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Restoration of Leukotriene B_4-12-Hydroxydehydrogenase/15-oxo-prostaglandin 13-Reductase (LTBDH/PGR) Expression Inhibits Lung Cancer Growth *in Vitro* and *in Vivo*

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Abstract

Leukotriene B_4 12-hydroxydehydrogenase/15-oxo-prostaglandin 13-reductase (LTBDH/PGR) is a bifunctional enzyme capable of inactivating leukotriene B_4 (LTB_4) and 15-oxo-prostaglandins (15-PGs). Its role in growth suppressive functions in lung cancer was studied in *in vitro* and *in vivo* systems. The LTBDH/PGR gene was expressed in lung cancer cell lines through recombinant adenovirus infection, and through a tetracycline-inducible expression system. After restoration of LTBDH/PGR expression in LTBDH/PGR-negative (H1299) or -low (A549) lung cancer cell lines, the restored enzyme induced apoptosis and growth inhibition *in vitro*. Ectopic expression of LTBDH/PGR caused also suppression of tumorigenicity of A549 cells in nude mice. In contrast, LTBDH/PGR over-expression in LTBDH/PGR-positive (H157) lung cancer cell line induced little apoptosis and growth inhibition. This study indicates that restoration of LTBDH/PGR expression is effective in preventing lung cancer growth *in vitro* and *in vivo*.

Keywords

Leukotriene B_4; Prostaglandin E_2; Dehydrogenase; Reductase; Tumor Suppressor; Lung Cancer; Dithiolethiones

1. Introduction

Available evidences indicate that the aberrant expression of enzymes involved in arachidonic acid metabolism appears to contribute significantly to the development of lung cancer [1,2]. Prostaglandins (PGs) and leukotrienes (LTs) are metabolites of arachidonic acid through cyclooxygenase (COX) and 5-lipoxygenase (5-LO) pathways respectively. COX catalyzes dioxygenation and cyclization of arachidonic acid to form PGH_2 which is an immediate precursor of prostaglandins such as PGE_2 and TXA_2. Two forms of COX have been recognized [3]. COX-1 is consititutive, whereas COX-2 is inducible by mitogens, phorbol esters, pro-inflammatory cytokines and some carcinogenic substances. Prostaglandins are rapidly

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catabolized *in vivo* and become inactivated. The two key enzymes involved in prostaglandin and lipoxin catabolism are 15-hydroxyprostaglandin dehydrogenase (15-PGDH) which catalyzes NAD^+-mediated oxidation of 15(S)-hydroxyl group of prostaglandins and lipoxins to 15-keto-metabolites with greatly reduced biological activities and 15-oxo-prostaglandin-13-reductase (PGR) which catalyzes NAD(P)H-mediated reduction of Δ^{13}-double bond to generate fully inactive metabolites [4]. 5-LO catalyzes 5-peroxygenation of arachidonic acid and subsequent dehydration to generate leukotriene A_4 (LTA_4) which is rehydrated to pro-inflammatory leukotriene B_4 (LTB_4) catalyzed by LTA_4 hydrolase (LTA_4H) [5]. Likewise, LTB_4 is also rapidly oxidized at the 12(R)-hydroxyl group and inactivated by LTB_4-12-hydroxydehydrogenase (LTBDH) which was found to be structurally identical to 13-PGR following the cloning of both enzymes [6,7]. Therefore, LTBDH/PGR is capable of inactivating prostaglandins and LTB_4 from either pathway. Both COX-2-derived prostaglandins (e.g. PGE_2 and TXA_2) and 5-LO-derived leukotrienes (e.g. LTB_4) have been shown to stimulate the proliferation of many cancer cell lines [8,9].

The first attempt to relate the expression of arachidonate metabolic enzymes to cancer is the discovery of over-expression of COX-2 in colorectal tumors [10]. It is now well established that prostaglandin synthetic enzyme COX-2 is over-expressed in many types of tumors including lung tumors [11,12]. Recent studies in our laboratory as well as others have demonstrated that catabolic enzyme 15-PGDH is down-regulated in lung, colon, breast and bladder tumors and may function as a tumor suppressor [13-17]. Up-regulation of COX-2 and down-regulation of 15-PGDH may allow levels of proliferative prostaglandins to be greatly elevated in tumors. Whether this is also true for synthetic and catabolic enzymes in 5-LO pathway remains to be determined. Expression of both synthetic and catabolic enzymes in 5-LO pathway in human cancers has not been explored extensively. 5-LO has been shown to express markedly in lung cancer [18] as well as in breast cancer [19] and pancreatic cancer [20]. LTA_4H has been shown to over-express in human lung tumors [21] and in rat esophageal tumors [22]. Furthermore, LTB_4 levels were found to be 10- to 30-fold higher in oral squamous cells cancers compared to control normal tissues, suggesting a role for LTB_4 in the pathogenesis of head and neck carcinoma [23]. There is no report showing that the expression of LTBDH/PGR is altered in tumors. Whether LTBDH/PGR behaves like 15-PGDH and functions as a growth suppressor remains to be determined.

To investigate growth suppression in lung cancer, we studied *in vitro* and *in vivo* effects of LTBDH/PGR expression in LTBDH/PGR-negative and -positive lung cancer cells by infection with adenovirus carrying the LTBDH/PGR gene and by stable transfection with an inducible LTBDH/PGR expression vector. The results indicate that LTBDH/PGR over-expression induced apoptosis and growth inhibition *in vitro* and suppressed tumorigenicity of lung cancer cells in an athymic nude mice model.

2. Materials and methods

2.1 Chemicals, antibodies, and constructs

15-oxo-PGE_1 and 15-oxo-PGE_2 were from Cayman Chemical Co. Ann Arbor, MI. Lipofectamine™ 2000, pcDNA6/V5-His A expression vector, T-REx (tetracycline-regulated expression) System, and restriction enzymes were from Invitrogen, Carlsbad, CA. β-Nicotinamide adenine dinucleotide (NAD^+), reduced form (NADH), DL-dithiothreitol (DTT), leupeptin, soybean trypsin inhibitor, phenylmethylsulfonyl fluoride (PMSF), RPMI 1640, DMEM were from Sigma (St Louis, MO). Blasticitidin S. HCl was from A.G. Scientific, Inc. (San Diego, CA). Fast-Link™ DNA Ligation Kit was purchased from Epicentre Biotechnologies, Madison, WI. The QIAprep Spin Miniprep Kit, QIAquick PCR Purification Kit and QIAquick Gel Extraction Kit were purchased from Qiagen, Valencia, CA. Electro-chemiluminescence (ECL^+) plus Western Blotting Detection System RPN 2132 was obtained

