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Integrins as Determinants of Genetic Susceptibility, Tumour Behaviour and Their Potential as Therapeutic Targets

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1. Introduction

Metastatic prostate tumours are responsible for the majority of deaths associated with prostate cancer. The most frequent site of prostate cancer metastasis is to bone; over 80% of men who die of the disease have metastatic bony lesions (Bubendorf et al., 2000). Early detection of prostate cancer remains crucial to effective treatment. However, we are still unable to identify with certainty those tumours requiring aggressive and immediate interventions, which are associated with considerable morbidity, from those where a "watchful waiting" approach may be more appropriate. Currently little is known about inherited determinants of an individual's propensity to develop tumours that rapidly progress and metastasise (Hunter, 2006). Experimental evidence investigating the role of integrins in tumourigenesis suggests that they play important roles in tumour progression and metastasis, particularly the development of metastatic lesions in bone. Notably, recent evidence indicates that genetic variants in selected integrins influence risk and/or prostate tumour behaviour.

The integrins represent a large family of cell surface receptors that are responsible for cell to cell adhesion and complex formation with ligands found within the extracellular matrix (ECM). The integrins have important roles in cell proliferation, cell survival, differentiation and cell migration. Thus, these adhesion receptors play important roles in many physiological processes including normal organ development and wound healing. Integrins are obligate, noncovalent, heterodimeric molecules, composed of a larger α subunit and a smaller β subunit. Each of the subunits extends from within the cytoplasm into the ECM. The ECM tail of the α and β subunits are approximately 700-1000 amino acid residues in length, and the cell membrane is spanned by approximately 20-24 residues. Integrin subunits have a short cytoplasmic tail, approximately 15-58 residues in length, with the exception of β_4 which has a cytoplasmic tail approximately 1000 residues (Alghisi and Ruegg, 2006). Within the cytosol they are attached to the cytoskeleton via talin-actin microfilaments. A detailed review on integrin structure can be found in the following publications (Buckley et al., 1999; Humphries et al., 2003; Ivaska and Heino, 2000; Lu et al., 2008). The conservation of integrin structure from more primitive through to higher order organisms highlights their importance to multicellular organisms (Burke, 1999). Upon

binding to extracellular ligands, the integrin molecules cluster at the membrane, permitting a focal cellular response and the transduction of signals from the ECM. Further, integrins respond to intracellular signalling resulting in conformational changes to the integrin molecules on the surface of cells, thus permitting modification of interactions with the ECM. Integrin signalling thus permits cells to be exquisitely sensitive to both extracellular ("outside-in") and intracellular ("inside-out") signalling (Burridge et al., 1988).

To date, 24 heterodimers have been identified, derived from 18 α and eight β subunits. Additional variation is provided by subunit 'variants' created by alternate mRNA splicing events. Selected integrins can also form multiple heterodimers; for example, α_v, β_2 and β_1 all form multiple αβ heterodimers (5, 4 and 12 respectively). Most, however, only form one or two heterodimers (Ivaska and Heino, 2000). There is promiscuity in integrin-ligand binding which may reflect the need to initiate different cellular processes using the same available ECM proteins. The ability of integrins to bind to multiple ligands is thought to be an advantage when elicitation of the response is more important than the ECM protein signalling it; for example, in cell migration and wound healing (Alghisi and Ruegg, 2006). The interaction of integrins with these ligands appears to be dependent upon signal transduction via the cytoplasmic tails to the ECM which is evoked by the focal adhesion kinase (FAK) pathway, although the exact mechanism regulating this process remains unclear. It is not surprising, then, that attempts to understand the mechanisms that underlie cell motility and adhesion have implicated the membrane spanning integrins as major regulatory molecules. Cell migration and motility are crucial to maintain and promote healthy cell development, wound healing and immunity. They are complex processes requiring tightly regulated and coordinated intra-cellular signal transduction with the ECM. Instances where uncontrolled cell adhesion and motility remain unchecked can lead to tumourigenesis and metastases (Wehrle-Haller and Imhof, 2003).

Given the physiological functions of integrins, it is not surprising that the role of integrins in neoplasms has been of intense interest over the past decade. Most pertinent to tumour development is the role of integrins in cellular processes, including cell survival and proliferation, cell migration, angiogenesis and lymphangiogenesis. Integrins are now known to play a key role in tumour biology and are implicated in tumour initiation, progression and metastasis. Aberrant expression of integrins also has been demonstrated to play a key role in cancer survival and proliferation. Integrins physically interact with the actin cytoskeleton which provides the traction required for migration to occur (Vicente-Manzanares et al., 2009). Integrin mediated matrix metalloproteinase (MMP) feedback systems control the degradation of ECM proteins. Importantly integrins play an important role in regulation of neovascularisation, required for metastases via the vascular endothelial growth factor (VEGF) pathway.

Much work has focussed on profiling changes in the expression of integrins at different stages of tumour development. In normal prostate tissue, integrins are expressed by normal basal epithelial cells, permitting interaction with the basal lamina comprising collagens, laminins, fibronectin, vitronectin and tenascin. During prostate tumour development, many studies report the overall down-regulation of both α and β integrin subunits. However, prostate tumourigenesis proceeds through defined stages of development from prostatic intraepithelial neoplasia (PIN), high-grade PIN lesions, prostate confined tumour, invasive tumour and finally to androgen-independent tumours. More rigorous examination of the integrin profiles at different stages of tumour development has revealed a more complex expression profile (Goel et al., 2008). Much research effort has focused on the identification

of an integrin expression profile as a prognostic marker for prostate cancer. In particular, integrins from the α_v group and in particular $\alpha_v\beta_3$ have been previously identified as a putative prognostic marker for a number of cancers including prostate cancer and its utility as a therapeutic target is under clinical trial (Beekman et al., 2006; Chen et al., 2008; McNeel et al., 2005). It is beyond the scope of this review to provide a detailed analysis of the role of all integrins in prostate tumour development. Overall reviews are available which profile integrin expression in prostate cancer (Alghisi and Ruegg, 2006; Goel et al., 2009; Goel et al., 2008; Lu et al., 2008).

