Peroxisome Proliferator-Activated Receptor-γ Activation Inhibits Tumor Metastasis by Antagonizing Smad3-Mediated Epithelial-Mesenchymal Transition

Ajaya Kumar Reka, Himabindu Kurapati, Venkata R. Narala, Guido Bommer, Jun Chen, Theodore J. Standiford, and Venkateshwar G. Keshamouni

Abstract

Epithelial-mesenchymal transition (EMT) was shown to confer tumor cells with abilities essential for metastasis, including migratory phenotype, invasiveness, resistance to apoptosis, evading immune surveillance, and tumor stem cell traits. Therefore, inhibition of EMT can be an important therapeutic strategy to inhibit tumor metastasis. Here, we show that activation of peroxisome proliferator-activated receptor γ (PPAR-γ) inhibits transforming growth factor β (TGF-β)-induced EMT in lung cancer cells and prevents metastasis by antagonizing Smad3 function. Activation of PPAR-γ by synthetic ligands (troglitazone and rosiglitazone) or by a constitutively active form of PPAR-γ prevents TGF-β–induced loss of E-cadherin expression and inhibits the induction of mesenchymal markers (vimentin, N-cadherin, fibronectin) and matrix metalloproteases. Consistently, activation of PPAR-γ also inhibited EMT-induced migration and invasion of lung cancer cells. Furthermore, effects of PPAR-γ ligands were attenuated by siRNA-mediated knockdown of PPAR-γ, indicating that the ligand-induced responses are PPAR-γ dependent. Selective knockdown of Smad2 and Smad3 by siRNA showed that TGF-β–induced EMT is Smad3 dependent in lung cancer cells. Activation of PPAR-γ inhibits TGF-β–induced Smad transcriptional activity but had no effect on the phosphorylation or nuclear translocation of Smads. Consistently, PPAR-γ activation prevented TGF-β–induced transcriptional repression of E-cadherin promoter and inhibited transcriptionsal activation of N-cadherin promoter. Finally, treatment of mice with troglitazone or knockdown of Smad3 in tumor cells significantly inhibited TGF-β–induced experimental metastasis in SCID-Beige mice. Together, with the low toxicity profile of PPAR-γ ligands, our data show that these ligands may serve as potential therapeutic agents to inhibit metastasis. Mol Cancer Ther; 9(12): 3221–32. ©2010 AACR.

Introduction

Epithelial-mesenchymal transition (EMT) is a complex manifestation of epithelial plasticity (1), which has been described in 3 major physiologic contexts: embryonic development and morphogenesis, chronic fibrotic disorders, and cancer progression. Oncogenic EMT is well documented in vivo and in vitro. It is characterized by a reversible conversion of polarized epithelial cells into highly motile fibroblastoid cells (2, 3). On the molecular level, EMT is defined by the loss of cell–cell adhesion molecules (e.g., E-cadherin), downregulation of epithelial differentiation markers, and induction of mesenchymal markers such as vimentin and N-cadherin. During EMT, cancer cells acquire self-sufficient autocrine growth signals to become autonomous entities with an invasive capacity to breach basement membrane, initiate the multistep process of metastasis, and spread throughout the host (2). In addition to making cancer cells highly invasive, EMT was shown to endow several additional abilities to promote metastasis. They include developing resistance to anoxia, senescence, chemotherapy, and avoid immune surveillance by promoting different immunosuppressive mechanisms (4). Cells undergoing EMT were also shown to acquire tumor stem cell–like properties (5). Together, these abilities allow cancer cells to successfully navigate the highly inefficient process of metastasis and link EMT to major clinical aspects that are responsible for cancer-related mortality. This also highlights the urgent need and potential impact of the compounds that can inhibit EMT.
Transforming growth factor β (TGF-β) is a multifunctional cytokine and a potent inducer of EMT (6). It acts as a tumor suppressor in early stages and as a tumor promoter in late stages of tumor progression (7). Most lung cancers have intact TGF-β signaling but develop resistant mechanisms against TGF-β-mediated growth inhibition (7), suggesting a tumor-promoting role of TGF-β. Expression of TGF-β is frequently upregulated in non–small cell lung cancer (NSCLC) and many other human cancers (8) and is correlated with enhanced invasion and metastasis (7). Elevated plasma levels of TGF-β confer a poor prognosis for patients with lung cancer (9). In recent years, a growing number of in vivo studies have shown that inhibition of TGF-β signaling and transcription reduces the metastatic and/or invasive properties of a variety of experimental cancers, presumably by preventing the induction of EMT in cancer cells (10, 11).

