Original Article

MOLECULAR METABOLISM

PPARγ is dispensable for clear cell renal cell carcinoma progression

Danielle J. Sanchez [1,4], David J. Steger [3], Nicolas Skuli [1,2], Ankita Bansal [1,2], M. Celeste Simon [1,4,*]

ABSTRACT

Objective: Clear cell renal cell carcinoma (ccRCC) is a subtype of kidney cancer defined by robust lipid accumulation, which prior studies have indicated plays an important role in tumor progression. We hypothesized that the peroxisome proliferator-activated receptor gamma (PPARγ), detected in both ccRCC tumors and cell lines, promotes lipid storage in ccRCC and contributes to tumorigenesis in this setting. PPARγ transcriptionally regulates a number of genes involved in lipid and glucose metabolism in adipocytes, yet its role in ccRCC has not been described. The objective of this study was to elucidate endogenous PPARγ function in ccRCC cells.

Methods and results: Using chromatin immunoprecipitation followed by deep sequencing (ChIP-seq), we found that PPARγ and its heterodimer RXR occupy the canonical DR1 PPAR binding motif at approximately 1000 locations throughout the genome that can be subdivided into adipose-shared and ccRCC-specific sites. CRISPR-Cas9 mediated, loss-of-function studies determined that PPARγ is dispensable for viability, proliferation, and migration of ccRCC cells *in vitro* and *in vivo*. Also, surprisingly, PPARγ deletion had little effect on the robust lipid accumulation that typifies the "clear cell" phenotype of kidney cancer.

Conclusion: Our results suggest that PPARγ plays neither a tumor suppressive nor oncogenic role in advanced ccRCC, and thus single-agent therapeutics targeting PPARγ are unlikely to be effective for the treatment of this disease. The unique cistrome of PPARγ in ccRCC cells demonstrates the importance of cell type in determining the functions of PPARγ.

Keywords Nuclear receptors; PPARγ; Kidney cancer; Cancer metabolism; Lipid metabolism

1. INTRODUCTION

Kidney cancer is the 8th most prevalent form of cancer diagnosed each year in the United States, with approximately 64,000 new diagnoses and 14,400 deaths annually [1]. While localized disease can be treated by surgical resection, 30% of patients initially present in the clinic with metastatic disease, which carries a poor prognosis due to limited efficacy of current standard-of-care therapies [2]. As such, a significant clinical need remains for therapeutics targeting unique genetic and metabolic vulnerabilities within this tumor type.

Clear cell renal cell carcinoma (ccRCC), the most common subtype of kidney cancer, is defined by constitutive hypoxia-inducible factor signaling as well as widespread changes in cellular metabolism of glucose, amino acids, and lipids [3]. Phenotypically, ccRCC is characterized by robust intracellular lipid and glycogen accumulation, resulting in "cleared" cytoplasm when prepared for common histologic analyses. Rather than simply reflecting a byproduct of increased anabolic metabolism, recent studies suggest that maintaining the integrity of neutral lipid droplets as well as abundant lipid uptake is critical to maintain ccRCC cell viability [4], particularly when oxygen is limiting in the tumor microenvironment [5]. However, factors imparting a lipogenic quality to ccRCC tumors remain to be fully elucidated.

The peroxisome proliferator-activated receptor gamma (PPARγ) along with its heterodimeric DNA-binding partner retinoid X receptor (RXR) promote the transcription of genes broadly important for lipid, glucose, and hormone metabolism, most notably in the context of adipose tissue [6]. PPARγ, the master regulator of adipogenesis, is both necessary and sufficient for this process *in vitro* and *in vivo* [7] [8]. Additionally, in non-adipose contexts including ischemic, diseased cardiomyocytes [9] and macrophages [10], PPARγ contributes to the regulation of genes involved in lipid metabolism. In a mouse model of high fat diet (HFD)-induced hepatosteatosis, PPARγ protein expression is elevated in the livers of mice fed HFD relative to controls [11], although the absolute level remains far below those observed in adipose tissues. Interestingly, conditional deletion of *Pparg* within hepatocytes abrogated liver steatosis, suggesting a link between PPARγ and lipid uptake, synthesis, and/or storage in this model.

