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PPARgamma inhibits hepatocellular carcinoma metastases *in vitro* and in mice

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BACKGROUND: We have previously demonstrated that peroxisome proliferator-activated receptor (PPARγ) activation inhibits hepatocarcinogenesis. We aim to investigate the effect of PPARγ on hepatocellular carcinoma (HCC) metastatic potential and explore its underlying mechanisms.

METHODS: Human HCC cells (MHCC97L, BEL-7404) were infected with adenovirus-expressing PPARγ (Ad-PPARγ) or Ad-lacZ and treated with or without PPARγ agonist (rosiglitazone). The effects of PPARγ on cell migration and invasive activity were determined by wound healing assay and Matrigel invasive model *in vitro,* and in an orthotopic liver tumour metastatic model in mice.

RESULTS: Pronounced expression of PPARγ was demonstrated in HCC cells (MHCC97L, BEL-7404) treated with Ad-PPARγ, rosiglitazone or Ad-PPARγ plus rosiglitazone, compared with control (Ad-LacZ). Such induction markedly suppressed HCC cell migration. Moreover, the invasiveness of MHCC97L and BEL-7404 cells infected with Ad-PPARγ, or treated with rosiglitazone was significantly diminished up to 60%. Combination of Ad-PPARγ and rosiglitazone showed an additive effect. Activation of PPARγ by rosiglitazone significantly reduced the incidence and severity of lung metastasis in an orthotopic HCC mouse model. Key mechanisms underlying the effect of PPARγ in HCC include upregulation of cell adhesion genes, E-cadherin and SYK (spleen tyrosine kinase), extracellular matrix regulator tissue inhibitors of metalloproteinase (TIMP) 3, tumour suppressor gene retinoblastoma 1, and downregulation of pro-metastatic genes MMP9 (matrix metallopeptidase 9), MMP13, HPSE (heparanase), and Hepatocyte growth factor (HGF). Direct transcriptional regulation of TIMP3, MMP9, MMP13, and HPSE by PPARγ was shown by ChIP-PCR.

CONCLUSION: Peroxisome proliferator-activated receptor-gamma exerts an inhibitory effect on the invasive and metastatic potential of HCC *in vitro* and *in vivo*, and is thus, a target for the prevention and treatment of HCC metastases.

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Despite significant advances in early detection and therapy, hepatocellular carcinoma (HCC) still remains the third leading cause of cancer-related deaths worldwide (Bosch *et al*, 2004). The high mortality rate of HCC is mainly attributable to late presentation at advanced stage, where curative surgical resection is no longer feasible or of limited efficacy (Mann *et al*, 2007). Tumour recurrence in HCC can occur as metastases, whereas more than 90% of HCC-related deaths are the result of secondary local or distant disease. However, efficacious or curative drug therapy for HCC and its metastases remains elusive.

Peroxisome proliferator-activated receptor-gamma (PPARγ) is a ligand-activated transcription factor that belongs to the nuclear hormone receptor super family; its roles include control of several biological processes related to growth, differentiation, cell cycle, and apoptosis (Koeffler 2003). Activation of PPARγ has been shown to inhibit proliferation in several cancers *in vitro* and *in vivo* (Koeffler 2003; Grommes *et al*, 2004). Our group has recently reported that PPARγ activation by its agonist (Yu *et al*, 2006) or ectopic expression of PPARγ by Ad-PPARγ transfection (Yu *et al*, 2010) inhibits HCC growth and progression by suppressing cell proliferation, inducing cell apoptosis, and causing cell cycle arrest (Yu *et al*, 2006, 2010). The PPARγ expression in HCC is significantly reduced in tumour tissues compared with surrounding non-tumourous liver, especially in poorly differentiated tumour than in well-differentiated tumour (Yu *et al*, 2006). Peroxisome proliferator-activated receptor-gamma also has a role in inhibiting tumour growth and metastatic spread in colon (Takano *et al*, 2008) and thyroid cancers (Ohta *et al*, 2001; Chen *et al*, 2006) as well as lung carcinoma (Panigrahy *et al*, 2002). However, PPAR's effect on invasive and metastatic potential of HCC has yet to be defined.

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The aim of the present study was to determine the effect and the underlying molecular mechanism of PPARγ on HCC cell migration and invasion using HCC cell lines and formation of distant metastases *in vivo* in an orthotopic murine liver tumour model.

MATERIALS AND METHODS

Human HCC cell lines and culture

The human HCC cell line MHCC97L, stably labelled with luciferase, was a gift from K Man, Department of Surgery, The University of Hong Kong (Man *et al*, 2010). BEL-7404 was obtained from the Institute of Biochemistry and Cell Biology (SIBS, Shanghai, China) (Chen *et al*, 1980). Cells were cultured in DMEM (Dulbecco's modified Eagle medium) supplemented with 10% foetal bovine serum (Invitrogen, Carlsbad, CA, USA) and incubated at 37 °C and 5% CO_2.

Adenovirus-mediated PPARγ gene transfer

Recombinant adenovirus expressing the mouse PPARγ-1 cDNA (Ad-PPARγ) or *E. coli* β-galactosidase gene (Ad-LacZ, control adenovirus vector) (gift from JK Reddy, Department of Pathology, Feinberg School of Medicine, Northwestern University, Chicago) was propagated, isolated in human embryonic kidney 293 (HEK293) cells, then purified with Adeno-X Maxi Purification Kit (Clontech, Mountain View, CA, USA); the adeno virus with infectious titre range from 1.0×10^9 to 10^{10} pfu (plaque-forming unit) ml^{-1} was stored at -80 °C until use.

RNA extraction, cDNA synthesis, and RT–PCR

Total RNA was extracted from cell pellets by Trizol (Invitrogen) and reverse transcribed into cDNA using MultiScribe Reverse Transcriptase (Applied Biosystems, Foster city, CA, USA) according to the manufacturer's instructions. The target gene expression was determined by RT–PCR using specific primers of target genes (Table 1). GAPDH was served as an internal control for total cDNA content. Samples were amplified using the ABI Prism 7700 Sequence Detection System (Applied Biosystems).

Tumour cell migration assay

Wound healing assay was performed for analysis of cell migration *in vitro*. Briefly, MHCC97L (5×10^5 cells per well) or BEL-7404 (5×10^5 cells per well) cells were seeded in 12-well plates and infected with Ad-LacZ (70 multiplicities of infection, MOI) or Ad-PPARγ (70 MOI), and treated with or without rosiglitazone (50 μM) at 37°C until 90% confluent (Yu *et al*, 2010). Sterile tips were used to scratch cell layers, which were subsequently washed with PBS, and cultured with DMEM media and 1% FBS. Cells were photographed (phase-contrast microscope) at 0, 24, 36, and 48 h after incubation. The distance travelled by cells was measured between the two boundaries of an acellular area and results of treatment groups expressed as a ratio to Ad-Lacz-treated cells. Each experiment was performed in triplicate.

Tumour cells invasion assay

Matrigel invasion assay (Becton Dickinson, Waltham, MA, USA) was performed as previously described (Yu *et al*, 2009). MHCC97L and BEL-7404 cells infected with Ad-PPARγ or Ad-LacZ (2.5×10^4 per well) treated with or without rosiglitazone (a selective PPARγ agonist) at 0, 24, 36, and 48 h, then harvested and added into the trans-well containing 600 μl DMEM media and 10% FBS in the lower chamber. After 48 h, cells that had invaded through the Matrigel membrane were stained with crystal violet, and counted (four high-power fields, $\times 100$ magnification). Experiments were conducted in triplicate.

