**FOXA1 Promotes Tumor Progression in Prostate Cancer and Represents a Novel Hallmark of Castration-Resistant Prostate Cancer**

Josefine Gerhardt,* Matteo Montani,† Peter Wild,* Marc Beer,* Fabian Huber,* Thomas Hermanns,‡ Michael Müntener,‡ and Glen Kristiansen§

From the Institute of Surgical Pathology* and the Department of Urology,† University Hospital of Zurich, Zurich, Switzerland; the Institute of Pathology,‡ University of Bern, Bern, Switzerland; and the Institute of Pathology,§ University Hospital Bonn, Bonn, Germany

Forkhead box protein A1 (FOXA1) modulates the transactivation of steroid hormone receptors and thus may influence tumor growth and hormone responsiveness in prostate cancer. We therefore investigated the correlation of FOXA1 expression with clinical parameters, prostate-specific antigen (PSA) relapse-free survival, and hormone receptor expression in a large cohort of prostate cancer patients at different disease stages. FOXA1 expression did not differ significantly between benign glands from the peripheral zone and primary peripheral zone prostate carcinomas. However, FOXA1 was overexpressed in metastases and particularly in castration-resistant cases, but was expressed at lower levels in both normal and neoplastic transitional zone tissues. FOXA1 levels correlated with higher pT stages and Gleason scores, as well as with androgen (AR) and estrogen receptor expression. Moreover, FOXA1 overexpression was associated with faster biochemical disease progression, which was pronounced in patients with low AR levels. Finally, siRNA-based knockdown of FOXA1 induced decreased cell proliferation and migration. Moreover, in vitro tumorigenicity was inducible by ARs only in the presence of FOXA1, substantiating a functional cooperation between FOXA1 and AR. In conclusion, FOXA1 expression is associated with tumor progression, dedifferentiation of prostate cancer cells, and poorer prognosis, as well as with cellular proliferation and migration and with AR signaling. These findings suggest FOXA1 overexpression as a novel mechanism inducing castration resistance in prostate cancer. (Am J Pathol 2012; 180:848–861; DOI: 10.1016/j.ajpath.2011.10.021)

Prostate cancer is the most common malignant tumor in men in more developed countries, and the molecular background of its tumor progression is still incompletely understood. Most prostate carcinomas require androgen stimulation to grow, and for nearly 70 years androgen ablation therapy has been one of the central therapeutic strategies against advanced prostate cancer. Although most tumors initially respond to this therapy, some will become resistant and progress to castration-resistant (CRPC) disease. CRPC clinically tends to progress more rapidly than earlier disease manifestations. Although novel chemotherapeutic agents have found wide acceptance in urooncological practice in recent years, a large proportion of patients with CRPC show only limited or no response to these therapies. The underlying molecular biology of CRPC is highly complex, and numerous mechanisms have been proposed that promote and retain androgen independence. Increased AR function/expression is one of the most important mechanisms in this context. Prostate cancer cells with high levels of AR expression are thought to survive androgen depletion and then are able to convert the disease into the hormone-refractory stage (CRPC). However, none of these proposed mechanisms have achieved therapeutic utility, and the analysis of new mechanisms of androgen independence remains of paramount importance. Prognostic markers that herald increased propensities of...

Supported in part by funds from the University of Bern and the University of Zurich.

Accepted for publication October 21, 2011.

J.G. and M.M. contributed equally to the present work.

Portions of these data have been presented at the annual meetings of the German Society of Pathology (DGP, Freiburg, Germany, June 2009) and the Swiss Society of Pathology (SGPath, Locarno, Switzerland, November 2009).

CME Disclosure: None of the authors disclosed any relevant financial relationships.

Supplemental material for this article can be found at http://ajp.amjpathol.org or at doi: 10.1016/j.ajpath.2011.10.021.

Address reprint requests to Glen Kristiansen, M.D., Institute of Pathology, University Hospital Bonn, Sigmund-Freud-Str. 25, D-53127 Bonn, Germany. E-mail: glen.kristiansen@ukb.uni-bonn.de.
tumors to become hormone refractory earlier are also needed.

In a previous study, we conducted an array-based transcript analysis of normal prostate tissue and matched cancer tissue to identify differentially expressed genes as candidates for further research, and forkhead box A1 (FOXA1) was among the top up-regulated genes in prostate cancer tissue. FOXA1 was originally detected in nuclear extracts of rat liver and hepatoma (HepG2) cells and was named hepatocyte nuclear factor 3α (HNF-3α). After analyses of the Drosophila forkhead gene, FOXA1 became the first identified member of the family of forkhead box transcription factors in vertebrates. Forkhead transcription factors are structurally characterized by a winged helix three-dimensional structure similar to that of linker histones. This enables FOX proteins to open highly packed chromatin and so to facilitate the binding of other transcription factors, which prompted researchers to give this class of proteins the name “pioneer factors.”

FOXA1 functionally promotes estrogen (ER) and androgen receptor (AR), among others. FOXA1 is found in various organs, including respiratory (lung), gastrointestinal (liver, pancreas, colon), and urogenital tract (bladder, prostate); it is also found in the breast. It can bind to the promoters of more than 100 genes and regulates metabolic processes, cell signaling, and the cell cycle. During embryogenesis, FOXA1 appears to be important in organogenesis of prostate, lung, liver, and pancreas. In the adult organism, FOXA1 may be centrally involved in glucose homeostasis; FOXA1 knock-out mice die soon after birth, because of hypoglycemia. Even after more than 20 years of FOXA1 research, the broad spectrum of FOXA1-related functions in physiological conditions is only incompletely understood. Nonetheless, a growing body of literature attests to the importance of FOXA1 in human neoplasia. The role of FOXA1 has been studied in tumors of breast, prostate, esophagus, and lung. Gene amplification and protein overexpression were observed in Barrett’s and lung adenocarcinomas. FOXA1 has been most extensively studied in breast cancer; a favorable prognosis is related to high FOXA1 levels, and there is a strong correlation between FOXA1 expression and luminal type A.

