PKA signaling pathway as a key driver of *SPP1*/OPN expression in prostate cancer bone metastasis.

Pablo Sanchis[1,2,3]; Agustina Sabater[1,2,3]; Julia Lechuga[1,2]; Mora Gatti[1,2,3]; Juan Bizzotto[1,2,3]; Peter DA. Shepherd[4]; Jun Yang[4]; Javier Cotignola[1,2]; Elba Vazquez[1,2]; Joaquin Mateo[5,6]; Pia Valacco[2]; Estefania Labanca[4;] Christopher Logothetis[4]; Geraldine Gueron[1,2]*; Nicolas Anselmino[4]*.

[1]Laboratorio de Inflamación y Cáncer, Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires C1428EGA, Argentina. [2]CONICET-Universidad de Buenos Aires, Instituto de Química Biológica de la Facultad de Ciencias Exactas y Naturales (IQUIBICEN), Buenos Aires C1428EGA, Argentina. [3]Universidad Argentina de la Empresa (UADE), Instituto de Tecnología (INTEC), Buenos Aires C1073AAO, Argentina. [4]Department of Genitourinary Medical Oncology and The David H. Koch Center for Applied Research of Genitourinary Cancers, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA. [5]Vall d'Hebron Institute of Oncology (VHIO), Vall d'Hebron Barcelona Hospital Campus, Barcelona, 08035, Spain. [6]Vall d'Hebron Institute of Research (VHIR), Vall d'Hebron Barcelona Hospital Campus, Barcelona, 08035, Spain. *Co-corresponding authors.

BACKGROUND: Bone metastatic (BM) dissemination of prostate cancer (PCa) cells is a key factor of disease progression and represents a major clinical challenge mainly because of PCa heterogeneity in treatment response and clinical outcomes. In this regard, serum levels of Osteopontin (*SPP1*/OPN) are elevated in a subset of PCa patients (11%) with advanced disease. However, the molecular mechanisms driving this observation have not been addressed. In this work we assessed the factors controlling *SPP1*/OPN expression in a subgroup of patients with BM.

METHODS: We performed a comprehensive transcriptomics analysis (DEG, Ingenuity Pathway Analysis (IPA)) of publicly available patients' datasets (GSE74685, SU2C-PCF, GSE32269, and Westbrooke et al), comparing PCa on different metastatic sites and/or treatment status. Using an indirect co-culture system (24h) to mimic the dialogue between PCa cells (PC3/C42B) and osteoblast precursors (MC3T3) *in vitro*, we integrated transcriptomics (RT-qPCR and RNAseq) and secretomic (ESI-MS/MS) of conditioned media (CM) data to dissect key players involved in the bi-directional crosstalk between PCa and bone cells. Protein Kinase A (PKA) pathway implication was evaluated by its induction (forskolin; FK; 1μM) or suppression (H89 10μM). AR expression plasmid was used to assess the role of AR in the PKA/*SPP1* axis, followed by dihydrotestosterone (DHT) treatment (10nM). Clinically relevant patient derived xenograft (PDX) models growing intrafemorally (*i.f.*) were used for *in vivo* validation. ANOVA/t-test were performed to assess statistical significance.

RESULTS: Our bioinformatics analysis revealed a subpopulation of PCa patients with BM exhibiting high *SPP1* expression. Interestingly, enrichment analysis showcased an association between active PKA and higher levels of *SPP1*. By modeling this response *in vivo* and *in vitro*, we showed that bone-released *Col1a1* and *Fn1* significantly induce *SPP1* expression in PCa cells by activating PKA ($P<0.05$). Moreover, we observed an induction of *SPP1* in longitudinal samples from a subpopulation of patients before starting enzalutamide (androgen receptor (AR) signaling inhibitor) treatment and at the time of progression, consistently correlating with active PKA signaling. To directly examine the interaction between AR and PKA/*SPP1* pathways, we transfected AR-negative PC3 cells with a human AR expression vector. Although, PKA-induced

SPP1 expression using FK 1 μM was unaffected in AR-transfected PC3 cells, DHT treatment significantly reversed FK-mediated *SPP1* upregulation, demonstrating the critical role of AR activity in this pathway. These results reinforce the critical role of PKA in response to the bone microenvironment and suggest that the AR modulates the PKA/*SPP1* axis.

CONCLUSIONS: In this study, we have identified a new regulatory axis through which the bone microenvironment increases *SPP1*/OPN expression in PCa cells, potentially contributing to the emergence of treatment resistance. Our results suggest that *SPP1*/OPN could serve as a valuable biomarker for detecting tumors with active PKA signaling, offering a promising approach to improving disease management strategies.

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