THE POSITIVE AND NEGATIVE TRANSCRIPTIONAL REGULATION OF N-CADHERIN EXPRESSION DURING THE PROGRESSION OF PROSTATE CANCER

by

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DEDICATION

This body of work is dedicated to all men that have died of prostate cancer, and to the women who loved them. Specifically, this work is dedicated to my Grandpa Alexander who died of an unknown cancer before I had the opportunity to develop a relationship with him. I dedicate this work in his memory, to all black men that have died of cancer due to lack of understanding of the disease, and I hope in the future to have an impact on the educational aspect of cancer. I would also like to dedicate this work to a good family friend, Bob Warner, who died of metastatic prostate cancer a decade after a prostatectomy. Because of Bob's passing, I hope to impact the disease of cancer by furthering the understanding of the metastastatic process. I also dedicate this work to my Grandma Goodwin, a strong woman and a breast cancer survivor.

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ABSTRACT

For cancer cells to initiate cell migration and progress to metastasize, epithelial genes must be silenced and the expression of mesenchymal genes must be upregulated. During prostate carcinogenesis, E-cadherin expression is downregulated through multiple mechanisms, the majority of which combine to silence E-cadherin expression through transcriptional regulation at the level of the E-cadherin promoter. Recently it has been discovered that there is transcriptional upregulation of the mesenchymal cadherin, *N-cadher*in during prostate cancer metastasis. Although N-cadherin expression can be detected in human prostate cancer and in prostate carcinoma cell lines, the mechanisms controlling the transcriptional regulation of N-cadherin in cancer are uncharacterized. This body of work offers the first evidence for the mechanisms controlling the transcriptional upregulation of N-cadherin expression in prostate carcinoma. We utilized anchorage independent culture to induce downregulation of N-cadherin expression, and then analyzed the necessary events for N-cadherin upregulation when cells attached to Fibronetin (FN). In order to determine the functional regions of the N-cadherin proximal promoter that were involved in the upregulation of N-cadherin expression, we cloned regions of the human *N-cadherin* 5' proximal promoter, and regions of the first intron of the N-*cadherin* gene into a luciferase reporter vector. It was determined that the bHLH transcription factor Twist1 controlled the upregulation of N-cadherin transcription in PC-3 cells, through β1 integrin dependent nuclear localization of Twist1. A cis-element located in the first intron of the N-cadherin gene was shown to be necessary for Twist1 mediated effects on the *N-cadherin* promoter. We then determined the requirements for

cell-type specific expression of the N-cadherin promoter. It was determined that an additional cis-element located in the first intron of the N-cadherin gene was necessary to repress N-cadherin promoter activity in cells lacking *N-cadherin*. Through deletion analysis of the N-cadherin promoter luciferase construct, a DNA binding site for the transcription factor FoxP1 was discovered. FoxP1 binds to the repressive cis-element *in vitro*, and mutation of the FoxP1 DNA binding site eliminated cell-type specific activity of the N-cadherin promoter. Therefore, we have documented that the aberrant expression of N-cadherin in prostate carcinoma involves alterations in both positive and negative transcriptional regulators.

I. INTRODUCTION

Prostate Cancer Statistics

It is predicted that in the year 2005 there will be 232,090 newly diagnosed cases of prostate adenocarcinoma and 30,350 deaths due to metastasis of the disease (1). This makes adenocarcinoma of the prostate the most diagnosed non-cutaneous cancer in American men, accounting for 33% of all diagnosed tumors. Of greater importance, prostate cancer is the second leading cause of cancer deaths in American men and therefore a significant source of cancer related mortality. Currently in America one man in six will be diagnosed with prostate cancer, and one in 34 will die of this disease; in comparison, during 2002 one man in 35 died in of an accidental injury, therefore a man has an equal chance of dying from an accidental injury or from prostate cancer (2).

There are striking differences in both the incidence and mortality rates of prostate cancer between African and Anglo American men. During the years 1996 through 2000, the average risk of developing prostate cancer was 272 per 100,000 in the African American population, compared with 164 per 100,000 in the Anglo American population (3). More disturbing are the mortality rates between these two populations, 73 and 30 per 100,000 for African and Anglo American men, respectively (4). The estimates for incidence and mortality rates for the year 2005 have not changed appreciably in the past five years, suggesting that the research and education about the disparities in prostate cancer incidence and mortality rates between African and Anglo American men must be continued (1).

It is difficult to suggest that genetic and cultural differences contribute to the increased incidence rates of prostate cancer in African American men, as no solid empirical data exists for genetic differences, and cultural differences are at best subjective. To counter the theory that genetic differences account for the increased rate of incidence and mortality of prostate cancer, Cher *et. al.* documented that clinically localized, untreated primary prostate cancers from African and Anglo American men shared the same pattern and frequency of chromosomal alterations (5). Yet, African American men seem to present with a higher Gleason score and more advanced disease (6;7). A disturbing study by Thatai *et. al.* concluded that in patients with androgen independent metastatic prostate cancer, race was an independent predictor of therapeutic outcome (8). One theory that might explain the differences in mortality rates between African and Anglo Americans are differences in the utilization of treatment options between these two populations. Data suggest that African American men may not chose definitive therapy (prostatectomy or external beam radiation therapy) as frequently as Anglo American men when diagnosed with prostate cancer (9-11). Interestingly, there are numerous reports of racial differences in the choices of therapy for prostate cancer even after the data is adjusted for insurance and economic status (12-14). Together, these data suggest that there is a dearth of research in this important aspect of prostate carcinogenesis, and that current campaigns to educate the African American community about prostate cancer must be expanded.

Current data concerning the risk factors for the development of prostate cancer are as equally nebulous as the differences in incidence rates between races. Age is the most

well documented risk factor for the development of prostate cancer. At least 70% of patients diagnosed with prostate cancer are above the age of 65 at the time of diagnosis (1), and these men account for more than 90% of all prostate cancer mortality (15). Interestingly, in a Spanish postmortem study of men that had not been diagnosed with nor died from cancer, 33% of men in their eighties had evidence of prostate cancer at death (16).

Although the data implicating diet as a risk factor is not as well defined as age, there have been many epidemiological studies attempting to correlate diet with prostate cancer development. Some of the most convincing data involves the analysis of prostate cancer incidence rates in the decedents of Asian immigrants. In these studies, the risk of developing prostate cancer in these families eventually, through time, equaled that of the members of their new society (17;18). Other studies have tried, therefore, to relate the consumption of a Western diet high in red meat and fat content with prostate cancer risk (19). Although the data is not free of caveats, on the whole it does support a role for the Western diet as a risk factor for the development of prostate cancer.

While highly penetrant genes conferring prostate cancer have yet to be found, there have been numerous genes that have been linked with hereditary prostate cancer. Genes that have been linked to familial prostate cancer include, but are not limited to; *RNASEL*, *NBS1* and *CHEK2* which encode an RNase, a protein involved in cell cycle regulation, and a regulator of p53, respectively (20-25). There is also a specific study evaluating gene linkage in the role of hereditary prostate cancer in African American men (26). Although some of the data is contradictory, the study of hereditary prostate cancer

is a relatively new field in prostate cancer research, and has the potential to discover important genes involved not only in familial inheritance of prostate cancer risk, but also determining genes involved in the carcinogenesis of sporadic prostate cancer.

Clinical Manifestations

Early detection of prostate cancer is, theoretically, one way in which the mortality associated with the disease can be minimized. The American Cancer Society and the American Urological Association recommend that men 50 years and older should be screened at least annually (27;28). The most widely used screening tool that has been shown to have a high positive predictive value detects blood serum levels of Prostate-Specific Antigen, or PSA. PSA is a serine protease that is readily detectable in prostatic secretions synthesized by luminal cells, and is thought to serve a role in the liquefaction of the ejaculate (29-31). The PSA assay is regularly used in the clinic, however it does result in false positives and negatives (32). The efficacy of the PSA test has been shown to be increased when used in combination with a digital rectal exam (DRE) (33). The DRE is used to detect both prostate cancer and benign prostatic hyperplasia (BPH), yet it is a subjective exam with variable sensitivity. The DRE has been shown, however, to detect prostate cancers in men that have normal PSA levels (34). Current research is ongoing to find alternative or additional screening tools for prostate cancer that will result in increases in selectivity and specificity.

At presentation, men with prostate cancer may complain of symptoms common to cancer such as lethargy, weight loss and pain. Clinical manifestations more specific to prostate cancer include loss of bladder control, pelvic soreness and bone pain, however

loss of bladder control and pelvic pain can be indicative of BPH (35). Bone pain is a particularly worrisome indication due to the possibility of bone metastasies, a frequent occurrence in late stage prostate cancer (36). Following results of abnormal PSA and DRE tests, biopsies are taken of the prostate so a proper diagnosis can be made. A biopsy consists of multiple corings of the apex, middle and the base of the prostate bilaterally guided by a transrectal ultrasound (37;38).

Prostate Intraepithelial Neoplasia (PIN) is now generally considered to be a precursor lesion to prostate cancer (39). While low-grade PIN has not been found to be associated with increased risk of prostate cancer (40;41), high-grade PIN is associated with a 25% to 35% risk of detecting prostate cancer on subsequent biopsies (42). It is hypothesized that possible changes in gene expression during the development of PIN serve as contributing factors to prostate carcinogenesis.

Prostate cancer is most frequently staged through the Gleason sum scoring system (43). In the Gleason scoring system, the two most predominant phenotypes of tumor are given a score from 1 to 5. These two scores are then added to get the Gleason score, where the lowest score of 2 is the most differentiated tumor, and the highest score of 10 would be the least differentiated tumor and signify the worst prognosis. The more universal cancer staging system known as TNM (where T stands for primary tumor, N for regional lymph nodes and M stands for distant metastasis) is also used often for prostate cancer staging. In TNM staging, the primary tumor is sub-classified into T1 through T4 based on the involvement of the tumor within the prostate itself (T1 and T2) and the extent of capsular penetration and involvement of regional organs (T3 and T4) such as

the seminal vesicles or bladder. Both the lymph node and distant metastasis scores are sub-classified into and X, 0 and 1 which describe unknown, no metastasis and positive metastasis respectively (44).

Prostate cancer is considered a hormone responsive disease, and therefore androgen deprivation is frequently the first line of therapy for advanced disease. Androgen deprivation consists of either chemical or surgical castration, thereby eliminating the production and release of androgens. The difficulty associated with androgen deprivation is that eventually the vast majority of tumors become androgen independent with regard to cell proliferation and tumor growth. Eventually these tumors must be either surgically removed or eliminated through radiation therapy.

The only curative therapy for prostate cancer is removal of the organ, or radical prostatectomy. More recently, the radical perineal prostatectomy was developed, the modifications of which enabled surgeons to preserve the nerves through the prostate thereby preserving sexual potency, one of the major side effects that contributes to lower quality of life (45). Radiation is considered an effective alternative to prostatectomy (46). Radiation can be applied in two forms, external beam or brachyotherapy, where radioactive beads are implanted into and surrounding the tumor in the prostate. To some, the efficacy of the various treatment options remain controversial, however radical prostatectomy is the standard to which all other therapies are compared.

Primary prostate cancer is a slow growing tumor, as such, over the past decade clinicians have offered an alternative to prostatectomy often referred to as “watchful waiting”. Watchful waiting assumes that the most significant benefit to this “therapeutic”

approach is quality of life; watchful waiting avoids the sexual side effects of a prostatectomy. However, in one of the first randomized trials that compared these two approaches to prostate cancer, Holmeberg *et. al.* found a significant reduction in disease associated mortality in men that had prostatectomies. Furthermore, the reported quality of life was similar between the two groups (47), suggesting that watchful waiting is a "therapeutic" option that should be intensely reviewed.

Death from prostate cancer begins when tumor cells metastasize to distant organs. Possible routes of metastasis include migration along nerves, blood and lymph vessels that enervate the prostate (48). The most frequent site of metastasis of prostate carcinoma is the regional lymph nodes. Tumor infiltration into lymph nodes has been shown to decrease five year survival rates from 71.4% to 53.9% (49). Tumor metastasis therefore, is of great clinical importance. Further evidence of this is the fact that the risk of developing prostate carcinoma in the general public outweighs the rates of death. Anglo American men are five times more likely to develop prostate cancer than they are to die from it (1). This suggests that there is a clinical difference in genotype and phenotype in the sub-set of metastatic tumors, that these genetic changes confer metastatic capabilities to tumors, and ultimately kill patients. Therefore, if researchers can determine the causes of the phenotypic changes in deadly variants of prostate carcinoma, clinicians will have new and better targets for treatment of prostate cancer.

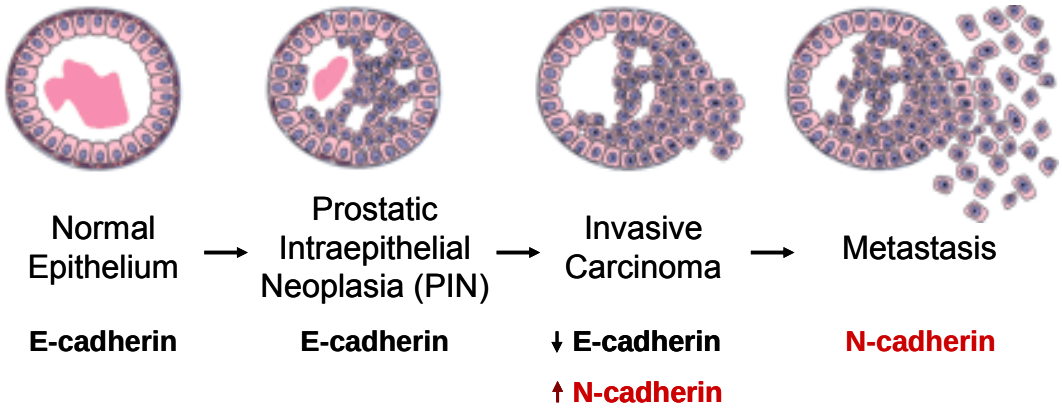

Figure 1. Schematic of the progression of prostate cancer and cadherin expression.

Normal prostate glands are composed of luminal cells surrounded by basal cells. Luminal cells in a normal prostate gland express high levels of E-cadherin. During PIN there is hyperproliferation of luminal cells, with a concomitant loss of basal cells and the surrounding basement membrane. E-cadherin expression is maintained during the development of PIN. E-cadherin expression becomes heterogenous during the progression of prostate cancer to an invasive carcinoma. Accordingly, N-cadherin expression can be detected in invasive prostate cancer. N-cadherin expression may facilitate the detachment of tumor cells from the tumor body, enabling prostate cancer metastasis to distant sites. Modified from reference (50).

Genetic Alterations During Prostate Carcinogenesis

The process of carcinogenesis involves the accumulation of genetic mutations in specific genes that regulate cell proliferation, apoptosis and migration (51). The genetic aberrations during carcinogenesis are cumulative, whereby additional mutations to genes that regulate the afore mentioned cellular processes further drive tumor cells towards metastatic disease. Mutations in genes during carcinogenesis change the expressed protein, increase the amount of protein product, or eliminate the production of the protein product. Not all mutations during carcinogenesis will lead to increases in tumorigenesis; deleterious mutations will be selected against whereas beneficial mutations will confer a selective advantage to a tumor cell. This process of carcinogenesis is under the selective pressures of the surrounding tissue where beneficial mutations confer a selective advantage and deleterious mutations are eliminated is called clonal expansion. The genes that contribute to prostate carcinogenesis can be generally classified into three groups; tumor suppressors, oncogenes and genes involved in invasion and metastasis.

One tumor suppressor that has been shown to be deleted in prostate carcinoma tissues is NKX3.1 (52). *NKX3.1*, located on chromosome 8p21, encodes a prostate specific homeobox transcription factor that has been shown to function as a transcriptional repressor *in vitro* (53). Target genes of NKX3.1 include members of the Sp family of transcription factors, and NKX3.1 seems to inhibit the transcriptional activity of the androgen receptor (54;55). In a study by Bowen *et. al.*, NKX3.1 was shown to be absent in 6% of low Gleason grade prostate cancers, 22% of Gleason high grade cancers, 34% of androgen independent cancers and 78% of metastatic cancers,

suggesting that loss of NKX3.1 correlates with prostate tumor progression (56). Accordingly, prostate specific deletion of NKX3.1 in mice leads to the development of a PIN-like lesion (57;58).

While NKX3.1 is one of the more interesting prostate specific tumor suppressors, the phosphatidylinositol 3,4,5-triphosphate phosphatase, PTEN, may be one of the more important tumor suppressors during prostate carcinogenesis. *PTEN* was first implicated in prostate cancer due to the frequent deletion of chromosome 10q23 where PTEN is located (59). The primary role of PTEN is the regulation of the activity of the serine/threonine kinase Akt, which has been shown to be an important regulator of proliferation and apoptosis (60). There seems to be a functional interaction between PTEN and NKX3.1 in prostate tumor carcinogenesis, as the dual heterozygous PTEN-NKX3.1 mouse knockout develops prostate carcinoma (61). An important characteristic of these mutant mice is that there is an age dependent development of PIN and cancerous lesions. Characterization of the changes in the tumors that develop in these mice show similarities to the human disease; interestingly, the tumors become androgen in-sensitive and loose E-cadherin expression. Moreover, the prostate specific PTEN mouse knockout develops prostate adenocarcinoma that mimics the phenotypic progression from PIN to metastatic cancer (62). These authors also presented evidence suggesting that PTEN may be epigenetic to NKX3.1 due to the loss of NKX3.1 in the prostate specific PTEN knockout. Although more research needs to be done to further define the functional interaction between PTEN and NKX3.1, these two tumor suppressors have a significant, possibly a fundamental role in prostate carcinogenesis.

Another tumor suppressor that may be involved in the progression of prostate carcinoma is p53. Although p53 is an integral regulator of both the cell cycle and a DNA repair machinery, and is mutated in the vast majority of human cancers (63), it is rarely mutated in early localized prostate carcinoma. On the contrary, 20% – 40% of all metastatic prostate cancers contained mutations in p53 (64;65), suggesting that p53 has a pivotal role in the progression of prostate carcinoma.

Of the oncogenes that have been documented to drive prostate carcinogenesis, the activity of the androgen receptor (AR) is fundamental to the development of the disease. Initially, most prostate cancers are sensitive to androgen deprivation, however most advanced tumors have progressed to an "androgen independent" state where cell proliferation is not dependent on the AR. It has been suggested that mutations of the AR which alter the ligand specificity, and that amplification of the AR gene both result in hypersensitivity to androgen activation (66-68). The androgen receptor is also controlled by multiple co-regulators, which is another mechanism by which tumor cells may adapt to alter AR activity (69). Although the mechanisms by which tumor cells develop androgen independence continue to be studied, recent evidence clearly indicate that androgen dependent signaling pathways remain activated during androgen independence (70;71).

Another oncogene that has been shown to play a role in prostate cancer progression is Bcl-2 (72;73). The Bcl-2 gene product is involved in the inhibition of programmed cell death, or apoptosis. Immunohistochemistry analysis of Bcl-2 indicates that it is not expressed in normal prostate luminal cells, but is aberrantly expressed in

metastatic, androgen independent disease (72;73). Moreover, forced expression of Bcl-2 has been shown to accelerate carcinogenesis of prostate cancer in a transgenic mouse model (74). The expression of Bcl-2 is regulated by Akt signaling to cyclic-AMP response element (CREB) sites located within the proximal promoter in the *Bcl-2* gene (75). Multiple signals upregulate the transcription of *Bcl-2*, including the signaling of N-cadherin (76), making Bcl-2 a vital component of the anti-apoptotic machinery.

Integrin Biology in Prostate Carcinoma

Integrins are heterodimeric transmembrane proteins that mediate cell adhesion to the extracellular matrix (ECM). Integrin molecules function as heterodimers, such that an α subunit pairs with a β subunit creating a functional integrin heterodimer. The major integrin subunits that are detectable (through immunolocalization) in normal human prostate epithelium include the α2, α3, α5, α6, αv, β1 and the β4 integrins (77;78). The expression of these integrins is maintained during PIN, but during the progression of prostate carcinoma integrin subunit expression changes such that the detectable α integrin subunits in cancer tissues are α2, α3, α5 and α6, with the α6 being the most detectable. Protein expression of the β subunits is also altered; the β4 subunit is lost while immunolocalization of the β1 integrin is maintained (77). These data, therefore, predict that the integrin receptors that are expressed during prostate carcinoma are a collagen receptor (α2β1), a laminin receptor (α3β1), to a lesser extent the fibronectin receptor (α5β1) and the α6β1 laminin receptor which is most likely the predominant ECM receptor.

The changes in integrin protein expression have significant impact on the progression of prostate carcinoma. In prostate epithelium, the α6β4 integrin heterodimer is the major component of the hemidesmosome. The hemidesmosome attaches epithelial cells to the laminin 5 rich basement membrane through the α6β4 integrin association with the cytokeratin based intermediate filament cytoskeleton (79). Mutations in the α6 or β4 integrin subunits has been shown to cause the skin blistering disease junctional epidermolysis bullosa, which is manifested by loss of skin integrity resulting in skin detachment with varying degrees of severity (80;81). These data suggest that one major function of the α6β4 integrin in epithelium is to maintain strong adhesion to the basement membrane. In the carcinogenesis of prostate cancer, loss of protein expression of the β4 integrin can be detected as early as PIN, indicating that the function of the α6β4 integrin heterodimer is selected against in prostate carcinoma. This is strong evidence for a role for the α6β4 in the maintenance of the epithelial phenotype in normal prostate.

While the α6β4 integrin connects with the intermediate filaments, the β1 integrin associates with the actin based cytoskeleton. The β1 integrin subunit is maintained during prostate carcinogenesis, thereby switching prostate cancer cells from the intermediate filament cytoskeleton, to the actin based cytoskeleton (77). The β1 integrin has been shown to be important in multiple cancer cell migration models *in vivo* and *in vitro*; more importantly the α6β1 integrin increases the invasive ability of the Du145 prostate cancer cell line in a mouse invasion model (82).

Both the β4 and β1 integrins, when bound to an ECM ligand, form multi-protein complexes through interactions with the cytoplasmic tails of the integrin heterodimers,

termed hemidesmosomes and focal adhesions or complexes for the β4 and β1 integrins respectively. The differences in the functions of the β4 and β1 integrins become evident when one analyzes the signaling proteins that associate with hemidesmosomes and focal adhesions. While there is more data concerning the characterization of the proteins within focal adhesions, hemidesmosomes have been shown to contain signaling molecules involved in MAPK and growth factor signaling (83).

The β1 integrin binds a wide variety of adapter proteins and actin binding molecules which combine to elicit a plethora of intracellular signals controlling both cell migration and transcription. The β1 integrin is expressed as multiple splice variants; β1A, β 1B, β 1C, and β 1D (84). Of interest are the β 1-A and β 1-C splice variants, the function of which is to stimulate and inhibit cell proliferation respectively. During prostate carcinogenesis, the mRNA of both splice variants are reduced, but only the β 1-C protein is reduced (85). These data suggest that in addition to adhering cells to the ECM, an additional role for the β 1-A integrin is to facilitate cell proliferation in prostate cancer.

One of the major integrin binding proteins which binds to the cytoplasmic tail of the β1 integrin is p125 focal adhesion kinase (FAK) (86). Following association with integrin following cell adhesion, the kinase domain of FAK phosphorylates tyrosine residue 397 (Y-397) creating an SH2 binding site for proteins such as Src (87). Phosphorylated Y-397 of FAK also serves to recruit the p85 subunit of PI3K (88), thereby regulating cell survival by activating Akt through the production of phosphatidyl inositol 3,4,5 phosphate, and the recruitment of PKB/ILK. In human prostate cancer tissues FAK expression is maintained throughout carcinogenesis, and possibly expressed

at elevated levels (89). In prostate carcinoma cell lines, FAK activity correlates with invasive potential. Interestingly, in this study, Slack *et. al.* noted that the Y-397 autophosphorylation site is dependent on cell adhesion to ECM, whereas the other Src dependent tyrosine phosphorylation sites were not. Moreover, attenuating the activity of FAK through the overexpression of the kinase deficient mutant FRNK significantly inhibited the migration of prostate carcinoma cell lines (90). FAK is, therefore, one of the more important signaling molecules associated with integrins in focal adhesions *in vitro*.

Multiple actin binding proteins associate directly with the β1 cytoplasmic tail, including talin, α-actinin and filamin, which link β1 integrin heterodimers with filamentous actin (91-94). Filamentous actin is mobilized to sites of integrin adhesion through the activities of the Rho family of GTPases which includes CDC42, Rac and Rho isoforms (this discussion will be limited to CDC42, Rac1 and RhoA). Rho GTPases cycle from an active GTP bound state, to an inactive GDP bound state following the hydrolysis of GTP. This cycling activity of Rho GTPases is catalyzed by guanosine exchange factors (GEFs) and guanosine activating proteins (GAPs), which catalyze the exchange of GTP for GDP, and catalyze the hydrolysis of GTP respectively. While much of the information of Rho family of GTPases has been documented in fibroblasts and tumor cells from various origins, the regulation of Rho GTPases downstream of integrin adhesion seems to be in agreement in the majority of cell lines. Of the Rho GTPases, CDC42 and Rac1 seem to be activated and inactivated independently of RhoA, and it is possible that the activation of CDC42 and Rac1 and RhoA may be mutually exclusive

(95). It is thought that Rac1 and CDC42 control lamellepodia and membrane extentions, while RhoA regulates stress fiber formation.

Following integrin adhesion, there is rapid activation of CDC42 and Rac1 which is catalyzed by the GEF Vav1 or 2, Tiam1 and DOCK 180 (96-99). The activities of CDC42 and Rac1 are important for early stages of integrin association with the actin cytoskeleton, and for cell spreading. In contrast to the activities of CDC42 and Rac1, there seems to be a slight inactivation of RhoA during the initial stages of integrin activation possibly mediated by p190RhoGAP (100), followed by an increase in RhoA activation in the hours following integrin activation through unknown RhoA GEFs (101), which is consistent with the possible exclusivity of Rho GTPase activation. However, the kinetics of RhoA activation downstream of integrins may depend on the cell model system used.

There are multiple effectors of the Rho GTPases that are activated downstream of integrins. A few of the more important CDC42 and Rac1 effectors are p21 activated kinase (PAK), Par-6 and IQGAP. PAK is a protein kinase that is activated upon binding of Rac1 or CDC42, and is involved in actin cytoskeletal dynamics and gene regulation (102-104). Par-6 associates with atypical PKC isoforms and may be a crucial mediator of CDC42 and Rac1 activity (105;106). IQGAP is an interesting effector of CDC42 and Rac1 which regulates cadherin based cell adhesion through association with β-catenin and inhibiting the cadherin link to actin (107). While the effectors of CDC42 and Rac1 are important mediators of GTPase signaling, and are logical pharmaceutical targets to

inhibit events downstream of these GTPases, their role(s) in prostate carcinogenesis remain poorly defined.

The most widely studied effector of RhoA is Rho-associated kinase (ROCK, RoK or Rho-Kinase; hereafter described ROCK). ROCK is a serine/threonine kinase that consists of an N-terminal kinase domain, a central putative coiled-coil domain which contains a Rho binding domain (RBD), and a C-terminal pleckstrin homology (PH) domain (108). The C-terminal PH domain of ROCK binds to and inhibits the activity of the kinase domain, ROCK is only active when the RBD domain is bound by active GTP-bound RhoA (109). A pharmacological inhibitor to ROCK, Y-27632, binds to the ATP pocket of the kinase domain and inhibits kinase activity (110).

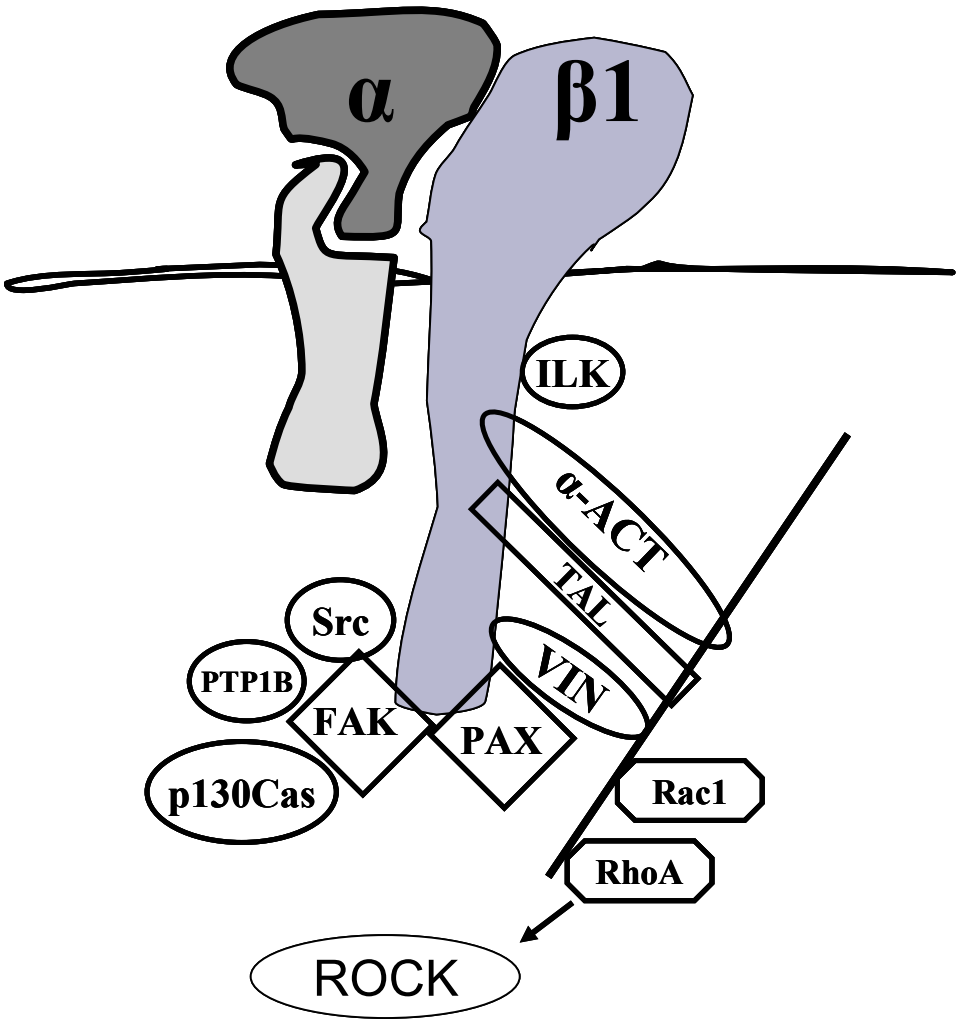

Figure 2. Simplified schematic of signaling from β1 integrin.

