Title: Somatostatin receptor subtype 1 as a potential diagnostic marker and therapeutic target in prostate cancer

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Shortened title: SSTR1 in prostate cancer

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Abstract

Background: Prostate cancer (PCa) is a highly prevalent neoplasia that is strongly influenced by the endocrine system. Somatostatin (SST) and its five receptors (sst1-5 encoded by *SSTR1-5* genes) comprise a pleiotropic system present in most endocrine-related cancers, some of which are successfully treated with SST analogs. Interestingly, it has been reported that *SSTR1* is overexpressed in PCa, but its regulation, functional role, and clinical implications are still poorly known.

Methods: PCa specimens (n=52) from biopsies and control prostates from cystoprostatectomies (n=12), as well as *in silico* databases were used to evaluate *SSTR1* and miRNAs expression. *In vitro* studies in 22Rv1 PCa cells were implemented to explore the regulation of *SSTR1*/sst1 by different miRNAs, and to evaluate the consequences of *SSTR1*/sst1 overexpression, silencing and/or activation [with the specific BIM-23926 sst1 agonist (IPSEN)] on cell-proliferation, migration, signaling-pathways and androgen-signaling.

Results: We found that *SSTR1* is overexpressed in multiple cohorts of PCa samples, as compared with normal prostate tissues, wherein it correlates with androgen receptor (AR) expression, and appears to be associated with aggressiveness (metastasis). Furthermore, our data revealed that *SSTR1*/sst1 expression might be regulated by specific miRNAs in PCa, including miR-24, which is downregulated in PCa samples and correlates inversely with *SSTR1* expression. *In vitro* studies indicated that treatment with the BIM-23926 sst1 agonist, as well as *SSTR1* overexpression, decreased, whereas *SSTR1* silencing increased, cell-proliferation in 22Rv1 cells, likely through the regulation of PI3K/AKT-CCND3 signaling-pathway. Importantly, sst1 action was also able to modulate androgen/AR activity, and reduced PSA secretion from PCa cell lines.

Conclusions: Altogether, our results indicate that *SSTR1* is overexpressed in PCa, where it can exert a relevant pathophysiological role by decreasing cell-proliferation and PSA secretion. Therefore, sst1, possibly in combination with miR-24, could be used as a novel tool to explore therapeutic targets in PCa.

Keywords: SSTR1, Prostate tumor, miRNA, Aggressiveness.

Introduction

Prostate cancer (PCa) is the second most common cancer in men, with an incidence of more than 160,000 estimated new cases in 2017 in United States alone [1] and the fifth-leading cause of cancer-related deaths worldwide [2,3]. Years of research have provided a wide number of available therapeutic strategies to tackle this pathology, including surgery, chemotherapy, radiotherapy and/or androgen-deprivation therapy (ADT), which together with the capacity of early detection due to the implementation of PSA screening, have permitted to reach almost 100% survival after 5 years when the tumor is localized [4-6]. Unfortunately, these therapeutic approaches are associated with serious side effects[7]. In addition, there is a lack of tools that can accurately guide treatment selection, which complicates options and choices for clinicians and patients[8]. Altogether, these facts demonstrate the necessity of identifying and characterizing novel therapeutic targets that can become useful for the development of new treatment and/or management strategies.

It is well known that PCa appearance and progression are highly influenced by androgens[9,10] and that other steroid hormones, such as estrogens or progesterone, are also involved in the progress of the disease[11]. It has also been shown that additional neuroendocrine systems may regulate prostate function, both under normal and tumoral conditions[12,13]. In particular, some components of the somatostatin (SST) system are expressed in both normal and tumoral prostatic tissue, where they may play a relevant role in the negative regulation of cell proliferation, as occurs in other tissues[14-16]. The SST system consists of a complex and pleiotropic set of hormones, receptors and associated proteins, widely distributed around the body, which control a number of physiological processes, such as hormone secretion, pancreatic and gut function, energy balance and the immune system[17-19]. SST exerts its functions by binding to a set of five different G-protein coupled receptors with seven transmembrane domains, named sst1-sst5 (gene names: *SSTR1-5*) [20,21]. Some components of this system have been previously associated with development and progression of tumoral pathologies, such as pituitary adenomas, thyroid cancer, neuroendocrine tumors (NETs) and breast cancer, where they may exert an important role as diagnosis/prognosis markers and therapeutic targets[22-24]. In fact, somatostatin analogs (e.g. the sst2-preferring

81 octreotide and lanreotide, and the pan-sst ligand pasireotide) are currently being used to treat some
82 of these pathologies, such as pituitary adenomas and NETs, highlighting the clinical relevance of
83 this system[25-27].

84 It has been demonstrated that the expression of some components of the SST system,
85 especially sst variants, are dysregulated in PCa tissue and associated with the aggressiveness of
86 tumor cells [28-30], which highlights the putative utility of these components as novel biomarkers
87 and therapeutic targets to develop pharmacologic strategies for this pathology. In this sense, SST
88 receptor subtype 1 (sst1, *SSTR1*) has been described as an important molecule in several types of
89 cancer, such as colon cancer, where it seems to play a significant role and is associated with some
90 aggressiveness features [31]. Furthermore, sst1 has been shown to be upregulated in PCa samples
91 and to correlate with aggressiveness features[32,33]. Nevertheless, the functional role of *SSTR1* and,
92 even more importantly, its putative therapeutic implications in PCa are still largely unknown.
93 Therefore, the aims of this study were to: **1)** further corroborate the presence and/or dysregulation
94 of *SSTR1* in PCa samples compared to control samples; **2)** evaluate the correlation of *SSTR1*
95 expression with clinical parameters; **3)** elucidate the functional role of *SSTR1* in PCa and its
96 putative therapeutic utility; and **4)** investigate the factors that could be regulating the expression
97 of *SSTR1* in PCa.

