PROSTATE CANCER
MOLECULAR ASPECTS TO DIRECT VISUALIZATION UTILIZING A BIOREACTOR

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ABSTRACT

Prostate cancer is the most common cancer in males, and the second leading cause of cancer deaths in American men. Most of the mortality associated with this disease is a result of widespread dissemination of tumors cells from the primary tumor mass. In order for metastasis to occur, the cancer cell must overcome multiple barrier which include development, neovascularization, intravastion, adherence or attachment, extravasation, and ectopic growth. As dissemination from the primary tumor mass is a rate-limiting step during metastasis, tumor cells undergo an epithelial to mesenchymal transition (EMT) to acquire enhanced invasiveness and increased motility. A key step within EMT is a loss of cadherin mediated cell-cell adhesion. Unfortunately, current understanding of the regulatory mechanism of this decreased cell-cell adhesion is poorly understood. Herein this work utilizes the LHRH antagonist Cetrorelix to investigate the regulation of E-cadherin expression in invasive prostate cancer cells. We provide direct evidence that E-cadherin expression can be reinstated upon abrogation of EGFR signaling via LHRH antagonist Cetrorelix or specific inhibitors of EGFR signaling thereby limiting the invasiveness of these cells.

In concert, we developed a microscale liver perfusion culture system that provides a tissue-relevant environment to assess metastasis behavior of human prostate cancer cell line DU-145 in the liver capillary bed as a model system. This system offers the currently unavailable features of real time observation of *in vivo* microenvironment with the manipulation of *in vitro* cultures. Within this system we were able to observe three dimensional growth and invasion of

prostate cancer cells juxtaposed to hepatic tissue, revealing an exceptionally defined cell border at the interface of prostate cancer cells and hepatic tissue. Although not completed defined within this system, we hypothesize that exists heterotypic cell-communication between prostate cancer cells and hepatocytes.

The very distinct cell border observed within our liver microreactor, coupled with our previous findings of reexpression of E-cadherin expression lead us to investigate the involvement of E-cadherin in this heterotypic communication. Consequently, prostate cancer cells utilize E-cadherin at the point of initial adherence to parenchymal hepatocytes (heterotypic interaction) and throughout the development of the metastatic tumor mass (homotypic interaction). Our observed expression pattern of E-cadherin has not been reported before. These findings constitute a new paradigm in the adhesiveness or lack there of in cancer cells during tumor invasion. The differentiation or redifferentiation (EMT) of the cancer cell during the pathophysiological events of metastasis is likely a characteristic of adaptability to the microenvironment. The term epithelial mesenchymal transition (EMT) only summates the dedifferentiation of epithelial cells to escape the primary tumor, although we have provided evidence of phenotypic reversion. Therefore we provide the impetus that Epithelial Mesenchymal Transition (EMT) should be renamed "Meschenymal Epithelial reverting Transition (MErT)" to underscore the dynamics of the cancer cell progression.

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PREFACE

Dedication

I would like to express his love and gratitude to my wife, Ronieta Yates, and daughter, Cionni Yates for their support, encouragement, and endurance during my training at the University of Pittsburgh.

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1. INTRODUCTION

Normal development, growth and survival of the prostate epithelium are regulated by androgen and paracrine production of growth factors from the surrounding stroma. Similarly early prostate cancer is dependent on androgen and locally synthesized growth factors. Androgen-deprivation therapy, usually with combined androgen blockade, is standard initial treatment for androgen dependent prostate cancer. However the effectiveness of this initial treatment invariably yields to the emergence of androgen independent cells with increased capability to disseminate from the encapsulated tumor to distal site such as lungs, bone, lymph nodes and liver (Ewing 1922; Shah, Mehra et al. 2004). This presents a significant medical problem as available therapies have shown little success and low patient survival.

The molecular basis for tumor progression is unclear despite years of study and effort. Although acquired processes such as invasiveness and migration of tumors have been the focus of recent studies, very few targeted approaches have lead to successful intervention. This is partially due to the limitations of available two-dimensional assays that follow the complete metastatic program within appropriate environments. Acquired cellular behavior such as increased growth factors, decreased cell adhesion, and the presence of angiogenic properties facilitate the progression of the cancer. For that reason, mechanisms of acquired cellular behavior in cancer cells should be examined within three dimensional environmentally appropriate assays.

1.1. THE PROSTATE

The prostate is an exocrine male accessory sex gland that is located just beneath the urinary bladder and is associated with the urethra (Cunha and Donjacour 1987). The prostate is found exclusively in mammals and produces a fluid rich in fructose, zinc ions, growth factors and prostaglandins. The mature mammalian prostate is a glandular organ consisting of epithelial and stromal cell types that are hormonally regulated. The epithelium consists of a single layer of polarized columnar epithelial cells together with basal and neuroendocrine cells. The epithelial cells supply secretions that empty through ducts into the urethra to form the major component of the seminal plasma of the ejaculate. The surrounding stromal compartment comprises of fibroblasts, smooth muscle cells and loose collagenous extracellular matrix (ECM), in addition to neuronal, lymphatic and vascular components. Despite the prostate's stable growth in adulthood, the gland may continue to become enlarged in size slightly after the age of fifty in men (Cunningham 1990; Morganstern and Abrahams 1994). Although enlargement of the prostate is not necessarily associated with benign prostatic hyperplasia (BPH) or prostate cancer, it could play a role in these expansions.

1.1.1. Prostate Cancer Epidemiology

In the United States, prostate cancer remains the most common solid tumor malignancy in men, causing ~30,000 deaths in 2004 (Jemal, Murray et al. 2003). In the year 2003, approximately 220,990 new prostate cancer cases were diagnosed (ACS: Cancer facts and figures, 2003), with more than 70 % of prostate cancers being diagnosed in men over 65. This equates to an incidence rate of 1 in 7 after the age of 60 as compared to a risk of 1 in 44 between the age 40 and 59 years (Haas and Sakr 1997; Bruckheimer, Gjertsen et al. 1999). These data highlight the prevailing

medical wisdom that progression of localized prostate cancer advances slowly, with survival lasting over a decade, even in untreated cases. As the probability of incidence greatly increases in elderly men, surgical and radiological ablation of the tumor (and the prostate) carry significant morbidity and even subsequent mortality. Although there has not been an identified cause of incidence, family history, age and ethnicity are well-established risk factors in prostate cancer.

African American males in the United States have the highest prostate cancer rates of any population in the world (Ross, Pike et al. 1998; Underwood, Jackson et al. 2004). Incidence of this disease is 37% higher than in white Americans, whereas mortality is an even more serious problem, with an increase of 140% in African Americans when compared to the mortality in Caucasian Americans (Farkas 1997). The disease, common in North America and Northwestern Europe, is rare in Asia, Africa, and South America. The geographical preference of prostate cancer incidence has led researchers to investigate genetic, environmental, and social factors as a basis for epidemiological differences noted between African Americans and whites (Cude, Dixon et al. 1999).

A major contributor to the increased identification rate is the development of more sensitive detection methods. Since the late 1940's, the rate of identification of prostate cancer cases has increased 67% or about 1.8% per year (Hsing, Tsao et al. 2000). This dramatic increase is in part due to the greater availability and use of detection methods, which include digital rectal examination, the use of a serum tumor marker called prostate-specific antigen (PSA), transrectal guided needle biopsy, and ultrasonography (Steenland, Rodriguez et al. 2004). PSA, the most widely practiced diagnostic test, has been attributed with the rise in cancer incidences. The data suggest that it is not so much the prevalence of prostate cancer that is increasing but that we may be diagnosing more cases from a pool of men with latent, previously

unsuspected disease and that these diagnoses occur at an earlier, more localized stage of the disease (Haas and Sakr 1997; Hernandez and Thompson 2004).

Options to managed diagnosed prostate cancers are limited. Among the effective treatments is chemical or physical castration to induce an androgen-withdrawal apoptosis of the tumor cells. Typically, an initial response to androgen ablation therapy is observed in 70% of patients but most patients relapse within 2 or 3 years. The failure of androgen ablation therapy is attributed to the growth of androgen-unresponsive tumors. The progression to invasion and metastasis is thus only slowed and not blocked.

As prostate tumors progress extracapsular invasion presents a significantly greater problem. Invasion of the adnexia results in compromised function of the renal and genital systems with significant physiological and psychological morbidity. Additionally, metastatic spread carries a high mortality burden. Bone (83%) was the most common site of metastasis, followed by liver (66%), lymph nodes (63%), lungs (50%), soft tissues (40%), dura (26%), and adrenal glands (23%) (Shah, Mehra et al. 2004). Once in bone, the prostate carcinoma cells induce an osteoblastic response that is responsible for debilitating bone pain. Liver metastasis, usually only detected at autopsy, also produces a conducive environment for sustained growth via liver's robust production of prostate cancer stimulating growth factor, EGF, and TGF-α. Brain metastasis is rare (1-2 %) but carries the worst prognosis, with a maximum life expectancy of 6 – 7 months.

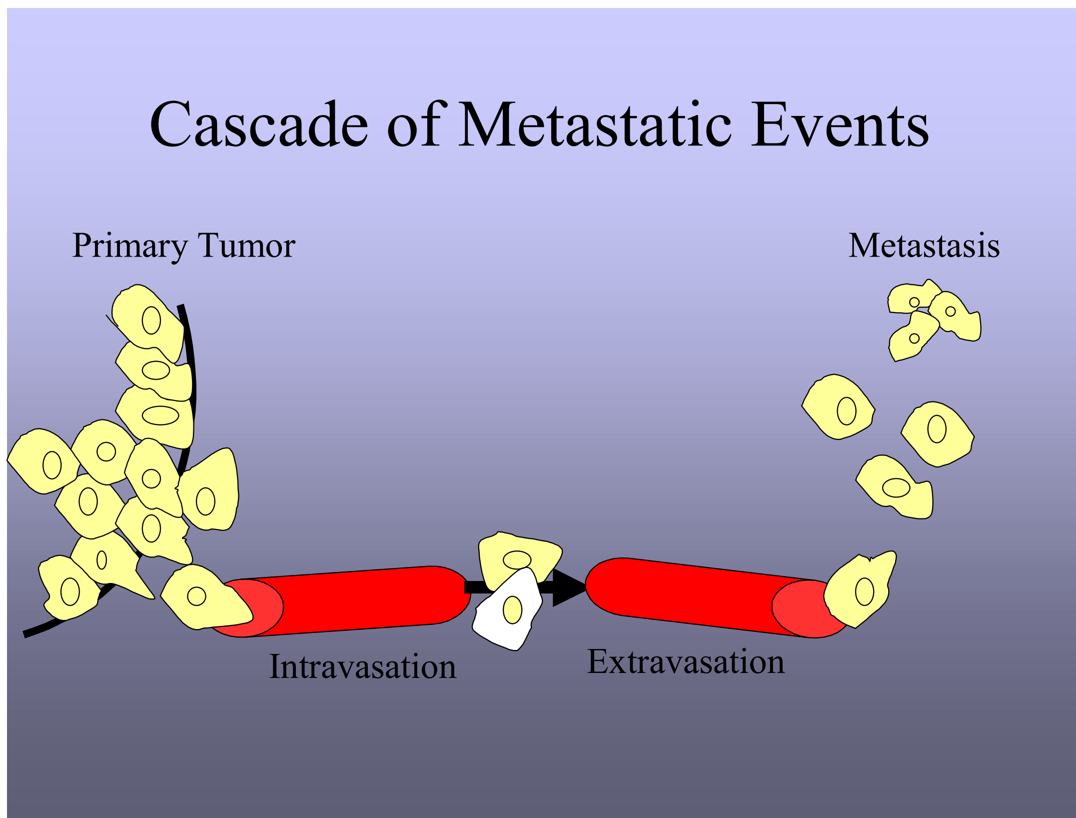

Figure 1. Cascade of Metastatic Events. Tumor cells invade basement membrane and intravasate into blood vessel or lymphatics. Tumor cells must then extravasate from circulation to colonize at distant metastatic foci.

1.1.2. Benign Prostatic Hyperplasia

Benign Prostatic Hyperplasia (BPH) is a disease in which the prostate resumes growth late in life. It is a common condition in men that increases steadily with age. Approximately 85% of all men older than 50 years have symptoms arising from BPH and 50% of all American men

require treatment for symptomatic relief of clinical BPH by the ninth decade of life. Benign prostatic hyperplasia (BPH) is defined as hyperplasia associated with both the stromal and epithelial compartments of the gland (Shah and Getzenberg 2004). Morphologically, it is characterized by the formation of new architecture by budding of the epithelium from preexisting ducts (Lee and Peehl 2004). This new architecture resembles the appearance of the mesenchyme in periurethral nodules, which during the earliest manifestation of BPH, is reminiscent of embryonic mesenchyme (Chatterjee 2003; Lee and Peehl 2004). In contrast, prostate carcinoma arises nearly exclusively from the peripheral zone and BPH from the transition zone of the gland (Castro, Giri et al. 2003).

The pathophysiology of BPH indicates that pharmacological agents that decrease the size of the prostatic adenoma or the tone of prostatic smooth muscle are effective treatment for intravesical obstruction in BPH (Diaz and Patterson 2004). Therefore most therapies target reduced of 5 alpha-dihydrotestosterone the major androgen in BPH for essentially shrinking the prostate's size or relaxing prostatic smooth muscle. Androgen receptor and its ligand have been shown to be extremely viable target for systematic regression of the prostate's size.

Despite current efforts directed toward establishing linkages between hyperplasia and clinical prostate cancer, BPH has been shown to be neither a premalignant lesion nor a precursor of prostate cancer. However, useful comparisons have been made using cell populations of BPH and prostate cancer to analyze differences or similarities between gene and phenotypic expression, in an effort to identify molecular markers that drive malignancy in prostate cancer but spares BPH.

1.2. Androgens

Androgens are central to prostate cancer progression, controling the development, growth, differentiation and function of the prostate gland (Cunha and Donjacour 1987; St-Arnaud, Poyet et al. 1988). The androgen receptor (AR) belongs to the superfamily of nuclear receptors that mediate the actions of steroids, retinoids, vitamin D3 and thyroid hormones (Zilliacus, Wright et al. 1995). The AR is a ligand activated transcription factor that mediates the biological responses of androgens. ARs have the ability to stimulate cell proliferation and inhibit cell death of normal prostatic glandular epithelial cells (Kyprianou, Martikainen et al. 1991), as well as maintain of cell morphology and functional activity in the adult prostate (Cunha and Donjacour 1987; Dirnhofer, Berger et al. 1998). In all stages of male development, androgens act upon specific intracellular AR (Cunha and Donjacour 1987; Wilding 1992). The AR is expressed throughout the pathophysiological process of prostate cancer progression. Results from immunohistochemistry studies have concluded that the AR is present in primary and metastatic prostate cancer regardless of stage and grade. It is also expressed in androgen independent cancers (Ruizeveld de Winter, Trapman et al. 1991) thus implicating it's relevance during prostate cancer progression.

Current therapies for prostate cancer are aimed at either reducing androgen levels or to prevent binding to the androgen receptor. The effectiveness of androgen ablation in the management of prostate cancer progression is hampered by limited duration; the median length of response is generally only 18-24 months (Diaz and Patterson 2004; Taplin and Balk 2004). Due to the heterogeneity of tumors consisting of various subpopulations of cells that respond differently to androgen withdrawal therapy, prostate cancer often progresses to a fatal, androgen independent or refractory state (Chatterjee 2003). The transition of the prostate cancer cell to an androgen independent phenotype is a complex process that involves selection and outgrowth of

pre-existing clones of androgen-independent cells (clonal selection) as well as adaptive up-regulation of genes that help the cancer cells survive and grow after androgen ablation (adaptation). The loss of androgen sensitivity is generally considered to have four causes: selection of cancer clones; adaptation of cells to an environment without androgen; an alternative pathway of signal transduction; and reduced involvement of ARs (Grossmann, Huang et al. 2001; Gelmann 2002; Navarro, Luzardo et al. 2002). Castrated neonatal mice demonstrated regression of prostate growth and maturation; however, administration of exogenous testosterone, the major physiological androgen, reversed this effect (Cunha and Donjacour 1987). During the pathophysiology of cancer in castrated humans, prostate cancer cells develop a growth advantage by amplifying or mutating AR, thus altering AR co-regulatory molecules and developing ligand-independent AR activation pathways (Suzuki, Ueda et al. 2003). Amplification of the AR gene or increased protein expression, in prostate tumors is a potential mechanism to utilize low levels of androgens, which are present in castrated patients. Therefore, ARs might play an important role in the progression of androgen independence in prostate cancer.

A cascade of events that begins in the hypothalamus and results in the production of testosterone achieves activation of the androgen receptor. The hypothalamus releases LHRH into the hypophyseal portal system, which provides the pathway for substances to enter the anterior pituitary gland. The pituitary controls the secretion of gonadotropins (Velduis 1991). The anterior pituitary is stimulated by LHRH to release follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Haas and Sakr 1997). In turn, LH stimulates the Leydig cells in the testicles to produce testosterone; FSH, in accordance with testosterone, stimulates the Sertoli cells and the spermatogonia causing spermatogenesis (Audersirk 1999).

Testosterone diffuses freely into the prostate gland via the bloodstream (Ross, Pike et al. 1998). Upon entrance, molecules of testosterone are metabolized by the enzyme 5-alpha (5-α) reductase into dihydrotestosterone (DHT). This active form of testosterone binds to androgen receptors due to selective preference. DHT acts to stimulate protein production, which results in cell division and inhibition of programmed cell death (Garnick 1997).

In summary, patients with metastatic prostate cancer will experience a predictable progression of their disease from an androgen responsive state to a relentless androgen-independent phenotype. The AR is central to growth signaling in prostate cancer cells and compiled data suggest that the AR remains active in progressive androgen-independent prostate cancer through a variety of mechanisms aimed at increasing the growth response to lower levels and a wider variety of compounds. Once androgen independence has been achieved, however, prostate cancer cells rely on other, often locally synthesized, factors to provide the proliferative signals required for growth (Dondi, Limonta et al. 1994; Suzuki, Ueda et al. 2003).

1.3. Growth Factors

Aggressive tumors consist of a multitude of perturbations including dsyregulation of oncogenes and tumor suppressor genes. Although genetic alterations are a precursor to a tumor lineage, growth factors and other extracellular signaling agents such as hormones and regulatory peptides drive the progression of tumor cell proliferation and differentiation. Invasive tumors secrete numerous growth factors and chemokines; commonly implicated are the epidermal growth factor (EGF), hepatocyte growth factor (HGF), fibroblast growth factor-1 (FGF-1, aFGF), keratinocyte growth factor (KGF, FGF-7), insulin like growth factor 1 (IGF1), interleukins (IL)-6, IL-8, and vascular endothelial growth factor (VEGF). The biology of these factors differ as neither their site of synthesis nor site(s) of action are restricted to defined tissues. Autonomous secretion of

ligands creates self-stimulatory autocrine signaling loops. Increased intracellular signaling stimulates tumor cell proliferation and migration, resulting in a more aggressive phenotype. Various strategies for treatment of prostate cancer involve blockage of EGF receptor and/or inhibition of it's intracellular signaling, thus implicating its importance in prostate tumor progression (Camp, Summy et al. 2005; Pal and Pegram 2005; Vallbohmer and Lenz 2005).

1.3.1. Epidermal Growth Factor Receptor and Activating

The epidermal growth factor receptor (EGFR) is a 170-kDa single chain transmembrane glycoprotein composed of 1186 amino acids (Carpenter and Cohen 1990; Sherwood, Van Dongen et al. 1998). It has been identified in normal, hyperplastic and malignant prostatic epithelium (Harari 2004). The EGFR consists of an extracellular domain with high cystine content and N-linked glycosylation, and binding sites for its ligands. Its intracellular domain consists of protein kinase activity and tyrosine residues (Singer, Hudelist et al. 2004).

EGFR has been implicated in epithelial cell malignant transformation and is found in all prostate cancer cell lines, with androgen-independent cells expressing 10 times more EGFR than the androgen dependent lines (De Miguel, Royuela et al. 1999). Enhanced expression of the EGFR on cancer cell coupled with paracrine/autocrine stimulation from activating ligands TGF-α and EGF has been associated with excessive proliferation and metastasis. In primary tumors, evidence of a paracrine situation between activating ligands and their receptors have been extensively studied (Kim, Turner et al. 1999; Maheshwari, Wiley et al. 2001; Singh and Harris 2005). The selective response of epithelial cells to paracrine stromal TGF-α and not autocrine EGF has led to the suggestion that EGFR is located on the basolateral surface of the cells, though the epithelial cell tight junctions prevent luminal EGF from reaching the surface. In the paracrine situation, TGF-α is expressed in the adjacent stromal cells while the EGFR location is in the

epithelial cells (Chen, Xie et al. 1994; Gioeli, Mandell et al. 1999). The transition from a paracrine to an autocrine stimulatory stage is correlative to advanced prostate cancer progression (Arteaga 2002). During the shift to the autocrine stage, EGFR and TGF-α are coproduced by the prostate tumor cells in order to support continuous proliferation, however this requires a disruption in cell tight junctions (Hazan and Norton 1998; Gioeli, Mandell et al. 1999; Thiery 2002). This has been shown to be a necessary step in aggressive cancers ability to acquire enhanced cell motility and invasiveness. It is worth noting that disruption of the TGF-α stimulated EGFR autocrine stimulatory loop by an EGFR antibody diminished EGFR-driven DU-145 cell invasion i*n vivo* (Turner, Chen et al. 1996); and *in vitro* (Xie, Turner et al. 1995).

1.4. Invasion and Metastasis

The process of cancer metastasis consists of a series of sequential interrelated steps, with the outcome depending on both the intrinsic properties of the tumor cells and the host. Metastatic dissemination of neoplasia cells to secondary sites is the primary cause of death among cancer patients (Hanahan and Weinberg 2000; Zijlstra, Mellor et al. 2002). Both experimental investigations and clinical observations have established that, in order for a tumor cell to hematogenously disseminate, it must intravasate into the circulation, arrest at a secondary site, and initiate secondary growth (Chambers, MacDonald et al. 1995; Al-Mehdi, Tozawa et al. 2000; Comoglio and Trusolino 2002) (Figure 1). Prostate tumor cells disseminate mainly via bloodstream or lymphatics. After dissemination, cancer cells must avoid ankiosis and reform tumor mass at target tissue. Secondary tumor formation is not a guaranteed process. The

sequential nature of this metastatic cascade implies that failure to complete even one of these steps eliminates the possible development of secondary colonization.

As cells commit to the metastatic paradigm, they have an increased capability to loosen their connections to the substratum and break cell-cell adhesions. Integrin receptors that mediate the cell-substratum adhesion and cadherins that mediate cell-cell adhesion are essential for cells to migrate and invade. While previous hypothesis contained the idea that cell-cell and cell-substratum adhesion were separate events, it has been recently proposed that these events are not mutually exclusive and in fact are coordinated events (Hinck, Nelson et al. 1994; Nelson and Nusse 2004). Integrins, act as adhesion receptors to the substratum during tumor cell migration through extracellular matrix (ECM), however cell-cell contacts must be decreased for invasion to occur resulting in matrix remodeling by various proteases including matrix metalloproteinases. This motile strategy is used both to invade local adnexia and gain access to conduits for distant dissemination.

After breaking off from the primary tumor, cancer cells travel through the blood vessels. Those that reach a secondary site, such as bone, lung, and liver, may colonize and form a metastasis. Recent imaging work by Chambers and colleagues (Chambers, Groom et al. 2002) have shown in skin cancer that only 1 in 40 melanoma cells arriving at the liver will form micrometastases, suggesting cell proliferation is a rate limiting factor at metastatic foci. Therefore, it raises the question as to what makes this subpopulation of cells capable of avoiding designed barriers of inhibition. The underlying mechanisms for metastasis and invasion are not well understood, but appear to surround the transition from epithelial to a mesenchymal phenotype. At the central core of this transitional process is the loss of cell-cell contact which enables enhanced cell migration. Therefore, any knowledge gained towards understanding the

metastatic process will promote the development of therapeutics that target the acquired tumor properties needed for metastasis and invasion to occur.

1.5. Adhesion Molecules in Prostate Cancer

As prostate cancer progresses and migrates to distal organs, cells undergo fundamental changes that allow them to ignore regulatory signals which tightly control their growth and motility, within their local environment. Unregulated growth and migration of cells is partially due to an alteration of integrin expression, accompanied with a loss of cell-cell adhesion molecule expression, characteristic of the phenotype of malignant tumors. The development of metastatic disease encompasses a complex cascade of events in which cells dislodge from the primary tumor mass, migrate through the extracellular matrix, and eventually enter and establish tumors at secondary sites in the body. At the core of this process lie the changing cell adhesion molecular profiles of the tumor cells that dictate their interactions with the surrounding extracellular matrix and neighboring cells.