The advent of new high-throughput genotyping technologies has permitted rapid and significant advances in our understanding of the genes contributing to prostate cancer. In particular, genome wide association studies (GWAS) have permitted the identification of common genetic susceptibility variants associated with a significantly increased risk of developing the disease. These discoveries are now providing insight into the biological pathways that determine how disease arises, tumour progression, and the acquired propensity to metastasise. Whilst the role of the vast majority of these variants in prostate cancer remains to be determined, these studies have identified biological pathways important in the development of disease. The integrin family of cell adhesion molecules has recently featured both in the search for prostate cancer susceptibility genes, and in comparative analyses of genetic susceptibility variants and gene expression profiling studies of prostate tumours. Further, studies using a variety of approaches to identify the key drivers of prostate tumour development and progression have identified selected integrins as key molecules in these processes. Indeed, these studies have highlighted integrins as potential therapeutic options against prostate cancer. Here we provide a new perspective on the role of the integrins α_2, α_6 and β_4 in prostate cancer risk and progression. Whilst these receptor subunits have not previously featured prominently in prostate tumour biology, their role in prostate tumour development has more recently come to the fore through the application of next generation molecular techniques to gene discovery in prostate cancer and prostate tumour stem cell studies.

2. Integrin genetic variants and prostate cancer

Family history remains the most frequently identified risk factor for developing prostate cancer, in addition to advancing age. A family history of disease is indicative of an underlying inherited genetic predisposition. Indeed, population studies have repeatedly identified increased risk to relatives of those diagnosed with this cancer. Despite intensive research over more than a decade, the genetic contributors to prostate cancer remained poorly understood. It was not until large scale genome-wide association studies became possible utilising high-throughput genotyping with commercially available arrays that rapid advances in our knowledge of the genetic contributors to prostate cancer and indeed other complex diseases became possible. These advances permitted the genotyping of hundreds of thousands of single nucleotide polymorphisms (SNPs) to test for association with a disease in large numbers of cases and controls. Using this approach, over 30 prostate cancer susceptibility variants have now been identified. These studies have identified genetic variants both in known genes and intergenic regions associated with risk of disease. However, it has become clear that for only for a select few is the role of the variant identified determining prostate cancer risk apparent. For example, the significantly associated variant, rs10993994, in the MSMB1 gene (Eeles et al., 2008) is located within 2bp of transcription

initiation site, and has been demonstrated to influence transcriptional activity (Chang et al., 2009). However, to date the functionality of the vast majority of the SNPs identified, many of which are not within or close to known genes, remains unknown. Thus, there are still significant gaps in our understanding of genetic contributors to prostate cancer susceptibility which is impeding translation of these exciting advances into the clinical setting.

Variants within the *ITGA6* gene, which codes for the α_6 integrins, has recently been identified as contributing to the genetic risk of prostate cancer. A large, multi-stage international collaborative GWAS utilising over 30,000 cases and controls has identified seven novel prostate cancer susceptibility loci. One variant identified as significantly associated with disease risk is in intron one of the *ITGA6* gene (Eeles et al., 2009). The SNP identified was rs12621278 on chromosome 2q31 (per allele odds ratio=0.75, confidence interval=95% $P=8.7x10^{-23}$) (Eeles et al., 2009). Most interestingly, Cheng et al., (2010) have since screened 26 SNPs identified by previous GWAS studies (Duggan et al., 2007; Eeles et al., 2009; Eeles et al., 2008; Gudmundsson et al., 2009; Gudmundsson et al., 2007; Gudmundsson et al., 2008; Thomas et al., 2008) in 788 patients that had undergone radical prostatectomies to test for association with aggressive cancer. Five of these SNPs were independently associated with prostate cancer progression; however, the most strongly associated risk variant identified was in *ITGA6* (rs12621278), where a 2.4 fold increase risk of prostate cancer progression (P=0.0003) was reported to be associated with the risk allele. This integrin is also known to play an important role in prostate cancer stem cell biology, vascularisation and metastasis. Thus, given the known role of this integrin in prostate tumour development, this may represent an opportunity for targeted therapy in those cases with *ITGA6* associated genetic susceptibility.

By employing a genome wide linkage approach in the study of familial prostate cancer, we have identified a second integrin as significantly associated with prostate cancer risk (FitzGerald et al., 2009). A region on chromosome 5p13q12 was highlighted by genetic linkage analysis of a large family with multiple, densely aggregated cases of prostate cancer. Two polymorphisms were subsequently found to be significantly associated with prostate cancer risk. The SNP rs3212649 was identified within the three prime untranslated region (3′-UTR), and a second polymorphism, rs1126643 (C807T), located in exon seven. Variant rs3212649 was the most strongly associated with disease in the familial cases (OR=2.43 CI=1.28-4.58) but also in the combined dataset comprising both sporadic and familial cases (OR=1.67 CI=1.07-2.60). This SNP had not been previously reported to be associated with cancer risk. The C807T SNP was also significantly associated with increased prostate cancer risk both in the familial (OR=2.16 CI=1.19-3.92) and combined datasets (OR=1.52 CI=1.01-2.28). Furthermore, the C807T variant has also been previously associated with an increased risk of oral cancers, and also advanced breast cancer (Langsenlehner et al., 2006; Vairaktaris et al., 2006). Whilst presence of the alternate allele at C807T does not alter amino acid sequence, it has been reported to be associated with altered levels of expression of the α_2 receptor on the cell surface (Jacquelin, 2001). It should be noted that there exists significant linkage disequilibrium between rs3212649 and rs1126643 and a number of other SNPs located in the 3′-UTR of the ITGA2 gene. ITGA2 is also known to play an important role in prostate cancer stem cells, angiogenesis and tumour spread and also represents an exciting opportunity for target therapy. The mechanism by which these susceptibility variants influence *ITGA2* gene expression is currently under investigation in our laboratory.

