High Aldehyde Dehydrogenase Activity Identifies Tumor-Initiating and Metastasis-Initiating Cells in Human Prostate Cancer

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Abstract
Metastatic progression of advanced prostate cancer is a major clinical problem. Identifying the cell(s) of origin in prostate cancer and its distant metastases may permit the development of more effective treatment and preventive therapies. In this study, aldehyde dehydrogenase (ALDH) activity was used as a basis to isolate and compare subpopulations of primary human prostate cancer cells and cell lines. ALDH-high prostate cancer cells displayed strongly elevated clonogenicity and migratory behavior in vitro. More strikingly, ALDH-high cells readily formed distant metastases with strongly enhanced tumor progression at both orthotopic and metastatic sites in preclinical models. Several ALDH isoforms were expressed in human prostate cancer cells and clinical specimens of primary prostate tumors with matched bone metastases. Our findings suggest that ALDH-based viable cell sorting can be used to identify and characterize tumor-initiating and, more importantly perhaps, metastasis-initiating cells in human prostate cancer. Cancer Res; 70(12); 5163–73. ©2010 AACR.

Introduction
Prostate cancer is the most commonly diagnosed cancer in males and the second leading cause of death. Although prostate cancer detected at early stage can be successfully eradicated by radical prostatectomy and radiotherapy, no curative treatment exists for metastatic disease. Bone metastases occur in ∼90% of patients with advanced disease and are leading causes of morbidity. The identification of the cell(s) of origin of prostate cancer as well as the neoplastic cells involved in the formation of distant metastases is, therefore, fundamental to a full understanding of this disease and the development of treatment and preventive therapies.

Most carcinomas comprise of a heterogeneous cell population with marked differences in their ability to proliferate and differentiate as well as their ability to reconstitute the tumor upon transplantation. This led to the hypothesis that the entire population of tumor cells might arise from a rare subpopulation of putative cancer stem/progenitor-like cells, also known as tumor-initiating cells (TICs; refs. 1–3). TICs share principle characteristics with adult stem cells namely self-renewal, high proliferative potential, clonogenicity, and multipotency. In addition, they have the ability to reproducibly form the same tumor phenotype as in the patient and to undergo differentiation into nontumorigenic cells (4–6). TICs were first isolated from patients with hematologic malignancies in which a few cells could initiate a new tumor (7). During the past few years, TICs were also identified and isolated from solid tumors such as breast, brain, colon, pancreatic, and prostate tumors (8–12). The cancer stem cell hypothesis has provided a paradigm shift in our understanding of carcinogenesis, metastasis, and tumor biology. The identification of TICs has important implications in the way cancer treatment should be conceived and future therapeutic approaches will be designed. Whether the subpopulation of TICs is involved in the formation of distant metastases, tumor dormancy, and therapy resistance has remained poorly understood.

Because of the heterogeneous nature of solid cancers, the prospective selection of cells based on cell surface markers such as CD44+/α2β1high/CD133+ cells (2) as the sole way to isolate TICs remains controversial (3–6). It also suggests that selection by (single) cell surface markers such as CD133 is unlikely to identify all putative stem or progenitor cell types (3, 4).

A complementary strategy for the functional identification and characterization of normal stem cells and their malignant counterparts involves the measurement of aldehyde dehydrogenase (ALDH) activity. ALDH enzymes have important functions in the development of epithelial homeostasis, and
as a result, deregulation of this class of enzymes has been implicated in multiple cancers (13). ALDH activity is important for drug resistance, cell proliferation, differentiation, and response to oxidative stress (14–16). In humans, 19 isoforms of ALDH enzymes have been identified thus far. To date, high ALDH activity has been used to identify and select stem-like subsets in hematopoietic cells (17–19), endothelial progenitor cells, and mesenchymal and epithelial stem cells (20–22). It is becoming increasingly clear that ALDH activity can be used, either alone or in combination with cell surface markers, to identify TICs in hematologic malignancies and a steadily increasing number of solid carcinomas, including those of the pancreas, lung, colon, and breast (14, 15, 23–25).

