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Abscisic acid signaling through LANCL2 and PPARγ induces activation of p38MAPK resulting in dormancy of prostate cancer metastatic cells

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Abstract. Prostate cancer (PCa) is one the most common malignancies in men. The high incidence of bone metastasis years after primary therapy suggests that disseminated tumor cells must become dormant, but maintain their ability to proliferate in the bone marrow. Abscisic acid (ABA) is a stress response molecule best known for its regulation of seed germination, stomal opening, root shoot growth and other stress responses in plants. ABA is also synthesized by mammalian cells and has been linked to human disease. The aim of the present study was to examine the role of ABA in regulating tumor dormancy via signaling through lanthionine synthetase C-like protein 2 (LANCL2) and peroxisome proliferator activated receptor γ (PPARγ) receptors. ABA signaling in human PCa cell lines was studied using targeted gene knockdown (KD), western blotting, quantitative PCR, cell proliferation, migration, invasion and soft agar assays, as well as co-culture assays with bone marrow stromal cells. The data demonstrated that ABA signaling increased the expression of p21, p27 and p16, while inhibiting viability, migration, invasion and colony size in a reversable manner without toxicity. ABA also induced p38MAPK activation and NR2F1 signaling. Targeted gene KD of LANCL2 and PPARγ abrogated the cellular responses to ABA. Taken together, these data demonstrate that ABA may induce dormancy in PCa cell lines through LANCL2 and PPARγ signaling, and suggest novel targets to manage metastatic PCa growth.

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Introduction

Prostate cancer (PCa) is the second leading cause of death from cancer in men in the United States (1). The majority of men who are diagnosed with localized PCa and who elect to undergo therapy are treated with surgery or radiation, by which they expect to be cured (2). A number of patients experience a prolonged period without evidence of biochemical recurrence or obvious disease, but some relapse with single or multiple metastases frequently detected in bone (3,4). Notably, ~90% of patients who succumb to advanced-stage PCa have bone metastases, whereas only ~10% of these individuals had bone metastases at the time of diagnosis (5-7). These observations suggest that disseminated tumor cells (DTCs) may have left the primary tumor site in the prostate and took up residence in distant sites. For PCa, it has been shown that DTCs may be present in the bone marrow at the time of initial diagnosis (8-11). In the bone marrow, DTCs maintain the ability to become reactivated and generate metastases by poorly understood processes (8-11). Further studies are needed in the context of PCa DTC dormancy to identify and develop improved therapeutic strategies to treat metastatic diseases.

One well-delineated pathway in the understanding of cellular dormancy is p38/ERK signaling. High levels of p38MAPK activity function as an inhibitory regulator of ERK, thus preventing cell proliferation by inducing G_0/G_1 phase arrest, or by initiating senescence and apoptosis (12-14). Transforming growth factor-β2 (TGF-β2), which is secreted from bone marrow-derived cells, is known to induce expression of a $p38^{high}/ERK^{low}$ phenotype in cancer (15). The subsequent activation of Smad1/5 and increased expression of p27 results in the downregulation of cyclin-dependent kinase (CDK)4, which collectively facilitates the transition into cellular quiescence (15,16). Additionally, $p38^{high}/ERK^{low}$ facilitates G_0/G_1 phase arrest via the regulation of various factors, including NR2F1, and CDK inhibitors p27 and p21. Therefore, the combined regulation of transcription factors by a $p38^{high}/ERK^{low}$ phenotype is responsible for quiescence (17). A second well-described pathway that regulates PCa dormancy in the bone marrow is activated by growth arrest-6 (GAS6) signaling. In bone marrow, GAS6 secreted by osteoblasts activates the TAM family of receptors (Tyro3, Axl and MerTK)

on PCa cells, which serve as molecular switches to turn on (Tyro3, Axl) and off (MerTK) the dormancy programs (18). Similarly, bone morphogenetic protein (BMP)7, which is produced by bone stromal cells, induces dormancy in PCa cells by activating p38 signaling (19). These studies have suggested that cells in the bone marrow microenvironment, including osteoblasts, serve key roles in regulating PCa dormancy.

The present study explored the signaling pathways activated by the dormancy-inducing phytohormone ABA in PCa cells. ABA has long been known as a regulator of plant growth and survival under stress conditions, but has more recently been detected in rat brain tissues (20). Since its discovery in mammals, ABA has been identified in blood plasma (21), and has been demonstrated to be produced by pancreatic β cells (22), adipocytes (21), keratinocytes (23), hematopoietic immune cells, such as granulocytes (24), monocytes (25) and macrophages (26), and human mesenchymal stem cells (27). ABA has also been shown to be linked to several human diseases, including type-2 diabetes and colitis (28,29). ABA signaling is mediated by two known receptors, lanthionine synthetase C-like protein 2 (LANCL2) and peroxisome proliferator activated receptor γ (PPARγ) (28-30). Numerous studies have demonstrated that LANCL2 serves a role in the regulation of stress responses, inflammation, and in metabolic and immune-related diseases (31,32). It has also been demonstrated that LANCL2 is expressed by DTCs in the bone marrow of patients with breast cancer (30). PPARγ is a nuclear hormone receptor, the signaling of which results in anti-proliferative activities by decreasing the levels of cyclin D1 and E, and increasing the expression of p21 and p27 in PCa (33-35). The present study further explored the role of ABA in dormancy and the molecular pathways activated by its signaling in PCa.

Materials and methods

Reagents. A number of the reagents used, their source and catalogue numbers are presented in Table I; short hairpin RNA (shRNA) targeting sequences are presented in Table II; primer sequences are presented in Table III.

Cell culture. As the present study aimed to study bone marrow DTCs, the following metastatic cell lines were used. The human androgen-independent bone metastatic PCa cell lines PC3 and LnCaP subline C4-2B were predominantly used in the present study, as they metastasize to bone (36-38). When injected into bone, PC3 cells result in predominantly lytic lesions, whereas C4-2B cells may produce mixed osteoblastic and osteolytic lesions (36-38). In some experiments, the human dura metastasis cell line DU145 and murine Myc-CaP cells were used. We recently demonstrated that Myc-CaP cells can also metastasize to bone (39). The human PCa cells were routinely grown in RPMI 1640, whereas the murine Myc-CaP cells were grown in DMEM. All cultures were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin, and were maintained in an incubator set to 5% CO_2 atmosphere at 37˚C (Marshall Scientific).

Cell viability assays. To evaluate viability, 3,000 PCa cells/well (PC3, C4-2B, Du145 and Myc-CaP) in 100 μl medium were added to 96-well plates in quintuplicate, and allowed to adhere overnight. The next day, the cells were washed in PBS (Thermo Fisher Scientific, Inc.) and fresh medium was added. ABA was diluted in dimethyl sulfoxide (DMSO), and was added at the following doses: 0 μM (DMSO only), 25, 50, 100 and 200 μM for 72 h at 37˚C. To determine viability, cells were either recovered from culture with trypsin (MilliporeSigma) and counted using a hemocytometer under a Nikon Eclipse Ts2 inverted phase light microscope (Nikon Corporation) following trypan blue staining (at 23˚C for 3 min), or were assessed using a colorimetric assay. For the colorimetric assays, a total of 70 h after the addition of ABA or control, 20 μl CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Reagent (cat. no. G5421; Promega Corporation) was added to the cultures for 2 h at 37˚C. The optical density (O.D.) of the cell culture plates was then measured using a plate reader (DU530 UV/Vis scanning spectrophotometer; BD Biosciences) at a wavelength of 490 nm. To determine if ABA permanently affected viability, cells were treated with ABA for 72 h at 37˚C, washed, trypsinized and counted. An equal number of cells ($1x10^5$ cells/well in 12-well plates, 1 ml of culture media) were then added back to culture for an additional 72 h at 37˚C and cell viability was evaluated by cell counting using a hemocytometer. Cell viability (%) was calculated as follows: (treatment group-background)/(control group-background)x100. The data are presented as the mean ± SEM.

Cytotoxicity assays. The cytotoxicity of ABA was assessed using a CytoTox96 Non-Radioactive Cytotoxicity Assay kit (Promega Corporation) according to the manufacturer's instructions. Briefly, PCa cell lines (PC3, C4-2B, DU145 and Myc-CaP) were seeded on 96-well plates ($1x10^4$ cells/well) in quintuplicate per group in 100 μl complete culture medium. After overnight incubation, the cells were treated with 25, 50, 100 and 200 μM ABA for 4 h at 37˚C; the control group was treated with DMSO. To measure lactate dehydrogenase (LDH) release, 50 μl supernatant was mixed with 50 μl reconstituted substrate mix solution in a new 96-well plate and was incubated for 30 min in the dark at room temperature. The reaction was stopped with 50 μl stop buffer and the O.D. was then measured at a wavelength of 490 nm. Each cytotoxicity assay was independently repeated two times.

shRNA and lentivirus preparation. pLKO.1 lentiviral vector-based shRNAs targeting specific candidate genes and the non-specific (NS) control shRNA were obtained from Horizon Discovery (NS control and shRNA LANCL2) and MilliporeSigma (shRNA PPARγ) (Table II). Lentivirus particles were prepared by transfecting 293T cells in 12-well plates with 0.5 μg either gene-specific shRNA plasmids (LANCL2, clone ID no. TRCN0000045403; and PPARγ, clone ID no. TRCN0000001670) or NS shRNA plasmids along with lentiviral packaging plasmids [2nd generation psPAX2 packaging (plasmid #12260; Addgene, Inc.) and pMD2.G envelope (plasmid #12259; Addgene, Inc.) in a 1:1 ratio for 48 h at 37˚C. All lentiviral transfections were performed using Effectene Transfection Reagent. Stable cell lines were generated by infecting PCa cells (PC3 and C4-2B) with a multiplicity of infection of ~250 viral particles per cell in 200 μl (total collection volume, 1 ml) in 12-well plates for 24 h, followed by selection in puromycin (1 μg/ml) at 37˚C for 1 week. Thereafter reverse

Table I. Reagents used in the present study.

A, Animals

Reagent or resource	Supplier	Identifier
Male 4-6 week old C57BL/6J mice	Jackson Laboratory	Strain no. 000664

B, Antibodies

Reagent or resource	Supplier	Identifier
β-actin	Cell Signaling Technology, Inc.	4970
Tubulin	ABclonal Biotech Co., Ltd.	AC021
p21	Cell Signaling Technology, Inc.	2947
p27	Cell Signaling Technology, Inc.	3686
LANCL2	Abcam	ab237520
PPARγ	Cell Signaling Technology, Inc.	2443
p-p38	Cell Signaling Technology, Inc.	4511
t-p38	Cell Signaling Technology, Inc.	9212
NR2F1	Abcam	ab181137

C, Chemicals, peptides and recombinant proteins

Reagent or resource	Supplier	Identifier
DMEM	Cytiva	SH30022.01
RPMI	Cytiva	SH30027.01
α-MEM	Gibco; Thermo Fisher Scientific, Inc.	12571-063
Fetal bovine serum	Gemini Bio Products	50-753-2984
0.25% Trypsin-EDTA	Gibco; Thermo Fisher Scientific, Inc.	25200-056
Penicillin-streptomycin	Gibco; Thermo Fisher Scientific, Inc.	15140-122
Effectene transfection reagent	Qiagen, Inc.	301425
ABA	Abcam	ab120860

D, Experimental cell lines

Reagent or resource	Supplier	Identifier
293T	ATCC	CRL-3216
PC3	ATCC	CRL-1435
C4-2B	ATCC	CRL-3315
DU145	ATCC	HTB-81
Myc-CaP	ATCC	CRL-3255

LANCL2, lanthionine synthetase C-like protein 2; p-, phosphorylated; PPARγ, peroxisome proliferator activated receptor γ; t, total.

transcription-quantitative PCR (RT-qPCR) was used to assess the mRNA expression changes and western blotting was used to validate protein expression changes. After the initial assessment of the efficiency of shRNA knockdown (KD) of the target genes, clone 1 was selected for both LANCL2 and PPARγ for further studies. Thereafter, frozen stocks were established and cells were used for experimentation after validation of the targeted KD within 2 weeks of transfection or thaw.

RNA extraction, cDNA preparation and RT-qPCR. Total RNA was extracted from target cells using TRIzol® Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and was purified using the RNeasy Mini Kit (Qiagen, Inc.). cDNA was synthesized using the ProtoScript First Strand cDNA Synthesis Kit (New England Biolabs, Inc.) according to the manufacturer's instructions. qPCR was performed with gene-specific primers using the iTaq Universal SYBR® Green Supermix (Bio-Rad

Table II. Short hairpin RNA sequences.