Whilst these GWAS are identifying genetic variants significantly associated with disease risk and progression, the functional implications of many of these variants and their relevance to disease remains to be determined (Manolio, 2010). Thus, the results of GWAS have been examined in combination with microarray gene expression data, providing a systematic approach to the examination of genetic variants associated with disease and observed changes in gene expression. Gorlov et al. (2009) performed a meta-analysis on gene expression data from normal prostate tissue and prostate tumour tissue, and combined it with a GWAS meta-analysis to identify sets of genes over-represented in both types of studies. Cell adhesion genes including α_2, α_6 and β_4 integrins feature prominently amongst the most significantly associated genes and also the most differentially expressed reported in the microarray study (Gorlov et al., 2009). Gorlov and colleagues (2009) also highlight cadherins and integrins as important modulators of prostate cancer development and further argue that whilst GWAS-identified genes are considered to be cancer susceptibility genes associated with tumour initiation, the detection of a tumour requires a minimum tumour size and thus those identified from GWAS are also likely be detecting variants association with tumour progression.

3. Integrin α_2 and α_6 in stem cells

The concept that most solid tumours, including prostate cancer, arise from cancer stem cells possessing the capacity for self-renewal and tumour initiating capacity has led to the search for tumour cell populations with "stem-cell like" properties. Collins et al. (2001) were the first to demonstrate a primary human prostate tumour cell subpopulation with the capacity for self renewal and high clonogenic potential. These cell populations were characterised by high expression of CD44, $\alpha_2\beta_1$, and CD133. Subsequent studies have also identified prostate cancer stem cell populations (Collins, 2005; Guzman-Ramirez et al., 2009; Li, 2008). Guzman-Ramirez et al., (2009) recently isolated cells from surgically excised human prostate tumour tissue and culture of these tumour derived cell populations termed "prostaspheres" resulted in sub-populations expressing high levels of both the α_2 and α_6 integrins. Furthermore, Eaton et al. (2010) have examined the tumour cell stem cell phenotype in 11 matched primary and bone metastasis specimens from prostate cancer patients for the presence of a number of putative stem cell markers. No definitive pattern was observed which established a single marker profile of the metastatic phenotype; integrins α_2 and α_6 were relatively widely expressed across the metastases series in nine of eleven tumours examined. Other groups have since characterised prostate cancer stem cell populations expressing stem cell markers including *OCT3/4, BMI1, β-catenin* and *SMOOTHENED* in addition to α_2 and α_6 integrins. Most recently, several studies have identified a link between prostate cancer stem cells and epithelial to mesenchymal transition (EMT) demonstrating the link between the biology of prostate cancer stem cells, EMT and the propensity to metastasise (Kong et al., 2010; Mathews et al., 2010). EMT occurs during normal embryonic development; however, more recently, it has been identified as one of the early stages in the transition from confined tumours to invasive malignancies. The loss of epithelial cell phenotype and the gain of mesenchymal phenotype is accompanied by increased cell motility and invasiveness. This is accompanied by the loss of markers of epithelial phenotype and gain of mesenchymal markers. Profiling of expression of α_2 and α_6 integrin expression and EMT in prostate tumours has only recently been examined (Neal et al., 2011). *SNAIL*, a key

factor promoting EMT decreases cell adhesion and increases α_2 and β_1 expression (Neal et al., 2011). However, there is evidence that known regulators of EMT, for example *ZEB1* influence expression of *ITGB4* (Drake et al., 2010), and down-regulation of $\alpha_6\beta_4$ in the prostate cancer cell line *SNAI2* knockdown studies in PC3 cells (Emadi Baygi et al., 2010) However, EMT in prostate cancer remains controversial.

4. Expression profiles of α_2 and α_6 integrins

Investigations of integrin expression patterns in prostate tumours stratified by tumour stage have produced inconsistent findings. Studies of $\alpha_2\beta_1$ expression in tumours report that expression patterns are abnormal in both primary tumours and lymph node metastases (Pontes-Junior et al., 2009). Whilst there have been several apparently conflicting reports, it appears that normal prostate basal epithelium expresses high levels of α_2 which is down regulated in primary tumours, with conflicting results in metastases. For example, studies of primary and metastatic prostate carcinomas have reported that $\alpha_2\beta_1$ expression is down-regulated in low grade tumours, heterozygous in intermediate grades, and up-regulated in lymph node metastases (Bonkhoff et al., 1993; Knox et al., 1994; Kostenuik et al. 1996). Mirtti et al., (2006) have reported differential expression levels in prostate tumour cell lines, and lower α_2 mRNA and protein expression in higher grade tumours when compared to benign lesions, although the levels of mRNA expression reported by Mirtti et al. (2006) again varied widely in tumours. Moreover, α_6 expression has been shown to be up-regulated in metastases and adenocarcinoma (Bonkhoff et al., 1993; Knox et al., 1994; Nagle et al., 1995; Schmelz et al., 2002), whereas α_2 has been shown to be down-regulated in adenocarcinomas and up-regulated in metastatic tumours (Bonkhoff et al., 1993; Nagle et al., 1995). This variable level of expression observed in prostate tumours is also evident in expression array analyses of $\alpha_2\beta_1$ in prostate tumours, published in the NCBI GEO database (Edgar R, 2002). Ramirez and colleagues (2011) combined previously published microarray studies (Lapointe et al., 2004; Tomlins et al., 2007) to identify that $\alpha_2\beta_1$ expression was markedly reduced or lost with tumour progression in prostate and breast cancer. In normal prostate tissue, α_2 is highly expressed and Ramirez et al., (2011) have shown that α_2 expression decreases progressively as prostate cancer proceeds from PIN lesions, to prostate cancer and then to metastatic prostate cancer where $\alpha_2\beta_1$ expression is completely absent. Ramirez et al. (2011) also suggests that $\alpha_2\beta_1$ may be a prognostic biomarker for identifying patients at risk of metastasis.