Peroxisome proliferator-activated receptor γ (PPAR-γ) is a ligand-activated transcription factor and belongs to the nuclear hormone receptor super family. It is highly expressed in adipose tissue and plays a crucial role in adipocyte differentiation (12). PPAR-γ is also expressed in a variety of tissues and cell types, regulates inflammatory responses (13) and cellular differentiation, and mediates antitumorigenic activity in various tumor types (14, 15). Ligands for PPAR-γ include a variety of compounds, both natural and synthetic. Most of the natural ligands are fatty acids or fatty acid derivatives. Thiazolidinediones (TZD) are synthetic ligands of PPAR-γ that include a class of insulin-sensitizing agents such as rosiglitazone (Rosi), pioglitazone, and troglitazone (Tro); ref. 16.

PPAR-γ activation is antiproliferative presumably by virtue of its differentiation-promoting effects. Consistent with this concept, treatment with PPAR-γ agonists inhibits cancer cell growth in various cancer types both in vitro and in vivo (17–19). We previously showed that PPAR-γ agonists inhibit NSCLC cell growth in vitro and in vivo by inducing G1–G2 cell-cycle arrest and promoting differentiation (20). We have also shown that treatment of mice with PPAR-γ agonists inhibits tumor progression of A549 xenografts in SCID-Beige mice (20). Recently, we showed that chemotherapeutic drugs induce PPAR-γ expression and show sequence-specific synergy with PPAR-γ ligands in inhibition of NSCLC (21). In this study, we show a novel mechanism of PPAR-γ–dependent inhibition of EMT in tumor cells by antagonizing Smad3 function. Smad3 knockedown or treatment of mice with PPAR-γ ligands inhibit experimental metastasis of lung cancer cells.

Materials and Methods

Cell culture

Human adenocarcinoma cell lines A549 (lung) and Panc-1 (pancreas) obtained from ATCC and were not independently authenticated by authors. Both the cell lines were maintained in RPMI-1640 medium with 10% FBS. In all experiments, cells at 40% to 50% confluence in complete medium were serum starved for 24 hours and treated with TGF-β (5 ng/mL) for 72 hours in the presence and absence of PPAR-γ ligands Rosi or Tro at indicated concentrations. PPAR-γ ligands were added to the cultures 30 minutes prior to TGF-β stimulation.

AdCMV-VP16-PPAR-γ transduction

A549 cells were transduced with adenovirus expressing constitutively active form of PPAR-γ (VP16-PPAR-γ) at 0.5 and 1.0 multiplicity of infection (MOI) for 6 to 8 hours, allowed to recover for 24 hours in the presence of complete medium, serum starved overnight, and treated with TGF-β (5 ng/mL). After 72 hours, cells were used for either assessing EMT markers or migration and invasion.

Live cell time-lapse imaging

Time-lapse images were acquired using IncuCyte, a live cell imaging microscope that fits within the standard carbon dioxide incubator (www.essenbioscience.com). Images were captured in 2-hour intervals and collated into a.wmv file using software from IncuCyte.

Cell migration and invasion assays

In vitro migration assay was done as previously reported (22). Briefly, cells were seeded in the top chamber of the cell culture inserts coated either with or without matrigel for invasion and migration assessment, respectively. Bottom chamber was filled with RPMI-1640 medium with 5% FBS. After 8 hours (for migration) or 24 hours (for invasion), cells that penetrated to the undersides of the inserts were fixed, stained, and counted under microscope.

