Stabilized β-catenin promotes hepatocyte proliferation and inhibits TNFα-induced apoptosis

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The human hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide. The mechanisms of liver cell oncogenic transformation are still unknown. The β-catenin mutations are identified in up to 30% of HCC and 80% of hepatoblastoma, suggesting a potential role of β-catenin in the pathogenesis of liver cancers. To define the biological role of the stabilized β-catenin in liver cell growth and transformation, we examined the effect of mutant β-catenin on an immortalized murine hepatocyte cell line, AML12. A cell line that stably expresses mutant β-catenin was established. The cell proliferation, apoptosis, and cell transformation of this cell line were characterized. Our data indicate that the stabilized β-catenin enhances hepatocyte proliferation, suppresses TNFα/Act D-induced cell apoptosis, and causes weak anchorage-independent cell growth. The stabilized β-catenin-containing cells did not develop tumor in immune-deficient mice. The target genes, *c-myc* and *cyclin D1*, were activated by β-catenin in the hepatocytes. Our study suggests that mutant β-catenin can promote cell proliferation and cell survival ability, but the stabilized β-catenin alone is insufficient for completely oncogenic transformation.

The β-catenin has two basic physiological roles inside the cells: cell-to-cell adhesion by association with E-cadherin, α-catenin, or γ-catenin;[1] serving as an important mediator in the Wnt/β-catenin signaling transduction pathway to regulate cell growth and cell differentiation.[2,3] This signaling pathway plays a critical role in the normal development of many organ systems, including the nervous system, the skin,[4] the gastrointestinal tract,[5] and the liver.[6] It also plays a role in liver regeneration.[7] Aberrant Wnt/β-catenin signaling pathway is believed to be involved in carcinogenesis.[8] Numerous studies have shown that many types of cancer cells contain mutations that may cause dysregulation of the Wnt/β-catenin signaling pathway, the key event of

which is the stabilization of β-catenin protein inside the cells.[9]

In a normal quiescent cell, the serine or threonine residuals located at the NH2 terminus of the β-catenin protein is phosphorylated by the glycogen synthase kinase β (GSK3β) in the context of a protein complex composed of Axin, GSK3β, adenomatous polyposis coli (APC), and β-catenin.[10] The phosphorylated β-catenin is then degraded by ubiquitin-proteasome pathway.[11] Mutations in any of these components may cause β-catenin accumulation in the cytoplasm and subsequent translocation into the nucleus. Mutations of APC and β-catenin have been well-documented in colon cancers.[12,13] These mutations are believed to play an important role in the development of colorectal cancer. Recent studies have also shown that liver cancers, including hepatocellular carcinoma (HCC) and hepatoblastoma, have β-catenin or Axin mutations.[14,15] Approximately 30–40% of HCC and 80% of the hepatoblastoma harbor β-catenin mutation, which suggests its potential role in the pathogenesis of these tumors.[14] In addition, β-catenin mutations have also been reported in thyroid cancer, prostate

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cancers, endometrial tumor, ovarian cancer, and skin tumors.[16–18] The role of β-catenin in the tumorigenesis is unknown. It is believed that the β-catenin exerts its function by interaction with members of the T-cell transcription factor (Tcf) or lymphoid enhancer factor (Lef) family to activate the downstream target genes.[19] Although the complete list of the target genes is still not available, cyclin D1 and c-myc, two essential transcription factors in cell growth and apoptosis regulation, are among the target genes.[20,21] The other recently identified potential targets include ITF-2 (immunoglobulin transcription factor-2),[22] glutamine synthetase,[23] and inositol hexakisphosphate kinase 2 (IP6K2).[24]

It is generally agreed that β-catenin is an important mediator in the Wnt/β-catenin signaling pathway. However, the precise biological function of β-catenin is still not fully defined. Several studies have suggested that the functional or stabilized β-catenin (N-terminal mutations) promotes cell survival, which is probably one of the underlying mechanisms for cancer development.[25] Although clinical studies and studies using transgenic mouse models have demonstrated the strong association between the β-catenin mutations and tumor formation, evidence for β-catenin direct neoplastic transformation is still sketchy. Kolligs et al have demonstrated that mutant β-catenin is capable of transforming RE3K cell, an adenovirus E1A-immortalized epithelial cell line derived from neonatal rat kidney.[26] The investigators have demonstrated that the transformation is associated with dysregulation of Tcf/Lef transcription activity. Aoki et al have shown that stabilized β-catenin was capable of transforming primary chicken fibroblasts and the transformation is associated with a novel target gene, IP6K2.[24] Transgenic mice expressing mutant β-catenin in keratinocytes developed epitheloid cysts and hair follicle tumors.[27] Overexpression of stabilized β-catenin in mouse colonic epithelial cells caused dysplasia and adenoma.[28] However, overexpression of a mutant β-catenin did not induce liver tumor; instead, the liver developed severe hepatomegaly immediately after birth.[29,30] Nevertheless, all these studies suggest that activated β-catenin (stabilized β-catenin) promotes cell growth and survival. A recent study by Monga et al demonstrated that β-catenin played a critical role in fetal liver cell proliferation and apoptosis.[6]

HCC is a common malignant tumor worldwide. Although chronic viral hepatitis (hepatitis B and hepatitis C) and long-term alcohol usage are important etiologic factors, the mechanisms of oncogenic transformation are still not well understood.[31] It is critical to investigate the molecular pathways that are involved in the tumorigenesis, in order to design better treatment for this fatal disease. A murine hepatocyte cell line AML12, established by Wu et al from a human transforming growth factor α (TGFα) transgenic mouse,[32] has been used as a cell model to study the tumorigenic activity of hepatitis

B viral pX protein.[33] In this study, the investigators demonstrated that the pX protein was capable of transforming the immortalized hepatocytes as determined by anchorage-independent cell growth assay. In another study, Dumenco et al[34] showed that introduction of mutant TP53 conferred AML12 cell growth advantage rather than cell transformation. These studies indicated that this cell line could serve as a model to study the molecular pathways in liver cell growth and transformation.

