The PPARγ Antagonist T0070907 Suppresses Breast Cancer Cell Proliferation and Motility *via* Both PPARγ-dependent and -independent Mechanisms

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Abstract. *Background: Peroxisome proliferator-activated receptor gamma (PPARγ) is overexpressed in many types of cancer, including breast cancer, and it is regulated by ligand binding and post-translational modifications. It was previously demonstrated that endogenous transactivation promotes an aggressive phenotype of malignant breast cells. This study examines whether selective antagonism of PPARγ with T0070907 is a potential strategy for breast cancer therapy. Materials and Methods: PPARγ activation was inhibited using both pharmacological and molecular approaches and proliferation, apoptosis, migration and invasion were measured in MDA-MB-231 and MCF-7 breast cancer cells. Results: T0070907 treatment inhibited proliferation, invasion and migration but did not significantly affect apoptosis. Molecular inhibition using a dominant negative (Δ462) receptor yielded similar results. T007 also mediated a dose-dependent decrease in phosphorylation of PPARγ, and its ability to bind to DNA, and may directly affect mitogen-activated protein kinase signaling. Conclusion: These data indicate that inhibiting endogenous PPARγ signaling may be a promising new approach to breast cancer therapy.*

Breast cancer is the most common malignancy and the second leading cause of cancer-related death among American women (1). Over the last few years advancements in treatment options, such as hormonal therapy, chemotherapy and radiation as well as development of unconventional approaches targeting the growth, migration, and invasion of cancer cells, have lead to significantly better outcomes for breast cancer patients. However,

the complexity and heterogeneity of this disease continues to pose difficult challenges not only to combat cancer growth at the primary site but also to prevent metastasis. Multiple signaling pathways have been under investigation in order to identify new, more specific targets for treatment of breast cancer.

Drugs that target the peroxisome proliferator-activated receptor gamma (PPARγ), a member of the nuclear-hormone receptor family, have been studied extensively as potential breast cancer therapies. There are two isoforms of the PPARγ protein that result from distinct transcriptional initiation sites in the same gene (2). PPARγ2 is predominantly expressed in adipose tissues (3), while PPARγ1 expression has been detected in many tissues of epithelial origin such as intestine, lung, breast, colon, and prostate (4, 5). It has been previously shown that the level of PPARγ expression is significantly higher in breast cancer cell lines compared to normal epithelial cells (5-9). High expression of PPARγ has also been reported in human breast cancer tissues (10, 11). Despite multiple, ongoing studies, the role of PPARγ in the development and progression of breast cancer remains unclear. As a ligand-activated transcription factor, PPARγ can be modulated by exogenous drugs such as the thiazolidinedione class of anti-diabetic drugs. Numerous groups have utilized this pharmacological approach to investigate the role of PPARγ activation in cancer cells (12-15). However, the use of exogenous PPARγ ligands raises concern about whether the observed effects are mediated through PPARγ or 'off-target' effects of the drugs (16-18). Moreover, one more layer of complexity has been added by the fact that in addition to classical regulation of PPARγ activity by ligands, PPARγ transcriptional activity can be affected by post translational modifications such as cross-talk with kinases and phosphatases (19, 20) and its interaction with other proteins in the cytoplasm that leads to non-genomic functions of PPARγ (21).

A genetic approach was employed to evaluate the consequences of PPARγ transactivation in breast cancer independent of exogenous stimulation. The results of these studies demonstrated that constitutive overexpression of

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PPARγ increases the incidence of breast cancer and accelerates mammary gland tumor development and death in mice already susceptible to the disease (22). It has also been shown that mice heterozygous for a null PPARγ mutation develop tumors with the same kinetics as those that carry two functional copies (22). Furthermore, ablation of PPARγ expression in the mouse mammary gland using a Cre-Lox recombination system has shown that no tumors developed in mammary glands lacking PPARγ, suggesting that PPARγ is not a tumor suppressor (23). These observations are supported by *in vitro* studies which demonstrated that inhibition of PPARγ expression by shRNA or inactivation by the dominant-negative mutant of PPARγ, Δ462, leads to a decrease in cellular proliferation and an increase in apoptosis in MFC-7 and T47D breast cancer cell lines (7). Together, these studies suggest that reduced PPARγ expression does not contribute to the initiation of breast cancer, but the acceleration of PPARγ signaling after tumor initiation markedly promotes breast cancer development. Thus, inhibition of endogenous activity of PPARγ may be beneficial in treatment of breast cancer.

T0070907 (T007; PubChem SID: 53790303) was identified as a potent and selective antagonist which shows a more than 800-fold preference for PPARγ over PPARα and PPARδ (24). The suppression of PPARγ activity by T007 has been demonstrated in cell-based reporter gene and functional assays (24-26). Moreover, antitumor activity was observed when cancer cells were treated with T007 (9, 27, 28). It has also been shown that inhibition of PPARγ by T007 suppressed pancreatic cell motility *in vitro* and invasion *in vivo* (26). Furthermore, T007 treatment inhibited cell adhesion and invasion, and induced anoikis in hepatocellular carcinoma cell lines, and these effects were PPARγ pathway-specific (25).

The present study investigates whether T007 treatment has significant antitumor effects in breast cancer cells and whether these effects are mediated by PPARγ. The data suggest that pharmacological inhibition of endogenous activity of PPARγ using selective antagonists such as T007 may provide a novel and effective therapeutic approach for breast cancer treatment.

Materials and Methods

Cell culture. MB-231 and MCF-7 breast cancer cells were obtained from the American Type Culture Collection (Rockville, MD, USA). MCF-7 cells were cultured in modified Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Hyclone/Thermo Scientific, Waltham, MA, USA). MDA-MB-231 cells were cultured in modified Iscove's modifed Eagle's medium (IMEM; Gibco/Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum. Cells were grown in medium lacking phenol red at 37°C in a 5% CO_2 atmosphere.

Reporter gene analysis. Cells were transiently transfected with 3.6 μg of pGL3 plasmid containing 3XPPRE-mTK-Luc and Renilla per plate and then co-transfected or treated according to the experimental design and protocol used. Cells were lysed in 80 μl of passive lysis buffer and treated according to the manufacturer's instructions (Promega Dual Luciferase assay kit; Promega, Madison, WI, USA). Luminometry was performed on a Berthold Technologies Lumat 9507 (Wildbad, Germany). Results were calculated as raw luciferase units divided by raw Renilla units. Data is presented as mean fold changes in treated cells as compared to control cells.

