate cancers are overexpressed (≥14) while ESE3 and PDEF are underexpressed (Nozawa et al. 2000; Tugores et al. 2001). Interestingly, each member of the ETS, ERG and PEA3 subfamilies is overexpressed in prostate tumours (Hu et al. 2008; Watson et al. 2010; Oh et al. 2012; Paulo et al. 2012a). Following the identification of ETS factors in humans (Watson et al. 1985), and their reported contribution to the induction of leukaemias (Wasylyk et al. 1993), ETS2 was identified to be overexpressed in a small cohort of malignant prostate specimens and in several prostate cancer cell lines (Liu et al. 1997). Subsequent examination of ETS factor expression in prostate tumours identified markedly increased levels of FLI1 and ELF1, elevated ERG1/2 and ETS2 expression and smaller increases in ELK1, PEA3 and PU.1 expression (Gavrilov et al. 2001), along with reduced levels
Chapter 7: General Discussion

of PDEF and ESE3 in comparison to nonmalignant prostate (Nozawa et al. 2000; Tugores et al. 2001).

In 2005, ERG was reported to be overexpressed in a high proportion of prostate tumours (Petrovics et al. 2005), while a study by Tomlins et al described for the first time chromosomal rearrangements in up to 80% of human prostate tumours that disrupted predominantly the ERG gene but also ETV1 (Tomlins et al. 2005). As a result of the chromosomal rearrangements, the coding regions of these ETS factors were fused to an androgen-responsive gene promoter generating high levels and androgen responsive expression of affected ETS factors that were otherwise not expressed or expressed at low levels in prostate tissues (Tomlins et al. 2005). Further characterisation of ETS factor rearrangements has indicated that this mechanism accounts for >50% of ETS factor abnormalities in prostate tumours, with a frequency of ≤1% (e.g. ETV1, FLI1) up to 50% (e.g. ERG) of all prostate tumours (Tanderfelt et al. 2014). Concurrently, immunohistochemical studies identified overexpression of factors such as ETS1 (Alipov et al. 2005), ETS2 (Gavrilov et al. 2001) and ESE2 (Rostad et al. 2007) in prostate cancers that was not associated with chromosomal rearrangements. Results from the present study of increased ETS1 protein levels in human prostate tumours in comparison to nonmalignant prostate support the previously reported immunohistochemical findings of ETS1 overexpression in prostate tumours (Alipov et al. 2005; Li et al. 2012a; Smith et al. 2012a) and for the first time identify that ETS1p51 is the isoform expressed in the nonmalignant prostate and overexpressed in prostate cancer. Additional mechanisms of dysregulated ETS factor expression or function have been reported and include gene amplification (ESE1) (Longoni et al. 2013), enhanced phosphorylation/activity (ETS1, NET) (Zheng et al. 2003; Smith et al. 2012a) and genomic loss/deletion (TEL) (Kibel et al. 2000) however, mechanisms underlying the aberrant expression of many ETS factors remain uncharacterised.

ETS factor dysregulation in prostate tumours has been characterised to correlate with clinical features of disease including the poorer survival of patients whose tumours carry TMPRSS2-ERG rearrangements (Hagglof et al. 2014), positive correlations between ETS1 expression and higher Gleason score or disease severity (Smith et al. 2012a), inverse correlation of PDEF expression with higher Gleason score and disease
severity (Ghadersohi et al. 2011), occurrence of metastases in patients with ESE1 overexpressing tumours (Longoni et al. 2013) and the rapid development of biochemical recurrence in patients with tumours expressing high ETS1 levels (Li et al. 2012a). In the present study, higher expression of ETS1 was not found to be associated with Gleason score, however numbers were too small for proper analysis and additional clinical details were not available. Regardless, accumulating evidence from published studies suggests that measurement of ETS factor expression in prostate tumours may be developed as prognostic or predictive markers that are useful for specific aspects of disease management (e.g. duration of response to androgen ablation therapies).

In addition to studies of expression of individual ETS factors in prostate tumours, a large amount of data on ETS factor expression may be retrieved from databases storing mass genomic and transcriptomic data (e.g. TCGA, OncomineTM) which frequently also include annotated patient and clinical data (Rhodes et al. 2007). The combination of genomic (mRNA) expression data and individual published studies of ETS factor (mRNA/protein) expression has emphasised that aberrant ETS factor expression is a prominent molecular feature of the majority of prostate tumours but also highlights discordant mRNA and protein expression in prostate tumours or prostate cancer cell lines. This includes the high levels of mRNA but undetectable protein expression of ETV1 in LNCaP cells (Selvaraj et al. 2014) or detectable PDEF protein in nonmalignant prostate but undetectable protein expression in prostate cancer cells (Nozawa et al. 2000), the low levels of ETS2 mRNA and high levels of ETS2 protein in prostate tumours (Gavrilov et al. 2001; Ernst et al. 2002) and detection of the TMPRSS2-ERG gene fusion with no detectable ERG protein products in up to 26% of prostate tumours (Gsponer et al. 2014). Findings from this thesis that indicate reduced ETS1 mRNA but elevated ETS1 protein levels in prostate tumours relative to nonmalignant tissue reinforce the divergent pattern of ETS factor mRNA and protein expression and suggest that post-transcriptional and/or post-translational regulation of ETS factor regulation is common. Although genomic databases centralise data and enhance the global study of dysregulated expression of ETS factors and other genes in prostate tumours, evidence of discordant mRNA and protein expression suggests that combinations of genomic, transcriptomic and proteomic databases may
better describe and be used to evaluate atypical ETS factor expression and function in human prostate tumours.

Similar to most solid tumours, prostate cancers are heterogeneous, thwarting generalisations regarding ETS factor expression, and are further complicated by evidence of abnormalities of multiple ETS factors within tumours (Gavrilov et al. 2001; Svensson et al. 2011), differential levels of expression of ETS factors in multiple foci of individual tumours (Furusato et al. 2008; Furusato et al. 2010) and discordant findings between studies (e.g. PDEF is reported to be either underexpressed or overexpressed in prostate tumours) (Tsujimoto et al. 2002; Sood et al. 2007). It is also evident following the identification and characterisation of ETS factor splice variants that differential expression of ETS factor isoforms may profoundly influence ETS factor function in tumour cells. The contribution of individual ETS factor isoforms to the abnormal expression of individual ETS factors may not be evident using conventional RT-qPCR or immunohistochemical methods but may be critical to the understanding of consequences of the aberrant expression of that ETS factor in prostate tumours. Several ETS factors including ESE3 (Sprater et al. 2012), TMPRSS2-ERG (Hu et al. 2008), ETS1 (Dittmer 2003; Laitem et al. 2009) and NET (Giovane et al. 1997) have been reported to undergo alternative splicing in prostate or other tissue types, with minimal studies further characterising their relative expression or the functional consequences of their differential expression in prostate tumours. Results from this thesis have demonstrated that the ETS1 isoforms, ETS1p51, ETS1p42 and ETS1p27 are differentially expressed at the mRNA and protein levels in both malignant and nonmalignant prostatic tissues, and that ETS1p51 was the sole protein isoform detected in either nonmalignant or malignant tissue. The predominant effects of individual ETS factor isoforms have been described for ESE3 in dendritic cells, where only ESE3b promoted the differentiation of cells, while the alternative isoforms, ESE3a and ESE3j did not (Sprater et al. 2012).

Studies characterising ETS1 isoform structure and function that have identified differential gene expression profiles induced by each of the isoforms have suggested that the predominant expression of ETS1p51 identified in this thesis will have specific effects on the expression of target genes that influence tumour growth and progression.
(Fisher et al. 1994; Laitem et al. 2009; Hahne et al. 2011). For example, ETS1 isoforms differentially regulate expression of the *MMP3* gene, with ETS1p42 reported to increase expression of *MMP3*, ETS1p51 not able to regulate *MMP3* levels and ETS1p27 outcompeting other isoforms to repress *MMP3* expression in mouse fibroblast and human breast cancer cells (Laitem et al. 2009; Hahne et al. 2011). Interestingly, ETS1p51, the sole ETS1 protein detected in LNCaP cells in this study strongly repressed *MMP3* expression, indicating that in addition to specific patterns of gene regulation resulting from the different structures of ETS factor isoforms, distinct cofactor expression profiles may alter ETS1 isoform-mediated regulation of target gene expression in prostate and other cell types.

ETS factors bind a core DNA sequence of 5'-GGA(A/T)-3' that is flanked by divergent sequences demonstrated to be differentially targeted by individual ETS factors, thereby conferring diverse functions of members of the ETS factor family in the regulation of target genes (Kodandapani et al. 1996; Hollenhorst et al. 2007; Wei et al. 2010). Mapping of ETS factor DNA binding sites in nonmalignant prostate cancer cell lines identified significant overlap between ERG, ETV1 and ETV4 occupancy of genes bioinformatically determined to mediate blood vessel development, cell proliferation and migration and regulation of RAS/MAPK signalling target genes (Hollenhorst et al. 2011a). Common ETS factor target genes include growth factors or growth factor receptors (e.g. *GM-CSF, TGF-β receptor 2*), cell cycle regulators (e.g. *CDK1, p16INK4a*), and mediators of apoptosis (e.g. *Bcl-XL, caspase-3*), differentiation (e.g. *c-kit, IL-3*) and angiogenesis (e.g. *VEGFR*) (Oikawa et al. 2003; Oikawa 2004) under normal physiological conditions and in cancer. Therefore, ETS factor overexpression in prostate cancer and particularly overexpression of members of the ERG and PEA3 subfamilies including ERG, ETV1, ETV4 and ETV5, have been associated with ‘oncogenic’ activity (Clark et al. 2009; Oh et al. 2012). Supporting this hypothesis, *in vivo* overexpression of ERG or ETV1 in mouse models leads to formation of the preneoplastic lesion PIN, but not cancer (Klezovitch et al. 2008; Tomlins et al. 2008; Shin et al. 2009), indicating that aberrant ETS factor expression may facilitate but is not sufficient for the development of prostate tumours.
ERG, ETV1, ETV4 and ETS1 also regulate expression of target genes encoding mediators of cell migration and invasion, cellular processes which promote prostate cancer progression (Tomlins et al. 2008; Shin et al. 2009; Hollenhorst et al. 2011a; Shaikhjibrahim et al. 2011; Smith et al. 2012a). Genes encoding central regulators of these processes, such as MMPs (Shin et al. 2013), uPA (Hollenhorst et al. 2011a), osteopontin (OPN, a secreted extracellular matrix protein) (Flajollet et al. 2011) and others are targets of multiple ETS factors that are commonly overexpressed in prostate cancers, including ETV1, ETV4, ETV5 and ERG, supporting the reported (ERG, ETV1) or proposed (ETV4, ETV5) associations between ETS factor overexpression and poor prognosis of prostate tumours (Nam et al. 2007; Attard et al. 2008). Similarly, ETS1p51 was found in this study to increase expression of SPARC, MMP2 and MMP9, which would facilitate cell migration and invasion. ETS factors are additionally characterised to promote chronic inflammation in the prostate (DeMarzo et al. 2007), a contributing factor in the aetiology of a proportion of prostate tumours. For example, in the prostate tumours of Pten^fl/fl;R26^ERG mice which are Pten deficient and overexpress TMRPSS2-ERG, increased expression of genes that encode pro-inflammatory mediators including HIF1A, IL1B and CCL7 was identified, with bioinformatics analysis predicting enhanced inflammation in the resulting prostate tumours (Chen et al. 2013). In addition, overexpression of ESE1 in LNCaP and 22Rv1 prostate cancer cells was proposed to drive inflammation through constitutive activation of NF-κB signalling (Longoni et al. 2013), and higher expression of ESE1 was identified to be correlated with sustained NF-κB signalling in human prostate tumours, where it was associated with poor prognosis.

A proportion of advanced prostate tumours display aberrant activation of EMT signalling pathways, which regulate EMT-associated processes including cell movement, invasiveness and tumour metastasis (Nauseef et al. 2011; Grant et al. 2013). These processes may also be associated with ETS factor overexpression, and recent studies have proposed that several ETS factors are able to induce EMT in prostate cancer cells. These include ERG and the TMRPSS2-ERG fusion, with ERG overexpression in nonmalignant RWPE1 cells activating WNT signalling and increasing cell invasion (Tomlins et al. 2008; Gupta et al. 2010) and bioinformatics-derived evidence of increased WNT signalling in TMRPSS2-ERG positive prostate tumours.
Chapter 7: General Discussion

(Iljin et al. 2006). Conversely, silencing of TMRPSS2-ERG in VCaP cells was correlated with reduced WNT signalling, increased cell adhesion, induction of active B1-integrin and E-cadherin expression. Similarly in RWPE1 cells, overexpression of ETV4 regulated cell proliferation, motility and anchorage-independent growth, with increased expression of the EMT transcription factors, TWIST1, SLUG, ZEB1 and ZEB2 (Pellecchia et al. 2012). PDEF and ESE3 are reported to repress expression of EMT transcription factors and EMT-associated cell motility in prostate cancer cell lines and their expression is found to be reduced in human prostate tumours (Gu et al. 2007; Albino et al. 2012).

Results derived from prostatic or prostate cancer cell lines have been supported by the findings from studies using other cell types, with PEA3 and ETS1 also identified to promote expression of EMT transcription factors and enhance EMT-mediated growth characteristics in breast cancer and non-small lung cancer cell lines (Kathuria et al. 2011; Yuen et al. 2011). In contrast, ELF5, PDEF and ETS2 repress EMT-associated transcription factor expression and mesenchymal-like growth characteristics in breast and gastric cancer cell lines (Findlay et al. 2011; Chakrabarti et al. 2012; Liao et al. 2012), although PDEF expression is frequently downregulated in prostate tumours (Nozawa et al. 2000). In combination with results of this study which identified that ETS1p51 overexpression promoted EMT in prostate cancer cells by upregulation of gene and/or protein expression of EMT-promoting transcription factors and TGF-β signalling components, it is apparent that aberrant ETS factor expression may promote EMT in prostate cancer cells via the regulation of different EMT-associated signalling pathways. Therefore in individual prostate tumours which would commonly exhibit abnormal expression of multiple ETS factors, EMT may be promoted by several pathways (e.g. activation of TGF-β and WNT signalling). This would need to be considered in the design of prostate cancer treatments that target ETS factors as targeting of individual ETS factors may not be effective if common pathways (e.g. EMT) are promoted by multiple ETS factors.

At the commencement of this thesis project, ETS1 had been shown to interact with the AR in a ligand-dependent manner and to enhance the transcriptional activity of the AR on a number of target gene promoters (Massie et al. 2007). PDEF had also been
reported to interact with the AR to regulate PSA expression (Oettgen et al. 2000) and subsequent to those initial studies, ERG, ETV1, ETV5 and GABPα were identified to interact with the AR and to modify gene expression profiles in prostate cancer cells (Massie et al. 2007; Yu et al. 2010; Baena et al. 2013; Chen et al. 2013; Sharma et al. 2014). ETS factors may bind to the same regulatory elements as the AR or may bind to different regulatory regions of common genes in prostate cancer cells, enhancing AR transcriptional activity and the expression of genes associated with a variety of cancer-associated processes including WNT and MAPK signalling, steroid biosynthesis and gene expression signatures identified in CRPC, amongst others (Sharma et al. 2014). In this study, ETS1p51 overexpression similarly enhanced the androgen-induced regulation of a number of target genes including ZEB1 and TGFβ1 as well as sustaining the upregulation of other factors including SNAIL1 and TWIST1 in DHT-treated LNCaP cells. AR signalling is a central regulator of prostate cancer development and tumour growth, with the maintenance of AR signalling in advanced and castrate-resistant cancers highlighting its importance at all disease stages (Heinlein et al. 2004; Yuan et al. 2014). A large number of AR co-regulators have been identified and characterised, with the critical roles of abnormal AR co-regulator expression in prostate and other cancer types well-established but incompletely described (Heemers et al. 2007; Heinlein et al. 2002). Interactions between abnormally expressed ETS factors including ETS1 and the AR as well as interactions between the AR or ETS1 and common co-regulators are likely to modify AR signalling in prostate tumours, potentially contributing to disease progression. Although additional analyses will be required to characterise regulation of genes including genes encoding EMT mediators in DHT-treated and DHT-treated, ETS1p51 overexpressing prostate cancer cells, findings from this and previous studies indicate that by interacting with the AR and enhancing its transcriptional activity, the elevated levels of ETS1 and other ETS factors in prostate tumours may enhance AR-mediated regulation of target gene expression and accelerate prostate cancer progression and the development of castrate-resistant disease.
7.2 Future Directions

In the present study, ETS1 isoform mRNA and protein levels were characterised in the prostate tumours and adjacent nonmalignant prostate of 45 patients. Identification of elevated ETS1p51 protein expression in prostate tumours was consistent with previous studies using immunohistochemical methods in which it was additionally determined that ETS1 overexpression correlated with disease severity and rapid development of biochemical recurrence (Alipov et al. 2005; Li et al. 2012; Smith et al. 2012). The validation of these collective results using large cohorts of specimens with a range of disease severity, patient treatment and outcomes data will be required in future studies in order that the predictive or prognostic usefulness of ETS1 or ETS1 isoform overexpression in prostate tumours can be determined. A number of ETS factors including ERG, PEA3 and FLI1 are reported to be aberrantly expressed in a high proportion of human prostate tumours and correlation of specific patterns of ETS factor expression (rather than expression of individual ETS factors) with clinical features of the prostate tumours or patient outcomes may yield stronger associations that are more useful clinically.

Mechanisms resulting in the overexpression of ETS1 and other ETS factors including ETS2 and ESE2 have not been characterised but once identified may be able to be targeted therapeutically. According to genomic data available in online datasets (e.g. OncomineTM), regions of chromosome 11 which contain both the ETS1 and FLI1 genes show high levels of methylation in prostate tumours. Specific examination of the methylation status of the ETS1 promoter, intronic/exonic as well as 5’ and 3’ untranslated regions (UTRs) may provide insight into genomic/transcriptional mechanisms of ETS1 regulation that account for the reduced ETS1 mRNA expression detected in this study. ETS1 has a long 3’ UTR which may be targeted by a number of microRNAs including those previously shown to be abnormally up- or down-regulated in human prostate tumours (Pang et al. 2010; Walter et al. 2013). Binding sites of miR200b (Chan et al. 2011), miR1 (Fleming et al. 2013) and miR145 (Zheng et al. 2013) have been identified in the ETS1 3’ UTR and expression of these miRNAs is upregulated (miR200b) or downregulated (miR145, miR1) in prostate tumours, potentially contributing to the altered levels of ETS1 mRNA (Mitchell et al. 2008;
Zaman et al. 2010; Hudson et al. 2012). Characterisation of specific microRNA interactions with ETS1 sequences and correlations between expression of ETS1 mRNA and miRNAs in prostate cancers will identify miRNAs that potentially regulate ETS1 mRNA expression and which may contribute to the development of microRNA targeted therapies (Broderick et al. 2011).

Regulation of ETS1p51 translation and protein stability are not well characterised, however phosphorylation of ETS1 by ERK/MAPK signalling has been reported to regulate its transcriptional activity and may affect protein stability (Callaway et al. 2010). Interaction between ETS factors and MAPK signalling has been identified in several studies, with treatment of cells with the MEK inhibitor, U0126 reducing ETS1 phosphorylation and ETS1-mediated invasiveness (Smith et al. 2012a). Conversely, overexpression of ERG or ETV1 restored expression of a number of RAS/MAPK target genes in U0126-treated RPWE-1 cells suggesting that ETS factor overexpression may be able to compensate for therapeutic downregulation of signalling pathways (Hollenhorst et al. 2011). However, identification of factors that modify ETS1p51 protein translation, turnover or activity, including kinases may indicate the usefulness of newer targeted agents including inhibitors of ERK2 (Ohori et al. 2005), RAF and MEK (Kohno et al. 2006), dual targeting of MEK and ERK (Hatzivassiliou et al. 2012) or PARP1 inhibitors (Brenner et al. 2011) in the management of prostate tumours that overexpress ETS1 or other ETS factors. These agents may be evaluated in cell culture as well as in prostate cancer xenograft models in immunosuppressed mice to determine the effects of inhibitor treatments on the levels, intracellular localisation and transcriptional activity of ETS1, and on tumour growth.

