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Invasive Prostate Cancer Cells Are Tumor Initiating Cells That Have A Stem Cell-Like Genomic Signature

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Abstract

Development of metastasis is a leading cause of cancer-induced death. Acquisition of an invasive tumor cell phenotype suggests loss of cell adhesion and basement membrane breakdown during a process termed epithelial-to-mesenchymal transition (EMT). Recently, cancer stem cells (CSC) were discovered to mediate solid tumor initiation and progression. Prostate CSCs are a subpopulation of CD44⁺ cells within the tumor that give rise to differentiated tumor cells and also self-renew. Using both primary and established prostate cancer cell lines, we tested the assumption that CSCs are more invasive. The ability of unsorted cells and CD44-positive and -negative subpopulations to undergo Matrigel invasion and EMT was evaluated, and the gene expression profiles of these cells were analyzed by microarray and a subset confirmed using QRT-PCR. Our data reveal that a subpopulation of CD44⁺ CSC-like cells invade Matrigel through EMT, while in contrast, CD44⁻ cells are non-invasive. Furthermore, the genomic profile of the invasive cells closely resembles that of CD44⁺CD24⁻ prostate CSCs and shows evidence for increased Hedgehog signaling. Finally, invasive cells from DU145 and primary prostate cancer cells are more tumorigenic in NOD/SCID mice compared with non-invasive cells. Our data strongly suggest that basement membrane invasion, an early and necessary step in metastasis development, is mediated by these potential cancer stem cells.

Keywords

cancer stem cells; metastasis; invasion; EMT; prostate cancer; hedgehog

Introduction

A critical factor in cancer survivability is whether or not cancer cells migrate from the site of the primary tumor and form metastases [1]. Several steps are required to develop metastases: cell detachment from the primary tumor and basement membrane invasion, dissemination by the blood or lymphatic systems while simultaneously surviving, and reinvasion of basement membrane and embedding itself in a new location (either randomly or through a receptor-

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mediated adhesion). Finally, the microenvironment (or niche) must be permissible so the cell propagates and establishes a metastatic secondary lesion [1-3].

The ability of a subset of cells to become locally invasive suggests loss of cadherin-mediated cell-cell adhesion within the tumor, integrin-mediated tumor/extracellular matrix (ECM) adherence, and basement membrane breakdown. These events collectively are termed epithelial-to-mesenchymal transition (EMT; [4,5]. EMT, conversion from an epithelial to a mesenchymal cell phenotype through loss of intracellular contacts and polarity, and morphologic changes, is both a fundamental process in embryonic development and required for cell motility and basement membrane invasion during metastasis [5,6]. EMT is accompanied by a loss of expression of the adhesion molecule E-cadherin through transcriptional repression by Snail and/or Twist. [6]. E-cadherin is essential in cell-cell junctions, and its loss contributes to invasive behavior, and allows β -catenin to enter the nucleus where it regulates the transcription of EMT- and proliferation-regulatory genes. Thus, EMT is a normal cellular process utilized by cancer cells in order to spread. Though millions of cancer cells are shed into the circulatory systems daily, metastasis is inefficient, with only 0.1% or fewer cells capable of producing a metastatic lesion [7]. Thus, only a subset of tumor cells are EMT- and metastatic-competent, suggesting only a small number of cells maintain inherent morphologic and phenotypic plasticity necessary to become invasive and home to the appropriate microenvironment, thereby ultimately establishing a proliferating tumor at a secondary site.

A relatively new hypothesis about cancer progression has suggested that cancer stem cells (CSCs) mediate solid tumor initiation and progression (reviewed in [8]). The theory suggests that CSCs are only a minor subpopulation of cells (generally <1%) within the tumor that self-renew and also give rise to differentiated tumor cells, while the bulk of tumor cells are highly differentiated, have limited proliferative potential and are non-tumorigenic. Several markers exist to identify prostate CSCs. For example CD44, CD133, $\alpha_2\beta_1$ integrin and flow cytometry-based 'side-populations' are all associated with CSCs (reviewed in [9]). Recently, within the field of prostate CSCs, mouse xenograft models have well established that CD44⁺ cells are more proliferative, clonogenic, tumorigenic and metastatic than the isogenic CD44⁻ [10]. Specifically, the CD44⁺ prostate cells isolated from LAPC-4 cells were shown to survive as long-term cells for 33 days in culture with BrdU labeling, and in both LAPC-4 and DU145 the CD44⁺ cells undergo asymmetric division and differentiate after injection into a host animal (the majority of the tumor cells isolated are CD44⁻ by FACS) [10]. Furthermore, as few as 1000 sorted CD44⁺CD24⁻ LNCaP prostate cells could initiate a tumor in mice, while 1000 cells depleted of CD44⁺CD24⁻ are non-tumorigenic [11]. Similarly, once these cells are injected into animals, the majority of the isolated tumor cells are CD44⁻, again demonstrating their ability to differentiate *in vivo* [11]. These studies clearly support that prostate cells isolated by these methods are in fact tumor-initiating cells. In addition, prostate CSCs express higher levels of 'stemness' genes such as *Oct-3/4*, *Bmi-1*, *β -catenin* and *Smo* [10,11]. Cells containing all, or some, of these markers (such as CD44) are much more tumorigenic when compared to the complete, unfractionated (bulk) tumor cell populations, where as few as 100 prostate CSCs form tumors in mice [10-12]. CSCs can also recapitulate the spectrum of cells contained within bulk tumors again suggesting that test are the cells responsible for tumor initiation and growth [11].

