Androgen-Regulated SPARCL1 in the Tumor Microenvironment Inhibits Metastatic Progression

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Abstract

Prostate cancer is a leading cause of cancer death in men due to the subset of cancers that progress to metastasis. Prostate cancers are thought to be hardwired to androgen receptor (AR) signaling, but AR-regulated changes in the prostate that facilitate metastasis remain poorly understood. We previously noted a marked reduction in secreted protein, acidic and rich in cysteine-like 1 (SPARCL1) expression during invasive phases of androgen-induced prostate growth, suggesting that this may be a novel invasive program governed by AR. Herein, we show that SPARCL1 loss occurs concurrently with AR amplification or overexpression in patient-based data. Mechanistically, we demonstrate that SPARCL1 expression is directly suppressed by androgen-induced AR activation and binding at the SPARCL1 locus via an epigenetic mechanism, and these events can be pharmacologically attenuated with either AR antagonists or HDAC inhibitors. We establish using the Hi-Myc model of prostate cancer that in Hi-Myc/Sparc1−/− mice, SPARCL1 functions to suppress cancer formation. Moreover, metastatic progression of Myc-CaP orthotopic allografts is restricted by SPARCL1 in the tumor microenvironment. Specifically, we show that SPARCL1 both tethers collagen in the extracellular matrix (ECM) and binds to the cell’s cytoskeleton. SPARCL1 directly inhibits the assembly of focal adhesions, thereby constraining the transmission of cell traction forces. Our findings establish a new insight into AR-regulated prostate epithelial movement and provide a novel framework whereby SPARCL1 in the ECM microenvironment restricts tumor progression by regulating the initiation of the network of physical forces that may be required for metastatic invasion of prostate cancer.

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Introduction

Despite progress in early detection and therapeutics, prostate cancer is a leading cause of cancer-related mortality among men in the United States. Androgen receptor (AR) signaling is integral to prostate cancer progression (1) and thus an increased understanding of AR-modulated barriers that typically constrain prostate cancer progression is critical for further development of prognostic biomarkers and therapeutics. Using an AR-induced developmental system to model cancer progression (2, 3), we previously identified SPARCL1 (also called Hevin and SC1) as a potential novel AR-regulated gene. SPARCL1 is markedly downregulated during androgen-induced invasion during prostate development (4). Consistent with this, SPARCL1 expression also inversely correlates with prostate cancer aggressiveness, and its loss in clinically localized prostate cancer is a significant and independent prognostic factor of metastatic recurrence following surgery (4, 5). The mechanisms triggering SPARCL1 downregulation during physiologic or pathologic growth in the prostate are not known; however, the paralleled loss of SPARCL1 mRNA and protein suggests that SPARCL1 loss in many prostate cancers may be attributed to deregulation of SPARCL1 gene expression. Collectively, this implicates SPARCL1 as a potential AR-regulated gene.

Although several functional studies support that SPARCL1 restricts tumor growth and progression (4, 6, 7), the role for SPARCL1 in prostate cancer remains poorly understood. The correlation between SPARCL1 loss and aggressiveness of clinically localized prostate cancer suggests that SPARCL1 may function as a barrier to tumor initiation and progression in the prostate (4). Consistent with this, overexpression of SPARCL1 in colon cancer cells suppressed growth of subcutaneous xenografts (6). While SPARCL1 has been shown to inhibit in vitro proliferation of colon cancer (6) and HeLa (8) cells, other studies support that SPARCL1 may not regulate cellular proliferation in the prostate (4, 7).
Alternatively, SPARCL1 has been shown in multiple models to inhibit processes integral to both local and metastatic progression, such as cancer cell adhesion, migration, and invasion (4, 6, 7, 9, 10). Two recent reports demonstrate that SPARCL1 suppresses tumor nodule formation in visceral organs following intravenous injection (6, 7). Although these studies collectively support that SPARCL1 constrains cancer growth, the precise role of SPARCL1 in the step-wise progression from prostate tumor initiation through localized progression has not been definitively examined in an autochthonous model. Thus, it remains to be determined if SPARCL1 functions as a bona fide metastasis suppressor gene by limiting metastatic progression without affecting primary tumor growth or if SPARCL1 functions as a barrier to both localized and metastatic tumor progression in the prostate.