SDS-PAGE, Western immunoblotting, and gelatin zymography

Cells were washed with PBS after treatment and lysed in radioimmunoprecipitation assay buffer containing a protease inhibitor cocktail. Samples containing 20 μg of total protein were separated by SDS-PAGE, transferred onto a polyvinylidene difluoride membrane, and probed with indicated primary antibodies with overnight incubation at 4°C, followed by horseradish peroxidase–conjugated secondary antibodies and developed using enhanced chemiluminescence reagents. Matrix metalloproteases (MMP) in the conditioned media were assayed as described previously by gelatin zymography (23). Equal amounts of proteins were separated on gelatin (2 mg/mL)-incorporated 10% SDS-PAGE gels. Proteins are renatured by incubating gels in a renaturing buffer for 30 minutes at 37°C, followed by overnight incubation in the developing buffer and stained with 0.25% Coomassie blue R250 for 3 hours at room temperature, and destained with 30% methanol and 10% acetic acid solution to visualize gelatin degradation.

siRNA transfection

siRNAs specific to PPAR-γ, Smad3, or Smad2 include a pool of 4 synthetic duplexes (Dharmacon’s SMARTpool).
A scrambled sequence from the same company is used as a control. Cells at 40% to 50% confluence were transfected with siRNA, using Lipofectamine 2000 and optiMEM medium. After 6 hours of transfection, cells were allowed to recover from transfection in RPMI-1640 medium with 10% FBS before assessing for EMT markers.

**Quantitative PCR and reverse transcriptase-PCR analyses**

Total RNA was isolated using 1 mL of TRIZOL following manufacturer’s protocol. Using the following primers and probe, 5'-GGCTTCTAGGACAGGAGTTTC-3' (forward), 5'-ACTCAAACTTGGGCTCCATAAGACG-3' (reverse), 5'-AAAGACGCTTGGAAAGCCCTTTTGTTG-3' (probe)—PPAR-γ expression was measured by quantitative PCR (qPCR) using ABI Prism (Applied Biosystems) and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. EMT markers shown in Fig. 5A were assessed using commercially available primers from SAB Biosciences by SYBR amplification.

Expression of fibronectin and GAPDH was assessed by reverse transcriptase-PCR (RT-PCR) using the following primers: for fibronectin: 5'-GAGGTGCCCCAACTCACTGTTG-3' (forward), 5'-GAGGTCCCCAACTCACTGTTG-3' (reverse), 5'-GGCTTCATGACAAGGGAGTTTC-3' (forward), 5'-GGCTTCATGACAAGGGAGTTTC-3' (reverse); and conditions: 30 seconds at 94°C, 1 minute at 50°C, and 45 seconds at 68°C. The amplification products after 40 cycles were analyzed in 2% agarose gel.

**Luciferase reporter gene assays**

Stable cell lines expressing A549-NcadPromo-Luc, A549-EcadPromo-Luc, and A549-SBE-Luc were developed for luciferase-based reporter assays. The full-length promoter sequences of human N-cad or E-cad were PCR cloned into the pGL4.14 vector (Promega) upstream of firefly luciferase gene, stably transfected into A549 cells, and stable transfectants were selected under hygromycin selection. For Smad-binding element (SBE) assays, a lentiviral-based Smad-responsive firefly luciferase reporter plasmid was purchased from SAB biosciences, transduced into A549 cells and stable transfectants were selected under puromycin selection. Cells serum starved overnight were treated with TGF-β (5 ng/mL) in the presence and absence of Tro pretreatment. At the end of 4 hours (SBE activity) or 72 hours (for N-cadherin and E-cadherin promoter activity), luciferase activity was measured using the steady-glo luciferase kit (Promega) as per the manufacturer’s instructions. Luciferase counts were normalized to the protein concentrations in the respective samples.

**Experimental lung metastasis**

Stable CMV-luciferase (A549-Luc) or SMAD3-shRNA (A549-Smad3 KD)-expressing clones of A549 cells were used in this study. Cells were serum starved for 24 hours and stimulated with or without TGF-β (5 ng/mL) for 72 hours, and 0.5 x 10⁶ cells in 100 μL of serum-free medium were injected into 8-week-old CB17/SCID-beige mice (Harlan Laboratories) through tail vein. The mice injected with TGF-β-treated A549 cells were randomly divided into a control and a treatment group. Mice in the treatment group received Tro (400 mg/kg) once daily by oral gavage. After 3 weeks, mice-harboring A549-Luc cells were anesthetized with isoflurane (2%) and injected with 100 μL of an aqueous solution of luciferin (5 mg/mL, intraperitoneally) 10 minutes before imaging. The animals were placed into the light-tight chamber of the bioluminescence imaging system (Xenogen) and the photons emitted from the luciferase-expressing cells were captured by CCD camera and quantitated using the software program Living Image (Xenogen) as an overlay on Igor (Wavemetrics). The mice with A549-Smad3 KD cells were sacrificed and the metastasis in the lungs was quantitated by counting the number of colonies in the hematoxylin and eosin (H&E)-stained section.