Both CSCs and metastatic cells share traits, such as migration to various sites and differentiation into different cell types (plasticity). Thus, the underlying hypothesis is that a subpopulation of cells, which may be CSCs, are responsible for migration from the site of the primary tumor and seed metastases. We tested the invasiveness of this subpopulation of cells using both primary and established prostate cancer cell lines, where Matrigel invasion was used as a model system to evaluate the ability of unsorted cells and CD44-positive and -negative

subpopulations to undergo basement membrane invasion and EMT. Simultaneously, this method of isolating potential CSCs has been investigated by another group who determined that the invasive population of the U87 glioma cell line expressed higher levels of the stem cell markers nestin and Oct4, and formed more spheres when cultured in serum-free media than the non-invasive cells [13]. Our data reveal that this subpopulation of prostate cancer cells invade Matrigel through an EMT process, and that these cells are CD44⁺ and exhibit gene expression profiles consistent with those of CD44⁺CD24⁻ prostate CSCs [11]. While in contrast, noninvasive cells do not express high levels of 'stemness' genes. Moreover, purified CD44⁺ cells, and not CD44⁻ cells, are invasive. Furthermore, the invasive cell subpopulation was tumorigenic in NOD/SCID mice whereas the non-invasive cells were only weakly tumorigenic. Thus, these data strongly suggest that the stem cell-like component of cancer cells is responsible for invasion, the first step in metastasis.

Results

Prostate Cancer Cells are Invasive

As a first step to determine if prostate cancer stem cells are responsible for initiating metastasis, we determined the overall ability of both established and primary prostate cancer cells to invade Matrigel, a basement membrane model used to estimate metastatic potential [14,15]. Human PCSCs were obtained from Celprogen (San Pedro, CA). The three cells lines (PCSC1-3) were selected by CD133 expression from prostate cancer tissues resected in the clinic using Florescent Activated Cell Sorting (FACS). Following isolation, cells were plated at a limiting dilution, expanded and then injected into mice for tumorigenicity studies. The most tumorigenic cells were then chosen for expansion and were subsequently shown to express Oct3/4, telomerase, SSEA3/4, AP and PSA (markers of 'stemness'). In addition, we know that these cells are over 97% CD44⁺ by FACS from studies in our lab (M.A. Duhagon, unpublished data). We acquired clones from three different patients in order to demonstrate reproducibility in our data, yet their stage/grade of their prostate cancer and other demographics about the patients are unknown. The cell lines LNCaP, DU145, PCSC1, 2, and 3 were analyzed for both chemotaxis/migration and invasiveness in modified Boyden chambers containing either a non-coated control membrane or a Matrigel-coated membrane, respectively. Each cell type crossed the control membrane in response to RPMI-1640/10% FBS (Fig. 1, "control" panels). With Matrigel-coated membranes, the total number of cells migrating to the underside of the membrane was less than the respective control in all cases (Fig. 1, "Matrigel" panels). LNCaP, DU145 and the three different PCSCs were all invasive (Fig. 1 A-C, respectively; PCSC3 shown as a representative example), though LNCaP cells were the least invasive (14%) while PCSC3 were the most invasive (33%). PCSC1, PCSC2 and DU145 were all just over 20% invasive (Fig. 1D). In all cases, the percentage of cells migrating across control membranes was estimated to be $\leq 25\%$ (data not shown). Thus, only a subpopulation of cells within each cell line has the inherent ability to invade a basement membrane.

Invasive Prostate Cancer Cells Undergo Epithelial-to-Mesenchymal Transition

Analysis of invasive cells indicates that they undergo EMT during Matrigel invasion. This was clearly demonstrated by loss of expression of the epithelial cell marker and adhesion protein E-cadherin as determined by immunofluorescence (IF) (Fig. 2). LNCaP cells had the highest E-cadherin expression of the cells analyzed, where it is preferentially localized near the cell membrane, and along with DU145 and PCSC3, showed a significant loss of E-cadherin upon Matrigel invasion. QRT-PCR analysis demonstrated a dramatic reduction in E-cadherin expression in the invading cells from both lines, and a significant increase in expression of vimentin was observed in the invading DU145 cells (Supplemental Fig.1A). In contrast, cells crossing control membranes did not show EMT hallmarks (data not shown).

Both populations of cells, i.e. those that invaded Matrigel and those that did not, were harvested, and RNA was arrayed using Agilent whole genome arrays. The molecular signature of EMT was confirmed (Fig. 3A) based on genes whose expression patterns reportedly change with EMT [4,6]. Genes coding for: claudins and occludin (important for tight junctions), mucins (adhesion), and were all down regulated after invasion in LNCaP and DU145, while the mesenchymal markers such as: fibronectin and vitronectin, Rhob (chemotaxis and actin polymerization) and Xr11 (survival), all increased after Matrigel invasion. These data strongly support the notion that Matrigel invasion by human prostate cancer cells is accompanied by EMT.

