Biology of Human Tumors

Clinical Cancer Research

T-box Transcription Factor Brachyury Is Associated with Prostate Cancer Progression and Aggressiveness

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Abstract

Purpose: Successful therapy of patients with prostate cancer is highly dependent on reliable diagnostic and prognostic biomarkers. Brachyury is considered a negative prognostic factor in colon and lung cancer; however, there are no reports on Brachyury's expression in prostate cancer.

Experimental Design: In this study, we aimed to assess the impact of Brachyury expression in prostate tumorigenesis using a large series of human prostate samples comprising benign tissue, prostate intraepithelial neoplasia (PIN) lesions, localized tumor, and metastatic tissues. The results obtained were compared with what can be inferred from the Oncomine database. In addition, multiple *in vitro* models of prostate cancer were used to dissect the biologic role of Brachyury in prostate cancer progression.

Results: We found that Brachyury is significantly overexpressed in prostate cancer and metastatic tumors when compared with normal tissues, both at protein and at mRNA levels. Brachyury expression in the cytoplasm correlates with highly aggressive tumors, whereas the presence of Brachyury in the nucleus is correlated with tumor invasion. We found that Brachyury-positive cells present higher viability, proliferation, migration, and invasion rates than Brachyury-negative cells. Microarray analysis further showed that genes co-expressed with Brachyury are clustered in oncogenic-related pathways, namely cell motility, cell-cycle regulation, and cell metabolism.

Conclusions: Collectively, the present study suggests that Brachyury plays an important role in prostate cancer aggressiveness and points, for the first time, to Brachyury as a significant predictor of poor prostate cancer prognosis. Our work paves the way for future studies assessing Brachyury as a possible prostate cancer therapeutic target. *Clin Cancer Res; 1–13. ©2014 AACR.*

Introduction

Prostate cancer is the most common malignancy in men and the second leading cause of cancer-related deaths worldwide. In the United States, prostate cancer is the leading cause of cancer-related mortality (1). Despite advances in prevention and early detection, refinements in surgical techniques, and improvements in adjuvant radiotherapy and chemotherapy, metastasis is a frequent event that hinders patients' cure. One important mechanism that governs cancer cell invasion and further metastasis is cellular epithelial–mesenchymal transition (EMT; ref. 2). The EMT is a complex process that involves downregulation of epithelial markers, such as E-cadherin, and upregulation of mesenchymal markers, such as Snail, Slug, and N-cadherin, among other alterations. These lead to loss of epithelial cell polarity and acquisition of more motile and invasive phenotypes, promoting cancer cell dissemination into distant sites (3).

The T-box protein Brachyury is a transcription factor required for mesoderm specification during embryo development (4), which is widely expressed in notochord cells and plays a pivotal role in notochord development (5). Recently, Brachyury was associated with tumor aggressiveness in several tumor types (6–11) and was found to be a significant predictor of poor prognosis in early colon cancer (8) and lung cancer (6). *In vitro* studies suggested that these associations are driven by EMT, accomplished by increased migratory and invasion capacity (12–14) and increased cancer stem cell features (10, 11). Different studies have reported divergent effects of Brachyury expression

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Translational Relevance

There is an emerging interest and demand to discover new robust biomarkers of prostate cancer development and prognostic. The presence of embryonic T-box transcription factor Brachyury has been recently associated with cancer aggressiveness and metastasis. Currently, the role of Brachyury in prostate cancer tumorigenesis is unknown. Using a large cohort of human prostate tissues with different malignancy grades (normal, intraepithelial lesions, primary tumors, and metastasis), *in silico* data, and *in vitro* studies, we provide the first evidence of aberrant Brachyury activation in primary and metastatic prostate cancer and its clinical relevance. In addition, we found that Brachyury nuclear expression predicts invasive and metastatic prostate cancer behavior. Herein, we suggest Brachyury as a novel biomarker of prostate cancer metastasis and a potential therapeutic target for patients with advanced prostate cancer.

on cell proliferation. In lung cancer cell lines, it was demonstrated that Brachyury blocks cell-cycle progression and, therefore, mediates tumor resistance (15). However, in adenoid cystic carcinoma cells, Brachyury promoted tumor growth and metastasis formation *in vivo* (11). Therefore, despite the described oncogenic role of Brachyury, some authors suggest that it can also act as a tumor suppressor gene (16).

A recent *in vitro* study showed that Brachyury overexpression promoted cell invasion in prostate cancer, probably mediated by TGFβ1 production (13). However, knowledge on the role of Brachyury in prostate cancer progression remains very limited. In the present work, we investigated the clinical impact of Brachyury expression in a well-characterized cohort of human prostate cancer samples and evaluated its biologic role in prostate cancer cell proliferation and invasiveness. We report that Brachyury is overexpressed in primary prostate cancer and metastatic tissues and that Brachyury expression is correlated with classic parameters of prostate cancer progression and aggressiveness. We also provide data that suggest Brachyury as a therapeutic target in prostate cancer treatment.

Materials and Methods

Tissue samples

Prostate tissues were obtained from 480 patients with a 64-year-old median age (range, 46–74), who performed radical prostatectomy as primary therapy (no preceding hormonal or radical therapy) from 1993 to 2010 at Centro Hospitalar do Porto and Centro Hospitalar do Alto Ave-Guimarães, Portugal. The series included a total of 211 nonneoplastic tissue, 143 high-grade prostate intraepithelial neoplasia (PIN) lesions, and 409 primary prostate carcinomas. High-grade PIN lesions and nonneoplastic tissues were obtained from tumor adjacency. Thirteen normal samples were obtained from patients undergoing radical cystoprostatectomy for transitional cell carcinoma of the bladder. Nine metastatic tissues were obtained from patients who performed biopsies for metastatic prostate cancer. Formalin-fixed and paraffin-embedded tumors and clinicopathologic data were retrieved from the files of the Department of Pathology of both the hospitals. Tumors were staged using the 2010pTNM American Joint Committee on Cancer (AJCC) classification (17) and graded using the Gleason grading system 2005 (18). Samples were organized into tissue microarray (TMA) as previously described (19). The histologic features of the sampled areas were representative of the final Gleason score for the case. The study was previously approved by Local Ethical Review Committee of Centro Hospitalar do Porto (ref. no. 017/08-010-DEFI/015-CES).

Cell lines and cell culture

Five human prostate cell lines representing *in vitro* models of prostate cancer progression and aggressiveness, PNT2, 22RV1, LNCaP, PC3, and DU145 (ATCC), were grown in RPMI-1640 medium supplemented with 10% FBS (GIBCO, Invitrogen) and 1% penicillin/streptomycin (P/S; GIBCO, Invitrogen). PNT2 is a normal prostate cell line, 22RV1 is a prostate epithelial carcinoma cell line, LNCaP is derived from lymph node metastasis and is hormone-sensitive, and DU145 and PC3 cell lines are derived from brain and bone metastasis, respectively, and represent poorly differentiated tumors.

