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Tumorigenesis and Neoplastic Progression

Epithelial-to-Mesenchymal Transition Leads to Docetaxel Resistance in Prostate Cancer and Is Mediated by Reduced Expression of miR-200c and miR-205

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Docetaxel is a standard chemotherapy for patients with metastatic prostate cancer. However, the response is rather limited and not all of the patients benefit from this treatment. To uncover key mechanisms of docetaxel insensitivity in prostate cancer, we have established docetaxel-resistant sublines. In this study, we report that docetaxel-resistant cells underwent an epithelial-to-mesenchymal transition during the selection process, leading to diminished E-cadherin levels and up-regulation of mesenchymal markers. Screening for key regulators of an epithelial phenotype revealed a significantly reduced expression of microRNA (miR)-200c and miR-205 in docetaxel-resistant cells. Transfection of either microRNA (miRNA) resulted in re-expression of E-cadherin. Functional assays confirmed reduced adhesive and increased invasive and migratory abilities. Furthermore, we detected an increased subpopulation with stem cell-like properties in resistant cells. Tissue microarray analysis revealed a reduced E-cadherin expression in tumors after neoadjuvant chemotherapy. Low E-cadherin levels could be linked to tumor relapse. The present study uncovers epithelial-to-mesenchymal transition as a hallmark of docetaxel resistance. Therefore, we suggest that this mechanism is at least in part responsible for chemotherapy failure, with implications for the development of novel therapeutics. *(Am J Pathol 2012, 181:2188–2201; http://dx.doi.org/10.1016/j.ajpath.2012.08.011)*

Systemic polychemotherapy, including the microtubule inhibitor docetaxel, is the state-of-the-art treatment for different types of cancer. Unfortunately, docetaxel-based chemotherapy often encounters several undesirable adverse effects, and many patients initially do not respond to docetaxel or acquire resistance during treatment. Little has been known about the molecular mechanisms responsible for initial or acquired docetaxel insensitivity in prostate cancer, even though recent studies suggested that STAT1,[1] the kinase PIM1,[2] and β3 tubulin[3] might be involved in this process. In castration-refractory prostate cancer, docetaxel treatment is the first chemotherapy that showed a significant benefit concerning survival,

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reduced pain, decreased prostate-specific antigen serum levels, and enhanced quality of life.[4,5] However, patient survival is prolonged just for a few months.[6] Therefore, the identification of molecular mechanisms underlying docetaxel resistance in prostate and other cancers becomes an issue of great interest.

It is well-known that cells in primary tumors that acquire increased invasive and migratory abilities, in combination with loss of adhesion molecule expression, are the source and origin of metastases. These cells change from an epithelial to a motile mesenchymal phenotype during a process called epithelial-to-mesenchymal transition (EMT). EMT is crucial for epithelial cancer cells to acquire an invasive phenotype and has already been linked to a metastatic state and poor prognosis in cancer.[7,8] Key signals that lead to EMT trigger expression of a variety of transcriptional repressors, including ZEB1, ZEB2/SIP1, Snail, and Twist.[9–11] These repressors are the intracellular mediators of EMT by binding to E-box elements of genes, such as *CDH1*, that encode for the adhesion protein E-cadherin. After binding, they recruit histone deacetylases and other corepressors to facilitate transcriptional repression of E-cadherin. Reduced E-cadherin expression is characterized by loss of cell-cell adhesion, notably by disruption of the cadherin-catenin complex along with other signaling events, which, in turn, lead to loss of cell polarization and the acquisition of a migratory mesenchymal phenotype.[9,12] In addition, increased expression of mesenchymal markers, such as vimentin and N-cadherin, is indicative of a morphological change.[13] Recent studies have identified miRNAs, especially members of the miR-200 family, as important regulators of EMT.[14,15] They repress ZEB1 and ZEB2 and enforce the epithelial phenotype. These miRNAs can be down-regulated in different types of cancer.[16,17] Those studies have also linked EMT and loss of E-cadherin with induction of stem cell-like phenotypes in various cancers.[18] In summary, EMT-driven E-cadherin loss and cell morphological change to a mesenchymal phenotype are hallmarks of progression to invasive and metastatic carcinomas. Therefore, we asked whether these cellular processes can be a source of docetaxel resistance and disease progression.

In the present study, we report that docetaxel resistance is characterized by the appearance of EMT, which is associated with a reduced expression of miR-200c and miR-205. Docetaxel-resistant cells showed a reduced E-cadherin and an increased vimentin expression accompanied by induced expression of stem cell markers compared with parental cells. These molecular changes resulted in the establishment of a highly aggressive docetaxel-resistant cancer cell phenotype *in vitro*. Furthermore, we demonstrate that E-cadherin expression *in vivo* decreases during chemotherapy and is associated with disease relapse.

Materials and Methods

Cell Culture and Chemicals

Human prostate cell lines PC3 and DU-145 were obtained from ATCC (Rockville, MD). Docetaxel-resistant cell lines PC3-DR and DU-145-DR were established in the presence of increasing concentrations of docetaxel (Sigma-Aldrich, St. Louis, MO) up to a final concentration of 12.5 nmol/L. Cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 20 mmol/L glutamine. Human bone marrow endothelial cells (HBMEC-60) were maintained in medium 199, including glutamine, and 25 mmol/L HEPES supplemented with 10% FCS and 10% human serum. Prostate fibroblasts (PFs; patient code 172) were established and cultured in Dulbecco's modified Eagle's medium, low glucose, supplemented with 10% FCS and 20 mmol/L glutamine. Images of cells were taken using IC Capture software version 2.2 with an Olympus CK2 microscope (Olympus, Vienna) equipped with an Imaging Source Camera DFK31F03 (Imaging Source, Bremen, Germany). The identity of the used cell lines was confirmed by short tandem repeat analysis.

Western Blot Analysis

Western blot analysis was performed as previously described.[19] The following antibodies were used: anti-glyceraldehyde-3-phosphate dehydrogenase (1:100,000; Chemicon, Vienna), anti-E-cadherin (1:500; Becton Dickinson, Heidelberg, Germany), anti-vimentin (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), anti–Twist-1 (1:500; Santa Cruz Biotechnology), anti-Snail (1:500; New England Biolabs, Ipswich, MA), and anti-cleaved poly (ADP-ribose) polymerase (1:1000; Promega, Madison, WI).

[^{3}H]Thymidine Incorporation and MTT Assay

Cells were seeded at a density of 3.5×10^3 per well in triplicate onto separate 96-well plates in the presence or absence of docetaxel and incubated for 72 hours. Thymidine incorporation was measured as previously described.[19] As an index of cell viability, an MTT assay (Biomedica, Vienna) was performed according to the manufacturer's protocol.

Apoptosis Measurement

The percentage of apoptotic cells was measured after incubation of cells in the absence or presence of docetaxel for 72 hours or after double transfection with miRNAs after 6 days by flow cytometry, as previously described.[19]

Clonogenic and Spheroid Assay

Colony formation assays were performed as previously described.[20] For a spheroid assay, six-well plates were coated with Matrigel (1:1 Matrigel/medium mix; Becton Dickinson). Cells, 1×10^4, were seeded onto coated wells, covered additionally with Matrigel, and incubated for 14 days. Formed spheroids were harvested and formalin-fixed and paraffin-embedded (FFPE) for immunohistochemistry (IHC).