Stem Cell Signature Associated with Matrigel Invasion

Closer analysis of gene expression changes revealed that genes important in stem cell maintenance are more highly expressed in Matrigel-invasive cells compared with non-invasive cells (Fig. 3B). For example, CD44 expression was slightly higher for invasive LNCaP cells and was significantly higher in both DU145 and PCSC1 invasive cells, while CD24 expression decreased in LNCaP and DU145 cells after invasion. These observations are compared with those of purified CD44⁺CD24⁻ LNCaP CSCs (Fig. 3B, right side) which were highly tumorigenic in xenograft models [11]. Like the LNCaP CSCs, the expression of *Nanog*, *BMI1* and *SHH* genes are greater in the invasive population of LNCaP, DU145 and the primary cell isolate PCSC1 (Figure 3B and supplementary figure 1B). *Nanog*, *BMI-1* and *SHH* are transcription factors important for embryonic, leukemic, and neural stem cell self-renewal (reviewed by [16]). In addition, these genes are also expressed to a greater degree in prostate CSCs relative to non-CSCs [10,11]. QRT-PCR analysis for a subset of altered genes further confirmed the microarray data for both non-invaded/invaded LNCaP and DU145 cells (Supplemental Fig. 1B).

To better understand how the gene signature of the invasive cells compared to other published data, Gene Set Enrichment Analysis (GSEA;[17,18]) was performed on genes in common to both DU145 and PCSC1 that showed a ≥ 1.8 -fold positive or negative change in expression between the invasive and non-invasive cell populations. Of the genes whose expression changed during invasion, 2465 genes were found and compared to 1892 curated gene sets (c2) from the GSEA molecular signature database (<http://www.broad.mit.edu/gsea/index.jsp>). The top 10 similar gene sets (based on total number of overlapping genes) are shown in Table 1 and the top 50 are available as Supplemental Table S2. There is a remarkable overlap with gene sets from either stem cells and/or comparisons with development or differentiation, with gene sets 3 and 5 not directly falling into these categories. There was significant overlap between our invasive cells and genes up regulated in neural stem cells compared with differentiated cells (243 genes), and with hematopoietic stem cells (184 genes; Table 1, set 1 and 2, respectively). The third gene set, Alzheimer's Disease, Down, seemed anomalous, however some of these genes are involved in proliferation and differentiation, adhesion and apoptosis [19]. Genes of set 10 change in response to loss of murine *POD1*, a transcription factor that negatively regulates differentiation of myoblasts and function similarly to known EMT regulators *Snail* and *Twist* [20]. The similarity of gene expression patterns in invasive prostate cancer cells and data sets from stem cells (Table 1) strongly imply that the Matrigel-invasive subpopulation of prostate cancer cells are relatively undifferentiated and stem cell-like.

CD44⁺ Subpopulation of Prostate Cancer Cells is Invasive

Because the gene expression analysis strongly supported the notion that the basement membrane-invasive cells display stem cell characteristics, the potential CSC-enriched component of the cell lines was directly evaluated for invasion ability. While prostate CSC markers include both CD44 and CD133, several reports have established that CD44⁺ cells

enrich for the tumor imitating cells [11]. In other systems, such as colorectal cancer, a single cell expressing CD44 (and not CD133, interestingly) produced a tumor sphere *in vitro* and this sphere was able to generate a xenograft tumor resembling the properties of the primary cells injected [21]. In addition, our lab has previously shown that 1000 CD44⁺CD24⁻ prostate CSCs form tumors in mice, while 1000 cells depleted of CSCs were non-tumorigenic [11]. Since nearly all CD44⁺ cells are also CD24⁻ [10] we purified ($\geq 90\%$) CD44⁺ and CD44⁻ subpopulations of LNCaP and DU145 cells were isolated by magnetic bead separation. Analysis of Matrigel invasion revealed that, while CD44⁺ and CD44⁻ cells migrate equally across the control membrane in response to serum, only the CD44⁺ cells invaded Matrigel (Fig. 4). Significant quantities of CD44⁺ LNCaP (Fig. 4A) and DU145 (Fig. 4B) invaded the Matrigel-coated membrane. Of the migration-competent cells, 75-80% were also Matrigel invasive (Fig. 4C). In contrast, less than 5% of migration-competent CD44⁻ DU145 cells were invasive, and CD44⁻ LNCaP had the same invasive potential as bulk LNCaP cells (Fig. 4C). The latter observation is not surprising since LNCaP bulk cells are $\sim 99.9\%$ CD44⁻ [11]. As expected, CD44⁺ cells go through EMT based on E-cadherin loss during invasion (data not shown). This evidence strongly supports the concept that the CSC subpopulation are the cells primarily responsible for basement membrane invasion, and that other populations of cells within these cell lines do not have a significant invasion ability.

