THE ROLE OF PODOCALYXIN IN BREAST AND PROSTATE CANCER AGGRESSIVENESS

A dissertation submitted to Kent State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

By

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CHAPTER ONE

Podocalyxin Increases The Aggressive Phenotype of Breast and Prostate Cancer Cells In vitro Through Its Interaction with Ezrin

INTRODUCTION

Podocalyxin Protein Structure and Transcriptional Regulation

Podocalyxin (PODXL, podocalyxin-like protein 1 [PCLP1], thrombomucin, or Myb-Ets-transformed progenitor 21 [(MEP)21]) is a 150-165 kDa transmembrane protein comprised of a highly charged extracellular mucin domain, a disulfide-bonded globular domain and a short cytoplasmic tail (Figure 1). Podocalyxin was originally cloned and characterized by Kershaw et al. (Kershaw et al., 1995; Kershaw et al., 1997a). Podocalyxin is translated as a 528-amino acid peptide including an N-terminal, 21 hydrophobic amino acid signal-peptide sequence. An internal span of 26 hydrophobic-amino acids predictive of a membrane-spanning region is located near podocalyxin's C-terminus. The amino acids C-terminal to this putative transmembrane region carry a net positive charge indicating that podocalyxin is a type I membrane protein with an extracellular N-terminal region and cytoplasmic C-terminus.

After cleavage of the N-terminal signal peptide, podocalyxin is calculated to have a molecular weight of 54 kDa, significantly smaller than the observed
molecular weight for podocalyxin (150-165 kDa), which suggests that the protein is highly glycosylated in vivo. Podocalyxin’s extracellular domain is comprised of the N-terminal 406 amino acids and contains five putative N-linked glycosylation sites, numerous serines and threonines for potential O-linked glycosylation, four prospective sites for glycosaminoglycan attachment, and four cysteine residues for potential disulfide bridges (Figure 1). The 75 amino acids nearest podocalyxin’s C-terminus make up podocalyxin’s intracellular domain. This region contains two potential phosphorylation sites for casein kinase II, a single prospective protein kinase C phosphorylation site, and a terminal DTHL motif for interaction with PDZ domain containing proteins (Figure 1).

Comparison of podocalyxin from human, rabbit, chicken, rat and mouse shows little sequence homology in the extracellular domain aside from a general mucin-like structure and conserved cysteine residues (Takeda et al., 2000; Kershaw et al., 1997a; Kershaw et al., 1995; McNagny et al. 1997). In contrast, the transmembrane and cytoplasmic domains are highly conserved between species suggesting functional importance of these regions (Takeda et al., 2000; Kershaw et al., 1997a; Kershaw et al., 1995; McNagny et al., 1997).

Regulation of podocalyxin expression at the transcriptional level is accomplished primarily by the transcription factor Sp1 and methylation of the PODXL promoter sequence (Butta et al., 2006). The podocalyxin promoter contains putative recognition sites for several transcription factors; however, super-shift and mutational analyses suggest that the Sp1 transcription factor is
responsible for the majority of the basic transcriptional activity of the *PODXL* promoter (Butta *et al.*, 2006). The DNA sequence of the *PODXL* promoter sequence is rich in G+C content and the percentage of methylated CpG islands in the PODXL promoter region correlates inversely with the expression of podocalyxin in various cell lines (Butta *et al.*, 2006). Expression of podocalyxin may also be repressed at the transcriptional level by tumor protein 53 (TP53) (Stanhope-Baker *et al.*, 2004).

**Podocalyxin in the kidney**

Podocalyxin was first described as a component of the renal glomerular epithelial cell’s (podocyte’s) polyanionic glycocalyx from which podocalyxin derives its name (Kerjaschki *et al.*, 1984). In addition to the podocyte, podocalyxin is expressed in a wide variety of cells including vascular endothelia cells, platelets, and hematopoietic progenitor cells (Kershaw *et al.*, 1995; Horvat *et al.*, 1986; McNagny *et al.*, 1997; Miettinen *et al.*, 1999). Podocalyxin is a member of the CD34 family of heavily sulfated and sialated proteins (McNagny *et al.*, 1997). Like CD34, podocalyxin is expressed in high endothelial venules (HEVs) where CD34 and podocalyxin act as adhesive ligands for L-selectin expressed by leukocytes (Kerjaschki *et al.*, 1984; Sassetti *et al.*, 1998).

The pro-adhesive function of podocalyxin in HEVs is in stark contrast to its role as an anti-adhesive molecule in the kidney. Kidney podocytes form interdigitating foot processes that support the glomerular capillaries. Neighboring foot processes are coupled by slit diaphragms, a specialized tight junction, that
Figure 1. Illustration of the Podocalyxin Protein Structure. Podocalyxin’s extracellular domain is composed of a highly glycosylated mucin domain and a disulfide-bonded globular domain. The mucin domain contains five N-linked glycosylation sites (black circles), multiple serines and threonines for O-linked glycosylation (lines) and four sites for glycosaminoglycan attachment (black arrows). The globular domain contains four cysteine residues for potential disulfide bridges (-S-). Podocalyxin’s short intracellular domain contains two potential phosphorylation sites for casein kinase II (black circle, white text), a single prospective protein kinase C phosphorylation site (white circle, black text), and a C-terminal DTHL motif for interaction with PDZ domain containing proteins.
delineates the podocyte's apical and basolateral surfaces. The apical podocyte surface is covered by a highly negative charged, sialic acid rich glycocalyx of which podocalyxin is the major component (Mohos and Skoza 1969; Kerjaschki et al., 1984).

Decreased expression of podocalyxin during podocyte development, such as in Minimal Change Disease Nephritic Syndrome, results in incomplete foot process formation and reduced glomerular filtration (Blau and Haas 1973; Gibson and More, 1998). Podocalyxin knockout mice generate normal numbers of glomerular epithelial cell precursors but do not develop mature podocytes or the specialized foot process architecture required for glomelular filtration and die soon after birth (Doyonnas et al., 2001). While expression of podocalyxin is unchanged in puromycin aminonucleoside (PAN) induced nephrosis, a rat model of nephrosis, sialylation of podocalyxin is significantly decreased providing proof that the anionic charge on podocalyxin’s extracellular domain is essential for normal glomerular function (Kerjaschki et al., 1985).

Glomerular function is believed to be dependent upon charge repulsion between neighboring podocytes to maintain the open foot process architecture required for filtration (Takeda et al., 2000; Andrews, 1988). Neutralization of the podocyte’s negative apical surface charge with polycations as well as treatment with sialidase induces nephrosis in the rat (Seiler et al., 1975; Seiler et al., 1977; Gelberg et al., 1996). The charge-dependent, anti-adhesive properties of podocalyxin have been demonstrated in Madin-Darby Canine Kidney (MDCK)}
cells as well as MCF7 breast carcinoma cells. Over-expression of podocalyxin in MDCK and MCF7 cells perturbs localization of cell junction proteins including E-cadherin and ZO-1, prevents normal cell-cell aggregation, and results in decreased tight junction-dependent transepithelial resistance (Takeda et al., 2000; Somasiri et al., 2004). Moreover, sialidase treatment to remove sialic acid residues from podocalyxin eliminates the anti-adhesive effect of podocalyxin in MDCK cells allowing for the cells to aggregate normally (Takeda et al., 2000).

**The Podocalyxin-Ezrin Complex**

The initial indication that podocalyxin forms a complex tied to the actin cytoskeleton came from observations in the puromycin aminonucleoside (PAN) nephrosis rat model. In addition to decreased sialylation of podocalyxin, actin in PAN-treated rats is dispersed within the cytoplasm rather than concentrated in membranous extensions as seen in the normal state (Whiteside et al., 1993). Further support for a link between podocalyxin and the actin cytoskeleton comes from immunoelectron microscopy experiments where the subcellular distribution of podocalyxin in podocytes was found to overlap with the distribution of the actin-binding protein ezrin (Kurihara et al., 1995). Ezrin is a member of the ezrin-radixin-moesin (ERM) family of proteins that are known to tie integral membrane proteins to the actin cytoskeleton (Tsukita and Yonemura 1997; Tsukita and Yonemura 1999; Bretscher, 1989). Among the integral membrane proteins that bind the cytoskeleton through ERMs are the hyaluronate receptor CD44, and members of the ICAM cell adhesion molecule family (Yonemura et al., 1999).
Podocalyxin and ezrin colocalize at the apical podocyte membrane and form a stable, co-immunoprecipitatable complex that remains associated with actin filaments following extraction with mild detergents such as Tween-20 or Triton X-100 (Orlando et al., 2001). The association between podocalyxin and ezrin does not require an intact actin cytoskeleton as treatment of MDCK or Chinese hamster ovary (CHO) cells with cytochalasin D or latrunculin B to depolymerize actin filaments does not prevent colocalization or coprecipitation of ezrin and exogenously expressed podocalyxin (Orlando et al., 2001).

In addition to ezrin, the actin-podocalyxin complex of the podocyte contains the PSD-95/Dlg/ZO-1 (PDZ) domain containing protein, Na\(^+\)/H\(^+\)-exchanger regulatory factor 2 (NHERF2, also known as E3KARP) (Orlando et al., 2001). NHERF2 was initially identified as a binding partner of Na\(^+\)/H\(^+\)-exchanger 3 (NHE3) in a yeast two-hybrid screen (Yun et al., 1997). In the podocyte, NHERF2 acts as a linker to connect podocalyxin and ezrin (Figure 2). Podocalyxin, through a DTHL motif at its extreme C-terminus, binds to the PDZ2 domain of NHERF2 (Yun et al., 1998). In turn, the C-terminal ERM binding region of NHERF2 binds to the FERM domain in the N-terminal half of ezrin (Yun et al., 1998). Ezrin links the complex to the actin cytoskeleton via an actin-binding domain in its C-terminal region (Matsui et al., 1998).

Two isotypes of NHERF have been identified: NHERF2 (E3KARP) and NHERF1 (ezrin binding protein 50 [EBP50]). The NHERFs share 48% of their amino acid sequence and both have two PDZ domains as well as an ERM
binding domain (Yun et al., 1997; Reczek et al., 1997). The NHERFs are expressed in different cell types but both are capable of interaction with the DTHL motif of podocalyxin and ERM family proteins (Nielsen et al., 2007; Schmieder et al., 2004; Takeda et al., 2000; Breton et al., 2000). The podocalyxin-NHERF-ezrin-actin complex depends upon the negative charge of podocalyxin's extracellular domain. Neutralization of podocalyxin's negative charge with polycation results in disruption of the interaction between podocalyxin's DHTL motif and PDZ2 of NHERF2 (Takeda et al., 2001). The interaction between NHERF2 and ezrin is not disrupted by polycation treatment leaving the NHERF2-ezrin complex bound to the actin cytoskeleton resulting in a cytoplasmic rather than membranous distribution of the NHERF2-ezrin complex (Orlando et al., 2001). Ectopic expression of podocalyxin in MDCK and MCF7 cells has been shown to recruit NHERF2 or NHERF1 respectively, ezrin and filamentous actin to the plasma membrane and induce the formation of microvilli (Nielsen et al., 2007). Interestingly, Nielsen et al., found that elimination of the DTHL motif of podocalyxin prevented NHERF recruitment but did not effect microvillus formation or recruitment of ezrin and filamentous actin by podocalyxin (Nielsen et al., 2007). This result may be explained by an earlier report of direct interaction between the juxtamembranous region of podocalyxin and the N-terminal half of ezrin (Schmieder et al., 2004).
Figure 2. Illustration depicting the Podocalyxin-NHERF-Ezrin-Actin Complex of the Podocyte. Podocalyxin binds NHERF through interaction of the C-terminal DTHL sequence of podocalyxin and the PDZ2 domain of NHERF. NHERF’s C-terminal ERM binding domain interacts with the uncovered FERM domain in the N-terminal half of phosphorylated ezrin. Ezrin links the complex to the actin cytoskeleton via an actin-binding domain in its C-terminal region.
**Podocalyxin in Cancer**

The gene encoding podocalyxin (*PODXL*) maps to chromosome 7q32-33 (Kershaw *et al.*, 1997b). This chromosomal region was linked to risk of prostate cancer aggressiveness following a Genome-wide linkage analysis of 513 brother pairs with prostate cancer using Gleason score as a quantitative trait (Witte *et al.*, 2000). Gleason grade is a prognostic measure of tumor histology, and was used as a means to quantitate prostate cancer aggressiveness. A follow-up study of 853 affected brother pairs strengthened the linkage to this region and narrowed the linkage peak to between markers D7S1804 and D7S2531 (Witte *et al.*, 2003). These two markers define a 1.1-Mb region that includes two plausible candidate genes, muskelin (*MKLN1*) and podocalyxin (*PODXL*). In a series of 48 primary prostate tumors this region was found to show a high frequency of allelic imbalance (indicating either the loss or amplification of a chromosomal region) (Neville *et al.*, 2002). Allelic imbalance at marker D7S1804 was also associated with early onset of prostate cancer and a high combined Gleason score and tumor stage, two measures associated with poor prognosis; while imbalance at D7S2531 positively correlated with a family history of prostate cancer (Neville *et al.*, 2002). Germ-line DNA from 17 men in families that showed the strongest linkage to chromosome 7q32-33 in the original linkage study was sequenced to identify mutations in either *MKLN1* or *PODXL*. No mutations were identified in *MKLN1*, but a variable in-frame deletion, four missense variants and two nonsense mutations were identified in the podocalyxin gene of linked men
(Casey et al., 2006). Follow up evaluation in a family based case-control population consisting of 439 cases and 479 controls found that the variable in-frame deletions in *PODXL* were positively associated with prostate cancer aggressiveness (Casey et al., 2006). These data strongly support a role for podocalyxin variants in risk of developing more aggressive forms of prostate cancer.

Other data implicating podocalyxin in cancer includes reports that *PODXL* is a downstream target of both Wilms tumor 1 (WT1) and tumor protein 53 (TP53) (Palmer et al., 2001; Stanhope-Baker et al., 2004). Gene expression studies have revealed a positive correlation between expression of *PODXL* and expression of ETS1, a regulator of angiogenesis, in human vascular endothelial cells (Teruyama et al., 2001). Furthermore, high levels of podocalyxin protein expression correlated with poor outcome in a subset of breast carcinomas (discussed in detail in Chapter Two) and podocalyxin is commonly expressed by blasts in acute myeloid and acute lymphoblastic leukemia (Somasiri et al., 2004; Kelley et al., 2005).

**Ezrin in Cancer**

While podocalyxin's anti-adhesive nature suggests one hypothetical mechanism through which it may increase cancer aggressiveness; podocalyxin's association with ezrin in the podocyte offers an alternative hypothesis. The ERM proteins (ezrin, radixin, moesin) are closely related members of the band 4.1 superfamily of proteins that link membrane proteins to the actin cytoskeleton...
(Bretscher et al., 2002). Through the formation of membrane-cytoskeleton complexes, ERM proteins regulate a number of processes important in tumor development and metastasis including cell adhesion, cell survival and cell migration (Takeuchi et al., 1994; Gautreau et al., 1999; Lamb et al., 1997; Bretscher et al., 2002, McClatchey, 2003). Activation of ERM proteins is accomplished by phosphorylation near the C-terminus, which weakens intermolecular associations between the C- and N-tails and is accompanied by translocation of the ERM to the plasma membrane-cytoskeletal interface (Bretscher et al., 2002). ERM phosphorylation is dependent on kinases activated by members of the small GTPase Rho family, which control actin cytoskeleton remodeling and cell movement, as well as AKT (PKB), a kinase important in cell survival (Bretscher et al., 2002; Shiue et al., 2005).

Ezrin, the product of the Vil2 gene, has been implicated as a critical determinant in metastasis of rhabdomyosarcomas and osteosarcomas (Yu et al., 2004; Khanna et al., 2004). Vil2 was consistently over-expressed in highly metastatic cell lines derived from rhabdomyosarcomas and osteosarcomas (Yu et al., 2004; Khanna et al., 2004). Transfection of dominant-negative ezrin (lacking phosphorylation sites required for activation) or an anti-sense ezrin construct dramatically inhibited the metastatic capacity of these cell lines in an experimental metastasis assay (Yu et al., 2004; Khanna et al., 2004). Furthermore, expression of exogenous ezrin in poorly metastatic rhabdomyosarcoma and osteosarcoma lines significantly increased the
metastatic potential of these cells in the experimental model of metastasis (Yu et al., 2004; Khanna et al., 2004). In addition to rhabdomyosarcoma and osteosarcoma, ezrin is associated with a more aggressive phenotype in melanomas and endometrial cancers (Leonard et al., 2003; Makitie et al., 2001; Ohtani et al., 2002). Furthermore, abnormal subcellular localization of ezrin has been associated with adverse tumor characteristics in breast carcinoma (Sarrio et al., 2006). The importance of ezrin as a positive regulator of metastasis has been further established in murine models of osteosarcoma, rhabdomyosarcoma and mammary carcinoma (Khanna et al., 2004; Yu et al., 2004; Elliott et al., 2005).

Ezrin interacts with several molecules that are known to be involved in cancer progression and metastasis including the hyaluronan receptor CD44, Rho GDP dissociation inhibitor (Rho-GDI), phosphatidylinositol 3-kinase (PI3K), and focal adhesion kinase (FAK) (Martin et al., 2003; Gautreau et al., 1999; Poullet et al., 2001; Takahashi et al., 1997, Crepaldi et al., 1997). CD44 has been implicated in metastasis in a number of studies (reviewed in Martin et al., 2003). Rho-GDI binds to inactive, GDP-bound, members of the Rho family to prevent their activation by exchange of GDP for GTP. By binding Rho-GDI, ezrin occupies the inhibitor allowing for increased Rho activity (Takahashi et al., 1997). Ezrin, when phosphorylated on Tyr-353, binds to the carboxyl-terminal SH2 domain of p85, the regulatory subunit of PI3K, to allow for increased activity of PI3K's catalytic subunit and subsequent activation of the pro-survival kinase AKT.
Ezrin also interacts with FAK, a kinase involved in cell adhesion, motility and cell survival, resulting in increased FAK phosphorylation and activity independently of cell-matrix adhesion (Poullet et al., 2001). Ezrin has been also found to be a component of lipid raft complexes containing both CD44 and PI3K suggesting that ezrin has the ability to bring multiple metastasis-associated factors together into a signaling complex (Ghatak et al., 2005).