Brachyury overexpression and knockdown in prostate cancer cell lines

22RV1 and DU145 cell lines were transfected with full-length human Brachyury in pcDNA4/TO vector, thus designated pcBrachyury. The empty vector (designated 4/TO) was used as control (12). Stable 22RV1 and DU145 cell pools with pcBrachyury expression were obtained following treatment with 50 μg/mL zeocin (Invitrogen). PC3 cells were transfected with Brachyury-specific shRNA construct (shBrachy.1) or empty vector alone (pLKO.1; Sigma-Aldrich) using X-tremeGENE HP transfection reagent (Roche) as recommended by the manufacturer. Stable PC3 cells with depleted endogenous Brachyury expression were obtained following treatment with 5 μg/mL of puromycin (Sigma-Aldrich).

Expression analysis by semiquantitative RT-PCR

Total RNA was extracted from cell lines using TRIzol Reagent (Invitrogen S.A.). One microgram of RNA was reverse-transcribed using Phusion RT-PCR Kit (Finnzymes), as recommended by the manufacturer. The primers used are presented in Supplementary Table S1. No amplification was obtained when RNA was mock-transcribed without adding reverse transcriptase.

Western blot analysis

Cells were lysed in buffer containing 50 mmol/L Tris, pH 7.6–8, 150 mmol/L NaCl, 5 mmol/L EDTA, 1 mmol/L Na_3VO_4, 10 mmol/L NaF, 10 mmol/L Na pyrophosphate, 1%

NP-40, and 1/7 of protease cocktail inhibitors (Roche). Proteins were resolved on standard 12% SDS-PAGE gels, transferred onto nitrocellulose membranes, and probed with antibody against Brachyury (AF2085, R&D Systems) and GAPDH (sc-69778, Santa Cruz Biotechnology, Inc.) at 4°C overnight. Blot detection was done by chemiluminescence (ECL Western Blotting Detection Reagents, GE Healthcare) using Chemidoc (Bio-Rad).

Immunofluorescence microscopy

Cells were plated on glass coverslips placed into 12-well plates and allowed to adhere overnight. Cells were fixed with 4% paraformaldehyde (PFA) in 1X PBS, washed, and permeabilized with 0.1% Triton X-100. Then, cells were blocked in 10% FBS, labeled for 1 hour at room temperature with primary anti-Brachyury antibody (sc-20109, Santa Cruz Biotechnology, Inc.), washed, and incubated at room temperature for 1 hour with a secondary anti-rabbit Alexa-488 antibody (Invitrogen-Molecular Probes). Coverslips were mounted on microscope slides with Vectashield Mounting Medium with DAPI (Vector Laboratories). Digital images were recorded with Olympus BX61 (Olympus Corporation).

Cell viability and proliferation assays

Colony formation assays were used to assess the survival capacity of 22RV1, DU145, and PC3 cells with and without Brachyury. A total of 1×10^3 cells per well were seeded into 6-well plates. After 15 days of culture, colonies formed were fixed in 4% PFA, washed, stained with 0.05% crystal violet, and manually counted.

MTS and bromodeoxyuridine (BrdUrd) assays were used to evaluate the viability and proliferation capacity over time. A total of 2×10^3 cells per well for 22RV1 and 1×10^3 cells per well for DU145 and PC3 were plated into 96-well plates in triplicate and allowed to adhere overnight. After 6 hours of starvation (RPMI only), viable or proliferative cells were quantified using the Cell Titer96 Aqueous cell proliferation (MTS, Promega) or Cell Proliferation ELISA, BrdUrd (colorimetric, Roche Applied Science) assay and this was the value for time 0. After 24, 48, and 72 hours, cell viability and proliferation were again assessed. The results were calibrated to the starting value (time 0 hours, considered as 100% of viability/proliferation) as previously described (20).

Wound-healing migration assay

Cells were seeded in 12-well plates and cultured to at least 95% of confluence. Monolayer cells were washed with 1X PBS and scraped with a plastic pipette tip and then incubated with fresh RPMI medium. The "wounded" areas were photographed by phase-contrast microscopy at different time points. The relative migration distance was calculated as described (20, 21).

Matrigel invasion assay

Matrigel invasion assays were performed using 8-μm pore size BD BioCoat Matrigel Invasion Chambers (BD Biosciences). Briefly, after rehydration with RPMI, 10% FBS, the upper compartment of the chamber received 2.5×10^4 cells per well grown in RPMI only, whereas the lower compartment contained fresh medium supplemented with 10% FBS and 10 ng/mL of EGF (Prepotech). After 22 hours of incubation, the upper surface of the filter was washed with 1X PBS and fixed with 4% PFA. Then, residual cells were cleared with a cotton swab, the filter washed with 1X PBS, and invasive cells attached to the lower filter surface were mounted in Vectashield Mounting Medium with DAPI (Vector Laboratories). Images were recorded on an Olympus BX61 microscope (Olympus Corporation), and invasive cells counted using ImageJ software.

IHC analysis

Histologic slides with 4-μm-thick tissue sections were subjected to IHC analysis according to the streptavidin–biotin peroxidase complex system (UltraVision Large Volume Detection System Anti-Polyvalent, HRP; LabVision Corporation), using the primary antibody raised against Brachyury (diluted 1:200; sc-20109, Santa Cruz Biotechnology, Inc.) or against AMACR (diluted 1:50; 504R-16, Cell Marque). CD44 staining was performed using an anti-human CD44 antibody (diluted 1:100; 156-3C11, AbD Serotec) and detected using Vectastain Universal Elite ABC kit PK-6200 (Vector Laboratories). The negative control was treated identically but with omitted primary antibody. Sections were scored in a double-blind fashion for cytoplasm expression following a semiquantitative criterion based on the intensity (0, negative; 1, weak; 2, moderate; 3, strong) and percentage of cells stained (0, 0% of immunoreactive cells; 1, <25% of immunoreactive cells; 2, 25%–50% of immunoreactive cells; and 3, >50% of immunoreactive cells). Both components were considered for an overall semiquantitative staining score (range, 0–6). Samples with scores 0, 1, and 2 were considered negative and those with scores 3–6 were considered positive. Tissues sections were separately evaluated for expression in the nucleus (≥25% nuclear staining was considered positive and cases with <25% of nuclear staining were considered negative).

***In silico* analysis of *Brachyury* expression in the Oncomine database**

Brachyury mRNA expression was assessed in 7 prostate cancer datasets (LaTulippe, ref. 22; Varambally, ref. 23; Grasso, ref. 24; Taylor, ref. 25; Glinsky, ref. 26; Yu, ref. 27; TCGA, ref. 28; and Arredouani, ref. 29) from the Oncomine database (30, 31). Categorization of patients with Brachyury-positive and Brachyury-negative prostate cancer was based on the $\log_2$ median-centered intensity values of *Brachyury* probes per study, and a linear model was fitted to estimate the association significance. Patient samples in each study with *Brachyury* expression values greater than its median intensity were grouped as *Brachyury*-positive and others were grouped as *Brachyury*-negative. *Brachyury* expression was further correlated with corresponding patient clinical data available.