To date, gene amplification and overexpression of FOXA1 protein have been observed in tumors of esophagus, lung, and breast. No overexpression was revealed in a small study on prostate cancer. Because FOXA1 is involved in androgen and estrogen signaling, a close analysis of FOXA1 in prostate carcinomas of various stages and its clinical and prognostic significance including correlations with AR and ER expression is of particular interest. This was our objective with the present study, in which expression data were complemented by experimental analyses, to unravel the functional role of FOXA1 in prostate cell line models.

We studied a large cohort of normal and cancerous prostate tissues (n = 324) to correlate FOXA1 expression with other prognostic parameters, relapse-free survival, and AR and ER expression. We demonstrated differential expression of FOXA1 in normal tissue, depending on the zonal origin, as well as a continuous up-regulation of FOXA1 during prostate cancer progression and an association with shorter relapse-free survival. Additionally, we revealed a close correlation between FOXA1 and hormone receptor expression. Furthermore, siRNA-based transient gene knockdown of FOXA1 led to a reduced cell proliferation and migration rate in prostate cancer cell lines. Finally, we determined that full in vitro tumorigenicity of the androgen-dependent prostate cancer cell line LNCaP is dependent on androgen stimulation as well as on FOXA1 expression. Thus, we have established for the first time a tumor-promoting function of FOXA1 in prostate cancer.

Materials and Methods

Patients and Tissue Microarray Construction

The initial study population was 529 patients who underwent radical prostatectomy, transurethral resection, or resection of organ metastasis between 1993 and 2006 at the Department of Urology of the University Hospital of Zurich because of either prostate cancer or benign prostatic hyperplasia (BPH), as described previously. In this study, only those cases were included that were evaluable for FOXA1 staining (n = 288). Within this group, the distribution of specimen materials was as follows: primary carcinomas (radical prostatectomy specimens; n = 207), castration-resistant prostate cancer (CRPC; n = 27), lymph node and distant metastases (n = 39), and benign prostatic hyperplasia (BPH; n = 15). Patient age among primary carcinomas ranged between 46 and 75 years (median, 65 years). Preoperative PSA levels ranged from 0.4 to 357 ng/mL (median, 11.1 ng/mL). Gleason scores in the primary prostate cancer cohort were distributed as follows: 35 (16.9%) cases with Gleason score 2 to 6, 119 (57.5%) cases with Gleason score 7, and 53 (25.6%) cases with Gleason score 8 to 10. The corresponding pT categories were 129 (62.3%) cases at pT2 and 78 (37.7%) cases at pT3/4.Margins were clear (R0) in 131 (63.9%) cases; the others had evidence of tumor at the inked margins (R1).

Follow-up data were obtained for the group of primary carcinomas after radical prostatectomy. In this group, 92 patients (44.4%) experienced a biochemical (PSA) relapse after a median time of 24 months, defined as a rising PSA level exceeding 0.1 ng/mL, from a nadir after surgery. The median follow-up time for these patients was 60 months. A tissue microarray (TMA) from these cases was constructed as described previously. Additionally, 23 cases of CRPC transurethrally resected for palliation were included, along with 1 bone trephine of a CRPC metastasis and 12 prostatectomy specimens with transitional zone carcinomas and benign prostate hyperplasia from the archives of the Institute of Surgical Pathology, University Hospital Zurich. The study was approved by the Cantonal Ethics Committee of Zurich (approval number StV 25-2007).
**Immunohistochemical and Immunofluorescence Staining and Analysis**

The TMA blocks were freshly cut (3 μm thick) and mounted on SuperFrost slides (Menzel Gläser, Braunschweig, Germany). Immunohistochemistry was conducted with a Benchmark automated staining system (Ventana Medical Systems, Tucson, AZ) using Ventana reagents for the entire procedure. Primary antibodies (FOXA1: anti-HNF3α/β, clone C-20, dilution 1:400 in a Bond diluent, Santa Cruz Biotechnologies, Santa Cruz, CA; AR: anti-androgen receptor, clone F39.4.1, dilution 1:500 in a Bond diluent, BioGenex, San Ramon, CA; ERα: anti-estrogen receptor, clone SP1, prediluted in Ventana diluent, Ventana Medical Systems; ERβ: anti-human estrogen receptor β2, clone 57/3, dilution 1:200 in bovine serum albumin/Tris, AbD Serotec, Oxford, UK; PSA: anti-human prostate-specific antigen, dilution 1:4000 in Ventana diluent, Dako, Glostrup, Denmark; Ki-67: anti-Ki-67 antigen, clone MIB-1, dilution 1:20 in Ventana diluent, Dako, Glostrup, Denmark; cyclin D1: anti-cyclin D1, clone SP4, dilution 1:40 in Ventana diluent, Thermo Scientific, Fremont, CA; and p27: anti-p27, clone C-19, dilution 1:30, Santa Cruz Biotechnologies) were detected with an UltraVIEW DAB detection kit (Ventana Medical Systems) using Benchmark CC1m heat-induced epitope retrieval. The signal was further enhanced with the Ventana amplification kit. Slides were counterstained with hematoxylin, dehydrated, and mounted. Immunofluorescence double stainings were performed as described previously, using the same antibodies as for immunohistochemistry.

Evaluation of the stained TMAs was done by two pathologists (M.M. and G.K.) simultaneously on a multiheaded microscope. Evaluation of nuclear staining intensities for FOXA1, AR, ERα/β, and p27 and the cytoplasmic staining intensity for PSA was evaluated according to a four-tiered scale: 0 (negative), 1+ (weak), 2+ (moderate), and 3+ (strong). For examples of FOXA1 staining, see Supplemental Figure S1 at http://ajp.amipathol.org. The proliferation index of tumors was estimated as the percentage of Ki-67 positive cells, and cyclin D1 expression was quantified as the percentage of positive cells.

