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Overexpression of Integrin β1 Inhibits Proliferation of Hepatocellular Carcinoma Cell SMMC-7721 Through Preventing Skp2-Dependent Degradation of p27 via PI3K Pathway

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Abstract Integrins may play important roles in many cellular events, such as cell proliferation, differentiation, and apoptosis. We showed previously that overexpression of integrin β1 inhibits cell proliferation in SMMC-7721 cells. Here we reported that one of the cyclin-dependent kinase (CDK) inhibitors, $p27^{Kip1}$ was involved in proliferation–inhibition induced by overexpression of integrin β1. Overexpression of integrin β1 upregulated $p27^{Kip1}$ at the protein level, but not mRNA level. The knock-down of $p27^{Kip1}$ expression restored cell growth in integrin β1-overexpressing cells. Cycloheximide (Chx) treatment and pulse-chase experiments revealed that overexpression of integrin β1 prolonged the half-life of $p27^{Kip1}$ by inhibiting its degradation. Proteasome inhibitor (MG132) treatment of the cells indicated that proteasome mediated degradation of p27, and Skp2-dependent degradation might be prevented. Overexpression of integrin β1 decreased Skp2 at mRNA level, which was regulated by cell adhesion and the subsequent adhesion-dependent signaling. Overexpression of integrin β1 reduced cell adhesion, accordingly, inactivated the phosphoinositide 3-kinase (PI3K) signaling. PI3K inhibitor LY294002 upregulated $p27^{Kip1}$ at post-translational level and downregulate Skp2 at mRNA level, which could mimic the effects of integrin β1 overexpression on $p27^{Kip1}$ and Skp2. Together, these results suggested that overexpression of integrin β1 inhibited cell proliferation by preventing the Skp2-dependent degradation of $p27^{Kip1}$ via PI3K pathway. J. Cell. Biochem. 102: 704–718, 2007. © 2007 Wiley-Liss, Inc.

Key words: integrin; p27; Skp2; PI3K; degradation

The regulation of cell proliferation is important not only in the development and normal homeostasis, but also in human disorders such as cancer and other diseases. Cell proliferation needs to progress through the cell cycle. Cell cycle progression is controlled by a series of kinase complexes composed of cyclins and cyclin-dependent kinases (CDKs) [Sherr, 1996]. These regulatory mechanisms include variations in cyclin levels, positive- and negative-acting phosphorylation of the kinase subunit, and the actions of CDK inhibitors (CKIs). Of these, the CKIs appear to be the most diverse and flexible regulators. Mammalian CKIs are classified into two families: the Cip/Kip and Ink4 families. The former comprises $p21^{Cip1/Waf1}$, $p27^{Kip1}$, and $p57^{Kip2}$ [Sherr and Roberts, 1999]. Disruption of cell cycle control is frequent in human cancer [Sherr, 1996]. Dysregulation of cell proliferation and failure to suppress tumor growth often result from alterations in the activity of Cdk inhibitors [Sherr and Roberts, 1999]. Ink4 Cdk inhibitors are lost through

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deletion, point mutations, and/or promoter methylation in a variety of human tumors and are thus true tumor suppressor genes [Ortega et al., 2002]. The Cip/Kip Cdk inhibitors $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ do not fit the classic tumor suppressor paradigm because mutations in these genes are rare [Shiohara et al., 1994; Sgambato et al., 2000]. Reduced expression of $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ is usually found in human tumors. However, $p21^{Cip1/Waf1}$ is regulated mainly at transcriptional level by p53-dependent or independent mechanisms [Gartel and Tyner, 1999]. Whereas $p27^{Kip1}$ is regulated predominantly at post-translational level, especially protein degradation [Sgambato et al., 1999].

Integrins are a large family of α/β heterodimeric transmembrane receptors binding to components of the extracellular matrix (ECM). Integrins participate in a number of signaling events in cells, including outside-in and inside-out signalings. The outside-in signaling events are induced by the ECM through integrins, including focal adhesion kinase (FAK), mitogen-activated protein kinases of the ERK and JNK types, phosphoinositide 3-kinase (PI3K), and integrin-linked kinase (ILK), all of which affect cell proliferation [Giancotti and Ruoslahti, 1999]. The inside-out signalings regulate the interaction of integrin-ligand to influence integrin function. By these two signaling events, integrins mediate many fundamental cellular processes such as proliferation, spreading, adhesion, migration [Harris et al., 2000; Giancotti, 2003]. Integrins often together with growth factor receptors, upregulate cyclins D and E, or downregulate CKIs $p21^{Cip1/Waf1}$, $p27^{Kip1}$, and $p57^{Kip2}$, resulting in cell cycle progression [Sherr and Roberts, 1999; Schwartz, 2001]. However, recently many studies have demonstrated that integrins give rise to growth inhibition rather than growth stimulation [Varner et al., 1995; Meredith et al., 1999], but the mechanism is still not completely understood.

