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Peroxisome proliferator-activated receptor γ in human breast carcinoma: a modulator of estrogenic actions

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Abstract

It has been reported that agonists of peroxisome proliferator-activated receptor γ (PPARγ) inhibit proliferation of breast carcinoma cells, but the biological significance of PPARγ remains undetermined in human breast carcinomas. Therefore, we immunolocalized PPARγ in 238 human breast carcinoma tissues. PPARγ immunoreactivity was detected in 42% of carcinomas, and was significantly associated with the status of estrogen receptor (ER) α, ERβ, progesterone receptor, retinoic X receptors, p21 or p27, and negatively correlated with histological grade or cyclooxygenase-2 status. PPARγ immunoreactivity was significantly associated with an improved clinical outcome of breast carcinoma patients by univariate analysis, and multivariate analysis demonstrated that PPARγ immunoreactivity was an independent prognostic factor for overall survival in ERα-positive patients. We then examined possible mechanisms of modulation by PPARγ on estrogenic actions in MCF-7 breast carcinoma cells. A PPARγ activator, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), significantly inhibited estrogen-responsive element-dependent transactivation by estradiol in MCF-7 cells, which was blocked by addition of a PPARγ antagonist GW9662. Subsequent study, employing a custom-made microarray focused on estrogen-responsive genes, revealed that mRNA expression was significantly regulated by estradiol in 49 genes, but this significance vanished on addition of 15d-PGJ_2 in 16 out of 49 (33%) genes. These findings were confirmed by real-time PCR in 11 genes. 15d-PGJ_2 significantly inhibited estrogen-mediated proliferation of MCF-7 cells, and caused accumulation of p21 and p27 protein. These results suggest that PPARγ is mainly expressed in well-differentiated and ER-positive breast carcinomas, and modulates estrogenic actions.

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Introduction

Peroxisome proliferator-activated receptor γ (PPARγ) is a member of the nuclear hormone receptor superfamily, and has also been designated NR1C3 (Lemberger *et al.* 1996, Schoonjans *et al.* 1996). PPARγ functions as a transactivation factor following heterodimerization with retinoic X receptors (RXRs), and binds to its specific response elements termed peroxisome proliferating responsive elements (PPREs) of various target genes (Mangelsdorf & Evans 1995). PPARγ is one of the ligand-activated transcription factors, and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) is currently considered a naturally occurring PPARγ ligand, which activates PPARγ at μM concentrations in the human (Koeffler 2003).

It is well known that PPARγ plays essential roles in adipogenesis (Tontonoz *et al.* 1994), insulin resistance (Celi & Shuldiner 2002), and development of various organs (Barak *et al.* 1999). In addition, various *in vitro* studies have demonstrated that PPARγ ligands have a potent antiproliferative activity against a wide variety

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of neoplastic cells (Koeffler 2003). For instance, PPARγ agonist inhibits the proliferation of human breast cancers (Elstner *et al.* 1998, Mueller *et al.* 1998, Yee *et al.* 1999), and a phase II clinical trial using PPARγ ligands has been recently performed as a novel therapy for advanced breast cancer patients (Burstein *et al.* 2003).

It then becomes very important to obtain a better understanding of the clinical and/or biological roles of PPARγ in breast cancer tissues in order to improve the potential clinical efficiency of PPARγ ligand therapy for breast cancer patients. Mueller *et al.* (1998) previously demonstrated the expression of PPARγ in human primary and metastatic cancers, and Wang *et al.* (2004) reported higher amounts of PPARγ expression in breast carcinoma cells than in normal human mammary epithelial cells. However, it has been also demonstrated that PPARγ expression is significantly lower in breast cancer tissues at both mRNA (Jiang *et al.* 2003) and protein (Watkins *et al.* 2004) levels than that in normal tissues. Expression of PPARγ has been examined in human breast carcinomas by several groups, but little information is available on the clinicopathological features of PPARγ-positive breast cancers. Therefore, the biological significance of PPARγ remains largely undetermined in human breast carcinoma. In this study, we examined immunolocalization of PPARγ in 238 cases of human breast carcinoma patients, and correlated these findings with various clinicopathological parameters. As the results of immunohistochemistry demonstrated a strong association between PPARγ and estrogen receptor (ER) α in breast carcinomas, we also examined a possible modulation by PPARγ on estrogenic actions in breast cancer cells for further characterization of PPARγ in human breast carcinoma.

Materials and methods

Patients and tissues

Two hundred and thirty-eight surgical pathology specimens of invasive ductal carcinoma of the breast were retrieved from pathology archives of the Department of Surgery, Tohoku University Hospital, Sendai, Japan. Breast tissue specimens were obtained from female patients who underwent mastectomy from 1982 to 1992 with a mean age of 54.1 years (range 22–82). The patients did not receive chemotherapy or irradiation prior to surgery. Review of the charts of patients revealed that 194 patients received adjuvant chemotherapy, and 43 patients received tamoxifen therapy after surgery. The mean follow-up time was 102 months (range 2–157 months). The histological grade of each specimen was evaluated based on the method of Elston & Ellis (1991). All specimens were fixed with 10% formalin and embedded in paraffin wax. Research protocols for this study were approved by the Ethics Committee at Tohoku University School of Medicine (approved number: 2000-142).

Antibodies

Rabbit polyclonal antibody for PPARγ was raised against a synthetic peptide corresponding to amino acids 60–79 of mouse PPARγ1 (accession number; AAA62110), which also corresponds to amino acids 62–81 of human PPARγ1 (CAA62152) or 90–109 of human PPARγ2 (AAB04028). This antibody therefore recognizes both human PPARγ1 and γ2. The characterization of this antibody has been previously confirmed by both immunoblotting and immunohistochemistry (Sato *et al.* 2004). The characteristics of polyclonal antibodies for RXRα, RXRβ and RXRγ have been previously reported by the authors (Sugawara *et al.* 1995, Suzuki *et al.* 2001). Monoclonal antibodies for ERα (ER1D5), progesterone receptor (PR; MAB429), Ki-67 (MIB1), p21 (6B6), p27 (1B4), c-Myc (1-6E10), pS2 (M7184), and cyclin D1 (P2D11F11) were purchased from Immunotech (Marseille, France), Chemicon (Temecula, CA, USA), DAKO (Carpinteria, CA, USA), Pharmingen (San Diego, CA, USA), Novocastra Laboratories (Newcastle, UK), Cambridge Research Biochemical (Cambridge, UK), DAKO and Novocastra Laboratories respectively. Rabbit polyclonal antibodies for ERβ (06-629), HER2 (A0485) and cathepsin D (A0561) were obtained from Upstate Biotechnology (Lake Placid, NY, USA), DAKO and DAKO respectively. Goat polyclonal antibody for cyclooxygenase-2 (COX2) (C-20) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Immunohistochemistry

A Histofine Kit (Nichirei, Tokyo, Japan), which employs the streptavidin-biotin amplification method was used in this study. Antigen retrieval for PPARγ, ERα,β, PR, RXRα,β,γ, HER2, Ki-67, p21, p27 and cyclin D1 immunostaining was performed by heating the slides in an autoclave at 120 °C for 5 min in citric acid buffer (2 mM citric acid and 9 mM trisodium citrate dehydrate, pH 6.0), and antigen retrieval for COX2 and pS2 immunostaining was done by heating the slides in a microwave oven for 15 min in the citric acid buffer. Dilutions of primary antibodies used in this study were as follows: PPARγ; 1/1500, ERα; 1/50,

ERβ; 1/50, PR; 1/30; RXRα; 1/4000, RXRβ; 1/4000, RXRγ; 1/2000, COX2; 1/500, HER2; 1/200, Ki-67; 1/50, p21; 1/250, p27; 1/150, c-Myc 1/600, pS2; 1/30, cyclin D1; 1/40 and cathepsin D; 1/300. The antigen–antibody complex was visualized with 3,3′-diaminobenzidine solution (1 mM, in 50 mM Tris–HCl buffer (pH 7.6) and 0.006% H_2O_2), and counterstained with hematoxylin. As a negative control, normal rabbit, mouse or goat IgG was used instead of the primary antibodies. For PPARγ immunohistochemistry, a preabsorption test was also performed as a negative control.

Scoring of immunoreactivity and statistical analysis

PPARγ, ERα,β, PR, RXRα,β,γ, Ki-67, p21, p27, c-Myc and cyclin D1 immunoreactivity was detected in the nucleus, and the immunoreactivity was evaluated in more than 1000 carcinoma cells for each case, and subsequently the percentage of immunoreactivity, i.e. labeling index (LI), was determined. Inter-observer differences were less than 5%, and the mean of the three values was obtained. Cases with PPARγ, ERα or c-Myc LIs of more than 10% were considered PPARγ-, ERα- or c-Myc-positive breast carcinomas in this study, according to a report for ERα by Allred *et al.* (1998). For p21 and p27 immunohistochemistry, the cut-off values used were 5 and 50% respectively, according to previous reports (Barbareschi *et al.* 2000, Pellikainen *et al.* 2003). Immunoreactivity for COX2 and cathepsin D was detected in the cytoplasm, and cases that had more than 10% of positive carcinoma cells were considered positive.