Matrigel-Invasive Cells are Tumorigenic

Since prostate CSCs are much more tumorigenic than non-stem cells contained within the cell lines [11], and cells responsible for metastasis must by definition be tumor-initiating, the ability of invasive and non-invasive prostate cancer cells to induce tumors in NOD/SCID mice was evaluated (Table 2). Either 100 or 1000 cells of each population were mixed with Matrigel and injected subcutaneously into NOD/SCID mice. Injection of 100 Matrigel-invasive DU145 cells resulted in tumor formation in 100% of the mice (5/5), while only 2/5 mice formed tumors resulting from injection of 100 non-invasive cells. Invasive cells formed tumors on average 50% earlier in the 5 mice compared with the 2 tumors detected after injection with non-invasive DU145 cells (day 38 vs. day 61; Table 2). Similarly, the invasive cells within the primary human prostate cancer cell isolate 1 (PCSC1) were also 100% tumorigenic when 1000 cells were injected (5/5; Table 2). The corresponding non-invasive cells formed tumors in only 2 out of 5 animals. Of the tumors that arose, each population of PCSC1 caused tumors within a similar time point, though at day 74 tumors were larger in animals injected with the Matrigel-invasive cells (Table 2). Interestingly, 1000 bulk PCSC1 resulted in tumor formation by ~ 27 days in 5/5 animals (Table 2), and the observation that 1000 non-invasive cells were significantly less tumorigenic than 1000 unsorted cells suggests that Matrigel invasion depleted most of the tumor-initiating cells from the PCSC1 cells that remained on the topside of the membrane. After recovery from the topside of the Matrigel-coated membrane, non-invasive cells grew similarly to the bulk cells in each case, ruling out the possibility that non-invasive cells were dead or in senescence. Thus, the cells that have the capacity of morphological and phenotypic plasticity (EMT) and exhibit stem cell-like gene expression are more tumorigenic than cells lacking these abilities.

Gene Expression Analysis of Invasive Cells

Since invasive cells show such a strong stem-cell hallmark, we determined to which functional categories these genes belonged. In total, 3833 genes common to both DU145 and PCSC1 were up or down-regulated by at least 1.8-fold. Of these, 2243 genes were found and analyzed using Ingenuity System's Pathways Analysis software. The top 20 functional categories are shown in Fig. 5, and the complete list of categories, which includes all the genes, can be found in Supplemental Table S3. Interestingly, the top two categories are cellular growth and proliferation and cellular development. These types of functions are known to be different between stem cells, progenitor cells and differentiated cells [11]. In addition, the categories of

cellular movement, tissue morphology and cell morphology are consistent with the observed EMT in the invasive cells.

To further understand which cell signaling and proliferation pathways are up-regulated in the Matrigel-invasive cells, the gene expression patterns for Wnt, Hedgehog (HH) and Notch pathways were analyzed (Fig. 6). These pathways are reported to contribute to EMT [6]. Individual *Wnt* genes are less expressed in the invasive cells, while Wnt pathway negative regulators *WIF1* and *DKK1* are up regulated (Fig. 6A). β -catenin expression also decreases after invasion. Taken together, the Wnt pathway, a known inducer of uncontrolled growth in cancer cell lines [22], is less active in the invasive cell subpopulation. In contrast, the Sonic Hedgehog pathway is clearly upregulated in the invasive cells. *SHH* and *GLI2* and/or *GLI4* are both more highly expressed in the invasive cells relative to the non-invasive LNCaP, DU145 and PCSC1 cells (Fig. 6B). *PTCH*, which normally blocks the pathway, is also downregulated. As noted earlier, *Bmi1* promotes self-renewal in CSCs and is also linked to the HH pathway [23], and is significantly elevated in the invasive cells. Finally, HH pathway-regulated genes, such as cyclins D1 and E, show some expression increase after invasion. This suggests that the HH pathway is important in either orchestrating Matrigel invasion and/or maintenance of the CSC subpopulation within the prostate cancer cell lines. Genes in the Notch pathway, a negative regulator of prostate progenitor cell proliferation [16], are all expressed to a lesser degree in the invasive cells (Fig. 6C). Finally, *Nanog*, *Sox2* and *Oct3/4* transcription factors are part of a complex transcriptional network important for self-renewal and inhibition of embryonic stem cell differentiation [24]. In each cell type analyzed, *Nanog*, which blocks differentiation, was up regulated after invasion while *Sox2* and *Oct 3* expression patterns were inconclusive (Fig. 6D). In summary, both *Nanog* and the HH pathway show significant positive changes in gene expression patterns in invasive cells compared with non-invasive cells, indicating they play key roles in either invasion and/or stem cell maintenance.

Discussion

The process of metastasis can be divided into a series of steps: 1. detachment from the primary tumor; 2. basement membrane invasion; 3. survival and transport; 4. extravasation, or homing to and re-invasion of the basement membrane, which is accompanied by mesenchymal-to-epithelial transition (MET); and, finally, growth and differentiation [2,3]. Each step is important, as failure of any single step blocks formation of secondary metastatic lesions. We recapitulated several steps of this complex and inefficient biological process, and found that only a subpopulation of cells within both established human prostate cancer cell lines and primary prostate cancer cells could invade a Matrigel-coated membrane through an EMT-mediated process (Fig. 1 and 2). A similar method for the isolation of potential CSCs based on their invasive properties has been examined by Yu et al. [13]. This assay demonstrated that the invasive population of the U87 glioma cell line expressed higher levels of the stem cell markers nestin and Oct4, and formed more spheres when cultured in serum-free media than the non-invasive cells [13]. In our assays, the invasive cells have a strong stem cell signature (Fig. 3B, Table 1), and purified CD44⁺ CSC-enriched subpopulations were confirmed to be the invasive prostate cancer cells (Fig. 4). Furthermore, CD44⁺ CSC cells did not contain a genotype with EMT prior to plating on Matrigel (Supplemental Fig. 2). Consistent with the observed CSC signature, bulk populations of injected cells did demonstrate tumor formation, however, invasive cells were much more tumorigenic in NOD/SCID mice than non-invasive or bulk cells (Table 2; results summarized in Fig. 7). However, in 2/5 mice injected with non-invasive cells (top) cells tumors were able to form, thus demonstrating that either the invasive cells are not the exclusive population of the tumor-initiating cells, or the separation of CD44^{hi} by magnetic beads not 100% efficient. We previously showed that 1000 CD44⁺CD24⁻ prostate CSCs form tumors in mice, while 1000 cells depleted of CSCs were non-tumorigenic [11]. Additionally, within the field of prostate CSCs, mouse xenograft models