These studies showed the feasibility of viable cell sorting based on ALDH enzyme activity for the isolation of “stem-like” cells based on a developmentally conserved stem/progenitor cell function. In this study, we show that a subpopulation of human prostate cancer cells with high ALDH activity correlates with enhanced clonogenicity and invasiveness in vitro. Furthermore, evidence is presented that ALDH-based viable cell sorting can be used to select tumor-initiating as well as metastasis-initiating cells in preclinical models of orthotopic growth and metastasis.

Materials and Methods

Cell lines and culture conditions

Human osteotrophic prostate cancer cell lines PC-3Iuc, PC-3M-Pro4IucA6, and PC-3M-Pro4IucBIII (PC-3M-Pro4Iuc passed thricre in the bone to generate highly metastatic clones that are capable of forming bone metastases) were maintained as previously described (26). LNCaP, C4, C4-2, and C4-2B (27) were maintained as described earlier (2). DU145 cells were grown in DMEM (Life Technologies) containing 4.5 g glucose/L supplemented with 10% FCS, 100 U/mL penicillin, and 50 μg/mL streptomycin (Invitrogen).

Primary cell cultures

Prostate tissue biopsies were obtained after radical prostatectomy with informed consent (CME P05.085). Single-cell suspension was generated by dissociating prostate tissue biopsies with collagenase I (200 IU/mL) in stem cell media at 37°C overnight according to a modified protocol (28). The epithelial and stromal fractions were separated by centrifugation and the epithelial cells were maintained in supplemented CnT52 stem cell medium (CELLnTEC). Tumorigenicity of the isolated cells was confirmed by qPCR of TMPRSS2/ERG (Supplementary Table S1). After being washed, sections were incubated with biotinylated secondary antibodies (1:300, 45 min) and Noble agarose (Becton Dickinson) and overlayed onto a 60-mm dish containing a solidified bottom layer of 0.6% agarose in medium. Once the top layer solidified, 1 mL of medium was placed on top of the cell layer. Plates were incubated for 1 to 3 weeks and colonies were measured and counted by using microscopy (ZEISS Axiosvert 200M). Colony area size of three representative fields was measured with the Image Pro plus software (Media Cybernetics). Only colonies with an area of >800 μm² were counted.

Colony-forming assay

On average, one cell per well was seeded into a 96-well plate and monitored as previously described by the use of light microscopy (ZEISS Axiosvert 200M; ref. 1).

Migration assay

Tumor cell migration was performed in 8-μm Transwell migration chambers (Costar). Prestarved cells (6 × 10⁴) were seeded in the upper chamber and allowed to migrate toward serum-containing medium in the lower chamber. Cells were fixed after 16 hours with 4% paraformaldehyde and stained with 0.1% crystal violet (2 mg/mL, Sigma-Aldrich). Three random fields were counted for each well, and mean numbers of migrated cells per field were calculated (29).

Immunohistochemistry

First, antibodies were incubated overnight at 4°C (Supplementary Table S1). After being washed, sections were incubated with biotinylated secondary antibodies (1:300, 45 min; DAKO) followed by streptavidin-conjugated peroxidase (1:200, 30 min room temperature; DAKO). Sections were washed and stained with AEC reagents (2 mg/mL) and Mayer’s hematoxylin (Merck). Pictures were taken using a Nikon Eclipse 610 microscope.

Tissue microarrays

For the tissue microarray (TMA), 30 tumor samples obtained by radical prostatectomy were collected at the University of Sheffield, United Kingdom. The TMA includes triplicate areas representing the largest carcinoma, high-grade prostate intra-epithelial neoplasia, as well as normal-appearing epithelium of each patient sample.

In vivo animal experiments

Mouse strains. Male nude (BALB/c nu/nu) mice were housed in individual ventilated cages under sterile condition according to the local guidelines for laboratory animals (DEC07026 and 09052).
Intraosseous inoculation. A single-cell suspension of $1 \times 10^4$ PC-3M-Pro4luc cells per 10 μL was injected into the right tibiae of anaesthetized 6-week-old mice as previously described (30).

