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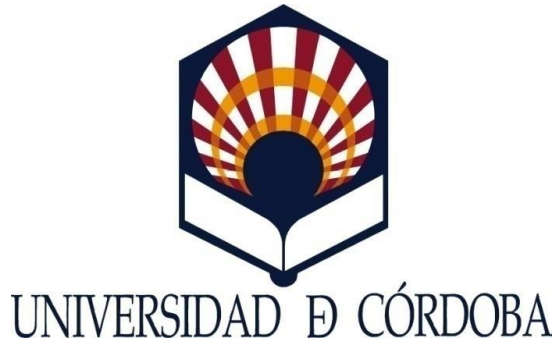

The somatostatin/cortistatin/neuronostatin system and RNA-exosome complex as novel sources for the identification of useful diagnostic, prognostic and therapeutic tools in prostate cancer

El sistema somatostatina/cortistatina/neuronostatina y el complejo ARN-exosoma como fuentes novedosas para la identificación de herramientas diagnósticas, pronósticas y terapéuticas útiles en el cáncer de próstata

Prudencio Sáez Martínez

Córdoba, enero 2024

TITULO: *The somatostatin/cortistatin/neuronostatin system and RNA-exosome complex as novel sources for the identification of useful diagnostic, prognostic and therapeutic tools in prostate cancer*

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The somatostatin/cortistatin/neuronostatin system and RNA-exosome complex as novel sources for the identification of useful diagnostic, prognostic and therapeutic tools in prostate cancer

Memoria de Tesis Doctoral presentada por **Prudencio Sáez Martínez**, Graduado en Biología, para optar al grado de **Doctor en Biomedicina**

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En Córdoba, a 23 de enero de 2024

UNIVERSIDAD DE CÓRDOBA

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D. Raúl Miguel Luque Huertas y D. Manuel David Gahete Ortiz, Catedrático y Profesor Titular, respectivamente, del Departamento de Biología Celular Fisiología e Inmunología de la Universidad de Córdoba,

INFORMAN

Que D. Prudencio Sáez Martínez, Graduado en Biología, ha realizado bajo nuestra dirección el trabajo titulado "**The somatostatin/cortistatin/neuronostatin system and RNA-exosome complex as novel sources for the identification of useful diagnostic, prognostic and therapeutic tools in prostate cancer**" y que, bajo nuestro juicio, reúne los méritos suficientes para optar al Grado de Doctor en Biomedicina.

Y para que conste, firmamos la presente en Córdoba, a 23 de enero de 2024.

Fdo.: Dr. Raúl Miguel Luque Huertas

Fdo.: Manuel David Gahete Ortiz

INFORME RAZONADO DE LAS/LOS DIRECTORAS/ES DE LA TESIS

Este documento se presentará junto con el depósito de la tesis en https://moodle.uco.es/ctp3/

DOCTORANDA/O

Prudencio Sáez Martínez

TÍTULO DE LA TESIS:

El sistema somatostatina/cortistatina/neuronostatina y el complejo ARN-exosoma como fuentes novedosas para la identificación de herramientas diagnósticas, pronósticas y terapéuticas útiles en el cáncer de próstata

INFORME RAZONADO DE LAS/LOS DIRECTORAS/ES DE LA TESIS
(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma)

Durante el desarrollo de la presente Tesis Doctoral, en el periodo comprendido entre octubre de 2018 y enero de 2024, el doctorando Prudencio Sáez Martínez no solo ha superado con creces los objetivos planteados al comienzo de la misma, sino que también ha desarrollado y validado técnicas experimentales de una gran utilidad para el grupo de investigación, que le han permitido obtener resultados muy relevantes en el campo clínico y molecular del cáncer de próstata, lo cual queda patente en diferentes publicaciones como primer autor.

Concretamente, como fruto de su trabajo durante este periodo, ha publicado 3 trabajos directamente relacionados con su Tesis Doctoral en las revistas “Journal of Clinical Medicine” [Cuartil 1 (39/167) del área de “Medicine, Research and Internal”; Factor de impacto: 4.242], “International Journal of Molecular Sciences” [Cuartil 1 (66/285) del área de “Biochemistry and Molecular Biology”; Factor de impacto: 5.6] y “Cancer Letters” [Cuartil 1 (34/241) del área de “Oncology”; Factor de impacto: 9.7], revistas de referencia dentro de nuestras áreas de investigación.

Por último, el doctorando ha presentado sus resultados en diferentes congresos de ámbito nacional e internacional, obteniendo diferentes distinciones y premios y de los que han derivado varios capítulos de libro. Además, actualmente está desarrollando una patente y otras publicaciones.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, a 6 de febrero de 2024

Las/los directoras/es

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Fdo.: Raúl M. Luque y Manuel D. Gahete

Esta Tesis Doctoral ha sido realizada en el Departamento de Biología Celular, Fisiología e Inmunología de la Universidad de Córdoba y en el Instituto Maimónides de Investigación Biomédica de Córdoba (IMIBIC), bajo la dirección de los Dres. Raúl Miguel Luque Huertas y Manuel David Gahete Ortiz. Dicho trabajo fue subvencionado mediante fondos obtenidos de una ayuda de Formación de Profesorado Universitario (FPU) del Ministerio de Educación y Ciencia (FPU17-00263) y de los proyectos/ayudas del MINECO (PID2019-105564RB-I00; PID2022-1381850B-I00), Instituto de Salud Carlos III FIS (PI16/00264; DTS18/00131; DTS20/00050), Junta de Andalucía (BIO-0139), Fundación La Caixa (CAIXAIMPULSE_003), Fundación para la Innovación y la Prospectiva en Salud en España (FIPSE-3188-2017), y del Centro de Investigación Biomédica en Red de la Fisiopatología de la Obesidad y Nutrición (CIBERobn). Durante el transcurso de la presente Tesis Doctoral se ha realizado una estancia de tres meses en el Wellcome Centre for Cell Biology de la Universidad de Edimburgo en Escocia bajo la supervisión del Prof. David Tollervey, financiada por una ayuda de movilidad internacional para estudiantes de FPU del Ministerio de Educación y Ciencia para la realización de estancias destinadas a la obtención de la Mención Internacional en el Título de Doctor de la Universidad de Córdoba, y de la cual ha derivado una estrecha colaboración que será parte de una futura publicación.

List of Publications

This Thesis is based on the research articles listed below, which will be referred in the test by their Roman numerals.

Article I: Sáez-Martínez P, Porcel-Pastrana F, Pérez-Gómez JM, Pedraza-Arévalo S, Gómez-Gómez E, Jiménez-Vacas JM, Gahete MD, Luque RM. Somatostatin, Cortistatin and Their Receptors Exert Antitumor Actions in Androgen-Independent Prostate Cancer Cells: Critical Role of Endogenous Cortistatin. Int J Mol Sci. 2022 Oct 27;23(21):13003. doi: 10.3390/ijms232113003.

[IF: 5.6; 66/285 (Q1) Biochemistry & Molecular Biology (JCR)]

Article II: Sáez-Martínez P, Jiménez-Vacas JM, León-González AJ, Herrero-Aguayo V, Montero-Hidalgo AJ, Gómez-Gómez E, Sánchez-Sánchez R, Requena-Tapia MJ, Castaño JP, Gahete MD, Luque RM. Unleashing the Diagnostic, Prognostic and Therapeutic Potential of the Neuronostatin/GPR107 System in Prostate Cancer. J Clin Med. 2020 Jun 2;9(6):1703. doi: 10.3390/jcm9061703.

[IF: 4.242; 39/167 (Q1) Medicine, Research & Internal (JCR)]

Article III: Sáez-Martínez P, Porcel-Pastrana F, Montero-Hidalgo AJ, Lozano de la Haba S, Sanchez-Sanchez R, González-Serrano T, Gómez-Gómez E, Martínez-Fuentes AJ, Jiménez-Vacas JM, Gahete MD, Luque RM. Dysregulation of RNA-Exosome machinery is directly linked to major cancer hallmarks in prostate cancer: oncogenic role of PABPN1. Cancer Letters. 2024. In Press. https://doi.org/10.1016/j.canlet.2023.216604.

[IF: 9.7; 34/241 (Q1) Oncology (JCR)]

List of abbreviations

4K: Four Kallikreins
AD: Androgen-Dependent
ADT: Androgen Deprivation Therapy
AI: Androgen-Independent
AR: Androgen Receptor
ARv7: Androgen Receptor Variant 7
BPH: Benign Prostatic hyperplasia
CLIA: Clinical Laboratory Improvement Amendments
CORT: Cortistatin
CRPC: Castration-resistant prostate cancer
CT: Computed Tomography
DDR: DNA Damage Response
DHT: Dihidrotestosterone
DRE: Digital Rectal Exam
DSF: Disease-free survival
EBRT: External-Beam Radiation Therapy
ERCs: Endocrine-related Cancers
eRNAs: Enhancer RNAs
FDA: Food and Drug Administration
GHS-R1a: Ghrelin Receptor 1a
GPCRs: G protein-coupled receptors
GPR107: G protein-coupled receptor 107
GS: Gleason Score
hK2: human Kallikrein-like peptidase 2
ISUP: The International Society of Urological Pathology
lincRNAs: Long intergenic RNAs
lncRNAs: Long non-coding RNAs
mCRPC: Metastatic castration-resistant prostate cancer
miRNAs: Micro RNAs
MRGPRX2: MAS-related GPR family member X2
MRI: Magnetic Resonance Imagin
mRNAs: Messenger RNAs
NGD: No-Go Decay
NMD: Non-sense mediated Decay
NSD: Non-Sense Decay
NST: Neuronostatin
PARP: Poly ADP-ribose polymerase
PCA3: Prostate Cancer Antigen 3
PCa: Prostate Cancer
PET: Positron Emission Tomography
PHI: Prostate Health Index
PLND: Pelvic Lymph Node Dissection
PROMPTs: Promoter upstream transcripts
PSA: Prostatic-Specific Antigen
ptRNAs: prematurely terminated RNAs
REC: RNA-Exosome Complex
ROC: Receiver Operating Characteristic
rRNAs: Ribosomal RNAs
snoRNAs: Small nucleolar RNAs
snRNAs: Small nuclear RNAs
SSAs: Somatostatin analogues
SST1-5: Somatostatin receptor 1-5
SST5TMD4: Somatostatin receptor 5 with 4 transmembrane domains splicing isoform
SST5TMD5: Somatostatin receptor 5 with 5 transmembrane domains splicing isoform
SST: Somatostatin
SSTR1-5: Somatostatin receptor gene 1-5
SSTRs: Somatostatin Receptors
TMDs: Transmembrane Domains
tRNAs: Transfer RNAs
tssRNAs: Transcription start site-associated RNAs
TURP: Transurethral resection of the prostate
uaRNAs: Upstream antisense RNAs

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Resumen

El cáncer de próstata (CaP) es una de las patologías tumorales más relevantes para el sistema sanitario, ya que cada año se registran 1.3 millones de nuevos casos y 400,000 muertes asociadas en todo el mundo. Los abordajes clínicos actuales de esta patología son similares en todo el mundo. En concreto, el tacto rectal (DRE), los niveles del antígeno prostático específico (PSA), y una biopsia con aguja gruesa son necesarios para diagnosticar un CaP. Sin embargo, diferentes técnicas basadas en la imagen [por ejemplo, la resonancia magnética (MRI), la tomografía por emisión de positrones (PET), la tomografía computarizada (CT), etc.] acompañan el proceso para clasificar y seleccionar el tratamiento más preciso para esta enfermedad. El tratamiento del CaP abarca desde la vigilancia activa y la cirugía (para estadios tempranos) hasta el uso de radioterapia, quimioterapia u hormonoterapia (para estadios tardíos), siendo este último enfoque el tratamiento más beneficioso del CaP avanzado debido a su progresión andrógeno-dependiente. Lamentablemente, entre el 10 y el 20 % de los pacientes desarrollan resistencia a la terapia hormonal, lo que conduce al fenotipo más agresivo, el CaP resistente a la castración (CPRC), que sigue siendo letal en la actualidad. Se están evaluando nuevos enfoques diagnósticos, pronósticos y terapéuticos para intentar mejorar el manejo clínico de esta patología. Sin embargo, el CaP sigue siendo una patología muy grave y se espera que el número de muertes asociadas al CaP se duplique para 2040, por lo que se necesita urgentemente un mayor conocimiento molecular sobre esta patología.

En este sentido, el CaP ha sido bien caracterizado principalmente desde el punto de vista genómico. En concreto, los reordenamientos genómicos estructurales a gran escala y las alteraciones del número de copias en diferentes genes relacionados con el control del crecimiento, la estabilidad genética, la señalización androgénica o la respuesta al daño del ADN contribuyen al desarrollo, la progresión y la resistencia farmacológica del CaP. De manera más reciente, diferentes estudios indican que otros sistemas hormonales, además del sistema androgénico, así como las maquinarias celulares implicadas en el control del metabolismo del ARN, están profundamente alterados en el CaP, donde parecen tener un papel fisiopatológico crítico. Sin embargo, el sistema somatostatina (SST)/cortistatina (CORT)/neuronostatina (NST) [un sistema hormonal compuesto por tres ligandos peptídicos naturales

(SST, CORT y NST) y diferentes receptores acoplados a proteínas G (SST_{1-5}, GPR107, SST_5TMD4/5)] y el Complejo ARN-Exosoma (REC; maquinaria celular estructuralmente compuesta por un núcleo, nucleasas y cofactores que interviene en el procesamiento y degradación 3'-5' de la mayoría de los tipos de ARN) han sido poco explorados en esta patología.

En consecuencia, el **objetivo general** de esta Tesis Doctoral fue determinar la desregulación, el papel fisiopatológico y la implicación clínica del sistema SST/CORT/NST (péptidos naturales, receptores y análogos químicos; los resultados se incluyen en el primer apartado) y del REC (nucleasas, cofactores y elementos centrales; los resultados se incluyen en el segundo apartado) en el CaP, con el objetivo último de descubrir nuevos biomarcadores y herramientas terapéuticas para mejorar el diagnóstico, el tratamiento y el manejo de los pacientes con CaP.

Los resultados derivados del primer estudio mostraron que el tratamiento con SST y CORT inhibió parámetros funcionales de agresividad clave en células de CaP andrógeno independiente (AI). Mecanísticamente, la capacidad antitumoral de SST/CORT se asoció con la modulación de rutas de señalización oncogénica (p-AKT y p-JNK), y con la reducción significativa de la expresión de genes críticos implicados en la proliferación/migración y agresividad del CaP (e.g., MKI67, MMP9, EGF). Curiosamente, la CORT se expresaba en altos niveles, mientras que la SST no se detectó en todas las líneas celulares de próstata analizadas, lo que sugería que la CORT podría presentar una función autocrina/paracrina en células de CaP. En consonancia con esto, la CORT endógena se encontró sobreexpresada en muestras de CaP (en comparación con la hiperplasia prostática benigna) y correlacionada con parámetros clínicos (i.e., metástasis) y moleculares (i.e., expresión de SST_2/SST_5) clave. Finalmente, el silenciamiento de CORT aumentó drásticamente la tasa de proliferación y redujo la actividad antitumoral de los análogos de la SST (octreotido/pasireotido) en las células de CaP AI.

Por otra parte, observamos que el receptor GPR107 se sobreexpresaba en CaP y se asociaba con parámetros clínicos relevantes (por ejemplo, estadio avanzado del CaP, presencia de invasión vascular y metástasis). Además, el silenciamiento de GPR107 inhibió las tasas de proliferación/migración en

células de CaP AI y alteró la expresión de genes clave en la patofisiología del CaP (KI67, CDKN2D, MMP9, PRPF40A, SST5TMD4, AR-v7, In1-ghrelin, EZH2) y la actividad de vías de señalización oncogénicas del CaP (p-AKT). Curiosamente, el tratamiento con NST también inhibió la proliferación/migración sólo en células AI y evocó una respuesta molecular idéntica al silenciamiento de GPR107. Por último, NST redujo la expresión de GPR107 exclusivamente en células de CaP AI, lo que sugiere que parte de los efectos antitumorales específicos de NST podrían estar mediados por una regulación a la baja de GPR107. En conjunto, los resultados descritos anteriormente (aceptados en 2 publicaciones científicas) proporcionan evidencias sólidas demonstrando que el sistema SST/CORT/NST y los análogos de SST podrían representar una potencial opción terapéutica para el CaP, especialmente para el CPRC.

Los resultados derivados de la segunda sección de esta Tesis Doctoral mostraron una desregulación específica de varios componentes del REC en tejidos de CaP en comparación de muestras control, identificando el factor Poly(A)-Binding-Protein-Nuclear 1 (PABPN1) como un regulador crítico de aspectos muy relevantes del CaP. Específicamente, PABPN1 se sobreexpresa consistentemente (a nivel de ARNm y proteína) en diferentes cohortes humanas de CaP y se asocia a una peor progresión, invasión y metástasis. Además, el silenciamiento de PABPN1 disminuyó características tumorales clave (proliferación, migración, tumoresferas, colonias, etc.) en diferentes modelos de CaP a través de la modulación de ARNs no codificantes largos (lncRNAs: PCA3, FALEC, DLEU2) y mRNAs (CDK2, CDK6, CDKN1A) claves relacionados con la agresividad tumoral. Además, los niveles plasmáticos de PABPN1 estaban alterados en pacientes con metástasis y recaída tumoral, lo que también muestra un potencial valor pronóstico para el CaP. Por último, la inhibición farmacológica de la actividad REC mediante el fármaco isoginkgetina inhibió drásticamente la agresividad de las células del CaP, lo que sugiere una potencial utilidad terapéutica para el tratamiento de esta patología. El conjunto de toda esta información revela que el REC está drásticamente desregulado en CaP, y que este nuevo mecanismo molecular, especialmente la alteración de PABPN1, podría ser potencialmente explotado como una

nueva herramienta pronóstica y terapéutica para el CaP.

En conjunto, los resultados globales obtenidos en esta Tesis Doctoral demuestran que componentes clave pertenecientes al sistema SST/CORT/NST y el REC están profundamente desregulados en el CaP, donde juegan un papel patofisiológico crucial en el desarrollo y progresión de esta enfermedad. Por lo tanto, la información aquí expuesta ofrece una mejor comprensión de la biología celular y molecular del CaP, y señala a estos sistemas reguladores como fuentes potenciales de nuevas herramientas diagnósticas, pronósticas y terapéuticas para el manejo de esta devastadora patología.

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Summary

Prostate cancer (PCa) is one of the most relevant tumoral pathologies for the health system since 1.3 million new cases and 400,000 associated deaths are registered globally every year. The current clinical approaches for this pathology are similar worldwide. Specifically, the digital rectal exam (DRE), the levels of the prostatic-specific antigen (PSA), and a core-needle biopsy are necessary to diagnose PCa. However, different image-based techniques [e.g., Magnetic Resonance Imagin (MRI), Positron Emission Tomography (PET), computed tomography (CT) scan, etc.] accompany this diagnostic process in order to classify and select the most accurate treatment for this disease. PCa treatment ranges from active surveillance and surgery (for early stages) to the use of radiation, chemotherapy or hormone therapy (for late stages), being the latter approach the most beneficial treatment of advanced PCa due to its androgen-dependent progression. Unfortunately, between 10-20 % of the patients develop resistance to hormone therapy leading to the most aggressive phenotype, the castration-resistant PCa (CRPC), which remains lethal nowadays. Novel diagnostic, prognostic, and therapeutic approaches are being evaluated to improve the clinical management of this pathology. However, PCa is still a challenging pathology, expecting the double of PCa-associated deaths by 2040; therefore, further molecular knowledge about this pathology is urgently needed.

In this sense, PCa has been well-characterized mainly from a genomic point of view. Specifically, large-scale structural genomic rearrangements and copy number alterations in different genes related to growth control, genetic stability, androgen-signalling or DNA damage response contribute to the development, progression and drug resistance of PCa. Most recently, different studies indicate that other hormonal systems, apart from the androgen system, as well as cellular machineries involved in the control of RNA-metabolism are profoundly altered in PCa, wherein they might have a critical pathophysiological role. However, the somatostatin (SST)/cortistatin (CORT)/neuronostatin (NST) system [a hormonal system composed of three natural peptide ligands (SST, CORT and NST) and different G-protein coupled receptor (SST_{1-5}, GPR107, SST_5TMD4/5)] and the RNA-Exosome Complex (REC; cellular machinery structurally comprised by a core, nucleases and cofactors which is involved

in the 3´-5´processing and degradation for most types of RNAs) have been poorly explored in this pathology.

Consequently, the **general aim** of this Doctoral Thesis was to determine the dysregulation, pathophysiological role and clinical implication of the SST/CORT/NST (natural peptides, receptors and chemical analogues; results included in the first section) system and of the REC (nucleases, co-factors and core elements; results included in the second section) in PCa, with the ultimate goal of discovering novel biomarkers and therapeutic tools to improve the diagnosis, treatment and management of PCa patients.

The results of the first section showed that SST and CORT treatment inhibited key functional/aggressiveness parameters in Androgen-independent (AI) PCa cells. Mechanistically, the antitumor capacity of SST/CORT was associated with the modulation of oncogenic signalling pathways (p-AKT and p-JNK), and with a significant down-regulation of critical genes involved in proliferation/migration and PCa-aggressiveness (e.g., MKI67, MMP9, EGF). Interestingly, CORT was highly expressed, while SST was not detected, in all prostate cell models analysed, suggesting that CORT could exert an autocrine/paracrine function in PCa cells. In line with this, endogenous CORT was overexpressed in PCa samples (compared with benign prostatic hyperplasia) and correlated with key clinical (i.e., metastasis) and molecular (i.e., SST_2/SST_5 expression) parameters. Remarkably, CORT-silencing drastically enhanced the proliferation rate and blunted the antitumor activity of SST-analogues (octreotide/pasireotide) in AI-PCa cells.

Additionally, we also found that GPR107 was overexpressed in PCa and associated with key clinical parameters (e.g., advanced stage of PCa, presence of vascular invasion and metastasis). Furthermore, GPR107-silencing inhibited proliferation/migration rates in AI-PCa cells, and altered key genes (KI67, CDKN2D, MMP9, PRPF40A, SST5TMD4, AR-v7, In1-ghrelin, EZH2) and oncogenic signalling pathways (p-AKT) involved in PCa aggressiveness. Interestingly, NST treatment also inhibited proliferation/migration only in AI-PCa cells and evoked an identical molecular response to GPR107-

silencing. Finally, NST decreased GPR107 expression exclusively in AI-PCa cells, suggesting that part of the specific antitumor effects linked to NST could be mediated through a GPR107-downregulation. Altogether, the results previously described (accepted in 2 scientific publications) provide solid evidence demonstrating that SST/CORT/NST system and SST-analogues could represent a potential therapeutic option for PCa, especially for CRPC.

The results included in the second section indicate a specific dysregulation of the REC components in PCa-tissues compared with control samples, identifying the Poly(A)-Binding-Protein-Nuclear 1 (PABPN1) factor as a critical regulator of crucial cancer hallmarks. Specifically, PABPN1 was consistently overexpressed (at mRNA and protein levels) in different human PCa cohorts and associated with poor progression, invasion and metastasis. Furthermore, PABPN1 silencing decreased relevant cancer hallmarks (proliferation, migration, tumourspheres, colonies, etc.) in multiple PCa models through the modulation of specific long non-coding RNAs (lncRNAs: PCA3, FALEC, DLEU2) and mRNAs (CDK2, CDK6, CDKN1A) that are relevant in the agresiveness of cancer. Interestingly, plasma PABPN1 levels were altered in patients with metastatic and tumour relapse showing also a potential prognostic value for PCa. Finally, pharmacological inhibition of the REC activity using isoginkgetin drastically inhibited PCa-cell aggressiveness suggesting a potential utility for the treatment of PCa. All this information indicates that the REC is drastically dysregulated in PCa, wherein this novel molecular mechanism, especially the alteration of PABPN1, may be potentially exploited as a novel prognostic and therapeutic tool for PCa.

Taken together, the results obtained from this Doctoral Thesis demonstrate that key components of the SST/CORT/NST system and the REC are deeply dysregulated in PCa, wherein they play a crucial pathophysiological role in the development and progression of this disease. Therefore, the information generated here offers a better understanding of the cellular and molecular biology of PCa and points out these regulatory systems as potential sources of new diagnostic, prognostic, and therapeutic tools for the management of this devastating pathology.

Introduction

1. Prostate Cancer Overview

The prostate is a male gland with the size of a nut involved in the production of seminal liquid under physiological conditions. However, when cellular homeostasis disruption occurs, prostate cells can lead to different prostate-derived pathologies such as Prostatitis, Benign Prostatic Hyperplasia (BPH), or Prostate Cancer (PCa), one of the commonest prostate-related pathologies in men worldwide (1). PCa is a heterogeneous and complex disease in which prostate cells multiply uncontrollably and spread to other parts of the body with a special affinity to the bone (2). According to the cell aetiology and molecular features acquired, it is possible to find different PCa subtypes such as squamous cell carcinoma, sarcoma, lymphoma, signet ring cell PCa or neuroendocrine tumours (2, 3). However, most PCa-related studies (including this doctoral thesis) are focused on adenocarcinomas, since they represent more than 94 % of all PCa cases (4). Currently, researchers aim to face PCa challenge through two different approaches: 1) Detecting the early and easy-curable stage of PCa and 2) fighting against the complex metastatic disease. Unfortunately, although some promising new diagnostic, prognostic, and therapeutic approaches have been established for the clinical management of this pathology, the PCa condition is still worrisome (the mortality is expected to be more than double by 2040), so further knowledge about PCa biology is urgently needed. In the following sections of this Doctoral Thesis, we have gathered the most recent information about the risk factors of PCa and how they could be influencing its epidemiology, the importance of early screening and diagnosis of this disease, the relationship between the PCa-stage and its treatment, the molecular features of this pathology as well as the last discoveries related with the management of this pathology. Furthermore, it is proposed how the approach from a hormonal (studying the Somatostatin/Cortistatin/Neuronostatin system) and cellular/molecular (studying the RNA-Exosome complex) point of view could offer a better understanding of PCa biology as well as a potential source of new diagnostic, prognostic, and therapeutic tools for the management of this devastating pathology.

1.1 Epidemiology and Risk Factors of Prostate Cancer

PCa is the second most common cancer type in men worldwide, with approximately 1.3 million new cases diagnosed yearly (2, 5) (Figure 1a). In addition, this tumour pathology is also the second cause of cancer-related death in this collective with more than 400,000 deaths registered annually (2, 5) (Figure 1b).

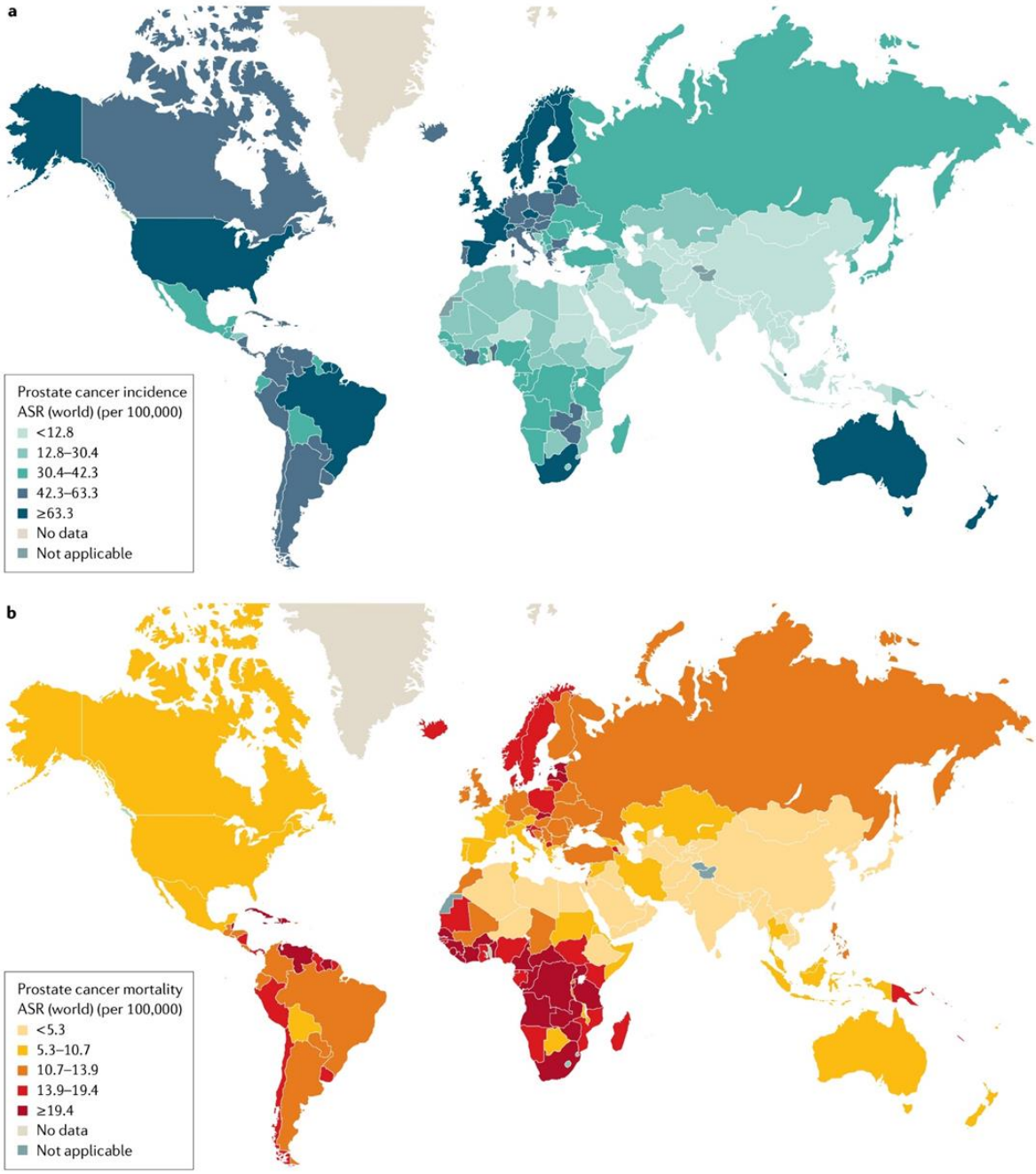

Figure 1. Global geographical incidence (a) and mortality (b) of Prostate cancer. Data are expressed as age-standardized rates (ASR; adjusted to World Standard Population) to account for differing age profiles among regions. Adapted from (2).

Different factors have been associated with higher risk of PCa (6). First, the incidence increases with **age**. Indeed, the chance of developing PCa increases in men older than 50 years of age, being 67 the median age at diagnosis. **Family history** represents another risk factor for PCa development [around 15% of men with a diagnosis of PCa have a first-degree relative (e.g., brother, father) with this disease] due to heritable susceptibility genes (7). Interestingly, **race** seems to be another risk factor for PCa having black men a higher risk of developing and dying from PCa (8). As will be discussed in this Doctoral Thesis, **hormones** also play a critical role in prostate regulation. Specifically, testosterone and dihydrotestosterone [DHT; a testosterone derived hormone with 4-50-fold greater affinity for the androgen receptor (AR) than testosterone (9)] play a key role in prostate physiology as well as in PCa progression, wherein the levels of these molecules in plasma are associated with long-term PCa death and aggressiveness (10, 11). Finally, alimentary habits such as **dietary fat**, **dairy**, **calcium**, and some **vitamin intakes** are also associated with the presence of PCa (12). New clinical trials are evaluating additional risk factors for this cancer (13).

1.2 Screening and Diagnosis of Prostate Cancer.

When men overpass 50 years old or have any initial PCa-associated symptoms (e.g., trouble starting the flow of urine, frequent urination, trouble emptying the bladder completely, etc.) they are submitted to standard screening/diagnostic tests which usually include the **Digital Rectal Exam (DRE)** and the **Prostate-Specific Antigen (PSA)** test (14, 15). On the one hand, DRE consists of introducing a gloved finger into the lower part of the rectum to check the presence of lumps or anything unusual in the prostate. On the other hand, PSA is highly secreted by PCa cells so high levels of PSA (> 4) in plasma are used as an indicative of the potential presence of PCa. However, physical prostate anomalies and an increase in serum PSA levels can occur due to other non-related PCa causes such as infection, HBP, or prostatitis, leading to false positives. For that reason, it is necessary to perform a **core-needle biopsy** to definitively confirm the diagnosis of PCa (16). Additional tests to detect regional or distal PCa such as **Magnetic Resonance Imagin (MRI)**, **Positron Emission Tomography (PET)**, **Pelvic Lymph Node**

Dissection (PLND), and **Computed Tomography (CT) scans** can be carried out during the diagnosis of PCa, especially when there are suspect of metastasis (e.g., PSA higher than 20 ng/mL, pain in the back, hips, or pelvis, etc.) (17).

1.3 Stage and Treatment of Prostate Cancer

Different imaging tools are used to classify PCa based on the **TNM staging system** (i.e., MRI, PET, PLND, CT, and Technetium Tc 99m-methylene diphosphonate bone scan), which considers the size of the primary tumour (T), whether it is spread to nearby lymph nodes (N), and the presence of distal metastasis (M) (18, 19) (Table 1).

Table 1. TNM system in prostate cancer. T: Tumour; N: Regional lymph nodes M: Distal metastasis.

PRIMARY TUMOUR (T)	
TX	Primary tumours cannot be assessed
T1	Clinically inapparent tumour that is not palpable
T1a	Tumour incidental histologic finding in 5% or less of tissue resected
T1b	Tumour incidental histologic finding in more than 5% of tissue resected
T1c	Tumour identified by needle biopsy found in one or both sides, but not palpable
T2	Tumour is palpable and confined within the prostate
T2a	Tumour involves one-half of 1 lobe or less
T2b	Tumour involves more than one-half of 1 lobe
T2c	Tumour involves both lobes
T3	Extraprostatic tumour that is not fixed or does not invade adjacent structures
T3a	Extraprostatic extension (unilateral or bilateral)
T3b	Tumours invade seminal vesicle(s)
T4	Tumour is fixed or invades adjacent structures other than seminal vesicles
Pathologic (pT)	
pT2	Organ confined
pT3	Extraprostatic extension
pT3a	Extraprostatic extension or microscopic invasion of the bladder neck
pT3b	Tumours invade seminal vesicle(s)
pT4	Tumour is fixed or invades adjacent structures other than seminal vesicles
REGIONAL LYMPH NODES (N)	
NX	Regional lymph nodes were not assessed

N0	No positive regional lymph nodes
N1	Metastases in regional lymph node(s)
DISTANT METASTASIS (M)	
M0	No distant metastasis
M1	Distant metastasis
M1a	Nonregional lymph nodes(s)
M1b	Bone(s)
M1c	Other site(s) with or without bone disease

Additionally, there is another critical information for the staging of PCa, the **Gleason Score (GS)** (20), which is a histopathological value that indicates how abnormal the cancer cells in a patient-derived biopsy look under a microscope and how quickly they are likely to grow and spread. Specifically, most PCa contains focus with different differentiation patterns, known as the Gleason patterns which range between 1 (well-differentiated tissue) to 5 (poorly differentiated tissue) (21, 22) (Figure 2).