In this report, we examined the effect of a mutant β-catenin on an immortalized murine hepatocyte cell line, AML12. Our study suggests that mutant β-catenin cannot completely transform the immortalized cells, although it significantly promotes cell proliferation, inhibits apoptosis, and induces overexpression of c-myc and cyclin D1.

Materials and methods

Cell Cultures

Murine immortalized hepatocyte cell line, AML12, was obtained from ATCC. The cells were maintained in Dulbecco's minimal essential medium (DMEM)/F-12 media (1:1) containing 10% fetal bovine serum, 5 μg/ml insulin, 5 μg/ml transferin, 5 ng/ml selenium, 100 U/ml penicillin, 100 μg/ml streptomycin, and 40 ng/ml dexamethasone. The cells were cultured at 37°C in humidified 5% CO_2–95% air, fed every 3 days, and passaged at 90% confluence.

Cell Transfection

The plasmid, pcDNA3S33Y (a gift from Dr Eric Fearon, University of Michigan), contains mutant β-catenin and the FLAG tag at its C-terminal. Plasmid pTOPFLASH and pFOPFLASH (provided by Dr Bert Volgestine, Johns Hopkins University) are luciferase reporter constructs containing three copies of wild-type Tcf-binding motifs and mutant Tcf motifis, respectively. The AML12 cells were transfected using Lipofectamine (Invitrogen) according to the manufacturer's instructions. In brief, in a six-well tissue culture plate, 1×10^5 AML12 cells were seeded in 2 ml of DMEM supplemented with serum and incubated at 37°C in an incubator overnight. Plasmid pcDNA3S33Y (2 μg) in 100 μl serum-free OptiMEM medium was gently mixed with 20 μl Lipofectamine in 100 μl of serum-free OptiMEM medium in a polystyrene tube and allowed to incubate at room temperature for 40 min to form complexes. Just before transfection, the cell culture supernatant was removed and cells were rinsed twice with serum-free OptiMEM medium. The lipid–DNA complex was diluted to 2 ml with serum-free OptiMEM medium. Approximately 48 h after the transfection, the selection medium containing G418 (500 μg/ml) was used to culture cells for 20

days. The isolated G418-resistant cell clones were then selected and amplified.

Western Blot Analysis

Western blot analysis was carried out according to the previously published protocol.[35] Briefly, cells were washed twice with PBS and lysed by lysis buffer (150 mM sodium chloride, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris-Cl [pH 8.0]) supplemented with $2 \mu g/ml$ of aprotinin, $2 \mu g/ml$ of leupeptin, $40 \mu g/ml$ of phenyl-methylsulfonyl fluoride, and 2 mM DTT. Cell lysates were cleared by centrifugation at $13000g$ for 15 min at $4^\circ C$. Approximately $50 \mu g$ of protein each well was loaded onto SDS-8% polyacrylamide gels and transferred onto polyvinylidene difluoride membrane (PVDF) (BIO-RAD, CA, USA) after electrophoresis. Membranes were incubated overnight at $4^\circ C$ in blocking buffer (TBS containing 0.1% Tween 20 and 5% fat-free milk power [wt/vol]). Anti-FLAG mouse monoclonal antibody (Sigma) was added at a dilution of 1:5000 in 1% bovine serum albumin (BSA) in TBS containing 0.5% Tween 20 for 1 h. After being washed three times for 10 min each with 0.1% Tween 20 in TBS, the membrane was incubated with goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, CA, USA) in a dilution of 1:1000 in 5% fat-free milk in TBS containing 0.1% Tween 20 for 45 min and washed three times as described above. The bound antibodies were detected by Western Blotting Luminol Reagent (Santa Cruz Biotechnology) according to the instructions.

Immunostaining

The cells growing on glass coverslips were fixed in 95% alcohol and 5% acetic acid at $-20^\circ C$ overnight. The coverslips were washed in PBS at room temperature twice for 5 min each. The slides were blocked by normal goat serum for 20 min at room temperature. The anti-FLAG monoclonal antibody or anti-β-catenin monoclonal antibody (Transduction Laboratories) was added at a dilution of 1:500 in PBS and incubated at $37^\circ C$ for 1 h. After being washed with PBS three times for 5 min each, the slides were incubated with FITC conjugate with goat anti-mouse IgG in a dilution of 1:200 in PBS for 30 min at $37^\circ C$ and washed as described. The cell slides were mounted using 25% glycerol and observed under a fluorescence microscope.

Cell Cycle Analysis

Cells were harvested, washed with PBS, and spun down at 2000 rpm for 5 min. The cells were resuspended in 1.0 ml cold 70% ethanol, and fixed for 30 min at $4^\circ C$. After being washed with PBS solution, the cells were resuspended in a solution containing RNase and propidium iodide, followed by flow cytometry (Becton Dickinson).

