Recombinant human decorin upregulates $p57^{KIP2}$ expression in HepG2 hepatoma cell lines

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Abstract. Increasing the expression of cyclin-cyclin-dependent kinase inhibitors (cyclin-CDK) using small molecule inhibitors is a therapeutic strategy used to suppress cancer cell growth. Decorin (DCN), a functional component of the extracellular matrix, has been implicated in the suppression of cell proliferation by upregulating p21, a cyclin-CDK inhibitor. The purpose of this study was to examine the effect of recombinant decorin on the reactivation of $p57^{KIP2}$, whose expression is silenced in hepatocellular carcinoma (HCC). Cell viability assay, cell cycle analysis, apoptosis assay and quantitative real time-PCR experiments were performed in three groups of HepG2 human cells: Uninfected HepG2 cells (control group), pcDNA3.1 vector-infected HepG2 cells (pcDNA3.1 group) and pcDNA3.1-DCN-infected HepG2 cells (pcDNA3.1-DCN group). Our results revealed that recombinant human decorin inhibited cell proliferation, induced G_0/G_1 phase arrest and induced apoptosis by increasing the expression of caspase-3 in the pcDNA3.1-DCN group. The expression of $p57^{KIP2}$ mRNA in the pcDNA3.1-DCN group was higher than in the pcDNA3.1 and control groups ($P<0.05$); however, there was no statistically significant difference between the control and pcDNA3.1 groups ($P>0.05$). In conclusion, recombinant human decorin reactivated $p57^{KIP2}$ expression in HepG2 cells. As the expression level of $p57^{KIP2}$ is downregulated in HCC, our finding may serve as a basis for the therapy and prognosis of HCC, although further studies are required.

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Introduction

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer. It is the third-leading cause of cancer-related mortality and the fifth most common type of cancer worldwide, with >600,000 cases diagnosed annually (1-3). HCC is normally diagnosed at an advanced stage and typically has a poor prognosis even following surgical resection and liver transplantation (4). Due to various etiologies, the prevention and treatment of HCC remains a challenge worldwide (5). HCC is more prevalent in developing countries with ~80% of the total incidence occurring in Asia and sub-Saharan Africa (6,7). The prevalence of HCC is increasing in western countries due to the increasing prevalence of hepatitis C virus (HCV) infection (8-10). Thus, with the global incidence of HCC on the rise, there have been increasing calls for the development of new and improved approaches for the treatment of HCC.

Decorin, a functional component of the extracellular matrix (ECM), has multiple biological functions, which include regulating matrix assembly and fibrillogenesis, and controlling cell proliferation (11-13). Recently, an *in vivo* investigation demonstrated that decorin-null mice developed severe liver fibrosis with a significantly delayed healing process (14). Decorin is often downregulated in various types of cancer of epithelial origin (15). In addition to its important biological functions, decorin inhibits cancer growth *in vitro* and *in vivo*. The mechanism for the suppression of tumor growth is independent of the functional p53 tumor suppressor gene; however, requires p21 to be functional (16). Decorin causes a rapid phosphorylation of the epidermal growth factor receptor (EGFR), leading to the activation of mitogen-activated protein kinase and the upregulation of p21, a cyclin-dependent kinase (CDK) inhibitor, and ultimately growth arrest (17). In an *in vivo* experiment, mice that were decorin and p53 null ($DCN^{-/-}$ and $p53^{-/-}$) developed a more aggressive form of lymphoma than those that were only p53 null (12), suggesting that decorin deficiency is permissive for tumorigenesis. Another study by Bi *et al*, using a decorin-deficient mouse model, demonstrated that the intestinal tumorigenesis in $DCN^{-/-}$ mice was linked to the downregulation of p21 and p27 (18), implying that the role of decorin in repressing tumorigenesis requires the upregulation of cyclin-dependent kinase inhibitors (CKIs).

