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Wnt/β-catenin aids in regulating the proliferation of hepG2 cells mediated by thy-1

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ABSTRACT. Cancer stem cells have been found to play important roles in carcinoma. Although thy-1 has been identified as a potential stem cell marker of liver cancer, whether the Wnt/β-catenin signaling pathway plays an important role in regulating hepatocarcinoma proliferation and apoptosis mediated by thy-1 remains unknown. Our results showed that high thy-1 expression caused hepG2 cells transfected with a pReceiver-M29/thy-1 eukaryotic expression vector to exhibit obvious heteromorphism, featuring double or multiple nuclei and weaker apoptosis. A high expression of β-catenin, as a critical signaling protein of Wnt/β-catenin, and its downstream transcription factor, cyclinD1, were detected in transfected hepG2 cells. We also used aspirin as an inhibitor of the Wnt signaling pathway in the treatment of hepG2 cells transfected with the pReceiver-M29/thy-1 expression vector to make detailed observations of apoptosis in hepG2 cells as well as the differential expression of β-catenin, cyclinD1, and thy-1. An increasing apoptosis rate was detected in the hepG2 cells and downregulated expression of the three proteins was detected. Hence, we suggest that thy-1 upregulation promotes the proliferation and inhibits apoptosis

of hepG2 cells, and that these processes are regulated by the Wnt/β-catenin signaling pathway.

Key words: Wnt/β-catenin; Thy-1; Proliferation; Aspirin; Apoptosis

INTRODUCTION

Hepatocarcinoma is the fifth most common type of cancer and one of the leading causes of cancer mortality worldwide. Although various treatments for hepatocellular carcinoma (HCC) are available, such as surgical removal and transplantation, the three year-survival rates are still not promising (Chiba et al., 2006; Ma et al., 2007; Visvader, 2011). The profound genetic changes identified in HCC include activation of proto-oncogenes, gene deletion, or mutation, reactivation of telomerase activity, and epigenetic abnormalities. However, these changes and existing theories of oncogenesis do not comprehensively elucidate the mechanisms underlying tumor development, progression, metastasis, and recurrence. Thus, other theories for the biological behavior of HCC have been formulated.

Over the past few decades, several lines of evidence have revealed the existence and importance of cancer stem cells (CSCs) in carcinogenesis. CSCs are considered root cause of cancer and are responsible for tumor growth as well as the differentiation of heterogeneous cell populations within tumors (Suetsugu et al., 2006; Lee et al., 2009; Colombo et al., 2011).

CSCs comprise a newly identified subpopulation that possesses stem cell properties and may differentiate into heterogeneous progenies of malignant cells. CSCs are most likely the progenitor cells that undergo unknown genetic mutations and lose potential for tissue repair. However, these cells retain stem cell characteristics, such as self-renewal and plasticity, which allow them to differentiate into different cell types in tumor tissues. CSCs are inferred to be the cells that are the least sensitive to chemotherapy or radiotherapy. These cells are also perceived to develop resistance to pharmacological, biological, and radiotherapeutic treatments. CSCs are also the most likely sources of tumor metastasis and relapse (Abou-Alfa, 2006; Zhou et al., 2009; Marquardt et al., 2010; Yam et al., 2010).

Thy-1 is a 25 to 37 kDa glycosylphosphatidylinositol-anchored glycoprotein that is expressed in bone marrow-derived mesenchymal stem cells and in hepatic stem/progenitor cells (Rege and Hagood, 2006). Thy-1 is primarily involved in cell-cell and cell-matrix interactions (Rege and Hagood, 2006). Recent studies have shown high-oncogenicity-marked thy-1+ liver cells, which were regarded as potential liver cancer stem cells (Yang et al., 2008a,b,c; Cohen et al., 2009; Conrad et al., 2009, Lingala et al., 2010; Lu et al., 2011; Ho et al., 2012). Results from our previous studies indicated that thy-1 is expressed in approximately 1% of hepG2 cells and thy-1+ hepG2 cells, but not in thy-1-cells, and demonstrated high tumorigenesis upon inoculation in 0.5×10^5 cells per BALB/C mouse after 2 months. High thy-1 expression was observed in 72% (36/50 cases) of neoplastic hepatic tissues compared to 40% (20/50 cases) of control tissues. In addition, thy-1 expression is higher in poorly differentiated liver tumors than in well-differentiated tumors (Cheng et al., 2012). Therefore, further understanding of the role of thy-1 in carcinogenesis is crucial for effective management of HCC.

The Wnt signaling pathway is involved in various differentiation events during embryonic development and could lead to tumor formation after the aberrant activation of its components. Beta (β)-catenin, a cytoplasmic component, plays a major role in the transduc-

tion of canonical signaling (Fevr et al., 2007; Neth et al., 2007; Takigawa and Brown, 2008; Yam et al., 2010). Aspirin could inhibit β-catenin activity and is used as a marker to degrade intestinal tract tumorigenesis. Thus, we investigated whether the Wnt/β-catenin signaling pathway might participate in regulating the proliferation of hepG2 cells mediated by thy-1, and we determined the role of aspirin in this regulating pathway.

