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Lack of epithelial PPAR γ causes cystic adenomatoid malformations in mouse fetal lung

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Abstract

Peroxisome proliferator-activated receptor- γ (PPAR γ) plays an important role in lipid and glucose metabolism. In this study, the function of PPAR γ on lung development was investigated. Lung-specific *Pparg* conditional knockout mice (*Pparg*^{LuEpC}) were developed using Cre-Lox system. *Pparg*^{LuEpC} mice showed abnormal lung development with enlarged airspaces and followed by increase of apoptotic cells at E14.5 to E18.5. Gene analysis revealed that expression of *Pmaip1*, a gene related to apoptosis, was significantly increased while expression of *Retnla*, a gene related to anti-apoptosis, was dramatically decreased in the fetal lung (E14.5) of *Pparg*^{LuEpC} mice. In addition, expression of *Pthlh*, a gene pheno-typically expressed in the congenital cystic adenomatoid malformation (CCAM), was increased at E14.5 to E18.5 in the lung of *Pparg*^{LuEpC} mice. Cell culture studies revealed that PPAR γ could bind to promoter region of *Pthlh* gene as a repressor in the immortalized mouse lung epithelial cell line MLE-15. Surprisingly, phenotypic changes in MLE-15-sh*Pparg* cells, stably transfected with sh*Pparg* plasmid, were similar to the *Pparg*^{LuEpC} mice model. In addition, MLE-15-sh*Pparg* cells were easily detached from the cultured plate when cold phosphate buffered saline was applied. Furthermore, expression of *Cdh1*, a gene related to cell adhesion, was significantly reduced in the MLE-15-sh*Pparg* cells. Taken together, PPAR γ may play an important role in fetal lung development via alveolar cell-to-cell adhesion system.

Keywords

PPAR γ ; Cystic adenomatoid malformation

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Conflict of interest

The authors have no financial conflicts of interest.

1. Introduction

Peroxisome proliferator-activated receptor- γ (PPAR γ , NPIX3), along with PPAR α (NR1C1) and PPAR β/δ (NR1C2), are ligand-activated transcription factor in the nuclear receptor superfamily [1,2]. PPAR γ is highly expressed in white and brown adipose tissue and to a lesser extent in macrophage, liver, colon, and kidney [3]. Together with the retinoid X receptor (RXR), PPAR γ binds to PPAR responsive-regulatory elements (PPRE) of genes involved in adipogenesis, lipid metabolism, inflammation, and glucose homeostasis [3,4]. PPAR γ is activated by endogenous metabolites such as 15-deoxy-delta 12, 14 prostaglandin J2 [5] and PPAR γ agonists including thiazolidinediones pioglitazone and rosiglitazone have been used for the treatment of metabolic disorders such as hyperlipidemia and diabetes mellitus type II [6].

Because PPAR γ is mostly distributed in adipose tissues, its roles in different tissues or cells have not received much attention. Lung tissue is composed of different cell types such as alveolar type I and type II epithelial cells, and club cells and ciliated cells in airway epithelia. Alveolar type II epithelial cells and club cells express PPAR γ . One study suggested a role for PPAR γ in lung development. Airway epithelial cell PPAR γ -targeted mice display enlarged airspaces resulting from insufficient postnatal lung maturation [7]. More recently, it was reported that PPAR γ activation enhances lung maturation in a neonatal rat model [8]. These findings suggest that PPAR γ is necessary for normal lung structure during developmental stage. In this study, the function of PPAR γ on fetal lung development was investigated using lung-specific *Pparg*-null mice.

2. Materials and methods

2.1. Mouse

Pparg-floxed (*Pparg*^{fl/fl}) mice containing *loxP* sites flanking exon 2 [9] were crossed with mice harboring the Cre recombinase under control of the surfactant protein C promoter [10] to generate *Pparg*^{LuEC} mice. Mice were interbred for over six generations to produce littermates with the same mixed genetic background (FVB). Mice, housed in temperature- and light-controlled rooms, were given water and pelleted chow ad *libitum*. All animal studies were carried out in accordance with Institute of Laboratory Animal Resources guidelines and approved by the National Cancer Institute Animal Care and Use Committee.