In human hepatocellular carcinoma (HCC), the expression of integrins α5β1 is much lower than in normal hepatocytes [Yao et al., 1997]. It implicated that the downregulation of integrins might promote proliferation of carcinoma cells, and overexpression of integrins might inhibit cell growth of cancers. In our previous studies, it has been demonstrated that overexpression of integrin β1 subunit induces S-phase delay, inhibits cell proliferation, and upregulates the expression of $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ in human HCC cell SMMC-7721 [Liang et al., 2003]. Furthermore, we have found that overexpression of integrin β1 in SMMC-7721 cells stimulates the promoter activity of $p21^{Cip1/Waf1}$ and enhances its transcription [Liang et al., 2004]. However, there are two questions remain unclear. Firstly, whether upregulation of $p27^{Kip1}$ (here referred to as p27) is really responsible to cell growth inhibition induced by integrin β1 overexpression? Secondly, how does overexpression of integrin β1 upregulate the level of p27, and whether integrin β1 overexpression affect the post-translational regulation of p27? If so, which signaling pathways are involved in this regulation?

To this end, we reduced the protein expression of p27 by siRNA in integrin β1—overexpressing cells, and found that cell growth-inhibition induced by integrin β1 overexpression was blocked by the siRNA. Further studies revealed upregulation of p27 by overexpression of integrin β1 was attributed to prevention of the Skp2-dependent proteasome degradation via PI3K pathway.

MATERIALS AND METHODS

Cell Culture and Reagents

The human HCC cell line (SMMC-7721) was obtained from the Liver Cancer Institute of Zhongshan Hospital, Fudan University (Shanghai, China). Cells were cultured in RPMI 1640 (Gibco, Carlsbad, CA) supplemented with 10% of calf bovine serum. The mock-7721 and β1-overexpressing (β1-7721) cells, respectively, with empty vector pcDNA3 and plasmid encoding a full-length integrin β1A subunit were described previously [Liang et al., 2004]. These cells were maintained in the same medium as above plus 500 μg/ml geneticin (G418, Gibco BRL, Carlsbad, CA), and incubated at the 37°C incubator with 5% CO_2.

pcDNA3-HA-PKB^{DD} was a gift from Dr. Jim Woodgett (University of Toronto). In this mutant, T308 and S473 have been substituted with aspartic acid, resulting in a constitutively active PKB [Hutchinson et al., 2001]. The dominant negative PKB plasmid (pcDNA3-DN-PKB) was kindly provided by Dr. Gu JX (Fudan university).

Antibodies against human p27 (F-8), Skp2 p45 (H-435), p-p27 (Thr187), GSK-3β (H-76),

p-GSK-3β (Ser9), Akt1/2 (H-136), FAK (H-1), α-tubulin (B-7), and actin (C-2) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and monoclonal antibody against integrin β1 subunit was from BD Transduction Laboratories (San Jose, CA). Anti-histone H1 antibody was from Upstate (clone AE-4). Anti-phospho-PKB (pSer473), anti-phospho-ERK1/2, anti-phospho-FAK (anti-phosphotyrosine clone PT-66), MG132, chloroquine, Fibronectin (FN), and digitonin were from Sigma (Saint Louis, MI). Horseradish peroxidase-labeled anti-mouse or rabbit IgG secondary antibodies and the specific inhibitor of PI3K, LY294002 were purchased from Calbiochem.

MTT Assay

Cells were seeded onto 96-well plates at a density of 2×10^4 cells/well in 200 μl RPMI 1640 medium, and grown for 0, 24, 48, 72 h, respectively. Meanwhile, the equal amount of fresh medium was added into wells without cells, and regarded as control. For each assay, 20 μl of MTT was added, and these plates were continued to culture for 4 h. Following incubation, culture medium was discarded and 150 μl DMSO was added to each well. These plates were vibrated gently for 10 min, which was followed by detection in the universal microplate reader at 490 nm.

Colony-Forming Ability Assay

To determine the effficiency of colony-forming, 2×10^4 cells were suspended in 1 ml of 0.3% select agar(GIBCO/BRL) in DMEM containing 10% fetal bovine serum and placed on top of a 1 ml gel composed of 0.8% agar in DMEM in 35 mm dishes (costar). The medium was changed twice a week. After 5 days the growth was estimated under a Nikon inverted phase-contrast microscope, and individual colonies of more than 50 cells were counted [Zhou et al., 2000].