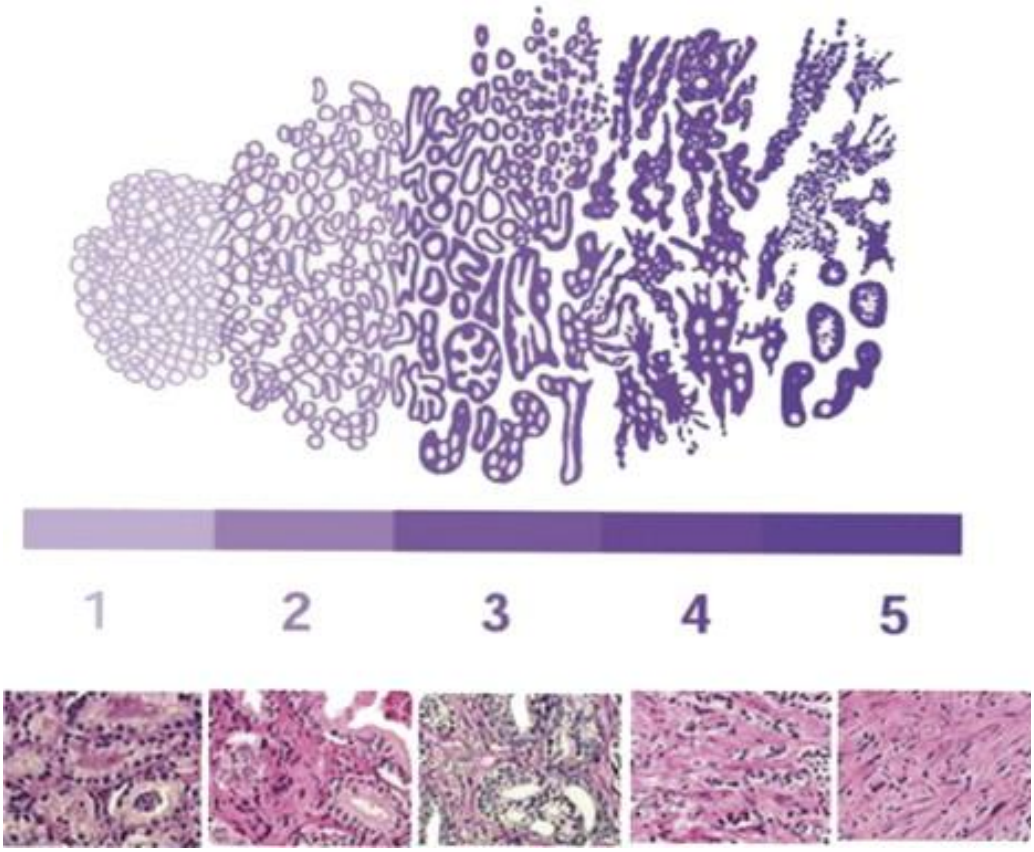

Figure 2. Schematic diagram of Gleason grading system. Numbers refer to Gleason's grades. Above are the original Gleason drawings of each grade and below are the corresponding stained micrographs for each grade. Adapted from (21).

Following the International Society of Urological Pathology (ISUP) recommendations, the GS is calculated by adding the two grades that comprise the largest areas of the biopsied tissue sample (e.g., if the main pattern found in a biopsy is 3 followed by 4, GS = 3 + 4 = 7). The GS usually ranges from 6 to 10 since a pattern categorized into 1 or 2 is considered benign tissue.

Finally, to select the most accurate treatment, the TNM staging system, the GS and the plasma levels of PSA are combined to categorize PCa into stage-treatment I to IV (23, 24) (Table 2).

Table 2. Classification and standard treatment of prostate cancer. EBRT: External-beam radiation therapy; ADT: Androgen deprivation therapy; TURP: Transurethral resection of the prostate; CRPC: Castration-resistant prostate cancer.

Stage	TNM	PSA	Gleason Score; Gleason pattern	Treatment
I	cT1a-c, cT2a, N0, Mo	<10	≤6; ≤3+3	Watchful waiting or active surveillance/active monitoring Radical prostatectomy EBRT Interstitial implantation of radioisotopes
	pT2, N0, M0	<10	≤6; ≤3+3	
IIA	cT1a-c, cT2a, pT2, N0, M0	10-20	≤6; ≤3+3	Watchful waiting or active surveillance/active monitoring Radical prostatectomy EBRT with or without ADT Interstitial implantation of radioisotopes
	cT2b–c, N0, M0	<20	≤6; ≤3+3	
IIB	T1-2, N0, M0	<20	7; 3+4	
IIC	T1–2, N0, M0	<20	7; 4+3	
	T1–2, N0, M0	<20	8; 4+4, 3+5, or 5+3	
IIIA	T1–2, N0, M0	≥20	≤6; ≤3+3	EBRT with or without ADT ADT with or without radiation therapy Radical prostatectomy with or without EBRT Watchful waiting or active surveillance/active monitoring
	T1–2, N0, M0	≥20	7; 3+4	
	T1–2, N0, M0	≥20	7; 4+3	
	T1–2, N0, M0	≥20	8; 4+4, 3+5, or 5+3	
IIIB	T3–4, N0, M0	Any value	≤6; ≤3+3	
	T3–4, N0, M0	Any value	7; 3+4	
	T3–4, N0, M0	Any value	7; 4+3	
	T3–4, N0, M0	Any value	8; 4+4, 3+5, or 5+3	

IIIC	Any T, N0, M0	Any value	9 or 10; 4+5, 5+4, or 5+5	
IVA	Any T, N1, M0	Any value	≤6; ≤3+3	ADT Bisphosphonates EBRT with or without ADT Palliative radiation therapy Palliative surgery with TURP Watchful waiting or active surveillance/active monitoring
	Any T, N1, M0	Any value	7; 3+4	
	Any T, N1, M0	Any value	7; 4+3	
	Any T, N1, M0	Any value	8; 4+4, 3+5, or 5+3	
	Any T, N1, M0	Any value	9 or 10; 4+5, 5+4, or 5+5	
IVB	Any T, Any N, M1	Any	Any	
CRPC	Any T, Any N, M1	Any	Any	Chemotherapy Immunotherapy radiopharmaceutical therapy

Stage I PCa usually is a clinically silent and prostate-confined disease, and most of them require no treatment other than **careful follow-up** (25). However, in younger patients (50 - 60 years old) **radical prostatectomy** (usually with pelvic lymphadenectomy), **external-beam radiation therapy (EBRT)** and **interstitial implantation of radioisotopes** (i.e., iodine I 125, palladium, and iridium Ir 192) could be considered (26-29). **Stage II** PCa presents **similar therapy approaches to stage I**. However, due to the importance of androgens in the progression of advanced PCa, **EBRT with adjuvant Androgen Deprivation therapy (ADT)** which includes the reduction of systemic testosterone [through physical (orchiectomy) or chemical (e.g., luteinizing hormone antagonist) castration] and the use of antiandrogens [molecules to block the synthesis (e.g., abiraterone) or function (e.g., enzalutamide) of testosterone] could be considered for patients with bulky T2b-T2c tumours (30, 31). Furthermore, **stage III** PCa is faced with **ADT with or without radiation therapy after treatment, EBRT with or without ADT after treatment or radical prostatectomy with or without radiation therapy after surgery** (32-34). Finally, the standard management of **stage IV** PCa consists of **ADT with or without chemotherapy, bisphosphonate therapy** (e.g., clodronate or zoledronate) to reduce the risk of bone fracture (the main metastatic tropism of PCa cells is the bone), **EBRT with or without hormonal**

therapy, **palliative radiation therapy** to relief bone pain, and **palliative surgery with transurethral resection of the prostate (TURP)** to reduce urinary obstruction (35-41). Importantly, although most patients with the advanced disease respond to ADT therapy correctly, some of them develop resistance to this approach leading to the most aggressive and lethal phenotype of PCa, the castration-resistant PCa **(CRPC)**. To improve the overall survival of CRPC patients, **chemotherapy**, **immunotherapy** (i.e., Sipuleucel-T, which consists of autologous peripheral blood mononuclear cells that have been exposed *ex vivo* to a recombinant fusion protein composed of prostatic acid phosphatase fused to granulocyte-macrophage colony-stimulating factor) and **radiopharmaceutical therapy** [i.e., Radium Ra 223, especially to treat bone metastatic CRPC (mCRPC)] are commonly employed (42-44). New potential therapy approaches for the different stages of PCa are under evaluation in clinical trials (13).

1.4 Molecular Features of Prostate Cancer

Genomic aberrations are the better characterized molecular alterations in PCa (45). Single nucleotide polymorphisms (SNPs) are not frequent in early-stage PCa. Instead, early PCa typically accumulates **large-scale genomic structural rearrangements and/or copy number alterations** such as TMPRSS2-ERG fusions (40 – 60 % of patients), **loss-of-function mutations** in SPOP (5 – 15 % of patients), and **gain-of-function mutations** in FOXA1 (3 – 5 % of patients) (46, 47). PTEN deletions and TP53 mutations are observed in 10 – 20 % of cases of localised PCa, and their frequency increases to more than 50 % of cases with advanced disease (47).

As mentioned above, hormones, especially androgens, play a key role in the pathophysiology of PCa so **alterations in AR signalling** are crucial during PCa-progression, particularly in resistance acquisition to ADT (45). mCRPC is commonly associated with enhanced AR signalling through different mechanisms such as amplification and/or gain-of-function mutations or increasing the transcription or post-translational modification to stimulate AR action (48). Notably, the generations of splicing variants with constitutive activity such as the AR variant 7 (AR-v7; which arises from an aberrant splicing process at the cryptic exon 3, resulting in a shorter protein without the ligand-binding domain) further

drive downstream AR signalling and is implicated in the resistance to AR-targeting agents (e.g., enzalutamide, apalutamide, darolutamide, etc.) (49). However, a loss of dependence on AR signalling occurs in 15 – 20 % of advanced, treatment-resistant PCa and can manifest as a transformation to castration-resistant neuroendocrine PCa, which is highly treatment-refractory.

Progression to mCRPC phenotype is also related to the **dysregulation of additional genes implicated in growth control and genetic stability**. Homozygous deletions and loss-of-function mutations in PTEN occur in more than 40 % of all cases of mCRPC, whereas gain-of-function mutations in PIK3CA, PIK3CB, or AKT1 occur in 5 % of all mCRPC patients (48, 50). Activation of the Wnt signalling pathway and overexpression of the MYC oncogene is also frequently observed (20 – 30 %) in mCRPC cases (48). Alterations in TP53 and RB1 are seen in 20 – 50% of cases. RB1 loss has been associated with poor prognosis in mCRPC. When concurrent with TP53 mutations, deletion of PTEN, or both, RB1 loss is also associated with lineage plasticity and acquired castration-resistant neuroendocrine PCa disease that is AR-indifferent (51, 52).

DNA damage response (DDR) genes also have a critical role in PCa. Men with germline BRCA1 or BRCA2 mutations have a three to eight times higher lifetime risk of PCa that can behave aggressively because of additional MYC activation in combination with the inactivation of TP53 and PTEN (53, 54). Inherited mutations in MLH1, MSH2, and PMS2 also result in an increased risk of PCa (55). The estimated prevalence of inherited DDR mutations in men with metastatic PCa is approximately 12 %, and these mutations are most commonly in BRCA1, BRCA2, ATM, CHEK2, RAD51D, and PALB2 (56). Somatic aberrations in DDR genes (most frequently, BRCA2, ATM, BRCA1, CHEK2, CDK12, and PALB2) occur in approximately 23% of metastatic PCa (56). Such changes, along with mismatch repair or CDK12 alterations, have led to targeted approaches, as will be described subsequently. Given the high incidence of germline and somatic DDR alterations in advanced PCa, the current recommendation is that all men with metastatic PCa should be offered germline and, if possible, tumour

genetic testing, regardless of family history of hereditary cancers or clinicopathological features (57, 58).

1.5 Latest Diagnostic, Prognostic, and Therapeutic Approaches for Prostate Cancer

A broad number of novel biomarker panel tests, as well as individual biomarkers, have been already approved by the Food and Drug Administration (FDA) or regulated by Clinical Laboratory Improvement Amendments (CLIA). Serum-based assays targeting kallikreins have been recently developed and proven their efficacy in the diagnosis of PCa, including the Prostate Health Index (PHI) test and the four kallikreins (4K) score test. PHI test is based on the levels of the total-, free- and pro-PSA, while the 4K score is an algorithm which includes free-, intact- and total PSA, kallikrein-like peptidase 2 (hK2) and clinical data (i.e., Age, DRE and prior biopsy status) (59, 60). Both tests show a similar capacity for detecting PCa but, unfortunately, their specificity remains low (approximately 30 %) (61, 62). On the other hand, the only FDA-approved urine-based test for reducing unnecessary biopsies is the Prostate Cancer Antigen 3 (PCA3) test (63). Specifically, this test is based on the evaluation of the urine PCA3 mRNA levels after DRE stimulation (64), which has shown high accuracy in detecting PCa (sensitivity: 67 %; specificity: 83 %) (64). Additional non-FDA-approved urine-based tests such as SelectMDX test, Mi-Prostate Score and ExoDx Prostate Intelliscore are being evaluated (65). Finally, there are also some commercially available biopsy-based tests, including OncotypeDx, Prolaris and Decipher, which are especially useful for predicting PCa outcomes (66-69).

In the same way, a significant improvement in the PCa treatment field has been observed during the last years, mainly due to the technological development that has been implemented at the surgical and pharmacological levels. In the case of localized PCa, novel and promising techniques are under research, such as high-intensity focused ultrasound and focal therapy (70). On the other hand, in the case of mCRPC, the results from PROfound phase III clinical trial have resulted in the approval by the FDA of olaparib [a Poly ADP-ribose polymerase (PARP) inhibitor (71)] as a novel treatment for CRPC patients with mutations in DNA repair genes, being the first example of personalized medicine in PCa patients

(72). Finally, other therapies to treat different stages of PCa such as photodynamic therapy, bicalutamide or Proton-beam therapy are also being evaluated obtaining promising results (73-75).

Nevertheless, despite the advances in recent years, PCa is still a challenging disease and it is essential to precisely unveil the molecular, cellular and endocrine-metabolic events underlying the development, progression and aggressiveness of PCa, in order to identify novel diagnostic, prognostic and therapeutic targets in PCa, and especially advanced PCa, which represents an urgent and unmet clinical need.

2. Somatostatin/Cortistatin/Neuronostatin System and Prostate Cancer

2.1 Structure and Organization

The Somatostatin (SST)/Cortistatin (CORT)/Neuronostatin (NST) system represents a complex hormone system composed of at least three peptide ligands and five G protein-coupled receptors (GPCRs) (76) (Figure 3).

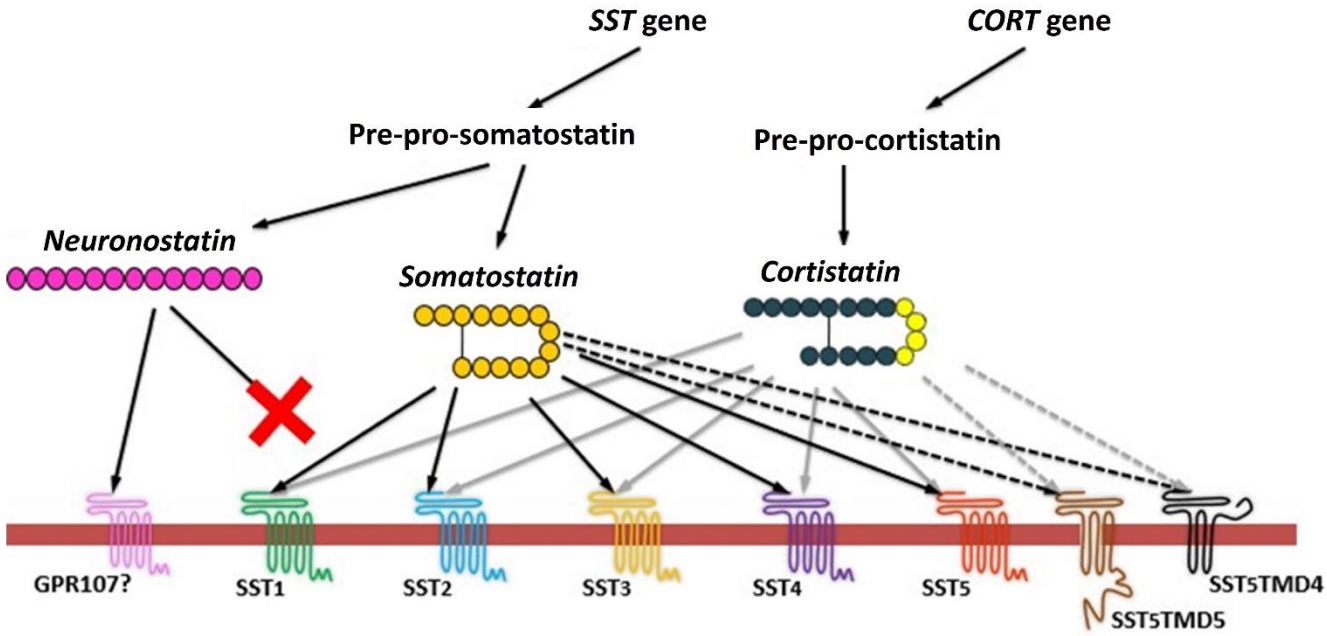

Figure 3. Schematical organization of the Somatostatin/Cortistatin/Neuronostatin system. SST: Somatostatin; CORT: Cortistatin; SST_{1-5}: Somatostatin receptor 1-5; SST_5TMD4-5: Somatostatin receptor 5 with 4/5 transmembrane domains splicing isoform; GPR107: G protein-coupled receptor 107.

Specifically, the *SST* gene encodes the pre-pro-SST polypeptide, which through proteolytic processing usually gives rise to two peptides with 14 and 19 amino acids, the **SST** and **NST**, respectively (77-79). Moreover, the *CORT* gene usually produces a peptide with 17 amino acids, the **CORT** (80). Importantly, SST and CORT share eleven amino acids and present similar three-dimensional structures (cyclic structure due to the formation of a disulfide bridge between two of their cysteines) so they can similarly bind to the five so-called SST-receptors (SSTRs), termed **SST_1-SST_5** (encoded by 5 different genes: *SSTR1-5*, respectively) (80, 81). However, CORT seems to be able to bind to additional receptors

including the ghrelin receptor 1a (GHS-R1a) and the MAS-related GPR family member X2 (MRGPRX2) (80). On the other hand, NST has a linear structure as well as an amide group at its N-terminus. With this structure, NST cannot bind to SSTRs, and the G protein-coupled receptor 107 (**GPR107)** is the only described potential receptor (78, 82).

It is noteworthy that some of the receptors mentioned can carry out ligand-independent functions, can form homo- and hetero-dimers with other receptors (SST_2-SST_2, SST_2-SST_5, etc.) to change their canonical signalling, and can present alternative splicing isoforms with different non-canonical functions [mRNA from the *SSTR5* gene generates two truncated splicing variants with 4 and 5 Transmembrane Domains (TMDs): the **SST_5TMD4** and **SST_5TMD5**, respectively]. In addition, differential proteolytic processing allows the generation of ligands with different sizes of the above-described (SST-28, NST-13, CORT-29), which translates into an enormous complexity and versatility of this system (76, 81, 83).

2.2 Role of Somatostatin/Cortistatin/Neuronostatin System in Prostate Cancer

The components of the SST, CORT and NST system are expressed in different tissues of the body (e.g., hypothalamus, stomach, pancreas, intestine, liver, etc.) where they interact to regulate a multitude of physiological processes, most of them through their inhibitory character (e.g., inhibition of growth hormone, insulin or glucagon secretion, control of gastric motility, etc.) (79, 80, 84). Additionally, certain members of this system are dysregulated in cancer (neuroendocrine and brain tumours, breast cancer, PCa, etc.), where they appear to play a critical role in the development and progression of these pathologies (85-89). In fact, due to its important pathological role, different synthetic **SST-analogues (SSAs)** with different affinities for the SSTRs (90) (Table 4), such as Octreotide (which acts primarily by binding to SST_2 and with less affinity to SST_3 and SST_5), Lanreotide (able to bind mainly to SST_2 and slightly to SST_3 and SST_5) or Pasireotide (a multi receptor ligand with high affinity for SST_1, SST_2, SST_3, and specially for SST_5), are used in clinical practice to manage certain tumour pathologies (90).

Table 3: Binding affinity (IC50, nM) of the native somatostatin and the currently available somatostatin analogues for the different somatostatin receptors.

Compound	SST_1	SST_2	SST_3	SST_4	SST_5
Somatostatin-14	2.26	0.23	1.43	1.77	0.88
Somatostatin-28	1.85	0.31	1.3	-	0.4
Octreotide	1140	0.56	34	7030	7
Lanreotide	2330	0.75	107	2100	5.2
Pasireotide	9.3	1	1.5	>100	0.16

Regarding PCa, certain components of this system, especially SSTRs, are expressed in both normal and tumour tissues, where they may play a relevant role in the development and progression of this disease. Specifically, some components of this system (e.g., SST_1 and SST_2) can contribute to reduce different tumour parameters, including cell proliferation and migration (91, 92), whereas other components (i.e., the truncated splicing SST_5TMD4 variant) promote aggressiveness features of PCa (89). As mentioned, SSAs have been used as valuable tools to manage different tumour pathologies, including pituitary and neuroendocrine tumours. However, attempts to apply SSAs in PCa have rendered controversial results, since the limited studies reported so far did not show improvement in overall survival. The mechanistic reasons for those clinical failures are still unknown but it has been suggested that one of the causes might be the overexpression of the spliced variant SST_5TMD4 in PCa cells, which hampers the normal response to these compounds (89).

Importantly, despite the information above described, some components of this system including the natural peptides SST, CORT and NST, the GPR107 receptor as well as the use and molecular mechanism underlying the resistance to some SSAs have not been evaluated in this pathology. Then, further knowledge about the pathophysiological relationship between SST/CORT/NST system and PCa could

represent a new avenue to find novel diagnostic/prognostic biomarkers and therapeutic targets for this disease.

3. The RNA-Exosome Complex and Prostate Cancer

3.1 Function, Structure, and Regulation

The **RNA-Exosome Complex (REC)** is a conserved, multi-subunit protein complex discovered by Tollervey and co-workers in the late 1990s, that contributes to 3´- 5´ processing and degradation of most types of RNAs present in eukaryotic cells (93, 94) (Figure 4). Specifically, the REC catalyses the 3´to 5´ trimming and/or degradation of certain stable RNA species, including messenger RNAs (mRNAs), transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), telomeric RNAs, small nuclear (snRNAs) and small nucleolar RNAs (snoRNAs), in order to ensure fidelity of these transcripts (95, 96). In addition, it is known that microRNAs (miRNAs) and different types of long-non-coding RNAs (lncRNAs) such as promoter upstream transcripts (PROMPTs), upstream antisense RNAs (uaRNAs), prematurely terminated RNAs (ptRNAs), transcription start site (TSS)-associated RNAs (tssRNAs), enhancer RNAs (eRNAs) and long intergenic RNAs (lincRNAs) are also under the surveillance of the REC (94, 97).

Structurally, the REC consists of a 9-protein inert barrel-shaped complex, known as Exo9 core (Figure 4a) (94, 98). This core is catalytically inactive, and it is differentiated into two different parts: i) The *S1/KH cap* which includes **EXOSC1, EXOSC2 and EXOSC3**, three proteins with the S1 and the KH RNA-binding domains; and, ii) the *PH-like Ring* which is composed by six RNase PH-like proteins called **EXOSC4, EXOSC5, EXOSC6, EXOSC7, EXOSC8 and EXOSC9** (Figure 4a). The Exo9 core serves as a scaffold for different ribonucleases (EXOSC10, DIS3 and/or DIS3L), which confer functionality to the complex (94, 98) (Figure 4a). Specifically, **EXOSC10** localizes at the top of the *S1/KH cap*, presenting a distributive 3′–5′ exoribonuclease activity. **DIS3** can be tethered at the bottom of the PH-like Ring and presents both processive 3′–5′ exoribonuclease and endonuclease activity. Finally, the localization of **DIS3L** is similar to DIS3, but this paralog enzyme only presents a processive 3′–5′ exoribonuclease activity. Importantly, the presence and abundance of these specific ribonucleases change according to the cellular localization (Figure 4b). For example, EXOSC10 is strongly enriched

in the nucleolus and a small amount of this protein has been found in cytoplasm; while, DIS3 is predominantly located in the nucleoplasm but absent in the nucleolus; and DIS3L is exclusively detected in the cytoplasm of human cells.

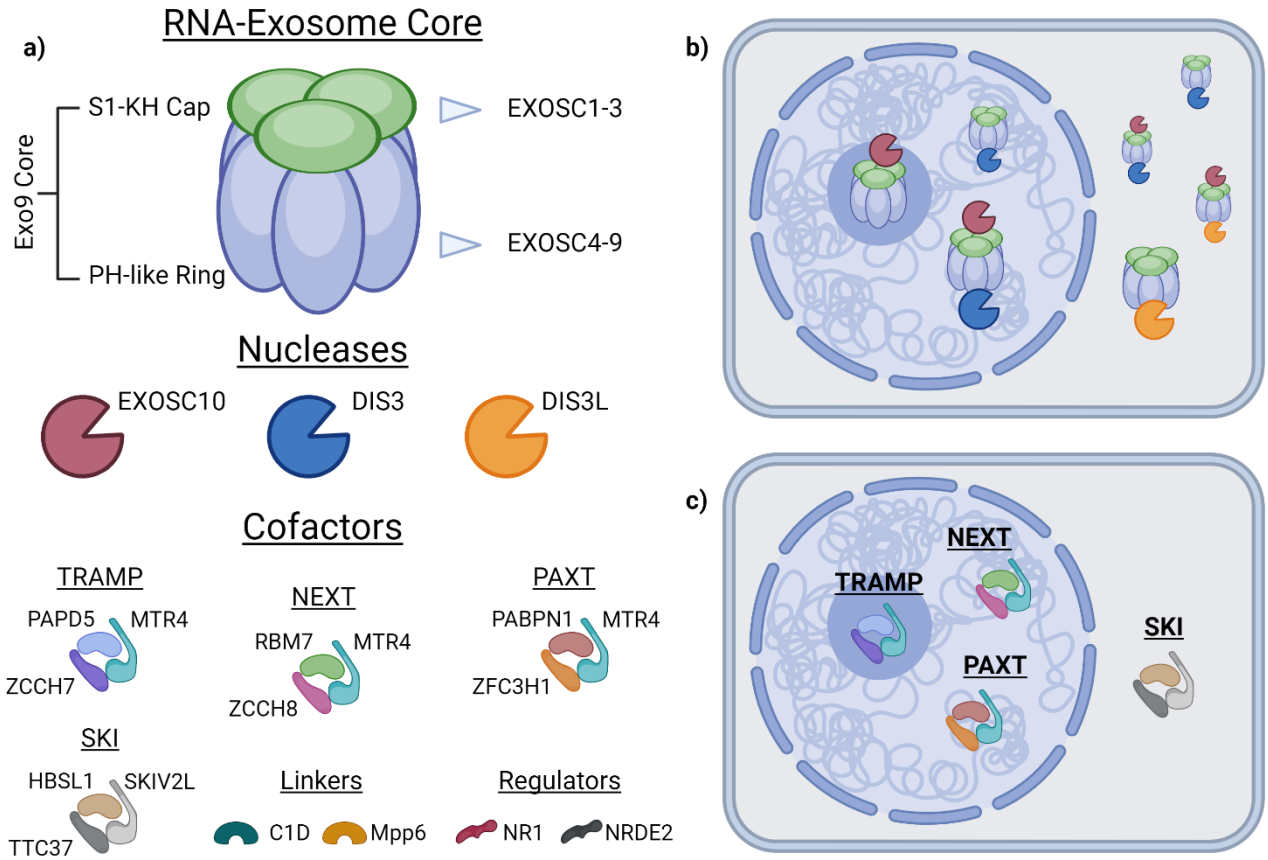

Figure 4. Organization and function of the RNA-Exosome Complex. a) Composition of the Core, Nucleases and cofactor belonging to the RNA-Exosome Complex. b) Distribution of the different nucleases along the nucleolus, nucleoplasm and cytoplasm of a human cell. A larger size of the representation in the figure indicates a larger proportion in the cellular compartment. c) Distribution of the different cofactors along the different cellular compartments.

The substrate-specificity of the REC is offered by different co-factors, which represent a group of 3 - 4 proteins that can recognize the sequences, structures and/or modifications of specific RNAs, unwind them and deliver these RNAs to the Exo9 core, in order to be processed by the different ribonucleases (99) (Figure 4a and c). To date, there are **three different paths for RNA substrates to reach the catalytic subunits** (100) (Figure 5).

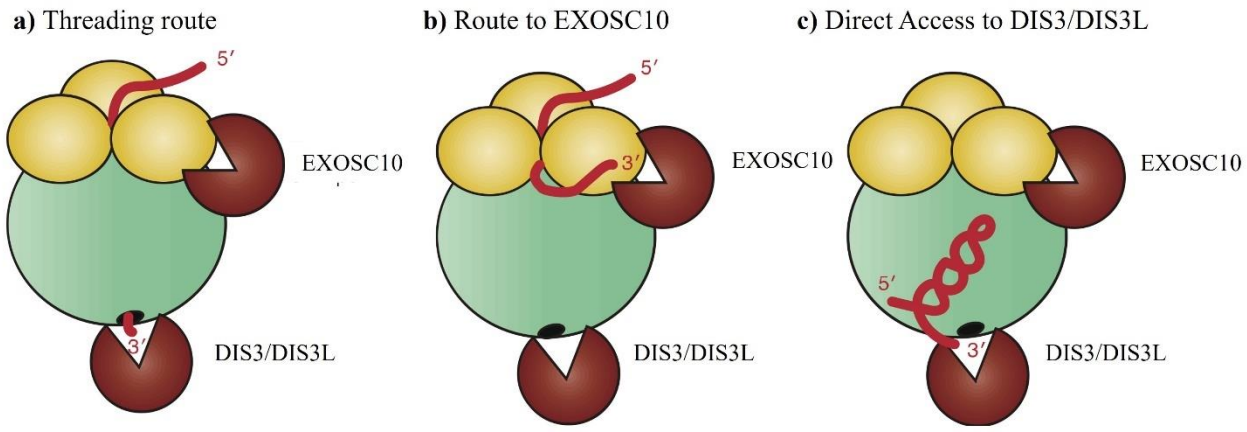

Figure 5. Pathways leading RNA substrates to the catalytic subunits of the RNA-Exosome Complex. In the threading route **(a)** the RNA crosses into the Exo9 core to reach DIS3 enzyme. In the EXOSC10 route **(b)** the RNA traverses the cap structure to reach EXOSC10. In the direct access to DIS3 **(c)** the RNA bypasses the core to reach its catalytic domain.

In the ***threading route*** (Figure 5a), the RNA is accommodated in the central channel of the core exosome and reaches the active site of DIS3/DIS3L. In the ***EXOSC10 route*** (Figure 5b), the RNA traverses the cap structure and reaches the active site of EXOSC10. Most recently, **new direct access to DIS3/DIS3L** (Figure 5c) has been found. In this case, the RNA bypasses the central channel and directly accesses to the catalytic site of DIS3/DIS3L.

As ribonucleases, the presence and abundance of a specific cofactor differ between cellular compartments (Figure 4c). The **TRAMP** co-factor (Trf4/5p-Air1/2p-Mtr4p polyadenylation; composed by MTR4, ZCCHC7, and PAPD5 proteins in humans) is localized mainly in the nucleolus and is associated with the maturation of pre-rRNA and turnover of mature snoRNAs mediated by DGCR8 (95, 101). Furthermore, the **NEXT** complex (Nuclear Exosome Targeting; composed by MTR4, ZCCHC8 and RBM7) is a nuclear cofactor involved in the turnover of introns of pre-mRNAs, intronic snoRNAs, PROMPTs, eRNAs, aberrant 3′ extended transcripts of snRNAs, snoRNAs, and telomerase RNA (95, 102). Otherwise, nuclear noncoding transcripts with longer poly(A) tails are channelled to **PAXT** nuclear co-factor (Poly(A) Tail Exosome Targeting; composed by MTR4, ZFC3H1 and PABPN1) (95, 103). Finally, **SKI** complex (Superkiller; composed by SKIV2L, TTC37 and HBSL1 proteins) is a cytoplasm

cofactor and it is associated with the mRNA decay [particularly that of short-lived A/U-rich element (ARE) RNAs] and the mRNA quality control related with ribosome stress (95, 104).

Importantly, other independent proteins such as C1D and Mpp6, NR1 or NRDE2 and different chemical modifications in the components of the REC contribute to activity control as well as the RNA targets of the complex conferring a huge combinatorial regulation (94, 95).

3.2 Potential Role of RNA-Exosome Complex in Prostate Cancer

The REC is critical for the surveillance of most RNA subtypes, contributing to important functions such as DDR, R-loop resolution, maintenance of genome stability, RNA transcription, modification, export and translation, or control of cell differentiation (96, 97). Then, the correct organization and function of this complex are crucial for cellular homeostasis. In this sense, recent evidence indicates that the disruption of the RNA metabolism is an emerging hallmark of cancer since it is involved in the development and progression of a multitude of cancer types (105). Indeed, it has been widely described that those components involved in RNA-related processes such as splicing, Nonsense-mediated Decay (NMD) or miRNA-biogenesis are frequently altered in tumour pathologies, and could be used to predict tumour initiation and progression as well as avoid critical steps of these diseases (106-108). Interestingly, genomic (mutations, deletions, amplification, fusion, methylations, etc.) and transcriptomic (up-/down-regulation in the expression, splicing isoform ratio changes, etc.) alterations along different core, cofactor and nuclease components of the RNA-exosome have been described in several tumour pathologies (109-112). However, the alterations and implications of the components of this complex have never been explored in PCa. Therefore, the study of this critical molecular complex might represent an interesting and novel source of useful diagnostic, prognostic and therapeutic tools for the clinical management of PCa.

Aims of study

The **GENERAL AIM of this Doctoral Thesis** was to determine the dysregulation, pathophysiological role and clinical implication of the SST/CORT/NST system (natural peptides, receptors and analogues) and of the REC (nucleases, co-factors and core elements) in PCa, with the ultimate goal of discovering novel biomarkers and therapeutic tools to improve the diagnosis, treatment and management of PCa patients.

To achieve this main aim, we proposed the following **SPECIFIC OBJECTIVES (SO):**

- **SO1)** To explore the presence and (dys)regulation of the entire SST/CORT/NST system [the natural peptides (SST, CORT and NST) and their receptors (SST_{1-5}, SST_5TMD4, GPR107, etc.)] in PCa tissues compared with control samples, and to explore their association with key clinical parameters of PCa patients.

- **SO2)** To perform a parallel comparison of the *in vitro* effects of SST, CORT and NST peptides on different normal and tumoral (PCa and CRPC) cell models, as well as a to explore the molecular mechanism underlying these effects.

- **SO4)** To determine the expression patterns of the REC components (nucleases, cofactors and core elements) in PCa tissues compared with control samples, and to explore its association with key clinical features of the patients.

- **SO5)** To unveil the functional and mechanistic role of the most relevant REC components identified in SO4, as well as to explore their usefulness as non-invasive biomarkers and therapeutic targets in PCa.

Results and general discussion

The results generated in the present Doctoral Thesis and their discussion have been summarized in this section and structured in three scientific manuscripts directly derived from this Doctoral Thesis [3 accepted manuscripts in top ranking journals of the "Biochemistry & Molecular Biology", "Medicine, Research & Internal", and "Oncology" fields) which can be found at the end of the Doctoral Thesis.

<u>Article I:</u> Somatostatin, Cortistatin and Their Receptors Exert Antitumor Actions in Androgen-Independent Prostate Cancer Cells: Critical Role of Endogenous Cortistatin.