Orthotopic inoculation. A single-cell suspension of $1 \times 10^4$ PC-3M-Pro4luc cells per 10 μL PBS was combined with 10 μL Matrigel (growth factor reduced, BD Biosciences) and surgically inoculated into the prostate of 6-week-old nude mice (26).

Intracardiac inoculation. A single-cell suspension of $1 \times 10^4$ PC-3M-Pro4luc cells per 100 μL PBS was injected into the left cardiac ventricle of anaesthetized 5-week-old male nude mice as previously described (26, 31).

Subcutaneous inoculation. A 10-μL single-cell suspension of either 100, 1,000 or $1 \times 10^4$ PC-3M-Pro4luc cells in PBS was combined with 10 μL Matrigel and injected s.c. in anaesthetized 6-week-old nude mice.

For all in vivo experiments, the progression of cancer cell growth was monitored weekly by bioluminescent imaging using the IVIS100 Imaging System (Caliper LifeSciences; ref. 26).

Statistical analysis
Statistical analysis was performed using the GraphPad Prism 4.0 software using either $t$ test or ANOVA. Data are

![Figure 1](image.png)

**Figure 1.** Characterization of ALDH$^{hi}$ and ALDH$^{lo}$ subpopulations in human prostate cancer cells. PC-3M-Pro4luc cells were FACS sorted to isolate ALDH$^{hi}$ and ALDH$^{lo}$ subpopulations using the ALDEFLUOR assay. A, mRNA expression levels of $\alpha^2$ integrin, CD44, and CD133 in ALDH$^{hi}$ and ALDH$^{lo}$ subpopulations as detected by qPCR ($n = 3$; *, $P < 0.05$). B, representative images of PC-3M-Pro4luc cells showing heterogeneous expression of CD44 and $\alpha^2$ integrin in PC-3M-Pro4luc cells. No staining of CD133 was detected. Scale bars, 80 μm. C, left, a representative color dot plot of PC-3M-Pro4 cells stained for both ALDH activity and $\alpha^6$ integrin. Cell surface expression of CD44, $\alpha^6$ integrin, $\alpha^6$ integrin, and EpCaM in ALDH$^{hi}$ versus ALDH$^{lo}$ cells as detected by FACS analysis. Data are representative for three independent experiments. N.d., not detectable.
presented as the mean ± SEM. P values of ≤0.05 were regarded as being statistically significant.

Results

ALDH\textsuperscript{hi} prostate cancer cells display enhanced clonogenicity and migration \textit{in vitro}

A potential strategy for the identification and isolation of cells with a stem cell–like phenotype in hematologic and several epithelial malignancies involves selection based on ALDH enzyme activity (15, 18) using ALDEFLUOR assays. Before the functional testing of clonogenicity, subsets of ALDH\textsuperscript{hi} and ALDH\textsuperscript{low} cells were isolated from PC-3M-Pro4-luc and C4-2B human prostate cancer cell lines by flow cytometry using the ALDEFLUOR assay. First, we investigated the presence of prostate cancer stem/progenitor-like cell markers α\textsubscript{2} integrin, CD44, and CD133 (2). As depicted in Fig. 1A, mRNA expression levels of α\textsubscript{2} integrin and CD44 were increased in the ALDH\textsuperscript{hi} compared with the ALDH\textsuperscript{low} population, whereas CD133 expression was not detected.

Immunohistochemical analyses revealed CD44 and α\textsubscript{2} integrin staining, but no detectable CD133 in PC-3M-Pro4luc and C4-2B cells (data not shown; Fig. 1B), which is in line with recent findings (3, 4).

In addition, relatively strong expression of other potential prostate tumor stem cell markers, α\textsubscript{6} integrin (32), α\textsubscript{v} integrin (33), and the Epithelial Cell Adhesion Molecule EpCAM (34) was observed in the ALDH\textsuperscript{hi} subpopulation compared with ALDH\textsuperscript{low} subpopulation (Fig. 1C). A representative example of PC-3M-Pro4-luc cells that were stained for both ALDH activity and α\textsubscript{6} integrin is shown as a color dot plot in Fig. 1C.

