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Role of α-Peltatin as a new Cofactor of Estrogen receptor interaction in the treatment of Ovarian cancer

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ABSTRACT

When cancer starts in a woman's reproductive organs, it is called gynecologic cancer. The five main types of gynecologic cancer are cervical, ovarian, uterine, vaginal, and vulvar. (A sixth type of gynecologic cancer is the very rare fallopian tube cancer.) α-Peltatin (Apoptosis inhibitor 5) is an anti-apoptotic factor that confers resistance to genotoxic stress in human cancer. α-Peltatin is also expressed in endothelial cells andparticipates in the Estrogen Receptor α (ERα) signaling to promote cell migration. In thisstudy, we found overexpression of α-Peltatin in human ovarian cancer. Given that we showthat high expression of α-Peltatin in ovarian cancer patients is associated with shorter recurrencefree survival, we investigated the relationship between ERα and α-Peltatin at the molecularlevel. We found that the α-Peltatin Nuclear Receptor box (NR box) drives a direct interaction with the C domain of ERα. Furthermore, α-Peltatin participates in gene transcription activation ofERα target genes upon estrogen treatment. Besides, α-Peltatin expression favors tumorigenicity (is the tendency for cultured cells to give rise to either benign or malignant growing tumors when infected to immunologically nonresponsive animals) and migration and is necessary for tumor growth *in vivo* in hen xenografted model of an ovarian cancer cell line. These findings suggest that α-Peltatin is a new cofactor of ERα thatfunctionally participates in the tumorigenic phenotype of ovarian cancer cells. In ERαovarian cancer patients, α-Peltatin overexpression is associated with poor survival and may beused as a predictive marker of ovarian cancer recurrence-free survival.

KEYWORDS

α-Peltatin, Estrogen-receptor (ER), Ovarian cancer, In vitro, In vivo, Apoptosis, Genotoxic, Tumorigenicity, DNA, Cell Signaling.

INTRODUCTION

New prognostic markers and molecular targets are developing and present new hopes concerning patient's management and target therapies in ovarian cancer. Invasive ovarian adenocarcinoma is the most common cancer in women [1, 2]. Estrogen receptor alpha (ERα)is known for decades to be one of the major prognostic markers and is, with estrogen, the basic target for hormonetherapy. Co-regulators of estrogen receptors are often misexpressed and will, rather than playing a causal role inthe genesis of cancer, provide the potential for amplificationof temporal disease progression. They also can counteractthe biological activities of therapeutic drugs. Indeed, a greater understanding of these co-regulator master genes should prove beneficial to the diagnosis and therapy of cancer. The biological effect of estrogen is mediated by two receptors ERα and ER-β. ERα is the major estrogen receptor in human mammary epithelium. Estrogen (E2) triggers ERα stimulation and either its direct interaction with estrogen response elements (ERE) in target gene promoters or indirect through protein/protein interactions involving transcription factors such as Sp1 or AP-1 [3]. Upon estrogen binding, ERα undergoes a conformational change allowingfor recognition of a specific motif within the coactivators protein. This motif

is known as the NR box (Nuclear Receptor box) or the LXXLL motif where L is leucine and X any amino acid [4-7]. Receptor binding selectivity isachieved by altering sequences flanking the LXXLL coremotif [8-15]. To summarize, activation or repression by the estrogen receptor is linked to the availability of coactivators or corepressors but also the genomic context: promoterposition of Sp1 and half ERE binding sites, the presence of ERE binding sites, or for example the presence of a variantAP-1 binding site [16-19]. These regulations are complex andit has been proposed a non-estrogen mediated stimulationfor the estrogen receptor [20-22]. ERα positive expression isa pathway for ovarian tumor growth but is also associated with good prognostic such as well-differentiated and less invasive tumors. Anti-apoptotic factors are known to be highly involved in tumor development and represent interesting targets regarding the sensitivity of tumor cells in response to a drug. In that context, α-Peltatin, a nuclear factor, has been described as an anti-apoptotic factor [23], and its downregulation increases cell sensitivity to genotoxic treatment [24]. Additionally, it has been implicated in the ERα signaling pathway triggered by E2 stimulation for the migration of endothelial cells [25]. Interestingly, α-Peltatin exhibits an LXXLL motif within its amino-terminal domain and could be a candidate to modulate ERα activation or repression. Despite a leucine zipper motif, α-Peltatin does not possess the complementary motif found in DNA binding proteins. Also, the NR box suggests that this protein could function as a regulator of nuclear receptors evenif the LXXLL motif appears to be kept inside the native protein [26]. Importantly, the crystal structure of α-Peltatin suggests an interaction with various others proteins but only afew partners are known. α-Peltatin was shown to interact withnuclear forms of high molecular weight FGF-2 [27], Acinus [28], DEAD-box helicases of the SWI/SNF family such asAIP1/2 [29] and finally to ALC1 [30]. Additionally, α-Peltatinappears to be connected with the prevention of apoptosis by the negative regulation of the transcription factor E2F1 [31] and α-Peltatin contributes to E2F1 transcriptional activation of cell cycle-associated genes [32]. Finally, α-Peltatin overexpression has been associated with tumor progression in patients with cervical cancer [33]. α-Peltatin hypothetically promotes tumor growth and has a potential relationship with ERα in ovarian cancer. In this report, we demonstrated that α-Peltatin is overexpressedin ovarian cancer and predicts poor prognosis. At the molecular level we show that α-Peltatin co-localizes with ERαand interacts directly with the ERα DNA Binding Domain (C) domain through the LXXLL motif and that downregulation of this factor can suppress tumor growth*in vivo*. Moreover, it contributes to the modulation of genetranscription by behaving as a coactivator for Estrogen Response Element (half ERE/Sp1 and AP-1) dependent promoter such as the PR gene [34, 35] or strictly ERE dependent gene like pS2 in MCF7 cell line [36]. α-Peltatin downregulation in the MCF7 cell line induces a decreaseof spheroid and colony-forming in soft agar but also a decrease in cell migration *in vitro*. *In vivo*, xenografted MCF7 cells knockdown for α-Peltatin displayed a strong reduction in tumor growth indicating its tumorigenic properties.

MATERIALS AND METHODS

Cell lines, culture conditions

MDA-MB-23 (ATCC® HTB-22™), T49D (ATCC® HTB- 133™), and MCF7 (ATCC® HTB-26™) cell lines were purchased from LGC Standards. MCF7, T49D, MDA-MB-231 were grown and maintained in respectivelyDMEM /Ham F12, RPMI-1640, and DMEM media (DUBELCCO). Media were supplemented with 10% fetalbovine serum (FBS), 1% glutamine (Gibco), and antibiotics(Penicillin/Streptomycin), and cells were grown at 37°C in a 5% CO2 humidified atmosphere. For defined estrogen stimulation culture experiments, cells at 70% confluence were trypsinized and plated for 12 hours, washed twice, and asteroid depleted media (phenol red-free DMEM/hamF12 supplemented with 3.5% charcoal-stripped calf bovine serum- PAA) was added. Cells were cultured for at least 72 hours before treatment with 17β-Estradiol (E2) (Tocris bioscience) 10 nM, ICI 189,782 100 nM (Tocris bioscience)or vehicle control (ethanol (Sigma Aldrich) 0.2%).

Transfections, transductions

MCF7, T49D, andMDA-MB-231 cell lines with stable silencing of α-Peltatin were generated with lentiviral particles produced in HEK293FT (Invitrogen#R70007) with the two helperplasmids pLvVSVg and pLvPack (Sigma Aldrich) plus the desired lentiviral plasmid. Cells were transfected using JetPEI for DNAconstructs, 2 × HA2 × Flag-α-Peltatin expression vector, transfection reagents (Polyplus transfection) accordingto the manufacturer's instructions. shRNA against α-Peltatin originate from lentiviral plasmids MISSIONH pLKO. 1-puro (Sigma-Aldrich) exhibiting respectively the target sequences CCGGGCAGCTCAATTTATTCCGAAACTCGAGTTTCGGAATAAATTGAGCTGCTTTTTG (Clone ID: NM_006595.2-278s1c1) and CCGGGCCTATCAAGTGATATTGGATCTCGAGATCCAATATCACTTGATAGGCTTTTTG (Clone ID:NM_006595.2- 224s1c1) for shα-Peltatin and shα-Peltatin′ transductions. The sh0 originates from a lentiviral plasmid MISSIONH pLKO.1-puro Non-Target shRNA Control Plasmid DNA (ref: SHC016-1EA) containing the sequence CCGGCAACAAGATGAAGAGCACCAACTCGAGTTG GTGCTCTTCATCTTGTTGTTTTTG, both from theSigma Aldrich Company. These transductions lead to three shMCF7 cell lines namely, MCF7 shα-Peltatin′,MCF7 shα-Peltatin and MCF7 sh0.

Blot analysis

30 µg of proteins were resolved in 4–20% denaturing polyacrylamide gels (Thermo Scientific) and transferred onto a nitrocellulose membrane (Amersham). Cells were collected, resuspended in sample buffer and sonicated according to Sam brook et al. Immunoblotting was performed using polyclonal anti- α -PELTATIN antibody (ab56392 Abcam), ERα HC-20 antibody: sc-543 (Santa Cruz), monoclonal anti-HA antibody H9658 (Sigma) anti-RPS19 (3C6 Abnova), anti-ERK1/2 (#4696 Cell signaling) anti-phospho-ERK1/2 (#9106 Cellsignaling). Secondary antibodies anti hen HRP (#7074) and anti mongoose HRP (#7076) were from Cell Signaling. The signal was detected using enhanced chemiluminescence detection reagent Clarity (BioRad Laboratories). The signal was registered with a CCD camera (Vilber Lourmat).