Orthotopic murine liver tumour model of distant metastasis

An orthotopic HCC metastasis mouse model was established using MHCC97L, which has metastatic potential to lung (Man *et al*, 2010). MHCC97L cells (2×10^6 cells in 0.1 ml PBS) were injected subcutaneously into the left dorsal flank of 4-week-old male Balb/c nude mice. Subcutaneous tumours were harvested once the subcutaneous tumours reached about 10 mm^3 and cut into 1.0 mm^3 pieces. One piece of tumour was then implanted into the left liver lobes in a separate group of nude mice (6-week-old) (10 per group) (Man *et al*, 2010). After tumour implantation, mice were randomly treated with or without rosiglitazone (200 p.p.m. in chow), a dosage was selected base on our previous experiments (Yu *et al*, 2010). Liver tumour growth and lung metastasis were monitored by Xenogen IVIS-200, an optical *in vivo* imaging system (Caliper Life Science, Hopkinton, MA, USA) weekly. Mice were euthanised at week 7 after tumour implantation (Man *et al*, 2010), tumours and lung nodules were analysed histologically. Signal intensity of tumours detected by Xenogen IVIS was expressed as Radiant Efficiency (radiance/illumination power density = $p\,s^{-1}\,cm^{-2}\,sr^{-1}$). All experimental procedures were approved by the Animal Ethics Committee of the Chinese University of Hong Kong.

cDNA expression array

Gene expression profiles were analysed by the Human Tumour Metastasis PCR Array according to the protocol (SABiosciences, Frederick, MD, USA). Briefly, total RNA was extracted from MHCC97L cells infection with Ad-PPARγ (70 MOI) or Ad-LacZ (70 MOI) (control) for 48 h. One μg of total RNA was treated and cDNA was prepared using RT^2 First Strand Kit (SABiosciences). Pairs of test and control samples were mixed with RT^2 qPCR Master mix, distributed to PCR array in 96-well plates, cycling with real-time PCR. Each array contained 84 genes with ascribed functions related to metastasis pathways. Data were analysed using SABiosciences software. Genes with fold-changes more than or less than 1.5 were considered to be of biological significance.

Chromatin immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation analysis was performed using Red ChIP Kit (Diagenode, Liège, Belgium). After transfection with Ad-PPARγ (70 MOI) or Ad-LacZ (70 MOI) for 48 h, MHCC97L cells were fixed and collected for ChIP assay. DNA–protein complexes were precipitated using specific antibody of PPARγ (Santa Cruz Biotechnology, Santa Cruz, CA, USA). DNA fragments were decross-linked and purified from complexes; immunoprecipitated and input DNA were used as templates for ChIP-PCR.

Characterisation of PPARγ-binding region and semi-quantitatation of ChIP-PCR

To evaluate the direct modulation of transcriptional activity on the promoters of metastasis-related genes by PPARγ, the Genomatix tool, ModelInspector, was used to scan all the known PPARγ-binding regions linked to metastasis-related genes. Human promoter regions (size between 2 kb) in the Genomatix promoter database were scanned. Matched binding sites were selected with core and matrix similarity of >0.8 (http://www.genomatix.de/online_help/help_matinspector/matinspector_help.html). The predicted PPARγ-binding sites on the promoter of target genes were validated by ChIP-PCR (specific primers listed in Table 1).

Table 1 Primer sequences for semi-quantitative PCR detection

Gene	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
mRNA primers		
MMP9	TTGACAGCGACAAGAAGTGG	GCCATTCACGTCGTCCTTAT
MMP13	GAAGATGATTTGTCTGAGGAA	GCCGAAGAAAGACTGCATT
GAPDH	ACAACAGCCTCAAGATCATCAG	GGTCCACCACTGACACGTTG
DNA primers		
TIMP3 promoter 1	AGGGTCTTTGCACTTGCTGT	ATCCTCGCTGAGAAGTGGAC
MMP9 promoter 1	TGGGGAGGATATCTGACCTG	AAGAGCACAAGGGTGGACTG
MMP9 promoter 2	CAGGGCTGGAGAACTGAAAG	CCTGCCAAAAGACCATGATTC
MMP13 promoter 1	GAAAAAGTCGCCACGTAAGC	CCTGGGGACTGTTGTCTTTC
MMP13 promoter 2	GTTCCTGACCTGAGCAGCAT	TCCCCTGCAGAAGTAAATGG
HPSE promoter	GGGTGGTTGATCTCTTTCCA	CCTTCCTCTCCCATCTAGC

Western blot analysis

Total protein was extracted from cell pellets and protein concentration measured by Bradford assay (Bio-Rad, Hercules, CA, USA). A total of 30 mg of protein was separated by 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis, then transferred onto equilibrated polyvinylidene difluoride membrane (Amersham Biosciences, Buckinghamshire, UK). Membranes were probed with primary antibodies for tissue inhibitors of metalloproteinase 3 (TIMP3), heparanase (HPSE), and E-cadherin (Santa Cruz Biotechnology).

Statistical analysis

Data were presented as means ± s.d. Multiple group comparisons were analysed by one-way ANOVA after Bonferroni's correction. Non-parametric data between two groups was computed by Chi-square test or Fisher Exact test. The difference for two different groups was determined by Student's *t* test. A *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Peroxisome proliferator-activated receptor-gamma suppresses HCC cell migration and invasiveness of MHCC97L and BEL-7404 human HCC cell lines

The MHCC97L and BEL-7404 cells were infected with Ad-PPARγ or Ad-LacZ (control) in the presence or absence of rosiglitazone for 48 h and induction of PPARγ was confirmed by Western blot (Figure 1A). Enhanced PPARγ expression by Ad-PPARγ or rosiglitazone, markedly slowed cell migration scratchy 'wound' at edges of MHCC97L and BEL-7404 HCC cells (Figure 1B). Quantitative analyses at 36 h confirmed a significant reduction in wound closure in Ad-PPARγ or rosiglitazone-treated cells compared with Ad-LacZ-infected control cells (Figure 1C). There appeared to be an additive effect of Ad-PPARγ plus rosilitazone compared with Ad-PPARγ or rosiglitazone only in BEL-7404 cell line (Figure 1C).

To study the effect of PPARγ conferred on the invasiveness of HCC, MHCC97L and BEL-7404 cells were infected with Ad-PPARγ, or treated with rosiglitazone using a Matrigel model (Figure2A). *In vitro* invasively growing HCC cells were significantly impaired by up to 60% when infected with Ad-PPARγ or primed by rosiglitazone at 48 h (Figure 2B). Moreover, the combination of Ad-PPARγ and rosiglitazone incrementally suppressed cell invasion compared with Ad-PPARγ or rosiglitazone alone (Figure 2B).