Studies performed in this thesis evaluated ETS1p51 function in the LNCaP cell line in vitro. Additional experiments to confirm these results may be performed using other prostate cancer cell lines that express a range of ETS1 isoforms and different levels of ETS1 expression, including the PC-3, DU145, 22Rv1 and LAPC-4 cell lines (Russell et al. 2003). It will also be important to generate prostate cancer cell lines that stably express low to high ETS1 levels, potentially using viral vectors which may be more easily introduced into cell lines, overcoming the technical problems encountered during this project (John et al. 2008; Stone et al. 2000). Growth of these cell lines in vivo in
nude or SCID mice will enable further evaluation of ETS1 effects on tumour growth, the roles of ETS1 in tumour responses to androgen ablation therapies including progression to castrate-resistant disease and responses to drugs that may modify ETS1 expression or activity, including RAS/MAPK inhibitors. As ETS1p51-induced cell migration and invasion were mediated in part by activation of TGF-β signalling, use of TGF-β pathway antagonists including drugs that target TGF-β mRNA or protein (e.g. Trabedersen, Fresolimumab) (Schlingensiepen et al. 2011; Morris et al. 2014), TGF-β receptor kinases (e.g. LY2109761) (Melisi et al. 2008) and SMADs (e.g. TRX-Sara) (Zhao et al. 2006) will also identify the role of TGF-β signalling in the more aggressive growth characteristics of ETS1-overexpressing tumours. Testing of additional strategies for attenuating the activities of ETS1 and other ETS factors in prostate cancers, including oligonucleotides incorporating EBS sequences to act as decoys (Miwa et al. 2005) may also be tested using these models, evaluating the efficacy of targeting aberrant ETS1 or ETS factor expression in the management of prostate cancer.

Results of this thesis have identified using PCR arrays that in prostate cancer cells, ETS1p51 regulates expression of EMT-associated genes. Future investigation to identify additional ETS1 targets in prostate cancer cells can employ a number of techniques including cDNA microarrays, ChIP-seq or ChIP-Chip using prostate cancer cells which express low or high levels of ETS1. These methods have been used to map DNA binding sites of multiple ETS factors including, ETS1, ELF1 and GABPa in Jurkat cells (Hollenhorst et al. 2009; Hollenhorst et al. 2007), ELK1 and GABPa in HeLa cells (Boros et al. 2009a) and ERG, ETV1, ETV4 and ETV5 in the nonmalignant prostate cell line RWPE-1 (Hollenhorst et al. 2011a). In addition, large scale gene expression screening may be performed using cDNA microarrays which have been previously employed to identify target genes of several ETS factors, including ETS1 in HUVEC (Teruyama et al. 2001) and MCF-7 cells (Jung et al. 2005), and following ChIP-seq for identification of ELK1 targets in the nonmalignant mammary cell line, MCF10A (Odrowaz et al. 2012). These data support enriched ETS factor DNA binding in regulatory regions of genes encoding mediators of cell proliferation, migration, differentiation, angiogenesis, activation of basal transcriptional machinery and T cell activation (Hollenhorst et al. 2011a; Boros et al. 2009a; Hollenhorst et al. 2007; Hollenhorst et al. 2009). Mapping of ETS1p51 DNA binding sites may be performed
using these techniques in prostate cancer cells that overexpress ETS1p51, for example the LNCaP model system developed in this thesis. Comparison of ETS1p51 DNA binding sites and the binding sites of other ETS factors will assist in the identification of common and unique target sequences as well as genes that are regulated by individual or multiple ETS factors and tissue-specific target genes of ETS1 or other ETS factors. Characterisation of ETS1p51-regulated genes in prostate cancer cells may indicate processes other than EMT that are induced or inhibited by ETS1p51 and which facilitate disease progression and the poor outcomes of ETS1-overexpressing tumours.

Although DNA binding sites or ETS1 target genes have not been specifically mapped in prostate cancer cells, in a study investigating AR promoter targets using ChIP-chip analysis of R1881-treated LNCaP cells, AREs were not identified in more than 50% of the androgen-responsive genes, however significant enrichment of EBS sequences and collaboration of ETS1 and the AR in the regulation of subsets of the genes were demonstrated (Massie et al. 2007). In the present study EMT-associated genes regulated following DHT treated of either LNCaP or ETS1p51-overexpressing LNCaP cells were identified using PCR arrays, which suggested enhanced regulation of a proportion of the genes by the combination of DHT treatment and ETS1 overexpression. To validate and extent these studies, bioinformatics analyses may be performed using similar programmes and strategies to those performed to evaluate gene expression following ETS1p51 overexpression (Chapter 5.2.7), with RT-qPCR and western blotting used to confirm regulation of expression of specific candidate target genes, including collaboration of DHT/AR and ETS1p51 overexpression in the regulation of gene expression. Results of the bioinformatics analyses will indicate biological assays (e.g. migration, invasion, adhesion) that may be used to investigate the functional consequences of ETS1p51 overexpression on the DHT responsiveness of prostate cancer cells. Additional experiments comparing gene expression following anti-androgen treatment or androgen deprivation of ETS1p51 overexpressing cells may also elucidate roles of ETS1p51 in the responses of prostate cancer cells to low androgen conditions, including development of castrate resistant disease. In combination with findings of this thesis and previous studies, these investigations will elucidate features of ETS1 function in prostate cancer cells both as a regulator of gene
expression and as a coregulator of AR function, contributing to disease progression and the poor outcomes of ETS1-overexpressing tumours.

7.3 Concluding remarks

This thesis has characterised *ETS1p51* and *ETS1p42* mRNA expression in nonmalignant and malignant prostatic tissues and determined that ETS1p51 is the predominant isoform expressed in the prostate and overexpressed in the majority of prostate tumours. The overexpression of ETS1p51 in LNCaP cells increased expression of genes which encode mediators of cell motility and invasiveness as well as components of the TGF-β signalling pathway, processes associated with the induction of EMT. ETS1p51-overexpression additionally increased the expression of EMT promoting transcription factors and mesenchymal markers, and upregulated phosphorylation of SMAD2, a principal mediator of TGF-β signalling. Increased ETS1p51 levels enhanced LNCaP cell migration and invasion, processes which were partially mediated by ETS1p51-induced activation of TGF-β signalling. These findings indicate that aberrant overexpression of ETS1p51 in prostate cancer cells induces an EMT programme which may promote the rapid progression of disease, leading to the poor prognosis of ETS1-overexpressing prostate tumours.
8.1 References


Chapter 8: References


Finger Protein SIP1 Downregulates E-Cadherin and Induces Invasion." Molecular Cell 7: 1267 - 1278.


and is of strong and independent importance for the progress of prostate cancer." Clinical Cancer Research 13(23): 7003 - 7011.


Chapter 8: References


proliferation of bone marrow-derived adult human mesenchymal stem cells." Genes and Development 20: 666 - 674.


Chapter 8: References

Colorectal Cancer Cells by Oncogenic Epidermal Growth Factor/Ras Signaling and Ets Transcription Factors." DNA and Cell Biology 31(8): 1403 - 1411.


receptor type I and type II dual inhibitor, as a therapeutic approach to suppressing pancreatic cancer metastasis." Molecular Cancer Therapeutics 7(4): 829 - 840.


Epithelium Is Dependent upon the Transcription Factor Ets Variant 5 and Contributes to Blood-Testis Barrier Function." Biology of Reproduction **81**: 871 - 879.


Prostate Cancer Cells is an Important Prognostic Factor for Cancer Progression."
selective ERK inhibitor and structural determination of the inhibitor-ERK2 complex." Biochemical and Biophysical Research Communications 336: 357 - 363.


Chapter 8: References


Chapter 8: References


Chapter 8: References


Chapter 8: References


Appendix 1 – Buffers and Solutions
Appendix 1: Buffers and Solutions

1. **Adenosine 5’ Triphosphate (ATP) (100mM)**

1g ATP
18mL ddH₂O
ATP was dissolved in ddH₂O and 1mL aliquots were stored at -20°C.

2. **Agarose Agar (1% or 2%) with Ethidium Bromide**

3g/6g Agarose (1%/2%)
300mL 1x TAE
8µL Ethidium Bromide
Agarose was dissolved in 1x TAE by heating in a microwave, ethidium bromide (10mg/mL) was added and the solution was stored at room temperature. Prior to use, the mixture was melted in a microwave, cooled to ~70°C, poured into a casting tray and allowed to set at room temperature.

3. **Ammonium Persulphate (APS) (10%)**

3mg Ammonium Persulphate
300µL ddH₂O
Ammonium persulphate was dissolved in ddH₂O and the solution was stored at 4°C for a maximum of one week.

4. **Ampicillin (100mg/mL)**

100mg Ampicillin Powder
1mL ddH₂O
Ampicillin was dissolved in ddH₂O, made up to 1mL and the solution was stored at -20°C.

5. **Bromophenol Blue (10mg/mL)**

100mg Bromophenol Blue
10mL ddH₂O
Bromophenol blue was dissolved in ddH₂O, made up to 10mL and the solution was stored at room temperature.

6. **Calcium Chloride (CaCl₂) (1M)**

203g Calcium Chloride
→1L ddH₂O
Calcium chloride was dissolved in ~800mL ddH₂O and made up to 1L using ddH₂O. The solution was sterilised using a 0.2µM filter and stored at room temperature.

7. **Co-Enzyme A Lithium Salt (100mM)**

100mg Co-Enzyme A Lithium Salt
1.3mL ddH₂O
Co-Enzyme A was dissolved in ddH₂O, the solution was divided into 25µL aliquots stored at -20°C protected from light.

8. **D-Luciferin (100mM)**

25mg D-Luciferin Potassium Salt
785µL ddH₂O
D-Luciferin was dissolved in ddH₂O, the solution was divided into 50µL aliquots and stored at -20°C protected from light.

9. **DEPC-Treated ddH₂O**

1mL Diethylpyrocarbonate (DEPC)
1L ddH₂O
DEPC was added to ddH₂O in a fumehood, the solution was shaken and left overnight to allow the DEPC to evaporate. The solution was autoclaved and stored at room temperature.
10. **DHT (10^{-2} M)**

0.0029g DHT
1mL Ethanol (100%)

DHT was dissolved in ethanol and the solution was diluted with 100% ethanol as required to prepare stock solutions. DHT solutions were stored at -20°C protected from light for a maximum of 6 months and used in cell culture at 1:1000 dilutions (final ethanol concentration = 0.1% v/v).

11. **Dithiothreitol (DTT) (1M)**

1.54g DTT
10mL ddH₂O

DTT was dissolved in ddH₂O, made up to 10mL and stored in 1mL aliquots at -20°C.

12. **dNTP Mix (10mM and 25mM)**

<table>
<thead>
<tr>
<th>dNTP</th>
<th>10mM dNTP</th>
<th>25mM dNTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>dATP</td>
<td>10µL</td>
<td>25µL</td>
</tr>
<tr>
<td>dTTP</td>
<td>10µL</td>
<td>25µL</td>
</tr>
<tr>
<td>dGTP</td>
<td>10µL</td>
<td>25µL</td>
</tr>
<tr>
<td>dCTP</td>
<td>10µL</td>
<td>25µL</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>60µL</td>
<td>-</td>
</tr>
</tbody>
</table>

Reagents were combined and the solution was stored at -20°C.

13. **Doxycycline (1mg/mL)**

0.1g Doxycycline
100mL ddH₂O

Doxycycline powder was dissolved in ddH₂O and the solution was sterilised using a 0.22µM filter, divided into 1mL aliquots and stored at -20°C protected from light.

14. **Ethylenediaminetetraactic Acid (EDTA) pH 8.0 (0.1M)**

100mL 0.5M EDTA pH 8.0\textsuperscript{15}
400mL ddH₂O

0.5M EDTA pH 8.0\textsuperscript{15} was diluted with ~400mL ddH₂O, the pH adjusted with sodium hydroxide, and the solution made up to 500mL, autoclaved and stored at room temperature.

15. **Ethylenediaminetetraactic Acid (EDTA) pH 8.0 (0.5M)**

186.12g EDTA
20g Sodium Hydroxide

EDTA was dissolved in 500mL ddH₂O, the pH was adjusted to 8.0 using sodium hydroxide, the solution made up to 1L with ddH₂O, autoclaved, stored at room temperature and diluted with ddH₂O as required.

16. **Enhanced Chemiluminescence Reagent (ECL)**

Equal quantities of solutions A and B (provided with Amersham ECL™ Prime or Select Western Blotting Detection Reagent kits) were combined immediately prior to use.

17. **Ethanol (EtOH)**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>95%</th>
<th>75%</th>
<th>70%</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% Ethanol</td>
<td>95mL</td>
<td>75mL</td>
<td>70mL</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>5mL</td>
<td>25mL</td>
<td>30mL</td>
</tr>
</tbody>
</table>

EtOH and ddH₂O were combined and the solutions were stored at room temperature.
Appendix 1: Buffers and Solutions

18. Ethidium Bromide (10mg/mL)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethidium Bromide</td>
<td>10mg</td>
<td>1mL ddH₂O</td>
</tr>
</tbody>
</table>

Ethidium bromide was dissolved in ddH₂O and the solution was stored at room temperature protected from light.

19. Glycerol/PIPES Buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂</td>
<td>1M</td>
<td>12mL</td>
</tr>
<tr>
<td>PIPES pH 7.0</td>
<td>0.5M</td>
<td>4mL</td>
</tr>
<tr>
<td>Glycerol</td>
<td>36</td>
<td>30mL</td>
</tr>
<tr>
<td>ddH₂O</td>
<td></td>
<td>138mL</td>
</tr>
</tbody>
</table>

Reagents were combined and the solution sterilised using a 0.2 μM filter then stored at 4°C.

20. Hydrochloric Acid (HCl) (1M)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCl</td>
<td>10M</td>
<td>10mL</td>
</tr>
<tr>
<td>ddH₂O</td>
<td></td>
<td>90mL</td>
</tr>
</tbody>
</table>

HCl was added to ddH₂O in a fume hood and the solution was stored at room temperature.

21. HCl (4mM)/Bovine Serum Albumin (BSA) (1mg/mL)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCl</td>
<td>4mM</td>
<td>40μL</td>
</tr>
<tr>
<td>BSA (40mg/mL)</td>
<td></td>
<td>250μL</td>
</tr>
<tr>
<td>ddH₂O</td>
<td></td>
<td>9.71mL</td>
</tr>
</tbody>
</table>

On the day of use, 5g of BSA was dissolved in 125mL of ddH₂O, 250μL of this mix (40mg/mL) was added to 40μL 1M HCl and made up to 10mL using ddH₂O. The solution was stored at 4°C and filter sterilised immediately prior to use.

22. Isopropyl β-D-1-thiogalactopyranoside (IPTG) (100mM)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPTG</td>
<td>1.2g</td>
<td>1.2g</td>
</tr>
<tr>
<td>ddH₂O</td>
<td></td>
<td>50mL</td>
</tr>
</tbody>
</table>

IPTG powder was dissolved in ddH₂O and the volume adjusted to 50mL. The solution was sterilised using a 0.2μM filter, divided into 1mL aliquots and stored at -20°C.

23. Kanamycin (100mg/mL)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kanamycin Powder</td>
<td>100mg</td>
<td>1mL</td>
</tr>
<tr>
<td>ddH₂O</td>
<td></td>
<td>ddH₂O</td>
</tr>
</tbody>
</table>

Kanamycin powder was dissolved in ddH₂O to a final volume of 1mL and the solution was stored at -20°C.

24. LB Agar

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB Broth</td>
<td>500mL</td>
<td>500mL</td>
</tr>
<tr>
<td>Agar</td>
<td>7.5g</td>
<td>7.5g</td>
</tr>
</tbody>
</table>

Agar was added to LB Broth and the solution was autoclaved and stored at room temperature. Prior to use, the agar was liquefied in a microwave and cooled to ~70°C.

25. LB Agar/Ampicillin (100µg/mL)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB Agar</td>
<td>15mL</td>
<td>15mL</td>
</tr>
<tr>
<td>Ampicillin (100mg/mL)</td>
<td>15µL</td>
<td>15µL</td>
</tr>
</tbody>
</table>

Ampicillin was added to liquefied LB Agar that had been cooled to ~70°C, the solution was poured into a petri dish and allowed to set at room temperature for ~1hr.

26. LB Agar/Ampicillin/IPTG/X-Gal

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPTG (100mM)</td>
<td>100uL</td>
<td>100uL</td>
</tr>
<tr>
<td>X-Gal (50mg/mL)</td>
<td>20uL</td>
<td>20uL</td>
</tr>
</tbody>
</table>

IPTG and X-Gal were spread onto LB Agar/Ampicillin plates and allowed to dry for 45 minutes at 37°C before use.
Appendix 1: Buffers and Solutions

27. LB Agar/Kanamycin

15mL LB Agar\textsuperscript{24}
15µL Kanamycin
(100mg/mL)\textsuperscript{23}

Kanamycin was added to liquefied LB Agar\textsuperscript{24} that had been cooled to ~70°C, the solution was poured into a petri dish and allowed to set at room temperature for ~1hr.

28. LB Broth

10g Bacto-Tryptone
5g Yeast Extract
10g NaCl

Reagents were dissolved in ~900mL ddH\textsubscript{2}O and the pH adjusted to 7.0. The solution was made up to 1L using ddH\textsubscript{2}O, autoclaved and stored at room temperature.

29. LB Broth/Ampicillin (100µg/mL)

10mL LB Broth\textsuperscript{28}
10µL Ampicillin (100mg/mL)\textsuperscript{4}

Ampicillin\textsuperscript{4} was added to LB Broth\textsuperscript{28} and the solution was used immediately.

30. LB Broth/Glycerol (10%)

100µL Glycerol
900µL LB Broth\textsuperscript{28}

LB Broth\textsuperscript{28} and glycerol were combined and the solution was stored at 4°C.

31. LB Broth/Kanamycin (100µg/mL)

10mL LB Broth\textsuperscript{28}
10µL Kanamycin
(100mg/mL)\textsuperscript{23}

Kanamycin\textsuperscript{23} was added to LB Broth\textsuperscript{28} and the solution was used immediately.

32. Loading Dye (6x)

3.5g Sucrose
100µL Bromophenol Blue
(10mg/mL)\textsuperscript{5}
5mL 0.1M EDTA (pH8.0)\textsuperscript{14}

Reagents were combined to a final volume of 5mL and the solution was stored at room temperature.

33. Luciferase Assay Reagent (LARI1)

500µL 1M Tris HCL pH7.8\textsuperscript{63}
300µL 0.5M MgSO\textsubscript{4}\textsuperscript{34}
2µL 0.5M EDTA pH8.0\textsuperscript{15}
25µL 100mM Co-enzyme A Lithium Salt\textsuperscript{2}
50µL 100mM D-Luciferin\textsuperscript{8}
50µL 100mM ATP\textsuperscript{1}
500µL Triton X-100 (10% v/v)\textsuperscript{71}
330µL 1M DTT\textsuperscript{11}

Reagents were combined and the final volume made to 10mL with ddH\textsubscript{2}O. Solution was made fresh prior to use and stored on ice protected from light until required.

34. Magnesium Sulphate (MgSO\textsubscript{4}) (1M)

24g MgSO\textsubscript{4}.7H\textsubscript{2}O
1L ddH\textsubscript{2}O

MgSO\textsubscript{4}.7H\textsubscript{2}O was dissolved in ddH\textsubscript{2}O to a final volume of 1L, the solution was autoclaved and stored at room temperature.
Appendix 1: Buffers and Solutions

35. PCR Buffer (5x)

5mL Taq 10X PCR Buffer (without MgCl₂)
(supplied with Platinum Taq DNA Polymerase)
200µL 25mM dNTP
4.8mL ddH₂O
Reagents were combined and the solution stored at -20°C in 1mL aliquots.

