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The activation of peroxisome proliferator-activated receptor γ is regulated by Krüppel-like transcription factors 6 & 9 under steatotic conditions

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ABSTRACT

Liver steatosis is characterised by lipid droplet deposition in hepatocytes that can leads to an inflammatory and fibrotic phenotype. Peroxisome proliferator-activated receptors (PPARs) play key roles in energetic homeostasis by regulating lipid metabolism in hepatic tissue. In adipose tissue PPARγ regulates the adipocyte differentiation by promoting the expression of lipid-associated genes. Within the liver PPARγ is up-regulated under steatotic conditions; however, which transcription factors participate in its expression is not completely understood. Krüppel-like transcription factors (KLFs) regulate various cellular mechanisms, such as cell proliferation and differentiation. KLFs are key components of adipogenesis by regulating the expression of PPARγ and other proteins such as the C-terminal enhancer binding protein (C/EBP). Here, we demonstrate that the transcript levels of *Klf6*, *Klf9* and *Pparγ* are increased in response to a steatotic insult *in vitro*. Chromatin immunoprecipitation (ChIp) experiments showed that *klf6* and *klf9* are actively recruited to the *Pparγ* promoter region under these conditions. Accordingly, the loss-of-function experiments reduced cytoplasmic triglyceride accumulation. Here, we demonstrated that KLF6 and KLF9 proteins directly regulate PPARγ expression under steatotic conditions.

1. Introduction

Hepatic steatosis is characterised by fatty acid droplet's deposition in the liver, that can lead to hepatic inflammation or steatohepatitis (NASH) and ultimately to fibrosis [1]. Several transcription factors including the peroxisome proliferator-activated receptors (PPARs), are key players in liver lipid metabolism [1]. The PPAR subfamily consists of three members, PPARα, PPARβ/δ and PPAR; they promote lipid catabolism (PPARα & PPARβ/δ) or lipogenesis (PPARγ) [2–4]. PPARγ is a pro-steatotic factor during early liver disease [5]; however, the transcription factors regulating its expression in this condition, has yet to be elucidated.

Several reports indicate that members of the Krüppel-like factor (KLF) family [6–8] actively regulate the expression of genes such as *C/ebpα*, *C/ebpβ* and *Pparγ*, which are key regulators of adipocyte differentiation [9–11]. Some KLF proteins are potential modulators of liver homeostasis [12]. Two splicing variants of the KLF6 (KLF6-IVSI-27G>A) (rs3750861) and *KLF6*-SV2, for example, are known because of its positive correlation with steatosis, inflammation and fibrosis in human subjects [13,14], and for its pro-apoptotic activity in response to the anticancer drug gemcitabine, respectively [15]. Krüppel-like factors appear to be crucial for many processes associated with liver homeostasis such as steatosis, fibrosis or hepatocarcinoma [17–19]. However, whether KLFs might regulate *Pparγ* gene expression during steatosis, remains to be determined.

Abbreviations: KLF6, Krüppel-like factor 6; KLF9, Krüppel-like factor 9; PPARγ, Peroxisome proliferator-activated receptor gamma.

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2. Materials and methods

2.1. Cell culture and treatment

HepG2 (human hepatocarcinoma) cells (kindly provided by Dr. J. Mass Instituto de Fisiología Celular, UNAM; México) were cultured as described [20]. Cells (0.4×10^6 cells/well) were seeded in 6-well culture plates. After 24 h, the cells were subjected to a 12 h fast in FBS-free DMEM, and then treated with palmitic acid (PA, 300 μM) (*Fisher Scientific*) or vehicle (3% BSA in PBS, *Sigma*) to induce steatotic conditions. The cells were harvested after 24 h and were used for either total RNA extraction or nuclear protein purification.