98

99 # Materials and methods

100 **Patients and samples**

101 Fresh PCa specimens (n=52) were obtained by core needle biopsies from patients
102 diagnosed with PCa (NCNN-guidelines). Control prostate samples were collected from patients
103 after cystoprostatectomy due to bladder cancer but without PCa (n=12). Expert pathologists
104 confirmed that all prostate samples were appropriately classified as normal or tumor (Table I).
105 Demographic and clinical parameters regarding tumor aggressiveness and metabolic status were
106 collected. This study was approved by the Hospital/University Ethics Committees and written

informed consents from patients were obtained through the Andalusian Biobank (Servicio Andaluz de Salud).

Reagents

Sst1 agonist BIM-23926[34,35] was provided by IPSEN (Milford, MA, USA). It was administered at 1μM and 10nM for proliferation and free cytosolic calcium concentration assays and, at 1μM for the rest of the experiments.

Cell culture

PCa cell-lines (androgen-dependent 22Rv1; and androgen-independent DU145 and PC3) were obtained from ATCC. 22Rv1, DU145 and PC3 were cultured in RPMI 1640 (Lonza) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, Madrid, Spain), 1% glutamine (Sigma-Aldrich) and 0.2% antibiotic (Gentamicin/Amphotericin B, Gibco, Thermo Fisher, Waltham, MA, USA). Cells were grown at 37C, in a humidified atmosphere with 5% CO_2. All the cell-lines were validated by STR system and verified for mycoplasma contamination as previously reported [36,37].

Stable transfection to overexpress *SSTR1*

22Rv1 cells was stably transfected with *SSTR1*-containing pCDNA3.1+ vector (1μg; cdna.org, Bloomsberg, PA, USA) and selected as previously reported [38]. Specifically, 22Rv1 cells were seeded in 6-well culture plates, transfected with *SSTR1* or empty (mock) vectors using Lipofectamine 2000 Transfection Reagent (Invitrogen, Thermo Fisher; following manufacturer's instructions) and selected by geneticin treatment (Gibco). The success of the transfection was validated by qPCR.

Alamar Blue proliferation assay

Cell proliferation was determined by the Alamar Blue fluorescent assay (Life Technologies, Thermo Fisher) as previously reported [36,37,39]. Briefly, cells were seeded in 96-wells

plates at a density of 5,000 cells/well. Cell viability in response to BIM-23926 sst1 agonist treatment (1μM and 10nM) was analyzed [at basal (0h), 24h, 48h and 72h] by measurement of fluorescent signal exciting at 560 nm and reading at 590 nm on the Flex Station 3 (Molecular Devices, Sunnyvale, CA, USA). Specifically, the day of the measurement, cells were incubated for 3h with 10% Alamar blue/serum free media and Alamar blue reduction was measured. Results are expressed as percentage of control. Medium was replaced by fresh 5% FBS-medium with treatment, if applicable, immediately after each measurement (every 24h). In all instances, cells were seeded per quadruplicate and all assays were repeated a minimum of three times.

Measurement of free cytosolic calcium concentration ($[Ca^{2+}]_i$)

Cells were plated on coverslips at a density of 100,000 cells per well and changes in $[Ca^{2+}]_i$ in response to sst1 BIM-23926 agonist treatment (1μM and 10nM) were analyzed in single cells using the fura-2/AM dye (Molecular Probes, Eugene, OR, USA), as described in detail previously [22,40,41].

RNA isolation and retrotranscription

Total RNA from control and PCa samples was obtained using AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Hilden, Germany), according to manufacturer's protocol. In the case of cell-lines, total RNA was isolated using TRIzol Reagent (Sigma-Aldrich) treated with DNase (Promega, Barcelona, Spain) following manufacturer's instructions. The amount of RNA recovered and its quality were determined using the NanoDrop2000 spectrophotometer (Thermo Fisher). One μg of RNA was reverse transcribed to cDNA using random hexamer primers with the First Strand Synthesis Kit (Thermo Fisher).

Quantitative real time PCR

Quantitative real time PCR (qPCR) reactions were performed using the Brilliant III SYBR Green Master Mix (Stratagene, La Jolla, CA, USA) in the Stratagene Mx3000p system.

Specifically, *SSTR1*, *p53*, *CCND3* and *PSA* (*KLK3* gene) expression was analyzed. For each reaction, 10µl of master mix, 0.3µl of each primer, 8.4µl of distilled H_2O and 1µl of cDNA (50 ng) in a 20µl total volume were mixed. The qPCR program consisted in the following steps: (1) 95C for 3 min; (2) 40 cycles of denaturing (95C for 20 sec) and annealing/extension (61C for 20 sec); and (3) a last cycle where final PCR products were subjected to graded temperature-dependent dissociation (55C to 95C, increasing 0.5C/30 sec), to verify that only one product was amplified. Specific primers for human transcripts were designed with Primer3 software and StepOne™ Real-Time PCR System software v2.3 (Applied Biosystems®, Foster City, CA, USA) (Supplemental Table I). Results were validated as previously reported [42], normalizing all genes with a normalization factor, calculated from values of beta-actin and *GAPDH* housekeeping genes, using Genorm Software, wherein the expression of the housekeeping genes did not differ between experimental groups (data not shown).

Microarray of gene expression profile

Microarray experiment was carried out using the Human Androgen Receptor Signaling Targets PCR Array PAHS-142Z (Qiagen). Three independent passages from 22Rv1 cells stably-transfected *SSTR1*-pCDNA3.1 and empty-pCDNA3.1 vector (used as control) were used. Total RNA was extracted using AllPrep DNA/RNA/Protein Mini Kit (Qiagen) and then, retrotranscribed using RT² First Strand Kit (Qiagen). Expression profile was measured using RT² qPCR SYBR Green ROX (Qiagen) in the Stratagene Mx3000p system. Results were analyzed with Data Analysis Center (Qiagen, http://www.qiagen.com/shop/genes-and-pathways/data-analysis-center-overview-page/), following the manufacturer's instructions.