Microarray co-expression studies were extracted from the Oncomine database. Microarray expression profiles were

clustered by functional importance and signaling pathways using DAVID v6.7 bioinformatic tool (The Database for Annotation, Visualization and Integrated Discovery; refs. 32, 33).

Statistical analysis

Univariate (χ^2 test) and multivariate analyses (linear regression model) were used to assess the correlations between Brachyury expression and clinicopathologic features from primary specimens. Pearson test was used to evaluate the correlation between gene expression profiles. Simple comparisons between 2 different conditions were analyzed using the Student *t* test and, for comparison of 2 conditions over time, we used the 2-way ANOVA (Bonferroni post-test). The statistical analysis was performed using SPSS software (version 19.0) or using Prism GraphPad software (version 5.0a). The level of significance in the statistical analyses is indicated as *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Results

Brachyury protein is overexpressed in prostate cancer and PIN lesions

Brachyury protein expression was assessed by IHC in a series of 784 prostate tissues, including normal tissues, PIN lesions, primary prostate cancer samples with different Gleason scores and prostate cancer metastasis. Figure 1 shows representative results of intensity scores observed for Brachyury expression. Normal prostate gland and adjacent nonneoplastic tissues presented absence or low levels of Brachyury staining when compared with neoplastic tissues (Fig. 1A; Supplementary Fig. S1) and were therefore clustered in a single group, designated nonneoplastic tissues. Brachyury was expressed in the nuclei and/or cytoplasm of epithelial cells in nonneoplastic tissues, PIN lesions, prostate cancer, and metastatic tissues (Fig. 1B–D; Supplementary Fig. S1). Overall, the number of cases presenting cytoplasm protein expression increased from nonneoplastic to prostate cancer and PIN lesions and to metastasis (33.9%, 55.2%, 61.5%, and 100% of positive cases, respectively; $P < 0.001$; Fig. 2A). Brachyury nuclear staining was present in a comparable number of cases in nonneoplastic (25.0%), PIN lesions (38.6%), and prostate cancer cases (25.4%), in contrast to 100% of metastatic tissue samples ($P < 0.001$; Figs. 1vi and 2A). Interestingly, Brachyury was also detected in the stroma (Supplementary Fig. S1) with a significant reduction of stroma-positive cases from nonneoplastic tissues (52.6%), to PIN lesions (44.2%), and to prostate cancer (14.2%; $P < 0.001$; Fig. 2A), indicating a possible role for Brachyury in prostate cancer tumor tissue microenvironment.

Heatmap analysis of overall Brachyury protein expression showed that Brachyury is remarkably overexpressed in PIN, prostate cancer, and metastatic prostate tissues when compared with nonneoplastic tissues (Fig. 2B). The higher expression profile was found in metastasis with scores consistently ≥ 4.

Brachyury protein overexpression is associated with poor prognosis in prostate cancer

The clinical impact of Brachyury protein expression levels was further explored in our cohort of 409 primary prostate

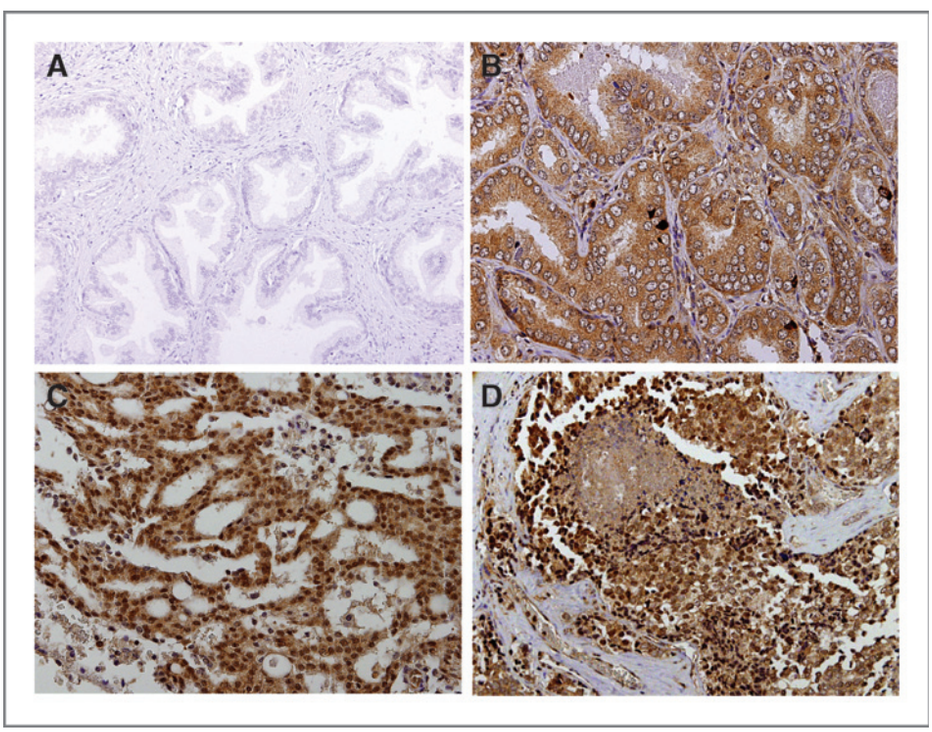
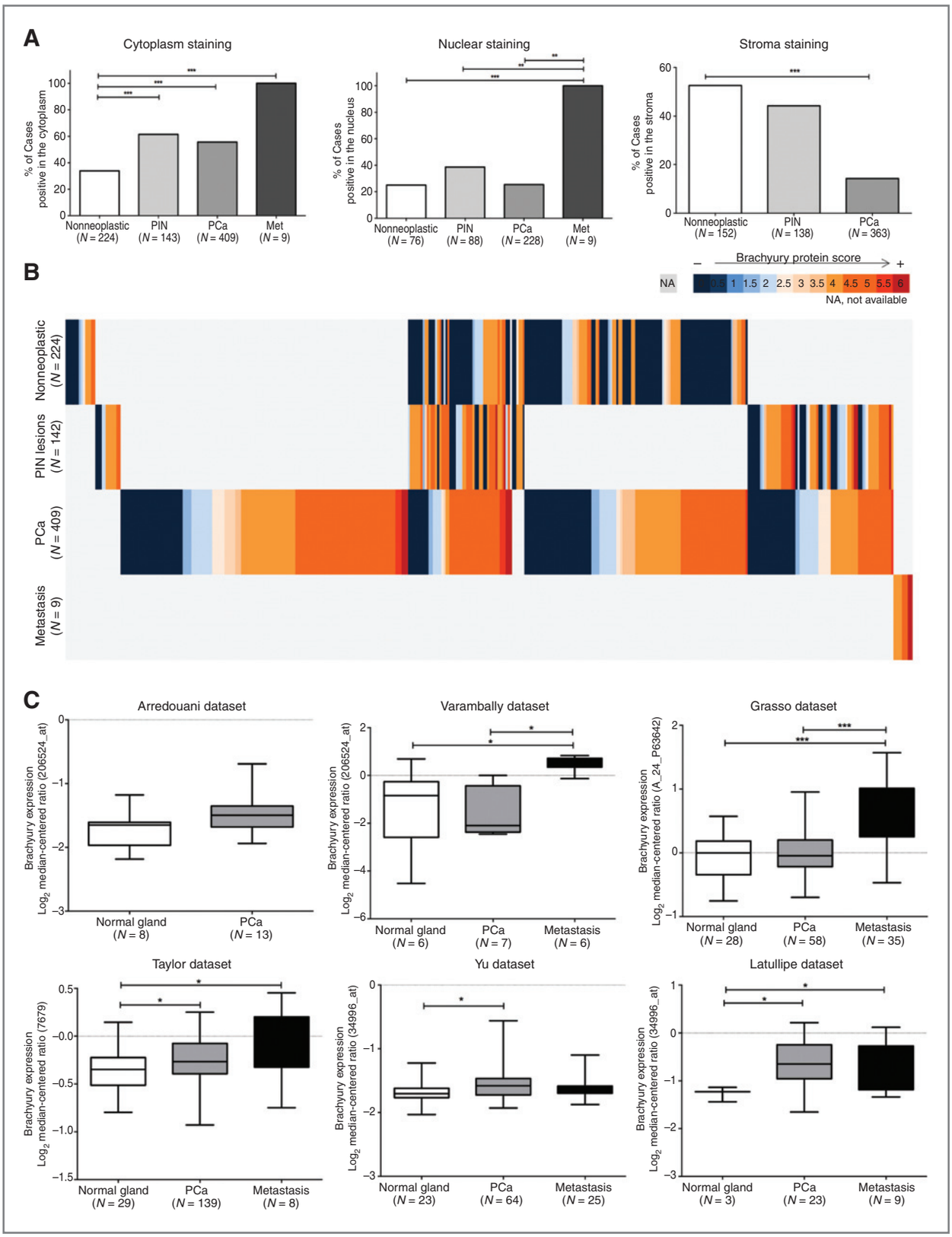