from Amersham Biosciences (Pittsburgh, PA). Rabbit polyclonal anti-PGR antibody was prepared as described earlier [7]. Mouse monoclonal anti-PARP [poly(ADP-ribose) polymerase] antibody was supplied by BD Biosciences, San Jose, CA. Rabbit polyclonal anti-p53 and mouse monoclonal anti-caspase-3 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-BAX, anti-Bcl-2 and anti-CD44 antibodies were from NeoMarkers (Fremont, CA). HRP-Goat anti-rabbit IgG(H+L) and HRP-Goat anti-mouse IgG (H+L) were from Zymed (San Francisco, CA). The pcDNA6-PGR construct was established by cloning porcine PGR cDNA (7) into *Eco*R I and *Xba* I sites of the pcDNA6/V5-His A vector. The correct insertion was confirmed by DNA sequencing. Human PGR cDNA was obtained by PCR cloning from human lung adenocarcinoma A549 cells. The inducible expression vector construct was established by cloning human PGR cDNA into *Eco*R I and *Xba* I sites of pcDNA4/TO vector using T-Rex system.

2.2 Cell culture

H1299, A549 and H157 cell lines were obtained from the American Type Culture Collection (Manassas, VA). H1299, A549 and H157 cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum. AD-293 cells from Stratagene (La Jolla, CA) were cultured in DMEM medium with 10% fetal bovine serum.

2.3 Recombinant adenovirus and in vitro transduction

The wild-type porcine PGR gene was cloned into an adenoviral shuttle vector (pshuttle-IRES-hrGFP1) in the AdEasy™ XL Adenoviral Vector System. The preparation of recombinant adenovirus-wild-type PGR (Ad-PGR) and the control adenovirus (Ad-GFP) were performed according to the manufacturer's instructions (Stratagene, La Jolla, CA). Adenoviruses were subsequently amplified by sequential rounds of infection on AD-293 cells and purified by the CsCl gradient centrifugation. Adenoviruses were titrated by optical density measurement and Adeno-X™ Rapid Titer Kit (BD Biosciences, Palo Alto CA). H1299, A549 and H157 cells were plated in a 6-well plate to about 50% confluent the next day of infection. Cells were infected with Ad-PGR or Ad-GFP at appropriate multiplicities of infection (MOI). Transduction efficiency was assessed by visualization of green fluorescent protein-expressing cells using fluorescent microscope to assure that equal amounts of recombinant and control adenovirus were used in all experiments.

2.4 PGR activity assay

The PGR activity was determined according to the chromophore method described earlier with minor modification [7]. Briefly, the cells were collected and lysed after transfection and transduction, and half of the cells were used for activity assay. The reaction mixture contained: 15-oxo-PGE_1 or 15-oxo-PGE_2 (10 μg/ml), 1 mM NADH and cell lysate in 1 ml of 50 mM Tris-HCl, pH 7.5. The reaction was incubated at 37°C for 30 min before termination by the addition of 200 μl of 2 N NaOH. The amount of 15-oxo-prostaglandins remaining was determined by reading the maximal absorption at 500 nm. This was reached about 1~2 min after the addition of NaOH. One unit of enzyme is defined as the amount of enzyme catalyzing the oxidation of 1μmol of substrate per min under the conditions of the assay.

2.5 Western blot analysis

Cells were harvested and lysed in a buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM DTT, 1% NP-40, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 1 mM PMSF for 20min on ice. Approximately 50~150 μg of protein extracts were loading on a 10% or 12% polyacrylamide gel, separated electrophoreticallly, and blotted from the gel to PVDF membrane (Millipore, Bedford, MA). The membranes were then immunoblotted with

the indicated antibodies followed by incubation with the second antibody conjugated with the horseradish peroxidase and detection of the enzyme with a Western Blotting Detection System.

2.6 Cell growth and cell cycle assay

For cell growth analysis, 2×10^5cells per well in 6-well plates in triplicate were infected with Ad-GFP or Ad-PGR at MOI of 100 and counted at the day 1, 2, 3, 4 and 5 under a light microscope. Alternatively, cell proliferation was determined by the MTT assay in which the reduction of MTT by viable cells to soluble formazan was quantified by measuring OD at 550 nm. For flow cytometry, 3×10^5 cells per 60-mm plate were infected with Ad-GFP or Ad-PGR at MOI of 100, harvested 72 h after infection, fixed in cold 70% ethanol, RNaseA-treated, and stained with propidium iodide (50 μg/ml). Cells were analyzed for DNA content by FACS Calibur cytometer (BD Biosciences, San Jose, CA). The data were analyzed using the software ModFitLT V2.0.

2.7 Cell transfection and colony formation assay

H1299, A549 and H157 cells in 12-well plates were transfected with pcDNA6 or pcDNA6-PGR using Lipofectamine™ 2000 Reagent. Forty-eight hours after transfection, the cells were split to 60-mm plates at 1:10~50 ratio and cultured in blasticitidin (5 μg/ml) medium for 2~3 weeks. Cells were then fixed with methanol and stained with MAY-GRUNWALD Stain (Sigma, St Louis, MO), and visible colonies ≥0.5mm were counted.

2.8 Inducible PGR Transfectants

The inducible PGR transfectants in H1299 cells were established with T-Rex (tetracycline-regulated mammalian expression) system according to the manufacturer's directions. In brief, H1299 cells were initially transfected with Tet repressor expression vector pcDNA6/TR by Lipofectamine™ 2000. Two days after transfection, transfectants were selected in the presence of 5 μg/ml blasticidin and tested for tetracycline-inducible expression by transient transfection with 15-PGDH reporter plasmid. Endogeneous expression of 15-PGDH was not detectable in H1299 cells. One stable clone showing highest levels and activity of 15-PGDH expression after addition of tetracycline was selected and designated H1299/pcDNA6/TR-1. This clone was subsequently transfected with pcDNA4/TO vector encoding LTBDH/PGR and cultured in zeocin (150 μg/ml). Tetracycline (2.5 μg/ml) was used for the induction of PGR. Positive clones were isolated and maintained in the presence of both blasticidin (5 μg/ml) and zeocin (150 μg/ml). The induction levels of LTBDH/PGR were examined in the absence and presence of tetracycline. The inducible transfectants designated H1299/I clone 3 was subjected to further analyses because the target proteins were induced most efficiently by Tet treatment in this clone.