**Cell Culture**

PC-3 and DU-145 cells from the American Type Culture Collection (ATCC, Manassas, VA) and LNCaP cells from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) were cultivated in Ham’s F-12 medium with Kaighn’s modification, Eagle’s minimal essential medium, and RPMI-1640 medium, respectively, each supplemented with 10% fetal bovine serum. RWPE-1 cells (ATCC) were grown in keratinocyte serum-free medium mixed with bovine pituitary extract and human recombinant epidermal growth factor (medium and supplements from Invitrogen, Carlsbad, CA). All cell lines were cultured at 37°C, 5% CO₂, and 100% humidity.

**Cell Lysis and Western Blot Analysis**

Cells were lysed in 60 mmol/L n-octyl-β-D-glucopyranoside (Sigma-Aldrich, St. Louis, MO) in the presence of protease inhibitors (complete, mini, EDTA-free, protease inhibitor cocktail tablets; Roche Applied Science). Cleared lysates (20 μg) were separated by SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA). After blocking with 1% bovine serum albumin in PBS-Tween buffer, membranes were probed with primary antibodies (FOXA1: clone C-20, Santa Cruz Biotechnologies; cyclin D1: clone 72-13G, Santa Cruz Biotechnologies; p27: clone C-19, Santa Cruz Biotechnologies; and actin: MAB1501, Millipore, Billerica, MA), followed by horseradish peroxidase-conjugated anti-mouse secondary antibody (Pierce; Thermo Scientific, Rockford, IL), incubation, and detection via Pierce SuperSignal West Dura extended duration substrate (Thermo Scientific).

**siRNA Transfection for Transient Gene Knockdown**

Generally, LNCaP cells were transfected immediately after seeding using HiPerFect transfection reagent (Qiagen) according to the FastForward protocol provided by the manufacturer. PC-3 cells were transfected using Lipofectamine 2000 (Invitrogen) as transfection reagent. Cells were transfected at the day of seeding with a final siRNA concentration of 10 nmol/L (target sequences: nonspecific 5`-AATTCTCGAAGCTGTCACTG-3`; FOXA1 siRNA #1 5`-CCAGACGCTTCTATCGATTAT-3`; FOXA1 siRNA #2 5`-CAACGGCCAGGCAAGCTATGA-3`; and FOXA1 siRNA #3 5`-CTCCTCCGTCGGGTTATG-3`, all from Qiagen). At 1 day after transfection, the medium of PC-3 cells was changed. Generally, the trans-
fection mix was prepared in Opti-MEM reduced-serum medium (Invitrogen). During androgen treatment, LNCaP cells were transfected using Lipofectamine 2000, as described for the PC-3 cells. Phenol red-free RPMI 1640 medium (Invitrogen) was used to prepare the transfection mix and no medium was changed. Knockdown efficiency was confirmed on the RNA and protein levels using qRT-PCR and Western blot analysis, respectively.

**DNA Synthesis Rate**

The DNA synthesis rate was measured based on bromodeoxyuridine (BrdU) incorporation into newly synthesized DNA strands by applying a cell proliferation ELISA BrdU kit (Roche Applied Science) according to the manufacturer’s instructions. Briefly, cells were seeded in 96-well plates; after 6 hours, BrdU labeling reagent was added to the medium, and cells were incubated at 37°C overnight. Subsequently, cells were fixed and stained with an anti-BrdU antibody, which was detected with substrate solution. The color reaction was stopped by adding sulfuric acid, and optical density was measured at 450 nm and at 595 nm as reference wavelength.

**Transmigration Assay**

Haptotactic cell migration was analyzed in Transwell chambers (Corning Life Sciences, Lowell, MA) that were coated at the bottom with 10 μg/mL fibronectin (Roche Applied Science) for 2 hours at room temperature. The lower chamber was filled with serum-free RPMI 1640 medium supplemented with 0.5% bovine serum albumin (Sigma-Aldrich). Cells were seeded in the upper chamber in the same medium. After 24 hours incubation at 37°C, nonmigrated cells were removed with a cotton swab, and the remaining cells were fixed with methanol/acetic acid and stained with DAPI (Sigma-Aldrich). Images were acquired at nine defined locations on the membrane and were analyzed with ImageJ software, version 1.4 (NIH, Bethesda, MD). The migration rate was quantified by determining the area of the membrane that was covered with stained nuclei.

**Scratch Wound Assay**

Cells were seeded in 6-cm cell culture plates and grown to 95% confluency, before they were transfected with siRNAs. A wound was introduced into the cell monolayer using a pipette tip and images were acquired every 24 hours. The area of the wound was measured using ImageJ 1.4 software.

**Cell Cycle Analysis**

Cells were detached, washed twice with sample buffer (1% glucose in PBS), and fixed with ethanol overnight at 4°C. The next day, cells were incubated with 50 μg/mL propidium iodide (Sigma-Aldrich), 0.3 mg/mL RNase A (Qiagen) and 0.05% Triton X-100 (Sigma-Aldrich) in sample buffer and then were analyzed by flow cytometry. The proportion of cells in G1, S, and G2 phases was determined applying the Dean/Jett/Fox model provided with the FlowJo version 6.3 software package (Tree Star, Ashland, Oregon).

**Androgen Treatment**

LNCaP cells were seeded in RPMI 1640 medium without phenol red, supplemented with 5% charcoal-stripped fetal bovine serum (Invitrogen). After 48 hours, dihydrotestosterone (DHT; Sigma-Aldrich) dissolved in ethanol was added, to a final concentration of 100 nmol/L.

