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Leptin-derived peptides block leptin-induced proliferation by reducing expression of pro-inflammatory genes in hepatocellular carcinoma cells

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ABSTRACT

The obesity-regulated gene, leptin, is essential for diet. Leptin resistance causes obesity and related diseases. Certain types of diet are able to decrease leptin resistance. However, leptin has been shown to be correlated with inflammation and stimulate proliferation of various cancers. Two synthetic leptin derivatives (mimetics), OB3 and [D-Leu-4]-OB3, show more effective than leptin in reducing obesity and diabetes in mouse models. OB3 inhibits leptin-induced proliferation in ovarian cancer cells. However, effects of these mimetics in hepatocellular carcinoma (HCC) have not been investigated. In the present study, we examined the effects of OB3 and [D-Leu-4]-OB3 on cell proliferation and gene expressions in human HCC cell cultures. In contrast to what was reported for leptin, OB3 and [D-Leu-4]-OB3 reduced cell proliferation in hepatomas. Both OB3 and [D-Leu-4]-OB3 stimulated expression of pro-apoptotic genes. Both compounds also inhibited expressions of pro-inflammatory, proliferative and metastatic genes and *PD-L1* expression. In combination with leptin, OB3 inhibited leptin-induced cell proliferation and expressions of pro-inflammation-, and proliferation-related genes. Furthermore, the OB3 peptide inhibited phosphoinositide 3-kinase (PI3K) activation which is essential for leptin-induced proliferation in HCC. These results indicate that OB3 and [D-Leu-4]-OB3 may have the potential to reduce leptin-related inflammation and proliferation in HCC cells.

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1. Introduction

Obesity is a metabolic disorder characterized by an excessive body mass index (BMI) of $\geq 30\,kg/m^2$. Obesity continues to be a worldwide health challenge at an increasing rate. The positive effects of appetite control have long been appreciated, but pharmacotherapeutic support appears to be necessary in order to stem the growth of this debilitating disease. The accumulation of adipose tissues can have serious adverse consequences on general health and is also associated with several diseases including cardiovascular diseases and cancers. Obesity is associated with increased secretion of cytokines and hormones from adipocytes (Jung and Choi, 2014). One such hormone is leptin, a polypeptide hormone also known as the "satiety hormone", a gene product of the *ob* gene. Physiologically, leptin regulates energy balance by inducing satiety, but this function is lost in obese persons who exhibit a certain resistance to the hormone despite high serum levels (Considine et al., 1996).

Increased serum leptin concentration has been shown to relate to inflammatory responses (Lee et al., 2007; van Dielen et al., 2001). Leptin stimulates expression of pro-inflammatory genes (Kang et al., 2016; Paz-Filho et al., 2012). Those genes play vital roles in cancer development and progression (Lu et al., 2006; Murata, 2018). Evidence has shown that leptin may promote initiation of various inflammation-related cancers including cholangiocarcinomas (Fava et al., 2008), ovarian cancer (Chin et al., 2017), endometrial cancer (Calle and Kaaks, 2004; Mendez-Lopez et al., 2017), and breast cancer (Pan et al., 2018). However, controversial results have been reported in other types of cancer. Leptin stimulates thyroid cancer growth in the studies by Hedayati (Hedayati et al., 2011; Park et al., 2016) but not by Yang' group (Yang et al., 2016). On the other hand, the leptin-related synthetic peptide mimetic, OB3, inhibits cell proliferation and also leptin-induced cancer cell growth in ovarian cancer (Chin et al., 2017).

Leptin activates AKT and Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathways to promote endometrial cancer growth and invasiveness (Sharma et al., 2006). Leptin induces invasion via activated extracellular signal-regulated kinase 1/2 (ERK1/2) and STAT3. In addition, leptin promotes cancer cell migration and is shown to be anti-apoptotic in cholangiocarcinomas (Chin et al., 2017; Fava et al., 2008). Leptin and its related biologically active synthetic peptides, OB3 and [D-Leu-4]-OB3, stimulate STAT3 activation via phosphorylation of ERK1/2 and phosphoinositide 3-kinase (PI3K) (Fava et al., 2008). OB3 induces STAT3 activation via ERK1/2 phosphorylation in HeLa cells (Lin et al., 2014). Besides, [D-Leu-4]-OB3-induced serine phosphorylation of STAT3 is predominantly via ERK1/2 activation (Lin et al., 2014). Activation of PI3K is primarily involved in tyrosine phosphorylation of STAT3 (Vogt and Hart, 2011) and may sequentially stimulate cancer cell proliferation by leptin and other growth factors (Procaccini et al., 2009; Somasundar et al., 2004). Additionally, studies have shown that Ser727 phosphorylated STAT3 translocating to mitochondria and increasing activity of electron transport and glycolysis leads to survival of the cell and inhibits the apoptosis (Ottani et al., 2013).

Hepatocellular carcinoma (HCC) is a disease with high mortality and unfortunately, there is no satisfactory treatment. Forkhead Box M1 (FOXM1), one of the cell-cycle proteins involved in mitotic progression, has shown to involve in hepatocellular carcinoma proliferation (Mukhopadhyay et al., 2015). Evidence indicates that FoxM1 is upregulated by obesity which is positively related to leptin concentration in serum (Davis et al., 2010). In the present study, we investigated the mechanisms involved in the anti-inflammation and anti-proliferation induced by the synthetic peptide leptin mimetics, OB3 and [D-Leu-4]-OB3, in two hepatocellular cancer (HCC) cell lines, HepG2 cells and Hep3B cells. Our results indicated that OB3 and [D-Leu-4]-OB3 inhibited expression of pro-inflammatory, proliferative and metastatic genes in HCC cells. Additionally, they also inhibited proliferation of HCC cells. [D-Leu-4]-OB3 and OB3 showed different effect on expression of pro-inflammatory genes. Inactivation of PI3K either by OB3 peptides or LY294002 reduced proliferative gene expressions. In combination with leptin, OB3 inhibited leptin-induced expression of pro-inflammatory and proliferative genes. Furthermore, it also inhibited leptin-induced proliferation in HCC cells. Therefore, inhibition of PI3K by OB3 peptide plays a crucial role in blocking leptin-induced gene expression and proliferation in HCC cells.

2. Materials and methods

2.1. Cell lines

Human HCC HepG2 (ATCC® HB-8065™) and Hep3B (ATCC® HB-8064™) cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) by the Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan). These cell lines were tested and authenticated by the BCRC. Cells purchased from the BCRC were passaged for less than 6 months after thawing and maintained for further study in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). All cell cultures were maintained in a 5% CO_2/95% air incubator at 37 °C. Prior to treatment, cells were placed in 0.25% hormone-stripped FBS-containing medium for 2 days.