have well established that CD44⁺ cells are more proliferative, clonogenic, tumorigenic and metastatic than the isogenic CD44⁻ [10]. In other systems, such as colorectal cancer, a single cell expressing CD44 (and not CD133, interestingly) produced a tumor sphere *in vitro* and this sphere was able to generate a xenograft tumor resembling the properties of the primary cells injected [21]. Furthermore, gene expression data revealed that the HH pathway and *Nanog* are upregulated in this subpopulation of cells (Fig. 6). These data suggest that it is the CSC population that has the plasticity to be EMT-competent, and it is also the CSC that is highly tumorigenic; whereas the other tumor cells are more differentiated and likely incapable of EMT and subsequent metastasis. Since EMT and invasion are necessary steps for metastasis, it follows that CSCs are most likely responsible for development of metastasis. More recently, groups have shown that isolated cancer stem cells are in fact generated by this EMT event [25,26] and are more migratory and invasive [27].

EMT and decreased expression of E-cadherin in prostate cancer is associated with a poor prognosis and indirectly associated with metastasis [28]. Loss of E-cadherin associated with EMT is due to direct repression by the transcription factors Snail and Twist [6], two proteins essential to EMT [29,30]. Recently, it was demonstrated that Twist is expressed in 90% of prostate cancer tissue, and its inactivation in DU145 cells blocked collagen invasion and increased cell membrane bound E-cadherin [31]. Our array data did not reveal any significant changes in either Twist or Snail mRNA levels after Matrigel invasion (data not shown). However it is possible that Snail is only activated during EMT initiation, and that Snail transcription had decreased when the invasive cells were harvested, as the EMT was complete. Furthermore, helix 1 of Snail is susceptible to phosphorylation, which influences its dimerization affinity and binding specificity [32], and the role, if any, of phospho-Snail in EMT has not been reported.

Of the CSC-related cell growth and signaling pathways analyzed, the SHH pathway was the most active in the invasive cells (Fig. 6). Secreted HH binds Patched (PTCH) and allows Smoothened (SMO) to eventually activate the GLI transcription factors, which subsequently regulate *cyclin D*, *E*, *c-myc* and other growth-related genes [16]. Activated HH signaling is an important contributor to prostate cancer cell growth, and in particular, metastases [33]. HH is also associated with stem cells: HH is expressed during fetal prostate development but not in mature cells [34], HH signaling is up regulated in prostate CSC [10,11], and the HH inhibitor cyclopamine both repressed prostate xenograft tumor growth and prevented tumor regrowth even after cyclopamine treatment ended, strongly suggesting that the tumor-initiating CSC were inhibited [33]. Furthermore, *Bmi-1*, which may mediate HH signaling in stem cells [23], is highly expressed in the invasive cells (Fig. 3B), and in prostate CSCs [11]. *Bmi-1* also promotes growth of breast CSC 'mammospheres' [23]. Cyclopamine also inhibited metastatic spreading of pancreatic cancer in an orthotopic xenograft model [35]. Taken together, these data are consistent with the idea that the HH pathway activation and CSCs promote invasiveness, EMT and metastasis [33].

The glycoprotein CD44 is expressed in both differentiated and hematopoietic stem cells, and in some tumors including breast and prostate (reviewed in [36,37]). Prostate CSCs and invasive prostate cells are enriched for CD44 (Fig 3B; [10,11]), and CD44 was previously linked to invasiveness in breast and prostate cancer [38-40], where its expression was positively correlated with circulating prostate cancer cells in the bloodstream [41]. Additionally, the primary ligand of CD44 is hyaluronic acid (HA), an ECM component important for cell adhesion, growth and regulation of cell trafficking [42]. MMP-9 is anchored to the cell surface via CD44 in breast cancer and melanoma cells [40], which suggests an intriguing mechanism for basement membrane degradation activity of CSCs. The cytoplasmic tail of CD44 physically associates with Ezrin, a protein important in cell morphology [37]. Ezrin regulates cell-cell and cell-ECM adhesion and is associated with changes in motility [43]. Moreover, elevated

Ezrin levels are linked with prostate cancer metastasis [44]. In support of this, our data indicate that Ezrin RNA levels are significantly greater in the invasive LNCaP and DU145 cells (Supplemental Table S1). Thus, the relation between CD44 as a stem cell marker and functionality is quite strong and satisfies some of the properties required for invasive behavior, motility and morphology, though the exact mechanism in which CD44 and Ezrin contribute to invasion and metastasis in prostate cancer is not fully understood. Furthermore, although the xenograft studies performed here (Table 1) do not directly examine the ability of the invasive cells to form gross metastases; our data has well established that the invasive cells are the tumorigenic cells. This is an important step since a metastatic cell must also be able to initiate a tumor once it is disseminated from its own environment, and furthermore we believe this data is the first step in understanding how invasive cells could be more metastatic overall. Additional experiments using orthotopic models and luciferase expressing cells would further validate the invasive CD44⁺ cells are not only more tumorigenic compared to their CD44⁻ counterpart, but are more metastatic as well.

