Chapter 7

EPITHELIAL-MESENCHYMAL MOLECULAR INTERACTIONS IN PROSTATIC TUMOR CELL PLASTICITY

Mary J.C. Hendrix[1,4], Jun Luo[5], Elisabeth A. Seftor [1,4], Navesh Sharma[1,6], Paul M. Heidger Jr.[1,4], Michael B. Cohen[2,4], Robert Bhatty[1], Jirapat Chungthapong[1], Richard E.B. Seftor[1,4] and David Lubaroff[3,4]

[1]*Departments of Anatomy and Cell Biology, University of Iowa, Iowa City, IA, USA*
[2]*Pathology, University of Iowa, Iowa City, IA, USA*
[3]*Urology, College of Medicine, University of Iowa, Iowa City, IA, USA*
[4]*The Holden Comprehensive Cancer Center, University of Iowa, Iowa City, IA, USA*
[5]*Brady Urological Institute, Johns Hopkins Medical Institutions, Baltimore, MD, USA*
[6]*University Health Sciences, College of Osteopathic Medicine, Kansas City, MO, USA*

Abstract: Tumor cell plasticity poses a significant clinical challenge in that the fate and function of tumor cells can be elusive until a tumor mass is evident. An overview of key molecular events in prostate cancer, initiated by an epithelial-to-mesenchymal transition, highlights the cooperative interactions of diverse prostatic subpopulations within heterogeneous tumors. A remarkable example of plasticity is demonstrated by subpopulations of E-cadherin-positive and E-cadherin-negative tumor cells working in concert to form *de novo* vasculogenic-like networks while expressing vascular-associated genes, called vasculogenic mimicry, resulting in acquisition of the metastatic phenotype. A better understanding of the molecular mechanisms underlying prostate tumor cell plasticity may provide new prognostic markers for clinical diagnosis and novel therapeutic intervention strategies for disease management.

Key words: prostate cancer, plasticity, E-cadherin, epithelial-mesenchymal transition

1. INTRODUCTION

Prostate cancer is the most commonly diagnosed type of cancer and the second-leading cause of cancer-related deaths in American men. With the advent of the prostate-specific antigen (PSA) test, the number of newly diagnosed cases reached 200,000 in 2000, accounting for over 35% of all

R.J. Ablin and M.D. Mason (eds.), Metastasis of Prostate Cancer, 127–141.

cancers affecting men (1). Statistics also indicate that after lung cancer, prostate cancer is the leading cause of cancer-related deaths among men in the U.S., with an estimated 30,000 deaths each year. Most remarkably, post-mortem examination has found that as many as 30% of men over age 50 harbor microscopic foci of prostate cancer, indicating the extremely high prevalence of latent prostate cancer which is not detected clinically during the life span of men (2, 3, 4). Although early-staged prostatic tumors are relatively benign, a subset of these tumors progresses to become invasive, metastatic, and life-threatening cancers. Thus, understanding the molecular mechanisms underlying prostate cancer progression is crucial to the development of therapeutic strategies to manage this disease.

Tumor metastasis involves a series of sequential steps, which include the acquisition of cellular motility and invasiveness (for review, see 5). A crucial event in prostate cancer progression is the transition from the noninvasive to the invasive phenotype, demonstrated by the epithelial-to-mesenchymal transition (6-8). This transition involves a series of molecular alterations, resulting in altered cell-substrate attachment, decreased cell-cell adhesion, and increased cellular motility and invasive ability.

Another significant clinical challenge in the detection and management of prostate cancer is tumor cell plasticity, in that the fate and function of tumor cells can be elusive until a tumor mass is evident. A remarkable example of tumor cell plasticity has recently been described as vasculogenic mimicry in aggressive uveal and cutaneous melanoma (9–13), ovarian (14) breast carcinoma (15, 16), and prostate cancer (17). This new concept suggests that aggressive tumor cells can mimic vascular cell phenotypes, such as endothelial cells, and possibly perform vascular functions (13). These observations may provide new prognostic markers for tumor detection, clinical diagnosis and novel therapeutic intervention strategies.