2.2. RT-PCR and Western Blotting

Total RNA was extracted using Trizol® reagent (*Life Technologies*, *USA*). Total RNA (1 μg) was reverse-transcribed using the *Transcriptor First Strand cDNA Synthesis Kit* (*Roche*, *USA*) and random hexamers. qRT-PCR was carried out using specific primers for PPARs, KLFs, lipogenic, and fatty acid catabolism-related genes, using the *LightCycler® TaqMan® Master Kit* (*Roche*, *USA*), and the *LightCycler 480* thermal cycler system (Roche, USA). The relative expression data were normalised to the expression of β-actin mRNA according to the $2^{\Delta\Delta}$CT method [21].

Western-blot was performed as described elsewhere [22] using 30 μg of nuclear extracts and the following antibodies: anti-KLF6 (sc-7158), anti-KLF5 (sc-31939), anti-POL-II (sc-9001), anti-*KLF9* (sc-12994), anti-PPARα (sc-9000), anti-PPARβ (sc-7197) anti-PPARγ (sc-7196) or anti-*H3*, (sc-10809) all from Santa Cruz Biotechnology.

2.3. Chromatin immunoprecipitation (ChIP)

ChIP was performed as described previously [22]. The chromatin (fragmented to 300–600 bp) was subjected to immunoprecipitation using 5 μg of anti-KLF5 (sc-20691), anti-KLF6 (sc-7158) or anti-KLF9 antibody (sc-12994), anti-POL-II or control IgG. Immunoprecipitated DNA was analysed by PCR using specific primers for the region encompassing the CACCC sequences of the *Pparγ* gene promoter. Coding region *Pparγ* gene primers were used as negative control.

2.4. Lipid content quantification by Oil Red O staining

Cellular neutral lipid droplet accumulation was determined using a previously reported quantitative staining method [23].

2.5. Short-hairpin-RNA (sh-RNA)-expressing cell line generation

Stable cell lines were generated using plasmids encoding sh-RNAs for KLF5 (sc-37718-SH) or KLF9 (sc-37716-SH), both from Santa Cruz Biotechnology, according to the manufacturer's instructions. Effective gene expression knockdown was assessed using RT-PCR and Western-blot. For KLF6 gene knockdown, we used a pool of three to five 19-25-nt si-RNAs particles (sc-38021, Santa Cruz Biotechnology, USA), according to the procedure described above, except that 80 pmol/ml of *si-Klf6* was used instead.

2.6. Transient plasmid transfection experiments

Confluent HepG2 cells were subjected to transient plasmid transfection as previously described [22]. The pIG-mKLF5 (kindly donated by Dr. J. M. Wells), pCMV-Tag2-KLF6 (A gift from Dr. G.J. Rosman & Dr. D. Miller) and the pTRIP-klf9-3xFlag-IRES-hrGFP (kindly donated by Dr. I. Dussart) vectors, were used for these experiments. The cells were cultured under the conditions described above. After 12 h, the cells were subjected to starvation to induce the steatotic conditions.