2.9 Animal study

All animals were maintained and animal experiments were performed under NIH and institutional guidelines established for the Animal Core Facility at the University of Kentucky Medical Center. A549 cells were infected with Ad-GFP or Ad-PGR at MOI of 100 viral particles per cell. After infection for 36 h, cells were harvested, washed twice with sterile PBS, counted and re-suspended in PBS. Six-week-old female athymic nude mice were injected subcutaneously in both flanks with infected A549 cells at a density of 5×10^6 viable cells/100 μl. Five individual mice were used in each group. Tumor size was measured with a linear caliper twice per week for up to 5 weeks, and the volume was estimated by using the equation $V=(a \times b^2)/2$, where a is the larger dimension and b the perpendicular diameter. At end point (day 32), animals were sacrificed, tumors were weighed.

2.10 Statistical analysis

Each experiment was preformed at least three times. The data were expressed as the mean ± SD or SE. Statistical significance was assessed by Student's *t* test. $P<0.05$ indicated significant difference.

3. Results

3.1 LTBDH/PGR expression in lung cancer cells

Western blot analysis showed that H1299 cells exhibited little endogenous expression of LTBDH/PGR and A549 cells showed only 15% of that in H157 cells (Fig.1).

3.2 LTBDH/PGR over-expression induces apoptosis

A549, H1299 and H157 lung cancer cell lines were infected with Ad-GFP or Ad-PGR at a MOI of 100. Western blot analysis 72 h after infection showed that the enzyme expressed in all Ad-PGR-transduced cells (Fig. 2A). The PGR protein band in the non-infected and Ad-GFP samples of A549 and H157 cells was not as visible as that in the infected samples due to a much shorter time of exposure of X-ray film to the membrane as compared to that in Fig. 1 since PGR was over-expressed in these Ad-PGR samples. At 72 h after infection, full-length PARP and pro-caspase 3 levels were reduced in Ad-PGR-infected A549 cells compared with Ad-GFP control cells. In H1299 cells, a decrease of full-length PARP was also observed. However, cleavage of pro-caspase 3 was not found in infected H1299 cells (Fig. 2A).

Cell cycle alterations induced by LTBDH/PGR over-expression were assessed with Ad-GFP or Ad-PGR after infection at 100 MOI for 72 h. Cells were collected for flow cytometry analysis to assess apoptosis population. An obvious apoptosis peak was observed after A549 and H1299 cells infected with Ad-PGR (Fig. 2B). After infection with Ad-PGR, the apoptosis population in A549 cells and H1299 cells was 8.59% and 9.57% respectively, whereas apoptosis population was not detected significantly in H157 infected cells.

3.3 Apoptotic pathways in LTBDH/PGR-over-expressed cells

To study LTBDH/PGR-induced apoptotic pathways, expression of downstream apoptotic markers was assessed *in vitro*. LTBDH/PGR over-expression after 72 h infection resulted in significant up-regulation of pro-apoptotic protein BAX, p53 and p21 in A549 cells (Fig. 3). Slight up-regulation of these proteins induced by Ad-GFP control could be due to minor virus-induced apoptosis. Interestingly, it appears that the expression of Bcl-2 and CD44 in A549 cells was not affected by the LTBDH/PGR over-expression. However, the expression of apoptosis-related proteins BAX, Bcl-2 and CD44 in H1299 and H157 cells was not changed by LTBDH/PGR over-expression except for BAX which was decreased in H157 cells. Endogenous p53 and p21 were not expressed in both H1299 and H157 cells.

3.4 LTBDH/PGR over-expression suppresses cell growth

A549, H1299 and H157 lung cancer cell lines were infected with Ad-GFP or Ad-PGR at a MOI of 100. Cell growth curve was carried out after infection. The adenoviral transgene was expressed in nearly 100% of cells of each cell line, as assessed by confocal microscopy of GFP fluorescence (data not shown). Infection with Ad-PGR but not with Ad-GFP suppressed cell growth of A549 and H1299 cells but had no apparent effect on cell growth of H157 cells (Fig. 4A).

A549, H1299 and H157 cell lines were also each transfected with pcDNA6 or pcDNA6-PGR expression plasmids. Colony formation assay revealed 74% reduction in the number and size

of surviving colonies following PGR expression in A549 cells, 62% reduction in H1299 cells, and no significant reduction in H157 cells (Fig. 4B).

3.5 Effects of induced expression of LTBDH/PGR in H1299 cells

H1299/I clones 3 and 8 expressed the LTBDH/PGR transgene only on induction with tetracycline (Fig. 5A) and clone 3 was used in subsequent experiments. LTBDH/PGR expression increased in a dose (Fig. 5B) and time (Fig. 5C) dependent manner after tetracycline treatment. Clone 3 H1299/I^- (uninduced) cells were plated and LTBDH/PGR expression was induced 24 h later (day 1) by tetracycline (2.5 μg/ml). Marked expression was observed at day 4 and significantly affected cell proliferation beginning day 5 causing reduction in cell number and suggesting that LTBDH/PGR expression inhibited growth of H1299 cells (Fig. 5D).

3.6 Tumorigenicity of Ad-PGR-infected A549 lung cancer cells

Athymic nude mice (5 mice in each group) were inoculated with 5×10^6 A549 cells infected with Ad-GFP or Ad-PGR *in vitro* and cultured for 36 h. At 15 days after injection, tumor growth appeared to be evident in mice inoculated with Ad-GFP but not with Ad-PGR. At 32 days after injection all mice inoculated with Ad-GFP exhibited significant tumor growth, while only 60% of the mice inoculated with Ad-PGR showed some tumor growth (Fig. 6 A and B). At day 32, two of the five mice inoculated with Ad-PGR-infected A549 cells showed no tumors, and the rest three mice had tumors with an average tumor weight of 0.30±0.26 g, which was significantly lower than that of tumors derived from Ad-GFP-infected A549 cells (1.06±0.15 g) ($P<0.01$).

4. Discussion

In this report we have provided evidence demonstrating that LTBDH/PGR exhibits growth inhibitory effects when ectopically expressed in lung cancer cell lines with low (A549 and H1299) levels of endogenous expression. We have shown that ectopic expression of LTBDH/PGR induced apoptosis and growth inhibition of A549 and H1299 cells but not H157 cells. We have determined if over-expression of LTBDH/PGR in lung cancer cells expressing low levels of the endogenous enzyme would reverse malignancy despite several cancer-associated genetic alterations that have accumulated in lung cancer cell lines. We have found that over-expression of LTBDH/PGR in A549 cells by infection with Ad-PGR resulted in a significant suppression of tumorigenicity of A549 cells in athymic mice. Therefore, several lines of evidence, both in cell culture and in athymic mice, suggest that LTBDH/PGR may function as a tumor suppressor. However, direct *in vivo* evidence is lacking. An ultimate proof of a tumor suppressor function of this gene is the development of tumors in knockout mice. Generation of mouse lacking LTBDH/PGR gene becomes warranted.