Figure 1. Brachyury expression in nonneoplastic tissues, PIN lesions, prostate cancer, and metastatic tissues. Brachyury is absent or expressed at low levels in nonneoplastic tissues (A, normal gland). Primary prostate cancer Brachyury-positive cases can exhibited only cytoplasm staining (B) or both cytoplasm and nuclear staining (C). Metastatic lesion showing both cytoplasm and nuclear (D). Magnification, ×200 (A) and ×400 (B–D).


A
Cytoplasm staining
% of Cases positive in the cytoplasm
Nonneoplastic (N = 224)
PIN (N = 143)
PCa (N = 409)
Met (N = 9)
Nuclear staining
% of Cases positive in the nucleus
Nonneoplastic (N = 76)
PIN (N = 88)
PCa (N = 228)
Met (N = 9)
Stroma staining
% of Cases positive in the stroma
Nonneoplastic (N = 152)
PIN (N = 138)
PCa (N = 363)
B
Brachyury protein score
NA
NA, not available
Nonneoplastic (N = 224)
PIN lesions (N = 142)
PCa (N = 409)
Metastasis (N = 9)
C
Arredouani dataset
Brachyury expression Log2 median-centered ratio (206524_at)
Normal gland (N = 8)
PCa (N = 13)
Varambally dataset
Brachyury expression Log2 median-centered ratio (206524_at)
Normal gland (N = 6)
PCa (N = 7)
Metastasis (N = 6)
Grasso dataset
Brachyury expression Log2 median-centered ratio (A_24_P63642)
Normal gland (N = 28)
PCa (N = 58)
Metastasis (N = 35)
Taylor dataset
Brachyury expression Log2 median-centered ratio (7679)
Normal gland (N = 29)
PCa (N = 139)
Metastasis (N = 8)
Yu dataset
Brachyury expression Log2 median-centered ratio (34996_at)
Normal gland (N = 23)
PCa (N = 64)
Metastasis (N = 25)
Latullipe dataset
Brachyury expression Log2 median-centered ratio (34996_at)
Normal gland (N = 3)
PCa (N = 23)
Metastasis (N = 9)

cancer. Univariate outcome analysis showed that Brachyury-positive cases (scores ≥ 3) are significantly ($P < 0.001$) associated with the prostate cancer biomarker α-methylacyl-CoA racemase (AMACR; Supplementary Table S2). Brachyury-positive cases correlated with highly undifferentiated prostate cancer tumors ($P = 0.007$, Table 1) and, concordantly, a strong tendency to be associated with stem cell marker CD44 was observed ($P = 0.054$; Supplementary Table S2). Importantly, Brachyury protein levels increased with the Gleason score ($P < 0.027$, Table 1; $P < 0.01$, Supplementary Fig. S2). To evaluate the clinical impact of the presence of Brachyury in the nucleus, a comparison between Brachyury nuclei–positive and nuclei-negative in prostate cancer–positive cases was performed (nonneoplastic tissues, $n = 76$; PIN lesion, $n = 88$; prostate cancer, $n = 228$; metastasis, $n = 9$). Primary prostate cancer tumors with nuclear Brachyury staining were significantly associated with perineural invasion ($P = 0.046$) and with capsular invasion ($P = 0.025$; Table 1), which is in agreement with predominant nuclear expression in metastasis (Figs. 1 and 2). Because Gleason scores and pT stage are known prognostic biomarkers, we performed multivariate analysis to determine whether high Brachyury expression has an independent statistical value. We observed that Brachyury is significantly associated with capsular invasion ($P = 0.027$, Table 1) on primary prostate cancer samples.

No significant correlations were found for Brachyury staining in stroma with clinicopathologic parameters by univariate analysis (Table 1). Yet, the multivariate analysis showed a significant association with capsular invasion ($P = 0.030$, Table 1), indicating the possible role of Brachyury on tumor microenvironment.

Altogether, these data suggest that high Brachyury levels are associated with patient's poor outcome and indicate that nuclear Brachyury staining in prostate cancer is an independent prognostic factor.

