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Cross-talk between β_1 Integrins and Insulin-like Growth Factor I Receptor in Prostate Cancer

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ABSTRACT

The progression of prostate cancer is strongly influenced by complex signaling pathways. The insulin-like growth factor I receptor (IGF-IR) and β_1 integrins promote various cellular events such as migration and proliferation that are important to cancer development. Both *in vitro* and *in vivo* techniques were implemented to investigate whether or not β_1 integrins are upstream regulators of IGF-IR expression and signaling. *In vitro*, downregulation of β_1 with small interfering RNA oligonucleotides (siRNA) causes subsequent downregulation of IGF-IR in human prostate cancer LNCaP cells. *In vivo*, using a mouse model for prostate cancer (TRAMP, transgenic prostate adenocarcinoma mice) generated by simian virus 40 large T antigen (SV40-Tag), expression of IGF-IR was found to be comparable in prostate sections from wild type and prostate specific β_1 conditionally ablated ($\beta_1^{pc-/-}$) TRAMP mice. Furthermore, the signaling downstream of IGF-IR was analyzed in LNCaP cells upon downregulation of β_1. Surprisingly, the levels of phosphorylated Akt, a kinase previously determined to be downstream of IGF-IR, were not affected by downregulation of β_1 integrins and IGF-IR. This study gives further insight to the regulatory relationship between β_1 integrins and IGF-IR, suggesting that β_1 integrins may play a key role in the expression of IGF-IR.

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1. BACKGROUND

1.1 Prostate cancer

Prostate cancer is the most frequently diagnosed cancer in men of Western countries with incidence rates on the rise worldwide (Lane *et al*., 2007). It is estimated, in 2008, that 186,320 American men will be diagnosed with prostate cancer, representing the majority of all male cancers. Because of its high prevalence, most men over 50 years old are screened for prostate cancer annually which helps with early detection and diagnosis. Even so, prostate cancer is still one of the most deadly types of cancer for men in various geographical locations. Nearly 1 out of every 6 prostate cancer cases develops invasive metastatic lesions that severely decrease the probability for survival. In fact, 2008 prostate cancer deaths are predicted at 28,660, 10% of all cancer related deaths in men, only surpassed in lethality by lung cancer (Jamel *et al*., 2008). Treatment options include chemotherapy, radiation, androgen deprivation therapy and surgery, yet none have proven significantly successful (Lane *et al*., 2007). However, ongoing prostate cancer research continues to aid in the development of more specific and effective treatments and contributes to the understanding of the mechanisms through which prostate cancer develops.

1.2 TRAMP mouse model

Before the mid-nineties, a reliable animal model for studying prostate cancer was not yet developed. Various attempts that have been previously made focused on using a variety of chemical or hormonal carcinogens for prostate transformation, which were inconsistent and unpredictable. Greenberg and colleagues generated transgenic mice that

accurately model the progression of malignant prostate disease, called TRAMP. Pathological states in TRAMP mice can range from prostate hyperplasia to invasive adenocarcinoma, closely similar to the progression of prostate cancer in human beings. The rat probasin promoter directs prostate-specific expression of the SV40-Tag that binds and inactivates tumor suppressors retinoblastoma (Rb) and p53. For this reason, cancer is only found in the prostate, even as early as 10 weeks of age (Greenberg *et al*., 1995). Today, the TRAMP model is frequently implemented for *in vivo* prostate cancer studies, such as those performed here.

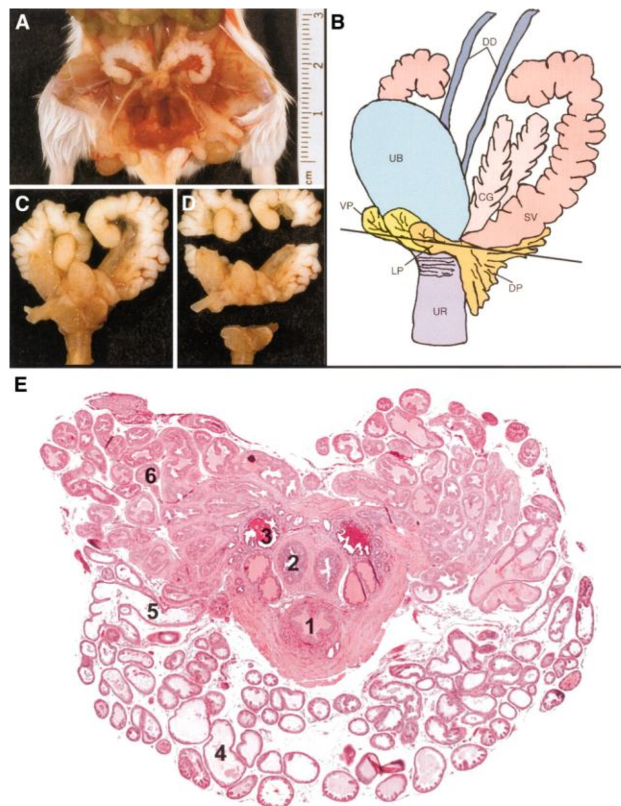

Figure 1. The normal murine prostate.
A. Intact mouse prostate, bladder, and seminal vesicles. *B*. Diagram of murine genitourinary (GU) bloc, urethra (UR) and prostate lobes: ventral prostate (VP), lateral prostate (LP), dorsal prostate (DP) and anterior prostate (CG); seminal vesicles (SV), ductus deferens (DD) and urinary bladder (UB) also shown. *C*. Removed GU bloc from A. *D*. Same GU block as in A and C, yet transversely cut through urethra and seminal vesicles. *E*. Histological cross-section of murine GU block; 1. urethra, 2. ductus deferens, 3. ampullary glands, 4. ventral prostate, 5. lateral prostate, 6. dorsal prostate (Shappell *et al*., 2004).