Gene/shRNA	Clone	ID sequence	Sequence, 5'-3'	Supplier
Nonspecific sequence	-	#RHS6848	-	Horizon Discovery
LANCL2	1	TRCN0000045403	TAAGGCATACAGATAACCTGC	Horizon Discovery
LANCL2	2	TRCN0000045404	AAATTATGAATGATCTTCCCG	Horizon Discovery
PPARγ	1	TRCN0000001670	GCCAACATTTCCCTTCTTCCA	MilliporeSigma
PPARγ	2	TRCN0000001671	CTGGCCTCCTTGATGAATAAA	MilliporeSigma

LANCL2, lanthionine synthetase C-like protein 2; PPARγ, peroxisome proliferator activated receptor γ.

Table III. Primer sequences.

Gene	Sequence, 5'-3'
ACTB	F: TCAGGACGGGAAGATCATTCA
	R: CAGAGCAGTCATGGGGATCAG
LANCL2	F: TCAGGACGGGAAGATCATTCA
	R: CAGAGCAGTCATGGGGATCAG
PPARγ	F: GGGATCAGCTCCGTGGATCT
	R: TGCACTTTGGTACTCTTGAAGTT
p21	F: TGTCCGTCAGAACCCATGC
	R: AAAGTCGAAGTTCCATCGCTC
p27	F: AACGTGCGAGTGTCTAACGG
	R: CCCTCTAGGGGTTTGTGATTCT
p16	F: GATCCAGGTGGGTAGAAGGTC
	R: CCCCTGCAAACTTCGTCCT
E-cadherin	F: CGAGAGCTACACGTTCACGG
	R: GGGTGTCGAGGGAAAAATAGG
Vimentin	F: GACGCCATCAACACCGAGTT
	R: CTTTGTCGTTGGTTAGCTGGT
ZEB2	F:CAAGAGGCGCAAACAAGCC
	R: GGTTGGCAATACCGTCATCC

LANCL2, lanthionine synthetase C-like protein 2; PPARγ, peroxisome proliferator activated receptor γ.

Laboratories, Inc.) on an Applied Biosystems 7500 thermocycler system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: Initial denaturation at 95˚C for 15 min; 40 cycles at 95˚C for 15 sec, annealing at 60˚C for 30 sec and 72˚C for 30 sec; and a final extension step at 72˚C for 7 min. Results were normalized against β-actin levels using the formula ΔCq=Cq of target gene-Cq of β-actin. The mRNA expression levels of the control group were used to establish the baseline; therefore, ΔΔCq was calculated using the formula ΔΔCq=ΔCq of target gene-ΔCq of the baseline. The fold change of mRNA expression was calculated as fold=$2^{-\Delta\Delta Cq}$ (40). The primer sequences for all the genes analyzed in the present study are provided in Table II.

Migration and invasion assays. Migration assays were performed using a Transwell with 8.0 μm pore polycarbonate membrane inserts (cat. no. 3422; Corning, Inc.), and invasion assays were performed using BioCoat Growth Factor Reduced Matrigel Invasion Chambers (cat. no. 354483; Corning, Inc.) in Transwell inserts. For these studies, PC3 and C4-2B cells infected with NS, LANCL2 and PPARγ shRNAs were serum-starved for 6 h, and were then recovered from culture and seeded in triplicate in the upper chamber of Transwell inserts ($5x10^4$ cells/well). The lower chambers were filled with medium containing 10% FBS as a chemoattractant. The cells in the upper chambers were treated with 100 μM ABA or DMSO as a control at 37˚C. After 24 h for PC3 cells and 48 h for C4-2B cells, the cells remaining in the top chambers (those cells that had not migrated or invaded) were removed with cotton-tipped applicators. The number of cells that had migrated or invaded into the lower chambers were quantified following DAPI staining at 23˚C for 3 min; 5-8 fields/membrane were evaluated under an All-in-one Fluorescence Microscope (BZ-X800/BZ-X810; Keyence Corporation; x10 magnification) and nuclei quantification was performed using ImageJ 1.53K software (National Institutes of Health). The percentage of migration or invasion was calculated as follows: (number of cells that migrated or invaded in the treated group)/(number of cells that migrated or invaded in the control group) x100.

Soft agar assays. PC3 and C4-2B cells ($5x10^3$/well) infected with LANCL2, PPARγ or NS shRNAs were seeded into 6-well plates on 0.4% low gelling point agarose (cat. no. A9045; MilliporeSigma) and layered on top of 0.8% agarose. The cultures were maintained for 15-18 days, with fresh medium replenished every third day containing DMSO (control) or 100 μM ABA. At the end of the experiment, the images of the cell colonies (>30 cells) in soft agar were captured using an inverted light microscope (Olympus Corporation; x10 magnification). Colony size was measured using ImageJ 1.53K software (National Institutes of Health) plotted as relative colony size (%) when compared with control cells.

Western blot analysis. Whole cell protein extracts were prepared using RIPA lysis buffer (Pierce; Thermo Fisher Scientific, Inc.) containing a Protease Inhibitor Cocktail (Roche Diagnostics) and Phosphatase Inhibitor (Thermo Fisher Scientific, Inc.). Lysed samples were centrifuged at 18,000 RCF for 15 min at 4˚C, and the clarified supernatants were collected and stored at -80˚C. Protein concentrations were determined using the Bradford Protein Assay Reagent (Bio-Rad Laboratories Inc.). Equal amounts of protein samples (25 μg) were separated by 10 or 12% SDS-PAGE and transferred onto polyvinylidene

difluoride membranes (Thermo Fisher Scientific, Inc.) using wet-transfer apparatus (Bio-Rad Laboratories, Inc.). The membranes were blocked with 5% skim milk (AmericanBio) and probed with primary antibodies (1:1,000 dilution) overnight at 4˚C. After washing with TBST buffer (25 mM Tris-HCl, 125 mM NaCl, 0.1% Tween 20), the membranes were incubated with IRDye®800CW- or IRDye®680-conjugated secondary antibodies (cat. nos. 926-32211 and 926-68071; 1:5,000 dilution; LI-COR Biosciences) for 1 h at room temperature. The results were visualized using an Odyssey Infrared Imager (LI-COR Biosciences). For loading controls, the membranes were stripped and reprobed for β-actin and/or tubulin. All primary antibodies used for western blotting are listed in Table I.

Ethics approval and consent to participate

Animal studies. The University of Alabama at Birmingham (UAB) Institutional Animal Care and Use Committee (IACUC) (Birmingham, AL, USA), approved the work (approval no. IACUC-21928). Male C57B/6J mice (age, 4-6 weeks; weight, 22 g) were housed for 2-3 weeks to permit acclimatization and were monitored daily. The animals were housed at 25˚C, under a 12-h light/dark cycle, with *ad libitum* access to food and water at 50% relative humidity. The ~22 g animals (n3) were exposed to 5% isoflurane for induction of anesthesia. Thereafter, the animals were maintained in 3% isoflurane until cardiac puncture and removal of the required blood volume (1.0 ml) was performed. To ensure euthanasia, the heart was removed. The long bone marrow of three animals was isolated by sterile dissection, and the cells from three animals were pooled.

Human studies. The studies were evaluated by the UAB Human Subjects Committee under IRB-300004457 'The Biology of Prostate Cancer Skeletal Metastases'. Given that no subject interactions were planned, and cell lines were to be purchased from commercial vendors, the studies were not deemed Human Subjects investigations and were therefore considered exempt.

Co-cultures of PC3-GFP cells with bone marrow cells and fluorescence-activated cell sorting (FACS) analyses. Murine primary bone marrow stromal cells were isolated by crushing the long bones from ~2-month-old male C57BL/6J mice. The cell clumps and debris were removed by filtering the cells using a 70-μm cell strainer (Thermo Fisher Scientific, Inc.). PC3 cells infected with GFP-labeled LANCL2 and PPARγ shRNA lentiviral vectors were placed into co-culture with confluent mouse primary bone marrow cells. Co-culture of PC3-GFP and bone marrow cells, with and without 100 μM ABA treatments were carried out for 3 days in an atmosphere containing 5% CO_2 at 37˚C. The proliferating PC3-GFP cells in the co-cultured (PC3-GFP/bone marrow) system were recovered using trypsin in PBS, and sorted using a BD FACS Melody™ Cell Sorter (BD Biosciences) to distinguish PC3 cells from murine bone marrow cells based upon GFP expression. To distinguish live versus dead cells or cellular debris, parallel cultures were used to establish gating parameters. A minimum of 20,000 events were recorded for each condition. The data were analyzed using BD FACSChorus™ 3.0 software (BD Biosciences). Cell proliferation percentages were established by comparing to control cells, and histograms were plotted using GraphPad Prism 5 (Dotmatics).

Statistical analyses. GraphPad Prism 5 was used for statistical analyses. All experiments were repeated at least three times and the results are presented as the mean ± SEM. For each data point, a two-tailed, unpaired, Student's t-test was performed to determine the significance of the differences between two groups, and one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test was performed to compare three or more groups. When analyzing multiple variables a two-way ANOVA analysis followed by Bonferroni's post hoc test was performed to determine significance. $P<0.05$ was considered to indicate a statistically significant difference.

Results

ABA treatment inhibits PCa cell viability. To determine the effects of ABA on PCa DTC viability, human and mouse PCa cells were exposed to increasing doses of ABA (0, 25, 50, 100 and 200 μM) for 72 h in culture medium. ABA significantly reduced the viability of the PCa cell lines in a dose-dependent manner (Fig. 1A-D). Specifically, ABA at concentrations of 100 and 200 μM decreased the viability of the PC3, C4-2B, DU145 and Myc-CaP cells by ~23, 33, 26 and 25%, and by ~24, 52, 36 and 48% compared with the control groups, respectively (Fig. 1A-D).

To determine if the inhibition of viability by ABA was reversible, a pulse-chase type of investigation was performed. Here, the metastatic PCa cells were treated with vehicle or ABA (100 μM) for 72 h. After the initial culture, the cells were washed, trypsinized and secondary cultures were established without additional ABA treatment. To account for the differences in viability that ABA induces, the secondary cultures were established with equal numbers of viable cells. As expected, the ABA-treated PC3 and C4-2B primary cultures had fewer cells relative to the vehicle-treated cultures (Fig. 1E and F). In the secondary cultures, the cells grew equally as well, regardless of their prior exposure in primary culture to ABA, thus demonstrating that the ABA-induced inhibition of viability was reversable (Fig. 1E and F). Notably, it was determined that ABA did not induce cell death of any of the PCa cells using a non-radioactive cytotoxicity assay to measure LDH release (Fig. 1G). These data collectively suggested that ABA induces reversible arrest of metastatic PCa cells.

ABA inhibits PCa cell viability by signaling through LANCL2 and PPARγ. To assess the molecular mechanisms by which ABA signaling influences PCa viability, stable KD of the ABA receptors LANCL2 and PPARγ were performed in metastatic PCa cell lines using shRNAs. To evaluate the efficiency of the KD, LANCL2 or PPARγ mRNA expression levels were confirmed by RT-qPCR (Fig. 2A-D) and protein expression levels were detected using western blotting (Fig. 2E-H). The targeting sequence #1 for LANCL2 and PPARγ were selected for all subsequent studies. After validation of the KD, the sensitivity of PC3 and C4-2B cells to ABA *in vitro* was assessed using cell viability assays. shRNA KD of LANCL2 or PPARγ conferred partial resistance to ABA in both PC3 and C4-2B cells (Fig. 2I and J).

