# A peroxisome proliferator-activated receptor $\gamma$ antagonist induces vimentin cleavage and inhibits invasion in high-grade hepatocellular carcinoma

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Received May 14, 2007; Accepted July 2, 2007

Abstract. Increased expression of vimentin in carcinomas correlates with parameters of malignant potential such as tumor grade and tumor metastasis. Peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) has been intensively evaluated as a potential target for the inhibition of cell growth and metastasis in cancer cells. In the present study, we examined whether PPAR $\gamma$  is a possible target molecule for the prevention of cell growth and invasion by treatment with agonists (troglitazone, rosiglitazone) and antagonists (T0070907, GW9662) in four different hepatocellular carcinoma (HCC) cell lines. We also evaluated the effects of the PPAR $\gamma$  agonists and antagonists on tumor cell migration and invasion. The expression level of PPARy protein was higher in the sarcomatoid SH-J1 and poorly differentiated HLE cell lines than that in the welldifferentiated HCC cell lines (HepG2 and Huh-7). Expression of vimentin was high in the SH-J1 HCC cell line and minimally detected in the HLE cell line. Treatment with low doses of the PPARy antagonists inhibited cell growth and colony formation of all four of the HCC cell lines. Vimentin in the high-grade HCC cells was cleaved by the treatment with the PPAR $\gamma$ antagonists. Furthermore, treatment with the PPARy antagonists also strongly inhibited migration and invasion of the SH-J1 and HLE cells. However, treatment with low doses of the agonists had no effect on vimentin expression, migration, and invasion of the high-grade HCC cells but cell growth was inhibited by treatment with high concentrations of the agonists. Our results indicate that treatment with a PPARy antagonist may prevent cell growth and invasion of highgrade HCC cells. Our findings also suggest that PPARy

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antagonists inhibit cell growth and invasion through vimentin disarrangement in high-grade HCC.

### Introduction

Human hepatocellular carcinoma (HCC) is the fifth most common type of malignant tumors and the third leading cause of cancer deaths worldwide (1). Despite the recent advances in diagnostic and therapeutic approaches in HCC, the prognosis of HCC patients is still poor. The poor prognosis of HCC has been associated with a high rate of recurrence and intrahepatic metastasis (2-4). Vimentin is a cytoplasmic intermediate filament usually expressed in mesenchymal cells but not in epithelial cells. The aberrant expression of vimentin and its relation to tumor metastasis have been reported for carcinoma of the prostate (5), breast carcinoma (6), uterine cervix carcinoma (7), and renal cell carcinoma (8). A previous study reported that overexpression of vimentin is associated with increased HCC metastasis (9). These findings strongly suggest that the aberrant expression of vimentin plays an important role in the invasion and metastasis of malignant epithelial tumor cells.

Peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) is a member of the nuclear hormone receptor superfamily and is expressed in many types of normal tissues and cancer cells (10-13). PPARy was originally characterized as a regulator of adipocyte differentiation and lipid metabolism (14,15). Previous studies showed that PPAR $\gamma$  is implicated as a putative therapeutic target for cancer in a variety of tumors, including HCC, as stimulation of PPARy function inhibits carcinogenesis and tumor cell growth (16-22). However, the exact role of PPARy in carcinogenesis and tumor growth is still controversial because of the many conflicting reports that provide evidence for a tumor promoter role (17,23,24). Moreover, recent studies have shown that blockage of PPARy by an antagonist caused cell death by the prevention of cell adhesion and the induction of anoikis (25,26). Furthermore, these studies have also indicated that antitumor effects of PPAR $\gamma$  antagonists are more potent than those of the PPAR $\gamma$ agonists.

In the present study, we examined the effects of PPAR $\gamma$  agonists and antagonists on cell growth and motility in highgrade HCC cells expressing vimentin.