Functional differences \textit{in vitro} between FACS sorted ALDH\textsuperscript{hi} and ALDH\textsuperscript{low} subpopulations for both cell lines were assessed by different clonogenic assays (see Supplementary Methods). The ALDH\textsuperscript{hi} subpopulations of PC-3M-Pro4luc and C4-2B cells formed significantly more colonies than their respective ALDH\textsuperscript{low} cell populations when grown anchorage independently in soft agar (Fig. 2A and B). In addition, the average colony size was significantly increased in the ALDH\textsuperscript{hi} compared with the ALDH\textsuperscript{low} subpopulation (respectively, 3,254 ± 520 μm\textsuperscript{2} versus 1,350 ± 96 μm\textsuperscript{2}). When plated \textit{in vitro} at low density (1 cell/well), ALDH\textsuperscript{hi} PC-3M-Pro4luc and ALDH\textsuperscript{hi} C4-2B subpopulations both showed significantly more colony formation than the cells with low ALDH activity.
(Fig. 2C). In addition to their clonogenic ability, ALDHhi and ALDHlow subpopulations were compared for their ability to migrate (Fig. 2D; ref. 29). ALDHhi PC-3M-Pro4luc cells were found to be significantly more migratory than the ALDHlow subpopulation.

**Human prostate cancer cell lines and primary cultures of clinical prostate cancer samples contain a subpopulation of cells with high ALDH enzyme activity**

The ALDEFLUOR assay was used to assess the presence and size of the population with high ALDH enzymatic activity in prostate cancer cell lines and primary prostate cancer cultures derived from patients that underwent radical prostatectomy (Table 1). The LNCaP-C4 series can be used as a model of human prostate cancer development and mimics the natural course of prostate cancer from androgen-dependent and nonmetastatic (LNCaP) to androgen-independent and metastatic (C4-2B), with the development of mixed osteoblastic/osteolytic bone metastases (27). Interestingly, the size of the ALDHhi population coincided with an increase in tumorigenicity from 0.8% in the poorly tumorigenic androgen-responsive LNCaP to 13% in the highly invasive C4-2B cells. The androgen-independent human prostate cancer cell line PC-3 was repeatedly passaged in vivo to produce clones that are highly metastatic and osteotropic, PC-3M-Pro4luc and PC-3M-Pro4lucBIII. Similar to the LNCaP series, enhanced tumorigenicity is associated with an increase in the size of the ALDHhi subpopulation in these cell lines.

Next, we evaluated the presence of an ALDHhi subpopulation in clinical specimens of primary human prostate cancer. Primary cell cultures from different clinical human prostate cancer samples, obtained from patients undergoing radical prostatectomy, indicated the presence of an ALDHhi population with an average size of 8% of all cancer cells (Table 1, second part).

**ALDHhi prostate cancer cells show enhanced tumorigenicity and metastatic ability in vivo**

Our in vitro data showed that cells with an increased ALDH activity in human prostate cancer cell lines have a higher clonogenic and migratory capability than cells with low ALDH activity. Subsequently, we analyzed and compared the tumorigenic and metastatic ability of the ALDHhi and ALDHlow populations of human prostate cancer cells in vivo.

First, the tumorigenic potential of ALDHhi and ALDHlow cell populations was assessed by s.c. inoculation of the different prostate cancer cell populations in immunocompromised mice. To monitor the progression of tumor growth, 100, 1,000, and 10,000 cells were implanted s.c. and measured weekly by bioluminescent imaging for 28 days (Fig. 3A). The tumor take after inoculation of ALDHhi PC-3M-Pro4luc cells was strikingly higher compared with the ALDHlow cell population (Fig. 3A). ALDHlow cells failed to reproducibly generate tumors. In contrast to ALDHlow cells, as little as 100 cells of the ALDHhi population were already able to form tumors in vivo. Enhanced tumorigenicity and subcutaneous growth of the ALDHhi population was observed at all three cell dosages tested (Fig. 3A).

To compare tumorigenicity and metastatic ability of both cell subpopulations in a more clinically relevant model system, we investigated the ability of the cells to grow orthotopically when inoculated into the mouse prostate. Total tumor burden was significantly elevated in the mice injected with the ALDHhi cell population compared with ALDHlow cells (Fig. 3B).