MATERIAL AND METHODS

Cell lines

The human cell line hepG2 (ADCC; USA) was maintained in an RPMI1640 (Hyclone; USA) medium with 10% fetal bovine serum (Hyclone) and 1% penicillin at 37°C in a humidified atmosphere of 5% CO_2 in air.

Vector construction and transfection

The pReceiver-M29 (Genecopoeia™, FulenGenCo., Ltd.); G418 (Sigma; China Mainland), Lipofectamine 2000™ (Invitrogen, Cat. No. 11668027), Opti-MEN I (Gibco, GO-31985); primer-amplified *thy-1* coding region sequence Forward: 5'-CCGACAACCACTACCTGA-3', Reverse: 5'-GTGGCACCTTCCAGGGTC-3', with a total length of 486 bp, was synthesized (Shang Hai Bio; China). Briefly, the *thy-1* coding region sequence was amplified using reverse transcription-polymerase chain reaction (RT-PCR) according to the following reaction conditions: i) an initial step of Taq-activation at 90°C for 5 min, ii) 35 cycles at 90°C for 5 s and at 58°C for 30 s, and iii) a final strand synthesis at 72°C for 5 min. After verifying the product, the pReceiver-M29 vector and the RT-PCR product were digested using a specific enzyme and were then linked using the T4DNA enzyme at 42°C overnight. The linkage product was then transfected on an agarose plate and set in a rocking bed at 37°C for 12 to 16 h. The positive clone was selected and transfected into a Luria-Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) with Acillin at 37°C for 12 to 16 h. The pReceiver-M29/thy-1 recombination vector was then extracted and identified using agarose gel electrophoresis. The pReceiver-M29/thy-1 recombination vector was transfected into the hepG2 cells in a 500 µL final concentration consisting of 10 µL Lipofectamine 2000™, 4 µg pReceiver-M29/thy-1 recombination vector, and 480 µL Opti-MEN I, according to manufacturer instructions. G418 was added to the hepG2 cells, and the positive clone was selected after transfection for 24 h and 15 days. RT-PCR and Western blot analysis for the gene and protein levels, respectively, were used to confirm whether the pReceiver-M29/thy-1 recombination vector was correctly constructed.

RT-PCR

Using an RT-PCR kit (TaKaRa, code: DRRO14A), total RNA was isolated using 1 x 10^7 cells/mL Trizol reagent (Invitrogen, Cat: 15596-026) according to manufacturer instructions. The total RNA concentration was measured using an Eppendorf Bio photometer. The first-strand cDNA was generated from 5 µg total RNA per sample using the 20 µL system, including 1 µL random 6 mers, 1 µL dNTP mixture, 0.5 µL prime Scrip™ RTase, and 0.5 µL RNase

inhibitor. The gene-specific primers for *thy-1* (primer A: 5'-AAGGTGACCAGCCTAACGG-3', primer B: 5'-CCCTCGTCCTTGCTAGTGAA-3', and for *β-actin* (primer A: 5'-GGAAATCGTGCGTGACATT-3', primer B: 5'-CGTCATACTCCTGCTTGCTG-3') were designed using the Primer 3 software and were then synthesized (Shanghai Biologic Company; China). The RT-PCR was run in 50 µL reactions with 1 µL specific primers mentioned above. The thy-1 gene, with a length of 293 bp, and the *β-actin* gene, with a length of 473 bp, were amplified. The reaction steps were as follows: 95°C for 5 min, 35 cycles at 95°C for 30 s, 58°C for 30 s, 72°C for 30 s, 72°C for 5 min, and then 4°C as the end temperature. The PCR product was confirmed using a single band in high-resolution agarose gel electrophoresis (USA).

Flow cytometry and immunofluorescence

The hepG2 cells were washed with phosphate-buffered saline and then digested with trypsogen containing 0.25% edetic acid (EDTA) according to manufacturer instructions for Annexin V-FITC (Biyuntian Jiangshu, C-1062; China). The hepG2 cells were collected after centrifugation at 1000 *g* for 5 min. Then, 195 µL Annexin V-FITC binding liquid was used to re-suspend the cells after the supernatant was discarded. Then, 5 µL Annexin V-FITC was added to the above mixture and incubated for 10 min away from the light and at room temperature. After centrifugation at 1000 *g* for 5 min, 190 µL Annexin V-FITC binding liquid was used to again resuspend the cells, and 10 iodionation (PI) was added to the mixture. Flow cytometry was used to detect the apoptosis rate of the hpG2 cells after various treatments by observing the number of cells at different stages of apoptosis and death.

A Hoechst 33258 kit (Biyuntian Jiangshu; China, c-0003) was used to observe the appearance of the apoptosis cells by immunofluorescence. hepG2 cells were implanted into a port plate containing a sterile coverslip overnight. Approximately 50 to 80% of the cells were treated to stimulate apoptosis. A fixation liquid was added to the cells for 10 min. The cells were then washed twice for 3 min. Hoechst 33258 staining was added for 10 min at room temperature, and then a drop of quenching liquid was added to the slide and coverslip containing the cells to protect the fluorescence. A fluorescence microscope was used to observe the blue nuclei. Apoptosis cells displayed pykno- and hyperchromic nuclei or broken bits of cell tissue.