2.2. Peptides, antibodies, and reagents

OB3 and [D-Leu-4]-OB3 were commercially prepared as C-terminal amides by Atlantic Peptides (Lewisburg, PA, USA). Goat anti-rabbit immunoglobulin G (IgG) and rabbit anti-mouse IgG were obtained from Dako (Carpentaria, CA, USA). Rabbit polyclonal and monoclonal antibodies to pERK1/2, pSTAT3 (Ser-727 or Tyr-705), and pPI3K p85 (Tyr458) were purchased from Cell Signaling Technology (Beverly, MA, USA). Rabbit polyclonal antibody to PI3K (p85) and mouse monoclonal antibody to GAPDH were purchased from GeneTex International (Hsinchu City, Taiwan). LY294002, a specific PI-3K inhibitor, was purchased from Calbiochem (San Diego, CA, USA).

2.3. Western blot analysis

This method was as described in our previous studies (Chin et al., 2015; Lin et al., 2016a, 2016b; Yang et al., 2016). In brief, protein samples were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A 40-μg quantity of protein was loaded in each well with 5x sample buffer, and protein samples were resolved by electrophoresis at 100 V for 2 h. The resolved proteins were transferred from the polyacrylamide gel to Millipore Immobilon-PSQ Transfer nitrocellulose membranes (Millipore, Billerica, MA, USA) with the Mini Trans-Blot® Cell (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked with a solution of 2% bovine serum albumin (BSA) in Tris-buffered saline. Membranes were incubated with primary antibodies to PI3K (p85), pPI3K (p85), pSTAT3 (Tyr705), pSTAT3 (Ser727), p44/42ERK (pERK1/2), or GAPDH at 4 °C overnight and washed, and proteins were detected with horseradish peroxidase (HRP)-conjugated secondary antibodies and the Immobilon™ Western HRP Substrate Luminol Reagent (Millipore). Images of the Western blots were visualized and recorded by Amersham Imager 600 (GE Healthcare Life Sciences, Pittsburgh, PA, USA).

2.4. Real-time quantitative (qPCR)

This technique was described previously (Chin et al., 2014; Lin et al., 2016a, 2016b; Yang et al., 2016). HCC cells were treated with OB3 or its derivatives in the presence or absence of inhibitors for 24 h. Total RNA was extracted with genomic DNA eliminated by an Illustra RNAspin Mini RNA Isolation Kit (GE Healthcare Life Sciences, Buckinghamshire, UK). Complementary (c)DNA was transcribed using 1 μg of DNase I-treated total RNA with a RevertAid H Minus First Strand cDNA Synthesis Kit (Life Technologies, Carlsbad, CA, USA). cDNA was used as a template for further real-time PCRs. The real-time PCRs were carried out using a QuantiNova™ SYBR® Green PCR Kit (Qiagen, Hilden, Germany) on a CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Initial denaturation was set to 95 °C for 5 min, followed by 40 cycles of denaturing at 95 °C for 5 s and combined annealing/extension at 60 °C for 10 s, as described in the manufacturer's instructions. The primer sequences were as follows: *Homo sapiens cyclin D1 (CCND1), forward 5′-CAAGGCCTGAACCTGAG GAG-3′ and reverse 5′-GATCACTCTGGAGAGGAAGCG-3′ (accession no.: NM_053056); Homo sapiens caspase-2, apoptosis-related cysteine peptidase (CASP2), forward 5′-GCATGTACTCCCACCGTTGA-3′ and reverse 5′-GACAGGCGGAGCTTCTTGTA-3′ (accession no.: NM_032982.3); Homo sapiens interleukin-1 β (IL-1β), forward 5′-CTTCGAGGCACAAGGCACA-3′ and reverse 5′-GCTTCAGACACTTGAGCAATGA-3′ (accession number NM_000576.2); Homo sapiens matrix metalloproteinase 9 (MMP9), forward 5′-TGTACCGCTATGGTTACACTCG-3′ and reverse 5′-GGCAGGGA CAGTTGCTTCT-3′ (accession no.: NM_004994.2); H. sapiens cyclin-dependent kinase inhibitor 1A (p21), forward 5′-CTGGGGATGTCCGTCAG AAC-3′ and reverse 5′-CATTAGCGCATCACAGTCGC-3′ (accession no.: BT006719.1); Homo sapiens proliferating cell nuclear antigen (PCNA), forward 5′-TCTGAGGGCTTCGACACCTA-3′ and reverse 5′-TCATTGCCG GCGCATTTTAG-3′ (Accession No.: BC062439.1); Homo sapiens PD-L1, forward 5′-GTTGAAGGACCAGCTCTCCC-3′ and reverse 5′-ACCCCTGCA TCCTGCAATTT-3′ (accession No. AY254342.1); Homo sapiens tumor necrosis factor-α (TNF-α), forward: 5′-CCTGCTGCACTTTGGAGTGA-3′, reverse: 5′-TCGAGAAGATGATCTGACTGCC-3′ (accession No. NM_000594.3); Homo sapiens vascular endothelial growth factor A (VEGF-A), forward 5′-TACCTCCACCATGCCAAGTG-3′ and reverse 5′-GATGAT TCTGCCCTCCTCCTT-3′ (accession No.: NM_001204384.1) and Homo sapiens 18S ribosomal RNA (18S), forward 5′-GTAACCCGTTGAACCCC ATT-3′ and reverse 5′-CCATCCAATCGGTAGTAGCG-3′ (*accession no. *NR_003286)*. Relative gene expressions (normalized to the *18s* reference gene) were calculated according to the ΔΔCT method. Fidelity of the PCR was determined by a melting point analysis.

2.5. Cell viability assay

HepG2 and Hep3B cells were plated at a density of 10^4 cells/well in 96-well plates. OB3 or leptin was refreshed daily with medium. Cell viability was determined using a CyQUANT® NF Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR, USA) at 96 h after treatment. Briefly, medium was removed, and cells were incubated with CyQUANT® NF reagent for 1 h at 37 °C according to the manufacturer's instructions. Plates were then analyzed using a microplate reader (Varioskan™ Flash Multimode Reader, Thermo Scientific, Waltham, MA, USA) (with excitation at 485 nm and emission at 530 nm).

2.6. Flow cytometric analysis

HepG2 cells were grown in 100-mm tissue culture dishes until 80% confluent and treated daily with different reagents for 48 h with refreshed medium containing reagents. Cells were harvested by trypsinization, washed with phosphate-buffered saline (PBS) and re-suspended in 200 µL PBS (10^5 to 10^6 cells). To quantify cellular DNA contents, cells were permeabilized by fixation with 70% ethanol for 30 min at 4 °C. Samples were stored in 70% ethanol at −20 °C for up to several weeks prior to propidium iodide (PI) staining and the flow cytometric analysis. DNase-free RNase (2.5 µL) was added to the cell suspension, and incubation was carried out at 37 °C for 1 h; cells were then collected and maintained in the dark at room temperature for 30 min. Flow cytometry was carried out on a FACSCalibur™ (Becton Dickinson, Franklin Lakes, NJ, USA) instrument, using CellQuest software to determine the DNA content. A fluorescence-activated cell sorting (FACS) analysis used Annexin V-FITC and PI. Relative percentages of cells in the G_0/G_1, S, and G_2/M phases were calculated from FL-2 histograms using ModFit LT software.