Luciferase Assay

The luciferase assay was carried out according to published protocol.[36] Briefly, cells (1×10^5 cells in 60 mm dish) were transiently transfected with $2 \mu g$ of pTOPFLASH or pFOPFLASH using lipofectamine according to the manufacturer's instruction (Invitrogen, CA, USA). At 48 h after transfection, the cells were lysed and the luciferase activity was quantified with a luciferase assay kit (Promega, Madison, WI, USA) according to the manufacturer's instruction.

Measurement of Cell Growth

The cell density was measured using a colorimetric assay system obtained from Promega (Promega, Madison, WI, USA). Approximately 1000 cells in $100 \mu l$ culture medium were placed into each well of a 96-well flat-bottom plate. A measure of $100 \mu l$ of phenazine methosulfate (PMS) solution was added to the 2.0 ml of tetrazolium compound (3-(4, 5dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, MTS) solution. The tube was gently swirled to ensure complete mixing of the combined MTS/PMS solution. A measure of $20 \mu l$ of the combined MTS/PMS solution into each well of the 96-well assay plate was pipetted. The plate was incubated for 4 h at $37^\circ C$ in a humidified, 5% CO_2 atmosphere. Cell proliferation was analyzed at 0, 24, 48, 72, 96, and 120 h. The absorbance at 490 nm was measured using an ELISA microplate reader.

Colony Formation in Soft Agar

Assays of colony formation in soft agar were carried out according to the standard protocol. Briefly, 2 ml underlayers of 0.6% agar medium were prepared by mix 1.2% Noble agar and 2 X DMEM with 40% fetal bovine serum (Difco, MI, USA), and added to a 6-well plate. The cells were trypsinized, centrifuged, and resuspended. Approximately 1×10^4 cells in 0.3% Difco agar per well were plated on the 2 ml 0.6% underlayer agar. The cells were fed once a week with small amounts of 0.3% agar during 3 weeks of incubation at $37^\circ C$ and macroscopically visible colonies were recorded.

Tumorigenicity Assays in SCID Mice

Tumorigenicity was determined by subcutaneous injection of 5×10^6 cells/site into the flanks of twelve SCID mice (two individual sites per cell line). Mice were divided into three groups: eight mice for AML12S33Y cell line, six mice for AML12 cell line, four mice for AML12S33Y-C, and four mice

for Huh7 cell line. Animals were monitored weekly for the development of any nodules or growth at the sites of injections for 3 months. Animals bearing tumors were killed, and a portion of each tumor was fixed in 10% formalin and stained with hematoxylin and eosin for histological studies. The remaining tumor tissue was frozen. If no tumors developed within that time period, the animals were sacrificed and examined. All animals received humane care in compliance with the guidelines established by the University Institutional Animal Care and Use Committee, and these studies were conducted in accordance with the NIH Guide for Care and Use of Laboratory Animals.

RNA Isolation and RT–PCR Analyses

Total cellular RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and our previous publication.[35] RT–PCR was performed using a SuperScript One-Step RT–PCR kit (Invitrogen). Total cellular RNA (1 μg for each sample) was used with the following pairs of primers: for c-myc, forward primer 5′-TCTCCACTCACCAGCACAAC-3′ and reverse primer 5′-TCGTCTGCTTGAATGGA-CAG-3′; for cyclin D1, forward primer 5′-GCG-TACCCTGACACCAATCT-3′ and reverse primer 5′-CCACTTGAGCTTGTTCACCA-3′; for GAPDH, forward primer 5′-AACTTTGGCATTGTGGAAGG-3′ and reverse primer: 5′-ACACATTGGGGGTAGGAA-CA-3′. The conditions of RT–PCR were as follows: (1) RT step 50°C for 30 min, (2) 94°C for 2 min, (3) 94°C for 15 s, (4) 55°C for 30 s, (5) 70°C for 1 min, (6) repeat steps 3–5 for 20 cycles, and (7) 72°C for 7 min. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel.

Detection of Apoptosis with Flow Cytometry and DAPI Stain

Cells were trypsinized, plated, and allowed to adhere and grow overnight at 90–95% confluence. The cells were treated with 20 ng/ml TNFα-alone or were pretreated with 250 ng/ml Act D in fresh medium for 30 min followed by 20 ng/ml TNFα for 20 h. The next day, cells were washed with PBS and removed with trypsin-EDTA and resuspended in 1× binding buffer (0.1 M HEPES/NaOH, PH 7.4:1.4 mM NaCl:25 mM CaCl$_2$). After centrifugation, cells were resuspend in 100 μl binding buffer and 5 μl Annexin V-FITC (BD Bioscience, CA, USA) was added. Incubation was carried out at room temperature for 10 min, and 400 μl binding buffer containing 2 μl PI (50 μg/ml) was added. Incubation was carried for 15 min at room temperature in the dark and analyzed by flow cytometry. Coverslips with cells treated by TNFα/ActD were fixed as described above. The cells were then stained using DAPI reagent to highlight the DNA in the nucleus.