The β1-A integrin associates with an α subunit and induces intracellular signaling. The actin binding proteins vinculin (VIN), tallin (TAL) and α-actinin (α-ACT) bind directly to the β1 integrin. Actin is mobilized to sites of integrin contact through the activities of the small GTPases Rac1 and RhoA. ILK also binds to the β1 integrin, and regulates E-cadherin expression. The scaffolding proteins FAK and paxillin (PAX) associate with β1

integrin, and recruit other signaling molecules such as src, p130cas and PTP1B to the signaling complex.

ROCK activity is integral to cell contractility and migration, and the substrates of ROCK are important regulators of myosin-based contractility and actin stabilization. Myosin light chain (MLC) must be phosphorylated to associate with actomyosin complexes and participate in cellular contraction and migration. MLC is phosphorylated by myosin light chain kinase (MLCK) and is dephosphorylated by myosin light chain phosphatase (MLC-P) (111). ROCK has been shown to inactivate MLC-P through direct phosphorylation (112;113), and to directly phosphorylate MLC (114). Another mechanism by which ROCK regulates cell migration is through the control of actin stabilization. ROCK phosphorylates and activates LIM-kinase which inhibits the actin depolymerizing protein cofilin (110;115). In addition to regulating cell contractility and actin dynamics, ROCK signaling has been shown to affect transcriptional events through the regulation of activity of c-jun N-terminal kinase (JNK) and c-jun expression (116). ROCK may further regulate nuclear events through the regulation of the LIM-kinase phosphorylation of CREB and CREB mediated transcriptional activity (117). Furthermore, LIM-kinase has recently been shown to be a pivotal factor regulating the invasion of prostate carcinoma cells in vitro (118). One possibility that explains the importance for RhoA-ROCK signaling in prostate cancer is that tumor cells may need more than one biochemical route to metastasis. While Rac1 and FAK signaling are undoubtedly important for the migration and invasion of prostate cancer, RhoA-ROCK signaling may offer divergent and distinct signaling pathways that upregulate metastatic

genes and increase cell contractility. RhoA-ROCK signaling is quickly becoming an area of intense research which is elucidating many novel functions of this signaling pathway, and will likely place RhoA-ROCK as key modulators of tumor cell biology.

Forkhead Transcription Factors in Prostate Carcinoma

The Forkhead transcription factor family is characterized by a DNA binding domain called the "winged helix" or Forkhead domain, based on the defect resulting from the deletion of the *fork head* gene in *Drosophila* (119-121). The structure of the winged helix domain was gleaned from the crystal structure of the mammalian founding member of the Forkhead family, hepatocyte nuclear factor-3 (HNF-3). The 110 amino acid DNA binding sequence of HNF-3, when bound a cognate DNA sequence, resembles the shape of a butterfly with two wings (122). The mammalian Forkhead family is quite large with an estimated 50 members of the family in humans, leading Kaestner *et. al.* to develop a unified nomenclature based on phylogenetic analysis which uses the symbol FOX (Forkhead box) followed by subclass and member, or FOXO1 for instance (123).

The FOXO family of FOX transcription factors has a well defined role in carcinogenesis of multiple tumor types (124). FOXO proteins are involved in the transcription of genes that drive apoptosis and inhibit cell cycle progression, and are therefore frequently inactivated through post-translational modification during carcinogenesis (125). FOXO family members are phosphorylated by the serine/threonine (ser/thr) kinase Akt, which creates binding sites for the scaffolding proteins 14-3-3 which sequester FOXO transcription factors in the nucleus (126-128). The phosphorylation which regulates FOXO nuclear localization tends to be in, or near nuclear localization

sites (NLS), which are amino acid sequences that are necessary and sufficient for nuclear import of proteins (129). However, it is important to note that phosphorylation of FOXO proteins within the DNA binding domain (which contains a NLS) has been shown to inhibit DNA binding of FOXO, but not to inhibit nuclear localization (130;131). More recently, acetylation of FOXO members on lysine residues has been documented to be an additional mechanism to inactivate transcriptional activity (132;133), while other reports provide evidence that de-acetylation is necessary for transcriptional activity (134).

In prostate cancer FOXO function is similar to that of other systems, promoting apoptosis and inhibiting the cell cycle (135-137). The mechanism of activity of FOXO proteins includes direct transcriptional activity and functional interactions with the androgen receptor, interactions which include FOXH1 (135;138). The regulation of FOXO members in prostate carcinogenesis involves inactivation through both ser/thr phosphorylation by Akt, and by acetylation (134-137). While Akt activation downstream of β1 integrin and N-cadherin activation may result in the inactivate FOXO members, the signaling pathway that regulates acetylation of FOXO members is, in part, regulated by RhoA (134).

A recently identified subclass of the FOX super family, FoxP1 through FOXP4, has unique structure and function (139). Originally isolated from mouse, mFOXP members are expressed in lung epithelium, and are involved in cardiac cushion morphogenesis (139;140). The novel feature of the FOXP family is that they act as transcriptional repressors. While other members of the FOX super family (notably FOXL members) also seem to repress in addition to activating transcription (124;141),

the FOXP proteins are uniquely specific to transcriptional repression. Both human and mouse FOXP transcription factors bind to the same DNA consensus sequence TTATTT(G/A)T (142;143). The most studied member of the FOXP family is FoxP1, while little is known about FoxP2-4. In mouse, FoxP1 is expressed as four splice variants, FoxP1A-D. The mFoxP1 protein is 90 Kilodaltons (KDa), and has multiple domains including N-terminal poly-glutamine and glutamine rich regions, zinc finger, lucine zipper and the Forkhead domain (143). The structure of the human FoxP1 is similar, however the protein lacks the poly-glutamine track at amino acids 78 – 108, making the human FoxP1 protein about 80 KDa (139). The structural differences in murine and human FoxP1 may result in differences in the protein domains necessary for transcriptional repression, however FoxP1 from both species have been shown to be potent transcriptional repressors (142;143). Interestingly, Shi *et. al.* documented that the human FoxP1 domain(s) necessary for transcriptional repression are located in the N-terminus of the protein, suggesting that the glutamine rich region of FoxP1 may interact with the transcriptional machinery (142). These data also imply that, although not yet documented, human splice variants of FoxP1 that lack the N-terminus may be transcriptionally inactive, or serve as dominant negative DNA binding proteins.

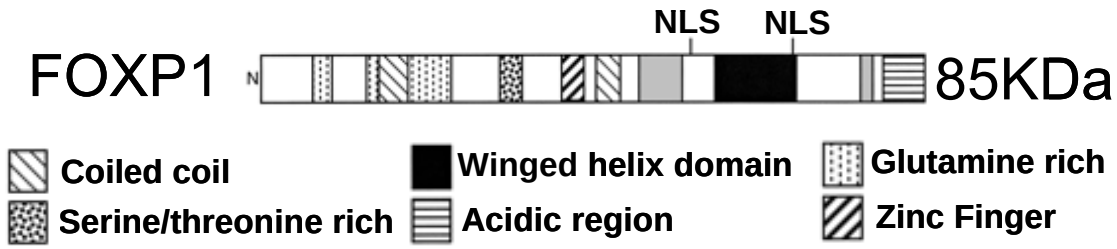

Figure 3. Protein motifs of the putative tumor suppressor FoxP1.

The human FoxP1 protein is ≈85 KDa, and has multiple protein domains including; glutamine rich sequences, two putative coiled coils, a serine/threonine rich region, a putative zinc finger, the Winged Helix consensus and an acidic region. Adapted from reference 139.

FoxP1 has been implicated as a tumor suppressor in multiple human tumor types. Expression of FoxP1 message can be detected in both normal and cancer from breast, prostate, colon, lung, rectum and uterine tissues (139). The immunolocalization of FoxP1 protein in these various tumors displays both reductions in staining intensity, and lack of nuclear localization, suggesting post-translational modification(s) of FoxP1. While *FoxP1* message seems to be steady, if not increased, in prostate carcinoma, the protein nuclear localization was absent in a metastatic lesion (133). These data highlight the importance of the post-translational modifications of the FOX super family in the regulation of FOX function. Accordingly, recent evidence suggests that murine FoxP isoforms homo- and hetero-dimerize through the leucine zipper domain (144). Expression of FoxP1 correlates with improved survival in human breast carcinoma, and reduced expression of FoxP1 has been documented in a rat lung adenocarcinoma model (145;146). While much of the ground work concerning the regulation of FoxP1 function has begun, the role of FoxP1 in prostate carcinogenesis has yet to be established.

Twist1 in Development and Prostate Carcinogenesis

The basic helix-loop-helix (bHLH) transcription factor Twist is an important regulator of developmental patterning in diverse species from fruit flies to humans. The Twist family is composed of Scleraxis, Paraxis, Dermo-1 (also called Twist2) and Twist (147). Originally discovered in *Drosophila*, mutant flies deficient in *D*Twist expression exhibit a severe twist of the torso, hence the name (147). Homozygous mutant flies fail to undergo proper gastrulation or develop any mesodermally derived tissues, indicating that DTwist regulates mesodermal patterning during *Drosophila* development (148;149).

In mice, heterozygous deletion of *mTwist* results in dose-related craniofacial deformities (150), which correlate to the human *Twist* haplo-insufficiency craniofacial mutations seen in Saethre-Chotzen syndrome (151). The mouse homozygous mutant differs from the *Drosophila* mutant in that mutant mice gastrulate normally, but die at day E10.5 – 11 due to severe defects in neural tube closure. Additionally, there are defects in the cranial mesoderm, deficiencies in the facial skeleton and stunted development of the limb buds (152). Interestingly, the failure of neural tube closure in the *mTwist* mutant is strikingly similar to the phenotype of the *N-cadherin* mutant mice (153). Moreover, analysis of the neural cells in *mTwist* mosaic mice reveal that the cells lacking *mTwist* expression fail to associate with neighboring cells, suggesting defects in cell-cell adhesion which act in a cell autonomous manner (152). In vivo, therefore, the *TWIST* gene is an important regulator of necessary developmental signals in invertebrates and vertebrates, regulating cell fates and possibly cell adhesion.

As a member of the bHLH class of transcription factors, the main feature of the twist protein is the bHLH region which is necessary for protein-protein interactions and for DNA binding. First characterized in the DNA binding proteins E12 and E47, the HLH region is composed of two amphipathic helices separated by a loop of varying length, while the basic region is composed of basic amino acids and precedes the first helix (154;155). bHLH transcription factors bind to a DNA consensus sequence called an E-box, CANNTG. *D*Twist has been shown to bind to the following E-box sequences: CATATG, CATGTG, and to a lesser extent CATCTG and CACGTG (156). Human twist is composed of 202 amino acids and the sequence of twist from all vertebrates

including human, frog and zebrafish is 85% - 100% identical in the bHLH domain, but diverges outside of this domain (147). There are two putative NLS sequences within the twist protein (aa 37 – 40, and 73 – 77), the importance of which has been documented in a naturally occurring mutation at aa 37 – 40 which prevents nuclear accumulation and results in mild craniosynostosis (157). Another important region of the twist protein is the "Twist box" which binds the transcription factor Runx2 (158).

Twist has been shown to interact with a number of bHLH and other transcription factors. The N-terminus of twist associates with other non bHLH transcription factors such as the histone acetyltransferases p300 and p300/CBP (159). Also, the N-terminus of twist was recently shown to interact with the homeobox transcription factor HOXA5 (160). Twist has been shown to homodimerize, and heterodimerize with other bHLH proteins such as E12, Mef2 and MyoD in Drosophila and mice (156;161-163). The C-terminal "Twist box" binds Runx2, which when mutated results in severe craniofacial deformities in mutant mice (158).

The effect of *Twist* overexpression *in vitro* highlights the importance of homo- and hetero-dimerization partners as regulators of twist activity, and twist regulation of other bHLH proteins. Analysis of Twist expression in mouse embryos indicates that Twist expression is absent in the myotome, and it is thought that twist may inhibit muscle differentiation (164;165). Accordingly, overexpression of Twist inhibits muscle differentiation in C2C12 and embryonic stem cells (166;167). However, homozygous Twist mutant mice do not develop any extra muscle tissue (166). While these data appear to conflict, they may be explained by work from Hebrok *et. al.* that illustrates the

difficulty in data interpretation when working with bHLH transcription factors. In this work, it was documented that the DNA binding domain was not necessary for muscle specific gene repression. When unable to bind DNA, overexpressed twist functioned in the same manner as an endogenous dominant negative HLH protein ID2, in that the twist mutant unable to bind DNA served as a decoy molecule binding up other important bHLH transcription factors and inhibiting their transcriptional activity. Mutation of the "Twist box" resulted in a similar but less obvious phenotype (158;168). Hebrok and colleagues argue that it is the interaction between E12 and twist that represses muscle specific transcription. When overexpressed, twist may interact with numerous transcription factors, and the experiments to determine the exact repertoire of interacting partners have not been done. It becomes difficult, therefore, to know *a priori* which interacting partner *Twist* is effecting when overexpressed. The only convincing way to determine whether a specific interaction between twist and another protein is regulating a gene is to complete experiments that increase and eliminate the expression of twist. With the increased efficiency of small interfering RNA (siRNA), these experiments have become much easier.

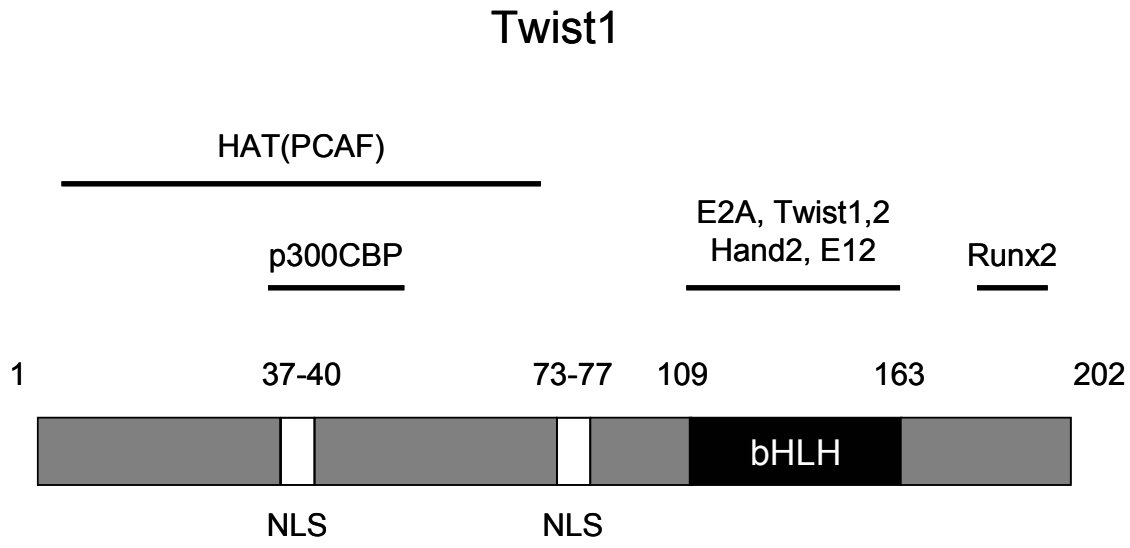

Figure 4. The bHLH transcription factor Twist1.

The transcription factor Twist1 is 202 aa long, contains two putative nuclear localization sequences and the basic helix-loop-helix domain. The regions of known interaction domains with other proteins (from multiple model systems) are indicated with lines, with the interacting protein(s) written above.

More recently, researchers have focused on a possible role for Twist in carcinogenesis. The seminal paper indicating that twist contributes to the progression of cancer was published in 2004 by Yang and colleagues (169). These researchers provided strong evidence that Twist regulates metastasis of human breast cancer cells in a mouse orthotopic tumor model. *Twist* expression was shown not to contribute to cell proliferation or inhibition of apoptosis, in contrast Yang *et. al.* suggest that *Twist* may have a function unique to the induction of metastasis through the regulation of cell migration and invasion. In this work, inhibition of *Twist* expression with siRNA eliminated tumor metastasis *in vivo*, and overexpression of *mTwist* in breast epithelial cells upregulated mesenchymal markers such as fibronectin, vimentin and importantly N-cadherin. Concomitant with the upregulation of mesenchymal markers, epithelial genes such as *E-cadherin* were down regulated, and cell migration was increased *in vitro*. *Twist* was also shown to be upregulated in human breast cancer tissues. Twist is also expressed in gastric cancer, and this expression correlated with N-cadherin expression (170). More recently, similar findings were found from prostate cancer tissues (171). An important discovery in prostate carcinoma was that Twist expression correlated with Gleason score, and that staining of tumor sections for twist detected both nuclear and cytoplasmic localization. These researchers also implicated twist in the regulation of chemotherapeutic sensitivity, which agrees with data from breast cancer cells *in vitro*. Yang *et. al.* did not evaluate apoptosis or proliferation in the presence of chemotherapies (169). These data suggest that twist is a potent transcriptional regulator of genes

involved in the metastatic spread of carcinoma, including N-cadherin. Whether twist regulates these genes directly or indirectly is unknown.

Cadherin Biology in Development and Prostate Carcinogenesis

Cadherins are transmembrane proteins that mediate calcium dependent cell-cell adhesion. The cadherin superfamily consists of five sub-families (the classical, non-classical, desmosomal, proto, fat-like and flamingo cadherins) with nearly 80 documented members (172). Of specific interest to the development and progression of prostate cancer are the classical cadherins E- and N-cadherin. Over the past two decades, the contribution of E- and N-cadherin to the carcinogenesis and metastatic spread of prostate cancer, and carcinomas in general, has shaped the biological understanding of cancer as a disease. Both E- and N-cadherin, and the cytoplasmic proteins that associate with classical cadherins, the catenins, are now known to contribute to fundamental changes in cancer progression, and to be essential regulators of tumor metastasis.

The extracellular portion of classical cadherins is composed of five cadherin domains (EC domains) that mediate homotypic interactions (173). Each EC domain binds calcium ions, inducing the conformational change required for functional adhesion (174-176). Cadherins function in cis on the surfaces of individual cells through an interaction between EC1 and EC2, that also involves EC3. These cis interactions may result in a long grouping of associated classical cadherin molecules that are oriented front to back, or two cadherin molecules may interact in the classical cis-homodimer. The cis-associated cadherin molecules on the surface of one cell then interact in-trans with cadherins on the surface of a neighboring cell. This trans link is mediated through

interactions between the N-terminal beta strands of the EC1 regions of adjoining cells (177). Importantly, it is the EC1 domain that provides specificity for homotypic interactions, such that distinct cadherin sub-types only interact with like molecules on the surfaces of neighboring cells (178).

There are two distinct regions within the cytoplasmic domain of classical cadherins; the juxtamembrane and the catenin binding domains. The catenin binding domain (CBD) binds β-catenin. β-catenin binds to the CBD through 12 armadillo repeats, each containing three alpha helicies, forming a structure similar to a cylinder (179). β-catenin binds α-catenin, an actin binding protein, which then links directly to the actin cytoskeleton (180). Inhibition of the binding of β-catenin to cadherin results in loss of cadherin mediated cell-cell adhesion (181). The juxtamembrane domain (JMD) of classical cadherins binds p120 catenin. All p120 catenin isoforms contain ten armadillo repeats that are structurally homologous to the β-catenin armadillo domains (182). The armadillo repeats of p120 catenin mediate an interaction with a region of ten amino acid residues within the JMD that are conserved throughout the classical cadherin family (183-186).

In addition to the role for β-catenin association with classical cadherins and functional cell-cell adhesion, β-catenin also plays a pivotal role in the canonical Wnt signaling pathway. When uncoupled from the CBD of classical cadherins, β-catenin is targeted for degradation by the proteosome through an interaction with the adenomatous polyposis coli (APC) tumor suppressor protein. However, upon activation of the Wnt signaling pathway, glycogen synthase kinase 3β (GSK3β) phosphorylates APC and

inactivates its ability to initiate degradation of β-catenin (187). Free cytoplasmic β-catenin is then able to interact with the TCF/LEF transcription factor complex, translocate into the nucleus, and activate target gene transcription (188). The β-catenin signaling pathway has been fundamental to the understanding of colon carcinogenesis, as APC is frequently mutated in colon carcinoma (189).

p120 catenin seems to have both positive and negative influences on cadherin mediated adhesion. Using a cadherin construct containing minimal mutations to the JMD of E-cadherin, it was shown that selective uncoupling of p120 catenin from E-cadherin inhibits strong cell-cell adhesion (186). p120 catenin has been implicated in the homodimerization of cadherin molecules. Using a cadherin construct lacking the CBD, Yap *et. al.* showed that this cadherin construct still retained adhesive ability (183). The adhesive ability of the cadherin construct lacking the CBD was dependent on cis-interactions. A cadherin construct lacking the JMD but retaining the CBD was not able to associate in cis, nor form cadherin clusters. It has also been demonstrated, through the use of laser trapping experiments, that associations of cadherins in cis, and trans associations of cadherins between adjoining cells is an active process not mediated by passive diffusion (190). As p120 catenin binds to the JMD of classical cadherins, these data strongly imply that p120 catenin is, in part, responsible for the cis association of cadherin molecules and the strength of cadherin cell-cell adhesion.

Other studies propose that p120 catenin may act to inhibit cadherin mediated cell-cell adhesion. In Colo205 cells E-cadherin is non-functional, despite the expression of all of the components of the cadherin-catenin complex. In these cells, p120 catenin is

hyperphosphorylated, and transfection of a p120 catenin construct containing N-terminal deletions that remove the majority of the phosphorylation sites restores E-cadherin cell-cell adhesion (191). This suggests that phosphorylation of p120 catenin modulates cadherin mediated cell-cell adhesion. p120 catenin was originally identified as a substrate for phosphorylation by oncogenic Src (192), and is a promiscuous target for phosphorylation by multiple receptor tyrosine kinases (193-195). Transient increases in phosphorylation of p120 catenin may be an effective way of down regulating cadherin cell-cell adhesion during periods of normal physiological cell migration, and aberrant hyper-phosphorylation of p120 catenin seems to be a mechanism to inhibit cadherin cell-cell adhesion. Thus, p120 catenin may be the mediator of growth factor inactivation of E-cadherin function by hepatocyte growth factor and epidermal growth factor through phosphorylation of p120, or the recruitment of kinases to the cadherin-catenin complex (196;197).

The activity of cadherins is regulated by the activities of the Rho family of GTPases. Expression of constitutively active Rac1, or a GEF for Rac, Tiam1, increases the adhesive activity of E-cadherin in vitro (198-200). CDC42 and Rac1 have been shown to regulate E-cadherin activity through the activity of IQGAP. IQGAP seems to bind directly to and inhibit the cadherin association with actin through α-catenin. Active CDC42 and Rac1 inhibit the ability of IQGAP to bind β-catenin, thereby maintaining cadherin adhesion (107;197). The effects of RhoA on E-cadherin activity are less consistent. This could be due to more recent findings that the activity of RhoA is inhibited following the ligation of E-cadherin (201). This suggests that the activity of E-

cadherin requires the precise regulation of RhoA activity. Both Rac1 and RhoA are necessary to initiate E-cadherin adhesion; Rac1 may be required to maintain the link to the cytoskeleton, and the continuous activity of RhoA may inhibit Rac1 activity.

Regulation of E-Cadherin Expression

The expression of E- and N-cadherin are regulated during embryogenesis and development. E-cadherin is the first adhesion molecule expressed during mouse embryogenesis and can be detected as early as the 8 cell stage (202). During development, E-cadherin is necessary for the compaction of the morula and to organize epithelial tissues (203;204). E-cadherin null mice fail to develop the trophectoderm epithelium, and therefore fail to implant into the uterus. The N-cadherin null mouse dies at embryonic day 10 due to severe cardiac defects. In addition, the neural tube fails to close in N-cadherin null mice and there are defects in other tissues that normally express N-cadherin (153). Interestingly, rescue of the N-cadherin null cells with forced expression of E-cadherin salvages much of the tissue and morphological defects due to lack of cell-cell adhesion, however tissues that normally expressed N-cadherin had high levels of apoptosis even though E-cadherin was expressed (205). Expression of E- and N-cadherin in cadherin null embryonic stem (ES) cells results in epithelial structures and neuroepithelium and cartilage, for E- and N-cadherin respectively (206). Therefore, genetic analysis of the function of E- and N-cadherin *in vitro* and *in vivo* clearly indicates that these two cadherins are required for development, and have non-redundant functional roles in cellular differentiation.

The biological functions of E- and N-cadherin have been elucidated primarily through *in vitro* analysis. The majority of studies concerning cadherin biology in tumors has focused on the function of E-cadherin as an invasion suppressor (207). The first evidence that E-cadherin contributed to the invasiveness of tumor cells was published in 1989 by Behrens *et. al.* when E-cadherin was known as uvomorulin (181). These same authors then correlated E-cadherin expression with the invasive capacity of numerous carcinoma cell lines (208). Experimental evidence provided by *in vivo* mouse models and zenograft studies further suggest that E-cadherin expression is important during tumor development and metastasis (209;210).

E-cadherin expression has been evaluated in many different carcinoma types; skin, lung, breast, gastric, pancreatic, colon, bladder, and prostate (211-218). In all cases, E-cadherin expression is downregulated in less differentiated and more aggressive disease. Interestingly, in prostate cancer, the expression of E-cadherin and the catenins can be correlated with patient survival rates (217;219). A mechanism for the inactivation of *E-cadherin* in prostate carcinoma is mutation of the gene, and has been correlated with aggressive disease (220-222). These data strongly support a role for E-cadherin in the suppression of tumor invasion and metastasis. Accordingly, during development of the primitive streak, the expression of E-cadherin is downregulated to facilitate cell migration (223).

One of the more important mechanisms to silence *E-cadherin* expression *in vivo* is through methylation of CpG islands located within the 5' promoter of the *E-cadherin* gene. The human E-cadherin gene is located on 16q22.1 and spans approximately 100

Kb (224). The Methylation of CpG islands recruits methyl-binding proteins that then associate with histone de-acetylases to promote an inactive chromatin structure (225;226). There are at least 138 CpG islands in the *E-cadherin* gene spanning a 3000+ base pair region, from -1200 base pairs 5' to exon 2 (227). Originally, hypermethylation of the CpG islands in the *E-cadherin* gene were reported in cell lines, including lines derived from prostate carcinoma, and correlated with transcriptional downregulation of the gene (228;229). Accordingly, methylation of *E-cadherin* 5' CpG islands has been detected in human prostate cancer, and correlates with progression of the disease (230).

The 5' promoter of the *E-cadherin* gene is fundamental to the regulation of *E-cadherin* transcriptional activity. The mouse *E-cadherin* promoter was first isolated in 1991 by Behrens *et. al.*, and has an a-typical TATA box, a CCAAT box (-65 bp), a GC-rich region (-30 bp to -58 bp) and contains a palandromic region composed of two E-boxes (CACGTGCAGGTG) (231). The transcription factors AP2 and SP1 bind to and drive the constitutive transcription of *E-cadherin* expression in epithelium *in vitro* (231;232). Interestingly, Hennig *et. al.* documented an epithelial-specific enhancer cis-element located within the first intron of the *E-cadherin* gene *in vitro* (232). While the mouse and human *E-cadherin* promoters display an extremely high degree of similarity, an additional fourth E-box element was found in the first intron of the human *E-cadherin* gene which is not present in mouse (233).

The E-boxes in the *E-cadherin* promoter have been shown to be important for transcriptional repression of E-*cadherin* during tumor progression. The E-boxes in the *E-cadherin* promoter have been shown to bind transcription factors through DNA

footprinting on tumor cells that lack *E-cadherin* expression (232;234). Accordingly, mutational analysis of these E-boxes has documented that these sequences are necessary for transcriptional repression of the *E-cadherin* promoter in tumor cell lines (235;236). The major transcriptional repressor that has been shown to interact with these E-boxes is the zinc finger transcription factor Snail/Slug (235;237). Transcriptional repression of the human *E-cadherin* gene by Snail *in vitro* has been shown to require the three E-boxes located in the 5' promoter of the human *E-cadherin* gene (235). Snail expression is inversely correlated with *E-cadherin* expression in breast cancer biopsies, and is also associated with lymph node metastasis (238;239). These data strongly suggest that Snail is one of the major nuclear proteins that binds to the E-boxes within the 5' promoter of the *E-cadherin* gene and represses transcriptional activation.