1.3 Integrins and growth factor receptors

Integrins are heterodimeric, transmembrane cell surface receptors that bind extracellular matrix (ECM) proteins to influence a number of cellular functions such as migration, differentiation, proliferation and survival. Combinations of 18 known α and 8 known β subunits are expressed based on cell type (Slack-Davis and Parsons, 2004). Members and ligands of the integrin family are listed in Table 1.

Table 1. Integrins and ligands.

Subunit		*Ligand*
β_{1A}, β_{1B}, β_{1C}, $\beta_{1C\text{-}2}$, β_{1D}	α_1	Laminin, collagen
	α_2	Laminin, collagen, thrombospondin, E-cadherin, tenascin
	α_{3A}, α_{3B}	Laminin, collagen, fibronectin, entactin, thrombospondin, uPAR
	α_4	Fibronectin, VCAM-1, osteopontin, ADAM, ICAM, MAdCAM-1, thrombospondin, Lu/BCAM, CD14, JAM-2, uPAR
	α_5	Fibronectin, L1, osteopontin, fibrillin, thrombospondin, ADAM, NOV
	α_{6A}, α_{6B}, α_{6X1}, α_{6X2}	Laminin, thrombospondin, Cyr61, ADAM, uPAR
	α_{7A}, α_{7B}	Laminin
	α_8	Fibronectin, tenascin, nephronectin, vitronectin, osteopontin, TGF β-LAP
	α_9	Tenascin, VCAM-1, osteopontin, uPAR, plasmin, angiostatin, ADAM, VEGF-C, VEGF-D
	α_{10}	Collagen, laminin
	α_{11}	Collagen
	α_V	Fibronectin, osteopontin, TGF β-LAP, L1
β_2	α_L	ICAM
	α_M	iC3b, fibrinogen, factor X, ICAM, heparin
	α_X	iC3b, fibrinogen, collagen, ICAM, heparin
	α_D	ICAM, VCAM-1, fibrinogen, fibronectin, vitronectin, Cyr61, plasminogen
β_{3A}, β_{3B}, β_{3C}	α_{IIb}, α_{IIbalt}	Fibrinogen, fibronectin, von Willebrand factor, vitronectin, thrombospondin, disintegrin, osteopontin, Cyr61, ICAM, L1
	α_V	Vitronectin, fibrinogen, fibronectin, von Willebrand factor, thrombospondin, fibrillin, tenascin, PECAM-1, BSP, ADAM, ICAM, FGF-2, uPA, angiostatin, TGF β-LAP, Del-1, L1, MMP, osteopontin, cardiotoxin, uPAR, plasmin, Cyr61, tumstatin, NOV
β_{4A}, β_{4B}, β_{4C}, β_{4D}	α_{6A}, α_{6B}	Laminin
β_{5A}, β_{5B}	α_V	Vitronectin, osteopontin, fibronectin, TGF β-LAP, NOV, BSP, MFG-E8
β_6	α_V	Fibronectin, tenascin, vitronectin, TGF β-LAP, osteopontin, ADAM
β_7	α_4, α_{IEL}	Fibronectin, VCAM, MAdCAM-1, osteopontin
	α_E	E-cadherin
β_8	α_V	Vitronectin, laminin, TGF β-LAP

Integrins have been shown to associate with receptor tyrosine kinases (RTKs i.e., growth factor receptors) to induce downstream signaling by a variety of mechanisms (Lee and Juliano, 2004) (Fig. 2). One such proposed mechanism states that the transmembrane domain of integrins can recruit specific adaptor molecules to the plasma membrane and concentrate them in the proximity of growth factor receptors (GFRs), which can influence their activity (Fig. 2). A second mechanism is that integrins can alter GFR localization. Subcellular focal contacts are concentrations of signaling kinases and cytoskeleton proteins, including paxillin, talin and vinculin. GFR signaling is, in turn, manipulated by these focal contacts (Fig. 2). In the third mechanism, integrins can alter the rate of internalization and degradation of GFRs via ubiquitination; a hypothesis that remains to be tested (Alam *et al*., 2007).

Integrin and GFR cross-talk has been widely investigated. One study has shown that insulin-like growth factor receptor substrate 1 (IRS-1) phosphorylation and signaling via the PI3-kinase (PI3K) pathway is correlated with increased cell adhesion and interaction of IRS-1 with $\alpha_5\beta_1$ (Reiss *et al*., 2001). Platelet-derived growth factor receptor (PDGFR) activation increases vitronectin-associated vascular endothelium migration (Woodard *et al*., 1998). PDGFR phosphorylation and protein levels decrease in response to cell detachment from the ECM (Baron and Schwartz, 2000). c-Met activation can be inhibited by blocking activity of an integrin-associated protein, KAI1/CD82 (Sridhar and Miranti, 2006). $\alpha_6\beta_4$ associates with c-Met receptor and assists in invasion (Trusolino *et al*., 2001). $\alpha_1\beta_1$ down-regulation has shown to increase epithelial growth factor receptor (EGFR) and downstream Rac activation (Chen *et al*.,

2007). Levels of activated EGFR are also influenced by cell attachment to the ECM (Reginato *et al*., 2003).