An association between immunoreactivity for PPARγ and clinicopathological factors was evaluated using a one-way ANOVA and a Bonferroni test or a cross-table using the chi-square test. Overall and disease-free survival curves were generated according to the Kaplan–Meier method and statistical significance was calculated using the log-rank test. An association between ERα LI and PR or cyclin D1 LI was performed utilizing a correlation coefficient (r) and regression equation. Univariate and multivariate analyses were evaluated by a proportional hazard model (Cox) using PROC PHREG in our SAS software. P values less than 0.05 were considered significant in this study.

Cell line, plasmids and chemicals

MCF-7 human breast cancer cell line was cultured in RPMI-1640 (Sigma-Aldrich, St Louis, MO, USA) with 10% fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, USA). MCF-7 cells were cultured with phenol red-free RPMI 1640 medium containing 10% dextran-coated charcoal (DCC)-FBS for 3 days before treatment in the experiment. In this study, we used estrogen-responsive reporter plasmids pERE-Luc, containing *Xenopus* vitellogenin A2 estrogen-responsive element (ERE) (Saji *et al.* 2001). The pRL-TK vectors were purchased from Promega (Madison, WI, USA). 15d-PGJ_2, ciglitazone and $PGF_{2\alpha}$ were purchased from Biomol Research Laboratories (Butler Pike, PA, USA), and GW1929 and GW9662 were purchased from Sigma-Aldrich.

Luciferase assay

The luciferase assay was performed according to a previous report (Sakamoto *et al.* 2002) with some modifications. Briefly, 1 μg ptk-ERE-Luc plasmids and 200 ng pRL-TK control plasmids were used to measure the transcriptional activity of endogenous ER. Transient transfections were carried out using *Trans*IT-LT Transfection Reagents (TaKaRa, Tokyo, Japan) in MCF-7 cells, and the luciferase activity of lysates was measured using a Dual-Luciferase Reporter Assay system (Promega) and Luminescencer-PSN (AB-2200) (Atto Co., Tokyo, Japan) after incubation with growth medium with the indicated concentrations of estradiol and/or 15d-PGJ_2 for 24 h. The cells were also treated with the same volume of ethanol (final dilution – 0.1%) for 24 h as controls. The transfection efficiency was normalized against *Renilla* luciferase activity using pRL-TK control plasmids, and the luciferase activity for each sample was evaluated as a ratio (%) compared with that of controls. The statistical analyses were performed using a one-way ANOVA and Bonferroni test.

Immunoblotting

The cell protein was extracted in triple detergent lysis buffer (LK-18) at 4 °C. Twenty micrograms of the protein (whole cell extracts) were subjected to SDS-PAGE (10% acrylamide gel). Following SDS-PAGE, proteins were transferred onto Hybond P polyvinylidene difluoride membrane (Amersham Biosciences, Piscataway, NJ, USA). The blots were blocked in 5% non-fat dry skim milk for 1 h at room temperature, and were then incubated with a primary antibody for ERα, p21, p27 or β-actin (Sigma-Aldrich) for 18 h at 4 °C. After incubation with anti-mouse IgG horseradish peroxidase (Amersham Biosciences) for 1 h at room temperature, antibody–protein complexes on the blots were detected using ECL-plus western blotting detection reagents (Amersham Biosciences). Immunointensity of

Table 1 Primer sequences used in real-time PCR in this study

cDNA (Accession no.)	Sequence (position in cDNA)	Size (bp)	Reference
pS2 (NM003225)	FWD: 53–72, REV: 240–260	208	Colombel *et al.* (1999)
PDZK1 (AF012281)	FWD: 725–746, REW: 824–847	123	Yoshida *et al.* (2004)
Cyclin D1 (NM053056)	FWD: 456–475, REV: 640–659	204	Paruthiyil *et al.* (2004)
IGFBP-4 (M62403)	FWD: 697–715, REV: 756–773	77	Yoshida *et al.* (2004)
SLC7A5* (M80244)	FWD: 816–835, REV: 1025–1044	206	—
TPD52L1 (U44427)	FWD: 450–469, REV: 561–580	130	Yoshida *et al.* (2004)
PR*† (XM006190)	FWD: 1987–2006, REV: 2163–2182	195	—
Cathepsin D (BT006910)	FWD: 697–715, REV: 804–822	126	Yoshida *et al.* (2004)
TAL6 (M90657)	FWD: 246–265, REV: 299–319	74	Kao *et al.* (2003)
IGFBP-5 (NM000599)	FWD: 1090–1108, REV: 1213–1232	143	Yoshida *et al.* (2004)
SELENBP1 (U29091)	FWD: 167–186, REV: 315–333	167	Yoshida *et al.* (2004)
p21* (NM000389)	FWD: 415–434, REV: 573–592	78	—
p27 (NM004064)	FWD: 829–848, REW: 929–938	120	Schonherr *et al.* (2001)
RPL13A (NM012423)	FWD: 487–509, REV: 588–612	125	Vandesompele *et al.* (2002)

PDZK1; PDZ domain-containing-protein, IGFBP-4; insulin-like growth factor-binding protein-4, SLC7A5; solute carrier family 7, member 5, TPD52L1; tumor protein D52-like 1, PR; progesterone receptor, TAL6; tumor-associated antigen L6, IGFBP-5; insulin-like growth factor-binding protein-5, SELENBP; selenium-binding protein-1, and RPL13A; ribosomal protein L 13a.
*Oligonucleotide primers for SLC7A5, PR and p21 were designed in the different exons in this study.
†The primers of PR recognize both PR-A and PR-B subtypes.

specific bands was measured by an LAS-1000 imaging system (Fuji Photo Film, Tokyo, Japan), and relative immunointensity of ERα, p21 or p27 was evaluated as a ratio (%) of β-actin immunointensity.

Microarray analysis

In this study, we used a custom-made microarray named EstrArray (InfoGenes, Tsukuba, Japan), which contains 175 estrogen-responsive genes identified in MCF-7 cells (Inoue *et al.* 2002, Hayashi *et al.* 2003). MCF-7 cells were cultured with phenol red-free RPMI 1640 medium containing 10% DCC-FBS for 3 days, and subsequently treated with estradiol (10 nM) with or without 15d-PGJ_2 (5 μM) for 72 h. The MCF-7 cells used as references were treated with the same volume of ethanol (final dilution – 0.1%) for 72 h.

Total RNA was isolated using TRIzol reagent (InVitrogen Life Technologies, Inc., Gaitherburg, MD, USA). Two micrograms of mRNA were reverse-transcribed with Cy3- or Cy5-dUTP (Amersham Biosciences, Bucks, UK) using a SUPERSCRIPT II Preamplification system (Gibco-BRL, Grand Island, NY, USA). Cy3- and Cy5-labeled cDNA probes were hybridized on the microarray slide for 16 h at 65 °C. The fluorescent signals were scanned by a GenePix 4000A (Axon Instruments, Foster City, CA, USA), and the ratio of Cy3 and Cy5 signal intensity of each spot was quantitatively calculated using GenePixPro 5.0 (Axon Instruments). The duplicated sets of values were averaged and normalized by subtracting the average of values for internal genes. The data from insufficient hybridization (signal areas below 100) were excluded from the analysis. Genes which showed a value of more than 2.0 or less than 0.5 were evaluated as significantly up-regulated or down-regulated respectively, in this study.

Real-time PCR

MCF-7 cells were cultured with phenol red-free RPMI 1640 medium containing 10% DCC-FBS for 3 days, and subsequently treated with the indicated concentration of estradiol and/or 15d-PGJ_2 for 72 h. As controls for the experiments, the cells were treated with the same volume of ethanol (final dilution – 0.1%) for 72 h. Total RNA was extracted using TRIzol reagent (InVitrogen Life Technologies, Inc.), and a reverse transcription kit (SUPERSCRIPT II Preamplification system (Gibco-BRL) was used in the synthesis of cDNA.

The Light Cycler System (Roche Diagnositics GmbH, Mannheim, Germany) was used to semi-quantify the mRNA expression levels by real-time PCR (Dumoulin *et al.* 2000). Characteristics of the primer sequences used in this study are summarized in Table 1 (Colombel *et al.* 1999, Schonherr *et al.* 2001, Vandesompele *et al.* 2002, Kao *et al.* 2003, Paruthiyil *et al.* 2004, Yoshida *et al.* 2004). Settings for the PCR thermal profile were: initial denaturation at 95 °C for 1 min followed by 40 amplification cycles of 95 °C for 1 s, annealing at 59 °C (PR), 60 °C (pS2, PDZ domain-containing protein (PDZK1), cathepsin D, selenium-binding protein 1 (SELENBP1), tumor protein D52-like 1 (TPD52L1), insulin-like growth