Cyclin-CDK inhibitors include three proteins: $p21^{CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$. CDK inhibitors regulate the cell cycle of mammalian cells by binding to cyclin-CDK complexes. In addition to cell cycle regulation, CKIs have CDK-independent functions, including regulating transcription, apoptosis, cell migration and the cytoskeleton (19). Unlike $p21^{CIP1}$ and $p27^{KIP1}$, $p57^{KIP2}$ has a unique role in embryogenesis, in which the genetic deletion of $p57^{KIP2}$ has been demonstrated to be lethal in $p57^{KIP2}$ null mice (20,21). Relative to $p21^{CIP1}$ and $p27^{KIP1}$, $p57^{KIP2}$ is the newest and least studied CIP/KIP member.

The $p57^{KIP2}$ human gene is located on chromosome 11 at the 11p15.5 locus and encodes a protein 316 amino acids long. Structurally, $p57^{KIP2}$ is almost identical to $p27^{KIP1}$ and functionally, it leads to cell cycle arrest in the G_1 phase. In addition, it has been reported that $p27^{KIP1}$ and $p57^{KIP2}$ play conducive roles in neuronal migration and may, when working together, coordinate the timing of neuronal differentiation, migration and, potentially, cell cycle arrest in neocortical development. $p57^{KIP2}$ may serve as an important domain for protein interactions implicated in functions other than the CDK-inhibitory role (21). Additionally, $p57^{KIP2}$, but not $p21^{CIP1/WAF1}$ or $p27^{KIP1}$, interacts *in vivo* and *in vitro* through its amino-terminal domain with transcription factor B-Myb. Mutations of $p57^{KIP2}$ occur in Beckwith-Wiedemann syndrome and its reduced protein expression in breast, lung, liver, prostate, colorectal and bladder cancer is suggestive of its tumor suppressive properties (22-26). There is mounting evidence that the $p57^{KIP2}$ protein level is normally downregulated in these types of cancer through several mechanisms, including maternal-specific loss of heterozygosity (LOH), loss of imprinting and promoter methylation (19). A recent study revealed that the downregulation of $p57^{KIP2}$ accelerates the growth and invasion of HCC (27), indicating that the upregulation of this tumor suppressor is essential for the prevention and therapy of HCC. Activating the cyclin-CDK inhibitors using a small molecule inhibitor to restore their regulatory role in the cell cycle, proliferation and differentiation is an attractive therapeutic strategy for cancer treatment. As the expression of $p57^{KIP2}$ is downregulated in several types of cancer, it may have therapeutic and prognostic uses. Hence, an investigation into upregulating its expression is essential. In this study, we demonstrated that recombinant human decorin upregulated the expression of $p57^{KIP2}$, a CDK inhibitor, in HepG2 cell lines.

Materials and methods

Cell culture. HepG2 cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA); cells were grown in DMEM (Invitrogen Life Technologies, Carlsbad, CA, USA) plus 10% fetal bovine serum, and supplemented with 100 μ/ml penicillin and 100 mg/ml streptomycin (Sigma, St. Louis, MO, USA). Cells were maintained at 37˚C in a humidified atmosphere containing 95% air and 5% CO_2. Recombinant human decorin was synthesized in our laboratory (28).

Transient transfection of HepG2 cell lines. HepG2 cells were divided into three groups: The pcDNA3.1-DCN group, the pcDNA3.1 group and the untransfected group (control group). Transient transfection was performed using Lipotap liposomal reagent according to the manufacturer's instructions (Beyotime Institute of Biotechnology, Haimen, Jiangsu, China).

Cell viability assay. Cells were seeded in 96-well plates (10^4 cells per well). The cell proliferation assay was performed in all three HepG2 groups using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] method (Sigma, 5 mg/ml). After 72 h of transfection, 20 μl (5 mg/ml) of MTT was added to each well for a 4-h incubation at 37˚C. The supernatant was removed and 150 μl DMSO (Sigma) was added following 10 min of oscillation. The optical density (OD) value was determined with an ELISA machine (Biotek Synergy HT, Winooski, VT, USA) at 490 nm and the assays were performed in triplicate. In each group, the cells were analyzed and the data are presented as the means ± SD.

