Castration-induced down-regulation of SPARC in stromal cells drives neuroendocrine differentiation of prostate cancer

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ABSTRACT

Fatal neuroendocrine differentiation (NED) of castration-resistant prostate cancer is a recurrent mechanism of resistance to androgen deprivation therapies (ADT) and anti-androgen receptor pathway inhibitors (ARPI) in patients. The design of effective therapies for neuroendocrine prostate cancer (NEPC) is complicated by limited knowledge of the molecular mechanisms governing NED. The paucity of acquired genomic alterations and the deregulation of epigenetic and transcription factors suggest a potential contribution from the microenvironment. In this context, whether ADT/ARPI induces stromal cells to release NED-promoting molecules and the underlying molecular networks are unestablished. Here, we utilized transgenic and transplantable mouse models and co-culture experiments to unveil a novel tumor-stroma crosstalk that is able to induce NED under the pressure of androgen deprivation. Castration induced upregulation of GRP78 in tumor cells, which triggers miR-29b-mediated downregulation of the matricellular protein SPARC in the nearby stroma. SPARC downregulation enabled stromal cells to release IL-6, a known inducer of NED. A drug that targets GRP78 blocked NED in castrated mice. A public, human NEPC gene expression dataset showed that Hspa5 (encoding for GRP78) positively correlates with hallmarks of NED. Finally, prostate cancer specimens from patients developing local NED after ADT showed GRP78 upregulation in tumor cells and SPARC downregulation in the stroma. These results point to GRP78 as a potential therapeutic target and to SPARC downregulation in stromal cells as a potential early biomarker of tumors undergoing NED.
STATEMENT of SIGNIFICANCE

Tumor-stroma crosstalk promotes neuroendocrine differentiation in prostate cancer in response to hormone therapy via a GRP78/SPARC/IL-6 axis, providing potential therapeutic targets and biomarkers for neuroendocrine prostate cancer.
INTRODUCTION

Prostate cancer is the second leading cause of cancer death and the most commonly diagnosed cancer in the US male population (1). Being a hormone-driven disease, advanced and metastatic tumors are treated with androgen deprivation therapy (ADT). However, this treatment eventually leads to castration-resistant prostate cancer (CRPC) in most patients (2). Despite the introduction of next-generation ADT such as enzalutamide or abiraterone, which blocks the androgen receptor (AR) pathway (named “androgen receptor pathway inhibitors”, or ARPI), prognosis remains dismal because of primary or acquired resistance (2). Therapeutic resistance of CRPC is often associated with the gain of neuroendocrine (NE) features (3). A form of “de novo” NE prostate cancer (NEPC), that resembles small cell NE tumors of lung and pancreas, occurs rarely (<2% of patients) in untreated prostate cancer patients (3,4). On the contrary, treatment-related NEPC is a relevant clinical entity (5) that is thought to occur through trans-differentiation of adenocarcinoma cells as a consequence of ADT/ARPI (6,7). The molecular mechanisms driving NE differentiation (NED) remain poorly defined. Intrinsic tumor features include (i) genomic alterations (loss of RB1 and P53 and amplification of MYCN (6)), (ii) deregulation of epigenetic regulators (up-regulation of Ezh2 (8), and down-regulation of REST (9)), and (iii) up-regulation of transcription factors (BRN2 (10), PEG10 (11), and FOXA2 (12)). The paucity of genomic aberrations suggests that the microenvironment contributes to NED through the production of paracrine factors (13), including the recently identified IL-6 (14), SFRP1 (15), glutamine (16) and neurotensin (17). However, the molecular networks that are activated in response to
ADT and trigger stromal cells to release these mediators remain largely unknown. Furtermore, no biomarkers that can predict risk of NED are currently available. The Secreted Protein Acidic and Rich in Cysteine (SPARC) is a matricellular protein involved in tissue remodeling and plasticity in physiological and pathological conditions. It exerts different functions when released from or retained within the cell (18,19). SPARC can be either up- or down-regulated in different tumor types, and can be produced by both the neoplasm and the neighboring stromal cells (20). Its role in prostate cancer is controversial. Tumor SPARC upregulation is associated with epithelial-mesenchymal transition (EMT) and metastasis (21), but stroma-derived SPARC can limit proliferation of prostate cancer cells (22). Deletion of the Sparc gene enhanced tumor growth in the TRAMP mouse model of prostate cancer (23), but the different roles of tumor versus stroma SPARC and the role of SPARC in NED were not investigated.

Here, we unveil that a crosstalk between tumor and stromal cells is necessary for adenocarcinoma-NEPC transition under the pressure of ADT. In response to therapy, tumor cells release \textit{miR29-b}, which in turn triggers the down-regulation of SPARC in stromal cells. We demonstrate that stromal SPARC down-regulation is a key event needed for NED of prostate cancer cells, and could represent a potential biomarker. We also prove that pharmacologic inhibition of GRP78, the driver of this crosstalk, restrains NED in castrated TRAMP mice, highlighting GRP78 as a promoter of NED and a potential therapeutic target in patients.
MATERIAL and METHODS

Mice and treatments

TRAMP mice on C57BL/6/J background (C57BL/6-tgN (TRAMP)8247Ng) (RRID:IMSR_JAX:003135) were kindly provided by V. Bronte (Verona University Hospital, Italy), under agreement with Dr. N.M. Greenberg (formerly at Fred Hutchinson Cancer Research Center, Seattle, WA, USA). They were maintained and screened as described (24). Mice deficient for Sparc on a mixed 129SV/C57BL/6 background (RRID:IMSR_JAX:003728) (25) were provided by C. Howe (The Wistar Institute, Philadelphia, PA, USA). They were backcrossed to C57BL/6 for 12 generations (Charles River Laboratories; RRID:SCR_003792) and then intercrossed over 12 generation with TRAMP mice (B6.tgN (TRAMP)8247Ng Sparc<tm1Hwe>Ptprc<tm1/J). TRAMP+/− and Sparc−/−TRAMP+/− mice were used for experiments.

Surgical castration was performed in 20 week old TRAMP mice, under anesthesia with ketamine (100 mg/Kg; Imalgene, Boeringher Ingheilm) and xylazine (5 mg/Kg; Rompun, Bayer) with carprofen given as an analgesic (5 mg/Kg; Norocarp, Norbrook). All transgenic mice were sacrificed at 30 weeks of age. For subcutaneous tumor challenge, 2x10⁶ T23 cells or 5x10⁵ RM1 cells were injected into the right flanks of C57BL/6 or Sparc−/− male mice. Mice were sacrificed when tumor diameters reached 10 mm. Anti-IL6-R antibody (200µg/mouse; clone 15A7; for in vivo studies purified from hybridoma cells as indicated in Table S1), isotype control (200µg/mouse, Table S1), isoliquiritigenin (Selleckem, Houston, TX. USA; cat.no. S2404, 10 mg/Kg) or vehicle, were injected intra-peritoneum twice a week. Treatments started one-week post castration in TRAMP mice or when tumors were palpable in subcutaneous models. Animal housing and experimentation were
performed following institutional guidelines and the Italian law (D.Lgs. 26/2014). In vivo experiments were approved by the Italian Ministry of Health (authorization numbers 664/2017-PR, 758/2018-PR and 256/2021-PR).

**Cell lines and in vitro experiments.**

T1525 and T23 prostate adenocarcinoma cell lines were isolated from TRAMP mice as described (26). Stable fibroblast cell lines were generated from muscle and dermis of newborn C57BL/6 or Sparc−/− mice as described (27). To restore SPARC expression, Sparc−/− fibroblasts were infected with the retroviral vector LXSPARCSH as in (28). RM1 murine prostate carcinoma cells (ATCC CRL-3310; RRID:CVCL_B459; (29)) and LNCaP human prostate cancer cells (clone FGC, ATCC CRL-1740; RRID:CVCL_1379) were purchased from ATCC (Manassas, VA. USA). The human WPMY-1 prostate stromal cell line was kindly provided by N. Zaffaroni in our Institute, who originally purchased it from ATCC (ATCC-CRL2854; RRID:CVCL_3814). All cell lines were routinely tested for Mycoplasma using the MicoAlert Mycoplasma Detection Kit (Lonza, cat no. LT07-118).

