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Nuclear Receptor 4A1 (NR4A1) Antagonists Target Paraspeckle Component 1 (PSPC1) in Cancer Cells

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Abstract

Paraspeckles compound 1 (PSPC1) is a multifunctional protein that plays an important role in cancer cells where PSPC1 is a master regulator of pro-oncogenic responses that includes activation of TGFβ (TGFβ1) and TGFβ-dependent EMT and metastasis. The pro-oncogenic activities of PSPC1 closely resembled those observed for the orphan nuclear receptor 4A1 (NR4A1, Nur77) and knockdown of NR4A1 decreased expression of PSPC1 in MDA-MB-231 breast, H1299 lung and SNU449 liver cancer cells. Similar results were observed in these same cell lines after treatment with bis-indole – derived (CDIMs) NR4A1 antagonists. Moreover, PSPC1-dependent regulation of TGFβ, genes associated with cancer stem cells and epithelial to mesenchymal transition (EMT) were also downregulated after NR4A1 silencing or treatment of breast, lung and liver cancer cells with CDIM/NR4A1 antagonists. Results of chromatin immunoprecipitation (ChIP) assays suggest that NR4A1 regulates PSPC1 through interaction with an NBRE sequence in the PSPC1 gene promoter. These results coupled with in vivo studies showing that NR4A1 antagonists inhibit breast tumor growth and downregulate PSPC1 in tumors indicate that the pro-oncogenic nuclear PSPC1 factor can be targeted by CDIM/NR4A1 antagonists.

Keywords

NR4A1; Nur77; PSPC1; ligands; antagonists

1. INTRODUCTION

Paraspeckle compound 1 (PSPC1) was initially identified as a multifunctional component of nuclear bodies called paraspeckles which also contain PSPC1, splicing factor proline and glutamine-rich (SFPQ) and non-POU-domain containing octamer binding protein

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(NONO) (1–6). These proteins form a complex with the long non-coding RNA Neat1 and their functions include regulation of the interleukin-6/STAT3 proinflammatory pathway in hepatocellular carcinoma (HCC) cells. Both NEAT1 and NONO mRNAs are more highly expressed in human HCCs compared to normal liver (7, 8). PSPC1 was overexpressed in breast, lung and liver tumors and was negative prognostic factor for survival of breast, lung and liver cancer patients (9). There was evidence from in vivo and tail-vein injection models (breast, lung and liver cancer) that PSPC1 plays a critical role in cancer cell growth and metastasis, and regulates genes associated with epithelial-mesenchymal transition (EMT) and cancer stem cells. This was confirmed in cell culture models by knockdown of PSPC1 and by subsequent analysis of changes in gene expression. PSPC1 also enhances secretion of TGFβ1 and enhances SMAD-dependent activation of pro-metastatic genes and PSPC1 interaction with PTK6 resulted in inhibition of PSPC1-mediated pro-oncogenic responses. Overexpression of PSPC1 or inhibition of PSPC1-PTK6 interactions results in nuclear export of PTK6 and nuclear import of β-catenin. This results in activation of PSPC1/β-catenin-regulated genes associated with EMT, stemness and metastasis (10). Thus, PSPC1 is a master regulator of pro-oncogenic genes and pathways (9–12) and clearly an important target for the development of novel cancer therapeutic drugs which have hitherto not yet been identified.

The orphan nuclear receptors NR4A1, NR4A2 and NR4A3 are immediate early genes induced by multiple stressors, and the NR4A receptors play an important role in maintaining cellular homeostasis and disease. There is increasing evidence for the role of these receptors in metabolic, cardiovascular and neurological functions as well as in inflammation and inflammatory diseases, immune functions and cancer (13–26). NR4A1 is overexpressed in lung, colon, liver and breast cancers and in Rhabdomyosarcoma and is a negative prognostic factor for lung, breast and colon cancer patient survival (15, 16, 22, 27–31). Ongoing research in our laboratories has been focused on the role of nuclear receptor 4A1 (NR4A1) in cancer and metabolic diseases. Results of either receptor knockdown or overexpression show that NR4A1 regulates cancer cell proliferation, survival, cell cycle progression, migration, and invasion in lung, melanoma, lymphoma, pancreatic, colon, cervical, ovarian, and gastric cancer cell lines, (14, 16–26, 32–38). Thus, like PSPC1, NR4A1 is overexpressed in various tumors, is a negative prognostic factor for some cancer patients and plays a role in several pro-oncogenic pathways. In addition, we have also previously identified a series of 1,1-bis(3’-indolyl)-1-(p-substituted phenyl)methane (C-DIM) compounds typified by the p-hydroxyphenyl analog (DIM8 or DIM-C-pPhOH) which are NR4A1 ligands that act as antagonists in cancer cells and inhibit NR4A1-mediated pathways/genes (19–26, 30, 32–38). The C-DIM compounds represent a unique group of mechanism-based agents that inhibit multiple NR4A1-regulated pro-oncogenic pathways and exhibit characteristics of selective receptor modulators which have been identified for other nuclear receptors (39, 40).

Thus, since both PSPC1 and NR4A1 regulate many of the same pro-oncogenic pathways in cancer cells and are overexpressed in many of these same tumors we hypothesized possible interactions between these two genes and this study reports that NR4A1 regulates expression of PSPC1 in cancer cell lines and PSPC1 can be targeted by CDIM/NR4A1 antagonists.

2. MATERIALS AND METHODS

2.1. Cell Lines, Reagents and Antibodies:

Human mammary MDA-MB-231 and MDA-MB468, lung A549, H1299 and H460, liver SNU449, HUH7 and HepG2, colon HCT116 and prostate PC3 cancer cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA) SUM158PT and HS159 breast cancer cells were provided by Dr. Weston Porter, Texas A&M University (College Station, TX); 4T1 mouse mammary cancer cells were provided by Dr. Mein-Chie Hung, MD Anderson Cancer Center (Houston, TX) and Ishikawa (Ish) and Hec1B endometrial cancer cells were provided by Dr. Russell Broaddus, MD Anderson Cancer Center. All cell lines originated from ATCC and were maintained in DMEM and RPMI growth medium supplemented with 10% FBS at 37˚C in CO2 incubator in an atmosphere of humidified 5% CO2 and 95% air and 1× antibiotic/antimycotic solution (Sigma-Aldrich, St Louis, MO). BioCoat Matrigel Invasion Chambers was purchased from Corning (Bedford, MA). NR4A1 (LS-C118011) antibody was purchased from LS Bio (Seattle, WA). Snail (3879) and Slug (9585) antibodies were purchased from Cell Signaling Technology (Danvers, MA, US). SOX2 (GTX101507), OCT4 (GTX101497), Twist1 (GTX60776) and Nanog (GTX100863) were purchased from GeneTex, Inc. (Irvine, CA, US). PSPC1 (sc-374181) was purchased from Santacruz Biotechnology (Santacruz, CA, US). β-actin (A5316) from Sigma Aldrich Corporation (Milwaukee, WI, US). Secondary antibodies for rabbit (7074) and mouse (7076) and anti-mouse (4408) Alexa Fluor conjugated were purchased from Cell Signaling Technology (Danvers, MA, US). siNR4A1 oligonucleotides used for NR4A1 in this study were siNR4A1 (1) 5'-CAGUGGCUCUGACUACUAU- 3' and siNR4A1 (2) 5'- GAGAGCUAUUCCAUGCCUA -3' and for PSPC1 were SASI_Hs02_00309618 and SASI_Hs02_00309619 and nontargeted scrambled small interfering ribonucleic acids (siRNA; iGL2) were used as controls (Sigma-Aldrich). The two ligands DIM-C-pPhOH and DIM-C-3-Br-5- OCF3 were synthesized by condensation of indole with p-hydroxybenzaldehyde or 3-bromo-5- trifluoromethoxy-p-benzaldehyde as described (13, 41, 42).