1.1 Importance of E-Cadherin in Prostate Biology

The majority of diagnosed prostate cancer remains localized and never produces dramatic clinical symptoms, while a subset of these cancers (roughly 1 in 5) progress to invasive and metastatic cancers that are life threatening. One of the key features of invasive and metastatic prostate cancer, as well as other carcinomas, is the downregulation of E-cadherin expression – a cell adhesion molecule responsible for normal epithelial maintenance. E-cadherin (originally called uvomorulin, L-CAM, cell-CAM 120/80, or Arc-1) is a 120 kDa glycoprotein mediating Ca^{2+}-dependent homophilic cell-cell adhesion between epithelial cells (18–20). As illustrated in Figure 1, the extracellular domain of the E-cadherin molecule

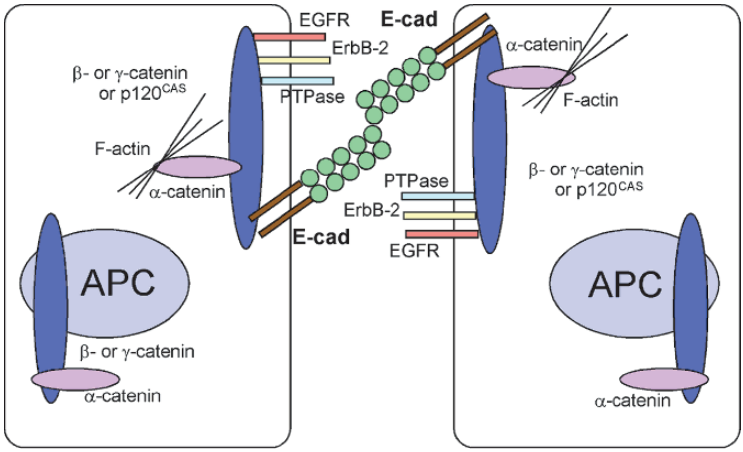


Figure 1. Molecules associated with the E-cadherin/catenin complex.

on the cell membrane binds to that of another molecule on the opposite membrane of another cell. Intracellularly, E-cadherin is linked to the cytoskeletal actin filaments through interactions with the catenins (α-, β-, γ-catenin/plakoglobin); (21). Interactions between E-cadherin and key cytoskeletal proteins through the catenins confer stability of the cell-cell adherens junctions. Disruption of these interactions results in the loss of cell adhesion and dissociation of epithelial sheets – the initiation of epithelial-mesenchymal molecular interactions. In fact, transgenic mice deficient in E-cadherin die *in utero* because of defective formation of the initial epithelium, the trophectoderm (22).

1.2 New Advances in the Dunning Rat Model for Prostate Cancer Biology

The field of prostate cancer research has been limited by the lack of reliable experimental models. Therefore, we devoted considerable effort to developing unique Dunning rat cancer cell lines that would allow the study of the heterogeneous components of prostate adenocarcinomas (23), using an integrated *in* vivo and *in* vitro experimental strategy (shown in Figure 2).

The heterogeneous tumor cell line R3327-5' arising from a subcutaneous injection of R3327-5, a cell line isolated from the original R3327 Copenhagen rat tumor, was generated and propagated in our laboratory. Cloning of individual populations from this heterogeneous cell line on a morphological basis yielded several homogeneous cell clones with different morphological,