Semi-Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and Real-Time PCR

The total RNAs were isolated using Trizol system (Watson Biotechnologies, Shanghai, China) according to the manufacturer's guidelines. RT-PCR was performed to quantify the mRNA levels of *p27* and *Skp2* genes. $(dT)_{15}$-primer and AMV-RTase were used for the first strand synthesis. Two microliter cDNA products were mixed with *Taq* DNA polymerase (SABC, Luoyang, China), 50 pM of each appropriate primer, 200 μM each dNTP in a reaction buffer containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.01% (w/v) BSA, 2 mM $MgCl_2$ in an end volume of 100 μl. The primers for *p27* and *β-actin* were as follows: 5′-AAG TGG CAT GTT TTG TGC ATT T-3′ (F) and 5′-GCT CAG TAT GCA ACC TTT TAA GCA-3′ (R). *β-actin*: 5′-TGG GCA TGG GTC AGA AGG AT-3′(F) and 5′-AAG CAT TTG CGG TGG ACG AT-3′ (R). The primers for *Skp2* were described previously [Radke et al., 2005]. The expected product sizes of *p27*, *Skp2*, and *β-actin* were 100, 271, and 991 bp, respectively. The samples were amplified for 25 cycles at cyclic temperature of 94°C 30 s, 55°C 30 s (for *p27* and *β-actin*), or 57°C 30 s (for *Skp2* and *β-actin*), 72°C 60 s. PCR products were analyzed through 1% agarose gel electrophoresis and following ethidium bromide staining. The band densitometry scanning of *p27* or *Skp2* was measured and normalized by that of *β-actin*.

The *p27* mRNA level was also measured using real-time PCR as described previously [Hernandez-Pigeon et al., 2004], and the primers for *p27* were described above. GAPDH was used as the internal control. The primers for GAPDH were 5′-AAG GTC GGA GTC AAC GGA TT-3′ (F) and 5′-CTG GAA GAT GGT GAT GGG ATT-3′(R). The product sizes of *p27* and GAPDH amplified were 100 and 222 bp, respectively. Real-time PCR was performed using ABI 7900 sequence detection system according to the manufacturer's instructions. Reactions were performed with 0.2 μM primers. SYBR Green I, dNTP, $MgCl_2$, and Taq DNA polymerase were included in 2 × SYBR Green PCR Master Mix for quantitative PCR. cDNA amplification consisted of one cycle at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. The threshold cycle (C_T) values were determined using ABI 7900 sequence detection system and the quantification data were analyzed following the $\Delta\Delta C_T$ method using GAPDH as reference.

SiRNA Construction

siRNA targeting p27 was designed according to the softwares "siRNA Target Finder" (http://www.ambion.com/techlib) and "Dharmacon siDESIGN Center" (http://www.dharmacon.com/sidesign). p27 siRNA targeted the regions

containing the 401–419 nt and 469–487 nt of human p27 mRNA (GenBank accession no. NM_004064). Two sequences were designed.

p27 siRNA1 (401—419): 5′-AGACTGATCCGT-CGGACAG-3′;
p27 siRNA2 (469—487): 5′-CCGACGATTCTT-CTACTCA-3′.

The selected sequences were submitted to BLAST search to assure only the selected gene was targeted.

Then these two sequences were cloned into pSilencer 2.0 (Ambion). In brief, the short-hairpin-RNA-encoding complementary single-stranded oligonucleotides, which hybridized to give overhangs compatible with *Hin*d III and *Bam*H I, were ligated into pSilencer 2.0. Bacterial colonies were pooled and used for plasmid preparation. The positive clones were confirmed by sequencing. The resulting plasmids were designated as pSi–p27-401 and pSi–p27-469. The control plasmid was pSi-NS (for nontargeted, obtained from Ambion), which is a plasmid with a similar structure but encoding a nonsense minigene with no homology to any known sequences in the human genome.

Transient Cell Transfection

Transient transfections were performed with Lipofectamine 2000 reagent (LF2000, Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. Cells were plated at 10^6 cells per well in 6-well culture dishes. After cells grow to 90–95% confluence, the transfection was usually performed with 4 or 6 μg of plasmid DNA mixed with 10 μl of Lipofectamine 2000 reagent. Cells were incubated for 6 h with this mixture in 2 ml antibiotic-free culture medium with 10% serum. Then cells were replaced with fresh culture medium containing 10% serum and harvested after appropriate time for assay transient gene expression.

Preparation of Whole Cell Lysates and Subcellular Fractionation

Cultured cells were harvested with trypsinization. After centrifugation the cells were rinsed twice in ice-cold PBS, and lysed in 1×SDS lysis buffer (50 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 100 μg/ml PMSF, 10 μg/ml leupeptin, and 5 mM Na_3VO_4) for 30 min on ice. The samples were boiled and clarified by centrifugation at 12,000*g* for 10 min at 4°C. The supernatants were transferred to a microcentrifuge tube and stored at −20°C.