Immunocytochemistry

hepG2 cells were placed on a plate overnight at a concentration of 1 x 10^5. The breast carcinoma cell line DAB-DA231 and non-transfected hepG2 were used as positive and negative controls, respectively. The hepG2 cells in the test group were transfected with the pReceiver-M29/thy-1 expression vector and treated with aspirin according to manufacturer instructions for the Ultrasensitive™ S-P (mouse/rabbit) kit (Maixin; Fujian China). Briefly, 4% paraformaldehyde was used to fix the cells for 10 min, 0.3% triton-100 was used for 10 min to break down the cell membrane structure, 3% H_2O_2 was used for 10 min to block non-specific binding, and normal non-immune goat serum was used for 10 min. All these steps were performed at room temperature. The primary antibody of β-catenin was incubated overnight at 4°C (1:200, sc-7963, Santa Cruz Biotechnology Inc.; Santa Cruz, CA, USA). An equivalent concentration normal mouse IgG1 (Santa Cruz) was used instead of a primary antibody as an additional negative control. The biotin-conjugated second antibody and

streptavidin-peroxidase were incubated for 10 min at room temperature. One DAB (Maixin) was used to stain the cytoplasm and hematoxylin (Maixin). The intensity of the β-catenin signal was detected, which was evaluated by an experienced pathologist and classified into 0 = negative, 1+ = weak positive, 2+ = intermediate, and 3+ = strong positive.

Western blot

Total protein was extracted using 200 μL western and IP cell lysate and 20 μL 100 mM phenylmethanesulfonyl fluoride (PMSF) (Biyuntian Jiangsu; China) per 1 x 10^7 cells, shaken at 12,000 rpm for 10 min, and the supernatant was collected. The concentration of the total protein was quantified using a bicinchoninic acid (BCA) protein assay (Biyuntian, Jiangsu). The solubilized proteins were resolved by electrophoresis on 12% or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. The resolved proteins were transferred onto nitrocellulose membranes and subjected to immunoblot analysis using a mouse anti-thy-1 monoclonal antibody (sc-53456, Santa Cruz Biotechnology Inc.) diluted to 1:500, a mouse monoclonal β-catenin (sc-7963, Santa Cruz Biotechnology Inc.) diluted to 1:500, a mouse monoclonal cyclin-D1 antibody (sc-8396, Santa Cruz Biotechnology Inc.), or a mouse monoclonal β-actin antibody diluted to 1:1000 as a control. The immunocomplexes were detected by enhanced chemiluminescence. The intensity of the immunoreactive bands was quantified using Quantity One Image (Bio-Rad; USA). The results were expressed in relation to the control, and the value of the control was arbitrarily set to one.

Statistical analysis

The results are reported as means ± SD. One-way analysis of variance (ANOVA) with the least significant difference (LSD) multiple comparison test was performed. Two group comparisons were performed using the Student *t*-test, and the SPSS software (version 11.5 for Windows; SPSS Inc.; Chicago, IL, USA) was used for all statistical analyses. P values less than 0.05 were considered to be significant.

RESULTS

Thy-1 expression

The non-transfected and transfected void vector hepG2 cells were regarded as the negative control groups. We evaluated thy-1 expression at the gene and protein levels using RT-PCR and Western blot analysis. Total RNA and total protein were extracted from the cells and used as substrates in the RT-PCR and Western blot analysis, respectively, according to manufacturer instructions. Compared with the two controls, higher thy-1 expression was detected in the hepG2 cells transfected with the pReceiver-M29/thy-1 eukaryote expression vector at both the gene (Figure 1) and the protein (Figure 2) levels. These differences from the two control groups were statistically significant ($F = 500.909, 17856.89$, $P = 0.000$). However, no significant difference in thy-1 expression was found between the two control groups ($P > 0.05$).