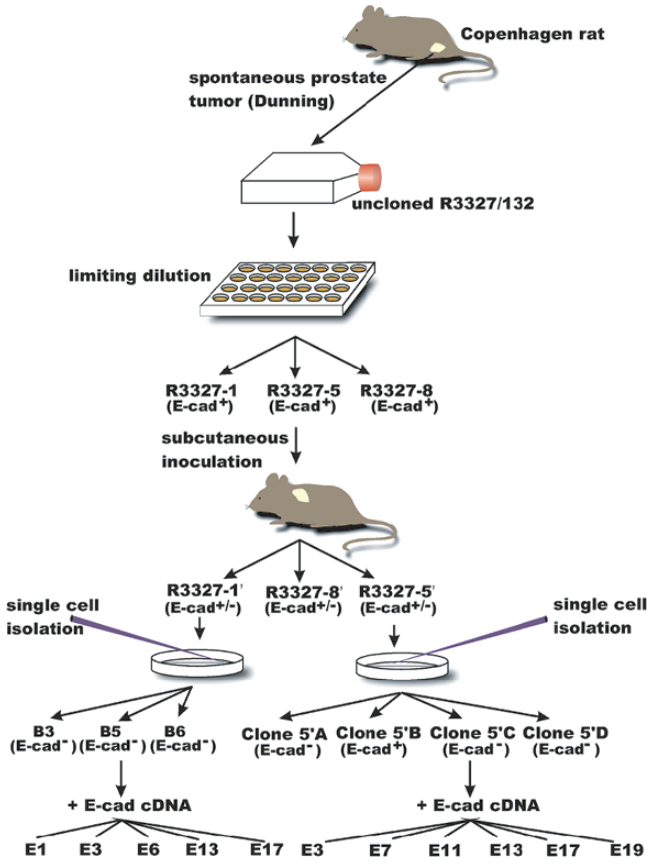
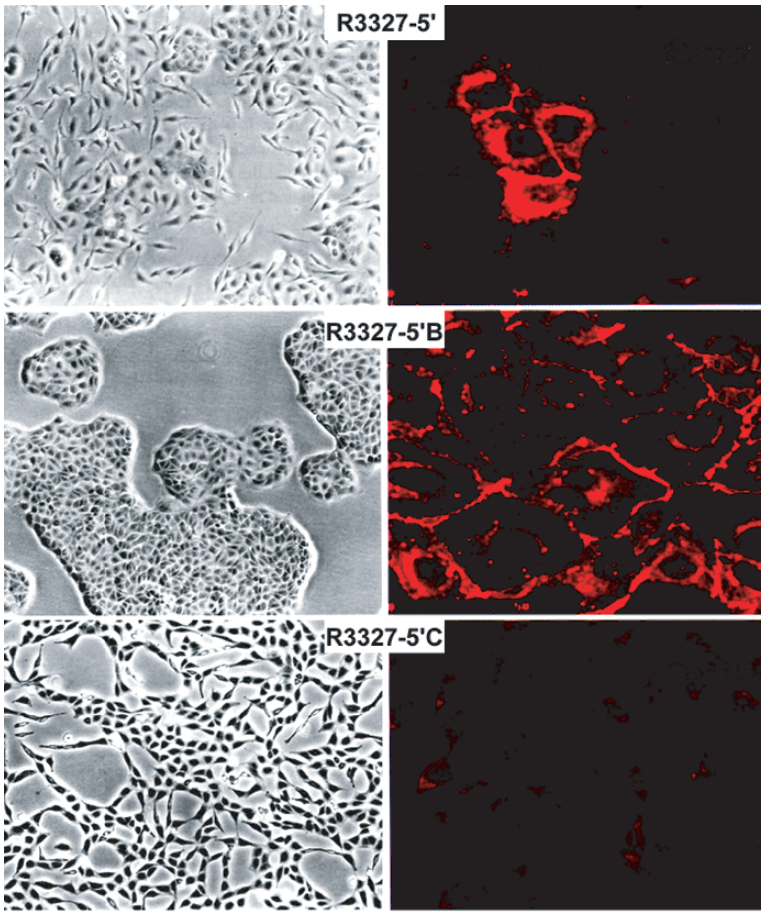


Figure 2. Experimental approach flow chart for the development of new Dunning rat cell lines. Cultured late passage cells from a spontaneous rat prostate tumor were cloned by a limiting dilution procedure. R3327-5, a poorly invasive E-cadherin-positive subline in vitro, was injected subcutaneously into the right flank of male Copenhagen rats. Single cells were cloned from the heterogeneous R3327-5' culture of the excised tumor to yield four morphologically distinct clones, which were subsequently assayed for differences in morphology, E-cadherin expression, and invasive and metastatic potential. In addition, select E-cadherin-negative clones were stably transfected with E-cadherin cDNA to yield several experimental cell clones.

tumorigenic, metastatic and invasive properties. The phenotypic characteristics distinguishing these subpopulations are demonstrated in Figure 3. R3327-5'B cells grow in clusters, are E-cadherin-positive, form large subcutaneous tumors but are poorly invasive and modestly metastatic compared with the R3327-5'A, R3327-5'C and R3327-5'D cells, which grow in individual fibroblast-like patterns, are E-cadherin-negative and are highly invasive and metastatic without being tumorigenic at subcutaneous injection sites.