**Statistical Analysis**

For analysis of the association of staining intensity with clinicopathological parameters, descriptive statistics were used (cross tables, two-tailed Fisher’s exact test, and two-tailed χ² test). For correlation significance, Spearman’s ρ test (two-tailed) was used. Univariate survival analyses were conducted according to the Kaplan-Meier log-rank test. These statistics were calculated with SPSS statistical software, version 19 (SPSS, Chicago, IL). Cell culture results are expressed as means ± SD. Each experiment was repeated at least three times, and significance was tested in a two-tailed paired or unpaired t-test, if necessary with Welch’s correction, depending on the data set. Significance was set at P < 0.05.

**Results**

**Immunohistochemistry of FOXA1**

FOXA1 was found nearly exclusively in nuclei of epithelial cells; no stromal immunoreactivity was observed, neither in normal nor in tumor tissues. In cases with strong FOXA1 expression, a mild degree of additional cytoplasmatic staining was noted but not recorded. In normal pros-tatic tissues from the peripheral zone (PZ) close to the tumor (adjacent normal; n = 204), the distribution of FOXA1 expression scores was as follows: 3 (1.5%) cases negative, 42 (20.6%) cases 1+, 146 (71.6%) cases 2+, and 13 (6.4%) cases 3+. The mean expression score of FOXA1 in normal tissue of the PZ was 1.8 (Figure 1A). FOXA1 was found in secretory cells; basal cells were either negative or only weakly and inconsistently positive. In contrast, hyperplastic normal tissues from the transitional zone (TZ; n = 15) showed less FOXA1 positivity (mean, 0.67); 7 (46.7%) cases negative, 6 (40%) cases 1+, and 2 (13%) cases 2+; in no case did tissues score 3+ (0%) (Figure 1B).

The differential expression of FOXA1 in normal tissues from PZ and TZ prompted us to differentiate tumors from both zones in the analysis. In primary carcinomas from the PZ (Figure 1C), the distribution of FOXA1 expression scores was as follows: 22 (10.6%) cases negative, 56 (27.1%) cases 1+, 114 (55.1%) cases 2+, and 15 (7.2%)
cases 3+. The mean expression score of FOXA1 in primary carcinomas of the PZ was 1.59. Of 12 TZ carcinomas, 11 were FOXA1-negative (Figure 1D) and 1 was weakly positive (mean expression, 0.08). Of these 12 cases, 7 had a coexisting PZ carcinoma (Figure 1, C and D) with significantly higher levels of FOXA1 (mean expression, 1.71; Wilcoxon test, \( P < 0.016 \)). In metastases, the distribution of FOXA1 expression scores was as follows: 7 (17.9%) cases negative, 4 (10.3%) cases 1+, 10 (25.6%) cases 2+, and 18 (46.2%) cases 3+. The mean expression score of FOXA1 in metastases was 2.0 (Figure 1E). In CRPC, the distribution of FOXA1 expression scores was as follows: 2 (3.9%) cases negative, 2 (3.9%) cases 1+, 23 (45.1%) cases 2+, and 24 (47.1%) cases 3+. The mean expression score of FOXA1 in CRPC was 2.35 (Figure 1F).

In summary, we saw little differences between normal tissue of the peripheral zone and peripheral zone cancer tissue, although a tendency to slightly decreased FOXA1 levels in cancer was observed. Significant up-regulation of FOXA1 was seen in metastases, which was more pronounced in CRPC. Surprisingly, normal or hyperplastic tissues from the transitional zone as well as carcinomas stemming from this region showed markedly and significantly lower FOXA1 expression levels (Table 1 and Figure 2).

---

**Table 1.** Staining Intensity of FOXA1 by Patient Group

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Staining intensity score [no. (%)]</th>
<th>Mean score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1+</td>
</tr>
<tr>
<td>Normal</td>
<td>15</td>
<td>7</td>
</tr>
<tr>
<td>TZ</td>
<td>204</td>
<td>3</td>
</tr>
<tr>
<td>Primary carcinoma</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>TZ</td>
<td>207</td>
<td>22</td>
</tr>
<tr>
<td>CRPC</td>
<td>39</td>
<td>7</td>
</tr>
<tr>
<td>CRPC</td>
<td>51</td>
<td>2</td>
</tr>
</tbody>
</table>

CRPC, castration-resistant prostate cancer; PZ, peripheral zone; TZ, transitional zone.
tases, and then decreased toward the hormone refractory state of CRPC.

Relationship of FOXA1 to Clinicopathological and Molecular Parameters

In primary carcinomas, FOXA1 expression was significantly associated with pT stage and Gleason score (Table 2). FOXA1 was further significantly correlated with epithelial AR ($\rho = 0.534$, $P < 0.001$), stromal AR ($\rho = 0.213$, $P = 0.002$), stromal ERα ($\rho = 0.444$, $P < 0.001$), epithelial ERβ ($\rho = 0.41$, $P < 0.001$), and stromal ERβ ($\rho = 0.354$, $P < 0.001$). No correlation with PSA expression or Ki-67 proliferation index was found. Other interdependencies are given in Supplemental Table S1 (available at http://ajp.amjpathol.org/).

The strong correlation of FOXA1 with epithelial AR prompted us to conduct a double immunofluorescence study of these two markers in a small subset of cases, which demonstrated an excellent concordance of immunoreactivity of both markers (Figure 4).

Survival Analyses

The analysis of patient follow-up data on serum PSA levels revealed a significant association between high FOXA1 expression levels and shorter relapse-free survival times in Kaplan-Meier analysis (mean, 87 versus 70 months; $P = 0.018$; Figure 5A). However, FOXA1 expression failed to represent an independent prognostic marker (Cox regression analysis, including preoperative PSA level, pT stage, Gleason score, and margin status; data not shown). To clarify whether this prognostic significance is dependent on AR expression, a stratified Kaplan-Meier analysis according to low (0 to 2) and high (3+) AR levels was performed. This analysis demonstrated an increased prognostic significance of FOXA1 in cases with low AR levels (mean, 86 versus 56 months; $P = 0.001$); in cases with high AR levels, no prognostic significance at all was observed.

