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Human invasive trophoblasts transformed with simian virus 40 provide a new tool to study the role of PPARγ in cell invasion process

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Invasive cytotrophoblasts play a key role in the development of human placenta and is therefore essential for subsequent development of the embryo. Human implantation is characterized by a major trophoblastic invasion that offers a unique model of a controlled and oriented tumor-like process. The ligand-activated nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ) modulates cell growth and differentiation and might be therefore considered as a tumor suppressor. We have recently reported that PPARγ, in synergy with its dimerization partner retinoid X receptor (RXR)α, controls the invasion of human primary cytotrophoblasts. Because these cells are unable to replicate in culture, we have, in the present study, transformed these primary cells with the simian virus 40 large T antigen for studying the role of PPARγ in cell invasion process. Our results show that the cell line human invasive proliferative extravillous cytotrophoblast (HIPEC) 65 expressed markers of human invasive primary cytotrophoblast as determined by immunocytochemistry, immunobloting and real-time RT–PCR, and were highly invasive *in vitro*. We have next studied the role of PPARγ/RXRα heterodimers in cell proliferation and invasion. Our results show that PPARγ and RXRα are co-expressed by HIPEC 65 and that, as commonly observed, activation of PPARγ/RXRα heterodimers with the specific PPARγ agonist rosiglitazone induced lipid droplet accumulation as revealed by oil red O staining. Treatment with rosiglitazone or with the natural PPARγ agonist 15-deoxy-δ-(12,14) PGJ_2 did not modify cell growth, but interestingly, activation of PPARγ by this synthetic (rosiglitazone) or natural (15d-PGJ_2) ligand markedly inhibited cell invasion in a concentration-dependent manner. Finally, we showed that other potential natural PPARγ ligand such as oxidized—but not native—low-density lipoprotein inhibited cell invasion. This proliferative and invasive human cytotrophoblast cell line from extravillous origin provides a new tool for studying specifically the role of PPARγ in the control of cell invasion.

Abbreviations: CK07, cytokeratin 7; 15d-PGJ_2, 15-deoxy-delta (12,14) prostaglandin J_2; EVCT, extravillous cytotrophoblast; HIPEC, human invasive proliferative extravillous cytotrophoblast; HLA, human leucocyte antigen; PAI, plasminogen activator inhibitor; PPARγ, peroxisome proliferator-activated receptor γ; PPIA, peptidylprolyl isomerase A; RXR, retinoid X receptor; TGF-β, transforming growth factor-β; SV40, simian virus 40.

Introduction

Implantation of the human conceptus involves the invasion of the uterine epithelium and the underlying stroma by extra embryonic trophoblastic cells, which undergo a complex process of proliferation, migration and differentiation. One particularity of human placentation is the very high degree of trophoblast invasion during the first trimester (1) unparalleled in other mammals. The trophoblasts named extravillous cytotrophoblasts (EVCT) invade the uterine wall and the associated arterioles where they replace the endothelial lining and most of the musculoelastic tissue of the vessel wall. This arteriole remodeling leads to low resistance vessels that provide adequate supply of maternal blood for fetal growth (2).

The human trophoblastic invasion, unlike tumor invasion, is precisely regulated. It is temporally restricted to early pregnancy and it is spatially confined to the endometrium, the first third of the myometrium and the associated uterine arterioles (3,4). Therefore, human trophoblast invasion offers a unique model of a controlled and oriented cell invasion process that may be used for understanding mechanisms by which cells become abnormally invasive during tumor formation.

Trophoblast migration and invasive capacity have been shown to be modulated by factors including oxygen concentration (5,6), transforming growth factor (TGF-β), IGF-II and IGFBP-1 (7,8), epidermal growth factor (9) and hepatocyte growth factor (10,11). We have reported recently that the ligand-activated nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ) played an important role in the control of human trophoblast invasion (12).

PPARγ is a member of the nuclear receptor superfamily that modulates the expression of a large array of genes involved in the control of cellular response such as cell differentiation, proliferation and death, as well as inflammation (for review see ref. 13). It has been suggested more recently that PPARγ might act as a tumor suppressor by inducing cell growth arrest in normal (14) and tumor cells (14–16). DNA binding of PPARγ to its response element requires obligate heterodimerization with another nuclear receptor, the retinoid X receptor (RXR). PPARγ is bound and activated by natural ligands such as fatty acids (17), oxidized-low-density lipoprotein (LDL) (18) and 15-deoxy-delta (12,14) prostaglandin J_2 (15d-PGJ_2) (19,20). In addition, synthetic ligands and agonists of PPARγ, such as the thiazolidinediones, which include rosiglitazone, have been developed and used recently in treatment of type 2 diabetes.

The aim of the present study was to develop a human cellular model to study mechanism that control human trophoblast invasion *in vitro* and the role of PPARγ in cell invasion process. Trophoblasts involved in the implantation process have been described and characterized by different authors. These cells are currently obtained after enzymatic digestion (21–23) or are derived from a placental explant (24–27). We have developed recently an *in vitro* model of purified EVCT primary cells isolated from first trimester human chorionic villi (28). These cells were shown to express *in vitro* the specific markers of human invasive EVCT described *in situ*: cytokeratin 07 (29), human leukocyte antigen-G (30), human placental lactogen (31), the proto-oncogene c-erbB2 (32) and α5 subunit of the fibronectin receptor α5β1 (33). These primary cells are unable to proliferate *in vitro* and require extracellular matrix components such as Matrigel for adhesion and differentiation toward a non-proliferative but invasive phenotype (28). We have, in the present work, transformed these purified and well-characterized invasive EVCT with the simian virus 40 (SV40) large T antigen. The construct used was shown to effectively establish mammalian cell lines *in vitro* (34,35). We obtained one cell line, HIPEC 65 (human invasive proliferative extravillous cytotrophoblast) that retained the phenotype of the parental invasive primary EVCT and the ability to invade extracellular matrix *in vitro*. This cell line co-expressed PPARγ and RXRα and activation of PPARγ/RXRα heterodimers, by PPARγ synthetic and natural ligands, modulate cell invasion without affecting proliferation. This easily accessible invasive human trophoblast cell line provides a new tool for studying specifically the role of PPARγ in cell invasion process.

Materials and methods

Transfection of SV40 large T antigen into primary EVCT

EVCT were isolated and purified from first trimester human placentas after enzymatic digestion as described recently (28). These purified primary EVCT were characterized using immunocytochemistry and real-time PCR as described (28). Transfection was performed as described by Schwartz *et al.* (34), using HuVim [830]-T/t recombinant DNA. Briefly, part of the human vimentin promoter, including 932 bp, was fused to SV40 Large T and small t coding sequences (2701 bp) with T4 DNA ligase (35). EVCT primary cells were transfected with this DNA recombinant using Lipofectin (Gibco, Grand Island, NY) as recommended by the manufacturer. The next day, cells were washed, cultured in HAM F12/DMEM supplemented with 10% FCS, 2 mM glutamine, 100 UI/ml penicillin and 100 μg/ml streptomycin and incubated in 5% CO_2 at 37°C. Then, cells were observed for the appearance of transformed cells.