Results

Establishment and Characterization of AML12 β-Catenin Stable Cell Line

The AML12 cell line is immortalized mouse hepatocyte derived from the liver of a transgenic mouse carrying human TGFα. The cells grow indefinitely but without anchorage-independent growth potential, such as colony formation in soft agar or tumor development in immune-deficient mice.[32] Using this cell line, we could determine whether mutant β-catenin is a transforming oncogene. We first established a cell line that stably expresses mutant β-catenin. The plasmid pCDNA3S33Y, containing serine mutation at aa. 33 of the N-terminal with a FLAG epitope tag at the C-terminal, was transfected into the AML12 cells, followed by G418 selection. The selected stable clone was referred to as AML12S33Y. A series of experiments were then carried out to confirm the presence and the function of β-catenin. The presence of mutant β-catenin expression in this cell line was confirmed by Western blot analysis (Figure 1a) using a monoclonal mouse anti-FALG antibody. As shown in Figure 1b and c, the mutant β-catenin can also be readily detected in all the cells using either anti-β-catenin or anti-FLAG antibody by the immunofluorescence method. Interestingly, the protein was predominantly expressed in the cytoplasm. Only occasional cells have nuclear β-catenin stain. To ascertain that the mutant β-catenin is functional in these cells, two reporter gene constructs were used for cell transfection. The pTOPFLASH contains three copies of optimal Tcf-binding sites, and the pFOPFLASH contains three copies of mutant Tcf-binding motifs. As shown in Figure 1d, the β-catenin in the AML12S33Y cells specifically activated the Tcf-driven-luciferase activity, while the mutant Tcf-binding motifs did not exhibit significant luciferase activity, implying that the β-catenin is functional in the hepatocyte cell line. These experiments indicate that AML12S33Y cells express functional mutant β-catenin.

Cell Growth Property of AML12S33Y Cells

To determine the biological function of the mutant β-catenin on cell growth property, the cells were first analyzed by flow cytometry. As shown in Figure 2a, the AML12S33Y cells have more S-phase population compared with the control AML12 cells, indicating that the AML12S33Y cells have a faster cell proliferation rate. We also compared the AML12 cell with the AML12GFP cells that stably express green fluorescence protein(GFP). There is no significant difference in cell cycle parameters between these two cell lines, which confirms that the effect is due to β-catenin (data not shown). To further test the cell growth property, a cell growth curve was generated by two independent methods: one with
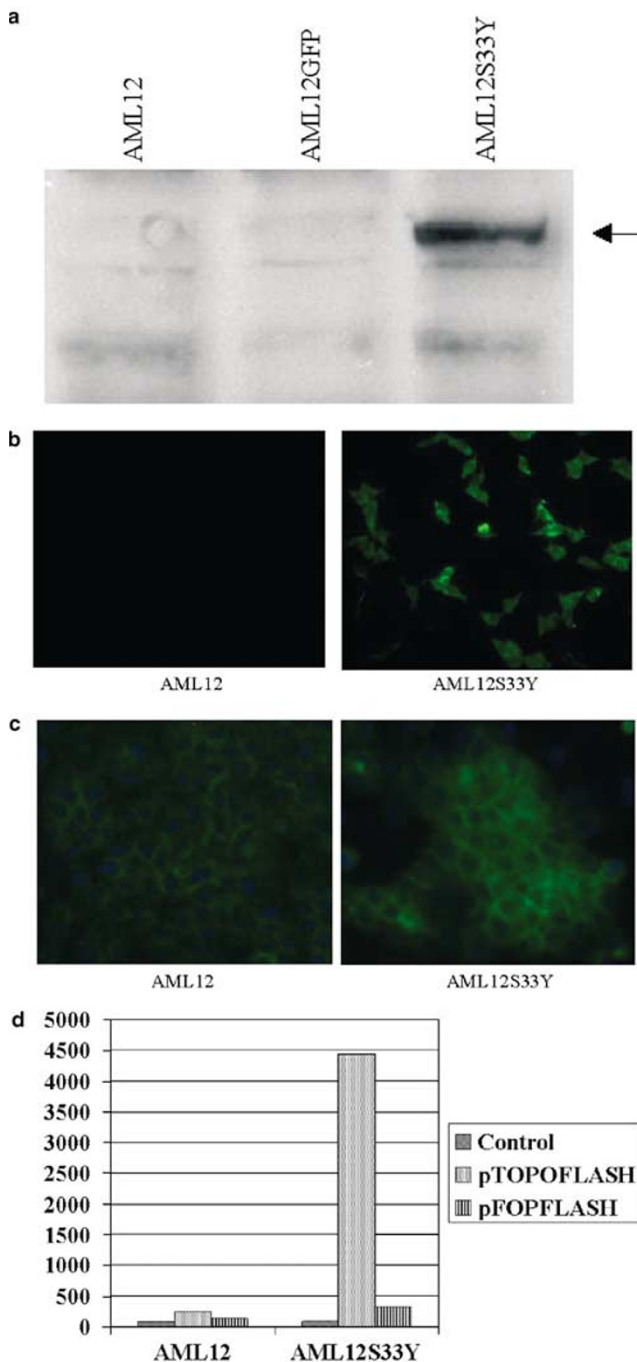
Figure 1 Expression and function of stabilized *β*-catenin in AML12S33Y cells. (**a**) Western blot analysis of control AML12 cells and AML12GFP cells, and the *β*-catenin-transfected AML12S33Y cells using a mouse monoclonal anti-FLAG antibody. The specific band is indicated by an arrow. (**b**) Immunofluorescence of AML12 cells and AML12S33Y cells using mouse anti-FLAG antibody. The original magnification is 100×. (**c**) Immunofluorescence of AML12 and AML12S33Y using anti-*β*-catenin antibody, the original magnification is 200×. The nuclei were counterstained by DAPI (4'6-diamidino-2-phenylindol). (**d**) Tcf motif-driven-luciferase reporter gene assay. The wild-type pTOPFLASH and the mutant pFOPFLASH were transfected into AML12 and AML12S33Y cells. The protein extracts were made from the cells 48 h after transfection. The luciferase activity was determined as described in the Material and methods. The graph represents the mean of three independent experiments.

conventional cell counting; the other with a colorimetric assay to measure cell number. The same number of AML12 and AML12S33Y cells were plated and cultured in a normal culture condition. Cell numbers were determined by two independent methods at different time points. As shown in Figure 2b and c, the mutant *β*-catenin-containing AML12S33Y cells grew faster than the control AML12 cells. These data support the notion that activated mutant *β*-catenin promotes cell proliferation.