2.2. Western Blotting:

MDA-MB-231, H1299 and SNU449 cells were plated on 6-well plates at $2X10^5$ per well in DMEM or RPMI supplemented with 2.5% charcoal-stripped FBS and allowed to attach for 24 hours and cells were then treated for 24 hours with either DMSO or different concentrations of DIM-C-pPhOH or DIM-C-3-Br-5-OCF3. Cells were then lysed and whole cell lysates were resolved in 10% SDS-PAGE gels and proteins were transferred using PVDF membrane by wet blotting followed by primary and secondary antibody incubation and detected using ECL reagent as described.

2.3. RNA Interference:

MDA-MB-231, H1299 and SNU449 cells were seeded in six-well plates and allowed to grow to 60% confluence (24 hours), then transfections were performed with Lipofectamine 2000 according to the manufacturer's protocol. Both siNR4A1 and siPSPC1 oligonucleotides and nontargeted scrambled small interfering RNAs were used. The sequences used were Six hours after transfection, the medium was replaced with fresh

medium and left for 72 hours and cells were harvested and protein expression was determined.

2.4. Boyden chamber assay:

MDA-MB-231, H1299 and SNU449 (2.0×10^5 per well) were seeded in DMEM or RPMI medium supplemented with 2.5% charcoal-stripped fetal bovine serum and allowed to attach for 24 hours and subsequently treated with various concentrations of DIMC-pPhOH or DIM-C-3-Br-5-OCF3 for 24 hours. The cells were then trypsinized, counted, placed in 24-well 8.0-μm-pore-size Matrigel Invasion Chambers from Corning (Bedford, MA), allowed to migrate for 24 hours, fixed with formaldehyde, and then stained with hematoxylin. The attractant was the medium with 10% FBS in the bottom chamber. Cells that migrated through the pores were counted.

2.5. Luciferase Reporter Assay:

Cells (MDA-MB-231, H1299 and SNU449) were plated on 12-well plates at 1×105 per well in DMEM or RPMI supplemented with 2.5% charcoal-stripped FBS. After overnight attachment and growth, various amounts of DNA [i.e., UASx5-Luc (500 ng), GAL4-NR4A1 (50 ng)] were co-transfected into each well using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. After 6 hours of transfection, cells were treated with plating medium (as above) containing either solvent (DMSO) or indicated concentrations of compound for 18 hours. Cells were then lysed and cell extracts were used for chemiluminescence quantification of luciferase activity. Luciferase activity values were normalized against corresponding protein concentration values determined by Bradford assay. The DNA constructs contain full length NR4A1 coding sequence and all the plasmids used in this study were previously described (19, 41).

2.6. Immunofluorescence:

MDA-MB-231, H1299 and SNU449 (0.7 × 105 per well) cells were seeded in Nunc chambered coverglass followed by various drug treatments. The cells were fixed with 4% paraformaldehyde in PBS for 20 minutes at 37°C. Cells were then blocked and incubated overnight with primary PSPC1 antibody in the buffer (5% bovine serum albumin in PBS) at 4°C, followed by incubation with Alexa Fluor®–conjugated secondary antibody at a dilution of 1:250 for 2 hours at room temperature. Finally, cells were observed using a Zeiss confocal fluorescence microscope.

2.7. Quantitative real-time polymerase chain reaction:

Total ribonucleic acid (RNA) was isolated from cultured cells according to the manufacturer's instructions (Zymo Research, Irvine, CA, US). The concentration and purity of the RNA samples were determined using a nanodrop spectrophotometer. Total RNA was reverse transcribed using iTaq Universal SYBR Green OneStep Kit (Thermo Fisher Scientific, Grand Island, NY, US) using the manufacturer's protocol with the CFX384 real-time polymerase chain reaction system (Bio-Rad). The comparative cycle threshold method was used for relative quantitation of samples. Values for each gene were normalized to expression levels of TATA-

binding protein. The sequences of the primers used for real-time polymerase chain reaction included the following: TGF-β1, 5'- GGAAATTGAGGGCTTTCGCC-3′ (sense) and 5′-CCGGTAGTGAACCCGTTGAT-3′ (antisense); TGF-β2, 5'-CTGTCCCTGCTGCACTTTTGT A-3′ (sense) and 5′- TGTGGAGGTGCCATCAAT ACC T-3′ (antisense); TGF-β3, 5'- TGGAAGTGGGTCCATGAAACCTA-3′ (sense) and 5′- GAT GCTTCAGGGTTAAGAGTGTTG-3′ (antisense); PSPC1, 5'-CTACGGATTCGCTTCGCTAC-3' (sense) and 5'-CTTTCGTGCAGGAGGTTTTG-3' (antisense); CHIP/PCR primers 5'- CGCCTGTAATCCCAACACTT-3' (sense) and 5'-CTGGGTTCAAGCAATTCTC −3' (antisense).

2.8. Chromatin Immunoprecipitation (ChIP) Assay:

MDA-MB-231, H1299 and SNU449 cells (5X106) were plated and treated with DIM-C-pPhOH or DIM-C-3-Br-5-OCF3 for 3 h and subjected to ChIP analysis using the ChIP-IT Express magnetic chromatin immunoprecipitation kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocol. The primers for detecting the PSPC1 promoter were 5'- CGCCTGTAATCCAACACTT-3' (sense) and 5'- CTGGGTTCAAGCAATTCTCC −3' (antisense). PCR products were resolved on a 2.5% agarose gel in the presence of ethidium bromide.

2.9. Athymic nude mouse xenograft:

Female athymic nu/nu mice of 4–6 weeks old were purchased from Charles River Laboratories (Wilmington, MA). MDA-MB-231 cells were harvested and suspended at a concentration of 1.5 × 106 cells in 100 μl of DMEM with ice-cold Matrigel (1:1 ratio) ratio) and these cells were implanted orthotopically into the mammary fat pad region and after 2 weeks, mice were divided in to two groups of 7 animals each. One group received 100 μL of vehicle (corn oil), and the other group received an injection of 35 mg/kg/day DIM-C-pPhOH in 100 μL volume of corn oil intraperitoneally for three weeks. All mice were weighed once a week over the course of treatment to monitor changes in body weight. Tumor volumes were measured using Vernier caliper over the period of treatment and later calculated. After three weeks of treatment, mice were sacrificed and tumor weights were determined. All animal studies were approved by and carried out according to the procedures mandated by the Texas A&M University Institutional Animal Care and Use Committee.

2.10. Statistical analysis:

All of the experiments were repeated a minimum of three times. The data are expressed as the mean ± standard error (SE). Analysis of variance was done using GraphPad Prism software was used to determine statistical significance. *$p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ values were considered statistically significant. Statistical significance is indicated in the Figures; *$p<0.05$, **$p<0.01$, ***$p<0.001$ and ****$p<0.0001$.