3. Results and discussion

3.1. PA increases Pparγ and Klf6 & Klf9 gene expression and promotes triglyceride accumulation in HepG2 cells

Previous reports indicated that PA treatment *in vitro* (final concentration 300 μM) generates a condition similar to that of fatty liver [19,24]. We performed a time-course assay at 3, 6, 12 and 24 h of treatment. We observed no significant transcript expression changes after 3 or 6 h of PA treatment (data not shown). However, the cells treated for 12 or 24 h, showed a marked increase in *Ppar* and *Klf* transcripts expression; the mRNA expression of *Pparγ* was significantly increased (1.47 ± 0.13 *vs* vehicle-treated cells; Fig. 1A & Supplementary Fig. 1A) at 12 h ($p < 0.01$), but that of *Pparα* and *Pparβ* was not altered at these time points (1.27 ± 0.16 & 1.05 ± 0.11, respectively, *vs* vehicle-treated cells; Fig. 1A & Supplementary Fig. 1A). By contrast, the expression of *Pparα* and *Pparγ* was down-regulated after 24 h of stimulus (Supplementary Fig. 1A), whereas that of *pparβ* was increased at this time point (Supplementary Fig. 1A). Because *klf5*, *klf6* and *klf9* are closely associated with the pro-adipogenic phenotype and with liver disease [10,12,13], we determined the *klf5*, *klf6 and klf9* transcript expression in response to PA. Here, we observed a significant effect within the first 12 h of treatment (*klf6* 1.9 ± 0.19 & *klf9* 1.87 ± 0.16, *vs* vehicle-treated cells, $p < 0.001$; Fig. 1A), which was more evident on *klf5* gene expression (4.14 ± 0.81 *vs* control cells, $p < 0.0001$; Fig. 1A). In contrast to the increased expression observed during the first 12 h of treatment, overall *klf* expression tended to decrease when PA exposure was extended to 24 h (Supplementary Fig. 1B). Moreover, HepG2 cells exposed to another steatotic insult using fructose, showed that the transcript levels of the *ppar* and *klf* genes also increased at 24 h of exposure, as determined by qPCR (Supplementary Fig. 2A). In addition, lipid storage was evaluated using Oil Red O staining based on a spectrophotometric assay [23]. Compared to those of vehicle-treated cells, whole extracts of PA-treated cells displayed significantly increased absorbance at 510 nm (1.55 ± 0.2-fold change *vs* vehicle, $p < 0.001$; Supplemental Fig. 1C), indicating that PA indeed induced a steatosis-like condition in HepG2 cells. Fructose treatment for 24 h also induced lipid storage (data not shown). These results are in line with those observed previously [25], and are in line with evidence indicating that KLF6, which regulates the *pparα* gene expression, is involved in the reduction of the liver triglyceride content, thereby improving glucose and insulin tolerance *in vivo* [26].

3.2. PA treatment induces the expression of lipogenic genes

We then determined the expression of genes associated with lipid and glucose metabolism in response to the PA stimulus. We observed that several lipid-related genes were up-regulated at 12 h; among the most significant were the fatty acid binding protein 1 (*fabp1*) and phosphoenolpyruvate carboxykinase 1 (*pck1*) (Fig. 1C); suggesting that a pro-adipogenic phenotype may have been activated. We also observed that the carnitine-palmitoyltransferase 1A (*cpt1a*) genes, was also up-regulated (Fig. 1C), suggesting that the cells were actively processing the excessive triglyceride content to return to the basal levels. By contrast, fructose treatment significantly increased the expression of the *pparγ*, *klf5*, *cpt1* and cytochrome P45 family 1 (*cyp1a*) genes (Supplementary Fig. 2A & B) and only slightly up-regulated lipogenic and gluconeogenic transcripts (Supplementary Fig. 2B). Similar results were obtained when *ppar* and *klf* gene expression was determined in the