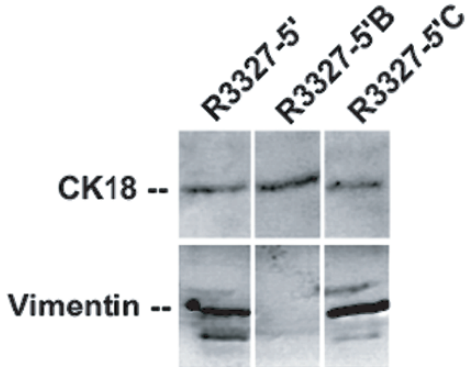


Figure 3. Left three panels show phase contrast, light micrographs of cell cultures: parental R3327-5'; and clones R3327-5'B and R3327-5'C. The cultures exhibited distinctive morphologies. Note the sparse, fibroblastic-like, spindle-shaped (continued on page 132)

Our previous studies utilizing these clones have shown differences in immunological response elicited by these tumor cells following inoculation into a syngeneic host (24). The parental cell lines and subpopulations were all cytokeratin positive; however, the fibroblastic-like subpopulation was vimentin-positive, while the E-cadherin-epithelial-like subpopulation was negative for the mesenchymal vimentin marker – indicating the phenotypic distinction between the two derivatives. Previous studies have shown the biological and clinical relevance of the coexpression of cytokeratin and vimentin intermediate filament proteins in several tumor types, including breast cancer and melanoma, which is indicative of an aggressive phenotype (25–28).

Although it is unlikely that E-cadherin is the sole determining factor for tumor invasion and metastasis, we have recently shown that E-cadherin plays a central role in reducing the cellular invasiveness of prostatic adenocarcinoma, due in part to the downregulation of matrix metalloproteinases-2 (MMP-2) activity (29), summarized in Table 1. These observations provide a direct connection between the re-expression of E-cadherin, a mesenchymal-to-epithelial phenotype transition, accompanied by changes in MMP activity – important biological targets for therapeutic intervention.

1.3 Prostatic Vasculogenic Mimicry

The recent observation of the cooperative interactions of the E-cadherin-positive (epithelial-like phenotype) and E-cadherin-negative (fibroblastic-like phenotype) subpopulations resulting in vasculogenic mimicry serve as an excellent example of prostatic plasticity – an epithelial-to-vascular phenotype transition (17), illustrated in Figure 4. In this study, Dunning rat R3327-5', R3327-5'A, R3327-5'B cells were assessed for their ability to form vasculogenic structures on 3-dimensional cultures of basement

Figure 3. (continued) cells mingled with epithelial-like, polygonal cells in the parental culture; the islands of closely adherent polygonal cells are the epithelial-like clone R3327-5'B; and the interlacing pattern of fibroblastic-like cells are characteristic of clone R3327-5'C cultures. (Magnification=220X). Right three panels show E-cadherin expression in cell cultures by immunofluorescence microscopy. Positive staining was only observed in R3327-5'B and in the epithelial clusters in the heterogeneous R3227-5' parental line. (Magnification=1400X). Western blot analysis, shown in the lowest right panel beneath the photomicrographs, demonstrated the presence of cytokeratin 18 (CK18) in all 3 cell lines tested, indicative of an epithelial marker. Vimentin, a mesenchymal marker, was found in the parental R3327-5' cells and in the fibroblastic-like R3327-5'C cells.

Table 1. Reduced invasive ability and MMP-2 activity in E-cadherin-transfected tumor cells

Cell Line	Invasive ability	MMP-2 activity
Clone5'C (sham)	20.1 ± 1.0[a]	1.00[b]
E7	6.8 ± 1.4	0.33
E11	6.7 ± 0.7	0.38
E13	6.6 ± 0.8	0.26
E19	6.0 ± 0.4	0.44

[a]Invasion is measured as the percentage of prostatic tumor cells capable of invading a basement membrane-coated polycarbonate membrane over 24 hours within a membrane invasion culture system (MICS) chamber compared with the total number of cells seeded (SE±; n = 6 wells per parameter and run in duplicate experiments). Clone 5'C (R3327-5'C) is an E-cadherin-negative Dunning cell clone that was stably transfected with E-cadherin cDNA and compared with the sham transfected control, as previously described [29]. Data from 4 of the experimental clones (E7, E11, E13 and E19) are presented.