Cell cycle detection by flow cytometry. Cells were plated (3x10^5 cells per well) in 6-well plates, treated with pcDNA3.1-DCN, pcDNA3.1 and/or with neither pcDNA3.1 nor pcDNA3.1-DCN for 72 h and then trypsinized, washed using 1X buffer A, fixed with 70% ice-cold ethanol and incubated overnight. RNase A (up to 0.25 mg/ml) was added and the DNA was labeled with propidium iodide (PI; 5 μl; Becton-Dickinson, Franklin Lakes, NJ, USA). The cells were analyzed using flow cytometry (FC500, Beckman Coulter, Miami, FL, USA). The experiment was performed in triplicate.

Analysis of apoptosis by annexin V-FITC/PI assay. Analysis was conducted by cultivating cells in 6-well plates (3x10^5 cells per well) treated with pcDNA3.1-DCN, pcDNA3.1 and/or with neither pcDNA3.1 nor pcDNA3.1-DCN for 72 h and then trypsinized. Apoptosis was determined using the Annexin V-FITC Apoptosis kit (Becton-Dickinson). The cells were analyzed using flow cytometry in triplicate. The experiment was performed in triplicate.

RNA extraction. RNA was extracted using TRIzol RNA reagent (Sangon Biotech Co. Ltd., Shanghai, China) from three groups of HepG2 cells after 72 h transient transfection according to the manufacturer's instructions. Briefly, adhering to protocol, cells (3x10^5 cells) from each group were washed three times using 0.1M PBS, trypsinized, and then transferred to an RNase-free Eppendorf tube and centrifuged at a low speed. The supernatant was discarded, 0.5 ml of TRIzol reagent was added to lyse the cells and they were thoroughly mixed. Samples were allowed to sit at room temperature for 5 min and 0.2 ml of chloroform was added. The samples were mixed by hand for 15 sec and allowed to stand for 5-10 min at room temperature. The resulting mixture was centrifuged at 12,000 x g for 15 min at 4˚C. The uppermost supernatant aqueous phase was transferred to a fresh RNase-free microcentrifuge tube, an equal amount of 70% ethanol was added, mixed and then transferred to a column. RPE solution (0.5 ml) was then added, incubated at room temperature and centrifuged. The purity and concentration of the RNA was checked using NanoDrop 1000 (Thermo Scientific, West Palm Beach, FL, USA) spectrophotometry and the OD 260/280 nm ratio was between 1.85-1.95 for each RNA sample group. The quality of the total RNA was verified by running an agarose gel and the total RNA was stored at -70˚C.

Complementary DNA (cDNA) synthesis. Total RNA (1 μg) from each HepG2 cell group was reverse transcribed into comple-

mentary DNA (cDNA) using the First Strand cDNA Synthesis kit (GeneCopoeia, Rockville, MD, USA). Briefly, 1 μl random primer was added, and ddH_2O (RNase/DNase free) was added up to 13 μl volume and the mixture was incubated for 10 min at 65˚C, cooled and centrifuged. The final volume of 25 μl was kept at 37˚C for 1 h followed by enzyme deactivation at 85˚C for 5 min. The final volume was stored at -20˚C.