Other important transcriptional repressors of *E-cadherin* expression in tumor cells are E47, Slug, Zeb1 and Zeb2 (235;236;239;240). These transcription factors have also been shown to bind to the E-boxes located within the 5' promoter of *E-cadherin*. More recently, E-box regulation of *E-cadherin* transcription by the bHLH Twist1 has proven to be an additional mechanism to down regulate the expression of E-cadherin (169). One of the remaining questions of *E-cadherin* transcriptional regulation by Twist1 is whether Twist1 directly binds to the E-boxes in the *E-cadherin* promoter, or if Twist1 regulates the expression of one, or more, of the other documented *E-cadherin* repressors.

While the data in the literature strongly points to E-box regulation of *E-cadherin* expression during tumor progression, the principal, and possibly the more important question of what bHLH proteins normally bind to the E-box sequences in epithelium has

not been addressed. The normal regulation of *E-cadherin* expression in epithelium is certainly very complex, as proposed by multiple publications from the Kemler group (241;242). These researchers have evaluated the necessary regions of the E-cadherin gene that regulate the *in vivo*, epithelial specific expression of E-cadherin. Through their work, it was determined that while regions of the 5' promoter (-6000 to +1) were necessary for the abundance of a transgene expression in mice, large regions of the first and second introns (nearly 12000 bp) of the *E-cadherin* gene were required for the epithelial specific expression of a transgene in mice. These data signify a shift in the understanding of cadherin transcriptional regulation. While the regulation of the 5' promoter of E-cadherin undoubtedly contributes to the downregulation of transcription during tumor progression, the proteins that bind to sequences within the introns of E-cadherin are entirely uncharacterized. The fact that the transcription factors that bind to the intronic cis-elements are necessary for epithelial expression of E-cadherin strongly suggests that they are regulated during tumor progression, possibly in a similar manner to the cis-elements located within the 5' promoter of E-cadherin. Experiments that evaluate this hypothesis, while difficult, will prove vital in furthering the understanding of E-cadherin transcriptional regulation during tumor progression and metastasis.

Regulation of N-Cadherin Function and Expression

The human *N-cadherin* gene, isolated in 1994, is located on chromosome 18q11.2, is composed of 15 introns and 16 exons and spans a region of nearly 250 Kb (Figure 5) (243). N-cadherin was originally defined as neural-cadherin, and much of the functional analysis of N-cadherin has been completed in neuronal cells. N-cadherin has

been shown to be required for neurite outgrowth and cell migration (244-247). In these studies, expression and function of N-cadherin is necessary for neurite extension and cell migration on both recombinant N-cadherin protein, and ECM component Laminin 1. Combined with the expression pattern of N-cadherin *in vivo*, these were some of the first evidence that N-cadherin expression and function did not inhibit cell migration. Rather, in stark contrast to the function of E-cadherin, N-cadherin enables, if not contributes to cell migration.

Further *in vitro* studies in both the fields of neuronal and cancer biology elucidated the molecular events that regulate N-cadherin function during cell migration. Evidence suggests that there is signaling between N-cadherin and the β1 integrin in neurons (244;248). This "crosstalk" may be mediated by a number of signaling molecules that function in both adhesion complexes. N-cadherin mediated neurite outgrown and cell migration is inhibited by inhibitors of the fibroblast growth factor receptor (FGFR), and extracellular portions of the two proteins interact (244;248). N-cadherin may be involved in both ligand dependent and independent activation of the FGFR. The cis-interactions of N-cadherin molecules associated with the FGFR may bring the kinase domains of the FGFR in close enough proximity to induce cross-phosphorylation. Conversely, it has been shown in breast cancer cells that N-cadherin stabilizes the FGFR2 and inhibits the internalization of the receptor, thereby enhancing activation potential (249). Although N-cadherin cooperation with the FGFR certainly has the potential to induce cell migration and cancer cell invasion through FGFR signaling

alone, many of the signaling partners with the FGFR, specifically kinases like src and phosphatases like PTP1B, associate with and regulate the activity of the β1 integrin.

N-cadherin association with the non-receptor tyrosine kinase Fer has been shown to dramatically increase the signaling of the β1 integrin. In chicken neurite cells, inhibition of the association between N-cadherin and Fer inhibits the phosphorylation of p130cas (246). This activity may be dependent on Fer mediated sequestration of phosphatases, such that when Fer is associated with integrin adhesion structures, it recruits a phosphatase, possibly PTP1B or PTP-PEST, which inactivates p130cas. Determining the regulatory mechanisms mediating the crosstalk between N-cadherin and the β1 integrin have proven difficult, a fact which is borne out in the paucity of publications on this subject.

N-cadherin ligation has been shown to activate the PI3K signaling cascade, in addition to activating RhoA GTPase (76;250). In prostate carcinoma cell lines, direct ligation of N-cadherin cell adhesion, both through calcium switch and antibody activation assays, induces the association of the p85 subunit of the PI3K with the cytoplasmic domain of N-cadherin (76). This then activates the serine/threonine kinase Akt, which activates the transcription of the anti-apoptotic protein Bcl-2. In fibroblasts, similar assays that activate N-cadherin ligation increase the GTP-loading of RhoA (250). Both of these signaling pathways are important for cell survival and migration, as previously mentioned. As a whole, the literature concerning N-cadherin function strongly indicates that N-cadherin expression is an important mediator of the mesenchymal phenotype. Accordingly, forced N-cadherin expression in epithelial tumor cells induces a migratory

phenotype which is dominant over the ability of E-cadherin to repress migration (251-253). Moreover, during transforming growth factor β (TGFβ) mediated epithelial to mesenchymal transformation (EMT), N-cadherin expression is necessary for cell migration (254).

The obvious role for N-cadherin in the facilitation of the mesenchymal phenotype and the regulation of transcription through the activation of PI3K and possibly RhoA suggests that N-cadherin expression in human tumors would confer a selective advantage and that such upregulation could be detected in aggressive and metastatic disease. This is in fact the case, as N-cadherin expression can be detected in numerous tumor biopsies from lung, breast, gastric, bladder and prostate carcinomas (211;255-258). In prostate carcinoma tissues, N-cadherin expression can be detected in aggressive disease and in tumors with and without E-cadherin expression (256). Moreover, resent data correlates N-cadherin expression with increasing Gleason score, and indicates that N-cadherin expression may be a marker for metastatic disease (259). These data suggesting that the switch in expression of E-cadherin to N-cadherin may be a critical regulator of metastasis in prostate cancer progression, and that the N-cadherin induced mesenchymal phenotype is dominant over the epithelial phenotype of E-cadherin. These data are also the foundation for determining the transcriptional regulation of the aberrant expression of N-cadherin in carcinoma cells.

Figure 5. Structure of the Human *E-* and *N-cadherin* genes.

The human *E-cadherin* gene (CDH1) is located on 16q22.1 and spans approximately 100 Kb. The *N-cadherin* gene (CDH2) is on chormosome 18q11.2 and spans a region of nearly 250 Kb. Both cadherin genes are composed of 16 exons and 15 introns. The major differences between the two genes are the differences in length of the first and second introns. The first and second introns of the *E-cadherin* gene are approximately 1 and 65 Kb, while the same introns in the *N-cadherin* gene are nearly 29 and 133 Kb long.

The difficulty when examining the transcriptional regulation of N-cadherin is that very little convincing data exists concerning the regulation of N-cadherin expression in any system. The original N-cadherin promoter was cloned out of the chicken genome, and was shown be regulated by GATA transcription factors in the chick heart (260). Unfortunately, the chicken N-cadherin promoter shows very little sequence homology with the mouse or human N-cadherin genes, and the GATA sites documented in the chicken promoter do not exist in the human N-cadherin promoter (data not shown). Hence, the few publications that have used mammalian cells to evaluate the activity of the chicken N-cadherin promoter have produced data that is very difficult to interpret given that the lack of homology between the species (260;261). Alternatively, studies evaluating the induction of N-cadherin expression in cancer cell lines suggest that HOXD3, PKC and gonadal steroids may be involved, although no links to the promoter of N-cadherin was offered in these studies (262-264). *N-cadherin* expression in osteoblasts has been shown to be regulated by SP1 and MZF-7 DNA binding sites between -161 bp and -131 bp in the 5' proximal promoter (265). This study difined the N-cadherin basal core promoter to be located between -335 bp and -18 bp in the 5' proximal promoter. The start of transcription is suggested to be at -42 bp 5' of the ATG start site. Very recent evidence suggests that Twist1 regulates the transcription of N-cadherin in breast epithelium (169). Undoubtedly, the transcriptional regulation of the N-cadherin gene in N-cadherin expressing cells will be as complex as the regulation of E-cadherin expression in epithelium.

Statement of the Problem

The fundamental changes that facilitate the progression of carcinoma to metastasis are alterations in the transcriptional regulation of genes involved in cell adhesion. During prostate carcnoma progression, changes in the expression of cadherins and integrins are central to the metastatic ablity of tumor cells. Loss of β4 integrin and downregulation of E-cadherin are well characterized epithelial genes that are regulated during prostate carcinogenesis (266;267). While it is unkown whether the loss of the β4 integrin is due to transcriptional silencing, it is predicted that loss of the β4 integrin and its cognate adhesion structure, the hemidesmosome, contributes to the loss of the epithelial phenotype . The transcriptional silencing of E-cadherin has been extensively studied in both *in vitro* models of prosate cancer, and in human prosate cancer. Recently it has been discovered that concomitant with the transcriptional silencing of E-cadherin in prostate carcinoma, there is transcriptional upregulation of the mesenchymal cadherin, N-cadherin (256). N-cadherin expression in carcinoma cells has been shown to regulate important signaling pathways that contribute to cell migration and invasion, and to inhibit apoptosis (268). The discovery of N-cadherin in prostate carcinoma, and its role in tumor metastasis has preceeded any knowledge of the mechanisms that control the transcription of this gene in carcinoma progression.

The initial experiments of this work were aimed at determining the molecular mechanisms that regulate N-cadherin expression during FN mediated EMT. The overall hypothesis of these experiments was: *The β1 integrin regulates N-cadherin gene expression through the regulation of Twist1 function*. The experiments are organized into

four specific aims: *(1) Determine whether cell adhesion to ECM regulates N-cadherin gene expression. (2) Establish whether Twist1 controls N-cadherin mRNA production. (3) Determine how the β1 integrin regulates the function of Twist1. (4) Verify whether the regulation of N-cadherin expression by Twist1 is at the level of the N-cadherin promoter.* It was determined through these studies that β1 integrin regulation of the nuclear localization of the bHLH transcription factor Twist1, controled N-cadherin expression in PC-3 prostate cancer cells.

The next studies were focused on determining the cis-elements within the promoter of the N-cadherin gene that regulated cell-type specific expression. The hypothesis of these studies was: *N-cadherin negative carcinoma cells contain a transcriptional repressor that inhibits the activation of the N-cadherin promoter.* The specific aims were: *(1) Create an N-cadherin promoter reporter that recapitulates N-cadherin mRNA expression in carcinoma cells. (2) Determine the necessary elements within the N-cadherin gene that inhibit N-cadherin promoter activation in non-N-cadherin expressing cells. (3) Determine the transcriptional repressor that inhibits N-cadherin promoter activation in cell that do not express N-cadherin*. These studies determined that the putative tumor suppressor FoxP1 regulates the transcriptional repression of N-cadherin in N-cadherin negative cells.

The final studies of this work evaluate the functional crosstalk between the β1 integrin and N-cadherin. The overal hypothesis was: *Expression of N-cadherin induces a migratory phenotype in E-cadherin expressing prostate cancer cells.* The specific aims were: *(1) Determine the effect of N-cadherin expression on cell adhesion and migration*

of prostate cancer cells. (2) Evaluate changes in intracellular signaling of integrins in E- and N-cadherin co-expressing prostate cancer cells. (3) Determine the mechanism for N-cadherin mediated increases in the activation of integrin signaling molecules. These experiments determined that N-cadherin expression in prostate carcinoma increases cell migration, and implicate a signaling molecule common to both adhesion structures in the regulation of prostate cancer cell adhesion and migration on Laminin 1.

II. MATERIALS AND METHODS

Cell Culture

The human prostate cancer cell lines LNCaP, PC-3, Du145, the human breast cancer cell line MCF7 and colon cancer cell line HT-27 were purchased from the American Type Culture Collection, and cultured in D-MEM (Gibco BRL) with 10% heat inactivated fetal bovine serum (Hyclone). PC-3N cells are a high N-cadherin expressing variant of PC-3 cells and were isolated previously in our lab (269). For suspension culture, cells were detached with 5 mM EDTA in PBS and plated at 10^5 cells/cm^2 in dishes coated with 25 mg/ml Poly-Hema (Poly 2-hydroxyethylmethacrylate; Sigma), for 2-3 days in serum-free media, for PC-3 cells, or for 1 to 2 hours for Du145 cells. Petri dishes were used to coat with ECM molecules, all of which were used at a final concentration of 10 μg/ml in 0.1 M $NaHCO_3$ (pH 9.6) overnight a 4º.

Antibodies and Reagents

Monoclonal anti-human E-cadherin (clone HEDC1) was purchased from Zymed Labs. Monoclonal antibodies against human N-cadherin, E-cadherin, β-catenin was purchased from Transduction Labs. Monoclonal anti N-cadherin (ACAM) was purchased from Sigma. Monoclonal anti-phosphotyrosine, anti-Rac 1, anti-PTP1B and anti-FAK (clone 4G10) antibodies were purchased from Upstate Biotechnology. Polyclonal anti Akt, Phospho-Akt Ser473, Erk1,2, Phospho Erk1,2 Thr202 and Tyr 204 were purchased from Cell Signaling. Polyclonal anti-Rho A, Lamin A, anti-p130cas and anti-Twist1 antibodies were obtained from Santa Cruz Biotechnology, Inc. Polyclonal

anti- phospho FAK was purchased from Bio Source. Monoclonal anti-myc antibody was obtained from Roche. Polyclonal anti-human alpha 6 integrin (AA6A) and monoclonal anti-human beta 1 integrin (clone A2BII used at a final concentration of 5 μg/ml) were gifts from Dr. Anne Cress (University of Arizona). The polyclonal anti-FoxP1 was a gift from Daniel Simon (Harvard). Laminin1 was purchased from American Type Culture Collection, poly-L-lysine was purchased from Sigma. Fibronectin was purchased from Roche and used at 10 μg/ml. The inhibitors Y-27632, latrunculin B and jasplakinolide were purchased from Calbiochem. Fluorescent tagged phaloidin was purchased from Molecular Probes.

Immunoprecipitation and Immunoblot

Cells were washed and scraped in ice-cold CMF-PBS containing 1mM PMSF, 10μg/ml leupeptin, 10μg/ml aprotinin, 20mM sodium fluoride and 2mM sodium vanadate, and lysed in ice cold NP-40 buffer (0.5% Nonidet P-40, 10mM Tris-HCl pH 7.6, 150mM NaCl, 1mM EDTA), containing 1mM PMSF, 10μg/ml leupeptin, 10μg/ml aprotinin, 20mM sodium fluoride and 2mM sodium vanadate. Lysates were clarified by centrifugation at 14,000 *g*, and the supernatants accessed for protein concentration with the BCA assay (Pierce Chemical Co.). Cell lysates (0.5 mg of total protein) were incubated with 5 μg of the anti-N-cadherin antibody ACAM-1 (Sigma), or anti-E-cadherin antibody HEDC-1 (Zymed) overnight at 4°C. Immune complexes were recovered through incubation with protein G-Sepharose (Sigma) for two hours at 4°C. Beads were washed once in NP-40 buffer and TBS (10mM Tris-HCl pH 7.6, 150mM

NaCl), boiled in SDS buffer separated by SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked in 5% non-fat milk for one hour at room temperature (RT), and primary and secondary antibodies were diluted in 5% non-fat milk, or 5% BSA in TBS-T (10 mM Tris-HCl pH 8.0, 150mM NaCl, 0.5% Tween-20) and incubated with membranes for one hour at RT, or overnight at 4°C. Bands were detected through enhanced chemiluminescence, and exposed to film (Kodak). Protein bands were quantified using Scion Image software (Scion Corporation).

Immunofluorescence

Glass coverslips were incubated in 10μg/ml of laminin in 0.1 M $NaHCO^3$ buffer, pH 9.6, overnight at 4°C, washed in water, and blocked in 1% heat denatured BSA in water for one hour at 37°C. Cells were allowed to attach to coverslips overnight in DMEM (Gibco BRL) without serum. Cells were then washed in CMF-PBS, fixed in 4% (w/v) paraformaldehyde in CMF-PBS and permeablized in CSK buffer (0.5% Triton X 100, 10mM PIPES pH 6.8, 50mM NaCl, 300mM sucrose, 3mM $MgCl_2$) for five minutes on ice. Cells were blocked with blocking buffer (2% BSA, 1% goat serum in CMF-PBS) for 30 minutes at RT, incubated with primary antibodies for 30 minutes at RT, washed with CMF-PBS, and incubated with secondary antibodies (Cy3 or Cy5 conjugated goat anti-mouse or goat anti-rabbit IgG) for 30 minutes at RT. Nuclei were labeled with BBI for five minutes at RT. The coverslips were mounted onto glass slides with mounting media (2% n-propyl gallate, 90% glycerol, pH 8.0). A Ziess confocal microscope was used to analyze fluorescence.

Nuclear Isolation

Cells were washed and detached with ice cold CMF-PBS containing 1 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 20 mM sodium fluoride and 2 mM sodium vanadate. Cells were pelleted and resuspended in hypotonic lysis buffer (10 mM Tris-HCl pH 7.4, 1 mM $MgCl_2$, 1 mM EDTA) and incubation on ice for 30 minutes. Nuclei were liberated with 50 strokes in a cell small glass homogenizer. Nuclei were pelleted through centrifugation at 2000 rpm for 5 minutes. Supernatant was kept as cytoplasmic fraction, while nuclear pellet was washed in hypotonic buffer, pelleted and lysed in Laemmli SDS sample buffer with inhibitors.

Calcium Switch Assay

Petri dishes were incubated with 10 µg/ml of Laminin in 10mM sodium bi-carbonate overnight at 4°C. Laminin coated dishes were washed in water and incubated in 3% BSA in water for 15 minutes. Cells were serum-starved (D-MEM without FBS) for 24 hours, detached in 5mM EDTA and plated at confluency in D-MEM without FBS. After attaching overnight, cells were briefly washed with CMF-PBS and incubated in D-MEM with 5mM EGTA for 45 minutes, at which time the cells were washed in CMF-PBS and incubated in D-MEM for various time points. Cells were then washed with ice cold CMF-PBS containing 1mM PMSF, 10µg/ml leupeptin, 10µg/ml aprotinin, 20mM sodium fluoride and 2mM sodium vanadate, and lysed in SDS sample buffer containing 1mM PMSF, 10µg/ml leupeptin, 10µg/ml aprotinin, 20mM sodium fluoride and 2mM sodium vanadate, and subjected to immunoblot analysis.

Adhesion Assay

Petri dishes were incubated with were incubated in 10μg/ml of laminin in 0.1 M $NaHCO_3$ buffer, pH 9.6, overnight at 4°C, washed in water, and blocked in 1% heat denatured BSA in water for one hour at 37°C. Cells were serum-starved (D-MEM without FBS) for 24 hours, detached in 5mM EDTA and plated at designated concentration in D-MEM without FBS. At various time points, cells were washed with ice cold CMF-PBS containing 1mM PMSF, 10μg/ml leupeptin, 10μg/ml aprotinin, 20mM sodium fluoride and 2mM sodium vanadate, and lysed in ice cold NP-40 buffer or SDS sample buffer containing 1mM PMSF, 10μg/ml leupeptin, 10μg/ml aprotinin, 20mM sodium fluoride and 2mM sodium vanadate, and subjected to immunoprecipitation, or immunoblot analysis, respectively.

***In vitro* Migration Assays**

The linear migration assay was preformed as previously described (270). Ten well Teflon coated slides were incubated in 10μg/ml of Laminin1 in 0.1 M $NaHCO_3$ buffer, pH 9.6, overnight at 4°C. The slides were washed in water and blocked in 1% BSA in water for one hour at 37°C. The cell-seeding manifold was then attached to the slide, and a confluent monolayer of 2000 cells was seeded in DMEM (Gibco BRL) without serum at 37°C. The cells were allowed to attach for 6-8 hours, at which time the radius of the confluent layer of cells was measured using a Nikon camera and Scion Image (Scion Corp.) software. At 18 and 32 hours, the radius of the migrated cells was again measured.

Boyden chambers/Transwell filters (Costar) were coated with Laminin1 at 10μg/ml of Laminin1 in 0.1 M $NaHCO_3$ buffer, pH 9.6, overnight at 4°C. The chambers were washed in water and blocked in 1% BSA in water for one hour at 37°C. Cells were seeded at sub-confluence (50 – 60%) and allowed to migrate for 18 hours at 37°C. The remaining cells that had not migrated were scrapped of the filter with a cotton swab, and the filter was removed. Cells were fixed in methanol, and stained with Crystal Violet (0.1% in 20% methanol) for 5 minutes. Background Crystal Violet was removed by 5 washes in water. Dye was then released from the migrated cells with 0.1M citric acid, and quantified through spectrophotometer analysis at 580 nm.

DNA Constructs and Transfections

The myc-tagged FoxP1 expression vector was described previously, and contains the entire coding sequence (142). The human Twist1 expression vector in pcDNA 3.1 contains the entire coding sequence of *Twist1* as previously described (271). The full length coding region of human N-cadherin cDNA, in the vector EGFP-N1 (Clonetech) was a gift from Dr. Brian McCray (University of Arizona). All plasmids were transfected using the FuGene 6 transfection reagent (Roche) according to manufacturer's instructions.

To select for stable clones, transfectants were cultured in 1 mg/ml of Genticin (Gibco BRL) for one week, followed by serial dilution into 96 well dishes, or clonal isolation with cloning rings by adding trypsin inside the rings and transferring cells to fresh plates. Isolated clones were placed under selection for an additional week with

750µg/ml antibiotic. Isolated clones expressing transgenes were passaged in 500µg/ml Genticin. Clones were characterized through immunoblot analysis for N-cadherin.

Northern Analysis

Total RNA was isolated from cultured cells using Trizol Reagent (Invitrogen) according to manufacture's protocol. Equal amounts of RNA were separated by electrophoresis in a 1% agarose gel containing 1.85% formaldehyde, and transferred to Hybond N+ nylon membranes (Amersham Life Sciences). Membranes were prehybridized for 18 hours at 42ºC in 6X SSC buffer with 0.05M NaH_2PO_4, 5X Denhardt's, 1% SDS, 50% formamide and 10µg/ml salmon sperm DNA. *N-cadherin* mRNA was detected using a 300 base pair *Eco* R1 fragment isolated from full length human *N-cadherin* (NM_001792) which was a gift from J. Hemperly. *E-cadherin* mRNA was detected with a 1.7 kilo-base *Sma* I fragment isolated from mouse *E-cadherin*. *GAPDH* mRNA was detected using a *Pst* I fragment isolated from full length human *GAPDH*. Probes were random primed with α-P32CTP using the Random Prime Kit (Invitrogen). Denatured probes were added to membranes and hybridized for 18 hours. Membranes were washed for 30 minutes with 0.1X SSC with 1.0 % SDS. Probe reactivity was detected by exposing membranes on X-OMAT AR film (Kodak).

Semi-Quantitative and Quantitative PCR

Total RNA was extracted from cells using TRIZOL (Invitrogen) and treated with RNAse-free DNAse I (Promega). One µg of the RNA from each sample was reverse transcribed for an hour at 42^{o}C using random hexamers and Superscript II (Invitrogen)

for the first-strand cDNA synthesis. For semi-quantitative PCR reactions contained 0.5 μCi of P^{32} labeled dATP per reaction. Reactions were ended during the linear range of amplification, typically following 18 cycles with an annealing temperature of 55º. PCR products were separated on a 5% PAGE, dried and viewed by autoradiography. Primers were

hFoxO1A-S:AACCTGGCATTACAGTTGGCC,

AS:AAATGCAGGAGGCATGACTACGT

hFoxO3A-S:TCAATCAGAACTTGCTCCACCCA,

AS:GGACTCACTCCAGCCCATGTTG

hFoxP1- S:TCAGTGGTAACCCTTCCCTTA, AS:GTACAGGATGCACGGCTTG

hFoxP2-S:CCACGAAGACCTCAATGGTT, S:TCACGCTGAGGTTTCACAAG

hFoxL1-S:TGTGGCGGATGCTGTTCTG, S:GCCCATGCTGTATCTGTACG

hFoxL2-S:CCGTAAGCGGACTCGTGC, S:AGTAGTTGCCCTTGCGCTC

hGAPDH-S:CAGTATGACTCCACTCACGG, AS:GTGAAGACACCAGTAGACTCC

hβ-actin-S: For quantitative PCR, master mixes were prepared according to the Quantitect SYBR Green kit (Qaigen). Real time PCR was set up according to instructions from Corbett Research and run for 35 cycles on the Rotorgene 3000. Expression levels were analyzed by the $2^{-\Delta\Delta Ct}$ method. Transcript levels were normalized to ribosomal *L19* gene expression. Values are the mean of three separate experiments. Primers were designed using the Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3).

Primers:

N-cadherin (X54315): 5’-TGTTTGACTATGAAGGCAGTGG-3’/ 5-‘TCAGTCATCACCTCCACCAT-3’

E-cadherin (MN004360): 5’-GAAAGCGGCTGATACTGACC-3’/5’-CGTACATGTCAGCCGCTTC-3’

Twist1 (NM000474): 5’-TGAGCAAGATTCAGACCCTCA-3’/5’-ATCCTCCAGACCGAGAAGG-3’

RPL19 (NM000981): 5’-ACCTGAAGGTGAAGGGGAAT-3’/5’-GCGTGCTTCCTTGGTCTTAG-3’

Slug (AF042001): 5’-TTCGGAACACATTACCT-3’/5’-TGACCTGTCTGCAAATGCTC-3’

siRNA

Small Interfering RNA (siRNA) against the *Twist1* target sequence 5’-ACUCCAAGAUGGCAAGCUG were purchased from Darmacon. siRNA were annealed and added to cells at a final concentration of 50 nM using (5 µl lipid per µl of oligonucleotidesnucleotide) the Oligonucleotidesfectamine transfection reagent according to manufacture’s protocol (Invitrogen). siRNA against firefly luciferase target sequence 5’-CTTACGCTGAGTACTTCGA was used as a negative control.

Gel Shift Analysis

EMSA was completed according to reference (156). Cell nuclei were released in sucrose buffer (0.32 M sucrose, 10mM Tris HCl pH 8.0, 3mM CaCl2, 2mM MgOAc, 0.1mM EDTA and 0.5% NP-40), pelleted with low centrifugation and washed in sucrose

buffer without NP-40. Isolated nuclei were swelled in Low Salt buffer (20mM HEPES pH 8.0, 1.5mM MgCl2, 20mM KCl, 0.2mM EDTA and 25% glycerol), followed by the addition of High Salt buffer (20mM HEPES pH 8.0, 1.5mM $MgCl_2$, 800mM KCl, 0.2mM EDTA, 25% glycerol and 1% NP-40). Nuclear extract was extruded for 30 minutes at 4 degrees, and nuclear debris was pelleted through centrifugation at 14,000g for 15 minutes.

Oligonucleotidesnucleotides were labeled with P32using T4 Polynucleotide Kinase (Invitrogen) according to manufactures instructions. Labeled oligonucleotidesnucleotides were purified with QIAquick Nucleotide Removal Kit (Qiagen), eluted in water and double strands were allowed to anneal overnight at room temperature. The N-cadherin +438-+468 oligonucleotides were 5'CTCCAATTTTTTGTTTATTTCTGCAGGGA3' and 5'TCCCTGCAGAAATAAACAAAAAAATTGGAG3'. Mutant oligonucleotidesnucleotides were 5'CTCCAATTTTTTGGATTAATTCTGCAGGGA3' and 5'TCCCTGCAGAATTAATCCAAAAAATTGGAG3'. The N-cadherin first intron E-box oligonucleotidesnucleotide sequence (+2619 to +2647) was 5'-GGTTAAGTGCAC**CA**ATGTGGATTGTACAACT, while the mutant sequence was 5'-GGTTAAGTGCAC**TT**TGTGGATTGTACAACT. Binding reactions with 10 µg of nuclear extract were carried out in Binding buffer (20mM HEPES, 0.1M KCl, 0.2M EDTA and 20% glycerol) with 100ng of poly dI-dC in a total volume of 20 µl with 1 – 2.5ng of labeled double stranded oligonucleotides. Reactions were incubated at room temperature for 30 minutes, and then loaded on a 5% polyacrylamide gel. Unlabeled

double stranded oligonucleotides competitors were added prior to incubation with labeled probe, while antibodies were added 15 minutes after the addition of radioactive probes. Complexes were resolved on a 5% non-denaturing polyacrylamide gel. The gels were dried, and exposed to film at -80 degrees.

