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Original Article

Pegylated interferon-α inhibits the proliferation of hepatic oval cells through down-regulation of Wnt/β-catenin pathway

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Abstract: Background and aims: Interferon alpha has shown potential benefit in patients with hepatocellular carcinoma (HCC), which could be mediated via inhibiting proliferation of hepatic oval cells. However, the detailed mechanism of anti-proliferative effect of IFNsremains obscure. In this study, we evaluate the effects of pegylated interfon-α2b (PEG-IFN) on the Wnt/β-catenin pathway, the major driving force behind the proliferation of hepatic oval cells. Methods: WB-F344 cells stimulated with Wnt3a were incubated with 16, 160, and 1600 ng/ml of PEG-IFN for 48 h in serum-free medium. Effects of PEG-IFN on cell proliferation and cell-cycle distribution were evaluated by Brdu incorporation assays and flow cytometry, Wnt3a-treated cells without PEG-IFN incubation were taken as controls. Effects of PEG-IFN on Wnt/β-catenin signal transduction were examined by qPCR, Western blot analysis,and immunofluorescence staining. Results: PEG-IFN significantly inhibited proliferation of WB-F344 cells induced by Wnt3a. Meanwhile, PEG-IFN at 1600 ng/ml could induce cell cycle arrest in the G1 phase. Furthermore, PEG-IFN could significantly suppress the expression of increased β-catenin induced by wnt3a in WB-F344 cells. These findings were confirmed by immunofluorescence staining for nuclear localization of β-catenin. Additionally, PEG-IFN could suppressthe transcription and protein expression of increased C-Myc and Cyclin D1 (encoded by a Wnt target gene) induced by Wnt3a. PEG-IFN could also increase the level of RanBP3 protein induced by Wnt3a, which resulted in decreased expression of β-catenin. Conclusions: PEG-IFN could inhibit hepatic oval cells proliferation through down-regulation of the Wnt/β-catenin pathway.

Keywords: PEG-IFN, Wnt/β-catenin, oval cells, cell proliferation, RanBP3, hepatocellular carcinoma

Introduction

Various studies have reported range of effects of IFN therapy on HCC in chronic viral hepatitis patients ranging from effectively preventing the development of HCC to reducing the late recurrence of HCC [1-4]. Furthermore, some studies have shown that interferon therapy could significantly reduce the risk ofHCC development, regardless of sustained virological response [5, 6]. Thus, it is conceivable that IFN-α may have anti-tumor effects beyond antiviral effects in these patients, and studies has shown that the effect may be mediated via inhibiting proliferation of hepatic oval cells [7, 8]. Such anti-proliferative activity makes IFN an intriguing option for chemoprevention and chemotherapy. The detailed mechanism of the anti-proliferative effect of IFNs, however, remains obscure.

The concept of oval cells as targets of malignant transformation is now widely accepted, and therapeutic control of proliferation of preneoplastic cells, is a plausible solution to overcoming their malignant potential [9, 10]. The Wnt/β-catenin signaling pathway plays an important role in the pathogenesis of hepatic adenoma and its progression to HCC [11] and also in self-renewal of stem cells in several tissue types [12]. β-catenin is a mediator of cancer stem cells [13] and plays an important role in early liver development at the stage of ongoing hepatic progenitor proliferation. Our previous study has demonstrated that activation of the Wnt/β-catenin signaling pathway in vitro is sufficient to induce proliferation of cultured hepatic oval cells, indicating that β-catenin targeting might be of essence in preneoplastic and early or late HCC as chemoprevention and

Table 1. Primer sequences used for Real-time PCR

Gene Name	Primer Sequences (5'→3')
β-catenin	F: CAGAGTTGCTCCACTCCAGG
	R: TCAGCAGTCTCATTCCAAGCC
C-Myc	F: TGACCGAGCTACTTGGAGGA
	R: GAAGCCGCTCCACATACAGT
CyclinD1	F: CTGCCGAGAAGTTGTGCATC
	R: CTGCTTGTTCTCATCCGCCT
β-actin	F: ATTGCTGACAGGATGCAGAA
	R: TAGAGCCACCAATCCACACAG

chemotherapymeasure [14]. However, whether IFN could inhibit the proliferation of hepatic oval cells by modulating Wnt/β-catenin pathway, remains to be elucidated.

