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MOLECULAR CHARACTERIZATION OF CADHERIN EXPRESSION AND FUNCTION IN PROSTATE CARCINOMA

By

Nhan Le Tran

A Dissertation Submitted to the Faculty of the

GRADUATE INTERDISCIPLINARY PROGRAM IN CANCER BIOLOGY

In Partial Fulfillment of the Requirements

For the Degree of

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SIGNED:

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DEDICATION

In loving memory of my grandfather, Le Phuoc, who succumbed to prostate cancer on December 31, 1989.

To my family for their love, support, and inspiration

To the people whose lives have been altered by prostate cancer

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ABSTRACT

The epithelial cytoarchitecture and function in the prostate gland are maintained in part by the E-cadherin/catenin complex. In human prostate adenocarcinoma, an association between the loss of E-cadherin, increased Gleason score, and extracapsular dissemination has been observed. Further characterizations of human prostate carcinoma cell lines show loss of E-cadherin and expression of N-cadherin in poorly differentiated prostate carcinoma cell lines. N-cadherin expression correlates with an invasive phenotype in cancer cells and mediates the interactions between malignant tumor cells and N-cadherin expressing cells, such as prostate stromal fibroblasts. Additionally, N-cadherin-mediated intercellular adhesions generate a compensatory mechanism that promotes anchorage-independent growth and suppresses apoptosis through a phosphatidylinositol 3-kinase /Akt/protein kinase B survival pathway. Activated Akt results in the phosphorylation of two downstream substrates, Bad and CREB, to regulate Bcl-2 protein stability and *bcl-2* transcription, respectively. Under serum deprivation, N-cadherin intercellular adhesion stimulates a 4-fold increase in *bcl-2* mRNA expression resulting in a 3.5-fold increase in Bcl-2 protein expression, while the cellular level of proapoptotic protein Bax remains constant. Following N-cadherin homophilic adhesion the phosphatidylinositol 3-kinase p85 subunit is found in immunoprecipitates of the N-cadherin/catenin complex. The recruitment of phosphatidylinositol 3-kinase is dependent on both N-cadherin homophilic adhesion and N-cadherin binding to an intact actin cytoskeleton. These results suggest that the association of the N-cadherin/catenin complex with the actin cytoskeleton acts as a scaffold to localize the activation of

phosphatidylinositol 3-kinase/Akt signaling pathway at adherens junctions. The identification of outside-in signal transduction mediated by N-cadherin adhesion provides new information on anti-apoptotic cell-cell adhesion mechanisms enhancing the activity of the phosphatidylinositol 3-kinase/Akt cell survival pathway in metastatic prostate carcinoma. Collectively, these observations indicate that alterations in cadherin expression play a role in prostate cancer progression that may have a profound affect on metastatic cell survival.

CHAPTER I. INTRODUCTION

Epidemiology of prostate cancer

Prostate cancer has become the most common visceral type of cancer diagnosed among men, and the second leading cause of cancer deaths in U.S. male(1). Current estimates suggest that approximately 180,000 men in North America will be diagnosed with prostate cancer and that close to 40,000 men will die from the disease(1). It is clear from epidemiological studies that the incidence of prostate cancer is greater than morbidity. Current investigational methods do not allow clinicians to differentiate between the progressive and quiescent tumors leading to potential over- and under-treatment of the disease(2). To date, the main serum tool for detecting early prostate cancer is the measurement of prostate specific antigen (PSA)(3).

PSA is a serine protease of the human glandular kallikrein family. This glycoprotein, previously regarded as being specific only to the prostate, has been shown to be secreted by the mammary and salivary gland(4, 5). The detectable amounts of PSA in the blood serum originate solely from the columnar epithelial cells of the prostate, thereby making it most specific for prostate(3). Since its discovery as a screening tool for prostate cancer in the late 70's and the large number of studies conducted, PSA today still continues to mature as a prognostic marker. For example, newer serum immunoassays measuring both free PSA and PSA complexed with alpha-1-antichymotrypsin (ACT) have been recently developed and are used to calculate 'percent free PSA' (%FPSA)(6, 7). Although several studies have strongly suggested that %FPSA may be valuable in differentiating prostate cancer from benign prostatic hypertrophy (BPH), its utility as a prognostic marker in established cancer is only now being evaluated(8). In one prospective study, Murphy and colleagues found that %FPSA had little prognostic significance(9). In a similar study, comprised of a select group of men for whom cancer was detected early via screening, a lower serum %FPSA independently predicted a higher probability of prostatic capsular penetration by invasive cancer cells and larger tumor volumes at radical prostatectomy(10).

In one third of men older than age 45, the formation of histological prostate cancer appears to be very frequent(11, 12). This incidence is roughly the same worldwide, however, the number of clinical cancers differs widely among various populations, suggesting important environmental factors in triggering this progression(13). For instance, studies have shown that environmental factors such as dietary fat play an important role in the etiology of prostate cancer. Age-adjusted prostate cancer incidence and mortality rates have risen along with per capita consumption of fat in studies in the US among both blacks and whites(14). In Japan and Puerto Rico(15) the incidence and mortality rates from prostate cancer have risen in conjunction with increases in dietary animal fat. Other case control studies, however, have shown no apparent association between fat intake and the risk of prostate cancer(16). Contradictions among these studies suggest the causes of latent prostate cancer are equally prevalent in the populations but that factors promoting growth of the disease to a clinically significant state are more prevalent in certain regions. Studies suggest that dietary fat intake may be promotional rather than causative for the development of prostate cancer(15). Nevertheless, within the US, regional age-adjusted prostatic cancer mortality rates have been reported as highest in areas with the highest per capita consumption of beef, milk products, pork, and eggs(17). Mortality rates for prostate cancer in the US are higher than in relatively low fat-consuming countries, but migration to the US from these countries results in rates intermediate between those of the native and host countries. In addition, prostate cancer incidence rates among ethnic groups in Hawaii also correlate positively with dietary fat intake(18). These data indicate an association between fat intake and prostate cancer; however further studies are required to establish a direct relation.

In relation to diet, oxidative damage has been suggested to play role in prostate carcinogenesis based on observations derived from the association between dietary fat consumption and prostate cancer coupled with data about oxidative biomarkers and glutathione-s-transferase activity in prostate tissue(19). High dietary fat consumption may contribute to the disease through an oxidative lipid peroxidation, in which fatty acids within the cell membrane are substrates for lipid oxidation. In turn, lipid peroxides and their products can lead to changes in functions and damage to membrane bound enzymes and DNA mutations(20-22). There is strong evidence that reactive oxygen species (ROS) generated both endogenously and from external sources, are associated with carcinogenesis and cancer progression. As byproducts of normal metabolic processes, for example, ROS generated endogenously cause oxidative damage to important biomolecules such as lipids, proteins and DNA. The oxidative modification of DNA bases leads to mutation and altered gene functions resulting in carcinogenesis(23, 24).

Moreover, ROS can affect transcription factor activity such as c-fos and c-jun, or they can alter conformational structures of proteins including p53, which then mimic a mutant phenotype. These ROS effects may also be true for prostate cancer(25-28).

Unlike diet, studies involving hereditary factors suggested a strong correlation to the incidence of prostate cancer independent of environmental exposure(29). The relative risk depends on the number of affected relatives. Statistically the risk of developing prostate cancer with one affected first-degree relative increases to 1.4, and the risk increases to over 3 with more than one. Studies of familial aggregation of prostate cancer have suggested the existence of an autosomal dominant allele that predisposes men to develop prostate cancer. The inheritance of this allele accounts for 5-10% of cases in all men and is generally associated with early onset disease(30, 31). At present, two familial susceptibility loci have been mapped to the X chromosome and to a region of chromosome 1q(32, 33). Candidate genes responsible for this predisposition are presently being investigated. As with other neoplasia, genetic and environmental factors have been implicated in the etiology of prostate cancer, with the interaction of both being the most significant.

In addition to hereditary factors, racial distribution also accounts for the difference in prostate cancer incidences(30, 34, 35). For example, the incidence is highest among African-Americans (188/100,000 person-years), intermediate among whites (136/100,000 person-years) and lowest among Asians (28-39/100,000 person-years)(36, 37). Asians living in the United States have a much higher incidence of prostate cancer than those residing in their country of origin, although still lower than whites and

African-Americans. An understanding of the risk factors that influence the incidences prostate cancer, based on racial distribution and nationality, may be useful in identifying variables important for the etiology and potential preventive treatment of the disease.

In summary, these epidemiological data designate strong factors that may contribute to the identification of the men at risk for developing prostate cancer such as hereditary, race, and geographical location. These factors, however, do not sufficiently define or identify individuals with either localized or malignant prostate cancer. An understanding and identification of biomarkers that recognize individuals with higher risk for prostate cancer may allow for early detection and better treatment of advanced disease. It is therefore essential to investigate the basic biology and biomolecular alterations of prostate tumor progression in order to distinguish between those cases that will progress rapidly to advance metastatic cancer and those with little likelihood of progressing.

Molecular advances in prostate cancer

The presence of multiple independent foci of prostatic adenocarcinoma within the same gland is a common finding in men with prostate cancer(38, 39). Within a given section of prostate cancer tissue, individual neoplastic leasions are described to be genetically distinct, which may have significant implications for the molecular mechanisms of disease progression. Because of this the identification of etiological factors and genetic alterations associated with aggressive prostate cancer has been complex and not well characterized. One early observation that led to an improvement of understanding the genetic changes during cancer progression was the finding that tumors undergo frequent genetic alteration. The genes affected by these alterations are usually categorized as oncogenes, tumor suppressor genes, and mutator genes. Although these changes may be inherited in a subset of prostate cancers, the majority of alterations are acquired as somatic mutations resulting from genetic instability and clonal selection in solid tumors(40-42). Identification of these molecules, and characterization of the mechanisms for their contribution to tumor formation and progression, is important because these studies are generating information for the improvement of diagnosis and therapy of prostate cancer. The most common chromosomal aberrations in this disease include deletions at chromosome regions 6q, 8p, 9q, 10q, 13q, 16q and 18q, and gains at 7p, 7q, 8q and Xq (43-47). Only a few target genes, associated with these aberrations are known.

Both 8p and 13q are two chromosome arms, which are most frequently lost in prostate cancer. Studies involving fluorescence *in situ* hybridization (FISH) and loss of heterozygosity (LOH) have indicated that deletion at these regions are commonly found in cases of high-grade prostatic intraepithelial neoplasia (PIN)(48-50). PIN is described as localized neoplasia that morphologically differs from normal prostate tissue. It is subdivided into low-grade and high-grade, with the latter being accepted as a true premalignant lesion for prostate cancer(51). Mapping of the 8p deleted site revealed that 8p12-p21 and 8p22 are two common regions lost in high-grade PIN, suggesting that there are tumor suppressor genes located at these regions. One of the tumor suppressor gene candidates that is targeted to 8p21 is *Nkx3.1*. *Nkx3.1* is a homeobox gene that is the earliest-known marker of prostate epithelium during embryogenesis and is subsequently expressed at all stages of prostate differentiation in vivo as well as in tissue recombinants(52). Interestingly, *Nkx3.1* knockout mice display prostatic epithelial hyperplasia and dysplasia that increases in severity with age. Since *Nkx3.1* is mapped to the prostate cancer hot spot 8p21, it is proposed to be a prostate-specific tumor suppressor gene and that loss of a single allele may predispose to prostate carcinogenesis(52).

In addition to chromosome 8p, loss of chromosome 13q, which contains the retinoblastoma (Rb) gene, occurs in about 50% of prostate tumors(53). Studies with *Rb*- prostatic carcinoma lines indicates that re-introduction of wild-type *Rb* inhibits tumorigenicity(54). In addition, Rb protein expression is lost in more advanced stages of prostate carcinoma, and also implicated in regulating apoptosis of prostate cells, particularly in response to androgens(54-58).

In advanced stages of prostate cancer and acquisition of metastatic phenotypes, chromosome 17p often displays loss of a region that includes the *p53* locus. It is known that mutations in *p53* occur early in most cancers. In prostate cancer, however, p53 mutations are seen later(59-61). In addition, over-expression of p53 is a predictive factor for poor prognosis and disease recurrence, when used in conjunction with Bcl-2 detection marker(62-64). In comparison with other cancers, p53 mutations are lower in prostate cancer. It is possible that the variability in p53 mutation rates reported is difficult to detect. Since p53 mutation is associated with more highly aggressive and recurrent forms of prostate cancer, the availability of these advance prostate cancer tissues is more difficult to obtained(65, 66).

Fifty percent of late stage prostate carcinoma display deletion at 10q(43, 46). This region houses candidate target genes such as *MXI1* and *PTEN*, located at 10q25 and 10q23 respectively. *MXI1* is a member of the helix-loop-helix-lucine zipper family and a negative regulator of the myc oncogene(67). Analysis of *MXI1* gene, however, showed that only a few prostate tumors possess mutation in this gene (68, 69). On the other hand, PTEN is a phosphatase that appears to have several functions in cells. The most important function is the negative regulation of the phosphoinositide 3-kinase (PI 3-kinase)/Akt pathway which in turn results in less cell death(70, 71). Somatic mutations of *PTEN* occur commonly in many human malignancies and also in other cancer predisposition syndromes, such as Cowden's Disease and the Bannayan-Zonana syndrome(72-75). In prostate cancer, 5-27% of localized and 30-58% of metastatic tumors display mutations in PTEN (76-78). Notably, *PTEN* heterozygous mice develop prostatic epithelial hyperplasia and dysplasia, consistent with the growth suppressive activities of PTEN in prostate carcinoma cell lines(79-82). *PTEN* can therefore be regarded as a candidate tumor suppressor gene, which plays a critical role in the development and progression of prostate cancer.

Allelic loss on chromosome 16q is also reported during prostate carcinoma progression. An invasion suppressor gene, E-cadherin, is mapped to chromosome 16q22.1(83). E-cadherin plays a critical part in embryogenesis and organogenesis by mediating epithelial cell-cell recognition and adhesion processes(84). Frixen and colleagues found that E-cadherin protein levels were frequently reduced or absent in cancer cell lines and such lines were often more fibroblastic in morphology an invasive in experimental assays(85). Examination of E-cadherin in prostate cancer tissues indicated that there was a strong association between high grade and aberrant E-cadherin staining. Tumors with Gleason sum scores higher than 9 showed complete loss of E-cadherin expression at cell-cell junctions(86). Furthermore, homozygous deletion of the gene that encodes for the E-cadherin cytoplasmic protein, α-catenin, located on chromosome 5q31 has been reported in prostate cancer cells, including the highly metastatic PC-3 cell line. α-catenin is involved in cell-cell adhesion by linking the E-cadherin molecule to the microfilament of the cytoskeleton(87). Re-expression of α-catenin into PC-3 cells represses tumorigenicity in nude mice(88). Mutation of β-catenin has also been reported. About 5% of prostate carcinoma contains β-catenin mutations. The role of β-catenin in tumorigenesis is distinct from cell adhesion. For example, gain of function mutations in β-catenin have been reported in several malignancies(89). Such activation mutations stabilize the protein, leading to accumulation of β-catenin, which then acts as transcriptional activator.

In addition to E-cadherin and catenins, alterations in the integrin family are also reported. In prostate cancer, reduction in the expression of most subunits of various integrins occurs during the progression of cancers(90, 91). There is a loss of expression of α2, α4, α5, αv and β4 in carcinoma. Moreover, higher expression of α6 is associated with higher invasive activity(91). Other alterations seen in invasive cancer are numerous. Among these are the modulation of the extracellular basement membrane proteins such as loss of the $\gamma 2$ subchain of laminin 5(92), increased expression of serine proteases(93) and matrix metalloproteases(94) and decreased expression of tissue inhibitors of metalloproteases (TIMPs), which inhibit the activity of the matrix metalloproteases(95).

Gain of chromosomal regions is also frequent in prostate carcinoma. For instance, Nupponen and colleagues have shown that the gain of the long arm of chromosome 8 is the most common aberration in advanced stages and metastatic prostate cancer(46) as a result of gene amplification. One putative target gene for amplification at this region is *c-myc*, a transcription factor that regulates cellular proliferation, differentiation, and apoptosis. Amplification of *c-myc* has been found in about 8% of primary and 11-30% of advance prostate cancers, and appears to be associated with poor prognosis among patients with locally advanced prostate cancer(96).

The progression and metastasis of human prostate cancer also depend on changes and alterations in the functions of multiple growth factors and their receptors(97). For example, the fibroblast growth factor (FGF) family has been implicated in the regulation of prostatic mitogenesis. FGFs, in particular, FGF7, FGF1, and to a lesser extent FGF2, are mitogenic for prostatic epithelial cells in culture, whereas FGF2 is mitogenic for prostatic stromal cells(98, 99). Over-expression of FGF7 and FGF2 has been reported in benign prostatic hyperplasia compared with normal tissue(100, 101). In addition, secretion of FGF9 primarily by prostatic stromal cells was found to be mitogenic for both prostatic epithelial and stromal cells in culture(99, 102).

In addition to FGF, the epidermal growth factor (EGF) and its receptor are also implicated in prostate cancer progression. Human prostatic carcinoma cells cultured *in* *vitro* showed that EGF is abundantly secreted. In addition, advanced and metastatic human prostate cancer cells express high levels of the epidermal growth factor receptor (EGFR or erbB1) and transforming growth factor alpha (TGFα), which also binds and activates the EGFR(98, 103). Furthermore, expression of erb family members (erbB1, erbB2 and erbB3) is often abnormal in PIN, as well as primary and metastatic carcinomas(51, 104-106).

Changes in transforming growth factor-β receptors (TGF-βR) are also detected in prostate cancer progression(107). In normal epithelium, TGF-β1 is a potent growth inhibitor. This inhibitory effect is primarily mediated by a heteromeric complex of two kinases called receptor I and II and are modulated by androgen, growth factors, and the extracellular matrix(108, 109). Inactivation of either receptor can result in resistance to TGFβ1 growth inhibition, a common and important step in prostate cancer progression(108). In normal prostate tissue, both receptors are located in epithelial cells. A decline in the levels of these receptors is correlated with advancing histological aggressiveness of prostate cancer(110). Advanced prostate carcinomas become insensitive to the inhibitory effect of TGF-β1 because of the defect in its receptors(108).

The progression of prostate cancer also depends upon other factors that act to recruit existing blood vessels to make new ones, a process known as angiogenesis. In general, angiogenesis is a critical element for solid tumor growth and metastasis(111). In clinical studies active neovascularization in primary tumors has been associated with a poor prognosis(112, 113). There is a strong correlation in prostate cancer between increased angiogenesis, disease stage and metastasis(114, 115). The most prominent and specialized agent mediating this process of blood vessel growth is vascular endothelial growth factor (VEGF). VEGF is a potent antigenic cytokine that stimulates endothelial cells and is involved in the angiogenesis of solid tumors including prostate cancer(116). Four human VEGF protein isoforms have been identified, composed of 121, 165, 189 and 206 amino acids, which is produced by alternative splicing from a single gene containing 8 exons. The most abundant secreted isoforms are VEGF 121 and VEGF165. In prostate tissues, the mRNA for VEGF121, VEGF165 and VEGF189 isoform are all detected(117). Only VEGF165 and VEGF189 mRNA expression, however, have a strong contribution to the establishment or progression of malignant prostatic disease(118).

Although VEGF exerts its pro-angiogenic properties by way of paracrine mechanisms in vivo by promoting several steps key to the development of neovascularization, there is recent evidence suggesting that VEGF may have some direct, but as yet poorly defined, role in regulating tumor cells themselves(119, 120). This action would occur as the result of self-regulation by tumor cells, also known as autocrine regulation. These paracrine and autocrine properties of VEGF are mediated by ligand binding to specific VEGF receptors on the target cell surface, such as neuropilin, Flt-1 and Flk-1(121). Flt-1 and Flk-1 receptors belong to a family consisting of 30 structurally similar cell-surface receptors(121). Analysis of the expressions of these receptors in prostate cancer shows that Flt-1 expression is detectable in all stages of prostate cancer cells *in vivo* and in prostate cancer cell lines *in vitro*. Flk-1 expression, however, was variable and related to tumor grade, in which high-grade tumors displayed little or no Flk-1 expression. Vascular endothelial cells within areas of prostate cancers consistently expressed both Flt-1 and Flk-1 receptor(122). However, the detection of Flt-1 in various prostate carcinoma cells themselves, rather than adjacent vascular epithelial cells, suggest that Flt-1 may also be an autocrine regulator of carcinoma cells. This is in addition to its well-characterized paracrine regulation of vascular endothelial cells by binding to VEGF. These findings suggest that a novel dual role may exist for VEGF, both as an autocrine growth factor for prostate tumor cells as well as a paracrine growth factor of endothelial cells. Therefore, there are a variety of growth factors that promote growth and differentiation of epithelial cells.

In the normal prostate gland, androgen plays a critical role in regulating the growth, differentiation and survival of epithelial cells(123). In prostate cancer, androgen-ablation therapy is one of the treatments for advanced prostate cancer. Removal of androgen from androgen-dependent carcinoma can result in tumor regression through an apoptotic mechanism(124, 125). In most cases, however, androgen-ablation therapy eventually results in the recurrence of highly aggressive and metastatic prostate cancer, which is androgen-independent(126, 127). This type of aggressive cancer is likely the result of selection of androgen-independent cells that may already exist in the heterogeneous tumor population. An understanding of how prostate cancer cells can escape their initial androgen dependent state to an aggressive androgen-independent state has been the focus of numerous clinical and basic science studies.

Several mechanisms are proposed by which prostate cancer cells may become androgen independent. It was originally thought that both protein and message expression for the androgen receptor (AR) were lost(128, 129) allowing cells to gain androgen independence. Current data suggests this may not be true since AR protein is expressed equally in tissues collected from different stages of prostate cancer(130, 131). This implies that the ability of cells to become androgen independent is through a mechanism other than down-regulation of the receptor expression.

There are many possibilities for acquiring androgen independence. One mechanism is through alterations of the AR. Normally, the formation of ligand-receptor complexes causes a conformational change in the AR, which exposes DNA binding sites. The activated complex is then able to bind to specific DNA sequences called androgen response elements to regulate gene transcription(132). Mutations in the androgen receptor are found in 10-30 percent of patients with hormone-refractory disease(133). These mutations occur throughout the coding region and within the hormone-binding domain, and may allow the AR to become permissive for binding other steroids hormones or highly sensitized for binding low levels of residual androgen. This in turn would allow cells to overcome a specific requirement for androgens(134, 135). Moreover, other growth factors can affect the activation of the androgen receptor pathways. For instance, growth hormones such as IGF-1, KGF and EGF can not only activate downstream intracellular kinase cascades, but also activate the androgen receptor pathway(136). Each of these growth factors can activate transcription from an androgen-responsive reporter construct in the absence of ligand or synergistically in conjunction with androgens. The fact that the androgen receptor antagonist complex can block this activation indicates that these effects required the androgen receptor. In synergistic activities with AR, these growth factors may bypass the requirement of hormones in cases where androgen is limiting(136).

In summary, there are many molecular alterations and changes that allow for the progression of prostate cancer (Figure1.1). An understanding and recognition of these molecular changes may allow for the development of better tools for the prevention, diagnosis, prognostic evaluation and treatment of prostate cancer. Only a subset of target genes, however, has been identified, and the continued identification of other suspected target genes are important. Additionally, it is imperative to continue to characterize the molecular alterations seen in prostate cancer, especially those that enable the tumor to readily metastasize. The elucidation of the changes seen in cell adhesion molecules and other relevant metastatic molecules will provide important advances in the understanding of the complex progression of prostate cancer. Such studies may also lead to the development of a comprehensive, diagnostic genetic screen for risk stratifying prostate cancer patients whereby early aggressive therapy will be provided to patients with the most aggressive form of the disease.

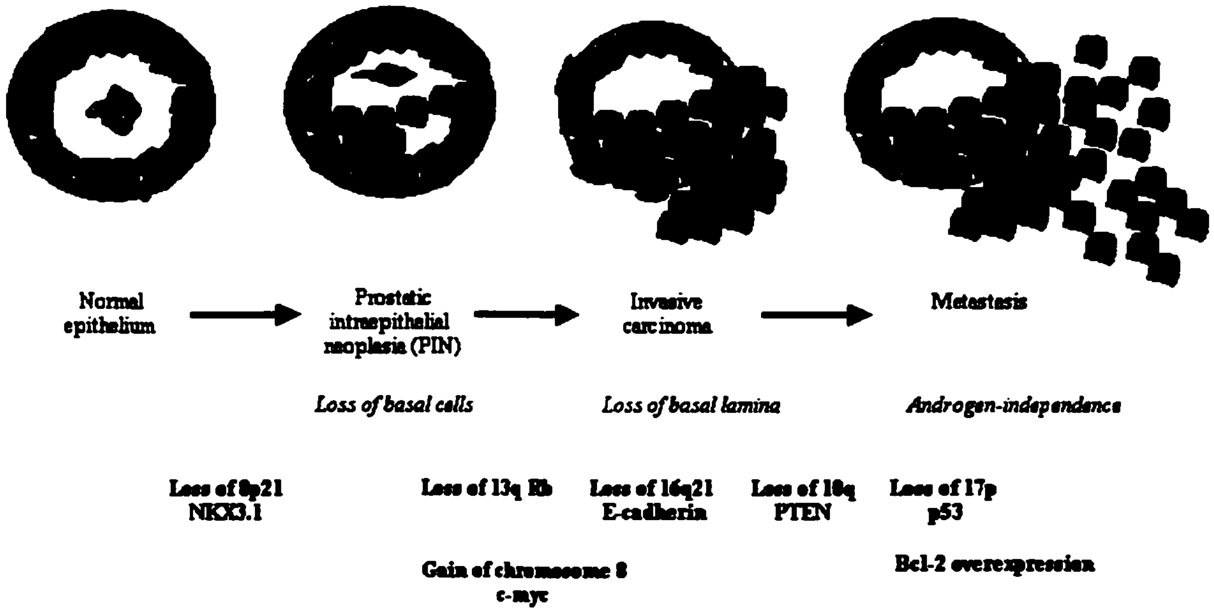
Normal epithelium
Prostatic intraepithelial neoplasia (PIN)
Invasive carcinoma
Metastasis
Loss of basal cells
Loss of basal lamina
Androgen-independence
Loss of 8p21
NKX3.1
Loss of 13q Rb
Loss of 16q21
E-cadherin
Loss of 10q
PTEN
Loss of 17p
p53
Gain of chromosome 8
c-myc
Bcl-2 overexpression

Figure 1.1: Schematic pathway for human prostate cancer progression [Adapted from Abate-Shen and Shen 2000. *Genes & Development* Vol. (14): 2410-2434]

Deregulation of the cell cycle and apoptosis in prostate cancer

The maintenance of normal epithelium is dependent upon a balance between a low rate of cell proliferation and a low rate of cell death(137). In high-grade PIN and early invasive carcinomas, cell proliferation is as high as 7- to 10-fold greater than normal. In contrast, advanced and metastatic carcinomas display an approximately 60% decrease in the rate of apoptosis. The difference between these two stages appears to be dependent upon altered cell-cycle regulation, in which the clinically localized disease is driven by altered cell-cycle control and deregulated apoptosis may be more important for advanced carcinomas.

Loss of function in the cell cycle regulatory genes such as $p27^{kip}$, a CDK4 inhibitor, is prevalent in prostate tumors. $p27^{kip}$ is localized to the chromosomal region 12p12-13.1(138). This region is frequently lost in advanced stages of prostate carcinoma and may possibly provide a prognostic marker of patient outcome based on studies comparing the stages of prostate cancer to $p27^{kip}$. In these studies, loss of $p27^{kip}$ protein expression strongly correlates with increased tumor grade(139, 140). Notably, homozygous knockout mice studies wherein $p27^{kip}$ has been deleted resulted in hyperplasia of multiple tissues including the prostate(141, 142).

Growth arrest also correlates with the dephosphorylation and activation of the retinoblastoma (Rb) protein (143). The regulation of cells from G1 to S phase is mediated by the Rb protein, in which its activity is modulated by G1-specific, cyclin-dependent kinases that phosphorylate and inactivate Rb in the late G1 phase of the cell cycle. Aggressive cancer with poor clinical outcome has been associated with mutational changes in the *Rb* gene. In prostate cancer, *Rb* mutations have been characterized as primary events in the early phases of tumor progression(144).

In addition to *Rb* mutation, alterations in the *p53* gene are also implicated in prostate cancer progression. The *p53* tumor suppressor gene is a transcription factor that regulates cell-cycle progression, DNA repair and apoptosis(145). In response to DNA damage, p53 functions to monitor genomic integrity by either inhibiting the cell from entering the cell cycle, enabling the cell to repair the DNA, or triggering apoptosis(146). Moreover, p53 may regulate apoptosis by modulating the ratio of Bcl-2 to Bax, an established, important determinant of apoptosis(147-149).

Somatic mutations in the *p53* gene are identified in diverse types of cancer. In the case of prostate cancer, studies indicated that *p53* mutations found in the primary tumor are clonally expanded in metastases and that these sites within the primary tumor define regions with high metastatic potential(150). In advance prostate cancer, increases in *p53* mutations occur, with the highest incidence in androgen-independent tumors. The *p53* gene is located on a region of chromosome 17, and loss of heterozygosity of this region occurs in approximately 20% of prostate cancer(151). Moreover, p53 protein expression in localized primary prostate carcinomas has been correlated with a higher Gleason score, nuclear grade, pathological stage, and proliferation(61, 152, 153).

In addition to deregulation of the cell cycle, resistance to apoptosis is important in prostate cancer progression. Epithelial cells normally require survival signals to prevent the process of apoptosis(154). Apoptosis resistance, however, leads to perturbations of cell growth involving cell accumulation, cell persistence, and altered growth factor and hormone sensitivities in various cancer systems, including prostate(155). Cell survival, *in vivo*, is mediated by signaling through engagement of surface receptors by soluble factors and by cell-cell and cell-matrix interactions. Members of the Bcl-2 family appear to function at a pivotal point in the decision process where cells become irreversibly committed to die (Figure 1.2). Some of this family, including Bcl-x, Bcl-2, Bcl-w, and Mcl-1 promote cell survival, whereas others, such as Bax, Bak, Bad, Bik, Bid, Bok, and Bim promote apoptosis(156). These molecules form both homo- and heterodimers, and one possible mechanism for their control on apoptosis is that the relative proportions of their interactions regulate the balance between apoptosis and survival(157, 158). For example, these members all possess at least one Bcl-2 homology domain(159). The Bcl-2 homology domain allows for hetero- and homodimerization between the Bcl-2 family members, which regulates the apoptotic response(147, 156, 160, 161). Homodimerization of Bcl-2 prevents the activation of caspase-9, Apaf-1 and cytochrome c(156). In contrast, heterodimerization of Bcl-2 and Bad prevents Bcl-2 functions and activates downstream pro-apoptotic signals. The balance between pro- and anti-apoptotic proteins is important in determining the fate of the cells when exposed to apoptotic signals(156).

Other data, however, have shown that the pro- and anti-apoptotic function of these proteins are at least partially independent of their ability to interact with each other(162-165). Based on crystallography, Bcl-x, Bcl-2, and Bax possess a transmembrane sequence at their carboxyl terminus which functions in membrane pore formation. A number of the Bcl-2 family members also are predicted to possess this carboxyl terminus sequence, which localizes them to internal cell membranes including mitrochodria and

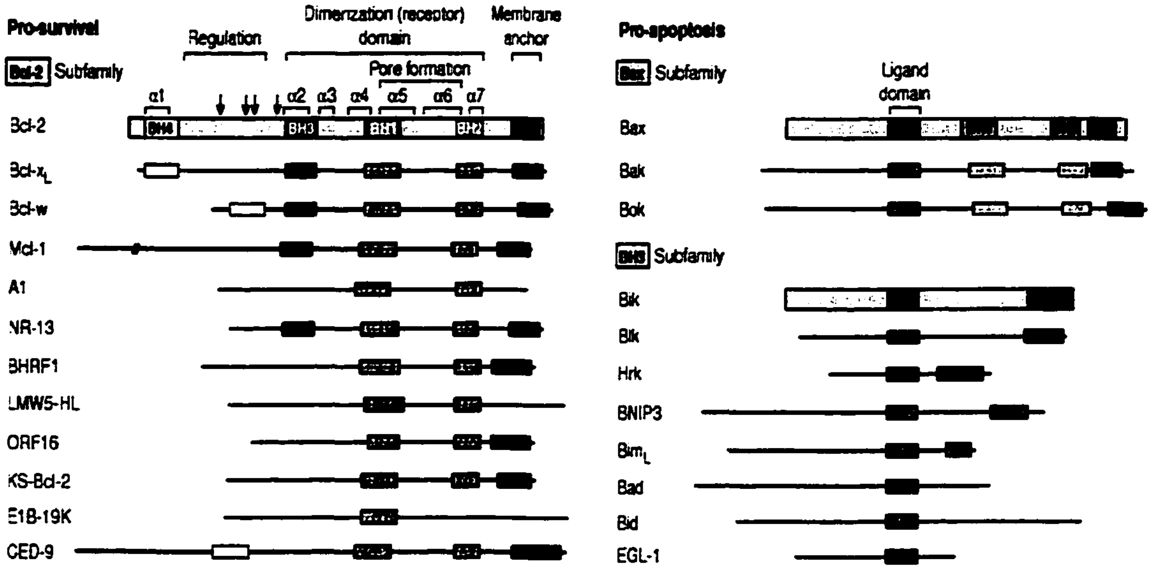
Pro-survival
Regulation
Dimerization (receptor) domain
Membrane anchor
Bcl-2 Subfamily
Pore formation
α1
α2
α3
α4
α5
α6
α7
Bcl-2
BH4
BH3
BH1
BH2
Bcl-xL
Bcl-w
Mcl-1
A1
NR-13
BHRF1
LMW5-HL
ORF16
KS-Bcl-2
E18-19K
CED-9
Pro-apoptosis
Bax Subfamily
Ligand domain
Bax
Bak
Bok
BH3 Subfamily
Bik
Blk
Hrk
BNIP3
BimL
Bad
Bid
EGL-1

Figure 1.2: Members of the Bcl-2 family and their structures. Schematic by Adams and Cory. *Science* 1998 Vol (281): 1322-1326.

the endoplasmic reticulum. Once in the membrane, these proteins may act on the mitochondria by inducing the release of cytochrome c(166). Thus, members of the Bcl-2 family of proto-oncogenes play a central role in the regulation of apoptosis. Apparently, signaling pathways that affect the functions of these proteins are critical during prostate cancer progression.

One mechanism that affects the balance of pro- to anti-apoptotic proteins is the Akt/protein kinase B (PKB) signaling pathway. Akt/PKB is a serine/threonine protein kinase that has been implicated in mediating a variety of biological responses including apoptosis inhibition, metabolism and cellular growth stimulation(167, 168). There are three mammalian isoforms of Akt: Akt1, Akt2 and Akt3(169, 170). In relation to cancer, Akt1 was found to be overexpressed in 20% of gastric adenocarcinomas, whereas, Akt2 was overexpressed in 3% of breast cancer, 15% of ovarian cancers, and 12% of pancreatic cancers(171, 172). Akt3 enzymatic activity and mRNA are elevated mainly in hormone-unresponsive breast and prostate carcinomas(173).

The activity of Akt is dependent on upstream activation of phosphatidylinositide 3'-OH kinase (PI 3K). PI 3K and its phospholipid products have been implicated in transmitting survival signals from extracellular cues such as growth factor receptor engagement or cell-matrix interactions. Activation of these receptors leads to the rapid recruitment of PI 3K to the membrane where it can catalytically generate phospholipid products such as PtdIns(3,4,5)P_3. These phospholipid products in turn are able to recruit signaling molecules such as Akt to the membrane. Translocation of Akt to the membrane brings Akt in proximity to regulatory kinases that phosphorylate and activate Akt(170, 174). One of these proteins, termed 3-phosphoinositide-dependent kinase-1 (PDK-1) can phosphorylate Akt on Thr-308(175), whereas PDK2 or ILK is capable of phosphorylating Akt on Ser-473(176, 177). Based on mutagenesis studies, both Ser-473 and Thr-308(178) phosphorylation are required for Akt activation.

The activity of Akt is also dependent upon the role of PTEN. PTEN is a lipid phosphatase that dephosphorylates PI 3K-generated 3'-phosphorylated phosphatidylinositides *in vivo*(179, 180). Overexpression of PTEN is sufficient to lower basal 3'-phosphorylated phosphoinositide levels in tumor cells and is an important tumor suppressor whose loss correlates with increased Akt activity(73-75). In prostate cancer, a frequent and prominent feature is the mutational inactivation of PTEN(82, 180). Loss of expression or mutational inactivation of PTEN results in the suppression of apoptosis and accelerated cell cycle progression(180, 181). Consequently, re-expression of PTEN induces apoptosis and G1 cell cycle arrest in PTEN null prostate cancer(182). These data suggest that Akt activity is dependent upon the balance between PI 3K and PTEN activities.

One of the down-stream substrates of Akt is the pro-apoptotic protein Bad. Upon exposure to growth factors, Bad is phosphorylated at two sites, Ser-112 and Ser-136. Phosphorylation at either site causes Bad to dissociate from Bcl-2 and interact with the cytoplasmic 14-3-3 proteins. 14-3-3 proteins are adaptor proteins that interact with a variety of phosphorylated signaling molecules(183). Interaction between Bad and 14-3-3 causes a sequestration of Bad from its target at the mitochondria and prevents its de-phosphorylation(184). The phosphorylation of Bad inactivates its ability to cause cell death and promotes cell survival. Bad can be phosphorylate on Ser-112 and Ser-136. However, Ser-136 is a preferential Akt target of Bad(167, 185). Mutation of Ser-112 to alanine had no affect on the ability of Akt to suppress Bad mediated apoptosis. This data supports the notion that in vivo, Akt phosphorylates Bad at Ser-136, which leads to the inactivation of Bad and thereby promotes cell survival by increasing Bcl-2 activity.

In the prostate, Bcl-2 is normally expressed in basal epithelial cells, seminal vesicles, ejaculatory ducts, and neurons. The majority of newly diagnosed prostate cancers do not express detectable levels of Bcl-2 protein, however, progression of prostate carcinoma following androgen-ablation therapy is associated with the upregulation of Bcl-2(186). Since hormone-unresponsive prostate carcinomas are generally more aggressive clinically with higher metastatic potential than hormone-responsive tumors(187), it is possible that enhanced PI 3K/Akt signaling transducing pathway may contribute to this phenotype via upregulation of Bcl-2 activity.

In summary, a common step during multistep carcinogenesis is an alteration of the genes, which function to determine the susceptibility of cells to undergo cell death. In general, this occurs by the upregulation of genes, which act to suppress apoptosis, such as *bcl-2*, and/or the inactivation of genes which function as tumor suppressors and enhance apoptosis, such as *bax* or *p53*. Many surface receptors can generate signals that affect the apoptotic pathway. These receptors include engagement of surface receptors by soluble factors, cell-matrix interaction mediated by integrins, and cell-cell adhesion mediated by cadherins. Analysis of these signaling pathways, which are involved in the regulation of apoptosis and cell-cycle progression, may yield important prognostic information in prostate cancer.

Factors involved in prostate cancer metastasis and tumor-stromal interaction

Although most prostate tumors will remain localized and confined to the prostate indefinitely, the issue of cellular invasion and metastasis is prevalent in advanced prostate carcinoma. Migration along the perineural spaces of the perineal nerve tracts and stromal invasion are common ways in which malignant prostate cells can penetrate the capsule(188, 189). Once outside the prostate, carcinoma cells enter lymphatic spaces and disseminate to local lymph nodes and preferentially to bone(188). This process of capsular penetration and dissemination involves changes in proteins critical for cell migration and invasion such as modulation of the extracellular matrices, proteases and adhesion molecules and interaction with the stromal compartment.

Alteration of the extacellular matrix, in prostate cancer, is reported at different stages. In normal prostate glands, the basal lamina that surrounds the epithelial ducts is composed of entactin, type IV collagen, and laminin(190). In tumors, there is a progressive loss of this basement membrane, particularly the alterations in the laminin subchains in association with increased histological grades(191). Laminin 5 is one of the laminin proteins integral to the formation of hemidesmosomes, which attach normal basal cells to the underlying basal lamina. Analysis of laminin 5 subchains in prostate carcinoma tissues showed that there is loss of expression of the both the β3 and γ2 subchains. Interestingly, the γ2 mRNA is present in these carcinoma tissues but the protein expression is lost. As a result, the failure to express this γ2 subchain of laminin 5 may contribute to the lack of hemidesmosomes formation in prostate cancer(92). Additionally, several other hemidesmosome proteins including BP230, HD1/plectin are lost (90, 92, 192). Thus, failure of hemidesmosome formation and alterations in the extracellular matrices can result in altered receptor/ligand interactions. This gives malignant cells more potential to invade into the underlying stromal compartment and spread through adjacent structures(92).

The interaction between epithelial cells and the underlying stromal cells in the prostate are essential for all stages of normal prostate growth and development(193). Transient changes in stromal-epithelial interactions occur in tissue remodeling during development, wound healing and regeneration(194). However it is likely that aberrant interactions play a significant role in neoplastic disease. For example, in benign prostatic hyperplasia (BPH), the tissue composition is heterogeneous where the ratio of epithelial to stromal cells may change from 1:2 to as much as 1:5(195). This change represents a loss of normal communication for growth regulation between epithelial and stromal cells.

Studies have shown that stromal cells modulate the differentiation pattern of normal prostatic epithelium (193). Growth factors produced by epithelial and stromal cell types can reciprocally regulate cell growth, and the role for stroma in prostate cancer progression has been noted(196, 197). In prostate carcinoma, changes in stromal-epithelial interactions are frequently observed at the invading front(198). For instance, normal epithelial cells do not breach the basement membrane. However, prostate cancer cells degrade and invade the basement membrane where they interact with the underlying stromal cells. Due to the altered characteristics of cancer cells, such as excessive production and secretion of growth factors and proteases, changes in stromal cell behavior are induced which in turn may enhance migration and invasion by cancer cells(198).

Remodeling of the ECM appears to be a necessary step in local invasion, which involves secretion or activation of proteases, such as metalloproteinases, or protease activators, such as urokinase(199, 200). Prostate cancer cells have been shown by a number of investigators to secrete high levels of matrix metalloproteinases (MMPs). In addition, there is also a positive correlation between the expression of MMPs and invasion or metastatic potential in animal models(94). One of the MMPs that is highly expressed in prostate cancer tissue is matrilysin or MMP-7. Transfection studies of MMP-7 in a noninvasive prostate cell line (DU-145) promote an invasive phenotype in the diaphragm model in SCID mice(94). This upregulation of MMP-7 expression in prostate cancer cells appears to be a result of factors contributed by the stromal environment and not by the invading tumor cells. Such factors may include both cytokines (such as IL1-1 and –6) as well as growth factor (such as FGF family members)(201-203). In addition to MMP-7, other MMPs such as MMP-2 and MMP-9 are also highly expressed in prostate cancer (204), along with newly identified class of membrane bound metalloproteinase, MT1-MMP. This membrane bound metalloproteinase can activate other MMPs such as MMP-2(205). It is proposed that the primary target of MMP-2 is laminin 5 and is critical for cell migration during tissue remodeling and tumor invasion(206). Cleavage of laminin 5 by MMP-2 is important in that it may provide a signaling mechanism involving the cell adhesion receptors known as integrin(199, 206). This integrin-mediated signal may cause cells to begin migration. Thus, MMPs are critical for tumor cell migration, and changes in the extracellular matrix by MMPs may be expected to modulate tumor cell motility via cell adhesion.