For co-culture experiments, cells were plated in transwell plates (0.4 μm pore size, Corning), at a 1:1 tumor cell: fibroblast ratio. Experiments were repeated to allow alternative seeding of tumor cells or fibroblasts on the bottom of the well, to ensure proper cell recovery. When indicated, IL-6 (50 ng/ml, Peprotech, London, UK), monoclonal antibody to IL6-R (50 ng/ml; D7715A7 clone, for in vitro experiments purchased from eBioscience, as indicated in Table S1), enzalutamide (10 μM; Selleckem, cat.no. S1250), isoliquiritigenin (25 μM, Selleckem, cat.no. S2404) or HA15 (10 μM Selleckem, cat no S8299) were added.
Human prostate cancer samples

Formalin-fixed paraffin-embedded (FFPE) human tumor samples from core-biopsies and prostatectomies from prostate cancer patients, either untreated or undergoing neoadjuvant ADT, were obtained from the European Institute of Oncology (IEO), Milan, Italy. Samples were collected in accordance with the Helsinki Declaration. Tissue collection, fixation and processing were performed according to standardized protocols adopted by the Uropathology and Intraoperative Consultation of IEO, as part of routine clinical activity. Immunofluorescence and immunohistochemistry performed in this study were approved by the Institutional Review Board of IEO (authorization number UID 2133; informed written consent was not obtained because according to Institutional rules it was not necessary for these analyses).

Immunohistochemistry and immunofluorescence

Murine and human FFPE tumor samples were cut in 5 µM sections. Slides were deparaffined, re-hydrated and stained with H&E (BioOptica).

For immunohistochemistry antigen retrieval was performed utilizing: (i) the Novocastra Epitope Retrieval Solution pH9 (Leica Biosystems), at 98°C for 30 minutes, for human and mouse SPARC; (ii), the Dako EnVision Flex Target Retrieval Solution High pH (Dako Omnis) for human AR and SYP and (iii) the Dako EnVision Flex Target Retrieval Solution Low pH (Dako Omnis) at 98°C for 30 minutes for human and mouse CgA. After neutralization of the endogenous peroxidase with 3% H2O2 and specific Fc blocking (Novocastra, Leica Biosystems), samples were incubated with primary antibodies (listed in Table S1). Staining was revealed using polymer detection kit (Novocastra, Leica Biosystems) or goat anti-rat IgG secondary antibody 1:500 (listed in Table S1) and 3’-Amino-9-ethylcarbazole or DAKO.
EnVison FLEX (Dako Omnis cat.no: GV800) as chromogenic substrate, followed by counterstaining with Harris hematoxylin (Novocastra, Leica Biosystems). Slides were analyzed under an Axioscope A1 microscope equipped with Axiocam 503 Color camera (Zeiss). Scan of whole human slides was obtained with Aperio Scan Scope (svs files) and pictures were extracted with ObjectiveView™ software.

For immunofluorescence, after antigen retrieval sections were blocked with PBS-Tween (0.1%) containing 5% of bovine serum albumin (BSA; Sigma). Primary antibodies were incubated overnight at 4°C; when necessary, staining with secondary antibodies was performed for 30 minutes at room temperature. All antibodies used are listed in Table S1. Slides were mounted with ProLong Diamond Antifade Mountant with DAPI (ThermoFisher), and acquired with a Leica DM4 B microscope equipped with a Leica DFC450 C digital camera, utilizing the LAS X software (Leica Biosystems; RRID:SCR_013673). Alternatively, for pSTAT3 immunofluorescence imaging was performed using a confocal laser-scanning microscope Leica TCS SP8 X (Leica Microsystems), Images were analyzed using the Image J (RRID:SCR_003070) software.

The “SPARC Score” was assigned comparing the staining intensity of all the slides, given 3 to the maximum intensity observed and 0 to the lowest, and then re-assigning the values to all the slides. For digital quantification we used the Aperio Image Scope (RRID:SCR_006355) and/or the the Image J (RRID:SCR_003070) softwares . We quantified staining intensity in 5 different pictures at 20x magnification for each slide and we reported in graph the average of the 5 measurements. Serial slides cut from the same FFPE sample containing patient’s prostatectomy or tumor biopsy were used for all the IHC and IF staining. Two board certified pathologists (G.R. and C.T.) analyzed IHC slides in a blind fashion and highlighted them with felt-tip areas.
interested by neoplasia. Slides used for IF were then superimposed and the same areas were marked. Pictures were taken within these areas. A similar procedure was adopted to analyze murine prostates.

**Mouse tumor histopathology.**

A board certified pathologist (C.T.) examined all the lobes of TRAMP prostates and classified lesions according to histopathological and phenotypic analyses as follows. Adenocarcinomas (ADENO) were characterized by CK8 positive atypical cells with high nuclear pleiomorphism, variably margined chromatin and prominent nucleoli, that formed distorted/ill-defined glands within the stroma. *De novo* NE in untreated TRAMP mice were composed by sheets and nests of medium-sized to large cells with high nuclear to cytoplasmic ratio, hyperchromatic nuclei, frequent mitotic/apoptotic figures and/or anaplastic morphology, in the absence of signs of glandular structures formation, immunoreactive for SYP and CgA, but negative for CK8. Tumors arising in castrated or Sparc-/- TRAMP mice and showing similar features as described above were also scored as “*de novo*” NE. NE differentiation (NED) was defined by the presence of tumor foci of atypical cells with less pleiomorphic nuclei and granular chromatin, which display a tendency to diffuse growth still maintaining the capability to form glandular remnants, with nuclear features of NE tumors and expression of both CK8, SYP and CgA. This definition of NED is in accordance with a published consensus (30) and with the proposed morphological classification of prostate cancer subtypes (31). Regression was marked by a variable degree of glandular involution within the context of stromal remodeling proliferation. When “*de novo*” NE tumors and adenocarcinoma were present in different lobes, we classified the sample as “*de novo*” NE.
RNA and BASE scope Assays

Mouse pre-Mir29b (Mm-pre-MIR29b-1zz-st targeting 2-52 of NR_029532.1; Cod. 713421; Advanced Cell Diagnostic, ACD) and mouse IL6 transcript (Mm-Il6; Cod. 315891, ACD) were detected using BaseScope Detection kit (ACD) or RNAscope 2.5 HD Detection Reagent-BROWN (ACD), respectively, according to the manufacturer’s protocol. The same sections that undergone RNA-scope hybridization were also stained for Synaptophysin or for PDGFRβ (Table S1) by immunohistochemistry. After specific Fc blocking (Novocastra, Leica Biosystems), primary antibodies were applied overnight and staining was revealed using Rabbit on Rodent AP-Polymer (BioCare) or SignalStain Boost IHC Detection Reagent (AP, Rabbit). Vulcan Fast Red was used as substrate-chromogen followed by counterstaining with Harris hematoxylin.

Transfection with miRNA Inhibitors

Fibroblasts or T23 cells were transfected mixing in 1:1 ratio Lipofectamine3000™ Reagent (Invitrogen cat no. L3000015) with an anti-miR specific for miR29b (mmu-miR-29b-3p cat. No. AM17000; Invitrogen mirVana™ by ThermoFisher) both diluted in Opti-MEM® (Gibco™ cat. no. 31985070). Positive and negative controls (Invitrogen mirVana™ miRNA Inhibitor, let-7c positive control, cat. No. 4464080 and negative control #1, cat. no. 4464076, respectively) were used to verify efficiency of transfection (by measuring Hmga2 transcript levels after two days).

Sparc reporter Assay

The sequence of Sparc 3’UTR, extrapolated on the Genome Browser website (RRID:SCR_004267) was synthetized, amplified in a pUC57 vector (Genscript
Biotech) and then cloned in a psiCHECK-2 vector (Promega cat. no. C8021), downstream of Firefly and Renilla luciferase genes, inserted as reporter (the vector was named psiCHECK-SPARC-3’UTR). Fibroblasts were transfected with psiCHECK-SPARC-3’UTR in presence or not of mimics of miR29b 3p or miR29b 5p (Invitrogen mirVana™ mimics cat. no. 4464066), using Lipofectamine3000™ Reagent (Invitrogen cat no. L3000015), following the manufacturer protocol. Dual-Luciferase Reporter Assay System kit (Promega cat. no. E1910) was used to assess Firefly and Renilla luciferase activity as readout of miRNA targeting the 3’UTR of Sparc.