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*Key words:* hepatocellular carcinoma, vimentin, peroxisome proliferator-activated receptor  $\gamma$ 

#### Materials and methods

Cell culture and treatment with PPARy agonists and antagonists. Four human HCC cell lines were used. The cell line HLE (poorly differentiated HCC) and Huh-7 (welldifferentiated HCC) were purchased from the Health Science Research Resources Bank (Osaka, Japan) and the line HepG2 (well-differentiated HCC) was obtained from the American Type Culture Collection (Manassas, VA, USA). In addition, we used the sarcomatoid HCC cell line, designated as SH-J1, which was previously established by us (27). HCC cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin and streptomycin (100 U/ml) containing 10% fetal bovine serum (Gibco BRL, Gaithersburg, MD, USA) and were grown at  $37^{\circ}$ C in a humidified 5% CO<sub>2</sub> incubator. After 24 h, cultures of 40-60% cell confluence were treated with the PPAR $\gamma$  antagonists T0070907, GW9662 or the PPAR $\gamma$  agonists troglitazone, rosiglitazone (Cayman, Ann Arbor, MI, USA), respectively.

Cell growth inhibition assay (XTT assay). Cell growth inhibition was determined by the use of the colorimetric tetrazolium derived XTT (sodium 3'-[1-(phenylaminocarbonyl)- 3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) assay (Roche Applied Science, Mannheim, Germany). HCC cells were grown in a 96-well plate at a density of  $5x10^3$  cells/well and treated with various concentrations of the PPAR $\gamma$  agonists (troglitazone, rosiglitazone) or antagonists (T0070907, GW9662), with concentrations ranging from 1  $\mu$ M to 100  $\mu$ M. After treatement for 24, 48, and 72 h, the XTT mixture solution (50  $\mu$ l) was added into each well. After incubation for 4-8 h, absorbance was measured using a microtiter plate (ELISA) reader at 470 nm. Independent experiments were repeated 3 times.

*Plating efficiency assay.* HCC cells were seeded on 12-well tissue culture plates at a density of  $2x10^4$  cells/well and were grown at 37°C in a humidified 5% CO<sub>2</sub> incubator for 10 days. The growth medium contained various concentrations of either the PPAR<sub>Y</sub> antagonists (ranging from 1  $\mu$ M to 30  $\mu$ M) or agonists (ranging from 10  $\mu$ M to 100  $\mu$ M); as controls, HCC cells of the same density were plated in wells containing growth medium with 0.1% dimethylsulfoxide (DMSO). HCC cells were seeded in triplicate, and the experiments were repeated 3 times.

Western blotting. Total protein was extracted in 1% Nonidet P-40 buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA), supplemented with protease inhibitor cocktail (Sigma, St. Louis, MO, USA) and phosphatase inhibitor cocktail I, II (Sigma), and was incubated on ice for 10 min, and centrifuged at 12,000 x g for 10 min at 4°C. Protein concentration was determined by use of the Coomassie blue method (Bio-Rad protein assay, Bio-Rad, Richmond, CA, USA). Protein samples were separated by electrophoresis on an 8% SDS-polyacrylamide gel, and the separated proteins were electrotransferred to a polyvinylidene difluoride (PVDF) membrane using a semidry transfer method (Bio-Rad). The membrane was then blocked with 5% nonfat dry milk in Tris-buffered saline (TBS)-0.1% Tween-20 (15 mM NaCl, 100 mM Tris-



Figure 1. Western blot analysis of peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) and vimentin expression in four different hepatocellular carcinoma cell lines. The expression level of PPAR $\gamma$  protein was higher in sarcomatoid SH-J1 (undifferentiated) and HLE cell (poorly differentiated) lines than in HepG2 and Huh-7 cell lines (well-differentiated). The expression of vimentin was high in the SH-J1 cell line and minimally detected in the HLE cell line.

HCl, pH 7.5) for 1 h to reduce nonspecific binding. The membrane was incubated with mouse anti-PPAR<sub>Y</sub> (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:1000 dilution), mouse anti-vimentin (Santa Cruz Biotechnology, 1:1000 dilution), rabbit anti-FAK (Cell Signaling Technology, Danvers, MA, USA; 1:1000 dilution) and rabbit p-FAK, overnight at 4°C. After washing 3 times, the membrane was incubated for 1 h at room temperature with a secondary antibody, and the immune complexes were visualized using enhanced chemiluminescence detection reagents (Amersham Biosciences, Buckinghamshire, UK) and exposed to a luminescent image analyzer (LAS-3000, Fuji Film, Tokyo, Japan). Equal loading of proteins in each lane was confirmed by probing the membrane with mouse anti- $\beta$ -actin (Sigma, 1:3000 dilution).