Western Blot

22Rv1 cells were processed to analyze protein phosphorylation after treatment with BIM-23926 sst1 agonist (1µM) during different times (5, 10 and 30min) by western blot, using standard procedures [38]. Briefly, proteins were extracted from cells seeded in 12-wells plates using pre-warmed SDS-DTT buffer (62,5mM Tris-HCl, 2% SDS, 20% glycerol, 100mM DTT and 0,005%

bromophenol blue), followed by sonication during 10 sec and boiling for 5 min at 95C. 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 with the specific antibodies for AKT (#9272S, Cell Signaling, Beverly, MA, USA), phospho-AKT (#4060S, Cell Signaling), ERK (sc-154, Santa Cruz Biotechnology, Dallas, TX, USA), phospho-ERK (#4370S, Cell Signaling), AR (ab133273, Abcam, Cambridge, UK), phospho-AR (ab71948, Abcam), JNK (AF1387, R&D Systems, Minneapolis, MN, USA) or phospho-JNK (AF1205, R&D Systems), as well as with the appropriate secondary antibodies: HRP-conjugated goat-anti rabbit IgG (#7074s; Cell Signaling) or HRP-conjugated rabbit-anti goat IgG (31753, Thermo Fisher). Proteins were detected using an enhanced chemiluminescence detection system (GE Healthcare, Madrid, Spain) with dyed molecular weight markers. A densitometry analysis of the bands obtained was carried out with ImageJ software, using total protein as normalizing factor of correspondent phosphorylated protein.

Evaluation of PSA secretion

To examine the effects of BIM-23926 sst1 agonist on PSA secretion in 22Rv1 cells, 200,000 cells per well were used. Cells were plated with RPMI1640 supplemented with 10% fetal bovine serum (FBS) for 48h, washed, serum starved for 1h and incubated with BIM-23926 sst1 agonist (1μM) for 24h in absence of FBS. After treatment, media were collected and stored at -20C until PSA measurement using commercial ELISA (Sigma-Aldrich). All the information about the assay can be accessed at the company website.

Silencing of *SSTR1* expression by siRNA

22Rv1 cells were transfected with a specific and previously validated siRNA against *SSTR1* (Ambion, Thermo Fisher). Specifically, 22Rv1 cells were seeded in 6-wells culture plates and transfected with *SSTR1* (100nM) or scramble siRNA, using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen, Thermo Fisher) following manufacturer instructions. Success of the silencing was validated by qPCR.

Transfection with miRNA mimics

22Rv1 cells were transfected with miRNA mimics of miR-24, miR-27, miR-383, miR-488 or with a negative control (20nM; GenePharma, Shanghai, China) and, 48h later, RNA was extracted using Trizol reagent as indicated above. Taqman probes for hsa-miR-24-3p, hsa-miR-27b-3p, hsa-mir-383-5p, hsa-miR-488-3p, and U6 (used as housekeeping) were purchased (Thermo Scientific, Wilmington, NC, USA). 2µl of extracted RNA were retrotranscribed with the Taqman microRNA Reverse transcriptase kit (Thermo Scientific, Wilmington, NC, USA) following the manufacturer's instructions. The validation of successful miRNA mimic transfection was determined by TaqMan® Universal PCR Master Mix (Thermo Scientific, Wilmington, NC, USA) in the Bio-Rad CFX PCR instrument (Bio-Rad, Hercules, CA, USA).

Bioinformatic analyses

Processed RNA-seq data from The Cancer Genome Atlas (TCGA, https://gdc-portal.nci.nih.gov/) and Memorial Sloan Kettering Cancer Center (MSKCC, https://www.mskcc.org/) were compiled for Prostate Cancer Adenocarcinoma (PRAD). Normalized expression of *SSTR1* and different miRNAs were obtained, and comparisons between expression in PCa vs. control samples and correlations in PCa samples were performed.

In vitro analyses were performed in order to predict potential miRNAs that regulate *SSTR1* expression. First, we used three different software packages (TargetScan, miRanda and DIANA) to predict the potential miRNAs that could target *SSTR1* (Table III). The criteria to choose the miRNA candidates were: **1)** predicted to bind the 3´UTR region in conserved sites (among conserved species); **2)** in, at least, two different software; **3)** number of poorly conserved sites (among conserved species); **4)** good score (Total Context score and Aggregate PCT in Target Scan, mirSVR score and PhastCons score in Miranda, miTG score in Diana); and **5)** miRNA already published showing functional effect (http://www.mirbase.org/) (Table III). Then, following these *in silico* results, we searched which of these miRNAs correlated inversely with *SSTR1* expression in PCa, using available data from TCGA database.

Statistical analysis

Statistical analysis was performed by unpaired parametric *t*-test and non-parametric Mann-Whitney *U* test, according to normality, assessed by Kolmogorov-Smirnov test. Spearman's bivariate correlations were performed for all quantitative variables. We compared the effect of BIM-23926 sst1 agonist, overexpression or silencing vs. respective controls (set at 100%). The *p*-values were two-sided and statistical significance was considered when $p < 0.05$. Statistical differences were assessed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA) and correlations were carried out using SPSS 22 (IBM SPSS Statistics Inc., Chicago, IL, USA).

Results

SSTR1 is overexpressed in PCa and associated with relevant markers

Expression levels of *SSTR1* were evaluated in samples from patients with PCa (n=52) and controls (n=12), whose clinical and demographic characteristics are summarized in **Table-I**. qPCR analyses revealed that *SSTR1* was expressed in a higher percentage of PCa samples compared with control samples (91% vs. 75%), and that its levels were significantly higher in PCa samples than in control tissues (**Fig-1A**). In addition, ROC curve analysis showed that *SSTR1* expression was able to distinguish significantly between tumor and control sample (p-value=0.048, AUC=0.68, **Fig-1B**). As expected, *AR* mRNA expression levels were significantly higher in PCa than control samples (**Fig-1C**). Interestingly, *SSTR1* mRNA levels correlated with *AR* expression in PCa patients but not in control individuals (**Fig-1D/1E**).