2.7. Quantification of results and statistical analysis

Densities of Western blots and gene expressions of the real-time qPCR were analyzed by IBM SPSS Statistics software version 19.0 (SPSS, Chicago, IL, USA). One-way analysis of variance (ANOVA) with Duncan's post-hoc test was used to analyze the differences between experimental groups followed by a Student's t-test. Student's t-tests for paired data were also used in some cases as indicated. $p < 0.05$ was considered statistically significant.

3. Results

The leptin derivatives, OB3 and [D-Leu-4]-OB3, suppress pro-inflammatory, proliferative and metastatic genes but stimulate expressions of anti-proliferative genes in HCC cells. The leptin derivative, OB3 peptide, inhibits ovarian cancer cell growth (Chin et al., 2017). To investigate the effects of OB3 and [D-Leu-4]-OB3 on expression of pro-inflammatory, proliferative and anti-proliferative genes, two hepatocellular cancer (HCC) cell lines, HepG2 cells and Hep3B cells were used. HepG2 is hepatitis B virus-negative and non-tumorigenic, whereas Hep3B is hepatitis B virus-positive and tumorigenic (Qiu et al., 2015). In addition, HepG2 cells are characterized with having more hepatocyte features, whereas Hep3B cells are more of fibrotic/fibroblasts type. Those two cell lines also show different gene expression with different drug effects and signal transduction pathways (Qiu et al., 2015). HepG2 cells were treated with OB3 or [D-Leu-4]-OB3 for 24 h 10 µM of [D-Leu-4]-OB3 but not OB3 suppress *IL-1β* expression in HepG2 cells (Fig. 1A). Both OB3 and [D-Leu-4]-OB3 suppress *TNF-α* significantly (Fig. 1A). Both inhibited expression of *PD-L1* and *CCND1* significantly as well (Fig. 1B). OB3 increased more expressions of the pro-apoptotic *CASP2* and *p21* genes than [D-Leu-4]-OB3 did (Fig. 1C).

Similar studies were conducted in another HCC cell line, Hep3B cells. Interestingly, only OB3 reduced expressions of *IL-1β*. On the other hand, [D-Leu-4]-OB3 and OB3 suppressed *TNF-α* expression (Fig. 2A). OB3 significantly reduced *PD-L1* expression and both OB3 and [D-Leu-4]-OB3 significantly repressed expressions of *CCND1* (Fig. 2B). Similar to the observation in HepG2, OB3 increased higher ratios for *CASP2* and *p21* than [D-Leu-4]-OB3 in Hep3B cells did (Fig. 2C).

Suppression of PI3K activation is essential for OB3 derivative-induced regulation of gene expressions in HCC cells. To investigate the activated signaling involved in OB3- and [D-Leu-4]-OB3-regulated gene expressions in HCC cells, HepG2 cells were treated with OB3 and [D-Leu-4]-OB3 in the presence or absence of PI3K inhibitor, LY294002, for 24 h. Total RNA was extracted and a qPCR was conducted to examine expressions of pro-apoptotic, proliferative, and metastatic genes. Both OB3 and [D-Leu-4]-OB3 activated the *p21* pro-apoptotic gene in two different HCC cell lines. Expression of *p21* was further enhanced when LY294002 was present (Fig. 3A and B). On the other hand, expression of both *CCND1* and *MMP9* was inhibited by OB3, and LY294002 enhanced the suppressive effect. Western blot analyses also indicated that constitutive PI3K phosphorylation was inhibited by both OB3 and [D-Leu-4]-OB3 (Fig. 3C). However, the two OB3 peptides showed difference in regulation of ERK1/2 activation and patterns of STAT3 phosphorylation in HepG2 and Hep3B cells. Results shown in Fig. 3 indicated that blockage of PI3K activity further enhanced OB3 derivative-suppressed expressions of proliferative and metastatic genes. Alternatively, it enhanced OB3 derivative-induced expression of a pro-apoptotic gene. Although LY294002 inhibits the secretion of the small surface antigen of HBV in a PI3K-AKT-independent manner (Xiang and Wang, 2018), there is no difference of LY294002-induced activities in hepatitis B virus-positive Hep3B and negative HepG2 cells.

Leptin and OB3 derivatives induce different cell cycle patterns in HCC cells. To further understand the mechanisms involved in OB3 peptide-induced anti-proliferation, HCC cells were treated with either