Transwell migration assays. Transwell inserts (8 μm pore size; Corning, NY, USA) were coated with collagen type I (BD Biosciences, Rockville, MD, USA). Cells were pre-treated with various concentrations of T007 or empty vehicle for 48 hours, then trypsinized and resuspended in serum-free DMEM containing 1% bovine serum albumin (BSA) to achieve a density of 3×10^5 cells/ml. Inserts were placed in wells containing normal growth media. A volume of 200 μl of cell suspension was added to the upper chamber and incubated at 37°C with 5% CO_2 for 6 hours. After incubation, the upper side of membrane was washed and wiped-off using cotton swabs and then cells on the lower membrane surface were fixed with methanol and stained with 0.5% crystal violet. The images of five random fields per well were taken using ×40 objective (Nikon Eclipse TE200, Nuance 2.8 Imaging System, Cri, Woburn, MA, USA) and the number of migrated cells was counted. The same protocol was applied for cells transiently transfected with Δ462 or control plasmids.

Transwell invasion assay. Matrigel inserts (BD Biosciences) were rehydrated for 2 hours and incubated at 37°C with 5% CO_2. Cells were pre-treated with various concentrations of T007 or empty vehicle for 26 hours, then trypsinized and resuspended in serum-free DMEM to achieve a concentration of 2×10^5 cells/ml. Inserts were placed in wells containing normal growth media. A volume of 500 μl of cell suspension with or without T007 was added to the upper chamber and incubated at 37°C with 5% CO_2 for 22 hours. After incubation, the upper side of membrane was cleaned by scrubbing using cotton swabs and then cells on the lower membrane surface were fixed with methanol and stained with 1% crystal toluidine blue. The images of five random fields per well were taken using a ×40 objective (Nikon Eclipse TE200, Nuance 2.8 Imaging System, Cri) and the number of invaded cells was counted. The same protocol was applied for cells transiently transfected with Δ462 or control plasmids.

PPRE-binding ELISA. Nuclear extracts from treated or untreated cells were prepared according the protocol using the Nuclear Extract Kit (# 40010; Active Motif; Carlsbad, CA, USA). A volume of 10 μg of nuclear extract per well was analyzed according the protocol for PPARγ TransAM Kit (# 40196; Active Motif).

Western blot analysis. Cell extracts were prepared in NP-40 lysis buffer plus Protease Inhibitor Cocktail (Complete Mini, Roche, Indianapolis, IN, USA) and Phosphatase Inhibitor Cocktail1 and 2 (Sigma, St. Louis, MO, USA). The antibodies PPARγ (E-8): sc-7273, RXR (C-20): sc-533, p-FAK (Y397): sc-11765-R, Erk1/2 sc-94 and p-Erk1/2(E-4): sc-7383 were obtained from Santa Cruz, Santa Cruz, CA, USA; FAK (#610088) was obtained from BD Bioscience. Western blot was performed as previously described (7).

Statistics. Data was analyzed either by one-way analysis of variance (ANOVA) or Welch's *t*-test as appropriate using the open statistical computing language and run-time environment, R (v 2.9.2) (29). In every ANOVA, Tukey's Honest Significant Difference pair-wise comparison test was used *post-hoc*. *P*-values of less than 0.05 were considered to be significant.
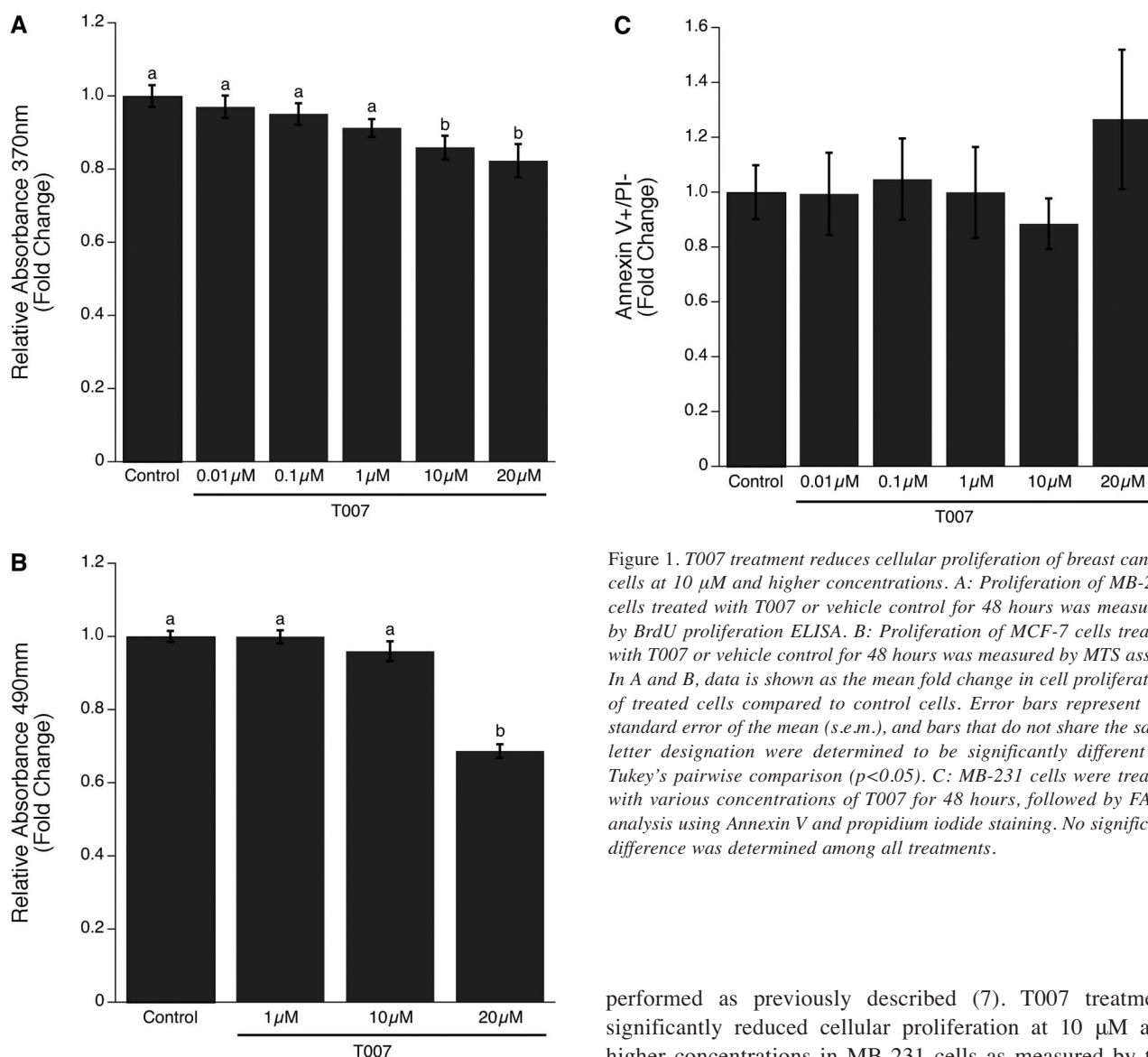
Figure 1. *T007 treatment reduces cellular proliferation of breast cancer cells at 10 μM and higher concentrations. A: Proliferation of MB-231 cells treated with T007 or vehicle control for 48 hours was measured by BrdU proliferation ELISA. B: Proliferation of MCF-7 cells treated with T007 or vehicle control for 48 hours was measured by MTS assay. In A and B, data is shown as the mean fold change in cell proliferation of treated cells compared to control cells. Error bars represent the standard error of the mean (s.e.m.), and bars that do not share the same letter designation were determined to be significantly different by Tukey's pairwise comparison (p<0.05). C: MB-231 cells were treated with various concentrations of T007 for 48 hours, followed by FACS analysis using Annexin V and propidium iodide staining. No significant difference was determined among all treatments.*