36. PIPES pH7.0 (0.5M)

15.15g PIPES
100mL ddH₂O
PIPES was dissolved in ~80mL ddH₂O, the pH adjusted to 7.0 using 1M NaOH and the volume made up to 100mL with ddH₂O. The solution was sterilised by passing through a 0.2µM filter and stored at room temperature.

37. Phosphate Buffered Saline pH 7.4 (PBS) (1x)

0.2g Potassium Chloride
0.24g Potassium Dihydrogen Orthophosphate
1.44g Disodium Hydrogen Orthophosphate
8g Sodium Chloride
→1L ddH₂O
Reagents were dissolved in ~800mL ddH₂O, the pH was adjusted to 7.4 using 1M HCl or 10M NaOH, the solution made up to 1L with ddH₂O, autoclaved and stored at room temperature.

38. Polyacrylamide Separating Gel (12%)

6.53mL ddH₂O
3.65mL 1M Tris HCl pH8.8
4.50mL 40% Acrylamide/ Bis-Acrylamide (37.5:1)
75µL 20% SDS
75µL 10% APS
7.5µL TEMED
Reagents were mixed in the order listed, the solution was poured immediately between prepared glass plates in a casting apparatus, overlayed with ddH₂O and incubated at room temperature for ~45 minutes to polymerise.

39. Polyacrylamide Stacking Gel (4%)

7.70mL ddH₂O
1.25mL 1M Tris HCL pH6.8
1mL 40% Acrylamide/ Bis-Acrylamide (37.5:1)
50µL 20% SDS
50µL 10% APS
10µL TEMED
Reagents were mixed in the order listed, the solution was poured immediately on top of the polymerised separating gel, well combs were inserted and the apparatus incubated at room temperature for ~30 minutes to polymerise.

40. Potassium Acetate (5M)

24.5g Potassium Acetate
50mL ddH₂O
Potassium acetate was dissolved in ddH₂O to a final volume of 50mL, the solution was autoclaved and stored at room temperature.
Appendix 1: Buffers and Solutions

41. **RNase A (10mg/mL)**

- 100mg RNase A
- 100µL 1M Tris pH 7.4
- 37.5µl 4M Sodium Chloride
- →10mL ddH₂O

Reagents were combined, the volume was made to 10mL using ddH₂O, heated at 100°C for 15 minutes, cooled to room temperature, divided into 1mL aliquots and stored at -20°C.

42. **RNase H (10mg/mL)**

- 10mg RNase H
- 10µL 1M Tris HCL pH 7.5
- 3.75µL 4M Sodium Chloride
- →10mL ddH₂O

Reagents were combined and the final volume adjusted to 1mL using ddH₂O. The solution was activated by heating to 95°C for 15 minutes and stored at -20°C.

43. **RPMI-1640**

- 104.5g RPMI 1640 Medium with L-Glutamine
- 2g Sodium Hydrogen Carbonate
- 1L ddH₂O

RPMI 1640 powder and sodium hydrogen carbonate were dissolved in ddH₂O, the solution was filtered through a 0.22μm filter into sterile bottles and stored at 4°C.

44. **RPMI 1640/PS (RPMI/PS)**

- 990mL RPMI 1640 Medium with L-Glutamine
- 10mL Penicillin-Streptomycin 10001U/mL (1mg/mL)

Reagents were combined and the solution was stored at 4°C.

45. **RPMI 1640/5% Charcoal Stripped Serum (CSS)/PS (RPMI/5%CSS/PS)**

- 190mL RPMI/PS
- 10mL CSS

Reagents were combined and the solution was stored at 4°C.

46. **RPMI 1640/10% Foetal Calf Serum/PS (Stock Medium) (RPMI/10%FCS/PS)**

- 450mL RPMI/PS
- 50mL FCS

Reagents were combined and the solution was stored at 4°C.

47. **RPMI 1640/10% Foetal Calf Serum/PS/10% Dimethyl sulphonyde (DMSO) (RPMI/10% FCS/PS/10% DMSO)**

- 9mL RPMI/10% FCS/PS
- 1mL DMSO

Reagents were combined and the solution was stored at 4°C protected from light.

48. **Running Buffer (10x) (Western Blotting)**

- 30g Tris
- 144g Glycine
- 10g SDS
- 1L ddH₂O

Tris and glycine were dissolved in ~800mL ddH₂O, SDS was added in a fume hood, the solution was made up to 1L with ddH₂O and stored at room temperature. 1x Running Buffer was prepared with ddH₂O as required.
Appendix 1: Buffers and Solutions

49. SB431542 (20mM)
10mg SB431542
1.2mL DMSO
SB431542 powder was warmed to room temperature and dissolved in DMSO. Mixture was aliquoted and stored at -20°C.

50. Sodium Acetate (3M) (pH4.6/5.0)
49.2g Sodium Acetate
200mL ddH₂O
Sodium acetate was dissolved in ~160mL ddH₂O and the pH adjusted to 4.6/5.0 using glacial acetic acid. The volume was adjusted to 200mL with ddH₂O and the solution autoclaved and stored at room temperature.

51. Sodium Chloride (NaCl) (4M)
23.38g Sodium Chloride
100mL ddH₂O
NaCl was dissolved in ~80mL ddH₂O and the solution made up to 100mL, autoclaved and stored at room temperature.

52. Sodium Dodecyl Sulphate (SDS) (20%)
10g SDS
50mL ddH₂O
SDS was dissolved in ~40mL ddH₂O and the solution was made up to 50mL and stored at room temperature.

53. Sodium Hydroxide (NaOH) (10M)
20g Sodium Hydroxide
50mL ddH₂O
NaOH was dissolved in ~40mL ddH₂O and the solution was made up to 50mL, autoclaved and stored at room temperature.

54. Solution I (Plasmid Isolation)
0.9g Glucose
2.5mL 1M Tris pH 8.8
2mL 0.5M EDTA pH8.0
95.5mL ddH₂O
Reagents were combined and the solution autoclaved and stored at 4°C.

55. Solution II (Plasmid Isolation)
250µL 20% SDS
100µL 10M Sodium Hydroxide
4.65mL ddH₂O
On the day required, reagents were combined and the solution was stored at room temperature until use.

56. Solution III (Plasmid Isolation)
60mL 5M Potassium Acetate
11.5mL Glacial Acetic Acid
28.5mL ddH₂O
Reagents were combined, the pH adjusted to 8.0 with 10M NaOH and the solution autoclaved and stored at 4°C.

57. Sucrose (20%)
2g Sucrose
10mL ddH₂O
Sucrose was dissolved in ddH₂O to a final volume of 10mL and the solution was stored at room temperature.
Appendix 1: Buffers and Solutions

58. SYBR Green qPCR Mastermix
2.5µL  10pmol/µL Sense Primer
2.5µL  10pmol/µL Antisense Primer
2.5µL  MgCl₂ (25mM)*
0.05µL  Platinum Taq DNA Polymerase
5µL  5x PCR Buffer³⁵
1.25µL  SYBR Green (0.25x)
9.2µL  ddH₂O
Reagents were combined as required in a light proof tube immediately prior to use. (*Supplied with Platinum Taq DNA Polymerase)

59. TAE (50x)
242g  Tris
57.1mL  Glacial Acetic Acid
100mL  0.5M EDTA pH 8.0¹⁵
→1L  ddH₂O
Tris was dissolved in ~600mL ddH₂O, glacial acetic acid and 0.5M ETDA pH 8.0¹⁵ were added, the volume was adjusted to 1L using ddH₂O and the solution was autoclaved and stored at room temperature. 1x TAE was prepared as required.

60. TGFβ1 (20μg/mL)
2μg  TGFβ1
100µL  4mM HCL/1mg/mL BSA²¹
Reagents were combined, the solution was divided into 20μL aliquots and stored at -20°C.

61. Toluidine Blue (1%)  
1g  Toluidine Blue O
1g  Borax
100mL  ddH₂O
Borax was dissolved in ~80mL ddH₂O, Toluidine Blue O was added, the volume adjusted to 100mL with ddH₂O, the solution stored at room temperature and filter sterilised on the day of use.

62. Transfer Buffer (Western Blotting)
14.4g  Glycine
3.03g  Tris
200mL  Methanol
800mL  ddH₂O
Glycine and Tris were dissolved in ~500mL ddH₂O, the volume was made up to 800mL with ddH₂O, methanol was added and the solution was stored at -20°C. Solution was prepared on the day of use.

63. Tris HCl (pH 6.8, 7.4, 7.5, 7.8, 8.0, 8.8) (1M)
60.55g  Tris
1L  ddH₂O
Tris was dissolved in ~400mL of ddH₂O and the pH adjusted as required with 1M HCl²⁰. The solution was made up to 1L with ddH₂O, autoclaved and stored at room temperature.

64. Tris Buffered Saline (TBS)
18.75mL  4M Sodium Chloride⁵¹
25mL  1M Tris HCL pH 7.5⁶³
→500mL  ddH₂O
NaCl and Tris were made up to 500mL with ddH₂O and the solution was stored at room temperature.

65. TBS/3% Blotto
10mL  TBS⁶⁴
0.3g  Skim Milk Powder
Skim milk powder was dissolved in TBS⁶⁴ and the solution stored on a horizontal shaker until use. Solution was prepared on day of use.

66. Tris Buffered Saline/(0.2%) Tween 20 (TBST)
500mL  TBS⁶⁴
1mL  Tween 20
Reagents were combined and the solution was stored at room temperature.
Appendix 1: Buffers and Solutions

67. TBST/1% Blotto

10mL TBST\textsuperscript{66}
0.1g Skim Milk Powder

Skim milk powder was dissolved in TBST\textsuperscript{66} and the solution stored on a horizontal shaker until use. Solution was prepared on day of use.

68. TBST/3% Blotto

10mL TBST\textsuperscript{66}
0.3g Skim Milk Powder

Skim milk powder was dissolved in TBST\textsuperscript{66} and the solution stored on a horizontal shaker until use. Solution was prepared on day of use.

69. TBST/5% Blotto

0.5g Skim Milk Powder
10mL TBST\textsuperscript{66}

Skim milk powder was dissolved in TBST\textsuperscript{66} and the solution stored on a horizontal shaker until use. Solution was prepared on day of use.

70. TBST/5% Bovine Serum Albumin (BSA) (TBST/5% BSA)

10mL TBST\textsuperscript{66}
0.5g BSA

Bovine serum albumin (BSA) was dissolved in TBST\textsuperscript{66} and the solution stored on a horizontal shaker until use. Solution was prepared on day of use.

71. Triton X-100 (10% v/v)

5mL Triton X-100
45mL ddH\textsubscript{2}O

Triton X-100 was combined with ddH\textsubscript{2}O, using a magnetic stirrer, and the solution was stored at 4°C protected from light.

72. Western Loading Dye (10x)

12.5mL Glycerol
2.5mL 1M Tris HCl pH 6.8\textsuperscript{63}
2.5mL 20% SDS\textsuperscript{52}
2.5mL β-Mercaptoethanol
0.25g Bromophenol Blue
20.25mL ddH\textsubscript{2}O

Reagents were combined and the solution was divided into 1mL aliquots and stored at -20°C.

73. Whole Cell Lysis Buffer

3mL ddH\textsubscript{2}O
5mL 20% Sucrose\textsuperscript{57}
1mL 20% SDS\textsuperscript{52}
0.5mL 1M Tris HCl pH 6.8\textsuperscript{63}
0.5mL β-Mercaptoethanol

Reagents were combined in a fume hood and the solution was wrapped in aluminium foil and stored in a fume hood for a maximum of 6 weeks.

74. X-gal (50mg/mL)

0.5g X-gal
10mL Dimethylformamide

X-gal powder was dissolved in dimethylformamide, the solution aliquoted and stored at -20°C.
Appendix 2 – Primer Sequences
# Appendix 2: Primer Sequences

## Table A2.1: Gene expression primers

<table>
<thead>
<tr>
<th>Gene (Isoform)</th>
<th>Oligonucleotide (5’ → 3’)</th>
<th>Amplicon length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Androgen Receptor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AR-S</td>
<td>CCTGGCTTCCCGCAACTTACAC</td>
<td>168bp</td>
</tr>
<tr>
<td>AR-AS</td>
<td>GGACTTGTGCATGCGGTACTC</td>
<td></td>
</tr>
<tr>
<td>ETS1 (p51)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ETS1p51-S</td>
<td>AGACCCTCTCCAGACAGAC</td>
<td>144bp</td>
</tr>
<tr>
<td>ETS1p51-AS</td>
<td>GCAGGCTTGGAAAGATGACTG</td>
<td></td>
</tr>
<tr>
<td>ETS1 (p42)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ETS1p42-S</td>
<td>CATCAGCTCGGAAGAGCTCC</td>
<td>192bp</td>
</tr>
<tr>
<td>ETS1p42-AS</td>
<td>TTGGTCCACTGCCCACGAC</td>
<td></td>
</tr>
<tr>
<td>ETS1 (p27)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ETS1p27-S</td>
<td>CCTCCCCCGGTAAGCTCGG</td>
<td>282bp</td>
</tr>
<tr>
<td>ETS1p27-AS</td>
<td>TTGGTCCACTGCTGTGTAG</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH-S</td>
<td>GTGAAGGTCGGAGTCAAACG</td>
<td>112bp</td>
</tr>
<tr>
<td>GAPDH-AS</td>
<td>TGAGGTCAATGAAAGGTC</td>
<td></td>
</tr>
<tr>
<td>NODAL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NODAL-S</td>
<td>TGTGGGGAAGGAGGTTTCATC</td>
<td>93bp</td>
</tr>
<tr>
<td>NODAL-AS</td>
<td>GCACAACAAGTGGAAGGGAC</td>
<td></td>
</tr>
<tr>
<td>SLUG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLUG-S</td>
<td>AAGCATTTTCAACGCTCCAAAA</td>
<td>118bp</td>
</tr>
<tr>
<td>SLUG-AS</td>
<td>GGATCTCTGGTTGTGTATGACA</td>
<td></td>
</tr>
<tr>
<td>SNAIL1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SNAIL1-S</td>
<td>TCGGAAGCCTAACTACAGCGA</td>
<td>140bp</td>
</tr>
<tr>
<td>SNAIL1-AS</td>
<td>AGATGAGCATTGGCAGCGA</td>
<td></td>
</tr>
<tr>
<td>SMAD2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SMAD2-S</td>
<td>GGAGCAGAATACCGAAGGCA</td>
<td>128bp</td>
</tr>
<tr>
<td>SMAD2-AS</td>
<td>CTTGAGCAACGCAGCTGAAGG</td>
<td></td>
</tr>
<tr>
<td>TGFβ1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGFβ1-S</td>
<td>GCGTGCTAATGGTGGAAC</td>
<td>276bp</td>
</tr>
<tr>
<td>TGFβ1-AS</td>
<td>CGGTGACATCAAAAGATAACCAC</td>
<td></td>
</tr>
</tbody>
</table>
Appendix 2: Primer Sequences

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>TWIST1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TWIST1-S</td>
<td>ATGTCCGCGTCCCACTAGCA</td>
<td>250bp</td>
</tr>
<tr>
<td>TWIST1-AS</td>
<td>TCTGGGAATCAGTGTCACAG</td>
<td></td>
</tr>
</tbody>
</table>

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ZEB1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZEB1-S</td>
<td>GCACCTGAAGAGGCCAGAG</td>
<td>72bp</td>
</tr>
<tr>
<td>ZEB1-AS</td>
<td>TGCACTGGGTTGCCATTTT</td>
<td></td>
</tr>
</tbody>
</table>

Table A2.2: ETS1p51 cloning primers

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ETS1p51 (w/SAL1 RE site)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ETS1SALIS</td>
<td>GTCGACCACCTCCTGGCACCAGAG</td>
<td></td>
</tr>
<tr>
<td>ETS1SAL1AS</td>
<td>GTCGACTCACTCCTGGGCATCTGGCTTG</td>
<td></td>
</tr>
</tbody>
</table>

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ETS1p51 (w/MLU1 RE site)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ETS1MLU1S</td>
<td>ACGCGTCACTCCTGGCACCAGAG</td>
<td></td>
</tr>
<tr>
<td>ETS1MLU1AS</td>
<td>ACGCGTTCACCTGTCGGCATCTGGCTTG</td>
<td></td>
</tr>
</tbody>
</table>
Appendix 3 – Plasmid Maps
Figure A3.1: Map of the pCMV-Tet3G plasmid (Clontech, USA). The vector constitutively expresses Tet-On 3G, a tetracycline-controlled transactivator that is highly active in the presence of doxycycline. The pCMV-Tet3G plasmid is required during the first stage of stable transfection using the Tet-On 3G Inducible kit to generate stable pCMV-Tet3G transfectants.
Appendix 3: Plasmid Maps

Figure A3.2: Map of the pcDNA3.1 (+) plasmid (Life Technologies, Australia). This plasmid (containing no insert) was used as a control for transfection studies.
Figure A3.3: Map of the pcDNA3.1/V5-His-TOPO expression vector (Life Technologies, Australia). This plasmid containing the ETS1p51 coding sequence cloned using the HindIII and Apal sites (Preece, 2009) was used as the template to PCR-amplify and clone the ETS1p51 coding sequence into the pEGFP-C2 or pTRE3G-IRES expression vectors.
Figure A3.4: Map of the pEGFP-C2 expression vector (Clontech, USA). The expression vector contains a constitutive pCMV promoter upstream of the EGFP (enhanced green fluorescent protein) and was used to generate pEGFP-C2-ETS1p51 that encoded ETS1p51 with a 5’ GFP tag or pEGFP-C2-ETS1p42 that encoded ETS1p42 with a 5’ GFP tag (Ms Ebony Rouse).
Figure A3.5: Map of the pGEM-T Easy Vector (Promega, Australia). The vector contains overhanging 5’ T cloning sites to insert PCR-amplified DNA fragments that contain 3’ A overhangs.
Figure A3.6: Map of the pTRE3G-IRES plasmid (Clontech, USA). The vector contains a Tet inducible promoter pTRE3G upstream of the MCS. Addition of doxycycline allows the Tet-On 3G to bind the pTRE3G promoter thereby inducing highly specific expression of the gene of interest cloned into the MCS. The pTRE3G-IRES plasmid was used to generate ETS1p51 overexpressing LNCaP cells.
Figure A3.7: Map of the pTRE3G-Luc plasmid (Clontech, USA). The vector contains a Tet inducible pTRE3G promoter upstream of firefly luciferase. The pTRE3G-Luc plasmid was used to transiently transfect LNCaP cells that had been stably transfected with pCMV-Tet3G to test the doxycycline inducibility of Tet-On 3G expression.
Appendix 4 – Prostate Specimens (Raw Data)
### Table A4.1: Prostate specimen demographics (and mRNA expression data)