Introduction of LTBDH/PGR gene in A549 and H1299 cells with low levels of the enzyme expression resulted in the induction of apoptosis *in vitro*, as shown by an increase in apoptosis population and by a suppression of cell growth in culture. The suppression of cell growth in H1299 cells was further demonstrated in an inducible system in which the enzyme was induced by tetracycline in a tetracycline repressor-expressing clone transiently transfected with a T-REx vector encoding the enzyme gene. However, transfection of the LTBDH/PGR gene in H157 cells having significant endogenous expression induced little apoptosis and growth inhibition. The fact that LTBDH/PGR suppressed tumorigenicity *in vivo* is most likely due to enzyme transduction which inhibited lung cancer cell growth and induced apoptosis *in vitro*.

Above observations that LTBDH/PGR induces growth inhibition and apoptosis in A549 and H1299 cells but not in H157 cells are consistent with the finding that cleavage of PARP occurred in A549 and H1299 cells but not in H157 cells. In A549 cells we further observed

increased expression of pro-apoptotic BAX, p53 and p21 as well as caspase 3-dependent induction of apoptosis through the intrinsic pathway. However, we did not observe any alteration in the expression of anti-apoptotic protein Bcl-2 and adhesion and invasion related protein CD44. This is in contrast to 15-PGDH, another tumor suppressor, in A549 cells in which over-expression of the enzyme decreased the expression of Bcl-2 and CD44 without altering the expression of p53 and BAX [13]. It appears that LTBDH/PGR and 15-PGDH induce apoptosis through different pathways. Although A549 and H1299 cells have similar growth curve after transfection with the enzyme as shown in Fig. 4A, we found significant cleavage of pro-caspase 3 in A549 cells but not in H1299 cells. This finding was rather reproducible. Cleavages of pro-caspase 3 and PARP were evident following PGR over-expression in A549 cells probably because these cells express wild type p53 which activates both intrinsic and extrinsic apoptotic pathways. The lack of cleavage of pro-caspase 3 in H1299 cells following PGR over-expression was probably related to the lack of expression of p53 in these cells (24). Consequently, the upstream caspases were not activated and the cleavage of PARP was achieved by other activated proteases. Alternatively, the time course of apoptosis could be different in two cell lines. It is likely that H1299 cells might undergo apoptosis later. Similar observations were made in H1299 cells over-expressing WWOX (25). We did not see any expression of p53 and p21 in H1299 and H157 cells because H1299 cells are p53 deleted and H157 cells are p53 mutated (24). Consequently, there is defective expression of p21 in both cell lines. On the other hand, A549 cells which have wild type p53 appear to undergo apoptosis in a p53 dependent manner since pro-apoptotic protein BAX was induced following PGR over-expression. Our finding with LTBDH/PGR as a potential tumor suppressor in A549 and H1299 cells is very much in similarity to that with WWOX as a tumor suppressor in these lung cancer cell lines. Fabbri et al. [25] showed that WWOX induced apoptosis and growth inhibition in A549 and H1299 cells which express no WWOX but not in U2020 cells which expresses endogenous WWOX. Cleavage of PARP was observed in both A549 and H1299 cells but not in U2020 cells, whereas activation of pro-caspase 3 was seen in A549 cells but not in H1299 and U2020 cells following Ad-WWOX infection of these cells. Tumorigenicity was also found to be dramatically attenuated in A549 cells infected with Ad-WWOX. A similar mechanism of suppressing tumorigenesis by either LTBDH/PGR or WWOX appears to be operative in these two tumor suppressor systems.

Our finding that LTBDH/PGR may function as a tumor suppressor has significant implications. Both 5-LO and LTA4H are known to over-express in human lung and other cancers [18-22]. Consequently, LTB_4 is over-produced in tumor tissues if the level of its catabolic enzyme remains unchanged or is decreased. How LTBDH/PGR expression alters the growth characteristics of cancer cells is not known. LTB_4 at nanomolar levels has been shown to stimulate proliferation of cancer cells [8,9,26,27], inhibit apoptosis of intestinal epithelial cells by regulating the expression of COX-2, β-catenin, and Bcl-2 [28], enhance oxidative stress [29], promote cell spreading [30], and stimulate hyperplasia in the epidermis [31]. Loss of LTBDH/PGR expression either by genetic or epigenetic mechanism can ensure elevated LTB_4 level in tumors. This is a reminiscence of up-regulation of COX-2 expression and down-regulation of 15-PGDH expression in lung and other cancers [13-17,32]. However, down-regulation of LTBDH/PGR expression in tumors remains to be established. In addition to oxidizing LTB_4, LTBDH/PGR also catalyzes the reduction of 15-oxo-PGEs to 15-oxo-13, 14-dihydro-PGEs. Recently, it was reported that 15-oxo-PGE_2 but not 15-oxo-13, 14-dihydro-PGE_2 is a PPARγ ligand and enhances adipogenesis in 3T3-L1 cells [33]. An isoform of LTBDH/PGR, designated as PGR-2, appears to be up-regulated in the late phase of 3T3-L1 adipocyte differentiation. PGR-2 may control the differentiation of adipocytes and other transitions. Whether this isoform may play a role in tumorigenesis remains to be determined.

The role of LTBDH/PGR in pathological consequences including cancer remains to be uncovered. Interestingly, the enzyme has also been identified as dithiolethione-inducible

gene-1 (DIG-1) [34]. Dithiolethiones belong to a structurally diverse family of compounds that are known to up-regulate the expression of phase 2 and anti-oxidative enzymes and to inhibit chemically induced carcinogenesis [35,36]. Members of this family of compounds, including isothiocyanates, mercaptans, trivalent arsenicals, α, β-unsaturated carbonyls, quinones and heavy metal salts, are sulfhydryl-reactive and thought to activate transcription of carcinogen detoxification enzymes via reaction with the cysteine-rich sensor protein Keap1 [37,38]. Recently, this enzyme was shown to catalyze the reduction of α, β-carbon=carbon double bond of a variety of α, β-unsaturated aldehydes and ketones to a single bond and was alternatively named alkenal/one oxidoreductase [39]. These compounds are capable of covalently binding to other cellular nucleophiles, including lysine, cysteine and histidine residues of proteins through a Michael addition mechanism. Furthermore, short chain aliphatic 2-alkenals and alkenones are thought to readily form exocyclic DNA adducts leading to mutations under physiological conditions [40]. The anti-oxidative function of this enzyme suggests that it may decrease the cancer risk associated with chemical carcinogens and exhibit anti-carcinogenic activity.