Immunocytochemistry. For the staining of vimentin, HCC cells were seeded on glass cover slides in 24-well culture plates. After 24 h, cultures of 50% cell confluence were treated with PPAR $\gamma$  antagonists (20  $\mu$ M) or agonists (30  $\mu$ M) for 48 h, and then the medium was removed, and the cells were fixed with phosphate-buffered saline (PBS) containing 3.7% paraformaldehyde for 10 min. Cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min, washed 3 times with PBS for 5 min, and were incubated with 2% bovine serum albumin in PBS containing 0.1% Tween-20 (PBS-T) for 30 min. A mouse anti-vimentin antibody (Santa Cruz) and the cells were incubated for 1 h at room temperature and were washed with PBS-T. The cells were then incubated with a secondary antibody (Alexa Fluor 488 rabbit anti-mouse IgG, Molecular Probes, Eugene, OR, USA) for 30 min at room temperature. To visualize the nucleus, the cells were incubated with Hoechst 33258 (Sigma) for 3 min. Cover slides were finally mounted and the cells were observed with the use of a fluorescence microscope.

*Migration assay*. Migration of HCC cells was assessed using a 48-well microchemotaxis chamber assay (Neuroprobe Inc., Gaithersburg, MD, USA). A polyvinylpyrrolidone-free poly-



B



 D
 T0070907 (µM)
 GW9662 (µM)
 Troglitazone (µM)
 Rosiglitazone (µM)

 CT
 1
 6
 CT
 1
 6
 CT
 10
 20
 CT
 10
 20

 SH-J1
 10
 20
 30
 10
 20
 30
 30
 50
 100
 30
 50
 100

 HLE
 10
 20
 30
 10
 20
 30
 30
 50
 100
 30
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 HLE
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 CT
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 10
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 CT
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 HLE
 10
 20
 30
 10
 20
 30
 30
 50
 100
 30
 50
 100

 CT
 1
 6
 CT
 1
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 CT
 10
 20
 CT
 10
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 HepG2
 10
 20
 30
 10
 20
 30
 30

Figure 2. PPAR<sub>Y</sub> antagonist inhibits the cell growth of HCC cells. (A) The time- and dose-dependent effects of PPAR<sub>Y</sub> agonists and antagonists on cell growth. A significant inhibition of cell growth by antagonists, but not by agonists, was observed. Cell growth was determined by use of the XTT assay as detailed in Materials and methods. The data represent the mean  $\pm$  SD of three experiments. \*P<0.01 vs. vehicle control. (B) Dose-dependent effects of the PPAR<sub>Y</sub> antagonists on colony formation assessed by the plating efficiency assay. HCC cells were seeded in 24-well plates at a density of 2x10<sup>4</sup> cells/plate for 10 days; the growth medium contained T0070907 (1-30  $\mu$ M/l) or GW9662 (1-30  $\mu$ M/l). Both T0070907 and GW9662 inhibited colony formation of HCC cells.

carbonate membrane with an 8- $\mu$ m pore size separated the upper and lower wells of the chamber. SH-J1 and HLE cells (3x10<sup>4</sup>) were suspended in DMEM medium containing 0.1% FBS and a PPAR<sub>Y</sub> antagonist, T0070907 (20  $\mu$ M) or GW9662 (20  $\mu$ M), and were placed in the upper chamber. In the lower chamber, DMEM with 5% FBS and a PPAR<sub>Y</sub> antagonist served as the source of a chemoattractant. After incubation for 10 h, the cells on the upper surface of the filter were wiped with a cotton swab. The cells on the lower surface of the filters were fixed and stained with Diff-Quik soulution (Dade Behring, Newark, NJ, USA) for 10 min. For each replicate, the HCC cells that migrated to the lower surface of the filter were counted under a light microscope at x100 magnification in five randomly selected fields per well.

