REGULATION OF THE ITGA2 GENE IN PROSTATE CANCER

A thesis submitted in fulfilment of the requirements of the degree of Doctor of Philosophy

by

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DECLARATIONS

Declarations of Originality

This thesis contains no material which has been accepted for a degree or diploma by the University of Tasmania or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

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Statement of Ethical Conduct

The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University.

Paulynn Chin Suyin

STATEMENT OF CO-AUTHORSHIP

At the time of writing, the following publications have arisen from the data presented in this thesis:

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ABBREVIATIONS

µg	microgram
µL	microlitre
µm	micrometre
µM	micromolar
Ac	acetylation
AP2	activator protein 2
APC	adenomatous polyposis coli
AR	androgen receptor
ARE	androgen response element
ASAP	atypical small acinar proliferation
AzaC	5-Aza-2'-deoxycytidine
B2M	beta-2 microglobulin
bHLH	basic helix-loop-helix
bp	base pair
BPH	benign prostatic hyperplasia
BRCA1	breast cancer 1
BSA	bovine serum albumin
CDH1	cadherin-1
cDNA	complementary DNA
CHART-PCR	chromatin accessibility by real-time PCR
ChIP	chromatin immunoprecipitation
CHX	cycloheximide
CpG	5' CG 3'
CTCF	CCTC-binding factor
DDR1	Discoidin domain receptor 1
DHT/ Androgen	5α-Androstan-17β-ol-3-one
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
Dnase	deoxyribonuclease
DNMT	DNA methyltransferase
DNMTi	DNA methyltransferase inhibitor
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
E-box	enhancer box
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epithelial growth factor
EGTA	ethylene glycol tetraacetic acid

EMT	Epithelial to mesenchymal transition
EZH2	enhancer of Zeste homologue 2
FAK	focal adhesion kinase
FCS	foetal calf serum
FGF	fibroblast growth factor
g	grams
g	relative centrifugal field
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GpC	5' GC 3'
GRP78	glucose-regulated protein, 78kD
GSTP1	glutathione S-transferase pi gene
H2A	histone H2A
H2B	histone H2B
H3	histone H3
H4	histone H4
HDAC	histone deacetylase
HDACi	histone deacetylase inhibitor
HDM	histone demethylating enzyme
HGF	hepatocyte growth factor
hKLK2	human glandular kallikrein-1
HMT	histone methyltransferase
hr	hour
ICAM	Intercellular Adhesion Molecule
ITGA2	integrin alpha 2, α2
ITGA3	integrin alpha 3, α3
ITGB1	integrin beta 1, β1
kb	kilobase
LINE-1	long interspersed nuclear element-1
LLC	Lewis lung carcinoma
LSD1	Lysine specific demethylase 1A
mg	milligram
MGMT	O-6-methylguanine-DNA methyltransferase
mL	millilitre
MLH1	mut homologue 1
mM	millimolar
MMPs	Matrix metalloproteinases
Mnase	micrococcal nuclease
mRNA	messenger RNA
NDR	nucleosome depleted region
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells

nm	nanometre
nM	nanomolar
nmol	nanomol
NoMe-seq	nucleosome occupancy and methylome sequencing
Notch-1	Notch homologue 1
NT	no treatment
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF-D	Platelet-Derived Growth Factor D
PIN	prostatic intraepithelial neoplasia
PLGF	placental growth factor
pmol	picomolar
PSA	prostate specific antigen
RASSF1A	Ras association domain family 1 isoform A
RGD	arginine-glycine-aspartic acid
RNA	ribonucleic acid
rRNA	ribosomal RNA
RTK	receptor tyrosine kinase
SCID	severe combined immunodeficiency
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
siRNA	small interfering RNA
SNPs	single nucleotide polymorphism
Sp1	specific protein 1
STK11	serine/ threonine kinase 11
TE	Tris/ EDTA buffer
TGF-β	transforming growth factor beta
TI	total input
TNT	Tris/ NaCl/ Tween buffer
Tris	Tris(hydroxymethyl)aminomethane
TSA	Trichostatin A
TSS	transcription start site
U	unit
uPA	Urinary plasminogen activator
V	volt
VCAM	vascular cell adhesion protein
VEGFR1	vascular endothelial growth factor receptor 1
VLA-2	very late activation antigen 2
WT	wild-type
WTI	Wilms' tumour gene 1

X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
ZEB	Zinc finger E-box-binding homeobox
ZEB1	Zinc finger E-box-binding homeobox 1
ZEB2	Zinc finger E-box-binding homeobox 2

ABSTRACT

Prostate cancer is the most commonly diagnosed cancer in males and is the second leading cause of cancer deaths in men after skin cancer. It is still associated with significant mortality and morbidity and statistics indicate that 80% of prostate cancer deaths have metastatic bony lesions. Previous work from our group has identified integrin alpha 2 (*ITGA2*) as a putative prostate cancer susceptibility gene through a familial genetic study. There is an increasing body of evidence suggesting it is involved in prostate cancer progression, particularly contributing to the preferential metastasis of prostate cancer cells to the bone. Integrins are surface receptors which play important roles in cell migration, invasion, survival and angiogenesis. Altered expression of integrins has been found to mediate tumour cell invasion and metastasis in a range of cancers including prostate cancer. Recently, it has become evident that epigenetic mechanisms play important roles in the progression of prostate cancer, affecting a large number of genes associated with the disease, which leads to the hypothesis that deregulation of *ITGA2* expression by epigenetic alterations may be associated with prostate tumour progression and metastasis. To examine this hypothesis, regulation of the *ITGA2* gene was investigated in a panel of prostate cell lines which represents different aspects of prostate cancer biology.

The *ITGA2* promoter is associated with a large CpG island and reduced methylation at the *ITGA2* promoter was observed in the bone metastatic cell line, PC3 when compared to the non-tumorigenic cell line, LNCaP and the benign prostate cell line, PWR-1E. Reduced methylation correlated with increased *ITGA2* expression levels in these cell lines. Chromatin accessibility and histone acetylation at the *ITGA2* promoter was found to be higher in the more highly expressing PC3 cell line. A lower percentage of nucleosome occupancy at the transcription start site also correlated with the higher expression of *ITGA2* in 22Rv1 as compared to LNCaP cells. In addition, inhibition of DNA methylation and histone acetylation in combination increased *ITGA2* expression in LNCaP cells. These data are consistent with DNA methylation, nucleosome occupancy and histone acetylation contributing to *ITGA2* regulation.

Higher *ITGA2* gene expression levels were also found to correlate with higher cell migration capacity. This was consistent with LNCaP cells displaying an epithelial-like phenotype while the highly expressing PC3 cells displayed a mesenchymal-like phenotype. Knockdown of *ITGA2* in the metastatic prostate cancer cell line, PC3, resulted in reduced cell migration, without affecting epithelial to mesenchymal transition (EMT). However, selection of cells with increased *ITGA2* expression by serial passaging of cells on collagen matrix correlated with altered expression of transcription factors known to modulate EMT. Higher *ITGA2* expression correlated with increased Twist and decreased Snail expression.

Further examination of the regulation of *ITGA2* gene by transcription factors suggests that the Sp1 transcription factor activates *ITGA2* expression. However, Sp1 is expressed at equivalent levels in both LNCaP and PC3 cells and thus, can not account for the differential *ITGA2* expression observed. While Twist showed inconsistent activity, androgen treatment and Snail overexpression repressed *ITGA2* promoter activity. Since Snail is more highly expressed in LNCaP as compared to PC3 cells, this could explain the lower *ITGA2* expression observed in LNCaP compared to PC3 cells.

Overall, *ITGA2* has been shown by others to be involved in the selective metastasis of prostate cancer to the bone, which is the major cause of prostate cancer related death. *ITGA2* may therefore represent a potential therapeutic target in metastatic prostate cancer. This study has shown that both epigenetic factors involving DNA methylation, histone acetylation and nucleosome occupancy and the transcription factors, Sp1, Snail and androgen receptor cooperate to regulate *ITGA2* gene expression. Further, the data presented suggest that alterations to epigenetic factors and/ or the EMT transcription factor Snail may contribute to aberrant *ITGA2* expression during prostate cancer metastasis.

Chapter 1

INTRODUCTION

1.1 PROSTATE CANCER

Prostate cancer is the most commonly diagnosed invasive cancer in men and is the second leading cause of cancer deaths in men after lung cancer. According to the report by the Australian Institute of Health and Welfare, in 2009, there were 21 808 new cases of prostate cancer and it is expected to reach 25 000 new cases per year in 2020. Despite considerable advances in prostate cancer research, this cancer is still associated with significant mortality and morbidity (Rider et al. 2012). The risk factors involved in the development of prostate cancer include advancing age, race and family history. If detected in the early stage of disease, prostate cancer is considered curable by surgical excision methods, radiotherapy and androgen deprivation therapy (Fleshner et al. 2010). However, in a percentage of men disease recurs, is frequently refractory to treatment and this is associated with poor prognosis. It is thought there is a population of prostate tumour cells that have the capacity to invade and metastasize, with bone being the most common metastatic site. Autopsy studies have found that more than 80% of men who die of prostate cancer have metastatic bony lesions (Bubendorf et al. 2000).

The current prostate specific antigen (PSA) screening tool has allowed early detection of prostate cancer, when still locally confined (Paquette et al. 2002). PSA is a protein produced by the cells in the prostate gland. The PSA screening tool measures the level of PSA in the blood where a high PSA level is indicative of the presence of cancer (Gann et al. 1995). However, benign conditions may also show elevated levels of PSA (Nadler et al. 1995). Therefore, the PSA screening tool has significant limitations resulting in a false positive rate of 80% when PSA cutoff is between 2.5 and 4.0 µg/L (Wolf et al. 2010). Further, it is unable to distinguish the aggressive tumours requiring immediate intervention from those that are more appropriately managed by regular surveillance. Thus, there is considerable interest in identifying and discovering new prognostic and

diagnostic markers for prostate cancer, particularly markers that can identify those tumours likely to progress to a more aggressive state.

Prostatic intraepithelial neoplasia (PIN), in particular high-grade PIN has been identified as a precursor to prostate cancer. High-grade PIN is an abnormal condition of the prostate gland and is considered a pre-malignant condition. Studies have reported that approximately 30% of men with high-grade PIN lesions will develop prostate cancer (Lefkowitz et al. 2002). Atypical small acinar proliferation (ASAP) is also a precursor to prostate cancer. ASAP lesions mimic cancer and have been found to be strongly predictive of subsequent prostate cancer, with approximately 60% of men with ASAP found to subsequently develop prostate cancer (Bostwick et al. 2006). The progression of prostate cancer may be driven by the accumulation of genetic and epigenetic changes, leading to the activation of oncogenes and inactivation of tumour suppressor genes (De Marzo et al. 2007). These changes lead to the development of PIN and ASAP which may progress into localised invasive cancer and finally metastatic tumours (Figure 1.1).

The process of the onset of invasion and metastasis is a multistep process. Firstly, this involves the detachment of tumour cells from the primary site by disruption of the basement membrane barrier followed by invasion through the basement membrane, intravasation into the blood vessels, extravasation from the blood vessels and finally, formation of secondary metastases (Geiger et al. 2009; Yang et al. 2004). Recently, the epithelial-mesenchymal transition (EMT) process has been proposed to be involved in facilitating tumour cell migration and many studies have suggested that activation of EMT is a critical event in the progression of a benign tumour into a malignant tumour (Thiery 2002).

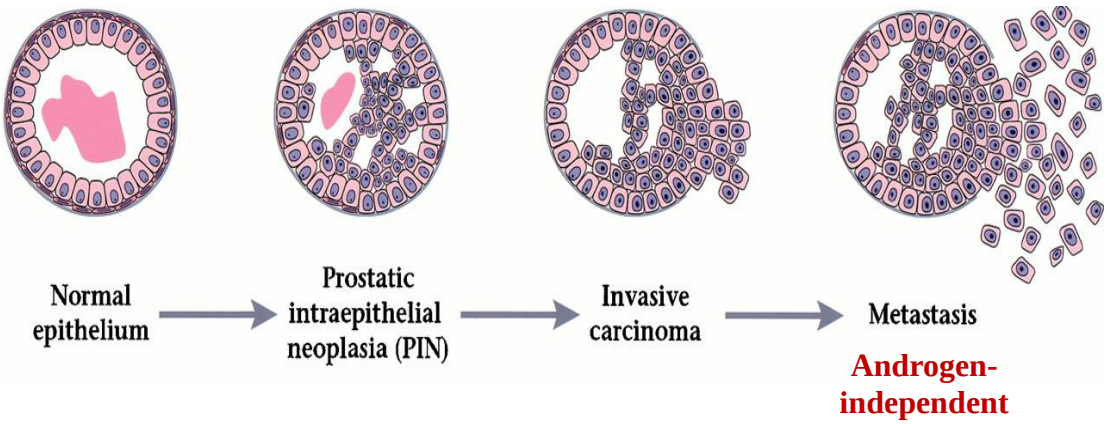

Figure 1.1 Development and progression pathway for human prostate cancer. Adapted from Abate Shen et al. (2000).

1.2 EPITHELIAL-MESENCHYMAL TRANSITION

(This section has been substantially published in Chin et al. 2013)

Epithelial cells are organised in layers of cells that are maintained by cell-cell interactions involving tight junctions and desmosomes. In addition, these cells have apical-basolateral polarity. In contrast, mesenchymal cells are irregular, elongated, spindle-shaped and do not have cell-cell contacts nor apical-basolateral polarity but have distinct cell-extracellular matrix (ECM) interactions and cytoskeletal structures and are highly motile. The transition of an epithelial cell into a mesenchymal cell is known as epithelial-mesenchymal transition (EMT). EMT involves a series of events where the cell-cell and cell-ECM interactions are altered by degradation of underlying basement membrane resulting in detachment of epithelial cells from the surrounding tissue followed by rearrangement of the cytoskeleton to confer the ability to move through a three-dimensional ECM and the induction of a series of new transcriptional signalling pathways to maintain the mesenchymal phenotype (Radisky 2005). This process is important in embryonic development, particularly in gastrulation and segment formation. Activation of EMT is also involved in tissue repair and more recently, EMT has been implicated in carcinogenesis. EMT is a reversible event, and the reverse process, known as mesenchymal-epithelial transition (MET) is involved in the formation of epithelial organs by mesenchymal cells (Thiery 2002). *In vivo* and *in vitro* studies have shown that tumour cells can undergo partial or complete EMT, showing loss of epithelial phenotype and gaining mesenchymal characteristics. EMT facilitates migration by disrupting the polarity of epithelial cells leading to the loss of cell adhesion, allowing tumour cells to invade through the basement membrane.

1.2.1 Regulation of EMT

EMT involves a series of signalling processes. Firstly, it involves the break-down of cell-cell interactions leading to loss of E-cadherin expression and the upregulation of mesenchymal markers such as N-cadherin, vimentin and the transcription factors Snail,

Twist and ZEB family members. Then, it is followed by a loss of cell polarisation and cytoskeleton remodelling. Finally, changes in cell adhesion occur leading to cell detachment and the activation of proteolytic enzymes, matrix metalloproteinases (MMPs) (Tomita et al. 2000). The initiation of EMT is tissue and context dependent and may not involve all EMT markers (Umbas et al. 1992). There are various stimuli from outside the cell which regulate EMT within the tumour microenvironment. These include the binding of transforming growth factor-β (TGFβ) to the TGFβ receptor (TGFβr), growth factors such as epidermal growth factor (EGF), fibroblast growth factor (FGF) and hepatocyte growth factor (HGF) which bind to their cognate kinase receptors (TKR), the highly conserved Wnt/β-catenin pathway and also integrin signalling which activates the focal adhesion kinase (FAK) signalling pathway (Gould Rothberg et al. 2006). Since integrins are involved in cell adhesion and signalling, it is possible that integrins can initiate and mediate EMT and invasion in tumour progression (Figure 1.2). These various signalling pathways then activate a range of EMT-inducing transcription factors such as Snail, Slug, Twist, zinc finger E-box binding homeobox 1 and 2 (ZEB1 and ZEB2).

1.3 INTEGRINS

(This section has been substantially published in Chin et al. 2013)

Integrins play important roles in normal prostate development where they are involved in the interaction of the prostate epithelial cells with the ECM and also influence cell signalling, growth, survival and differentiation (Koistinen et al. 2000). In addition, the transition from a normal prostate gland to the formation of PIN and to invasive and metastatic cancers involves alterations in these cell surface adhesive receptors, integrins (Bonkhoff et al. 1993; Haywood-Reid et al. 1997; Knox et al. 1994; Murant et al. 1997). During metastasis, changes in integrin expression result in changes in the tumour cell adhesion to adjacent cells and to the ECM leading to increased cell motility. Thus, integrins are key players in metastatic events since they mediate cell to cell (homotypic) and cell to ECM (heterotypic) interactions of prostate cells and EMT is likely important in this process. During EMT, tumour cells switch from cell-cell to cell-matrix adhesion

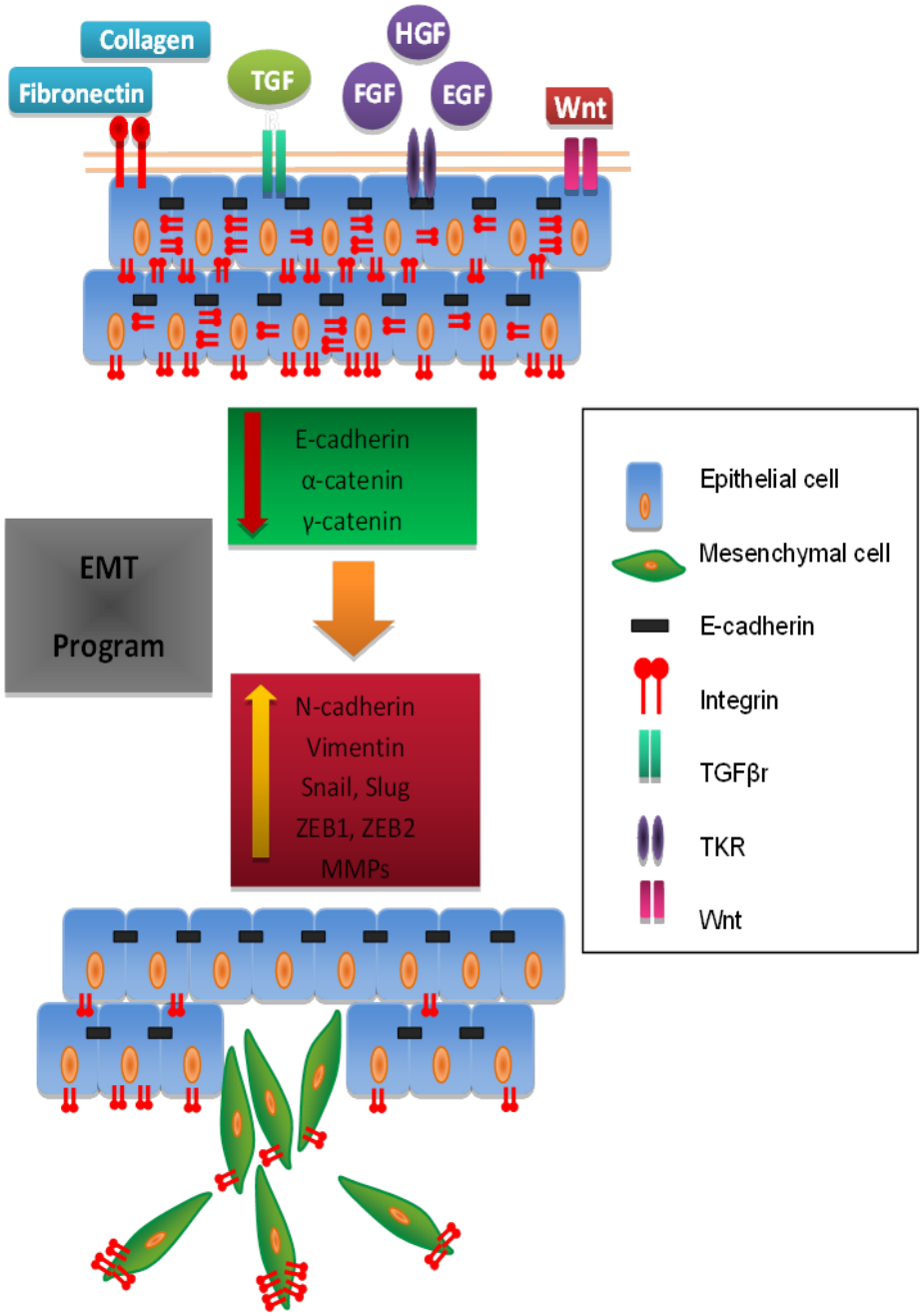

Figure 1.2 Schematic representation of the EMT process and the roles of integrins in cell adhesion and migration. Adapted from Chin et al. (2013).

thus mediating cell migration. As integrins are involved in cell-matrix adhesion, changes in integrins may occur during EMT.

Integrins belong to a superfamily of transmembrane glycoprotein receptors involved in mediating cell to cell and cell to ECM interactions. They exist as heterodimers composed of α and β subunits bound by non-covalent bonds. To date, 18 α subunits and 8 β subunits have been identified in humans, which can associate to form 24 unique complexes (Table1.1) with the different αβ combinations possessing distinct ligand binding specificities (Hynes 1992; Rosales et al. 1995). There are three distinct regions in each integrin subunit with each subunit containing an extracellular domain, a transmembrane domain and a short intracellular domain.

The extracellular regions of the α and β subunits together form the ligand binding site. The most common ligands for integrins are large ECM proteins such as laminin, fibronectin, collagen and vitronectin. These ECM proteins (except for laminin and collagen) have a common arginine-glycine-aspartic acid (RGD) motif, whereas integrins recognise laminin and collagen through cryptic RGD sites. In addition, there are some integrins that interact with other adhesion molecules such as cadherins, intracellular adhesion molecules (ICAMs) and vascular adhesion molecules (VCAMs), expressed on leukocytes and endothelial cells. However, integrins can frequently bind several ligands (as outlined in Table 1.1), permitting redundancy in signalling as multiple integrins are generally present on any particular cell surface.

As an integrin binds to its ligand, it undergoes structural changes which affect the ligand binding affinity (Hughes et al. 1997). This affinity is also determined by the cytoplasmic signals from within the cell which affects the molecular interactions at the integrin cytoplasmic domain influencing the degree of cell adhesion. This is referred to as inside-out signalling. Integrins also play a role in signal transduction where they transduce extracellular signals to the interior of the cell, referred to as outside-in signalling. Such signalling can affect cell migration, differentiation, survival and proliferation (Giancotti et al. 1999; Hood et al. 2002; Lee et al. 2004). When bound to the ECM proteins,

Integrin	Ligand	Ligand receptors
α1β1	Collagen, Laminin	Collagen receptors
α2β1	Collagen, Laminin	
α10β1	Collagen	
α11β1	Collagen	
α3β1	Laminin, Collagen, Fibronectin	Laminin receptors
α6β1	Laminin, Merosis, Kalinin	
α7β1	Laminin	
α6β4	Laminin	
α4β1	Fibronectin, VCAM	RGD receptors
α5β1	Fibronectin	
α8β1	Fibronectin	
α9β1	Fibronectin, Tenascin, Laminin	
αvβ1	Fibronectin, Vitronectin	
αvβ3	Fibronectin, Vitronectin	
αvβ5	Vitronectin	
αvβ6	Fibronectin	
αvβ8	Fibronectin, Collagen, Laminin	
$\alpha_{IIb}\beta3$	Fibronectin, Vitronectin	
α4β7	Fibronectin, VCAM	Leukocyte binding
αEβ7	E-cadherin	
αDβ7	ICAM3, VCAM	
αLβ2	ICAM1-5	
αMβ2	ICAM1, VCAM, fibronogen	
αXβ2	Fibrinogen	

Table 1.1 List of integrins and their ligands. Adapted from Chin et al. (2013).

integrins recruit a range of adaptor proteins, and activate various signalling pathways. For example, integrin clustering activates the FAK, Src family kinases, Rac and Rho GTPases leading to the recruitment of cytoskeleton proteins such as talin, α-actinin, vinculin, paxillin and tensin (Ren et al. 2007). Activation of these kinase pathways and cytoskeleton proteins contributes to changes in cell architecture, adhesion and migration on the ECM (Desgrosellier et al. 2010).

1.3.1 Roles of Integrins in cancer progression

While integrins mediate cell attachment, ligation of integrins by the ECM proteins induces cell migration by generating the traction required for invasion. In cancer, expression of integrins that are involved in cell adhesion is frequently altered, leading to cell proliferation, migration and metastasis. Previous studies in which integrin expression levels were correlated to the different stages of human tumours and the pathological outcomes (metastasis, recurrence, survival), implicated a number of integrins in cancer progression (Gillan et al. 2002; Hall et al. 2008; Mitchell et al. 2010; Pontes-Junior et al. 2009; Ramirez et al. 2011; Reinmuth et al. 2003; Rolli et al. 2003; Saito et al. 2010; Sung et al. 1998; Tsuji et al. 2002; Zutter et al. 1995). These integrins include αvβ3, α2β1, α3β1 and α6β1. In contrast integrin α4β1 is associated with tumour suppression (Saramaki et al. 2006).

Integrin αvβ3 has been associated with tumour progression in a range of cancers including lung cancer, gastric cancer, breast cancer and prostate cancer (Mitchell et al. 2010; Pontes-Junior et al. 2009; Saito et al. 2010; Tsuji et al. 2002). Integrin αvβ3 remains the most well-studied integrin involved in tumour progression. Interestingly, integrin αvβ3 is usually only expressed in activated leukocytes, macrophages, platelets and osteoclasts and is not normally expressed in epithelial cells. It has been found to mediate adhesion of breast cancer cells to bone matrix and also facilitate migration of breast cancer cells on bone sialoprotein (Sung et al. 1998; van der et al. 1997). In colon cancer, blocking integrin αvβ3 resulted in a decrease in tumour metastasis and improved survival in mice (Reinmuth et al. 2003). This integrin was also found to bind to periostin,

which is upregulated in epithelial ovarian cancer cells, and to promote cell adhesion and migration (Gillan et al. 2002).

Changes in integrin α2β1 have also been associated with tumour progression where high expression of α2β1 was observed in ductules of normal breast, low or undetectable expression was observed in poorly differentiated adenocarcinomas and intermediate expression levels were detected in more well-differentiated adenocarcinomas (Zutter et al. 1990). These observations suggest that in breast cancer, decreased expression of α2β1 contributes to the malignant phenotype. In another study, loss of integrin α2β1 resulted in the induction of breast cancer cell metastasis *in vivo*, suggesting that integrin α2β1 is a metastasis suppressor (Ramirez et al. 2011). The re-expression of α2β1 in breast cancer cells reversed some of the tumorigenic properties of the cells (Zutter et al. 1995). In contrast, in prostate cancer, integrin α2β1 was found to induce prostate cancer cell metastasis to the bone (Hall et al. 2008). Thus, these studies suggest that integrin function is cell type and context dependent.

The development of integrin α2 knockout mice (Chen et al. 2002) has provided further insight into the role of α2β1 in tumorigenesis. These mice were viable, fertile and showed normal development most likely due to the compensatory mechanisms of other collagen-binding integrins (Zhanf et al. 2006b). However, when the α2-null mice were challenged with B16F10 melanoma cells, tumours in these mice grew rapidly with larger tumours and increased tumour angiogenesis correlating with upregulation of Vascular Endothelial Growth Factor Receptor 1 (*VEGFR1)* expression when compared to wild type mice. However, α2 knockout mice bearing Lewis Lung carcinoma (LLC) cells displayed no difference in tumour angiogenesis. Further analysis showed that integrin α2β1-dependent angiogenesis involves the secretion of placental growth factor (PLGF) which was produced by B16F10 cells but not the LLC cells. These data add weight to the notion that integrin expression is cell type and context dependent, where it depends on the interactions of the host factors with the surrounding microenvironment. Following this study, the α2 knockout mice were crossed with mice carrying the MMTV-*c-erbB2*/Neu oncogene, a spontaneous, clinically relevant mouse model of breast cancer, to generate

α2-null/Neu mice (Ramirez et al. 2011). α2-null/Neu mice displayed decreased mammary tumour latency, increased cancer metastasis and enhanced tumour cell intravasation, supporting the idea that α2β1 is a metastasis suppressor.

Knockdown of α2β1 expression by transfecting the human breast carcinoma cells T47D, with antisense mRNA resulted in decreased cell adhesion on collagen I and IV but adhesion to fibronectin and laminin were not affected (Keely et al. 1995). α2β1 expression has also been associated with cell migration. Cell attachment and migration were analysed in melanoma cell lines with cells that did not express α2β1 showing weak cell attachment and low cell migration ratse on both laminin and type IV collagen (Etoh et al. 1993). Blocking of either α2 or β1 using monoclonal antibodies resulted in inhibition of cell migration but no detectable cell detachment. Other integrin blocking antibodies such as anti-α3 and anti-α6 did not affect cell migration and attachment, suggesting that cell migration on laminin and collagen IV is mainly mediated by α2β1.

1.3.2 Roles of integrins in prostate cancer progression

Integrins are expressed in normal prostate basal cells and are required for the interaction of the cells with surrounding stroma which influences their growth, survival and differentiation potential. These integrins include α2β1, α3β1, α5β1 and α6β4 (Bonkhoff et al. 1993; Collins et al. 2001; Davis et al. 2001b; Knox et al. 1994; Nagle et al. 1995). Altered expression of integrins affects cell adhesion to adjacent cells and to the ECM and such effects have been observed in solid tumours and prostate cancer cell lines. Table 1.2 highlights the best characterised integrins involved in prostate cancer progression, migration and invasion, and these integrins are discussed below.

A microarray study was conducted on 111 individuals with localised prostate cancer who had undergone radical prostectomy, including 60 individuals who had tumour recurrence after a follow-up of 123 months (Pontes-Junior et al. 2010). In this study increased integrin α3 and α3β1 expression was found to be related to worse outcome with strong

Integrin	Integrin expression	Reference
α3β1	↑ Associated with higher recurrence	Pontes-Junior et al. (2010)
$\alpha_{IIb}\beta3$	↑ Metastatic prostate cell lines	Trikha et al. (1998)
α4	↓ Metastatic prostate cancer cell lines ↓ Xenograft samples	Saramaki et al. (2006)
α7	↓ Metastatic xenograft samples	Ren et al. (2007)
αvβ3	↑ Prostate cancer tissue samples, metastatic prostate cancer cell lines	Zheng et al. (1999), Nameth et al. (2003)
α5β1	↑ Metastatic prostate cancer cell line	Stachrurska et al. (2012)
α6β1	↑Metastatic prostate cancer tissue ↑ Metastatic prostate cancer cell lines	Davis et al, (2001), Bonkhoff et al, (1993), Trikha et al. (1998), King et al. (2008)
α6β4	↓ Metastatic prostate cancer tissue	Davis et al. (2001)

Table 1.2 Summary of publications which have reported altered expression of integrins in prostate cancer progression. Adapted from Chin et al. (2013).

α3 and α3β1 expression associated with higher incidence of recurrence. In another microarray study performed on four prostate cancer cell lines (LNCaP, DU145, PC3 and 22Rv1) and 13 prostate cancer xenografts, decreased expression of integrin α4 was observed and was found to be associated with deletion of the integrin α4 locus (Saramaki et al. 2006). Since all samples were derived from metastases, it suggests that integrin α4 could be a tumour suppressor. Interestingly, integrin α7 has also been identified as a tumour suppressor (Ren et al. 2007). In this study, the prostate cancer cell lines, PC3 and DU145 were transfected with an integrin α7 expression vector and implanted in SCID mice. After six weeks, the volume of the tumours was measured and compared to tumour volume in mice injected with cell that had been transfected with control vector. Reduced tumour volume and fewer metastases were observed in the integrin α7 vector transfected mice. Further analysis of metastatic potential using a wound-healing assay recorded reduced rates of migration in both PC3 and DU145 cells overexpressing integrin α7. Thus, these studies support the notion that integrin α7 inhibits cell migration and acts as a tumour suppressor.

An early study using DU145 and PC3 cells, which express integrin $\alpha_{IIb}\beta3$, suggested that integrin $\alpha_{IIb}\beta3$ is also involved in prostate cancer metastasis (Trikha et al. 1998). Although both cell lines express integrin $\alpha_{IIb}\beta3$, immunofluorescence data displayed different localisation patterns of the integrin. In DU145 cells the integrin localises to focal contact sites whereas in PC3 cells, it is mainly intracellular. Interestingly, when both the tumorigenic cell lines were injected intraprostatically into SCID mice, only the DU145 cells metastasised. Further analysis by flow cytometry with an antibody to $\alpha_{IIb}\beta3$ detected higher expression of $\alpha_{IIb}\beta3$ in DU145 cells isolated from the prostate when compared to DU145 cells from the subcutaneous tissue. Therefore, the data suggests that integrin $\alpha_{IIb}\beta3$ is involved in the metastatic progression of prostate tumours. Recently, integrin α5β1 also has been found to be important in cell adhesion in prostate cancer cells (Stachurska et al. 2012). When integrin α5β1 was blocked with an antibody, a decrease in the number of adherent PC3 cells to fibronectin was observed. Partial inhibition of PC3 cell migration and the formation of quasi-spherical cell shape changes were observed, suggesting a reversal to a less mesenchymal phenotype. In addition, the blocking of α5β1

resulted in weak expression of the cytoskeletal proteins F-actin and α-actinin suggesting a weak cell-fibronectin interaction. Thus, these results support the idea that integrin α5β1 plays an important role in the adhesion of PC3 cells to fibronectin and the migration of PC3 cells.

Integrin αvβ3 has also been identified to be involved in prostate cancer metastasis. Zheng et al. (1999) found expression of integrin αvβ3 in 16 prostate cancer specimens but not in normal prostate epithelial cells. The highly metastatic and invasive PC3 cell line also expresses integrin αvβ3 but not the non-invasive LNCaP cell line (Zheng et al. 1999). These αvβ3 expressing PC3 cells and the primary prostate cancer cells were found to adhere and migrate on vitronectin. When LNCaP cells were transfected with an αvβ3 expression plasmid to induce αvβ3 expression, LNCaP cells also adhered to and migrated on vitronectin. Thus, this study suggests that αvβ3 is potentially involved in prostate cancer invasion and metastasis. A following study found integrin αvβ3 to be involved in bone metabolism and angiogenesis (Nemeth et al. 2003). To investigate how inhibition of integrin αvβ3 in cells native to the bone would affect prostate cancer bone metastasis, a prostate cancer cell line that expresses little or no integrin αvβ3 was chosen. Interestingly, in this study, PC3 cells were used as they found undetectable levels of αvβ3 by FACS analysis and antibody staining. This is conflicting with the previous study which reported expression of αvβ3 in PC3 cells and it is possible that this is due to the use of different types of antibodies. Regardless, PC3 cells were injected directly into human bone fragments which were previously implanted subcutaneously in SCID mice and the mice were treated with anti-β3 antibody fragment (m7E3 $F(ab')_2$). This antibody only blocks the human bone-derived αvβ3. After two weeks of treatment, inhibition of integrin αvβ3 resulted in a reduced proportion of antigenically-human blood vessels within tumour-bearing bone implants. In addition, a reduction in the rate of tumour cell proliferation within the bone implants, reduced osteoclast number and degradation of calcified bone tissue were observed.

Integrin α6 can pair with either β1 or β4 subunits and it binds to laminin. The integrin α6β4 is a laminin receptor and is known as a hemidesmosome complex, mediating cell

attachment to the ECM. It acts as a junctional complex on the basal cell surface and is involved in the attachment of epithelial cells to the adjacent basement membrane. In contrast integrin α6β1 has been found to be involved in the cell migratory phenotype. The expression and distribution of integrin α6β1 in normal, hyperplastic and neoplastic prostate tissue and lymph node metastases was therefore examined (Bonkhoff et al. 1993). Approximately 85% of the grade I and grade II tumours and also the lymph node metastases showed upregulation of integrin α6β1, compared to normal and hyperplastic samples. Staining showed clusters of α6β1 receptors in acinar basement membranes which suggests that integrin α6β1 is important in mediating cell attachment to the basement membrane. In a later study, Nagle et al. (1994), found that while most of the prostate carcinoma tissues they tested displayed downregulation of integrins, the majority of these samples expressed α6β1 (Nagle et al. 1994). This is consistent with the loss of integrin β4 in the carcinoma samples. In a separate study, integrin β4 was found to be absent in prostate carcinoma tissues and only present in normal prostate glands and PIN lesions (Davis et al. 2001a), supporting the previous study. Therefore, these data suggest that integrin β4 is lost during cancer progression and therefore, integrin α6 is preferentially paired with the β1 subunit, forming α6β1. A following study found a variant form of integrin α6, α6p which was expressed in DU145, LNCaP and PC3 prostate cancer cell lines but not expressed in the normal prostate cells, PrEC (Davis et al. 2001b). This α6p variant also binds to both the β1 and the β4 subunits and has three times longer half-life than α6. Recently, King et al. (2008) investigated the role of integrin α6β1 in prostate cancer migration and bone pain in a novel xenograft mouse model. The human prostate cancer cells (PC3N), were stably transfected to overexpress either the cleavable wild type (PC3N-α6-WT) which forms the α6p variant or the uncleavable (PC3N-α6-RR) form of integrin α6. The α6 subunit can be cleaved via Urokinase-type Plasminogen Activator (uPA) treatment and the cells were directly injected and sealed into the femur of a mouse. After 21 days, tumour cells expressing wild-type integrin α6 (non-cleavable) showed a significant decrease in bone loss, unicortical or bicortical fractures and decreased ability of tumour cells to reach the epiphyseal plate of bone and prevented movement evoked pain, compared to the cleavable α6 integrin. Thus, these results suggest thaTargetedg of integrin α6 cleavage in

prostate tumour cells results in decreased tumour cell migration within the bone and reduced bone fractures and pain.

Targeted deletion of integrin β1 (which binds to many α subunits) in the mammary epithelium of the MMTV/Cre mice breast cancer model using the Cre/LoxP1 recombination system has shown that integrin β1 expression plays a role in the initiation of mammary tumorigenesis and for maintaining the cell proliferative capacity of late-stage tumour cells (White et al. 2004). Similarly, targeted disruption of integrin β1 in ErbB2-induced mammary tumours resulted in impaired tumour progression (Huck et al. 2010). Further investigation revealed that this was associated with increased apoptotic cell death and impaired angiogenic infiltration. In addition, during prostate cancer progression, integrin β1 expression has been shown to be upregulated and mis-localised (Knox et al. 1994; Murant et al. 1997). Targeted deletion of integrin β1 in the transgenic adenocarcinoma of the mouse prostate (TRAMP) model resulted in decreased animal survival, decreased retention of normal prostate morphology, increased the percentage of tissue with poorly differentiated carcinoma and increased cell proliferation (Moran-Jones et al. 2012). Taken together, these results suggest that integrin β1 plays an important role in tumour initiation and maintaining cell proliferative capacity.

1.4 PUTATIVE PROSTATE CANCER SUSCEPTIBILITY GENES

Advances in high-throughput genotyping assays have allowed genotyping of thousands of single nucleotide polymorphism (SNPs) and thus, lead to the identification of cancer susceptibility variants. Genome wide association studies have identified more than 50 variants associated with prostate cancer risk. One such variant within the α6 gene has been associated with prostate cancer susceptibility (Eeles et al. 2009). As family history is a significant risk factor for developing prostate cancer, many studies have been focused on identifying these susceptibility genes. Family history of disease is important in identifying inherited genetic predisposition.

Familial linkage analysis of a large Tasmanian family affected by multiple, densely aggregated cases of prostate cancer has identified a linkage on chromosome 5q13q2 (Fitzgerald 2009). Two polymorphisms within the *ITGA2* gene were found to be associated with prostate cancer risk in both familial and sporadic cases.

The *ITGA2* gene encodes for the α2 subunit which only heterodimerises with the β1 subunit to form the collagen/laminin receptor. The α2β1 complex is known as CD49b or Very Late Antigen 2 receptor (VLA-2). The α2β1 integrin is expressed on a wide variety of cell types including epithelial, mesenchymal, endothelial and fibroblast cells as well as platelets and megakaryocytes (Kirchhofer et al. 1990; Santoro et al. 1988; Takada et al. 1988). The α2β1 complex is involved in mediating cell differentiation, invasion and metastasis (Chan et al. 1991; Keely et al. 1995).

1.5 ROLES OF *ITGA2* IN PROSTATE CANCER

Bonkhoff et al. (1993) investigated the expression of integrin α2β1 in normal, hyperplastic and neoplastic human prostate tissue as well as lymph node metastasis samples. Results showed downregulation of α2β1 in 70% of the hyperplastic samples compared to normal prostate tissues. However, α2β1 was upregulated in the lymph node metastases compared to primary lesions (Table 1.3). In contrast, immunohistochemistry of α2β1 showed loss of expression in lymph node compared to their respective primary prostate tumour (Pontes-Junior et al. 2009). In another study, the role of integrin α2 in prostate cancer metastasis was investigated (Van Slambrouck et al. 2009). Immunofluorescence staining showed the presence of α2 and β1 subunit clusters in the bone metastatic prostate cancer cell line (C4-2B) but not in the lymph node metastatic prostate cancer cells (LNCaP), consistent with findings of Pontes-Junior et al. (2009), but in contrast to the findings of Bonkhoff et al., which reported α2β1 upregulation in lymph node metastasis. The functional blocking of the integrin α2 subunit with antibodies in the C4-2B bone metastatic prostate cancer cell line resulted in reduced adhesion and inhibition of invasion to collagen I (Van Slambrouck et al. 2009). Consistent with this,

flow cytometry showed an increase of α2 in LNCaP compared to C4-2B cells (Stewart et al. 2004). This correlated with C4-2B cells spontaneously metastasising to the bone whereas LNCaP cells failed to do so. Ramirez et al. (2011) found high expression of α2β1 in normal prostate and decreased expression in PIN and significantly decreased expression in lymph node metastases thus, supporting the findings of Van Slambrouck et al. (2009) and Pontes-Junior et al. (2009). More recently, α2β1 protein was found to be elevated in prostate cancer skeletal metastases compared to prostate cancer primary lesions or soft tissue metastases (Sottnik et al. 2012).

The α2β1 integrin has been shown to be involved in adhesion of metastatic prostate cells to the bone. PC3 cells were found to rapidly adhere in a dose-dependent manner to immobilised human type I collagen but not to fibronectin (Festuccia et al. 1999). In addition, PC3 cells were found to show greater adherence and spread on collagen 1 than on fibronectin and poly-L-lysine (Kiefer et al. 2001). In both studies, blocking of the α2β1 integrin using anti-α2 and anti-β1 antibodies inhibited PC3 cell adhesion on collagen, suggesting adherence of PC3 cells to collagen 1 was through α2β1. Treatment of PC3 cells with transforming growth factor-β1 (TGF-β1), a major bone derived growth factor, increased the *de novo* synthesis of α2 and β1 and resulted in a 2-3 fold increase in cell adhesion and spreading on collagen. These data suggest that α2β1 and TGF-β synergistically facilitate adhesion of metastatic prostate cells to the bone. In another study, prostate epithelial cells derived from both malignant and benign tissues showed greater preference of adhesion to bone marrow stromal cells than to benign prostatic fibroblasts, skin fibroblasts or plastic tissue culture plates, in keeping with data suggesting that prostate cancer cells have a propensity to metastasise to the bone (Lang et al. 1997). Adhesion of prostate epithelial cells to bone marrow stromal cells was inhibited by anti-α2 and anti-β1 antibodies.

The role of α2β1 in bone metastasis is further supported by a study by Hall et al. (2006). A collagen-binding LNCaP cell line was derived ($LNCaP_{col}$) which showed increased levels of α2β1 with associated increased migration towards collagen I (Hall et al. 2006).

Primary tumour	Secondary tumour	Reference
↓ Prostate hyperplastic tissue	↑ Lymph node metastases	Bonkhoff et al. (1993)
-	↓ Lymph node metastases	Pontes-Junior et al. (2009)
-	↓ Lymph node metastatic prostate cells ↑ Bone metastatic prostate cancer cells	Van Slambrouck at al. (2009)
↓ PIN	↓ Lymph node metastases	Ramirez et al. (2011)
-	↓ Soft tissue metasteses ↑ Bone metasteses	Sottnik et al. (2012)
-	↓ Soft tissue metastatic prostate cells ↑ Bone metastatic prostate cells	Hall et al. (2006)

Table 1.3 Summary of publications which have reported altered expression of α2β1 in prostate cancer samples.

In an *in vivo* analysis of these cells, $LNCaP_{col}$ cells were injected into the tibia of nude mice and the $LNCaP_{col}$ injected mice developed bone tumours. In addition, SCID mice implanted with $LNCaP_{col}$ cells with knockdown of *ITGA2* displayed a 69% decrease in osseous tumour burden.

Overall, these studies suggest that *ITGA2* is involved in prostate cancer progression playing a role in adhesion, invasion and migration to collagen type I, which is the predominant protein in the bone. Bone is the most common and frequent target for prostate cancer metastasis and thus, suggests that *ITGA2* plays an important role in prostate cancer metastasis.

1.5.1 Prostate cancer stem cells

According to the cancer stem cell hypothesis tumours are initiated by a rare subpopulation of putative cancer stem cells or progenitor cells, sharing similar properties to normal adult stem cells that have the ability to self-renew and also give rise to differentiated tissue cells (Tan et al. 2006). Whilst the cellular origin of prostate cancer remains controversial, putative prostate cancer stem cells originating from primary tumours have been shown to have the ability for self-renewal and high clonogenic potential (Collins et al. 2001).