In silico validation of the role of Brachyury expression in prostate cancer aggressive behavior

To corroborate our findings, we extended the analysis to microarray profiling datasets of prostate cancer tissues available on the Oncomine database (30, 31). *Brachyury* mRNA expression was analyzed in 6 independent prostate cancer datasets (LaTulippe, ref. 22; Varambally, ref. 23; Grasso, ref. 24; Taylor, ref. 25; Yu, ref. 27; and Arredouani, ref. 29) comprising a total of 97 normal prostate gland, 304 prostate cancer, and 83 prostate cancer metastasis samples. We found that *Brachyury* was significantly overexpressed in prostate tissues in multiple microarray cancer profiling datasets, in particular in metastatic prostate cancer (Fig. 2C). This was concordant with our protein analysis reported above. Importantly, although multiple probes were used to determine *Brachyury* mRNA levels in these datasets (23996_at, 206524_at, A_24_P63642, 7679), they all consistently showed that *Brachyury* overexpression is a common event in primary and metastatic prostate cancer (Fig. 2C).

We next assessed the impact of Brachyury in prostate cancer prognosis at the mRNA level, exploring the microarray profiling datasets of localized prostate tumors with clinical data from Oncomine (Table 2). Univariate statistical analysis revealed that high levels of *Brachyury* expression correlated with higher (>7) Gleason scores for the Taylor (25), Glinsky (26), and LaTulippe (22) datasets ($P = 0.043$, $P = 0.042$, and $P = 0.049$, respectively; Table 2). In agreement with protein IHC levels, the percentage of *Brachyury*-positive cases directly increased with the Gleason score ($P < 0.05$; Supplementary Fig. S2). In addition, high *Brachyury* mRNA levels correlated with pT (Yu, ref. 27; $P = 0.033$) and N stage (Taylor, ref. 25; $P = 0.043$), biochemical recurrence (Taylor, ref. 25; $P = 0.048$), capsular invasion and extraprostatic extension (Glinsky, ref. 26; $P = 0.002$ and 0.007, respectively; Table 2). A similar tendency could also be observed in the other datasets. In the multivariate analysis, we found that high *Brachyury* mRNA levels still correlate with capsular invasion and extraprostatic extension (Glinsky, ref. 26; $P = 0.001$ and 0.032, respectively) and with biochemical recurrence (TCGA, ref. 28; $P = 0.004$; Table 2).

Prostate cell lines recapitulate Brachyury expression profiles of human prostate cancer tissues

To explore the biologic role of Brachyury in prostate cancer aggressiveness, 5 prostate cancer cell lines (PNT2, 22RV1, LnCaP, PC3, and DU145), representative of different degrees of prostate cancer progression, were screened for Brachyury expression by semiquantitative RT-PCR and Western blot analyses. Brachyury protein subcellular localization was additionally evaluated by immunofluorescence. We observed an absence of Brachyury at both mRNA and protein levels in the nonmalignant prostate cell line (PNT2) and in the primary prostate cancer cell line (22RV1; Supplementary Fig. S3A). In contrast, the metastatic cell lines LNCaP and PC3 showed strong nuclear and cytoplasm Brachyury expression both at mRNA and at protein levels (Supplementary Fig. S3A). The metastatic DU145 cell line was negative for mRNA by conventional RT-PCR but still

Figure 2. Brachyury is overexpressed in prostate cancer (PCa) and metastatic tissues. A, representation of Brachyury-positive cases according to Brachyury localization; left, overall score for cytoplasm staining; middle, presence in nucleus; right, presence in stromal cells. B, heatmap of protein levels in tissue microarray prostate samples (range, 0–6). There is a predominant blue staining (negative, score < 3) in normal tissues and orange/red staining (positive, score ≥ 3) in PIN, prostate cancer, and metastasis tissues. Each column represents a single case and it is possible verify the respective normal adjacent tissue or PIN lesion of a specific prostate cancer case. In the majority of the cases, an increased expression from normal to PIN and to prostate cancer can be appreciated in the same patient. C, analysis of microarray expression data for *Brachyury* levels from the Oncomine database. Log_2 median-centered ratio expression is present for 6 different datasets (Yu, ref. 27; Varambally, ref. 23; Grasso, ref. 24; Taylor, ref. 25; Latullipe, ref. 22; and Arredouani, ref. 29) representing 4 different probes for *Brachyury* detection (34996_at, 206524_at, A_24_P63642, and 7679). *Brachyury* is commonly overexpressed in prostate cancer tissues and PCa metastasis. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Q5 **Table 1.** Correlation between Brachyury subcellular localization and clinicopathologic features in prostate cancer tissues by univariate and multivariate analyses

	Overall staining				Nuclear staining in positive cases				Stromal staining			
			P				*P*				*P*	
Clinical parameters	*n*	Positive (%)	Univariate	Multivariate[a]	*m*	Positive (%)	Univariate	Multivariate[a]	*n*	Positive (%)	Univariate	Multivariate[a]
Age, y												
<64	221	52	0.134	0.220	112	27.7	0.900	0.974	195	13.3	0.545	0.496
≥64	187	59.9			109	28.4			167	15.6		
PSA, ng/mL												
≤6.0	106	59.4	0.279	0.087	63	27	0.485	0.557	99	13.1	0.479	0.530
>6.0	204	52.5			102	25.5			182	10.4		
Gleason score (grouped)												
<7	110	49.1	**0.027**	Variable	53	22.6	0.536	Variable	95	41.1	0.123	Variable
7	261	55.2			140	30.7			242	47.9		
>7	30	76.7			22	27.3			27	63		
pT stage												
pT2	268	55.2	0.171	Variable	148	21.7	0.081	Variable	268	12.7	0.794	Variable
pT3	84	61.9			52	32.7			84	14.3		
Gleason differentiation												
Differentiated	330	54.8	**0.007**	0.060	181	24.9	0.791	0.340	329	14.4	0.251	0.411
Undifferentiated	26	80.8			22	27.3			22	7.4		
Biochemical recurrence												
Absent	359	55.2	0.518	0.843	193	29	0.243	0.237	314	14.6	0.425	0.857
Present	50	60			29	20.7			49	12.2		
Perineural invasion												
Absent	102	54.9	0.883	0.561	55	18.2	**0.046**	0.194	87	14.9	0.802	0.573
Present	305	55.7			165	30.9			274	13.9		
Capsular invasion												
Absent	229	57.6	0.586	0.591	131	29	**0.025**	**0.027**	218	18.3	0.055	**0.030**
Present	65	53.8			30	50			30	33.3		
Extraprostatic extension												
Absent	313	54.6	0.259	0.951	167	25.7	0.107	0.490	267	15.2	0.594	0.375
Present	93	59.1			53	35.8			83	12		

[a]For multivariate analysis, Gleason score and pT stage were used as variables to determine whether high Brachyury indicates poor outcome.