In this work, we observed that the treatment with SST and CORT peptides was able to reduce different key functional parameters linked to tumour growth and metastasis (i.e., proliferation, migration, and colonies formation) only in androgen-independent (AI) PCa cells (22Rv1 and PC-3 cells, two representative models of CRPC pathology), but not in normal prostate and androgen-dependent (AD) PCa cells, suggesting a potential and specific antitumor capacity of these peptides in the most aggressive phenotype of PCa. Mechanistically, these antitumor effects of SST and CORT were associated with the alteration of the expression levels of critical genes and the activity of oncogenic signalling pathways that have been reported to be frequently associated with the functional and cellular control of the SST system in multiple endocrine-related cancers (ERCs) (e.g., proliferation, migration, and PCa-aggressiveness features) (87, 89, 113, 114). Specifically, we found that SST and CORT could exert their antitumor actions in AI-PCa cells through the modulation of AKT, JNK, MKI67, CDK2, CDK4, CDK6, CDKN1A, CDKN1B, CDKND, MMP3, MMP9, MMP10, CDH2, EGF, EZH2, C-MYC, PTEN, and VEGFR levels. All these molecular events might be associated with the reduction in the proliferation, migration and colonies formation previously described in response to SST and CORT treatments, wherein some of these changes (especially the alteration of CDK2/4/6 and CDKN1A/1B) might be probably linked to an alteration in the cell cycle arrest (interruption of G1 to S transition), cellular matrix degradation and stem-like cell status (115-118). However, it should be mentioned that the modulatory actions of SST and CORT were, in some cases, cell line dependent, which might be explained by the specific phenotypic

differences between the two AI-PCa cell models used (i.e., mutation profile, aggressiveness, metabolic rate, etc.) (119, 120). Additionally, these differences could be also attributed to the differential SSTRs expression profile found between 22Rv1 and PC-3 cells (i.e., SSTR1 = SSTR5 >>> SSTR2 > SST_5TMD4 > SSTR3 in 22Rv1 cells vs. SSTR5 >>> SSTR2 > SSTR1 > SST_5TMD4 in PC-3 cells) since it has been reported that each SSTR-subtype can be linked to a different signalling pathway profile (81, 121, 122).

In addition, we analysed in parallel the expression pattern of all SSTR-subtype by a quantitative PCR method in a representative cohort of PCa tissues, which revealed that SSTR1, SSTR2 and SSTR5 were highly expressed in PCa tissues (i.e., SSTR1 ≥ SSTR2 = SSTR5). Our results are in accordance with a previous study from our group indicating that SSTR1 is highly expressed in PCa tissues (89, 91). Notably, these data might be considered as an important clinical finding because it might suggest that PCa tissues could be sensitive to the actions of SSAs, as the responsiveness of SSAs is critically dependent on the presence of SSTRs, and because the treatment with available SSAs [both, first generation (e.g., octreotide) and second generation (i.e., pasireotide)] has become the mainstay of medical therapy for tumour control in different ERCs expressing SSTRs, such as pituitary and gastroenteropancreatic neuroendocrine tumours (90, 114, 123-125). In fact, we demonstrated that octreotide (which acts primarily by binding the SSTR2 and with less affinity the SSTR5) and pasireotide (a multi-receptor ligand with high affinity for SSTR1, SSTR2, SSTR3, and SSTR5) significantly reduced proliferation rate in 22Rv1 cells (a cell model with an expression profile of SSTR1 = SSTR5 >>> SSTR2), while only pasireotide, but not octreotide, inhibited proliferation rate in PC-3 (a cell model with an expression profile of SSTR5 >>> SSTR2 > SSTR1), which reinforces the idea that PCa patients, especially patients with CRPC, could be sensitive to the antitumor actions of SSAs, opening new avenues to explore their potential as targeting therapy (alone or in combination with other drugs) for patients with CRPC.

This study also demonstrated that endogenous CORT is highly expressed in 22Rv1 and PC-3 cells, as well as in human PCa tissues compared with BPH samples. Remarkably, endogenous CORT expression was higher in metastatic PCa samples compared to primary tumours and non-tumour samples. As a result, Receiver Operating Characteristic (ROC) analysis revealed that endogenous CORT expression could discriminate between patients with PCa vs. BPH, and between patients that developed metastasis vs. those that did not. Moreover, the expression of endogenous CORT was positively correlated with the expression of SSTR1, SSTR2 and SSTR5 in metastatic PCa tissues but not in non-metastatic tissues. All these results suggest a causal link between dysregulation of endogenous CORT expression and PCa progression/aggressiveness and, therefore, that endogenous CORT may play a significant autocrine/paracrine pathophysiological role in AI-PCa cells, being its expression functionally linked to the dominant SSTR-subtypes expressed in PCa tissues. This hypothesis was confirmed when we silenced endogenous CORT levels in AI-PCa cells which resulted in a significant increase in proliferation rate in these cells, and in the modulation of the expression/levels of critical genes and oncogenic signalling pathways, including the reduction in the expression of different cell cycle inhibitors (e.g., CDK2, CDKN1A, CDKN1B, and/or CDKND). Moreover, our data are consistent with previous reports showing that SST, the other SSTR-subtypes ligand, also plays an important autocrine/paracrine role in several cellular models including colorectal cancer cells (126, 127). In fact, a constitutive activation of different SSTRs has been also reported since various SSTRs can display a relevant degree of ligand-independent constitutive activity in different cell systems (128). However, our results have particular relevance because, to the best of our knowledge, this is the first evidence demonstrating a potential autocrine/paracrine regulatory function for endogenous CORT in cancer cells, which might be functionally linked to the expression of the dominant receptors expressed in PCa cells (i.e., SSTR1, SSTR2 and SSTR5).

Remarkably, as previously mentioned, this study demonstrates that the proliferation rate of AI-PCa cells was significantly inhibited in response to octreotide and/or pasireotide; however, when CORT

expression was silenced, the treatment with these SSAs was completely inefficient in decreasing the proliferation rate, suggesting that the reduction in CORT levels could desensitize AI-PCa cells to the antitumour actions of SSAs treatment. Interestingly, we found that CORT-silencing was able to significantly down-regulate the expression of the dominant SSTR-subtypes expressed in 22Rv1 (i.e., SSTR1, SSTR2 and SSTR5) and in PC-3 (i.e., SSTR5), which might in part explain the desensitization observed in CORT-silenced AI-PCA cells to the antiproliferative effects of SSAs. Therefore, it seems plausible that additional factors, besides the simple abundance of endogenous CORT, might critically influence the SSAs response in PCa cells, including the presence of the truncated splicing SST_5TMD4 as has been previously suggested by our group in PCa and other tumour pathologies (83, 86, 89).

These results have been published in "Int J Mol Sci" journal (2022, Article I)

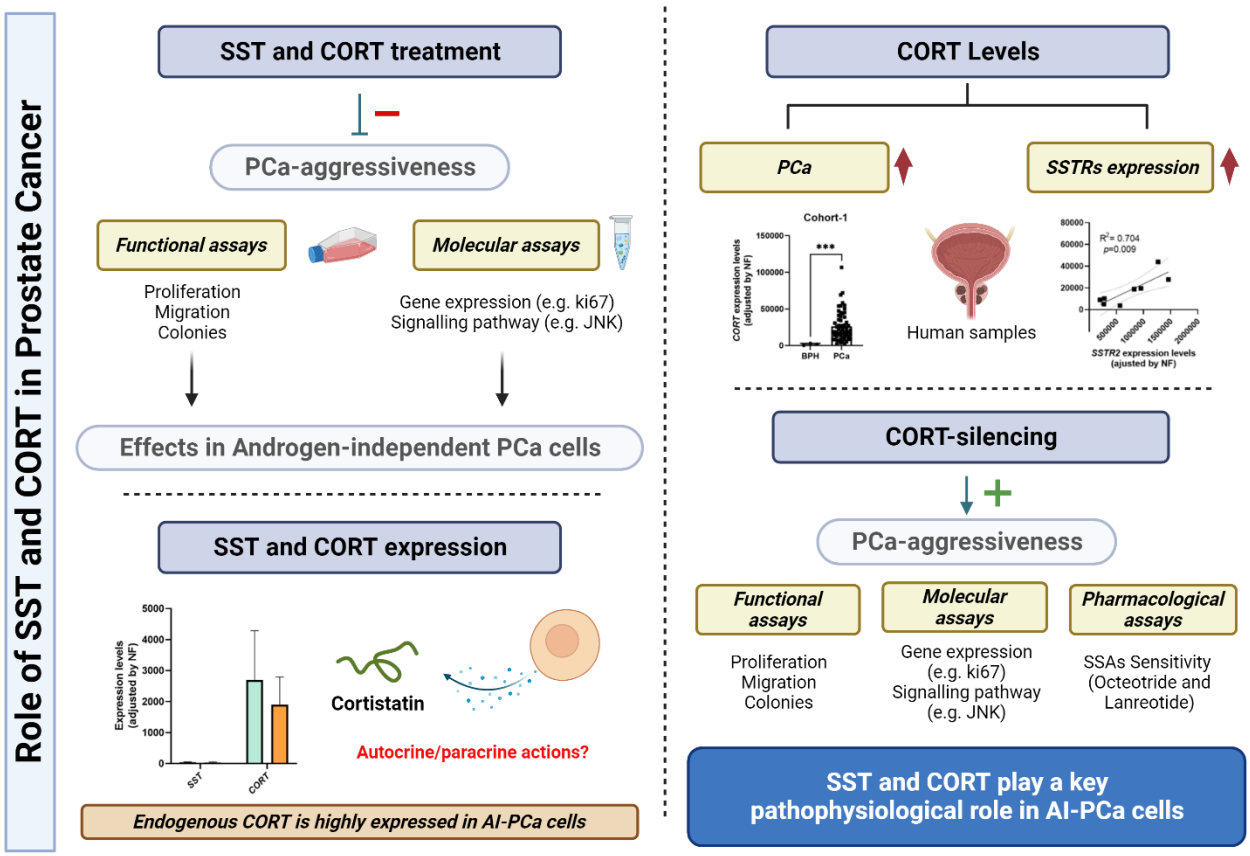

Figure 6: Graphical Abstract of Article I

<u>Article II:</u> Unleashing the Diagnostic, Prognostic and Therapeutic Potential of the Neuronostatin/GPR107 System in Prostate Cancer.

This work exploring the GPR107 presence and functional relevance in PCa revealed that GPR107 is present in a high proportion of PCa samples, and is overexpressed, at both mRNA and protein levels, in PCa tissues, as compared to non-tumour tissues in two independent cohorts of human samples. Most importantly, GPR107 overexpression was evidenced in samples from patients with metastasis compared to those without metastasis, a result that was compared favourably with data from two independent external *in silico* cohorts of patients (Grasso and Varambally datasets). As a result, ROC analysis revealed that GPR107 expression could discriminate between patients who developed metastasis vs. those who did not. Furthermore, GPR107 expression levels were directly associated with other relevant clinical parameters of PCa aggressiveness (i.e., tumour stage, vascular invasion and presence of metastasis), as well as with the expression levels of key molecular markers of PCa aggressiveness (e.g., CDK2, VEGFR, IL6R). These results reinforce the notion of a causal link between dysregulation of GPR107 expression and PCa aggressiveness, suggesting that this receptor may play a significant pathophysiological role in PCa cells. The contention of the potential oncogenic role of GPR107 in PCa is in line with a previous report indicating that GPR107 drives self-renewal and tumorigenesis of liver tumour-initiating cells (129).

Then, these results led us to further explore the functional pathophysiological role of GPR107 in PCa cell models. Silencing of GPR107 decreased proliferation and migration in two representative models of CRPC pathology (i.e., 22Rv1 and PC-3 cells), demonstrating that GPR107 is functionally active in AI-PCa cells, and that its presence is directly associated with their aggressive features. These results are in agreement with previous evidence indicating that the knock-down of GPR107 expression decreased aggressive features in liver tumour-initiating cells (i.e., impaired tumour initiation, self-renewal and invasion capacities) (129). Additionally, the functional data generated in the present study in response to GPR107-silencing could suggest that GPR107 displays a constitutive functional activity in PCa cells.

Remarkably, this is neither the sole nor the first time that constitutive activation of receptors belonging to the somatostatin-related regulatory system has been reported since various SSTRs have been demonstrated to display a relevant degree of ligand-independent constitutive activity in different cell systems (128).

Next, we interrogated the signalling pathways and molecular elements mediating GPR107 actions in PCa cells. This revealed that GPR107 might exert its tumour-associated functions through modulation of several molecular/signalling pathways, including a decreased activation (basal phosphorylation) of the AKT signalling pathway, which has been shown to be a key oncogenic-signalling pathway and cooperate in different tumour pathologies, including PCa, to promote malignancy, drug resistance and CRPC development (130). Moreover, silencing of GPR107 in AI-PCa cells decreased the expression levels of MKI67, a well-known proliferation marker associated with biochemical recurrence in PCa (131), and, in PC-3 cells, tended to increase the expression of CDKN2D, a cell-cycle inhibitor involved in the growth arrest at senescence of PCa cells (116). Similarly, GPR107-silencing resulted in a decrease of the expression of two genes (MMP9 and PRPF40A) involved in the process of migration and cytoskeletal regulation, respectively (132, 133). Interestingly, we also found that the decrease in the aggressiveness of PCa cells in response to GPR107-silencing could involve a diminished expression of the splicing variants SST_5TMD4, In1-ghrelin, AR-v7, and EZH2, four elements previously reported as key oncogenic factors in PCa and/or main drivers of CRPC (49, 89, 134, 135). Interestingly, GPR107 expression was correlated with SST_5TMD4 in highly aggressive PCa samples, which reinforces the idea of a role for GPR107 as a potential therapeutic target in PCa, in that we have reported that SST_5TMD4 is a key pathophysiological component in this cancer type. Moreover, this last result has special relevance because GPR107 silencing was able to consistently decrease SST_5TMD4 expression in all the AI-PCa models tested herein, in which we previously demonstrated that overexpression of SST_5TMD4 is directly associated with the inefficiency of SSAs therapy (i.e. octreotide treatment) in PCa cells and other tumour types (83, 89, 113).

Finally, in order to further explore the potential utility of the NST-GPR107 axis as a therapeutic target in PCa, functional and mechanistic studies were performed in response to NST treatment in PCa cells. Our results revealed for the first time that NST treatment evoked virtually similar antitumor effects (i.e., reduction of proliferation and migration capacity) to those previously observed with GPR107-silencing in AI-PCa cells. Similarly, treatment with NST induced a signalling and molecular regulatory response comparable to that of the GPR107-silencing treatment (i.e. inhibition of AKT signalling pathway and modulation of the expression of MKI67, CDKN2D, MMP9, PRPF40A, AR-v7, SST_5TMD4, In1-ghrelin, AR-v7 and EZH2), which reinforces the idea that the antitumor actions observed in response to GPR107-silencing or NST treatment might be functionally connected and mediated through similar mechanisms and/or signalling pathways. Moreover, in support of this notion is the fact that the combined treatment of NST and GPR107-silencing did not modify the anti-proliferative/migratory actions of both treatments individually in AI-PCa cells. Lastly, we also found that NST administration significantly decreased GPR107 levels in AI-PCa cells, which might indicate that the antitumor actions of NST might be exerted, at least in part, by decreasing the expression levels of GPR107 in AI-PCa cells.

These results have been published in "J Clin Med" journal (2020; Article II)

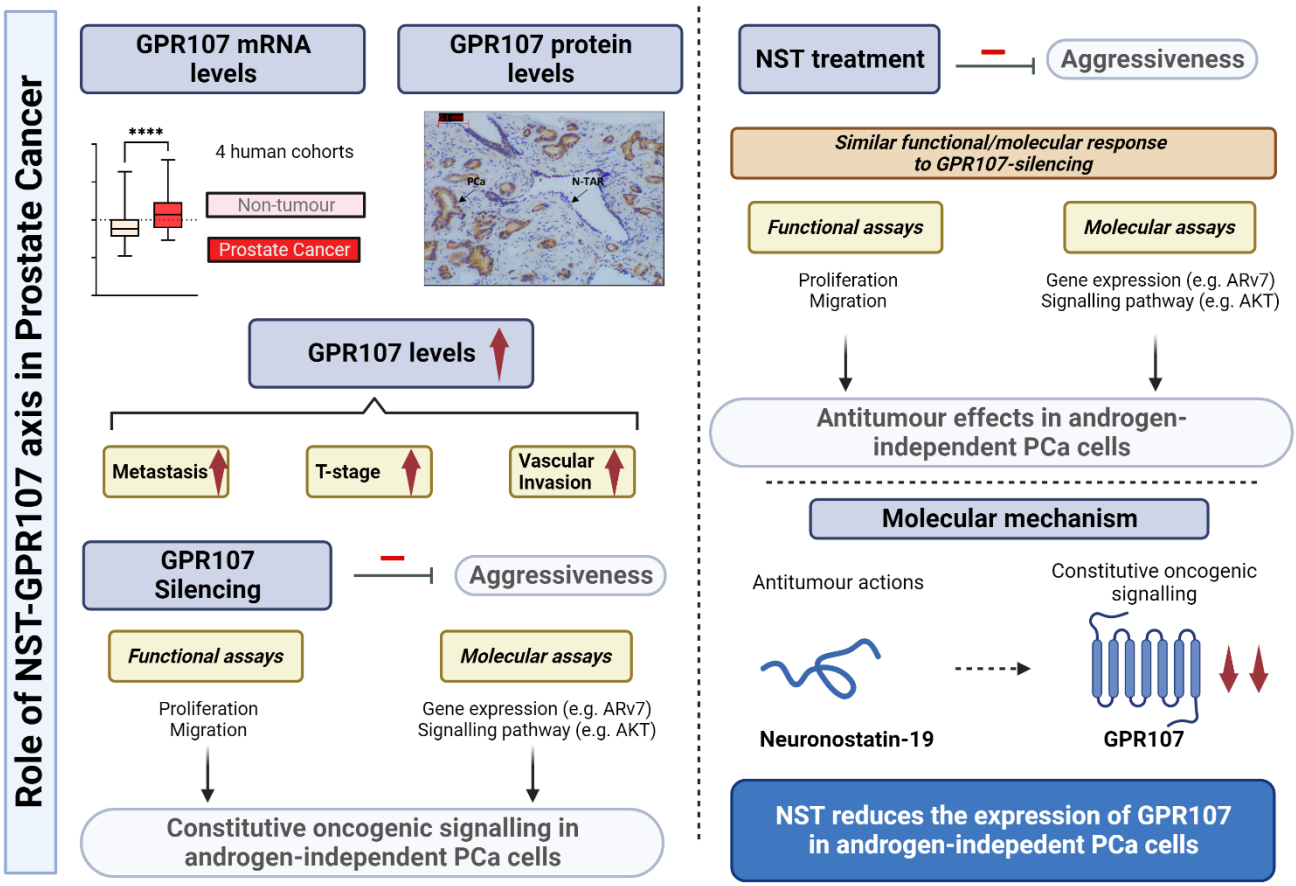

Figure 7: Graphical Abstract of Article II

<u>Article III:</u> Dysregulation of RNA-Exosome machinery is directly linked to major cancer hallmarks in prostate cancer: oncogenic role of PABPN1.

This study demonstrated for the first time a drastic dysregulation of the expression profile of the components of the RNA-exosome machinery in a well-characterized cohort of PCa samples vs. healthy-tissues, wherein a representative set of cofactors and core-elements was markedly altered (73% and 45%, respectively). Notably, our analyses revealed that PABPN1 had the higher capacity to discriminate between PCa and control tissues, and that the overall PABPN1 overexpression (at mRNA and protein level) found in different PCa sample cohorts was positively correlated with key clinical parameters (see below).

PABPN1 is a critical element of the REC since interacts with the ZFC3H1 protein to form the PAXT complex in the nucleus (136), wherein it plays two well-known REC-dependent functions: the nuclear decay of mRNAs, and the turnover of several lncRNAs (137). Additionally, PABPN1 has other REC-independent intracellular functions including the polyadenylation and length-control of the polyadenine-tail, the nuclear export of polyadenylated-RNAs, and the regulation of the alternative polyadenylation process (137-139). Remarkably, we found that high PABPN1 levels in PCa was positively correlated with key clinical (T-stage/perineural-invasion), molecular (EZH2/CDK4-levels) and aggressiveness [metastasis/poor clinical-outcome/shorter Disease-free survival (DFS)] parameters. This later finding is consistent with a recent study indicating that PABPN1 is overexpressed in PCa and positively associated with shorter progression-free survival of the patients (140). All these observations suggested a causal link between PABPN1-dysregulation and PCa aggressiveness, which might represent a useful tool as a diagnostic/prognostic biomarker and a potential therapeutic target to tackle PCa.

Indeed, PABPN1-silencing in metastatic PCa cell models induced marked reductions in aggressiveness features (i.e., proliferation and migration). Most notably, PABPN1-silencing also strikingly decreased the number of PCa stem/progenitor-cells of tumourspheres and colonies, a relevant functional result that may open new research avenues to overcome the well-known resistance of metastatic PCa cells to different current drugs (36). In line with our results, previous studies have also indicated a potential oncogenic role of PABPN1 *in vitro* in cervical cancer (141) and breast cancer (142). This study also demonstrates that PABPN1 is an effective target in PCa *in vivo*, since PABPN1-silencing effectively blocks PCa progression of already established PCa tumours, and decreased tumour-volume, mitosis numbers and KI67 expression, thus further demonstrating the clinic-pathophysiological relevance of the antitumour role of PABPN1-silencing in PCa, and its potential value as a future therapeutic target in this disease. However, it should be noted that these data are not in accordance with a recent report showing that PABPN1-silencing increased aggressiveness features in bladder cancer *in vitro* and *in vivo* (111). This dual function is not surprising since other components of the REC such as

DIS3 might exert antitumour action in several haematological cancers but facilitates the progression of breast, liver, colorectal, ovarian, and pancreatic tumour pathologies (143).

To interrogate the molecular mechanisms linked to the functional role of PABPN1 in PCa, we explored an ample set of cancer-related lncRNAs and mRNAs involved in different pathophysiological processes in response to PABPN1-silencing in PCa cells (DU145 and LNCaP) since a pivotal role of the REC has been previously demonstrated in controlling the expression of key lncRNAs (144, 145) and mRNAs (146, 147) in human cells. Our data revealed, for the first time, a striking alteration in multiple lncRNAs in PCa cells. Of note, PABPN1-silencing led to a downregulation of FALEC and DLEU2 lncRNAs in both PCa cell lines which have been shown to promote important oncogenic processes in PCa (proliferation, migration, invasion and resistance to hormonal blockade) (148-150). Furthermore, PABPN1-silencing also significantly induced the expression of SPRY4-IT1 and DHRS4-AS1 lncRNAs in both PCa cell lines, which have been associated with apoptosis induction, inhibition of stemness capacity and epithelial-mesenchymal transition in liver, lung, and gastric cancers (151-153).

Furthermore, our report also demonstrated that PABPN1-silencing induced an alteration in the expression levels of some critical mRNA-coding genes. Interestingly, PABPN1-silencing reduced CDK2 and increased CDKN1A and APC mRNA levels in both PCa cell lines, being CDK2 a regulator of the G1-phase progression and the G1 to S transition during the cell-cycle (115), and CDKN1A and APC negative regulators of CDK2/CDK4 function and Wnt-signalling (115, 154). However, it is also important to mention that molecular mechanisms linked to the critical role of PABPN1 in PCa cells are partially cell-line dependent (i.e., specific lncRNAs/mRNAs types were differentially altered in DU145 and LNCaP cells), which might be due to specific phenotypic differences between the two PCa cell-models used (mutation profile, chemical modifications, protein-interactions, etc.) (155). In this sense, similar divergences in response to the modulation of REC-components were found in other tumour cell-models (i.e., colorectal and pancreatic cancer) (110, 156), which have been also attributed to the distinct nature of the cell models. Nonetheless, our data clearly demonstrate that PABPN1 is functionally active

in PCa cells, and that its presence is directly associated with their progression and/or aggressiveness features.

The results of this study open a new research avenue in the study of PCa since this is the first report demonstrating that: 1) PCa cells express and release PABPN1; 2) PABPN1 protein can be detected in human plasma, and; 3) PABPN1 levels were significantly lower in PCa patients with biochemical recurrence vs. those without recurrence, and tended to be lower in PCa patients with metastatic vs. those without metastatic disease. Therefore, these results would suggest the potential utility of PABPN1 levels as a novel prognostic biomarker for PCa patients by using non-invasive biopsies. Supporting our results is the report demonstrating that the PABPC1 (a homolog protein of PABPN1) has also a potential use as a biomarker for human metastatic duodenal cancer since it is actively secreted within exosome by these cells (157). Furthermore, we demonstrate that metastatic PCa cells (DU145/LNCaP) secrete lower PABPN1 protein levels to possibly maintain significant higher PABPN1 levels inside metastatic PCa cells vs. healthy prostate cells as an oncogenic feature. Supporting this idea are our data demonstrating that PABPN1-overexpression increase the migration rate of metastatic PCa cells, as well as a previous report indicating that mouse models of carcinoma PCa with a higher rate and shorter latency of tumour recurrence after castration presented high levels of antibody against PABPN1 (158). Obviously, further work will be required to complete our understanding about this oncogenic process and to fully elucidate the translational potential behind these interesting and potentially relevant observations.

Finally, our study also provides an initial, unprecedented proof-of-concept on the suitability of RNA-exosome dysregulation as a novel potential target for PCa treatment by demonstrating that the pharmacological inhibition of the activity of the RNA-exosome machinery [by isoginkgetin; a flavonoid obtained from Ginkgo Biloba (159)] exerts clear antitumour effects on PCa cells (inhibition of proliferation, migration and colonies formation]. Some reports have associated the *in vitro* treatment of this compound with antitumour properties in different cancers (liver, brain, haematological cancers and fibrosarcoma (160-163), but its actions in PCa were still unknown. To the best of our knowledge, no

clinical trials have been carried out to explore the utility of this specific compound in cancer, but the translational and clinical relevance of flavonoids as a promising therapeutic approach in cancer has been recently reviewed elsewhere (164). Therefore, when viewing together our data add compelling evidence demonstrating that targeting the RNA-exosome machinery might translate into a beneficial effect in patients with PCa, an observation that certainly warrants further investigation.

These results have been published in "Cancer Letters" journal (2024; Article III)

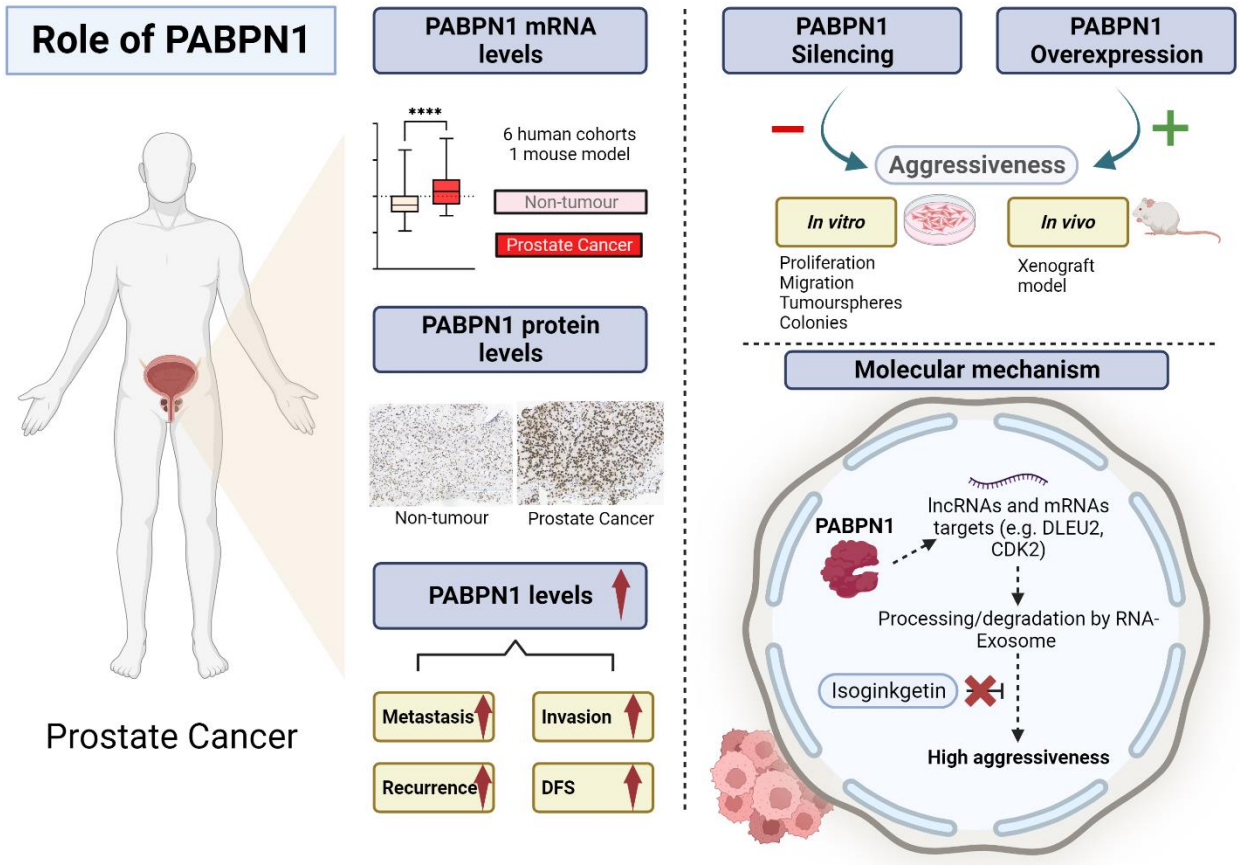

Figure 8: Graphical Abstract of Article III

General conclusions

The **MAIN CONCLUSIONS** associated with each section/article of this Doctoral Thesis are described below:

Article I

1) SST and CORT treatment reduces different tumour parameters in AI PCa cells through the modulation of key genes (e.g., MKI67, CDK2, CDKN1B, MMP9, EZH2, etc.) and classical oncogenic signalling pathways (i.e., PTEN-PI3K-AKT and JNK).

2) Endogenous CORT levels are increased in PCa patients and associated with key clinical (e.g., metastasis) and molecular (SSTR2 and SSTR5 expression) features.

3) Endogenous CORT silencing increased the proliferation rate of AI PCa cells and unsensitized these cells to the actions of SSAs, suggesting that CORT could exert potential autocrine/paracrine actions in a CRPC phenotype.

Article II

4) The GPR107 receptor is overexpressed in PCa, especially in metastatic PCa, and its expression levels are associated with key aggressiveness features of PCa (e.g., T-Stage, vascular invasion, etc.).

5) GPR107-silencing and NST treatment altered key pathophysiological parameters in AI PCa cells, including a reduction of cell proliferation and migration as well as the modulation of relevant molecular markers (e.g., MKI67, SST_5TMD4, AR-v7, etc.) and oncogenic signalling pathways (e.g., p-AKT, p-ERK).

6) NST significantly reduces the expression level of GPR107 in AI PCa cells, suggesting that the antitumor effects of NST might be exerted, at least in part, through the downregulation of this receptor.

Article III

7) Different components of the REC (cofactors, nucleases, and core elements) are drastically altered in PCa compared with non-tumour tissues, being this dysregulation especially evident in the case of PABPN1 which is directly associated with the progression and aggressiveness of PCa (i.e., T-stage, perineural invasion, metastasis, recurrence, and shorter disease-free survival).

8) The modulation of PABPN1 plays a crucial pathophysiological *in vivo* and *in vitro* role (i.e., cell proliferation, migration, tumourspheres/colonies formation, mitosis number, etc.) through the modulation of key lncRNAs (PCA3, FALEC, DLEU2) and mRNAs (CDK2, CDK6, CDKN1A) involved in important oncogenic processes.

9) Plasma PABPN1 levels are altered in patients with metastatic and tumour relapse, suggesting a potential utility of this factor as a non-invasive prognostic tool for PCa progression.

10) Pharmacological inhibition of the REC activity (using isoginkgetin) reduces aggressiveness features of PCa cells, opening a potential therapeutic avenue for the treatment of this devastating disease.

Taken together, the results of this Doctoral Thesis unveiled new conceptual and functional avenues in PCa with potential clinical implications, by demonstrating that key components of the SST/CORT/NST system and of the REC are deeply dysregulated in PCa, wherein they play a crucial pathophysiological role in the development, progression and aggressiveness of this disease, representing a source of novel diagnostic, prognostic, and therapeutic targets that could be used to improve the diagnosis, management, and survival of PCa patients.

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Articles I-III

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Somatostatin, Cortistatin and Their Receptors Exert Antitumor Actions in Androgen-Independent Prostate Cancer Cells: Critical Role of Endogenous Cortistatin

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Abstract: Somatostatin (SST), cortistatin (CORT), and their receptors (SSTR1-5/sst5TMD4-TMD5) comprise a multifactorial hormonal system involved in the regulation of numerous pathophysiological processes. Certain components of this system are dysregulated and play critical roles in the development/progression of different endocrine-related cancers. However, the presence and therapeutic role of this regulatory system in prostate cancer (PCa) remain poorly explored. Accordingly, we performed functional (proliferation/migration/colonies-formation) and mechanistic (Western-blot/qPCR/microfluidic-based qPCR-array) assays in response to SST and CORT treatments and CORT-silencing (using specific siRNA) in different PCa cell models [androgen-dependent (AD): LNCaP; androgen-independent (AI)/castration-resistant PCa (CRPC): 22Rv1 and PC-3], and/or in the normal-like prostate cell-line RWPE-1. Moreover, the expression of SST/CORT system components was analyzed in PCa samples from two different patient cohorts [internal (n = 69); external (Grasso, n = 88)]. SST and CORT treatment inhibited key functional/aggressiveness parameters only in AI-PCa cells. Mechanistically, antitumor capacity of SST/CORT was associated with the modulation of oncogenic signaling pathways (AKT/JNK), and with the significant down-regulation of critical genes involved in proliferation/migration and PCa-aggressiveness (e.g., *MKI67*/*MMP9*/*EGF*). Interestingly, CORT was highly expressed, while SST was not detected, in all prostate cell-lines analyzed. Consistently, endogenous CORT was overexpressed in PCa samples (compared with benign-prostatic-hyperplasia) and correlated with key clinical (i.e., metastasis) and molecular (i.e., *SSTR2*/*SSTR5* expression) parameters. Remarkably, CORT-silencing drastically enhanced proliferation rate and blunted the antitumor activity of SST-analogues (octreotide/pasireotide) in AI-PCa cells. Altogether, we provide evidence that SST/CORT system and SST-analogues could represent a potential therapeutic option for PCa, especially for CRPC, and that endogenous CORT could act as an autocrine/paracrine regulator of PCa progression.

Keywords: somatostatin; cortistatin; prostate cancer; somatostatin analogues; therapeutic tool

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1. Introduction

Prostate cancer (PCa) represents the most common cancer type among men worldwide and the second cause of cancer-related death in this collective [1]. The main problem associated with this pathology is the management of the advanced disease, which consequently represents more than 80% of PCa-related deaths [2]. Due to its hormone dependency, the diagnosis of advanced PCa is followed by androgen deprivation therapy (ADT), usually combined with certain drugs such as Abiraterone or Enzalutamide/Daroluamide/Apalumatied,

which block the androgen receptor signaling pathway in PCa cells [3,4]. Unfortunately, between 10–20% of the patients develop resistance to these approaches, leading to the development of the most aggressive PCa phenotype, called castration-resistant prostate cancer (CRPC) [5,6]. Currently, this stage is mainly treated with androgen-synthesis inhibitors (e.g., Abiraterone), androgen receptor (AR)-inhibitors (e.g., Enzalutamide), and/or taxanes (e.g., Docetaxel) [7]. In addition, new therapies are being introduced to manage the advanced stage of PCa including platinum-based therapies (e.g., Cisplatin, carboplatin, etc.) or PARP-inhibitors (e.g., Olaparib, Rucaparib, etc.) [8–11]. However, despite the improvement in the overall survival associated with the aforementioned therapies, CRPC remains lethal nowadays [5,6]. Therefore, it is necessary to further understand the biology of PCa, in order to develop novel or optimize available medical therapeutic approaches to tackle this disease, especially the CRPC phenotype.