![Figure 3](image.png)

**Figure 3.** Mean staining intensity of FOXA1, androgen (AR) and estrogen receptors (ER), and PSA in the different patient subgroups.

![Figure 4](image.png)

**Figure 4.** Double immunofluorescence of FOXA1 and AR in human prostate cancer. Original magnification, ×400.

### Table 2. Relationship Between FOXA1 Expression and Clinicopathological Data for Primary Prostate Carcinoma Specimens (Radical Prostatectomies)

<table>
<thead>
<tr>
<th>Clinicopathological feature</th>
<th>FOXA1 expression [no. (%)]</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preoperative PSA, ng/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;10</td>
<td>35 (17.7)</td>
<td>0.769</td>
</tr>
<tr>
<td>&gt;10</td>
<td>40 (20.2)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>75 (37.9)</td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td>0.018*</td>
</tr>
<tr>
<td>pT2a–c</td>
<td>57 (27.5)</td>
<td></td>
</tr>
<tr>
<td>pT3–4</td>
<td>21 (10.1)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>78 (37.7)</td>
<td></td>
</tr>
<tr>
<td>Margins</td>
<td></td>
<td>0.094†</td>
</tr>
<tr>
<td>R0</td>
<td>53 (25.9)</td>
<td></td>
</tr>
<tr>
<td>R1</td>
<td>24 (11.7)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>77 (37.6)</td>
<td></td>
</tr>
<tr>
<td>Gleason score</td>
<td></td>
<td>0.017</td>
</tr>
<tr>
<td>5–6</td>
<td>20 (9.7)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>42 (20.3)</td>
<td></td>
</tr>
<tr>
<td>8–10</td>
<td>16 (7.7)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>78 (37.7)</td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td></td>
<td>0.774</td>
</tr>
<tr>
<td>&lt;64</td>
<td>37 (17.9)</td>
<td></td>
</tr>
<tr>
<td>&gt;64</td>
<td>41 (19.8)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>78 (37.7)</td>
<td></td>
</tr>
</tbody>
</table>

*P < 0.05.
†P < 0.1.
discerned (Figure 5, B and C). Furthermore, in the patient subgroup with low AR levels, FOXA1 expression failed prognostic significance in a multivariate analysis (Cox regression analysis, including preoperative PSA level, pT stage, Gleason score, and margin status; data not shown).

Characterization of FOXA1 Expression in Prostatic Cell Lines

We next sought to test whether FOXA1 overexpression has a functional relevance in prostate cancer cell lines. Expression on the transcript and protein levels was determined in an immortalized prostatic epithelial cell-line (RWPE-1) and in three metastasis-derived prostate cancer cell lines (LNCaP, DU-145, and PC-3). In Western blot analysis, FOXA1 protein was strongly expressed in LNCaP and PC-3 cells, but was undetectable in DU-145 and RWPE-1 cells. mRNA expression was strongest in LNCaP cells. Medium levels of FOXA1 mRNA were detected in PC-3 and DU-145 cells, but levels in RWPE-1 cells were very low (Figure 6A). The discrepancy between FOXA1 mRNA and protein levels in DU-145 and PC-3 cells indicates differential post-transcriptional regulation of protein expression in these cell lines. In light of the strong protein expression of FOXA1, LNCaP and PC-3 cells were chosen for initial tumorigenicity studies. A knockdown protocol based on RNA interference was established for both cell lines. qPCR and Western blot analysis revealed a potent FOXA1 knockdown on the mRNA and the protein level, respectively, 72 hours after transfection of three different siRNAs (Figure 6, B and C).

FOXA1 Knockdown Reduces Cell Proliferation

One of the main characteristics of tumor cells is uncontrolled proliferation.36 To determine whether FOXA1 expression has an influence on prostate cancer cell proliferation, LNCaP and PC-3 cells were transfected with FOXA1-specific siRNAs and cells were counted. Compared with corresponding nonspecific siRNA-transfected cells, the cell number of FOXA1 siRNA #1, #2 and #3 transfected LNCaP cells was reduced significantly (by 24%, 18%, and 20%, respectively). Although siRNA treatment itself strongly affected the propagation of PC-3 cells, the number of cells transfected with FOXA1 siRNA #1, #2 and #3 was further decreased by a respective 32%, 44%, and 36%, compared with control cells (Figure 7, A and B). To test whether the reduction of cell number after FOXA1 knockdown was caused by a reduction of cell proliferation rate, DNA synthesis rate was determined by measuring BrdU incorporation; 44%, 47%, and 29% less BrdU was detected after transfection of LNCaP cells with FOXA1 siRNA #1, #2 and #3, respectively. The DNA synthesis rate of PC-3 cells was decreased by a highly significantly ($P < 0.0008$) by 18%, 48%, and 28% in cells transfected with FOXA1 siRNA #1, #2, and #3, compared with nonspecific siRNA-transfected cells (Figure 7, C and D). Taken together, these results show a significant reduction of proliferation of LNCaP and PC-3 cells after FOXA1 knockdown.
Reduced Cell Proliferation Is Associated with G1 Arrest

The observed reduction of cell proliferation on FOXA1 knockdown involved the question of whether this is reflected in a change of cell cycle distribution. We therefore determined the proportion of cells in G1, S, and G2/M phases by flow cytometry of propidium iodide-stained cells. The proportion of LNCaP cells in G1 phase increased from 65% in control cells to 70% to 75% in FOXA1 knockdown cells, whereas the proportion of cells in S phase declined from 22% in control cells to 15% to 20% in FOXA1 knockdown cells; the proportion of cells in G2/M-phase also decreased slightly, from 13% to 10% (Figure 8, A, C, and E). The differences were even more pronounced in PC-3 cells. Of the cells treated with nonspecific siRNA, 63% were in G1 phase, in contrast to 72% to 81% of the cells treated with FOXA1-specific siRNA #1, #2, and #3. In accord with this ≤20% of increase of cells in G1 phase, the proportions of cells in S phase decreased in FOXA1 knockdown PC-3 cells from 19% to 6% to 14%, and those in G2/M decreased from 10% to 17% (Figure 8, B, D, and F).