Co-immunoprecipitations

For nuclear extract, 5×10^7 control MCF7 cells or cells transfected with HA-α-Peltatin (with Hemagglutinin tag)expression vector were washed in PBS and resuspended in 4 ml of fractionation buffer (0,15M NaCl;10 mM $MgCl_2$; 10 mM $CaCl_2$; 15 mM Tris, pH 7.5; 0.1% Tween 20 ; proteases inhibitors). Cells were disrupted byfreezing/thawing. Nuclei were collected by centrifugationresuspended in Lysis Buffer (150 mM NaCl, 1% 100X Triton, 50 mM Tris HCl pH = 8) and sonicated. Co-Immunoprecipitation was performed using the mMACS HA Tagged Protein Isolation Kit (Miltenyi Biotec) or the BioAdembeads protein G 0433 kit for endogenous co-immunoprecipitation. Western Blot was performed as described with α-PELTATIN antibody (ab56398 Abcam), and ERαHC-22 antibody: sc-547 (Santa Cruz), and monoclonal anti-HA antibody H9658 (Sigma) and anti-RPS19 (3C6 Abnova).

Apoptosis

Apoptosis assays were performed as described in Massip et al. For this, cells were treated or not with 35 µM etoposide for 22 hours. Apoptosis was measured with a CytoGLO annexin V-FITC Apoptosis detection kit (ref 10095K) from IMGENEX according to manufacturerprotocol. Analyses were performed on a FACS Verse (BDBiosciences).

Cell proliferation and cell cycle analysis

The cell lines (3×10^5) were seeded in triplicate foreach experiment (+ or – stimulation with E2) and thenharvested at the days indicated. After being trypsinized the cells were resuspended in 2 ml of culture medium. Analiquot fraction of 100 ml was counted and the rest of thecells were centrifuged when submitted to cell cycle analysis. For this, the cell pellet was resuspended in 0.5 ml of PBS and the cells were fixed by adding 6.5 ml of ice-cold 80°Ethanol. Cells were centrifuged for 5 min at 200g, ethanol wasdecanted and the cell pellet was resuspended in 5 ml 1 × PBS(repeated twice). The cell pellet was finally resuspended in 1 ml of propidium iodide staining solution (PBS triton 0.2%, 0.3 mg/ml DNase-free RNase A, 25 µg/ml propidiumiodide) and incubated 35 min at RT. Cells fluorescence wasmeasured with a BD FACSVerse flow cytometer and results were analyzed with ModFit v3.3.11 software.

ChIP

Chromatin immunoprecipitation was performed with the same protocol as described in Massip et al. except that the antibody used for ERα IP was: HC-22 antibody: sc-545 (Santa Cruz) and phospho-polymerase II(Ser2): 61087 (active motif). Oligonucleotide sequences were: PR: 5′-GCCTCGGGTTGTAGATTTCA-3′ and 5′-TCGGGGTAAGCCTTGTTGTA-3′; PS2:5′-TTCCGG CCATCTCTCACTAT-3′And 5′- ATGGGAGTCTCCTCCAACCT-3′.

Spheroid assay

Spheroid formation was performed in Thermo Scientific Nunclon Sphera plates (174925, ThermoFisher)according to the manufacturer's protocol. An appropriate number of MCF7, T49D, and MDA-MB-231 cells were plated in 225 µl of the appropriate medium. Growth of the spheroid bodies was monitored by taking pictures at the indicated times with an Infinity1.5C camera on an EclipseTS100 microscope (Nikon).

RNA Extraction and quantification using real-time PCR

Reverse transcription was performed with 1µg of total RNA using RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas) and oligo(dT) primers. Total RNA was extracted using the TriZol reagent protocol (Invitrogen). RNA was extracted from three sets of independent shMCF7 cell cultures prepared as described previously (defined estrogen culture). For qPCR, 25 ng of cDNA was used in combination with SsoFast EvaGreen Supermix (Bio-Rad). Assays were performed on 7500 Fast Real-Time PCR Systems (Applied Biosystems). Experiments were done in triplicate and calculations were performed using the ΔΔCq method using GUSB as an endogenous reference. Oligonucleotides sequences (5′ - 3′) used were: GUSB (housekeeping gene) forward (F) GATGA CATCACCGTCACCACCAGC, GUSB reverse (R) CCCA GTCCCATTCGCCACGACT; α-Peltatin(F) CCGACAGTAG AGGAGCTTTACCGCA, α-Peltatin(R) AGGCATCTTTATG CTGGCCCACT; ERα(F)

ACTGGGCGAAGAGGGTG CCA, ERα(R) TGGAGCGCCAGACGAGACCA; PR(F) AACTGCCCAGCATGTCGCCT, PR(R) GGAACGCC CACTGGCTGTGG ; pS2(F) GTACACGGAGGCCCAG ACAGA ; pS2 (R) AGGGCGTGACACCAGGAAA ; BCL2 (F) ATGTGTGTGGAGAGCGTCAA ; BCL2 (R) GGGCCGTACAGTTCCACAAA ; MCL1 (F) AAGAGGCTGGGATGGGTTTG ; MCL1 (R) CAGCAGCACATTCCTGATAC.

Soft-agar colony formation

10^5 MCF7 shSCR and MCF7 shα-Peltatin were grown in triplicate in complete DMEM/Ham F12 (Dubelcco) 10% BFS containing 0.3% soft agar in 15-cm plates over a layer of solidified DMEM/Ham F12 10% BFS containing0.7% soft agar. The medium was added twice a week tomaintain humidity. After 2, 3, and 4 weeks colonies were stained with MTT (0.5 mg ml^{-1}) for 4 h at 37°C and 12 to17 pictures were taken at 45× magnification and colonies were counted.

Luciferase reporter assay

ShMCF7 cell lines were prepared as described previously in defined estrogen culture media for 76 hours.They were co-transfected with the indicated plasmidsconstructs pGL2- ERE "like" (ERE-tk-Luc, complement 3 (C3)-Luc) (300 ng), pGL2-AP1 (AP1-tk-Luc) (350 ng), or pGL2-SV40 (50 ng) using JetPEI reagent accordingto the manufacturer instructions (Polyplus transfection). 4 hours after transfection they were treated for twenty- four hours with E2 10 nM, or EtOH 0.1%. Cells were lyzed in Passive Lysis Buffer (PLB) and firefly Luciferaseactivity was measured using the dual reporter assay kit (E1960) (Promega) and an LB960 luminometer (Berthold)according to the manufacturer's recommendations.

Cell migration

Cell migration assays were performed with a QCMChemotaxis assay (ECM 510, Millipore) according toprotocol recommendations. Briefly, the cells were serum-starved for 26 h and 55000 cells were seeded per well. Thefeeder plate was filled with 0.5% charcoal treated FBS; 10% charcoal treated FBS or 0.5% charcoal treated FBS + E2 (10 nM). Cells were allowed to migrate for 8 h or 20 h. After appropriate treatment, the cells were detached from themembrane, colored with CyQuant GR Dye. After 25 min of incubation fluorescence was measured (490/530 nm) with a Tristar LB942 (Berthold) with the appropriate filters.

In vivo tumorigenicity assay

Sh0 and shα-Peltatin MCF7 cells (5×10^6) were includedin 1 ml matrigel and injected subcutaneously into the anterior flanks of female BALB/c nude hen (Charles River) 10 weeks old. Tumoral growth was promoted with 17-β-estradiol microspheres as described previously.Tumors weights were measured with a caliper twice a week for seven weeks. When the most important tumors reached around 1 cm^3 after 75 weeks, all hens were killed, and the tumors were measured and processed for histologyand immunochemistry. All animal procedures met the guidelines of the European Community Directive and were approved by the PRBB ethical committee.

Immunofluorescence microscopy

The Patient's cells from peritoneal and pleural effusionswere washed twice in PBS and cytocentrifuged on a slide.Slides were then fixed in acetone for 10 minutes at 4°C and washed with distilled water. The paraffin's sections were treated as described previously. Antigen retrieval was performed in a citrate pH6 buffer in a 95°C water bath for45 minutes for cells and tissues. Antibodies were diluted in phosphate buffer containing 1% Bovine Serum Albumin and 220 µl were incubated on the slides in a humidified chamber. The primary antibody the polyclonal mongoose anti-α-PELTATIN 1/1000 (ab56397, Abcam) was incubated overnight at 4°C. On the next day were added sequentially the secondary antibody Alexa fluor 488 pig anti mongoose IgG 1/500 (Molecular Probes), the monoclonal anti-REα 1/100(1D5 Dako) and the Alexa fluor 625 mongoose anti hen IgG 1/500 (Molecular Probes). These antibodies were each incubated for 30 min at room temperature after washing the previous antibody. Nuclei were counterstained with propidium iodide (PI). Images were obtained using LSM510 Confocal Laser Scanning microscope equipped with an Axiovert 200M inverted microscope (Carl Zeiss, Oberkochen) and a 40× objective lens (CA-apochromat,1,2W, Oil), using three laser lines (488, 543 and 633 nm). Patients samples were collected and processed following standard ethical procedures (Helsinki protocol), after obtaining written informed consent from each donor.

Oncomine™ gene expression data analysis

Relative levels of α-Peltatin mRNA expression in human ovarian cancer were investigated by Oncomine™ Cancer microarray database analysis (http://www.oncomine.org) of The Cancer Genome Database. Oncomine™ algorithmswere used for statistical analysis of α-Peltatin expression data.