In this sense, the somatostatin, cortistatin and somatostatin-receptors (SST/CORT/SSTRs) system represents a useful source of diagnostic/prognostic biomarkers and therapeutic targets to manage and treat various endocrine-related cancers (ERCs), owing to its pleiotropic functional role encompassing whole body homeostasis to cancer cell functioning in different tumor types, wherein this system commonly acts to inhibit multiple processes, such as hormone secretion and cell proliferation, migration and invasion [12–17]. In fact, we have recently demonstrated that certain components of the SST system, especially some SSTR-subtypes [SSTR1-5, encoded by the somatostatin receptor 1–5 genes (*SSTR1-5*)], are dysregulated in PCa tissues and cells, wherein they play a relevant role in the pathophysiology of this disease [18–20]. Specifically, the presence of SSTR1 and the truncated splicing variant sst5TMD4 could exert relevant pathophysiological roles by regulating different tumor parameters in PCa cells [e.g., cell proliferation, migration and PSA secretion; disruption of the normal response to somatostatin analogs (SSAs)] [18–20]. However, the presence and/or functional roles of other key components of this hormonal system, including the endogenous ligands SST and CORT (both able to bind all SSTRs with comparable affinities [21]), have hitherto not been fully explored in PCa. Moreover, due to the relevance of this hormonal system in cancer, synthetic SSAs [e.g., first generation (octreotide, lanreotide), and second generation (pasireotide)] have been developed and are widely used as valuable tools to treat multiple ERCs, including pituitary and neuroendocrine tumors [12,22,23]. However, attempts to apply SSAs in PCa have yielded controversial results since the limited studies reported so far did not show improvement in overall survival [24], and the mechanistic reasons for those clinical failures are not fully known.

Based on the information described above, the current study was aimed at exploring, for the first time, the presence of the entire SST/CORT/SSTRs system in PCa, and to perform a parallel comparison of the in vitro effects of SST and CORT peptides on different normal-prostate and prostate tumor (PCa and CRPC) cell models, in order to design new diagnostic, prognostic, and therapeutic approaches that could impact the management of PCa, especially CRPC.

2. Results

2.1. SST and CORT Treatment Exert Antitumor Actions Exclusively in Androgen-Independent PCa Cells, but Not in Androgen-Dependent PCa Cells or Normal Prostate Cells

Treatments with SST or CORT peptides [10^{-7} M; dose based on previous reports (see Material and Methods sections below)] did not alter the proliferation rate in normal-prostate (RWPE-1) or AD-PCa (LNCaP) cell models (Figure 1A); however, they significantly decreased proliferation rate in two AI-PCa cell models (22Rv1 and PC-3; Figure 1A). Additionally, SST treatment considerably decreased the number of colonies formed in 22Rv1 and PC-3 cells (Figure 1B, top-panel), while this inhibition was also significantly observed in response to CORT in 22Rv1, but not in PC-3, cells (Figure 1B, bottom-panel). Similarly, SST treatment significantly reduced the migration rate in PC-3 cells, while CORT only tended to reduce ($p = 0.09$) this capacity (Figure 1C).

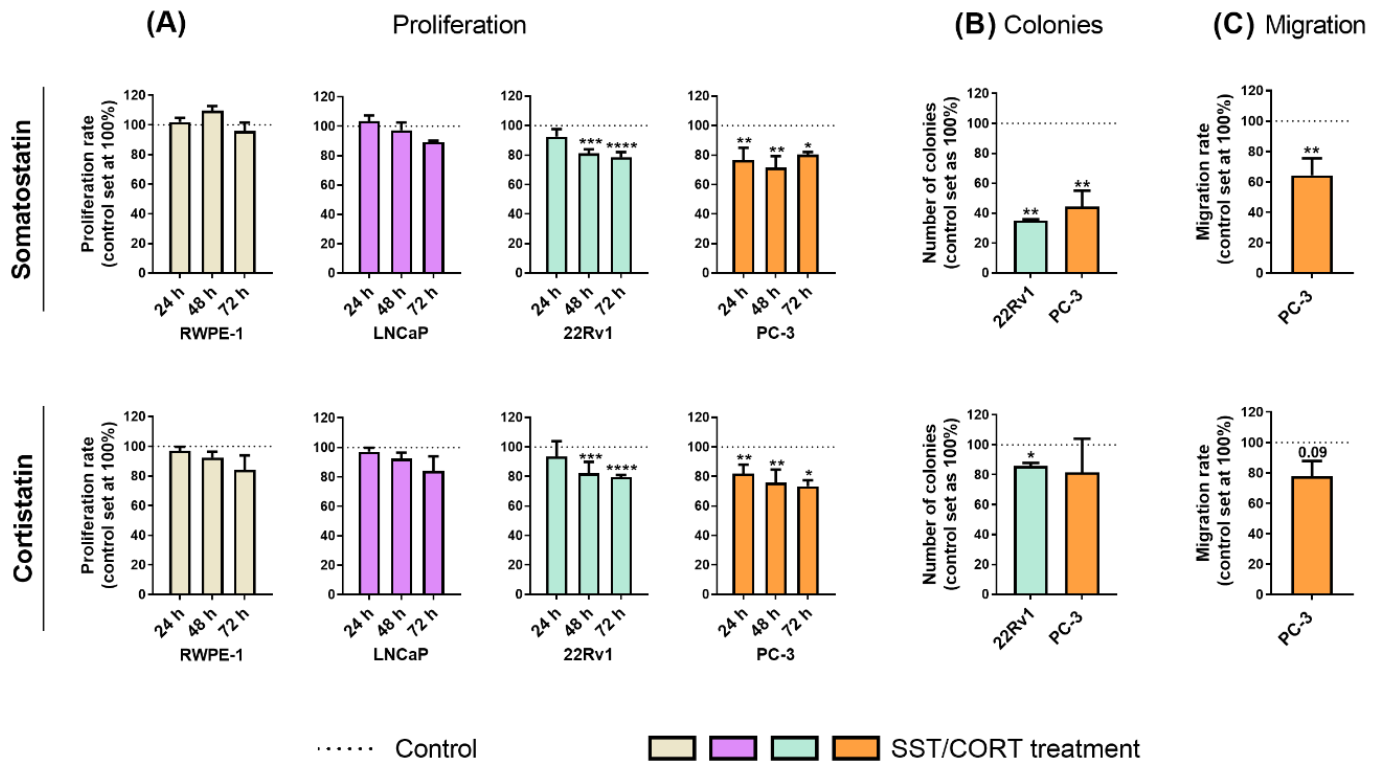

Figure 1. Functional effects after somatostatin (SST) and cortistatin (CORT) treatment in prostate cells. (**A**) Proliferation rate of normal prostate (RWPE-1) and prostate cancer (PCa) cells [androgen-dependent (LNCaP) and androgen-independent (AI; 22Rv1 and PC-3)] in response to SST and CORT treatment (after 24, 48 and 72 h). (**B**) Colonies formation in response to SST and CORT treatment in AI-PCa cells. (**C**) Migration rate of PC-3 cells after 16 h of SST and CORT treatment. Data were represented as percent of vehicle-treated cells (set at 100%). Asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$) indicate statistically significant differences between groups.

2.2. SST and CORT Treatment Modulates the Levels of Key Oncogenic Signaling Pathways and Tumor-Related Genes in Androgen-Independent PCa Cells

Based on the results previously showed, we next explored the potential signaling-pathways modulated in response to SST and CORT treatment in AI-PCa cells. Firstly, phosphorylation levels of key proteins belonging to different oncogenic signaling pathways and/or associated with PCa development/aggressiveness (i.e., Protein kinase B (AKT), extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), Phosphatase and Tensin Homolog (PTEN) and Androgen Receptor (AR)] were determined by Western blotting after 30 min of SST and CORT exposition (Figure 2A,B).

This analysis revealed that treatment with SST and CORT cells significantly decreased the phosphorylation levels of AKT, but not of ERK, PTEN or AR, in 22Rv1 and PC-3 cells (Figure 2A,B, respectively). Moreover, CORT (but not SST) also decreased the phosphorylation levels of JNK in 22Rv1 and PC-3 cells (Figure 2A,B, respectively). As previously reported elsewhere [25], PC-3 did not express PTEN nor AR at a protein level (Figure 2B).

To further understand the molecular mechanisms underlying the SST and CORT effects, we also evaluate the expression levels of key genes related to proliferation/cell-cycle, migration, and aggressiveness in AI-PCa models after 24 h of SST and CORT exposition (Figure 2C). In 22Rv1 cells, SST treatment significantly decreased the expression levels of the cyclin-dependent kinase 4 (*CDK4*), the cyclin-dependent kinase inhibitor D (*CDKND*), the matrix metallopeptidase 9 (*MMP9*), and the enhancer of Zeste homolog 2 (*EZH2*) (Figure 2C, left-panel). Moreover, SST treatment increased the expression levels of the cyclin-dependent kinase inhibitor 1A and 1B (*CDKN1A* and *CDKN1B*) and *PTEN* in 22Rv1 cells (Figure 2C, left-panel). Likewise, CORT treatment also decreased in 22Rv1 cells the

expression levels of *MKI67*, N-Cadherin 2 (*CDH2*), *EGF*, and Proto-Oncogene C-Myc (*MYC*) (Figure 2C, right-panel); and increased the expression of *CDKN1A*, *CDKN1B*, and *CDKND* (Figure 2C, right-panel).

Similarly, SST and/or CORT treatment significantly reduced in PC-3 cells the expression levels of the proliferation markers *MKI67*, *CDK2*, *CDK4*, *CDK6*, *MMP3* (only CORT), *MMP9* (only SST), *MMP10* (only SST), the endothelial grow factor (*EGF*), *EZH2*, *MYC*, and the vascular endothelial growth factor receptor (*VEGFR*) (Figure 2C).

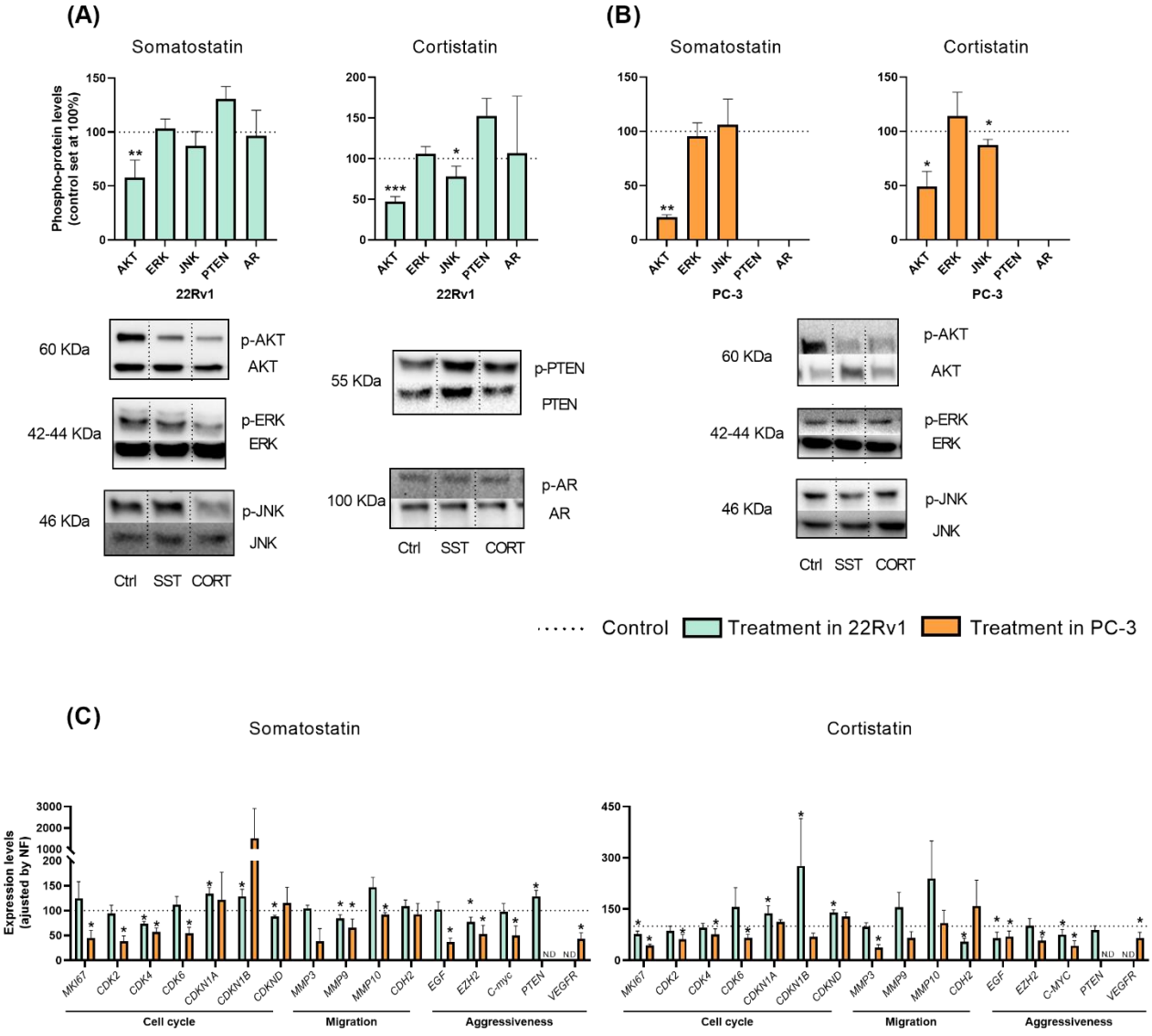

Figure 2. Molecular consequences of somatostatin (SST) or cortistatin (CORT) treatment in androgen-independent (AI) prostate cancer (PCa) cells. (**A**,**B**) Phosphorylation levels of protein belonging to different oncogenic signaling pathways (AKT, ERK, JNK, PTEN and AR) in response to SST and CORT treatment in AI-PCa cells. Phospho-protein levels were normalized by the total amount of each respective protein. Protein data were represented as percent of vehicle-treated cells (set at 100%). (**C**) Fold change in markers of proliferation, migration, and PCa-aggressiveness in response to SST and CORT treatment in AI-PCa cells. Gene expression was represented as the percentage of vehicle-treated cells (set at 100%). Asterisks (* $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$) indicate statistically significant differences between treatment and vehicle-treated cells. N.D: Non-detected. Ctrl: Control.

2.3. Expression of Somatostatin Receptors in Androgen-Independent PCa Cells and in PCa Tissues

We next interrogated the expression of all SSTR-subtypes in AI-PCa cells in order to identify which receptors might be mediating the antitumor actions and molecular-related events previously observed (Figures 1 and 2) in response to SST and CORT treatment. A variable expression level for each of the SSTR-subtypes was found in 22Rv1 and PC-3 (Figure 3A). Specifically, the present work revealed that *SSTR1* and *SSTR5* are the dominant SSTR-subtypes expressed in 22Rv1 cells (mean ± SEM: 1.751 ± 592.9, and 2.172 ± 856.9 mRNA copy number, respectively), followed by significant lower levels of *SSTR2* > *sst5TMD4* > *SSTR3* (345.3 ± 90.65; 24.99 ± 7.902; 11.74 ± 5.84; respectively; *SSTR4* and *sst5TMD5* expression levels were very low or undetectable) (Figure 3A).

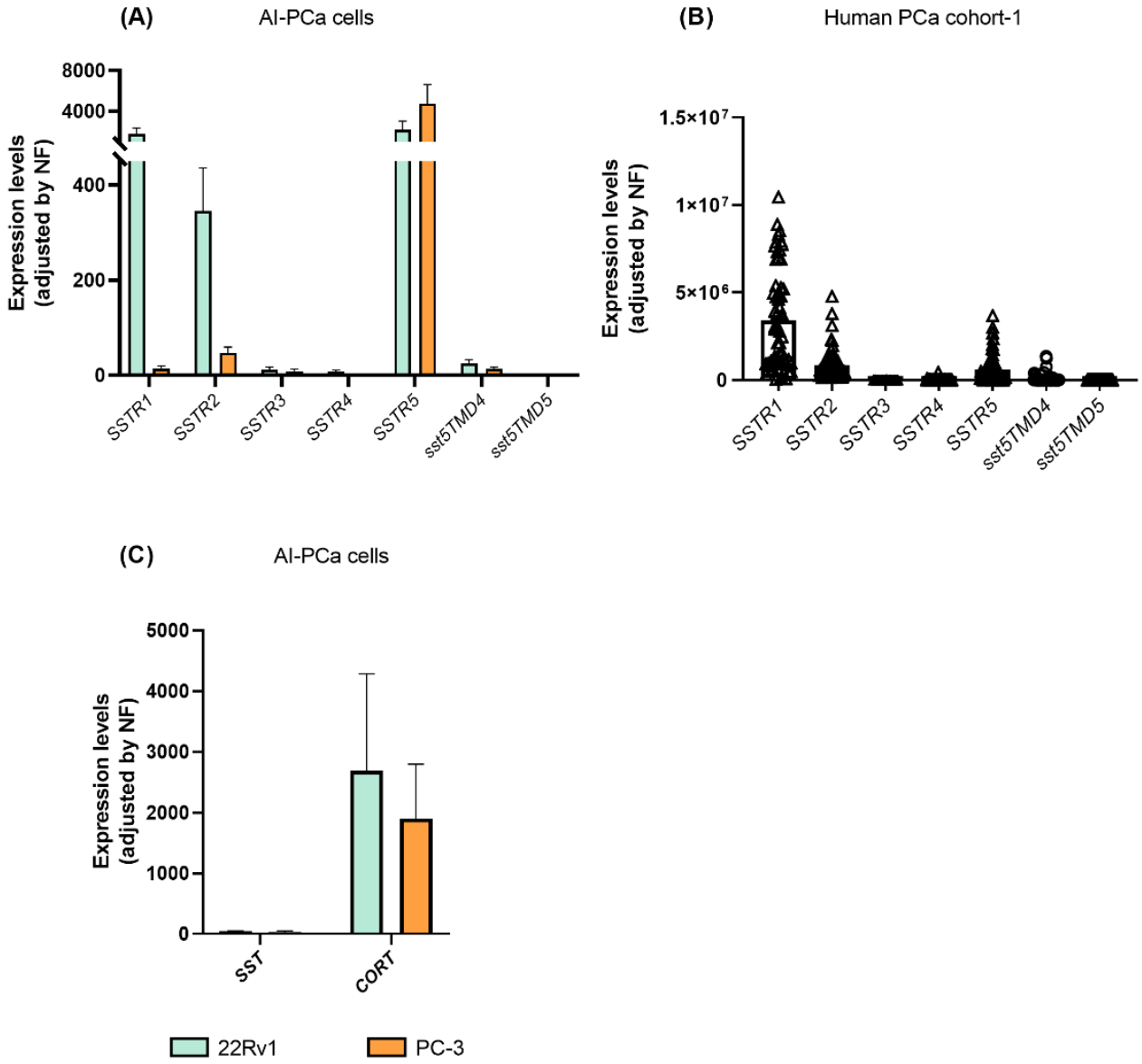

Figure 3. Expression profile of somatostatin-system [receptors (SSTRs), and ligands (somatostatin-SST and cortistatin-CORT)] in androgen-independent (AI) prostate cancer (PCa) cells and fresh prostate tissue (n = 69; cohort-1). (**A**) Expression of SSTRs in 22Rv1 and PC-3 AI-PCa cells. (**B**) Expression of SSTRs in PCa fresh samples. (**C**) Expression of SST and CORT in AI-PCa cells. Data represent the mean of mRNA copy number ± Standard Error of the Mean (SEM). mRNA levels were determined by quantitative polymerase chain reaction and adjusted by normalization factor (NF).

In PC-3 cells, *SSTR5* is the dominant SSTR-subtype expressed (mean ± SEM: 4,743 ± 1,893 mRNA copy number), followed by significant lower levels of *SSTR2* > *SSTR1* > *sst5TMD4* (46.70 ± 12.69; 14.12 ± 5.44; 13.17 ± 4.48, respectively, *SSTR3*, *SSTR4* and *sst5TMD5* expression levels were very low or undetectable) (Figure 3A).

Moreover, we also explored which SSTR-subtypes are expressed in human PCa tissues using the available samples from cohort-1 (Figure 3B). Specifically, we found that *SSTR1*, *SSTR2* and *SSTR5* were highly expressed in human PCa tissues (*SSTR1* ≥ *SSTR2* = *SSTR5*; mean ± SEM: 3,422,432 ± 362,369; 846,092 ± 110,588; and 602,159 ± 108,931 mRNA copy number, respectively). In contrast, expression levels of *sst5TMD4* were low, and *SSTR3*, *SSTR4* and *sst5TMD5* levels were very low or undetectable.

When viewing the results of Figure 3A,B together, it might be suggested that: (1) 22Rv1 and PC-3 were appropriate PCa cell models to perform the functional assays presented in this study (i.e., similar expression profile between AI-PCa cell models and human PCa tissues); and (2) human PCa might be sensitive to the actions of SST and CORT peptides as well as to different SSAs [first generation (octreotide; with high-affinity binding to SSTR2 and SSTR5) but specially to second generation (Pasireotide; a multireceptor-targeted SST with high affinity for SSTR1, SSTR2, SSTR3, and SSTR5)].

Finally, we also sought to determine whether endogenous *SST* and *CORT* were expressed in AI-PCa cells (Figure 3C). Interestingly, we found that endogenous *CORT*, but not *SST*, was highly expressed in 22Rv1 and PC-3 cells [mean ± SEM: CORT (2690 ± 1,595 and 1905 ± 889.9) vs. SST (44.12 ± 10.55 and 33.86 ± 16.21) mRNA copy number in 22Rv1 and PC-3, respectively; Figure 3C].

2.4. CORT Is Overexpressed in Human PCa Samples and It Is Associated with Aggressive Features

Based on the previous results, we next examined whether PCa tissues also express high levels of endogenous *CORT*. Our results revealed that, similar to the AI-PCa cell models previously analyzed (Figure 3C), *CORT* was also highly expressed in human PCa tissues. In fact, we demonstrated that *CORT* expression was significantly higher in PCa samples compared with BPH samples (used as controls; Figure 4A; cohort-1: see Materials and Methods below). Moreover, this differential expression was corroborated by Receiver Operative Characteristic (ROC) analyses since *CORT expression* levels was able to significantly discriminate between PCa vs. BPH samples, with an AUC (area under the curve) of 0.988 (p = 0.0045; Figure 4A).

Interestingly, although we did not observe any difference in the expression levels of endogenous *CORT* between primary tumors obtained from patients with metastasis compared to those without metastasis (cohort-1; Figure 4B), the expression of CORT was positively correlated with the expression of *SSTR2* and *SSTR5* in primary tumors obtained from patients with metastasis but not in those without metastasis [Figure 4C; a trend for significant (p = 0.1) was also observed for *SSTR1*]. Strikingly, analysis from the available Grasso in silico cohort revealed that endogenous *CORT* expression was higher in metastatic PCa samples compared to primary tumors and non-tumor samples (Figure 4D). Indeed, ROC analysis indicated that *CORT* expression significantly discriminated between metastatic vs. non-metastatic samples (AUC = 0.644, p = 0.023; Figure 4D).

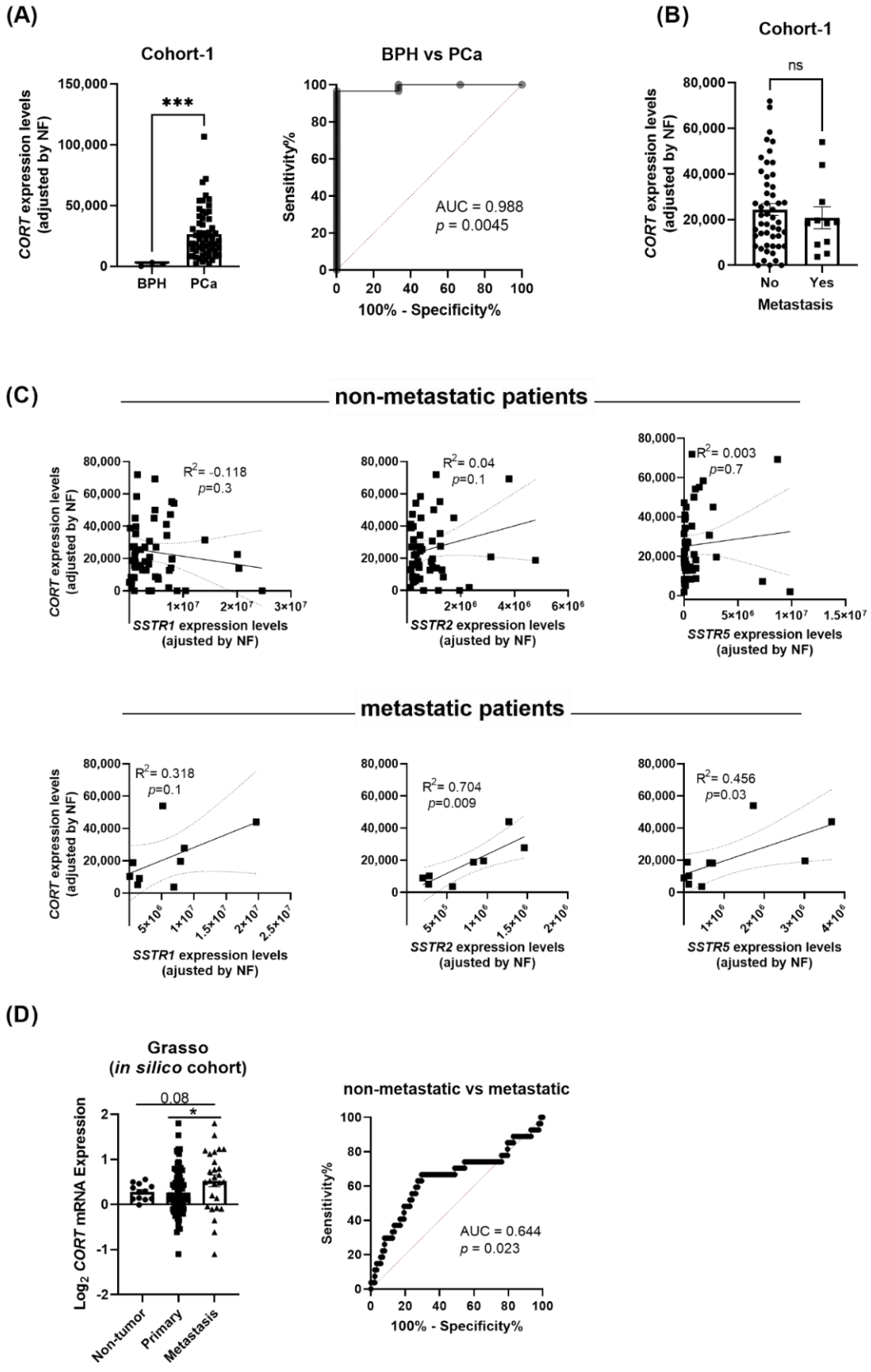

Figure 4. Expression levels of cortistatin (CORT) in prostate tissue. (**A**) Comparison of CORT expression levels between benign prostatic hyperplasia (BPH) and prostate cancer (PCa) samples (cohort-1).

ROC curve analysis comparing CORT expression in PCa vs. non-tumor BPH tissues, and associated AUC, is also indicated. (**B**) Comparison of CORT expression between primary tumors obtained from patients with metastasis vs. those without metastasis. (**C**) Correlation between CORT-expression and SSTR1, SSTR2, and SSTR5 expression in primary tumors obtained from patients without and with metastasis (cohort-1). (**D**) Comparation of CORT expression between non-tumor, primary tumor and metastatic samples obtained from the Grasso in silico cohort. ROC curve analysis comparing CORT expression and associated AUC from Grasso cohort is also indicated. mRNA levels were determined by quantitative polymerase chain reaction and adjusted by normalization factor (NF). Asterisks (* $p < 0.05$; and *** $p < 0.001$) indicate statistically significant differences between groups.

2.5. Endogenous CORT Modulates the Functional and Pharmacological Response of Androgen-Independent PCa Cells

To determine whether the high levels of endogenous CORT found in PCa cells/tissues could exert an autocrine/paracrine regulatory function in AI-PCa cells, we silenced the expression of endogenous *CORT* using a specific and validated siRNA (Figure 5A).

Our results indicate that CORT silencing increased the proliferation rate of 22Rv1 (after 48–72 h) and PC-3 cells (after 24–48–72 h; Figure 5B). However, it should be mentioned that this increase in the proliferative rate seemed to be cell line dependent [i.e., more sustained in time in 22Rv1 (maximum increment after 48–72 h) than in PC-3 cells (maximum increase at 24 h and then, a gradually decrease was observed at 48 and 72 h)], which might be explained in part to a potential different sensitivity to the transient transfection of the two PCa cell lines used (i.e., a loss of function over time is expected in all cell models after a transient transfection), and/or to specific phenotypic differences of the two PCa cell models (i.e., metabolic rate, aggressiveness, etc.). Additionally, no significant changes were observed in the number of colonies formed and in the migration rate in response to endogenous CORT silencing in PC-3 cells (see response to reviewer 1). However, we also explored the phosphorylation levels of AKT and JNK proteins in response to CORT silencing (pathways previously altered in response to CORT peptide treatment) which revealed that levels of JNK were up-regulated only in PC-3 cells (Figure 5C). In addition, gene expression levels of key cell cycle/proliferation markers and SSTRs were also evaluated in response to CORT silencing (Figure 5D). Specifically, a down-regulation in the expression of *CDK2* (in 22Rv1 and PC-3), of *CDKN1B* and *CDKND* (in 22Rv1 cells), and of *CDKN1A* and *CDKN2B* (in PC-3 cells) was observed after CORT silencing (Figure 5D). Interestingly, the silencing of CORT also reduced the expression of *SSTR1*, *SSTR2*, and *SSTR5* in 22Rv1 and the expression of *SSTR5* in PC-3 cells (Figure 5D).

Finally, we also evaluated whether the silencing of endogenous CORT could influence the responsiveness of AI-PCa cells to different SSAs [first generation (octreotide) and second generation (pasireotide)]. Specifically, we found that octreotide and pasireotide significantly reduced proliferation rate in scramble-intact 22Rv1 cells (Figure 5E). Similarly, pasireotide (but not octreotide) also inhibited proliferation rate in scramble-intact PC-3 cells (Figure 5E). In contrast, CORT silencing was able to completely block the antiproliferative effects of octreotide and pasireotide in both AI-PCa cell models (Figure 5E). These results suggest that altered endogenous CORT expression may influence selectively the antitumor response of SSAs in AI-PCa cells.

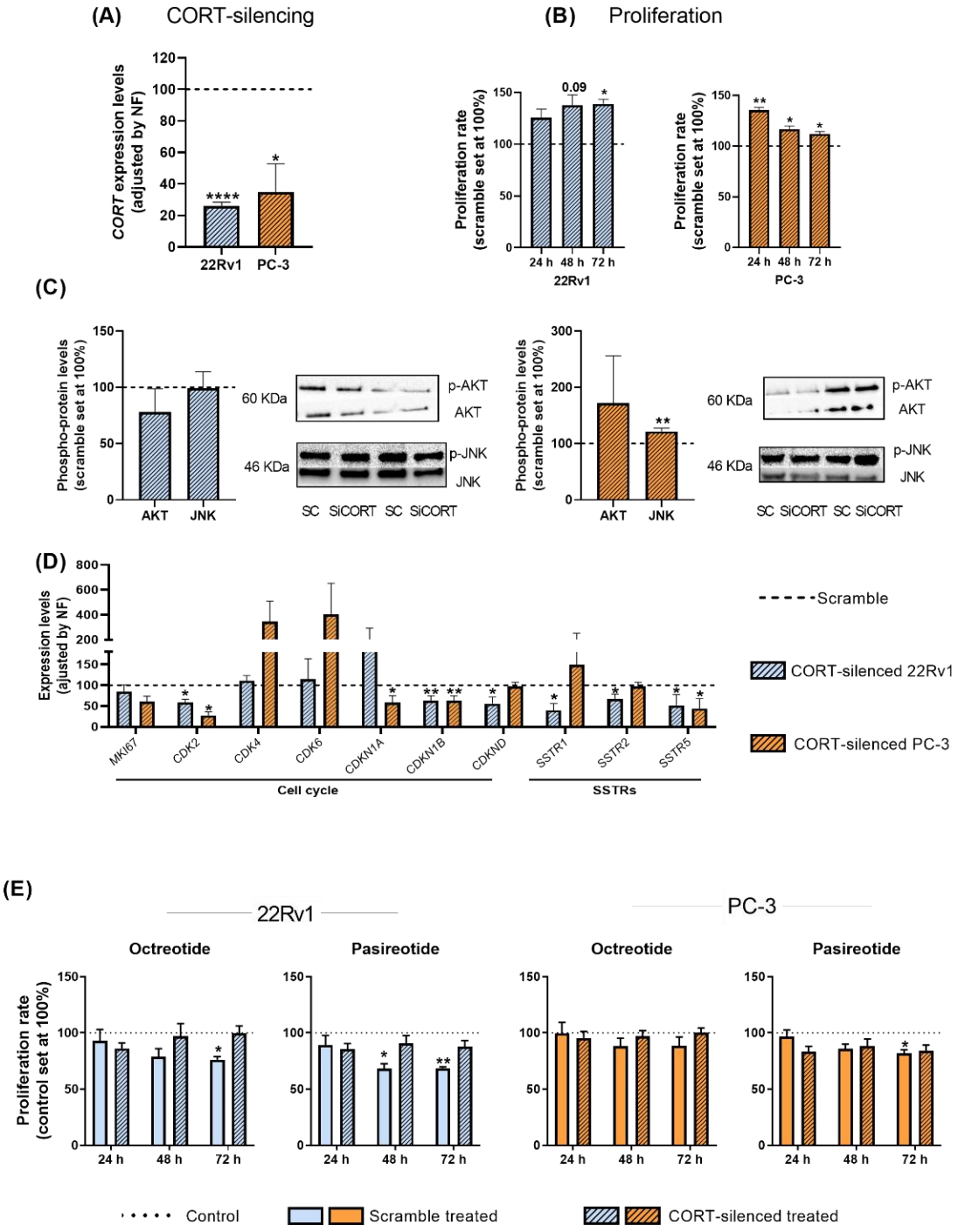

Figure 5. Functional and pharmacological consequences of cortistatin (CORT)-silencing in androgen-independent (AI) prostate cancer (PCa) cells. (**A**) Validation of CORT-silencing in 22Rv1 and PC-3 cells. (**B**) Proliferation rate in response to CORT-silencing in AI-PCa cells. Data were represented as percent of scrambled cells (set at 100%). (**C**) Phosphorylation levels of protein belonging to different oncogenic signaling pathways (AKT, JNK) in response to CORT-silencing in AI-PCa cells. (**D**) Expression of proliferation/cell-cycle and somatostatin receptors genes in response to CORT-silencing in AI-PCa cells. Data were represented as percent of scrambled cells (set at 100%). (**E**) Proliferation rate of scrambled AI-PCa cells or CORT-silenced AI-PCa in response to octreotide and pasireotide. Data were represented as the percent of vehicle-treated cells (set at 100%). Asterisks (* $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$) indicate statistically significant differences between groups. SC: Scramble. SiCORT: small interference RNA CORT.

3. Discussion

Despite new advances in clinical practice, the management of PCa remains one of the world's leading health problems [1,26]. In contrast to localized PCa, advanced disease represents the main cause of PCa-related death, causing more than 350,000 new deaths worldwide per year [1,26,27]. Then, new diagnostic, prognostic, and therapeutic alternatives are urgently needed to improve the clinical management of this pathology. In this sense, it is widely described that some components belonging to the SST/CORT-system are frequently altered and play a critical role in different ERCs, including PCa [18,19,28–30]. However, to the best of our knowledge, the pathophysiological role of the two natural ligands belonging to this system, SST and CORT, and their receptors has not been explored in parallel so far in PCa. Therefore, since this system has been very useful in other ERCs to identify new molecular biomarkers to better diagnose, predict prognosis and tumor behavior, and has provided tools to develop novel therapeutic strategies (i.e., SSAs), we aimed to explore the presence of this system (ligands and receptors) and the actions of these peptides and SSAs in PCa cells.

In this work, we observed that the treatment with SST and CORT peptides was able to reduce different key tumor parameters linked to tumor growth and metastasis (i.e., proliferation, migration, and colonies formation) only in AI-PCa cells (22Rv1 and PC-3 cells, two representative models of CRPC pathology), but not in normal prostate and AD-PCa cells, suggesting a potential and specific antitumor capacity of these peptides in the most aggressive phenotype of PCa. Interestingly and in line with these results, our group has recently described that neuronostatin (NST; a recently discovered peptide contained in the preproSST precursor polypeptide encoded by the SST gene but not sharing amino-acid homology to SST) also exerts a specific antitumor capacity in AI-PCa cells [18]. Therefore, all these results might suggest that this complex set of natural ligands (SST, CORT and NST) might exert antitumor actions exclusively in the most aggressive PCa phenotype, which could be considered an important clinical finding as will be discussed below.