Cell adhesion molecules bind specifically to cell surface molecules which in turn can bind specifically to additional cell surface molecules (receptors) on another cell (Mendelson, Howley et al. 1995). A cell adhesion molecule can bind to itself (homophilic interaction) or to unrelated cell surface molecules (heterophilic interaction) and many cell adhesion molecules can do both (Takeichi 1993). Likewise, adhesion molecules mediate adhesion between two cells of the same type (homotypic adhesion) as well as between cells of different tissue origin (heterotypic adhesion). Most cell adhesion molecules have multiple receptors and these are functionally identified as mediating cell-cell binding or aggregation. Structurally, many different

kinds of molecules can mediate cell adhesion, however many studies involving cancer cell adhesion have concentrated on the cell-cell and cell substratum adhesion molecules known as integrins and cadherins (Mareel, Behrens et al. 1991; Lowy, Knight et al. 2002; Chunthapong, Seftor et al. 2004). For this study, we will concentrate on the cadherin superfamily and its associated molecules.

1.5.1. Cadherins

Cadherins are a superfamily of molecules that form a group of cell-cell adhesion molecules which are calcium-dependent transmembrane glycoproteins (Mason, Davies et al. 2002). These molecules are required for cell-cell recognition, tissue morphogenesis, inhibition of apoptosis (Alahari, Reddig et al. 2002), cell signaling, and maintenance of tissue integrity in both vertebrates and invertebrates (Bogenrieder and Herlyn 2003; Perez-Moreno, Jamora et al. 2003; Takeichi 2004). They mediate cell-cell adhesion mainly through homotypic interactions, although heterotypic binding between different cadherin molecules is possible (Jiang 1996). The typical mammalian cadherin is a transmembrane glycoprotein consisting of between 723 to 748 amino acids; the fully mature forms of which have a molecular mass of approximately 120 kDa (Behrens 1999). Cadherins are classified according to their structural and functional similarities: classical or type-I cadherins, atypical or type-II cadherins, desmocollins, desmogleins, and protocadherins. Types I cadherins are: 4-11, B-(Mila glia), C-(blastula and early gastula), EP-(homologue to both E- and P-cadherin), N-(neural), P-(placental), R-(retinal), T- (truncated), OB-(osteblast), K-(kidney), M-(muscle), desmogleins 1-3 and desmocollins 1-3 (desmosomal cadherins) (Jiang 1996). These mediate homophilic adhesion between cells in a Ca2+-dependent manner. Type 2 cadherins include VE-, OB, F and cadherins 6, 7, 8, 10, 11, 12, among others.

Although all of these cadherins play essential roles in cell adhesions, E-cadherin is one of the most frequently identified cadherins of the metastatic cascade in most human cancers.

E-cadherin is confined to all epithelia originating from ectodermal, mesodermal and endodermal tissue. It is synthesized as a precursor polypeptide that is processed to the mature polypeptide (120kDa) shortly after the addition of complex carbohydrate groups in the late Golgi complex. The mature form is then delivered to the cell surface. Induction of Ca^{2+}-dependent cell-cell contact results in the rapid localization of surface E-cadherin molecules to the regions of contact, where they form homophilic junctions with neighboring cells. Structural analysis of cadherin-mediated cell-cell adhesion indicates that cadherins act as a cell adhesion zipper (Shapiro, Fannon et al. 1995). This zipper is made by E-cadherin forming cell-to-cell adhesion complexes in the cellular membrane with itself and the following cytoplasmic proteins, α-, β-,γ-catenins, and p120 (Shibamoto, Hayakawa et al. 1995; Jiang 1996). However, loss of this E-cadherin mediated zipper is common to most carcinomas. No real consensus exists on how inactivation occurs, however the following epigenetic mutations have been implicated: mutational inactivation of the E-cadherin gene, hypermethylation of the promoter, transcriptional repression by SIP1 or snail, transactivation of other cadherins, tyrosine phosphorylation of intercellular catenins, and ectodomain shedding of E-cadherin by matrix metalloproteinases (MMP) (Van Aken, De Wever et al. 2001; Hajra, Chen et al. 2002; Reynolds and Carnahan 2004; Wheeler 2005).

α-Catenin is a 102-kDa multifunctional protein with multiple interaction sites, including amino-terminal β-catenin-binding site, homodimerization sites, and amino-terminal as well as the carboxyl-terminal actin-binding sites (Ozawa 1998). α-Catenin influences adhesive stability through linking the β-catenin-E-cadherin complex to the actin cytoskeleton and promoting cell-

cell interactions (Richmond, Karayiannakis et al. 1997; Kallakury, Sheehan et al. 2001). Decreased expression results in instability of the complex and decreased cell adhesion (Kallakury, Sheehan et al. 2001; Kallakury, Sheehan et al. 2001; Moon, Choi et al. 2001). Moreover, α-catenin expression inhibits ß-catenin-dependent activation of TCF-mediated transcription (Simcha, Shtutman et al. 1998; Giannini, Vivanco et al. 2000; Giannini, Vivanco et al. 2000).

The gene for β-catenin, a 92-kD protein, has been localized to chromosome 3p22.21. β-Catenin binds directly to the cytoplasmic tail of E-cadherin followed by the linkage by α-catenin to the actin microfilaments of the cytoskeleton. β-Catenin is a multifunctional protein that plays a complex pivotal role in orchestrating various cell responses, thus can be categorized as an oncogene (Kim, Crooks et al. 2002; Minamoto, Ougolkov et al. 2002; Kielhorn, Provost et al. 2003; Schneider, Finnerty et al. 2003). Its cellular behavior includes cell-adhesion at the plasma membrane and involvement in the Wnt signaling cascade. When Wnt receptors are not engaged, kinases in the APC complex phosphorylate β-catenin, thus targeting the latter protein for rapid destruction. However when the Wnt receptors are activated by their ligands or integrin linked kinase (ILK) (Wu 1999), the intrinsic kinase activity of the APC complex is inhibited. As a consequence, stable non-phosphorylated β-catenin accumulates and makes its way into the nucleus triggering inappropriate activation of transcription factors, oncogenes, and cell cycle regulators causing tumor cell proliferation and promoting oncogenesis. β-catenin sequestering from the E-cadherin complex is in part a consequence of increased accumulation in the nucleus. This is accomplished by tyrosine phosphorylation of the cytoplasmic kinases Src or Fer. Fer phosphorylation causes disruption of b-catenin to α-catenin (Piedra, Miravet et al. 2003) whereas phosphorylation by Src or the epidermal growth factor receptor (EGFR) disrupts binding to E-

cadherin (Gomez, del Mont Llosas et al. 1999; Roura, Miravet et al. 1999). Therefore, Wnt signaling and E-cadherin mediated adhesion both act as negative regulators of β-catenin translocation to the nucleus (Nelson and Nusse 2004). The dynamics of β-catenin expression and signaling in human malignancies such as carcinomas of esophagus, head and neck, and prostate offers promising avenues for therapeutic intervention.

The membrane-associated protein termed p120, originally identified as a tyrosine kinase substrate (Anastasiadis and Reynolds 2000), phosphorylated at the tyrosine, serine, and threonine residues in src-transformed cells, or in response to growth factor stimulation (Jawhari, Farthing et al. 1999). It shares structural similarity with the *Drosophila* Armadillo protein and the vertebrate β-catenin and α-catenin proteins. This is evidenced by its characteristic Arm domain that is composed of repeats of a 42-amino acid motif. In the cell, p120 is localized to the E-Cadherin/catenins cell adhesion complex (Shibamoto, Hayakawa et al. 1995). Like α and β-catenin, p120 is directly associated with the cytoplasmic C-terminus of E-Cadherin via its Arm domain and may similarly interact with other cadherins (Behrens 1999). Initially, the importance of p120 in the cell adhesion complex was understated as only being required for clustering of cadherins and strong cell–cell adhesion (Yap, Stevenson et al. 1997; Thoreson, Anastasiadis et al. 2000). However, recent evidence suggests that p120's core function in the complex is to regulate cadherin turnover (Ireton, Davis et al. 2002; Davis, Ireton et al. 2003). Evidence supporting the dynamic relationship was discovered when restoring p120 expression efficiently rescues proper epithelial morphology by stabilizing E-cadherin and increasing its abundance approximately 10-fold (Ireton, Davis et al. 2002).

1.5.2. Expression of Cadherins in Cancer Progression

EMT (epithelial-mesenchymal transition or EMT) has been shown to be a necessary step in the dissemination of cancer cell from the primary tumor mass. During this progress there have been documented changes in the phenotypic expression of the cancer cells including a reduction in the cell adhesiveness. The mechanisms responsible for such changes in adhesion include mutations in the *E-cadherin* gene (*CDH1*) that compromise the adhesive capacity of E-cadherin (Hajra and Fearon 2002), hypermethylation of the *E-cadherin* promoter (Graff, Herman et al. 1995; Hennig, Behrens et al. 1995), or a combination of mutations in one allele with loss or inactivation (by DNA methylation) of the remaining allele (Berx, Becker et al. 1998; Machado, Oliveira et al. 2001). However, in many types of cancer including breast and prostate cancers, E-cadherin expression is lost without mutations in the gene (Hirohashi 1998), due to transcriptional repression of *E-cadherin* (Batlle, Sancho et al. 2000; Cano, Perez-Moreno et al. 2000; Grooteclaes and Frisch 2000; Comijn, Berx et al. 2001; Perez-Moreno, Locascio et al. 2001; Poser, Dominguez et al. 2001; Hajra and Fearon 2002. A family of zinc finger proteins of the Slug/Snail family, EF1/ZEB1, SIP-1, and the basic helix–loop–helix E12/E47 factor that interact with E-box sequences in the proximal E-cadherin promoter have been implicated as the transcription factors triggering repression [Bolos, 2003 #488; Nieto 2002). Of the transcriptional repressors mentioned above, Slug expression demonstrated a much stronger correlation with loss of E-cadherin in breast cancer cell lines than did SNAIL expression, suggesting Slug is a likely *in vivo* repressor of *E-cadherin* expression in breast carcinoma (Come, Arnoux et al. 2004).

During EMT, cancer cells acquire phenotypic advantages that are imperative to their dissemination from the primary tumor mass (Comoglio and Boccaccio 2001; Conacci-Sorrell, Zhurinsky et al. 2002). Decreased cell-cell adhesion in many cancers may not only be genetic but a consequence of receptor tyrosine kinase signaling. Autocrine stimulation of EGFR signaling

and c-Met receptor by HGF have been show to influenced the downregulate E-cadherin expression with subsequent increased cell proliferation, dedifferentiation, and induction of cell motility (Downing and Reynolds 1991; Hazan and Norton 1998; Andl, Mizushima et al. 2003). As noted earlier, EGFR mediated downregulation of E-cadherin in breast carcinoma and prostate carcinoma cells are a direct result of phosphorylated catenins. Extensive investigations have revealed that increased phosphorylation of the preferential catenins, β-catenin and p120, destabilize the cadherin complex thus inducing scattering of cancer cell lines to a more invasive phenotype (Nakashiro, Okamoto et al. 2000). The underlying pathophysiology of these events reveal a situation where decreased E-cadherin levels concede the tight junctions and enable apically-secreted EGF to establish an autocrine loop with the basolaterally sequestered EGFR (Kassis, Moellinger et al. 1999). Decreased E-cadherin levels also promote increased HGF production, in a reinforcing cycle. In addition to disrupting the cell-cell junctions and enabling a more migratory phenotype (Hiscox and Jiang 1999), HGF/SF upregulates secretion of matrix metalloproteinases that degrade the extracellular matrix aiding in tumor dissemination. HGF upregulates matrilysin (MMP-7) that mediates extracellular cleavage of E-cadherin, thereby further disrupting cell-cell adhesion and switching of prostate cells from a lesser to a highly invasive phenotype (Davies, Jiang et al. 2001).

Concomitant with the loss of E-cadherin, N-cadherin levels increase during the EMT noted in carcinomas. This increased expression of N-cadherin has also been observed in invasive prostate cancer cell lines (Tran, Nagle et al. 1999; Seidel, Braeg et al. 2004). The decreases in E-cadherin expression and increases in N-cadherin expression have been shown to correlate with increased metastatic ability (Tran, Nagle et al. 1999; Suyama, Shapiro et al. 2002; Hazan, Qiao et al. 2004; Kang and Massague 2004). Although several reports have implied a switch from E

to N-cadherin during cancer progression, N-cadherin functions have primarily been associated with angiogenesis (Nakashima, Huang et al. 2003).

1.6. Luteinizing Hormone Releasing Hormone

Among the therapeutic approaches to prostate cancer are bilateral orchiectomy, treatment with synthetic estrogens, diethylstilbestrol (DES), prostatectomy, radiation therapy, chemotherapy and androgen deprivation. However, each of these approaches incurres side effects that reduce their therapeutic efficiency. Clinicians have begun to carefully weigh the potential benefits of recommending hypothalamic luteinizing hormone releasing hormone (LHRH) analogs as an approach to therapy for advanced prostate cancer (Teillac, Bono et al. 2005) GnRH (also known as luteinizing hormone-releasing hormone or LHRH) is produced in the hypothalamic area of the brain under the influence of norepinephrine, dopamine, histamine, and other neurotransmitters (Cook and Sheridan 2000). The practice of hormone replacement began in the 1700s with the discovery a short-lived decapeptide known as Gonadotropin-releasing hormone (GnRH). Modifications in the amino-acid sequence of the native decapeptide resulted in the production of literally thousands of derivatives to maximize various potent, long-acting analogs that have the potential to be used therapeutically.

LHRH analog consists of both agonist and antagonist. LHRH receptor agonists such as leuprolide, bruserelin, and goserelin (with or without an antiandrogen) have been used for the treatment of prostate cancer (Moretti, Marelli et al. 1996; Schally, Comaru-Schally et al. 2001; Wells, Souto et al. 2002). Chronic administration of these LHRH agonists exhibited eventual decreases in the number of GnRH receptors (termed down-regulation) and suppresses

gonadotropin synthesis. However, this is preceded by a variable period ‘flare’ of pituitary gonadal stimulation in which existing gonadotropin stores are liberated. This flare period generally lasts for 10–20 days and may be associated with a 10-fold rise in luteinizing hormone (LH), resulting in increased testosterone levels. LHRH agonists are therefore combined with antiandgrogens to minimize these effects. To alleviate the initial and harmful surge in testosterone seen with administration of LHRH agonists, LHRH antagonists which bind immediately and competitively to GnRH receptors in the pituitary gland were developed. Within 8 to 24 hours after the initial dose of LHRH antagonists, LH concentrations are reduced by 51–84%, the FSH concentrations by 17 to 42%. The competitive blocking of the GnRH receptor results in a rapid, but reversible decrease in LH, FSH and testosterone without any flare. This lack of testosterone surge prevents a temporary worsening of the cancer (Schally, Comaru-Schally et al. 2001; Moul and Chodak 2004).

Although the signaling mechanisms of these analogs has not yet been elucidated, new investigations into mode of action of these drugs been initiated. Initially the effectiveness of LHRH analogs were thought to be limited to decreased LH secretion from the hypothalamic-hypophyseal portal blood system with a subsequent decrease serum testosterone levels (McDougal and Skerrett 1996) making LHRH agonists (Dondi, Limonta et al. 1994; Mongiat-Artus and Teillac 2004) and antagonists (Jungwirth, Pinski et al. 1997) highly effective for the treatment of the androgen-dependent prostatic carcinoma. However, these studies did not explain LHRH analog’s reciprocal effectiveness in androgen- independent prostate cells. It was later discovered that LHRH receptors were located directly on the prostate and could possibly offer therapeutic value (Qayum, Gullick et al. 1990; Halmos, Arencibia et al. 2000). More recently, numerous reports have shown that LHRH analogs directly inhibit tumor cell

proliferation in androgen-dependent and androgen-independent prostate cancer cell lines (Dondi, Limonta et al. 1994; Jungwirth, Pinski et al. 1997; Wells, Souto et al. 2002).

The LHRH antagonist Cetrorelix, now used in clinical trials to treat patients with prostate cancer (Verschraegen, Westphalen et al. 2003), also inhibits the pituitary gonadal axis without the initial surge in LH leading to a dramatic fall in serum testosterone levels (Stricker 2001). This hormone has also inhibited the growth of the androgen-independent rat prostate cancer cell line Dunning R-3327 AT-1 and the OV-1063 human epithelial ovarian cancer cell line *in vivo* and *in vitro*, highlighting the direct effects of the LHRH antagonist on tumors (Jungwirth, Pinski et al. 1997; Jungwirth, Schally et al. 1997).

Evidence that the effects of the LHRH antagonist are through interactions with the EGFR was provided by the work of Dondi and colleagues (Moretti, Marelli et al. 1996; Dondi, Moretti et al. 1998; Limonta, Pratesi et al. 1998). In these studies, they demonstrated that EGF stimulation of androgen-dependent prostatic cell line LNCaP causes cell proliferation; however, simultaneous treatment with the LHRH antagonist Cetrorelix counteracts this effect (Moretti, Marelli et al. 1996). Moreover it was later discovered that a LHRH agonist mediating downregulation of EGFR was much less effective than the LHRH antagonist Cetrorelix (Jungwirth, Pinski et al. 1997; Jungwirth, Schally et al. 1997). Since these data demonstrate that the LHRH antagonist, Cetrorelix, has more significant inhibition of EGFR levels, this author believes a cascade of events resulting from this may in turn decrease phosphorylation of catenins, thus positively affected cell adhesion. The eventual outcome of such a cascade could lead to the decreased detachment of cell adhesion molecule E-cadherin, which may ultimately result in inhibition of prostate tumor progression.

1.6.1. Mechanisms of LHRH Signaling

The rationale for our proposed mechanism of LHRH signaling stemmed from signaling carried out in the pituitary gland or with cultured pituitary cells (Mobbs, Kaplitt et al. 1991). In these studies, binding of LHRH to its receptor induces very rapid hydrolysis of phosphatidylinositol 4, 5-biphosphate (PIP_2) by phospholipase C (PLC); thus yielding the two messengers, inositol triphosphate (IP_3) and diacylglycerol (DAG) that commence two distinct paths (Berridge 1985; Mobbs, Kaplitt et al. 1991). IP_3 causes an increase in intracellular Ca^{2+} (Blobe, Stribling et al. 1996); DAG activates protein kinase C (PKC) (al-Mazidi, Kleine et al. 1998). PKC then phosphorylates select enzymes that, along with the elevated intracellular Ca^{2+}, are responsible for the final biological effects of the hormone (Berridge 1985). Although the exact mechanism by which LHRH directly affects prostate cancer cell proliferation is controversial, it is likely that LHRH activates identical pathways in prostate cells as these enzymes are found in all tissues. Thus, from our experimental evidence, we have generated a model whereby Cetrorelix will activate LHRH receptors which result in disruption of known EGFR-mediated cell proliferation through activation of PKC. PKC activate results in negative transmodulation the EGFR, subsequently resulting in decreased kinase activity, inducing increased stability catenin expression. This results in re-expression of E-cadherin promoting cell-cell adhesiveness (Figure 2).

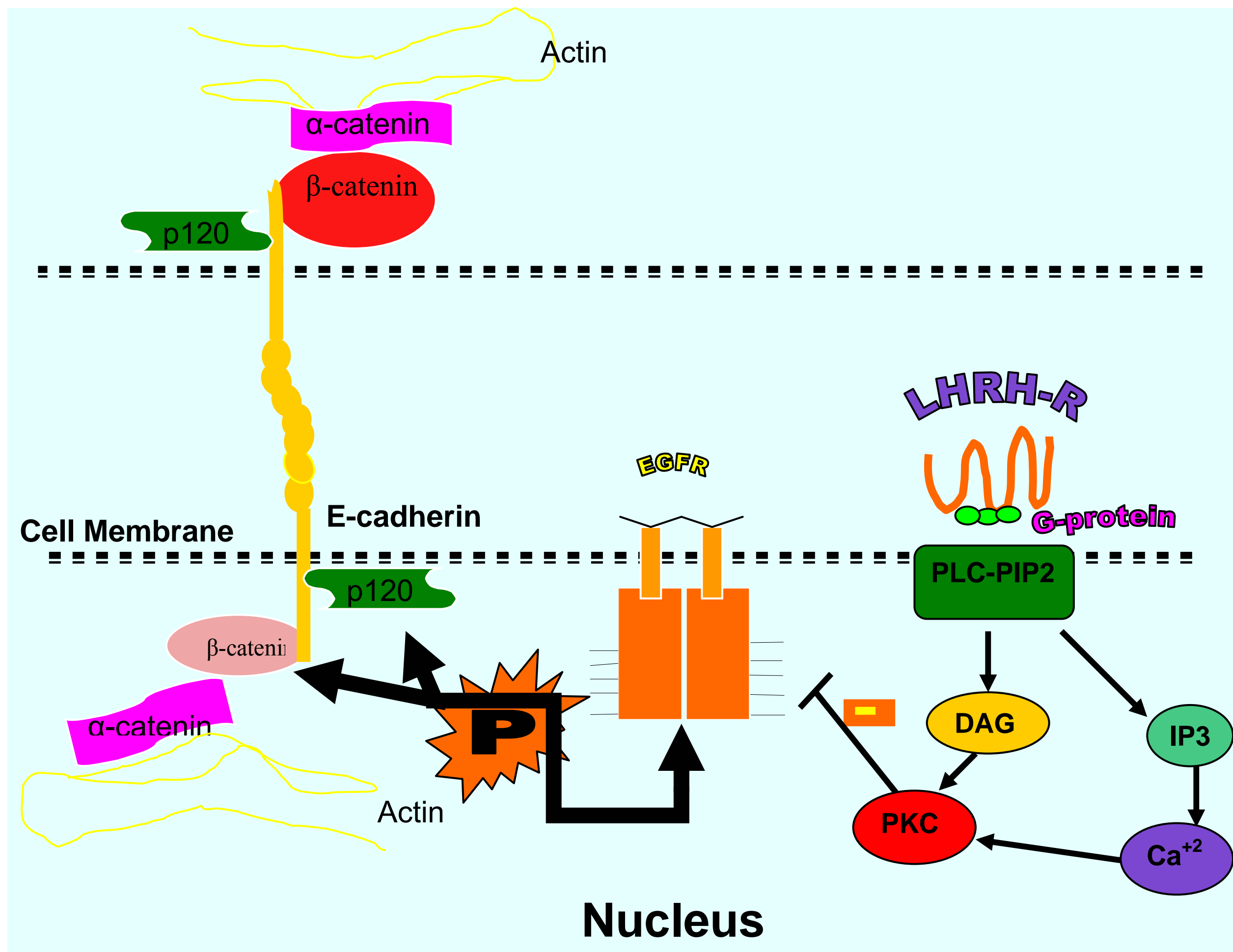

Figure 2. Proposed mechanism of action of the EGFR and cell adhesion molecules in prostate cell

1.7. SUMMARY

In summary, cancer cell metastasis is a result of a multistep mechanism which is not completely understood at the cellular level. The importance of growth factors and their interactions with cell-cell adhesion molecules has been shown to be critical to the maintenance of normal and pathogenic epithelial function. A variety of adhesion molecules and growth factor receptors communicate via signal transduction pathways to either maintain epithelial homeostasis or induce its pathological transition (Jawhari, Farthing et al. 1999; Roura, Miravet et al. 1999; Andl, Mizushima et al. 2003). Thus use of the LHRH antagonist, Cetrorelix which is currently under evaluation for clinical treatment of prostate cancer, should provide insight into possible mechanism of pathopysiological events leading to increase aggressiveness of prostate cancer cells.

2. LUTEINIZING HORMONE RELEASING HORMONE (LHRH) ANALOG REVERSES THE CELL ADHESION PROFILE OF EGFR OVER-EXPRESSING DU-145 HUMAN PROSTATE CARCINOMA SUBLINE

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2.1. ABSTRACT

Cetrorelix, a luteinizing hormone releasing hormone (LHRH) analog, has been shown to limit growth of the human androgen-independent prostate cell line DU-145, though other inhibitory actions may also be effected. Both growth and invasion of DU-145 cells are linked to autocrine epidermal growth factor receptor (EGFR) signaling. Invasiveness requires not only cells to migrate to conduits, but also reduced adhesiveness between tumor cells to enable separation from the tumor mass. Thus, we investigated whether Cetrorelix alters the DU-145 cell-cell adhesion and if this occurs via altered EGFR signaling. Pharmacologic levels of Cetrorelix limited the invasiveness of a highly invasive DU-145 subline overexpressing full-length EGFR (DU-145 WT). Extended exposure of the cells to Cetrorelix resulted in increased levels of the cell-cell adhesion complex molecules E-cadherin, α- and β-catenin, and p120. Puromycin blocked the increases in E-cadherin and β–catenin levels, suggesting that *de novo* protein synthesis is required. The Cetrorelix effect appears to occur via transmodulation of EGFR by a protein kinase C (PKC)-dependent mechanism, as there were no changes in DU-145 cells expressing EGFR engineered to negate the PKC trans-attenuation site (DU-145 A654); down-regulation of EGFR signaling produced a similar up-regulation in adhesion complex proteins further suggesting a role for autocrine signaling. Cetrorelix increased the cell-cell adhesiveness of DU-145 WT cells to an extent similar to that seen when autocrine EGFR signaling is blocked; as expected DU-145 A654 cell-cell adhesion also was unaffected by Cetrorelix. The increased adhesiveness is expected as the adhesion complex molecules moved to the cells' periphery. These data offer direct insight into the possible cross-talk pathways between the LHRH and EGFR receptor signaling. The

ability of Cetrorelix to downregulate EGFR signaling and subsequently reverse the anti-adhesiveness found in metastatic prostate cancer highlights a novel potential target for therapeutic strategies.