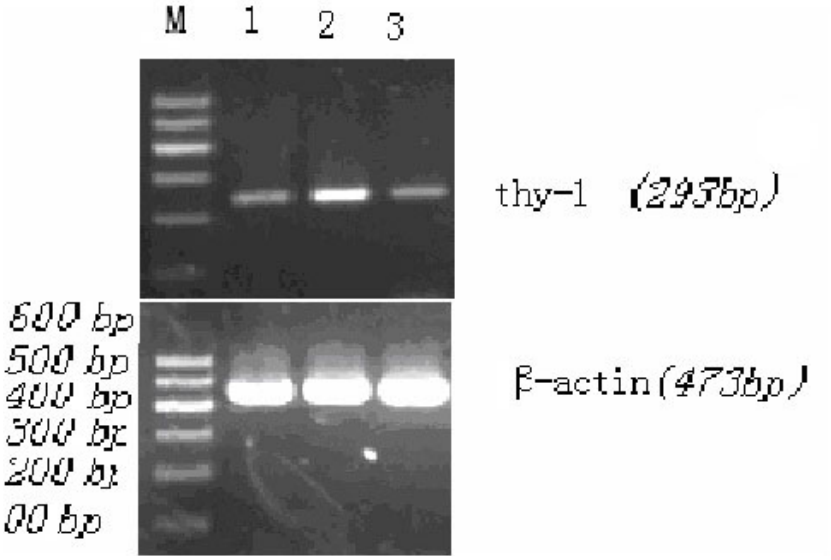

Figure 1. Higher expression in pReceiver-M29/thy-1 eukaryote expression vector by RT-PCR. *Lane 1* = non-transfected hepG2 cells showing weak expression of Thy-1; *lane 2* = the highest expression for Thy-1showing in transfected pReceiver-M29/thy-1 eukaryote expression vector; *lane 3* = transfected pReceiver-M29 vector hepG2 cells showing weak expression of Thy-1; *lane M* = biomaker.

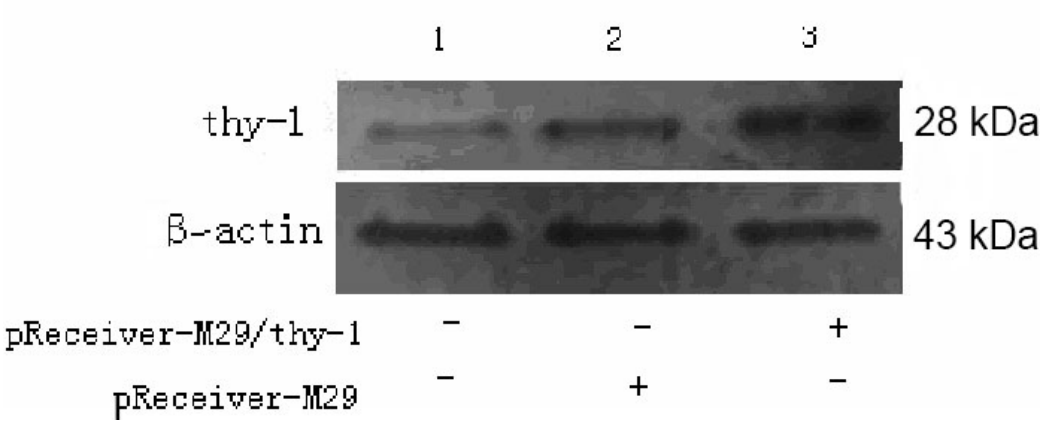

Figure 2. Thy-1 demonstrating higher expression in pReceiver-M29/thy-1 eukaryote expression vector by Western blot. *Lane 1* = non-transfected hepG2 cells showing weak expression of Thy-1; *lane 2* = transfected pReceiver-M29 vector hepG2 cells showing weak expression of Thy-1; *lane 3* = the highest expression for thy-1 showing in transfected pReceiver-M29/thy-1 eukaryote expression vector.

Apoptosis ratio

To understand the biological function of thy-1 in hepG2 cells, we detected hepG2 cells apoptosis after the cells were transfected with the pReceiver-M29/thy-1 eukaryote expression

vector and treated with aspirin by flow cytometry. The results showed that the apoptosis ratio of hepG2 cells transfected with the thy-1 expression vector was remarkably reduced compared to that of the non-transfected hepG2 cells. Subsequently, 0.5 μmol/mL aspirin was added to the hepG2 cells transfected with the thy-1 expression vector. We detected an increasing apoptosis ratio. Dead hepG2 cells increased in number compared with hepG2 cells that were not treated with aspirin and untransfected cells (Figure 3). A significant difference was found among the three batches of hepG2 cells treated with the methods described above (Table 1).