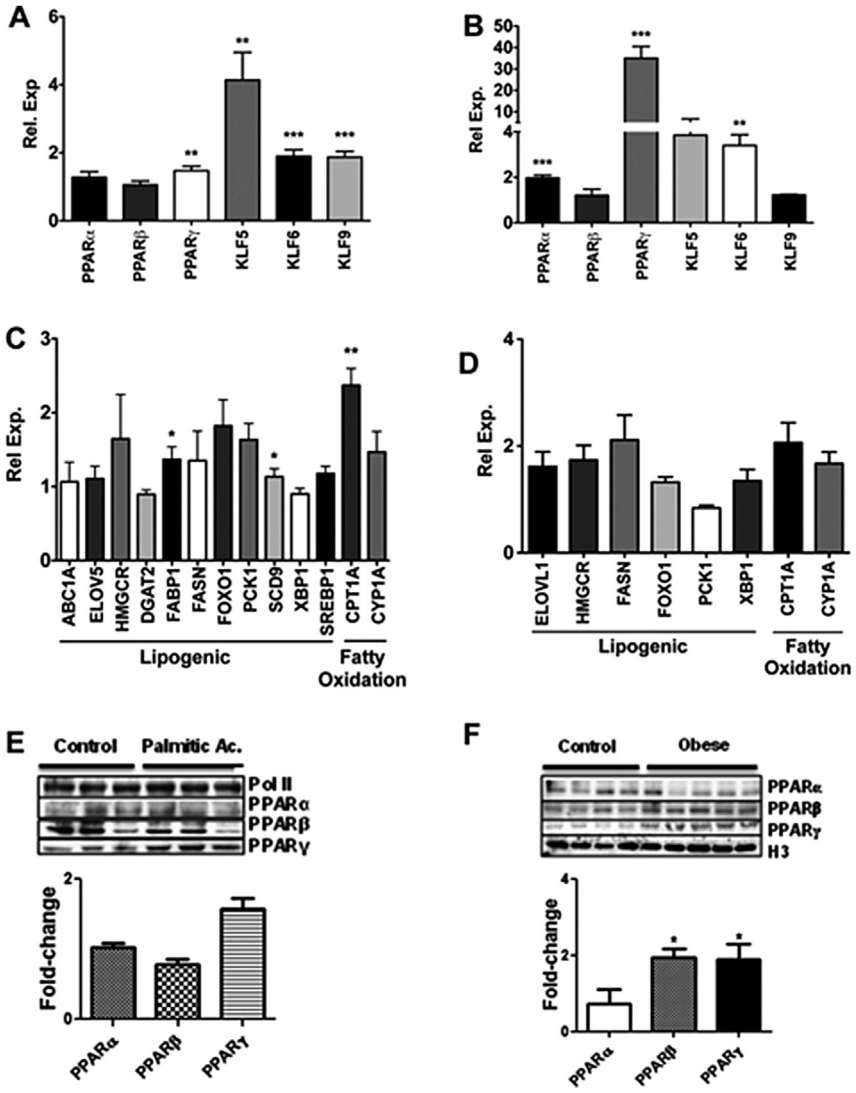

Fig. 1. Palmitic acid increases ppar and klf expression *in vitro* e *in vivo*. The expression of *ppar*, *klf*, lipogenic and fatty acid oxidation genes in the HepG2 cells (**A**, **C**), or mice liver samples (**B**, **D**), are shown. Data indicate the mean ± SEM of the three independent experiments. (**E** & **F**) The PPAR protein fold-change compared with control conditions *in vitro* (**E**) or *in vivo* (**F**) is shown. Data represents the mean fold change of at least two independent experiments. Pol II, polymerase II; H3 histone 3. ***p < 0.0001, **p < 0.01, *p < 0.05; *vs* control conditions.

liver from diet-induced obesity (DIO) model mice. Despite the increased levels observed *in vitro*, *pparγ* was significantly up-regulated in the obese mice (34.97 ± 5.5 *vs* control mice, Fig. 1B). Evaluation of the *klf5* and *klf6* transcripts revealed the same tendency (Fig. 1B). The *klf9* transcript was not altered in the obese mice (Fig. 1B). Histological analysis of the liver tissue from the DIO mice, showed both, a strong Oil Red O staining, and positive *pparγ* immunoreactivity (Supplementary Fig. 1D). The mRNA and protein levels of *ppar* and *klf* and *hmgcr*, *fasn*, and *foxo1* genes, in the DIO mice, were consistent with the corresponding *in vitro* data (Fig. 1C–F). Although the expression of *pparα* and *pparβ* at the protein level was opposite to that at the mRNA level, suggesting the existence of a post-transcriptional regulatory mechanism.