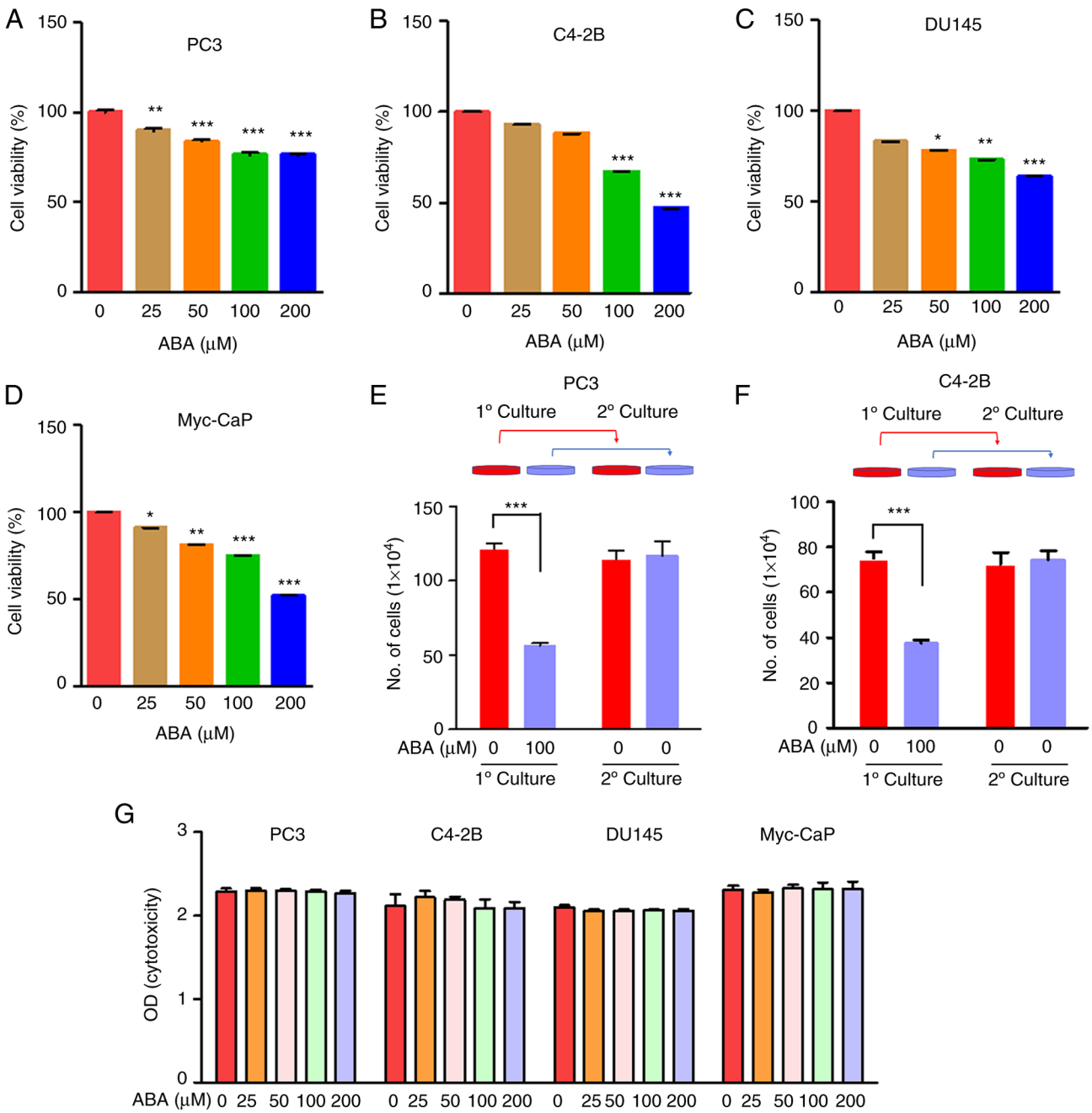

Figure 1. ABA inhibits PCa cell viability. (A) PC3, (B) C4-2B, (C) DU145 human and (D) Myc-CaP mouse PCa cells were plated in 96-well plates, and were treated with 0, 25, 50, 100 and 200 μM ABA for 72 h in quintuplicate. Changes in cell density were determined colorimetrically using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay. Data are presented as the mean ± SEM. Significance was calculated by one-way ANOVA followed by Bonferroni's post hoc test (*P<0.05, **P<0.01, ***P<0.001 vs. control). (E) PC3 and (F) C4-2B cells were seeded in 12-well plates and treated with 100 μM ABA for 72 h. Viable cell numbers were assessed using trypan blue exclusion staining and a hemocytometer. To determine if ABA-induced inhibition of viability was reversible, ABA-treated and control cultures were established. After 72 h, the cells were recovered from culture and equal numbers of cells were plated in secondary cultures for an additional 72 h without ABA supplementation and were quantified. Data are presented as the mean ± SEM. Significance was calculated by one-way ANOVA followed by Bonferroni's post hoc test (***P<0.001). (G) Cytotoxicity assays on PCa cells were conducted by treating them with ABA at concentrations of 0, 25, 50, 100 and 200 μM for 4 h, and subsequently assessing toxicity using the CytoTox96 Non-Radioactive Cytotoxicity Assay to detect lactate dehydrogenase release into the medium. ABA, abscisic acid; O.D., optical density; PCa, prostate cancer.

As a surrogate assay for estimating *in vivo* tumorigenesis, anchorage-independent growth in soft-agar assays was performed to evaluate the effects of ABA and its signaling through its receptors (41,42). ABA significantly decreased the ability of PCa PC3 and C4-2B cells to form colonies in the NS groups; however, when LANCL2 or PPARγ expression was downregulated by shRNA, ABA treatment did not affect colony formation in soft agar (Fig. 3A-D). These observations suggested that LANCL2 or PPARγ are required for ABA signaling to induce anchorage-independent growth. Given the functional link between colony formation and cancer-stem cell (CSC) activities (43), these data also indicated that ABA, and its signaling though its receptors, may downregulate CSC proliferation; however, the impact on CSCs was not formally tested.

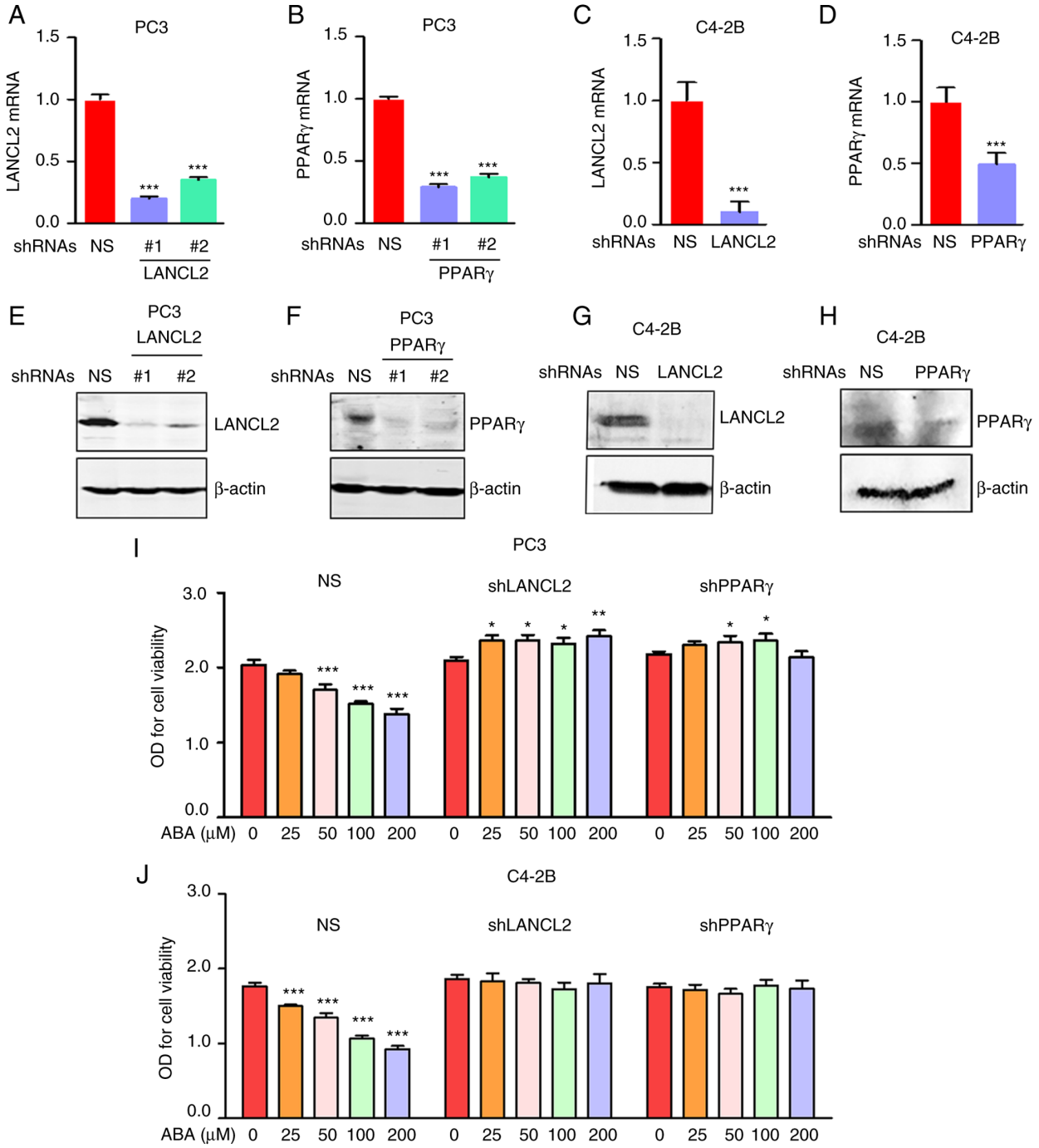

Figure 2. ABA inhibits PCa cell viability through LANCL2 and PPARγ. For all studies, PCa cells were infected with NS shRNA, or shRNAs targeting (A) LANCL2 or (B) PPARγ for PC3 cells, and (C) LANCL2 or (D) PPARγ for and C4-2B cells. The cells were then analyzed for LANCL2 and PPARγ mRNA expression by reverse transcription-quantitative PCR. Expression levels of LANCL2 and PPARγ mRNA were plotted relative to those in the NS group. β-actin was used for normalization. Data are presented as the mean ± SEM. (A and B) Significance was calculated by one-way ANOVA followed by Bonferroni's post hoc test (***P<0.001 vs. shRNA NS). (C and D) Significance was calculated using a two-tailed, unpaired Student's t-test, (***P<0.001 vs. shRNA NS). (E) LANCL2 and (F) PPARγ expression in PC3 cells, and (G) LANCL2 and (H) PPARγ expression in C4-2B cells were analyzed via western blotting after SDS-PAGE and blot transfer to membranes with specific antibodies to the receptors. β-actin was used as a loading control. (I) Viability of PC3 and (J) C4-2B cells infected with NS shRNA, or shRNAs targeting LANCL2 or PPARγ, and treated with ABA (0, 25, 50, 100 and 200 μM) for 72 h. Cell density was enumerated colorimetrically using CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay Kit. Data are presented as the mean ± SEM. Significance was calculated by one-way ANOVA followed by Bonferroni's post hoc test (*P<0.05, **P<0.01, ***P<0.001 vs. control). ABA, abscisic acid; LANCL2, lanthionine synthetase C-like protein 2; NS, non-specific; O.D., optical density; PCa, prostate cancer; PPARγ, peroxisome proliferator activated receptor γ; shRNA, short hairpin RNA.

ABA inhibits PCa cell migration and invasion through LANCL2 and PPARγ receptors. Given the effects of ABA on viability and colony formation, the impact of ABA on PCa invasion was next evaluated. For these studies, the migration of cells treated with or without ABA was detected in response to FBS as a chemoattractant in Transwell plates. ABA decreased the invasion of PCa cells, whereas targeted KD of LANCL2 or PPARγ expression reduced the effects of ABA treatment on migration (Fig. 4A-D).