In addition, we investigated the capacity of both ALDHhi and ALDHlow subpopulations to grow in bone marrow, the preferred site of prostate cancer metastasis. We observed enhanced tumor growth in the bone marrow after intraosseous injection of 10,000 ALDHhi PC-3M-Pro4luc cells compared with ALDHlow cells. Total tumor burden of the mice injected with ALDHhi cells was higher at all time points compared with the mice injected with the ALDHlow subpopulation (Fig. 3C).

**ALDHhi subpopulation in prostate cancer cell display increased metastatic ability in vivo**

We have previously shown that the inoculation of 100,000 PC-3M-Pro4luc cells into the left cardiac ventricle of nude mice, a model for prostate cancer bone metastasis, results in the formation of multiple bone metastases. After FACS sorting using the ALDEFLUOR assay, metastasis formation and tumor growth was significantly increased in mice inoculated intracardiacally with only 10,000 ALDHhi prostate cancer cells versus ALDHlow cell subpopulation (Fig. 4A–D). It is important to note that ALDHlow cells failed to reproducibly generate metastases, although limited growth was observed in

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**Table 1. Flow cytometric analysis of cell lines and primary prostate cancer cultures based on ALDH enzymatic activity**

<table>
<thead>
<tr>
<th>PCa cell lines</th>
<th>ALDH expression (%) ± SEM</th>
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<tbody>
<tr>
<td>LNCaP</td>
<td>0.8 ± 0.4</td>
</tr>
<tr>
<td>C4</td>
<td>2.6 ± 0.2</td>
</tr>
<tr>
<td>C4-2</td>
<td>6.2 ± 0.6</td>
</tr>
<tr>
<td>C4-2B</td>
<td>13.7 ± 0.3</td>
</tr>
<tr>
<td>PC-3</td>
<td>6.3 ± 1.0</td>
</tr>
<tr>
<td>Pro4luc</td>
<td>25.7 ± 1.9</td>
</tr>
<tr>
<td>Pro4lucBIII</td>
<td>31.0 ± 1.3</td>
</tr>
<tr>
<td>DU145</td>
<td>2.4 ± 0.3</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>PCa primary cultures</th>
<th>ALDH expression (%) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>7123</td>
<td>8.0 ± 0.5</td>
</tr>
<tr>
<td>567</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>025</td>
<td>5.5 ± 0.3</td>
</tr>
<tr>
<td>585</td>
<td>10.4 ± 0.8</td>
</tr>
<tr>
<td>021</td>
<td>12.4 ± 0.2</td>
</tr>
<tr>
<td>601</td>
<td>12.5 ± 0.2</td>
</tr>
</tbody>
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NOTE: Human prostate cancer cell lines and primary cultures were assayed with an ALDEFLUOR kit. Data are presented as percentage of cells with high ALDH enzymatic activity. Means of three individual experiments ± SEM are shown.
25% of the animals. In addition to metastatic tumor burden, the number of both bone and visceral metastases was significantly elevated in the mice injected with the ALDH\textsuperscript{hi} cells versus the ALDH\textsuperscript{low} cell subpopulation (Supplementary Fig. S3).

Expression of ALDH isoforms in human prostate cancer cell lines and primary tumors

We evaluated the mRNA expression levels of different ALDH isoforms in several cell lines (Supplementary Table S2).