Mutant *β*-Catenin Protects Cells from Apoptosis

The other putative function of *β*-catenin is a role in regulating cell apoptosis. Both proapoptotic and antiapoptotic activities, depending on different cell types studied, were reported in the literature. To determine the role of mutant *β*-catenin in hepatocyte apoptosis, we decided to develop a protocol to induce apoptosis in this hepatocyte cell line. It has been reported that the combination of TNF*α* and Actinomycin-D (Act D) induced more than 50% of AML12 cell apoptosis. We first treated both the control cells and the mutant *β*-catenin AML12S33Y cells by recombinant murine tumor necrosis factor alpha (TNF*α*) alone. After 24 h treatment, the cells were harvested for annexin V detection by flow cytometric analysis and DAPI stain for nuclear morphology. As shown in Figure 3, treatment by TNF*α* alone induced mild apoptosis in AML 12 cells, but not in the AML12S33Y cells. This is consistent with the findings of others that AML12 cells are not very sensitive to TNF*α*. We then treated the cell by both TNF*α* and actinomycin D, this treatment caused significant apoptosis in both AML12 and AML12S33Y cells, but the degree of apoptosis in AML12S33Y cells was much less compared to the AML12 cells. These data suggest that expression of the mutant *β*-catenin protects cells from apoptosis induced by TNF*α*/Act D.

AML12S33Y Renders Limited Anchorage—Independent Cell Growth but not Tumorigenic in SCID Mice

Cell immortalization is a prerequirement for cancer development. The AML12 is an immortalized cell line that does not have anchorage-independent cell growth ability, suggesting that other genetic changes are required. To test whether the mutant *β*-catenin can transform the AML12 cells, we performed the soft-agar colony assay, a commonly used method to determine the anchorage-independent cell growth. As shown in Figure 4, the AML12/S33Y cells formed small colonies, while AML12 cells essentially did not form any colony after 4 weeks of culture. However, compared to the control Huh7 cells, human liver cancer cells, the size of the colonies was much smaller. The largest colony was
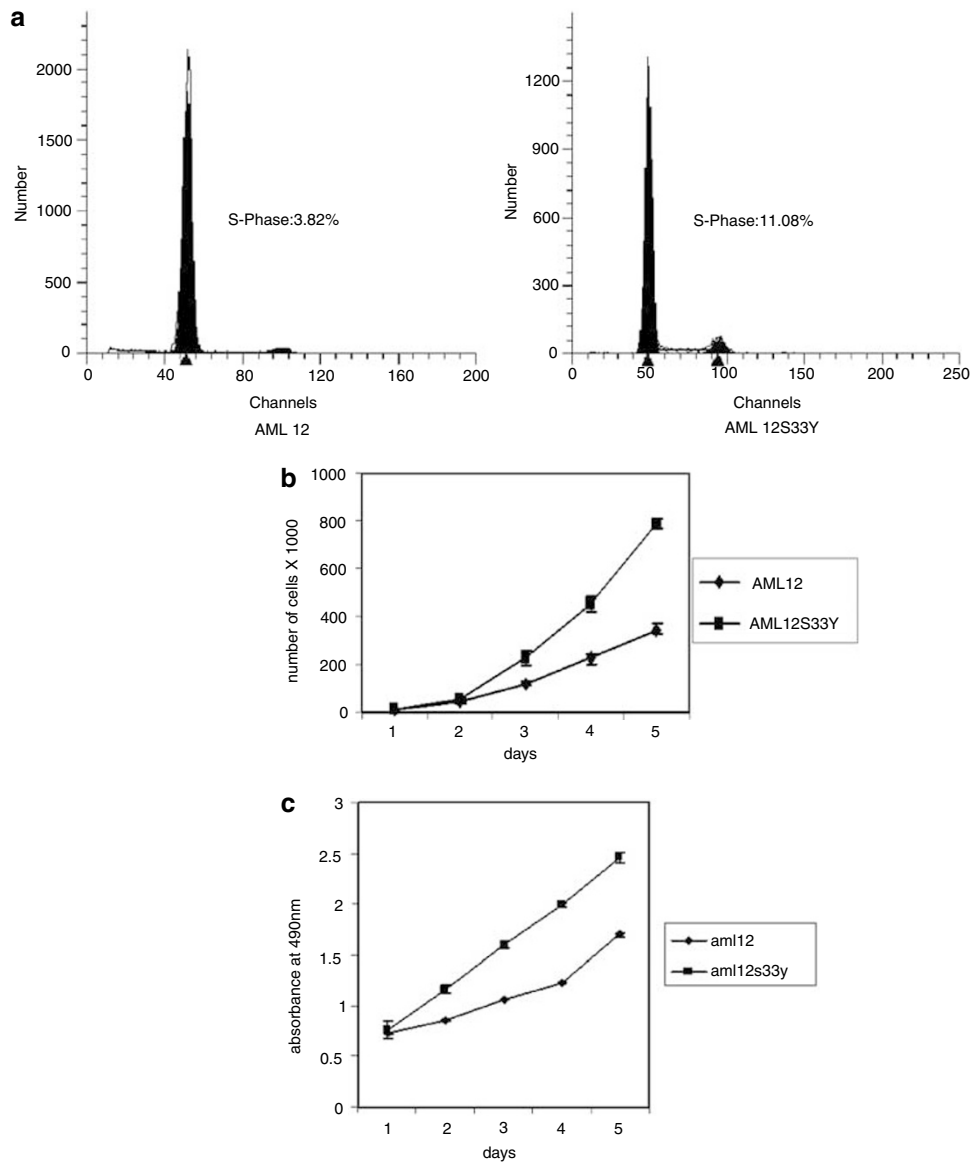
Figure 2 Determination of cell growth property of AML12 cells and β-catenin-transfected cells. (**a**) Analysis of the effect of β-catenin on cell cycle. AML12 and AML12S33Y cells were cultured under a normal condition. The cells were harvested, washed, and stained by PI, followed by flow cytometric analysis. The different proportions of the cell cycle are as labeled. The number indicates the percentage. (**b**) Cell growth curve of the control cells and β-catenin-transfected cells. Cells were counted after trypsin digestion at different time points as indicated. (**c**) Cell growth curve generated by colormetric assay as described. The OD value correlates with the cell number in the linear range.