N-cadherin Promoter Constructs and Luciferase Reporter Assays

The E-cadherin promoter construct was a gift from Antonio García de Herreros, and contains the -178 bp to +92 bp fragment of the *E-cadherin* promoter (235). The proximal promoter sequence for human *N-cadherin* -860 bp to +20 bp was amplified from human genomic DNA (Promega) using the following primers s5'-ACGGAGCCGCGGCGGGAGAGACCGC and as5'-GGAGGCGGAGAGGGGCCGAGCGGAGA. The PCR product was ligated into the *Sma I* site in pBS (Stratagene) following blunting with T4 polymerase (Invitrogen), and sequenced. The -860 to +20 fragment of the N-cadherin promoter was then sub-cloned into pGL3b (Promega) using the *KpnI* and *HindIII* restriction sites, creating NP860pGL3b. This construct was then cut using the *Sac I* restriction enzyme which cuts the N-cadherin proximal promoter at -463, and re-ligated using T4 Ligase (Roche), creating NP460pGL3b. Regions of the first intron of the human *N-cadherin* gene (+373 bp to +2822 bp) downstream of firefly luciferase in NP-860pGL3 were amplified by PCR from a BAC obtained from the University of California Santa Cruz. The following primers, which contained 5' engineered *Bam HI* and 3' engineered *Sal I* restrictions sites, were used to amplify regions of the first intron of *N-cadherin*: +373-+1338s5'-CGCCTAGGGTGAGGCAGGATAACTCC and as5'-

ACGCCAGCTGATCCACACCACCTGTCTC, +373-+2822 as5'-ACGCCAGCTGGACAGCCACTTTCACC. Intronic sequences were then cloned into pBS and sequenced, and subcloned into NP860pGL3b at the *Bam HI* and *Sal I* sites.

Intron deletion mutants were created by restriction of the pBS+373-+1338 with *Sma* I, re-ligated and sequenced. The deletion fragments of the N-cadherin first intron were +373-+757, +757Δ+988, and +988-+1338. The deletion mutants were subcloned into NP860pGL3b at the *Bam HI* and *Sal I* sites. Point mutations of the N-cadherin first intron were created using the QuickChange mutagenesis kit (Stratagene) mutating the Twist1 E-Box sequence at +2627 (5'-CATGTG to 5'-TTTGTG) and the FoxP1 binding site at + 460 (TGTTTAT to GGATTAA), and were verified by sequencing.

For lucifierase assays, cells were plated at 60% confluence in triplicate. 250 ng of promoter construct and 25 ng of the pTKRenilla transfection control were used to transfect cells with the Fugene6 Transfection Reagent (Roche) according to manufacture's protocol. The Dual Luciferase Kit (Promega) was used in luciferase assays according to manufacture's protocol, although protein was determined and only 5 µg of total protein was added to 25 µl of LARII, and only 25 µl of Stop-and-Glow was added.

III. *N-CADHERIN* EXPRESSION IN PROSTATE CARCINOMA IS MEDIATED BY INTEGRIN DEPENDENT NUCLEAR TRANSLOCATION OF TWIST1

Introduction

During tumor progression genetic and epigenetic alterations contribute to the complex process of metastasis. The *E-cadherin* gene is a common target for transcriptional repression and is considered a key step in the metastasis of carcinomas (272). Concomitant with the silencing of *E-cadherin* transcription, the expression of *N-cadherin* is upregulated, suggesting a role for N-cadherin during tumor progression (256). Aberrant N-cadherin expression in prostate carcinoma cell lines has been shown to upregulate anti-apoptotic signaling pathways (76), enhance the signaling of the FGF receptor (249) and is required for cell migration during TGFβ1 induced epithelial-to-mesenchymal transformation (EMT) (254). Although the mechanisms regulating the aberrant expression of *N-cadherin* in carcinoma progression remain unknown, the signaling of RhoA has been shown to be necessary for N-cadherin expression during TGFβ1 induced EMT (273).

Twist1 is a basic helix-loop-helix transcription factor that is preferentially expressed in mesoderm-derived cells, but expression has also been detected during carcinoma progression (171). Recent evidence implicates Twist1 in the regulation of *E-cadherin* expression in prostate and breast carcinoma (169;171), presumably through the transcriptional regulation of the zinc finger transcriptional repressor Snail. Mouse Twist has been shown to induce the expression of *N-cadherin* in human breast epithelial cells

(169), but it is not known whether the expression of human *Twist1* directly activates *N-cadherin* transcription in carcinoma cells.

Integrin are transmembrane heterodimeric adhesion molecules that mediate cell adhesion to extracellular matrix (ECM) proteins. Integrin adhesion activates specific intracellular signaling pathways that reorganize the actin cytoskeleton and regulate the nuclear/cytplasmic shuttling of certain transcription factors, such as Elk-1 and SRF (274;275). Recent studies of human cancer metastasis indicate a dynamic regulation of *E-cadherin* transcription through integrin mediated cell adhesion and signaling by integrin linked kinase (276). In this study we document a β1 integrin dependent upregulation of *N-cadherin* mRNA expression in the prostate carcinoma cell line PC-3. We show that Twist1 is necessary for *N-cadherin* transcription, and that Twist1 nuclear accumulation is dependent on β1 integrin mediated cell adhesion. Furthermore, we demonstrate that Twist1 regulation of *N-cadherin* expression is through a direct interaction with an E-box located within the first intron of the human *N-cadherin* gene.

Results and Discussion

Reciprocal Regulation of E-cadherin and N-cadherin by Integrin Adhesion to Fibronectin

We have utilized the prostate adenocarcinoma cell line PC-3, which expresses both E and N-cadherin (76), to investigate the molecular events that contribute to the regulation of N-cadherin in prostate carcinoma. To examine the relative cadherin transcript levels in PC-3 cells cultured in the absence of attachment (suspension) or attached to Fibronectin

(FN), total RNA was isolated from cells cultured in both conditions in serum free media. *E-cadherin* mRNA was high in suspended PC-3 cells and decreased when the cells attached to a FN substrate. In contrast, *N-cadherin* mRNA was four-fold lower when PC-3 cells were cultured in suspension as compared to FN attached PC-3 cells (Figure 6A). The protein levels of E- and N-cadherin in PC-3 cells cultured in both conditions recapitulated the cadherin transcript levels. The adhesion dependent increase in N-cadherin expression is not dependent on growth factor signaling as N-cadherin expression levels were unaffected when serum was present during cell adhesion (Figure 6B). These results suggest that the adhesion dependent signaling of integrins is necessary for the dynamic expression of *E-* and *N-cadherin* upon cell attachment to FN.

The induction of *N-cadherin* mRNA expression when suspended PC-3 cells were replated onto FN substrate is two-fold at 6 hours, and maximal at 12 hours (Figure 6C). β1 Integrin colocalization with F-actin was a rapid event, visible at 30 minutes following adhesion to FN (Figure 6D). Activation of *N-cadherin* expression was dependent on β1 integrin mediated cell adhesion, as pre-incubation with a β1 integrin function blocking monoclonal antibody prior to cell adhesion to FN abrogated the increase in *N-cadherin* mRNA (Figure 6C). These data indicate that N-cadherin expression is upregulated by β1 integrin mediated cell adhesion to fibronectin. Interestingly, re-expression of β1 integrin in β1 integrin-null epithelial cells was shown to differentially regulate cadherin gene expression and induce a transformation from epithelium-to-mesenchyme (277), suggesting that β1 integrin signaling may be a mechanism regulating aberrant N-cadherin expression in epithelium.

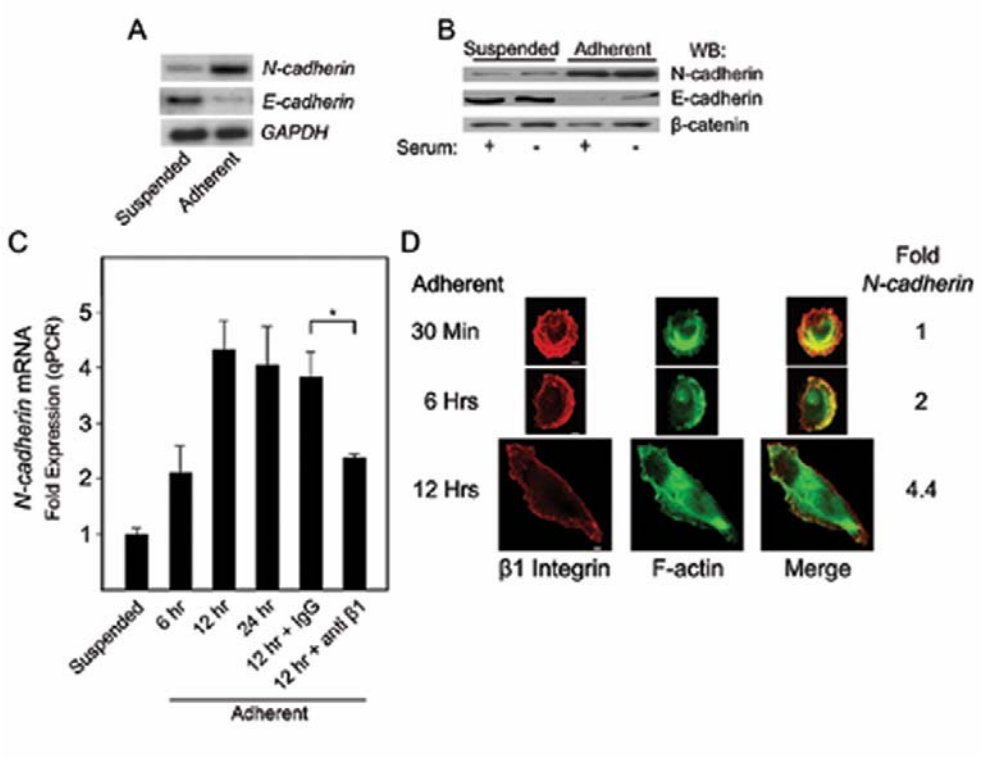

Figure 6. Anchorage dependent adhesion reciprocally regulates *E-* and *N-cadherin*.

(A), Northern analysis from total RNA for *E-cadherin*, *N-cadherin* and *GAPDH* from PC3 cells grown in suspension for 2 days or adherent to FN (10 µg/ml) for 24 hrs (10^4 cell/cm^2). **(B)**, western blot analysis of E-cadherin, N-cadherin and β-catenin from PC-3 cells with or without the addition of serum (10%). **(C)**, time course of *N-cadherin* expression following adherence to FN by quantitative PCR analysis. Non-immune mouse IgG or the function blocking anti β1integrin antibody (A2BII) were added at 5 µg/ml prior to adherence. Data is represented as mean fold increase over N-cadherin expression in suspended cells ±SD. * denotes statistical significance ($p = < 0.05$). **(D)**, Immunofluorescence of β1 integrin and F-actin in adherent PC-3 cells fixed at the indicated times.

Depletion of Twist1 with siRNA affects N-cadherin mRNA Expression

We next examined whether Twist1 was necessary for N-cadherin expression in PC-3 prostate cancer cells. siRNA depletion of *Twist1* resulted in decreased expression of both *Twist1* and *N-cadherin* mRNA (Figure 7A) and protein levels (Figure 7B). Transfections of *Twist1* siRNAs reduced the *Twist1* mRNA levels about 60% and *N-cadherin* transcript levels to 50%. Twist1 protein levels were reproducibly reduced nearly 90% in each experiment, and N-cadherin levels were 50% of the control samples. In contrast, a control Luciferase siRNA did not affect levels of Twist1 or N-cadherin. These findings, together with previous studies, suggest that Twist1 is necessary for the expression of *N-cadherin* in carcinoma cells (169).

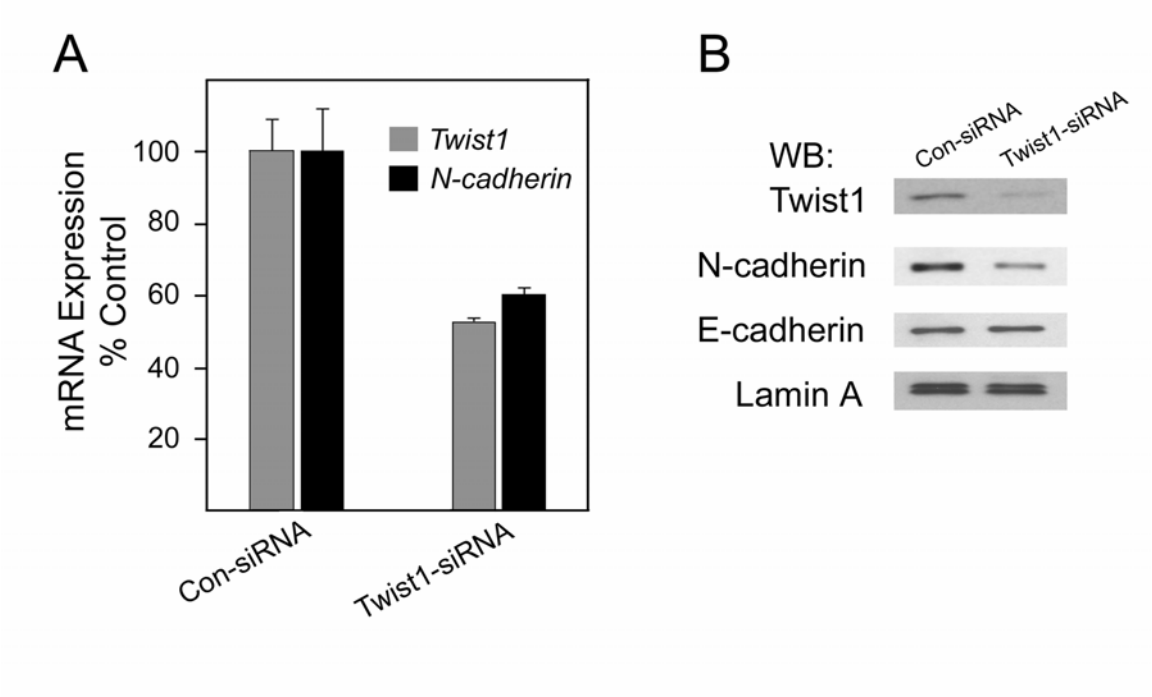

Figure 7. *N-cadherin* transcript levels are dependent on *Twist1* expression.

(A), Quantitative PCR analysis of *N-cadherin* and *Twist1* mRNA levels following treatment with siRNA against Twist1 or a control luciferase siRNA for 24 h. **(B)**, western blot analysis of N-cadherin, E-cadherin, and TWIST1 protein levels following siRNA treatment.

Nuclear Accumulation of Twist1 is Inhibited in Non-adherent Cells

Since inhibition of β1 integrin engagement decreased *N-cadherin* transcript levels, we determined whether changes in *Twist1* transcript levels could account for the decrease in *N-cadherin* expression when cells were grown in suspension. Analysis of *Twist1* mRNA levels showed no change in the transcript level in suspended or adherent cells, while *N-cadherin* expression was upregulated in adherent cells. In comparison, the zinc finger transcriptional repressor Slug, which silences *E-cadherin* expression, showed increased mRNA expression in adherent cells (Figure 8A). We next determined whether Twist1 was present in the nuclei of suspended cells. Although the expression levels of *Twist1* mRNA did not change in suspended or adherent PC3 cells, the nuclear accumulation of Twist1 protein was absent in cells cultured in suspension (Figure 8B). When suspended PC-3 cells were replated onto a FN substrate, there was a rapid translocation of Twist1 into the nucleus from the cytoplasm which was maximal at 30 minutes and inhibited by the addition of β1 integrin function blocking antibodies (Figure 8B and C). To determine the adhesion dependent signals downstream of the β1 integrin that regulate Twist1 nuclear accumulation, pharmacological inhibitors to signaling pathways downstream of the β1 integrin were added to PC-3 cells prior to attachment to FN. As shown in figure 7C, an inhibitor of RhoA associated Kinase (ROCK), Y27632, and the actin destabilizing drugs latrunculin B and jasplakinolide inhibited the nuclear accumulation of Twist1. Consistent with the inhibition of Twist1 nuclear accumulation by an inhibitor of ROCK and by jasplakinolide, the levels of N-cadherin in PC3 cells were also reduced (Figure 8D). Together these results suggest that while *Twist1* expression is necessary for *N-*

cadherin expression, the regulation of *N-cadherin* transcript levels is not due to changes in *Twist1* mRNA levels, rather the β1 integrin dependent nuclear accumulation Twist1 in adherent cells is the adhesion dependent signal that regulates *N-cadherin* expression in prostate carcinoma cells.

This work and recent data propose a functional role for Twist1 in the process of tumor metastasis and as a transcriptional regulator of cell adhesion molecules (169;171). We offer the first evidence for the regulation of Twist1 activity in tumor cells; a β1 integrin dependent nuclear accumulation of Twist1 through the activation of ROCK and a functional actin-based cytoskeleton. Integrin activation and cytoskeleton dynamics have been shown to regulate the nuclear accumulation of multiple transcription factors (275), which link the extracellular environment with nuclear signaling. Twist1 may, therefore, be a novel transcription factor modulating transcriptional events downstream of the β1 integrin.

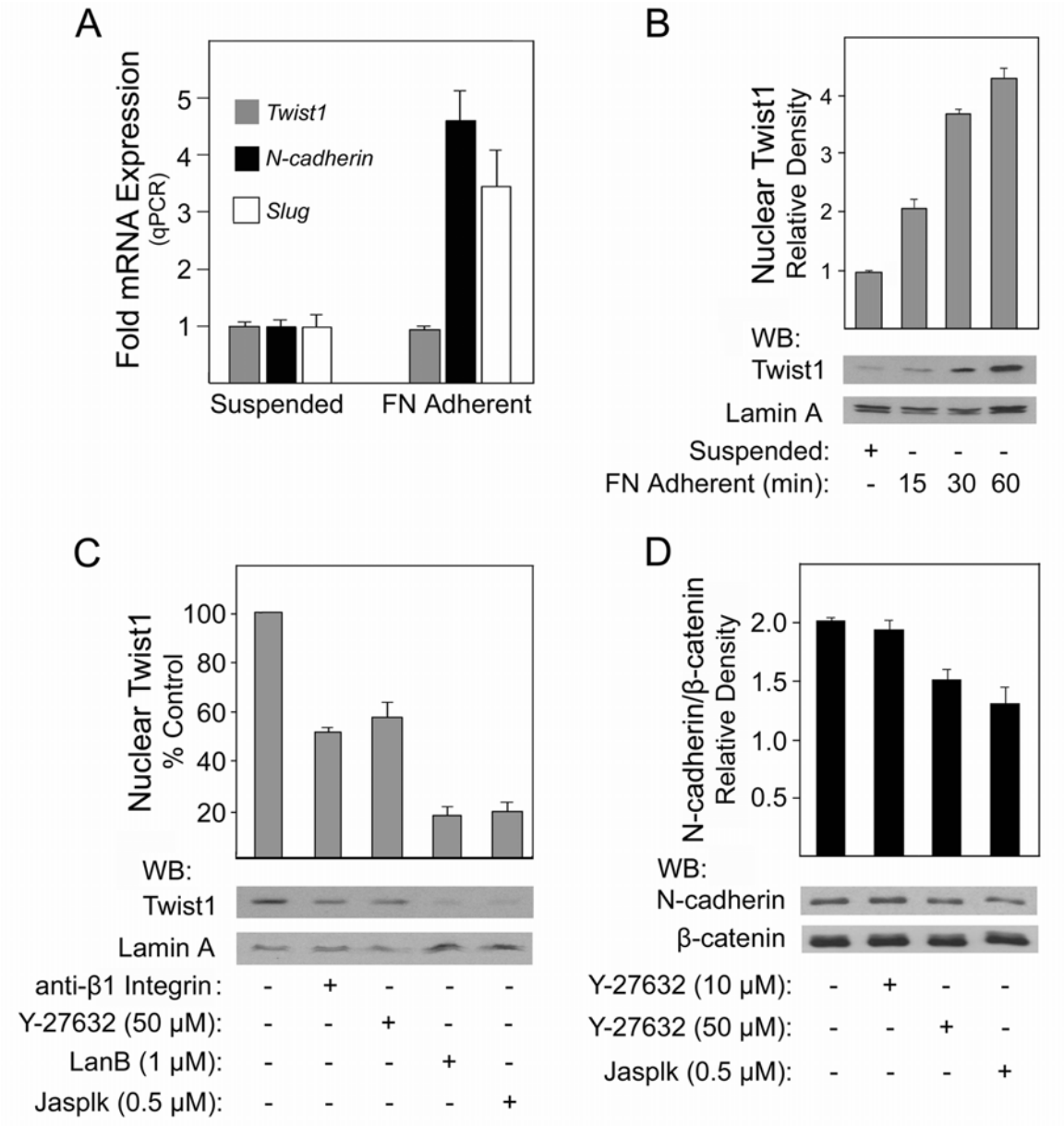

Figure 8. Adhesion to fibronectin regulates Twist1 nuclear accumulation.

(A), Quantitative PCR analysis of *Twist1* mRNA in adherent and non-adherent cells. **(B)**, western blot analysis of Twist1 nuclear accumulation following adherence to FN. **(C)**, western blot analysis of twist1 nuclear accumulation following adherence to FN for 30 minutes following preincubation with β1 integrin function blocking antibodies (5 μg/ml) and the pharmacologic inhibitors Y-27632 (25 μM), latrunculin B (1 μM) and jasplakinolide (5 μM). **(D)**, N-cadherin expression following treatment with Y-27632 (10 and 25 μM) and jasplakinolide (5 μM), β-catenin was used as a loading control. Relative densities of bands in western blots are represented as graphs.

Twist1 Upregulates N-cadherin promoter activity

To determine whether the activation of *N-cadherin* expression by Twist1 in PC-3 cells involves direct binding of Twist1 to the promoter of N-cadherin, we cloned the -860 to +20 region of the *N-cadherin* 5' proximal promoter into the luciferase reporter construct pGL3basic (Figure 9A), which contains the minimal basal core promoter region -335 to -18 (265). Co-transfection of the 5' *N-cadherin* promoter construct and *Twist1* showed no increase in promoter activity in PC-3 cells, nor in the *N-cadherin* and *Twist1* negative cell line MCF-7 (Figure 9B). This suggests that Twist1 has little direct or indirect effect on the 5' promoter of the human *N-cadherin* gene. Of the two documented Twist1 E-box sequences [CATATG or CATGTG (156)], we found the E-box sequence CATGTG located within the first intron of the human *N-cadherin* gene at +2627 (Figure 9A). Incorporation of the +373- +2822 region of the first intron of the *N-cadherin* gene downstream of luciferase in the 5' *N-cadherin* promoter reporter construct resulted in Twist1 mediated increases in *N-cadherin* promoter activity (Figure 9B). Mutation of the E-box sequence at +2627 in the *N-cadherin* promoter construct eliminated the ability of Twist1 to increase *N-cadherin* promoter activity.

EMSA was then performed to determine the ability of Twist1 to directly bind the putative Twist1 E-box element in the *N-cadherin* first intron. Three DNA-protein complexes (Figure 9C lane 2; C1, C2 and C3) were formed when nuclear extracts from adherent PC-3 cells were incubated with labeled N-cadherin first intron oligonucleotides containing the putative Twist1 E-box. Addition of a 50-fold excess unlabeled wild type oligonucleotides efficiently competed for complex formation, while excess mutant

oligonucleotides (**TT**TGTG) did not inhibit complex formation (lanes 3 and 4 respectively). To define Twist1 as the nuclear protein associating with the putative Twist1 E-box, Twist1 antibodies were added to the shifting reactions which resulted in a supershifted band (lane 5). Moreover, purified GST-Twist1 also binds to and shifts the putative Twist1 E-box (lane 7). As expected, nuclear extracts from PC-3 cells grown in suspension failed to shift the putative Twist1 E-box oligonucleotides (lane 6). These data suggest that Twist1 regulates adhesion dependent expression of *N-cadherin* in prostate carcinoma through a direct interaction with an E-box located within the first intron of the human *N-cadherin* gene.

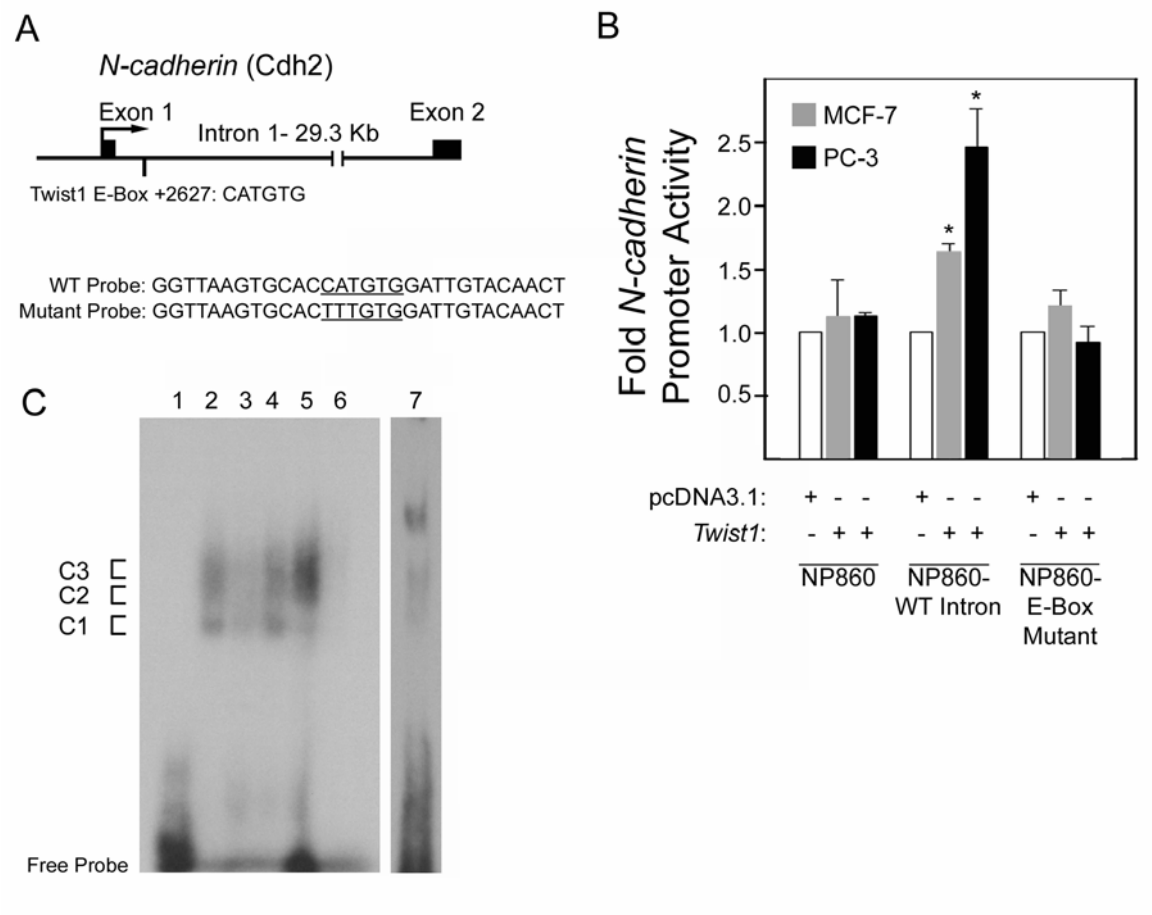

Figure 9. Twist1 regulates *N-cadherin* expression through binding to an E-box within the N-cadherin gene.

(A) Schematic of the N-cadherin 5' promoter region. **(B)** human *N-cadherin* promoter activity in MCF7 and PC3 cells co-transfected with empty vector (pcDNA 3.1), or human *Twist1*. The promoter activity was normalized to the activity of the pTK*Renilla* control and represented as fold over control (pcDNA 3.1) for each cell line. * denotes statistical significance of both cell lines ($p = < 0.05$). **(C)**, EMSA analysis with the N-cadherin intron E-box oligonucleotides and nuclear extracts from adherent (lanes 2-5) and suspended PC-3 cells; lanes are as follows: lane 1- probe only, lane 2- adherent PC-3 nuclear extract, lane 3- 50x WT competitor, lane 4- 50x mutant (**CA**TGTG to **TT**TGTG) competitor, 5- supershift with Twist antibody, 6- suspended PC-3 cell nuclear extract, 7- 100ng of GST-Twist1.

Together our data implicate Twist1 as a direct regulator of the *N-cadherin* promoter at a cis-element located within the first intron. Transcriptional regulation of E-cadherin expression through cis-elements located within introns was first documented by Hennig and colleagues (232), while the tissue specific regulation of E-cadherin transcription involves both the first and second introns *in vivo* (241;242). While the sequences of the first and second introns of the E- and N-cadherin genes are quite dissimilar, it seems as though the intronic regulation of transcription has been conserved between the two genes.