PCR Array and RT-qPCR

Total RNA was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany), and cDNA synthesis was performed using a cDNA RT^2 first-strand kit (Qiagen). EMT array was performed on an ABI Prism 7900HT system (Applied Biosystems, Carlsbad, CA) with four biological replicates for each cell line on RT^2 Profiler PCR-array PAHS-090 plates (Qiagen), following the manufacturer's protocol. Quantitative RT-PCR (RT-qPCR) for confirmation of regulated genes was performed as previously described.[19] TATA-Box–binding protein was chosen as an endogenous expression standard. For E-cadherin, vimentin, ZEB1, and ZEB2, TaqMan gene expression assays (Hs01013955_m1, Hs00185584_m1, Hs00232783_m1, and Hs00207691_m1) were used.

miRNA Screening and Transfection

Total RNA was isolated using the miRNeasy mini kit (Qiagen), and cDNA synthesis was performed using a universal cDNA synthesis kit (Exiqon, Vedbaek, Denmark) following the manufacturer's protocol. Specific primers for miR-200c and miR-205 and primers for SNORD44 and SNORD48, used for normalization, were obtained from Exiqon. PCR products were measured using the ABI Prism 7500 Fast RT-PCR System (Applied Biosystems). For miRNA transfection, hsa-miR-200c, hsa-miR-205, and miRNA Mimic Negative Control were obtained from Dharmacon (Epsom, UK). Cells were transfected twice during a period of 6 days with 25 nmol/L of miRNAs using Lipofectamine 2000 (Invitrogen, Vienna) reagent, according to the manufacturer's protocol, and harvested for Western blot and fluorescence-activated cell sorting (FACS) analyses, respectively.

TMA and IHC Data

To evaluate differences in E-cadherin expression between malignant and benign prostate tissue, we constructed a tissue microarray (TMA) of FFPE tissue blocks of 14 patients with prostate cancer who underwent chemotherapy with docetaxel before radical prostatectomy and 14 untreated patients with prostate cancer. Both groups were matched for Gleason score and age (see Supplemental Table S1 at *http://ajp.amjpathol.org*). The use of the archive samples was approved by the Ethics Committee of Innsbruck Medical University. For each selected case, three cancer tissue cores and three benign cores were punched. The TMA was assembled using a manual tissue arrayer (Beecher Instruments, Sun Prairie, WI). H&E and p63/α-methylacyl-CoA racemase IHC double staining to control the histological diagnosis and E-cadherin and vimentin IHC were performed on a Discovery-XT staining device (Ventana, Tucson, AZ). The following antibodies were used: anti-E-cadherin (1:50; Dako), anti-vimentin (1:300; Dako, Glostrup, Denmark), anti-p63/α-methylacyl-CoA racemase (1:200; Dako), and anti-p63 (1:100; Sigma-Aldrich). In addition, FFPE PC3, DU-145, PC3-DR, and DU-145-DR were also stained with E-cadherin (1:50; Dako) and vimentin (1:300; Dako) antibodies. Microscope images were taken with a Zeiss Imager Z2 microscope (Zeiss, Vienna) equipped with a Pixelink PL-B622-CU camera (Canimpex Enterprises Ltd, Halifax, NS, Canada). The IHC evaluation was performed by a uropathologist (G.S.) using the semiquantitative scoring system, quick score, combining the proportion of positive cells and the average staining intensity.[21] E-cadherin expression of PC3, DU-145, PC3-DR, and DU-145-DR cells was quantified using HistoQuest software version 3.0 (Tissue Gnostics, Vienna).

FACS Analysis of Stem Cell Markers

For the measurement of stem cell markers, cultivated cells were harvested and washed in PBS (PAA Laboratories, Pasching, Austria). Before staining, the cells were blocked in FACS buffer containing 6 μg/mL Human IgG1 (Dinova, Königswinter, Germany). The cells were centrifuged at room temperature. The cell pellets were resuspended in 300 μL of FACS buffer. For each staining, 10 μL of the antibody and 25 μL of the cell suspension were used and incubated for 30 minutes at 4°C. After incubation, the cells were washed with 1.5 mL of FACS buffer and centrifuged at 300 $\times$ *g* for 10 minutes at 4°C. The supernatant was aspirated, and cells were resuspended in 500 μL of FACS buffer. Measurement was performed in a BD FACS Calibur flow cytometer (Becton Dickinson). The analysis was performed by Cell Quest software version 4.0.1 (Becton Dickinson). Areas were determined by analysis of isotype-specific stains and single stains. The following antibodies were used for analysis: anti-human CD133/1 (ACC133)–phycoerythrin (PE; Miltenyi Biotec, Bergisch Gladbach, Germany), anti-human CD44-PerCP-Cy5.5 (488/695 nm) (Becton Dickinson), fluorescein isothiocyanate mouse anti-human CD24 (Becton Dickinson), fluorescein isothiocyanate mouse IgG1 κ isotype control (Becton Dickinson), monoclonal anti-human integrin α 2, anti-human CD49b-PE (R&D Systems, Minneapolis, MN), mouse IgG1-PE (Miltenyi Biotec), PerCP-Cy5.5 mouse IgG2b, isotype control (Becton Dickinson), and anti-human prostate stem cell antigen (PSCA; 7F5)–PE (Santa Cruz Biotechnology).

Re-Attachment Assay

The 96-well plates were coated with laminin (coverage, 1.5 μg/cm^2; Sigma-Aldrich), fibronectin (coverage, 3 μg/cm^2; Becton Dickinson), Matrigel (4%; Becton Dickinson), collagen IV (coverage, 6 μg/cm^2; Sigma-Aldrich), HBMEC-60, or PF-172. The cells were trypsinized and stained with Calcein AM Solution (Sigma-Aldrich) for 1 hour. Stained cells were washed twice with PBS and counted using a CASY cell counter system (Schärfe System, Reutlingen, Germany). A total of 3 $\times$ 10^4 cells per well were seeded onto coated or uncoated wells and allowed to attach for 1 hour. Subsequently, the plates were washed twice with PBS. The cells were then lysed in 100 μL of PBS containing 10% Triton X-100 for 15

minutes, with gentle shaking, and subjected to measurement with extinction/emission at approximately 494/517 nm using a Chameleon plate reader (HVD Life Sciences, Vienna). The results were normalized to the light emission of 3×10^4 stained cells of each cell line counted.

Adhesion Assay

Cells were seeded onto 12-well plates and allowed to attach for 24 hours. On the next day, the cells were stained with Calcein AM Solution (Sigma-Aldrich) for 1 hour and washed with PBS. Trypsin, 200 μL, was added for 120 seconds. The reaction was stopped by adding 500 μL of medium. After 1 minute of shaking, detached cells were removed. The remaining cells were lysed in 700 μL of PBS/10% Triton X-100 for 15 minutes. Each lysate, 100 μL, was transferred onto 96-well plates in triplicate and subjected to measurement with extinction/emission at approximately 494/517 nm using a Chamaeleon plate reader. The results were normalized to lysates of non-trypsinized control wells of the respective cell line.

Migration and Invasion Assays

Migration and invasion were assayed in Fluoro Blok individual inserts (pore size, 8 μm thick) in a 24-well companion plate (both from Becton Dickinson). For invasion, inserts were coated with 30 μL of Matrigel (30% in medium; Becton Dickinson) for 2 hours before use. A total of 2.5×10^4 cells were seeded onto the apical surface of the inserts in full growth medium. To attract cells to migrate or invade, the basal chamber of the wells was filled with 650 μL of medium containing 30% FCS. The cells were allowed to migrate or invade for 48 hours and subsequently stained with Calcein AM Solution (Sigma-Aldrich) for 1 hour. Migration or invasion was measured using a microplate reader (Tecan, Grödig, Austria) with extinction/emission of 494/517 nm. To calculate the percentage of migrated or invaded cells, the light emission of wells without insert, but the same number of cells, was set to 100%.

Statistical Analysis

The analysis was performed with Graph Pad Prism software version 4.0 (Graph Pad Software, La Jolla, CA). For all experiments, gaussian distribution was determined using a Kolmogorov-Smirnov test. Differences between treatment groups were analyzed using a Student's *t*-test. $P < 0.05$ was considered significant. In addition, statistical analysis was controlled using the Mann-Whitney *U*-test, a nonparametric test identifying statistically significant differences independent of sample distribution and variance between the samples. All differences highlighted by asterisks were statistically significant and encoded in figures. Data are presented as mean + SD unless otherwise stated.