WB-F344, a rat hepatic stem-like epithelial cell line, isolated from the liver of an adult male Fischer-344 rat, can express a phenotypic repertory of both hepatocytes and bile duct epithelial cells compared with those of normal hepatocytes, biliary epithelial (ductal) and "oval" cells isolated from liver treated with chemical carcinogens. The phenotypic properties of cultured liver epithelial cell line most resemble those of the "oval" cells. Thus, it is considered to be an in vitro model of bipotent oval cells as it shares their phenotype [15, 16].

In the present study, the molecular mechanism of anti proliferative effects induced by PEG-IFN was investigated in association with Wnt/β-catenin signaling in WB-F344 cells in vitro. Our results showed that PEG-IFN could inhibit Wnt3a induced HOC proliferation through down-regulation of the Wnt/β-catenin pathway. A better understanding of its role in hepatic oval cell proliferation can lead to the successful manipulation of liver biology for therapeutic purposes.

Materials and methods

Cell culture

The rat hepatic oval cell line (WB-F344) was obtained from Academy of Military Medical Sciences. The cells were cultured in Dulbecco's modified Eagle's medium/F12 (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (Gibco BRL, USA), 10 mL of 200 mmol/L L-glutamine and 0.5 mL of penicillin-streptomycin mixture. All cultures were maintained at 37°C in 50 mL/L CO_2. WB-F344 Cells were plated with the maintenance medium and the next day were starved by incubation in serum-free medium (SFM) for an hour at 37°C. SFM is composed of Dulbecco's modified Eagle's medium/F12, 20 IU/mL penicillin, 20 mg/mL streptomycin and Insulin-Transferring-Selenium supplement (Gibco, Carlsbad, CA, USA). In order to stimulate Wnt/β-catenin pathway, cells were stimulated with 160 ng/mL Wnt3a for 24 h after starvation.

Proliferation test (Brdu incorporation assays)

Cell proliferation was detected by Brdu incorporation assays. Briefly, 24 h prior to the experiment, a total of 6000 WB-F344 cells were seeded into 96-well culture plates. Cells were brought to 90% confluence and then fasted overnight in serum-free media before addition of Brdu to a final concentration of 10 μmol/L. After starvation, cells wereincubated with 160 ng/ml Wnt3a for 24 h and then treated with escalating doses of PEG-IFNα2b (0, 16, 160, and 1600 ng/mL) for 48 h, washed free of BrdU and harvested. The cells were fixed with 4% paraformaldehyde for 30 min, blocked with 1% BSA in phosphate-buffered saline containing 0.2% Triton X-100 for 30 min, incubated with a HRP-Brdu antibody (1:200) for 2 h at 37°C, and washed. A TMB substrate solution was added to the wells and color developed in proportion to the amount of Brdu bound. The stop solution changed the color from blue to yellow, and intensity of the color was measured at 450 nm with a spectrophotometer.

RNA isolation and quantitative real-time polymerase chain reaction (qPCR)

The cells treated with Wnt3a were stimulated with or without different concentrations PEG-IFN (16, 160, 1600 ng/ml) in serum-free media for 48 h. Total RNA was extracted from Wnt3a treated cells with Trizol according to the manufacturer's instructions. RNA (1 μg) was reverse transcribed. For qPCR, the number of cycles corresponded to the mid-logarithmic phase. Primers were designed using GenBank sequences (**Table 1**). Real-time PCR analysis was performed using CyBRGreen PCR Master Mix (Applied Biosystems, Darmstadt, Germany) under standard conditions.