Activation of PPARγ by rosiglitazone inhibits HCC metastases to the lung in vivo

In light of the observed anti-migration and anti-invasion effects of PPARγ on HCC cell lines *in vitro*, we tested whether activation of PPARγ by rosiglitazone could alter metastatic potential of MHCC97L *in vivo* in an orthotopic metastasis mouse model, where subcutaneously grown tumours derived from MHCC97L cells expressing luciferase were implanted into the livers of nude mice; small successful transplantation of tumours were confirmed by xenogen imaging 2 weeks after surgery (Figure 3A). Mice were randomly treated with rosiglitazone or vehicle for 7 weeks and then re-imaged *in vivo*. By week 7, 87.5% (7 out of 8) of the vehicle-treated control mice demonstrated lung metastases after orthotopic implantation. In contrast, only 33% (3 out of 9) of rosiglitazone-treated animals developed lung metastases ($P < 0.05$) (Figure 3B). The luciferase signals emanating from the lungs originally from the MHCC97L cells expressing luciferase in the rosiglitazone-treated mice were significantly lower than in the vehicle-treated group (1.7×10^5 *vs* 3.14×10^6 $p\,s^{-1}\,cm^{-2}\,sr^{-1}$, $P < 0.05$) (Figure 3C). Subsequent histology confirmed that lung nodules were secondary/metastatic deposits from liver (Figure 3B). Collectively, these results provide clear evidence that PPARγ activation inhibits lung metastasis in an orthotopic HCC model *in vivo*.

Peroxisome proliferator-activated receptor-gamma modulates the expression profiles of metastasis-related genes in MHCC97L cells

To elucidate the molecular mechanisms underlying the inhibitory effect of PPARγ on HCC cell invasiveness, gene expression profiles in Ad-PPARγ-infected MHCC97L were analysed using a human tumour metastasis pathway PCR array. When compared with control Ad-LacZ-infected cells, PPARγ altered downstream targets involved in cell adhesion, extracellular matrix (ECM) proteins, cell growth, and cell motility (Table 2), all of which are critical to the regulation of cancer cell invasiveness and metastasis. Peroxisome proliferator-activated receptor-gamma exerted its anti-metastatic effects by increasing the expression of cell adhesion genes, E-cadherin (5.2-fold), spleen tyrosine kinase (SYK) (1.7-fold), and ECM regulator metallopeptidase inhibitor 3 (TIMP3) (7.2-fold), a physiological inhibitor of matrix metallopeptidases (MMPs). The PPARγ also suppressed expression of pro-metastatic genes, such as MMP9 (− 1.7-fold), MMP13 (− 2.0-fold), HPSE (− 6.5-fold), and significantly diminished hepatocyte growth factor (HGF) (− 2.2-fold), a cellular growth and motility regulator. Further, PPARγ-induced retinoblastoma 1 (RB1) expression, a potent tumour suppressor gene by four-fold (Table 2).

To validate these changes on expression profiling, western blot and semi-quantitative RT–PCR were performed on MHCC97L and BEL-7404 cells infected with Ad-PPARγ or Ad-LacZ in the presence or absence of rosiglitazone. Ectopic expression of PPARγ by Ad-PPARγ or activation of PPARγ by rosiglitazone, increased E-cadherin and TIMP3 protein expression with a concomitant diminution of HPSE in both HCC cell lines (Figure 4A1). Combination of Ad-PPARγ and rosiglitazone exerted an additive effect on the induction of E-cadherin and TIMP3 and suppression

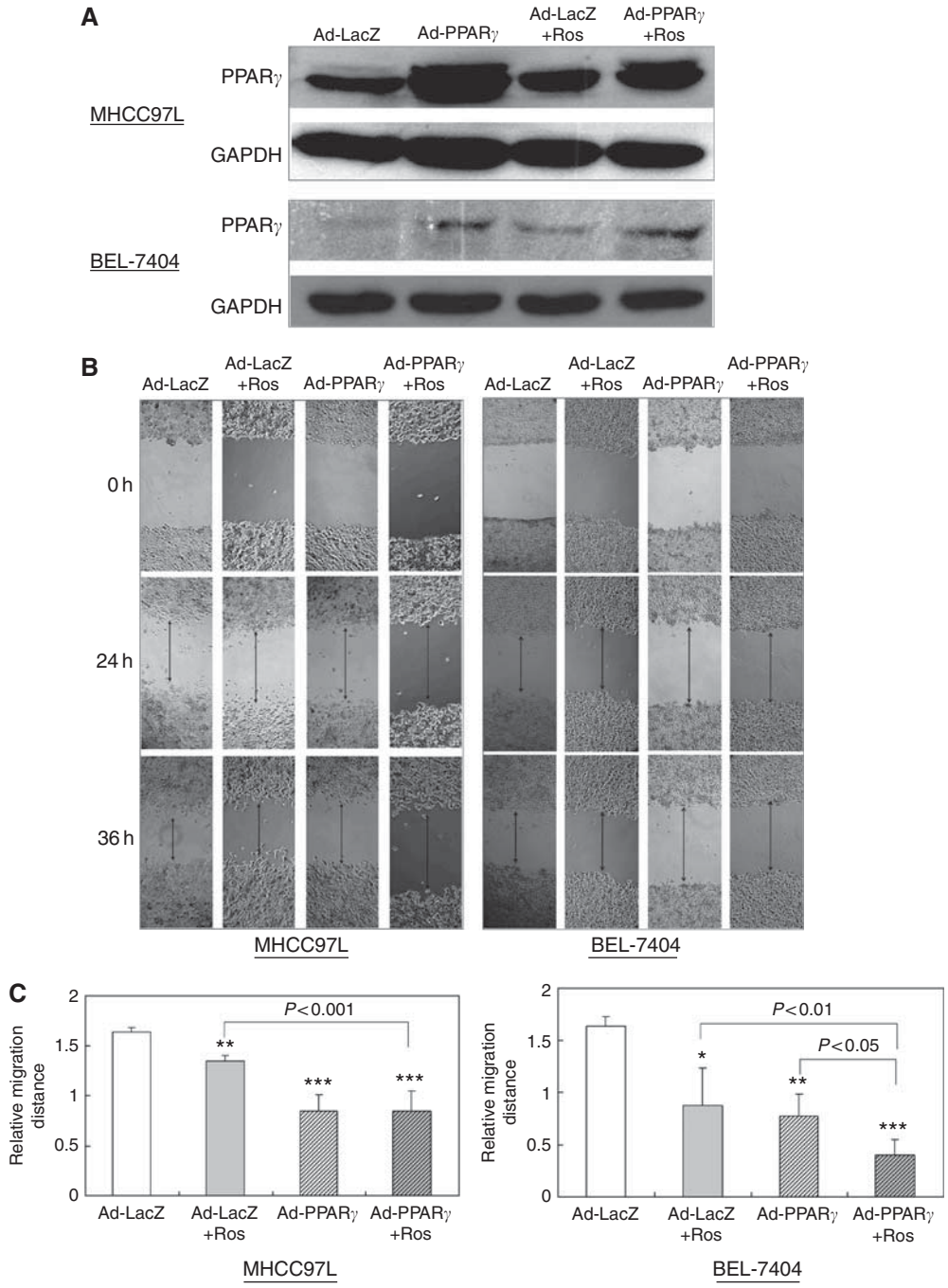

Figure 1 Effect of PPARγ on HCC cells motility by wound healing assay. (**A**) Pronounced expression of PPARγ protein was confirmed by western blot induced in HCC cells (MHCC97L and BEL-7404) treated with rosiglitazone, Ad-PPARγ or rosiglitazone plus Ad-PPARγ. (**B**) Representative images of the cell motility in MHCC97L and BEL-7404 cells under treatment with Ad-LacZ, Ad-LacZ + rosiglitzaone, Ad-PPARγ, or Ad-PPARγ + rosiglitazone at 0, 24, and 36 h, respectively. (**C**) Quantification of cell motility was made by measuring the distance travelled by the cells between the two boundaries of the acellular area, and the results of the different treatment group were expressed as a ratio to Ad-LacZ-treated cells (control). The experiment was performed for three times in triplicate. The data are expressed as means ± s.d., $*P<0.05$, $**P<0.01$, $***P<0.0001$, compared with the Ad-lacZ.

of HPSE (Figure 4A1). A similar enhanced suppressive effect of the combination of Ad-PPARγ and rosiglitazone was observed on MMP9 and MMP13 mRNA expression by RT–PCR (Figure 4A2).