Using the profile of overall integrin expression as a predictive tool to measure patient outcomes has received some investigatory attention. Pontes-Junior et al. (2010) measured expression levels of eight integrins ($\alpha_2\beta_1$, $\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$, α_v, $\alpha_3\beta_1$, α_3, β_4 and α_6) in resected tumour tissue from patients with localised prostate cancer, and found that the expression level of integrins α_3 and $\alpha_3\beta_1$ were associated with poor outcomes after a follow up period of ten years (2.5 and 3 fold higher respectively). The majority of integrins were down-regulated in tumour tissue with the exception of α_6, consistent with previous studies (Cress et al., 1995; Edlund et al., 2001; Rabinovitz et al., 1995; Schmelz et al., 2002) where its ability to bind ligands such as laminins and collagens is associated with increased invasiveness and metastases to bone. Down-regulation of $\alpha_2\beta_1$ in the early stages of prostate and breast tumour development has been reported to be associated with poor outcomes, with up-regulation of this integrin in metastasis. Interestingly, increased expression has been

reported in colorectal cancer (CRC) and ovarian cancer to be associated with tumour cell spread (Luque-Garcia et al., 2010).

It should perhaps be noted that studies examining integrin expression profiles at different stages of tumour development vary in the integrin subunits targeted for immunohistochemical analysis, some targeting single subunits, others heterodimeric complexes. Edlund et al. (2001) observed a change from normal prostate tissue expressing $\alpha_6\beta_1$ and $\alpha_6\beta_4$ to tumour tissue expressing only $\alpha_6\beta_1$. This loss of the β_4 subunit in carcinomas of the prostate was attributed to differential binding caused by the two different isoforms of α_6 (α_{6A} and α_{6B}). Integrin α_{6A} which is expressed by the prostate cancer cell line LNCaP, preferentially binds to β_1, which is known to have high metastatic ability (Edlund et al., 2001). Therefore, examining changes in heterodimer ratios combined with isoform data may provide a more detailed profile of the α_2 and α_6 contribution to the prostate cancer biomarker model. The variable expression of these integrins in prostate tumour samples is perhaps reflective of the recognised heterogeneous nature of prostate tumours. Further, this observation is supported by the ability to derive sub-population cells expression high levels of α_2 and α_6 and with "stem cell-like" properties from prostate tumours expression. It is also worthwhile noting that examining the integrin expression profiles in tumours may not take into account individual genotype-driven variation in gene expression.

5. Animal models of prostate tumour metastasis and angiogenesis

Animal models have proven useful in the examination of the role of integrins in driving prostate tumour metastasis. In a study aiming to identify the critical molecules regulating prostate tumour metastasis, Hall et al. (2006) utilised LNCaP cells, a cell line of low tumourigenic potential that does not form metastases in mouse models. Selection by growth of LNCaP cells on type I collagen resulted in the generation of the derived LNCaPcol subline with enhanced chemotactic capacity. This subline expressed high levels of $\alpha_2\beta_1$ receptor (in contrast to the parent line) and chemotactic capacity was inhibited by $\alpha_2\beta_1$-specific antibodies. Further, upon injection of the derived LNCaPcol cell line into the tibia of nude mice, 53% of mice developed bony lesions compared with 0% of those injected with parental LNCaP cells. In similar studies, van Slambrouck et al. (2009) have demonstrated that the high bone metastatic potential of the subline, C4-2B, derived from LNCaP cells, is mediated by α_2 signalling. This signalling results in down-stream activation of the critical FAK/src/paxillin/Rac/JNK pathway and activation of metalloproteinases, MMP2 and MMP9, known to play a central role in tumour invasion. Furthermore, van Slambrouck et al. (2009) argue that it is redistribution and clustering of α_2 on the cell surface in their model that activates downstream signalling, rather than altered receptor level as observed by Hall et al. (2006). King et al. (2008) have also utilised a derivative of the prostate cancer cell line PC3 in a mouse xenograft model to demonstrate a role for α_6 in the growth of prostate tumours in bone. PC3N cells were transfected with wild-type and functional mutant α_6 subunits and stable transfectants were injected into femurs of severe combined immuno-deficient (SCID) mice. Whilst both wild-type and mutant transfected PC3N cells established bone tumours, the study showed the PC3N-α_6 mutant transfected cells showed dramatically reduced invasion of bone marrow and less tumour associated bone loss.

The $\alpha_2\beta_1$ receptor also plays a key role in developmental angiogenesis signalling via the VEGF pathway. In a Lewis lung carcinoma (LLC) xenografts model, $\alpha_2\beta_1$ null mice exhibited no response to angiostatic agents targeting $\alpha_2\beta_1$ in contrast to wild-type mice (Woodall et al., 2008). Interestingly, it appears that this effect may be tumour cell dependent, a phenomenon also reported by Zhang et al. (2008), with current evidence suggesting that tumour cell characteristics, such as $\alpha_2\beta_1$ integrin expression, and the interactions with the surrounding tissue environment, under the influence of host genetic factors, determine tumour development and/or metastasis. This is in keeping with the "soil determining the seed" hypothesis of tumour metastasis.

6. Epigenetic regulation of integrins α_2 and α_6

Events causing up-regulation or down-regulation of genes, whether they are DNA based sequence changes or epigenetic events can function to disrupt key genes in cancer development. It is now clear that epigenetic alterations feature prominently in abnormal growth states and there is strong evidence that these alterations can predict tumour behaviour. The apparent paucity of functional loci identified by GWAS and gene expression studies in prostate cancer has also led many research groups to focus on the epigenetic mechanisms influencing gene expression. Evidence that some epigenetic regulatory mechanisms are uniquely sensitive to environmental factors, such as diet and oxidative stress, may be an important mechanism by which environmental factors influence cancer risk (Dobosy et al., 2007). These mechanisms include DNA methylation, histone modification and micro RNAs (miRNA).

Segments of DNA rich in CpG motifs, also known as CpG islands, are the targets for DNA methylation. CpG islands, when present in gene promoters, are an important mechanism by which genes can be regulated by the addition or removal of methyl groups at key CpG motifs. This permits differential expression of genes in different cell types. DNA methylation occurs at cytosine-guanine repeats where a methyl group is attached to a cytosine residue by a DNA methyltransferase or DNMT. DNA methylation can affect gene transcription in two ways: either by preventing the transcriptional machinery from binding or by becoming targets for chromatin remodelling proteins. Global de-methylation or hypo-methylation has been reported in the promoter region of genes causing dysregulation and thus overexpression of genes including oncogenes (Ehrlich, 2009). Dysregulation of the genes that control for normal cellular function, such as integrins, can cause an over proliferation and thus tumour growth.