Real time-PCR. The relative expression levels of mRNA $p57^{KIP2}$ and caspase-3 from each group of cells were determined by quantitative PCR using the SYBR All-in-One qPCRMix (GeneCopoeia) with GADPH as a reference (Takara Bio, Inc., Shiga, Japan). Samples were run in separate tubes on an ABI Prism 7500 according to the manufacturer's instructions. In brief, the 25 μl samples were treated at 95˚C for 10 min followed by 40 cycles of 95˚C for 20 sec and 60˚C for 30 sec, and a final extension of 5 min at 72˚C. The real time-PCR (RT-PCR) primers were synthesized by Sangon Biotech Co. Ltd., and the sequences (5' to 3') were as follows: $p57^{KIP2}$, forward: 5'-CAGAACCGCTGGGAT TACGA-3', reverse: 5'-CACCGAGTCGCTGTCCACTT-3' and caspase-3, forward: 5'- GAGTGCTCGCAGCTCATACCT-3', reverse: 5'-CCTCACGGCCTGGGATTT-3'. GAPDH was purchased from Takara Bio, Inc., and was used as an endogenous reference and its primer sequence was as follows: forward: 5'-TGCACCACCAACTGCTTAGC-3' and reverse: 5'-GGCATGGACTGTGGTCATGAG-3'. The mRNA expression of $p57^{KIP2}$ and caspase-3 was determined from each group of HepG2 cell cultures and performed in triplicate. Relative quantitation using the comparative CT method was performed for each sample group.

Statistical analysis. The Student's t-test was used to identify statistically significant differences among the samples for cell proliferation, cell cycle, apoptosis and quantitative PCR assays. The experiments were performed with three replicates and repeated three times. $P<0.05$ was considered to indicate a statistically significant result.

Results

Recombinant human decorin inhibits cell growth in HepG2 hepatoma cells. To investigate the cell proliferation inhibitory role of recombinant human decorin, an MTT assay was performed. As shown in Fig. 1, the control group cells and pcDNA3.1 group cells exhibited a higher OD after 72 h transient transfection, whereas in the pcDNA3.1-DCN group, cell proliferation was markedly inhibited at a statistically significant level. This result revealed that recombinant human decorin represses cell growth in HepG2 cells after 72 h of transient transfection and so all of the following experiments were conducted after culturing for 72 h.

Recombinant human decorin induces G_0/G_1 cell cycle arrest in HepG2 cells. Flow cytometry was used to investigate the cell cycle regulatory role of recombinant human decorin in HepG2 cells. Our result revealed that recombinant human decorin caused cell cycle arrest at the G_0/G_1 phase in HepG2 cells at 72 h following transient transfection (Fig. 2). More HepG2 cell cultures that were treated with pcDNA3.1-DCN accumulated in

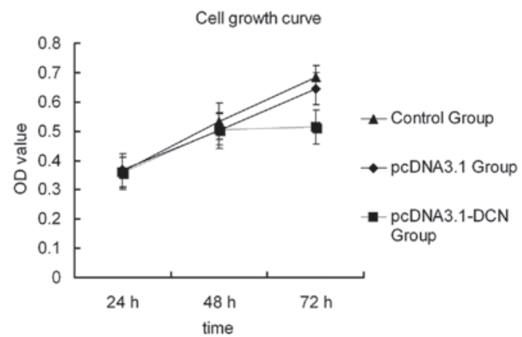

Figure 1. Effect of recombinant human decorin on proliferation of three groups of HepG2 cells. Three groups of HepG2 cells were cultured for 24, 48 and 72 h after transient transfection, and the proliferation rate of the three groups was measured by the MTT reagent. The pcDNA3.1-DCN group cell growth rate was significantly lowered compared with the pcDNA3.1 and control group cells ($P<0.05$). The assay was performed in triplicate. Data are expressed as the means ± SD of independent experiments. OD, optical density; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide

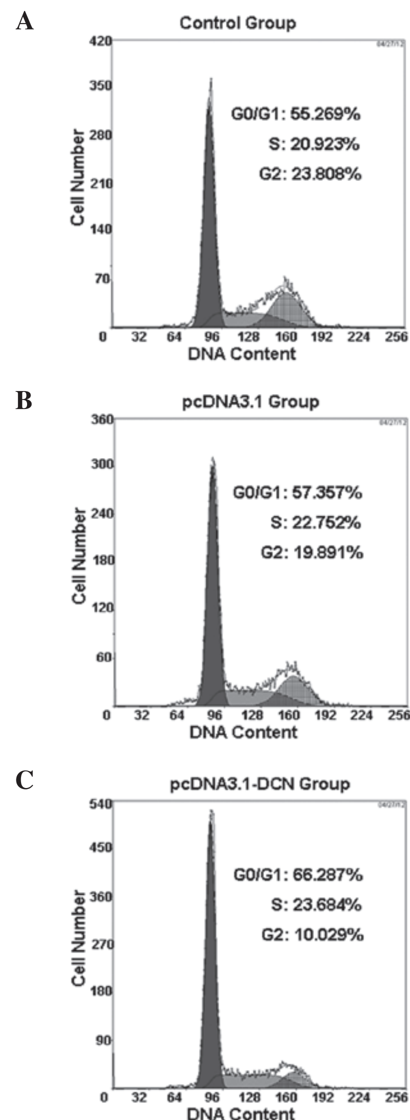

Figure 2. Results of the cell cycle for the three groups. After 72 h transient transfection, cells from each HepG2 group were harvested separately and subjected to flow cytometry for cell cycle analysis. Histograms are representative of three independent experiments. (A) Control group. (B) pcDNA3.1 group. (C) pcDNA3.1-DCN group.

Table I. Cell cycle distribution of pcDNA3.1-DCN, pcDNA3.1 and control group cells (n=3).

	Cell cycle distribution (%)		
Group	G_0/G_1	S	G_2/M
Control	55.323±1.641	21.045±1.442	23.632±1.815
pcDNA3.1	57.116±1.421	21.284±1.735	21.602±1.912
pcDNA3.1-DCN	66.126±2.701[a]	22.108±1.915	11.776±1.043[a]

[a]P<0.05, compared with the control group.

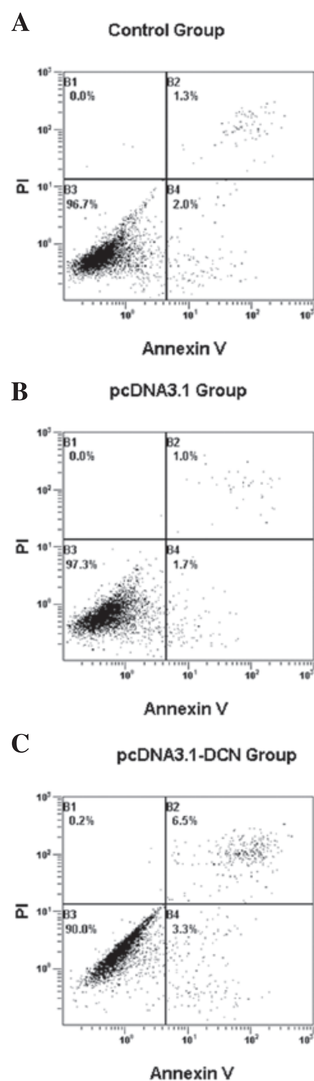

Figure 3. Results of the apoptosis analysis for the three HepG2 groups. Cultured cells from each group were allowed to grow after 72 h transient transfection, stained with annexin V and propidium iodide, and subjected to flow cytometry. The right quadrant, including the lower right quadrant and the upper right quadrant, revealed apoptotic cells. The results of the apoptosis are representative of three repeated experiments. (A) Control group. (B) pcDNA3.1 group. (C) pcDNA3.1-DCN group.

the G_0/G_1 phase compared with the control and pcDNA3.1 groups. The percentage of cells in the G_0/G_1 phase was 66.126±2.701, 57.116±1.421 and 55.323±1.641% in the pcDNA3.1-DCN, pcDNA3.1 and control groups, respectively (Table I).