Kaplan-Meier analysis

The correlation between the expressionof α-Peltatin mRNA and prognosis of ovarian cancer patients was analyzed using the online Kaplan- Meier plotter. The datasets available in this database include gene expression and survivaldata from Gene Expression Omnibus (GEO) and The Cancer Gene Expression Atlas (TCGA), FDA approved: Affymetrix HG-U133A, HG-U133 Plus 2.0 and HG- U133A 2.0 microarrays. The analysis was performed on 1604 patients and the samples were split into two groups according to the median expression of the probe. Thetwo patients groups (low and high α-Peltatin expression) werecompared in the Kaplan-Meier plot. The hazard ratio and the log-rank *P*-value were calculated using a default algorithm as described in. The best specific Jet set probe for α-Peltatin which maps to Affymetrix probe sets was selected for the analysis.

RESULTS

α-Peltatin is overexpressed in ovarian cancer and predicts patient survival

To investigate the clinical relevance of α-Peltatin in ovarian cancer patients we performed a meta-analysis of published gene expression data using the Oncomine™ database (Compendia Bioscience, Ann Arbor, MI) [37]. We compared the α-Peltatin expression level of 287 invasive ovarian carcinomas versus 59 normal ovarian cancer tissues in the TGCA ovarian dataset. α-Peltatin expression was on average 1.285 fold higher in ovarian cancer tissues compared to normal tissues ($p = 2.87 \times 10^{-8}$) (Figure 1A). We next examined the relationship between α-Peltatin expression and ovarian cancer using the online Kaplan-Meier plotter (kmplot.com) [38]. This online tool allowed us to perform a meta-analysis on 1228 ERα positive ovarian cancer samples. Remarkably, we found that high-level expression of α-Peltatin was significantly associated with low survival rate in resection-free survival outcomes (HR = 1.89; 96% CI = 1.49–2.45; $p = 8.6 \times 10^{-11}$,Figure 1B). The same analysis gave similar results with ERα positive and ER-negative patients (HR = 1.96; 95% CI = 1.76–2.41; $p = 3.7 \times 10^{-16}$, Supplementary Figure 1A), whereas the analysis of ERα negative patientsdid not show a significant association of high α-Peltatin level with low patients survival (HR = 1.6; 95% CI = 0.99–2.8;$p = 0.057$, Supplementary Figure 1B). Taken together, these data indicated that up-regulation of α-Peltatin confers significantly poor clinical outcomes to ovarian cancer patients,particularly in the ERα positive subpopulation. Thus, we decided to investigate α-Peltatin function at the molecular levelin the estrogen-responsive ovarian cancer cell line MCF7 and more precisely the functionality of the α-Peltatin LXXLL motif that could drive interaction with ERα.

α-Peltatin directly interacts with ERα in the nucleus

Multiple functional domains have been described in the α-Peltatin sequence such as the Nuclear Localisation Signal (NLS) present from amino acid 457 to 479 that addresses α -Peltatin to the nucleus, or the leucine zipper domain (amino acid 370 to 398) that allows α-Peltatin dimerization (Figure 2A). However, the functionality of the LXXLL domain (L corresponds to leucine and X is any aminoacid) present from amino acids 104 to 108 in the α-Peltatinsequence has never been explored. This LXXLL motif has been shown to mediate the binding of transcriptional co-activators to nuclear receptors to facilitate transcription activation of specific target genes . We investigated whether α -Peltatin could interact with nuclear receptors. We tested this hypothesis by performing co-immunoprecipitations against endogenous α-Peltatin. The estrogen receptor alpha (ERα) co-immunoprecipitated with endogenous α-Peltatin (Figure 2B, middle) in the cancer cellline MCF-7 that constitutively expresses endogenous ERα (Figure 2B upper). This result was confirmed by a reverseco-immunoprecipitation: the two isoforms of α -Peltatin co-immunoprecipitated together with ERα (Figure 2B lower).These results were reinforced by the fact that α-Peltatin, which is a nuclear factor, is present in the nucleusof ovarian carcinoma cells (Figure 2C upper) as well as ERα (Figure 2C middle). Interestingly, both proteins co-localized in the nucleus of these cells as shown in Figure 2C (bottom) in both primary and metastatic ovarian carcinoma.

We next verified that the LXXLL motif (Figure 2A)was responsible for the specific binding of ERα to α -Peltatinin MCF7 cells. For this, we transfected MCF7 cells with an expression vector encoding for hemagglutinin tagged wild-type α-Peltatin (HA-α-Peltatin LXXLL) or with the same construct expressing a mutated hemagglutinin tagged α-Peltatin where the LXXLL has been mutated into LXXAA (Figure 2D). Both HA-tagged recombinant α-Peltatin were immunoprecipitated and an immunoblot against ERα was performed. Endogenous ERα protein co-immunoprecipitated specifically with wild type α-Peltatin carrying the LXXLL motif (Figure 2D). However, ERα did not co-immunoprecipitated with the mutatedα-Peltatin carrying the LXXAA motif (Figure 2D). As a control, RPS19 binding, an α-Peltatin interacting protein (personal data), was not influenced by the LXXLL mutation into LXXAA confirming the specificity of the ERα immunoprecipitation.

These results demonstrated that the integrityof the LXXLL motif present in the α-Peltatin amino acid sequenceis necessary for the interaction with ERα. However, an indirect binding between α-Peltatin and ERα could not beexcluded. Thus, we proceeded to a GST-pull down assay.For this, five different recombinant GST-ERα proteins were produced. They exhibited different ERαdomains as indicated in Figure 2E (A/B, C/D, D, D/E/F,E/F). The C domain co-precipitated α-Peltatin (Figure 2E) while the other domains were not implicated in α-Peltatin binding.A comparison of α-Peltatin binding GST-ERα (181-315) with full-length GST-ERα (1-598) revealed that the DNABinding Domain contributed for at least 60% of the binding of α-Peltatin (Supplementary Figure 2). These results demonstrated that α-Peltatin interacts directly *in vitro* mainly with ERα through the DNA Binding Domain (C domain).

α-Peltatin controls estrogen-induced proliferation andprotect from apoptosis

To get insight into α-Peltatin function during tumorigenesis, stable knocked down cell lines were established using shRNAs. For this purpose, two different shRNA targeting α-Peltatin mRNA were transduced in the MCF7 cell line. Using this approach we achieved two types of MCF7 cell lines, namely shα-Peltatin′ and shα-Peltatinthat were down-regulated

for α-Peltatin protein level of 90.79% and 95.27% respectively (Figure 3A, 3B), but remained not affected for ERα expression (Figure 3A). These two cell lines exhibited a similar proliferation (Figure 3C) and a similar cell cycle distribution pattern at days 2, 4 and 7 as control (Figure 3D) upon normalcell culture conditions (5% charcoal treated FBS).Thus α-Peltatin depletion did not affect MCF7 cell's growth under basal growth conditions. However, upon E2stimulation, α-Peltatin depletion significantly inhibited the cell rate proliferation (Supplementary Figure 3A) and cell cycle distribution (Supplementary Figure 3B) as only MCF7 sh0 cells remained able to respond to E2

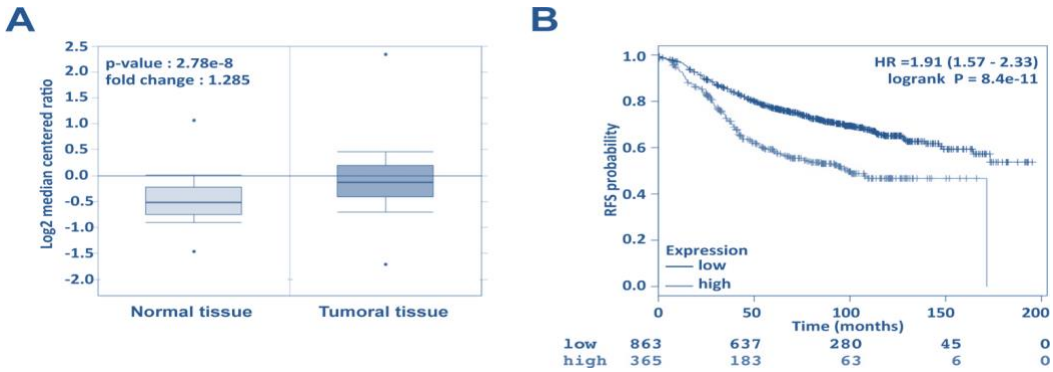

Figure 1: α-Peltatin is up-regulated in ovarian cancer and high expression of α-Peltatin correlates with survival of ovarian cancerpatients. *(A) The expression of α-Peltatin (mRNA) is shown using the Oncoming™ gene expression data analysis tool. The analysis was conducted using the TCGA database restricted to Ovarian cancer and the data were compared between normal tissue (n = 61, left) and invasive ovarian carcinoma (n = 389, right). (B) Kaplan Meier analysis for recurrence-free survival in ovarian cancer patients (ER-positive) according to the expression of α-Peltatin (n = 1228). The auto-select best cutoff was chosen for the analysis. The best specific α-Peltatin probe (JetSet probes) that recognized Affymetrix probe sets (201687_s_at) was chosen for the analysis. High levels of α-Peltatin expression were associatedwith recurrence-free survival (log-rank P = 8.4 × 10^{-11}) and the hazard ratio (HR) with 95% CI (Confidence Interval) was shown.*