2.2. Isolation of lung epithelial cells

Lung epithelial cells were isolated from *Pparg*^{LuEC} and *Pparg*^{fl/fl} fetuses at E18.5 as described previously [11] with minor modifications. Briefly, minced lung was incubated in Dulbecco's Modified Eagle Medium (DMEM)/F12 (Cellgro, Herndon, VA) containing 10% heat-inactivated fetal bovine serum (FBS, Gemini Bio-Products, Woodland, CA), 1 U/ml Dispase® (Invitrogen, Grand Island, NY) and 100 U/ml collagenase type I (Invitrogen) at 37 °C for 30 min. Cells were washed three times in DMEM/F12 containing 10% fetal bovine serum. Cells were seeded and incubated at 37 °C for 20 min to separate epithelial cells from mesenchymal cells. Epithelial cells that remained in the media were transferred to a 12-well plate and incubated at 37 °C for 24 h in 5% CO₂ incubator.

2.3. Cell culture and transfection

Immortalized mouse lung epithelial MLE-15 cells (kindly provided by Dr. Jeffrey Whitsett, Cincinnati Children's Hospital [12]) were maintained at 37 °C in a humidified atmosphere of 5% CO₂/95% air in DMEM supplemented with 10% heat-inactivated FBS and 50 U/ml of penicillin/streptomycin mixture (Invitrogen). Cells were grown to 60–80% confluence and trypsinized with 0.05% trypsin containing 2 mM EDTA. For silencing of PPAR γ in the MLE-15 cells, cells were transfected with 3 μ g of the pGFP-V-RS-GI56785 construct (Origene) using JetPEI transfection reagent (Polyplus transfection) or transfected with mouse *siPparg* (Santa Cruz biotechnology) using Lipofectamine RNAiMAX reagent (ThermoFisher).

2.4. Reporter gene assays

For *Pthlh*-promoter luciferase assays, MLE-15 cells were transfected with different sizes of *pGL3-ptlh* constructs (−7648/+442, −6055/+442, −3767/+442, and −1876/+442) in the presence of *pSG5-Pparg* and *pSG5-RXR α* plasmids or rosiglitazone. The assay was analyzed using luciferase assay kit (Promega).

2.5. DNA-protein pulldown assay

For protein-DNA pulldown assay, MLE-15 cells were plated in 100-mm culture dishes at 70% confluence and cultured overnight. Then, the cells were transfected with pEGFP-*Pparg* plasmid (3 μ g) for 24 h and nuclear protein was isolated using NE-PER kit (Thermo Fisher Scientific). The nuclear proteins (50 μ g) were incubated with biotinylated *Pthlh* promoter DNA fragments (1 μ g) in the incubation buffer (10 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1 mM DTT; 1 mM EDTA, 0.1% NP-40, 0.1 mg/ml sonicated salmon sperm DNA) and the protein-DNA complexes were pull-downed with 50 μ l of streptavidin-labelled dynabead (Thermo Fisher Scientific). After washing the beads three times with same buffer, samples were subjected to Western blotting. To analyze the PPAR γ binding sites in the *Pthlh* promoter, chromatin immunoprecipitation (ChIP) assay was executed as previously described [13]. Briefly, cultured MLE-15 cells were fixed with 1% formaldehyde for 10 min at room temperature and the reaction was stopped by 500 μ l of 1.25 M glycine. The cells were then scraped and lysed with SDS lysis buffer [1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.1) and protease inhibitor cocktail], the samples were sonicated using the Biorupter system (Cosmo Bio, Tokyo, Japan) for 15 min (30 s ON/30 s OFF mode) in an ice-water bath and centrifuged at 12,000 r.p.m. for 5 min at 4 °C. Each sample was mixed with ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl and protease inhibitor cocktail) in the presence of anti-PPAR γ antibody (Santa Cruz Biotechnology). After washing and reverse cross-linking, eluted DNA was subjected to PCR reaction using the following primers for the two different regions of PPREs and one negative control: A region (89 bp): 5' - GAGACAGGGTTTCTCTGTATAGCCC-3' and 5' - CAGAGGCAGGTGGATTCTGAGTT-3'; B region (105 bp): 5' - TAACTGAAGTAGCACAGTGGGCA-3' and 5' - CCACTCGGCTGTATTTCCCATGGAT-3'; negative control (99 bp): 5' - ATGGCAGAACACCTGCAGTTCCAA-3' and 5' -

AGGGTCTCTCAATGAGGTCTAGGA-3'. PCR was performed using KOD Hot start DNA polymerase (EMD Milipore) under the following reaction conditions: an initial activation step at 95 °C for 2 min, 32 cycles of 95 °C for 20 s, 60 °C for 20 s and 72 °C for 20 s, and a final extension at 72 °C for 5 min. PCR amplicons were visualized by running the gel electrophoresis.