3. RESULTS

3.1. NR4A1 knockdown or NR4A1 antagonists decrease PSPC1 protein

Since PSPC1 and NR4A1 exhibit comparable pro-oncogenic activities in cancer cells we initially investigated the expression of PSPC1 in multiple cancer cell lines and compared the

results with NR4A1 expression in the same cell lines (Fig. 1A). Western blots of cell lysates from breast (HS159, SUM158PT, MDA-MB-231, MDA-MB-468 and 4T1), lung (A549, H1299, andH460), liver (SNU449, HUH7 and HepG2), endometrial (Ishikawa and Hec 1B), colon (HCT116) and prostate (PC3) cancer cells show that both NR4A1 and PSPC1 are expressed in these cell lines. Relative NR4A1 and PSPC1 band intensities are summarized in Supplemental Figure 1. The potential relationship between NR4A1 and PSPC1 was further investigated in MDA-MB-231 (breast), H1299 (lung) and SNU449 (liver) cancer cells and knockdown of NR4A1 by RNA interference (RNAi) decreased expression of PSPC1 protein in MDA-MB-231, H1299 and SNU449 cells (Fig. 1B). Knockdown of PSPC1 in MDA-MB-231, H1299 and SNU449 cells by RNAi also decreased expression of the target gene however, levels of NR4A1 were unchanged (Fig. 1C) suggesting that NR4A1 plays a role in regulation of PSPC1.

Previous studies have identified 1,1-bis(3′-indolyl)-1-(4-hydroxy-phenyl)methane (DIM-C-pPhOH, DIM8) and 1,1-bis(3′-indolyl)-1-(3-bromo-5-trifluoromethoxyphenyl)methane (DIM-C-3-Br-5-OCF3) as NR4A1 ligands (16, 18, 19, 42) and Figures 2A–2C illustrate effects of both CDIMs on NR4A1-dependent transactivation. Cells were transfected with a GAL4-NR4A1 (ligand binding domain) chimera and a UAS-Luc reporter construct containing 5 tandem GAL4 response elements and both CDIM compounds decreased NR4A1-dependent transactivation in MDA-MB-231, H1299 and SNU449 cells (Figs. 2A–2C). Treatment of MDA-MB-231, H1299 and SNU449 cells with DIM-C-pPhOH and DIM-C-3-Br-5-OCF3 decreased expression of PSPC1 protein (Fig. 2D). We also examined the effects of CDIMs on PSPC1 expression and location by immunostaining and showed that PSPC1 is nuclear and both CDIMs decreased nuclear staining of PSPC1 which colocalizes with Hoechst staining in MDA-MB-231 (Fig. 3A) H1299 (Fig. 3B) and SNU449 (Fig. 3C) cells. Both NR4A1 antagonists significantly decreased PSPC1 staining in all 3 cell lines. These results are consistent with PSPC1 being an NR4A1 regulated gene that can be targeted by either NR4A1 knockdown or treatment with NR4A1 antagonists.

3.2. NR4A1 knockdown or antagonists decrease PSPC1 regulated genes/gene products.

Previous studies show that PSPC1 regulates TGFβ expression in breast, liver and lung cancer cells (9–12) and Figure 4A illustrates that PSPC1 knockdown by RNA interference significantly decreased expression of TGFβ mRNA levels in MDA-MB-231, H1299 and SNU449 cells. Similar results were observed in cells transfected with oligonucleotides targeting knockdown of NR4A1 by RNAi (Fig. 4B) or by treatment with the NR4A1 antagonists DIM-C-pPhOH and DIM-C-3-Br-5-OCF3 (Fig. 4C). Parallel experiments were determined for the effects of siPSPC1, siNR4A1 and DIM-C-pPhOH/DIM-C-3Br-5-OCF_3 on TGFβ2 (Supplemental Figures 2A–2C) and TGFβ3 (Supplemental Figure 3A–3C). Coordinate downregulation of TGFβ2 by all 3 treatments was observed only in SNU449 cells whereas knockdown of NR4A1, PSPC1 or treatment with NR4A1 antagonist did not coordinately decrease TGFβ3 in MDA-MB-231, H1299 and SNU449 cells. However, with the exception of TGFβ3 in SNU449 cells knockdown of PSPC1 decreased TGFβ2 in all 3 cell lines and decreased TGFβ3 in MDA-MB-231 and H1299 cells demonstrating that PSPC1 also regulated TGFβ2 and TGFβ3 mRNAs. The PSPC1-TGFβ pathway also regulates genes associated with epithelial to mesenchymal transition (EMT) (Snail, Slug and

Twist) and cancer stem cells (Nanog, Oct4 and Sox2) (9) and results in Figure 5A show that knockdown of PSPC1 in MDA-MB-231 breast cancer cells decreased expression of Snail, Slug, Twist, Nanog, Oct4 and Sox2. In parallel, studies we observed that DIM-C-pPhOH (20 μM) and DIM-C-3-Br-5-OCF3 (10 μM) also decreased expression of the same set of genes associated with EMT and cancer stem cells and the latter compound was the more potent NR4A1 antagonist in this cell line (Fig. 5B). PSPC1 knockdown by RNAi in H1299 cells was approximately 50% efficient with both oligonucleotides, however, with the exception of Oct4, the gene products associated with EMT and cancer stem cells were decreased by siPSPC1 and they were also decreased after treatment with CDIM/NR4A1 antagonists (Fig. 5A and 5B). In SNU449 cells, knockdown of PSPC1 and treatment with NR4A1 antagonists decreased expression of Snail, Slug, Twist, Nanog, Oct4 and Sox2 proteins (Fig. 5A and 5B). Decreased expression of the EMT genes in these cell lines was observed after treatment with CDIM/NR4A1 antagonists and this same treatment decreased invasion of MDA-MB-231, H1299 and SNU449 cells in a Boyden chamber assay (Fig. 5C). These results are consistent with the decreased expression of the PSPC1 gene which regulates pro-invasion genes (7–12).

The PSPC1 gene promoter contains an NGF1β response element (NBRE) sequence that binds NR4A1 monomer and treatment of MDA-MB-231, 1H1299 and SNU99 cells with DIM-C-pPhOH or DIM-C-3-Br-5-OCF3 significantly decreased expression of PSPC1 mRNA in all 3 cell lines (Fig. 6A). A ChIP assay was used to investigate NR4A1 interactions with the PSPC1 promoter and using primers that encompass the NBRE sequence in the PSPC1 gene promoter (Fig. 6B). After treating cells with DIM-C-pPhOH or DIM-C-3-Br-5-OCF_3 the ChIP assay showed that there was decreased binding of NR4A1 to the promoter and this was also accompanied by a decrease in polII. These results are consistent with a mechanism of PSPC1 regulation through a classical receptor interaction with cognate cis elements and contrasts to previous studies in this laboratory showing that NR4A1/Sp binding to GC-rich elements regulates expression of PAX3-FOX01, survivin, PD-L1 and several integrins in cancer cells (21–23, 43, 44).