Herein, we delineate a specific AR-regulated pathway that facilitates prostate cancer progression. We demonstrate that direct AR binding at the SPARCL1 locus inhibited SPARCL1 expression through epigenetic modifications and that this could be pharmacologically modulated by either AR antagonists or HDAC inhibitors. In two independent patient-based cohorts, we note that loss of SPARCL1 expression in the prostate significantly co-occurred with AR amplification or overexpression. Using an animal model that recapitulates human prostate cancer progression, we demonstrate that SPARCL1 functions to suppress adenocarcinoma formation in the prostate. Although temporal loss of SPARCL1 in invading epithelial buds has been shown to be necessary for prostate development (4), we show that constitutive absence of SPARCL1 did not lead to a hyperplastic phenotype. In the context of oncogenic activation such as c-MYC, SPARCL1 functioned to suppress tumor formation and limit metastatic progression. As a matricellular protein, SPARCL1 is secreted in organ culture and UGE and UGM isolation. In vitro cell micromechanical methods (13) have assessed SPARCL1 expression using quantitative real-time PCR using primers specific to the target region in SPARCL1 (F-5'-TGTTGGCCCTAAAGTCAT, R-5’-GATGATGGCCAGTTAGG). The relative methylation-index normalized to in vitro completely methylated white blood cell DNA (M.Sssl WBC DNA) is shown color-coded according to the displayed methylation heatmap. Note that none of the prostate cancer cell lines show significant CpG methylation at the SPARCL1 locus.

Materials and Methods

Quantitative real-time PCR (4). Johns Hopkins University prostate cancer antiandrogen therapy tissue microarray (11), androgen gene regulation (12), chromatin immunoprecipitation assay (12), immunohistochemistry (4), cell proliferation (4), live cell micromechanical methods (13–16), Fourier transform traction microscopy (15–18), and immunofluorescence (4) have been described previously and are detailed in Supplementary Materials and Methods.

In vitro organ culture and UGE and UGM isolation

The protocol was approved by the Johns Hopkins University Animal Care and Use Committee. Similar as described previously (4), the urogenital sinus was harvested from embryonic day (E) 15.5 females (androgen naive), placed ventral side up on a 0.4-μm Millicell filter (Millipore) in a six-well plate with UGS media: DMEM-F12 (Invitrogen), nonessential amino acids (Cellgro), ITS media (Sigma), pen/strep (Invitrogen), 1 g/L d-glucose (Sigma), 1-glutamine (Invitrogen), and 10^-8 mol/L dihydrotestosterone or vehicle for 0, 24, or 48 hours. UGS were digested in 1% trypsin (Gibco) in Hank’s Balanced Salt Solution (Gibco) at 4°C for 90 minutes. UGS were washed with DMEM (Gibco) containing 10% FCS (Gibco). UGE and UGM were separated using fine gauge needles.

Pharmacologic epigenetic modulation experiments

LNCaP, VCaP, 22RV1, and PC3 cells were treated with vehicle or 1 μmol/L 5-Aza-2'–deoxycytidine (Sigma-Aldrich) for 3 days. Similarly, LNCaP, VCaP, 22RV1, and PC3 cells were treated with 1 to 5 nmol/L vorinostat (SelleckChem) or vehicle for 48 hours. Media with vehicle or vorinostat were changed daily. LNCaP cells were treated with 1 nmol/L Panobinostat (SelleckChem) or vehicle for 24 hours.

Cell line methylation status

The DNA methylation status of the putative regulatory region in the first intron of SPARCL1 was evaluated from genomic DNA isolated from prostate cancer cell lines (LNCaP, C42B, LAPC4, VCaP, PC3, and CWR22Rv1) and normal prostate epithelial cells (PrEC) grown in 10% serum-containing media using a combined restriction enzyme digestion and methylation-specific affinity enrichment (COMPARE-MS assay) as described previously (19). In brief, after digestion of genomic DNA with AluI and Hhal (New England Biolabs), methylated DNA fragments were enriched using the Epixplore Methylated DNA Enrichment Kit (Clontech). The abundance of the enriched methylated DNA was assessed by quantitative real-time PCR using primers specific to the target region in SPARCL1 (F-5’-TGTTGGCCCTAAAGTCAT, R-5’-GATGATGGCCAGTTAGG). The relative methylation-index normalized to in vitro completely methylated white blood cell DNA (M.Sssl WBC DNA) is shown color-coded according to the displayed methylation heatmap. Note that none of the prostate cancer cell lines show significant CpG methylation at the SPARCL1 locus.