Results

T007 treatment reduces cellular proliferation, but does not affect apoptosis of breast cancer cells. It was previously demonstrated that PPARγ is highly expressed in breast cancer cells compared to normal epithelial cells and that its inhibition using molecular approaches might be beneficial in treatment of breast cancer (6, 7). To investigate whether pharmacological inhibition of PPARγ using its selective antagonist, T007, can affect cellular proliferation and apoptosis of breast cancer cells, two breast cancer cell lines, MB-231 and MCF-7, were treated with various concentrations of T007 or vehicle for 48 hours and then proliferation or apoptosis assays were

performed as previously described (7). T007 treatment significantly reduced cellular proliferation at 10 μM and higher concentrations in MB-231 cells as measured by the BrdU proliferation assay (Figure 1A). A decrease in cellular proliferation was also observed when MCF-7 cells were treated with T007 at 20 μM and higher concentrations, as measured by the MTS assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) (Figure 1B) and BrdU (5-bromo-2-deoxyuridine) proliferation assays (data not shown). However, the tested concentrations of T007 had no significant effect on apoptosis in either MB-231 cells (Figure 1C) or MCF-7 cells (data not shown).

Inhibition of PPARγ activity by both T007 and the dominant-negative PPARγ mutant, Δ462, significantly reduces migration of breast cancer cells. In order to determine whether T007 treatment affects breast cancer cell migration, the wound-healing assay was performed. MB-231 cells were scratched using a pipette tip, rinsed with phosphate buffered saline
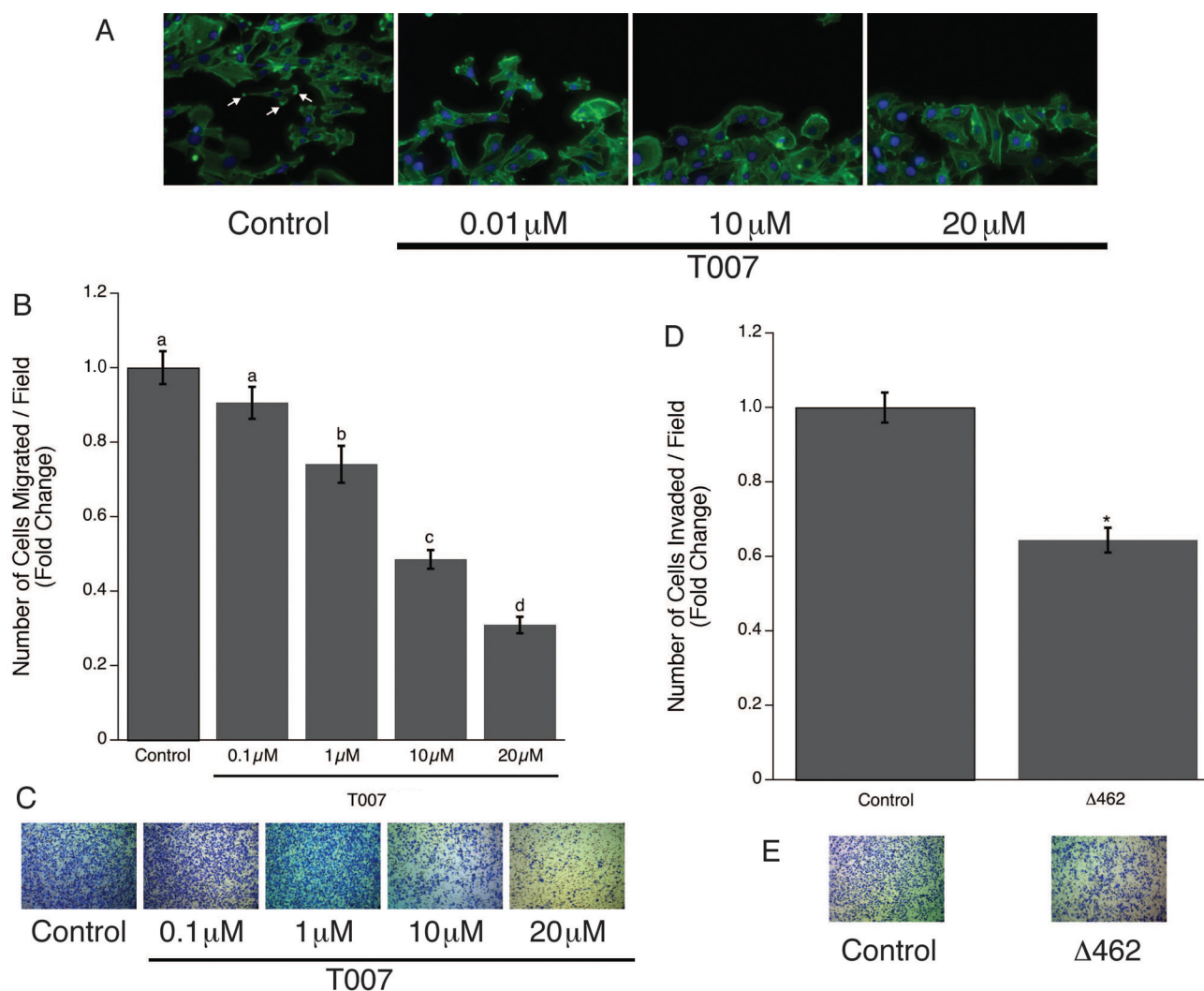
Figure 2. *Pharmacological and molecular suppression of PPARγ activity reduces migration of breast cancer cells. A: Changes in migration of MB-231 cells treated with T007 or vehicle control for 48 hours were visualized using immunofloresence analysis of F-actin. Arrows indicate the accumulation of F-actin at the leading edges of migrating cells. B: MB-231 cells were pretreated with T007 or vehicle control for 48 hours and then migrated through a collagen-coated membrane for 6 hours (transwell migration assay). The number of migrated cells was counted on images taken using a ×40 objective (five random fields for each well). The experiment was repeated four times. Data is shown as the mean fold change in number of treated cells which migrated through the membrane compared to control cells. Error bars represent the standard error of the mean (s.e.m.) and bars that do not share the same letter designation were determined to be significantly different by Tukey's pairwise comparison (p<0.05) C: Representative images of MB-231 cell migration were taken using a ×10 objective. D:MB-231 cells were transiently transfected with Δ462 or control plasmids and then migrated through a collagen coated membrane for 6 hours. Migrated cells were counted on images taken using a 40× objective (5 random fields for each well). The experiment was repeated five times. Data is shown as the mean fold change in number of treated cells migrated through the membrane compared to control cells. *Significantly different from the appropriate control at p<0.01 (Welch's t-test). E: Representative images of MB-231 cell migration were taken using a ×10 objective.*