[b]The zymograms were digitized and the area associated with the MMP-2 activity determined for each cell line and compared to the value for Clone 5'C (normalized to a value of 1.00).

membrane matrix (matrigel) or collagen I gels. Heterogeneous populations of rat parental R3327-5' cells formed vasculogenic-like tubular structures, which proceeded to anastomose, forming networks similar to embryonic vascular networks. Microscopic observations of the network evolution over three weeks revealed steady outgrowths which developed into tubular patterns interconnecting spheroidal nests of cells. Co-cultures of GFP-labeled R3327-5'B plus unlabeled R3327-5'A cells resulted in vasculogenic-like tubular networks identical in structure to those formed by the heterogeneous parental cell line, with the GFP-labeled R3327-5'B cells forming the tubular structures and the R3327-5'A cells forming the support background. Furthermore, microinjection of fluorescent dye into the tubular networks demonstrated that the dye followed the course of the vasculogenic-like networks, thus demonstrating the perfusability of the tubular networks. Most noteworthy was the finding that 3-D cultures of R3327-5'A and R3327-5'B, grown independently, did not yield vasculogenic-like networks until they were co-cultured to reflect the heterogeneous composition found in the parental R3327-5' cell line (17).

Scanning electron micrographs of the 3-D cultures demonstrated the presence of cell-lined tubular networks with widely varying lumenal diameters interconnecting nests of dense tumor cell clusters. Transmission electron microscopic analysis of these extending structures revealed that the tubular structures consisted of a lumen bound by tumor cells exhibiting a polarity