The PI3K-AKT and Mitogen-activated protein kinase (MAPK) pathways have been shown to be important in the prevention of anoikis by ezrin (Louvet-Vallee, 2000). Both of these pathways are thought to be involved in permitting cancer cell survival during the initial steps of metastasis (Ward et al., 2001). Highly metastatic rhabdomyosarcoma and osteosarcoma cell lines that express elevated levels of ezrin also demonstrate increased activity of the PI3K and MAPK pathways (Yu et al., 2004; Khanna et al., 2004). Furthermore, reduction of ezrin expression by an anti-sense construct or short hairpin RNA (shRNA) is accompanied by reduced activity of these pathways implicating PI3K and MAPK signaling as important mechanisms in ezrin dependent cancer aggressiveness (Yu et al., 2004; Khanna et al., 2004). One of the mechanisms through which the PI3K and MAPK signaling pathways increase the metastatic potential of cancers cell is via induction of Matrix metalloproteases (MMPs) (Fukaya et al., 2005; O-charoenrat et al., 2004; Kunapuli et al., 2004). MMPs are endopeptidases capable of degrading extracellular matrix (ECM) and are known promoters of
tumor cell invasion (Yu et al., 1997; Parsons et al., 1997). The relevant signaling pathways influenced by ezrin are summarized in Figure 3.
**Figure 3. Illustration of the Putative Signaling Mechanisms Involved in Ezrin-Dependent Cancer Aggressiveness.** Ezrin interacts with p85, the regulatory subunit of PI3K, to allow for increased activity of PI3K's catalytic subunit and subsequent activation of the pro-survival kinase AKT. The PI3K pathway is inhibited by LY294002. Ezrin also interacts with FAK leading to activation of the MAPK pathway. Ezrin may also indirectly activate PI3K through activation of FAK (dashed line). PD98059 is an inhibitor of MEK1, part of the MAPK pathway.
Rationale and Purpose of Study

Our group originally identified PODXL as a candidate genetic risk factor for more aggressive forms of prostate cancer through linkage and genetic association studies. Subsequent work by our lab and others implicated podocalyxin as a determinant of aggressiveness in breast and prostate cancers. We hypothesized that the mechanism through which podocalyxin increases cancer aggressiveness involves its interaction with ezrin, a protein implicated in the metastatic potential of several cancers, and increased activity of the ezrin-influenced PI3K and MAPK signaling pathways. To test this hypothesis, podocalyxin was stably expressed in MCF7 breast cancer and PC3 prostate cancer cells to determine if expression of podocalyxin increased cancer aggressiveness in vitro and subsequently to determine if ezrin and ezrin-influenced signaling pathways were required for the increased aggressiveness imparted by podocalyxin expression.

We show that in MCF7 breast cancer and PC3 prostate cancer cells podocalyxin expression correlates with increased migration and invasion, increased MMP expression and increased MAPK and PI3K activity. We also demonstrate that podocalyxin and ezrin form a complex in these cells and that over-expression of podocalyxin correlates with changes in the phosphorylation state and subcellular localization of ezrin. Finally, we show that podocalyxin-induced changes in signaling events and invasiveness are ezrin-dependent.
MATERIALS AND METHODS

Cell lines and Culture Conditions

MCF7 and MDA-MB-231 breast cancer cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% fetal calf serum (FCS), 1% L-glutamine, 1% penicillin and 1% streptomycin in 5% CO₂ at 37°C. PC3 prostate cancer cells were grown in DMEM supplemented with 10% FCS, 1% L-glutamine, 1% penicillin and 1% streptomycin in 5% CO₂ at 37°C. Cells were routinely passaged at sub-90% confluence using a solution of 0.05% trypsin, 0.53 mM EDTA-4Na in Hank’s Balanced Salt Solution without calcium or magnesium. Full-length PODXL cDNA was cloned into pcDNA3.1 (Invitrogen, Carlsbad, California) and stably transfected into MCF7 and PC3 cells by electroporation using a BioRad GenePulser followed by clonal selection in media containing 500 µg/mL G418 (Invitrogen, Carlsbad, California). Control cells were transfected with empty pcDNA3.1 vector and selected in the same manner. Transient transfections with a full-length anti-sense ezrin construct (gift from Dr. Chand Khanna, CCR National Cancer Institute, National Institutes of Health) (Khanna et al., 2004) and/or empty pcDNA3.1 (Invitrogen, Carlsbad, California) were performed using the cationic liposome-based transfection reagent, Lipofectamine 2000 (Invitrogen, Carlsbad, California), in accordance with the manufacturer’s recommendations. The total concentration of DNA used in all transient transfections was maintained at 10 µg by titration of full-length anti-
sense ezrin construct with empty vector DNA. Transfection efficiencies were 40% for MCF7 and 45% for PC3 as determined by transfection with GFP vector pcDNA3.1/NT-GFP-Topo (Invitrogen, Carlsbad, California).

**Western Blotting and Immunodetection**

Cells at 70% confluence were rinsed in 1x PBS and lysed by repeated freeze-thaw in buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 1% Triton X-100, 1 x proteinase inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), 1 mM sodium fluoride, and 1µM sodium orthovanadate. To detect protein phosphorylation state, cells were deprived of serum for 20 hours prior to lysis. For detection of Tyr$^{353}$ phosphorylated ezrin, cells at 70% confluence were deprived of serum for 20 hours then treated with 50 µM sodium orthovanadate for 3 hours prior to cell lysis. To determine the phosphorylation state of ezrin at Thr$^{567}$, cell lysates were immunoprecipitated with 2 µg ezrin antibody (BD Biosciences, San Jose, CA) and 20 µL pre-blocked Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) for 16 hours at 4ºC. The immunoprecipitate was then separated by SDS-PAGE, electroblotted and probed with Phospho-Ezrin (Thr$^{567}$/Radixin (Thr$^{564}$/Moesin (Thr$^{558}$) antibody (1:1000 dilution; Cell Signaling, Danvers, MA). Protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL), a colorimetric assay that determines protein concentration by reaction of bicinchoninic acid (BCA) with cuprous cation generated from reduction of Cu$^{2+}$ to Cu$^{+}$ by protein in alkaline solution. In experiments utilizing protein lysates from cells in which ezrin was
transiently knocked down by the introduction of anti-sense construct, lysates were collected 56 hours post-transfection.

Proteins were separated using 10% SDS-PAGE gels followed by electroblotting to Immobilon-P membranes (Millipore, Bedford, MA) using a transfer buffer containing 25 mM Tris, 192 mM glycine, 0.01% SDS, and 20% methanol at pH 8.3. Membranes were blocked by incubation in 1x PBS containing 5% fat-free dry milk for 1 hour at room temperature.

Blots were incubated with the following primary antibodies: podocalyxin (1:10,000 dilution; clone 3D3; Santa Cruz Biotechnology, Santa Cruz, CA), ezrin (1:5000 dilution; BD Biosciences, San Jose, CA), actin (1:10,000 dilution; Clone AC-15; Sigma, St. Louis, MO), phospho-ezrin (Tyr\textsuperscript{353}) (1:1000 dilution; Cell Signaling, Danvers, MA), Phospho-Ezrin (Thr\textsuperscript{567})/Radixin (Thr\textsuperscript{564})/Moesin (Thr\textsuperscript{558}) antibody (1:1000 dilution; Cell Signaling, Danvers, MA), Ezrin/Radixin/Moesin (1:1000 dilution; Cell Signaling, Danvers, MA), phospho-ERK1/2 (Thr\textsuperscript{202}/Tyr\textsuperscript{204}) (1:1000 dilution; clone E10; Cell Signaling, Danvers, MA), total ERK1/2 (1:1000 dilution; Cell Signaling, Danvers, MA), phospho-AKT (Ser\textsuperscript{473}) (1:1000 dilution; Cell Signaling, Danvers, MA), or total AKT (1:1000 dilution; Cell Signaling, Danvers, MA). Incubation with primary antibodies specific to podocalyxin, ezrin, and actin were carried out for 1 hour at room temperature in a dilution buffer containing 1x PBS, 0.1% tween-20 and 5% fat-free dry milk. Incubations with antibodies against phospho-ezrin, phospho-ERK1/2, total ERK1/2, phospho-AKT, and total AKT, were carried out for 16 hours at 4ºC in a dilution buffer containing
5% BSA in 1x PBS and 0.1% tween-20. Blots were subsequently probed with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (Amersham Biosciences, Buckinghamshire, England) diluted 1:5000 in 1x PBS, 0.1% tween-20 and 5% fat-free dry milk. Signals were visualized using the ECL Plus detection system (Amersham Biosciences, Buckinghamshire, England) according to the manufacturer’s instructions. All immunodetection experiments were repeated to ensure reproducibility. Relative quantitations of representative Western blots were performed using Scion Image Software for Windows (Scion Corporation, Frederick, Maryland).

**Invasion Assays**

Matrigel-precoated transwell chambers with PET membranes containing 8 µm pores (BD Bioscience, San Jose, CA) were soaked in DMEM and incubated for 60 minutes at 37°C. After the chambers were rehydrated, 1x 10^5 cells in 0.5 ml of serum-free culture medium were added to the upper compartment of the transwell chamber in triplicate. DMEM (0.5 mL) supplemented with 10% FCS as a chemo-attractant was added to the lower chamber. As a control, an equal number of uncoated BD control chambers were seeded with cells in parallel. After 48 hours (MCF7 cells) or 24 hours (PC3 cells) of incubation, non-invaded cells in the upper compartment were removed using a cotton-tipped swab. Invaded cells were stained with xanthene and thiazine dyes by the Diff-Quik stain kit (BD Bisciences, San Jose, CA), and photographed (10x magnification). Cells were counted in three unique fields of each membrane and averaged to provide
a single value. Data were expressed as the percentage of cells that invaded through the Matrigel matrix coated membrane relative to the cells that migrated through the control membrane and normalized to a control sample (Invasion Index). For inhibitor studies, cells were treated with 10 µM LY 294002 (Sigma, St. Louis, MO), 30 µM PD98059 (Calbiochem, San Diego, CA), or DMSO (Sigma, St. Louis, MO) as a vehicle control and invasion was assayed after 48 hours (MC7 cells) or 24 hours (PC3 cells) of incubation. For experiments utilizing transiently transfected clones, invasion assays were initiated 16 hours following transfection. Two independent experiments were performed for each set of conditions to provide n = 6.

**Wound Migration Assays**

Confluent MCF7 or PC3 cells grown in 10 cm² dishes were wounded using sterile pipette tips (T = 0 hours), washed twice with 1 x PBS, and grown in DMEM with 5% FCS. At T = 0, 24, 48, and 72 hours the cells were photographed under a phase contrast microscope (10x magnification). PC3 cells were monitored for migration at T = 0, 24 and 48 hours. Results were confirmed in at least two independent experiments.

**Migration Assays**

Migration was analyzed in BD Falcon 24-well plates receiving 8 µm pore size PET membrane BD Biocoat cell culture inserts (BD Biosciences, San Jose, CA). 1x 10⁵ in DMEM without serum were seeded to the upper compartment of
the transwell chamber in triplicate. The lower compartment was filled with 0.5 mL DMEM supplemented with 10% FCS as a chemoattractant. After 48 hours (MCF7 cells) or 24 hours (PC3 cells) at 37°C, remaining cells were removed from the upper side of the membrane with a cotton swab, and cells that had migrated and attached to the lower side were stained with Diff-Quik kit (BD Biosciences, San Jose, CA) and photographed (10x magnification). Cells were counted in three fields of each membrane and averaged to provide a single value. Two independent experiments were performed for each set of conditions to provide n = 6.

**Soft Agar Growth Assays**

Cells (1x10^6) were grown in triplicate on 10 cm^2 dishes in a suspension of 0.6% low melting point agarose (Gibco, Carlsbad, CA) and DMEM supplemented with 10% FCS. After 2 weeks the plates were photographed under a phase contrast microscope and assayed for colony number and size.

**Proliferation Assays**

5x10^3 cells were dispensed in triplicate to 96-well plates containing serum supplemented with 5% FCS. Cell proliferation was measured over a 7-day period using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay Kit (Promega, Madison, WI). This assay is based on the conversion of the tetrazolium salt MTT into a formazan product. The quantity of the formazan
product is measured by the amount of 490nm absorbance and is directly proportional to the number of live cells.

**co-Immunoprecipitation**

Cells at 70% confluence were rinsed in 1x PBS and lysed in buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 1% Triton X-100, 1x proteinase inhibitor cocktail (Boehringer, Ingelheim, Germany), 1 mM sodium fluoride, and 1 µM sodium orthovanadate. Protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL). 500 µg of total protein was immunoprecipitated with 2 µg anti-ezrin rabbit polyclonal antibody (Upstate, Charlottesville, VA), 2 µg anti-podocalyxin mouse monoclonal antibody Clone 3D3 (Santa Cruz Biotechnology, Santa Cruz, CA), 2 µg rabbit IgG (Rockland Immunochemicals, Gilbertsville, PA) or 2 µg mouse IgG (Rockland Immunochemicals, Gilbertsville, PA) and 20 µL pre-blocked Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) for 16 hours at 4º C.

Complexes immunoprecipitated by anti-ezrin rabbit polyclonal antibody were separated by SDS-PAGE followed by immunoblotting with anti-ezrin mouse monoclonal antibody (1:1000 dilution; BD Bioscience, San Jose, CA) and antipodocalyxin mouse monoclonal antibody Clone 3D3 (1:2000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). Complexes immunoprecipitated by antipodocalyxin mouse monoclonal antibody Clone 3D3 were separated by SDS-PAGE followed by immunoblotting with anti-ezrin rabbit polyclonal antibody (1:1000 dilution; Upstate, Charlottesville, VA).
**Subcellular co-Localization**

Cells were grown on glass cover slips until 30% confluent and fixed with acetone for 5 minutes. After blocking, cells were incubated with anti-ezrin rabbit polyclonal antibody (1:100 dilution; Upstate, Charlottesville, VA) alone or in combination with anti-podocalyxin mouse monoclonal antibody (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies were Texas red-conjugated anti-rabbit IgG and FITC conjugated anti-mouse IgG used at 1:200 dilutions. Cells were counter-stained with DAPI and visualized under fluorescent microscopy (100x magnification). Results were confirmed in multiple independent experiments.

**Gelatin Zymography**

Cells were grown to 70% confluence, washed twice with 1 x PBS and once with serum-free DMEM and incubated in serum-free medium. After 24 hours, conditioned media was collected and concentrated 20-fold using Amicon Ultra 10,000 MWCO centrifugal filter devices (Millipore, Bedford, MA). Media protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL). Protein samples were prepared in non-reducing sample buffer containing 0.625 mM Tris-HCl, 10% glycerol, 2% SDS and 2% bromphenol blue. Proteins, 40 µg per lane, were separated by SDS-PAGE on gels containing 2 mg/mL gelatin type A (Sigma, St. Louis, MO). Gels were renatured for 1 hour at room temperature in a solution containing 50 mM Tris/HCL (pH 8.0), 5 mM CaCl$_2$ and 2.5% Triton X-100. Gels were incubated overnight at 37° in a buffer
containing 50 µM Tris/HCl (pH 8.0), 5 µM CaCl₂, 0.5% Triton X-100, and 0.02% NaN₃. The gels were fixed in a solution of 20% methanol and 5% acetic acid for 30 mins at room temperature. Gels were stained with Coomassie Blue R-250 (Sigma, St. Louis, MO) and destained in a solution of 40% methanol and 15% acetic acid to reveal gelatinolytic activity. For inhibitor studies, cells were treated with 10 uM LY294002, 30 uM PD98059, or DMSO (as vehicle control) and conditioned media were collected after 24 hours of incubation. For studies in which ezrin protein levels were transiently knocked down, culture media was changed 32 hours post transfection and collected after conditioning for 24 hours. Results were confirmed by multiple independent experiments.

**Real-time PCR Quantification**

Total RNA was extracted from cell lines with TRIzol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer’s instructions. Following DNase I treatment using the DNA-free kit (Ambion, Austin, TX), cDNA was generated from total RNA using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen Carlsbad, CA). RTQ-PCR experiments were performed in quadruplicate using the SYBR Green PCR Core kit (Applied Biosystems, Foster City, CA) according to the vendor’s instructions utilizing an ABI 7900HT (Applied Biosystems, Foster City, CA) real-time PCR instrument. The SYBR Green kit utilizes an asymmetrical dye that fluoresces when bound non-specifically to double-stranded DNA. The fluorescence may be monitored as PCR product is generated over a number of PCR cycles.
The following primer pairs were used to amplify MMP1, MMP2, MMP9, MT1-MMP (MMP14) and ACTB (β-ACTIN): MMP1 (f) 5’-TGG ACC TGG AGG AAA TCT TGC-3’, MMP1 (r) 5’-TCC AAG AGA ATG GCC GAG TTC-3’, MMP2 (f) 5’-AGC TGC AAC CTG TTT GTG CTG-3’, MMP2 (r) 5’-CGC ATG GTC TCG ATG GTA TTC T-3’, MMP9 (f) 5’-TTC TAC GGC CAC TAC T GT GCC T-3’, MMP9 (r) 5’-AAT CGC CAG TAC TTC CCA TCC T-3’, MT1-MMP (f) 5’-CCA TCA TGG CAC CCT TTT ACC-3’, MT1-MMP (r) 5’-TTA TCA GGA ACA GAA GGC CGG-3’, ACTB (f) 5’-TTG CCG ACA GGA TGC AGA A-3’, ACTB (r) 5’-TTG CTG ATC CAC ATC TGC TGG-3’. All primers were designed using Primer Express v2.0 software (Applied Biosystems, Foster City, CA). Primer efficiencies were determined by plotting cDNA template dilution versus ΔCt. Amplification of all targets was similar in efficiency to amplification of the endogenous reference ACTB.

Transcript expression levels were normalized using ACTB levels as an endogenous reference. Cycle conditions were 95°C for 10 minutes followed by 45 cycles of 95°C for 10 seconds and 60°C for 60 seconds. Relative quantitation (RQ) of gene transcripts was accomplished using the Comparative C_{T} method (ΔΔC_{T} = ΔC_{T, sample} - ΔC_{T, reference}). Data are presented as RQ ± SD. Student’s t-test was used for statistical comparisons between ΔCt values. $P < 0.05$ was considered to be statistically significant.
EXPERIMENTAL PROTOCOLS

Protocol 1A

Hypothesis: Podocalyxin Expression increases the aggressiveness of breast and prostate cancer cells in vitro. Full-length, sequence-verified PODXL was stably expressed in MCF7 breast cancer and PC3 prostate cancer cells. Three independent MCF7/PODXL and two independent PC3/PODXL clones were chosen for use in subsequent experiments. MCF7 and PC3 cells stably transfected with empty pcDNA3.1 vector served as controls. Cell invasion was assayed using Matrigel-coated culture chamber inserts. Migration was assessed by wound healing assays and movement through uncoated culture chamber inserts. Anchorage-independent growth was measured by culture in soft agar. Cell proliferation was analyzed by colorimetric determination of tetrazolium salt (MTT) conversion into a formazan product.