To analyze whether G1 arrest is caused by a decrease in cyclin D1 expression or an increase in p27 expression, we measured levels of these two proteins in FOXA1 knockdown cells, as well as in our patient cohort. Western blot analysis showed that the expression of cyclin D1 was clearly reduced in PC-3 cells treated with FOXA1 siRNA #2 and #3, but expression of p27 did not change in these cells (Figure 9). Furthermore, we reanalyzed the expression data of cyclin D1 in our cohort, which we had studied recently. Additionally, the TMA slides were stained with an anti-p27 antibody. We found a strong correlation between FOXA1 and cyclin D1 (r = 0.183, P = 0.02) and also p27 (r = 0.295, P < 0.001) in primary prostate carcinomas. In summary, these data clearly show a G1 arrest on FOXA1 knockdown, which is very prominent in PC-3 cells.

Cell Migration Is Inhibited on FOXA1 Knockdown

An elevated migration rate is another important feature of malignant cells. The relevance of FOXA1 expression during cell migration was elucidated in a transmigration assay. In particular, migration toward fibronectin was investigated. Down-regulation of FOXA1 reduced migration of LNCaP cells through the porous membrane significantly, by 18%, 12% and 25% for siRNA #1, #2, and #3, compared with control cells. In addition to the nonspecific transfection effect, the FOXA1 siRNA-specific reduction of PC-3 cell migration accounted for 12%, 20%, and 29% for the respective siRNAs #1, #2, and #3, compared with corresponding control cells (Figure 10, A and B). Migration of PC-3 cells on an uncoated surface was assessed in a scratch wound assay, which is less reliably applicable to LNCaP cells, because these cells do not tend to form dense monolayers. The area not covered with migrated PC-3 cells within 24 hours after wounding was 1.9, 2.1, and 1.6 times larger after FOXA1 down-regulation with siRNA #1,
#2, and #3, respectively, compared with nonspecific siRNA treatment (Figure 10C), indicating a diminished cell motility. Altogether, these data show that the reduction of FOXA1 expression induces considerable inhibition of LNCaP and PC-3 cell migration.

Androgen Inducibility of Cell Proliferation and Migration Is Dependent on FOXA1

Because we observed an increased prognostic significance of FOXA1 expression in patients with low AR levels but no prognostic value of FOXA1 expression in patients with high AR levels, we asked whether FOXA1 is important in mediating the androgen response. We therefore reanalyzed the proliferation and migration behavior of FOXA1\textsuperscript{high} and FOXA1\textsuperscript{low} LNCaP cells on androgen treatment. In nontransfected and nonspecific siRNA-transfected cells, DNA synthesis rate was inducible by androgen treatment (fold change H11005 2.5 and 3.2, respectively). However, in FOXA1 siRNA-treated cells, DHT did not change the cell proliferation rate (Figure 11A). Similarly, the number of transmigrated cells was elevated in nontransfected and nonspecific siRNA-transfected LNCaP cells (fold change H11005 1.5 and 1.4, respectively). In contrast, the migration rate of FOXA1 siRNA-transfected cells was unaffected by DHT treatment (Figure 11B).

We further analyzed the expression of known androgen-dependent genes. PSA mRNA levels were strongly inducible by DHT treatment, independent of FOXA1 expression status of LNCaP cells (average fold change = 10.3; Figure 11C). In contrast, expression of AGR2, which has been shown to be stimulated by FOXA1,\textsuperscript{38} was not inducible by DHT treatment in FOXA1 siRNA-treated LNCaP cells, although its expression was strongly increased in FOXA1 high LNCaP cells (fold change = 3.3 and 5.1; Figure 11D). Thus, proliferation, migration, and AGR2 expression of androgen-dependent cells is inducible by DHT treatment only in the presence of FOXA1.
Discussion

A previous study on whole-mount prostatectomy sections from 20 primary prostate carcinomas, which included benign and malignant tissue, revealed a strong expression of FOXA1 in normal tissue and no up-regulation in prostatic intraepithelial neoplasia or carcinoma tissue.\(^{30}\) Similar results were obtained in mouse models of prostate cancer.\(^{39,40}\) However, one study analyzing FOXA1 expression in mouse models indicated a consistent up-regulation of FOXA1 in prostatic intraepithelial neoplasia and prostate adenocarcinoma.\(^{30}\)

Here, we have presented data from a large patient cohort (to our knowledge, the largest yet) for analysis of the role of FOXA1 in prostate tissues, including benign
Figure 11. Cell proliferation and migration with androgen treatment in wild-type and FOXA1 knockdown LNCaP cells. 1,000,000 cells were seeded in phenol red-free medium with charcoal-stripped fetal bovine serum in 6-cm dishes and transfected with nonspecific siRNAs (unspec) or siRNAs #1, #2, or #3. After 48 hours, ethanol (EtOH) or DHT was added to cells. Another 24 hours later, 20,000 cells were reseeded in quadruplicate into 96-well plates and the DNA synthesis rate was measured (A) and 50,000 cells were reseeded in triplicate into fibronectin-coated Transwell chambers and a transmigration assay was performed (B). mRNA expression of PSA (C) and AGR2 (D) was determined by qPCR. *P < 0.05.

tissues from different prostatic zones and a large set of clinically characterized primary prostate cancer cases, metastases, and advanced castration-resistant prostate cancer. The main findings are a zonal dependence of FOXA1 expression in normal and tumor tissues, increased FOXA1 levels with tumor progression (with the highest levels in CRPC), a prognostic value in primary tumors, a very strong correlation of FOXA1 with AR and ER, and finally experimental data to endorse the functional relevance of FOXA1 in prostate cancer cell lines.