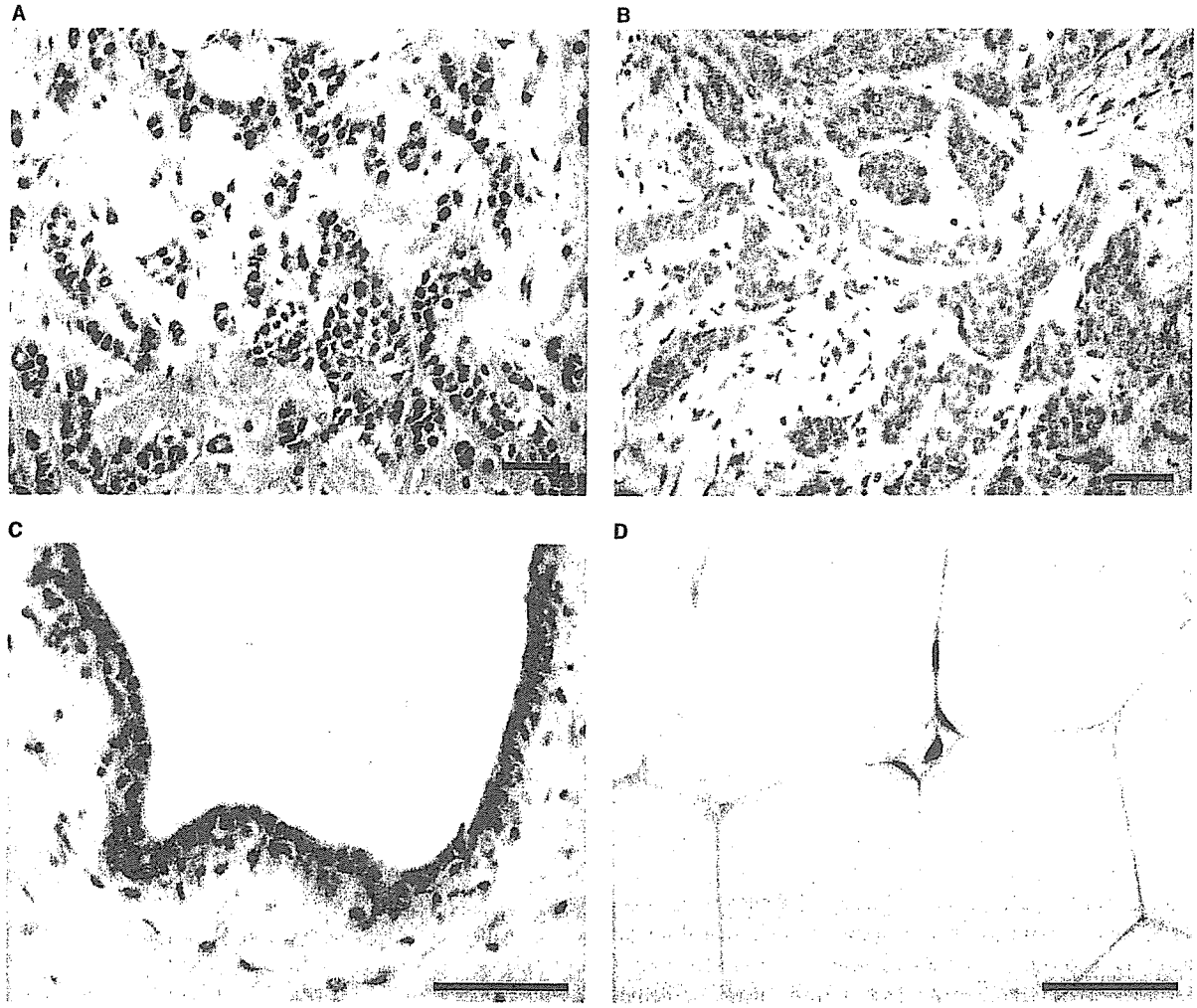

Figure 1 Immunohistochemistry for PPARγ in invasive ductal carcinoma. (A) PPARγ immunoreactivity was detected in the nuclei of carcinoma cells. (B) No significant immunoreactivity of PPARγ was detected in the sections where an immunohistochemical preabsorption test was performed as a negative control. (C) Immunoreactivity for PPARγ was detected in the nuclei of epithelial cells in morphologically normal mammary glands. (D) PPARγ immunoreactivity was positive in the nuclei of adipocytes. Bar = 50 μm.

factor-binding protein-5 (IGFBP-5), PDZ domain-containing-protein (PDZK1), and p21), 64 °C (cyclin D1, and tumor-associated antigen L6 (TAL6)), 66 °C (solute carrier family 7, member 5 (SLC7A5), and p27), or 68 °C (ribosomal protein L 13a (RPL13A)) for 15 s, and elongation at 72 °C for 15 s. To verify amplification of the correct sequences, PCR products were purified and subjected to direct sequencing. Negative control experiments lacked cDNA substrate to check for the possibility of exogenous contaminant DNA. The mRNA levels were summarized as a ratio of RPL13A, and subsequently evaluated as a ratio (%) compared with that of controls. The statistical analyses were performed using a one-way ANOVA and Bonferroni test.

Cell proliferation assay and apoptosis analysis

The status of cell proliferation of MCF-7 cells was measured using a WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2-H-tetrazolium, monosodium salt) method (Cell Counting Kit-8; Dojindo Inc., Kumamoto, Japan)) (Isobe *et al.* 1999). We also examined apoptosis status of MCF-7 cells using an apoptosis screening kit (Wako, Osaka, Japan), which employed a modified TdT-mediated dUTP nick-end labeling (TUNEL) method (Gavrieli *et al.* 1992). Optical densities (OD = 450 nM for cell proliferation assay, and OD = 490 nM for

apoptosis analysis) were obtained with a Model 680 microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). The cell number and apoptosis index were calculated according to the equation (Cell OD value after test material treatment)/(Vehicle control cell OD value), and subsequently evaluated as a ratio (%) compared with that of controls.

Results

Immunohistochemistry for PPARγ in breast carcinoma tissues

Immunoreactivity for PPARγ was detected in the nuclei of invasive ductal carcinoma cells (Fig. 1A and B). A mean value of PPARγ LI in 238 breast carcinoma cases examined was 15% (range 0–74%), and the number of PPARγ-positive breast carcinomas (i.e. PPARγ LI ≥ 10%) was 99 out of 238 cases (42%). Immunoreactivity of PPARγ was also detected in epithelia of morphologically normal mammary glands (Fig. 1C), and adipocytes (Fig. 1D).

Associations between PPARγ immunoreactivity and clinicopathological parameters in 238 breast carcinomas were summarized in Table 2. PPARγ immunoreactivity was significantly associated with ERα status ($P = 0.0003$), ERα LI ($P < 0.0001$), ERβ LI ($P = 0.0255$), PR LI ($P = 0.0012$), RXRα LI ($P = 0.0365$), RXRβ LI ($P < 0.0001$), RXRγ LI ($P = 0.0005$), p21 immunoreactivity ($P = 0.0057$) or p27 immunoreactivity ($P = 0.0019$). PPARγ immunoreactivity was negatively correlated with histological grade ($P = 0.0035$) or COX2 immunoreactivity ($P = 0.0178$). No significant association was detected between PPARα immunoreactivity and other clinicopathological parameters examined, including patient age, menopausal status, clinical stage, tumor size, lymph node status, HER2 status, Ki-67 LI, and c-Myc immunoreactivity in this study. The association between PPARγ immunoreactivity and RXRβ LI, RXRγ LI, COX2 immunoreactivity, p21 immunoreactivity or p27 immunoreactivity was significant regardless of ERα status of these cases (Table 3). PPARγ immunoreactivity was positively correlated with RXRα LI ($P = 0.0469$) and inversely with lymph node status ($P = 0.0303$) or Ki-67 LI ($P = 0.0485$) only in the ERα-positive group.

Influence of PPARγ immunoreactivity on association between ERα and estrogen-responsive genes in breast cancer tissues

pS2, cyclin D1, PR and cathepsin D are all well recognized as estrogen-responsive genes in human breast cancers. As shown in Table 4, a significant positive association was detected between ERα LI and the status of these immunoreactivity in the 238 breast cancer tissues examined ($P < 0.0001$), which is in good agreement with previous immunohistochemical studies

Table 2 Association between PPARγ immunoreactivity and clinicopathological parameters in 238 breast carcinomas

	PPARγ immunoreactivity		
	Positive (n=99)	Negitive (n=139)	*P* value
Age (years)*	53.1±1.2	55.1±1.1	0.0850
Menopausal status			
Premenopausal	46 (19.3%)	54 (22.7%)	
Postmenopausal	53 (22.3%)	85 (35.7%)	0.2983
Stage			
I	30 (12.6%)	33 (13.9%)	
II	57 (24.0%)	75 (31.5%)	
III	12 (5.0%)	31 (13.0%)	0.1032
Tumor size (mm)*	32.6±0.4	29.4±0.2	0.3808
Lymph node status			
Positive	38 (16.0%)	68 (28.6%)	
Negative	61 (25.6%)	71 (29.8%)	0.1389
Histological grade			
1	35 (14.7%)	31 (13.0%)	
2	40 (16.8%)	46 (19.3%)	
3	24 (10.1%)	62 (26.1%)	**0.0035**
ERα status			
Positive	85 (35.7%)	89 (37.4%)	
Negative	14 (5.9%)	50 (21.0%)	**0.0003**
ERα LI*	54.5±3.1	34.8±2.9	**<0.0001**
ERβ LI*	19.6±2.4	13.4±1.6	**0.0255**
PR LI*	49.6±3.3	34.9±3.0	**0.0012**
RXRα LI*	27.1±3.5	18.4±2.4	**0.0365**
RXRβ LI*	36.0±4.7	10.5±2.2	**<0.0001**
RXRγ LI*	17.5±5.1	3.0±1.0	**0.0005**
COX2 immunoreactivity			
Positive	29 (12.2%)	72 (30.3%)	
Negative	70 (29.4%)	67 (28.2%)	**0.0178**
HER2 status			
Positive	46 (19.3%)	53 (22.7%)	
Negative	53 (22.7%)	85 (35.7%)	0.7836
Ki-67 LI*	22.7±1.6	26.7±1.6	0.0916
p21 immunoreactivity			
Positive	56 (23.5%)	45 (18.9%)	
Negative	43 (17.3%)	91 (38.2%)	**0.0057**
p27 immunoreactivity			
Positive	58 (24.4%)	47 (19.7%)	
Negative	41 (17.2%)	92 (38.7%)	**0.0019**
c-Myc immunoreactivity			
Positive	49 (20.6%)	62 (26.1%)	
Negative	50 (21.0%)	77 (32.4%)	0.5715

*Data are presented as means±95% confidence interval. All other values represent the number of cases and percentage. *P* values less than 0.05 were considered significant, and are in boldface.