(PBS), and then allowed to migrate in serum-free media in the presence or absence of various concentrations of T007 for 48 hours. T007 treatment significantly reduced the ability of MB-231 cells to close the wound at 1 μM and higher concentrations. Interestingly, the difference between treatments was visually detected at 48 hours but not at 24 hours (supplementary Figure 1). Morphological changes associated with a less migratory phenotype when cells were treated with T007 were also observed. To investigate if these changes correlate with the ability of these cells to migrate F-actin staining was used. Figure 2A demonstrates that an accumulation of F-actin at the leading edges of control cells or cells treated with low concentrations of T007 is more prominent than that in cells treated with high concentrations
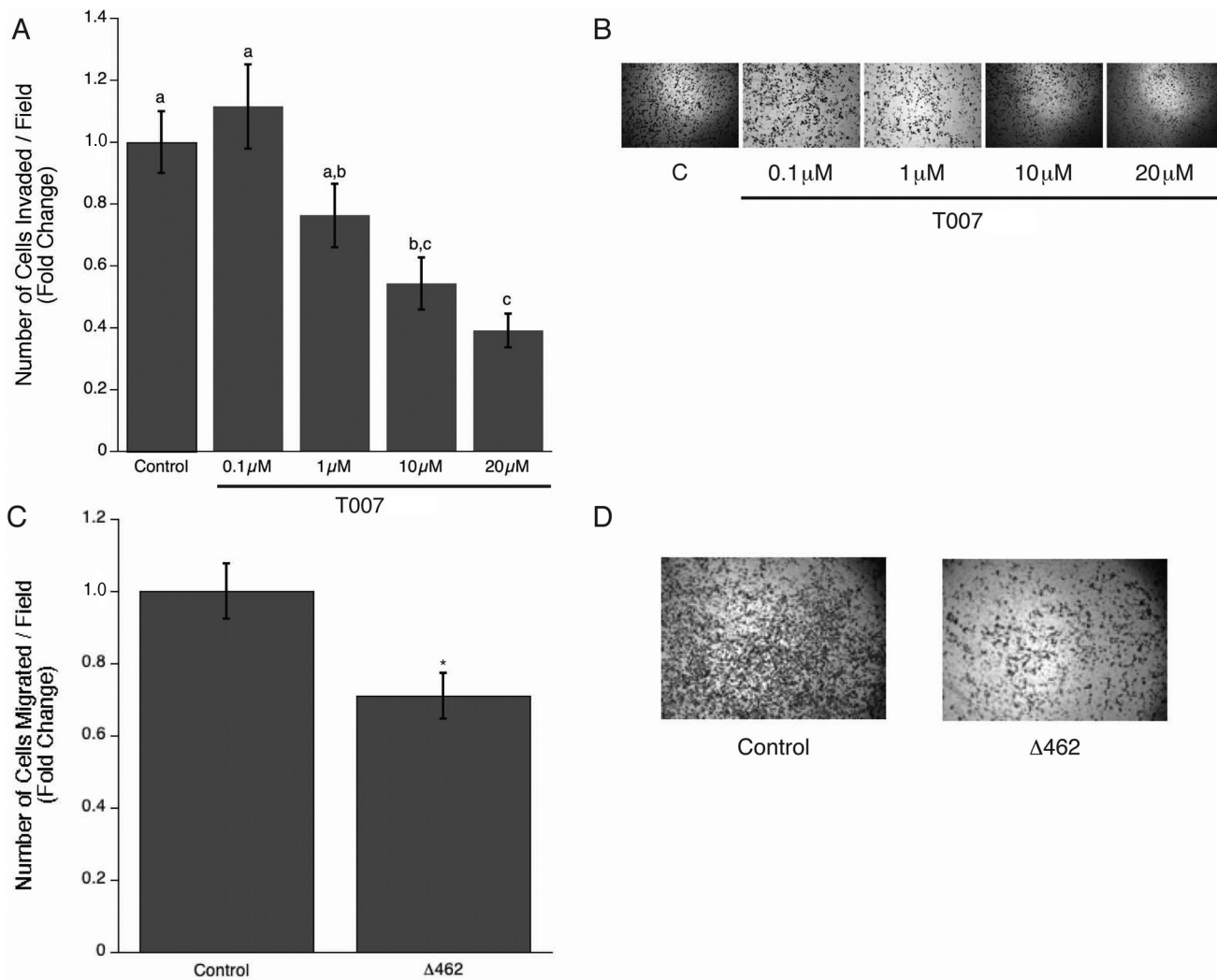
Figure 3. *Pharmacological and molecular suppression of PPARγ activity reduces invasion of breast cancer cells. A: MB 231 cells were pretreated with various concentrations of T007 or vehicle for 26 hours and then allowed to invade through Matrigel for 22 hours. The number of invaded cells was counted on images taken using a ×40 objective (5 random fields for each well). The experiment was repeated three times. Data is shown as mean fold change in number of treated cells invaded through Matrigel compared to control cells. Error bars represent the standard error of the mean (s.e.m.) and bars that do not share the same letter designation were determined to be significantly different by Tukey's pairwise comparison (p<0.05). B: Representative images of MB-231 cell invasion taken by using a ×10 objective. C: MB-231 cells were transiently transfected with Δ462 expression plasmid or control plasmid and then allowed to invade through Matrigel for 22 hours. The number of invaded cells was counted on images taken using a ×40 objective (5 random fields for each well). The experiment was repeated three times. Data is shown as mean fold change in number of treated cells invaded through Matrigel compared to control cells. *Significantly different from the appropriate control at p<0.01 (Welch's t-test). D: Representative images of MB-231 cell invasion were taken using a ×10 objective.*