In disease states such as cancer, disrupted methylation can permit aberrant silencing or re-expression of key genes contributing to tumour development. Epigenetic alterations are a feature of both benign prostatic hyperplasia and prostate tumour development. There is a significant body of evidence highlighting the role of DNA methylation in prostate cancer. However, it is evident from these studies that these changes are complex, with both global loss of methylation across the genome occurring in addition to localised hyper-methylation of gene promoters. Global hypo-methylation is associated with chromosomal instability and activation of proto-oncogenes whilst gene-specific hyper-methylation frequently results in silencing of tumour suppressor genes. In particular, the research has focussed on the identification of a panel of genes that are hyper-methylated in prostate cancer with several key genes identified for example *GSTP1* (Dobosy et al., 2007). As discussed later in this chapter, therapies attempting to reverse silencing of tumour suppressor genes in cancer are of current interest.

However there is also some evidence suggesting that agents promoting de-methylation may actually enhance tumour development, by increasing the expression of genes promoting tumour development (Shukeir et al., 2006).

6.1 DNA methylation and chromatin remodelling

Whilst there has been significant advances in our knowledge of a range those genes altered by epigenetic changes in prostate cancer, at present studies examining epigenetic alteration of integrin gene expression and its role in prostate cancer are limited (Chen et al., 2009; Park et al., 2004; Uhm et al., 2010; Yang et al., 2009). In experiments utilising bisulphite sequencing of cloned DNA derived from prostate cancer cell lines conducted in our laboratory, we have observed that altered methylation of the *ITGA2* promoter is associated with altered gene expression (data not shown). At present, there is no evidence that the α_6 gene promoter is regulated by altered methylation. However, Yang et al. (2009) have reported the β_4 integrin is regulated by both methylation changes and histone modifications. Further, the loss of β_4 expression in mammary gland cells is associated with increased methylation of the β_4 promoter and an increase in repressive histone modifications and EMT (Yang et al., 2009). In addition, enzyme specific assays and bisulphite sequencing reveal that the LNCaP cell lines displayed higher methylation levels than PC3s and this is correlated with gene expression. Our results strongly suggest that *ITGA2* expression may in fact be regulated by epigenetic mechanisms (data not shown).

While yet to be reported in prostate cancer, another mechanism by which integrin epigenetics can regulate gene expression is by chromatin remodelling. Chen et al. (2009) report that integrin $\alpha_6\beta_4$ indirectly affects gene transcription by chromatin remodelling. Several genes including *FST, S100A4*, *NKx2.2*, *PDLIM4* and *CAPG* in which expression was previously shown to be controlled by DNA methylation were significantly up-regulated by the expression of $\alpha_6\beta_4$. In addition the inhibition of DNMTs in cells lacking the $\alpha_6\beta_4$ cell line stimulated the expression of these genes. These results suggest that $\alpha_6\beta_4$ can alter the expression of particular genes by stimulating de-methylation at their promoters. Further studies are required to characterise the role of epigenetic mechanisms in the regulation of gene expression of integrins in prostate cancer.

6.2 Micro RNAs

Perhaps the most recent and exciting area of epigenetic regulation in prostate cancer is the field of micro RNAs (miRNA). MiRNAs are small molecules typically 17-22bp in length which binds to the 3'-UTR and either degrade the mRNA directly or prevent it from being translated. This post transcriptional regulation is particularly dependent on the 'seed region' of the miRNA which constitutes the first 7bp-9bp of the miRNA. A number of software programs are available (DIANA, mictoT and PITA), which vary in their predictions depending on the parameters and algorithms used in their design. Polymorphisms within the seed region can alter whether a miRNA will bind or change the energy with which it will bind to the mRNA. These allele specific changes indicate that miRNAs can act as either oncomiRNAs or tumour suppressors, and array studies can contribute to increasing the precision with which tumours are characterised (Zhang et al., 2007). MiRNAs are normally associated with decreased levels of gene expression, as one of the established mechanisms of action is to suppress translation and promote degradation of their target mRNAs. However, up-regulation of genes following transfection of miRNAs predicted to bind to sequences in

their 3′-UTRs has been previously reported in a number of studies reviewed by Khan et al. (2009). The potential role of miRNAs in the regulation of integrins in breast cancer was examined by Brendle et al. (2008). In a novel approach, miRNA binding site predictive software was used to predict altered miRNA binding to the 3′-UTRs of several integrins at the location of known genetic variants within these regions. A miRNA binding site was predicted to be altered by the alternate allele at rs743354. Upon subsequent examination it was observed that this SNP within the 3′-UTR of *ITGB4* was significantly associated with oestrogen receptor status and survival in breast cancer patients. In an approach similar to that taken by Brendle et al. (2008), we have determined that several of the SNPs associated with prostate cancer risk in the 3′-UTR of the ITGA2 gene are located in miRNA binding sites. The presence of the alternate allele alters the binding affinity of the miRNA. Our current work is investigating the role of the specific miRNAs in ITGA2 gene regulation.

7. Integrin α_6 and α_2 in prostate cancer therapeutics

Integrins represent ideal therapeutic targets as they are cell surface receptors that interact with extracellular ligands (Lu et al., 2008). The notion of integrins as therapeutic targets has been explored over a number of years, and there are two main mechanisms by which they have been targeted. Integrins are potentially important diagnostic biomarkers for cancer detection and progression, as it has been particularly relevant for prostate cancer which is historically difficult to diagnose with the inherent variability associated with the prostate-specific antigen (PSA) test and associated risks that accompany the collection of biopsy tissue. Secondly, they continue to be targeted directly as a method of preventing oncogenesis. In particular, they have been targeted as therapeutic options for metastases, as they are key mediators of cell dissemination and tumour growth via the angiogenesis pathways. Several of these integrin based therapies have shown promise in late stage cancers, extending life expectancies by several months in some cases. Further, it is often in conjunction with other adjuvant therapies such as chemotherapy and radiotherapy that integrins show the most promise as therapeutic agents. As we move forward into an era of personalised medicines it seems likely that integrins will have a major role to play.