With our first TMA analysis, we found significantly lower FOXA1 expression in non-neoplastic tissues, compared with prostate cancer tissues, a finding in striking contrast to the data of a previous study revealing no differences between normal and cancerous tissues.30 When we noted a high variability of FOXA1 expression levels in non-neoplastic tissues, we reanalyzed our tissues. It turned out that normal tissues from the transitional zone (TZ), where benign prostate hyperplasia occurs, showed very low levels of FOXA1, but normal tissues from the peripheral zones (PZ) showed levels similar to those of primary carcinomas, which resolves the apparent discrepancy between our current initial analysis and the above-mentioned study. Because primary carcinomas arise mostly in the peripheral zone and show staining intensities similar to the adjacent non-neoplastic tissue from the same zone, we later included 12 prostatectomy specimens with transitional zone carcinomas and noticed very low FOXA1 expression, similar to the expression levels of the adjacent transitional zone glands. This finding suggests a need to analyze the diagnostic validity of FOXA1 loss as a marker for transitional zone differentiation of prostate cancer in further studies, and also emphasizes the importance of more accurately defining the origins of normal tissue for comparative analyses.

Because some degree of BPH can be found in most prostatectomy specimens, BPH tissues are commonly used as benign prostate tissues for research. This was also the case in our previous array-based transcript profiling study of prostate cancer, which revealed FOXA1 mRNA as strongly up-regulated.6 Because the transitional and the peripheral zones of the prostate are known to follow distinctly different regulatory molecular pathways, many findings based on comparison of tissue of both zones might be misleading.41–43 The more appropriate normal tissue to compare to peripheral zone prostate carcinomas should be normal tissue of the very same zone. However, peripheral zone normal tissue is commonly affected by inflammation, atrophy, and preneoplastic lesions, which also explains the popularity of hyperplastic but otherwise morphologically normal-appearing transitional zone tissue from the prostate.

The present study not only extends our knowledge of FOXA1 in normal prostatic tissues, but also offers a more detailed analysis in primary carcinomas, metastases, and especially CRPC, for which previous study has been largely lacking. In primary carcinomas after radical prostatectomy, FOXA1 correlated positively with conventional parameters of tumor progression (ie, higher Gleason scores and higher pT stage). FOXA1 also possessed a limited prognostic relevance, but this was lost in multivariate analyses. This finding is in contrast to the prognostic value of FOXA1 in breast cancer, for which a favorable prognosis has been related to high FOXA1 levels, particularly in ER+ cases.20,27–29 Prostate cancer metastases and CRPC showed significantly higher levels of FOXA1 than primary carcinomas, further indicating a role of FOXA1 in prostate cancer progression.

We then asked whether FOXA1 influences the behavior of tumor cells in in vitro studies based on RNA interference. FOXA1 knockdown slowed down propagation of LNCaP and PC-3 cells. This slowing was due to a reduced cell proliferation rate caused by an arrest in G1 phase, as demonstrated by DNA synthesis rate measurement and cell cycle analysis. A reduction of the S-phase cell population was also observed in the cell cycle study. These data substantiate an enhanced mitotic activity and stimulation toward proliferation of cancer cells with high FOXA1 levels. FOXA1 has been shown previously to promote expression of the negative cell cycle regulator p27, as well as of the cell cycle promoter cyclin D1, and thus is suggested to balance cell proliferation and maintenance of differentiation.14 We observed a corresponding correlation between FOXA1 and expression of both p27 and cyclin D1 in our patient cohort. However, on FOXA1 knockdown in PC-3 cells, levels of p27 were unaffected, whereas the expression of cyclin D1 was reduced. The reduction was strongest in the samples with the strongest FOXA1 knockdown, substantiating a direct relationship between expression of FOXA1 and cyclin D1. Thus, in the advanced prostate cancer cell line PC-3, FOXA1 knockdown causes G1 cell cycle arrest via down-regulation of cyclin D1. In human primary carcinomas (represented by our TMA study), cell cycle regulation seems to be more complex. Additionally, a decreased motility of both cell lines on FOXA1 down-regulation was observed in a haptotactic transmigration assay, which could be verified for PC-3 cells in the scratch wound assay. Similar to our
present data, strong expression of FOXA1 has been detected in HER2+ breast cancer cell lines, which are a model of aggressive (HER2+) breast cancer; in these cells, knockdown of FOXA1 inhibited cell proliferation and promoted response to trastuzumab (Herceptin) by induction of apoptosis. In contrast, forced expression of FOXA1 in ER+ breast cancer cell lines inhibited clonal growth, and FOXA1 expression levels correlated negatively with growth stimuli. These observations indicate different roles of FOXA1 in HER2+ and ER+ breast cancer cells, which explains the partially conflicting data. Our cell culture experiments clearly substantiate the proposed FOXA1 dependency of tumor progression in the prostate. However, the details of FOXA1 and hormone receptor interplay in prostate cancer cells remain to be elucidated.

Steroid hormone receptors are among the best described transcription factors whose activity is enhanced by the pioneer transcription factor FOXA1. In the present study, therefore, AR, ERα, and ERβ were analyzed in parallel. Close and significant correlation with FOXA1 expression levels in primary carcinomas was found for all three hormone receptors, and was strongest for AR (see Supplemental Table S1 at http://ajp.amjpathol.org). Furthermore, a subsequent loss of stromal AR expression toward CRPC disease has been observed (Figure 3), which cannot be explained by current knowledge and understanding of the disease. However, a stromal-epithelial interaction (and androgen-mediated induction) in the setting of the development of the organ has been discussed (see review by Bosland).