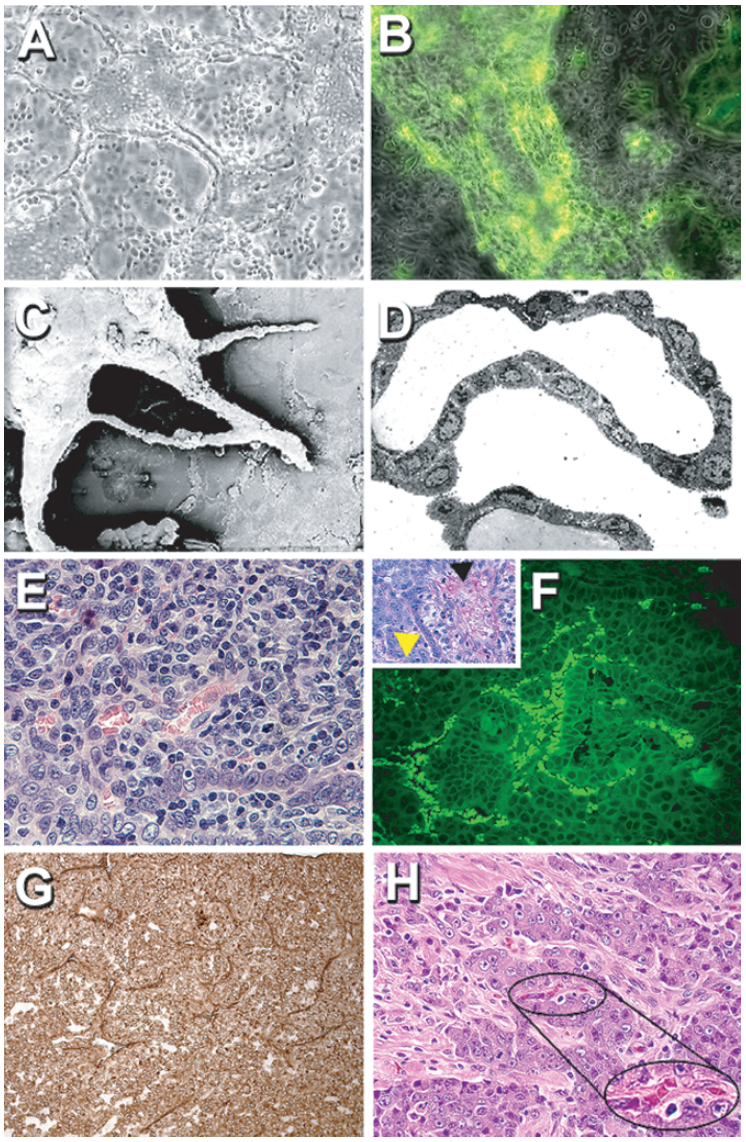


Figure 4. Microscopic demonstration of vasculogenic mimicry by rat prostatic tumor cells on 3-D matrices in culture and *in situ*. Phase and fluorescence microscopy of in vitro tubular network formation by the rat heterogeneous cancer cell line R3327-5' (A) and a co-culture of R3327-5'A with GFP-labeled R3327-5'B cells (B). Scanning electron microscopy (SEM; C) and transmission electron microscopy (TEM; D) micrographs of R3327-5' cells grown on a 3-D matrix. SEM shows the presence of tubular, cell-lined extensions or protrusions from a cluster of tumor cells (C), while TEM shows the presence of lumen within the tubular, cell-lined extensions (D). Evidence for putative *in vivo* vasculogenic mimicry following injection of Dunning tumor cells into Copenhagen rats is shown in E, F and G.

reversed from that expected in glandular epithelium. Polarized tumor cells were arranged with their basal aspects toward the tubular lumen and their microvillous apical surfaces toward the exterior. The lining cells were connected by desmosomal junctions on the outer aspect of the tubular wall, forming tubular, blood vessel-like structures.

In the *in vivo* studies, circumstantial evidence was presented in support of *in situ* vasculogenic mimicry by the presence of tumor cell-lined, erythrocyte-containing channels within rat tumors and various grades of human prostatic adenocarcinoma. Rat tumors arising from s.c. injection of the Dunning R3327-5' cell line into shoulder flanks averaged 15–20 mm in diameter and exhibited little-to-no necrosis. Several of the tumor-lined channels could be seen in the vicinity of conventional endothelial-cell lined vasculature. Presence of plasma and erythrocytes in rouleaux formation within many of these channels was clearly revealed using short wavelength fluorescence. Localization of laminin within the tumors arising from the injection of Dunning R3327-5' cells into nude mice demonstrated laminin-stained channels in regions of high vascularity (*i.e.*, erythrocytes) between clusters of laminin-positive prostatic tumor cells. In addition, eighteen histological sections of low and high grade human prostatic adenocarcinoma were examined for the presence of erythrocyte-containing channels or spaces. There was little-to-no evidence of tumor-lined channels in Gleason grades 2, 3 and 4 tumors. However, Gleason grade 5 tumors revealed some tumor-lined channels containing erythrocytes in rouleaux formation. Endothelial-lined blood vessels were identified in all cases, regardless of disease severity.

A multi-probe ribonuclease protection assay was used to characterize the expression of a series of vascular-associated markers by the Dunning rat prostate cancer cells. All of the cell lines assessed expressed at least some of

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Figure 4. (continued) Subcutaneously (s.c.)injected R3327-5' tumor cells generated large tumors with little-to-no necrosis. Microscopic study of the tumors using brightfield (E and F, inset) and short wavelength fluorescence (F) to highlight erythrocyte containing spaces, revealed the presence of tumor cell-lined channels containing erythrocytes in rouleaux formation and plasma. These tumor cell-lined channels (yellow arrowhead) often occurred in close proximity to traditional endothelial-lined vessels (black arrowhead; F, inset). Localization of laminin expression in s.c. tumors arising from injection of R3327-5' cells into nude mice revealed the presence of laminin stained networks between clusters of laminin-positive tumor cells (G). While the presence of conventional endothelial-lined vasculature was predominantly seen in histopathological sections of human prostatic adenocarcinoma tumors from Gleason grades 2 to 4, erythrocyte containing tumor cell-lined channels were observed in Gleason grade 5 tumors (H). Original magnifications, X 100 (A), X 200 (B, F and F inset), X 300 (C), X 2250 (D), X 630 (E), X 400 (G and H).

the markers to varying degrees. Specifically, the results demonstrated that TIE-1, thrombin-receptor, TIE-2, CD-31, endoglin, angiopoietin, and VEGF were expressed by R3327-5'B cells, whereas endoglin, angiopoietin and VEGF were expressed at the message level by R3327-5'A and R3327-5'C cells at varying levels, as summarized in Table 2.