Results

Alterations in Proliferation and Apoptosis in Docetaxel-Resistant Cells

The resistance of the established sublines PC3-DR and DU-145-DR was determined by testing cell proliferation and viability in the presence of increasing concentrations of docetaxel (up to 100 nmol/L) by [^{3}H]thymidine incorporation and MTT assay, respectively (see Supplemental Figure S1, A and B, at *http://ajp.amjpathol.org*). Both parental cell lines were sensitive to docetaxel and showed a decreased viability and proliferation at a concentration of 5 nmol/L docetaxel. In contrast, PC3-DR cells showed no changes in cell viability and proliferation, even with the highest docetaxel concentration. When parental and resistant PC3 cells were treated with 12.5 nmol/L docetaxel (Figure 1, A and B), we detected a significant decrease in proliferation and cell viability in PC3, but not in PC3-DR, cells. DU-145-DR cells showed a decreased basal proliferation and viability compared with DU-145 cells in the absence of docetaxel. However, we observed a significantly higher proliferation and cell viability compared with their parental counterparts in the presence of 12.5 nmol/L docetaxel. The MTT assay results were confirmed by apoptosis measurement (Figure 1C). After treatment with 12.5 nmol/L docetaxel for 3 days, light microscopy images (see Supplemental Figure S1D at *http://ajp.amjpathol.org*) were taken and the percentage of apoptotic cells was assessed and quantified (Figure 1C; see also Supplemental Figure S1C at *http://ajp.amjpathol.org*). Propidium iodide staining and FACS analysis revealed an increase in apoptosis (from 2.95% to 15.53% in PC3 and from 2.26% to 52.02% in DU-145 cells). In contrast, PC3-DR cells showed no change in the apoptosis rate (from 1.13% to 1.15%), and in DU-145-DR cells, there was a slight increase in the percentage of apoptotic cells (from 3.49% to 8.01%).

EMT Occurs during the Selection Process with Docetaxel

Resistant cells, especially PC3-DR, were, in general, smaller and showed a more elongated shape than their parental counterparts. Therefore, we hypothesized that docetaxel-resistant cells changed their morphological characteristics to a mesenchyme-like phenotype (Figure 1D). The screening of 84 EMT-associated genes by an EMT-specific PCR array revealed 10 genes that were significantly up- or down-regulated in both docetaxel-resistant cell lines (see Supplemental Table S2 at *http://ajp.amjpathol.org*). Of these 10 genes, *CDH1* (E-cadherin) was down-regulated, whereas several EMT markers, *VIM* (vimentin), *ZEB1* (zinc finger E-box–binding homeobox 1), and *ZEB2* (zinc finger E-box–binding homeobox 2),

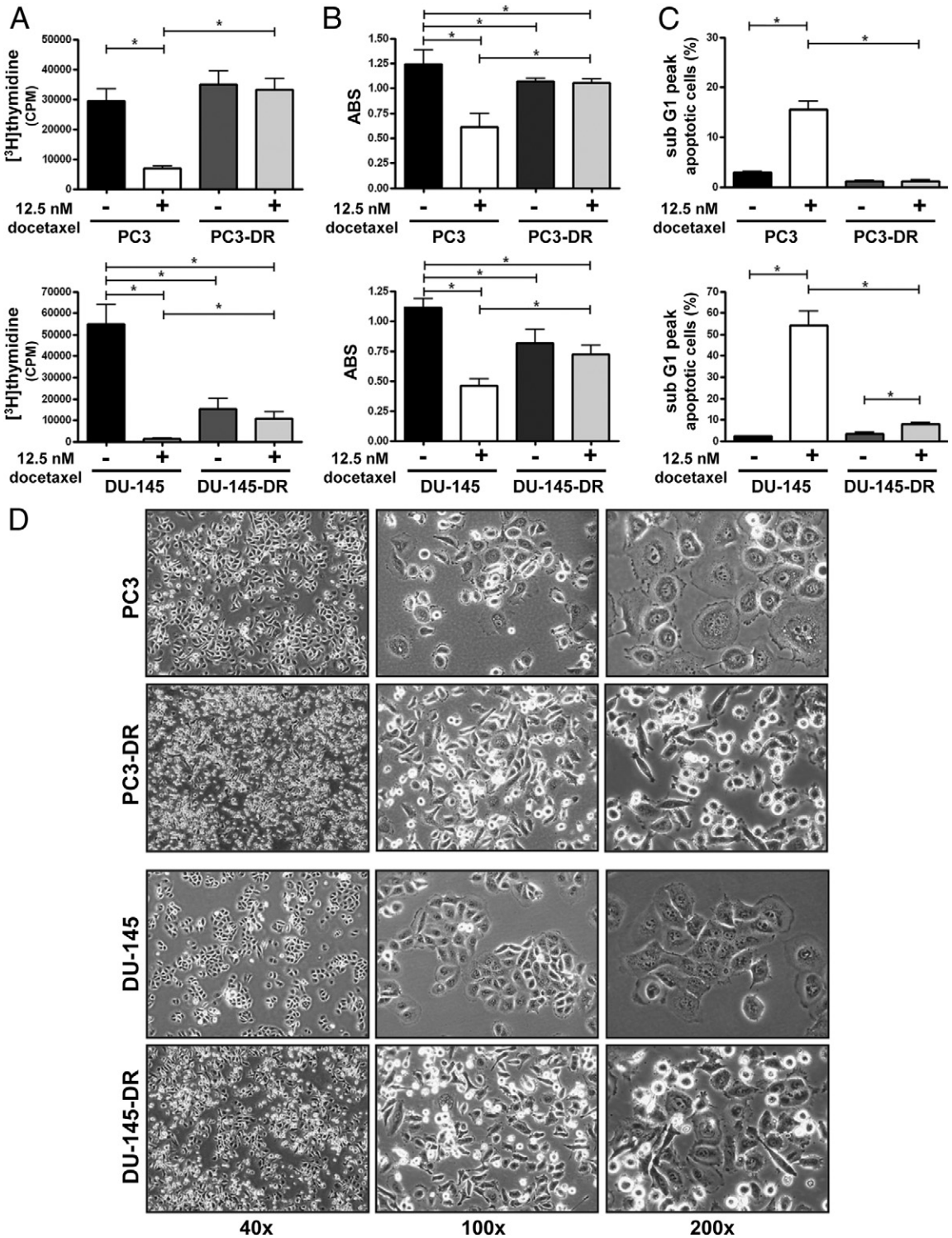

Figure 1. PC3-DR and DU-145-DR cells are less sensitive to docetaxel and have mesenchyme-like morphological characteristics. The measurement of proliferation (**A**) and cell viability (**B**) in parental and docetaxel-resistant PC3 and DU-145 cells is shown. Cellular proliferation was assessed by measurement of [^{3}H]thymidine incorporation. Cell viability was determined by the MTT assay. **C:** Confirmation of reduced cell viability. The percentage of apoptotic cells was assessed by flow cytometry after propidium iodide staining. The results represent mean values + SEM from at least three independent experiments. *$P < 0.05$. **D:** Docetaxel-resistant cells have different cell morphological characteristics compared with the parental cell lines. ABS, absorbance; sub, standard for apoptosis analysis.

were up-regulated in docetaxel-resistant cells (Table 1). Therefore, PCR array results were, in addition, verified with a second set of primers by RT-qPCR (Figure 2A). Moreover, Western blot analysis revealed a similar change in E-cadherin and vimentin expression at the protein level (Figure 2B). Furthermore, reduced E-cadherin and increased vimentin protein expression levels were confirmed with a second set of antibodies by IHC

Table 1. Selected Significantly Up- and Down-Regulated Genes in Docetaxel-Resistant Prostate Cancer Cells Compared with Their Parental Counterparts

Gene of interest	Fold regulation	
	PC3-DR	DU-145-DR
VIM	4.2136	2.3057
ZEB1	4.7775	4.1611
ZEB2	5.4294	12.4165
CDH1	−12.1887	−48.2364

staining of FFPE parental and docetaxel-resistant cells (Figure 2C).