A correlation between FOXA1 and ERβ has not been reported previously in cells of any tissue. Such a correlation contradicts the current assumption that ERβ is the good estrogen receptor in prostate cancer development, because we identified FOXA1 as an unfavorable prognostic factor. However, other recent studies also question this hypothesis. To date, four studies have investigated expression of ERβ and association with aggressiveness in human prostate carcinomas. Three studies indicated a general loss of ERβ mRNA and/or protein expression in prostate carcinomas, and one of these revealed up-regulation of ERβ in metastases. The fourth study indicated decreased expression of ERβ in 40% of HG-PIN, compared with normal tissue, high expression in hormone-dependent carcinomas and their metastases and again a loss of ERβ expression in CRPC. Poor prognosis was associated with low ERβ as well as with high ERβ expression. Two cell culture studies dealing with the role of ERβ in prostate cancer cells, however support the theory of ERβ being the good guy, as seen from the perspective of the patient. Induction of apoptosis and down-regulation of the fusion protein TMPRSS2:ERG on treatment with ERβ agonists have been reported. Furthermore, ERβ (together with its ligand 5alpha-androstane-3beta,17beta-diol) maintains the epithelial phenotype and represses epithelial-mesenchymal transition. Conversely, ERβ expression was reduced on epithelial-mesenchymal transition induction by TGFβ or hypoxia. Analysis of ERβ expression in various independent patient cohorts is needed to clarify the role of this steroid hormone receptor in prostate cancer development.

Although a functional relationship between FOXA1 and AR has been described from several previous studies, no correlation was revealed between the expression values of the two transcription factors. We further verified the coexpression of FOXA1 and AR on the single-cell level by double immunofluorescence. This is the more intriguing, because androgen signaling is the core pathway of prostate carcinogenesis and tumor progression. Moreover, similar to FOXA1, strong AR expression was significantly more often encountered in metastatic and CRPCs than in hormone-sensitive primary tumors (Figure 3), which fits with the observation that amplification of AR occurs more frequently in CRPC than in primary prostate carcinomas. Nonetheless, the influence of FOXA1 on AR transactivation and androgen-dependent gene expression remains to be elucidated, because conflicting (activating and repressing) properties of FOXA1 on AR-dependent functions have been proposed.

Very recently, during our final work on the present article, Jain et al published a smaller immunohistochemical expression study on FOXA1 in primary prostate cancer and prostate cancer metastases and compared FOXA1 to AR levels. Their findings are in line with our present data, showing a correlation of FOXA1 with AR and increased FOXA1 levels in metastases. The authors speculated that FOXA1 might be a prognostic marker for disease progression, although they did not conduct a survival analysis, and their study did not address the clinically most critical prostate cancer subgroup of hormone refractory cases.

FOXA1 is known to be involved in the recruitment of AR to the corresponding co-regulatory elements. Also, FOXA1 binding to androgen-responsive elements independent of androgen concentrations has been reported, as well as a direct physical interaction between FOXA1 and AR. Notably, the correlation between strong FOXA1 expression and shorter relapse-free survival was particularly significant in the subgroup of primary prostate carcinomas with low AR expression levels. In contrast, relapse-free survival did not depend on FOXA1 expression levels in the subgroup of carcinomas with high AR levels. These findings might indicate that high AR expression promotes prostate cancer progression in a FOXA1-independent way, whereas in cancers with low AR expression, FOXA1 is needed to maintain AR signaling (as described above) and thus to promote prostate cancer progression. This points to the possibility of elevated androgen sensitivity by up-regulation of AR expression in the AR high-expression subgroup on the one hand and the requirement of FOXA1 to maintain a basal responsiveness of AR to its ligand in the AR low-expression subgroup on the other hand. Such a functional cooperation of FOXA1 and AR on in vitro tumorigenicity has not been analyzed to date. Here, we have clearly demonstrated that the inducibility of LNCaP cell proliferation and migration by androgens is dependent on the presence of FOXA1. This observation was substantiated by synchronous changes in AGR2 mRNA lev-
els, which has been shown to promote migration of LNCaP cells. Moreover, two studies revealed that this gene is regulated by androgens on the one hand and by FOXA1 on the other hand. Here, we show for the first time that indeed a cooperation of FOXA1 and androgens is needed to induce AGR2 expression.

In summary, the present findings demonstrate that FOXA1 expression increases from primary prostate carcinomas of the peripheral zone and metastases to CRPC, which is reflected by a correlation with higher Gleason scores, higher tumor stages, and faster biochemical disease progression (the last particularly in cases with lower AR levels). Because carcinomas from the TZ showed invariably low FOXA1 expression, a FOXA1-independent development of this subtype of cancer can be assumed. Functionally, a reduced cell proliferation and migration rate was observed on FOXA1 knockdown in androgen-dependent as well as androgen-independent prostate cancer cell lines. On knockdown of the pioneer transcription factor in androgen-dependent cells, in vitro tumorigenicity was not inducible by androgen treatment. This demonstrates that, in the case of reduced androgen levels (eg, due to hormonal therapy), FOXA1 can amplify androgen signaling and thus promote cell proliferation and migration. Altogether, both our in vivo and our in vitro data suggest that increased FOXA1 levels represent a late event in tumor development, contributing more to tumor progression than to tumor initiation. Therefore, we suggest FOXA1 as a novel mechanism of castration resistance and a novel candidate therapeutic target for CRPC, to be substantiated in further studies.

Acknowledgments

We thank Silvia Behnke, Martina Storz, Oraelea Büchi, and Annette Bohnert for excellent technical assistance, Stefan Runz and Alfred E. Neumann for intense discussions and counseling, and Peter Schraml for providing patient material from the tissue biobank.

References