In this work we have provided the first evidence of direct transcriptional regulation of human N-cadherin expression in cancer cells, through a Twist1 consensus E-box located within the first intron of the *N-cadherin* gene. Analysis of *D*N-cadherin in Drosophila shows a correlation between *D*N-cadherin and Twist1 expression during mesoderm development (20), and there also seems to be a correlation between N-cadherin and Twist1 expression in gastric carcinoma (278). mTwist1 has been shown to inhibit mouse mammary cell differentiation, and upregulate mesenchymal markers including N-cadherin (169;279). Twist1 expression has recently been shown to correlate with an increased Gleason score in human prostate cancer, and displayed both nuclear and cytoplasmic accumulation (171). The work presented in this report documents the β1 integrin mediated control of nuclear accumulation of Twist1, and thus transcriptional regulation of *N-cadherin*. Taken together, these data indicate that TWIST1 is a pivotal transcription factor that regulates the expression of cell adhesion molecules during

normal EMT process and cancer metastasis through multiple mechanisms including the direct transcriptional regulation of *N-cadherin*.

IV. THE PUTATIVE TUMOR SUPPRESSOR FOXP1 REPRESSES *N-CADHERIN* EXPRESSION IN PROSTATE CARCINOMA

Introduction

During development, the complex process of cell migration involves changes in the expression patterns of genes involved in cell adhesion and motility (280). Instances of organized cell movement that involve the downregulation of epithelial genes, and the upregulation of mesenchymal genes are often refered to as epithelial to mesenchymal transitions (EMT) (281;282). It has been suggested that the changes in gene expression seen during EMT involve the upregulation of mesenchymal transcription factors that either repress, or activate the basal transcriptional machinery. Examples of such transcription factors are the zinc finger transcriptional repressor *Snail/Slug*, and the bHLH transcription activator *Twist*, both of which have been shown to regulate the transcription inherent in EMT during tumor metastasis (281;283). Given that transcription factors that suppress epithelial genes and activating mesenchymal genes are integral to EMT, it is logical then, that transcriptional repressors that repress the expression of mesenchymal genes must also be inactivated to initiate processes of EMT. Therefore, in contrast to transcriptional activators of mesenchymal genes, this work aims at identifying transcriptional repressors that inhibit the expression of mesenchymal genes preceding processes of EMT.

In prostate cancer disease progression is thought to proceed through multiple steps to metastatic disease and is associated with changes in glandular cytostructure, such

as alteration of epithelial cellular organization, and disruption of basement membrane composition. The integrity of the epithelial phenotype is maintained in part through the ability of E-cadherin to establish strong cell:cell adhesion through the interactions with the catenins α, β and $p120^{ctn}$ and the actin cytoskelton. The *E-cadherin* gene at 16q22.1 is a common target for transcriptional repression which is considered a key step in EMT and the metastasis of carcinomas (284). Studies indicate that the silencing of *E-cadherin* occurs through genetic and epigenetic pathways. Several transcription factors have been implicated in such repression including the zinc finger proteins Slug/Snail, Zeb1, SIP-1 and the basic helix loop helix factors E12/E47 through interaction with E-box sequences (285-288). The proximal 5' promoter region (-178-+92) in the human *E-cadherin* gene contains four E-boxes which bind the transcriptional repressors Snail, and this small region in the proximal promoter is sufficient for *E-cadherin* transcriptional repression *in vitro* (289). While the core promoter region of the *E-cadherin* gene is sufficient to recapitulate *in vitro* expression, enhancers and/or repressors in the first and second introns of *E-cadherin* gene are necessary for tissue specific expression *in vivo* (241).

The neuronal cadherin, N-cadherin, is upregulated in carcinomas during both normal cell migration and pathologic tumor metastasis (256;290), and has been detected in multiple human carcinomas including prostate, colon, gastric, esophageal, pancreatic, and breast (256;291). Aberrant N-cadherin expression in metastatic carcinoma has been shown by our lab to upregulate anti-apoptotic signaling pathways (76). Expression of N-cadherin in breast cancer potentiates the signaling of the FGF receptor 1 (249) and may actively contribute to enhanced cell migration and invasion in multiple tumor types

(251;252;254). While the function of N-cadherin in carcinoma progression has received much attention, the molecular mechanisms underlying the aberrant upregulation of N-cadherin during metastasis are unknown.

N-cadherin expression is repressed in normal epithelium, therefore, aberrant expression of N-cadherin during cancer progression is due to significant changes in both the positive and negative regulation of the *N-cadherin* promoter. The *N-cadherin* gene located at 18q11.2 and consists of 16 exons which covers ≈226.3 kb of genomic DNA (243). Characterization of the human *N-cadherin* 5' promoter region reveals that SP1 and MZF cis-regulatory elements, located in the core promoter region (bases -335 to -18), are important for normal expression of *N-cadherin* in osteoblasts (265). Although transcription factors such as SP1 are important for core promoter activation in cells which express N-cadherin, we are interested in negative cis-regulatory elements in the *N-cadherin* promoter that control cell-type specific expression in epithelial derived tumors.

To identify transcriptional repressors of N-cadherin, we have characterized a 1 Kb region of the first intron of the human *N-cadherin* gene that contains the necessary information for cell-type specific expression. We document that deletion or mutation of this silencing cis-element eliminates the repression of the activity of the *N-cadherin* core promoter in carcinoma cell lines. Furthermore, we report the specific association of a Forkhead Family transcription factor FoxP1 with the *N-cadherin* silencing cis-element. Mutation of the FoxP1 consensus DNA binding site within the first intron of the N-cadherin gene eliminates cell-type specific activity of the N-cadherin core promoter. We,

therefore, implicate the putative tumor suppressor FoxP1 in the transcriptional repression of *N-cadherin* in prostate carcinoma cell lines.

Results

A First Intron Cis-Regulatory Element in the N-cadherin Gene is Necessary for Expression in Epithelial Tumors

We are interested in the molecular mechanisms that control the upregulation of N-cadherin during prostate tumorigenesis, and repression of *N-cadherin* expression in epithelium. The transcriptional repression of *N-cadherin* is maintained in less aggressive tumor cell lines, while more migratory and invasive tumor cell lines have upregulated N-cadherin expression. In order to develop reporter assays that recapitulated the expression of *N-cadherin*, we determined the mRNA levels of *N-cadherin* in carcinoma cell lines. Figure 11A shows semiquantitative RT-PCR analysis of *N-cadherin* and *E-cadherin* mRNA from PC-3, Du145, MCF-7 and HT-29. The prostate carcinoma cell lines PC-3 and the variant PC-3N express abundant levels of *N-cadherin*. The cell lines Du145, MCF-7 and HT29 of prostate, breast and colon origins do not express *N-cadherin*, yet express high levels of *E-cadherin* transcripts. These data predict, therefore, that N-cadherin reporter constructs should show high levels of activity in PC-3 and PC-3N cells, and display significantly less activity in Du145, MCF-7 and HT-29 cells.

To determine the cis-elements necessary for the regulation of *N-cadherin* expression in N-cadherin negative cell lines, we cloned regions of the human *N-cadherin* 5' promoter and segments of the first intron into pGL3basic. The +1 bp to +2822 bp

sequence of the first intron of N-cadherin is shown in figure 10. Two luciferase constructs that contain only 5' information, NP-1900 (-1900 bp to -11 bp) and NP -860 (-860 bp to +20 bp), do not recapitulate cell type specific *N-cadherin* expression in carcinoma cell lines, as determined by luciferase assays (Figure 11C and 12B). It was determined that a 1 Kb fragment of the first intron of the human *N-cadherin* gene (+373 to +1338), inserted 3' of luciferase, was necessary for cell type specific activity of the NP-860 reporter construct (NP-860/+1338, Figure 11C and NP-1900/+1338, Figure12B). The NP-860/+1338 reporter construct dramatically decreases the activity of the N-cadherin core promoter in luciferase assays in non-*N-cadherin* expressing Du145, MCF-7 and HT-27 cells, while having no statistically significant effect on *N-cadherin* promoter activity in PC-3 and PC-3N cells which endogenously express *N-cadherin* (Figure 11C). The *N-cadherin* promoter activity of the NP-860/+1338 reporter construct recapitulates the mRNA expression shown in figure 11A. Interestingly, sequences in the first intron of the *N-cadherin* gene did not enhance the expression of the *N-cadherin* core promoter in N-cadherin expressing cell lines. These data suggest that the cell-type specific activity of the NP-860/+1338 *N-cadherin* promoter construct is due to the presence of a repressive cis-element(s) located with in the first intron of the *N-cadherin* gene. *E-cadherin* promoter activity with the 5' proximal promoter region (-178 bp to +92 bp) is shown in figure 12 A.

In order to identify the cis element in the *N-cadherin* first intron that is necessary for cell-type specific expression of the *N-cadherin* core promoter, we created deletion mutants of the first intron (+373 bp to +1338 bp) of the *N-cadherin* gene through *Sma* I

restriction. When transfected into Du145 prostate carcinoma cells, only the intron deletion mutants that contained the region +373 bp to +757bp consistently repressed the activity of the *N-cadherin* core promoter. The same deletion mutants had little effect on the activity of the *N-cadherin* core promoter when transfected into PC-3 cells (Figure 13), suggesting that the deletion mutants that repressed *N-cadherin* promoter activity contain the nessary elements for cell-type specific expression of *N-cadherin*. These data also predict that the cis-element located in the first intron of the *N-cadherin* gene necessary for repression of the *N-cadherin* core promoter resides within a 384 base pair region between +373 bp to +757 bp.

Figure 10. Sequence of the *N-cadherin* first intron from +1 bp to +2822 bp.

The *N-cadherin* gene, located on on chormosome 18q11.2, spans ≈ 250 Kb. This figure shows the DNA sequence of the first intron of the N-cadherin gene from +1 bp to +2822 base pairs. Underlined is the 60 bp first intron. Highlighted in green is the ATG start of translation. The red and red boxed sequence at +450 bp is the putative FoxP1 consensus DNA binding sequence. The blue and blue boxed sequence at +2627 bp is the putative Twist1 consensus DNA binding sequence. These two transcription factor binding sites lie within regions of homology between the human and mouse first introns of the two *N-cadherin* genes.

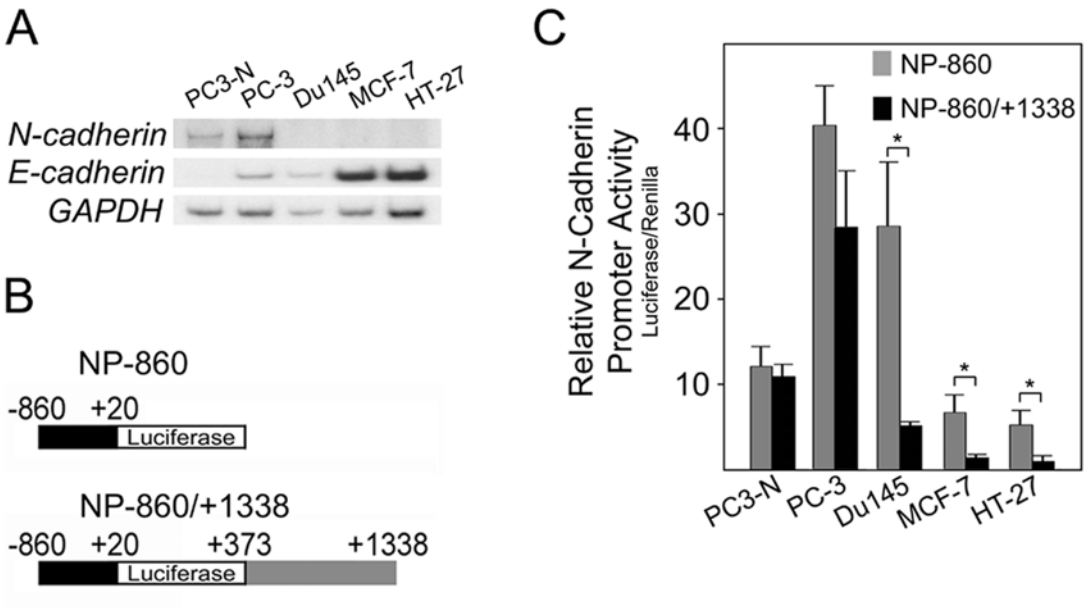

Figure 11. A silencing cis-element in the first inron of the N-cadherin gene is necessary for repression of the N-cadherin promoter in N-cadherin negative tumor cell lines.

A) Semi-quantitative analysis of *N-cadherin* mRNA expression in total RNA from tumor cell lines using RT-PCR. (B) Representation of the human N-cadherin promoter constructs. (C) A cis-element located in the first intron of the N-cadherin gene is necessary for cell type specific expression of the N-cadherin luciferase promoter construct. Data is representative of three independent experiments. Values represent the mean and standard deviation. * indicates statistical significance ($p = < 0.05$)

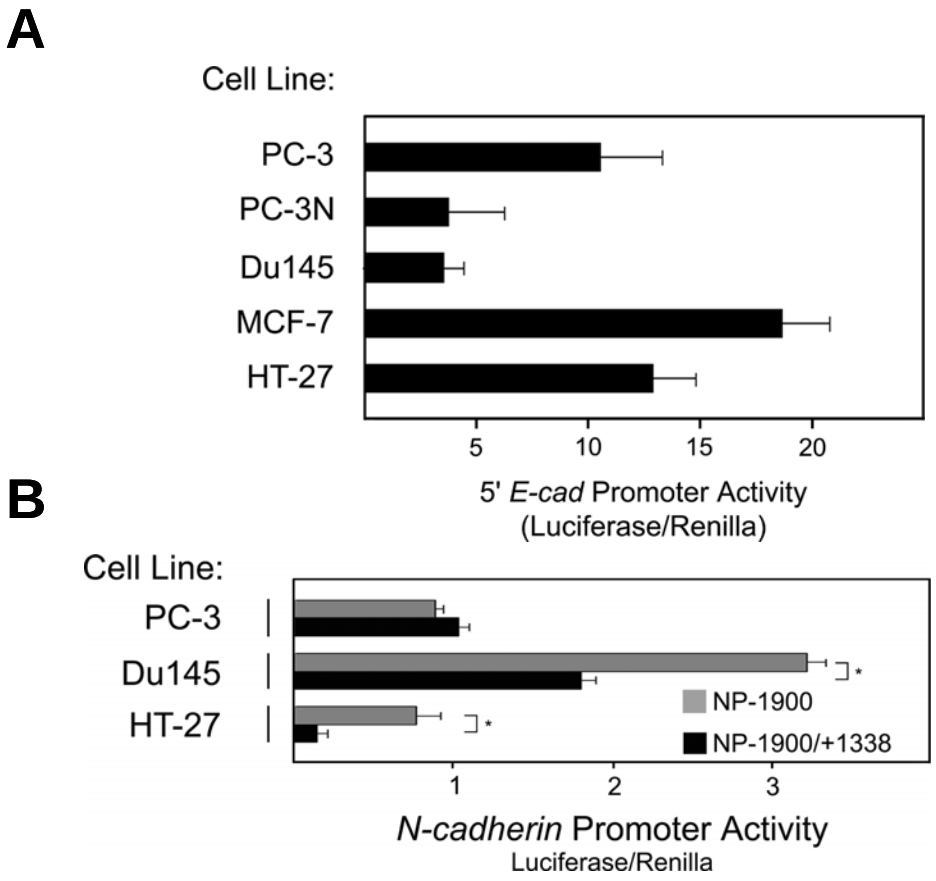

Figure 12. E-cadherin and N-cadherin promoter activity in carcinoma cell lines.

A) *E-cadherin* promoter activity using a luciferase construct containing -178 bp to +92 bp fragment of the *E-cadherin* promoter. (B) *N-cadherin* promoter activity with the NP-1900 construct which contains the -1900 bp to -11 bp fragment of the *N-cadherin* promoter, or with NP-1900/+1338 that contains the +373 bp to +1338 bp fragment of the first intron of the *N-cadherin* gene. Note that only when the ≈1 Kb fragment of the intron is included in the construct do non *N-cadherin* expressing cells show reduced levels of *N-cadherin* promoter activity. Data is representative of three independent experiments. Values represent the mean and standard deviation. * indicates statistical significance ($p = < 0.05$)

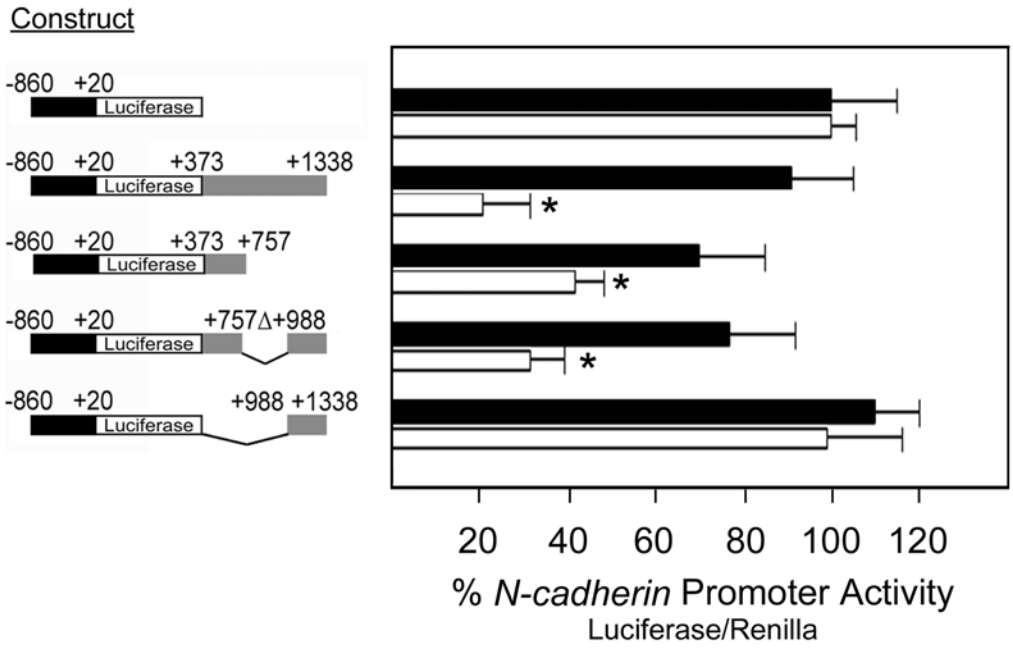

Figure 13. The silencing cis-element is located with in the +373 to +757 region of the first inron of the N-cadherin gene.

Representations of the deletions to the intron region of the -860/+1338 *N-cadherin* luciferase reporter, and experimental results from three independent experiments in Du145 (white bars) and PC-3 (black bars) cells. Data is presented as % promoter activity, where the NP-860 promoter construct was set as 100%. Values represent the mean and standard deviation. * indicates statistical significance ($p = < 0.05$)

The Putative Tumor Suppressor FoxP1 Binds to the N-cadherin First Intron

To focus our efforts on the identification of specific transcription factors, we used the MatInspector V2-2 software, and the Transfac 4.0 database was used to interrogate the +373-+757 region of the first intron of the *N-cadherin* gene for putative transcription factor binding sites (292). A putative binding site for a transcriptional repressor of great interest was located at +450, and proved to be a complete consensus DNA recognition site for the Forkhead transcription factor family member FoxP1 (TGTTTAT). Figure 14 shows RT-PCR analysis of Forkhead family members (*FoxO1A*, *FoxO3A*, *FoxP1*, *FoxP2*, *FoxL1*, and *FoxL2*) expressed in prostate carcinoma cells. The prostate carcinoma cell lines Du145 and PC-3N express FoxP1 as determined by semiquantitative RT-PCR using primers directed against a 3' 250 bp region of the message outside of the Winged helix domain, and immunoblotting with an antibody against the 17 most carboxy terminal amino acids (CDHDRDYEDEPVNEDME) (Figure 15). Differential expression of N-terminally truncated variants of FoxP1 were detected in Du145, PC-3 and PC-3N cells; Du145 express more abundant levels of slower migrating bands at 82 and 80 KDa, while PC-3 and PC-3N cells express high levels of a faster migrating band at 63 KDa (figure 15). Interestingly, deletion of the N-terminus of FoxP1 has been shown to eliminate transcriptional repression (142). As murine FoxP1 transcription factors may form homo- and heterodimers through interactions involving the leucine zipper domain (293), the expression of an N-terminally truncated form of FoxP1 may be a mechanism by which tumor cells inactivate the repressive activity of full length FoxP1. This

suggests that post-translational modifications involving cleavage, or differential mRNA splicing may regulate the activity of FoxP1.

To determine whether FoxP1 actively bound the consensus DNA binding site located at +450 bp to +456 bp in the first intron of the *N-cadherin* gene, an electromobility shift assay (EMSA) was preformed. Nuclear extracts were incubated with radio-labeled oligonucleotides correlating to +438 bp to +468 bp of the *N-cadherin* gene. As shown in figure 16, proteins from the Du145 nuclear extract form specific DNA:protein complexes with the +438-+468 oligonucleotides which are eliminated when a 50 fold excess of unlabeled oligonucleotides is added. To implicate FoxP1 as the transcription factor binding to the *N-cadherin* +438 bp to +468 bp oligonucleotides, nuclear extracts from Du145 cells overexpressing myc-tagged FoxP1 were incubated with labeled *N-cadherin* +438 bp to +468 bp oligonucleotides. The pattern of DNA:protein complexes from nuclear extracts from cells expressing the myc-tagged FoxP1 is the same as the parental Du145 cell nuclear extract. Anti-myc monoclonal antibodies were added to extracts from Du145 cells overexpressing myc-tagged FoxP1 which resulted in a super-shifted band (Figure 16, complex 2), suggesting that FoxP1 can indeed bind and occupy its consensus DNA binding site located in the *N-cadherin* first intron at +450.

While nuclear extracts from PC-3 cells also form DNA:protein complexes with the *N-cadherin* +438 bp to +468 bp oligonucleotides, proteins from PC-3N cell nuclear extracts were unable to complex with labeled oligonucleotides. These data suggest that FoxP1 is competent to bind DNA in PC-3 cells, but that FoxP1 does is not able to bind its consensus DNA site in PC-3N cells. These data also predict potential post-translational

modifications (eg. phosphorylation or acetylation) of FoxP1 in PC-3N cells that are distinct from modifications of FoxP1 in Du145 cells, such that the modifications of FoxP1 in PC-3N cells renders the protein unable to interact with its cognate DNA binding sequence.

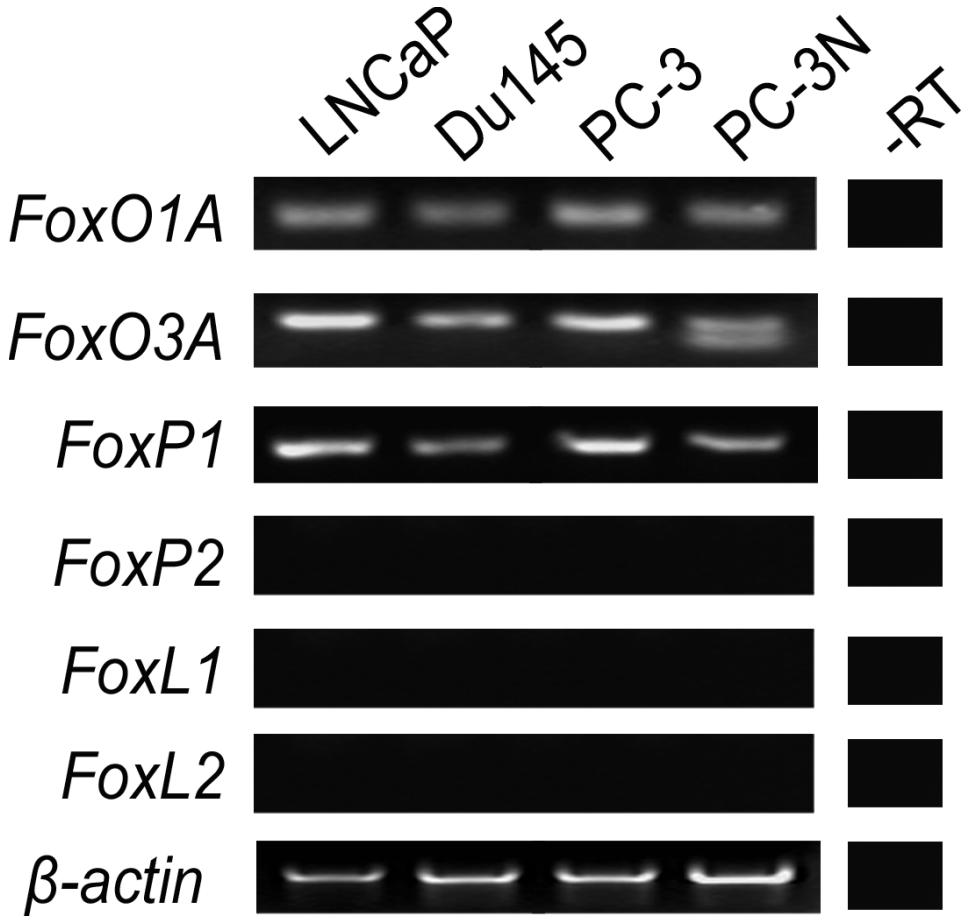

Figure 14. Forkhead family member expression in prostate carcinoma cell lines.

RT-PCR analysis of *FoxO1A*, *FoxO3A*, *FoxP1*, *FoxP2*, *FoxL1*, and *FoxL2* mRNA from prostate cancer cell lines. All primers were for sequences outside of the conserved Winged Helix domain, such that the bands are specific for one Forkhead family member. Messages could not be detected for *FoxP2*, *FoxL1*, or *FoxL2* even when multiple annealing temperatures were tried (60°, 55° and 50°)

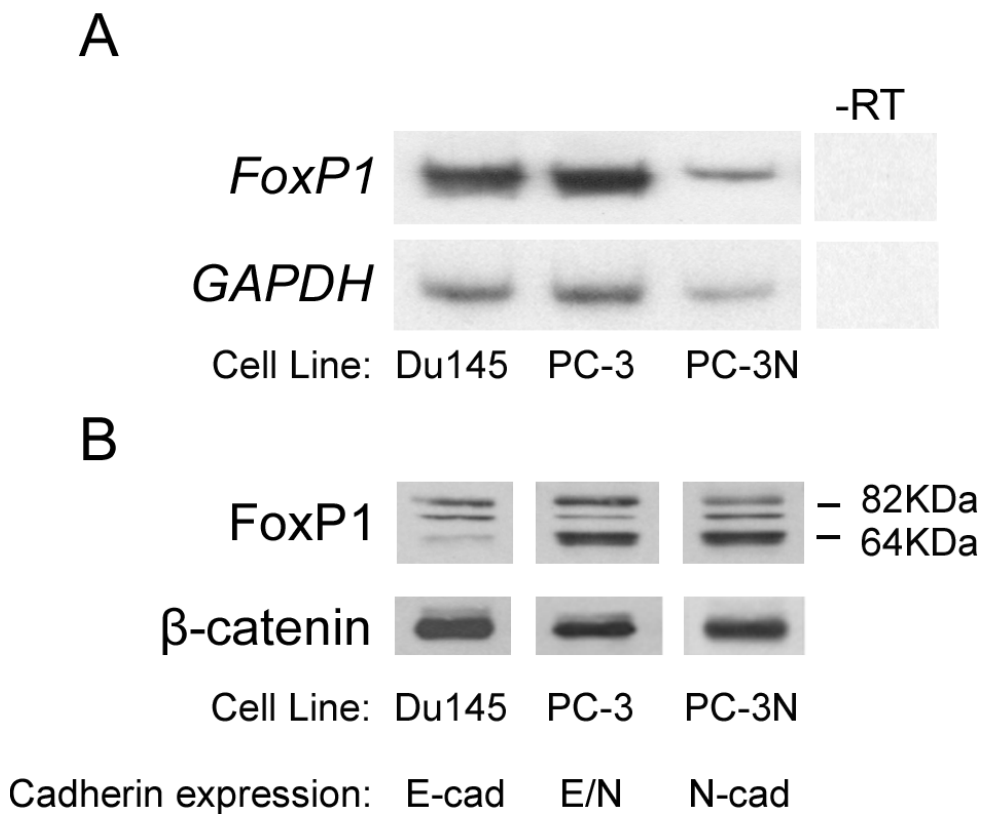

Figure 15. FoxP1 expression in prostate carcinoma cell lines

(**A**) Semi-quantitative RT-PCR analysis of *FoxP1* mRNA expression in the prostate carcinoma cell lines Du145, PC-3 and PC-3N. The primer was directed to the 3' portion of the message, and -RT denotes negative control (**B**) Immunoblot analysis of FoxP1 protein from prostate carcinoma cell lines with a polyclonal antibody against the C-terminus of the protein. B-cateinin eas used as a loading control. The expression of E- and N-cadherin is listed under the immunoblot analysis.