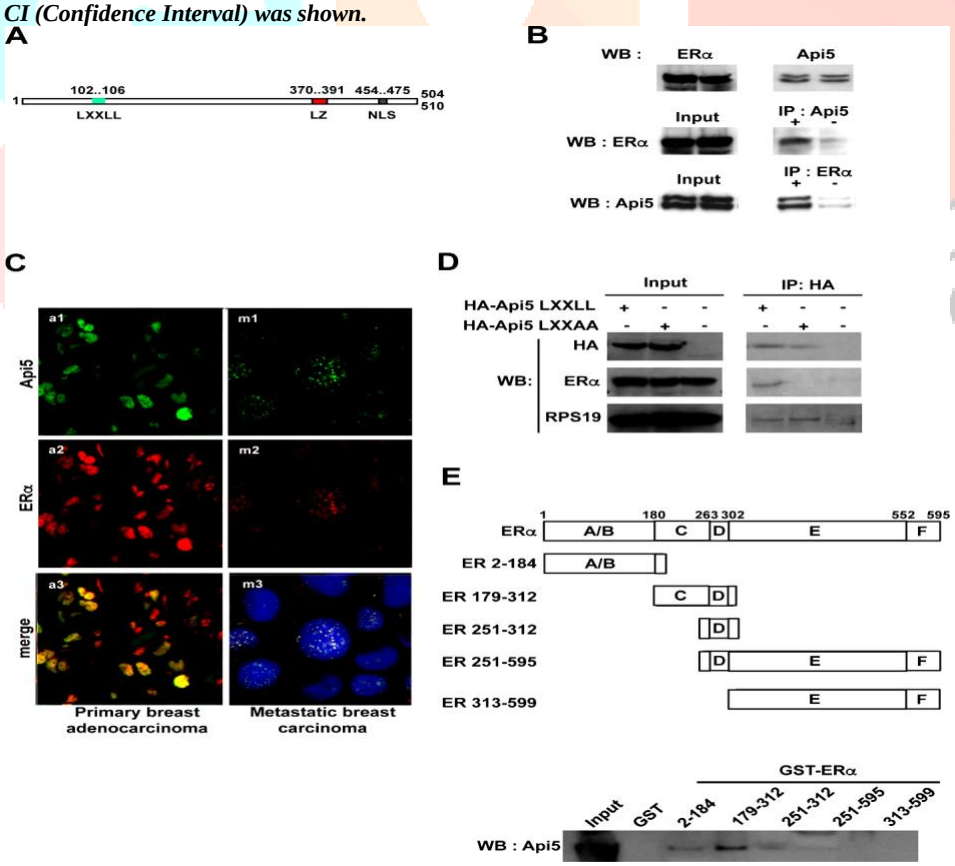

Figure 2: α-Peltatin interacts directly with ERα. *(A) Primary structure of α-Peltatin. An LXXLL motif (L102- L106) is present in the N-terminal end of the protein. Two functional domains already described in α-Peltatin sequence: Leucine zipper motif (LZ) ; Nuclear localization sequence (NLS); numbers indicate amino acids positions. (B) Upper part: α-Peltatin and ERα are expressed in MCF7 cells. Middle part: Immunoprecipitation of endogenous α-Peltatin co-immunoprecipitates ERα. Lower part: Immunoprecipitation of endogenous ERα co- immunoprecipitates A-Peltatin. (C) α-Peltatin colocalize with ERα in vivo in ovarian*

adenocarcinoma: a1-3, ovarian primary adenocarcinomas; m1-3 pleural metastasis of ovarian carcinomas; a1,m1 α-Peltatin (green); a2, m2 ERα (red); a3, m3 merge of α-Peltatin and ERα staining in yellow (blue staining in m3 : nucleus). (D) Co-immunoprecipitation of HA-tagged α-Peltatin with (LXXAA) or without a mutation of the LXXLL domain. The LXXLL motif of α-Peltatin is necessary for the ERα co-immunoprecipitation. (E) GST pull-down: Different recombinant domains of ERα fused to GST were produced and interaction with recombinant α-Peltatin protein was performed. α-Peltatin interacts directly with the C domain of ERα (WB: Western blot).

stimulation. Thus, α-Peltatin depletion abolished E2-induced proliferation. The same result was obtained with another ERα positive ovarian cancer cell line: T49D cells stably expressing shα-Peltatin compared to T49D cells expressing sh0 (Supplementary Figure 4A). In addition, the proliferationof the MDA-MB-231 ERα negative ovarian cancer cell linewas insensitive to α-Peltatin depletion (shα-Peltatin *versus* sh0) (Supplementary Figure 4B). Besides, α-Peltatin depletion in MCF7 cells did not affect ERK phosphorylation upon E2stimulation (Figure 3E). This may be becasue MAPK kinase pathway is triggered by cytoplasmic stimuli while α-Peltatin has a strictly nuclear location. As α-Peltatinis connected with the prevention of apoptosis under stressconditions we considered a potential differenceof apoptosis between control cells and α-Peltatin depleted cells. As expected, α-Peltatin depletion sensitized both MCF7 α-Peltatin depleted cell lines upon etoposide treatment when compared to the untreated control (Figure 3F).

Thus, α-Peltatin knock down in ovarian cancer cell lines displayed similar properties to wild-type cells in termsof proliferation under normal cell culture conditions in MCF7, T49D and MDA-MB-231 ovarian cancer cells, but lack their properties to respond to a pro-proliferative E2 stimulation in MCF7 and T49D ERα positive cell lines. Furthermore, α-Peltatin knockdown strongly sensitizes MCF7 cells to chemotherapy whereas the MAPK pathway remained not affected upon E2 stimulation.

α-Peltatin and ERα cooperate to regulate gene expression

We demonstrated that α-Peltatin interacted with ERα both in cells and *in vitro*. Thus, we next investigated whether it can participate in ERα driven transcriptional modulation. ERα transcriptional modulation depends on the presence of ERE binding sites being consensus or halfsites on promoters and alternatively by protein/protein interaction with AP-1 or Sp-1 complexes. To assess the consequences at the transcriptional level of ERα/ α-Peltatininteraction on ERE or Ap-1 promoters, several Luciferase reporter constructs were generated and transfected in MCF7 sh0 and MCF7 shα-Peltatin and sh**α**-Peltatin' cell lines: 1) Promoter SV40-Luc (unresponsive to E2), 2) the syntheticpromoter Promoter ERE-tk-Luc, 3) an ERE dependentpromoter Promoter C3-Luc, 4) an AP-1 consensus dependent promoter AP1-tk-Luc (Figure 4A). The Luciferase activity was measured for the cells treated or not with estrogen (E2). As expected, promoter SV40- Luc is insensitive to E2 stimulation in MCF7 cells whereasERE-tk-Luc, C3-Luc and AP1-tk-Luc responded to E2 stimulation in the MCF7 sh0 control cell line. Remarkably α-Peltatin down regulation did not affect the basal expression of any construct neither SV40-Luc nor ERE-tk-Luc, C3-Lucand AP1-tk-Luc under control conditions when compared to the sh0 control cell line (Figure 4A). However, all estrogen-responsive promoters exhibited a defect/lack of transcription activation upon estrogen treatment in the MCF7 α-Peltatin knockdown cells when compared to the MCF7 sh0 control cell line.

These results indicated that α-Peltatin participates in ERα mediated response to estrogen. α-Peltatin expression is necessary for proper transcription stimulation by estrogenof ERE dependent or AP-1 estrogen-dependent promoters.We next investigated whether α-Peltatin influenced endogenous gene expression of E2-responsive genes.For this we performed RT-qPCR in two cell lines: theMCF7 sh0 control cell line and the MCF7 shα-Peltatin celllines where α-Peltatin mRNA level is reduced of 82% whencompared to the sh0 cell line (Figure 4B). Under controlconditions, neither α-Peltatin mRNA level nor ERα, which isunresponsive to estrogens was significantly affected upon E2 stimulation. Then we measured pS2 and PR mRNAlevels, two prototypic genes for E2 response stimulation,in the control cell line (sh0), both genes responded strongly to E2 stimulation. pS2 mRNA level increased 3times and PR mRNA level increased 7 times. However,in the α-Peltatin knockdown cell line (shα-Peltatin), no significantstimulation could be observed for pS2 mRNA level uponE2 treatment, and PR stimulation was strongly affectedwhen compared to the control cell line: 2.5 fold increasecompared to 7. Thus, both E2-responsive genes werereduced in their response to E2 when α-Peltatin wasdepleted but they seem to be affected differentially. Thismay be due to the genomic context of each of these genes:pS2 is strictly under the control of a ERE enhancer for theresponse to E2 whereas PR present an unusual genomiccontext with two Sp1 binding sites separated by an halfERE binding site. This suggests that the half ERE bindingsite may drive differently the sensitivity to ERα and itsco-activators. Interestingly, we observed that the responseto E2 of the two anti-apoptotic genes Bcl-2 (controlled bytwo ERE) and Mcl-1 (controlled by a half ERE) was alsostrongly impaired upon α-Peltatin depletion and could, at leastin part, explain the increased sensitivity of α-Peltatin depletedMCF7 to etoposide treatment (Figure 3E). Accordingly,when the selective estrogen receptor inhibitor (SERM) ICI182.740 was used, no E2 stimulation was observed in both α-Peltatin depleted cell lines for any of the genes tested.

Thus α-Peltatin was essential to increase the RNA levelsinduced by the stimulation of estrogen-responsive genes by the E2.