2.2. INTRODUCTION

Adhesion between normal epithelial cells is usually strong and stable limiting cell movement. In carcinomas, these tight cell associations must first be disrupted or prevented from forming before tumor cells are able to disseminate and metastasize. Cell-cell association is often disorganized in tumors, and has been linked to tumor invasiveness and metastasis (Pignatelli and Vessey 1994; Shino, Watanabe et al. 1995; Richmond, Karayiannakis et al. 1997). Acquisition of invasive potential by malignant cancer cells results from an accumulation of characteristics, including increased cell motility, secretion of proteolytic enzymes, and alterations of cell-substrate and cell-cell adhesion (Fidler 2003; Grunert, Jechlinger et al. 2003). The molecular mechanisms responsible for this latter process, altered cell-cell adhesion in invasive cancer cells are poorly understood (Comoglio and Trusolino 2002). However, the net result is a reduction in cadherin/catenin complexes at the cells' periphery (Morita, Uemura et al. 1999; Davies, Jiang et al. 2000). Thus, to better understand the mechanisms of tumor cell dissociation, the role of cadherins must be taken into account, as they are crucial in cell-cell adhesion (Takeichi 1993; Kim, Turner et al. 1999; Suyama, Shapiro et al. 2002).

Cadherins comprise a family of transmembrane cell surface glycoproteins that mediate calcium (Ca^{++})-dependent, homotypic cell–cell interactions through their extracellular domains, and regulate a variety of biological processes during development, morphogenesis, and tumor metastasis (Gumbiner 1996; Yap, Stevenson et al. 1997; Conacci-Sorrell, Zhurinsky et al. 2002). Ca^{++}-dependent cell-cell adhesion usually consists of rapid localization of surface E-cadherin molecules to the regions of contact resulting in homotypic binding that fosters the maintenance

of normal cellular structure. However, metastatic cancer cells are able to override or avoid contact inhibition signals employed by normal epithelial cells to control proliferation and cell movement.

The linkage between E-cadherin and the cellular cytoskeleton is a complex interaction involving a number of structural and signaling cytoplasmic proteins such as α- and β-catenin and p120 (Van Aken, De Wever et al. 2001; Mason, Davies et al. 2002). Early studies identified E-cadherin/catenin interactions as imperative for cell-cell adhesion (Chitaev and Troyanovsky 1998). β-catenin binds with high affinity to the carboxyl-terminal region of the cadherin cytoplasmic tail while α-catenin serves as an anchor, by bridging to α-actinin, to link the complex to the actin cytoskeleton (Aberle, Butz et al. 1994; Hulsken, Birchmeier et al. 1994; Funayama, Fagotto et al. 1995; Jou, Layhe et al. 1995; Rimm, Sinard et al. 1995). These molecules not only play structural roles but also alter cell responses and phenotypes. β-Catenin is also found to immunoprecipitate with the APC tumor suppressor protein (Su, Vogelstein et al. 1993; Hulsken, Birchmeier et al. 1994; Shibata, Gotoh et al. 1994), and has been recently identified as an oncogene (Kim, Crooks et al. 2002; Minamoto, Ougolkov et al. 2002; Kielhorn, Provost et al. 2003; Schneider, Finnerty et al. 2003). It is also central to cell signaling, as upon dissociation from E-cadherin, it transits to the nucleus to alter transcriptional profiles (van de Wetering, Sancho et al. 2002). Reduction in β-catenin expression decreases the stability of the adhesion complex and likely results in impairment in E-cadherin function (Willert and Nusse 1998; Lowy, Knight et al. 2002). Similarly, a reduction in E-cadherin often results in β-catenin degradation (Liu, Ikeguchi et al. 2002). Another protein associated with E-cadherin, p120 (Thoreson, Anastasiadis et al. 2000), is phosphorylated on both tyrosine and serine residues in response to a variety of growth factors such as epidermal growth factor (EGF), platelet derived

growth factor (PDGF), and colony stimulating factor (CSF)-1, suggesting involvement in active signaling (Downing and Reynolds 1991; Shibamoto, Hayakawa et al. 1995). Thus, cell-cell adhesion serves not only a structural role but dictates cellular behavior.

As carcinomas progress to the invasive and metastatic stages, select adhesive epithelial cells usually undergo a mesenchymal-like transition that enables their movement from the primary tumor mass (Comoglio and Boccaccio 2001; Conacci-Sorrell, Zhurinsky et al. 2002). During this process in breast, gastric, and pancreatic metastatic carcinomas, E-cadherin expression is frequently downregulated or even undetectable (Birchmeier and Behrens 1994; Lowy, Knight et al. 2002). This pattern of E-cadherin expression also persists in disseminated prostate carcinomas when compared to non-metastatic prostate cells (Umbas, Schalken et al. 1992; Davies, Jiang et al. 2000). In addition, the loss of E-cadherin expression has been shown as a consequence of autocrine activation of epidermal growth factor receptor (EGFR) signaling (Jawhari, Farthing et al. 1999). This combination of autocrine EGFR signaling and loss of E-cadherin expression leads to cell proliferation, dedifferentiation, and induction of cell motility (Hazan and Norton 1998). Such an association has been suggested in the progression of breast carcinoma cells to a more invasive phenotype, which correlates with downregulation of E-cadherin and overexpression of EGFR (Sorscher, Green et al. 1995; Hazan and Norton 1998). On a molecular level, EGFR signaling leads to tyrosine phosphorylation of the catenin complex with subsequent breakdown of cell adhesion (Jawhari, Farthing et al. 1999; Mariner, Davis et al. 2004).

In this study, we examined whether the beneficial anti-cancer effects of Cetrorelix include effects in addition to the established anti-proliferative effects. LHRH receptors have increased expression in many cancers compared to normal cells (Emons, Muller et al. 1998;

Schally, Comaru-Schally et al. 2001; Straub, Muller et al. 2001), with increased expression in begin prostate hyperplasia (BPH) as well (Straub, Muller et al. 2001). The presence of these receptors enables LHRH analogs to directly affect prostate tumor cells (Qayum, Gullick et al. 1990; Halmos, Arencibia et al. 2000) in addition to the indirect central androgen suppression. In addition, it has been shown that LHRH agonists directly inhibit cell proliferation of DU-145 and LNCaP prostate cancer cell lines (Dondi, Limonta et al. 1994; Dondi, Moretti et al. 1998; Limonta, Montagnani Marelli et al. 2001). In line with these observations, the LHRH analog Cetrorelix has been shown to have direct antiproliferative actions on DU-145 cells (Jungwirth, Pinski et al. 1997). As a consequence of this exposure, LHRH analogs have caused decreased levels of EGFR expression (Moretti, Marelli et al. 1996; El-Bahrawy and Pignatelli 1998; Lamharzi, Halmos et al. 1998). Previously we have shown DU-145 WT, a subline of the human prostate carcinoma cell line DU-145, presents autocrine EGFR signaling that is critical to both cell proliferation and invasion (Xie, Turner et al. 1995; Turner, Chen et al. 1996). Recently we demonstrated under both *in vivo* and *in vitro* conditions that a LHRH agonist inhibited enhanced invasiveness of EGFR-dependent proliferation in DU-145 WT through interference with EGFR signaling (Wells, Souto et al. 2002). Therefore, these data taken together lead us to hypothesize that the LHRH analog, Cetrorelix, would abrogate EGFR signaling. This abrogation would in turn decrease phosphorylation of the associated catenins; thus leading to upregulation of the cell adhesion molecule E-cadherin, which may ultimately result in inhibition of prostatic tumor progression.

2.3. MATERIAL AND METHODS

LHRH analog Cetrorelix ([Ac-D-Nal (2)^{1}, D-Phe (4Cl)^{2}, D-Pal (3)^{3}, D-Cit^{6}, D-Ala^{10}] LH-RH) was obtained from ASTA Medica (Frankfurt/Main, Germany) and dissolved in serum-free DMEM media. The primary antibodies used were mouse monoclonal antibodies to E-cadherin, α- and β–catenin, and p120 (Transduction Laboratories, California), phosphorylated-MARCKS (Cell Signaling, Massachusetts), phosphorylated-EGFR (Cell Signaling, Massachusetts) and EGFR (Zymed Laboratories, California). FITC conjugated secondary antibodies were obtained from (BD Biosciences). Secondary antibodies for the Immunofluorescence were obtained from (Molecular Probes, Oregon). Inhibitors included the EGFR specific tyrosine kinase inhibitor PD153035 (CalBiochem, California), monoclonal antibody (528) EGFR, (Oncogene, Massachusetts), EGFR siRNA (Upstate, Virginia) and the transcriptional and translational inhibitor puromycin (Sigma, Missouri). Other reagents were obtained from Sigma.

2.3.1. DU-145 Cell lines

The cell line DU-145 was originally derived from a brain metastasis of a human prostate adenocarcinoma (Stone, Mickey et al. 1978); it retains the androgen independence of the original tumor and does not express a functional androgen receptor (Dondi, Moretti et al. 1998). This cell line possesses both LHRH and EGF receptors and produces EGFR ligands, TGF-α and EGF (Xie, Turner et al. 1995; Jungwirth, Pinski et al. 1997). We have expressed exogenously-encoded EGFR in DU-145 cells (Xie, Turner et al. 1995). Utilizing established protocols, DU-145 cells were transfected by retroviral-containing EGFR constructs (Wells, Welsh et al. 1990). The Wild Type (WT) EGFR construct is a full-length cDNA derived from a placental cDNA library. Cells

expressing WT EGFR at levels which escape down-regulation, demonstrate enhanced invasiveness *in vitro* (Xie, Turner et al. 1995) and *in vivo* (Turner, Chen et al. 1996).

The DU-145 WT subline express EGFR that are phosphorylated and negatively modulated by PKC; thus, we have generated an additional DU-145 subline which is not negatively modulated by PKC (Wells, Souto et al. 2002). This subline is identical to DU-145 WT except it contains a full length EGFR in which the target site for PKC phosphorylation, amino acid threonine 654 (T654), has been replaced with alanine (DU-145 A654) by site directed mutagenesis; this construct is resistance to PKC phosphorylation and negative transmodulation (Welsh, Gill et al. 1991; Chen, Xie et al. 1996).

The DU-145 WT and A654 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (4.5g/ml glucose) (Cellgro, Virginia) containing 10 % FBS and supplemented with L-glutamine (2 mM), penicillin/streptomycin (100 units/ml), nonessential amino acids (0.1 mM), and sodium pyruvate (1 mM) (37˚C, 90% humidity, 5% CO_2 and 95 % air). For stable selection of WT or A654 EGFR, cells were grown in G418 (1000μg/ml) (Gibco, New York), though all experiments were performed in the absence of G418.

2.3.2. Invasion Assay

Cell invasiveness *in vitro* was determined by the ability of cells to transmigrate a layer of extracellular matrix, Matrigel, in a Boyden Chamber assay. Matrigel invasion chamber plates were obtained from Becton Dickinson Labware (Bedford, Massachusetts). 20,000 cells were plated in the Matrigel-containing chamber in serum-free media containing 1% BSA for the first 24 hours; this was then replaced with Cetrorelix serum-free media for the remaining 24 hours. Enumeration of the cells that invaded through the matrix over a 48 hour-period was

accomplished by visually counting cells on the bottom of the filter. All experiments were performed in triplicate chambers.

2.3.3. Flow Cytometry

3 x 10^5 cells were grown for 2 days or to 80% confluency in 60mm plates. LHRH analog Cetrorelix (10^{-5}M) was added for time intervals of 6, 12, and 24 hours and compared to diluent alone. Samples were washed with PBS and fixed with paraformaldhyde, and permeabilized with 1% Triton X 100. Samples were blocked with 5% BSA and incubated with the appropriate FITC conjugated primary antibody or primary antibody (anti-EGFR, anti-E-cadherin, anti-α−catenin, anti-β−catenin, and anti-p120) at 37˚C for 1 hour. For unconjugated samples FITC-conjugated secondary antibody was added. Fluorescence was measured by a flow cytometer (Coulter, Florida).

2.3.4. Immunoblotting

3 x 10^5 cells were grown for 2 days or to 80% confluency in six-well plates. LHRH analog Cetrorelix (10^{-5}M) was incubated for 6, 12, and 24 hour time intervals and compared to diluent alone. Protein lysates were prepared from cultured cells in the following buffer: 50 mM Tris, pH 7.5, 120 mM NaCl, 0.5% Nonidet p-40, 40 μM phenylmethylsulfonylfluoride (PMSF), 50 μg/ml leupeptin, and 50 μg/ml aprotinin (all from Sigma). Cells were allowed to lyse for 1 hour on ice; the lysed cell solution was centrifuged and the resulting supernatants extracted and quantitated using a Bradford assay. 30 μg of protein lysates were separated by 7.5% SDS PAGE, immunoblotted and analyzed using chemiluminescence (Amersham Biosciences, New Jersey). Primary antibodies used included anti-EGFR (Zymed Diagnostics, California), anti-E-cadherin, anti-β-catenin, and anti-p120 (Transduction Laboratories, Kentucky), and anti-α-catenin (Santa

Cruz Biotechnology, California). The staining was visualized by a secondary anti-mouse IgG or anti-rabbit antibody linked to horseradish peroxidase (Promega, Wisconsin).

2.3.5. siRNA for EGFR

2×10^5 cells were plated in six well plates equaling 60-70% confluency. 160 pmole of EGFR siRNA was diluted in 200 µl of Opti-MEM (Invitrogen, California). 4µl of Lipofectamine 2000 (Invitrogen, California) was diluted in 200µl of Opti-MEM and incubated for 5minutes at room temperature. The diluted siRNA and Lipofectamine 2000 were mixed and incubated for 20 minutes at room temperature. Complexes were added to each well and incubated for 24 hours. Media was changed and incubated for an additional 24 hours. Cells were lysed according to established protocols.

2.3.6. Immunofluorescence Microscopy

3×10^5 cells were grown for 2 days or to 80% confluency on glass coverslips and then treated with or without Cetrorelix (10^{-5}M) and compared to diluent alone. Cells were then fixed in 4% paraformaldehyde, permeabilized with 100 mM Tris-HCl pH 7.4, 150 mM NaCl, 10 mM EGTA, 1% Triton X-100, 1 mM PMSF, and 50 µg/ml aprotinin (all from Sigma), and subsequently blocked with 5% BSA for 1 hour at room temperature. Samples were incubated with indicated primary antibodies diluted in blocking buffer at 4°C overnight. FITC-conjugated secondary antibody was then added (BD Biosciences, California). Cells were then stained with propidium iodine for nuclear staining. Cells were analyzed with laser confocal microscopy using a Leica TCSNT 3 laser 4 PMT system (Olympus, NY).

2.3.7. Cell Aggregation Assay

Calcium-dependent aggregation of the DU-145 sublines was measured as previously described by (Shibamoto, Hayakawa et al. 1995) with the following modifications. Cell monolayers grown to 80% confluence were incubated for 24 or 48 hours in 10 % FBS in DMEM with or without 10^{-5} M Cetrorelix. Cell monolayers were detached from the culture dishes by incubating in cell stripper (Cell Gro, Virginia) for 5-10 minutes at 37˚C. Any remaining cells were detached using a rubber policeman, washed once with PBS and collected by centrifugation. Cells were resuspended in 10% FBS in DMEM and single cell suspensions made by trituration with a Pasteur pipette. Cell number was determined in the Coulter Counter Z1 (Coulter, Florida). Cells were plated in triplicate wells of a 24-well plate at 2 x 10^5 cells/well in 10% FBS in DMEM with 1 mM $CaCl_2$ and allowed to aggregate for 60 minutes on a gyratory shaker at 80 rpm at 37˚C. Assays were stopped at 0 and 60 minutes by fixing the cells in 0.5% paraformaldehyde. The extent of cell-cell binding was monitored by measuring the disappearance of single cells using the Coulter Counter Z1. The index of the degree of aggregation was measured utilizing the formula 100 x (N_0/N_{60}), where N_0 is the total cell number per well and N_{60} is the total number of particles after 60 minutes of incubation as determined by counting in a Coulter Counter Z1.

2.3.8. Statistical Analysis

Statistics for all experiments were performed using the Sigma Plot statistical program (Jandel Scientific, California). Independent Student's T-test was utilized to determine a statistical difference between experimental and the controls for individual experiments.

2.4. RESULTS

2.4.1. LHRH Analog Cetrorelix Decreases Invasion in DU-145 Sublines

To confirm and extend the inhibitory effects of Cetrorelix on prostate carcinomas we utilized a genetically engineered human androgen-independent prostate carcinoma cell line that over expresses a full length EGFR, DU-145 WT. This subline is highly invasive in response to upregulation of autocrine EGFR signaling (Xie, Turner et al. 1995; Turner, Chen et al. 1996) that exists in practically all prostate carcinomas (Kassis, Moellinger et al. 1999). In determining the utilized dose of Cetrorelix, we selected the pharmacologic dose of 10^{-5}M based on literature reports for Cetrorelix (Tang, Yano et al. 2002) and a related LHRH analog goserelin (Dondi, Limonta et al. 1994; Jungwirth, Pinski et al. 1997; Dondi, Moretti et al. 1998; Wells, Souto et al. 2002). In addition, growth studies from our laboratory utilizing Cetrorelix at 10^{-5}M inhibited DU-145 WT proliferation without causing cell death (data not shown).

To probe the extent of effectiveness of Cetrorelix against prostate cancer progression, we determined whether invasion was abrogated. Cetrorelix exposure reduced the invasiveness of the DU-145 WT sublines through a Matrigel barrier from 100% down to 23 ± 14 % (Figure 8; $n = 4$, $P < 0.05$). This level of inhibition is comparable to the decreases noted when either EGFR motility signaling via PLCγ or calpain signaling is abrogated (Xie, Turner et al. 1995; Turner, Chen et al. 1996; Kassis, Moellinger et al. 1999; Mamoune, Luo et al. 2003).

2.4.2. Cetrorelix Increases Levels of Cell Adhesion Molecules

To determine to the effectiveness of Cetrorelix treatment on altering protein expression levels, we measured EGFR, E-cadherin and its associated adhesion molecules (α- and β-catenins, and

p120) by flow cytometry. After six hours of Cetrorelix exposure, EGFR levels were significantly reduced in DU-145 WT cells when compared to non-treated, control levels. This significant reduction in EGFR levels continued throughout the 24 hour experimental time-period (Figure 3A; $P < 0.05$). While Cetrorelix decreased EGFR surface expression, it induced an increase in E-cadherin levels (Figure 3B). Likewise, the E-cadherin associated molecules α-catenin, β-catenin and p120 also demonstrated a continual increase in their expression, with all showing significant increases after 24 hours of Cetrorelix exposure (Figures 3C-E; $p<0.05\%$).

To confirm results obtained from the flow cytometry experiments, we immunoblotted for whole cell protein content of total EGFR and adhesion molecules E-cadherin and β-catenin. Again a similar pattern was seen with a reduction in EGFR levels and an increase in E-cadherin and β-catenin levels (data not shown).

To thoroughly examine if the increase in protein and expression levels of E-cadherin and β-catenin were associated with upregulation in transcription, we used the protein synthesis inhibitor puromycin. Puromycin exposure was able to completely block the enhanced ability of Cetrorelix to restore the E-cadherin and β-catenin expression levels (Figure 4).

2.4.3. Reversal in Adhesion Molecule Profile is Related to EGFR Signaling

A role for Cetrorelix in the stimulation of PKC activity was determined by phosphorylation of the MARCKS substrate for classical and novel PKC isoforms or by probing for generalized increased phosphorylation of canonical PKC-target serines (Figure 5) (Fujise, Mizuno et al. 1994; Nishikawa, Toker et al. 1997). This was further confirmed through the use of chelerythrine, a pan-PKC inhibitor (Wells, Souto et al. 2002), to prevent such phosphorylation (data not shown).

If Cetrorelix acts via PKC-mediated attenuation of EGFR signaling, then an EGFR variant lacking the PKC target site should be resistant. We utilized a DU-145 subline expressing

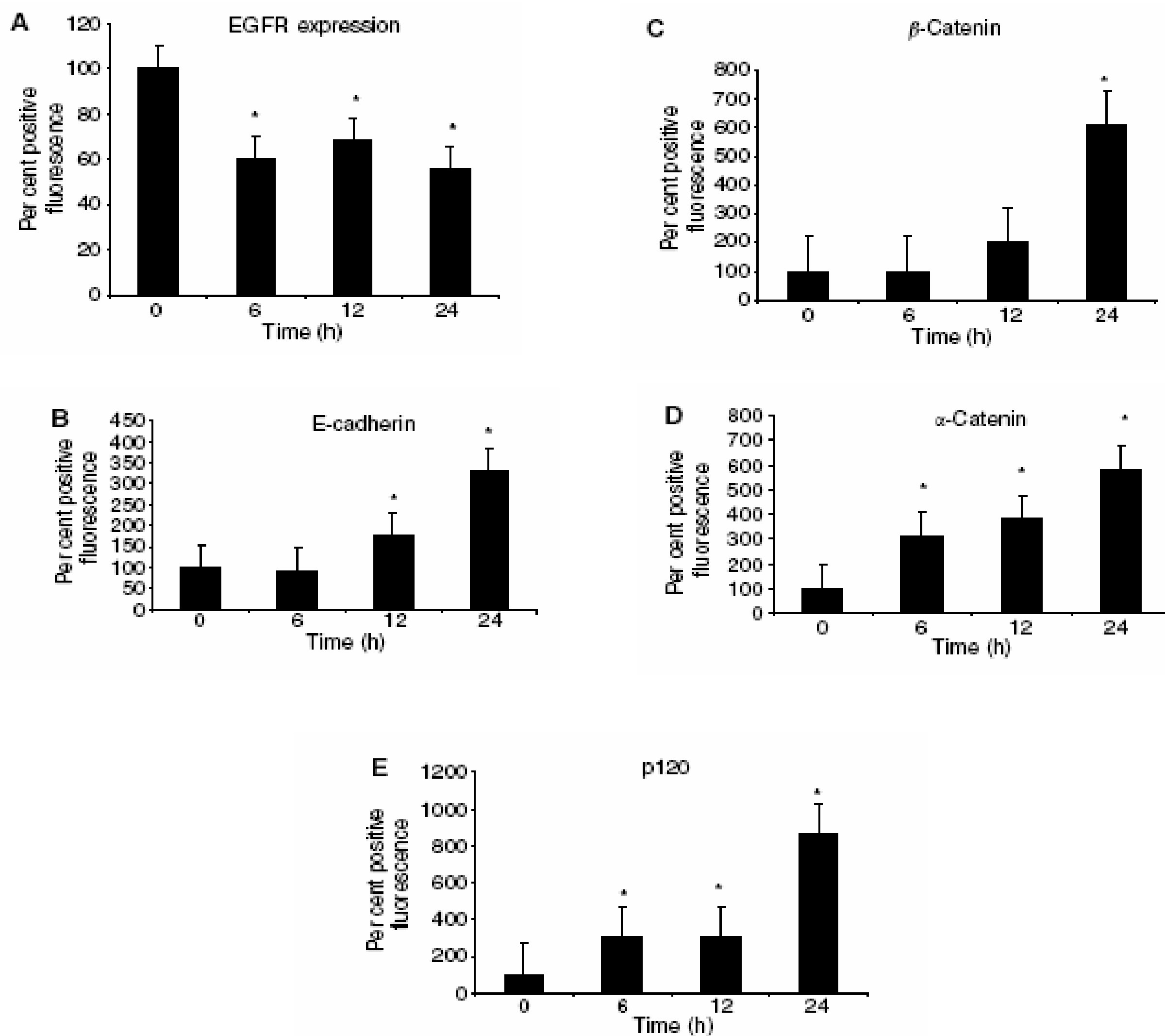

Figure 3. Expression levels were measured as the mean of percent positive fluorescence at time zero ± SEM at various time intervals. A, DU-145 WT cells labeled with FITC conjugated anti-E-cadherin were analyzed by flow cytometry. B, DU-145 WT cells labeled with FITC conjugated anti-alpha catenin were analyzed by flow cytometry. C, DU-145 WT cells labeled with FITC conjugated anti-beta-catenin were analyzed by flow cytometry. D, DU-145 WT cells labeled with FITC conjugated anti-p120 were analyzed by flow cytometry. E, DU-145 WT cells labeled with FITC conjugated anti-EGFR were analyzed by flow cytometry. Data are the mean ± SEM of 3 experiments each performed in triplicate. * indicates $P < 0.05$ compared to untreated.

an EGFR construct in which the target PKC site, threonine 654, was replaced by an alanine (DU-145 A654). Since Cetrorelix decreased EGFR surface levels (Figure 3A) and increased surface levels and protein levels of cell adhesion molecules (Figures 3B-E & 4), cells expressing this EGFR A654 construct should be at least partly resistant to Cetrorelix. Through the use of immunoblotting techniques, we examined the protein levels of the cell adhesion molecules after 24 hours of Cetrorelix exposure. Phosphorylated and total EGFR levels, as well as total E-cadherin, and β-catenin levels were not extensively altered in the DU-145 A654 cells when compared to changes observed in DU-145 WT cells (Figure 6). These findings indicate that direct abrogation of EGFR signaling by various means should yield a similar increase in E-cadherin and β-catenin levels. Both the specific tyrosine kinase inhibitor, PD153035, and the anti-EGFR antibody (mb528), increased E-cadherin and β-catenin levels similarly to those observed in DU-145 WT after Cetrorelix treatment (Figure 7 A, B). Finally, exposure of DU-145 WT cells to EGFR siRNA resulted in the down-regulation of EGFR levels and an increase in E-cadherin levels when compared to cells exposed to the non-relevant siGFP (Figure 7 C).