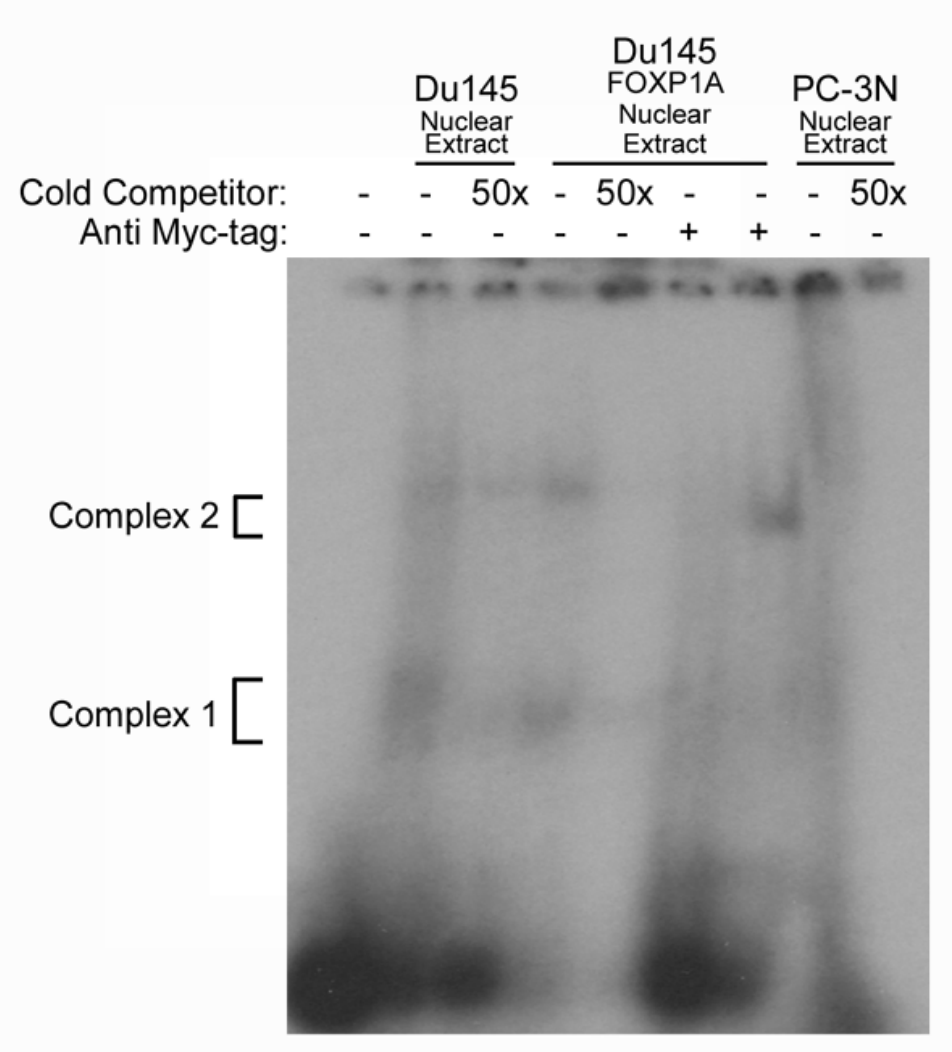

Figure 16. FoxP1 binds to a putative FoxP1 consensus site in the first intron of the human *N-cadherin* gene.

EMSA analysis with the N-cadherin +438 bp to +468 bp oligonucleotides using nuclear extracts from Du145 cells, Du145 cells transfected with myc-FoxP1, and PC-3N cells. Complex 1 indicates the major nucleo-protein complex, and complex 2 indicates supershift with anti-myc antibodies. Note that nuclear extracts from PC-3N cells fail to form protein:DNA complexes with labeled oligonucleotides.

FoxP1 is Necessary for Repression of the N-cadherin Promoter in Prostate Carcinoma

We next mutated the putative FoxP1 DNA binding site in the NP-860/+1338 luciferase reporter. Changing the FoxP1 consensus sequence from TGTTTAT to GGATTAA (NP-860/ΔFoxP1) eliminates the ability of the +373 bp to +1338 bp segment of the first intron of the N-cadherin gene to repress the activity of the core promoter of *N-cadherin* in Du145 cells (Figure 17). As FoxP1 binds to this site in the N-cadherin first intron in vitro (Figure 16), together these data implicate FoxP1 as a transcriptional repressor of N-cadherin expression in prostate epithelium.

Interestingly, although PC-3N cells express FoxP1, the ability of nuclear extracts from PC-3N cells to form complexes with labeled N-cadherin +438 bp to +468 bp oligonucleotides is significantly decreased compared to nuclear extracts from Du145 cells (Figure 16). This suggests that the DNA binding of FoxP1 is attenuated in PC-3N cells. A common pathway to inhibit Forkhead transcription factor activity is the PI3K-Akt signaling cascade, and it has been suggested that kinases downstream of this pathway may regulate the activity of FoxP1. While PC-3N cells have activated PI3K (76), we could not detect serine or tyrosine phosphorylation of FoxP1 (data not shown).

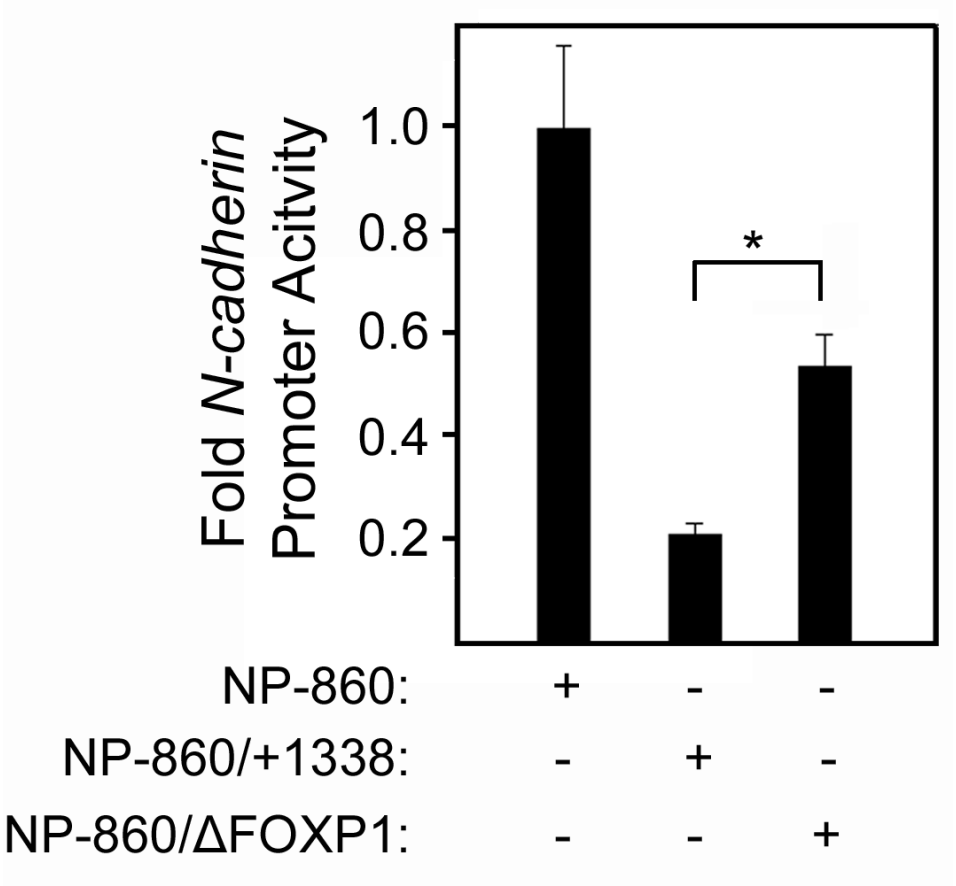

Figure 17. Deletion of FoxP1 consensus DNA binding site reduces cell-type specific activation of the *N-cadherin* promoter.

Luciferase analysis of human N-cadherin promoter activity with N-cadherin 5' promoter alone (NP-860), the 5' promoter containing a one Kb region of the first intron of the N-cadherin gene (NP-860/+1338), or 5' promoter containing a mutant FoxP1 DNA binding site at +450. The N-cadherin promoter construct NP-860/ΔFoxP1 contains three point mutations, TGTTTAT to GGATTAA, within the FoxP1 DNA binding site. Data is representative of three independent experiments, and presented as fold over control where the N-cadherin core promoter (NP-860) has been set at one. * indicates statistical significance ($p = < 0.05$)

Discussion

During the process of EMT, the expression of cell adhesion molecules is altered such that genes encoding for adhesion molecules involved in the maintenance of the epithelial phenotype are downregulated, and genes encodeing cell adhesion molecules that facilitate cell migration and invasion are upregulated (280). It is known that the changes in gene regulation during EMT are regulated by transcription factors that either repress epithelial gene expression, or upregulate mesenchymal gene expression. The N-cadherin cell:cell adhesion molecule is frequently upregulated during normal processes of EMT, and during pathological tumor metastasis (268;294). As N-cadherin is never expressed in normal epithelium, the aim of this work was to determine the transcription factor(s) that functions to repress N-cadherin expression in epithelium, and determine how such transcription factors are regulated during EMT and tumor metastasis.

We and others have documented the functional importance of N-cadherin during EMT and carcinoma progression (76;249;252;254). The role of N-cadherin during EMT and during carcinoma progression seems to be two fold: the upregulation of a migratory phenotype, and the initiation of intercellular signaling primarily through the recruitment and activation of PI3K in the adhesion complex. Although the function of N-cadherin has received much attention, the genetic regulation of human *N-cadherin* gene expression has only recently been investigated. Lee *et. al.* have recently determined the functional units of the *N-cadherin* core promoter in osteoblasts *in vitro* (265). While these authors have determined the necessity of the core promoter for sustained activity of the N-

cadherin promoter, we have investigated the cis-elements in and around the N-cadherin core promoter that are necessary for cell-type specific activity of the *N-cadherin* promoter. We report in this publication that the transcriptional repression of the *N-cadherin* promoter in N-cadherin negative carcinoma lines is, in part, due to a negative regulatory cis-element located in the first intron of the *N-cadherin* gene. We characterized the binding of the putative tumor suppressor FoxP1 to its consensus DNA recognition site at +450 bp in the first intron of the *N-cadherin* gene, and the importance of the FoxP1 DNA binding site for repression of *N-cadherin* promoter activity in N-cadherin negative cell lines.

The experiments in this study were designed to examine *N-cadherin* promoter regulation in cells that do not express *N-cadherin*. As such, these data suggest that the repressive cis-element located in the first intron of the *N-cadherin* gene may be a common means by which epithelial cells silence *N-cadherin* gene expression. While FoxP1 is expressed in N-cadherin positive cell lines, we believe there may be epithelial specific repressors of N-cadherin transcription and are in the process of characterizing other regions of the first intron of N-cadherin for such factors.

The activity of tissue specific cis-elements located within introns in the genes of classical cadherins is not unprecedented. Multiple publications from the Kemler lab have eloquently proven the necessity of large regions of the first and second introns of the murine *E-cadherin* gene for tissue specific expression *in vivo* (241;295). There are 16 introns and 15 exons in both *E-* and *N-cadherin*, with striking size similarities in intron length in all but the first two introns (243;296). The first and second introns of E-

cadherin are 1 and 65 Kb respectively, while the first and second introns of N-cadherin are 27 and 129 Kb respectively. Although there is no sequence similarity, there is regional similarity, at least in the fact that there are cis-elements located within the first introns of the E- and N-cadherin genes. Notably, there is an epithelial specific transcriptional enhancer located at +456 bp to +574 bp in the first intron of the murine E-cadherin gene (232).

FoxP1 was originally identified through a search for tumor suppressor genes, and has been shown to be differentially expressed at the mRNA and protein levels in a number of tumors including breast, stomach, colon and prostate (139). Work by Shi, C. *et. al.* documented the necessity for the amino-terminus of FoxP1 for transcriptional repression, showing that an amino-terminal truncated version of FoxP1 (≈60 KDa) displays little transcriptional repression on a physiological gene target *in vitro* (142). FoxP1 has been shown to interact with the co-repressor protein CtBP-1 (C-termial Binding Protein) (297). CtBP-1 can associate with several transcription factors and chromatin remodeling proteins including members of the class II histone deacetylases (298). We believe that inhibition of the FoxP1, and its transcriptional repression, is a key event nessesary for the initiation of *N-cadherin* expression. Lack of FoxP1 repression may change the availability of the heterochromatin around the *N-cadherin* core promoter, allowing for transcription factors such as SP1 and MZF family members to activate *N-cadherin* transcription.

FoxP1 caontains a Forkhead domain together with a zinc finger motif and a lucine zipper (139). Members of the FoxP subfamily of Forkhead transcription factors in mice

are able to interact with each other through the leucine zipper domain (143). Through this work we find that the putative tumor suppressor FoxP1 is differentially expressed at the protein level in prostate carcinoma cell lines; inasmuch as the N-cadherin positive carcinoma lines PC-3 and PC-3N produce an N-terminal variant of FoxP1 protein (Figure 15). An important report by Li and colleagues describes the necessity of the FoxP1/2/4 family of transcription factors to homo- and heterodimerize through the conserved leucine zipper domain for DNA binding, and suggest that the heterodimerization pattern of the FOXP family is a novel means by which cells regulate the transcriptional activity of these transcriptional repressors (144). Under this hypothesis, it is likely that heterodimerization between FoxP1 N-terminal variants could regulate the repressive activity of FoxP1 heterodimers, such that an N-terminally truncated FoxP1 heterodimer would yield lower transcriptional repression than would a full-length FoxP1 homodimers. N-terminal truncation of FoxP1 in PC-3N cells may be one of the mechanisms N-cadherin expressing prostate carcinoma cells utilize to inactivate the transcriptional repressive activity of full-length FoxP1. We are currently determining whether the truncation of FoxP1 is due to differential splicing or proteolytic processing.

In summary, we have documented a negative cis-element located in the first intron of the N-cadherin gene which represses N-cadherin promoter activity in a cell-type specific manner *in vitro*. The consensus DNA binding site for the putative tumor suppressor FoxP1 specifically interacts with high affinity to FoxP1 in N-cadherin negative prostate cancer cells. The N-cadherin positive prostate cancer cell line PC-3N may regulate FoxP1 activity at the protein level through the production of N-terminal

truncated FoxP1 variant. This report provides evidence for acetylation of FoxP1 in the regulation of N-cadherin transcription in prostate carcinoma. Synthesis of this report with the existing data suggests a complex genetic regulation of N-cadherin expression through the utilization of cis-elements located within introns, similar to that of E-cadherin.

V. N-CADHERIN CROSSTALK WITH THE β1-INTEGRIN INDUCES A MIGRATORY PHENOTYPE AND ACTIVATES INTEGRIN SIGNALING

Introduction

The complex process of tumor metastasis requires changes in the expression and function of cell adhesion molecules, and increased activation of the molecular machinery that facilitates cell migration. During the metastasis of prostate cancer changes in the gene expression of integrins and cadherins are hallmarks of aggressive disease (266;299). A very early change in the expression of the β4 integrin subunit, documented as early as Prostate Intraepithelial Neoplasia (PIN), results in the functional downregulation of the hemidesmosome (300;301). Characteristic of epithelial cells, the hemidesmosome is an adhesion structure that maintains a strong connection with the basement membrane by linking the cytokeratin based cytoskeleton to the α6β4 integrin heterodimer (302). Downregulation in the expression of the β4 integrin subunit may result in a higher heterodimerization pattern of the α6 subunit with the β1 subunit. This change in integrin heterodimerization pattern, also known as integrin switching, shifts cells from cytokeratin based adhesion structures, to the more motile actin based adhesion structures. Evidence suggests that this switch in integrin heterodimerization changes the outside-in signaling in response to ligand binding and actin cytoskeleton organization.

Cadherins are transmembrane glycoproteins that mediate cell:cell adhesion through an interaction with the catenins. During carcinoma progression, E-cadherin is either functionally downregulated through defects in catenin expression or function, or

transcriptionally downregulated through gene silencing at the level of the E-cadherin promoter (272). In prostate carcinoma, the downregulation of E-cadherin transcription is frequently associated with the upregulation of N-cadherin expression (303). An interesting discovery by Tomita *et. al.* was the co-expression of E- and N-cadherin in the same cells in tissue sections of prostate carcinoma (256), an observation that holds true in the prostate carcinoma cell line PC-3 (290). We are interested in the functional consequence of N-cadherin expression in tumor cells that endogenously express E-cadherin.

Cadherins appear to directly affect each other's function. Suppression of N-cadherin function in invasive squamous cell carcinoma cells results in the induction of E- and P-cadherin expression and a reversion to an epithelial phenotype. In contrast, forced expression of N-cadherin in squamous carcinoma cells with an epithelial phenotype causes the downregulation of E- and P-cadherin and induces an invasive phenotype (251). This implies that the expression of N-cadherin during tumor progression might be necessary and sufficient to overcome the E-cadherin mediated epithelial phenotype, and to promote cancer progression.

This chapter is a detailed analysis of experiments that evaluate the effect of the overexpression of N-cadherin in the E-cadherin expressing prostate carcinoma cell line Du145. Du145 cells overexpressing a GFP-tagged N-cadherin allele display increased rates of cell migration and adhesion. At the protein level, N-cadherin expressing Du145 cells show lower levels of α6 integrin, and increased association of the α3 integrin subunit with the β1 integrin. Moreover, the cellular machinery that drives cell migration

is activated at higher rates in Du145 cells expressing N-cadherin. These data represent the identification N-cadherin-integrin cross-talk that may contribute to the migration of prostate carcinoma.

Results

N-cadherin/GFP localizes to cell:cell contacts and associates with the catenins

To evaluate the effect of co-expression of E and N-cadherin on the migratory phenotype, an N-cadherin/GFP fusion construct, containing the entire coding region of *N-cadherin* fused to GFP, was transfected into Du145 PCa cells. Clones expressing the N-cadherin/GFP or GFP empty vector were isolated through serial dilution, and selected under neomycin resistance. Several clones were isolated expressing the N-cadherin/GFP fusion protein were selected and designated Ncad1, Ncad2, and Ncad3, while the clone expressing the GFP empty vector was designated GFP1. Immunoflourescence for E-cadherin in the Ncad clones, GFP clones and Du145 parental cells reveals that the N-cadherin/GFP fusion protein localizes to areas of cell:cell contact in the Ncad clones, as does E-cadherin in the GFP clone and Du145 cells (Figure 18C).

Expression of the N-cadherin/GFP fusion protein results in a ≈160 kDa product which accounts for the size of N-cadherin and the GFP tag (Figure 18A). Expression of the N-cadherin/GFP fusion protein and the GFP control had no effect on the cellular levels of the catenins alpha, beta and p120 (data not shown). Immunoprecipitation of E and N-cadherin followed by immunoblotting for the associated catenins in these same cells demonstrates that the N-cadherin/GFP fusion protein associates with p120, beta and

alpha catenin in the Ncad clones, as does E-cadherin in the GFP clone and Du145 cells (Figure 18B).

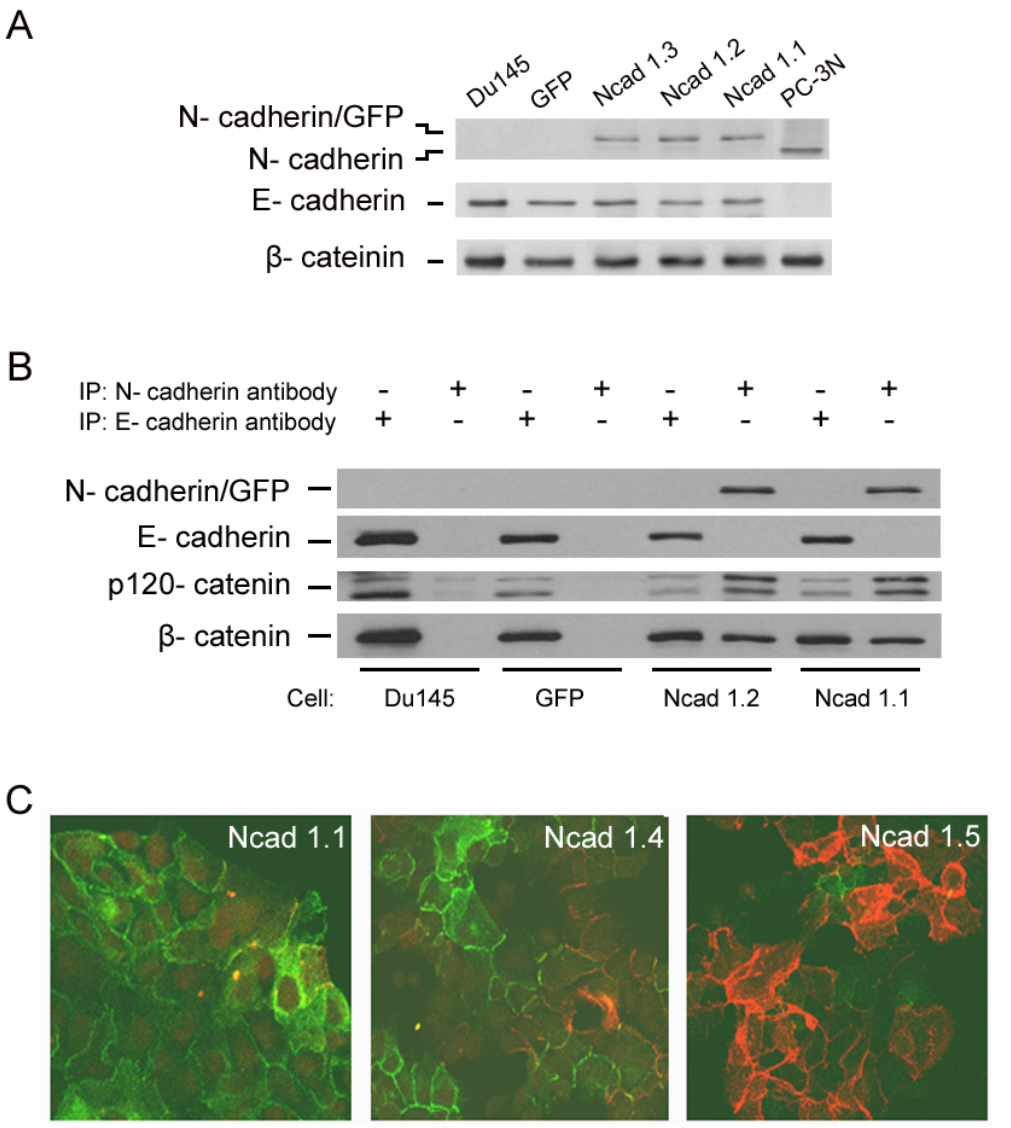

Figure 18. Characterization of Du145 transfectants co-expressing E- cadherin and N-cadherin

(A) Immunoblots of total protein from parental (Du145), empty vector control (GFP) and three isolated clones (N-cad 1.1- 1.3) expressing the N-cadherin/GFP fusion protein (≈145 KDa) using monoclonal antibodies against N-cadherin, E-cadherin and β-catenin. (**B)** Immunoprecipitation of E- and N- cadherin from Du145 cells, GFP control and N-cad clones, was followed by immunoblot analysis for E- and N- cadherin, $p120^{ctn}$ and β-catenin. **(C)** N-cad 1, N-cad 4 and N-cad 5 cells were grown on glass coverslips and labeled with a monoclonal antibody against E-cadherin (red). The N-cadherin/GFP fusion protein fluoresces green.

Analysis of Cell Migration and Adhesion in Carcinoma cells Miss-expressing N-cadherin

Data suggests that N-cadherin expression in carcinomas contributes to increased tumor cell migration. Aberrant expression of N-cadherin has been demonstrated to increase the migratory ability of carcinoma cells *in vitro* (252;254), furthermore N-cadherin expression can be detected in human metastatic prostate carcinoma (256). In this work, we have attempted to determine whether forced miss-expression of N-cadherin in Du145 prostate cancer cells enhances cell motility. The ability of N-cad clones to migrate away from a confluent monolayer of cells was evaluated in a linear migration assay, adapted from Berens *et. al.* (270). The clones expressing the highest levels of the N-cadherin/GFP fusion protein migrated the farthest in this assay, and cell motility correlated with N-cadherin/GFP expression levels in all clones tested (Figure 19). The GFP empty vector control cells migrated at rates similar to that of the Du145 parental cell line in these assays. Transwell migration chambers were utilized to determine the ability of the N-cadherin clones to migrate in three dimensions on Laminin 1. As evident in figure 20, cell migration through the transwell filters correlated with N-cadherin expression in the Du145 N-cad clones. This suggests that both linear migration and haptotactic migration is increased.

In addition to increases in rates of cell migration, N-cadherin expression in Du145 cells also display faster rates of cell adhesion to Laminin 1. Following detachment of cells with 5 mM EDTA and suspension for 30 minutes, cells were allowed to attach to coverslips that were coated with Laminin 1. At the indicated times, cell attachment was quantified through crystal violet staining. As shown in figure 21, N-cadherin expressing

cells have a six fold faster rate of cell attachment at 15 minutes than does the parental cell line Du145.

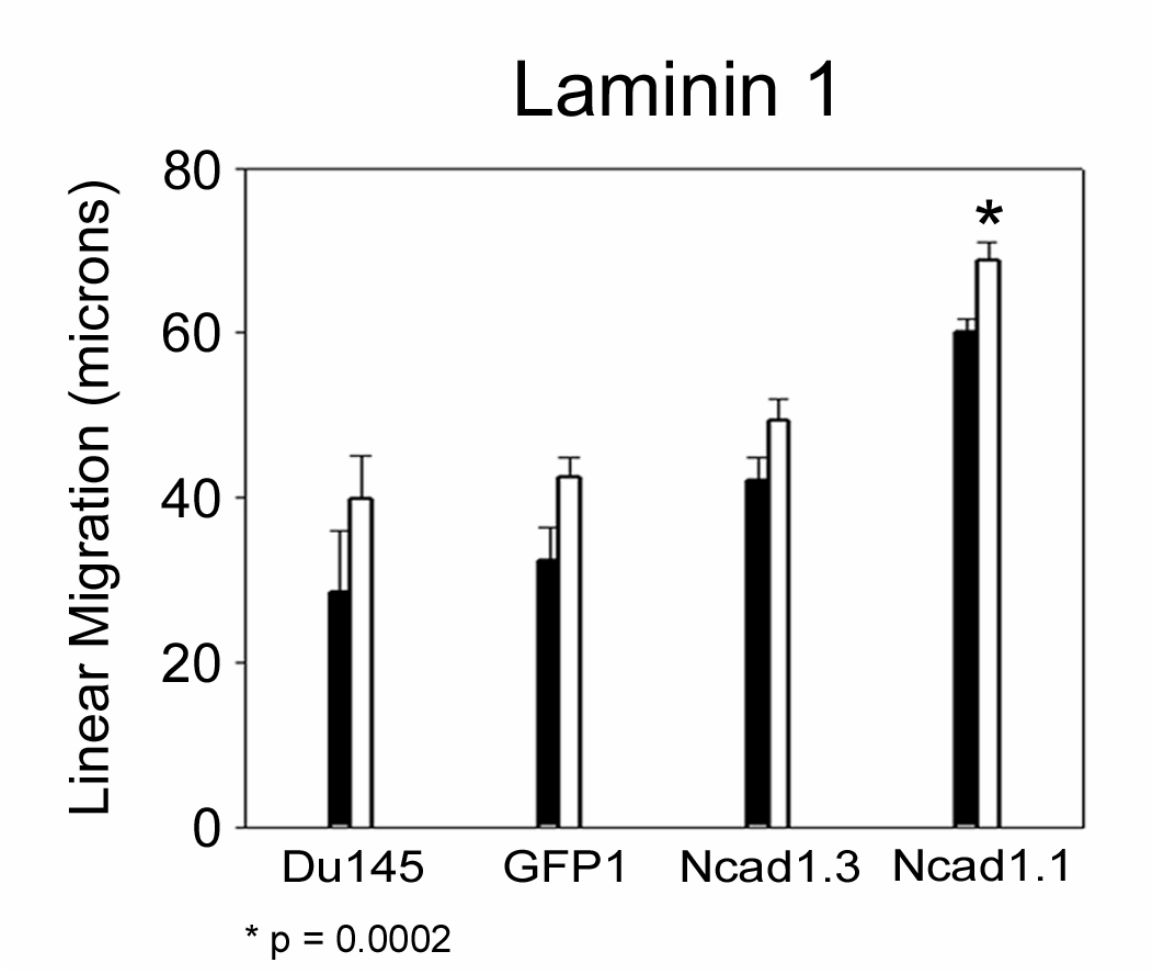

Figure 19. Co-expression of E- cadherin and N- cadherin induces cell migration in prostate carcinoma cells

Representative graph of migration data from linear migration assay on Laminin1 (10µg/ml) for the indicated cell lines. Black bars indicate 12 hours post cell attachment, white bars indicate 18 hours post cell attachment. Data is reported as radius migrated, in microns.