**Sparc and Hspa5 siRNA transient transfection**

Fibroblasts or T23 cells were transfected with 10µM of mouse siRNA specific for Sparc or Hspa5, (ThermoFisher, siRNA ID: 150942 and s68084, respectively) or a scramble control, using Lipofectamine3000™ Reagent (Invitrogen cat no. L3000015).

**Murine prostate digestion and sorting of luminal and stromal populations**

We adapted the procedure from a published protocol (32). Prostates lobes were digested with collagenase I (1 mg/ml GIBCO, cat. no. 17018-029) for 2 h at 37°C. After washing, pellets were dissociated with Trypsin-EDTA (Euroclone), p1000 pipette and 18G needle. Obtained cell suspensions were filtered through a 40 µm strainer, washed in PBS and then stained for 15 min at 4°C with fluorochrome-labeled monoclonal antibodies (listed in Table S1). 7AAD (eBioscience) was added to exclude dead cells. Samples were acquired with a BD FACSAnia instrument. Cells were sorted as Lineage (CD45/CD31/Ter119) negative, and CD49f<sup>+</sup>Sca-1<sup>neg</sup> (luminal)
or CD49fnegSca1+ (stromal). For each experiment, cells were sorted from a pool of at least three mice per group. Real time PCRs were performed on samples obtained in three independent sorting, for a total of 3 independent biological replicates per group.

**Flow cytometry and western blot.**

Detailed protocols are reported in supplementary material and methods; antibodies used are listed in table S1.

**Exosomes purification**

Exosomes were purified from cell culture supernatants utilizing the ExoQuick-TC kit (System Biosciences, Palo Alto, CA, USA; cat.no EXOTC50A-1), following the manufacturer’s protocol. RNA extraction followed the protocol described for cell supernatants.

**Real time PCR**

Total RNA from cells was extracted using the Quick RNA micro prep kit (Zymo Research). For the supernatants, 500 µL of supernatant were admixed 1:1 with TRIzol Reagent (ThermoFisher; cat. 15596026), and then 200 µL of chloroform were added. After the phase separation, the clear upper aqueous layer containing RNA was admixed 1:1 to 95% ethanol; then miRNeasy Micro Kit (QIAGEN, cat. 217084) was used to purify total RNA. cDNA was obtained using the MultiScribe™ Reverse Transcriptase kit (Applied Biosystems by ThermoFisher). Real-time PCR was performed in a volume of 20 µl using the Taqman® Fast Universal PCR Master Mix (Applied Biosystems), 20 ng of cDNA and specific probes (all from Applied Biosystems, listed in supplementary material and methods). Values were normalized to Gapdh and analyzed using the ∆CT or ∆∆CT method as indicated. For the
detection of miR-29b1, cDNA was obtained using Taqman® MicroRNA Reverse Transcription Kit (Applied Biosystems), with a custom RT primer pool consisting in specific primer set for miR-29b1 and U6 snRNA (used as internal control) for analysis of cell lysates, or with a custom RT primer pool consisting in specific primer set for miR-29b1 and cel-miR-39 (used as internal control) for supernatants. Real-time PCR was performed in a volume of 10 µL using the Taqman® Fast Advanced Master Mix (Applied Biosystems), 10 ng of cDNA and specific probes for miR-29b1, U6 or cel-miR-39, all from Applied Biosystems (listed in supplementary methods). Values were normalized to internal control and analyzed using the ΔCT or ΔΔCT method as indicated.

RNA-Sequencing
Detailed protocols are reported in supplementary material and methods. RNAseq data have been deposited to GEO (RRID:SCR_004584), the accession number is GSE156033. Log2Fold-changes and Adjusted p-values were generated for each class comparison (Table S2).

Functional analyses
We used: the functional annotation tool available within DAVID 6.8 (RRID:SCR_001881) (https://david.ncifcrf.gov/) to investigate over-represented biological processes and molecular functions; MetaCore version 19.4 (Clarivate Analytics, Filadelfia, PA, USA; RRID:SCR_008125) for network and pathway analysis; MeV version 4.9.0 for Heatmap representation.
cBioPortal and correlation analyses

Copy number alteration and mutation data of the \textit{Hspa5} gene were analyzed with cBioportal (https://www.cbioportal.org/ ref. (33,34)), comparing data from prostate adenocarcinoma (from TCGA), metastatic CRPC (from the SU2C/PCF Dream Team data set (35)), and CRPC and NECP samples (from the Kumar data set (36)).

Median normalized RNAseq data of the Beltran data set, including 34 CRPC samples and 15 CRPC-NE (NEPC) samples (6) was downloaded from cBioPortal (33,34) and imported in R software. Spearman correlation between \textit{Hspa5} and all other genes was calculated and genes with a positive correlation (spearman coefficient $\geq 0.7$; Table S3) were used to calculate pathway enrichment through package pathfindR with Reactome database (https://reactome.org/). Among significantly enriched pathways (p-value < 0.05) we chose those with biological relevance. The average of expression of each gene within each pathway was then correlated with the expression of \textit{Hspa5} through Spearman correlation. Spearman correlation was also calculated between \textit{Akt1} or \textit{Cdk5} and \textit{Hspa5}.

Statistical analyses and reproducibility

Statistical analyses were performed with the GraphPad Prism8 software (RRID:SCR_005375). For \textit{in vitro} and \textit{ex-vivo} experiments, histograms report means $\pm$ standard deviation of biological replicates, which are represented by dots. We applied One-Way Anova followed by Tukey’s tests, Two-Way Anova or Student’s t test, as indicated. For ethical reasons, the number of animals used for \textit{in vivo} studies, randomly assigned to different groups, was the minimum necessary to ensure significance of the results. Based on previous experience with the model (26), sample size was selected to obtain an effect size of 0.4 with 80% power and 5% error.
(α=0.05). We used Fisher’s Test to compare categorical variables indicating the phenotypes of tumor lesions.

In all statistical comparisons, differences were considered significant when \( P < 0.05 \), and were indicated as: * \( P < 0.05 \), ** \( P < 0.01 \), *** \( P < 0.001 \), **** \( P < 0.0001 \). Numbers and types of replicates are indicated in each figure legend.
RESULTS

Absence of SPARC and castration induce comparable NED

We compared tumors collected from TRAMP and Sparc−/−TRAMP mice of 30 weeks of age in order to identify linkages between SPARC expression and tumor types. Consistent with the literature (24,37,38), the majority of TRAMP mice (11/13 mice, 84.6%) developed multifocal invasive adenocarcinoma, whereas a small fraction (2/13 mice, 15.4%) developed de novo small cell NE tumors (Fig. 1A and 1B). Adenocarcinoma lesions selectively expressed the luminal marker cytokeratin 8 (CK8) whereas de novo NE lesions selectively expressed NE markers synaptophysin (SYP) and cromogranin A (CgA) (Fig. 1C-E and Fig. S1A-B). Pathological examination revealed areas of NED within adenocarcinoma in 38.5% of Sparc−/−TRAMP mice (5/13 mice) and 35.7% of TRAMP mice previously castrated at 20 weeks of age (5/14 mice). NED areas were not observed in untreated TRAMP mice (Fig.1A). Tumor cells in NED areas had large morphology (Fig. 1B) and co-expressed CK8, SYP and CgA (Fig. 1C-E and Fig. S1A-B; please refer to methods for detailed criteria for tumor lesion classification). In the castrated cohort, we also found mice with CRPC without features of NED (2/14 mice, 14.3%) and mice with tumor regression (5/14 mice, 35.7%). No changes in the frequency of tumors showing features of de novo small cell NE tumors were observed in comparing TRAMP, castrated TRAMP or Sparc−/−TRAMP mice (frequency was 15.4%, 14.3% and 15.4%, respectively; Fig. 1A). We concluded that SPARC genetic deficiency mimics NED induced by late-stage castration in TRAMP mice. Castration in Sparc−/−TRAMP mice slighted enhanced the frequency of NED (7/15 mice, 47%) if compared to untreated Sparc−/−TRAMP or castrated TRAMP mice (38.5% and 35.7%, respectively; Fig 1A).
suggesting that additional factors, unrelated to SPARC, might contribute to NED upon castration.