Mechanistically, these antitumor effects of SST and CORT were associated with the alteration in the levels of critical genes and oncogenic signaling pathways that have been reported to be frequently associated with the functional and cellular control of the SST system in multiple ERCs (e.g., proliferation, migration, and PCa-aggressiveness features) [14,15,18,20,28,31,32]. Specifically, we found that SST and CORT could exert their antitumor actions in AI-PCa cells through the modulation of AKT, JNK, *MKI67*, *CDK2*, *CDK4*, *CDK6*, *CDKN1A*, *CDKN1B*, *CDKND*, *MMP3*, *MMP9*, *MMP10*, *CDH2*, *EGF*, *EZH2*, *C-MYC*, *PTEN*, and *VEGFR* levels. All these molecular events might be associated with the reduction in the proliferation, migration and colonies formation previously described in response to SST and CORT treatments, wherein some of these changes (especially the alteration of CDK2/4/6 and CDKN1A/1B) might be probably linked to an alteration in the cell cycle arrest (interruption of G1 to S transition), cellular matrix degradation and stem-like cell status [33–36]. However, it should be mentioned that the modulatory actions of SST and CORT were, in some cases, cell line dependent, which might be explained by the specific phenotypic differences between the two AI-PCA cell models used (i.e., mutation profile, aggressiveness, metabolic rate, etc. [25,37,38]). Additionally, these differences could be also attributed to the differential SSTRs expression profile found between 22Rv1 and PC-3 cells in the present study (i.e., *SSTR1* = *SSTR5* >>> *SSTR2* > *sst5TMD4* > *SSTR3* in 22Rv1 cells vs. *SSTR5* >>> *SSTR2* > *SSTR1* > *sst5TMD4* in PC-3 cells) since it has been reported that each SSTR-subtype can be linked to a different signaling pathway profile [12,39,40]. Nonetheless, our data clearly demonstrate that SST and CORT are functionally active inhibitors of proliferation, migration, and colonies formation exclusively in AI-PCa cells through the modulation of the levels of multiple key signaling molecules related to cancer development, progression and aggressiveness.

In this study, we also had the opportunity to analyze in parallel the expression pattern of all SSTR-subtype by a quantitative PCR method in a representative cohort of PCa tissues, which revealed that *SSTR1*, *SSTR2* and *SSTR5* were highly expressed in PCa tissues (i.e.,

SSTR1 ≥ *SSTR2* = *SSTR5*). Our results are in accordance with a previous study from our group indicating that SSTR1 is highly expressed in PCa tissues [18–20]. Notably, these data might be considered an important clinical finding because it might suggest that PCa tissues could be sensitive to the actions of SSAs, as the responsiveness of SSAs is critically dependent on the presence of SSTs, and because the treatment with available SSAs [both, first generation (e.g., octreotide) and second generation (i.e., pasireotide)] has become the mainstay of medical therapy for tumor control in different ERCs expressing SSTRs (such as pituitary and gastroenteropancreatic neuroendocrine tumors [22,23,41–43]). In fact, we demonstrated that octreotide (which acts primarily by binding to SSTR2 and with less affinity to SSTR5) and pasireotide (a multi-receptor ligand with high affinity for SSTR1, SSTR2, SSTR3, and SSTR5) significantly reduced proliferation rate in 22Rv1 cells (a cell model with an expression profile of *SSTR1* = *SSTR5* >>> *SSTR2*), while only pasireotide, but not octreotide, inhibited proliferation rate in PC-3 (a cell model with an expression profile of *SSTR5*>>>*SSTR2* > *SSTR1*), which reinforce the idea that PCa patients, especially patients with CRPC, could be sensitive to the antitumor actions of SSAs, opening new avenues to explore their potential as targeting therapy for patients with CRPC. Obviously, additional work will be required to evaluate the efficiency of SSAs alone or in combination with other drugs currently used for the treatment of CRPC (i.e., abiraterone or enzalutamide) in patients with CRPC.

Another relevant finding of our study is that we demonstrated that endogenous CORT is highly expressed in 22Rv1 and PC-3 cells, as well as in human PCa tissues compared with BPH samples. Remarkably, we also found that endogenous *CORT* expression was higher in metastatic PCa samples compared to primary tumors and non-tumor samples. As a result, ROC analysis revealed that endogenous *CORT* expression could discriminate between patients with PCa vs. patients with BPH, and also between patients that developed metastasis vs. those that did not. Moreover, we observed that the expression of endogenous CORT was positively correlated with the expression of *SSTR1*, *SSTR2* and *SSTR5* in metastatic PCa tissues but not in non-metastatic tissues. All these results suggest a causal link between dysregulation of endogenous *CORT* expression and PCa progression/aggressiveness and, therefore, that endogenous *CORT* may play a significant autocrine/paracrine pathophysiological role in AI-PCa cells, being its expression functionally linked to the dominant SSTR-subtypes expressed in PCa tissues. This hypothesis was confirmed when we silenced endogenous *CORT* levels in AI-PCa cells which resulted in a significant increase in proliferation rate in these cells, and in the modulation of the expression/levels of critical genes and oncogenic signaling pathways, including the reduction in the expression of different cell cycle inhibitors (e.g., *CDK2*, *CDKN1A*, *CDKN1B*, and/or *CDKND*). Moreover, our data is consistent with previous reports showing that SST, the other main ligand of SSTR-subtypes, also plays an important autocrine/paracrine role in several cellular models including colorectal cancer cells [44,45]. In fact, a constitutive activation of different SSTRs has been also reported since various SSTRs can display a relevant degree of ligand-independent constitutive activity in different cell systems [46]. However, our results have particular relevance because, to the best of our knowledge, this is the first evidence demonstrating a potential autocrine/paracrine regulatory function for endogenous *CORT* in cancer cells, which might be functionally linked to the expression of the dominant receptors expressed in PCa cells (i.e., *SSTR1*, *SSTR2* and *SSTR5*). In support of this idea is the fact that CORT silencing in AI-PCa cells induced significant changes in the expression levels of key cell cycle/proliferation markers and SSTR-subtypes, such as modulation of *CDK2*, *CDKN1A*, *CDKN1B*, *CDKND*, *SSTR1*, *SSTR2*, and/or *SSTR5*.

Remarkably, as previously mentioned, we also demonstrated that the proliferation rate of AI-PCA cells was significantly inhibited in response to octreotide and/or pasireotide in AI-PCa cells; however, when *CORT* expression was silenced, the treatment with these SSAs was completely inefficient in decreasing the proliferation rate, suggesting that the reduction in the levels of *CORT* could desensitize AI-PCa cells to the antitumor actions of SSAs treatment. Interestingly, we found that CORT-silencing was able to significantly

down-regulate the expression of the dominant SSTR-subtypes expressed in 22Rv1 (i.e., *SSTR1*, *SSTR2* and *SSTR5*) and in PC-3 (i.e., *SSTR5*), which might in part explain the desensitization observed in CORT-silenced AI-PCA cells to the antiproliferative effects of SSAs. We acknowledge that the limitations of our study are the lack of in vivo preclinical studies analyzing the actions of SST and CORT in the prostate gland physiology under normal and pathological-PCa conditions, the lack of analyzed metastatic CRPC samples in our internal cohort of patients, and that further work will be required to evaluate whether the levels of *CORT* expression could be used as a predictive molecular biomarker to select patients with PCa, especially CRPC, susceptible to being treated with SSAs. Moreover, it seems plausible that additional factors, besides the simple abundance of endogenous *CORT*, might critically influence the SSAs response in PCa cells, including the presence of the truncated splicing sst5TMD4 as has been previously suggested by our group in PCa and other tumor pathologies [14,17,20].

4. Materials and Methods

4.1. Patients and Samples

This study was approved by the Reina Sofia University Hospital Ethics Committee and conducted in accordance with the ethical standards of the Declaration of Helsinki and the World Medical Association. Core needle biopsies from patients with significant PCa (n = 66) and benign prostatic hyperplasia (BPH; n = 3; used as control) were collected (cohort-1; results included in Figure 4A-C). The presence or absence of tumor was histologically confirmed by expert uropathologists. Clinical information of patients is provided in Table 1.

Table 1. Demographic, biochemical, and clinical parameters of patients with significant PCa. PSA: Prostate-specific antigen.

Patients [n]	66
Age, years [median (IQR)]	75 (69–81)
PSA levels, ng/mL [median (IQR)]	62.0 (36.2–254.5)
Gleason score $\geq$ 7 (%)	66 (100%)
Metastasis (%)	11 (17%)

The Andalusian Biobank (Córdoba Node) coordinated the collection, processing, management, and assignment of the biological samples used in the present study according to the standard procedures established for this purpose. Written informed consent was obtained from all patients.

In addition, expression levels and clinical data were obtained from the publicly available Grasso cohort [6], which includes metastatic CRPC (n = 27), localized prostate adenocarcinomas (n = 49), and non-tumor prostate tissue specimens (n = 12) (results included in Figure 4D). The data were downloaded from the CANCERTOOL portal [47].

4.2. Cell Cultures and Reagents

The normal-like prostate cell line RWPE-1, the Androgen-Dependent (AD) PCa cell model LNCaP, and the two Androgen-Independent (AI) PCa cell models 22Rv1 and PC-3 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA), and maintained according to manufacturer instructions as previously described [19,20,48]. These cell lines were validated by analysis of short tandem repeats sequences (STRs) using GenePrint 10 System (Promega, Barcelona, Spain), and monthly checked for mycoplasma contamination by polymerase chain reaction (PCR) as previously reported [20]. Human somatostatin-14 (SST-14) and cortistatin-17 (CORT-17) were purchased from Polypeptide Group (Neuhofstrasse, Switzerland). SSAs (octreotide and pasireotide) were obtained from Polypeptide Group and Novartis Pharmaceuticals Corporation, respectively. All these treatments were resuspended in water and used at 10^{-7} M based on previous reports [13,14,49].

4.3. Cell Proliferation Assay

As previously described [18,32], cell proliferation was assessed by Resazurin Reagent (# CA035; Canvax Biotech, Córdoba, Spain). Briefly, cells were seeded in 96-well plates at a density of 3000 cells/well, serum-starved overnight, and then fluorescence (540 nm excitation and 590 nm emission) was measured after 3 h incubation with 10% resazurin using the FlexStation III system (Molecular Devices, Sunnyvale, CA, USA). This process was repeated after 24, 48, and 72 h of incubation in response to SST, CORT, octreotide, and pasireotide treatment and/or CORT-silencing (see below) in RWPE-1, LNCaP, 22Rv1, and/or PC-3 cell lines. All the data were normalized to values obtained in day 0 and represented as fold change compared to vehicle-treated controls or scramble-transfected cells.

4.4. Cell Migration Assay

Cell migration was evaluated in PC-3 cells, given its high invasiveness nature, as previously reported [50]. Specifically, 30,000 cells were seeded in an Incucyte Imagelock 96-well plate (Cat. No. 4379, Sartorius, Goettingen, Germany). Then, when confluence was reached, cells were starved for 3 h, a scratch was made using Incucyte® Woundmaker Tool (Cat. No. 4563, Sartorius) in each well and the media was replaced by fresh serum-free media. Images of the wound were taken at 0 and after 16 h of incubation with the different treatments. Wound-healing was calculated as the area observed 16 h after the wound was made vs. the area observed just after wounding, using ImageJ software [51].

4.5. Colonies Formation

To determine the clonogenic capacity of 22Rv1 and PC-3 PCa cells in response to different treatments, 2000 cells were seeded into 6-well plates, as previously reported [52]. Then, after 10 days, the medium was removed, the colonies washed with PBS, stained with crystal violet solution (crystal violet at 0.05% and glutaraldehyde at 6%) for 30 min, and air-dried. The number of individual colonies was determined by ImageJ software (colony area plugin) [51].

4.6. RNA Isolation, Quantitative Real-Time PCR (qPCR), and Customized qPCR Dynamic Array Based on Microfluidic Technology

AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Hilden, Germany) and TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA) were used to isolate RNA from fresh tissues and PCa cell lines, respectively. RNA was DNase-treated using RNase-Free DNase Kit (Qiagen). Total RNA concentration and purity were assessed using Nanodrop One Spectrophotometer (Thermo Fisher Scientific, Madrid, Spain). Total RNA (1 µg) was reverse transcribed using random hexamer primers and the cDNA First-Strand Synthesis kit (Thermo Scientific). Details regarding the development and validation of primers and for the standard real-time qPCR and qPCR microfluidic-based dynamic array technology have been previously reported by our laboratory [53,54]. Detailed information about the primers used herein can be found in Table S1. To control for variations in the efficiency of the retrotranscription reaction, mRNA copy numbers of the different transcripts analyzed were adjusted by the expression level of a normalization factor (calculated with ACTB and GAPDH expression levels, using GeNorm 3.3) [55].

4.7. Western Blotting

22Rv1 and PC-3 cell lines were processed to analyze protein levels by Western-blot after 30 min of SST or CORT exposure or after 48 h of CORT silencing (siRNA transfection) as previously reported by our group [18,53]. Briefly, 300,000 cells were seeded in 6-well plates, and after the experimental procedure described above, proteins were extracted using pre-warmed Sodium Dodecyl Sulfate-Dithiothreitol (SDS-DTT) buffer (62.5 mM Tris-HCl, 2% SDS, 20% glycerol, 100 mM DTT, and 0.005% bromophenol blue). Then, proteins were sonicated for 10 s and boiled for 5 min at 95 °C. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA).

Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline/0.05% Tween-20 and incubated overnight with the specific primary antibodies at 1:1000 dilution [phospho-AKT (p-AKT; #4060S; Cell-Signaling, Barcelona, Spain), AKT (#9272S; Cell-Signaling, Barcelona, Spain), phospho-ERK (#4370S; Cell-Signaling), ERK (#9102S; Cell-Signaling), phospho-JNK (#AF1206; RD system), JNK (#AF1387; RD system), phospho-PTEN (#S380; Cell-Signaling), PTEN (9552S; Cell-Signaling), phospho-AR (#16969; Cell-Signaling), AR (ab133273; Abcam)]. Secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit Immunoglobulin G (IgG) (# 7074S; Cell-Signaling, Barcelona, Spain) or anti-mouse IgG (#7076S; Cell-Signaling) were used at 1:2000 dilution. Proteins were detected using an enhanced chemiluminescence detection system (GEHealthcare, Madrid, Spain) with dyed molecular weight markers (Bio-Rad, Madrid, Spain). Phosphorylation levels of specific proteins were calculated as the ratio between the levels of a specific phospho-protein and its total protein levels detected. Densitometry analysis of the bands obtained was carried out with ImageJ software [51].

4.8. Silencing of Endogenous CORT Gene Expression

22Rv1 and PC-3 cells were used for silencing experiments as previously reported [18,20]. Briefly, 300,000 cells were seeded in 6-well plates and grown until 70–90% confluence was reached. Then, cells were transfected (transient transfection) with a specific and validated small-interfering RNA oligo (siRNA) for knockdown of endogenous levels of CORT (#s194341; Thermo Fisher Scientific, Madrid, Spain), along with the SilencerVR Select Negative Control siRNA (#4390843, Thermo Fisher Scientific) at 75 nM, using Lipofectamine-RNAiMAX (#13778-150, Thermo Fisher Scientific), following the manufacturer's instructions. After 48 h of incubation, cells were collected for validation and seeded to measure proliferation rate.

4.9. Statistical Analysis

All the experiments were performed in at least 3 independent experiments ($n \geq 3$) and with at least 2 technical replicates. Statistical differences between two conditions were calculated by unpaired parametric t-test or nonparametric Mann–Whitney U test, according to normality, assessed by Kolmogorov–Smirnov test. For differences among three conditions, a One-Way ANOVA or Kruskal–Wallis analysis was performed. Spearman's or Pearson's bivariate correlations were performed for quantitative variables according to normality. Statistical significance was considered when $p < 0.05$. A trend for significance was considered when p values ranged between > 0.05 and < 0.1. Data represent means ± SEM. All the analyses were assessed using GraphPad Prism 9 (GraphPad 9 Software, La Jolla, CA, USA).

5. Conclusions

Taken together, our results unveiled new conceptual and functional avenues in PCa with potential clinical implications, by demonstrating a therapeutic potential of the SST/CORT/SSTRs system and of different SSAs (i.e., octreotide and pasireotide) in AI-PCa cells. Moreover, our results offer original evidence demonstrating that endogenous *CORT* levels are significantly overexpressed in PCa compared with BHP tissues, and in metastatic vs. non-metastatic tissues, and that the modulation of its expression could be a potential therapeutic avenue that should be explored in the future in PCa since its silencing altered the proliferation rates in AI-PCa cells and desensitized these cells to the antitumor effect of octreotide and pasireotide.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms232113003/s1.

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content. P.S.-M.; F.P.-P.; J.M.P.-G. and J.M.J.-V. performed the statistical analysis. R.M.L.; obtained funding's. M.D.G. and R.M.L. supervised the work. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, of the World Medical Association, and with the approval of the Hospital Ethic Committee (reference local committee: 4499; 25 May 2020).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Unleashing the Diagnostic, Prognostic and Therapeutic Potential of the Neuronostatin/GPR107 System in Prostate Cancer

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Abstract: Certain components of the somatostatin-system play relevant roles in Prostate Cancer (PCa), whose most aggressive phenotype (Castration-Resistant-PCa (CRPC)) remains lethal nowadays. However, neuronostatin and the G protein-coupled receptor 107 (GPR107), two novel members of the somatostatin-system, have not been explored yet in PCa. Consequently, we investigated the pathophysiological role of NST/GPR107-system in PCa. GPR107 expression was analyzed in well-characterized PCa patient's cohorts, and functional/mechanistic assays were performed in response to GPR107-silencing and NST-treatment in PCa cells (androgen-dependent (AD: LNCaP) and androgen-independent (AI: 22Rv1/PC-3), which are cell models of hormone-sensitive and CRPC, respectively), and normal prostate cells (RWPE-1 cell-line). GPR107 was overexpressed in PCa and associated with key clinical parameters (e.g., advance stage of PCa, presence of vascular invasion and metastasis). Furthermore, GPR107-silencing inhibited proliferation/migration rates in AI-PCa-cells and altered key genes and oncogenic signaling-pathways involved in PCa aggressiveness (i.e., KI67/CDKN2D/MMP9/PRPF40A, SST_5TMD4/AR-v7/In1-ghrelin/EZH2 splicing-variants and AKT-signaling). Interestingly, NST treatment inhibited proliferation/migration only in AI-PCa cells and evoked an identical molecular response than GPR107-silencing. Finally, NST decreased GPR107 expression exclusively in AI-PCa-cells, suggesting that part of the specific antitumor effects of NST could be mediated through a GPR107-downregulation. Altogether, NST/GPR107-system could represent a valuable diagnostic and prognostic tool and a promising novel therapeutic target for PCa and CRPC.

Keywords: prostate cancer; castration resistant prostate cancer; neuronostatin; G protein-coupled receptor GPR107; diagnostic/prognostic biomarker; therapeutic target; somatostatin-system; splicing

1. Introduction

Prostate cancer (PCa) is one of the most diagnosed tumor pathologies among men worldwide and represents one of the leading causes of cancer-related death among the male population in developed countries [1]. One of the main characteristics of PCa is the strong influence of the endocrine-metabolic environment, which contributes to the high heterogeneity of this pathology and hinders the development of new, useful, diagnostic and/or therapeutic strategies [2]. In fact, the current available pharmacological approaches used in clinical practice to combat this tumor pathology consist in the use of drugs (e.g., Abiraterone, Enzalutamide, etc.) that avoid the steroid-hormones synthesis and/or disrupts the androgen signaling pathway in PCa cells [3,4]. However, although the development of these drugs has significantly improved the overall survival, some PCa patients acquire resistance to these compounds and develop the most aggressive phenotype, named Castration-Resistant PCa (CRPC), which remains lethal nowadays [3–5]. For this reason, new therapeutic targets in order to tackle PCa and CRPC are urgently required.

In this sense, certain components of the somatostatin system, especially somatostatin-receptors ($SST_{1\text{-}5}$, encoded by the somatostatin receptor 1-5 genes (SSTR1-5)), are expressed in both normal and tumor prostate tissues, where they may play a relevant role in the development and progression of this disease [6–9]. Specifically, some components of this system (e.g., SST_1 and SST_2) can contribute to reduce different tumor parameters, including cell proliferation and migration, whereas other components [i.e., the truncated splicing variant of SST_5 with four transmembrane domains (SST_5TMD4 variant)] promote aggressiveness features of PCa [10–12]. Synthetic somatostatin analogs (i.e., octreotide and/or pasireotide) have been used as valuable tools to treat different tumor pathologies, including pituitary and neuroendocrine tumors [13]. However, attempts to apply somatostatin analogs in PCa have rendered controversial results, since the limited studies reported so far did not show improvement in overall survival [14]. The mechanistic reasons of those clinical failures are still unknown, but it has been suggested that one of the causes might be the overexpression of the spliced variant SST_5TMD4 in PCa cells, which hampers the normal response to somatostatin-analogs in PCa-cells [11].

Interestingly, the complexity and versatility of the somatostatin system has been lately increased by the discovery of a new peptide contained in the preprosomatostatin precursor polypeptide encoded by the somatostatin gene, which shares no amino-acid homology to somatostatin, named neuronostatin (NST) [15]. NTS seems to bind to the G protein-coupled receptor 107 (GPR107) to exert its actions [16]. Specifically, it has been reported that NST and/or GPR107 are expressed in different tissues (e.g., brain, pancreas, gut, etc.) wherein they exert important pathophysiological functions, being some of these actions similar, but others also unique, to those exerted by other members of the somatostatin/SSTRs-system [17–19]. Despite the tight genetic and putative functional link between NST and somatostatin, the presence and/or functional role of the NST/GPR107-system has hitherto not been explored on PCa. Accordingly, the current study sought to explore for the first time the functional actions and therapeutic potential of the NST/GPR107-system in PCa cells by investigating the direct effects of NST treatment on normal and tumor (PCa and CRPC) cells and the pathophysiological role of endogenous GPR107 in this severe disease.

2. Experimental Section

2.1. Patients and Samples

This study was approved by the Hospital Ethic Committee (approval number: 2461) and conducted in accordance to the principles of the Declaration of Helsinki. Written informed consent was obtained from all patients. Two different cohorts of prostate samples obtained through the Andalusian Biobank (Cordoba Node) were included:

Cohort 1: formalin-fixed, paraffin-embedded (FFPE) PCa tissues (n = 84) and their adjacent non-tumor region (N-TAR; used as control tissues; n = 84), which were obtained from radical

prostatectomies from patients who were diagnosed with localized PCa, without metastasis and with Gleason Score (GS) 6–8 (Table 1).

Table 1. Demographic, biochemical and clinical parameters of the patients who underwent radical prostatectomies (Cohort 1).

Parameter	
Patients (*n*)	84
Age, years (median (IQR))	61 (57–66)
PSA levels, ng/mL (median (IQR))	5.2 (4.2–8.0)
GS (*n*; %)	GS 6 (8; 9.52%), GS 7 (73; 86.90), GS 8 (3; 3.57%)
SigPCa (*n* (%))	76 (90.5%)
pT ≥ 3a (*n* (%))	59 (70.2%)
PI (*n* (%))	72 (85.7%)
VI (*n* (%))	8 (9.52%)
Recurrence (*n* (%))	35 (41.7%)
Metastasis (*n* (%))	0 (0%)

PSA: Prostate specific antigen; GS: Gleason Score; SigPCa: Significant prostate cancer, defined as Gleason score ≥ 7; pT: Pathological primary tumor staging; PI: Perineural invasion; VI: Vascular invasion.

Cohort 2: fresh PCa samples (n = 67) that were obtained by core needle biopsies from patients with high suspect of presenting palpable significant PCa, which was further confirmed histologically by a specialized pathologist. This cohort includes more aggressive PCa, presenting metastasis in some cases (metastatic hormone-sensitive PCa or mHSPC) and with GS 7–10 (Table 2).

Table 2. Demographic, biochemical and clinical parameters of the patients who underwent prostate biopsy (Cohort 2).

Parameter	
Patients (*n*)	67
Age, years (median (IQR))	75 (69–81)
PSA levels, ng/mL (median (IQR))	62.0 (36.2–254.5)
GS (*n*; %)	GS 7 (18; 26.86%), GS 8 (20; 29.85%) GS 9 (24; 35.82%), GS 10 (5; 7.46%)
SigPCa (*n* (%))	67 (100%)
Metastasis (*n* (%))	27 (40.3%)

PSA: Prostate specific antigen; GS: Gleason Score; SigPCa: Significant prostate cancer, defined as Gleason score ≥ 7.

Computed tomography scan and bone scan were performed in these patients to determine the presence of metastasis. Available clinical parameters of tumor aggressiveness were collected from each patient, such as presence of metastasis, Gleason score (analyzed by specialist uro-pathologists following the 2005, 2010 and 2014 International Society of Urological Pathology (ISUP) criteria, based on the sample collection date [20–22]) and prostatic specific antigen (PSA) levels (cohort 1 (Table 1) and cohort 2 (Table 2)). In addition, expression and clinical data of interest for this study were downloaded from different available in silico cohorts using cBioPortal (Grasso/Varambally cohorts) [23–25] or CANCERTOOL (Lapointe/Taylor/Tomlins) [26–29]. Specifically, Grasso cohort includes 35 metastatic Castration Resistant Prostate Cancer (mCRPC), 59 localized prostate carcinomas and 28 benign prostate tissue specimens; Varambally cohort includes 6 mCRPC, 7 primary prostate carcinomas and 6 normal prostate samples; Lapointe cohort includes 9 mHSPC, 62 localized prostate carcinomas and 41 matched normal prostate tissues; Taylor cohort includes 19 mHSPC, 131 localized prostate carcinomas and 29 paired normal adjacent prostate tissue specimens and Tomlins cohort includes 19 mHSPC, 49 localized prostate carcinomas and 23 normal prostate glands.

2.2. Cell Cultures and Reagents

The androgen-dependent metastatic PCa LNCaP cell line, the androgen-independent 22Rv1 and PC-3 (non-metastatic and metastatic, respectively) PCa cell lines and the normal-like prostate cell line RWPE-1 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained according to manufacturer instructions as previously described [10,11,30]. These cell lines were validated by analysis of short tandem repeats sequences (STRs) using GenePrint 10 System (Promega, Barcelona, Spain) and checked for mycoplasma contamination by polymerase chain reaction (PCR) as previously reported [11]. For functional assays, selected cell lines were used as indicated. For mechanistic assays, 22Rv1 and PC-3 were used as representative models of androgen-independence with and without AR-v7 expression, respectively. Human amidated NST-19(Ala-Pro-Ser-Asp-Pro-Arg-Leu-Arg-Gln-Phe-Leu-Gln-Lys-Ser-Leu-Ala-Ala-Ala-Ala-NH_2) was purchased from Phoenix Pharmaceuticals (Burlingame, CA, USA), resuspended in water and used at 10^{-7} M based on previous reports [19].

2.3. Transfection with Specific siRNA

For silencing assays, 22Rv1 and PC-3 cell lines were used. Specifically, 200,000 cells were seeded in 6-well plates and grown until 70% confluence was reached. Then, cells were transfected with specific small interferent RNA (siRNA) against GPR107 (Catalog # AM16708; Thermo Fisher Scientific, Madrid, Spain) at 15 nM or scramble control (Catalog # 4390843, Thermo Fisher Scientific, Madrid, Spain) using Lipofectamine-RNAiMAX (Catalog # 13778-150, Thermo Fisher Scientific, Madrid, Spain) following the manufacturer's instructions. After 48 h, cells were collected for validation (quantitative-PCR (qPCR) and western blot) and seeded for proliferation and/or migration assays.

2.4. Measurements of Cell Proliferation and Migration Rates

Both cell proliferation and migration were measured as previously reported [11,31]. Briefly, cell proliferation was assessed by Resazurin Reagent (# CA035; Canvax Biotech, Córdoba, Spain) following the manufacturer's instructions. Cells were seeded in 96-well plates at a density of 3000–5000 cells/well and serum-starved for 24 h, then cell proliferation was evaluated using FlexStation III system (Molecular Devices, Sunnyvale, CA, USA) until 72 h in response to NST-19 treatment and/or GPR107-silencing. All experiments were performed at least with three independent cell preparations.

Cell migration was evaluated by wound-healing assay in RWPE-1 and PC-3 cells due to the inability of LNCaP and 22Rv1 to migrate. Images of the wound were taken at 0 and 24 h, and wound healing was calculated as the area of a rectangle centered in the picture 24 h after the wound was made vs. the area of the rectangle just after doing the wound. Results were expressed as percentage referred to control. All experiments were performed at least with three independent cell preparations.

2.5. RNA Isolation, Reverse Transcription and Quantitative Real-Time PCR (RT-qPCR)

Total Ribonucleic acid (RNA) from FFPE samples was isolated and treated with Deoxyribonuclease (DNase) using the Maxwell 16 LEV RNA FFPE Kit (Promega, Madison, WI, USA) according to manufacturer instructions in the Maxwell MDx 16 Instrument (Promega, Madison, WI, USA). Additionally, total RNA was extracted from fresh samples using the AllPrep DNA)/RNA/Protein Mini Kit (Qiagen, Madrid, Spain) and from prostate cell lines using TRIzol Reagent (Thermo Fisher Scientific, Madrid, Spain), followed, in both cases, by DNase treatment using Ribonuclease (RNase)-Free DNase Kit (Qiagen, Hilden, Germany). Total RNA concentration and purity was assessed using Nanodrop One Spectrophotometer (Thermo Fisher Scientific, Madrid, Spain). Total RNA was retrotranscribed using random hexamer primers and the cDNA First Strand Synthesis Kit (Thermo Fisher Scientific, Madrid, Spain).

Details regarding the development, validation, and application of the RT-qPCR to measure expression levels of the transcripts of interest have been previously reported by our laboratory [32–35].

Specific and validated primers set used to measure the expression levels of genes of interest in this study (absolute mRNA copy number/50 ng of sample) are described in Table S1. To control for variations in the amount of RNA used and the efficiency of the retro-transcription, messenger RNA (mRNA) copy numbers of the different transcripts analyzed were adjusted by a normalization factor, which was calculated with by the expression levels of Actin Beta (*ACTB)* and Glyceraldehyde-3-Phosphate Dehydrogenase (*GAPDH)* using GeNorm 3.3 (CMMG, Ghent, Belgium) [36] or by the expression levels of *ACTB* (the most appropriated housekeeping gene), wherein *ACTB* and *GADPH* mRNA levels did not significantly vary among the different experimental conditions.

2.6. Western-Blotting

Prostate cell lines were processed to analyze protein levels by western-blot after 5, 10 and 15 min of NST-19 exposure and after 48 h of GPR107 siRNA transfection. These processes have been previously described by our group [11,30,34]. Briefly, 150,000 cells were seeded in 12-well plates, and after the experimental procedure describe above, proteins were extracted using pre-warmed Sodium Dodecyl Sulfate-Dithiothreitol (SDS-DTT) buffer (62.5 mM Tris-HCl, 2% SDS, 20% glycerol, 100 mM DTT, and 0.005% bromophenol blue). Then, proteins were sonicated for 10 s and boiled for 5 min at 95 °C. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA). Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline/0.05% Tween-20 and incubated overnight with the specific primary antibodies for GPR107 (orb161193; byorbyt, Cambridge, United Kingdom), Tubulin Beta (TUBB; # 2128S; Cell-Signaling, Barcelona, Spain), phospho-AKT (p-AKT; #4060S; Cell-Signaling, Barcelona, Spain) and total-AKT (#9272S; Cell-Signaling, Barcelona, Spain). Secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit Immunoglobulin G (IgG) (# 7074S; Cell-Signaling, Barcelona, Spain) were used. Proteins were detected using an enhanced chemiluminescence detection system (GEHealthcare, Madrid, Spain) with dyed molecular weight markers (Bio-Rad, Madrid, Spain). A densitometry analysis of the bands obtained was carried out with ImageJ 2 software (Madison, Wisconsin, USA) [37], using total protein levels of TUBB (for GPR107) or AKT (for phospho-AKT) as normalizing factors. All experiments were performed at least with three independent cell preparations.

2.7. GPR107 Immunohistochemistry (IHC) Analysis

GPR107 Immunohistochemistry (IHC) analysis was performed on FFPE samples from cohort 1 ($n = 16$; randomly selected samples) and cohort 2 ($n = 4$; metastatic patients) using standard procedures [11,38,39]. In both cases, the staining of the tumor tissue was compared to non-tumor adjacent regions. Briefly, deparaffinized sections were incubated overnight (4 °C) with the primary antibody against GPR107 (# 161193; Biorbyt, Cambridge, United Kingdom) at 1:200 dilution, followed by incubation with an anti-rabbit horseradish peroxidase-conjugated secondary antibody (# 7074; Cell-Signaling, Barcelona, Spain). Finally, sections were developed with 3.39-diaminobenzidine (Envision system 2-Kit Solution DAB, Thermo Fisher Scientific, Madrid, Spain) and contrasted with haematoxylin. Two independent pathologists performed histopathologic analysis of the tumors following a blinded protocol. In the analysis, 1, 2, 3 indicate low, moderate and high staining intensities.

2.8. Statistical Analysis

Statistical differences between two conditions were calculated by unpaired parametric t-test or nonparametric Mann Whitney U test, according to normality, assessed by Kolmogorov–Smirnov test. For differences among three conditions, One-Way ANOVA analysis was performed. Spearman's or Pearson's bivariate correlations were performed for quantitative variables according to normality. All the experiments were performed in at least 3 experiments ($n \geq 3$) and with at least 2 technical replicates. The receiver operating characteristic (ROC) curve was performed for evaluation of the accuracy of GPR107 as a discriminator marker between metastatic vs. non-metastatic patients. Statistical significance was considered when $p < 0.05$. A trend for significance was indicated when p values

ranged between >0.05 and <0.1. All the analyses were assessed using GraphPad Prism 7 (GraphPad 7 Software, La Jolla, CA, USA).

3. Results

3.1. GPR107 is Overexpressed in PCa and Associated with Aggressive Features

Analysis of *GPR107* mRNA expression in FFPE-prostate pieces from patients diagnosed with localized PCa (n = 84; Gleason score 6–8; Table 1) revealed that GPR107 expression was significantly higher in tumor vs. non-tumor adjacent regions (N-TAR; Figure 1a). GPR107 IHC analysis was performed on 16 FFPE pieces (Figure 1b), which revealed that GPR107 staining was negligible in benign prostate gland epithelium (N-TAR; Figure 1b), while it was always more, and highly, intense in the cancerous prostate glands (N-TAR vs. PCa/tumor-tissue; Figure 1b).

