



*Toxicomics Report*

## Cyclin-dependent kinase inhibitor p21/Cip1 down-regulates the expression of proteasome activator PA28 $\gamma$ in normal hepatocyte-derived cells

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**ABSTRACT** — We previously reported that knockdown of the farnesoid X receptor (FXR), a bile acid-activated nuclear receptor, increases mRNA level of cyclin-dependent kinase inhibitor p21/Cip1 in normal hepatocyte-derived cell line Fa2N-4 and hepatocellular carcinoma cell line HepG2. Although p21/Cip1 protein levels of HepG2 cells are also increased by FXR knockdown, elevated levels of p21/Cip1 mRNA does not cause an increase in p21/Cip1 protein levels of Fa2N-4 cells, indicating post-transcriptional suppression of p21/Cip1 expression in Fa2N-4 cells. Given that degradation of p21/Cip1 by proteasomes is mediated by PA28 $\gamma$ , an activator of the 20S proteasome, we examined whether p21/Cip1 regulates the expression of PA28 $\gamma$ , a proteasome activator, in HepG2 and Fa2N-4 cells. In Fa2N-4 cells, ectopic expression of p21/Cip1 increased the mRNA and protein levels of PA28 $\gamma$ . PA28 $\gamma$  expression was down-regulated by knockdown of p21/Cip1. In contrast, in HepG2 cells, neither ectopic expression nor knockdown of p21/Cip1 affected the expression of PA28 $\gamma$ . Therefore, p21/Cip1 likely down-regulates its own expression in a post-transcriptional manner by stimulating the expression of the proteasome activator PA28 $\gamma$  in normal hepatocyte-derived cells, while hepatocellular carcinoma cells lack such feedback regulation of p21/Cip1 expression.

**Key words:** Farnesoid X receptor, Cyclin-dependent kinase inhibitor p21/Cip1, Proteasome activator PA28 $\gamma$

### INTRODUCTION

We previously reported that knockdown of the farnesoid X receptor (FXR), a bile acid-activated nuclear receptor, effectively suppressed the proliferation of the human hepatocellular carcinoma cell line HepG2, and that this effect was accompanied by elevated protein expression of cyclin-dependent kinase (CDK) inhibitors p16/INK4a and p21/Cip1 (Fujino *et al.*, 2012; Fujino *et al.*, 2015). In contrast, growth of the primary human hepatocyte-derived cell line Fa2N-4 was not affected by treatment with FXR siRNA. Interestingly, the protein expres-

sion levels of p16/INK and p21/Cip1 were unchanged while mRNA levels of these CDK inhibitors were elevated in Fa2N-4 cells that were treated with FXR siRNA (Fujino *et al.*, 2015). Because the protein expression levels of these CDK inhibitors in FXR-knockdown Fa2N-4 cells were elevated in the presence of the proteasomal inhibitor MG132, these CDK inhibitors may be subjected to proteasomal degradation, thereby counteracting the increased expression of their cognate mRNAs, leading to similar levels of p16 and p21 proteins in control and FXR knockdown Fa2N-4 cells. Given that degradation of p21/Cip1 by proteasomes is mediated by PA28 $\gamma$ , an activator

of the 20S proteasome (Masson *et al.*, 2009), we examined whether p21/Cip1 regulates the expression of PA28 $\gamma$ , a proteasome activator, in Fa2N-4 and HepG2 cells.

## MATERIALS AND METHODS

### Materials

Antibodies (Abs) specific for  $\beta$ -actin (C-2) and p21 (H-164) were purchased from Santa Cruz Biotechnology, Santa Cruz, Dallas, USA. Abs specific for PA28 $\gamma$  (N-term) was purchased from Invitrogen, Camarillo, USA. ECL<sup>TM</sup> anti-mouse IgG, horseradish peroxidase linked whole antibody (from sheep) and ECL<sup>TM</sup> anti-rabbit IgG, horseradish peroxidase linked whole antibody (from donkey) were purchased from GE Healthcare, Buckinghamshire, UK.

### Cell culture

The human hepatocellular carcinoma cell line HepG2 and primary human hepatocyte-derived cell line Fa2N-4 were obtained from ATCC. These cell lines were maintained in Dulbecco's modified eagle medium containing 10% fetal calf serum, 50 units/mL penicillin G sodium salt, and 50  $\mu$ g/mL streptomycin sulfate and cultured in a humidified atmosphere of 8.5% CO<sub>2</sub> at 37°C.