We also investigated the effects of DIM-C-pPhOH (35 mg/kg/d) on tumor growth in an orthotopic model in athymic nude mice bearing MDA-MB-231 cells. Compared to corn oil controls DIM-C-pPhOH treatment inhibited tumor weight (Fig. 7A and 7B), tumor volume (Fig. 7C) and decreased expression of PSPC1 and some downstream genes in tumor lysates. We have previously reported that DIM-C-3-Br-5-OCF3 (1 mg/kg/d) also inhibited breast tumor growth in the same mouse model (42) and lysates from these tumors also showed that PSPC1 and downstream gene products were decreased (Fig. 7D). These results demonstrate that the multiple anticancer activities reported for NR4A1 antagonists (45) are due, in part, to antagonism of NR4A1-dependent regulation of PSPC1 and that CDIM/NR4A1 antagonists represent a novel class of drugs that target PSPC1.

4. DISCUSSION

Both PSPC1 and NR4A1 play pro-oncogenic roles in cancer and influence expression of genes and pathways associated with cancer cell proliferation, survival and migration/invasion (7–13, 45). A recent study showed that high expression of PSPC1 is a negative

prognostic factor for liver, lung and breast cancer patient survival (9) and similar results have been reported for NR4A1 in the latter two tumor types (14, 29). In addition, PSPC1 and NR4A1 play a role in TGFβ signaling where PSPC1 induces TGFβ expression and enhances activation of prometastic pathways including EMT (9). Moreover, in hepatocellular carcinoma, upregulation of PSPC1 promotes EMT and stemness via nuclear export of PTK6 and nuclear import of β-catenin (10). In breast and lung cancer cells TGFβ-induced EMT and invasion is due to increased phosphorylation of NR4A1 (S351) which results in nuclear export of the receptor which in turn forms a complex with Axin2, Arkadia and RNF12 to activate proteasome-dependent degradation of inhibitory SMAD7 (25, 26). In breast cancer cells TGFβ activates MKK3/MKK6 and p38 which in turn phosphorylates NR4A1 (25) whereas in lung cancer cells phosphorylation of NR4A1 is dependent on TGFβ activation of MKK4/7 and JNK (26). The CDIM/NR4A1 antagonists block TGFβ-induced pro-oncogenic pathways in lung and breast cancer cells by inhibiting nuclear export of NR4A1.

Thus, both PSPC1 and NR4A1 are modulators of TGFβ signaling and highly expressed in multiple tumors and solid-tumor derived cell lines (Fig. 1A) and in this study we investigated possible interrelationships between these two pro-oncogenic factors. Results illustrated in Figures 1B and 1C demonstrate that knockdown of NR4A1 and RNAi decreased PSPC1 expression whereas decreased PSPC1 had minimal effects on NR4A1 levels demonstrating that NR4A1 either directly or indirectly regulated PSPC1 expression in MDA-MB-231, H1299 and SNU449 cells. Previous studies show that CDIM/NR4A1 ligands such as DIM-C-pPhOH and DIM-C-3-Br-5-OCF3 act as NR4A1 antagonist in cancer cells (16, 18, 19 40) and both compounds decreased NR4A1-dependent transactivation and decreased expression of PSPC1 as observed in western blot and immunostaining in MDA-MB-231, H1299 and SNU449 cells (Figs. 2 and 3). Previous studies have shown that NR4A1 regulates expression of several genes including PAX3-FOX01, survivin and several integrins through an NR4A1/Sp1 or NR4A1/Sp4 complex bound to GC-rich promoter sequences that bind Sp transcription factor. Thus, NR4A1 acts as a cofactor of Sp-dependent transactivation and this has previously been observed as a mode of action of several other nuclear receptors (46). Although both DIM-C-pPhOH and DIM-3-Br-5-OCF3 decrease expression of PSPC1 mRNA, the proximal region of the PSPC1 promoter does not contain GC-rich sites and results of ChIP assays suggest that NR4A1 regulates PSPC1 through binding on NBRE site at −498 to −492 in the PSPC1 promoter (Fig. 6). Further confirmation of NR4A1-PSPC1 interaction was observed in studies showing that NR4A1 antagonists also inhibited expression of TGFβ mRNA. Regulation of PSPC1 by NR4A1 was observed for TGFβ2 (but not TGFβ3) in SNU449 cells but not in MDA-MB-231 or H1299 cells and regulation of TGFβ2 and TGFβ3 by PSPC1 is cell context-dependent (Supplemental Figures 2 and 3). NR4A1-PSPC1 interactions also regulate EMT and stem cell related gene products in cancer cells (Figs. 4 and 5) and in tumors (EMT/stem cell gene products) (Fig. 7). These responses have previously been characterized as PSPC1-regulated genes/gene products (9–11) and our results indicate that they can be targeted by CDIM/NR4A1 antagonist.

In summary this study confirms previous reports on the pro-oncogenic activities of PSPC1 and its regulation of TGFβ and TGFβ pathways in cancer cells (9–12). It was previously concluded that PSPC1 is a master activator of prometastic switches and a potential target

for anti-metastasic drugs (9). Our results now show that PSPC1 can be targeted by CDIM/NR4A1 antagonists which decrease expression of PSPC1 and PSPC1-mediated responses by inhibiting NR4A1, the upstream regulator of PSPC1. These results add to the number of important NR4A1-regulated pro-oncogenic factors that can be inhibited in cancer cells by CDIM/NR4A1 antagonist which also enhance immune surveillance by downregulating PD-L1 (44).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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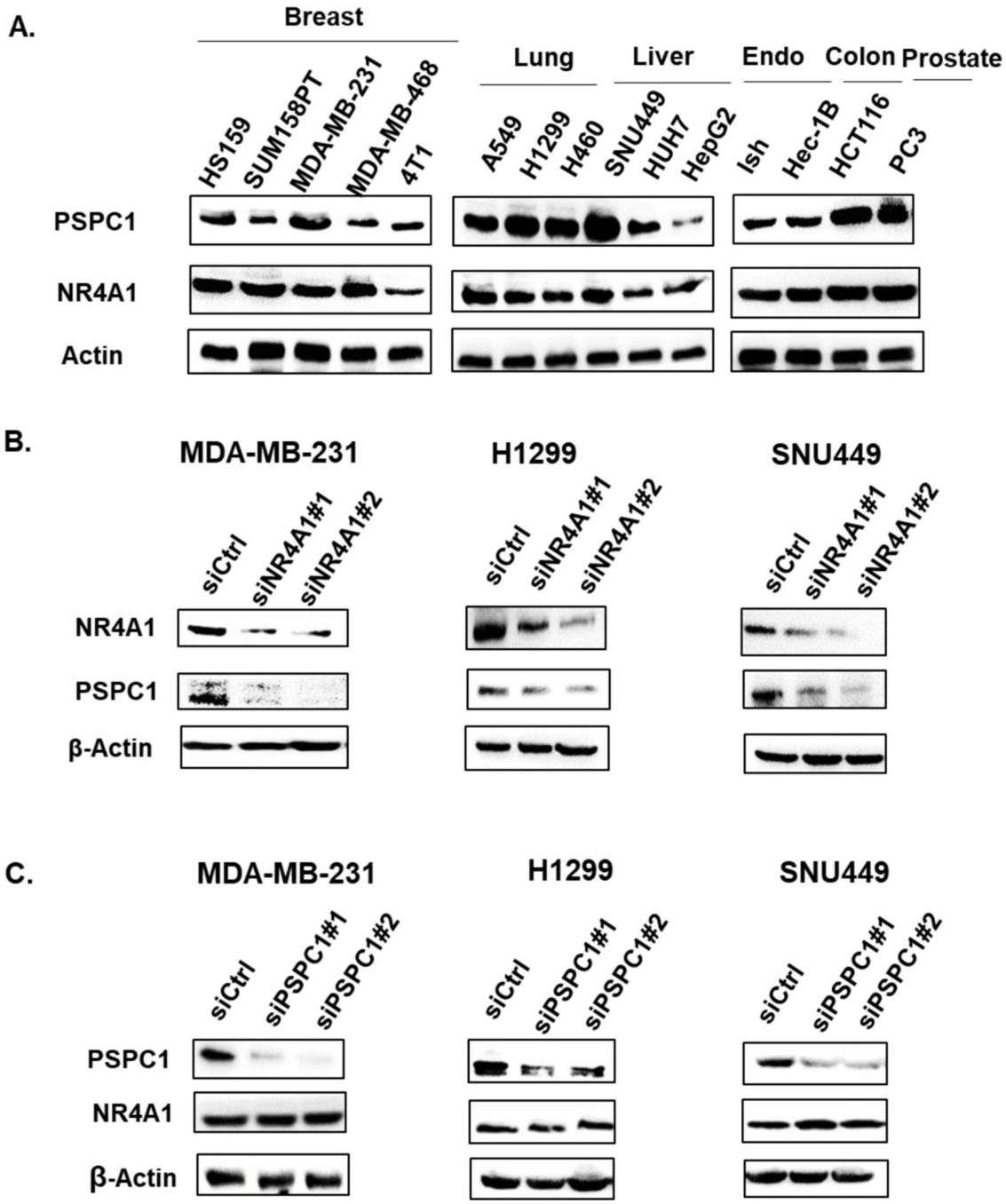