**Statistical analysis**

Data are represented as mean ± SEM and were analyzed with the Prism 4.0 statistical program (GraphPad Software). Groups were compared using 1-sided ANOVA. Differences were considered significant if P < 0.05.

**Results**

**PPAR-γ activation inhibits loss of E-cadherin and acquisition of mesenchymal phenotype during EMT**

Earlier we reported that TGF-β induces EMT in A549 cells within 72 hours. A549 cells change their shape from cuboidal to a more elongated fibroblastoid form and become more motile and migratory in response to TGF-β (22). To study the effect of PPAR-γ activation on EMT, A549 cells were stimulated with TGF-β in the presence and absence of PPAR-γ ligands Rosi or Tro (Fig. 1A) at indicated concentrations and assessed the expression of epithelial and mesenchymal markers by Western immunoblotting or RT-PCR. TGF-β treatment completely suppressed the E-cadherin expression by 72 hours. Rosi or Tro treatment significantly rescued TGF-β-induced E-cadherin suppression (Fig. 1B). As expected, mesenchymal markers vimentin, N-cadherin, and fibronectin (Fig. 1B and C) were upregulated with TGF-β stimulation. However, TGF-β stimulation, in the presence of Rosi or Tro, prevented upregulation of these mesenchymal markers (Fig. 1B and C). Similarly, Tro and Rosi also inhibited TGF-β-induced EMT in a human pancreatic adenocarcinoma cell line, Panc-1. This indicates that the effect of PPAR-γ ligands on EMT is not restricted to lung cancer cells alone (Fig. 1B).

Given the effects on epithelial and mesenchymal markers, we further assessed the effect of PPAR-γ activation on TGF-β-induced change in morphology by time-lapse imaging in real time. TGF-β-stimulated cells loose cell-to-cell contact, show a scattering response throughout
the culture dish, and acquire a spindle-shaped fibroblastoid morphology in a time-dependent manner (Fig. 1D; Time-lapse Movie 1 and 2). Cells stimulated with TGF-β in the presence of Rosi seem to undergo a slight change in morphology but largely stayed within the colony without scattering and maintaining cell-to-cell contacts (Fig. 1D; Time-lapse Movie 3), consistent with the effects of Rosi on biochemical markers of EMT (Fig. 1B). These observations together show that PPAR-γ activation completely blocks EMT by preventing both the loss of epithelial phenotype and acquisition of mesenchymal phenotype.

Inhibition of EMT by PPAR-γ ligands correlates with inhibition of cell migration and invasion

Because PPAR-γ activation modulates biochemical markers of EMT, we assessed functional consequence of such an effect on TGF-β-induced tumor cell migration and invasion. Interestingly, treatment of both Rosi and Tro significantly reduced TGF-β-induced migration and invasion (Fig. 2A) in A549 cells. The inhibition of tumor cell migration and invasion correlates with the ability of PPAR-γ ligands to inhibit mesenchymal markers. At the concentrations used, Rosi or Tro alone had no effect on tumor cell migration or invasion, suggesting that the effects of Rosi and Tro are specific to TGF-β-induced responses.

Consistent with the increase in tumor cell invasion, TGF-β substantially induced MMP2 and MMP9 secretion from A549 cells. Stimulating A549 cells with TGF-β, in the presence of Rosi or Tro, significantly inhibited the secretion of both proteases (Fig. 2B). These results suggest that PPAR-γ activation attenuates the TGF-β–induced invasion of A549 cells, involving inhibition of MMP2 and MMP9 secretion.

Effects of PPAR-γ ligands on EMT are PPAR-γ dependent

Fusion of the potent viral transcription factor VP16 with PPAR-γ cDNA results in a constitutively active form of PPAR-γ (VP16-PPAR-γ) with a potent ligand-independent transcriptional activity (24). We used an adenovirus expressing VP16-PPAR-γ to assess whether the effects of PPAR-γ ligands on EMT are PPAR-γ dependent or independent. A549 cells were transduced with adenovirus-expressing VP16-PPAR-γ or an empty virus at different

![Figure 1](image-url).