(Horwitz & McGuire 1978, Barbareschi *et al.* 1997, Gillesby & Zacharewski 1999, Ioachim *et al.* 2003). When the breast cancers were classified into two groups according to their PPARγ status, no significant association was detected between ERα LI and pS2 ($P = 0.3785$) or cyclin D1 LI ($P = 0.1978$) in PPARγ-positive breast carcinomas, although significant association ($P < 0.0001$ for pS2, and $P = 0.0018$ for cyclin D1) was detected in PPARγ-negative breast carcinomas. On the other hand, ERα LI was significantly associated with PR LI ($P = 0.0008$ in PPARγ-positive cases, and $P < 0.0001$ in PPARγ-negative cases) or cathepsin D ($P = 0.0006$ in PPARγ-positive cases, and $P = 0.0003$ in PPARγ-negative cases) regardless of the PPARγ status in the breast carcinoma cases examined.

Table 3 Statistical association between PPARγ immunoreactivity and clinicopathological parameters according to the ERα status in 238 breast carcinomas

	PPARγ immunoreactivity (positive/negative)	
	ERα-positive group (n=173)	ERα-negative group (n=64)
Age	0.1107	0.1073
Menopausal status	0.6702	0.6616
Stage	0.1272	0.7195
Tumor size	0.6077	0.6063
Lymph node status	**0.0303**	0.7192
Histological grade	0.3691	0.2452
ERβ LI	0.0963	0.1541
PR LI	0.2826	0.5011
RXRα LI	**0.0469**	0.5403
RXRβ LI	**0.0005**	**0.0002**
RXRγ LI	**0.0108**	**0.0185**
COX2 immunoreactivity	**0.0205**	**0.0411**
HER2 status	0.7702	0.7616
Ki-67 LI	**0.0485**	0.7103
p21 immunoreactivity	**0.0113**	**0.0481**
p27 immunoreactivity	**0.0142**	**0.0205**
c-Myc immunoreactivity	0.3999	0.5404

Data are presented as P values. P values less than 0.05 were considered significant, and are in boldface.

Correlation between PPARγ immunoreactivity and clinical outcome of the patients

No significant association was detected between PPARγ immunoreactivity and a risk of recurrence ($P = 0.8715$) (Fig. 2A). PPARγ immunoreactivity was significantly associated with a better clinical outcome of the 238 breast cancer patients ($P = 0.0257$) (Fig. 2B). This significant association was detected in the ERα-positive group ($P = 0.0057$) (Fig. 2C), but not in the ERα-negative group ($P = 0.6405$) (Fig. 2D). The significant correlation between PPARγ immunoreactivity and overall survival of ERα-positive breast cancer patients was not influenced by tamoxifen therapy after the surgery (Fig. 2E and F).

Utilizing a univariate analysis (Table 5), lymph node status ($P < 0.0001$), histological grade ($P < 0.0001$), tumor size ($P = 0.0002$), HER2 status ($P = 0.0029$), c-Myc immunoreactivity ($P = 0.0066$), and PPARγ immunoreactivity ($P = 0.0287$) turned out to be significant prognostic factors for overall survival in this study. Multivariate analysis revealed that only lymph node status ($P < 0.0001$) and c-Myc immunoreactivity ($P = 0.0024$) were independent prognostic factors with a relative risk over 1.0 (Table 5). When we examined a univariate analysis in the ERα-positive breast cancer patients ($n = 174$), lymph node status ($P < 0.0001$), histological grade ($P < 0.0001$), tumor size ($P < 0.0001$), HER2 status ($P = 0.0008$), PPARγ

Table 4 Correlation between ERα and estrogen-responsive gene immunoreactivity associated with PPARγ status in 238 breast carcinomas

	Total (n=238)		**PPARγ-positive** (n=99)		**PPARγ-negative** (n=139)	
	ERα LI	P value	ERα LI	P value	ERα LI	P value
pS2						
Positive	53.2±3.3		57.3±4.4		52.4±4.8	
Negative	31.8±3.7	**<0.0001**	49.0±6.7	0.3785	22.0±4.1	**<0.0001**
Cyclin D1 LI*		**<0.0001** (r=0.355)		0.1978 (r=0.231)		**0.0018** (r=0.313)
PR LI*		**<0.0001** (r=0.512)		**0.0008** (r=0.402)		**<0.0001** (r=0.566)
Cathepsin D						
Positive	54.2±3.7		58.0±4.4		51.2±5.6	
Negative	17.5±4.4	**<0.0001**	24.8±8.5	**0.0006**	12.2±4.2	**0.0003**

P values less than 0.05 were considered significant, and are in boldface.
*The association was statistically evaluated utilizing a correlation coefficient (r) and regression equation.

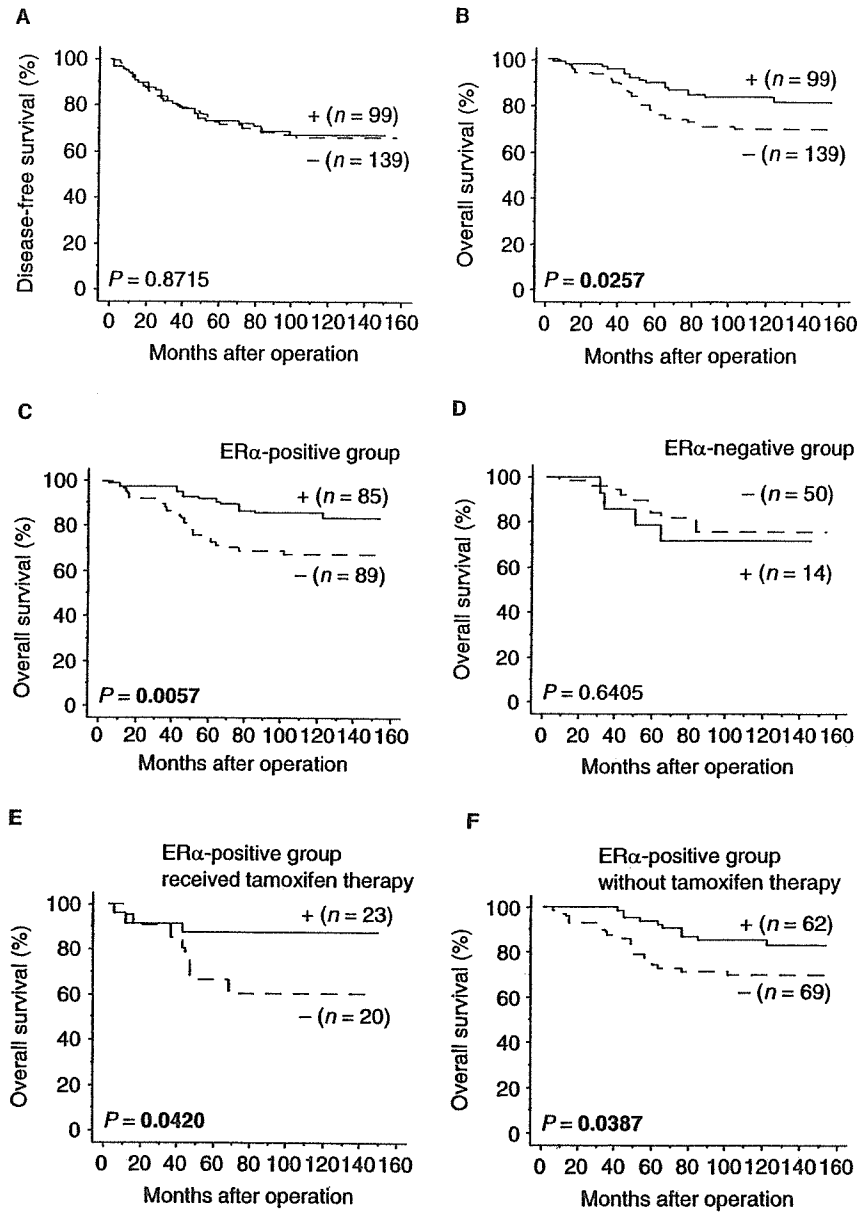

Figure 2 Disease-free (A) and overall (B–F) survival of 238 patients with breast carcinoma according to PPARγ immunoreactivity (Kaplan–Meier method). PPARγ immunoreactivity was not correlated with a risk of recurrence (P=0.8715 in log-rank test) (A), but significantly associated with an improved overall survival (P=0.0257 in log-rank test) (B). The significant association was detected in the ERα-positive group (n=174; P=0.0057 in log-rank test) (C), but not in the ERα-negative cases (n=64; P=0.6405 in log-rank test) (D). PPARγ immunoreactivity was significantly associated with an improved prognosis regardless of tamoxifen therapy after surgery in the ERα-positive breast cancer patients (E, F). P values less than 0.05 are in boldface.

immunoreactivity ($P = 0.0076$) and c-Myc immunoreactivity ($P = 0.0252$) were demonstrated as significant prognostic factors for overall survival (Table 6). A multivariate analysis revealed that lymph node status ($P < 0.0001$) and PPARγ immunoreactivity ($P = 0.0372$) were independent prognostic factors with relative risks over 1.0.