For SV40 large T antigen immunodetection, cells were cultured for 48 h, fixed for 5 min in methanol/acetone (7/3, v/v) at −20°C, washed and incubated for 1.5 h with a monoclonal antibody raised against large T SV40. Then, cells were incubated for 1 h with a fluorescein-conjugated donkey anti-mouse antibody (1/150 dilution, Jackson Immunoresearch Laboratories, West Groove, PA), washed and the slides were cover slipped in a drop of mounting medium for fluorescence (Vector Laboratories, Burlingame, CA). To ensure the specificity of the immunological reactions, negative controls were performed by substituting the primary antibody with a non-immune mouse serum.

Immunodetection of invasive EVCT specific markers

For HIPEC 65 immunolabelling, all experiments were realized on subconfluent cultures at passage 8. Cells were fixed for 20 min with 4% paraformaldehyde and permeabilized for 4 min in 0.3% Triton X-100, except for studying the EGF receptor, c-erbB2 and human leucocyte antigen (HLA)-G. For PPARγ, cells were permeabilized for 30 min in 0.3% Triton X-100 as described previously (12). After pre-incubation with 7% goat, sheep or donkey serum, monoclonal or polyclonal antibodies, respectively, diluted in PBS containing 1% BSA, were applied for 2 h at room temperature (Table I).

Then cells were incubated for 1 h with a 1/200 dilution of a biotinylated donkey IgG anti-rabbit antibody or with a biotinylated goat IgG anti-mouse antibody [species specific F (ab') 2 fragment; Amersham, Les Ulis, France]. Bound antibodies were revealed after 1 h incubation in the dark with streptavidin–fluorescein (Amersham) diluted 1/400 or, for α6 detection, with a fluorescein-conjugated donkey anti-rat antibody (1/50, Jackson Immunoresearch Laboratories). In all cases, cells were extensively washed between each step with 0.1% Tween 20 in PBS. Finally, slides were cover slipped in a drop of fluorescent Dapi mounting medium and analyzed under an epifluorescent microscope. To ensure the specificity of the immunological reactions, negative controls were performed by substituting primary antibody with a non-immune mouse or rabbit serum.

Immunoblot analysis

Exponentially growing cells were harvested by mild trypsinization and low-speed centrifugation, then cell pellets were lysed by sonication in a phosphate buffer (K_2HPO_4/KH_2PO_4 10 mM, pH 8.6). After centrifugation (15 min at 12 000 *g*), the resulting extracts were heated at 100°C for 3 min in electrophoresis sample buffer and subjected to SDS–PAGE on a 12% gel. Then, proteins were electrotransferred onto nitrocellulose membrane using a liquid blotting apparatus (Bio-Rad Laboratories, Hercules, CA). Transfer was performed in 25 mM Tris, 192 mM glycine, 20% ethanol (v/v) and 0.1% SDS (w/v) at pH 8.3 for 1 h. The membranes were then washed in 10 mM Tris, 150 mM NaCl, 0.05% Tween, incubated for 2 h with HLA-G antibody (MEM-G/1, Exbio Praha, Czech Republic, diluted 1/500) or overnight with RXRα (1/500) or PPARγ (1/200) antibodies, followed by a mouse or rabbit peroxydase-conjugated secondary antibody and developed using an enhanced chemiluminescence kit (Pierce, Rockford, IL). To correct for differences in protein loading, the membranes were washed and reprobed with 1 to 1000 dilution polyclonal antibody against human actin (Sigma, Saint Louis, MO).

Quantification of specific transcripts by real-time RT–PCR

Total RNA was extracted from 48 h-cultured HIPEC 65 or primary EVCT, using Qiagen RNeasy mini kit (Courtabeuf, France). cDNA synthesis and PCR amplification were performed as described previously (37). All PCR reactions were performed using an ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA) and the SYBR Green PCR Core Reagents kit (Perkin-Elmer Applied Biosystems) (Table II).

The gene peptidylprolyl isomerase A (PPIA) coding for human peptidylprolyl isomerase A (cyclophilin A) was used as the endogenous RNA control and each sample was normalized on the basis of its PPIA content.

PPARγ ligands

Rosiglitazone (BRL49653) was from Cayman Chemical (Ann Arbor, MI), 15d-PGJ_2 from Calbiochem (Darmstadt, Germany). Preparation and

Table I. Antibodies used for immunocytochemistry

Antibody	Species	Isotype	Dilution	Source
CK 07 (OV-TL 12/30)	Mouse	IgG1κ	1:100	Dako, Trappes, France
HLA-G (W6/32)	Mouse	IgG2α	1:50	Leinco Technologies, St Louis, MO, USA
CD9 (ALB6)	Mouse	IgG1κ	1:50	Coulter, Margency, France
α5 (SAM1)	Mouse	IgG2β	1:200	Coulter, Margency, France
c-erbB2 (9G6)	Mouse	IgG1	1:20	Santa Cruz, CA, USA
TGFβ2 (V)	Rabbit	–	1:200	Santa Cruz, CA, USA
c-erbB1 (6F1)	Mouse	IgG2bκ	1:100	Coulter, Margency, France
Vimentin (V9)	Mouse	IgG1	Ready to use	Coulter, Margency, France
α6 (GOH3)	Rat	IgG2α	1:200	Coulter, Margency, France
PPARγ (E8)	Mouse	IgG1	1:100	Santa Cruz, CA, USA
RXRα (4RX3A2)	Mouse	IgG	1:100	(36)

Table II. Primers used for real-time RT–PCR

Gene	Primers	Protein
PPIA	(+) 5′-GTC AAC CCC ACC GTG TTC TT-3′	Cyclophilin A
	(−) 5′-CTG CTG TCT TTG GGA CCT TGT-3′	
KRT7	(+) 5′-GGA CAT CGA GAT CGC CAC CT-3′	Cytokeratin 07
	(−) 5′-ACC GCC ACT GCT ACT GCC A-3′	
HLA-G	(+) 5′-TTG CTG GCC TGG TTG TCC TT-3′	HLA-G
	(−) 5′-TTG CCA CTC AGT CCC ACA CAG-3′	
TGFβ2	(+) 5′-AGA GTG CCT GAA CAA CGG ATT-3′	TGFβ2
	(−) 5′-CCA TTC GCC TTC TGC TCT T-3′	
ERBB1	(+) 5′-GGA GAA CTG CCA GAA ACT GAC C-3′	c-erbB1
	(−) 5′-GCC TGC AGC ACA CTG GTT G-3′	
ERBB2	(+) 5′-AGC CGC GAG CAC CCA AGT-3′	c-erbB2
	(−) 5′-TTG GTG GGC AGG TAG GTG AGT T-3′	
PAI	(+) 5′-CAC AAA TCA GAC GGC AGC ACT-3′	PAI-1
	(−) 5′-CAT CGG GCG TGG TGA ACT C-3′	
PPARγ	(+) 5′-AGT GGG GAT GTC TCA TAA TGC C-3′	PPARγ
	(−) 5′-AGC TCA GCG GAC TCT GGA TTC-3′	

characterization of oxidized-LDL were performed as described (38). Briefly, LDL were isolated by sequential ultracentrifugation of pooled human plasma, and LDL samples were oxidized at 37°C in the presence of 5 μM $CuSO_4$ for up to 24 h. The dialyzed native- or oxidized-LDL were used in cell culture at the concentration of 50 μg protein/ml in PBS.