2.4.4. Cetrorelix Diminished Prostate Cancer Cell Invasiveness

The functional consequences of EGFR signaling cross attenuation by Cetrorelix extend to the invasive potential of the prostate carcinoma cells. While Cetrorelix significantly reduced the invasiveness of the DU-145 parental and WT cells, the invasiveness of DU-145 A654 was limited to a lesser extent (Figure 8: $P < 0.05$, comparing Matrigel invasion after Cetrorelix treatment of DU-145 A654 and WT cells). These findings suggest that the effects of Cetrorelix on both cell-cell adhesion molecules and cell invasiveness are mediated through its interference with the EGFR signaling cascade.

2.4.5. Cetrorelix Exposure Increases Cell-Cell Aggregation

To further assess the functional consequences of the concurrent Cetrorelix-related decrease in EGFR levels and the increase in E-cadherin and its associated proteins observed in the DU-145 WT subline, a calcium-dependent aggregation assay was used after 48 hours of Cetrorelix exposure (Figure 9). In these experiments, the aggregation index of DU-145 WT and A654 cells treated with Cetrorelix was compared to that of non-treated cells. We observed that DU-145 WT cells exposed to Cetrorelix formed significantly more cell-cell aggregates compared to either non-treated WT cells or treated and non-treated A654 cells, while Cetrorelix-induced DU-145 A654 aggregation was indistinguishable from non-treated cells (Figure 9; $P < 0.05$). We were also able to see similar results when we exposed DU-145 WT cells to PD153035 to block EGFR signaling (Figure 9B: $p<0.05\%$).

Cell-cell aggregation requires E-cadherin to be present on the cell surface and its associate molecules at the inner face of the plasma membrane. In DU-145 WT cells, these adhesion complex molecules were distributed throughout the cytosol (Figure 10). Upon Cetrorelix treatment, not only did the levels increase, but the molecules were redistributed to the cells' periphery; this was particularly evident at sites of cell-cell contacts, regardless of the degree of cell confluence. In aggregate, these data further confirmed with functional application that the increases observed in E-cadherin, $\alpha-$ and β-catenins, and p120 levels in Cetrorelix-exposed DU-145 WT cells are the results of a reversal of the cells invasive phenotype to one that resembles a more normal phenotype and that Cetrorelix exerts at least some of its effects via abrogation of autocrine EGFR cell signaling.

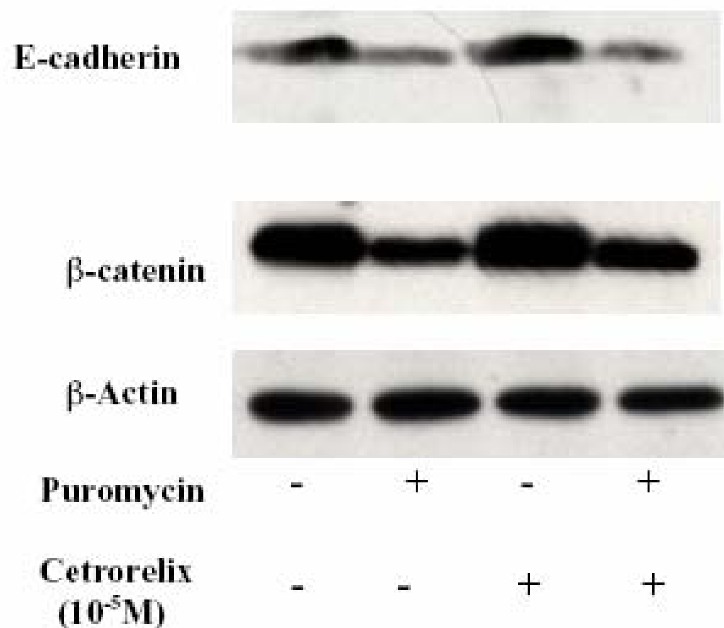

Figure 4. A, DU-145 WT cells were challenged with ± puromycin (40 M) in the presence of Cetrorelix (10-5M) for 24 hours. Lysates were collected and separated by SDS-PAGE, transferred, and immunoblotted with antibody to E-cadherin. B, DU-145 WT cells were challenged as in A, except immunoblotted with antibody to β-catenin. Shown are representative blots of two experiments.

2.5. DISCUSSION

The LHRH analog Cetrorelix is undergoing evaluation for prostate cancer treatment. While initially considered for treatment due to its central androgen suppression mechanism, direct cancer cell efficacy has been shown. Cetrorelix has been demonstrated to limit proliferation of a variety of human cancer cell lines, including breast, ovarian, endometrial [Schally, 1999 #64;Yap, 1997 #98] and prostate cancer cell lines [Qayum, 1990 #60;Halmos, 2000 #29]. Herein, we examined whether Cetrorelix altered an important phenotype of tumor cells, decreased cell-cell adhesion. We found that Cetrorelix exposure increased the levels of cell adhesion molecules and enhanced the resultant cell-cell adhesion. Furthermore, Cetrorelix appears to function, at least in part, by cross attenuation of signaling from the EGFR.

Several studies have long established that the loss of the homotypic E-cadherin binding machinery correlates with an invasive phenotype in prostate carcinomas [Behrens, 1989 #6;Bussemakers, 1992 #9;Vleminckx, 1991 #89]. Thus, it is logical that this cell-cell zipper would disappear concomitant with increased cellular invasion [Shibata, 1994 #69]. This disappearance of E-cadherin and/or any of the major adhesion components affiliated with it, is noted in most advanced carcinoma cells [Takeichi, 1977 #79;Takeda, 1999 #78;Hazan, 1998 #31]. In fact, re-expression of E-cadherin has been shown to reduce the tumorigenicity of some carcinoma cell lines [Lowy, 2002 #47;Jawhari, 1999 #33]. Interestingly, Cetrorelix exposure increases the levels of all of the major adhesion molecules probed; this could be secondary to either increased transcription or decreased degradation. This should subsequently lead to the reforming of the zipper. This was corroborated in our invasion (Figure 8) and aggregation studies

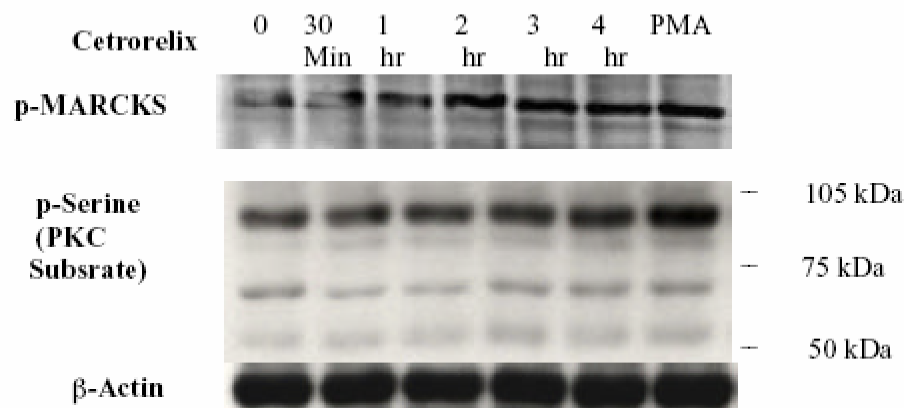

Figure 5. Top immunoblot, DU-145 WT cells were exposed to Cetrorelix (10-5M) from 30 min to 4 hours. Lysates were collected and separated by SDS-PAGE, transferred, and immunoblotted with antibody recognizing phosphorylated MARCKS. Bottom immunoblot, DU-145 WT cells were challenged as in A, except immunoblotted with antibody recognizing phosphorylated serine in the context of

canonical PKC target sites. Increases observed in figures A and B are comparable to PMA positive control. Shown are representative blots of three experiments.

(Figure 9A,B) where, after extended Cetrorelix exposure, the highly invasive WT cell line became less invasive and aggregated to a greater extent than non-treated cells.

The ability to exploit the findings that Cetrorelix-treatment increases both cell-cell adhesion and the levels of the key molecules involved in the adhesion process is vastly improved by defining the underlying basis for this occurrence. Other LHRH analogs have been shown to limit prostate carcinoma cell growth secondary to down-regulation of EGFR (Moretti, Marelli et al. 1996; Jungwirth, Pinski et al. 1997) or through interference with signaling pathways initiated by the EGFR (Wells, Souto et al. 2002). This occurred via PKC-mediated cross attenuation (Wells, Souto et al. 2002) secondary to phosphorylation on threonine 654 of EGFR (Lin, Chen et al. 1986; Welsh, Gill et al. 1991). In this study, we show direct activation of PKC substrates MARCKS by LHRH receptors in a time-dependent manner (Figure 5). These findings led us to believe that DU-145 cells engineered to express the PKC-resistant A654 EGFR should be impervious to Cetrorelix treatment. This was borne out by our findings that EGFR levels remained high and cell adhesion molecule levels low in these cells in the face of Cetrorelix exposure (Figure 6). The importance of EGFR signaling was further demonstrated in a time dependent manner from the exposure of the DU-145 WT subline to an EGFR specific tyrosine kinase inhibitor, PD153035 and a monoclonal antibody against EGFR (mb528) (Figure 7 A,B). Cetrorelix and PD153035 both increased cell-cell adhesion in DU-145 WT, but had little effect on DU-A654 cells (Figure 6). The results of all of our findings taken together indicates that the ability of the LHRH analog, Cetrorelix to alter the adhesive profile of these cells is at least partly mediated through altered EGFR signaling.

That Cetrorelix restores cell-cell adhesion secondary to disrupting EGFR signaling would be supported if EGFR signaling could be shown to down-regulate cell-cell adhesion. This was shown to occur at least in the DU-145 WT cells by their increased aggregation upon disruption of autocrine EGFR signaling (Figure 9). EGFR signaling, upregulated in an autocrine manner in prostate carcinomas (Kassis, Moellinger et al. 1999), was shown to be responsible, at least in part, for the down-regulation of cadherin-mediated adhesion and levels of molecules noted in these tumors as it is in many other carcinomas (Sorscher, Green et al. 1995; Wilding, Vousden et al. 1996; Jawhari, Farthing et al. 1999; Andl, Mizushima et al. 2003). Another report indicated

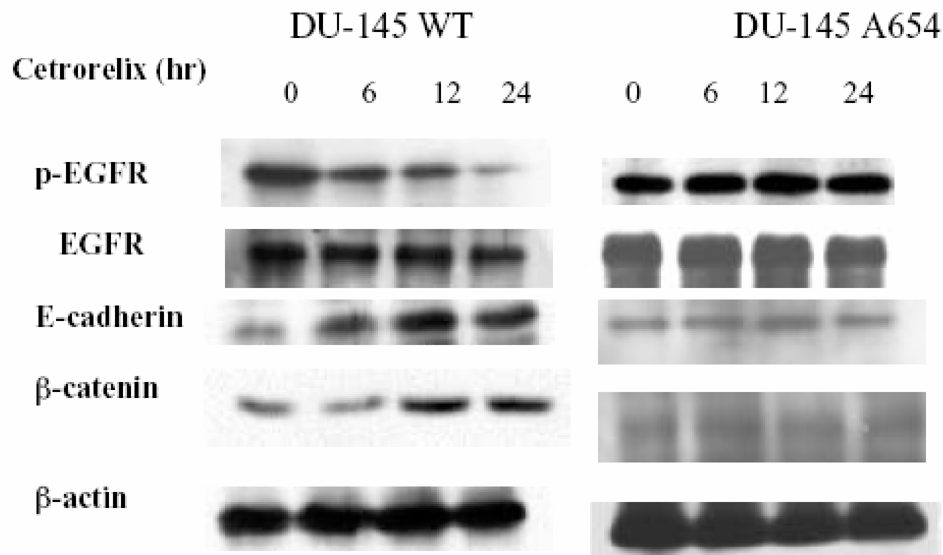

Figure 6. DU-145 WT (left immunoblots) and A654 (right immunoblots) cells were expose Cetrorelix (10-5M) for up to 24 hours. Lysates were collected and separated by SDS-PAGE, transferred, and immunoblotted with antibodies to E-cadherin, -catenin, and EGFR respectively. Similar data were seen with -catenin and p120 (data not shown). Shown are representative examples of 3 experiments.

that EGFR down-regulation resulted in decreasing E-cadherin and catenins in ovarian carcinoma cells (Alper, De Santis et al. 2000). Although the reason for this opposite effect in these cells was not obvious, it may be related to the distinct nature of some ovarian cell types. Presumably, such a reduction in the levels of adhesion molecules plays a major role in prostate cancer progression (Wells 2000). How EGFR signaling limits cadherin-mediated adhesions is still being deciphered (Ackland, Newgreen et al. 2003; Cozzolino, Stagni et al. 2003). However, this appears to involve both acute phosphorylation of PKC and the dissociation and subsequent degradation of key adhesion components. Regardless of the actual mechanism, the end result is witnessed in the long-term downregulation of these molecules.

In summary, we found Cetrorelix restored the adhesiveness of the human prostate carcinoma cells (and significantly inhibited cellular proliferation) at similarly high pharmacologic doses used by others (Jungwirth, Pinski et al. 1997; Tang, Yano et al. 2002). Additionally, the LHRH agonist Zoladex was shown to only inhibit in vitro cell proliferation of androgen-dependent (LNCaP) and androgen-independent (DU-145) cell lines at similarly high concentrations (Moretti, Marelli et al. 1996; Wells, Souto et al. 2002).

Thus it seems that higher concentrations of LHRH analogs are needed to accomplish direct cell growth inhibition than to achieve androgen suppression. There are obvious speculative reasons for this, but regardless of the mechanism, these studies serve as proofs of concepts that this signaling axis can be exploited to limit prostate tumor progression. It remains to be determined whether therapeutic interventions will exploit this using higher affinity analogs or indirect augmentation of the described pathway that cross attenuates the autocrine EGFR signaling pathway in tumor promotion.

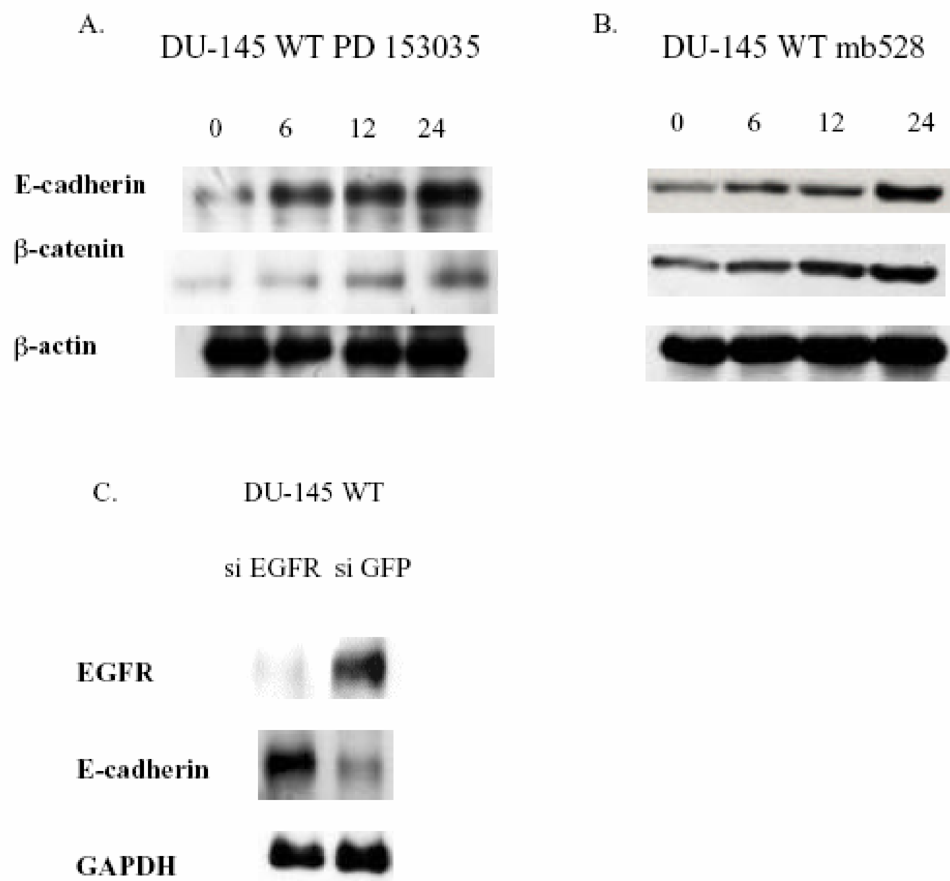

Figure 7. A, DU-145 WT cells were exposed to PD153035 for 6, 12, and 24 hours. B. DU-145 WT cells were exposed to monoclonal antibody (528) against EGFR for 6, 12, 24 hours. C, EGFR siRNA was exposed to cells for 24 hours and compared to GFP siRNA. Lysates were collected and separated by SDS-PAGE, transferred, and immunoblotted with antibodies recognizing EGFR and E-cadherin and β-catenin. Shown is one of two experiments.

2.6. ACKNOWLEDGEMENTS

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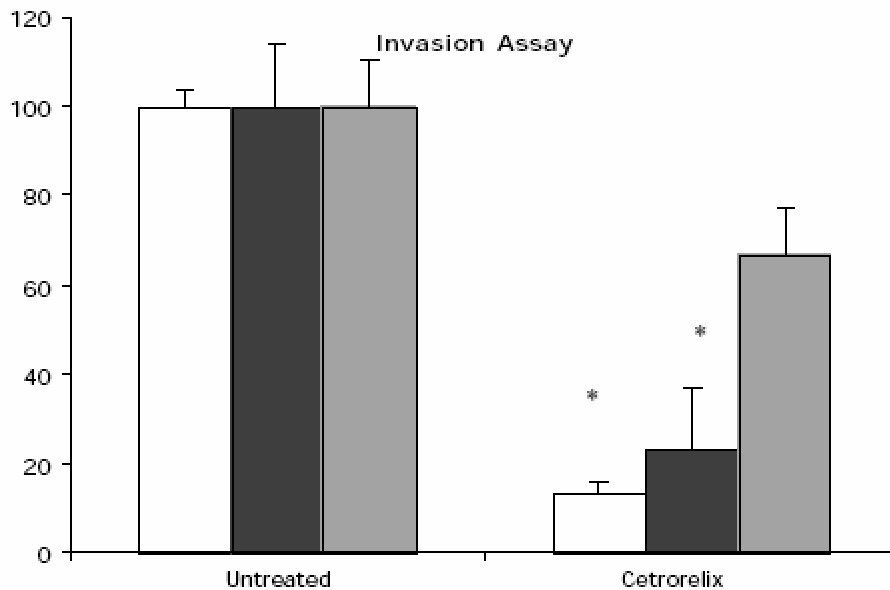

Figure 8. Cetrorelix reduced the invasiveness of the DU-145 Parental (□) and DU-145 WT (■) cells while only partly affecting that of DU-145 A654 (▧) cells. Invasiveness was measured by (continued Figure 8) the cells' ability to transmigrate the extracellular matrix, Matrigel, in a Boyden Chamber assay. Data is the mean ± SEM (n=4). *, $P < 0.05$, Cetrorelix-treated (48 hours) groups versus Controls (Diluent only), without drug; also $P < 0.05$ between the extent of decreased invasiveness of WT and A654 cells in the presence of Cetrorelix.

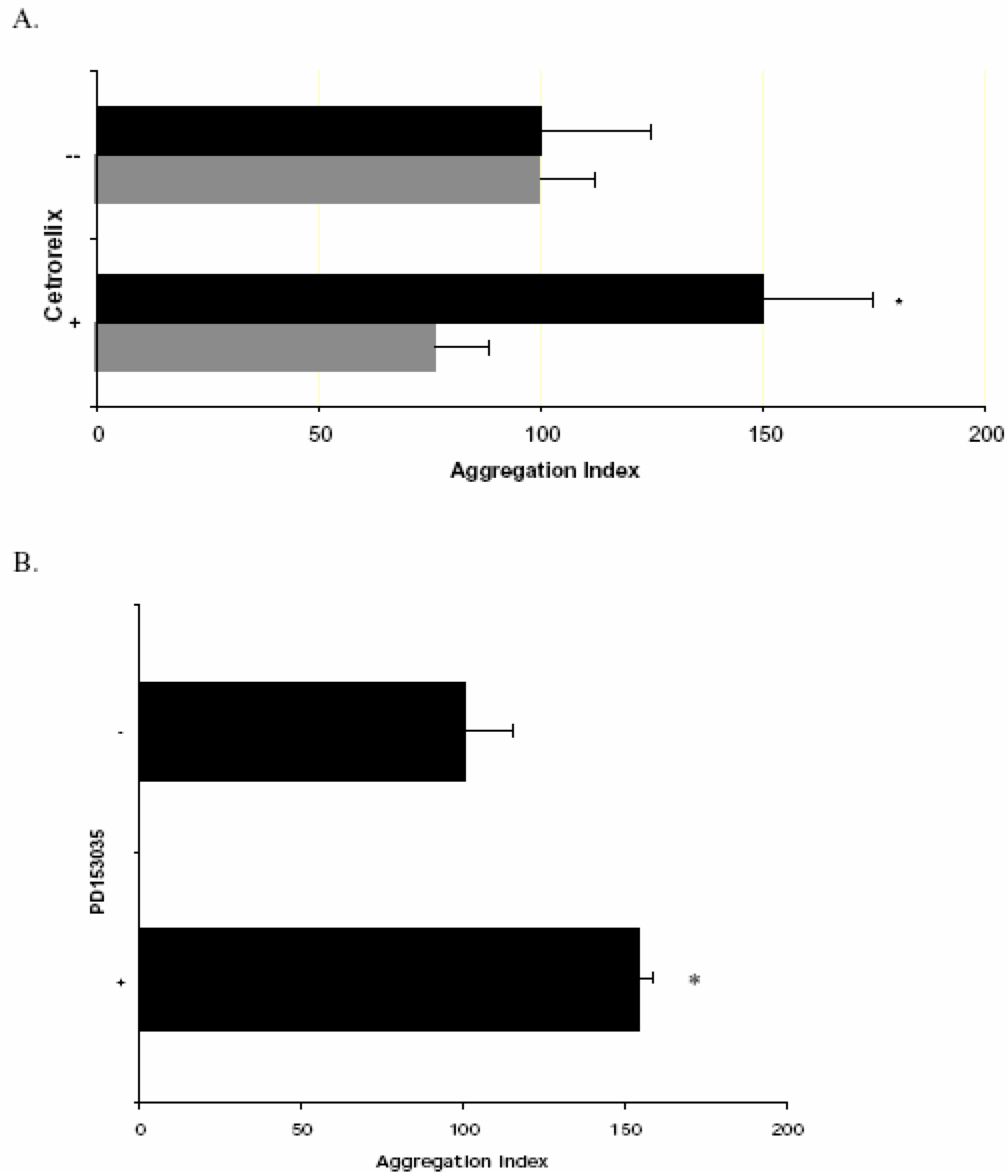

Figure 9. A, Cetrorelix increased the cell-cell aggregation of the DU-145 WT (■) cells after 48 hours of exposure, while not effecting DU-145 A654 (▧) cells. B, EGFR inhibitor PD153035 increased the cell-cell aggregation of the DU-145 WT after 48 hours of exposure. Results are expressed as the mean of the index of the degree of aggregation versus time zero ± SEM at one hour (n = 3, each in triplicate). *, $P < 0.05$, Cetrorelix-treated (+) groups versus Controls (-), without drug.