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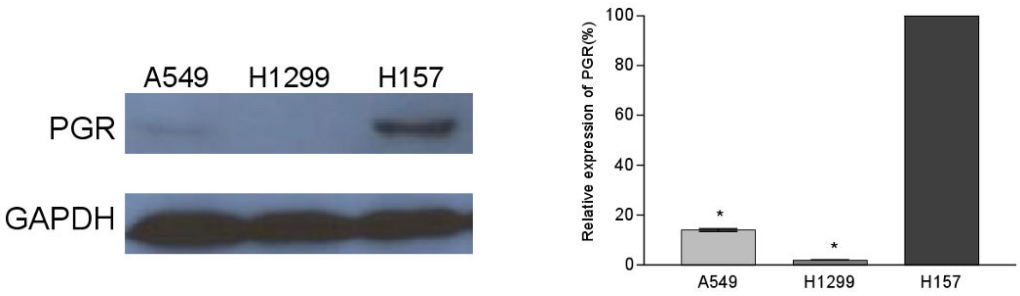


Figure 1.

LTBDH/PGR expression in A549, H1299 and H157 cells by Western blot analysis. Equal amount of proteins (100 μg) was loaded in each lane. SDS/PAGE and Western blot analyses were carried out as described in **Materials and Methods.** GAPDH was used as a loading control. Each column is represented by % relative expression ± SD (n=3). Level of expression in H157 cells was normalized as 100%. *, $P<0.01$ when level of expression in A549 or H1299 cells was compared with that in H157 cells.

Figure 2A

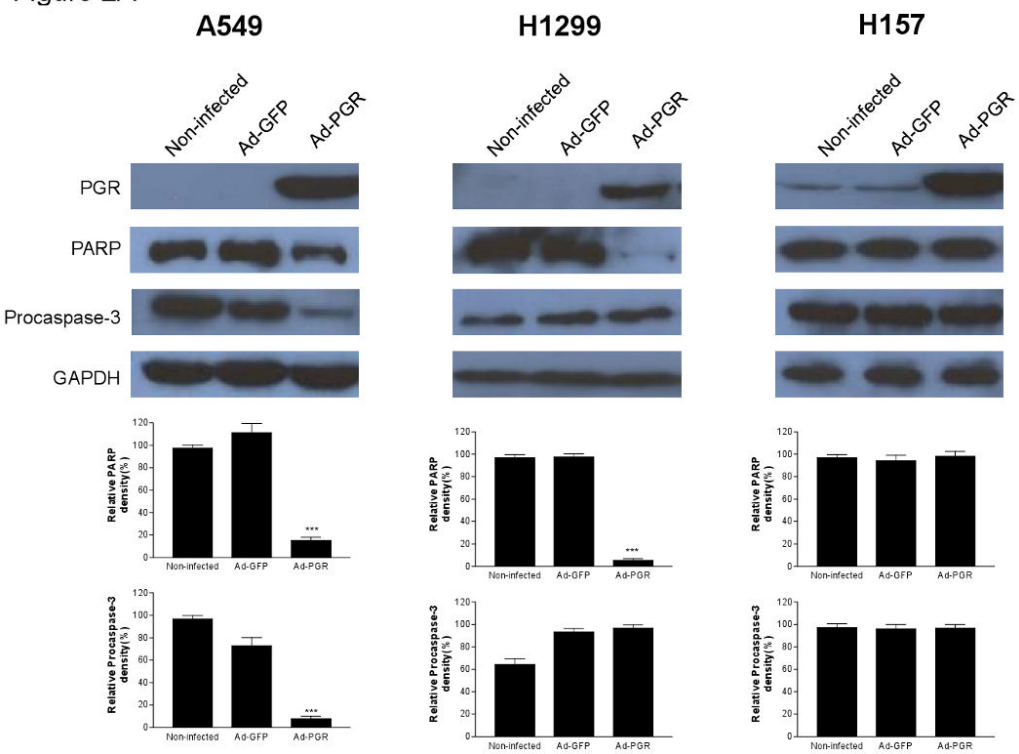


Figure 2B

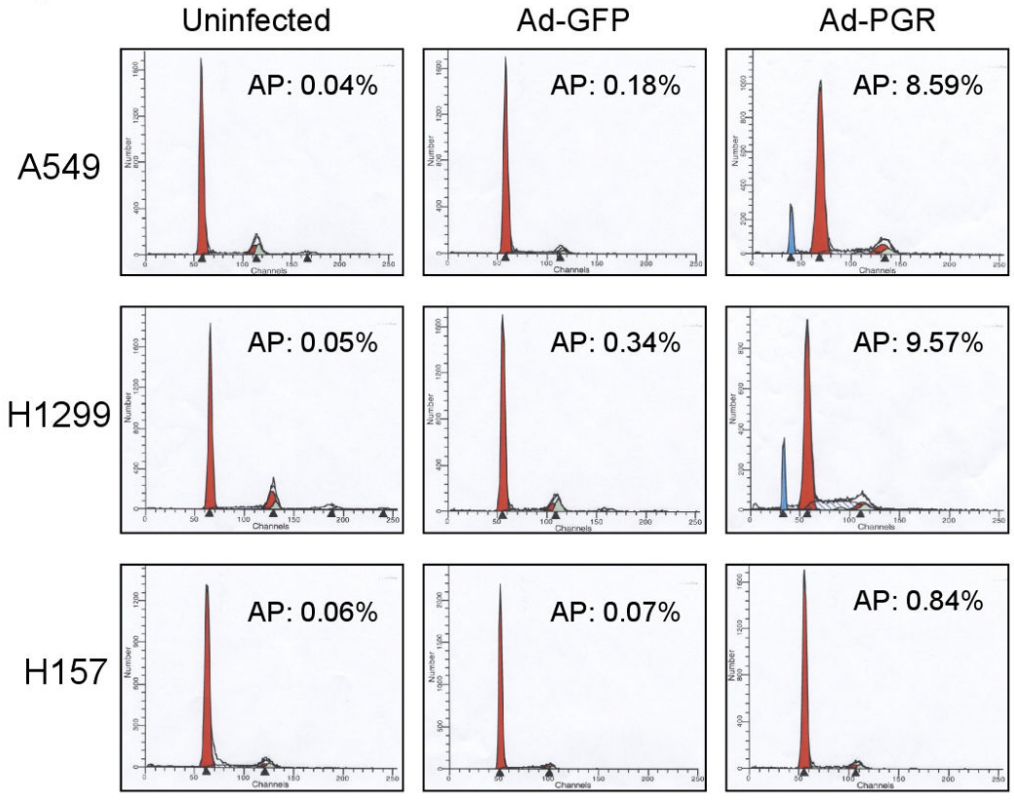


Figure 2.