Oil red O staining

Cultured HIPEC 65 were washed in PBS, fixed in 60% isopropanol for 1 min and incubated at room temperature for 10 min with 0.3% oil red O (Sigma Chemical, St Louis, MO) in isopropanol (w/v). After additional 30 s incubation in 60% isopropanol, cells were washed in water and the nuclei were counter-stained in hematoxylin for 2 min.

Cell growth and cytotoxicity analysis

Measurement of proliferation and cell toxicity was performed using MTT assay as described in the manual (Sigma Chemical). Briefly, HIPEC 65 were cultured as described above in tissue culture 96 well plates and incubated with or without PPARγ ligands for 24 h. After an additional 3 h incubation with MTT (soluble tetrazolium), the converted dye (insoluble purple formazan) was solubilized with acidic isopropanol (0.04 N HCl in absolute isopropanol). Absorbance was measured at 570 nm with background subtraction at 630 nm.

Invasion assays

To assess the invasive potential of cytotrophoblasts, transwell inserts (6.5 mm; Costar, Cambridge, MA) containing polycarbonate filters with 8 μm pores were used as described previously (12). The upper side was coated with 10 μl of 5 mg/ml Matrigel and the cells (5×10^4 cells) were plated in 200 μl of HAM F12/DMEM supplemented with 2% FCS, 2 mM glutamine, 100 UI/ml penicillin and 100 μg/ml streptomycin. The bottom well was filled with 600 μl of the same medium supplemented with 20% FCS. After a 24 h culture period (5% CO_2 at 37°C), the transwell inserts were washed three times with PBS and cells were fixed for 20 min in 4% paraformaldehyde. Samples were rinsed and fixed for 10 min in methanol at −20°C. The cells were then incubated with 7% donkey serum in PBS for 30 min to reduce non-specific binding. Vimentin antibody was added for 2 h at room temperature. Cells were washed in PBS–Tween 0.1% and incubated with FITC-conjugated donkey anti-mouse IgG (Jackson Immunoresearch Laboratories) for 1 h and washed in PBS–Tween 0.1%. Filters were dissected with a scalpel and the upper side of the filter was placed in contact with a superfrost slide, mounted in a fluorescent Dapi mounting medium and examined and photographed on an Olympus BX60 epifluorescence microscope.

Scanning electron microscopy

Cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 1 h at room temperature. The cultures were then dehydrated with increasing concentrations of acetone and dried with a critical-point-drying apparatus (Balzers Union) using acetone and liquid CO_2. The dried specimens were coated with a 30 nm layer of gold in a vacuum evaporator.

Statistical analysis

Results are presented as the mean ± SD. Significant differences were identified by using analysis of variance (ANOVA) with $P < 0.05$.

Results

Morphology and characteristics of human EVCT primary cells transformed with SV40 large T antigen

Human extravillous cytotrophoblasts isolated from first trimester chorionic villi were purified and characterized as described previously by Tarrade *et al*. (28). These purified primary cells were then cultured and transformed with SV40 large T antigen as described in the Materials and methods. In contrast to primary EVCT that need extracellular matrix (Matrigel) for adhesion and primary culture, transformed cells HIPEC 65 were cultured on tissue culture plastic in the absence of the reconstituted basement membrane Matrigel. Figure 1A shows the morphological aspect of the cells at two different densities: subconfluent culture of HIPEC 65 (center panel); confluent culture (right panel) and primary EVCT (left panel). HIPEC 65 proliferate on tissue culture plastic with a doubling time of ~42 h in exponential growth phase. A plateau was reached at day 6 when cells were plated at 20×10^3 cells/cm^2, whereas at lower density (10×10^3 and 15×10^3) cells still grow even after a 10 day culture period. It is to note that HIPEC 65 still proliferate after 100 passages and could be considered as immortalized cells. Detection of the Human Vim 830 T/t recombinant protein in the nuclei of transformed cells was done by immunocytochemistry using a specific anti-large T antibody and Dapi counter staining (Figure 1C). The entire cell population expressed the large T antigen after four passages and was still positive for this antigen after 22 passages.

Expression of parental primary EVCT specific markers by transformed cell HIPEC 65

Immunodetections of main EVCT markers were performed at passage 8 and are presented in Figure 2A. The entire population of HIPEC 65 cells express the three major antigens considered as specific markers of EVCT as recently established (39,40): cytokeratin 07, human leukocyte antigen G and CD9. For HLA-G immunodetection, we used the w6/32 antibody, which is widely used in immunocytochemistry but cannot discriminate between the trophoblast HLA class I molecules HLA-C, HLA-G and HLA-E (39). In order to confirm that the transformed cells expressed specifically HLA-G, we analyzed HLA-G expression by immunoblotting using the mouse MEM-G/1 monoclonal antibody that reacts specifically with the denatured HLA-G heavy chain. As shown in Figure 2B, a very weak signal at the expected apparent molecular weight (45 kDa) was detected in HIPEC 65 (50 mg total protein loaded) in comparison with primary EVCT (10 mg loaded), which highly express this antigen. In addition to the HLA-G-specific band, and as described by others in EVCT cell lines HT-116 and HTR-8 (41), a band at ~62–64 kDa was observed for both primary and transformed cells.

We next examined whether HIPEC 65 exhibited the specific phenotype of invasive EVCT. Immunodetection of α5 subunit of the fibronectin receptor α5β1 is illustrated in Figure 2A, and expression of other markers in comparison with invasive primary cells (28) are summarized in Table III. α5β1 was the major integrin produced by these invasive cells as HIPEC 65 and their parental EVCT failed to express αV integrin and α6 subunit of the laminin receptor (Table III). In agreement with primary cells, HIPEC 65 expressed the proto-oncogenes c-erbB2, c-erbB1 and TGFβ2, but in contrast, they also express vimentin whereas parental primary cells do not (Table III).