Modifications of integrin expression majorly contribute to tumor invasion and metastasis through altered affinity to ECM proteins, cytoskeleton stability changes and recruitment of proteolytic activity for basement membrane degradation. In many different cancers, the integrin profile is modified due to selective pressure changes: alterations in ECM secretions and oncogene or tumor suppressor activity. Integrin and RTK signaling can also disrupt cell-cell adhesion, largely contributing to tumor invasion and metastasis. Integrins and RTKs are thought to regulate the internalization and transcription of E-cadherin, a molecule important in cell adhesion (Guo and Giancotti, 2004).

The polymerization of actin cytoskeleton molecules influences the mobility of cells. Additionally, this is affected by integrin and RTK signaling to focal adhesion kinase (FAK) and Shc. Signaling to Ras and subsequently ERK/MAPK cooperates with signaling from transforming growth factor β receptor to induce the transitional movement of malignant cells from epithelium to mesenchyme. Some integrins, including $\alpha_V\beta_3$, have been shown to recruit degradative enzymes such as matrix-metalloproteinase-2 (MMP2) and its activator urokinase plasminogen activator (uPA) to the cell surface, destroying the basement membrane as the tumor progresses (Guo and Giancotti, 2004). Similarly in prostate carcinoma, the normal basal cell layer diminishes until the tumor cells contact the substratum. Integrins interact with the substratum layer, potentiating tumor cell survival. Tumor substratum is marked by the loss of laminin 5 and collagen

VII, playing a part in the modification of integrin profile in malignant prostate epithelium (Knudsen and Miranti, 2006).

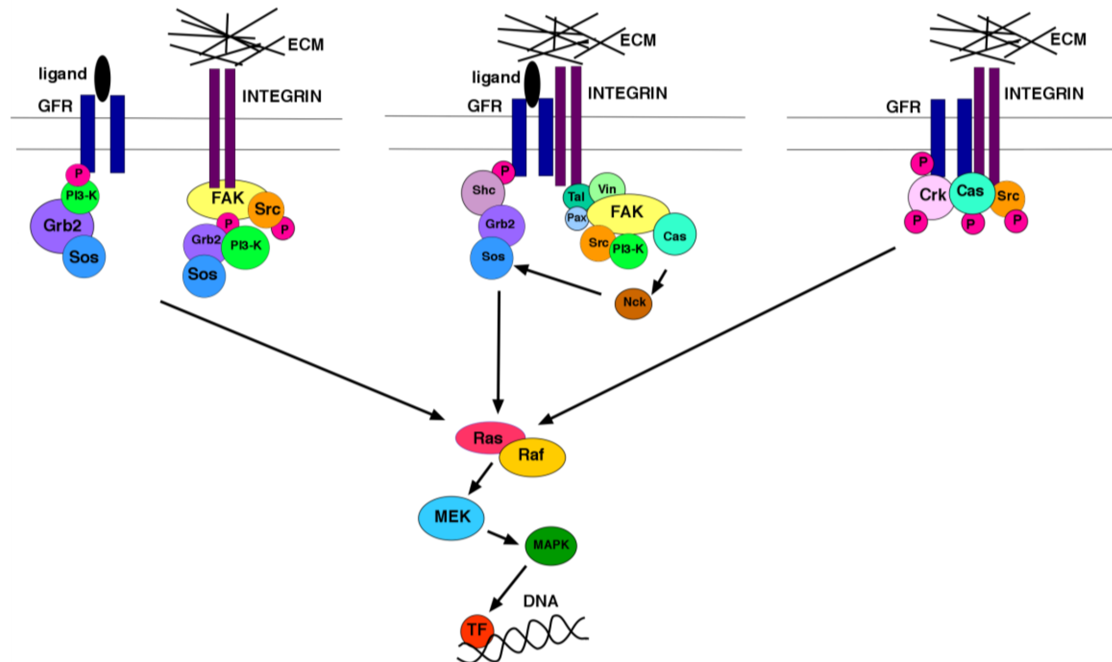

Figure 2. Regulation of growth factor receptor signaling by integrins.
The interaction of $\alpha_v\beta_1$ with the ECM and PDGFR, IGF-IR or VEGFR results in FAK stimulation and cytoskeleton rearrangement, inducing collaborative downstream signaling to the MAPK pathway. Recruitment of Shc to PDGFR or EGFR causes additional recruitment of Grb2/Sos complex (left). Association of β_1 integrin and adaptor molecules and activation of FAK leads to downstream signaling to the MAPK pathway (middle). Adhesion of $\alpha_v\beta_3$ and β_1 integrins to the ECM phosphorylates c-Src and recruits other adaptor molecules like Cas and Crk. This complex of $\alpha_v\beta_3$ and β_1 integrins, EGFR and adaptor molecules allows for activation of the MAPK pathway (right).