Cell-matrix interactions are mediated via integrin receptors, and changes in integrin receptor expression have been associated with prostate cancer progression. In prostate cancer, the hemidesomosmal integrins $\alpha6\beta4$ pair is lost, due mainly to the loss of $\beta4$ expression(207, 208). Normally, $\alpha6$ integrin can pair with $\beta1$ or $\beta4$ to bind to laminin, though $\alpha6\beta4$ pairing is usually dominant. While $\alpha6\beta4$ integrin associates with the hemidesmosome formation and intermediate filaments, $\alpha6\beta1$ is associated with focal contact formation. Thus, the loss of $\beta4$ provides a selective pairing of $\alpha6$ with $\beta1$ forming more $\alpha6\beta1$. The interaction of $\alpha6$ with $\beta1$ and increase $\alpha6$ expression correlates positively with the progression of prostate carcinoma cells to a motile and invasive phenotype. Selection of DU145 cells with high $\alpha6$ expression demonstrated both increased cell motility on laminin matrix and increase invasion *in vivo*(91). In addition to alterations in alpha and beta pairing, changes within the structure of the integrin molecules may also influence the interaction of the tumor cell with the ECM. For instance, alternative splicing of the αII_b can result in a truncated receptor, which lacks the transmembrane region and cytoplamic tail of the αII_b light chain. This isoform is detected in the prostate carcinoma cell lines PC-3 and DU-145 but not in normal epithelial cells(209, 210). Recently, the $\alpha6$ integrin was discovered to be post-transcriptionally modified, in which a portion of the α6 extracellular domain is lost. This variant was also detected in various prostate cancer cell lines but not in normal epithelial cells. Although the function of this newly defined variant, α6p, is not known, it is hypothesized that this molecule could influence intracellular signaling to promote cellular migration and invasion(211).

In addition to integrins, alterations in other cell adhesion molecules, particularly cell-cell adhesion receptors, are critical during metastatic progression. In prostate tumors, cell-cell associations are often disorganized, and this loss is thought to be a cause of the unregulated behaviors of tumor cells, including invasion and metastasis. Prostate tumor metastasis involves two independent processes relevant to cell adhesion: detachment of cells from primary tumors, and reattachment of cells to metastatic sites. To understand the mechanisms of metastasis, the role of cadherins must be taken into account, as they are crucial for cellular differentiation and cell-cell interaction. Therefore, I have focused my attention on the role of one of the major families of cell-cell adhesion molecules, the cadherins and their associated catenins in prostate carcinoma metastasis.

Cadherins and catenins

Cadherins are transmembrane glycoproteins that interact in a Ca^{2+}-dependent homotypic fashion and play an important role in tissue maintenance as well as cell differentiation, migration and regulation(212). Common to all cadherins are three protein domains: the extracellular amino terminus, the transmembrane domain and the intracellular carboxyl terminus (Figure 1.3). The cadherin-binding site lies within the first

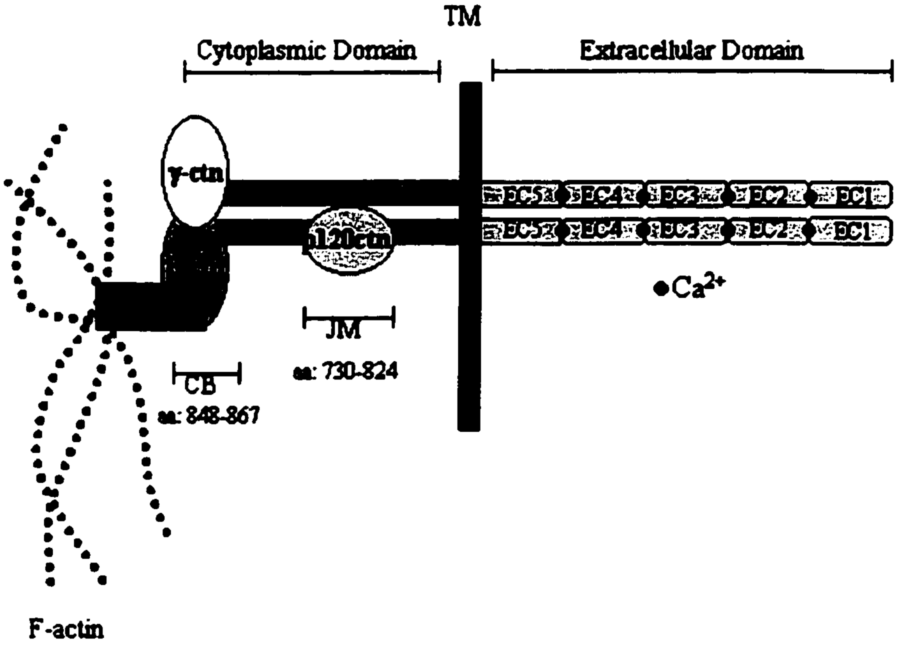
TM
Cytoplasmic Domain
Extracellular Domain
γ-ctn
p120ctn
EC5
EC4
EC3
EC2
EC1
EC5
EC4
EC3
EC2
EC1
●Ca2+
JM
aa: 730-824
CB
aa: 848-867
F-actin

Figure 1.3: The basic structure of cadherins and intracellular protein association. EC=extracellular cadherin repeat, JM=juxtamembrane region consisting of 94 amino acids, a putative $p120^{ctn}$ binding site, CB=cadherin binding region, a putative β-catenin binding site.

extracellular domain and is responsible for selective, homotypic calcium-dependent cadherin binding(213). Homotypic binding of identical cadherins is preferred, and experiments have shown that self-sorting of a mixed cell population is dependent on the cadherin expressed on the cells(214).

The most conserved region of the cadherin molecule is the cytoplasmic domain. This region contains an important binding site for the cadherin-associated proteins known as catenins(215) and is highly conserved among the members of the cadherin family. Four catenins have been identified: α-, β-, plakoglobin, and p120^{ctn}. The interaction of these catenins with the cadherins is necessary for mediating the contact between the cell adhesion molecules and the cytoskeletal microfilaments(216). Truncation of cadherins at the carboxyl terminus results in a substantial loss of the adhesive properties. Co-transfection of cadherin/α-catenin fusion proteins reverses this process, demonstrating that the association of catenins with cadherins is a key step in the formation of intact adhesion complexes(217).

The association of cadherins with catenins is essential for intercellular Ca^{2+}-dependent adhesiveness(216, 218). α-catenin contains consensus amino acid repeats characteristic of vinculin(219), an intracellular protein first identified at cell-substrate focal contact sites where it associates with actin and the integrin complex. Experimental evidence demonstrates that α-catenin links the cadherin complex to the actin cytoskeleton through interaction with β-catenin(220). Unlike α-catenin, β-catenin and plakoglobin share high sequence identity with the *Drosophila* segment polarity gene product, *armadillo*. The *armadillo* gene encodes a signaling molecule required for cell fate decisions that underlie patterning in the embryonic segments in *Drosophila*(221). Biochemical analysis also places the Armadillo protein in the epithelial zonula adherence junction with both α-catenin and cadherins(222). Like *armadillo*, β-catenin was identified in a complex with cadherins by binding to the cytoplasmic domain of cadherins and to α-catenin, which in turn links the cadherin-catenin complex to the actin cytoskeleton(223). Disruption of β-catenin binding to cadherin or deletion of the β-catenin gene results in loss of cell-cell adhesion and disorganization of cells and tissues(224).

β-catenin is also part of a signaling pathway initiated by wingless/Wnt(225). Activation of this pathway by Wnt signaling results in association of β-catenin with lymphocyte enhancer binding factor-1 (LEF-1)/T-cell factor-4 (TCF-4), a DNA-binding transcription factor, and subsequently, localization of the β-catenin/TCF-4 complex to the nucleus, indicating a role for β-catenin in regulating gene expression(226, 227). In addition, β-catenin has been found to complex with adenomatous polyposis coli (APC) protein(228-230). First identified as a tumor suppressor in human colon epithelium, APC controls the cytoplasmic pool of β-catenin. In colon cancer, inactivation or deletion of APC results in cellular dedifferentiation caused by β-catenin over-expression(231).

A number of target genes regulated by β-catenin/LEF-1 have been identified in the past several years, and these include developmental regulatory genes such as *siamois*, *twin*, and *Xnr-3* in *Xenopus*(232-235), and *ultrbiothorax* in *Drosophila* (236). Additional targets include genes involved in cell growth and prolifteration, *c-myc* and *cyclin D-*

1(237-239), a regulator of cell-cell communication, *connexin-43*(240), and the *metalloproteinase*, *matrilysin*(241). These findings demonstrate that β-catenin is not only important in linking the cadherins to the actin cytoskeleton, but additionally plays a role in transcriptional events.

Recent studies demonstrated that β-catenin is highly mutable in various types of cancer(242). This highlights the relationship between regulation of β-catenin stability and oncogenesis. The majority of the identified mutations are missense mutations in one of the putative GSK-3β phosphorylation sites such as serine 33 and 37(243). In addition to missense mutations, a number of deletion mutants that remove one or more of the putative GSK-3β sites have been isolated. Together, these data strongly argue that mutations in β-catenin that enable it to evade regulation by the ubiquitination pathway play an important role in the tumorigenic transformation of many cell types.

The fourth catenin, $p120^{ctn}$(244) was originally identified as a major target for the oncogenic Src tyrosine kinase(245, 246). $p120^{ctn}$ shares significant sequence identity with the members of the Armadillo gene family, including β-catenin and plakoglobin(247, 248). Reynolds et al. (1994) defined $p120^{ctn}$ as a member of the cadherin-based cell-cell adhesion complex. Biochemical studies suggest that $p120^{ctn}$ interacts with E-cadherin at a different site than β-catenin or plakoglobin(244). In addition, $p120^{ctn}$ co-precipitated with other classical cadherin family members, such as N- and P-cadherin. This suggests that there is a broad interaction between $p120^{ctn}$ and the cadherins. Several data speculate the role of $p120^{ctn}$ in transformation and signaling. For instance, $p120^{ctn}$ may play a role in modulation of adhesion since the association of tyrosine phosphorylated $p120^{ctn}$ with E-cadherin is elevated in ras-transformed breast epithelial cell lines(249). p120^{ctn} is phosphorylated on tyrosine residues in response to ligand-induced stimulation of several receptor tyrosine kinases, including those for epidermal growth factor (EGF) and platelet-derived growth factor (PDGF)(250, 251). The phosphorylation of p120^{ctn} is speculated to modify cadherin affinity thus resulting in a weaker cadherin-cadherin interaction. In addition to its role in binding cadherins, p120^{ctn} has been recently identified to interact with a Zn finger protein known as Kaiso(252). This interaction requires p120^{ctn} Arm repeats 1-7 and the carboxy-terminal 200 amino acids of Kaiso that encompass the Zn finger. Like other Zn finger proteins, Kaiso/p120^{ctn} complex also localizes to the nucleus indicating a role for p120^{ctn} in gene regulation(252). Recently, p120^{ctn} has been shown to regulate the actin cytoskeleton via Rho family GTPases(253, 254). Overexpression of p120^{ctn} disrupts stress fibers and focal adhesions and results in a decrease in RhoA activity. This suggests that p120^{ctn} can shuttle between cadherin-bound state and a cytoplasmic pool in which it can interact with regulators of Rho family. Inhibition of Rho family GTPases is predicted to elevate active Rac1 and Cdc42, thereby promoting cell migration(255). Together, these data suggest that p120^{ctn} has several functions including cadherin binding, modulation of the actin cytoskeleton and signaling.

Regulation of cadherin/catenin complex and evidence for cadherin outside-in signaling

There are a number of different signaling pathways that can regulate the cellular adhesive junctions. Several studies have suggested that tyrosine phosphorylation plays a role in controlling junctional integrity, and evidence has pointed to components of the cadherin/catenin complex as possible molecular targets. The tyrosine kinases c-Yes, c-

Src, and c-Lyn have been found at the cadherin/catenin complex(256), where levels of phosphotyrosine can, under certain conditions, become elevated(256, 257). Transfection of epithelial cells with oncogenic Src or Ras leads to tyrosine phosphorylation of components of the cadherin/catenin complex, and this correlates with loss of cell-cell-adhesion(258), an important step in metastasis(259, 260).

β- γ- and p120-catenin are all subjected to tyrosine phosphorylation. Increased tyrosine phosphorylation of all these catenins have been correlated with the decreased cell adhesion which occurs upon malignant transformation or mitogenic growth factor stimulation(258, 261, 262). For instance, $p120^{ctn}$ is rapidly phosphorylated in response to EGF, PDGF and CSF-1(250, 251). Likewise, tyrosine phosphorylation of β- and γ-catenin has been reported in response to EGF and TGFα(261).

One of the important mechanistic issues is whether the receptor tyrosine kinases (RTKs) of these ligands phosphorylate the catenins directly, or indirectly through the recruitment of other protein tyrosine kinases. Upon activation by ligand, RTKs dimerize and autophosphorylate themselves at specific tyrosine residues, which then serve as binding sites for phosphotyrosine binding proteins(263). A subset of the phosphorylated tyrosines on certain activated RTKs are known to interact with the Src-family tyrosine kinases through specific SH2-mediated interaction(264, 265). As a result, growth factor-induced tyrosine phosphorylation of catenins could happen indirectly through the recruitment of the Src-family tyrosine kinases. On the other hand, β-catenin has been shown to interact directly with the EGFR, which suggests that direct phosphorylation of catenins by RTKs is also possible(266). It is not yet known whether EGFR directly phosphorylates p120^{ctn} directly or indirectly. In addition to their well-studied roles in promoting cell proliferation, RTKs may regulate cell-cell adhesion and thereby contribute to metastasis by directly or indirectly regulating cadherin function.

Since cadherin function is regulated by its association with the actin containing cytoskeleton, the stability of this association must be regulated by a balance of tyrosine phosphorylation and dephosphorylation of the catenins. Cadherin immunoprecipitation studies have identified certain protein tyrosine phosphatases associated with the complex. From these result, the non-receptor protein tyrosine phosphatase PTP1β and the receptor protein tyrosine phosphates PTPμ have been shown to bind directly to the cytoplasmic domain of cadherins and modulate function by regulating tyrosine phosphorylation of the cadherin and/or catenin(267, 268). Because several pathways can regulate the activity and/or association of tyrosine kinases and phosphatases with the cadherin/catenin complex, understanding these pathways may add a new dimension to the potential for cadherin function and signaling.

There is now emerging evidence that cadherin can modulate outside-in signaling transduction pathways affecting biological processes in cells. These signaling events can be generated upon cadherin mediated cell-cell receptor recognition or in conjunction with RTKs. For instance, the extracellular domain of N-cadherin has been shown to interact with FGFR(269). The validity of this work resulted from both dominant negative FGFR experiments and the use of a highly selective inhibitor to the tyrosine kinase activity of the FGFR. Use of these techniques showed that N-cadherin mediated neurite outgrowth was dependent upon FGFR activity. It is hypothesized that N-cadherin might act as a surrogate ligand for the FGFR and/or sensitize the FGFR to low levels of FGF present in the culture(270). Mechanistically, this may explain how N-cadherin through syngergistic interaction with FGFR activity can promote processes such as contact-dependent survival of ovarian granulose cells, or more recently, promote the motility of cancer cells(271-273).

RTKs can also function with other cadherins such as E-cadherin. Pece et al.(274) showed that E-cadherin dependent adhesion could regulate the activity of MAPK through the ligand independent activation of the EGFR kinase. Evidence for MAPK stimulation by E-cadherin was supported by the use of both function-perturbing antibodies to E-cadherin and antibody-induced clustering of E-cadherin. This activation of MAPK by E-cadherin was abolished by addition of EGFR selective inhibitors and functional blocking antibodies(274). In addition, E-cadherin-mediated adherens junction formation initiates other signal transducing pathways such as the PI 3-kinase/Akt pathway. The assembly of E-cadherin-mediated adherens junction resulted in a rapid PI 3-kinase dependent activation of Akt and the subsequent translocation of Akt to the nucleus. Moreover, the activation of PI 3-kinase in response to E-cadherin mediated cell-cell contact involves recruitment of the p85 subunit of PI 3-kinase to the E-cadherin/catenin complex and subsequently the phosphorylation of PI 3-kinase(275). Therefore, these findings suggest that E-cadherin can transduce outside-in signaling that can affect biological processes including growth, survival and differentiation upon cell-cell contacts.

In addition to cadherin modulation of signal transducing pathways, there is evidence that E-cadherin mediated cell-cell attachment can regulate the activities of the

Rho family of small GTPases(276). The Rho family of GTPases, including Rac1, Cdc42 and RhoA, regulate the reorganization of the actin cytoskeleton(277, 278). In fibroblasts, the activation of RhoA stimulates both stress fibers and focal adhesion formation, whereas activation of Rac1 and Cdc42 promote extensions of lamellipodia and filopodia(277). However in epithelial cells, overexpression of constitutively active Rac1 and Cdc42 increases E-cadherin localization and actin assembly at the cell-cell junction(279, 280). In recent studies E-cadherin mediated adherens junction assembly results in triggering a decrease in RhoA activity and the recruitment and stimulation of both Rac1 and Cdc42(276, 278, 281). This direct interaction and stimulation of Rac1 and Cdc42 provides evidence that cadherins themselves are able to relay information from adhesion to the organization of the cytoskeleton which are critical determining the fate of cell migration and differentiation. Thus, the regulation of Rho family GTPases through cadherin-mediated adhesion may in part play a role in the progression of metastatic prostate cancer.

Synergy and cross talk between cadherin and integrin

The mechanism of tumor cell dissemination requires communication among various cell adhesion molecules. For example, downregulation of E-cadherin activity is necessary for cells to break away from the primary tumor mass(282). This is followed by increased involvement of integrins in cell-substrate interactions during increased motility. These diverse requirements may be met as a consequence of interactions between the different classes of adhesion receptors. The functions performed by integrins are often dependent upon the presence and/or activity of other membrane-associated molecules, such as cadherins. Consequently, evidence is emerging that there may be cross-talk between cadherins and integrins. Functional blocking antibodies to cadherins have been shown to prevent the loss of α5 and β1 integrins in terminally differentiating keratinocytes(283). In contrast, in Xenopus-derived fibroblasts, introduction of E-cadherin caused downregulation of α3β1 integrin and reduced adhesion to fibronectin and laminin(284). In addition, interfering with β1 or β3-integrin-mediated interactions with the ECM blocks migration. This effect correlates with the enhanced localization of N-cadherin to adherens junctions and cell aggregation, indicating N-cadherin cellular distribution is restrained by integrin-mediated signals(285). The signals that cause downstream repression of this integrin action involve calcium fluxes and the inhibition of serine/threonine kinases(285). One possible mechanism concerning cadherin and integrin crosstalk involves the integrin-linked kinase (ILK)(286). ILK is a serine/threonine kinase that interacts with both the β1 and β3 integrin cytoplasmic domains. ILK can inhibit GSK-3β by direct phosphorylation(287). Inactivation of GSK-3β by ILK enhances β-catenin translocation to the nucleus and increases β-catenin/LEF-1 transcriptional activity (288). Interestingly, E-cadherin is one of the possible targets of the LEF-1 transcriptional activity, and cells overexpressing ILK show dramatically reduced levels of E-cadherin and loss of cell-cell adhesion(288, 289). These data provide interesting potential connections between integrins and cadherins, in which cross-talk between these two adhesion receptors has particular relevance to malignant epithelial cells, which show decreased levels of adhesion molecules and are highly motile and invasive.

Epithelial adhesion and cadherin expression in prostate cancer

One of the most important types of adhesive interactions required for the maintenance of solid tissues is that mediated by the classic cadherin adhesion molecules. In epithelial tissues, E-cadherin forms the basis of the epithelial architecture(212). Continued expression and functional activity of E-cadherin are required for cells to remain tightly associated in the epithelium; in its absence the many other cell adhesion and cell junction proteins such as integrins and occludens are not capable of supporting intercellular adhesion(290). In addition, E-cadherin is the major adhesion receptor of the *zonula adherens* junctions of epithelial cells, where it co-localizes with a prominent actin filament bundle. The *zonula adherens* junction and associated actin filament bundle are necessary in epithelial tissues that experience strong contractile or mechanical forces such as in the digestive tract or tissues undergoing wound closure or invagination(291).

Like other epithelial tissues, prostate glandular epithelial architecture consists of E-cadherin mediated cell-cell adhesion(292). In prostate carcinoma, down-regulation of E-cadherin protein expression correlates with increased malignancy(293-295). Bussemakers *et al.* (1992) was the first to report a strong correlation between lack of E-cadherin expression and metastatic potential in the Dunning rat model of prostate cancer(296). In human prostate cancers, half the tumors examined showed reduced or absent E-cadherin compared to the uniformly intense expression of the glandular epithelium in normal prostate. There was also a strong correlation between E-cadherin expression and Gleason grade of the prostate carcinomas. Tumors with Gleason sum scores <6 expressed normal levels of E-cadherin; tumors with Gleason sum scores of 6-8 had heterogeneous expression of E-cadherin, while there was a total loss of E-cadherin expression was in high-grade tumors (Gleason 9 or 10)(294). The loss of E-cadherin expression may be due to factors such as decreased levels of expression, deletion or mutation(297) in the E-cadherin gene. In other cases, decreased adhesive function was observed as a result from mutations in the catenin genes that encode the catenin; e.g., mutations in β-catenin that disrupt the association of E-cadherin with α-catenin and result in a non-adhesive phenotype(298). Mutations in the α-catenin gene are reported to effectively inactivate E-cadherin function by disrupting association of the cadherin complex with the cytoskeleton(299). Further, inactivation of E-cadherin in this manner results in the conversion of tumor cells to a metastatic phenotype. In fact, re-expression of E-cadherin in invasive carcinoma cells, which lack E-cadherin, results in restriction of cell migration and invasion and a reverse transformation from a less differentiated phenotype to a normal epitheloid morphology(300). Therefore, E-cadherin is coined an invasive suppressor. In addition to being an invasive suppressor, E-cadherin also function as a proliferation suppressor. St. Croix et al. (1998)(301) demonstrated the importance of E-cadherin in contact inhibition of growth of normal epithelial cells. Expression of E-cadherin in the null E-cadherin EMT/6 mouse mammary carcinoma cells resulted in tighter adhesion of multicellular spheroids and a reduced proliferative fraction in three-dimensional culture. Interestingly, the expression of E-cadherin also resulted in an increase in the cyclin-dependent kinase (cdk) inhibitor $p27^{kip1}$ and a decrease in both Rb dephosphorylation and cyclin D1 protein. This suggests that $p27^{kip1}$ plays a significant role in E-cadherin-dependent growth inhibition, which may explain why solid tumors, despite harboring multiple mutant oncogenes and tumor suppressor genes, often contain low proliferative fractions in vivo(301). Thus, disruption of the function of the E-cadherin/catenin complex frequently results in the formation of invasive tumorigenic cells.

Recently, our data and studies by Bussemaker *et al.*(302) showed that the loss of E-cadherin in prostate epithelial carcinoma cell lines is associated with an unexpected expression of another classical cadherin, N-cadherin in prostate carcinoma cell lines(302, 302, 303). N-cadherin plays an important role in maintaining cell-cell adhesion in certain cell types such as neurons, skeletal muscle, and cardiac muscles, while it is not expressed in normal epithelial cells(304). Analysis of prostate tissues by Bussemaker et al. (2000) reveal expression of N-cadherin is prevalent in high grade tumors, Gleason sum score >7 (302). In addition the gain of N-cadherin is found in other tumor types such as breast, melanoma, and squamous cell carcinoma(273, 305, 306). The expression of N-cadherin in these tumors also positively correlates with advance metastatic tumor stages.

There is also an emerging data, which suggests that N-cadherin expression in advanced tumors may function as a potent inducer of invasion and metastasis. Exogenous expression of N-cadherin in tumorigenic, but weakly metastatic, E-cadherin-expressing MCF-7 cells results in the ability of these cells to metastasize in vivo(272, 273). This motility and invasion in vitro appears to be greatly enhanced by FGF-2 and is accompanied by upregulation in MMP-9 activity. Mechanistically, N-cadherin expression may activate a metastatic pathway that is coordinated by FGFR and MMP-9 signals in these cells, which can bypass the suppressive signals mediated by E-cadherin(272, 273).

Interestingly, the exogenous expression of E-cadherin into invasive and N-cadherin-expressing breast cancer cells did not revert their invasive phenotype(307) although both N- and E-cadherin were colocalized at the invasive edge of squamous tumors(306). Notably, deletional analysis of the extracellular region of N-cadherin shows that the 69-amino acid portion of EC-4 of N-cadherin was necessary and sufficient to promote an epithelial to mesenchymal transition in squamous epithelial cells and increased cell motility(308). This segment of N-cadherin includes the sequences proposed by Doherty and Walsh (1996) to interact with the FGF receptor family(269). Thus, the coordinate interaction of N-cadherin with the FGFR may play a functional role in inducing cellular invasion.

In summary, the early stages of cancer metastasis requires detachment of cells from the primary tumor mass. Changes in cell-cell interactions must be a reflection of alterations in the levels, activity, or function of the cadherins that mediate this characteristic. In prostate cancer, the loss of E-cadherin and a gain of N-cadherin appear to play important roles in the progression of a metastatic disease. Therefore, understanding the basis for further characterization of the molecular mechanism of N-cadherin-mediated metastasis in prostate cancer could yield potential insights for diagnostic or therapeutic applications.

Statement of the problem

Prostate cancer is now the most common cancer of males in the United States, accounting for 21% of all newly diagnosed cancers, and a subset of these tumors metastasize and give rise to cancer-related deaths(309). The other subgroup of patients will harbor prostate cancer that remains silent during their lifetimes. To date, a series of molecular alterations in prostate cancer have been analyzed with the goal of identifying changes that may be of predictive value(310). One focus has been on the invasive and adhesive properties of malignant prostate cancer cells, which are two of the main characteristics that distinguish benign from malignant disease. Prostate tumor cells acquire increased invasive potential by a complex pathway that includes altered cell-substrate attachment and cell-cell adhesion as well as increasing cell motility and cell survival(311). Understanding the changes in the proteins that lead to metastasis and how these changes affect cellular motility in malignant cells would provide clear prognostic indicators to identify individuals who are more likely to develop aggressive prostate cancer.

Cadherins and catenins participate in the regulation of the epithelial phenotype, changes of which have profound consequences for tissue differentiation and malignant transformation. In prostate carcinoma progression, changes in cadherin expression and function are linked to malignancy. Identification of the role of cadherins and characterization of the mechanisms for their contribution to metastasis is one important area of prostate cancer research because these studies are generating information for the improvement of diagnosis and therapy of prostate cancer. Thus, exploring mechanism for

N-cadherin mediated cell-cell adhesion and signaling in malignant prostate carcinoma may explain in part one of the crucial steps to metastasis, a fundamental concept in cancer biology for solid tumor growth and survival which has yet to be explained at the molecular level.

Hypotheses and Specific Aims

Previous studies have implicated the downregulation of E-cadherin function as one of the important factors for prostate cancer metastasis. The central hypothesis of this dissertation is that **the switch in the expression of cadherins plays a critical role in prostate cancer progression and survival.** Four specific aims were designed to investigate this hypothesis:

Specific Aim 1: Identify and characterize the expression of cadherin/catenin in prostate carcinoma cell lines. **Hypothesis 1: Loss of E-cadherin expression is accompanied by a gain of N-cadherin.** RT-PCR was used to identify N-cadherin expression in prostate carcinoma cells, and a human xenograph model of intraperitoneal inoculation in SCID mice was used to characterize cellular invasion.

Specific Aim 2: Investigate the role of N-cadherin in promoting prostate carcinoma cell survival. **Hypothesis 2. N-cadherin homophilic interactions between prostate carcinoma cells or with prostate stromal fibroblasts facilitate a signal transduction pathway involved in promoting cell survival through Bcl-2 protein stability.** The Calcium Switch Assay was used to evaluate N-cadherin ligation, and immunoprecipitation studies along with phospho-specific antibodies to various proteins was used to evaluate activation of signaling molecules. PARP cleavage and Bcl-2/Bax protein ratio were used to evaluate cell survival.

Specific Aim 3: Extend the investigation of N-cadherin signaling in the regulation of *bcl-2* anti-apoptotic gene expression. **Hypothesis 3: 'Outside-In' signal transduction by N-cadherin mediated cell-cell adhesion results in upregulation of *bcl-2* transcription for cell survival.** The Calcium Switch Assay was used to investigate N-cadherin ligation and phospho-specific antibodies to various signaling molecules were used to examine the signaling components. In addition, different promoter regions of the *bcl-2* gene linked to luciferase reporter were used to analyze bcl-2 transcription following N-cadherin ligation.

Specific Aim 4: Examine the cadherin expression and regulation using a multicellular spheroid system. **Hypothesis 4: Regulation of cadherin-mediated cell-cell adhesion is dependent on tumor microenvironment such as integrin signaling or metabolic stress due to hypoxia.** An *in vitro* multicellular spheroid system was used along with a variety of methods to examine the expression and regulation of cadherins.

CHAPTER 2: N-CADHERIN EXPRESSION IN HUMAN PROSTATE CARICNOMA CELL LINES: AN EPITHELIAL-MESENCHYMAL TRANSFORMATION MEDIATING ADHESION WITH STROMAL CELLS[1]

Introduction

Prostate carcinomas display a high degree of biological diversity(312), and can be present as localized disease within the prostate, or can become highly invasive and metastasize to regional lymph nodes and bone. Although localized prostate carcinoma can be treated successfully(313), the treatment success diminishes significantly when prostate tumor cells metastasize beyond the confines of the gland, mainly through perineurial and stromal invasion(188, 314, 315). The process of metastasis is multifaceted; however, the molecular mechanisms that promote dissemination of the metastatic phenotype in prostate carcinoma are not well understood. An early invasion event in prostate carcinoma is the loss or disruption of the basal cell component, followed by the gain or loss of genetic and biochemical functions in transformed luminal epithelial cells(316). Some of the gains or losses of functions during prostate carcinoma progression are due to alterations in tissue organization between cells themselves, mediated by cell-cell adhesion receptors of the cadherin superfamily, and cell-extracellular matrix adhesion receptors of the integrin family and components of the extracellular matrix(317).

Epithelial cytoarchitecture and function in the prostate gland are maintained in part by the E-cadherin/catenin complex(318). E-cadherin is a member of a family of

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Ca^{2+}-dependent integral membrane cell-cell adhesion receptors(319). E-cadherin is localized at the *zonula adherens* junction between epithelial cells(320) and is associated with peripheral basal-lateral actin filaments in a multiprotein complex with kinases, phosphatases, and catenins(321). The cytoplasmic complex, which anchors E-cadherin to the actin cytoskeleton(322), includes the intracellular proteins α-catenin, which has homology to vinculin(323), and the *armadillo* family members β-catenin, γ-catenin/plakoglobin(324), and $p120^{ctn}$(244). The $p120^{ctn}$ binding site in E-cadherin is different from the β-catenin/plakoglobin binding site, and it does not bind to α-catenin(325). Following cell-cell contact, adhesion of the E-cadherin/catenin complex functions to establish epithelial cellular architecture by initiating formation of desmosomes, tight junctions, and gap junctions(326).

Alteration in E-cadherin/catenin function or expression is found in the neoplastic process as a step in metastasis(327-330) . This loss, in part, results in a transformation from the normal epitheloid morphology toward an invasive and less differentiated mesenchymal phenotype(331, 332). E-cadherin levels are reduced or absent in the more invasive tumor cell lines; this phenotype is reversed by transfection with full-length E-cadherin cDNA(327). Immunohistochemical analysis of highly invasive tumors (breast, melanoma, prostate, non-small cell lung carcinomas) indicates that these tissues have decreased E-cadherin levels, suggesting a decreased function for E-cadherin in organization of tissue structure(333). Umbas *et al.*(300) found that human prostate carcinomas with a Gleason score above 6 had decreased E-cadherin immunoreactivity compared to normal glandular epithelium, and tumors with a Gleason score of 9 and 10 had low E-cadherin immunoreactivity. Moreover, in prostate tumor cell lines (DU145, PC-3, PPC-1 and TSU-PR1), E-cadherin expression was also found to be decreased or absent(295). These studies demonstrate that the down regulation of the E-cadherin/catenin adhesion pathway is associated with loss of differentiation, and an increase in the invasive behavior of tumor cells observed in prostate carcinomas.

To further understand the molecular basis of variability of prostate carcinoma invasiveness, the protein and mRNA expression levels of the E-cadherin/catenin complex were examined in four human prostate adenocarcinoma cell lines: LNCaP(334), DU145(335), PC-3(336), JCA1(337). The prostate cell line JCA1 and PC-3N, a derivative of PC-3, both of which lacked E-cadherin, expressed instead a larger molecular mass cadherin. This additional cadherin was identified as N-cadherin, and N-cadherin is expressed in more invasive prostate adenocarcinomas cell lines and in prostate stromal fibroblasts. Moreover, the isoform expression (p120 and p100) of the cadherin associated protein $p120^{ctn}$ was found to be dependent upon whether E- or N-cadherin was expressed in the prostate carcinoma cell lines. These results suggest that the loss of the epithelial phenotype in invasive prostate adenocarcinoma cell lines is followed by a gain of a N-cadherin/$p120^{ctn}$ phenotype.

Materials and Methods

Cell Culture

LNCaP and DU145 human prostate tumor cell lines were obtained from American Type Tissue Culture Collection (Rockville, MD). Isolation of JCA1 cells was previously described and established by Muraki *et al*(337). The PC-3 cell line was originally obtained from American Tissue Culture Collection, and long-term passage in cell culture resulted in selection of a cell population with different growth, adhesive, and morphological phenotype (PC-3N). All cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% heat inactivated fetal bovine serum (FBS; Intergen, Purchase, NY) and penicillin/streptomycin in a 37°C, 5% CO_2 atmosphere at constant humidity.

Human prostate stromal fibroblasts (PSF) were cultured from surgical samples. Fibroblasts were isolated by cutting the prostate tissue into 1 mm^3 pieces, which were placed in 100 mm culture dishes, and allowed to attach overnight. The tissue was maintained in DMEM with 10% FBS as cells migrated from the explants. After two passages with trypsin/EDTA, only prostate fibroblast cells remained in culture, which was shown by the absence of cytokeratin positive cells. The cells were then maintained for another two passages before use in these studies.

Antibodies

A polyclonal antiserum (anti-pan cadherin) was made to the deduced amino acid sequence of the COOH-terminus of mouse N-cadherin (residues 883-906)(338) using the peptide CDYDYLNDWGPRFKKLADMYGGGDD (Peptide Express, Fort Collins, CO). The peptides were coupled to keyhole limpet hemocyanin as described by Marcantonio and Hynes (1988)(339) and injected into New Zealand white rabbits with Freund's incomplete adjuvant. Serum was harvested after booster injections of the antigen.

Mouse monoclonal antibodies used in the experiments were as follows: α-catenin clone 5 (Transduction Laboratories, Lexington, KY), E-cadherin clone HECD-1 (Zymed laboratories, Inc, San Francisco, CA), N-cadherin (A-CAM clone GC-4; Sigma Chemical Co., St. Louis, MO), p120^{ctn} clone 98 (Transduction Laboratories). A monoclonal mouse antibody to plakoglobin, PG5.1(340) was provided by Drs. Franke and Schmeltz (Institute of Cell and Tumor Biology, German Cancer Research Center). Rabbit cytokeratin 18A antibody was previously described by Nagle et al(341). Secondary antibodies used in the experiments are as followed: Cy3-conjugated affinipure goat anti-mouse IgG (H+L) and Fluorescein (FITC)-conjugated affinipure donkey anti-rabbit IgG (H+L) were purchased from Jackson ImmunoResearch Laboratories, Inc., (West Grove, PA). Anti-mouse IgG+HRP conjugate was purchased from Promega (Madison WI) and anti-rabbit IgG-peroxidase conjugate was obtained from Boehringer Mannheim (Indianapolis, IN).

SDS-PAGE and Western Blot

Cell lysates were prepared and separated by 7% SDS-PAGE(342), and electrophoretically transferred to nitrocellulose. Monolayers of cells were washed with calcium and magnesium free phosphate buffered saline (CMF-PBS) containing 1 mM phenylmethylsulfonyl fluoride (PMSF). Cells were scraped in CMF-PBS, transferred to a microcentrifuge tube, and centrifuged. The pellet was lysed with 2X SDS Sample Buffer (0.25M Tris-HCl pH 6.8, 10% SDS, 25% glycerol) and 30 μg of cellular protein was loaded per lane. Protein concentrations were measured using the BCA assay procedure (Pierce Chemical Co., Rockford, IL), with bovine serum albumin as a standard. Antigens were detected by primary antibodies, followed with peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG. Protein bands were identified by chemiluminescence (NEN, Boston, MA) exposed on X-OMAT AR film (Kodak, Rochester, NY). Images of Western Blots were captured using Metamorph Version 3.0 (Hollis, NH), and quantitative densitometry was carried out by using One-D Scan Version 1.0 (Scanalytics, CSP Inc.).

To examine non-ionic detergent solubility of N-cadherin, PC-3N cells were grown to 90% confluency. Cells were washed 3X with CMF-PBS and cytoskeletal stabilization buffer (CSK buffer; 0.5% Triton X-100, 10 mM PIPES, pH6.8, 50 mM NaCl, 3 mM $MgCl_2$, 0.3M sucrose) was added for 5 min 4°C on a rocking platform. The cells were scraped, centrifuged, and detergent soluble protein fractions collected by acetone precipitation. The precipitate were collected by centrifugation at 10,000 rpm for 20 min, air-dried and resuspended in 2X SDS sample buffer. The insoluble fraction was collected by adding hot 2X SDS sample buffer to the cellular components remaining on the plate and syringing.

For immunoprecipitation, cells were lysed according to procedures by Reynolds *et al.* (1994)(244) with 0.5% Nonidet P-40 (NP-40) in a buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 0.1 mM sodium vanadate, 10 µg/ml aprotinin and 10 µg/ml leupeptin (Sigma Co.). Proteins were immunoprecipitated from the lysates, separated by SDS-PAGE, transferred to nitrocellulose, and antigen was detected as described above.

RNA Extraction and Northern Analysis

Total RNA was prepared from cultured cells by acid guanidinium thiocyanate-pheno-chloroform extraction(343). Twenty µgs of each RNA sample was separated by electrophoresis in 1% agarose gel containing 1.85% formaldehyde and transferred onto a Hybond N+ nylon membrane (Amersham Life Science, Arlington Heights, IL). N-cadherin mRNA was detected by Northern blot analysis with a 300 bp *EcoR*I cDNA fragment isolated from full length N-cadherin (GenBank Accession X54315), which was obtained from Dr. John Hemperly (Becton Dickinson Research Center, Research Triangle Park, NC)(344). E-cadherin mRNA was detected using 1.7 kb *Sma*I fragment of mouse E-cadherin (GenBank Accession X06115)$^{(322)}$. Detection of plakoglobin (GenBank Accession M23410) was with a human cDNA obtained from Dr. Werner Franke$^{(345)}$. Probes were random-primed with labeled α-^{32}PdCTP (Amersham Life Science). Membranes were prehybridized for 18 hr at 42°C in a 6X SSC buffer consisting of 0.05M NaH_2PO_4, 5X Denhardt's (50X=1.0 Ficoll, 1% polyvinylpyrrolidone, 1% bovine serum albumin), 1% SDS, 50% formamide and 10 µg/ml salmon sperm DNA(346). A denatured probe was added to blots and hybridized overnight. Blots were sequentially washed for 30 min at 65°C using the following conditions: 2X SSC/0.1% SDS, 0.3X SSC/0.5% SDS, and 0.1X SSC/1.0% SDS. Blots were then exposed to X-OMAT AR film (Kodak). Normalization for loading was compared to hybridization of a 1.2 kb *Pst*I fragment of human GAPDH (GenBank Accession J04038).

RT/PCR and DNA Sequencing

A polymerase chain reaction (PCR) with cDNA generated by reverse transcription of total RNA from PC-3N cells was performed, using degenerate primers to amplify multiple cadherin subtypes(347). PC-3N cDNA was amplified from 1 μg of DNase I treated total RNA in 40 μl reaction mix containing random hexamer primers, 10 mM DTT, 0.5 mM dNTPs, 10 U RNasin, and 200 U of Maloney murine leukemia virus reverse transcriptase (Gibco BRL) for 60 min at 42°C. The cDNA product was then diluted with 80 μl of H_2O, and 2.5 μl of this PC-3N cDNA product was used in a 25 μl PCR reaction using the 5' oligonucleotide primer AATGAATTCGTNTTYGAYTAYGARGG and the 3' primer AATGAATTCRTCNGCNAGYTTYTTRAA. The reaction products were next separated by a 4% agarose gel electrophoresis (3% Nusieve GTG agarose, and 1% Seakem ME agarose, FMC BioProducts, Rockland, ME), and a cDNA fragment of about 150 bp was extracted, digested with *Eco*R1, and ligated into pBluescript (Stratagene, La Jolla, CA). Ligated products were transformed into XL-1 Blue E. coli (Stratagene), and sequences of thirty-one cDNA inserts were determined by dideoxy chain termination (Sequenase 2.0, United States Biochemical, Cleveland, OH).

SCID Mouse Model

BALB-c/B-17/IcrACCscid mice (Arizona Cancer Center SCID Colony) were maintained in a specific pathogen-free environment in compliance with USPHS guidelines governing the care and maintenance of animals. Five weeks old male SCID mice were each inoculated intraperitoneally with 5×10^6 DU-145 or PC-3N cells resuspended in 0.25 ml of DMEM serum-free medium. Forty-two days after inoculation, mice were sacrificed, and diaphragm tissues were fixed, and processed according to

McCandless *et al.*(348) Xenograft fixed tissues were sectioned at 5 µm thickness. Sections were deparaffinized and stained with hematoxylin and eosin.

Preparation of Tissues and Immunocytochemistry

Human frozen prostate tissue was obtained from the University of Arizona Pathology Tissue Bank. Specimens were obtained at the time of surgery or autopsy snap frozen in isopentane, cooled by freon, and stored at -80°C. Frozen sections of 6 µm were placed onto poly-L-lysine-coated slides and fixed in acetone for 10 min at -20°C. Sections were then blocked with 2% BSA and 2% goat serum in CMF-PBS for 1 hr, then incubated with both rabbit polyclonal anti-cytokeratin 18 antibody and murine anti-N-cadherin for 1 hr. Following washing, Cy3-conjugated anti-mouse IgG and FITC-conjugated donkey anti-rabbit IgG were applied for 1 hr. The slides were mounted with 2% n-propyl gallate/ 90% glycerol, pH 8.0. Human prostate tissues were viewed by laser scanning confocal microscope, LSM 410, equipped with He, Ne and Ar lasers (Zeiss).

For immunofluorescence, cells were grown on glass coverslips to confluence. Cells were fixed for 5 min in 4% (w/v) paraformaldehyde in CMF-PBS and permeabilized in CSK buffer for 5 min at 4°C. Coverslips were incubated with 2% BSA and 2% goat serum in CMF-PBS and exposed to antibodies for 1 h at 25°C. After washing, Cy3-conjugated secondary anti-mouse IgG was applied for 1 h. For detection of N-cadherin in PC-3N and prostate stromal fibroblasts (PSF) co-cultures, PSF cells were grown to 50% confluency on glass coverslips overnight. PC-3N cells were labeled with 40 µg/ml of DiO (3,3'-dioctadecyloxacarbocyanine perchlorate [Molecular Probes, Eugene, OR] in ethanol) for 1 hr and washed extensively with CMF-PBS. Labeled PC-

3N cells (10^4) were seeded with the PSF culture for 24 hr. Cells were then fixed, permeabilized with CSK buffer and stained for N-cadherin as described above.