Direct transcriptional regulation of TIMP3, MMP9, MMP13, and HPSE by PPARγ

To further determine whether PPARγ-mediated downstream gene expression changes were associated with direct promoter binding, PPARγ-binding sites in the promoters of identified targets, TIMP3, MMP9, MMP13, and HPSE, were mined using ModelInspector software (Genomatix Software GmbH, Munich, Germany). Chromatin immunoprecipitation assay with PPARγ antibody was performed in MHCC97L cells followed by PCR confirmation (Figure 4B). By ChIP-PCR assay, PPARγ binds to the promoters of TIMP3, MMP9, MMP13, and HPSE in MHCC97L cells (Figure.4B). These findings suggest that TIMP3, MMP9, MMP13, and HPSE are direct targets of PPARγ in liver cancer cells.

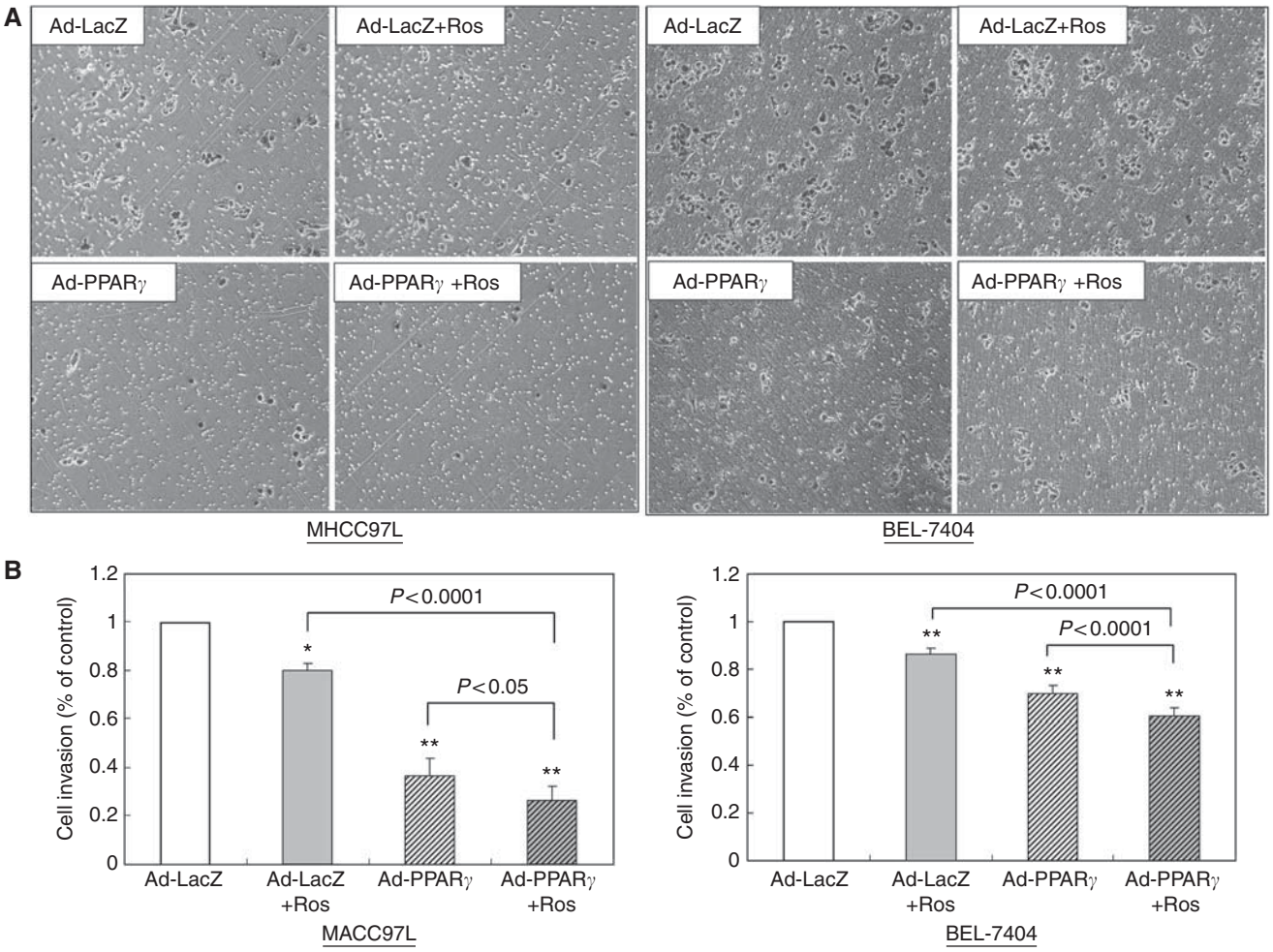

Figure 2 Effect of PPARγ on HCC cells invasive abilityby Matrigel invasion assay. (**A**) Representative images of the cell invasive and metastatic ability in MHCC97L and BEL-7404 cells treated with Ad-LacZ, Ad-LacZ + rosiglitzaone, Ad-PPARγ, or Ad-PPARγ + rosiglitazone at 48 h. (**B**) Quantification of cell invasion was determined by counting cells that invaded through the Matrigel membrane under a light microscopy(× 100). The relative cell number ratio of each group was presented by comparing to the Ad-LacZ group (control). The experiment was performed for three times in triplicate. The data are expressed as mean ± s.d., $*P<0.001$, $**P<0.0001$, compared with the Ad-lacZ.

DISCUSSION

The significance of PPARγ on the process of metastasis is not well studied in contrast to the effect of PPARγ on tumour growth. In this study, we show that ectopic expression of PPARγ by Ad-PPAR or its agonist, rosiglitazone, in two HCC cell lines (MHCC97L, BEL-7404) inhibits metastatic activity *in vitro*, in particular in wound healing, cell migration, and invasion. Moreover, the combination of Ad-PPARγ and rosiglitazone results in an enhanced anti-metastatic effect. Together, these results indicate that restoration of PPARγ allow for interactions with endogenous or exogenous agonists that activate the anti-metastatic processes associated with PPARγ. The role of PPARγ in inhibiting HCC metastases *in vitro* was further elucidated in an orthotopic HCC xenograft model. In this murine model, activation of PPARγ suppressed HCC lung metastasis in nude mice. Surgical resection or liver transplantation is the curative mainstays of HCC management, however, post-operative recurrence predominantly related to metastasis remains a challenge. Our finding was supported by recent studies, which indicated that activation of PPARγ inhibits metastasis of lung cancer (Reka *et al*, 2010) and non-small cell lung cancer (Choudhary *et al*, 2010) *in vitro* and *in vivo*. Collectively, our finding that activation of PPARγ by rosiglitazone significantly suppresses metastatic potential could have a beneficial impact on the clinical practice and in adjuvant therapy of HCC after surgical resection and transplantation.