<table>
<thead>
<tr>
<th>Sample Pair</th>
<th>Sample</th>
<th>Weight (mg)</th>
<th>Sample Type</th>
<th>Gleason Score</th>
<th>ETS1p51 Ave Ct (value)</th>
<th>ETS1p51 Ave Melt (Tm)</th>
<th>ETS1p42 Ave Ct (value)</th>
<th>ETS1p42 Ave Melt (Tm)</th>
<th>GAPDH Ave Ct (value)</th>
<th>GAPDH Ave Melt (Tm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AA</td>
<td>240</td>
<td>Nonmalignant</td>
<td>7</td>
<td>27.43</td>
<td>84</td>
<td>29.74</td>
<td>84.95</td>
<td>18.46</td>
<td>82.78</td>
</tr>
<tr>
<td>1</td>
<td>AB</td>
<td>190</td>
<td>Tumour</td>
<td>7</td>
<td>30</td>
<td>84.47</td>
<td>40</td>
<td>-</td>
<td>22.83</td>
<td>82.51</td>
</tr>
<tr>
<td>2</td>
<td>AC</td>
<td>90</td>
<td>Nonmalignant</td>
<td>7</td>
<td>30</td>
<td>84.37</td>
<td>40</td>
<td>-</td>
<td>25.17</td>
<td>82.5</td>
</tr>
<tr>
<td>2</td>
<td>AD</td>
<td>110</td>
<td>Tumour</td>
<td>7</td>
<td>29.71</td>
<td>84.35</td>
<td>40</td>
<td>-</td>
<td>21.73</td>
<td>82.56</td>
</tr>
<tr>
<td>3</td>
<td>AE</td>
<td>140</td>
<td>Nonmalignant</td>
<td>8</td>
<td>30</td>
<td>84.38</td>
<td>40</td>
<td>-</td>
<td>24.72</td>
<td>82.35</td>
</tr>
<tr>
<td>3</td>
<td>AF</td>
<td>170</td>
<td>Tumour</td>
<td>8</td>
<td>26.14</td>
<td>84.22</td>
<td>30</td>
<td>85</td>
<td>16.81</td>
<td>82.51</td>
</tr>
<tr>
<td>4</td>
<td>AG</td>
<td>150</td>
<td>Nonmalignant</td>
<td>7</td>
<td>28.45</td>
<td>84.44</td>
<td>30</td>
<td>84.81</td>
<td>21.87</td>
<td>82.52</td>
</tr>
<tr>
<td>4</td>
<td>AH</td>
<td>150</td>
<td>Tumour</td>
<td>7</td>
<td>23.77</td>
<td>84.22</td>
<td>31.65</td>
<td>84.99</td>
<td>16.06</td>
<td>82.56</td>
</tr>
<tr>
<td>5</td>
<td>AI</td>
<td>350</td>
<td>Nonmalignant</td>
<td>9</td>
<td>30</td>
<td>84.51</td>
<td>40</td>
<td>-</td>
<td>21.50</td>
<td>82.69</td>
</tr>
<tr>
<td>5</td>
<td>AJ</td>
<td>150</td>
<td>Tumour</td>
<td>9</td>
<td>29.01</td>
<td>84.51</td>
<td>30</td>
<td>84.82</td>
<td>19.65</td>
<td>82.68</td>
</tr>
<tr>
<td>6</td>
<td>AM</td>
<td>100</td>
<td>Nonmalignant</td>
<td>9</td>
<td>28.87</td>
<td>84.42</td>
<td>40</td>
<td>-</td>
<td>21.80</td>
<td>82.68</td>
</tr>
<tr>
<td>6</td>
<td>AN</td>
<td>180</td>
<td>Tumour</td>
<td>9</td>
<td>29.70</td>
<td>84.38</td>
<td>30</td>
<td>84.81</td>
<td>21.38</td>
<td>82.75</td>
</tr>
<tr>
<td>7</td>
<td>AO</td>
<td>190</td>
<td>Nonmalignant</td>
<td>8</td>
<td>26.23</td>
<td>84.45</td>
<td>31.55</td>
<td>85.15</td>
<td>19.3</td>
<td>82.68</td>
</tr>
<tr>
<td>7</td>
<td>AP</td>
<td>210</td>
<td>Tumour</td>
<td>8</td>
<td>27.42</td>
<td>84.47</td>
<td>31.69</td>
<td>85.55</td>
<td>19.44</td>
<td>82.63</td>
</tr>
<tr>
<td>8</td>
<td>AQ</td>
<td>130</td>
<td>Nonmalignant</td>
<td>7</td>
<td>25.56</td>
<td>84.37</td>
<td>32.33</td>
<td>85.07</td>
<td>18.43</td>
<td>82.45</td>
</tr>
<tr>
<td>8</td>
<td>AR</td>
<td>160</td>
<td>Tumour</td>
<td>7</td>
<td>26.6</td>
<td>84.47</td>
<td>31.05</td>
<td>84.92</td>
<td>18.66</td>
<td>82.68</td>
</tr>
<tr>
<td>9</td>
<td>AS</td>
<td>120</td>
<td>Nonmalignant</td>
<td>7</td>
<td>25.92</td>
<td>84</td>
<td>30.58</td>
<td>85.28</td>
<td>18.1</td>
<td>82.58</td>
</tr>
<tr>
<td>9</td>
<td>AT</td>
<td>100</td>
<td>Tumour</td>
<td>7</td>
<td>25.48</td>
<td>84.33</td>
<td>32.13</td>
<td>85</td>
<td>18.76</td>
<td>82.58</td>
</tr>
<tr>
<td>10</td>
<td>AU</td>
<td>110</td>
<td>Nonmalignant</td>
<td>7</td>
<td>27.40</td>
<td>84.62</td>
<td>30</td>
<td>84.68</td>
<td>20.83</td>
<td>82.43</td>
</tr>
<tr>
<td>10</td>
<td>AV</td>
<td>150</td>
<td>Tumour</td>
<td>7</td>
<td>27.43</td>
<td>84</td>
<td>31.49</td>
<td>84.92</td>
<td>16.46</td>
<td>82.5</td>
</tr>
<tr>
<td>11</td>
<td>AW</td>
<td>120</td>
<td>Nonmalignant</td>
<td>7</td>
<td>27.57</td>
<td>84.47</td>
<td>32.78</td>
<td>85.06</td>
<td>20.32</td>
<td>82.7</td>
</tr>
<tr>
<td>11</td>
<td>AX</td>
<td>110</td>
<td>Tumour</td>
<td>7</td>
<td>28</td>
<td>84</td>
<td>32.99</td>
<td>85.15</td>
<td>19.7</td>
<td>82.75</td>
</tr>
<tr>
<td>12</td>
<td>AY</td>
<td>170</td>
<td>Nonmalignant</td>
<td>9</td>
<td>26.48</td>
<td>84</td>
<td>30</td>
<td>84.81</td>
<td>20.86</td>
<td>82.75</td>
</tr>
</tbody>
</table>
## Appendix 4: Prostate Specimens – Raw Data

<p>| | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>AZ</td>
<td>120</td>
<td>Tumour</td>
<td>9</td>
<td>26.36</td>
<td>84.26</td>
</tr>
<tr>
<td>13</td>
<td>B1</td>
<td>260</td>
<td>Nonmalignant</td>
<td>7</td>
<td>30</td>
<td>84.51</td>
</tr>
<tr>
<td>13</td>
<td>B2</td>
<td>220</td>
<td>Tumour</td>
<td>7</td>
<td>30</td>
<td>84.53</td>
</tr>
<tr>
<td>14</td>
<td>7K</td>
<td>290</td>
<td>Nonmalignant</td>
<td>9</td>
<td>24.98</td>
<td>84.36</td>
</tr>
<tr>
<td>14</td>
<td>7L</td>
<td>190</td>
<td>Tumour</td>
<td>9</td>
<td>24.56</td>
<td>84.55</td>
</tr>
<tr>
<td>15</td>
<td>7M</td>
<td>620</td>
<td>Nonmalignant</td>
<td>8</td>
<td>22.68</td>
<td>84.15</td>
</tr>
<tr>
<td>15</td>
<td>7N</td>
<td>230</td>
<td>Tumour</td>
<td>8</td>
<td>28.39</td>
<td>84.19</td>
</tr>
<tr>
<td>16</td>
<td>7O</td>
<td>450</td>
<td>Nonmalignant</td>
<td>7</td>
<td>23.09</td>
<td>84.39</td>
</tr>
<tr>
<td>16</td>
<td>7P</td>
<td>250</td>
<td>Tumour</td>
<td>7</td>
<td>28.39</td>
<td>84</td>
</tr>
<tr>
<td>17</td>
<td>7Q</td>
<td>250</td>
<td>Nonmalignant</td>
<td>7</td>
<td>25.76</td>
<td>84</td>
</tr>
<tr>
<td>17</td>
<td>7R</td>
<td>330</td>
<td>Tumour</td>
<td>7</td>
<td>26.45</td>
<td>84</td>
</tr>
<tr>
<td>18</td>
<td>7U</td>
<td>220</td>
<td>Nonmalignant</td>
<td>7</td>
<td>24.78</td>
<td>83.7</td>
</tr>
<tr>
<td>18</td>
<td>7V</td>
<td>180</td>
<td>Tumour</td>
<td>7</td>
<td>23.8</td>
<td>84</td>
</tr>
<tr>
<td>19</td>
<td>7E</td>
<td>280</td>
<td>Nonmalignant</td>
<td>9</td>
<td>23.57</td>
<td>84.35</td>
</tr>
<tr>
<td>19</td>
<td>7F</td>
<td>100</td>
<td>Tumour</td>
<td>9</td>
<td>27.89</td>
<td>84.47</td>
</tr>
<tr>
<td>20</td>
<td>7I</td>
<td>170</td>
<td>Nonmalignant</td>
<td>7</td>
<td>26.75</td>
<td>84.39</td>
</tr>
<tr>
<td>20</td>
<td>7J</td>
<td>190</td>
<td>Tumour</td>
<td>7</td>
<td>26.91</td>
<td>84</td>
</tr>
<tr>
<td>21</td>
<td>7G</td>
<td>490</td>
<td>Nonmalignant</td>
<td>7</td>
<td>25.63</td>
<td>84.29</td>
</tr>
<tr>
<td>21</td>
<td>7H</td>
<td>350</td>
<td>Tumour</td>
<td>7</td>
<td>27.43</td>
<td>84.52</td>
</tr>
<tr>
<td>22</td>
<td>7S</td>
<td>240</td>
<td>Nonmalignant</td>
<td>9</td>
<td>23.41</td>
<td>84.29</td>
</tr>
<tr>
<td>22</td>
<td>7T</td>
<td>220</td>
<td>Tumour</td>
<td>9</td>
<td>25.2</td>
<td>84.27</td>
</tr>
<tr>
<td>23</td>
<td>7W</td>
<td>150</td>
<td>Nonmalignant</td>
<td>7</td>
<td>30</td>
<td>84.51</td>
</tr>
<tr>
<td>23</td>
<td>7X</td>
<td>250</td>
<td>Tumour</td>
<td>7</td>
<td>26.86</td>
<td>84</td>
</tr>
<tr>
<td>24</td>
<td>BA</td>
<td>170</td>
<td>Nonmalignant</td>
<td>7</td>
<td>25.64</td>
<td>84.45</td>
</tr>
<tr>
<td>24</td>
<td>BB</td>
<td>140</td>
<td>Tumour</td>
<td>7</td>
<td>26.76</td>
<td>84.3</td>
</tr>
<tr>
<td>25</td>
<td>BC</td>
<td>200</td>
<td>Nonmalignant</td>
<td>7</td>
<td>26.73</td>
<td>84.24</td>
</tr>
<tr>
<td>25</td>
<td>9I</td>
<td>240</td>
<td>Tumour</td>
<td>7</td>
<td>28.71</td>
<td>84.32</td>
</tr>
<tr>
<td>No.</td>
<td>Code</td>
<td>Value 1</td>
<td>Value 2</td>
<td>Value 3</td>
<td>Value 4</td>
<td>Value 5</td>
</tr>
<tr>
<td>-----</td>
<td>------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>26</td>
<td>92</td>
<td>290</td>
<td>Nonmalignant</td>
<td>5</td>
<td>25.98</td>
<td>84.39</td>
</tr>
<tr>
<td>26</td>
<td>93</td>
<td>260</td>
<td>Tumour</td>
<td>5</td>
<td>27.28</td>
<td>84.41</td>
</tr>
<tr>
<td>27</td>
<td>94</td>
<td>240</td>
<td>Nonmalignant</td>
<td>7</td>
<td>25.57</td>
<td>84.44</td>
</tr>
<tr>
<td>27</td>
<td>95</td>
<td>180</td>
<td>Tumour</td>
<td>7</td>
<td>27.57</td>
<td>84.38</td>
</tr>
<tr>
<td>28</td>
<td>96</td>
<td>260</td>
<td>Nonmalignant</td>
<td>7</td>
<td>24.66</td>
<td>84.51</td>
</tr>
<tr>
<td>28</td>
<td>97</td>
<td>120</td>
<td>Tumour</td>
<td>7</td>
<td>25.96</td>
<td>84.54</td>
</tr>
<tr>
<td>29</td>
<td>98</td>
<td>310</td>
<td>Nonmalignant</td>
<td>7</td>
<td>29.32</td>
<td>84.58</td>
</tr>
<tr>
<td>29</td>
<td>99</td>
<td>180</td>
<td>Tumour</td>
<td>7</td>
<td>28.22</td>
<td>84.57</td>
</tr>
<tr>
<td>30</td>
<td>9A</td>
<td>290</td>
<td>Nonmalignant</td>
<td>7</td>
<td>27.9</td>
<td>84.31</td>
</tr>
<tr>
<td>30</td>
<td>9B</td>
<td>130</td>
<td>Tumour</td>
<td>7</td>
<td>27.88</td>
<td>84.3</td>
</tr>
<tr>
<td>31</td>
<td>9C</td>
<td>190</td>
<td>Nonmalignant</td>
<td>7</td>
<td>26.2</td>
<td>84.22</td>
</tr>
<tr>
<td>31</td>
<td>9D</td>
<td>140</td>
<td>Tumour</td>
<td>7</td>
<td>26.66</td>
<td>84.43</td>
</tr>
<tr>
<td>32</td>
<td>9E</td>
<td>170</td>
<td>Nonmalignant</td>
<td>7</td>
<td>25.00</td>
<td>84.35</td>
</tr>
<tr>
<td>32</td>
<td>9F</td>
<td>180</td>
<td>Tumour</td>
<td>7</td>
<td>28.98</td>
<td>84.33</td>
</tr>
<tr>
<td>33</td>
<td>9G</td>
<td>160</td>
<td>Nonmalignant</td>
<td>7</td>
<td>27.44</td>
<td>84.4</td>
</tr>
<tr>
<td>33</td>
<td>9H</td>
<td>140</td>
<td>Tumour</td>
<td>7</td>
<td>29.48</td>
<td>84.42</td>
</tr>
<tr>
<td>34</td>
<td>9K</td>
<td>120</td>
<td>Nonmalignant</td>
<td>7</td>
<td>27.2</td>
<td>84.4</td>
</tr>
<tr>
<td>34</td>
<td>9L</td>
<td>190</td>
<td>Tumour</td>
<td>7</td>
<td>25.35</td>
<td>84.49</td>
</tr>
<tr>
<td>35</td>
<td>9M</td>
<td>130</td>
<td>Nonmalignant</td>
<td>7</td>
<td>26.79</td>
<td>84.55</td>
</tr>
<tr>
<td>35</td>
<td>9N</td>
<td>180</td>
<td>Tumour</td>
<td>7</td>
<td>26.55</td>
<td>84.62</td>
</tr>
<tr>
<td>36</td>
<td>9O</td>
<td>110</td>
<td>Nonmalignant</td>
<td>7</td>
<td>24.48</td>
<td>84.32</td>
</tr>
<tr>
<td>36</td>
<td>9P</td>
<td>160</td>
<td>Tumour</td>
<td>7</td>
<td>25.63</td>
<td>84.37</td>
</tr>
<tr>
<td>37</td>
<td>9R</td>
<td>250</td>
<td>Nonmalignant</td>
<td>9</td>
<td>28.24</td>
<td>84.27</td>
</tr>
<tr>
<td>37</td>
<td>9T</td>
<td>170</td>
<td>Tumour</td>
<td>9</td>
<td>26.09</td>
<td>84.17</td>
</tr>
<tr>
<td>38</td>
<td>9U</td>
<td>180</td>
<td>Nonmalignant</td>
<td>7</td>
<td>26.2</td>
<td>84.3</td>
</tr>
<tr>
<td>38</td>
<td>9V</td>
<td>110</td>
<td>Tumour</td>
<td>7</td>
<td>26.27</td>
<td>84.25</td>
</tr>
<tr>
<td>39</td>
<td>9W</td>
<td>340</td>
<td>Nonmalignant</td>
<td>7</td>
<td>26.46</td>
<td>84.5</td>
</tr>
</tbody>
</table>
### Appendix 4: Prostate Specimens – Raw Data

<p>| | | | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>39</td>
<td>9X</td>
<td>130</td>
<td>Tumour</td>
<td>7</td>
<td>24.13</td>
<td>84.4</td>
<td>29.29</td>
<td>84.59</td>
</tr>
<tr>
<td>40</td>
<td>9Y</td>
<td>300</td>
<td>Nonmalignant</td>
<td>8</td>
<td>27.18</td>
<td>93.98</td>
<td>28.98</td>
<td>84.5</td>
</tr>
<tr>
<td>40</td>
<td>9Z</td>
<td>150</td>
<td>Tumour</td>
<td>8</td>
<td>27.71</td>
<td>84.34</td>
<td>40</td>
<td>-</td>
</tr>
<tr>
<td>41</td>
<td>A1</td>
<td>240</td>
<td>Nonmalignant</td>
<td>7</td>
<td>24.59</td>
<td>84.31</td>
<td>30.93</td>
<td>84.05</td>
</tr>
<tr>
<td>41</td>
<td>A2</td>
<td>110</td>
<td>Tumour</td>
<td>7</td>
<td>26.03</td>
<td>84.18</td>
<td>31.78</td>
<td>84.2</td>
</tr>
<tr>
<td>42</td>
<td>A3</td>
<td>390</td>
<td>Nonmalignant</td>
<td>9</td>
<td>29.57</td>
<td>84</td>
<td>40</td>
<td>-</td>
</tr>
<tr>
<td>42</td>
<td>A4</td>
<td>170</td>
<td>Tumour</td>
<td>9</td>
<td>28.02</td>
<td>84</td>
<td>40</td>
<td>-</td>
</tr>
<tr>
<td>43</td>
<td>A5</td>
<td>170</td>
<td>Nonmalignant</td>
<td>7</td>
<td>29.36</td>
<td>84.27</td>
<td>30</td>
<td>84.81</td>
</tr>
<tr>
<td>43</td>
<td>A6</td>
<td>160</td>
<td>Tumour</td>
<td>7</td>
<td>29.09</td>
<td>84.51</td>
<td>40</td>
<td>-</td>
</tr>
<tr>
<td>44</td>
<td>A7</td>
<td>130</td>
<td>Nonmalignant</td>
<td>8</td>
<td>28.37</td>
<td>84</td>
<td>31.62</td>
<td>84.85</td>
</tr>
<tr>
<td>44</td>
<td>A8</td>
<td>180</td>
<td>Tumour</td>
<td>8</td>
<td>30</td>
<td>84.51</td>
<td>40</td>
<td>-</td>
</tr>
<tr>
<td>44</td>
<td>A9</td>
<td>290</td>
<td>Tumour</td>
<td>8</td>
<td>28.24</td>
<td>84.23</td>
<td>32.95</td>
<td>84.83</td>
</tr>
</tbody>
</table>
Table A4.2: Prostate specimens demographics (and protein expression)