Decreased Expression of miR-200c and miR-205 Is Responsible for E-Cadherin Loss in Chemotherapy-Resistant Cells

The expression of ZEB1 and ZEB2, which, in turn, regulated the expression of E-cadherin, was under tight control by miR-200 family members, especially miR-200c and miR-205.[14,15] To better understand the mechanism of the observed E-cadherin decrease in docetaxel-resistant cells, we measured miR-200c and miR-205 expression levels (Figure 2D) in parental and docetaxel-resistant sublines. PC3-DR cells showed a significantly reduced expression of miR-200c and miR-205 compared with parental PC3 cells. The expression of miR-200c was also reduced in DU-145-DR cells, whereas the expression of miR-205 was under the detection limit in DU-145 and DU-145-DR cells. To investigate the possibility that short-term docetaxel treatment caused down-regulation of both miRNAs, we measured their expression after 48 and 72 hours of treatment. Although a minor inhibitory effect was observed, statistical significance was notable only at one time point in PC3 cells (see Supplemental Figure S2B at *http://ajp.amjpathol.org*). Therefore, we concluded that inhibition of both miR-200 family members occurred after long-term treatment with docetaxel during the selection process. Transfection of both miRNAs for 3 days resulted in a significantly increased expression of E-cadherin and a significantly diminished expression of ZEB1 and ZEB2 mRNAs in both resistant sublines (Figure 3A). A prolonged treatment with miRNAs for 6 days resulted in elevated E-cadherin protein levels (Figure 3B) and an increase in the percentage of apoptotic cells (see Supplemental Figure S2, C and D, at *http://ajp.amjpathol.org*). Apoptosis was determined by measurement of cleaved poly (ADP-ribose) polymerase (Figure 3B), confirmed, and quantified (Figure 3C) by flow cytometry.

Docetaxel-Resistant Cells Are Able to Form Colonies and Spheroids

Despite changed cell morphological characteristics and low E-cadherin expression caused by the EMT, docetaxel-resistant cells were still able to form colonies in a two-dimensional colony formation assay. Colonies of docetaxel-resistant cells were, in general, smaller than those of parental cells. DU-145-DR cells formed fewer colonies than their parental counterparts in the absence of docetaxel. However, in contrast to parental cells, PC3-DR and DU-145-DR cells formed colonies in the presence of 12.5 nmol/L docetaxel (Figure 4, A and B). In addition, single cells were seeded in Matrigel and grown spheroids were harvested. In spheroids, E-cadherin and vimentin protein expression levels were determined (Figure 4, C and D) by IHC and revealed a similar E-cadherin and vimentin staining pattern compared with single cells grown in a monolayer (Figure 2C).

Increased Stemness Is a Characteristic Feature of Docetaxel-Resistant Cells

Cells that undergo EMT might display an increased expression of stem cell markers. To investigate this issue in docetaxel-resistant cells, we determined the expression of CD24, CD44, CD49b, CD133, and PSCA. Parental and docetaxel-resistant cells expressed the stem cell markers CD44 and CD49b (Figure 5A). Docetaxel-resistant cells contained an increased $CD24^{low}$-$CD44^{high}$ subpopulation compared with their parental counterparts (Figure 5B). Moreover, cells of the $CD44^{high}$ subgroup showed a more intense CD49b staining compared with the $CD44^{low}$ subgroup in parental and docetaxel-resistant cells (see Supplemental Figure S3A at *http://ajp.amjpathol.org*). All screened cell lines were negative for CD133 (see Supplemental Figure S3B at *http://ajp.amjpathol.org*). The prostate stem cell marker, PSCA, was expressed in all cell lines. However, no significant difference in its expression was observed in docetaxel-resistant cells (see Supplemental Figure S3C at *http://ajp.amjpathol.org*).

Increased Invasive Potential of Docetaxel-Resistant Prostate Cancer Cells

Because of their morphological characteristics, docetaxel-resistant cells detach more easily from the surface in a specifically established adhesion assay (Figure 6A). At the same time, they had the same or even higher ability to attach to extracellular matrix proteins, such as laminin, fibronectin, collagen IV, and Matrigel (Figure 6B). Moreover, PC3-DR and DU-145-DR cells had a similar or higher affinity to attach to HBMEC-60 and PF-172 compared with parental cells (Figure 6C). The ability to detach from surfaces, combined with the higher re-attachment potential, resulted in an increased migratory and invasive behavior (Figure 6D).

E-Cadherin Expression Is Reduced in Patients after Neoadjuvant Docetaxel Chemotherapy

To prove that a lower E-cadherin expression after docetaxel treatment was not limited to *in vitro* conditions, we determined E-cadherin expression in benign and malig-