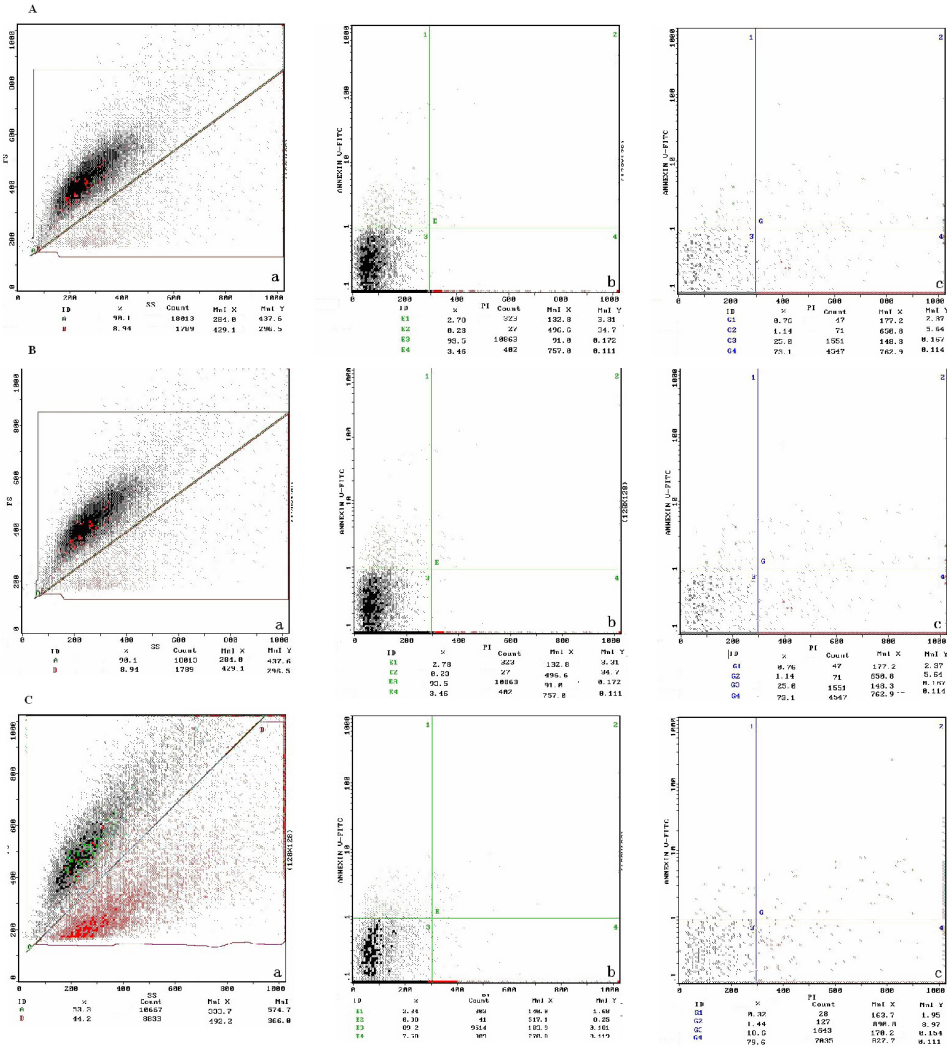

Figure 3. Apoptosis of hepG2 treated with different methods by flow cytometry. **A.** Non-transfected hepG2 cells demonstrating higher apoptosis ratio; **B.** hepG2 cells transfected pReceiver-M29/thy-1 eukaryote expression vector showing the lowest apoptosis ratio; **C.** hepG2 cells transfected pReceiver-M29/thy-1 eukaryote expression vector and treated with aspirin showing the highest apoptosis ratio. **a.** Scatter plot of flow cytometry; **b.** further analysis for figure **a** showing almost normal and apoptotic cells; **c.** further analysis for figure **b** showing almost died and apoptotic cells.

Table 1. Apoptosis of hepG2 treated by different methods by flow cytometry (means ± SD).

Apoptosis state	Groups (hepG2 cells)				
	Non-transfected	Transfected and aspirin	Transfected	F	P
Early	2.55 ± 0.01	3.27 ± 0.10	0.49 ± 0.06	1986.69	0.000
Advanced and died	77.59 ± 0.93	88.56 ± 1.22	54.33 ± 2.08	409.60	0.000
t	-137.57	-119.833	-44.783		
P	0.000	0.000	0.000		

Significant difference showing in the three above groups from early and advanced and died apoptosis state (P = 0.000). Significant difference also showing in apoptosis states in the three above groups (P = 0.000).

Using a fluorescence microscope, we observed further differences in the appearance of hepG2 cells after treatment. The apoptosis proportion was 10% in the hepG2 cells after being transfected with the pReceiver-M29/thy-1 eukaryote expression vector, compared with the 30% proportion in the untransfected cells, and the 90% proportion in the hepG2 cells treated with aspirin. A significant difference in the rate of apoptosis was found among the three hepG2 cells (F = 821.053, P = 0.000). From the fluorescence images, we observed that apoptotic hepG2 cells exhibited nucleolus pykno- or fragment status, whereas normal hepG2 cells were blue in color (Figure 4).

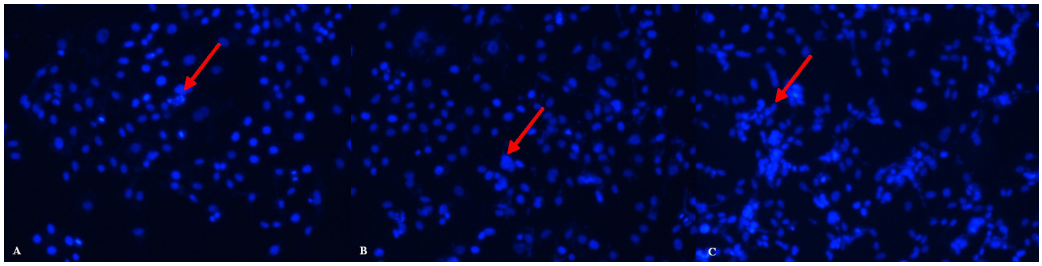

Figure 4. Apoptosis ratio of hepG2 cells treated with differenct methods by Fluorescence Microscope. **A.** Higher apoptosis ratio showing in non-transfected hepG2 cells (magnification 100X); **B.** the lowest apoptosis ratio showing in transfected pReceiver-M29/thy-1 eukaryote expression vector (magnification 100X); **C.** the highest apoptosis ratio showing in transfected pReceiver-M29/thy-1 eukaryote expression vector and treated with aspirin (magnification 100X). Apoptosis cell shape showing as arrow.