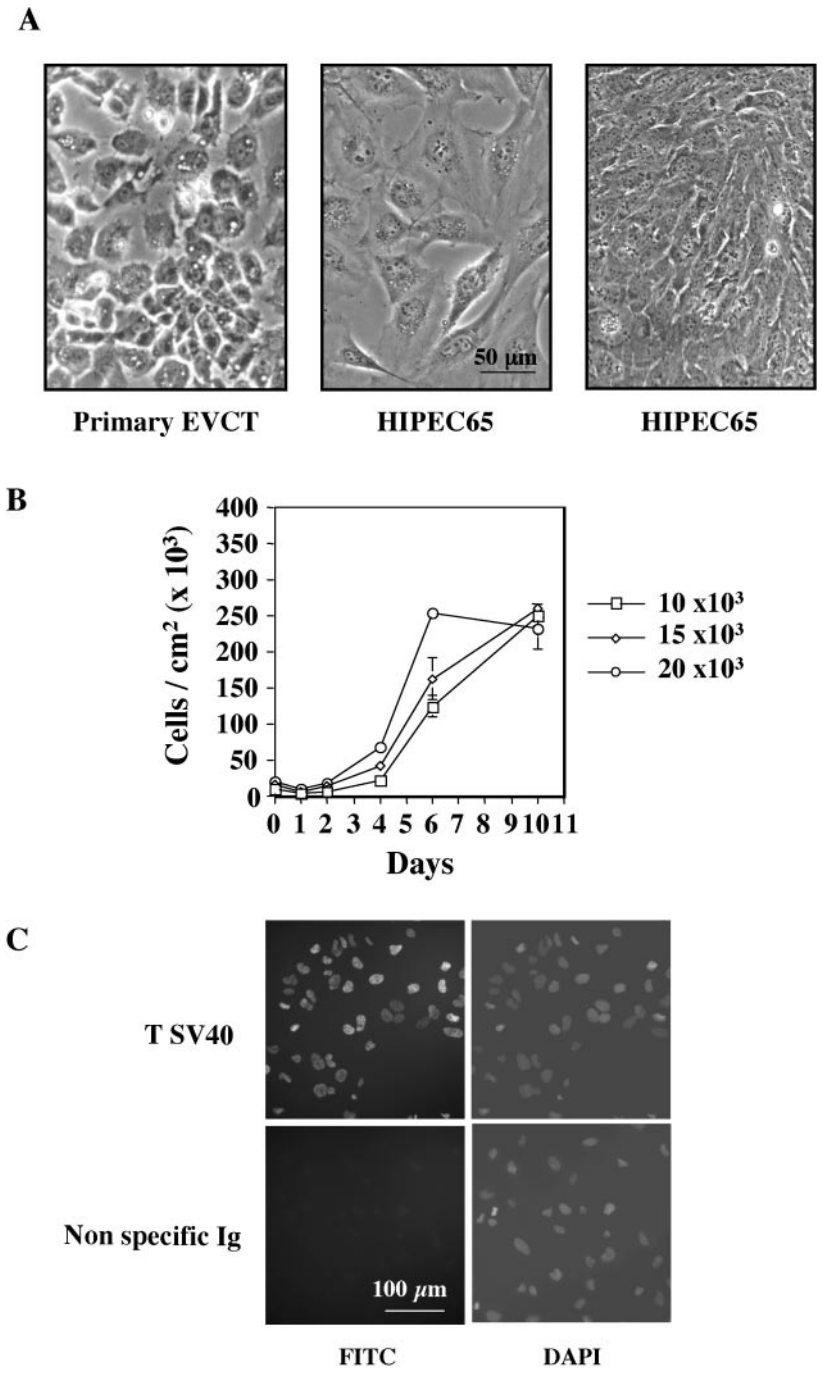

Fig. 1. Morphology and growth characteristics of SV40 large T antigen-transformed human EVCT. (**A**) EVCT isolated and purified from human first trimester placenta (left panel) were transformed by SV40 large T antigen and cultured on plastic dishes (center and right panels). (**B**) For proliferation studies, cells were plated at 10, 15 or 20 $\times$ 10^3 cells/cm^2. Values are expressed as mean $\pm$ SD of triplicates from a representative culture and represent numbers of alive cells as assayed by trypan blue exclusion. (**C**) Immunodetection of SV40 large T antigen in the nuclei of transformed EVCT. Nuclei were counter stained with Dapi.

Finally, the expression of EVCT markers was confirmed by real-time RT–PCR and the results are in agreement with the immunocytochemistry and immunoblot data. Values are expressed as levels of each marker transcript normalized to cyclophilin A (PPIA) transcript levels and comparison between HIPEC 65 and primary EVCT are shown in Table IV. As shown in western analysis (Figure 2B) HLA-G transcript levels are very low in HIPEC 65 compared with primary EVCT. In addition, we found that HIPEC 65 expressed the inhibitor of plasminogen activator type 1 (PAI-1), which is specific to invading trophoblasts (42) and expressed by parental primary EVCT in the same order of magnitude.

All together, our data demonstrated that HIPEC 65 are human invasive trophoblasts from EVCT origin.

Invasive properties of HIPEC 65

To analyze cellular invasion we used Matrigel-coated transwells (Figure 3A). Cells were added into the upper well and a serum gradient was used between the upper and the lower well in order to accelerate cell invasion. Figure 3B(a) shows vimentin immunostained and Dapi counterstained HIPEC 65

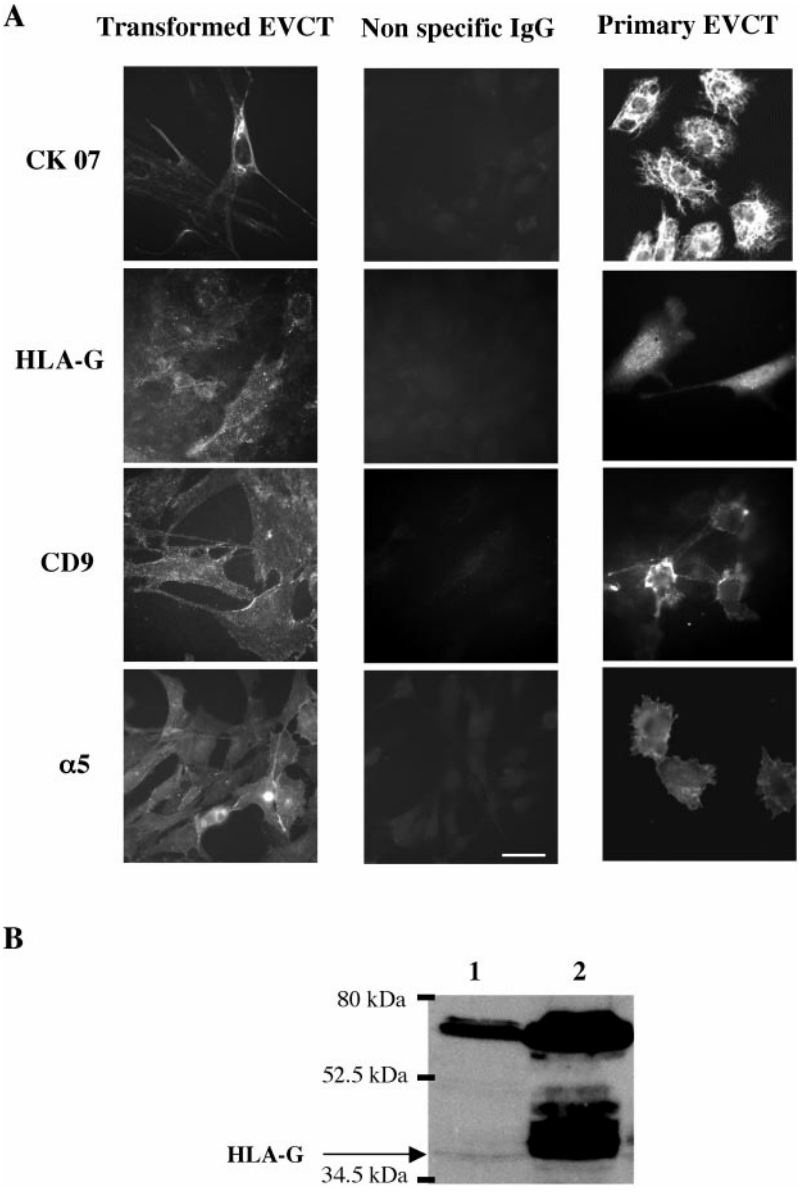

Fig. 2. Immunophenotyping of transformed human EVCT cell line HIPEC 65. (**A**) Immunodetections of CK07, HLA-G, CD9 and α5 subunit of the fibronectin receptor were performed on transformed EVCT at passage 8 and on primary EVCT after 48 h of culture. No staining was observed when incubated with non-immune antibodies. Scale bar: 50 μm. (**B**) Immunoblot analysis of HLA-G (45 kDa) expression. HIPEC 65 (50 mg of protein loaded) weakly expressed a 45 kDa protein similar to the HLA-G protein present in primary EVCT (10 mg of protein loaded). The 62 kDa protein recognized by the anti HLA-G antibody remains unidentified as described by others.