### Construction of pcDNA-p21/Cip1 expression vector

The pcDNA-p21/Cip1 expression vector was prepared as follows: cDNA was obtained using ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan) using total RNA extracted from ACHN cells. Polymerase chain reaction (PCR) was then performed using the cDNA as a template (forward primer: CGGGATCCGAAGTCAGTTCCTTGTGGA; reverse primer: GGAATTC AAGGCAGAA-GATGTAGAGC), and the amplified product corresponding to the open reading frame of p21/Cip1 (GenBank no. NM000389) was subcloned into the *Bam*HI/*Eco*RI site of the pcDNA3.1 (+) vector.

### RNA interference experiments

To knockdown endogenous p21/Cip1, cells were seeded on 60-mm dishes at a density of  $3.0 \times 10^5$  Fa2N-4 cells or  $2.0 \times 10^5$  HepG2 cells per dish and transfected with siRNA against p21/Cip1 (50 nM) using HiPerfect Transfection Reagent (Qiagen, Hülsterweg, NLD) according to the manufacturer's instructions. After incubating for 24 hr, protein extracts for immunoblotting analyses or total RNAs for real-time PCR analyses were prepared. In the RNA interference experiments, "Nonsilencing Control" siRNA (#1022076) from Qiagen was used as a con-

trol.

### Quantification of mRNA

Quantification of mRNA was performed using real-time PCR. Briefly, 5  $\mu$ g of total RNA was reverse-transcribed using ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan). The resultant cDNA was subjected to real-time PCR analysis using a TaqMan Gene Expression Assay kit (Applied Biosystems, Tokyo, Japan). mRNA levels were determined using TaqMan assay mixtures for PA28 $\gamma$  (Hs00195072) and  $\beta$ -actin (4310881E). Amplification and quantification were performed using the StepOne Real-Time PCR System (Applied Biosystems). PA28 $\gamma$  mRNA levels were normalized to those of  $\beta$ -actin as an internal control. Data were analyzed using Student's t-test.

### Immunoblotting

Cells were washed with PBS, and cell extracts were prepared using SDS sample buffer without loading dye. After normalization of protein content via the protein assay, the dye was added to samples. The samples were subjected to SDS-PAGE and immunoblotting analyses. For detection of p21/Cip1, PA28, and  $\beta$ -actin, PVDF membranes were incubated with primary antibody (1:200) (Santa Cruz Biotechnology) for 2 hr. Immunocomplexes on the PVDF membranes were visualized using enhanced chemiluminescence western blotting detection reagents (GE Healthcare).