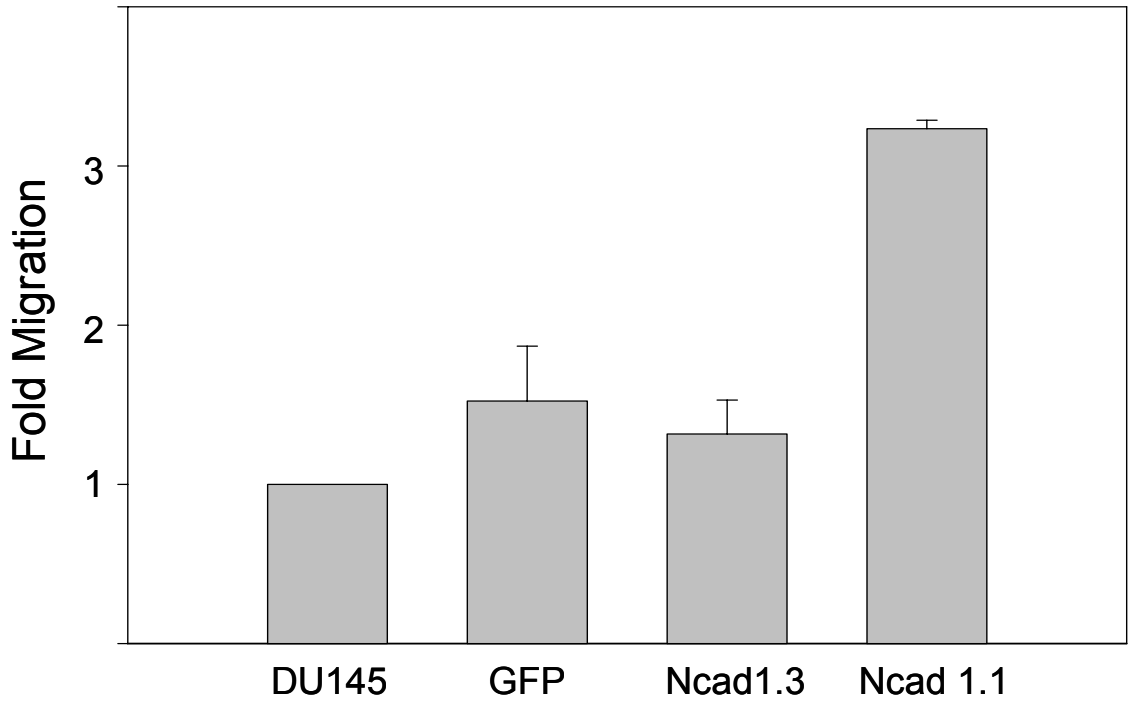

Figure 20. N-cadherin expression in Du145 cells increases 3 dimensional migration through transwell filters.

Analysis of cell migration for 18 hours through Transwell filters coated with Laminin 1 (10μg/ml). Data is the average of 3 independent experiments with migration assays in triplicate. Data is reported as the average number of cells migrated to the bottom side of the filter, and the average of Du145 cells has been set as 1.

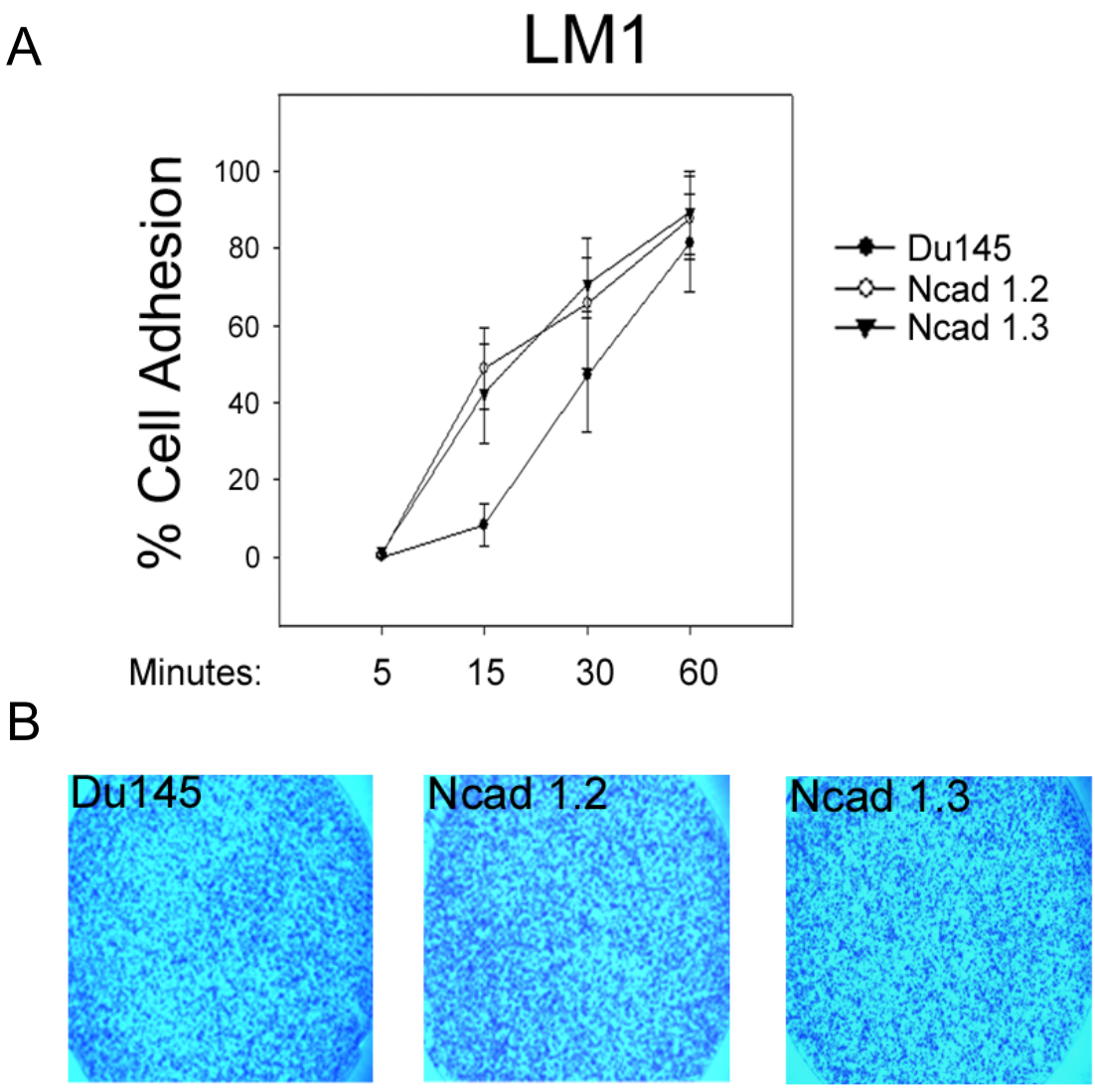

Figure 21. Expression of N-cadherin in Du145 cells increases the rate of cell adhesion to Laminin 1.

(A) Cells adhesion to Laminin 1 following 1 hour culture in suspension. Note that N-cadherin expressing Du145 clones 1.2 and 1.3 display a more rapid rate of adhesion to Laminin 1 through 15 minutes, but is not statistically significant at 30 minutes. Data is reported as percent cell adhesion, where cell adhesion at 2 hours was considered 100%. Data is representative of 3 independent experiments. **(B)** Picture of Crystal Violet stained cells at 2 hours.

The difference in cell attachment is modest at 30 minutes, and by one hour all cells have attached for all cell lines tested.

Activation of Rho family GTPases following cadherin ligation

To determine the activation of Rac1 and RhoA following cadherin ligation in N-cadherin expressing Du145 and parental cells, calcium switch assays were performed. Following incubation with 4 mM EGTA for 30 minutes to separate cadherin molecules, normal media without serum was added back, and the cells were lysed at the indicated times. Following cell lysis, equal amounts of total protein were incubated with GST fused PAK binding domain and Rhotekin to affinity purify GST bound Rac1 and Rho isoforms, respectively (101;304). Following affinity purification, immunoblot analysis determined the relative levels of GTP bound GTPase. As shown in figure 22, following cadherin ligation Rac1 GTPase is loaded with GTP more efficiently in N-cadherin expressing Du145 cells compared to the parental Du145 cells. While attempts were made to determine the activation of RhoA, no GTP-RhoA was detected under any condition, with any Du145 N-cadherin expressing clones. These results are consistent with previous studies indicating that cadherin ligation activates Rac1 and reorganizes the actin cytoskeleton (305).

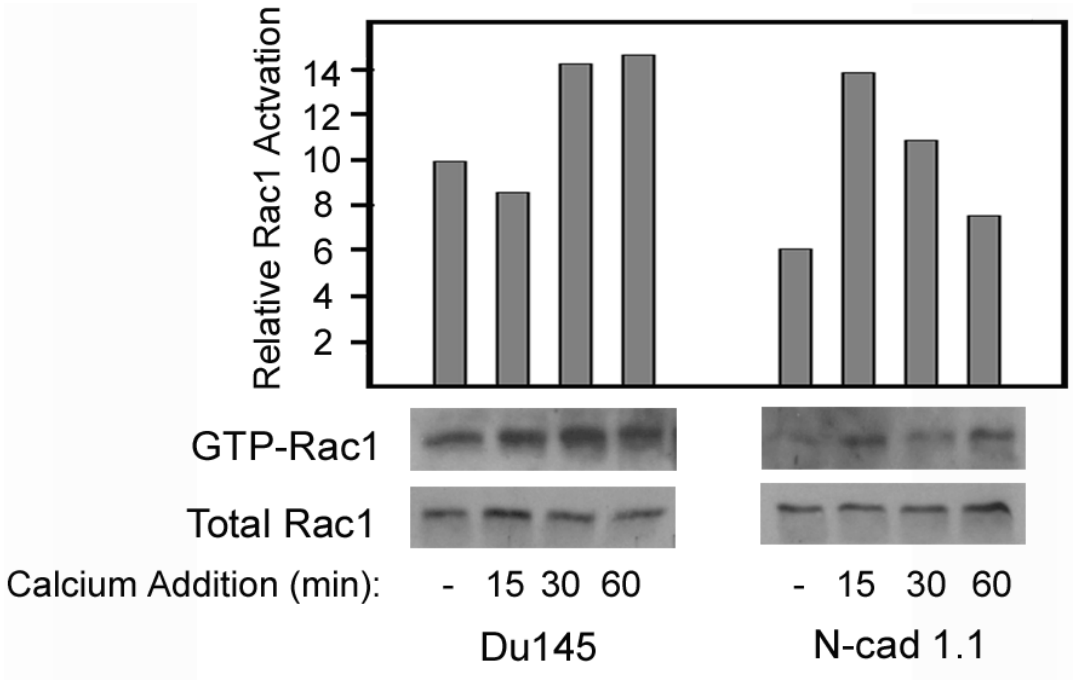

Figure 22. Rac1 is activated following cadherin ligation in N-cadherin expressing Du145 cells.

Representative immunoblots of GTP loaded Rac1, and of total Rac1 following calcium switch assay in Du145 and N-cadherin expressing Du145 clone Ncad 1.1. The graph above depicts densitometry analysis of the immunoblots, and is reported as the density of GTP-Rac1/the density of Total Rac1.

Immunofluorescence of phosphorylated tyrosinein E- and N-cadherin co-expressing cells

The steady state levels of phosphorylated tyrosine residues throughout the cell were analyzed in Du145 cells expressing N-cadherin, and in parental cells through immunofluorescence with monoclonal phospho-tyrosine specific antibody. Cells were plated onto glass coverslips in media containing serum, serum starved overnight, fixed and stained with the monoclonal phospho-tyrosine specific antibody PY20. As shown in figure 23, while the total levels of phosphorylated tyrosine does not appear to change, the distribution of phospho-tyrosine is dramatically changed. There is abundant staining of phospho-tyrosine at cell junctions in the parental cell line Du145. Conversely there is only very weak phospho-tyrosine staining at cell junctions in N-cadherin expressing Du145 cells; the majority of phospho-tyrosine staining is at the cell periphery. This suggests that there may be inactivation of kinase signaling at cell:cell junctions in Du145 cells, and that kinase signaling may be enhanced at the cell periphery in Du145 cells miss-expressing N-cadherin.

Co-expression of E and N-cadherin decreases the steady state activation of Erk 1,2 in Du145 cells

In attempts to ascertain the effects of co-expression of E and N-cadherin in Du145 cells, we analyzed the phosphorylation status of a number of proteins involved in signal transduction. Expression of the N-cadherin/GFP fusion protein has no observable effect on the steady state activation levels of GSK-3β, Akt/PKB or beta-catenin, as accessed by immunoblot using phospho-specific antibodies (data not shown). Conversely, expression of N-cadherin/GFP in Ncad clones does decrease the steady state activation levels of Erk

1,2 as analyzed by immunoblot with a phospho-specific polyclonal antibody. Figure 24 shows that while Du145 and the GFP clone have moderate levels of phosphorylated Erk 1,2 in DMEM without serum, Ncad clones have significantly reduced levels of phosphorylated Erk 1,2 under the same conditions. The ability of growth factors to activate Erk 1,2 is not affected by the expression of the N-cadherin/GFP fusion protein (Figure 24). To determine whether the reduction in phosphorylated Erk 1,2 is dependent on N-cadherin ligation, calcium was removed from the culture medium which prevents the function of classical cadherins. Following 24 hours incubation in calcium free media, the basal levels of Erk 1,2 phosphorylation in Ncad clones returns to that of the empty vector control and parental Du145 cells.

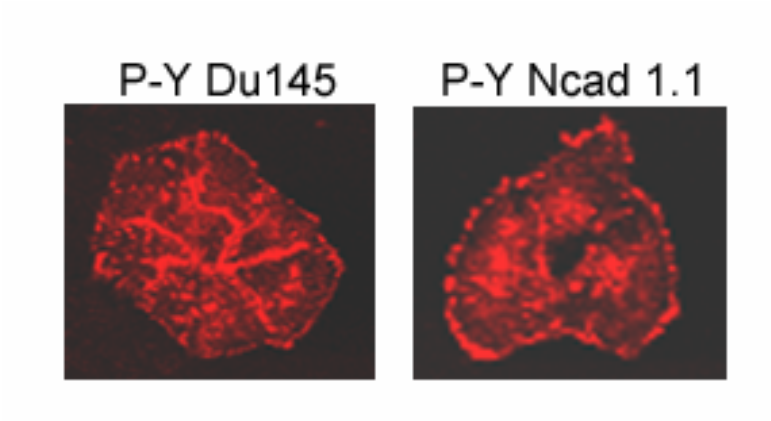

Figure 23. N-cadherin expression in Du145 cells alters the immunolocalization of tyrosine phosphorylation.

Representative confocal pictures of Du145 and N-cadherin expressing clone Ncad1.1 stained with the phosphotyrosine specific antibody PY-20. Note the absence of phosphotyrosine staining at cell-cell contacts, and increase staining at the cell periphery in Ncad 1.1 N-cadherin expressing clone.

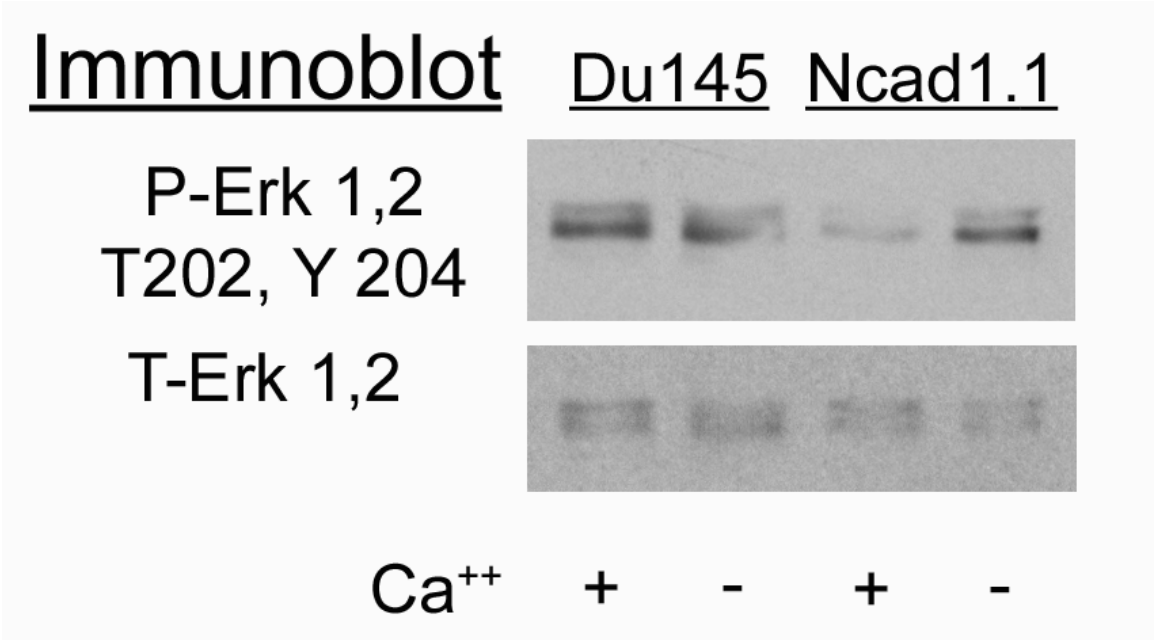

Figure 24. Co-expression of E- cadherin and N- cadherin decreases the levels of phosphorylated Erk kinase.

Whole cell lysates were separated through 12% PAGE, and membranes were immunoblotted with phospho-specific antibodies detecting both threonine 202 and tyrosine 204. The membrane was stripped and re-probed with a polyclonal antibody detecting total Erk 1,2.

N-cadherin expression in Du145 cells results in decreased expression levels of α6 integrin and increases in the α3β1 integrin heterodimer

Onishi *et. al.* have demonstrated that Sp1 sites in the promoter of the α6 integrin promoter are necessary for constitutive gene expression of the α6 integrin in Du145 cells (306). Upon examination of α6 integrin protein levels, the Du145 clones expressing the N-cadherin/GFP fusion protein have reduced protein levels of the α6 integrin, while GFP1 clones expressing the empty vector and Du145 parental cells express equivalent and robust levels of the α6 integrin (Figure 25A).

The α6 integrin subunit pairs with the β4 and β1 integrin subunits in Du145 cells (307). We were interested in determining whether the decrease in expression of the α6 integrin resulted in changes in the integrin expression and heterodimerization profile in the Du145 Ncad clones expressing the N-cadherin/GFP fusion protein. To this aim, the β1 integrin was immunoprecipitated from N-cad clones, GFP empty vector clones and the parental Du145 cells. Figure 25B shows that overexpression of N-cadherin in Du145 cells decreases the amount of α6 integrin that functionally associates with the β1 integrin. Accordingly, levels of the α3 integrin that associate with the β1 subunit are dramatically increased in the N-cad clones. These data suggest that the miss-expression of N-cadherin in prostate carcinoma may play a role in coordinating the protein turnover or gene expression levels of integrins.

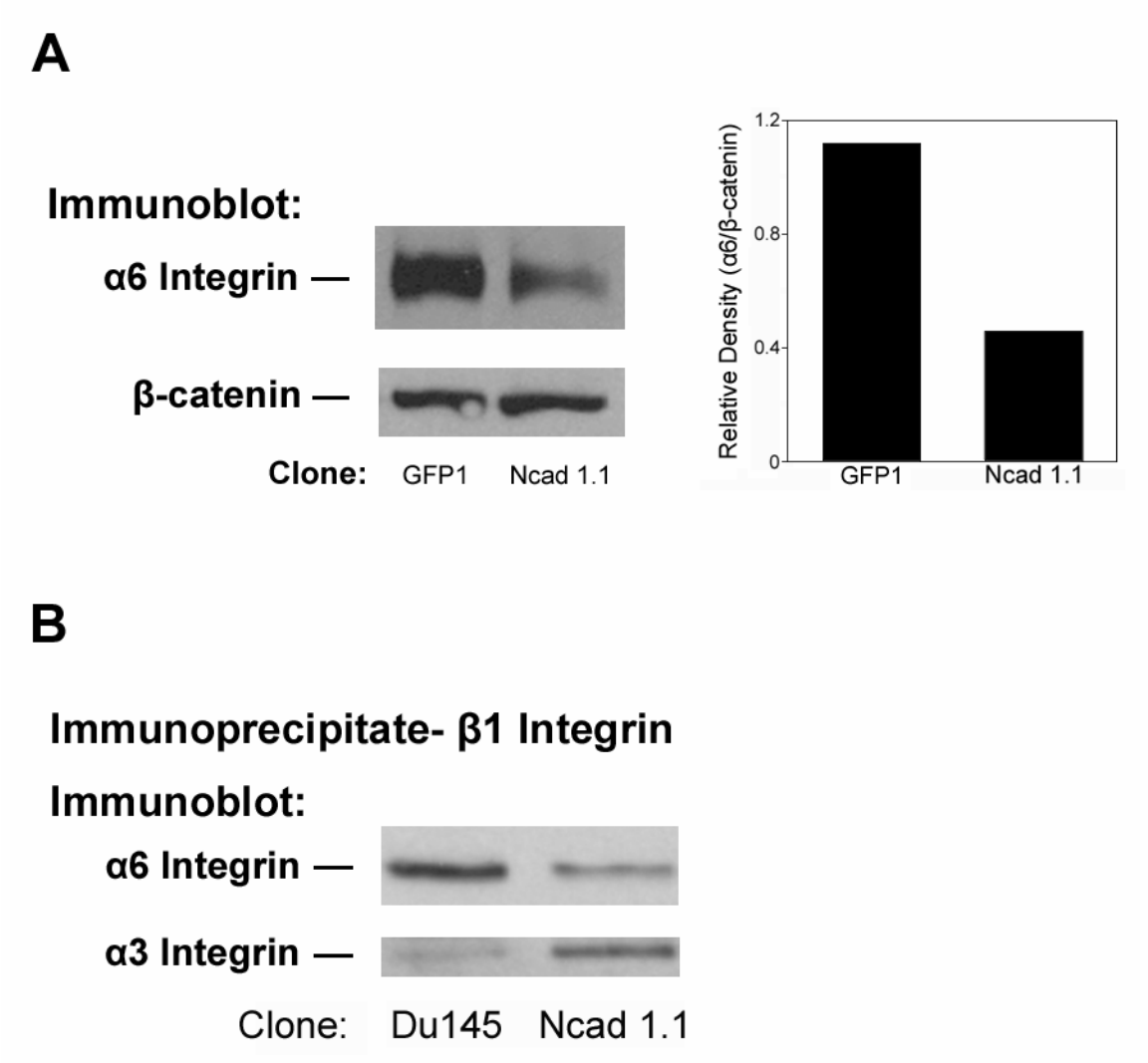

Figure 25. Co-expression of E- and N- cadherin changes the integrin expression and heterodimerization profile.

A. Immunoblot analysis of total cellular protein from GFP control and N-cadherin expressing clone 1.1 using polyclonal antibodies against the α6. **B.** Immunoprecipitation of the β1 integrin from parental cells, and N-cadherin expressing clone 1.1, was followed by immunoblot analysis with polyclonal antibodies for the α6 and α3 integrins.

N-cadherin suppresses FAK dephosphorylation and induces AKT activation following cell detachment

In addition to mediating cell anchorage to ECM molecules, integrins serve as nucleation sites for intercellular signaling (204). Although the β subunits contain the majority of the docking sites for proteins that initiate signaling pathways, it has been hypothesized that the α subunits augment the conformation of the β subunit cytoplasmic tail, thereby specifying which signaling molecules will bind and become activated. It is likely, therefore, that a switch in integrin heterodimerization pattern from the α6β1 to the α3β1 alters the integrin dependent signaling profile in the Du145 N-cad clones. Initially the levels of FAK phosphorylation were assessed in two ways, immunoblotting with a phosphorylation specific antibody that recognizes auto phosphorylation on tyrosine residue 397 and immunoprecipitation of FAK followed by immunoblotting with an antibody that recognized phosphorylated tyrosine. Using these two methods to detect tyrosine phosphorylation of FAK in cells attached to Laminin 1, neither tyrosine 397 nor total tyrosine phosphorylation differed between the parental Du145 cells or the N-cad clones (data not shown). Nor did immunoprecipitation of the β1 integrin reveal any changes in FAK tyrosine 397 phosphorylation.

In prostate cancer cell lines FAK tyrosine 397 is normally dephosphorylated by phosphatases following cell detachment, while the total levels of tyrosine phosphorylation remain unchanged (90). The levels tyrosine 397 phosphorylation of FAK were assessed in Du145, empty vector control and N-cad clones following culture in suspension for 15, 30 and 60 minutes. While tyrosine 397 was rapidly

dephosphorylated in Du145 and empty vector cells, there was a severe delay in tyrosine 397 dephosphorylation in the N-cad clones (Figure 26). This defect in dephosphorylation was dependent on functional N-cadherin adhesion as addition of N-cadherin functional blocking antibodies to suspended N-cad clones rescued the dephosphorylation defect. The dephosphorylation of FAK tyrosine 397 in Du145 and empty vector cells could be inhibited by the addition of the tyrosine phosphatase inhibitor sodium vanadate.

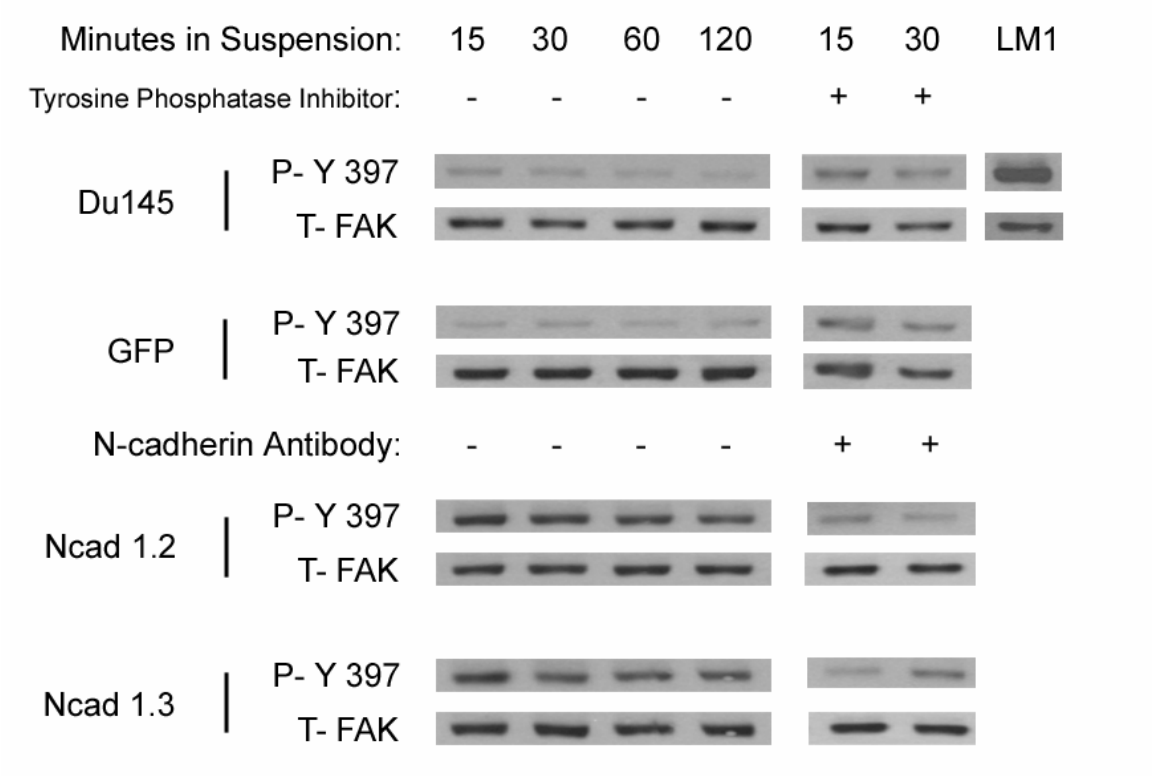

Figure 26. N-cadherin expressing Du145 cells fail to inactivate FAK during anchorage independent culture.

Immunoblot analysis of whole cell lysates from cells cultured in suspension for the indicated times. Tyrosine phosphatase inhibitor (2 μM Sodium Vanadate) or anti N-cadherin monoclonal antibodies (10 μg/ml) were added to cells at the onset of suspension culture. Du145 cell adhesion to Laminin 1 is used as a FAK activation control.

Akt has been shown to be activated following the phosphorylation of tyrosine 397 of FAK (88). To determine a consequence of sustained tyrosine 397 phosphorylation of FAK, the activation of Akt was determined using a phosphorylation specific antibody. Cells grown in suspension were lysed, and equal amounts of protein were immunoblotted for serine 473 phosphorylation of Akt. The activation of Akt in N-cad clone cells grown in suspension is dramatically upregulated, whereas no Akt phosphorylation is detectable in the parental Du145 cells (Figure 27). Interestingly, there is no consistent difference in activation of Erk1,2 between the N-cad clones and parental Du145 cells when grown in suspension.

Another signaling molecule that associates with β1 integrin complexes follwing cell attachment, that plays a role in activation of Rac1, is p130cas (296). To determine whether the dephosphorylation of p130cas was deficient in N-cadherin expressing Du145 cells, p130cas was immunoprecipitated and phosphotyrosine was analyzed through immunoblotting. As shown in figure 28, p130cas tyrosine phosphorylation is maintained when N-cad clones are cultured in suspension, whereas p130cas is efficiently dephosphorylated in the parental Du145 cells. These data indicate that the expression of N-cadherin in Du145 cells potentiates the anchorage independent signaling downstream of the β1 integrin when β1 integrin is no longer engaged to an ECM ligand. This suggests that Du145 cells co-expressing E- and N-cadherin are deficient in the inactivation of tyrosine phosphorylation, and implicates the dysregulation of a tyrosine phosphatase in the inactivation of tyrosine phosphorylation.