2.6. Western blotting

MLE-15 cell lysates were subjected to Western blotting as previously described [13]. Briefly, 30 µg of whole cell extracts were run on the SDS PAGE gels and transferred to nitrocellulose membranes. The membranes were blocked with 5% fat-free dry milk in phosphate-buffered saline containing 0.1% Tween-20 (PBST) for 1 h, and incubated with primary antibodies and horseradish peroxidase-conjugated secondary antibodies using standard western blotting procedures. Proteins were visualized using the Femto signal chemiluminescent substrate (Thermo Fisher Scientific) under an image analyzer (Bio-Rad).

2.7. Southern blot analysis

Southern blotting was performed as described previously [9].

2.8. RNA analysis

Total RNA was extracted from MLE-15 cells or lung of *Pparg*^{LuEC} and *Pparg*^{fl/fl} mice using TRIzol reagent (Invitrogen). Quantitative real-time PCR (qPCR) was carried out using cDNA generated from 1 µg total RNA with SuperScript III Reverse Transcriptase Kit (Invitrogen). qPCR was performed using an Applied Biosystems Prism 7900HT Sequence Detection System (Foster City, CA). All reactions were performed in a 10 µl volume consisting of 25 ng of cDNA, 300 nM of each primer and 5 µl of SYBR Green PCR Master Mix (Applied Biosystems) at 95 °C for 10 min and 40 cycles of 95 °C for 3 s and 60 °C for 30 s. Expression levels of mRNA were normalized to *Actb* mRNA. Primers for the qPCR were designed using qPrimer Depot (<http://mouseprimerdepot.nci.nih.gov>).

2.9. Lung histology

The lungs of *Pparg*^{LuEC} and *Pparg*^{fl/fl} fetal mice were fixed in 10% neutral phosphate-buffered formalin (Fisher, Pittsburgh, PA) overnight. The tissues were processed through conventional paraffin embedding. Four-mm sections were cut, deparaffinized with xylene, and hydrated in an ethanol gradient. The sections were stained with hematoxylin-eosin. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay was carried out with a DeadEnd™ Fluorometric TUNEL System (Promega) according to the manufacturer's instructions.

2.10. Data analysis

All data are expressed as the mean ± S.D. Statistical significance was determined by the Student's *t*-test for unpaired samples.

3. Results and discussion

3.1. PPAR γ deficiency causes cystic adenomatoid malformations in mouse fetal lung

Since PPAR γ expression in mouse fetal lung has hitherto not been reported, developmental expression of *Pparg* in the fetal lung was examined. A lung-specific PPAR γ knockout mice was developed using the Cre-lox system (Fig. 1A) and the level of *Pparg* mRNA was measured in both fetal lung epithelial cells and mesenchymal cells (Fig. 1B). Only epithelial cells expressed the *Pparg* mRNA. *Pparg* mRNA increased in a time-dependent manner and reached a plateau at E16.5. Expression of *Spc* mRNA, which is a marker of alveolar type II epithelial cell and its progenitor cells, progressively increased and reached a plateau at E17.5 (Fig. 1C). As the expression of *Pparg* and *Spc* appeared in different developmental stages of the fetus, the phenotypic change at E18.5 in *Pparg*^{LuEC} fetal lung was monitored. Enlarged alveolar sacs were noted grossly in the *Pparg*^{LuEC} fetal lung, but not in the *Pparg*^{fl/fl} fetal lung (Fig. 1D). This abnormal feature of the fetal lung was confirmed by H&E staining (Fig. 1E). Based on these results, epithelial PPAR γ may play an important role in fetal lung development.

3.2. Apoptotic cell death in the alveolar sac is increased in *Pparg*^{LuEC} fetal lung

Based on the data above, the abnormal fetal alveolar formation appears to be derived from the lack of PPAR γ in lung epithelial cells. However, mechanism of alveolar sac enlargement is not clear. Some cells at E18.5 were found in the alveolar sac of *Pparg*^{LuEC} fetal lung (Fig. 2A). To investigate the phenomena, apoptotic cell death was examined in fetal lung tissues by fluorometric TUNEL staining, which revealed that cells in the lumen were positive (Fig. 2B). Expression of *Retnla* (resistin like beta), a gene related to anti-apoptosis, was dramatically decreased (Fig. 2C), while *Pmaip1* (phorbol-12-myristate-13-acetate-induced protein 1), a gene related to apoptosis, was significantly increased (Fig. 2D) at E14.5 of *Pparg*^{LuEC} fetal lung.