of T007. Suppression of cell migration in response to T007 treatment was also observed in both MCF-7 and MB-231 cells using the Oris migration assay (Supplementary Figure 2). To confirm these observations and to quantify changes in cell migration in response to various concentration of T007 a transwell migration assay was performed. Equal numbers of breast cancer cells pre-treated with T007 for 48 hours were seeded into the top chambers of the transwell assay. MB-231 cells were allowed to migrate through a porous membrane coated with collagen for 6 hours. A dose-dependent decrease in cell migration in response to T007 treatment was observed (Figure 2B, C). To test whether changes in cell migration in T007 treated cells are PPARγ dependent, the transwell assay was performed using MB-231 cells transiently transfected with control or Δ462 expression plasmids as previously described (7). Figures 2D and 2E demonstrate that inhibition of PPARγ
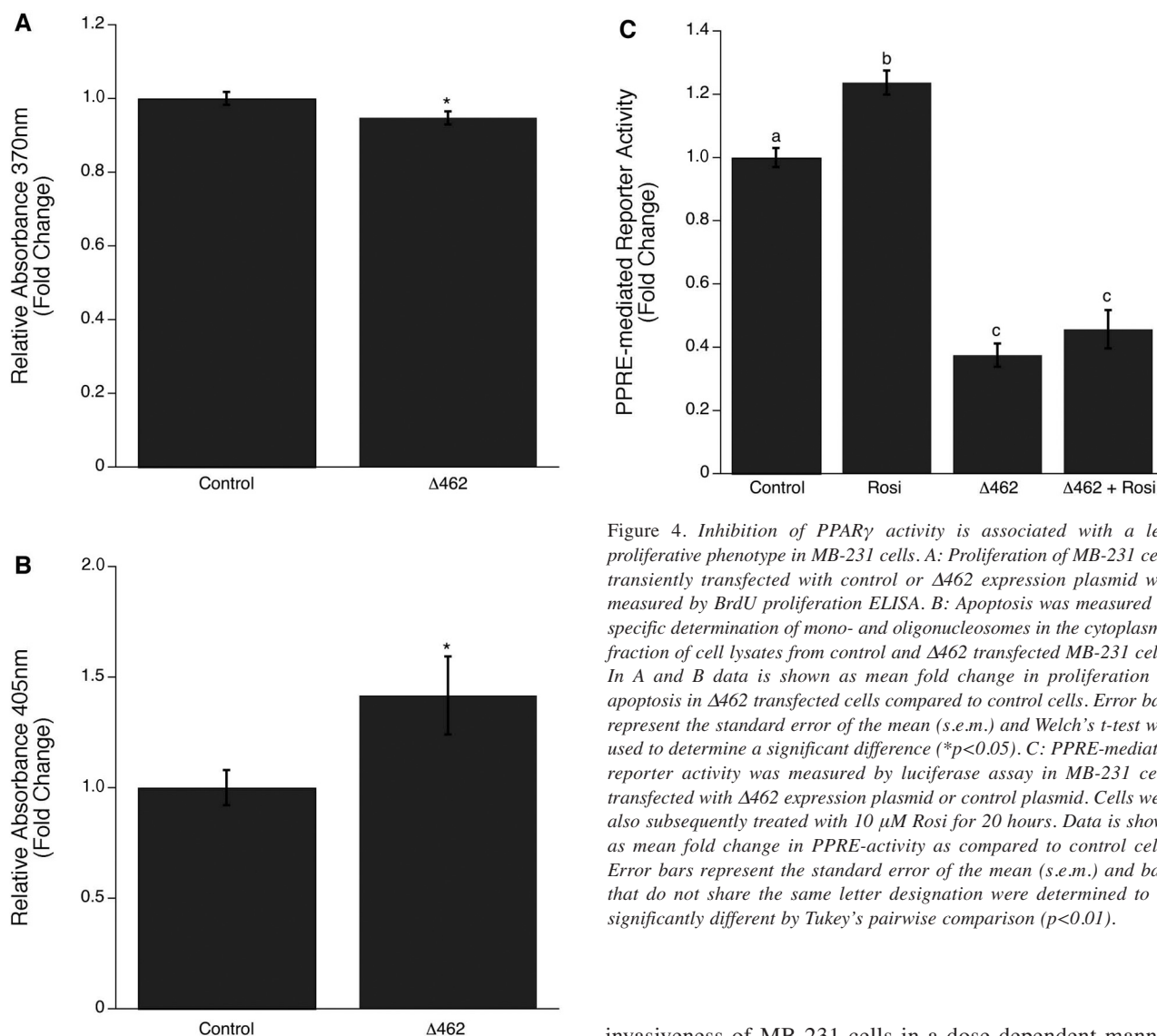
Figure 4. *Inhibition of PPARγ activity is associated with a less proliferative phenotype in MB-231 cells. A: Proliferation of MB-231 cells transiently transfected with control or Δ462 expression plasmid was measured by BrdU proliferation ELISA. B: Apoptosis was measured by specific determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates from control and Δ462 transfected MB-231 cells. In A and B data is shown as mean fold change in proliferation or apoptosis in Δ462 transfected cells compared to control cells. Error bars represent the standard error of the mean (s.e.m.) and Welch's t-test was used to determine a significant difference (*p<0.05). C: PPRE-mediated reporter activity was measured by luciferase assay in MB-231 cells transfected with Δ462 expression plasmid or control plasmid. Cells were also subsequently treated with 10 μM Rosi for 20 hours. Data is shown as mean fold change in PPRE-activity as compared to control cells. Error bars represent the standard error of the mean (s.e.m.) and bars that do not share the same letter designation were determined to be significantly different by Tukey's pairwise comparison (p<0.01).*

activity significantly suppresses migration of breast cancer cells, suggesting that T007 acts through a PPARγ-dependent mechanism and that endogenous transactivation of PPARγ might play an important role in breast cancer cell migration. These results were confirmed using transfections with scrambled or PPARγ shRNAs (Supplementary Figure 3).

Inhibition of PPARγ activity by both T007 and the dominant-negative PPARγ mutant, Δ 462, significantly reduces invasion of MB-231 breast cancer cells. To determine whether inhibition of PPARγ has an effect on cell invasion, MB-231 cells were pre-treated with T007, as described, and then allowed to invade through Matrigel for 22 hours in the presence or absence of antagonist. These data revealed that T007 significantly reduces the

invasiveness of MB-231 cells in a dose-dependent manner (Figure 3A, B). Similarly, inhibition of PPARγ activity by Δ462 also suppressed the invasion of MB-231 cells (Figure 3C, D) suggesting that the effect of T007 on breast cancer cell invasion is mediated by PPARγ.