Previous reports indicate that PPARγ is functionally expressed [12] in ccRCC and that increased PPARγ abundance correlates with reduced patient survival [13], suggesting a possible oncogenic function. *In vitro* studies investigating the role of PPARγ in ccRCC and other cancers have largely employed natural and synthetic activating ligands including the insulin-sensitizing thiazolidinediones, yet many used super-physiologic concentrations, which can cause off-target effects

[1]Abramson Family Cancer Research Institute, USA [2]Department of Cancer Biology, USA [3]Institute for Diabetes, Obesity and Metabolism, USA [4]Department of Cell and Developmental Biology, USA

*Corresponding author. Perelman School of Medicine at the University of Pennsylvania, 456 BRB II/III, 421 Curie Boulevard, Philadelphia, PA, 19104-6160, USA. E-mail: celeste2@pennmedicine.upenn.edu (M.C. Simon).

Received May 1, 2018 • Revision received May 11, 2018 • Accepted May 17, 2018 • Available online xxx

https://doi.org/10.1016/j.molmet.2018.05.013

Original Article

and confound interpretation of results [14,15]. In this study, our goal was to investigate endogenous PPARγ function through ChIP-seq and a number of *in vitro* and *in vivo* assays of tumor progression using loss-of-function models in established ccRCC cell lines.

2. MATERIALS AND METHODS

2.1. Primary patient samples and gene expression data

Matched tumor/normal samples were obtained from the Cooperative Human Tissue Network (CHTN). Tumors were homogenized in TRIzol (see quantitative real-time PCR) or whole cell elution buffer (see western blot) and analyzed for *PPARG* mRNA and protein expression. Gumz et al. microarray dataset was downloaded from Oncomine. RNA-seq data for 480 ccRCC and 69 normal kidney samples were downloaded from TCGA on April 2, 2013. Differential gene expression analysis of tumor and normal samples was performed using DeSeq (Bioconductor Version 2.12). TCGA mutation and copy number data for 418 sequenced patients/cases were downloaded from cBioPortal for Cancer Genomics [16].

2.2. Cell culture, plasmids, lentiviral production, and viral transduction

Human ccRCC cell lines (RCC10, UMRC2, Caki2, 786-O, A498, 769-P) were obtained from the American Type Culture Collection (ATCC) and were cultured in DMEM (ThermoFisher Scientific, cat. 11965092) supplemented with 10% FBS (Gemini Bio-Products, cat. 900-108). Immortalized renal epithelial cells (HK2) obtained from ATCC and cultured in Keratinocyte Serum Free Media with appropriate supplements (ThermoFisher Scientific, cat. 17005042). Human single-guide RNAs (sgRNA) targeting *PPARG* #1 (ctccgtggatctctccgtaa) and #3 (cattacgaagacattccatt) along with control gRNA targeting mouse *Rosa26* locus (aagatgggcgggagtcttct) were cloned into LentiCRISPRv2 plasmid [17]. Mature antisense human *PPARG* shRNA #3 sequence (clone ID: TRCN0000001673) along with scrambled (SCR) control were cloned into a doxycycline-inducible pLKO lentiviral plasmid (AddGene, cat. 21915, [18]). Lentivirus was prepared by co-transfection of 293T cells with shRNA or CRISPR plasmid of interest along with packaging plasmids pVSVg (AddGene, cat. 8454), psPAX2 (AddGene, cat. 12260) and Fugene6 transfection reagent (Promega). Lentivirus-containing media was collected from plates at 24 and 48 h post-transfection, filtered using a 0.45 μm filter, and stored at −80 °C. For viral transduction, cells were incubated with lentivirus-containing medium and 8 μg/mL polybrene for 24 h. Cells were allowed to recover for another 24 h before selection with puromycin. All experiments were performed with cells that survived puromycin selection and displayed knockdown/knockout of *PPARG* as assayed by western blot.

2.3. Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated using TRIzol reagent (ThermoFisher Scientific, cat. 15596026) and RNeasy mini kit (Qiagen, cat. 74104). Reverse transcription was performed using High-Capacity RNA-to-cDNA (Applied Biosystems, cat. 4387406). qRT-PCR was performed using ViiA7 Real-Time PCR system (Applied Biosystems) with TaqMan master mix (Life Technologies). TaqMan probes were used to quantitate expression of *PPARG* (cat. Hs01115513_m1), *FABP4* (cat. Hs01086177_m1), *CD36* (cat. Hs01567185_m1) *SLC38A4* (Hs00394339_m1) and normalized to housekeeping genes *HPRT1* (cat. Hs02800695_m1) and *TBP* (Hs00427620_m1).