The recent prostatic vasculogenic mimicry findings lend further credence to previous reports on melanoma, breast and ovarian cancers showing vasculogenic-like networks formed by tumor cells *in vitro* and tumor cell-lined channels *in vivo* (9–17), which may account for a subcategory of highly aggressive, non-angiogenic tumors. *In situ*, the presence of tumor cell-lined channels containing erythrocytes in rouleaux formation (not random leakage from vasculature) or just plasma was confirmed in advanced rat and human prostatic adenocarcinoma, and may represent a potential alternative mode of dissemination and possibly tumor perfusion. Recent studies by Shirakawa and colleagues (16) have demonstrated unique hemodynamic imaging associated with vasculogenic mimicry in aggressive inflammatory breast cancer. This is the first study to provide experimental evidence characterizing the microcirculatory differences between angiogenesis and vasculogenesis in a cancer model. The molecular underpinnings of the vascular phenotype observed in various tumor models is slowly becoming elucidated through various molecular analyses. Studies with melanoma, ovarian and breast carcinoma, and most recently prostate cancer, are collectively revealing the expression of multiple molecular phenotypes by the aggressive tumor cells indicative of a genetically deregulated genotype, similar to an embryonic phenotype (9–15, 17, 30, 31). Interestingly, the formation of a microcirculation in the absence of endothelial cells has been observed in normal embryonic tissue. Adoption of an endothelial phenotype by cytotrophoblasts, including the expression of vascular markers, as they participate in the establishment of the human placenta and primordial circulation has been reported

Table 2. Expression of vascular/angiogenic markers by rat prostatic tumor cells

Cells	TIE-1	Thrombin Receptor	TIE-2	CD31	Endoglin	Angio-poietin	VEGF
R3327-5'A	−[a]	−	−	−	++	+	+++
R3327-5'B	++	++	+	+	++	++	++
R3327-5'C	−	±	−	−	++	+	+++

[a]The amount of RNAse-protected mRNA was determined by digitizing the exposed film and determining the area of the markers that were then ranked from + → +++ relative to the most abundantly expressed mRNA (VEGF).

(32–34), which indicates the ability of more differentiated cells to perform embryonic vasculogenic-like functions (called pseudovasculogenesis).

Indeed, the intriguing differences in the expression of vascular genes, such that the R3327-5'B cells expressed more of an endothelial phenotype while the R3327-5'A cells produced higher levels of VEGF, may suggest important changes in the heterogeneous tumor phenotype as it evolves from a less aggressive state to a more aggressive state. These results strongly support the contention that cooperativity of multiple cell phenotypes is necessary for successful prostatic tumor development.

2. CONCLUSIONS

A hypothetical model depicting prostate tumor cell plasticity is illustrated in Figure 5, based on the interpretation of data derived from *in vitro* and *in vivo* models of prostatic adenocarcinoma. The importance of E-cadherin in maintaining the integrity of the epithelial phenotype is well known, and its transient downregulation coincides with observations of epithelial-to-mesenchymal transition. What is not well understood is the molecular trigger that catalyzes this event leading to sequential steps in the metastatic cascade, including acquisition of the invasive/aggressive phenotype involving the coordinate regulation of MMP activity and the initiation of remodeling the tumor microenvironment. The molecular signals involved in the dynamic reciprocity between the tumor cells and their microenvironment are the focus of intense investigation, and these studies are key to the development of new therapeutic strategies. The heterogeneity of prostatic adenocarcinomas poses an experimental challenge as well as an opportunity for experimental manipulation. Our investigative team has attempted to address this challenge by isolating distinct subpopulations of tumor cells with fibroblastic and epithelial phenotypes. The data indicate that these subpopulations cooperate to engage in vasculogenic mimicry, in which the epithelial-like subpopulation expressesendothelial/vascular-associated molecules, and the fibroblastic-like subpopulation expresses vascular-stimulating growth factors. It is tempting to speculate that these tumor cells have the potential to revert to an embryonic-like phenotype, based on their *de novo* formation of primitive vasculogenic-like networks reminiscent of embryologic events. However, the biological significance of this observation remains to be elucidated. In this regard, further ambiguity may result from studies attempting to document the increase in microvascularity with a corresponding increase in tumor burden and severity, in which the results have been somewhat perplexing and inconclusive. A contributing factor to