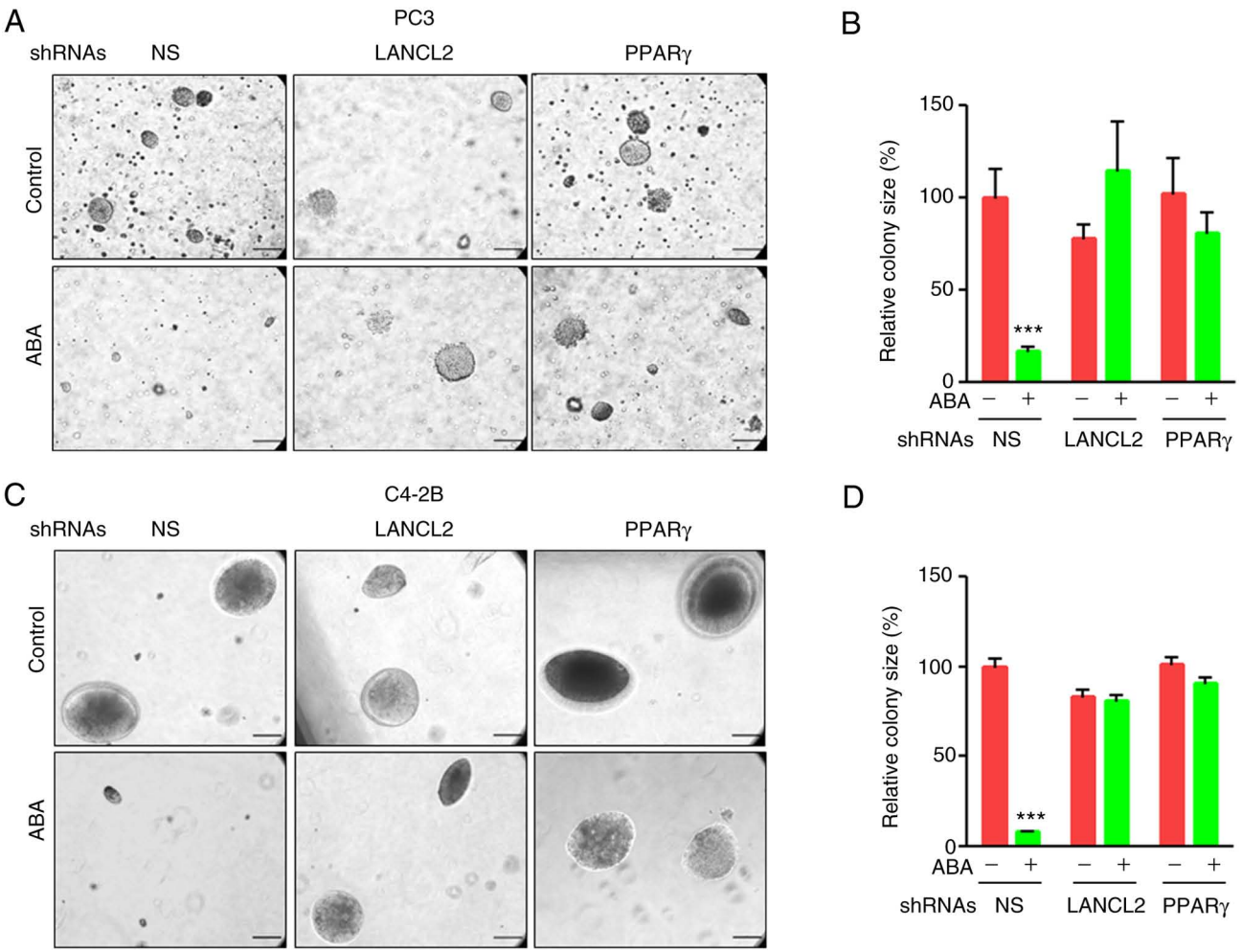

Figure 3. ABA inhibits prostate cancer cell viability through LANCL2 and PPARγ receptors. (A) Representative images (x10 magnification) of anchorage-independent cell growth as determined by soft agar assay for PC3 cells stably expressing NS shRNA, or shRNAs targeting LANCL2 or PPARγ in the presence or absence of ABA (100 μM). (B) Plot showing the relative colony size (%) for data presented in (A). (C) Representative images (x10 magnification) of anchorage-independent cell growth as determined by soft agar assay for C4-2B cells stably expressing NS shRNA, or shRNAs targeting LANCL2 or PPARγ in the presence or absence of ABA (100 μM). (D) Plot showing the relative colony size (%) for data presented in (C). Data are presented as the mean ± SEM. Significance was calculated by two-way ANOVA followed by Bonferroni's post hoc test (***P<0.001 vs. shRNA NS control). ABA, abscisic acid; LANCL2, lanthionine synthetase C-like protein 2; NS, non-specific; PPARγ, peroxisome proliferator activated receptor γ; shRNA, short hairpin RNA.

To evaluate the effects of ABA and the respective receptor KD on invasion, Transwell plates covered in Matrigel were utilized. As with the migration assays, ABA decreased cell invasion in response to FBS, whereas KD of LANCL2 or PPARγ expression abrogated this effect (Fig. 4E-H).

To evaluate the mechanisms by which ABA affects invasion, the present study evaluated the mRNA expression levels of E-cadherin, Vimentin and ZEB2 by RT-qPCR. The results showed that ABA increased E-cadherin, and decreased Vimentin and ZEB2 expression levels in PCa cells in the NS group (Fig. S1). However, shRNA KD of LANCL2 or PPARγ receptors mitigated the effects of ABA on the majority of the cells. The exception being that the mRNA expression levels of Vimentin in ABA-treated shRNA PPARγ KDC4-2B cells were reduced compared with in those cells not treated with ABA, which could be due to a compensatory effect of the LANCL2 receptor (Fig. S1). Collectively, these results indicated that LANCL2 and PPARγ are necessary for the effects of ABA on PCa cell migration and invasion, and suggested that these receptors play a critical role in the metastatic attributes of PCa cells.

Inhibition of PCa cell viability in co-culture is enhanced by ABA. To explore the role that ABA and its receptors play in promoting dormancy in bone marrow, *in vitro* studies were performed. For these studies, PC3 cells were cultured in the presence of confluent monolayers of bone marrow stromal cells isolated from C57BL/6J mice. The co-cultures were grown for 3 days in the presence or absence of ABA. To distinguish the PC3 cells from the stromal layers, PC3-GFP cells were used. The viability of PC3-GFP cells was evaluated upon recovery of the cells from culture with trypsin by FACS probing for GFP. In co-culture, PC3-GFP viability was significantly decreased when ABA was included in the co-cultures (Fig. 5A, B and G). Notably, when the PCa cells with KD of LANCL2 or PPARγ were cultured with the stromal cells, they exhibited increased viability relative to the NS control group (Fig. 5C-G). As expected, the addition of exogenous ABA in the absence of LANCL2 or PPARγ expression led to little to no impact on cellular viability in the co-cultures (Fig. 5C-F). These results indicated that ABA inhibited PCa cell viability in co-culture, whereas

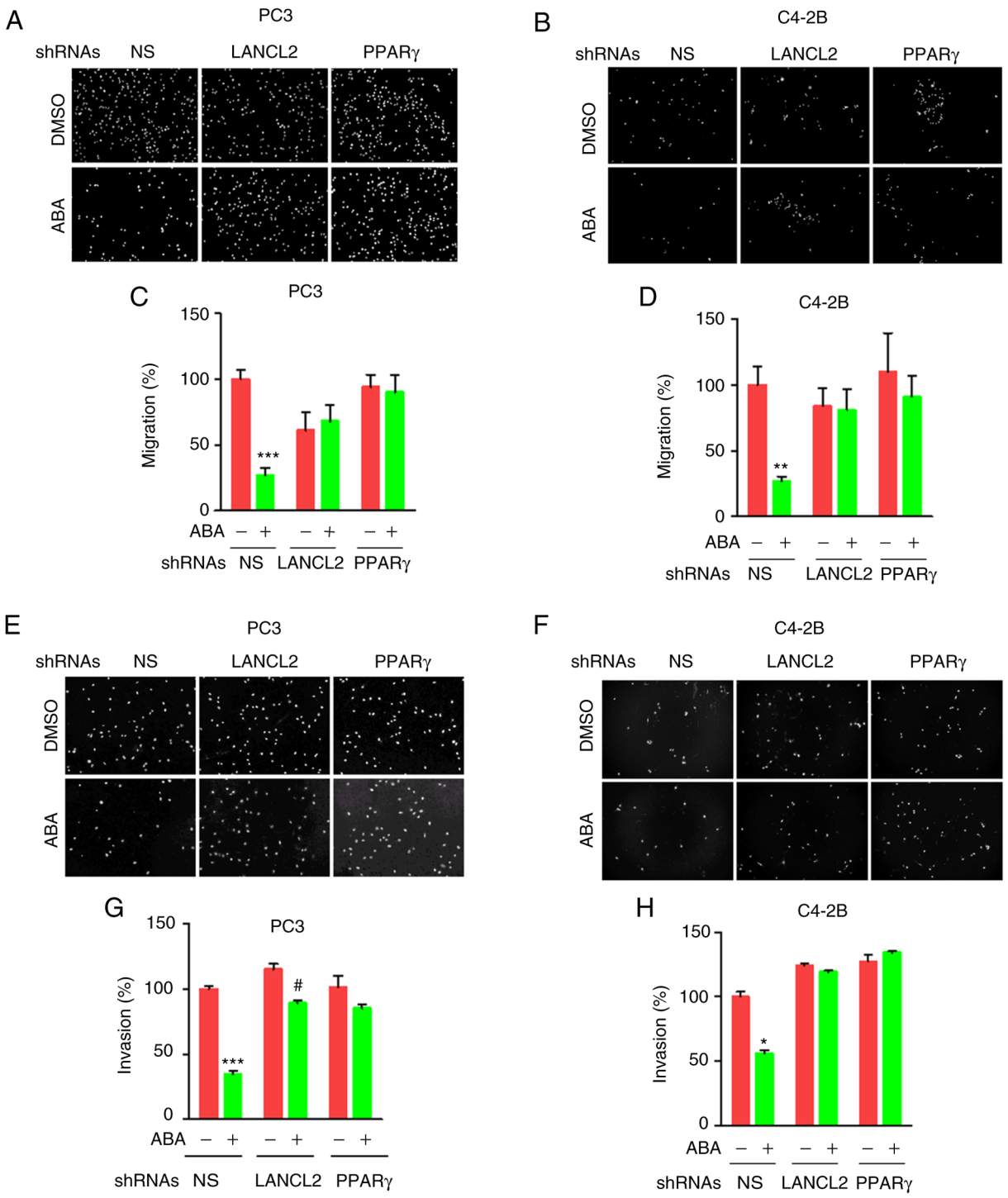

Figure 4. ABA inhibits prostate cancer cell migration and invasion through LANCL2 or PPARγ. Migration of (A) PC3 or (B) C4-2B cells infected with NS shRNA, or shRNAs targeting LANCL2 or PPARγ, across a Transwell membrane in response to 10% FBS. Cells were treated with ABA (100 μM) for 24 h (PC3) and 48 h (C4-2B), and were analyzed at the end of the culture period. Representative images (x10 magnification) at the indicated times are shown. Relative cell migration (%) of (C) PC3 and (D) C4-2B cells. Invasion assay of vehicle- and ABA-treated (E) PC3 and (F) C4-2B cells across a Transwell membrane covered with Matrigel (x10 magnification). Invasion through the matrix was evaluated at (G) 24 h (PC3) and (H) 48 h (C4-2B). Invasion (%) is shown. Data are presented as the mean ± SEM. Significance was calculated by two-way ANOVA followed by Bonferroni's post hoc test (*P<0.05, **P<0.01, ***P<0.001 vs. shRNA NS control; $^{\#}$P<0.05 vs. shRNA LANCL2 control). ABA, abscisic acid; LANCL2, lanthionine synthetase C-like protein 2; NS, non-specific; PPARγ, peroxisome proliferator activated receptor γ; shRNA, short hairpin RNA.

when LANCL2 or PPARγ expression was reduced, either the PCa cells were no longer sensitive to ABA expressed by the bone marrow cells (44), or they become insensitive to another negative cell growth regulator produced by stomal cells which signals through LANCL2 or PPARγ.