Table 2. Correlation of Brachyury microarray expression profiles with clinicopathologic features available in different datasets from the Oncomine database by univariate and multivariate analyses

Dataset	Taylor (25) [-7679]				Glinsky (26) [34966_at]				TCGA (28) [06_166496598]				LaTulippe (22) [34966_at]				Yu (27) [34966_at]			
Probe			*P*				*P*				*P*				*P*				*P*	
Clinical parameters	*n*	Positive (%)	Univariate	Multivariate[a]	*n*	Positive (%)	Univariate	Multivariate[a]	*n*	Positive (%)	Univariate	Multivariate[a]	*n*	Positive (%)	Univariate	Multivariate[a]	*n*	Positive (%)	Univariate	Multivariate[a]
Age, y																				
<61	83	18 (21.7)	0.565	0.723	50	30 (60.0)	0.219	0.268	53	9 (17.0)	0.904	0.441	14	4 (28.6)	0.471	0.362	NA	NA	NA	NA
≥61	67	18 (26.9)			29	22 (75.9)			73	13 (17.8)			18	8 (44.4)			NA	NA		
Gleason score (grouped)																				
<7	81	13 (16.0)	**0.043**	Variable	17	10 (58.8)	**0.042**	Variable	7	1 (14.3)	0.101	Variable	2	0 (0.0)	**0.049**	Variable	18	10 (55.6)	0.074	Variable
7	50	16 (32.0)			44	26 (59.1)			94	13 (13.8)			15	5 (33.3)			27	23 (85.2)		
>7	19	7 (36.8)			18	16 (88.9)			25	8 (32)			6	5 (83.3)			19	12 (63.2)		
pT stage																				
pT1	NA	NA	0.055	Variable	34	23 (67.6)	0.868	Variable	46	5 (10.9)	0.835	Variable	NA	NA	0.59	Variable	NA	NA	**0.033**	Variable
pT2	86	17 (19.8)			43	28 (65.1)			41	5 (12.2)			10	5 (50.0)			25	14 (56.0)		
pT3	47	16 (34.0)			2	1 (50.0)			5	1 (20.0)			11	5 (45.5)			37	30 (81.1)		
N stage																				
N0	105	21 (20.0)	**0.043**	Variable	76	49 (64.5)	0.279	Variable	100	18 (18.0)	0.185	Variable	19	8 (42.1)	0.596	Variable	NA	NA	NA	NA
N1	16	7 (43.8)			3	3 (100.0)			12	4 (33.3)			4	2 (50.0)			NA	NA		
Biochemical recurrence																				
No	104	21 (20.2)	**0.048**	0.416	42	26 (61.9)	0.294	0.867	108	16 (14.8)	0.087	**0.004**	NA	NA	NA	NA	NA	NA	NA	NA
Yes	36	13 (36.1)			37	26 (70.3)			7	3 (42.9)			NA	NA			NA	NA		
Capsular invasion																				
No	50	10 (20.0)	0.219	0.573	23	9 (39.1)	**0.002**	**0.001**	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Yes	91	25 (27.5)			56	43 (76.8)			NA	NA			NA	NA			NA	NA		
Vesicle invasion																				
No	119	26 (21.8)	0.055	0.654	69	43 (62.3)	0.080	0.133	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Yes	22	9 (40.9)			10	9 (90.0)			NA	NA			NA	NA			NA	NA		
Extraprostatic extension																				
No	108	30 (27.8)	0.105	0.167	36	18 (50.0)	**0.007**	**0.037**	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Yes	33	5 (15.2)			43	34 (79.1)			NA	NA			NA	NA			NA	NA		

Abbreviation: NA, not available.

[a]For multivariate analysis, Gleason score, pT and N stages were used as variables to determine whether high Brachyury indicates poor outcome.

exhibited low levels of nuclear protein expression (Supplementary Fig. S3A). These findings indicate that prostate cell lines are good models to study the functional role of Brachyury in prostate cancer cells, as they recapitulate the expression profiles found in human clinical samples.

Brachyury promotes prostate cancer aggressiveness *in vitro*

To address whether the modulation of Brachyury expression influences the tumorigenic properties of prostate cancer cells, Brachyury was overexpressed in primary (22RV1) and metastatic (DU145) prostate cancer cell lines. Successful ectopic overexpression was obtained upon transfection of both cell lines with the pcBrachyury expression vector and Brachyury protein exhibited nuclear localization (Supplementary Fig. S3B). To investigate the effect of Brachyury inhibition, a specific short-hairpin clone (shBrachy.1) was used to deplete Brachyury in a positive metastatic prostate cell line (PC3; Supplementary Fig. S3B).

We initially studied the biologic role of Brachyury on prostate cancer cell viability and proliferation (Fig. 3A; Supplementary Fig. S4A). pcBrachyury prostate cells had a significant ($P < 0.05$) viability advantage over time (MTS assay) compared with the cells transfected with the empty vector (4/T0). Colony formation assays revealed a significant ($P < 0.05$) increase in the number of the colonies formed in the pcBrachyury-transfected cells when compared with the control cells (Fig. 3A, Supplementary Fig. S4A). The opposite findings were obtained with Brachyury depletion in shBrachy.1-PC3 cells ($P < 0.05$; Fig. 3A). To determine whether this viability advantage was due to higher proliferation rates, we analyzed BrdUrd incorporation during S-phase of the cell cycle. The presence of Brachyury, whether endogenous or exogenously overexpressed, promoted higher rates of proliferation over time ($P < 0.05$; Fig. 3A; Supplementary Fig. S4A). We further performed wound migration and Matrigel invasion assays in the transfected cell lines and observed that both 22RV1 and DU145 pcBrachyury cells had a higher migratory capacity over time and increased cell invasion capability than the empty vector cells ($P < 0.05$; Fig. 3B; Supplementary Fig. S4B). When Brachyury was depleted in an endogenously positive cell line, we were able to attenuate the aggressive behavior ($P < 0.05$; Fig. 3B).

To characterize the molecular players underlying prostate aggressiveness *in vitro*, we studied the expression profile of some key genes involved in EMT, migration, and stemness processes. We observed that Brachyury expression was associated with a decrease of the epithelial marker E-cadherin and concomitant increased expression of mesenchymal genes (N-cadherin, fibronectin, and Snail), as well as upregulation of metalloprotease MMP14 (Supplementary Fig. S5). Concordantly with IHC analysis in human prostate cancer (Supplementary Table S2), Brachyury overexpression was associated with an increased expression of the stem cell marker CD44 (Supplementary Fig. S5).