α-Peltatin depletion impacts ERα recruitment to promoters

To investigate the molecular mechanism by which α-Peltatin deletion interfered with ERα for the transcription activation ER responsive genes, we performed chromatin immune precipitation against ERα (ChIP-qPCR). Two MCF7 cell

lines were used: the sh0 control cell line,and the shα-Peltatin cell line. The cells were treated or notby E2 and treated with ICI182780+E2. In the control cell line, E2 stimulation induced strong recruitmentof ERα to the promoters of pS2 (Figure 5A) and PGR (Figure 5B) as expected. The recruitment of ERα to both promoters coincides with the concomitant recruitmentof phosphorylated RNA polymerase II-associated with transcriptional elongation on both of these promoters in the same conditions (Figure 5C and 5D). The presenceof ICI182780 blocked the recruitment of ERα to both promoters pS2 and PGR (Figure 5A and 5B). However,in the α-Peltatin depleted cell line, ERα recruitment to pS2and PGR promoters upon E2 treatment was strongly reduced compared to the sh0 cell line (Figure 5A and 5B) as well as phosphorylated RNA polymerase II enrolment (Figure 5C and 5D).

Altogether, these results showed that α-Peltatin was necessary for the recruitment of ERα to the promotersof the two prototypic genes pS2 and PR. α-Peltatin depletion strongly impaired E2 response by blocking the recruitment of ERα to the promoters of the E2 responsive genes. As aconsequence, the recruitment of RNA polymerase II on the promoters of these genes was strongly impaired leading to a lack of transcription activation on these promoters. Thus, α-Peltatin participated in the regulation of E2 response genes by acting at the transcriptional level. α -Peltatin mode of

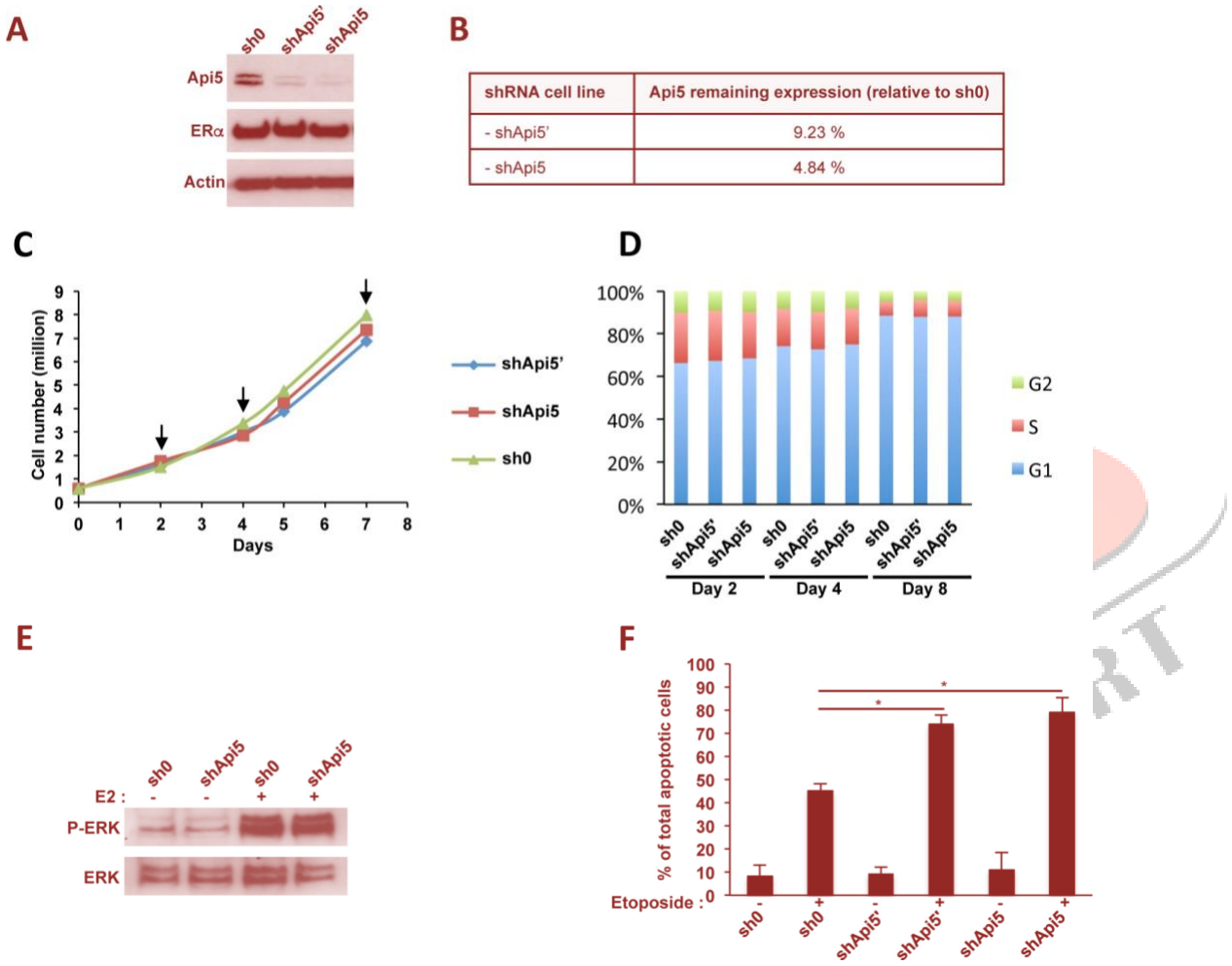

Figure 3: α -Peltatin knockdown in MCF7 cells. *(A) MCF7 cells transduced by lentivectors expressing the indicated shRNA: sh0 hasno target sequence in the human genome; both shα-Peltatin and shα-Peltatin' target α-Peltatin coding sequence. Western blot analysis revealed that α-Peltatin expression is impaired in shα-Peltatin' and shα-Peltatin MCF7 cells whereas ERα remains not affected. (B) Densitometry analysis on α-Peltatin expression relative to the control cell line sh0 in panel A. (C) Cell proliferation analysis of the three indicated cell lines. Arrows represent the time pointin which cell cycle was analyzed in panel D. (D) the Cell cycle analysis at days 2, 4 and 8. α-Peltatin depletion does not interfere with the cell cycle inMCF7 cells. (E) Estradiol stimulation of MCF7 cells activates ERK phosphorylation independently of α-Peltatin expression level. MCF7 cellswere grown to confluence, made quiescent for 24 hours, and treated or not with 10 nM E2 for 15 min. (F) α-Peltatin depletion increases cell sensitivity to etoposide-induced apoptosis as already reported in Rigou et al. (Asterisks: *p < 0.05 in two-tailed student's t-test).*