Figure 1.

NR4A1 and PSPC1 expression in cancer cells. A. whole cell lysates from several solid tumor derived cancer cell lines were analyzed by western blots for expression of NR4A1 and PSPC1. MDA-MB-231, H1299 and SNU449 cells were transfected with small interfering oligonucleotides targeting NR4A1 (siNR4A1 #1 and siNR4A1 #2) (B) and PSPC1 (siPSPC1 #1 and siPSPC1 #2) (C) and after 72 hours whole cell lysates were analyzed by western blots.

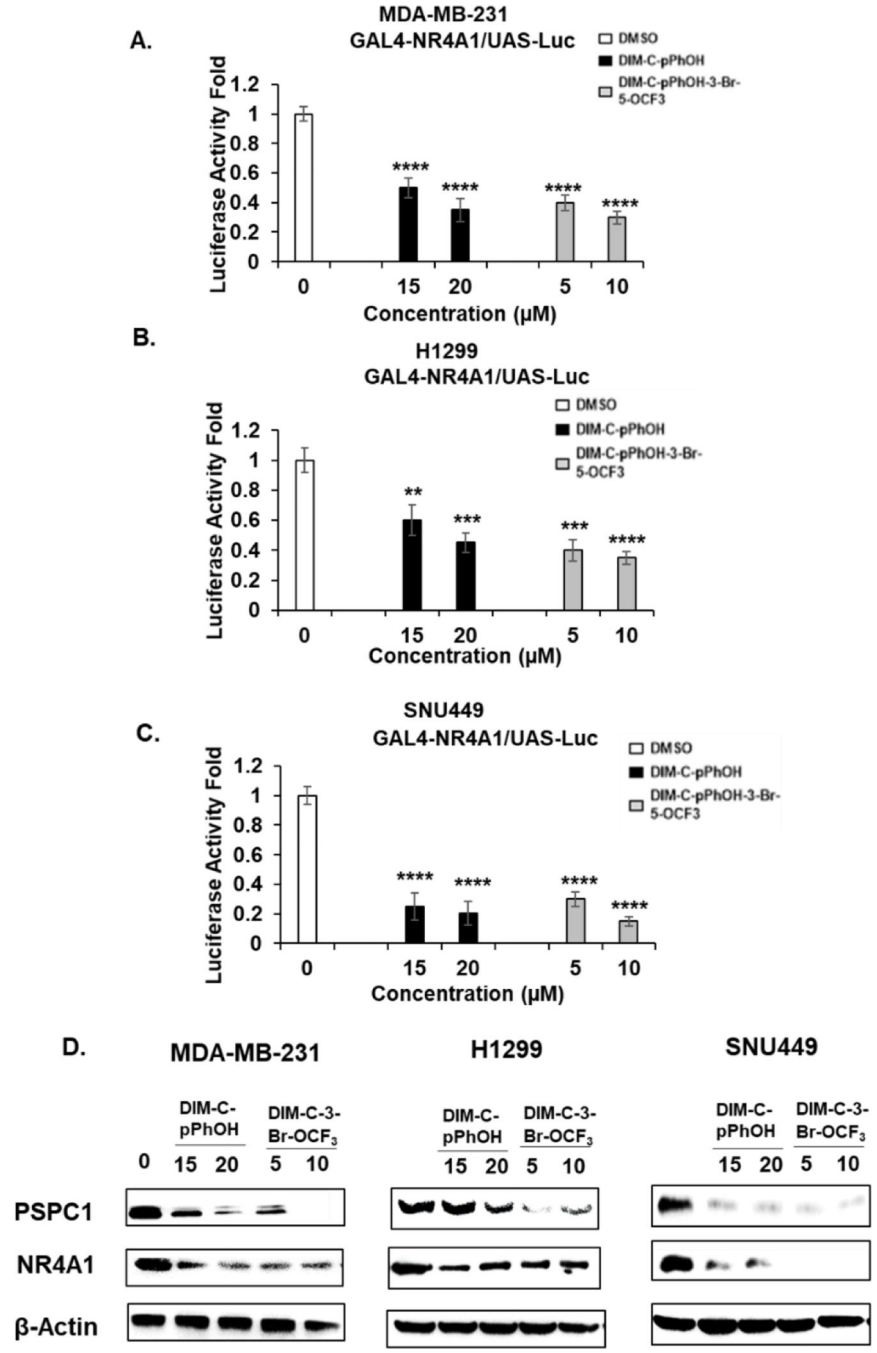

Figure 2.

CDIM/NR4A1 antagonists inhibit transactivation and PSPC1. MDA-MB-231 (A), H1299 (B) and SNU449 (C) cells were transfected with GAL4-NR4A1 (LBD) and UAS-luc constructs and after treatment DIM-C-pPhOH or DIM-C-3-Br-5-OCF3 luciferase activity was determined as outlined in the Methods. D. Cells were treated with DIM-C-pPhOH and DIM-C-3-Br-5-OCF3 for 24 hours and whole cell lysates were analyzed by western blots. Results (A-C) are expressed as means ± SE for at least 3 determinations and significant ($p<0.05$) decreases are indicated (*).