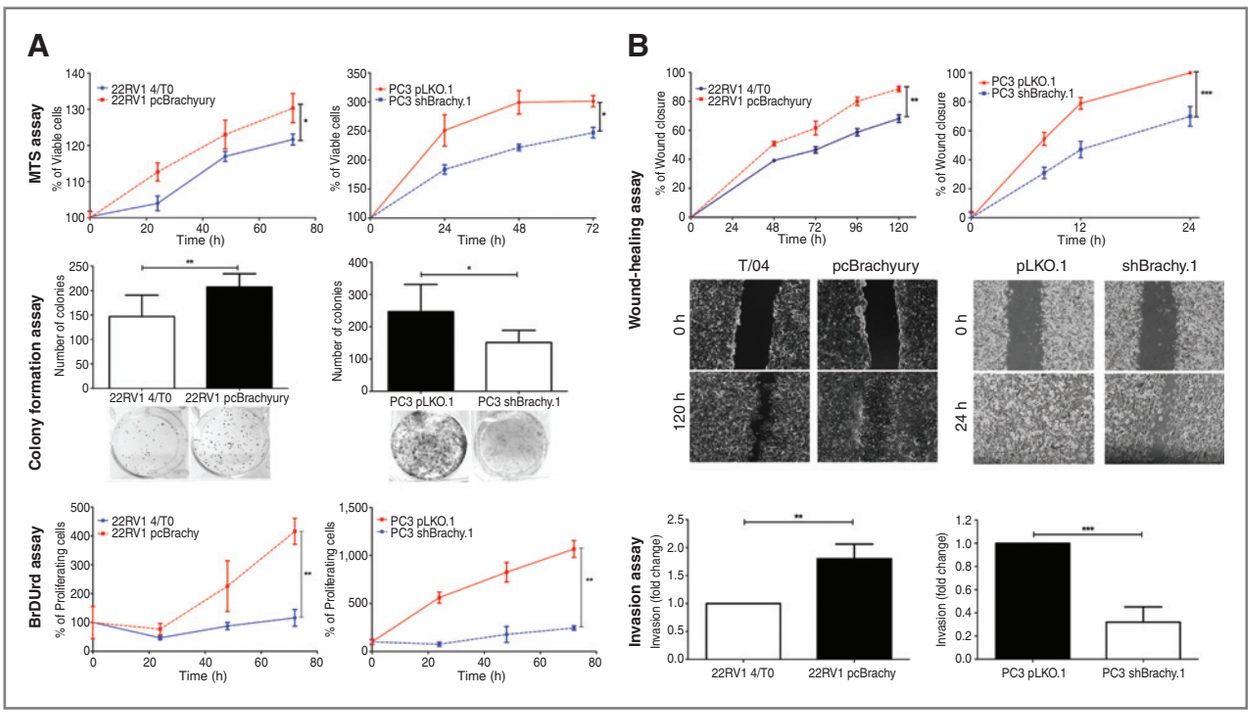


Figure 3. Evaluation of biologic role of Brachyury in prostate cancer cell lines. A, effect of Brachyury on viability and proliferation of prostate cells (22RV1 and PC3) evaluated by MTS, colony formation, and BrdUrd assays. B, wound-healing and Matrigel invasion assays were used to evaluate the role of Brachyury in migration and invasion, respectively. The presence of Brachyury correlated with increased cell viability, proliferation, migration, and invasion. Red lines and black bars, Brachyury-positive cell lines; blue lines and white bars, Brachyury-negative or Brachyury-depleted cell lines. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Genes co-expressed with Brachyury in microarray analyses are associated with tumorigenic clusters

We clustered the genes co-expressed with Brachyury in prostate tissues available at the Oncomine database (30, 31) by their functional role and importance in signal transduction pathways using the DAVID bioinformatic tool (Fig. 4A). We found that the majority of genes co-expressed with *Brachyury* were functionally clustered in the categories of immune response, cell membrane/receptor activity, development, cell motility, and chemotaxis pathways in cancer and response to hormone stimulus. A subanalysis by KEGG signaling pathways revealed that *Brachyury*