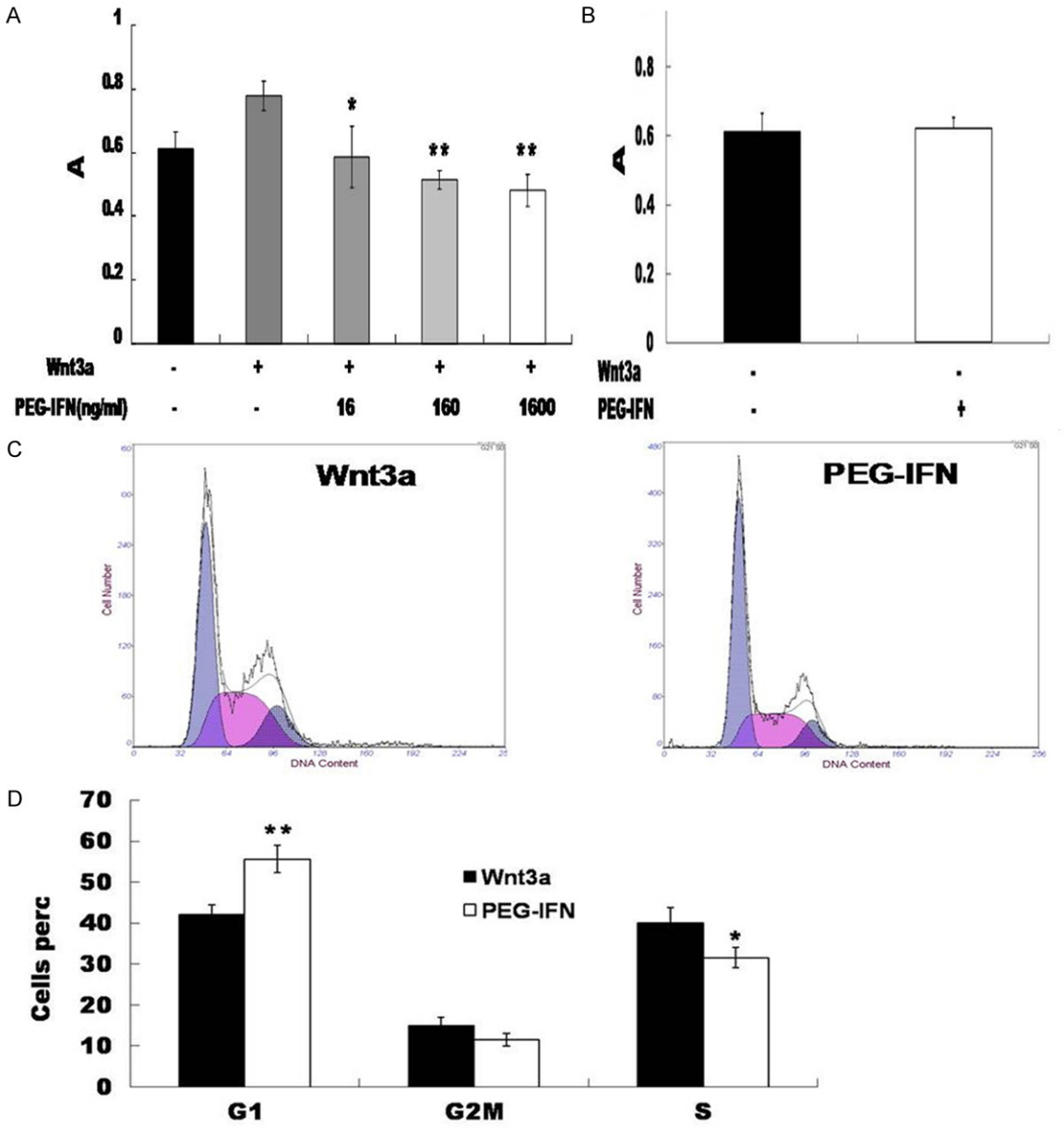

Figure 1. PEG-IFN inhibits the proliferation of WB-F344 cells induced by Wnt3a. A: The inhibitory effect of PEG-IFN on Wnt3a-induced cell proliferation. Cell proliferation was evaluated using Brdu incorporation assays(**P<0.05, **P<0.01*, vs. Wnt3a-treated cells not given PEG-IFN). B: No obvious effect of PEG-IFN on quiescent WB-F344 cell proliferation. (*P=0.294* vs. quiescent WB-F344 cells). C: Flow cytometric analyses of the cell cycle of Wnt3a-treated WB-F344 cells following treatment with PEG-IFN (1600ng/ml). D: Cell cycle quantitative analysis. Histograms indicating the fraction of Wnt3a-treated cells not given PEG-IFN and treated with PEG-IFN in the G1, G2/M and S phases. The data represent the means ± SD derived from three independent experiments. (**P<0.05, **P<0.01*, vs. Wnt3a-treated cells not given PEG-IFN).