<table>
<thead>
<tr>
<th>Sample Pair</th>
<th>Sample</th>
<th>Weight (mg)</th>
<th>Sample Type</th>
<th>Gleason Score</th>
<th>Normalised ETS1p51 (Protein)</th>
<th>Relative ETS1p51 Protein (T/N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AA</td>
<td>240</td>
<td>Nonmalignant</td>
<td>7</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>AB</td>
<td>190</td>
<td>Tumour</td>
<td>7</td>
<td>0.05</td>
<td>1.18</td>
</tr>
<tr>
<td>2</td>
<td>AC</td>
<td>90</td>
<td>Nonmalignant</td>
<td>7</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>AD</td>
<td>110</td>
<td>Tumour</td>
<td>7</td>
<td>0.19</td>
<td>10.18</td>
</tr>
<tr>
<td>3</td>
<td>AE</td>
<td>140</td>
<td>Nonmalignant</td>
<td>8</td>
<td>0.20</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>AF</td>
<td>170</td>
<td>Tumour</td>
<td>8</td>
<td>0.07</td>
<td>0.33</td>
</tr>
<tr>
<td>4</td>
<td>AG</td>
<td>150</td>
<td>Nonmalignant</td>
<td>7</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>AH</td>
<td>150</td>
<td>Tumour</td>
<td>7</td>
<td>0.26</td>
<td>1.31</td>
</tr>
<tr>
<td>5</td>
<td>AI</td>
<td>350</td>
<td>Nonmalignant</td>
<td>9</td>
<td>0.64</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>AJ</td>
<td>150</td>
<td>Tumour</td>
<td>9</td>
<td>1.64</td>
<td>2.53</td>
</tr>
<tr>
<td>6</td>
<td>AM</td>
<td>100</td>
<td>Nonmalignant</td>
<td>9</td>
<td>0.21</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>AN</td>
<td>180</td>
<td>Tumour</td>
<td>9</td>
<td>0.50</td>
<td>2.42</td>
</tr>
<tr>
<td>7</td>
<td>AO</td>
<td>190</td>
<td>Nonmalignant</td>
<td>8</td>
<td>0.53</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>AP</td>
<td>210</td>
<td>Tumour</td>
<td>8</td>
<td>1.00</td>
<td>1.87</td>
</tr>
<tr>
<td>8</td>
<td>AQ</td>
<td>130</td>
<td>Nonmalignant</td>
<td>7</td>
<td>0.77</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>AR</td>
<td>160</td>
<td>Tumour</td>
<td>7</td>
<td>0.92</td>
<td>1.19</td>
</tr>
<tr>
<td>9</td>
<td>AS</td>
<td>120</td>
<td>Nonmalignant</td>
<td>7</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>AT</td>
<td>100</td>
<td>Tumour</td>
<td>7</td>
<td>0.19</td>
<td>0.82</td>
</tr>
<tr>
<td>10</td>
<td>AU</td>
<td>110</td>
<td>Nonmalignant</td>
<td>7</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>AV</td>
<td>150</td>
<td>Tumour</td>
<td>7</td>
<td>0.40</td>
<td>3.17</td>
</tr>
<tr>
<td>11</td>
<td>AW</td>
<td>120</td>
<td>Nonmalignant</td>
<td>7</td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>AX</td>
<td>110</td>
<td>Tumour</td>
<td>7</td>
<td>0.12</td>
<td>0.83</td>
</tr>
<tr>
<td>12</td>
<td>AY</td>
<td>170</td>
<td>Nonmalignant</td>
<td>9</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>--------</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>12</td>
<td>AZ</td>
<td>120</td>
<td>Tumour</td>
<td>9</td>
<td>0.38</td>
<td>5.52</td>
</tr>
<tr>
<td>13</td>
<td>B1</td>
<td>260</td>
<td>Nonmalignant</td>
<td>7</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>B2</td>
<td>220</td>
<td>Tumour</td>
<td>7</td>
<td>1.41</td>
<td>5.46</td>
</tr>
<tr>
<td>14</td>
<td>7K</td>
<td>290</td>
<td>Nonmalignant</td>
<td>9</td>
<td>0.74</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>7L</td>
<td>190</td>
<td>Tumour</td>
<td>9</td>
<td>1.87</td>
<td>2.51</td>
</tr>
<tr>
<td>15</td>
<td>7M</td>
<td>620</td>
<td>Nonmalignant</td>
<td>8</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>7N</td>
<td>230</td>
<td>Tumour</td>
<td>8</td>
<td>1.02</td>
<td>2.66</td>
</tr>
<tr>
<td>16</td>
<td>7O</td>
<td>450</td>
<td>Nonmalignant</td>
<td>7</td>
<td>0.49</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>7P</td>
<td>250</td>
<td>Tumour</td>
<td>7</td>
<td>0.37</td>
<td>0.75</td>
</tr>
<tr>
<td>17</td>
<td>7Q</td>
<td>250</td>
<td>Nonmalignant</td>
<td>7</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>7R</td>
<td>330</td>
<td>Tumour</td>
<td>7</td>
<td>0.34</td>
<td>0.67</td>
</tr>
<tr>
<td>18</td>
<td>7U</td>
<td>220</td>
<td>Nonmalignant</td>
<td>7</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>7V</td>
<td>180</td>
<td>Tumour</td>
<td>7</td>
<td>0.25</td>
<td>3.02</td>
</tr>
<tr>
<td>19</td>
<td>7E</td>
<td>280</td>
<td>Nonmalignant</td>
<td>9</td>
<td>1.84</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>7F</td>
<td>100</td>
<td>Tumour</td>
<td>9</td>
<td>2.66</td>
<td>1.44</td>
</tr>
<tr>
<td>20</td>
<td>7I</td>
<td>170</td>
<td>Nonmalignant</td>
<td>7</td>
<td>0.49</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>7J</td>
<td>190</td>
<td>Tumour</td>
<td>7</td>
<td>0.77</td>
<td>1.58</td>
</tr>
<tr>
<td>21</td>
<td>7G</td>
<td>490</td>
<td>Nonmalignant</td>
<td>7</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>7H</td>
<td>350</td>
<td>Tumour</td>
<td>7</td>
<td>0.24</td>
<td>1.07</td>
</tr>
<tr>
<td>22</td>
<td>7S</td>
<td>240</td>
<td>Nonmalignant</td>
<td>9</td>
<td>0.57</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>7T</td>
<td>220</td>
<td>Tumour</td>
<td>9</td>
<td>1.95</td>
<td>3.37</td>
</tr>
<tr>
<td>23</td>
<td>7W</td>
<td>150</td>
<td>Nonmalignant</td>
<td>7</td>
<td>0.49</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>7X</td>
<td>250</td>
<td>Tumour</td>
<td>7</td>
<td>0.93</td>
<td>1.88</td>
</tr>
<tr>
<td>24</td>
<td>BA</td>
<td>170</td>
<td>Nonmalignant</td>
<td>7</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>BB</td>
<td>140</td>
<td>Tumour</td>
<td>7</td>
<td>1.45</td>
<td>4.32</td>
</tr>
<tr>
<td>25</td>
<td>BC</td>
<td>200</td>
<td>Nonmalignant</td>
<td>7</td>
<td>0.45</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>9I</td>
<td>240</td>
<td>Tumour</td>
<td>7</td>
<td>0.32</td>
<td>0.72</td>
</tr>
</tbody>
</table>
### Appendix 4: Prostate Specimens – Raw Data

<p>| | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>26</td>
<td>92</td>
<td>290</td>
<td>Nonmalignant</td>
<td>5</td>
<td>1.01</td>
</tr>
<tr>
<td>26</td>
<td>93</td>
<td>260</td>
<td>Tumour</td>
<td>5</td>
<td>1.70</td>
</tr>
<tr>
<td>27</td>
<td>94</td>
<td>240</td>
<td>Nonmalignant</td>
<td>7</td>
<td>0.31</td>
</tr>
<tr>
<td>27</td>
<td>95</td>
<td>180</td>
<td>Tumour</td>
<td>7</td>
<td>0.12</td>
</tr>
<tr>
<td>28</td>
<td>96</td>
<td>260</td>
<td>Nonmalignant</td>
<td>7</td>
<td>0.25</td>
</tr>
<tr>
<td>28</td>
<td>97</td>
<td>120</td>
<td>Tumour</td>
<td>7</td>
<td>0.15</td>
</tr>
<tr>
<td>29</td>
<td>98</td>
<td>310</td>
<td>Nonmalignant</td>
<td>7</td>
<td>0.34</td>
</tr>
<tr>
<td>29</td>
<td>99</td>
<td>180</td>
<td>Tumour</td>
<td>7</td>
<td>0.23</td>
</tr>
<tr>
<td>30</td>
<td>9A</td>
<td>290</td>
<td>Nonmalignant</td>
<td>7</td>
<td>1.00</td>
</tr>
<tr>
<td>30</td>
<td>9B</td>
<td>130</td>
<td>Tumour</td>
<td>7</td>
<td>1.14</td>
</tr>
<tr>
<td>31</td>
<td>9C</td>
<td>190</td>
<td>Nonmalignant</td>
<td>7</td>
<td>0.68</td>
</tr>
<tr>
<td>31</td>
<td>9D</td>
<td>140</td>
<td>Tumour</td>
<td>7</td>
<td>0.63</td>
</tr>
<tr>
<td>32</td>
<td>9E</td>
<td>170</td>
<td>Nonmalignant</td>
<td>7</td>
<td>0.41</td>
</tr>
<tr>
<td>32</td>
<td>9F</td>
<td>180</td>
<td>Tumour</td>
<td>7</td>
<td>0.37</td>
</tr>
<tr>
<td>33</td>
<td>9G</td>
<td>160</td>
<td>Nonmalignant</td>
<td>7</td>
<td>0.81</td>
</tr>
<tr>
<td>33</td>
<td>9H</td>
<td>140</td>
<td>Tumour</td>
<td>7</td>
<td>3.47</td>
</tr>
<tr>
<td>34</td>
<td>9K</td>
<td>120</td>
<td>Nonmalignant</td>
<td>7</td>
<td>0.09</td>
</tr>
<tr>
<td>34</td>
<td>9L</td>
<td>190</td>
<td>Tumour</td>
<td>7</td>
<td>0.14</td>
</tr>
<tr>
<td>35</td>
<td>9M</td>
<td>130</td>
<td>Nonmalignant</td>
<td>7</td>
<td>0.11</td>
</tr>
<tr>
<td>35</td>
<td>9N</td>
<td>180</td>
<td>Tumour</td>
<td>7</td>
<td>0.81</td>
</tr>
<tr>
<td>36</td>
<td>9O</td>
<td>110</td>
<td>Nonmalignant</td>
<td>7</td>
<td>0.10</td>
</tr>
<tr>
<td>36</td>
<td>9Q</td>
<td>160</td>
<td>Tumour</td>
<td>7</td>
<td>0.48</td>
</tr>
<tr>
<td>37</td>
<td>9R</td>
<td>250</td>
<td>Nonmalignant</td>
<td>9</td>
<td>0.44</td>
</tr>
<tr>
<td>37</td>
<td>9T</td>
<td>170</td>
<td>Tumour</td>
<td>9</td>
<td>1.85</td>
</tr>
<tr>
<td>38</td>
<td>9U</td>
<td>180</td>
<td>Nonmalignant</td>
<td>7</td>
<td>0.57</td>
</tr>
<tr>
<td>38</td>
<td>9V</td>
<td>110</td>
<td>Tumour</td>
<td>7</td>
<td>1.36</td>
</tr>
<tr>
<td>39</td>
<td>9W</td>
<td>340</td>
<td>Nonmalignant</td>
<td>7</td>
<td>0.82</td>
</tr>
</tbody>
</table>

274
<p>| | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>39</td>
<td>9X</td>
<td>130</td>
<td>Tumour</td>
<td>7</td>
<td>0.49</td>
<td>0.59</td>
</tr>
<tr>
<td>40</td>
<td>9Y</td>
<td>300</td>
<td>Nonmalignant</td>
<td>8</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>9Z</td>
<td>150</td>
<td>Tumour</td>
<td>8</td>
<td>0.17</td>
<td>1.41</td>
</tr>
<tr>
<td>41</td>
<td>A1</td>
<td>240</td>
<td>Nonmalignant</td>
<td>7</td>
<td>0.89</td>
<td></td>
</tr>
<tr>
<td>41</td>
<td>A2</td>
<td>110</td>
<td>Tumour</td>
<td>7</td>
<td>0.65</td>
<td>0.73</td>
</tr>
<tr>
<td>42</td>
<td>A3</td>
<td>390</td>
<td>Nonmalignant</td>
<td>9</td>
<td>1.20</td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>A4</td>
<td>170</td>
<td>Tumour</td>
<td>9</td>
<td>0.01</td>
<td>0.001</td>
</tr>
<tr>
<td>43</td>
<td>A5</td>
<td>170</td>
<td>Nonmalignant</td>
<td>7</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>43</td>
<td>A6</td>
<td>160</td>
<td>Tumour</td>
<td>7</td>
<td>0.13</td>
<td>0.39</td>
</tr>
<tr>
<td>44</td>
<td>A7</td>
<td>130</td>
<td>Nonmalignant</td>
<td>8</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>44</td>
<td>A8</td>
<td>180</td>
<td>Tumour</td>
<td>8</td>
<td>0.24</td>
<td>1.25</td>
</tr>
<tr>
<td>44</td>
<td>A9</td>
<td>290</td>
<td>Tumour</td>
<td>8</td>
<td>0.56</td>
<td>2.90</td>
</tr>
</tbody>
</table>
Appendix 5 – Prostate Specimens (Western Blots)
Figure A5.1: Relative ETS1p51 protein levels between matched (1 - 15) nonmalignant prostate tissue (N) adjacent to prostate tumour tissue (T). Protein lysates were separated by SDS-PAGE, transferred to nitrocellulose and analysed by immunoblotting using antibodies against ETS1 and β-actin. The experiment was performed twice with the representative displayed. ETS1p51 levels were normalised using respective β-actin levels. Relative ETS1 levels >1 indicates higher ETS1 protein expression in tumour samples compared with matched normal sample.
Figure A5.2: Relative ETS1p51 protein levels between matched (16 - 30) nonmalignant prostate tissue (N) adjacent to prostate tumour tissue (T). Protein lysates were separated by SDS-PAGE, transferred to nitrocellulose and analysed by immunoblotting using antibodies against ETS1 and β-actin. The experiment was performed twice with the representative displayed. ETS1p51 levels were normalised using respective β-actin levels. Relative ETS1 levels >1 indicates higher ETS1 protein expression in tumour samples compared with matched normal sample.
Appendix 5: Prostate Specimens – Western Blots

**Figure A5.3:** Relative ETS1p51 protein levels between matched (31 - 45) nonmalignant prostate tissue (N) adjacent to prostate tumour tissue (T). Protein lysates were separated by SDS-PAGE, transferred to nitrocellulose and analysed by immunoblotting using antibodies against ETS1 and β-actin. The experiment was performed twice with the representative displayed. ETS1p51 levels were normalised using respective β-actin levels. Relative ETS1 levels >1 indicates higher ETS1 protein expression in tumour samples compared with matched normal sample. *Note: nonmalignant sample 44 is the matched tissue for tumour samples 44 and 45.
Appendix 6 – RT² Profiler PCR Array Gene Table
### Table A6.1: RT² Profiler PCR Array Human Epithelial-To-Mesenchymal Transition (EMT) (PAHS-090A)