In order to select the most appropriate PCa cell lines to carry out functional assays with *SSTR1*, we screened the expression of *SSTR1* in a panel of PCa cell lines previously used in our laboratory [12,30,37,43]. Androgen-dependent 22Rv1 cell-line expressed considerable mRNA levels of *SSTR1* compared with PC3, DU145 and VCaP cell-lines (i.e. 1751 vs 14, 1, 0 mRNA copy number, respectively) (**Fig-1F**). Moreover, the levels of expression found in 22Rv1 cells (**Fig-**

1F) compared well to those measured in PCa samples (**Fig-1A**). Therefore, we used the 22Rv1 model to explore the functional role of sst1 in PCa.

Functional role of *SSTR1* in 22Rv1 cells

The potential functional role of *SSTR1* in PCa was first assessed by measuring proliferation of 22Rv1 cells in response to BIM-23926 sst1 agonist treatment (at 1µM and 10nM; 24-72 h; **Fig-2A**). Treatment with 1µM, but not 10nM, of BIM-23926 sst1 agonist significantly decreased cell proliferation at 48h and 72h, which was accompanied by a reduction in PSA secretion (**Fig-2B**). Supporting these previous results, overexpression of *SSTR1* also decreased cell proliferation at 48h and 72h (**Fig-2C**), while the silencing of *SSTR1* with a specific siRNA significantly increased cell proliferation in 22Rv1 cells at 24h, 48h and 72h (**Fig-2D**).

Signaling pathways associated to sst1 in PCa cells

We next explored the signaling-pathways associated to sst1 activation in PCa cells. Firstly, measurement of free cytosolic calcium concentration ($[Ca^{2+}]_i$), a second messenger tightly coupled to SST/sst signaling, was determined in response to treatment with BIM-23926 sst1 agonist at 1µM and 10nM in 22Rv1 cells. Our results revealed that only a low percentage of cells responded to BIM-23926 sst1 agonist (**Table-II**), with a limited response, indicating that sst1 may not be primarily connected to this signaling pathway in PCa.

Additionally, phosphorylation of key proteins in pathways related with proliferation (AKT, ERK, JNK) and PCa development (AR) was determined by Western blotting. This analysis revealed that treatment with BIM-23926 sst1 agonist at 1µM decreased the phosphorylation of AKT at 30min (**Fig-3A**). In contrast, no changes were found in the level of ERK, AR or JNK phosphorylation in response to BIM-23926 sst1 agonist treatment.

Furthermore, in order to explore a putative association between sst1 activation and key factors associated with PCa, we measured the mRNA expression levels of *KLK3* (the gene encoding PSA), *CCND3*, *p53,* and *SSTR1* itself, by qPCR, after 24h of incubation with the BIM-23926 sst1 agonist (**Fig-3B**). Remarkably, *KLK3* and *CCND3* expression was decreased, which

supports the previous results observed in terms of PSA secretion and proliferation (**Fig-2**), while *p53* expression was not altered. Finally, we found a homologous downregulation of *SSTR1*, since its expression was decreased by BIM-23926 sst1 agonist treatment.

We then performed a PCR Array of Human Androgen Receptor Signaling Pathways, which facilitates the measurement of the mRNA levels of a wide number of key genes involved in pathways related with AR, which are crucial in PCa development and progression. This array was used to find alterations in *SSTR1* stable-transfected 22Rv1 cells compared to mock transfected cells. Interestingly, we found that several genes, mainly related with tumor progression, were dysregulated when *SSTR1* was overexpressed (**Fig-4A**). Specifically, when considering significant differences in genes with a fold change higher than 2, the array revealed the upregulation of *IGFBP5, KLK3* and *NDRG1* and the downregulation of *ADAMTS1*, *IRS2*, *VIPR1*, *SLC45A3*, *LIFR* and *TSC22D* (**Fig-4A**); however, further analysis of these changes by qPCR only confirmed the upregulation of *IGFBP5* and the downregulation of *ADAMTS1*, *IRS2*, *VIPR1*, *SLC45A3* and *LIFR* (**Fig-4B and Supplemental Fig-1**).

SSTR1 **expression is regulated by miR-24 in PCa**

In order to understand how *SSTR1* could be regulated in PCa, we performed *in silico* and *in vitro* analyses to identify putative *SSTR1*-targeting miRNAs. *In silico* analysis revealed many miRNAs that could putatively recognize the 3´UTR of *SSTR1* with high affinity. To filter this list, we identified miRNAs that exhibited a negative correlation with *SSTR1* in the TCGA PCa cohort, and found four miRNAs (miR-24, miR-27b, miR-383, miR-488) of interest (**Fig-5A**). We tested the capacity of these miRNAs to reduce *SSTR1* expression by using specific miRNA mimics in 22Rv1 cells. *In vitro* transfection with these miRNAs showed that only miR-24 was able to significantly decrease both *SSTR1* mRNA (**Fig-5B**) and protein (sst1; **Fig-5C**) expression.

By exploring another PCa dataset from MSKCC, we found that miR-24 was downregulated in primary tumors compared to normal prostate and its expression was even lower in metastases (**Fig-5D**). This expression pattern was inverse to that of *SSTR1*, which was elevated in malignant samples in this cohort (Fig-5D), further validating our earlier findings (Fig-1A).

These results suggest that miR-24 targeting might be an important mechanism regulating the expression of *SSTR1* in PCa.

Discussion

PCa represents the second most common cancer amongst men worldwide, and thousands of new cases and deaths are associated with this disease annually [2,3]. Unfortunately, the high heterogeneity and complexity of PCa hampers the identification and development of new and more sensitive and specific diagnostic and therapeutic strategies for this pathology. Thus, it is crucial to broaden our knowledge regarding PCa pathophysiology, in order to identify new biomarkers and therapeutic targets that could be useful for the management of PCa.