Protein extraction and Western-blot

The cells treated with Wnt3a were stimulated with or without different concentrations PEG-IFN (16, 160, 1600 ng/ml) in serum-free media for 48 h. Protein isolation was carried out using lysis buffer (50 mM Na_2HPO_4; 50 mM NaH_2PO_4; 0.2 M NaCl; 5 mM EDTA; 1% Triton X-100, pH 6.0). After a 30-minute incubation on ice, samples were centrifuged (13.000 g, 20 minutes, 4°C), and 2_dithiothreitol (DTT) loading buffer (0.4 M Tris, pH 6.8; 4% SDS; 20% glycerol; 10% DTT) was added to the sample supernatants, with subsequent incubation for

5 minutes at 95°C. Following electrophoretic separation by SDS-polyacrylamide gel electrophoresis, proteins were electroblotted on nitrocellulose membranes. The membranes were blocked in NET buffer (150 mM NaCl; 5 mM EDTA, pH 8.0; 50 mM Tris/HCl, pH 7.5; 0.05% Triton X-100) containing 2.5% gelatin (Merck) for 1 hour at room temperature. Polyclonal antibodies against β-catenin (R & D, Systems, Inc), clyclin D1 (Santa Cruz Biotechnology Inc), C-Myc (Santa Cruz Biotechnology Inc), RanBP3 (Santa Cruz Biotechnology Inc). Incubation was performed for 1 hour at room temperature. Thereafter, membranes were washed in NET buffer, and a further incubation was carried out with a peroxidase-conjugated antibody at a dilution of 1:20,000. Antibody binding was visualized by hydrogen peroxidase-chemiluminescent detection kit (Frontier Laboratories, Koriyama, Japan). Semiquantitative evaluation of the bands was performed by densitometric analysis. The protein expression levels of β-catenin werethereby normalized to that of the housekeeping gene β-actin.

Immunofluorescence staining

Cells treated with recombinant Wnt3a protein in the presence or absence of PEG-IFN (1600 ng/ml) were fixed with 4% paraformaldehyde for 30 min at 37°C. Permeabilization of the cells was achieved after incubation for 30 min at 37°C with PBS containing 0.2% Triton X-100. To minimize nonspecific binding of the antibody, blocking was carried out with a buffer containing 1% bovine serum albumin for 1 h. β-catenin antibody (R & D, Systems, Inc) was applied at a 1:25 dilution for 90 min at 37°C. As a negative control, PBS was used instead of the primary antibody to exclude the unspecific binding of the secondary antibody. No fluorescent labeling was observed in the negative control. After repeated washing with PBS, the cells were incubated with a goat-anti-mouse antibody labeled with fluorescein isothiocyanate (1:10) for an additional 30 min. Finally, cell nuclei were counterstained with Hoechst 33258. Images were obtained using a confocal laser scanning microscope.

Flow cytometry (FACS)

The cell cycle analysis was performed as previously described. Briefly, aliquots of cells (1×10^6) were pelleted (1300 rpm ×5 minat 4°C) and washed twice in ice-cold phosphate-buffered saline (PBS). The cells were fixed in 70% ethanol overnight at 4°C, washed in PBS and then digested with DNase-free RNase A (10 μg/mL) at 37°C for 30 min. Prior to FACS analysis, the cells were resuspended in 200 μL propidium iodide (PI, 10 μg/mL; Sigma, St Louis, MO, United States) for DNA staining. A BD FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, United States) flow cytometer was used to analyze cellular DNA contents.

Statistical analysis

All results were expressed as mean ± SD. Measurement data were analyzed using one-way analysis of variances (ANOVA, SPSS 11.5). $P < 0.05$ was considered statistically significant.

Results

PEG-IFN inhibited proliferation of WB-F344 cells induced by Wnt3a

We detected the inhibitory effects of PEG-IFN on the proliferation induced by Wnt3a using Brdu method. The Wnt3a-induced proliferation was largely affected by treatment of PEG-IFN even at low concentrations, and reached its peak at 1600 ng/mL within 48 h (**Figure 1A**). To estimate the genotoxicity of PEG-IFN on quiescent WB-344 cells, the quiescent WB-344 cells were exposed to 1600 ng/mL PEG-IFN for 48 h. No obvious inhibitory effect on the growth of quiescent WB-344 cells was observed (**Figure 1B**). To further investigate the impact of PEG-IFN on the proliferation induced by Wnt3a, the cell cycle profile was analyzed using propidium iodide staining and flow cytometry after PEG-IFN (1600 ng/ml) treatment for 48 h. We observed that treatment with 1600 ng/mL PEG-IFN increased the G1 subpopulation to 55.6% within 48 h, compared to 42.1% in unexposed cells (**Figure 1C**). In addition, PEG-IFN decreased the size of the G2/M phase and S phase subpopulations compared with unexposed cells. These results indicated that PEG-IFN could inhibit DNA replication, leading to cell cycle arrest in the G1 phase. In summary, these results indicated that PEG-IFN inhibited the Wnt3a-induced proliferation in WB-F344 cells.