β-catenin

β-catenin is a critical signaling protein in the Wnt/β-catenin signaling pathway. Dephosphorylation could shift to the nucleus and bind with the nuclear transcription factor. In this study, using immunocytochemistry, we detected the β-catenin expression level in hepG2 cells after transfection with the pReceiver-M29/thy-1 eukaryote expression vector. The β-catenin protein in breast carcinoma AM293 cells was stained as a positive control (Figure 5A). Stronger β-catenin staining was observed in the transfected cells (Figure 5B) compared to the untransfected hepG2 cells (Figure 5C). We also found significant heteromorphism in the hepG2 cells transfected with double and multiplied nuclei, but not in the untransfected hepG2 cells. Weak staining was detected in the hepG2 cells treated with 0.5 μmol/mL aspirin compared with untreated hepG2 cells (Figure 5D).

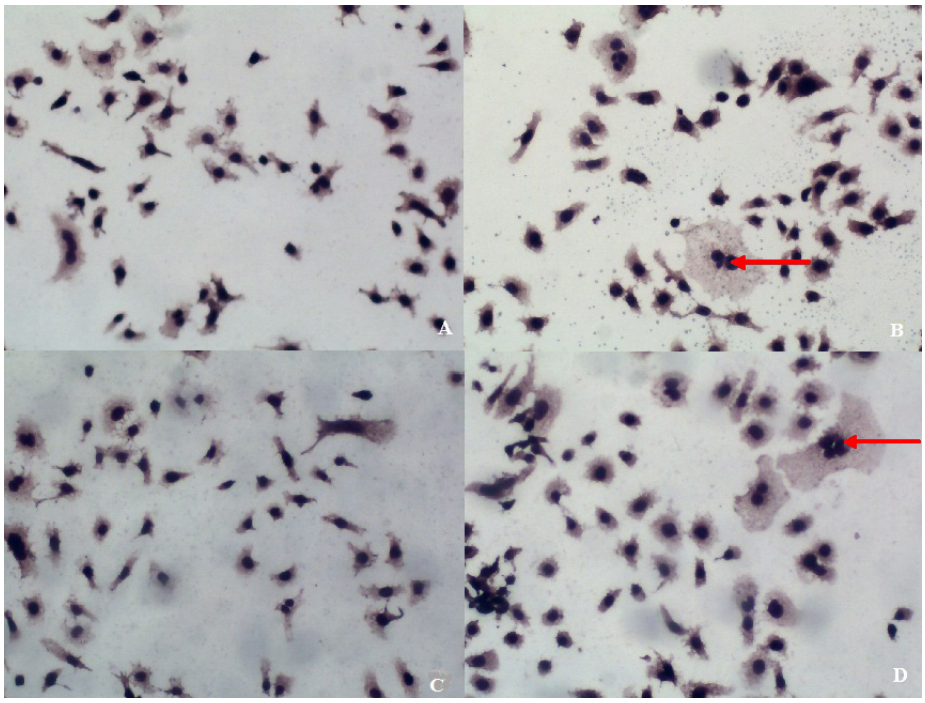

Figure 5. Immuocytochemistry staining for b-catenin in hepG2 cells after treated with different methods. **A.** positive staining for b-catenin in breast carcinoma AM293 cells (magnification 200X); **B.** strong staining for b-catenin in hepG2 cells transfected pReceiver-M29/thy-1 eukaryote expression vector (magnification 200X); **C.** weak staining for b-catenin in hepG2 cells (magnification 200X); **D.** weak staining for b-catenin in hepG2 cells transfected pReceiver-M29/thy-1 eukaryote expression vector with aspirin (magnification 200X); hepG2 cells transfected pReceiver-M29/thy-1 eukaryote expression vector demonstrating double and multipled nucleus as arrow hinting in (B) and (D).

β-catenin and cyclinD1 expression

The β-actin expression was used as an internal control. Western blot results showed that a higher β-catenin expression was detected in the hepG2 cells transfected with the pReceiver-M29/thy-1 eukaryote expression vector than in the untransfected hepG2 cells and in the pure transfected pReceiver-M29 vector (Figure 6A). Significant differences were detected between the first group and the other two groups ($F = 194.743$, $P = 0.000$). The detected expression of cyclinD1 was similar to that of β-catenin (Figure 6B) ($F = 19846.94$, $P = 0.000$).