Cell Aggregation Assay

Cell-cell aggregation experiments were performed as described by Urushihara and Takeichi (1976)(349). Monolayer cultures were treated with 0.01% trypsin (Worthington Biochemical Corp., Freehold, NJ) in the presence of 2 mM calcium for 2 min. The trypsinized cells were washed gently by centrifugation in Hank's Balanced Salt Solution (HBSS) containing 10 mM HEPES pH 7.4, 1 % BSA and free of calcium and magnesium. Cells were dissociated thoroughly by trituration 10 times with a Pasteur pipette. 5×10^5 cells were then transferred to 24-well dishes in a final volume of 0.5 ml HEPES-buffered HBSS containing 1% BSA and 100 µg/ml of DNAse I with or without 2 mM $CaCl_2$. The plates were previously coated with poly-hema (Sigma). Cell-cell adhesion was initiated with addition of calcium and the plates were rotated at 80 rpm at 37°C for 1 h, and cells were then fixed with an equal volume of 8% paraformaldehyde in CMF-PBS, pH 7.4. For the mixed aggregation experiments, PC-3N cells were labeled for 1 hr at 37°C with 40 µg/ml DiO. Cell aggregation was done in the presence or absence of $CaCl_2$ and/or N-cadherin specific blocking monoclonal antibody (A-CAM; clone GC4) at 80 rpm at 37°C for 1 h. Cells were then fixed as previously described above. For analysis, 50 µl of the fixed aggregates were removed, placed on a slide and covered with a coverslip. Aggregates were photographed under epifluorescence optics using the 20X objective (Zeiss) with both a FITC filter set and phase contrast.

Results

Invasive characteristics of prostate carcinoma cell lines.

The human prostate adenocarcinoma cell lines LNCaP, DU145, PC-3, JCA1, and a sub-line PC-3N show distinct cellular morphologies in vitro(334-337). DU145 and LNCaP cells display an epithelial phenotype, while PC-3, PC-3N and JCA1 cells have to varying degrees a less organized and elongated, spindle-shaped, mesenchymal phenotype. PC-3N cells are a variant of parental PC-3 cell line(336) that displayed a more fibroblast-like phenotype after extensive sub-culturing. PC-3 has been previously shown to contain two distinct sub-populations(350). One population expresses E-cadherin, which displays an epithelial phenotype, and the other lacking E-cadherin expression, which possesses a more scattered spindle-shaped phenotype, similar to PC-3N. Since the human prostate carcinoma cell lines PC-3N and DU145 have distinctly different growth characteristics, I sought to characterize the invasiveness using a human xenograph model of intraperiteneal inoculation in SCID mice(348). Cross sections of the diaphragm stained with hematoxylin and eosin show that after five weeks, PC-3N and DU145 cells had randomly attached to mesothelial surface of the diaphragm. PC-3N cells grew as small solid tumors on the surface of the diaphragm. There was also PC-3N cell invasion into the striated muscle of the diaphragm. Small clusters of invading PC-3N cells were detected at multiple sites within the diaphragm muscle (Figure 2.1A). These invading colonies of PC-3N human carcinoma were consistently only a few millimeters in diameter. DU145 grew as large highly vascularized tumors on the diaphragm surface, but did not invade the diaphragm muscle (Figure 2.1B).

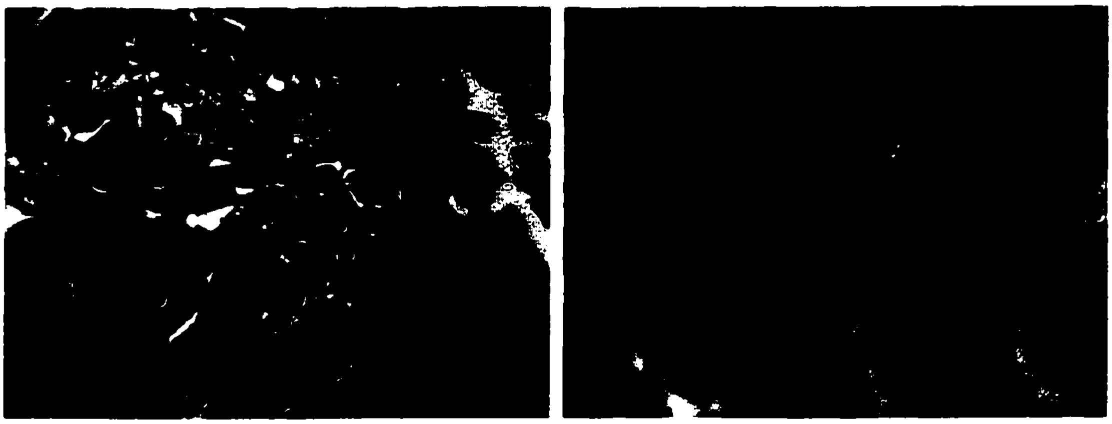


Figure 2.1: Photomicrographs of tumors of the prostate carcinoma cell lines PC-3N (panel A) and DU-145 (panel B) on the surface of diaphragms of SCID mice. SCID mice (n=4) were injected intraperitoneally with 5 X 10^6 cells, sacrificed 5 weeks after injection, and the diaphragms fixed and processed in paraffin. DU145 tumors have penetrated the basement membrane, and PC-3N have penetrated through the murine straited muscle. Five-µm sections were cut and deparaffinized for hematoxylin and eosin staining. Bar = 60 µm.

Expression of an N-cadherin in PC-3, PC-3N and JCA1 prostate cell lines.

Since the growth and invasive characteristics of PC-3N prostate carcinoma cells in the SCID mouse diaphragm suggested weak cell-cell adhesion, E-cadherin/catenin expression of PC-3N prostate carcinoma cells and the other four prostate carcinoma cell lines was characterized. The expression level of E-cadherin and α-,β-, and γ-catenins was assessed by immunoblotting equivalent amounts of cellular protein. Data are shown in Figure 2.2, and summarized in Table 1. To examine the cadherin expression, total prostate cell lysates were immunoblotted with a polyclonal cadherin antibody directed against the conserved cytoplasmic region of the classical cadherin family(351)(Figure 2.2A). Antibodies prepared to this region have been shown to be immunoreactive with several members of the cadherin family. Immunoblot analysis indicated E-cadherin (MW=125 kDa) was present in LNCaP, DU145, and PC-3, but was absent from PC-3N and JCA1 cell lines (Figure 2.2A and C). In addition, a higher molecular weight cadherin (138 kDa) was detected in PC-3, PC-3N, and JCA1 adenocarcinoma cell lines. PC-3 cells showed expression of a mixed cadherin phenotype containing both E-cadherin and the larger cadherin. This unknown cadherin was not detected in LNCaP and DU145 (Figure 2.2A).

Since the loss of catenins, especially α-catenin, has been shown to decrease E-cadherin mediated cell-cell interaction during the malignant progression of prostate cancer(295, 352), we characterized α-catenin, β-catenin, γ-catenin/plakoglobin (immunoblot not shown) and p120^{ctn} expression levels in prostate carcinoma cell lines by immunblotting of equivalent amounts of protein. The results are summarized in Table 1.

Table 1: Relative Expression Levels of E-cadherin and Catenins in Human Prostate Adenocarcinoma Cell Lines

	DU-145[*]	LNCaP	PC-3	PC-3N	JCA1
E-cadherin	1.0	3.7	0.4	0.0	0.0
α-catenin	1.0	5.5	ND[§]	0.0	0.2
β-catenin	1.0	3.1	ND	0.9	0.0
plakoglobin	1.0	1.7	ND	0.1	0.3
$p120^{ctn}$ (p120+ p100)[†]	1.0	2.5	0.5	0.4	0.8
p120/p100[‡]	0.5	0.3	1.1	2.3	2.9

ND, not determined.

[*] DU-145 is a differentiated prostate carcinoma cell line which expresses equal levels of all catenins. Thus, all densitometry values are normalized to DU-145. Representative values are shown from at least three independent lysates.

[†] Immunoblot of $p120^{ctn}$ is shown in Figure 2.2B.

[‡] Values represent a ratio of p120 to p100.

[§] Loss of α-catenin has been previously reported by Morton *et al.* (1992)

Since DU145 expressed approximately equal levels of all catenins and E-cadherin, densitometry values were normalized to DU145. Both LNCaP and DU145 expressed all catenins, consistent with a more differentiated phenotype. However, the catenin expression levels in both JCA1 and PC-3N cells showed dramatic differences. JCA1 lacked detectable β-catenin, and the level of α-catenin and plakoglobin was lower than in DU145. On the other hand, PC-3N cells showed reduced plakoglobin protein, and in agreement with Morton *et al.*(295) α-catenin was also not present.

Since the expression of the other catenins was abnormal in half of the prostate carcinoma cell lines studied, the expression of p120^{ctn} isoforms was assessed by immunoblotting. The p120^{ctn} monoclonal antibody used recognizes a common epitope in both the p120 and p100 isoforms. Both isoforms were present in all cell lines (Figure 2.2B). However, expression of p100 was lower in PC-3N and JCA1 as compared to DU145 (Table 1). This suggests expression of p120 and p100 is dependent on the type of cadherin present in the prostate cell lines. In the prostate carcinoma cell lines expressing only E-cadherin (DU145, LNCaP), p100 was the dominant isoform, approximately two folds higher than p120. In contrast, p120 was the dominant isoform in PC-3N and JCA1, which lack E-cadherin and express a different cadherin. In PC-3 cells, which display a mixed cadherin population, both p120 and p100 isoforms were equally expressed (Table 1).

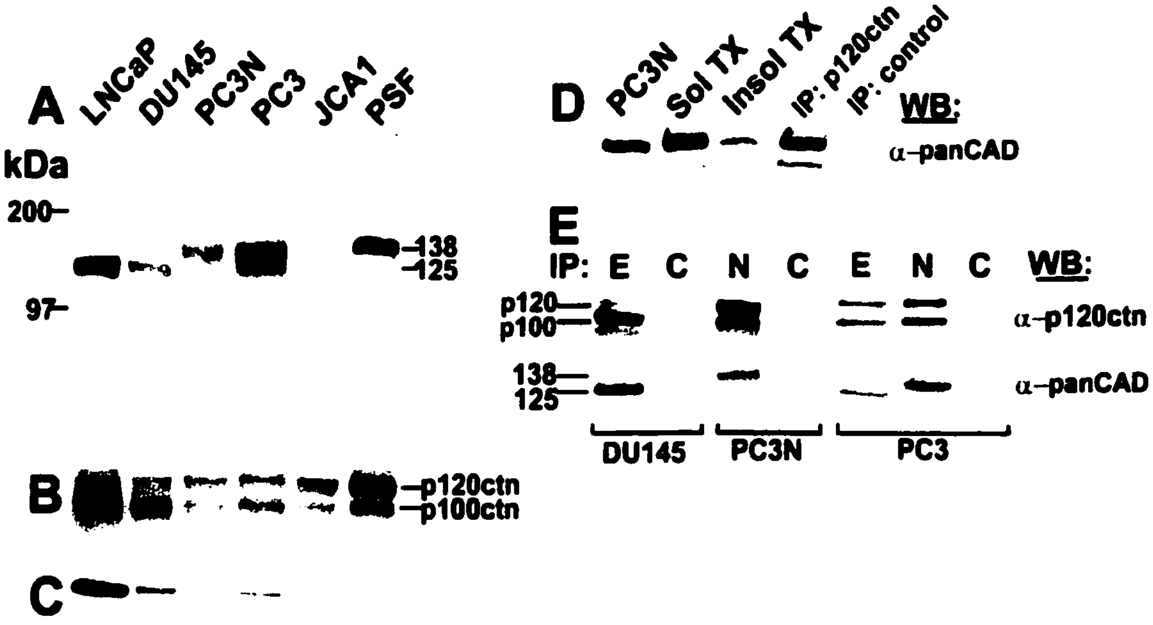
To identify the unknown cadherin in PC-3N, PC-3, and JCA1 carcinoma cells, we amplified cadherin cDNAs by RT-PCR using degenerate oligonucleotide primers based on well-conserved amino acid sequences of the cadherin cytoplasmic domain(347). A single cDNA band of approximately 150 bp was amplified from PC-3N cDNA, gel purified, subcloned and sequenced. The nucleotide sequence of 42% of the independent clones demonstrated 100% sequence identity with human N-cadherin(344). None of the remaining clones demonstrated homology to E-cadherin or any other cadherin. These results suggest that the cadherin (MW=138 kDa) detected in PC-3N cells with the anti-pan cadherin polyclonal antibody is N-cadherin.

p120^{ctn} isoform binds to N-cadherin PC-3N Cells.

We assessed whether N-cadherin was distributed in the Triton X-100 insoluble fraction of confluent PC-3N cells, which presumably reflects N-cadherin associated with the cytoskeleton (Figure 2.2D). Densitometric analysis showed approximately 25% of the N-cadherin was present in the detergent insoluble fraction of PC-3N cells (Figure 2.2D; lane 2 and 3), suggesting that N-cadherin maybe associated with proteins of the cytoskeleton. Immunoprecipitation of p120^{ctn} from detergent lysates of PC-3N was performed, transferred the immunoprecipitate to nitrocellulose, and blotted with a polyclonal pan-cadherin antibody. A cadherin of 138 kDa was detected in the p120^{ctn} immunoprecipitate, indicating the presence of N-cadherin (Figure 2.2D; lane 4). No cadherin band was detected in the non-immune control.

To further determine if the ratio of p100 to p120 isoforms was associated with a particular cadherin subtype, we immunoprecipitated PC-3, DU145, and PC-3N cells with monoclonal antibodies specific to E-cadherin or N-cadherin and immunoblotted for p120^{ctn} (Figure 2.2E). In DU145 cells, which express only E-cadherin, p100 was the predominant isoform. In PC-3N cells, which express only N-cadherin, p120 was the

Figure 2.2: Immunoblot analysis of E-cadherin and p120^{ctn} catenin in prostate carcinoma cell lines (LNCaP, DU-145, PC-3, PC-3N, JCA-1). Total cell lysates were extracted in 2X SDS-sample buffer, and 30 μg of protein/lane was analyzed by SDS-PAGE. After electrophoresis, proteins were transferred to nitrocellulose membranes and the filters were treated with either a pan cadherin polyclonal antibody which recognizes all classical cadherins (A), a mouse monoclonal antibody to p120^{ctn} which recognizes both isoforms, p120^{ctn} and p100^{ctn} (B), or a mouse monoclonal antibody to E-cadherin (C). Non-ionic detergent solubility was determined by treating cultured PC-3N cells with CSK buffer. Triton X-100 soluble and insoluble fractions were collected. Equivalent amounts of total protein fractions were analyzed by SDS-PAGE and immunoblotted for N-cadherin using a polyclonal pan-cadherin antibody (D). PC-3N cells were extracted and immunoprecipitated with anti-p120^{ctn} or normal mouse IgG. The immunoprecipitates were immunoblotted with anti-pan cadherin. Lane 1, PC-3N total protein lysate. Lane 2, soluble protein fraction of PC-3N. Lane 3, insoluble fraction of PC-3N cells. Lane 4, immunoprecipitation of p120^{ctn} and immunoblot of N-cadherin. Lane 5, control immunoprecipitation using mouse IgG antibody. (E) Immunoprecipitation of E- or N-cadherin in prostate carcinoma cell lines (DU-145, PC-3N, PC-3) using either a mouse monoclonal antibody to E-cadherin (E), a mouse monoclonal antibody to N-cadherin (N), or an irrelevant mouse IgG antibody (C). The immunoprecipitation fraction was separated on SDS-PAGE, transferred to a nitrocellulose membrane and immunoblotted with a monoclonal antibody to p120^{ctn} (α-p120ctn) or a polyclonal cadherin antibody (α-panCAD). All immunoblots were developed with a chemiluminescence detection reagent.


A
LNCaP
DU145
PC3N
PC3
JCA1
PSF
kDa
200
97
138
125
B
p120ctn
p100ctn
C
D
PC3N
Sol TX
Insol TX
IP: p120ctn
IP: control
WB:
α-panCAD
E
IP: E C N C E N C
WB:
p120
p100
α-p120ctn
138
125
α-panCAD
DU145
PC3N
PC3

predominate isoform. However, in PC-3 cells, which express both E- and N-cadherin, there were no differences in the binding of p100 vs. p120 in lysates immunoprecipitated with either E-cadherin or N-cadherin antibodies. This suggests that while both p120 and p100 isoforms bind N-cadherin and E-cadherin, the switch in the ratio of $p120^{ctn}$ isoforms in PC-3N and DU145 is due to differences in the isoform expression and not to differences in binding affinity.

Immunolocalization of N-cadherin in PC-3N cells.

N-cadherin was localized to sites of cell-cell adhesive contacts in confluent cultures of PC-3N cells (Figure 2.3A). The spindle-shaped PC-3N cells formed loose and extensive cellular contacts with their neighbors. The majority of N-cadherin immunoreactivity was localized in the cellular projections (arrows). In addition, catenin $p120^{ctn}$ immunolabeling was similar to that of N-cadherin (Figure 2.3B). $p120^{ctn}$ localized to similar regions as N-cadherin, with specific immunolocalization in the cellular projections. No E-cadherin immunoreactivity was observed in PC-3N as expected (Figure 2.3C). N-cadherin was also localized at cell-cell junctions in JCA1 and a sub-population of PC-3 (data not shown). Moreover, E-cadherin was localized in the cellular junctions of DU145, which showed an epithelial morphology (Figure 2.3D). E-cadherin expression is also similar in LNCaP cells, and no N-cadherin immunoreactivity was not detected in either DU145 or LNCaP cells (data not shown).

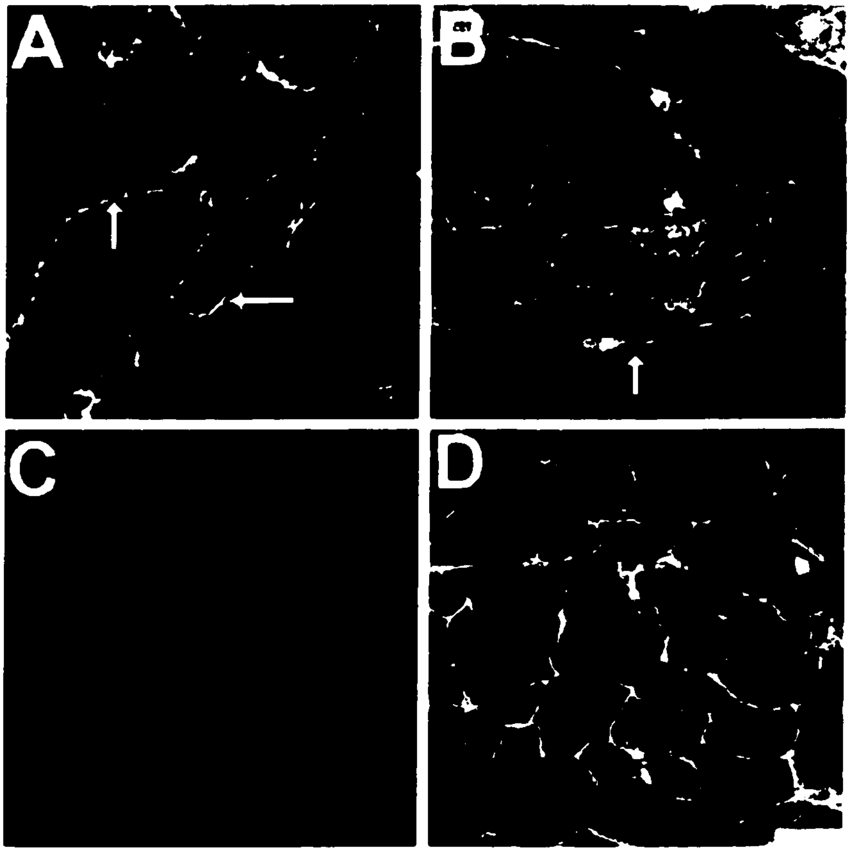


Figure 2.3: Immunolocalization of N-cadherin, E-cadherin and p120^{ctn} in PC-3N and DU-145 carcinoma cell lines. Cells were grown on coverslips until confluency, then fixed with 4% PFA in CMF-PBS and permeabilized with CSK buffer. Immunofluoresence was carried out using either a mouse monoclonal antibody to N-cadherin on PC-3N (A), mouse monoclonal antibody to p120^{ctn} on PC-3N cells (B), or mouse monoclonal antibody to E-cadherin on PC-3N (C) and DU-145 (E) cells. Rhodamine-coupled secondary antibody was used to reveal immunoreactivity, and photographs were taken by confocal microscopy. Arrows indicate cellular junctions and localization of N-cadherin and p120^{ctn} to cellular extensions. Bar = 40 μm.

N-cadherin mRNA is expressed in PC-3, PC-3N, JCA1 and Prostate Stromal Fibroblasts.

Differential expression of steady-state N-cadherin mRNA levels in the prostate carcinoma cell lines was determined by Northern analysis (Figure 2.4). Similar to N-cadherin protein data, a N-cadherin mRNA transcript (4.2 kb) was detected in PC-3, PC-3N and JCA1 cells, but was not detected in DU145 and LNCaP cells, even after long-term (1 week) exposure. In contrast, E-cadherin mRNA was expressed in LNCaP and DU145 cells, but not in PC-3N and JCA1 cells. Both E- and N-cadherin mRNAs were detected in PC-3 cells. Thus, in agreement with the protein expression, both PC-3N and JCA1 lack expression of E-cadherin, but alternatively express N-cadherin. N-cadherin mRNA, but not E-cadherin mRNA, was also detected in cultured prostate stromal fibroblasts (Figure 2.4).

Moreover, steady-state mRNA expression levels of both β-catenin and plakoglobin mRNAs were not consistent with the protein expression in prostate cell lines. All prostate lines expressed detectable levels of plakoglobin mRNA (Figure 2.4). However, while plakoglobin protein levels were lower in JCA1 than DU145 (Table 1), plakoglobin mRNA appeared to be three-fold higher in JCA1 vs. DU145. Moreover, equal levels of β-catenin mRNA were expressed by all prostate carcinoma cell lines, even though protein levels varied (Figure 2.4).

Immunolocalization of N-cadherin in prostate stromal fibroblasts in vitro and in situ.

Immunolocalization of N-cadherin showed that both PC-3N cells and prostate stromal fibroblasts (PSFs) expressed N-cadherin and formed N-cadherin adherens junction contacts at cell-cell borders (arrow, Figure 2.5). Figure 2.5A shows that the

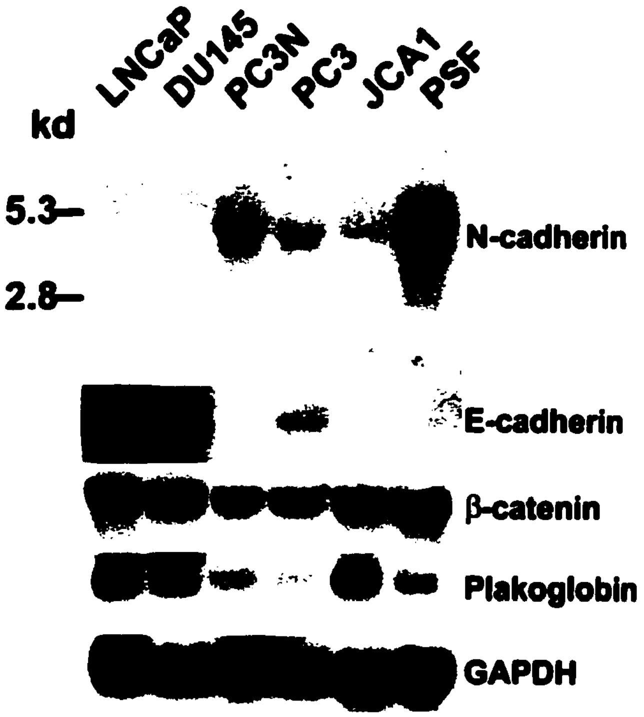

LNCaP
DU145
PC3N
PC3
JCA1
PSF
kd
5.3
2.8
N-cadherin
E-cadherin
β-catenin
Plakoglobin
GAPDH

Figure 2.4: Northern blot analysis of E- and N-cadherins and plakoglobin in prostate carcinoma cell lines and prostate stromal fibroblasts. Twenty μgs/lane of total RNA from each cell line was blotted on a nylon membrane. A 300 bp EcoRI fragment to N-cadherin was used as a probe for N-cadherin detection. A 1.7 kb fragment to mouse E-cadherin was used as a probe for E-cadherin expression. Full-length plakoglobin and β-catenin cDNA were used to detect plakoglobin and β-catenin expression. A 1.2 kb GAPDH cDNA fragment was used as normalization standard. The hybridized membranes were exposed to x-ray films for 1 day (E-cadherin, β-catenin, plakoglobin, GAPDH) and 2 days (N-cadherin). This is a representative result of two independent experiments.

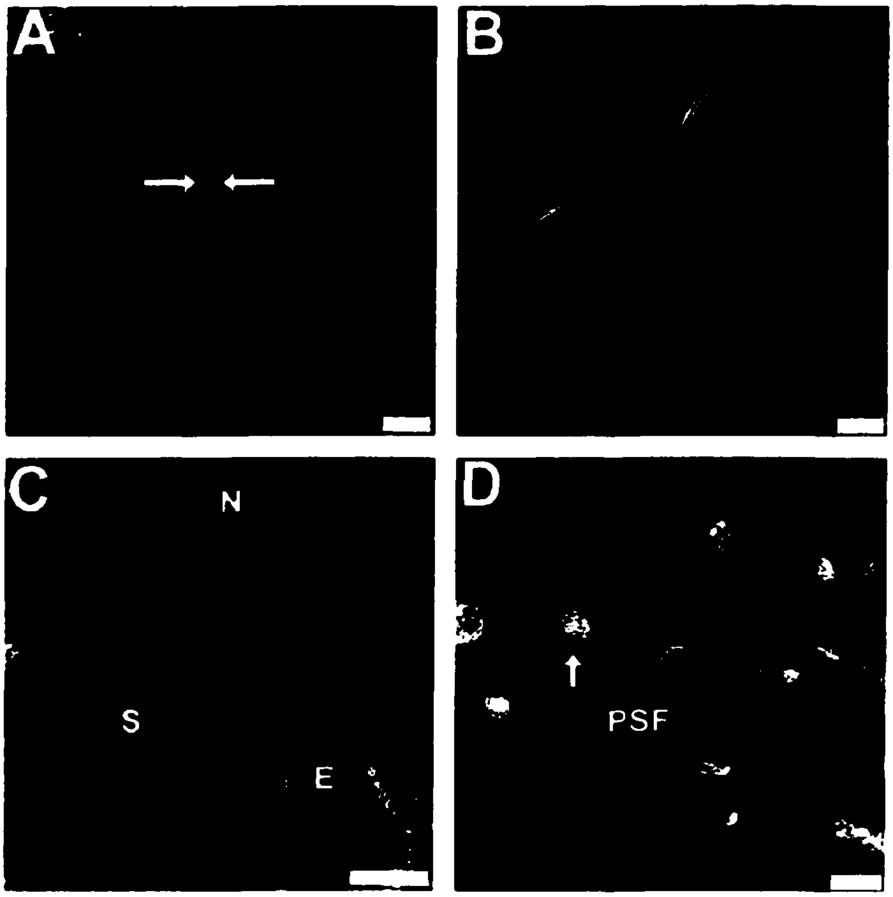
expression of N-cadherin in cell-cell junctions of PSFs maintained *in vitro*. All cells were positive for N- cadherin and α-smooth muscle actin, which confirms the presence of only PSFs in the culture(353, 354) (Figure 2.5B). In addition, N-cadherin was immunolocalized in frozen sections of normal human prostate tissue. Immunoreactivity of N-cadherin in the prostate tissue sections was strongly detected in stromal cells, and in nerve bundles that penetrate the gland, but N-cadherin was not detected in normal prostate epithelial glands as shown by co-immunolocalization with antibodies to keratin 18 (Figure 2.5C).

PC-3N prostate carcinoma cells and prostate stromal fibroblasts were co-cultured to determine if N-cadherin was localized at sites of cell-cell contact. Stromal fibroblasts were cultured overnight before fluorescent dye DiO labeled PC-3N cells were added, and cultured for an additional 24 hours. Immunolocalization of N-cadherin showed that there were sites of N-cadherin immunoreactivity at cell-cell contacts between PC-3N cells and stromal fibroblasts (arrow; Figure 2.5D). There was no immunostaining detected in samples incubated with secondary antibodies alone (data not shown).

Cell-cell aggregation of PC-3N cells and PSFs is mediated by N-cadherin

To examine whether the interaction between prostate carcinoma cells and prostate stromal fibroblasts was mediated by N-cadherin, a cell-cell aggregation assay(349) was performed. After dissociation of PC-3N cells by trypsinization in the presence of calcium into a single cell suspension, PC-3N cells labeled with DiO were mixed with unlabeled PC-3N cells and allowed to aggregate in the presence of calcium (Figure 2.6). The calcium-dependent aggregation of PC-3N cells was time dependent and blocked in the

Figure 2.5: Cellular localization of N-cadherin in prostate stromal fibroblast in vitro (A,B,D) and in situ (C). PSF cells were grown on a coverslip until confluency. Cells were fixed with 4% PFA in CMF-PBS, permeabilized with CSK buffer and reacted with a mouse monoclonal antibody to N-cadherin (A) or mouse monoclonal antibody to α-smooth muscle actin (B). Arrow indicates N-cadherin mediated cellular junctions. (C) N-cadherin immunoreactivity in stromal fibroblasts of normal prostate tissue as indicated by S. Fresh-frozen normal prostate tissues were sectioned at 6 µm and fixed with cold acetone. Co-expression of epithelial high molecular weight keratin and N-cadherin was performed by co-immunoreacting the tissue with both monoclonal antibody to N-cadherin and polyclonal keratin antibody (clone 18A). N-cadherin was detected by rhodamine-coupled secondary antibody while keratins were detected by FITC-conjugated secondary antibody. N indicates N-cadherin expression in nerve and E indicates keratin 18 in a normal epithelial gland. (D) Expression of N-cadherin in co-culture of PC-3N and PSF. PC-3 cells were labeled with 40 µg/ml of DiO (green membrane-labeled cells) before seeded on top of PSF cultured coverslip for 24. The arrow represents localization of N-cadherin in the cell-cell borders between PC-3N and PSF cells. Images were captured by confocal microscopy. Bar = 60 µm (A,B,D) and 100 µm (C).


A
B
C
N
S
E
D
PSF

absence of Ca^{2+}. Addition of function blocking antibodies to N-cadherin inhibited the calcium-dependent aggregation of PC-3N carcinoma cells (data not shown).

The homotypic interactions between PC-3N prostate carcinoma cells and stromal fibroblasts were also mediated by N-cadherin. Equivalent numbers of prostate stromal fibroblasts were mixed with PC-3N cells labeled with DiO. Large cell-cell aggregates of PC-3N cells and PSF cells were observed in the presence of calcium (Figure 2.6). The calcium-dependent cell-cell aggregates consisted of all possible cell interactions: PC-3N/PC-3N, PC-3N/PSF, and PSF/PSF. This calcium-dependent cell-cell aggregation was largely abrogated in the presence of functional blocking antibody to N-cadherin. No aggregation was observed between DU145/PSF cells and DU145/PC-3N cells (data not shown). These finding demonstrate that homotypic interaction of N-cadherin mediates the interaction between the PC-3N prostate carcinoma cell line and prostate stromal fibroblasts.

Discussion

The results of this study demonstrate expression of N-cadherin in human prostate carcinoma cell lines (PC-3N and JCA1) that lack E-cadherin, and in PC-3 cells, which have a mixed expression of E-cadherin and N-cadherin. In addition, the expression of N-cadherin in prostate stromal fibroblasts was found both *in vitro* and *in situ*. Co-aggregation of the prostate stromal fibroblasts with PC-3N carcinoma cells indicates that N-cadherin can mediate homotypic adhesion between these two cell populations. Prostate carcimona invasion proceeds through stroma(188) with subsequent perineural migration

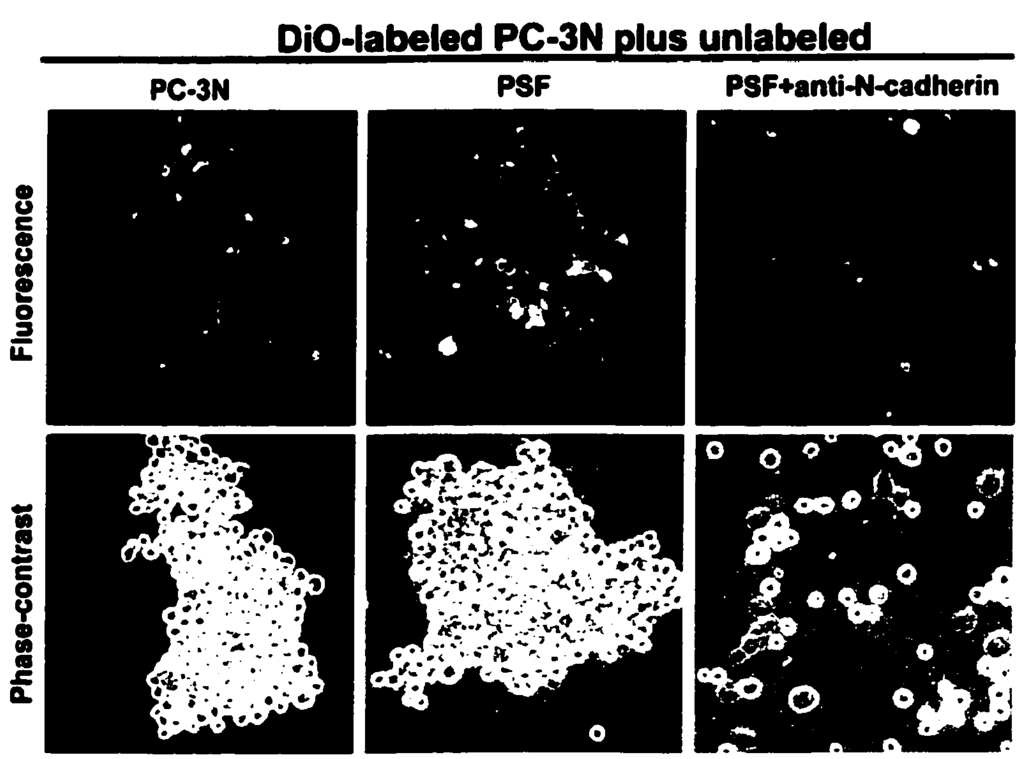

DiO-labeled PC-3N plus unlabeled
PC-3N
PSF
PSF+anti-N-cadherin
Fluorescence
Phase-contrast

Figure 2.6: Cell-cell aggregation assay of PC-3N and prostate stromal fibroblasts. To distinguish PC-3N from PSF cells, PC-3N cells were labeled with 40 μg/ml of DiO for 1 hr. All cells were trypsinized in the presence of 2 mM Ca^{2+} to prevent destruction of cadherins. Labeled PC-3N cells were mixed in an aggregation assay with either unlabeled PC-3N or PSF in the presence of Ca^{2+}. Additionally, labeled PC-3N and PSF cells were mixed in an aggregation assay in the presence of anti-N-cadherin blocking antibody (N=3). Cells were allowed to aggregate for 1 hr at 37°C on a gyrator shaker. Pictures of cell aggregation were taken both under fluorescence (top row) and phase contrast (bottom row) within same fields. Bar = 100 μm

and penetration of the capsule and escape from the prostate gland(314, 317). These data suggest that the presence of N-cadherin in stromal cells surrounding glandular epithelium and in nerve bundles extending into the prostate could facilitate prostate carcinoma cell invasion, and extracapsular metastasis. Furthermore, the less differentiated prostate cell lines that expressed only N-cadherin, PC-3N and JCA1, also demonstrated a shift in isoform of the cadherin associated catenin, p120^{ctn}, a major substrate for *src* kinase and tyrosine kinase growth factor receptors(244). Intercellular communication between stromal and carcinoma cells, through cell-cell adhesion molecules and growth factors, have been shown to be important factors in neoplastic progression(355, 356). Umbas *et al.*(300) found that sixty-three percent of prostate tumors that extended beyond the prostate capsule had decreased E-cadherin expression compared to 33% of the tumors confined to the prostate(300).

Associated with the expression of N-cadherin in prostate carcinoma cell lines was the shift in p120^{ctn} isoform expression of p120/p100. These p120^{ctn} isoforms are generated by alternative splicing at the NH_2-terminal end of p120^{ctn}(244, 357, 358). Immunoprecipitation of PC-3, PC-3N and DU145 cells indicated that although both p120 and p100 isoforms can bind E- and N-cadherin, the shift is a result of an increase in steady state isoform protein level. In addition, high levels of the p120 isoform were also reported in cells that are highly motile, such as fibroblasts, whereas p100 is more abundant in epithelial cells(357). p120^{ctn} is localized in the cellular extensions of PC-3N cells, and immunoprecipitated N-cadherin with a monoclonal antibody against p120^{ctn} using conditions that preserve cadherin/catenin interactions. As p120^{ctn} modulates cadherin adhesion, the differences in isoform expression may be important in the regulation of cadherin adhesion and metastatic potential of tumors. Reynolds *et al.* (1996)(359) demonstrated that p120^{ctn} was associated with all classical cadherin subtypes, and that over-expression of the p120 isoform in fibroblasts leads to changes in cellular morphology with development of dendrite-like extension, with p120 isoform localized in these extensions.

The down-regulation of E-cadherin and/or catenins is a critical step for the progression of epithelial tumor invasion and metastasis(360-362). In prostate adenocarcinomas, the aggressiveness of the tumor has been related to loss of E-cadherin(300). The mechanism by which E-cadherin expression was lost may be by mutation(363), deletion(364), hypermethylation(362), and/or lost or altered catenin expression(352). In this chapter the results demonstrate that the loss of E-cadherin in prostate cancer cell lines is accompanied by an unexpected expression of another classical type I cadherin subtype, N-cadherin. While human E-cadherin has been mapped to chromosome 16q22.1(365) and is frequently deleted in prostate cancer(366, 367), N-cadherin has been mapped to human chromosome 18q11.2(368).

N-cadherin expression was not detected in normal prostate glandular epithelium, but it is found in neurons, and stroma of the prostate. N-cadherin is found in a wide variety of cell types including neurons, skeletal and cardiac myocytes, fibroblasts, mesothelial cells and some neoplastic epithelial cells(305, 369-372). Although growth factors and extracellular matrix are important contributors to prostate tumor progression, N-cadherin mediated prostate carcinoma-stroma interaction may promote metastasis.

Stromal mesenchymal-epithelial interactions reciprocally mediate the embryonic development and differentiation of the prostate(373). In addition, prostate fibroblasts co-inoculated in athymic mice with prostate carcinoma cells have been found to accelerate tumor growth (355, 356). The role of N-cadherin in epithelial derived tumor cell invasion is not restricted to prostate carcinomas. Islam *et al.*(306) reported that there was an inverse expression of N- and E-cadherin in squamous cell carcinomas, and cells expressing high levels of N-cadherin were more invasive.

N-cadherin homotypic adhesion functions in distinct roles in different cell types. N-cadherin plays an important role in maintaining strong cell-cell adhesion in certain non-motile cell types, such as in the intercalated discs of the myocardium(304). In contrast, N-cadherin also plays an adhesive role in the dynamic growth of neurites, and the expression is also spatially diffuse throughout the cell surface of the neuron body(374). Similar to neurons, N-cadherin localization in PC-3N carcinoma cells appears to be spatially diffuse and highly expressed in cellular extensions. A majority of the N-cadherin molecules are expressed diffusely throughout the cell membrane, and only a fraction of the N-cadherin molecules is concentrated at sites of cell-cell contact. This is similar to the reported distribution of N-cadherin(375) in neural crest cells migrating from the neural epithelium.

The expression of N-cadherin in invasive prostate carcinoma cell lines may be indicative of an epithelial/mesenchyme transition. In prostate carcinoma, the transformation of epithelium to invasive mesenchyme appears to involve a number of events in which certain carcinoma cells undergo the loss and gain of functions, including cell-cell and cell-extracellular matrix interactions. Loss of E-cadherin and certain integrins are associated with loss of epithelial differentiation in prostate carcinoma(317, 376). This loss may lead to a gain of other adhesion molecules that may advance the development and aggressiveness of prostate carcinoma, as indicated by alterations in N-cadherin and catenin expression in the present studies. Further in prostate carcinoma, loss of β_4 integrin with high expression of α_6 and β_1 integrins is associated with high invasive activity, implicating the heterodimer $\alpha_6\beta_1$ integrins as leading candidates for conferring the invasive phenotype(377).

The stable adhesion for cadherins requires the homotypic protein-protein binding of the extracellular domains, and in addition, the cadherin cytoplasmic domain forms a complex with the actin cytoskeleton (Figure 2.7A). Since α-catenin has actin binding activity, it probably links the cadherin/catenin complex to the actin cytoskeleton(378) and plays an important role in formation of a tight epithelial morphology(379). The armadillo family members β-catenin and γ-catenin/plakoglobin function as an intermediate in linkage to α-catenin and the carboxy terminal cadherin cytoplasmic domain(380). $p120^{ctn}$ binds in the juxtamembane region(381) of the cadherin cytoplasmic domain and is likely to function in a different role than the other catenins.

This work suggests that while expression of N-cadherin may in part play a role in the progression of prostate carcinoma from epithelium to mesenchyme. It is likely that N-cadherin mediates a less stable cell-cell adhesion and may allow for carcinoma cell invasion and stromal interactions (Figure 2.7B). Expression of N-cadherin in normal epithelial cells results in down regulation of E-cadherin expression and a scattered mesenchymal phenotype(306). The PC-3N cell line has a spindle shaped mesenchymal morphology, which expresses N-cadherin at sites of cell-cell contact. Since α-catenin is absent(295) the N-cadherin adhesion between PC-3N cells is likely to be less stable. Our results show that N-cadherin mediates adhesion between α-catenin deficient PC-3N cells and stromal fibroblasts, which contain normal levels of all of the catenins. N-cadherin in PC-3N cells may regulate the cellular outgrowth through cell-cell interactions, which may allow PC-3N to interact with surrounding prostate stromal fibroblasts. The adhesion by N-cadherin may explain the PC-3N invasive phenotype in the diaphragm striated muscle of xenograft tumors in SCID mice. Both PC-3N cells and the myocytes of the diaphragm express N-cadherin potentially allowing the two cell populations to establish homotypic interactions. The future direction is to determine whether N-cadherin is expressed in high-grade prostate carcinomas associated with capsular penetration through the perineural space, and is associated with metastasis.