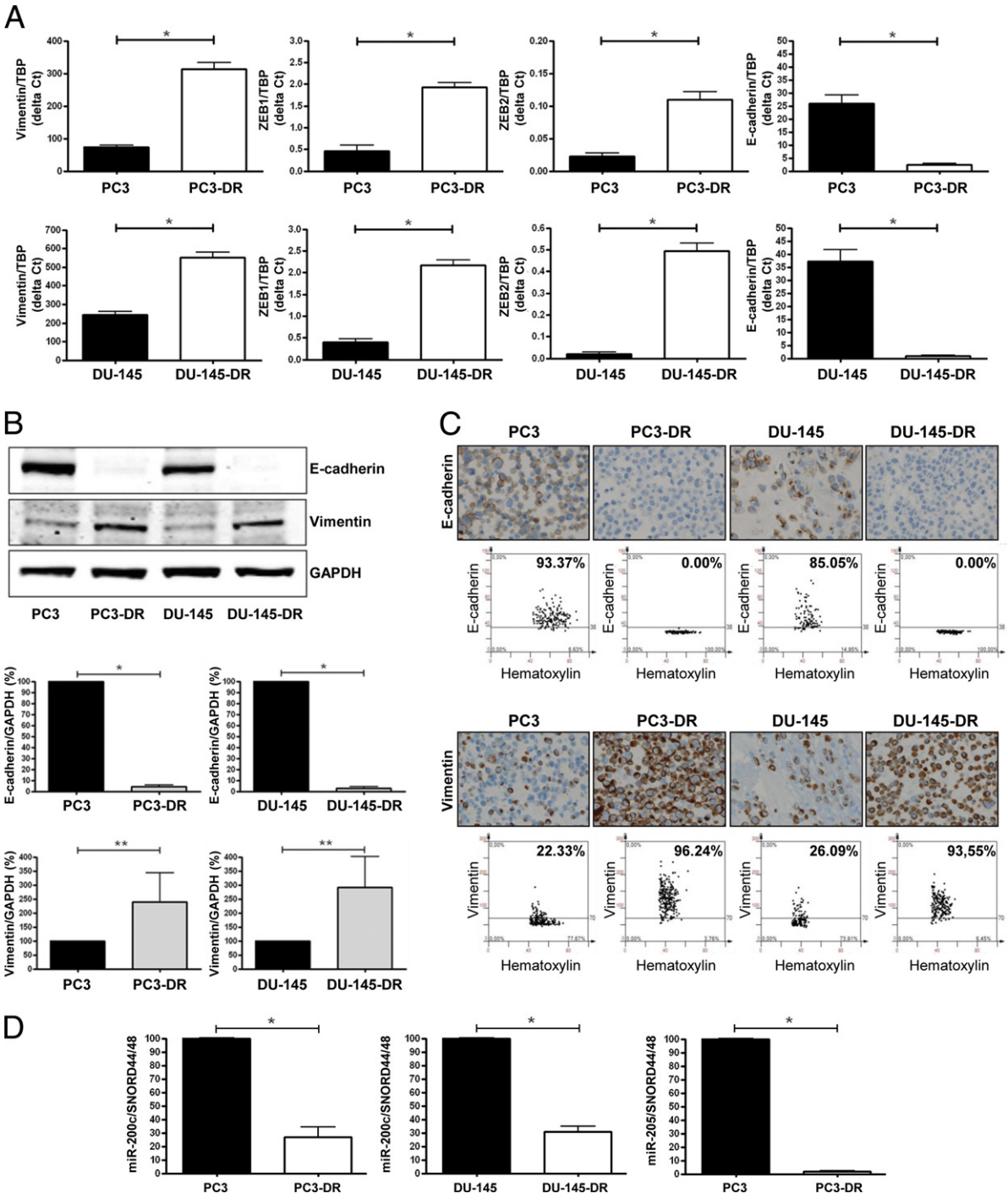

Figure 2. Altered expression of EMT markers in docetaxel-resistant cells. **A:** Confirmation of PCR array results by RT-qPCR. The results represent mean values + SEM from at least three independent experiments. **B:** E-cadherin and vimentin protein expression was determined and quantified by using Western blot analysis. **C:** Different E-cadherin and vimentin protein expression was confirmed by IHC. The percentage of stained cells was determined by using HistoQuest software version 3.0. **D:** Total RNA was isolated, and miR-200c and miR-205 expression was determined by RT-qPCR and normalized to small nucleolar RNAs, SNORD44 and SNORD48. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TBP, TATA-Box–binding protein. $^{*}P < 0.05$ (**A**, **B**, and **D**), $^{**}P < 0.01$ (**B**).

nant cores of patients with prostate cancer. For this purpose, a TMA was established. In total, 28 patients were selected (see Supplemental Table S1 at *http://ajp.amjpathol.org*). Of these patients, 14 received neoadjuvant docetaxel chemotherapy before radical prostatectomy. For analysis, both patient groups were divided into low (≤7) and high (>7) Gleason score subgroups. In control patients without chemotherapy, no significant difference in E-cadherin expression was detected. However, patients with a high Gleason score in the chemotherapy group showed significantly lower E-cadherin expression in malignant compared with benign areas and with malignant areas of patients with low Gleason scores (Figure 7, A and C). Strikingly, a direct comparison be-

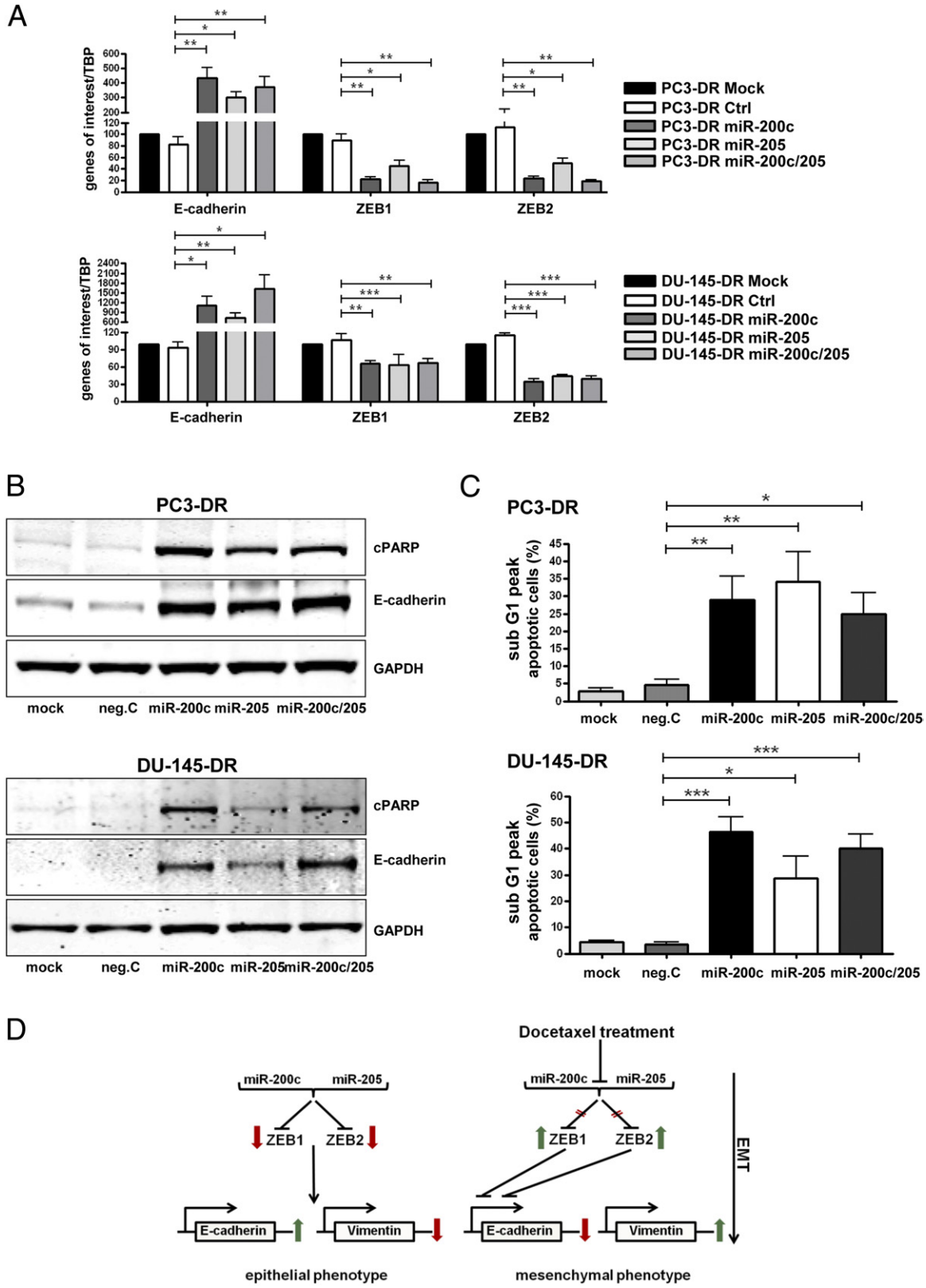

Figure 3. Decreased expression of miR-200c and miR-205 is responsible for E-cadherin loss and a crucial factor to circumvent apoptosis. **A:** The expression of E-cadherin, ZEB1, and ZEB2 mRNA in docetaxel-resistant PC3 and DU-145 cells after transfection with miR-200c and/or miR-205 for 3 days. **B:** Representative Western blot analyses for E-cadherin and cleaved poly(ADP-ribose) polymerase (PARP) expression after double transfection with 25 nmol/L miR-200c and miR-205 for 6 days in the presence of docetaxel. **C:** The percentage of apoptotic cells was assessed by flow cytometry after propidium iodide staining. RT-qPCR and FACS analysis results represent mean values + SEM from at least three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. **D:** Proposed model for docetaxel-triggered EMT in docetaxel-resistant cells. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; neg, negative; sub, standard for apoptotic analysis; TBP, TATA-Box–binding protein.