Table II. Role of recombinant human decorin in inducing apoptosis in HepG2 group cells (n=3).

Group	Apoptosis (%)
Control	3.1±0.3
pcDNA3.1	2.8±0.2
pcDNA3.1-DCN	10.2±0.6[a]

[a]P<0.05, compared with the control group.

Recombinant human decorin induces apoptosis in HepG2 cells. We examined the effect of recombinant human decorin on the induction of apoptosis and, as shown in Fig. 3, recombinant decorin induced apoptosis. The proportion of cells stained with annexin V and PI was higher in the pcDNA3.1-DCN group compared with the control and pcDNA3.1 groups (Table II). We measured caspase-3 expression by quantitative RT-PCR in the three human HepG2 groups. As illustrated in Fig. 4, caspase-3 expression was markedly increased in the pcDNA3.1-DCN group compared with the pcDNA-3.1 and control groups. There was no statistically significant difference in the expression of caspase-3 between the control group and pcDNA3.1 group (P>0.05).

$p57^{KIP2}$ expression in the three HepG2 cell groups. To determine the effect of human recombinant decorin on the relative expression of $p57^{KIP2}$ mRNA between each cell group, quantitative RT-PCR was used. GAPDH was used as an endogenous reference for normalization. The $p57^{KIP2}$ mRNA level was more highly expressed in the pcDNA3.1-DCN group than in the control and pcDNA3.1 groups.

Discussion

To the best of our knowledge, our findings demonstrate for the first time that recombinant human decorin upregulates $p57^{KIP2}$ mRNA levels in HepG2 cells. By using quantitative RT-PCR, we examined the expression of $p57^{KIP2}$ transcriptional mRNA levels and identified that expression was higher in recombinant human decorin-treated HepG2 cells compared with the control and pcDNA3.1 groups.

Earlier studies have revealed that decorin, a member of the family of small leucine-rich proteoglycans, inhibits the growth of cancer cells. For instance, Hu *et al* demonstrated that decorin

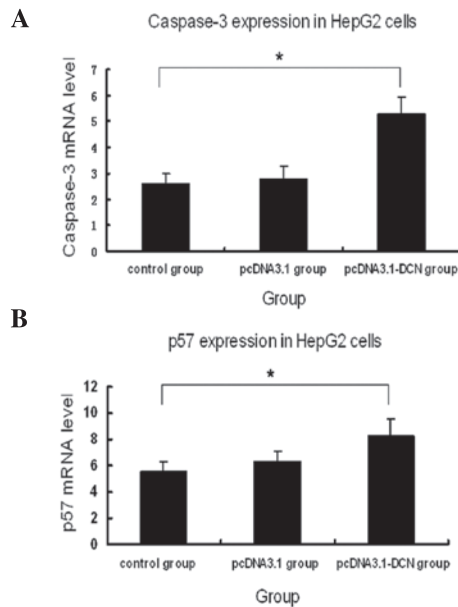

Figure 4. Effects of recombinant decorin on caspase-3 and p57KIP2 expression. RNA from each group was extracted, transcribed and evaluated via quantitative PCR. GAPDH was used as an internal reference gene. Assays were performed in triplicate, P<0.05. (A) Caspase-3 mRNA expression. (B) p57 mRNA expression.

suppressed prostate cancer cells through the EGFR and androgen receptor pathways (29). In addition, a study conducted by De Luca *et al* revealed that the inhibitory effect of decorin was correlated with the overexpression of p21, a CDK inhibitor (30).

Furthermore, previous studies have revealed that the decorin protein core causes cell death in both *in vivo* and *in vitro* experiments by activating the caspase-3 enzyme (31). In agreement with this, our study demonstrated that recombinant human decorin induced apoptosis via the activation of caspase-3 in the HepG2 cell line. In this study, the caspase-3 gene was significantly overexpressed in the recombinant human decorin-transfected HepG2 cells compared with the control and pcDNA3.1 groups. Caspase-3 is a crucial enzyme for apoptosis (32). Thus, one of the tumor suppressive properties of recombinant decorin in HepG2 cells promotes cell death via activation of the caspase-3 enzyme.