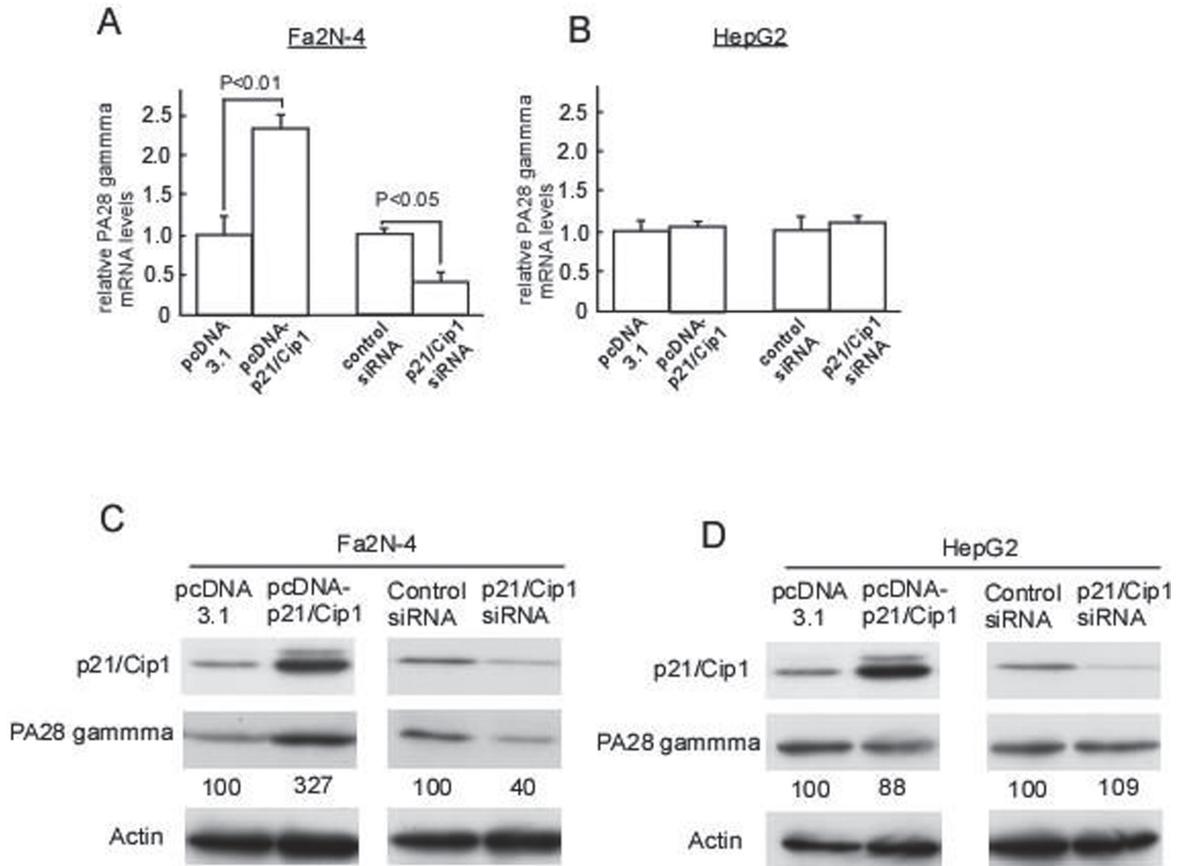
### Statistical analysis

Data are presented as the mean  $\pm$  S.E.M. of three experiments performed in triplicate and were analyzed using Student's t-test.

## RESULTS AND DISCUSSION

The normal human hepatocyte-derived cell line Fa2N-4 was transfected with a p21/Cip1 expression vector and PA28 $\gamma$  levels were determined. As shown in Fig. 1A and C, ectopic expression of p21/Cip1 increased the mRNA (Fig. 1A) and protein (Fig. 1C) levels of PA28 $\gamma$  in Fa2N-4 cells. This result was supported by findings from the p21/Cip1 knockdown assay, which showed that PA28 $\gamma$  expression was down-regulated by knockdown of p21/Cip1 (Fig. 1A and C). In contrast, in the human hepatocellular carcinoma cell line HepG2, neither ectopic expression nor knockdown of p21/Cip1 affected the expression of PA28 $\gamma$  (Fig. 1B and D). Therefore, p21/Cip1 likely down-regulates its own expression in a post-transcriptional manner by stimulating the expression of the proteasome activator