<table>
<thead>
<tr>
<th>Position</th>
<th>Unigene</th>
<th>GeneBank</th>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A01</td>
<td>Hs.502756</td>
<td>NM_024060</td>
<td>AHNAK</td>
<td>AHNAK nucleoprotein</td>
</tr>
<tr>
<td>A02</td>
<td>Hs.525622</td>
<td>NM_005163</td>
<td>AKT1</td>
<td>V-akt murine thymoma viral oncogene homolog</td>
</tr>
<tr>
<td>A03</td>
<td>Hs.1274</td>
<td>NM_006129</td>
<td>BMP1</td>
<td>Bone morphogenetic protein 1</td>
</tr>
<tr>
<td>A04</td>
<td>Hs.473163</td>
<td>NM_001719</td>
<td>BMP7</td>
<td>Bone morphogenetic protein 7</td>
</tr>
<tr>
<td>A05</td>
<td>Hs.490203</td>
<td>NM_004342</td>
<td>CALD1</td>
<td>Caldesmon 1</td>
</tr>
<tr>
<td>A06</td>
<td>Hs.197922</td>
<td>NM_018584</td>
<td>CAMK2N1</td>
<td>Calcium/calmodulin-dependent protein kinase II inhibitor 1</td>
</tr>
<tr>
<td>A07</td>
<td>Hs.212332</td>
<td>NM_001233</td>
<td>CAV2</td>
<td>Caveolin 2</td>
</tr>
<tr>
<td>A08</td>
<td>Hs.461086</td>
<td>NM_004360</td>
<td>CDH1</td>
<td>Cadherin 1, type 1, E-cadherin (epithelial)</td>
</tr>
<tr>
<td>A09</td>
<td>Hs.464829</td>
<td>NM_001792</td>
<td>CDH2</td>
<td>Cadherin 2, type 1, N-cadherin (neuronal)</td>
</tr>
<tr>
<td>A10</td>
<td>Hs.489142</td>
<td>NM_000089</td>
<td>COL1A2</td>
<td>Collagen, type I, alpha 2</td>
</tr>
<tr>
<td>A11</td>
<td>Hs.443625</td>
<td>NM_000090</td>
<td>COL3A1</td>
<td>Collagen, type III, alpha 1</td>
</tr>
<tr>
<td>A12</td>
<td>Hs.445827</td>
<td>NM_000393</td>
<td>COL5A2</td>
<td>Collagen, type V, alpha 2</td>
</tr>
<tr>
<td>B01</td>
<td>Hs.476018</td>
<td>NM_001904</td>
<td>CTNNB1</td>
<td>Catenin (cadherin-associated protein), beta 1, 88kDa</td>
</tr>
<tr>
<td>B02</td>
<td>Hs.95612</td>
<td>NM_004949</td>
<td>DSC2</td>
<td>Desmocollin 2</td>
</tr>
<tr>
<td>B03</td>
<td>Hs.519873</td>
<td>NM_004415</td>
<td>DSP</td>
<td>Desmplakin</td>
</tr>
<tr>
<td>B04</td>
<td>Hs.488293</td>
<td>NM_005228</td>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>B05</td>
<td>Hs.118681</td>
<td>NM_001982</td>
<td>ERBB3</td>
<td>V-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)</td>
</tr>
<tr>
<td>B06</td>
<td>Hs.208124</td>
<td>NM_000125</td>
<td>ESR1</td>
<td>Estrogen receptor 1</td>
</tr>
<tr>
<td>B07</td>
<td>Hs.517293</td>
<td>NM_016946</td>
<td>F11R</td>
<td>F11 receptor</td>
</tr>
<tr>
<td>B08</td>
<td>Hs.1690</td>
<td>NM_005130</td>
<td>FGFBP1</td>
<td>Fibroblast growth factor binding protein 1</td>
</tr>
<tr>
<td>B09</td>
<td>Hs.203717</td>
<td>NM_002026</td>
<td>FN1</td>
<td>Fibronectin 1</td>
</tr>
<tr>
<td>B10</td>
<td>Hs.436448</td>
<td>NM_005251</td>
<td>FOXC2</td>
<td>Forkhead box C2 (MFH-1, mesenchyme forkhead 1)</td>
</tr>
<tr>
<td>B11</td>
<td>Hs.173859</td>
<td>NM_003507</td>
<td>FZD7</td>
<td>Frizzled family receptor 7</td>
</tr>
<tr>
<td>B12</td>
<td>Hs.83381</td>
<td>NM_004126</td>
<td>GNG11</td>
<td>Guanine nucleotide binding protein (G protein), gamma 11</td>
</tr>
<tr>
<td>C01</td>
<td>Hs.440438</td>
<td>NM_173849</td>
<td>GSC</td>
<td>Goosecoid homeobox</td>
</tr>
<tr>
<td>C02</td>
<td>Hs.445733</td>
<td>NM_002093</td>
<td>GSK3B</td>
<td>Glycogen synthase kinase 3 beta</td>
</tr>
<tr>
<td>C03</td>
<td>Hs.462998</td>
<td>NM_001552</td>
<td>IGFBP4</td>
<td>Insulin-like growth factor binding protein 4</td>
</tr>
<tr>
<td>C04</td>
<td>Hs.81134</td>
<td>NM_000577</td>
<td>IL1RN</td>
<td>Interleukin 1 receptor antagonist</td>
</tr>
<tr>
<td>C05</td>
<td>Hs.5158</td>
<td>NM_004517</td>
<td>ILK</td>
<td>Integrin-linked kinase</td>
</tr>
<tr>
<td>C06</td>
<td>Hs.505654</td>
<td>NM_002205</td>
<td>ITGA5</td>
<td>Integrin, alpha 5 (fibronectin receptor, alpha polypeptide)</td>
</tr>
<tr>
<td>C07</td>
<td>Hs.436873</td>
<td>NM_002210</td>
<td>ITGAV</td>
<td>Integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)</td>
</tr>
<tr>
<td>C08</td>
<td>Hs.643813</td>
<td>NM_002211</td>
<td>ITGB1</td>
<td>Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)</td>
</tr>
<tr>
<td>C09</td>
<td>Hs.728907</td>
<td>NM_000214</td>
<td>JAG1</td>
<td>Jagged 1</td>
</tr>
<tr>
<td>C10</td>
<td>Hs.654380</td>
<td>NM_00526</td>
<td>KRT14</td>
<td>Keratin 14</td>
</tr>
<tr>
<td>C11</td>
<td>Hs.654568</td>
<td>NM_002276</td>
<td>KRT19</td>
<td>Keratin 19</td>
</tr>
<tr>
<td>C12</td>
<td>Hs.411501</td>
<td>NM_005556</td>
<td>KRT7</td>
<td>Keratin 7</td>
</tr>
<tr>
<td>D01</td>
<td>Hs.335079</td>
<td>NM_005909</td>
<td>MAP1B</td>
<td>Microtubule-associated protein 1B</td>
</tr>
<tr>
<td>D02</td>
<td>Hs.166017</td>
<td>NM_000248</td>
<td>MITF</td>
<td>Microphthalmia-associated transcription factor</td>
</tr>
<tr>
<td>D03</td>
<td>Hs.513617</td>
<td>NM_004530</td>
<td>MMP2</td>
<td>Matrix metalloproteinase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)</td>
</tr>
<tr>
<td>D04</td>
<td>Hs.375129</td>
<td>NM_002422</td>
<td>MMP3</td>
<td>Matrix metalloproteinase 3 (stromelysin 1, progelatinase)</td>
</tr>
<tr>
<td>D05</td>
<td>Hs.297413</td>
<td>NM_004994</td>
<td>MMP9</td>
<td>Matrix metalloproteinase 9 (gelatinase B,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td><strong>D06</strong></td>
<td>Hs.87752</td>
<td>NM_002444</td>
<td>92kDa gelatinase, 92kDa type IV collagenase)</td>
<td></td>
</tr>
<tr>
<td><strong>D07</strong></td>
<td>Hs.517973</td>
<td>NM_002447</td>
<td>Moesin</td>
<td></td>
</tr>
<tr>
<td><strong>D08</strong></td>
<td>Hs.370414</td>
<td>NM_018055</td>
<td>Nodal homolog (mouse)</td>
<td></td>
</tr>
<tr>
<td><strong>D09</strong></td>
<td>Hs.495473</td>
<td>NM_017617</td>
<td>Notch 1</td>
<td></td>
</tr>
<tr>
<td><strong>D10</strong></td>
<td>Hs.533657</td>
<td>NM_015901</td>
<td>Nudix (nucleoside diphosphate linked moiety X)-type motif 13</td>
<td></td>
</tr>
<tr>
<td><strong>D11</strong></td>
<td>Hs.592605</td>
<td>NM_002538</td>
<td>Occludin</td>
<td></td>
</tr>
<tr>
<td><strong>D12</strong></td>
<td>Hs.509067</td>
<td>NM_002609</td>
<td>Platelet-derived growth factor receptor, beta polypeptide</td>
<td></td>
</tr>
<tr>
<td><strong>E01</strong></td>
<td>Hs.170473</td>
<td>NM_016445</td>
<td>Pleckstrin 2</td>
<td></td>
</tr>
<tr>
<td><strong>E02</strong></td>
<td>Hs.570455</td>
<td>NM_015704</td>
<td>PPPDE peptidase domain containing 2</td>
<td></td>
</tr>
<tr>
<td><strong>E03</strong></td>
<td>Hs.395482</td>
<td>NM_005607</td>
<td>PTK2 protein tyrosine kinase 2</td>
<td></td>
</tr>
<tr>
<td><strong>E04</strong></td>
<td>Hs.227777</td>
<td>NM_003463</td>
<td>Protein tyrosine phosphatase type IVA, member 1</td>
<td></td>
</tr>
<tr>
<td><strong>E05</strong></td>
<td>Hs.413812</td>
<td>NM_006908</td>
<td>Rac1</td>
<td></td>
</tr>
<tr>
<td><strong>E06</strong></td>
<td>Hs.78944</td>
<td>NM_002923</td>
<td>RGS2</td>
<td></td>
</tr>
<tr>
<td><strong>E07</strong></td>
<td>Hs.414795</td>
<td>NM_000602</td>
<td>Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1</td>
<td></td>
</tr>
<tr>
<td><strong>E08</strong></td>
<td>Hs.708127</td>
<td>NM_003616</td>
<td>Survival of motor neuron protein interacting protein 1</td>
<td></td>
</tr>
<tr>
<td><strong>E09</strong></td>
<td>Hs.12253</td>
<td>NM_005901</td>
<td>SMAD2</td>
<td></td>
</tr>
<tr>
<td><strong>E10</strong></td>
<td>Hs.48029</td>
<td>NM_005985</td>
<td>Snail homolog 1 (Drosophila)</td>
<td></td>
</tr>
<tr>
<td><strong>E11</strong></td>
<td>Hs.360174</td>
<td>NM_003068</td>
<td>Snail homolog 2 (Drosophila)</td>
<td></td>
</tr>
<tr>
<td><strong>E12</strong></td>
<td>Hs.253790</td>
<td>NM_178310</td>
<td>Snail homolog 3 (Drosophila)</td>
<td></td>
</tr>
<tr>
<td><strong>F01</strong></td>
<td>Hs.376984</td>
<td>NM_006941</td>
<td>SOX10</td>
<td></td>
</tr>
<tr>
<td><strong>F02</strong></td>
<td>Hs.111779</td>
<td>NM_003118</td>
<td>SPARC</td>
<td></td>
</tr>
<tr>
<td><strong>F03</strong></td>
<td>Hs.313</td>
<td>NM_000582</td>
<td>SPP1</td>
<td></td>
</tr>
<tr>
<td><strong>F04</strong></td>
<td>Hs.463059</td>
<td>NM_003150</td>
<td>Signal transducer and activator of transcription 3 (acute-phase response factor)</td>
<td></td>
</tr>
<tr>
<td><strong>F05</strong></td>
<td>Hs.61635</td>
<td>NM_012449</td>
<td>STEAP1</td>
<td></td>
</tr>
<tr>
<td><strong>F06</strong></td>
<td>Hs.371282</td>
<td>NM_003200</td>
<td>Transcription factor 3 (E2A immunoglobulin enhancer binding factors E12/E47)</td>
<td></td>
</tr>
<tr>
<td><strong>F07</strong></td>
<td>Hs.644653</td>
<td>NM_003199</td>
<td>TCF4</td>
<td></td>
</tr>
<tr>
<td><strong>F08</strong></td>
<td>Hs.438231</td>
<td>NM_006528</td>
<td>Tissue factor pathway inhibitor 2</td>
<td></td>
</tr>
<tr>
<td><strong>F09</strong></td>
<td>Hs.645227</td>
<td>NM_000660</td>
<td>Transforming growth factor, beta 1</td>
<td></td>
</tr>
<tr>
<td><strong>F10</strong></td>
<td>Hs.133379</td>
<td>NM_003238</td>
<td>Transforming growth factor, beta 2</td>
<td></td>
</tr>
<tr>
<td><strong>F11</strong></td>
<td>Hs.592317</td>
<td>NM_003239</td>
<td>Transforming growth factor, beta 3</td>
<td></td>
</tr>
<tr>
<td><strong>F12</strong></td>
<td>Hs.522632</td>
<td>NM_003254</td>
<td>TIMP1</td>
<td></td>
</tr>
<tr>
<td><strong>G01</strong></td>
<td>Hs.598100</td>
<td>NM_003690</td>
<td>Transmembrane protein with EGF-like and two follistatin-like domains 1</td>
<td></td>
</tr>
<tr>
<td><strong>G02</strong></td>
<td>Hs.118552</td>
<td>NM_178031</td>
<td>Transmembrane protein 132A</td>
<td></td>
</tr>
<tr>
<td><strong>G03</strong></td>
<td>Hs.364544</td>
<td>NM_014399</td>
<td>Tetraspanin 13</td>
<td></td>
</tr>
<tr>
<td><strong>G04</strong></td>
<td>Hs.66744</td>
<td>NM_000474</td>
<td>Twist homolog 1 (Drosophila)</td>
<td></td>
</tr>
<tr>
<td><strong>G05</strong></td>
<td>Hs.643801</td>
<td>NM_004385</td>
<td>Versican</td>
<td></td>
</tr>
<tr>
<td><strong>G06</strong></td>
<td>Hs.642813</td>
<td>NM_003380</td>
<td>Vimentin</td>
<td></td>
</tr>
<tr>
<td><strong>G07</strong></td>
<td>Hs.459790</td>
<td>NM_033305</td>
<td>Vacuolar protein sorting 13 homolog A (S. cerevisiae)</td>
<td></td>
</tr>
<tr>
<td><strong>G08</strong></td>
<td>Hs.108219</td>
<td>NM_004626</td>
<td>Wingless-type MMTV integration site family, member 11</td>
<td></td>
</tr>
<tr>
<td><strong>G09</strong></td>
<td>Hs.696364</td>
<td>NM_003392</td>
<td>Wingless-type MMTV integration site family, member 5A</td>
<td></td>
</tr>
</tbody>
</table>
## Appendix 6: RT² Profiler PCR Array Gene Table

<table>
<thead>
<tr>
<th>Gene Id</th>
<th>Hs.</th>
<th>NM_</th>
<th>Gene Name and Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>G10</td>
<td>306051</td>
<td>032642</td>
<td>WNT5B</td>
</tr>
<tr>
<td>G11</td>
<td>124503</td>
<td>030751</td>
<td>ZEB1</td>
</tr>
<tr>
<td>G12</td>
<td>34871</td>
<td>014795</td>
<td>ZEB2</td>
</tr>
<tr>
<td>H01</td>
<td>534255</td>
<td>004048</td>
<td>B2M</td>
</tr>
<tr>
<td>H02</td>
<td>412707</td>
<td>000194</td>
<td>HPRT1</td>
</tr>
<tr>
<td>H03</td>
<td>728776</td>
<td>012423</td>
<td>RPL13A</td>
</tr>
<tr>
<td>H04</td>
<td>592355</td>
<td>002046</td>
<td>GAPDH</td>
</tr>
<tr>
<td>H05</td>
<td>520640</td>
<td>001101</td>
<td>ACTB</td>
</tr>
<tr>
<td>H06</td>
<td>N/A</td>
<td>00105</td>
<td>HGDC</td>
</tr>
<tr>
<td>H07</td>
<td>N/A</td>
<td>00104</td>
<td>RTC</td>
</tr>
<tr>
<td>H08</td>
<td>N/A</td>
<td>00104</td>
<td>RTC</td>
</tr>
<tr>
<td>H09</td>
<td>N/A</td>
<td>00104</td>
<td>RTC</td>
</tr>
<tr>
<td>H10</td>
<td>N/A</td>
<td>00103</td>
<td>PPC</td>
</tr>
<tr>
<td>H11</td>
<td>N/A</td>
<td>00103</td>
<td>PPC</td>
</tr>
<tr>
<td>H12</td>
<td>N/A</td>
<td>00103</td>
<td>PPC</td>
</tr>
</tbody>
</table>
Appendix 7 – PCR Array Data
Appendix 7: PCR Array Data

Comments:

Fold-Change ($2^{(- \Delta \Delta Ct)}$) is the normalized gene expression ($2^{(- \Delta Ct)}$) in the Test Sample divided the normalized gene expression ($2^{(- \Delta Ct)}$) in the Control Sample.

Fold-change values greater than one indicate a positive- or an up-regulation, and the fold-regulation is equal to the fold-change.

Fold-change values less than one indicate a negative or down-regulation, and the fold-regulation is the negative inverse of the fold-change.

Fold-change and fold-regulation values greater than 1.5 are indicated in red; fold-change values less than 0.5 and fold-regulation values less than -1.5 are indicated in blue.

A: This gene’s average threshold cycle is relatively high (> 30) in either the control or the test sample, and is reasonably low in the other sample (< 30).

B: These data mean that the gene’s expression is relatively low in one sample and reasonably detected in the other sample suggesting that the actual fold-change value is at least as large as the calculated and reported fold-change result.

C: This gene’s average threshold cycle is either not determined or greater than the defined cut-off value (default 35), in both samples meaning that its expression was undetected, making this fold-change result erroneous and un-interpretable.
Table A7.1: Array 1

<table>
<thead>
<tr>
<th>Position</th>
<th>Symbol</th>
<th>10^8M DHT</th>
<th>GFP-ETSi1p51</th>
<th>DHT + GFP-ETSi1p51</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>fold</td>
<td>regulation</td>
<td>average ct</td>
<td>fold regulation</td>
</tr>
<tr>
<td>A01</td>
<td>AHNAK</td>
<td>1.6245</td>
<td>OKAY 25.56</td>
<td>-1.2746 OKAY 27.34</td>
</tr>
<tr>
<td>A02</td>
<td>AKT1</td>
<td>-1.2058</td>
<td>OKAY 22.01</td>
<td>-1.2658 OKAY 22.81</td>
</tr>
<tr>
<td>A03</td>
<td>BMP1</td>
<td>-2.4116</td>
<td>OKAY 27.61</td>
<td>-1.0792 OKAY 27.18</td>
</tr>
<tr>
<td>A04</td>
<td>BMP2</td>
<td>-1.2397</td>
<td>C 35</td>
<td>1.3379 C 35</td>
</tr>
<tr>
<td>A05</td>
<td>BMP7</td>
<td>-7.1602</td>
<td>B 35</td>
<td>-1.181 B 33.13</td>
</tr>
<tr>
<td>A06</td>
<td>CALD1</td>
<td>-1.1892</td>
<td>OKAY 21.43</td>
<td>-1.0792 OKAY 22.02</td>
</tr>
<tr>
<td>A07</td>
<td>CAMK2N1</td>
<td>-69.071</td>
<td>OKAY 28.27</td>
<td>1.014 OKAY 22.87</td>
</tr>
<tr>
<td>A08</td>
<td>CAV2</td>
<td>-3.1821</td>
<td>B 33.47</td>
<td>-1.1487 B 32.73</td>
</tr>
<tr>
<td>A09</td>
<td>CDH1</td>
<td>-1.3947</td>
<td>OKAY 20.74</td>
<td>-1.2311 OKAY 21.29</td>
</tr>
<tr>
<td>A10</td>
<td>CDH2</td>
<td>-1.2397</td>
<td>C 35</td>
<td>1.3379 C 35</td>
</tr>
<tr>
<td>A11</td>
<td>COL1A2</td>
<td>-1.2397</td>
<td>C 35</td>
<td>1.3379 C 35</td>
</tr>
<tr>
<td>A12</td>
<td>COL3A1</td>
<td>-10.411</td>
<td>A 32.62</td>
<td>-1.7901 A 30.81</td>
</tr>
<tr>
<td>B01</td>
<td>COL5A2</td>
<td>-8.5742</td>
<td>OKAY 29.53</td>
<td>-1.2924 OKAY 27.53</td>
</tr>
<tr>
<td>B02</td>
<td>CTNNB1</td>
<td>-1.4845</td>
<td>OKAY 22.57</td>
<td>-1.6358 OKAY 23.44</td>
</tr>
<tr>
<td>B03</td>
<td>DSC2</td>
<td>-1.2924</td>
<td>OKAY 20.9</td>
<td>-1.3013 OKAY 21.64</td>
</tr>
<tr>
<td>B04</td>
<td>DSP</td>
<td>-1.4948</td>
<td>OKAY 21.99</td>
<td>-1.0644 OKAY 22.23</td>
</tr>
<tr>
<td>B05</td>
<td>EGFR</td>
<td>1.1487</td>
<td>OKAY 24.09</td>
<td>-1.1892 OKAY 25.27</td>
</tr>
<tr>
<td>B06</td>
<td>ERBB3</td>
<td>-1.2142</td>
<td>OKAY 22.2</td>
<td>-1.2834 OKAY 23.01</td>
</tr>
</tbody>
</table>
### Appendix 7: PCR Array Data