ABA regulates transcription of p21, p27 and p16 through LANCL2 and PPARγ in PCa cells. The role of p21, p27 and p16 in cell cycle progression is well established. Activation of p21, p27 and p16 inhibits phosphorylation of CDK1 and CDK2 resulting in cell cycle arrest. To explore whether ABA

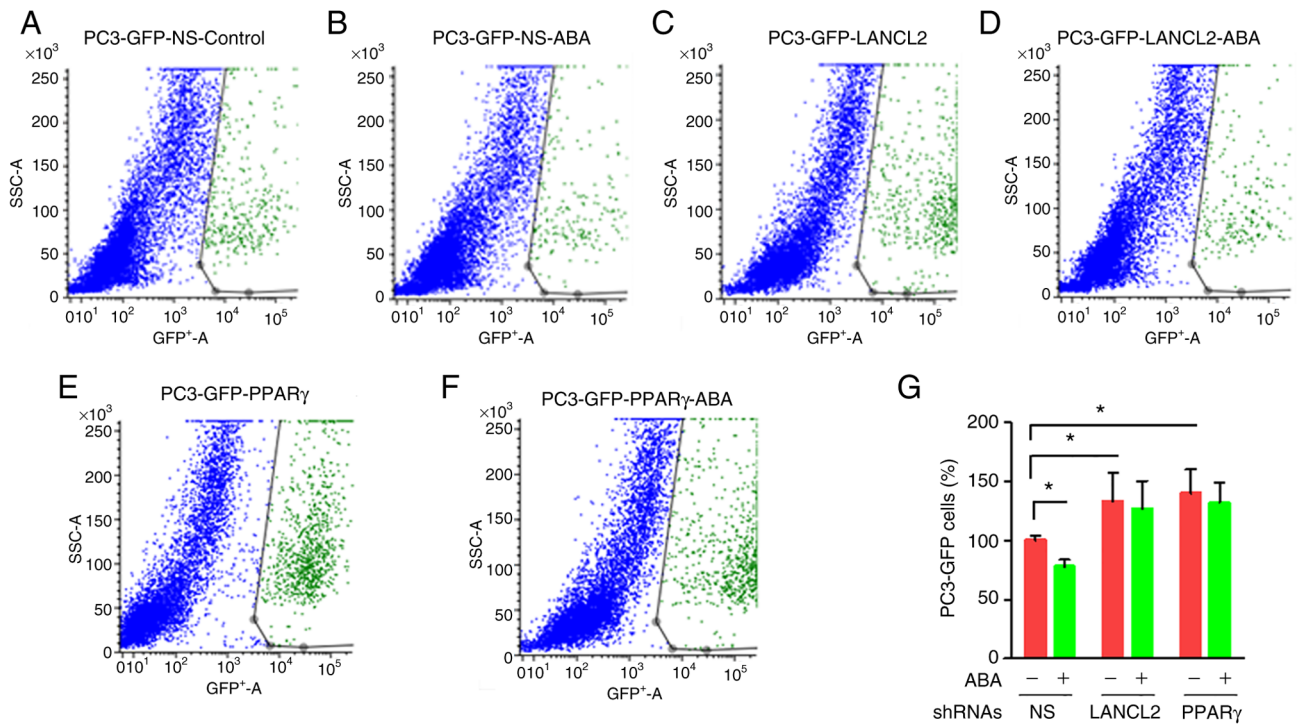

Figure 5. Effect of bone marrow cells on the viability of prostate cancer cells with and without ABA. Murine bone marrow stromal cells were cocultured with GFP-labeled PC3 cells with reduced expression of LANCL2 or PPARγ in the presence or absence of exogenous ABA. At 72 h, the cultures were trypsinized and the GFP-labeled PC3 cells were distinguished and enumerated by FACS gating on GFP. Representative FACS plots of the recovered cells, with PC3 cells shown in green and stromal cells in blue. In each case the shRNA contained a GFP expression cassette. (A) Control NS PC3 cells treated with vehicle, (B) control PC3 cells treated with ABA, (C) KD LANCL2 PC3 cells treated with vehicle, (D) KD LANCL2 PC3 cells treated with ABA, (E) KD PPARγ PC3 cells treated with vehicle and (F) KD PPARγ PC3 cells treated with ABA (G) Percentage of PC3-GFP cells. Data are presented as the mean ± SEM. Significance was calculated by two-way ANOVA followed by Bonferroni's post hoc test (*P<0.05). ABA, abscisic acid; FACs, fluorescence-activated cell sorting; LANCL2, lanthionine synthetase C-like protein 2; NS, non-specific; PPARγ, peroxisome proliferator activated receptor γ; shRNA, short hairpin RNA.

signaling through LANCL2 and PPARγ impacts the expression of p21, p27 or p16, the PCa control or LANCL2 and PPARγ KD cells were treated with ABA and were examined by RT-qPCR analysis. Unexpectedly, when PPARγ expression was knocked down by shRNA ABA increased the expression levels of LANCL2 (Fig. 6A and F), and had variable impact on PPARγ expression when LANCL2 expression was knocked down by shRNA (Fig. 6B and G), thus suggesting that the expression of these ABA receptors establish a feedback loop in PC3 (Fig. 6A and B) and C4-2B cells (Fig. 6F and G). Notably, ABA induced the mRNA expression levels of p21, p27 and p16 in both PC3 (Fig. 6C-E) and C4-2B (Fig. 6H-J) cells. In the predominance of the studies, KD of LANCL2 and PPARγ by shRNA markedly abrogated the effect of ABA on the mRNA expression levels of p21, p27 and p16 (Fig. 6C-E and H-J). Collectively, these results suggested that ABA induces transcriptional regulation of several cell cycle inhibitor proteins in PCa cells through LANCL2 and PPARγ signaling.

ABA induces PCa dormancy through LANCL2 and PPARγ and p38MAPK pathway activation. Phosphorylation of p38MAPK is one of the major signaling pathways involved in the induction of dormancy by molecules that inhibit cell proliferation, including TGFβ, BMPs and GAS6 (15,17). Notably, activation of p38MAPK signaling by phosphorylation, and enhanced expression of the cell cycle inhibitors p21 and p27, are considered as dormancy markers in cancer cells (15,19,45). Therefore, the present study evaluated the effects of ABA signaling on p38MAPK activation. Treatment of the PCa cells with ABA resulted in phosphorylation 38MAPK (Fig. 7A, B, D and E), as well as stimulation of NR2F1 expression, a dormancy marker (Fig. 7A, C, D and F). ABA also enhanced the expression levels of the downstream targets of p38MAPK, including p21 and p27 (Fig. 7G and H). As expected, KD of the ABA receptors LANCL2 and PPARγ negated the ABA-induced effects on p21, p27 and NR2F1 expression (Fig. 7A-H). Collectively, these findings indicated that ABA affects the viability arrest of PCa cells through LANCL2 and PPARγ receptors, in part by signaling through p38MAPK.

Discussion

PCa is a heterogenous disease, which has variable clinical outcomes ranging from early-stage, curable, advanced and terminal disease. Although the majority of men are cured soon after diagnosis at an early stage, a subset of men develop recurrent disease, or present with *de novo* metastatic disease (46). The bone is a common site for PCa metastasis (47), where metastatic cells that have escaped the prostate early in disease are likely to have entered a dormant state for years or even decades. Notably, ~90% of patients who succumb to metastatic PCa have some level of bone involvement (48). Several new therapeutic strategies, including androgen receptor-targeted therapies, chemotherapy, poly ADP-ribose polymerase inhibitors, sipuleucel-T and radium-223, have been approved for the treatment of patients with advanced

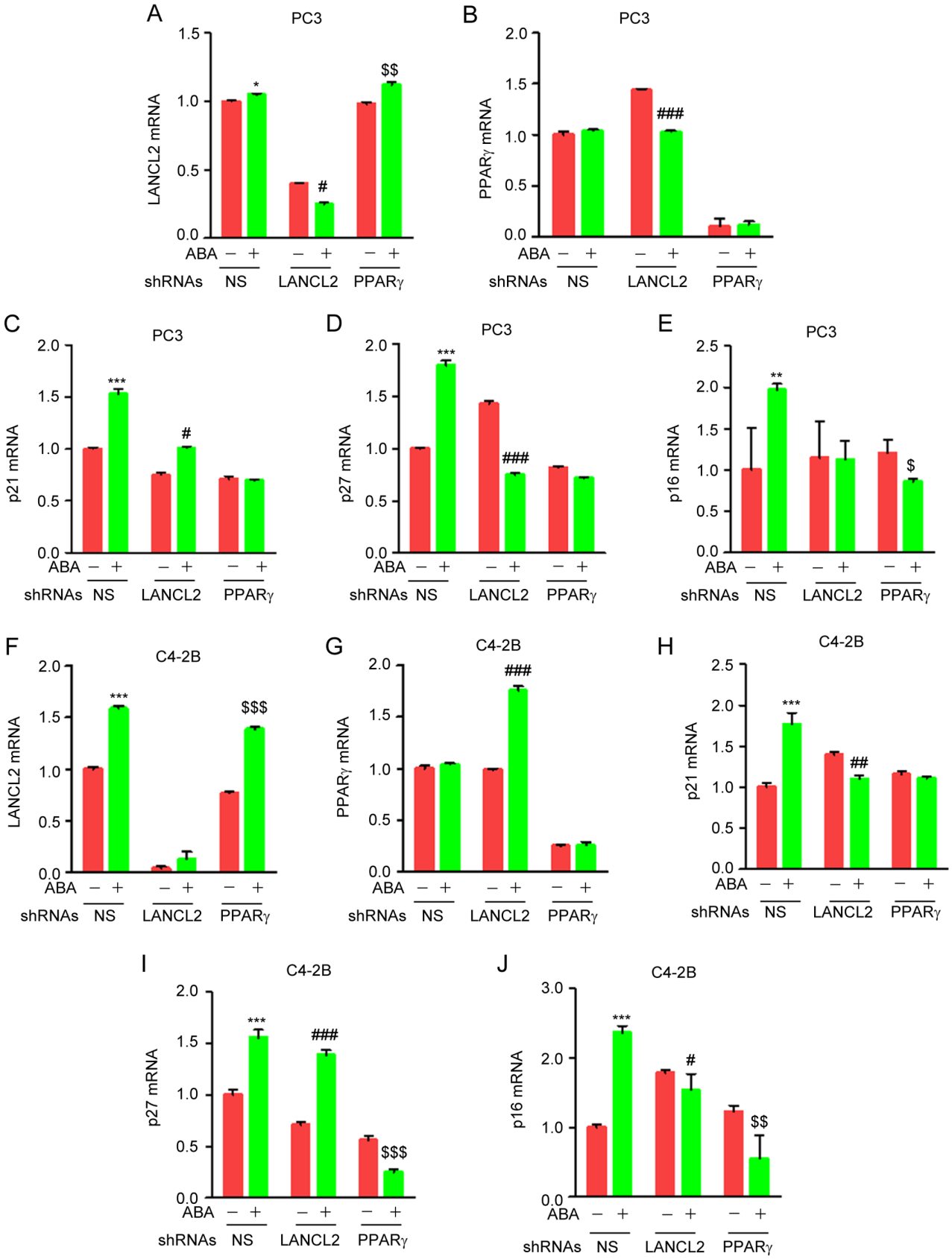

Figure 6. ABA increases the mRNA expression levels of cell cycle-regulating genes through LANCL2 and PPARγ. PC3 prostate cancer cells infected with NS shRNA, or shRNAs targeting LANCL2 or PPARγ, were treated with 100 μM ABA and (A) LANCL2, (B) PPARγ, (C) p21, (D) p27 and (E) p16 expression was evaluated. The mRNA expression levels of the indicated genes were analyzed by quantitative PCR. For C4-2B prostate cancer cells, they too were infected with NS shRNA, or shRNAs targeting LANCL2 or PPARγ, and were treated with 100 μM ABA, and (F) LANCL2, (G) PPARγ, (H) p21, (I) p27 and (J) p16 expression was evaluated. Fold changes were calculated based on normalization to β-actin levels and using the untreated control. Data are presented as the mean ± SEM. Significance was calculated by two-way ANOVA followed by Bonferroni's post hoc test (*P<0.05, **P<0.01, ***P<0.001 vs. shRNA NS control; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. shRNA LANCL2 control; $^{\$}$P<0.05, $^{\$\$}$P<0.01, $^{\$\$\$}$P<0.001 vs. shRNA PPARγ control). ABA, abscisic acid; LANCL2, lanthionine synthetase C-like protein 2; NS, non-specific; PPARγ, peroxisome proliferator activated receptor γ; shRNA, short hairpin RNA.