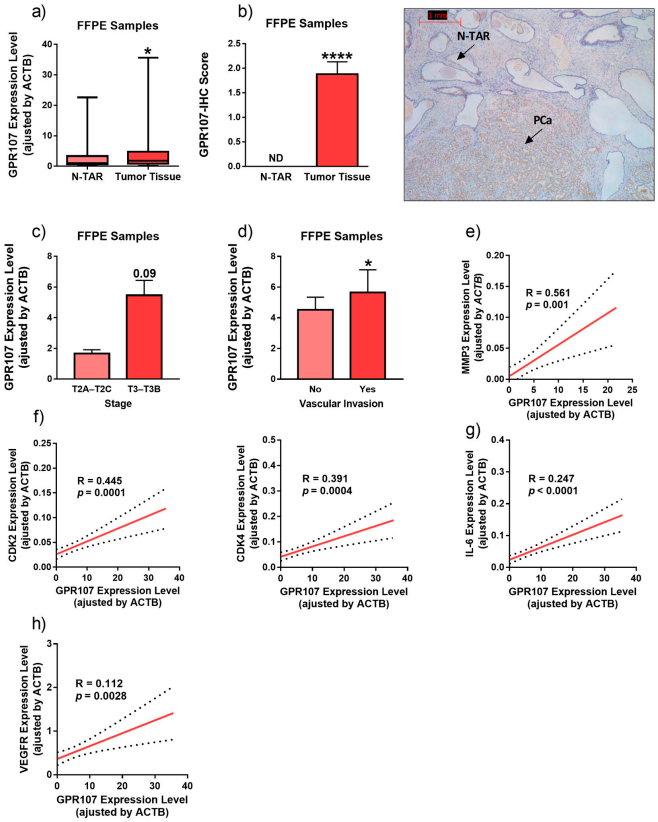

Figure 1. Expression levels of G protein-coupled receptor 107 (GPR107) in human prostate cancer samples. (**a**) Comparison of GPR107 expression levels between formalin-fixed paraffin embedded (FFPE) samples from Prostate Cancer (PCa) tissues and non-tumor adjacent regions (N-TAR) (n = 84). (**b**) Comparation of GPR107 protein levels by Immunohistochemistry (IHC) between a representative set of PCa samples (n = 16) and its N-TAR (n = 16). A representative image is also included. (**c**,**d**) Association between GPR107 expression levels and advance stage of PCa (**c**) and vascular invasion (**d**). (**e**–**h**) Correlation of GPR107 expression levels and Matrix Metallopeptidase 3 (MMP3; (**e**), Cyclin Dependent Kinase 2 and 4 (CDK2 and CDK4, respectively; (**f**), Interleukin 6 Receptor (IL6-R; (**g**) and Vascular endothelial growth factor Receptor (VEGFR; (**h**) expression levels in the same cohort of FFPE samples. Messenger RNA (mRNA) levels were determined by quantitative Polymerase Chain Reaction (qPCR) and adjusted by Actin Beta (ACTB) expression levels. Asterisks (* $p < 0.05$; **** $p < 0.0001$) indicate statistically significant differences between groups. ND: Non-detected.

Interestingly, GPR107 expression was associated with key clinical and molecular features of aggressiveness. Specifically, we found a positive association between GPR107 expression and an

advance stage of PCa (Tumor Stage 2A-2C vs. Tumor Stage 3-3B (T2A–T2C vs. T3–T3B; Figure 1c)) and with the presence of vascular invasion (Figure 1d), while no association was found with GS. Moreover, GPR107 expression was positively correlated with the expression of key genes related to migration (Matrix Metallopeptidase 3 (MMP3); Figure 1e), cell-cycle control (Cyclin Dependent Kinase 2 and 4 (CDK2 and CDK4, respectively); Figure 1f), inflammatory state (Interleukin 6 Receptor (IL6R); Figure 1g) and angiogenesis process (Vascular endothelial growth factor Receptor (VEGFR); Figure 1h).

3.2. GPR107 is Overexpressed in Patients with Metastasis

We also analyzed the expression of GPR107 in an independent cohort of more aggressive PCa (n = 67; Gleason score 7–10; Table 2). We found that GPR107 expression was significantly higher in primary PCa samples from patients with mHSPC compared to those without metastasis (Figure 2a). Indeed, ROC analysis indicated that GPR107 expression significantly discriminated between metastatic vs. non-metastatic patients (p = 0.0064; Figure 2b). These observations were also corroborated at the protein level by GPR107 IHC, which clearly indicated that GPR107 staining was negligible in benign prostate gland epithelium (N-TAR; Figure 2c), while it was highly intense in the metastatic regions (Figure 2c). Remarkably, GPR107 overexpression was also corroborated in metastasis from metastatic CRPC (mCRPC) compared to primary prostate tumors using two independent external in silico cohorts of patients obtained from different databases available online (Grasso (Figure 2d) and Varambally (Figure 2e)), while this overexpression was not found in mHSPC samples obtained from Lapointe, Taylor and Tomlins in silico cohorts [26–28] (Figure S3).

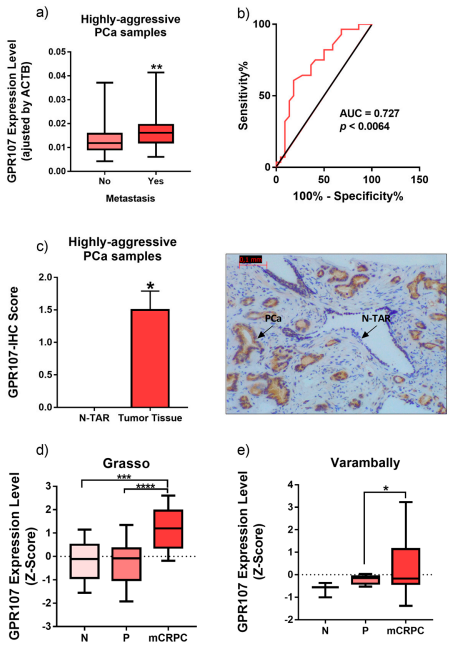

Figure 2. Expression levels of G protein-coupled receptor 107 (GPR107) in primary prostate cancer samples from patients with metastasis. (**a**) Association between GPR107 expression levels and the presence of metastasis in a cohort of fresh samples from patients with Prostate Cancer (PCa) n = 67). (**b**) Operating characteristic (ROC) curve analysis to determine the accuracy of GPR107 to discriminate between metastatic vs. non-metastatic patients' tumor. (**c**) Comparison of GPR107 protein level by Immunohistochemistry (IHC) between primary PCa samples from patients with metastasis and its non-tumor adjacent regions (N-TAR). Messenger RNA (mRNA) levels were determined by quantitative Polymerase Chain Reaction (qPCR) and adjusted by Actin Beta (ACTB) expression levels. (**d**-**e**) Comparison of GPR107 expression levels between normal prostate (n), primary prostate tumor (P) and metastatic Castration Resistant Prostate Cancer (mCRPC) samples obtained from two in silico databases (Grasso/Varambally; (**d**) and (**e**), respectively). Asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$) indicate statistically significant differences between groups. AUC: Area Under the Curve.

3.3. Silencing of GPR107 Reduces Aggressiveness Parameters in Androgen-Independent PCa Cells

To examine the possible functional role of GPR107 on PCa cell malignant features, we initially examined its expression levels on different prostate cell lines (normal (RPWE-1) and PCa (androgen-dependent LNCaP, as well as androgen-independent 22Rv1 and PC-3 cells)). Specifically, GPR107 expression was significantly higher in androgen-independent, 22Rv1 and PC-3, cells compared to the LNCaP and the normal RWPE-1 cells (Figure 3a). Based on these results, 22Rv1 and PC-3 were selected as suitable models to analyze the functional consequences of GPR107 silencing. Interestingly, silencing of GPR107, confirmed by qPCR and western-blot (Figure S1), clearly decreased cell proliferation in both 22Rv1 (Figure 3b) and PC-3 (Figure 3c) cells (at 24, 48 and 72 h vs. scramble-transfected control). Moreover, GPR107 silencing markedly decreased migration rate in PC-3 cells (Figure 3d).

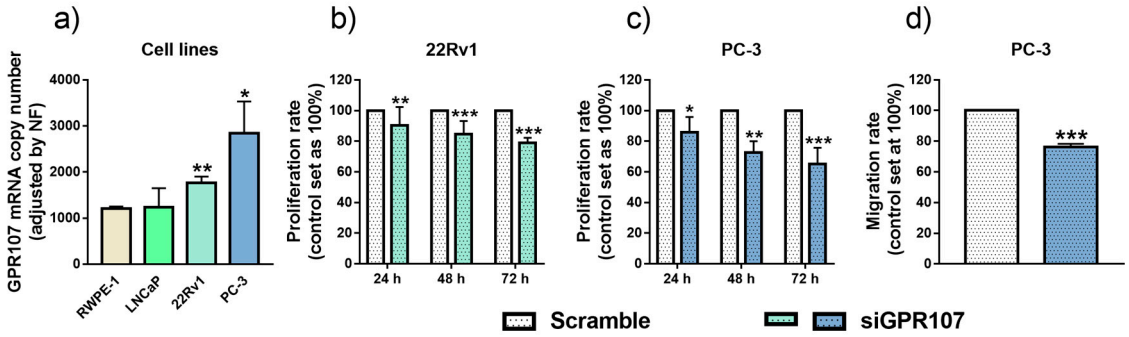

Figure 3. Screening of G protein-coupled receptor 107 (GPR107) expression level and functional effects of its silencing in normal and tumor prostate cell lines. (**a**) Comparison of GPR107 expression levels between a non-tumor prostate cell line (RWPE-1) and different Prostate Cancer (PCa) cell lines (LNCaP, 22Rv1 and PC-3). Messenger RNA (mRNA) levels were determined by quantitative Polymerase Chain Reaction (qPCR) and adjusted by a normalization factor (NF) generated by the combination of Actin Beta (ACTB) and Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) expression levels. (**b**)–(**c**) Proliferation rate of 22Rv1 (**b**) and PC-3 (**c**) cells after 24, 48 and 72 h of GPR107-silencing. (**d**) Migration rate of PC-3 cells after 24 h of GPR107-silencing. In (**b**)–(**d**), data were represented as percent of scrambled cells (set at 100%). Asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$) indicate statistically significant differences between groups. siGPR107: small interferent RNA against GPR107.

3.4. Silencing of GPR107 Modulates the Expression of Key Genes and Oncogenic Signaling Pathway in Androgen-Independent PCa Cells

To identify the molecular consequences of GPR107 silencing in androgen-independent PCa cells, we analyzed the expression levels of key genes related to proliferation/cell-cycle, migration and aggressiveness. Specifically, we found that the silencing of GPR107 in 22Rv1 cells significantly decreased the expression levels of the proliferation marker Ki-67 (MKI67), of genes involved in migration process (Matrix Metallopeptidase 9 and Pre-MRNA Processing Factor 40 Homolog A (MMP9 and PRPF40A, respectively)) as well as of key genes associated to PCa aggressiveness such as the oncogenic splicing variants AR-v7, SST_5TMD4, Intron 1-retained ghrelin splicing variant (In1-ghrelin) and the Enhancer Of Zeste Homolog 2 (EZH2) (Figure 4a). In PC-3 cells, a significant decrease in the expression levels of MKI67 and SST_5TMD4 and a trend for a significant decrease of MMP9, In1-ghrelin and EZH2 and for a significant elevation in the expression of cell-cycle suppressor Cyclin Dependent Kinase Inhibitor 2D (CDKN2D) was also found in response to GPR107 silencing (Figure 4a).

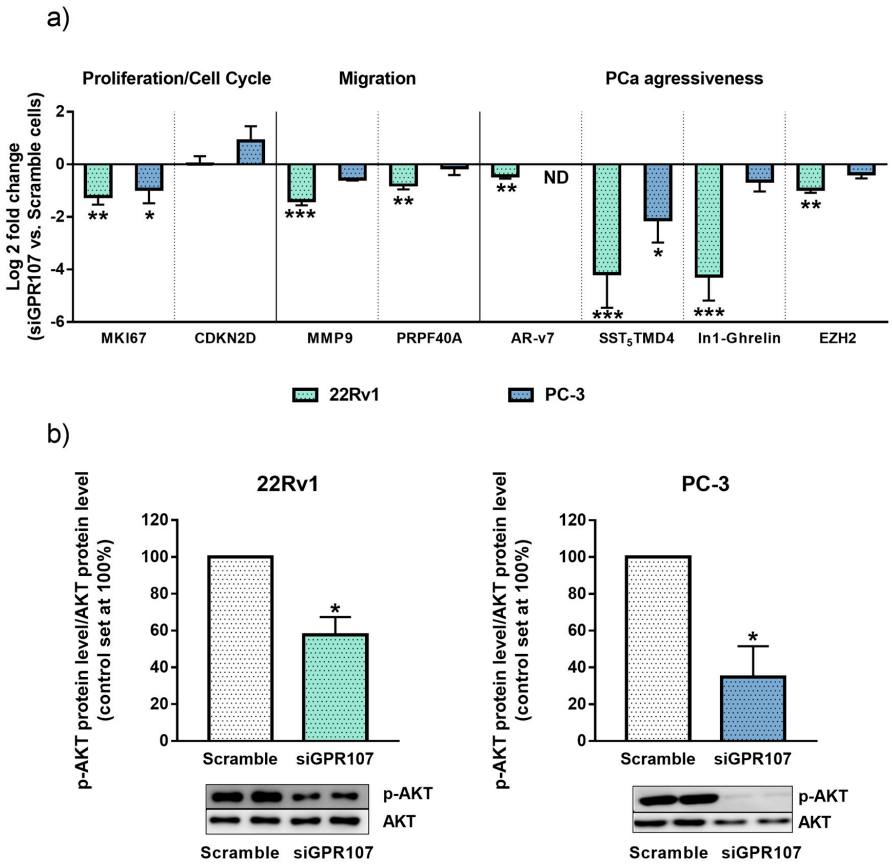

Figure 4. Molecular consequences of G protein-coupled receptor 107 (GPR107)-silencing in androgen-independent Prostate cancer (PCa) cells. (**a**) Fold change of markers of proliferation (Marked of Proliferation Ki-67 (MKI67)), cell cycle inhibition (Cyclin Dependent Kinase Inhibitor 2D (CDKN2D)), migration (Matrix Metallopeptidase 9 and Pre-MRNA Processing Factor 40 Homolog A (MMP9 and PRPF40A, respectively)) and aggressiveness (Androgen Receptor variant 7 (AR-v7), Somatostatin Receptor 5 Transmembrane Domain 4 variant (SST_5TMD4), Intron 1-retained ghrelin variant (In1-Ghrelin) and Enhancer Of Zeste Homolog 2 (EZH2)) in androgen-independent cells (22Rv1 and PC-3) in response to GPR107-silencing compared to scrambled cells. Messenger RNA (mRNA) levels were determined by quantitative Polymerase Chain Reaction (qPCR), adjusted by Actin Beta (ACTB) expression levels and represented as log 2 of fold change of expression levels small interferent RNA(siRNA)-treated/scrambled-cells). (**b**) Protein levels of phospho-AKT (p-AKT) in response of GPR107-silencing in androgen-independent PCa cells (22Rv1 and PC-3). p-AKT levels were normalized by total AKT protein levels. Protein data were represented as percent of scrambled cells (set at 100%). Asterisks (* p <0.05; ** p < 0.01 and *** p < 0.001) indicate statistically significant differences between GPR107-silencing and scrambled cells. siGPR107: small interferent RNA against GPR107.

Moreover, we sought to identify the downstream consequences of GPR107 silencing by analyzing the AKT route, a key signaling pathway in PCa cells. Specifically, phosphorylation levels of AKT were down-regulated in response to GPR107 silencing in both 22Rv1 and PC-3 cells (Figure 4b). We also analyzed the modulation of ERK pathway in response to GPR107 silencing but this signaling pathway was not significantly altered by this experimental intervention.

3.5. NST Treatment Exerts Antitumor Effects in Androgen-Independent PCa Cells

We next tested the direct effects of NST on proliferation and migration of normal and PCa cells (Figure 5). Incubation with NST did not alter proliferation or migration rate in normal (RWPE-1) cells (Figure 5a,b, left panels). Likewise, NST treatment did not alter proliferation of androgen-dependent LNCaP cells (Figure 5a). However, similar to that previously found with GPR107 silencing (Figure 3),

NST treatment significantly decreased proliferation rate of androgen-independent 22Rv1 and PC-3 cells (Figure 5a), as well as the migration of PC-3 cells (Figure 5b).

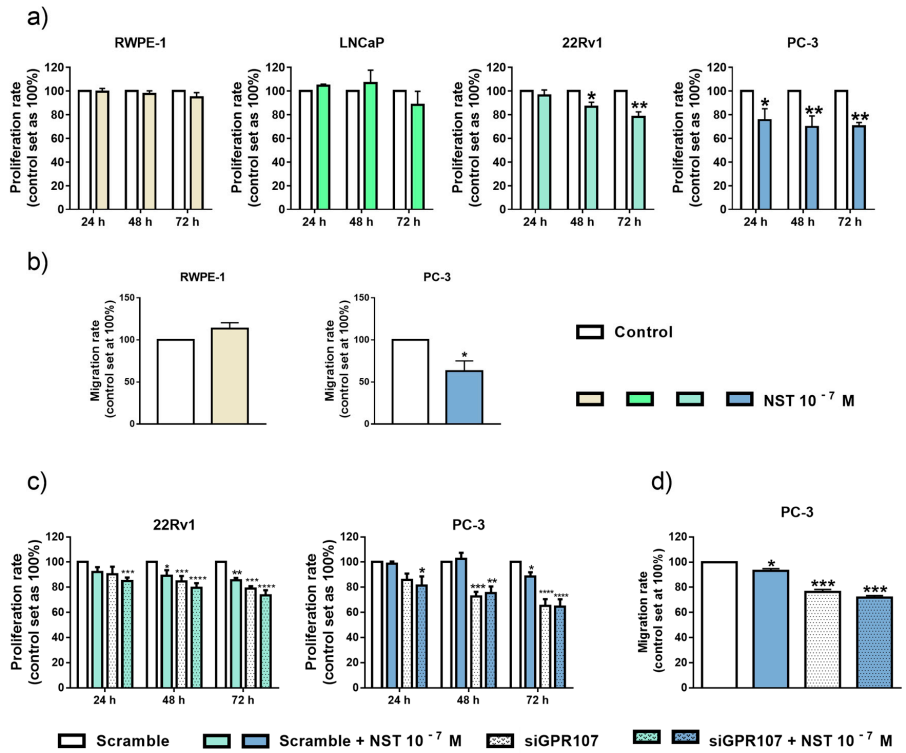

Figure 5. Functional effects after neuronostatin (NST) treatment and after the combination of NST and G protein-coupled receptor 107 (GPR107)-silencing treatment in prostate cell lines. (**a**) Proliferation rate of normal prostate (RWPE-1) and Prostate Cancer (PCa) (LNCaP, 22Rv1 and PC-3) cells in response to NST treatment (10^{-7} M; after 24, 48 and 72 h). (**b**) Migration rate of normal prostate (RWPE-1) and PCa (PC-3) cells after 24 h of NST treatment (10^{-7} M). Proliferation (**c**) and migration (**d**) rate of androgen-independent PCa cells in response to NST and GPR107-silencing alone or in combination. Data were represented as percent of vehicle-treated cells (set at 100%). Asterisks (* $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$) indicate statistically significant differences between groups. siGPR107: small interferent RNA against GPR107.

3.6. Actions of NST Treatment and GPR107 Silencing are Similar, and Functionally Connected, in Androgen-Independent PCa Cells

In order to analyze whether the actions of NST in the proliferation and/or migration rates of androgen-independent PCa cells could be functionally associated to GPR107, we next tested the direct effects of NST alone or in combination with GPR107 silencing. As observed previously, NST treatment or GPR107 silencing alone inhibited the proliferation of 22Rv1 and PC-3 (Figure 5c), as well as the migration of PC-3 cells (Figure 5d). These inhibitory actions of NST treatment or GPR107 silencing were virtually similar in 22Rv1 cells, while the actions of GPR107 silencing seem to be higher compared to NST treatment in PC-3 cells (Figure 5c,d). Furthermore, the combination of NST treatment and GPR107 silencing did not exert higher, additive or synergistic, effects compared with both experimental conditions alone (Figure 5c,d), which might suggest that the inhibitory actions evoked by NST treatment and GPR107 silencing could be mediated through similar mechanisms and/or signaling pathways.

Supporting this notion, we found that treatment with NST induced a molecular response virtually similar to that previously observed with GPR107 silencing in term of the modulation of the expression levels of key genes related to proliferation/cell cycle, migration and aggressiveness (MKI67, CDKN2D, MMP9, PRPF40A, AR-v7, SST_5TMD4, In1-ghrelin and EZH2; Figure 6a), as well as inhibition of AKT (Figure 6b), but not ERK, signaling pathway.

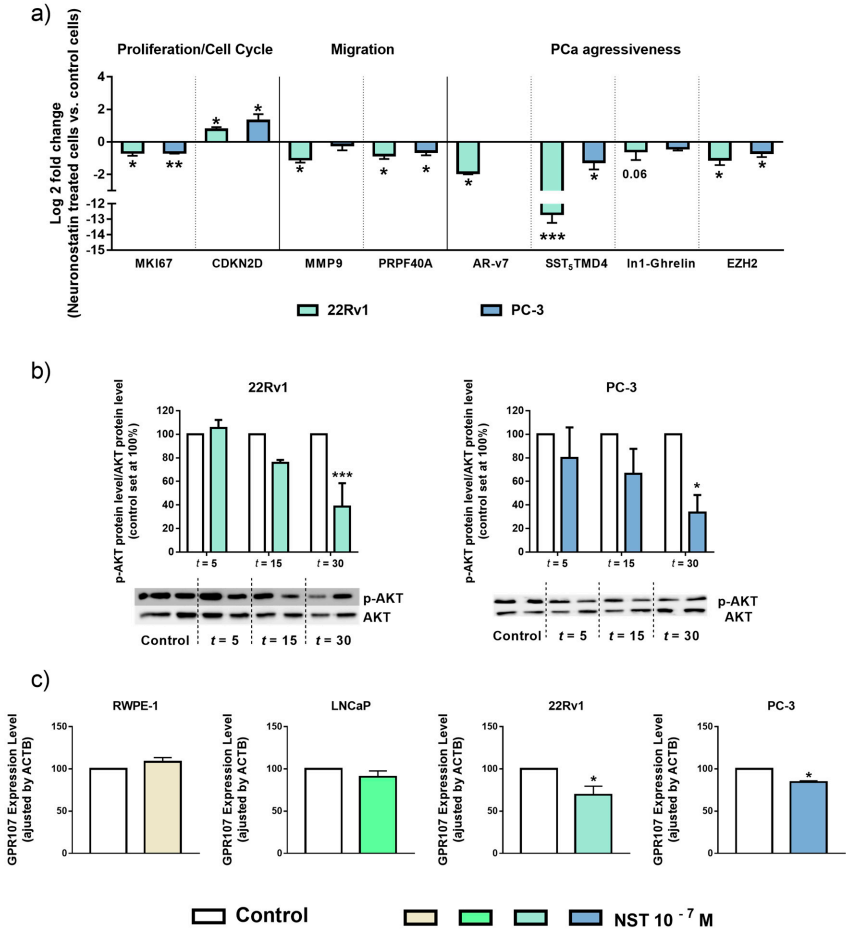

Figure 6. Molecular consequences of neuronostatin (NST) treatment in prostate cell lines. (**a**) Fold change of markers of proliferation (Marked of Proliferation Ki-67 (MKI67)), cell cycle inhibition (Cyclin Dependent Kinase Inhibitor 2D (CDKN2D)), migration (Matrix Metallopeptidase 9 and Pre-MRNA Processing Factor 40 Homolog A (MMP9 and PRPF40A, respectively)) and aggressiveness (Androgen Receptor variant 7 (AR-v7), Somatostatin Receptor 5 Transmembrane Domain 4 variant (SST_5TMD4), Intron 1-retained ghrelin variant (In1-Ghrelin) and Enhancer Of Zeste Homolog 2 (EZH2)) in androgen-independent Prostate Cancer (PCa) cells (22Rv1 and PC-3) after NST treatment (10^{-7} M) compared to vehicle-treated cells. Messenger RNA (mRNA) levels were determined by quantitative Polymerase Chain Reaction (qPCR), adjusted by Actin Beta (ACTB) expression levels and represented as log2 of fold change of expression levels (NST-treated/vehicle-treated cells). (**b**) Protein levels of phospho-AKT in response of NST treatment in androgen-independent PCa cells (22Rv1 and PC-3) after 5 ($t = 5$), 15 ($t = 15$) and 30 ($t = 30$) minutes of exposition. Phospho-AKT (p-AKT) levels were normalized by total AKT protein levels. Protein data were represented as percent of vehicle-treated cells (set at 100%). (**c**) GPR107 expression levels after 24 h of NST treatment in prostate cell lines. mRNA levels of GPR107 were determined by qPCR and adjusted by ACTB expression levels. Asterisks (* $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$) indicate statistically significant differences between NST treatment and vehicle-treated cells.

Finally, we found that NST administration did not alter the expression levels of GPR107 in normal RWPE-1 cells or in androgen-dependent LNCaP cells, whereas it significantly decreased GPR107 levels in androgen-independent 22Rv1 and PC-3 cells (Figure 6c).

4. Discussion

PCa is the most prevalent form of cancer and the second cause of death in men worldwide [1,5]. The management of PCa has improved in recent years with the use of novel imaging and treatment

procedures; however, locally advanced or metastatic PCa still has the potential to develop often into a lethal phase as no curative paradigm yet exists. Thus, new molecular avenues are urgently needed to better diagnose, predict their prognosis and tumor behavior and to provide tools to develop better therapeutic tools that prolong patient survival. In line with this, the somatostatin-SSTRs system represents a useful source of therapeutic targets and tools to treat various endocrine-related tumors, owing to its pleiotropic role encompassing from whole body homeostasis to cancer cell functioning in different tumor types, where this system commonly acts to inhibit multiple processes, such as hormone secretion and cell proliferation, migration and invasion [10,13,32,40,41]. Notwithstanding, earlier, limited studies using somatostatin-analogues (SSAs) found no benefits in overall survival in PCa patients [14,42]. More recently, we reported that one of the mechanistic reasons of this clinical failure might be the presence and relevant oncogenic role of the spliced SST_5TMD4 variant in PCa cells [11]. In this scenario, the recently discovered functional system associated to the somatostatin regulatory axis comprised by NST and GPR107 has been shown to exert diverse physiologic activities at the central and peripheral level [15,19,43]; however, their presence and possible functional role in the pathophysiology of PCa is still unknown.

Accordingly, we initially explored this issue by testing for the first time the GPR107 presence and functional relevance in PCa, using diverse experimental and analytical approaches. This revealed that GPR107 is present in a high proportion of PCa samples and is overexpressed, at both mRNA and protein levels, in PCa tissues, as compared to non-tumor tissues in two independent cohorts of human samples. Moreover, elevated expression of GPR107 was found in primary tissues from patients diagnosed with localized PCa and in patients with more aggressive, metastatic PCa. Most importantly, GPR107 overexpression was evidenced in samples from patients with mHSPC compared to those without metastasis. In this line, although we acknowledge that a limitation of our study is the lack of analyzed mCRPC samples, the results presented herein compare favorably with data from two independents external in silico cohorts of patients with mCRPC (Grasso and Varambally datasets). As a result, ROC analysis revealed that GPR107 expression could discriminate between patients that developed metastasis vs. those that did not. Even more important is the fact that GPR107 expression levels were directly associated with other relevant clinical parameters of PCa-aggressiveness (i.e., tumor stage and vascular invasion and presence of metastasis) as well as with the expression levels of key molecular markers of PCa-aggressiveness (e.g., CDK2, VEGFR, IL6R) [44–46]. These results reinforce the notion of a causal link between dysregulation of GPR107 expression and PCa aggressiveness, suggesting that this receptor may play a significant pathophysiological role in PCa cells. The contention of the potential oncogenic role of GPR107 in PCa is in line with a previous report indicating that GPR107 drives self-renewal and tumorigenesis of liver tumor initiating cells [47]. Thus, our results offer original evidence to suggest that GPR107 dysregulation may play a relevant functional pathophysiological role in PCa and could provide new tools as a diagnostic and prognostic biomarker and/or therapeutic target for PCa, especially for metastatic PCa, given its association with clinical and molecular features of aggressiveness.

These initial results led us to further explore the functional pathophysiological role of GPR107 in PCa cell models. The first approach was to assess the effect of GPR107-silencing on cell proliferation and migration, two parameters tightly linked to tumor growth and metastasis, some of the main clinical problems associated to PCa. Silencing of GPR107 decreased proliferation and migration in two representative models of CRPC pathology (i.e., 22Rv1 and PC-3 cells), demonstrating that GPR107 is functionally active in AI-PCa cells and that its presence is directly associated with their aggressiveness features. These results are in agreement with a previous evidence indicating that GPR107 expression knock-down decreased aggressiveness features in liver tumor initiating cells (i.e., impaired tumor initiation, self-renewal and invasion capacities) [47]. Additionally, the functional data observed in the present study in response to GPR107-silencing (i.e., decreased proliferation and migration capacity) could suggest that GPR107 bears a constitutive functional activity in PCa cells. Remarkably, this is neither the sole nor the first time that a constitutive activation of receptor belonging to the

somatostatin-related regulatory system has been reported since various SSTRs have been demonstrated to display a relevant degree of ligand-independent constitutive activity in different cell systems [48]. The mechanisms underlying the effect of GPR107 are yet unknown, and future studies should ascertain whether they are mediated by ligand-dependent or -independent (e.g., receptor constitutive activity) actions. Nonetheless, these observations unveiled new conceptual and functional avenues in PCa, with potential therapeutic implications, which warrant further investigation.

To interrogate the signaling pathways and molecular elements mediating GPR107 actions in PCa cells, we used AI-PCa cells (22Rv1 and PC-3 cells) as model. This revealed that GPR107 might exert its tumor-associated functions through modulation of several molecular/signaling pathways, including a decreased activation (basal phosphorylation) of AKT signaling pathway, which has been shown to be a key oncogenic-signaling pathway and cooperate in different tumor pathologies, including PCa, to promote malignancy, drug resistance and CRPC development [49,50]. In fact, this result indicating that silencing of GPR107 may inhibit cell proliferation/migration via negative regulation of AKT pathway is a common mechanism that has been previously reported with other components of the somatostatin system in different tumor types, including PCa [10,41,51–53]. Moreover, silencing of GPR107 in AI-PCa cells decreased the expression levels of MKI67, a well-known proliferation marker associated to biochemical recurrence in PCa [54], and, in PC-3 cells, tended to increase the expression of CDKN2D, a cell-cycle inhibitor involved in the growth arrest at senescence of PCa cells [55]. Similarly, GPR107-silencing resulted in a decrease of MMP9 and PRPF40A, genes involved in the process of migration and cytoskeletal regulation, respectively [56,57]. Interestingly, we also found that the decrease in the aggressiveness of PCa cells in response to GPR107-silencing could also involve a diminished expression of the splicing variants SST_5TMD4, In1-ghrelin and AR-v7, as well as EZH2, four elements previously reported as key oncogenic factors in PCa and/or main drivers of CRPC [11,30,58,59]. Interestingly, GPR107 expression was correlated with SST_5TMD4 but not with SST_5, In1-ghrelin or AR-v7 expression in the more aggressive cohort of PCa samples (Cohort 2, Figure S2), which reinforces the idea of a role for GPR107 as potential therapeutic target in PCa, in that we have recently reported that SST_5TMD4 is a key pathophysiological component in this cancer type [11,53]. This is consistent, actually, with the fact that GPR107 silencing in AI-PCa cells induced significant changes in key factors involved in and associated with SST_5TMD4-related pathways, such as modulation of AKT-signaling pathway and MKI67 expression [11,53]. Moreover, these results have special relevance because GPR107 silencing was able to consistently decrease SST_5TMD4 expression in all the AI-PCa models tested herein, in which we previously demonstrated that overexpression of SST_5TMD4 is directly associated to the inefficiency of SSA therapy (i.e., octreotide treatment) in PCa cells and other tumor types [11,32,60–62] as well as of other drugs currently used for the treatment of PCa (i.e., abiraterone or enzalutamide) [11].

Finally, in order to further explore the potential utility of the NST-GPR107 system as therapeutic target, functional and mechanistic studies were performed in response to NST treatment in PCa cells [16,17,19]. Our results revealed for the first time that NST treatment evoked virtually similar antitumor effects (i.e., reduction of proliferation and migration capacity) to those previously observed with GPR107-silencing in AI-PCa cells. Similarly, treatment with NST induced a signaling and molecular regulatory response comparable to that of the GPR107-silencing treatment (i.e., inhibition of AKT signaling pathway and modulation of the expression of MKI67, CDKN2D, MMP9, PRPF40A, AR-v7, SST_5TMD4, In1-ghrelin, AR-v7 and EZH2), which reinforces the idea that the antitumor actions observed in response to GPR107-silencing or NST treatment might be functionally connected and mediated through similar mechanisms and/or signaling pathways. Moreover, in support of this notion is the fact that the combined treatment of NST and GPR107-silencing did not modify the anti-proliferative/migratory actions of both treatments individually in AI-PCa cells. Furthermore, we also found that NST administration significantly decreased GPR107 levels in AI-PCa cells, which might indicate that the antitumor actions of NST might be exerted, at least in part, by decreasing the expression levels of GPR107 in AI-PCa cells. Therefore, although further studies would be necessary

before a precise and unequivocal conclusion can be reached in this regard, all these in vitro experiments suggest that GPR107 may exert its oncogenic role through the induction of a constitutive activation of AKT signaling pathway, which may lead to changes in the expression of prostate cancer-related genes (i.e., the overexpression of MKI67, MMP9, PRPF40A, SST5TMD4, AR-v7, In1-ghrelin, EZH2 and the reduction of CDKN2D gene expression) and that NST may be exerting its anti-tumor actions by decreasing GPR107 expression, at least in AI-PCa cells.

5. Conclusions

Taken together, our results provide the first identification of the presence and functional role of the NST-GPR107 system in PCa, which enabled to demonstrate a relevant pathological function and therapeutic potential of this regulatory system in PCa cells in vitro and in vivo. Indeed, our results demonstrate that GPR107 is overexpressed in PCa, especially in metastatic-PCa, and its expression levels are associated to key aggressiveness features of PCa suggesting that this receptor could represent a valuable diagnostic tool and a promising prognostic biomarker in PCa patients. Moreover, we have demonstrated that both GPR107-silencing and NST treatment altered key pathophysiological parameters in PCa in vitro, including a reduction of cell proliferation and migration and modulation of the expression levels of relevant molecular markers (e.g., MKI67, SST_5TMD4, AR-v7, etc.), possibly through the modulation of the key AKT pathway. Altogether, the translational research implications of these findings indicate that GPR107 has a functional role in the pathophysiology of PCa and invites to suggest that pharmacological treatments specifically targeting this receptor, including NST treatment, could become a promising option to treat patients with PCa, specially metastatic PCa, providing a relevant clinical conclusion, which should be soon tested for their use in humans.

Supplementary Materials: The following are available online at http://www.mdpi.com/2077-0383/9/6/1703/s1. Figure S1: Validation of GPR107-silencing in androgen-independent PCa cell line 22Rv1 after 48h of GPR107 siRNA transfection. Figure S2: Correlation between GPR107 expression levels and different key oncogenic factors in primary PCa samples from patients with metastasis. Figure S3. Comparison of GPR107 expression levels between non-Tumoral (N), primary tumor (PT), mHSPC specimens (M) obtained from three in silico databases (Lapointe, Taylor and Tomlins). Table S1: Specific primers for human transcripts used in this study.

Author Contributions: R.M.L. conceived and designed the project. P.S.-M., J.M.J.-V., A.J.L.-G., V.H.-A., A.J.M.H., E.G.-G., R.S.-S., M.J.R.-T. and R.M.L. acquired the data. P.S.-M., J.M.J.-V., A.J.L.-G., V.H.-A., A.J.M.H., E.G.-G., R.S.-S., M.J.R.-T., J.P.C., M.D.G. and R.M.L. performed the analysis and interpretation of data. P.S.-M. and R.M.L. wrote the manuscript. J.M.J.-V., A.J.L.-G., V.H.-A., A.J.M.H., E.G.-G., R.S.-S., M.J.R.T., J.P.C. and M.D.G. revised the manuscript for important intellectual content. P.S.-M., J.M.J.-V., V.H.-A., A.J.M.H. and R.M.L. performed the statistical analysis. R.M.L., M.D.G. and J.P.C. obtained funding's. E.G.-G., R.S.-S. and M.J.R.-T. provided technical and material support. R.M.L. supervised the work. All authors have read and agreed to the published version of the manuscript.