Invasion assay. Cell invasion assays were performed using a 24-transwell BD BioCoat Matrigel invasion chamber with an 8- $\mu$ m pore size (BD Biosciences, San Jose, CA, USA). SH-J1 and HLE cells (3x10<sup>4</sup>) were suspended in DMEM medium containing 0.1% FBS and T0070907 (20  $\mu$ M) or GW9662 (20  $\mu$ M) and were placed in the upper chamber. DMEM with 5% FBS and PPAR $\gamma$  antagonists were also added to the lower chamber. After incubation for 24 h, the cells on the Matrigel

were mechanically removed with a cotton swab. The cells on the lower surface of the filters were fixed and stained for 10 min using Diff-Quik solution. The invaded cells were counted under a light microscope at x100 magnification in five randomly selected fields per well.

Statistical analysis. Data are presented as the mean  $\pm$  SD. The unpaired t-test or one-way ANOVA test was used to determine statistical significance. A P-value of <0.05 was considered as statistically significant.

#### Results

*Expression of PPARy and vimentin in HCC cell lines*. We evaluated the expression of PPARy in four different human HCC cell lines using Western blotting. Western blot analysis revealed a different level of PPARy protein expression in each cell line (Fig. 1). The expression level of the PPARy protein was higher in the SH-J1 (undifferentiated) and HLE (poorly differentiated) cell lines than that in the HepG2 and Huh-7 (well-differentiated) cell lines. The expression of vimentin was high in the SH-J1 cell line and barely detected in the HLE cell line (Fig. 1). However, expression of vimentin was not evident in the HepG2 and Huh-7 cell lines.





Α



HLE



Figure 3. A PPAR<sub>Y</sub> antagonist induces disarrangement of vimentin filaments in HCC cells. (A) Western blot analysis shows that the treatment of HCC cells with a PPAR<sub>Y</sub> antagonist induced cleavage of vimentin. (B) In the T0070907-treated SH-J1 and HLE cells, vimentin filaments are condensed along the cytoplasmic membrane and the regular arrangement from the cytoplasmic membrane to the adjacent nucleus was destroyed. (C) Expression of p-FAK decreased in HCC cells incubated with a PPAR<sub>Y</sub> antagonist. CT, control; TGZ, troglitazone (30  $\mu$ M/l); RGZ, rosiglitazone (30  $\mu$ M/l); T0070907 (20  $\mu$ M/l); GW9662 (20  $\mu$ M/l).

Inhibition of HCC cell growth by PPAR $\gamma$  antagonists in vitro. Use of the XTT assay showed that the PPAR $\gamma$  antagonists significantly inhibited growth of all four of the different HCC cells in a time- and dose-dependent manner (Fig. 2A). A significant growth inhibition of all HCC cells by the PPAR $\gamma$  antagonists (T0070907, GW9662) was seen at concentrations of 20  $\mu$ M and higher. At this concentration (20  $\mu$ M) of the antagonists, the growth inhibition of the cell lines was 28.6% (SH-J1), 37.6% (HLE), 18.8% (HepG2), and 18.5% (Huh-7), respectively. However, the PPAR $\gamma$  agonists troglitazone and rosiglitazone did not show any effects on the growth of all four HCC cell lines up to a dose of 50  $\mu$ M. A plating efficiency assay revealed that the PPAR $\gamma$  antagonists effectively inhibited colony formation of the HCC cells (Fig. 2B). At a concentration of 20  $\mu$ M, T0070907 or GW9662 exhibited a strong ability to prevent colony formation of HCC cells. In contrast, only a high concentration of the PPAR<sub> $\gamma$ </sub> agonists (>50  $\mu$ M) affected the plating efficiency of HCC cells, which was similar to that observed with the XTT assay.

PPAR<sub>Y</sub> antagonists induce vimentin disarrangement and reduce focal adhesion kinase (FAK) activation. Treatment of the HCC cells with the PPAR<sub>Y</sub> antagonists (20  $\mu$ M) for 48 h resulted in an induction of the cleavage of vimentin. The amount of cleaved vimentin isoforms with lower molecular weight increased with treatment with T0070907 or GW9662 in both the SH-J1 and HLE cell lines (Fig. 3A). However, treatment with troglitazone and rosiglitazone (30  $\mu$ M) did not affect the cleavage of vimentin. Immunocytochemical staining for vimentin showed that HCC cells treated with T0070907