Through various signaling mechanisms, GFRs and integrins can activate ERK/MAPK, contributing to cell proliferation (Fig. 2). GFR mutation or overexpression can result in phosphorylation of integrin cytoplasmic domain, creating adaptor protein docking sites and thereby amplifying and activating ERK/MAPK (Fig. 2, middle). Integrins also have the ability to enhance GFR signaling through signaling intermediates such as p130Cas (Fig. 2, right). It has been demonstrated, *in vitro*, that GFR activation of

Raf or MEK can be prevented by inhibition of integrin binding to ECM (Hood and Cheresh, 2002).

1.4 β_1 integrins, IGF-IR and prostate cancer

The β_1 integrin subunit exists as several cytoplasmic domain splice isoforms that directly affect integrin-mediated cell processes and signaling (Fornaro and Languino, 1997). Previous research has demonstrated that the commonly expressed and highly conserved β_{1A} splice variant promotes and enhances cell proliferation and migration (Marcantonio and Hynes, 1988; Fornaro and Languino, 1997). Antagonistically, the β_{1C} splice variant, which contains an exclusive 48 amino acid sequence in its carboxy terminus, inhibits cell proliferation and is frequently upregulated in differentiated epithelium (Fornaro *et al*., 1995, 1998). β_{1C} was found to be selectively downregulated in prostate adenocarcinoma, a consequence of transcriptional as well as pre- and post-translational regulation (Fornaro *et al*., 1996; Moro *et al*., 2004).

The IGFs are known to be important to cellular growth and differentiation. Variations in this signaling axis are thought to be involved in malignant transition and are relevant to cancer grade and prognosis (Cardillo *et al*., 1996; Baserga, 2000). Elevated serum levels of IGF-I have been proposed to be a risk factor in the development of malignant prostate disease (Chan *et al*., 1998). Previous reports introduce contradictory evidence of IGF axis expression in prostate cancer, some pointing towards its downregulation (Tennant *et al*., 1996; Chott *et al*., 1999). Other more recent studies have shown that IGF-IR protein and mRNA levels are increased in prostate cancer compared to benign prostate (Nickerson *et al*., 2001; Hellawell *et al*., 2002).

The synergistic relationship between GFRs and integrins applies also to IGF-IR and β_1 in prostate carcinoma. β_{1A} integrins can influence basement membrane adhesion via IGF signaling, indicating that these molecules are involved in tumor migration. β_{1A} was shown to promote IGF-IR phosphorylation and recruitment of IRS-1 for the activation of IGF downstream signaling. On the other hand, β_{1C} does not associate with IGF-IR or promote its phosphorylation. β_{1C} inhibits cell growth by enhancing adaptor protein Gab1/Shp2 recruitment to the plasma membrane. This research has shown that cytoplasmic variation in β_1 integrins can modulate IGF-IR activities (Goel *et al*., 2004). The β_{1A} integrin splice variant was demonstrated as necessary to prostate cancer cell proliferation and anchorage-independent growth through IGF-I signaling. In early prostate transformation in TRAMP mice, both β_{1A} and IGF-IR are upregulated, suggesting essential roles in tumor initiation. β_{1A} integrins have the ability to direct IGF-IR to focal contacts and support downstream IGF signaling, whereas β_{1C} integrins do not have this effect, potentiating IGF-IR inactivation in normal prostate (Goel *et al*., 2005). β_1 integrins have been demonstrated as important effectors in IGF-IR localization and signaling that fundamentally contribute to prostate cancer growth and migration. Despite these current findings, little is still known about the influence of β_1 on IGF-IR expression in prostate cancer.

1.5 Akt and prostate cancer

In addition to downstream mitogenic signaling via the Ras-MAPK/ERK pathway, IGF-IR also activates Akt (Pollack *et al*., 2004). Akt is a serine/threonine kinase that is

most widely accepted as a cell survival mediator, but also functions in cell proliferation and growth. For optimal activation of Akt, phosphorylation on both Thr308 and Ser473 is required. After activation, Akt signals to multiple components of the cell-death cycle, including BAD. By phosphorylating BAD, Akt prevents BAD interaction with BCL-X, contributing to cell survival. Akt phosphorylation of caspase-9 inhibits its proteolytic activity in apoptosis. Through Akt signaling, both NF-κB transcription and p53 degradation are indirectly promoted; further augmenting Akt impact on cell survival. Akt also affects G1/S transition by preventing the degradation of cyclin D1. Expression of cell cycle inhibitors, p27 and p21 are inhibited by Akt signaling. It is mainly the inactivation of the tumor suppressor PTEN in prostate cancer that causes the deregulation of Akt signaling, ultimately resulting in reduced cell apoptosis and prolonged cell survival (Vivanco and Sawyers, 2002). Deregulation of Akt through IGF-IR expression may contribute to cell survival in prostate cancer.

1.6 Project purpose

The purpose of this project was to evaluate the cross-talk between β_1 integrins and IGF-IR in prostate cancer. Since both β_1 and IGF-IR are upregulated in prostate cancer and form a complex, further investigation into the interaction of these molecules may reveal more information about the initiation and progression of prostate cancer. However, not much is known regarding the mechanism that controls the expression of these molecules. Specifically, β_1 integrins were analyzed *in vitro* and *in vivo* as potential upstream regulators of IGF-IR expression and signaling. Although constitutive Akt activation often occurs in prostate cancer due to disabling of PTEN, it is not known

whether or not β_1 or IGF-IR control Akt activation. Therefore, the activation of Akt was studied upon downregulation of β_1 and IGF-IR expression.