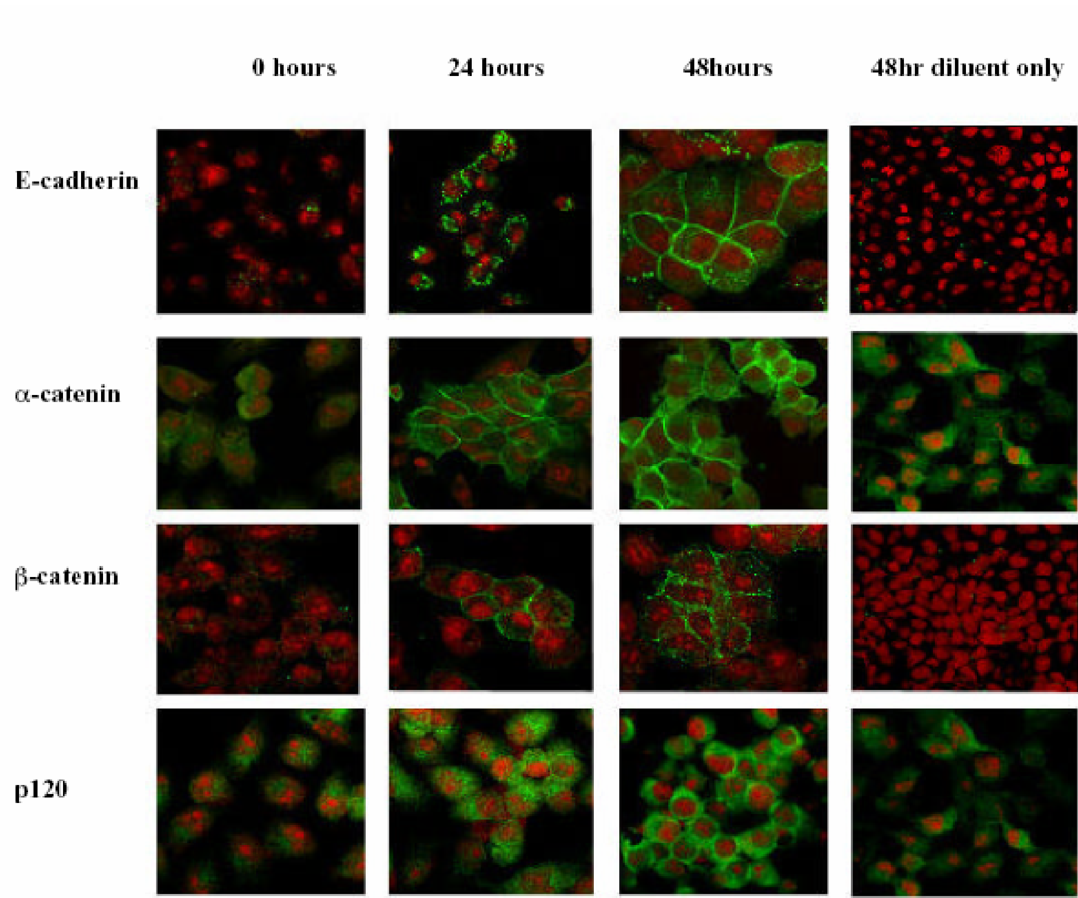

Figure 10. Cells were exposed to Cetrorelix for up to 48 hours prior to immunofluorescent localization of E-cadherin (top panels), - and –catenins (second and third panels, respectively), or p120 (bottom panels) and compared to 48 hours diluent alone (right panel). Shown are representative photomicrographs of two independent experiments; the target molecules are green and nuclei are red.

3. IMAGING INVASION AND METASTASIS EX VIVO

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A variety of *in vivo* animal models are used to study metastasis. The most common models involve tumors in animal hosts; these are often xenografts of human tumor cells or fragments,

animal allografts, or *in situ* tumors. In vivo assays provide for the integrated process or subparts thereof (such as for tail-vein seeding of lung fields) and have revealed many general phenomena involved in metastasis, such as the roles of endothelial cells and resident macrophages in host tissue invasion and for evaluation of potential inhibitors (Mook, Van Marle et al. 2003; Liang, Wu et al. 2004; Timmers, Vekemans et al. 2004; Wyckoff, Wang et al. 2004). The advent of new imaging methods to follow behavior of individual cells and metastases in vivo in real time offers even greater possibilities. Still, in vivo models do not completely fulfill all the desired features for replicating human metastasis. First, in xenografts, many key cytokine and matrix signals do not cross species barriers, and most rodent tumors are of limited generalizability to human tumors (Rangarajan, Hong et al. 2004). Thus, some phenomena representative of human metastasis may be easily found and studied in animal models, while others may be missed. In vivo assays, especially those that involve imaging at the individual cell level, are cumbersome, often inefficient for metastases, and time-consuming limiting the number of parameters that can be studied. Still, we have learned much from these models as a result of great effort on the part of the individual investigators.

Intravital and whole body imaging are increasingly being used to study establishment and early growth of metastases *in situ* in animals. Intravital imaging relies on confocal or multi-photon imaging to follow the behavior of individual fluorescently-labeled cells within a particular target organ, allowing dissection of cell-cell interactions involved in penetration of the target tissue and providing a dynamic picture of tumor cell morphology as it moves between the vascular space and the tissue. The depth of focus is less than 0.5 mm and thus to gain images a portion of a target organ is exposed and placed on the microscopy viewing platform. This approach has been especially useful for characterizing primary-tumor, properties, growth rates

and mechanisms of metastasis to target organs (Condeelis and Segall 2003). With fluorescence markers, direct imaging of intravasation at the single-cell level within the primary tumor has been observed and related mechanistically to metastatic potential. For example, this approach has revealed that tumor cell arrest in the vasculature is distinct from the hematopoietic rolling mechanism (Chambers, MacDonald et al. 1995). Segall and Condeelis and colleagues (Condeelis, Wyckoff et al. 2001; Condeelis and Segall 2003) have imaged cells with confocal microscopy after establishment of a mass in the tissue and demonstrated that tumor cell lamellipodia are oriented towards vasculature and have increased activity and motility. They have also further used intravital imaging to reveal cross talk between macrophages and tumor cells in tissue invasion (Wyckoff, Wang et al. 2004). Multiphoton laser scanning microscope allows for deeper penetration of tissue than does confocal imaging, and has been used to follow vascular and lymphatic vessel size and tortuosity in a quantitative during growth of a tumors arising in a dorsal skin flap from single cells injected into the animal (Jain, Munn et al. 2002).

Whole body imaging is also emerging as a powerful tool in determining metastatic behavior of cancer cells and is becoming more widely used as the instrumentation becomes more available and the probes and host systems become more varied and well-characterized (Hoffman 2004; Iyer, Salazar et al. 2004; Gross and Piwnica-Worms 2005; Michalet, Pinaud et al. 2005). Typically the cancer cells are manipulated to express a reporter gene that allows tracking position, and in some cases functional behavior, following metastasis from the primary tumor. Reporter genes include GFP, RFP and other variants detected by fluorescence imaging; luciferase genes from firefly and other organisms detected by luminescence of the products of the oxidative cleavage of their substrates; endogenous transferrin receptor, detected by magnetic resonance imaging of iron accumulation; and a variety of metabolic genes that allow intracellular

trapping of radiolabelled substrates that can be detected with high resolution by positron emission spectroscopy (Gross and Piwnica-Worms 2005). Reporter genes can also provide a real-time window into the effectiveness of treatment modalities, including gene therapy approaches (Yaghoubi, Barrio et al. 2004). Whole body imaging can also provide insights into the role of host stromal and immune tissues, either by introduction of labeled cells that home to tumor (Kim, Dubey et al. 2004), tissue level imaging of nonluminous angiogenic blood vessels that appear as sharply defined dark networks (Hoffman 1999; Hoffman 2002) or using animals engineered to express a contrasting fluorescent label in stromal cells, (Yamamoto, Jiang et al. 2004).

Our focus here is the advent of ex vivo metastasis assays that seek to recapitulate features of the in vivo environment. Such assays allow often provided enhanced access to molecular-level information, are more accessible (particularly in a context of drug discovery and development), and offer the potential to provide a window into how human tumor cells behave in the context of underlying human tissue. We highlight recent advances in complex 3D in vitro models involving heterotypic cell cultures with an emphasis on tissue engineering approaches.

3.1. Static Culture Invasion and Growth Assays

The most accessible approaches to modeling metastasis employ static culture assays as a means to dissect molecular events in a reasonably controlled way. An example of an invasion assay is the invasion chamber, which in its simplest form is a thin (~0.1 mm) matrix barrier (typically collagen or Matrigel) on a large pore-containing support in a modified Boyden chamber, with the tumor cells placed on top of this barrier. After a period of time, and in response to factors in the

originating top or targeting bottom compartment, the number of tumor cells that transmigrate this barrier is determined. These assays can be used to assess response to soluble factors or to parse the roles of various cell-matrix interactions; for example, Mercurio and co-workers used fluorescent beads embedded in Matrigel to assess the tractile forces exerted by cancer cells on the extracellular matrix, thus implicating basement remodeling during tumor invasion (Rabinovitz, Gipson et al. 2001). Although, a predominance of in vitro metastasis assays have concentrated on metastatic cancer cells invading the basement membrane in situ, a similar design has been successful in identifying molecular cues during extravasation. Extravasation is evaluated by forming an endothelial cell monolayer, typically with intact endothelial cells; tight junctions are verified by electrical resistance or dye exclusion. Both assays have been useful in defining cells that have invasive potential and parsing key regulatory switches and cell behaviors.

Obviously, each *in vitro* assay is limited by simplification. The matrices presented are not truly representative of target organs having different components and growth factors. The endothelial cell barriers are not necessarily organ-specific. Other cellular elements, chiefly stromal cells consisting of underlying epithelial cells, are lacking. Lastly, only invasiveness is evaluated since there is no underlying parenchyma for assessment of metastatic growth.

Beyond initial tumor cell invasion events, subsequent events in the early stages of metastatic growth are also being examined in 3D monocultures and cocultures of normal cells with tumor cells. One of the most universally accepted forms of 3D model systems for cancer research are the monotypic 3D cell culture assays, which are possible to reproduce in almost any laboratory. Spheroidal aggregates of cells have been used for decades to create 3-D spheroids of tumor cells, using the spheroids as models of the primary tumors and also to model the process

of metastasis by shedding of tumor cells. (Deakin 1975; Durand and Olive 1976; Mueller-Klieser and Sutherland 1982; Franko and Koch 1983; Santini and Rainaldi 1999). Spheroidal aggregates of tumor cells in suspension are an enduring model, and whereas early methods typically resulted in a broad range of spheroid sizes, new methods of creating near monodisperse-sized spheroids in a reasonably high throughput fashion have recently been described (Kelm, Timmins et al. 2003), and these and other methods are being adapted to early stage screening of anticancer drug efficacy (Kunz-Schughart, Freyer et al. 2004).

While monocultures of spheroids in suspension provide a more realistic phenotype than monolayer culture, the interplay between tumor cells and matrix is also a strong determinant of tumor phenotype and thus culture systems that provide an in vivo-like matrix milieu are becoming favored for dissection of basic disease processes (Muthuswamy, Li et al. 2001; Jacks and Weinberg 2002) A central theme of these assays is the use of ECM to create a tissue relevant environment. Matrigel is typically used for assays of epithelial behavior as includes many components of basement membrane. A number of cell models have been coupled with appropriate 3D matrices and show promising results in recapitulating tissue functions in 3D (Schmeichel and Bissell 2003). Studies have been reported for liver, salivary gland, vasculature, bone, lung, skin, intestine, kidney, and mammary and thyroid glands, but arguably the most well characterized models have been with the mammary gland. Both mouse and human mammary cells embedded in or cultured on Matrigel adopt a spherical, polarized structure that resembles the normal mammary alveolus (or acinus) that is capable of mammary-gland-specific function (e.g., producing milk in response to lactogenic hormones) (Stoker, Streuli et al. 1990; Petersen, Ronnov-Jessen et al. 1992; Howlett, Bailey et al. 1995; Weaver, Petersen et al. 1997; Fata, Werb et al. 2004). Pathophysiological behaviors that are similar to those observed in vivo are also

captured in this in vitro system, for example loss of polarization and aberrant proliferation in the center of acini when signaling by epidermal growth factor receptor (EGFR) family members is perturbed through overexpression or mutations (Muthuswamy, Gilman et al. 1999; Muthuswamy, Li et al. 2001). The mechanical environment – rigidity of the matrix – can also be systematically controlled using synthetic gels to which matrix proteins are cross-linked, and changes in cell signaling and down-stream behaviors resulting from matrix compliance changes are now being correlated with in vivo changes in tumor mechanical properties (Val Weaver, personal communication). A powerful aspect of this assay is the potential to adapt it to moderate or high-throughput screens for metastasis (Martin, Wendt et al. 2004).

Tumor-stromal interactions are emerging as a critical factor in growth of metastatic tumors (Bhowmick, Chytil et al. 2004; Bhowmick and Moses 2005). At a basic histological level, stroma appears as a matrix-rich tissue populated by fibroblasts and permeated by a blood vessel network. Many approaches have been described in the literature for creating 3D cultures of either fibroblasts or endothelial cells and using these cultures to examine the interactions with cancer cells. To model tumors, the stromal cells are typically mixed in a gel or cultured in a 3D scaffold, and the tumor cell might be added directly on top of the gel containing fibroblasts or endothelial cells, mixed in another gel layer on top, or various other configurations (Donovan, Brown et al. 2001; Feraud, Cao et al. 2001; Masso-Welch, Zangani et al. 2002; Parmar, Mahadeva et al. 2002; Velazquez, Snyder et al. 2002). 3D in vitro co-cultures have been particularly useful in revealing the profound effects of tumor-derived versus normal stroma in contributing to malignant behavior of epithelial tumors (Atula, Grenman et al. 1997; Kunz-Schughart, Freyer et al. 2004; Bhowmick and Moses 2005), and in identifying profiles of

molecules secreted by tissue-specific stroma that may foster tissue-specific growth of certain kinds of carcinomas (Nakashiro, Okamoto et al. 2000; Martin, Ridgeway et al. 2004).

3.2. 3D Organiods Culture Under Flow Conditions

Most tissues comprise a hierarchical arrangement of cells permeated by capillary blood vessels. Tissue homeostasis is maintained in part by a symphony of communication between the different cell types in tissue; each cell receives signals from neighbors via direct cell-cell interactions, cell-matrix interactions, and via soluble signaling molecules (cytokines and growth factors). In addition, mechanical forces -- such as shear stress on endothelium from flowing blood -- are converted to chemical signals that are necessary for normal tissue function. As discussed above, both 3D culture and heterotypic cell cultures are useful tools in dissecting dynamic processes in tumor progression. Such models fulfill an important connection between the well-defined cultures of single cell types and the complexity of the whole animal. They also provide experimental models of human tissue responses, where in vivo models are usually unavailable. A distinguishing feature of the in vivo cell environment is that cells are typically within a few tens or hundreds of microns from a nutrient capillary perfused with blood.

Thus various bioreactor configurations have been developed to provide enhancement of mass transfer, shear stress, or both, by providing fluid flow on or through cells. Bioreactors also offer the possibility of monitored and tightly controlled environmental and operating conditions (e.g. pH, temperature, pressure, nutrient supply and waste removal) (Martin, Wendt et al. 2004).

They also have been proposed as the excellent models to more fully recreate the complex in vivo environment *ex vivo*.

Tissue engineered model systems provide and promote a fundamental understanding of structure–function relationships in normal and pathological conditions, with possible commercial applications in molecular therapeutics (e.g. drug screening) (Powers, Domansky et al. 2002). Recent advances have been made in the development of engineered tissue for repair of cartilage, bone, liver, kidney, skeletal muscle, blood vessels, the nervous system, and urological disorders (Atala 2001; MacNeill, Pomerantseva et al. 2002; Powers, Domansky et al. 2002; Vunjak-Novakovic 2003; Sharma, Lansdell et al. 2004). These systems, repair being their primary function, also offer relevant target organs study cancer metastasis and invasion.

Among the simplest bioreactors are fluid-filled spinner flasks, which have been used for decades to create 3-D spheroids of tumor cells and create environments which provide controlled mass transfer to the outside of the spheroid (Deakin 1975; Durand and Olive 1976; Mueller-Klieser and Sutherland 1982; Franko and Koch 1983; Margolis, Hatfill et al. 1999; Santini and Rainaldi 1999) (Schmeichel and Bissell 2003). Spheroid formation of cancer cells are cell type specific and time-dependent, since smaller spheroids may be relatively homogenous when small or may have a necrotic center due to nutrient and oxygen deprivation when larger (Kunz-Schughart 1999; Santini and Rainaldi 1999)

The rotating wall vessel bioreactor (RWV) is an alternative spinner flask design for three-dimensional culture of cells, and was developed to simulate microgravity conditions. In this system, a cylindrical vessel filled with cells and culture medium is rotated about an axis parallel to the ground, thus balancing gravity with fluid drag forces and creating a low-shear stress, high mass transfer environment (Schwarz, Goodwin et al. 1992). The environment in the RWV has

been shown to foster tissue-like structures of mullerian tumor cells of the ovary and prostate (Margolis, Hatfill et al. 1999) and melanoma cancer cells (Goodwin, Prewett et al. 1997) as assessed by histology. Inclusion of human umbilical vein endothelial cells with cervical tumor cell cultures resulted in tubular structures penetrating the tumor cell masses, forming aggregates larger in size than the monocultures and typically with greater cell mass and number (Chopra, Dinh et al. 1997) cervical tumor cells and human umbilical vein endothelial cells (HUVEC). Of particular interest was that these 3D cell cultures to show biochemical markers known to be involved with cellular function and cancer progression. Cell aggregates formed in RWV bioreactors have also been used as model systems to test therapeutic options which include radiation resistance, phenotypic differentiation, and response to anti-cancer drugs. Among the potential limitations of the RWV are the polydispersity of spheroid sizes and the barriers to in situ imaging of cell behavior.

Microfluidic bioreactors offer the potential for more homogeneous, controlled formation of 3D structures and thus potentially better-controlled exposure of cells to agents that affect cell behavior in the context of a 3D environment. A step in this direction is the recent report by Torisawa and co-workers, where 3D culture of MCF-7 breast cancer cells in a collagen gel was controlled in tiny chambers in a microfluidic device, and could be used in a multiplexed format to assess efficacy of anti-tumor compounds (Torisawa, Shiku et al. 2005). Although it remains to be demonstrated that this approach will work well on a large scale, the success of "lab on a chip" chemical assays suggests the barriers are not insurmountable.

3.3. Flow Perfusion Bioreactors

Although a rich array of three dimensional co-culture models have been developed for investigations of behaviors ranging from tumor angiogenesis and embryonic differentiation to skin permeation of drugs, the available models lack one crucial feature of most tissues - a perfused microvasculature. Inclusion of endothelial cells in the RWV or in static cultures provides some features of tissue structure, but the lack of flow through the vessels limits the ability of these systems to mimic tissue physiology.

Thus an organotypic system would ideally include several features 1.) an integrated epithelial/stromal/endothelial cell architecture representing the key target organ; 2.) long-term (weeks) stability to allow tumor cell survival and growth; 3.) controlled local perfusion of the organoid structures over length scales comparable to a capillary bed; 4.) direct visualization throughout the process to discern subprocesses such as extravasation versus growth; 5.) easy manipulation and intervention; and 6.) assay robustness in reproducibility and moderate or high throughput.

As a step toward creating true physiological mimics of human and animal tissues that recapitulate the features of a capillary bed, we have developed a microfabricated bioreactor system that facilitates perfusion of 3D heterotypic co-cultures at the length scale of the capillary bed in an arrangement that also allows in situ analysis of cell behavior via microscopy (Powers, Domansky et al. 2002; Torisawa, Shiku et al. 2005). This system circumvents rapid loss of liver specific functions that normally occurs when hepatocytes are maintained under standard culture conditions thus providing a reasonable model system for the testing of tumor-host interactions in

ex vivo environment. The liver is a major site of metastasis for many carcinomas, and its anatomically relevant for metastasis models due to a simplified architecture of hepatocytes covered with an endothelial lining, and can be recreated in vivo via selective cell adhesion and cell self-assembly (Powers and Griffith 1998; Poser, Dominguez et al. 2001; Powers, Domansky et al. 2002).

Our cross-flow perfusion reactor is designed to address several needs for 3D liver tissue culture (Powers, Domansky et al. 2002). The classical challenges in reactor design for three dimensional perfusion culture – ensuring a relatively homogeneous distribution of flow and mass transfer throughout the system to meet the metabolic demands of the cells – are augmented in the case of three dimensional cultures of primary cells by the need to provide a scaffold appropriate for tissue morphogenesis. Varying degrees of histotypic reorganization have been observed in several types of three dimensional liver cell cultures, particularly those incorporating perfusion through the tissue mass (Gerlach, Schnoy et al. 1995; Bader, Knop et al. 1996; Griffith, Wu et al. 1997; Michalopoulos, Bowen et al. 1999; Kaihara, Kim et al. 2000). Distinguishing features of our design include: an appropriate scaffold for tissue morphogenesis; uniform distribution of fluid flow and nutrients throughout the 3D culture; and an optical window to allow repeated *in situ* observation of cells via light or 2-photon microscopy during perfusion culture.

A photo and schematic cross-section depicting the major design features of our current reactor (as used for liver culture) is shown in Figure 11. The heart is the cell scaffold, comprising a thin (~230 μm) silicon sheet permeated from top to bottom by a regular array of ~300 μm channels (created by deep reactive ion etching) and seated atop a microporous filter, which is in turn mechanically supported by a second scaffold. The morphogenesis of cells into tissue-like structures following seeding into the channels is guided in part by scaffold surface chemistry,

which controls the relative values of cell-cell and cell-substrate adhesion strength, and by the channel geometry and dimensions. The scaffold is maintained between the upper and lower chambers of a flow-through housing. Each chamber has a pair of ports to allow flow of culture medium across the surfaces of the chip. The arrangement of the ports allows for several modes of operation. Under our current mode of operation for liver tissue, the fluid in the upper chamber is initially maintained at a higher pressure than that in the lower chamber, thus creating a driving force for perfusion of culture medium through the tissue in the channels immediately after cells are introduced. Cells seeded into the channels are initially held in place by the filter, and after initial attachment and reorganization (~ 1 day), by adhesion to the channel walls. After 1-2 days in culture, fluid flow through the channel is reversed (i.e., to flow upward through the filter and then through the tissue mass), dislodging any dead or unattached cells. This reverse flow is controlled by a peristaltic pump throughout the remainder of the culture period, and in the case of liver, stable tissue structures are maintained for weeks, thus creating appropriate environment for cancer cells.

Metastasis is a highly orchestrated series of events comprised of many "sub-processes" whereby cancer cells must attach, proliferate, and invade the parenchyma of specific target tissue (Figure 12a). Using our cross-flow perfusion bioreactor we were able to recreate the progression of invasion and metastasis with the added advantage of real-time visualization by fluorescence microscopy over several weeks. GFP expressing cancer cells were introduced into previously seeded, and established hepatic parenchymal spheroids. Initially, individual cancer cells occupy the hepatic wells, but overgrow the hepatic wells and the rest of the chamber by day 14 (Figure 12b). During this rapid and visually apparent growth of cancer cells, invasion into hepatic

parenchyma also occurs, with very distinct heterotypic cell-cell interactions observed upon histology and electron microscopy (Yates, Wells et al. 2005; Yates, Papworth et al. Submitted).