The abnormal phenotype observed in *Pparg*^{LuEC} fetus resembles congenital cystic adenomatoid malformation (CCAM), which is a relatively rare lesion characterized by an increase in terminal respiratory structures forming varying-sized cysts. It was previously reported that localized overexpression of *Fgf10* (fibro-blast growth factor 10), *Bmp4* (bone morphogenetic protein 4), and *Pthlh* (parathyroid hormone like hormone) in fetal lungs induces a phenotype of CCAM [14,15]. Thus, expression of these genes was investigated in whole lungs from *Pparg*^{LuEC} fetuses. Interestingly, like other genes such as *Fgf10* and *Bmp4* (data not shown), *Pthlh* was significantly increased during E14.5 to E18.5 in *Pparg*^{LuEC} fetal lung (Fig. 2E). Based on the fetal lung histology and its gene analysis, CCAM may be characterized by apoptotic cell death in alveolar epithelial cells in the absence of PPAR γ .

3.3. PPAR γ binds to *Pthlh* promoter regions as repressor in MLE-15 cells

To investigate whether PPAR γ could bind to *Pthlh* promoter, the possible PPAR γ binding site was predicted using Genomatix software. MLE-15 cells, immortalized mouse lung epithelial cells [12], were transfected with various *Pthlh*-luciferase constructs having different promoter sequences between -7648 and +442 bp relative to the transcription start

site in the presence of both PPAR γ and RXR α expression plasmids or treated with rosiglitazone after transfection with *Pthlh*-luciferase constructs (Fig. 3). *Pthlh*-luciferase activity was dramatically decreased in all sizes of *Pthlh*-luciferase constructs by PPAR γ /RXR α in dose dependent manner. In addition, the longer size of 5' flanking region of *Pthlh*-promoter-luciferase resulted in a significant decrease of the luciferase activity (Fig. 3A). Rosiglitazone, a PPAR γ agonist, showed an inhibitory effect on *Pthlh*-luciferase activity (Fig. 3B). Furthermore, the binding study showed that PPAR γ could bind to the *Pthlh* promoter region from the 5' flanking region (Fig. 3C) and possible PPAR γ binding sites were found in the *Pthlh* promoter by ChIP assay (Fig. 3D).

Previously, it was suggested that parathyroid hormone-related protein (PTHrP/PTHLH), known as a paraneoplastic protein, may play an important role in the non-small lung carcinomas (NSCLC) and its receptor is frequently present in lung cancers. Also the protein has been implicated in cell growth, apoptosis and invasion [16–19]. Thus, increased *Pthlh* by ablation of *Pparg* may change the fetal lung architecture.

3.4. PPAR γ may contribute cell-cell adhesion in MLE-15 cells

Due to the limitation of molecular studies using fetal lung epithelial cells, immortalized mouse lung epithelial cells (MLE-15) was employed. When MLE-15 cells were transfected with a *Pparg* silencing construct MLE-15-*shPparg*, *Pparg* silencing in MLE-15 cells resulted in a pattern of colonization growth (Fig. 4A). Colony size from single cell culture was significantly reduced in the MLE-15-*shPparg* stable cells (Fig. 4B). Interestingly, monolayer growth pattern of MLE-15-*shPparg* cells was similar to the abnormal phenotype of *Pparg*^{LuEC} fetal lung. Furthermore, when the MLE-15-*shPparg* cells were rinsed with cold PBS, the cell layer was easily lifted compared to the parent cells (Fig. 4C). These results revealed that silencing of *Pparg* can inhibit cell-cell or cell-matrix interactions and cause abnormal fetal lung growth similar to CCAM. To support this conclusion, *E*-cadherin protein levels were measured after transfection with the *siPparg*. *E*-cadherin and its mRNA were significantly reduced by *siPparg* or *shPparg* in MLE-15 cells, respectively (Fig. 4D–E). Because cell adhesion molecules may play a critical role not only in maintaining tissue architecture or morphogenesis [20] but also in cancer cell invasion [21], if the adhesion system in the fetal epithelium is weakened, it can lead to abnormal fetal lung development. Previously, it was demonstrated that *E*-cadherin can be regulated by PPAR γ and inhibit growth and invasion of prostate cancer or MCF-7 cells [22,23]. Based on these results, *Pparg* silencing may lose the epithelial architecture of fetal lung, resulting in CCAM.

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Abbreviations:

PPAR γ peroxisome proliferator-activated receptor gamma

Retnla	Resistin-like alpha
Pthlh	parathyroid hormone-like hormone
Cdh1	Cadherin-1

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Fig. 1.