Figure 3. ALDH\textsuperscript{hi} cells show increased tumorigenicity in vivo. A, tumor take after s.c. injection of 100, 1,000, and 10,000 ALDH\textsuperscript{hi} PC-3M-Pro4luc cells in ALDH\textsuperscript{hi} and ALDH\textsuperscript{low} subpopulations (n = 8/group; *, P < 0.05). Total tumor burden of the mice injected with 10,000 ALDH\textsuperscript{hi} PC-3M-Pro4luc cells (●) compared with the mice injected with 10,000 ALDH\textsuperscript{low} cells (○). Inset, the first 20 d after inoculation. B, representative images of mice 7, 14, 21, and 28 d after orthotopic injection with either 10,000 ALDH\textsuperscript{hi} or ALDH\textsuperscript{low} PC-3M-Pro4luc cells. Total tumor burden for the mice injected with the ALDH\textsuperscript{hi} population (●) or the ALDH\textsuperscript{low} cell subpopulation (○) (n = 8/group; *, P < 0.05). Data are representative of two independent experiments. C, representative images of mice intraosseously inoculated with either ALDH\textsuperscript{hi} or ALDH\textsuperscript{low} PC-3M-Pro4luc cells (10,000) at different days after inoculation. Total tumor burden of the mice injected with ALDH\textsuperscript{hi} (●) or ALDH\textsuperscript{low} (○) subpopulation (n = 6/group; **, P < 0.01). Inset, the first 20 d after inoculation.
Different ALDH isoforms (e.g., ALDH3A2, ALDH4A1, ALDH7A1, ALDH9A1, and ALDH18A1) were found to be highly expressed at the transcriptional level in the cell lines.

Similar to established cell lines, the mRNA expression levels of several ALDH isoforms were evaluated in clinical specimens and primary cultures of human prostate cancer (Supplementary Table S3). We observed relatively high expression of certain ALDH isoforms (e.g., ALDH3A2, ALDH4A1, ALDH7A1, ALDH9A1, and ALDH18A1) in primary cultures in a comparable manner as in established human prostate cancer cell lines.

Next, we investigated the potential use of these ALDH isoforms as diagnostic and prognostic markers in prostate cancer by immunostaining of paraffin-embedded sections of prostate primary tumors and matched bone metastases. We investigated a TMA (n = 30 samples) as well as 10 primary prostate tumors and their 10 matching bone metastases for ALDH expression. In contrast to previous published reports describing a role for ALDH1 in other solid cancers (14, 15, 23–25), no significant ALDH1 expression was found in primary prostate tumors, matching bone metastases and noncancerous tissue (Supplementary Fig. S4; Fig. 5). ALDH7A1 was expressed in the majority of primary tumors on the TMA (25 of 30; Fig. 5). Interestingly, strong ALDH7A1 staining was found in other series of matching bone metastases, whereas the surrounding bone marrow was negative (Supplementary Fig. S5; Fig. 5B). In our TMA, no correlation was found between Gleason score and ALDH7A1 expression (Supplementary Fig. S5B). No significant immunohistochemical localization was observed in noncancerous prostate tissue, high-grade prostate intraepithelial neoplasia, or prostate carcinoma of ALDH3A2 and ALDH18A1 (Fig. 5). In addition to ALDH7A1, other ALDH isoforms can potentially contribute to the strong ALDH activity as detected by the ALDEFLUOR assay (Supplementary Tables S1 and S2). Immunohistochemical staining was indeed observed for ALDH4A1 and ALDH9A1 in primary prostate cancer specimens (Supplementary Fig. S6).

Figure 4. ALDHHi cells show increased metastatic growth in vivo. A, representative images of mice inoculated with either ALDHHi or ALDHHilo PC-3M-Pro4luc cells (10,000) at day 21, 28, and 35 after inoculation (n = 15/group). B, total tumor burden of ALDHHi (○) and ALDHHilo (○) subpopulation. Inset, the first 28 d. C, total number of metastases per mouse in the mice injected with either ALDHHi (○) or ALDHHilo (○) cells (*, P < 0.05; ***, P < 0.001). D, bones of mice intracardially inoculated with either ALDHHi or ALDHHilo cells stained with Goldner staining. B, bone; T, tumor; BM, bone marrow; GP, growth plate. Data are representative of three independent experiments. Scale bars, 80 μm.
Discussion

Prostate cancer is a molecularly, phenotypically, and clinically heterogeneous disease. In addition to primary tumors, rapid autopsy programs have revealed a remarkable degree of heterogeneity among tumor cells within bone metastatic sites when comparing different patients as well as multiple sites within individual prostate cancer patients (35, 36).

Due to the observed heterogeneity in primary tumors and metastases, it has been a major challenge to distinguish and identify subtypes of prostate cancer. ALDH (aldehyde dehydrogenase) expression has been a potential biomarker for identifying cancer stem cells and metastatic potential in prostate cancer. The figure illustrates the expression of ALDH isoforms in human prostate tissue, primary prostate tumor, and bone metastases.