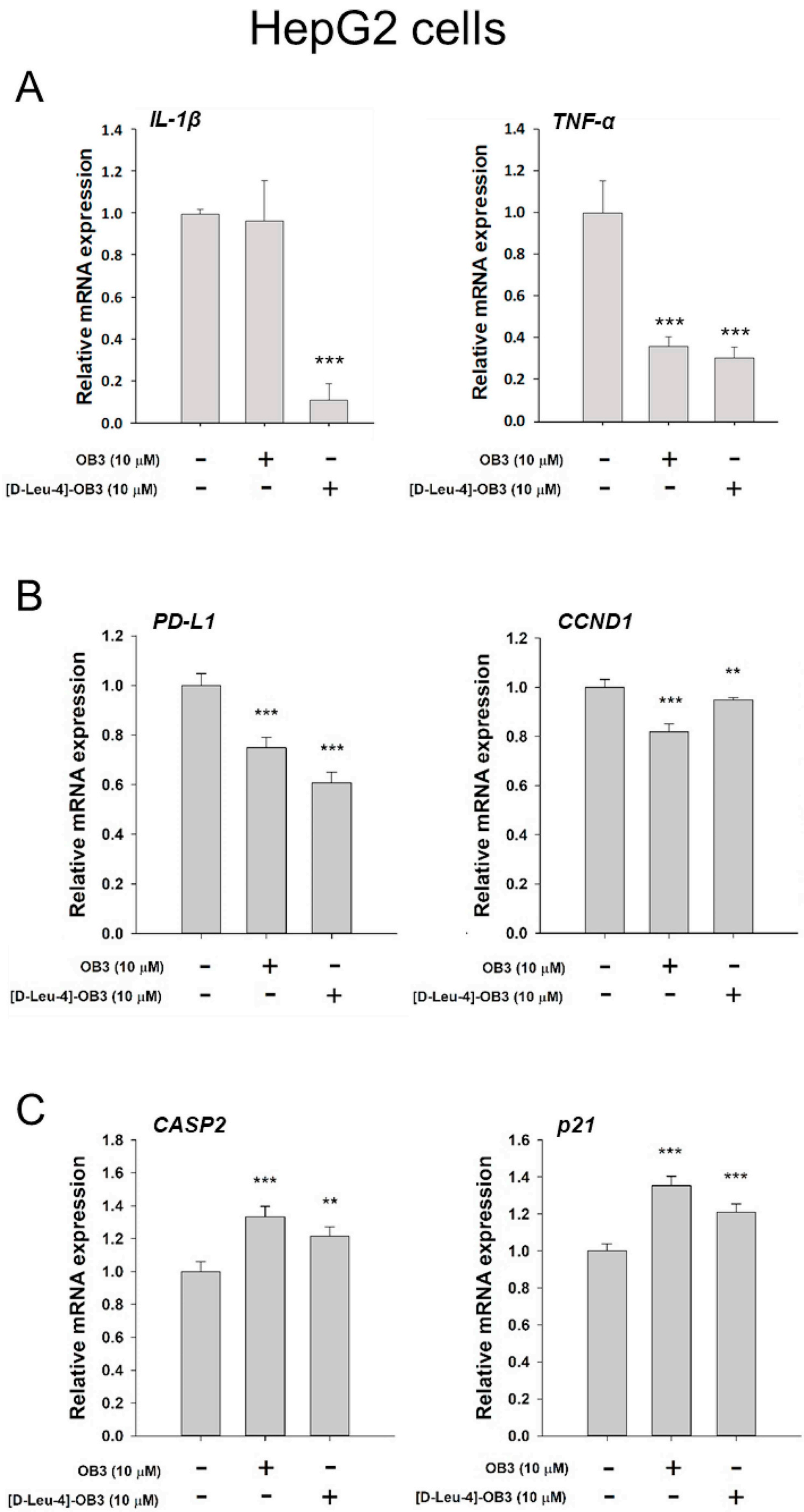

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Fig. 1. OB3 derivatives regulate the expression of cancer-related genes in hepatocellular carcinoma HepG2 cells. HepG2 cells were seeded in 6-well trays and treated with 10 μM of OB3 or [D-Leu-4]-OB3 for 24 h before being harvested. Total RNA was extracted, and qPCR was conducted for (A) *IL-1β* and *TNF-α*; (B) *PD-L1* and *CCND1*; and (C) *CASP2* and *p21*. Number of independent experiments (N) = 3. Data are expressed as mean ± SD; $^{**}p < 0.01$, $^{***}p < 0.001$, compared with untreated control.).

10 μM OB3, [D-Leu-4]-OB3 or 0.1 μM leptin for 48 h and harvested for a flow cytometric assay. Treatment with leptin or OB3 but not [D-Leu-4]-OB3 on cells for 48 h significantly decreased cell population in S phase. Both OB3 and [D-Leu-4]-OB3 decreased cell population in G_2/M phase. On the other hand, OB3 and [D-Leu-4]-OB3 affected cell population in G_0/G_1 phase with 1.35-fold and 1.45-fold of increase respectively (from 30.12 ± 5.62% in control to 40.89 ± 0.93% in (OB3), 43.79 ± 2.14% in ([D-Leu-4]-OB3) (Fig. 4). Conversely, leptin only increased cell population in G_2/M slightly. The results indicated that OB3 peptides may induce G_0/G_1 phase arrest in HCC cells.

OB3 derivatives inhibit leptin-induced gene expressions in HCC cells. In order to determine if OB3 peptide derivatives can block leptin-induced gene expressions in hepatoma cells, studies were conducted to investigate the effect of the combination of OB3 peptides and leptin on gene expressions in HCC cells. HepG2 cells were treated with either OB3 (10 μM), [D-Leu-4]-OB3 (10 μM), leptin (100 nM), or a combination of the two agents for 24 h. Cells were harvested, and total RNA was extracted. qPCR was conducted for *TNF-α*, and *PCNA*, pro-apoptotic genes, *CASP2* and *p21*, *VEGF,* and checkpoint gene, *PD-L1*. OB3 and [D-Leu-4]-OB3 induced expression of both *CASP2* and *p21* and suppressed expression of *TNF-α*, proliferative and metastatic genes in HepG2 cells (Fig. 5). On the other hand, leptin activated expression of *TNF-α*, *PCNA* and *PD-L1* and suppressed expression of *CASP2* and *p21* in HCC cells (Fig. 5). Co-treatment of cells with OB3 or [D-Leu-4]-OB3 and leptin inhibited leptin-induced expression of *TNF-α*, *PCNA* and *PD-L1*. Alternatively, co-treatment also reversed leptin-suppressed expression of *CASP2* and *p21*. These results further support OB3 derivatives being able to suppress leptin-regulated biological activities.

The OB3 peptide inhibits leptin-induced proliferation in HCC cells. To further investigate the inhibitory effect of OB3 on leptin-induced proliferative gene expression correlated with leptin-induced proliferation of hepatoma cells, studies were conducted on the proliferative effects of leptin and the OB3 peptide in HCC cells. HepG2 and Hep3B cells were treated with leptin (100 nM) or different concentrations of OB3 (0.1 and 10 μM) with refreshed medium and peptides daily for 4 days. Results from the CyQUANT® NF Cell Proliferation Assay showed that leptin stimulated the proliferation of HepG2 cells, but treatment with OB3 reduced cell proliferation, even though the OB3 concentration was 100-fold higher than that of leptin. Moreover, 10 μM of OB3 significantly inhibited HepG2 cell proliferation around 50.67 ± 3.56%. Remarkably, the proliferative effect of leptin was reduced by co-treatment of cells with the OB3 peptide (Fig. 6A). Similar results were observed in Hep3B cells that OB3 was able to reduce effect of leptin (Fig. 6B).

In summary, OB3 and its derivatives inhibited PI3K to activate the expression of pro-apoptotic genes and inhibit expressions of pro-inflammatory, proliferative and metastatic genes. These results suggest that OB3 and [D-Leu-4]-OB3 have the potential to inhibit the proliferation of HCC cells.

4. Discussion

In this manuscript, we demonstrate that OB3, a leptin-derived peptide, not only inhibited cell growth but also reduced leptin-induced gene expression and cell growth of HCC cells. In addition, studies were conducted on the signal transduction pathways and gene expressions related to proliferation and metastasis induced by OB3 derivatives in HCC cells. Inflammation plays a vital role in obesity-related diseases including cancers. Leptin induces not only expression of pro-inflammatory genes but also growth of related-cancers.