3.3. The pparγ gene promoter recruits KLF6, and KLF9 under steatotic conditions

Our expression profile experiments indicated that the expression of *pparγ*, *klf5*, *klf6*, and *klf9* was significantly increased *in vitro*, in response to the steatotic insult. Therefore, we examined whether PA modulated the occupancy of the *pparγ* gene promoter region by these KLFs. First, we performed an *in silico*-based analysis using the algorithm described in RSA-tools [27] and the reported *klf* frequency matrix (FM) for the consensus binding site CACCC (Jaspar database: http://jaspar.genereg.net/; [28]). We identified four potential binding sites in the *pparγ* gene promoter relative to the transcription start site (TSS) (Fig. 2), which likely explains some of the previously reported KLF-mediated steatotic effects [29]. Once identified, we designed specific oligonucleotides encompassing these Krüppel sites (KEM) [22] (Fig. 2 & Supplementary Fig. 3). To evaluate their functionality we performed ChIP experiments using specific antibodies against *klf5*, *klf6* or *klf9*. Analysis of the *pparγ* promoter region under basal conditions revealed that KEM1 did not interact with *klf6* or *klf9* but slightly interacted with *klf5*, whereas the KEM2 site, interacted with all three of these genes (Fig. 2). The KEM3 site displayed no difference in the promoter occupancy under this condition (Fig. 2). By contrast, we observed that the KEM1 site significantly recruited the three KLFs (KLF5, KLF6 & KLF9) at 12 h of treatment (Fig. 2). However, the KEM2 and KEM3 sites displayed no significant changes in their occupancy (Fig. 2). We used a polymerase II antibody as a positive control for poised transcriptional genes [30]. The identical experiments were

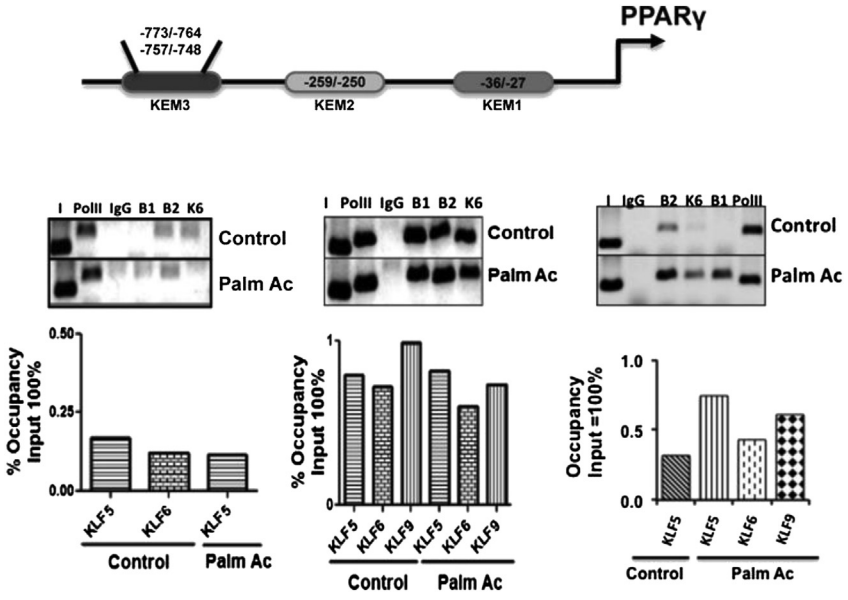

Fig. 2. PPARγ proximal gene promoter region recruits klf6 & klf9 in steatotic conditions. A schematic representation of the PPARγ gene proximal promoter region is shown (Top), and the putative KLF binding sites (KEM1-3) are indicated. Band pixel density quantitation was determined, and the percentage of enrichment is shown compared to the input signal (bottom). Numbers indicate relative position of each KEM element respect to the transcriptional initiation site (arrow). I, input; IgG, immunoglobulin; B1, Krüppel-like factor 9; Krüppel-like factor 5; K6, Krüppel-like factor; Palm Ac., palmitic acid.

performed using specific oligonucleotides for the *pparα* and *pparβ* gene promoters. Here, we observed that KLF6 significantly interacted with KEM1 on *pparα* promoter, while KEM2 and KEM3 sites, displayed no differences between the basal and stimulated conditions (Supplementary Fig. 3A). On the other hand, KLF9 was released from the KEM3 site on the *pparβ* promoter, suggesting a potential repressive effect of this gene in the presence of PA (Supplementary Fig. 3B). Taken together, these results strongly suggest that KLF6 and KLF9 play a negative role over the *pparγ* gene expression in basal conditions, as they tend to be recruited to the