The regulatory system comprised by SST and its receptors (sst1-sst5) represents a relevant and useful source of therapeutic targets for the treatment of different endocrine-related tumors, including pituitary and neuroendocrine tumors[22-24]. Indeed, the utility of SST analogs (SSAs) for treatment of additional types of cancer, including PCa, has been extensively explored experimentally and also, to a lesser extent, clinically, with analogs targeted to sst2 and sst5. However, although the experimental results were most promising[46], the clinical findings were disappointing in prostate, breast, colorectal, lung, and other cancers. Specifically, in the case of PCa, previous attempts to use clinically analogs for subtype sst2 and 5 leaded to poor results in the case of relapsed PCa [47,48], or even negative results, worsening the outcome, in terms of tumor development [49]. However, although sst2 and sst5 represent the main targets for the currently available SST analogs (e.g. octreotide, lanreotide) [25], additional studies have suggested that other ssts could also represent promising therapeutic alternatives in certain pathologies [44]. In this sense, some studies have revealed that sst1 could be a key SST receptor in terms of mediating the stimulation of tyrosine phosphatases and the inhibition of cell proliferation in response to SSAs [45]. For these reasons, in this study, we focused on assessing the implications and potential utility of sst1 on PCa. Indeed, we found herein that *SSTR1* is present in a high percentage of PCa samples, and is overexpressed in those samples compared to normal prostate tissues, which is

consistent with previous studies[32]. Furthermore, we discover herein, for the first time, that the expression of *SSTR1* could be regulated by four different miRNAs (miR-24, miR-488, miR-383 and miR-27b) and that miR-24 could be specifically playing an important role in PCa, inasmuch as we have demonstrated that miR-24 can down-regulate *SSTR1* expression in 22Rv1 cells. In addition, *in silico* analysis of the MSKCC database demonstrated that miR-24 expression inversely correlated with that of *SSTR1*, wherein miR-24 expression was progressively reduced while that of *SSTR1* progressively increased from normal prostates to PCa and finally to metastatic samples: these results reinforce the view that *SSTR1*, alone or in combination with miR-24, may play a relevant role in this pathology and could represent novel biomarkers and/or suitable therapeutic target options in future studies.

Our results suggest that sst1 is anti-proliferative in PCa. This finding is in line with previous studies, where sst1 has been described as an antiproliferative effector in other tumoral pathologies, such as pancreatic cancer [50-52]. To further characterize the underlying mechanisms mediating the anti-proliferative capacity of sst1, we assessed multiple signaling pathways in response to BIM-23926 sst1 agonist treatment. While sst1 activation did not affect $[Ca^{2+}]_i$ kinetics, ERK activation or JNK activation, it caused a clear inhibition of AKT phosphorylation, a widely known effector involved in the activation of cell proliferation [53]. This indicates that sst1 activation may inhibit cell proliferation via negative regulation of PI3K/AKT pathway, which is a common mechanism that has been previously reported with other components of the SST system [54]. In line with this concept, treatment with this sst1 agonist also inhibited *CCND3* expression, which has been previously linked with proliferation promotion [55,56] and is modulated by AKT phosphorylation [57], which could suggest a novel mechanism of action for BIM-23926 sst1 agonists in PCa (i.e. sst1/pAKT/CCND3). On the other hand, the effect exerted by this sst1 agonist on 22Rv1 cells does not seem to be mediated by the promotion of apoptosis since *p53* mRNA expression levels were not altered by this treatment; however, further studies would be necessary before a clear conclusion can be reached in this regard.

Besides controlling cell proliferation, our results indicate that sst1 could play additional relevant roles in PCa, including the modulation of the androgen/AR system. Indeed, *SSTR1*

385 expression was correlated with *AR* expression in PCa samples, which reinforces the idea of a role
386 for *SSTR1* as potential therapeutic target, in that AR is a key pathophysiological component in
387 this type of cancer. This is consistent, indeed, with the fact that *SSTR1* overexpression in 22Rv1
388 cells induced significant changes in key factors involved in and associated with AR-related
389 pathways, such as upregulation of *IGFBP5* and downregulation of *ADAMTS1*, *IRS2*, *VIPR1*,
390 *SLC45A3* and *LIFR*. Interestingly, *IGFBP5*, mainly because of its function as repressor of *IGF-I*,
391 is described as a tumor suppressor[58]. Thus, the overexpression of *SSTR1* could have crucial
392 implications by activating tumor suppression programs. Consistently, downregulated genes were
393 found to be related with tumor progression, as is the case of *ADAMTS1*, which has been reported
394 to be overexpressed in tumor cells and tissues and is related to the activation of *TGF-β* [59]. Also,
395 *IRS2* is a known oncogene related with insulin and *IGF-I* function [60], and *VIPR1* is overexpressed
396 in several tumors, acting as receptor of vasoactive intestinal peptide (VIP), which shows anti-
397 apoptotic and pro-angiogenic function [61]. This might be an important observation since other
398 growth factors, such as GHRH [62] or gastrin-releasing peptide [63], have also been described as PCa
399 enhancer, being even used as therapeutic targets [64]. Additionally, *SLC45A3* has been shown to be
400 usually present in malignant prostate tissue [65] and *LIFR* has been previously reported as an
401 unfavorable prognosis marker in melanoma, and to be associated with reduced drug response in
402 breast cancer [66,67]. Therefore, the downregulation of these genes highlights the anti-tumoral
403 function of sst1. However, treatment with BIM-23926 sst1 agonist did not alter AR
404 phosphorylation, which could suggest that *SSTR1* is tightly associated with AR pathway, but not
405 directly through the activation of AR phosphorylation. Furthermore, treatment with BIM-23926
406 sst1 agonist during 24h decreased PSA secretion in 22Rv1 cell line, which reinforces the potential
407 role of *SSTR1* in the prostate, since PSA is over-secreted in PCa [68] and clearly associated to AR
408 activity[69]. In this sense, the role of sst1 in the modulation of AR activity in PCa, as well as its
409 putative role in castration resistant PCa warrant further investigation.
410

411 # Conclusions

Altogether, the *in vitro* and *in vivo* results presented in this study reveal, for the first time, that *SSTR1*, which is overexpressed in PCa, can be controlled by specific miRNAs and could have relevant implications in this pathology. Indeed, our results indicate that sst1 is directly involved in the inhibition of cell proliferation and PSA secretion in 22Rv1 PCa cells, likely by modulating the PI3K/AKT-CCND3 pathway and the androgen-AR signaling. Thus, when taken together, our results support the notion that *SSTR1*, and its possible combination with miR-24, could be used as a novel biomarker and therapeutic target and sst1 agonist as therapeutic agent for PCa.