Sparcl1-deficient mice and Hi-Myc mice

This protocol was approved by the Johns Hopkins University Animal Care and Use Committee. Sparcl1+/−/129/SvEv mice were gifted to our laboratory by Cagla Eroglu, PhD at Duke University (20). Sparcl1+/−/129/SvEv mice were backcrossed greater than seven generations to FVB/N. FVB-Tg(ARR2/Pbsn-MYC) 7Key (Hi-Myc/FVB/N) were obtained from Jackson Labs. Sparcl1+/−/FVB/N mice were crossed with Hi-Myc/FVB/N transgenic mice to obtain Sparcl1+/−/Hi-Myc mice. Sparcl1+/−/Hi-Myc were mated to generate the crosses used in this study. Prostates were harvested at 4.5 months (138 ± 2 days), formalin-fixed, paraffin embedded, and sectioned using standard methods. Two hematoxylin and eosin (H&E)–stained slides placed 100 μm apart were examined for evidence of adenocarcinoma by the study pathologist.

Myc-CaP cells

Stable clones of Myc-CaP cells overexpressing mouse SPARCL1 or empty vector were generated. Mouse Sparcl1 cDNA (Open Biosystems) was subcloned into pIREsNeo3 (Clontech) and then transfected into Myc-CaP cells using Fugene HD (Promega). Cells were grown on type I collagen plates (BD Biosciences) in selection media: DMEM (Invitrogen) supplemented with 10% FBS (Gemini Bioproducts), penicillin and streptomycin (Invitrogen), 25 μg/mL bovine pituitary extract (Invitrogen), 6 ng/mL recombinant human epidermal growth factor (R&D), and 5 μg/mL.
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Oranginal allografts

Myc-CaP cells (1 × 10⁶) in Matrigel were grafted into the anterior prostate of male wild-type (WT) and Sparcl1⁻/⁻ FVB mice that were greater than 10 weeks in age. 1 × 10⁶ Myc-CaP-Neo (B) or Myc-CaP–mSPARCL1 (A) cells in Matrigel were grafted into the anterior prostate of male WT FVB mice that were greater than 10 weeks in age. An isolurane gas anesthetic system (Caliper Life Sciences) was used for surgical anesthesia. A midline incision was made in the lower abdomen to externalize the seminal vesicles, bladder, and prostate. Following injection of 10 μL of cells 1:1 in Matrigel into the anterior lobe of the prostate, the wound was closed with surgical sutures and surgical metal clips. Mice were monitored daily for distress. Mice were euthanized at day 21, necropsied, and inspected for gross evidence of metastatic disease. Organs were removed, formalin-fixed, paraffin embedded, and sectioned using standard methods. H&E-stained slides were examined for evidence of metastatic disease by the study pathologist.

Statistical analysis

Statistical analyses were performed using Prism GraphPad and SAS Software. Statistical tests were two sided, and P values less than 0.05 were considered statistically significant.

Results

Androgen suppresses Sparcl1 expression during prostate development

We previously reported a marked suppression of Sparcl1 gene expression during invasive phases of androgen-induced prostate development and regeneration in vivo (4). To determine if androgen signaling mediates Sparcl1 gene repression, we examined Sparcl1 expression during prostate development in response to 5α-dihydrotestosterone (DHT). DHT treatment of androgen-responsive prostate epithelium resulted in decreased expression compared with control (4). As SPARCL1 has been shown to inhibit epithelial bud outgrowth (4), these results suggest that androgen facilitates epithelial bud invasion by suppressing Sparcl1 gene expression.

Androgen suppresses SPARCL1 expression in prostate cancer

We previously reported that SPARCL1 expression inversely correlates with Gleason grade with the most pronounced loss seen in metastatic lesions (4). Herein, we postulated that, similar to prostate development, androgens also repress SPARCL1 expression in prostate cancer. In canonical androgen signaling, androgens regulate gene transcription through AR. Analysis of human gene expression data showed that AR gene amplification and overexpression occurred concurrently with the loss of SPARCL1 expression with an OR of 9.23 [95% confidence interval (CI), 3.92–21.74; P = 0.000001 by Fisher exact test; refs. 21–23; Fig. 1D]. We validated this finding in a prospectively designed study of high-risk men who underwent radical prostatectomy (RP) at the Mayo Clinic (4, 24, 25). We found that the loss of SPARCL1 expression cooccurred with overexpression of AR with an OR of 2.78 (95% CI, 1.59–4.90; P = 0.0001485 by Fisher exact test; Fig. 1D). To determine if androgen directly suppressed SPARCL1 gene expression, we assayed for SPARCL1 expression in response to androgen depletion. Androgen was removed by culturing androgen-responsive prostate cancer cell lines, LNCaP and VCaP, in media with charcoal-dextran-stripped FBS (C/D serum). Compared with cells grown with serum, LNCaP and VCaP cells grown with C/D serum showed a significant increase in SPARCL1 gene expression (Fig. 1E and F). SPARCL1 gene expression in turn decreased with the addition of DHT, whereas KLK3 (PSA), a known androgen-induced gene, was increased with similar kinetics (Fig. 1E and F). Furthermore, chemical inhibition of AR with MDV3100/Enzalutamide, an AR antagonist approved for the treatment of metastatic prostate cancer, significantly increased SPARCL1 gene expression in prostate cancer cells in the presence of androgen (Fig. 1G). Consistent with these results, men treated with antiandrogen therapy (ADT) prior to RP for localized prostate cancer (n = 46) had significantly higher SPARCL1 expression in their prostate at RP by IHC compared with men who did not have ADT (n = 18; Fig. 1H). SPARCL1 expression was also increased in an additional cohort of men treated with ADT compared with untreated men (Supplementary Fig. S1A; ref. 26). Complementary to data in humans, treatment of castrated mice with DHT decreased SPARCL1 levels in the prostate (Supplementary Fig. S1B). Collectively, these results demonstrate that androgen functions through AR to suppress SPARCL1 gene expression in prostate cancer.