Inhibition of PPARγ activity by the dominant-negative PPARγ mutant, Δ 462, significantly reduces cellular proliferation and induces apoptosis in MB-231 breast cancer cell line. It was previously shown that knockdown of PPARγ using PPARγ shRNA or inhibition by the dominant-negative mutant, Δ462, leads to a decrease in cellular proliferation and an increase in apoptosis in MFC-7 and T47D breast cancer cell lines (7). To test whether the observed changes in motility of breast cancer cells following PPARγ inactivation are due to changes in cellular proliferation, MB-231 cells were transfected with Δ462 or control plasmids and then BrdU proliferation and cell death assays were performed.
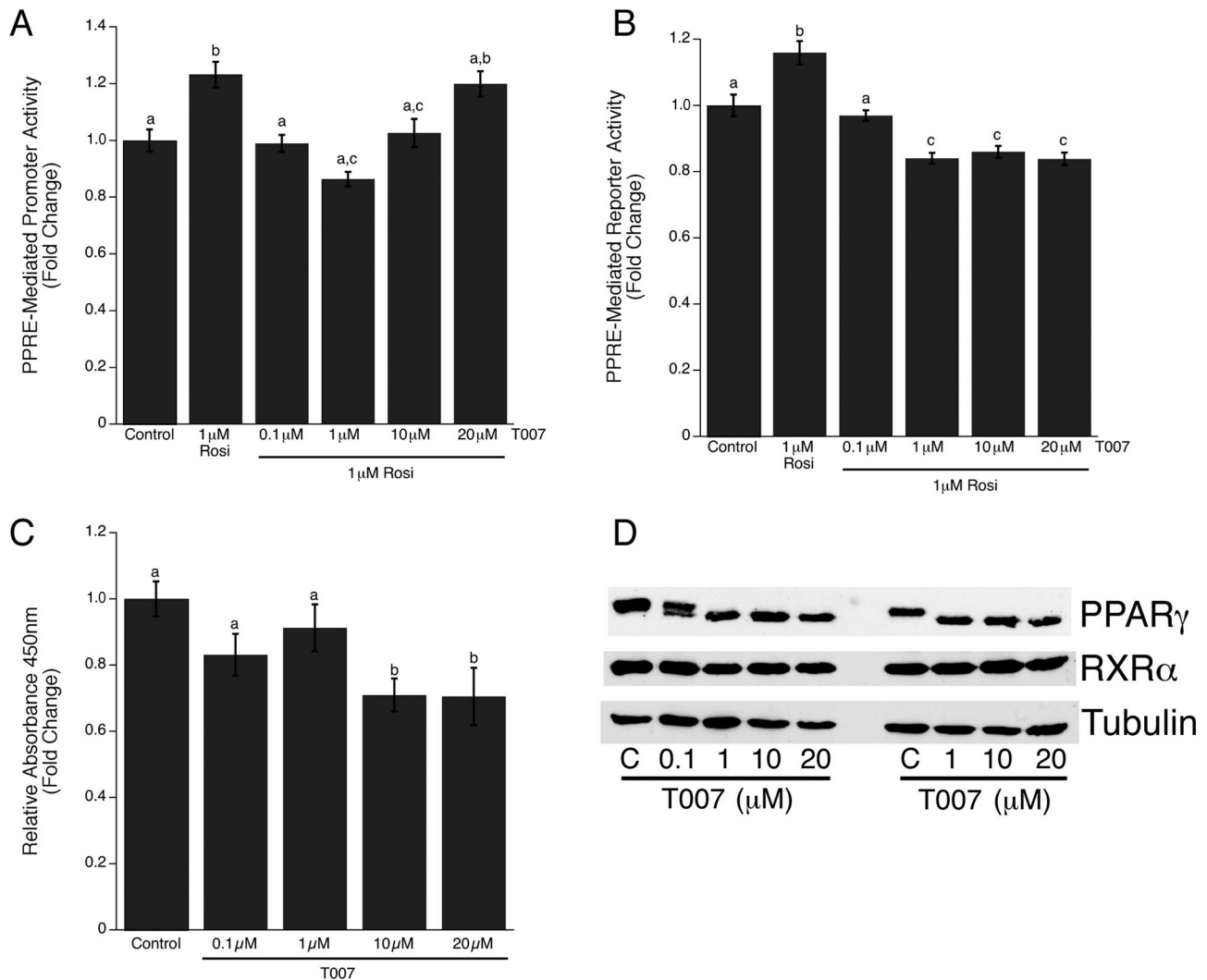
Figure 5. *Effect of T007 treatment on PPARγ transcriptional activity and phosphorylation of the receptor. A: PPRE-mediated reporter activity was measured in MB-231 cells transiently transfected with a 3XPPRE-mTK-pGL3 reporter plasmid and then treated with 1 μM Rosi alone or 1 μM Rosi with various concentration of T007. B: PPRE-mediated reporter activity was measured in MCF-7 cells transiently transfected with a 3XPPRE-mTK-pGL3 reporter plasmid and then treated with 1 μM Rosi alone or 1 μM Rosi with various concentration of T007. In A and B, data is shown as mean fold change in luciferase to renilla ratios compared to control. Error bars represent the standard error of the mean (s.e.m.) and bars that do not share the same letter designation were determined to be significantly different by Tukey's pairwise comparison (p<0.05). C: MB-231 cells were treated with T007 for 48 hours, nuclear extracts were isolated, and then the amount of PPARγ bound to its response element was analyzed. D: Western blot analysis of nuclear extracts used to perform the PPARγ TransAM ELISA then was analyzed for immunoreactive PPARγ, RXR and tubulin.*