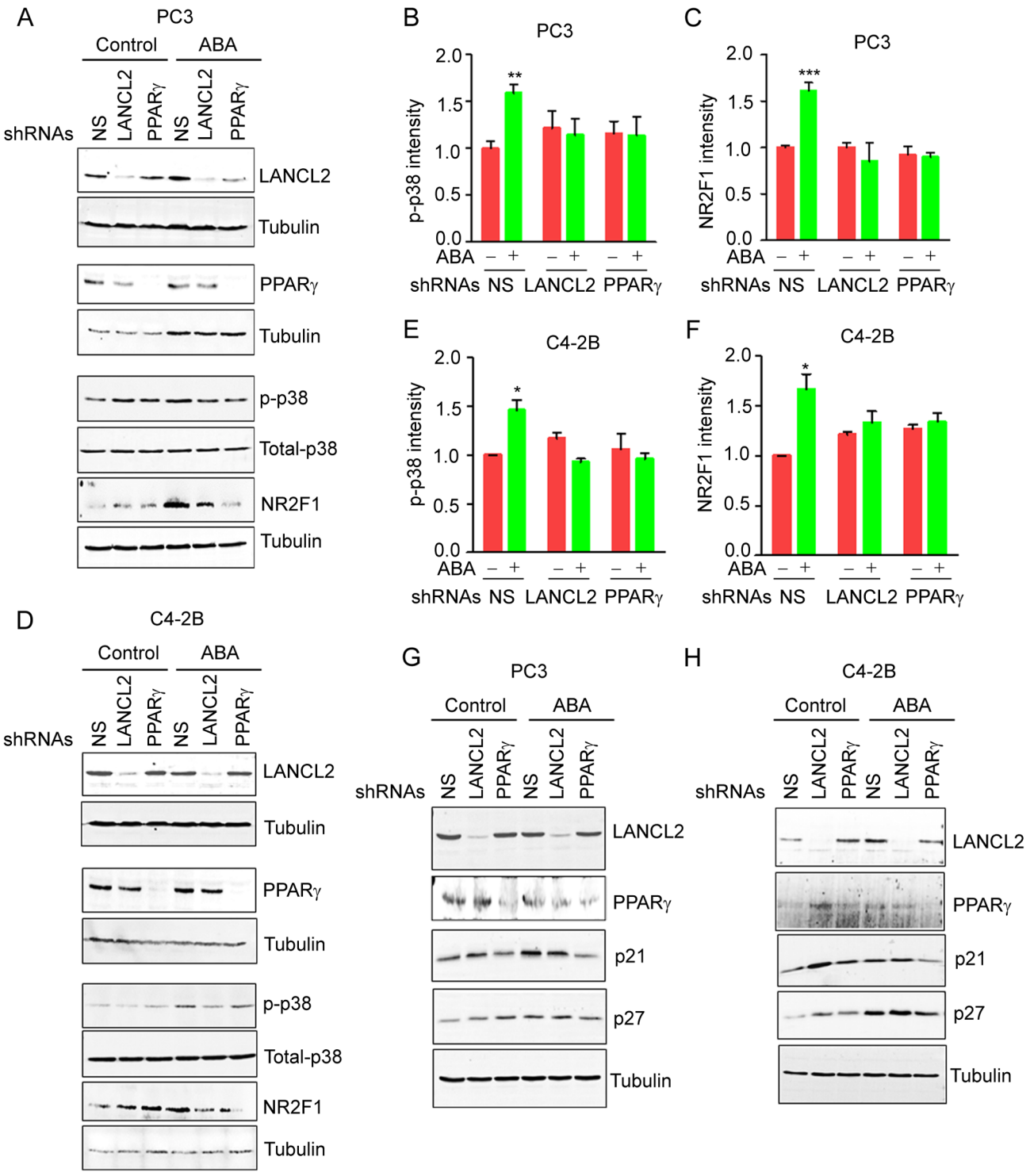

Figure 7. ABA induces changes in PCa cell dormancy via signaling through LANCL2 or PPARγ to activate the p38MAPK pathway in PCa. PC3 and C4-2B cells infected with either NS shRNA, or shRNAs targeting LANCL2 or PPARγ, were treated with ABA and analyzed by western blotting for the indicated proteins. (A) Expression of LANCL2, total p38 and p-p38 in PC3 cells. (B) p-p38 expression was semi-quantified, and (C) NR2F1 band intensity was measured and normalized to the loading control tubulin using ImageJ software in PC3 cells. (D) Expression of LANCL2, total p38 and p-p38 expression in C4-2B cells. (E) p-p38 was semi-quantified, and (F) NR2F1 band intensity was evaluated using ImageJ software in C4-2B cells. In (G) PC3 and (H) C4-2B cells, the expression levels of LANCL2, PPARγ, p21 and p27 were detected in response to ABA. Significance was calculated by two-way ANOVA followed by Bonferroni's post hoc test (*P<0.05, **P<0.01, ***P<0.001 vs. shRNA NS control). ABA, abscisic acid; LANCL2, lanthionine synthetase C-like protein 2; NS, non-specific; p-, phosphorylated; PCa, prostate cancer; PPARγ, peroxisome proliferator activated receptor γ; shRNA, short hairpin RNA.

PCa and bone metastasis. However, this disease still represents the second most frequent cause of cancer-related death in men in the United States (49). The challenges in treating bone metastases at the advanced stage include a high level of genomic heterogeneity (50), a pro-immunosuppressive environment (51) and several emerging mechanisms of androgen independence (52). Therefore, new therapeutics are urgently needed.

ABA is known to regulate cell responses and biological processes in plants, mammals and other organisms (53,54). ABA is a key hormone that promotes seed dormancy during development in plants and after seed dispersal (55). ABA controls the induction of dormancy, and ultimately the release and germination of seeds in response to environmental signals (55). ABA has also been recognized as an endogenous hormone in humans (29). It has a role in the inflammatory response, as well as in immunoregulatory activities, antioxidant properties and the maintenance of glycemic control in pre-clinical models of diabetes and inflammatory diseases (56,57). Despite the advances in the understanding of the role that ABA plays in several physiological processes, the role of ABA in tumor dormancy remains to be elucidated.

The present study demonstrated that ABA suppressed the viability of four distinct PCa cell lines (PC3, C4-2B, DU-145 and Myc-CaP) in a dose-dependent manner; however, exposure to ABA was not in and of itself cytotoxic to the cancer cells. Notably, ABA-induced inhibition of PCa cell viability was reversible, as cells which were removed from ABA treatment were able to resume their proliferation. There are at least two receptors that are responsible for transducing ABA signaling; LANCL2 and PPARγ. To explore how ABA induced proliferative quiescence, targeted KD of LANCL2 and PPARγ was established using shRNAs in PC3 and C4-2B cell lines. In almost every instance where ABA signaling was examined, including viability, migration, invasion and downstream signaling, reduced expression of LANCL2 and PPARγ abrogated the impact of ABA on the cancer cells. Furthermore, it was observed that ABA inhibited the formation of PCa colonies in soft agar, whereas LANCL2 and PPARγ KD alone did not. Mechanistically, ABA treatment induced p21, p27 and p16 mRNA and protein expression, whereas LANCL2 and PPARγ shRNA KD did not significantly inhibit either p21, p27 or p16 mRNA or protein expression relative to the controls. These data suggested that ABA may inhibit viability through inducing the expression levels of p21, p27 and p16, but when the expression of ABA receptors is reduced, there is little impact on p21, p27 and p16 expression.

LANCL2 and PPARγ are known to mediate a range of physiological responses of ABA in a number of systems, including granulocytes and rat insulinoma cells (31,58). ABA signaling in mammalian immune cells, keratinocytes and pancreatic cells requires LANCL2 expression, as small interfering RNA targeting of LANCL2 has been shown to abrogate cellular responses (23,26,58). Several studies have reported that activation of PPARγ by agonists markedly reduces cell proliferation, including in hepatocellular carcinoma (59), PCa (60) and gastric cancer (61). Other studies have demonstrated that PPARγ agonists promote terminal differentiation, inhibit cell proliferation and increase the apoptosis of human cancer cell lines, as well as tumor growth in animal models (33,62). Notably, in some cases, PPARγ agonists have demonstrated modest efficacy as chemopreventives in clinical trials (33,62). It is not surprising that clinical studies have reported that the overall survival of patients with colorectal cancer is better when PPARγ expression is detectable in the primary tumors (63). In part, this may be due to the findings that PPARγ modulates the expression of different cell cycle regulators, including decreasing the expression of cyclin D1 (64), and increasing the expression of the CDK inhibitors p21 (65) and p27 (66).

The present study reported on notable findings in co-culture studies of PCa with bone marrow stromal cells. In the presence of marrow stroma, ABA is known to inhibit PCa cell viability (44). However, when PCa cells with reduced expression of LANCL2 and PPARγ were grown on the bone marrow stromal cells, the cells proliferated at the same rate as the control cells. These results suggested that PCa cells with decreased expression of LANCL2 and PPARγ may no longer be sensitive to ABA produced by the mesenchymal stromal cells, as shown in a previous study (67), or are no longer sensitive to growth inhibitors secreted by the stomal cells, which also signal through LANCL2 and PPARγ. Very little is known as to what activates LANCL2. In glioma it has been demonstrated that LANCL2 expression may be co-regulated by epidermal growth factor receptor (68). Activation of PPARγ can inhibit cell proliferation, induce differentiation and promote metastasis in PCa cells (60). In other contexts, PPARγ is activated by fatty acids and derivatives including polyunsaturated fatty acids, derivatives of arachidonic acid and prostaglandins (69). However, what other negative regulators of PCa cell viability are produced by bone marrow stromal cells, which signal through LANCL2 and PPARγ remain unclear.

In the PCa cell lines tested there appears to be some diversity in signaling in response to ABA through PPARγ and LANCL2 . For example, p27 activity was enhanced by ABA treatment in both cell lines, but when LANCL2 was knocked down by shRNA, the p27 response was altered in the C4-2B and PC3 cells, although the response was in the opposite direction. Currently, there is no specific explanation as to the basis of the differences in these observations. A reasonable possibility is that the cells have other receptors for ABA in addition to LANCL2 and PPARγ. In other studies, LANCL1 and CD38 have been suggested to serve as receptors or co-stimulatory molecules for LANCL2 (58,70). Unfortunately, we were not able to achieve functional reductions of CD38 in the cell lines used in the present study (C4-2B and PC3); therefore, it is possible that other players participate in the signaling. Furthermore, there are known ABA inhibitors in plants, including gibberellins, which can in some cases serve as allergens and of which derivatives may have anti-neoplastic activities (71-77). Whether equivalent proteins function in human systems remains unclear. However, in preliminary studies we did not observe any significant impact of gibberellin on the cell cycle of metastatic cancer cell lines (Jung *et al*, unpublished data). Further studies are needed to determine the basis of the variations in cell cycle inhibitors.

To further understand the mechanisms by which ABA signals through LANCL2 and PPARγ to induce dormancy, western blot analysis was performed to detect p38 phosphorylation in PCa cells. It was revealed that KD of LANCL2 and PPARγ inhibited the activation of p38 by ABA. In addition, NR2F1 has been reported to act as a master regulator of tumor cell dormancy (78). By binding to DNA and recruiting coactivator or corepressor complexes, NR2F1 is able to serve

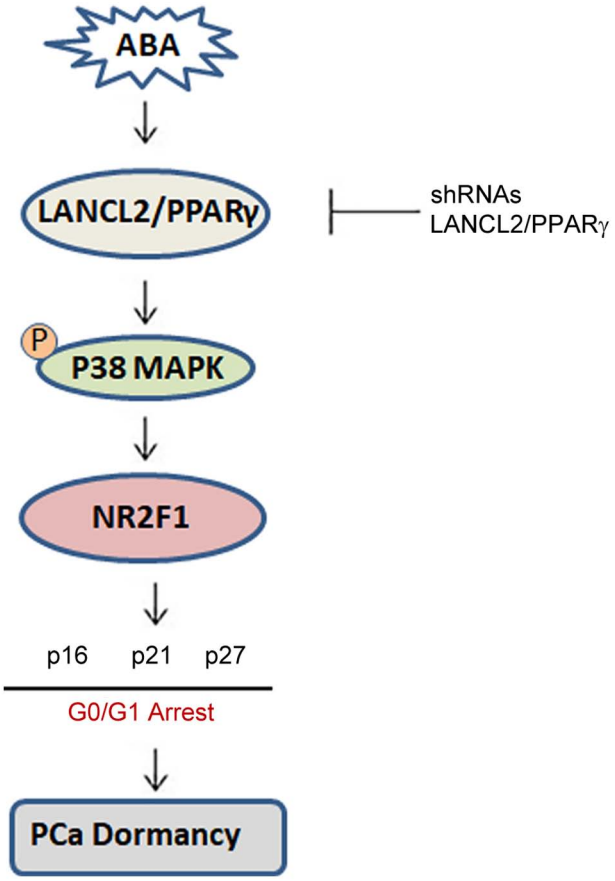

Figure 8. A schematic diagram showing the signaling pathways by which ABA induces PCa dormancy. ABA induces activation of p38MAPK via LANCL2 and PPARγ, which triggers NR2F1, which finally stimulates the upregulation of cell cycle genes and induces PCa dormancy. ABA, abscisic acid; LANCL2, lanthionine synthetase C-like protein 2; PCa, prostate cancer; PPARγ, peroxisome proliferator activated receptor γ; shRNA, short hairpin RNA.

as a cofactor to other nuclear receptors. Notably, NR2F1 expression, one of the best known dormancy markers, has been reported to be directly regulated by ABA (78), and its expression in this previous study was associated with p38 signaling (78). Activation of p38 signaling induces expression of the cell cycle inhibitors p21 and p27, also considered as dormancy markers in cancer cells (15,19,45). These findings make sense given the proposed hypothesis that the ratio between the p38 MAPK stress response signaling pathway and ERK guides cancer cells into a proliferative or dormant state (79).