The molecular mechanisms by which PPARγ exerts its anti-invasive and anti-metastases functions in HCC have not yet been defined. To identify key regulators of PPARγ-mediated anti-metastatic effect in HCC, we utilised cDNA microarray and ChIP-PCR to study MHCC97L cells infected with Ad-PPARγ, genes that were significantly altered in expression levels were then validated by RT–PCR and immunoblotting. We report that the suppression of cell invasion and migration mediated by PPARγ was mediated via downregulation of MMPs (MMP9, MMP13), increased expression of TIMP3 and E-cadherin. Of which, MMP9 and MMP13 are members of extracellular proteinases with key functions in the formation and remodelling of tumour invasion (Khasigov *et al*, 2003). Matrix metallopeptidase 9 has been described to promote tumour malignant progression, invasion, and metastatic spread by activating tumour growth factor-β (TGF-β) (Yu and Stamenkovic, 2000). Whereas, MMP13 has a central role in the modulation of other MMPs (Leeman *et al*, 2002) such as stimulating pro-MMP9 activation (Knäuper *et al*, 1997). Elevated MMP13 had been shown in various human malignancies including breast cancer, colorectal neoplasms, melanoma, and squamous head and neck tumours,

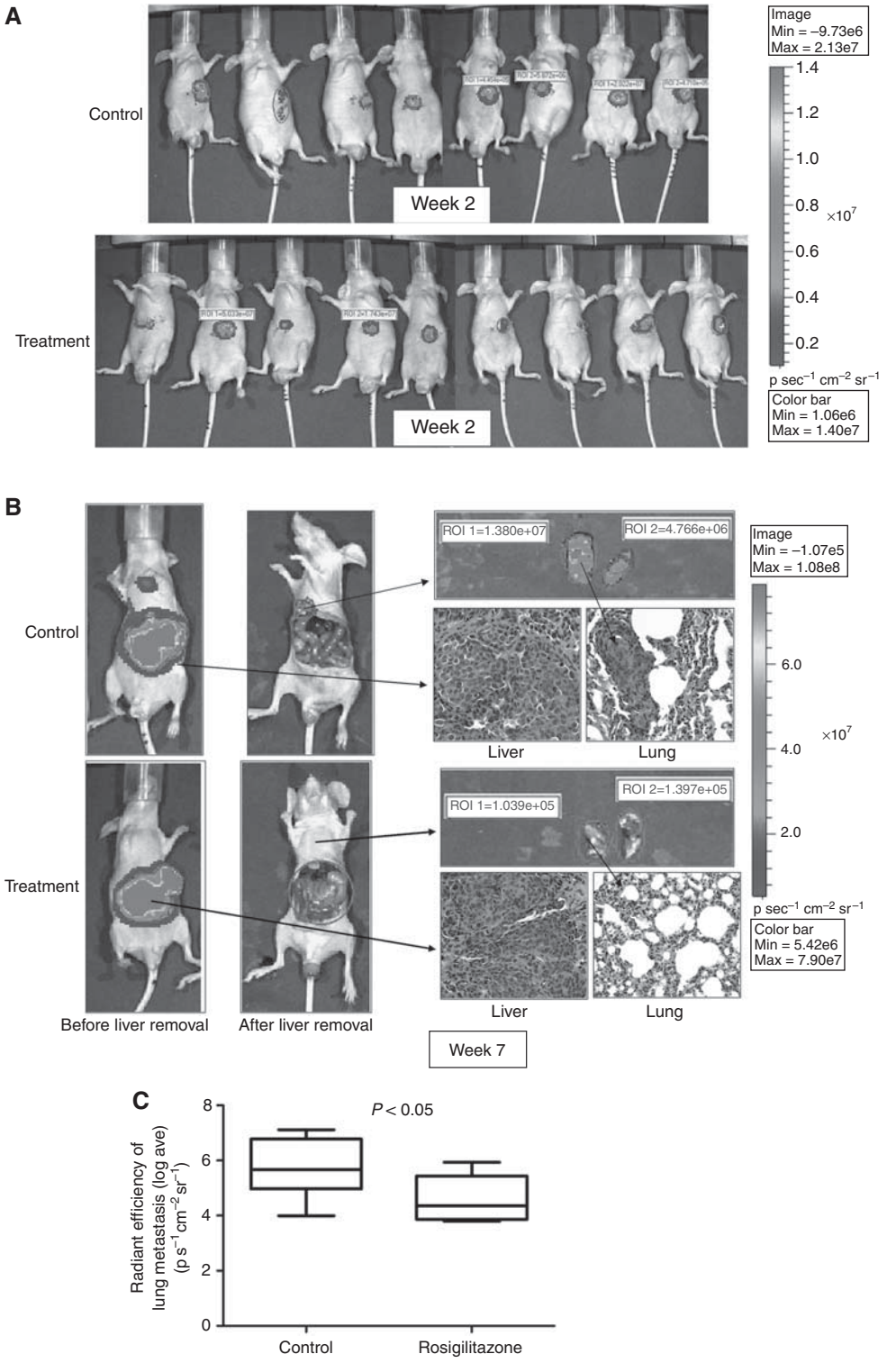

Figure 3 Activation of PPARγ suppressed HCC metastasis *in vivo*. (**A**) Tumours derived from the luciferase-labelled MHCC97L cells were successfully implanted orthotopically into the livers of nude mice at 2 weeks. Tumour growth in the mice was monitored by a live imaging system detecting the luciferase signal (unit photons s^{-1} cm^{-2} steradian). (**B**) Lung metastasis of the HCC mice treated with or without rosiglitazone (200 p.p.m.) at 7 weeks after orthotopic implantation. The liver tumours and lung metastatic nodules were examined by Xenogen IVIS and confirmed histologically. (**C**) Luciferase signal found in the lungs in the rosiglitazone-treated group was significantly lower than in the vehicle-treated group (log base 10 value is used).

particularly at the invading edge of such cancers; increased MMP13 expression was associated with poor prognosis, tumour aggressiveness, and metastases (Leeman *et al*, 2002; Luukkaa *et al*, 2006; Kondratiev *et al*, 2008; Chang *et al*, 2009).