**Figure 1.** PPAR-γ ligands inhibit acquisition of mesenchymal markers and loss of E-cadherin expression during EMT. Chemical structures of Tro and Rosi (A). Cells serum starved for 24 hours were stimulated with TGF-β (5 ng/mL) in the presence or absence of PPAR-γ ligands Rosi or Tro at indicated concentrations for 72 hours. E-cadherin, vimentin, N-cadherin, and GAPDH were assessed in by Western immunoblotting in A549 and Panc-1 cells (B) and fibronectin 1 by RT-PCR in A549 cells (C). In a separate experiment, A549 cells treated with or without Rosi in the presence of TGF-β (5 ng/mL) stimulation were monitored continuously using IncuCyte (a live cell imaging system that sits within the standard cell culture incubator) and images were captured once every 2 hours and collated into movie files (Supplementary data). Representative images at 0, 48, and 72 hours are presented in panels for control, TGF-β, and TGF-β + Rosi (D).
MOI (0.5 and 1.0). After 24 hours, cells were serum starved and stimulated with TGF-β for 72 hours and assessed for epithelial and mesenchymal markers by Western immunoblotting. Mimicking the effects of PPAR-γ ligands, constitutively active VP16-PPAR-γ inhibited TGF-β–induced mesenchymal markers (vimentin and N-cadherin; Fig. 3A), tumor cell migration (Fig. 3B), invasion (Fig. 3B), secretion of MMP2 and MMP9 (Fig. 3C), as well as rescued TGF-β–induced loss of E-cadherin expression (Fig. 3A). The slight increase observed in invasion with VP16-PPAR-γ was not statistically significant compared with cells treated with control adenovirus (Fig. 3B). These observations indicate that the effects of Rosi and Tro on TGF-β–induced EMT are mediated by PPAR-γ.

To confirm that the observed effects of PPAR-γ ligands on EMT are PPAR-γ dependent, we knocked down PPAR-γ expression in A549 cells by siRNA (SMARTpool; Dharmacom). We were able to achieve ~70% knockdown of PPAR-γ expression at mRNA and protein levels (Supplementary Fig. S1). After transfection with scrambled siRNA or PPAR-γ siRNA, A549 cells were stimulated with TGF-β for 72 hours in the presence and absence of Rosi and assessed for E-cadherin and vimentin expression by Western immunoblotting. Absence of PPAR-γ in the siRNA transfected cells significantly impaired ability of Rosi to inhibit TGF-β–induced vimentin expression and to rescue TGF-β–induced loss of E-cadherin expression (Fig. 3D). In a similar fashion, PPAR-γ siRNA also significantly inhibited the ability of Rosi to block TGF-β–induced MMP2 and MMP9 secretion (Fig. 3E). This clearly shows that the inhibitory effects of PPAR-γ ligands on EMT are PPAR-γ dependent.

**PPAR-γ activation inhibits TGF-β–induced Smad transcriptional activity without affecting its phosphorylation or nuclear translocation**

Earlier studies have suggested that PPAR-γ ligands may block TGF-β–induced Smad phosphorylation (25) and in some cases their nuclear translocation (26, 27). Smad signaling is required for TGF-β–induced EMT in various cell systems including A549 cells (28). Hence, we examined whether PPAR-γ activation affects TGF-β–induced Smad phosphorylation using phospho-specific Smad2 and Smad3 antibodies by Western immunoblot analysis. TGF-β induces a robust phosphorylation of Smad2 and Smad3 within 60 minutes. Rosi or Tro or VP16-PPAR-γ had no effect on TGF-β–induced Smad2 and Smad3 phosphorylation (Fig. 4A and B). However, at 10 μmol/L concentration, Tro slightly inhibited phos-
phorylation of Smad3 but of not Smad2. This odd inhibition of only Smad3 phosphorylation with 10 μmol/L of Tro seems to be nonspecific and potentially PPAR-γ independent, as neither Rosi nor VP16-PPAR-γ had any effect on Smad phosphorylation. After TGF-β stimulation, phosphorylated Smad2 or Smad3 translocates into the nucleus as Smad2/4 or Smad3/4 heterodimers, binds to the SBE in the promoters of the target genes, and triggers gene transcription. Interestingly, we also observed that Tro had no effect on TGF-β–induced nuclear translocation of Smads (Fig. 4C). Finally, we assessed whether PPAR-γ activation regulates TGF-β–induced Smad transcriptional activity using a Smad-specific promoter assay. A549 cells stably transfected with an SBE-luciferase reporter plasmid were stimulated with TGF-β in the presence and absence of Tro and assessed for luciferase activity in the cell lysates. After 4 hours of TGF-β stimulation, as expected, TGF-β significantly increased the Smad-dependent luciferase reporter activity. In contrast, Tro treatment significantly attenuated both the basal and TGF-β–induced Smad-mediated promoter activation even at 5 μmol/L (Fig. 4D), a concentration at which there was no effect on TGF-β–induced Smad3 phosphorylation. These results show that PPAR-γ activation inhibits EMT by attenuating transcriptional activity of Smads without effecting their phosphorylation or nuclear translocation.
Smad3 but not Smad2 mediates TGF-β-induced EMT