Figure 5. Expression of ALDH isoforms in human prostate tissue, primary prostate tumor, and bone metastases. A, representative images of TMA stained for several ALDH isoforms showing ALDH7A1-positive prostate carcinoma (25 of 30). No staining was found in ALDH1-, ALDH3A2-, or ALDH18A1-stained primary tumors (0 of 30) or normal prostate tissue. As a positive control, either liver or colon tissue was used. B, representative images of ALDH7A1 staining in primary prostate cancer and matching bone metastases (right, magnifications of the images). Scale bars, 80 μm.
select prostate cancer cells with tumor- and metastasis-initiating ability. Identification of the cell(s) of origin of prostate cancer as well as the neoplastic cell(s) involved in the formation of distant metastases is, therefore, fundamental to the understanding of carcinogenesis and metastasis. Furthermore, the functional identification of metastasis-initiating cells is a prerequisite for properly targeted therapy of metastatic disease in advanced prostate cancer.

The exact role and nature of TICs in prostate cancer (and the supportive stroma) in the formation of distant metastases has remained largely elusive. The development of more effective cancer therapies in advanced prostate cancer may, thus, benefit from the outcome of new studies and require selective targeting of this specific subpopulation of metastasis-initiating cells.

In prostate cancer, pioneering studies have led to the identification of CD44+/a2b1high/CD133+ prostate cancer stem cells (2). Because of the observed heterogeneity in prostate cancer, the use of single-cell markers for the selection, characterization/identification, and functional evaluation of stem/progenitor-like prostate cancer cells has been a major impediment and the reliability of cell surface markers such as CD133 as the sole way to isolate TICs remains controversial to date (3, 37). Deregulation of ALDH enzyme activity is implicated in the pathophysiology of various hematologic and epithelial cancers (13). The introduction of FACS-based viable cell sorting for ALDH activity (ALDEFLUOR assays) in tumor biology has further substantiated a role of ALDHhi subpopulations of cancer cells in carcinogenesis (15, 18). High ALDH activity, as detected by the ALDEFLUOR assay, can thus be used as a functional marker to isolate TICs in several types of epithelial cancers, including those of breast, lung, and colon (14, 15, 25). The applicability of the ALDEFLUOR assay for the functional identification in human prostate cancer is, however, not known.

We show here for the first time that high ALDH activity can be used to isolate human prostate cancer cells with significantly enhanced clonogenic and migratory properties in vitro as well as elevated tumorigenic and metastasis-initiating abilities in vivo. The percentage of ALDHhi cells in prostate cancer cell lines also seems to be related to tumorigenicity and metastatic behavior.

Our observations in human prostate cancer are in line with recent data showing elevated ALDH activity in adult murine prostate stem cells. Murine ALDHhi prostate epithelial cells also displayed a higher proliferative potential and were more effective in generating prostatic tissue in an in vivo prostate reconstitution assay (38).

Strikingly, our data show that the ALDHhi subpopulation of human prostate cancer cells display not only enhanced clonogenicity, migration, and tumorigenicity but also readily form metastases in vivo using preclinical models of intraprostastic growth and experimental metastasis. Importantly, significantly higher numbers of metastases were formed by the ALDHhi subpopulation and (10- to 1,000-fold) less cells are required to induce experimental metastasis in these models (26, 31). Our observations in prostate cancer are in line with the observed decrease in tumor growth of the ALDHhi cell population in breast cancer coinciding with diminished lung metastases (39). Limited growth of the ALDHhi population in prostate cancer was observed in our in vivo models as ALDHhi cells failed to reproducibly generate large tumors and (bone) metastases. Our in vivo observations together with the in vitro data further support the notion that the ALDHhi population predominantly contains restricted numbers of transit-amplifying cells (limited proliferative capacity) and postmitotic, differentiated cells, whereas the ALDHlo population is enriched for stem/progenitor-like prostate cancer cells (high proliferative capacity). In agreement with other studies describing the functional identification of TICs in prostate cancer, we show that the ALDHhi subpopulation expresses the potential stem/progenitor-like cell markers a2, a6, and av integrins, and CD44 (2, 32), whereas CD133 was not detected (3, 4, 37). It is believed that the lack of CD133 expression, as described recently also by others, may be due to the observed heterogeneity in prostate cancer. Strikingly, CD44 and integrin adhesion receptors (particularly a2, a6, and av) are significantly upregulated in these tumor- and metastasis-initiating prostate cancer cells. It has been firmly established that a2, a6, and av integrins, and CD44 (and their interplay with urokinase receptor and matrix metalloproteinases) play pivotal roles in the acquisition of an invasive phenotype and bone metastasis in several osteotrophic cancers, including prostate tumors (40–43). Taken together, we strongly feel that the elevated expression of the integrins and CD44 may underlie the observed enhanced migratory, clonogenic, tumor initiation, and metastasis initiation abilities of the ALDHhi/a2+/a6+/av+/ CD44+ subpopulation of human prostate cancer cells.