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Figures

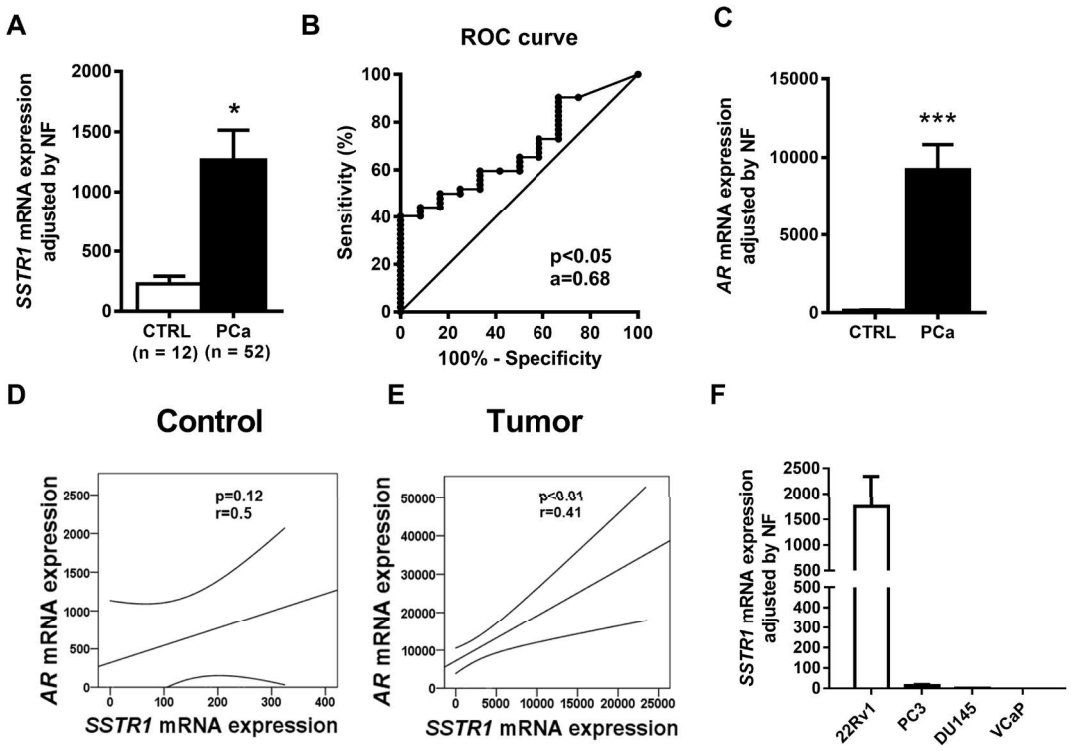


Figure 1: Presence of *SSTR1* in PCa and clinical correlations. **A**. Comparison of *SSTR1* mRNA expression in PCa biopsy samples (n=52) vs. non-tumor control cystoprostatectomy samples (n=12). Absolute mRNA levels were determined by qPCR and adjusted by normalization factor (NF) calculated from the expression levels of three housekeeping genes (*ACTB*, *GAPDH* and *HPRT*). **B**. Receiver operating characteristic (ROC) curve analysis to determine the accuracy of *SSTR1* expression to discriminate between patients with PCa and controls. **C**. Comparison of *AR* mRNA expression in PCa biopsy samples (n=52) vs non-tumor control cystoprostatectomy samples (n = 12). Absolute mRNA levels were determined by qPCR and adjusted by normalization factor (NF) calculated from the expression levels of three housekeeping genes (*ACTB*, *GAPDH* and *HPRT*). **D**. Correlation between *SSTR1* expression and *AR* mRNA expression in control individuals without PCa. **E**. Correlation between *SSTR1* expression and *AR* mRNA expression in PCa samples. **F**. *SSTR1* mRNA expression in PCa cell lines (androgen-dependent: 22Rv1 or androgen-independent: PC3 and DU145). Asterisks (*, $p < 0.05$) indicate values that significantly differ from controls. In all cases, date represent mean ± SEM.