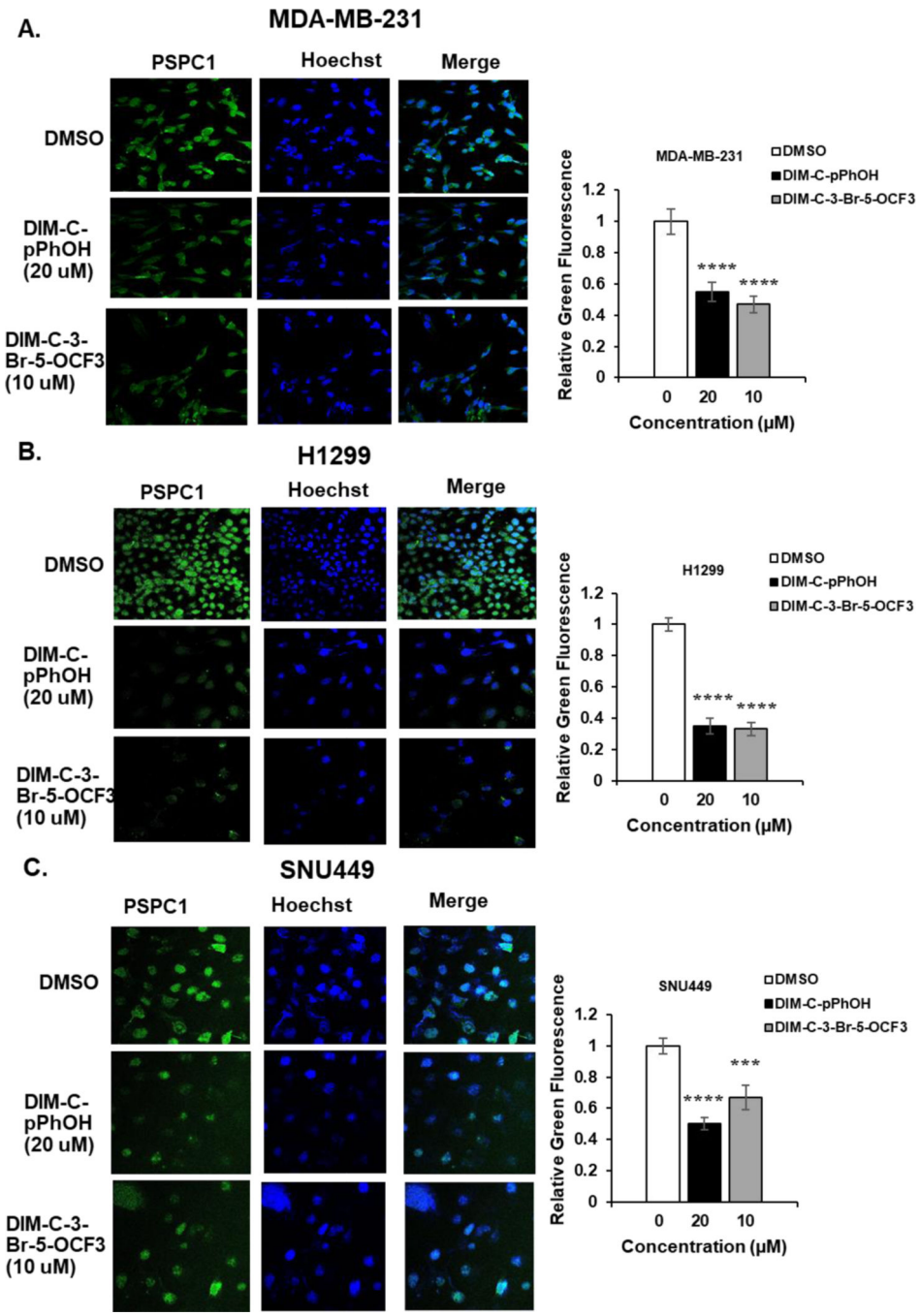

Figure 3.

CDIM/NR4A1 antagonists decrease nuclear PSPC1 expression in cancer cells. MDA-MB-231 (A), H1299 (B) and SNU449 (C) cells were treated with DMSO, 20 μM DIM-C-pPhOH and 10 μM DIM-C-3-Br-5-OCF3 for 24 hours, cells were fixed and stained with PSPC1 antibodies or Hoechst dye.

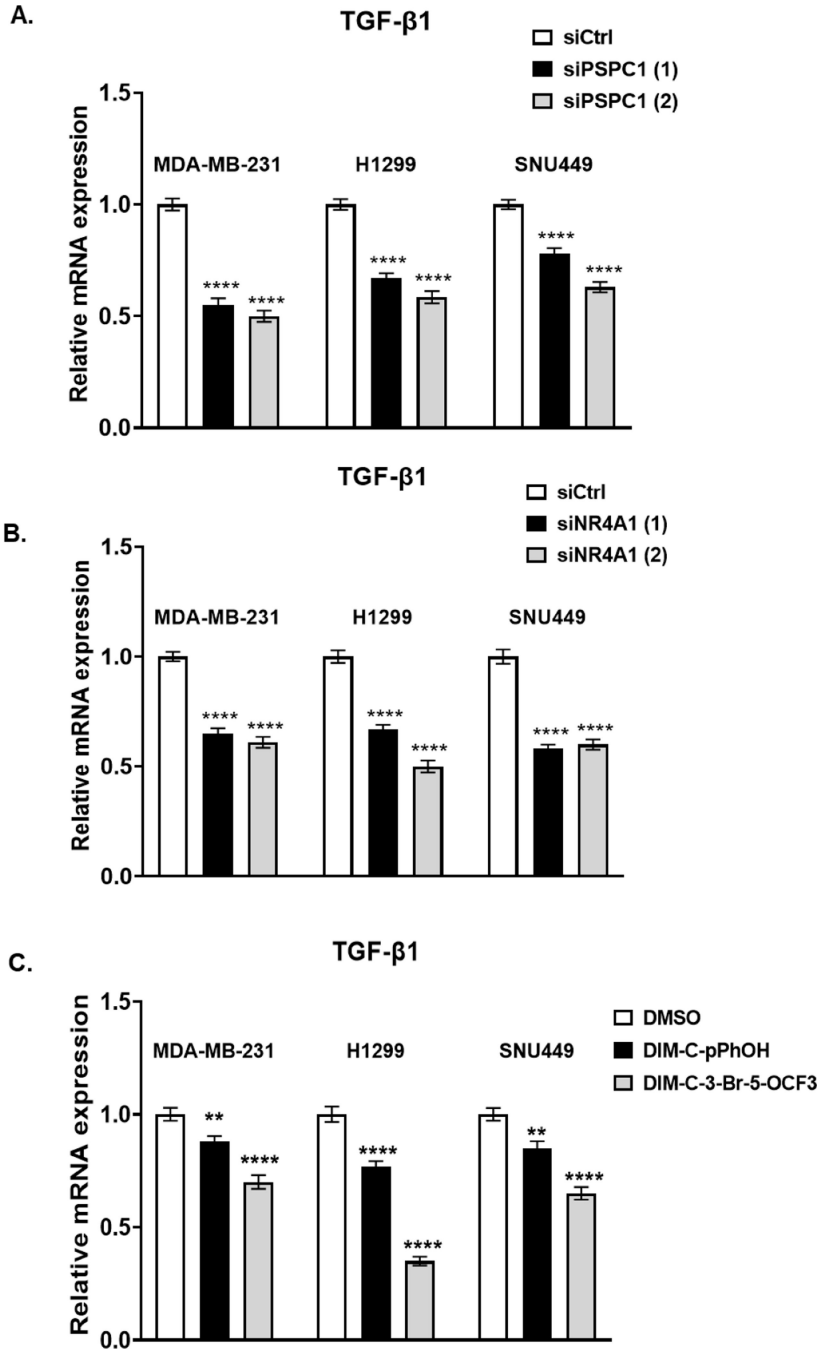

Figure 4.