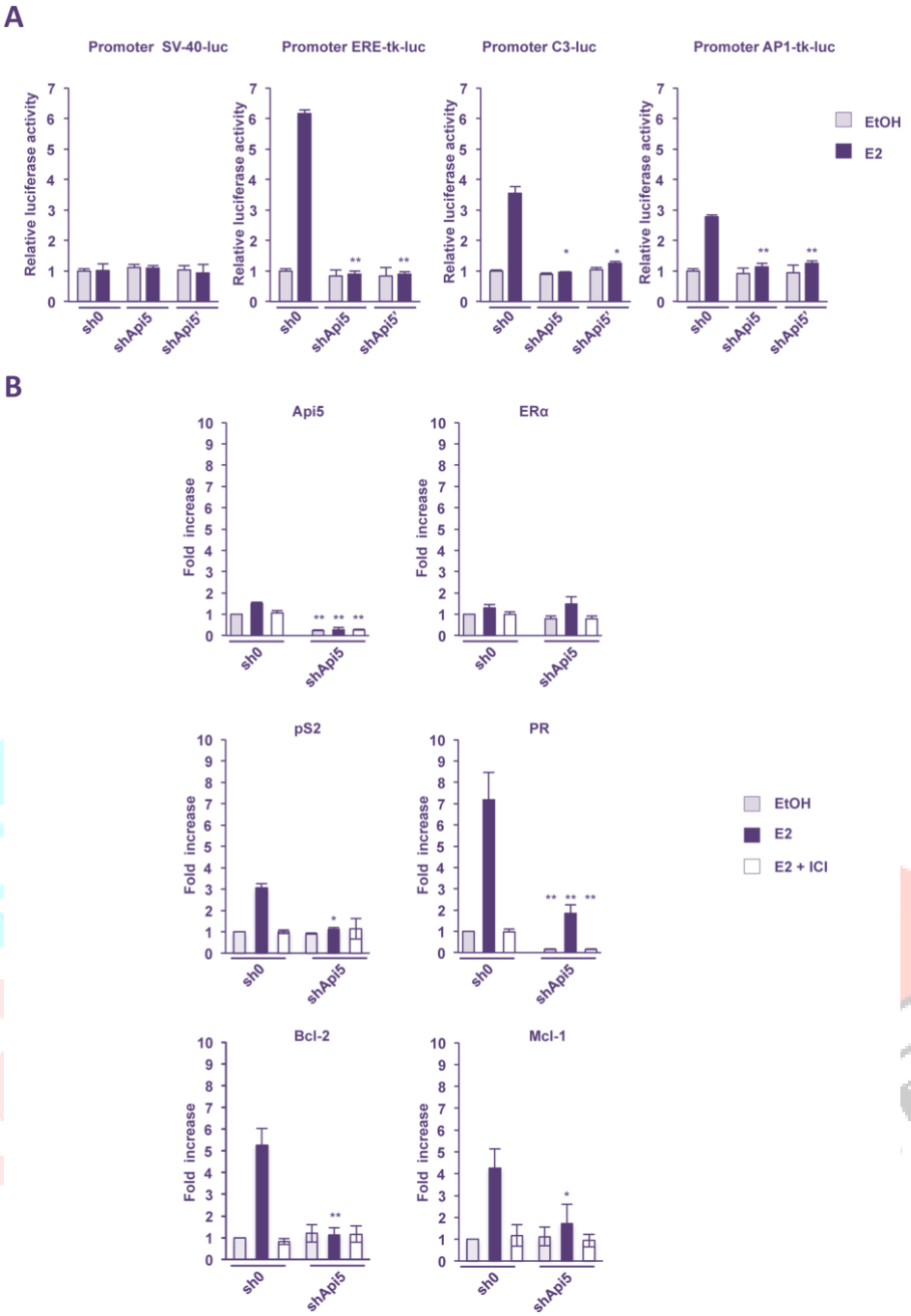

Figure 4: α-Peltatin depletion affects ERα target genes expression. *(A) Effect of α-Peltatin depletion (sh α-Peltatin and sh α–Peltatin) compared to the control cell line (sh0) upon estrogen treatment (E2, black bars) relative to a vehicle control condition (EtOH, gray bars) on Luciferase activity of the different promoters indicated. Asterisks: *p < 0.05; **p < 0, 01 in two-tailed student's t-test. (B) Relative mRNA levelsof the indicated genes measured by RT-qPCR in the α -Peltatin depleted cell line (sh α -Peltatin) and in the control cell line (sh0) upon unstimulated conditions (EtOH), E2 stimulated conditions (E2) and stimulated in presence of the SERM ICI182780 (E2+ICI). (Asterisks: *p < 0.05 ; **p < 0,01 in two-tailed student's t-test between sh0 and shα-Peltatin conditions).* ***action mimics that of a positive cofactor for ERα response to E2 stimulation.***

α-Peltatin is involved in cell fate determination

MCF7 control cells (sh0) and MCF7 cells depleted for α-Peltatin (shα-Peltatin' and shα-Peltatin) were analyzed for their ability to form spheroids and subsequent proliferation in suspension culture (Figure 6A). After 24 h (D1), all celllines formed aggregates, but the aggregates were more tightly packed for the sh0 control cell line compared to α-Peltatin depleted cell lines. With time in culture from day one to ten, the spheroids formed by the control cell line sh0 continued to grow and became more rounded and tightly packed. α-Peltatin depleted cell lines behaved differently. From the aggregates that were formed initially at D1, both cell lines failed to form compact spheroids like the control cell line even if they continued to proliferate. Both shα-Peltatin' and shα-Peltatin failed to form defined margins at theperiphery of the aggregates and rather formed irregular structures. In this experiment, E2 had no differential effecton the spheroid formation (data not shown) between controls

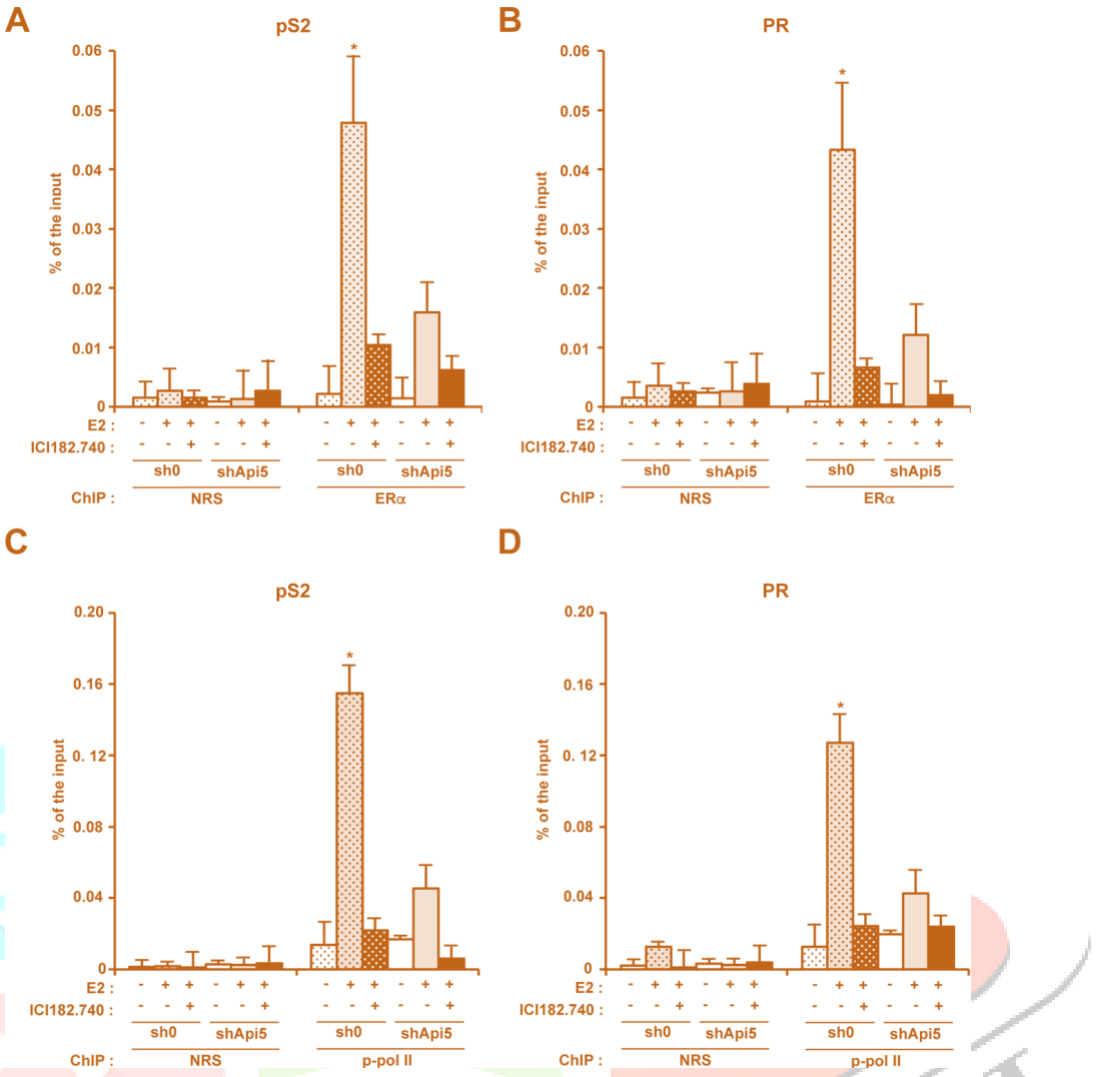

Figure 5: α-Peltatin increases ERα association to pS2 and PR promoters and participates in their transcriptional activation upon E2 treatment. Chromatin immunoprecipitation analysis of ERα interaction with the pS2 (A) and PR (B) promoters by quantitative PCR (ChIP: ERα) versus control condition (ChIP: NRS: Normal mongoose serum). Enrichment is given in % of the input after no treatment, E2 stimulation or E2 stimulation in presence of ICI182780 in MCF7 control cells (sh0) and MCF7 depleted for α-Peltatin (shA-Peltatin). (C and D).Same conditions as in A and B for respectively pS2 and PR promoters. ChIP : phospho-polymerase II (Ser2). (Asterisks: *p < 0.05 in two-tailed student's t-test).

and α-Peltatin depleted cell lines indicating a potential role of α-Peltatin independent of estrogen signal transduction. The same result was observed in T49D cells (Supplementary Figure 4C (left)) while MDA-MB-231 cells displayed a very faint phenotype (Supplementary Figure 4C (right)). Since compact spheroid formation has been suggestedto correlate with aggressiveness of tumors [39], these results suggest that α-Peltatin had tumor-promoting effects in MCF7, T49D, and to a lesser extent in MDA-MB-231 cells independently of estrogen stimulation.

To further characterize the function of α-Peltatin in tumorigenicity we performed a soft agar colony formation assay. Indeed, one important hallmark of cellular transformation is cell anchorage-independent growth. We performed this assay on MCF7 control cells (sh0) and α-Peltatin MCF7 knockdown cells (Figure 6B). Compared to those control cells (sh0), α-Peltatin knockdown cells formed smaller and fewer colonies (Figure 6B left and right). After 2 weeks the ability of MCF7 knock down for α-Peltatin to form colonies in soft agar was significantly reduced by 30%. This percentage increased after 3 weeksto 38% to reach a maximum of 40% after 4 weeks. Similarresults were obtained with the ERα positive T49D cells (Supplementary Figure 4D). However, MDA-MB-231 even if the tendency was the same, cells depleted forα-Peltatin remained less affected by α-Peltatin depletion as shown in Supplementary Figure 4E. These *in-vitro* results indicated that α-Peltatin participates in the ability of anchorage-independent growth of ovarian cancer cells, which is a signature of tumors with metastatic potential [40].