ITGA2 has been found to be an important marker of these prostate cancer stem cells. Isolation of cells with higher surface expression of *ITGA2* from the human prostate epithelium displayed the ability to form colonies and regenerate a fully differentiated prostate epithelium *in vivo* and thus behave equivalent to the prostate stem cell (Collins et al. 2001). Further studies have found that human basal cells expressing $\alpha2\beta1^{hi}/CD133^{+}$ have epithelial stem cell properties and thus are used as a marker for selecting human prostatic epithelial stem cells (Richardson et al. 2004). Following this study, it was discovered that prostate cancer stem cells have a $CD44^{+}/\alpha2\beta1^{hi}/CD133^{+}$ phenotype (Collins et al. 2005). CD44 and CD133 are well chracterised cell surface markers for cancer stem cells in brain, breast, pancreas, ovarian and liver cancers (Curley et al. 2009;

Lee et al. 2008; Singh et al. 2004; Wright et al. 2008; Zhu et al. 2010b). These cells were shown to have the capacity for extensive proliferation, self-renewal, differentiation and invasion. These were consistent with data presented in Patrawala et al. (2006) where 80% of the $CD44^+$ prostate cancer cells were of a $CD44^+/\alpha2\beta1^{hi}/CD133^+$ tumour progenitor phenotype and they therefore concluded that in prostate cancer, the tumour is likely to contain multiple populations of tumorigenic cells which are able to give rise to tumours with differing efficiencies.

1.5.2 Regulation of *ITGA2* gene expression

Numerous studies have demonstrated altered expression of *ITGA2* during carcinogenesis. *ITGA2* expression appears to be regulated primarily at the transcriptional level and its regulation has been examined in megakaryocytes (Zutter et al, 1995), epithelial cells (Ye et al. 1996) and fibroblasts (Xu et al. 1998a). Our understanding of its regulation is currently limited but, promoter and enhancer regions have been identified 5' to the transcription start site (TSS; Zutter et al. 1994). Thus far, several Sp1 transcription factor binding sites have been identified and studies have shown that the Sp1 transcription factor is responsible for the basal activity of *ITGA2* in megakaryocytic cells (Jacquelin et al. 2001). In addition to transcription factors, epigenetic factors have also been shown to be important in the regulation of integrin gene expression.

1.6 EPIGENETIC REGULATION

(This section has been substantially published in Chin et al. 2011)

Epigenetic alterations are heritable changes in gene expression that occur without changes in DNA sequence, with the broadest definition including all factors other than DNA sequence changes, that heritably influence gene expression (Berger et al. 2009). While the best described of these mechanisms is DNA methylation, other epigenetic mechanisms include physical and chemical changes to chromatin and regulation of gene expression by microRNAs (miRNAs).

DNA methylation plays an important role in DNA repair, recombination and replication, as well as regulating gene activity (Figure 1.3). DNA methylation involves the addition of a methyl group to the 5'-carbon of cytosine in CpG dinucleotide sequences, catalysed by a family DNA methyltransferases (DNMTs). CpG rich regions, known as CpG islands are commonly found associated with the 5' region of vertebrate genes (Gardiner-Garden et al. 1987) and are generally protected from methylation (Bird 2002). For many years CpG islands have been implicated in gene regulation with their methylation strongly correlated with gene silencing (Illingworth et al. 2009). DNA methylation can regulate gene activity via two mechanisms. Firstly, methylation of CpG dinucleotides within transcription factor binding sites can inhibit transcription factor binding and therefore directly influence gene activity (Hark et al. 2000b). Secondly, methylated CpG dinucleotides act as binding sites for methyl CpG binding proteins, which are associated with other factors such as histone deacetylases, involved in establishing repressive chromatin structures (Jones et al. 1998; Nan et al. 1998).

Changes in DNA methylation patterns have been linked with cancer for many years now (Jones et al. 2007). However, the methylation changes are complex, with both hypomethylation and hypermethylation occurring in cancer cells. Aberrant DNA hypermethylation occuring at gene promoters, can lead to gene inactivation and localised hypermethylation of gene promoters has been reported in virtually all types of cancers, including prostate cancer. In contrast, DNA hypomethylation is the demethylation of normally methylated DNA and can lead to chromosomal instability and activation of proto-oncogenes (Dunn 2003; Eden et al. 2003; Sharma et al. 2010). Both global and gene-specific hypomethylation events have also been implicated in prostate cancer.

While alterations in DNA methylation have long been linked to cancer, there is also mounting evidence that other epigenetic changes, such as changes to chromatin composition or structure, contribute to cancer. Within the eukaryotic nucleus DNA is assembled into chromatin, the basic unit of which is the nucleosome. Nucleosomes are composed of approximately 147 base pairs of DNA wrapped around an octamer of core histone proteins, containing two each of histones H2A, H2B, H3 and H4 (Kornberg et al.

1999). The N-termini of the core histones protrude from the nucleosome and are subjected to a range of covalent modifications, catalysed by various histone modifying enzymes. At least ten different histone modifications have now been reported, including acetylation, methylation, phosphorylation and ubiquitination (Gardner et al. 2011; Kouzarides 2007). Each of these modifications affects chromatin structure and function in a different way by either disrupting chromatin contacts or affecting the recruitment of other proteins to the chromatin (Kouzarides 2007). Acetylation of lysine residues in histone H3 and H4 (H3Ac, H4Ac) is in general associated with transcriptional activity, whereas histone methylation is associated with transcriptional activation or repression depending on the site of modification, and the number of methyl groups added (Figure 1.3). For example, histone H3 lysine 4 (H3K4) methylation is generally associated with transcriptional activation, whereas histone H3 lysine 9 (H3K9) and histone H3 lysine 27 (H3K27) di- and tri-methylation is generally associated with transcriptional repression (Kouzarides 2007). As with DNA methylation, there is an increasing body of evidence that changes in histone modifications due to aberrant activity or mis-targeting of chromatin-modifying enzymes is involved in carcinogenesis (Hake et al. 2004).

1.6.1 Epigenetic alterations in prostate cancer progression

1.6.1.1 DNA Hypomethylation

There is considerable evidence that changes in DNA methylation patterns occur in prostate cancer with DNA hypomethylation in tumour samples first documented more than twenty years ago. In 1987, Bedford and van Helden analysed DNA 5'-methylcytosine content in human prostate samples and reported a correlation between global hypomethylation and development of benign prostatic hyperplasia (BPH) and metastatic tumours (Bedford et al. 1987). Comparison of DNA methylation levels in tumour and normal prostate tissue by immunohistochemistry similarly detected global hypomethylation in prostate cancer (Brothman et al. 2005). In keeping with this Santourlidis et al. (1999) examined methylation of *LINE-1* repetitive sequences in prostate adenocarcinomas and found that *LINE-1* methylation tended to decrease with tumour stage. A further study analysing tumour samples similarly demonstrated an

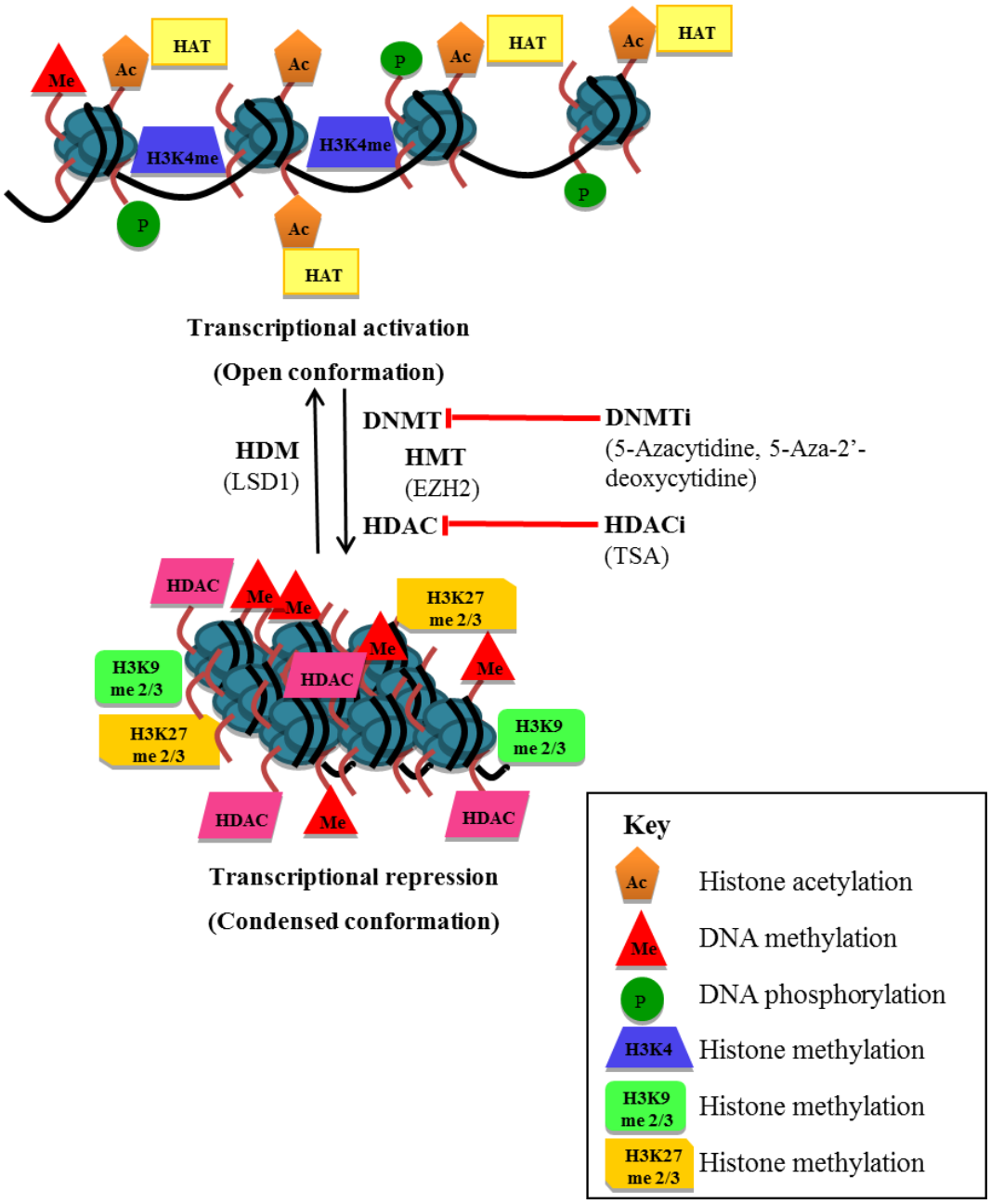

Figure 1.3 A model depicting epigenetic modifications associated with different transcriptional states and some of the inhibitors that target epigenetic modifiers and, therefore, influence transcriptional activity. Adapted from Chin et al. (2011).

association between DNA hypomethylation, tumour state and metastasis, with extensive hypomethylation of *LINE-1* observed in 64% of cases with lymph node or distant metastases, but only in 21% of cases without detectable metastases (Schulz et al. 2002). All cases of hormone-refractory locally recurrent tumours displayed extensive *LINE-1* hypomethylation. The study by Schulz et al. (2002) also found a strong association between *LINE-1* DNA hypomethylation and chromosomal alterations, in support of the notion that DNA hypomethylation increases genomic instability (Schulz et al. 2002). Most prostate cancer samples with prominent DNA hypomethylation also exhibited a large number of chromosomal alterations, and vice versa. These findings were supported by a further study which found increased prevalence of *LINE-1* hypomethylation in later stage prostate cancers and lymph-node positive prostate cancers (Florl et al. 2004). In contrast gene-specific hypermethylation events did not correlate with tumour stage, being present in both early and late stage prostate cancer. These data therefore suggest that hypomethylation is a later event in prostate cancer progression compared to gene-specific hypermethylation. Consistent with this, a recent study examined *LINE-1* methylation in primary prostate cancers compared to normal prostate tissues and found a significantly higher level of *LINE-1* hypomethylation in metastatic prostate cancer tissues, suggesting that global hypomethylation occurs late in prostate cancer progression, particularly at the metastatic disease stage (Yegnasubramanian et al. 2008).

While global DNA hypomethylation can contribute to cancer by promoting genomic instability, the hypomethylation of individual gene promoters can also contribute to cancer development and progression by directing aberrant gene expression. Hypomethylation of a number of genes has been linked to prostate cancer. For example, a study by Yegnasubramanian et al. (2008), found that CpG islands associated with a class of cancer testis antigen genes were hypomethylated in prostate cancer, correlating with their overexpression in primary prostate cancers, and more so in metastatic prostate cancer. Similarly hypomethylation of the promoter of the heparanase gene has been reported in prostate cancer, compared to BPH (Ogishima et al. 2005). This correlates with increased mRNA expression of the heparanase gene, which is associated with tumour invasion and metastasis (Hulett et al. 1999; Vlodavsky et al. 1988). Further investigation

of the *WNT5A* gene revealed three CpG sites in the promoter region which were consistently methylated in a normal prostate cell line and normal prostate tissues, but not in a prostate cancer cell line and primary prostate cancer tissues (Wang et al. 2007). Therefore hypomethylation and consequent upregulation of genes involved in metastasis and cell invasion may be an important factor in prostate cancer progression. In support of this, treatment of the PC-3 prostate cancer cell line with reagents that prevent DNA hypomethylation has an inhibitory effect on cell invasion *in vitro* and tumour growth *in vivo* (Shukeir et al. 2006).

1.6.1.2 DNA Hypermethylation

DNA hypermethylation is the most commonly reported epigenetic alteration observed in prostate cancer. Many genes have been identified as aberrantly hypermethylated in prostate cancer and these genes include tumour-suppressor genes, DNA damage repair genes, hormonal response genes and genes involved in cell cycle control, tumour cell invasion and metastasis (Phé et al. 2010). Hypermethylation of DNA can lead to inappropriate gene silencing, disrupting gene function and thus contributing to tumour initiation, progression and metastasis (Li et al. 2004).

The most frequently reported hypermethylated gene in prostate cancer is the π-class glutathione-S-transferase (*GSTP1*) gene. *GSTP1* is an enzyme involved in the metabolism, detoxification and elimination of reactive chemical compounds and in this way protects cells from DNA damage and cancer initiation (Lee 2007). Hypermethylation of the *GSTP1* gene in prostate cancer was first reported by Lee et al. (1994) who found hypermethylation of the gene in all 20 human prostate cancer tissue samples examined, but not in normal tissues or BPH. Following this, *GSTP1* gene expression was analysed in 60 high-grade PIN samples, with all samples showing a loss of *GSTP1* gene expression (Brooks et al. 1998). Further investigation found that this loss of *GSTP1* expression was due to hypermethylation of the gene promoter. A large number of studies have now reported *GSTP1* hypermethylation in prostate cancer samples, with methylation detected in up to 90% of samples, suggesting *GSTP1* hypermethylation is a common epigenetic

alteration in prostate cancer (Bastian et al. 2004; Chu et al. 2002; Goessl et al. 2001; Harden et al. 2003; Jeronimo et al. 2002; Nakayama et al. 2003).

Promoter hypermethylation of genes involved in tumour invasion has also been observed in prostate cancer. The maintenance of the normal cell architecture is regulated by the cadherin-catenin system. Loss of E-cadherin gene expression is associated with the transition from adenoma to carcinoma and the acquisition of metastatic potential (Perl et al. 1998). E-cadherin hypermethylation was observed in prostate cancer cell lines and treatment with the demethylating agent, 5-aza-2'-deoxycytidine restored E-cadherin mRNA and protein expression in the cell lines, suggesting that promoter hypermethylation was responsible for E-cadherin silencing in these cells (Graff et al. 1995). Furthermore, Kallakury et al. (2001) reported that the E-cadherin gene promoter was methylated in 8 out of 10 prostate cancer tissues examined. Interestingly, the degree of E-cadherin promoter methylation correlated with the pathological stage of the prostate tumour tissues, with E-cadherin promoter methylation occurring in 30% of low-grade prostate cancer tissues, but increasing to 70% in high-grade tumours (Li et al. 2001). These results suggest that methylation of the E-cadherin promoter is associated with prostate tumour progression.

CD44, which is a cell surface glycoprotein involved in cell matrix adhesion and signal transduction is also silenced in prostate cancer by methylation of the gene promoter. Lou et al. (1999), examined methylation levels of the *CD44* gene in 84 matched normal and prostate cancer samples and found hypermethylation of *CD44* in 31 out of 40 of the primary prostate cancer samples. Further investigation by Verkaik et al. (1999) showed that in CD44 negative prostate cancer cell lines (LNCaP and PC346C) the *CD44* promoter was hypermethylated compared to CD44 positive prostate cancer cell lines (DU145, PC3 and TSU). A further study of *CD44* silencing was conducted on human tissue samples and demonstrated that 9 out of 11 lymph node metastases of prostate cancer displayed *CD44* gene promoter methylation. These data therefore suggest that hypermethylation of the *CD44* promoter resulting in downregulation of *CD44* gene

expression may be involved in prostate cancer progression and metastasis (Verkaik et al. 2000).

DNA methylation is also involved in regulation of the androgen receptor (AR). The AR is activated by androgen, which plays a critical role in the development, growth and maintenance of the prostate (Jenster 1999). In the initial stages, prostate cancer is androgen dependent, but eventually becomes androgen independent, due to the loss of AR expression (Jarrard et al. 1998; Suzuki et al. 2003; Takahashi et al. 2002; Tekur et al. 2001). Data from a number of studies suggest that this loss of AR expression is at least partly due to hypermethylation of the AR gene promoter. Jarrard et al. (1998) found AR promoter hypermethylation in AR negative prostate cancer cell lines (DU145, DuPro, TSU-PR1 and PPC1) whereas the promoter was unmethylated in AR positive cell lines (LNCaP and PC3). Expression of the AR gene was restored in the AR negative cell lines by treatment with the demethylating agent 5-aza-2'deoxycytidine, suggesting that promoter methylation was responsible for AR gene silencing in the AR negative cell lines. Further, Suzuki et al. (2003) reported that promoter hypermethylation of AR leading to loss of AR expression occurs in 30% of hormone refractory prostate cancers, suggesting that DNA hypermethylation contributes to loss of AR expression in at least some prostate cancers.

The silencing of cell cycle regulation genes by DNA hypermethylation has also been observed in prostate cancer. The Ras associated domain family 1A gene (*RASSF1A*) is highly methylated in several human cancers, including prostate cancer (Aitchison et al. 2007; Dammann et al. 2005; Hesson et al. 2007; Kang et al. 2004a; Liu et al. 2002; Pfeifer et al. 2005; Serth et al. 2008). Liu et al. (2002), examined methylation of the *RASSF1A* promoter in primary prostate tumours and reported methylation of the *RASSF1A* promoter in over 70% of the tumours. Further investigation found a correlation between the frequency of methylation and the Gleason score of the tumour, with highly aggressive tumours displaying more frequent DNA methylation compared to less aggressive tumours. Similarly, Kang et al. (2004a) reported methylation of a number of

genes, including *RASSF1A* in prostate cancer and PIN samples, with more frequent methylation correlating with higher PSA levels and Gleason score.

Silencing of tumour suppressor genes by DNA methylation is also often observed in prostate cancer. DNA hypermethylation of the adenomatous polyposis coli (*APC*) gene in prostate cancer individuals was observed in a study by Rosenbaum et al (2005), which examined promoter methylation of a number of genes. Hypermethylation of *APC* alone, and hypermethylation of *APC* and the cell cycle regulation gene *cyclin* D2 in combination were found to be significant predictors of prostate cancer progression. In keeping with this, Henrique et al (2007) analysed a small panel of gene promoters in prostate biopsy samples and similarly found that hypermethylation of *APC* is an independent predictor of poor prognosis in prostate cancer. Subsequent studies have similarly found *APC* hypermethylation to be a predictor of prostate cancer progression (Liu et al. 2011; Richiardi et al. 2009).

1.6.1.3 Histone modifications

Compared to DNA methylation the involvement of histone modifications in prostate cancer is relatively poorly understood, even though these two epigenetic mechanisms are closely related (Sharma et al. 2010). However, some data relating particular histone modifications to prostate cancer have emerged in recent years. Seligson et al. (2005), analysed a range of histone modifications by immunohistochemistry including acetylated histone H3 lysine 9 (H3K9Ac), H3K18Ac, H4K12Ac, dimethylated H4 arginine 3 (H4R3me2) and dimethylated H3 lysine 4 (H3K4me2) in 183 primary prostate cancer tissue samples. In the individuals with low-grade tumours, the study found two subgroups with different risks of tumour recurrence based on the presence of similar combinations of global histone modifications. Individuals with a lower risk of tumour recurrence were those who were above the 60^{th} percentile staining for H3K4me2 or above the 35^{th} percentile staining for H3K18Ac and H3K4me2. However, these histone modification patterns did not correlate with the Gleason score. In contrast, a more recent study of primary and metastatic prostate cancer samples found that high global levels of H3K18Ac and H3K4me2 correlated with a 3-fold increased risk of prostate cancer

recurrence (Bianco-Miotto et al. 2010). A further study showed that global levels of H3K4me1, H3K9me2, H3K9me3, H3Ac and H4Ac were significantly reduced in prostate cancer compared to BPH and normal prostate tissue (Ellinger et al. 2010), with H3Ac and H3K9me2 in particular discriminating between the malignant and non-malignant samples. They also found that individuals with high H3K4me1 levels were more likely to experience recurrence of the prostate cancer and thus suggested that analysis of H3K4me1 may provide predictive information regarding likelihood of tumour recurrence.

In addition to the studies outlined above that have documented changes in histone modifications associated with prostate cancer, there is accumulating evidence that expression of histone modifying enzymes is altered in prostate cancer. Bianco-Miotto et al. (2010) identified a candidate gene signature consisting of six genes encoding epigenetic modifiers, including both DNA methyltransferases and histone methyltransferases (HMT), that was associated with prostate cancer progression. In addition a number of studies have documented alterations in the HMT EZH2, which is responsible for the repressive H3K27me3 modification, in prostate cancer. EZH2 is upregulated in hormone-refractory metastatic prostate cancer (Varambally et al. 2002). Overexpression of EZH2 in prostate cancer cell lines increases the invasive characteristics of the cells, while knockdown of EZH2 decreases the proliferative capacity of the cells, and more so in hormone independent cell lines (Karanikolas et al. 2010; Varambally et al. 2002). Furthermore, microarray analysis of metastatic prostate cancer tissue identified a group of EZH2 repressed genes that were associated with prostate cancer progression (Yu et al. 2007).

A number of studies have also implicated lysine-specific demethylase 1 (*LSD1*) in prostate cancer. *LSD1* was originally identified as a H3K4 demethylating enzyme (HDM). This enzyme was thought to function as a transcriptional co-repressor by reversing the H3K4me modifications associated with transcriptional activation (Shi et al. 2004). However, genome-wide studies of *LSD1* homologues in yeast suggest that these enzymes can act as both co-activators and co-repressors by targeting H3K4 or H3K9,

respectively (Opel et al. 2007). In keeping with this, *LSD1* has been demonstrated to form ligand-dependent, chromatin-associated complexes with AR, stimulating AR-dependent transcription (Metzger et al. 2005). In this case *LSD1* acts by demethylating histone H3K9, thus activating AR target genes by removing repressive H3K9me modifications. This study found that LSD1 and AR were co-localised in both normal prostate and prostate cancer. A further study by Kahl et al (2006), correlated the expression patterns of AR, *LSD1* and the AR co-activator FHL2 (four and a half LIM-domain protein 2) with Gleason score, Gleason grade and p53 expression in 153 prostate tumour samples, from patients which relapse after radical prostatectomy. The study found that increased *LSD1*, nuclear expression of FHL2, high Gleason score and grade and high levels of p53 in tumours strongly associated with relapse during follow-up. In addition, upregulation of both *LSD1* mRNA and protein levels was associated with high risk of relapse. While *LSD1* demethylates H3K9me1 and H3K9me2, a further study found that the Jumonji C domain containing protein JMJD2C can demethylate H3K9me3 (Wissmann et al. 2007). JMJD2C associates with AR and *LSD1* in prostate cells, and this complex acts to demethylate H3K9me3 and increase AR-dependent gene transcription. A second Jumonji protein, JHDM2A, which also demethylates H3K9me1/2, has similarly been found to stimulate transcription of AR dependent genes (Yamane et al. 2006). Furthermore, Gaughan et al (2011) showed that AR interacts with, and is methylated by, the HMT enzyme SET9. SET9 was originally identified as an enzyme responsible for catalysing the activating histone modification H3K4me1, and therefore was associated with transcriptional activation (Nishioka et al. 2002). However, Gaughan et al (2011) found that by methylating AR SET9 increased the transcriptional activation of AR itself. In doing so SET9 was found to have pro-proliferative and anti-apoptotic activity in the AR-dependent LNCaP prostate cancer cell line.

While the studies outlined above have highlighted differences in histone modifications and histone modifiers correlating with prostate cancer stage or recurrence, it should be noted that many of these studies have described global changes and further studies are therefore needed to investigate how these global changes relate to gene-specific loci, particularly at genes that have already been implicated in prostate cancer.

1.6.2 Epigenetic modulation and transcription factor binding

Epigenetic factors are clearly involved in the regulation of gene expression. However, these factors do not operate in isolation, but rather operate with transcription factors within the chromatin landscape. DNA methylation can mediate gene silencing by directly inhibiting the binding of transcription factors. The insulator CTCF protein is an example of a factor that is affected by CpG methylation (Bell et al. 2000; Hark et al. 2000a; Holmgren et al. 2001; Szabó et al. 2000). CTCF is responsible for insulating the promoter from the influence of enhancers and the binding of this protein between the promoter and its downstream enhancer in the maternal *Igf2* gene results in silencing of that gene. Contrary to this, paternal *Igf2* is highly methylated and thus, prevents the binding of CTCF which leads to gene activation by the downstream enhancer. Interestingly, there are also factors that are responsible for blocking epigenetic inactivation. Binding of the Sp1 transcription factor at the promoter appears to confer resistance to CpG methylation (Mancini et al. 1999; Mummaneni et al. 1998). However, whether methylation at the Sp1 binding site affects Sp1 binding remains unresolved. Several studies have found that Sp1 binding and gene expression are not affected by methylation at Sp1 binding sites both *in vitro* and *in vivo* (Ghosh et al. 1999; Harrington et al. 1988; Höller et al. 1988; Song et al. 2002). In contrast, others have found that methylation of the CpG dinucleotide affects Sp1 binding which depends on the configuration of methylated cytosines within the Sp1 consensus sequence (Clark et al. 1997; Mancini et al. 1999). These results suggest that the effect of methylation at the Sp1 binding site may be dependent on context. Further investigations have reported that methylation of CpG sites outside of Sp1 consensus sites can directly reduce the binding of Sp1 (Zhu et al. 2003).

It is still unclear how histone modifications directly affect transcription factor binding but it is proposed that histone modifications regulate chromatin organisation and thus promoter accessibility, which results in either an accessible open chromatin, allowing transcription factor binding or inaccessible chromatin states, which are inhibitory to transcription factor binding (Struhl 1998). Overall, both epigenetic regulation and transcription factors play important roles in the regulation of gene expression.

1.7 RESEARCH AIMS

The *ITGA2* gene, encoding the α2 subunit of the collagen receptor (α2β1), has been found to be involved in the progression of several cancers, including prostate cancer. Altered expression of α2β1 in prostate cancer has been correlated with a poor outcome and progression of prostate tumours to an invasive, metastatic and highly aggressive tumour phenotype in a highly cell-dependent manner. A previous study by our research group has identified *ITGA2* as a prostate cancer susceptibility gene through a familial prostate cancer study (FitzGerald et al. 2009). There is evidence that α2β1 expression changes during tumour progression and we hypothesise that deregulation of *ITGA2* expression by epigenetic alterations may be associated with prostate tumour progression.

Therefore, the main aim of this study is to examine the regulation of the *ITGA2* gene in prostate cancer. More specifically, the aims are to:

1. Determine whether the *ITGA2* gene is regulated by epigenetic factors in prostate cancer cell lines.

2. Determine whether the *ITGA2* gene is regulated by/ during EMT and if modulation of *ITGA2* affects cell migration.

3. Identify the transcription factors involved in regulation of *ITGA2* expression in prostate cells.

Chapter 2
MATERIALS & METHODS

2.1 Mammalian cell cultures

2.1.1 Cell culture

PWR-1e, LNCaP, 22Rv1, VCaP and PC3 cell lines were purchased from the American Type Culture Collection (Rockville, MD). All cell lines were cultured in a humidified incubator at 37 ºC and 5% CO_2. LNCaP and 22Rv1 cells were cultured in Roswell Park Memorial Institute 1640 medium (RPMI 1640; Sigma-Aldrich Corporation, USA) supplemented with 10% foetal calf serum (FCS; Sigma-Aldrich Corporation, USA) and 1% Penicillin/Streptomycin solution (containing 5000 U/mL Penicillin G and 5000 μg/mL Streptomycin) (Sigma-Aldrich Corporation, USA). The LNCaP cells were further supplemented with 2.0 g/L D-glucose and 1.0 mM sodium pyruvate. PC3 cells were cultured in Ham's F12K nutrient mixture Kaighn's modification 1X liquid (Sigma-Aldrich Corporation, USA), VCaP cells were cultured in Dulbecco's Modified Eagle Medium, (DMEM; Invitrogen, USA) and PWR-1e cells were cultured in Keratinocyte Serum Free Media (Invitrogen, USA) which were all supplemented with 10% FCS and 1% Penicillin/Streptomycin solution.

2.1.2 Derivation of $LNCaP_{col}$

$LNCaP_{col}$ cells were derived from parental LNCaP cells by successive panning on type I collagen according to Hall et al. (2006). LNCaP cells (1×10^5 cells/mL) in 10 mL binding buffer [serum free RMPI 1640 supplemented with 0.5% bovine serum albumin (Sigma-Aldrich Corporation, USA)] were plated in a 75 cm^2 BD Falcon collagen I coated flask (Becton Dickinson Biosciences, USA). After 1 hour, non-adherent cells were removed by washing three times with binding buffer. Fresh culture medium was added and the collagen-adherent cells were allowed to expand. Once the cells reach 80% confluency, the cells were removed in 1.0 mmol/L ethylenediaminetetraacetic acid (EDTA) in phosphate buffered saline (PBS), washed in PBS and 1×10^5 cells/mL were repeatedly panned while the rest of the cells were harvested for RNA and DNA extraction.

2.1.3 Cell treatments

Where indicated in the results, LNCaP, and 22Rv1 cells were treated with 0.5 µM 5-Aza-2'-deoxycytidine (AzaC; Sigma-Aldrich Corporation, USA). Cells were grown to approximately 60% confluency in their respective media for 24 hours and then washed with PBS before the addition of OPTI-MEM medium with AzaC for 5 days, during which AzaC was added on alternate days with fresh media. In addition, the cells were treated with 200 ng/mL Trichostatin A (TSA) for 4 hours on the final day of AzaC treatment. After 5 days, the cells were trypsinised and harvested by centrifuging at 500 g for 5 minutes at 20 ºC to pellet cells. The medium was aspirated and cell pellets were stored at -80 ºC.

Where indicated in the text, cells were treated with 10 nM 5α-Androstan-17β-ol-3-one (DHT; Sigma-Aldrich Corporation, USA) in OPTI-MEM® (Gibco®, USA) medium for 3, 6 and 24 hours. Cells were seeded at 2 x 10^5 cells/mL in OPTI-MEM media without FCS and left to adhere for 24 hours before the addition of DHT. The cells were then pelleted as described above. Where specified in the relevant chapters, cells were also pre-treated with 10 µg/mL Cyclohexamide (CHX; Sigma-Aldrich Corporation, USA) for 30 minutes before DHT treatment.

2.2 Gene expression analysis

2.2.1 RNA extraction

Cell pellets (from 5 x 10^5 cells) which were stored at -80 ºC were resuspended in 1mL TRI® reagent (Sigma-Aldrich Corporation, USA) and transferred to microcentrifuge tubes. Cells were thoroughly resuspended in TRI® reagent to lyse cells, and then incubated at room temperature for 5 minutes. Next, 200 µL chloroform was added, followed by vigorous shaking for 15 seconds and incubation at room temperature for 10 minutes. The samples were then centrifuged at 13000 g for 15 minutes at 4 ºC. The clear, aqueous upper layer was transferred to a fresh microcentrifuge tube, and 250 µL isopropanol was added. This was followed by an overnight incubation at -20 ºC to precipitate the RNA. The RNA was then pelleted by centrifugation at 13000 g for 20

minutes at 4 ºC. The pellet was then washed in 1mL 70% ethanol, and then re-pelleted by centrifugation at 13000 g for 5 minutes at room temperature. All residual ethanol was removed and the pellet was allowed to dry at room temperature, after which it was resuspended in 20 μL MilliQ® water. The RNA samples were quantified (Section 2.2.2) then frozen in liquid nitrogen and stored at -80 ºC until further analysis.

2.2.2 RNA quantitation

The RNA was quantified by measuring the optical density of the absorbance at 260 nm using a NanoDrop® ND-1000 spectrophotometer. The absorbance at 260 nm was converted to a concentration of RNA by the conversion factor of 1 $O.D._{260}$ = 40 μg/mL. The purity of each sample was also assessed by the absorbance at 260 nm relative to the absorbance at 280 nm (OD_{260}/OD_{280}), with a ratio between 1.8 and 2.0 indicating high purity.

2.2.3 RNA quality assessment

The quality of RNA can not be assessed by spectrophotometry (Section 2.2.2). Therefore, RNA quality was evaluated by agarose gel electrophoresis. The RNA samples (0.5-1.0 μg) were diluted to 12.0 μL with MilliQ® water. Agarose gel loading dye (3 μL) was then added to a total volume of 15 μL, and the RNA sample was then loaded onto a 1% agarose gel. The λHindIII molecular weight marker (λHindIII Marker; New England Biolabs, USA, 1 μg) was electrophoresed alongside the RNA, in order to visualise the amount and quality of the RNA. The gel was electrophoresed for 60 minutes at 100 V in 1X Tris Acetic acid EDTA (TAE) buffer containing 0.03 μg/μL SYBR safe DNA gel stain 10000X (Invitrogen, USA). Finally, the gel was visualised using a Safe Imager (Invitrogen, USA). Presence of 2 rRNA bands (18s, 28s) was used a guide to the quality of the RNA.

2.2.4 Synthesis of cDNA

An aliquot of 1μg of RNA was used in preparing cDNA using SuperScript™ reverse transcriptase III (Invitrogen, USA). The RNA was first treated with 1 U of DNase I (Invitrogen, USA) in 1X First Strand Buffer (Invitrogen, USA). Samples were incubated

at 37 ºC for 30 minutes, followed by 5 minutes of heating at 75 ºC to inactivate DNase I. Then, 5 µM of oligo dT (Invitrogen, USA) was added and the samples were incubated at 70 ºC for 10 minutes. mRNA was then reverse transcribed to cDNA with 100 U of SuperScript™ reverse transcriptase III enzyme (Invitrogen, USA), in 1X First Strand Buffer supplemented with 0.1 M DTT (Invitrogen, USA) and 10 µM dNTPs (Invitrogen, USA) at 42 ºC for 50 minutes. This was followed by a 70 ºC incubation for 15 minutes to inactivate the enzyme. cDNA was stored at -20 ºC until further analysis.

2.2.5 Real-time PCR

Gene expression was analysed using real-time PCR. cDNA (50 ng) prepared as described above (Section 2.2.4), was used as template in the PCR reaction, and amplified using QuantiTect® SYBR® Green PCR Mastermix (Qiagen, USA).

Forward and reverse primer sets for each mRNA (designed as outlined in Section 2.2.6) were added to the mastermix. In each PCR tube, 1x QuantiTect® SYBR® green PCR mastermix, 0.3 µM of forward primer, 0.3 µM of reverse primer and 50 ng of either diluted cDNA (for each sample) or nuclease free water (for the no template control, NTC) were added, in a total volume of 25 µL.

The polymerase was activated by an initial incubation at 95 ºC for 10 minutes and then amplified for 40 cycles consisting of 95 ºC for 15 seconds, 60 ºC for 30 seconds. Amplification and analysis were carried out in a Rotorgene® real-time PCR machine using the accompanying Rotorgene® software (Corbett Research).

Melt curve analysis was undertaken to ensure that the PCR was specific and was amplifying a single product. Analysis from 60 ºC to 95 ºC, increasing by 1 degree every 5 seconds was undertaken to verify amplification of a single product. In addition, PCR products were subjected to agarose gel electrophoresis (Section 2.2.3) to ensure the correct fragment size.

2.2.6 Primer design

PCR primers were designed to amplify each cDNA using the Primer3 program at http://frodo.wi.mit.edu/primer3/ and were designed to span across exons to ensure that these primers would amplify only the RNA. The selected primers were then analysed using the BLAST program at http://blast.ncbi.nlm.nih.gov/Blast.cgi to confirm specificity. The primer sequences are shown in Table 2.1.

Primer Name	Primer sequence	Amplicon size
ITGA2 Forward	5' –CTCACCAGGAACATGGGAAC– 3'	99 bp
ITGA2 Reverse	5' –GTCAGAACACACACCCGTTG-3'	
E-Cadherin Forward	5' – CGCCTGGGACTCCACCTACAGAA– 3'	97 bp
E-Cadherin Reverse	5' – AGAAACGGAGGCCTGATGGGGC– 3'	
Twist1 Forward	5' – TGGTCCATGTCCGCGTCCCA– 3'	74 bp
Twist1 Reverse	5' – AATGACATCTAGGTCTCCGGCCC– 3'	
N-Cadherin Forward	5' – TCCGCCTCCATGTGCCGGATA– 3'	89 bp
N-Cadherin Reverse	5' – CCAGAAGCCTCTACAGACGCCTGA– 3'	
Snai1 Forward	5' – GCCCTCCGACCCCAATCGGA– 3'	77 bp
Snai1 Reverse	5' – AGGGCTGCTGGAAGGTAAACTCTGG– 3'	
Vimentin Forward	5' – AGGCGAGGAGAGCAGGATTTCT – 3'	98 bp

Vimentin Reverse	5’ – AGTGGGTATCAACCAGAGGGAGTGA– 3’	
PSA Forward	5’ – TCTGCGGCGGTGTTCTG– 3’	87 bp
PSA Reverse	5’ – GCCGACCCAGCAAGATCA– 3’	
hGAPDH Forward	5’ – AAATATGATGACATCAAGAAGG – 3’	68 bp
hGAPDH Reverse	5’ – AGCCCAGGATGCCCTTGAGGG – 3’	
h18s Forward	5’ – GTAACCCGTTGAACCCCATT – 3’	147 bp
h18s Reverse	5’ – CCATCCAATCGGTAGTAGCG – 3’	
hB-Actin Forward	5’ – GGCTGGCCGGGACCTGACTGA – 3’	106 bp
hB-Actin Reverse	5’ – CTTCTCCTTAATGTCACGCACG – 3’	
hB2M Forward	5’ – ACTGAATTCACCCCCACTGA – 3’	114 bp
hB2M Reverse	5’ – CCTCCATGATGCTGCTTACA – 3’	

Table 2.1 Real-time PCR primer sequences.

2.3 Bisulphite methods

2.3.1 Bisulphite treatment

Genomic DNA isolated from prostate cancer cell lines (see Section 2.5.1) was subjected to bisulphite modification using an EZ DNA Methylation-Lightning™ Kit (Zymo Research Corporation, USA) according to the manufacturer’s instructions.

2.3.2 Bisulphite primers

The bisulphite treated DNA was then subjected to nested PCR to amplify the regions of interest. Primers were designed using the Methyl Primer Express program (ABI). Primer

sequences are summarised in Table 2.2 and the primer binding positions in the *ITGA2* promoter are shown in Figure 2.1.