Effects of PPARγ activator 15d-PGJ$_2$ on estrogen-mediated transcription in MCF-7 cells in luciferase assay

Results of PPARγ immunoreactivity demonstrated a strong association between PPARγ and ERα in breast carcinoma tissues, suggesting a possible

Table 5 Univariate and multivariate analyses of overall survival in 238 breast cancer patients examined

		Multivariate	
Variable	**Univariate *P***	*P*	Relative risk (95% CI)
Lymph node status (positive/negative)	**<0.0001**	**<0.0001**	12.319 (3.233–51.07)
Histological grade (3/1, 2)	**<0.0001**	0.1248	—
Tumor size (>20 mm/<20 mm)	**0.0002**	0.1248	—
HER2 status (positive/negative)	**0.0029**	0.4669	—
c-Myc immunoreactivity (positive/negative)	**0.0066**	**0.0024**	2.168 (1.079–4.356)
PPARγ immunoreactivity (negative/positive)	**0.0287**	0.2558	—
p27 immunoreactivity (negative/positive)	0.0797	—	—
Adjuvant chemotherapy (no/yes)	0.1458	—	—
Ki-67 LI (>10/<10)	0.2818	—	—
Tamoxifen therapy (no/yes)	0.5807	—	—
p21 immunoreactivity (negative/positive)	0.6751	—	—
ERα status (negative/positive)	0.9532	—	—

Data considered significant ($P<0.05$) in the univariate analyses are in boldface, and were examined in the multivariate analyses.

Table 6 Univariate and multivariate analyses of overall survival in 174 ER α-positive breast cancer patients examined

		Multivariate	
Variable	**Univariate *P***	*P*	Relative risk (95% CI)
Lymph node status (positive/negative)	**<0.0001**	**<0.0001**	19.006 (4.402–68.06)
Histological grade (3/1, 2)	**<0.0001**	0.0512	—
Tumor size (>20 mm/<20 mm)	**<0.0001**	0.0724	—
HER2 status (positive/negative)	**0.0008**	0.3899	—
PPARγ immunoreactivity (negative/positive)	**0.0076**	**0.0372**	2.799 (1.128–8.264)
c-Myc immunoreactivity (positive/negative)	**0.0252**	0.2891	—
p27 immunoreactivity (negative/positive)	0.0532	—	—
Adjuvant chemotherapy (no/yes)	0.1057	—	—
Ki-67 LI (>10/<10)	0.1123	—	—
Tamoxifen therapy (no/yes)	0.1497	—	—
p21 immunoreactivity (negative/positive)	0.2231	—	—

Data considered significant ($P<0.05$) in the univariate analyses are in boldface, and were examined in the multivariate analyses.

interaction of these two nuclear receptors in human breast carcinoma cells. Previously, Keller *et al.* (1995) reported that PPARα/RXRβ heterodimer can bind to ERE and possibly modulate the ER-signaling pathway, but this has not been examined in breast cancers.

In order to examine this hypothesis, we used MCF-7 breast cancer cells in the following *in vitro* experiments, because MCF-7 cells were associated with expression of ERα, PPARγ, and RXRα, β, γ (data not shown). When MCF-7 cells were transiently transfected with ptk-ERE-Luc plasmids and treated with 10 nM estradiol, the luciferase activity of the cells was 17-fold increased compared with their basal level (Fig. 3A). PPARγ activator 15d-PGJ_2 significantly inhibited ERE-dependent transactivation by estradiol in a dose-dependent manner, and the luciferase activity of MCF-7 cells treated with 10 nM estradiol and 5 μM 15d-PGJ_2 was decreased to 53% of that treated with 10 nM estradiol alone ($P<0.001$). 15d-PGJ_2 (5 μM) alone did not significantly change the luciferase activity compared with their basal level ($P=0.8837$). 15d-PGJ_2, however, did not significantly inhibit the ERE-dependent transactivation by estradiol, when these cells were treated with a potent PPARγ antagonist GW9662 (Leesnitzer *et al.* 2002).

The ERE-dependent transactivation by estradiol was also inhibited by other PPARγ agonists such as GW1929 and ciglitazone, in a dose-dependent manner ($P<0.001$, on addition of 5 μM GW1929 or ciglitazone), but was not altered by treatment with $PGF_{2\alpha}$, which does not activate PPARγ (Kliewer *et al.* 1995) (Fig. 3B). Results of immunoblotting analysis revealed that relative immunointensity of ERα was not significantly ($P=0.7749$) altered by the treatment with 15d-PGJ_2 in MCF-7 cells (Fig. 3C).

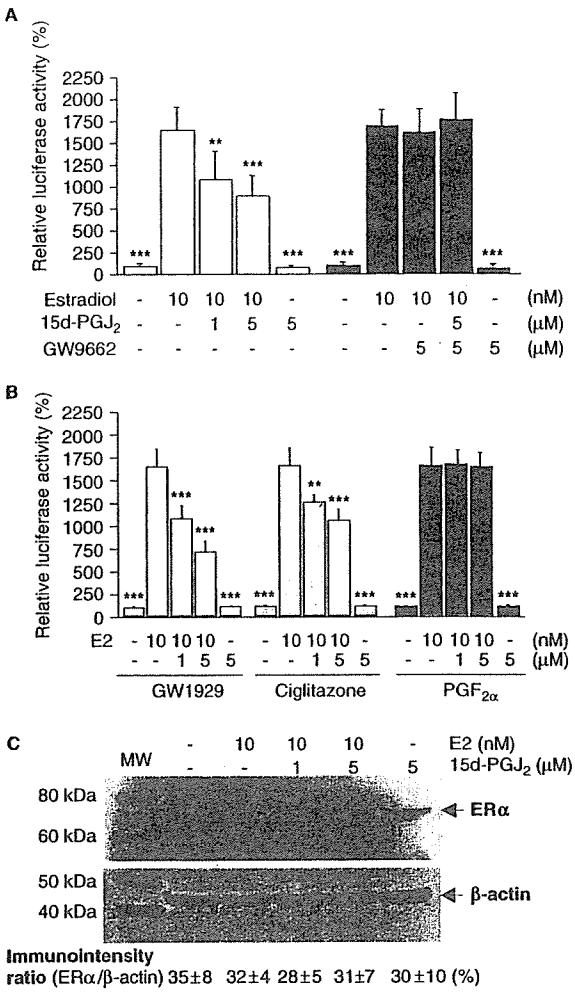

Figure 3 Effects of PPARγ activator 15d-PGJ_2 on ERE-dependent transactivation in MCF-7 cells. (A) MCF-7 cells were transiently transfected with pERE-Luc plasmids, and treated with the indicated concentrations of estradiol, 15d-PGJ_2 and GW9662 (PPARγ antagonist) for 24 h. The luciferase activity was evaluated as a ratio (%) compared with that of controls (treatment without estradiol, 15d-PGJ_2 or GW9662 for 24 h (left column)). Data are presented as means±S.D. (n=3). $^{**}P<0.01$ and $^{***}P<0.001$ vs 10 nM estradiol alone. (B) MCF-7 cells transfected with pERE-Luc plasmids were treated with the indicated concentrations of estradiol and GW1929 (PPARγ agonist), ciglitazone (PPARγ agonist), or $PGF_{2\alpha}$ (no activator of PPARγ) for 24 h. Data are presented as means±S.D. (n=3). $^{**}P<0.01$ and $^{***}P<0.001$ vs 10 nM estradiol alone respectively. (C) Immunoblotting for ERα in MCF-7 cells. ERα and β-actin immunoreactivities were detected as specific bands (approximately 66 and 42 kDa respectively); 20 μg of protein were loaded in each lane. Data of immunointensity ratio (ERα/β-actin) are presented as means±S.D. (n=3). No significant association was detected.

Table 7 The number and percentage of genes classified into four groups according to the effects of 15d-PGJ_2 on the estrogen-mediated mRNA expression in the microarray analysis

	Regulation of mRNA expression by estradiol with 15d-PGJ_2		
	Not significant	Significant	**Total**
Estrogen up-regulated genes	Group A n=9 (18%)	Group B n=23 (47%)	— n=32 (65%)
Estrogen down-regulated genes	Group C n=7 (14%)	Group D n=10 (20%)	— n=17 (35%)
Total	n=16 (33%)	n=33 (67%)	n=49 (100%)

A custom-made microarray (EstrArray; InfoGenes, Co. Ltd, Tsukuba, Japan), which contains 175 estrogen-responsive genes (Inoue *et al.* 2002, Hayashi *et al.* 2003), was used in this study.
MCF-7 cells were treated with estradiol (10 nM) with or without 15d-PGJ_2 (5 μM) for 72 h. Genes showing the value of more than 2.0- or less than 0.5-fold compared with the control (treated without estradiol or 15d-PGJ_2 for 72 h) were regarded as significantly regulated.