Immunohistochemistry on prostates from untreated TRAMP mice revealed mild SPARC expression by tumor cells and a stronger positivity in infiltrating fibroblasts and myeloid elements (Fig 1F). Notably, SPARC was down regulated in the stromal cell compartment in castrated mice (Fig. 1F). Results were confirmed by relative quantification of Sparc transcript levels on stromal or luminal cells that had been sorted by FACS from the prostates of untreated or castrated TRAMP mice (Fig. 1G and Fig. S1C-D). Data so far suggest that the absence of SPARC in stromal cells is associated with NED

**SPARC-null stroma induces NED via IL-6**

In order to better understand the source and role of SPARC in NED, we co-cultured prostate adenocarcinoma cells with SPARC-sufficient or deficient immortalized fibroblasts (Fig S1E-F) under transwell physical separation. We used two different TRAMP-derived adenocarcinoma cell lines: T1525, a well-differentiated adenocarcinoma that does not express endogenous SPARC, and T23, a poorly differentiated adenocarcinoma (26) that produces and secretes high levels of SPARC (Fig. S1E-F). In co-cultures, SPARC-proficient fibroblasts did not alter tumor cell phenotypes, but tumor cells co-cultured with SPARC-deficient fibroblasts acquired Syp and retained Ck8 and Ar expression (Fig. 2A and 2B). Immunofluorescence confirmed the upregulation of SYP in these cells at the protein level (Fig. 2C-F and Fig. S2A-B) and the maintained expression of AR (Fig. S2C). To better mimic the tumor microenvironment, we cultured adenocarcinoma cells with stromal or myeloid cells isolated from the prostates of TRAMP or Sparc−/−TRAMP mice. T1525 cells
increased SYP expression when in the presence of either stromal or myeloid cells from Sparc−/− TRAMP mice (Fig. S2D-F). These data indicate that SPARC-deficient fibroblasts, and myeloid cells can induce NED of prostate adenocarcinoma cells, regardless of whether or not SPARC is produced by the tumor.

We tested whether IL-6 was produced in our co-cultures since (i) loss of SPARC correlates with IL-6 production in bladder cancer-associated fibroblasts (39), (ii) IL-6 can induce NED (14), and (ii) T1525 and T23 tumor cells express the IL-6 receptor (IL-6R/CD126) and its co-receptor CD130 (Fig. S3A). ELISA detected IL-6 in the supernatant of cultures containing SPARC-deficient fibroblasts either alone or with T1525 or T23 cells, but not in cultures containing SPARC-proficient fibroblasts, regardless of the presence of tumor cells (Fig. 2G). Real time PCR for Il6 transcript levels in fibroblasts and tumor cells, collected separately because of transwell segregation, confirmed that SPARC-deficient fibroblasts were the only source of IL-6 (Fig. 2H). In line with published evidence (39), IL-6 production correlated with constitutive activation of the canonical and non-canonical NF-κB pathway in SPARC-deficient fibroblasts, here detected as an increase in IkBα and p52 (Fig. S3B-C).

Retroviral-mediated Sparc-replacement in Sparc-null fibroblasts strongly reduced Il6 expression (Fig. S3D). Conversely, transient Sparc down-regulation induced by siRNA resulted in increased Il6 expression in wild type fibroblasts (Fig. S3E). Finally, SYP expression (associated with NED) by T1525 and T23 cells that were co-cultured with SPARC-deficient fibroblasts was abolished when a blocking antibody to IL-6R was added to the co-culture (Fig. 2I-L Fig. S3F and Fig S4A-B). Specificity of the blocking antibody was confirmed in vitro for its capacity to reduce phosphorylation of STAT3 (pSTAT3) in tumor cells stimulated with IL-6 (Fig. S3G).
We then subcutaneously injected SPARC-proficient T23 adenocarcinoma cells into SPARC competent or deficient syngeneic C57BL/6 mice, to determine if host SPARC expression affected tumor phenotype. We found no apparent effect on growth rate (Fig. S5A). Tumors that developed in Sparc<sup>−/−</sup> hosts acquired SYP expression, and this acquisition was blocked if mice were treated with anti-IL-6R antibody (Fig. 2M-N and Fig. S5C). Histologically, wild type C57BL/6 mice developed high-grade tumors, with sarcomatoid (Fig. S5B, black arrows) and epithelioid elements (Fig. S5B, red arrows). The latter appeared to be replaced by small-size cells (Fig. S5B, cyan arrows) in tumors from Sparc<sup>−/−</sup> mice, either untreated or treated with isotype control. This phenotypic change was reduced in Sparc<sup>−/−</sup> mice treated with anti-IL-6R antibody whose tumors retained marked epithelioid features (Fig. S5B, red arrows). Also, the expression of pSTAT3 was significantly increased in tumors from Sparc<sup>−/−</sup> mice, in comparison to tumors from wild type C57BL/6 and anti-IL-6R antibody-treated Sparc<sup>−/−</sup> mice (Fig. S5D-E). Similarly, tumors generated by RM1, a murine prostate carcinoma not related to the TRAMP mouse (29), acquired SYP expression only when grown in isotype treated Sparc<sup>−/−</sup> recipients, and not when the latter were treated with anti-IL-6R antibody (Fig. S6A-B). Histopathology indicated highly undifferentiated and EMT-like features of RM1 tumors grown in wild type mice. In Sparc<sup>−/−</sup> mice RM1 tumors had a contrasting appearance of a syncytial pattern with apoptotic and mitotic figures, compatible with an anaplastic NE phenotype, whereas in Sparc<sup>−/−</sup> mice treated with anti-IL-6R antibody tumors gained epithelioid features (Fig. S6C).

Together these results show that stroma defective in SPARC expression can promote the adenocarcinoma-NE transition through IL-6 release. Since castration induces stromal SPARC down-regulation in the prostates of TRAMP mice (Fig. 1F-G), we
tested their IL-6 production. RNAscope (ACDbio), which enables the analysis of RNA expression on FFPE tissues, detected high Il6 positivity in stromal cells of prostates of castrated TRAMP (Fig. 3A-B) and Sparc−/−TRAMP mice (Fig. S6D), and not in untreated TRAMP mice (Fig. 3A-B). Il6 positive stromal cells co-expressed the fibroblast marker PDGFRβ (Fig. S6E), and were adjacent to SYP-positive tumor cells (Fig. S6F). Real time PCR on luminal and stromal cells, that had been FACS-sorted from prostates of TRAMP mice, confirmed no Il6 expression in the luminal compartment, and its upregulation in the stroma after castration (Fig. 3C). Both luminal and stromal cells of TRAMP mice, either castrated or not, expressed IL-6R (Fig. 3D and Fig. S6G). Consistently, anti- IL-6R antibody reduced the frequency of NED in castrated TRAMP mice (16.7% of anti-IL-6R treated versus 35.7% of untreated castrated mice (Fig. 3E and Fig. S6H). These data support the proposal that castration-induced down-regulation of SPARC expression in tumor-infiltrating stromal cells is responsible for their secretion of IL-6, the final mediator of NED.

**Tumor cells trigger stromal SPARC down regulation**

We wanted to identify the mechanism that causes stromal SPARC down-regulation in response to castration (Fig. 4A-E and Fig. S7A-I). Fibroblasts and immune cells infiltrating prostate cancer and fibroblasts used in our in vitro experiments express AR (ref (40) and Fig. S7A-B). However, in vitro treatment with the AR inhibitor enzalutamide or co-culture with tumor cells alone did not alter SPARC levels in wild type fibroblasts. Notably, in culture conditions combining tumor cells, fibroblasts and enzalutamide, fibroblasts down-regulated SPARC (Fig. 4A-C), and up-regulated IL6 (Fig. 4D-E). This suggested a tumor-stroma crosstalk, activated by AR inhibition, as responsible for SPARC down-regulation and consequent IL6 production by stroma.
Both steps are necessary to induce NED in tumor cells. According to this hypothesis, T23 cells did not acquire the expression of the NE markers SYP and CgA after treatment with enzalutamide, unless wild type fibroblasts (Fig. S8A-D) or exogenous IL-6 (Fig. S9A-D) were also added in the culture. Notably, IL-6 alone was sufficient to induce upregulation of NE markers, albeit at a lower extent (Fig S9A-D). T23 cells did not autonomously produce Il6 upon enzalutamide treatment (Fig S9E), highlighting the need of fibroblasts as a source of IL-6 in our model.