7.1 Antibody based therapies

Integrin-targeted therapies have been of interest for a number of years, however few have targeted α_6 and α_2 heterodimers, and even fewer have been examined in relation to prostate cancer. The majority of studies have focussed on preventing angiogenesis and thus tumour growth, with most examining the α_v heterodimers. However, recently, studies have begun to shift towards the role of α_2 and α_6 in cancer models (Table 1). One of the more promising studies that has undergone human trial utilised α_2 as a platelet biomarker for an anti-integrin drug E7820, a derivative of an aromatic sulphonamide compound (Funahashi et al., 2002). E7820 inhibits angiogenesis via the VEGF or basic fibroblast growth factor (bFGF) pathways, and has been shown to inhibit the α_2 mRNA transcription in human umbilical vein endothelial cell (HUVEC) cultures and, importantly for prostate cancer, it has also been shown to inhibit vascular formation in a type I collagen matrix (Semba et al., 2004). E7820 administered orally twice daily by mice with sub-cutaneous KP-1 tumours displayed a decreasing expression level of α_2 on platelets which was positively correlated with anti-tumour activity (Semba et al., 2004). Stage I trials of E7820 have been completed and stage II trials have commenced, examining the efficacy of E7820 in conjunction with Cetuximab (a

monoclonal antibody, targeting the epidermal growth factor receptor EGFR) in metastatic colorectal cancer (mCRC)(ClinicalTrials.gov, 2011b). To date, E7820 combined with Cetuximab, has been well tolerated in patients with advanced metastatic CRC. Integrin α_2 expression levels decreased by 82.1% without significant disruption of platelet function, with median progression-free survival increasing by 1.9 months and median overall survival increasing by 9.6 months (Sawyer, 2010). In addition, stage II trials of E7820 administered in conjunction with FOLFIRI (FOL-folinic acid, F-5-flourouracil and IRI-irinotecan), a traditional chemotherapeutic agent have also commenced in patients with mCRC. This study has begun with the premise of testing the tolerability and efficacy on mCRC for those who have failed first round treatment, before moving on to a larger cohort of patients (ClinicalTrials.gov, 2011a).

Integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ play a significant role in driving angiogenesis (Lu et al., 2008) and antibodies targeting the α_2 receptor have shown promising results *in vivo*. Anti α_2 antibody Ha1/29 inhibited endothelial cells in an immobilised collagen gradient assay by approximately 40% (Senger et al., 2002). In addition, Senger et al. (1997; 2002) also applied a combination of anti-$\alpha_1\beta_1$ and $\alpha_2\beta_1$ antibodies to mice harbouring the human A431 squamous cell xenografts which resulted in decreased tumour growth <60% and angiogenesis <40%. The inhibitory effect of anti-α_2 antibodies on endothelial cells in a collagen matrix is likely to be particularly pertinent to prostate cancer where 80% of metastases are to bone; where collagen, the most abundant ligand of α_2, is abundant. Alghisi and Ruegg (2006) suggest that a humanised form of Ha1/29 may be a useful anti-angiogenesis target, and this may be particularly relevant for prostate cancer. Furthermore, antibodies against α_2 have been shown to reduce the invasive capability of mouse mammary carcinoma cells across the basement membrane (Lochter et al., 1999). This was found to be regulated by the expression of the matrix metalloproteinase stromelysin-1. The relationship between MMPs and integrins has been well documented and may represent another avenue for targeted cancer therapy. Murine based anti-α_6 antibodies have also been utilised by Ruiz et al. (1993) who illustrated a lower ability for human melanoma cells to metastasise in nude mice. Metastases were inhibited when the antibody EA-1 was injected in to the mice either before or simultaneously with the melanoma cells (Ruiz et al., 1993).

7.2 RGD peptide / disintegrin based therapies

Integrin $\alpha_2\beta_1$ has been implicated in mediating the effects of peptides designed to target the matrix bound tumour associated protein, angiocidin. Angiocidin is a protein that is found in the sera of patients with melanoma, colon, prostate and breast cancer, in levels that correlate with the progression of the disease, indicating that angiocidin may regulate tumour progression (Gaurnier-Hausser et al., 2008). Disintegrins or RGD based peptides are small, soluble molecules that originate from viper venom toxin and target the RGD (arginine-glycine-aspartate) motif. A 20 amino acid N–terminal peptide disintegrin of angiocidin has been shown to bind to integrin $\alpha_2\beta_1$ in K562 cells (a myelogenous leukaemia cell line) and ligate type I collagen on epithelial and tumour cells in a mouse model (Sabherwal et al., 2006). Soluble peptides that bind to $\alpha_2\beta_1$ integrins at the RGD motif are therefore able to prevent cell attachment and thus induce apoptosis (Buckley et al., 1999). *In vitro* angiocidin-inhibitory peptide trials have been demonstrated to be well tolerated and to reduce cancer burden in murine colon cancer models, with reductions in primary tumour volume and tumour burden (Liebig et al., 2007).