Table III. Expression of trophoblast markers by the human invasive trophoblast cell line HIPEC 65 at passage 8 in comparison with their parental primary EVCT

Antigens	Primary EVCT	HIPEC 65
CK07	+	+
HLA-G	+	+
CD9	+	+
α5 subunit	+	+
TGFβ2	+	+
c-erbB2	+	+
c-erbB1	+	+
Vimentin	−	+
α6 subunit	−	−
PPARγ	+	+
RXRα	+	+

+, expressed; −, not expressed.

Table IV. Real-time PCR analysis of EVCT transcript expression

Gene/PPIA	Primary EVCT	HIPEC 65
CK07	395.3	110
HLA-G	3297	4.6
c-erbB1	105.9	28.4
c-erbB2	80.3	6.6
TGFβ2	16.4	4.6
PAI-1	6.3	4.9
PPARγ	532.6	20.1
RXRα	6.1	4.6

Transcript levels were normalized to PPIA transcript levels.

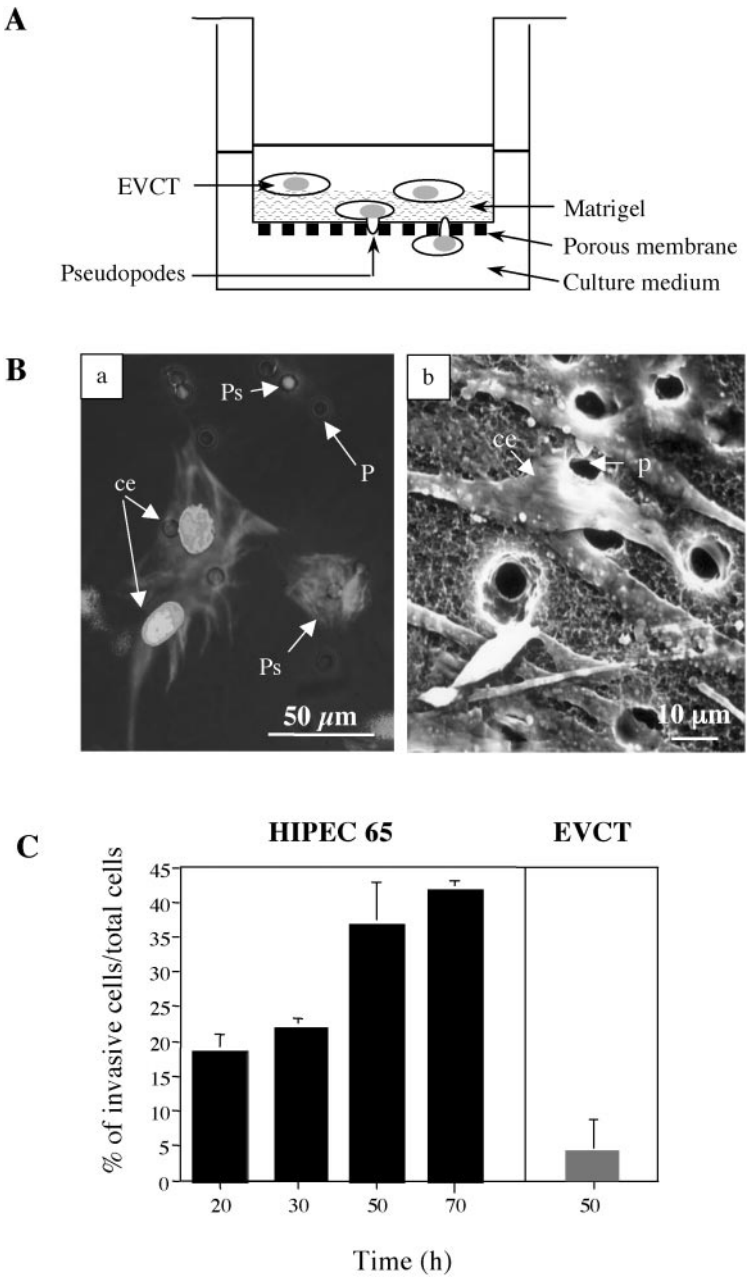

Fig. 3. Transformed EVCT cell line HIPEC 65 are highly invasive *in vitro*. (**A**) Scheme of a Matrigel-coated Transwell used for invasion assay. (**B**) Epifluorescent (a) and scanning electron (b) micrographs of transformed EVCT after 24 h (a) or 48 h (b) of culture on Matrigel-coated filters. (a) Cells were immunostained using anti-vimentin antibody and nuclei counter stained with Dapi. Pseudopodia and cells crossing the membrane visible through the 8 μm diameter pores were quantified and normalized to the number of total nuclei. (b) After crossing the membrane, cells are visible on the inferior side of the porous membrane (faces up) where they plated. Ce: cell; P: pore; Ps: pseudopodia. (**C**) Cell invasion time course. Results are expressed as the percentage of invasive cells per number of total nuclei. Values represent the mean ± SD of triplicate determinations from a representative experiment.

invading Matrigel and emitting pseudopodia through the membrane pores after 24 h of culture. Dapi staining of the nuclei [Figure 3B(a)] and scanning electron micrograph of HIPEC 65 [Figure 3B(b)] show cells on the lower surface of the porous membrane, after invading the Matrigel (24 and 48 h of culture, respectively). To quantify cell invasion we determined for each condition an invasion index corresponding to the percentage of cell observed on the lower surface of the membrane normalized to the number of total nuclei. We next realized an invasion time course showing an invasion index of ~20 and 35% of invading cells at 24 and 50 h, respectively (Figure 3C). In comparison with parental primary cells assayed in the same conditions (invasion index of ~5% at 50 h), HIPEC 65 was found to be more invasive.

We next investigated the expression and the role of PPARγ/RXR heterodimers in HIPEC 65 invasion and proliferation.