The expression of SPARC can be dampened by miR-29b, which targets the Sparc transcript (41). Notably, miR-29b can be up-regulated through the activation of the Wnt/β-catenin pathway (42), which is often altered in prostate cancer and associated with castration resistance (43). Moreover, RNA-seq data on paired prostate cancer samples collected from patients before and after ADT showed up-regulation of the Wnt/β-catenin pathway in response to treatment (44). Both T1525 and T23 cells treated with enzalutamide up-regulated β-catenin (Fig S7C-E) and miR-29b1 (Fig. 5A), which was also released into their supernatants (Fig. S7F). In these supernatants, miR-29b1 was encapsulated in exosomes (Fig 5B and S7G). The same supernatants caused SPARC down-regulation in fibroblasts, but not if fibroblasts were pre-transfected with an anti-miR specific for miR-29b (Fig. 5C-E). Furthermore, we found that intracellular levels of miR-29b1 increased in fibroblasts incubated with supernatants from T23 cells treated with enzalutamide. Previous transfection of T23 cells with an anti–miR specific for miR-29b abolished this effect (Fig 5F and S7H). These data indicate T23 cells can release miR-29b encapsulated in exosomes; miR-29b is then transferred to fibroblasts. The specificity of miR-29b for Sparc in fibroblasts was confirmed by transfecting them with a vector containing two luciferase reporter genes followed by the 3’UTR region of Sparc, in presence or not
of miRNA mimic specific for miR-29b-3p or miR-29b-5p. The mimic specific for miR-29b-3p reduced luciferase activity, confirming its targeting Sparc expression in fibroblasts (Fig. 5G).

We confirmed the up-regulation of β-catenin (Fig. S7I) and miR-29b1 (Fig. 5H) in luminal cells FACS sorted from the prostates of castrated TRAMP mice versus non-castrated controls. We also confirmed miR-29b1 up-regulation in tumor cells in FFPE prostate samples of castrated TRAMP mice, utilizing the BASEscope Assay (ACDBio; Fig. 5I-J). A plausible explanation for these results is that inhibition of AR signaling leads to activation of the β-catenin pathway that up-regulates miR-29b in tumor cells. miR-29b is then transferred in neighboring stromal cells, in which it causes down-regulation of SPARC; this event consequently induces IL-6 production, leading to NED of tumor cells.

**Drug to GRP78 blocks SPARC down-regulation and NED**

β-catenin signaling can be promoted by GRP78 (45), a protein involved in the unfolded protein response and regulation of cancer cell survival (46). GRP78 is also up-regulated in prostate cancer in correlation with CRPC (47) and NE features (48). We found increased levels of GRP78 in luminal cells of castrated TRAMP mice (Fig. 6A-C and Fig. S10A) and in T1525 or T23 cells treated with enzalutamide (Fig. 6D-E and Fig. S10B-C). Fibroblasts expressed only low levels of GRP78, not further increased by enzalutamide (Fig. S11A-B).

We hypothesized that the up-regulation of GRP78 in tumor cells in response to enzalutamide induces the production of miR-29b, through stimulation of β-catenin activity. Therefore, blocking GPR78 could prevent the pathway, here described, that leads to NED. We then tested the efficacy of isoliquiritigenin, a natural compound...
that inhibits the activity of GRP78 in stimulating the β-catenin pathway (45). Expression of miR-29b1 in enzalutamide-treated T1525 and T23 tumor cells was reduced by co-treatment with isoliquiritigenin (Fig. 6F). We found a significant reduction of miR-29b production and secretion also in enzalutamide-treated T23 cells transfected with a siRNA specific for GRP78, or co-treated with HA15 a specific chemical inhibitor of GRP78 (Fig. S11C-D). Furthermore, Sparc down-regulation that occurred in fibroblasts cultured with tumor cells in the presence of enzalutamide was inhibited by the addition of isoliquiritigenin (Fig. 6G-I).

As shown above (Fig. S8), tumor cells cultured with enzalutamide expressed SYP and CgA only when fibroblasts were present in the culture, indicating that NED of adenocarcinoma cells required tumor-stroma crosstalk. The inhibition of GRP78 activity with isoliquiritigenin prevented SYP upregulation in tumor cells in this setting (Fig. 6J-K, Fig. S11E-G and Fig. S12A-D). As a final proof of our proposed mechanism, NED was reduced in castrated TRAMP mice treated with isoliquiritigenin (1/13 mice with isoliquiritigenin vs 5/14 untreated mice; Fig. 6L and Fig. S11H). Notably, immunohistochemistry on prostate sections and real-time PCR on FACS-sorted cells showed that castrated mice treated with isoliquiritigenin did not down modulate stromal SPARC (Fig. 6M-N and Fig. S11I).

In summary, these results indicate that ADT up-regulates GRP78 expression, which triggers miR-29b upregulation and tumor-stroma crosstalk leading to down-modulation of stromal SPARC to mediate NED of prostate cancer cells (Graphical Abstract).

To gain further molecular insights on NED occurring via crosstalk with stroma cells we performed RNAseq on T23 adenocarcinoma cells, in different co-culture conditions, as for Fig. 2I-L or Fig. 6J-K. When cultured with Sparc+ fibroblasts, T23
cells up-regulated genes involved in the IL-6 pathway, in pathways related to NEPC (i.e. HIF-1 (12), WNT (13), IGF (49)) and to NE tumors in general, as HGF receptor (50). In the same condition, T23 cells down-regulated genes related to AR and Notch signaling, the latter needed down-regulated to promote NED (51) (Fig. S13A and Table S2). Similarly, when cultured with wild type fibroblasts and enzalutamide T23 cells up-regulated genes related to IL-6, β-catenin, tumor stroma interaction and neurogenesis, and known pathways related to NEPC such as Ezh2 (8) and sonic-hedgehog (52), whereas they down-regulated genes related to Notch and YAP-TAZ pathway (Fig. 13A and Table S2). Indeed, it is known that AR and YAP can co-localize in the nucleus of tumor cells and down-regulation of YAP results in down-regulation of AR-target genes (53). Adding isoliquiritigenin to the culture between T23 and wild type fibroblasts, in presence of enzalutamide, reverted most of the enzalutamide effects, causing in T23 cells the upregulation of transcription targets of AR and the down-regulation of NE-related pathways such as HIF-1, IGF and hedgehog and pathways related to general neurogenesis (Fig. S13B and Table S2). Results indicate that blocking GRP78 activity with isoliquiritigenin can restrain, at least in part, the NED program triggered by the interaction between tumor and stroma in response to enzalutamide.

**GRP78 correlates with NED in human prostate cancer**

We replicated our *in vitro* experiments with mouse cells with the human cell lines LNCaP and WPMY-1, which are representative of prostate cancer and prostate stroma, respectively. As predicted by our mouse studies, enzalutamide caused the upregulation of GRP78, β-catenin and miR-29b1, but not of IL-6 in LNCaP cells (Fig. 7A-E and Fig. S14A-B). GRP78 expression in human fibroblasts was negligible and
unaffected by enzalutamide (Fig. S14C-D). Addition of enzalutamide to co-cultures of the two human cell lines caused SPARC down-regulation in WPMY-1 cells (Fig. 7F-H) and up-regulation of the NE markers CgA and Eno2 in LNCaP cells (Fig. 7I and S14E-F). In LNCaP cells cultured alone, upregulation of CgA and SYP was caused by stimulation with exogenous IL-6, and further increased by concomitant addiction of enzalutamide (Fig. S15A-D). Finally, in co-cultures in the presence of enzalutamide, isoliquiritigenin increased SPARC expression in fibroblasts and decreased CgA and Eno2 expression in tumor cells (Fig. 7I and S14E-F), confirming the role of GRP78 in driving NED in LNCaP cells.