PEG-IFN inhibited the expression of increased β-catenin induced by wnt3a

There were numerous ways to activate the Wnt pathway, such as adding wnt3a protein to cell

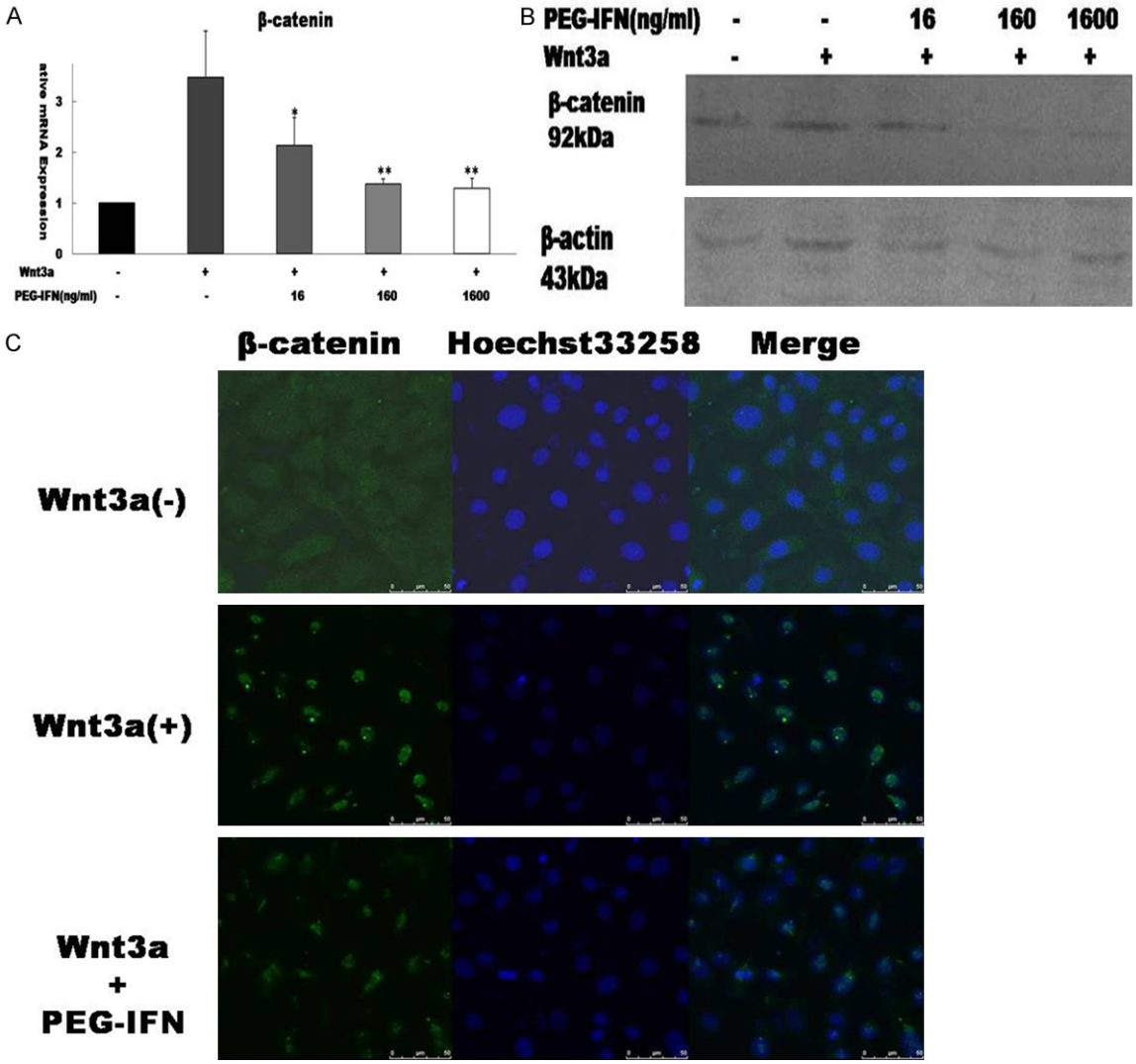

Figure 2. PEG-IFN inhibits the expression of increased βcatenin induced by wnt3a. A: Real-time PCR for the expression of β-catenin mRNA levels. Data represent mean ± SD from three independent experiments. (**P<0.05, **P<0.01*, vs. Wnt3a-treated cells not given PEG-IFN). B: Changes of β-catenin protein after treatment of PEG-IFN for 48 h. Representative images from three independent experiments are shown. C: PEG-IFN impairs the β-catenin nuclear accumulation induced by Wnt3a in WB-F344 cells, as shown by immunofluorescence confocal microscopy. WB-F344 cells treated with Wnt3a with or without PEG-IFN (1600 ng/ml). β-Catenin signal is in green. Nuclei are in blue (Hochst33258). Scale bars: 50 μm.

culture media or increasing the expression of β-catenin. In order to investigate the inhibitor role of PEG-IFN, WB-F344 cells were stimulated with the well-established Wnt/β-catenin pathway activator Wnt3a [17]. In our study, we pretreated WB-F344 cells with Wnt3a for 24 h and then incubated them with PEG-IFN for 48 h. β-catenin is one of the hallmarks of Wnt/β-catenin pathway activation and the most straight forward method of inhibiting Wnt pathway is to target β-catenin. Therefore, we performed this protocol to assess whether PEG-IFN could inhibit the expression of β-catenin induced by Wnt3a. In our study, qPCR and western blot analysis showed that incubation with PEG-IFN resulted in a dose-dependent decrease of the increased β-catenin induced by Wnt3a (**Figure 2A** and **2B**).

To determine whether PEG-IFN interacts with Wnt3a and affects Wnt signaling, the accumulation of β-catenin was assessed in WB-F344 cells treated with Wnt3a in the presence or absence of PEG-IFN. Confocal microscopy of