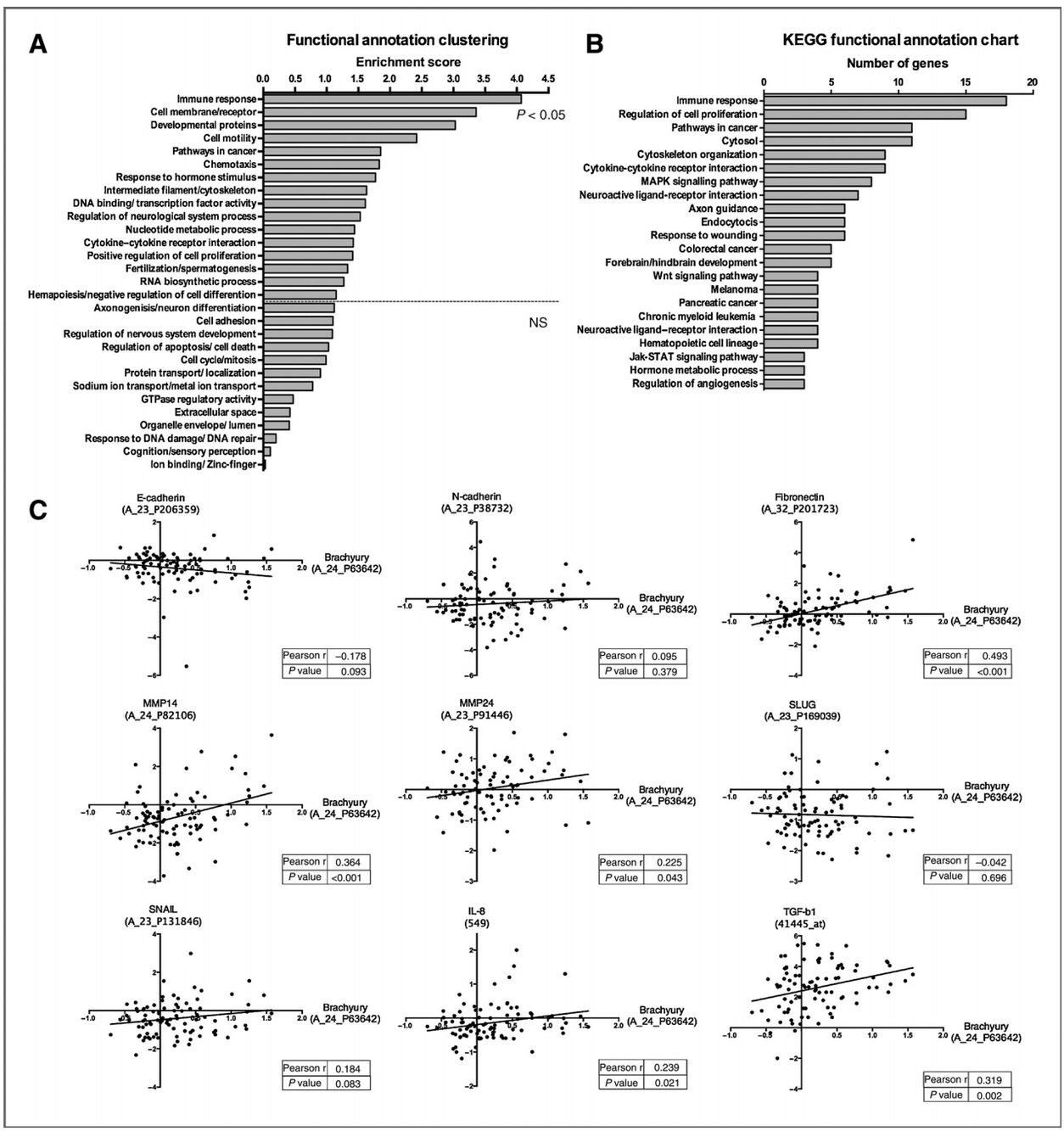


Figure 4. *In silico* analysis of genes co-expressed with Brachyury in prostate cancer. Microarray expression profiles of Brachyury co-expressed genes were clustered by functional role and signaling pathways using DAVID *in silico* tool. The functional clusters organized by enrichment score (A) and the KEGG signaling pathway analysis (B). Brachyury co-expressed genes are associated with pathways involved in tumor aggressiveness, namely, in immune cell response, positive cell-cycle regulation, cell motility, and chemotaxis. C, *in silico* analysis indicates an inverse correlation between *Brachyury* and epithelial marker *E-cadherin* and a direct correlation with several genes involved with EMT (*fibronectin*, *MMP14*, *MMP24*, *Snail*, *IL8*, and *TGFβ1*). NS, not significant.

co-expressed genes are grouped in pathways associated with higher aggressiveness, namely, pathways in cancer, positive cell-cycle regulation, and immune response (Fig. 4B). These analyses strongly point to a role of Brachyury, not only in cell migration and invasion but also as a regulator of the cell cycle and in cancer microenvironment metabolism. Importantly, we found that the levels of *Brachyury* expression in prostate cancer tissues are directly correlated with those of IL8 and TGFβ1 (Fig. 4C), which are involved in EMT and cancer microenvironment modulation, as previously described (13, 14). Accordingly, there is a correlation of *Brachyury* expression with the expression of genes associated with EMT process (like *fibronectin*) and migration (*MMP14*; *MMP24*; Fig. 4C) that support our expression analysis (Supplementary Fig. S5).

Discussion

The T-box transcription factor Brachyury was initially discovered for its role in mouse mesoderm development and differentiation (34). This involves massive conversion of epithelial cells into migratory and invasive mesenchymal cells during gastrulation via a process known as EMT (2, 4). Several reports have demonstrated that EMT is critical for prostate cancer progression, as acquisition of mesenchymal features may favor dissemination and resistance to therapy (35). High levels of Brachyury have previously been reported in various types of cancer (5–9, 11) and a phase I clinical trial of a vaccine targeting Brachyury-positive tumors (GI-6301) is currently under way (36, 37). Although prostate cancer is a leading cause of cancer-related deaths in men worldwide, a characterization of Brachyury biologic role in prostate tumorigenesis is missing.

Our study reports for the first time that the transcription factor Brachyury is aberrantly overexpressed across prostate malignancy and, in particular, nuclear Brachyury staining is associated with prostate invasion and prostate metastatic tissues. Our findings indicate Brachyury as an independent prognostic factor in prostate cancer. The role of Brachyury nuclear staining in metastasis was demonstrated in other tumor types, such as colorectal, lung cancer, and oral squamous cell carcinoma (6–9). Herein, we also found that cytoplasm immunostaining in prostate cancer is associated with prostate cancer biomarker AMACR and with highly aggressive tumors. The role of Brachyury in the cell cytoplasm remains to be elucidated, yet, we can hypothesize that it interacts with other proteins and in this way regulates cell behavior in a nontranscription manner.

It has been shown that Brachyury expression can influence tumor microenvironment through the release of soluble factors that could induce adjacent epithelial tumor cells to undergo an EMT and acquire metastatic potential (14). In prostate cancer, the activation and secretion to the extracellular environment of soluble factors that mediate the cross-talk between tumor cells and tumor stroma, such as interleukins and growth factors, has been reported to play a role in tumor progression (13, 14, 38–40). Our analysis confirm a previously result that demonstrated that Brachyury increases the expression and secretion of TGFβ1 in a prostate cell line (13). However, the influence of Brachyury expression in stromal cells has not yet been characterized. To our knowledge, we provide the first evidence for decreased Brachyury expression in stromal cells with prostate malignancy, at variance with the reported upregulation in tumor cells. Therefore, we can hypothesize that Brachyury has different roles in stromal and tumor cells and that it could be involved in the regulation of tumor microenvironment. In addition, we found that the majority of Brachyury co-expressed genes are involved in immune or metabolic processes.

By *Brachyury* overexpression and downregulation in prostate cancer cell lines, we demonstrated its role in tumor cell migration and invasion, as well as in cell viability and proliferation. Our findings were further corroborated by an *in silico* analysis with multiple genes functionally clustered in pathways related with cell motility and cell proliferation. A study performed by Shomoda and colleagues demonstrated that ablation of *Brachyury* in adenoid cystic carcinoma (ACC) cells decreased the number of metastasis and tumor size *in vivo* (11). Moreover, depletion of *Brachyury* in chordoma cells promotes a complete block of cell proliferation (41). An opposite role for Brachyury in cell proliferation was demonstrated in lung and colorectal cell lines by Huang and collaborators, where Brachyury blocks cell cycle progression and mediates tumor resistance to conventional antitumor therapies (15). Therefore, it can be deduced that the role of Brachyury may be tissue-specific or cell-type–dependent.

Brachyury seems to be a key driver of EMT in various human tumors by increasing expression of genes such as *Slug*, *Snail*, *MMPs*, *IL8*, and *TGFβ1* (6, 9, 13–15, and current study). A possible link between cells undergoing EMT and cells with "stem cell–like" properties was recently described (42). The role of Brachyury in conferring stemness properties was already demonstrated in colorectal cancer cells (10) and in ACC cells (11). The present study shows that Brachyury is more represented in $CD44^+$ prostate tissues, and ectopic Brachyury overexpression *in vitro* promotes CD44 expression. Because $CD44^+$ prostate tumors are more resistant to the currently used therapies (43, 44), we speculate that Brachyury could have a role in prostate cancer therapy resistance. Future studies are warranted to elucidate this hypothesis.

In conclusion, the present work reports increased levels of Brachyury expression in localized and metastatic prostate cancer, with clinicopathologic significance and evidences a role for Brachyury in promoting prostate cancer cell growth and invasion. Our work further suggests new roles for Brachyury in prostate cancer, namely, in tumor microenvironment regulation and possibly in immune response. Clinical applicable prognostic biomarkers are needed for clinical management of patients with prostate cancer and our study positions Brachyury as a putative independent prognostic biomarker in prostate cancer and a possible therapeutic target for advanced prostate tumor patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: F. Pinto, P. Andrade, R.M. Reis
Development of methodology: F. Pinto, R.M. Reis
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): F. Pinto, N. Pértega-Gomes, M.S. Pereira, J.R. VizcaÚno, R.M. Reis
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