Induction of apoptosis in A549 and H1299 cells infected with Ad-PGR. **A.** Western blot detection of PGR, full length PARP, and procaspase-3. A549 and H1299 cells were infected with or without Ad-GFP or Ad-PGR for 72 h and then processed for Western blot analysis as described in **Materials and Methods. B.** Flow cytometry analysis of uninfected, Ad-GFP and Ad-PGR-infected cells. A549, H1299 and H157 cells were infected with Ad-GFP or Ad-PGR for 72 h and then analyzed by a FACSCalibur cytometer as described in **Materials and Methods.** AP: apoptosis.

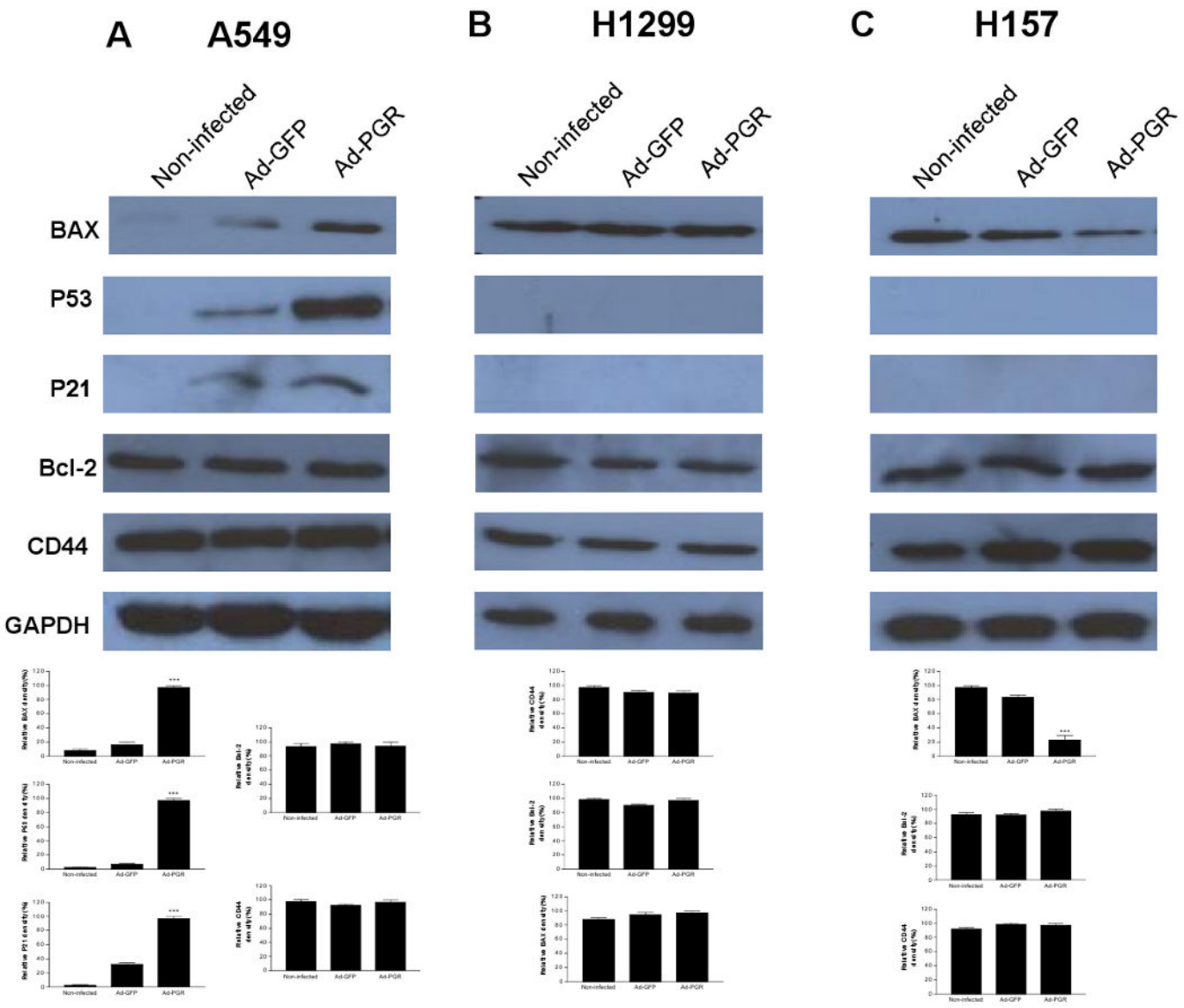


Figure 3.

Effect of ectopic expression LTBDH/PGR on the expression of apoptotic markers of BAX, p53, p21, Bcl-2 and CD44. A549, H1299 and H157 cells were infected with or without Ad-GFP or Ad-PGR for 72 h and then processed for Western blot analysis for each marker as described in **Materials and Methods.**