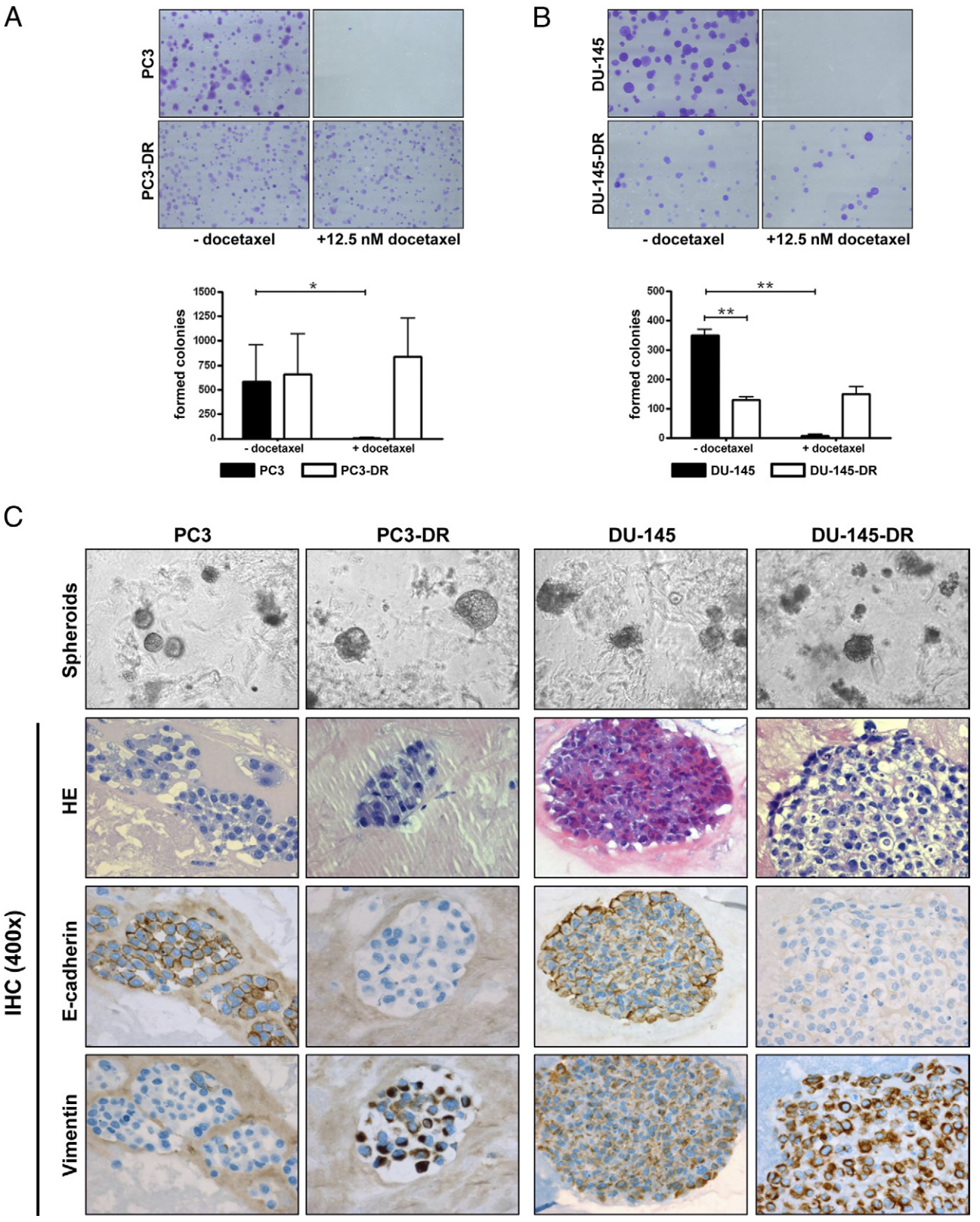

Figure 4. Despite decreased E-cadherin expression, docetaxel-resistant cells are still able to form colonies and spheroids. Clonogenic assay for parental and docetaxel-resistant PC3 (**A**) and DU-145 (**B**) cells in the presence or absence of 12.5 nmol/L docetaxel. Colony formation ability was assessed by counting the number of colonies formed after 12 days. PC3-DR (**C**) and DU-145-DR (**D**) cells are also able to form spheroids. Original magnification, ×40. IHC reveals a similar E-cadherin and vimentin staining pattern compared with single cells grown in a monolayer. Original magnification, ×400. $^{*}P < 0.05$, $^{**}P < 0.001$.

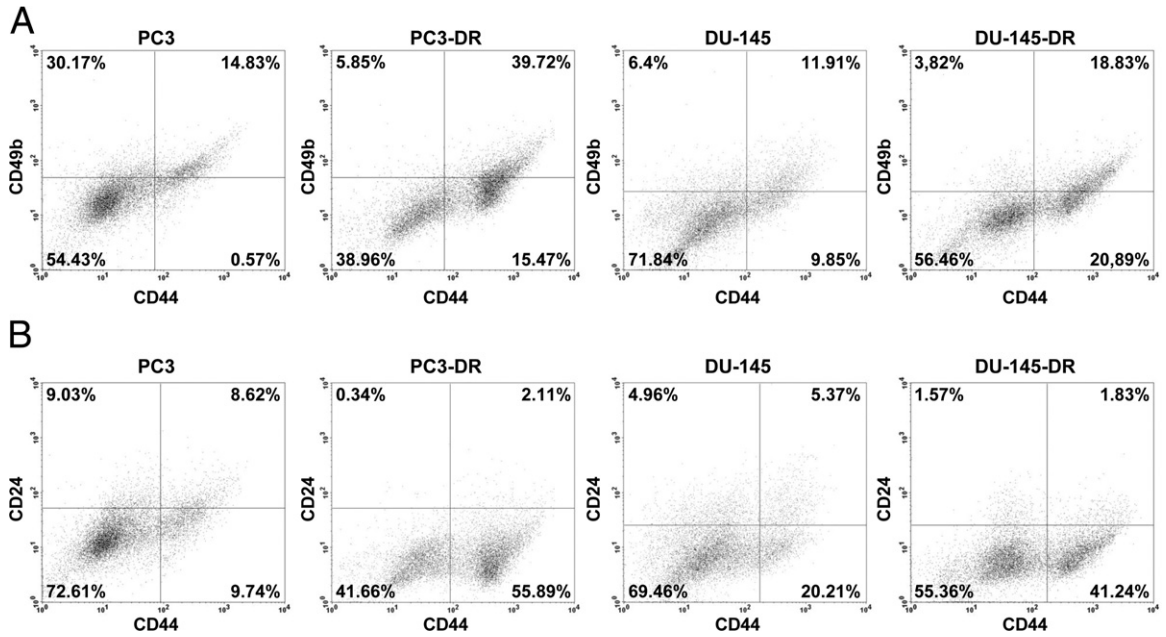

Figure 5. Increased expression of stem cell markers CD44 and CD49b. Analysis of expression of specific stem cell markers by FACS analysis. **A:** PC3-DR and DU-145-DR cells have an increased $CD24^{low}$ and $CD44^{high}$ cell subpopulation compared with parental cells. **B:** $CD44^{high}$ cells have a more intense CD49b staining compared with $CD44^{low}$ cells.

tween patients with a high Gleason score of both groups revealed significantly lower E-cadherin expression in patients treated with docetaxel (Figure 7, B and C). Moreover, when patients were compared according to tumor recurrence, a reduced E-cadherin expression was observed in tissues from individuals who experienced relapse (Figure 7D). Furthermore, patients were stratified according to tumor relapse in the control and chemotherapy groups. E-cadherin expression was significantly lower in specimens from patients who received docetaxel treatment (Figure 7D). We also performed vimentin staining of our TMA obtained from patients with prostate cancer (see Supplemental Figure S4B at *http://ajp.amjpathol.org*). Vimentin was expressed in benign tissue in the stromal compartment and in both malignant epithelium and adjacent stroma.

Discussion

Treatment with docetaxel is the preferred chemotherapeutic option for different types of cancer. However, it provides only a modest survival benefit, with most patients experiencing tumor progression. To gain more insight into the molecular changes that lead to or are associated with docetaxel insensitivity, we have generated and characterized resistant PC3 and DU-145 sublines. In several laboratories, docetaxel-resistant cells have been established to address the role of other mechanisms in the development of chemotherapy resistance.[1,22,23] In those publications, increased clusterin expression was observed in their docetaxel-resistant cell lines. Patterson et al[1] also reported an induced expression of STAT1 and hypothesized a novel role for both proteins in chemotherapy resistance. Other researchers showed an association between high clusterin protein levels and increased Akt phosphorylation. The findings of all three groups are in line with our observations. We were able to confirm an increased expression of STAT1 and clusterin and an increased basal phosphorylation of Akt in docetaxel-resistant PC3 cells (see Supplemental Figure S2A at *http://ajp.amjpathol.org*), thus suggesting that observed changes are a common phenomenon and independent of cell culture conditions in individual laboratories.