Growing evidence indicates that CDK inhibitors, including $p57^{KIP2}$, are important in regulating cell proliferation and differentiation, cell cycle and cell apoptosis (19,20). Enhancing the expression of CKIs in order to suppress the activity of CDK in cancer has become a focus of cancer therapy research. Thus, reactivating the cyclin-CDK inhibitors using a small tumor inhibitor molecule, such as recombinant human decorin, provides an attractive therapeutic strategy for cancer treatment. We recently demonstrated that recombinant human decorin represses the growth of HepG2 cells by upregulating p21 via the p53-independent pathway (28). Ma and Cress demonstrated that $p57^{KIP2}$ was significantly upregulated using small molecule CDK inhibitors, for instance BMS-387032 (SNS-032), in a breast cancer cell line (33). These findings led us to examine the role of recombinant decorin in the reactivation of $p57^{KIP2}$, a family member of the CDK inhibitors in HepG2 culture cells. It is well documented that $p57^{KIP2}$ is a potential tumor suppressor gene (34). However, the expression of this multifunctional CDK inhibitor is generally silenced in many types of cancer. A recent study by Guo *et al* demonstrated that the downregulation of $p57^{KIP2}$ accelerates the growth and invasion of HCC (27). It has also been reported that $p57^{KIP2}$ expression correlates with the malignant transformation of hepatocytes (35). Furthermore, another recent study revealed that the decreased expression of decorin and $p57^{KIP2}$ correlated with poor survival rates and lymphatic metastasis in lung cancer patients (36). These studies suggest that $p57^{KIP2}$ has a role in tumor inhibition. Notably, besides the possible function in tumorigenesis, the decreased expression of $p57^{KIP2}$ may provide important prognostic implications for patients with ovarian, hepatocellular and colorectal cancer, and acute lymphoblastic leukemia (23,37-39). Previously, it had been reported that the marked loss of $p57^{KIP2}$ expression is a frequent event in HCC and so it may be important in the differentiation of HCC (25). Taken together, these observations imply that the reactivation of $p57^{KIP2}$ suppresses the growth of cancer cells. Thus, restoring the normal function of $p57^{KIP2}$ by increasing its expression using small molecule inhibitors may have therapeutic value. The results from our study demonstrated an increase in the expression of $p57^{KIP2}$ in the pcDNA3.1-DCN HepG2-infected group compared with the control and pcDNA3.1 groups. Statistical analysis of the changes in $p57^{KIP2}$ mRNA levels revealed a significant induction of $p57^{KIP2}$ expression by recombinant decorin in HepG2 culture cells.

In this study, our results demonstrate that recombinant decorin induces the reactivation of $p57^{KIP2}$ transcriptional mRNA in the HepG2 cell line. Therefore, it may suppress cell growth in the HepG2 cell line by upregulating p21 and also by reactivating $p57^{KIP2}$, a cyclin-CDK inhibitor identified in pcDNA3.1-DCN-infected HepG2 cells.

In conclusion, our results demonstrated that recombinant human decorin increases the expression of $p57^{KIP2}$ mRNA in the HepG2 cell line. Since p57 protein expression is silenced in various types of cancer, its reactivation may have a therapeutic use in clinical practice, in addition to other prognostic implications. In the present study, the statistical analysis of the changes in $p57^{KIP2}$ mRNA levels revealed a significant induction of $p57^{KIP2}$ expression by recombinant decorin in the HepG2 culture cells. However, for a more detailed understanding, particularly regarding the mechanism of recombinant decorin in the upregulation of $p57^{KIP2}$ in the HepG2 cell line, further studies are required.

Acknowledgements

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