p21/Cip1 down-regulates PA28γ expression

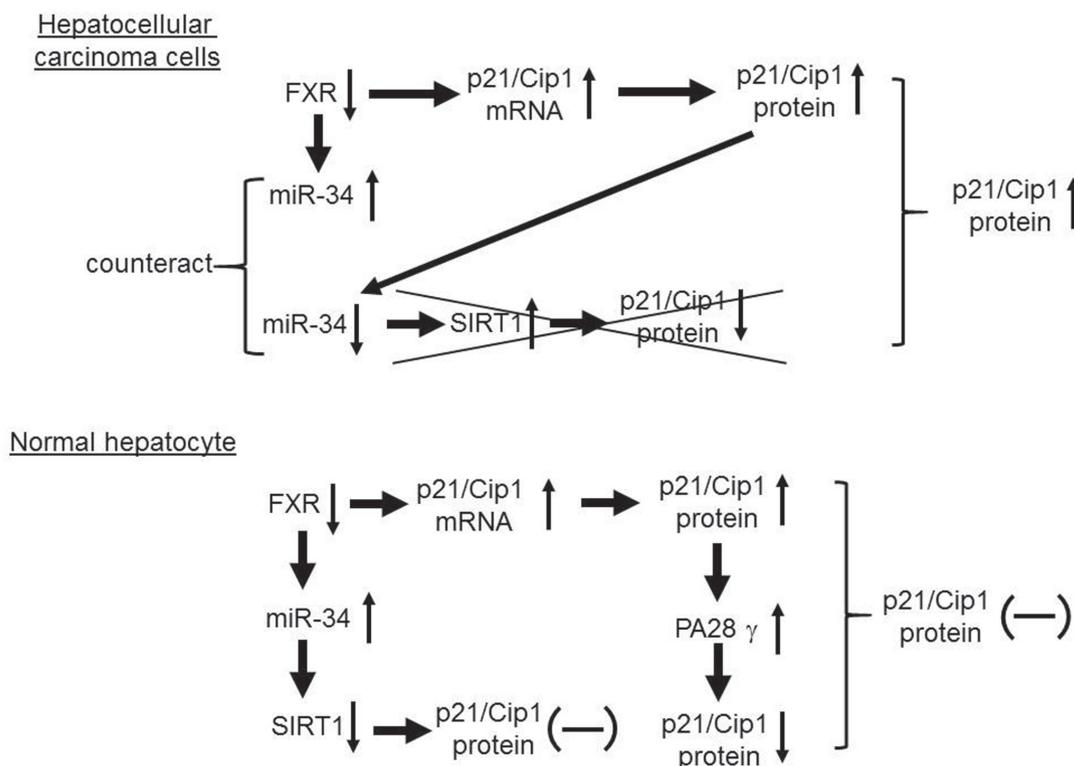


**Fig. 1.** p21/Cip1 stimulates the expression of the proteasome activator PA28γ in normal hepatocyte-derived cells without affecting expression in hepatocellular carcinoma cells. Fa2N-4 and HepG2 cells were seeded at  $4.0 \times 10^5$  and  $2.0 \times 10^5$  cells/60-mm dish, respectively. For ectopic expression of p21/Cip1, these cells were transfected with pcDNA 3.1 (+) or pcDNA-p21/Cip1 expression vector (left side of A-D). For knockdown of p21/Cip1, these cells were transfected with control or p21/Cip1 siRNA (right side of A-D). (A and B) After 24 hr, total RNA was extracted, and quantification of PA28γ mRNA was performed as described in Materials and Methods. Data are presented as the mean  $\pm$  S.E.M. of three experiments performed in triplicate and analyzed using Student's t-test. (C and D) After 24 hr, cell extracts were subjected to immunoblotting to detect p21/Cip1, PA28γ, and  $\beta$ -actin protein. Quantification of the bands was conducted using densitometric analysis (IMAGE GAUGE 4.0).

PA28γ in normal hepatocyte-derived cells, while hepatocellular carcinoma cells lack such feedback regulation of p21/Cip1 expression.

Importantly, we previously reported that p21/Cip1 protein down-regulates its own expression in microRNA miR-34- and NAD<sup>+</sup>-dependent deacetylase SIRT1-dependent manner (Fujino *et al.*, 2017). That is, p21/Cip1 down-regulates the expression of miR-34, resulting in an increase in SIRT1, a direct target of miR-34 (Yamakuchi *et al.*, 2008), and SIRT1 reduces p21/Cip1 protein levels. This mechanism raises the question of why the FXR knockdown-mediated increase in p21/Cip1 protein levels is not a transient event in hepatocellular carcinoma cells (Fujino *et al.*, 2015). Interestingly, Lim *et al.* reported

that FXR down-regulates the expression of miR-34 (Lim *et al.*, 2018), suggesting that FXR knockdown in hepatocellular carcinoma cells up-regulates the expression of miR-34. Therefore, the p21/Cip1-mediated down-regulation of miR-34 is likely counteracted by the FXR knockdown-mediated increase in miR-34. As a consequence, in hepatocellular carcinoma cells, FXR knockdown-mediated up-regulation of p21/Cip1 mRNA expression results in an increase in p21/Cip1 protein levels. In normal hepatocyte-derived cells, p21/Cip1 does not down-regulate miR-34 (Fujino *et al.*, 2017). Moreover, although SIRT1 level are reduced by FXR knockdown in a miR-34-dependent manner, SIRT1 down-regulation does not cause an increase in p21/Cip1 level (Fujino *et al.*, 2018). There-



**Fig. 2.** Regulation of p21/Cip1 expression by FXR knockdown in normal hepatocyte-derived cells and hepatocellular carcinoma cells. In hepatocellular carcinoma cells, FXR knockdown up-regulates p21/Cip1 mRNA expression. Because FXR knockdown also up-regulates the expression of miR-34, this counteracts the p21/Cip1-mediated down-regulation of miR-34 and subsequent decrease in p21/Cip1 protein levels. Therefore, in hepatocellular carcinoma cells, FXR knockdown-mediated up-regulation of p21/Cip1 mRNA expression results in an increase in p21/Cip1 protein levels. In normal hepatocyte-derived cells, p21/Cip1 does not down-regulate miR-34 (Fujino *et al.*, 2017). Moreover, although SIRT1 level are reduced by FXR knockdown in a miR-34-dependent manner, SIRT1 down-regulation does not cause an increase in p21/Cip1 levels (Fujino *et al.*, 2018). Therefore, stimulation of p21/Cip1 mRNA expression is simply counteracted by stimulated degradation of p21/Cip1 caused by p21/Cip1 itself in a PA28 $\gamma$ -dependent manner.

fore, stimulation of p21/Cip1 mRNA expression is simply counteracted by stimulated degradation of p21/Cip1 by p21/Cip1 itself in a PA28 $\gamma$ -dependent manner. The mechanism by which FXR knockdown regulates the expression of p21/Cip1 in normal hepatocyte-derived cells and hepatocellular carcinoma cells is summarized in Fig. 2.

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**Conflict of interest**---- The authors declare that there is no conflict of interest.

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