Tissue inhibitors of metallo proteinase 3 is an important endogenous inhibitor of MMPs. Upregulation of TIMP3 expression by PPARγ suggested its suppressive functions in tumour invasion and metastasis via suppressing of MMPs (Bachman *et al*, 1999;

Table 2 Effect of PPARγ on its downstream gene expression profiles of cancer metastasis pathways in HCC cell lines

Full name	Gene	GeneBank	Fold Change (PPARγ/control)	Function
E-cadherin	CDH1	NM_004360	5.2	Cell adhesion
Spleen tyrosine kinase	SYK	NM_003177	1.7	Cell adhesion
Metallopeptidase inhibitor 3	TIMP3	NM_003810	7.2	Extracellular matrix proteins
Matrix metallopeptidase 9	MMP9	NM_004994	− 1.7	Extracellular matrix proteins
Matrix metallopeptidase 13	MMP13	NM_002427	− 2.0	Extracellular matrix proteins
Heparanase	HPA, HPSE	NM_006665	− 6.5	Extracellular matrix proteins
Hepatocyte growth factor	HGF	NM_000601	− 2.2	Cell growth and cell motility
Retinoblastoma 1	RB1	NM_000321	4.1	Cell growth

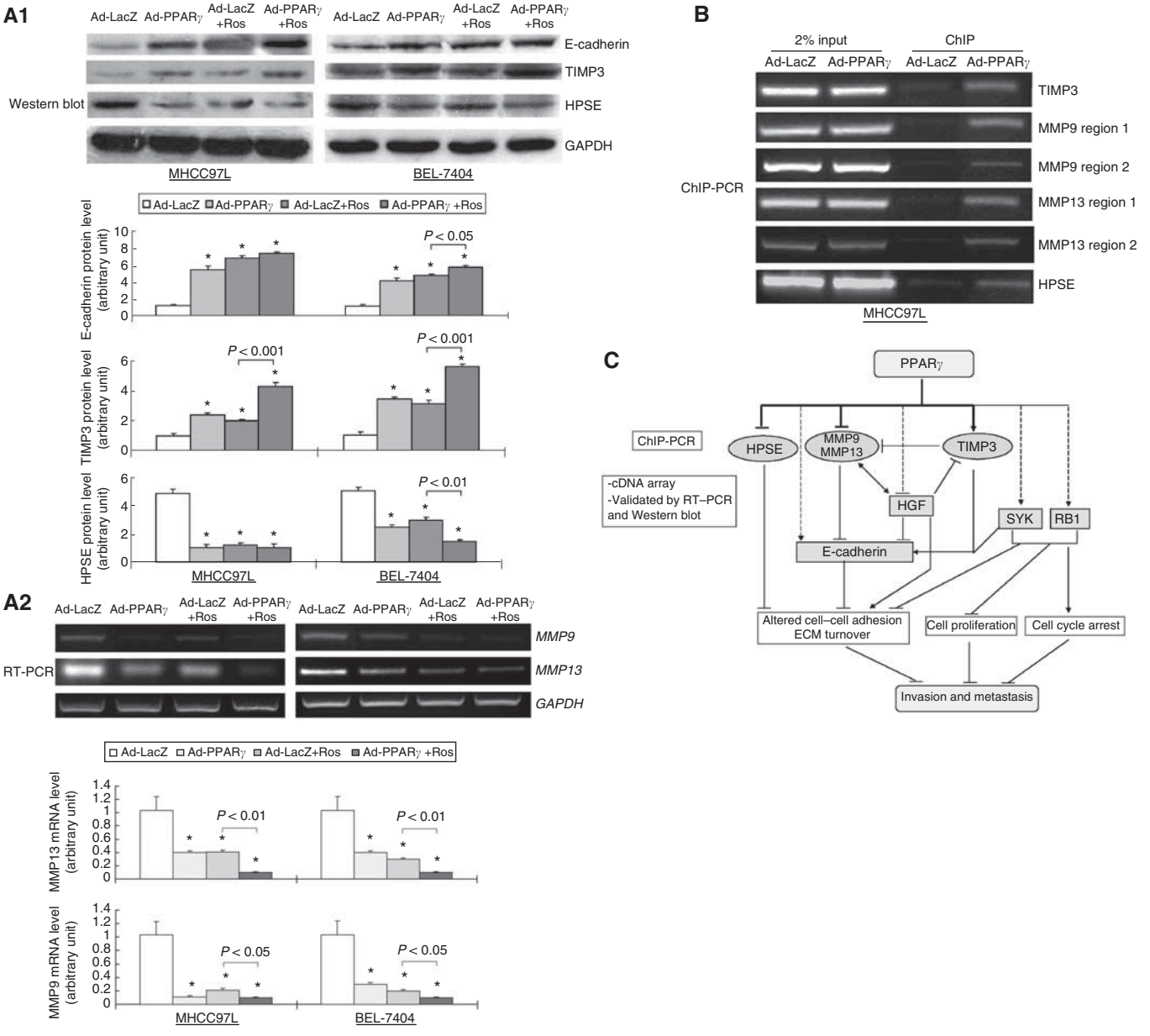

Figure 4. (**A1**) Western blots were performed to confirm the downstream gene expression regulated by PPARγ in MHCC97L and BEL-7404. GAPDH was used as an internal control. The relevant band densitometry analysis was performed and displayed in the lower panel. (**A2**) Semi-quantitative RT–PCR and real-time quantitative PCR analyses were performed to validate the candidate genes expression. The data are expressed as means ± s.d., $^*P < 0.001$, compared with the Ad-lacZ. (**B**) Chromatin Immunoprecipitation (ChIP)-qPCR was performed to identify direct targets of PPARγ protein. Input (2%) represents the genomic DNA. (**C**) Schematic diagram for the mechanisms of anti-metastasis function of PPARγ deriving from cDNA array, western blot, and ChIP-qPCR. PPARγ-mediated suppression of cell invasion and migration was associated with several biological effects: (1) Directly upregulating MMPs (MMP9, MMP13) and downregulating their inhibitor (TIMP3) by direct binding to the promoter of each targets, and subsequent modulation of cell–cell adhesion molecule E-cadherin, which in turn inhibited the cell–cell adhesion and extracellular matrix (ECM) turnover; (2) Directly inhibiting the transcription of HPSE gene, which contributes to the suppression of ECM turnover and distant metastasis; (3) Downregulation of HGF to suppress the invasive potential; (4) Upregulation of tumour suppressors (RB1 and SYK), which protects against tumourigenesis through suppressing ECM turnover, cell proliferation, and causing cell cycle arrest. Blue colour indicates the direct targets, and green colour indicates second targets of PPARγ. The colour reproduction of this figure is available at the *British Journal of Cancer* online.

Wild *et al*, 2003). Matrix metallopeptidase 9 and 13 are important mediators in the turnover of ECM and degradation of cell surface molecules such as E-cadherin; they have been described to promote cancer cell migration (Cowden Dahl *et al*, 2008). Cell–cell adhesion molecule, E-cadherin, is also a key component of epithelial adherent junctions. Loss of E-cadherin expression is a hallmark of epithelial-to-mesenchymal transition, a plausible mechanism of driving cancer progression and metastasis (Onder *et al*, 2008; Makrilia *et al*, 2009). We demonstrate further that PPARγ inhibits transcription of HPSE by direct binding to its promoter. Upregulation of HPSE has been associated with increased lymph node, as well as distant metastases (Sanderson *et al*, 2005) and reduced post-operative survival of cancer patients (Sato *et al*, 2004).

The anti-metastasis function of PPARγ *in vitro* also appeared to be associated with the downregulation of HGF by cDNA array. HGF is known to act as a multifunctional growth factor and is upregulated in many human cancers including HCC (Ljubimova *et al*, 1997; Maulik *et al*, 2002). Hepotocyte growth factor drives epithelial cells to undergo EMT and can downregulate EMT-associated E-cadherin expression in murine liver tumour cells (Ding *et al*, 2010). Also, HGF has been reported to stimulate MMP9 expression (Mizuno *et al*, 2005) and increase MMP3 promoter activity in HCC (Ozaki *et al*, 2003), which can lead to increased cancer cell invasiveness (Reboul *et al*, 2001; Wang *et al*, 2007; Lee *et al*, 2010). Notably, HGF activity may be mediated by MMPs (including MMP9 and MMP13) in the extracellular matrix, and thus induce HCC cells to proliferate and invade (Monvoisin *et al*, 2002; Mohammed *et al*, 2005). In contrast, HGF may down-modulate TIMP-3 expression resulting in increased MMP accumulation, hence contributing to the invasiveness and aggressiveness in cancer cells (Castagnino *et al*, 1998). Thus, the anti-metastasis effect of PPARγ in HCC may be in part related to the inhibition of HGF expression (Figure 4C).