Protocol 1B

Hypothesis: Podocalyxin and Ezrin form a complex in breast and prostate cancer cell lines. Lysates from MCF7 pcDNA and PODXL clones were immunoprecipitated with ezrin antibody or podocalyxin antibody. The immunoprecipitated complexes were separated by SDS-PAGE and immunoblotted for podocalyxin and/or ezrin. MCF7 and PC3 clones were examined by fluorescent microscopy for localization of podocalyxin and ezrin.
Changes in ezrin phosphorylation in response to podocalyxin expression were assayed by immunoblotting with phosphorylation site-specific ezrin antibodies.

**Protocol 1C**

_Hypothesis: Expression of podocalyxin induces increased activity of the ezrin-influenced PI3K and MAPK signaling pathways._ MAPK pathway activity was determined by normalized comparison of phosphorylated, active ERK1/2 kinase detected by immunoblot of pcDNA and PODXL clone lysates. PI3K activity was analyzed by comparison of normalized levels of phosphorylated, active AKT in lysates from pcDNA and PODXL clones as detected by immunoblot. The requirement of ezrin in increased activity of the MAPK and PI3K in PODXL expressing cells was determined by transient knockdown of ezrin by transfection of an antisense-ezrin construct followed by immunoblot based determination of ERK1/2 and AKT activity.

**Protocol 1D**

_Hypothesis: The increased invasive capacity of podocalyxin expressing cells requires increased expression of proteins that degrade the extracellular matrix._ Conditioned media from MCF7 and PC3 clones were analyzed by gelatin zymography for MMP activity. Expression of MMP family-members was measured by quantitative PCR. To determine if ezrin was required for increased expression of MMPs in PODXL-expressing cells, MMP activity and expression was measured in MCF7/PODXL and PC3/PODXL cells in which ezrin had been
knocked down with an anti-sense ezrin construct. To determine if MMP activity and expression was dependent upon increased activity of the MAPK and/or PI3K pathways, MMP activity and expression was measured in MCF7/PODXL and PC3/PODXL cells following treatment with the MEK1 inhibitor PD98059 or the PI3K inhibitor LY294002.

**Protocol 1E**

*Hypothesis: Ezrin and the ezrin-influenced PI3K and MAPK signaling pathways are required for increased invasion by podocalyxin expressing cells:* Invasion of MCF7 and PC3 clones through Matrigel was determined following knockdown of ezrin with an anti-sense ezrin construct or chemical inhibition of the MAPK and PI3K pathways by treatment with the MEK1 inhibitor PD98059 or the PI3K inhibitor LY294002

**Statistical Analysis**

For invasion and migration data each set of experimental conditions was performed in triplicate and repeated to provide $n = 6$. Invasion data are presented as Invasion Indexes ± SD. Migration data are presented as mean number of migrating cells ± SD. Quantitative Real-time PCR experiments were performed in quadruplicate to provide $n = 4$. Quantitative Real-time PCR data are presented relative expression values +/- SD. Statistical comparisons were made by Student's $t$-test. A value of $P < 0.05$ was considered to indicate statistical significance.
RESULTS

*Podocalyxin expression increases the in vitro invasive and migratory potential of cancer cells.*

To investigate the potential role of podocalyxin in invasion and migration we compared the phenotype of MCF7 and PC3 cells stably expressing podocalyxin or empty pcDNA3.1 vector. pcDNA3.1/PODXL or empty pcDNA3.1 vector was stably introduced into MCF7 and PC3 cells. Three independent podocalyxin-expressing MCF7 clones (MCF7/PODXL1, MCF7/PODXL2, and MCF7/PODXL3), two independent podocalyxin-expressing PC3 clones (PC3/PODXL1 and PC3/PODXL2) along with one MCF7/pcDNA and one PC3/pcDNA control clone were selected for use in further experiments (Figure 4). Podocalyxin expression had no effect on ezrin expression (Figure 4, middle panels).

The effect of podocalyxin expression on the invasive potential of MCF7 and PC3 cells was examined using matrigel-coated transwell chamber assays. As shown in Figure 5A, podocalyxin expression increased the *in vitro* invasive potential of MCF7 cells by 2.5 to 4-fold in the three MCF7/PODXL transfectants compared to the empty vector control. Podocalyxin expression also increased the *in vitro* invasive potential of PC3 cells 1.8 to 2.7 fold compared to the vector control (Figure 5B).
Figure 4. Western Blot Demonstrating Expression Levels of Exogenous Podocalyxin and Endogenous Ezrin Proteins in MCF7/PODXL and PC3/PODXL cells. (A) Cell lysates from three MCF7/PODXL transfectants (PODXL1, 2, and 3) and MCF7/pcDNA3.1 empty vector control cells (pcDNA) along with (B) two PC3/PODXL transfectants (PODXL1 and 2) and PC3/pcDNA3.1 empty vector control cells (pcDNA) were subjected to Western blot analysis to determine podocalyxin and ezrin protein expression. Alpha actin was used as a protein loading control.
Figure 5. Podocalyxin Increases Invasion of MCF7 and PC3 Cells.

Histograms summarizing the Invasion Indexes of MCF7/pcDNA and PODXL cells (A) and PC3/pcDNA and PC3/PODXL cells (B). Invasion was analyzed using a Matrigel-coated cell culture insert system. After 48 hours (MCF7 clones) or 24 hours (PC3 clones), cells were fixed, stained and counted. Data are expressed as Invasion Indexes ± SD. *$P < 0.05$ compared to respective pcDNA control. $n = 6$. 
In a wound-healing assay, cells were grown to confluence prior to wounding with a sterile pipette tip and monitored every 24 hours for 3 days for migration into the wound area (Figure 6A). All MCF7/PODXL transfectants showed increased wound healing at each time point compared to the pcDNA vector control cells. Migration was also assayed quantitatively using culture inserts with 8 µm pores (Figure 6B and 6C). Migration of MCF7/PODXL and PC3/PODXL cells was increased 1.6 to 1.9-fold compared to their respective vector control cells.

The effect of podocalyxin on anchorage independent growth of MCF7 cells was measured by growth in soft agar. Podocalyxin expression had no effect on the number or size of colonies developing over a two-week period (data not shown). Podocalyxin expression was also found to have no effect on the proliferation of MCF7 cells grown in DMEM media supplemented with 5% FCS or PC3 cells grown in DMEM media supplemented with 10% FCS. Cell doubling times for the MCF7 clones was determined to be 40 hours while the PC3 clones doubled in 24 hours.
Figure 6. Expression of Podocalyxin Increases Migration of MCF7 and PC3 Cells. A. Photographs showing wound closure of MCF7/pcDNA and MCF7/PODXL clones over 72 hours. Representative fields were photographed at 0, 24, 48, and 72 hours. B and C. Migration of MCF7 clones (B) and PC3 clones (C) were also analyzed using a cell culture insert system. After 48 hours (MCF7 clones) or 24 hours (PC3 clones), cells that had migrated to the lower side of the culture insert were stained, photographed and counted. Data are expressed as mean Cell Count ± SD. *P < 0.05 compared to respective pcDNA control. n = 6.
Podocalyxin co-Immunoprecipitates and co-Localizes with Ezrin.

Podocalyxin is known to interact with the cytoskeletal linker protein ezrin in kidney podocytes but this complex has not been demonstrated in cancer cells (Orlando et al., 2001). We first tested for the presence of podocalyxin:ezrin complexes in MCF7 transfectants by co-immunoprecipitation. Lysates from MCF7/PODXL transfectants and empty vector controls were immunoprecipitated with ezrin antibody. The precipitates were separated by SDS-PAGE and probed for either ezrin or podocalyxin (Figure 7A). Podocalyxin was co-immunoprecipitated with ezrin in all MCF7/PODXL transfectants but was not detectable in immunoprecipitates from MCF7.pcDNA vector control cells (Figure 7A, lane 2). Neither ezrin nor podocalyxin were precipitated with a non-specific (IgG) antibody (Figure 7A, lane 1) showing the interaction to be specific. To ensure that podocalyxin and ezrin could form a complex in cancer cells that endogenously express both of these proteins we confirmed the co-immunoprecipitation of podocalyxin and ezrin in MDA-MB-231 cells (Figure 7B). A reciprocal set of experiments using podocalyxin antibody to immunoprecipitate ezrin confirmed the presence of complexes containing podocalyxin and ezrin in MCF7/PODXL cells (data not shown).

To determine whether ezrin and podocalyxin co-localized, MCF7 and PC3 clones were examined by fluorescent microscopy. Ezrin and podocalyxin were found to co-localize in both MCF7/PODXL (Figure 7B, upper panels) and PC3/PODXL (Figure 7B, bottom panels) cells.
Figure 7. Podocalyxin and Ezrin co-Immunoprecipitate and co-Localize in Cancer Cells. A. Western blot showing co-immunoprecipitation of exogenous podocalyxin and endogenous ezrin in MCF7/PODXL cells. Cell lysates were immunoprecipitated with anti-ezrin antibody (lanes 2-5) or a control IgG (lane 1). The immunoprecipitates were separated and blotted with antibodies to ezrin or podocalyxin. B. Western blot showing co-immunoprecipitation of endogenous podocalyxin and ezrin in MDA-MB-231 cells. Cell lysates were immunoprecipitated with anti-ezrin antibody (lane 2) or a control IgG (lane 1). C. Immunofluorescence analyses showing co-localization of podocalyxin and ezrin in MCF7/PODXL1 (upper panels) and PC3/PODXL1 (lower panels) cells. Representative results are shown.
Cytoplasmic ezrin staining was seen in both pcDNA control and PODXL expressing cells (Figure 7C and Figure 8A), with additional strong, focal ezrin staining primarily at cell boundaries evident in PODXL expressing clones but not in pcDNA control cells (Figure 7C and Figure 8A). Focal staining of podocalyxin was seen primarily along cellular boundaries in MCF7/PODXL and PC3/PODXL cells, and these areas of staining overlapped with areas of intense ezrin staining (Figure 7C).

Phosphorylation of Tyr$^{353}$ is associated with changes in the sub-cellular localization of ezrin, regulates the interaction between ezrin and the p85 subunit of PI3K, and is required for activation of the PI3K/AKT cell survival pathway by ezrin (Yu et al., 2004; Bretscher, 1989). We therefore tested whether phosphorylation of ezrin at Tyr$^{353}$ was changed in response to podocalyxin expression. Western blots with an antibody specific for ezrin phosphorylated on Tyr$^{353}$ showed that phosphorylation of this residue was increased in MCF7/PODXL and PC3/PODXL cells (Figure 8B, upper panels). Ezrin may also be phosphorylated at threonine 567. Phosphorylation of this residue is reported to disrupt intramolecular association and is associated with changes in ezrin localization and the formation of actin rich structures near the cell membrane (Chambers and Bretscher, 2005; Gautreau et al., 2000). Expression of podocalyxin was also found to lead to increased phosphorylation of ezrin on Thr$^{567}$ in both MCF7 and PC3 cells (Figure 8B, middle panels).
Figure 8. Expression of Podocalyxin is Associated with a Change in the Sub-Cellular Localization of Ezrin and Increased Ezrin Phosphorylation. **A.** Cells were stained with rabbit anti-ezrin antibody followed by Texas red coupled anti-rabbit secondary antibody. Representative results for MCF7/pcDNA and MCF7/PODXL1 cells are shown. Arrows indicate areas of intense ezrin staining where neighboring cells are in close proximity. **B.** Western blots of lysates from MCF7/pcDNA, MCF7/PODXL1, PC3/pcDNA and PC3/PODXL1 transfectants demonstrating that expression of podocalyxin correlated with increased phosphorylation of ezrin on tyrosine 353 (upper panels) and threonine 567 (middle panels).
**Podocalyxin Expression Increases the Activity of the MAPK and PI3K Pathways.**

Interaction between podocalyxin and ezrin in cancer cells suggests that podocalyxin may increase cancer cell aggressiveness by modulating signaling pathways influenced by ezrin. To test this theory, we analyzed the activity of two pathways influenced by ezrin, the PI3K and MAPK pathways (Gautreau et al., 1999; Khanna et al., 2004). Activity of the MAPK pathway was assayed by measuring the amount of phosphorylated, active ERK1/2. PI3K pathway activity was determined by measuring the amount of phosphorylated, active AKT. Western blot analysis showed that ERK1/2 phosphorylation was increased by 1.6 to 2.3-fold in MCF7/PODXL cells compared to MCF7/pcDNA control cells (Figure 9A). Expression of podocalyxin in PC3 cells resulted in a 2.6 to 2.7-fold increase in ERK1/2 phosphorylation (data not shown). AKT phosphorylation was also enhanced 2.4 to 2.5 fold in MCF7/PODXL cells compared to MCF7/pcDNA cells (Figure 9A) and 7.5 to 10 fold in PC3/PODXL cells compared to PC3/pcDNA cells (data not shown). Ezrin has been shown to directly interact with PI3K to increase activity of the PI3K-AKT pathway; activation of the MAPK pathway by ezrin is hypothesized to depend on activation of focal adhesion kinase (FAK) and subsequent activation of the MAPK cascade (Gautreau et al., 1999; Poullet et al., 2001; Yu et al., 2004; Khanna et al., 2004).

To determine if PODXL expression correlated with increased activity of FAK we measured the amount of phosphorylated, active FAK. Western blot
analysis showed that FAK phosphorylation was increased by 2.8 to 4.2-fold in MCF7/PODXL cells compared to MCF7/pDNA control cells (Figure 9A). Expression of podocalyxin in PC3 cells resulted in a 3.5 to 4.4-fold increase in FAK phosphorylation (data not shown).

We next tested whether ezrin was required for the increase in ERK1/2 and AKT phosphorylation seen in MCF7/PODXL and PC3/PODXL cells. Ezrin expression was transiently knocked-down using an anti-sense ezrin construct kindly provided by Dr. Chand Khanna (CCR National Cancer Institute, National Institutes of Health). This construct has previously been used successfully to reduce ezrin protein levels in osteosarcoma cells (Khanna et al., 2004). When podocalyxin expressing MCF7 or PC3 transfectants were transiently transfected with 5 or 10 µg of full-length anti-sense ezrin construct, ezrin protein levels were reduced by 40 or 80% respectively in MCF7 cells and 40 or 60% respectively in PC3 cells (Figure 9B, top panels). Knock-down of ezrin protein using 5 or 10 µg of the anti-sense ezrin construct resulted in 40 and 70% reductions in ERK1/2 phosphorylation in MCF7/PODXL cells along with 40% reduction in PC3/PODXL cells (Figure 9B, middle panels). Transient knockdown of ezrin resulted in 50 and 90% reductions in AKT phosphorylation in MCF7/PODXL cells and 60 and 70% reductions in AKT phosphorylation in PC3/PODXL cells (Figure 9B, lower panels). These data indicate that the increased activity of the MAPK and PI3K pathways induced by podocalyxin expression is mediated through ezrin.
Figure 9. Podocalyxin Increases Activation of MAPK and PI3K Pathways in an Ezrin-Dependent Manner. A. Western blots demonstrating correlation of podocalyxin expression with increased phosphorylation of ERK, AKT and FAK in MCF7/PODXL cells. Lysates from MCF7/PODXL transfectants were probed with the indicated antibodies. Proteins were quantitated using Scion Image for Windows. Similar data were seen in PC3 cells (data not shown). B. Western blots illustrating that transient knockdown of ezrin protein levels abrogated ERK and AKT phosphorylation in MCF7/PODXL and PC3/PODXL clones. Ezrin protein levels were knocked-down by transient transfection of an anti-sense ezrin construct. Lysates were probed with the indicated antibodies. Representative results are shown for MCF7/PODXL1 and PC3/PODXL1. Proteins were quantitated using Scion Image for Windows.
Podocalyxin Expression Results in Increased Expression and Activity of MMPs.

Increased cellular invasion is often accompanied by increased activity of proteins that degrade the extracellular matrix such as members of the matrix metalloprotease (MMP) family. In addition, the MAPK and PI3K pathways have been shown to regulate expression of MMP family members (Fukaya et al., 2005; O-charoenrat et al., 2004; Kunapuli et al., 2004). Therefore, we assessed the activity and expression of several members of the MMP family in MCF7/PODXL and PC3/PODXL cells. Supernatants from cells grown in the absence of serum were concentrated 20-fold and subjected to gelatin zymography to measure the activity of MMP2 and MMP9. MCF7/PODXL and PC3/PODXL cells showed an increase in MMP9 activity compared to pcDNA control cells (Figure 10A). MMP2 activity was unaffected (data not shown).

We next measured mRNA expression levels of MMP9, MMP2, MMP1 and MT1-MMP (MMP14) by real time quantitative PCR. MMP9 gene expression was increased 2.5 to 5.5 fold in the MCF7/PODXL cells compared to empty vector control cells (Figure 10B) and transcript levels correlated well with observed MMP9 activity levels. In addition, MMP1 mRNA expression was upregulated 7.5 to 14 fold in PODXL transfectants (Figure 10C). There were no changes in expression of MMP2 and MT1-MMP (MMP14) mRNAs between MCF7/PODXL cells compared to empty vector control cells (data not shown).
Figure 10. Podocalyxin Expression Leads to Increased MMP9 and MMP1 Activity and Expression. A. Zymogram showing increased secretion of active MMP9 by MCF7/PODXL and PC3/PODXL transfectants. Cells were grown for 24 hours in the absence of serum. Supernatants were collected, concentrated and used for gelatin zymography. B and C. Histograms illustrating the correlation between podocalyxin expression and increased production of MMP9 (B) and MMP1 (C) mRNA. mRNA was extracted from the same MCF7/pcDNA and MCF7/PODXL cells as in (A) and real-time quantitative PCR analysis were performed in triplicate. Data are expressed as relative expression ± SD. *P < 0.05 compared with pcDNA control. n = 4.
Increased MMP Expression and Activity in Podocalyxin-Expressing Cells Depends on Ezrin.