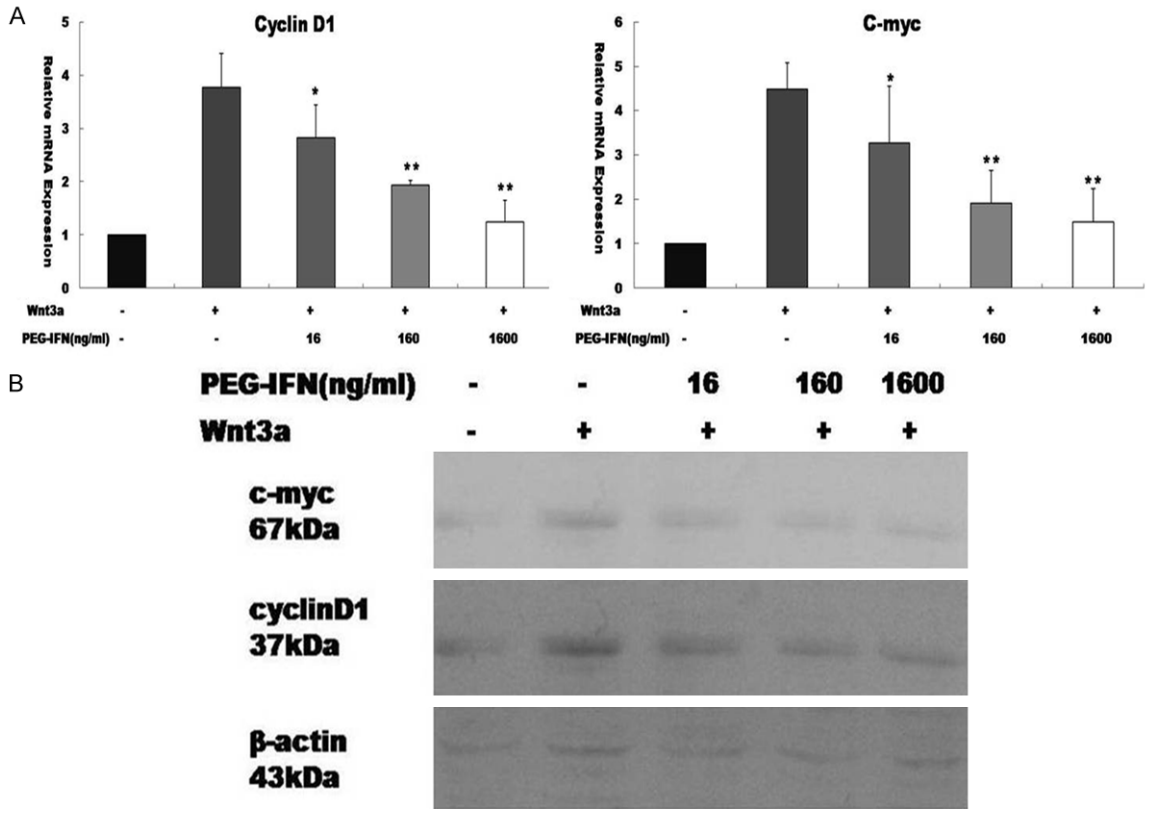

Figure 3. PEG-IFN inhibits increased C-myc and Cyclin D1 induced by Wnt3a. A: Real-time PCR for the expression of cyclinD1 and c-Myc mRNA levels. Data represent mean ± SD from three independent experiments. (**P<0.05, **P<0.01*, vs. Wnt3a-treated group not given PEG-IFN). B: Western blot assay showing dose-dependent effect of PEG-IFN on cyclin D1 and c-Myc protein levels induced by Wnt3a.

our immunofluorescence staining of WB-F344 cells revealedβ-catenin accumulation in most of the nuclei after treatment with Wnt3a (**Figure 2C**). Whereas, the intensity of nuclear β-catenin was dramatically reduced by PEG-IFN treatment, as determined by indirect immunofluorescence (**Figure 2C**). These findings indicated that PEG-IFN could inhibit the Wnt3a-induced increase of β-catenin and impair the β-catenin nuclear accumulation.

PEG-IFN suppressedthe upregulation of C-Myc and Cyclin D1 induced by Wnt3a

Activation of β-catenin can enhance expression of Wnt target genes. We examined two of the better characterized Wnt signal pathway target genes, C-Myc and Cyclin D1 by real-time PCR and Western-blot. C-Myc, cyclin D1 are two of the better characterized Wnt/β-catenin signal pathway direct downstream targets genes, and they encode a transcription factor that has major roles in cell proliferation. Therefore, C-Myc and Cyclin D1 upregulation occurs when β-catenin is hyperactivated [18]. As