approximately 1 mm, even after 7 weeks of incubation. The data suggest that the mutant β-catenin allows the cells to have a limited anchorage-independent growth property, but it does not fully transform the cells. To test the tumorigenic property of these cells, the colonies were selected and amplified, referred to as AML12S33Y-C. Approximately 5×10^6 AML12S33Y cells, the same number of AML12 cells and AML12S33Y-C cells, were injected subcutaneously into SCID mice. Four mice were injected with Huh7 cells to serve as positive control. As shown in Table 1, none of the mice injected with AML12 cells, AML12S33Y cells, and its agar colony-derived cells developed tumor in the

SCID mice 12 weeks after injection, while all the mice injected with Huh7 cells developed tumor after 3 weeks. These data suggest that mutant β-catenin by itself cannot fully transform the murine-immortalized cell line.

Mutant β-Catenin Activates c-myc and cyclin D1 in Murine Hepatocytes

It has been demonstrated that the c-myc and cyclin D1 are among the target genes of mutant β-catenin in colonic epithelial cells. The activation of c-myc and cyclin D1 is dependent on the β-catenin/Tcf
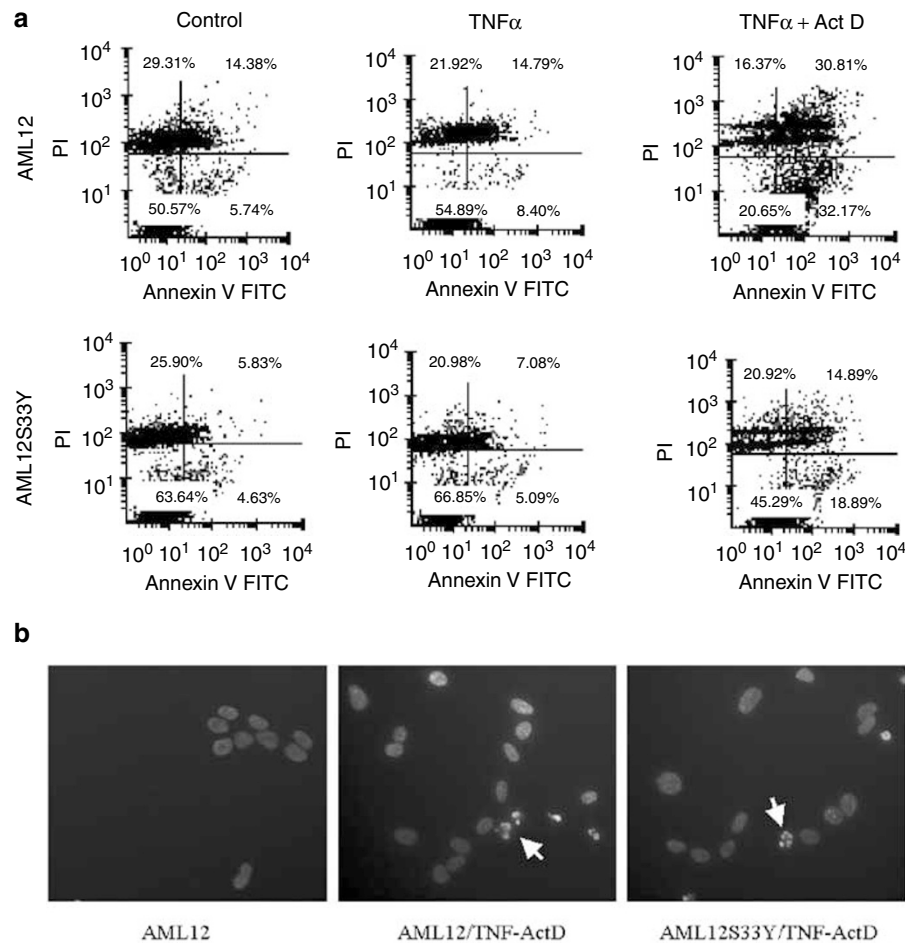
Figure 3 Effect of mutant β-catenin on TNFα-induced apoptosis. (**a**) The control AML12 cells and mutant β-catenin AML12S33Y were treated with either TNFα alone or TNFα and actinomycin D combination. The cells were stained with both PI and Annexin V, followed by flow cytometric analysis. Only the PI-negative and Annexin V-positive cells were considered as the early stage of apoptosis (right-lower quadrant). The numerical number indicates the percentage of total cells analyzed. (**b**) The cells were stained by DAPI, which showed the nuclear morphology under UV-light. The condensed and fragmented nuclei indicate apoptosis as marked by arrows.