To explore the possibility of anti-inflammation-related proliferation by OB3 derivatives, we examined expressions of pro-inflammatory, proliferative, anti-proliferative genes in HCC cells. OB3 and [D-Leu-4]-OB3 suppressed different pro-inflammatory genes in different HCC cells (Figs. 1 and 2). However, they suppressed proliferative gene but activated expressions of anti-proliferative genes in almost the same patterns (Figs. 1 and 2). On the other hand, studies of signaling activated by OB3 and [D-Leu-4]-OB3 indicated that inhibition of PI3K activation by PI3K inhibitor, LY294002 enhanced OB3- and [D-Leu-4]-OB3-induced expressions of anti-proliferative gene, *p21*. In addition, PI3K inhibitor, LY294002 further enhanced their ability to inhibit the expression of proliferative and angiogenic genes (Fig. 3A and B). Indeed, Both OB3- and [D-Leu-4]-OB3 inhibited PI3K activation (Fig. 3C). These results indicate that inhibition of PI3K activity plays a vital role in OB3 derivative-induced cellular activities.

Studies have indicated that leptin and OB3/[D-Leu-4]-OB3 have different receptor (Lin et al., 2014). Therefore, OB3 won't compete with leptin on leptin receptor to block leptin-induced signal transduction and proliferative activity. It is likely that OB3 and [D-Leu-4]-OB3 suppress PI3K activation which is essential for leptin-induced biological functions. This inhibitory effect of OB3/[D-Leu-4]-OB3 on leptin-induced activities was reproduced by the PI3K inhibitor, LY294002. Studies indicated that the ERK1/2 and PI3K signaling pathways contribute to the proliferative effect of leptin in prostate cells (Hoda et al., 2012; Huang et al., 2011). OB3 peptide activated ERK1/2 but inhibited activation of PI3K in both cancer cell lines (Fig. 3C). On the other hand, [D-Leu-4]-OB3 peptide inhibited ERK1/2 in HepG2 cells but activated ERK1/2 in Hep3B cells. However, it inhibited activation of PI3K which was shown to be involved in leptin-induced cancer proliferation (Fig. 3C).

Interestingly, results presented in Fig. 3 indicate that OB3 derivatives inhibited proliferation of HCC cells via inhibiting PI3K activity, and LY294002 further enhanced this anti-proliferative effect. In adipocyte, insulin-stimulated leptin releasing is mediated by a Ca^{2+}-required PI3K/Akt pathway (Wang et al., 2014). In addition, the leptin-induced increase in hepatic sympathetic outflow is also PI3K-dependent (Tanida et al., 2015). Furthermore, leptin-induced neuroprotection is mediated by the PI3K/Akt signal transduction pathway (Zhang et al., 2013).

Previous studies revealed that obesity-induced thyroid tumor growth and cancer progression are mediated by activated phosphorylation of the oncogenic Janus kinase 2 (JAK2) and STAT3 transcription factors in prostate cancer cells (Hoda et al., 2012; Huang et al., 2011; Kim et al., 2013; Park et al., 2016). Constitutively activated STAT3 stimulates cancer progression in different types of human cancers (Carpenter and Lo, 2014; Lo et al., 2008). Leptin activates ERK1/2 and STAT3 to stimulate STAT3 binding to Estrogen receptor alpha (ERα) and increase the binding of ERα to ERα-responsive promoters in ovarian cancer cells (Chin et al., 2017). Interestingly, the crosstalk was inhibited by the ERα-specific antagonist, ICI 182,780, the specific STAT3 inhibitor, AG490, and to a lesser extent, by PI3K inhibition (Carpenter and Lo, 2014). These observations suggest that signal activation by leptin is via the STAT3-ERα axis to the ERα-responsive gene promoter in ovarian cancer cells. OB3 derivative increased tyrosine and serine phosphorylation of STAT3 in HepG2 but not in Hep3B cells, whereas [D-Leu-4]-OB3 inhibited tyrosine phosphorylation in both cell lines (Fig. 3C). However, OB3 was shown to activate STAT3 and inhibit leptin-induced proliferation in ovarian cancer cells (Chin et al., 2017). These results suggest that activation of STAT3 might not be essential for OB3's regulation of cell proliferation.