To determine whether the ability of podocalyxin to induce MMP expression in PODXL expressing cells was dependent on ezrin, we measured MMP activity and expression in MCF7/PODXL and PC3/PODXL cells in which ezrin protein levels were transiently knocked-down via transfection with a full-length anti-sense ezrin construct (see Figure 9B). Transient knockdown of ezrin protein levels resulted in a dose-dependent reduction in the amount of active MMP9 secreted by MCF7 and PC3 podocalyxin-expressing clones (Figure 11A). Furthermore, knockdown of ezrin resulted in a dose-dependent decrease in MMP9 and MMP1 transcript levels (Figure 11B and C). There was strong concordance between MMP9 mRNA levels measured by real-time quantitative PCR, MMP9 activity levels observed in gelatin zymography and ezrin protein levels measured by immunoblot (Figure 11A and B and Figure 9B top panels). These data indicate that ezrin is required for the increased MMP expression and activity observed in PODXL expressing cells.
Figure 11. Ezrin is Required for Increased MMP Activity and Expression in MCF7/PODXL and PC3/PODXL Cells. A. Zymogram demonstrating that transient knockdown of ezrin protein levels resulted in decreased secretion of MMP9 in MCF7 and PC3 PODXL transfectants. Representative results are shown for MCF7/PODXL1 and PC3/PODXL1 cells. B and C. Histograms demonstrating the correlation between knockdown of ezrin protein levels and decreased expression of MMP9 (B) and MMP1 (C) mRNA. mRNA was extracted from the MCF7/PODXL1 cells used in (A) and real-time quantitative PCR analysis were performed. Data are expressed as relative expression +/- SD. *P < 0.05 compared to 0 µg anti-sense Ezrin control. **P < 0.05 compared to 5 µg anti-sense Ezrin control. n = 4.
Inhibition of MAPK or PI3K Activity Attenuates MMP Expression and Activity in MCF7 and PC3 Cells.

We next sought to determine if either of the ezrin influenced signaling pathways, MAPK and PI3K, were responsible for the induction of MMP expression in PODXL expressing cells. To accomplish this we used inhibitors targeted to each of these pathways; 10 µM LY294002 and 30 µM PD98059 specifically and effectively inhibited the PI3K or MAPK (MEK1) pathways in MCF7 and PC3 cells (data not shown). Treatment of MCF7/PODXL (Figure 12A) and PC3/PODXL (data not shown) cells with LY 294002 resulted in a decrease in MMP9 activity. Likewise, when MCF7 (Figure 12B) and PC3 (data not shown) cells were treated PD98059, a decrease in MMP9 activity was observed by zymography in all transfectants. LY 294002 treatment resulted in a decrease in MMP9 mRNA expression of 45 to 53% in MCF7/PODXL cells (Figure 12C). Treatment with PD98059 resulted in a decrease of between 33 and 50% in MMP9 expression MCF7/PODXL cells (Figure 12D). MMP1 expression was also significantly decreased in MCF7/PODXL cells treated with either LY 294002 (Figure 12E) or PD98059 (Figure 12F). Together these results suggest that signaling through both the MEK1 and PI3K pathways are required for the increased expression and activity of MMP1 and MMP9 seen in PODXL expressing cells.
Figure 12. Podocalyxin Increases MMP1 and MMP9 Expression in a MEK1 and PI3K-Dependent Manner. A and B. Zymograms demonstrating that secretion of MMP9 by MCF7/PODXL cells is dependent on PI3K and MEK1 activity. Conditioned media was collected from cells grown for 24 hours in serum-free medium in the presence of 10 µM LY294002 (A) or 30 µM PD98059 (B). DMSO was used as a vehicle control. Similar results were seen in PC3 clones. RNA extracted from the MCF7/pDNA and MCF7/PODXL cells used in A and B was subsequently used to generate cDNA for real-time quantitative PCR analysis to assay expression of MMP9 (C and D) and MMP1 (E and F) mRNA. Data are expressed as relative expression +/- SD. *P < 0.05 compared with pDNA vehicle control. **P < 0.05 compared to respective sample vehicle control. n = 4
Ezrin and activities of the MAPK and PI3K Pathways are required for Podocalyxin-Dependent Increased Invasiveness of MCF7/PODXL and PC3/PODXL cells.

We next asked if increased activity of the ezrin–related signaling pathways (MAPK and PI3K) was responsible for the enhanced in vitro invasive potential of PODXL expressing cells. PODXL expressing cells and empty vector control cells were seeded onto Matrigel-coated Transwell chambers and treated with PD98059, LY294002 or DMSO and allowed to invade. The presence of either PD98059 or LY294002 significantly inhibited invasion of podocalyxin transfectants (Figure 13A and B). Invasion of MCF7/pcDNA and PC3/pcDNA vector control cells was also affected by treatment with either inhibitor as may be expected since these cells do exhibit some invasive capacity and MAPK and AKT activity in the absence of podocalyxin expression.

Finally, to confirm that ezrin is required for the increased invasiveness exhibited by podocalyxin over expressing cells we measured the ability of MCF7/PODXL and PC3/PODXL cells to invade when ezrin was transiently knocked down. Transient knockdown of ezrin with anti-sense ezrin construct reduced the invasive ability of MCF7/PODXL and PC3/PODXL clones in a dose-dependent manner (Figure 13C and D). Transient knockdown of ezrin also reduced the invasive capacity of PC3/pcDNA although not in a dose-dependent manner (Figure 13D). This is not unexpected as ezrin is likely required, at least in part, for the invasive capacity displayed by these cells in the absence of
podocalyxin. The lack of a dose-dependent response to anti-sense ezrin in PC3/pcDNA can likely be attributed to the fact that these cells have less phosphorylated, active ezrin than do the PC3/PODXL clones and are therefore more sensitive to reductions in ezrin protein levels. Invasion of MCF7/pcDNA was unaffected by anti-sense ezrin treatment which likely reflects the poor invasive capacity of these cells in the absence of podocalyxin expression (Figure 13C).
Figure 13. Inhibition of MEK1 or PI3K or Knockdown of Ezrin Decreases Invasion of MCF7 and PC3 Cells. A and B. Histograms showing that inhibition of MEK1 or PI3K decreases invasion of MCF7 (A) and PC3 clones (B). Data are normalized to DMSO-treated MCF7/pcDNA or PC3/pcDNA cells, respectively. C and D. Histograms illustrating that ezrin knockdown decreases invasion of MCF7 (C) and PC3 clones (D). Data are normalized to MCF7/pcDNA or PC3/pcDNA cells treated with 0 µg of anti-sense ezrin construct, respectively. Data are expressed mean Invasion Indexes ± SD. *P < 0.05 cf. DMSO or 0 µg anti-sense treated pcDNA control. **P < 0.05 cf. respective DMSO or 0 µg anti-sense treated sample. †P < 0.05 cf. respective 5 µg anti-sense ezrin treated sample. n = 6.
DISCUSSION

Recent studies have implicated podocalyxin in aggressive forms of breast and prostate cancers (Somasiri et al., 2004; Casey et al., 2006). Here we show for the first time that podocalyxin expression increases the in vitro migratory and invasive properties of MCF7 breast cancer and PC3 prostate cancer cells, induces increased MMP1 and MMP9 expression and leads to increased MAPK and PI3K activity. Furthermore, we show that these podocalyxin-dependent effects are mediated at least in part by ezrin.

The known anti-adhesive properties of podocalyxin suggest one mechanism through which podocalyxin may increase the aggressiveness of cancer cells. Recent work has shown that over-expression of podocalyxin in breast cancer cells disrupts cell-cell contact (Somasiri et al., 2004). A second possible mechanism through which podocalyxin may increase cancer aggressiveness relies on the property of podocalyxin to form a complex with the metastasis promoter ezrin, an interaction that has been demonstrated in kidney podocytes (Orlando et al., 2001). Here we establish for the first time that podocalyxin is able to form a complex with ezrin in breast and prostate cancer cells. In MCF7 and PC3 cells over-expressing podocalyxin both podocalyxin and ezrin localize to cell boundaries. The anti-adhesive properties of podocalyxin suggest that high concentrations of podocalyxin along cellular boundaries might inhibit the formation of proper cell-cell interactions. Ezrin is thought to play an active role in targeting molecules to specific cell surface areas (Serrador et al.,
Our data suggests that the redistribution of podocalyxin to cell boundaries relies on interaction with ezrin and the underlying cytoskeleton. Interaction between podocalyxin and ezrin in cancer cells may occur by direct contact of the juxtamembranous domain of podocalyxin and ezrin or through the linking protein NHERF. Both of these mechanisms of interaction were demonstrated in MCF7 cells soon after our study was submitted for publication (Nielsen et al., 2007).

Podocalyxin expression was also found to result in changes in the phosphorylation state of ezrin at tyrosine residue 353 and threonine residue 567. Phosphorylation of ezrin Tyr$^{353}$ has been seen in response to treatment with epidermal growth factor followed by a change in the sub-cellular localization of ezrin (Bretscher, 1989). Phosphorylation at Tyr$^{353}$ is also required for interaction of ezrin and the p85 subunit of PI3K and ezrin-regulated modulation of the PI3K pathway (Gautreau et al., 1999). Phosphorylation at Thr$^{567}$ is thought to allow for activation of ezrin by reduction of intramolecular association and is associated with changes in localization of ezrin and the formation of actin rich structures (Chambers and Bretscher, 2005; Gautreau et al., 2000).

Our observation that podocalyxin expression correlated with a change in the sub-cellular localization of ezrin and changes to ezrin phosphorylation suggests that podocalyxin might influence ezrin-regulated signaling pathways (i.e. the PI3K and MAPK pathways). In support of this, we found that podocalyxin expression correlated with increased PI3K and MAPK activity. Transient knock-down of ezrin protein by expression of anti-sense ezrin RNA abrogated the
increase in podocalyxin-dependent changes in MAPK and PI3K activities, providing further support for the hypothesis that the ability of podocalyxin to increase migration and invasiveness of MCF7 and PC3 cells is dependent upon its interaction with ezrin.

The PI3K and MAPK pathways are believed to affect metastasis through a number of mechanisms including induction of members of the MMP family (O-charoenrat et al., 2004, Kunapuli et al., 2004; Ruhul Amin et al., 2003). We observed an increase in MMP9 secretion as well as an increase in MMP9 and MMP1 mRNA levels in PODXL-overexpressing cells. This podocalyxin-dependent induction of MMP expression required ezrin and ezrin-related signaling as shown following knockdown of ezrin protein and chemical inhibition of the PI3K and MAPK pathways. Inhibition of either the MAPK or PI3K pathways or knockdown of ezrin levels not only led to a decrease in MMP expression but also to a decrease in the in vitro invasive potential of MCF7/PODXL and PC3/PODXL cells.

**Summary and Conclusions**

Taken together our results support the hypothesis that podocalyxin increases the in vitro migration and invasive properties of breast and prostate cancer cells through its interaction with ezrin. This complex may be important for targeting podocalyxin to specific membrane sites where the anti-adhesive properties of podocalyxin prevent cell-cell interaction. Moreover, by binding to ezrin and tethering this protein near the plasma membrane, podocalyxin may
also affect ezrin-dependent signaling events leading to increased activities of the MAPK and PI3K pathways, induction of MMPs, and ultimately leading to a more invasive phenotype. Our findings suggest that podocalyxin and the podocalyxin:ezrin complex represent potential targets for the treatment of some metastatic breast and prostate cancers. The mechanisms through which podocalyxin and ezrin may increase cancer aggressiveness are summarized in Figure 14.
Figure 14. Illustration Depicting the Potential Mechanisms Through Which Podocalyxin and Ezrin may Increase Cancer Aggressiveness. Podocalyxin's heavily negative charged extracellular domain might encourage cancer dissemination by reducing cell-cell adhesion. By forming a complex with ezrin, either through direct interaction between podocalyxin's juxtamembranous domain and ezrin or via the bridging protein NHERF, podocalyxin increases the concentration of ezrin near the plasma membrane. Ezrin in close proximity to the plasma membrane may be more likely to interact with FAK and PI3K resulting in activation of signaling pathways that ultimately encourage migration and invasion of cancer cells.
CHAPTER TWO

Podocalyxin Expression is Associated with the Basal-like Breast Cancer Phenotype and Metastasis to the Brain

INTRODUCTION

Prognostic Factors in Breast Cancer

Breast cancers are a heterogeneous group of tumors both in terms of their behavior and their therapeutic response. Clinical management of breast cancer has traditionally relied on histological markers for prognosis and prediction of therapeutic response. Scarff-Bloom-Richardson Classification, often referred to as Bloom-Richardson Grade or BRG, is a widely accepted system to assign a standardized histological grade to invasive ductal and lobular carcinomas (Bloom and Richardson, 1957; Elston and Ellis, 1991). Bloom-Richardson Classification takes into account three morphologic features to assign tumor grade: degree of tumor tubule formation, pleomorphism (nuclear grade), and mitotic index. Each feature is assigned a score ranging from 1 to 3 with higher scores representing greater deviation from the normal breast morphology. The combined score, ranging from 3 to 9, is then used to classify the tumor into one of three grades. Grade I tumors have a combined score of 3-5 and are well-differentiated tumors, tumors with a combined score of 6 or 7 are moderately differentiated Grade II tumors, Grade III tumors are poorly differentiated and have a combined score of
8 or 9. Patients with higher Bloom Richardson Grade tumors have a higher risk of recurrence and reduced 5-year survival rate than those with lower Grade tumors (Fisher et al., 1988; Contesso et al., 1987). Amongst the strongest predictors of breast cancer recurrence is the presence or absence of axillary lymph node metastases (LN status) (Carter et al., 1989). Tumor size at diagnosis is also a strong prognostic indicator of overall survival for breast cancer patients (Carter et al., 1989).

In addition to Bloom Richardson Grade, tumor size and nodal status, hormone receptor status is a strong prognostic indicator and predictor of response to adjuvant endocrine therapy (Fisher et al., 1988; McGuire et al., 1990). Estrogen receptor (ER) status is the most powerful marker in predicting response to hormone therapy; progesterone receptor status (PR) is also a widely used predictive marker of hormone therapy response although its predictive value is weaker than ER (McGuire et al., 1990; Payne et al., 2008; Bast, Jr. et al., 2001).

Her-2 status is also a widely used prognostic indicator and predictor of response to Trastuzumab-based chemotherapy (Slamon et al., 1987, McKeage and Lyseng-Williamson, 2008; Iwata, 2007). Her-2/neu/ERBB2 is an epithelial growth factor receptor family member that is amplified or over-expressed in 20-30% of breast cancers (Schechter et al., 1984; Bange et al., 2001). Her-2 positive breast cancers are associated with a poor prognosis and distant metastases (Fan et al., 2006; Hu et al., 2006)
Cytokeratin (KRT) expression also has prognostic value in breast cancers. Breast cancers that express cytokeratins associated with the basal/myoepithelial of the normal breast duct including KRT 5, 6 and 14 can be categorized as "basal" breast cancers. These have a poorer prognosis than "luminal" breast cancers that express cytokeratins (KRT 8, 18, and 19) associated with the luminal epithelial layer of the normal duct (Nagle et al., 1986; Nielsen et al., 2004; Rakha et al., 2006). Breast cancers that express basal KRTs also tend to be negative for ER, PR and Her-2 (the so-called "triple negative" breast cancer phenotype). The triple-negative phenotype is predictive of breast cancer relapse and distant metastases (Haffty et al., 2006).

The list of prospective prognostic molecular markers continues to expand as the mechanisms involved in breast cancer progression are elucidated. Like Her-2, these molecules may represent novel therapeutic targets. One putative molecular prognostic marker for breast carcinoma is fascin, an actin-bundling protein involved in cell motility (Kureishy et al., 2002). Fascin expression correlated with hormone receptor negative, Bloom Richardson Grade 3 breast carcinomas and decreased disease free survival in one patient population (Yoder et al., 2005) and with the basal breast cancer phenotype in a second population (Rodriguez-Pinilla et al., 2006).
Podocalyxin is a Predictor of Breast Cancer Progression

Like fascin, podocalyxin has been found to be a prognostic indicator in breast carcinoma. Somasiri et al. reported that podocalyxin was an independent predictor of breast cancer progression in a population of 272 patients with invasive breast carcinoma diagnosed at Vancouver General Hospital between 1974 and 1995 (Somasiri et al., 2004). Of the patient tumor samples in the Somasiri study population, 6% (15 of 272) showed intense staining for podocalyxin and comprised subset of breast carcinomas with poor prognosis. High expression of podocalyxin was found to correlate with higher Bloom Richardson Grade ($P = 0.001$) and negative ER status ($P = 0.003$) but was not correlated with tumor size, lymph node status or Her-2 staining (Somasiri et al., 2004). Patients in the high podocalyxin group also had significantly decreased disease-free ($P = 0.01$) and overall ($P = 0.025$) survival. Furthermore, high expression of podocalyxin was found to be an independent predictor of poor outcome by Cox regression proportional hazard analysis ($P = 0.0005$) with predictive power equal to or exceeding lymph node status and Her-2 overexpression. While this study indicates that podocalyxin is a predictor of poor outcome in one moderately sized patient population, these results must be confirmed in independent patient populations.
Factors that Predict Breast Cancer Metastasis to the Brain

In addition to prediction of survival rate and therapy response, some prognostic factors may be able to predict breast carcinomas that are likely to metastasize and even forecast metastatic pattern (Ramaswamy et al., 2003; Patanaphan et al., 1988; Arriagada et al., 2008). The primary sites of distant metastases from breast carcinoma are the liver, lung, bone and brain (Maki and Grossman, 2000; Patanaphan et al., 1988). The incidence of brain metastases in women with metastatic breast cancer has been reported as 10-16% but may be twice that when asymptomatic cases are included (Lin et al., 2004; Patanaphan et al., 1988; Miller et al., 2003). Recent data suggest that involvement of brain metastases may be increasing in breast cancer as more effective chemotherapeutic strategies afford breast cancer patients longer survival after initial diagnosis (Weil et al., 2005; Lin et al., 2004). The blood-brain barrier is an obstruction to effective delivery of most chemotherapeutic agents limiting treatment options for breast cancer patients with brain metastases (Peacock and Lesser, 2006; Ballabh et al., 2004). Survival for breast carcinoma patients with brain metastases is extremely poor with a mean 1-year survival rate of only 20% (Engel et al., 2003). Identification of factors that are predictive of metastasis to the brain could increase breast cancer survival by identifying patients that would most benefit from pre-emptive adjuvant therapy that might not otherwise be recommended. Metastasis predicting markers may themselves represent targets of novel therapies.
The high morbidity of breast cancer patients with brain metastases is particularly devastating given that brain metastases are more frequent in patients presenting with breast cancer at a young age (Evans et al., 2004; Hicks et al., 2006). In addition to early age at diagnosis, features such as high Bloom-Richardson Grade, tumor size and lymph node status are also predictive of brain metastasis (Gabos et al., 2006, Pestalozzi et al., 2006, Hicks et al., 2006). A number of studies have shown patients with ER-negative breast carcinomas are significantly more likely to develop brain metastases than patients with ER-positive breast carcinomas (Hicks et al., 2006; Gabos et al., 2006 Pestalozzi et al., 2006; Chang et al., 2003a). Patients with breast carcinomas positive for Her-2 have also been reported to be more likely to develop brain metastases (Emi et al., 2002; Kirsch and Hochberg, 2003). In addition, the basal breast cancer phenotype has been found to be predictive of increased incidence of brain metastases (Hicks et al., 2006).