transcription complex in the nucleus, because promoters of both genes contain Tcf-binding motifs. To test if a similar pathway exists in the hepatocyte cell lines, we examined the RNA expression level of c-myc and cyclin D1 in AML12 cells and AML12S33Y cells. As shown in Figure 5, the c-myc and cyclin D1 were both activated in the AMLS33Y cell, suggesting that the β-catenin activates similar target genes in hepatocytes.

Discussion

The β-catenin is an important mediator for the Wnt signaling pathway. This pathway plays a crucial role in the normal development and carcinogenesis. Mutations along this pathway, including APC, Axin, and β-catenin, have been implicated in a number of cancer cells. It is postulated that the critical outcome of these mutations causes β-catenin accumulation, nuclear translocation, binding of Tcf/Lef, and target gene activation, which potentially cause accelerated

cell growth and suppression of cell apoptosis. Several laboratories have investigated the transformation ability of β-catenin in different cell lines,[24,26] and the results were variable.

In this report, we have shown that the stabilized β-catenin promotes hepatocyte cell growth by increasing the cell proliferation potential. By transfecting the mutant form of β-catenin (stabilized form) into a hepatocyte cell line, we found that the cells expressed stabilized β-catenin, and the β-catenin expression activated the Tcf-derived transcription as determined by a reporter construct. The cells that expressed β-catenin also have a greater proliferation potential. In the normal culture condition, 11% of the β-catenin-containing cells were in the S phase, while 4% of the control AML12 cells were in the S phase. When we compared the two cell lines by generating cell growth curves, we found that the β-catenin-containing cells grew faster that the control cells. These data support the notion that the stabilized β-catenin has the capacity to stimulate cell proliferation. Our study was carried out using a
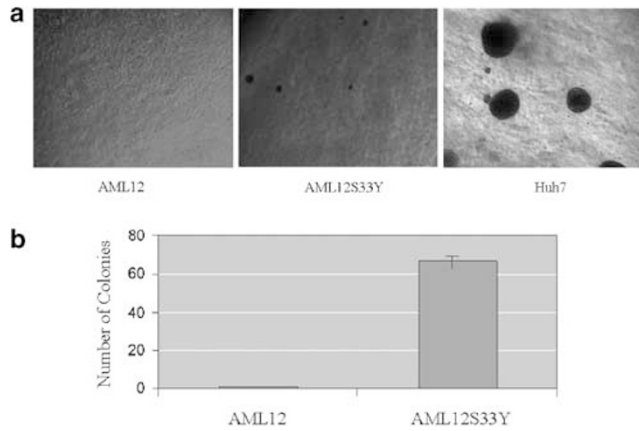
Figure 4 Soft-agar colony assay. Approximately 1×10^4 of each cell line were cultured in 0.3% of noble agar. After 4 weeks, the colonies were examined and counted. (**a**) Microscopic photographs of the colonies. Magnification, $20 \times$. (**b**) Results of the colony counting per 1×10^4 cells.

Table 1 Tumor formation in SCID mice

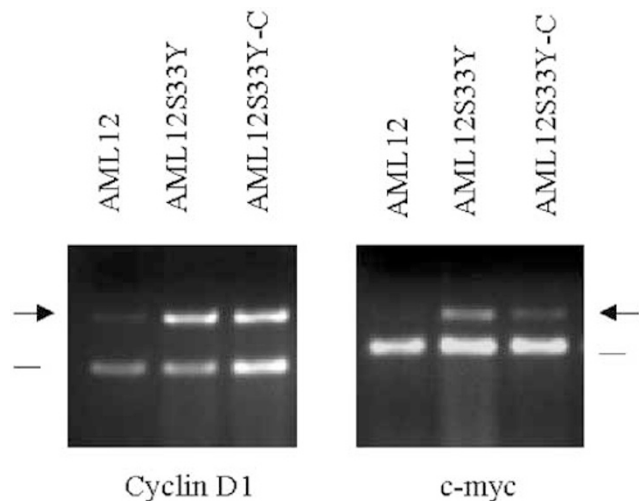| Cells	Number of mice injected	Number of mice developed tumor
AML12	6	0
AML12S33Y	8	0
Huh7	4	4

Figure 5 Semiquantitative RT–PCR amplification of c-myc and cyclin D1. Total RNA was isolated from control AML 12 cells and AML12S33Y cells, followed by one-step RT–PCR amplification using gene-specific primers and the internal control GADPH primers. The PCR products were resolved in 1.5% agarose gel. The arrow indicates the gene-specific product and the bar indicates the internal control GADPH.

stable cell line, which may have biased selection. To rule out this possibility, we also established a stable cell line that expressed GFP; similar results were obtained (data not shown). Therefore, it is unlikely that the faster growth potential is due to selection bias. A recent study has shown that the transgenic mice that expressed liver-specific mutant β-catenin developed liver hypertrophy, suggesting that β-catenin promotes hepatocyte proliferation *in vivo*.[29,30] Another report has also demonstrated that β-catenin is required for hepatoblast proliferation during liver development.[6] Our results derived from the *in vitro* cell culture study are supportive of this notion.