Primer Name	Primer sequence
BSITGA1 Forward	5' – CTGATGAGTTAGTTTCTAACCTGG – 3'
ITGA2NoMe2 Reverse	5' – CCCATCCTAAATCTAAC – 3'
BSITGA2 Reverse	5' – AAAAAATACCCCAATCCC – 3'
BSITGA2 Forward	5' – TTAGGTATTGTGGTTTAGGGTT – 3'
BSITGA46 Reverse	5' –AGTTTCTGGGCAGCTCCTGCA– 3'

Table 2.2 Bisulphite PCR primer sequences.

```
-611 cacacacagctcttgcagcaggtattgcttaaatatcaccttggataatc
-561 ataacttgtgagcagatcttctttcctgatgagttagtttctaacctggt BSITGA1F
-511 cattctgcgcttatttttgtccctttctccacccacttaggaaaaacaga
-461 gaaagggacgcaccgcgcagcccctaggcactgtggtttagggctagtgc BSTIGA2F
-411 cctcggcacccgctgccaggagccgggcgctgccaagggctgcggagggg
-361 ccacgttctcccggggactggggcatctcctgcgtgctggcgacaggctc BSITGA2R
-311 gcgggggcggagtggtgccagggcgggcgctcgcccgtccggatatgccc
-261 acccgtcccgtccaggcaggaaagcctgccagggcgccatccccatcccc
-211 accgcctccaggctgccggggctgggccgctgtacgggagccaaggtcgg
-161 tgccccgcgtgtggacgagccgaggtgcagcccgcggggccgcagggccg
-111 gggtggggcgggcgcggccggagcagatccggtgtttgcggaatcaggag
 -61 gggcgggctggggcgggccctcggcgctgcaggagctgcccagaaactt BSTGA46R
 -11 tccctgctctcaccgggcgggggagagaagccctctggacagcttctaga
 +40 gtgtgcaggttctcgtatccctcggccaagggtatcctctgcaaacctct
 +90 gcaaacccagcgcaactacggtccccggtcagacccaggatggggccag ITGA2NoMe2R
+140 aacggacaggggccgcgccgctgccgctgctgctggtggtagcgctcagt
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Figure 2.1 Bisulphite PCR primer position in the *ITGA2* promoter.

2.3.3 PCR amplification of bisulphite converted DNA

The regions of interest were amplified using PCR from 100 ng of bisulphite converted DNA using primers listed in Table 2.2. PCR was carried out using GoTaq® Green 2X Master Mix (Promega Corporation, USA) in 20 µl reaction volumes. Each PCR contained 1X GoTaq® Green 2X Master Mix, 0.25 µM of forward primer, 0.25 µM of reverse primer and 100 ng of bisulphite converted DNA (from Section 2.3.1) or nuclease free water (for the no template control, NTC).

Amplifications were carried out in a Veriti Thermal Cycler (Applied Biosystems, USA). The polymerase was activated by an initial incubation at 95 ºC for 5 minutes and then DNA amplified for 35 cycles consisting of 95 ºC for 15 seconds, annealing at 52-56 ºC (depending on the primer set) for 30 seconds, 60 ºC for 30 seconds and 72 ºC for 10 minutes. The PCR products were visualised by agarose gel electrophoresis (Section 2.2.3) and products to be cloned were excised from the gels.

2.4 Cloning

2.4.1 PCR purification

The PCR fragments were excised from agarose gels and purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Australia) according to the manufacturer's instructions. All samples were eluted in 10 µL of nuclease free water.

2.4.2 Ligation

As for bisulphite sequencing and NoMe-seq (Section 2.9.3), isolated PCR fragments were ligated into the pGEMT-easy vector (Promega, USA) whereas for cloning promoter deletion mutants (Section 2.12), pXPG (provided by Prof Peter Cockerill, Bert et al. 2000) vector was used. The purified PCR products (Section 2.4.1) were quantitated by gel electrophoresis. The amount of PCR product needed to be ligated to 50 ng of vector in a 1:3 ratio was calculated and the determined amount was added to 0.5 µl of T4 Ligase and 2.5 µl of 2x ligation buffer in a total volume of 5 µl. The reaction was incubated at room temperature for 1 hour and then overnight at 4 ºC.

2.4.3 Transformation of plasmid

The ligated plasmids were transformed into JM109 High Efficiency Competent Cells (Promega, USA) according to the manufacturer's instructions and plated on agar plates containing 100 µg/mL ampicillin (Sigma-Aldrich Corporation, USA), 0.5 mM IPTG (Sigma-Aldrich Corporation, USA) and 80 µg/mL X-Gal (Promega, USA). Plates were incubated at 37 ºC overnight.

Isolated colonies were selected from the agar plate and inoculated into 10 µl of MilliQ® water and screened for recombinants by PCR by using 2 µL of the MilliQ® water as DNA template in a PCR reaction. If the colonies were positive in the PCR, the colonies were grown overnight in L-broth and DNA was harvested using a DNA Purification System SV Minipreps kit (Promega, USA) according to the manufacturer's instructions. The DNA was digested with appropriate restriction enzymes to confirm the presence of the insert (Section 2.6.2).

2.5 DNA sequencing

2.5.1 DNA isolation

The nucleotide sequence of recombinant DNA constructs was determined by DNA sequencing. In order to sequence the construct, the plasmid was purified using a DNA Purification System SV Minipreps kit (Promega, USA) according to the manufacturer's instructions. The plasmid DNA was stored at -20 ºC.

2.5.2 DNA sequencing

Sequencing were conducted using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) with the SP6 reverse primer in Table 2.3. In each sample, 1.75 µl of BigDye® Terminator sequencing buffer, 0.25 µl of BigDye® Terminator, 0.32 µM of primer, 100-150 ng of DNA isolated by DNA Purification System SV Minipreps (Promega, USA; Section 2.5.1) were added and were made up to 10 µl in total with MilliQ® water.

The reactions were amplified in a Veriti Thermal Cycler (Applied Biosystems, USA) for 25 cycles consisting of 96 ºC for 10 seconds, 50 ºC for 5 seconds and 60 ºC for 4 minutes. The products were then purified to remove the dye-terminator using the Agencourt® CleanSEQ® kit (Agencourt Bioscience Corporation, Beckman Coulter®, USA) according to manufacturer's instructions.

After purification, 30 µl of the sample was subjected to sequencing on an ABI Prism® 310 Genetic Analyzer (Applied Biosystems, USA). The sequencing data was retrieved using 310 Data Collection and Sequence Analysis software (Applied Biosystems, USA) and analysed using the computer software program Sequencher™ (Gene Codes Corporation, USA).

As for bisulphite sequencing, the sequences were further analysed using BiQ Analyzer software (Max Planck Institute Informatik, Germany) and the information used to generate bisulphite bubble maps using CpG Bubble Chart Generator, Version 20061209 Alpha (created by Mark A. Miranda).

Primer	Primer sequence
Sp6 Reverse	5' –TATTTAGGTGACACTATAG– 3'

Table 2.3 Sequencing primer sequences

2.6 Reporter assays

2.6.1 Plasmid preparation

Plasmids used in reporter assays are summarised in Table 2.4. The plasmids were transformed into JM109 cells (Promega, USA) plated on agar plates; single colonies were selected and grown in 1 mL of L-broth containing 50 µg/mL ampicillin starter culture at 37 ºC overnight with shaking. The starter cultures were inoculated into 100 mL L-broth and were incubated overnight with shaking at 37 ºC (200 mL in total for each plasmid). The plasmid DNA was extracted from the bacterial culture using a Plasmid Maxi Kit

(Qiagen, USA) according to the manufacturer's instructions. Plasmid DNA pellets were resuspended in 100 µL of Tris/EDTA (TE) buffer. The plasmid DNA was then quantified using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, USA). The DNA was stored at -20 ºC.

Plasmid	Description	Source
pGL3 Control	*SV40* promoter and enhancer driving luciferase expression with ampicillin resistance gene	Promega
pXPG ITGA2	874 bp of human *ITGA2* promoter (-791 to +83) with ampicillin resistance gene	Cloned by Alison West
pCpGL ITGA2	874 bp of human *ITGA2* promoter (-791 to +83) with zeocin resistance gene	Cloned by Annabel Short
RcCMV	Contains neomycin and ampicillin resistance gene	Invitrogen
pCMV-Flag SNAIL WT	800 bp of Human *SNAII* gene with kanamycin resistance gene	Addgene
pCMV6 TWIST1	884 bp of human *TWIST1* gene with ampicillin resistance gene	Origene
pXPG ITGA2-E-Boxm	As for pXPG ITGA2 with putative E-Box site mutated with ampicillin resistance gene	Refer to Chapter 5
pXPG ITGA2 Del1	652 bp of human ITGA2 promoter (-569 to +83) with ampicillin resistance gene	Refer to Chapter 5
pXPG ITGA2 Del2	353 bp of human ITGA2 promoter (-270 to +83) with ampicillin resistance gene	Refer to Chapter 5

EF1α-Sp1Neo	Sp1 cDNA with neomycin resistance gene	Professor Merlin Crossley, Ref Crossley et al. 1995

Table 2.4 Plasmids used in transient transfection

2.6.2 Restriction enzyme digests

Restriction enzyme digestions were used to confirm the identity of the plasmids. In general, the concentration of DNA used was between 100 ng and 1 µg. The digestion reaction consisted of the 1X recommended buffer (New England Biolabs, USA), 100 µg/mL BSA (New England Biolabs, USA), 1 µL of enzyme (20 U/µL, New England Biolabs, USA) and MilliQ® water to a total volume of 20 µL. The samples were incubated at the optimal temperature of the enzyme overnight, then analysed by agarose gel electrophoresis.

2.6.3 Methylation of vector

CpG methyltransferase (*M.SssI*) (New England Biolabs, USA) was used to methylate the pCpGL ITGA2 vector. Approximately 1 µg of vector was methylated with $1/10^{th}$ volume of NEB2, $1/50^{th}$ volume of 32 mM SAM and 100 U of *M.SssI*. The reaction was incubated for 4 hours at 37 ºC. This was followed by an inactivation step at 60 ºC for 20 minutes. To confirm methylation, the vector was digested with *AciI* methylation sensitive enzymes.

2.6.4 Transfection of prostate cancer cell lines

LNCaP and/or PC3 cells (2×10^6 cells) in 500 µL of PBS were used for each transfection. Transfections were conducted in duplicate. Purified plasmid DNA was added to a Gene Pulser® electroporation cuvette (4 mm; BioRad, USA) along with 500 µL of cells and then electroporated at 300 V with a capacitance of 500 µF using a BioRad Gene Pulsar® PLUS™ electroporator unit. Next, 1 mL of medium was added into each cuvette and the cells were allowed to recover for 5 minutes at room temperature. Cells were removed from the cuvette with a sterile Pasteur pipette and duplicate transfections

were combined into a flask containing 7.5 mL medium. The cells were allowed to recover for 24 hours.

2.6.5 Preparation of cell lysates

Following appropriate treatment, the transfected cells were harvested in PBS using a cell scraper (TPP®, Switzerland) and then centrifuged at 500 g for 5 minutes to pellet the cells. The supernatant was discarded and the cell pellets were resuspended in 100 µL of 1X cell lysis buffer (Promega, USA). The cell lysates were subjected to a freeze thaw cycle by placing them at -80 ºC for a minimum of 15 minutes and then returning them to room temperature. This was followed by vortexing for 15 seconds and then centrifuging at 10000 g for 15 seconds. The supernatant containing cellular proteins was removed and an aliquot of the lysate was diluted 1:10 to measure protein concentration using a Bradford assay (Section 2.6.6). The cell lysate was stored at -80 ºC, until analysis.

2.6.6 Bradford assays

Bradford protein assay was used to ascertain the concentration of proteins in cell lysates prepared in Section 2.6.5. A standard curve created using bovine serum albumin (BSA; New England Biolabs, USA) was used to determine the concentration of the protein. The Protein Assay Dye Reagent Concentrate (Bio-Rad, USA) was diluted 1:5 and 1 mL was added to 10 µL of each standard or protein extract, mixed well and allowed to stand at room temperature for 5 minutes. The absorbance at 595 nm was measured for each standard relative to a blank using the BioSpec-mini spectrophotometer (Shimadzu Corporation, USA). Concentrations of samples were determined using standard curves generated using BSA standards.

2.6.7 Luciferase assays

The activity of the integrin promoter cloned into reporter plasmids (Section 2.4) was determined using a Luciferase Assay System (Promega, USA). Protein extracts (30 µg) were added into each well of a 96 well plate in triplicate for each sample. Then, 100 µL luciferase assay reagent (Promega, USA) was added into each well. Luciferase activity

was measured using a Veritas™ Microplate Luminometer (Turner Biosystems, USA) program with 2 seconds integration.

2.7 Small interfering RNAs (siRNA)

2.7.1 siRNA transfection

Specific human *ITGA2* siRNA (ON-TARGETplus SMARTpool *ITGA2*), negative control siRNA (ON-TARGETplus Non-Targeting pool) and siGLO® siRNA (to measure transfection efficiency) were purchased from Dharmacon (USA). These siRNAs were resuspended to 100 µM stocks, according to the manufacturer's instructions. The *ITGA2* siRNA target sequences are summarised in Table 2.5.

ON-TARGETplus SMARTpool *ITGA2*
GAACGGGACUUUCGCAUCA
GAACGCCCUUGAUACUAA
GUUCAGACCUACUAAGCAA
AAACAAGGCUGAUAAUUUG

Table 2.5 *ITGA2* siRNA sequences

The siRNAs were diluted to 40 ρmol per well and added to Opti-MEM® (Gibco®, USA) medium to a total of 100 µL (per well). A 1.5 µL aliquot of Attractene Transfection Reagent (Qiagen, USA) was added to complete the transfection reaction and incubated for 15 minutes at room temperature. PC3 and 22Rv1 cells were harvested at approximately 70% confluency and the cell pellet was resuspended in 1 mL of medium and counted. A 1 mL aliquot of cells (1.5×10^5 cells/mL) was added to each well in a 24-well plate. A 100 µL aliquot of the transfection complex was then added with pipette mixing. Each siRNA transfection was conducted in duplicate wells and the cells were incubated for 24 to 48 hours.

2.7.2 Harvesting transfected cells

To harvest the siRNA transfected cells, medium was removed and the cells were washed with PBS. A cell scraper (TPP®, Switzerland) was used to detach the adherent cells in 250 µL of 0.05% trypsin in PBS solution. A 250 µL aliquot of medium was added before pipette mixing and combining the replicates. Cells were pelleted at 500 g for 5 minutes, cell pellets were stored at -80 ºC and RNA was extracted according to Section 2.2.1.

2.8 Cell migration methods

2.8.1 Cell migration assays

LNCaP, 22Rv1 and PC3 cells as well as cell lines transfected with siRNA were plated onto circular glass coverslips (13 mm^2) pre-coated overnight with 0.01% poly-l-lysine at a cell density of 3 x 10^5 cells/mL in 500 µL medium per well. Cells were incubated for 24 hours. After 24 hours, the attached cells were scratched with a P20 pipette tip and replicate wells fixed at 0 hour, 4 hour and 6 hour by removing the medium and incubating in 4% paraformaldehyde for 20 minutes at room temperature with agitation. The fixed cells were washed 3 times by incubating with PBS for 10 minutes at room temperature with agitation. After the last wash, the coverslips were stored in PBS at 4 ºC until further analysis.

2.8.2 Immunostaining

To visualise cell migration, the cell nuclei were stained with 0.01% Nuclear Yellow (Invitrogen, USA) in 250 µL PBS per well in a 24-well plate for 10 minutes with agitation. This was followed by 3X 10 minutes PBS washes with agitation and then the coverslips were rinsed in MilliQ® water. Coverslips were mounted onto glass slides using Permafluor mounting medium (Immunotech, Marseilles, France). Fluorescence was viewed with an Olympus BX-50 microscope and images were acquired with a Magnafire CCD camera (Optronics, Goleta, CA). Photos were taken at 20x magnification.

2.9 Chromatin methods

2.9.1 Chromatin accessibility assay (CHART-PCR)

Chromatin accessibility to digestion with micrococcal nuclease (MNase) was analysed using CHART-PCR as described in Rao et al., (2001). To isolate the nuclei, LNCaP, 22Rv1 and PC3 cells at 5 x 10^5 cells/mL were centrifuged for 5 minutes at 500g, washed with PBS and resuspended in 1 mL of ice cold nuclei buffer (10 mM Tris pH7.5, 10 mM NaCl, 3 mM $MgCl_2$, 0.1 mM EDTA and 0.5% Igepal, 0.15 mM spermine, 0.5 mM spermidine). The cells were incubated on ice for 5 minutes and the nuclei were recovered by centrifugation at 3000 g for 3 minutes, washed with 1 mL of ice cold MNase buffer (10 mM Tris pH7.5, 15 mM NaCl, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine), centrifuged at 3000 g for 3 minutes and resuspended in 200 µL MNase buffer. Nuclei (94 µL) were treated with 25 U MNase (Sigma-Aldrich Corporation, USA) and 1 µL 0.1 M $CaCl_2$ for 5 minutes at room temperature, as determined empirically. For each sample, a control (without the enzyme) was incubated in a similar way to monitor the endonuclease activity. An aliquot of 20 µL stop buffer (0.1 M EDTA pH8, 0.05 M EGTA pH8) was added to quench the reaction. Genomic DNA was isolated using a QIAamp blood kit (Qiagen, USA) according to the manufacturer's instructions. Genomic DNA (50 ng) was analysed by real-time PCR (Section 2.2.5). The primer sets used are summarised in Table 2.6.

CHART-PCR primers		Product size
-555 Forward	CACAGCTCTTGCAGCAGGTA	106 bp
-555 Reverse	GCGCAGAATGACCAGGTTAG	
1hm Forward	GCAGCCCCTAGGCACTGTGGT	85 bp
1hm Reverse	GCCCCTCCGCAGCCCTTG	
2hm Forward	GCTCGCCGTCGGATATG	120 bp
2hm Reverse	CACCTTGGCTCCCGTACAGC	

-126 Forward	CTGTACGGGAGCCAAGGTG	112 bp
-126 Reverse	GCAAACACCGGATCTGCTC	
52hrm#2 Forward	GGTGTTTTGCGGAATCAG	78 bp
52hrm#2 Reverse	AGCAGGGAAAAGTTTC	
3hm Forward	GAAACTTTTCCCTGCT	77 bp
3hm Reverse	AGGGATACGAGAACCTGCAC	
+210 Forward	GTCAAGGTAAGCGGGGATTT	95 bp
+210 Reverse	CTCCCTAGTTCCGCCCAAT	

Table 2.6 CHART-PCR primer sequences

2.9.2 Chromatin immunoprecipitation assay (ChIP)

DNA-protein interactions were examined by ChIP analysis. LNCaP, 22Rv1 and PC3 (5 x 10^6 cells) were treated with 1% formaldehyde for 15 minutes with agitation at room temperature to crosslink the proteins and DNA then quenched by addition of 0.125 M glycine and incubated for 10 minutes with agitation. Cells were then pelleted by centrifugation at 500 g for 5 minutes and washed twice with ice cold PBS. Cell pellets were resuspended in 1 mL lysis buffer (20 mM Tris-HCl pH8, 8.5 mM KCl, 0.5% Igepal) and incubated on ice for 10 minutes. After cell lysis, nuclear extracts were pelleted by centrifugation at 500g for 5 minutes, washed twice with ice cold PBS. Nuclei (9 x 10^6) were lysed with 250 µL of nuclei lysis buffer (1% SDS, 10 mM 0.5M EDTA, 50 mM 1M Tris pH8) and incubated for 10 minutes on ice. Nuclei were sheared into 100-500 bp fragments by sonification on high power setting and two runs of 10 times (30 seconds 'on', 30 seconds 'off') with a Diagenode Bioruptor sonicator (Diagenode, USA), as determined empirically. The solute was pre-cleared for 2 hours at 4 ºC on a rotating wheel with 1 mL ChIP dilution buffer (0.01% SDS, 1.2 mM EDTA, 16.7 mM Tris-HCL pH8, 1% Triton X-100, 167 mM NaCl) and 60 µL salmon sperm DNA/protein A agarose slurry (Millipore, USA). Solubilised chromatin was immunoprecipitated with 1.8 µg anti-

H3 (1791 Abcam, USA) and 4 µg anti-acetyl H3 (06-599 Millipore, USA) by incubating overnight at 4 ºC on a rotating wheel.

The immune complexes were recovered using 60 µL salmon sperm DNA/ protein A agarose for 4 hours at 4 ºC on a rotating wheel and then pelleted by centrifugation at 2000 g for 1 minute at 4 ºC and the supernatant discarded. The slurry was the washed with 1 mL each of low salt buffer (2 mM EDTA pH 8, 0.1% SDS, 1% Triton X-100, 20 mM Tris-HCl pH 8.1, 150 mM NaCl), high salt buffer (2 mM EDTA pH 8, 0.1% SDS, 1% Triton X-100, 20 mM Tris-HCl pH 8.1, 500 mM NaCl), washed twice with LiCl buffer (1 mM EDTA pH 8, 10 mM Tris-HCl pH 8.1, 250 mM LiCl, 1% Igepal, 1% sodium deoxycholate) and washed twice with TE buffer (1 mM EDTA pH 8, 10 mM Tris-HCl pH 8.1). DNA/ protein complexes were eluted from the slurry with 200 µL of elution buffer (100 mM $NaHCO_3$, 1% SDS), incubated on a rotating wheel for 15 minutes at room temperature and the slurry pelleted. To reverse the cross-links, 0.2 M NaCl was added to the supernatant and the proteins were degraded with proteinase K (Qiagen, USA) treatment overnight. DNA was purified by phenol/chloroform extraction (50% phenol, 50% chloroform), followed by ethanol precipitation for at least 4 hours and resuspended in 50 µL MilliQ water. DNA (5 µL) was amplified using real-time PCR (Section 2.2.5) and levels determined as a percentage of the total input DNA with no antibody control immunoprecipitates were analysed in parallel. The -555 and the 3hm primer sets (refer to Table 2.6) were used to analyse regions of the *ITGA2* promoter.

2.9.3 Nucleosome occupancy and methylome sequencing (NoMe-seq)

Analysis of both nucleosome occupancy and DNA methylation on the same DNA was undertaken by NoMe-seq (You et al., 2011). Nuclei were extracted by modification of a previously published method (Schreiber et al. 1989). Briefly, cells were trypsinised and centrifuged for 3 minutes at 500 g. Cell pellets were washed in ice-cold PBS and resuspended in 1 mL ice-cold Nuclei Buffer (10 mM Tris pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.1 mM EDTA and 0.5% NP-40, plus protease inhibitors) per 5 x 10^6 cells, and incubated on ice for 5 minutes. Nuclei were recovered by centrifugation at 900 g for 3 minutes, washed in Nuclei Wash Buffer (10 mM Tris pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$

and 0.1 mM EDTA containing protease inhibitors) and resuspended at a concentration of 2 x 10^5 cells/mL in 1X M.CviPI reaction buffer. Nuclei were treated with 200 U of *M.CviPI* for 15 minutes at 37 ºC. Reactions were quenched by the addition of an equal volume of Stop Solution (20 nM Tris-HCl pH 7.9, 600 mM NaCl, 1% SDS, 10 mM EDTA) and genomic DNA was isolated using a QIAamp blood kit (Qiagen, USA) according to the manufacturer's instructions. Bisulphite conversion was performed using an EZ DNA Methylation-Lightning™ Kit (Zymo Research Corporation, USA; refer to Section 2.3) according to the manufacturer's instructions and regions of interest were amplified using primers summarised in Table 2.7. PCR fragments were cloned into the pGEMT easy vector (Promega, USA) according to Section 2.4.

Primer Name	Primer sequence
-488NoMe Forward	5' – TTTTTTTATTTATTTAGGAAAAATAGAGAAAGGGA-3'
ITGA2NoMe2 Reverse	5' – CCCATCCTAAATCTAAC – 3'
GRP78 Forward	5' – GAGAAGAAAAAGTTTAGATTTTATAG – 3'
GRP78 Reverse	5' – AAACACCCCAATAAATCAATC – 3'

Table 2.7 NoMe-seq primer sequences

2.10 Protein analysis

2.10.1 Nuclear extracts

LNCaP and PC3 cells at 5 x 10^5 cells/mL were centrifuged for 5 minutes at 500 g, washed with PBS and resuspended in 1 mL of ice cold buffer A (10 mM Tris, pH7.4, 10

mM NaCl, 3 mM $MgCl_2$, 0.1 mM EDTA and 0.5% Igepal). The cells were incubated on ice for 5 minutes and the nuclei were recovered by centrifugation at 900 g for 5 minutes, washed with buffer A without Igepal and resuspended in 75 µL buffer C (400 mM NaCl, 7.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1 mM EGTA, 1 mM DTT and protease inhibitors) and incubated on ice with shaking for 15 minutes. The nuclear debris was removed by centrifugation at 13000 g for 5 minutes. Protein concentrations were determined by Bradford Assay (Section 2.6.6).

2.10.2 Western blot

Protein extracts were resolved by SDS-PAGE through 12% polyacrylamide, transferred to a nitrocellulose and subjected to western analysis with 1:1000 anti-Sp1 antibody (sc-59, Santa Cruz Biotechnology, USA) and 1:1000 anti-H3 antibody (1791 Abcam, USA). Proteins were visualised using a Supersignal West Pico Chemiluminescent kit (Pierce, USA).

2.11 Site-directed mutagenesis methods

2.11.1 Primer design

The QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, USA) was used to introduce mutations into specific sites of the *ITGA2* promoter. Mutagenesis primer sequences are listed in Table 2.8.

Primers	Sequences
E-BoxMutF	CGGTGCCCCGCG**AC**TGTGGACGAGCC
E-BoxMutR	GGCTCGTCCACA**GT**CGCGGGGCACCG
T_m : 87.1˚C; GC% : 76.9%	
E-BoxMut2F	GGTCGGTGCCCCGC**T**T**A**TGGACGAGCCGAC

E-BoxMut2R	GTCGGCTCGTCCA**T**A**A**GCGGGGCACCGACC
T_m : 82.5˚C; GC% : 70%	

Table 2.8 E-Box site-directed mutagenesis primer sequences

2.11.2 Mutagenesis

Mutagenesis reactions were set up according to the manufacturer's instructions. Briefly, the plasmid was constructed with the mutation primers to produce the mutant plasmid containing staggered nicks. The reaction was then digested with *DpnI* enzyme, specific for methylated DNA which digests the parental plasmid as DNA isolated from *E. coli* strains is dam methylated and thus, susceptible to *DpnI*. This results in the mutated plasmid remaining behind. These plasmids were then transformed into the XL10-Gold ultracompetent cells supplied with the QuikChange II XL kit, according to the manufacturer's instructions. Colony screening was then carried out by isolating plasmid DNA (Section 2.4.3) from each clone and subjecting them to restriction enzyme analysis (Section 2.6.2) or sequencing (Section 2.5).

2.12 Promoter analysis

2.12.1 Primer design

Primers were designed to amplify two different lengths of the *ITGA2* promoter. Restriction enzyme sites for *BamH*I and *XhoI* were added to the ends of the primers. Primer sequences are listed in Table 2.9.

Promoter deletion mutant construct primers	
ITGA2cl F1	CTACGGATCCGGATAATCATAACTTGTGAGC
ITGA2cl F2	CTACGGATCCGATATGCCCACCCGTC
ITGA2cl R1	CATCCTCGAGTTTGCAGAGGATACCCTTG

Table 2.9 Deletion mutagenesis primer sequences

Primers were designed to amplify 2 sizes of the *ITGA2* promoter as summarised in Table 2.10.

Construct sizes	
ITGA2 WT	-791 to +83
ITGA2 Del1	-569 to +83
ITGA2 Del2	-270 to +83

Table 2.10 Deletion constructs sizes

2.12.2 Cloning

Cloning methods in Section 2.4 were followed but instead of ligation into pGEMT vector, pXPG vector was used for these deletion mutant constructs. Screening and sequencing were performed as mentioned previously in Section 2.5.

2.13 Statistical analysis

All statistical analysis of data was performed using GraphPad Prism® 5.

Chapter 3

REGULATION OF *ITGA2* GENE EXPRESSION BY EPIGENETIC FACTORS IN PROSTATE CANCER

3.1 Introduction

3.1.1 Genetic and epigenetic events in prostate cancer

Traditionally, it was thought that cancer arises by progressive genetic mutations in tumour-suppressor genes and oncogenes as well as chromosomal abnormalities (Hahn et al. 1999; Hanahan et al. 2000, 2011). This is supported by Knudson's two hit hypothesis that cancer arises by two successive genetic 'hits', where both alleles of a gene are required to be inactivated to cause the loss of function of tumour-suppressor genes. Knudson's model explains development of some tumours, for example, the 'genetically' arising Wilms' tumours which involve Wilms' tumour gene (*WTI*) mutations. However, a second group/class of 'late-arising' Wilms' tumours are usually not a result of a second hit in a tumour suppressor gene, but due to inactivation of the second allele by epigenetic mechanisms (Moulton et al. 1994; Steenman et al. 1994). This has subsequently been shown for many other cancers, for example, the breast cancer 1 (*BRCA1*) gene in breast cancer and serine/ threonine kinase 11 (*STK11*) gene in colon cancer (Jones et al. 2002).