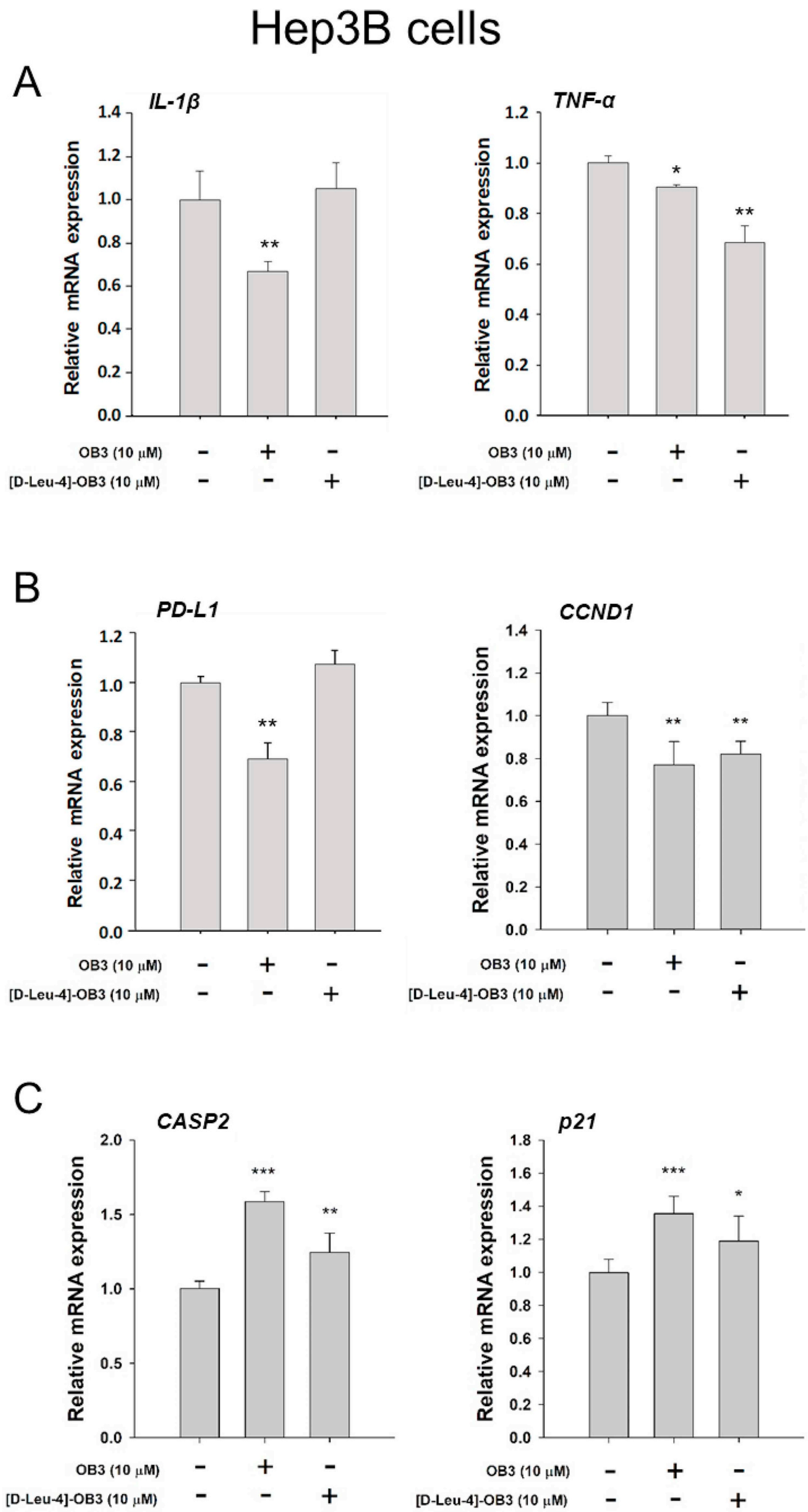

Fig. 2. OB3 derivatives regulate expressions of cancer-related genes in hepatocellular carcinoma Hep3B cells. Hep3B cells were seeded in 6-well trays and treated with 10 μM of OB3 and [D-Leu-4]-OB3 respectively for 24 h before being harvested. Total RNA was extracted, and qPCR was conducted for (A) *IL-1β* and *TNF-α*; (B) *PD-L1* and *CCND1*; and (C) *CASP2* and *p21*. N = 3. Data are expressed as mean ± SD. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared with untreated control.).

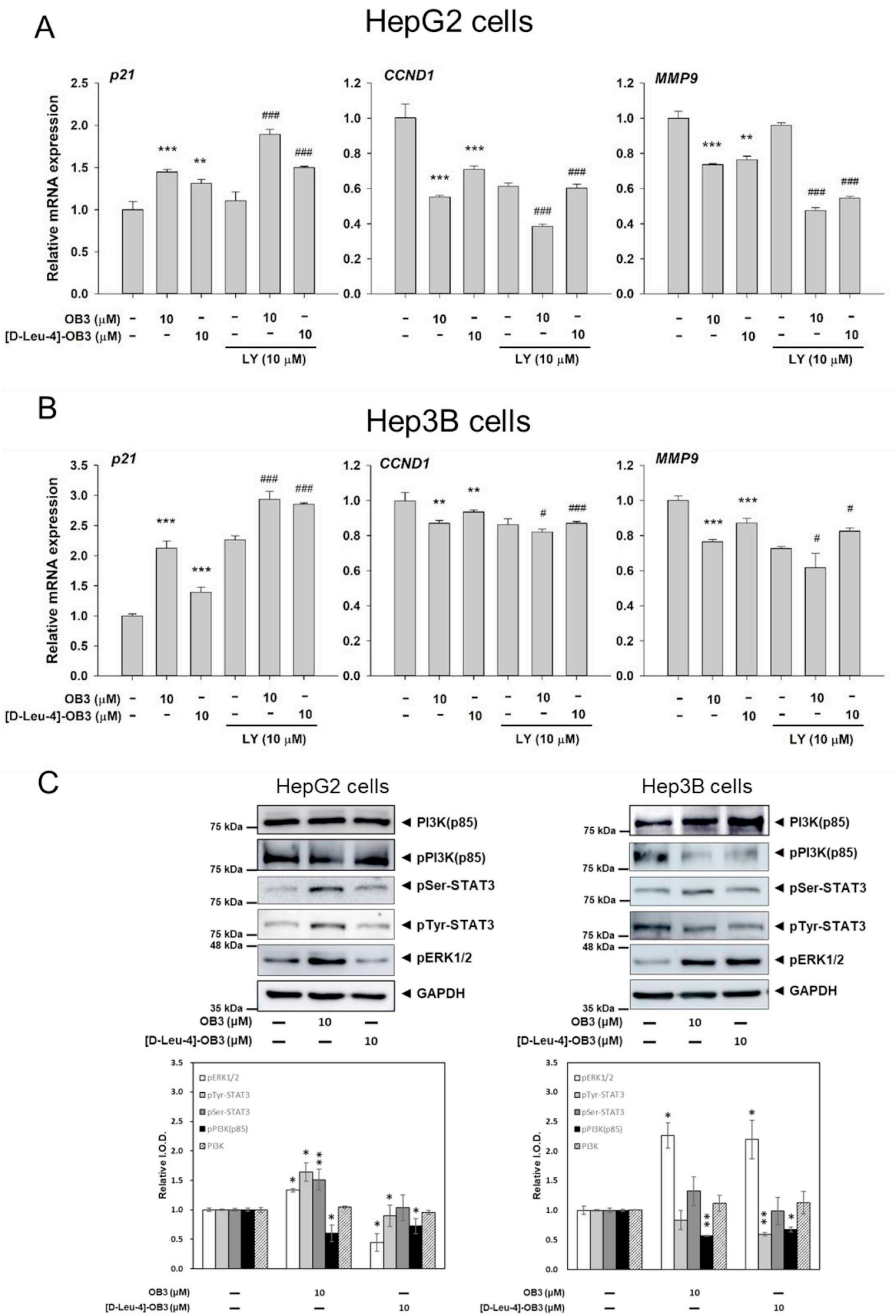

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Fig. 3. OB3 derivatives inactivate phosphoinositide 3-kinase (PI3K) activity to regulate gene expressions in hepatocellular carcinoma cells. (A) HepG2 and (B) Hep3B cells were seeded in 6-well trays and treated with 10 μM of OB3 or [D-Leu-4]-OB3 in the presence or absence of the PI3K inhibitor, LY294002, for 24 h prior to being harvested. Total RNA was extracted, and qPCR was conducted for *p21*, *CCND1*, and *MMP9.* **(C)** Cells were treated with 10 μM of OB3 or [D-Leu-4]-OB3 for 24 h prior to being harvested. Total proteins were harvested, and Western blot analyses were conducted for pPI3K, pSTAT3s and pERK1/2. N = 3. Data are expressed as mean ± SD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, compared with untreated control. $\#p < 0.05$, $\#\#\#p < 0.001$, compared with LY294002.