Cancer development occurs in the setting of increased cell proliferation and decreased apoptosis. The role of β-catenin in cell apoptosis is still not fully understood. Several studies have suggested that stabilization of β-catenin could induce cell apoptosis.[37] One such example is the mutation of presenilin in familiar early-onset Alzheimer's disease, which causes apoptosis of neural cells via stabilization of β-catenin.[38] Olmeda *et al* have recently shown that stabilized β-catenin could promote keratinocyte apoptosis.[39] However, a number of other studies have demonstrated that stabilization of β-catenin could suppress cell apoptosis.[6,40] It is most likely that the disparity is related to different cell types, suggesting that the stabilization of β-catenin may have different effects on cell apoptosis depending on the other cellular factors or cellular pathways. Our data indicated that overexpression of the β-catenin does not appear to affect hepatocyte apoptosis in normal cell culture conditions. However, the stabilized β-catenin seems to protect cell apoptosis induced by TNFα alone or TNFα/Actinomycin D combinations. Previous reports indicated that TNFα alone does not induce AML12 cell apoptosis by morphology examination.[41] Here, we showed that the apoptosis is detectable by annexin V flow cytometry examination, a more sensitive method for early detection of apoptosis. The degree of apoptosis induced by TNF alone is marginal. The addition of actinomycin D dramatically increased the percentage of the apoptosis. The β-catenin containing cells were relatively resistant to these agents. These data suggest that the activated β-catenin has an impact on TNFα signaling pathway. TNF/Act D induces cell apoptosis by inhibition of NF-κB activation.[41] Interestingly, recent reports suggested that β-catenin could interact with NF-κB and IKK pathways, which might potentially affect cell apoptosis.[42,43] The interface between Wnt/β-catenin signaling pathway and TNFα pathway remains to be defined.

Although β-catenin increases cell proliferation and suppresses apoptosis in some cell types, its transformation function is still controversial. It appears that β-catenin can only transform certain cell types *in vitro*. The cell lines that can be transformed include NIH3T3,[44] L cells,[45] RK3E,[26] and chicken fibroblasts.[24] There are also reports suggesting that β-catenin does not transform the cell lines studied, which include NIH3T3,[26] Rat-1 cells,[46] and 1811 human epithelial cells.[26] The

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inconsistency may be related to the genetic differences among the tested cell lines, suggesting that additional factors are probably required for stabilized β-catenin transformation. Whether β-catenin transforms liver cells has not been previously studied. In our report, we tested the cell line AML12, which is an immortalized murine hepatocyte cell line. Presumably, these cells carry the first genetic hit toward cancer development by TGFα-induced pathways. We investigated whether the stabilized β-catenin can deliver the second hit to transform the cells fully. A study by Tarn *et al* [33] has shown that HBV pX gene product could transform AML12 cells, supporting the feasibility of using this cell line as a model system for cell transformation. Using soft agar colony assay, we have found that the β-catenin-containing cells form colonies in soft agar. But these colonies were small and did not grow into full-scale colonies as the transformed cells such as Huh7 cells. Moreover, none of the mice injected with AML12S33Y cells or the cells isolated from the colonies developed tumor after 12 weeks of observation. These data indicate that the stabilized β-catenin alone cannot fully transform murine hepatocytes, which suggests that β-catenin cannot deliver an efficient second genetic hit to transform the liver cells fully. Other yet unidentified factors must be required to accomplish this mission. The findings are consistent with the results using transgenic mice. The transgenic mice carrying mutant β-catenin did not develop liver cancer, implying that the β-catenin signaling pathway alone may not be sufficient for liver cancer development.

The identification of target genes of oncogenic transcription factors is essential for understanding molecular mechanisms. Several target genes associated with stabilized β-catenin have been identified. But their role in cell transformation is not fully defined. The c-myc and cyclin D1 are among the target genes. Transcription factor Tcf-binding sites are present in the promoter region of these genes. Our study shows that both c-myc and cyclin D1 are elevated in mutant β-catenin cells (Figure 5). The cells that contain the stabilized β-catenin did not exhibit full transformation potential, suggesting that the activation of both c-myc and cyclin D1 is insufficient for oncogenic transformation of liver cells. Aoki *et al* have identified another target gene, IP6K2, from β-catenin-transformed chicken fibroblasts. This target gene activation is associated with all the transformation phenotypes in their study. We examined the IP6K gene expression in the AML12 and AML12S33Y cells using an RT–PCR approach. Preliminary result indicates that the IP6K is not activated in AML12S33Y cells (data not shown). It will be interesting to see whether IP6K is essential to assist β-catenin transformation in mammalian cells. Forced expression of this gene in the AML12S33Y cell line may be informative.

In conclusion, our study demonstrate that the stabilized β-catenin enhances hepatocyte proliferation, suppresses TNFα/Act D-induced cell apoptosis, and causes weak anchorage-independent cell growth. The stabilized β-catenin-containing cells do not develop tumor in immune-deficient mice. The target genes, c-myc and cyclin D1, are activated by β-catenin in the hepatocytes. Our study suggests that the stabilized β-catenin alone is sufficient for promoting cell proliferation, but other factors are required for oncogenic transformation.

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