**Gene Expression Signatures Define Breast Cancer Subgroups**

Recently, gene expression analysis of breast tumors has been used to identify distinct gene signatures associated with clinical outcome (Chang et al., 2003b; Sorlie et al., 2001; van de Vijver et al., 2002; van't Veer et al., 2002; Perou et al., 2000). At least five clinically relevant subtypes of breast cancer have been identified through hierarchical clustering analysis: luminal A, luminal B, Her-2+, normal-like and basal (Sorlie et al., 2001; Perou et al., 2000).
The clearest distinction between the subtypes occurs between the luminal A and basal subgroups (Sorlie et al., 2006; Sorlie et al., 2001; Perou et al., 2000). Luminal A-type tumors are characterized by high expression of genes typically expressed in the luminal epithelium that lines the inner layer of the breast lobular duct while basal-like tumors are characterized by expression of genes associated with the myoepithelial cells of the outer (basal) layer of the breast duct. Among the genes that define the luminal A cluster are *ESR1* and *PGR1*, which encode for estrogen receptor-alpha (ERα) and progesterone receptor (PR) respectively, as well as *GATA3* and *XBP1*, two transcription factors associated with the luminal lineage, and genes encoding cytokeratins of the luminal epithelia including *KRT8*, *KRT18* and *KRT19* (Perou et al., 2000; Sorlie et al., 2001; Bertucci et al., 2003; Lopez et al., 2004).

The basal subgroup is defined by reduced expression of genes associated with the luminal A subgroup and increased expression of *LAMB3*, *ANXA8*, *FSCN1* and several cytokeratins associated with breast duct myoepithelial cells including *KRT5*, *KRT6*, *KRT14* and *KRT17* (Perou et al., 2000; Sorlie et al., 2001; Bertucci et al., 2003; Hicks et al., 2006; Rodriguez-Pinilla et al., 2006). In addition to reduced expression of *ESR1* and *PGR1*, cancers of the basal subgroup tend not to express Her-2 (Yehiely et al., 2006; Sorlie et al., 2006; Sorlie et al., 2001; Perou et al., 2000).

The Her-2+ subgroup is primarily defined by increased expression of *HER-2/ERBB2* as well as a group of genes found on chromosome region 17q12
near HER-2 that are co-amplified in many Her-2+ tumors (Perou et al., 2000; Sorlie et al., 2001; Bertucci et al., 2003).

Like the luminal A subgroup, those of the luminal B group tend to express ESR1, GATA3 and other markers of the breast duct luminal epithelia and tend not to express markers of the basal subgroup. However, expression of ESR1 and GATA3 and luminal genes regulated by ERα and GATA3 is decreased in luminal B cancers compared to cancers of the luminal A subgroup (Perou et al., 2000; Sorlie et al., 2001). Tumors of the luminal B subgroup are also more often Her-2+ than those of the luminal A group (Perou et al., 2000). Cancers of the normal-like subgroup tend to express markers of the basal epithelia (although at a lower level than the basal subgroup) and tend not to express markers of the luminal subgroups but do not cluster into any of the four other subgroups (Perou et al., 2000; Sorlie et al., 2001).

Of the five subtypes, luminal A tumors have the best prognosis, best response to hormone therapy and lowest rates of recurrence (Carey et al., 2006; Hu et al., 2006; Calza et al., 2006). Luminal B and normal-like breast cancers also have relatively good prognosis, although not as promising as the luminal A subgroup (Carey et al., 2006). Breast cancers falling into the Her-2+ and basal subgroups are associated with a poor prognosis and are prone to distant metastases (Carey et al., 2006; Fan et al., 2006). In particular, breast cancers of the basal and Her-2+ subgroups have been associated with metastasis to the brain (Hicks et al., 2006; Emi et al., 2002). Cancers of the Her2+ subgroup can
be treated with trastuzumab, a humanized antibody against Her-2, to extend disease-free survival; however, relapse is common (Fan et al., 2006; Calza et al., 2006). While targeted therapy for treatment of breast cancers of the basal subgroup does not exist, potential therapeutic targets include EGFR, which is expressed in a portion of basal-like cancers, have been identified (Yehiely et al., 2006).

**Breast Tumors from Familial BRCA1 Breast Tumors are Basal-like**

Recent studies indicate that hereditary breast cancers in *BRCA1* mutation carriers are similar to sporadic basal-like tumors (Turner et al., 2004). *BRCA1*-related breast cancers are typically triple negative (ER-, PR-, Her-2-) and express basal markers including KRT5, KRT6 and KRT17 (Lakhani et al., 2002; Foulkes et al., 2004). Tumors from *BRCA1* carriers also cluster with the basal subgroup by gene expression profiling (van de Vijver et al., 2002; Sorlie et al., 2003). Like sporadic basal-like breast cancers, *BRCA1*-related cancers have a poor prognosis and are prone to distant metastases particularly brain metastases (Turner et al., 2004; Foulkes et al., 2003). While somatic mutations in *BRCA1* have not been detected in sporadic basal-like breast cancers, recent studies indicate that most sporadic basal-like tumors have reduced expression of *BRCA1* (Catteau et al., 1999; Abd El-Rehim et al., 2004). *BRCA1*-promoter methylation has also been reported in sporadic basal-like tumors (Catteau et al., 1999; Weil et al., 2005).
Breast Cancer Cell Lines also Cluster into Subgroups

Like breast cancers, breast cancer cell lines can be divided into subgroups by hierarchical clustering (Charafe-Jauffret et al., 2006; Neve et al., 2006). Whereas breast cancers cluster into at least five distinct subgroups, the majority of breast cancer cell lines cluster into one of two major groups: luminal or basal-like. The luminal cluster is defined by expression of a set of genes that includes $ESR1$, $GATA3$, $KRT8$, $KRT18$ and $MUC1$. Commonly used breast cancer cell lines derived from ductal carcinomas including MCF7, ZR75-1 and T47D fall into the luminal breast cancer cell line cluster (Charafe-Jauffret et al., 2006; Neve et al., 2006). Cell lines derived from Her-2+ breast tumors are split across the luminal and basal subgroups with the Her-2+/ER+ lines, SKBR3 and BT474, falling into the luminal group while the Her-2+/ER- line HCC1954 clusters with the basal group (Charafe-Jauffret et al., 2006; Neve et al., 2006).

The basal group of breast cancer cell lines includes HCC38, MCF10A and BT20. Basal-like breast cancer cell lines tend to show low expression of genes that define the luminal cluster and express high levels of the basal cyokeratins $KRT5$, $KRT6A$, $KRT6B$ along with other genes including LAMB3 and ANXA8 (Charafe-Jauffret et al., 2006; Neve et al., 2006). Charafe-jauffret et al. defined a mesenchymal-like subcluster of the basal group that included the MDA-MB-231 cell line and was distinguished by the highest expression of a set of genes including $VIM$, $MMP2$, $MMP14$ and $PLAU$ (Charafe-Jauffret et al., 2006). Neve et al. also found that the basal group of breast cancer cell lines divided into two
subclusters which they termed basal A and basal B (Neve et al., 2006). Like Charafe-Jauffret’s mesenchymal subcluster, expression of VIM was highest in the basal B subcluster that included the MDA-MB-231 cell line (Neve et al., 2006). Breast cancer cell lines in the basal B subcluster were significantly more invasive in vitro as measured by modified Boyden chamber assays than were cell lines of the basal A and luminal groups (Neve et al., 2006).

**Epithelial-to-Mesenchymal Transition in Breast Cancer Cell Lines**

The mesenchymal/basal B breast cancer cell line subgroup is identified by elevated expression of markers that are associated with a phenomenon known as epithelial-to-mesenchymal transition (EMT). EMT is an essential developmental process whereby cells of epithelial origin lose their epithelial characteristics and acquire a motile, fibroblastic-like phenotype to form the primary mesenchyme (Duband et al., 1995; Prindull and Zipori, 2004). EMT can be defined molecularly as reduced expression of the epithelial markers E-cadherin, KRT8, KRT 18 and KRT19 and increased expression of the mesenchymal markers vimentin (VIM), smooth muscle actin (SMA) and N-cadherin among others. Several transcription factors including SNAIL1, SNAIL2/SLUG and TWIST are associated with induction of EMT in vitro (Cano et al., 2000; Bolos et al., 2003; Comijn et al., 2001).

In breast cancer cell lines, spontaneous EMT has been observed in vitro and the transition has been induced by overexpression of EMT-associated transcription factors as well as constitutive activation of Ras and transforming
growth factor-β (Sarrio et al., 2008; Janda et al., 2002; De et al., 2005; Moreno-Bueno et al., 2006, Maschler et al., 2004). EMT is most commonly associated with breast cancer cell lines that display the basal-like phenotype but EMT has been observed in the luminal-like MCF7 cell line (Sarrio et al., 2008; Blick et al., 2008). While EMT has not been convincingly demonstrated in human cancers, the expression of some EMT markers has been associated with poor outcome in breast carcinomas (Peinado et al., 2007). Given that breast cancer cell lines that have undergone EMT demonstrate increased migratory behavior and increased invasive capacity in vitro and in animal models, elucidating the mechanisms of EMT and identifying factors that induce EMT in breast cancer cell lines may provide insight into the metastatic process (Jechlinger et al., 2003; Sarrio et al., 2008).

**Rationale and Purpose of Study**

Breast cancers are a heterogeneous group of tumors both in terms of their behavior and their therapeutic response. Molecular markers that predict breast cancer progression are valuable tools for determining the appropriate course of therapy and identifying novel therapeutic targets. Our previous work implicated podocalyxin in increased breast cancer aggressiveness. We next sought to determine the value of podocalyxin as a prognostic indicator of breast cancer progression. Utilizing tissue microarrays (TMAs) containing tumor samples from 248 patients with invasive breast carcinoma diagnosed at the Cleveland Clinic
we found that high podocalyxin protein expression correlated with higher Bloom Richardson Grade and expression of fascin, a protein associated with the more-aggressive, basal-like breast cancer phenotype. Furthermore, in a TMA of primary breast cancer tumor samples from 55 patients who developed brain metastases, 40 patients that developed a mix of visceral and skeletal metastases and 254 patients that remained free of metastases (mean follow up of 67 months), podocalyxin expression was associated with tumors that metastasized to the brain.

Aggressive, metastatic breast cancers are associated with unique gene expression signatures that are also reflected in breast cancer cell lines. We next sought to determine if expression of podocalyxin in relatively non-aggressive, luminal-like breast cancer cell lines induced a change in gene expression signature to that of a more aggressive, basal-like cancer. We found that expression of podocalyxin in luminal-like breast cancer cells lines was met with a concomitant decrease in expression of luminal-type markers including the estrogen and progesterone receptors and an increase in expression of basal-type markers including KRT5/6, caveolin-1 and fascin. Podocalyxin expression also inversely correlated with BRCA1 expression. These data support a critical role for podocalyxin in the development of basal-like breast cancer and metastasis of breast cancers to the brain.
MATERIALS AND METHODS

Cell lines and Culture Conditions

MCF7, T47D, and MDA-MB-231 breast cancer cells were routinely grown in phenol red-free DMEM (MCF7 and T47D) or DMEM (MDA-MB-231) supplemented with 5% FCS, 1% L-glutamine, 1% penicillin, and 1% streptomycin in 5% CO$_2$ at 37°C. Cells were passaged at sub-90% confluence using a solution of 0.05% trypsin, 0.53 mmol/L EDTA-4Na in HBSS without calcium or magnesium.

Transfections and siRNA Knockdown of PODXL Expression

Full length, sequence verified, human PODXL cDNA was cloned into pcDNA3.1 (Invitrogen, Carlsbad, CA) and stably transfected into MCF7 and T47D cells by electroporation using a Bio-Rad GenePulser followed by clonal selection in media containing 500 µg/mL G418 (Invitrogen, Carlsbad, CA). Control cells were transfected with empty pcDNA3.1 vector and selected in the same manner. MCF7/pDNA and MCF7/PODXL cells have been described previously (Sizemore et al., 2007). Transient transfections of MDA-MB-231 with siRNAs directed against podocalyxin (Dharmacon, Lafayette, CO) were performed using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s recommendations. Control siRNA-B (Santa Cruz, Santa Cruz, CA) was used as a control in all siRNA experiments.
**Construction of Tissue Microarrays**

Breast cancer tissue microarrays (TMA) from tumors of 248 breast cancer patients diagnosed at the Cleveland Clinic with a mean clinical follow-up time of 90 months were constructed as previously described (Hicks *et al.*, 2006; Yoder *et al.*, 2005). These TMAs consist of 8 x 12 arrays of 1.5-mm tissue cores from archived formalin-fixed, paraffin-embedded tumor blocks. Two separate tumor tissue cores represent each clinical case in the TMA. Each tissue core is assigned a unique TMA location number linked to an Institutional Review Board approved database containing information on clinicopathological features, treatment, and outcome data. A summary of the clinical characteristics of tumors from this patient population is shown in **Table 1**.

A second series of TMAs containing primary tumor tissue from 55 breast cancer patients who developed brain metastases, 40 patients who developed a mixture of visceral and bone metastatic disease without CNS metastases and 254 patients who remained free of metastases for an average follow-up of 67 months has also been described previously (Hicks *et al.*, 2006). These TMAs consist of 12 x 13 arrays of 0.6-mm tissue cores from archived formalin-fixed, paraffin-embedded tumor blocks. Two separate tumor tissue cores represent each clinical case in this TMA series. A summary of the clinical characteristics of tumors of each sub set of patients is shown in **Table 3**.
**Immunohistochemistry**

Staining for podocalyxin on both TMAs was performed using the automated Ventana Benchmark system and mouse anti-podocalyxin antibody (clone 3D3, Santa Cruz, Santa Cruz, CA; 1:1000 dilution) as previously described (Hicks *et al.*, 2006; Yoder *et al.*, 2005). Briefly, a 4µm-thick unstained section of each TMA was placed onto electrostatically charged glass slides and baked. The glass slides were pretreated for tissue deparaffinization and antigen retrieval. After primary antibody incubation, antigen detection was performed by peroxidase/3,3′-diaminobezidine following a secondary biotinylated antibody-streptavidin amplification step. A hematoxylin counterstain was then applied prior to immunohistochemistry scoring by two independent observers. Each separate tissue core was scored on a 0 to 3+ intensity scale (0 = no staining, 1 = weak staining, 2 = moderately intense staining, 3+ = strong staining) for podocalyxin. Cases scored as 0 or 1 were considered negative for podocalyxin while cases scored as 2 or 3+ were considered positive for podocalyxin. Biavariate analysis was done via the $\chi^2$ method. Disease-free survival data was calculated via Kaplan-Meier analysis. Deaths due to causes other than breast cancer were censored. Statistical significance was assumed if $P < 0.05$.

**Gene Expression Array Analyses**

Gene expression changes between MCF7/pcDNA and MCF7/PODXL transfectants were examined using the Illumina Human-6 V2 Expression BeadChip whole genome array. Briefly, total RNA was extracted from cell lines
with TRIzol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer’s instructions and quantified by 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Total RNA was biotinylated and amplified using Illumina TotalPrep™ RNA Amplification Kit (Ambion Inc, Austin, TX) as per manufacturer’s instructions. Briefly, total RNA was converted to cDNA by reverse transcription of total RNA using an oligo(dT) primer bearing a T7 promoter followed by second-strand synthesis. The cDNA product was then used as template for in vitro transcription with T7 RNA Polymerase in the presence of biotin UTP to create biotin labeled cRNA for use in microarray experiments. cRNA quality was checked by agarose gel electrophoresis prior to use for further gene expression studies. Samples were hybridized to Sentrix Human-6 V2 Expression BeadChip (Illumina, San Diego, CA) according to manufacturer’s protocols. Hybridized chip images were then obtained using Illumina’s BeadArray Reader. RNA from three MCF7/pcDNA transfectants and three MCF7/PODXL transfectants were used in these studies. Gene expression data was analyzed by BeadScan 3.0 software (Illumina, San Diego, CA) with normalization by the Rank invariant method. A differential expression score exceeding ±13 is equivalent to $P < 0.05$ and was considered to be statistically significant.

**Real-time PCR Quantitation**

Total RNA was extracted and used to generate cDNA as described in the Materials and Methods section of *Chapter One*. Quantitative Real-time PCR
experiments were performed in triplicate using commercially available, validated TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) according to the vendor’s instructions utilizing an ABI 7900HT (Applied Biosystems, Foster City, CA) real-time PCR instrument. A summary of the TaqMan Gene Expression Assays utilized in this study can be found in Table 5. Transcript expression levels were normalized using 18S ribosomal RNA levels as an endogenous control. Relative quantitation (RQ) of gene transcripts was accomplished using the Comparative \( C_T \) method. Data are presented as RQ ± SD. Student’s t-test was used for statistical comparisons between ΔCt values. \( P < 0.05 \) was considered to be statistically significant.

**Western Blotting and Immunodetection.**

Protein extraction and Western blot protocols are described in the Materials and Methods section of Chapter One. The following primary antibodies were used: mouse anti-podocalyxin (clone 3D3; Santa Cruz, Santa Cruz, CA), rabbit anti-caveolin-1 (Santa Cruz, Santa Cruz, CA), mouse anti-cytokeratin 5/6 (Clone D5/16 B4; Dako, Glostrup, Denmark), mouse anti-fascin (Chemicon, Temecula, CA), mouse anti-ERα (Santa Cruz, Santa Cruz, CA), mouse anti-GATA3 (Santa Cruz, Santa Cruz, CA), mouse anti-cytokeratin 8/18 (clone C51, Cell Signaling, Boston, MA), and mouse anti-actin (Rockland, Gilbertsville, PA). Blots were then probed with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (Rockland, Gilbertsville, PA). Signals were
visualized using the ECL or ECL Plus detection systems (Amersham Biosciences, Piscataway, NJ) depending on the desired signal strength.