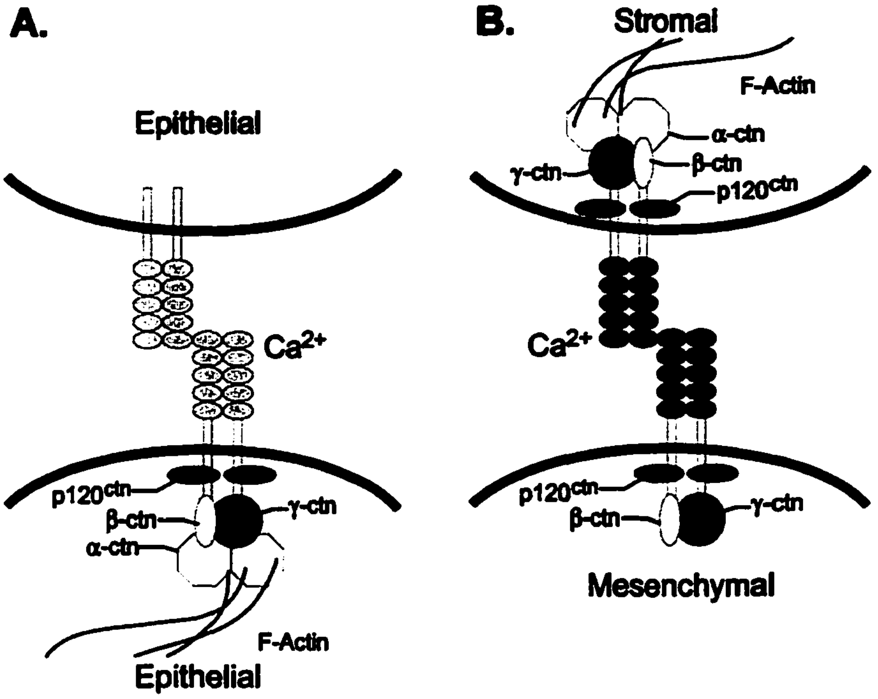

A.
Epithelial
Ca2+
p120ctn
β-ctn
γ-ctn
α-ctn
F-Actin
Epithelial
B.
Stromal
F-Actin
α-ctn
γ-ctn
β-ctn
p120ctn
Ca2+
p120ctn
β-ctn
γ-ctn
Mesenchymal

Figure 2.7: Two forms of cadherin cell-cell adhesion in human prostate carcinoma. E-cadherin mediated epithelial cell-cell adhesion (A) and N-cadherin mediated stromal:mesenchymal cell adhesion (B) are depicted. The cadherins are shown as a dimer. The armadillo catenins $p100/p120^{ctn}$, β-catenin (β-ctn) and γ-catenin/plakoglobin (γ-ctn) anchor E-cadherin to α-catenin (α-ctn), which links the complex to the peripheral actin cytoskeleton. In panel A stable cadherin homotypic adhesion is depicted. Panel B shows the known components of the N-cadherin/catenin complex in PC-3N carcinoma cells, which preferentially expresses the $p120^{ctn}$ isoform, and stromal fibroblasts. This cell-cell interaction is likely to be less stable and have a weaker affinity with the loss of α-catenin.

CHAPTER 3: SIGNALING FROM N-CADHERIN INCREASES BCL-2: REGULATION OF THE PHOSPHATIDYLINOSITOL 3-KINASE/AKT PATHWAY VIA ASSOCIATION WITH THE ACTIN CYTOSKELETON[2]

Introduction

The cadherins constitute one of the major families of structural transmembrane cell adhesion receptors and have an important functional role in development and maintenance of tissue integrity(84). Functional adhesion by cadherins requires both the coordinated homophilic binding of the extracellular domain and the cadherin cytoplasmic domain with the actin cytoskeleton(382). Calcium binds to cadherin extracellular domains inducing a conformation that initiates and stabilizes the binding of cadherin subtypes on adjacent cells(383, 384). The conserved cadherin cytoplasmic domain is associated with the cytoplasmic plaque of actin microfilaments through the catenins(385). Either β-catenin or γ-catenin binds the complex to α-catenin, which either interacts directly with the peripheral actin cytoskeleton, or indirectly through actin associated proteins, such as vinculin or α-actinin(386, 387). In addition, $p120^{ctn}$ binds to the juxtamembrane region of cadherins and controls the strength of cadherin-mediated adhesion(381).

Although cadherins and catenins lack intrinsic enzymatic activity, they can associate with kinases and phosphatases in adherens junctions(388-390). Homophilic E-cadherin adhesion initiates the activation of phosphatidylinositol 3-kinase (PI 3-kinase) followed by down-stream activation of Akt/protein kinase B(275). The serine/threonine kinase

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Akt is an important regulator of metabolic pathways, as well as apoptotic pathways. Akt is activated in response to diverse extracellular stimuli, such as EGF and other growth factors and integrin adhesion(168). Akt activity is regulated through the production of phosphatidylinositol 3,4 biphosphate and phosphatidylinositol 3,4,5-triphosphate by PI 3-kinase, which bind to the pleckstrin homology domain of Akt altering its conformation at the plasma membrane(168). For full activation, Akt requires phosphorylation on Threonine-308 (Thr-308) and Serine-473 (Ser-473) by 3-phosphoinositide-dependent kinase 1 (PDK1)(178) or the phosphoinositide-dependent kinase 2/integrin linked kinase(391). Phosphorylation of Thr-308 occurs when Akt is recruited to the membrane, but is not sufficient for activation of kinase activity, which requires phosphorylation of Ser-473(175).

Phosphorylated Akt can phosphorylate several substrates that result in suppression of apoptosis(168). It is proposed that upon exposure to apoptotic signals, cell fate is determined by the relative balance between pro- and anti-apoptotic protein interactions of the Bcl-2 family(158). Members of this family include the pro-apoptotic proteins Bad, Bik, Bid, and the anti-apoptotic cell survival proteins Bcl-2 and Bcl-xL(156). While homodimers of Bcl-2 in the mitochondrial membrane prevent the activation of caspase-9, heterodimerization of Bcl-2 and Bad induces the activation of caspase-9 and initiates an irreversible pathway(392). One mechanism by which Akt prevents apoptosis is through the phosphorylation of the pro-apoptotic protein Bad. Akt phosphorylates Bad on Ser-136, and inhibits the heterodimer formation of Bad and Bcl-2 in the mitochondrial membrane(167). Phosphorylated Bad is sequestered in the cytoplasm by interacting with scaffolding 14-3-3 proteins, which in turn suppresses apoptosis(393).

Among the molecular alterations that facilitate prostate cancer progression and the development of androgen-independence is the increased resistance to apoptosis(29). E-cadherin is essential for adhesion of glandular epithelium to each other, and homophilic cell-cell adhesion results in formation of adherens junctions, which in turn regulates differentiation, growth, and survival within the tissue microenvironment(394). Decreased expression of E-cadherin is an important factor in the regulation of carcinoma invasiveness and metastasis(395). The dysregualtion of cell adhesion in prostate cancer is, in part, facilitated by the gain in expression of N-cadherin and members of the integrin family(302, 303). Evidence indicates that disruption of cadherin adhesion can initiate programmed cell death (apoptosis) in both normal and cancer cells(396, 397) similar to the inhibition of integrin adhesion to extracellular matrix ligands(398). Several studies have partly delineated the molecular mechanisms that link integrin-mediated signal transduction pathways to the intracellular apoptotic machinery, but the mechanism whereby cadherin adhesion contributes to cell survival is poorly understood.

In the present study, we investigated whether N-cadherin-mediated cell adhesion could initiate outside-in signal transduction that may account for prostate carcinoma survival. N-cadherin is upregulated in dedifferentiated, invasive prostate carcinomas lacking E-cadherin expression(302, 303). N-cadherin expression is reported to induce a motile scattered phenotype in breast carcinomas(272) and squamous cell carcinoma(399). Through Ca^{2+}-dependent modulation of N-cadherin-dependent adherens junctions, this chapter provide evidence that N-cadherin engagement can induce an outside-in signal transduction pathway. The results demonstrate that N-cadherin homophilic adhesion recruits PI 3-kinase to the N-cadherin/catenin/actin complex and results in an increased Bcl-2/Bax expression level. Overexpression of Bcl-2 has been observed in androgen-independent prostate adenocarcinomas, which correlated with the presence of metastases(400, 401). These findings suggest that the gain of N-cadherin expression by prostate carcinomas is a regulator of cell survival through stabilization of Bcl-2 in advanced stages of metastasis.

Materials and Methods

Cell Culture—PC-3 (American Type Culture Collection; ATCC), PC3N(303) and DU-145 human prostate carcinoma cell lines (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS; Hyclone Laboratories, Inc., Logan, UT) and penicillin/streptomycin at 37°C in 5% CO_2 atmosphere at constant humidity. PC3N cells, which only express N-cadherin, were derived from PC-3 carcinoma cell line, which expresses both N-cadherin and E-cadherin(303).

Expression Plasmids and Transfection--An expression vector for hemagglutinin- (HA) tagged AKT [pCMV6-AKT-HA (WT)] and K179M kinase deficient AKT [pCMV6-AKT-HA (KD)] was a gift from Dr. Gabriel Nunez (U. of Mich, Ann Arbor, MI)(185). PC3N cells in 60 mm cultured dishes were grown to 70% confluence and transfected with 4 μg of either HA-tagged AKT or kinase-deficient AKT(KD) HA-tagged construct using

10 μg of DOTAP Liposomal Transfection Reagent (Roche Molecular Biochemicals; Indianapolis, IN) in OptiMEM according to the manufacture's protocol. After 16 hrs, the transfected cells were placed in DMEM without serum for additional 16 hours prior to analysis. Transfection efficiency of PC3N cells with DOTAP was found to be 80% (data not shown).

A full-length cDNA encoding Bad was constructed using RT-PCR. Total RNA isolated from a 15-day mouse embryo was used to prepare cDNA with Superscript Reverse Transcriptase II (Gibco/BRL) and random hexamer primers. In a PCR reaction to obtain the full-length Bad the following primers were used: sense 5'-GCC TCC AGG ATC CAA ATG GGA ACC-3' containing a *Bam*H1 endonuclease site and antisense, 5'-TCC GGG ATG TCG ACC AGA AGA TCA CTG-3' containing *Sal*1 endonuclease site. The resulting PCR product was subcloned into pGEM-T (Promega, Madison, WI) and sequenced. Subsequently, the Bad cDNA was excised from pGEM-T with *Bam*H1 and *Sal*1 and ligated into pGEX-5X-1 digested with *Bam*H1 and *Sal*1. The Bad/pGEX-5X-1 plasmid DNA was transformed into the E. *coli* strain, BL21(DE3)LysS for production of recombinant GST-Bad. The Lamin/pGEX-5X-1 was constructed by excising the lamin cDNA from lamin/pBTM116 generously provided by Dr. Anne Vojtek (402). Lamin cDNA was excised from pBTM116 using *Eco*R1 and *Sal*1 endonuclease sites and ligated into pGEX-5X-1 cut with the same enzymes. Lamin/pGEX-5X-1 was transformed into the *E. coli* strain, BL21(DE3)LysS for production of recombinant GST-lamin. Purification of recombinant GST-Bad and GST-lamin was carried out according to the manufacture's protocol using Glutathione Sepharose 4B beads (Pharmacia, Peapack, NJ).

Antibodies and Immunoblotting —A mouse monoclonal antibody against the extracellular domain of N-cadherin (A-CAM clone GC-4; St. Louis, MO), human vinculin monoclonal antibody (hVN1) and non-immune mouse IgG were purchased from Sigma Chemical Co. Antibodies to N-cadherin, β-catenin, p120^{ctn}, and Bcl-2 were purchased from Transduction Laboratory (Lexington, KY). Rabbit polyclonal phospho-specific antibodies to Akt Ser-473 and Bad Ser-136, and rabbit polyclonal antibodies to Akt and Bad, and were purchased from New England Biolabs (Beverly, MA). Rabbit polyclonal antibodies against the p85 regulatory subunit of PI 3-kinase were from Upstate Biotechnology Inc. (Lake Placid, NY). A monoclonal E-cadherin antibody was obtained from Zymed Laboratories (clone HECD-1; South San Francisco, CA). Poly(ADP-ribose)polymerase (PARP) antibodies were purchased from Enzymes System Products (Livermore, CA). Bax antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). A transferrin receptor monoclonal antibody was obtained from Becton Dickenson (Flanders, NJ). Anti-mouse IgG and anti-rabbit IgG antibodies coupled to horseradish peroxidase were purchased from Promega (Madison, WI). Mouse monoclonal antibody to hemagglutinin (clone 12CA5) was purchased from Roche Molecular Biochemicals.

Immunoblotting and protein determination experiments were performed as described previously(303). Briefly for immunoblotting, cells were lysed in 2X SDS-Sample Buffer (0.25M Tris-HCl pH 6.8, 2% SDS, 25% glycerol) containing 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.7 μg/ml pepstatin, 20 mM NaF, 20 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride

(PMSF). Protein concentrations were determined using the BCA assay procedure (Pierce Chemical Co., Rockford, IL)(403), with bovine serum albumin as a standard. Thirty micrograms of total cellular protein were loaded per lane and separated by SDS-PAGE(404). After transfer at 4°C, the nitrocellulose (Schleichter & Schuell, Keene NH) was blocked with 5% non-fat Milk in Tris-buffered saline pH 8.0 with 0.1 % Tween-20 (TBST) prior to addition of by primary antibodies and followed with peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG. For detection of phosphorylated proteins, antigens were detected with phospho-specific antibodies diluted in 5% BSA in TBST at 4°C overnight, washed, and followed by peroxidase-conjugated anti-rabbit IgG. Protein bands were identified by chemiluminescence (NEN, Boston, MA) and exposed on X-OMAT AR film (Kodak, Rochester, NY). Images of immunoblots were captured and quantitative densitometry for protein bands was carried out using Scion Image (Scion Corporation, Frederick, MD). The results shown are the mean of three independent experiments, and each exposed film was quantitated twice.

Cell-Cell Adhesion Survival Assay—Cells were grown to 75% confluence, deprived of serum for 24 hours, and detached from the culture dishes with 4 mM EDTA in Calcium Magnesium-Free PBS (CMF-PBS) to preserve cells surface expression of cadherin subtypes. Cells were dissociated thoroughly to single cells by trituration 10 times with a Pasteur pipette. After centrifugation the cells were suspended in a final volume of 0.5 ml in calcium-free Suspension Modified Eagle's Medium (SMEM; Gibco/BRL) in the absence of serum and 1 X 10^6 cells were maintained in suspension on 25 μg/ml Poly-HEMA (Poly[2-hydroxyethylmehtacrylate]; Sigma Chemical Co.) coated 2 cm^2 culture dishes to prevent cell attachment. Cells were compared in the presence of Ca^{2+} (1.8 mM) to induce cell-cell aggregation or without Ca^{2+} to prevent cell-cell adhesion. At various times, the cells were washed once in phosphate-buffered saline (PBS) and the cell pellet was lysed in 2X SDS Sample Buffer, equivalent protein was separated on a 7% SDS-PAGE, and analyzed for PARP cleavage by immunoblotting.

Calcium Switch Assay(384)—Confluent culture cells were deprived of serum overnight, and N-cadherin mediated cell-cell contacts were disrupted by treatment with 4 mM EGTA in DMEM for 40 min at 37°C. The calcium-free medium was removed and N-cadherin adherens junctions were allowed to re-establish by addition of DMEM medium, which contains 1.8 mM [Ca^{2+}]. In the Calcium Switch experiments the time of addition of Ca^{2+} was considered as 0 min. Following calcium restoration at 37°C, cells were harvested and lysed in 2X SDS-Sample Buffer containing 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.7 μg/ml pepstatin, 20 mM NaF, 20 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1 mM PMSF. In individual experiments, a N-cadherin monoclonal antibody, wortmannin (50 nM; Tocris Cookson, Inc., Ballwin, MO), LY294002 (Calbiochem; San Diego, CA) or cytochalasin D (Sigma) were included in the medium for pretreatment of cells.

Antibody Immobilization—Bacteriological 60 mm petri dishes were coated for 2 h at 37°C with goat anti-mouse FAB fragments (50 μg/ml) in 100 mM $NaHCO_2$ pH 9.6 (final volume 1.5 ml). Plates were rinsed and then incubated overnight with mAbs specific for N-cadherin or E-cadherin (10 μg/ml) at 4°C and blocked with heat-denatured 1% BSA in PBS, pH 7.4. Serum starved PC3N cells were harvest with EDTA treatment, washed in

CMF-PBS, and resuspended in two ml of serum-free medium without calcium (SMEM). Cells ($1X10^6$) were permitted to adhere to the antibody-coated dishes at 37°C or poly-L-Lysine (Sigma) coated dishes as control after which nonadherent cells were removed with washing. Attached cells were lysed, and subjected to immunoblot analyses as described above.

Immunoprecipitation and GST-Bad Pull Down— For immunoprecipitation, cells were lysed 0.5% on ice for 10 min in a buffer containing 10 mM Tris-HCL (pH 7.4), Nonidet P-40 (NP-40), 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 2 mM sodium vanadate, 10 μg/ml aprotinin and 10 μg/ml leupeptin (Sigma Co.). Equivalent amounts of protein (500 μg) were immunoprecipitated from the lysates, washed with lysis buffer followed by S1 buffer (10 mM HEPES, pH 7.4, 0.15 M NaCl, 2 mM EDTA, 1.5 % Triton X-100, 0.5% deoxycholate, 0.2% SDS)(405). Samples were then resuspended in 2 X SDS Sample Buffer and boiled in the presence of 2-mercaptoethanol (Sigma), separated by SDS-PAGE, transferred to nitrocellulose overnight at 4°C, and proteins were detected as described above.

For Akt co-precipitation with Bad, cell extracts from confluent cells were prepared as described above. Pre-clearing was first carried out with agarose conjugated GST at 4°C for 1 hr. Supernatants were then incubated with an agarose conjugated GST fusion protein containing full-length Bad for 4 hr at 4°C. The GST-Bad sepharose beads were washed five times with 1 mL lysis buffer, and the precipitated proteins were boiled in SDS Sample Buffer. The samples were separated on SDS-PAGE and transferred onto nitrocellulose membrane.

Immunofluoresence Microscopy—Cells were grown on glass coverslips to confluence, fixed for 5 min in 4% paraformaldehyde in PBS. Cells were then permeabilized with 0.5% Triton X-100 in 10 mM PIPES, pH 6.8, 50 mM NaCl, 3 mM $MgCl_2$, 0.3 M sucrose for 5 min at 4°C. After washing with PBS, cells were blocked with 2% bovine serum albumin and 1% goat serum, and incubated with primary antibody for 1 h at 25°C. Following washing with 0.1% BSA in PBS and incubation with Cy3-conjugated anti-mouse IgG or FITC-conjugated anti-rabbit, cell nuclei were stained with bis-Benzimide (BBI; Molecular Probes, Inc., Eugene, OR) in PBS for 5 min, washed, and mounted in 2% n-propyl gallate/90% glycerol, pH 8.0. Immunofluorescent samples were examined under laser scanning confocal microscope, LSM 410, equipped with He, Ne and argon lasers (Zeiss) using the appropriate filters. Images were processed using Adobe Photoshop.

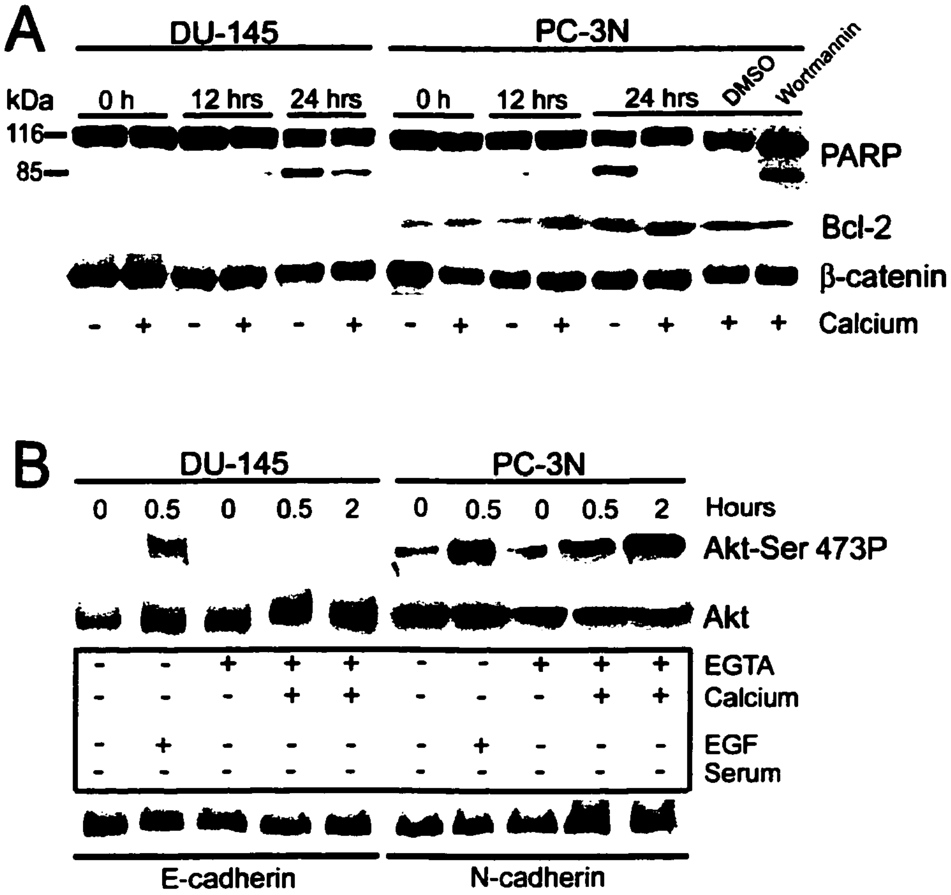
Results

Ca^{2+} Dependent Cell Adhesion Inhibits Programmed Cell Death

Previous studies have indicated that E-cadherin homophilic adhesion could regulate cell cycle progression and cell survival during anchorage independent growth(397, 406). To further examine the mechanism of cadherin adhesion in cell survival signal transduction, human prostate carcinoma cell lines were cultured in serum-free anchorage-independent conditions. By varying the concentration of extracellular calcium in the culture medium DU-145 and PC3N cells were cultured in suspension as single cells in low calcium containing medium, or allowed to form multicellular aggregates in the presence of a normal physiological concentration of calcium. Apoptosis in the suspension cultures of single cells or multicellular aggregates was assessed at different time points by immunoblot analysis examining poly(ADP-ribose)polymerase (PARP) proteolytic cleavage (Figure 3.1A). PARP is cleaved by caspase 3 between Asp-213 and Gly-214 to produce two fragments of apparent molecular weights of 29-kDa and 85-kDa(407). When cultured as single cells in the absence of integrin-mediated adhesion, PARP cleavage was detected by the appearance of an 85-kDa fragment at 12 hours in both DU-145 and PC3N cells. At 24 hours the level of 85-kDa proteolytic fragment of PARP in DU-145 and PC3N was increased and determined to represent approximately 40% cleavage of PARP (Figure 3.1A). Ca^{2+}-dependent multicellular aggregates of PC3N cells in suspension did not undergo PARP cleavage, while multicellular aggregates of DU-145 cells showed reduced PARP proteolytic cleavage (18.6%; lane 6) compared to single DU-145 cells (42.0%; lane 5). Substrate attached cells showed no PARP cleavage when subjected to low calcium medium (data not shown). These results suggest that in the absence of integrin engagement and serum growth factors, Ca^{2+}-dependent PC3N multicellular aggregates are able to induce a cell survival pathway.

Since Bcl-2 plays a central role in prostate carcinoma cell survival, we determined the level of Bcl-2 expression in the suspension cultures. Bcl-2 protein levels were downregulated in suspension cultures of single PC3N cells and no detectable expression of Bcl-2 was detected in DU145 cells (Figure 3.1A). Immunoblot analysis showed that Bcl-2 expression levels were increased in multicellular aggregates of PC3N cells. The involvement of activated PI 3-kinase in regulating Bcl-2 level was further explored in

Figure 3.1: Calcium-Dependent Cell-Cell Adhesion Inhibits PARP Cleavage and Induces Akt Phosphorylation. (A) Serum-starved DU-145 and PC3N cells were detached from the substrate with EDTA. Single cells were seeded in poly-Hema coated dishes in serum-free medium with or without calcium. PARP cleavage was analyzed by Western blotting of equivalent protein from cell lysates at various time points along with Bcl-2 and β-catenin. Serum starved PC3N cells were pretreated with 50 nM for 1 hour of Wortmannin prior to detachment. Suspended cells treated with Wortmannin in the presence calcium were also treated with 50 nM of Wortmannin (last lane). (B) Calcium was chelated with 4 mM EGTA to disrupt adherens junctions in DU-145, which express E-cadherin and PC3N cells, which express N-cadherin. Calcium containing medium [1.8 mM] was added back to reform adherens junctions. Akt Ser-473 phosphorylation was analyzed on a 10% SDS-PAGE by Western blotting using phospho-specific Akt antibodies to Ser-473 and Enhanced Chemiluminescence. Membranes were then stripped and Western blotted for N-cadherin or E-cadherin. As controls, serum starved cells were stimulated with 100 nM EGF for 30 min. Similar results were obtained in three independent experiments.


A
DU-145
PC-3N
kDa
0 h
12 hrs
24 hrs
0 h
12 hrs
24 hrs
DMSO
Wortmannin
116
85
PARP
Bcl-2
β-catenin
- + - + - + - + - + - + + +
Calcium
B
DU-145
PC-3N
0 0.5 0 0.5 2 0 0.5 0 0.5 2
Hours
Akt-Ser 473P
Akt
- - + + + - - + + +
EGTA
- - - + + - - - + +
Calcium
- + - - - - + - - -
EGF
- - - - - - - - - -
Serum
E-cadherin
N-cadherin

PC3N multicellular aggregates. PC3N multicellular aggregates in the presence of 50 nM Wortmannin showed that PARP was cleaved to a similar level found in single cells in suspension. Cleavage of PARP was not observed in PC3N multicellular aggregates treated with DMSO as a solvent control. These results suggest the involvement of Ca^{2+}-dependent cell-cell adhesion in the activation of PI 3-kinase, which can regulate the cellular level of Bcl-2(168).

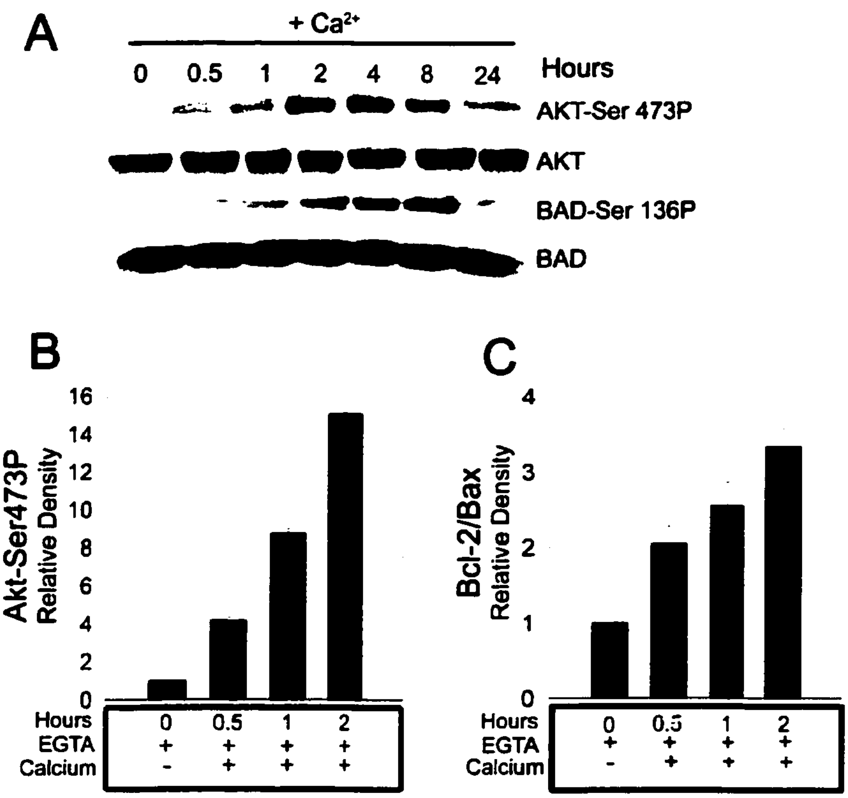
Since the protein kinase Akt/PKB is both a critical regulator of cell survival and one of the down-stream substrates of PI 3-kinase signaling, we evaluated whether formation of Ca^{2+}-dependent cell-cell adhesion in adherent PC3N cells could affect the activation state of Akt by assessing phosphorylation of Akt on Ser-473 using phospho-specific antibodies. Cadherin homophilic adhesion in a confluent cells is Ca^{2+}- dependent and is disrupted when extracellular calcium is reduced (384). Restoration of calcium re-establishes the adherens junction cell-cell contacts in a Calcium Switch Assay (383, 384). Previously we had shown that DU145 prostate carcinoma cells express E-cadherin, while PC3N cells express N-cadherin(303). Dissociation of cell-cell contacts reduced the basal phosphorylation level of Akt on Ser-473 as compared to control cells in normal calcium (Figure 3.1B). As shown in Figure 3.1B, engagement of cell-cell adhesion by calcium restoration [1.8 mM] induced Akt Ser-473 phosphorylation in a time dependent manner (approximately three-fold at 30 min.). The magnitude of the increase in phosphorylation of Akt Ser-473 by PDK at 2 hr in the Ca^{2+} Switch Assay was similar to that induced by addition of 100 nM EGF (lane 7 and 10). However, even though EGF stimulates Akt Ser-473 phosphorylation in DU-145 cells, no phosphorylation was detected upon adherens junction reassembly (Figure 3.1B lane 1-5). In addition, when PC3N cells form multicellular aggregates in the presence of Ca^{2+} they display an induction of Akt Ser-473 phosphorylation in a time dependent manner, and no tyrosine phosphorylation of the focal adhesion kinase (FAK) was detected (data not shown). These observations suggest that cadherin-mediated cell-cell contacts trigger a signal transduction pathway involving PI 3-kinase and Akt that is independent of integrin-mediated signaling pathways.

N-cadherin Engagement Increases the Bcl-2/Bax Protein Ratio

One role of Akt kinase activity in promoting cell survival is its ability to regulate the cellular level of Bcl-2. Cell survival is favored by a high Bcl-2/Bax protein ratio(408). To examine the whether N-cadherin ligation could regulate the Bcl-2/Bax ratio, we again utilized the Calcium Switch Assay(384). As shown in Figure 3.2 A and B, the relative phosphorylation of Akt Ser-473 was elevated in a time dependent manner following restoration of N-cadherin intercellular adhesion. Phosphorylation of Akt Ser-473 was maximal at 2 hours showing a 14-fold increase. Analyses of equivalent protein from the same lysates were then used to measure the steady state level of Bcl-2 and Bax protein expression by immunoblotting. In Figure 3.2C, the relative Bcl-2/Bax protein ratio increased upon N-cadherin engagement with approximately 2.5-fold induction after 2 hours. The protein level of Bax remained constant and the level of Bcl-2 increased upon N-cadherin engagement.

One mechanism by which Akt activation increases Bcl-2/Bax ratio is through the phosphorylation of the pro-apoptotic protein Bad on Ser-136(167). The Bcl-2 protein level has been shown to increase when Bad is phosphorylated on Ser-136(168). The

Figure 3.2: N-cadherin Adhesion Increases the Bcl-2/Bax Protein Ratio and the Phosphorylation of Bad on Ser-136. (A) Immunoblot analyses of Bad phosphorylated Ser-136, and Akt phosphorylated Ser-473 following a Calcium Switch Assay. Cellular lysates were separated on a 15% SDS-PAGE, transferred onto nitrocellulose and immunoblotted with antibodies to phospho-specific Bad on Ser-136, phosphor-specific Akt on Ser-473, total Bad and total Akt. (B) PC3N cells were serum-starved overnight, treated with EGTA and normal calcium was restored in serum-free media. Equivalent protein was immunoblotted with antibodies to Akt phosphorylated on Ser-473, anti-Akt, anti-Bcl-2 (C), or anti-Bax (C). Relative protein densities of Akt on Ser-473 and Bcl-2/Bax ratio were determined. Each relative protein values was further normalized to 0 hours. Data represents the average of three independent experiments.

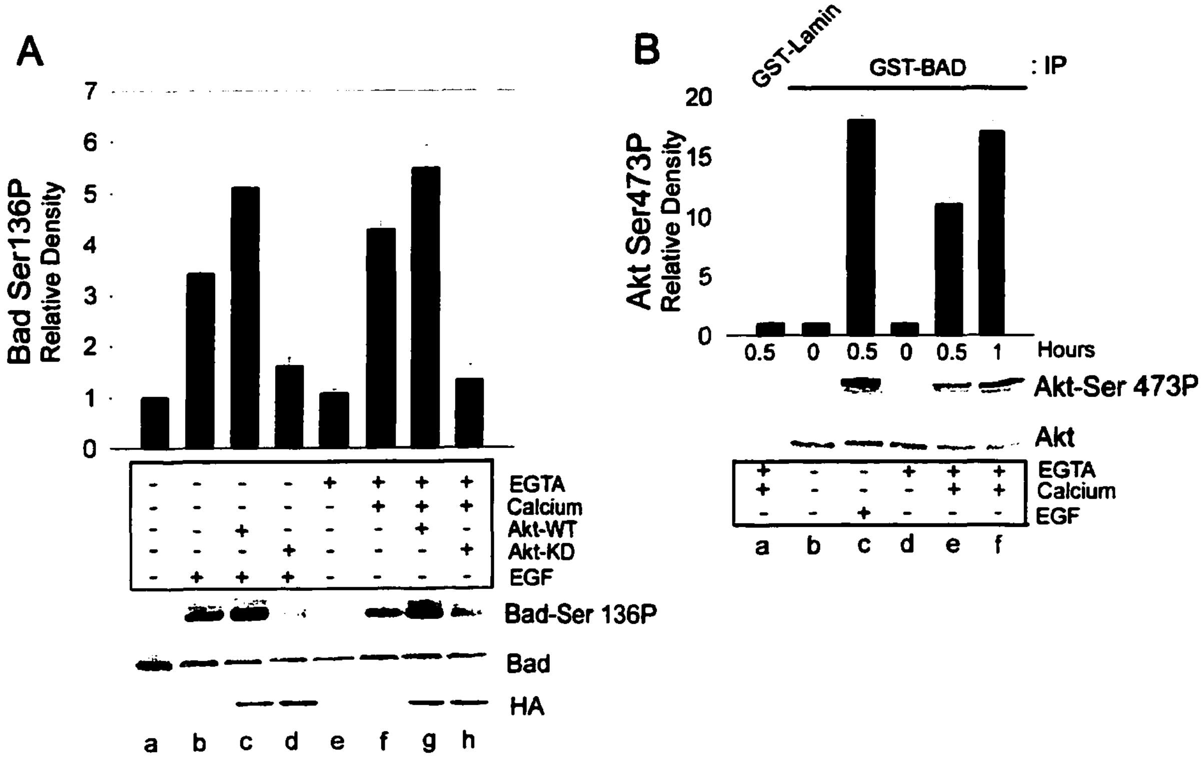

A
+ Ca2+
0 0.5 1 2 4 8 24 Hours
AKT-Ser 473P
AKT
BAD-Ser 136P
BAD
B
Akt-Ser473P Relative Density
0 2 4 6 8 10 12 14 16
Hours 0 0.5 1 2
EGTA + + + +
Calcium - + + +
C
Bcl-2/Bax Relative Density
0 1 2 3 4
Hours 0 0.5 1 2
EGTA + + + +
Calcium - + + +

possible involvement of Bad as a downstream substrate of N-cadherin-mediated Akt kinase activity was determined by using phospho-specific antibodies to Bad Ser-136. Figure 3.2A shows that N-cadherin ligation resulted in increased Bad phosphorylation on Ser-136. This elevation of Bad phosphorylation on Ser-136 was detected in a time dependent manner, and Bad phosphorylation was maximal at 8 hours.

To examine whether Bad Ser-136 phosphorylation following N-cadherin ligation was dependent upon Akt kinase activity, cells were transiently transfected with a dominant-negative kinase deficient mutant of Akt, K179M Akt (AktKD)(185) and analyzed Bad Ser-136 phosphorylation following N-cadherin ligation. PC3N cells transiently transfected with the AktKD construct prevented the induction of phosphorylation of Bad on Ser-136 (Figure 3.3A, lane h). However, following N-cadherin engagement Bad Ser-136 phosphorylation was readily detected in cells containing the transfected Akt Wild Type expression construct (Figure 3.3A, lane g). Thus, Bad Ser-136 phosphorylation induced by N-cadherin mediated cell-cell adhesion is dependent on Akt kinase activity. These findings demonstrate that assembly of N-cadherin mediated adherens junction induces a distinct cell-cell adhesion-dependent signal transduction pathway.

To determine the phosphorylation state of Akt following Akt/Bad interaction upon N-cadherin engagement a GST-Bad pull down assay was performed. Following N-cadherin engagement, protein extracts from cell lysates were prepared at different time points and incubated with an excess of GST-Bad sepharose followed by washing and immunoblotting for Akt Ser-473 phosphorylation. In all samples equivalent amount of Akt was affinity precipitated with GST-Bad (Figure 3.3B). Approximately 17-fold

Figure 3.3: Akt phosphorylates Bad following N-cadherin engagement. (A) Suppression of Bad Ser-136 phosphorylation by expression of dominant-negative Akt. Serum starved PC3N cells were transiently transfected with 4 μg of either Wild-Type or dominant-negative Akt, and 2 days later subjected to a Calcium Switch experiment and analyzed by immunoblotting for Bad Ser-136 phosphorylation and total Bad protein expression. Transfection of Wild Type Akt or Akt Kinase Dead construct was detected by monoclonal antibodies against HA. (B) Precipitation of PC3N lysates after Calcium Switch Assay with GST-Bad and immunoblots of Akt phosphorylated Ser-473 and endogenous Akt. Relative densities of Akt Ser-473 phosphorylation were normalized to endogenous Akt and further corrected to 0 hour protein lysate. The quantitation data represented are the mean of three independent experiments.


A
Bad Ser136P
Relative Density
0
1
2
3
4
5
6
7
- - - - + + + + EGTA
- - - - - + + + Calcium
- - + - - - + - Akt-WT
- - - + - - - + Akt-KD
- + + + - - - - EGF
Bad-Ser 136P
Bad
HA
a b c d e f g h
B
GST-Lamin
GST-BAD
: IP
Akt Ser473P
Relative Density
0
5
10
15
20
0.5 0 0.5 0 0.5 1 Hours
Akt-Ser 473P
Akt
+ - - + + + EGTA
+ - - - + + Calcium
- - + - - - EGF
a b c d e f

phosphorylation of Akt on Ser-473 was affinity purified with GST-Bad following 1 hr after reformation of the adherens junction as compared to EGF control (Figure 3.3B, compare lanes c and f). This increase in Akt Ser-473 phosphorylation was not seen in samples maintained in low calcium medium (Figure 3.3B; lane b). In addition, Akt was not affinity precipitated in cell lysates with control GST-lamin (Figure 3.3B; lane a). Together, these data support the role of N-cadherin engagement in inducing Bad Ser-136 phosphorylation mediated by increased Akt kinase activity.

Ligation of N-cadherin Elicits Recruitment of PI 3-kinase to the Adhesion Complex

To confirm that Akt activation was due to N-cadherin homophilic engagement and not to calcium manipulation, we utilized a N-cadherin monoclonal antibody. As shown in Figure 3.4A, pretreatment of cells with a neutralizing antibody that inhibits N-cadherin mediated formation of cell-cell contacts led to a suppression of Akt Ser-473 phosphorylation upon adherens junction reformation. The N-cadherin monoclonal antibody did not suppress EGF stimulated phosphorylation of Akt. This indicates that in the Calcium Switch Assay, N-cadherin ligation is responsible for Akt Ser-473 phosphorylation. To confirm that PI 3-kinase is required for the activation of Akt kinase activity in response to N-cadherin ligation, cells were pretreated with 50 nM of Wortmannin for 30 minutes prior to calcium restoration in the presence or absence of neutralizing N-cadherin antibody. Wortmannin completely suppressed Akt Ser-473 phosphorylation following N-cadherin ligation and EGF stimulated cells.

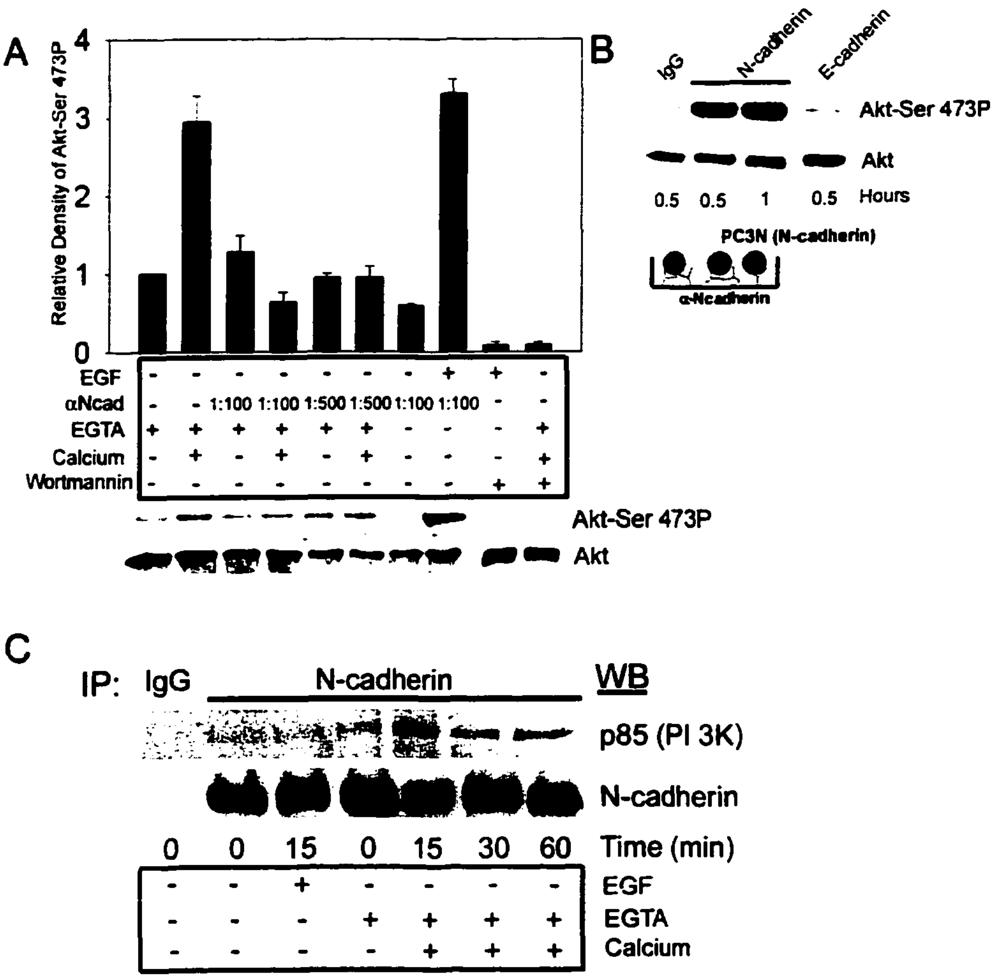
Antibody immobilization assays have been reported to cluster cell surface adhesion receptors, thereby acting as agonists and eliciting downstream signaling pathway(409). To further examine whether the ligation of N-cadherin is sufficient to promote Akt phosphorylation on Ser-473, cells were plated in serum-free conditions on a substrate consisting of an immobilized N-cadherin monoclonal antibody. As shown in Figure 3.4B, the phosphorylation of Akt on Ser-473 expression was determined to be ten-fold higher in PC3N cells seeded on immobilized N-cadherin antibody as compared to non-adherent carcinoma cells seeded on an immobilized non-immune mouse IgG or E-cadherin antibody. These results further confirm that N-cadherin ligation induces Akt phosphorylation on Ser-473.

Since N-cadherin mediated activation of Akt is dependent upon PI 3-kinase activity, we explored whether PI 3-kinase is recruited to the N-cadherin/catenin complex upon engagement of cell-cell adhesion. After restoration of adherens junction, cell lysates were immunoprecipitated with an antibody to N-cadherin and immunoblotted with an antibody to the p85 subunit of PI 3-kinase. The p85 subunit of PI 3-kinase associated with the N-cadherin complex in a time dependent manner reaching a maximal level at 15 minutes following N-cadherin adhesion (Figure 3.4C). Together with our studies of PI 3-kinase inhibition, these results suggest that engagement of N-cadherin forms a novel complex with PI 3-kinase at the plasma membrane which results in the activation of Akt.