In contrast to parental cells, PC3-DR and DU-145-DR sublines have different morphological characteristics. We prove that the transformation to a mesenchymal phenotype occurs during the selection process with docetaxel. The expression of the main EMT marker, E-cadherin, was significantly reduced in docetaxel-resistant cells *in vitro* and in patients with prostate cancer who received neoadjuvant chemotherapy with docetaxel. Several groups have already reported a reduced E-cadherin expression during cancer progression. In addition, they observed an inverse correlation between E-cadherin expression and patient survival.[24,25] In line with these findings, our TMA data also indicate significantly reduced E-cadherin expression in patients after neoadjuvant chemotherapy. These data are additionally supported by observations that patients with tumor relapse had a significant reduction of E-cadherin expression. Therefore, this kind of therapy may have previously unrecognized disadvantages. However, we are aware of the low patient number in our study. In concordance with our conclusions, recent findings linked high E-cadherin expression within the tumor with a good prognosis and sensitivity to therapy. In the literature, high E-cadherin expression and an epithelial cancer cell phenotype are associated with sensitivity to epidermal growth factor receptor inhibitors in urothelial

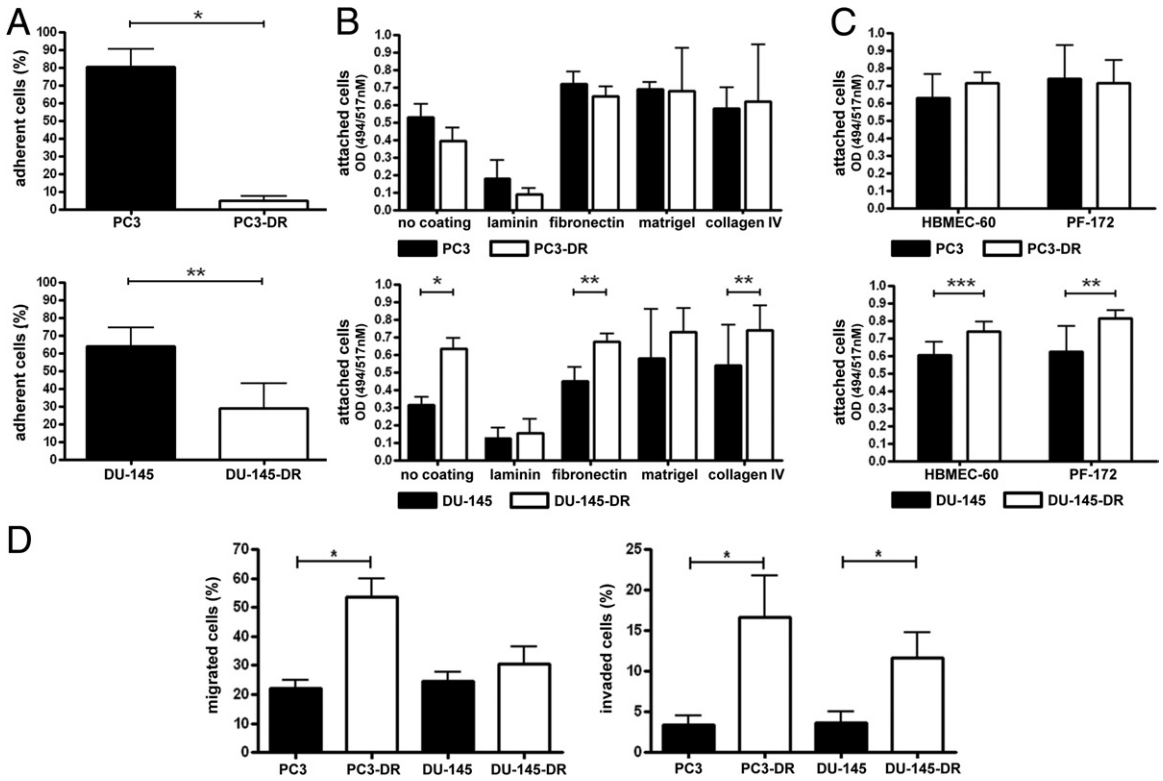

Figure 6. Docetaxel-resistant cells have a highly invasive potential. **A:** Compared with parental cell lines, docetaxel-resistant cells detach easier from the surface in an adhesion assay. **B** and **C:** Docetaxel-resistant cells have the same or even higher ability to attach to extracellular matrix proteins, HBMEC-60, and PF-172 in an attachment assay. **D:** Migration and invasion assays prove an altered invasive and migratory behavior of docetaxel-resistant cells. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

and lung cancer cells.[26,27] We observed significantly reduced E-cadherin expression in patient samples after chemotherapy, thus suggesting that the therapy itself can have a negative influence on E-cadherin expression. Similar to our results, cisplatin treatment of primary and metastatic epithelial ovarian carcinomas has generated residual cells with reduced E-cadherin expression and a mesenchymal stem cell-like profile.[28] However, to our knowledge, we are the first group that demonstrated a direct influence of prolonged docetaxel treatment on E-cadherin expression *in vitro* and *in vivo*.

Tumors with a mesenchymal-like phenotype are associated with poor prognosis and high mortality rates. These tumors frequently express the E-cadherin repressor, ZEB2, that at the same time protects tumor cells from DNA damage-induced apoptosis.[29] In line with those observations, we were able to detect an increased expression of *ZEB1*, *ZEB2*, and vimentin in docetaxel-resistant cells. ZEB1 and ZEB2 are repressors of E-cadherin, whereas ZEB2 has directly interacted with the vimentin promoter, thus increasing vimentin expression.[30] On the other hand, it is well-known that the transcriptional repressors Snail and Twist1 can also block expression of E-cadherin.[9] However, Twist1 was not expressed in DU-145 or DU-145-DR cells, and expression of Snail was not significantly changed. PC3-DR cells had a slight increase of Twist1 and Snail (see Supplemental Figure S4A at *http://ajp.amjpathol.org*). Because of these different expression patterns, we propose that ZEB1 and ZEB2 are the main drivers of E-cadherin repression in our docetaxel-resistant cell lines. Recent publications have identified miR-200 family members, miR-200c and miR-205, as key regulators of EMT. They enforce the epithelial phenotype by repression of ZEB1 and ZEB2. After selection with docetaxel, miR-200c expression was significantly reduced in both docetaxel-resistant cell lines. Furthermore, miR-205 levels declined in chemotherapy-resistant PC3 cells, whereas miR-205 expression was undetectable in DU-145 and DU-145-DR cells. These results are consistent with the observations of Bhatnagar et al,[31] who also did not observe miR-205 expression in the DU-145 cell line. Mechanistically, transfection of both miRNAs resulted in restored E-cadherin expression, accompanied by increased apoptosis, in docetaxel-resistant cells. These data suggest that reduced miR-200c and miR-205 levels during chemotherapy are crucial for cancer cell survival and drug resistance. Furthermore, we show that the levels of transcription factors ZEB1 and ZEB2 are down-regulated on transfection of either miR-200c or miR-205. Based on these experiments, our proposed mechanism of miRNA reduction, causing EMT in docetaxel-resistant cells, is shown in Figure 3D. Similar to our observations, miR-200c and miR-205 functioned as tumor suppressors in various neoplasms.[32–34] This was supported by observations that both miRNAs have inhibitory functions on cellular invasiveness and migration. miR-205 can influence migration and invasion by down-regulation of the protein kinase Cε or low-density lipoprotein receptor–related protein 1.[35,36] Furthermore, Jurmeister et al[37] showed that miR-200c is able to