It is therefore clear that cancer is a disease that also involves epigenetic changes (Baylin et al. 2006). These epigenetic changes involve DNA hypo- and hypermethylation and altered histone modifications, which affect the chromatin structure and thus, regulation of genes. Many studies have shown that in cancer, epigenetic events complement genetic mutations (Esteller et al. 2000b; Feinberg 2004; Sailasree et al. 2008; Wong et al. 2003). It has been shown that mutations can be maintained in one allele of a gene while the other allele can be hypermethylated and this leads to the functional inactivation of both alleles. This had been observed in the $p16^{ink4A}$ gene in colon carcinoma cells (HCT116) and E-cadherin gene in gastric cancer (Grady et al. 2000; Myohanen et al. 1998).

In addition to complementing genetic mutations, epigenetic silencing in cancer can lead to mutational events during tumour progression. This has been observed for the mismatch-repair gene, mutL homologue 1 (*MLH1*), which is frequently hypermethylated and this is associated with microsatellite instability in colorectal cancer (Nakagawa et al. 2001), in gastric cancers (Fleisher et al. 1999) and in sporadic endometrial cancers (Simpkins et al. 1999). Further, silencing of the O^6-methylguanine DNA methyltransferase (*MGMT*) gene (a DNA repair gene) was found to predispose cells to mutation in the K-RAS oncogene in colon cancer (Esteller et al. 2000a). The review by Baylin et al. (2006) highlighted that epigenetic changes might predispose tumour cells to signalling pathway abnormalities during early stages of tumour development and dependence on these pathways will lead to acquisition of genetic mutations. Therefore, it is now well accepted that both genetic mutations and epigenetic events can affect tumour progression.

A previous familial genetic study by FitzGerald et al. (2009) identified two polymorphisms (rs3212649 and rs1126643) within the *ITGA2* gene that are associated with prostate cancer risk. Further, the differential expression of the *ITGA2* gene in normal prostate samples as compared to prostate tumour samples suggests that *ITGA2* plays an important role in prostate cancer progression, as outlined in Section 1.5. In addition, studies have also shown that the α2β1 complex is involved in adhesion of metastatic prostate cells to the bone (Hall et al. 2006; Sottnik et al. 2012).

Given this it was hypothesised that deregulation of *ITGA2* expression by epigenetic alterations may contribute to prostate tumour development and progression. Analysis of the *ITGA2* promoter revealed a highly CpG rich region (Figure 3.1) and therefore, led to the examination of whether altered methylation and chromatin environment is involved in dysregulation of *ITGA2* expression in a prostate cancer cell model. The aim of this chapter is therefore to investigate the regulation of *ITGA2* by epigenetic factors in prostate cancer.

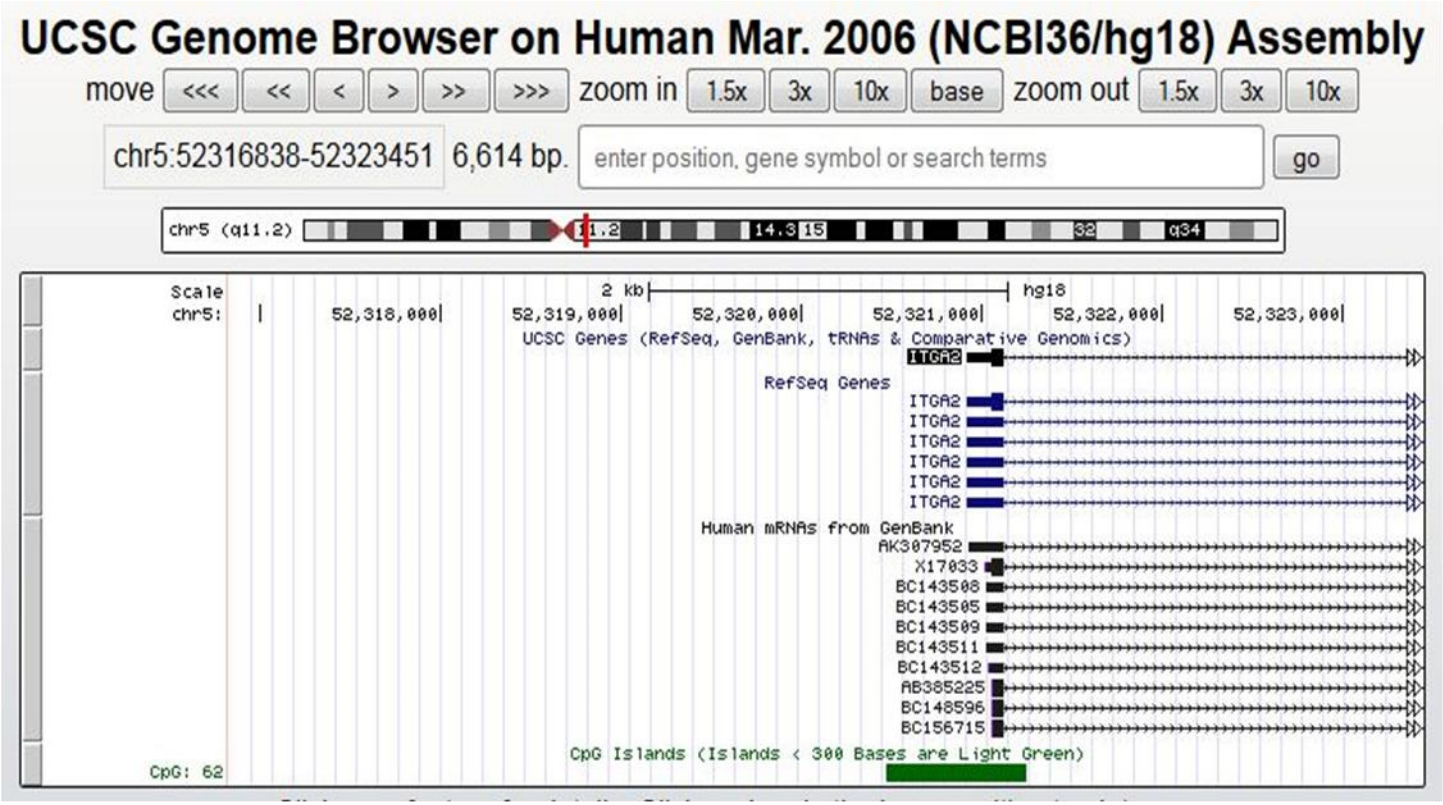


Figure 3.1 CpG island at the *ITGA2* promoter

Screenshot from the UCSC Genome browser showing the 774 bp CpG island consisting of 62 CpG dinucleotides (labelled in green) at the *ITGA2* promoter exon 1, intron 1. The blue bars above are the locations of known human protein-coding and non-protein coding genes taken from NCBI RNA reference (RefSeq). The black bars are the alignments between human *ITGA2* mRNA in GenBank and the genome.

3.2 Results

3.2.1 Increased ITGA2 gene expression correlates with increased cell migration capacity

In order to investigate the role of *ITGA2* in prostate cancer, a panel of prostate cell lines was used in this study which represents different aspects of prostate tumour biology. These cells include a benign prostate cell line (PWR-1e), lymph node metastasis-derived cell line (LNCaP), localised prostate cancer cell line (22Rv1), vertebral metastasis-derived cell line (VCaP) and bone metastatic prostate cancer cell line (PC3), as summarised in Table 3.1. The PWR-1e cell line is classified as non-tumorigenic cells as these cells do not develop tumours when injected into nude mice (Webber et al. 1996; Wu et al. 1994). Conversely, LNCaP, 22Rv1, VCaP and PC3 cell lines are classified as tumorigenic as they develop tumours in nude mice (Kaighn et al. 1979; Korenchuk et al. 2000; Sramkoski et al. 1999). Interestingly, LNCaP cells alone are nontumorigenic, however, for LNCaP cells to grow on xenografts in nude mice, the cells must be injected with Matrigel or stromal cells, which support cell viability and promote formation of a blood supply as tumour establishes (Tuxhorn et al. 2002). The latter cell lines have also been reported as having differing migratory potential. In order to assess comparative migratory capabilities, the LNCaP, 22Rv1 and PC3 cell lines were seeded onto poly-lysine coated coverslips, grown to confluency and a scratch in the monolayer was created across the coverslip using a plastic pipette tip. Replicate coverslips were fixed at the time of injury (0 hour) and 24 hours post-injury and the cell nuclei were stained with Nuclear yellow. A qualitative assessment of migratory potential was made following visualising the cells by fluorescence microscopy (Figure 3.2). LNCaP cells showed little or no migration at 24 hours whereas 22Rv1 cells showed some cell migration with the border of the scratch becoming irregular. Conversely, PC3 cells showed higher migration capacity with cells clearly localised within the scratch. Therefore, these results confirm that these cell lines have different cell migration capacity.

To determine whether *ITGA2* gene expression correlates with migration capacity, *ITGA2* mRNA levels were determined by real-time PCR and using the Pfaffl method. This method is a widely used method which produces reproducible and highly accurate results

Cell lines	PWR-1e	LNCaP	22Rv1	VCaP	PC3
Origin	Benign prostate	Lymph node metastasis	Localised to prostate	Vertebral metastasis	Bone metastasis
Metastatic potential	No	Low	Yes	Yes	Yes
Tumourigenic	No	Yes	Yes	Yes	Yes

Table 3.1 Summary of the characteristics of the prostate cell lines used in this study.

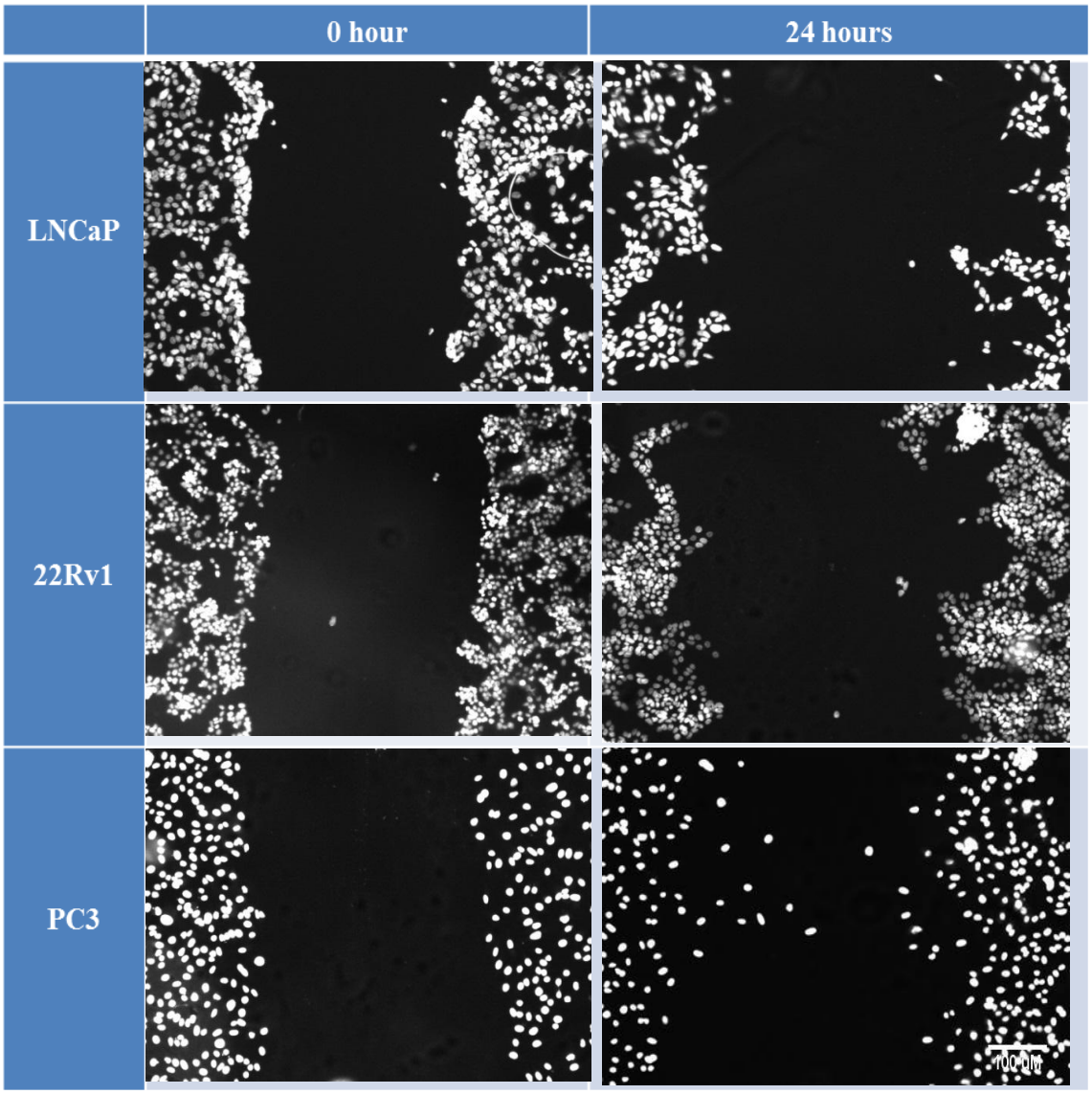


Figure 3.2 PC3 cells display higher cell migration capacity than 22Rv1 and LNCaP cells

Images of confluent LNCaP, 22Rv1 and PC3 cells scratched with a P20 pipette tip and fixed at 0 hour and 24 hours as indicated. Cell nuclei were stained with 1:100 dilution of nuclear yellow. Scale bar, 100 µM.

where the relative expression ratio is calculated only from the real-time PCR efficiencies and the crossing point deviation of an unknown sample versus control (Pfaffl 2001). Expression of four different putative housekeeping genes was also analysed in the cell lines in order to identify a gene whose expression could be used to normalise expression of *ITGA2* across the cell lines. Interestingly, *β-actin* (Figure 3.3a) and *β-2 microgrobulin* (*β2M*; Figure 3.3b) expression was not consistent across the different cell lines, with higher expression levels in PC3 cells compared to LNCaP cells in both cases. However, levels of the 18S ribosomal RNA (Figure 3.3c) and the *Glyceraldehyde 3-phosphate dehydrogenase* mRNA (*GAPDH*; Figure 3.3d) were relatively consistent across the cell lines. As a much higher abundance of the 18S RNA was detected compared to the other genes, *GAPDH* was selected for normalisation of *ITGA2* levels across the cell lines.

The data presented here show that, the PC3 cell line expresses higher levels of *ITGA2* mRNA while 22Rv1 cells showed moderate levels of expression compared to LNCaP cells, which displayed low levels of *ITGA2* expression (Figure 3.3e). Therefore, *ITGA2* expression in these cells correlated with the migration capacity observed with higher expression of *ITGA2* correlating with higher migration capacity and lower expression of *ITGA2* correlating with lower or no migration capacity.

3.2.2 *The ITGA2 promoter is differentially methylated in prostate cancer cell lines*

Given the differential *ITGA2* expression patterns across the prostate cancer cell lines, mechanisms that might regulate this differential expression were investigated. As noted previously, the *ITGA2* gene promoter is located within a large CpG island (Figure 3.1). This 774 bp island contains 62 CpG dinucleotides and therefore, could potentially be regulated by DNA methylation. The bisulphite sequencing method was therefore utilised to analyse *ITGA2* promoter methylation across the cell lines. Genomic DNA was bisulphite converted and the promoter region of *ITGA2* from -500 to -37 bp relative to the TSS, containing 47 CpG dinucleotides was amplified by PCR (Figure 3.4a). Amplified fragments were then cloned and representative clones were selected for sequencing. Methylation of each CpG from -500 bp to the transcription start site (TSS) was analysed in 6-10 clones from each of the prostate cell lines (Figure 3.4b-f). The *ITGA2* promoter

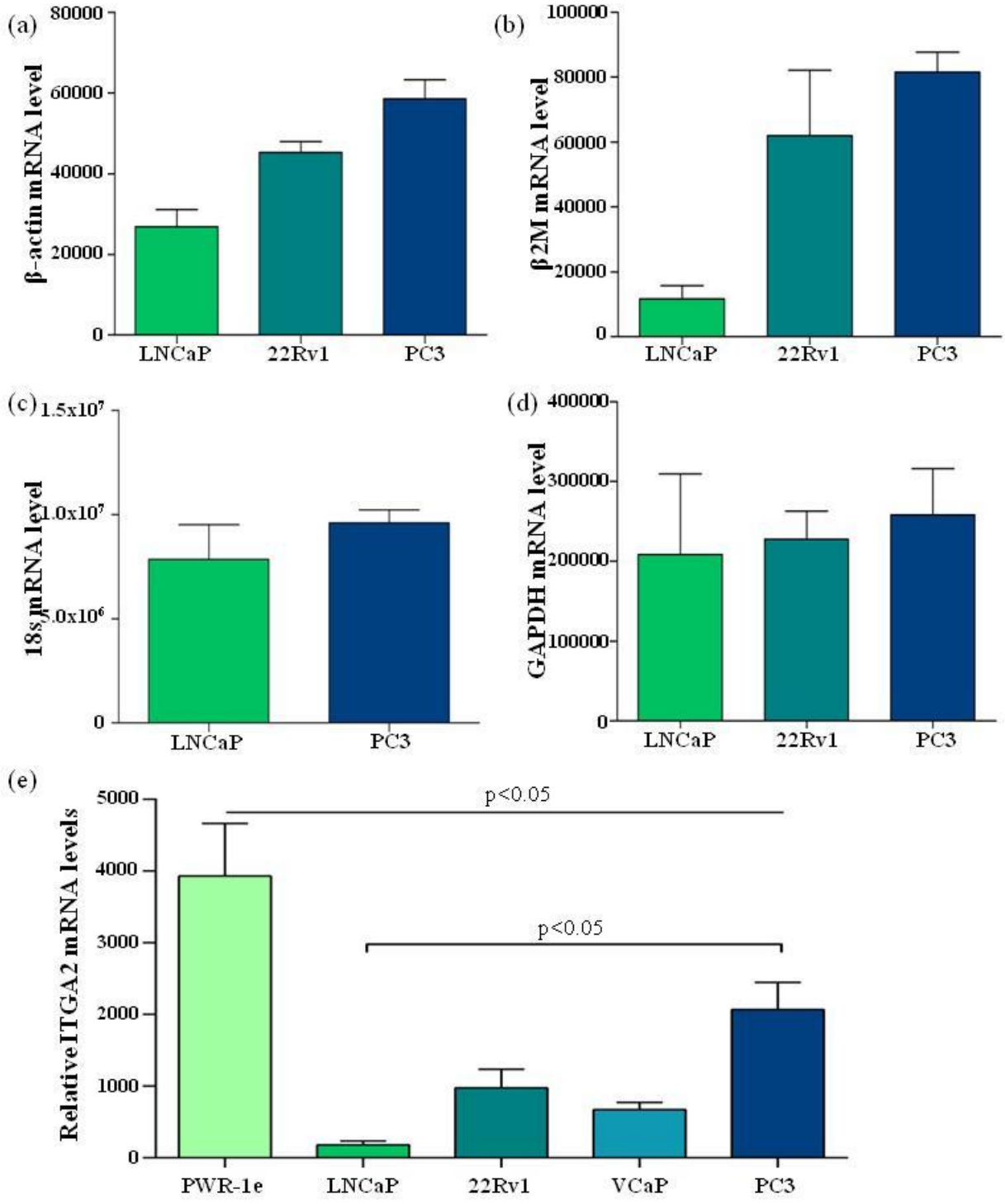


Figure 3.3 Differential *ITGA2* expression levels in the prostate cell lines

Total mRNA isolated from the prostate cell lines as indicated was reverse transcribed and amplified by real-time PCR using primers designed to amplify (a) *β-actin* (b) *β2M* (c) *18s* ribosomal RNA (d) *GAPDH* (e) *ITGA2*. Raw levels of expression were graphed in (a)-(d) and expression relative to *GAPDH* were graphed in (e). Values expressed as mean ± SEM (n=3). Statistical significance was determined using one-way ANOVA with Neuman-Keuls multiple comparison test.

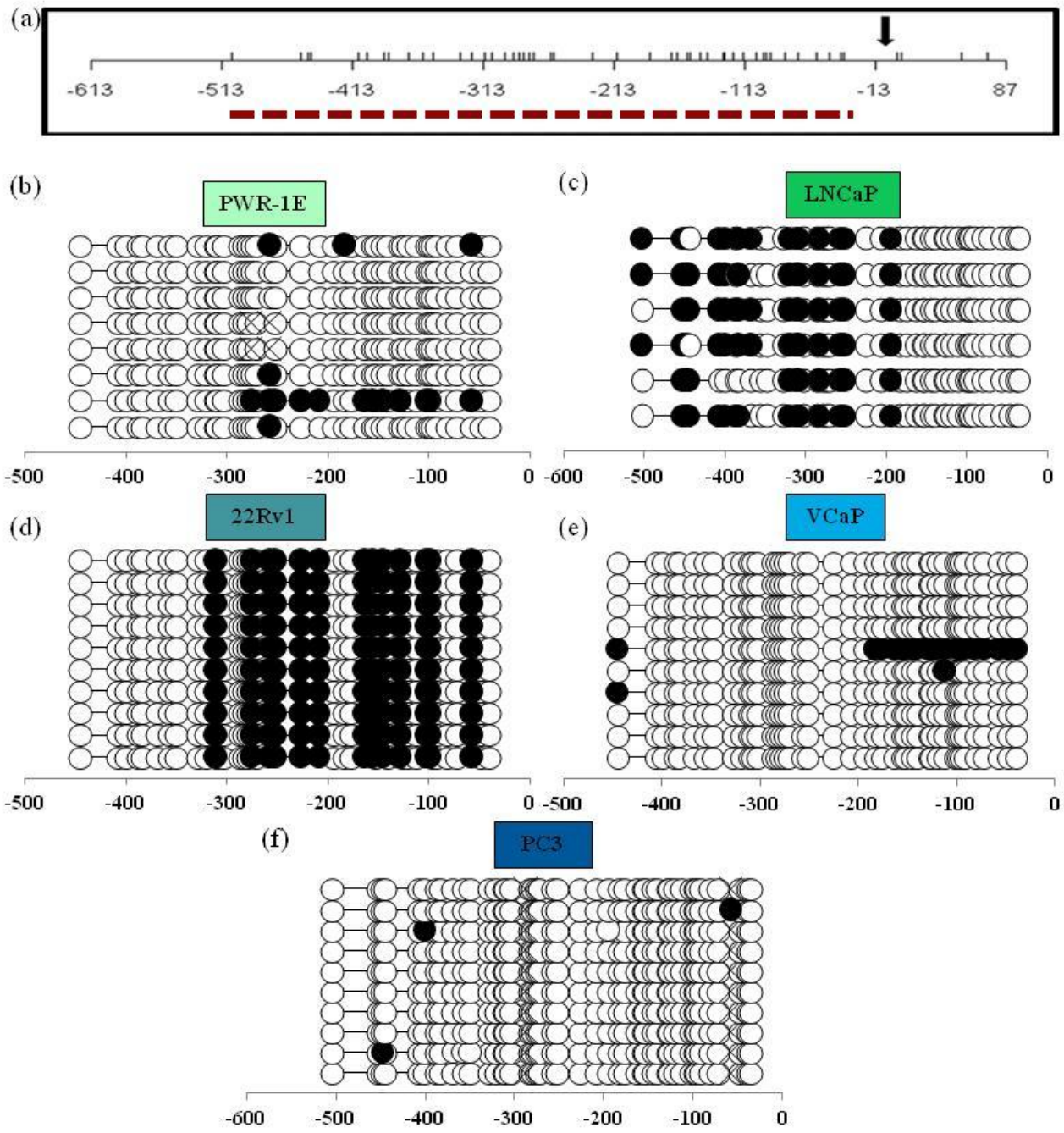


Figure 3.4 A putative CpG island within the *ITGA2* promoter is densely methylated in LNCaP and 22Rv1 but not in PWR-1e. VCaP and PC3 cells

(a) CpG plot of the *ITGA2* promoter region analysed in this study. Each vertical line represents a CpG dinucleotide and the transcription start site is indicated by an arrow. The region analysed by bisulphite sequencing is represented by the dashed line in red; (b-f) Methylation pattern of the *ITGA2* promoter analysed by bisulphite sequencing in (b) PWR-1e, (c) LNCaP, (d) 22Rv1, (e) VCaP and (f) PC3 cells. White circles represent unmethylated CpG sites and black circles represent methylated CpG sites. Each line of circles represents 1 clone. **X** indicates nucleotides where the sequence where nucleotides could not be determined because of sequencing quality.

was largely unmethylated in PWR-1e, VCaP and PC3 cell lines whereas in LNCaP and 22Rv1 cells, high levels of DNA methylation were observed across the promoter. Interestingly, distinctly different methylation patterns were observed in LNCaP and 22Rv1 cells with high methylation at the -500 to -250 bp region in LNCaP cells whereas in 22Rv1 cells, high methylation at the -300 to -50 bp region was observed. However, overall the methylation levels displayed at the *ITGA2* promoter in these cells correlated well with ITGA2 gene expression levels. Higher expression of *ITGA2* was observed in PWR-1e and PC3 cells correlating well with lower DNA methylation levels, while lower expression of *ITGA2* was observed in LNCaP and 22Rv1 cells correlating with higher DNA methylation at the promoter. These data suggest that DNA methylation may be involved in regulating *ITGA2* expression. However, in VCaP cells, *ITGA2* expression was relatively low despite the promoter being almost completely unmethylated, suggesting that other factors are also involved in its regulation.

3.2.3 Increased chromatin accessibility and histone H3 acetylation at the ITGA2 promoter correlates with increased ITGA2 expression

Differences in DNA methylation are also often associated with differences in chromatin status (reviewed in Jones et al. 2002). Therefore, to determine whether chromatin accessibility across the *ITGA2* gene promoter in the different cell lines also correlates with expression levels, accessibility of DNA to micrococcal nuclease (MNase) was measured using a real-time PCR based accessibility assay (CHART-PCR; Rao et al. 2001). Nuclei were isolated from LNCaP, 22Rv1 and PC3 cells and digested with a limiting amount of MNase. Seven PCR primer sets amplifying regions from -555 to +210, with the approximate locations shown in Figure 3.5, were used to measure the degree of chromatin accessibility across the *ITGA2* promoter. In this assay, the amount of PCR product generated is inversely proportional to the level of MNase digestion within the region. An increase in the level of MNase digestion and thus, decrease in PCR product generated indicates increased chromatin accessibility. A ratio of PCR product generated from undigested DNA compared to the amount generated from digested DNA for each primer set was determined and a line graph representing the relative accessibility was generated by plotting the centre position of each primer set (Figure 3.5).

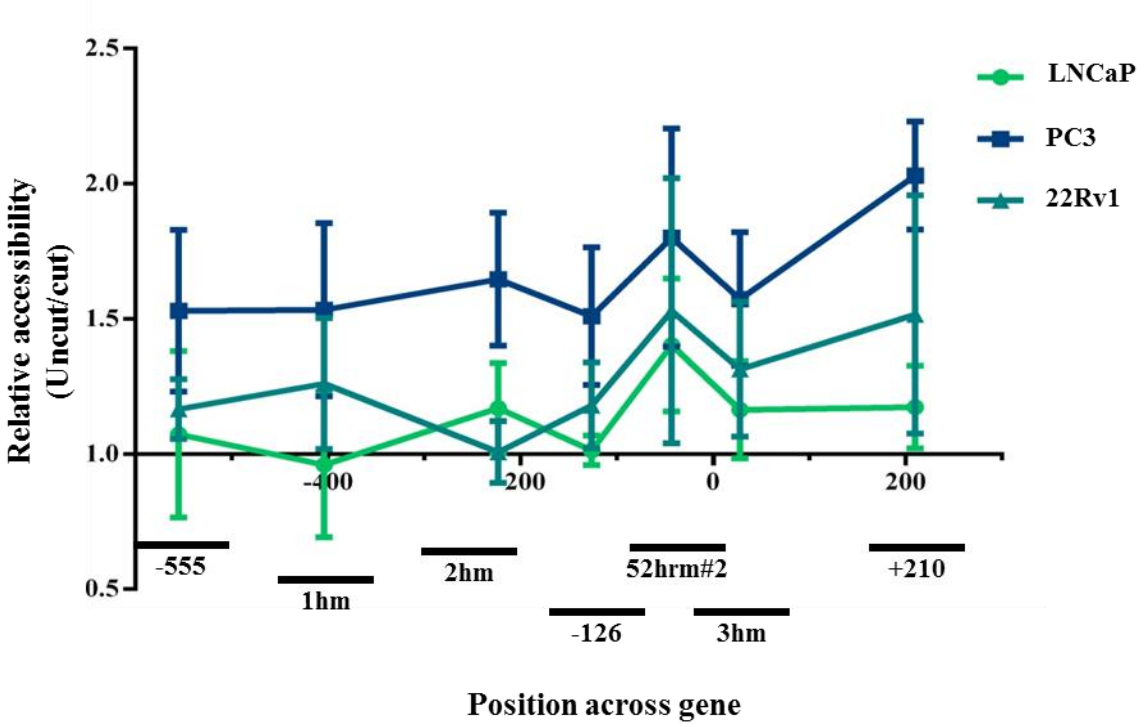


Figure 3.5 Chromatin accessibility at the *ITGA2* promoter in prostate cancer cell lines Relative accessibility (uncut/cut) determined in LNCaP, 22Rv1 and PC3 cells by CHART-PCR assay was plotted against the position on the *ITGA2* gene promoter corresponding to the midpoint of each primer set indicated below the x-axis. Values expressed as mean ± SEM (n=3).

PC3 cells displayed relatively high chromatin accessibility across the *ITGA2* promoter compared to 22Rv1 cells, which displayed moderate levels of chromatin accessibility, while low levels of chromatin accessibility were detected in LNCaP cells. Thus, the chromatin accessibility levels correlated with *ITGA2* gene expression. In addition, increased chromatin accessibility in all three cell lines was observed at the -52 bp region near the TSS. The region downstream of the TSS (0 to +210) showed increased accessibility as compared to the region upstream of the TSS (-555 to -126). The accessibility levels at the 0 to +210 region, also positively correlated with *ITGA2* gene expression in the cell lines, with a statistically significant difference in accessibility at this region in LNCaP compared to PC3 cells (Figure 3.6). Although accessibility between 22Rv1 and PC3 cells was not statistically significant, the data still suggest a trend of increasing chromatin accessibility correlating with increased *ITGA2* gene expression.

Further examination of the chromatin environment at the *ITGA2* gene promoter using ChIP analysis was performed to determine histone occupancy at the promoter. The level of promoter H3 occupancy was determined by analysing DNA immunoprecipitated with anti-H3 antibodies and quantified using real-time PCR with two primer sets that amplified the region 0.5kb upstream of the TSS and the region at the TSS (Figure 3.7a). H3 occupancy at the 0.5kb 5' region was relatively similar in all cell lines (Figure 3.7b). H3 occupancy of both regions were similar in LNCaP cells, however in 22Rv1 and PC3 cells, a trend of lower H3 occupancy was observed at the TSS as compared to the 0.5kb 5' region, although this was not statistically significant. This is in keeping with the increased chromatin accessibility observed at these regions in the cell lines.

Histone acetylation levels were also examined at both regions by ChIP analysis with anti-acetyl H3 antibodies. At the 0.5kb 5' region in 22Rv1 cells, higher levels of histone H3 acetylation was observed as compared to both LNCaP and PC3 cells (Figure 3.7c). In LNCaP cells, similar levels of histone H3 acetylation were observed at both the 0.5kb 5' region and the TSS. However, histone H3 acetylation was significantly higher at the TSS as compared to the 0.5kb 5' region in both 22Rv1 and PC3 cells. This higher level of

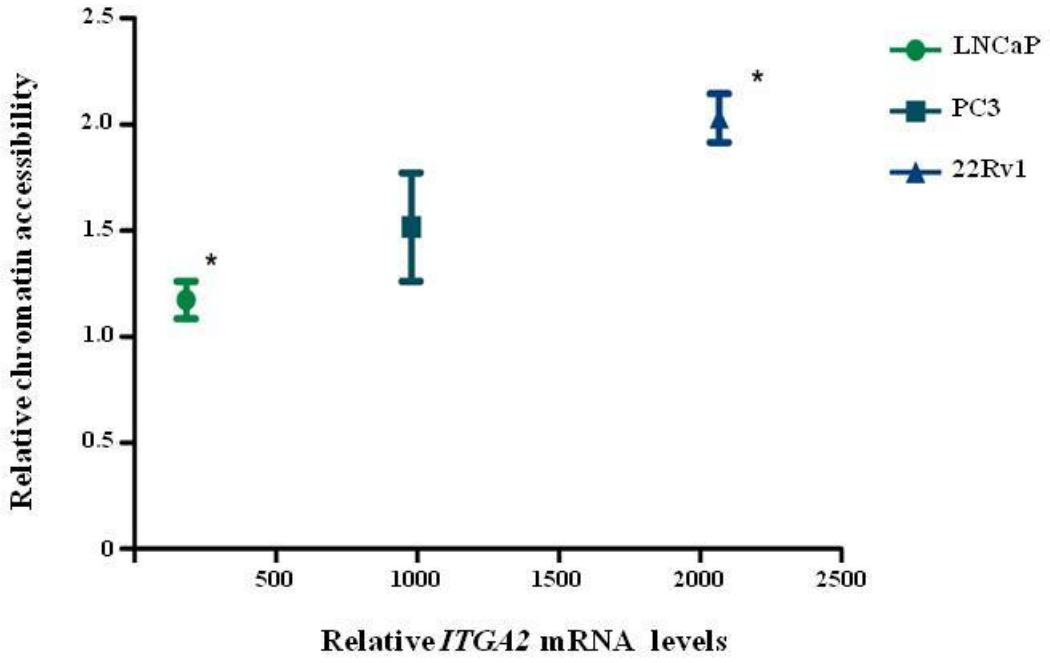


Figure 3.6 Increased chromatin accessibility correlates with increased *ITGA2* expression
Relative chromatin accessibility (as depicted in Figure 3.5) at the +210 bp region was plotted against the average *ITGA2* mRNA expression in LNCaP, 22Rv1 and PC3 cells (as determined in Figure 3.3e). Values expressed as means ± SEM (n=3). Statistical significance (* $p<0.05$) was determined using one-way ANOVA with Neuman-Keuls multiple comparison test.

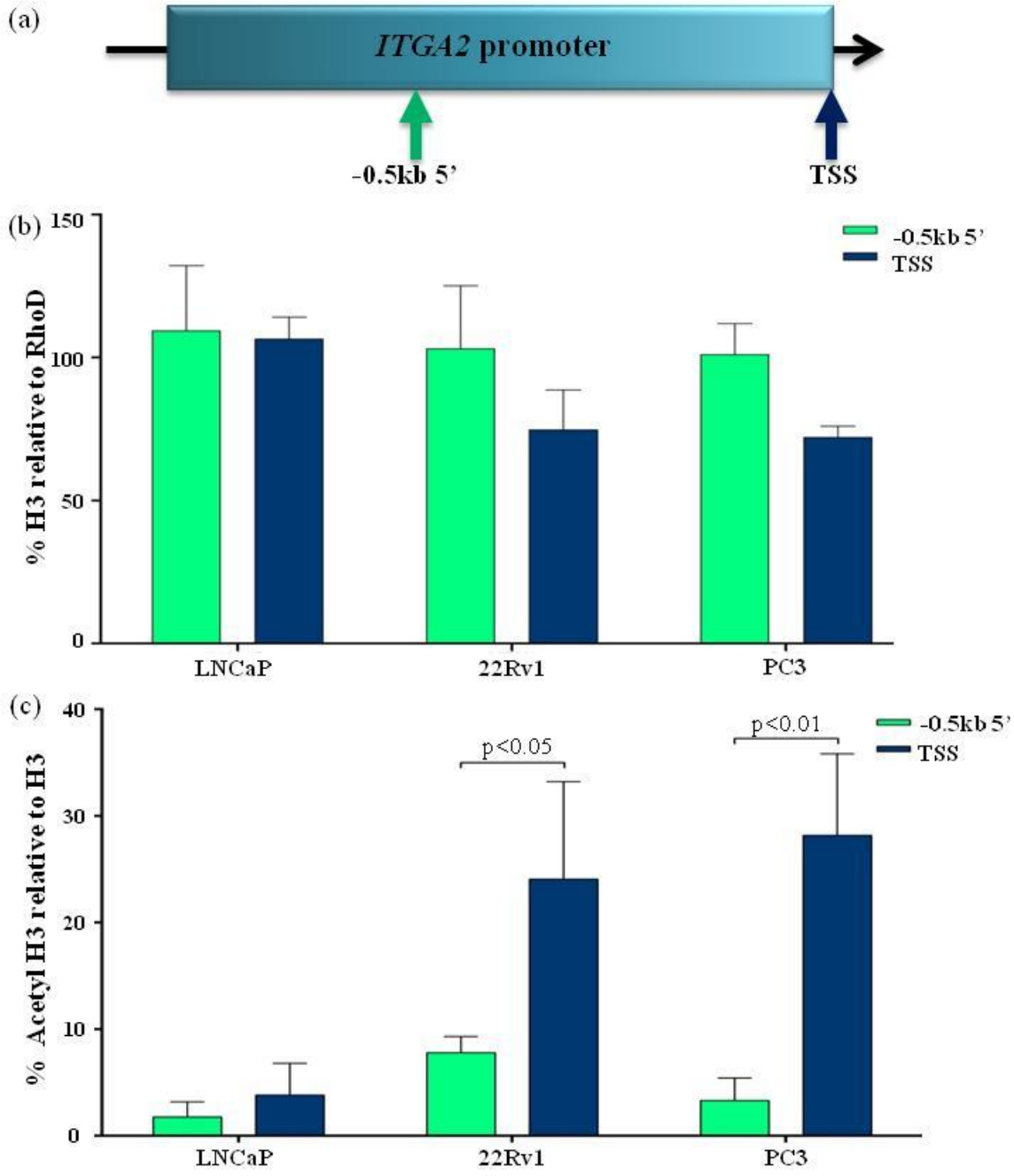


Figure 3.7 Decreasing trend of histone H3 occupancy and increased histone H3 acetylation at the TSS of the *ITGA2* gene in higher expressing cell lines, 22Rv1 and PC3
(a) Schematic diagram of the *ITGA2* promoter with positions of primer sets at 0.5kb 5' and TSS indicated by arrows; (b) ChIP analysis of histone H3 occupancy analysed in the cell lines as indicated and (c) acetylated H3 levels relative to total H3. Values expressed as mean ± SEM (n=3). Statistical significance was determined using student's *t* test.

histone H3 acetylation at the TSS may be contributing to the higher expression of the *ITGA2* gene in these cells. Overall, these results suggest that increased chromatin accessibility and increased levels of histone acetylation at the *ITGA2* promoter correlates with higher *ITGA2* gene expression.

3.2.4 A nucleosome depleted region at the promoter correlates with higher ITGA2 gene expression

Nucleosome depleted regions (NDRs) have been described previously at the TSS of active genes and also enhancer regions (Jiang et al. 2009; Kelly et al. 2012; You et al. 2011). The methylation data presented here showed a reciprocal pattern of DNA methylation in LNCaP and 22Rv1 cells and also higher levels of histone H3 acetylation at the TSS in 22Rv1 cells. However, DNA methylation and histone modifications work coordinately with nucleosome positioning to alter the accessibility of promoter regions to the transcription machinery and thus regulate gene expression (Lin et al. 2007).

A further, recently developed method known as the nucleosome occupancy and methylome sequencing (NoMe-seq; You et al. 2011) approach was therefore also used to determine accessibility at the promoter in these two cell lines. A major advantage of this method is that it provides information regarding both endogenous DNA methylation as well as the distribution of nucleosomes on the same DNA strand (You et al. 2011). Nuclei from LNCaP and 22Rv1 cells were treated with 200 U *M.CviPI* enzyme which methylates GpC dinucleotides not protected by nucleosomes or tight binding proteins. DNA was then extracted and subjected to bisulphite conversion which allows differentiation between methylated and unmethylated cytosines residues. Endogenous methylation data is derived from the cytosine of CpG dinucleotides while nucleosome positioning is determined from analysis of cytosines within GpC dinucleotides. The data generated provides information on DNA accessibility, with regions of inaccessibility less than 146 bp suggestive of DNA binding proteins and regions greater than 146 bp indicating the presence of nucleosomes (Kelly et al. 2010). As GpCs are endogenously unmethylated in humans, the *M.CviPI* enzyme can provide an accurate footprint of

nucleosome positioning, except when it is occurring at GpCpG sites and thus, these sites are excluded from the analysis.

To determine if the amount of enzyme used provided accurate footprinting results, the *GRP78* gene promoter was used. This has been used commonly as a 'control' for the NoMe-seq method (Gal-Yam et al. 2006; Kelly et al. 2012; Kelly et al. 2010). The *GRP78* gene promoter contains a CpG island and thereby provides a dense CpG grid that allows endogenous methylation analysis. The promoter is endogenously unmethylated and has a TATA box with a well-defined TSS that has a NDR region of 350 bp (Gal-Yam et al. 2006). Analysis of the GRP78 gene promoter (from -350 to +102) in both LNCaP and 22Rv1 cells using 200 U enzyme provided accurate data as expected. The *GRP78* gene promoter was unmethylated in both LNCaP and 22Rv1 cells and showed a NDR region upstream of the promoter (Figure 3.8a-d).

From the same bisulphite converted DNA, the *ITGA2* promoter was amplified from both cell lines and 9-10 clones were selected and analysed for DNA methylation and accessibility to the GpC enzyme from -350 to +120 (Figure 3.9a-d). Interestingly, the *ITGA2* promoter in LNCaP cells showed two different methylation patterns. Half of the clones were either unmethylated or sporadically methylated, suggesting monoallelic methylation. These results were in contrast to the previous bisulphite sequencing pattern observed in Section 3.2.2 and these differences will be discussed later. However, unmethylated clones had minimal accessibility to *M.CviPI* with regions greater than 146 bp, indicating nucleosome occupancy at regions both upstream and downstream of the TSS. In contrast, the other 50% of clones in LNCaP cells showed heavy methylation upstream of the TSS but also displayed a NDR throughout the promoter. Similarly in 22Rv1 cells, the *ITGA2* promoter displayed heavy methylation upstream of the TSS (7 out of 9 clones) and displayed an NDR throughout the promoter. Two of the clones were not methylated but displayed nucleosome occupancy upstream of the TSS. Therefore, from both cell lines, the data suggest that clones that were unmethylated displayed nucleosome occupancy, suggesting a poised or repressed promoter (Kelly et al. 2012). However, methylated DNA was found to have NDR regions upstream of the TSS, which

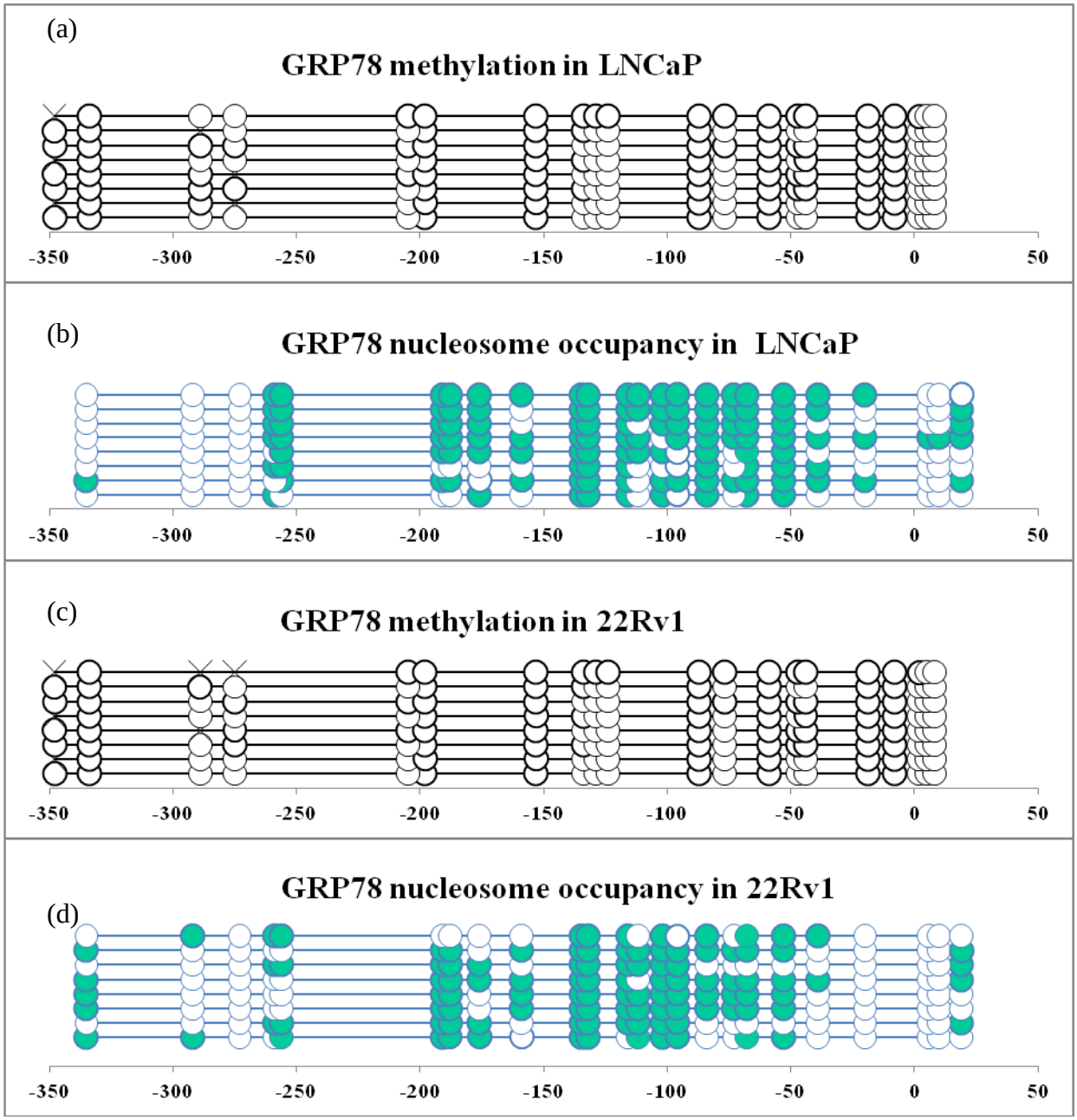


Figure 3.8 NoMe-seq footprint of the active *GRP78* promoter
Cell nuclei were treated with 200 U of *M.CviPI*, DNA extracted, bisulphite converted and sequenced. Analysis of CpG sites at the *GRP78* promoter in (a) LNCaP cells and (c) 22Rv1 cells. Analysis of GpC sites which were either protected or unprotected by nucleosomes in (b) LNCaP cells and (d) 22Rv1 cells. White circles represent unmethylated CpG and black circles represent methylated CpG sites. Unfilled blue circles represent GpC sites that are inaccessible to *M.CviPI* and green filled blue circles represent GpC sites that are accessible to *M.CviPI*. Each line of circles represents 1 clone. **X** indicates nucleotides where the sequence where nucleotides could not be determined because of sequencing quality.

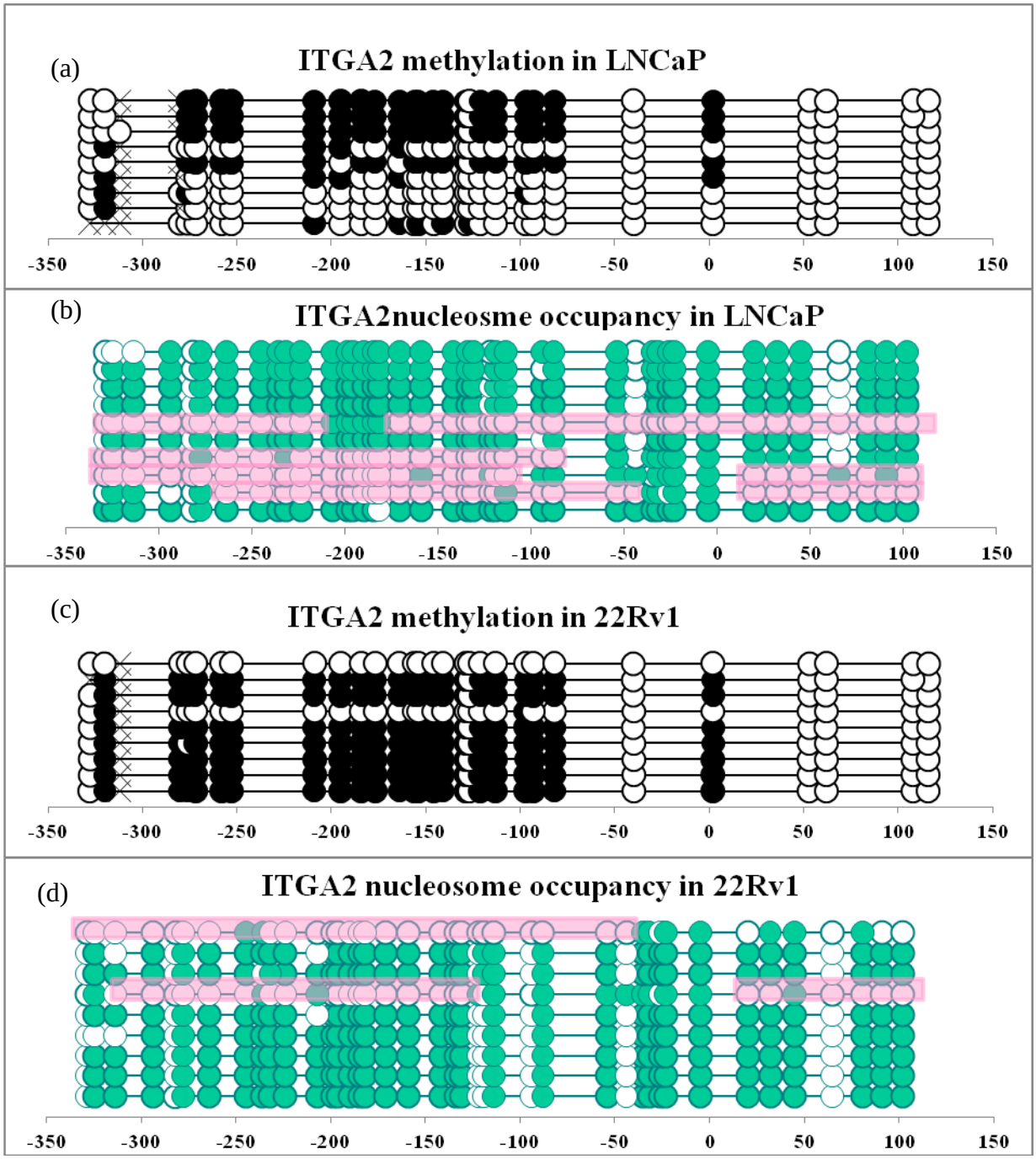


Figure 3.9 NoMe-seq analysis of the *ITGA2* promoter

Cell nuclei were treated with 200 U of *M.CviPI*, DNA extracted, bisulphite converted and sequenced. Analysis of CpG sites at the *ITGA2* promoter in (a) LNCaP cells and (c) 22Rv1 cells. Analysis of GpC sites which were either protected or unprotected by nucleosomes in (b) LNCaP cells and (d) 22Rv1 cells. White circles represent unmethylated CpG and black circles represent methylated CpG sites. Unfilled blue circles represent GpC sites that are inaccessible to *M.CviPI* and green filled blue circles represent GpC sites that are accessible to *M.CviPI*. Pink bars represent regions of inaccessibility large enough to accommodate a nucleosome. Each line of circles represents 1 clone. **X** indicates nucleotides where the sequence where nucleotides could not be determined because of sequencing quality.

has not previously been reported. The percentage of *ITGA2* promoter clones in both LNCaP and 22Rv1 cells which displayed nucleosome occupancy as assessed by the lack of *M.CviPI* methylation was determined and compared to *ITGA2* expression levels in these cells (Figure 3.10). LNCaP cells which have low *ITGA2* expression displayed higher levels of nucleosome occupancy at the TSS while 22Rv1 cells which express *ITGA2* at higher levels have a lower nucleosome occupancy at the TSS. Therefore, decreased nucleosome occupancy at the TSS correlates with increased expression of the *ITGA2* gene.

3.2.5 Combined treatment with 5-Aza-2'-deoxycytidine and Trichostatin A upregulates ITGA2 gene expression in a lower expressing cell line

The data presented so far suggest that *ITGA2* is regulated by DNA methylation and histone H3 acetylation. Unlike genetic mutations, epigenetic alterations are chemically reversible by epigenetic modifiers and these agents allow the effect of modulating epigenetic modifications to be determined (Chin et al. 2011). To investigate the effects of DNA demethylation on *ITGA2* gene expression, both LNCaP and 22Rv1 cells were treated with a DNA methyltransferase inhibitor (DNMTi), 5-Aza-2'-deoxycytidine (AzaC) on alternate days for 5 days. This treatment regime has been shown to be the lowest dose sufficient to demethylate the *GSTP1* promoter (a well described hypermethylated gene in prostate cancer) and was previously shown to permit reexpression of *GSTP1* protein in LNCaP cells (Chiam et al. 2011). Hence, as a control, *GSTP1* expression was analysed in these cells. *GSTP1* mRNA levels were determined by real-time PCR. Interestingly, AzaC treatment was found to affect the house-keeping gene, *GAPDH*, with increased expression following AzaC treatment (Figure 3.11a). Therefore, another house-keeping gene, β-actin was used for normalisation in the AzaC treated cells as the expression of this gene was unchanged between non-treated (NS) and AzaC treated cells (Figure 3.11b). As expected, upregulation of the *GSTP1* gene was observed in both cell lines following AzaC treatment (Figure 3.11c) suggesting that AzaC was effectively demethylating DNA. *ITGA2* expression was then analysed in the same samples and the results showed that treatment of both LNCaP and 22Rv1 cells with AzaC did not

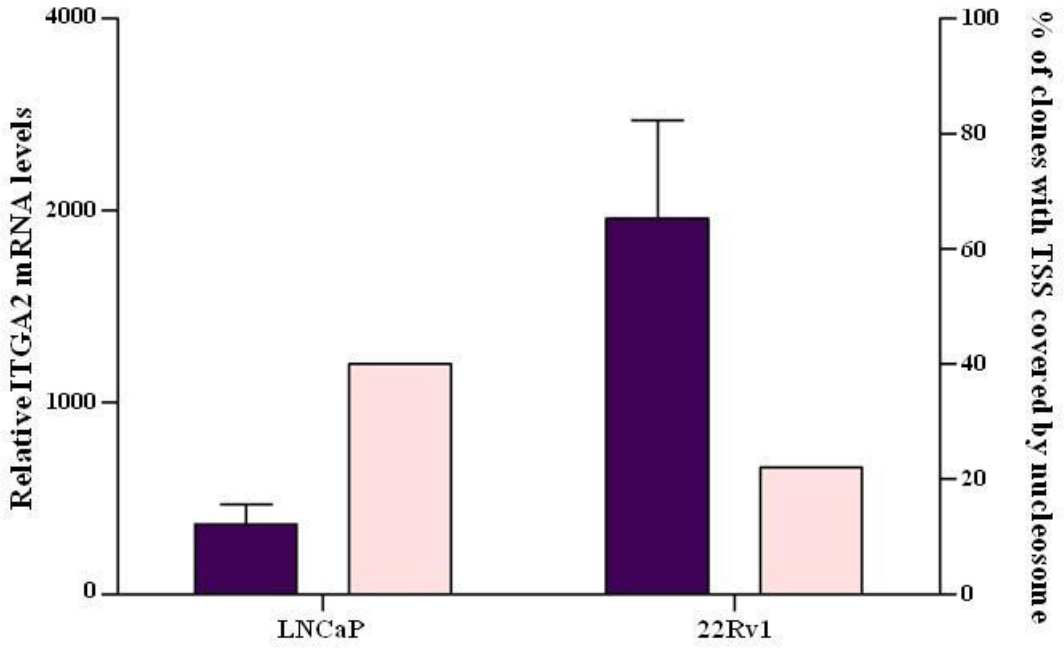


Figure 3.10 Nucleosome occupancy at the TSS of the *ITGA2* gene inversely correlated with *ITGA2* expression

The percentage of promoter clones that contained a nucleosome at the TSS (pink bar; as depicted in Figure 3.9) is plotted along with *ITGA2* expression (purple bar; as determined in Figure 3.3e). Expression is shown relative to *GAPDH*. Values expressed as mean ± SEM (n=3).

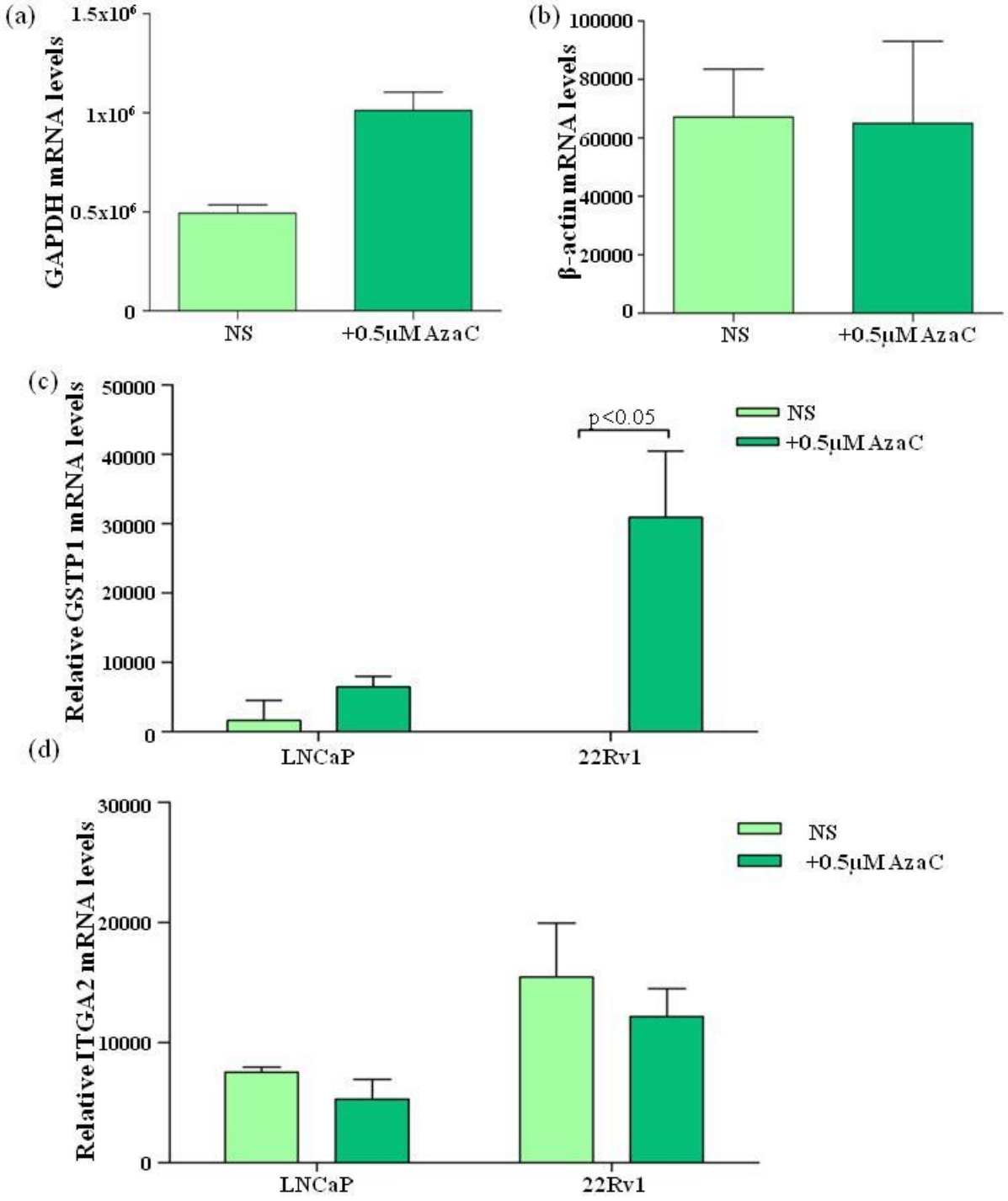


Figure 3.11 AzaC treatment did not upregulate *ITGA2* expression in prostate cancer cells
Total mRNA isolated from either untreated (NS) cells or cells treated with 0.5 μM AzaC as indicated, reverse transcribed and amplified by real-time PCR primers designed to amplify: (a) *GAPDH* (b) *β-actin* (c) *GSTP1* (d) *ITGA2*. Raw levels of expression are shown in (a) and (b) and expression relative to *β-actin* is depicted in (c) and (d). Values expressed as mean ± SEM (n=3). Statistical significance was determined relative to the indicated control using student's *t* test.