Another way that CD44 may be important to metastasis lies with homing and microenvironment. In order for stem cells to survive and maintain the balance between self-renewal and differentiation, a niche or protective microenvironment is required to anchor the stem cells [45-47]. Cell anchoring proceeds via adhesion factors, such as integrins and E-cadherin, which are re-expressed after MET. CD44 plays an important role in lymphocyte homing via stromal derived factor (SDF-1) and its receptor CXCR4 [48,49]. It is well documented that disseminated prostate cancer cells home to bone and lymph nodes, sources of SDF, and that prostate cancer cells express CXCR4 [50,51]. Thus, prostate CSCs may utilize CXCR4 to locate a permissive microenvironment, and once there, the combination of SDF, and cell surface CD44/HA interaction, E-cadherin and integrins [50] facilitate adhesion to the ECM.

In summary, we demonstrated that an invasive prostate CSC-like subpopulation exists within both established cell lines and in primary prostate cancer isolates. These are the only cells capable of EMT and Matrigel invasion, and they are also tumor initiating, potentially suggesting that metastasis is mediated by CSCs. Recently this hypothesis has been supported when it was found that CD133⁺ pancreatic cancer cells and CD44⁺CD24⁻ breast cancer cells were much more invasive than their non-stem-like counterparts [39,52]. Collectively, our data support the hypothesis that CSCs may be the principle subpopulation of cells responsible for invasion, and since CSCs are more resistant to conventional chemotherapies than differentiated tumor cells [53], it is likely that these cells are responsible for both cancer reoccurrence and metastasis. However, it remains to be determined if invasive prostate CSCs survive dissemination through the body, and if these cells, which go through EMT, also go through MET. Moreover, since the *in vivo* efficiency of metastasis is so poor [7], it suggests an additional subpopulation may exist within the invasive cells that is capable of each step necessary in metastatic development.

Materials and Methods

Cell lines

LNCaP and DU145 human prostate cancer cell lines were obtained from ATCC (Manassas, VA), and maintained in RPMI supplemented with 10% FBS, 2 mM L-glutamine, and penicillin/streptomycin. Primary human prostate cancer cells (PCSC1-3) were obtained from Celprogen (San Pedro, CA) and maintained in the recommended prostate cancer stem cell media also from the company.

Matrigel Invasion Assay

Matrigel-coated 24-well inserts (8 μ M pore size) and non-coated control inserts purchased from BD Biosciences Clontech (Palo Alto, CA) were used according to manufacturer's instructions. A range of 50-70,000 cells were seeded for the invasion and gene expression assays, while only 10-30,000 cells were seeded for immunofluorescence based experiments to allow for adequate imaging. Cells were seeded in serum-free RPMI and migrated toward RPMI +10% FBS. Three to five microscopic fields (20 $\times$) were photographed and counted for each sample. Percent invasion was calculated as average number of cells/field (Matrigel) divided by average number of cells/field (control insert). Values were averaged from 2-5 independent experiments. For experiments involving isolation of top 'non-invading' and bottom 'invading' cells, parallel invasion chambers were setup. For non-invading cells, the bottom of the membrane was scrubbed with a cotton swab and cells on top were harvested using 500 μ L of trypsin incubated at 37°C for 5 minutes. To obtain the invading cells, the top of the membrane was scrubbed with a cotton swab and the chambers were placed into another 24- well plate containing 500 μ L of trypsin incubated at 37°C for 5 minutes.

Quantitative real time polymerase chain reaction (QRT-PCR)

Total RNA was isolated using TRIzol (Invitrogen Corporation, Carlsbad, CA). RNA from 'top' cells was isolated using a cell pellet acquired from trypsinizing cells from one membrane after bottom cell were removed with a cotton swab. Conversely, RNA from the bottom cells was isolated by combining three membranes where the top cells were removed using a cotton swab. The membranes were pooled and placed in TRIzol for 10 minutes at room temperature, and the conventional procedure for isolation of RNA was then followed. To increase the yield of RNA, 5 μ g of linear arylamide (Ambion, Austin, TX) was added prior to precipitation of RNA with isopropanol. cDNA was prepared using the SuperScript®III First-Strand Synthesis System (Invitrogen Corporation, Carlsbad, CA). Quantitative real time polymerase chain reaction (QRT-PCR) analysis was performed using a StepOne Real-time PCR machine (Applied Biosystems, Foster City, CA) with TaqMan Gene Expression Assay reagents and probes (Applied Biosystems). A total of 4 μ L of cDNA was used in a 20 μ L reaction resulting in a 1:5 dilution. The following SYBR based human probes were used: CD44 (Hs00174139_m1), CD24 (Hs00273561_s1), CDH1 (E-cadherin) (Hs01023894_m1), vimentin (Hs00185584_m1), BMI1 (Hs00180411_m1), Nanog (Hs02387400_g1), FGF2 (Hs00266645_m1), TGFB1 (Hs00171257m_1), SHH (Hs00179843_m1) and 18S rRNA (Hs99999901_s1). Relative fold induction of mRNA was compared between top and bottom cells using the Delta-Delta CT method of quantitation, and 18S rRNA was used as a loading control.