To further isolate the point of interaction between TGF-β and PPAR-γ pathways, we assessed the role of Smad2 and Smad3 in the regulation of EMT by using siRNA approach. We were able to achieve 70% to 90% knock-down of Smad3 or Smad2 expression at the protein level, as assessed by Western immunoblotting using Smad2- or Smad3-specific siRNA (Supplementary Fig. S2A). After transfection with scrambled or Smad3- or Smad2-specific siRNA, A549 cells were stimulated with or without TGF-β for 72 hours and assessed for the gene expression of several epithelial and mesenchymal markers using qPCR. Smad3 inhibition significantly blocked the TGF-β-induced activation of mesenchymal gene expression while attenuating the TGF-β-induced repression of epithelial gene expression when compared with corresponding scrambled siRNA controls (Fig. 5A). On the contrary, Smad2 knockdown had little effect on TGF-β-induced EMT and gene expression (Fig. 5A). Interestingly, in some cases, Smad2 knockdown has increased TGF-β-modulated response, suggesting that Smad2 might be a negative regulator of TGF-β-induced EMT. These results clearly show that Smad3 is a critical regulator of EMT and suggest that inhibition of Smad3 functional activity might be the mechanism by which PPAR-γ activation blocks EMT.

PPAR-γ activation inhibits Smad3-regulated E- and N-cadherin promoter activity

Previous studies have shown that several genes involved in EMT are regulated at transcriptional level by Smad signaling. Hence, we assessed the effects of Tro on TGF-β-induced transcriptional regulation of N-cadherin and E-cadherin by luciferase-based reporter assays. We developed stable cell clones of A549 cells in which the expression of luciferase gene was under the control of promoters of either N-cadherin (A549-NcadPromo-Luc) or E-cadherin (A549-EcadPromo-Luc). As expected, TGF-β treatment reduced the E-cadherin promoter activity and enhanced the N-cadherin promoter activity as assessed by luciferase activity. Consistent with the inhibition of Smad activity, Tro attenuated the TGF-β-induced N-cadherin promoter activity (Fig. 5B) and TGF-β-mediated repression of E-cadherin promoter activity (Fig. 5B), indicating that PPAR-γ activation modulates the Smad-dependent gene transcription. This suggests that the inhibition of Smad3...
functional activity acts as a mechanism for PPAR-γ-mediated inhibition of EMT.

**Activation of PPAR-γ or knockdown of Smad3 inhibits EMT-induced experimental metastasis of lung cancer cells in vivo**

To determine the in vivo relevance of EMT inhibition either by PPAR-γ activation or by blocking Smad3 function, we evaluated the effects of Tro and Smad3-shRNA on experimental metastasis of lung cancer cells in SCID-Beige mice. Luciferase-expressing (A549-Luc) or Smad3-shRNA–expressing A549 (A549-Smad3 KD) cells were cultured with TGF-β for 72 hours to induce EMT, and control cells were cultured in the absence of TGF-β for the same time. To assess experimental metastasis, control and EMT-induced cells were injected into 4 groups (control, EMT, EMT + Tro, and EMT + Smad3 KD) of mice via tail vein. A group of mice injected with EMT-induced cells is treated with Tro (EMT + Tro; 400 mg/kg) once daily by oral gavage. Except for the Smad3 KD group, lung metastasis was assessed by monitoring bioluminescence from luciferase on day 20. Because Smad3-shRNA construct had no luciferase in it, metastasis in the Smad3 KD group was assessed by counting the number of colonies formed in the lung by serial sectioning and H&E staining. As expected, 5 of 5 mice injected with EMT-induced A549-Luc cells showed metastasis in lungs by bioluminescence imaging (Fig. 6A) compared with control group mice, which showed significantly little bioluminescence activity (2/5 mice; Fig. 6A). These results show that TGF-β–induced EMT in fact enhances the metastatic capabilities of cancer cells in vivo.