upregulate *ITGA2* gene expression (Figure 3.11d), suggesting that demethylation does not affect *ITGA2* gene expression. Next, to investigate the effects of modulating histone acetylation, a widely used histone deacetylase inhibitor (HDACi), Trichostatin A (TSA) was used to treat both LNCaP and 22Rv1 cells for 4 hours either with or without prior AzaC treatment. RNA was isolated from the cell lines, converted to cDNA and *ITGA2* mRNA levels were determined by real-time PCR. TSA treatment did not upregulate *ITGA2* gene expression in either cell line (Figure 3.12). However, combination treatment of both AzaC and TSA upregulated *ITGA2* expression in the lower expressing cell line, LNCaP, but not in the higher expressing, 22Rv1 cell line suggesting that DNA demethylation and inhibition of histone acetylation in combination modulates *ITGA2* expression in LNCaP cells.

Overall, the data presented here suggests that the *ITGA2* gene is regulated by both DNA methylation and histone H3 acetylation.

3.2.6 Increased ITGA2 expression corresponds with demethylation at the ITGA2 promoter

Experiments were then conducted to determine whether upregulation of *ITGA2* necessarily involves DNA demethylation. To do this, a strategy was employed in which higher *ITGA2* expressing cells, $LNCaP_{col}$ were derived by successive panning of LNCaP cells on type I collagen. This method has been used previously to generate LNCaP cells that displayed characteristics of high affinity for collagen type I with increased α2β1 expression (Hall et al. 2006). $LNCaP_{col}$ cells were derived as previously described (Hall et al. 2006) and summarised in Figure 3.13. After each panning, RNA was isolated from some of the cells, converted to cDNA and *ITGA2* mRNA levels were determined by real-time PCR. DNA was also isolated from the cells to perform bisulphite sequencing while the rest of the cells were replated on collagen. Cells were panned for two rounds, with $LNCaP_{col}$ from the second panning ($LNCaP_{col}2$) displaying a significant increase in *ITGA2* mRNA levels as compared to the $LNCaP_{col}$ from the first panning ($LNCaP_{col}1$)

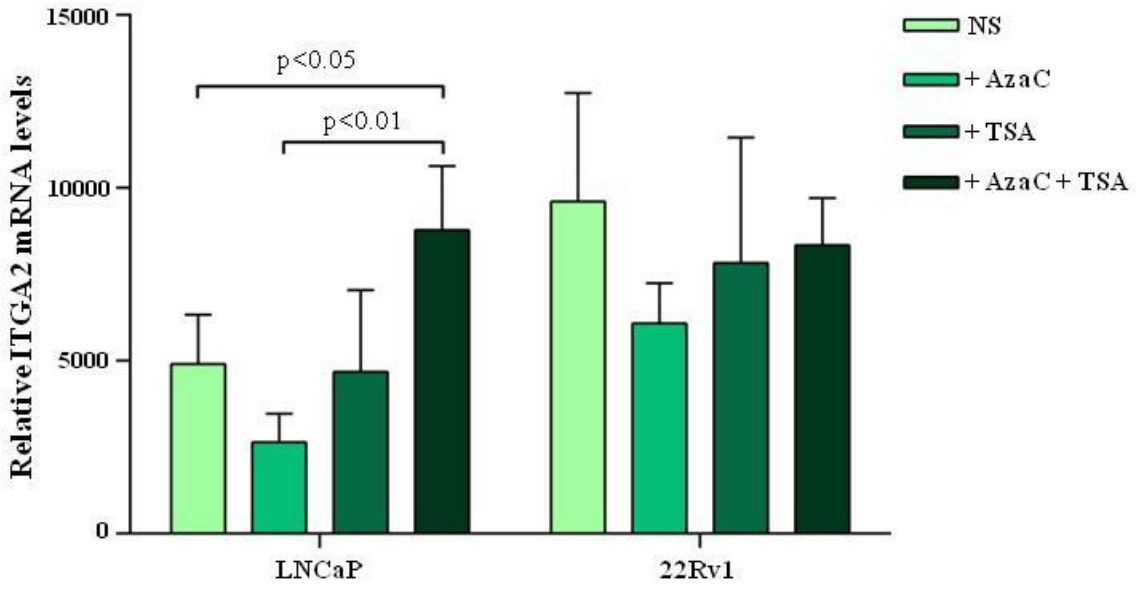


Figure 3.12 DNA demethylation and histone acetylation upregulated *ITGA2* expression in LNCaP but not 22Rv1 cells

Total mRNA isolated from cells either untreated (NS), treated with 0.5 µM AzaC, treated with 200 ng/mL TSA or treated with both AzaC and TSA, was reverse transcribed and amplified by real-time PCR using primers designed to amplify *ITGA2* mRNA. The data show *ITGA2* expression in LNCaP and 22Rv1 relative to *β-actin*. Values expressed as mean ± SEM (n=3). Statistical significance was determined using one-way ANOVA with Neuman-Keuls multiple comparison test.

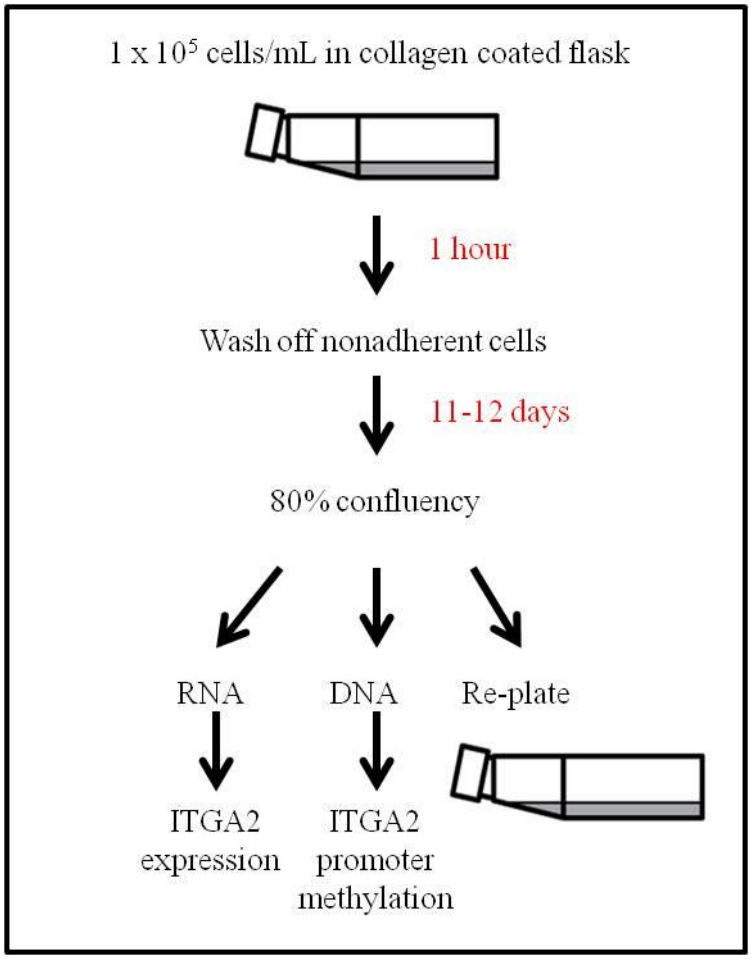


Figure 3.13 Flow chart depicting the derivation of the LNCaP$_{col}$ cells and subsequent *ITGA2* analysis.

and the parental LNCaP cells (Figure 3.14a). Analysis of *ITGA2* promoter methylation of $LNCaP_{col}1$ and $LNCaP_{col}2$ cells showed demethylation at regions -325 to -190 when compared to the parental LNCaP cells (Figure 3.14b and c). However, although demethylation was also observed in $LNCaP_{col}1$ cells, no changes in *ITGA2* gene expression were detected, suggesting perhaps a delay in upregulation of gene expression following demethylation. Demethylation observed in $LNCaP_{col}2$ cells correlated with increased *ITGA2* expression.

3.3 Discussion

Bone is the most frequent site of prostate cancer metastases with 90% of all men who die of prostate cancer having metastatic bony lesions (Bubendorf et al. 2000). The collagen receptor, α2β1 has been shown to play an important role in the normal development of the prostate and also progression of prostate cancer by facilitating tumour migration and bone metastasis. Studies have shown variable *ITGA2* expression at different stages of tumour development as outlined in Section 1.5. In particular, down regulation of *ITGA2* was observed as an early event in prostate cancer, followed by up regulation as a late event in prostate cancer (Bonkhoff et al. 1993; Pontes-Junior et al. 2009; Ramirez et al. 2011; Van Slambrouck et al. 2009). Since *ITGA2* encodes for the α subunit of the α2β1 complex and has been identified as a prostate cancer susceptibility gene through a previous study (FitzGerald et al. 2009), the *ITGA2* gene would appear to be a logical therapeutic target in prostate cancer metastasis. However, little is known about the regulation of this gene in prostate cancer or during tumorigenesis.

In order to investigate the epigenetic regulation of the *ITGA2* promoter, a panel of prostate cell lines which represent non-tumorigenic and tumorigenic aspects of prostate cancer were used. Although LNCaP cells are derived from lymph node metastasis, these cells routinely fail to bind collagen type I in *in vitro* attachment assays and are considered non-tumorigenic in nude mice (Hall et al. 2006). The *ITGA2* expression pattern observed in these cells suggests that in benign prostate cells (represented by PWR-1e), high expression of *ITGA2* could be involved in growth and cell attachment. This is similar to the expression pattern observed by Bonkhoff et al. (1993) where similar staining patterns

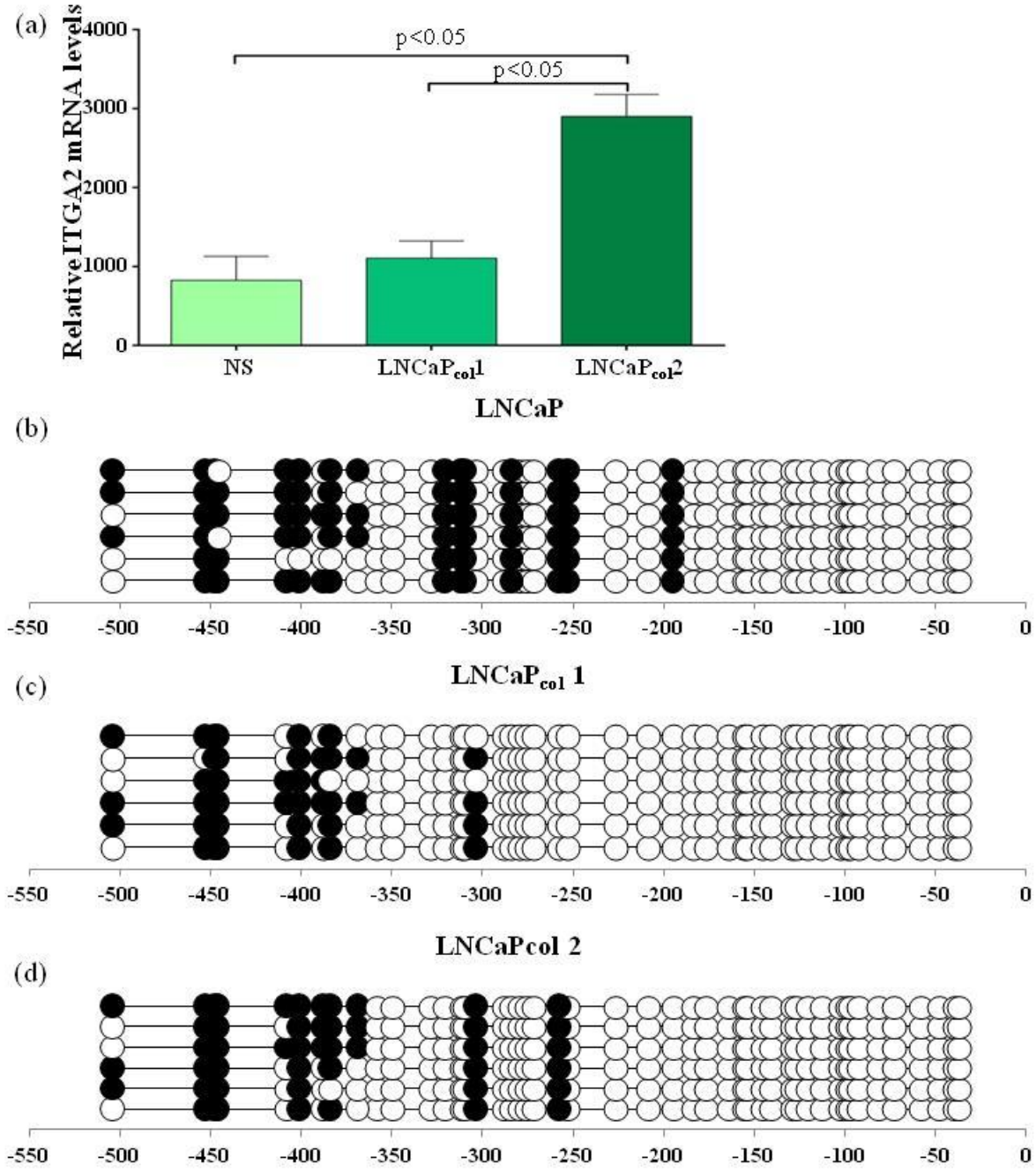


Figure 3.14 Increased *ITGA2* expression in LNCaP$_{col}$ cells correlated with demethylation at regions -325 to -190 of the *ITGA2* promoter

(a) RNA from the parental LNCaP, LNCaP$_{col}$1 and LNCaP$_{col}$2 cells was reverse transcribed and amplified by real-time PCR using *ITGA2* primers. *ITGA2* expression was graphed relative to *GAPDH*. Values expressed as mean ± SEM (n=3). Statistical significance was determined using one-way ANOVA with Neuman-Keuls multiple comparison test; (b-d) Methylation pattern of the *ITGA2* promoter analysed by bisulphite sequencing in (b) parental LNCaP, (c) LNCaP$_{col}$1, and (d) LNCaP$_{col}$2 cells. White circles represent unmethylated CpG sites and black circles represent methylated CpG sites. Each line of circles represents 1 clone.

for α2β1 were observed in both normal and hyperplastic prostate samples. Downregulation of its expression (in keeping with the relatively low expression detected in 22Rv1 cells) may be involved in the development of prostate cancer and also may facilitate cell detachment, necessary for tumour cells to escape from the primary site.
This type of gene expression pattern was observed by Gorlov et al. (2009) where *ITGA2* downregulation was observed in localised prostate cancer tissue compared to normal tissue using microarray assays. Re-expression of *ITGA2* in bone metastatic prostate cancer (in keeping with the higher expression levels observed in PC3 cells) may be involved in facilitating tumour cell relocation and in this case, attachment to the bone, where collagen I, the ligand of α2β1, is highly expressed. This is consistent with the *ITGA2* staining that was reported in 9 out of 11 bone metastasis tissue samples as compared to the matched normal samples (Eaton et al. 2010).

Higher *ITGA2* gene expression also correlated with increased cell migration capacity as observed in PC3 cells which support the idea that *ITGA2* may play a role in prostate cancer metastasis. Differential expression of the *ITGA2* gene also correlated with the differential methylation pattern observed in these cells, suggesting *ITGA2* could be regulated by DNA methylation. Previous analysis of *ITGA2* promoter methylation by our group, from -504 to -350 upstream of the TSS in localised prostate tumour samples showed lower methylation levels as compared to normal prostate samples (Short AK, unpublished data; Figure 3.15a, b and c). *ITGA2* expression was not examined in these tumour samples but since lower DNA methylation is associated with increase gene expression, this would suggest higher *ITGA2* expression in tumour samples as compared to normal prostate samples and thus, does not correlate with these findings. Nevertheless, only 11 of 47 CpG sites were analysed in the tumour samples and analysis of the CpGs further downstream and particularly at the TSS may provide more informative data. It is interesting however that this region is also demethylated in the 22Rv1 cell line which represents a localised prostate cancer cell lines. In this study, increased *ITGA2* expression correlated with low DNA methylation, increased chromatin accessibility at the promoter and increased histone H3 acetylation at the TSS. Interestingly, while the *ITGA2* promoter of the VCaP cells remained unmethylated, these cells did not express high levels of the

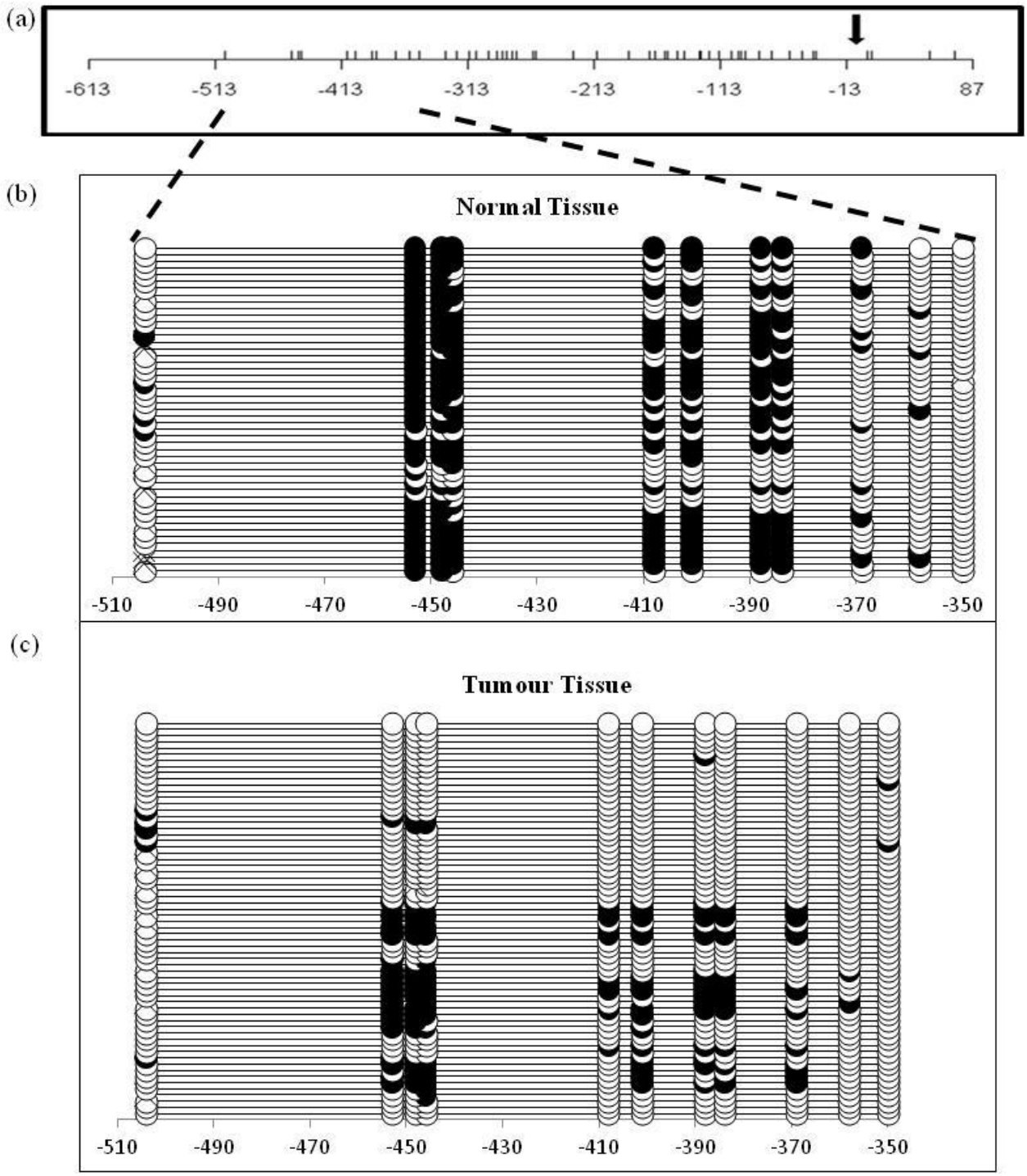


Figure 3.15 The *ITGA2* promoter is more densely methylated in normal tissue samples as compared to tumour tissue samples

(a) CpG plot of the *ITGA2* promoter region analysed in this study. Methylation pattern of the *ITGA2* promoter analysed by bisulphite sequencing in (b) normal tissue samples and (c) tumour tissue samples. White circles represent unmethylated CpG sites and black circles represent methylated CpG sites. Each line of circles represents 1 clone. Data adapted from Short, AK 2009 (unpublished data).

ITGA2 gene, suggesting these cells may be regulated by an alternative mechanism involving other histone or chromatin modifications. However, further experiments using CHART-PCR, ChIP and NoMe-seq may be required in order to elucidate the alternative mechanisms regulating the *ITGA2* gene expression observed in this cell line. Difficulties in growing these cells were encountered during the study, which prevented the further experiments to be carried out.

The NoMe-seq method was utilised to analyse the nucleosome occupancy and DNA methylation at the *ITGA2* promoter of the same DNA strand. The NoMe-seq footprinting data for the *GRP78* promoter suggests that the amount of enzyme used is enough to provide accurate footprinting results. Nucleosome occupancy footprinting at the *ITGA2* promoter in LNCaP and 22Rv1 cells was analysed using PCR primers lacking CpG or GpC sites to avoid complications due to endogenous methylation. Therefore, these primers were different from the normal bisulphite primers used previously. The *ITGA2* promoter methylation observed in the LNCaP cells in the bisulphite data in Figure 3.4c showed methylation from -500 to -250 whereas NoMe-seq endogenous methylation data in Figure 3.9a showed monoallelic methylation, where 5 of 9 clones sequenced showed unmethylated or sporadically methylated CpGs from -300 to +100 and this matches the methylation pattern observed using the normal bisulphite primers. However, 4 of 9 clones sequenced showed high methylation from -300 to +3, which in contrast to the bisulphite sequencing data from the earlier study. This discrepancy may be due to the normal bisulphite primers selecting for the unmethylated allele. This may also be due to fact that the earlier bisulphite sequencing only involved 6 clones whereas for the NoMe-seq, 10 clones were selected and thus, if more clones were selected in the earlier study, the monoallelic effect may be observed. Therefore, an alternate approach using the next generation sequencing would likely result in rapid sequencing of larger number of DNA strands and thus reveal the heterogeneicity in these cells. In addition, growth behaviour of these cells could change over time with continuous culturing and thus, may affect these results. Interestingly, discrepancies in methylation levels in LNCaP cells were previously observed when using two different methods, bisulphite sequencing and restriction enzyme based analysis (West AC, unpublished data). Further investigation suggested the

presence of an inhibitory element affecting the enzyme based assay in 50% of the DNA, which was not present during the examination of LNCaP DNA by bisulphite sequencing, again, suggesting allelic differences at the *ITGA2* loci in this cell line.

Interestingly, NoMe-seq data showed that the two different alleles also have different nucleosome occupancy conformation at the promoter, with unmethylated alleles having nucleosome occupancy while methylated alleles have a NDR. Therefore, NoMe-seq provides more information than average levels observed in a population of cells because different chromatin states can exists on the two alleles in a single cell or in different subpopulation of cells within a sample (Kelly et al. 2012). It is also well described now that active promoters typically have a NDR immediately upstream of the TSS whereas transcriptionally inactive promoters usually lack this NDR. Comparing percentage of nucleosome occupancy at the TSS in both LNCaP and 22Rv1 cells, 40% of the clones in LNCaP cells displayed nucleosome occupancy at the TSS, correlating with lower *ITGA2* expression. On the other hand, 22Rv1 cells displayed 22% nucleosome occupancy at the TSS, correlating with higher *ITGA2* expression as compared to LNCaP cells. These results suggest that the presence of NDR at the TSS may be contributing to increase *ITGA2* gene expression. Overall, these results strongly suggest that *ITGA2* expression may be regulated by epigenetic factors. However, in is also interesting that methylated DNA was found to have NDR regions upstream of the TSS, which has not previously been reported.

To assess whether *ITGA2* expression could be induced using epigenetic modifiers, the lower expressing cell lines, LNCaP and 22Rv1 were treated with the demethylating agent, AzaC. However, promoter demethylation was not sufficient to induce gene re-expression. Treatment of cells with the HDACi, TSA was also not sufficient to induce gene re-expression. However, co-treatment with both AzaC and TSA induced upregulation of the *ITGA2* gene in the lower expressing cells (LNCaP), suggesting a possible interaction between AzaC and TSA where demethylation and increased histone acetylation in combination leads to changes in chromatin structure and thus, facilitates increased gene expression. Interestingly, upregulation of *ITGA2* gene expression with

both AzaC and TSA was only observed in LNCaP cells but not in 22Rv1 cells, although the level of expression was still not as high as the *ITGA2* expression observed in normal PC3 cells. This could be because while treatment with AzaC in both cells leads to demethylation at the promoter, treatment with TSA had more effect in LNCaP cells compared to 22Rv1 cells (Figure 3.16) as the endogenous histone H3 acetylation levels were much higher in 22Rv1 as compared to LNCaP cells (Figure 3.7c). The idea that the *ITGA2* gene is regulated by DNA methylation was further supported by the collagen panned cells, $LNCaP_{col}2$, where upregulation of *ITGA2* expression correlated with demethylation at regions -325 to -190 as compared to parental LNCaP cells. However, demethylation at the same region was also observed in the first round collagen panned cells, $LNCaP_{col}1$ but expression of *ITGA2* was similar to parental LNCaP, suggesting a delay in re-expression of *ITGA2* after demethylation has occurred. Recently, You et al. (2011) showed that nucleosome occupancy precedes DNA methylation in gene silencing while during gene re-expression, loss of methylation occurs first and is followed by NDR formation. This is consistent with the idea that nucleosomal DNA is the preferred substrate for DNA methylation (Jones PA 2013). Therefore, the delay in re-expression of the *ITGA2* gene despite demethylation at the promoter occurring could be due to the presence of nucleosome occupancy. Although treatment of cells with both AzaC and TSA did not produce as marked changes in *ITGA2* expression as expected, an alternative approach to highlight these effects to a greater extent may be conducting the AzaC and TSA treatment of LNCaP cells during its growth on collagen.

In summary, the data presented here provide evidence that epigenetic factors are involved in the differential expression of ITGA2 observed in the prostate cell lines. Loss of methylation and increased histone H3 acetylation at the *ITGA2* promoter may be associated with increased *ITGA2* expression. NoMe-seq data also suggests that nucleosome occupancy at the TSS results in lower *ITGA2* expression. Treatment with epigenetic modifiers, AzaC and TSA resulted in upregulation of *ITGA2* expression. However these levels were not as high as the *ITGA2* expression observed in PC3 cells. In addition, inducing *ITGA2* expression in LNCaP cells only resulted in slight demethylation at the promoter. Overall, these results support the notion that *ITGA2* is

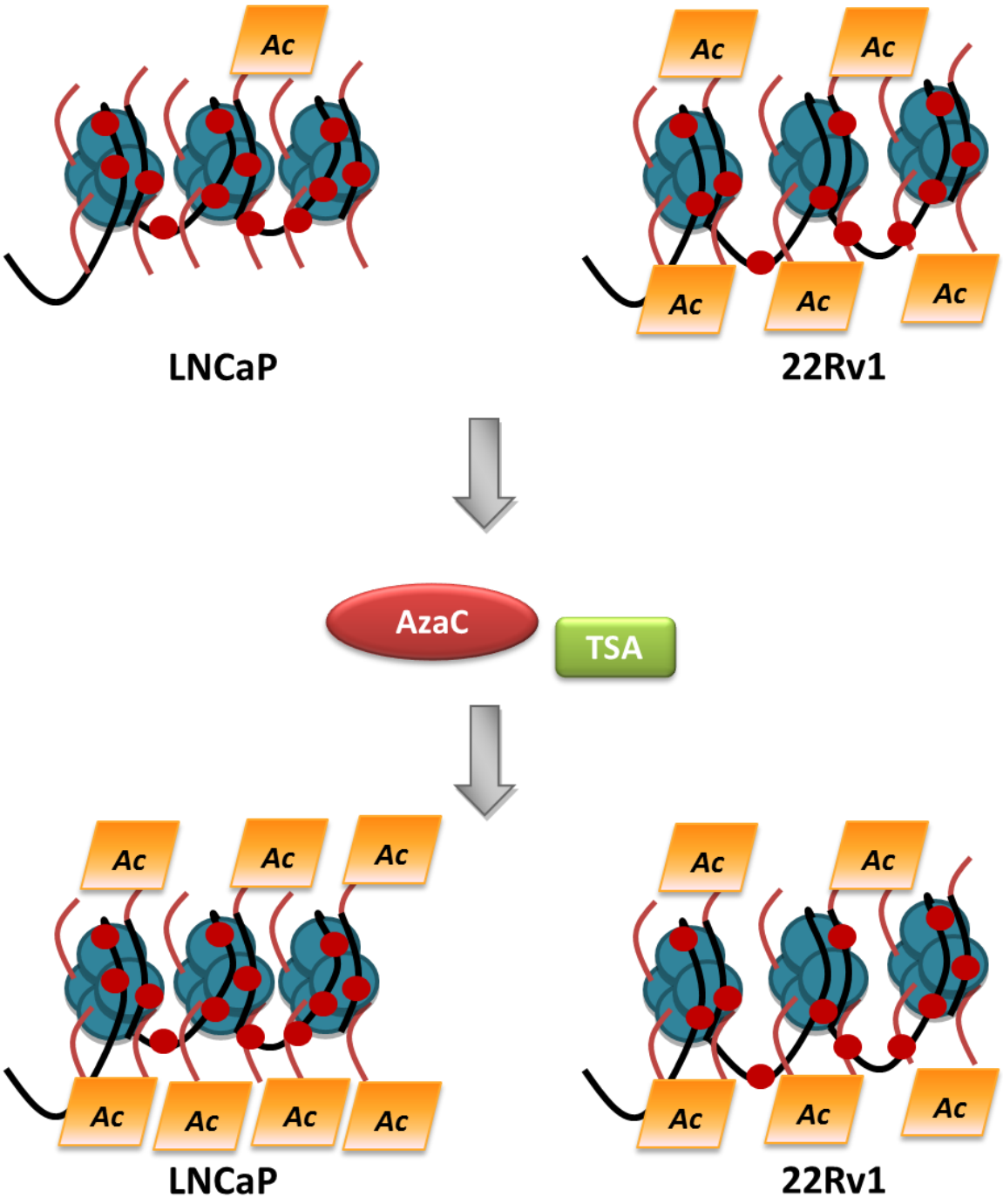


Figure 3.16 Model of activation of *ITGA2* expression in LNCaP
Schematic diagram of the DNA methyalation and histone acetylation marks present at the *ITGA2* promoter in LNCaP and 22Rv1. Treatment with AzaC leads to demethylation on both cells however, TSA treatment only leads to increase histone H3 acetylation in LNCaP, which has low histone H3 acetylation levels.

regulated by epigenetic factors but suggests that other factors are also involved in regulating *ITGA2* expression. This is also supported by the data generated in VCaP cells, in which the *ITGA2* promoter is not methylated but the gene displayed relatively low expression.

Chapter 4

POSSIBLE INVOLVEMENT OF EMT FACTORS IN REGULATING *ITGA2*

4.1 Introduction

4.1.1 EMT in prostate cancer

Tumour progression and metastasis involves the invasion and migration of tumour cells from the primary site into the surrounding tissue. In order to acquire invasive and metastatic abilities, the tumour cells must convert from a polarised epithelial cell to a highly motile mesenchymal phenotype and this process is also known as epithelial-mesenchymal transition, EMT (Thiery 2002). This transition is characterised by changes in gene expression programs and cell surface molecules, with some of these such as E-cadherin and N-cadherin now commonly regarded as markers of EMT. E-cadherin is an adheren junction protein which is widely expressed in epithelial cells and is involved in cell-cell adhesion (Oda et al. 1994). In contrast, N-cadherin is associated with a less stable and more dynamic form of cell-cell adhesion and it is found in mesenchymal cells (Bixby et al. 1990). Several transcription factors have also been characterised as playing supporter roles in EMT. Twist is a basic helix-loop-helix (bHLH) transcription factor that is involved in initiating mesoderm development during gastrulation and plays a role in E-cadherin repression and EMT induction (Kang et al. 2004b; Yang et al. 2004), while Snail is a zinc finger transcription factor involved in EMT and responsible for inducing tumour invasion through the repression of E-cadherin (Batlle et al. 2000; Cano et al. 2000).

EMT has now been found to contribute to metastasis of several tumour types, including breast cancer, colon cancer and prostate cancer (Beach et al. 2007; Burk et al. 2008; Karnoub et al. 2007; Spaderna et al. 2006; Tomita et al. 2000). In prostate cancer, decreased E-cadherin expression due to loss of cell-cell adhesion is a feature of progression of the disease and is associated with poor prognosis (Umbas et al. 1994; Gravdal et al. 2007; Richmond et al. 1997). Loss of E-cadherin expression has been correlated with increased tumour grade, with 46 out of 92 prostate tumour samples

showing reduced or absence of E-cadherin staining when compared to non-malignant prostate samples (Umbas et al. 1992). In contrast N-cadherin, was not expressed in normal prostate tissue but expressed in the poorly differentiated areas of prostate cancer specimens, where E-cadherin was absent (Tomita et al. 2000). Therefore, the loss of E-cadherin and the upregulation of N-cadherin suggest that cadherin switching occurs during tumour progression. The switch to N-cadherin expression was found to occur in higher grade prostate cancer and this correlated with increasing Gleason grade and tumour stage (Bussemakers et al. 2000; Jaggi et al. 2006). These studies suggest that switching of cadherin expression correlates with prostate cancer metastasis. Further study has shown that cells in the centre of a prostate tumour maintain an epithelial phenotype with high E-cadherin expression whereas cells at the invasive tumour front have a mesenchymal phenotype with higher expression of vimentin, Platelet Derived Growth Factor D (*PDGF-D*), Notch homologue 1 (*Notch-1*), *ZEB1* and nuclear factor kappa-light-chain-enhancer of activated B cells (*NF-κB*) (Sethi et al. 2011).

EMT has been shown to be involved in tumour metastasis using the inducible ARCaP prostate cancer cell line model. ARCaP cell subclones were derived by dilution cloning, forming two morphologically distinct subclones, where the $ARCaP_E$ subline have a cobblestone epithelial-like morphology, while the $ARCaP_M$ subline have a spindle-shape mesenchymal-like phenotype (Xu et al. 2006). $ARCaP_E$ cells expressed epithelial markers including E-cadherin, whereas $ARCaP_M$ cells expressed mesenchymal markers such as N-cadherin and vimentin and showed higher migration rate. Interestingly, the $ARCaP_E$ cells can be induced to undergo EMT to form $ARCaP_M$ cells by treating the cells with TGFβ1 plus EGF, IGF-1, β-2 microglobulin or subjecting them to a bone microenvironment by orthotopic injection into SCID mice. $ARCaP_M$ cells showed upregulation of mesenchymal markers including N-cadherin and vimentin and also formed bone metastases (Zhau et al. 2008). These studies suggest that EMT can be induced by soluble factors by activating a specific cell signalling pathway and the transition of $ARCaP_E$ to $ARCaP_M$ can be correlated with prostate cancer progression. In addition, in the bone microenvironment, growth factors that are released during bone

turnover may play a critical role in the initiation of EMT and prostate cancer growth, survival and bone colonisation.

4.1.2 ITGA2 and EMT

During EMT, tumour cells switch from cell-cell to cell-matrix adhesion thus mediating cell migration. As integrins are involved in cell-matrix adhesion, changes in integrins may occur during EMT. To date, studies on the involvement of integrins in EMT during cancer progression have been limited, particularly in prostate cancer. Integrin αvβ6 (Bates et al. 2005), αvβ3 (Haraguchi et al. 2008) and α5 (Nam et al. 2012) were upregulated during EMT while integrin β4 was downregulated in metastatic prostate tumour samples as compared to primary tumours (Drake et al. 2010).

Collagen type I, which is a ligand of integrin α2β1, was found to induce the disruption of E-cadherin adhesion complexes in pancreatic cancer (Koenig et al. 2006). The study suggested that binding of collagen type I to α2β1 activates FAK phosphorylation which enhances tyrosine phosphorylation of β-catenin and causes the disassembly of the E-cadherin complex. In addition, Shintani et al. (2008) showed that activation of integrin α2β1 by collagen type I together with activation of the discoidin domain receptor 1 (*DDR1*) induces N-cadherin expression (Shintani et al. 2008). Furthermore, high E-cadherin was observed in suspended PC3 cells and the expression decreased as cells attached to a fibronectin substrate, whereas N-cadherin expression was 4-fold lower in suspension cells compared with attached cells (Alexander et al. 2006). Blocking of integrin β1 by the AIIB2 antibody resulted in no increase of N-cadherin expression in PC3 cells, suggesting that integrin β1-mediated cell adhesion to fibronectin is involved in regulating N-cadherin expression in prostate cancer. The study also investigated the regulation of N-cadherin by Twist1. Knockdown of Twist1 expression in PC3 cells resulted in decreased N-cadherin expression and inhibition of cell migration. Interestingly, blocking of integrin β1 correlated with inhibition of nuclear accumulation of Twist1 following cell attachment. Therefore, these data suggest that the integrin β1-

mediated adhesion is regulated through Twist1 accumulation and activation of N-cadherin.

Since the previous results support that increased *ITGA2* expression in the prostate cell lines correlates with increased cell migration capacity and it is well described that EMT is associated with tumour progression and metastasis, it was hypothesised that *ITGA2* expression may change during EMT. In addition to cadherins, transcription factors such as Snail, Slug and members of ZEB and bHLH families are involved in EMT. A microarray study was conducted to determine the transcriptional consequences of exogenous expression of Snail and Slug at a global level in the MCF-7 breast cancer cell line (Dhasarathy et al. 2011). Interestingly, while downregulation of E-cadherin expression was observed, *ITGA2* gene expression was also downregulated by Snail. In addition, when the prostate cancer cell line ARCaP was stably transfected with Snail, decreased E-cadherin and also decreased *ITGA5*, *ITGA2* and *ITGB1* expression was observed (Neal et al. 2011). Therefore, these studies suggest that integrin expression and particularly *ITGA2* may change during EMT.

Since EMT and *ITGA2* are involved in tumour progression and metastasis, the aim of this study is to determine whether modulation of *ITGA2* expression in the prostate cancer cell lines affects EMT marker expression and thus, determine the potential role of *ITGA2* in EMT.

4.2 Results

4.2.1 Increased ITGA2 expression correlates with a mesenchymal-like phenotype

The prostate cancer cell lines were previously shown to have different migration capacity (Chapter 3). Since EMT is associated with tumour progression and metastasis, expression analysis was undertaken to characterise the EMT phenotype of the various cell lines. In order to determine the epithelial or mesenchymal phenotype of the different prostate cancer cell lines, a panel of EMT markers was analysed by real-time PCR. E-cadherin which represents an epithelial marker was selected along with the mesenchymal marker N-cadherin and the transcription factors, Twist and Snail. Expression of these EMT