Immunofluorescence

Immunofluorescent staining of invasive or non-invasive cells was performed directly on the membrane. Cells invaded as described above, except serum replacement media (SCM; DMEM:F12 (1:1), 10 ng/ml bFGF, 20 ng/ml EGF, 5 μ g/ml insulin and 0.4% BSA; [54] was used as the chemoattractant to ensure invasive cells maintain 'stemness' properties, because upon serum exposure, CSCs differentiate and lose stem cell characteristics [11]. Analysis revealed similar invasion percentage toward SCM or RPMI-1640/serum.

Magnetic Bead Separation of CD44 positive cells

The basic direct technique protocol was suggested by Invitrogen (Frederick, MD)) with the following changes: washing buffer was PBS/1% BSA, and 2 separate pools of cells were used to enrich for CD44⁺ (20 $\times$ 10⁶ cells) and CD44⁻ (10 $\times$ 10⁶) cells. Unconjugated mouse anti-human CD44 antibody (Caltag/Invitrogen) was used at 1- μ g/1 $\times$ 10⁶ cells. Dynabeads (Invitrogen; Frederick, MD) were pre-coated with goat anti-mouse IgG. Both CD44⁺ and CD44⁻

populations were $\geq 90\%$ pure based on flow cytometry. Cells were incubated in SCM overnight before invasion.

Mouse Xenograft Studies

NCI-Frederick is accredited by AAALAC International and follows the Public Health Service Policy for Care and Use of Laboratory Animals. Animal care was provided in accordance with the procedures outlined in the "Guide for Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources 1996). Following Matrigel invasion (48hr) membrane-associated cells were isolated using trypsin/EDTA or Accutase (Millipore, Billerica, MA) and grown in SCM for 48 hr. Invasive and non-invasive cells (100 or 1000 in 50 μ l of SCM) were mixed with 50 μ l of Matrigel (BD Biosciences, San Diego, CA) and injected subcutaneously into opposite flanks in male NOD/SCID mice (Jackson Labs, Bar Harbor, ME). Animals were monitored daily, tumor size was measured with calipers, and volume was determined with the formula: $V = \pi/6 \times L \times W \times H$ [55]. Mice were sacrificed when tumors reached >1.5 cm in one dimension.

Microarray analysis

RNA was isolated and labeled as previously described [11], with the following modifications. Reverse transcriptase was heat inactivated at 65°C for 10 minutes followed by RNase A RNA degradation at room temperature for 30 min. Universal Reference RNA (Stratagene, La Jolla, CA) was labeled with Cy3-dUTP and experimental samples were labeled with Cy5-dUTP. Samples were hybridized to an Agilent whole genome gene expression array following manufacturer's directions. Arrays were scanned using a GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA) and analyzed using Cluster and Treeview offered by Michael B. Eisen as freeware (<http://rana.lbl.gov/EisenSoftware.htm>). The complete list of genes whose expression changed ≥ 1.8 - or ≤ 1.8 -fold is available in Supplemental Table S1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Prostate Cancer Cell Invasiveness. Representative photos of prostate cancer cell migration across an uncoated, porous membrane, "control" or invasion across a Matrigel-coated membrane, "Matrigel". A. LNCaP cells (25,000-50,000) were plated in 500 μ l RPMI-1640 in the upper chamber, and 750 μ l of RPMI/10% heat inactivated fetal bovine serum (Gemini Bioproducts; West Sacramento, CA) was used as the attractant in the lower reservoir. After 22-48 hr at 37°C, 5% CO₂, 90% humidity, non-invasive cells were removed from the upper surface of the membrane with cotton swabs, and invasive cells were stained with the Richard-Allen Scientific 3 Step Stain Kit (Kalamazoo, MI). B. DU145 cells (25,000) were plated and treated as in "A", C. PCSC3 cells (25,000) were plated as described in "A", D. Percent invasion

relative to control migration $\pm$ standard deviation, calculated as described in “Materials and Methods.”

Fig. 2.

Invasive Prostate Cancer Cells go Through Epithelial-to-Mesenchymal Transition. LNCaP, DU145 and PCSC3 staining for E-cadherin. Duplicate Matrigel invasion chambers were used for each antibody; one each for staining invasive cells non-invasive cells. Cells not being stained were removed from each insert, and cells of interest were fixed to the membrane in ice-cold methanol for 5 min followed by 3 PBS washes. Non-specific antibody binding sites were blocked for 30 min with PBS/0.3% fish skin gelatin (Sigma, St. Louis, MO) and briefly washed. Cells were incubated with a mouse antibody (25°C, 1.5-2 hrs) to E-Cadherin (Abcam; 1:60 dilution). Following 3 PBS washes, rabbit anti-mouse antibody (Texas Red conjugated; Abcam) was added for 30 min at room temp and membranes received 3 PBS washes and then

air-dried. Membranes were mounted on glass slides with Vectashield containing DAPI (Vector Laboratories, Burlingame, CA). Cells were visualized with an Olympus IX70 fluorescent microscope, separate images were obtained for Texas Red and DAPI fluorescence, and images were overlaid with Adobe Photoshop (Adobe Systems, Inc; San Jose, CA). “Non-invasive” cells were stained on the topside of the membrane, while “Invasive cells” were stained on the underside of the membrane. Controls using the Texas Red-coupled secondary antibody and no primary antibody indicated that little, if any, fluorescence was contributed by non-specific binding of this antibody (data not shown).