Expression of PPARγ and RXRα by HIPEC 65

PPARγ and its heterodimer partner RXRα were detected by immunocytochemistry in the nuclei of HIPEC 65 (from passages 13 to 40) and co-located with Dapi staining as observed in primary EVCT (Figure 4A). Figure 4B depicts western analysis of PPARγ and RXRα. In addition to the 50 kDa protein observed in both HIPEC 65 and primary EVCT, two

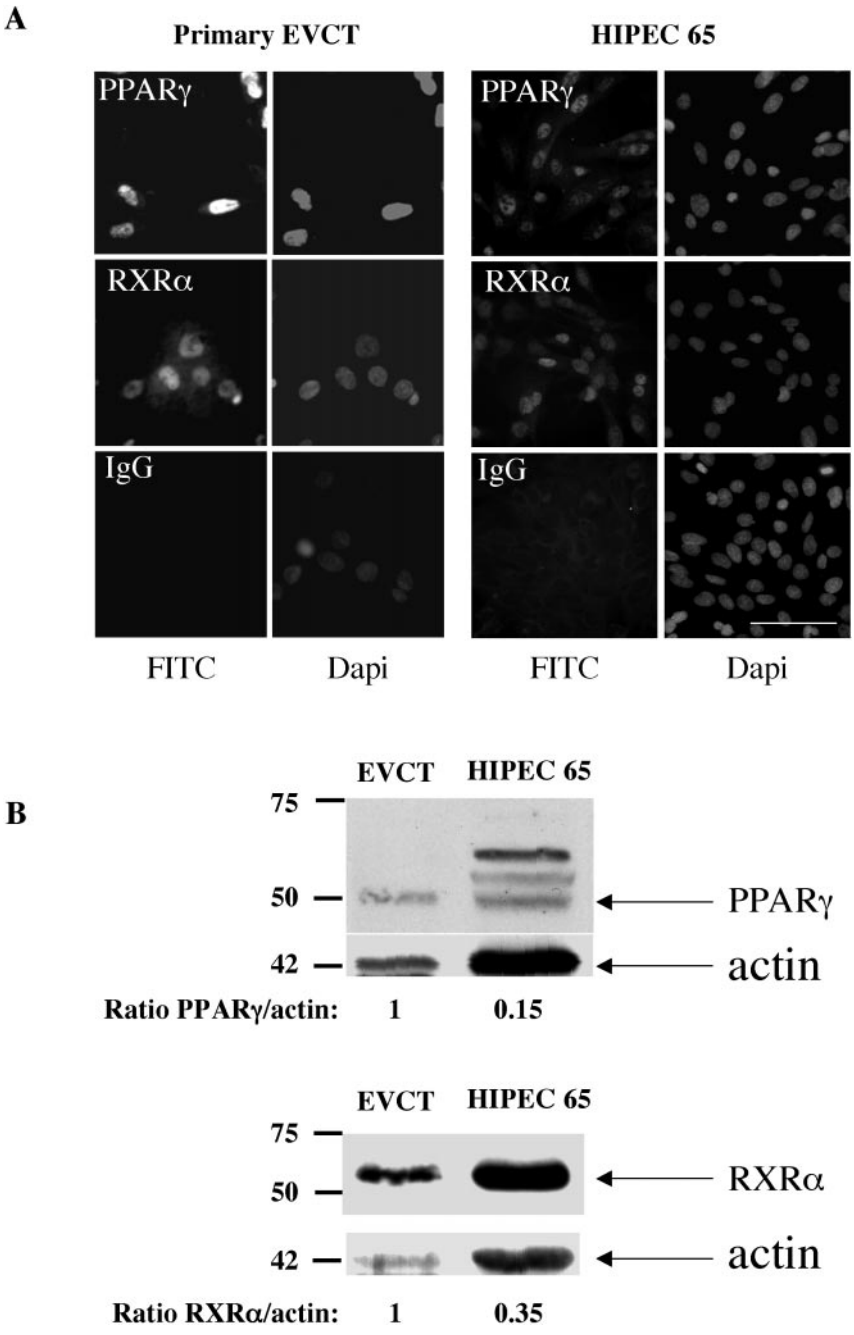

Fig. 4. HIPEC 65 co-expressed the nuclear receptors PPARγ and RXRα. (**A**) PPARγ and RXRα were immunolocalized in the nuclei of HIPEC 65 and primary EVCT while no staining was observed when incubated with nonimmune antibodies. Nuclei were counter stained with Dapi. Scale bar: 100 μm. (**B**) Immunoblot analysis of PPARγ and RXRα expression. HIPEC 65 expressed a 50 kDa and a 54 kDa protein similar respectively to the PPARγ and RXRα proteins present in primary EVCT. Two other bands that might correspond to PPARγ isoforms or use of different translation sites were found in HIPEC 65.

other bands at ~55 and 60 kDa were also detected in HIPEC 65. Two of these bands might correspond to PPARγ isoforms as the antibody we used recognized the two isoforms PPARγ 2 and PPARγ 1 or PPARγ 3, which are identical proteins. Similarly, two to three bands in PPARγ western analysis of hepatocyte cell lysates have been described by others (43). The high molecular bands found in HIPEC 65 might also correspond to different translation sites as described by Mukherjee *et al*. (44) who found two bands (57 and 53 kDa) for human PPARγ2 analyzed by PAGE. RXRα protein (54 kDa) was detected in both HIPEC 65 and primary EVCT. PPARγ and RXRα protein are both expressed to a lesser extent in HIPEC 65 in comparison with primary EVCT (six and three times less, respectively) after normalization to actin. Finally, the presence of each nuclear receptor transcripts was confirmed by real-time RT–PCR (Table IV).

We next examined the effects of synthetic or natural PPARγ ligands on lipid accumulation, cell viability, proliferation and invasion (Figure 5).

PPARγ activation induced lipid accumulation in HIPEC 65

As shown in Figure 5A, staining of cultured cells with oil red O, which detects triglycerides, unsaturated cholesterids and unsaturated free fatty acids (45) revealed lipid accumulation only in cells treated for 24 h with 1 μM of the synthetic PPARγ agonist BRL49653 (rosiglitazone). This observation