The regulation of cell proliferation in the bone marrow is a critical function of the marrow microenvironment to protect and promote proliferation of hematopoietic and mesenchymal stem cells, and its integration across an organism is critical to the survival of mammals. The present study assessed the role of ABA and its signaling pathways in regulating cell proliferation/quiescence of cells that do not belong in the marrow, specifically cells of epithelial origin. Given that there are a number of pathways that regulate the proliferation and dormancy of stem and progenitor cells in the marrow, it is not surprising that there are multiple pathways that can regulate stem cell proliferation (16,80-82). That tumor cells exploit the niches used by the bone marrow to regulate stem and progenitor cells is not surprising and makes sense from an ecological perspective (80). For example, osteoblasts that participate in forming hematopoietic stem cell (HSC) niches produce GAS6, which not only regulates HSC function, but limits the proliferation of PCa cells *in vitro* (83) and *in vivo* (84-86). Other studies have shown that GAS6 and its receptor AXL are required for TGFβ2-mediated cell proliferation suppression in PCa, where AXL positively regulates the expression of TGFβ and TGFβ receptor 2 (18). In the current study, it was demonstrated that ABA in co-culture of PCa cells with murine bone marrow significantly inhibited PCa cell viability. However, LANCL2 and PPARγ KD abrogated the response to ABA. Together, these data suggest the following model: ABA signaling through LANCL2 or PPARγ receptors activates signaling via p38MAPK, NR2F1, p27, p16 and p21, which ultimately leads to dormancy (Fig. 8). These results suggest that interference with ABA signaling under the appropriate conditions, or perhaps even from dietary sources such as fruits and vegetables, may prove beneficial in treating men with prostate cancer, by either keeping DTCs as dormant cells or by interfering with ABA signaling together with chemotherapy to selectively kill DTCs.

In conclusion, the present findings suggested that ABA may induce cell cycle arrest in PCa. ABA does so by signaling through LANCL2 and PPARγ, which activate p38MAPK and the CDK inhibitors, p21, p16 and p27, and NR2F1 resulting in PCa cell dormancy. Further investigation into how the ABA signaling pathway results in dormancy may reveal novel opportunities for eradicating dormant cancer cells and/or keeping them in a perpetual dormant state, thus preventing PCa metastasis.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

KRP, YJ and RST conceived or designed the study. KRP collected the data. KRP and RST analyzed and interpreted the data. KRP drafted the article. YJ and RST critically revised the article. KRP and RST confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approved the present study (approval no. IACUC-21928).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Siegel RL, Miller KD, Fuchs HE and Jemal A: Cancer statistics, 2021. CA Cancer J Clin 71: 7-33, 2021.
2. Taichman RS, Loberg RD, Mehra R and Pienta KJ: The evolving biology and treatment of prostate cancer. J Clin Invest 117: 2351-2361, 2007.
3. Wenzel M, Garcia CC, Hoeh B, Jorias C, Humke C, Koll F, Tselis N, Rödel C, Graefen M, Tilki D, *et al*: Real-world evidence of outcomes of oligometastatic hormone-sensitive prostate cancer patients treated with metastasis-directed therapy. Prostate 83: 1365-1372, 2023.
4. Rogowski P, Trapp C, von Bestenbostel R, Schmidt-Hegemann NS, Shi R, Ilhan H, Kretschmer A, Stief C, Ganswindt U, Belka C and Li M: Outcomes of metastasis-directed therapy of bone oligometastatic prostate cancer. Radiat Oncol 16: 125, 2021.
5. Bubendorf L, Schöpfer A, Wagner U, Sauter G, Moch H, Willi N, Gasser TC and Mihatsch MJ: Metastatic patterns of prostate cancer: An autopsy study of 1,589 patients. Hum Pathol 31: 578-583, 2000.
6. Berruti A, Dogliotti L, Bitossi R, Fasolis G, Gorzegno G, Bellina M, Torta M, Porpiglia F, Fontana D and Angeli A: Incidence of skeletal complications in patients with bone metastatic prostate cancer and hormone refractory disease: Predictive role of bone resorption and formation markers evaluated at baseline. J Urol 164: 1248-1253, 2000.
7. Sartor O and de Bono JS: Metastatic prostate cancer. N Engl J Med 378: 645-657, 2018.
8. Uhr JW and Pantel K: Controversies in clinical cancer dormancy. Proc Natl Acad Sci USA 108: 12396-12400, 2011.
9. Ruppender NS, Morrissey C, Lange PH and Vessella RL: Dormancy in solid tumors: Implications for prostate cancer. Cancer Metastasis Rev 32: 501-509, 2013.
10. Aguirre-Ghiso JA: Models, mechanisms and clinical evidence for cancer dormancy. Nat Rev Cancer 7: 834-846, 2007.
11. Phan TG and Croucher PI: The dormant cancer cell life cycle. Nat Rev Cancer 20: 398-411, 2020.
12. Aguirre-Ghiso JA, Estrada Y, Liu D and Ossowski L: ERK(MAPK) activity as a determinant of tumor growth and dormancy; regulation by p38(SAPK). Cancer Res 63: 1684-1695, 2003.
13. Dhillon AS, Hagan S, Rath O and Kolch W: MAP kinase signalling pathways in cancer. Oncogene 26: 3279-3290, 2007.
14. Zhang W and Liu HT: MAPK signal pathways in the regulation of cell proliferation in mammalian cells. Cell Res 12: 9-18, 2002.
15. Bragado P, Estrada Y, Parikh F, Krause S, Capobianco C, Farina HG, Schewe DM and Aguirre-Ghiso JA: TGF-β2 dictates disseminated tumour cell fate in target organs through TGF-beta-RIII and p38α/β signalling. Nat Cell Biol 15: 1351-1361, 2013.
16. Prunier C, Baker D, Ten Dijke P and Ritsma L: TGF-β family signaling pathways in cellular dormancy. Trends Cancer 5: 66-78, 2019.
17. Sosa MS, Avivar-Valderas A, Bragado P, Wen HC and Aguirre-Ghiso JA: ERK1/2 and p38α/β signaling in tumor cell quiescence: Opportunities to control dormant residual disease. Clin Cancer Res 17: 5850-5857, 2011.
18. Yumoto K, Eber MR, Wang J, Cackowski FC, Decker AM, Lee E, Nobre AR, Aguirre-Ghiso JA, Jung Y and Taichman RS: Axl is required for TGF-β2-induced dormancy of prostate cancer cells in the bone marrow. Sci Rep 6: 36520, 2016.
19. Kobayashi A, Okuda H, Xing F, Pandey PR, Watabe M, Hirota S, Pai SK, Liu W, Fukuda K, Chambers C, *et al*: Bone morphogenetic protein 7 in dormancy and metastasis of prostate cancer stem-like cells in bone. J Exp Med 208: 2641-2655, 2011.
20. Le Page-Degivry MT, Bidard JN, Rouvier E, Bulard C and Lazdunski M: Presence of abscisic acid, a phytohormone, in the mammalian brain. Proc Natl Acad Sci USA 83: 1155-118, 1986.
21. Bruzzone S, Ameri P, Briatore L, Mannino E, Basile G, Andraghetti G, Grozio A, Magnone M, Guida L, Scarfì S, *et al*: The plant hormone abscisic acid increases in human plasma after hyperglycemia and stimulates glucose consumption by adipocytes and myoblasts. FASEB J 26: 1251-1260, 2012.
22. Bruzzone S, Bodrato N, Usai C, Guida L, Moreschi I, Nano R, Antonioli B, Fruscione F, Magnone M, Scarfì S, *et al*: Abscisic acid is an endogenous stimulator of insulin release from human pancreatic islets with cyclic ADP ribose as second messenger. J Biol Chem 283: 32188-32197, 2008.
23. Bruzzone S, Basile G, Mannino E, Sturla L, Magnone M, Grozio A, Salis A, Fresia C, Vigliarolo T, Guida L, *et al*: Autocrine abscisic acid mediates the UV-B-induced inflammatory response in human granulocytes and keratinocytes. J Cell Physiol 227: 2502-2510, 2012.
24. Bruzzone S, Moreschi I, Usai C, Guida L, Damonte G, Salis A, Scarfì S, Millo E, De Flora A and Zocchi E: Abscisic acid is an endogenous cytokine in human granulocytes with cyclic ADP-ribose as second messenger. Proc Natl Acad Sci USA 104: 5759-5764, 2007.
25. Magnone M, Bruzzone S, Guida L, Damonte G, Millo E, Scarfì S, Usai C, Sturla L, Palombo D, De Flora A and Zocchi E: Abscisic acid released by human monocytes activates monocytes and vascular smooth muscle cell responses involved in atherogenesis. J Biol Chem 284: 17808-17818, 2009.
26. Magnone M, Sturla L, Jacchetti E, Scarfì S, Bruzzone S, Usai C, Guida L, Salis A, Damonte G, De Flora A and Zocchi E: Autocrine abscisic acid plays a key role in quartz-induced macrophage activation. FASEB J 26: 1261-1271, 2012.
27. Scarfi S, Ferraris C, Fruscione F, Fresia C, Guida L, Bruzzone S, Usai C, Parodi A, Millo E, Salis A, *et al*: Cyclic ADP-ribose-mediated expansion and stimulation of human mesenchymal stem cells by the plant hormone abscisic acid. Stem Cells 26: 2855-2864, 2008.
28. Li HH, Hao RL, Wu SS, Guo PC, Chen CJ, Pan LP and Ni H: Occurrence, function and potential medicinal applications of the phytohormone abscisic acid in animals and humans. Biochem Pharmacol 82: 701-712, 2011.
29. Sakthivel P, Sharma N, Klahn P, Gereke M and Bruder D: Abscisic Acid: A phytohormone and mammalian cytokine as novel pharmacon with potential for future development into clinical applications. Curr Med Chem 23: 1549-1570, 2016.
30. Fresia C, Vigliarolo T, Guida L, Booz V, Bruzzone S, Sturla L, Di Bona M, Pesce M, Usai C, De Flora A and Zocchi E: G-protein coupling and nuclear translocation of the human abscisic acid receptor LANCL2. Sci Rep 6: 26658, 2016.
31. Bassaganya-Riera J, Guri AJ, Lu P, Climent M, Carbo A, Sobral BW, Horne WT, Lewis SN, Bevan DR and Hontecillas R: Abscisic acid regulates inflammation via ligand-binding domain-independent activation of peroxisome proliferator-activated receptor gamma. J Biol Chem 286: 2504-2516, 2011.
32. Leber A, Hontecillas R, Zoccoli-Rodriguez V and Bassaganya-Riera J: Activation of LANCL2 by BT-11 Ameliorates IBD by supporting regulatory T cell stability through immunometabolic mechanisms. Inflamm Bowel Dis 24: 1978-1991, 2018.
33. Koeffler HP: Peroxisome proliferator-activated receptor gamma and cancers. Clin Cancer Res 9: 1-9, 2003.
34. Hisatake JI, Ikezoe T, Carey M, Holden S, Tomoyasu S and Koeffler HP: Down-Regulation of prostate-specific antigen expression by ligands for peroxisome proliferator-activated receptor gamma in human prostate cancer. Cancer Res 60: 5494-5498, 2000.
35. Sikka S, Chen L, Sethi G and Kumar AP: Targeting PPARγ signaling cascade for the prevention and treatment of prostate cancer. PPAR Res 2012: 968040, 2012.
36. Fizazi K and Navone NM: Preclinical models of prostate cancer. Bull Cancer 92: 129-141, 2005 (In French).
37. Rosol TJ, Tannehill-Gregg SH, LeRoy BE, Mandl S and Contag CH: Animal models of bone metastasis. Cancer 97 (Suppl 3): S748-S57, 2003.