To the best of our knowledge, our study is the first to address the issue of multiple ALDH enzymes in tumor progression and, more importantly perhaps, metastasis initiation. At present, it is not entirely clear to what extent the various ALDH isoforms contribute to the high ALDH activity observed in highly tumorigenic and metastatic prostate cancer cells. It is important to note that the ALDEFLUOR kit used here and in previous reports (15, 44) has only been validated for ALDH1A1 (StemCell Technologies), whereas 19 different isoforms have been identified to date (13). In contrast to other epithelial cancers, ALDH isoforms (other than ALDH1) may contribute to ALDH activity and may be indicative of tumor-initiating and metastasis-initiating cells in human prostate cancer (14, 15, 25, 44). Moreover, ALDH3A2 and ALDH18A1 are not likely involved in the observed high ALDH activity in human prostate cancer as well. Instead, relatively high expression of ALDH7A1 in prostate cancer cell lines, primary cultures, and in primary prostate cancer tissue and matched bone metastases was found.

At present, it is unclear if high ALDEFLUOR activity is functionally involved in stemness (normal and cancer) or if it is a useful biomarker ("flag") to identify tumor-initiating and metastasis-initiating cells in prostate cancer. Further research is warranted to identify which of the 19 isoforms, besides ALDH7A1, are contributing to the observed high ALDEFLUOR activity of highly tumorigenic and metastatic human prostate cancer cells.

Given the fact that there is no real consensus in stem cell surface markers in prostate cancers, implementation of
markers of universal stemness based on intrinsic properties instead of phenotype seems invaluable for the stem cell research field.

It should also be noted that ALDH enzyme activity is frequently required for the detoxification of compounds, thus serving to protect (stem) cells, and maintaining cellular integrity. Indeed, several studies involving human cancers showed the ability of this class of enzymes to cause resistance to chemotherapeutic agents such as cyclophosphamide (45, 46). In addition, ALDH enzymes also play important roles in retinoic acid metabolism (47) and androgen receptor binding (48), which are both involved in prostate development, prostate cancer progression, and metastasis formation.

In summary, the overall results of this study show that ALDH-based sorting of human prostate cancer cells by ALDEFLUOR can be potentially used simultaneously to select for metastasis-initiating cells and TICs. Analysis of ALDH activity of clinical prostate cancer samples may thus become useful for the stratification of prostate cancer patients at risk of developing metastatic disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank the Department of Pathology (University of Sheffield, Sheffield, United Kingdom) for supplying the TMA, Maggy van Wijk (Department of Endocrinology, LUMC, the Netherlands) for the technical support, Caroline Jong-Mom (Department of Urology, LUMC, the Netherlands) for the clinical sampling, and Prof. N. Maitland and Dr. A. Collins (University of York, York, United Kingdom) for their technical support with primary prostate cancer cultures and helpful suggestions.

Grant Support

PROMET, a European Specific Targeted Research Project.

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Received 10/15/2009; revised 03/23/2010; accepted 03/31/2010; published OnlineFirst 06/01/2010.

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*Cancer Res* 2010;70:5163-5173. Published OnlineFirst June 1, 2010.

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