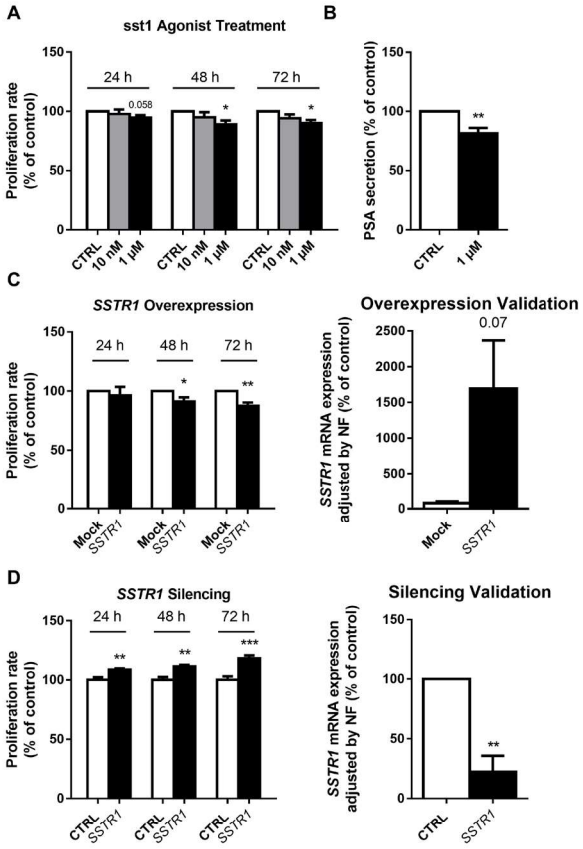


Figure 2: *SSTR1* has a crucial functional role in the regulation of proliferation and PSA secretion in PCa cell line 22Rv1. A. Proliferation rate of 22Rv1 cells after treatment with BIM-23926 sst1 agonist at two different concentrations (10nM and 1µM), during 24, 48 and 72 h, compared with non-treated control. **B**. PSA secretion from 22Rv1 cells determined by specific ELISA kit after 24h treatment with BIM-23926 sst1 agonist. **C**. Overexpression proliferation rate and validation of 22Rv1 cells stably-transfected with *SSTR1*-pCDNA3.1 compared with empty-pCDNA3.1 (mock), at 24, 48 and 72 h. **D**. Silencing proliferation rate and validation of 22Rv1 cells after transfection with *SSTR1* specific siRNA, compared with scramble siRNA as negative control (NC), and siRNA validation by qPCR. Asterisks (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$) indicate values that significantly differ from controls. In all cases, data represent mean ± SEM of $n \geq 3$ independent experiments.

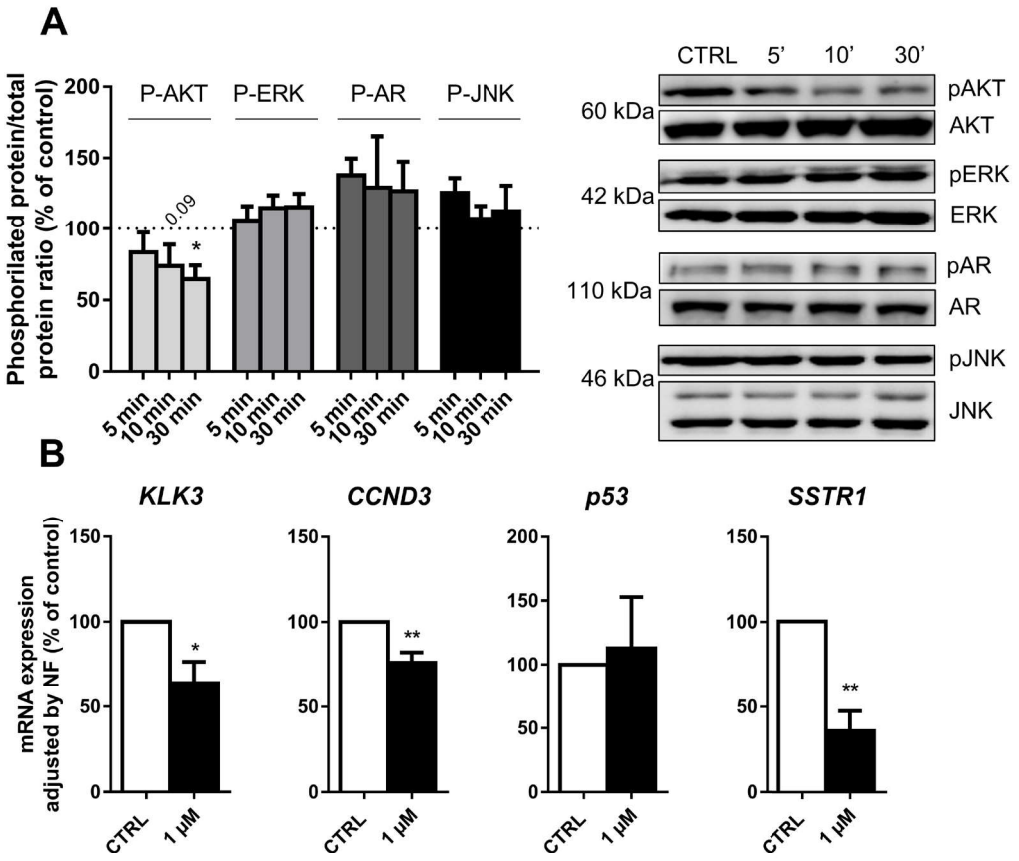
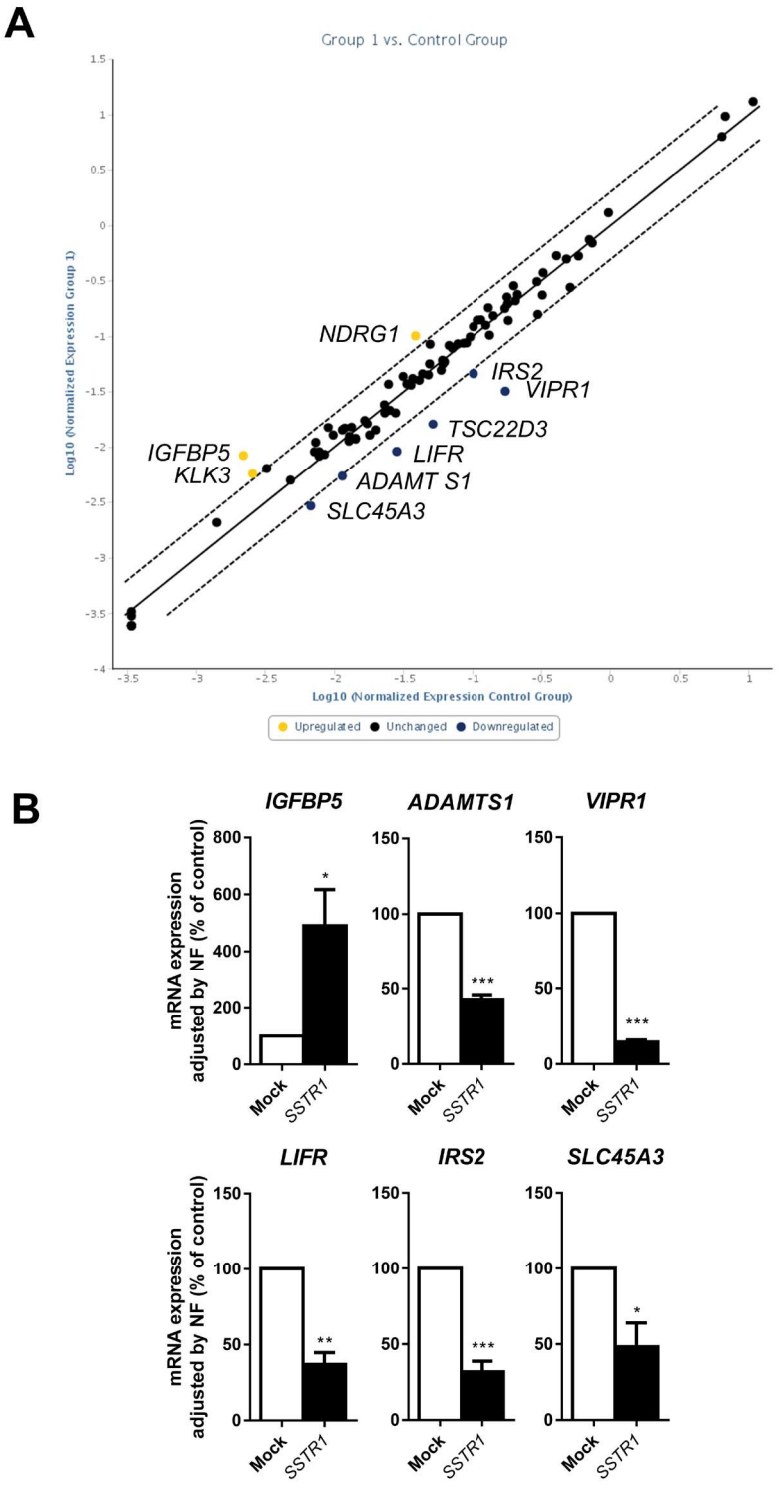