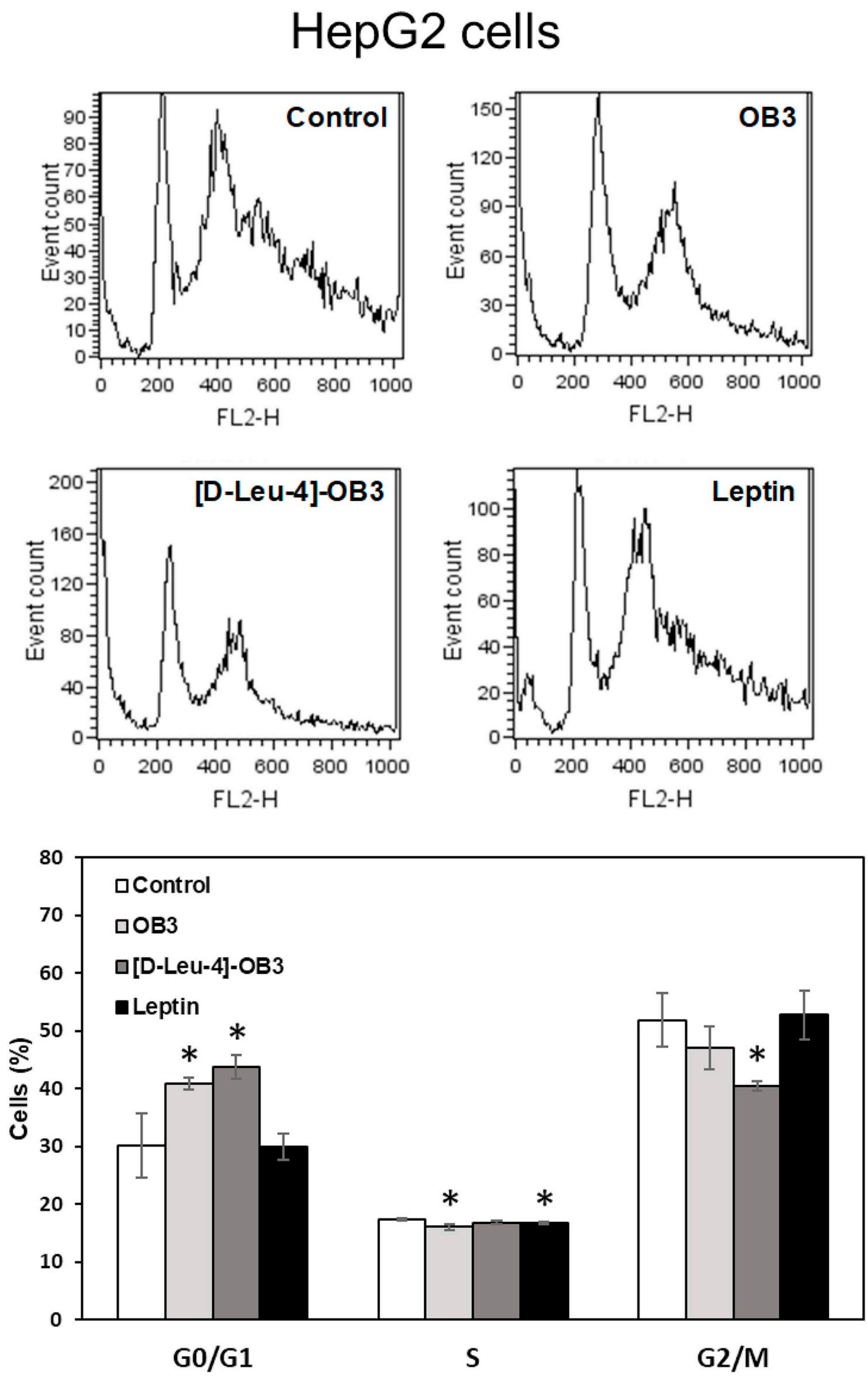

Fig. 4. Leptin and OB3 derivatives induce different cell cycles in HCC cells. HepG2 cells were treated with leptin, OB3, [D-Leu-4]-OB3 or leptin for 48 h. Cell cycle analysis was conducted as described. Cells were harvested, and flow cytometric assay was conducted as described in "Materials and Methods". N = 3. Data are expressed as mean ± SD. $*p < 0.05$ compared with untreated control.