Treatment of mice with Tro significantly inhibited the ability of EMT-induced cells to form metastatic colonies in the EMT + Tro group (Fig. 6A). Of 5 mice, only 3 mice had detectable bioluminescence activity, which was significantly less than the activity in EMT group (Fig. 6A), showing the efficacy of Tro treatment. Because the A549-
Smad3 KD cells do not contain any luciferase expression plasmid, for comparison, we sacrificed the animals in all groups and counted the number of lung metastases in each mouse after H&E staining (Fig. 6B and C). EMT+Smad3 KD group showed significantly lower number of lung metastases than the EMT group, indicating that Smad3 inhibition attenuates EMT-induced ability to metastasize in lung cancer cells. Together, these results clearly show that PPAR-\(\gamma\) activation inhibits metastasis by antagonizing Smad3-mediated EMT.

**Discussion**

In addition to conferring migratory and invasive capabilities in tumor cells, EMT is implicated in increased resistance to apoptosis resulting in acquired drug resistance (4), enabling evasion of host immune surveillance (29) and, in some cases, conferring stem cell traits (5). Together, these observations established the role of EMT in tumor progression by putting a long-standing debate about its physiologic relevance to rest. Given these clinically relevant phenotypes acquired by tumor cells in the mesenchymal state, targeting EMT can be an attractive strategy with a potential to make a significant impact on the management of metastatic disease. Here, we showed one such strategy to inhibit EMT by means of activating PPAR-\(\gamma\) with its synthetic ligands Tro and Rosi (TZDs). Of the 2 ligands, Rosi, along with another PPAR-\(\gamma\) ligand pioglitazone, is currently in clinical use for the treatment of type II diabetes. Tro was also used for the treatment of...
diabetes for several years before it was withdrawn from the market for a rare liver toxicity. Interestingly, epidemiologic evidence suggests that clinical use of PPAR-γ ligands significantly lowers the risk of developing lung cancer in patients who received these drugs for the treatment of type II diabetes (30).

In contrast to the effects of PPAR-γ ligands on tumor growth, their effects on the process of metastasis is not well studied. A recent report by Tan et al. in the context of fibrotic EMT showed the inhibition of TGF-β–induced EMT marker expression by PPAR-γ agonists (31). Here, we showed that activation of PPAR-γ by its ligands completely inhibited TGF-β–induced cancer cell EMT, as assessed by the expression of epithelial and mesenchymal markers. This was observed in 2 different cell types, that is, a lung and a pancreatic adenocarcinoma cell line, showing the relevance of these observations to more than one tumor type. Furthermore, PPAR-γ ligands also blocked functional consequences of EMT by inhibiting change in morphology, cellular migration, invasion, and secretion of MMPs. PPAR-γ ligands are known to exert their effects utilizing both PPAR-γ–dependent and -independent pathways (32, 33). In this study, the effects of constitutively active VP16-PPAR-γ along with the effects of PPAR-γ siRNA clearly show that the inhibition of EMT by TZDs is mostly due to the activation of PPAR-γ.

In an earlier study, overexpression of PPAR-γ in NSCLC cells was shown to inhibit metastasis by inducing a differentiated epithelial phenotype (32). This is consistent with our PPAR-γ–dependent inhibition of EMT, which may result in maintaining differentiated epithelial phenotype. In another study, Onder et al. showed that the inhibition of E-cadherin expression, by shRNA, was sufficient to induce N-cadherin and vimentin expression in H-ras–transformed human breast epithelial cells (34). They also showed that cells expressing E-cadherin shRNA were more migratory and invasive in vitro and more metastatic in vivo (34). This suggests that preventing the loss of E-cadherin expression by PPAR-γ ligands might be one of the reasons for blocking the gain of mesenchymal markers and subsequent functional phenotype of increased motility and invasion during EMT.