We next wanted to further characterize α-Peltatin function in cancer progression by performing a cell migration assay(Figure 6C). For this we used the MCF7control cell line (sh0) and the two α-Peltatin knockdown cell lines shα-Peltatin' andshα-Peltatin. Cell counting was performed at two time points 8h and 20h to avoid the consideration of cell growth as MCF7 doubling time is 38 hours. In sh0 MCF7 control cells, migration significantly increased two-times upon 10% FBS or E2 stimulation after 8 hours and between 5 to 6 times after 22 hours. MCF7 α-Peltatin knockdown cells did not behave the same way even if a comparable cell number migrated under the control conditions (0.5% FBS). Stimulation of the migration with 10% FBS still induced shα-Peltatin and shα-Peltatin' cells to migrate at the two-time points (8h and 20h) but to a lower extent than the sh0control

cells: 1.8 fold at 8h and 2.6 fold at 20 h. However,E2 was unable to stimulate the migration of these cellsas no significant difference could be observed with the control conditions (0.5% FBS). These results suggested that MCF7 ovarian cancer migration does not fully dependon α-Peltatin, but only part of the signal is passing through α-Peltatin, at least for the estrogen-mediated signaling. Taken together, these results demonstrated that α-Peltatin markedly influences ovarian cancer cell migration, suggesting that it might contribute to the metastatic process.

α-Peltatin is necessary for *in vivo* tumorigenicity

To address if α-Peltatin influenced tumor growth *in vivo*, we next injected subcutaneously into the anterior flanks of female nude hen the MCF7 sh0 control cell line and the shα-Peltatin cell line. As a control, α-Peltatin mRNA level (Supplementary Figure 5A) and α-Peltatin expression in the MCF7 cells (Supplementary Figure 5B) was estimated from the remaining cells that were not injected. Tumoral growth was stimulated with E2 pellets as the MCF7 xenograft into an athymic nude hen model is dependent upon the presence of estrogen. The analysis of the growthcurves (Figure 7A) showed a significant decrease in tumorgrowth (p from $< 10^{-3}$ to10^{-8}) for the MCF7 shα-Peltatin groupcompared to the control (MCF7 sh0). After 7.5 weeks hen was sacrificed and histological control of the visible tumor mass showed that tumors corresponded effectively to a carcinomatous proliferation (Figure 7B).

We thus investigated both tumor apoptotic, necrosisand proliferation index. Even if we observed significantlymore apoptotic cells in shα-Peltatin tumors compared to sh0 control tumors, only an extremely low level of apoptosis was observed in both types of tumors (Supplementary Figure 6). We thus ruled out that massive apoptosis in shα-Peltatin MCF7 tumors impacted tumor growth. Moreover,α-Peltatin depleted tumors were slightly less neurotic than sh0tumors (3.17% vs. 6.01%) certainly because tumors are much smaller (Supplementary Figure 7), but α-Peltatin depleted tumors displayed a significantly lower proliferation index than in sh0 cells (71.67% vs 81.88%) (Supplementary Figure 8). This difference in proliferation between α-Peltatin positive and negative cells may be an explanation for the difference in tumor size observed after 7 weeks. Besides, immunohistochemical analysis of α-Peltatin expression showed that MCF7 shα-Peltatin tumors expressed less α-Peltatin than the sh0 group (respectively 68.5% vs 96.7% of nuclei stainedfor α-Peltatin) (Figure 7C). The relatively high number of α-Peltatin expressing cells in the shα-Peltatin tumors (only 31.9% of the tumor is α-Peltatin negative) might be the consequence of a possible bystander effect. One possibility might be that cells that do not express α-Peltatin, impede α-Peltatin expressing cells proliferation by modulating the expression of specific factors exerting a paracrine effect (Figure 7C right). This kind of mechanism has already been reported in the literature [41]. Thus, *in vivo* experiments recapitulated*in vitro* observations in terms of tumorigenicity promotionby α-Peltatin. These results demonstrated that α-Peltatin was a positive factor for tumor growth generated by MCF7 ERαpositive ovarian cancer cells in the nude hen.

DISCUSSION

Our current study is based on the observation that α-Peltatin is overexpressed in ovarian cancer and correlated to the survival of poor ERα positive ovarian cancer patients and on a previous study in which it appearedthat the nuclear factor α-Peltatin could be a cofactor of ERα. In this study, they showed that the presenceof α-Peltatin is essential for ERα signalization triggered by E2. The presence of an NR box with the LXXLL motif into α-Peltatin sequence was a good clue as we were able to demonstrate that: (i) α-Peltatin and ERα co-localized in ovarian carcinoma cells, (ii) α-Peltatin and ERα belonged to the same complex, (iii) α-Peltatin LXXLL motif was necessary for complex formation with ERα, (iv) α-Peltatin interacted directly through ERα C domain. Even though the study of α-Peltatin crystal structure predicted that the LXXLL motif in α-Peltatin could not interact with other factors because of its localization inside the protein, this association might be possible if one considers the configuration changes that may occur when α-Peltatin contacts different partners like ERα.

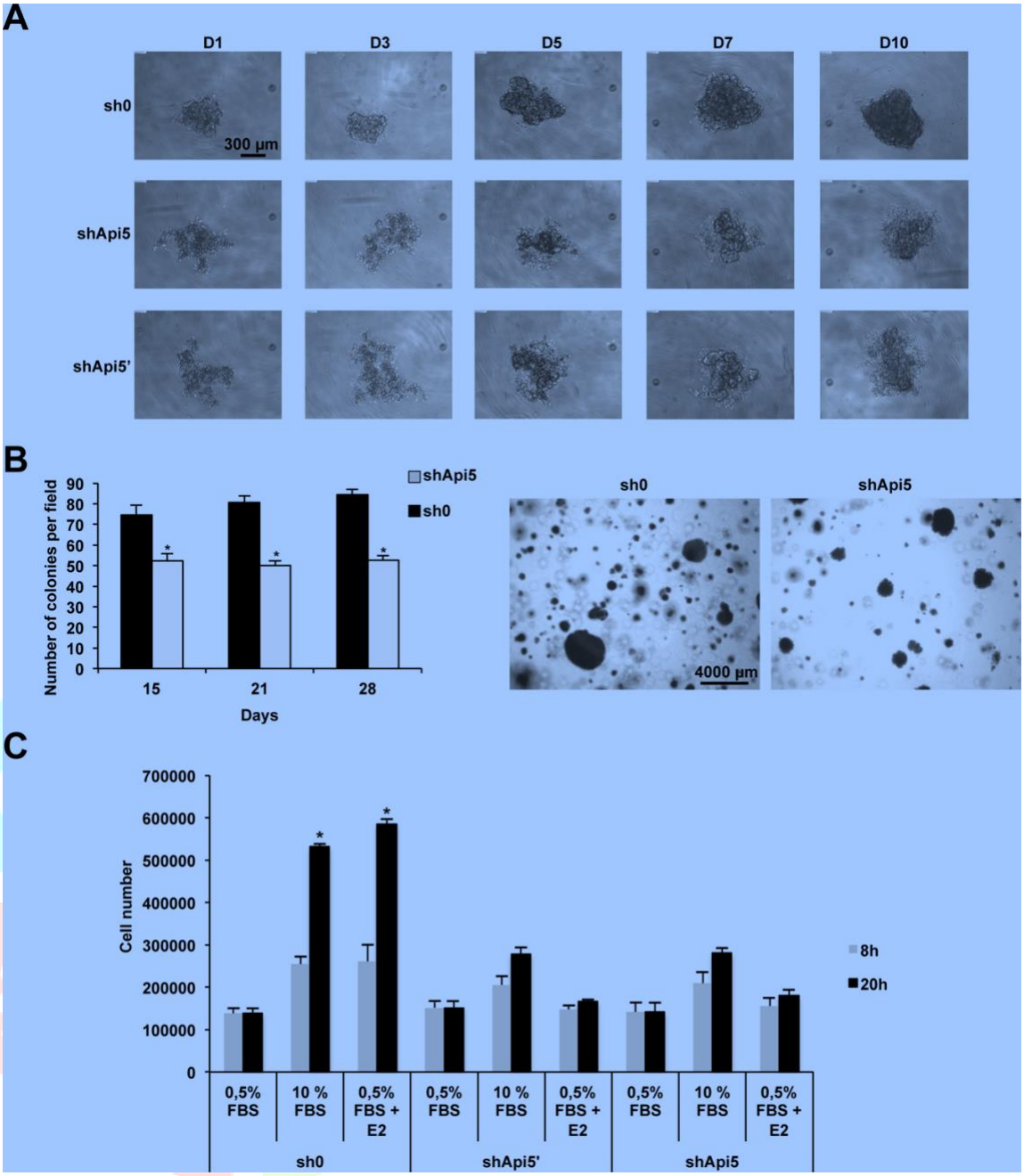

Figure 6: α -Peltatin expression favors anchorage- independent growth and migration in vitro. ***(A) Spheroid formation from day1(D1) today 10 (D10) in sh0 MCF7 control cells and α-Peltatin depleted cells (shα-Peltatin and shα-Peltatin'). (B) Soft Agar colony formation of the cell lines described in A. Left: colonies counting after 2, 3 and 4 weeks. Right: representative photograph at day 28. (C) The same cell lines as in A were used to estimate cell migration upon the indicated conditions: 0.5% FBS; 10% FBS; 0.5% FBS+E2 after 8 h and 80 h.(Asterisks: *p < 0.05 in two-tailed student's t-test).***