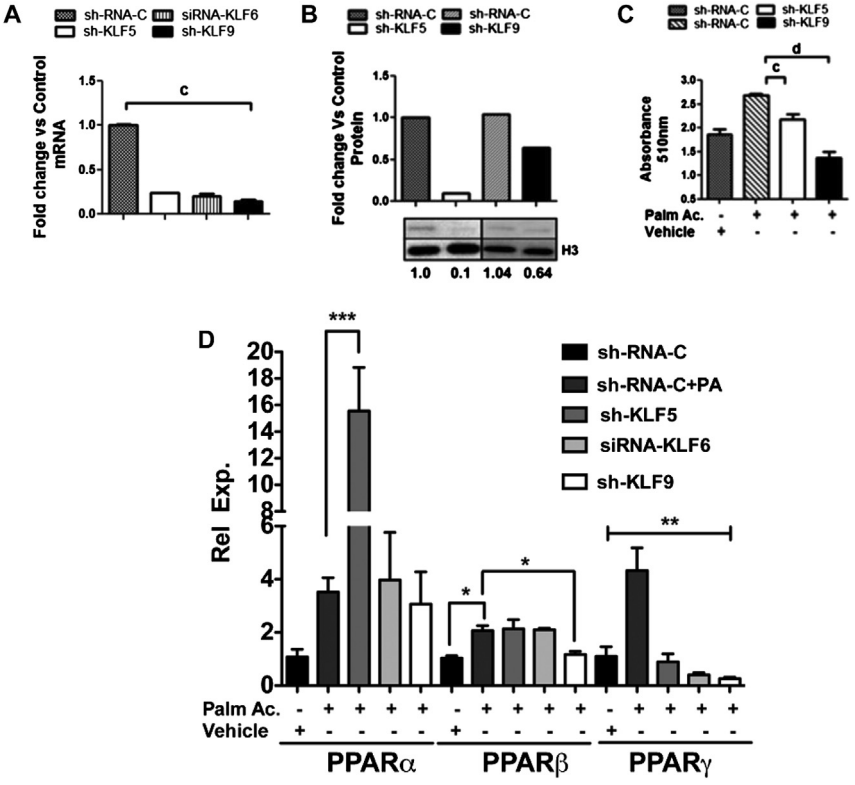

Fig. 3. Klf5, Klf6, and Klf9 lost-of-function repress the pparγ expression *in vitro*. HepG2 cells were stably transfected with short-hairpin RNAs (sh-RNA). The *klf5*, *klf6* or *klf9* transcripts (**A**), or proteins (**B**), are indicated. The triglyceride content was determined by Oil Red staining and the absorbance at 510 nm is indicated (**C**). The PPAR expression was determined by qPCR (**D**). All the experiments show the mean ± SEM of three independent experiments, at 12 h of exposure. Numbers in (**B**) indicate the fold-change using the sh-control RNA cells as 1. H3, histone 3. Palm Ac, palmitic acid. *p < 0.05; **p < 0.005; ***p < 0.001 *vs* sh-RNA-C + PA.

promoter during a steatotic insult. It is possible that the increase in *pparα* expression, occurred in response to the fasting period that we performed prior to the treatment; in this context, the increased expression of one of *pparα* gene targets, *cpt1*, was consequently also observed (Supplementary Fig. 4).