Both Smad-dependent as well as -independent pathways have been implicated in the regulation of TGF-β–induced EMT (35, 36). Among the receptor Smads (Smad2 and Smad3), Smad3 was shown to be an important mediator of EMT whereas Smad2 was suggested as a potential negative regulator (37, 38). Consistently, we observed a primary role for Smad3 in regulating TGF-β–induced EMT in A549 cells. Smad2 knockdown has exaggerated certain TGF-β responses consistent with its potential role as a negative regulator. Activation of PPAR-γ has been shown to antagonize TGF-β signaling in other biological contexts. However, the precise mechanism of this cross talk is still not clear. Our analysis clearly shows that activation of PPAR-γ either by ligands or by constitutively active form of PPAR-γ does not affect phosphorylation of Smads by TGF-β receptor or the subsequent translocation of Smads into nucleus, as suggested by studies in nonmalignant cells (26, 27). Interestingly, PPAR-γ activation by Tro completely abrogated Smad-dependent transcriptional activity. Given the primary role of Smad3 in EMT, abrogation of Smad transcriptional activity by PPAR-γ ligands suggests that activation of PPAR-γ might be selectively antagonizing Smad3 function to inhibit EMT. When we assessed the effect of PPAR-γ activation on the promoters of 2 Smad3–dependent genes, E- and N-cadherin, we observed a reversal of E-cadherin promoter repression and a robust inhibition of N-cadherin promoter activity. More important, either knockdown of Smad3 or treatment with PPAR-γ ligand Tro significantly inhibited EMT–induced experimental metastasis in vivo. These observations further strengthened the notion that PPAR-γ activation inhibits EMT by antagonizing Smad3-dependent transcriptional activity. It would be interesting to decipher the precise molecular mechanism by which PPAR-γ antagonizes Smad transcriptional activity.

Many studies have shown the potent anticancer effects of PPAR-γ ligands in different preclinical models in a variety of human cancers including lung. Particularly, in lung, this is also supported by strong epidemiologic evidence reporting a decreased risk of lung cancer in patients receiving PPAR-γ ligands for type II diabetes (30). However, there were no clinical trials testing the efficacy of PPAR-γ ligands in lung cancer, with the exception of one recently initiated trial (NCT00923949). Earlier trials, testing PPAR-γ ligands as monotherapy, in a very small number of advanced stage prostate and breast cancer patients failed to show a therapeutic benefit (39–41). Interestingly, recent studies (42, 43), including ours (21), assessing the use of PPAR-γ ligands in combination with standard chemotherapeutic agents, showed synergistic effect. Colorectal cancer cells resistant to oxaliplatin or ovarian cancer cells resistant to paclitaxel both exhibit EMT phenotype (44, 45). Similarly, NSCLC cell lines with mesenchymal morphology were shown to be resistant to epidermal growth factor receptor inhibitors gefitinib and erlotinib compared with cells with epithelial morphology (46). The observation along with the ability of EMT to confer drug resistance and induce tumor stem cell traits suggests that blocking EMT with PPAR-γ ligands may have much broader impact on the clinical management of metastatic disease. It is worth noting that in our in vivo experimental metastasis assay, Tro inhibited the metastasis of cells that are already undergone EMT and in circulation, mirroring the condition most often encountered in human patients. Together with the low toxicity profile of PPAR-γ ligands, this observations strongly suggests that it is worthwhile to test PPAR-γ ligands at least as adjuvant in cases wherein the tumors show resistance to standard or targeted chemotherapies either due to EMT or due to other mechanisms.
Disclosure of Potential Conflicts of Interest

The authors declare no conflict of interest.

Acknowledgment

We thank Dr. Mitchell Lazar (University of Pennsylvania) for providing V16-PPAR-γ cDNA construct.

References


Molecular Cancer Therapeutics

Peroxisome Proliferator-Activated Receptor-γ Activation Inhibits Tumor Metastasis by Antagonizing Smad3-Mediated Epithelial-Mesenchymal Transition


Updated version
Access the most recent version of this article at:
http://mct.aacrjournals.org/content/9/12/3221

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2011/01/04/9.12.3221.DC1

Cited articles
This article cites 45 articles, 19 of which you can access for free at:
http://mct.aacrjournals.org/content/9/12/3221.full.html#ref-list-1

Citing articles
This article has been cited by 6 HighWire-hosted articles. Access the articles at:
/content/9/12/3221.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.