To determine if AR directly suppresses SPARCL1 gene expression, we examined AR binding at the SPARCL1 locus. Androgen binding to AR facilitates recruitment of coactivators, such as SRC-3 and p300, to its upstream region. Dynamics of AR binding at SPARCL1 were similar to other known AR targets, such as PSA and TMPRSS2 (Supplementary Fig. S2C and S2D). These data, taken together, support the conclusion that androgen directly suppresses SPARCL1 expression through AR binding at the SPARCL1 locus.

AR mediates SPARCL1 suppression through modulation of histone acetylation

How AR negatively regulates transcriptional activation by direct DNA binding is not fully understood. Because suppressors of tumor and metastatic progression can be epigenetically repressed in cancer (30, 31), and aberrant epigenetic modifications such as DNA methylation and histone acetylation often occur in prostate cancer (32), we considered if SPARCL1 expression is governed by epigenetic modifications at the SPARCL1 locus. In order to assess the role of promoter hypermethylation on SPARCL1 expression, we treated prostate cancer cell lines with a DNA methyltransferase inhibitor (5-Aza-2′-deoxycytidine). 5-Aza-2′-deoxycytidine treatment did not increase SPARCL1 expression (Fig. 2A), but did
increase expression of the control gene GSTPI (Supplementary Fig. S2E), which is known to be epigenetically repressed by promoter DNA hypermethylation in prostate cancer cells. Notably, CpG methylation patterns at the SPARCL1 locus in cancer cells were comparable with that of benign primary PrEC (Fig. 2B). These findings are consistent with prior studies in cancers of other origins (9, 33) and indicate that promoter CpG methylation is not primarily responsible for the observed suppression of SPARCL1 in prostate cells.

Another important mechanism involved in gene regulation is chromatin remodeling through histone acetylation. Histone acetylation leads to formation of open chromatin, which thereby allows for transcriptional activation. In the absence of acetylation, transcription is hindered due to formation of closed chromatin conformations. HDACs remove acetyl groups from ε-N-acetyl lysine (K) in histones to regulate gene transcription. Inhibition of HDACs with vorinostat, an HDAC inhibitor currently in clinical trials for the treatment of metastatic prostate cancer, or a broad spectrum HDAC inhibitor, Panobinostat, significantly increased SPARCL1 expression, even in the presence of androgen, suggesting that HDACs function downstream of AR binding to the SPARCL1 locus (Fig. 2C and Supplementary Fig. S2F).
ChIP-Seq data from ENCODE and analyzed through the UCSC genome browser (hg19) support that a region downstream of the NC-ARE at the \textit{SPARCL1} locus is enriched for the Histone H3 acetyl Lys27 (H3K27Ac) marks (34), which are active transcription marks often found at transcriptional promoters and/or enhancers (Fig. 2D; ref. 35). In the absence of androgen, ChIP for H3K27Ac demonstrated that this region in \textit{SPARCL1} was acetylated (Fig. 2E). Upon androgen stimulation, H3K27Ac was lost at the \textit{SPARCL1} locus (Fig. 2E and Supplementary Fig. S2G). Collectively, these results are consistent with a model of androgen-induced AR binding and HDAC-mediated deacetylation at the \textit{SPARCL1} locus thereby functioning to suppress \textit{SPARCL1} expression (Fig. 2F).