Drug	Type	Target	Tumour/ cell type	Reference
E7820	Peptide	α_2	Broad spectrum anti-tumour activity in seven cell lines and murine models	(Funahashi et al., 2002), (Semba et al., 2004)
E7820 & cetuximab*	Peptide and monoclonal antibody	α_2	Metastatic colorectal cancer	(Sawyer, 2010), (ClinicalTrials.gov, 2011b)
E7820 & FOLFIRI*	Peptide and chemotherapeutic agent	α_2	Metastatic colorectal cancer	(ClinicalTrials.gov, 2011a)
Angiocidin	Disintegrin/ RGD based peptide	$\alpha_2\beta_1$	Myelogenous leukaemia cell line, murine colon cancer models	(Sabherwal et al., 2006), (Liebig et al., 2007)
HYD-1	D-amino acid peptide	$\alpha_6\beta_1$	DU145 prostate carcinoma cell line	(DeRoock et al., 2001), (Cheresh and Spiro, 1987)
Endorepellin	C-terminus of perlecan protein	α_2, $\alpha_2\beta_1$	Lewis lung carcinoma model, HT1080 and A431 cell lines	(Bix et al., 2007), (Woodall et al., 2008)
Perlecan	Heparan sulfate proteoglycan	$\alpha_2\beta_1$	C4-2B and HT1080 cell lines	(Savore et al., 2005), (Mathiak et al., 1997)
HA 1/29	Monoclonal antibody	α_2, $\alpha_2\beta_1$	Human dermal endothelial cells	(Senger et al., 1997, 2002)
EA-1	Monoclonal antibody	α_6	Murine model of melanoma cells	(Ruiz et al., 1993)
SiRNAs	Synthetic oligonucleotides	$\alpha_6\beta_4$	MDA-MB-231 breast carcinoma cell line	(Lipscomb et al., 2003)
Valproic acid (VPA) & RAD001	Histone deacetylase (HDAC) inhibitor and mamallian target of rapamycin (mTOR) inhibitor	α_2, α_6, β_4	PC3 and LNCaP prostate carcinoma cell lines	(Wedel et al., 2011)
Pabinostat & rapamycin	HDAC and mTOR inhibitors	α_2, β_1	PC3 and C2 prostate and renal carcinoma cell lines	(Verheul et al., 2008)

Table 1. A summary of agonists targeting the α_2, α_6 and β_4 integrins. Treatments that have reached phase II of human trial are denoted *. Efforts to reduce tumour progression by targeting angiogenesis with small peptide based molecules have proven promising. The protein fragment endorepellin which is derived from the C-terminus of perlecan a basement membrane and scaffold protein displays remarkable anti-angiogenic properties (Bix et al., 2007). Bix et al. (2007) have reported a significant decrease in vasculature in an LLC mouse model (P=<.001) and interestingly observed that the administration of endorepellin immediately prior to the appearance of the tumours almost completely blocked their growth. Furthermore, Woodall et al. (2008) identified that the anti-angiogenic effects of endorepellin did not occur in the absence of $\alpha_2\beta_1$. The experiment showed that $\alpha_2\beta_1$ knockout mice displayed significantly less vasculature than wild-type mice (P=<.0001). Integrin $\alpha_2\beta_1$ was necessary for the recruitment of endorepellin to the vasculature where it conjugated with the α_2 domain in a cation-independent manner and supressed angiogenesis (Woodall et al., 2008).

Preceding, and contrary, to these findings is that perlecan the precursor molecule from which endorepellin is derived was also found to have anti-angiogenic and tumour suppressing qualities in prostate cancer (Savore et al., 2005). The suppression of perlecan expression with siRNA in the androgen independent bone targeted cell line C4-2B caused a reduction in colony size and the cohesiveness of transfected sub-clones in anchorage–independent growth assays. Further, when injected into athymic mice they showed a reduced growth rate, tumour size, vasculature and a failure to elevate PSA levels (Savore et al. 2005). Similar results have also been reported by Mathiak et al., (1997) in the HT-1080 fibrosarcoma cell line where perlecan cDNA transfected in antisense orientation caused an increased invasiveness through matri-gel coated filters, increased migratory ability through 8μm filters and elevated adhesiveness to type IV collagen substrate. These results are seemingly incongruent with the more recent findings of Bix et al. (2007) and Woodall et al. (2008) that a reduction of perlecan should in theory reduce the endogenous levels of the derivative endorepellin and thus increase tumour growth. Woodall et al., (2008) suggests that is quandary can be reconciled by "the unique dependence" of HT-1080 cell line on integrin $\alpha_2\beta_1$.

While RGD based peptides represent a good target for the collagen binding integrins such as α_2, unfortunately the laminin binding integrins such as α_6 and β_4, which bind in a RGD independent manner, are not clinically useful agonists for RGD mimicking peptides (Cheresh and Spiro, 1987). Therefore, additional therapeutic development is required to target this group of integrins. A D-amino acid peptide KIKMVISWKG (HYD-1) has been shown to bind to integrin $\alpha_6\beta_1$ in the human prostate carcinoma cell line DU-145 (DeRoock et al., 2001). The HYD-1 peptide was illustrated to prevent cellular attachment to ECM proteins and dermal cell fibroblasts (DeRoock et al., 2001). The finding that α_2 and α_6 can be bound by peptides is important and preliminary evidence suggests that they are well tolerated in murine models, intriguingly, DeRoock et al. (2001) also suggests that peptide administration can 'sensitise' cancer cells to radiotherapy, and aid the removal of cancer cells refractory to primary treatment.

7.3 siRNA based therapies

Antisense and siRNA oligonucleotides have enormous potential as therapeutic agents in prostate cancer and cancers in general (Juliano et al., 2011). Currently, siRNA oligonucleotides which target the $\alpha_6\beta_4$ subunit have been shown to reduce migratory and decrease the invasive capability of the breast carcinoma cell line MDA-MB-231 (Lipscomb et al., 2003). It is now clear that targeting one integrin with siRNAs can also lead to increased expression in another integrin. For example Defilles et al. (2009), knocked out $\alpha_v\beta_5$ and $\alpha_v\beta_6$ using several antibodies and disintegrins, which caused the expression of $\alpha_2\beta_1$ to increase. This negative crosstalk between $\alpha_v\beta_5$ and $\alpha_v\beta_6$ integrin also unexpectedly interfered with P13Kinase regulated $\alpha_2\beta_1$ mediated migration (Defilles et al., 2009). This dynamic crosstalk between integrins needs to be considered when administering integrin-targeted therapies. It may be that a suite of different disintegrins will need to be developed to target the numerous different integrins expressed on the cellular surface to effectively combat cell migration and oncogenesis. While the results using siRNAs *in vitro* are extremely promising, difficulties have arisen attempting to deliver these molecules to targeted regions in the animal model. Delivering oligonucleotides into cells in the absence of transfecting reagents is extremely difficult, given their potential as therapeutic agents a

suite of mechanisms have been attempted including lipoplexes, polymers and dendrimers (reviewed by Juliano *et al*., 2011).