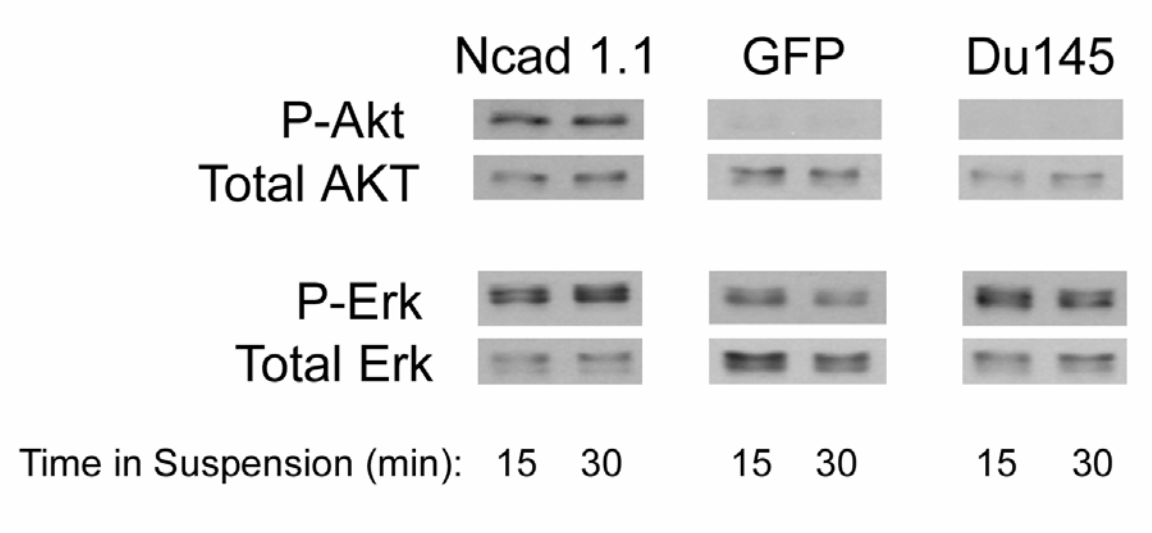

Figure 27. Failure to inactivate Akt activation during anchorage independent culture in N-cadherin expressing Du145 cells.

Immunoblot analysis of phospho-Akt (ser x), and phospho-Erk1,2 (T-202, Y-204) in lysates from cells in suspension for the indicated times. Note the inconsistent phospho-Erk activity.

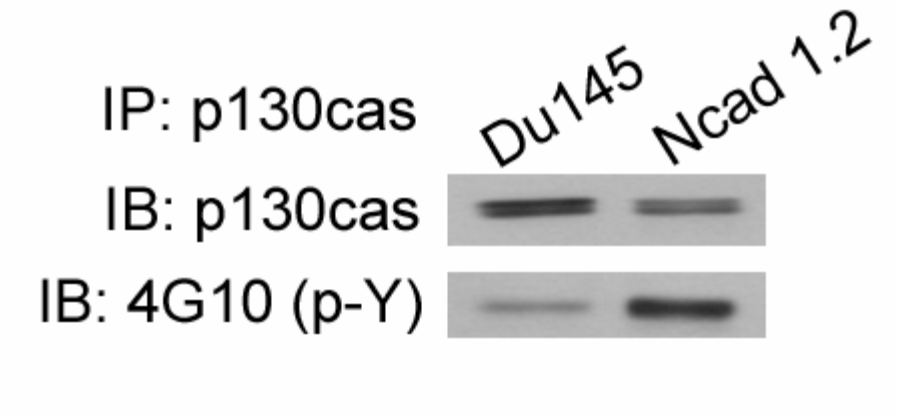

Figure 28. p130cas remains tyrosine phosphorylated during anchorage independent culture in N-cadherin expressing clones

Immunoprecipitation followed by immunoblot analysis of phosphotyrosine residues of p130cas, from cells cultured in suspension for 30 minutes.

The tyrosine phosphatase PTP1B fails to localize properly in N-cadherin expressing Du145 cells

The literature suggests that cross-talk between cadherins and integrins may, in part, be mediated by the tyrosine phosphatase PTP1B (246;308;309). In attempts to resolve the differences in the phosphorylation patterns in Du145 cells expressing N-cadherin, the association of the tyrosine phosphatase PTP1B with cadherins and integrin adhesions was analyzed through immunoprecipitation. Immunoblot for PTP1B following immunoprecipitation of E- and N-cadherin from Du145 cells and Ncad 1.1 cells shows that PTP1B associates only with N-cadherin and not E-cadherin (Figure 29B). To determine whether, in addition to differences in PTP1B association with cadherins, there was concomitant differences in PTP1B association with integrin adhesions we immunoprecipitated FAK from both parental cells and Ncad clones. As shown in figure 29A, there is a dramatic decrease in the association of PTP1B with FAK in N-cadherin expressing Du145 cells as compared to the parental cell line. These data argue that PTP1B localization at sites of integrin adhesion is impaired due to the re-distribution of PTP1B to sites of N-cadherin cell:cell adhesion.

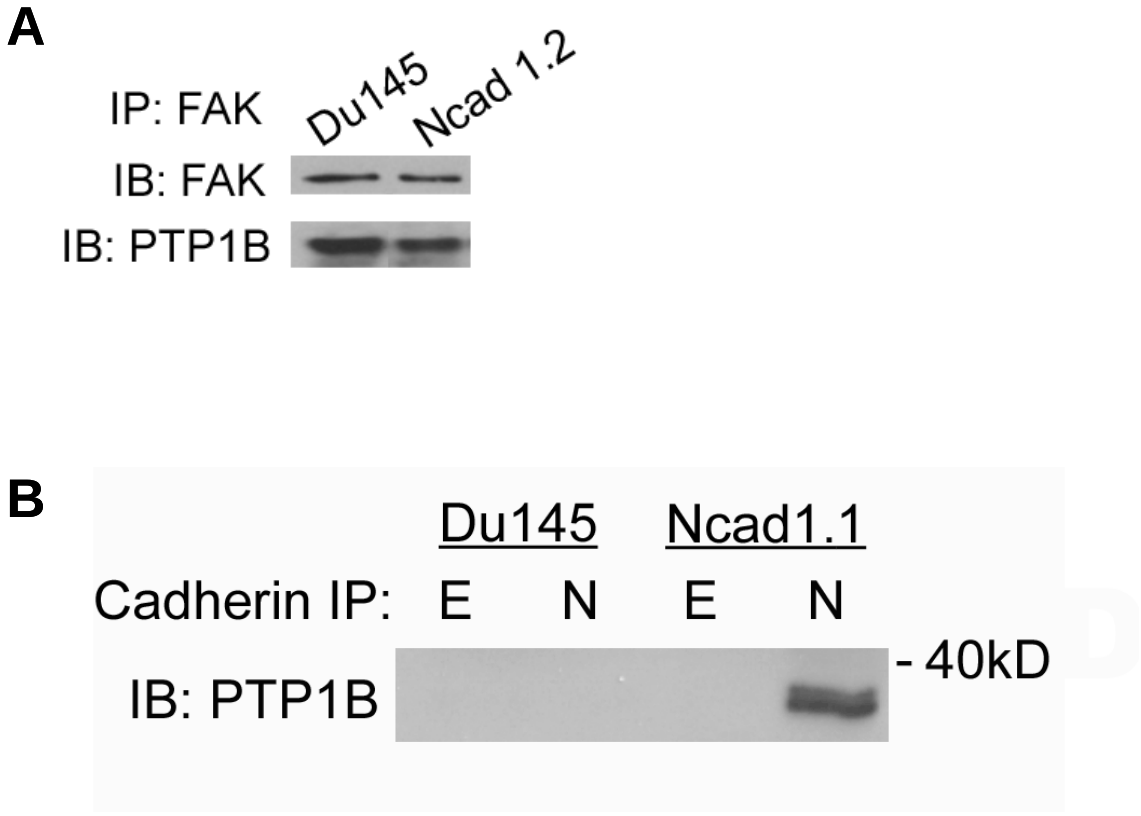

Figure 29. Different protein-protein interactions of PTP1B in E- and N- cadherin co-expressing Du145 cells.

(A) Immunoprecipitation of FAK from Du145 and N-cad clone 1.2 shows less PTP1B associated with FAK in the N-cadherin expressing clone, following immunoblot analysis for PTP1B and FAK. **(B)** Immunoprecipitation of cadherins from Du145 cells and Ncad clone 1.1 shows that PTP1B only associates with N-cadherin in E- and N-cadherin co-expressing Du145 cells.

Discussion

One of the hallmarks of metastatic carcinoma is the downregulation of E-cadherin, either at the level of function or expression (272). Functional destabilization of the E-cadherin adhesion complex occurs through the downregulation of catenin expression, or through inactivation of catenins through phosphorylation. A more frequent event is the downregulation of the expression of the E-cadherin gene through transcriptional repression by Snail, or through methylation of CpG islands in the 5' promoter of the E-cadherin gene (229;237). As E-cadherin has been shown by many groups to be essentially a suppressor of tumorigenesis and metastasis, it was originally surprising when N-cadherin expression was detected in prostate carcinomas that had downregulated E-cadherin expression. It is counterintuitive that a tumor cell would downregulate one cell:cell adhesion molecule while upregulating the expression of another. However, rather that simply mediating cell:cell adhesion in tumor cells, our lab and others have documented the ability of N-cadherin to serve as an initiator of intracellular signaling (76;249;310), activating distinct pathways from E-cadherin. Moreover, recent evidence suggests that N-cadherin mediated cell:cell adhesion is required for cell migration during an EMT process (254). N-cadherin is, therefore, a cell adhesion molecule that yields a selective advantage for tumor cells during the complex process of tumor metastasis. An interesting finding in human tissue sections was that both E- and N-cadherin can be detected in the same prostate cancer cell (256). Given that E-cadherin is a tumor/metastasis suppressor, we were interested in determining whether

or not the mesenchymal characteristics of N-cadherin in prostate carcinoma are dominant over the epithelial qualities of E-cadherin.

An N-cadherin/GFP fusion protein was stably expressed in the E-cadherin expressing prostate carcinoma cell line Du145. As shown in figure 18, when over expressed in Du145 cells, the N-cadherin/GFP fusion protein is expressed at similar levels to that of E-cadherin, and does not result in appreciable decreases in the expression of E-cadherin as compared to both parental Du145 and GFP transfected cells and both are junctionally localized. When both E- and N-cadherin are immunoprecipitated, both cadherins associate with p120ctn and β-catenin; in short, the N-cadherin/GFP fusion protein is biochemically functional.

To determine whether the mesenchymal characteristics of N-cadherin were dominant in the E- and N-cadherin expressing Du145 cells, the migration rates of the cells were tested in both a linear migration assay, and Boyden transwell filters. In both assays, Du145 cells co-expressing E- and N-cadherin migrate faster that Du145 cells with E-cadherin only (Figures 19 and 20). These data agree with studies in breast carcinoma cell lines forced to express N-cadherin (252). A novel finding of this study is that Du145 cells overexpressing N-cadherin display increased adhesion rates to Laminin 1 (Figure 21). Although integrin adhesion rates do not necessarily correlate with increased cell migration, it is assumed that the increased rates of both cell adhesion and cell migration are downstream effects of the mesencymal characteristics of N-cadherin mediated cell adhesion.

In attempts to ascertain why N-cadherin expression in Du145 cells results in increased rates of cell adhesion and migration on Laminin 1, activities of cell signaling molecules following cadherin ligation were analyzed. Following cadherin activation, N-cadherin expressing Du145 cells were found to have an increased activation of Rac1 (Figure 22). There was no detectable activation of RhoA following cadherin activation (data not shown). As shown in figure 14, the N-cadherin/GFP fusion protein may have a higher affinity for p120ctn than does E-cadherin. This agrees with the findings of Seidel *et. al.*, who showed that N-cadherin has a higher affinity for p120ctn *in vitro* (311). This may explain the differences in the activity of Rac1, a member of the Rho family of GTPases. Although these experiments did not differentiate the contribution of E- or N-cadherin to the activation of Rac1, it is arguable that N-cadherin expression in Du145 cells results in additional activation of these two GTPases. It would be interesting to determine what the impact of N-cadherin molecule that lacks the ability to bind p120ctn would be on the phenotype of Du145 cells; these experiments would specify the role of p120ctn in the regulation of the activation of the Rho family of GTPases following N-cadherin ligation.

Due to the inconsistency which activation levels of Rac1, the expression of integrins, and signaling downstream of integrins was analyzed. Levels of the α6 integrin are decreased in Du145 cells expressing the N-cadherin/GFP fusion protein (Figure 25), which results in increased heterodimerization of the α3 and β1 integrins. While no loading control was included in the β1 integrin immunoprecipitation (due primarily to the lack of an anti- β1 integrin antibody that works in immunoblots) the same membrane was

striped and reprobed, assuring that the differences in heterodimerization must be real. Changes in integrin heterodimerization may be a means by which tumor cells switch from integrin subunits that either suppress migration, or to integrin subunits that enhance cell migration (307).

Expression of the α6 integrin is regulated by the MAPK transcription factor Erk1,2 at the level of the α6 integrin promoter (312). Furthermore, inhibition of the signalingof Erk1,2 was shown to decrease α6 integrin mRNA levels. Du145 cells expressing N-cadherin have decreased expression levels of the α6 integrin, and accordingly display decreased activity of Erk1,2 as detected by immunoblotting with phospho-specific antibodies (Figure 24). Although the mechanism by which N-cadherin expression suppresses the activation of Erk was never determined, activation levels of Erk following cadherin ligation are consistent between N-cadherin expressing Du145 cells and parental Du145 cells (data not shown). It is possible that E-cadherin mediated activation of the EGF receptor (313) is inhibited due to the presence of N-cadherin in cell:cell junctions. As N-cadherin is localized to cell:cell junctions, the interaction between E-cadherin and the EGF receptor may be decreased due to high expression levels of N-cadherin which may push E-cadherin out of cell junctions. Accordingly, immunofluorescence with an antibody that recognizes phospho-tyrosine reveals less phospho-tyrosine in the cell:cell junctions of Du145 cells expressing N-cadherin. Moreover, the majority of the phosphorylated tyrosine residues appear to be at the cell periphery, most likely associated with integrin adhesion structures (Figure 23). These data suggest that not only may the decrease in Erk1,2 activation, which leads to

downregulation of the α6 integrin, be caused by the lack of phosphorylated tyrosine at the cell junctions of N-cadherin expressing Du145 cells, but it also implies that the changes in integrin adhesion structures results in increased signaling downstream of integrin adhesion.

To address whether or not signaling downstream of integrins is altered in Du145 cells expressing N-cadherin, the activity of the integrin associated tyrosine kinase FAK was determined. Previous work by Slack *et. al.* documented that only through changes in cell adhesion could one detect differences in FAK tyrosine phosphorylation in prostate carcinoma cell lines (90). Importantly, only the levels of tyrosine residue 397, or the autophosphorylation site of FAK, change when prostate carcinoma cell lines are incubated in suspension culture, while the total levels of tyrosine do not change. Taking this data into account, the phosphorylation of Y-397 was analyzed in suspension cells. As shown in figure 26, FAK Y-397 is rapidly dephosphorylated in both GFP control and Du145 parental cells in suspension. This dephosphorylation of FAK Y-397 is inhibited when sodium vanadate, an inhibitor of tyrosine phosphatases, is added to the culture medium. In stark contrast to this observation, Y-397 is not at all dephosphorylated in N-cadherin expressing Du145 cells in suspension; although FAK Y-397 is fully dephosphorylated following 18 hours growth in suspension. The lack of suspension dependent FAK Y-397 dephosphorylation is dependent on functional N-cadherin adhesion, as addition of N-cadherin function blocking antibodies result in the rapid dephosphorylation of FAK Y-397.

One of the immediate events downstream of tyrosine residue 397 of FAK is the activation of PI3K through the association of the p85 subunit with the Y-397 SH2 domain (88). An indirect measure of this activation is PI3K dependent serine phosphorylation of Akt. Whole cell lysates analyzed for serine 473 phosphorylation of Akt from N-cadherin expressing, empty vector and parental Du145 cells shows robust activation of Akt in N-cadherin expressing Du145 cells, but a complete lack of Akt activation in empty vector and parental cells (Figure 27). These data show that the maintenance of FAK Y-397 phosphorylation in N-cadherin expressing Du145 cells is functional and that integrin dependent signaling is continuing in the absence of integrin ligation. A caveat to this experiment is that N-cadherin homo-typic adhesion has been shown to activate Akt in prostate carcinoma cell lines (76). One way to eliminate the possibility of an N-cadherin dependent contribution to Akt activation is to repeat these experiments with cells expressing mutant FAK lacking Y-397. An interesting implication of these data is that lack of cell adhesion results in the improper function or localization of the PI3K phosphatase PTEN. While Akt activation can not be detected in adherent Du145 cells expressing N-cadherin under any conditions measured (data not shown), it would be interesting to knock-down levels of PTEN with si-RNA, and analyze N-cadherin dependent activation of Akt in adherent N-cadherin expressing Du145 cells.

Another protein that is activated by integrin adhesion is the scaffolding protein p130cas (314). p130cas serves to recruit both kinases and GEFs for Rac1 to sites of integrin adhesion, particularly at the leading edge of migrating cells (315-317). To analyze the activity of p130cas in Du145 cells expressing N-cadherin, p130cas was

immunoprecipitated and immunoblotted for phosphorylated tyrosine residues. While there is no difference in tyrosine phosphorylation of p130cas in adherent cells, there is a striking difference in phosphorylated p130cas between N-cadherin expressing Du145 cells and parental cells grown in suspension for 30 minutes (Figure 28). These data suggest that N-cadherin expression in prostate carcinoma induces a migratory and mesenchymal phenotype through the disruption of the proper regulation of integrin adhesion dependent signaling.

It is known that the phosphatase PTP1B regulates proper integrin activity by dephosphorylating tyrosine residues on many signaling molecules including FAK and the many tyrosine residues of p130cas (309;318;319). I determined the association of PTP1B with FAK through immunoprecipitation assays. As shown in figure 29, there is less PTP1B associated with FAK in N-cadherin expressing Du145 cells as compared to the parental cell line. There may also be a delocalization of PTP1B from FAK to N-cadherin, as immunoprecipitation of E- and N-cadherin in Du145 cells and N-cadherin expressing Du145 cells shows that PTP1B may preferentially associate with N-cadherin. Although there may be some technical issues in the cadherin immunoprecipitation (lack of loading control) these data point to the possibility of N-cadherin regulating integrin function in prostate carcinoma through the sequestration of PTP1B from sites of integrin adhesion, and aberrant localization of PTP1B to sites N-cadherin cell:cell adhesion. PTP1B has been implicated as one of the factors that regulates the potential cross-talk between N-cadherin and the β1 integrin (309), it would be interesting to determine

whether mutation of the PTP1B binding site in N-cadherin would eliminate the increases in cell migration and FAK signaling in the N-cadherin expressing Du145 cells.

More research needs to be completed to determine whether N-cadherin increases cell migration through crosstalk with integrin based signaling. Much of this preliminary data hinges on determining a phosphatase that both dephosphorylates FAK and associates with N-cadherin and not E-cadherin. Although PTP1B is an attractive candidate based on the preliminary data, mutation of the putative PTP1B binding site must result in the proper dephosphorylation of FAK and p130cas, and return the migration of the PTP1B mutant N-cadherin expressing cells to that of E-cadherin expressing controls. Furthermore, the increased signaling of FAK in suspension culture must be correlated to adhesion dependent migration. Although an increased rate of cell adhesion is a start, rates of adhesion do not necessarily correlate with rates of migration. Ultimately, determining the signaling pathways that regulate the crosstalk between cadherins and integrins has proven to be a difficult endeavor, a fact that is bears true in the literature. The data presented in this chapter will contribute to furthering the understanding of the complex signaling involved in cadherin-integrin crosstalk in prostate carcinoma.

VI. CONCLUSION

N-cadherin expression has been detected in both human prostate cancer and in prostate carcinoma cell lines (256;290). The functional relevance of N-cadherin in prostate cancer was originally defined in our lab to be the inhibition of apoptosis (76). Other labs have provided evidence implicating N-cadherin in the regulation of cell migration and invasion in carcinoma (249;251;252;254). While the contribution of N-cadherin to carcinogenesis has been established, these data preceded even a cursory understanding of the transcriptional regulation of *N-cadherin* gene expression in carcinoma progression. The data presented in this work is the first attempt to determine the molecular events that contribute to the upregulation of *N-cadherin* expression, through the activation of the human *N-cadherin* promoter. Through this work it was determined that the β1 integrin regulates the nuclear localization of the bHLH transcription factor Twist1, and that Twist1 in turn regulates the transcriptional activation of *N-cadherin* upon β1 integrin activation following cell adhesion. The mechanisms underlying the cell-type specific expression of N-cadherin *in vitro* was also evaluated in this work; the putative tumor suppressor FoxP1 binds to a cis-element located within the first intron of the *N-cadherin* gene, and this site is necessary for repression of *N-cadherin* promoter activity in *N-cadherin* negative cell lines. Additional experiments were aimed at determining the functional cross-talk between the β1 integrin and N-cadherin, and this work implicates the phosphotyrosine phosphatase PTP1B in the regulation of this cross-talk.

The regulation of *N-cadherin* expression and function is of principle importance to the understanding of the molecular events that regulate the metastatic process in prostate carcinoma. The hypothesis of the third chapter was: *The β1 integrin regulates N-cadherin gene expression through the regulation of Twist1 function*. The aims of the third chapter were: *(1) Determine whether cell adhesion to ECM regulates N-cadherin gene expression. (2) Establish whether Twist1 controls N-cadherin mRNA production. (3) Determine how the β1 integrin regulates the function of Twist1. (4) Verify whether the regulation of N-cadherin expression by Twist1 is at the level of the N-cadherin promoter.*

Determining the regulatory factors controlling N-cadherin expression in prostate carcinoma has proven the importance of Twist1 in governing the expression of genes involved in the cell migration and survival of prostate cancer (This work and (171). It would be interesting to determine the genetic profile (i.e. gene array analysis) of prostate cancer cells in which Twist1 expression levels have been eliminated through the use of siRNA. Moreover, it would be of clinical importance to determine the extent to which Twist1 expression correlates with metastatic prostate cancer. While Twist1 has been shown to correlate with Gleason grade (171), Twist1 expression has not been linked to distant metastasis. A link between Twist1 expression and distant metastatic prostate cancer would certainly put Twist1 on the short list of viable prognostic markers for aggressive prostate cancer.

Twist1 expression is absent in normal prostate epithelium (171). It would be interesting to determine whether the putative nuclear localization sites in Twist1 (aa 37 –

40, and 73 – 77) are necessary for nuclear import. It would also be interesting to determine wheter serine phosphorylation by ROCK can control the nuclear and cytoplasmic localization of Twist1 The fact that Twist1 can heterodimerize with multiple members of the bHLH family (162;163), and that Twist1 can associate with other transcriptional regulators (159;160), suggests that Twist1 may interact with novel transcription factors when expressed in epithelial cells. As the E-box DNA binding of Twist1 is determined by the bHLH transcription factor that Twist1 associates with, determining the full scope of Twist1 interacting proteins will, in addition to advancing the understanding of the role of Twist1 in cell biology, predict additional genes that may be regulated by Twist1. These experiments have begun in our lab with the purification of GST-bound Twist1, which will be used to determine Twist1 interacting proteins through proteomics. Another means by which Twist1 interacting proteins may be affinity purified with the use of biotinylated double stranded DNA oligonucleotides that contain E-box sequences. These specific assays would provide insightful clues to how Twist1 E-box recognition changes with dimerization partner choice.

While the positive effect of Twist1 on the transcriptional upregulation of N-cadherin expression during prostate cancer progression is critical to the aberrant regulation of N-cadherin expression, this work also asked whether negative regulators of N-cadherin expression must be inactivated for the aberrant expression of N-cadherin. The hypothesis for chapter 4 was: *N-cadherin negative carcinoma cells contain a transcriptional repressor that inhibits the activation of the N-cadherin promoter.* The specific aims of this chapter were: *(1) Create an N-cadherin promoter reporter that*

recapitulates N-cadherin mRNA expression in carcinoma cells. (2) Determine the necessary elements within the N-cadherin gene that inhibit N-cadherin promoter activation in non-N-cadherin expressing cells. (3) Determine the transcriptional repressor that inhibits N-cadherin promoter activation in non-N-cadherin expressing cells.

In addition to determining the transcriptional regulators that control the increase in N-cadherin expression in prostate carcinoma, the data presented in this work has also implicated a transcriptional repressor, FoxP1, in the cell-type specific activation of the *N-cadherin* promoter *in vitro*. FoxP1 is expressed in various tissues, and differentially regulated in a number of tumor types (139). It may be that FoxP1 is more of an invasion suppressor, due to its role in suppressing *N-cadherin* gene expression, rather than a typical tumor suppressor. It is logical that in contrast to Twist1 which induces mesenchymal gene expression during tumorigenesis and EMT, FoxP1 may repress mesenchymal gene expression prior to tumorigenesis and EMT and therby inhibit the mesenchymal phenotype. This hypothesis could be evaluated through inhibition of FoxP1 expression in epithelial and non-invasive cancer cell lines through siRNA against FoxP1.

A novel finding of our work is N-terminal truncations to the FoxP1 protein in the metastatic prostate tumor cell lines PC-3 and PC-3N. An important continuation of this work is the sequencing of these N-terminal truncations of FoxP1, as there are multiple regions of FoxP1 that contribute to transcriptional function. It is unknown whether the N-terminal variants of FoxP1 are created through protein processing, or are splice

variants. Determining the regions of FoxP1 which are deleted will predict whether the N-terminal deletions create a FoxP1 protein that is able to bind DNA, but not able to repress transcription, i.e. a dominant negative variant. It would also be relevant to evaluate the transcriptional profile of prostate cancer cells following perturbation of FoxP1 expression. These experiments would implicate additional genes that FoxP1 may regulate.

Forkhead transcription factors are known to be regulated by both PI3K-Akt mediated phosphorylation and by acetylation on lysine residues (124). While attempts have been made to ascertain the extent of serine phosphorylation of FoxP1, threonine phosphorylation was never analyzed. A more powerful way of determining the possible post-translational modifications to FoxP1 would be immunoprecipitation of FoxP1, followed by proteomic analysis that could evaluate many different types of protein modifications in any given experiment. These data would quickly suggest to which signaling pathways may be involved in the regulation of FoxP1 activity.

The data presented evaluating the transcriptional regulation of N-cadherin is certainly a very small portion of the global regulation of the N-cadherin gene. If N-cadherin transcriptional regulation is anywhere near as complicated as the regulation of E-cadherin expression, then mouse model systems may be the most effective way of determining the more complicated elements of N-cadherin expression. The major differences between E- and N-cadherin at the genetic level are the sizes of the first and second introns. When evaluating the contribution of the first intron of the E-cadherin gene to the *in vivo* regulation of E-cadherin expression, Stemmler *et. al.* included the

entire first intron of the E-cadherin gene (241). This would prove very difficult for N-cadherin as the first intron of N-cadherin is closer to 30 Kb, whereas the first intron of the E-cadherin gene is closer to 1 Kb. Although daunting, determining the regions of the N-cadherin gene that control the expression of N-cadherin in an organism would be ground breaking research that would certainly advance the understanding of not only N-cadherin biology, but these experiments could provide insight into global gene regulation in general.

The literature suggests that there may be functional inhibition between N-cadherin and E-cadherin in squamous carcinoma cells (251), and that N-cadherin expression in breast cancer cells induces cell migration (244-247). The hypothesis of the fifth chapter of this dissertation was: *Expression of N-cadherin induces a migratory phenotype in E-cadherin expressing prostate cancer cells.* The specific aims of this chapter were: *(1) Determine the effect of N-cadherin expression on cell adhesion and migration of prostate cancer cells. (2) Evaluate changes in intracellular signaling of integrins in E- and N-cadherin co-expressing prostate cancer cells. (3) Determine the mechanism for N-cadherin mediated increases in the activation of integrin signaling molecules.*

Data regarding the potential cross-talk between the β1 integrin and N-cadherin adhesion structures suggests that PTP1B, or other phosphatases, may regulate the activities of both of these adhesion structures. The principal question that this study did not address is how the signaling molecules that are shared between integrin and cadherin based adhesion structures are differentially localized. There are two possibilities: the signaling molecules are shuttled between the two adhesion structures located at different

micro-domains within the plasma membrane, or integrin and cadherin molecules themselves may be co-localized within exceedingly large adhesion structures. Of the two possibilities, the latter is much more interesting, and there is data suggesting that integrins and cadherins may co-localize within the same macro-molecular organizations (320;321). It would, therefore, be very intriguing to determine the components of such a macro-molecular structure that could contain cell adhesion molecules from very divergent families. An attractive hypothesis is that such a large complex would have obvious differences in organization and composition that would give researchers and clinicians new targets for therapeutic intervention.

APPENDIX A: PUBLISHED MANUSCRIPTS AND ABSTRACTS

Alexander NR, Tran NL, Rekapally H, Summers CE, Glackin C, Heimark RL. *N-cadherin* Expression in Prostate Carcinoma is Mediated by Integrin Dependent Nuclear Translocation of Twist1. Cancer Research (in press).

Alexander NR, Tran NL, Heimark RL. "β1 Integrin Signaling Upregulates the Expression of N-cadherin in Prostate Carcinoma Progression" Therapeutic Targeting of Human Prostate Cancer Symposium 2004, Tucson, AZ.

Alexander NR, Tran NL, Heimark RL. "Forced Expression of N-cadherin in Prostate Carcinoma Induces a Migratory Phenotype" Abstract # 809 American Association for Cancer Research 2002, San Francisco, CA.

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