2.4. Western blot

Cells were washed with PBS prior to lysis in whole cell elution buffer (150 mM NaCl, 10 mM Tris pH 7.6, 0.1% SDS, and 5 mM EDTA) containing Roche ULTRA protease inhibitor cocktail (cat. 05892791001). Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotting was performed by incubating with primary antibodies overnight at 4 °C. The next day, membranes were incubated with secondary antibody and Western Lightning Plus-ECL, Enhanced Chemiluminescence Substrate (PerkinElmer, cat. NEL103E001EA) was used to visualize proteins. All primary antibodies were diluted of 1:1000 in 5% w/v nonfat milk (except GAPDH, 1:10,000), and secondary antibodies were diluted 1:2000 in 5% w/v nonfat milk. PPARγ (cat. 2435), FASN (cat. 3180), GAPDH (cat. 2118), anti-Rabbit IgG, HRP-linked (cat. 7074), anti-Mouse IgG, HRP-linked (cat. 7076) were purchased from Cell Signaling Technology. SREBP1 (cat. 13551) was purchased from Santa Cruz Biotechnology. SCD (cat. 19862) was purchased from Abcam.

2.5. Chromatin immunoprecipitation (ChIP) and ChIP-seq

ChIP was performed with whole cell extracts isolated from UMRC2 PPARγ WT and PPARγ KD cell lines using 10 μg PPARγ (Santa Cruz, cat. sc-7196) or 10 μg RXRα/β/γ (Santa Cruz, cat. sc-774) antibodies for immunoprecipitation (IP). Briefly, confluent 10 cm dishes of cells were prepared by crosslinking with 1% formaldehyde for 15 min at room temperature and quenched with 125 mM glycine for 5 min at room temperature. Cells were harvested by scraping and pellets were resuspended in 200 μl SDS lysis buffer (50 mM HEPES/NaOH pH 7.5, 1% SDS, 10 mM EDTA, 1 mM PMSF, and Roche ULTRA protease inhibitor cocktail (cat. 05892791001)) on ice for 10 min. Sonication was performed using Bioruptor Pico (Diagenode, cat. B01060010) on high setting for 30 s, followed by centrifugation of lysates to remove cellular debris. 100 μl of sheared chromatin from each tube was then diluted 10X, with 5% saved as Input DNA and the rest prepared for either PPARγ or RXR IP.

PPARγ WT, PPARγ KD, RXR, and Input libraries were prepared in duplicate from two independent biological replicates. For ChIP-seq, sequencing data was mapped to the human genome (GRCh38) using STAR [19] with parameters appropriate for ungapped alignments. Peaks were called for each sample with input samples as background by HOMER [20]. HOMER was also used for differential peak calling (PPARγ WT vs. KD and RXR WT vs. KD) and to annotate peaks to proximal genes as described in Ensembl v85 (http://www.ensembl.org/index.html). For bioinformatics analyses displayed in Figure 2C, F, 1031 "high-confidence sites" were defined by the following criteria: peak score ≥10 (≥1 read per million), fold change (PPARγ WT vs. KD) ≥ 2, RXR peak called with strict overlap. Motif enrichment analysis was performed on this filtered peak list (1031 peaks) using HOMER against the standard list of known motifs; *de novo* motif discovery included consideration of lengths 8, 10, 12, 15,18 bp.

2.6. Annexin V-PI apoptosis assay

30,000 cells of each cell line were plated in triplicate on 6-well plates. Four days later, cells were prepared using the FITC—Annexin V, PI Kit (BD Biosciences, cat. 556547) according to the manufacturer's instructions. Flow cytometry was performed using the BD Accuri C6 instrument, with viable cells represented as the double-negative population.

2.7. 2D and 3D proliferation assays

For 2D proliferation assays, 30,000 cells of each cell line were plated in triplicate on 6-well plates. The following day (represented as Day 0), cells were trypsinized and counted using the Countess Automated Cell Counter (Invitrogen, cat. C10281), as per the manufacturer's instructions with Trypan blue. Cells were then counted again at the