<p>| | | | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>B07</td>
<td>ESR1</td>
<td>-2.2815</td>
<td>B</td>
<td>35</td>
<td>-1.3755</td>
<td>B</td>
<td>35</td>
<td>2.4116</td>
</tr>
<tr>
<td>B08</td>
<td>F11R</td>
<td>-1.3472</td>
<td>OKAY</td>
<td>22.83</td>
<td>-1.1096</td>
<td>OKAY</td>
<td>23.28</td>
<td>-1.4845</td>
</tr>
<tr>
<td>B09</td>
<td>FGFBP1</td>
<td>-3.3173</td>
<td>B</td>
<td>35</td>
<td>2.4284</td>
<td>B</td>
<td>32.72</td>
<td>1.9725</td>
</tr>
<tr>
<td>B10</td>
<td>FN1</td>
<td>-3.249</td>
<td>OKAY</td>
<td>25.14</td>
<td>-1.2058</td>
<td>OKAY</td>
<td>24.44</td>
<td>-2.8879</td>
</tr>
<tr>
<td>B11</td>
<td>FOXC2</td>
<td>1.007</td>
<td>B</td>
<td>34.68</td>
<td>1.3379</td>
<td>C</td>
<td>35</td>
<td>1.1019</td>
</tr>
<tr>
<td>B12</td>
<td>FZD7</td>
<td>-1.5801</td>
<td>OKAY</td>
<td>29.09</td>
<td>1.007</td>
<td>OKAY</td>
<td>29.15</td>
<td>-2.1735</td>
</tr>
<tr>
<td>C01</td>
<td>GNG11</td>
<td>-1.2397</td>
<td>C</td>
<td>35</td>
<td>1.3379</td>
<td>C</td>
<td>35</td>
<td>1.1019</td>
</tr>
<tr>
<td>C02</td>
<td>GSC</td>
<td>-2.1435</td>
<td>OKAY</td>
<td>26.45</td>
<td>-1.4241</td>
<td>OKAY</td>
<td>26.59</td>
<td>-3.249</td>
</tr>
<tr>
<td>C03</td>
<td>GSK3B</td>
<td>-1.3379</td>
<td>OKAY</td>
<td>22.56</td>
<td>1.0425</td>
<td>OKAY</td>
<td>22.81</td>
<td>-1.257</td>
</tr>
<tr>
<td>C04</td>
<td>IGFBP4</td>
<td>-1.2397</td>
<td>C</td>
<td>35</td>
<td>1.3379</td>
<td>C</td>
<td>35</td>
<td>1.1019</td>
</tr>
<tr>
<td>C05</td>
<td>IL1RN</td>
<td>-8.8766</td>
<td>OKAY</td>
<td>28.49</td>
<td>-1.0425</td>
<td>OKAY</td>
<td>26.13</td>
<td>-10.928</td>
</tr>
<tr>
<td>C06</td>
<td>ILK</td>
<td>1.1647</td>
<td>OKAY</td>
<td>22.59</td>
<td>1.0425</td>
<td>OKAY</td>
<td>23.48</td>
<td>1.2311</td>
</tr>
<tr>
<td>C07</td>
<td>ITGAV</td>
<td>-1.6472</td>
<td>OKAY</td>
<td>25.49</td>
<td>-1.007</td>
<td>OKAY</td>
<td>25.51</td>
<td>-1.3566</td>
</tr>
<tr>
<td>C08</td>
<td>ITGAV</td>
<td>1.3947</td>
<td>OKAY</td>
<td>19.97</td>
<td>-1.257</td>
<td>OKAY</td>
<td>20.93</td>
<td>-1.3379</td>
</tr>
<tr>
<td>C09</td>
<td>JAG1</td>
<td>2.7321</td>
<td>OKAY</td>
<td>21.23</td>
<td>-1.2058</td>
<td>OKAY</td>
<td>23.68</td>
<td>2.114</td>
</tr>
<tr>
<td>C10</td>
<td>KRT14</td>
<td>5.0281</td>
<td>B</td>
<td>30.28</td>
<td>1.3195</td>
<td>B</td>
<td>32.94</td>
<td>4.5315</td>
</tr>
<tr>
<td>C12</td>
<td>KRT19</td>
<td>4.2281</td>
<td>OKAY</td>
<td>26.62</td>
<td>1.1329</td>
<td>OKAY</td>
<td>29.25</td>
<td>2.6759</td>
</tr>
<tr>
<td>D01</td>
<td>KRT7</td>
<td>-1.2311</td>
<td>B</td>
<td>33.57</td>
<td>1.7171</td>
<td>B</td>
<td>33.22</td>
<td>3.5064</td>
</tr>
<tr>
<td>D02</td>
<td>MAP1B</td>
<td>-1.4641</td>
<td>OKAY</td>
<td>21.86</td>
<td>-1.1019</td>
<td>OKAY</td>
<td>22.18</td>
<td>-1.1975</td>
</tr>
<tr>
<td>D03</td>
<td>MMP2</td>
<td>-1.2397</td>
<td>C</td>
<td>35</td>
<td>1.8532</td>
<td>B</td>
<td>34.53</td>
<td>1.3851</td>
</tr>
<tr>
<td>D04</td>
<td>MMP3</td>
<td>-1.7777</td>
<td>B</td>
<td>33</td>
<td>-3.4822</td>
<td>B</td>
<td>34.7</td>
<td>-2.0279</td>
</tr>
<tr>
<td>D05</td>
<td>MMP9</td>
<td>-1.1096</td>
<td>B</td>
<td>32.32</td>
<td>2.2658</td>
<td>B</td>
<td>31.72</td>
<td>1.7777</td>
</tr>
<tr>
<td>D06</td>
<td>MSN</td>
<td>-1.0943</td>
<td>OKAY</td>
<td>23.64</td>
<td>-1.257</td>
<td>OKAY</td>
<td>24.57</td>
<td>-1.257</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>----</td>
<td>--------</td>
<td>-------</td>
<td>-----</td>
<td>-------</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>D07</td>
<td>MST1R</td>
<td>-2.4794</td>
<td>B</td>
<td>33.26</td>
<td>-1.1892</td>
<td>B</td>
<td>32.93</td>
<td>1.1647</td>
</tr>
<tr>
<td>D08</td>
<td>NODAL</td>
<td>-3.0738</td>
<td>B</td>
<td>31.8</td>
<td>2.2346</td>
<td>A</td>
<td>29.75</td>
<td>-1.815</td>
</tr>
<tr>
<td>D09</td>
<td>NOTCH1</td>
<td>-2.5491</td>
<td>OKAY</td>
<td>27.97</td>
<td>-1.1329</td>
<td>OKAY</td>
<td>27.53</td>
<td>-2.042</td>
</tr>
<tr>
<td>D10</td>
<td>NUDT13</td>
<td>-1.057</td>
<td>OKAY</td>
<td>27.08</td>
<td>-1.021</td>
<td>OKAY</td>
<td>27.76</td>
<td>-1.3755</td>
</tr>
<tr>
<td>D11</td>
<td>OCLN</td>
<td>-3.0738</td>
<td>OKAY</td>
<td>23.23</td>
<td>1.0867</td>
<td>OKAY</td>
<td>22.22</td>
<td>-3.0314</td>
</tr>
<tr>
<td>D12</td>
<td>PDGFRB</td>
<td>1.057</td>
<td>B</td>
<td>32.51</td>
<td>1.3472</td>
<td>B</td>
<td>32.89</td>
<td>-1.1251</td>
</tr>
<tr>
<td>E01</td>
<td>PLEK2</td>
<td>-2.1287</td>
<td>B</td>
<td>31.62</td>
<td>1.454</td>
<td>B</td>
<td>30.72</td>
<td>-1.2924</td>
</tr>
<tr>
<td>E02</td>
<td>PPPDE2</td>
<td>-1.2226</td>
<td>OKAY</td>
<td>22.58</td>
<td>-1.0425</td>
<td>OKAY</td>
<td>23.08</td>
<td>-1.2483</td>
</tr>
<tr>
<td>E03</td>
<td>PTK2</td>
<td>-1.5052</td>
<td>OKAY</td>
<td>23.56</td>
<td>-1.1728</td>
<td>OKAY</td>
<td>23.93</td>
<td>-1.6245</td>
</tr>
<tr>
<td>E05</td>
<td>RAC1</td>
<td>-1.1251</td>
<td>OKAY</td>
<td>20.71</td>
<td>-1.0497</td>
<td>OKAY</td>
<td>21.34</td>
<td>-1.0718</td>
</tr>
<tr>
<td>E06</td>
<td>RGS2</td>
<td>-5.4264</td>
<td>B</td>
<td>33.15</td>
<td>-1.5263</td>
<td>B</td>
<td>32.05</td>
<td>-6.9644</td>
</tr>
<tr>
<td>E07</td>
<td>SERPINE1</td>
<td>-1.2397</td>
<td>C</td>
<td>35</td>
<td>1.3379</td>
<td>C</td>
<td>35</td>
<td>2.2501</td>
</tr>
<tr>
<td>E08</td>
<td>SIP1</td>
<td>-1.8277</td>
<td>OKAY</td>
<td>24.31</td>
<td>-1.2483</td>
<td>OKAY</td>
<td>24.49</td>
<td>-1.8532</td>
</tr>
<tr>
<td>E09</td>
<td>SMAD2</td>
<td>-1.2834</td>
<td>OKAY</td>
<td>22.8</td>
<td>-1.0497</td>
<td>OKAY</td>
<td>23.24</td>
<td>-1.3013</td>
</tr>
<tr>
<td>E10</td>
<td>SNAI1</td>
<td>-4.4076</td>
<td>B</td>
<td>32.49</td>
<td>-1.4845</td>
<td>B</td>
<td>31.65</td>
<td>-2.5847</td>
</tr>
<tr>
<td>E11</td>
<td>SNAI2</td>
<td>28.6408</td>
<td>OKAY</td>
<td>23.45</td>
<td>1.2746</td>
<td>OKAY</td>
<td>28.67</td>
<td>26.5382</td>
</tr>
<tr>
<td>E12</td>
<td>SNAI3</td>
<td>1.0718</td>
<td>OKAY</td>
<td>28.62</td>
<td>-1.2142</td>
<td>OKAY</td>
<td>29.73</td>
<td>-1.7532</td>
</tr>
<tr>
<td>F01</td>
<td>SOX10</td>
<td>-1.1728</td>
<td>B</td>
<td>33.15</td>
<td>1.8277</td>
<td>B</td>
<td>32.78</td>
<td>-1.4439</td>
</tr>
<tr>
<td>F02</td>
<td>SPARC</td>
<td>2.1886</td>
<td>A</td>
<td>28.64</td>
<td>6.774</td>
<td>A</td>
<td>27.74</td>
<td>9.4479</td>
</tr>
<tr>
<td>F03</td>
<td>SPP1</td>
<td>-1.2397</td>
<td>C</td>
<td>35</td>
<td>1.8921</td>
<td>B</td>
<td>34.5</td>
<td>1.5583</td>
</tr>
<tr>
<td>F04</td>
<td>STAT3</td>
<td>1.1567</td>
<td>OKAY</td>
<td>21.45</td>
<td>-1.1096</td>
<td>OKAY</td>
<td>22.54</td>
<td>1.057</td>
</tr>
<tr>
<td>F05</td>
<td>STEAP1</td>
<td>1.1567</td>
<td>OKAY</td>
<td>19.22</td>
<td>-1.0497</td>
<td>OKAY</td>
<td>20.23</td>
<td>1.0792</td>
</tr>
<tr>
<td>F06</td>
<td>TCF3</td>
<td>-1.9053</td>
<td>OKAY</td>
<td>23.95</td>
<td>-1.0718</td>
<td>OKAY</td>
<td>23.85</td>
<td>-2.0562</td>
</tr>
<tr>
<td></td>
<td>Gene ID</td>
<td>Expression</td>
<td>Status</td>
<td>Fold Change</td>
<td>Gene ID</td>
<td>Expression</td>
<td>Status</td>
<td>Fold Change</td>
</tr>
<tr>
<td>---</td>
<td>---------</td>
<td>------------</td>
<td>--------</td>
<td>-------------</td>
<td>---------</td>
<td>------------</td>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>F07</td>
<td>TCF4</td>
<td>-3.5554</td>
<td>OKAY</td>
<td>28.83</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F08</td>
<td>TP12</td>
<td>-1.2397</td>
<td>C</td>
<td>35</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F09</td>
<td>TGFB1</td>
<td>-3.4822</td>
<td>B</td>
<td>34.92</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F10</td>
<td>TGFB2</td>
<td>1.9588</td>
<td>B</td>
<td>32.69</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F11</td>
<td>TGFB3</td>
<td>-2.639</td>
<td>B</td>
<td>32.15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F12</td>
<td>TIMP1</td>
<td>-1.2397</td>
<td>C</td>
<td>35</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G01</td>
<td>TMEFF1</td>
<td>-1.9588</td>
<td>OKAY</td>
<td>29.79</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G02</td>
<td>TMEM132A</td>
<td>1.057</td>
<td>OKAY</td>
<td>23.32</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G03</td>
<td>TSPAN13</td>
<td>1.4948</td>
<td>OKAY</td>
<td>20.85</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G04</td>
<td>TWIST1</td>
<td>5.0281</td>
<td>OKAY</td>
<td>22.31</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G05</td>
<td>VCAN</td>
<td>-1.2397</td>
<td>C</td>
<td>35</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G06</td>
<td>VIM</td>
<td>-1.1975</td>
<td>OKAY</td>
<td>26.59</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G07</td>
<td>VPS13A</td>
<td>-1.5583</td>
<td>OKAY</td>
<td>23.17</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G08</td>
<td>WNT11</td>
<td>-1.5583</td>
<td>B</td>
<td>35</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G09</td>
<td>WNT5A</td>
<td>-1.3472</td>
<td>OKAY</td>
<td>25.96</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G10</td>
<td>WNT5B</td>
<td>3.0314</td>
<td>B</td>
<td>32.66</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G11</td>
<td>ZEB1</td>
<td>-2.8481</td>
<td>B</td>
<td>33.83</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G12</td>
<td>ZEB2</td>
<td>-1.2397</td>
<td>C</td>
<td>35</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H01</td>
<td>ACTB</td>
<td>1.3195</td>
<td>OKAY</td>
<td>16.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H02</td>
<td>B2M</td>
<td>2.7702</td>
<td>OKAY</td>
<td>18.67</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H03</td>
<td>GAPDH</td>
<td>-1.4845</td>
<td>OKAY</td>
<td>17.69</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H04</td>
<td>HPRT1</td>
<td>-1.7901</td>
<td>OKAY</td>
<td>23.84</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H05</td>
<td>RPLP0</td>
<td>-1.3755</td>
<td>OKAY</td>
<td>15.73</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H06</td>
<td>HGDG</td>
<td>-1.2397</td>
<td>C</td>
<td>35</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
## Appendix 7: PCR Array Data

<p>| | | | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>H07</td>
<td>RTC</td>
<td>-1.0425</td>
<td>OKAY</td>
<td>24.08</td>
<td>1.434</td>
<td>OKAY</td>
<td>24.23</td>
<td>1.1728</td>
</tr>
<tr>
<td>H08</td>
<td>RTC</td>
<td>-1.1728</td>
<td>OKAY</td>
<td>24.16</td>
<td>1.3472</td>
<td>OKAY</td>
<td>24.23</td>
<td>1.1329</td>
</tr>
<tr>
<td>H09</td>
<td>RTC</td>
<td>-1.2397</td>
<td>OKAY</td>
<td>24.17</td>
<td>1.1019</td>
<td>OKAY</td>
<td>24.45</td>
<td>-1.021</td>
</tr>
<tr>
<td>H10</td>
<td>PPC</td>
<td>-1.2746</td>
<td>OKAY</td>
<td>20.34</td>
<td>1.2311</td>
<td>OKAY</td>
<td>20.42</td>
<td>1.1487</td>
</tr>
<tr>
<td>H11</td>
<td>PPC</td>
<td>-1.3566</td>
<td>OKAY</td>
<td>20.43</td>
<td>1.3013</td>
<td>OKAY</td>
<td>20.34</td>
<td>1.007</td>
</tr>
<tr>
<td>H12</td>
<td>PPC</td>
<td>-1.3472</td>
<td>OKAY</td>
<td>20.45</td>
<td>1.3287</td>
<td>OKAY</td>
<td>20.34</td>
<td>1.0425</td>
</tr>
</tbody>
</table>
Table A7.2: Array 2

<table>
<thead>
<tr>
<th>Position</th>
<th>Symbol</th>
<th>Fold Regulation</th>
<th>Comments</th>
<th>Average Ct</th>
<th>Fold Regulation</th>
<th>Comments</th>
<th>Average Ct</th>
<th>Fold Regulation</th>
<th>Comments</th>
<th>Average Ct</th>
</tr>
</thead>
<tbody>
<tr>
<td>A01</td>
<td>AHNAK</td>
<td>1.6245</td>
<td>OKAY</td>
<td>27.02</td>
<td>-1.2746</td>
<td>OKAY</td>
<td>28.16</td>
<td>1.014</td>
<td>OKAY</td>
<td>26.99</td>
</tr>
<tr>
<td>A02</td>
<td>AKT1</td>
<td>-1.2058</td>
<td>OKAY</td>
<td>22.71</td>
<td>-1.2658</td>
<td>OKAY</td>
<td>23.17</td>
<td>-1.5476</td>
<td>OKAY</td>
<td>22.95</td>
</tr>
<tr>
<td>A03</td>
<td>BMP1</td>
<td>-2.4116</td>
<td>OKAY</td>
<td>27.96</td>
<td>-1.0792</td>
<td>OKAY</td>
<td>35</td>
<td>-1.6935</td>
<td>OKAY</td>
<td>28.09</td>
</tr>
<tr>
<td>A04</td>
<td>BMP2</td>
<td>-1.2397</td>
<td>C</td>
<td>35</td>
<td>1.3379</td>
<td>C</td>
<td>35</td>
<td>1.1019</td>
<td>C</td>
<td>35</td>
</tr>
<tr>
<td>A05</td>
<td>BMP7</td>
<td>-7.1602</td>
<td>B</td>
<td>34.34</td>
<td>-1.181</td>
<td>B</td>
<td>34.88</td>
<td>-5.2416</td>
<td>B</td>
<td>35</td>
</tr>
<tr>
<td>A06</td>
<td>CALD1</td>
<td>-1.1892</td>
<td>OKAY</td>
<td>21.89</td>
<td>-1.0792</td>
<td>OKAY</td>
<td>22.25</td>
<td>-1.3472</td>
<td>OKAY</td>
<td>22</td>
</tr>
<tr>
<td>A07</td>
<td>CAMK2N1</td>
<td>-69.071</td>
<td>OKAY</td>
<td>28.56</td>
<td>1.014</td>
<td>OKAY</td>
<td>22.69</td>
<td>-59.302</td>
<td>OKAY</td>
<td>27.6</td>
</tr>
<tr>
<td>A08</td>
<td>CAV2</td>
<td>-3.1821</td>
<td>B</td>
<td>32.73</td>
<td>-1.1487</td>
<td>B</td>
<td>32.76</td>
<td>-1.3379</td>
<td>B</td>
<td>33.47</td>
</tr>
<tr>
<td>A09</td>
<td>CDH1</td>
<td>-1.3947</td>
<td>OKAY</td>
<td>21.25</td>
<td>-1.2311</td>
<td>OKAY</td>
<td>21.57</td>
<td>-1.8404</td>
<td>OKAY</td>
<td>21.5</td>
</tr>
<tr>
<td>A10</td>
<td>CDH2</td>
<td>-1.2397</td>
<td>C</td>
<td>35</td>
<td>1.3379</td>
<td>C</td>
<td>35</td>
<td>2.1886</td>
<td>B</td>
<td>34.63</td>
</tr>
<tr>
<td>A11</td>
<td>COL1A2</td>
<td>-1.2397</td>
<td>C</td>
<td>35</td>
<td>1.3379</td>
<td>C</td>
<td>35</td>
<td>1.1019</td>
<td>C</td>
<td>35</td>
</tr>
<tr>
<td>A12</td>
<td>COL3A1</td>
<td>-10.411</td>
<td>A</td>
<td>31.71</td>
<td>-1.7901</td>
<td>A</td>
<td>30.45</td>
<td>-5.3889</td>
<td>A</td>
<td>32.72</td>
</tr>
<tr>
<td>B01</td>
<td>COL5A2</td>
<td>-8.5742</td>
<td>OKAY</td>
<td>29.05</td>
<td>-1.2924</td>
<td>OKAY</td>
<td>27.72</td>
<td>-7.21</td>
<td>OKAY</td>
<td>29.34</td>
</tr>
<tr>
<td>B02</td>
<td>CTNNB1</td>
<td>-1.4845</td>
<td>OKAY</td>
<td>23.03</td>
<td>-1.6358</td>
<td>OKAY</td>
<td>23.7</td>
<td>-2.2974</td>
<td>OKAY</td>
<td>23.67</td>
</tr>
<tr>
<td>B03</td>
<td>DSC2</td>
<td>-1.2924</td>
<td>OKAY</td>
<td>21.51</td>
<td>-1.3013</td>
<td>OKAY</td>
<td>21.91</td>
<td>-1.7532</td>
<td>OKAY</td>
<td>21.83</td>
</tr>
<tr>
<td>B04</td>
<td>DSP</td>
<td>-1.4948</td>
<td>OKAY</td>
<td>22.66</td>
<td>-1.0644</td>
<td>OKAY</td>
<td>22.6</td>
<td>-1.9588</td>
<td>OKAY</td>
<td>22.44</td>
</tr>
<tr>
<td>B05</td>
<td>EGFR</td>
<td>1.1487</td>
<td>OKAY</td>
<td>24.85</td>
<td>-1.1892</td>
<td>OKAY</td>
<td>25.58</td>
<td>-1.2058</td>
<td>OKAY</td>
<td>24.9</td>
</tr>
<tr>
<td>B06</td>
<td>ERBB3</td>
<td>-1.2142</td>
<td>OKAY</td>
<td>22.86</td>
<td>-1.2834</td>
<td>OKAY</td>
<td>23.35</td>
<td>-1.6245</td>
<td>OKAY</td>
<td>22.97</td>
</tr>
</tbody>
</table>
Appendix 7: PCR Array Data