Ablation of *Pparg* gene resulted in abnormal development of mouse fetal lung. (A) Southern blotting of the recombination of *Pparg* allele in lung (Lu), small intestine (Si), caecum (Ce), colon (Co), liver (Li), kidney (Ki) and brown adipose tissue (BAT) genomic DNA isolated from *Pparg*^{fl/fl} and *Pparg*^{ΔLuEpC} mice. (B) qPCR analysis of *Pparg* mRNA in epithelial and mesenchymal cells isolated from *Pparg*^{fl/fl} (open circles, n = 11) and *Pparg*^{ΔLuEpC} (closed circles, n = 5) lungs at E18.5. Data points and bars represent individual values and the means, respectively. (C) qPCR quantitation of *Pparg* (closed circles) and *Spc* (open circles) mRNAs using whole lungs from normal mice at gestational days of 13.5 to 18.5 and postnatal weeks of 8. Individual values represent as mean ± S.D. of three lungs. (D) Fetal lung morphology is shown at gestational days of 18.5 in *Pparg*^{fl/fl} and *Pparg*^{ΔLuEpC} mice. (E) Fetal lung histology at different gestational days is shown in *Pparg*^{fl/fl} and *Pparg*^{ΔLuEpC} mice. Scale bars: 200 μm. **, p < 0.001.

Fig. 2.

Ablation of lung *Pparg* gene shows signature of apoptotic cell death. (A) Sections of *Pparg*^{fl/fl} and *Pparg*^{ΔLuEpC} lungs at E16.5 and E18.5 were subjected to H&E staining. Note the presence of cellular debris (arrowheads). Scale bars: 50 mm. (B) Immunofluorescence for TUNEL and DAPI (nuclei). Note the presence of apoptotic cells (arrowheads). Scale bars: 100 μm. Expression of anti-apoptotic *Retnla* mRNA (C) and apoptotic *Pmaip1* mRNA (D) were measured by qPCR at E14.5. (E) *Pthlh* mRNA measured from *Pparg*^{fl/fl} and *Pparg*^{ΔLuEpC} fetus lung at E14.5 (n = 6 and 10, respectively), E16.5 (n = 13 and 10, respectively) and E18.5 (n = 6 and 10, respectively). Individual values represent as mean ± S.D. **p* < 0.05 and ***p* < 0.01 vs *Pparg*^{fl/fl} at the corresponding days.

Fig. 3.

PPAR γ binds to *Pthlh* promoter regions as repressor. (A) *Pthlh*-luciferase activity analyzed after transfection of different sizes of pGL3-*Pthlh*-promoter constructs with plasmid expressing PPAR γ or RXR α proteins in the MLE-15 cells. (B) *Pthlh*-luciferase activity analyzed after transfection with pGL3-*Pthlh*-promoter constructs (-7648/+442) with different concentrations of rosiglitazone, a PPAR γ agonist in the MLE-15 cells. (C) MLE-15 cells were transfected with plasmids expressing eGFP-PPAR γ or RXR α protein and then, nuclear proteins were incubated with DNA fragments from the *Pthlh* promoter and subjected to Western blotting. The bottom gel shows PCR amplicons for the study. *a*, *b*, *c*, *d*: DNA fragment covering different areas used in pull down assays. *CE*: cytosolic extract, *NE*: nuclear extract. (D) PPRE sites were monitored by ChIP assay. Predicted PPRE sites were analyzed using Genomatix software (www.genomatix.de). γ , PPAR γ binding site; NC, negative control. ***, $p < 0.0001$.

Fig. 4. Cellular adhesion of MLE-15 cells was reduced by knock-down of *Pparg* gene. (A) Colony shape of MLE15 cells is shown after transfection with *shPparg* plasmid for 4 days. Transfected cells were visualized by turbo GFP (tGFP) protein. Scale bars: 100 μ m. (B) Colony areas from stable MLE-15-*shPparg* and parental cells. (C) MLE-15-*shPparg* and parental cells were grown to 100% confluence and challenged with cold PBS. Lifted cells are shown in MLE-15-*shPparg* cells. Scale bars: 300 μ m. (D) MLE-15 cells were transfected with *siPparg* or *scramble RNA* and total protein extracts were subjected to Western blotting. (E) Expression of *Cdh1* in MLE-15-*shPparg* cells was measured by qRT-pCR. *Gapdh* mRNA was used as internal control. ***, $p < 0.0001$.