**EXPERIMENTAL PROTOCOLS**

**Protocol 2A**

_Hypothesis:_ Podocalyxin expression correlates with poor prognosis in breast cancer. Breast cancer tissue microarrays (TMA) containing tumor samples from 248 breast cancer patients diagnosed at the Cleveland Clinic with a mean clinical follow-up time of 90 months were stained for podocalyxin using the automated Ventana Benchmark system and mouse anti-podocalyxin antibody (clone 3D3, Santa Cruz; 1:1000 dilution). Each separate tissue core was scored on a 0 to 3+ intensity scale (0 = no staining, 1 = weak staining, 2 = moderately intense staining, 3+ = strong staining) for podocalyxin by two independent observers. Cases scored as 0 or 1 were considered negative for podocalyxin while cases scored as 2 or 3+ were considered positive for podocalyxin. Statistical associations between Podocalyxin staining and clinical and pathological features were assessed.

**Protocol 2B**

_Hypothesis:_ Podocalyxin expression is predictive of breast cancer metastasis. A series of TMAs containing primary tumor tissue from 55 breast cancer patients who developed brain metastases, 40 patients who developed a mixture of visceral and bone metastatic disease without CNS metastases and
254 patients who remained free of metastases for an average follow-up of 67 months were stained for podocalyxin using the automated Ventana Benchmark system and mouse anti-podocalyxin antibody (clone 3D3, Santa Cruz; 1:1000 dilution). Each separate tissue core was scored on a 0 to 3+ intensity scale (0 = no staining, 1 = weak staining, 2 = moderately intense staining, 3+ = strong staining) for podocalyxin by two independent observers. Cases scored as 0 or 1 were considered negative for podocalyxin while cases scored as 2 or 3+ were considered positive for podocalyxin. Statistical associations between Podocalyxin staining and metastasis class were assessed.

**Protocol 2C**

*Hypothesis: Expression of podocalyxin in breast cancer cell lines correlates with the basal-like gene expression signature.* To determine if podocalyxin expression could induce a basal-like gene expression signature in luminal-like breast cancer cell lines, we examined differences in the expression of genes reported to be associated with the luminal or basal-like breast cancer phenotypes between MCF7/pcDNA and MCF7/PODXL cells using the Illumina Human-6 V2 whole genome array platform. Changes in gene expression between MCF7/pcDNA and MCF7/PODXL cells were confirmed by Real-time quantitative PCR and Western blot analyses. Our results were further verified using a second luminal-like breast cancer cell line stably expressing podocalyxin and by knockdown of endogenous podocalyxin in a basal-like breast cancer cell line.
Protocol 2D

**Hypothesis:** Podocalyxin-associated changes in gene expression in breast cancer cell lines are not indicative of an epithelial-to-mesenchymal transition. The Epithelial-to-mesenchymal transition (EMT) phenomenon is associated with global changes in gene expression and increased invasion in breast cancer cell lines. To determine if the changes in gene expression observed in correlation with podocalyxin expression were the result of EMT we examined differences in the expression of genes reported to be associated with EMT between MCF7/pcDNA and MCF7/PODXL cells. Western blot analysis was used to confirm our results.

**Statistical Analysis**

Associations between Podocalyxin staining and clinical and pathological features and metastasis class were assessed by the $\chi^2$ method. Disease-free survival data was calculated via Kaplan-Meier analysis. Three MCF7/pcDNA and three MCF7/PODXL clones were used in gene expression analyses. Gene expression data was normalization by the Rank invariant method. Differential expression of genes was determined using Illumina's custom differential expression score (a combined t-test/fold-change algorithm). Differential expression scores $> |13|$ are equivalent to $P < 0.05$ while those $> |20|$ are equivalent to $P < 0.01$. Statistical analysis of Real-time PCR data was
accomplished using Student's t-test to compare ΔCt values. $P < 0.05$ was considered to be statistically significant.

RESULTS

*Podocalyxin expression correlated with high Bloom Richardson Grade and fascin expression.*

The study population represented on the tissue microarray is summarized in Table 1 and has been described previously (Yoder et al., 2005). Of the 248 breast cancers analyzed, 13% were Bloom Richardson Grade (BRG) I, 42% were Grade II, 28% were Grade III and 17% were reported as unknown including breast cancers of the infiltrating lobular and infiltrating ductal type. Of the breast cancers, 79% were ER+, 62% were PR+, and 13% showed overexpression of HER2.

Podocalyxin immunoreactivity was seen along the apical surface of the ductal lumen and in the vascular endothelium in normal breast control samples (Figures 15A and 15B). Of the 248 breast cancers, 189 (76%) were negative for podocalyxin (scored as 0 or 1 on a 3+ scale) (Figure 15C) while 59 (24%) were positive (scored as 2 or 3+) (Figure 15D).

The relationship between podocalyxin expression and clinical and pathologic factors is summarized in Table 2. A statistically significant correlation was found between increased podocalyxin protein expression and high Bloom Richardson Grade ($P = 0.012$; Figure 16). There was a significant positive
correlation between podocalyxin and fascin staining ($P = 0.002$; Figure 17). Fascin is an actin binding protein that is associated with aggressive breast cancers and the basal-like breast cancer phenotype (Rodriguez-Pinilla et al., 2006; Yoder et al., 2005). Podocalyxin expression also showed a trend towards an inverse association with ER expression but this relationship did not reach statistical significance ($P = 0.09$). No association was seen between podocalyxin expression and age at diagnosis ($P = 1.00$), tumor size ($P = 0.14$), nodal status ($P = 0.7$), PR expression ($P = 0.7$), or HER2 expression ($P = 0.5$). While Kaplan-Meier analysis revealed only a non-significant trend between podocalyxin expression and reduced disease-free survival ($P = 0.0872$, data not shown), the mean survival time for the podocalyxin positive staining group (71.49 ±4.64, 95% CI = 62.38 - 80.59 months) was significantly shorter than for the podocalyxin negative group (90.77 ± 2.58 months, 95% CI = 85.71 - 95.83 months).
**Table 1.** Clinical and pathologic characteristics for the study population (248 patients).

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Number</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age at Diagnosis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 50</td>
<td>62</td>
<td>25%</td>
</tr>
<tr>
<td>&gt; 50</td>
<td>165</td>
<td>67%</td>
</tr>
<tr>
<td>Unknown</td>
<td>21</td>
<td>8%</td>
</tr>
<tr>
<td><strong>Bloom Richardson Grade</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>33</td>
<td>13%</td>
</tr>
<tr>
<td>II</td>
<td>103</td>
<td>42%</td>
</tr>
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<td>III</td>
<td>69</td>
<td>28%</td>
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<td>6%</td>
</tr>
<tr>
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<td>4%</td>
</tr>
<tr>
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<td>11%</td>
</tr>
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<td></td>
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<tr>
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<td>119</td>
<td>48%</td>
</tr>
<tr>
<td>N1</td>
<td>56</td>
<td>23%</td>
</tr>
<tr>
<td>N2</td>
<td>22</td>
<td>9%</td>
</tr>
<tr>
<td>N3</td>
<td>11</td>
<td>4%</td>
</tr>
<tr>
<td>LN dissection not performed</td>
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<td>9%</td>
</tr>
<tr>
<td>Unknown</td>
<td>17</td>
<td>7%</td>
</tr>
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</tr>
<tr>
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<td>196</td>
<td>79%</td>
</tr>
<tr>
<td>Negative</td>
<td>52</td>
<td>21%</td>
</tr>
<tr>
<td><strong>Progesterone Receptor</strong></td>
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<td></td>
</tr>
<tr>
<td>Positive</td>
<td>153</td>
<td>62%</td>
</tr>
<tr>
<td>Negative</td>
<td>95</td>
<td>38%</td>
</tr>
<tr>
<td><strong>HER2 status (IHC)</strong></td>
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<tr>
<td>Positive</td>
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</tr>
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<td>207</td>
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</tr>
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<td>3%</td>
</tr>
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<td><strong>Podocalyx</strong></td>
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<tr>
<td>Negative</td>
<td>189</td>
<td>76%</td>
</tr>
<tr>
<td>Positive</td>
<td>59</td>
<td>24%</td>
</tr>
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</table>

* Infiltrating lobular and rare infiltrating ductal variants were not assigned a Bloom-Richardson grade.
Figure 15. Immunohistochemical analysis of podocalyxin protein expression in breast tumor specimens. A and B, normal breast tissue with podocalyxin staining restricted to the ductal epithelium and vascular endothelium (x400). C, an invasive breast carcinoma tissue section showing weak (1+) staining for podocalyxin (x400). D, an invasive breast carcinoma tissue section showing strong (3+) staining for podocalyxin (x400).
### Table 2. Clinical and Pathologic characteristics of the study population as a function of Podocalyxin expression.

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Podocalyxin (+) (n = 59)</th>
<th>Podocalyxin (-) (n = 189)</th>
<th>P</th>
</tr>
</thead>
<tbody>
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<td><strong>Age at Diagnosis</strong></td>
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<td></td>
</tr>
<tr>
<td>&lt;50</td>
<td>15</td>
<td>47</td>
<td>25%</td>
</tr>
<tr>
<td>&gt;50</td>
<td>40</td>
<td>125</td>
<td>66%</td>
</tr>
<tr>
<td>Unknown</td>
<td>4</td>
<td>17</td>
<td>9%</td>
</tr>
<tr>
<td><strong>Bloom Richardson Grade</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>3</td>
<td>30</td>
<td>16%</td>
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<tr>
<td>II</td>
<td>25</td>
<td>78</td>
<td>41%</td>
</tr>
<tr>
<td>III</td>
<td>25</td>
<td>44</td>
<td>23%</td>
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<tr>
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<td>6</td>
<td>37</td>
<td>20%</td>
</tr>
<tr>
<td><strong>Tumor size</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>30</td>
<td>92</td>
<td>49%</td>
</tr>
<tr>
<td>T2</td>
<td>18</td>
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<td>T3</td>
<td>1</td>
<td>15</td>
<td>8%</td>
</tr>
<tr>
<td>T4</td>
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<td>6</td>
<td>3%</td>
</tr>
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<td>12%</td>
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<td>90</td>
<td>48%</td>
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<td>N1</td>
<td>11</td>
<td>45</td>
<td>24%</td>
</tr>
<tr>
<td>N2</td>
<td>7</td>
<td>15</td>
<td>8%</td>
</tr>
<tr>
<td>N3</td>
<td>2</td>
<td>9</td>
<td>5%</td>
</tr>
<tr>
<td>LN dissection not done</td>
<td>4</td>
<td>19</td>
<td>10%</td>
</tr>
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<td>11</td>
<td>6%</td>
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<tr>
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<td>Positive</td>
<td>42</td>
<td>154</td>
<td>81%</td>
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<td>19%</td>
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<tr>
<td><strong>Progesterone Receptor</strong></td>
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<tr>
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<td>118</td>
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<td>12</td>
<td>6%</td>
</tr>
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<td>89%</td>
</tr>
<tr>
<td>Unknown</td>
<td>1</td>
<td>8</td>
<td>4%</td>
</tr>
</tbody>
</table>

**Note:** The table shows the distribution of clinical and pathologic characteristics for the study population, split by Podocalyxin expression status. The P values are given for statistical significance differences between Podocalyxin (+) and (-) groups.
Figure 16. Podocalyxin Staining Correlates with Higher Bloom-Richardson Grade. A statistically significant correlation was observed between positive podocalyxin staining and higher Bloom-Richardson Grade ($P = 0.012$).
Figure 17. Podocalyxin Staining Correlates with Fascin Positivity. A statistically significant correlation was observed between podocalyxin and fascin staining ($P = 0.002$).
**Podocalyxin expression correlated with increased incidence of breast cancer metastasis to the brain.**

A summary of the clinical and pathologic characteristics for the metastasis study populations is shown in Table 3 as described previously (Hicks et al., 2006). Breast cancer patients with brain metastases (BM) had received radiation therapy for CNS metastasis at the Cleveland Clinic (Hicks et al., 2006). The remaining cases were selected from the study population described in Table 1 and previously described (Yoder et al., 2005). Of these 294 cases, 254 patients remained free of any distant metastases for an average follow-up of 67 months (No metastases subgroup), while 40 patients developed a mixture of visceral and skeletal metastases but remained free of brain metastases during the same follow-up period (V/B metastases subgroup).

Patients with primary breast cancers that showed strong immunoreactivity for podocalyxin (scored as 2 or 3+) were more likely to have metastasis to the brain ($p = 0.001$; Figure 18). After excluding cases scored as unknown due to lack of sufficient tumor tissue for evaluation, 45% of the brain metastases (BM) group were scored negative (0 or 1 on a 3+ scale) for podocalyxin while 54% were scored positive (2 or 3+) for podocalyxin. In contrast, after excluding unknowns, only 20% of the tumors in the V/B and 22% of the No metastases subgroups were positive for podocalyxin immunoreactivity.
### Table 3. Summary of clinical and pathologic factors for each metastasis subset.

<table>
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<tr>
<th>Clinical Characteristics</th>
<th>Brain Mets (n = 55)</th>
<th>Visceral and/or Bone Mets (n = 40)</th>
<th>No Mets (n = 254)</th>
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<tbody>
<tr>
<td></td>
<td>Patient number</td>
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<td>Patient number</td>
</tr>
<tr>
<td>Age at diagnosis</td>
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<td></td>
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</tr>
<tr>
<td>&lt; 50</td>
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<td>55%</td>
<td>13</td>
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<td>45%</td>
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</tr>
<tr>
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<td>0%</td>
<td>2</td>
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<tr>
<td>Bloom Richardson Grade</td>
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</tr>
<tr>
<td>I</td>
<td>0</td>
<td>0%</td>
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</tr>
<tr>
<td>II</td>
<td>9</td>
<td>16%</td>
<td>14</td>
</tr>
<tr>
<td>III</td>
<td>14</td>
<td>26%</td>
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<td>58%</td>
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<tr>
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</tr>
<tr>
<td>T1</td>
<td>6</td>
<td>11%</td>
<td>13</td>
</tr>
<tr>
<td>T2</td>
<td>9</td>
<td>16%</td>
<td>15</td>
</tr>
<tr>
<td>T3</td>
<td>4</td>
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</tr>
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<td>73%</td>
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Figure 18. Podocalyxin Staining Predicts Brain Metastasis. Patients with brain metastasis (BM) were more likely ($P = 0.001$) to have primary breast cancers that stained strongly for podocalyxin than were patients with visceral/bone metastasis (V/B) or patients with no metastasis (No). There was no statistically significant difference in the frequency of tumors with strong podocalyxin staining between the V/B and No mets groups ($P = 1.00$).
Podocalyxin expression in MCF7 breast cancer cells leads to a change in gene expression pattern from a luminal to basal-like phenotype.

To directly examine the relationship between podocalyxin expression and the basal-like phenotype we examined the effect of stably over-expressing podocalyxin in MCF7 luminal breast cancer cells. A gene expression array analysis was performed using the Illumina Human-6 V2 whole genome array. RNA from three MCF7/pcDNA transfectants and three independent MCF7/PODXL transfectants were used in these studies. Signals from the transfectants were pooled and averaged to determine average fold-changes in gene expression as well as differential expression scores using Illumina’s Beadscan software (Table 4).

Changes in expression of genes reported associated with the luminal or basal-like breast cancer phenotypes were examined using recent literature (Sorlie et al., 2001; Sorlie et al., 2003; Charafe-Jauffret et al., 2005; Charafe-Jauffret et al., 2006; Neve et al., 2006). Emphasis was placed on luminal and basal subgroup markers known to be relevant in both breast cancer cell lines and breast cancers and markers for which suitable antibodies were available. The luminal and basal-like associated genes used in this study are summarized in Table 4.

Of the 12 basal-like associated genes examined (KRT5, KRT6A, KRT6B, KRT14, KRT17, LAMB3, FSCN1, CRYAB, CAV1, TRIM29, ANXA8, and SLPI), 8 were significantly upregulated in MCF7/PODXL cells compared to control
MCF7/pcDNA cells. The basal cytokeratins, \textit{KRT17} and \textit{KRT6B} were increased in MCF7/\textit{PODXL} cells compared to parental cells (+23.29 and +12.42-fold respectively). Two other basal cytokeratins, \textit{KRT5} and \textit{KRT6A}, also showed a significant increase in expression in MCF7/\textit{PODXL} cells (+3.89 and +.56 fold). Other basal-like markers that were upregulated in our MCF7/\textit{PODXL} group included \textit{LAMB3} (+2.2 fold), \textit{FSCN1} (+1.95 fold), \textit{CRYAB} (+2.47 fold) and \textit{CAV1} (+1.7 fold). Three of the basal-like markers showed no significant change in expression between the MCF7/\textit{PODXL} and control cells. These include the basal cytokeratin \textit{KRT14} (-1.15 fold), \textit{TRIM29} (-1.07 fold) and \textit{ANXA8} (-1.20 fold). Expression of one of the twelve basal-like markers, \textit{SLPI}, was significantly decreased in MCF7/\textit{PODXL} cells compared to the MCF7/pcDNA control group (-1.92 fold).

Of the 14 luminal-like subgroup markers examined (\textit{ESR1}, \textit{PGR1}, \textit{FOXA1}, \textit{ZNF42}, \textit{GATA3}, \textit{XBP1}, \textit{KRT8}, \textit{KRT18}, \textit{KRT19}, \textit{CLDN7}, \textit{CRABP2}, \textit{DDR1}, \textit{CLDN4}, and \textit{MUC1}), 12 were significantly downregulated in the MCF7/\textit{PODXL} group compared to the control MCF7/pcDNA group. The significantly down-regulated genes include the estrogen receptor alpha gene, \textit{ESR1} (-3.31 fold) as well as the gene encoding the progesterone receptor, \textit{PGR1} (-1.98 fold). The genes encoding several DNA binding proteins associated with ERα function including \textit{FOXA1}, \textit{ZNF42}, \textit{GATA3}, and \textit{XBP1} were also significantly downregulated (-2.17, -2.11, -1.91, and -1.71 fold respectively) in MCF7/\textit{PODXL} cells compared to the pcDNA controls. Other luminal-like subgroup associated
genes that were also statistically significantly downregulated in MCF7/PODXL cells were: $KRT18$ (-2.11 fold), $CLDN7$ (-1.98 fold), $CRABP2$ (-1.55 fold), $DDR1$ (-1.52 fold), $CLDN4$ (-1.59 fold) and $MUC1$ (-1.12 fold). Of the luminal cytokeratins only $KRT18$ was significantly downregulated in MCF7/PODXL cells. Two other luminal cytokeratins, KRT19 and KRT8, showed no significant change in expression between MCF7/PODXL cells and control MCF7/pcDNA cells (-1.08 and +1.07 fold respectively). Two housekeeping genes, $GAPDH$ and $ACTB$, showed no significant change in expression between MCF7/PODXL transfectants and the MCF7/pcDNA controls (-1.05 and -1.04 fold) while expression of $PODXL$ was increased 8.96-fold in MCF7/PODXL cells.
Table 4. Comparison of differential expression of basal and luminal breast cancer subgroup associated genes in MCF7/pcDNA and MCF7/PODXL cells

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### Housekeeping Genes and PODXL

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Real-time quantitative PCR analyses confirms luminal to basal-like changes associated with PODXL expression.