NR4A1 and PSPC1 regulate TGFβ gene expression. MDA-MB-231, H1299 and SNU449 cells were transfected with oligonucleotides (2) that targeted PSPC1 (A) and NR4A1 (B) or treated (for 24 hours) with DIM-C-pPhOH and DIM-C-3-Br-5-OCF3 (C) and RNA was isolated and analyzed for expression of TGFβ mRNA levels by real time PCR. Results are expressed as means ± SE for at least 3 separate determinations for each treatment group and significant ($p<0.05$) decreases in gene expression are indicated (*).

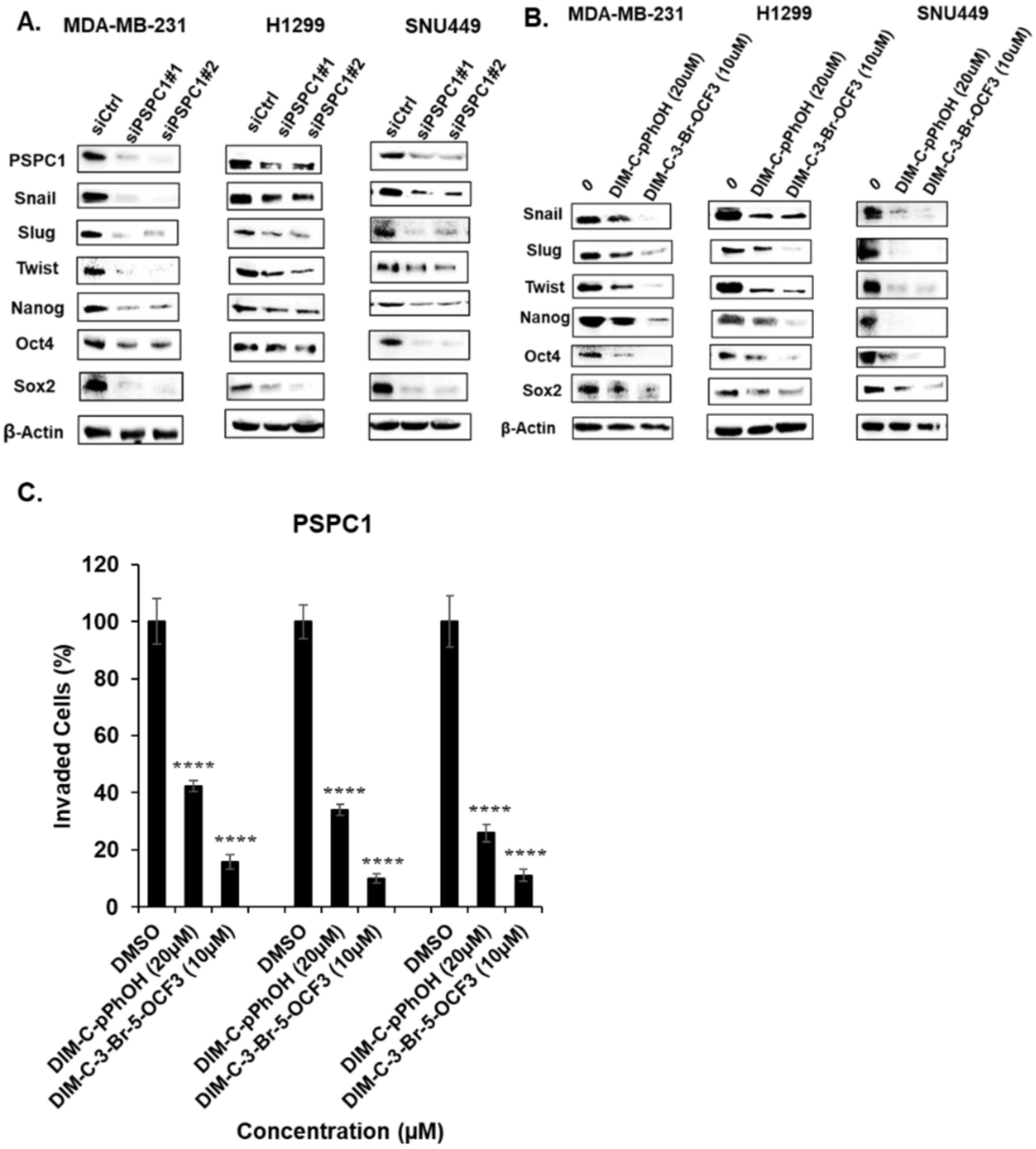

Figure 5.

CDIM/NR4A1 antagonists decrease PSPC1-regulated EMT and cancer stem cell gene products and cancer cell invasion. MDA-MB-231, H1299 and SNU449 cells were transfected with oligonucleotides targeting PSPC1 (A) or treated with the NR4A1 antagonists DIM-C-pPhOH (20 μM) or DIM-C-3-Br-5-OCF3 (10 μM) (B) and whole cell lysates were analyzed by western blots as outlined in the Methods. C. MDA-MB-231, H1299 and SNU449 cells were treated with DMSO, 20 μM DIM-C-pPhOH and 10 μM DIM-C-3-Br-5-OCF3 and inhibition of cancer cell invasion was determined in a Boyden

chamber assay. Results are expressed as means ± SE for at least 3 determinations per treatment group and significant ($p<0.05$) inhibition is indicated (*). Western blots of lysates from siPSPC1 studies outlined in Figures 1C and 5A were derived from the same experiment.