To add translational relevance to our data, we interrogated publicly available data sets of prostate cancer patients through the cBioPortal tool (33,34). Copy number alterations and mutation data were available for the Kumar data set (36), which included 156 CRPC and 20 NEPC samples, however with no available clinical information to distinguish the two subtypes. Nevertheless, in this data set we found an increased frequency of mutations in Hspa5 (the gene encoding for GRP78) in comparison with data sets including only CRPC patients (SU2C/PCF data set, ref (35)) or patients with primary adenocarcinoma (TCGA). Furthermore, we found that Hspa5 is strongly amplified in CRPC/NEPC patients (Fig. 7J). We then focused on the Beltran data set of NEPC (6), finding an increase, albeit not statistically significant, of mRNA levels of Hspa5 in NEPC compared to CRPC patients (Fig. S16A). In this data set, we further analyzed genes correlated with Hspa5 mRNA expression (Table S3), finding its positive correlation with two genes, Akt1 and Cdk5, involved in the NED promoting activity mediated by MCYN (8,54) (Fig. 7K), and with pathways related to FOXO transcription factors, AURKA, DNA damage response, and down-regulation of cyclin D1 and of Notch signaling, all hallmarks of
NEPC (6,12,51,55,56) (Fig. S16B-C). These analyses further highlight GRP78 as a possible therapeutic target to prevent NED of prostate cancer.

**SPARC down-regulation relates to NED in patients**

Additional translational relevance of our results comes from the analysis of paired FFPE specimens from prostate core-biopsies (pre-ADT) and radical prostatectomies (post-ADT) of eight prostate cancer patients (Pt) who underwent neo-adjuvant ADT (Fig.8). We also analyzed prostatectomies from six untreated prostate cancer patients (Fig. S17A-B). All patients had comparable Gleason scores (Gleason Grade Group 2 or 3; Fig. S18A). Immunofluorescence revealed low to intermediate levels of GRP78 in tumor cells in untreated patients (UntrPt; Fig. S17A, Fig. S18B, S19A-B and Fig. S20A-B) and in pre-ADT core-biopsies (Fig. 8A, Fig. S18B and Fig. S19A-B). GRP78 was up-regulated in the majority of prostates collected after ADT, excluding Pt#5 and Pt#14 (Fig. 8B, Fig. S18B and Fig. S19C-D). Conversely, SPARC was highly expressed by tumor and stroma cells in prostatectomies from untreated patients (Fig. S17B and Fig. S18B-C) and in core-biopsies pre-ADT, except for Pt#14 (Fig. 8A and Fig. S18B-C), and variably down-modulated in all patients after ADT (Fig. 8B and Fig. S18B-C). Besides digital quantification (Fig. S18B), examination by board-certified pathologists identified differences between the expression of SPARC in tumor and stroma with the latter being the primary source of SPARC in the crosstalk we are describing. They assigned empirical scores (0 to 3) for distinct SPARC expression in stroma and tumor cells (Fig. S18C). This enabled us to quantify the evidence in the IHC images (Fig. 8A-B) that showed that all patients down-modulated SPARC in stroma cells after ADT, except Pt#5 and Pt#14. Notably, in these two patients the levels of GRP78 had not increased after ADT (Fig. 8A, Fig.
S18B and Fig. S19), in line with its presumed upstream role in the network regulating SPARC down-regulation.

We further used IHC to evaluate the expression of AR, SYP and CgA on prostatectomies. All patients’ tumor samples showed mild to high AR staining, except for Pt#3, UntrPt#7 and UntrPt#8. All specimens from control untreated patients and prostatectomies collected after ADT from Pt#1, Pt#3, Pt#5 and Pt#14 showed no expression of the NE markers SYP and CgA in tumor cells. Curiously, Pt#5 showed SYP positivity in stromal but not tumor cells. Tumor cells in Pt#4 and Pt#13 showed mild SYP and CgA upregulation only in a few scattered cells. On the contrary, in Pt#2 and Pt#6, we found a mild-moderate staining for SYP and a strong positivity for CgA in tumor cells and only in the areas showing stromal SPARC down-regulation (Fig. 8B Fig. S17B and Fig. S18B). Results demonstrated that stromal SPARC down-regulation in tumor microenvironment occurs also in prostate cancer patients after ADT (in 6/8 patients, 75%), and that this event correlated with focal NED of adenocarcinoma cells. Indeed, we detected clear positivity of NE markers in 2/6 patients that modulated GRP78 and SPARC (corresponding to 33% of them, and to 25% of the total of 8 patients analyzed). Two additional patients showed scattered upregulation of NE markers in correlation with stromal SPARC loss. These results confirm the correlation between tumor GRP78 gain, stroma SPARC down-regulation and NED, following ADT, in human patients and support the translational relevance of the mechanism we here detailed. Results cannot exclude the existence of additional microenvironment-related mechanisms, to be further investigated, that can drive or prevent NED.
DISCUSSION

NED of CRPC occurs as a mechanism of resistance to ADT/ARPI and remains an obstacle to effective therapies. Cellular plasticity permits NED with the complicity of tumor microenvironment that provides the necessary paracrine factors (13,15,16). Nevertheless, the molecular pathways activated by ADT that induce stromal cells to release NED-promoting molecules are undefined. Here, we unveil that castration triggers a tumor-stroma crosstalk leading to stromal SPARC down-regulation, that we found to be a crucial step for NED of prostate cancer cells (Graphical Abstract).

Either genetic inactivation of SPARC or its castration-induced down-regulation led to increased occurrence of NED within adenocarcinoma in TRAMP mice, but the frequency of tumors with features mirroring de novo small-cell NEPC remained unaltered. The cellular origin of de novo NEPC is still debated (37,57) and out of the scope of this manuscript. On the contrary, it is widely accepted that NED of prostate adenocarcinoma results from a process of lineage plasticity triggered by ADT/ARPI (6,7,13). The final outcome of such plasticity is associated with the loss of AR expression and AR-related transcriptional activity but the gain of NE features. However, the timing and molecular steps of this transition are not well understood (30). “Hybrid tumors” expressing AR (and related genes) and NE markers, as well as “double negative” tumors, can be different transition steps of this process (30). Notably, it has recently been shown that patients with treatment-related NEPC have low expression of AR-regulated genes despite nuclear AR positivity (5). Similarly, in our small set of patients, we found expression of NE markers in post-ADT samples in the presence of nuclear AR staining. Also, in our system, prostate cancer cells up-

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regulated NE markers and down-regulated AR-target genes despite persistent AR expression when cultured with fibroblasts.

The molecular steps leading to NED detailed here reinforce the fundamental role of stromal accessory cells in modulating tumor cell plasticity in response to therapy. We show that SPARC produced by stromal cells needs to be down-regulated to allow NED of tumor cells, and we excluded any contribution of SPARC released by tumor cells in this process. This suggests that SPARC may have intracellular functions, not yet described, in regulating the production of cytokines in response to external signals. It was recently described that, in the cytoplasm of colorectal cancer cells, physical interaction between SPARC and GRP78 results in increased apoptosis in response to endoplasmic reticulum stress (58). Our data point out an inverse correlation between these two players when expressed on different cells within the tumor microenvironment. Indeed, we show that up-regulation of GRP78 in tumor cells is the first step of a network that leads to down-regulation of SPARC in stromal cells and consequent IL-6 release. The fine mechanism that explains how SPARC can limit IL-6 production remains elusive and will be investigated in future studies. Consistent with published data (39), we found a constitutive, mild up-regulation of the NF-κB pathway, which promotes IL-6 production, in Sparc−/− fibroblasts. Being SPARC a chaperone protein, it could be conceivable that it could sequester members of the NF-κB pathway, preventing its activation. An alternative explanation could be that intracellular SPARC could influence the expression of AR, which in cancer-associated fibroblasts actively represses the transcription of key effector genes, including Il6 (59). In support of this second hypothesis, we found that AR was down regulated in Sparc−/− fibroblasts.
The molecular findings that we detailed here in preclinical models are corroborated by *in silico* analyses of human data sets, showing that the *Hspa5* gene, encoding for GRP78, positively correlates with genes involved in key pathways related to NED. The role of the gain of GRP78 expression and loss of stromal-SPARC expression in driving NED was further validated analyzing tumor tissues of patients collected before and after ADT. Despite the shortness of the cell exposure to the ADT in the neo-adjuvant setting, we detected evidence of NED in two of them (corresponding to 25% of total patients and the 33% of those who down-regulated SPARC). Although we analyzed only a few cases, this frequency is in the range of those reported in the literature for treatment-related NEPC (5,60). The relatively low number of tissues that we collected post-ADT is due to the common clinical practice in Italy that spares patients with advanced/metastatic prostate cancer from biopsies, in favor of non-invasive imaging techniques for follow-up. As ARPI in neo-adjuvant settings is now being tested in clinical trials (61), our data indicate that evaluating NED as a possible drawback of these treatments could be informative and could provide a rationale for Italian physicians to collect biopsies from metastatic sites in patients at risk of NED to facilitate diagnosis and inform therapy.