Fig. 3.

Gene Expression Analysis of Invasive Prostate Cancer Cells. RNA from non-invasive “top” and invasive “bottom” cells was isolated and analyzed using microarrays as described in “Materials and Methods.” Changes in gene expression are relative to the “top” data sets for each cell line. A. Invasive DU145 and LNCaP cells go through EMT as judged by change in expression of the EMT-related genes, where green indicates a decrease and red indicates an increase, black indicates no change, and gray indicates missing data due to array imperfections or data that fell below cut-offs. B. ‘Stemness’ genes are preferentially expressed in the invasive subpopulation of PCSC1, DU145 and LNCaP cells. “Depleted” are LNCaP cells depleted of the CD44⁺CD24⁻ cells. “Stem” are LNCaP CD44⁺CD24⁻ cells.

Fig. 4. Purified Prostate Cancer Stem Cells are Invasive. Magnetic bead purified CD44⁺ and CD44⁻ cells were evaluated for migration across control membranes and Matrigel-coated membranes. A. LNCaP B. DU145 C. Invasion percentage relative to control migration (average of 3 experiments $\pm$ standard deviation). “Bulk” refers to the unsorted cell population.

Fig. 5. Top 20 functional categories of genes as determined by Ingenuity Pathway analysis showing an equal to or greater than 1.8-fold change in Matrigel-invasive cells compared with non-invasive cells. Y-axis shows functional category while X axis are the number of genes in each category.

Fig. 6. Cell Signaling Pathways Active in the Invasive Subpopulation. Data extracted from the microarrays to examine key genes in the A. Wnt signaling pathway; B. Hedgehog pathway; C. Notch pathway; and D. Nanog/Sox2/Oct3 network. Fold changes in expression are depicted for the invasive cells relative to the non-invasive cells. The asterisk indicates data averaged from multiple oligonucleotides that bind different regions of the same gene.

Fig. 7.
Depiction of Cellular Changes Upon Matrigel Invasion

Table 1

GSEA Overlaps

Gene Set Name	Description	Number of Genes in Overlap	P value
1. STEMCELL_NEURAL_UP [1838]	Enriched in mouse neural stem cells, compared to differentiated brain and bone marrow cells	243	6.77×10^{-3}
2. STEMCELL_HEMATOPOIETIC_UP [1452]	Enriched in mouse hematopoietic stem cells, compared to differentiated brain and bone marrow cells	184	3.35×10^{-3}
3. ALZHEIMERS_DISEASE_DN [1121]	Downregulated in correlation with overt Alzheimer's Disease, in the CA1 region of the hippocampus	140	4.59×10^{-5}
4. BYSTRYKH_HSC_TRANS_GLOCUS [977]	Trans-regulated hematopoietic stem cell (HSC) transcripts detected in bone marrow tissue (high likelihood ratio statistic (LRS) value and genome-wide linkage $P < 0.005$)	103	3.21×10^{-5}
5. BRCA_ER_POS [527]	Genes whose expression is consistently positively correlated with estrogen receptor status in breast cancer - higher expression is associated with ER-positive tumors	56	1.53×10^{-3}
6.HSC_EARLYPROGENITORS_ADULT [458]	Up-regulated in mouse hematopoietic early progenitors from adult bone marrow (Early Progenitors Shared + Adult)	53	1.01×10^{-2}
7. HSC_EARLYPROGENITORS_FETAL [455]	Up-regulated in mouse hematopoietic early progenitors from fetal liver (Early Progenitors Shared)	53	1.12×10^{-2}
8. MATSUDA_VALPHAINKT_DIFF [493]	Differential gene expression between developmental stages of Val4i NKT cells	48	3.37×10^{-4}
9. KUMAR_HOXA_DIFF [468]	Genes that were significantly different between wild-type, preleukemic, and leukemic mice	48	1.28×10^{-3}
10. POD1_KO_UP [416]	Up-regulated in glomeruli isolated from Pod1 knockout mice, versus wild-type controls	47	9.19×10^{-3}

Table 2

Tumorigenicity of invasive prostate cancer cells

Cell Type	Number of animals with tumors	Ave. date of detection (days)	Ave. tumor volume (mm ³) ¹
100 DU145 cells (Matrigel invasive)	5/5	38±3	495±217 ²
100 DU145 cells (Non-invasive)	2/5	61±19	137±176 ²
1000 PCSC1 (Matrigel invasive)	5/5	≤40	506±549 ³
1000 PCSC1 (Non-invasive)	2/5	44±5	197±161 ³
1000 PCSC1 (bulk)	5/5	27±8	596±497 ⁴

¹
 $v=\pi/6 \times L \times W \times H$ ² Measurement at day 70³ measurement at day 74⁴ measurement at day 56