Figure 4A

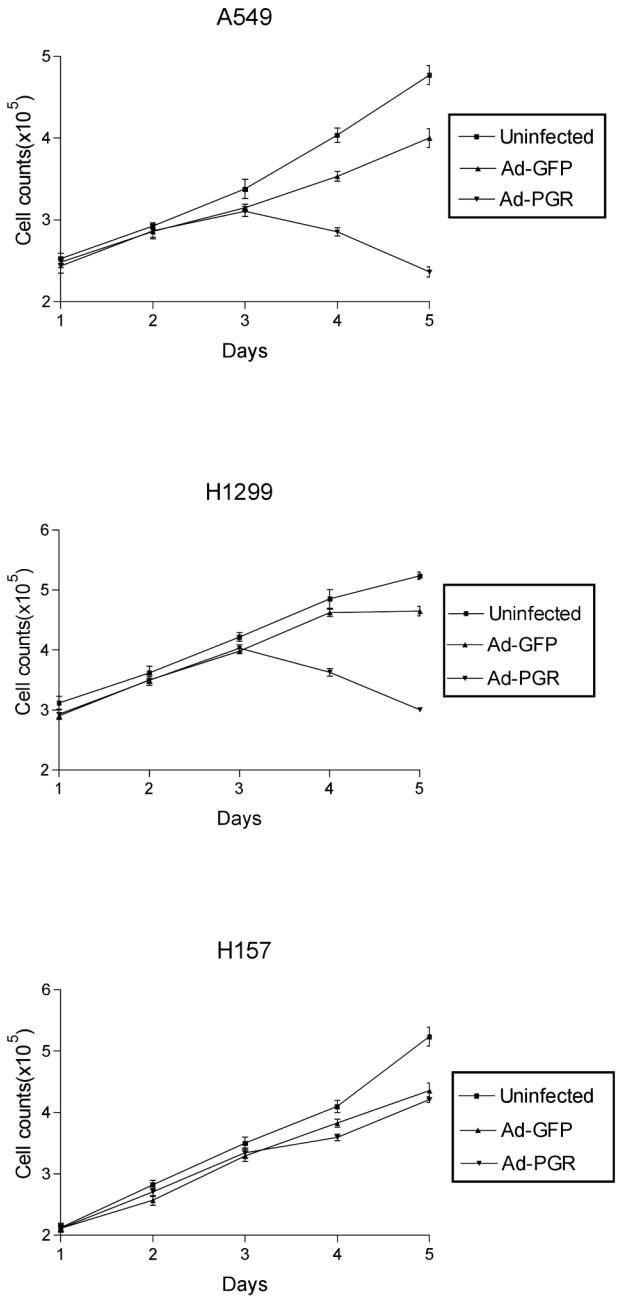


Figure 4B

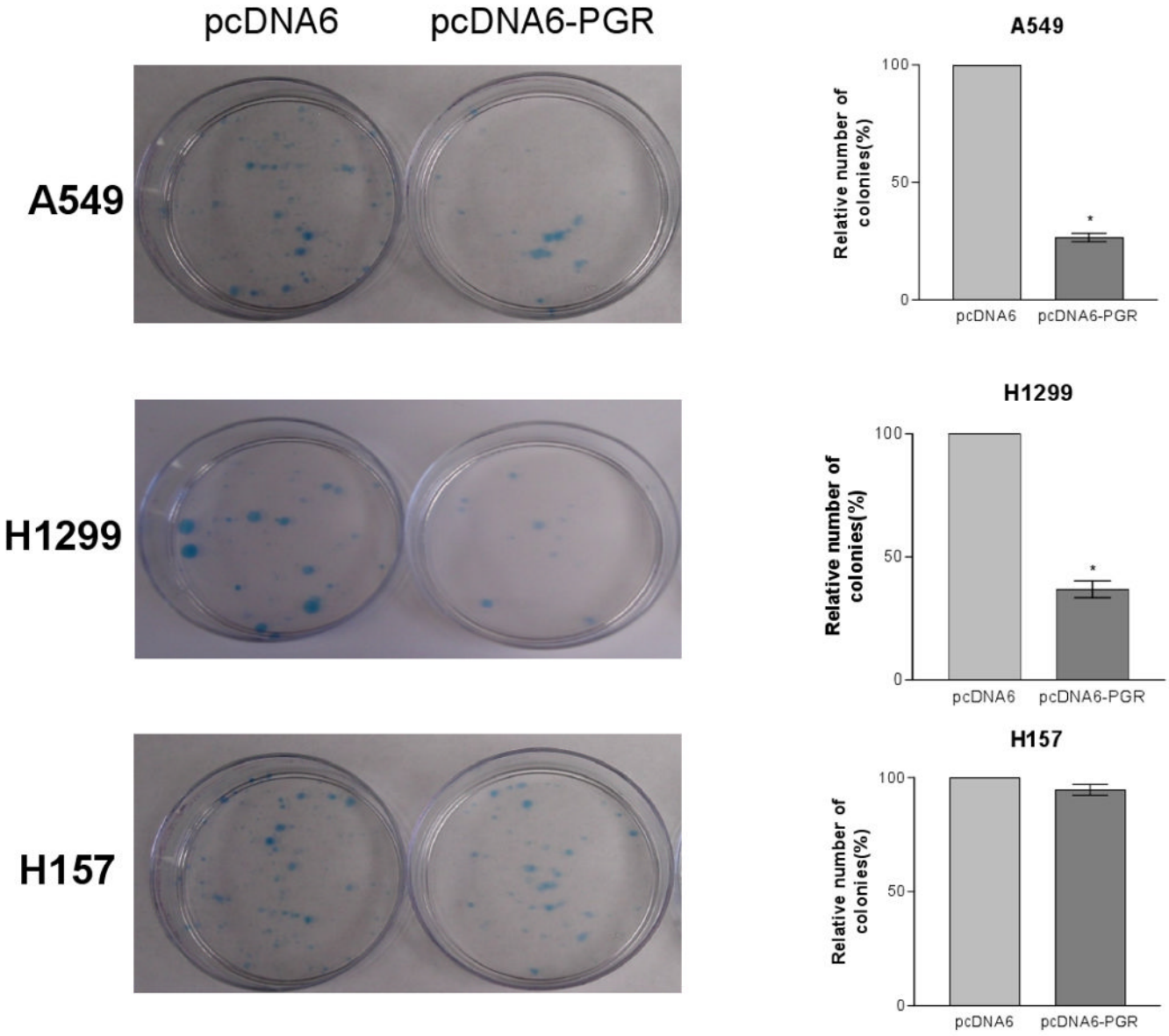


Figure 4.

Effect of ectopic expression of LTBDH/PGR on the growth of A549, H1299 and H157 cells. **A.** A549, H1299 and H157 cells were infected with or without Ad-GFP or Ad-PGR and the growth of each cell line was followed each day for a period of 5 days as described in **Materials and Methods. B.** Colony formation assay in pcDNA6 and pcDNA6-PGR-trandfected A549, H1299 and H157 cells. Quantification of colonies per 60-mm plate for pcDNA6 and pcDNA6-PGR-trandfected A549, H1299 and H157 cells was made. Each column was represented by mean ± SD (n=3). The number of colonies from cells transfected with vector control pcDNA6 was normalized as 100%. *, *P<0.01* when the relative number of colonies from cells transfected with pcDNA6-PGR was compared to that from cells transfected with vector control pcDNA6.