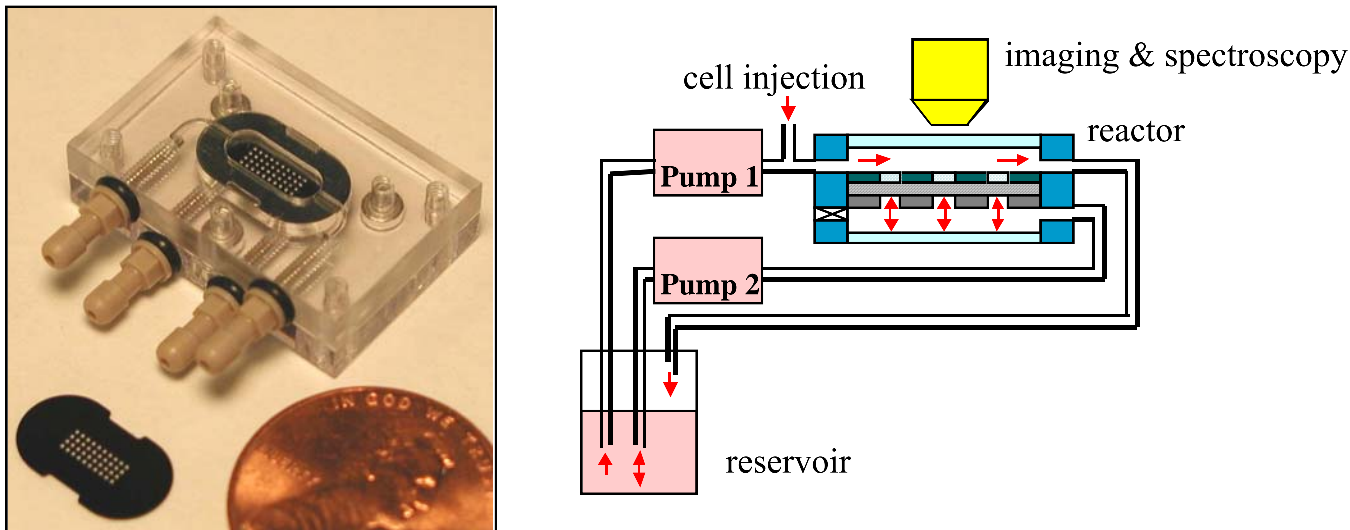


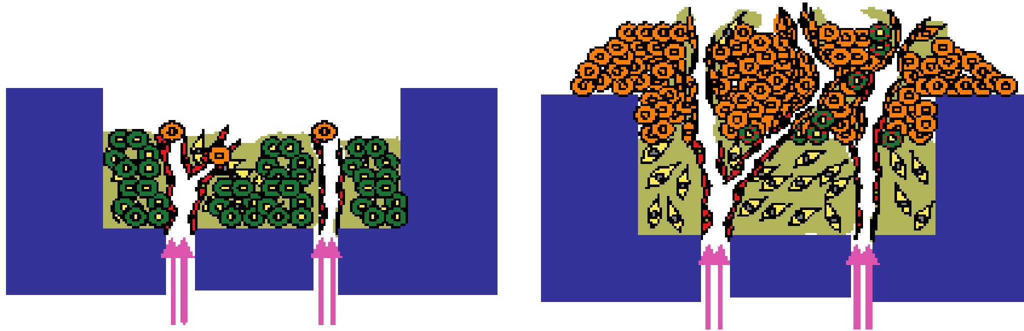
Figure 11: Photo of the silicon-chip scaffold (lower left) and polycarbonate microreactor housing that holds the scaffold and provides four ports for continuous flow of culture medium across the top of the scaffold and through the tissue mass held within the scaffold. An optical window at the top allows in-situ observation of the tissues by two-photon microscopy. Right: Schematic of scaffold and reactor housing shown in cross-section, indicating the connections to recirculation loop and observation window. The main flow of culture medium is across the top of the chip in the upper chamber (pump 1). Flow through the tissue mass in the chip is maintained at a constant rate by either pulling medium through or pumping medium in reverse flow through with the second pump.

3.4. SUMMARY

Unlike enclosed bioreactors, this bioreactor allows for repeated visualization and affords the opportunity to examine critical processes underlying the metastatic program over time, such as cell specific proliferation, death and tissue organization. Interaction of metastases with the parenchymal cells of the target organ, as well as resident ECM and endothelial cells are required to fully recapture the microenvironment presented to and modified by the metastatic cancer. As

more advanced tissue engineered models are generated to determine cellular responses of many organs, research should include alternative applications, as these systems could possibly represent the leading models for metastasis and invasion.

a.



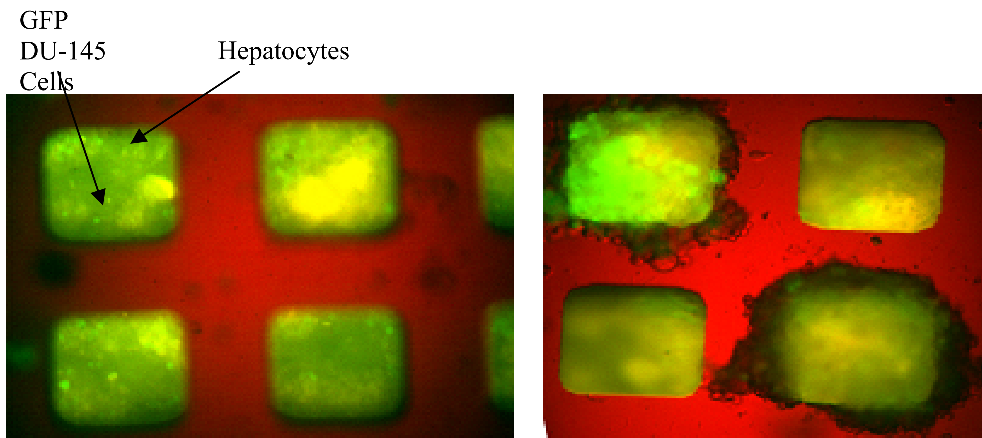


Figure 12 (a) Illustration of Metastatic Events that occur within individual channels of the Micro Perfusion Flow Bioreactor from initial seeding of cancer among parenchymal tissue to overgrowth of cancer after continued coculture. (b) Left Day 2 light micrograph of 4 channels of coculture. Dark grass green is hepatic tissue, bright yellow is auto fluorescence from underlining filter, and bright green are GFP DU-145 WT cells. (Right) 14 Days of DU-145 GFP growth.

4. DIRECT VISUALIZATION OF PROSTATE CANCER PROGRESSION UTILIZING A NOVEL ORGANOTYPIC LIVER BIOREACTOR AS METASTIC TARGET ORGAN

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4.1. ABSTRACT

Most methods available to study the behavior of metastatic tumors at the resolution of individual cell events are indirect or capture only brief periods. Therefore we developed a microscale perfusion culture system that provides a tissue-relevant environment to assess metastasis behavior, using the human prostate cancer cell line DU-145 in the liver capillary bed as a model system. Real-time behavior of the co-culture system was observed by 2-photon microscopy of RFP-labeled DU-145 cells against a GFP-labeled hepatic tissue bed over a 14 day period. Fluorescent intensity of co-cultures revealed ongoing cell proliferation of the DU-145 cells, which resulted in tumors visible to the naked eye by day 25. Histological analysis of the resulting tumors revealed intact cellular structures and no evident necrosis in the ~0.5 mm tumor mass, which was perfused at a local level during culture. The DU-145 cells failed to grow in the absence of the supporting hepatic tissue structure, suggesting a paracrine or stromal support function for the liver microtissue in tumor progression. The overt tumor mass resulted in a decline in hepatocyte tissue structure. TEM also revealed DU-145 cells invading the hepatocyte parenchyma by 14 days with very distinct cell-cell interaction. Therefore, our 3D ex vivo organotypic liver tissue system presents a critical vehicle to examine tumor-host interactions during cancer metastasis/and or invasion. It also circumvents current limitations in metastasis assays, and provides new approaches to the problem of tumor progression.

4.2. INTRODUCTION

Metastasis leads to the major part of mortality and morbidity in cancer patients. As novel therapies are being developed to target molecular steps that contribute to metastasis, the development of new analytical methods to study development of metastatic lesions at the cellular level are needed. Currently, few modes are available to evaluate this critical progression in the target organ. Newer three-dimensional, mixed tumor and stromal cell models have yielded invaluable information about carcinogenic transformation (Kenny and Bissell 2003; Seton-Rogers and Brugge 2004), but these do not elucidate the events at the sites of metastasis. End-point animal models of metastasis, which have been the most common assays thus far, have yielded important information for the size of metastases and the number of cells at the target organ, but have failed to provide information about the cellular processes that occur during the development of metastasis. This information has evaded study due to the difficulties in identification of single cancer cells or micrometastases in tissues and the ability to follow their evolution over time. To overcome this limitation, some investigators have developed real time *in vivo* systems that allow for short-term imaging and evaluation of the tumor cell behavior (Chambers, MacDonald et al. 1995; Condeelis, Wyckoff et al. 2001). These have lead to new appreciation for the roles played by non-tumor cells and the matrix (Wyckoff, Wang et al. 2004). Still, the window of examination is in hours at most and the systems are not easily manipulated preventing higher throughput investigations.

Herein, we report using a microscale bioreactor that fosters three dimensional liver tissue formation and culture under perfusion that mimics flow through the liver capillary bed and

allows for *in situ* observation of the interactions of human prostate cancer cells within the capillary bed-sized tissue units. This system affords for the recreation of an *in vivo* environment for *in vitro* observation and provides for an optimal device for the study of physiological events (Powers, Domansky et al. 2002). With the liver being a preferred ectopic site for metastasis of many cancers including prostate cancer (Paget 1989; Fidler 2003; Shah, Mehra et al. 2004), we propose that tumor growth and invasion at the metastatic target organ can be observed at the cellular level in real-time with the use of fluorescent markers for visualization. Further, tumors grown under perfusion conditions, where fluid flow through the tumor mass is sited within the tissue and controlled at the scale of tens of microns, have non-necrotic cores, unlike tumor cells grown in standard spheroid culture. We demonstrate our approach using rodent cells as the host tissue, however, the ultimate advantage of this system is the potential to create human perfused tissue structures for supporting human tumor growth allowing for an easily manipulatable procedure for visualizing in real-time invasion and growth of a target organ capillary bed during metastasis.

4.3. Materials and Methods

The cell line, DU-145, was originally derived from a brain metastasis of a human prostate adenocarcinoma (Stone, Mickey et al. 1978); it retains the androgen independence of the original tumor, does not express a functional androgen receptor, and forms invasive and metastatic tumors in athymic mice (Turner, Chen et al. 1996; Dondi, Moretti et al. 1998). This cell line possesses EGF receptors and produces EGFR ligands, TGF-α and EGF (Xie, Turner et al. 1995; Jungwirth, Pinski et al. 1997). DU-145 cells were made to express the red fluorescence protein (RFP) by stably transfecting cells with dsRED vector from Clontech (Palo Alto). The vector expresses rapid expression of RFP and neomycin resistance gene. For selection, the cells were

sorted by flow cytometry and then maintained in the presence of 1000 mg/ml G418 until used for experimentation.

4.3.1. Seeding and Maintenance of Liver Microtissue Bioreactor

Hepatocytes were obtained from EGFP-transgenic and WT Sprague-Dawley (SD) rats, originally generated by Dr. Masaru Okabe (Genome Information Research Center, University of Osaka, Osaka, Japan) and were generously provided by Japan SLC, Inc. (Hamamatsu, Japan). The expression of EGFP was under the control of the cytomegalovirus enhancer and the chicken β-actin promoter derived from an expression vector, pCAGGS (Ito, Suzuki et al. 2001). Animals were maintained in laminar flow cages in a specific pathogen-free animal facility at the University of Pittsburgh, fed a standard diet, and provided water ad libitum. All procedures in this experiment were performed according to the guidelines of the Council on Animal Care at the University of Pittsburgh and the National Research Council's Guide for the Humane Care and Use of Laboratory Animals.

Hepatocytes were isolated from 150-g to 230-g male rats by a modification of Seglen's two-step collagenase perfusion procedure as described previously (Block, Locker et al. 1996). The resulting cell suspension was centrifuged three consecutive times at 50 X *g* (2 min each). After the final centrifugation the pellet was resuspended in hepatocyte growth medium (HGM), based on the medium described (Block, Locker et al. 1996) with the exception of hepatocyte growth factor and several typographical errors in the original medium formulation. The correct formulation for HGM is as follows: 0.03g/l L-Proline; 0.10g/l L-Ornithine; 0.305g/l Niacinamide; 2.25g/l D-(+)-Glucose; 2g/l D-(+)-Galactose; 2g/l Bovine Serum Albumin Fraction V; 0.0544 mg/l $ZnCl_2$, 0.0750mg/l $ZnSO_4 7H_2O$, 0.020mg/l $CuSO_4 5H_2O$, 0.025mg/l $MnSO_4$;10

mM Hepes; 0.1μM Dexamethasone; 5mg/l Insulin-Transferrin-Sodium Selenite; 20ng/ml Epidermal Growth Factor; penicillin/streptomycin (100 units/ml), 5mM L-Glutamine.

Cell viability before cell seeding was 90–95% as determined by trypan blue exclusion. The fraction of non-parenchymal cells that was typically observed in this suspension was approximately 5%. Spheroids were formed in suspension culture similar to the methods of Powers and co workers (Powers, Domansky et al. 2002). Cells were suspended in HGM immediately after isolation at a concentration of 3 X 10^5 viable hepatocytes per milliliter. One hundred milliliters of this solution was then added to a 250-mL spinner flask (Bellco Glass, Vineland, NJ), which was stirred at 85 rpm for up to 72h. After the prescribed culture period, 100- to 300-μm spheroids were selected from the suspension by filtration through 300- and 100-μm nylon meshes (SEFAR America, Kansas City, MO). Spheroids of the desired size were resuspended in 25 mL of rinse medium and centrifuged at 40 X *g* for 3 min, and then resuspended in 30 mL of HGM.

4.3.2. Three Dimensional Co-culture Assembly

The heart of the reactor is a 230-mm-thick silicon scaffold containing an array of 100 round-edge square channels; each of cross-sectional dimensions 300 X 300 mm (Powers, Domansky et al. 2002). The walls of the channels are cell adhesive whereas the top and bottom of the silicon scaffold are poorly adhesive for liver cells. A microporous filter and supporting scaffold beneath the cell scaffold provide for initial retention of cells in the channels under perfusion flow through the cell mass; cell retention at times greater than 1 day is governed by cell adhesion to the channel walls. Reactors were primed with rinse solution to passivate the reactor, connector, and tubing surfaces, and to remove bubbles from the flow paths. The rinse solution comprised phenol red-free Dulbecco's modified Eagle's medium (DMEM) with sodium

pyruvate (110 mg/mL) and glucose (1 g/L) (Life Technologies, Rockville, MD) supplemented with bovine serum albumin (2 g/L; Sigma, St. Louis, MO) and penicillin–streptomycin (100 U/mL). Before seeding, reservoir bottles were aspirated and refilled with 25 mL of HGM.

For seeding, a syringe filled with 1 mL of cell suspension was placed at the inlet of the upper reactor chamber. Cells or spheroids were manually injected into the upper chamber of the reactor at a flow rate of 1 mL/min and allowed to enter the scaffold channels through a combination of settling and the slight amount of pressure introduced in the upper chamber from the resistance in the waste syringe, which drove fluid into the lower chamber. After seeding, medium was pumped into the chamber at 0.5mL/min and collected in a waste container for 2 min to clear off the cells from the top surface of the chip. The upper recycle tubing was then reconnected to the reactor. After 1 h the reservoir bottle was replaced with a new reservoir bottle containing 25 mL of fresh HGM (removing any residual cells/debris). Following introduction of the cells, flow was maintained in a forward direction (down through the chip and filter) for 24 hours, and then reversed to provide a constant flow rate of 40 ml/min through the cell mass throughout the culture period Reactor cell medium was changed every 4 days by replacing with 25 mL of fresh HGM.

After five days of hepatic tissue morphogenesis, human prostate cancer cells ~150,000 DU-145 endogenously expressing the RFP, were introduced in the microreactor by microsyringe using HGM media containing cells in the mode of forward flow and cross flow stopped. Forward flow resulted in variable attachment of a low number of DU-145 cells to hepatocytes. DU-145 RFP cells were imaged at various time periods. The cross flow fluid was restored to the direction of hepatocytes after 24 hours. The shear stress in the channels under normal flow conditions is at the lower end of the physiological microvascular range (Powers, Domansky et al. 2002). At the

first imaging after attachment, channels that contained only a few (<10) individual tumor cells were chosen for sequential imaging.

4.3.3. In Situ Imaging of DU-145 – Hepatic Tissue Interactions

Tissue structures were imaged using a two-photon microscope comprising a titanium-sapphire ultra fast tunable laser system (Coherent Mira Model 900-F), Olympus Fluoview confocal scanning electronics, an Olympus IX70 inverted system microscope, and custom-built input-power attenuation and external photomultiplier detection systems. Single-plane image acquisition used two-photon excitation at 850 nm with Olympus water-immersion objective dry X20 LMPlan IR, 0.4NA. Emission filters (Chroma, Brattleboro, VT) comprised a Q535/50m filter (green emission), a 565dclp dichroic mirror, and a HQ610/75m filter (red emission).

4.3.4. Two Dimensional Cell Proliferation Assay

2000 Prostate cancer cells per cm^2 were measured for fluorescent intensity by fluorescent microplate reader (Tecan) excitation 558 and emission 620. The reported values for each well were measured and correlated to growth of cells. Experiments were performed in triplicate, and repeated at least two times.

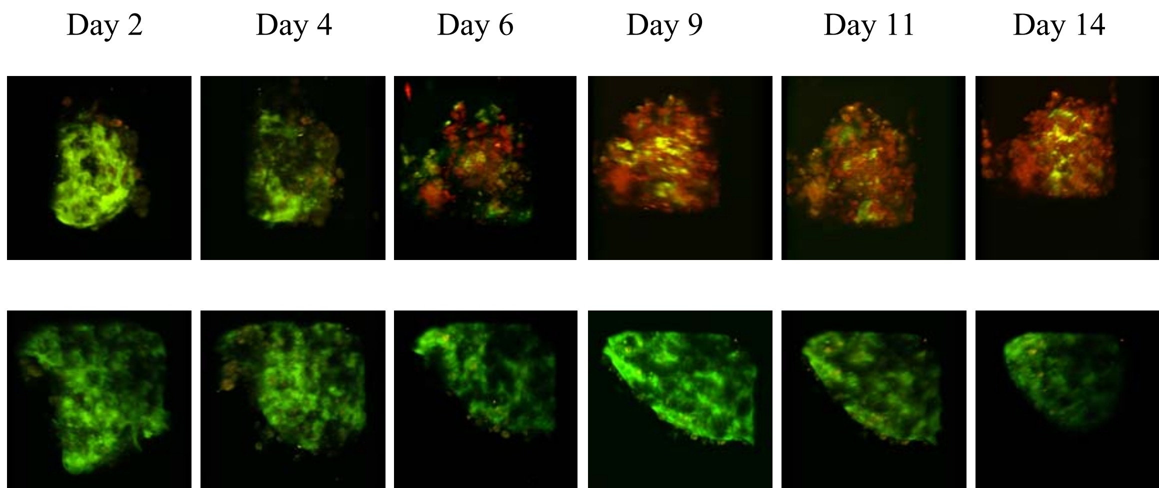
4.3.5. Two Dimensional Co-cultures

We attempted to create a co-culture environment to mimic our three dimensional culture system. Intimate co-cultures consisting of 50, 000 cells per cm^2 hepatocytes and 2,000 cells per cm^2 prostate cancer cells were plated on 1% collagen and compared to non-collagen co-cultures. Transwell co-culture consisting of .4um pore inserts were utilized with prostate cancer cells in the insert and hepatocytes on the 2D surface and assayed for 6 days. Cell viability was measured by fluorescence intensity using a Tecan according to above protocol.

4.3.6. TEM Protocol

Bioreactor scaffolds were fixed in 2.5% glutaraldehyde in PBS for 24 h, washed three times with PBS, and postfixes for 1 h with aqueous 1% osmium tetroxide. After three PBS washes, scaffolds were dehydrated through a graded ethanol series, and then further dehydrated in four 15-min changes of 100% ethanol. Scaffolds were subjected to two 10-min incubations in propylene oxide, and then preembedded with a 1:1 mix of propylene oxide:Polybed 812 epoxy resin (Polysciences, Warrington, PA) for 1 h. Scaffolds were then incubated in 100% Polybed overnight at 4°C. The following day, resin was changed four times before embedding chips in a thin layer of resin, just enough to fill the channels, and curing at 37°C overnight and then at 65°C for an additional 2 days. Cells growing within the channels were removed from embedded chips by rapid, alternating treatments in liquid nitrogen and boiling water. This treatment shattered the scaffolding and allows for embedded cells and channels to be removed intact. Blocks of cells were reembedded in rubber molds and cross-sectioned perpendicular to channel flow. Thin sections (60 nm) were collected on copper grids and stained with 4% uranyl acetate in 50% methanol for 7 min and with 1% lead citrate for 10 min. Cells were viewed with a JEOL (Tokyo, Japan) JEM 1210 transmission electron microscope (TEM) at 80 kV.

(a)



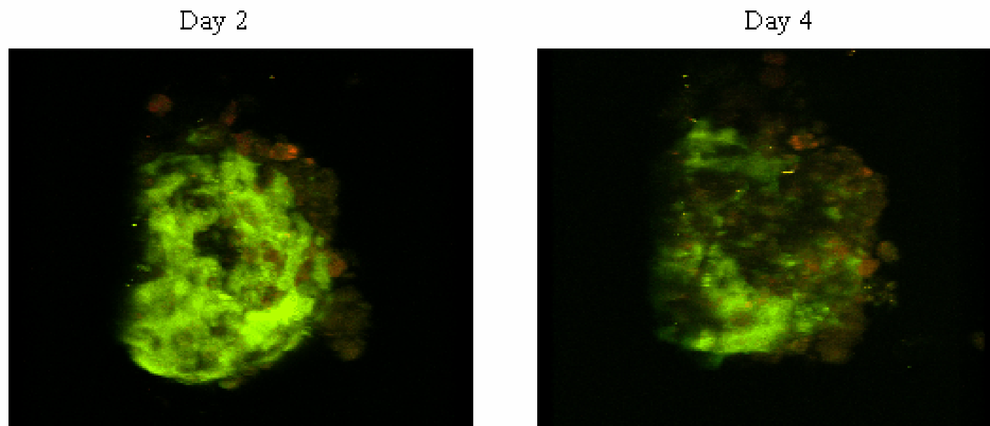


Figure 13. RFP-expressing DU-145 human prostate cancer cells were introduced to primary hepatocytes obtained from GFP transgenic rats. (a) 2-photon Images were taken of DU-145 prostate cancer (red) in the presence of hepatic tissue (green). DU-145 growth was assessed over a 14 day period. (b) Bioreactors with only liver cells demonstrate hepatocyte structure and function stabilility over this time period. Shown are one of more than a dozen experiment. (c) Magnified view of days 2 and 4 to demonstrate presence of single cells as points of origin of subsequent tumor mass. Shown are two representative channels from experiments repeated five times.

4.4. RESULTS

To determine whether human prostate cancer cells could grow in serum-free medium in the context of the three dimensional liver bioreactor, we introduced a single cell suspension of RFP-expressing DU-145 carcinoma cells into a bioreactor containing stable liver microtissue units

formed 5 days previously (Powers, Domansky et al. 2002). These bioreactor cocultures were then imaged at the same sites with 2-photon microscopy at regular intervals from day 2 until day 14 (Figure 13, upper row). Initial attachment of RFP expressing DU145 is apparent by day 2. Three dimensional reconstruction of day 2 and day 4 images demonstrate that expanded growth is from single cell origin (Supplementary Figure 13 c). By day 6 there was apparent growth and invasion into the parenchyma of the hepatic tissue, which resulted in overgrowth of individual channels in the bioreactor by cancer tissue by day 14. As a control, bioreactors that had not been seeded with DU-145 cells were imaged through the 14 day period (Figure 13, lower row) and beyond to 30 days (data not shown); these bioreactors showed stable persistence of the liver microtissue units.

After 14 days of growth and invasion, the cocultures were stopped and assessed for tissue morphology and integrity of the tumor cell mass by transmission electron microscopy (TEM) and Immunofluorescence. By differential color expression of the endogenous GFP and RFP proteins, we were able to detect prostate cancer cells invading the hepatic parenchyma (Figure 14a). Histologically, viable prostate cancer cells alone with viable hepatocytes were observed after 14 days. Although we observed, as expected, obvious overgrowth of prostate cancer cells, we also observed necrotic hepatocytes surrounded by non-necrotic cancer cells (Figure 14b).