Figure 4A shows that inhibition of PPARγ activity using the Δ462 significantly reduces cellular proliferation in MB-231 breast cancer cell. However, the effect of PPARγ inactivation is not as prominent as was observed in MCF-7 cells (7). Data also revealed that MB-231 cells transfected with the Δ462 mutant had a significantly higher level of apoptosis (Figure 4B). To confirm that changes in cellular proliferation and apoptosis are indeed in response to the inhibition of PPARγ activity by the Δ462, PPRE reporter activity was measured using luciferase assays. Cells were transfected with a 3XPPRE-mTK-pGL3 reporter plasmid and co-transfected with control or Δ462 expression plasmids. Following transfection, cells were treated with 10 μM rosiglitazone (Rosi). In Rosi-treated and -untreated cells, overexpression of Δ462 resulted in a significantly lower level of PPRE-mediated reporter activity (Figure 4C), thus, demonstrating that Δ462 efficiently inhibits endogenous and ligand-mediated activity of PPARγ1 in MB-231 cancer cells.
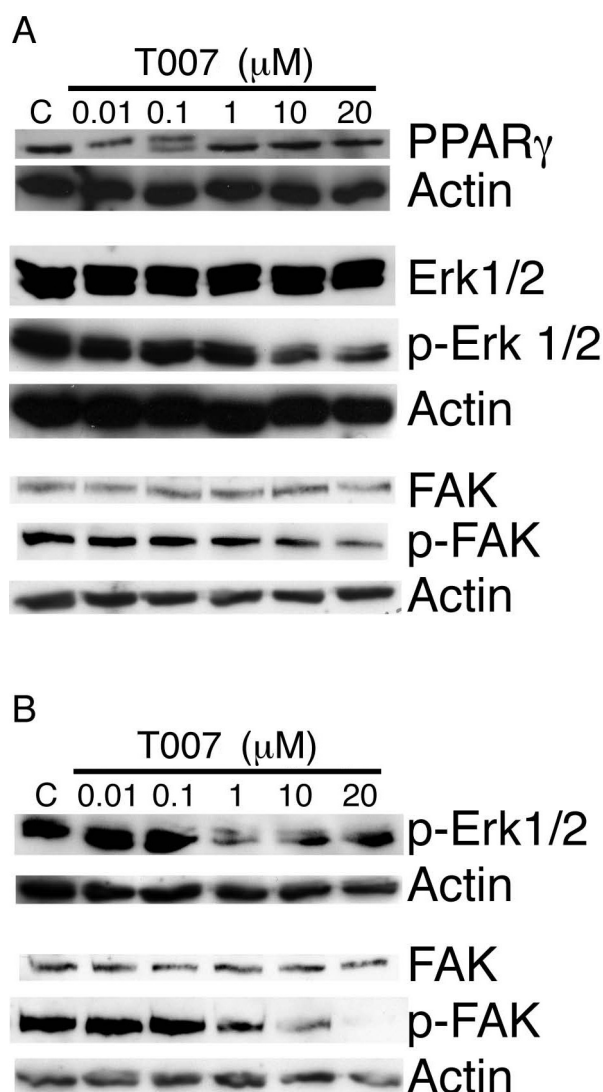
Figure 6. *Effect of T0070907 on the FAK-MAPK pathway. A:Western blot analysis was performed on total cell lysis from MB-231 cells treated T0070907 or vehicle control and analyzed for immunoreactive PPARγ, Erk1/2, p-Erk1/2, FAK, p-FAK (Tyr 397) and actin. B: Western blot analysis was performed on total cell lysis from MCF-7 cells treated T0070907 or vehicle control and analyzed for immunoreactive Erk1/2, p-Erk1/2, FAK, p-FAK (Tyr 397) and actin.*

T007 alters phosphorylation of PPARγ and significantly reduces PPARγ binding to the DNA response element and its activation. T007 has been reported to be a selective PPARγ antagonist that decreases transcriptional activity of the receptor (24); however, numerous studies have demonstrated that PPARγ antagonists also may act *via* PPARγ independent pathways (27, 30). To elucidate the mechanism of T007 action in breast cancer cells, PPRE functional response was measured in MB-231 and MCF-7 cells. Cells transfected with a

3XPPRE-mTK-pGL3 reporter plasmid were pre-treated with 1 μM of Rosi and then treated with various concentrations of T007. Luciferase reporter assay data revealed that T007 treatment reduced PPRE-mediated reporter activity in both MB-231 (Figure 5A) and MCF-7 cells (Figure 5B). Interestingly, no dose-dependent inhibition of PPRE activity was observed in either of these cell lines. Moreover, we did not see a significant decrease in PPRE-mediated reporter activity between MB-231 control cells and cells treated with 10 μM and 20 μM of T007. These data suggest that at high concentrations, T007 might interfere with other signaling pathways and lead to PPARγ-independent activation of PPRE. To verify that T007 treatment reduces transcriptional activity of PPARγ and measure the ability of PPARγ to bind to a PPRE in response to various concentrations of T007, a DNA binding ELISA was employed. As shown in Figure 5C, T007 treatment reduced the ability of PPARγ to bind to the PPRE at 10 μM and higher concentrations in MB-231 cells but did not have any effect on PPARγ binding to the PPRE in MCF-7 cells (data not shown). In order to test whether a decrease in PPARγ binding to the PPRE is due to a decrease in PPARγ expression, Western blot analysis of nuclear extracts from MB-231 cells used in the PPRE-binding assay was performed. T007 treatment did not affect the protein level of PPARγ or RXR at any tested concentrations, but did alter phosphorylation of PPARγ at 0.1 μM and higher concentrations in MB-231 cells. (Figure 5D). No changes in PPARγ phosphorylation were seen in MCF-7 cells treated with T007 (data not shown).

T007 treatment affects FAK- MAPK pathway. The fact that T007 alters phosphorylation of PPARγ suggests that this compound might affect the activity of upstream kinases. Erk/Erk2 MAP kinases were previously reported to modulate PPARγ activity (19, 21). An increase in Erk phosphorylation was also linked to an enhanced migration of breast cancer cells (20). To test whether T007 affects the MAPK pathway in breast cancer cells, MB-231 and MCF-7 cells were treated with various concentrations of T007 for 48 hours and total cell lysates were analyzed by Western blot analysis. The representative immunoblot for total Erk1/2 and p-Erk1/2 demonstrate that although no difference was observed in total Erk1/2 expression between treated and untreated cells, T007 treatment did decrease Erk1/2 phosphorylation in a dose-dependent manner in both MB-231 and MCF-7 cell lines (Figure 6). It was also investigated whether T007 affects phosphorylation of FAK. FAK, a nonreceptor tyrosine kinase, is an important regulator of cell migration and invasion (31-33). It has also been shown that phosphorylation of FAK is associated with cytoskeleton changes and activation of the MAPK pathway (34, 35). In Figure 6A and B, the representative immunoblots for total FAK and p-FAK demonstrate a dose-dependent decrease in phosphorylation of FAK in response to T007 treatment in both MCF-7 and MB-

231 cell lines. Expression of Δ462 did not affect the level of Erk1/2 and FAK phosphorylation (Supplementary Figure 4). It supports aforementioned data and suggests that in addition to its action as a PPARγ antagonist, T007 might target the FAK-MAPK pathway directly.