Effects of 15d-PGJ_2 on estrogen-mediated transcription in MCF-7 cells in microarray analysis

We further examined the effects of 15d-PGJ_2 on estrogen-mediated transcription in MCF-7 cells using a custom-made microarray. Significant alterations of mRNA expression by estradiol treatment (10 nM for 72 h) were detected in 49 genes in this study, and these genes were classified into the following four groups (Table 7): Group A; mRNA expression was significantly up-regulated by estradiol, but the significance vanished on addition of 15d-PGJ_2 (5 μM) (nine genes; Table 8), Group B; mRNA expression was significantly up-regulated by estradiol with or without 15d-PGJ_2 (23 genes; Table 9), Group C; mRNA expression was significantly down-regulated by estradiol, but the significance vanished on addition of 15d-PGJ_2 (seven genes; Table 10), and Group D; mRNA expression was significantly down-regulated by estradiol with or without 15d-PGJ_2 (ten genes; Table 11).

Effects of 15d-PGJ_2 on estrogen-mediated transcription in MCF-7 cells using real-time PCR analysis

In order to confirm the results from microarray analysis, we performed real-time PCR analyses for the representative 11 genes in MCF-7 cells (Fig. 4A–K).

Table 8 Summary of genes classified into Group A in the microarray analysis examined

Accession no.	Gene symbol	Estradiol	Estradiol +15d-PGJ_2
NM003225	**TFF1 (pS2)**	7.5	1.7
AA143530	SCD	3.4	1.5
AF012281	**PDZK1**	3.1	1.6
AI660571	ASS	2.3	1.2
AB012664	STC2	2.3	1.1
X16396	MTHFD2	2.2	1.6
NM053056	**CCND1 (cyclin D1)**	2.2	0.8
M62403	**IGFBP4**	2.1	0.9
X63741	EGR3	2.0	1.1

Values are presented as fold change compared with controls. Boldface: mRNA expressions were examined by real-time PCR analysis (Fig. 4).

Table 9 Summary of genes in Group B

Accession no.	Gene symbol	Estradiol	Estradiol +15d-PGJ_2
M80244	**SLC7A5**	4.4	3.9
AI151190	S100P	4.0	3.0
U44427	**TPD52L1**	4.0	2.4
XM006190	**PGR (PR)**	3.8	2.0
XM041014	—	3.5	2.2
X74837	HUMM9	2.9	3.7
L02785	SLC26A3	2.8	2.6
N39944	ATF3	2.8	4.1
AB028974	PEG10	2.6	2.2
AA587912	PSAT1	2.5	2.6
BT006910	**CTPD (cathepsin D)**	2.4	2.9
AA216685	GDF15	2.4	2.8
NM003458	BSN	2.3	2.4
D90070	PMAIP1	2.3	2.5
U95626	CCRL2	2.3	3.6
AF039022	XPOT	2.3	2.9
X89773	ISG20	2.2	2.3
X72875	BF	2.2	3.0
D30658	GARS	2.2	2.1
M90516	GFPT1	2.1	2.3
U72066	RBBP8	2.1	2.1
AJ011972	HDAC6	2.0	2.0
X16706	FOSL2	2.0	2.8

Values are presented as fold change compared with controls. Boldface: mRNA expressions were examined by real-time PCR analysis (Fig. 4).

mRNA expression of pS2 (Fig. 4A), PDZK1 (Fig. 4B) cyclin D1 (Fig. 4C) and IGFBP-4 (Fig. 4D), which were tentatively classified into Group A in microarray analysis as above, was significantly ($P<0.001$) increased by estradiol treatment (10 nM, for 72 h) compared with the control (neither estradiol nor 15d-PGJ_2),

Table 10 Summary of genes in Group C

Accession no.	Gene symbol	Estradiol	Estradiol +15d-PGJ_2
AI337192	SH3BGR	0.073	1.1
M90657	**TM4SF1 (TAL6)**	0.23	0.71
JQ1035	—	0.35	0.66
XM006424	—	0.36	1.1
M59828	HSPA1A	0.40	1.8
AA481712	CDKN1A	0.42	1.4
L25081	RHOC	0.43	0.57

Values are presented as fold change compared with controls. Boldface: mRNA expressions were examined by real-time PCR analysis (Fig. 4).

Table 11 Summary of genes in Group D

Accession no.	Gene symbol	Estradiol	Estradiol +15d-PGJ_2
D11428	PMP22	0.074	0.075
NM000599	**IGFBP5**	0.23	0.27
D87993	PCSK6	0.26	0.23
U69263	MATN2	0.30	0.20
M19922	FBP1	0.32	0.17
U29091	**SELENBP1**	0.33	0.34
U30246	LC12A2	0.34	0.24
NM033001	GTF2I	0.42	0.27
U97276	QSCN6	0.43	0.42
AA482422	ENO1	0.43	0.62

Values are presented as fold change compared with controls. Boldface: mRNA expressions were examined by real-time PCR analysis (Fig. 4).

but not by treatment with estradiol (10 nM) with 15d-PGJ_2 (5 μM). mRNA expression of SLC7A5 (Fig. 4E), TPD52L1 (Fig. 4F), PR (Fig. 4G), and cathepsin D (Fig. 4H) in Group B was, however, significantly up-regulated by the treatment with estradiol with or without 15d-PGJ_2 (1 or 5 μM). Estradiol-mediated mRNA expression of PR was also demonstrated to be inhibited by addition of 15d-PGJ_2 in a dose-dependent manner ($P<0.01$, between estradiol alone and estradiol with 15d-PGJ_2 (5 μM)). The mRNA level of TAL6 in Group C (Fig. 4I) was significantly lower ($P<0.001$) in estradiol alone than that in the control group, but was not significantly different under treatment with estradiol with 15d-PGJ_2 (5 μM). mRNA expression of IGFBP-5 (Fig. 4J) and SELENBP1 (Fig. 4K) in Group D was significantly down-regulated by the treatment with estradiol (10 nM) with or without 15d-PGJ_2 (1 or 5 μM). mRNA expression in these 11 genes was not significantly altered by treatment with 15d-PGJ_2 (5 μM) alone in this study.

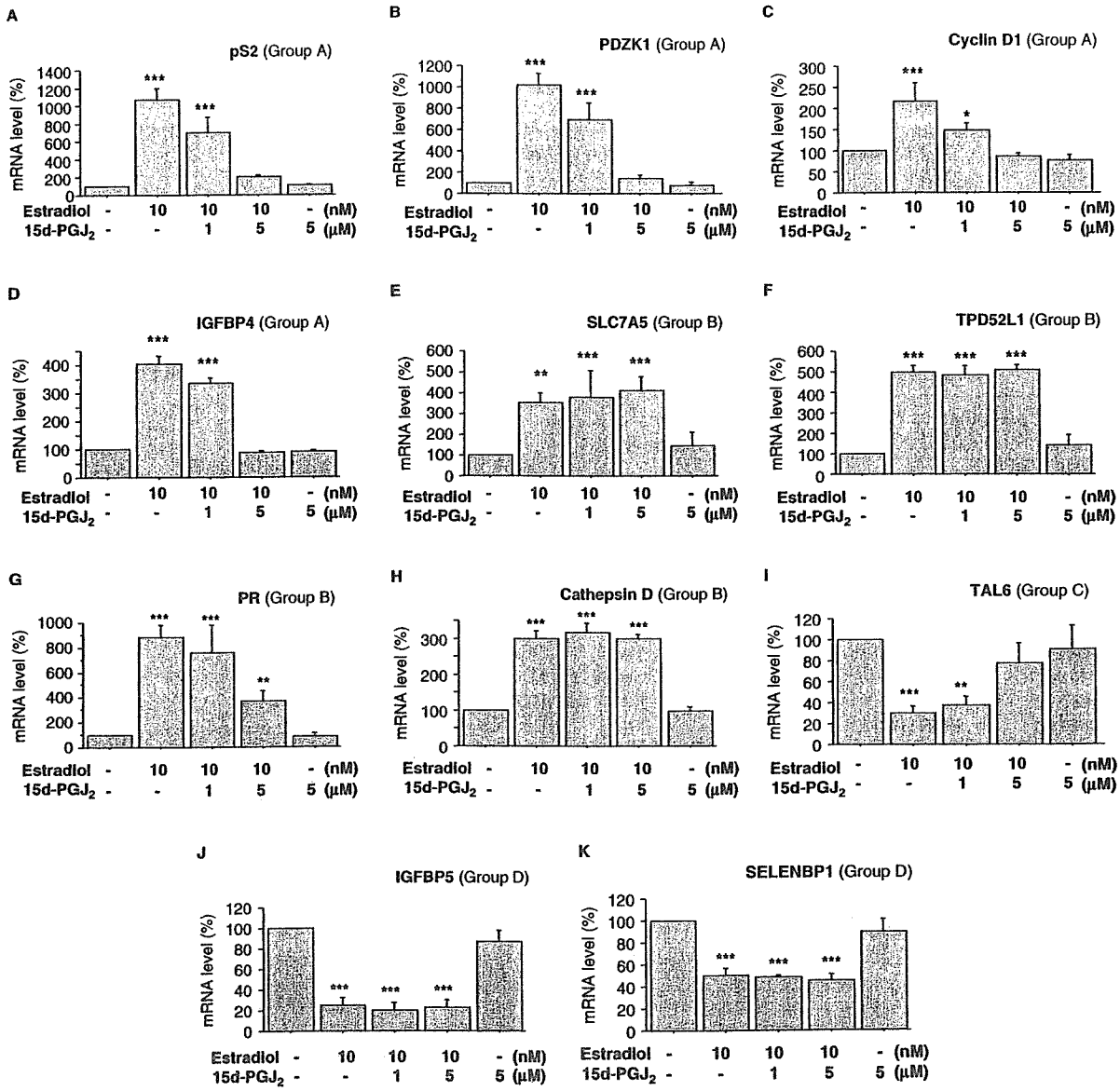

Figure 4 Effects of estrogen and 15d-PGJ_2 on mRNA expression of estrogen-responsive genes in MCF-7 cells by real-time PCR. (A) pS2, (B) PDZK1, (C) cyclin D1, (D) IGFBP-4, (E) SLC7A5, (F) TPD52L1, (G) PR, (H) cathepsin D, (I) TAL6, (J) IGFBP-5 and (K) SELENBP1. MCF-7 cells were treated with the indicated concentrations of estradiol and/or 15d-PGJ_2 for 72 h, and mRNA expression was evaluated by real-time PCR. The mRNA level was summarized as a ratio of RPL13A, and subsequently evaluated as a ratio (%) compared with that of controls (treatment without estradiol or 15d-PGJ_2 for 72 h (left column)). Data are presented as means ± s.d. (n=3). *P<0.05, **P<0.01 and ***P<0.001 vs controls