Figure 4. Effect of a PPAR $\gamma$  antagonist on migration and invasion of HCC cells *in vitro*. (A) Effect of a PPAR $\gamma$  antagonist on the migration of HCC cells *in vitro*. (A) Effect of a PPAR $\gamma$  antagonist on the migration of HCC cells *in vitro*. HCC cells were pretreated with or without a PPAR $\gamma$  antagonist for 24 h as described in Materials and methods. After incubation for 10 h, cells on the lower surface membrane were fixed and stained using Diff-Quik solution. Experiments were repeated independently 3 times with similar results. \*P<0.01 vs. control vehicle. (B) Effect of a PPAR $\gamma$  antagonist on the invasion of HCC cells *in vitro*. HCC cells were pretreated with or without of a PPAR $\gamma$  antagonist for 24 h as described in Materials and methods. After incubation for 24 h, cells on the lower surface membrane were fixed and stained using Diff-Quik solution. Experiments were repeated independently 3 times with similar results. \*P<0.05 vs. control vehicle.

were shrunken and vimentin filaments were condensed along the cytoplasmic membrane. The regular architectural arrangement of vimentin that stretched from the cytoplasmic membrane to the nuclear membrane was disarranged in the T0070907- or GW9662-treated cells (Fig. 3B). In the vehicle control, vimentin was clearly observed and the cell shapes remained normal (Fig. 3B). Western blot analysis revealed a decreased expression of phosphorylated FAK (p-FAK) by treatment with the antagonists (20  $\mu$ M) (Fig. 3C).

 $PPAR\gamma$  antagonists inhibit migration and invasion of HCC cells. To determine the effect of the PPAR<sub>Y</sub> antagonists on HCC cell migration and invasion, a cell migration and invasion assay was performed. Treatment with both PPAR<sub>Y</sub> antagonists at a concentration of 20  $\mu$ M dramatically inhibited the migration and invasion ability of SH-J1 and HLE cells

(Fig. 4). T0070907 at 20  $\mu$ M significantly decreased the migration of SH-J1 and HLE by 48% and 45%, respectively (p<0.001). Similarly, PPAR<sub>Y</sub> antagonists at 20  $\mu$ M significantly decreased the invasion of SH-J1 and HLE by 27-43% and 30-46%, respectively (p<0.05).

## Discussion

This study demonstrates the following points: i) High-grade HCC cells showed high expression of PPAR $\gamma$  and the SH-J1 cell line exhibited high expression of vimentin. ii) Low concentrations of specific PPAR $\gamma$  antagonists, but not agonists, induced cell growth inhibition of the HCC cell lines in a timeand dose-dependent manner. iii) PPAR $\gamma$  antagonists were able to induce cleavage of vimentin filaments in the highgrade HCC cell lines. iv) The PPAR $\gamma$  antagonists inhibited

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the migration and invasion of the high-grade HCC cells. These findings indicate that the antitumor effect of the PPAR $\gamma$  inhibitors in vimentin-expressing high-grade HCC cells is partly due to the disruption of vimentin filaments.

Our results showed that PPAR $\gamma$  was expressed at various levels in all of the different liver cancer cell lines. The PPAR $\gamma$ expression was detected in the HepG2 and Huh-7 cell lines, in good agreement with previous studies (25,28,29). The expression level of PPAR $\gamma$  was much higher in the poorly differentiated HCC cell lines (SH-J1 and HLE) than in the two other well-differentiated HCC cell lines (HepG2 and Huh-7), a previously unreported observation. In agreement with our findings, previous studies have demonstrated that the expression of PPARy was higher in poorly differentiated esophageal cancer cells (30,31) and had a tendency toward an increased frequency of positive nuclear staining according to the degree of dedifferentiation of gastric cancer cells (32). However, a relationship between the expression level of PPARy and tumor differentiation remains somewhat controversial. Schaefer et al reported that the expression level of PPARy did not show a significant immunohistochemical difference in HCC according to the grade of tumor differentiation (25). Another study has shown that welldifferentiated adenocarcinomas of the lung increased the frequency for PPARy positivity compared with moderately and poorly differentiated ones (33). Hence, in human cancers, PPAR $\gamma$  expression level does not seem to show a consistent tendency according to the tumor grade, suggesting a tumorspecific expression of PPARy.