\textit{Sparcl1} is dispensable for prostate development

Prostate development occurs in an undifferentiated UGS when androgens induce proliferation and invasion of UGE into the surrounding mesenchyme to form epithelial prostate buds. Prior studies support that discrete loss of \textit{SPARCL1} expression within the invading epithelial bud tip is required for bud migration and invasion during bud elongation (Fig. 1C; ref. 4). It is not known, however, if \textit{SPARCL1} expression elsewhere in the UGE is necessary to restrict appendicular patterning. When compared with WT male UGS in organ culture, \textit{Sparcl1}/+/C0 UGS had similar spatial patterning, length, and number of buds (Fig. 3A–C). Analogous to early development, \textit{SPARCL1} was also dispensable for adult prostate maturation. \textit{Sparcl1}/+/C0 adult prostates were grossly indistinguishable from WT prostates as measured by appearance and weight (Fig. 3D and E). Histologic and immunohistological analyses of \textit{Sparcl1}/+/C0 prostates at 21 months (n = 20) demonstrated comparable gland architecture, including basal (p63) and luminal (CK8) cell number (Fig. 3F) and ratio (Fig. 3G) as WT. Consistent with prior studies demonstrating that \textit{SPARCL1} does not regulate cellular proliferation in the prostate (4, 7), \textit{Sparcl1}/+/C0 prostates did not have elevated proliferation as measured by Ki67 (Fig. 3F). Consistent with this, \textit{Sparcl1}/+/C0 prostates did not have
histologic evidence of hyperplasia, cancer precursor lesions [murine prostatic intraepithelial neoplasia (mPIN)], or invasive prostate adenocarcinoma formation (Fig. 3D–F). These data demonstrate that, while the temporal loss of SPARCL1 has been shown to be necessary for bud migration and invasion (4), its constitutive absence does not disrupt early prostate development or adult maturation and is not sufficient to initiate prostate carcinogenesis. This is consistent with the notion that while SPARCL1 loss is necessary to release a “brake” to physiologic prostate development, its loss is not sufficient to activate a corresponding “acceleration” switch.

**SPARCL1 functions to suppress adenocarcinoma formation in the prostate**

In contrast with prostate cancer initiation, compelling data suggest that SPARCL1 functions as a tumor suppressor by restricting local invasion (6, 9). To test the hypothesis that SPARCL1 inhibits local invasion driving the transition between cancer precursor lesions and invasive adenocarcinoma, we modeled dynamics of human prostate cancer in mice. **MYC** is one of the most commonly amplified and overexpressed oncogenes in human prostate cancer. Hi-Myc transgenic mice develop mPIN lesions as early as 2 weeks that progress to locally invasive adenocarcinoma of the prostate by 6 months (36). Consistent with human prostate cancer (Fig. 4A), SPARCL1 loss correlates with tumor progression in Hi-Myc mice (Fig. 4B and C), with the most pronounced loss observed in Myc-CaP cells (37), a spontaneously immortalized cell line derived from a Hi-Myc prostate. To determine the role of SPARCL1 in cancer progression, we examined prostate carcinogenesis in Hi-Myc mice with heterozygous (Hi-Myc/Sparcl1+/−) and homozygous (Hi-Myc/Sparcl1−/−) genetic deletions of **Sparcl1**.

Consistent with prior studies demonstrating that SPARCL1 does not restrict cellular proliferation in prostate cells (4, 7), prostate weight was comparable between Hi-Myc and Hi-Myc/Sparcl1−/− mice at 4.5 months (Fig. 4D). However, pathologic examination demonstrated that homozygous loss of **Sparcl1** in Hi-Myc mice led to a significant increase (by over 50%) in invasive adenocarcinoma incidence at 4.5 months (Fig. 4E and F). An intermediate phenotype was seen with the loss of one copy of **Sparcl1** with an increase in incidence of nearly 20% over Hi-Myc mice at 4.5 months (Fig. 4E). These results support that SPARCL1 restricts the transition from precancerous lesions to invasive carcinoma.

As SPARCL1 expression is inversely associated with cancer aggressiveness as measured by Gleason grade, we tested the
hypothesis that SPARCL1 restricts tumor progression. To examine this, Myc-CaP cells overexpressing murine SPARCL1 (Myc-CaP-mSPARCL1) or empty-vector (Myc-CaP-EV) were orthotopically allografted into WT prostates (Fig. 4G and Supplementary Fig. S4A). Although in vitro proliferation was not significantly different between Myc-CaP-EV and Myc-CaP-mSPARCL1 cells, Myc-CaP-mSPARCL1 allografts were significantly smaller compared with Myc-CaP-EV allografts (Fig. 4H–I). Collectively, these results suggest that SPARCL1 functions as a barrier to tumor formation and that its loss contributes to tumor progression.