Effects of 15d-PGJ_2 on estrogen-mediated proliferation of MCF-7 cells

The number of MCF-7 cells was significantly increased after the treatment with estradiol (10 nM) in a time-dependent manner, and was 1.4-fold higher than the basal level (control: no treatment with estradiol or 15d-PGJ_2) at 5 days after the treatment (Fig. 5A). The estrogen-mediated proliferation of MCF-7 cells was significantly inhibited by addition of 5 μM 15d-PGJ_2 (P<0.05 and P<0.001 for 3 and 5 days respectively). The apoptosis index of MCF-7 cells was not significantly altered under the same treatments for 3 days (Fig. 5B). The treatment with 5 μM 15d-PGJ_2

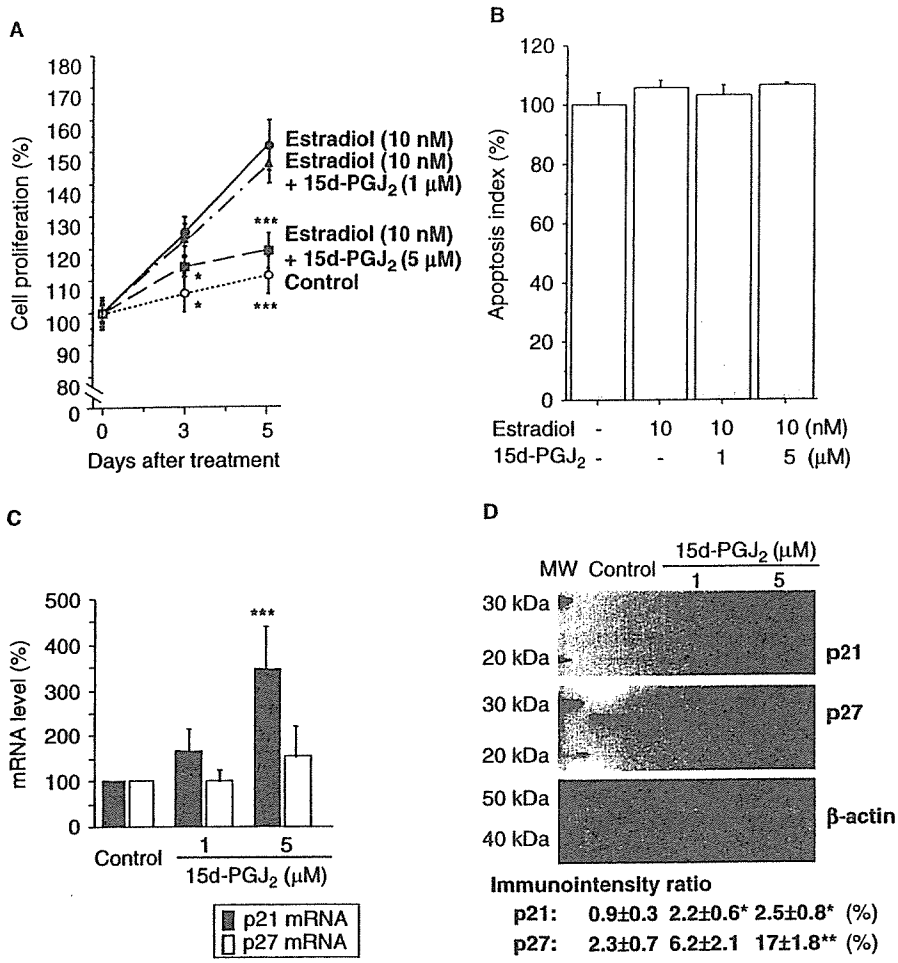

Figure 5 Effects of 15d-PGJ_2 on estrogen–mediated proliferation in MCF-7 cells. (A) MCF-7 cells were treated with the indicated concentrations of estradiol and 15d-PGJ_2 for 0, 3 or 5 days, and the status of cell proliferation was measured using a WST-8 method. The cell number was evaluated as a ratio (%) compared with that at 0 day after the treatment. Control; no treatment with estradiol or 15d-PGJ_2. Data are presented as means±S.D. (n=3). *P<0.05 and ***P<0.001 vs 10 nM estradiol alone respectively. (B) MCF-7 cells were treated with the indicated concentrations of estradiol and 15d-PGJ_2 for 3 days, and apoptosis was evaluated by an apoptosis screening kit. The apoptosis index was evaluated as a ratio (%) compared with that of controls (no treatment with estradiol or 15d-PGJ_2 for 3 days (left column)). Data are presented as means±S.D. (n=3). No significant association was detected. (C) Real-time PCR for p21 and p27 in MCF-7 cells. MCF-7 cells were treated with the indicated concentrations of 15d-PGJ_2 for 72 h. The mRNA level was summarized as a ratio of RPL13A, and subsequently evaluated as a ratio (%) compared with that of controls (no treatment with 15d-PGJ_2 for 72 h (left column)). Data are presented as means±S.D. (n=3). ***P<0.001 vs controls). (D) Immunoblotting for p21 and p27 in MCF-7 cells. MCF-7 cells were treated with the indicated concentrations of 15d-PGJ_2 for 72 h; 20 μg of protein were loaded in each lane. Data of immunointensity ratio (p21 or p27/β-actin) are presented as means±S.D. (n=3). *P<0.05 and **P<0.01 vs controls (no treatment with 15d-PGJ_2 for 72 h).

alone did not significantly influence the proliferation or apoptosis of MCF-7 cells compared with the basal level (data not shown).

We also examined effects of 15d-PGJ_2 on the expression of p21 and p27 in MCF-7 cells. Results of real-time PCR analyses demonstrated significant (P<0.001) stimulation of p21 mRNA by 15d-PGJ_2 (5 μM for 3 days) (Fig. 5C). In immunoblotting analyses, relative immunointensities of p21 and p27 were significantly (P<0.05) increased by the treatment with 15d-PGJ_2 in a dose-dependent manner (Fig. 5D).

Discussion

In our present study, PPARγ immunoreactivity was detected in carcinoma cells in 99 out of 238 human

breast carcinomas (42%), and was significantly associated with the histological grade or ERα status of the cases. Expression of PPARγ has been previously reported in breast cancer cases by several groups (Mueller *et al.* 1998, Jiang *et al.* 2003, Watkins *et al.* 2004). Mueller *et al.* (1998) reported that ligand-activated PPARγ in cultured breast cancer cells resulted in extensive lipid accumulation, and transformed the breast epithelial gene expression to a more differentiated and less-malignant state. In addition, both Jiang *et al.* (2003) and Watkins *et al.* (2004) reported that PPARγ expression was significantly lower in breast cancer tissues than in normal tissues, suggesting that PPARγ has a possible protective role against development of breast cancers (Jiang *et al.* 2003, Koeffler 2003). Results of our present study are generally consistent with these previously reported findings, and PPARγ may be mainly expressed in well-differentiated breast carcinomas with hormonal regulatory mechanisms maintained.

It then becomes important to know whether PPARγ is colocalized with RXRs and its natural ligands or not in breast cancers, because these factors play essential roles in activation of PPARγ function. PPARγ immunoreactivity was significantly associated with all the subtypes of RXR. RXRα and RXRβ are known to be major subtypes of RXR in breast cancer tissues (Suzuki *et al.* 2001), and PPARγ/RXRα heterodimer was reported to be biologically active in human breast cancer cells (Crowe & Chandraratna 2004). Therefore, PPARγ is mainly expressed in RXR-overexpressing breast cancer tissues, and possibly heterodimerizes with RXRα and/or RXRβ in breast cancer cells. Previously, Badawi & Badr (2003) reported that concentration of 15d-PGJ_2, which is considered a natural ligand of PPARγ, was inversely correlated with mRNA expression of COX2 or concentration of PGE_2, and was marginally associated ($P = 0.081$) with PPARγ mRNA levels in breast cancer tissues. In addition, estrogen is known to influence PG synthesis in estrogen target tissues (Ham *et al.* 1975). Ma *et al.* (1998*a*) reported that estrogen induced enzymatic conversion of PGD_2 and the metabolites of PGD_2 potently activated PPARγ, although estrogen did not directly induce the mRNA expression of PPARγ (Ma *et al.* 1998*b*). In our present study, PPARγ immunoreactivity was inversely associated with COX2 immunoreactivity and positively associated with ERα, although we could not examine the tissue concentrations of natural PPARγ ligands in breast cancer tissues. These data suggest that PPARγ is biologically activated in human breast cancer tissues.