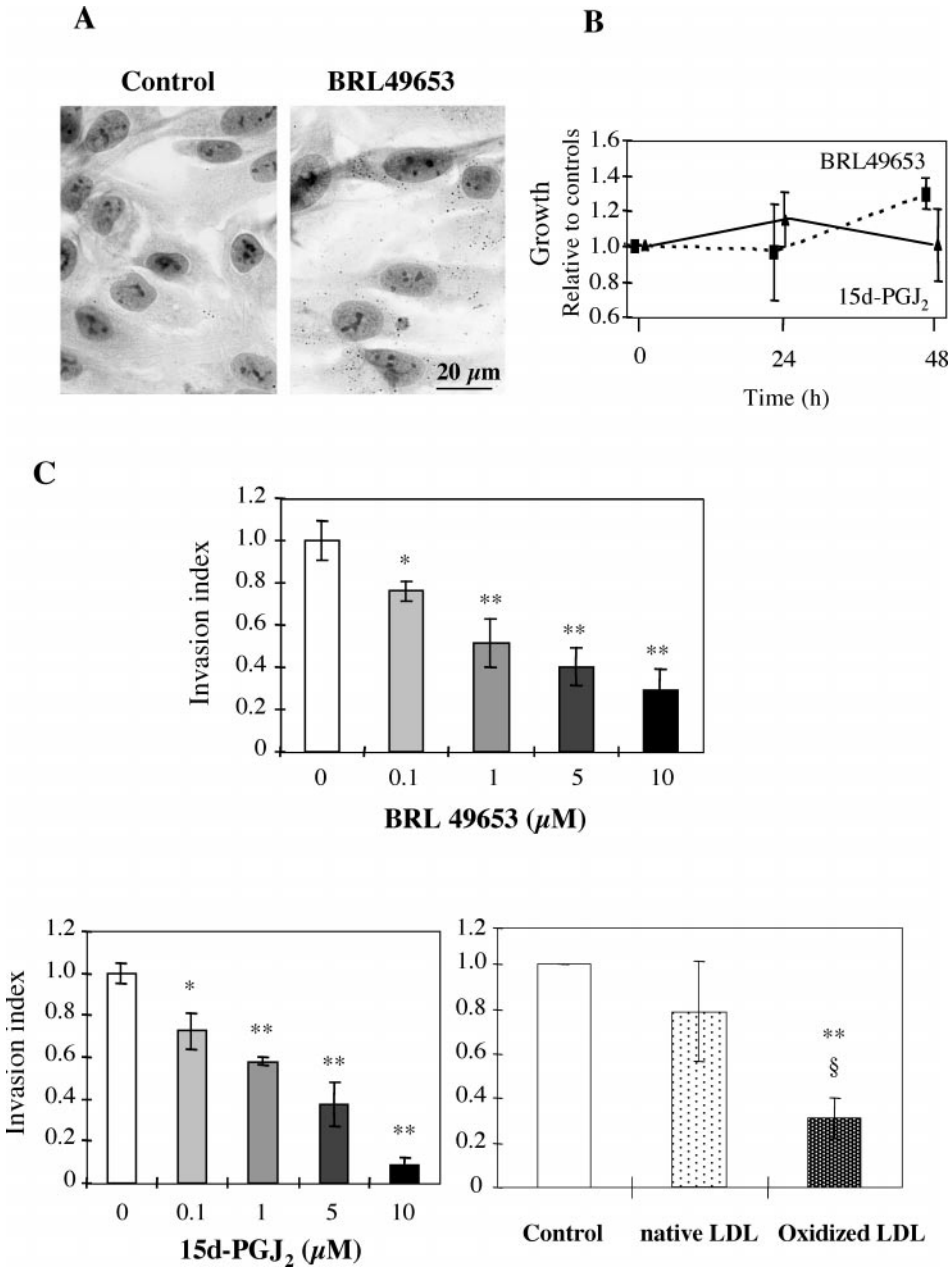

Fig. 5. Effect of PPARγ agonists on cell differentiation, growth and invasion. (**A**) Detection by oil red O staining of unsaturated fatty acids and triglycerides in HIPEC 65 incubated for 24 h with 1 μM rosiglitazone (BRL49653); no staining was observed in untreated cells. (**B**) Treatment of HIPEC 65 with PPARγ agonists (1 μM BRL49653 or 5 μM 15d-PGJ_2) over a 48 h-culture period did not significantly modify cell growth. Results are expressed as mean ± SD from three separate cultures and represent number of alive cells normalized to control values. (**C**) HIPEC 65 were cultured on Matrigel-coated transwells for 24 h with increasing concentrations of BRL 46653 or 15d-PGJ_2, and with 50 μg/ml native- or oxidized-LDL. Cell invasion was quantified as described in Figure 3 and is expressed as invasion index relative to control. Results represent the mean ± SD of triplicates from a representative experiment. $^{*}P < 0.05$, $^{**}P < 0.01$, treated versus control cells, $^{\S}P < 0.01$, oxidized versus native-LDL.

corroborates the function of PPARγ in inducing genes involved in lipogenesis as described for adipocyte differentiation.

HIPEC 65 proliferation was not affected by PPARγ activation

Because PPARγ agonists have been shown to regulate cell growth, we measured cell viability and proliferation using trypan blue exclusion test and conversion of water-soluble tetrazolium salt by active mitochondrial dehydrogenases of living cells (MTT assay). As shown in Figure 5B, the cell number of BRL49653 (1 μM) or 15d-PGJ_2 (5 μM)-treated cultures was not significantly different from control cells over a 48 h incubation time. In accordance, the cell viability as assayed by trypan blue uptake remained unchanged: controls (24 h, 87.7 ± 3.6; 48 h, 89.8 ± 4.3), BRL49653 (24 h, 85.5 ± 3.5; 48 h, 89.6 ± 1), 15d-PGJ_2 (24 h, 92.6 ± 0.4; 48 h, 88.4 ± 2.2). In agreement with the above results, incubation of HIPEC 65 for 48 h with concentrations of BRL49653 or 15d-PGJ_2 ranging from 0.1 to 10 μM did not modify cell proliferation as assayed by MTT test (Table V).

HIPEC 65 invasion was markedly abrogated by PPARγ synthetic or natural ligands

We next investigated whether activation of PPARγ by synthetic as well as natural ligands might alter the invasive

Table V. PPARγ agonists did not modify cell toxicity or growth after a 48 h-culture as assayed by MTT test

Culture conditions	Alive cell number index
Untreated	0.406 ± 0.026
BRL49653: 0.1 μM	0.454 ± 0.034
1 μM	0.392 ± 0.073
5 μM	0.390 ± 0.034
10 μM	0.420 ± 0.026
15d-PGJ_2: 0.1 μM	0.386 ± 0.044
1 μM	0.461 ± 0.053
5 μM	0.435 ± 0.012
10 μM	0.387 ± 0.041

OD at 570 nm were measured (MTT assay) and values correspond to the mean ± SD ($n = 3$).

properties of HIPEC 65 (Figure 5C). Therefore, HIPEC 65 were cultivated for 24 h in the *in vitro* system described in Figure 3, and incubated with increasing concentration of synthetic (BRL49653) or natural (15d-PGJ_2) PPARγ agonists. These two PPARγ agonists used at concentrations ranging from 0.1 to 10 μM inhibited cell invasion in a concentration-dependent manner reaching a 50% inhibition at 1 μM. Interestingly, a moderate but significant 25% inhibition was reached at concentrations as low as 0.1 μM for both BRL49653 and 15d-PGJ_2. This observation suggests that PPARγ plays a key role in the control of HIPEC 65 invasion. Oxidized lipids and oxidized-LDL have been shown to activate PPARγ in different cell types including macrophages (46) and human term trophoblasts (47). Thus, we have incubated HIPEC 65 for 24 h with non-oxidized (native) and oxidized-LDL at the concentration of 50 μg protein/ml and measured cell invasiveness as described above. Oxidized-LDL decreased cell invasiveness by ~70% whereas native-LDL did not significantly modify invasion. An inhibition of the same order of magnitude was obtained with the PPARγ agonists BRL49653 or 15d-PGJ_2 used at 5 μM. Cell viability as assayed by trypan blue uptake remained unchanged irrespective of the treatment: controls 97.5 ± 2.5, native-LDL 92.8 ± 1.1, oxidized-LDL 94.3 ± 1.1.

Discussion

Human trophoblast invasion provides a unique model of tumor-like controlled cell invasion process and the ligand-dependent nuclear receptor PPARγ has been shown to play a role in tumorigenesis by controlling cell growth and differentiation as well as apoptosis. We have recently reported that PPARγ/RXRα heterodimers are involved in human trophoblast differentiation (48) and invasion (12). Hence, in an attempt to obtain a suitable culture model to study the role of PPARγ in cell invasion and metastasis processes, we have in the present work immortalized primary invasive cytotrophoblasts and generated a human invasive trophoblast cell line whose invasion but not proliferation is regulated by PPARγ ligands.