expected, our pretreatment of WB-F344 cells with Wnt3a for 24 h significantly raised C-Myc and Cyclin D1 gene and protein levels compared to untreated WB-F344 cells, whereas the Wnt3a-induced upregulation of C-Myc and Cyclin D1 was significantly repressed by PEG-IFN (**Figure 3A** and **3B**), confirming impeded β-catenin activity. Altogether, the results above demonstrated PEG-IFN restrains cell survival through repressing Wnt/β-catenin pathway activity.

Peg-IFN increased the level of RanBP3 protein induced by Wnt3a

One well-characterized inhibitor of the Wnt signaling was Ran Binding Protein 3 (RanBP3), which was involved in the nuclear export of β-catenin and capable of acting on both wild-type and mutant forms of β-catenin [19]. Therefore, to determine the possible mechanisms through which PEG-IFN inhibited the

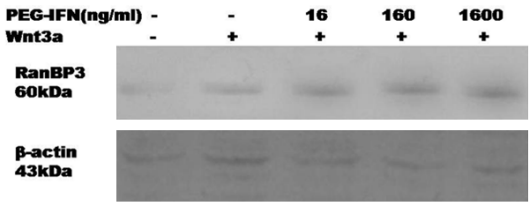

Figure 4. Peg-IFN increases transcription of RanBP3 induced by Wnt3a. Western blot assay showing dose-dependent effect of PEG-IFN on RanBP3 protein levels induced by Wnt3a.

expression of increased βcatenin induced by wnt3a, we explored its effect on RanBP3 induced by Wnt3a. Our results revealed that a dose-dependent increase in protein levels of RanBP3 induced by Wnt3a occurred after PEG-IFN treatment (**Figure 4**). These findings could partially explain the negative effect on the Wnt pathway exhibited by PEG-IFN therapy through upregulation of RanBP3 induced by Wnt3a.