Although the role of IL-6 in cellular plasticity and NED in prostate cancer is established (13), clinical trials attempting to modulate the IL-6 signaling pathway have not shown significant efficacy in CRPC (62). Our data suggest the possibility of future testing of SPARC down-regulation in stromal cells in CRPC patients who relapse after ADT, as a biomarker to identify patients who are likely undergoing NED and who could benefit from anti-IL-6 therapy in combination with ARPI.

A different, conceivable, therapeutic approach could include the concomitant use of GRP78 inhibitors and ADT/ARPI. This is based on our demonstration that (i) GRP78
is a triggering factor of the newly identified crosstalk between tumor and stromal cells that is activated in response to ADT and (ii) pharmacologic targeting of GRP78 with isoliquiritigenin prevents NED in castrated TRAMP mice. We show that inhibition of GRP78 prevents the production of miR-29b in enzalutamide-treated tumor cells, and consequently SPARC down-regulation in fibroblasts. We hypothesize that GRP78 could promote the expression of miR-29b by fostering the expression/activity of β-catenin, because (i) miR-29b expression can be promoted by the β-catenin pathway (42) and (ii) isoliquiritigenin can prevent the binding of GRP78 to β-catenin, which consequently is no more protected from proteasome degradation (45).

Isoliquiritigenin showed in vitro activity against prostate cancer cells (63), but it has not been tested relative to NED nor tested in cancer patients to date. Several other drugs that block GRP78 are under preclinical or clinical evaluation (46). The MAb159 antibody against cell-surface GRP78 demonstrated anti-tumor activity in the PTEN-null mouse model of prostate cancer, and the PAT-SM6 antibody is being clinically tested in patients with multiple myeloma. The ruthenium-based drug, NKP-1339, against GRP78 was tested in phase I with different solid tumors, but prostate, and showed a manageable safety profile but limited anti-tumor activity (46) which might suggest that it should be further tested in combination therapies.

In conclusion, we have unveiled tumor-stroma crosstalk that is triggered by ADT that is responsible for the adenocarcinoma-NE transition in prostate cancer. Additive/alternative mechanisms of NED might co-exist, as suggested by our in vivo data showing further increase of NED in SPARC-deficient TRAMP mice subjected to castration, and in vitro data showing that NED caused by administration of exogenous IL-6 to tumor cells was slightly increased by co-administration of enzalutamide. These mechanisms could be cell autonomous or mediated by microenvironment...
signals. Since only roughly one third of mice and patients that/who experience down-regulation of stromal-derived SPARC have evidence of NED, we also hypothesize that other mechanisms exist that can counteract NED or have different threshold levels that enable the activation of pathways described in this paper. Inter- and intra-patient heterogeneity of tumors and stroma may be based in different pathways toward NED and should be considered when translating our results to the clinic.

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REFERENCES


FIGURE LEGENDS

Figure 1. NED similarly occurs in SPARC-deficient or castrated TRAMP mice. 
A. Percentage of prostatic lesions scored as adenocarcinoma (ADENO), neuroendocrine differentiation (NED), de novo neuroendocrine (NE), or regression in 30 week old TRAMP (n = 13), Sparc-/-TRAMP mice (n = 13), TRAMP mice subjected to surgical castration (n = 14) and Sparc-/-TRAMP mice subjected to surgical castration (n = 15). Fisher’s test: **** P < 0.0001, * P < 0.05. B -E. Representative images of prostates of mice affected by adenocarcinoma, NED or de novo NE. Hematoxylin and eosin (B); immunofluorescence staining for CK8 (green), SYP (red) and DAPI (blue) (C); immunohistochemistry for CgA (D); immunofluorescence staining for CK8 (green), CgA (red) and DAPI (blue) (E). F. Representative immunohistochemistry for SPARC in prostates of TRAMP and castrated TRAMP mice. Red and black arrows highlight stromal and tumor cells, respectively. G. Real time PCR for Sparc on stromal and luminal cells sorted from the prostates of untreated and castrated TRAMP mice. Histograms depict mean ± s.d. of biological replicates (indicated by dots, n=3/group). One-Way Anova followed by Tukey’s test: ** P < 0.01. B, D and F. Top panels magnification x200, bottom panels magnification x400 of the same areas.

Figure 2. SPARC-deficient fibroblasts mediate NED of adenocarcinoma cells via IL-6. A-H. T1525 or T23 prostate adenocarcinoma cell lines were co-cultured with wild type (Fibro) or SPARC-deficient (Fibro Sparc-/-) fibroblasts. Cells were divided by a 0.4 µm pore transwell and analyzed after 7 days of culture. A and B. Real time PCR for Ar, Syp and CK8 on tumor cells. C and D. Representative immunofluorescence for SYP (red) on tumor cells. Blue: DAPI. E and F.
Quantification of immunofluorescence in panel C and D. **G.** ELISA for IL-6 on cell culture supernatants. **H.** Real time PCR for *IL-6* on tumor cells (black bars) or fibroblasts (white bars). **A-H.** Data are a pool of three independent experiments. Legends indicate the cell type plated on the bottom of the well, and further analyzed, and the cell type plated in the transwell insert is reported in the brackets. **I and J.** T1525 (I) or T23 (J) cells were cultured as in (A) with *Sparc-/-* fibroblasts, in presence or not of anti-IL6 receptor antibody (αIL6R). SYP (red) was analyzed by immunofluorescence. Blue: DAPI. Experiment was repeated three times. **K.** Quantification of immunofluorescence in panel I. **L.** Quantification of immunofluorescence in panel L. **M.** Representative immunofluorescence (red: SYP, blue: DAPI) of T23-derived tumors grown in wild type (B6) or *Sparc-/-* mice, treated or not with αIL6R or isotype control as indicated. Experiment was repeated two times, each with 3 mice/group, with comparable results. One of the two experiments is shown. **N.** Quantification of immunofluorescence in panel M.

All histograms depict mean ± s.d of biological replicates (represented by dots). One-Way Anova followed by Tukey’s test: * * * * P < 0.0001.

**Figure 3.** Blocking IL-6 receptor inhibits NED in castrated TRAMP mice. **A.** Representative pictures showing *Il6* evaluation by RNAscope on prostate tissues of TRAMP and castrated TRAMP mice. Black arrows highlight positive cells. Top panels magnification x200, bottom panels magnification x400 of the same areas. **B.** Quantification of staining in panel 3A and in panel S6D. Histogram depicts mean ± s.d. of biological replicates (represented by dots; n=4/group). Anova followed by Tukey’s test: **** P < 0.0001 **C.** Real time PCR for *Il6* on stromal and luminal cells...
sorted from the prostates of untreated and castrated TRAMP mice. Histogram depicts mean ± s.d. of biological replicates (represented by dots, n=3/group). Anova followed by Tukey’s test: ** P < 0.01. D. Flow cytometry evaluation of IL-6R (CD126) and CD130 on stromal and luminal cells in prostates of TRAMP and castrated TRAMP mice. E. Relative percentage of prostatic lesions, scored as in Fig.1, in 30 weeks old TRAMP mice subjected to surgical castration at 20 weeks of age and left untreated (n = 14; the same cohort reported in Fig. 1A) or treated weekly with αIL6R (n = 12). Fisher’s test: * P < 0.05.