2. METHODS

2.1 Cells and culture conditions

Human lymph node prostate cancer metastasis derived LNCaP cells (ATCC) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS, Gemini Bioproduct), 100 units/mL penicillin, 100 μg/mL streptomycin, sodium pyruvate and non-essential amino acids (all from Invitrogen). The cells were stimulated with non-metabolizable synthetic androgen analogue, R1881, and then transfected with siRNA (100 nM) using oligofectamine (Invitrogen) in serum-free media. Following 4 hours of incubation, cells were fed complete medium with serum and incubated an additional 48 hours. The sequence of β_{1A} siRNA was: sense strand 5'—AUGGGACACGGGUGAAAAUTT—3' and antisense strand 5'—AUUUUCACCCGUGUCCCAUTT—3'. The sequence of β_{1C} siRNA was: sense strand 5'—CCUCUGACUUCCAGAUUCCTT—3' and antisense strand 5'—GGAAUCUGGAAGUCAGAGGTT—3'. The sequences were submitted to BLAST search to ensure that only β_{1A} integrin was targeted by the siRNA and that the control sequences were not homologous to any known genes. The siRNA specific to β_{1A} and β_{1C} integrin were both synthesized by Dharmacon.

2.2 Mice

TRAMP (B6), $\beta_1^{loxP/loxP}$ (B6;129) and PB-Cre4 (B6.D2) mice were generated and characterized by Dr. Goel in Dr. Languino's lab. $\beta_1^{loxP/loxP}$ / TRAMP / PB-Cre4 (TRAMP mice with conditional ablation of β_1) and $\beta_1^{loxP/loxP}$ / TRAMP (TRAMP mice

with wt β_1) were generated as described below. The male PB-Cre4 and female $\beta_1^{loxP/loxP}$ mice were mated and male offsprings expressing PB-Cre4 were backcrossed with female $\beta_1^{loxP/loxP}$ to generate male $\beta_1^{loxP/loxP}$ / PB-Cre4 mice designated as $\beta_1^{pc-/-}$. Similarly, female TRAMP and male $\beta_1^{loxP/loxP}$ were bred, resulting in the female offsprings which express TRAMP; these female mice were backcrossed with male $\beta_1^{loxP/loxP}$ to generate female $\beta_1^{loxP/loxP}$ / TRAMP. Male $\beta_1^{pc-/-}$ mice were crossed with female $\beta_1^{loxP/loxP}$ / TRAMP to generate male $\beta_1^{loxP/loxP}$ / TRAMP designated as β_1^{wt} / TRAMP and male $\beta_1^{pc-/-}$ / TRAMP. Only male β_1^{wt} / TRAMP and male $\beta_1^{pc-/-}$ / TRAMP littermates were used in this project.

2.3 Immunohistochemistry (IHC)

Prostate lobes from β_1^{wt} / TRAMP and $\beta_1^{pc-/-}$ / TRAMP mice were isolated, fixed in buffer-neutral formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin for histological analysis. The mouse tissue sections prepared for IHC were placed on charged glass slides from paraffin-embedded blocks. After baking in a 60°C oven for 2 hours, the slides were deparaffinized in three xylene washes and rehydrated in ascending concentrations of ethanol. Antigen retrieval was achieved by boiling the slides in 10 mM sodium citrate, pH 6.0, for 23 minutes. The slides were washed in 3% hydrogen peroxide to remove endogenous peroxidase activity. Sections were blocked in 50% goat serum in TBS for 1 hour. Primary antibody to IGF-IR β subunit (2 μg/mL, Santa Cruz), and negative control, rabbit IgG, were added to the sections for overnight incubation at 4°C. Following several TBS washes, biotinylated

anti-rabbit IgG (Vector) was applied to the sections for 30 minutes at room temperature. Immunoperoxidase staining was carried out with diaminobenzidene solution (2.5 mg DAB powder in 5 mL H_2O, plus 100 μL 3% H_2O_2). The slides were counterstained with Mayer's hematoxylin and then dehydrated in decreasing ethanol concentrations. The sections were fixed with a solution of 55% toluene and 45% dissolved polymers under a glass coverslip. All the slides were examined and photographed by an Olympus BX41TF optical microscope equipped with an Evolutions MP 5.0 RTV digital camera. The stained slides were reviewed by Dr. Garlick, a veterinary pathologist from Charles River Laboratories, for intensity of the staining dependent on lobe (anterior, dorsal, lateral and ventral) or by pathological state (prostatic intraepithelial neoplasia or PIN and invasive adenocarcinoma). Staining intensity was given a rating on a scale of +1 (weak staining), +2 (moderate staining) or +3 (strong staining) (Armes *et al*., 1999).

2.4 Immunoblotting

LNCaP cells, 48 hours after siRNA transfection, were lysed in 20 mM Tris pH 7.5, 150 mM NaCl, 10% glycerol, 1% NP-40, 10 mM NaF, 1 mM $NaVO_4$, 1 mM $Na_4O_7P_2$, 2 μM leupeptin, 2 μM aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF). Protein concentration was quantified using the BCA protein assay (Pierce Biotechnology). Total cell lysate was resolved by 10% SDS-PAGE under reducing conditions and transferred onto a PVDF membrane. The membrane was blocked with 5% non-fat dry milk in TBS-T for 1 hour at room temperature. The membrane was immunoblotted with monoclonal antibody to β_1 integrin (BD Transfection Laboratories), and polyclonal antibodies to IGF-IR β subunit, phosphorylated Akt and total Akt (all

from Santa Cruz). Proteins were visualized with ECL reagent (Boston Bioproducts) and developed using autoradiography.