markers was determined in the prostate cancer cell lines LNCaP, 22Rv1 and PC3, which differ in their *ITGA2* expression and migration ability (Figure 4.1). PWR-1e was excluded as this cell line has different growth characteristic and it requires the addition of EGF in the growth media. Since EGF is a known EMT inducer (Ackland et al. 2003; Docherty et al. 2006; Schlessinger et al. 2004), this may affect EMT marker expression. RNA was isolated from the cell lines, converted to cDNA and mRNA levels of EMT markers was analysed by real-time PCR. The LNCaP cells displayed a statistically significantly higher expression of the epithelial marker E-cadherin as compared to the 22Rv1 and PC3 cells, with PC3 cells expressing very low levels of E-cadherin. In contrast, LNCaP cells displayed very low expression of Twist and undetectable N-cadherin. Conversely, PC3 cells displayed a statistically significant higher expression of the mesenchymal markers, Twist and N-cadherin. The 22Rv1 displayed higher levels of Twist but low levels of N-cadherin. Therefore, decreased E-cadherin expression observed in these cell lines was consistent with increased Twist and N-cadherin expression. Unexpectedly, although it is documented that Snail represses E-cadherin (Batlle et al. 2000; Cano et al. 2000), Snail expression was higher in LNCaP cells as compared to 22Rv1 and PC3 cells, and thus showed a positive correlation with E-cadherin, rather than the expected inverse correlation.

Therefore, LNCaP cells displayed a more epithelial-like expression pattern (with high E-cadherin and low N-cadherin expression), while PC3 cells displayed a more mesenchymal-like phenotype (with low E-cadherin and high N-cadherin) and 22Rv1 cells displayed an expression pattern intermediate between the two. When comparing these phenotypes to the *ITGA2* expression documented earlier (Figure 3.2), higher *ITGA2* expression in PC3 is correlated with a mesenchymal-like phenotype whereas lower expression of *ITGA2* is correlated with an epithelial-like phenotype.

4.2.2 Downregulation of ITGA2 leads to suppression of migration but does not modulate EMT

Previously, differential expression of *ITGA2* was shown to correlate with differential cell migration capacity, with higher *ITGA2* expression correlating with higher migratory

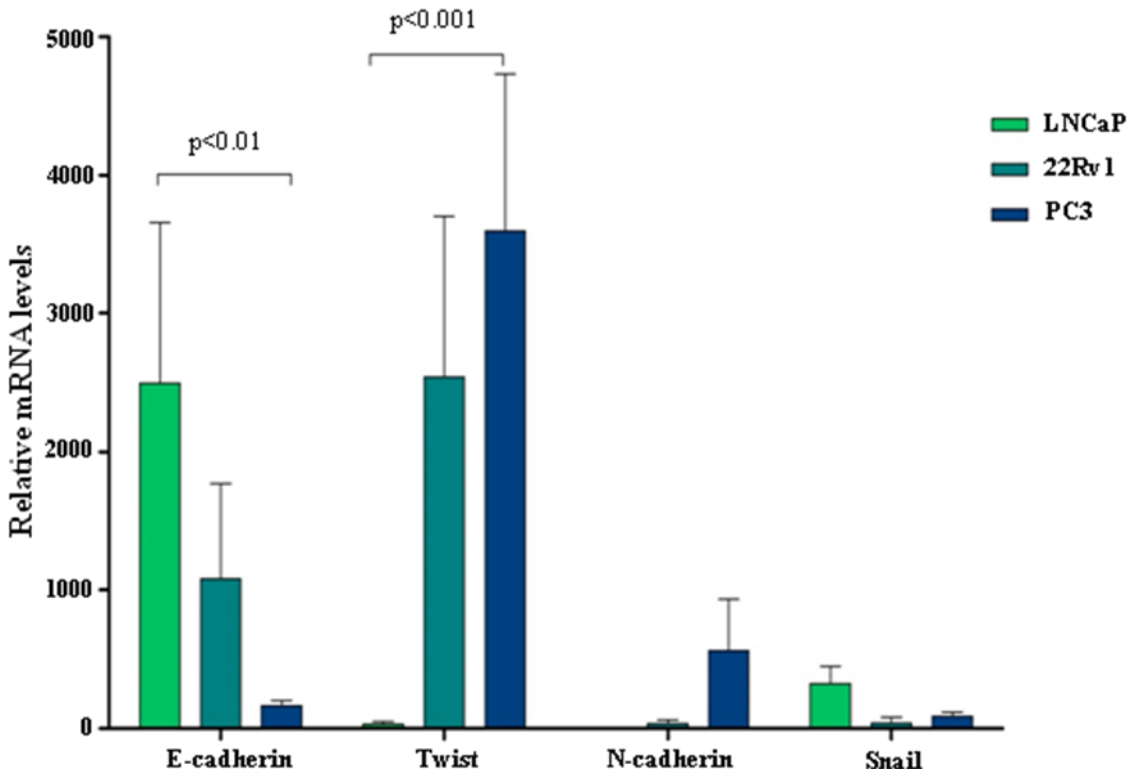


Figure 4.1 PC3 cells display a mesenchymal-like phenotype while LNCaP cells display an epithelial-like phenotype

Total RNA isolated from LNCaP, 22Rv1 and PC3 cells was reverse transcribed and amplified by real-time PCR using primers designed to amplify the EMT markers E-cadherin, Twist, N-cadherin and Snail as indicated. EMT marker mRNA levels are shown relative to *GAPDH*. Values expressed as mean ± SEM (n=3). Statistical significance was determined using one-way ANOVA with Neuman-Keuls multiple comparison test.

capacity (Chapter 3). Therefore, to determine whether changes in *ITGA2* expression may influence the epithelial or mesenchymal phenotype, *ITGA2* expression was modulated in PC3 and 22Rv1 cells. In order to modulate *ITGA2* expression, *ITGA2* siRNA was used to knockdown *ITGA2* expression in these cells. As transfection efficiency varies across cell lines, a fluorescence transfection indicator, siGLO was used. siGLO is a red fluorescently labelled siRNA which localises to the nucleus and thus, enables visual assessment of uptake into cells. This was used as an alternative to flow cytometry given that the cell lines are adherent. Both PC3 and 22Rv1 cells were transfected with 25 nM siGLO. After 24 hours, images of the cells were captured (Figure 4.2). Analysis of the fluorescence images, suggests almost 100% transfection efficiency in the PC3 cells whereas 22Rv1 cells displayed approximately 70% transfection efficiency.

To knockdown *ITGA2* expression, the cells were transfected with 40 ρmol of either a negative control siRNA containing a scrambled sequence of non-targeting siRNA or a siRNA which is specifically designed to knockdown *ITGA2* expression. To determine the amount of *ITGA2* gene knockdown in the transfected cells, *ITGA2* mRNA levels were measured in both PC3 and 22Rv1 cells at 24 hours and 48 hours post-transfection. This was compared to the *ITGA2* mRNA levels in the cells transfected with the non-targeting control siRNA. PC3 cells showed approximately 57% *ITGA2* knockdown at 24 hours and 60% at 48 hours after transfection, which was statistically significantly different to the control (Figure 4.3a). *ITGA2* knockdown was also observed in 22Rv1 cells, although only 27% knockdown was observed at 24 hours and 23% at 48 hours, which was not statistically significantly different to the control, and may reflect the lower transfection efficiencies of these cells (Figure 4.3b). Interestingly, *ITGA2* knockdown cells appeared to display a different cell morphology as compared to the cells transfected with the control siRNA (Figure 4.4). The *ITGA2* knockdown PC3 cells (Figure 4.4b) displayed rounded cell morphology while the control transfected PC3 cells (Figure 4.4a) displayed a more elongated cell morphology.

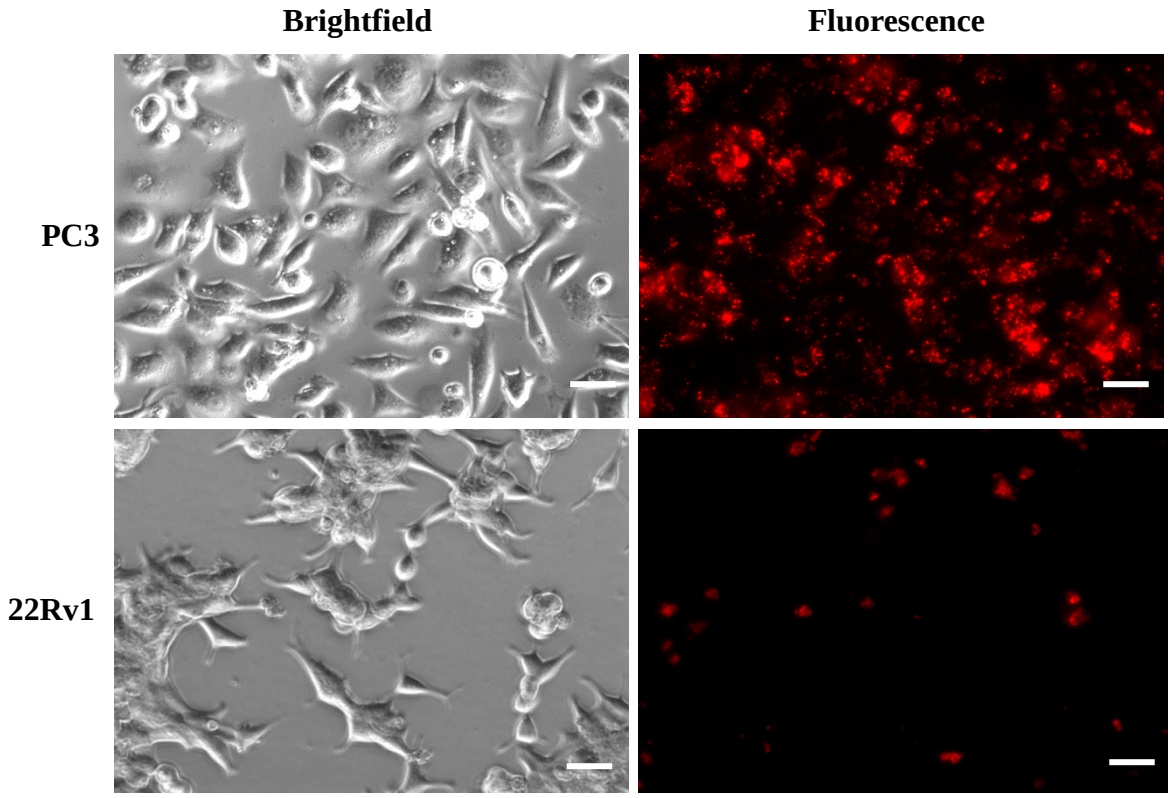


Figure 4.2 siGLO transfection in PC3 and 22Rv1 cells
Cells were transfected with 25 nM siGLO for 24 hours. Representative images were taken at 20X magnification under brighfield (left) and florescence (right) to show transfection efficiency after 24 hours using siGLO. Scale bar, 50 µM.

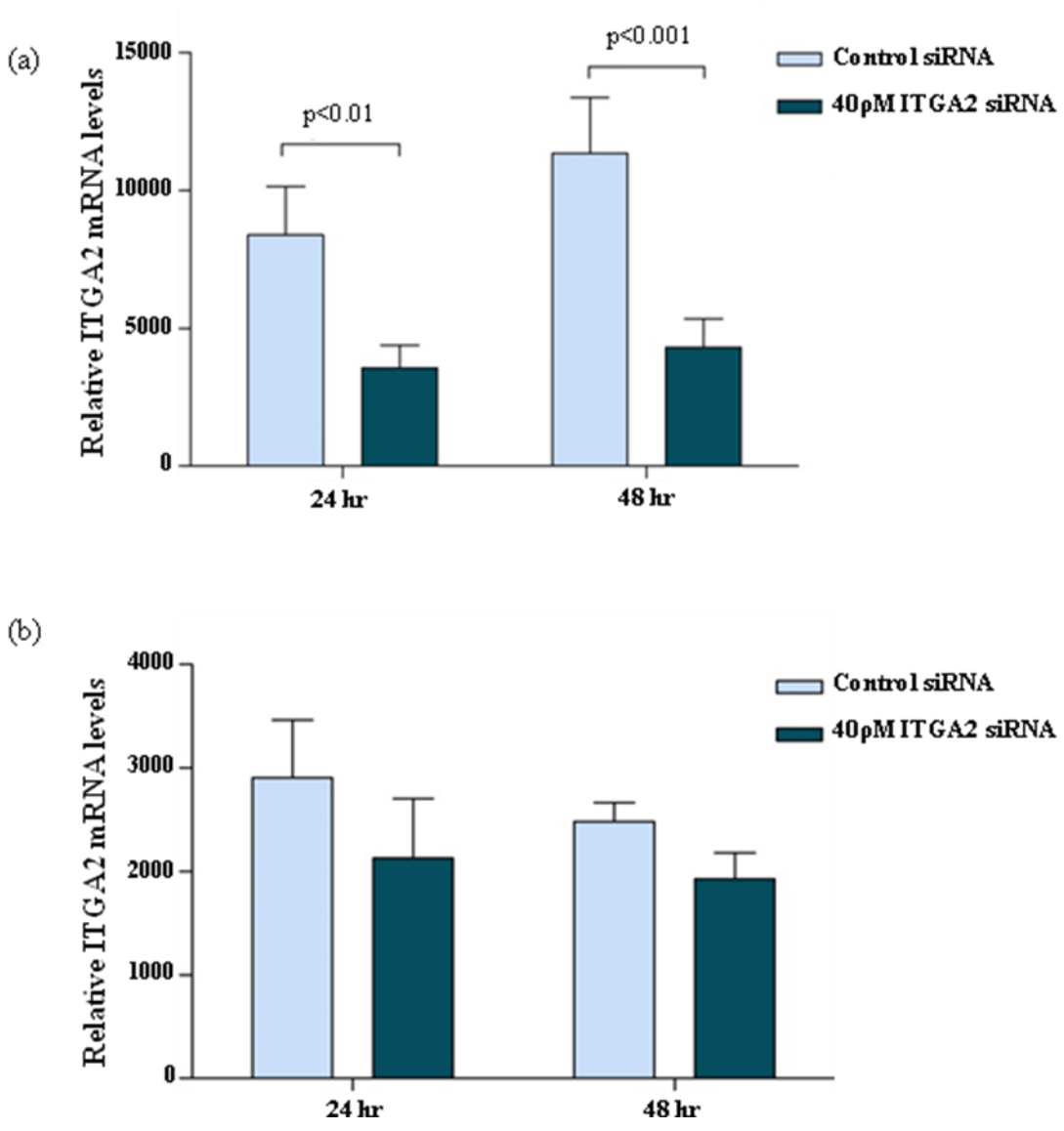
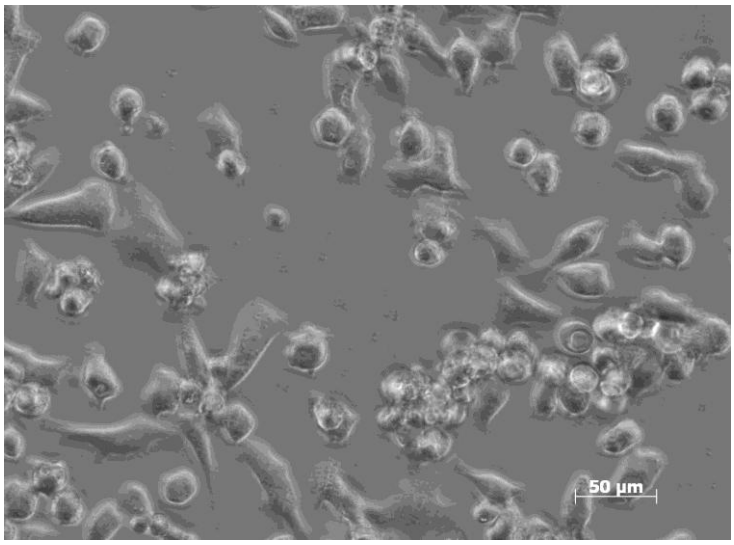


Figure 4.3 Knockdown of *ITGA2* gene expression in prostate cancer cell lines
Total RNA isolated from cells transfected with control siRNA or *ITGA2* siRNA was reverse transcribed and amplified by real-time PCR using primers designed to amplify *ITGA2*. (a) *ITGA2* mRNA levels relative to *GAPDH* in PC3 cells at 24 hour and 48 hours after transfection and (b) *ITGA2* mRNA levels are shown relative to *GAPDH* in 22Rv1 cells at 24 hours and 48 hours after transfection. Values expressed as mean ± SEM (n=3). Statistical significance was determined relative to the indicated control using student's *t* test.

(a)



(b)

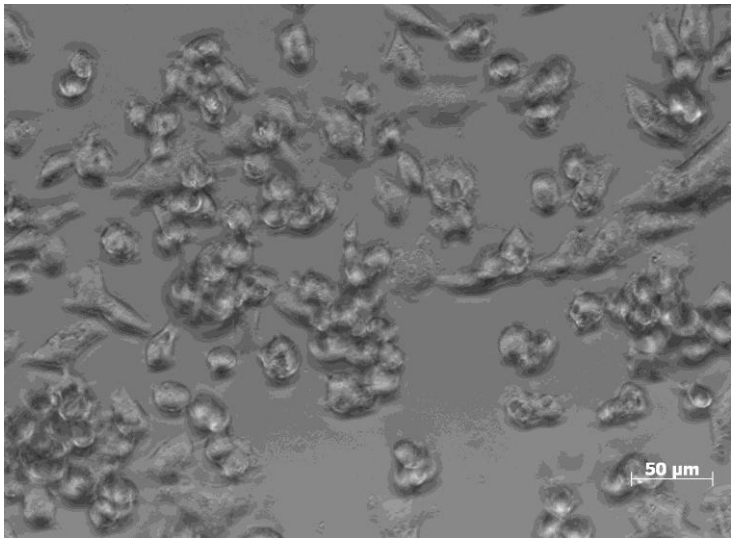


Figure 4.4 *ITGA2* knockdown in PC3 changes cell morphology
PC3 cells were transfected with (a) control siRNA and (b) *ITGA2* siRNA and representative images were taken 24 hours after transfection at 20X magnification. Scale bar, 50 µM.

To determine whether altering *ITGA2* expression affects cell migration, the effect of *ITGA2* knockdown on cell migration was determined. PC3 cells transfected with either the control siRNA or the *ITGA2* siRNA were grown overnight on poly-L-Lysine coated coverslips. After 24 hours, a scratch was created in the middle of the coverslip using a P20 pipette tip and the cells were fixed at the time of scratching (0 hour) as well as 4 hours and 6 hours post-scratching (Figure 4.5). The cells were stained with nuclear yellow and the migration of cells into the scratch site assessed as a measure of migration capacity of the cells. While PC3 cells transfected with the control siRNA showed migration into the scratch site at 6 hours, PC3 cells transfected with *ITGA2* siRNA appeared to show fewer cells migrating into the scratch site. This suggests that reduced *ITGA2* expression impaired PC3 cell migration.

Since knockdown of *ITGA2* expression is consistent with a decrease in cell migration, it was asked whether knockdown of *ITGA2* also results in a change in EMT phenotype. Therefore, EMT marker expression was examined in cells transfected with *ITGA2* siRNA compared to cells transfected with the control siRNA. RNA isolated from PC3 and 22Rv1 cells transfected with control or *ITGA2* siRNA was analysed by real-time PCR, which determined that there was no significant change in the EMT markers E-cadherin, N-cadherin, Snail or Twist in either PC3 (Figure 4.6a) or 22Rv1 cells (Figure 4.6b) transfected with *ITGA2* siRNA. Taken together, these results indicate that while reduced *ITGA2* expression impairs cell migration, it does not affect the EMT phenotype of these cells.

4.2.3 LNCaP$_{col}$ cells with increased ITGA2 expression display higher Snail and lower Twist expression

To further validate whether modulation of *ITGA2* expression affects EMT, EMT marker expression was determined in LNCaP$_{col}$ cells. Previously (Section 3.2.6), the derivation of LNCaP$_{col}$ cells, a collagen binding variant of LNCaP cells showed a significant upregulation of *ITGA2* expression in cells following the second panning (LNCaP$_{col}$2), as compared to the first panning (LNCaP$_{col}$1) and the parental LNCaP cells. This was

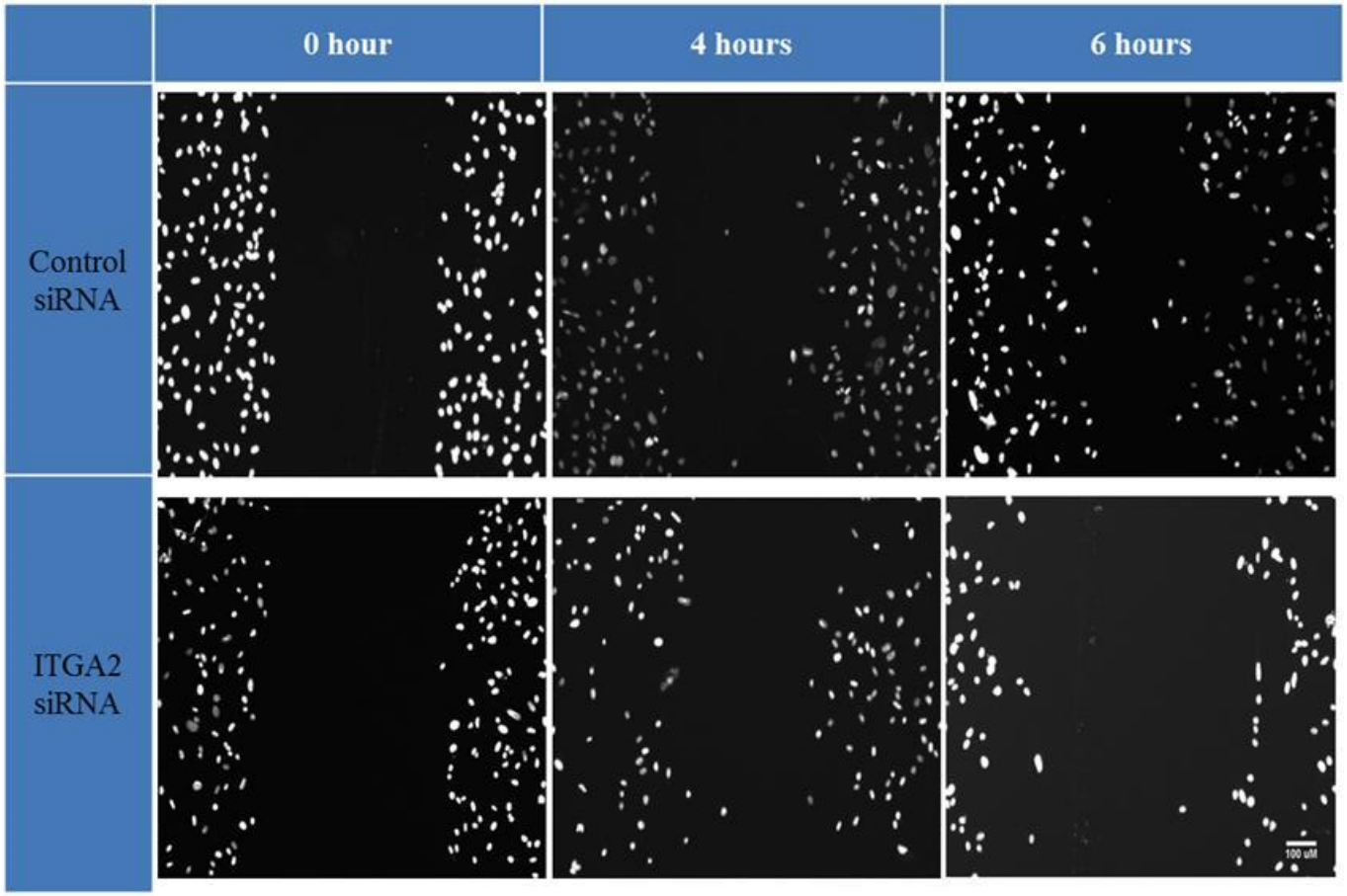


Figure 4.5 Reduced cell migration in *ITGA2* knockdown cells
Representative images of confluent PC3 cells transfected with control siRNA or *ITGA2* siRNA, scratched with a P20 pipette tip and fixed at 0 hour, 4 hours and 6 hours as indicated. Cell nuclei were stained with 1:100 dilution of nuclear yellow. Scale bar, 100 µM.

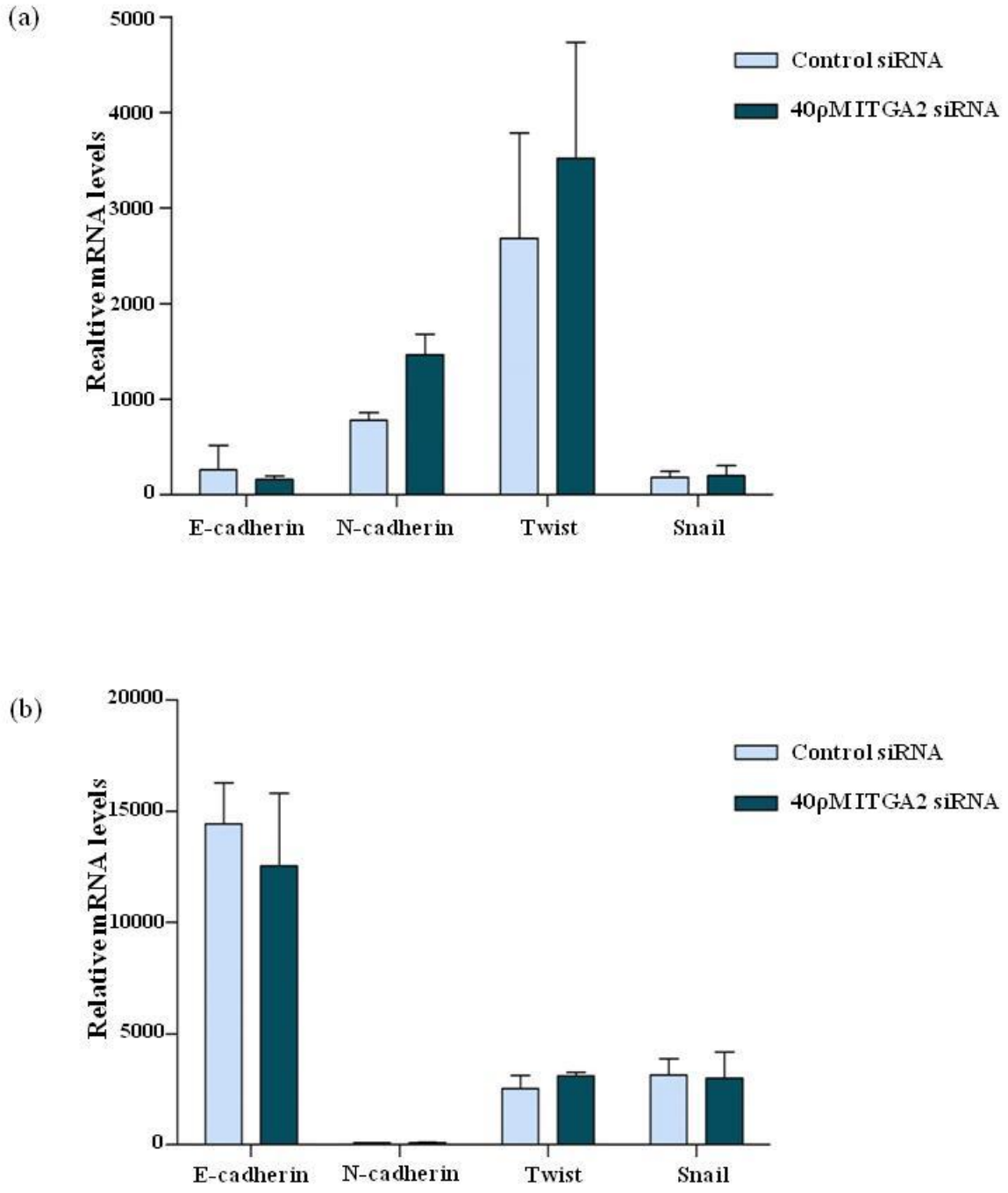


Figure 4.6 *ITGA2* knockdown does not affect EMT marker expression
Total RNA isolated from cells transfected with control siRNA or *ITGA2* siRNA was reverse transcribed and amplified by real-time PCR using primers designed to amplify EMT markers. (a) EMT marker mRNA levels are shown relative to *GAPDH* in PC3 cells at 24 hours after transfection; (b) EMT marker mRNA levels are shown relative to *GAPDH* in 22Rv1 cells at 24 hours after transfection. Values expressed as mean ± SEM (n=3).

consistent with a previous report (Hall et al. 2006) where these $LNCaP_{col}$ cells were shown to display increased collagen I binding associated with increased α2β1 expression. These cells were shown to be capable of growth within the bone. Therefore, since these cells showed acquisition of a bone metastatic phenotype (Hall et al. 2006) and this correlated with increased *ITGA2* expression, the expression of EMT markers was examined by real-time PCR in the $LNCaP_{col}$ cells. A statistically significant increase in E-cadherin expression in $LNCaP_{col}2$ as compared to $LNCaP_{col}1$ and the parental LNCaP cells was observed (Figure 4.7a). Twist also showed a statistically significant increased expression in $LNCaP_{col}2$ as compared to $LNCaP_{col}1$ and LNCaP cells (Figure 4.7b). Expression of Snail was not statistically significantly different although it showed a trend of increased expression in $LNCaP_{col}1$ but not in $LNCaP_{col}2$ cells (Figure 4.7c). Interestingly, Snail and Twist which are transcription factors involved in downregulating E-cadherin did not show any correlation with E-cadherin expression. However, increased *ITGA2* expression in the $LNCaP_{col}2$ cells correlated with higher Twist and lower Snail expression. Although knockdown of *ITGA2* expression did not directly affect EMT marker expression, the collagen binding variant $LNCaP_{col}$ cells showed increase in *ITGA2* expression which correlates with changes in expression of EMT markers.

4.3 Discussion

Both EMT and changes in *ITGA2* expression have been well described in tumour progression and metastasis (Alexander et al. 2006; Dhasarathy et al. 2011; Koenig et al. 2006; Neal et al. 2011; Shintani et al. 2008). However, it is unclear whether *ITGA2* is modulated by EMT. Several studies have shown that tumour cells switch from cadherin-mediated to integrin-mediated adhesion during EMT, with downregulation of E-cadherin and upregulation of *ITGA2* (Chen et al. 2011; Zhang et al. 2006a). However, contrary to this in another study, overexpression of the mesenchymal transcription factor Snail, resulted in downregulation of E-cadherin and downregulation of *ITGA2* was also observed (Dhasarathy et al. 2011; Neal et al. 2011).

In this study, LNCaP, 22Rv1 and PC3 prostate cancer cell lines were used, which have different tumorigenicity and metastatic potential with the metastatic potential of these

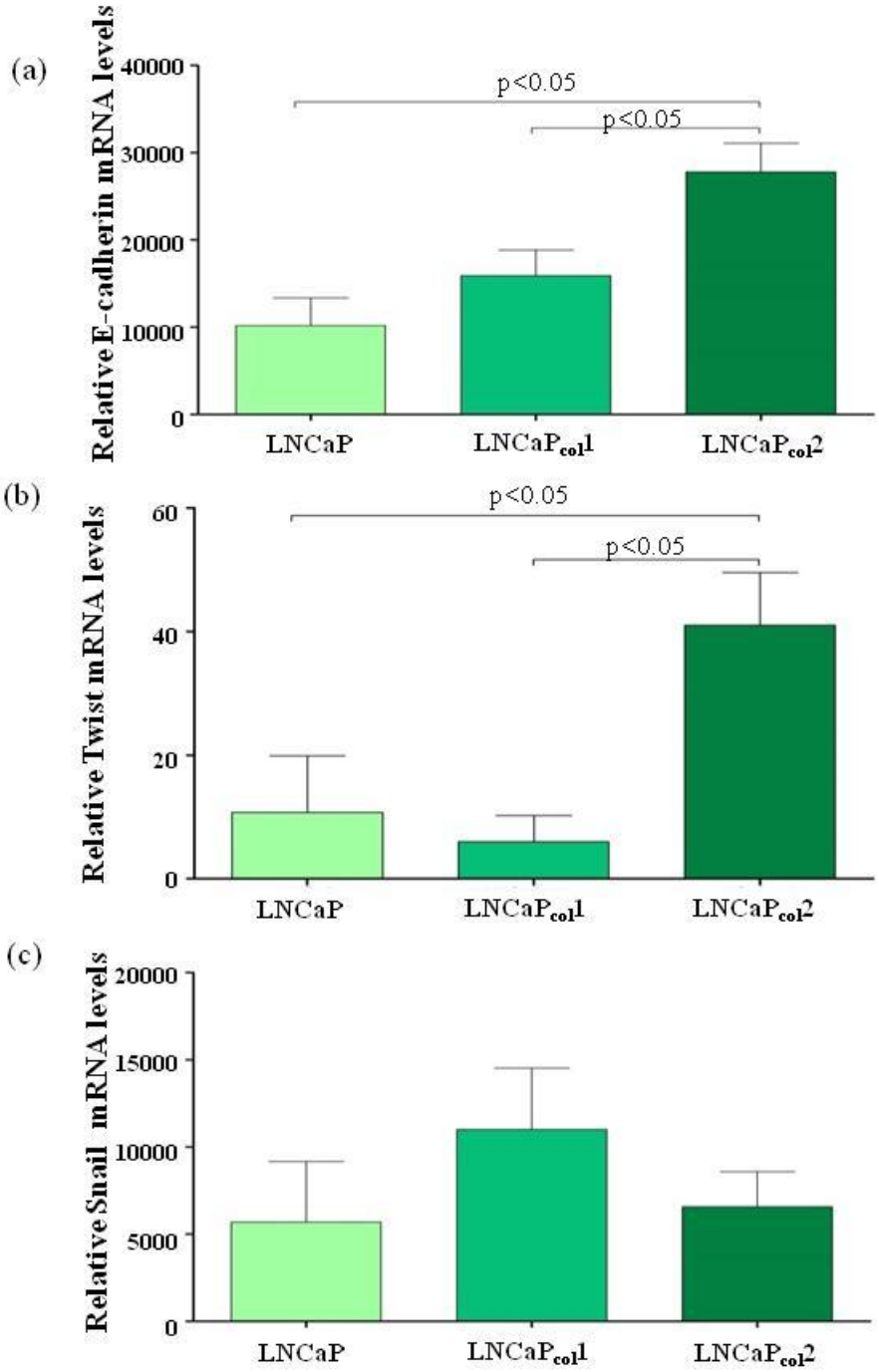


Figure 4.7 Increased *ITGA2* expression in LNCaP$_{col}$ cells correlated with changes in EMT markers expression

Total RNA isolated from the parental LNCaP, LNCaP$_{col}$1 and LNCaP$_{col}$2 cells was reverse transcribed and amplified by real-time PCR using primers designed to amplify EMT markers. (a) E-cadherin mRNA levels are shown relative to *GAPDH*; (b) Twist mRNA levels are shown relative to *GAPDH*; and (c) Snail mRNA levels are shown relative to *GAPDH*. Values expressed as mean ± SEM (n=3). Statistical significance was determined using one-way ANOVA with Neuman-Keuls multiple comparison test.

cells correlating with *ITGA2* expression (Chapter 3). The data presented here suggest that the less tumorigenic and less metastatic cell line, LNCaP which displayed low *ITGA2* expression has an epithelial-like phenotype with high expression of E-cadherin and low or no expression of the mesenchymal markers, Twist and N-cadherin. On the other hand, the highly metastatic and tumorigenic cell line, PC3 which displayed high expression of *ITGA2* has a mesenchymal-like phenotype with low E-cadherin and high Twist and N-cadherin expression. The epithelial-like and mesenchymal-like phenotypes observed in these cells correlated with cell morphology. LNCaP and 22Rv1cells displayed a more flattened shape with pseudopodium-like extensions, consistent with an epithelial-like phenotype, while PC3 cells display an elongated spindle shape cell morphology, consistent with a mesenchymal-like phenotype. However, expression of the Snail transcription factor which has been well described as a repressor of E-cadherin expression (Batlle et al. 2000; Cano et al. 2000), did not correlate with E-cadherin expression levels displayed in these cells. Nevertheless, it has also been shown that in instances where there was no difference in protein levels, colocalisation of E-cadherin from the nucleus to cytosol was observed in Snail transfected LNCaP cells as compared to the control transfected LNCaP cells (McKeithen et al. 2010). Therefore, E-cadherin localisation rather than expression levels may be a better indicator of EMT. In this study, although Snail expression levels did not correlate with E-cadherin expression, the Snail expression pattern displayed by these cells showed an inverse correlation with *ITGA2* expression. This expression pattern is consistent with the findings of Neal et al. (2011), where overexpression of Snail in ARCaP cells resulted in decreased cell adhesion and increased cell migration on collagen I with downregulation of *ITGA2* and *ITGB1* expression.

Interestingly, knockdown of *ITGA2* expression using siRNA further confirms the involvement of *ITGA2* in cell migration. *ITGA2* knockdown in PC3 cells resulted in reduced cell migration capacity consistent with morphological changes from elongated to rounded cells, suggesting the cells are less adhesive. However, while changes in *ITGA2* expression were consistent with changes in cell migration, knockdown of *ITGA2* did not

change epithelial and mesenchymal marker expression which suggests that they are not a downstream pathway of *ITGA2* signalling (Figure 4.8). This is consistent with the idea that tumour progression involves switching from cadherin-mediated to integrin-mediated adhesion (Chen et al. 2011; Zheng et al. 2000) and thus, *ITGA2* expression may change as a result of EMT, rather than being a driver of EMT.

While knockdown of *ITGA2* did not affect the EMT phenotype, the collagen binding variant $LNCaP_{col}$ cells displayed changes in EMT marker expression, which correlated with upregulation of *ITGA2* expression (Chapter 3). $LNCaP_{col}$ cells have previously been found to display increased α2β1 surface expression, with increased collagen mediated migration and capability to grow within the bone (Hall et al. 2006). Therefore, changes in *ITGA2* expression in these cells with bone tumour formation capability are possibly due to changes in EMT marker expression.

Taken together, these results emphasise that EMT is a dynamic and reversible response to environmental conditions. As such, modification of expression of an individual marker or small set of markers, especially ones that change as a result of EMT rather than being an initiator of the EMT process, may not be expected to promote induction of EMT if disrupted. However, it is possible that changes in EMT affect *ITGA2* expression which leads to cell migration and the data presented here suggest that Snail and/ or Twist may regulate *ITGA2* expression. Alternatively, both EMT and *ITGA2* may independently affect cells migration, and this warrants further study.

Chapter 5

REGULATION OF THE *ITGA2* GENE EXPRESSION BY TRANSCRIPTION FACTORS IN PROSTATE CANCER

5.1 Introduction

5.1.1 ITGA2 expression and malignancy

α2β1 integrin is expressed on epithelial cells, endothelial cells and fibroblasts in numerous tissues and its altered expression has been associated with many cancers, including prostate cancer (Bonkhoff et al. 1993; Kirchhofer et al. 1990; Pontes-Junior et al. 2009; Santoro et al. 1988; Takada et al. 1988; Van Slambrouck et al. 2009). The role of *ITGA2* expression in tumour malignancy is controversial with both upregulation and downregulation of the α2β1 integrin having been correlated with a poor outcome and the progression of tumours to acquire an invasive, metastatic and aggressive tumour phenotype (Zutter et al. 1995; Klein et al. 1991; Koretz et al. 1991; Ramirez et al. 2011). In breast cancer, decreased expression of α2β1 integrin is associated with poorly differentiated adenocarcinoma and re-expression of the α2β1 integrin resulted in a reversed phenotype from an invasive fibroblastoid to a less motile epitheloid cell (Zutter et al. 1995). Consistent with this, loss of α2β1 integrin expression is associated with an invasive tumour type in colon cancer (Koretz et al. 1991). In contrast, increased expression of α2β1 integrin is associated with a more malignant phenotype in anaplastic thyroid carcinoma (Dahlman et al. 1998), melanoma (Klein et al. 1991) and prostate cancer (Hall et al. 2006; Ramirez et al. 2011; Van Slambrouck et al. 2009). It has been shown that increased α2β1 integrin expression is due to an increase in steady-state levels of *ITGA2* mRNA as a result of transcriptional activation of the *ITGA2* gene (Zutter et al. 1990). Discovering the contributions of changing levels of expression on tumour development necessarily requires an understanding of how the gene is regulated.

5.1.2 Characterisation of the ITGA2 promoter

Interestingly, the promoter and enhancer regions of the *ITGA2* gene were found to be involved in cell type-specific and differentiation-induced *ITGA2* gene expression. Elements within the first 961 bp upstream of the TSS were found to be important in driving *ITGA2* expression in cells of epithelial origin (Zutter et al. 1995). Deletion mutants then displayed an enhancer region between -776 and -92 bp and a repressor region between -961 and -776 bp in epithelial cells, while in megakaryocytic cells, the region from -961 to -92 bp displayed the presence of repressor elements (Figure 5.1). The core promoter for both cell types appears to be from -92 to -30 bp and thus it was suggested that this core promoter is not cell type specific (Zutter et al. 1995). Therefore, these results suggest a combination of positive and negative regulatory elements present at the *ITGA2* promoter regulating its expression in a cell-type and differentiation specific manner.

Characterisation of the promoter in the haematopoietic cell line K562 also identified four Sp1 transcription factor binding sites within the core promoter with two of these sites overlapping a potential AP-2 site and an additional two Sp1 sites further upstream (Zutter et al. 1995). Follow on studies by the same group then found that the two tandem Sp1 binding sites (-61 to -53 and -51 to -43), but not the AP-2 site, are responsible for the core promoter activity in the haematopoietic cell line (Zutter et al. 1997). Although Sp1 is generally ubiquitously expressed, Sp1 is involved in transcription of many cell type specific genes (Block et al. 1996; Faber et al. 1993). Sp1 is also an activator of promoters that lack a TATA box (Emami et al. 1997). Since the *ITGA2* promoter also lacks a TATA and CAAT boxes, Sp1 may play an important role in activation of the *ITGA2* promoter. Later studies also confirmed that Sp1 binds to the -107 to -99 region and mutation of these Sp1 sites abolishes basal activity of the promoter (Jacquelin et al. 2001). Interestingly, the Sp1 binding sites also contain CpG sites and evidence suggests that methylation of CpG islands that surround the Sp1 binding site can inhibit Sp1 activity and therefore affect gene expression (Zhu et al. 2003). However, whether Sp1 activity is affected by methylation of the CpG island at the *ITGA2* promoter remains unstudied.

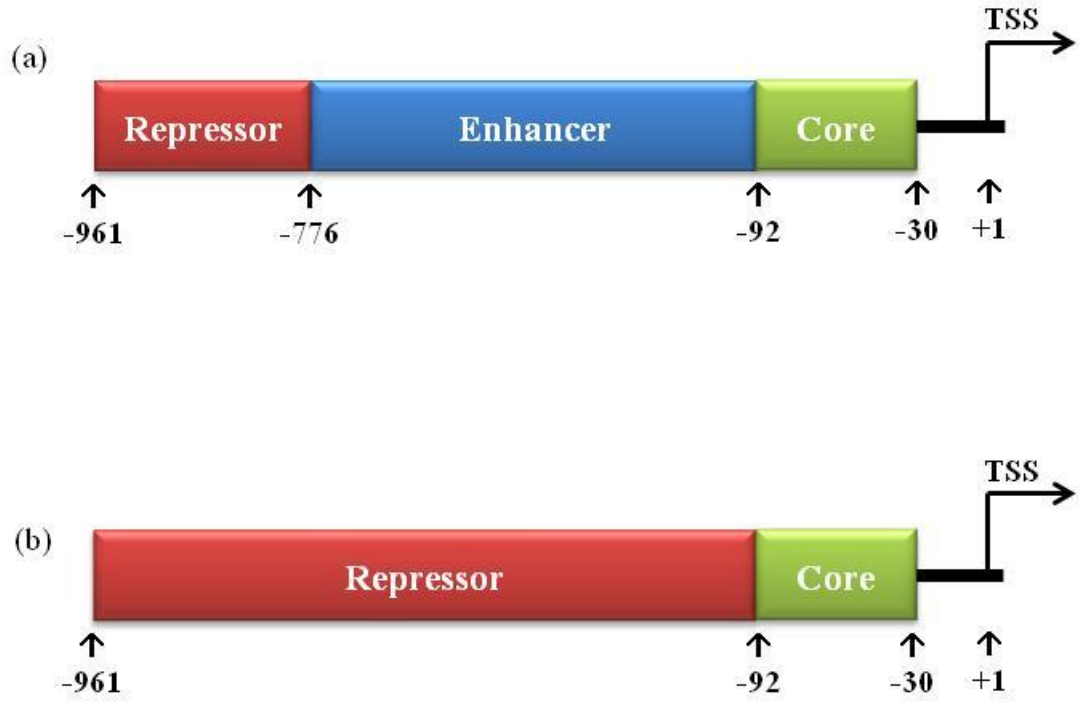


Figure 5.1 Schematic representation of *ITGA2* regulatory elements
Regulatory elements identified upstream of the TSS of the *ITGA2* gene in (a) the epithelial cell line, T47-D and (b) the megakaryocytic cell line, K562. The core promoter (-92 to -30 bp) is not cell-type specific. Enhancer function within -761 to -92 bp is specific to cells of epithelial origin while repressor activity is present from -961 to -92 bp in megakaryocytic cells. Figure constructed based on the findings of Zutter et al. (1995).

A number of other factors have been documented as regulating *ITGA2* activity include collagen, a number of growth factors; platelet-derived growth factor, PDGF (Åhlén et al. 1994), EGF (Fujii et al. 1995), TGF-β (Riikonen et al. 1995), the NF-κB transcription factor (Xu et al. 1998b) and the oncogenes Erb-B2 and V-ras (Ye et al. 1996). Furthermore two estrogen half sites have been identified in the enhancer region, suggesting a possible role for steroid hormones in the regulation of *ITGA2* expression (Zutter et al. 1993).

Overall, the regulation of *ITGA2* expression plays a key role in the processes of cell differentiation, and altered adhesion, invasion and migration of tumour cells. Characterisation of the *ITGA2* promoter has shown that the regulatory elements present at the promoter confer cell type specific expression. Although the regulatory regions of the *ITGA2* promoter have been characterised in epithelial cells, the transcription factors that bind to these regions of the promoter in epithelial cells have not been investigated. Results presented in Chapter 3 suggest that epigenetic factors are involved in regulation of the *ITGA2* gene in prostate cancer. However, only small effects were observed when cells were treated with epigenetic modulators, suggesting the involvement of other factors also.

It is well documented that transcription factors and epigenetic factors cooperate to regulate gene expression (Jaenisch et al. 2003; Reik 2007). Therefore, the aim of this chapter was to identify the transcription factors predicted to be important in regulating *ITGA2* expression in prostate cancer cell lines.

5.2Results

5.2.1 Putative transcription factor binding sites at the ITGA2 promoter

The MatInspector bioinformatics tool that uses the TRANSFAC database of transcription factors was utilised for screening of potential transcription factor binding sites within the *ITGA2* promoter (http://www.genomatix.de/online_help/help_matinspector/matinspector

_help.html). This tool places emphasis on sequences with experimentally verified binding capacity that have been shown to be useful in estimating functional putative binding sites that can be used as a basis for designing appropriate experiments (Quandt et al. 1995). The region from -1000 bp to the TSS of the *ITGA2* promoter was screened for potential transcription factor binding sites, and the list of putative transcription factor binding sites was further narrowed based on prostate related and EMT related transcription factors. Therefore, Sp1, an androgen response element (ARE) half-site and enhancer box (E-box) transcription factor binding sites were selected for further analysis, with the predicted positions and recognition sequences of these transcription factors outlined in Figure 5.2a and b.

5.2.2 Sp1 transactivates the ITGA2 promoter regardless of methylation

Sp1 is an ubiquitously expressed zinc finger transcription factor and was initially thought to regulate expression of housekeeping genes. However, it is now known to regulate expression of genes involved in many cellular processes such as differentiation (Opitz et al. 2000), cell growth (Santiago et al. 2007), angiogenesis (Mazure et al. 2003), apoptosis (Kaczynski et al. 2003) and immune responses (Jones et al. 1986). The Sp1 transcription factor specifically binds to GC rich sites and can regulate both TATA-containing and TATA-less promoters via interactions with other transcription factors (Näär et al. 1998). Since the *ITGA2* promoter is a TATA-less promoter, it has been suggested that Sp1 plays an important role in regulating basal *ITGA2* gene expression. However, the role of Sp1 in regulating *ITGA2* promoter activity in epithelial cells has not been investigated. Given *ITGA2* gene expression is cell-type specific, the role of Sp1 in the different cell types is of interest.

To determine the effect of the Sp1 transcription factor on the *ITGA2* promoter in prostate cancer cells, transient luciferase reporter assays were performed. A construct containing -791 to +83 bp of the *ITGA2* promoter cloned upstream of a luciferase gene, pXPG *ITGA2*, was transfected into PC3 and LNCaP cells either with or without an Sp1 expression plasmid, EF1α-Sp1Neo. This region of the promoter has previously been

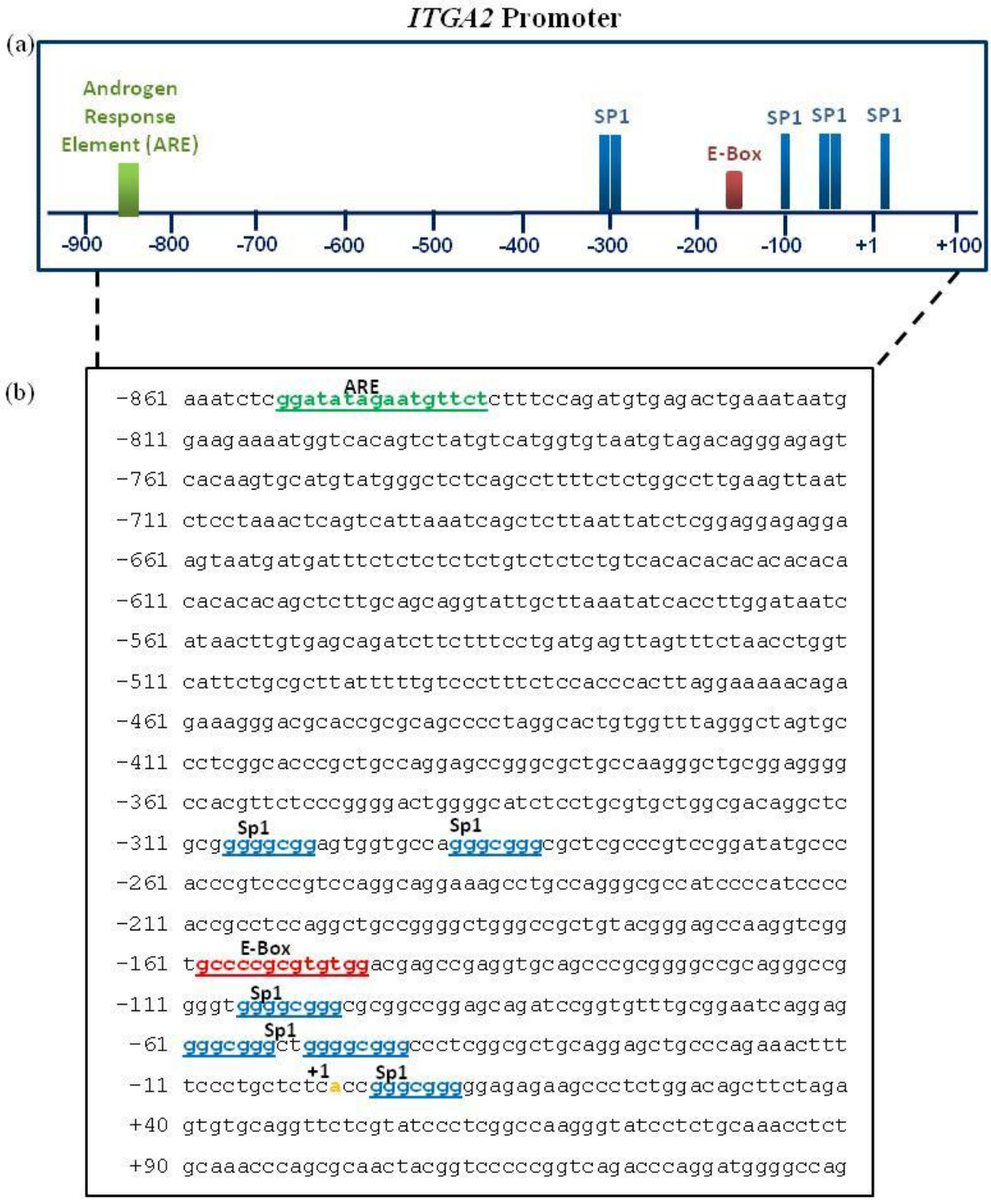


Figure 5.2 (a) Schematic representation of the positions of putative transcription factor binding sites and (b) recognition sequences identified by the MatInspector tool in the region upstream of the *ITGA2* TSS