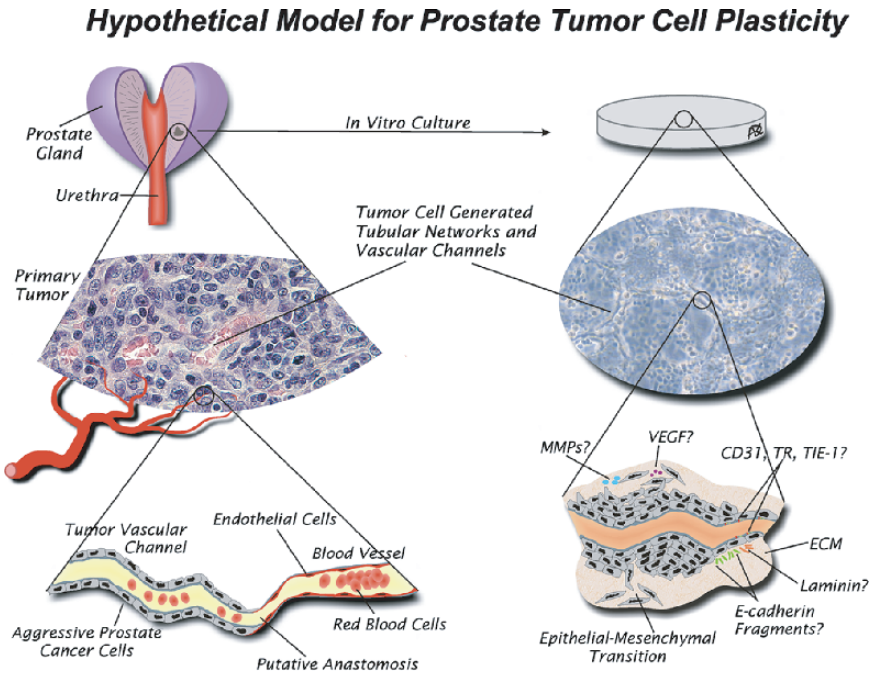

Figure 5. Hypothetical model for prostate tumor cell plasticity, including epithelial-mesenchymal molecular transition and embryonic-like vasculogenic mimicry by aggressive heterogeneous prostatic tumor cells. Synergistic biomechanical and molecular interactions of individual components in a primary, heterogeneous tumor result in the formation of tumor-lined, channel-like structures. These occur within the tumor (shown containing erythrocytes in rouleaux formation) in the vicinity of endothelial-lined vasculature. This phenomenon is observed in 3-D cultures *in vitro* where perfusable tumor cell-lined tubular networks connecting spheroids of tumor cells express laminin in addition to vascular-associated genes.

these mixed results has been the reliance on conventional endothelial and other vascular markers to identify tumor neovasculature (35) and tendency to ignore the staining of tumor-lined structures whose presence was hitherto unexplained. Our results suggest that a growing tumor mass may contain tumor-cell lined channels which express vascular markers that would stain for typical endothelial markers, in addition to conventional endothelial-lined vasculature. Whether this observation reflects vessel co-option, mosaic vasculature (tumor and endothelial cells; (36)), or putative anastomosis between tumor- and endothelial-lined vasculature remains enigmatic, and will require more precise tools of investigation. Also, investigations addressing the potential clinical relevance of tumor cell-lined vasculature are critical to our understanding of their significance. The field of tumor vascularization is rapidly evolving, and offers ample opportunity for new insights

(37). For example, recent observations showing aggressive melanoma cells forming new vasculature in ischemic tissues uniquely demonstrated the importance of the microenvironment in cell fate determination (13). Similar experimental approaches are encouraged in prostate cancer research to explore the extent of tumor cell plasticity and its profound ramifications, particularly with respect to detection, diagnosis and management.

ACKNOWLEDGEMENTS

This research has been supported by grants from the National Cancer Institute CA88043 and the Wallace Research Foundation. The authors gratefully acknowledge Dr. Dawn Kirschmann for her artistic contributions, and Katherine Walters of the University of Iowa Central Microscopy Research Facility for her electron microscopic expertise.

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