The transfected pReceiver-M29/thy-1 eukaryote expression vector and the untransfected hepG2 cells were used as negative controls. After these two groups were treated with aspirin, we further detected the expression of β-catenin, cyclinD1, and thy-1. The lowest β-catenin expression level was observed in the transfected pReceiver-M29/thy-1 eukaryote expression vector after aspirin treatment. By contrast, no significantly weak β-catenin expression was found in the untransfected hepG2 cells after aspirin treatment (Figure 6C). Significant differences were found among the four groups ($F = 1316.963$, $P = 0.000$). A significant difference was also found between two of the groups ($P < 0.05$).

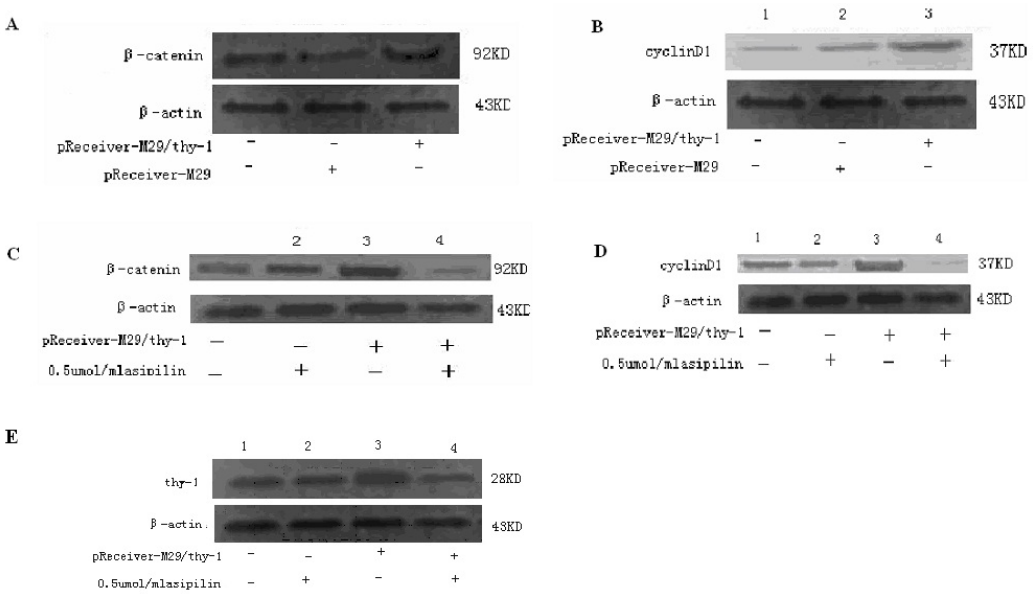

Figure 6. B-catenin and cyclinD1 expression by Western blot. **A.** The highest expression of B-catenin demonstrating inv pReceiver-M29/thy-1 eukaryote expression vector than non-transfected hepG2 cells. **B.** The highest expression of cyclinD1 demonstrating in pReceiver-M29/thy-1 eukaryote expression vector than non-transfected hepG2 cells. **C.** The highest expression of B-catenin demonstrating in pReceiver-M29/thy-1 eukaryote expression vector and groups treated by aspirin. **D.** The highest expression of cyclinD1demonstrating in pReceiver-M29/thy-1 eukaryote expression vector and groups treated by aspirin. **E.** The highest expression of thy-1demonstrating in pReceiver-M29/thy-1 eukaryote expression vector and groups treated by aspirin.

A significantly weak cyclinD1 expression level was detected in the transfected pReceiver-M29/thy-1 eukaryote expression vector after aspirin treatment. By contrast, a slightly weak cyclinD1 expression level was detected in the untransfected hepG2 cells after aspirin treatment (Figure 6D). Significant differences were found among the four groups above ($F = 7480.75$, $P = 0.000$). A significant difference was also found between two of the groups ($P < 0.05$).

To investigate the relationship between the Wnt/β-catenin signaling pathway and the thy-1 protein, thy-1 expression was further detected in the hepG2 cells treated with β-catenin. The lowest expression level was detected in the transfected pReceiver-M29/thy-1 eukaryote expression vector after aspirin treatment, but the expression was slightly weak in the untransfected hepG2 cells with aspirin treatment (Figure 6E). Significant differences were found among the four groups ($F = 805.571$, $P = 0.000$). A significant difference was also observed between two of the groups ($P < 0.05$), except in the untransfected hepG2 cells, regardless of aspirin treatment ($P = 0.446$).

DISCUSSION

The distinct characteristics of stem cells include self-renewal and multi-directional differentiation potency, both of which are normally strictly regulated by multiple signaling con-

duction pathways. Once a component of a signaling pathway is subjected to mutation or disproportionate expression, this regulatory mechanism becomes abnormal or destructive. Stem cells could thus undergo dysdifferentiation, unconditional growth, and development to form tumor stem cells (Fevr et al., 2007; Neth et al., 2007; Takigawa and Brown, 2008; Yam et al., 2010).