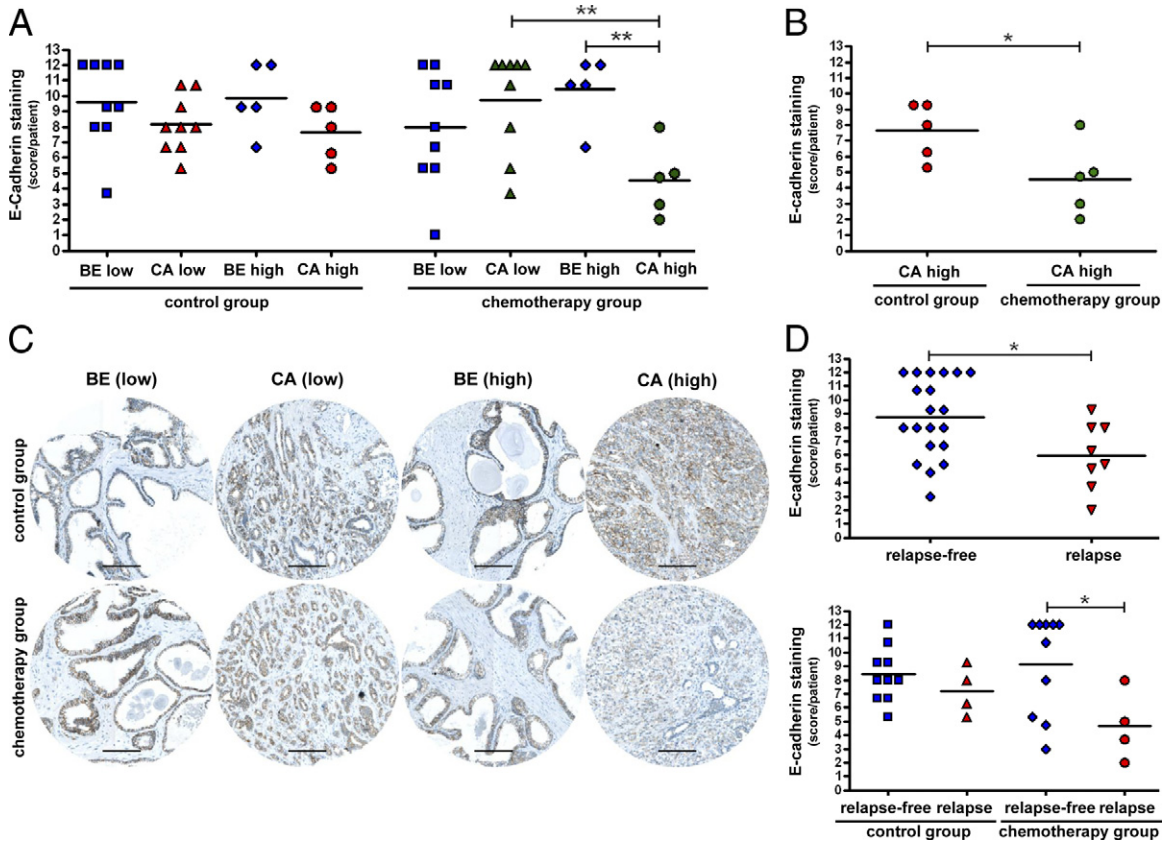

Figure 7. E-cadherin protein expression is significantly lower in malignant tissue of patients with prostate cancer after chemotherapy. TMA results for E-cadherin expression. **A:** In the patient control group, no significant difference in E-cadherin expression is detected. Patients with a high Gleason score who received chemotherapy have a significantly lower E-cadherin expression in malignant areas compared with benign areas and with malignant areas of patients with a low Gleason score. **B:** Patients with a high Gleason score in the chemotherapy group have a significantly lower E-cadherin expression compared with patients with a high Gleason score in the control group. **C:** Representative cores of the TMA for all groups. Original magnification, ×20/0.5 digital camera. Scale bar = 100 μm. **D:** E-cadherin expression is significantly lower in patients with tumor relapse. Patients with tumor relapse have a significantly reduced E-cadherin expression compared with relapse-free patients in the chemotherapy group. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. BE, benign; CA, cancer.

antagonize invasion and migration by inhibiting the actin-regulatory proteins FHOD1 and PPM1F. In contrast to findings by Zemskova et al,[38] who report on up-regulation of the prosurvival PIM1 kinase after some hours of docetaxel treatment, miRNA regulation seems to be a rather long-term effect of docetaxel. The exact underlying mechanism should be a subject of further studies. However, epigenetic changes might be considered responsible for this long-term regulation of miRNAs by docetaxel, as described in pancreatic stem cells.[39]

An important finding of the present study is that the percentage of cells with a stem cell-like phenotype is increased in chemotherapy-resistant prostate cancer models. Several reports provided evidence that EMT is a critical factor for invasion and migration and a key event in tumor recurrence, which is believed to be tightly linked to cancer stem cells.[18,40,41] EMT can favor prostate cancer cells with stem cell–progenitor-like properties that differentially express markers, such as CD24, CD44, CD49b, CD133, and PSCA.[41–44] CD49b^{high}/CD44^{high}/CD133^{high} prostate cancer cells show enhanced clonogenic potential. Furthermore, Patrawala et al[45] reported that CD44^{high} prostate cancer cells have an increased invasive and metastatic potential compared with CD44^{low} cells. Those findings were supported by Li et al,[46] who detected CD24^{low}-CD44^{high} breast cancer cells that were resistant to neoadjuvant chemotherapy and human epidermal growth factor receptor-2 inhibition. In addition, they reported that chemotherapy resulted in an increase of CD24^{low}-CD44^{high} cells and suggested that such an increase is a potentially important mechanism of acquired drug resistance.[46] Our results are in line with those observations. We were able to detect an increased CD24^{low}-CD44^{high} cell population in both docetaxel-resistant models. Moreover, CD44^{high} cells had more intense CD49b staining compared with CD44^{low} cells in parental and docetaxel-resistant cells. Elevated CD49b expression is also associated with increased invasiveness. Yang et al[47] proposed that CD49b is a key regulator of hepatocarcinoma cell invasion across the fibrotic matrix microenvironment. In functional assays, we were able to confirm an increased migratory and invasive behavior, as well as a strong affinity of docetaxel-resistant cells to bind to extracellular matrix proteins, endothelial cells, and prostate fibroblasts. However, we could not observe any differences in PSCA expression between parental and docetaxel-resistant cells, whereas CD133 staining was negative for all investigated cells. The role of CD133 in prostate cancer biological characteristics is a subject of discussion. CD133^{high} cells express the andro-

gen receptor.[48] Our observations show that neither androgen receptor negative parental nor docetaxel-resistant cells express CD133. The androgen signaling pathway is active in patients in whom chemotherapy failed. On the other hand, manipulations in androgen levels are associated with the appearance of a mesenchymal phenotype in prostate cancer.[49,50] EMT reported herein cannot be attributed to any influence of androgen because both cell sublines are androgen receptor negative. In addition, enhancement of a stem cell-like population by chemotherapeutics is not limited to docetaxel. Doxorubicin-selected breast cancer cells also displayed a $CD24^{low}$-$CD44^{high}$ phenotype.[51] Therefore, new generations of chemotherapeutics should be examined for their effects on EMT. For example, CD44-positive cell populations were reduced by the dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor, NVP-BEZ 235, in prostate cancer.[52] The appearance of CD44 splice variants has been well documented in human prostate cancer.[53] Based on the data available in the literature, quantitative analysis of CD44 expression in a subpopulation of stem cells in human specimens may not necessarily yield reliable data.[53] Such analysis may be complicated by the fact that standard CD44 isoform expression is down-regulated in prostate cancer, whereas some of the isoforms may be expressed at a higher level. Our TMA findings on expression of the mesenchymal marker, vimentin, are in concordance with results reported by others.[54,55] The expression of vimentin in a small percentage of tumor cells was observed solely in a subgroup of poorly differentiated prostate cancers, whereas it was much higher in metastatic samples.[55] Previous studies on CD44 and vimentin expression in prostate cancer indicate differences in expression of these markers between cellular models and tissue specimens.

Taken together, the results of the present and other studies are relevant for development of novel experimental therapies in prostate cancer. Our findings prove that docetaxel treatment leads to a clonal selection of highly invasive prostate cancer cells characterized by a mesenchymal and stem cell-like phenotype. Furthermore, we speculate that this mechanism is at least in part responsible for chemotherapy failure, with implications for the development of novel therapeutics.

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