Integrins are recycled by endocytotic mechanisms and thus represent a particularly attractive mechanism in which siRNAs and small oligonucleotide delivery and efficacy can be improved. To date, delivery improvement has been made by utilising RGD peptides conjugated to oligonucleotides in the presence of polyethylene glycol (PEG). PEG acts as a protective mechanism against phagocytosis and also allows the peptides to remain functional for a longer period by reducing the interaction with negatively charged cell surface proteoglycans (Juliano et al., 2011). Additional PEG-RGD mediated cellular entry mechanisms have incorporated oligonucleotides into cationic lipoplexes and utilised nano-carrier technology, where upon cellular entry siRNAs are released and transported via cellular pathways (Juliano et al., 2011).

7.4 Epigenetic therapies

Genes altered by epigenetic modification in cancer are of current interest both as diagnostic markers and as targets for epigenetic modifiers as therapeutic agents. Histone modification or altered DNA methylation of α_2 and α_6 integrins as potential targets for such therapies in prostate cancer have not been widely investigated; however, there is preliminary evidence that their activity is targeted by such therapeutic targets. Recently, Wedel and colleagues (2011) have demonstrated *in vitro* that two drugs, Valproic acid (VPA) and RAD001 reduce tumour cell invasion, migration and adhesion of the prostate cancer cell lines PC3 and LNCaP. Valproic acid is a histone deacetlyase inhibitor and RAD001 is a mammalian target of rapamycin (mTOR) inhibitor. Previous studies have shown that mTOR and Akt phosphorylation is highly correlated with Gleason grade and mTOR has been shown to be a significant prostate cancer biomarker (Dai et al., 2009; Kremer et al., 2006) The separate application of RAD001 or VPA significantly reduced cell adhesion, migration and invasion in PC3 and LNCaP cells, and combination therapy proved to have an additive effect both on migration and invasion but curiously not adhesion. Furthermore, the addition of VPA and RAD001 also changed the cell surface expression of integrins in both cell lines, significantly altering the expression of α_2, α_6 and β_4 integrins. More specifically, the addition of VPA caused a significant down-regulation of α_6 and β_4 integrins in PC3 cells and in LNCaP cells α_2 and α_6 were significantly up-regulated. Given the differential response to the application of VPA to the androgen independent PC3 and androgen dependent LNCaP cell lines, the authors speculate that early and late stage tumours may vary in their response to the application of VPA (Wedel et al., 2011). The simultaneous use of pabinostat (LBH589), also a HDAC inhibitor with rapamycin, also had an additive effect in another prostate cancer model, in particular on the α_2 and β_1 integrins, key drivers of the metastasis (Verheul et al., 2008).

8. Conclusion

Approximately one in seven men are now diagnosed with prostate cancer over their lifetimes; clinicians still lack the ability to identify those tumours that are likely to be aggressive with a propensity to metastasise. There have been significant advances in our understanding of the genetics of prostate cancer, with genetic analyses and gene expression studies highlighting the integrins as key molecules driving prostate cancer and

susceptibility and progression. While many early studies have examined the α_v integrins, here we have highlighted the importance of α_2, α_6 and β_4 in prostate cancer. The characterisation of prostate cancer stem cells has identified α_2 and α_6 integrins as important markers in stem cells and may suggest an emerging role for these integrins in EMT, a key event in tumour progression. Further, the integrins α_2 and α_6, which bind to collagen and laminin, have been identified as key drivers of prostate cancer metastasis, particularly to bone. Given that the vast majority of prostate cancer deaths are associated with metastatic disease, with over 80% of these metastases occurring in bone, this represents an exciting avenue for therapeutic development. Advances in technology, such as next generation sequencing, have provided new tools for mapping genetic and epigenetic changes associated with tumour development. Over 30 prostate cancer susceptibility loci have been identified to date, contributing approximately 25% relative risk; however, critical questions remain as to how these genetic changes identified influence gene function and thus prostate cancer. Therefore, there remains a need to address these current gaps in our understanding of genetic susceptibility and how these variants actually influence disease risk and progression, as we are still waiting for the translation of the vast majority of these findings into the clinical setting. Studies of cancer genes have generally focused on elucidation of mutations, deletions or amplification of critical growth promoting or suppressor genes. It is now clear that small molecules that control post transcriptional gene expression such as miRNAs, or aberrant DNA methylation, and chromatin remodelling are also known to contribute to prostate cancer development and progression and are proving attractive targets for future cancer therapies. Knowledge of how therapies targeting DNA methylation and histone modification influence the behaviour of key cancer genes is a key area for future research. Thus, there is the potential for selected integrins such as α_2, α_6 and β_4 to be utilised as therapeutic targets, with several of these having already reached phase II in human clinical trials.

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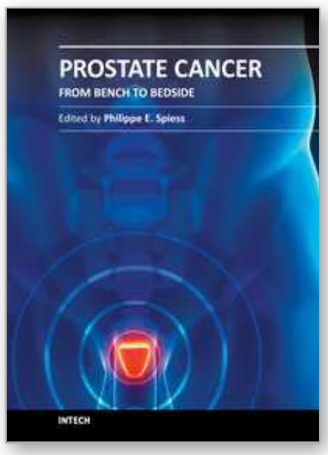
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The present textbook highlights many of the exciting discoveries made in the diagnosis and treatment of prostate cancer over the past decade. International thought leaders have contributed to this effort providing a comprehensive and state-of-the art review of the signaling pathways and genetic alterations essential in prostate cancer. This work provides an essential resource for healthcare professionals and scientists dedicated to this field. This textbook is dedicated to the efforts and advances made by our scientific community, realizing we have much to learn in striving to some day in the not too distant future cure this disease particularly among those with an aggressive tumor biology.

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