38. Chung LW, Kao C, Sikes RA and Zhau HE: Human prostate cancer progression models and therapeutic intervention. Hinyokika Kiyo 43: 815-820, 1997.
39. Wang Y, Herroon MK, Zielske SP, Ellis L, Podgorski I, Taichman RS and Cackowski FC: Use of FVB Myc-CaP cells as an immune competent, androgen receptor positive, mouse model of prostate cancer bone metastasis. J Bone Oncol 30: 100386, 2021.
40. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
41. Westbrook TF, Martin ES, Schlabach MR, Leng Y, Liang AC, Feng B, Zhao JJ, Roberts TM, Mandel G, Hannon GJ, *et al*: A genetic screen for candidate tumor suppressors identifies REST. Cell 121: 837-848, 2005.
42. Lin L, Chamberlain L, Pak ML, Nagarajan A, Gupta R, Zhu LJ, Wright CM, Fong KM, Wajapeyee N and Green MR: A large-scale RNAi-based mouse tumorigenesis screen identifies new lung cancer tumor suppressors that repress FGFR signaling. Cancer Discov 4: 1168-1181, 2014.
43. Oktem G, Bilir A, Uslu R, Inan SV, Demiray SB, Atmaca H, Ayla S, Sercan O and Uysal A: Expression profiling of stem cell signaling alters with spheroid formation in CD133high/CD44high prostate cancer stem cells. Oncol Lett 7: 2103-2109, 2014.
44. Jung Y, Cackowski FC, Yumoto K, Decker AM, Wang Y, Hotchkin M, Lee E, Buttitta L and Taichman RS: Abscisic acid regulates dormancy of prostate cancer disseminated tumor cells in the bone marrow. Neoplasia 23: 102-111, 2021.
45. Sharma S, Xing F, Liu Y, Wu K, Said N, Pochampally R, Shiozawa Y, Lin HK, Balaji KC and Watabe K: Secreted protein acidic and rich in cysteine (SPARC) mediates metastatic dormancy of prostate cancer in bone. J Biol Chem 291: 19351-19363, 2016.
46. Rebello RJ, Oing C, Knudsen KE, Loeb S, Johnson DC, Reiter RE, Gillessen S, Van der Kwast T and Bristow RG: Prostate cancer. Nat Rev Dis Primers 7: 9, 2021.
47. Roudier MP, Corey E, True LD, Hiagno CS, Ott SM and Vessell RL: Histological, immunophenotypic and histomorphometric characterization of prostate cancer bone metastases. Cancer Treat Res 118: 311-339, 2004.
48. Mehra R, Kumar-Sinha C, Shankar S, Lonigro RJ, Jing X, Philips NE, Siddiqui J, Han B, Cao X, Smith DC, *et al*: Characterization of bone metastases from rapid autopsies of prostate cancer patients. Clin Cancer Res 17: 3924-3932, 2011.
49. Viale PH: The American Cancer Society's facts & figures: 2020 edition. J Adv Pract Oncol 11: 135-136, 2020.
50. Ku SY, Gleave ME and Beltran H: Towards precision oncology in advanced prostate cancer. Nat Rev Urol 16: 645-654, 2019.
51. Patel VG and Oh WK: The evolving landscape of immunotherapy in advanced prostate cancer. Immunotherapy 11: 903-912, 2019.
52. Watson PA, Arora VK and Sawyers CL: Emerging mechanisms of resistance to androgen receptor inhibitors in prostate cancer. Nat Rev Cancer 15: 701-711, 2015.
53. Takezawa D, Komatsu K and Sakata Y: ABA in bryophytes: How a universal growth regulator in life became a plant hormone? J Plant Res 124: 437-453, 2011.
54. Lievens L, Pollier J, Goossens A, Beyaert R and Staal J: Abscisic acid as pathogen effector and immune regulator. Front Plant Sci 8: 587, 2017.
55. Sano N and Marion-Poll A: ABA metabolism and homeostasis in seed dormancy and germination. Int J Mol Sci 22: 5069, 2021.
56. Chaqour J, Lee S, Ravichandra A and Chaqour B: Abscisic acid-an anti-angiogenic phytohormone that modulates the phenotypical plasticity of endothelial cells and macrophages. J Cell Sci 131: jcs210492, 2018.
57. Baliño P, Gómez-Cadenas A, López-Malo D, Romero FJ and Muriach M: Is there a role for abscisic acid, a proven anti-inflammatory agent, in the treatment of ischemic retinopathies? Antioxidants (Basel) 8: 104, 2019.
58. Sturla L, Fresia C, Guida L, Bruzzone S, Scarfì S, Usai C, Fruscione F, Magnone M, Millo E, Basile G, *et al*: LANCL2 is necessary for abscisic acid binding and signaling in human granulocytes and in rat insulinoma cells. J Biol Chem 284: 28045-28057, 2009.
59. Cao LQ, Shao ZL, Liang HH, Zhang DW, Yang XW, Jiang XF and Xue P: Activation of peroxisome proliferator-activated receptor-γ (PPARγ) inhibits hepatoma cell growth via downregulation of SEPT2 expression. Cancer Lett 359:127-135, 2015.
60. Bolden A, Bernard L, Jones D, Akinyeke T and Stewart LV: The PPAR gamma agonist troglitazone regulates Erk 1/2 phosphorylation via a PPARγ-Independent, MEK-dependent pathway in human prostate cancer cells. PPAR Res 2012: 929052, 2012.
61. Cho SJ, Kook MC, Lee JH, Shin JY, Park J, Bae YK, Choi IJ, Ryu KW and Kim YW: Peroxisome proliferator-activated receptor γ upregulates galectin-9 and predicts prognosis in intestinal-type gastric cancer. Int J Cancer 136: 810-820, 2015.
62. Grommes C, Landreth GE and Heneka MT: Antineoplastic effects of peroxisome proliferator-activated receptor gamma agonists. Lancet Oncol 5: 419-429, 2004.
63. Ogino S, Shima K, Baba Y, Nosho K, Irahara N, Kure S, Chen L, Toyoda S, Kirkner GJ, Wang YL, *et al*: Colorectal cancer expression of peroxisome proliferator-activated receptor gamma (PPARG, PPARgamma) is associated with good prognosis. Gastroenterology 136: 1242-1250, 2009.
64. Wang C, Fu M, D'Amico M, Albanese C, Zhou JN, Brownlee M, Lisanti MP, Chatterjee VK, Lazar MA and Pestell RG: Inhibition of cellular proliferation through IkappaB kinase-independent and peroxisome proliferator-activated receptor gamma-dependent repression of cyclin D1. Mol Cell Biol 21: 3057-3070, 2001.
65. Elnemr A, Ohta T, Iwata K, Ninomia I, Fushida S, Nishimura G, Kitagawa H, Kayahara M, Yamamoto M, Terada T and Miwa K: PPARgamma ligand (thiazolidinedione) induces growth arrest and differentiation markers of human pancreatic cancer cells. Int J Oncol 17: 1157-1164, 2000.
66. Itami A, Watanabe G, Shimada Y, Hashimoto Y, Kawamura J, Kato M, Hosotani R and Imamura M: Ligands for peroxisome proliferator-activated receptor gamma inhibit growth of pancreatic cancers both in vitro and in vivo. Int J Cancer 94: 370-376, 2001.
67. Jabbar ZR and Sahib HB: The effects of abscisic acid on angiogenesis in both ex vivo and in vivo assays. Asian Pac J Cancer Prev 23: 4193-4203, 2022.
68. Zhao HF, Zhou XM, Wang J, Chen FF, Wu CP, Diao PY, Cai LR, Chen L, Xu YW, Liu J, *et al*: Identification of prognostic values defined by copy number variation, mRNA and protein expression of LANCL2 and EGFR in glioblastoma patients. J Transl Med 19: 372, 2021.
69. Yousefnia S, Momenzadeh S, Seyed Forootan F, Ghaedi K and Nasr Esfahani MH: The influence of peroxisome proliferator-activated receptor γ (PPARγ) ligands on cancer cell tumorigenicity. Gene 649: 14-22, 2018.
70. Spinelli S, Begani G, Guida L, Magnone M, Galante D, D'Arrigo C, Scotti C, Iamele L, De Jonge H, Zocchi E and Sturla L: LANCL1 binds abscisic acid and stimulates glucose transport and mitochondrial respiration in muscle cells via the AMPK/PGC-1α/Sirt1 pathway. Mol Metab 53: 101263, 2021.
71. Biagioni B, Tomei L, Valleriani C, Liccioli G, Barni S, Sarti L, Citera F, Giovannini M and Mori F: Allergy to Gibberellin-Regulated Proteins (Peamaclein) in Children. Int Arch Allergy Immunol 182: 1194-1199, 2021.
72. Nakagawa M, Hanada M, Inomata N and Amano H: A case of a gibberellin-regulated protein-positive patient allergic to various fruits. Eur J Dermatol 31: 88-90, 2021.
73. Inuo C, Okazaki F, Shiraki R, Tanaka Y, Momma K, Kondo Y and Narita H: Generalized allergic reaction in response to exercise due to strawberry gibberellin-regulated protein: a case report. Allergy Asthma Clin Immunol 18: 49, 2022.
74. Mo J, Kang M, Ye JX, Chen JB, Zhang HB and Qing C: Gibberellin derivative GA-13315 sensitizes multidrug-resistant cancer cells by antagonizing ABCB1 while agonizes ABCC1. Cancer Chemother Pharmacol 78: 51-61, 2016.
75. Egbewande FA, Sadowski MC, Levrier C, Tousignant KD, White JM, Coster MJ, Nelson CC and Davis RA: Identification of gibberellic acid derivatives that deregulate cholesterol metabolism in prostate cancer cells. J Nat Prod 81: 838-845, 2018.
76. Mukherjee A, Gaurav AK, Singh S, Yadav S, Bhowmick S, Abeysinghe S and Verma JP: The bioactive potential of phytohormones: A review. Biotechnol Rep (Amst) 35: e00748, 2022.
77. Shuai HW, Meng YJ, Luo XF, Chen F, Qi Y, Yang WY and Shu K: The roles of auxin in seed dormancy and germination. Yi Chuan 38: 314-322, 2016.
78. Sosa MS, Parikh F, Maia AG, Estrada Y, Bosch A, Bragado P, Ekpin E, George A, Zheng Y, Lam HM, *et al*: NR2F1 controls tumour cell dormancy via SOX9- and RARbeta-driven quiescence programmes. Nat Commun 6: 6170, 2015.
79. Chambard JC, Lefloch R, Pouysségur J and Lenormand P: ERK implication in cell cycle regulation. Biochim Biophys Acta 1773: 1299-1310, 2007.

80. Yu C, Shiozawa Y, Taichman RS, McCauley LK, Pienta K and Keller E: Prostate cancer and parasitism of the bone hematopoietic stem cell niche. Crit Rev Eukaryot Gene Expr 22: 131-148, 2012.
81. Morrissey C, Vessella RL, Lange PH and Lam HM: The biology and clinical implications of prostate cancer dormancy and metastasis. J Mol Med (Berl) 94: 259-265, 2016.
82. Cackowski FC and Taichman RS: Minimal residual disease in prostate cancer. Adv Exp Med Biol 1100: 47-53, 2018.
83. Shiozawa Y, Pedersen EA, Patel LR, Ziegler AM, Havens AM, Jung Y, Wang J, Zalucha S, Loberg RD, Pienta KJ and Taichman RS: GAS6/AXL axis regulates prostate cancer invasion, proliferation, and survival in the bone marrow niche. Neoplasia 12: 116-127, 2010.
84. Jung Y, Shiozawa Y, Wang J, McGregor N, Dai J, Park SI, Berry JE, Havens AM, Joseph J, Kim JK, *et al*: Prevalence of prostate cancer metastases after intravenous inoculation provides clues into the molecular basis of dormancy in the bone marrow microenvironment. Neoplasia 14: 429-439, 2012.
85. Jung Y, Decker AM, Wang J, Lee E, Kana LA, Yumoto K, Cackowski FC, Rhee J, Carmeliet P, Buttitta L, *et al*: Endogenous GAS6 and Mer receptor signaling regulate prostate cancer stem cells in bone marrow. Oncotarget 7: 25698-25711, 2016.
86. Cackowski FC and Taichman RS: Parallels between hematopoietic stem cell and prostate cancer disseminated tumor cell regulation. Bone 119: 82-86, 2019.