3.4. Silencing of klf5, klf6, and klf9, represses pparγ gene expression under steatotic conditions

The KLF5, KLF6 and KLF9 are recruited to occupy the gene promoter region of *pparγ* under steatotic conditions, likely facilitating its expression (see Fig. 1). To further corroborate this idea, we interfered with *klf5*, *klf6* or *klf9* gene expression by using a sh-RNA strategy. HepG2 cells stably expressing short-hairpin RNAs (sh-RNA) displayed significantly reduced the expression of KLF5, KLF6 and KLF9, at the transcript and protein levels (Fig. 3A and B). The cells transfected with a scrambled short-hairpin control vector and exposed to PA (sh-RNA-C + PA), markedly increased *pparα* and *pparγ* gene expression (3.52 ± 0.5 & 4.32 ± 0.9, respectively, *vs* the control cells; Fig. 3D); the expression of *pparβ* was also significantly increased, although to a lesser extent (2.06 ± 0.2 *vs* the control cells; Fig. 3D). Conversely, PA treatment in the sh-RNA-*klf5* cells significantly reduced *pparγ* gene expression by approximately 60% compared with the sh-RNA-C + PA cells (Fig. 3D). A similar result was obtained for *pparβ* (2 ± 0.3 *vs* the sh-RNA-C + PA cells). Moreover, the sh-RNA-*klf6* or sh-RNA-*klf9* under steatotic conditions displayed a stronger repression of *pparγ* expression (0.4 ± 0.08 & 0.26 ± 0.05-fold, respectively, *vs* the sh-RNA-C + PA cells; $p < 0.001$). Interestingly, in the presence of PA, the sh-RNA-*klf5* cell line displayed a remarkable increase in *pparα* gene expression, which was more than 14-fold greater than that of the PA-stimulated control cells (sh-RNA-C + PA), indicating that *klf5* might repress the *pparα* expression under basal conditions (Fig. 3D). These results suggested that *klf5* might regulate the beta-oxidation process by reducing the expression of *pparα*. Alternatively, *klf6*, and *klf9* positively regulate the expression of *pparγ*, a pro-lipogenic gene that is closely associated with lipogenesis. KLF9 has also been associated with the pre-adipocyte differentiation process by regulating *c/ebpβ* expression in 3T3-L1 cells via its direct interaction with a KEM-like responsive element located at −874 bp in the *c/ebpβ* regulatory region [31].

We next measured the transcript levels of lipogenic, gluconeogenic, and fatty acid oxidation genes. This analysis indicated that the PA-treated sh-RNA-*klf5* HepG2 cell line notably increased in the expression of the lipogenic gene *fasn* and augments the expression of *dgat2*; both genes play key roles in triglyceride synthesis. Fatty acid oxidation-related genes, such as *cpt1a* and *cyp1a1*, were markedly up-regulated in this cell line (Supplementary Fig. 4), whereas the transcript expression of pro-lipogenic and oxidative genes, such as *fabp1*, *srebp1c*, *cpt1a*, and *cyp1a1*, were notably higher in the sh-RNA-*klf6* and sh-RNA-*klf9* cell lines (Supplementary Fig. 4). These results suggest that KLFs induce a pro-lipogenic phenotype in response to the PA treatment *in vitro*.

3.5. The gain-of-function of KLFs counteracts the pro-steatotic effect of by pparγ on HepG2 cells

Because KLF6 and KLF9 regulate the expression of *pparγ* under steatotic conditions *in vitro*, we sought to investigate whether *ppars* are indeed, gene targets of the *klfs*. To this end we transiently transfected HepG2 cells with *klf5*-, *klf6*-or *klf9*-cDNA-encoding plasmids (see Methods). PCR and Western-blot analysis indicates that *klf5*, *klf6*, and *klf9* were significantly overexpressed in our cells at the transcript and protein levels, respectively (Fig. 4A-B). Interestingly, the overexpression of the *klf6* or *klf9* proteins resulted in