SPARCL1 in the tumor microenvironment suppresses metastatic progression in the prostate

As a matricellular protein, SPARCL1 is secreted into the ECM and regulates cellular function. We postulated that if SPARCL1 inhibited tumor invasion, then prostate cancer cells would be more invasive in Sparcl1–/– prostates compared with WT prostates. To examine the role of SPARCL1 as a matricellular protein in the tumor microenvironment, Myc-CaP cells, which do not express SPARCL1 (Fig. 4C), were orthotopically allografted into WT mice (Fig. 4A). Although in vitro proliferation was not significantly different between Myc-CaP-EV and Myc-CaP-mSPARCL1 cells, Myc-CaP-mSPARCL1 allografts were significantly smaller compared with Myc-CaP-EV allografts (Fig. 4H–I). Collectively, these results suggest that SPARCL1 functions as a barrier to tumor formation and that its loss contributes to tumor progression.

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metastases in Sparc1−/− mice compared with WT mice (Fig. 5C and D). Similar to prior studies in WT mice, most metastases in both WT and Sparc1−/− mice were to regional and distant lymphatic tissues; however, hematogenous spread to the lungs was observed in one Sparc1−/− mouse (Fig. 5C). These results suggest that SPARCL1 in the ECM tumor microenvironment restricts metastatic progression.

**SPARCL1 modulates collagen-regulated mechanical properties of prostate cancer cells**

Collagen has been shown to regulate both biophysical and biochemical dynamics of the tumor microenvironment. How SPARCL1 functions within the framework of a collagen matrix to inhibit cellular migration and invasion, and ultimately tumor progression, is not fully understood (4, 6, 7). To begin to understand the mechanism(s), we used RGD-coated ferrimagnetic microbeads anchored to the cytoskeleton through cell surface integrin receptors (13–16) and probed the material properties of PC3 cells adhered on collagen matrix versus collagen matrix containing SPARCL1 (collagen+rSPARCL1). Applying forced bead motions with magnetic twisting cytometry (MTC), we first measured cytoskeletal stiffness (g′) and internal friction (g″) of PC3 cells over a wide frequency range (Fig. 6A and B). Throughout the measurement range (oscillatory frequencies from 10−1 to 103 Hz), stiffness of PC3 cells increased with frequency as a weak power law (Fig. 6B). The internal friction also followed a weak power law at low frequencies (below ~10 Hz), but showed stronger frequency dependence at higher frequencies (above ~10 Hz). These classic cellular responses were observed under both conditions (collagen vs. collagen+rSPARCL1). Nevertheless, SPARCL1 in the collagen matrix appreciably decreased PC3 cell stiffness in physiologic range of probing frequencies (Fig. 6B). The internal friction also significantly differed between cells adhered on collagen versus collagen+rSPARCL1 at both low (1 Hz and below) and high frequencies (100 Hz and above; Fig. 6B).

To determine the underlying dynamics of the cytoskeletal network, we then assessed spontaneous motions of the same functionalized microbeads (13). In both conditions (collagen vs. collagen+rSPARCL1), the mean square displacements (MSD) of unforced beads increased with time as a power law with an exponent α greater than unity (Fig. 6C and D). As we have defined elsewhere (13), such anomalous bead motions are not characteristics of Brownian motions that are thermally-driven but are rather characteristics of a discrete molecular-level rearrangements (remodeling) of actin cytoskeleton driven by myosin motors. The exponent α did not differ between cells adhered on collagen versus cells on collagen+rSPARCL1 (Fig. 6C). Compared with cells adhered on collagen, however, PC3 cells adhered on collagen+rSPARCL1 showed marked decreases in diffusion coefficient D* (a measure of the speed of cytoskeleton remodeling) and computed MSDs (Fig. 6D–F). Similar to PC3 cells grown on collagen+rSPARCL1, Myc-CaP-mSPARCL1 cells had a significantly reduced rate of cytoskeletal remodeling compared with Myc-CaP parental and Myc-CaP-EV controls when adhered to a collagen matrix (Supplementary Fig. S4B). Collectively, these data indicate that SPARCL1 in the collagen matrix acts to slow down the rate at which cells remodel their internal network of cytoskeleton. These biophysical analyses, taken together, suggest that SPARCL1 attenuates collagen-regulated mechanical correlates that may be required for metastatic invasion of prostate cancer.