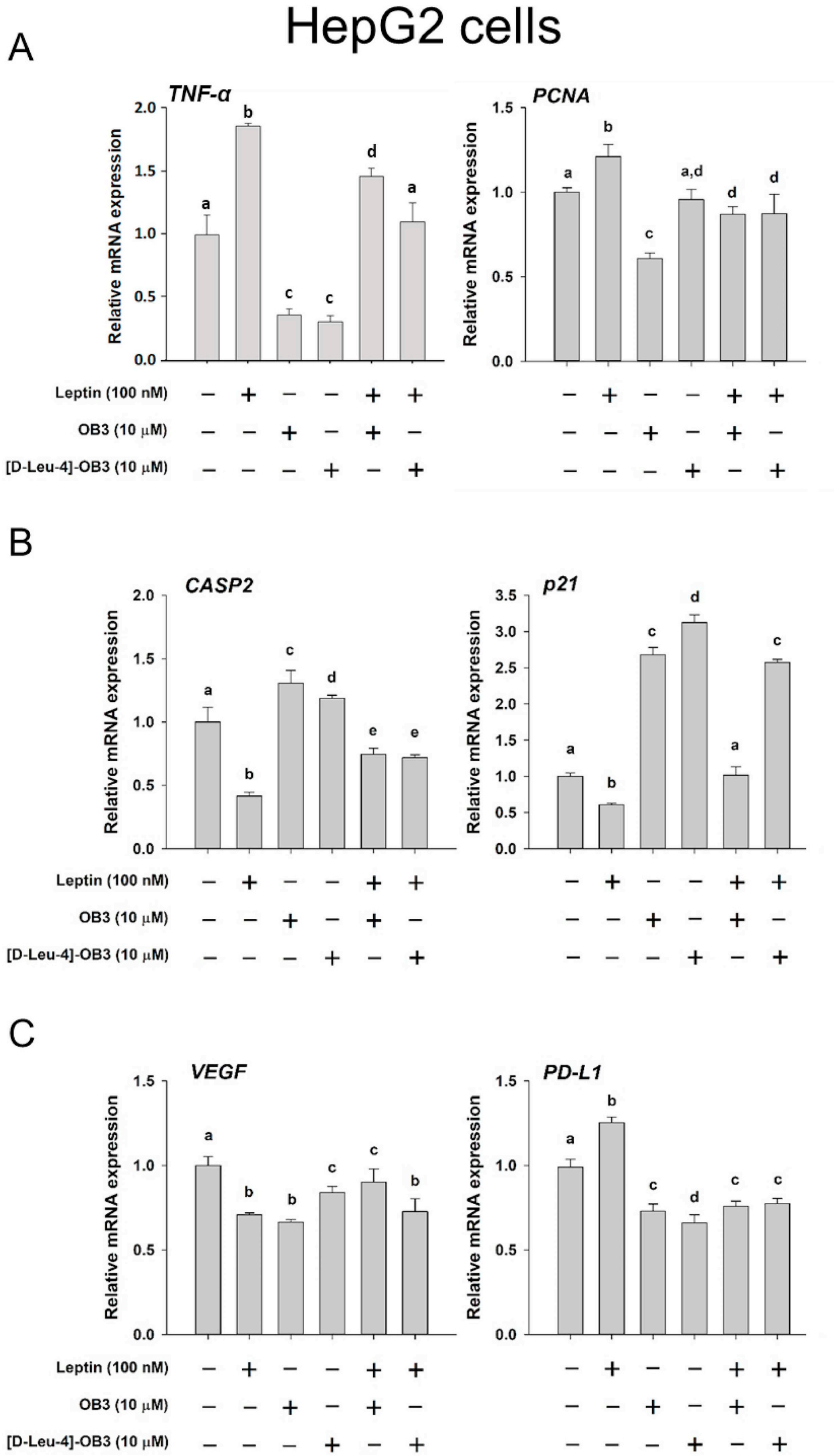

Fig. 5. OB3 and [D-Leu-4] -OB3 reverse expression of leptin-regulated genes in hepatocellular carcinoma cells. HepG2 cells were seeded in 6-well plates and treated with different concentrations of leptin peptide, OB3 peptide, or their combination for 24 h. Cells were harvested, and total RNA was extracted. qPCR experiments were conducted to examine expressions of **(A)** *TNF-α* and *PCNA* **(B)** *CASP2* and *p21* **(C)** *VEGF* and *PD-L1*. N = 3. Data are expressed as the mean ± SD of separate independent experiments, a–d: indicated statistical difference by post hoc test after the significant differences of ANOVA.

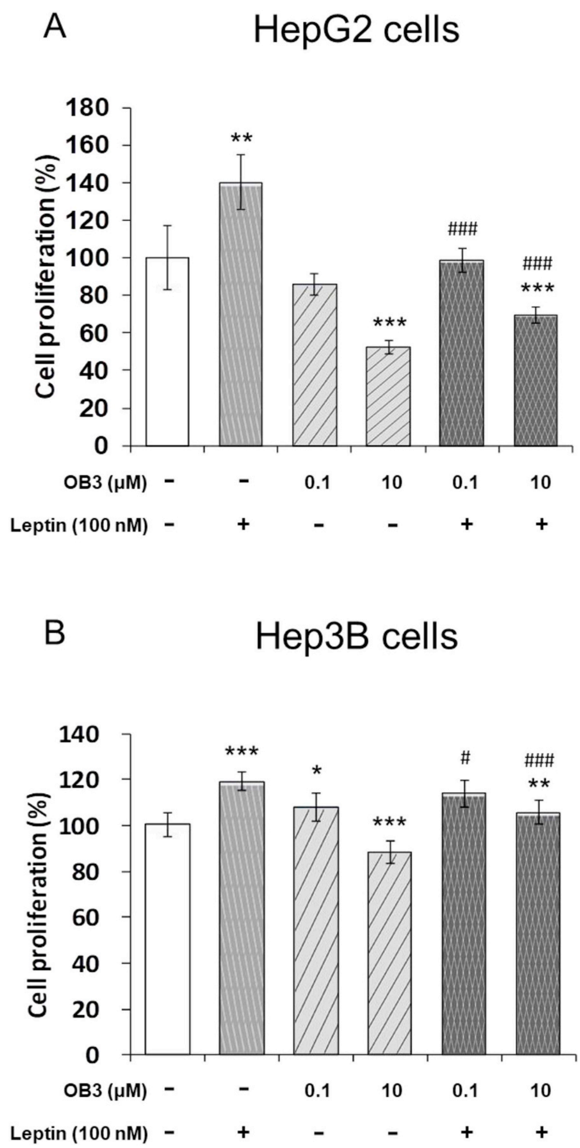

Fig. 6. OB3 peptide inhibits leptin-induced cell proliferation in hepatocellular carcinoma cells. (A) HepG2 and **(B)** Hep3B cells were seeded in 6-well plates and treated with 100 nM leptin peptide, OB3 peptide (0.1 μM or 10 μM), or their combination as indicated, with refreshed medium daily for 4 days. Cell proliferation was examined by cell counting. N = 5. Data are expressed as the mean ± SD of separate independent experiments, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to the control, non-treatment group, $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ compared to 100 nM leptin group.

The OB3 peptide was shown to induce phosphorylation of ERK1/2 and PI3K and phosphorylation of Ser727 and Tyr705 of STAT3 in cervical cancer HeLa cells and ovarian cancer cells. However, OB3 did not induce activation of ERK1/2, PI3K, or STAT3 in anaplastic thyroid cancer or papillary thyroid cancer cells (Yang et al., 2016). On the other hand, leptin induces activation of STAT3 via phosphorylation of ERK1/2 and Tyr705 of STAT3. Indeed, pharmacologic inhibition of ERK1/2 and STAT3, but not PI3K, signaling blocked leptin-induced invasion in anaplastic thyroid cancer cells (Yang et al., 2016). Leptin activates ERK1/2 to phosphorylate Tyr705 in STAT3 which is essential for triggering leptin-induced expression of *MMP2*, *MMP9*, and *VEGF* and consequent cell invasion. Hormones and growth factors activate ERK1/2 which supports cancer cell proliferation and metastasis. On the other hand, resveratrol inhibits cancer proliferation, which is also via ERK1/2 activation (Lin et al., 2013). Activated STAT3 was shown to support the progression of many types of human cancers (Carpenter and Lo, 2014; Lo et al., 2008). Obesity-related cancer progression is facilitated by activated phosphorylation of the oncogenic transcription factors, JAK2 and STAT3 (Mullen and Gonzalez-Perez, 2016). However, OB3 induces Tyr705 phosphorylation of STAT3 but inhibits cell proliferation in human ovarian cancer cells (Chin et al., 2017). OB3 derivatives inhibited PI3K activation (Fig. 3C) and induced cell cycle arrest in G_0/G_1 phase (Fig. 4). Studies conducted by others have shown that blockage of PI3K activation suppresses tumor growth via G_0/G_1 arrest (Dan et al., 2009; Li et al., 2015). Our results also indicated that OB3 reduced leptin-induced gene expressions and cell proliferation (Figs. 5 and 6). Therefore, inactivated PI3K plays a vital role in the OB3-induced inhibitory effect on leptin-induced proliferation in HCC cells.

Taken together, these results indicate that inhibition of the expressions of inflammatory and proliferative genes in HCC cells by OB3 derivatives via inhibiting PI3K is involved in suppressing leptin-induced proliferation in human hepatocellular carcinoma.

Author contributions

YR Chen, ZL Li, YJ Shih, and HY Lin conceived the experiments.

Y Ho, YT Chin, HY Lin, YC Yang, P Grasso, PJ Davis, and K Wang designed the experiments.

Y Ho, SH Wang, ZL Li, YR Chen, HR Chu, and YJ Shih performed the experiments.

YT Chin, KW Su, ZL Li, HC Chiu, YH Wu, DR Crawford, YJ Shih, and P Grasso analyzed the data.

Y Ho, YR Chen, HY Tang, HY Lin, PJ Davis, K Wang, and J Whang-Peng prepared the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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