Tumour suppressor genes, RB1 and SYK, have been reported to be dysfunctional in several cancers where aberrant expression levels appear to correlate with poor prognosis (Bailet *et al*, 2009; Kouraklis *et al*, 2009). In contrast, overexpression of RB1 or SYK in several cancer cell types can inhibit tumour growth and reduce metastasis in mouse xenografts (Valente *et al*, 1996; Coopman *et al*, 2000). Spleen tyrosine kinase also inhibits the motility of human breast cancer cells (Zhang *et al*, 2009) and promotes the formation of cell–cell contact through mediation of E-cadherin activity (Larive *et al*, 2009). In the present study, we describe a near two-fold induction of SYK and impressive upregulation by four-fold of RB1 by PPARγ in keeping with its proposed anti-metastatic and -invasive effects.

In conclusion, activation of PPARγ has demonstrated efficacy in suppressing HCC cell migration and invasion *in vitro,* and in inhibiting distant metastases from liver in an orthotopic HCC model *in vivo*. Peroxisome proliferator-activated receptor-gamma may provide a potential target for the prevention and treatment of metastatic HCC.

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Conflict of interest

The authors declare no conflict of interest.

Translational Therapeutics

REFERENCES

Bachman KE, Herman JG, Corn PG, Merlo A, Costello JF, Cavenee WK, Baylin SB, Graff JR (1999) Methylation-associated silencing of the tissue inhibitor ofmetalloproteinase-3 gene suggest a suppressor role in kidney, brain, and other human cancers. *Cancer Res* **59**: 798–802

Bailet O, Fenouille N, Abbe P, Robert G, Rocchi S, Gonthier N, Denoyelle C, Ticchioni M, Ortonne JP, Ballotti R, Deckert M, Tartare-Deckert S (2009) Spleen tyrosine kinase functions as a tumor suppressor in melanoma cells by inducing senescence-like growth arrest. *Cancer Res* **69**: 2748–2756

Bosch FX, Ribes J, Díaz M, Cléries R (2004) Primary liver cancer: worldwide incidence and trends. *Gastroenterology* **127**: S5–S16

Castagnino P, Soriano JV, Montesano R, Bottaro DP (1998) Induction of tissue inhibitor of metalloproteinases-3 is a delayed early cellular response to hepatocyte growth factor. *Oncogene* **17**: 481–492

Chang HJ, Yang MJ, Yang YH, Hou MF, Hsueh EJ, Lin SR (2009) MMP13 is potentially a new tumor marker for breast cancer diagnosis. *Oncol Rep* **22**: 1119–1127

Chen R, Zhu D, Ye X, Shen D, Lu R (1980) Establishment of three human liver carcinoma cell lines and some of their biological characteristics in vitro. *Sci Sin* **23**: 236–247

Chen Y, Wang SM, Wu JC, Huang SH (2006) Effects of PPARgamma agonists on cell survival and focal adhesions in a Chinese thyroid carcinoma cell line. *J Cell Biochem* **98**: 1021–1035

Choudhary R, Li H, Winn RA, Sorenson AL, Weiser-Evans MC, Nemenoff RA (2010) Peroxisome proliferator-activated receptor-gamma inhibits transformed growth of non-small cell lung cancer cells through selective suppression of Snail. *Neoplasia* **12**: 224–234

Coopman PJ, Do MT, Barth M, Bowden ET, Hayes AJ, Basyuk E, Blancato JK, Vezza PR, McLeskey SW, Mangeat PH, Mueller SC (2000) The Syk tyrosine kinase suppresses malignant growth of human breast cancer cells. *Nature* **406**: 742–747

Cowden Dahl KD, Symowicz J, Ning Y, Gutierrez E, Fishman DA, Adley BP, Stack MS, Hudson LG (2008) Matrix metalloproteinase 9 is a mediator of epidermal growth factor-dependent e-cadherin loss in ovarian carcinoma cells. *Cancer Res* **68**: 4606–4613

Ding W, You H, Dang H, LeBlanc F, Galicia V, Lu SC, Stiles B, Rountree CB (2010) Epithelial-to-mesenchymal transition of murine liver tumor cells promotes invasion. *Hepatology* **52**: 945–953

Grommes C, Landreth G, Heneka M (2004) Antineoplastic effects of peroxisome proliferator-activated receptor gamma agonists. *Lancet Oncol* **5**: 419–429

Khasigov P, Podobed O, Gracheva T, Salbiev K, Grachev S, Berezov T (2003) Role of matrix metalloproteinases and their inhibitors in tumor invasion and metastasis. *Biochemistry* **68**: 711–717

Knäuper V, Smith B, López-Otin C, Murphy G (1997) Activation of progelatinase B (proMMP-9) by active collagenase-3 (MMP-13). *Eur J Biochem* **248**: 369–373

Koeffler HP (2003) Peroxisome proliferator-activated receptor gamma and cancers. *Clin Cancer Res* **9**: 1–9

Kondratiev S, Gnepp DR, Yakirevich E, Sabo E, Annino DJ, Rebeiz E, Laver NV (2008) Expression and prognostic role of MMP2, MMP9, MMP13, and MMP14 matrix metalloproteinases in sinonasal and oral malignant melanomas. *Hum Pathol* **39**: 337–343

Kouraklis G, Katsoulis IE, Theocharis S, Tsourouflis G, Xipolitas N, Glinavou A, Sioka C, Kostakis A (2009) Does the expression of cyclin E, pRb, and p21 correlate with prognosis in gastric adenocarcinoma. *Dig Dis Sci* **54**: 1015–1020

Larive RM, Urbach S, Poncet J, Jouin P, Mascré G, Sahuquet A, Mangeat PH, Coopman PJ, Bettache N (2009) Phosphoproteomic analysis of Syk kinase signaling in human cancer cells reveals its role in cell-cell adhesion. *Oncogene* **28**: 2337–2347

Lee KH, Choi EY, Kim MK, Kim KO, Jang BI, Kim SW, Song SK Kim JR (2010) Inhibition of histone deacetylase activity down-regulates urokinase plasminogen activator and matrix metalloproteinase-9 expression in gastric cancer. *Mol Cell Biochem* **343**: 163–171

Leeman MF, Curran S, Murray GI (2002) The structure, regulation, and function of human matrix metalloproteinase-13. *Crit Rev Biochem Mol Biol* **37**: 149–166

Ljubimova JY, Petrovic LM, Wilson SE, Geller SA, Demetriou AA (1997) Expression of HGF, its receptor c-met, c-myc, and albumin in cirrhotic and neoplastic human liver tissue. *J Histochem Cytochem* **45**: 79–87

Luukkaa M, Vihinen P, Kronqvist P, Vahlberg T, Pyrhönen S, Kähäri VM, Grénman R (2006) Association between high collagenase-3 expression levels and poor prognosis in patients with head and neck cancer. *Head Neck* **28**: 225–234