Cytoskeletal Organization is Necessary for Formation of the N-cadherin/catenin complex and Signaling Activity

Functional adhesion of the N-cadherin/catenin complex requires association with peripheral filamentous actin(215, 216, 410), although previous studies have shown that

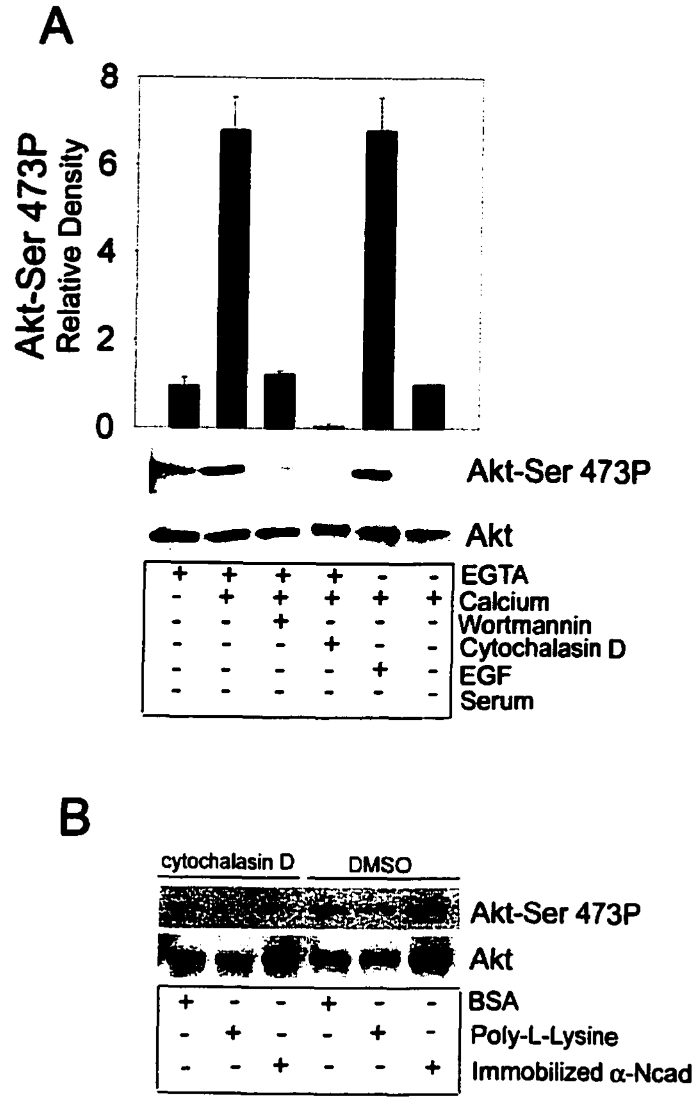
Figure 3.4: N-cadherin ligation mediates recruitment of the p85 subunit of PI 3-kinase to the N-cadherin/catenin complex and Akt activation. (A) Serum-starved PC3N cells were treated with 4 mM EGTA to disrupt cell-cell contacts. Cells were treated with the N-cadherin neutralizing monoclonal antibodies for 30 min, and lysed 30 min after calcium restoration. Controls included serum-starved cells either untreated or treated with 100 nM EGF for 5 min in the presence of N-cadherin antibodies. Treatment with 50 nM of Wortmannin suppressed Akt activation in cells treated with EGF or cells following restoration of calcium. Equivalent cellular lysates were immunoblotted with phospho-specific Akt or anti-Akt. Relative density of phospho-Ser-473 of Akt was normalized to total Akt using NIH Scion Imaging. Relative densities of each values was then normalized to EGTA treated PC3N cells with no calcium addition (first bar) (B) For N-cadherin antibody engagement studies, serum starved PC3N cells were detached with EGTA, and seeded on plates immobilized with N-cadherin antibodies in buffered medium. Akt activity was assayed as described above. Data analysis represents the average of 3 independent experiments. (C) After a Calcium Switch experiment, N-cadherin was immunoprecipitated from PC3N cell lysates (500 µgs) with an anti-N-cadherin monoclonal antibody. The presence of the p85/PI 3-kinase regulatory subunit in anti-N-cadherin immunprecipitation was detected by immunoblotting with anti-p85. Untreated cells and EGF stimulated cells (100 nM) were included as controls.


A
Relative Density of Akt-Ser 473P
0
1
2
3
4
EGF - - - - - - - + + -
αNcad - - 1:100 1:100 1:500 1:500 1:100 1:100 - -
EGTA + + + + + + - - - +
Calcium - + - + - + - - - +
Wortmannin - - - - - - - - + +
Akt-Ser 473P
Akt
B
IgG
N-cadherin
E-cadherin
Akt-Ser 473P
Akt
0.5 0.5 1 0.5 Hours
PC3N (N-cadherin)
α-Ncadherin
C
IP: IgG N-cadherin
WB
p85 (PI 3K)
N-cadherin
0 0 15 0 15 30 60 Time (min)
- - + - - - - EGF
- - - + + + + EGTA
- - - - + + + Calcium

clustering of the cadherin extracellular domains by homophilic binding is independent of a direct attachment to the actin cytoskeleton(215, 381). This initial contact of cadherins is remodeled and strengthened by association with the actin cytoskeleton(410, 411). We investigated whether the association of N-cadherin/catenin with F-actin was necessary for stimulating phosphorylation of Akt on Ser-473. Cells were treated with 1 μM of cytochalasin D, which disrupts actin microfilaments and cytoskeletal structure, prior to adherens junction formation, and Akt Ser-473 phosphorylation was determined by immunoblot analyses. As shown in Figure 3.5A&B, disruption of the actin cytoskeletal integrity by pretreatment with cytochalasin D suppressed Akt Ser-473 phosphorylation following calcium restoration and N-cadherin ligation to immobilized anti-N-cadherin antibodies. An additional approach was used to ligate N-cadherin that does not depend on the functional adhesion to the cytoskeleton. In Figure 3.5B, cells treated with cytochalasin D were able to bind and adhere to the N-cadherin immobilized antibodies, but were not able to induce Akt Ser-437 phosphorylation similar to cells seeded on poly-L-Lysine or BSA coated dishes. However, cells treated with DMSO as solvent control adhere to the immobilized N-cadherin antibodies and induce a 10-fold phosphorylation of Akt on Ser-473. This indicates that a functional actin cytoskeleton is necessary for Akt activation induced by N-cadherin ligation. In contrast, activation of Akt by addition of 100 nM EGF was not inhibited by pretreatment with cytochalasin D.

Since the actin binding protein α-catenin is deleted in PC3N cells, I investigated whether vinculin is associated with the N-cadherin/catenin complex. Immunoprecipitation of N-cadherin from PC3N cells as shown in Figure 3.6A indicates

Figure 3.5: Disruption of F-actin inhibits N-cadherin mediated Akt Ser-473 phosphorylation. Prior to calcium restoration, serum starved PC3N cells were treated with the indicated inhibitors (cytochalasin D 1 μM; Wortmannin 50 nM for 60 min). Cells were lysed and immunoblotted with anti-phospho Akt Ser-473. The relative density of Akt phosphorylation was normalized to total Akt using NIH Scion Imaging. Data were further normalized to 0 hr protein level. Data represents a mean of three independent experiments. (B) Serum deprived PC-3N cells were treated with 1 μm cytochalasin D or DMSO as a control for 30 min and cells were lifted with EDTA. Single cells (1 X 10^6) were added to immobilized N-cadherin antibody plate (α-Ncad), poly-L-Lysine coated plate (PLL) or BSA. Attached cells were lysed and immunoblotted for Akt Ser-473 phosphorylation and Akt. Since cells did not attach to BSA coated dishes, suspended cells were lysed and analyzed as stated above.

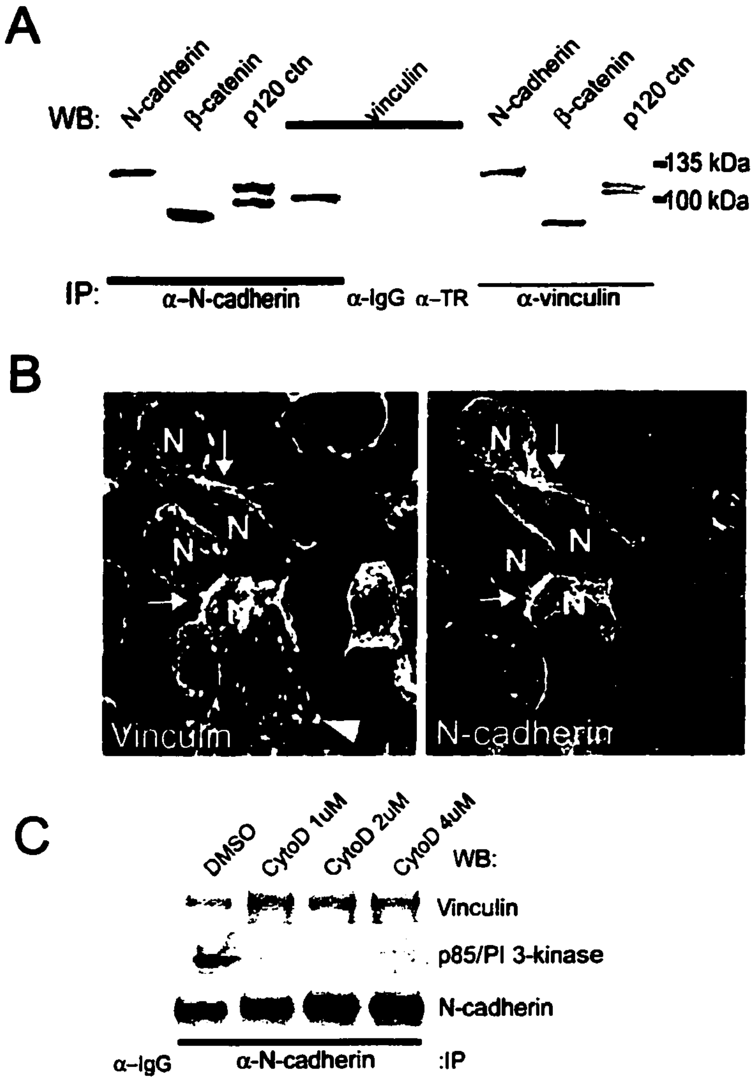

A
Akt-Ser 473P
Relative Density
0
2
4
6
8
Akt-Ser 473P
Akt
+ + + + - - EGTA
- + + + + + Calcium
- - + - - - Wortmannin
- - - + - - Cytochalasin D
- - - - + - EGF
- - - - - - Serum
B
cytochalasin D
DMSO
Akt-Ser 473P
Akt
+ - - + - - BSA
- + - - + - Poly-L-Lysine
- - + - - + Immobilized α-Ncad

that in addition to β-catenin and p120^{ctn}, vinculin was also detected in the N-cadherin/catenin complex. Co-precipitation of N-cadherin with vinculin was verified in immunoprecipitations with an anti-vinculin antibody. N-cadherin was also found in the anti-vinculin immunoprecipitations associated both β-catenin and p120^{ctn} (Figure 3.6A). These results suggest that vinculin is present with N-cadherin/catenin complex in prostate carcinoma cells similar to what has been observed in breast cancer cell lines(386), and this interaction may mediate N-cadherin/catenin binding to the actin cytoskeleton complex.

To further confirm the association of vinculin with the N-cadherin complex, immunolocalization studies were performed to determine the localization of N-cadherin and vinculin. N-cadherin immunofluorescence showed strong localization at cell-cell contacts as anticipated (Figure 3.6B; arrow). When cells were co-immunolabeled with vinculin and N-cadherin, two patterns for vinculin localization were observed. One pattern was similar to that for N-cadherin at cell-cell contacts, and merging of the two confocal images revealed identical patterns of co-localization (data not shown). In addition, vinculin but not N-cadherin was also detected in focal adhesion sites that were associated with stress fibers (Figure 3.6B; arrow head). In summary, the interaction and localization of vinculin with N-cadherin mediated cell-cell contacts can replace α-catenin in the complex and may play a role in linking the N-cadherin/catenin complex to the peripheral actin cytoskeleton.

Since a functional F-actin upon N-cadherin ligation is required for Akt phosphorylation on Ser-473, we investigated whether the recruitment of p85 subunit of PI

Figure 3.6: Vinculin interacts with the N-cadherin/β-catenin complex. (A) N-cadherin was immunoprecipitated from PC3N cells and immunoprecipitates were immunoblotted for β-catenin, p120ctn and vinculin. Negative controls include cells immunoprecipitated with irrelevant antibodies to mouse IgG or the transferrin receptor. (B) Co-immunolocalization of N-cadherin (FITC stained) and vinculin (Cy-3 stained) and sites of cell-cell contacts (arrow) and vinculin at focal adhesion sites (arrowhead). N represents location of the nucleus within PC3N cells that had been stained with BBI. (C) Cells were pretreated with the indicated concentrations of cytochalasin D and calcium was restored for 15 min. N-cadherin was immunoprecipitated from total cellular lysates with monoclonal antibodies against N-cadherin or irrelevant mouse IgG as control, separated on 7% SDS-PAGE, transferred onto nitrocellulose membrane, and immunoblotted for vinculin or p85 subunit of the PI 3-kinase.


A
WB:
N-cadherin
β-catenin
p120 ctn
vinculin
N-cadherin
β-catenin
p120 ctn
135 kDa
100 kDa
IP:
α-N-cadherin
α-IgG
α-TR
α-vinculin
B
N
N
N
N
N
N
N
N
Vinculin
N-cadherin
C
DMSO
CytoD 1uM
CytoD 2uM
CytoD 4uM
WB:
Vinculin
p85/PI 3-kinase
N-cadherin
α-IgG
α-N-cadherin
:IP

3-kinase to the N-cadherin/catenin complex was also dependent upon a functional actin cytoskeleton. Cells were pretreated 1 μM of cytochalasin D or DMSO as solvent control prior to adherens junction reformation. Immunoblot analysis of N-cadherin immunoprecipitates showed that the p85 regulatory subunit of PI 3-was not able to associate with the N-cadherin/catenin complex in prostate carcinoma cells treated with cytochalasin D (Figure 3.6C). However, the vinculin interaction with the N-cadherin/catenin complex was not disrupted by cytochalasin D.

N-cadherin ligation induces phosphorylation of a 102 kDa protein

The activation of PI 3-kinase involves the translocation of this enzyme to the plasma membrane where it can gain access to its lipid substrate. Although is not known how the PI 3-kinase is activated upon N-cadherin ligation, one might speculate that a tyrosine kinase, yet to be identified, when activated plays a role in the recruitment of PI 3-kinase to the N-cadherin complex at the plasma membrane. To investigate whether a tyrosine kinase interacts with the N-cadherin complex, immunoprecipitation of N-cadherin upon adherens junction reformation was performed at various time points. The immunoprecipitation complex of N-cadherin was then immunoblotted for phospho-tyrosine using a phospho-specific monoclonal antibody to phosphorylated tyrosine. As shown in Figure 3.7, a 102-kDa protein, which interacts with N-cadherin upon ligation, is highly phosphorylated. The phosphorylation of this 102-kDa protein is observed early within 5 min of N-cadherin ligation.

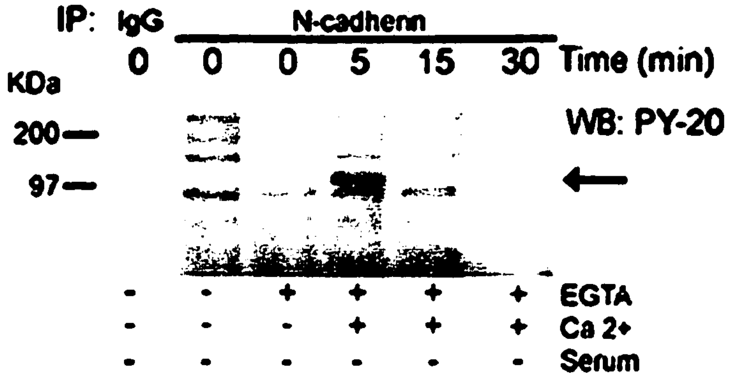

IP: IgG
N-cadherin
KDa
0 0 0 5 15 30 Time (min)
200—
97—
WB: PY-20
- - + + + + EGTA
- - - + + + Ca 2+
- - - - - - Serum

Figure 3.7: N-cadherin ligation induces tyrosine phosphorylation of a 102-kDa protein. Serum deprived PC-3N cells were treated with 4 mM EGTA to chelate exogenous calcium. Calcium was added back to induce adherens junction reformation. At various time courses, cells were lysed and immunoprecipitated with anti-N-cadherin antibody and immunoblotted for phospho-tyrosine using a phospho-specific antibody to phosphorylated tyrosine.

Discussion

This study describes a role for N-cadherin-dependent adhesion in regulating the protein level of Bcl-2 in invasive prostate carcinoma cells. Inhibition of homophilic binding of the N-cadherin extracellular domain correlates with decreased cellular levels of activated Akt (Ser473 phosphorylation) and Bcl-2 protein expression. N-cadherin engagement between adjacent cells has been shown to have an essential role in the recruitment of the actin cytoskeleton to adherens junctions complex(410, 411) and the results show that complex formation leads to the recruitment of PI 3-kinase. Subsequent activation of the PI 3 Kinase/Akt pathway leads to phosphorylation of Bad at Ser-136 and stabilization of Bcl-2. Thus, these results suggest an overlap in the cell survival signal transduction cascades that are stimulated by growth factors, adhesion to extracellular matrix, and intercellular adhesion by cadherins(168, 412).

Several lines of evidence suggest that the suppression of apoptosis following cellular attachment is mediated not solely by integrin engagement but, rather, by the ability of the cells to spread and adopt an optimal cell shape(413). Cell-cell adhesion is an additional mechanism to control cell shape. For example, the expression of E-cadherin in L-cells, which lack cadherins, converts L-cells with an elongated mesenchymal morphology to an epithelial phenotype. These changes in cell volume or cell shape are also governed by cell surface adhesion molecules and the actin cytoskeleton, which is known to affect signal transduction(413, 414). Cell rounding induced by disruption of actin cytoskeletal organization is sufficient to induce endothelial apoptosis under defined extracellular matrix conditions(415).

The cell adhesion and signaling properties of N-cadherin depends on both the formation of lateral homodimers of the extracellular domains and on the cytoplasmic domain, which through the catenins is connected to the actin cytoskeleton (215, 216, 385). The membrane proximal region of the N-cadherin cytoplasmic domain is involved in regulation of cell adhesion activity and binds $p120^{ctn}$(416). The carboxyteminal region of the cytoplasmic domain contains the binding site for α-catenin and β-catenin and is required for cell-cell adhesion. The actin binding proteins, α-catenin or vinculin, which show nearly a 30% overall sequence homology to α-catenin, have been found to link the cadherin/catenin adhesion complex to the actin cytoskeleton(417). While vinculin has been identified in E-cadherin/catenin complexes in epithelial cells lines along with β-catenin, the replacement of α-catenin by vinculin in cells that do not express α-catenin is suggested to mediate a less stable cell-cell adhesion(386). The results indicate that in PC3N carcinoma cells vinculin has substituted for α-catenin to link the N-cadherin/β-catenin/p120ctn complex to the peripheral actin cytoskeleton. Vinculin is associated at the membrane with actin filaments in both integrin-mediated focal contacts and in N-cadherin adherens junctions in PC3N cells. Vinculin remained associated with the N-cadherin adhesion complex when the actin cytoskeleton was dissociated by cytochalasin D. Together these results suggest that vinculin can function in the N-cadherin/catenin complex to link the complex to the actin cytoskeleton.

Formation of intercellular adherens junctions is a dynamic process that involves actin reorganization and polymerization initiated by cadherin adhesion(411, 418). Binding of calcium ions to the cadherin extracellular domains converts the conformation of the cadherin to become competent for binding to identical dimers on neighboring cells(383, 384). Cadherin homophilic binding initiates clustering of the extracellular domains, and interaction of the cytoplasmic domain with the actin cytoskeleton is an important mechanism to strengthen the weak forces provided by the homophilic binding(416). The association of the N-cadherin/catenin/vinculin complexes with peripheral actin cytoskeleton provides sites for attachment of the cytoskeleton at the membrane, which are important for the development of mechanical stress during epithelial polarization and control of cell shape(418). This cadherin based interaction also transmits signals from the plasma membrane to the actin cytoskeleton, ranging from activation of the Rho family of small GTPases(254, 281, 419) to production of phosphoinositides(420) and activation of tyrosine kinases(421). Homophilic E-cadherin adhesion activates the GTPase Cdc42, which is involved in regulation of cytoskeletal rearrangement and can modulate cadherin function(276). Moreover, E-cadherin has been found immediate signal pathways in multicellular aggregates that can regulate cell cycle progression and cell survival(397, 406). The N-cadherin survival-signaling pathway in PC3N cells appears to be independent of ERK1/2 MAP kinase activity. Inhibition of the MAPK pathway in PC3N cells had no effect on the regulation of Bcl-2, and we were unable to detect phosphorylation of ERK1/2 following N-cadherin ligation (data not shown). Studies by Pece *et al.*(274) previously showed that E-cadherin adhesion can activate the MAPK pathway through the recruitment of the EGF receptor to the E-cadherin-catenin complex. Thus, cadherin outside-in signaling may initiate different signal transduction cascades depending on the cell type.

Following homophilic adhesion of the N-cadherin/catenin complex, the association with F-actin to the N-cadherin/catenin complex recruits the p85 regulatory subunit of PI 3-kinase with the subsequent activation of Akt. In addition to recruitment and remodeling of the actin cytoskeleton at the membrane, Lambert *et al.*(421) have demonstrated the local accumulation of tyrosine phosphorylated proteins. These tyrosine phosphorylated sites apparently serve as binding sites for the SH2 domain of the p85 regulatory subunit of PI 3-kinase. The recruitment of PI 3-kinase to the N-cadherin/catenin adhesion complex following ligation appears to be dependent upon F-actin, since the inhibition of F-actin polymerization prior to N-cadherin mediated adherens junction reformation disrupts PI 3-kinase recruitment to the cadherin complex and activation. We demonstrated that cell attachment to anti-N-cadherin and N-cadherin initiated cell-cell adhesion failed to activate PI 3-kinase and Akt following treatment with cytochalasin D. Conversely, disruption of the actin cytoskeleton with cytochalasin D in PC3N cells was found to have no effect on Akt-dependent EGF signal transduction (data not shown). The activation of the EGFR by its ligand elicits remodeling of F-actin(422) which results in the formation of lamellipodia(423) and requires the activity of PI 3-kinase. Distinct signaling properties have been shown for the isoforms of the p110 catalytic subunit of PI 3-kinase have been demonstrated(424). EGF-stimulated lamellipodia extension was shown to be a function of the α isoform and not the β isoform of the catalytic subunit(423), although both p110 catalytic subunits are capable of leading to downstream Akt activation(425). The differential utilization of the p110 isoforms could reflect the specific signaling properties that distinguish between the N-cadherin and EGF receptor signal transduction complexes.

Increased Akt activity has been suggested to function in the progression of prostate cancer. Graff *et al.*(426) have shown that in an androgen-independent prostate carcinoma line, there is increased activation of the kinase Akt/PKB. In addition, overexpression of activated Akt was sufficient to facilitate increased tumor growth in a murine xenograft model(426). Since the protein and lipid phosphatase PTEN is a negative regulator of the PI 3-kinase pathway and is lost in advanced prostate cancer, tumor progression is likely to be associated with increased responsiveness to extracellular signals, such as cadherin or integrin-mediated adhesion that activates the PI 3-kinase pathway. The studies show that the level of Bcl-2 protein expression is enhanced in a *PTEN* negative prostate carcinoma cells line that forms N-cadherin mediated adherens junctions.

The loss of normal epithelial ductal architecture and the breakdown or altered synthesis of the basement membrane are frequent occurrences in the progression to metastatic prostate cancer(376). *In vivo* carcinomas grow as multicellular masses that are selected to survive in the absence of integrin-mediated adhesion to an organized basement membrane. Evidence suggests that suppression of the resistance to apoptosis in cultured transformed epithelial cells inhibits their tumorgenicity(156). The phenotypic conversion from E-cadherin to N-cadherin expression in prostate carcinoma cells is a molecular alteration that is proposed to facilitate tumor progression allowing cancer cells to metastasize, and to survive and grow in ectopic sites (302, 303). The accumulation of experimental evidence from these studies and by others indicates that, even in the absence of anchorage-dependent or matrix interactions, intercellular adhesion may provide compensatory signals that promote cell viability(397). As such, this aberrant cell survival might be expected to contribute to neoplastic development. Thus, it is likely that the accumulated mutations such as loss of α-catenin and PTEN and a gain of N-cadherin expression facilitate survival signaling through a direct pathway.

In summary, we have identified N-cadherin mediated cell-cell adhesion as an additional mechanism that determines the regulation of the balance of the apoptotic pathway as summarized in Figure 3.8. Signaling starts with N-cadherin homophilic adhesion and is associated with the transient recruitment of the p85 subunit of PI 3-kinase to the N-cadherin/catenin/vinculin/actin complex. Subsequent recruitment and activation of PI 3-kinase to the N-cadherin complex leads to the production of phosphidylinositol 3,4,5 triphosphate, which mediates Akt activation by promoting association with the membrane at the adherens junction and activation of PDK1/2. The increased Akt kinase activity is associated with the Ser-136 phosphorylation of the pro-apoptotic protein Bad. In the unphosphorylated state Bad forms heterodimers with anti-apoptotic Bcl-2 homologs and promotes cell death by allowing Bax-Bax homodimers in the mitochondrial outer membrane. Bax is predominantly cytoplasmic in cells under normal conditions, but translocates to the outer mitochondrial membrane in response to apoptotic signals(166). During apoptosis the activation of caspases is a critical step and may contribute to the loss of anti-apoptotic signaling from cadherin since many of the substrates of caspases have been shown to be components of adherens junctions including plakoglobin, β-catenin and E-cadherin(427, 428). These studies provide new insights into the function of cadherin survival signaling pathway and may provide new approaches for the therapeutic treatment of uncontrolled growth and metastasis of malignant prostate cancer cells.

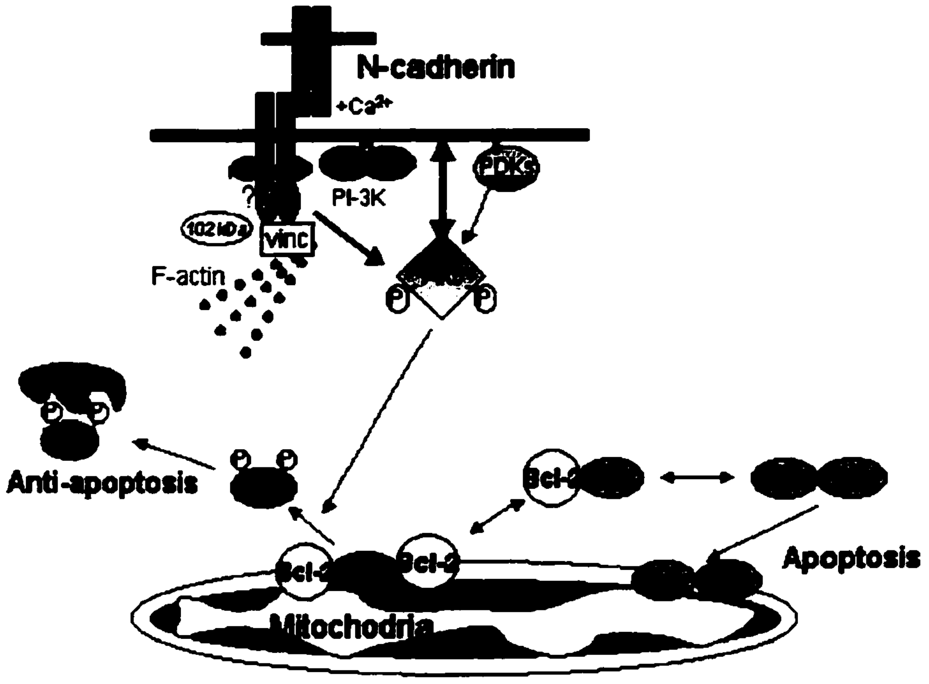

N-cadherin
+Ca2+
PI-3K
?
102kDa
vinc
PDKs
F-actin
P
P
Anti-apoptosis
Bcl-2
Bcl-2
Bcl-2
Apoptosis
Mitochondria

Figure 3.8: Illustrated model summarizing the outside-in signaling of N-cadherin ligation for Bcl-2 protein stability. Upon N-cadherin engagement, the p85 regulatory subunit of PI 3-kinase is recruited to the N-cadherin/catenin complex followed by induction of Akt kinase activity by phosphorylation of Akt on Ser-473. Activated Akt in turn phosphorylates Bad on Ser 133, sequestering Bad in the cytoplasm by interaction with 14-3-3 proteins. This results in the increased Bcl-2/Bax ratio, which promote cell survival. This cell survival signal induced by N-cadherin also depends on the association of the N-cadherin/catenin complex to the actin cytoskeleton.

CHAPTER 4: REGULATION OF *BCL-2* TRANSCRIPTION BY N-CADHERIN ADHESION THROUGH THE CREB SIGNALING PATHWAY

Introduction

Cadherin mediated cell-cell adhesion is important for the morphogenesis of tissues and the maintenance of tissue function(319). The adhesive function of the cadherin requires the homophilic binding between extracellular domains in a Ca^{2+}-dependent manner. This intracellular adhesion is controlled by the cytoplasmic domain, which strengthens the homophilic adhesion by association with catenins and the actin cytoskeleton(429). The interaction of the cadherin complex with the actin cytoskeleton is important in eliciting certain signaling pathway that affect cell behavior, including proliferation(430, 431)and differentiation(432-434).

In epithelial cells, E-cadherin is a major determinant of the cyto-architecture. Altered regulation of E-cadherin expression or function leads to alteration in epithelial cell growth and differentiation. Loss of E-cadherin, in cancer, is strongly correlated with increase cell invasiveness *in vitro* and tumor progression *in vivo*(48, 85, 435, 436). While integrin anti-apoptotic pathways have been described, current evidence suggests that E-cadherin mediated cell-cell contact is also an important regulator of epithelial cell survival, although the molecular basis underlying the resistance to apoptosis are still largely unknown. Recently, outside-in signaling from E-cadherins mediated cell-cell adhesion was described to activate cell survival pathways(275). A study by Pece *et al.*(275) demonstrated that engagement of E-cadherin in kidney epithelial cells leads to a rapid activation of Akt/Protein Kinase B on Ser-473 through a PI 3-kinase dependent pathway. In addition, my previous studies have indicated that ligation of abnormal expressed N-cadherin in malignant prostate carcinoma cells lacking E-cadherin expression also leads to Akt Ser-473 phosphorylation(437). N-cadherin mediated Akt activation was able to regulate Bcl-2 protein stability by Bad phosphorylation on Ser136 suggesting a role in prostate tumor progression. Akt can prevent apoptosis by inactivating Bad function by phosphorylation of Bad on Ser136, which sequesters Bad in the cytoplasm through an association with 14-3-3 proteins. Bad association with 14-3-3 prevents its heterodimerization with Bcl-2, thereby stabilizing Bcl-2. In the normal prostate, Bcl-2 is expressed only in the basal epithelial cells(186), whereas overexpression of Bcl-2 is frequent in advanced androgen independent prostate carcinomas(401, 438). However, it is still unclear whether the regulation of *bcl-2* transcription could be mediated by cadherin cell-cell adhesion.

One mechanism by which *bcl-2* transcription is regulated is through the activation of transcription factors, such as CREB. CREB is a 43-kDa nuclear protein that binds to CRE [cAMP response element], and its activity is regulated by phosphorylation(439). One of the known mechanisms of CREB transcriptional activation is phosphorylation of Ser133 by the catalytic subunit of cAMP-activated protein kinase A(440) or other Ser/Thr kinases, such as Akt/protein kinase B(439). Since CREB is identified as a down-stream substrate of Akt(439), there is evidence that the Akt/CREB signaling pathway upregulates *bcl-2* transcription. A study by Pugazhenthi *et al.*(441) has shown that the CRE-site in the promoter region of *bcl-2*, in conjunction with the transcription factor

CREB, was identified as a positive regulator of Bcl-2 expression through an IGF-I signaling cascade mediated by PI 3-kinase/PDK1/Akt. In addition, Matter and Ruoslahti(442) reported that upon activation of $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins in CHO cells, *bcl-2* transcription is elevated. This integrin-mediated regulation of *bcl-2* transcription is Shc- and FAK-dependent, which are necessary for Ras/PI 3-kinase/Akt signaling cascade. Thus, the up-regulation of *bcl-2* has been identified as a critical mechanism by which both growth factors and cell-matrix interaction through integrins promote cell survival.

Signals that can activate Akt can also inactivate GSK-3β by phosphorylation on Ser-9. Normally, activation of GSK-3β results in the inhibition of several transcription factors including AP-1(443), TCF/LEF(444), and CREB(445) activities, in part through phosphorylation-dependent events that reduce transcription factor binding to their cognate DNA sequences. GSK-3β can phosphorylate Ser-129 of CREB thus inactivating CREB transcriptional activity(445). Thus, CREB activity is dependent not only on Ser-133 phosphorylation by Akt, but also the ability of Akt to inactivate GSK-3β.

This study describes a novel regulation of *bcl-2* transcription by N-cadherin mediated cell-cell adhesion. Homophilic adhesion of N-cadherin in a dedifferentiated prostate cancer cell line leads to a rapid activation of Akt and subsequently the phosphorylation of CREB on Ser-133. Both dominant negative forms of Akt and CREB were able to block N-cadherin mediated induction of *bcl-2* mRNA and the induction of the CRE site-containing promoter of *bcl-2,* thus suppressing *bcl-2* transcription. Re-expression of wildtype PTEN into PC-3N prostate carcinoma cells suppresses N-cadherin stimulation of CREB phosphorylation and *bcl-2* mRNA induction. Moreover, N-cadherin engagement results in the activation of Akt and the phosphorylation of GSK-3β on Ser-9, inactivating GSK-3β kinase activity. The objective of the present study is to examine whether N-cadherin mediated signaling through PI 3-kinase/Akt leads to a CREB dependent increase in *bcl-2* promoter activity and mRNA expression.

Materials and Methods

Cell Culture and Transfection— The human prostate carcinoma cell line PC-3N, which only expresses N-cadherin, was used in this study. PC-3N cells were derived from PC-3 cell line(303). PC-3N cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS; Hyclone Laboratories, Inc., Logan, UT) and penicillin/streptomycin in a 37°C, 5% CO_2 atmosphere at constant humidity. Cells were transiently transfected with hemagglutinin (HA) tagged AKT [pCMV6-AKT-HA (WT)], kinase deficient AKT [pCMV6-AKT-HA K179M (KD)] (U. of Michigan, Ann Arbor, MI)(185), dominant-negative CREB [pCREBM1 containing a serine to alanine substitution at position 133; Dr. Marc Montminy; The Salk Institute for Biological Studies, La Jolla, California](446), GFP-PTEN wild-type construct (Kenneth Yamada, National Institute of Health)(447) or wild-type GSK-3β plasmid (Dr. Woodgett, Ontario Cancer Institute, Toronto, Ontario, Canada)(169). Transfection of PC-3N cells was performed according to manufacture's protocol by using FUGENE 6 Liposomal Transfection Reagent (Roche Molecular Biochemicals; Indianapolis, IN). Control cells were transfected with an empty vector pcDNA3.1. After 16 hrs, the transfected cells were placed in DMEM without serum twenty-four hours prior to analysis. PC-3N transfection efficiency with FUGENE 6 was shown to be greater than 80%.

Western Blot and Antibodies —Equivalent amounts of protein were separated by SDS-PAGE and transferred to nitrocellulose membrane (SS). Western blot analysis using chemiluminescence detection method (NEN, Boston, MA) and exposure on X-OMAT AR film (Kodak, Rochester, NY) was carried out as described previously(303). Images of immunoblots were captured and quantitative densitometry for protein bands was carried out using Scion Image (Scion Corporation). The results shown are the average of three independent experiments, and each exposed film was quantitated three times. Antibodies used in this study are as followed: a mouse monoclonal antibody against N-cadherin and non-immune mouse IgG were obtained from Sigma Chemical Co. (A-CAM clone GC-4; St. Louis, MO). A monoclonal antibody against N-cadherin was purchased from Transduction Laboratory (Lexington, KY). Rabbit polyclonal phospho-specific antibodies to Akt Serine-473, CREB-Serine-133, and GSK-3α/β Ser 21/9 and rabbit polyclonal antibodies to Akt and CREB were purchased from New England Biolabs (Beverly, MA). GSK-3β (clone 7) monoclonal antibody was purchased from Transduction Laboratories (Lexington, KY) and PARP monoclonal antibody was obtained from Enzyme Systems Products (Livermore, CA). Anti-mouse IgG and anti-rabbit IgG antibodies coupled to horseradish peroxidase were purchased from Promega (Madison, WI).

Calcium Switch Assay—Confluent PC-3N cells were serum-starved overnight and N-cadherin cell-cell contacts were disrupted by treatment with 4 mM EGTA in DMEM for 40 min at 37°C. Thereafter, intercellular contacts were allowed to reform by addition of calcium containing medium, [DMEM; 1.8 mM Ca^{2+}] at 37°C. Cells were harvest after calcium restoration and lysed in 2X SDS-Sample Buffer containing 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.7 μg/ml pepstatin, 20 mM NaF, 20 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF). In certain experiments, cells were treated with kinase inhibitors PD98059 (40 μM), SB203580 (10 μM), Rapamycin (10 ng/ml, or LY294002 (20 μM) (Calbiochem; San Diego, CA) 30 min prior to calcium restoration.

Antibody Immobilization—Bacteriological petri dishes were coated for 2 h at 37°C with goat anti-mouse FAB fragments (50 μg/ml) in 100 mM $NaHCO_2$ pH 9.6. Plates were rinsed and then incubated overnight with mAbs specific for N-cadherin or E-cadherin (10 μg/ml) at 4°C and blocked with heat-denatured 1% BSA in PBS, pH7.4. Serum starved PC-3N cells were harvest with EDTA treatment, washed in calcium/magnesium free PBS, and resuspended in two ml of serum free medium without calcium. Approximately, $1X10^6$ cells were permitted to adhere to the antibodies at 37°C after which nonadherent cells were removed with washing. Attached cells were lysed and subjected to Western blot analyses as described above.

Luciferase Assay—Dual Luciferase reporter assays were performed according to manufacture's protocols (Promega). Cells were transiently transfected with CRE luciferase reporter gene (Stratagene, La Jolla, CA) or *bcl-2* promoter/luciferase reporter gene (0.5 μg) and a control pTKReneilla reporter construct (50 ng) using Fugene (Roche). Different promoter regions of the bcl-2 gene (full-length, -3934 to –1287; truncated with CRE site, -1640 to –1287; truncated without CRE site, -1526 to –1287; and the CRE mutated site, -1640 to 1287) were linked to luciferase reporter as described previously(448). In certain experiments, cells were also co-transfected with Akt-WT, Akt-KD, GSK-3βWT, GFP-PTEN or CREBM1 or pcDNA3.1 control vector. Cells were then switched to serum free, calcium free medium (SMEM) for 24 hours. Cells were then incubated in medium containing calcium (DMEM) for 8 hours. The promoter activity was normalized to the activity of the pTKReneilla control and the results were expressed as relative light units of luciferase activity to reneilla activity.

RNA Extraction and Northern Blot Analysis—Total RNA was prepared from cultured cells by acid guanidinium thiocyanate-pheno-chloroform extraction(343). Poly(A)RNA was then isolated from the total RNA extract by using FastTrack mRNA Isolation Kit Version 3.0 (Invitrogen Corp., Carlsbad, CA) according to manufacturer's protocol. Five micrograms of each poly(A)RNA samples were then separated by electrophoresis in 1% agarose gel containing 1.85% formaldehyde and transferred onto a HybondN+ nylon membrane (Amersham Life Science, Arlington Heights, IL). *bcl-2* mRNA was detected by Northern blot analysis with a 600 bp B*amHI*/E*coRI* cDNA fragment isolated from full length human *bcl-2* (GenBank Accession M14745) provided by Dr. Roger Meisfeld (University of Arizona, Tucson, AZ). Probes were random-primed and membranes were prehybridized as described previously (303). Blots were then washed at 42°C in 2X SSC/0.1% SDS and 2X 30 min at 42°C in 0.3X SSC/0.5% SDS and then exposed to X-

OMAT AR film (Kodak). Normalization for loading was compared to hybridization of the 500 bp fragment of human *β-actin* (GenBank Accession X00351).

Cell-Cell Adhesion Survival Assay—Cells were grown to 75% confluency and serum deprived for 24 hours. Cells were then detached by EDTA to preserve cells surface expression of cadherin and dissociated into single cells by tituration 10 times with a Pasteur pipette. After washing the cells two times with calcium-free Suspension Modified Eagle's Medium (SMEM; Gibco/BRL) in the absence of serum, the cells were resuspended in a concentration of 1 X 10^6 cells/cm^2 in SMEM and maintained in suspension on 25 μg/ml Poly-HEMA (poly[2-hydroethylmethacrylate]; Sigma Chemical Co.) coated 2 cm^2 culture dishes to prevent cell attachment. Cells were compared in the presence of Ca^{2+} (1.8mM) to induce cell-cell contacts or without Ca^{2+} to prevent cell-cell contacts. After 12 or 24 hours in suspension, the cells were washed once in PBS and the cell pellets were lysed in 2X SDS Sample Buffer. Thirty micrograms of protein were separated on a 7% SDS-PAGE and analyzed for PARP cleavage by immunoblotting.