**SPARCL1 inhibits transmission of cell traction force**

The ability of a single living cell, or cell ensemble, to exert traction force upon its surrounding is the necessary prerequisite for diverse biologic processes, such as local cellular migrations in development to metastatic invasion of cancer (15–18). Using Fourier transform traction microscopy (17), we measured the ability of an individual PC3 cell to exert traction force upon its surrounding (collagen vs. collagen+rSPARCL1). On a collagen matrix containing SPARCL1, PC3 cells showed slower adhesion dynamics than on collagen, but exhibit similar dispersion of cell size on both matrices for 24 hours (4). Consistent with this, we found no significant differences in the computed cell-projected area between cells on collagen versus collagen+rSPARCL1 (Fig. 7A and B). Strikingly, however, cells on collagen+rSPARCL1 exhibited a much muted cell traction force than on collagen (Fig. 7C and D). Traction root mean square
average over the entire cell-projected area was significantly reduced by SPARCL1 in the collagen matrix (Fig. 7C). In addition, cells adhered on collagen+rSPARCL1 showed a 2-fold reduction in net contractile moment (Fig. 7D), which is a scalar measure of the cell's contractile strength (17). These results suggest that SPARCL1 inhibits transmission of cell traction force, presumably via focal adhesions.

In order to test this postulate, we coated microbeads (~4.5 μm in diameter) with rSPARCL1 or RGD, allowed the beads to first adhere to the collagen matrix, and then plated PC3 cells. Using both unconstraint and constraint Fourier transform traction cytometry (17), we measured cell traction forces of individual PC3 cells encompassing the beads (rSPARCL1 vs. RGD), as well as the localized tractions near the beads (Supplementary Fig. S5A).
Compared with cells adhered directly to collagen (without beads underneath) or through uncoated beads, PC3 cells adhered to collagen through RGD-coated beads tended to increase cell spreading (Supplementary Fig. S5B). In contrast, cells adhered to collagen through SPARCL1-coated beads tended to decrease cell spreading (Supplementary Fig. S5B). Interestingly, cells adhered to collagen through RGD-coated beads show reinforcement of contractile strength, whereas those adhered through SPARCL1-coated beads did not (Supplementary Fig. S5C). Moreover, localized RMS tractions around SPARCL1-coated beads were significantly lower than those around RGD-coated beads (Fig. 7E and Supplementary Fig. S5D and S5E). Consistent with the notion that SPARCL1 inhibits transmission of cell traction force via focal adhesions, we also observed decreased focal adhesion assembly as measured by immunofluorescent staining of paxillin and vinculin in cells adhered on collagen matrix containing SPARCL1 (Fig. 7F and G and Supplementary Fig. S5F). These results, taken together, support the conclusion that SPARCL1 inhibits collagen-induced formation of focal adhesions and transmission of cell traction forces that are required for metastatic invasion of prostate cancer (15, 16, 40, 41).

SPARCL1 engages cell–ECM interactions

We previously reported that SPARCL1 loss increases the migratory and invasive properties of prostate cancer cells through Ras homolog gene family, member C (RHOC), a known mediator of metastatic progression (42–46). Hence, the reduction in focal adhesions and cell traction forces by SPARCL1 might be due to
progression holds promise for clinically translatable targets. Our elucidation of novel AR-driven programs that mediate metastatic cell surface integrin receptors, showed super-diffusive motions that tended to grow with incubation time (Supplementary Fig. S6A–S6C). In contrast, rSPARCL1-coated beads showed slower ligation to cell surface and exhibited largely diffusive motions that tended to decrease with incubation time (Supplementary Fig. S6A–S6C). Strikingly, at 24 hours, motions of rSPARCL1-coated beads emerged as super-diffusive and did not differ from those of RGD-coated beads (Supplementary Fig. S6A–S6C), indicating ligation of SPARCL1 to the cell’s interior cytoskeletal network. These responses were concentration dependent (Supplementary Fig. S7A–S7C) and distinct from iSPARCL1-coated beads bound to collagen, suggesting ligation of SPARCL1 to the CSK is not due to time-dependent embedding of the beads but rather specific and selective ligation through cell surface receptors. Collectively, these results suggest that SPARCL1 not only tethers directly to collagen ECM but also binds to cell surface through as yet unidentified receptor class.

Discussion

Prostate cancer is exquisitely reliant on AR signaling, and elucidation of novel AR-driven programs that mediate metastatic progression holds promise for clinically translatable targets. Our findings presented herein establish insight into a novel AR-regulated pathway. We define an AR-mediated mechanism in the prostate for both physiologic and pathologic suppression of SPARCL1. We show both in vitro and in vivo pharmacologic modulation of SPARCL1 expression with antiandrogen therapies, including Enzalutamide. This is consistent with previous findings showing decreased Sparc1 gene expression during phases of androgen-induced physiologic growth in the prostate (4). We mechanistically demonstrate that androgens directly suppress SPARCL1 expression through recruitment of AR to the SPARCL1 locus. In support of this, we demonstrate that SPARCL1 loss concurrently occurs with AR amplification or overexpression in patient-based data. We further show that androgens mediate epigenetic regulation of SPARCL1 and that this can be pharmacologically modulated with HDAC inhibitors, which are currently in clinical trials for metastatic prostate cancer. Thus, our data establish a model whereby androgen-induced AR signaling inhibits SPARCL1 gene expression through chromatin remodeling.