These human invasive and proliferative extravillous cytotrophoblasts (HIPEC 65) were generated from our well-characterized primary EVCT purified from human first trimester chorionic villi (28), after transformation by SV40 large T and small t genes. Cell transformation was achieved by transfection of a recombinant DNA construct composed of the most efficient deletion mutant prepared from the promoter of the human vimentin gene, controlling the expression of the SV40 large T and small t genes. This human vimentin 830-T/t recombinant was used to obtain cell lines from numerous primary cells of different origins that retained characteristics of differentiated cells (34,35,49). Using immunocytochemistry and real-time PCR, we demonstrated in the present study that HIPEC 65 at passage 8 expressed the main markers that define human extravillous cytotrophoblast, i.e. expression of CK07, CD9 and HLA-G. These three phenotypic criteria were recently suggested as the minimal characteristic requirements for an extravillous cell line, even though very few of the cell lines tested fulfilled these criteria (39,40). The antibody used for HLA-G immunocytochemistry (W6/32) cross react with HLA-C and HLA-E, whereas the one used for western analysis is supposed to be specific for HLA-G. These might explain the discrepancy observed in HIPEC 65 HLA-G expression between immunocytochemistry and western blot where a very weak expression was observed. All together, these results show that HIPEC 65 are clearly derived from human extravillous cytotrophoblast. However, some of the *in vivo* differentiation characteristics can be lost in culture due to the insertion of the immortalizing sequence in the DNA and also due to the absence of cell–cell interactions that are essential for the proper expression of differentiated functions. Thus, a very weak expression of HLA-G, loss of CK07 expression on ongoing passages and acquisition of vimentin expression, was observed in HIPEC 65. Indeed, it has been shown that EVCT can co-express vimentin and cytokeratin in culture (50) and vimentin expression has already been described in other extravillous trophoblastic lines (50,51). This could be linked to the invasive phenotype of these cells as suggested by Gilles *et al*. (52) who clearly established a correlation between vimentin expression and the acquisition of an invasive and metastatic phenotype in epithelial cervical carcinoma.

We next analyzed the expression of the main markers of invasive cytotrophoblasts. In the present work, we showed that, like parental primary EVCT (28), our cell lines expressed TGFβ2 and the inhibitor of PAI-1, which is the predominant PAI present in invading trophoblasts (42). It was suggested that PAI (42) as well as TGFβ (53,54) might play a role in modulating trophoblast differentiation and invasion. We also showed that HIPEC 65 expressed markers of the invasive cytotrophoblast phenotype such as the α5 integrin subunit of the fibronectin receptor (7,28) and the proto-oncogene c-erbB2 (28,32). We next demonstrated that HIPEC 65 had the ability to invade an extracellular matrix in our Matrigel-coated transwell invasion assays. This was tested from passage 11 to 33. Interestingly, it appeared that transformed cells were more invasive than their parental primary cells. The fact that transcript levels of TGFβ and PAI-1, factors that regulate trophoblast invasion (55), were lower in HIPEC 65 than in primary cells corroborate this observation. Together, our results demonstrate that HIPEC 65 is a human invasive cell line of invasive EVCT origin.

One prominent feature found in the data so far reported is the high expression of the ligand-activated nuclear receptor PPARγ in tumor cells from human origin compared with normal tissue (15,56). These results suggest that PPARγ play an important role in the tumor process and in the control of tumor formation. We have recently reported high levels of PPARγ expression in human primary cytotrophoblasts compared with 3T3-L1 adipocytes (12). In the present study, we showed that our transformed cells HIPEC 65 also co-expressed

the nuclear receptors PPARγ and RXRα from passage 13 to 40. We next investigated the effects triggered by the ligand-induced activation of PPARγ/RXRα heterodimers on cell growth and invasion. As described for PPARγ-induced adipocyte differentiation (19,57,58), incubation of HIPEC 65 with the PPARγ agonist rosiglitazone, at concentrations as low as 1 μM, led to accumulation of intracellular unsaturated fatty acids and triglycerides that stain positive with oil red O. This observation demonstrates that PPARγ is activated by its ligand in HIPEC 65 and that ligand-activated PPARγ/RXRα heterodimers trigger the transcription of target genes involved in lipid metabolism.

PPARγ has been shown to induce differentiation and/or to inhibit growth in a number of cell types including cancer cells and is therefore considered as a tumor suppressor. Thus, it has been reported that thiazolidinedione reduced the proliferation rate of Caco2 colorectal cancer cells (59) and MCF7 breast cancer cells (60). They had potent antitumor effects against prostate cancer both *in vitro* and *in vivo* (15), and they caused cell cycle arrest of growing fibroblasts and of SV40 large T antigen-transformed adipogenic cells (14). In contrast, we showed that in human invasive trophoblast line HIPEC 65, activation of PPARγ by rosiglitazone did not affect cell growth. This lack of effects was reported by others in certain tumor lines *in vitro* (60–62) suggesting that tumor cells can acquire resistance to direct thiazolidinedione effect. Other proliferation studies showed that different cell types displayed different sensitivities to the same thiazolidinedione concentration (63). In HIPEC 65, it is unlikely to be the case because no effect on cell growth was observed irrespective of the ligand (rosiglitazone or the natural PPARγ agonist 15d-PGJ2) or the concentrations (0.1 to 10 μM) used.

Recent studies have suggested that in mice, rosiglitazone might prevent the establishment and progression of metastatic disease through indirect antiangiogenic effect and through direct modulation of regulators of metastatic invasion (63). In the present study, we have investigated the role of PPARγ in the regulation of HIPEC 65 invasion *in vitro*. Our results showed that activation of PPARγ/RXRα heterodimers with various PPARγ ligands from synthetic or natural origins abrogated the invasive properties of HIPEC 65 in a concentration-dependent fashion. This was observed from passage 13 to 40. A significant inhibition was induced with rosiglitazone or 15d-PGJ_2 used at concentrations as low as 0.1 μM, reaching a 50% inhibition for 1 μM. Interestingly, these results confirmed those obtained with non-proliferative primary EVCT (12), but HIPEC 65 appeared to be more sensitive to lower concentrations of PPARγ ligands. Oxidized-LDL has been shown to stimulate PPARγ expression in macrophage (46) and to activate a PPARγ-controlled reporter gene construct in trophoblasts (47). Nagy *et al*. (18) showed that certain oxidized lipid components of the oxidized-LDL particle function as endogenous PPARγ activators. In order to investigate the effect of this PPARγ activator on cell invasion, we incubated HIPEC 65 with native- or oxidized-LDL. As described for rosiglitazone and 15d-PGJ_2, oxidized- but not native-LDL markedly decreased cell invasion up to 70%. Our results show for the first time that various PPARγ ligands and activators, such as thiazolidinedione, 15d-PGJ_2 or oxidized-LDL, modulate cell invasion without affecting proliferation. In conclusion, human trophoblast implantation offers a unique model to study invasion of normal cells. In contrast to cancer cell, our human transformed trophoblast model provides a sensitive tool to study specifically the mechanism by which PPARγ directly control the metastatic invasion process, independently of its effect on cell growth.

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