Results

N-cadherin ligation induces CREB phosphorylation on Ser-133

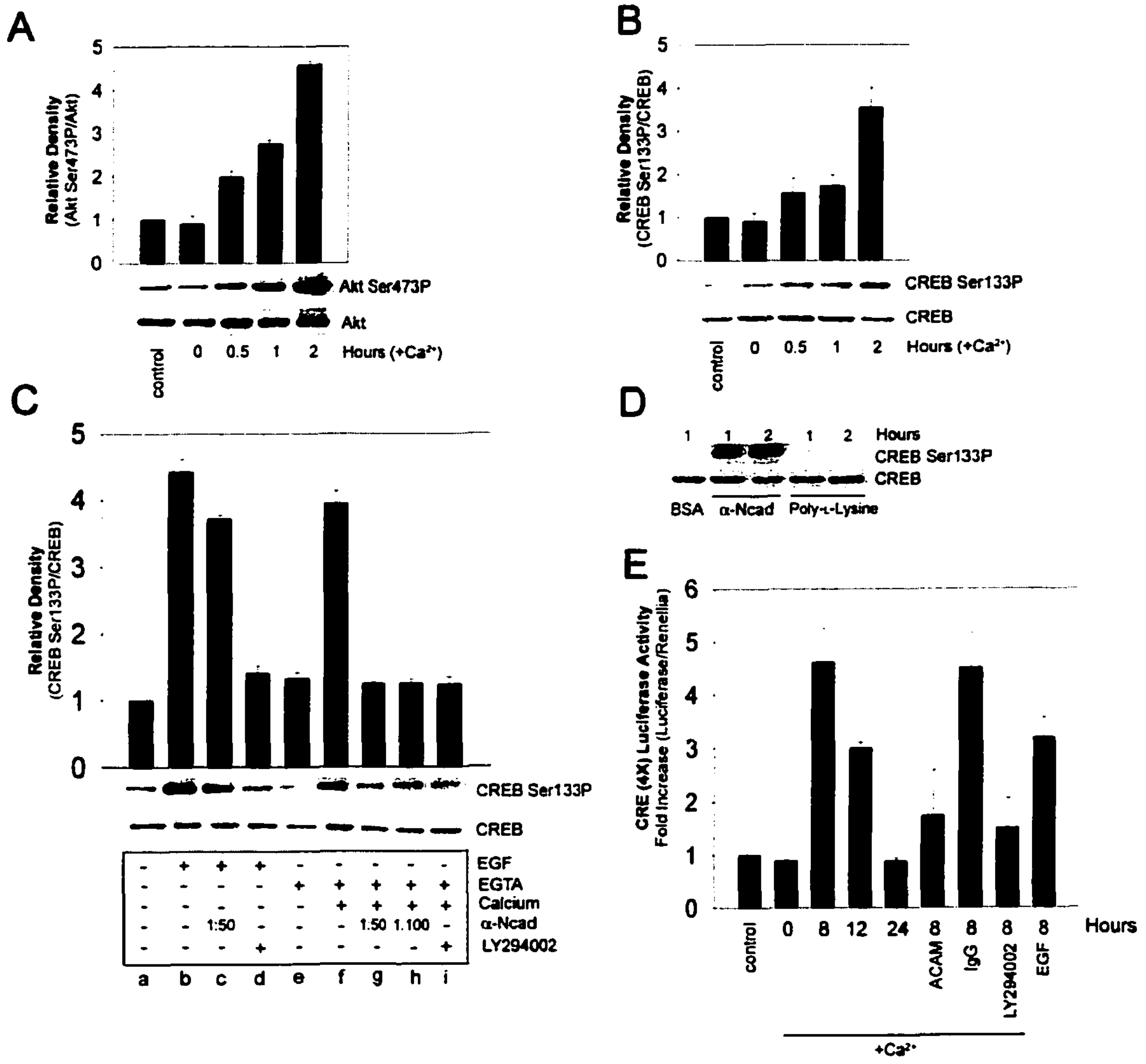
To determine whether N-cadherin mediated adhesion which results in Akt activation could upregulate CREB activity, the phosphorylation of CREB on Ser-133 was measured. Using a Calcium Switch Assay to manipulate cadherin mediated adherens junction reformation, Figure 4.1A shows that upon calcium restoration (1.8 mM) after EGTA treatment (4 mM), Akt phosphorylation on Ser-473 occurred in a time dependent fashion. When the same lysates were analyzed for CREB phosphorylation, the phosphorylation of CREB on Ser-133 (MW=43 kDa) is also increased in a time dependent manner, similar to the time course of Akt Ser-473 phosphorylation. Nearly a three-fold increase in CREB Ser-133 phosphorylation is detected after 2 hours as compared to no calcium addition (Figure 4.1B). To confirm that CREB activation is not due to the manipulation of calcium levels but to the ability of calcium restoration to mimic the physiological engagement of N-cadherin in the adherens junction, a functional blocking antibody to the extracellular domain of N-cadherin was utilized to perturb N-cadherin cell-cell adhesion. As illustrated in Figure 4.1C, pretreatment of PC-3N cells with anti-N-cadherin antibodies prior to calcium restoration results in suppression of CREB phosphorylation on Ser-133 (lane g and h). As a control, serum deprived cells were treated with EGF in the presence and absence of anti-N-cadherin antibody (lane b and c). The presence of an anti-N-cadherin antibody does not effect EGF stimulation of CREB Ser-133 phosphorylation. This indicates that N-cadherin mediated stimulation of CREB Ser-133 phosphorylation requires the homotypic binding of the extracellular domains on adjacent cells to stimulate CREB kinase activity.

To confirm that N-cadherin ligation leads to CREB Ser-133 phosphorylation, anti-N-cadherin antibodies were immobilized onto cell culture dishes (10 μg/ml). Serum deprived cells were detached with EGTA to maintain N-cadherin expression on cell surface and seeded onto N-cadherin antibody immobilized culture dishes. The antibody immobilization experiment has been shown to mimic cadherin receptor activation by causing lateral dimerization and clustering of N-cadherin, which can mimic N-cadherin activation by calcium-dependent homophilic interaction(275, 437). As shown in Figure 4.1D, phosphorylation of CREB on Ser-133 was dramatically induced when cells were seeded on anti-N-cadherin immobilized antibodies as compared to cells seeded on BSA or poly-L-lysine coated cell culture dishes. In addition, pre-treatment of the cells with a PI 3-kinase inhibitor LY294002 (20 μm) or Wortmannin (50 nM) suppresses CREB Ser133 phosphorylation after calcium restoration (Figure 4.1C; lane i). These data suggests that engagement of N-cadherin leads to CREB phosphorylation on Ser133 and is dependent upon the upstream activity of PI 3-kinase.

To determine whether CREB Ser-133 phosphorylation induced by N-cadherin adhesion increases transcriptional activity by binding to a CRE DNA consensus sequence, PC-3N cells were transfected with a luciferase reporter gene containing a TATA box joined to tandem repeats of CRE cis element (4X). Following calcium restoration, CRE luciferase activity can be detected at eight hours following reformation of N-cadherin adhesion junction with approximately a four-fold induction over basal level and decreases over a twenty-four hour period (Figure 4.1E). Cells treated with either an anti-N-cadherin neutralizing antibody or the PI 3-kinase inhibitor LY294002 suppresses CRE driven luciferase activity. This data suggests that N-cadherin mediated CREB Ser-133 phosphorylation increases DNA binding activity and can drive gene expression by interacting with CRE DNA consensus sequence, and that this event is dependent upon the activity of PI 3-kinase.

Figure 4.1: N-cadherin-mediated intercellular adhesion induces CREB activation. Confluent PC-3N cells grown at confluence were serum-starved and treated with 4mM EGTA, and calcium was restored. As controlled, serum starved cells were left untreated (control). After calcium restoration, total cellular protein lysates were separated on a 10% SDS-PAGE and immunoblotted with a polyclonal antibody against phosphospecific Akt Ser473 (A) or phosphospecific CREB Ser133 (B). Relative protein densities of phosphorylated Akt and CREB were normalized to total Akt and CREB respectively. The values were further normalized to untreated serum-deprived cells (control) and are represented as fold of induction over basal phosphorylation level. (C) Serum-deprived confluent PC-3N cells were treated with 4mM EGTA. Fifteen minutes prior to calcium restoration, anti-N-cadherin antibody (anti-ACAM) was added at the indicated dilutions, and calcium was restored for 30 min. As controlled, serum starved cells were left untreated or stimulated with 100 nM EGF for 30 min. In certain experiments, cells were treated with 20 μM of LY294002 for 30 min prior to calcium takeaway. Total cellular proteins were separated on 10% SDS-PAGE and immunoblotted with phospho-specific antibodies to CREB Ser133. Relative CREB Ser133 phosphorylation was normalized to total CREB protein and values were further normalized to untreated controlled cells. Data represents the average of three independent experiments. (D) Antibody immobilization assay. Serum starved PC-3N cells were suspended into single cells and placed on immobilized anti-N-cadherin antibodies, on poly-L-Lysine or BSA coated dishes. Cells were allowed to attach for 1 or 2 hour and gently washed with PBS. Cellular lysates were

subjected to CREB Ser133 phosphorylation analysis using phosphospecific antibodies to CREB Ser133. (E) PC-3N cells cultured in 12-well culture plates to 80%, transfected with CRE (4X) luciferase construct and pTKReneilla. After 16-hour transfection, cells were serum-deprived in calcium free medium (SMEM) for addition 24 hours. Calcium was restored for the indicated time periods. In certain experiments, anti-N-cadherin antibodies (1:50 dilution) were added 15 min prior to calcium addition or treated with LY294002 (20 µM) for 30 min prior to calcium addition. As a control, serum starved cells were treated with 100 nM EGF. At the end of incubation period, cell lysates were prepared for the assay of luciferase and reneilla and data represents fold increases of luciferase to reneilla. The values represent means +/- S.E. of three observations, each being the average of duplicate measurements.


A
Relative Density (Akt Ser473P/Akt)
0
1
2
3
4
5
Akt Ser473P
Akt
control
0
0.5
1
2
Hours (+Ca²⁺)
B
Relative Density (CREB Ser133P/CREB)
0
1
2
3
4
5
CREB Ser133P
CREB
control
0
0.5
1
2
Hours (+Ca²⁺)
C
Relative Density (CREB Ser133P/CREB)
0
1
2
3
4
5
CREB Ser133P
CREB
EGF
EGTA
Calcium
α-Ncad
LY294002
1:50
1:50
1:100
a
b
c
d
e
f
g
h
i
D
1
1
2
1
2
Hours
CREB Ser133P
CREB
BSA
α-Ncad
Poly-L-Lysine
E
CRE (4X) Luciferase Activity Fold Increase (Luciferase/Renellia)
0
1
2
3
4
5
6
control
0
8
12
24
ACAM 8
IgG 8
LY294002 8
EGF 8
Hours
+Ca²⁺

PTEN suppresses CREB activation in response to N-cadherin adherens junction assembly.

In a variety of human cancers including prostate, PTEN is frequently inactivated by somatic mutations(73). Although PTEN functions in dephosphorylating a variety of phosphoproteins, its primary substrates are the 3' phosphoinositides, PtdIns-3,4-P2 and PtdIns-3,4,5-P3(449). Genetic and biochemical studies have demonstrated that PTEN can regulate the activity of PI 3-kinase and subsequently, Akt kinase activity(81, 180). To confirm that CREB Ser-133 phosphorylation by N-cadherin mediated cell-cell adhesion is a result of Akt kinase activity, we utilize a kinase deficient Akt (Akt-KD) expression construct to evaluate CREB Ser133 phosphorylation. PC-3N cells were transfected with either Akt-KD or wildtype Akt (Akt-WT) constructs and subjected to the Calcium Switch Experiment. Upon calcium restoration, cells transfected with the Akt-KD construct had no elevated CREB Ser133 phosphorylation as compared to a two and a half fold increase in CREB Ser133 phosphorylation in empty vector (pcDNA3.1) transfected cells after thirty minutes of calcium addition (Figure 4.2A, compare lane b and d). In contrast, cells transfected with controlled Akt-WT display an increase in CREB Ser-133 phosphorylation (Figure 4.2A; lane c).

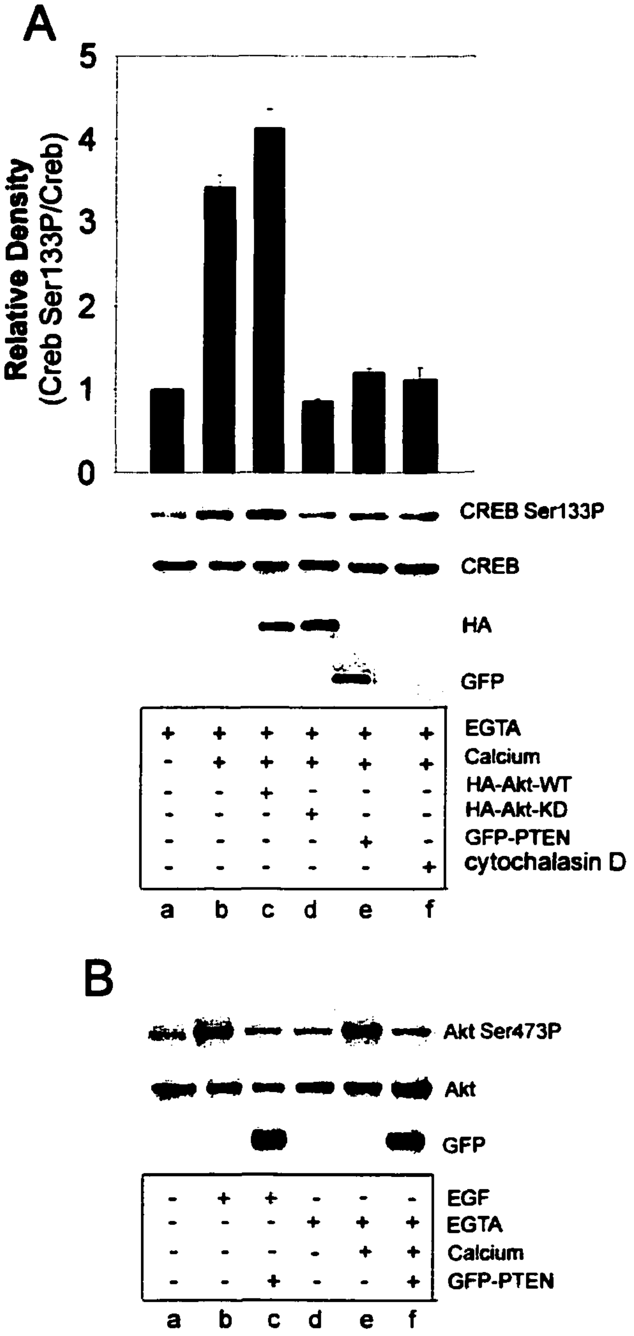
Since PC-3N cells lack PTEN expression(450), we sought to determine whether re-expression of wild-type PTEN, using a GFP-PTEN construct, could suppress the N-cadherin dependent phosphorylation of CREB phosphorylation on Ser-133 by PI 3-K/Akt pathway. As shown in Figure 4.2A (lane e), re-expression of PTEN in PC-3N cells suppresses CREB Ser-133 phosphorylation upon N-cadherin mediated adherens junction reformation. Moreover, re-expression of wildtype PTEN suppresses Akt Ser-473 phosphorylation in both calcium restoration and EGF treatment (Figure 4.2B; lane c and f).

Previous data indicated that N-cadherin mediated Akt activation requires stable N-cadherin/catenin association to the actin cytoskeleton. To determine whether actin cytoskelatal organization at the N-cadherin/catenin complex is also required for CREB phosphorylation on Ser-133, PC-3N cells were treated with 1 μM of cytochalasin D for 15 min prior to calcium addition. As indicated in Figure 4.2A (lane f), disruption of F-actin organization by cytochalasin D prevents N-cadherin mediated CREB Ser-133 phosphorylation. This indicates that in addition to stable homophilic adhesion of the extracellular domain N-cadherin signaling also requires stable association of the cytoplasmic domain with the actin cytoskeleton.

N-cadherin mediated cell-cell adhesion upregulates bcl-2 mRNA expression via an Akt/CREB pathway

Since enhanced CREB DNA binding activity results in the up-regulation of *bcl-2* transcription in response to external stimuli such as growth factor ligand/receptor(441, 451) or integrin/matrix engagement(442), we next determined whether N-cadherin engagement would effect *bcl-2* transcription resulting from the activation of CREB. In Figure 4.3A, the protein expression of CREB Ser-133 phosphorylation upon N-cadherin ligation peaks at eight hours, corresponding to Akt Ser-473 phosphorylation. To determine whether *bcl-2* mRNA expression is also increased, poly(A)RNA was isolated from PC-3N cells after calcium restoration at various time points and analyzed

Figure 4.2: CREB phosphorylation is suppressed by PTEN in response to N-cadherin/PI 3-kinase/Akt signaling pathway. PC-3N cells were transiently transfected with the indicated constructs. After 16 hours of transfection, cells were serum deprived for an additional 24 hours, treated with 4 mM EGTA and calcium was restored for 30 min. Cellular lysates were analyzed for CREB Ser-133 phosphorylation (A) or Akt Ser 473 phosphorylation (B) and relative density was normalized to total CREB and Akt proteins respectively. Fold of CREB Ser-133 phosphorylation in (A) was determined by further normalization of all samples to control, no calcium addition (panel A, lane 1). Relative phosphorylation values represent the average of three independent experiments. Immunoblots were also reacted with antibodies against the epitope-tagged Akt (HA) or PTEN (GFP).

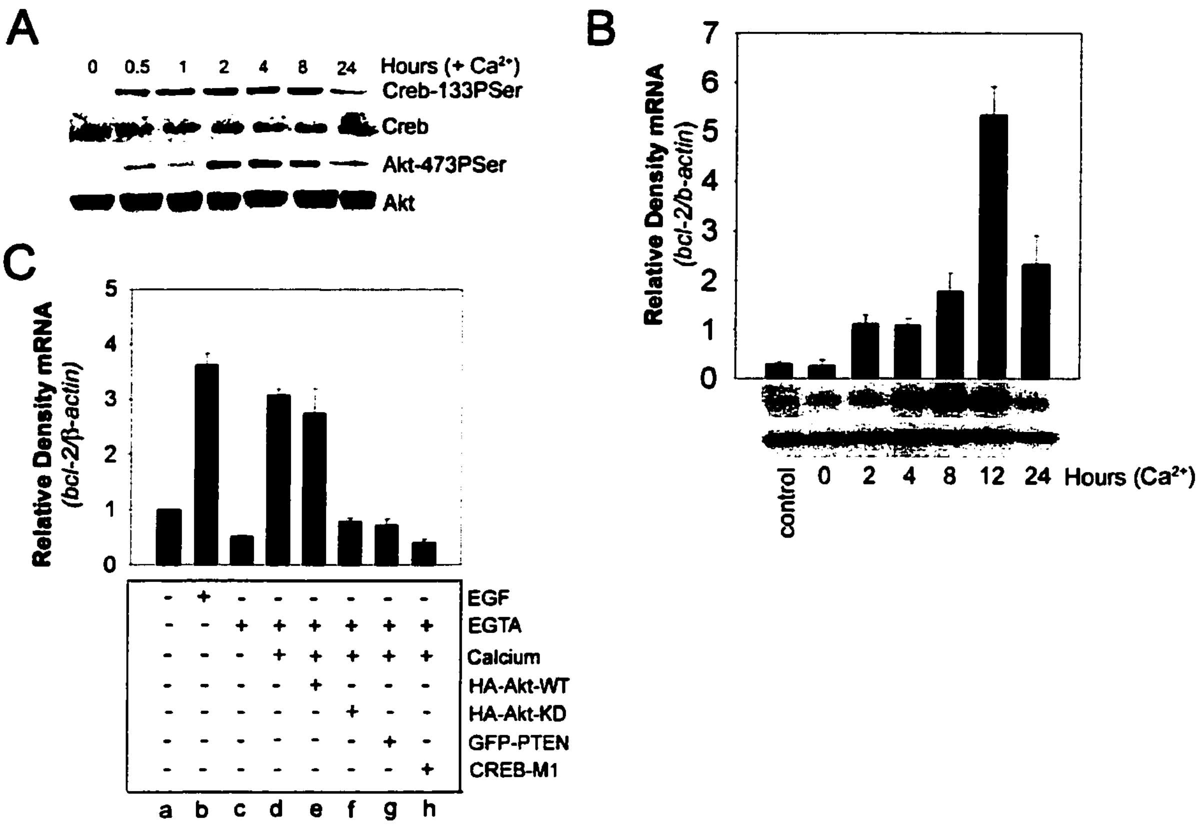

A
Relative Density
(Creb Ser133P/Creb)
0
1
2
3
4
5
CREB Ser133P
CREB
HA
GFP
EGTA
Calcium
HA-Akt-WT
HA-Akt-KD
GFP-PTEN
cytochalasin D
+ + + + + +
- + + + + +
- - + - - -
- - - + - -
- - - - + -
- - - - - +
a b c d e f
B
Akt Ser473P
Akt
GFP
EGF
EGTA
Calcium
GFP-PTEN
- + + - - -
- - - + + +
- - - - + +
- - + - - +
a b c d e f

by Northern blot for *bcl-2* expression. As shown in Figure 4.3B, the expression of *bcl-2* mRNA is increased at 8 hours and peaks at 12 hours following N-cadherin mediated adherens junction reformation, with approximately 6 fold increase. Over-expression of a dominant negative kinase deficient Akt or dominant-negative CREB (CREBM1) suppresses *bcl-2* mRNA expression upon calcium restoration (Figure 4.3C; lane f and h). These results provide evidence that PI 3-kinase/Akt/CREB signaling activity mediates elevation of *bcl-2* mRNA expression. In addition, re-expression of PTEN prevents the induction of *bcl-2* mRNA (Figure 4.3C; lane g). These results suggest that N-cadherin cell-cell adhesion is capable of upregulating *bcl-2* mRNA by a PI 3-K/Akt/CREB dependent pathway.

Bcl-2 promoter activity is positively regulated by the CREB dependent activation of N-cadherin adhesion and protects cells from apoptosis.

Earlier studies have shown that the regulation of *bcl-2* promoter activity is dependent upon the cAMP-response element (CRE)(448, 452). To determine the importance of CRE in N-cadherin mediated *bcl-2* expression, the luciferase reporter driven by the promoter region of *bcl-2* was utilized as described previously(448). Serum deprived cells were transfected with luciferase reporters driven by different sizes of the *bcl-2* promoter sequences. After 16 hours post-transfection, cells were placed in serum-free, calcium-free medium for an additional 24 hours to disrupt cadherin-mediated cell-cell contacts. Cells were then placed in calcium containing medium to restore the adherens junctions for an additional 8 hours. The full-length *bcl-2* promoter sequence from –3934 to –1287 is able to drive expression of luciferase upon calcium restoration

Figure 4.3: Modulation of bcl-2 mRNA expression by N-cadherin mediated cell-cell adhesion. through CREB. (A) Immunoblots of CREB Ser133 and Akt Ser473 phosphorylation upon N-cadherin mediated adhesion junction formation. (B) *bcl-2* mRNA expression upon adherens junction reformation. β-actin was used as loading control. Relative density of bcl-2 mRNA expression is normalized to β-actin. (C) PC-3N cells were transfected with indicated constructs, treated with 4 mM EGTA and calcium was restored for 12 hours. Poly(A)RNAs were extracted blotted for *bcl-2* message. *β-actin* was used as loading control and relative *bcl-2* mRNA expression was normalized to β-actin and presented as fold of induction over controlled unstimulated cells. Values are means +/- S.E. of three independent experiments.


A
0 0.5 1 2 4 8 24 Hours (+ Ca²⁺)
Creb-133PSer
Creb
Akt-473PSer
Akt
B
Relative Density mRNA
(bcl-2/b-actin)
0 1 2 3 4 5 6 7
control 0 2 4 8 12 24 Hours (Ca²⁺)
C
Relative Density mRNA
(bcl-2/β-actin)
0 1 2 3 4 5
- + - - - - - - EGF
- - + + + + + + EGTA
- - - + + + + + Calcium
- - - - + - - - HA-Akt-WT
- - - - - + - - HA-Akt-KD
- - - - - - + - GFP-PTEN
- - - - - - - + CREB-M1
a b c d e f g h

(Figure 4.4A). Truncation of the 5'-end from -3934 to –1640, which retained the CRE site between –1611 and –1526 shows a 37% increase in luciferase activity in promoter activity as compared to full-length *bcl-2* promoter driven luciferase construct. This increase appears to be due to the loss of negative regulatory regions identified by previous studies(453). Mutation of the CRE site in the *bcl-2* promoter showed no luciferase activity similar to the truncated *bcl-2* promoter without the CRE from –1526 to –1287 upon N-cadherin engagement (Figure 4.4A). These experiments clearly show the importance of CRE in *bcl-2* expression upon reformation of the adherens junctions.

To determine whether the increase in *bcl-2* promoter activity is an effect of N-cadherin homotypic adhesion and not a manipulation of calcium, a functional blocking antibody to the extracellular domain of N-cadherin was utilized as described in the previous section. Pre-treatment of cells with N-cadherin antibodies prior to calcium restoration suppresses the expression of a luciferase gene driven by a truncated *bcl-2* promoter containing CRE (-1640 to –1287; Figure 4.4B). However, cells incubated with the irrelevant monoclonal IgG antibodies show a 4-fold stimulation of luciferase activity, similar to no antibody treatment. These experiments suggest that the activity of the *bcl-2* promoter is a direct effect of N-cadherin mediated cell-cell adhesion.

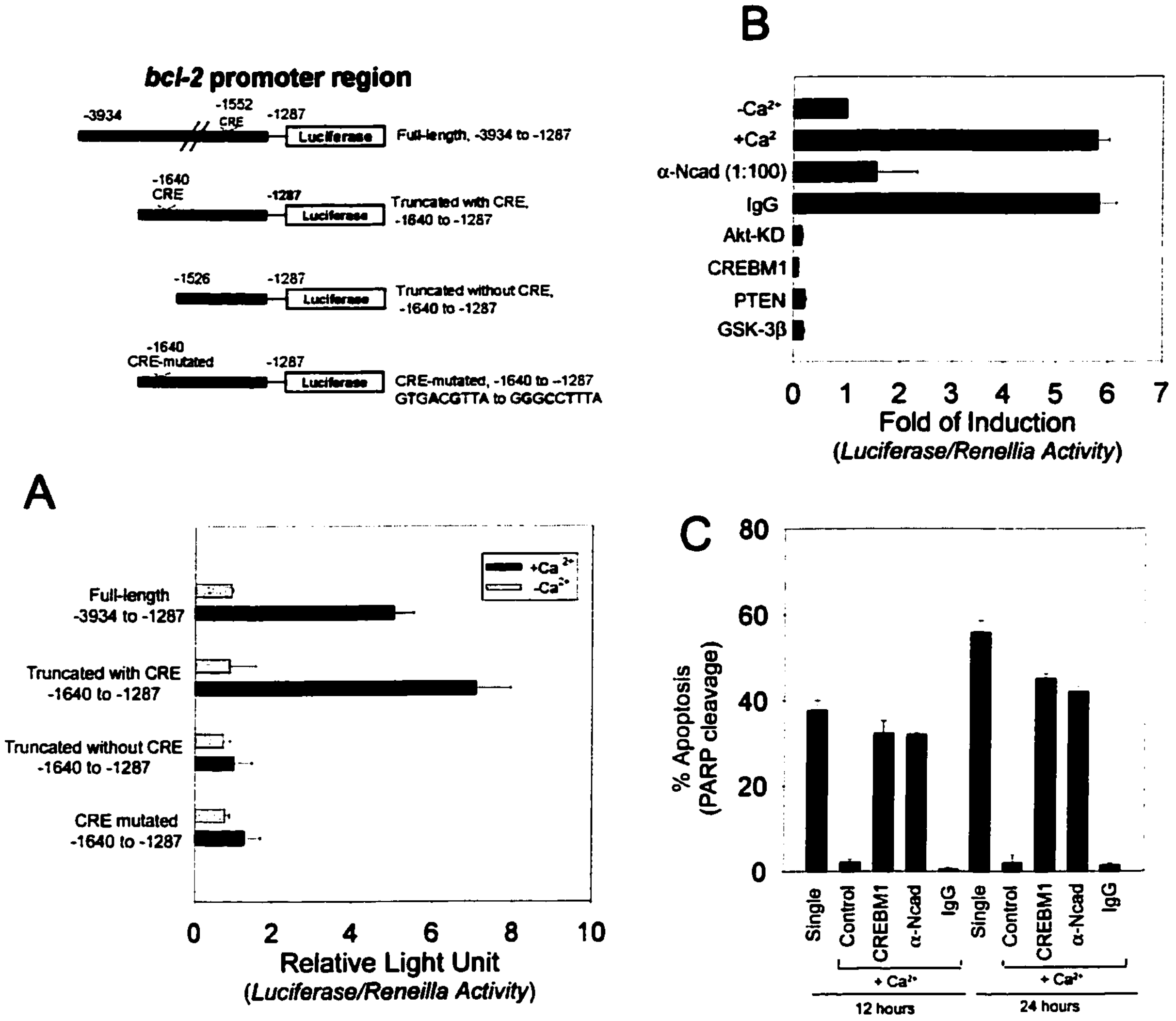
To further examine the role of CREB in the *bcl-2* promoter activity, a dominant-negative CREB mutant construct, CREBM1, was utilized. Cells transfected with the CREBMl constructs, show no induction of the *bcl-2* promoter (-1640 to –1287) containing CRE luciferase activity upon adherens junction reformation (Figure 4.4B). CREBMl suppresses the basal activity of this promoter, along with dominant negative

Akt and wild-type PTEN. This suggests that the activity of *bcl-2* promoter is dependent upon the action of CREB, and the upstream events of Akt and PTEN.

GSK-3β has been previously shown to abrogate CREB functional activity(445). To determine whether GSK-3β can inhibit *bcl-2* promoter activity, cells were transfected with wild-type GSK-3β constructs. Interestingly, no induction of the *bcl-2* promoter activity was observed, and this expression also appeared to suppress the basal activity of the *bcl-2* promoter (Figure 4.4B). This suggests that GSK-3β is able to repress CREB activation of the *bcl-2* promoter.

To determine if the increased bcl-2 transcription upon N-cadherin ligation was able to protect cells from apoptosis, poly(ADP-ribose)polymerase (PARP) proteolytic cleavage in suspended cells were examined. PARP is a DNA repair enzyme that is cleaved by caspase 3 between Asp213 and Gly214 in the late phase of programmed cell death. This produces two fragments of apparent molecular weights of 29 kDa and 85 kDa(407). PC-3N cells were cultured in suspension as single cells in low calcium or multicellular aggregates in normal physiological calcium. When cultured as single cells in suspension, approximately 40% of PARP is cleaved at 12 hr, which is detected by the presence of the 85 kDa PARP protein, and cleavage of PARP increases to 58% after 24 hr (Figure 4.4C). No PARP cleavage was detected in multicellular aggregates. To confirm that cell survival is an effect of N-cadherin mediated cell-cell adhesion, cells were pretreated with an anti-N-cadherin antibody and cell-cell adhesion was initiated by addition of calcium. As shown in Figure 4.4C, the anti-N-cadherin antibody disrupted N-cadherin mediated cell-cell adhesion, and approximately 30% of PARP cleavage is

Figure 4.4: Activation of bcl-2 promoter by N-cadherin engagement. (A) PC-3N cells grown to 80% confluence in 12 well culture dishes were transfected with *bcl-2* reporter constructs and pTKReneilla. Cells were then serum deprived in calcium free medium for 24 hours to disrupt cell-cell adhesion and adherens junction was restored by addition of calcium containing medium. After 8 hours cell lysates were prepared and assayed for luciferase and reneilla activity. (B) The truncated CREB containing *bcl-2* reporter (-1640 to –1287) was cotransfected with constitutively active AktWT, GFP-PTEN, and GSK-3β, dominant negative CREB or AktKD. In certain experiments, after 24 hours of transfection, cells with only the *bcl-2* reporter and pTKReneilla were incubated with anti-N-cadherin antibodies or irrelevant mouse IgG antibodies prior to calcium restoration. Eight hours after calcium restoration, cell lysates were prepared and assayed for firefly luciferase and reneilla activity. Values represent mean +/- S.E. of observations from three independent experiments, each carried out in duplicate. (C) Percent cellular apoptosis was measured by immunoblot analyses of PARP cleavage of PC-3N suspended cultures in the presence or absence of calcium. This is represented by the presence of the cleaved 85 kDa PARP protein (%PARP cleavage=85 kDa/116 kDa + 85 kDa). In certain experiments, cells were treated with an anti-N-cadherin antibody or an irrelevant mouse IgG antibody. For CREB study, cells were transfected with the CREBM1 construct or empty vector (pcDNA3.1; control).


bcl-2 promoter region
-3934
-1552
CRE
-1287
Luciferase
Full-length, -3934 to -1287
-1640
CRE
-1287
Luciferase
Truncated with CRE,
-1640 to -1287
-1526
-1287
Luciferase
Truncated without CRE,
-1640 to -1287
-1640
CRE-mutated
-1287
Luciferase
CRE-mutated, -1640 to -1287
GTGACGTTA to GGGCCTTTA
A
+Ca2+
-Ca2+
Full-length
-3934 to -1287
Truncated with CRE
-1640 to -1287
Truncated without CRE
-1640 to -1287
CRE mutated
-1640 to -1287
0
2
4
6
8
10
Relative Light Unit
(Luciferase/Reneilla Activity)
B
-Ca2+
+Ca2
α-Ncad (1:100)
IgG
Akt-KD
CREBM1
PTEN
GSK-3β
0
1
2
3
4
5
6
7
Fold of Induction
(Luciferase/Renellia Activity)
C
80
60
40
20
0
% Apoptosis
(PARP cleavage)
Single
Control
CREBM1
α-Ncad
IgG
Single
Control
CREBM1
α-Ncad
IgG
+ Ca2+
+ Ca2+
12 hours
24 hours

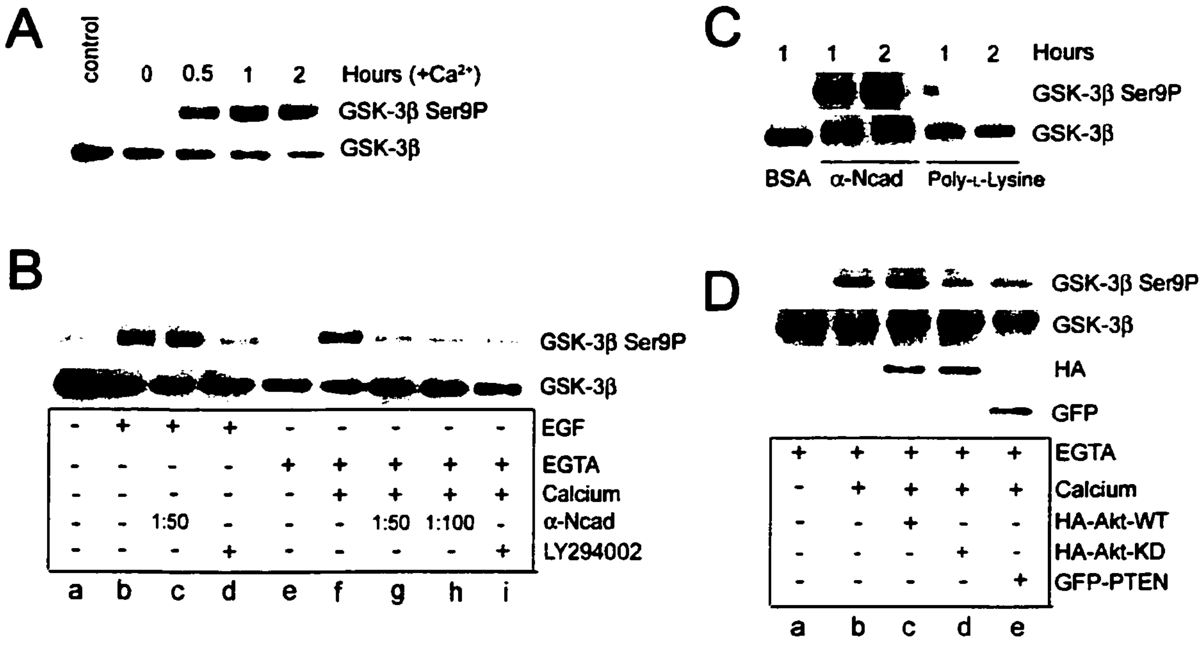
observed after 12 hours and 40% PARP cleavage after 24 hours. No PARP cleavage is observed when cells are treated with irrelevant mouse IgG. To confirm that CREB plays a functional role in N-cadherin mediated cell survival, cells were transfected with a dominant negative CREBM1. Although transiently transfected cells expressing CREBM1 are able to form cell-cell aggregates (data not shown) they were unable to induce cell survival as represented by 30 % PARP cleavage after 12 hours and 45% after 24 hours (Figure 4.4C). These data indicate that N-cadherin mediated cell-cell adhesion induces cell survival and is dependent upon the functional activity of CREB.

Inactivation of GSK-3β by N-cadherin mediated cell-cell adhesion by phosphorylation of GSK-3β on Ser9

In the Wnt signaling pathway, one of the major substrates of Akt is GSK3β. Akt phosphorylates GSK3β at Ser-9, thus inactivating GSK-3β function. To determine whether N-cadherin dependent Akt activation induces GSK-3β phosphorylation on Ser-9, the protein level of GSK-3β Ser-9 phosphorylation was assessed using a polyclonal antibody that recognizes GSK-3β Ser-9 phosphorylation. Serum deprived cells upon calcium restoration showed an increase in the level of phosphorylation on Ser-9 of GSK-3β in a time dependent manner as shown in Figure 4.5A. To confirm that inactivation of GSK-3β is a result of N-cadherin engagement, cells were pretreated with anti-N-cadherin antibodies prior to calcium restoration. As shown in Figure 4.5B, anti-N-cadherin antibodies suppress the phosphorylation of GSK-3β on Ser-9 upon calcium restoration, and do not affect EGF dependent induction of GSK-3β Ser-9 phosphorylation (lane g and h), whereas N-cadherin clustering antibody assay induces high level of GSK-3β Ser-9 phosphorylation (Figure 4.5C). Moreover, the inhibition of PI 3-kinase activity with LY294002 compound suppresses GSK-3β phosphorylation on Ser-9 (Figure 4.5B; lane i). These data suggests that N-cadherin mediated cell-cell adhesion results in the inactivation of GSK-3β by phosphorylation of GSK-3β on Ser-9 residue.

To further determine whether GSK-3β Ser-9 phosphorylation by N-cadherin mediated cell-cell adhesion is due to N-cadherin dependent Akt kinase activity, PC-3N cells were transfected with the Akt-KD construct. Upon re-addition of calcium, induction of GSK-3β Ser-9 phosphorylation was not detected in cells transfected with Akt-KD (Figure 4.5 D; lane d), as compared to cells transfected with Akt-WT (Figure 4.5D; lane c) or empty control vector (Figure 4.5D; lane b). In addition, re-expression of PTEN results in the suppression of GSK-3β Ser-9 phosphorylation (Figure 4.5D; lane e). Thus, the activation of PI-3 kinase/Akt signaling pathway by N-cadherin mediated cell-cell adhesion leads to the inactivation of GSK-3β by phosphorylation of GSK-3β on Ser-9.

Figure 4.5: N-cadherin engagement induces GSK-3β phosphorylation on Ser9. (A) Induction of GSK-3β Ser9 phosphorylation upon adherens junction reformation detected by phosphospecific antibody to GSK-3β Ser9. (B) Serum starved PC-3N cells were treated with 4mM EGTA for 40 min. Prior to calcium addition, cells were treated with either anti-N-cadherin antibodies at the indicated dilution (15 min pre-treatment) or LY294004 (20uM; 30 min pre-treatment). Cellular lysates were analyzed for GSK-3β phosphorylation on Ser 9 using phosphospecific antibodies to GSK-3β Ser9. (C) Serum deprived PC-3N cells were lifted with EDTA. Single suspended cells were seeded on top of plates coated with anti-N-cadherin antibody, BSA, or poly-L-Lysine. Attached cells were lysed and analyzed for GSK-3β phosphorylation. (D) PC-3N cells grown to 80% confluency were transfected with AktWT, AktKD, or GFP-PTEN. As a control cells were transfected with pcDNA3.1 empty vector. After 16 hours of transfection, cells were serum deprived for additional 24 hours, treated with 4 mM EGTA and calcium as restored for 30 min. Cell lysates were analyzed for phosphorylation of GSK-3β.


A
control
0 0.5 1 2 Hours (+Ca²⁺)
GSK-3β Ser9P
GSK-3β
B
GSK-3β Ser9P
GSK-3β
EGF - + + + - - - - -
EGTA - - - - + + + + +
Calcium - - - - - + + + +
α-Ncad - - 1:50 - - - 1:50 1:100 -
LY294002 - - - + - - - - +
a b c d e f g h i
C
1 1 2 1 2 Hours
GSK-3β Ser9P
GSK-3β
BSA
α-Ncad
Poly-L-Lysine
D
GSK-3β Ser9P
GSK-3β
HA
GFP
EGTA + + + + +
Calcium - + + + +
HA-Akt-WT - - + - -
HA-Akt-KD - - - + -
GFP-PTEN - - - - +
a b c d e

Discussion

Cell survival signals, particularly the PI 3-kinase/Akt signaling pathway are involved in the survival effects of many growth factors, some transformed oncogenes, and cell-matrix interactions mediated by integrins(167, 454-457). Recent findings demonstrate that cell-cell adhesion mediated by cadherins can initiate outside-in signaling through a PI 3-kinase/Akt dependent pathway(275, 437). Disruption of N-cadherin adhesion has been shown to reduce apoptosis in normal and cancer cells(458-460). From my previous work, activation of kinase activity of Akt dependent upon N-cadherin ligation resulted in the phosphorylation of BAD on Ser-136, thereby stabilizing Bcl-2 protein function and promoting cell survival(437). The present study provides evidence that the activity of another cellular substrate of Akt, CREB, is involved in mediating cell survival upon N-cadherin ligation. In addition to the post-translational modification of the apoptotic machinery by Bcl-2 protein stability, the N-cadherin dependent PI 3-kinase/Akt pathway can upregulate *bcl-2* mRNA expression at a transcriptional level as a result of CREB activation.

The transcriptional activation of CREB is dependent on phosphorylation of Ser133 by such proteins as protein kinase A (PKA), Ca2+-activated calmodulin kinases, ribosomal S6 kinase2, mitogen-activated protein-kinase-activated protein kinase 2, and Akt(461). In our study, it appears that the CREB dependent transcriptional regulation of *bcl-2* is a direct result of the PI 3-kinase/Akt pathway since pharmacological inhibitors to MEK, p38 MAP kinase, and p70 S6 kinase do not suppress CREB Ser133 phosphorylation upon N-cadherin ligation (Figure 4.6). In contrast, phosphorylation of

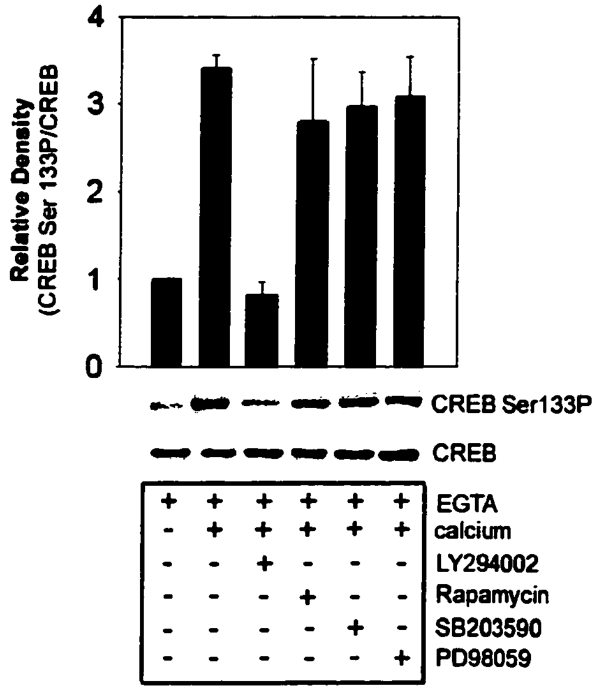


Figure 4.6: N-cadherin ligation induces CREB phosphorylation through a direct PI 3-kinase/Akt pathway. Serum deprived PC-3N cells were treated with various pharmacological inhibitors to PI 3-kinase (LY294002; 20 μM), MEK (PD98059; 40 μM), p38 MAPK kinase (SB203590, 10 μM), and p70 S6 kinase (Rapamycin 10 ng/ml) or control vehical (DMSO; lane 2) for 30 min. prior to Calcium Switch Assay. Cells were then treated with 4 mM EGTA to chelate exogenous calcium for 40 min and adherens junction was allowed to reform for 30 min by the addition of medium containin calcium (1.8 mM). Total cellular lysates were then immunoblotted for CREB Ser-133 phosphorylation and CREB. Fold of CREB Ser-133 phosphorylation is determined by normalizing relative CREB Ser-133 phosphorylation to endogenous CREB. These values were further normalized to no calcium addition control (lane 1). Values are means +/- S.E. of three independent experiments.

CREB at Ser-133 is decreased by treatment of cells with an inhibitor to PI 3-kinase, and expression of a dominant negative kinase deficient Akt. A study by Du and Montminy(439) provides evidence that Akt can directly phosphorylate CREB on Ser-133 *in vitro* and promote the recruitment of the coactivator CREB-binding protein. In addition, using the GAL4 CREB construct, Du and Montminy(439) showed that the induction of the CRE-driven gene expression by Akt could be suppressed by a serine to alanine mutation in CREB at Ser-133.