SPARCL1 loss is associated with disease progression in multiple cancer types (4, 6, 47, 48), suggesting that its loss may be a conserved and critical step for metastatic invasion. Alternate mechanisms governing SPARCL1 expression have yet to be fully defined; however, conservation may exist among steroid-responsive cancers as Estradiol has been shown to inhibit SPARCL1 gene expression (49). Studies in non–small cell lung cancer support that steroid-independent pathways may similarly regulate SPARCL1 at this locus (33). Aberrant epigenetic modulation at this locus via HDACs in androgen-independent cells suggests that this mechanism may also occur in castration-resistant prostate cancer and in steroid-independent cancers. Although these studies indicate that aberrant methylation at this locus is not the driving force for SPARCL1 suppression in the prostate, this does not preclude that epigenetic modulation by DNA methylation does not occur in other cancers. In addition to epigenetic regulation of SPARCL1 expression, genomic alterations and loss of heterozygosity (48) may account for a small percentage of SPARCL1 loss observed in advance cancers.

We establish a model whereby SPARCL1 secreted in the ECM microenvironment restricts the invasive pathways necessary for both local and metastatic disease progression. Temporal loss of SPARCL1 is necessary for epithelial bud invasion during prostate development (4), yet constitutive loss of SPARCL1 in Sparcl1+/− mice was not sufficient to initiate carcinogenesis. Consistent with prior studies (4), our in vivo findings demonstrate that SPARCL1 loss does not lead to aberrant proliferation or activation of oncogenic signaling. Instead, our data suggest that in the context of oncogenic activation, SPARCL1 functions to restrict the invasive steps required for the transition from precancerous lesions (PIN) to cancer. We show that, similar to human prostate cancers, Sparcl1 gene expression was increasingly suppressed during Hi-Myc–induced prostate tumor progression. Genetic deletion of Sparcl1 in Hi-Myc mice led to a significant increase in early prostate cancer incidence with an intermediate phenotype seen in Hi-Myc/Sparcl1+/− mice. Complementary to these studies, overexpression of SPARCL1 in Mcc-CaP cells restricted orthotopic allograft size. Interestingly, Mcc-CaP orthotopic allografts in WT and Sparcl1−/− mice were similar in size, suggesting that SPARCL1 secreted by benign adjacent prostate cells was not sufficient to suppress tumor growth. In contrast with tumor size, SPARCL1 from the host mitigated metastatic progression of Myc-CaP orthotopic allografts in both WT and Sparcl1−/− mice (50). These data, taken together, support the conclusion that SPARCL1 functions to suppress adenocarcinoma formation and progression to metastases by attenuating the invasive pathways necessary for local and metastatic cancer invasion.

Our data offer new understanding into how SPARCL1 in the ECM microenvironment mechanistically functions as a barrier to tumor initiation and progression. Cellular migration and invasion through the ECM are dependent on the transmission of mechanical and regulatory signals between the matrix and the cell through focal adhesions. Our data demonstrate that SPARCL1 in the ECM limited cellular ability to form focal adhesions on the collagen matrix and thereby attenuated corresponding biophysical dynamics of the cytoskeletal framework, which have been shown to potentiate migration. This is consistent with prior reports showing that SPARCL1 mitigates mediators of focal adhesion activation such as RHO family members (4, 50). Integration of ECM-induced biochemical and biophysical signals in the cell ultimately translates into its ability to exert traction force upon the ECM with increasing traction associated with enhanced metastatic potential (40). Our data support that SPARCL1 inhibits the transmission of cell traction force on the matrix. Similar to other matricellular proteins (51), we determined that SPARCL1 in the ECM readily tethers to the collagen ECM (52) while also binding with slower kinetics to the cell cytoskeleton. This suggests that SPARCL1 may restrict cellular invasion by binding to collagen as well as through cell receptor interactions. From the data presented herein, we proposed a model in which cells secrete SPARCL1 in the microenvironment to limit cellular traction and consequent migration and invasion, and thus cancers may optimize their metastatic potential by suppressing SPARCL1 expression.
Disclosure of Potential Conflicts of Interest

E. Davicioni has ownership interest (including patents) in GenomeDx. E.M. Schaeffer is a consultant/advisory board member for GenomeDx. No potential conflicts of interest were disclosed by the other authors.

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Androgen-Regulated SPARCL1 in the Tumor Microenvironment Inhibits Metastatic Progression

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