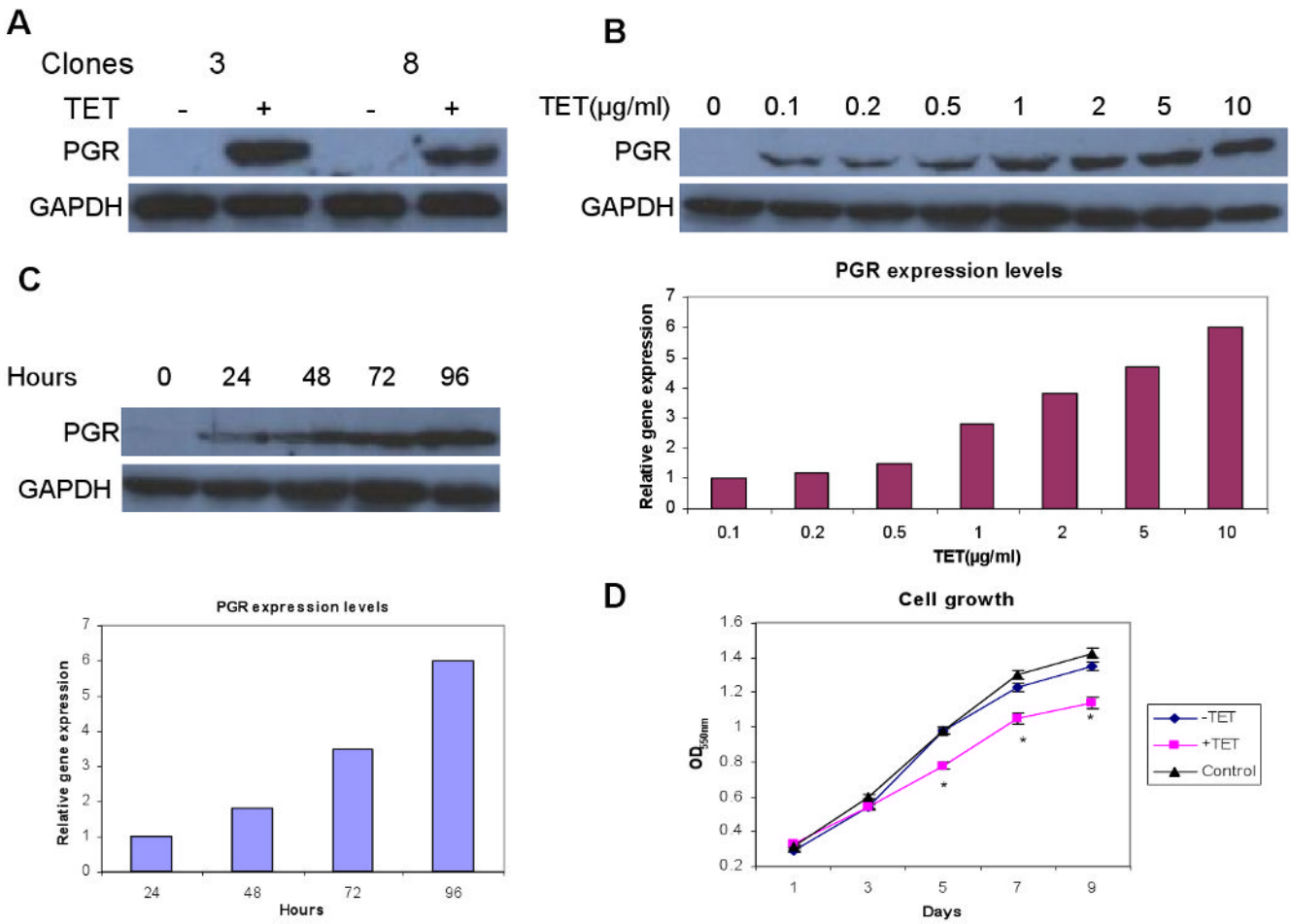
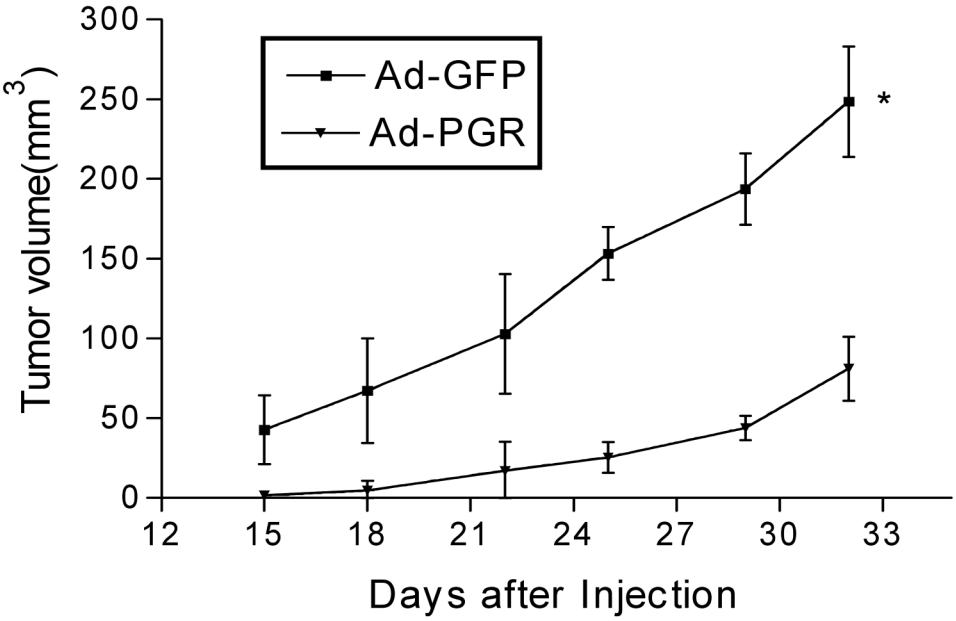


Figure 5.

Inducible expression of LTBDH/PGR in H1299/I cells. **A.** The inducible expression system in H1299 cells was constructed as described in **Materials and Methods.** Cells were cultured in the presence(+) or absence(-) of tetracycline (TET, 2.5 μg/ml) for 48 h and then tested for LTBDH/PGR expression in clones 3 and 8 by Western blot analysis. GAPDH expression served as a loading control. **B.** Dose-dependent induction of LTBDH/PGR in H1299/I clone 3 cells. Cells were incubated in the absence or presence of increasing concentrations of TET for 48 h. LTBDH/PGR expression at different levels of TET was quantified by densitometry and normalized to LTBDH/PGR expression at 0.1μg/ml TET. **C.** Time dependent induction of LTBDH/PGR in H1299/I clone 3 cells. Cells were incubated in the presence of TET (2.5 μg/ml) for 24 to 96 h. LTBDH/PGR expression at different time was quantified by densitometry and normalized to LTBDH/PGR expression at 24 h. **D.** Effect of TET on the growth of H1299/I clone 3 cells. H1299/I clone 3 cells were incubated in the absence or presence of TET (2.5 μg/ml) for a period of 9 days. TET was added after Day 1. Control: untreated H1299 cells. Cell growth was monitored by MTT assay as described in **Materials and Methods.** The experiment was done in triplicate.

A



B

Treatment	Tumor Incidence(%)	Tumor weight(g) ±SD
Ad-GFP	5/5(100%)	1.06±0.15
Ad-PGR	5/3(60%)	0.3±0.26

Figure 6.

Suppression of tumorigenesis of A549 cells in athymic nude mice by ectopically increased expression of LTBDH/PGR by Ad-PGR infection. **A.** Athymic female mice (5 in each group) were injected with A549 cells infected with Ad-GFP or Ad-PGR as described in **Materials and Methods.** Tumor growth was monitored for 5 weeks, and caliper measurements were conducted twice per week. The tumor volume was plotted against time. Results were expressed as the mean ± SE at each time point. *P<0.01 when Ad-PGR was compared with Ad-GFP. **B.** Tumor incidence and tumor weight are detailed in the table.