Estrogens are well-known to contribute immensely to the development of hormone-dependent breast carcinomas, and biological estrogenic actions are mainly mediated by ERα (Korach 1994). Estrogens stimulate the transactivation of activation function 2 domain of ERα in a ligand-dependent manner (Kumar *et al.* 1987), and subsequently ERs activate transcription of various target genes by direct DNA interaction through EREs or by tethering to other transcription factors (Tsai & O'Malley 1994, Acconcia & Marino 2003). Previously, Keller *et al.* (1995) demonstrated that PPARα/RXRβ heterodimer could bind to ERE using the artificial promoter context. In this report, PPARα/RXRβ strongly bound with EREs of pS2 and vitellogenin A2 (vitA2) genes, but did not induce these ERE-dependent transactivations (Keller *et al.* 1995). The binding affinity between PPARα/RXRβ and ERE of very-low-density apolipoprotein II gene was, however, very low, despite containing the same ERE consensus sequence as vitA2. In our study, ligand-mediated PPARγ activation significantly inhibited estrogen-mediated ERE transactivation in MCF-7 cells. These data are in good agreement with the report by Keller *et al.* (1995), and suggest that PPARγ suppresses the estrogen-signaling pathway through inhibition of the binding of ERs with the target genes in breast cancer cells. In our microarray analysis, inhibition of estrogen-mediated mRNA expression by PPARγ was detected in 33% of estrogen-responsive genes, including ERE-containing genes such as pS2 (Stack *et al.* 1988) and early growth response 3 (Bourdeau *et al.* 2004). However, 15d-PGJ_2 did not significantly regulate the estrogen-mediated transactivation of a proportion of ERE-containing genes, such as SLC7A5 (Bourdeau *et al.* 2004), cathepsin D (Wang *et al.* 1997), retinoblastoma-binding protein 8 (Bourdeau *et al.* 2004), and Fos-like antigen 2 (Bourdeau *et al.* 2004). On the other hand, 15d-PGJ_2 inhibited the estrogen-mediated expression of cyclin D1 and IGFBP-4 (Group A), in which functional ERE has not been identified and indirect gene regulation by ER is suggested (Qin *et al.* 1999, Acconcia & Marino 2003, O'Lone *et al.* 2004). Therefore, inhibition of PPARγ in estrogen-mediated transactivation is considered to vary among the target genes, and may influence not only ERE-containing genes but also some genes which are induced by an interaction between ER and other DNA-binding transcription factors.

In our immunohistochemical analysis (Table 4), significant associations were detected between ERα and estrogen-responsive genes, such as pS2, cyclin D1, PR and cathepsin D, as reported previously

(Barbareschi *et al.* 1997, Gillesby & Zacharewski 1999, Ioachim *et al.* 2003). However, the significant association between ERα and pS2 or cyclin D1 was not detected in the group of PPARγ-positive breast cancers, while correlation between ERα and PR or cathepsin D was not influenced by PPARγ status in those breast cancer patients examined. These data are in good agreement with our results of microarray and real-time PCR analyses. Recently, Qin *et al.* (2003) reported that PPARγ agonists induced proteasome-dependent degradation of cyclin D1, which may be partly involved in the present immunohistochemical results of cyclin D1.

In this study, PPARγ immunoreactivity was correlated with immunoreactivity of p21 and p27 in breast carcinoma tissues, and expression of p21 and p27 was significantly induced by 15d-PGJ_2 at mRNA and/or protein levels in MCF-7 cells. Previous studies demonstrated that PPARγ ligands induced cyclin-dependent kinase inhibitors such as p21 and p27 in various types of cancer cells (Chung *et al.* 2002, Han *et al.* 2004, Motomura *et al.* 2004), and Lapillonne *et al.* (2003) reported the induction of p21 by a novel synthetic ligand for PPARγ (2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid) in breast carcinoma cells. A potential conserved consensus PPRE was detected in the promoter region of p21 gene (Lapillonne *et al.* 2003, Qin *et al.* 2003), and Motomura *et al.* (2004) have reported that accumulation of p27 by ligand-activated PPARγ was caused by induction of ubiquitination of p27 and reduction of degradation activity of p27 by proteasomes in hepatocellular carcinoma cells. Results of our present study are consistent with these previous reports, and suggest that PPARγ regulates the expression of p21 and p27 in breast cancer tissues.

PPARγ immunoreactivity was demonstrated as an independent improved prognostic factor for overall survival in ERα-positive breast carcinoma patients in our study, although it may not be as robust as lymph node status, a well-established diagnostic modality (Dowlatshahi *et al.* 1997). In addition, 15d-PGJ_2 significantly inhibited the estrogen-mediated proliferation in MCF-7 cells. Recently, Jiang *et al.* (2003) reported that mRNA levels of PPARγ in patients with local recurrence or those who died of breast cancer were significantly lower than those who remained disease free, which is generally consistent with our immunohistochemical results. An antiproliferative effect of PPARγ is considered to be, at least in part, due to overexpression of p21 and/or p27 in carcinoma cells, but this mechanism still remains largely unknown. Immunoreactivities of p21 and p27 are not necessarily associated with improved clinical outcomes of breast cancer patients (Barbareschi *et al.* 2000, Pellikainen *et al.* 2003), which is consistent with the findings in our present study (Table 5). PPARγ modulates estrogenic actions in breast carcinoma cells, through the suppression of a part of estrogen-mediated transactivation as described above, which may be also involved in an improved prognosis in breast carcinoma patients positive for PPARγ and ERα. Further examinations are required to clarify detailed functions of PPARγ as a modulator of estrogenic actions in breast carcinoma tissues.

In summary, PPARγ immunoreactivity was detected in carcinoma cells in 42% of breast cancer tissues. PPARγ immunoreactivity was positively associated with ERs, PR, RXRs, p21, or p27, and negatively correlated with histological grade or COX2. Moreover, PPARγ immunoreactivity was a better independent prognostic factor in ERα-positive breast carcinoma patients. Ligand-mediated PPARγ activation caused the suppression of a portion of estrogen-mediated transactivation or inhibition of estrogen-mediated proliferation in MCF-7 cells. These findings suggest that PPARγ is mainly expressed in well-differentiated and ER-positive breast cancers, and in part, plays a role as a modulator of estrogenic actions.

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Cardiovascular, Pulmonary and Renal Pathology

MDM2: A Novel *Mineralocorticoid-Responsive* Gene Involved in Aldosterone-Induced Human Vascular Structural Remodeling

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Aldosterone has been demonstrated to play an important role in the pathogenesis of various cardiovascular diseases. Vascular structural remodeling, including vascular smooth muscle cell (VSMC) proliferation, has been also reported in small resistance arteries of patients with primary aldosteronism. Therefore, in this study, we examined whether genes involved in the regulation of the cell cycle were induced by aldosterone alone in cultured human VSMCs and in human small resistance arteries. Results of these studies eventually demonstrated that *MDM2*, one of the genes involved in anti-apoptosis and cell growth, was markedly increased in mineralocorticoid receptor (MR)-positive VSMCs by aldosterone in all microarray, reverse transcriptase-polymerase chain reaction, immunoblotting, and immunofluorescence analyses. In addition, an analysis using small interfering RNA demonstrated that this gene product was involved in cell proliferation of VSMCs induced by aldosterone. Eplerenone, a specific MR antagonist, inhibited this gene induction by aldosterone in VSMCs. MDM2 protein was also more abundant in VSMCs of small resistance arteries in patients with primary aldosteronism compared with a control population. *MDM2* is therefore considered one of the mineralocorticoid-responsive genes that regulates cell proliferation of VSMCs induced by MR-mediated aldosterone stimulation, possibly playing an important role in aldosterone-induced vascular structural remodeling. *(Am J Pathol 2006, 169:362–371; DOI: 10.2353/ajpath.2006.051351)*

Aldosterone is a steroid hormone synthesized in the zona glomerulosa of human adrenal cortex as a result of stimulation by angiotensin II and others.[1,2] Aldosterone has been demonstrated to bind to the mineralocorticoid receptor (MR) and to increase systemic blood pressure by regulating systemic electrolytes and volume balance in kidney, subsequently resulting in various human cardiovascular diseases.[1] However, aldosterone has also been demonstrated to directly exert its effects on cardiovascular systems via MR.[1,3] For instance, aldosterone has been reported to induce expression of some genes involved in vascular fibrosis, calcification, and inflammation, which are all considered important in pathology of vascular injuries.[1] Aldosterone also induce mitogenesis of vascular smooth muscle cells (VSMCs), resulting in vascular structural remodeling under the presence of angiotensin II.[4,5] However, aldosterone itself without the presence of angiotensin II is also considered to cause cardiovascular injuries.[6] Vascular structural remodeling in small resistance arteries has been reported in patients with primary aldosteronism, where serum aldosterone levels were elevated but serum angiotensin II level is markedly down-regulated.[7] In addition, aldosterone itself has been also demonstrated to stimulate proliferation of VSMCs.[8] Therefore, aldosterone may directly induce some MR-responsive gene associated with regulation of the cell cycle in VSMCs, although inflammatory reaction and fibrosis are also very important features for aldosterone-induced vascular injuries and alterations.[1] Jaffe and Mendelsohn recently reported that some MR-mediated genes were associated with vascular injuries in VSMCs

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