3. RESULTS

3.1 β_1 integrins modulate IGF-IR expression

Cell lysates from human prostate cancer LNCaP cells were analyzed for protein expression of the IGF-IR. Immunoblotting was performed on three sets of LNCaP lysate: cells treated with non-metabolizable, synthetic androgen analogue, R1881, cells treated with R1881 and transfected with siRNA to β_{1C} and cells treated with R1881 and transfected with siRNA to β_{1A}. LNCaP cells do not express the β_{1C} integrin splice variant, so β_{1C} siRNA does not interfere with β_1 integrin expression. For this reason, β_{1C} siRNA was used as a siRNA negative control. However, the cells do express the β_{1A} variant, the expression of which is susceptible to downregulation by siRNA specifically targeting the β_{1A} sequence.

Detection of β_1 integrins with a monoclonal antibody revealed a 130-140 KDa protein expressed in cells only supplemented with R1881 as well as cells that were transfected with the control siRNA. In cells with siRNA to β_{1A}, the level of β_1 was significantly decreased (Fig. 3). When the membrane was probed with a polyclonal antibody to IGF-IR, the results show that IGF-IR expression was markedly reduced in cells transfected with siRNA to β_{1A} (Fig. 3). Comparing the band intensities with that of the Akt loading control, both β_1 and IGF-IR were both significantly downregulated (Fig. 3). The results suggest that IGF-IR and β_1 integrins are closely associated and more specifically that β_1 integrins may play a key role in regulating the expression of IGF-IR.

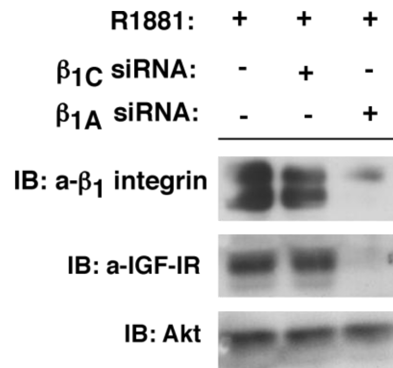

Figure 3. Decrease of β_1 expression by siRNA in LNCaP cells subsequently downregulate expression of IGF-IR. LNCaP cells transfected with siRNA to β_{1C} or β_{1A} were stimulated with R1881 (synthetic androgen) and cell lysate was immunoblotted using an antibody to β_1 integrin, IGF-IR or Akt. β_{1C} siRNA was used as a negative control. The results of this experiment were reproduced several times.

3.2 IGF-IR expression in $\beta_1^{pc-/-}$/TRAMP mice

After determining that IGF-IR expression decreases as a result of β_1 downregulation *in vitro*, the focus was shifted to an *in vivo* approach. Tissue sections from the murine prostate cancer model, TRAMP, were stained with an antibody specific for IGF-IR. IGF-IR expression was compared in β_1^{wt} / TRAMP and $\beta_1^{pc-/-}$ / TRAMP mice to determine the influence of β_1 on IGF-IR *in vivo*. Semi-quantitative immunohistochemical analysis of prostate tissue from these mice indicates that IGF-IR expression is localized in the prostate epithelium cytoplasm and, in some cases, nucleus (Fig. 4A-H).

Histological examination of the immunostained slides was performed by a veterinary pathologist who compared overall staining intensity between the four major lobes as well as the intensity of non-invasive prostate intraepithelial neoplasia (PIN) and invasive adenocarcinoma lesions to baseline staining in normal glands (Fig. 4I and J). For the majority of mouse cases (19 total), staining was light and minimal, a rating of +1

(Fig. 4I and J). Only a few cases actually displayed some higher, moderate intensity of staining, a rating of +2 (Fig. 4I and J). Since only a few cases, independent of β_1 expression by lobe or lesion type, attained a higher staining intensity of IGF-IR, it was concluded that there was no significant correlation in these results.