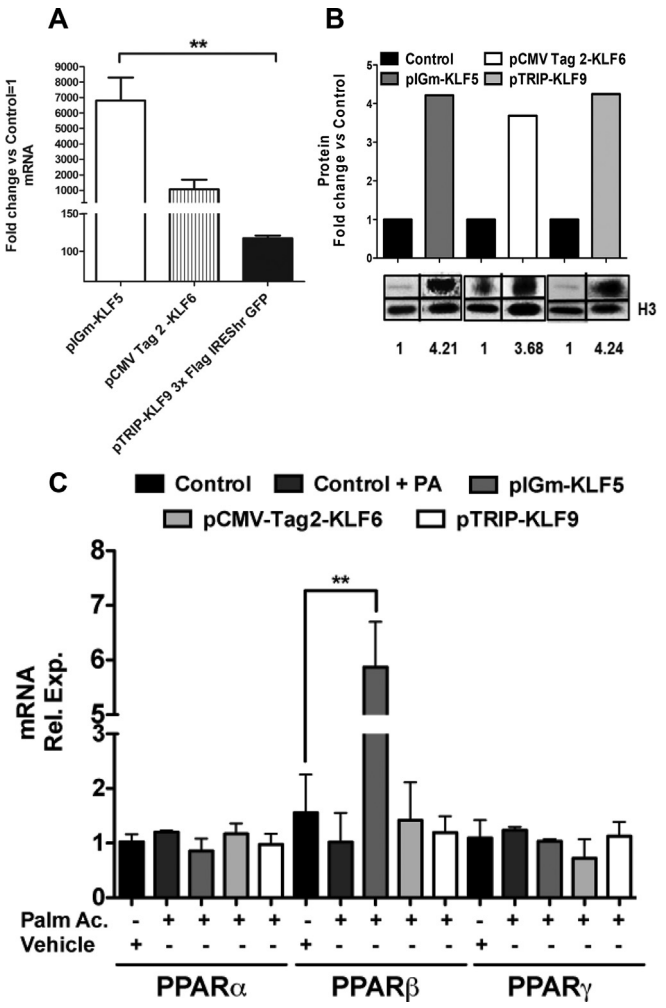

Fig. 4. KLF gain-of-function prevents the pro-steatotic effect mediated by palmitic acid over the HepG2 cells. The cells were treated for 12 h with PA. The expression of klf5, klf6 or klf9 at the transcript (A) or the protein (B) levels, are indicated. The pparα, pparβ/δ, and pparγ gene expression levels are indicated in presence of KLF5, KLF6 or KLF9 overexpression (C). Each plot represents the mean ± SEM of three independent experiments. C+PA, empty-vector cells treated with palmitic acid; PA, palmitic acid; *p < 0.05; **p < 0.01 vs C+PA control cells.

the restoration of *pparγ* expression to its basal levels. Conversely, the gain-of-function of *klf5* did not reverse the effect observed in the corresponding gene silencing experiments (Fig. 4C). However, *pparβ* expression was highly induced when *klf5* was overexpressed (5.87 ± 0.8 *vs* control conditions; $p < 0.001$). We also observed that increasing the expression of *klf6* or *klf9* resulted in a modest but not significant induction of *pparβ* (Fig. 4C). By contrast, the *pparα* gene did not display any significant change in the context of *klf5*, *klf6* or *klf9* overexpression. However, when *klf6* was overexpressed, *pparα* tended to be down-regulated (Fig. 4C), although this effect was not significant. KLFs are subjects to regulation via post-translational modifications [32]. In this regard, it is known that KLF5 and KLF6 are subject to ubiquitination *in vitro* in response to DNA damage [33]. Thus, it is possible that HepG2 cells might activate an ubiquitin-based mechanism that regulates the expression level of KLF5 or KLF6, thereby muting the responses observed in our gain-of-function experiments. Hepatocytes are known to express the WWP1 ligase gene, which is a key enzyme in the ubiquitination mechanism [34]; it remains to be established whether this ligase is also expressed under our *in vitro* conditions.

In conclusion, our data clearly established that in response to the palmitic acid, hepatocyte recruit members of the KLF family proteins to the promoter region of the *pparγ* gene, increasing its transcriptional activity. The increased expression of this receptor contributes to the development of steatosis, as *pparγ* activity promotes triglyceride accumulation within the hepatocytes.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.01.145.

Transparency document

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