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Original Articles

Dysregulation of RNA-Exosome machinery is directly linked to major cancer hallmarks in prostate cancer: Oncogenic role of PABPN1

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Therapeutic targets

ABSTRACT

Novel biomarkers and therapeutic strategies for prostate-cancer (PCa) are required to overcome its lethal progression. The dysregulation/implication of the RNA-Exosome-complex (REC; cellular machinery controlling the 3′-5′processing/degradation of most RNAs) in different cancer-types, including PCa, is poorly known. Herein, different cellular/molecular/preclinical approaches with human PCa-samples (tissues and/or plasma of 7 independent cohorts), and *in-vitro/in-vivo* PCa-models were used to comprehensively characterize the REC-profile and explore its role in PCa. Moreover, isoginkgetin (REC-inhibitor) effects were evaluated on PCa-cells. We demonstrated a specific dysregulation of the REC-components in PCa-tissues, identifying the Poly(A)-Binding-Protein-Nuclear 1 (PABPN1) factor as a critical regulator of major cancer hallmarks. PABPN1 is consistently overexpressed in different human PCa-cohorts and associated with poor-progression, invasion and metastasis. PABPN1 silencing decreased relevant cancer hallmarks in multiple PCa-models (proliferation/migration/tumourspheres/colonies, etc.) through the modulation of key cancer-related lncRNAs (*PCA3/FALEC/DLEU2*) and mRNAs (*CDK2/CDK6/CDKN1A*). Plasma PABPN1 levels were altered in patients with metastatic and tumour-relapse. Finally, pharmacological inhibition of REC-activity drastically inhibited PCa-cell aggressiveness. Altogether, the REC is drastically dysregulated in PCa, wherein this novel molecular event/mechanism, especially PABPN1 alteration, may be potentially exploited as a novel prognostic and therapeutic tool for PCa.

1. Introduction

Prostate cancer (PCa) represents one of the most common cancer types in men worldwide, and the second cause of cancer-related death in this collective [1]. Currently, two main strategies are employed to combat this pathology: 1) early detection followed by radical therapeutic approaches, including surgery and radiotherapy; and 2) managing advanced disease primarily through hormonal therapy and chemotherapy [2]. Although new diagnostic (e.g., phi-test, 4kscore-test, etc.) and therapeutic (e.g., PARP-inhibitors, immunotherapy, etc.) approaches have been established to manage PCa [3–7], the mortality associated to this pathology is expected to increase almost double by 2040 [2]. Therefore, identifying novel diagnostic and prognostic biomarkers, as well as therapeutic targets for PCa, is an urgent clinical unmet need.

The disruption of RNA metabolism has emerged as a key hallmark of cancer, being involved in the development/progression of different cancer types [8]. Thus, components of macromolecular machineries involved in the regulation of cellular processes controlling the RNA metabolism, including splicing and nonsense-mediated decay (NMD)

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among others, are frequently altered in PCa and hold diagnostic and prognostic potential [9–14]. Likewise, the RNA-Exosome Complex (REC; a multi-subunit ribonucleolytic complex organized into nucleases, co-factors and core elements) plays a pivotal role in the regulation of RNA homeostasis. Specifically, the REC carries out the 3′-5′processing, quality control and degradation of virtually all classes of nuclear and cytoplasmic RNAs, thus representing one of the most versatile RNA-machinery in eukaryotes, and being a potential source of clinical tools for cancer [15,16]. However, despite its relevance, to the best of our knowledge, the levels, potential alterations and clinical significance of the components of the REC (cofactors, nucleases, and core elements) have not yet been explored in PCa.

For all the reasons mentioned above, the present study aimed to explore the dysregulation and potential clinical implication of the REC in PCa to identify key components that could serve as biomarkers and/or therapeutic targets, and therefore be valuable for the clinical management of this devastating disease. In fact, this study underscores the significance of molecular RNA-exosome machinery components, especially PABPN1, as potential exploitable tools for PCa diagnosis, prognosis, and therapy.

2. Materials and methods

2.1. Ethics statement

This study was approved by the Reina Sofia University Hospital Ethics Committee and was conducted in accordance with the principles of the Declaration of Helsinki. The biobank of the public health system of Andalusia coordinated the collection of the biological samples according to the standard procedures established for this purpose. Written informed consent was obtained from all individuals included in the study. All *in vivo* experiments with mice were performed according to the European Regulations for Animal Care under the approval of the university/regional government research ethics committees.

2.2. Human samples

Two different cohorts of prostate tissues were used in the present study: **Cohort-1**) formalin-fixed, paraffin-embedded (FFPE) PCa tissues ($n = 84$) and their non-tumour adjacent region (N-TAR; used as control tissues; $n = 84$), taken from radical prostatectomies from patients diagnosed with clinically-localized PCa (Table-1); and, **Cohort-2**) Significant PCa fresh samples ($n = 66$) obtained by core needle biopsies (Table-2). Available clinical parameters were Gleason-Score, T-Stage, perineural invasion, lymphovascular invasion, and/or presence of metastases at diagnosis (determined by computed tomography and bone scan). Additionally, an independent cohort of plasma samples (**Cohort-3)** from patients diagnosed with PCa ($n = 158$) and from patients with PCa suspicion but negative results in their biopsies ($n = 111$; controls) was also analysed (Table-3). Finally, transcriptomic/clinical information from four external cohorts (**Cohorts 4–7: TCGA**, **Grasso**, **Varambally**, and **Glinsky**) was downloaded from the CANCERTOOL-portal [17].

Table 1
Biochemical and clinical parameters of the patients from cohort 1. PSA: Prostate specific antigen; pT: Pathological primary tumor staging; PI: Perineural invasion; VI: Vascular invasion.

Patients [*n*]	84
Age, years [median (IQR)]	61 (57–66)
PSA levels, ng/mL [median (IQR)]	5.2 (4.2–8.0)
Gleason score ≥ 7 [*n* (%)]	76 (90.5%)
pT ≥ 3a [*n* (%)]	59 (70.2%)
PI [*n* (%)]	72 (85.7%)
VI [*n* (%)]	8 (9.52%)
Recurrence [*n* (%)]	35 (41.7%)
Metastasis [*n* (%)]	0 (0%)

Table 2
Biochemical and clinical parameters of the patients from cohort 2. PSA: Prostate specific antigen; SigPCa: Significant PCa defined as Gleason score ≥7.

Patients [*n*]	66
Age, years [median (IQR)]	75 (69–81)
PSA levels, ng/mL [median (IQR)]	62.0 (36.2–254.5)
Gleason score ≥ 7 [*n* (%)]	66 (100%)
Metastasis at diagnosis [*n* (%)]	11 (17%)

Table 3
Biochemical and clinical parameters of the patients from cohort 3. PSA: Prostate specific antigen; SigPCa: Significant PCa defined as Gleason score ≥7.

Control patients [*n*]	111
Age, years [median (IQR)]	63 (57–69)
PCa Patients [*n*]	**158**
Age, years [median (IQR)]	67 (61–72)
PSA levels, ng/mL [median (IQR)]	6.64 (4.49–11.32)
Gleason score ≥7 [*n* (%)]	91 (57.59%)
Metastasis at diagnosis [*n* (%)]	9 (5.69%)

2.3. Cell cultures and reagents

The normal-like prostate epithelium cell-line PNT2 was kindly provided by Prof. Johann De Bono (London). The PCa cell-models (DU145 and LNCaP) were obtained from American-Type-Culture-Collection (ATCC, Manassas, VA, USA), maintained according to manufacturer instructions, validated by analysis of short tandem repeats sequences (STRs), and monthly checked for mycoplasma contamination, as previously reported [9,18,19]. Isoginkgetin (#416154-10 MG, Calbiochem, Frankfurter, Germany) was dissolved in DMSO (Applichem, Chicago, USA) and used at 35 μM (concentration as reported elsewhere [20]) to block the REC activity in PCa cells.

2.4. RNA isolation, retrotranscription, quantitative real-time PCR (qPCR), and customized qPCR dynamic array

RNA from FFPE samples, fresh tissues, and cell-lines was isolated as previously reported [9]. Briefly, Maxwell 16 LEVRNA FFPE Kit (Promega, Madison, USA) was used in the Maxwell MDx16 Instrument (Promega, Madrid, Spain) to isolate RNA from FFPE samples. AllPrep DNA/RNA/Protein Mini Kit (Qiagen) and TRIzol Reagent (Thermo-Fisher, Waltham, MA, USA) were used to isolate RNA from fresh human and mouse tissues and from the different cell-lines, respectively. RNA concentration/purity was assessed using Nanodrop One Spectrophotometer (Thermo-Fisher). Total RNA was retrotranscribed using random hexamer primers and the cDNA First-Strand Synthesis kit (Thermo-Fisher). Details regarding the development and validation of primers, standard real-time qPCR, and qPCR microfluidic-based dynamic array technology to explore mRNA levels have been previously reported by our laboratory [9,21]. Detailed information about the primers used to quantify the expression levels of the components of the RNA-exosome machinery (including 11 co-factors, 9 core elements, and 3 nucleases), as well as critical mRNA-encoding genes involved in PCa pathophysiology can be found in Supplemental Tables 1 and 2, respectively. Gene expression levels were adjusted by a normalization factor (calculated from the expression of *ACTB* and *GAPDH* using GeNorm 3.3 [22]).

2.5. Immunohistochemistry (IHC)

A representative set of PCa tissue samples from cohort-1 [PCa and their N-TAR ($n = 10$)] and cohort-2 [PCa fresh samples with ($n = 5$) and without ($n = 5$) metastasis at diagnostic] were used for IHC analyses

following a protocol previously described [10]. Briefly, deparaffinized sections were incubated overnight (4 °C) with the anti-PABPN1 antibody (ab75855, Abcam; 1:100), followed by incubation with anti-rabbit horseradish peroxidase-conjugated secondary antibody (#7074; Cell-Signalling). Finally, sections were developed with 3,39-diaminobenzidine (Envision-system 2-Kit Solution DAB) and contrasted with hematoxylin. The nuclear H-score was calculated as the sum of the percentage of stained nuclei with low, moderate, and high intensity following a blinded protocol as described elsewhere [23].

2.6. Generation of PABPN1 silenced and overexpressed cell lines

DU145 and LNCaP cells were used for silencing experiments as previously reported [21,24]. Briefly, 400,000 cells were seeded and grown until 70–90% confluence was reached. Then, cells were independently transfected during 48 h with two different small-interfering RNAs (siRNAs) for knockdown of PABPN1 endogenous levels, along with the Silencer Select Negative Control siRNA (Trilencer-27 kit #SR30537 Origene, Rockville, USA), at 15 nM (Manufacture recommended dose) using Lipofectamine-RNAiMAX (#13778-150, Thermo-Fisher), following the manufacturer's instructions.

DU145 cells were transiently transfected with plasmids as previously reported [10]. Briefly, 200,000 cells were seeded and incubated until reach 60–80% of confluence. Then, cells were transfected with 0.7 μg of pCDNA3.1-PABPN1 plasmid (OHu22259D, Genscript, New Jersey, USA) or pCDNA3.1-empty plasmid (mock-vector used as control) using Lipofectamine-2000 at 1:3 ratio (Gibco, Barcelona, Spain), following manufacturer's instructions. Completed media was refreshed after 24 h of transfection and cells were evaluated after 48 h of transfection.

2.7. Cell proliferation

Proliferation was assessed by Resazurin-reagent (#CA035; Canvax Biotech, Córdoba, Spain) in response to PABPN1-silencing and isoginkgetin treatment, as previously described [25,26]. Briefly, cells were seeded at a density of 3500 cells/well, serum-starved overnight, and then fluorescence was measured (560 nm excitation and 590 nm emission) after 3 h incubation with 10% resazurin using the FlexStation III system (Molecular-Devices, Sunnyvale, CA, USA). Resazurin measurement was repeated after 24 h, 48 h, and 72 h of incubation. Isoginkgetin treatment was also refreshed at these points. All the data were normalized to values obtained on day 0 and represented as fold change compared to control-experimental cells.

2.8. Colonies formation

Clonogenic assay was performed in DU145 and LNCaP cells in response to PABPN1-silencing and isoginkgetin treatment, as previously described [27,28]. Briefly, 3000 cells were seeded and 24 h later treatments were administered, and cells were incubated for 10 days (in the case of isoginkgetin, treatment was refreshed at day 5). Then, cells were washed with PBS1X and crystal violet 0.5% plus glutaraldehyde 6% was added and incubated for 30 min at room temperature. Finally, cells were rinsed 3 times with distilled water and left to dry at room temperature. Colonies were measured by ChemiDoc-XRS + System (Bio-Rad, Hercules, CA; SCR_019,690), and analysed using ImageJ-software [29].

2.9. Tumourspheres formation

Tumourspheres formation assay was performed with DU145 and LNCaP cells in response to PABPN1-silencing and isoginkgetin treatment, as previously described [30,31]. Briefly, 2000 cells/well were seeded and cultured in Corning Costar 24-well ultra-low attachment plates (Merck, Madrid, Spain) with DMEM F-12 medium supplemented with EGF (20 ng/mL; Sigma-Aldrich), FGF (10 ng/mL; #100-18B Peprotech, London, UK) and B271X (#12587010, Thermo-Fisher) for 14 days. All supplements were refreshed every three days. Isoginkgetin treatment was added when cells were seeded and at day 7. Photographs were taken to visualize and measure the tumourspheres number and size after 14 days of incubation with ImageJ-software [29].

2.10. Cell migration

Migration was evaluated by wound-healing assay only in DU145 cells given its high invasiveness nature, and not in the LNCaP cells due to its incompatibility to obtain reliable data with this assay, as previously reported [21,28]. Briefly, 40,000 cells were seeded in an Incucyte Imagelock 96-well plate (Cat.No. 4379, Sartorius, Goettingen, Germany). Then, when confluence was reached, cells were starved for 3 h to achieve cell-synchronization, and then, the wound was made using Incucyte® Woundmaker-Tool (Cat.No. 4563, Sartorius) in each well, and the media was replaced by fresh serum-free media (in the case of isoginkgetin, treatment was added at this moment). Images of the wound were taken at time 0 and 24 h after the wound and analysed using ImageJ-software [29].

2.11. Long non-coding RNA (lncRNAs) analysis

The Human Long-non-coding RNA and Cancer SignArray (#PMS2-Z2, AnyGenes) was used to analyse the expression levels of 84 lncRNAs and 8 internal controls (Housekeeping genes) in *PABPN1*-silenced PCa cells after the samples retrotranscription using the StaRT reverse-transcription kit (#StaRT-10, AnyGenes, France), following the manufacturer's instructions. Gene expression levels were adjusted by a normalization factor (calculated from the expression of *TBP* and *HPRT1*) using GeNorm 3.3 [22].

2.12. Xenograft mouse models

A preclinical xenograft mouse model was developed to test the antitumour action of PABPN1-silencing in PCa cells using methods previously described [18,32]. Specifically, 6-week-old ATHYMFoxn1 nu/nu mice ($n = 4$; Janvier-Labs) were injected subcutaneously with 3.5×10^6 DU145 cells in both flanks [resuspended in 100 μL of basement membrane extract (Trevigen, #3432-010-01)]. Once the tumours reached ~100 mm [3] (~one month later), each mouse received an injection with PABPN1 siRNA into one flank and a negative-control siRNA (scramble-control) into the other flank using AteloGene® (KOKEN Co, #KKN1394). Tumour-growth was monitored 2 days per week using a digital calliper. Sixteen days after injection, mice were sacrificed and each tumour was dissected, fixed, and sectioned for histopathological examination after H&E-staining. Mitosis number examination and Ki67-staining by IHC was performed. Additional tumours pieces were placed in liquid nitrogen and frozen at −80 °C until RNA isolation using Trizol-reagent, as previously reported [18].

2.13. Western blotting

DU145 and LNCaP cells were processed to analyse protein levels by western-blot after PABPN1-modulation using SDS-PAGE method, as previously reported [21,24]. Briefly, total protein extract and dyed molecular weight markers (Bio-Rad, Madrid, Spain) were separated by SDS-PAGE and transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA). Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline/0.05% Tween-20, and incubated overnight with anti-PABPN1 antibody (1:1000; ab75855; Abcam). Secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit Immunoglobulin G was used (1:2000; #7074S; Cell-Signaling). Proteins were detected using an enhanced chemiluminescence detection system (GEHealthcare, Madrid, Spain). PABPN1 protein levels were normalized with total protein detected (Ponceau-stain), as previously reported [9]. A

densitometric analysis of the bands was carried out with ImageJ-software [29].

2.14. PABPN1 protein levels determination in plasma and secreted media

Commercial ELISAs (#MBS280784-96, MyBioSource, San Diego, USA) were used to determine PABPN1 levels in plasma from patients with and without PCa (Cohort-3), and from secreted media obtained from normal and tumour prostate cell-models (PNT2 and DU145/LNCaP, respectively), following the manufacturer's instructions. The sensitivity of this assay is 7.8 pg/mL, and the detection range is between 15.6 and 1,000 pg/mL. The intra-/inter-assay accuracy showed a coefficient of variations lower than 8% and 12%, respectively. To obtain the secreted media, 400,00 cells were seeded, and 24 h later washed with PBS1X and serum-free media was added. 24 h later, secreted media was collected, centrifuged for 10 min at 3000 rpm, aliquoted, and stored at −80 °C. Optical density at 450 nm was measured to determine PABPN1 protein levels using the FlexStation III system (Molecular-Devices).

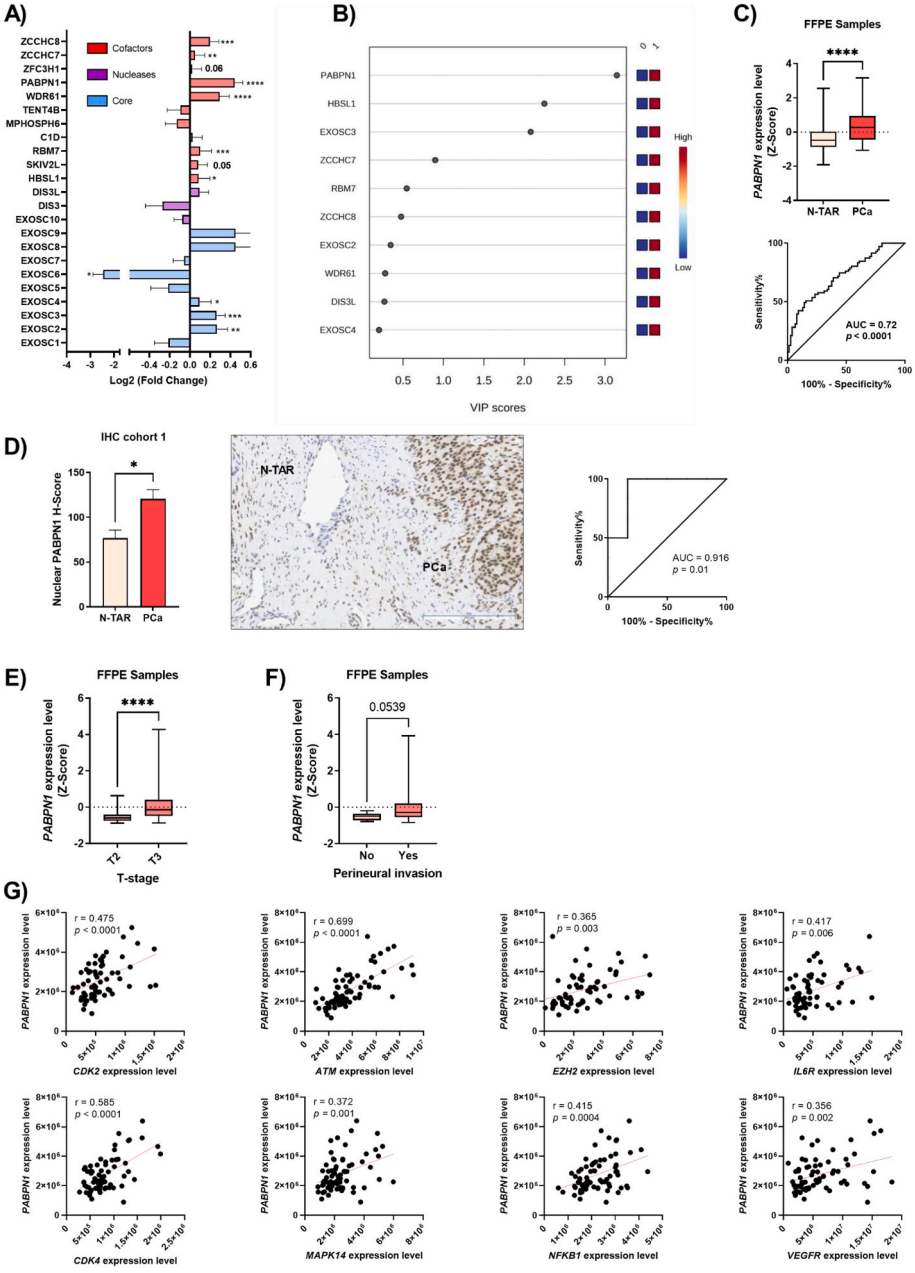

Fig. 1. The RNA-Exosome complex dysregulation in prostate cancer. (A) Individual fold-change of the expression of all the RNA-Exosome complex (REC) components analysed in prostate cancer (PCa) and non-tumour adjacent region (N-TAR) from patients belonging to cohort 1 ($n = 84$). **(B)** Variable importance in projection (VIP) score obtained from partial least squares discriminant analysis (PLS-DA) of all the REC components studies. **(C)** Comparison of *PABPN1* mRNA expression between PCa samples and N-TAR and its associated ROC curve. **(D)** Comparison of PABPN1 protein levels by immunohistochemistry (IHC) between PCa tissue and N-TAR in a representative set of samples (IHC cohort 1; $n = 10$). Representative image is shown in the middle panel. **(E-F)** Association between *PABPN1* mRNA levels and clinical parameters (T-stage and perineural invasion) in PCa samples from cohort 1 ($n = 84$). Data are represented as mean ± SEM of mRNA levels adjusted by a normalization factor (calculated from *ACTB* and *GAPDH* expression levels). Normalized mRNA levels were standardized by Z-score. **(G)** Correlation between *PABPN1* mRNA levels and expression levels of *CDK2*, *ATM*, *EZH2*, *IL6R*, *CDK4*, *MAPK14*, *NFKB1* and *VEGFR*. Correlations are represented by mean (connecting line) of expression levels. Asterisks (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) indicate statistically significant differences between groups.

2.15. Bioinformatics and statistical analysis

All the experiments were performed in at least 3 independent experiments ($n \geq 3$) and with at least 2 technical replicates. Statistical differences between two conditions were calculated by unpaired parametric *t*-test or nonparametric Mann-Whitney *U* test, according to normality, assessed by Kolmogorov-Smirnov test. For differences among three conditions, a One-Way ANOVA or Kruskal-Wallis analysis was performed. Spearman's or Pearson's bivariate correlations were performed for quantitative variables according to normality. The receiver-operating-characteristic (ROC) curve to obtain the Area under Curve (AUC) was performed for the evaluation of the PABPN1 accuracy as a discriminator marker between different conditions. All these analyses were assessed using GraphPad-Prism 9 (La Jolla, CA, USA). Moreover, clustering analyses including Partial Least Squares-Discriminant Analysis (PLS-DA) and its variable importance in projection (VIP) score were performed using MetaboAnalyst-Software v.4.0 (McGill University, Quebec, Canada). PrediSi informatic software was used to predict potential secretion sequences of PABPN1. $P<0.05$ were considered statistically significant. A trend for significance was indicated when p-values ranged between >0.05 and < 0.1. Data represent means ± standard error of the mean (SEM).

3. Results

3.1. The RNA-Exosome complex is drastically dysregulated in PCa

REC components (cofactors, nucleases, and core-elements) expression levels were initially analysed in PCa vs. control (N-TAR) tissues in cohort 1 (Table-1). Specifically, expression levels of six cofactors (*ZCCHC8*, *ZCCHC7*, *PABPN1*, *WDR61*, *RBM7*, and *HBSL1*) and 3 core-elements (*EXOSC4*, *EXOSC3*, and *EXOSC2*) were significantly elevated in PCa tissue vs. N-TAR (Figure-1A). Moreover, two additional cofactors (*ZFC3H1* and *SKIV2L*) also tended to be higher in PCa tissues, while the expression of the core-element *EXOSC6* was significantly downregulated in PCa vs. N-TAR (Figure-1A). Indeed, application of the variable VIP score of PLS-DA based on the expression pattern of all the REC-components revealed that PABPN1 was the top hit at discriminating between PCa and control tissues (VIP score > 3; Figure-1B). Indeed, ROC-curve analysis corroborated the capacity of PABPN1 to finely discriminate between PCa and control samples, suggesting a potential diagnostic capacity of this REC-component in PCa confirmed by the AUC = 0.72 ($p < 0.0001$; Figure-1C). Actually, ROC-curves obtained from the expression of the rest of REC-components confirmed a worse ability to discriminate between these conditions (Supplemental Figure-1). Based on these results, PABPN1 was selected for further analyses.

3.2. PABPN1 protein levels are elevated in PCa

Consistent with the mRNA levels, IHC analysis revealed that PABPN1 protein levels were also significantly higher in PCa tissue vs. N-TAR (Figure-1D), and that these levels could significantly discriminate between these conditions based on the ROC curve analysis (Figure-1D, right-panel; AUC = 0.916; $p = 0.01$). As expected, the IHC-staining was observed in the nucleus of PCa cells (Figure-1D), according to PABPN1 function [33].

3.3. PABPN1 levels are overexpressed in additional patient cohorts and associated with relevant molecular and clinical pathophysiological features, as well as with disease-free survival in PCa

High *PABPN1* expression found in cohort-1 was associated with relevant clinical parameters [i.e., advanced T-stage (Figure-1E), and perineural invasion (Figure-1F)], and positively correlated with the expression of key genes involved in cell-cycle (i.e., *CDK2*, *CDK4*, *ATM*, *MAPK14*) and tumour-aggressiveness (i.e., *EZH2*, *IL6R*, *NFKB1*, *VEGFR*) (Figure-1G).

We next investigated a cohort of patients with more aggressive tumours (Cohort-2; Table-2). Specifically, PABPN1 mRNA and protein levels were overexpressed in primary tumours from patients with metastasis compared with those without metastasis at diagnosis [Figure-2A and 2B, respectively; ROC analysis of PABPN1 protein levels showed an AUC = 1 ($p = 0.03$; Figure-2B, right-panel)]. Additionally, we could corroborate that *PABPN1* expression levels were significantly elevated and could discriminate between metastatic PCa samples and non-metastatic samples in two external cohorts [Varambally (Figure-2C; AUC = 0.83, $p = 0.02$), and Grasso (Figure-2D; AUC = 0.72, $p = 0.001$)]. Moreover, *PABPN1* levels were also higher and could discriminate in samples from patients with and without recurrent disease [TCGA (Figure-2E; AUC = 0.59, $p = 0.003$), and Glinsky (Figure-2F; AUC = 0.61, $p = 0.07$) datasets]. Importantly, we also observed that high *PABPN1* expression levels in PCa samples were associated with shorter Disease-Free patient Survival (DFS) [Figure-2G; TCGA ($p = 0.001$), and Glinsky ($p = 0.06$) datasets]. Moreover, we also confirmed the previously observed correlations between *PABPN1-EZH2* and *PABPN1-CDK4* across all datasets (Varambally, Grasso, TCGA, and Glinsky; Figure-2H). Additionally, the expression levels of PABPN1 according to the Gleason grade of the patients [i.e. patients with non-significant Gleason-score ($GS \leq 6$) and significant GS ($GS \geq 7$)] was also analysed in all cohorts but no significant associations were found (Supplemental Figure-2).

3.4. PABPN1-silencing decreases relevant functional parameters in PCa-cells in vitro

We found that *PABPN1* expression levels were significantly higher in DU145 and LNCaP cells compared to PNT2 cells (Figure-3A), indicating that they are appropriate PCa cell-models to study the functional role that PABPN1. Specifically, *PABPN1*-silencing using two different siRNAs in DU145 and LNCaP cells [Figure-3B; Supplemental Figure-3A] decreased proliferation-rate in both models (Figure-3C/-3D). Silencing was more efficient with siRNA-A vs. siRNA-B in both cell-models (Supplemental Figure-3A). Therefore, siRNA-A was selected for further analyses. *PABPN1*-silencing decreased the number and size of tumourspheres in DU145 (Figure-3E) and tumourspheres size in LNCaP (Figure-3F) cells. Moreover, *PABPN1*-silencing significantly decreased the number of colonies and tended to reduce the colonies-covered area ($p = 0.08$) in LNCaP, but not in DU145 cells (Figure-3H/-3G). Finally, *PABPN1*-silencing markedly decreased the migration-rate of DU145 cells (Figure-3I).

3.5. PABPN1-silencing reduces tumour growth in vivo

PABPN1-silencing *in vivo* (Figure-4A) reduced tumour-volume (Figure-4B) in a preclinical xenograft PCa-model [PABPN1-silenced (red-line) vs. scramble-transfected tumours (black-line)]. Moreover, the mitosis number and %KI67 IHC-staining was significantly decreased in the PABPN1-silenced model vs. scramble-transfected group (Figure-4C/-4D), supporting the previously observed anti-tumour potential of PABPN1-silencing in PCa-cells *in vitro* (Figure-3) and *in vivo* (Figure-4).

3.6. Molecular landscape in response to PABPN1-silencing revealed that PABPN1 alters the expression pattern of critical lncRNAs and mRNAs

Given the pivotal role of the RNA-exosome machinery (including PABPN1) in controlling the expression of key lncRNAs in human cells [34, 35], we investigated the potential relationship between the PABPN1-silencing, the observed decrease in oncogenic features, and the regulation of 84 critical lncRNAs in PCa cells (DU145 and LNCaP; Figure-5). *PABPN1*-silencing in DU145 cells significantly decreased the expression of nine lncRNAs (*LINC00312/PCA3/BACE1-A/DLEU2/SNHG1/LNCRNA-ATC/NPTN-IT1/BANCR/BCAR4*), increased four lncRNAs expression (*MALAT1/UCA1/SPRY4-IT1/FOXCUT*), and tended to alter

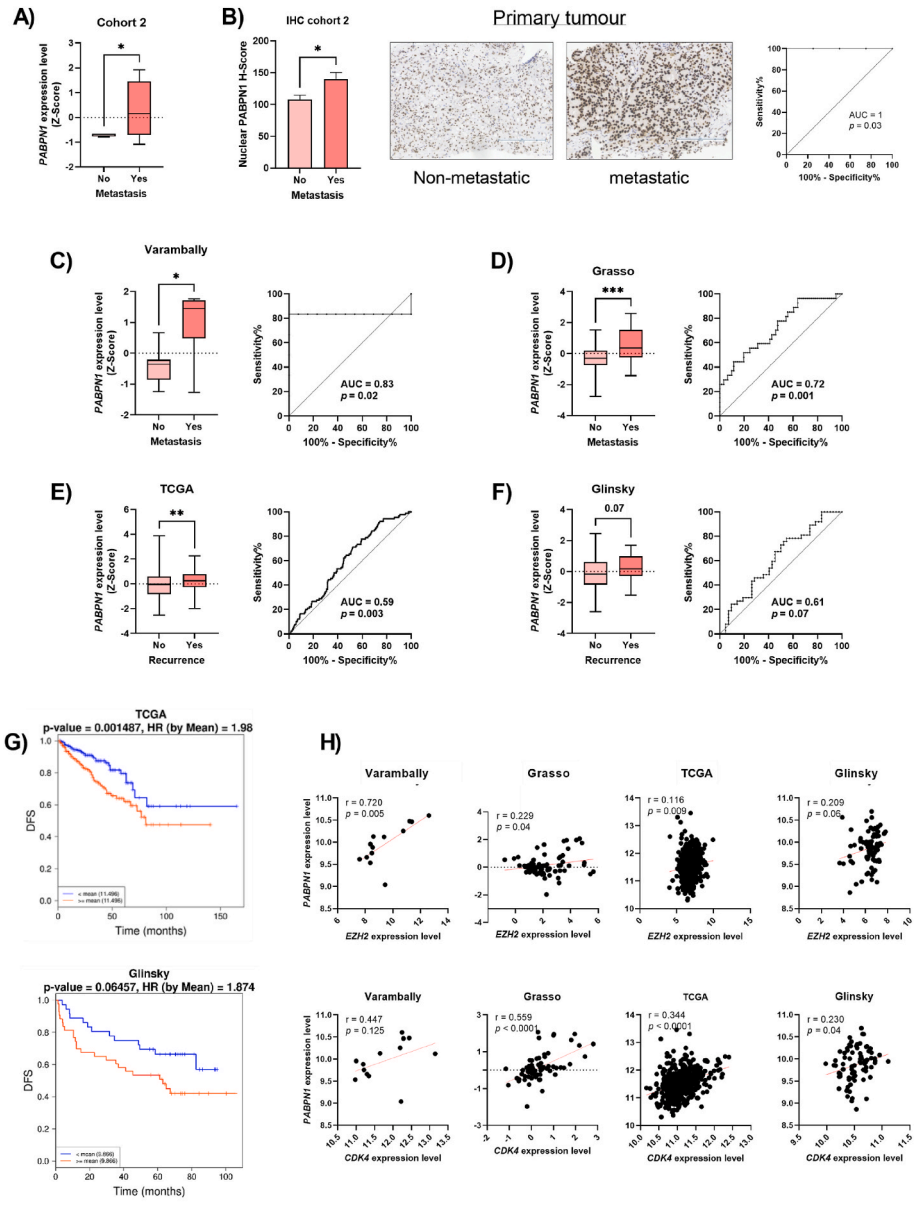

Fig. 2. PABPN1 is associated with aggressive prostate cancer. Comparison of PABPN1 mRNA **(A)** and protein **(B)** levels between primary tumours of patients with and without metastasis at diagnosis obtained from biopsies belonging to cohort 2 ($n = 66$). mRNA data represent the mean ± SEM of mRNA expression levels adjusted by a normalization factor (calculated from *ACTB* and *GAPDH* expression levels) and standardized by Z-score. IHC was used for PABPN1 protein levels comparation. **(C-D)** Comparison of PABPN1 mRNA levels between metastatic and non-metastatic PCa samples obtained from Varambally and Grasso *in silico* cohorts. mRNA expression levels normalized were standardized by Z-score. **(E-G)** Association between *PABPN1* mRNA levels and clinical parameters (presence of recurrence and Disease-free survival) in PCa samples obtained from TCGA and Glinsky *in silico* cohorts. mRNA expression levels normalized were standardized by Z-score. **(H)** Correlation between *PABPN1* mRNA levels and expression levels of *CDK4* and *EZH2* in PCa samples obtained from all *in silico* cohorts explored. Correlations are represented by mean (connecting line) of expression levels. Associated ROC curves of the above-mentioned analysed are represented right to its graphs. The Area under the curve (AUC) and p-value (p) are depicted in the plots. Asterisks ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$) indicate statistically significant differences between groups.

four additional lncRNAs expression (down-regulation of *FALEC/HOXA-AS2*, and up-regulation of *HIF1A-AS1/DHRS4-AS1*; Figure-5A). In LNCaP cells, *PABPN1*-silencing significantly reduced six lncRNAs levels (*FALEC/TERC*, *PCGEM1/UCA1/DLEU2/MEG3*), increased three lncRNAs expression (*FAS-AS1/CCAT1/NRON*), and tended to alter five additional lncRNAs expression (down-regulation of *MALAT1/HULC*, and up-regulation of *DHRS4-AS1/SPRY4-IT1/NKILA*); Figure-5B). The results of the non-dysregulated lncRNAs can be found in Supplemental Figure-4.