shown to direct high levels of reporter gene activity in the breast cancer cell line, T47-D (epithelial cell model).After a 24 hour recovery period, cell lysates were harvested and luciferase activity was then measured. Cells were transfected in parallel with the pGL3 control plasmid (Promega), containing the constitutively active Simian virus 40 (SV40) promoter and enhancer upstream of the luciferase gene, to monitor transfection efficiency in the two cell lines.

Transfection of cells with the control plasmid, pGL3, resulted in a significant difference in luciferase activity between LNCaP and PC3 cells, with an approximately 10-fold higher level of luciferase activity observed in PC3 cells (Figure 5.3a). These results indicate that PC3 cells transfect more efficiently than LNCaP cells. In keeping with this, different levels of *ITGA2* promoter activity were observed in the two cell lines with approximately 10-fold lower activity in LNCaP (Figure 5.3b) compared to PC3 cells (Figure 5.3c). In both cell types, Sp1 overexpression resulted in a two-fold increase in *ITGA2* promoter activity. Given the differences in transfection efficiencies, in subsequent luciferase activity analysis, the luciferase activity of cells co-transfected with both *ITGA2* and the transcription factor of interest were graphed relative to the luciferase activity of cells transfected with only *ITGA2* plasmid, which was adjusted to 100. This will account for the differences in transfection efficiency in these cells and allow a more direct comparison of the effect of transcription factors on the promoter between cell lines. Sp1 transcription factor showed a relatively similar level of activation of the *ITGA2* promoter in both LNCaP and PC3 cells (Figure 5.4a), although this was only statistically significant in PC3 cells.

To determine whether the differences in endogenous *ITGA2* expression in these cell lines (Chapter 3) can be accounted for by differences in Sp1 levels in these cells, western blot analysis was performed to examine protein levels. Nuclear extracts were isolated from both LNCaP and PC3 cells and separated by SDS-PAGE. The proteins were transferred to a nitrocellulose membrane and probed with antibodies for Sp1 and histone H3, as a control (Figure 5.4b). Relatively equivalent levels of Sp1 protein were observed in the

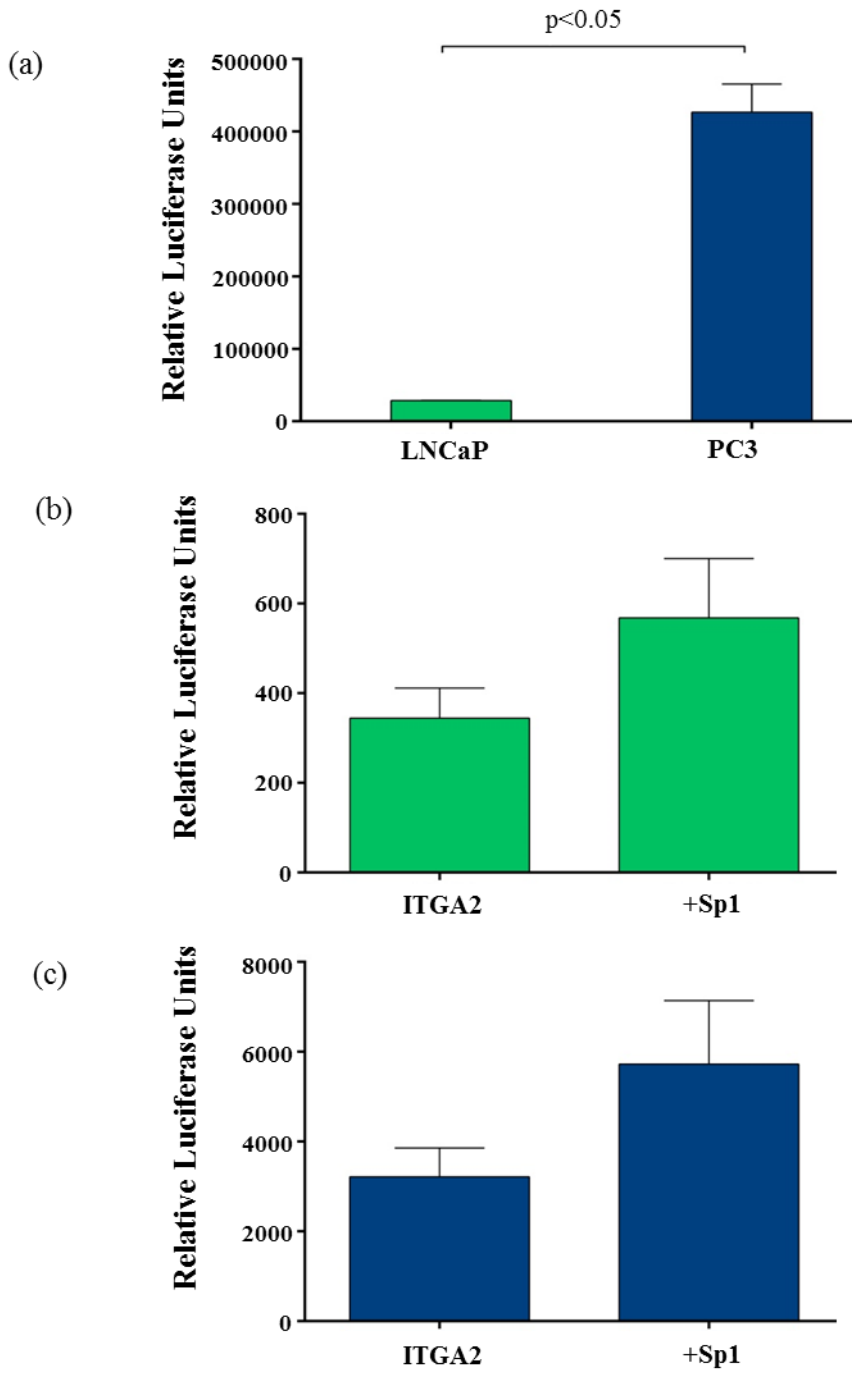


Figure 5.3 Sp1 activates the *ITGA2* promoter in both LNCaP and PC3 cells
(a) Relative luciferase activity of the pGL3 vector (5µg) in LNCaP and PC3 cells. (b) Relative luciferase activity of the pXPG ITGA2 promoter (5µg) in cells transfected with or without EF1α-Sp1Neo (5µg) in LNCaP cells and (c) in PC3 cells. Values expressed as mean ± SEM (n=3). Statistical significance was determined using student's *t* test.

nucleus of LNCaP and PC3 cells, with approximately equivalent levels of histone H3 protein, demonstrating relatively equal loading of proteins in each lane. Therefore, while Sp1 activates the *ITGA2* promoter, LNCaP and PC3 cells contain equivalent endogenous Sp1 protein levels and thus, Sp1 cannot account for the different expression levels of the *ITGA2* gene observed in these cell lines (Figure 3.3e).

As all six of the putative Sp1 binding sites are located in the CpG island of the *ITGA2* promoter and the Sp1 consensus site contains a CpG dinucleotide, it is possible that the ability of Sp1 to activate the *ITGA2* promoter is affected by methylation of the CpG sites at the *ITGA2* promoter. In order to investigate whether methylation of the *ITGA2* promoter affects its activity and transactivation by the Sp1 transcription factor, a construct was generated containing the region from -791 to +83 bp of the *ITGA2* promoter region cloned into a CpG free luciferase reporter, pCpGL (Klug et al. 2006). This construct was either left unmethylated or methylated with the CpG methylase *M.SssI,* before transfection into PC3 cells.

To confirm that methylation was complete, methylated and unmethylated plasmid were digested with *AciI* restriction enzyme and separated by agarose gel electrophoresis. This enzyme cuts at the recognition site 5' C^CGC 3', however, methylation at the CpG dinucleotide prevents digestion by this enzyme. As can be seen in Figure 5.5a, the unmethylated vector is digested by *AciI* whereas the *M.SssI*-methylated vector remains undigested, indicating that the vector had undergone complete CpG methylation. Transfection of the unmethylated and methylated vector into PC3 cells showed that methylation of the *ITGA2* promoter significantly reduced promoter activity by 4-fold as compared to the unmethylated vector (Figure 5.5b) thus, suggesting that DNA methylation represses *ITGA2* promoter activity. Consistent with the previous results, a 4-fold activation of the unmethylated *ITGA2* promoter by Sp1 transcription factor was observed. In addition, 6-fold activation of the methylated *ITGA2* promoter by the Sp1 transcription factor was observed, suggesting methylation of the CpG dinucleotides, while affecting basal activity of the promoter, does not affect the ability of Sp1 to transactivate the promoter. Together, these results suggest that Sp1 transcription factor

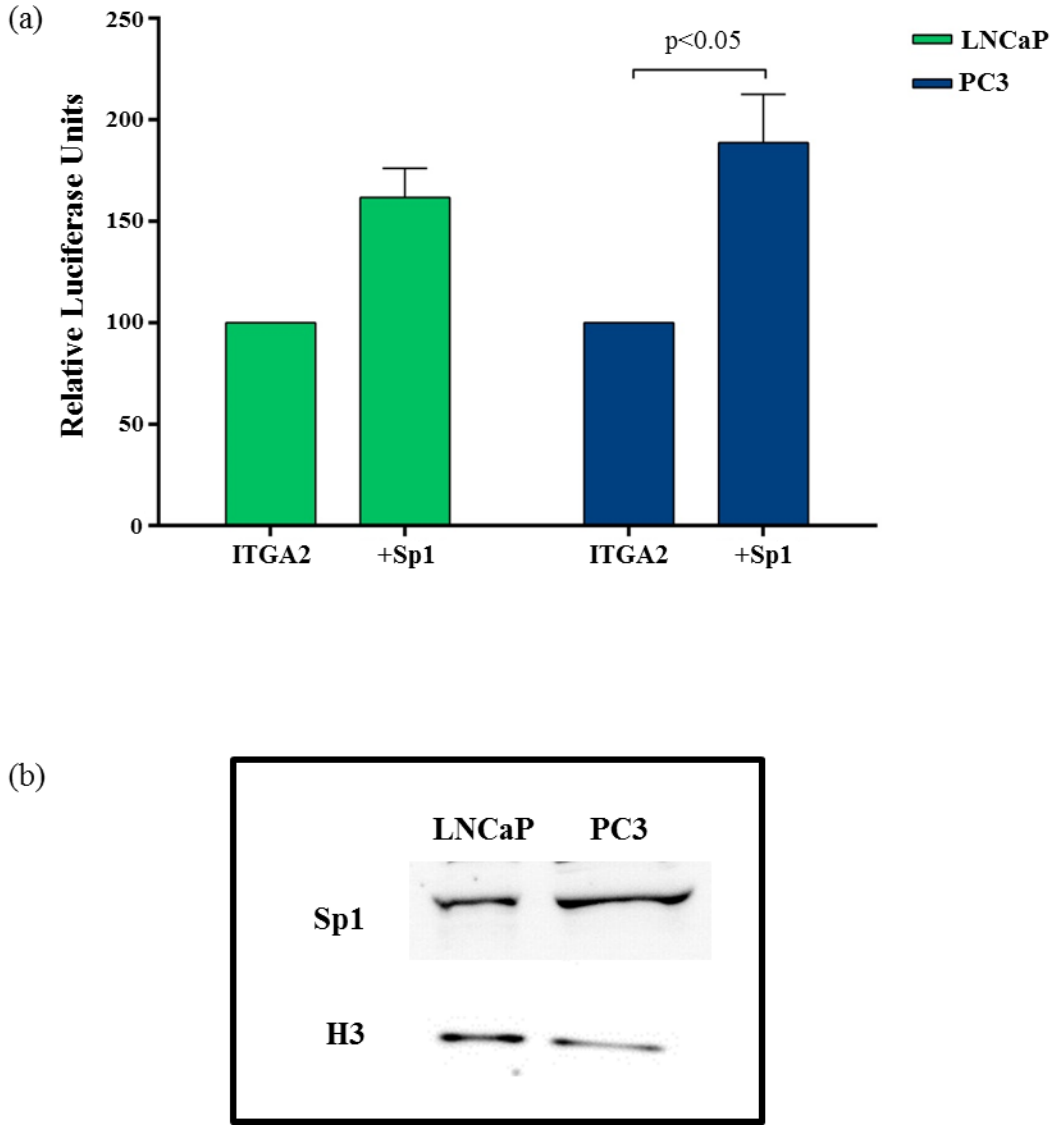


Figure 5.4 Sp1 activates the *ITGA2* promoter in both LNCaP and PC3 cells
(a) Relative luciferase activity of the pXPG ITGA2 promoter (5µg) in cells transfected with or without EF1α-Sp1Neo (5µg) was measured and adjusted relative to pXPG ITGA2 promoter activity which was set at 100. Values expressed as mean ± SEM (n=3). Statistical significance was determined using two-way ANOVA with Bonferonni post test. (b) Nuclear proteins isolated from LNCaP and PC3 cells were subjected to Western blotting with antibodies as indicated.

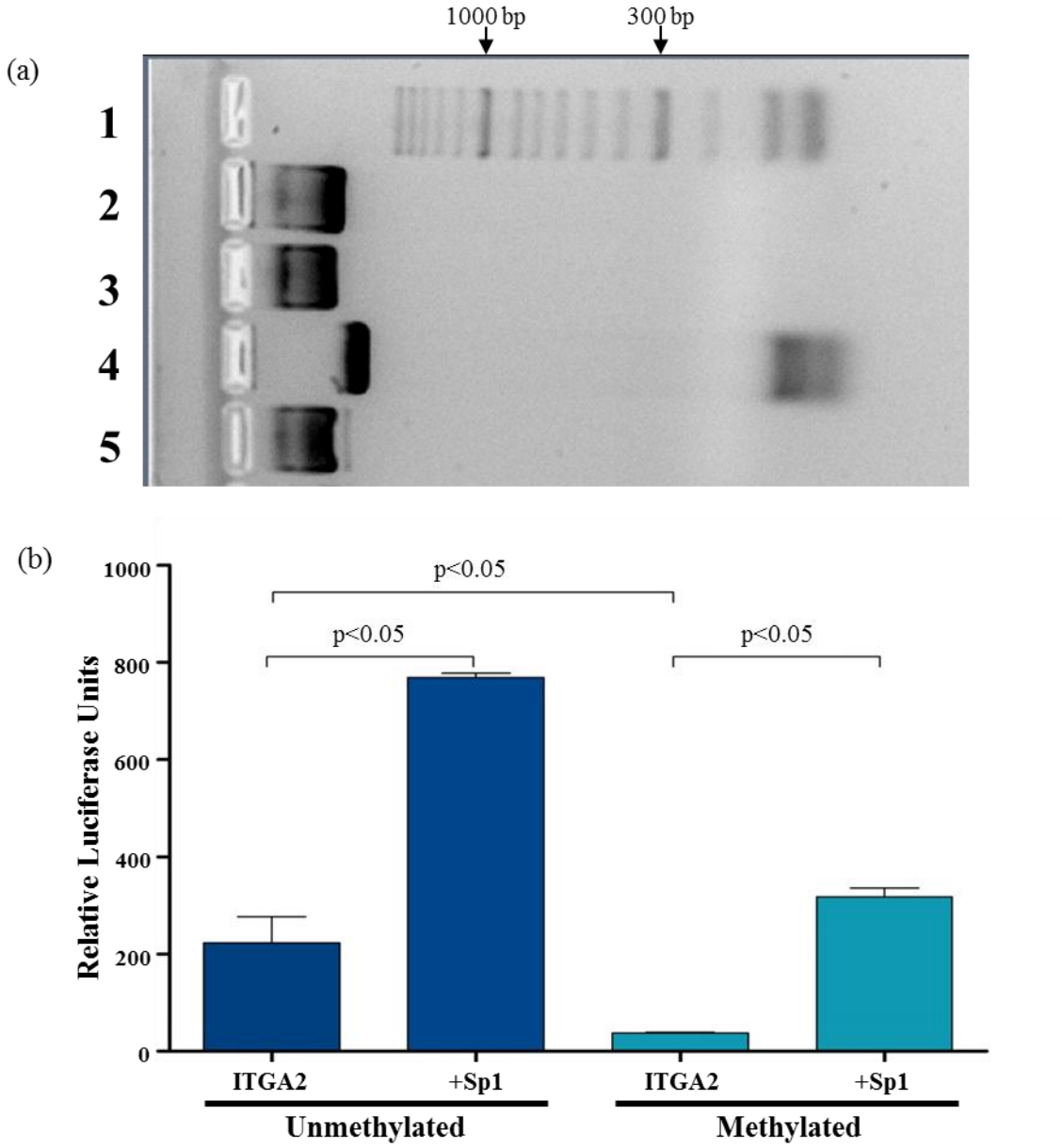


Figure 5.5 Sp1 activates the *ITGA2* promoter in PC3 cells regardless of DNA methylation level

(a) The pCpGL ITGA2 vector was either treated with *M.SssI* methylase or left untreated, digested with *AciI* or left undigested, and analysed by agarose gel electrophoresis. Lanes contain (1) HyperladderII size marker; (2) non-*M.SssI* treated, non-*AciI* digested plasmid; (3) *M.SssI* treated, non-*AciI* digested plasmid; (4) non-*M.SssI* treated, *AciI* digested plasmid and (5) *M.SssI* treated, *AciI* digested plasmid. Sizes of selected marker bands are indicated. (b) Relative luciferase activity of unmethylated and methylated *(M.SssI*-treated) pXPG ITGA2 vector transfected into PC3 cells either with or without EF1α-Sp1Neo was measured. Values expressed as mean ± SEM (n=3). Statistical significance was determined one-way ANOVA with Neuman-Keuls multiple comparison test.

plays a role in activation of the *ITGA2* promoter regardless of methylation. However, these data suggest that Sp1 drives basal activity of the promoter but does not contribute to the differential expression of *ITGA2* observed between the prostate cancer cell lines.

5.2.3 Androgen treatment represses the ITGA2 mRNA expression through an indirect effect

Prostate cancer is initially androgen-dependent and can be treated by androgen ablation therapy (Wang et al. 2007). However, the tumour eventually progresses into an androgen-independent state that leads to metastases with no effective therapy available. Androgens which activate androgen receptor are required for normal growth and function of the prostate gland (So et al. 2003). Androgens have been shown to regulate gene expression through the ARE site in the well described androgen regulated genes, human glandular kallikrein-1 (*hKLK2*) and *PSA* (Murtha et al. 1993). Given that an ARE half site was present in the *ITGA2* promoter region, experiments were undertaken to determine whether the promoter is androgen responsive.

The predicted ARE half site in the *ITGA2* promoter contains the consensus sequence of the classical ARE, 5' TGTTCT 3', although a functional ARE usually consists of tandem sites. To determine if androgen is regulating the *ITGA2* promoter, the androgen hormone, 5α-Androstan-17β-ol-3-one (dihydrotestosterone; DHT) was used to treat the androgen-sensitive cell line, LNCaP. LNCaP cells were treated with DHT in a serum-free medium, OPTI-MEM and RNA isolated at 3, 6, and 24 hours post-treatment. RNA was isolated, cDNA prepared and *ITGA2* mRNA expression analysed by real-time PCR. A statistically significant downregulation of *ITGA2* mRNA levels upon DHT treatment was observed at all three timepoints (Figure 5.6). This repressive effect was unexpected as androgen is generally reported to be an activator of gene expression (Heemers et al. 2001; Murtha et al. 1993).

In order to determine whether the effect of the DHT treatment on *ITGA2* is dependent on protein synthesis and therefore likely to be an indirect effect, cells were pre-treated with cycloheximide (CHX). Given that androgen receptor is pre-existing in the cells, CHX is

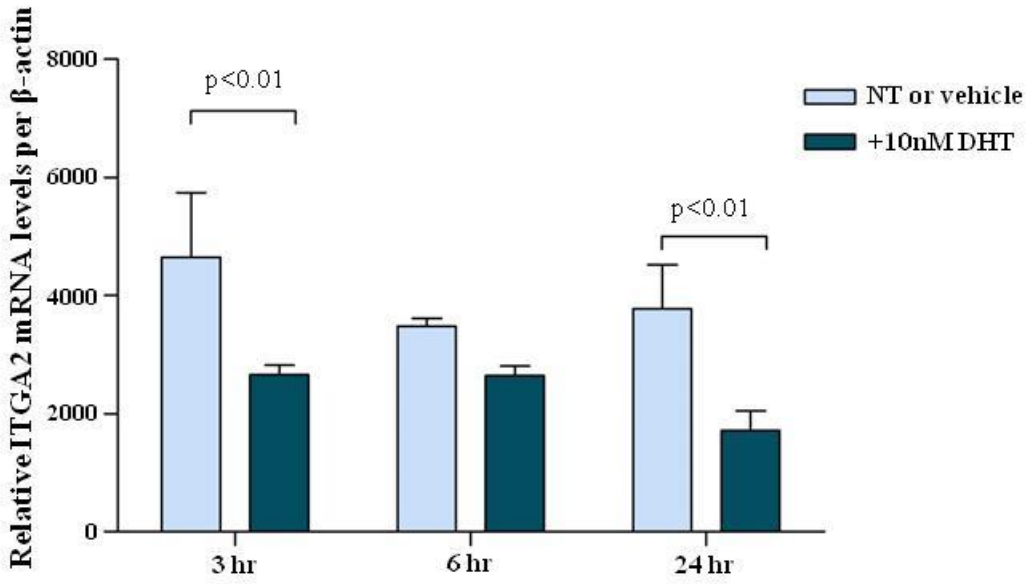


Figure 5.6 DHT treatment represses *ITGA2* mRNA expression
Total RNA isolated from LNCaP cells treated either with 95% ethanol NT (no treatment or vehicle) or 10nM DHT (5α-Androstan-17β-ol-3-one) for 3, 6 and 24 hours, was reverse transcribed and amplified by real-time PCR using primers designed to amplify *ITGA2* mRNA. *ITGA2* mRNA levels are shown relative to *β-actin*. Values expressed as mean ± SEM (n=3). Statistical significance was determined relative to the indicated control using student's *t* test.

used to inhibit *de novo* protein synthesis and if repression of *ITGA2* gene expression upon DHT treatment is still observed with CHX pre-treatment, this will indicate that it is independent of *de novo* protein synthesis. Conversely, if no repression of *ITGA2* gene expression upon DHT treatment with CHX pre-treatment is observed, this will indicate that it is dependent on *de novo* protein synthesis. DHT treatment in LNCaP cells for 3 hours demonstrated repression of *ITGA2* mRNA expression thus this treatment timepoint was used to determine if the DHT effect is dependent on protein synthesis (Figure 5.7a). In the CHX pre-treated cells, DHT failed to repress *ITGA2* expression, suggesting that repression of *ITGA2* by androgen is protein synthesis dependent and thus a secondary effect. CHX treatment alone decreased *ITGA2* expression, suggesting that basal ITGA2 expression is protein synthesis dependent.

Since the *PSA* gene is an extensively studied direct target gene of androgen receptor and contains a well characterised ARE in its promoter (Cleutjens et al. 1997; Murtha et al. 1993; Wang et al. 1999), *PSA* mRNA levels were analysed in the same samples to validate the DHT treatment. In the cells without CHX pre-treatment, *PSA* mRNA levels were statistically significantly upregulated upon DHT treatment (Figure 5.7b), consistent with previously reported findings by others. This was also observed in cells treated with DHT in the presence of CHX pre-treatment, which supports that upregulation of *PSA* mRNA expression is a direct effect of androgen receptor and thus, is consistent with the findings of others. Therefore, these results suggest that *ITGA2* is repressed by androgen through an indirect mechanism.

5.2.4 *E-box binding factors: Snail represses ITGA2 promoter activity*

The bHLH transcription factors are known to bind to E-box recognition sites and mediate target gene transcription. This family of transcription factors includes the EMT transcription factors, Snail and Twist, which for example bind to the E-box of E-cadherin leading to downregulation of this gene (Batlle et al. 2000; Cano et al. 2000). Given that a putative E-box binding site was identified in the *ITGA2* promoter and the results presented in Chapter 4 suggests the possible involvement of Snail and Twist in regulating

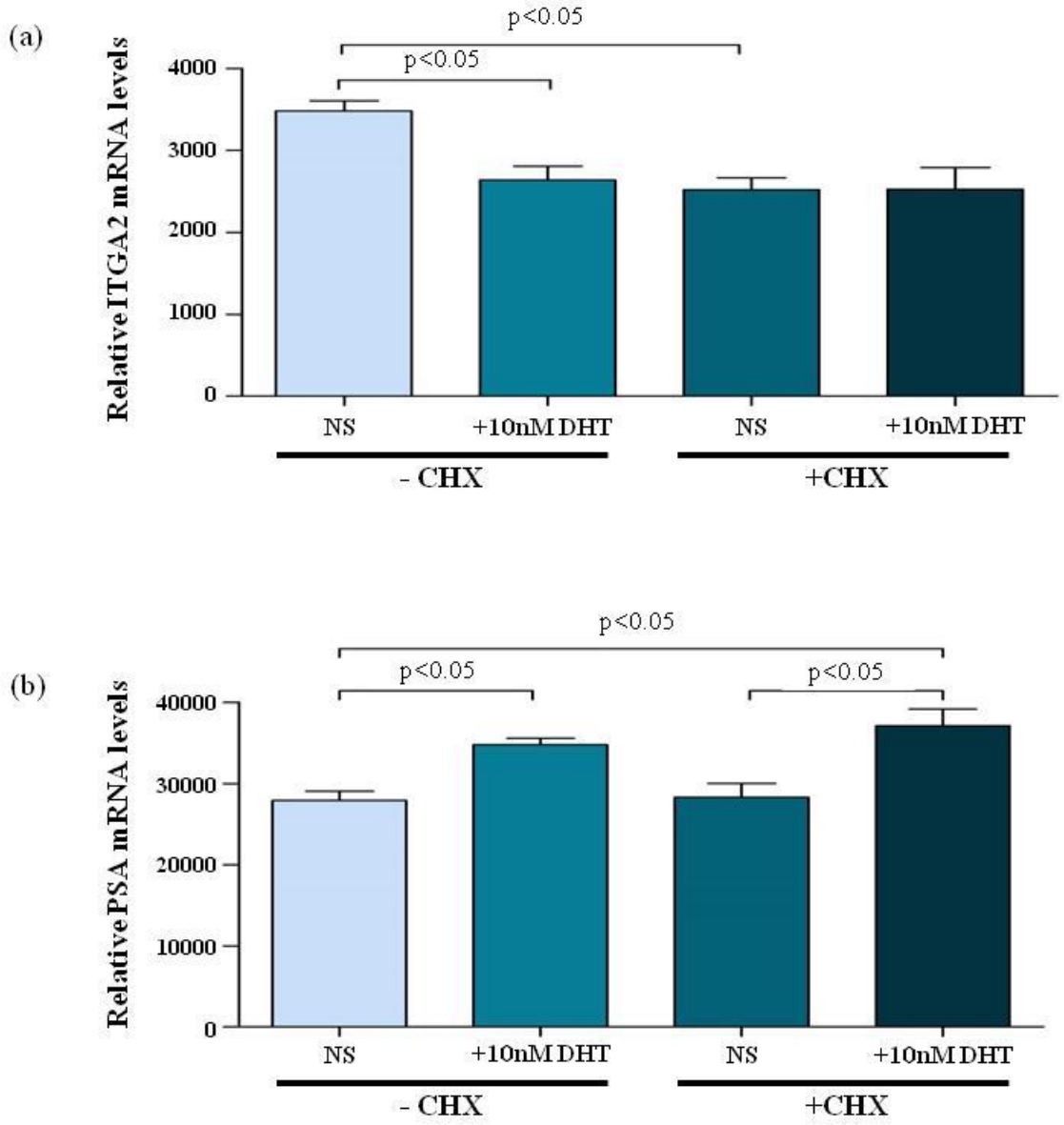


Figure 5.7 Repression of *ITGA2* mRNA expression by DHT is an indirect effect
LNCaP cells were treated with or without cycloheximide (CHX) for 30 minutes, followed by treatment with or without DHT for 3 hours. Total RNA was isolated from cells, reverse transcribed and amplified by real-time PCR using primers designed to amplify *ITGA2* mRNA or *PSA* mRNA. (a) *ITGA2* mRNA levels are shown relative to *β-actin*; (b) *PSA* mRNA levels are shown relative to *β-actin*. Values expressed as mean ± SEM (n=3). Statistical significance was determined using one-way ANOVA with Neuman-Keuls multiple comparison test.

ITGA2 expression, further investigation of the potential role of these transcription factors in *ITGA2* regulation was undertaken.

LNCaP and PC3 cells were transfected with pXPG *ITGA2* vector as previously described (Section 5.2.2), either with or without a Snail expression vector, pCMV-Flag SNAIL WT (Addgene, USA). After a 24 hour recovery period, cell lysates were harvested and luciferase activity was then measured. To account for the differences in transfection efficiency in these cells and to allow a direct comparison between both cell lines, the relative luciferase activity observed from cells transfected with only the *ITGA2* plasmid was adjusted to 100 and the relative luciferase activity from cells co-transfected with Snail was adjusted accordingly. As previously, a basal level of *ITGA2* promoter activity was detected in both LNCaP and PC3 cells, and this was repressed upon co-transfection with Snail expression vector. A greater repressive effect, which was statistically significant, was observed in PC3 cells as compared to LNCaP cells (Figure 5.8a). This could be due to the fact that LNCaP cells express higher levels of endogenous Snail mRNA levels as compared to the PC3 cells (Figure 5.8b). Overall, these results suggest that Snail represses *ITGA2* promoter activity.

Twist, another EMT transcription factor, is known to also bind to E-box elements and the results in Section 4.2.3 suggest a possible involvement of this transcription factor in regulating *ITGA2* gene expression also. Therefore, as with the previous experiment, LNCaP and PC3 cells were transfected with pXPG *ITGA2* vector either with or without a Twist expression vector, pCMV6 TWIST1 (Origene, USA) and cell lysates were harvested after 24 hours to assay for the luciferase activity. Interestingly, Twist activates *ITGA2* promoter activity in LNCaP cells but did not affect *ITGA2* promoter activity in PC3 cells (Figure 5.9a). These differences in activity observed could be due to the differences in Twist endogenous mRNA expression levels (Figure 5.9b). A statistically significantly higher level of Twist mRNA levels was observed in PC3 cells compared to LNCaP cells, which showed very low or no expression of Twist (Figure 5.9b). Since activation of *ITGA2* promoter activity by Twist was only observed in LNCaP cells, Twist effects on *ITGA2* promoter activity was further investigated. While the initial four

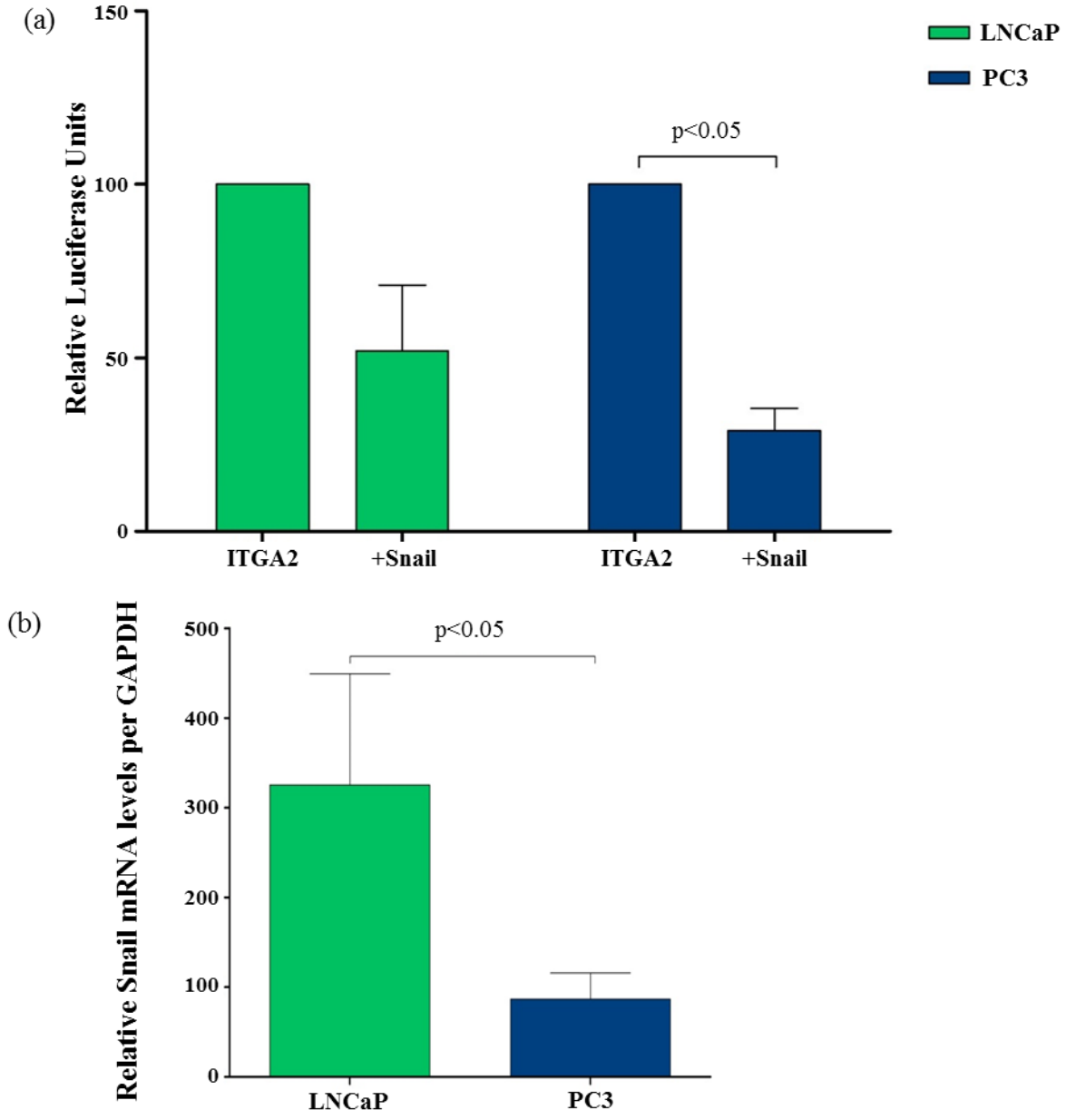


Figure 5.8 Snail represses *ITGA2* promoter activity in prostate cancer cells
(a) Relative luciferase activity of the pXPG ITGA2 promoter (5µg) in cells transfected with or without pCMV-Flag SNAIL WT (5µg) were measured and adjusted relative to pXPG ITGA2 promoter activity and (b) total RNA isolated from LNCaP and PC3 cells was reverse transcribed and amplified by real-time PCR using primers designed to amplify Snail mRNA. Snail mRNA levels are shown relative to *GAPDH* (adapted from Figure 4.1). Values expressed as mean ± SEM (n=3). Statistical significance was using student's *t* test.

experiments represented in Figure 5.9 were consistent with activation of the promoter by Twist, further experimental replicates showed either no change or slight repression of *ITGA2* promoter activity (Figure 5.10). These results suggest inconsistency in Twist activity and thus, no further studies on Twist were undertaken at this stage, although this warrants further investigation.

5.2.5 *Sp1 and Snail regulate ITGA2 promoter activity in PC3 cells*

Thus far, Sp1 has been found to activate whilst Snail represses *ITGA2* promoter activity, and therefore the effect of the combination of these factors was determined. As this experiment requires transfection of multiple vectors into the cells, PC3 cells were selected for these experiments because this cell line showed higher and more consistent transfection efficiency. PC3 cells were transfected with the pXPG ITGA2 vector and either Sp1, Snail or both Sp1 and Snail expression constructs. Cell lysates were harvested after 24 hours to assay for luciferase activity.

Consistent with the previous findings, Sp1 activated *ITGA2* promoter activity while Snail represses *ITGA2* promoter activity (Figure 5.11). Further, Sp1 was able to partially relieve the repressive effect of Snail. Therefore, these results suggest that Sp1 and Snail combine to regulate *ITGA2* promoter.

5.2.6 *Snail potentially regulates ITGA2 through an E-box site within the -791 to -569 bp region of the promoter*

To determine if Snail acted via the putative E-box binding site -154 bp upstream of the TSS identified by the MatInspector bioinformatics tool, site-directed mutagenesis of the E-box was performed. The well characterised E-box recognition sequence is 5' CANNTG 3' (where N can be substituted by any nucleotide), however, the recognition sequence identified by the MatInspector bioinformatics tool as an E-box is 5' GCCCGCGTGTGG 3' (with the core recognition bases underlined). Therefore, in order to mutate the E-box site, the core recognition sequence was mutated from CGTG to CTTA to generate the construct, pXPG ITGA2-E-boxm (Figure 5.12a). Sequencing confirmed that the E-box mutation was present in the vector.

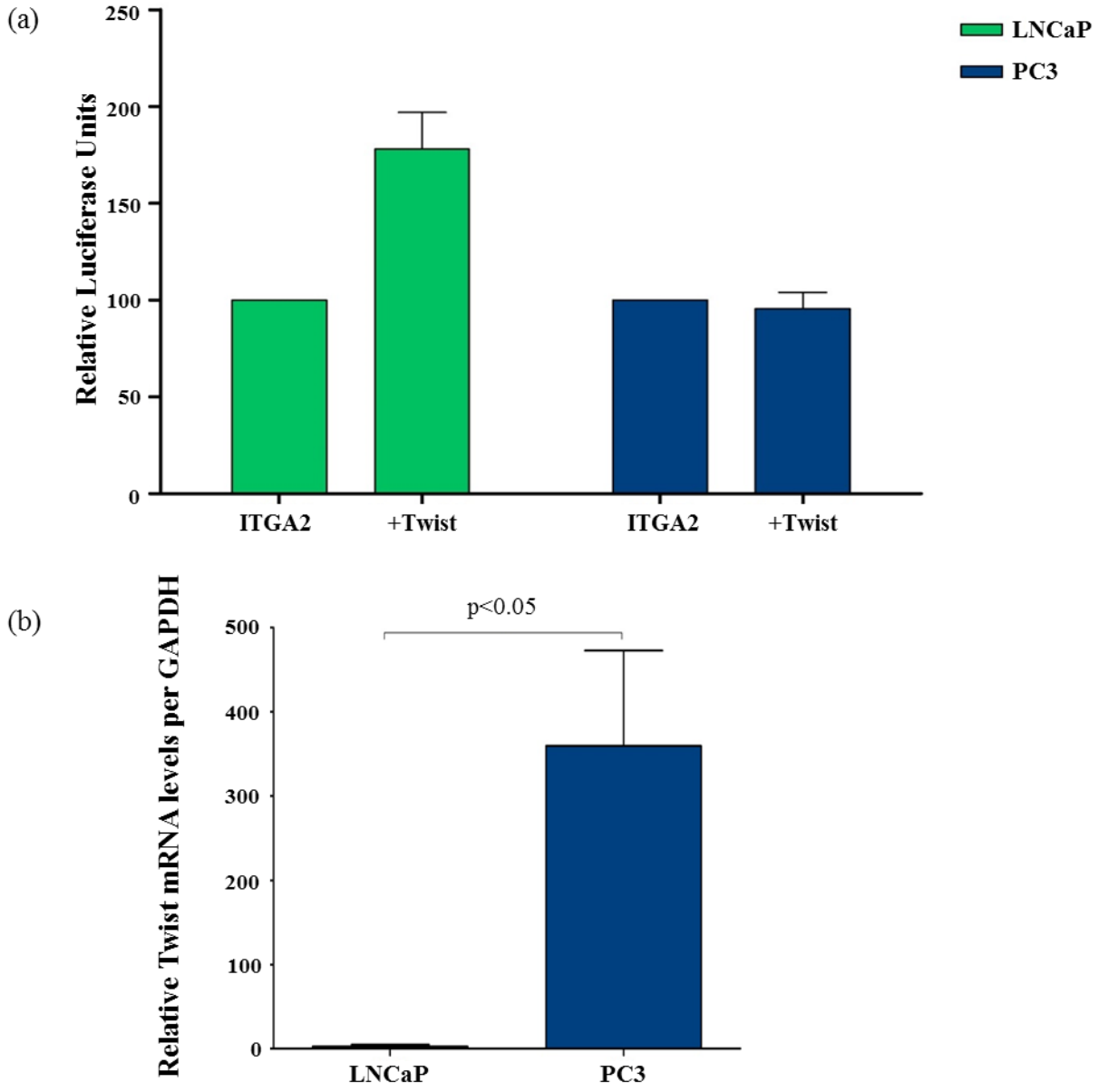


Figure 5.9 Twist activates *ITGA2* promoter activity in LNCaP but not PC3 cells
(a) Relative luciferase activity of the pXPG ITGA2 promoter (5µg) in cells transfected with or without pCMV6 TWIST1 (5µg) were measured and adjusted relative to pXPG ITGA2 promoter activity and (b) total RNA isolated from LNCaP and PC3 cells was reverse transcribed and amplified by real-time PCR using primers designed to amplify Twist mRNA, Twist mRNA levels are shown relative to *GAPDH* (adapted from Figure 4.1). Values expressed as mean ± SEM (n=3). Statistical significance was determined using student's *t* test.

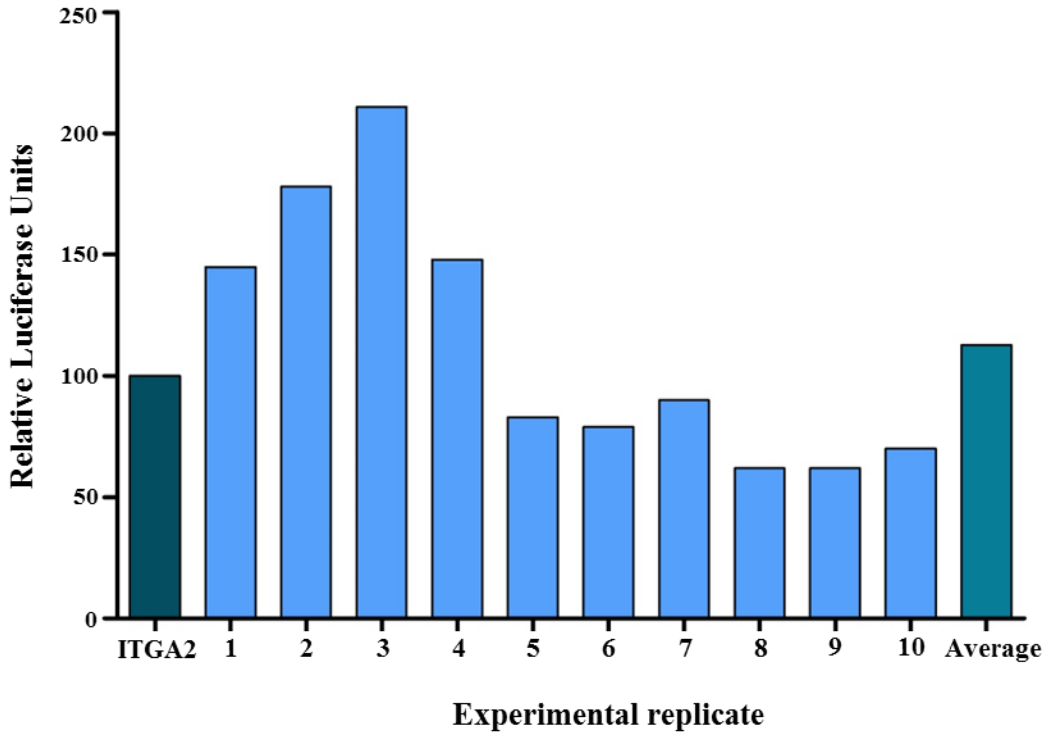


Figure 5.10 Effects of Twist on the *ITGA2* promoter were inconsistent
Relative luciferase activity of the pXPG ITGA2 promoter (5µg) was measured in cells transfected with the *ITGA2* promoter construct alone or with pCMV-Flag pCMV6 TWIST1 (5µg). Ten individual experiments are shown (1-10) and the average value of these 10 experiments were measured and adjusted relative to pXPG ITGA2 promoter activity in each case.

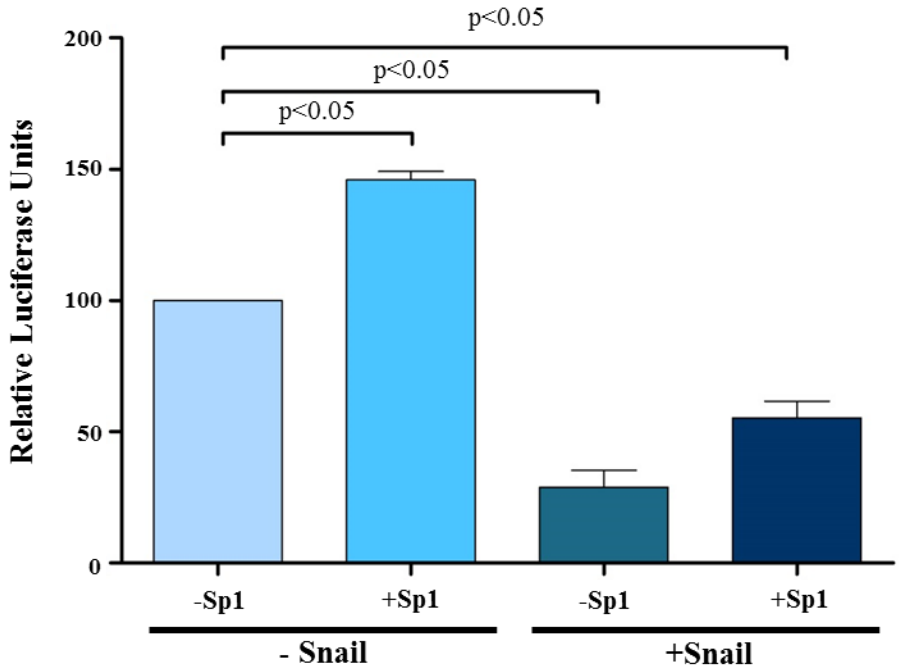


Figure 5.11 Sp1 is able to partially relieve Snail repression of the *ITGA2* promoter in PC3 cells

Relative luciferase activity of the pXPG ITGA2 promoter (5µg) in cells transfected with or without EF1α-Sp1Neo (5µg) or pCMV-Flag SNAIL WT (5µg), or both. Values were measured and adjusted relative to pXPG ITGA2 promoter activity. Values expressed as mean ± SEM (n=3). Statistical significance was determined using one-way ANOVA with Neuman-Keuls multiple comparison test.

LNCaP cells were then transfected with the wild type (WT) ITGA2 vector either with or without Snail expression vector and cell lysates were harvested after 24 hours to assay for the luciferase activity. At the same time, LNCaP cells were also transfected with the ITGA2 vector containing the mutant E-box either with or without Snail transcription factor (Figure 5.12b). As expected, Snail repressed *ITGA2* promoter activity. Cells transfected with the mutant vector showed equivalent levels of luciferase activity as compared to the cells transfected with the WT ITGA2 vector. Interestingly, repression of *ITGA2* promoter activity by Snail was still observed when the E-box site was mutated, indicating that Snail does not act through the putative E-box at -154 bp. Since this experiment showed negative results, these transfections were not repeated and the possibility of an alternative E-box binding site at the *ITGA2* promoter region was investigated.

The MatInspector tool predicted E-box site at -154 bp recognition sequence did not match the classical 5' CANNTG 3', however, using the classical consensus recognition site, a search of the *ITGA2* promoter revealed a second putative E-box site at -759 bp with the recognition sequence 5' CAAGTG 3' (Figure 5.13a and b). The possibility exists that the repression of *ITGA2* promoter activity observed with Snail could be through binding to this putative E-box site.

To further determine if the second predicted E-box at -759 bp plays a role in Snail transcription factor binding and thus, drives the repression of *ITGA2* expression, deletion mutants pXPG ITGA2 Del1 (-569 to +83) and pXPG ITGA2 Del2 (+270 to +83) were constructed (Figure 5.14a). Primers were designed to amplify the regions of interest from the WT vector, then cloned and screened for inclusion of the inserts by digestion with the same restriction enzymes used for cloning, *BamHI* and *XhoI*. After digestion, the products were separated using agarose gel electrophoresis. Results showed the expected sizes of insert for pXPG ITGA2 Del1 (652 bp) and pXPG ITGA2 Del2 (353 bp; Figure 5.14b).

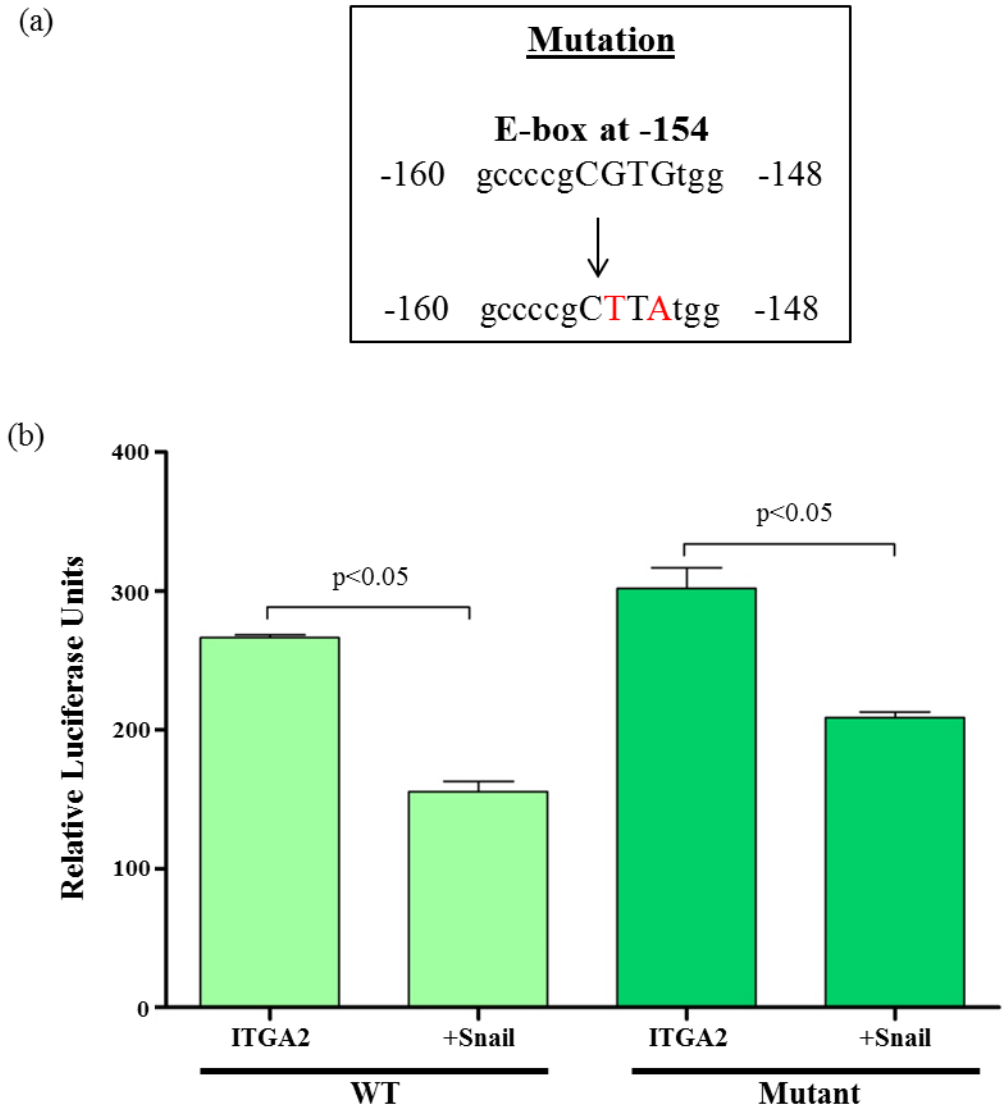


Figure 5.12 Mutation of the predicted E-box site at -154 bp did not affect Snail repression of the ITGA2 promoter in LNCaP cells

(a) Schematic representation showing the potential E-box at -154 of the *ITGA2* promoter and the nucleotides mutated, as indicated in red. (b) Relative luciferase activity of the pXPG ITGA2 promoter (5µg) or the pXPG ITGA2 Eboxm, with or without pCMV-Flag SNAIL WT (5µg) were measured. Values expressed as mean ± SEM for one independent experiment with three repeated measures. Statistical significance was determined using student's *t* test.

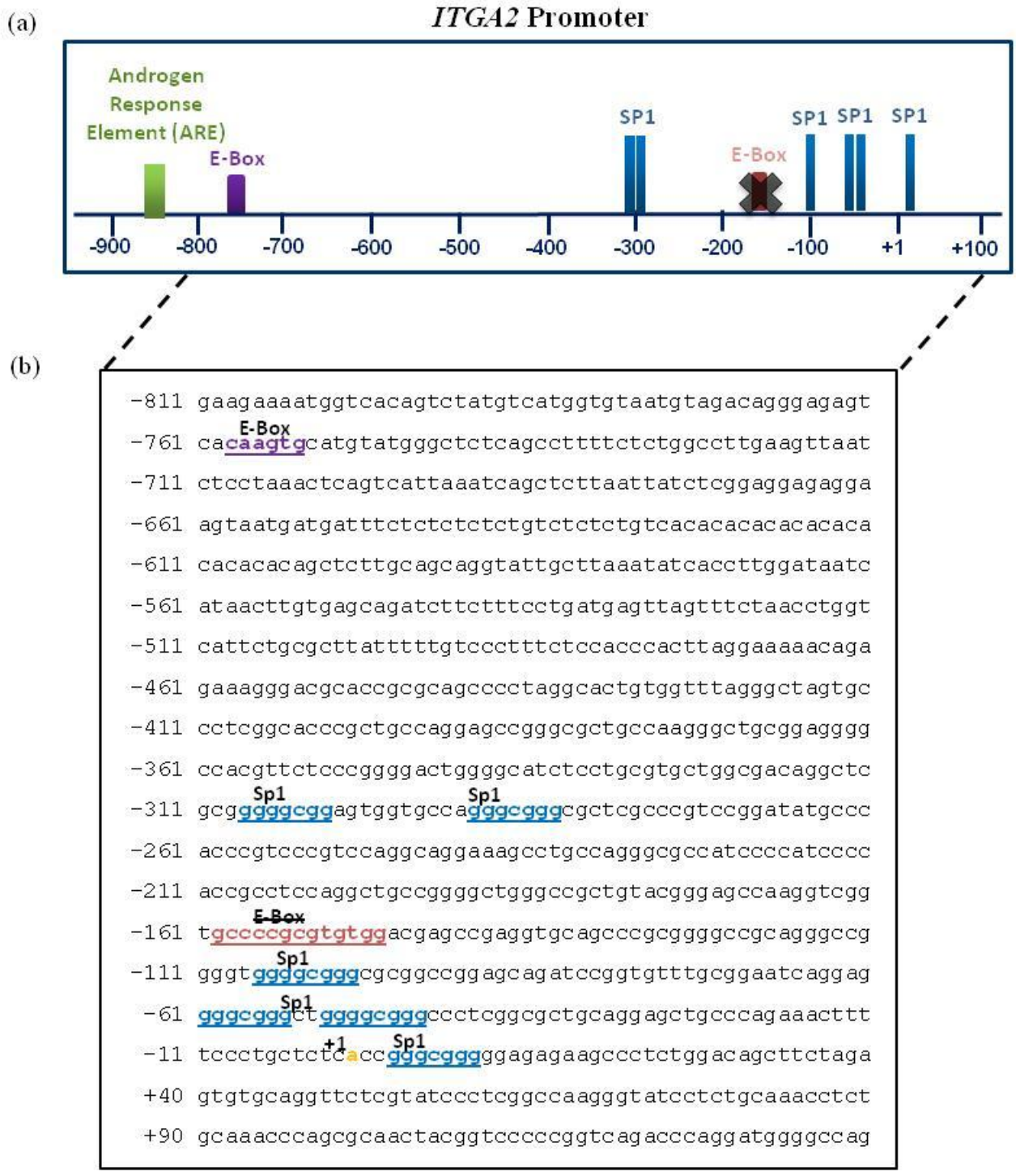


Figure 5.13 (a) Schematic representation of the positions of putative transcription factor binding sites in the *ITGA2* promoter. Predicted E-box sites are shown at position -759 bp (in purple) and -154 bp. (b) Recognition sequences of the transcription factor binding sites are shown with the new predicted E-box site at position -759 indicated in purple.

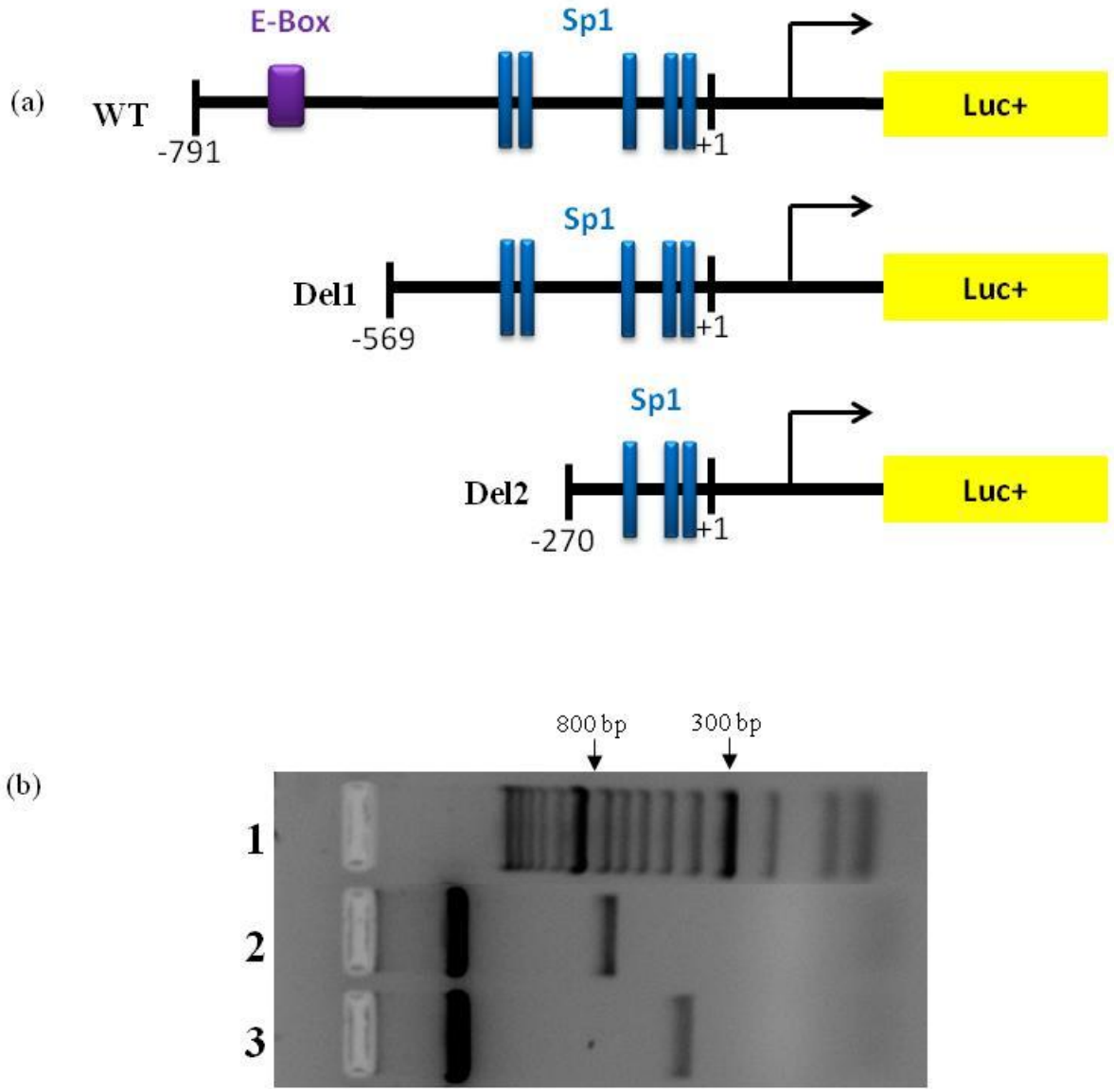


Figure 5.14 (a) Schematic representation of the *ITGA2* promoter deletion constructs showing the putative transcription factor binding sites in the promoter and (b) deletion mutants were cloned and checked for the expected insert sizes by digestion with *BamHI* and *XhoI* enzymes and analysed by agarose gel electrophoresis.
Lanes contain (1) HyperladderII size marker; (2) digested pXPG ITGA2 Del1 vector and (3) digested pXPG ITGA2 Del2 vector. Sizes of selected marker bands are indicated.

LNCaP cells were then transfected with the WT, Del1 and Del2 constructs, either with or without Snail expression vector, and cell lysates were harvested after 24 hours to assay for the luciferase activity. Both of the shorter constructs, Del1 and Del2 showed higher *ITGA2* promoter activity as compared to the full length, WT construct (Figure 5.15). Therefore, these results suggest that a repressor element could be present within the -791 to -569 bp region and an enhancer element within the -569 to -92 bp region of the *ITGA2* promoter.

As would be expected, Snail repressed WT *ITGA2* promoter activity in the transfected cells (Figure 5.15). Interestingly, Snail did not have an effect on the *ITGA2* Del1 promoter. This suggests the predicted E-box site lies within the region of -791 to -569 bp which contains the putative E-box at -759 bp. Unexpectedly, Snail activated the *ITGA2* Del2 promoter. Due to time constraints, this experiment was conducted only once, however, the luciferase activity was measured in triplicate. Overall, the results suggest that the E-box where Snail binds and represses *ITGA2* promoter activity is present within the -791 to -569 bp region although this remains to be confirmed.

5.3 Discussion

Previously, the results presented in Chapter 3 suggest that epigenetic factors, mainly involving promoter DNA methylation and histone acetylation, are at least in part responsible for the differential expression of *ITGA2* observed in the prostate cancer cell lines. Since both epigenetic factors and transcription factors play important roles in regulation of gene expression, the data presented here in this chapter demonstrate that *ITGA2* activity can be regulated by a combination of transcription factors. Utilising the MatInspector bioinformatics tool several putative transcription factor binding sites were indentified in the *ITGA2* gene promoter, including Sp1 binding sites, an ARE and an E-box element.

Sp1 was found to activate the *ITGA2* promoter in both LNCaP and PC3 cells. It is well established that methylation of CpG dinucleotides can directly inhibit promoter function

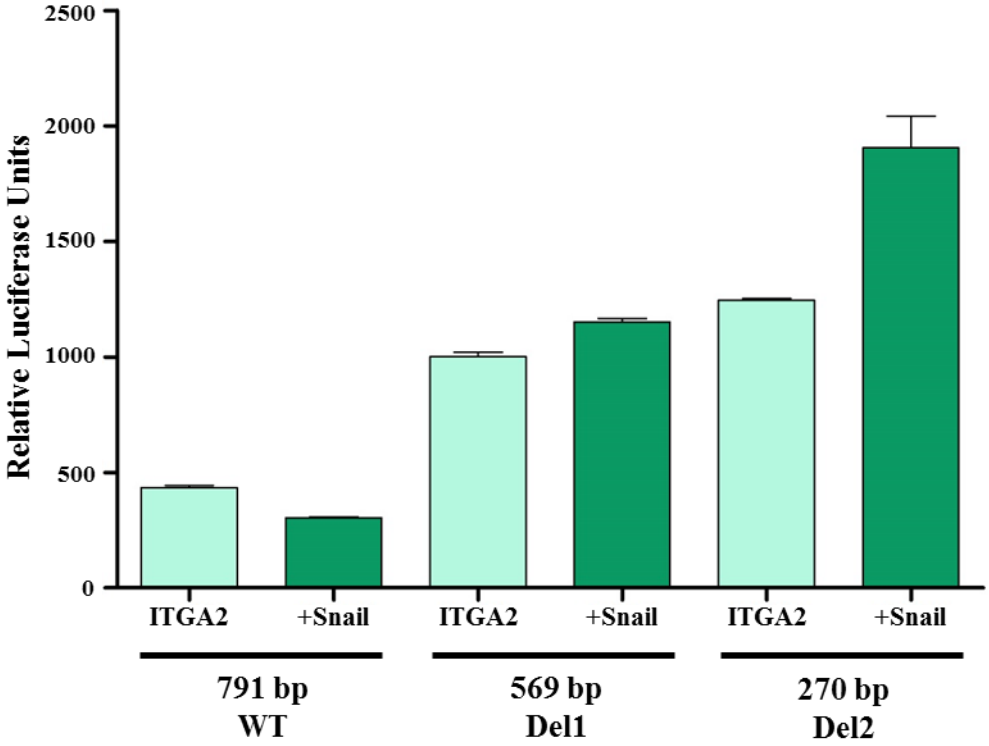


Figure 5.15 Identification of the Snail responsive region of the *ITGA2* promoter
Relative luciferase activity of the pXPG ITGA2 promoter (5µg), pXPG ITGA2 Del1 (5µg) and pXPG ITGA2 Del2 (5µg), with or without pCMV-Flag SNAIL WT (5µg) in LNCaP cells were measured. Values expressed as mean ± SEM for one independent experiment, with three repeated measures.

by preventing the binding of transcription factors (Bell et al. 2000; Hark et al. 2000a; Holmgren et al. 2001; Szabó et al. 2000). Whilst the Sp1 binding site contains a CpG dinucleotide, whether Sp1 binding is affected by methylation remains controversial. Interestingly, one of the six Sp1 binding sites showed differential methylation at the CpG dinucleotide at position -58 bp region between the prostate cancer cell lines. This site is unmethylated in LNCaP and PC3 cells while it is methylated in 22Rv1 cells (Figure 3.4). However, reporter assays suggest that Sp1 still activates *ITGA2* promoter activity regardless of methylation. Hence Sp1 activation of the *ITGA2* promoter is unlikely to be affected by the differential methylation pattern observed in the different cell lines. This suggests that the Sp1 transcription factor can not account for the differences in *ITGA2* expression observed in LNCaP and PC3 cells. Therefore, the Sp1 transcription factor may be important in regulating basal *ITGA2* expression in these prostate cancer cells, consistent with other studies which have found Sp1 to be important for *ITGA2* expression in megakaryocytic cells (Jacquelin et al. 2001). However, when the *ITGA2* endogenous gene is methylated, this is also associated with decreased chromatin accessibility which may affect Sp1 binding at the promoter. Additionally, the reporter assay results support that methylation represses *ITGA2* promoter activity and thus, are consistent with the methylation analysis and gene expression presented in Chapter 3.