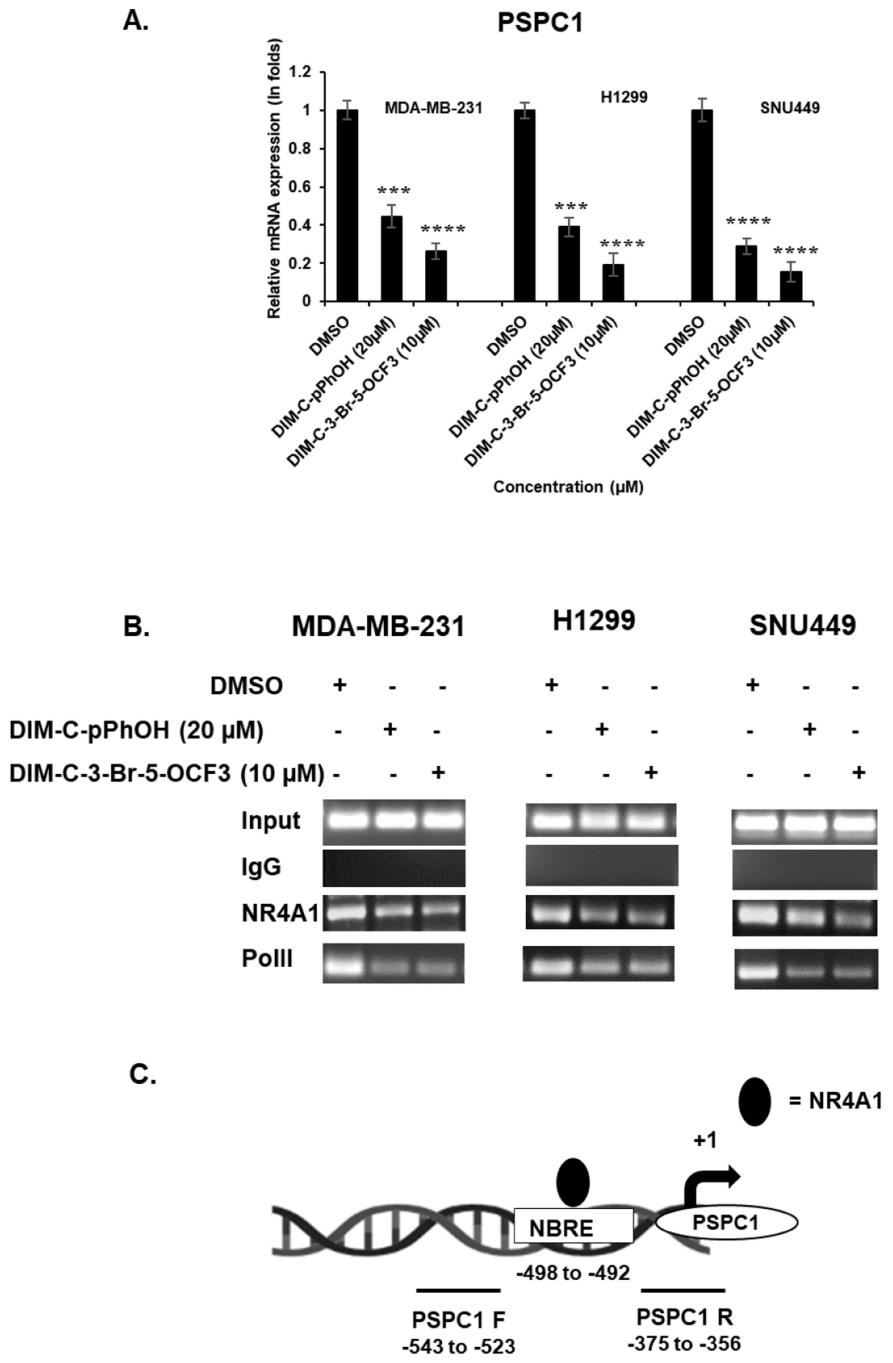

Figure 6.

Mechanism of NR4A1 regulation of PSPC1. A. Location of an NBRE in PSPC1 gene promoter. B. MDA-MB-231, H1299 and SNU449 cells were treated with 20 μM DIM-C-pPhOH and 10 μM DIM-C-3-Br-5-OCF3 for 24 hours and decreases in PSPC1 mRNA levels were determined by real time PCR. C. MDA-MB-231, H1299, and SNU449 cells were treated with 10 μM DIM-C-3-Br-5-OCF3 and 20 μM DIM-C-pPhOH for 3 hours and primers encompassing the NBRE region in the PSPC1 promoter were used in a ChIP assay to detect NR4A1 and polII interactions with the NBRE promoter regions. Results (A) are

expressed as means ± SE for at least 3 determinations per treatment group and significant ($p<0.05$) decreases are indicated (*).

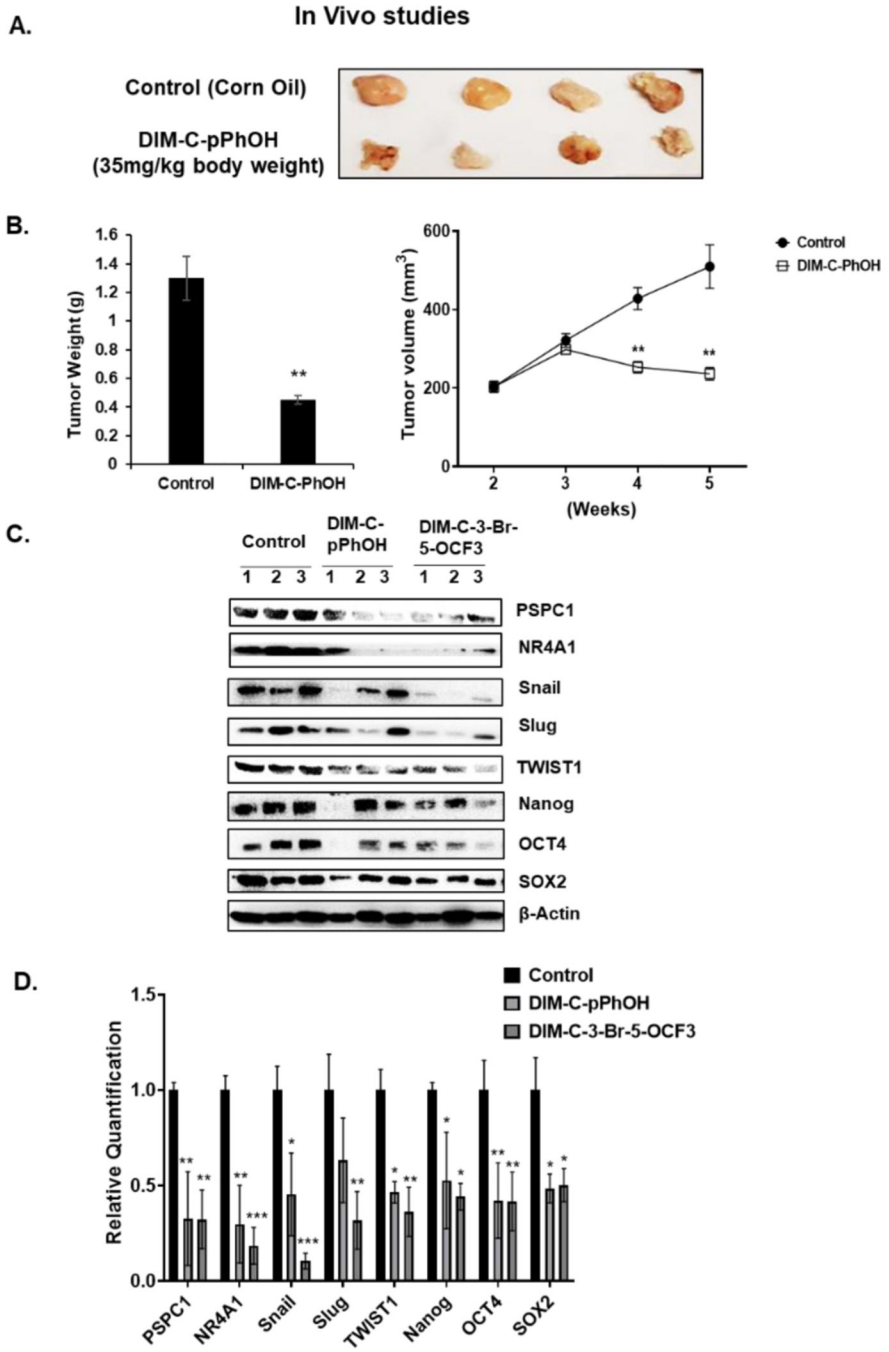

Figure 7.

In vivo studies. Athymic nude mice bearing MDA-MB-231 cells (orthotopic) were treated with corn oil (control) or DIM-C-pPhOH (35 mg/kg/d) for 21 days and effects on tumor size (A) weight (B) and volume (C) were determined as outlined in the Methods. D. Tumor lysates from control and DIM-C-pPhOH treated mice were analyzed by western blots. Lysates were also obtained from a separate study using the same cells/animal protocol (42) but treated with DIM-C-3-Br-5-OCF3 (1 mg/kg/d) were also analyzed in the same western blot. E. Protein levels were determined from replicate samples and quantitated relative to

β-actin and levels for control values were set at 1.0. At least 4 mice were used in each treatment group and significant ($p<0.05$) decreases are indicated (*).