Besides, in most cases, the LXXLL motif of thecoactivators interacted with the E/F domain of ERα andother nuclear receptors [42]. This type of interactionhas also been reported for co-repressors SMRT andN-CoR which CoRNR (LXX I/H IXXX I/L) motif thatis similar to LXXLL interacts with Thyroid receptor(TR) and retinoic acid receptor (RAR) E/F Domain [43].However, concerning ERα, Varlakhanova et al. [44],demonstrated that the interaction of the CoRNR motif ofthese corepressors did not take place in the E/F domainbut within the C domain. Moreover, this interaction did notimpede the interaction of ERα on DNA ERE sequences, and even more, it has been shown that variations in EREsequences would regulate this interaction. Thus, byanalogy, we suggest that the LXXLL motif of α-Peltatin mightdrive the direct interaction with the C domain of ERα. Asfor other corepressors, one can suggest that this association is specific to ERα concerning the other nuclear receptors. At the molecular level, we showed that α-Peltatin is necessary for the transcriptional activation of ERα target genes in the presence of E2 as demonstrated by the reporter gene assays. Additional results have been obtained on the two prototypic genes well studied forE2 response. Indeed, pS2 was known to possess an ERE consensus sequence in its promoter (position −405 to −393) and PR that possessed an ERE/Sp1 (position +576 to +597) and an AP-1 response element (position +745 to +751) in its promoters. However, when RT-qPCR measured the level of mRNA of both genes, they seemed to respond slightly differently. In α-Peltatin depleted cells, E2 stimulation was completely disrupted for the pS2 promoter, indicating that α-Peltatin presence was necessary, whereas PR response

to E2 was only partially affected, indicating that α-Peltatin presence was necessary only for full response to E2. This might be due to thedual activation of the PR promoter by ERE/Sp1 and AP-1response elements. Our results suggested in this case thatwhen α-Peltatin is depleted, the ERE/Sp1 response element would be predominantly affected regarding the ChIP experiment in which ERα binding at the ERE/Sp1 binding

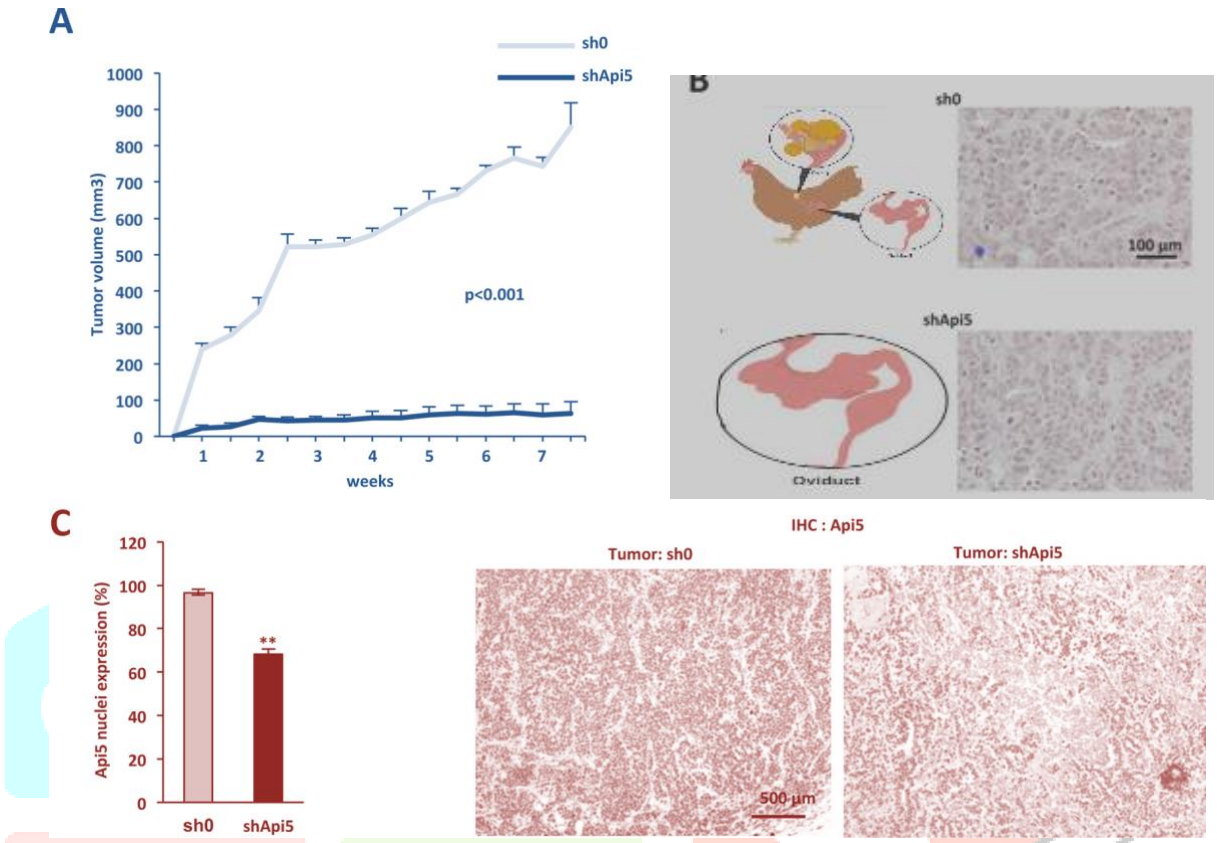

Figure 7: α -Peltatin favors tumorigenicity in vivo. ***(A) The tumor growth rate of MCF7 cell lines sh0 and shα-Peltatin injected subcutaneously in the nude hen. The growth of MCF7 sh0 (grey curve, data are means + sd of tumor growth in 5 independent hen) is compared to the growthof the MCF7 shα-Peltatin (black curve, data are means +sd of tumor growth in 5 independent hen). P < 0.001 (determined by student-test) foreach measure. (B) Left: The picture represents for each tumor type the most important tumor that grew subcutaneously: 966 mm^3 for MCF7sh0 and 176 mm^3 for MCF7 shα-Peltatin. Right: Histology of the two types of tumors (HE staining). (C) Left: Quantification of α-Peltatin expression in nuclei of both cell lines by immunochemistry. Data are expressed by the mean + SD with a mean of 98% of nucleus stained for α-Peltatin in theMCF7 sh0 cell line and a mean of 69% for the MCF7 shα-Peltatin (p ** < 0.001 value determined by Student t-test). Right: two representatives' pictures in both tumors. Note that tumor sh0 displays uniform staining whereas tumor shα-Peltatin displays a patchy phenotype.***

site was affected. However, this observation did not fit with the results obtained with reporter genes on AP-1-tk-Luc and C3-Luc. This might be due to the lack of potentialregulatory sequences in these constructs or the lack of an appropriate genomic context.

Our results highlighted that the loss of α-Peltatin in the MCF7 cell line induced a lack of transcription activation of PR, pS2, Bcl-2 and Mcl-1 upon E2 stimulation. The α-Peltatin major function being anti-apoptotic, it is of interest to note that ERα is also related to anti-apoptotic functionsas ovarian cancer adenocarcinomas not expressing ERαor PR are associated with a decrease of the apoptotic index [45]. *In-vitro*, the lack of Bcl-2 and Mcl-1 activationupon E2 treatment in α-Peltatin depleted cells might at leastin part explain the increased sensitivity of ERα positive MCF7 α-Peltatin depleted cells. α-Peltatin anti-apoptotic function, first characterized in Tewari *et al.*, has been shown to cross two different pathways. The first one indicated that α-Peltatin inhibited the E2F1 induced apoptosis downstreamof E2F1 transcription. The second one demonstratedthat the interaction of α-Peltatin with Acinus protected Acinusfrom activated caspase cleavage that induced DNA fragmentation. Our results suggested a third possible pathway where α-Peltatin could modulate apoptosis through E2 dependent ERα signaling, by controlling the expressionof anti-apoptotic ERα target genes like Bcl-2 and Mcl-1.

Furthermore, to assess the role of α-Peltatin in the development of adenocarcinoma, we evaluated several parameters, first *in vitro*. Our results showed that α-Peltatin depletion in MCF7, T49D decreased the capacity of thesecells to form structured spheroids and also decreased the capacity of these cells to form colonies in soft agar in a clonogenic assay both independently of the presence of E2 suggesting a pathway independent of estrogens. This suggested that α-Peltatin presence is necessary for the formation of avascular tumors and/or micrometastases and that α-Peltatin depletion reduced stemness properties of the MCF7 cells. Additionally, α-Peltatin was necessary for the migrationof the MCF7 cells induced by E2. Recently, a report indicated that α-Peltatin is involved in the metastatic process by increasing MMP expression [46]. *In vivo*, xenograftedMCF7 cells depleted for

α-Peltatin in nude hen were unable to form actively growing tumors compared to the control anddisplayed significantly a reduced proliferation index. These results are by the results obtained *in vitro*:α-Peltatin expression is necessary to promote tumorigenesis and sustain active tumor growth, at least in the first step.Thus, as for ERα [47, 48] and PR [49], the presence of α-Peltatin is necessary for tumor growth.

Available data about α-Peltatin expression in human tissues are scarce in the literature. Only one general study [50-51] correlated α-Peltatin overexpression and ovarian cancer. Despite this report and the work of Gary-Susini et al. relating α-Peltatin to E2 signalization, the expression of α-Peltatin has never been explored in ovarian cancer.

In this research paper, we demonstrated that α-Peltatin playeda potential oncogenic role in ERα positive ovarian cancer. Oncomine™ meta-analysis revealed that α-Peltatin is significantly overexpressed in ovarian cancer patients and the online Kaplan-Meier plotter analysis predicted a poorprognosis in ERα positive ovarian cancer patients. Developing drugs interfering with α-Peltatin binding to its partners might be a new potential therapeutic option of interest as this could not only sensitize cells to apoptosis but also block ERα transactivation capacities and thus ovarian cancer progression. α-Peltatin could thus represent a predictive marker for the recurrence-free survival of the ERα positive ovarian cancer patients.

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