The nuclear transcription factor PPAR $\gamma$  has been considered as a putative therapeutic cancer target in a variety of epithelial cell tumors, including HCC (16-22). Initial investigations favor activation of PPAR $\gamma$  by demonstrating that PPARy agonists induce growth arrest and/or apoptosis in many different epithelial tumor cell lines (16-22). Despite the findings that PPAR $\gamma$  stimulation might favor cancer remission, clinical trials with PPARy agonists have shown modest to little efficacy (34-36). In this study, we demonstrated that a low concentration (20  $\mu$ M) of the PPAR $\gamma$  antagonists induced the inhibition of growth and invasive properties of HCC cells. However, high concentrations of PPARy agonists were required for growth inhibition. Our findings are consistent with the results of Schaefer et al (25), who reported that the PPAR $\gamma$  inhibitors T0070907 and GW9662, but not the thiazolidenediones, troglitazone and rosiglitazone, were able to prevent reattachment of HepG2 and Hep3B cells to extracellular matrix proteins and resulted in apoptotic cell death subsequent to cell detachment (anoikis). Furthermore, in other types of cancer, for example breast, esophageal and colorectal carcinoma cells, inhibition of PPARy induces growth inhibition and apoptosis (31,37,38). Taken together with the findings of the present study, these results indicate that PPAR $\gamma$  inhibitors can be considered as important candidates for further development as anticancer agents of HCC.

In various cancers, vimentin expression is associated with a dedifferentiated malignant phenotype, increased motility, invasive ability, and poor prognosis (5-8). It has also been shown that the aberrant expression of vimentin is significantly associated with metastasis in HCC (9,39). The increased motility and invasive behavior of cancer cells could be transiently down-regulated by treatment with vimentin antisense nuleotides (40). In this study, we showed that treatment with PPARy antagonists induced decreased activation of focal adhesion kinase in HCC cells. In addition, we showed that the expression of vimentin in the high-grade HCC cells was disrupted and that vimentin was cleaved by the treatment with PPAR $\gamma$  antagonists. Furthermore, treatment with a low dose of the PPAR $\gamma$  antagonists strongly inhibited cell migration and invasion of the SH-J1 and HLE cells. In agreement with our findings, previous studies have demonstrated that PPARy antagonists inhibit cell migration and invasion of various cancer cells (31,37,38). In the HCC cell lines, PPARy inhibitors cause a lack of adhesion and morphologic changes prior to their commitment to apoptosis, and caspase inhibition does not prevent these changes (25). Similar to our results, the loss of cellular adhesion is associated with decreased focal adhesion kinase (FAK) activation in hepatocellular, esophageal, and squamous carcinoma cell lines (25,26,31). PPARy inhibitors also reduce the levels of the microtubule precursor proteins  $\alpha$ and ß tubulin by a post-transcriptional regulatory mechanism and result in alterations in the morphology and defects in microtubule structure (37). In addition, PPARy antagonists inhibit colorectal cancer cell migration and reduce metastatic tumor formation in vivo (37). Intact tubulin networks are required to support focal adhesion complexes that bind the actin cytoskeleton. Since focal adhesion kinase (FAK) activation is dependent on the intact actin cytoskeleton, reduced FAK activation may also be a direct result of a decreased microtubule network (37,41). Previous studies have demonstrated that treatment of DU-145 human prostate cancer cells and Hep3B HCC cells with retinoic acid results in a marked decrease in vimentin expression as well as in cell motility and invasive ability (42,43). In addition to these findings, cleavage of vimentin by caspase disrupts its cytoplasmic network of intermediate filaments and induces apoptosis (44). Loss of the vimentin network results in reduced mechanical stability, motility and directional migration towards chemo-attractive stimuli (45). Collectively, our findings suggest that the PPARy antagonists inhibit cell motility and invasiveness of high-grade HCC through the disarrangement of vimentin filaments, including inactivation of FAK or the loss of microtubule network.

In conclusion, our findings provide further evidence for the important and novel role of the inhibition of PPAR<sub> $\gamma$ </sub> function and support the use of specific antagonists in the treatment of high-grade HCC and the prevention of tumor invasion and metastasis.

#### Acknowledgements

This study was supported by the Korea Research Foundation Grant funded by the Korean government (MOEHRD) (The Regional Research Universities Program/Center for Healthcare Technology Development).

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