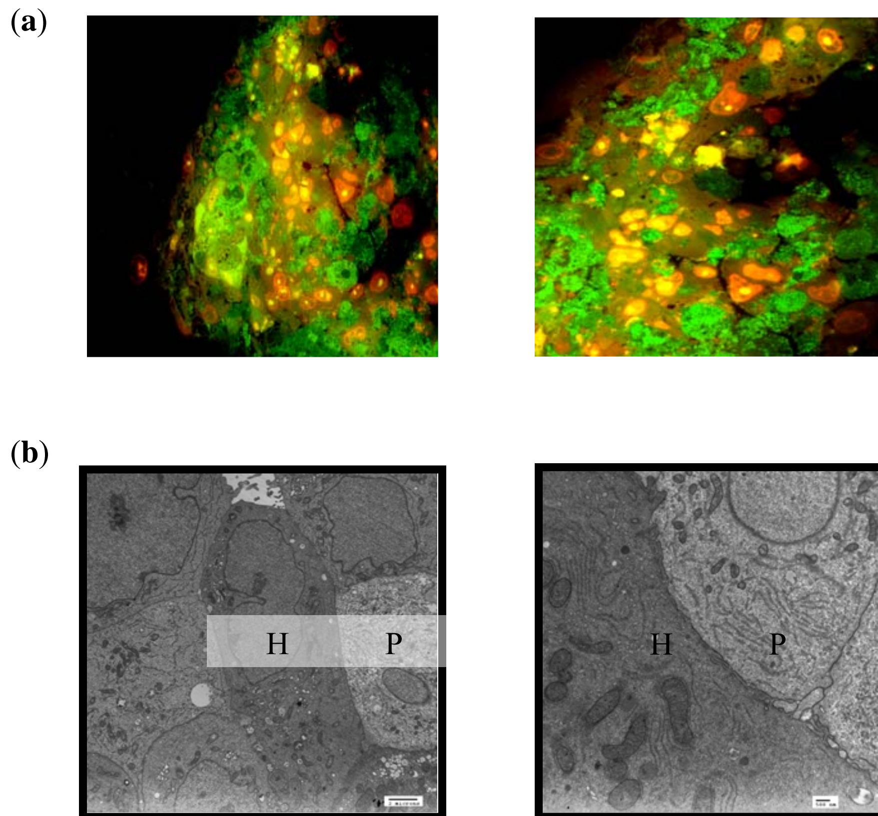


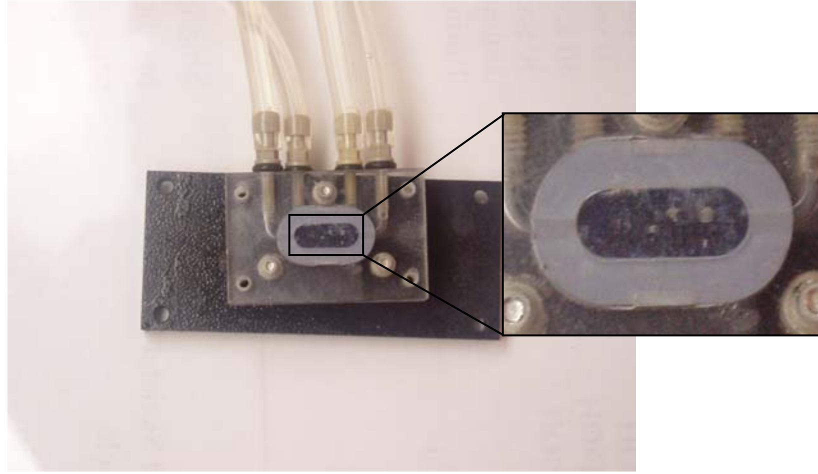
Figure 14. The tumor masses are non-necrotic even when >100 micron diameter. (a) Images were prepare by sectioning through the silicon scaffold at day 14 when the tumors overgrew the 260 micron3 chambers. Immunofluorescence shows DU-145 prostate cancer (red) invasion of the hepatocytes parenchyma (green). (b) Silicon scaffolds were also sectioned and imaged by electron micrography. P=Prostate cancer cell, H=Hepatocytes. Shown are one of three similar experiments.

In addition to cancer cell invasion, overt tumor formation was also observed. Cocultures that were monitored for 25 days resulted in a distinguishable tumor mass (Figure 15a). Upon closer examination by toluidine blue and EM, we found that the tumor cell mass did not possess

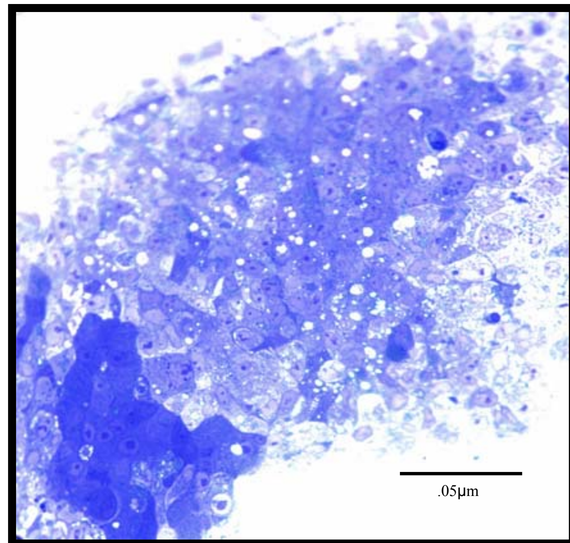
necrosis from the center to the perimeter, despite exceeding 300um in diameter (Kunz-Schughart, Freyer et al. 2004) (Figure 15b). This demonstrated the existence of an adequate supply of oxygen and nutrients, avoiding the formation of necrotic centers in experimental tumors of this mass.

With the demonstration that prostate cancer cells grow in the experimental hepatic environment we asked whether this obvious growth was intrinsic to structural environment provided by our three dimensional system, or simply due to the ability of a tumor cell line to grow autonomously. Seeding the prepared, but liver-devoid, bioreactor with DU-145 prostate tumor cells failed to result in any tumor cell adhesion let alone growth, with the cross-flow clearing all the tumor cells. Therefore we sought to recapture the environment found in the three dimensional cultures in two-dimensional cultures (2D). Prostate cancer cells were plated in identical growth media used for the hepatocytes bioreactors. As expected, prostate cancer cells in their usual 10% FBS supplemented DMEM media showed significant growth as early as day 2, however prostate cancer cells in the HGM media exhibited only marginal growth throughout the six day period examined (Figure 16a).

(a)



(b)



(c)

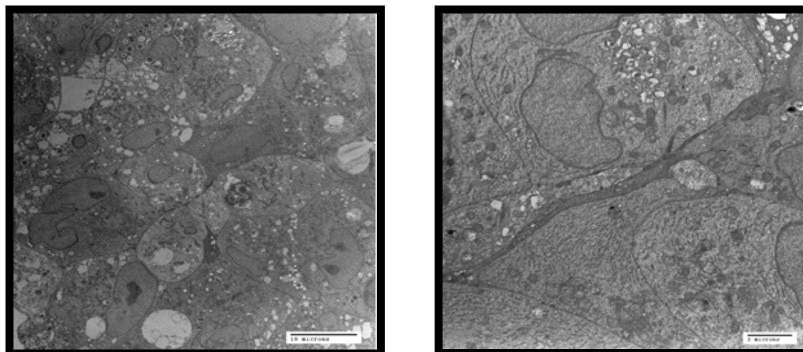
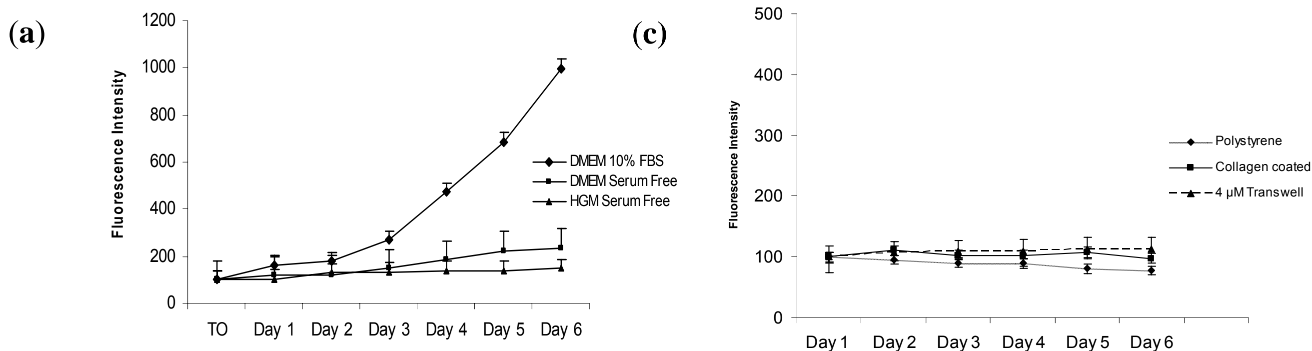


Figure 15. (a) Digital image of visible tumor in bioreactor >300um in diameter. (b). Tumors were removed from reactor and stained with toludine blue. (c) Removed tumor were also imaged by electron microscopy. Tumor-parenchymal heterogeneity remained intact throughout tumor. Shown are images from one of three similar experiments.

To further demonstrate the requirement of a three dimensional environment for cancer cells to elicit their in vivo characteristics we utilized two different 2D co-culture systems consisting of transwells co-culture and direct cell contact co-culture. In the transwell and direct contact co-cultures, RFP-expressing prostate cancer growth was measured by fluorescence intensity. Neither assay report any significant growth up to six days (Figure 16b). However, during intimate co-cultures, “homing” of the cancer cells to the hepatocytes could be seen as early as day 2 and migration of the prostate cancer cells directionally to the hepatocytes was evident by day 4 (Figure 16c). This occurrence was enhanced in the presence of collagen coated plates, suggesting a matrix is necessary to induce at least a migratory response of the prostate cancer cells. Notably, however, cell proliferation was missing, similar to that seen in the bioreactor.



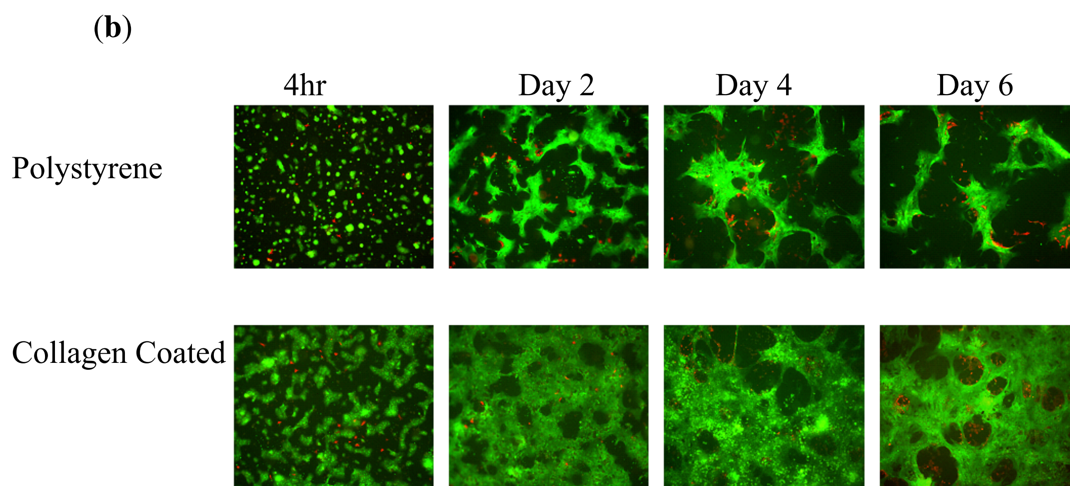


Figure 16. (a) Growth of DU-145 human prostate cancer cells was assessed by fluorescent intensity in the presence of Hepatocyte Growth Medium (HGM), 10% FBS DMEM, or serum-free DMEM over a 6-day period. (b) Fluorescence images of hepatocyte (green) and DU-145 cells (red) on polystyrene or collagen-coated surfaces in the presence of HGM. (c) Growth of DU-145 prostate cancer cells in HGM on polystyrene, collagen-coated or 4μM pore transwell plates in co-culture systems. All experiments were performed in triplicate and repeated three times; in the graphs the data were normalized to Day 1 and are presented as percent growth ±SEM.

4.5. DISCUSSION

Currently, most methods available to study the behavior of metastatic tumor cells are outside of target organ systems or are snapshots in time that capture only brief periods. To better understand the cell biology of metastasis, the key events must be isolated in appropriate organ context and over the dynamic periods that are critical to establishment and growth of metastases.

Studies in experimental metastasis systems and correlative evidence from human cancer patients strongly suggest that dissemination to ectopic sites is not the only or even main rate-limiting step (Luzzi, MacDonald et al. 1998; Cameron, Schmidt et al. 2000; Condeelis, Wyckoff et al. 2000). Rather, establishment in the ectopic parenchyma and further tumor cell expansion appear to select the rare metastatically-capable cells. In this study we utilized the liver, second only to the bone for prostate cancer metastases (Ewing 1922; Shah, Mehra et al. 2004), in the context of a bioreactor that provides an appropriate environment for the morphogenesis of hepatocytes into functional liver tissue (Powers, Domansky et al. 2002). Such a system puts the tumor cells in the appropriate organ environment.

Attempts have been made to dissect the individual aspects of the metastatic process. There are established assays for cell proliferation, migration, adhesion and survival, as well as specific assays for key regulatory molecules. However, these are limited in that the process of metastasis requires a constellation of individual cell properties and molecular activations; no one assay is full predictive either positive or negative. Furthermore, as these cellular and molecular events are interdependent, assays designed to isolate each may provide a panel of responses not indicative of the integrated situation. For this reason, investigators seek experimental systems that recapitulate this integrated process.

Our bioreactor system, upon establishment of a functional liver parchencyma including non-hepatocyte support cells, addresses the concerns of an appropriate environment to study molecular events of metastasis (Powers, Domansky et al. 2002). DU-145 human prostate cancer cells, stably expressing RFP, were introduced into the established liver bioreactor and attachment was seen by day 2 with subsequent growth noticeable by day 4 (Figure 13a). This was not observed on 2D culture plates, when DU-145 cells were incubated in the presence of the

bioreactor medium HGM alone or co-cultured with hepatocytes. Possibly the inefficient growth of DU-145 cells is a result of the absence of fetal calf serum in the HGM, leaving the DU-145 cells at the quiescent stage, although this media is adequate for hepatocytes function. Interestingly, DU-145 cells exhibited increased proliferation in the bioreactor, with growth dominating the visually observed events (Figure 13a). Not only was cell proliferation supported but other integrated cell responses such as the relocation/migration of DU-145 cells across the tissue mass was seen in the early days after inoculation.

The fact that tumor cells only proliferated in the bioreactor and not under tissue culture conditions, even in the presence of close contact co-cultures (Figure 16), suggests a form of liver-tumor communication. TEM investigations revealed invasion of DU-145 cells juxtaposed to hepatic tissue. Distinct cell-cell interactions among the hepatocytes and cancer cells were observed (Figure 14b). While the nature of such communications are under examination, and lie beyond the scope of the present communication, these micrographs provide evidence for heterotypic cell communication during establishment of metastases.

As expected, after 14 days cancers cells completely engulfed the bioreactor and hepatic tissue (Figure 13a). This corresponds with previously published reports that inoculation of DU-145 into the peritoneal cavity result in invasion into the parenchyma of various organs such as lung, diaphragm, and liver by day 14 (Turner, Chen et al. 1996). What was not expected was that tumor growth would become visible with naked eye, by day 25, with intact non-necrotic cellular structures throughout the tissue mass (Figure 15a). Central necrosis has plagued spheroid cultures and other approaches to generating large tumor or liver structures *ex vivo*. For a tumor of this size (>350 μm diameter) to avoid central necrosis suggests maintenance of an adequate supply of nutrient, which has previously not been accomplishable in *in vitro* environments.

Our successful establishment of this organotypic liver system that supports tumor cell invasion and/or metastasis, opens many avenues for future investigation. Current approved therapies aim at cell proliferation and do not expressly target the stages of metastasis establishment and progression. Thus, an integrative model of tumor progression including the target environment would be a significant advancement to highlight total systemic responses. Lastly, by utilizing a fully functional liver bioreactor, the ability to intimately link drug metabolism in real time to target actions opens up new possibilities for the development and testing of agents.

4.6. ACKNOWLEDGEMENTS

These studies were supported by grants from the US Army Medical Research Program in Breast Cancer and the National Cancer Institute (NIH, USA). We thank Ajit Dash, the Wells Lab, and the Griffith Lab for technical assistance and helpful discussions.

5. REEXPRESSION OF E-CADHERIN MEDIATED CELL ADHESION IN PROSTATE CANCER PROGRESSION

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5.1. ABSTRACT

Metastasis is a multi-step processes were tumor cells must be able to detach, migrate, gain access to conduits and disseminate throughout the body. At the molecular level, during invasion cells undergo epithelial mesenchymal-like transition where there is loss of E-cadherin accompanied by increased motility (Savagner 2001; Thiery 2002). Because this transition is a requirement in prostatic neoplasia, correlations to epithelial differentiation during embryogenesis have been made (Bates, DeLeo et al. 2004; Bates and Mercurio 2005). However, we and others have shown that abrogated growth factor signaling results in reexpression of E-cadherin, highlighting the plasticity of these cells (Jawhari, Farthing et al. 1999; Yates, Wells et al. 2005). Furthermore, previous unpublished data from a three dimensional perfusion microreactor show heterotypic communication between prostate cancer cells and hepatic tissue. Therefore we hypothesize that there is phenotypic variability throughout prostate cancer progression. Immunofluorescence staining for E-cadherin in co cultures of GFP expressing hepatocytes and RFP DU-145 prostate cancer cells reveal E-cadherin expression at the peripheral regions of contact by day 2 of coculture with eventual gain of homotypic expression as the cancer cluster become denser. These findings were supported by significant reexpression of E-cadherin in paraffin embedded tissue from human prostate metastasis to the liver. Therefore we conclude that the term epithelial mesenchymal transition only summarizes the transient down regulation E-cadherin for invasion, and that reexpression of E-cadherin is physiological consequence of metastatic tumor development. Therefore we propose that Meschenymal Epithelial reverting Transition (MErT) as the term that fully describes this process in tumor progression.

5.2. INTRODUCTION

Metastatic cancers are responsible for most of the morbidity and mortality associated with cancer patients. However our understanding of the multistep cascade of events that must occur for successful attachment and subsequent metastasis has not been completely elucidated. An essential step for cells to migrate from the primary tumor mass is a loss of epithelial differentiation and a gain of mesenchymal-like phenotype generally defined as epithelial mesenchymal transition (EMT) (Bates, DeLeo et al. 2004; Bates and Mercurio 2005). This transition is necessary for cells to invade their surrounding adnexia and gain access to conduits (Mareel, Behrens et al. 1991; Thiery 2002; Ackland, Newgreen et al. 2003). E-cadherin is a calcium (Ca^{++})-dependent transmembrane cell surface glycoprotein and is most notably known as a suppressor of mesenchymal transition. Normal E-cadherin function usually consists of rapid localization of surface E-cadherin molecules to the regions of contact, resulting in homotypic binding, thus fostering the maintenance of normal cellular structure. Aberrant loss of E-cadherin expression has been well documented and even clinically defined as an invasive marker for many cancers including lung cancer, prostate cancer, gastric cancer, colorectal carcinoma, and breast cancer (Al-Mehdi, Tozawa et al. 2000; Davies, Jiang et al. 2000; Lowy, Knight et al. 2002; Lind, Thorstensen et al. 2004).

Altered E-cadherin expression, which is a key factor during epithelial mesenchymal transition (EMT) has been shown to be the result of epigenetic reversible (Graff, Herman et al. 1995; Lind, Thorstensen et al. 2004) or growth factor mediated down regulation (Kallakury, Sheehan et al. 2001; Hurtubise and Momparler 2004; Wheeler 2005). However, abrogation of

EGFR signaling, reverses decreased E-cadherin expression, thus highlighting the plasticity of cancer cells (Hazan and Norton 1998; Jawhari, Farthing et al. 1999; Yates, Wells et al. 2005). Previous unpublished data utilizing a real-time microtissue perfusion culture demonstrate that DU-145 prostate cancer cells that invade the hepatic tissue are juxtaposed to the hepatic interface, thus creating a very distinct cell border (unpublished data). This would suggest some type of heterotypic communication, at the least, if not involving E-cadherin interactions. Therefore we sought to determine the expression pattern of E-cadherin throughout the progression of prostate cancer cells within an appropriate metastatic microenvironment, the liver.

Herein we propose that decreased E-cadherin expression; a necessary process to disseminate beyond the encapsulated primary tumor mass, is reverted in order for cancer cells to adhere and develop at metastatic target organ. In order to examine the phenotypic transition, we utilized *in vitro* cocultures of primary rat hepatocytes and DU-145 human prostate cancer cells, as liver is a preferred ectopic site for metastasis of many cancers including prostate cancer (Paget 1989; Fidler 2003; Shah, Mehra et al. 2004) demonstrate that as DU-145 cells “home” to the liver cells, there is an increase in E-cadherin at the point of peripheral cell-cell contact, resulting in DU-145 cells adhering to the host hepatocytes. As the prostate cancer cells progress to more dense clusters surrounding the hepatocytes we observed homotypic binding of prostate cancer cells among DU-145 cells and hepatocytes. As a physiological correlate to our in vitro findings, human liver tissue, with prostate cancer metastasis, were analyzed for reexpression of cell adhesion molecules. These tumors we observed significant reexpression of E-cadherin and associated catenins, with a lack of the differentiation marker, vimentin. Therefore we propose that modulation of E-cadherin expression is a result of the cancer cells environment that can be reverted to allow acquired or diminished cellular behavior.

5.3. MATERIAL AND METHODS

The primary antibodies used were mouse monoclonal antibodies to E-cadherin, α- and β−catenin, and p120 (Transduction Laboratories, California). FITC conjugated secondary antibodies were obtained from (BD Biosciences). Secondary antibodies for the Immunofluorescence were obtained from (Molecular Probes, Oregon). Cy5-conjugated secondary antibody was then added (Jackson Laboratories). Other reagents were obtained from Sigma

5.3.1. DU-145 Cell Lines

The cell line DU-145 was originally derived from a brain metastasis of a human prostate adenocarcinoma (Stone, Mickey et al. 1978); it retains the androgen independence of the original tumor and does not express a functional androgen receptor (Dondi, Moretti et al. 1998). This cell line possesses both LHRH and EGF receptors and produces EGFR ligands, TGF-α and EGF (Xie, Turner et al. 1995; Jungwirth, Pinski et al. 1997).

The DU-145 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (4.5g/ml glucose) (Cellgro, Virginia) containing 10 % FBS and supplemented with L-glutamine (2 mM), penicillin/streptomycin (100 units/ml), nonessential amino acids (0.1 mM), and sodium pyruvate (1 mM) (37˚C, 90% humidity, 5% CO_2 and 95 % air). For stable selection of RFP cells, cells were grown in G418 (1000μg/ml) (Gibco, New York), though all experiments were performed in the absence of G418.

5.3.2. Tissue Specimens

Formalin-fixed paraffin-embedded tissues were obtained from the University of Pittsburgh tumor bank. Only well-differentiated prostate adenocarcinomas with liver metastastis were included, irrespective of other criteria.

5.3.3. Immunohistochemistry/Immunocytochemistry

Frozen tissue or cells from culture were stained by established protocol with anti-E-cadherin, anti-α and β-catenin, p120 catenin, vitmentin antibodies. Appropriate secondary antibodies was used for primary antibody.

5.3.4. Two Dimensional Co-cultures

Intimate co-cultures consisting of 50, 000 cells per cm^2 hepatocytes and 2,000 cells per cm^2 prostate cancer cells were plated on 10% collagen. Co-cultures were processed for immunofluorescence at designated time intervals.

5.3.5. Immunofluorescence Microscopy

Cocultures were then fixed in 4% paraformaldehyde, permeabilized with 100 mM Tris-HCl pH 7.4, 150 mM NaCl, 10 mM EGTA, 1% Triton X-100, 1 mM PMSF, and 50 μg/ml aprotinin (all from Sigma), and subsequently blocked with 5% BSA for 1 hour at room temperature. Samples were incubated with indicated primary antibodies diluted in blocking buffer at 4°C overnight. Cy5-conjugated secondary antibody was then added (Jackson Laboratories). Cells were analyzed with laser confocal microscopy using a Leica TCSNT 3 laser 4 PMT system (Olympus, NY).