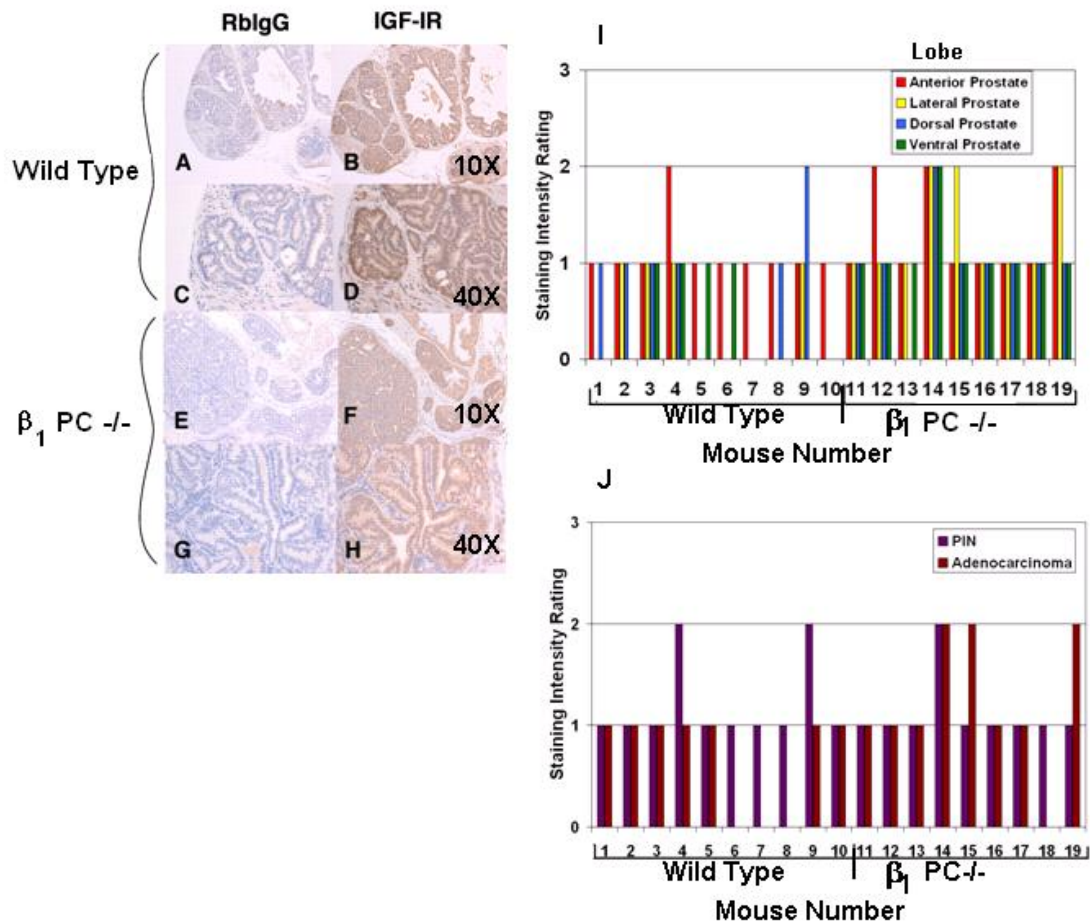

Figure 4. ***In vivo*** **IGF-IR expression is unaffected in $\beta_1^{pc-/-}$ TRAMP mice.**
A-H. Representative images of TRAMP mouse prostate sections stained using an antibody to IGF-IR, wild type (A-D) and $\beta_1^{pc-/-}$ TRAMP mice (E-H). Panels A, C, E and G show negative control staining with Rabbit IgG (RbIgG). *I*. Rating of overall IGF-IR staining intensity in prostate lobes (anterior, lateral, dorsal or ventral). *J*. Rating of IGF-IR staining intensity in PIN or adenocarcinoma lesions compared to normal gland as baseline staining [33].

3.3 Downregulation of β_1 and IGF-IR does not affect activated Akt levels

Additionally, the downstream signaling pathways of IGF-IR were examined. LNCaP cell lysates were analyzed for phosphorylated Akt (p-Akt) using the same conditions in which β_1 and IGF-IR were proven to be downregulated. Downstream of insulin and IGF pathways, Akt is phosphorylated on serine residue 473. A polyclonal antibody that detects this phosphorylation event, and therefore, Akt activation as a consequence of IGF-IR signaling, was used. A 60 KDa protein band was revealed after probing for p-Akt (Fig. 5). The results show that p-Akt levels were unmodified when comparing band intensities with the total Akt loading control even under β_1 and IGF-IR downregulation conditions (Fig. 5).

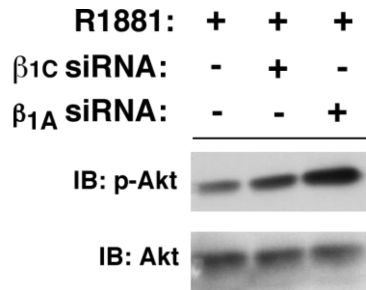

Figure 5. Phosphorylated levels of Akt (p-Akt) are not altered when β_1 and IGF-IR expression is downregulated by siRNA to β_1 in LNCaP cells. LNCaP cells transfected with siRNA to β_{1C} or β_{1A} were stimulated with R1881 and the cell lysate was immunoblotted using an antibody to p-Akt or Akt. These results are representative of three experiments.

4. DISCUSSION

In this study, it was found that the β_{1A} integrins were essential in the regulation of IGF-IR expression in prostate cancer LNCaP cells (Fig. 6). Downregulation of β_{1A} by siRNA caused reduced expression of IGF-IR as well as β_{1A}. Similar results were obtained using siRNA to all β_1 integrins (data not shown). Past reports indicate the importance of β_{1A} for IGF-IR mitogenic and tumorigenic activities in prostate cancer. It was also shown that β_{1A} and IGF-IR are both upregulated in TRAMP mice during the initial stages of the disease: PIN and well differentiated (WD) tumors, suggesting β_{1A} and IGF-IR are involved in the uncontrolled cell division at the start of cancer transformation (Goel *et al*., 2005). By discovering the mechanism behind prostate cancer progression, possible therapeutic targets may be identified for treatment or prevention of the disease. The full potential of RNA interference (RNAi) in treating human diseases is just beginning to be unraveled. By directing RNAi towards the downregulation of β_{1A} integrin expression during the pre-cancer or early cancer stages in the prostate, tumor progression may be inhibited before a more aggressive form develops and the likelihood of survival diminishes.