Likewise, given also the central role of the RNA-exosome machinery (including PABPN1) in controlling the expression of key mRNAs in human cells [36,37], we also analysed the expression levels of 40 critical genes involved in different pathophysiological processes of PCa in response to *PABPN1*-silencing. A significant decreased in *CDK2* mRNA levels, and an increased in *CDKN1A* expression was observed in both, DU145 and LNCaP, cell-models (Figure-5C/-5D, respectively). Additionally, *PABPN1*-silencing significantly increased *ANGPT4* mRNA levels in DU145 (but not in LNCaP) cells (Figure-5E). Moreover, *PABPN1*-silencing increased *APC* mRNA levels in LNCaP, and tended to up-regulate in DU145 cells (Figure-5F). *PABPN1*-silencing also tended to up-regulate *CDKN1B* and *ATM* expression levels, and to down-regulate *CDK6* levels in DU145 (but not in LNCaP; Figure-5G/-5H/5I, respectively). Finally, *PABPN1*-silencing tended to increase *TP53* expression in

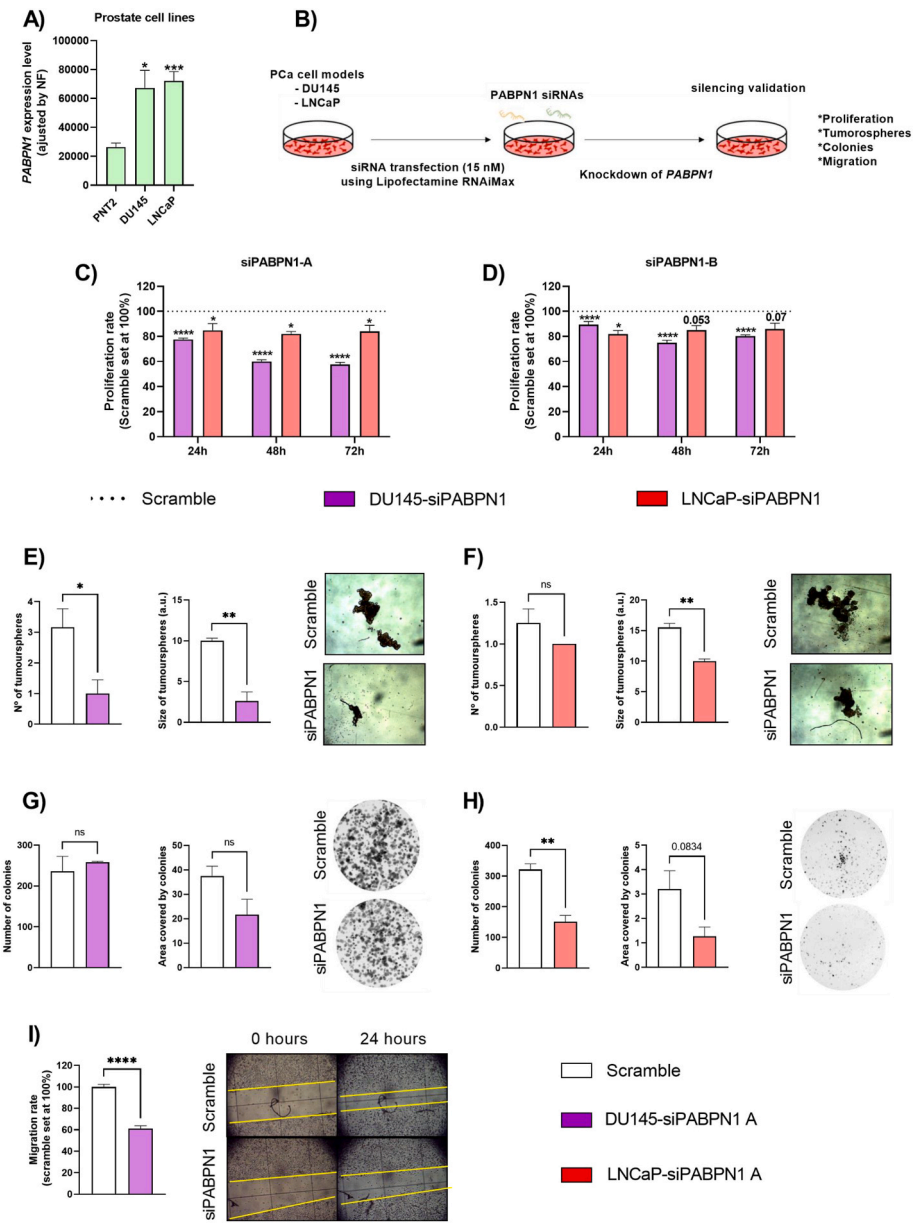

Fig. 3. Functional consequences of *PABPN1* silencing in prostate cancer cell lines. **(A)** Comparison of *PABPN1* mRNA expression levels between non-tumour (PNT2) and tumour (DU145 and LNCaP) prostate-derived cell lines. Data are represented as mean ± SEM of mRNA levels adjusted by a normalization factor (calculated from *ACTB* and *GAPDH* expression levels). **(C-D)** Proliferation rate of DU145 and LNCaP cells in response to *PABPN1* silencing compared to scramble-transfected cells (indicated with the dotted line at 100%). Effect of *PABPN1* silencing in DU145 **(E)** and LNCaP **(F)** tumoursphere number (left panel) and size (right panel) compared to scramble-transfected cells. Representative images of tumourspheres are depicted right on the graphs. Effect of *PABPN1* silencing in DU145 **(G)** and LNCaP **(H)** colony number (left panel) and area (right panel) compared to scramble-transfected cells. Representative images of colonies are depicted right on the graphs. **(I)** Migration rate of DU145 cells in response to *PABPN1* silencing compared to scramble-transfected cells. Representative images are depicted right on the graph. Asterisks (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) indicate statistically significant differences between groups.

LNCaP (but not in DU145; Figure-5J). The results of the non-dysregulated mRNAs can be found in Supplemental Figure-5.

Based on the above results, we found that 20.24% and 16.67% of the lncRNAs analysed were altered (or tended to be altered) in DU145 (n = 17/84) and LNCaP (n = 14/84) *PABPN1*-silenced cells, while 17.5% (n = 7/40) and 10% (n = 4/40) of the mRNAs analysed were altered (or tended to be altered) in the same *PABPN1*-silenced PCa cell-models [DU145 (Figure-5K) and LNCaP (Figure-5L)]. Interestingly, we could conclude that among all genes explored, only four lncRNAs and three mRNAs were similarly altered in both *PABPN1*-silenced PCa-cells [DU145 and LNCaP; lncRNAs (downregulation of *FALEC/DLEU2*, and up-regulation of *DHRS4-AS1/SPRY4-IT1*); mRNAs (downregulation of *CDK2*, and up-regulation of *CDKN1A/APC*)].

3.7. *PABPN1* plasma levels are reduced in advanced PCa patients

The PrediSi informatic tool predicted that PABPN1 protein has a potential N-terminal peptide secretion sequence of 16 amino-acids (Figure-6A); therefore, we next investigate the potential diagnostic and/or prognostic utility of PABPN1 as a non-invasive biomarker by evaluating PABPN1 levels in plasma from patients with and without PCa, with and without biochemical recurrence, and with a without metastasis development (Cohort-3; Table-3). Although plasma PABPN1 levels were not altered in patients with and without PCa (Figure-6B), lower plasma PABPN1 levels were observed in patients with biochemical recurrence vs. without recurrence (Figure-6C), and also a trend was observed in patients with metastatic PCa vs. without metastatic disease

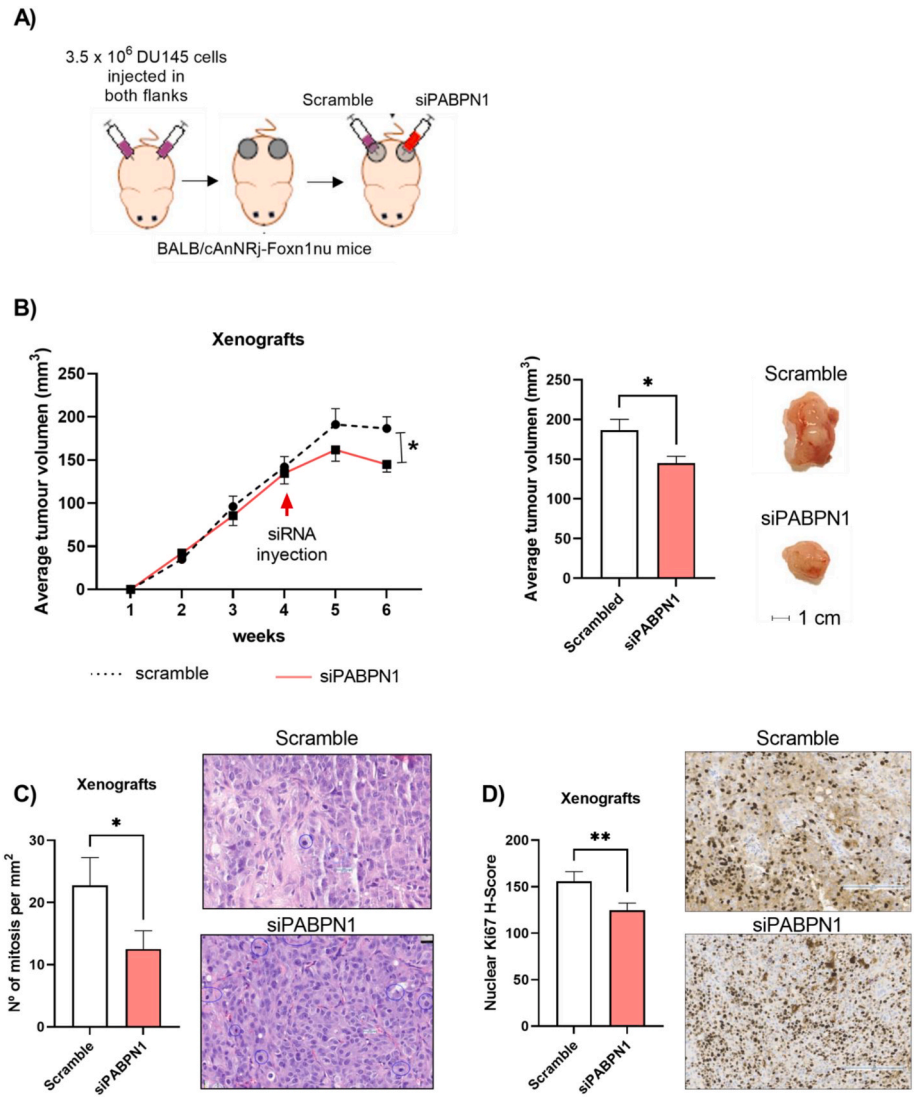

Fig. 4. PABPN1 silencing *in vivo*. (A) Generation of a preclinical-xenograft PCa-model by subcutaneously inoculating DU145 cells in both flanks; once the tumours reach 100 mm^3 siRNA against PABPN1 or scramble was injected (4 tumours/condition). Comparison between the growth over time **(B)** and the tumour volume **(C)** mitotic **(D)** and ki67 **(E)** index at the end of the experiment of xenograft tumours derived from scramble-transfected cells and *PABPN1*-silenced cells. Asterisks (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) indicate statistically significant differences between groups.

(Figure-6D). In line with this, plasma levels of PABPN1 were negatively correlated with the plasma testosterone levels in PCa patients (Figure-6E). Additional analyses revealed that plasma levels of PABPN1 were not altered in control vs. PCa patients ranging from 3 to 10 ng/mL of PSA (the so-called PSA grey-zone, wherein PSA diagnostic capacity is significantly worse), nor in patients with different Gleason Score [i.e. patients with non-significant Gleason-score (GS $\leq$ 6) and significant GS (GS $\geq$ 7)] (Supplemental Figure-6A-B, respectively).

Based on these previous results, we wondered if PCa cells secrete lower protein PABPN1 levels to maintain high protein PABPN1 levels inside PCa cells as an oncogenic feature compared to healthy prostate cells (results previously demonstrated using different patients-cohorts: Figure-1D/-2B). We demonstrated that metastatic DU145 and LNCaP cells secrete lower protein PABPN1 levels vs. healthy prostate PNT2 cells (Figure-6F), and that the PABPN1 overexpression significantly increased the migration-rate and the tumourspheres formed in DU145 cells (Figure-6G).

3.8. Pharmacologic blockade of RNA-exosome activity decreases aggressiveness parameters

Finally, we evaluated the functional effect of isoginkgetin (inhibitor of REC-activity [20]) in LNCaP and DU145 cells (Figure-7). Isoginkgetin treatment decreased proliferation-rate of DU145 and LNCaP cells after 48 h (Figure-7A/-7B), completely abolished colonies-formation in DU145 (Figure-7C), significantly reduced colonies-formation in LNCaP cells (Figure-7D), tended to reduce the number of tumourspheres formed in DU145 (Figure-7E), but not in LNCaP, cells (Figure-7F), and reduced migration-rate of DU145 cells (Figure-7G).

4. Discussion

Despite significant advances over the last years in PCa clinical management, this cancer type is still one of the most serious health problems worldwide, representing 1.3 million new cases and leading to more than 400,000 deaths every year [1]. Then, further knowledge about PCa-biology is necessary to overcome this devastating pathology. Thus, an important cancer-hallmark involved in the development/progression of different cancers is the disruption of cellular machineries that are responsible for the surveillance and control of RNA-metabolism [8–10,38,39]. Surprisingly, despite playing a key role in the processing and surveillance of a whole host of RNA types, the dysregulation of the molecular components of the RNA-exosome machinery, a highly conserved

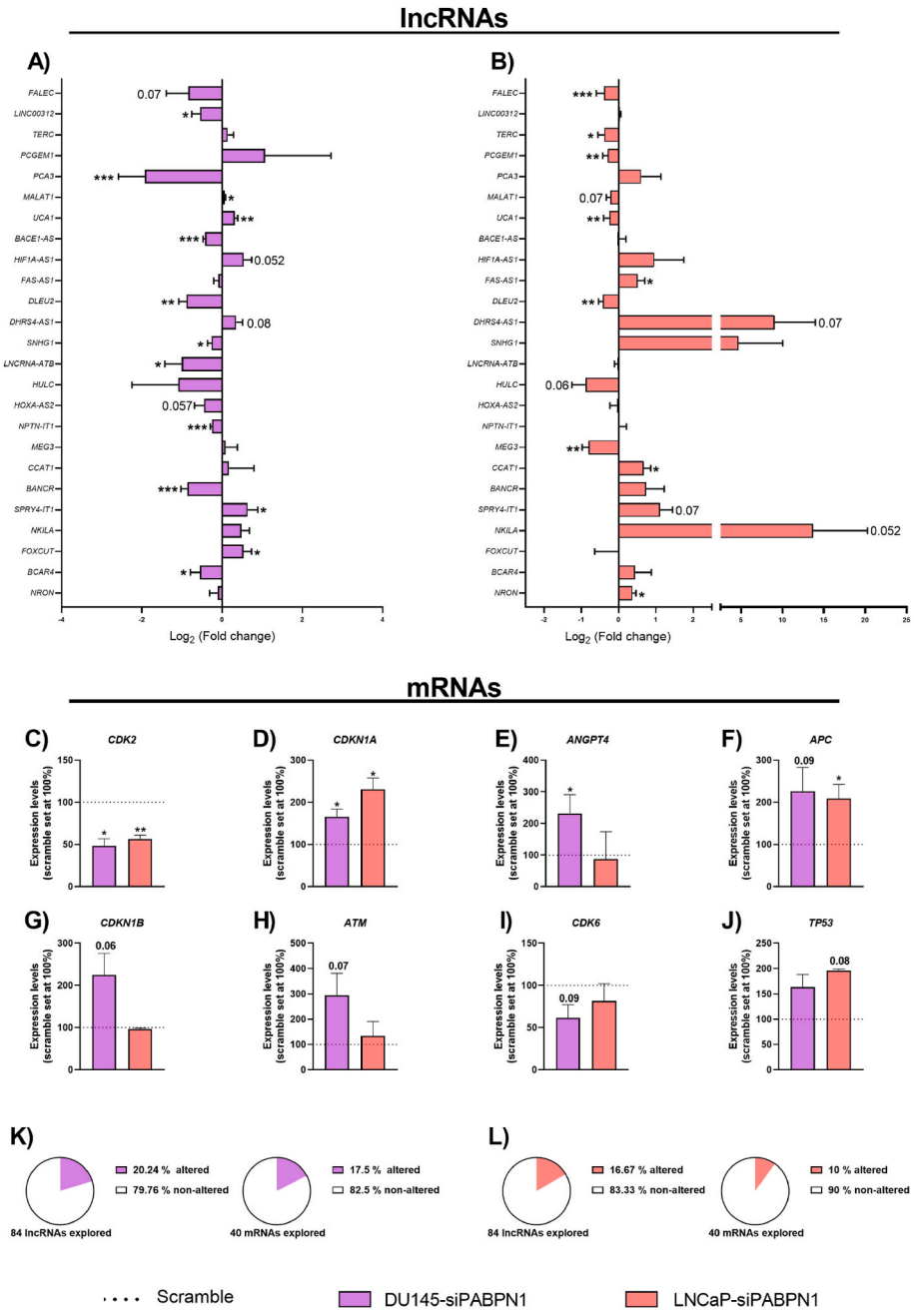

Fig. 5. Molecular consequences of *PABPN1* silencing in prostate cancer cell lines. Comparation of cancer-related lncRNAs **(A-B)** and mRNAs **(C-J)** levels between scramble and *PABPN1*-silenced DU145 and LNCaP cells. Gene expression was obtained by qPCR and adjusted by normalization factor [calculated from *TBP* and *HPRT1* (lncRNA analyses) or *ACTB* and *GAPDH* (mRNA analyses) expression levels]. Data are showed as fold change (lncRNA analyses) or percentage (mRNA analyses; control set at 100%) and represented as mean ± SEM. **(K-M)** Percentage of altered lncRNAs and mRNAs with respect to the total in DU145 and LNCaP cells. Asterisks (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) indicate statistically significant differences between groups.

eukaryotic RNA processing/degradation-complex [40], has not been profoundly explored in cancer. Indeed, although some alterations in specific components belonging to this RNA-exosome machinery have been described in different cancers, including liver, breast, colorectal, or hematological cancers [41–45], most studies focused on determining the function of this machinery in eukaryotic cells. Here, we demonstrate for the first time a drastic dysregulation of the expression profile of the components belonging to the RNA-exosome machinery in a well-characterized cohort of PCa samples vs. healthy-tissues, where a representative set of cofactors and core-elements was markedly altered (73% and 45%, respectively). Notably, our analyses revealed that PABPN1 had the higher capacity to discriminate between PCa and control-tissues, and that the overall PABPN1-overexpression (at mRNA/protein-level) found in different PCa sample cohorts was positively correlated with key clinical parameters (see below).

PABPN1 is a critical element of the REC since co-works with the Zinc-finger C3H1-type containing (ZFC3H1) protein to form the poly(A)-tail exosome targeting (PAXT) complex in the nucleus [33], wherein it plays two well-known REC-dependent functions: the nuclear decay of mRNAs, and the turnover of several lncRNAs [46]. Additionally, PABPN1 has other REC-independent intracellular functions including the poly-adenylation and length-control of the polyadenine-tail, the nuclear export of polyadenylated-RNAs, and the regulation of the alternative polyadenylation (APA) process [46–48]. Remarkably, we found that

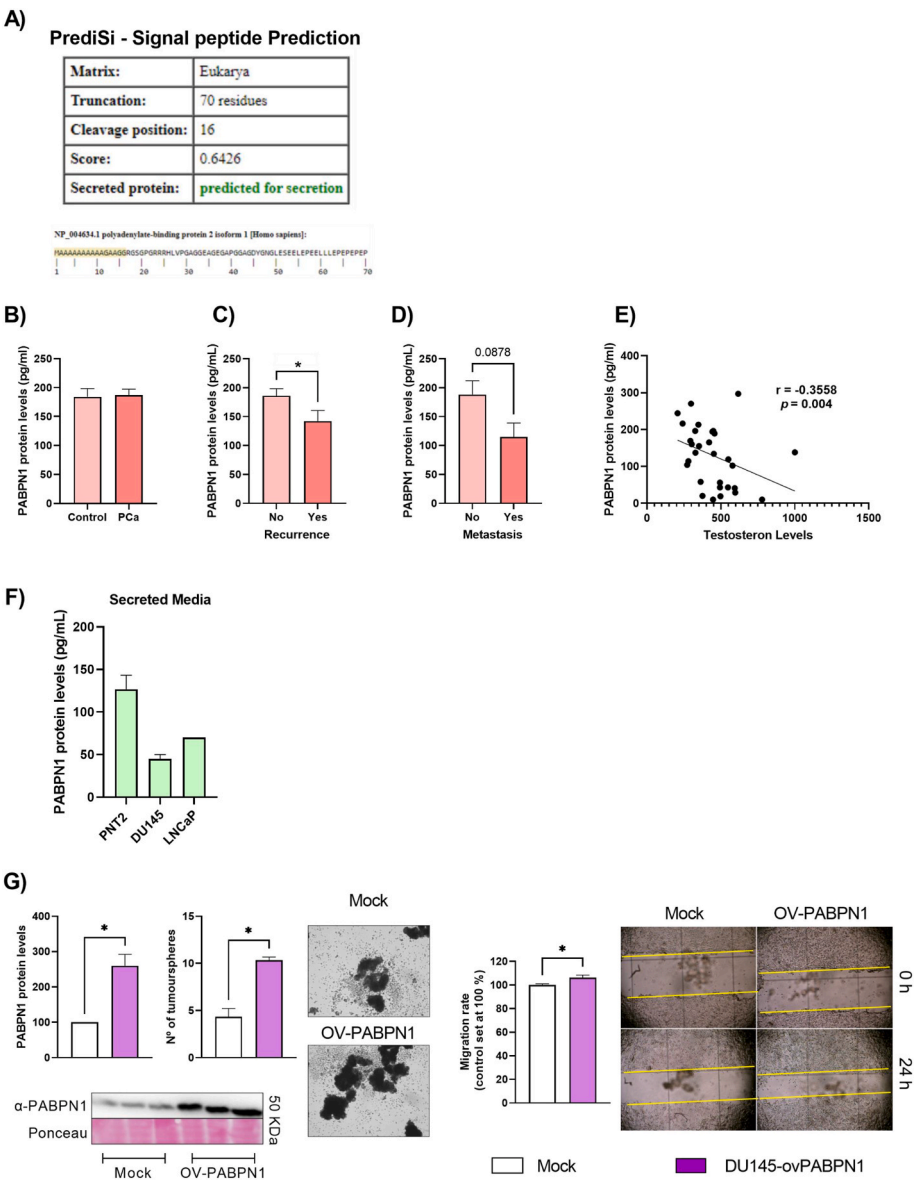

Fig. 6. PABPN1 utility as a prognostic biomarker. (A) Prediction of a signal peptide in the PABPN1 sequence by prediSi software. (**B-D)** Association between PABPN1 protein levels and clinical outcome (presence of PCa, recurrent disease and metastasis) in plasma samples from PCa patients belonging to cohort 3. **(E)** Correlation between PABPN1 and testosterone plasma levels from patients belonging to cohort 3. **(F)** Determination of PABPN1 protein by ELISA technique in secreted media derived from PCa cells. **(G)** Validation of PABPN1-overexpression at protein level and its effect on the tumourspheres formation and migration rate in DU145 cells. Representative images of tumourspheres and migration are depicted. Asterisks ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$) indicate statistically significant differences between groups.

high PABPN1 levels in PCa was positively correlated with key clinical (T-stage/perineural-invasion), molecular (EZH2/CDK4-levels) and aggressiveness (metastasis/poor clinical-outcome/shorter DFS) parameters. This later finding is consistent with a recent study indicating that PABPN1 is overexpressed in PCa and positively associated with shorter progression-free survival of the patients [49]. Interestingly, we could observe that the expression pattern of ZFC3H1 (which, as above-mentioned, co-works with PABPN1 to form the PAXT complex and drives lncRNAs and mRNAs to the REC) is quite similar to that found for PABPN1 in cohort 1 (Fig. 1A), and also in all external cohorts used (Supplemental Figure-7), supporting the idea that, during the progression of PCa, PABPN1 could be controlling the levels of key oncogenic lncRNAs and mRNAs in an RNA-Exosome-dependent way by coworking with ZFC3H1 to form the PAXT complex. All these observations suggested a causal link between PABPN1-dysregulation and PCa aggressiveness, which might represent a useful tool as a diagnostic/prognostic biomarker and a potential therapeutic target to tackle PCa.

Indeed, PABPN1-silencing in metastatic PCa cell-models induced marked reductions in aggressiveness features (i.e., proliferation/migration). Most notably, PABPN1-silencing also strikingly decreased the number of PCa stem/progenitor-cells of tumourspheres and colonies, a relevant functional result that may help to explore how to overcome the well-known resistance of metastatic PCa cells to different current drugs [50]. In line with our results, previous studies have also indicated a potential oncogenic role of PABPN1 *in vitro* in cervical-cancer [51] and breast-cancer [52]. Importantly, we also demonstrate that PABPN1 is an effective target in PCa *in vivo*, since PABPN1-silencing effectively blocks PCa progression of already established PCa tumours, and decreased tumour-volume, mitosis numbers and KI67 expression, thus further demonstrating the clinico-pathophysiological relevance of the anti-tumour role of PABPN1-silencing in PCa, and its potential value as a future therapeutic target in this disease. However, it should be noted

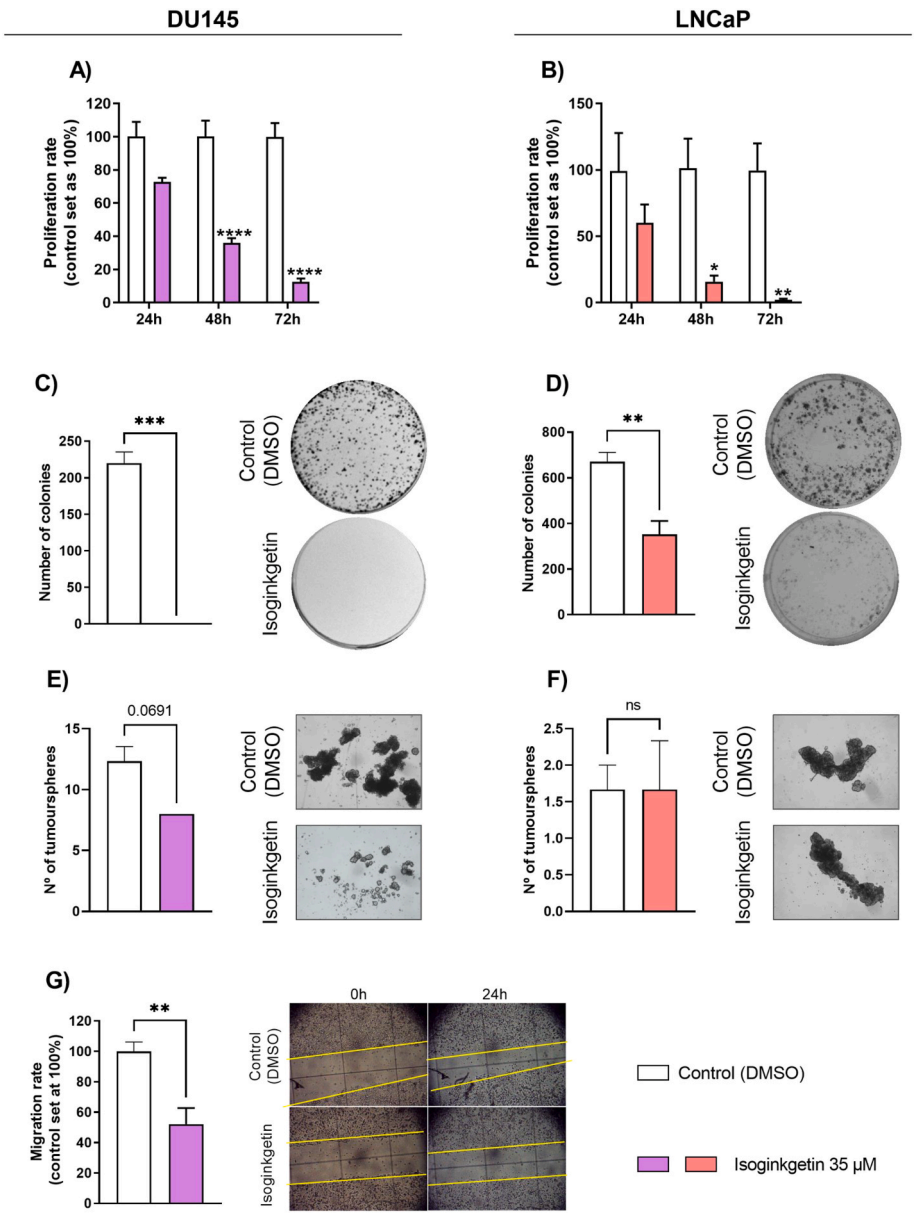

Fig. 7. Functional consequences of isoginkgetin treatment *in vitro*. (A-B) Proliferation rate of DU145 and LNCaP cells in response to isoginkgetin treatment compared to DMSO-treated cells. **(C-D)** Effect of isoginkgetin treatment in DU145 and LNCaP colony number compared to DMSO-treated cells. **(E-F)** Effect of isoginkgetin treatment in DU145 and LNCaP tumourspheres number compared to DMSO-treated cells. **(G)** Migration rate of DU145 cells in response to isoginkgetin treatment compared to DMSO-treated cells. Representative images are depicted right on the graphs. Asterisks ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$) indicate statistically significant differences between groups.

that these data are not in accordance with a recent report showing that PABPN1-silencing increased aggressiveness features in bladder-cancer *in vitro/in vivo* [53]. This dual function is not surprising since others components of the REC such as DIS3 might exert antitumour action in several hematological cancers but facilitates the progression of breast, liver, colorectal, ovarian, and pancreatic tumour pathologies [54].

To interrogate the molecular mechanisms linked to the critical role of PABPN1 in PCa, we explored an ample set of cancer-related lncRNAs and mRNAs involved in different pathophysiological processes in response to PABPN1-silencing in PCa-cells (DU145/LNCaP) since a pivotal role of the REC has been previously demonstrated in controlling the expression of key lncRNAs [34,35] and mRNAs [36,37] in human cells. Our data revealed, for the first time, a striking alteration in multiple lncRNAs in PCa cells. Of note, PABPN1-silencing led to a downregulation of *FALEC* and *DLEU2* lncRNAs in both PCa cell-lines which have been shown to promote important oncogenic processes in PCa (proliferation/migration/invasion/resistance to hormonal blockade) [55–57]. Furthermore, PABPN1-silencing also significantly induced the expression of *SPRY4-IT1* and *DHRS4-AS1* lncRNAs in both PCa cell-lines, which have been associated with apoptosis induction, inhibition of stemness capacity and epithelial-mesenchymal transition in liver, lung, and gastric cancers [58–60].

Furthermore, our report also demonstrated that PABPN1-silencing induced an alteration in the expression levels of some critical mRNA-coding genes. Interestingly, PABPN1-silencing reduced *CDK2* and increased *CDKN1A* and *APC* mRNA levels in both PCa cell-lines, being CDK2 a regulator of the G1-phase progression and the G1 to S transition during the cell-cycle [61,62], and CDKN1A and APC negative regulators of CDK2/CDK4 function and Wnt-signalling [63]. In line with this mechanistic results, a previous study also indicated that PABPN1-depletion in HEK293T-cells leads to a dysregulation in the expression of several lncRNAs and specific mRNAs [35], supporting the

key role of PABPN1 in the expression control of critical lncRNAs and mRNAs in cancer. However, at this point, it is also important to mention that molecular mechanisms linked to the critical role of PABPN1 in PCa cells are partially cell-line dependent (i.e., specific lncRNAs/mRNAs types were differentially altered in DU145 and LNCaP cells), which might be due to specific phenotypic differences between the two PCa cell-models used (mutation-profile/chemical-modifications/protein-interactions, etc. [64]). In this sense, similar divergences in response to the modulation of REC-components were found in other tumour cell-models (i.e., colorectal and pancreatic cancer) [43,65], which have been also attributed to the distinct nature of the cell-models. Nonetheless, our data clearly demonstrate that PABPN1 is functionally active in PCa-cells, and that its presence is directly associated with their progression/aggressiveness features.

The results of this study open a new research avenue in the study of PCa since, to the best of our knowledge, this is the first report demonstrating that: 1) PCa cells express and release PABPN1; 2) PABPN1 protein can be detected in human plasma, and; 3) PABPN1 levels were significantly lower in PCa patients with biochemical recurrence vs. those without recurrence, and tended to be lower in PCa patients with metastatic vs. those without metastatic disease. Therefore, these results would suggest the possible utility of PABPN1 levels as a novel prognostic biomarker for PCa patients by using non-invasive biopsies. Supporting this idea is the fact that patients with low levels of plasmatic PABPN1 have high levels of testosterone, which has been associated with higher risk of death and progression in some studies [66], as well as the report demonstrating that the Poly(A) Binding Protein Cytoplasmic 1 (PABPC1; a homolog protein of PABPN1) has also a potential use as a biomarker for human metastatic duodenal cancer since it is actively secreted within exosome by these cells [67]. Furthermore, we demonstrate that metastatic PCa cells (DU145/LNCaP) secrete lower PABPN1 protein levels to possibly maintain significant higher PABPN1 levels inside metastatic PCa cells vs. healthy prostate cells as an oncogenic feature. Supporting this idea are our data demonstrating that PABPN1-overexpression increase the migration-rate and the tumourspheres formation of metastatic PCa cells, as well as a previous report indicating that mouse models of carcinoma PCa with a higher rate and shorter latency of tumour recurrence after castration presented high levels of antibody against PABPN1 [68]. Obviously, further work will be required to complete our understanding about this oncogenic process and to fully elucidate the translational potential behind these interesting and potentially relevant observations.

Finally, our study also provides an initial, unprecedented proof-of-concept on the suitability of RNA-exosome dysregulation as a novel potential target for PCa treatment by demonstrating that the pharmacological inhibition of the activity of the RNA-exosome machinery (by isoginkgetin; flavonoid obtained from *Ginkgo Biloba* [20]) exerts clear antitumour effects on PCa cells (inhibition of proliferation/migration/colonies-formation). Some reports have associated the *in vitro* treatment of this compound with antitumour properties in different cancers (liver/brain/hematological-cancer/fibrosarcoma [69–72]), but its actions in PCa were still unknown. To the best of our knowledge, no clinical trials have been carried out to explore the utility of this specific compound in cancer, but the translational and clinical relevance of flavonoids as a promising therapeutic approaches in cancer has been recently review elsewhere [73]. Therefore, when viewing together our data add compelling evidence demonstrating that targeting the RNA-exosome machinery might translate into a beneficial effect in patients with PCa, an observation that certainly warrants further investigation.

Taken our evidence together, our results unveiled new conceptual and functional avenues in PCa, with potential translational implications, by demonstrating for the first time a drastic dysregulation of the RNA-exosome machinery (cofactors, nucleases, and core-elements; especially PABPN1) in PCa vs. non-tumour tissues (see graphical abstract). This is likely relevant clinically, because the dysregulation of PABPN1 directly associates with the progression and aggressiveness features of PCa and plays a crucial role in pathophysiological processes of PCa *in vivo* and *in vitro*. These actions are likely mediated through the modulation of critical lncRNAs and mRNAs. Moreover, our study also provides an initial, unprecedented proof-of-concept on the suitability of blocking the activity of the RNA-exosome machinery as a novel therapeutic avenue in PCa by demonstrating that its pharmacological disruption with isoginkgetin may have antitumour effects in PCa cells. Therefore, these findings provide new insights into the relatively unknown role of RNA-exosome components in cancer and suggest a putative window of opportunity for the components belonging to this molecular machinery as potential new diagnostic, prognostic and therapeutic tools for the management of human PCa.

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CRediT authorship contribution statement

Prudencio Sáez-Martínez: Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Francisco Porcel-Pastrana:** Writing – review & editing, Visualization, Validation, Methodology, Formal analysis, Data curation. **Antonio J. Montero-Hidalgo:** Writing – review & editing, Visualization, Project administration, Methodology, Data curation. **Samanta Lozano de la Haba:** Visualization, Validation, Methodology, Formal analysis, Data curation. **Rafael Sanchez-Sanchez:** Visualization, Validation, Methodology, Formal analysis, Data curation. **Teresa González-Serrano:** Resources, Data curation. **Enrique Gómez-Gómez:** Writing – review & editing, Resources, Data curation. **Antonio J. Martínez-Fuentes:** Writing – review & editing, Visualization. **Juan M. Jiménez-Vacas:** Writing – review & editing, Visualization, Data curation, Conceptualization. **Manuel D. Gahete:** Writing – review & editing, Validation, Supervision, Investigation, Conceptualization. **Raúl M. Luque:** Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2023.216604.

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