The Wnt/β-catenin signaling pathway is a recently discovered signaling pathway that plays an important role in maintaining self-renewal and suppressing differentiation, proliferation, migration, polarity, and apoptosis. The Wnt/β-catenin signaling pathway occurs in various types of tissues, including the epidermis, intestinal, haemopoietic, nerve, and embryonic stem cells. Recent studies have reported that Wnt/β-catenin can cause liver cancer and development by activating the downstream target gene (Yang et al., 2008c; Cohen et al., 2009; Cheng et al., 2012). β-catenin is a critical regulatory factor that can bind to T-cell factors (TCFs) to form β-catenin/TCF heterodimers, which could activate many proto-oncogenes, including c-myc, c-jun, cylinD1, and VEGF. Although numerous studies have confirmed the relationship between the Wnt/β-catenin signaling pathway and hepatocarcinoma, no detailed reports are available on the role of this pathway in the regulation of the biological function of liver stem cells to hepatocarcinoma. Thus, this study was intended to elucidate whether the thy-1 biological function in liver cancer hepG2 cells might be related with the Wnt/β-catenin signaling pathway.

Based on the approximately 1% thy-1 expression in hepG2 cells according to the flow cytometry results, we propose that the pReceiver-M29/thy-1 eukaryote expression vector upregulates thy-1 expression in hepG2 cells. Untransfected and only retransfected pReceiver-M29vector hepG2 cells were used as controls. The RT-PCR and Western blot results confirmed that thy-1 was upregulated in hepG2 cells transfected with the pReceiver-M29/thy-1 eukaryote expression vector compared with the two controls.

The relationship between the proliferation and apoptosis of hepG2 cells and thy-1 was recently discovered. Flow cytometry was used to detect the apoptosis of thy-1. A lower apoptosis ratio was detected in the transfected pReceiver-M29/thy-1 hepG2 cells than in the untransfected hepG2 cells. The significant difference between the two hepG2 cells demonstrated that thy-1 possibly inhibits apoptosis. Hoechst 33258 staining was used to confirm the lower apoptosis ratio in the transfected hepG2 cells, which showed nucleolus pykno- or fragment status, unlike normal hepG2 cells, which showed a blue color.

The position of β-catenin in hepG2 was investigated using immunocytochemistry. Strong staining was detected in the transfected hepG2 cells compared with the non-transfected cells. Significant heteromorphism was also observed in the hepG2 cells transfected with pReceiver-M29/thy-1, featuring double and multiple nuclei. No heteromorphism was observed in the untransfected hepG2 cells. This phenomenon was possibly caused by the excess proliferation of hepG2 cells or by the hepG2 cells displacing β-catenin from the cytoplasm to the nucleus by stimulated thy-1. The β-catenin and cyclinD1 expressions were further detected using Western blot, and similar results were obtained. Higher thy-1 expression was found in the transfected pReceiver-M29/thy-1 hepG2 cells than in the control cells, possibly because thy-1 activated the β-catenin protein, with the latter displaced into the nucleus, and combined with cyclinD1 to cause the proliferation of hepG2 cells.

Non-steroidal anti-inflammatory drugs (NSAIDs) could prevent intestinal tract tumorigenesis (Chan et al., 2005). According to Haile et al. (2005), NSAIDs could inhibit the growth of many kinds of malignant tumors, which depend on the COX pathway and other mechanisms, including the Wnt/β-catenin pathway, which has attracted intense research inter-

est. Some reports have confirmed that aspirin and indometacin could suppress the activity of certain signaling proteins in the Wnt/β-catenin signaling pathway. Inhibiting β-catenin expression and changing its cell position could hinder the transcription and translation of cyclinD1 and c-myc, the latter of which suppresses cell proliferation and induces apoptosis (Hawcroft et al., 2002; Dihlmann et al., 2003).

Based on the findings of Dihlmann et al. (2001), 0.5 μmol/mL aspirin was used in this study to interfere with the activity of β-catenin to investigate whether the Wnt/β-catenin signaling pathway might regulate the biological function of thy-1 in hepG2 cells. After 48 h aspirin treatment, the hepG2 cells transfected with pReceiver-M29/thy-1 exhibited higher apoptosis, i.e., the number of cells in the advanced stage of apoptosis and the number of dead cells were obviously increased compared with the untreated hepG2 cells. Slight, weak staining for β-catenin, and obviously low β-catenin and cyclinD1 expressions were also detected. Thy-1 expression was downregulated after the transfected pReceiver-M29/thy-1 hepG2 cells were treated with aspirin.

Therefore, we conclude that aspirin could inhibit β-catenin activity and restrain cyclinD1 transcription, which in turn depressed the thy-1 proliferative function of hepG2 cells. We can therefore draw a preliminary conclusion that the Wnt/β-catenin signaling pathway might participate in the proliferation and apoptosis regulation of hepG2 cells mediated by thy-1.

This study is the first to demonstrate the relationship between Wnt/β-catenin and thy-1 in liver cancer. Further research should be performed to confirm this relationship in liver cancer tissues. Moreover, designing short-interfering RNA to inhibit β-catenin activity could downregulate thy-1 expression and possibly reduce the occurrence and recurrence of liver cancer in the future.

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