This study showed that in the TRAMP mouse model, IGF-IR expression was unaffected by loss of β_1 in the prostate of these mice. TRAMP mice were generated by expression of the SV40-Tag driven by a prostate specific rat probasin promoter. The SV40-Tag interacts with tumor suppressor genes Rb and p53, causing their inactivation. This results in the development of adenocarcinoma in the prostate of these mice between 10 and 20 weeks of age (Greenberg *et al*., 1995). Kaplan *et al*. have previously analyzed

IGF expression in TRAMP mice, observing an increase in prostatic IGF-I mRNA levels in early PIN or WD carcinoma formation. However, IGF-IR levels were found to be significantly decreased in metastatic and androgen-independent prostate cancer in TRAMP mice (Kaplan *et al*., 1999). SV40-Tag has been shown to inhibit the binding of E2F complex to the IGF-I promoter, leading to an increase in IGF-I transcription (Porcu *et al*., 1994). IGF-IR expression in SV40-Tag transformed cells has been proposed to be involved in maintaining proliferative, differentiated cells (Plymate *et al*., 1997). A recent study by Bocchetta and colleagues revealed that Tag and p53 complex binds the IGF-I promoter, along with several other molecules including pRb. It was noted that when p53 was absent from SV40-Tag transformed cells, IGF-IR expression was decreased even though no association was found between Tag-p53 and IGF-IR promoter (Bocchetta *et al*., 2008). Although IGF-IR may not be directly influenced by SV40-Tag expression in TRAMP mice, it may be the increase in IGF-I expression that is directing an autocrine positive feedback loop on IGF-IR expression in both $\beta 1^{wt}$/TRAMP and $\beta 1^{pc-/-}$ /TRAMP mice. Further experimentation is necessary to uncover the mechanism behind the indirect effects of SV40-Tag complexes on IGF axis expression.

Finally, this study found that when β_{1A} integrins and IGF-IR are downregulated by siRNA, the levels of phosphorylated Akt remain unaltered in LNCaP cells (Fig. 6). In many cases, constitutive Akt activation in prostate cancer is a result of PTEN tumor suppressor inactivation or growth factor stimulation (i.e., IGF-I) (Mujamder and Sellers, 2004). Akt activation in prostate cancer has been positively correlated with an increase in tumor grade (Kreisberg *et al*., 2004). Androgen has been shown to activate Akt through the PI3K signaling pathway. Androgen receptor (AR) interaction with the p85α

regulatory subunit of PI3K as well as Src contributes to Akt activation (Sun *et al*., 2003). Through tyrosine kinase and small GTPase interaction with p85α, PI3K activation can occur. Src phosphorylation of p85α inactivates an inhibitory region on p85α, allowing for activation of PI3K by small GTPases (Chan *et al*., 2002). Androgen stimulation enhances the association between AR and p85α and activates Src kinase activity (Sun *et al*., 2003). The non-metabolizable synthetic androgen analogue, R1881, used to stimulate the androgen-sensitive LNCaP cells may be activating Akt through a β_1- or IGF receptor-independent pathway, which may include signaling pathways acted upon by other integrins. Various reports in the literature demonstrate how androgen can activate the Akt survival pathway, yet the role of R1881 stimulation in prostate cancer LNCaP cells is not fully understood and still needs to be investigated.

In conclusion, the results of this project show that β_1 integrins directly modulate IGF-IR expression and that Akt activation does not require β_1 and IGF-IR in prostate cancer cells.

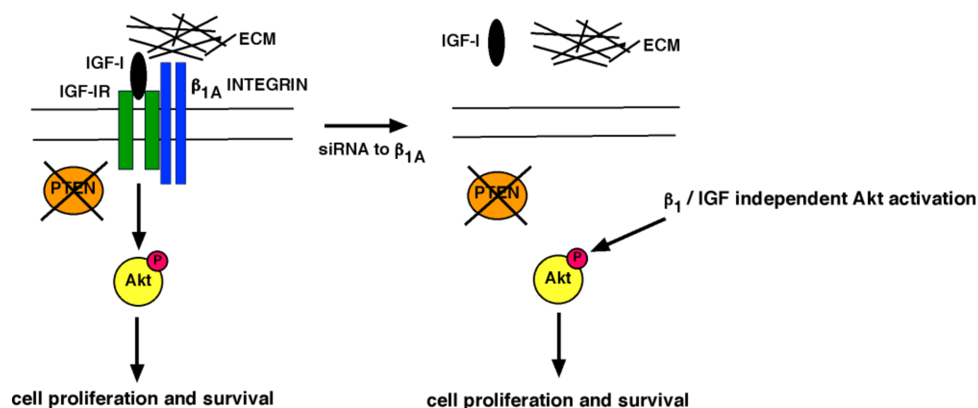

Figure 6. β_1 integrins are essential to IGF-IR expression and are not required for Akt activation. Upon downregulation of β_1 by siRNA, IGF-IR is not expressed in prostate cancer cells. Phosphorylation of Akt occurs both dependently and independently of β_1 and IGF-IR in prostate cancer cells

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