To confirm gene expression data, we performed real-time quantitative PCR (RTQ-PCR) in two independent MCF7/PODXL transfectants (MCF7/PODXL1 and MCF7/PODXL3) and MCF7/pcDNA cells using a panel of genes that have been identified as markers of luminal and basal-like breast cancers (Charafe-Jauffret et al., 2006; Neve et al., 2006). The TaqMan Gene expression assays used in this study are listed in Table 5. The results for this experiment are summarized in Figure 19. Values listed in this RESULTS section are an average of the values from the two independent PODXL transfectants relative to MCF7/pcDNA. The data show a strong concordance between RTQ-PCR and gene array data.

Of the 12 basal-like genes analyzed in our gene expression array, nine were significantly increased in both MCF7/PODXL transfectants compared to MCF7/pcDNA control cells. These included CAV1 (+4.17 fold), CRYAB (+49.41 fold), FSCN1 (+5.36 fold), KRT17 (+234.12 fold), KRT5 (+615.55 fold), KRT6A (+99.26 fold), KRT6B (+214.77 fold), LAMB3 (+7.66 fold) as well as ANXA8 (+3.81 fold) that was not increased in our gene expression array analyses. SLPI, which we found to be significantly downregulated in the MCF7/PODXL cells in our gene expression array analysis, was unchanged (-1.11 fold) by RTQ-PCR analyses. We could not detect any expression of the remaining basal-like marker KRT14, in MCF7/PODXL or MCF7/pcDNA cells.
Of the 14 luminal–associated transcripts, 11 were significantly downregulated by RTQ-PCR in MCF7/PODXL transfectants compared to the MCF7/pcDNA cells (Figure 19), including CLDN4 (-1.92 fold), CLDN7 (-2.18 fold), ESR1 (-2.45 fold), FOXA1 (-1.78 fold), GATA3 (-2.67 fold), KRT18 (-2.05 fold), MUC1 (-4.55 fold), PGR1 (-2.57 fold), XBP1 (-1.73 fold), ZNF42 (-2.83 fold) and DDR1 (-1.34 fold). CRABP2 expression was unchanged (1.00 fold), in agreement with our gene expression array results. KRT19 (-1.06 fold) and KRT8 (-1.10 fold) also showed no significant changes in expression between the MCF7/PODXL transfectants and the MCF7/pcDNA cells.

To provide independent validation of these findings we examined PODXL-dependent gene expression changes in T47D, another luminal cell line. We compared transcript levels in two independent T47D/PODXL transfectants (T47D/PODXL1 and T47D/PODXL6) and T47D/pcDNA control cells (Figure 20). Values listed in this RESULTS section are averages of the values obtained from two independent T47D/PODXL transfectants relative to the T47D/pcDNA control. As we observed in MCF7 cells, there was a shift in gene expression pattern in T47D cells consistent with a change from a luminal to a basal-like breast cancer cell. There was a high concordance between gene expression changes seen between MCF/PODXL and T47D/PODXL transfectants.

Of the 12 basal-like breast cancer genes, 8 were upregulated in the T47D/PODXL transfectants compared to the MCF7/pcDNA cells, including ANXA8 (+27.98 fold), CAV1 (+2.25 fold), CRYAB (+4.23 fold), FSCN1 (+3.37
fold), *KRT17* (+9.42 fold), *KRT5* (+54.79 fold), *KRT6A* (+18.32 fold), and *LAMB3* (+8.37 fold). Among the remaining genes examined, *TRIM29* (-1.10 fold) and *SLPI* (-1.03 fold) transcript levels remained unchanged in T47D cells, while *KRT14* and *KRT6A* were not expressed in T47D cells (Figure 20).

Of the 14 luminal-associated transcripts, 10 were significantly downregulated in T47D/*PODXL* transfectants, including *CLDN4* (-2.32 fold), *CLDN7* (-2.27 fold), *ESR1* (-2.87 fold), *FOXA1* (-1.56 fold), *GATA3* (-1.63 fold), *MUC1* (-5.08 fold), *PGR1* (-2.91 fold), *XBP1* (-4.05 fold), *ZNF42* (-3.94 fold), and *DDR1* (-1.69 fold). *CRABP2* transcripts were unchanged (-1.01 fold). Unexpectedly, *KRT18* (+1.38 fold) and *KRT8* (+1.49) expression was increased in T47D/*PODXL* cells. Expression of *KRT19* was inconsistent between the two T47D/*PODXL* transfectants (Figure 20).
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</table>
Figure 19. RTQ-PCR Analysis Confirms that Podocalyxin Expression Leads to Decreased Expression of Luminal-Subtype Associated Genes and Increased Expression of Basal-Subtype Associated Genes in MCF7 Cells. Histogram showing relative changes of luminal and basal-associated gene expression in MCF7/pDNA (used as baseline), MCF7/PODXL1 (dark gray bar) and MCF7/PODXL3 (light gray bar) cells. The data were normalized with 18S ribosomal RNA. *P < 0.05; (t-test, n = 3).
Figure 20. RTQ-PCR Analysis Confirms Podocalyxin-Associated Changes in Gene Expression in a Second Luminal Breast Cancer Cell Line. Histogram showing the relative changes in expression of luminal and basal-subtype associated genes in T47D/pcDNA (used as baseline), T47D/PODXL1 (dark gray bar) and T47D/PODXL6 (light gray bar) cells. The data were normalized with 18S ribosomal RNA. *P < 0.05; (t-test, n = 3).
Western blot analysis confirmed changes in gene expression associated with PODXL.

To confirm that changes in gene expression of basal-like and luminal-associated genes resulted in changes in protein levels we performed Western blot analysis for a subset of the basal and luminal markers (Figure 21). We were able to confirm that MCF7/PODXL and T47D/PODXL transfectants showed increased expression of the caveolin-1 (encoded by CAV1), KRT5/6, and fascin (encoded by FSCN1) proteins. Expression of the luminal proteins ERα and GATA3 were decreased in both MCF7/PODXL and T47D/PODXL transfectants compared to MCF7/pcDNA and T47D/pcDNA cells, respectively. Expression of the luminal cytokeratin KRT18 was also decreased at the protein level in MCF7/PODXL transfectants. However, the expression of KRT18 protein was increased in both T47D/PODXL clones compared to the pcDNA control.

Transient knockdown of PODXL in MDA-MB-231 cells by siRNA decreases expression of basal-like markers FSCN1 and CAV1.

We looked to confirm these findings in a breast cancer cell line with high endogenous expression of podocalyxin. Hierarchical clustering of breast cancer cell lines in two recent studies showed that MDA-MB-231 cells separated into the mesenchymal-like (Charafe-Jauffret et al., 2006) or basal-B (Neve et al., 2006) sub-clusters of basal-like breast cancer that show similarities in gene expression profiles. MDA-MB-231 cells express high levels of endogenous podocalyxin as well as the basal-like markers FSCN1 and CAV1. To confirm the relationship
between podocalyxin expression and the basal-like phenotype we examined changes in \textit{FSCN1} and \textit{CAV1} expression following the knock down of podocalyxin expression in MDA-MB-231 cells, and determined that \textit{FSCN1} and \textit{CAV1} showed decreased expression in these cells. Of the four siRNAs targeted to \textit{PODXL}, three (\textit{PODXL}si1, \textit{PODXL}si2, and \textit{PODXL}si4) significantly reduced \textit{PODXL} mRNA expression to 35\%, 39\%, and 30\%, respectively, of the control after 48 hours (\textbf{Figure 21A}). \textit{PODXL}si3 did not significantly change expression of podocalyxin at the RNA or protein level and was used as an additional control (\textbf{Figure 21A and 21B}). The three siRNAs that significantly knocked down \textit{PODXL} expression also led to significantly reduced expression of \textit{CAV1} by 39\%, 34\%, and 39\% and \textit{FSCN1} expression by 35\%, 34\%, and 38\% respectively as measured by RTQ-PCR (\textbf{Figure 22A}). Western blot analysis confirmed that knockdown of podocalyxin resulted in decreased expression of caveolin-1 and fascin proteins in MDA-MB-231 breast cancer cells (\textbf{Figure 22B}).
**Figure 21.** Western Blot Analysis Confirms Decreased Expression of Luminal Markers and Increased Expression of Basal Markers in MCF7/PODXL and T47D/PODXL Transfectants Compared to Empty Vector Parental Controls. Western blot analysis shows a correlation between PODXL expression and increased expression of basal-like markers caveolin-1, KRT5/6, and fascin along with decreased expression of luminal markers ERα and GATA3 in MCF7 clones (left panels) and T47D clones (right panels). KRT18 expression was decreased in the MCF7/PODXL clones (MCF7/PODXL1 and MCF7/PODXL3) compared to MCF7/pcDNA but increased in the T47D/PODXL clones (T47D/PODXL1 and T47D/PODXL6) compared to their pcDNA control.
Figure 22. Knockdown of PODXL Expression in MDA-MB-231 Results in Decreased Expression of the Basal Markers Caveolin-1 and Fascin. A, histogram showing RTQ-PCR results for expression of PODXL, CAV1, and FSCN1 in MDA-MB-231 cells treated with control siRNA (used as baseline), PODXL si1 (black bars), PODXL si2 (white bars), PODXL si3 (solid gray bars), or PODXL si4 (hatch-marked bars). Data were normalized with 18S ribosomal RNA. *P < 0.05 (t-test, n = 3). B, Western blot analysis confirms decreased expression of fascin and caveolin-1 in response to PODXL knockdown.
Expression of podocalyxin correlates with reduced BRCA1 expression.

Recent studies have implicated BRCA1 in the basal-like breast cancer phenotype. The majority of BRCA1-deficient breast tumors have a basal-like phenotype, low ER and PR expression and express basal cytokeratins (Rakha et al., 2008c; Turner et al., 2004, Turner and Reis-Filho 2006). In addition, loss of BRCA1 expression is frequently associated with the basal-like phenotype in sporadic breast cancer (Turner and Reis-Filho 2006; Turner et al., 2007; Rakha et al., 2008b). To examine the relationship between podocalyxin and BRCA1 expression we examined expression and found that BRCA1 expression was significantly decreased (-1.51 fold) in MCF7/PODXL compared to MCF7/pcDNA cells (Table 6). We confirmed these findings using RTQ-PCR in both MCF7 and T47D cells (Figures 19 and 20). BRCA1 expression was decreased 1.64 fold in MCF7/PODXL cells compared to MCF7/pcDNA cells (Figure 19), and was decreased 1.8 fold in T47D/PODXL cells compared to T47D/pcDNA cells (Figure 20).

Expression of podocalyxin does not result in an epithelial to mesenchymal transition (EMT) in MCF7 cells.

Epithelial to mesenchymal transition (EMT), the loss of epithelial markers and gain of mesenchymal markers, is associated with decreased cell adherence and increased breast cancer aggressiveness (Guarino et al., 2007; Kokkinos et al., 2007). Given the role of podocalyxin in tumor aggressiveness as well as podocalyxin’s role as an anti-adhesive molecule, we hypothesized that the
changes in gene expression induced by podocalyxin expression might, at least partially, be explained by podocalyxin expression leading to EMT in breast cancer cells. In addition to decreased expression of epithelial markers, EMT is typically characterized by increased expression of mesenchymal markers including vimentin (VIM) and smooth muscle actin (SMA, ACTA2), increased expression of N-cadherin, and transcription factors SNAIL1, SLUG (SNAIL2) and TWIST (Comijn et al., 2001; Thiery, 2002; Thiery and Sleeman 2006; Yang et al., 2004; Thompson et al., 2005; Gupta and Massague 2006).

To assess the ability of podocalyxin to trigger EMT in MCF7 cells we compared the effect of podocalyxin expression on these targets (VIM, ACTA2, N-cadherin, E-cadherin, SNAIL1, SLUG (SNAIL2), and TWIST) (Table 6, Figure 23). Although expression of the EMT associated N-cadherin (CDH2) was significantly increased in the MCF7/PODXL cells (+2.06 fold), there was little evidence based on gene expression data that podocalyxin was involved in epithelial-mesenchymal transition. This is in agreement with the observation that MCF7 and T47D cells expressing PODXL do not show the elongated fibroblastic morphology associated with cells that have undergone EMT (unpublished observation). Furthermore, expression of the mesenchymal marker, vimentin, was unchanged at the protein level in response to siRNA-mediated knockdown of PODXL (data not shown).
Table 6. Comparison of differential expression of EMT markers and BRCA1 in MCF7/pcDNA and MCF7/PODXL cells

<table>
<thead>
<tr>
<th>PROBE_ID</th>
<th>Symbol</th>
<th>MCF7/pcDNA pool Ave. Signal</th>
<th>MCF7/PODXL pool Ave. Signal</th>
<th>Fold change</th>
<th>Differential Expression Score</th>
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<tr>
<td>ILMN_1671703</td>
<td>ACTA2</td>
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<td>229.4322</td>
<td>-1.419349594</td>
<td>-45.4813125</td>
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<tr>
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<td>CDH1</td>
<td>1337.807</td>
<td>1460.12945</td>
<td>1.09143505</td>
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<tr>
<td>ILMN_1672611</td>
<td>CDH11</td>
<td>45.88302</td>
<td>39.25398</td>
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<td>-3.8900035</td>
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<tr>
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<td>723.31145</td>
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<tr>
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<tr>
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<tr>
<td>ILMN_1672908</td>
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<tr>
<td>ILMN_1782538</td>
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<td>100.76571</td>
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<td>-44.591987</td>
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<tr>
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<td>92.514765</td>
<td>-1.510588067</td>
<td>-27.248495</td>
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Figure 23. Western Blot Analysis Shows a Correlation Between PODXL Expression and Increased Expression of N-cadherin. N-cadherin protein was increased in the MCF7/PODXL clones (MCF7/PODXL1 and MCF7/PODXL3) compared to MCF7/pcDNA as well as in the T47D/PODXL clones (T47D/PODXL1 and T47D/PODXL6) compared to their pcDNA control. E-cadherin expression was unchanged by PODXL expression either cell line.
DISCUSSION

Podocalyxin has been implicated in the development of aggressive forms of cancer, and we recently showed that forced stable podocalyxin expression in both MCF7 breast cancer and PC3 prostate cancer cells led to increased invasion and migration, and activation of MAPK and AKT/PI3K signaling in an ezrin-dependent manner (Casey et al., 2006; Sizemore et al., 2007). Here we have extended these studies in breast cancer and report that podocalyxin expression is associated with a basal-like breast cancer phenotype both in vitro and in vivo.

Clinically basal-like breast cancers are associated with high grade, and increased metastasis, including metastases to the brain (Nielsen et al., 2004; Hicks et al., 2006; Gaedcke et al., 2007; Rakha et al., 2008c; Rakha et al., 2007; Rakha et al., 2008a). In this study, we found that podocalyxin staining in primary breast tumors was statistically significantly associated with increased Bloom Richardson grade ($P = 0.012$) and with reduced mean survival time in podocalyxin positive cases (71.49 ±4.64, 95% CI = 62.38 - 80.59 months) compared to podocalyxin negative cases (90.77 ± 2.58 months, 95% CI = 85.71 - 95.83 months). In addition, we found that podocalyxin expression was statistically significantly associated with breast cancer metastasizing to the brain ($P = 0.001$).

The central nervous system is a common site of metastasis for breast cancer, and brain metastases have been estimated to be involved in between 10-16% of breast cancers (Lin et al., 2004). Furthermore, recent data suggest that
involvement of CNS metastases may be increasing in breast cancer with the improvements in survival due to the use of new and more effective chemotherapies (Lin et al., 2004; Weil et al., 2005; Souglakos et al., 2006; Stemmler and Heinemann, 2008). Breast cancers that metastasize to the brain are more likely to be estrogen and progesterone receptor negative and be either HER2 positive or display characteristics of the basal-subtype (Hicks et al., 2006; Gaedcke et al., 2007).

There are many similarities between the pathological features of basal-like and triple-negative breast cancers, including loss of estrogen and progesterone receptor expression. While we only observed a trend towards and association between podocalyxin staining in primary tumors and loss of estrogen receptor expression, this relationship has been independently observed in a previous independent study (Somasiri et al., 2004). Moreover, we did find a strong inverse relationship between podocalyxin mRNA and protein expression and both the estrogen and progesterone receptors in vitro.

A strong association between podocalyxin expression and the basal-like phenotype was also seen in our in vitro studies of breast cancer cell lines stably over-expressing full-length exogenous PODXL cDNA. We found that stable expression of podocalyxin in two independent luminal breast cancer cell lines (MCF7 and T47D) led to changes in gene expression consistent with a shift from a luminal to a basal-like phenotype. Generally, concordance was seen between PODXL expression and increased expression of basal-like markers in both cell
lines including the basal cytokeratins KRT5, KRT6A, KRT6B and KRT17, and basal-like markers FSCN1, CAV1 and CRYAB. In addition to decreased ESR1 and PGR1 expression in PODXL-expressing cells, we observed a concomitant decreased expression of luminal cell markers of several DNA binding proteins associated with ER function, including GATA3, XBP1, and FOXA1. Consistent with these data, we saw a significant decrease in the expression of the two basal-like markers FSCN1 and CAV1 in the basal-like/mesenchymal breast cancer cell line MDA-MB-231 following siRNA knock down of endogenous podocalyxin expression.

The observation that not all of the luminal or basal markers demonstrated complete concordance with the basal or luminal types is consistent with that found in hierarchical clustering of breast cancer cell lines and primary breast cancers (Charafe-Jauffret et al., 2006; Neve et al., 2006; Perou et al., 2000; Sorlie et al., 2001; Sorlie et al., 2003). In a large TMA-based study, the majority of breast cancers positive for basal cytokeratins also expressed one or more luminal cytokeratins (Abd El-Rehim et al., 2004). The cytokeratin profile seen in MCF7/PODXL is similar to that seen in a population of glandular stem cells that have been identified in the breast (Bocker et al., 2002). This combined basal and luminal phenotype is associated with steroid receptor negative breast cancer, poor outcome and frequent metastases (Abd El-Rehim et al., 2004; Malzahn et al., 1998). A complex pattern of co-expression luminal and basal cytokeratins has also been reported in the highly invasive, estrogen receptor negative, tamoxifen-
resistant MCF7 variant cell line TMX2-28 (Gozgit et al., 2006, Gozgit et al., 2007).