Discussion

It has previously been shown that PPARγ is highly expressed in breast cancer cells as compared to normal epithelial cells (7, 8). High expression of PPARγ has also been detected in human breast cancer tissues (10, 23). However, the role that PPARγ plays in initiation and progression of breast cancer still remains unclear. Several animal studies have been performed in an attempt to elucidate the role of endogenous activation of PPARγ in breast cancer (22, 23). It has been shown that bigenic mice prone to mammary gland cancer and expressing a constitutively active form of PPARγ in mammary tissue have an increase in incidence of breast cancer and metastasis as compared to control mice (22). Results from this study strongly suggest that an increase in PPARγ expression and activation is not sufficient for malignant transformation but once an initiating event has taken place aberrant PPARγ signaling promotes a more malignant phenotype. Previously published data have demonstrated that inhibition of PPARγ expression and activity in breast cancer cells leads to a significant decrease in cellular proliferation and an induction of apoptosis (7). Taken together, these results suggest that breast cancer cells require PPARγ transactivation for survival and that targeted inhibition of PPARγ might be beneficial and can be used as a therapeutic approach for breast cancer treatment. PPARγ antagonists have exhibited anticancer effects in a broad range of epithelial cancer cell lines as well as in *in vivo* systems and might be used as a potential therapy for breast cancer (36, 37). T007, a selective PPARγ antagonist, has been demonstrated to have anticancer effects in hepatocellular carcinoma, esophageal carcinoma, squamous cell carcinoma, and pancreatic cancer cell lines (26, 28). To test whether T007 has an anticancer effect in breast cancer cells, two breast cancer cell lines, invasive MB-231 and non-invasive MCF-7, were treated with various concentrations of antagonist. In both cell lines, T007 treatment exerted anti-proliferative effects at 10 μM or higher concentrations after 24 (data not shown) and 48 hours of treatment. No effect on apoptosis was observed. These results were similar to the results observed in pancreatic and esophageal cancer cells (9, 26). Although no changes in cell death were detected at any of the concentrations tested, it has been previously reported that T007 affects apoptotic pathways by inducing anoikis at 50 μM and higher concentrations in hepatocellular carcinoma cells (25).

In order to investigate whether T007 affects migration of breast cancer cells, MB-231 cells were treated with various concentrations of T007 and the differences in migration

between untreated and treated cells were estimated by a wound-filling assay. Interestingly, a significant difference in the wound closure between control and T007-treated cells was observed at 48 hours, but not at 24 hours, suggesting the complexity of signaling necessary to elicit changes in cell migration. The similar inhibitory effect of T007 on cell migration was observed in both MB-231 and MCF-7 breast cancer cell lines using the Oris migration assay. The more migratory phenotype of control cells compared to T007-treated cells was also confirmed by using F-actin staining. In order to quantify changes in cell migration, MB-231 cells were pre-treated for 48 hours with T007 or vehicle and then analyzed by transwell assay. A dose-dependent decrease in cell migration was observed in response to T007 treatment. Moreover, an effect on cell migration was exhibited at lower concentrations of antagonist than the effect on cellular proliferation. This disparity might be explained by the concept that T007 treatment might affect other targets than PPARγ and its effect on proliferation and migration is educed *via* different signaling pathways. It was also demonstrated that T007 at 1 μM and higher concentrations inhibits the invasive properties of MB-231 cells in a dose-dependent manner. A decrease in migration and invasion was also observed when MB-231 cells were transfected with a dominant-negative mutant of PPARγ, Δ462. Knock-down of PPARγ expression in MB-231 cells using PPARγ shRNA has shown similar results and confirmed that a decrease in PPARγ signaling suppresses migration of breast cancer cells. These data strongly suggest that PPARγ might be involved in regulation of breast cancer cell motility and invasion and that T007, at least in part, acts through a PPARγ-dependent mechanism to affect these processes.

T007 has been reported to decrease transcriptional activity of PPARγ *via* modulation of the receptor interaction with cofactor proteins (24). To confirm that T007 treatment reduces transcriptional activity of PPARγ and to elucidate the mechanism of T007 action in breast cancer cells, the PPRE-mediated reporter activity was analyzed when breast cancer cells were treated with various concentrations of antagonist. T007 treatment significantly reduced the PPRE-mediated reporter activity in both MB-231 and MCF-7 cells stimulated with Rosi. No dose-dependent decrease in the PPRE activity was observed in either of the cell lines, as was expected based on the fact that T007 treatment affected cellular proliferation, migration, and invasion in a dose-dependent manner. Moreover, the fact that no significant inhibtion of endogenous PPRE activation has been seen at 10 μM and 20 μM of T007 treatment as compared to control in MB-231 cells supports the concept that high concentrations of T007 might have some 'off-target' effect and lead to PPARγ-independent activation of the PPRE. The fact that a significant decrease in PPARγ binding to the PPRE was observed at the concentrations which did not affect the PPRE activity also suggests PPARγ-independent mechanisms of T007 action. To test whether the

observed decrease in PPARγ binding to DNA is due to a decrease in protein expression, the nuclear extracts used in the DNA-binding ELISA were immunoblotted for PPARγ. Although T007 did not affect the protein level of PPARγ in nuclear extracts or total lysates from both MB-231 and MCF-7 cells, alterations in the phosphorylation state of PPARγ was observed in MB-231 cells. The fact that the lower band of PPARγ was predominantly observed on the SDS gel when cells were treated with T007 suggests that this compound might lead to a decrease in phosphorylation of the receptor or to an increase in protein phosphatases activity. Regulation of PPARγ by phosphorylation has been previously reported (19, 21). PPARγ has multiple phosphorylation sites, although the consequences of its phosphorylation have not been thoroughly investigated (19). To investigate whether T007 affects PPARγ phosphorylation directly or *via* upstream kinases, changes in p-Erk were examined. The MAP kinase was previously reported to modulate PPARγ activity (19, 21). An increase in Erk1/2 phosphorylation has also been linked to enhanced migration of breast cancer cells (20). Although no change has been observed in total Erk1/2 expression, this study demonstrated a dose-dependent decrease in p-Erk1/2 in response to T007 treatment. A dose-dependent decrease in p-FAK, a non-receptor tyrosine kinase and an important marker of cell migration and invasion has also been shown (33). Thus, these data suggest that in addition to direct effects on PPARγ activity, T007 might affect other signaling pathways such as the FAK-MAPK pathway. Furthermore, it is not clear whether T007 alters phosphorylation of PPARγ directly or indirectly *via* upstream kinases or whether changes in phosphorylation of PPARγ have an effect on migration and invasion. More detailed studies are required to answer these questions.

Taken together, these results demonstrated that T007, a selective PPARγ antagonist, significantly reduces cellular proliferation, migration and invasion in two breast cancer cell lines, MB-231 and MCF-7. It was confirmed that its action in these cell lines is at least in part through a PPARγ-dependent mechanism. It was also demonstrated that physiological responses such as changes in proliferation and migration to T007 treatment in breast cancer cells might be caused by 'off-target' action of this compound *via* suppression of the FAK-MAPK pathway. This data suggest that inhibition of endogenous PPARγ signaling could be used to slow growth and metastasis of breast cancer and, therefore, PPARγ antagonists such as T007 should be further evaluated as a potential therapy for breast cancer.

Acknowledgements

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