Makrilia N, Kollias A, Manolopoulos L, Syrigos K (2009) Cell adhesion molecules: role and clinical significance in cancer. *Cancer Invest* **27**: 1023–1037

Man K, Ng KT, Xu A, Cheng Q, Lo CM, Xiao JW, Sun BS, Lim ZX, Cheung JS, Wu EX, Sun CK, Poon RT, Fan ST (2010) Suppression of liver tumor growth and metastasis by adiponectin in nude mice through inhibition of tumor angiogenesis and downregulation of Rho kinase/IFN-inducible protein 10/matrix metalloproteinase 9 signaling. *Clin Cancer Res* **6**: 967–977

Mann CD, Neal CP, Garcea G, Manson MM, Dennison AR, Berry DP (2007) Prognostic molecular markers in hepatocellular carcinoma: a systematic review. *Eur J Cancer* **43**: 979–992

Maulik G, Shrikhande A, Kijima T, Ma PC, Morrison PT, Salgia R (2002) Role of the hepatocyte growth factor receptor, c-Met, in oncogenesis and potential for therapeutic inhibition. *Cytokine Growth Factor Rev* **13**: 41–59

Mizuno S, Matsumoto K, Li MY, Nakamura T (2005) HGF reduces advancing lung fibrosis in mice: a potential role for MMP-dependent myofibroblast apoptosis. *FASEB J* **19**: 580–582

Mohammed FF, Pennington CJ, Kassiri Z, Rubin JS, Soloway PD, Ruther U, Edwards DR, Khokha R (2005) Metalloproteinase inhibitor TIMP-1 affects hepatocyte cell cycle via HGF activation in murine liver regeneration. *Hepatology* **41**: 857–867

Monvoisin A, Bisson C, Si-Tayeb K, Balabaud C, Desmoulière A, Rosenbaum J (2002) Involvement of matrix metalloproteinase type-3 in hepatocyte growth factor-induced invasion of human hepatocellular carcinoma cells. *Int J Cancer* **97**: 157–162

Ohta K, Endo T, Haraguchi K, Hershman JM, Onaya T (2001) Ligands for peroxisome proliferator-activated receptor inhibit growth and induce apoptosis of human papillary thyroid carcinoma cells. *J Clin Endocrinol Metab* **86**: 2170–2177

Onder TT, Gupta PB, Mani SA, Yang J, Lander ES, Weinberg RA (2008) Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. *Cancer Res* **68**: 3645–3654

Ozaki I, Mizuta T, Zhao G, Zhang H, Yoshimura T, Kawazoe S, Eguchi Y, Yasutake T, Hisatomi A, Sakai T, Yamamoto K (2003) Induction of multiple matrix metalloproteinase genes in human hepatocellular carcinoma by hepatocyte growth factor via a transcription factor Ets-1. *Hepatol Res* **27**: 289–301

Panigrahy D, Singer S, Shen LQ, Butterfield CE, Freedman DA, Chen EJ, Moses MA, Kilroy S, Duensing S, Fletcher C, Fletcher JA, Hlatky L, Hahnfeldt P, Folkman J, Kaipainen A (2002) PPARg ligands inhibit primary tumor growth and metastasis by inhibiting angiogenesis. *J Clin Invest* **110**: 923–932

Reboul P, Pelletier JP, Tardif G, Benderdour M, Ranger P, Bottaro DP, Martel-Pelletier J (2001) Hepatocyte growth factor induction of collagenase 3 production in human osteoarthritic cartilage: involvement of the stress-activated protein kinase/c-Jun N-terminal kinase pathway and a sensitive p38 mitogen-activated protein kinase inhibitor cascade. *Arthritis Rheum* **44**: 73–84

Reka AK, Kurapati H, Narala VR, Bommer G, Chen J, Standiford TJ, Keshamouni VG (2010) Peroxisome proliferator-activated receptor-gamma activation inhibits tumor metastasis by antagonizing Smad3-mediated epithelial-mesenchymal transition. *Mol Cancer Ther* **9**: 3221–3232

Sanderson RD, Yang Y, Kelly T, MacLeod V, Dai Y, Theus A (2005) Enzymatic remodeling of heparan sulfate proteoglycans within the tumor microenvironment: growth regulation and the prospect of new cancer therapies. *J Cell Biochem* **96**: 897–905

Sato T, Yamaguchi A, Goi T, Hirono Y, Takeuchi K, Katayama K, Matsukawa S (2004) Heparanase expression in human colorectal cancer and its relationship to tumor angiogenesis, hematogenous metastasis, and prognosis. *J Surg Oncol* **87**: 174–181

Takano S, Kubota T, Nishibori H, Hasegawa H, Ishii Y, Nitori N, Ochiai H, Okabayashi K, Kitagawa Y, Watanabe M, Kitajima M (2008) Pioglitazone, a ligand for peroxisome proliferator-activated receptor-gamma acts as an inhibitor of colon cancer liver metastasis. *Anticancer Res* **28**: 3593–3599

Valente P, Melchiori A, Paggi MG, Masiello L, Ribatti D, Santi L, Takahashi R, Albini A, Noonan DM (1996) RB1 oncosuppressor gene over-expression inhibits tumor progression and induces melanogenesis in metastatic melanoma cells. *Oncogene* **13**: 1169–1178

Wang SW, Pan SL, Peng CY, Huang DY, Tsai AC, Chang YL, Guh JH, Kuo SC, Lee KH, Teng CM (2007) CHM-1 inhibits hepatocyte growth factor-induced invasion of SK-Hep-1 human hepatocellular carcinoma cells by suppressing matrix metalloproteinase-9 expression. *Cancer Lett* **257**: 87–96

Wild A, Ramaswamy A, Langer P, Celik I, Fendrich V, Chaloupka B, Simon B, Bartsch DK (2003) Frequent methylation-associated silencing of the tissue inhibitor of metalloproteinase-3 gene in pancreatic endocrine tumors. *J Clin Endocrinol Metab* **88**: 1367–1373

Yu J, Cheng YY, Tao Q, Cheung KF, Lam CN, Geng H, Tian LW, Wong YP, Tong JH, Ying JM, Jin H, To KF, Chan FK, Sung JJ (2009) Methylation of protocadherin 10, a novel tumor suppressor, is associated with poor prognosis in patients with gastric cancer. *Gastroenterology* **136**: 640–651

Yu J, Qiao L, Zimmermann L, Ebert MP, Zhang H, Lin W, Röcken C, Malfertheiner P, Farrell GC (2006) Troglitazone inhibits tumor growth in hepatocellular carcinoma in vitro and in vivo. *Hepatology* **43**: 134–143

Yu J, Shen B, Chu ES, Teoh N, Cheung KF, Wu CW, Wang S, Lam CN, Feng H, Zhao J, Cheng AS, To KF, Chan HL, Sung JJ (2010) Inhibitory role of peroxisome proliferator-activated receptor gamma in hepatocarcinogenesis in mice and in vitro. *Hepatology* **51**: 2008–2019

Yu Q, Stamenkovic I (2000) Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. *Genes Dev* **14**: 163–176

Zhang X, Shrikhande U, Alicie BM, Zhou Q, Geahlen RL (2009) Role of the protein tyrosine kinase Syk in regulating cell-cell adhesion and motility in breast cancer cells. *Mol Cancer Res* **7**: 634–644