There is growing evidence of a role for BRCA1 in basal-like breast cancer. A striking relationship between BRCA1 mutation-associated tumors and the basal-like phenotype has been reported, with approximately 90% of breast tumors arising in BRCA1-mutation carriers exhibiting a basal-like phenotype (Foulkes et al., 2003; Lakhani et al., 2005). As with basal-like breast cancers, BRCA1 mutation-related breast tumors are generally cytokeratin 5/6 and 14 positive (Lakhani et al., 2005), are high grade, highly proliferative (Rodriguez-Pinilla et al., 2006; Turner and Reis-Filho 2006; Turner et al., 2007, Honrado et al., 2006a; Honrado et al., 2006b), and are estrogen and progesterone receptor negative (Turner and Reis-Filho 2006, Rodriguez-Pinilla et al., 2007, Honrado et al., 2006b). BRCA1 mutation-associated breast cancers are also highly aggressive, and lung and brain metastases are common (Tischkowitz and Foulkes 2006; Albiges et al., 2005). The molecular mechanisms underlying the development of sporadic basal-like breast cancers remain poorly understood. However, studies indicate that most basal-like breast cancers show reduced expression of BRCA1 (Turner et al., 2007; Rakha et al., 2008a). While somatic mutations in BRCA1 do not appear to contribute significantly to these cancers, BRCA1 promoter hypermethylation has been reported (Hedenfalk et al., 2001). However, recent studies suggest that promoter hypermethylation of BRCA1 is not a common mechanism underlying reduced BRCA1 expression in basal-like
tumors (Turner and Reis-Filho, 2006). The inverse relationship between *PODXL* expression and *BRCA1* expression in both MCF7 and T47D cells suggests that podocalyxin may either directly or indirectly down regulate *BRCA1* expression.

We also examined the possibility that podocalyxin expression induced EMT in breast cancer cell lines but found no evidence to support this hypothesis. Although both SLUG, an EMT associated transcription factor, and N-cadherin expression were increased MCF7/*PODXL* and T47D/*PODXL* cells, there was no corresponding increased expression of the vimentin, smooth muscle actin, SNAIL or TWIST; nor was E-cadherin expression lost in response to podocalyxin expression. These data suggest the changes in gene expression corresponding to over-expression of podocalyxin are not the result of EMT.

Taken together these data suggest that podocalyxin plays a critical role in the development of basal-like breast cancer, possibly through the down-regulation of *BRCA1*. These data also implicate the cytoskeletal-binding protein ezrin in basal-like breast cancer as we have previously shown that the podocalyxin-dependent increase in migration and invasion, as well as activation of PI3K-AKT, MAPK-ERK1/2 signaling pathways in MCF7 cells was ezrin-dependant. This finding is consistent with the known role of ezrin as a metastasis-promoter. These data support a critical role for podocalyxin and ezrin-dependent PI3K-AKT, MAPK-ERK1/2 signaling pathways in the development of basal-like breast cancer.
Summary and Conclusions

In summary, our *in vitro* and *in vivo* data strongly implicate podocalyxin in the development of basal-like breast cancer. Many of the features of podocalyxin expressing breast cancers including loss of estrogen and progesterone receptor expression, expression of basal-like cytokeratins, high grade, and association with poor outcome and the development of brain metastases are all consistent with a basal-like phenotype. Our *in vitro* data shows that podocalyxin correlates with a shift in gene expression from a luminal to basal-like phenotype in breast cancer cell lines suggesting a causal role for podocalyxin in the development of the basal-like phenotype. Together these data strengthen the hypothesis that podocalyxin and downstream ezrin-dependent PI3K-AKT, and MAPK signaling is involved in breast cancer aggressiveness and suggest that high levels of podocalyxin expression represents a marker of basal-like breast cancer and that podocalyxin, ezrin, and related signaling pathways represent potential targets for treatment of some metastatic breast cancers.
OVERALL SUMMARY AND CONCLUSIONS

The unifying goal of this work was to determine the molecular mechanisms underlying the role of podocalyxin in cancer aggressiveness. Our group originally identified \textit{PODXL} as a candidate genetic risk factor for more aggressive forms of prostate cancer through linkage and genetic association studies. Subsequent work by our lab and other groups further implicated podocalyxin as a determinant of aggressiveness in breast and prostate cancers. Little or no published work focused on trying to understand the underlying biology of action of podocalyxin in cancer aggressiveness. I hypothesized that podocalyxin increased cancer aggressiveness through interaction with ezrin and was a marker of a clinically relevant subset of highly aggressive cancers.

I demonstrated that expression of podocalyxin in MCF7 breast cancer and PC3 prostate cancer cells correlated with increased cellular migration and invasion. Podocalyxin formed a complex with the metastasis-associated protein ezrin in these cells and over-expression of podocalyxin correlated with changes in the phosphorylation state and subcellular localization of ezrin. Podocalyxin-independent changes in the subcellular localization and phosphorylation of ezrin suggested that podocalyxin might influence ezrin-influenced signaling pathways such as the PI3K and MAPK pathways. I next demonstrated that podocalyxin expression correlated with increased PI3K and MAPK activity in breast and prostate cancer cells and that ezrin was required for the observed podocalyxin-independent increase in PI3K and MAPK activity. The MAPK and PI3K pathways...
have been shown to influence cancer aggressiveness by a variety of mechanisms. One way in which these pathways may increase cancer invasion is via induction of MMP expression. I found that expression of podocalyxin in breast and prostate cancer cells resulted in increased expression of MMP1 and 9. Finally, I found that the podocalyxin-dependent increase in migration and invasiveness and MMP expression were dependent on ezrin expression and activity of the PI3K and MAPK pathways.

I next sought to determine the value of podocalyxin as a prognostic marker of cancer progression in a patient population. Utilizing tissue microarrays (TMAs) containing tumor samples from 248 patients with invasive breast carcinoma diagnosed at the Cleveland Clinic I found that high podocalyxin protein expression correlated with higher Bloom Richardson Grade and expression of fascin, a protein associated with the more-aggressive, basal-like breast cancer phenotype. Furthermore, in a TMA of primary breast cancer tumor samples from 55 patients who developed brain metastases, 40 patients that developed a mix of visceral and skeletal metastases and 254 patients that remained free of metastases (mean follow up of 67 months), podocalyxin expression was associated with tumors that metastasized to the brain.

As aggressive, metastatic breast cancers are associated with unique gene expression signatures that are also reflected in breast cancer cell lines, I next sought to determine if expression of podocalyxin in relatively non-aggressive, luminal-like breast cancer cell lines could induced a change in gene expression
signature to that of a more aggressive, basal-like breast cancer. Utilizing gene expression arrays, real-time quantitative PCR and immunoblots, I found that expression of podocalyxin in luminal-like MCF7 and T47D breast cancer cells lines was met with a concomitant decrease in expression of luminal-type markers including the estrogen and progesterone receptors and an increase in expression of basal-type markers including KRT5/6, caveolin-1 and fascin. Furthermore, podocalyxin expression also inversely correlated with \textit{BRCA1} expression. Significantly, \textit{BRCA1} has recently been strongly implicated in the development of basal-like breast cancer.

Together these findings lend insight into the mechanisms through which podocalyxin may increase cancer aggressiveness and help to establish podocalyxin and the podocalyxin:ezrin complex as markers of aggressive cancers and possible therapeutic targets. Questions remain regarding the mechanism through which podocalyxin comes to be over-expressed in some cancers. Expression of podocalyxin is thought to be controlled primarily by methylation of the podocalyxin promoter region (Butta \textit{et al.}, 2006). Our group has identified a variable in-frame deletion within the podocalyxin-coding region that is positively associated with prostate cancer aggressiveness (Casey \textit{et al.}, 2006). The deletion lies in close proximity to the podocalyxin promoter in a CG-rich area (Kershaw \textit{et al.}, 1995; Casey \textit{et al.}, 2006). It is tempting to hypothesize that these deletions are associated with more aggressive forms of cancer due to reduced methylation-dependent suppression of podocalyxin expression. Better
understanding of the mechanisms which result in increased expression of podocalyxin may lead to novel treatment options for patients with cancers that over express podocalyxin. It also remains unclear whether or not members of the NHERF-family are required for interaction between podocalyxin and ezrin and the resulting increased activity of the PI3K and MAPK pathways in cancer cells. Podocalyxin has been show to interaction with ezrin both directly and indirectly through members of the NHERF1 and NHERF2 (Takeda et al., 2000; Schmieder et al., 2004; Nielsen et al., 2007). It is possible that a complex comprised of only podocalyxin and ezrin may more readily activate the PI3K and MAPK pathways than a complex that also contains NHERF1 or 2; or it may be that a NHERF family member is required for podocalyxin to bring sufficient quantities of ezrin to the plasma membrane for activation of signaling pathways to occur. A more detailed picture of the podocalyxin complex in cancer cells should identify novel molecular targets of therapeutic intervention for the treatment of podocalyxin-associated cancers.

My work, as well as the work of others (Somasiri et al., 2004), establishes podocalyxin as a marker of aggressive breast cancers in two moderately sized patient populations in agreement with my observations that podocalyxin expression increases cancer cell aggressiveness in vitro. These studies also provided the initial data to suggest that podocalyxin expression correlates with the basal subtype of breast cancers. Subsequent studies utilizing panel of markers of the breast cancer subtypes are needed to confirm and expand upon
this observation in breast cancer patient populations. Reliable molecular markers that identify aggressive cancers and predict therapeutic response are vital tools in the treatment of cancers. My observation that podocalyxin correlates with breast cancers that metastasize to the brain but does not correlate with metastasis to the bone or viscera suggests that podocalyxin may be useful in the diagnosis of breast cancer as well as a potential novel therapeutic target for treatment of a group of cancers that currently have a poor prognosis and few targeted treatment options. Of future interest will be determining the role podocalyxin may play in actively targeting disseminated cancer cells to the brain. Currently little is know of the mechanisms that determine metastatic pattern; podocalyxin and the molecular pathways influenced by podocalyxin may shed light on the factors that encourage metastasis to the brain. Furthermore, while abnormal localization of ezrin has been associated with poor prognosis in breast cancer, the ability of podocalyxin to influence the localization of ezrin has not been demonstrated in patient populations. Studies designed to test the hypothesis that breast cancers that express high levels of podocalyxin also show abnormal localization of ezrin are needed to illuminate the role of the podocalyxin:ezrin complex in the clinical setting and may allow for identification of other ezrin binding molecules that increase cancer aggressiveness by similar means to podocalyxin. Furthermore, ezrin has been implicated in the aggressiveness of a wide array of cancers, it is unlikely that podocalyxin’s role in cancer aggressiveness is limited to cancers of the breast. Studies utilizing tissue
microarrays comprised of cancers other than invasive breast carcinomas are needed to explore the role of podocalyxin in other cancer types.

Finally, my data indicate that expression of podocalyxin in breast cancer cells elicits global changes in gene expression. Expression of podocalyxin in luminal-like breast cancer cell lines resulted in decreased expression of genes associated with the luminal-subtype and increased expression of genes associated with the more aggressive basal subtype. To date little is known of the mechanisms that give rise to breast cancers of the basal subtype and only a few potential therapeutic targets, such as EGFR, exist for treatment of these highly aggressive cancers. EGFR, like the podocalyxin:ezrin complex, elicits its effects in part through the PI3K and MAPK pathways (Yehiely et al., 2006) which may indicate that these pathways are vital for the formation of basal-like breast cancers. A better understanding of the role of podocalyxin, ezrin, along with the PI3K and MAPK pathways in the basal-like phenotype should allow for a more complete picture of how basal-like breast cancers arise and how to best treat these cancers. The ability of podocalyxin to induce a more basal-like phenotype in luminal-like breast cancer cell lines is in contrast to the current popular thought that breast cancers with the basal-like phenotype arise from basal/myoepithelial cells of the normal breast duct or their progenitor cells (Malzahn et al., 1998; Bocker et al., 2002). While there has been previous evidence to suggest the possibility that luminal-like cancer cell lines can progress to a more basal-like phenotype (Gozgit et al., 2006), my data is the first to thoroughly demonstrate
this phenomenon. My data suggests that basal-like breast cancers may arise from more luminal-like cells given the proper molecular milieu and that the molecules responsible for this transition are promising targets for the treatment of basal-like breast cancers. Finally, my data establishes a negative correlation between podocalyxin expression and expression of BRCA1. Hereditary breast cancers in BRCA1 mutation carriers are basal-like in nature and sporadic basal-like breast cancers are reported to have reduced BRCA1 expression (Turner et al., 2004; Foulkes et al., 2004; Catteau et al., 1999; Abd El- Rehim et al., 2004). Currently, the mechanism through which BRCA1 is down regulated in sporadic basal-like tumors is not understood. My results suggest that expression of podocalyxin, perhaps through increased activity of the PI3K and/or MAPK pathways, as a possible explanation for decreased BRCA1 expression in sporadic basal-like breast cancers. My results also imply that cancers over expressing podocalyxin may be susceptible to DNA damaging chemotherapeutic agents such as cisplatin since BRCA1 is involved in the repair of DNA damage induced by these agents. Future studies will clarify the mechanism through which podocalyxin down regulates BRCA1 expression and may lead to better understanding and improved therapy for both hereditary BRCA1-related breast cancers and sporadic basal-like breast cancers.

The known and hypothetical roles of podocalyxin in increased cancer aggression and metastasis are summarized in Figure 23.
**Figure 24. Illustration Summarizing the Means Through Which Podocalyxin may Increase Cancer Aggressiveness.** Decreased methylation of the PODXL promoter and/or increased activity of the Sp1 transcription factor may result in increased expression of podocalyxin. Podocalyxin's antiadhesive properties encourage cancer dissemination by reducing cell-cell adhesion. Through formation of a complex with ezrin, either by direct interaction or via the bridging protein NHERF, podocalyxin increases PI3K and MAPK activities. Increased activity these pathways may encourage metastasis through multiple means including inhibition of apoptosis. Podocalyxin associated changes in gene expression including decreased expression of BRCA1 and increased expression of MMPs and FSCN1 may also encourage cancer metastasis. These changes in expression may be dependent upon activation of the PI3K and MAPK pathways through ezrin, as demonstrated for MMP1 and MMP9, or may depend on yet to be discovered mechanisms.
CHAPTER THREE

REFERENCES


Appendix: Abbreviations

AKT - V-akt murine thymoma viral oncogene homolog
ANXA8 - annexin A8
BCA - bicinchoninic acid
BRCA1 - breast cancer 1, early onset
BRG - Scraff-Bloom-Richardson Grade
cDNA - complementary deoxyribonucleic acid
C - cytidine
CAV1 - caveolin-1
CHO - Chinese hamster ovary
CLDN4 - claudin 4
CLDN7 - claudin 7
CNS - central nervous system
CpG - cytidine-phosphodiester bond-guanosine
CRABP2 - Cellular retinoic acid binding protein 2
cRNA - complementary ribonucleic acid
CRYAB - alpha B Crystallin
DDR1 - discoidin domain receptor family, member 1
DES - differential expression score
DMEM - Dulbecco’s Modified Eagle’s Medium
DTHL - aspartic acid-threonine-histidine-leucine
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<tr>
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<td>extracellular matrix</td>
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<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EMT</td>
<td>epithelial-to-mesenchymal transition</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor alpha</td>
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<td>ERα</td>
<td>see ER</td>
</tr>
<tr>
<td>ERBB2</td>
<td>see Her-2</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal regulated kinase</td>
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<tr>
<td>ERM</td>
<td>ezrin-radixin-moesin</td>
</tr>
<tr>
<td>ESR1</td>
<td>estrogen receptor alpha gene</td>
</tr>
<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
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<tr>
<td>FCS</td>
<td>fetal calf serum</td>
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<td>FERM</td>
<td>band 4.1 (F)-ezrin-radixin-moesin protein-protein interaction domain</td>
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<tr>
<td>FOXA1</td>
<td>forkhead box A1</td>
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<td>FSCN1</td>
<td>fascin gene</td>
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<tr>
<td>G</td>
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<td>GATA3</td>
<td>GATA binding protein 3</td>
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<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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</table>
GTP - guanosine triphosphate
HBSS - Hank’s balanced salt solution
HER-2 - V-erb-b2 erythoblastic leukemia viral oncogene homolog 2
HEV - high endothelial venule
HGF - hepatocyte growth factor
HGFR - see Met
ICAM - intercellular adhesion molecule
KRT - cytokeratin
LAMB3 - laminin β3
LN - axillary lymph node
MAPK - mitogen activated protein kinase
MDCK - Madin-Darby canine kidney
Met - hepatocyte growth factor receptor
MEK1 - mitogen activated protein kinase kinase 1
MMP - matrix metalloprotease
mRNA - messenger ribonucleic acid
MTT - dimethyl thiazolyl diphenyl tetrazolium salt
MUC1 - mucin 1
MWCO - molecular weight cut-off
Neu - see Her-2
NHE3 - Na+/H+ exchanger
NHERF1 - Na+/H+ exchanger regulatory factor 1
NHERF2 - Na⁺/H⁺ exchanger regulatory factor 2
PAN - puromycin aminonucleoside
PBS - phosphate buffered saline
PCLP1 - podocalyxin-like protein1; see PODXL
PDZ - PSD-95/Dlg/ZO-1 protein-protein interaction domain
PET - polyethylene terephthalate
PGR1 - progesterone receptor gene
PI3K - phosphatidylinositol 3-kinase
PKB - see AKT
PLAU - plasminogen activator, urokinase
PODXL - podocalyxin gene
PR - progesterone receptor
Rho-GDI - Rho guanosine diphosphate dissociation inhibitor
RNA - ribonucleic acid
RQ - relative quantitation
RT-PCR - reverse transcription-polymerase chain reaction
RTQ-PCR - real-time quantitative polymerase chain reaction
SD - standard deviation
SDS - sodium dodecyl sulfate
SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser - serine
SH2 - src homology-2
**shRNA** - short hairpin ribonucleic acid

**siRNA** - small interfering ribonucleic acid

**SLPI** - Secretory leukocyte peptidase inhibitor

**SMA** - smooth muscle actin

**Sp1** - Selective Promoter Factor 1

**TGF-β** - transforming growth factor beta

**Thr** - threonine

**TMA** - tissue microarray

**TP53** - tumor protein 53

**TRIM29** - Tripartite motif-containing 29

**Tyr** - tyrosine

**V/B** - visceral/bone

**VIM** - vimentin

**WT1** - wilms tumor 1

**XBP1** - X-box binding protein 1

**ZNF42** - zinc finger protein 42