<table>
<thead>
<tr>
<th>B07</th>
<th>ESR1</th>
<th>-2.2815</th>
<th>B</th>
<th>35</th>
<th>-1.3755</th>
<th>B</th>
<th>34.71</th>
<th>2.4116</th>
<th>B</th>
<th>35</th>
</tr>
</thead>
<tbody>
<tr>
<td>B08</td>
<td>F11R</td>
<td>-1.3472</td>
<td>OKAY</td>
<td>23.33</td>
<td>-1.1096</td>
<td>OKAY</td>
<td>23.63</td>
<td>-1.4845</td>
<td>OKAY</td>
<td>23.56</td>
</tr>
<tr>
<td>B09</td>
<td>FGFBP1</td>
<td>-3.3173</td>
<td>B</td>
<td>35</td>
<td>2.4284</td>
<td>B</td>
<td>32.03</td>
<td>1.9725</td>
<td>B</td>
<td>35</td>
</tr>
<tr>
<td>B10</td>
<td>FN1</td>
<td>-3.249</td>
<td>OKAY</td>
<td>25.97</td>
<td>-1.2058</td>
<td>OKAY</td>
<td>24.87</td>
<td>-2.8879</td>
<td>OKAY</td>
<td>25.9</td>
</tr>
<tr>
<td>B11</td>
<td>FOXC2</td>
<td>1.007</td>
<td>B</td>
<td>35</td>
<td>1.3379</td>
<td>C</td>
<td>35</td>
<td>1.1019</td>
<td>C</td>
<td>35</td>
</tr>
<tr>
<td>B12</td>
<td>FZD7</td>
<td>-1.5801</td>
<td>OKAY</td>
<td>29.44</td>
<td>1.007</td>
<td>OKAY</td>
<td>29.62</td>
<td>-2.1735</td>
<td>A</td>
<td>30.91</td>
</tr>
<tr>
<td>C01</td>
<td>GNG11</td>
<td>-1.2397</td>
<td>C</td>
<td>35</td>
<td>1.3379</td>
<td>C</td>
<td>35</td>
<td>1.1019</td>
<td>C</td>
<td>35</td>
</tr>
<tr>
<td>C02</td>
<td>GSC</td>
<td>-2.1435</td>
<td>OKAY</td>
<td>26.99</td>
<td>-1.4241</td>
<td>OKAY</td>
<td>26.86</td>
<td>-3.249</td>
<td>OKAY</td>
<td>27.47</td>
</tr>
<tr>
<td>C03</td>
<td>GSK3B</td>
<td>-1.3379</td>
<td>OKAY</td>
<td>22.79</td>
<td>1.0425</td>
<td>OKAY</td>
<td>23</td>
<td>-1.257</td>
<td>OKAY</td>
<td>22.93</td>
</tr>
<tr>
<td>C04</td>
<td>IGFBP4</td>
<td>-1.2397</td>
<td>C</td>
<td>35</td>
<td>1.3379</td>
<td>C</td>
<td>35</td>
<td>1.1019</td>
<td>C</td>
<td>35</td>
</tr>
<tr>
<td>C05</td>
<td>IL1RN</td>
<td>-8.8766</td>
<td>OKAY</td>
<td>28.32</td>
<td>-1.0425</td>
<td>OKAY</td>
<td>26</td>
<td>-10.928</td>
<td>OKAY</td>
<td>28.21</td>
</tr>
<tr>
<td>C06</td>
<td>ILK</td>
<td>1.1647</td>
<td>OKAY</td>
<td>23.09</td>
<td>1.0425</td>
<td>OKAY</td>
<td>23.83</td>
<td>1.2311</td>
<td>OKAY</td>
<td>23.3</td>
</tr>
<tr>
<td>C07</td>
<td>ITGA5</td>
<td>-1.6472</td>
<td>OKAY</td>
<td>26.01</td>
<td>-1.007</td>
<td>OKAY</td>
<td>25.89</td>
<td>-1.3566</td>
<td>OKAY</td>
<td>25.87</td>
</tr>
<tr>
<td>C08</td>
<td>ITGAV</td>
<td>1.3947</td>
<td>OKAY</td>
<td>22.84</td>
<td>-1.3195</td>
<td>OKAY</td>
<td>24.11</td>
<td>1.1975</td>
<td>OKAY</td>
<td>23.24</td>
</tr>
<tr>
<td>C09</td>
<td>ITGB1</td>
<td>-1.0718</td>
<td>OKAY</td>
<td>20.09</td>
<td>-1.257</td>
<td>OKAY</td>
<td>20.96</td>
<td>-1.3379</td>
<td>OKAY</td>
<td>20.65</td>
</tr>
<tr>
<td>C10</td>
<td>JAG1</td>
<td>2.7321</td>
<td>OKAY</td>
<td>21.81</td>
<td>-1.2058</td>
<td>OKAY</td>
<td>23.93</td>
<td>2.114</td>
<td>OKAY</td>
<td>22.43</td>
</tr>
<tr>
<td>C11</td>
<td>KRT14</td>
<td>5.0281</td>
<td>B</td>
<td>30.83</td>
<td>1.3195</td>
<td>B</td>
<td>33.68</td>
<td>4.5315</td>
<td>B</td>
<td>31.08</td>
</tr>
<tr>
<td>C12</td>
<td>KRT19</td>
<td>4.2281</td>
<td>OKAY</td>
<td>28.05</td>
<td>1.1329</td>
<td>OKAY</td>
<td>29.72</td>
<td>2.6759</td>
<td>OKAY</td>
<td>27.66</td>
</tr>
<tr>
<td>D01</td>
<td>KRT7</td>
<td>-1.2311</td>
<td>B</td>
<td>33.04</td>
<td>1.7171</td>
<td>B</td>
<td>34.93</td>
<td>3.5064</td>
<td>B</td>
<td>33.06</td>
</tr>
<tr>
<td>D02</td>
<td>MAP1B</td>
<td>-1.4641</td>
<td>OKAY</td>
<td>22.9</td>
<td>-1.1019</td>
<td>OKAY</td>
<td>22.51</td>
<td>-1.1975</td>
<td>OKAY</td>
<td>22.74</td>
</tr>
<tr>
<td>D03</td>
<td>MMP2</td>
<td>-1.2397</td>
<td>C</td>
<td>35</td>
<td>1.8532</td>
<td>B</td>
<td>35</td>
<td>1.3851</td>
<td>B</td>
<td>35</td>
</tr>
<tr>
<td>D04</td>
<td>MMP3</td>
<td>-1.7777</td>
<td>B</td>
<td>33.16</td>
<td>-3.4822</td>
<td>B</td>
<td>34.44</td>
<td>-2.0279</td>
<td>B</td>
<td>32.58</td>
</tr>
<tr>
<td>D05</td>
<td>MMP9</td>
<td>-1.1096</td>
<td>B</td>
<td>33</td>
<td>2.2658</td>
<td>B</td>
<td>31.89</td>
<td>1.7777</td>
<td>B</td>
<td>33.09</td>
</tr>
<tr>
<td>D06</td>
<td>MSN</td>
<td>-1.0943</td>
<td>OKAY</td>
<td>24.21</td>
<td>-1.257</td>
<td>OKAY</td>
<td>24.86</td>
<td>-1.257</td>
<td>OKAY</td>
<td>24.47</td>
</tr>
</tbody>
</table>
### Appendix 7: PCR Array Data

<table>
<thead>
<tr>
<th>ID</th>
<th>Gene</th>
<th>Fold Change</th>
<th>p-value</th>
<th>Adjusted p-value</th>
<th>Expression</th>
<th>ID</th>
<th>Gene</th>
<th>Fold Change</th>
<th>p-value</th>
<th>Adjusted p-value</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>D07</td>
<td>MST1R</td>
<td>-2.4794</td>
<td>B</td>
<td>33.77</td>
<td>-1.1892</td>
<td>B</td>
<td>33.18</td>
<td>1.1647</td>
<td>B</td>
<td>34.05</td>
<td></td>
</tr>
<tr>
<td>D08</td>
<td>NODAL</td>
<td>-3.0738</td>
<td>B</td>
<td>32.69</td>
<td>2.2346</td>
<td>A</td>
<td>30.31</td>
<td>-1.815</td>
<td>B</td>
<td>30.71</td>
<td></td>
</tr>
<tr>
<td>D09</td>
<td>NOTCH1</td>
<td>-2.5491</td>
<td>OKAY</td>
<td>28.31</td>
<td>-1.329</td>
<td>OKAY</td>
<td>28.21</td>
<td>-2.042</td>
<td>OKAY</td>
<td>28.2</td>
<td></td>
</tr>
<tr>
<td>D10</td>
<td>NUDT13</td>
<td>-1.057</td>
<td>OKAY</td>
<td>27</td>
<td>-1.021</td>
<td>OKAY</td>
<td>27.91</td>
<td>-1.3755</td>
<td>OKAY</td>
<td>27.89</td>
<td></td>
</tr>
<tr>
<td>D11</td>
<td>OCLN</td>
<td>-3.0738</td>
<td>OKAY</td>
<td>23.69</td>
<td>1.0867</td>
<td>OKAY</td>
<td>22.9</td>
<td>-3.0314</td>
<td>OKAY</td>
<td>23.45</td>
<td></td>
</tr>
<tr>
<td>D12</td>
<td>PDGFRB</td>
<td>1.057</td>
<td>B</td>
<td>33.56</td>
<td>1.3472</td>
<td>B</td>
<td>33.89</td>
<td>-1.1251</td>
<td>B</td>
<td>34.14</td>
<td></td>
</tr>
<tr>
<td>E01</td>
<td>PLEK2</td>
<td>-2.1287</td>
<td>B</td>
<td>31.79</td>
<td>1.454</td>
<td>B</td>
<td>31.67</td>
<td>-1.2924</td>
<td>B</td>
<td>32.63</td>
<td></td>
</tr>
<tr>
<td>E02</td>
<td>PPPDE2</td>
<td>-1.2226</td>
<td>OKAY</td>
<td>22.83</td>
<td>-1.0425</td>
<td>OKAY</td>
<td>23.21</td>
<td>-1.2483</td>
<td>OKAY</td>
<td>22.98</td>
<td></td>
</tr>
<tr>
<td>E03</td>
<td>PTK2</td>
<td>-1.5052</td>
<td>OKAY</td>
<td>23.97</td>
<td>-1.1728</td>
<td>OKAY</td>
<td>24.19</td>
<td>-1.6245</td>
<td>OKAY</td>
<td>24.03</td>
<td></td>
</tr>
<tr>
<td>E05</td>
<td>RAC1</td>
<td>-1.1251</td>
<td>OKAY</td>
<td>20.69</td>
<td>-1.0497</td>
<td>OKAY</td>
<td>21.28</td>
<td>-1.0718</td>
<td>OKAY</td>
<td>20.9</td>
<td></td>
</tr>
<tr>
<td>E06</td>
<td>RGS2</td>
<td>-5.4264</td>
<td>B</td>
<td>32.65</td>
<td>-1.5263</td>
<td>B</td>
<td>32.97</td>
<td>-6.9644</td>
<td>B</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>E07</td>
<td>SERPINE1</td>
<td>-1.2397</td>
<td>C</td>
<td>35</td>
<td>1.3379</td>
<td>C</td>
<td>32.92</td>
<td>2.2501</td>
<td>B</td>
<td>33.89</td>
<td></td>
</tr>
<tr>
<td>E08</td>
<td>SIP1</td>
<td>-1.8277</td>
<td>OKAY</td>
<td>24.29</td>
<td>-1.2483</td>
<td>OKAY</td>
<td>24.31</td>
<td>-1.8532</td>
<td>OKAY</td>
<td>24.62</td>
<td></td>
</tr>
<tr>
<td>E09</td>
<td>SMAD2</td>
<td>-1.2834</td>
<td>OKAY</td>
<td>23.18</td>
<td>-1.0497</td>
<td>OKAY</td>
<td>23.48</td>
<td>-1.3013</td>
<td>OKAY</td>
<td>23.24</td>
<td></td>
</tr>
<tr>
<td>E10</td>
<td>SNAI1</td>
<td>-4.4076</td>
<td>B</td>
<td>32.81</td>
<td>-1.4845</td>
<td>B</td>
<td>32.29</td>
<td>-2.5847</td>
<td>B</td>
<td>32.92</td>
<td></td>
</tr>
<tr>
<td>E12</td>
<td>SNAI3</td>
<td>1.0718</td>
<td>OKAY</td>
<td>30.81</td>
<td>-1.2142</td>
<td>OKAY</td>
<td>30.24</td>
<td>-1.7532</td>
<td>OKAY</td>
<td>30.06</td>
<td></td>
</tr>
<tr>
<td>F01</td>
<td>SOX10</td>
<td>-1.1728</td>
<td>B</td>
<td>35</td>
<td>1.8277</td>
<td>B</td>
<td>35</td>
<td>-1.4439</td>
<td>B</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>F03</td>
<td>SPP1</td>
<td>-1.2397</td>
<td>C</td>
<td>33.91</td>
<td>1.8921</td>
<td>B</td>
<td>33.87</td>
<td>1.5583</td>
<td>B</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>F04</td>
<td>STAT3</td>
<td>1.1567</td>
<td>OKAY</td>
<td>21.9</td>
<td>-1.1096</td>
<td>OKAY</td>
<td>22.71</td>
<td>1.057</td>
<td>OKAY</td>
<td>22.12</td>
<td></td>
</tr>
<tr>
<td>F05</td>
<td>STEAP1</td>
<td>1.1567</td>
<td>OKAY</td>
<td>19.12</td>
<td>-1.0497</td>
<td>OKAY</td>
<td>20.03</td>
<td>1.0792</td>
<td>OKAY</td>
<td>19.58</td>
<td></td>
</tr>
<tr>
<td>F06</td>
<td>TCF3</td>
<td>-1.9053</td>
<td>OKAY</td>
<td>23.97</td>
<td>-1.0718</td>
<td>OKAY</td>
<td>23.83</td>
<td>-2.0562</td>
<td>OKAY</td>
<td>23.97</td>
<td></td>
</tr>
</tbody>
</table>
## Appendix 7: PCR Array Data

<table>
<thead>
<tr>
<th>Code</th>
<th>Gene</th>
<th>Fold Change</th>
<th>P Value</th>
<th>Fold Change</th>
<th>P Value</th>
<th>Fold Change</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>F07</td>
<td>TCF4</td>
<td>-3.5554</td>
<td>OKAY</td>
<td>28.8</td>
<td>-1.1975</td>
<td>OKAY</td>
<td>28.42</td>
</tr>
<tr>
<td>F08</td>
<td>TFPI2</td>
<td>-1.2397</td>
<td>C</td>
<td>35</td>
<td>1.3379</td>
<td>C</td>
<td>35</td>
</tr>
<tr>
<td>F09</td>
<td>TGFβ1</td>
<td>-3.4822</td>
<td>B</td>
<td>33.59</td>
<td>2.9282</td>
<td>B</td>
<td>31.8</td>
</tr>
<tr>
<td>F10</td>
<td>TGFβ2</td>
<td>1.9588</td>
<td>B</td>
<td>32.98</td>
<td>5.7358</td>
<td>B</td>
<td>32.63</td>
</tr>
<tr>
<td>F11</td>
<td>TGFβ3</td>
<td>-2.639</td>
<td>B</td>
<td>33.54</td>
<td>1.5052</td>
<td>B</td>
<td>31.44</td>
</tr>
<tr>
<td>F12</td>
<td>TIMP1</td>
<td>-1.2397</td>
<td>C</td>
<td>35</td>
<td>1.8921</td>
<td>B</td>
<td>35</td>
</tr>
<tr>
<td>G01</td>
<td>TMEFF1</td>
<td>-1.9588</td>
<td>OKAY</td>
<td>30.02</td>
<td>1.2746</td>
<td>OKAY</td>
<td>29.12</td>
</tr>
<tr>
<td>G02</td>
<td>TMEF132A</td>
<td>1.057</td>
<td>OKAY</td>
<td>25.14</td>
<td>-1.3195</td>
<td>OKAY</td>
<td>26.33</td>
</tr>
<tr>
<td>G03</td>
<td>TSPAN13</td>
<td>1.4948</td>
<td>OKAY</td>
<td>21.09</td>
<td>1.007</td>
<td>OKAY</td>
<td>22.14</td>
</tr>
<tr>
<td>G04</td>
<td>TWIST1</td>
<td>5.0281</td>
<td>OKAY</td>
<td>22.96</td>
<td>-1.0718</td>
<td>OKAY</td>
<td>25.78</td>
</tr>
<tr>
<td>G05</td>
<td>VCAN</td>
<td>-1.2397</td>
<td>C</td>
<td>35</td>
<td>4.6589</td>
<td>B</td>
<td>35</td>
</tr>
<tr>
<td>G06</td>
<td>VIM</td>
<td>-1.1975</td>
<td>OKAY</td>
<td>26.69</td>
<td>1.1647</td>
<td>OKAY</td>
<td>27.03</td>
</tr>
<tr>
<td>G07</td>
<td>VPS13A</td>
<td>-1.5583</td>
<td>OKAY</td>
<td>23.61</td>
<td>-1.1487</td>
<td>OKAY</td>
<td>23.65</td>
</tr>
<tr>
<td>G08</td>
<td>WNT11</td>
<td>-1.5583</td>
<td>B</td>
<td>35</td>
<td>1.0644</td>
<td>B</td>
<td>35</td>
</tr>
<tr>
<td>G09</td>
<td>WNT5A</td>
<td>-1.3472</td>
<td>B</td>
<td>26</td>
<td>-1.5692</td>
<td>OKAY</td>
<td>27.03</td>
</tr>
<tr>
<td>G10</td>
<td>WNT5B</td>
<td>3.0314</td>
<td>B</td>
<td>35</td>
<td>6.6807</td>
<td>B</td>
<td>35</td>
</tr>
<tr>
<td>G11</td>
<td>ZEB1</td>
<td>-2.8481</td>
<td>B</td>
<td>35</td>
<td>2.9282</td>
<td>B</td>
<td>32.78</td>
</tr>
<tr>
<td>G12</td>
<td>ZEB2</td>
<td>-1.2397</td>
<td>C</td>
<td>35</td>
<td>1.8025</td>
<td>B</td>
<td>35</td>
</tr>
<tr>
<td>H01</td>
<td>ACTB</td>
<td>1.3195</td>
<td>OKAY</td>
<td>16.55</td>
<td>-1.1173</td>
<td>OKAY</td>
<td>17.6</td>
</tr>
<tr>
<td>H02</td>
<td>B2M</td>
<td>2.7702</td>
<td>OKAY</td>
<td>18.76</td>
<td>1.1647</td>
<td>OKAY</td>
<td>20.59</td>
</tr>
<tr>
<td>H03</td>
<td>GAPDH</td>
<td>-1.4845</td>
<td>OKAY</td>
<td>18.04</td>
<td>-1.0644</td>
<td>OKAY</td>
<td>18.42</td>
</tr>
<tr>
<td>H04</td>
<td>HPRT1</td>
<td>-1.7901</td>
<td>OKAY</td>
<td>23.94</td>
<td>-1.1251</td>
<td>OKAY</td>
<td>23.86</td>
</tr>
<tr>
<td>H05</td>
<td>RPLP0</td>
<td>-1.3755</td>
<td>OKAY</td>
<td>15.87</td>
<td>1.1487</td>
<td>OKAY</td>
<td>15.79</td>
</tr>
<tr>
<td>H06</td>
<td>HGDC</td>
<td>-1.2397</td>
<td>C</td>
<td>35</td>
<td>1.3379</td>
<td>C</td>
<td>35</td>
</tr>
</tbody>
</table>
## Appendix 7: PCR Array Data

<p>| | | | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>H07</td>
<td>RTC</td>
<td>-1.0425</td>
<td>OKAY</td>
<td>25.64</td>
<td>1.434</td>
<td>OKAY</td>
<td>25.6</td>
<td>1.1728</td>
</tr>
<tr>
<td>H08</td>
<td>RTC</td>
<td>-1.1728</td>
<td>OKAY</td>
<td>25.56</td>
<td>1.3472</td>
<td>OKAY</td>
<td>25.68</td>
<td>1.1329</td>
</tr>
<tr>
<td>H09</td>
<td>RTC</td>
<td>-1.2397</td>
<td>OKAY</td>
<td>25.63</td>
<td>1.1019</td>
<td>OKAY</td>
<td>25.78</td>
<td>-1.021</td>
</tr>
<tr>
<td>H10</td>
<td>PPC</td>
<td>-1.2746</td>
<td>OKAY</td>
<td>20.46</td>
<td>1.2311</td>
<td>OKAY</td>
<td>20.48</td>
<td>1.1487</td>
</tr>
<tr>
<td>H11</td>
<td>PPC</td>
<td>-1.3566</td>
<td>OKAY</td>
<td>20.45</td>
<td>1.3013</td>
<td>OKAY</td>
<td>20.5</td>
<td>1.007</td>
</tr>
<tr>
<td>H12</td>
<td>PPC</td>
<td>-1.3472</td>
<td>OKAY</td>
<td>20.44</td>
<td>1.3287</td>
<td>OKAY</td>
<td>20.5</td>
<td>1.0425</td>
</tr>
</tbody>
</table>
Appendix 8 – PCR Array Amplification and Melt Curves
Figure A8.1: RT-qPCR amplification and melt curves of (A) EMT PCR array 1 and (B) EMT PCR array 2. (i) Amplification curves indicated Ct values between 16 and 40. (ii) Melt curve analysis of the amplified PCR products identified single products in all cases. Positive amplification (red), unsure/late amplification (green) and negative amplification (blue) are indicated. mRNA samples were reverse transcribed twice with qPCR performed in singlelicate per each gene/treatment. Internal array quality control (i.e. reverse transcription, positive and negative PCR, housekeeping controls) for each PCR array was verified using the RT² Profiler™ PCR array data analysis (Version 3.5).
Appendix 9 – ETS1p51 Sequencing

Chromatograms