**Figure 4. Interaction with tumor cells causes SPARC down-regulation in fibroblasts.** Fibroblasts were co-cultured in 0.4 μm pore transwell system with T1525 or T23 cells (indicated in brackets) and treated or not with enzalutamide (ENZA). A and B. After 48h we measured SPARC expression in fibroblasts by real-time PCR (A) and western blot (B). C. Quantification of B. The western blot was validated twice D and E. We also measured Il6 transcript in fibroblasts (D) and protein by ELISA on culture supernatants collected after 72h (E). A/D/E: Data are a pool of three independent experiments. In all panels histograms depict mean ± s.d. of biological replicates (represented by dots). One-Way Anova followed by Tukey’s test or Two-tailed Student’s t test: * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

**Figure 5. miR29b upregulation by enzalutamide-treated tumor cells causes SPARC down regulation in fibroblasts.** A. Real time PCR for miR29b1 in T1525 or T23 cells, treated or not with enzalutamide (ENZA) for 24h. B. Real time PCR for miR29b1 in exosomes isolated from supernatants of T23 cells, treated as in A.
Experiment was repeated twice. C-E. Fibroblasts were incubated for 48h with supernatants (ratio 1:1 with fresh DMEM) collected from either untreated or enzalutamide treated T1525 cells (as shown in brackets). Where indicated we previously transfected fibroblasts with an anti-miR specific for miR29b (anti-miR29b), or with an unspecific sequence as negative control (scramble). We measured SPARC by real time PCR (C) or western blot (D-E). Vinculin was used as internal control. The western blot was validated twice. F. Fibroblasts were incubated with supernatants of enzalutamide (ENZA) – treated T23 cells that had been transfected with a negative control (scramble) or with anti-miR specific for miR29b. Histogram shows real time PCR for miR29b1 in recipient fibroblasts. G. Histogram shows luciferase activity of fibroblasts that were transfected with a vector containing two luciferase reporter genes followed by the 3’UTR region of Sparc. Transfection was made in presence or not of miRNA mimic specific for miR29b-3p or miR29b-5p. A-C and F-G. Data are a pool of two independent experiments. H. Real time PCR for miR29b1 on stromal and luminal cells sorted from the prostates of untreated and castrated TRAMP mice (n= 3 biological replicates/group). I. Representative pictures showing miR29b1 evaluation by BaseScope on prostate tissues of TRAMP and castrated TRAMP mice. Black arrows highlight positive cells. J. Quantification of staining in panel H (n=4/group).

In all panels histograms depict mean ± s.d. of biological replicates (represented by dots). One-Way Anova followed by Tukey’s test or Two-tailed Student’s t test: * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

Figure 6. GRP78 inhibition reduces NED in castrated TRAMP mice. A. Representative immunofluorescence for GRP78 (red), CK8 (green) and DAPI (blue)
on prostate tissues of TRAMP and castrated TRAMP mice. Top panels magnification x200, bottom panels magnification x400 of the same areas. **B.** Quantification of staining in panel A. Dots indicate biological replicates (n=3/group). **C.** Real time PCR for **Hspa5** (GRP78) on luminal cells sorted from the prostates of untreated and castrated TRAMP mice. Dots indicate biological replicates (n=3/group). **D.** Representative immunofluorescence for GRP78 (red) in T1525 or T23 tumor cells treated for 24h with enzalutamide (ENZA). Blue: DAPI. **E.** Quantification of staining in panel D. **F.** Real time PCR for **miR29b1** in T1525 or T23 cells, treated with enzalutamide (ENZA), isoliquiritigenin (ISO) or their combination for 24h. **G.** Real time PCR for **Sparc** on fibroblasts co-cultured with T23 cells (indicated in brackets) and treated with ENZA, ISO or their combination. Cells were analyzed after 48h of culture. **H** Western blot for **SPARC** in fibroblasts treated as in G. Vinculin was used as internal control. The western blot was validated twice. **I.** Quantification of G. **J.** T23 cells were co-cultured in transwell system with fibroblasts (fibro, indicated in brackets) as in Fig.2, also adding enzalutamide (ENZA), isoliquiritigenin (ISO) or their combination. Pictures show representative immunofluorescence for SYP (red) on tumor cells. Blue: DAPI. **K** Quantification of staining in panel J. **F/G/K.** Data are a pool of at least two independent experiments. **L.** Relative percentage of prostatic lesions, scored as in Fig1, in 30 weeks old TRAMP mice subjected to surgical castration at 20 weeks of age and left untreated (n = 14; the same cohort reported in Fig. 1a) or treated weekly with isoliquiritigenin (ISO; n = 13). Fisher’s test: *** P < 0.001. **M.** Quantification of staining in panel N. Biological replicates are indicated by dots (n=4/group). **N.** Representative immunohistochemistry for SPARC on prostate of untreated TRAMP and castrated TRAMP mice, either untreated or not with ISO. Red arrows highlight stromal cells.
In all panels, histograms depict mean ± s.d. of biological replicates (represented by dots). Anova followed by Tukey’s test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

**Figure 7. The tumor-GRP78/stroma-SPARC network in human NE prostate cancer.**

**A.** Representative immunofluorescence for GRP78 (red) in LNCaP tumor cells treated for 24h with enzalutamide (ENZA). Blue: DAPI. **B.** Quantification of staining in panel A. Experiment was repeated two times. **C-D.** Real time PCR for β-catenin (**Ctnbb1** gene; panel D) and **miR29b1** (panel E) in LNCaP cells treated with enzalutamide (ENZA) for 8h or 24h, respectively. **E.** ELISA for IL-6 in LNCaP cells treated with for 24h with enzalutamide (ENZA), alone or in co-culture with human prostatic fibroblasts (the WPMY-1 cell line, indicated as Human Fibro). **F.** Real time PCR for **Sparc** in Human Fibro co-cultured or not with LNCaP cells (indicated in brackets) and treated with enzalutamide (ENZA), isoliquiritigenin (ISO) or their combination. Cells were analyzed after 48h of culture. **G.** Western blot for SPARC in human fibro cultured as in F. Vinculin was used as internal control. The western blot was validated twice. **H.** Quantification of G. **I.** Real time PCR for **Cga** or **Eno2** on LNCaP cells co-cultured or not with Human Fibro (indicated in brackets) and treated with ENZA, ISO or their combination. Cells were analyzed after 48h of culture. **F and I.** Data are a pool of three independent experiments. In all panels histograms depict mean ± s.d. of biological replicates (represented by dots). Two-tailed Student’s t test or One-Way Anova followed by Tukey’s test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. **J.** Mutation burden for **Hspa5** gene (encoding for GRP78) in human prostate cancer, obtained on cBioportal analyzing data from the Kumar data set (NEPC and CRPC) (36), the SU2C/PCF Dream Team data set of CRPC (35) and
the TCGA. K. Spearman correlation between transcript levels of Hspa5 and Akt1 or Cdk5 in the Beltran data set of NEPC (6).

Figure 8. Stromal SPARC loss after ADT correlates with NED in human prostate cancer patients. Immunofluorescence for GRP78 (red) and DAPI (blue), H&E staining and immunohistochemistry for SPARC, CgA, SYP and AR, in biopsies collected pre treatment (A) and in prostatectomies obtained post treatment (B) from prostate cancer patients undergoing neo-adjuvant ADT. Except for H&E staining (provided at x200 magnification only), for each staining we provide both x200 magnification (top) and x400 magnification of the same area (bottom).
ENRIQUEZ, FIGURE 2
ENRIQUEZ, FIGURE 3
ENRIQUEZ, FIGURE 4
ENRIQUEZ, FIGURE 5
ENRIQUEZ, FIGURE 7
Castration-induced down-regulation of SPARC in stromal cells drives neuroendocrine differentiation of prostate cancer

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