Figure 3. Downstream consequences of sst1 activation in 22Rv1 cells. **A**. Phosphorylation of key signaling pathways (AKT, ERK, AR and JNK) after BIM-23926 sst1 agonist treatment (1µM) at 5, 10 and 30 min (from left to right), compared with non-treated control. **B**. mRNA expression levels of *KLK3*, *CCND3*, *p53* and *SSTR1* genes after 24h of BIM-23926 sst1 agonist treatment at 1µM, compared with non-treated control. Absolute mRNA levels were determined by qPCR and adjusted by normalization factor (NF) calculated from the expression levels of three housekeeping genes (*ACTB*, *GAPDH* and *HPRT*). Asterisks (*, $p<0.05$; **, $p<0.01$) indicate values that significantly differ from controls. In all cases, data represent mean ± SEM of $n \geq 3$ independent experiments.



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673 **Figure 4. PCR array and validation of human AR signaling pathway. A**. Representation in
674 scatter plot of difference (2-fold change) between mock and *SSTR1* stably-transfected 22Rv1
675 cells. Upregulated genes are at the top of the image and downregulated genes at the bottom. **B**.

Validation by qPCR of changes in genes found to be altered in the array. Absolute mRNA levels were determined by qPCR and adjusted by normalization factor (NF) calculated from the expression levels of three housekeeping genes (*ACTB*, *GAPDH* and *HPRT*). Asterisks (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$) indicate values that significantly differ from controls. In all cases, data represent mean ± SEM of $n \geq 3$ independent experiments.

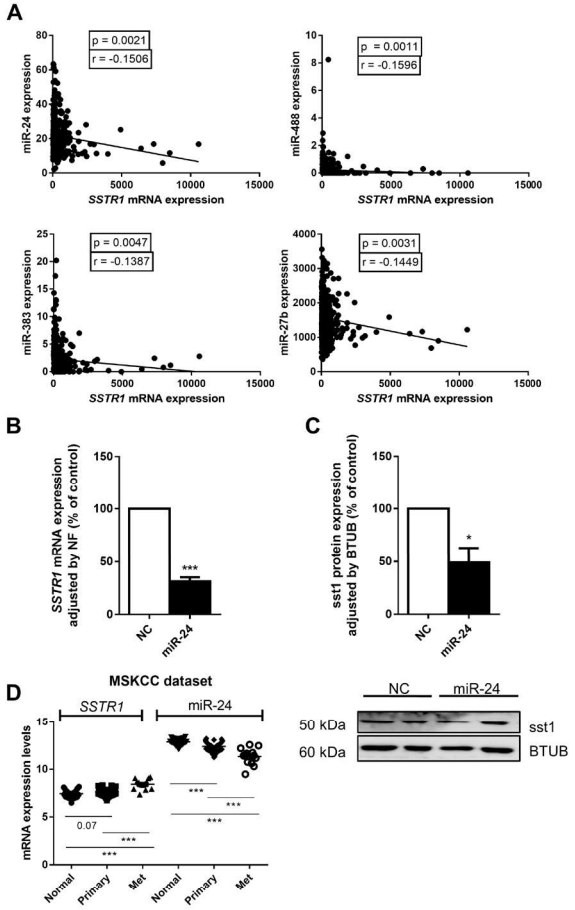

Figure 5. *SSTR1* expression is regulated by different miRNAs and correlated with its expressions in PCa. A. Correlations between *SSTR1* mRNA expression levels and expression of 4 different miRNAs (miR-24, miR-488, miR-383 and miR-27b) in PCa patients, from TCGA database. **B**. mRNA expression of *SSTR1* after transfection with miR-24 compared with negative control (NC) in 22Rv1 PCa cell lines. Absolute mRNA levels were determined by qPCR and adjusted by normalization factor (NF), calculated from the expression levels of three housekeeping genes (*ACTB*, *GAPDH* and *HPRT*). **C**. sst1 protein level after transfection with miR-24 or NC in 22Rv1 cells. Protein level was determined by western blot and normalized by

690 BTUB. **D**. Differences in the expression of *SSTR1* and miR-24 in normal prostate, primary PCa
691 and metastatic tissue, from MSKCC dataset. Asterisks (*, $p<0.05$; ***, $p<0.001$) indicate values
692 that significantly differ from controls. In all cases, data represent mean ± SEM of $n \geq 3$
693 independent experiments.
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695