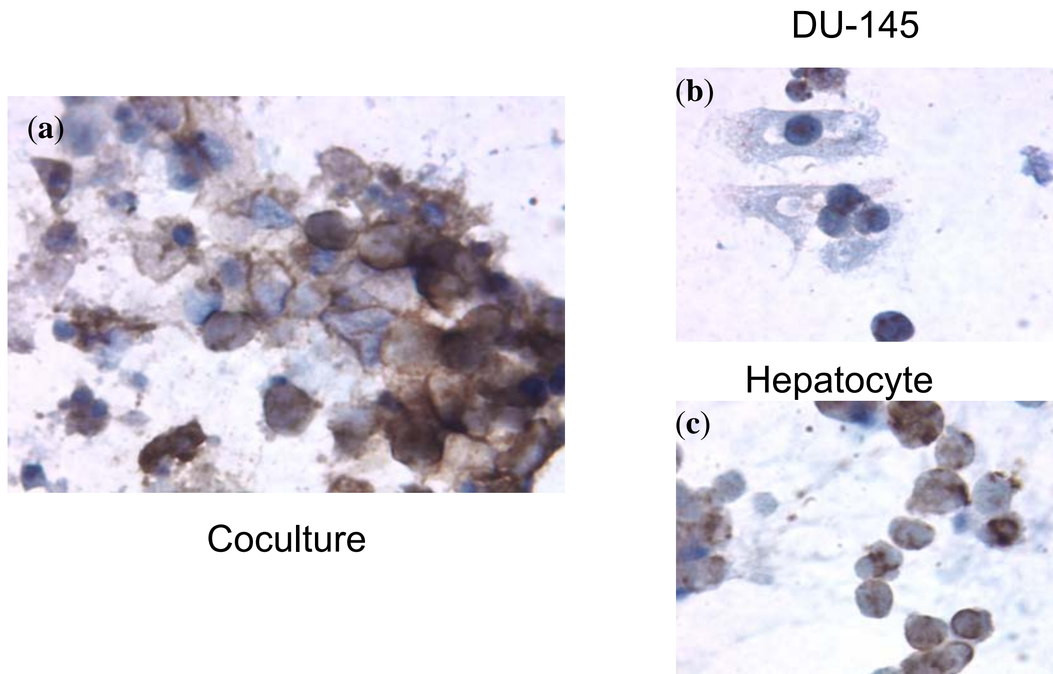

Figure 17. (a) E-cadherin staining of coculture of primary rat hepatocytes and human DU- 145 prostate cancer cells after 14 days in presence of HGM (b) E-cadherin staining of single culture of DU-145 cells after 14 days of in HGM (c) E-cadherin staining of hepatocytes only culture after 14 days in HGM.

5.4. RESULTS

To determine the intimate interactions between prostate cancer cells and hepatocytes we utilized an *in vitro* co-culture assay. Freshly isolated GFP expressing primary rat hepatocytes were allowed to adhere 24 hours prior to seeding of the RFP expressing prostate cancer cells. To determine if DU-145 cells reexpress E-cadherin only in the presence of hepatocytes we allowed coculture to progress to day 14. These cultures revealed heterogeneous staining for E-cadherin throughout the cluster mass, although staining was irrespective of cell type (Figure 17a). However, DU-145 cells that were not juxtaposed to the hepatocytes lacked E-cadherin expression (Figure17b). To determine to if our observed heterogeneous E-cadherin expression was cancer cells driven, we analyzed the cocultures by differential color expression. After 2 days, reexpression of E-cadherin was observed as DU-145 cells directionally extend toward the hepatocytes (Figure 18a). E-cadherin binding among different cells has not been seen before, and therefore we characterized this interaction as heterotypic binding. During the co-culture period, differential color expression of E-cadherin in hepatocytes and DU-145 cells revealed that DU-145 cells predominately expressed E-cadherin in these co-cultures (Figure 18b).

To confirm our *in vitro* coculture findings that E-cadherin is reexpressed during the progression of DU-145 cells, we obtained human liver tissue with prostate cancer metastasis and examined the expression of E-cadherin in these tumors by immunohistochemistry. E-cadherin expression was significantly upregulated, when compared to adjacent normal hepatic tissue (Figure 19c). This increased expression was accompanied by increased in E-cadherin associated adhesion molecules α, β, and p120 as well (Figure 19 d-f). To determine whether these cells reverted from the mesenchymal phenotype, we stained for mesenchymal marker vimentin.

Vimentin was negatively expressed in these tumors suggesting the reversion to an epithelial phenotype (Figure 19b).

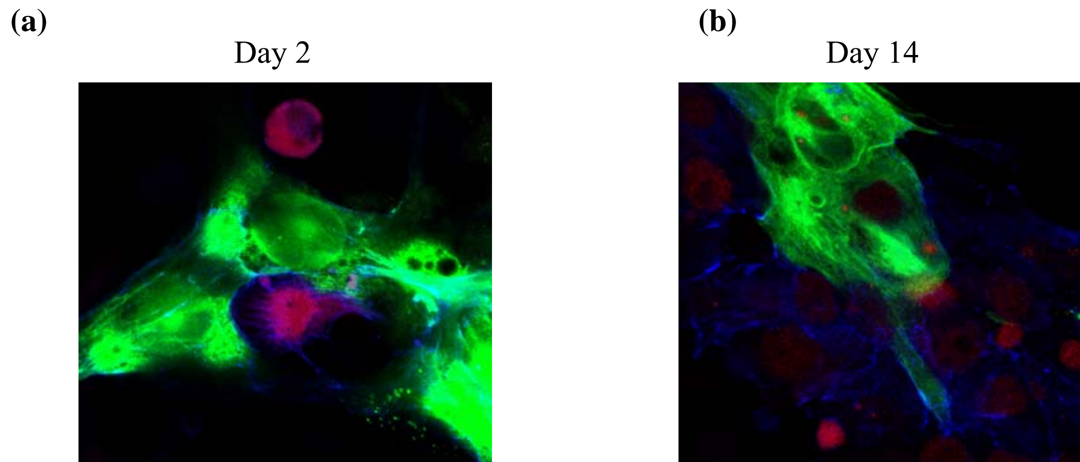

Figure 18 (a) Day 2 fluorescence image of endogenous GFP hepatocytes (green) and RFP DU-145 cells (red) with staining for E-cadherin (blue) in the presence of HGM show heterotypic binding of DU-145 cells to hepatocytes. (b) Day 14 fluorescence image of endogenous GFP hepatocytes (green) and RFP DU-145 cells (red) with staining for E-cadherin (blue) show homotypic binding among RFP DU-145 cells and in the presence of HGM

5.5. DISCUSSION

The multistep process of metastasis suggests that cancer cell must undergo changes at the molecular level that influence phenotypic behavior. This transition can be compared to the central theme of migrating cells during embryogenesis (EMT) (Bates and Mercurio 2005). Mesenchymal cells have an increased advantage to migrate and invade local surrounding. Imperative to migrating cells is the loss of cell-cell adhesion. The role of E-cadherin expression in various cancers has been inversely correlated to increased invasive and metastatic behavior. This is impart due to it's malignancy- associated parameters such as loss of differentiation, invasiveness and metastatic potential of a broad range of carcinomas that are often associated with down-regulation of E-cadherin expression or function (Mareel, Behrens et al. 1991; Mareel, Boterberg et al. 1997). Herein we report that decreased cell adhesion is transient during cancer progression and reexpression is required for reestablishment at the metastatic target organ. In order to examine these events, we utilized differential color expression of RFP DU-145 prostate cancer cells and GFP hepatocytes. Immunofluorescence staining for E-cadherin revealed reexpression of E-cadherin as DU-145 prostate cancer cells extend to adhere to parenchymal hepatocytes, thus exhibiting heterotypic binding (Figure 18a). However as these cocultures continue to day 14, there is clustering of the RFP DU-145 cells around the hepatocytes with mainly E-cadherin homotypic binding of the RFP DU-145 cells (Figure 18b). This is in direct contrast to previously published results from our lab that confluent monolayers of DU-145 cells lack E-cadherin staining (Yates, Wells et al. 2005).

Since *in vitro* cocultures of DU-145 cells and hepatocytes emphasize the transient expression of E-cadherin during prostate cancer progression we sought to correlate this with human tissue specimens of prostate cancer metastasis to the liver. Two tissue specimens were examined and reveal significant expression of E-cadherin, even greater than that of adjacent parenchymal hepatocytes (Figure 19c). The increased E-cadherin expression was further supported by increases in E-cadherin intercellular associated molecules α-catenin, β-catenin, and p120 catenin (Figure 19 d-f). Since E-cadherin is a marker of normal epithelium, we sought to determine if this reexpression of cell adhesion is associated with a complete reversion to epithelial phenotype. Vimentin, a well-defined marker of differentiation was deficient in both tumors (Figures 19b). With E-cadherin and the E-cadherin/complex completely present and vimentin absence, these tumors closely resemble a more normal epithelial phenotype.

On the basis of these observations, we propose a model for the progression of differentiated tumors, which includes the transition from epithelial to mesenchymal phenotype, and takes into consideration the ability of tumor cells to detach and migrate form the primary tumor mass into the circulation. What follows is at least a partial reversion of the epithelial phenotypic, necessary to allow E-cadherin heterotypic binding and avoid ankosis. This then progresses to a complete redifferentiation to epithelial phenotype to form the tumor mass (Figure 20). Therefore we propose that we formally name this MErT (Mesenchymal epithelial reverting Transition).

Although are model has effectively identified that redifferentiation of prostate cancer cells at metastatic organ is a pathophysiological occurrence, determining if reexpression of cell cell adhesion is a requirement to attach the liver parenchyma is a point of interest. Unpublished data from our lab using a centrifugal adhesion of fluorescent cell adhesion assay (CAFCA) demonstrates that MCF-7 breast cancer cells adhere to hepatocytes is as early as 10 min and maximizes at 60 minutes. However, inactivation of E-cadherin binding by depletion of Ca^{2++} or knock down of E-cadherin levels by siRNA significantly decreases the adhesiveness to hepatocytes (personal communication Christopher Shepherd). This would propel that

reexpression of E-cadherin is an early event of disseminated prostate cancers and a requirement for ectopic seeding. It if of interest to note that normal homophilic binding of cadherins results in an increase in survival signals initiated by nascent cadherin interaction (Kovacs, Ali et al. 2002; Verma, Shewan et al. 2004). If the newly reexpressed E-cadherin on the cancer cells operates in a similar function as normal E-cadherin binding, then this could possibly be the initial survival signal required to establish residence among the parenchymal tissue.

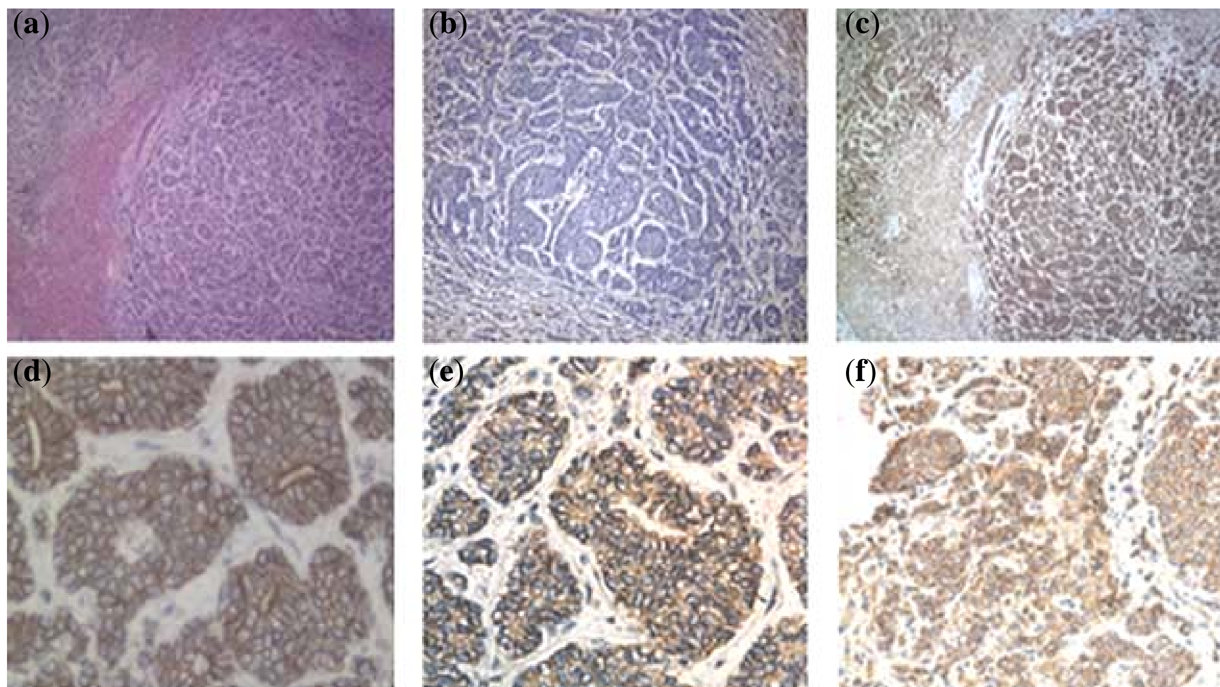

Figure 19. Correlation of the expression patterns of cell adhesion molecules in distant metastasis of prostate adenocarcinomas. Shown are central areas (first column) (a) H&E, (b) Vimentin and (c) and E-cadherin staining of tumor metastasis at the liver. Images are 1.4 mm2. (c-e) are immunohistochemical staining for E-cadherin, β-catenin, and p120. Images are 300um2.

In summary, our proposed model of cancer redifferentiation (MErT) presents a novel view of the progression of cancer in its entirety. Previous reports have identified the reexpression of cell adhesion during metastatic tumor formation in other cancer types (Brabletz, Jung et al. 2001; Kowalski, Rubin et al. 2003), yet none have identified the role of E-cadherin in the establishment of cancer cells after dissemination. Although, these results are preliminary and need further investigation, it correlates with reports that a large percentage of cancers downregulate E-cadherin expression through epigenetic mechanisms (Machado, Oliveira et al. 2001; Lind, Thorstensen et al. 2004),. Therefore other environmental factors, including the modulation of growth factor signaling, transcription factors, and matrix components also need to be deciphered and considered in the context of our findings.

5.6. ACKNOWLEDGEMENTS

The authors would like to thank the Wells Lab for insightful comments and helpful discussions. We would also like to thank the Pittsburgh Department of Pathology Tumor Bank for providing the human tissue specimens.

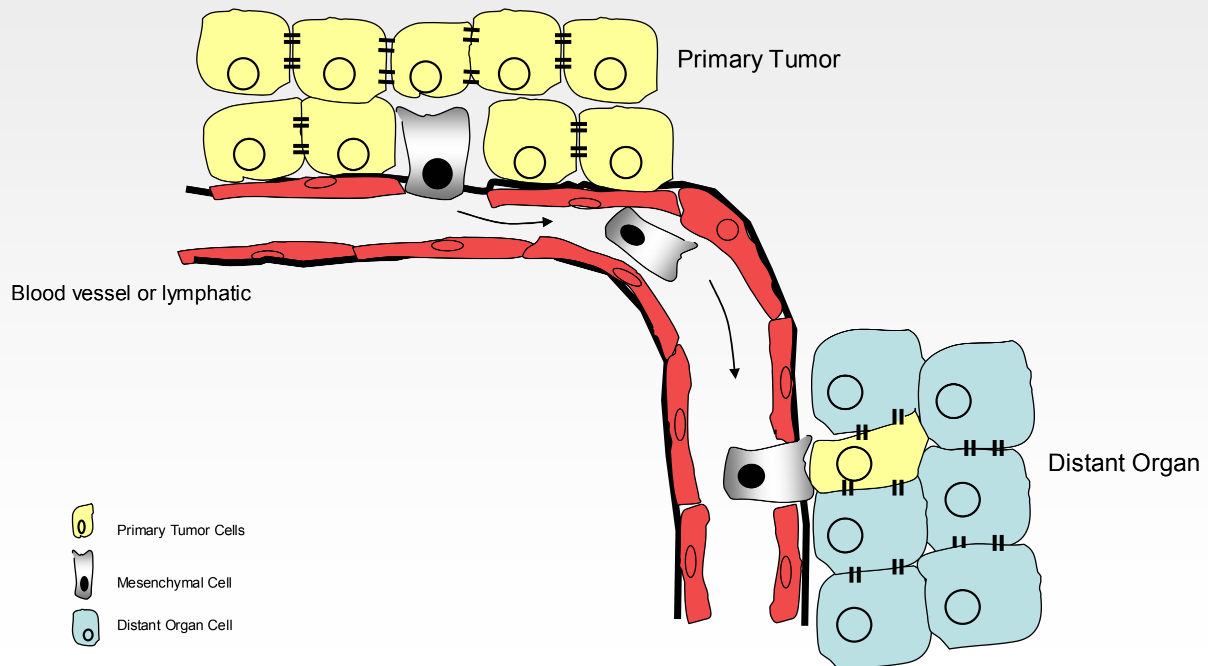

Figure 20. Proposed model for Metastatic events during Prostate Cancer Progression

6. SUMMARY AND DISCUSSION

The totality of these studies outlines significant advances to our understanding of cancer progression. To this author's knowledge, the current study is the first to investigate the reversible expression of E-cadherin in aggressive prostate cancers. Our first approach to elucidate the signaling pathways associated with these changes utilized Cetrorelix, a hormonal treatment under clinical evaluation. We were able to demonstrate that activation of the LHRH receptor by Cetrorelix, results in up-regulation cell adhesion molecules E-cadherin, α-, β-, and p120 catenins, but also restores the cell adhesiveness in human DU-145 prostate cancer cells. We were further able link growth factor signaling, specifically EGFR, with decreases in cell-adhesion in aggressive prostate cancer. This work provided the basis for identifying a mechanism of reversible E-cadherin expression, and serves as a proof of concept that the EGFR signaling axis can be exploited for therapeutic intervention in prostate tumor progression.

As in vivo verification of any molecular pathways identified in vitro is required, available in vivo systems seemed inappropriate to monitor the molecular changes of individual cells over time. This prompted us to develop a new ex vivo system that replicates a relevant microtissue environment to better assess cancer cell behavior. This system, formally named a Micro-fabricated Array Bioreactor, affords for the recreation of an *in vivo* environment for *in vitro* observation in real-time and provides for an optimal device for the study of physiological events. Within our system, proliferation coupled with invasion of DU-145 cells juxtaposed to hepatic tissue was observed. The juxtaposition of the DU-145 cells was also present in close contact co-cultures suggesting a form of heterotypic liver-tumor communication. To elucidate the nature of this communication, we first utilized the close contact DU-145/ hepatocyte cocultures and stained for

E-cadherin expression. Positive staining for E-cadherin at the regions of contact was apparent as DU-145 cells immediately adhered to hepatocytes. As the cancer progressed, DU-145 cells surrounded and isolated the hepatocytes. These events were observed in the later stages with mainly homotypic expression of E-cadherin among the DU-145 cells the coculture clusters. This was an unexpected observation given that heterotypic cell-cell adhesion of cancer cells to the target metastatic organ has not been seen before. The homotypic reexpression of E-cadherin along with absence of mesenchymal marker, vimentin, was verified in human liver tissue with prostate cancer metastasis obtained from autopsy patients. Lack of vimentin staining emphasizes a reversion from the mesenchymal phenotype to epithelial phenotype. Although we are not the first to report reexpression of E-cadherin in distant organ tumor development (Weaver, Petersen et al. 1997; Kowalski, Rubin et al. 2003), these finding are significant in that we show that reexpression of E-cadherin is an initial requirement in the reversion of dedifferentiated cancer cells to a more normal phenotype. Loss of E-cadherin is critical during embryonic development as cells adopt a more amenable phenotypic for increased cell movement (Bates and Mercurio 2005). Although comparisons have been made to cancer, regain of epithelial phenotype has not been included in these comparisons. If cancer cell plasticity is a consequence of aggressive cancer progression, then it seems very applicable that developing tumors at the metastatic organ be viewed in this context. It would also suggest that the cancer cells plasticity would extend to various other regulators of cancer differentiation based on situational or environmental ques.

6.1. Prospective Uses of LHRH Analogs

Hormonal intervention has been the choice of therapy for many cancer patients to avoid prostatectomy. Since the introduction of LHRH analogs as a viable cancer therapeutic numerous clinical trials have found the efficacy of the drug to be safe and show signs of management of progression. However the molecular pathways that are targeted by these hormones and the mechanism of action have not been fully revealed. We show activation of LHRH receptor with the LHRH analog, Cetrorelix results in up-regulation of cell adhesion via PKC mediated down-regulation of EGFR. This report confirmed with previous experiments from our lab that mice, inoculated with a DU-145 subline resistance to PKC negative attenuation of EGFR, show minimal effects after administration of LHRH analog Zoladex. However, PKC mediated downregulation of EGFR is not the only downstream signaling pathway activated. Previous reports have suggested that LHRH administration results in activation of the cAMP dependent pathway with subsequent activation of PKA (Limonta, Montagnani Marelli et al. 2001) mediates the antiproliferative effects of Cetrorelix. Although observed decreases in EGFR expression are consistently reported, a correlation to the mechanism by which this occurs has not been made. Our findings are significant in that we highlight a direct pathway of EGFR mediated down-regulation via activation of PKC. This was further supported with establishment of the linkage between EGFR and E-cadherin**.** If Cetrorelix does indeed activate both the PKC mediated pathway and the cAMP pathway, the question would be to determine are the pathways functioning independently of, or in synergy. Determination of cellular response to activation of LHRH receptor would be a very helpful in the identification of specific downstream molecules that are involved in the cancer progression.

6.2. Prospective Applications for the Bioreactor

Within our present three dimensional perfusion system, there is a broad spectrum of applications were this system would be applicable. Models systems that incorporate environmental quest are more relevant to assay different aspects in tumor biology. Compounded with direct visualize of cellular responses to inhibitory drugs such as our above described LHRH analog Cetrorelix, will certainly highlight previously identifiable processes of tumor behavior. Also individual cancer cells and or hepatic tissue within the system (personal communication Artemis Kalezi) can be manipulated prior to introduction into our system. Assays such as these, should provide an invaluable tool to gain insight into the immediate and long-term effectiveness of therapeutics relative to tumor behavior and host environment.

Future experiments in development of the model will include the addition of a greater fraction of non parenchymal endothelial cells. The importance of vasculature has been understated in numerous cancer assays. Addition of endothelial cells would be a step toward creating true physiological mimics of tissues that recapitulate the features of a capillary bed. The relationship of tumor cells; endothelium and stromal hepatocytes would hopefully open avenues for novel investigations of tumor cell behavior relative to their metastatic microenvironment with molecular level sensitivity. A similar combination of factors is not currently available.

Although in vivo models will ultimately have to be utilized to assess systemic responses, this system offers a quick manipulitable analysis with real-time visualization. With liver being a site of metastasis for many cancers, our successful establishment of this organotypic liver system will be quickly amenable to tumor cell invasion and/or metastasis of other cancers. Although the

greatest advantage of this bioreactor system is the ability to scale to an all human system. With access to primary human hepatocytes and primary human cancer cells, an integrative all-humanized bioreactor system would provide the most applicable model system available. Other possibilities such as initial testing of therapeutic agents and drug metabolism would also increase clinically applicable correlates to tumor biology.

6.3. Prospective: Transient expression of E-cadherin

Herein we have presented a new hypothesis of reexpression of E-cadherin during the process of metastasis. We have previously shown evidence that E-cadherin expression can be regulated via EGFR signaling. In our hands, abrogation of EGFR mediated downregulation of E-cadherin has been reversed by addition of antibody, siRNA to EGFR, and LHRH receptor activation by Cetrorelix. This would suggest that control of expression is at least in part due to growth factor signaling. We have also shown preliminary evidence that E-cadherin expression is involved in the initial adhesion/attachment of prostate cancer cells to hepatocytes with increased expression in human prostate cancer metastasis to the liver. This would imply that E-cadherin expression is transient and subject to therapeutic control. These new findings provide valuable insight into the metastatic process, but are preliminary and need further examination. Further questions worth addressing, surround the dynamic interplay of molecules transiently expressed during transformation and implementation of oncogenesis. If previously noted EGFR-E-cadherin interactions are the primary control of E-cadherin expression, then mediators of this signaling pathway need to be examined to determine the dynamics of E-cadherin regulation. As these

interactions are likely acute extending from days to weeks, our microtissue system would be a very appropriate assay to observe these processes.

Many different signaling pathways have been found to inhibit cell-cell adhesion during cancer progression, although none have been shown to support cancer cell survival. For this we turn investigations involving homophilic cadherin interactions in normal cells. During normal cell-cell contact E-cadherin binding is considered as a ligand-receptor interaction, with a net result of increased signals for survival (Kovacs, Ali et al. 2002; Pang, Kraemer et al. 2005). Homotypic ligation of E-cadherin mediates signals through its cytoplasmic domain seems to recruit PI 3-kinase to the membrane. PI 3 kinase signaling is reflected through its phosphorylation of Akt and translocation to the nucleus. Although cell type specific events have impeded the designation of a single mode action of E-cadherin ligation in homophlic interactions (Kovacs, Ali et al. 2002), it would be helpful to determine the involvement of these implicated molecules in cancer heterotypic adhesion.

6.3.1. Final Word

This research represents the culmination of years of effort on the part of various Investigators and myself. Within it, we have discerned a new role for cell adhesion during the progression of prostate cancers. We have developed an *ex vivo* model for real time determination of metastasis related events and uncovered a signaling cascade that can be exploited for therapeutic intervention. Future investigations will hopefully utilize this microreactor system to map key regulator events previously unavailable in *in vitro* systems and imaging of whole body animal models. Finally, further identification the role of cell-cell adhesion at metastatic target organ,

will hopefully uncover future rate limiting steps in cancer progression. Hopefully these results will lead to new innovations in the battle against cancer and cancer related deaths.

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