Previous studies indicate that the function of CREB is important in promoting cell survival. For instance, targeted disruption of the CREB gene results in a defect in spermatogenesis secondary to germ cell apoptosis(462-464). In addition, suppression of CREB function with a dominant-negative CREB induces apoptosis in T cells in response to growth factor stimulation(465). A recent study by Pugazhenthi *et al.*(441) showed that enhanced CREB activity can increase *bcl-2* transcription expression levels in cells stimulated with insulin-like growth factor-1, providing evidence by which CREB can promote cell survival. This study is supported by the fact that the *bcl-2* promoter region contains a CRE site, and the identification of CREB as a positive regulator of *bcl-2* expression(448, 452). In addition, integrin-matrix interactions were recently shown to enhance *bcl-2* transcription. Matter and Ruoslahti(442) showed that signals from the α5β1 and αvβ3 integrins supports cell survival on fibronectin and increases *bcl-2* transcription. It is interesting to note that this integrin-mediated regulation of *bcl-2* transcription appears to be Shc/FAK/RAS/PI 3-kinase driven pathway, and even though the MAPK is active, the MAPK pathway does not mediate *bcl-2* transcription.

Furthermore, re-expression of PTEN in PC-3N cells also suppresses not only CREB phosphorylation on Ser-133 but also *bcl-2* transcription upon N-cadherin mediated cell-cell adhesion. Somatic mutation of PTEN is a common event in diverse human cancers including prostate cancer(78, 79). Recent studies suggest that the lipid phosphatase activity in PTEN mediates tumor suppression(82, 180, 181). PTEN lipid phosphatase catalyzes the dephosphorylation of phosphatidylinositol 3,4,5-triphosphate [PI93,4,5)P3](449), resulting in the inactivation of the downstream Akt activity(180, 181). Re-expression of PTEN in PTEN-null prostate cancer cells induces cell cycle arrest and apoptosis(81, 181) by inhibition of Bcl-2 expression(466). In prostate cancer, loss of PTEN expression and Bcl-2 over-expression occurs frequently and both events are associated with androgen-independent prostate cancer(77, 186). Since N-cadherin expression is also detected late in prostate cancer progression(302), it is possible that the increase in Bcl-2 expression is directly related to N-cadherin adhesion in malignant prostate carcinoma in conjunction with alteration in PTEN expression.

In addition to CREB, Akt targets other cytosolic proteins for phosphorylation such as GSK-3β at Ser-9(467). Phosphorylation of Ser-9 residue of GSK-3β results in its inability to inhibit the activity of several transcription factors, including CREB. GSK-3β phosphorylation of CREB on Ser-129 occurs after CREB is phosphorylated at Ser-133, and this results in decreased ability of CREB to bind to the somatostain gene CRE(445). This study demonstrates that the phosphorylation of GSK-3β on Ser-9 upon N-cadherin ligation is dependent on the activities of PI 3-kinase/Akt. The inactivation of GSK-3β enhances the CREB driven transcription of *bcl-2* given that over-expression of wildtype

GSK-3β suppresses N-cadherin mediated CREB dependent transcription of *bcl-2*. Thus, the regulation of *bcl-2* mRNA by 'Outside-In' signaling of N-cadherin requires both the activation of CREB and the inactivation of GSK-3β by Akt.

In summary, this chapter provides evidence that the engagement of N-cadherin in prostate carcinoma cells can regulate *bcl-2* transcription through a CREB dependent manner as depicted in Figure 4.7. It appears that the activation of CREB upon N-cadherin ligation is through a distinct PI 3-kinase/Akt signaling pathway. It has recently been described that both matrix-derived signals and growth factor regulation of cell survival *in vitro* can converge in a pathway involving PI 3-kinase and Akt. This study provides evidence that cell-cell adhesion mediated by N-cadherin also activates this particular pathway to regulate *bcl-2* transcription through the CREB transcription factor, thus, providing a mechanism by which cell-cell contact regulate cell survival.

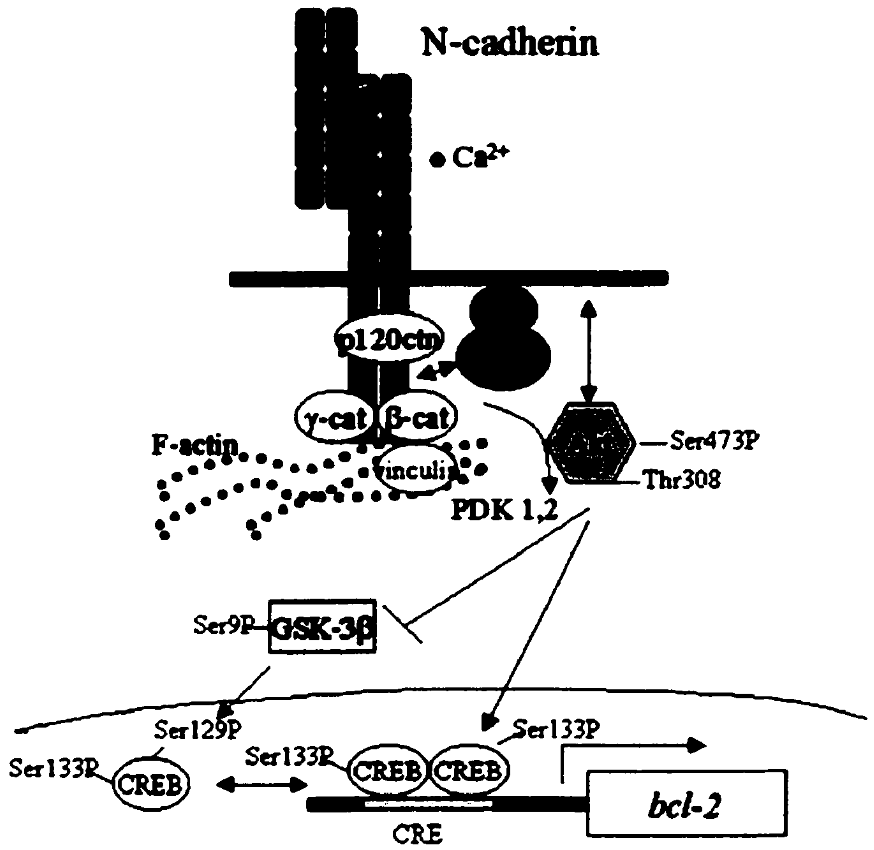

N-cadherin
Ca²⁺
p120ctn
γ-cat
β-cat
F-actin
vinculin
Ser473P
Thr308
PDK 1,2
Ser9P
GSK-3β
Ser129P
Ser133P
CREB
Ser133P
CREB
CREB
Ser133P
CRE
bcl-2

Figure 4.7: Illustrated model summarizing the regulation of *bcl-2* trancription by N-cadherin engagement. Ligation of N-cadherin induces a rapid PI 3-kinase/Akt activation and subsequently CREB phosphorylation on Ser-133. Activation of CREB results in the binding of CREB to the CRE DNA consensus site of the *bcl-2* promoter to drive *bcl-2* transcription. The transcriptional activity of CREB and subsequently transcription of *bcl-2* is also dependent upon Akt phosphorylation of GSK-3β on Ser-9 upon N-cadherin engagement, thus inactivating the kinase activity of GSK-3β.

CHAPTER 5: REGULATION OF CADHERIN EXPRESSION IN MULTICELLULAR PROSTATE SHEROIDS

Introduction

Classical cadherins constitute a family of transmembrane glycoproteins that mediate calcium-dependent homotypic cell-cell adhesion and play an important role in the maintenance of normal tissue architecture(468). These proteins consist of a long extracellular domain, a transmembrane domain, and a short and highly conserved cytoplasmic domain. The cytoplasmic domain is essential for association with catenins, which link cadherins to the actin cytoskeleton(469, 470). This linkage is required for full cadherin adhesive activity, in which either β-catenin or plakoglobin binds directly to the cadherin and α-catenin. In turn, α-catenin is able to interact directly or indirectly with the actin cytoskeleton(387, 417, 471-473). The ability of cadherins to self-associate and interact with the actin cytoskeleton enables these proteins to mediate both the cell recognition required for cell sorting and the strong cell-cell adhesion need to form tissues.

In the last decade, there has been a growing number of new cadherins identified. As a result, the cadherin superfamily is divided into two sub-groups, type I and type II, based on their overall sequence similarities and the conversation of several motifs and aromatic amino acid residues in their extracellular domains. The human classic cadherins E-, N-, P- cadherin, and cadherin-4 and -5 have been classified as type I, while cadherin-6, -8, -11, -12, and –14 are classified as type II (328, 347, 474-479). The type I classic cadherins have been well characterized both functionally and structurally. By contrast, there is little characterization of the non-type I, and it is not clear whether they behave as classical cadherins due to the fact that they are expressed in loosely associated cells(319).

The downregulation or loss of E-cadherin and the catenins are proposed to be a critical step in the development of cancers of epithelial origin(282, 435). Downregulation or lost of E-cadherin expression is common in various tumors, including stomach, colon, head and neck, bladder, breast, and prostate(436, 480-484). Both genetic and epigenetic alterations in the E-cadherin/catenin complex contribute to the modulation in expressions of these proteins (480, 485-487). Furthermore, numerous studies have demonstrated the importance of the E-cadherin/catenin complex in maintaining the normal phenotype of epithelial cells (319). Inhibition of E-cadherin activity with function perturbing antibodies altered the morphology of MDCK cells and promote cellular invasion in vitro(217). Re-expression of E-cadherin into invasive carcinoma, lacking E-cadherin, results in a suppression of cell motility and invasion(85). These studies support the role of E-cadherin as an 'invasion suppressor gene'.

Differential E-cadherin expression has also been shown to correlate with tumor grade. Human prostate carcinoma tissues with a Gleason score above 6 have been shown to have decrease E-cadherin immunoreactivity, whereas, no E-cadherin expression is found in Gleason score 9 and higher(86). Analysis of advance prostate carcinoma tissues also reveals a gain of another cadherin, N-cadherin. N-cadherin expression can be detected in tumors with Gleason score above 7(302). Prostate carcinoma cell lines in culture also show differential cadherin expression, displaying more than one type of cadherin. For example, certain cell lines such as PC-3 cells possess type II cadherins, notably cadherin-6 and –11, in addition to the type I cadherins, E- and N-cadherin(302, 302, 303, 303). It has been suggested that unlike E-cadherin, N-cadherin and cadherin-11 may promote motility and invasion in carcinoma cells. Over-expression of N-cadherin or cadherin-11 in E-cadherin expressing breast carcinoma cell line promotes cellular migration and invasion(273, 488). In addition, expression of N-cadherin by prostate carcinoma cells lines correlates with invasion and suggests that invasion is mediated by N-cadherin-mediated interactions between the prostate cancer cells and stromal cells(302, 303).

Stromal-epithelial interactions are vital to the development, proliferation, and spread of prostate cancer(489). Numerous studies have established that communication between tumor cells and their surrounding stroma contributes to the growth and dissemination of prostate cancer(197, 355, 489). These interactions are necessary for the maintenance of the functional integrity of the normal adult prostate gland. Abnormalities in the constituents of the stromal-epithelial milieu or aberrations in their interactions can induce genomic instability, enhance tumor cell proliferation, and enhance metastasis and progression to an androgen-independent state(490). One of the key mediators of a stromal induced prostate carcinoma response is the transforming growth factor (TGF-β) superfamily. Members of the TGF-β superfamily include TGF-βs, activins and bone morphogenetic proteins (BMPs). This family regulates growth, differentiation, and epithelial transformation in the multistep processes of tumorigenesis, wound healing, and embryogenesis. Several reports have shown the elevation of TGFβ-1 in human prostate cancer(491, 492). Although TGF-β acts as a tumor suppressor during early benign stages of carcinoma, it potently aggravates the malignancy at later stages. TGF-β1 has been shown to down-regulate E-cadherin expression through the regulation of a zinc finger transcription repressor, Snail or SIP1, which binds the E boxes in the E-cadherin promoter(493, 494). This down-regulation of E-cadherin is accompanied by an epithelial to mesenchymal cell transition, in which cells acquire a spindle-shaped morphology, delocalize E-cadherin from cell junctions, and elevate N-cadherin expression levels. These cellular changes are hallmarks of the dedifferentiation in mammary and prostate epithelium in cell culture and in tumor invasion(273, 302, 303).

Many groups have implicated alterations in cell-matrix interactions mediated by integrins in prostate cancer progression, invasion and metastasis(90, 207, 208, 495). For example, the laminin receptor of α6β1 is up regulated during the invasive stages of prostate carcinoma. Conversely, α6β4, which is usually located basolaterally in the hemidesmosomes of prostate epithelial cells, is often lost in prostate cancer(207). Interestingly, recent evidence suggests that the activities of integrins and cadherins are linked. For instance, the use of anti-E-cadherin antibodies in terminally differentiating keratinocytes has been shown to prevent the loss of α5 and β1 integrins(283). Moreover, expression of an E-cadherin dominant-negative construct in keratinocytes resulted in reduced levels of α2β1 and α3β1 integrin expression, while blocking β1 and β3 integrin activities through functional blocking antibodies resulted in N-cadherin-dependent clustering of quail embryo neural crest cells(285). For example, the upstream events that lead to a downregulation of E-cadherin activity in cells breaking away from the primary tumor mass are not fully known. Similarly, how the increased involvement of integrins is initiated in cell-substrate interactions during increased motility is also not know. However, it is postulated that these two events may interconnect, since more evidence is emerging that there may be cross-talk between cadherins and integrins. Thus, it seems that in normal cells alterations in the integrin activities can have substantial effects upon the activity, level and function of cadherins under certain circumstances. These examples of the cross talk between cadherins and integrins may provide insight into the metastatic behavior of cancer cells.

In this chapter, we utilized the multicelllular spheroid (MCS) model to examine coordinate changes in the expression of E-cadherin and N-cadherin cell-cell adhesion sytem mediated by intrinsic events of tumor microenvironment such integrin signaling or metabolic stress. Here, we provide evidence that the regulation of E- and N-cadherin in PC-3 cells may in part be generated from β1 integrin signaling. When PC-3 cells are grown as spheroids, E-cadherin expression is highly up regulated, whereas N-cadherin expression is repressed. Conversely, when PC-3 cells are grown as subconfluent monolayer, N-cadherin is highly expressed whereas E-cadherin and $p27^{kip}$ expression is suppressed. When β1 integrin function in PC-3 monolayers is blocked through the use of a dominant negative β1 cytoplasmic tail, an overexpression of E-cadherin and suppression of N-cadherin is seen. Thus, the regulation of E- and N-cadherin in spheroids, in part, appears to be dependent upon signals generated from β1 integrin. In addition, MCS have been used to investigate the gene regulation in response to hypoxia. These genes include VEGF, which reflect a shift to an angiogenic phenotype. This shift promotes both tumor growth and metastasis. Type II cadherins are also up regulated in

PC-3 spheroids, including both cadherin 6 and cadherin 11. Their expression appears to be dependent upon hypoxic signals, generated from either spheroid culturing or molecular agents that mimic hypoxic conditions in monolayer cell culture. However, hypoxia alone is not sufficient to induce the vascular endothelial growth factor (VEGF) expression in a MCS system. A co-regulatory factor is required. Evidence provided in this chapter showed that the TGFβ-1 family members such as TGF-β1 and BMP-4, secreted by prostate stromal fibroblasts, act as co-factors in the regulation of VEGF expression in prostate cancer. The objective of the present study is to examine the effect of tumor microenvironment on the regulation of cadherin mediated cell-cell adhesion.

Materials and Methods

Cell Culture:

The human prostate carcinoma cell line PC-3, isolated from a metastatic lesion in the bone(336), was obtained from ATCC (Manassas, VA). Human prostate-derived stromal fibroblasts (PSF) were isolated and cultured as previously described(303). PC-3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS; Intergen, Purchase, NY) and penicillin/streptomycin in a 37°C, 5% CO_2 atmosphere at constant humidity.

For formation of multicellular spheroids, 70-80% confluent PC-3 cells were detached with 5 mM EDTA in calcium/magnesium free phosphate buffered saline (CMF-PBS) and plated at a density of $1X10^5$ cells/cm^2 in tissue culture dishes. The dishes were previously coated with 50 µg/ml poly-hema (Sigma Chemical Co., St. Louis, MO). Cells were grown in suspension for 7-10 days to ensure large spheroid formation.

For PC-3 spheriods and PSF co-culture, PSF cells (1X10^5 cells/cm^2) were seeded on top of the Transwell plates (0.4 micron pores, 24mm diameter; Corning Coster Corporation). Transwells were previously coated with 0.1% gelatin at 37°C for 60 min. PSF cells were grown in the presence of serum for 24 hours then serum-deprived for 48 hours. Serum-deprived PC-3 spheriods (48 hours) were then added to the bottom of the poly-hema coated transwell culture dish. RNA isolation was carried out after 24 hours of co-culture.

RNA Extraction and Northern Blot Analysis

Total RNA was prepared from cultured cells by acid guanidinium thicyanate-pheno-chloroform extraction (343). Twenty micrograms of total RNA were separated on a 1% agarose gel containing 1.85% formaldehyde and transferred to Hybond N+ nylon membrane (Amersham Life Science, Arlington Heights, IL) and cross-linked by ultraviolet light. The blots were probed overnight as previously described(303) at 42°C with a random primed α-^{32}P-labeled probe of either the 400-bp fragment of human VEGF, 1.7-kb SmaI fragment of mouse E-cadherin (GenBank Accession X06115), 300-bp EcoRI human N-cadherin fragment (GenBank Accession X54315), rat Cadherin-11, rat-cadherin-6. Blots were washed for 30 minutes at temperature ranging form 42°C to 56°C using the following conditions: 2X SSC/0.1% SDS, 0.3X SSC/0.1% SDS, and 0.1X SSC/0.1% SDS. Blots were then exposed to X-OMAT AR film (Kodak, Rochester, NY).

A 1.2-kb PstI fragment of human GAPDH (GenBanK Accession JO4038) was used for normalization of loading and quantification.

Antibodies and Reagents:

Mouse monoclonal antibodies used in this experiments are as followed: E-cadherin clone HECD-1 was purchased from Zymed Laboratories (San Francisco, CA). N-cadherin clone 32 and β-catenin clone 14 were obtained from Transduction Laboratories (Lexington, KY), and monoclonal antibodies recognizing both keratin 8 and 18 was obtained from Dr. Ray Nagle (University of Arizona, Tucson, AZ). Rabbit polyclonal antibodies to $p27^{kip}$ clone C19 and cyclin D clone H292 were purchased from Santa Cruz Biotechnology (Santa Cruz, Ca). The secondary antibody Cy-3 conjugated affinipure goat anti-mouse IgG was obtained from Jackson ImmunoResearch Laboratories, Inc., (West Grove, PA) and goat anti-mouse and anti-rabbit IgG + HRP conjugated were purchased from Promega (Madison, WI). The adenovirus expressing either the mutant or wildtype cytoplasmic domain of β_{1a} fused with the TAC subunit of the human IL-2 receptor (TAC-β_{1a}, Adβ_{1a} respectively) was obtained from Dr. Joseph C. Loftus (Mayo Clinic Scottsdale, Scottsdale, AZ).

SDS-PAGE, Western Blotting and Immunohistochemistry:

Cells were washed with CMF-PBS containing 1 mM phenylmethslsulfonyl fluoride (PMSF) and lysed in 2X SDS sample buffer (0.25 mol/L Tris-HCL, pH 6.8, 10% SDS, 25% glycerol) containing 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.7 μg/ml pepstatin, 1 mM PMSF and 20 mM NaF. Thirty micrograms of total cellular proteins were separated by SDS/PAGE and transferred at 4C overnight to nitrocellulose membranes. The membranes were then probed with specific primary antibodies, followed with peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG. Bound proteins were detected by incubation with a chemiluminescent substrate (SuperSignal, Pierce, Rockford, IL) according to manufacture protocol and exposed on X-OMAT AR film (Kodak, Rochester, NY). For immunofluorescence, PC-3 spheroids were embedded in Tissue-Tek O.C.T. Compound (Sakura Finetechnical Co., Torrance, CA), snap frozen by liquid nitrogen, and stored at –80°C. Frozen sections of 15-20 micron thick were placed onto poly-L-lysine-coated slides and fixed with 4% (w/v) paraformaldehyde for 5 min, and permeabilized on ice with cytoskeletal stabilization buffer (CSK buffer; 0.5 % Triton X-100, 10 mmol/L PIPES, pH 6.8, 50 mmol/L NaCl, 3 mmol/L $MgCl_2$, 0.3 mol/L sucrose). Immunohistochemistry was carried out as previously described(303).

RT-PCR Analysis

RT-PCR was performed using 1 µg of total RNA, isolated using the guanidinium isothiocyanate method (Chomczynski P, 1987). The following primers were used: BMP-4 sense, 5'-CCT AAG CAT CAC TCA CA GCG-3'; BMP-4 antisense, 5'-GTT CAG TGG GCA CAC AAC AG-3'; TGFβ1 sense, 5'-GCC CTG CAC ACC AAC TAT TGC T-3'; TGFβ1 antisense 5'-AGG CTC CAA ATG TAG GGG CAG G-3'; β-actin sense, 5'-CTT CTA CAA TGA GCT GGG TG-3'; β-actin antisense, 5'-TCA TGA GGT AGT CAG TCA GG-3'. cDNA synthesis and PCR was performed as described previously(303). The PCR product was separated by a 4% agarose gel electrophoresis (3% Nusieve GTG agarose and 1% Seakem ME agarose, FMC BioProducts, Rockland, ME).

Results

Differential expression of E- and N-cadherin in PC-3 multicellular spheroids and monolayer culture

The human prostate adenocarcinoma cell line, PC-3, displays a distinct cellular morphology consisting of both epitheloid and mesenchymal characteristics with loose cell-cell contacts and numerous cellular processes(336). There are two sub-populations within PC-3, in which one population expresses E-cadherin and display an epithelial differentiated phenotype, whereas the second population possesses a more scattered spindle-shape mesenchymal phenotype consisting of N-cadherin expression(303, 336, 350). Previously, PC-3 cells were shown to display equal protein expression of E-cadherin and N-cadherin(303). To examine the regulation of these cadherins during the progression of prostate carcinoma, PC-3 cells were grown as multi-cellular spheroids (MCS) for seven to ten days in suspension. As shown in Figure 5.1, PC-3 cells formed smooth and irregular spheroids, containing approximately 5,000-7,000 cells/spheroid, in the presence and absence of serum. However, in the presence of serum, the spheroids appear to be denser compared to spheroids grown in the absence of serum (Figure 5.1 panel A&B). Interestingly, sections of spheroids grown in the absence of serum display a hollow, glandular morphology similar to epitheloid glands observed in prostate tissue. Immunolocalization of β-catenin shows β-catenin staining at cell-cell contacts and suggest that cell-to-cell contacts were greater and tighter than cells grown as monolayer culture (Figure 5.1 panel c&d). Since increased cell-cell contacts were observed in MCS, cadherin/catenin protein expressions were compared in PC-3 spheroids

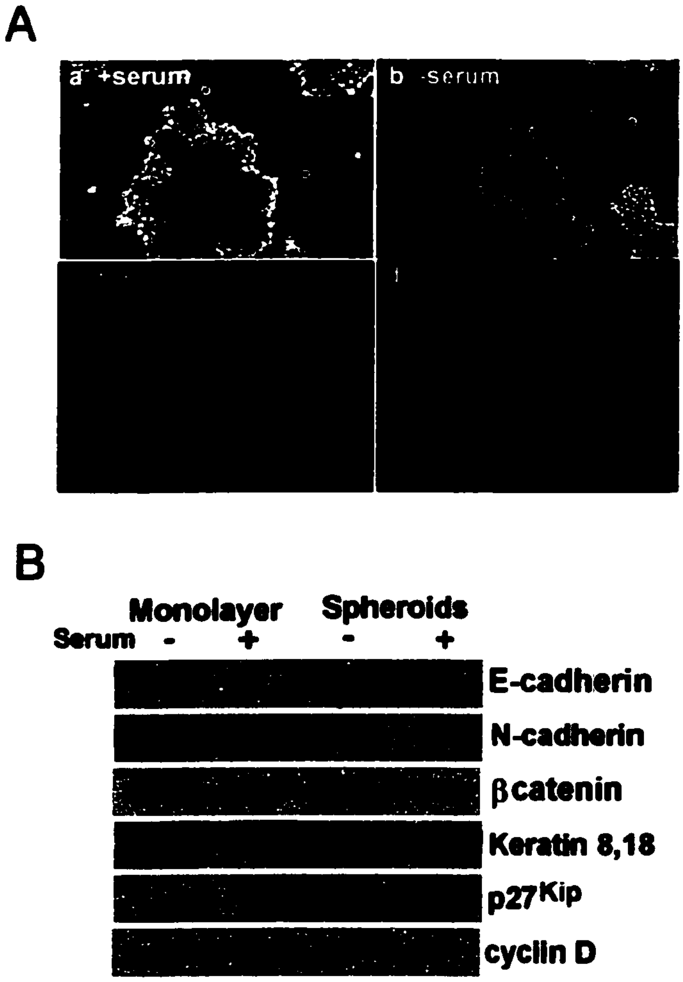

A
a +serum
b -serum
B
Monolayer
Spheroids
Serum - + - +
E-cadherin
N-cadherin
βcatenin
Keratin 8,18
p27Kip
cyclin D

Figure 5.1: Characterization of PC-3 multicellular spheroids and monolayer culture. (A) Morphology of PC-3 cells grown as multicellular spheroids. Phase contrast of PC-3 spheroids in the presence (a) and absence of serum (b). Immunohistochemical analyses of β-catenin in sections of PC-3 spheroids in the presence (c) and the absence (d) of serum. (B) Immunoblot analysis of total levels of E-cadherin, N-cadherin, β-catenin, keratin 8,18, $p27^{kip}$, and cyclin D in PC-3 spheroids and monolayer culture.

with PC-3 cells grown as sparse monolayer. In Figure 5.1B, the expression of E-cadherin is higher in MCS, compared to the low expression observed in PC-3 cells grown as sparse attached cells at a density of 1 X 10^4 cells/cm^2. In addition, characterization of N-cadherin expression of PC-3 spheroids reveals low expression of N-cadherin, whereas high expression of N-cadherin is detected in PC-3 monolayer cells. This indicates that E-cadherin and N-cadherin expressions are inversely correlated and independent of serum, and that MCS appears to be more differentiated with increased E-cadherin mediated cell-cell contacts.

Since E-cadherin mediated cell-cell adhesion is reported to inhibit cell growth by up-regulation of the cell cycle inhibitor, $p27^{kip}$, we also examined $p27^{kip}$ protein expression. PC-3 cells grown as attached sparse culture show no detectable $p27^{kip}$ protein (Figure 5.1B). These results suggest that multi-cellular spheroids display characteristics of glandular differentiation by increased E-cadherin mediated cell-cell contacts and up regulation of a cell cycle inhibitor $p27^{kip}$. Finally, the expression of β-catenin appears unchanged in both cell-culturing conditions, as does keratin 8 and 18. Interestingly, no change in cyclin D expression is detected between cells cultured as MCS or attached sparse cells.

Regulations of E-cadherin and N-cadherin are dependent upon cell density and the activity of β1 integrin

Since the upregulation of N-cadherin expression is associated in PC-3 cells with loose cell-cell contacts, and forced cellular contacts in multicellular spheroids resulted in increased E-cadherin expression, we assessed whether cell density alters the regulation of the expression of these two cadherins. PC-3 cells were seeded at various densities in a monolayer culture ranging from sparse cells with little cell-cell contacts to highly dense cell-cell interactions. In Figure 5.2A, as cell density increases, E-cadherin expression also increases, approximately three fold at confluency ($1X10^5$ cells/cm^2). However, as cell becomes increasingly dense ($1X10^5$ cells/cm^2), N-cadherin protein expression decreases six fold as compared to low cell density culture ($1X10^4$ cells/cm^2). This suggests that increased cell-cell contacts as a result of high cell density play a significant role in regulating the expression of E- and N-cadherin.

It has been proposed that the activation of integrin signaling pathways regulate junctional complex stability, cell-cell interactions and cell migration. In prostate cancer, the integrin $\alpha6\beta1$ subunit expression correlates positively with cellular invasion. Characterization of the integrins in PC-3 cells showed that PC-3 cells express primarily $\alpha3$, $\alpha5$, and $\alpha6$ integrin at similar levels, and $\beta1$, $\beta3$, and $\beta4$, with $\beta1$ predominant(496). However, the invasive tumors formed by PC-3 cells in SCID mice were found to express $\alpha6\beta1$ and does not contain surface $\beta4$, thus suggesting primarily $\alpha6\beta1$ complexes(496, 497). In addition, studies by Novak et al. (1998) showed that overexpression of ILK, the $\beta1$ and $\beta3$ integrin associated protein kinase, results in the loss of cell-cell adhesion, particularly E-cadherin expression. Based on these studies, we explored the possibility that signals generated from $\beta1$ integrin receptor effected the regulation of cadherin expression(288). Confluent cultures of PC-3 cells were infected with adenoviruses expressing either the wildtype $\beta1$ ($\beta1$-WT) or mutated $\beta1$ ($\beta1$-MT) cytoplasmic tail at two multiplicity of infection (MOI). Characterization of cadherin expression indicated

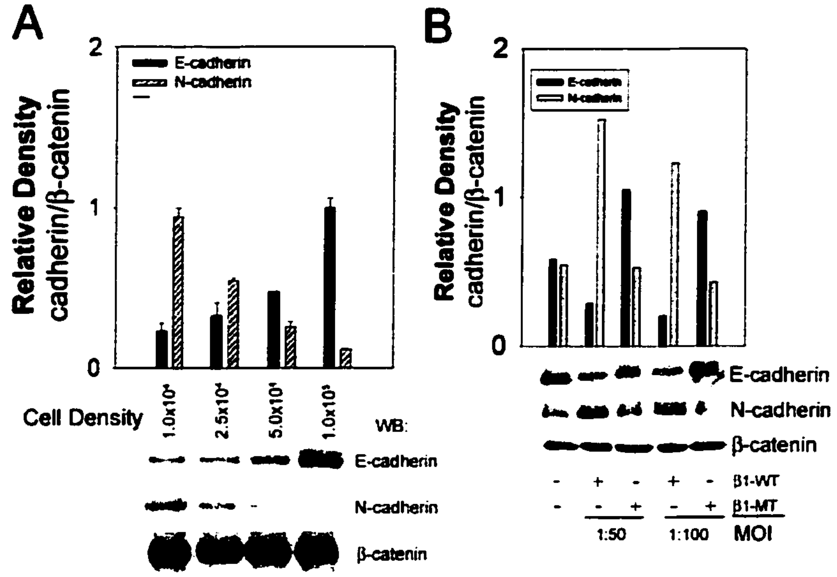


Figure 5.2: Expression of E- and N-cadherin in PC-3 cells is dependent upon cell density and activation of β1 integrin. (A) PC-3 cells were plated at different density as indicated. Immunoblots of cadherin expression were carried out using monoclonal antibodies specific to either E-cadherin or N-cadherin. Densitometry of E-cadherin and N-cadherin proteins were normalized to β-catenin expression. Data represents an average of three independent experiments. (B). Expression of a dominant-negative β1 construct in PC-3 cells grown as sparse monolayer. PC-3 cells were infected with adenoviruses at different multiplicity of infection (MOI), carrying either a wildtype β1 cytoplasmic domain construct (β1-WT) or a mutant β1 cytoplasmic domain construct (β1-MT), followed by immunoblot analysis of E-cadherin and N-cadherin protein expression. Relative protein densitometry of E- and N-cadherin were normalized to β-catenin protein expression.

that PC-3 cells infected with the β1-WT viruses at a MOI of 1:50 or 1:100 show a reduction of E-cadherin protein expression and an increase expression of N-cadherin protein (Figure 5.2B). In contrast, E-cadherin expression is increased in PC-3 cells infected with the β1-MT viruses, whereas N-cadherin expression is decreased. These data indicate that the signal generated from active β1 integrin is able to regulate cadherin expressions, particularly the suppression of E-cadherin protein and an increase in N-cadherin protein.

Type II cadherins mRNAs are up regulated in PC-3 multicellular spheroids and are induced under hypoxic condition

Differential expression of E- and N-cadherin steady state mRNA levels is compared in PC-3 cells grown as multicellular aggregates or as sparse attached culture. Similar to the protein data, PC-3 cells, when grown as multicellular spheroids expressed high levels of E-cadherin mRNA, whereas N-cadherin mRNA was significantly increased in PC-3 cells grown as a sparse attached cells in culture (Figure 5.3A). Thus, the mRNA levels of E-cadherin and N-cadherin correlate with the protein expression.

Previously, Bussemakers et al (2000) reported that PC-3 cells express low mRNA levels of type II cadherins, cadherin-11 and cadherins-6(302). Figure 5.3A shows that PC-3 cells in the monolayer culture system express low detectable levels of mRNAs for cadherin-6 and 11. When examining the mRNA levels for these type II cadherins in PC-3 multicellular spheroids, it was found that both mRNA transcripts for cadherin-6 and 11 are significantly upregulated. This data indicates that in addition to upregulation of E-

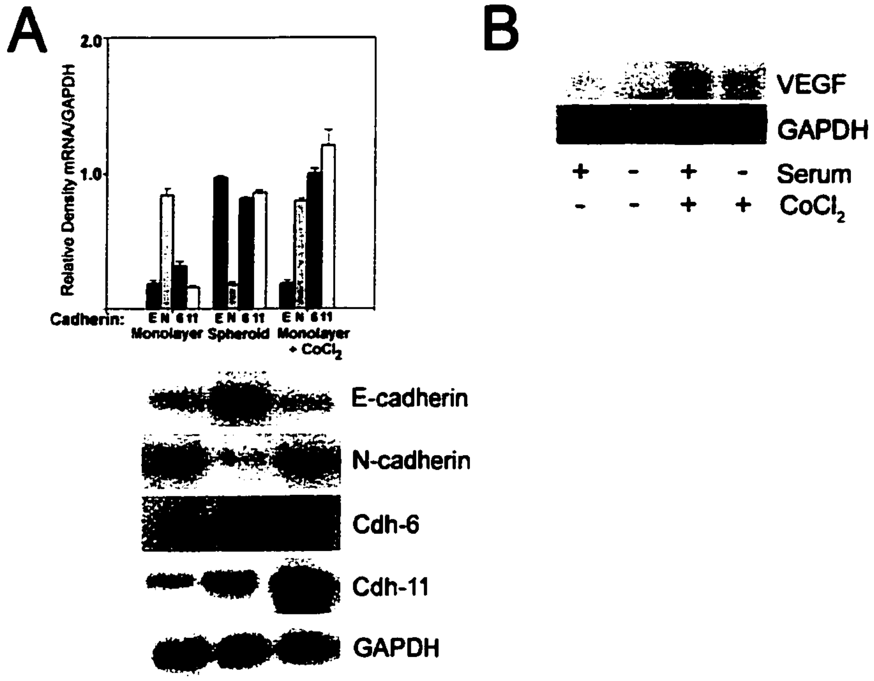


Figure 5.3: Northern blot analysis of cadherin expressions in PC-3 spheroids and monolayer culture. (A) Total RNA was isolated from each condition and twenty micrograms per lane of total RNA were blotted on a nylon membrane. For $CoCl_2$ study, PC-3 cells were treated for 24 hours with 100 μM of $CoCl_2$ prior to RNA isolation. A 1.7-bp fragment to mouse E-cadherin was used to probe for E-cadherin expression. A 300-bp EcoRI fragment to N-cadherin was used as a probe for N-cadherin detection. The 500bp fragments rat cadherin 6 and cadherin 11 were used to detect human cadherin 6 and cadherin 11, respectively. A 1.2-kb GAPDH cDNA fragment was used as normalization standard. (B) Monolayer cultures of PC-3 cells were treated with 100 μM of CoCl2 for 24 hours. Total RNA was extracted and VEGF mRNA expression was analyzed using a human 400 bp fragment of VEGF cDNA. GAPDH was used as normalization standard.

cadherin mRNA level, formation of PC-3 multicellular spheroids induces other type II cadherins, particularly cadherin-6 and cadherin-11.

Multicellular spheroids, like solid tumors, experience metabolic stress such hypoxia due to low pO_2. Changes in O_2 levels can lead to alteration of gene expression within a solid tumor. To determine whether the expressions of these cadherins in multicellular spheroids are regulated by hypoxia, we utilize a hypoxic mimicking agent $CoCl_2$. The Co^{2+} ion substitutes for ferrous ions at an oxygen sensing iron center and mimics hypoxia(498). PC-3 monolayer cultures were treated with 100 μm of $CoCl_2$ 24 hours prior to mRNA analysis. As shown in Figure 5.3A, treatment of PC-3 cells with $CoCl_2$ has no effect on E-cadherin or N-cadherin mRNA expression. However, mRNA expression of cadherin-6 and cadherin-11 are highly induced under low O_2 condition, similar to cells grown in multicellular spheroids. These data suggest PC-3 cells under hypoxic conditions are able to regulate cadherin mRNA transcripts, specifically the upregulation of cadherin 6 and 11.

To verify that PC-3 multicellular spheroids experience hypoxia, we analyzed the VEGF mRNA transcript, a gene that is known to be regulated by hypoxic conditions. As shown in Figure 5.3B, PC-3 VEGF mRNA is expressed at low levels in PC-3 subconfluent attached cultures and is independent of serum. When treated with 100 μM of $CoCl_2$ for 24 hours, VEGF expression is highly induced. However, a four-fold VEGF mRNA transcript of 4.5 kb was only detected in MCS when they were grown in the presence of serum (Figure 5.4A). No VEGF mRNA was detected in spheroids deprived

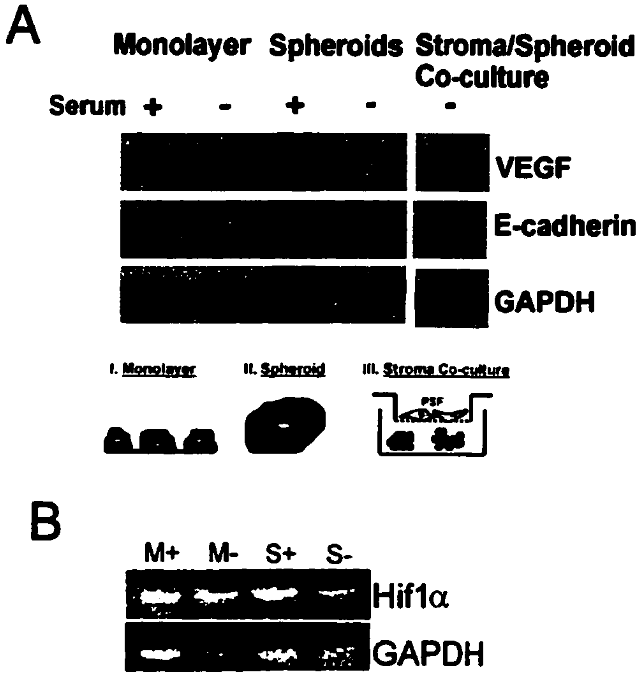

A
Monolayer
Spheroids
Stroma/Spheroid Co-culture
Serum + - + - -
VEGF
E-cadherin
GAPDH
I. Monolayer
II. Spheroid
III. Stroma Co-culture
PSF
B
M+ M- S+ S-
Hif1α
GAPDH

Figure 5.4: Northern blot analysis of VEGF mRNA in PC-3 spheroids and monolayer culture. (A) A 400 bp fragment of VEGF cDNA was used to blot for VEGF mRNA expression in PC-3 cells grown as spheroids or sparse monolayer. For stromal/spheroid co-culture, serum deprived prostate stromal cells were plated on the top chamber of a transwell plate and incubated for 24 hours with PC-3 spheroids which have been serum-starved for 48 hours. Total RNA was isolated and twenty micrograms were blotted on a nylon membrane for VEGF mRNA detection. (B) RT-PCR analysis of Hif1α mRNA in PC-3 cells grown as monolayer (M) or multicellular spheroids (S) in the presence (+) or absence (-) of serum.

of serum. Since VEGF expression is also dependent upon the expression and activation of Hif1α, a transcription factor induced under hypoxic conditions, we analyzed by RT-PCR Hif1α mRNA expression. The result suggests that Hif-1α mRNA is expressed at similar levels in both multicelllular spheroids grown in the presence and absence of serum (Figure 5.4B). Thus, this suggests that Hif1α transcription activity is not sufficient for the regulation of VEGF mRNA expression in PC-3 multicellular spheroids, and that signaling from a co-regulatory growth factor is required for VEGF mRNA expression.

Regulation of VEGF mRNA is dependent upon stromal-derived factors for expression in PC-3 multicellular spheroids

Numerous studies in prostate cancer indicate that growth factors released by the stromal cells of the tumor microenvironment are able to regulate tumor gene expression during the progression of prostate cancer through a paracrine effect. To determine whether this co-regulatory growth factor involved in VEGF mRNA regulation are produced by prostate stromal cells, a co-culture experiments was performed using prostate stromal fibroblasts (PSF) and PC-3 multicellular spheroids. PSF were placed on top of a transwell system and cultured in the absence of serum with PC-3 spheroids, which are placed on the bottom of the transwell system (Figure 5.4A). This system allows for the separation of the two cell types in a defined medium, where only soluble factors are exchanged through the 0.4-micron pore. After 24 hour, VEGF mRNA transcript was analyzed in the co-cultured PC-3 multicellular spheroid. As shown in Figure 5.4A, VEGF mRNA expression is increased significantly in PC-3 spheroid, suggesting that PSF cells are producing factor(s) that co-regulate VEGF expression.