Examination of the influence of androgens, which activate the androgen receptor that binds to the ARE, in the regulation of the *ITGA2* gene expression revealed that androgen represses *ITGA2* gene expression. This is consistent with a study where DHT treatment of PC3 cells transfected with androgen receptor resulted in down regulation of α2β1 surface expression (Evangelou et al. 2002) and was followed by decreased PC3 cell adhesion to collagen type I. Further examination of this repressive effect confirmed that it is not a direct effect and thus, androgen treatment could be activating a repressor, resulting in repression of *ITGA2* expression. Treatment of LNCaP cells with CHX alone resulted in repression of *ITGA2* basal expression, which is potentially due to CHX treatment inhibiting synthesis of the Sp1 transcription factor which contributes to basal *ITGA2* promoter activity. However, the effect of androgen treatment on *ITGA2* promoter activity was not determined in this study as the pXPG ITGA2 promoter construct was only cloned

from -791 bp, which did not include the ARE site. A future study of the effects of androgen treatment on *ITGA2* promoter activity can be determined by assessing the luciferase activity of the ITGA2 promoter construct (containing the ARE site) with DHT treatment.

Studies have shown that upon binding to the E-box, Snail family members act as transcriptional repressors (Batlle et al. 2000; Cano et al. 2000). Consistent with these observations, Snail had a repressive effect on *ITGA2* promoter activity in both LNCaP and PC3 cells. The greater repression observed in PC3 cells following Snail overexpression is consistent with the lower endogenous Snail expression in this cell line. Interestingly, Sp1 was able to partially relieve the repressive effect of Snail and therefore, while Sp1 is expressed at equivalent levels in both PC3 and LNCaP cells, which confers the basal *ITGA2* expression, the higher endogenous Snail expression in LNCaP cells as compared to PC3 cells may be responsible for the lower *ITGA2* expression observed in LNCaP cells.

While studies have shown that Twist directly downregulates E-cadherin expression through binding to an E-box site (Kang et al. 2004b; Vesuna et al. 2008), there are also studies which have demonstrated the Twist can also act as a transcriptional activator of some genes, for example, GLI1 and N-cadherin (Alexander et al. 2006; Villavicencio et al. 2002; Yang et al. 2008). Initially, Twist activation of the *ITGA2* promoter was detected. However, this result was inconsistent across a large number of experiments. Therefore, one possibility is that the Twist effect is cell cycle dependent or otherwise influenced by the growth characteristics of the cells. Although this has not previously been reported, there are studies that have shown that Twist regulates genes involved in cell differentiation (Lee et al. 1999; Maestro et al. 1999). Future studies to further verify the role Twist transcription factor can be carried out using well-characterised cell lines with very high transfection transfection efficiencies and high levels of co-factors, such as the Cos-7 cell line, although this can not be used to investigate the prostate cancer cell type specific promoter regulagtion.

Since the predicted E-box site at -154 bp initially identified bioinformatically in the *ITGA2* promoter was different to the classical E-box consensus sequence, site-directed mutagenesis experiments were undertaken and indicate that Snail does not act through this site. However, further analysis using deletion mutants demonstrated that a possible E-box site could be present within the -791 to -569 bp region and a classical E-box consensus sequence was found at position -759 bp. Consistent with this, the second putative E-box site at -759 bp lies within the repressor region of the *ITGA2* promoter. This repressor region from -961 to -569 bp may be specific for prostate cancer (Figure 5.16a). In previous study the *ITGA2* promoter was characterised in the breast cancer cell line, T47-D (Figure 5.16b), although the repressor region was identified from -961 to -776 bp and therefore does not encompass the potential E-box at -759 (Zutter et al. 1994), it is likely these positive and negative regulatory elements play important roles in determining cell type specific expression.

Interestingly, Snail activated *ITGA2* promoter activity of the Del2 construct. As this short construct does not include the putative E-box site, it is possible that this effect by Snail is an indirect effect and since this region contains the Sp1 transcription factor binding sites, Snail may be associating with Sp1 transcription factor to activate *ITGA2* activity. Although conventionally Snail has been described as a transcriptional repressor (Batlle et al. 2000; Cano et al. 2000) there are now studies that have found a possible role for Snail as a transcriptional activator of the matrix methalloproteinase 9 (*MMP-9*) gene (Jordà et al. 2005; Miyoshi et al. 2005; Sun et al. 2008; Whiteman et al. 2008) and the cyclin-dependent kinase 4 inhibitor b ($p15^{ink4b}$) gene (Hu et al. 2010). While the mechanisms by which Snail activates MMP-9 were not investigated, Snail was found to associate with the Sp1 and early growth response gene 1 (*EGR-1*) transcription factors to upregulate transcription of $p15^{ink4b}$. Interestingly, using the MatInspector tool, two putative *EGR-1* binding sites are identified, which coincide with the Sp1 binding sites at the -109 bp and -59 bp regions, and are highlighted in Figure 5.16c and d. Although the classical recognition sequence for a Snail/ E-Box binding site could not be found in both these regions, it is possible that Snail may be binding near one or both of these sites together

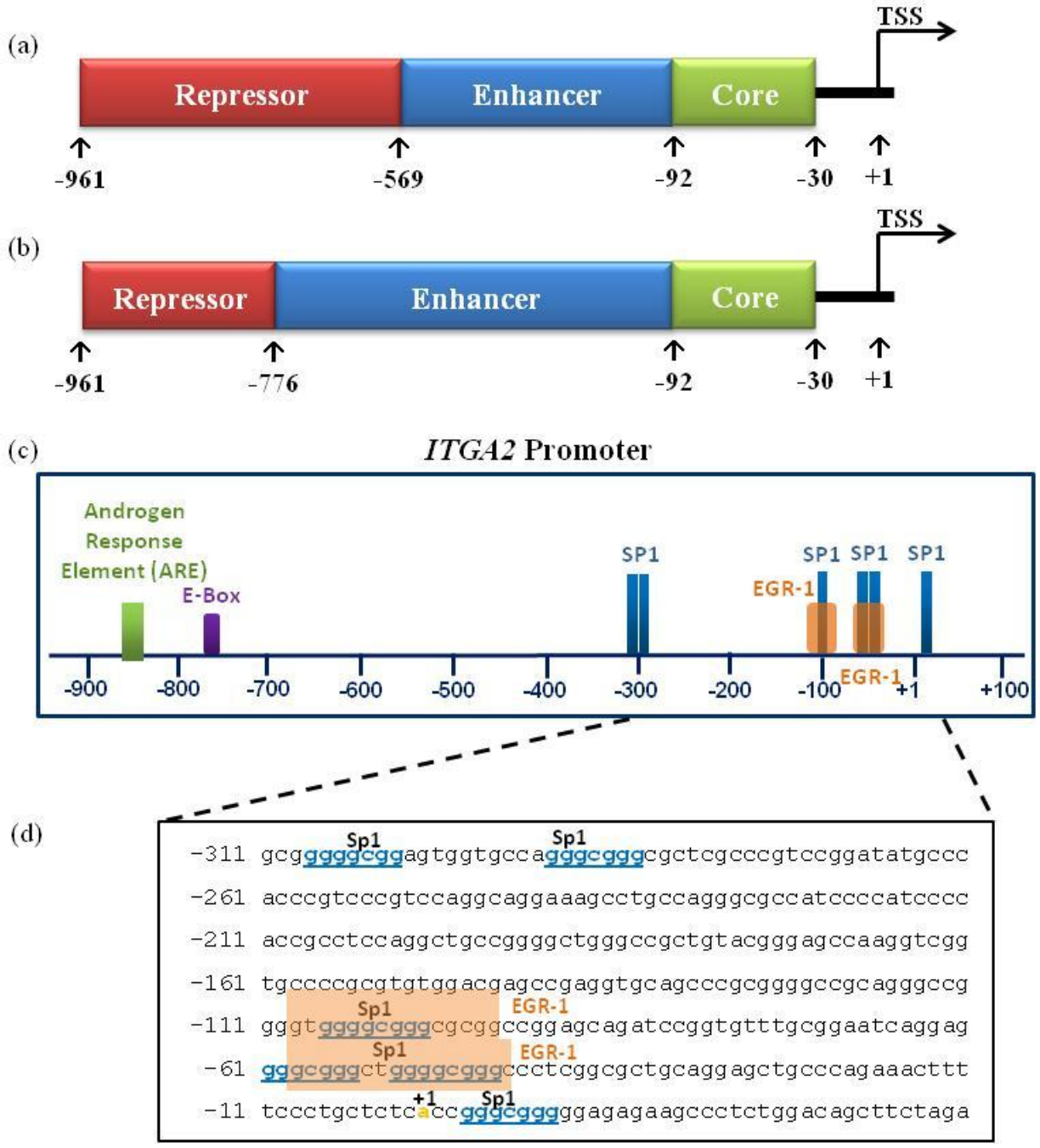


Figure 5.16 Schematic representation of *ITGA2* putative regulatory elements
Regulatory elements identified upstream of the TSS of the *ITGA2* gene in (a) the prostate cancer cell line, LNCaP and (b) the epithelial cell line, T47-D. A repressor region from -961 to -776 was identified in a breast cancer model and similarly a repressor region from -961 to -569 was identified using prostate cancer cells here. (c) Schematic representation of the positions of putative transcription factor binding sites in the *ITGA2* promoter, including the putative EGR-1 binding sites (orange) and (d) recognition sequences identified by the MatInspector tool, with the putative EGR-1 binding sites as shown in orange.

with the Sp1 and EGR-1 transcription factor and thus leading to the activation of *ITGA2* promoter activity when the repressor region is absent. Interestingly, Snail binding at the $p15^{ink4b}$ promoter was through a sequence element which did not match the classical recognition site.

In summary, Sp1 activates *ITGA2* promoter activity regardless of methylation and since it is expressed at equivalent levels in both LNCaP and PC3 cells, Sp1 may be regulating basal expression of *ITGA2* in these cells. Additionally, Snail is involved in the repression of *ITGA2* promoter activity and since endogenous Snail is expressed in higher levels by LNCaP cells, Snail may be responsible for the repression of *ITGA2* observed in LNCaP cells. Furthermore, androgens indirectly repress *ITGA2* promoter activity and several studies have shown that DHT can activate Snail (Zhu et al. 2010; Chen et al. 2006) and therefore androgen may be activating Snail leading to the repression in *ITGA2* promoter activity. Finally, Twist displayed inconsistent activity on the *ITGA2* promoter and thus suggests that it could be regulating other genes which indirectly affect *ITGA2* promoter activity.

Future studies to further verify the mechanism of Snail regulation of the *ITGA2* promoter can be carried out using more extensive bioinformatics analysis of the *ITGA2* promoter using the ever expanding gene expression, SNP and ChIP databases now available. In addition, methods such as DNase 1 footprinting and electromobility shift assays (EMSAs) could be used to further characterise the *ITGA2* promoter.

Chapter 6
FINAL DISCUSSION AND FUTURE DIRECTIONS

Bone metastasis is the major cause of prostate cancer related death (Bubendorf et al. 2000). There is an increasing body of evidence suggesting that the *ITGA2* gene is involved in prostate cancer progression, particularly mediating the preferential metastasis of prostate cancer cells to the bone (Hall et al. 2006; Hall et al. 2008; Sottnik et al. 2012). In addition, differential levels of *ITGA2* expression have been reported in prostate cancer tissue samples from different stages of disease thus, suggesting that altered expression of *ITGA2* may contribute to the progression of prostate cancer. This gene has also been identified as a putative prostate cancer susceptibility gene through a previous familial genetic study (FitzGerald et al. 2009). *ITGA2* is then a rational therapeutic target in prostate cancer metastasis. However, little is known about the regulation of the *ITGA2* gene and how this gene is dysregulated during tumorigenesis. The data presented here expands our understanding of the regulatory mechanisms that control *ITGA2* gene expression in prostate cancer.

A panel of prostate cell lines were used in this study, with the cells displaying different migration capacity and tumorigenicity. These cells displayed differential *ITGA2* expression with high expression observed in the benign cell line, PWR-1e and bone metastatic cell line PC3 while lower expression was observed in the non-tumorigenic cell line, LNCaP. Although other studies suggest that high expression of *ITGA2* is associated with increased prostate cancer cell metastasis, high *ITGA2* expression would also be expected in normal tissue as integrins are involved in cell growth and cell attachment and high integrin expression is associated with maintenance of cell-cell interactions and tissue organisation. Consistent with this, Bonkhoff et al. (1993) found *ITGA2* to be expressed in normal and benign prostate tissue samples. The lower *ITGA2* expression in LNCaP cells and high expression in PC3 cells is consistent with other studies where decreased *ITGA2* expression was observed in soft tissue metastasis while high expression was observed in skeletal tissue metastasis (Ramirez et al. 2011; Sottnik et al. 2012; Van Slambrouck et al. 2009) and also normal prostate tissue (Ramirez et al. 2011). Therefore, while high

expression of *ITGA2* is important for prostate cell growth, downregulation during prostate cancer progression may facilitate cell detachment and tumour escape from the primary site. Re-expression of this gene may then facilitate tumour cell relocation and particularly the preferential binding of prostate cancer cells to the bone.

Gaining a thorough understanding of the mechanisms underlying differential expression of the *ITGA2* gene and the regulation of this gene is therefore of warranted. The *ITGA2* promoter contains a 700 bp CpG island 5' to the TSS. Examination of this region using bisulphite sequencing showed differential methylation patterns, which inversely correlated with the expression levels in these cells. LNCaP cells displayed high levels of methylation at the promoter, correlating with lower *ITGA2* gene expression while PC3 cells displayed low levels of methylation, correlating with higher *ITGA2* expression. Increased chromatin accessibility and histone H3 acetylation at the *ITGA2* promoter also correlated with increased *ITGA2* expression as observed in PC3 cells. Interestingly, LNCaP cells and 22Rv1 cells displayed similar levels of methylation but opposing methylation patterns at the *ITGA2* promoter with 22Rv1 cells displaying methylation closer to the TSS and expressing higher *ITGA2* as compared to LNCaP cells. Further examination using the NoMe-seq assay showed that methylation observed in LNCaP cells is a monoallelic effect and the non-methylated clones were occupied by nucleosomes. Therefore, the higher percentage of nucleosome occupancy at the TSS may account for the lower *ITGA2* expression in LNCaP cells while a lower percentage of nucleosome occupancy at the TSS in 22Rv1 may account for the higher *ITGA2* expression.

Taken together, these results are suggestive that epigenetic factors involving DNA methylation, histone acetylation and nucleosome occupancy are involved in regulation of *ITGA2* expression. Changes in epigenetic modifications are increasingly being associated with prostate cancer but unlike genetic mutations, epigenetic alterations are potentially reversible. 5-azacytidine and 5-aza-2'-deoxycytidine (Figure 1.3) are the most clinically advanced DNMTi and have been examined extensively in clinical trials in a range of cancers, with 5-aza-2'-deoxycytidine receiving US Food and Drug Administration (FDA)

approval for the treatment of myelodysplastic syndrome (Kaminskas et al. 2005). However, a small number of clinical trials conducted in individuals diagnosed with prostate cancer suggest these inhibitors may have more limited use in the treatment of this cancer. Although the *in vitro* studies of nucleoside inhibitors have been successful in restoring silenced gene expression in prostate cancer cell lines, as reviewed in Chin et al. (2011), a phase II study of 5-aza-2'-deoxycytidine conducted in 14 men with androgen independent metastatic prostate cancer had limited success with only 2 of the 12 individuals displaying stable disease with delayed time to progression (Thibault et al. 1998). This study concluded that while 5-aza-2'-deoxycytidine is well-tolerated it has only moderate effects on hormone-independent prostate cancer. Further, although these inhibitors may reverse aberrant DNA hypermethylation, they act through nonspecific mechanisms and have previously been found to have side effects such as myelosuppression (Appleton et al. 2007; Batty et al. 2010; Cashen et al. 2008; Chuang et al. 2010; Kantarjian et al. 2003; Schrump et al. 2006), tumorigenesis (Hamm et al. 2009; Schnekenburger et al. 2011; Walker et al. 1986) and mutagenesis (Jackson-Grusby et al. 1997; Lavelle et al. 2007; Perry et al. 1992; Sauntharajah et al. 2003), which may limit their usefulness as a therapeutic drug in prostate cancer. In addition, remethylation occurs upon withdrawal of this drug, suggesting a continual need for administration (Egger et al. 2004). While agents that inhibit DNA methylation are being actively investigated as potential therapeutic agents in prostate cancer, one issue that also needs to be considered is their potential to hypomethylate and therefore upregulate genes involved in metastasis.

LNCaP and 22Rv1 cells were treated with the DMNTi, 5-Aza-2'-deoxycytidine (AzaC) and/ or in combination with the HDACi, Trichostatin A (TSA). Interestingly, treatment with AzaC alone or TSA alone did not affect *ITGA2* expression but, the combination treatment of both resulted in upregulation of *ITGA2* in the lower expressing cell line, LNCaP. Therefore, treating prostate cancer with these epigenetic modifiers may actually be upregulating genes that are involved in mediating tumour cell metastases. Nonetheless, the upregulation resulting from AzaC treatment was not as high as the *ITGA2* expression observed in PC3 cells. In addition, cells that were selected for increased *ITGA2* expression ($LNCaP_{col}$) by serial passaging of cells on collagen showed

only some demethylation at specific regions however, whether there are changes in nucleosome occupancy remains to be examined. Overall, these data suggest that while epigenetic factors contribute to *ITGA2* regulation in prostate cancer, other factors not surprisingly also contribute to the regulation of the *ITGA2* gene.

Cell migration assays have shown a correlation between *ITGA2* expression and cell migration capacity with higher expression of *ITGA2* correlating with increased cell migration. EMT has been found to be associated with several tumour metastases including prostate cancer (Alexander et al. 2006; Thompson et al. 2005; Tomita et al. 2000). In breast cancer, classification of breast cancer cell lines based on the expression profile of 24 gene products was shown to be predictive of their invasive and migratory properties (Zajchowski et al. 2001). Cell lines with an epithelial-like phenotype were found to be noninvasive while a mesenchymal-like phenotype was associated with invasive, more motile, more tumorigenic and more metastatic cell lines. In keeping with this, examination of EMT markers in prostate cancer cell lines in this present study showed that an epithelial-like phenotype correlates with low or no cell migration with low levels of *ITGA2* as observed in LNCaP while mesenchymal-like phenotype correlated with high cell migration capacity with high levels of *ITGA2* as observed in PC3 cells. Therefore, these EMT-like phenotypes correlated with heightened metastatic potential in prostate cancer. Knockdown of *ITGA2* expression using siRNA impaired cell migration potential. However, no significant changes in EMT marker expression was detected and while this suggests that this change in cell migration driven by *ITGA2* changes does not involve EMT changes, it may also be due to only the 'invasive front' cells showing changes in EMT-like signatures, which are then not detectable when examining expression in the entire population of cells. Nonetheless, collagen binding variant $LNCaP_{col}$ cells that have been shown by other studies to have increased collagen mediated migration and bone tumour growth capabilities (Hall et al. 2006; Sottnik et al. 2012), displayed increased *ITGA2* expression which correlated with lower Snail and higher Twist expression.

It is well described that epigenetic factors and transcription factors cooperate to regulate gene expression (Li 2002; Reik 2007; Struhl 1998). DNA methylation can directly inhibit the binding of methylation sensitive transcription factors while histone modifications affect chromatin organisation and thus, modulate accessibility of DNA to transcription factors. Putative transcription factor binding sites in the *ITGA2* promoter were identified using the MatInspector bioinfomatics tool. Six Sp1 binding sites are present in the *ITGA2* promoter and Sp1 activated an *ITGA2* promoter reporter in both LNCaP and PC3 cells. However, since the Sp1 transcription factor is endogenously expressed at equivalent levels in both cell lines, this transcription factor can not account for the differential *ITGA2* expression observed in these cells. Therefore, consistent with the role determined for Sp1 in megakaryocytic cells (Jacquelin et al. 2001), Sp1 may be involved in the basal expression of *ITGA2* in prostate cells. In addition, although one out of six of these Sp1 sites were differentially methylated across the cell lines, the reporter data presented confirmed that methylation does not affect the ability of Sp1 to activate the *ITGA2* promoter.

While androgen treatment was shown to repress *ITGA2* expression, this was found to be an indirect effect. Snail, a transcription factor involved in EMT was also found to repress *ITGA2* promoter activity with deletion mutant analysis strongly suggesting that the -791 to -569 bp region of the *ITGA2* promoter contains a repressor element and is required for the Snail repressive effect. Interestingly, LNCaP cells express higher levels of endogenous Snail as compared to PC3 and thus, this higher expression of Snail may be responsible for the lower expression of the *ITGA2* gene in LNCaP cells, and the data presented suggest that this is a direct effect potentially through a putative E-box located at -759 bp although this remains to be confirmed by site directed mutagenesis.

Snail activation triggers EMT with epithelial cells converted into mesenchymal type cells through direct repression of E-cadherin. Interestingly, investigation of the endogenous E-cadherin promoter in Snail-expressing cells showed enrichment in deacetylated H3/H4 and H3K9me2 and thus, repression of E-cadherin by Snail involve HDAC activity which was subsequently shown to be reversed by TSA treatment (Peinado et al. 2004). The

recruitment of HDAC1, HDAC2 and the corepressor mSin3A have been found to be required for the repression of E-cadherin by Snail. Further, a microarray study showed 167 genes which were repressed by Snail while only 23 genes were upregulated when Snail was overexpressed. These data suggest that Snail mainly acts as a transcriptional repressor by repressing genes that are involved in epithelial phenotype, signalling and metabolism (De Craene et al. 2005). Further analysis of a subset of potential Snail target genes showed the presence of E-boxes at the gene promoters and ChIP analysis confirmed the binding of Snail at these gene promoters.

Other studies have shown that androgens can activate Snail expression (Chen et al. 2006; Zhu et al. 2010a). Since androgen was found to repress *ITGA2* expression through an indirect effect, and given that androgen is usually an activator (Chen et al. 2000; Cleutjens et al. 1997; Murtha et al. 1993; Wang et al. 1999), androgen may be activating Snail expression and thus, leading to the repression of *ITGA2* expression observed in this study. In addition, while in the present study Sp1 was able to partially relieve the repressive effect of Snail, the higher endogenous Snail expression may be responsible for the downregulation of *ITGA2* expression in LNCaP cells.

Taken together the data presented here from analysis of prostate cancer cell lines is consistent with a model in which the Sp1 transcription factor binds to the *ITGA2* promoter, regardless of methylation and is responsible for basal *ITGA2* expression. In contrast, the Snail transcription factor represses *ITGA2* promoter activity and androgen leads to the activation of Snail expression and thus, further represses *ITGA2* promoter activity, resulting in lower *ITGA2* gene expression as observed in LNCaP cells. In addition, the higher DNA methylation as observed in LNCaP cells, consistent with a higher percentage of nucleosome occupancy at the TSS and low levels of histone H3 acetylation at the *ITGA2* promoter may be responsible for the lower *ITGA2* expression. This low *ITGA2* expression is consistent with little or no cell migration potential. Therefore, data from this study broadens our understanding of how epigenetic factors cooperate with transcription factors to regulate *ITGA2* gene expression in the different prostate cancer cell lines, resulting in different cell migration capacity.

This model can now be expanded upon (Figure 6.1). High *ITGA2* expression observed in normal prostate cells may be due to low DNA methylation and regulation by the Sp1 transcription factor (Figure 6.1a). In early stage prostate cancer, the lower expression of the *ITGA2* gene reported may be due increased DNA methylation, decreased H3 acetylation and lower chromatin accessibility at the promoter, correlating with a higher percentage of nucleosome occupancy at the TSS (Figure 6.1b). Although Sp1 activity is not affected by methylation and thus can still activate *ITGA2* promoter activity, these early stage tumour cells are androgen responsive and therefore, the presence of androgen may activate Snail expression leading to the repression of *ITGA2* expression. In contrast, in late stage prostate cancer (bone metastatic prostate cancer), upregulation of *ITGA2* expression has been observed and this may be due to the lower DNA methylation and higher chromatin accessibility correlating with higher histone H3 acetylation at the promoter (Figure 6.1c). Since late stage prostate cancer cells are androgen independent, Snail expression may be reduced relieving repression of *ITGA2* expression. However, in this model what remains to be determined are the triggering events that lead to prostate cancer progression. For example it is currently not clear whether epigenetic changes, such as those observed at the *ITGA2* gene promoter, are a cause or consequence of the progression of prostate cancer.

Elucidating the regulation mechanism of the *ITGA2* gene in prostate cancer as presented in this study may open new perspectives and possibilities to explore *ITGA2* as a therapeutic target to prevent prostate cancer bone metastasis. Future work involving integration of DNase I hypersensitive sites sequencing (DNase-seq), RNA sequencing (RNA-seq), ChIP sequencing (ChIP-seq) and motif analysis may further elucidate the epigenetic platform and transcriptional regulation mechanism at this promoter which will allow the development of a more specific and targeted therapy for prostate cancer treatment.

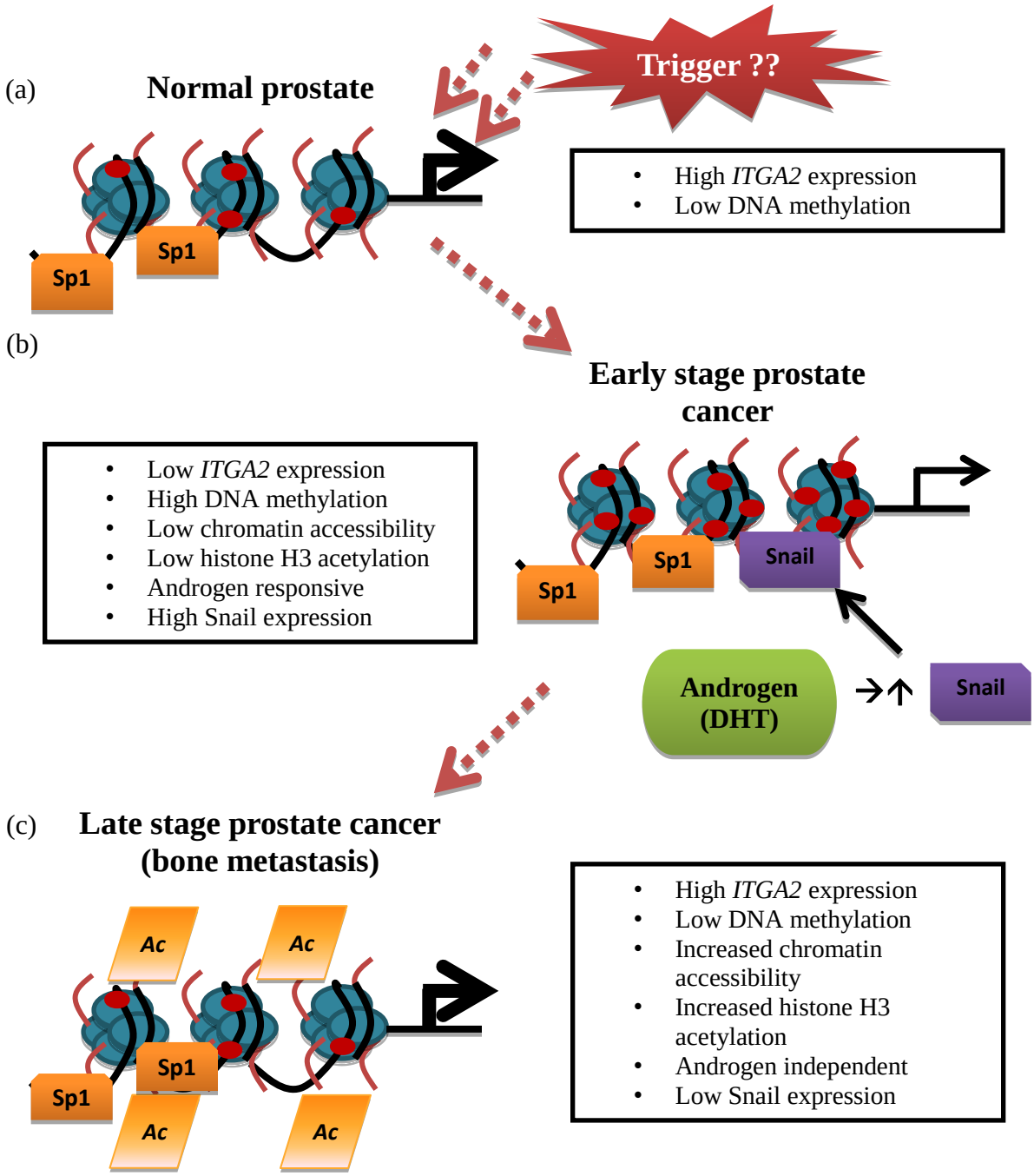


Figure 6.1 Model of regulation of the *ITGA2* promoter by epigenetic factors and transcription factors in different stages of prostate cancer.

(a) In the normal prostate, the *ITGA2* promoter is marked by relatively low levels of DNA methylation and Sp1 binding, resulting in high expression of *ITGA2*. (b) In early stage prostate cancer, the *ITGA2* promoter is marked by high DNA methylation (red circles), low histone H3 acetylation, Sp1 binding and Snail binding, resulting in repression of *ITGA2* gene expression. Androgen activates Snail which can further repress *ITGA2* gene expression. (c) In late stage prostate cancer, the *ITGA2* promoter is marked by relatively low levels of DNA methylation, high histone H3 acetylation and Sp1 binding, resulting in permissive state and thus, allowing high expression of *ITGA2*. However, the factors and events that trigger the progression of prostate cancer in this model are not clear.

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