Discussion

Sustained proliferation is an important part of cancer development and progression [20]. Oval cell activation is important to the pathogenesis of HCC, maintaining oval cell quiescence and inhibiting their proliferation may have therapeutic value. This may prevent or delay the onset of primary tumors, and help to prevent metastasis or relapse [21-23]. Understanding the molecular mechanisms will be crucial for the development of new therapeutic strategies to cope with this challenge. The present study demonstrated that PEG-IFN could inhibit the proliferation of HOC through down-regulating the Wnt/β-catenin pathway in vitro.

We performed this study in serum-free conditions to minimize other confounding factors. To activate Wnt/β-catenin pathway, WB-F344 cells were stimulated with the well-established Wnt/β-catenin pathway activator Wnt3a in serum-free conditions. Our initial results demonstrated that the Wnt3a-induced proliferation was largely affected by treatment of PEG-IFN even at low concentrations, and this effect was more obvious following dose increase. Whereasno obvious inhibitory effect on the growth of quiescent WB-344 cells was observed. In agreement with above observations, cell cycle analyses performed in wnt3a treatment of WB-F344 cells demonstrated that the cell cycle distribution was changed following PEG-IFN treatment. The S phase fraction was significantly reduced compared with control, and the proportion of cells in G1 phase was significantly increased, indicating that an inhibition of wnt3a induced proliferation may be involved in the anti-proliferative effects of PEG-IFN.

Aberrant activation of the Wnt/β-catenin pathway has been implicated in multiple hepatic tumors, especially including hepatoblastomas and HCCs. Nuclear and cytoplasmic localization of β-catenin were reported in 90% to 100% of all hepatoblastomas, which mainly originate from immature liver precursor cells and presents morphologic features recapitulating some of the developmental aspects of the liver [24]. Consistently, expression of stabilized β-catenin promotes the self-renewal and proliferation of hepatic stem/progenitor cells and leads to tumorigenesis in the liver [25, 26]. In the present study, we observedthat the wnt3a up-regulated expression of β-catenin decreased in a dose-dependent manner following PEG-IFN treatment. This reduction in protein levels was confirmed by immunofluorescence. Nuclear β-catenin acts as a transcription factor activating downstream target genes, such as oncogenes cyclin D1 and C-Myc [27, 28]. C-Myc is an important target effector of Wnt mitogenic signal that could induce cell growth, proliferation and apoptosis [29]. As a downstream component of C-Myc pathway, amplification and overexpression of cyclin-D1 can cause cell cycle changes [30]. Deregulation of C-Myc activity is one of the most common alterations reported in human malignancy [31]. Recent studies indicated that C-Myc may also regulate expression of long non-coding RNAs in both normal and cancer cells, suggesting a unique, novel route by which C-Myc may exert additional control over the proliferation of both normal and cancerous tissue [32, 33]. Based on these specific characteristics of regulatory molecules, we investigated the further mechanism of PEG-IFN-associated inhibition against wnt3a-induced proliferation. As a consequence, PEG-IFN contributed to down-regulation of intracellular wnt3a induced increase of C-Myc, cyclin D1 in a dose-dependent manner, consistent with down-regulation of β-catenin expression. RanBP3 is a novel downstream target of PEG-IFN and mediates its negative impact on Wnt signaling. Overexpression of RanBP3 inhibited Wnt-me-

diated transcriptional activation [34]. Our results showed that PEG-IFN treatment led toincreased wnt3a induced expression of β-catenin nuclear export factor RanBP3, indicating this effect maybe in part due to suppression ofβ-catenin through upregulation of RanBP3. These evidences illuminated the molecular mechanisms that PEG-IFN inhibited wnt3a induced proliferation via down-regulation of Wnt/β-catenin pathways, secondary to the decreased expression of downstream targets such as Cyclin D1, C-Myc, which are critical in proliferation, indicating that the Wnt/β-catenin signaling pathway may be an attractive target for directed anti-progenitor cell therapeutics. Whether PEG-IFN can directly control the expression of RanBP3 need further study.

In conclusion, our data demonstrated that PEG-IFN exerted an inhibitory effect on the wnt3a induced proliferation of WB-F344 cells through suppression of the Wnt/β-catenin pathway, which may be a contributory mechanism of delayed HCC and to improve overall tumor control with cancer therapy. This observation might have chemo preventive or chemotherapeutic implications in tumor with aberrant Wnt pathway activation. The next step could be carried on in vivo test to confirm their effectiveness.

Acknowledgements

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Disclosure of conflict of interest

None.

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