Transforming growth factor-β family members are mesenchyme-derived co-factors regulating VEGF expression in prostate cancer

PSF cells produce many soluble growth factors that have an impact on the behavior of the epithelium, such as members of the transforming growth factor-β family (TGFβ). Since TGFβ family members such as TGFβ1 and BMP-4 have been shown to regulate VEGF mRNA, we investigated whether these two soluble factors are produced by PSFs and are responsible for the VEGF mRNA induction seen in PC-3 MCS. In Figure 5.5A, serum-deprived PC-3 multicellular spheroids were treated with various soluble growth factors. Treatment of PC-3 multicellular spheroids with BMP-4 induced high expression of VEGF mRNA. In addition, TGFβ1 and IGF also increases VEGF mRNA but not to the extent as BMP-4. As controls, both serum conditions and conditioned medium derived by PSF cells also induces VEGF mRNA as expected, while no VEGF mRNA transcripts were detected in untreated cells and cells treated with EGF. we next determined the time course of BMP-4 induction of VEGF mRNA transcript in PC-3 multicellular spheroids. Treatment of BMP-4 on PC-3 MCS induces VEGF in a time dependent manner. The expression of VEGF mRNA appears to peak around 16 hours (Figure 5.5B). To verify that BMP-4 and TGFβ1 are produced by PSF cells, we performed RT-PCR for BMP-4 and TGFβ1 using primers specifics to these two factors. As shown in Figure 5.5C, PSF cells express very high message for both soluble factors, while these factors are not easily detected in PC-3 cells. we also compared the expression pattern of BMP-4 and TGFβ1 in a variety of smooth muscle cells isolated from different organs. As shown in Figure 5C, it appears that both BMP-4 and TGFβ1 are also

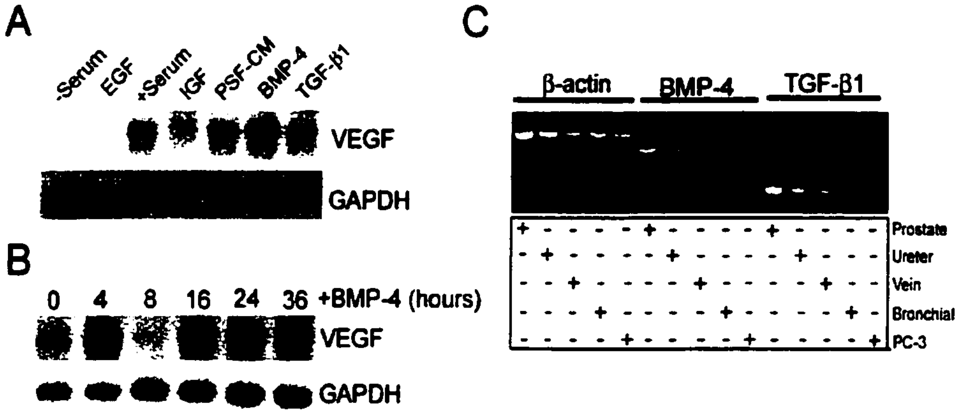

A
-Serum
EGF
+Serum
IGF
PSF-CM
BMP-4
TGF-β1
VEGF
GAPDH
B
0 4 8 16 24 36 +BMP-4 (hours)
VEGF
GAPDH
C
β-actin
BMP-4
TGF-β1
Prostate
Ureter
Vein
Bronchial
PC-3

Figure 5.5: Identification of BMP-4 and TGF-β1 as co-regulatory factors in the regulation of VEGF mRNA expression in PC-3 multicellular spheroids. (A) Serum-deprived multicellular spheroids were treated with various growth factors as indicated 24 hours prior to total RNA extractions. VEGF mRNA analysis was performed using 400 bp cDNA fragment of human VEGF. GADPH cDNA fragment was used as normalization standard. (B) Time course induction of VEGF mRNA expression by BMP-4 treatment. Serum-deprived PC-3 cells were treated with 5 ng/ml of BMP-4 and total RNA was collected at various time courses as indicated. VEGF mRNA analysis was then performed as described previously. (C) Comparison of BMP-4 and TGF-β1 mRNAs in various smooth muscle cells isolated from different organs as indicated. Total RNA from different smooth muscle cell cultures isolated from various organs were collected and subject to RT-PCR analysis using primers specific for BMP-4 and TGF-β1. PCR products were separated and analyzed with EtBR on a 3% agarose gel.

expressed in smooth muscle cells in different organs such as the ureter, vein and bronchus, but not in PC-3 cells. Thus, these data suggests that soluble factors, mainly BMP-4 and TGFβ1 produced by PSF cells acts as co-factors in regulating VEGF in PC-3 MCS.

Discussion

In prostate carcinoma, loss or down-regulation of E-cadherin expression is often associated with increased malignancy of the tumor(395). Previously, it was demonstrated that changes in cadherin expression patterns such that loss of E-cadherin and a gain of N-cadherin, may play a role in the process of cellular invasion and is indicative of dedifferentiation. Here, the data provided in this chapter show that the prostate cell line PC-3, displays characteristics of glandular differentiation when grown as multicellular spheroids. PC-3 cells are unique in that they express both E-cadherin and N-cadherin. However, the expression of E-cadherin protein is dominant over N-cadherin in MCS, whereas N-cadherin expression is more highly expressed in sparse attached cells.

Alterations of the integrin composition have been observed to correlate with metastatic potential. In prostate cancer, loss of the hemidesmosomal α6β4 integrin pair is accompanied by the preferential pairing of α6β1, which is implicated promoting cancer cell invasion(496, 497). Since, over-expression of ILK, which associates with β1 integrin is able to suppress E-cadherin expression(289), it is possible that, in our system, the changes in cadherin expressions in part may be a result of the alteration of β1 activity.

For example, cells that are plated as a sparse attached culture may have more active β1 integrin and thus is able to suppress E-cadherin protein expression, whereas N-cadherin expression is upregulated. However, in MCS where β1 integrins in PC-3 cells may be inactive, E-cadherin expression is up regulated and N-cadherin expression is low. To verify this, we used well-characterized chimeric β1 molecules that cannot bind ECM, and act to compete for β1 signaling molecules to disrupt β1 integrin signaling(499-501). Blocking β1 integrin signal in sparse attached cultures with a dominant-negative β1 construct resulted in re-expression of E-cadherin and suppression of N-cadherin. Although this data supports that signals from β1 integrin in part may regulate the cadherin expression in prostate carcinoma cells, the mechanism by which β1 integrin signals to regulate cadherin expressions is still unknown.

It is well recognized that nontransformed cell lines are contact-inhibited when they reach confluence in monolayer tissue culture. However, data is now emerging to show that in both normal fibroblasts and epithelial cells, intercellular adhesion molecules such as NCAM and E-cadherin respectively, are involved in this process(502-504). Recently, St. Croix *et al.* have demonstrated that E-cadherin mediated cell-cell adhesion results in an increase in the level of the cyclin-dependent kinase inhibitor $p27^{kip}$ and a late reduction in cyclin D1 protein. In addition, tightly adherent spheroids in their system also increase levels of $p27^{kip}$ bound to the cylin E-cdk2 complex, and a reduction in cyclin E-cdk2 activity. Exposure of these spheroids to E-cadherin neutralizing antibodies resulted in an increase cell proliferation and prevention of cell-cell adhesion(301). In agreement with their data, $p27^{kip}$ protein expression increased in PC-3 cells when grown as MCS.

Interestingly, $p27^{kip}$ expression was not detected in high N-cadherin expressing cells grown as sparse monolayer culture. This suggests that signaling generating from of E- and N-cadherin in PC-3 cells are different, in which $p27^{kip}$ protein expression is most pronounced when cells are cultured as multicellular masses as a result of E-cadherin mediated cell-cell contacts. In prostate cancer, loss of $p27^{kip}$ expression strongly correlates with increase tumor grade(140). Notably, $p27^{kip}$ homozygous knockout mice studies result in hyperplasia of multiple tissues including the prostate(141, 142).

It has been reported that solid tumors in vivo and spheroids grown from established tumor cell lines experience metabolic changes in which the center of the spheroid are deprived of O_2, glucose and other substrates. Under these conditions, certain genes are activated to accommodate these changes. In a recent study by Bussemakers et al. (2000), low expression of type II cadherins such as cadherin-6 and 11 is reported in PC-3 cells(302). In these studies, we also detect low levels of cadherin-6 and 11. However, the mRNA levels for these two cadherins are induced when PC-3 cells are grown as MCS which experiences increased hypoxic stress. Interestingly, we found that both cadherins are upregulated due to alteration in the O_2 content. Using $CoCl_2$ to mimic hypoxic conditions in monolayer system, we determined that the upregulation of cadherin-6 and -11 mRNA expressions are dependent upon signals generated from hypoxia, which does not appear to affect E- or N-cadherin expression. In both breast and prostate carcinoma cells, the expression of cadherin-11 has been implicated in facilitating cellular invasion and migration. Forced expression of cadherin-11 in non-invasive breast carcinoma cell lines resulted in a high migratory phenotype(273, 488). This data implicates that cadherin-11 could be involved in cell-cell recognition between tumor cells and heterotypic interactions between other cadherin-11 expressing cells such as the stroma that may facilitate cell motility and may also be essential for the loose aggregation of cell types that is necessary in tissue morphogenesis. These hypotheses are further supported by that fact that cadherin-11 expression is associated with invasive cells during both normal stages of embryogenesis and in invasive tumor cells(476, 478, 505). The association of the invasive cadherin-11 cells with the surrounding mesenchymal cells, which also express cadherin-11, may facilitate this invasion. In addition, cadherin-6 expression is also highly expressed in migrating neural crest cells, but mainly at an early stage. Based on these data, we would like to speculate that under hypoxic condition, prostate carcinoma cells might alter their cell-cell adhesion processes by inducing these type II cadherins. These type two cadherins may play a role in cellular invasion and migration through a heterotypic interaction with other cadherin-6 and -11 expressing cells such as endothelial and smooth muscle cells during prostate cancer metastasis.

Solid tumor cannot grow to more than 1 mm^3 in vivo without a new blood supply(506). In the PC-3 MCS model, we hypothesized that changes in the metabolic behavior of these cells would stimulate production of VEGF, a potent angiogenic cytokine that stimulates endothelial cells migration and proliferation(506). We further hypothesized that VEGF mRNA would be dramatically upregulated in serum deprived MCS, which experience increased hypoxic stress. Surprisingly, VEGF mRNA was highly expressed in PC-3 MCS grown in the presence of serum, compared to serum deprived multicellular spheroids or PC-3 grown as sparse monolayer culture. In addition, conditioned medium collected from cultured prostate stromal fibroblasts (PSF) also induces VEGF mRNA expression in PC-3 multicellular aggregates, suggesting that soluble factor(s) released by PSF cells may play a role as a cofactor in upregulating VEGF mRNA in PC-3 multi-cellular aggregates. In these studies, we identified the soluble factors to be members of the TGFβ1 family, TGFβ1 and BMP-4. It is not clear how these factors act to increase VEGF mRNA production. Analysis of Hif1α mRNA indicates that there is no difference in expression between serum-cultured or serum-deprived PC-3 MCS. Since Hif1α protein has a high turnover rate(507), it is possible that signals generated from TGFβ1 and BMP-4 may stimulate the stability of Hif1α protein for VEGF production. Clearly, more work needs to be done to investigate the role of TGFβ family members of VEGF regulation in prostate carcinoma.

Many questions still remain as to the nature of the type of genes that are expressed in prostate tumor cells grown as MCS as compared to sparse attached culture. In summary, this chapter provides evidence that cadherin expression in PC-3 cells are altered in when grown as multicellular aggregates as compared to sparse attached culture, and that the tumor microenvironment such as altered integrin signaling and metabolic changes plays a significant role in cadherin expression. In addition, hypoxia alone is not sufficient to stimulate VEGF expression, rather, additional signaling from the TGFβ family members, such as TGFβ1 and BMP-4, is required for VEGF mRNA expression. Thus, the use of MCS also allow for investigation of the effects of soluble factors such as TGFβ1 and BMP-4 released from the microenvironment on the behavior of prostate carcinoma in vivo. Since the majority of the research in VEGF expression is done in cultured cell lines in the presence of serum, it would be of interest to examine carefully the mechanism of VEGF regulation in prostate cancer by these factors.

CHAPTER 6: CONCLUDING STATEMENTS

One of every three cancers diagnosed in America is of prostatic origin, making prostate cancer a significant cause of cancer mortality in men in the United States(1). Most deaths from prostate cancer are due to metastases outside of the prostate and their ability to develop resistance to therapy. Accordingly, the development of methods to treat solid tumors that are localized are selectively successful; however, there is much less success in the attempts to eradicate metastatic disease. Current methods of predicting whether prostate cancer will acquire metastatic potential and progress from an androgen-dependent to an androgen-independent state are unreliable. Consequently, proteins specifically expressed in prostate cancer must be identified in order to serve as reliable diagnostic and prognostic markers as well as targets for effective therapy. Cadherins are particularly important because changes in cadherin expression and function result in the loss of normal epithelial morphology and loss of differentiation in carcinomas, thus resulting in metastatic consequences. The overall objective of this dissertation has been to investigate the changes in expression and function of cadherins in the progression and survival of prostate carcinomas.

The development of prostate cancer is a complex process influenced by various factors such as growth factors and cytokines from the tumor microenvironment. Cross-talk between benign precursor cells, malignant cells, and surrounding stromal host cells also influences tumor development. One family of the molecules involved in the intercellular communication is the cadherin superfamily, which has been shown to play a critical role in prostate tumor progression. For example, downregulation of E-cadherin expression have been noted in malignant degeneration of prostate epithelium, metastasis, and decreased patient survival(86, 485-487). Restoration of E-cadherin in cancer cells results in decreased invasiveness(508), growth suppression(406), and terminal differentiation(509). The addition of antibodies that inhibit E-cadherin-mediated cell-cell junctions(406), incubation of the cells in low-calcium media (397), or modulation of the cell density(280) abrogates the growth-inhibitory effects mediated by E-cadherin and induces cellular migration and invasion. Because of these characteristics, E-cadherin is proposed to be potent invasion suppressor gene.

Our first hypothesis was that downregulation of E-cadherin expression correlates with the gain of an additional cadherin, which was identified to be N-cadherin. In our studies we identified three classes of prostate carcinoma cells lines: 1) cells isolated from metastatic lesions that express only E-cadherin (LNCaP, DU-145); 2) metastatic cells expressing both E-cadherin and N-cadherin (PC-3); 3) metastatic cells that have loss E-cadherin expression but gained N-cadherin expression (PC-3N). Evidence presented in Chapter 2 indicates that a gain of expression of N-cadherin in prostate tumor cells lacking E-cadherin expression is associated with an increase in invasive potential. This gain in expression of N-cadherin in prostate carcinoma results in cells with a more scattered, less adhesive phenotype, which is typical of invasive tumor cells. Analyses of cadherin mediated cell-cell aggregation *in vitro* and laser confocal microscopic analysis of N-cadherin immunolocalization showed that invasive prostate carcinoma cells not only mediates cell-cell homophilic interactions between adjacent cancer cells, but also mediates cell-cell interaction between carcinoma cells and prostate derived stromal fibroblasts. In addition, the isoform expression of the cadherin binding protein p120^{ctn} differed in relation to the expression of E- *verses* N-cadherin by the prostate carcinoma cell lines. The p100 isoform was more highly expressed in E-cadherin-positive carcinoma cell lines, whereas p120 was predominantly expressed only in N-cadherin-positive prostate carcinoma cell lines and prostate stromal fibroblasts. Studies have shown that overexpression of p120 isoform in fibroblast cells leads to changes in cellular morphology with development of cellular processes(510). The N-cadherin expressing prostate carcinoma cells (PC-3N) were also able to form small tumors and invade into diaphragms of SCID mice as compared to non-invasive E-cadherin prostate carcinoma cells (DU-145), which formed larger vascularized tumors but do not invade. While these data suggest that there is a correlation between expression of N-cadherin in prostate carcinoma cells and the ability of these cells to invade into the diaphragm, there are several gene expression differences between these prostate carcinoma cells lines that may facilitate the invasive phenotype. Additionally, these data suggest that the expression of N-cadherin might be an indicator of a potentially aggressive tumor. In fact, recent clinical data by Bussemakers and colleagues(302) indicated that the expression of N-cadherin in invasive prostate carcinoma correlates with increased Gleason Grade. N-cadherin expression is detected in 50% of human prostate cancer Gleason Grade 7 and above that were examined. Notably, immunohistochemical analysis of the expression of N-cadherin in human prostate cancer specimens indicated staining of N-cadherin in poorly differentiated areas, the same areas which showed mainly aberrant or negative E-cadherin staining(511). Furthermore, N-cadherin has been postulated to promote both stable and motile cellular interactions(512-514), while maintaining a functional adherens junction(515). However, it is likely that N-cadherin promotes a state of dynamic adhesion similar to functional roles of cadherin-6 and cadherin-11 in migrating neural crest cells(375) that allows both attachment and detachment of cells from the primary tumor and selective association with N-cadherin expression cells such as the stromal fibroblasts as depicted in Figure 6.1.

Not only is the downregulation of E-cadherin important for malignant prostate cancer cells to dissociate from the primary tumor, but also the ability of these cancer cells to survive and proliferate as metastases in the absence of attachment to extracellular matrices. Normal epithelial cells rely on the association with the extracellular matrix and their ability to spread and adopt a distinct cell shape for their survival(413). These interactions are mediated primarily by integrins, particularly the β1 and β3 integrins(516-518). Tumor cells early in prostate cancer progression appear to lose their dependence on cell-matrix interaction, but maintain their requirement for cell-cell interactions. Later in prostate cancer progression, cell-cell interactions often become weaker, which is a requirement for metastasis. However, late-stage prostate tumors maintain some level of intercellular adhesion suggesting that the loss and gain of cell-cell adhesion may be a dynamic process(519).

Based on data provided in Chapter 2 and evidence that cell-cell adhesion may compensate for cell survival(519, 520), we hypothesized that N-cadherin homophilic interactions between prostate carcinoma cells could initiate a signal transduction pathway

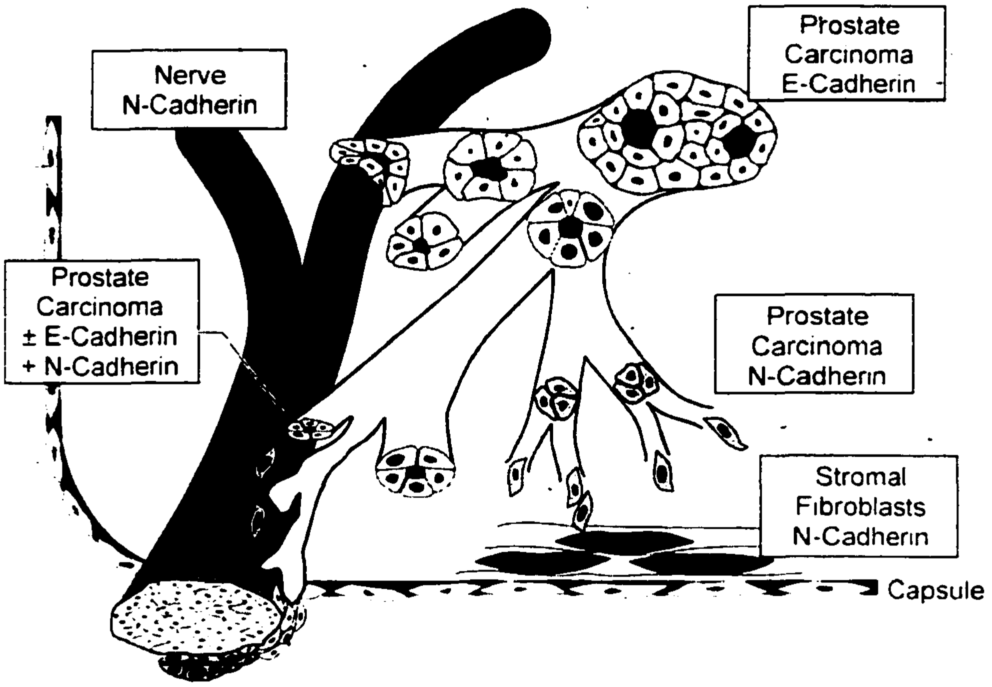

Nerve
N-Cadherin
Prostate
Carcinoma
E-Cadherin
Prostate
Carcinoma
± E-Cadherin
+ N-Cadherin
Prostate
Carcinoma
N-Cadherin
Stromal
Fibroblasts
N-Cadherin
Capsule

Figure 6.1: Model of cadherin expression in human prostate tumor invasion and metastasis. More differentiated, low-grade prostate tumors are shown as expressing E-cadherin (shown in upper right-hand corner; represented by DU-145 cells in the experiment). High-grade prostate carcinomas showing variable expression of E-cadherin and development of expression of N-cadherin are depicted in the lower left-hand corner (e.g. PC-3 cells). Invasive prostate tumors are also shown lacking E-cadherin expression and only expressing N-cadherin (lower right-hand corner; e.g. PC-3N). Expression of N-cadherin is also in both nerve bundles and stromal myofibroblasts. Perineural invasion of prostate carcinoma cells is a major route of extracapsular dissemination, and N-cadherin could be critical in the invasion of prostate carcinoma into the stroma, interaction of prostate carcinoma cells with stromal fibrolasts and capsular penetration and metastasis.

that is involved in promoting cell survival as describe in the second specific aim. The fate of cells exposed to apoptotic signals is determined by the balance between pro- and anti-apoptotic proteins of the Bcl-3 family(156). Evidence presented in Chapter 3 suggests that N-cadherin ligation in prostate carcinoma cells results in the generation of a survival signal through the PI 3-kinase/Akt pathway. This outside-in signaling property appears to require both actin association and recruitment of PI 3-kinase to the N-cadherin/catenin complex. The results indicate that Akt dependent N-cadherin activation results in the phosphorylation of Bad on Ser136. In the unphosphorylated state BAD forms heterodimers with anti-apoptotic Bcl-2 homologs, which promotes cell death by allowing Bax-Bax homodimers. Bax homodimers accelerate cell death by inserting into the mitochondrial membrane and facilitating opening of the voltage-dependent anion channel in the outer mitochondrial membrane(521, 522). This activity is inhibited by phosphorylation of Bad on Ser136, which sequesters Bad in the cytoplasm as a complex with 14-3-3 proteins, repressing its inhibition of Bcl-2(523). The increased Bcl-2/Bax ratio following N-cadherin engagement indicates that prostate carcinoma cells may benefit from N-cadherin-mediated aggregation with increased viability and resistance to apoptosis. It is not known whether N-cadherin mediated heterotypic interaction between prostate carcinoma cells and stromal fibroblasts can also elicit a similar survival response. However, based on data presented in Chapter 2 and 3 we would predict that a similar signal-transducing pathway might occur, although more investigation is required to assess this hypothesis. In addition, a surprising result we found was that even though Ca^{2+}-dependent adhesion protects DU-145 cells from apoptosis, these cells appears to initiate a different survival signal other than the Akt pathway observed upon N-cadherin ligation. This Ca^{2+}-dependent adhesion mediated survival pathway in DU-145 possibly could be initiated through the MAPK signal transduction pathway; however more research is needed to verify this assumption.

Agonist activation of PI 3-kinase involves the translocation of this enzyme to the plasma membrane where it can regulate the function of its lipid substrates(168). Thus, we hypothesized that a similar mechanism may underlie the activation of Akt upon organization of N-cadherin ligation. Immunoprecipitation of N-cadherin from cellular lysates after adherens junction reformation described in Chapter 3 showed that the p85 regulatory subunit of PI 3-kinase was recruited to the N-cadherin complex. Although the mechanism whereby N-cadherin engagement stimulates the recruitment of PI 3-kinase to the complex is unknown, it is most likely involves a yet to be identified tyrosine kinase, which, when activated in response to N-cadherin mediated-cellular adhesion, might facilitate the recruitment of PI 3-kinase to N-cadherin/catenin complex at the plasma membrane. It is possible that this 102 kDa protein, which interacts with the N-cadherin complex (described in Chapter 3), becomes rapidly autophosphorylated upon N-cadherin ligation. It is possible that the phosphorylation of this 102-kDa protein could be responsible for PI 3-kinase recruitment and activation at the N-cadherin/catenin complex.

Furthermore, growth factor and integrin signaling pathways have been shown to induce phosphorylation of the transcription factor CREB on Ser133, which in turn stimulate CREB dependent cellular gene expression. Transcriptional regulation of *bcl-2* was shown for both integrin and growth factor signaling and is dependent upon CREB activation. Transcription of *bcl-2* is important to sustain survival of prostate carcinoma cells, since *in vitro* studies have shown that Bcl-2 expression protects prostate cancer cells from apoptosis. Further characterization of N-cadherin mediated cell survival signals which affects the upregulation of Bcl-2 expression was tested in the third hypothesis. This hypothesis states that outside-in signal transduction through N-cadherin ligation results in the upregulation of *bcl-2* transcription. In Chapter 4 we tested this hypothesis by investigating the role of CREB as a downstream substrate of Akt activity. The homotypic adhesion by N-cadherin leads to the activation of CREB through the PI 3-kinase/Akt pathway. Activated CREB increases both *bcl-2* promoter and transcription activity. These studies have demonstrated that the gain of N-cadherin mediated cell-cell adhesion in prostate cancer is an important mechanism through which these cancer cells acquired cell survival.

Although this dissertation did not assess whether outside-in survival signals generated by N-cadherin ligation would promote drug resistance in prostate cancer cells, these studies suggest that it is possible that N-cadherin mediated cell-cell adhesion in a solid tumor may confer chemotherapeutic resistance of advanced prostate cancer by enhancing Bcl-2 expression. Exploring this hypothesis may be important in the development of future therapy against N-cadherin expression in prostate cancer. In fact, increased level of adhesion is thought to facilitate survival of tumor emboli, and in some cases allow tumor cells to evade the cytotoxic effects of anticancer therapy(524, 525). An earlier study by Durand and Sutherland(520) suggested that cell-cell contacts might promote resistance of solid tumor cells to anticancer agents. This was based on the fact that tumor cells grown as multicellular spheroids were highly resistant to anticancer agents compared to same cells grown as monolayer cultures. This finding led them to postulate that cell-cell contact was responsible for the drug resistance. Thus, cell-cell adhesion may protect cells from undergoing drug-induced programmed cell death.

In addition to CREB, Akt can phosphorylate other proapoptotic proteins. One of these proteins includes the forkhead transcription factor FKHRL-1, which activates genes such as Fas ligand(526) and IGF binding protein 1(527). Phosphorylation of FKHRL-1 leads to association with 14-3-3 proteins and loss of FKHRL-1 function(526). Akt also phosphorylates procaspase 9, and this phosphorylation prevents cleavage, which is required for activation(528). Data have shown that decreased Akt activity, due to loss of contact with matrix, results in the activation of proapoptotic proteins and apoptosis ensues. However, in spite of the extensive investigation described in both Chapter 3 and 4, it is still not known whether activation of Akt by N-cadherin ligation results in the phosphorylation of these proapoptotic proteins; an interesting hypothesis that is worthy of future investigation.

Pece *et al.* (274) have shown that E-cadherin homotypic binding can also result in the activation of both Akt and MAPK pathways, implicating the function of E-cadherin in eliciting cell survival and proliferation. In our studies, we did not detect activation of the ERK 1/2 MAPK pathway using specific phospho-antibodies to ERK 1/2. Inhibitors of the MAPK did not alter N-cadherin mediated Akt Ser-473 phosphorylation suggesting that N-cadherin does not signal through the MAPK pathway. In addition, based on studies in Chapter 4, it appears that the activity of CREB upon N-cadherin ligation is dependent upon Akt activation. Several pathways can converge on the transcriptional activation of CREB such as protein kinase A, Ca^{2+}-activated calmodulin kinases, ribosomal S6 kinase2 and the mitogen-activated protein-kinase-activated protein kinase 2. In our study, it appears that CREB activity upon N-cadherin ligation is a direct result of PI 3-kinase/Akt pathway since pharmacological inhibitors to MEK, p38 MAP kinase, and p70 S6 kinase do not suppress CREB Ser133 phosphorylation and subsequent CREB dependent transcriptional activation of *bcl-2*.

Moreover, prostate carcinoma cells have been reported to display altered basement membrane deposition resulting from accelerated degradation, defects in matrix assembly or protein translation(92, 191). It appears that these malignant cells have escaped the normal pattern of regulated growth control found in normal epithelium and, in the absence of anchorage to the basement membrane, may utilize cell-cell adhesive interactions through N-cadherin for cell survival and proliferation. It is reported that normal epithelium that proliferate as adherent cells fail to survive in suspension even when permitted to form cell aggregates(398). Thus, in the case of normal epithelial cells, formation of cell-cell contacts was not sufficient to prevent apoptosis, suggesting that E-cadherin signaling is distinct from that of N-cadherin in prostate carcinoma cells. This suggests that transformation may be a necessary requirement for N-cadherin mediated cell-induced survival and growth (Figure 6.2).

To date, there is no cure for advanced prostate carcinoma. One factor that may contribute to the resistance of prostate cancer to chemotherapy and androgen deprivation is the overexpression of Bcl-2. Re-expression of PTEN in the PTEN-null prostate cancer

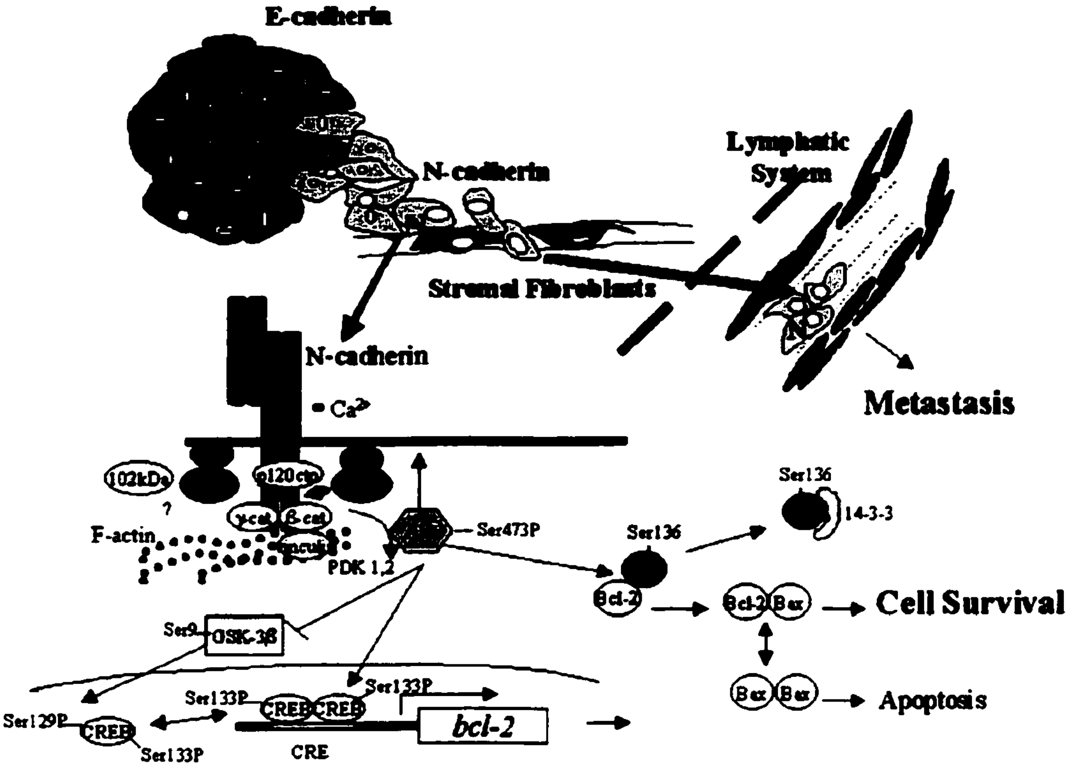

E-cadherin
N-cadherin
Lymphatic System
Stromal Fibroblasts
N-cadherin
Ca2+
Metastasis
102kDa
p120ctn
?
F-actin
γ-cat
β-cat
α-catenin
PDK 1,2
Ser473P
Ser136
Ser136
14-3-3
Bcl-2
Bcl-2 Bax
Cell Survival
Bax Bax
Apoptosis
Ser9 GSK-3β
Ser133P
CREB CREB
Ser133P
Ser129P CREB
Ser133P
CRE
bcl-2

Figure 6.2: Model of cadherin expression and function in prostate cancer progression. The loss of E-cadherin is accompanied by a gain of N-cadherin by invasive prostate cancer cells. Homotypic cell-cell interaction mediated by N-cadherin elicits a cell survival signal in invasive prostate cancer cells through a PI 3-kinase/Akt dependent pathway to stabilze Bcl-2 protein and increases *bcl-2* transcription. The induction of cell survival by N-cadherin ligation may be important for the progression of prostate carcinoma metastasis.

cells induces a decrease of Bcl-2 expression, and the lipid phosphatase function of PTEN is required for the downregulation of Bcl-2(466). In addition, my study showed that the effect of PTEN on *bcl-2* transcription is accomplished by inhibiting the activities of Akt and CREB and subsequently, down regulation of *bcl-2* transcription. We have demonstrated that N-cadherin homotypic binding and PTEN reconstitution results in a decrease of *bcl-2* transcription. As previously discussed, N-cadherin expression is detected late during the progression of prostate carcinoma, since expression of N-cadherin was found in prostate cancer tissues Gleason grade of 7 and above(511). In advanced and androgen independent prostate carcinoma, loss of PTEN and overexpression of Bcl-2 are also inversely correlated(81, 466). It is possible that the expression of N-cadherin and loss of PTEN observed at a later stage during prostate cancer progression may be indicative of Bcl-2 overexpression (Figure 6.3).

Currently, it is not known how cadherins are regulated during the progression of metastatic cancer. It is thought that the downregulation of E-cadherin activity by malignant prostate cancer cells is necessary to break away from the primary tumor mass, followed by the gain of a mesenchymal cadherin, N-cadherin. In specific aim 4, we hypothesized that the regulation of cadherin-mediated cell-cell adhesion is dependent upon the intrinsic events of tumor microenvironment such as altered integrin signaling or metabolic stress due to hypoxia. Evidence provided in Chapter 4 showed that the coordinated expression between E-cadherin and N-cadherin cell-cell adhesion in prostate carcinoma cells are dependent in part by the activity of β1 integrin. Expression of a dominant-negative β1 integrin subunit appears to upregulate E-cadherin expression and

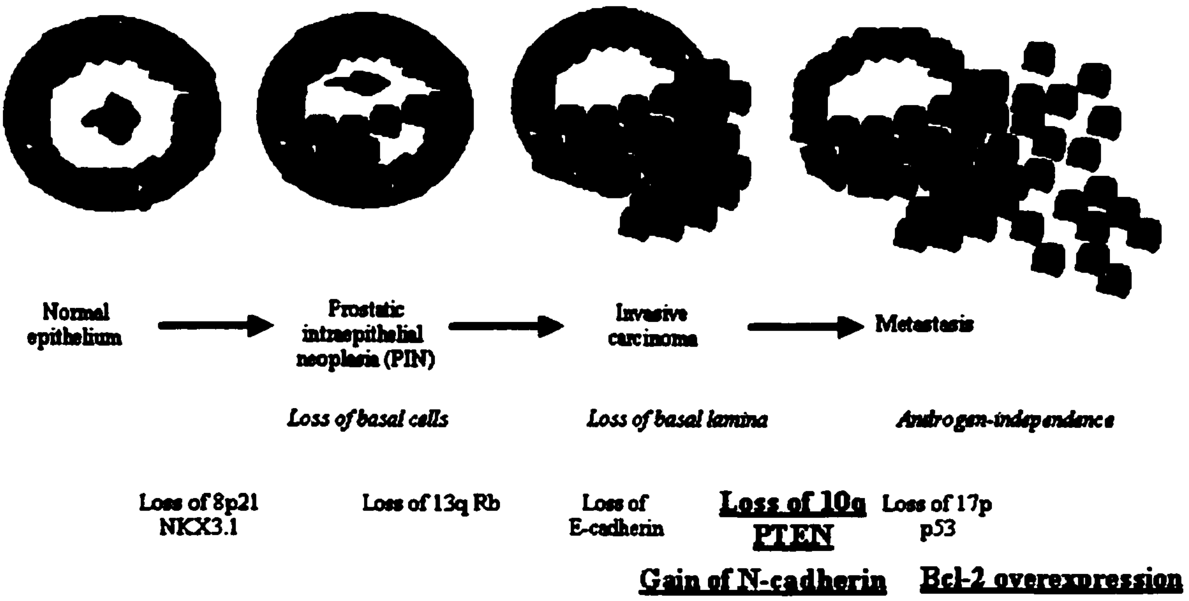

Normal epithelium
Prostatic intraepithelial neoplasia (PIN)
Invasive carcinoma
Metastasis
Loss of basal cells
Loss of basal lamina
Androgen-independence
Loss of 8p21 NKX3.1
Loss of 13q Rb
Loss of E-cadherin
Loss of 10q PTEN
Loss of 17p p53
Gain of N-cadherin
Bcl-2 overexpression

Figure 6.3: Model depicting changes in cadherin expression during prostate cancer progression. Loss of E-cadherin is observed in early invasive carcinoma, whereas, the gain of N-cadherin expression is observed late in prostate cancer progression. This gain of N-cadherin along with loss of PTEN may be indicative of Bcl-2 overexpression. Bcl-2 expression is observed in advanced androgen-independent prostate cancer.

downregulate N-cadherin expression. Although these data provide evidence of cross-talk between the two adhesion systems, it is not clear whether the cadherin regulation is a result of protein stability or a transcriptional event.

One of the key molecules that interacts with the β1 cytoplasmic tail is ILK. Dedhar and colleagues have shown that the overexpression of ILK in epithelial cells results in the increased phosphorylation of Akt and GSK-3β, activating the former and inhibiting the later(529, 530). One of the hallmarks of over-expressing ILK in normal epithelial cells is the loss of cell-cell adhesion, due to the downregulation of E-cadherin expression(531). The downregulation of E-cadherin expression may involve ILK-mediated activation of the E-cadherin repressor Snail(286). Snail was shown to bind to three E-boxes present in the human E-cadherin promoter and represses transcription of E-cadherin(493). Recently, the up-regulation of the transcription factor Snail was reported to mediate significant negative regulation of E-cadherin expression in bladder, colorectal, and pancreatic carcinoma(493, 532). Mechanistically, it is possible that the inactivation of β1 function in prostate carcinoma cells may inactivate the ILK-mediated activation of Snail, thus upregulating E-cadherin expression. However, it is not known whether the repressor activity of Snail has the same or opposite effect on N-cadherin expression.

Although the mechanism is unknown, my studies show that signal(s) generated during hypoxic conditions can upregulate the expression of the type II cadherins, cadherin-6 and –11. It is proposed that these type II cadherins are essential for the loose aggregation of cell types and associated with migrating populations of neural crest cells(273, 476, 478, 488). For example, during embryogenesis, the expression of both cadherin 6 and cadherin 11 are increased dramatically in cells undergoing the epithelial to mesenchymal transition that precedes the formation of various structures and also in regions of mesenchymal condensation(533, 534). The association and loose aggregation of cells may depend upon these type II cadherins and could be extremely significant in cancer, yet more studies are need to define this. For example, it is possible that cadherin-11, which was originally described as OB-cadherin in osteoblasts, may act to specifically target metastatic tumor cells to sites that express cadherin-11. Consequently, expression of cadherin-11 may facilitate association of metastatic cells with cadherin-11 expressing osteoblasts in the bone, thereby establishing a bone metastasis.

In summary, investigating the cross-talk between cell-cell adhesion molecules and cell matrix adhesion molecules within the context of prostate tumor and its microevironment can have profound effects on cellular function. To date, it is still unknown how metastatic cells resolve their requirements for variations in cell adhesion environments such as down-regulation of E-cadherin and expression of other adhesion molecules in cell-substrate and cell-cell interactions during increased motility. Interactions between the different classes or subclasses of adhesion receptors exert an effect on cell motility. It is possible that differences in signaling capabilities of the individual cadherins might contribute to the differences in cellular behavior and phenotypes. Therefore, understanding the regulation of cadherins and their signaling properties might provide a step toward development of treatments that decrease the survival and invasiveness of malignant prostate carcinoma cells.

APPENDIX A: ABSTRACTS AND PUBLICATIONS

PUBLICATIONS

Tran NL, Nagle RB, Cress AE, Heimark RL. N-cadherin expression in human prostate carcinoma cell lines: An epithelial-mesenchymal transformation mediating adhesion with stromal cells. *American Journal of Pathology*. 155(3):787-98, 1999 Sept.

Nhan L. Tran, Deanna G. Adams, Richard R. Vaillancourt, and Ronald L. Heimark. Signaling from N-cadherin increases Bcl-2: regulation of the Phosphatidylinositol 3-Kinase/Akt pathway via association with the actin cytoskeleton. *Journal of Biological Chemistry* (Submitted Jan. 2002).

Nhan L. Tran, Linda M. Boxer, and Ronald L. Heimark. Regulation of *bcl-2* Transcription by N-cadherin Adhesion Through the CREB Signaling Pathway. (Manuscript in preparation).

Eliszabeth L. Bair, Chad P. Massey, **Nhan L. Tran**, Alexander H. Borchers, Ronald L. Heimark, Anne E. Cress, G. Tim Bowden. Integrin and cadherin mediated induction of the matrix metalloproteinase matrilysin in co-cultures of malignant oral squamous carcinoma cells and dermal fibroblasts. *Experimental Cell Research*. 2001 Nov. 1; 270(2):259-267.

Brent D. Butts, **Nhan L. Tran**, Margaret M Briehl. Identification of a functional PPRE in the 3' UTR of human *bcl-2* gene. *Journal of Biological Chemistry* (Submitted Dec. 2001).

Expression of N-cadherin in Invasive Human Prostate Carcinoma Cell Lines: Transformation from an Epithelial to a Mesenchymal Phenotype

Nhan L. Tran, Raymond B. Nagle, Anne E. Cress, and Ronald L. Heimark. Molecular Biology of the Cell (Abstract#277) Vol 9, Nov. 1998. American Society for Cell Biology, San Franciso, CA.

In human prostate adenocarcinoma, an association between loss of E-cadherin, increased Gleason score and extracapsular dissemination has been observed. Further characterization of the E-cadherin/catenin phenotype of human prostate carcinoma cell lines showed loss of E-cadherin and expression of a larger cadherin with a molecular weight of 138 kDa in poorly differentiated prostate carcinoma cell lines (PC-3N derived from PC-3, PC-3, and JCA1). We identified this cadherin as N-cadherin, and showed that it is concentrated at sites of cell-cell contact in PC-3N cellular extensions. N-cadherin was also expressed in prostate stromal fibroblasts both *in vitro* and in prostate tissue. Co-cultures of prostate stromal fibroblasts and PC-3N cells showed the immunolocalization of N-cadherin in intercellular contacts. In addition, the isoform expression of the cadherin binding protein $p120^{ctn}$ differed in relation to the expression of E- vs. N-cadherin by the prostate carcinoma cell lines. The p100 isoform was more highly expressed in E-cadherin positive carcinoma cell lines while p120 was predominantly expressed in only N-cadherin positive prostate carcinoma cell lines. The N-cadherin positive carcinoma cell line, PC-3N, displayed aggressive invasion into the of the diaphragm muscle after intraperitoneal injection of SCID mice. The gain of N-cadherin and loss of E-cadherin by invasive prostate carcinoma cell lines suggests a progression from an epithelial to a mesenchymal phenotype, which may allow for their interaction with surrounding stromal fibroblasts and facilitate metastasis.

Akt Mediates Prostate Carcinoma Survival by Adhesion of N-cadherin

Nhan L. Tran and Ronald L. Heimark. Proceedings of the American Association for Cancer Research (Abstract #4523) Vol 42, Mar. 2001. American Association for Cancer Rearch, New Orleans, LA.

The E-cadherin/catenin complex has been implicated in a number of biological functions including regulation of cell growth and differentiation, cell recognition, and cellular survival. We recently showed that the more invasive human prostate carcinoma cell lines have down-regulated E-cadherin and gained expression of N-cadherin, which can form stable junctional complexes with N-cadherin on prostate-derived stromal fibroblasts (Tran et al., 1999 AJP 155:787-798). To determine whether N-cadherin has a role in prostate cancer progression, we examined N-cadherin adhesion as a potential mechanism to activate cellular survival signaling. The PI-3 Kinase/Akt signaling cascade is a central mediator in activation of cell survival pathways initiated by extracellular factors. Using the human prostate carcinoma cell line, PC-3N, we found that engagement of N-cadherin in a Calcium Switch Assay involving Ca^{2+}-dependent cell-cell interactions, or by antibody ligation resulted in the activation of Akt in a rapid PI 3-Kinase dependent manner. The activation of Akt sequentially leads to an increase in the cellular Bcl-2Bax ratio. In response to formation of cell-cell contacts, Akt phosphorylated Bad on serine-136, which has been shown to result in stabilization of Bcl-2. Following N-cadherin homophylic adhesion, Akt translocation from membrane to the nucleus was observed. N-cadherin occupancy also leads to phosphorylation of the transcription factor, Creb, at serine-133 by Akt. Phosphorylated Creb regulates transcription of Bcl-2 mRNA by interacting with the Creb responsive element in the Bcl-2 promoter region. These findings suggest that N-cadherin adhesion can initiate Outside-In signal transducing pathways that controls prostate carcinoma cell fate during metastasis.

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