Subcellular fractionation was performed as described [Ishida et al., 2002]. Briefly, cells were lysed in an ice-cold solution containing 0.02% digitonin, 5 mM sodium phosphate (pH 7.4), 50 mM NaCl, 150 mM sucrose, 5 mM KCl, 2 mM dithiothreitol, 1 mM MgCl2, 0.5 mM CaCl2, and 0.1 mM phenylmethylsulfonyl fluoride. The cytoplasmic fraction was collected after centrifugation of lysates at 1,000*g* for 10 min at 4°C. The resulting pellet was resuspended in the lysis solution without digitonin and loaded onto a cushion of a solution containing 30% (w/v) sucrose, 2.5 mM Tris-HCl (pH 7.4), and 10 mM NaCl. After centrifugation at 1,000*g* for 10 min at 4°C, nuclei were collected and extracted for 30 min at 4°C with an ice-cold solution containing 0.5% (v/v) Triton X-100, 50 mM Tris-HCl (pH 7.5), and 300 mM NaCl. After centrifugation of the extract at 12,000*g* for 10 min at 4°C, the supernatant was collected as the nuclear fraction.

Western Blotting

Western blot analysis was carried out as described elsewhere [Liang et al., 2004]. Equal proteins were resolved by SDS–polyacrylamide gel electrophoresis and transferred onto PVDF membranes. The membranes were blocked in 5% nonfat dry milk in phosphate buffer saline-0.05% Tween-20 (PBST) and incubated with primary antibody overnight at room temperature. Following three washes in PBST, the blots were incubated with the appropriate HRP-conjugated secondary antibody, then detected using the ECL kit (Boxin, China).

Cycloheximide Treatment and Pulse-Chase Analysis

To measure protein stability, cells were incubated in the presence of 50 μg/ml cycloheximide (Chx, an inhibitor of protein synthesis). Pulse-chase analysis was carried out as described previously [Pagano et al., 1995]. Mock-7721, β1-7721 cells in 60-mm tissue culture plates were pre-incubated for 1 h at 37°C with methionine- and cysteine-free medium containing 10% calf bovine serum. Then [^{35}S] methionine/cysteine (Amersham, Piscataway, NJ) was added at a concentration of 100 μCi/ml to the culture medium, incubation for 30 min for protein labeling. Cells were then incubated for

0, 1, 2, 4, and 6 h in RPMI 1640 supplemented with 2 mM methionine and 2 mM cysteine. At the various incubation times, cell lysates were collected and protein concentration was assayed by the Lowry method. The cell lysates (0.5 mg) were immunoprecipitated with anti-p27 monoclonal antibody at 4°C for 3 h. Pre-equilibrated protein A/G PLUS-agarose beads (20 μl) were then added and incubated overnight. They were collected by centrifugation and then gently washed three times with the lysis buffer. The concentration of the bound proteins were determined again, then the proteins were eluted by boiling in 2 × SDS sample buffer and resolved on a 10% SDS–PAGE gel. After electrophoresis, gels were dried, and the radioactivity was measured with a Fujifilm FLA-5100 Bio-Imaging Analyzer system (Fuji Photo Film).

Cell Adhesion Assay and Plating Experiments

Cell adhesion assay was carried out as previously reported [Busk et al., 1992]. Briefly, 96-well plates (Nunc) were coated with or without 10 μg/ml FN at 37°C for 1 h. Meanwhile, the plates were coated with 100 μg/ml polylysine or 10 mg/ml BSA as positive or negative controls, respectively. Then the plates were washed twice with PBS and blocked with 10 mg/ml BSA for 1 h at 37°C. Wells were washed twice with PBS and stored at 4°C before use. Cells were collected and resuspended in complete RPMI without FCS. A total of 10,000 cells in 100 μl medium were added into each substrate coated well, and plates were incubated for 30 min at 37°C in 5% CO_2. Unadhered cells were gently washed away by PBS for three times. The attached cells were fixed with 4% formaldehyde, stained with 0.5% crystal violet overnight, destained with distilled water, solubilized in 36% acetic acid and quantified by the microtiter plate reader.

Plating experiments were performed as described [Liang et al., 2003]. Cells were seeded on the tissue culture plates coated with or without 10 μg/ml FN, grown for 60 min, then followed by RNA and protein extraction.

RESULTS

Overexpression of Integrin β1 Inhibits Cell Proliferation by Upregulation of p27 Protein Level

To observe the influence of integrin β1 overexpression on cell proliferation, MTT assay, and colony-forming ability assay were employed. MTT result was shown in Figure 1A (the upper panel). At the time points of 0 and 24 h, the mock-7721 and β1-7721 cells were grown at a similar rate. However, growth inhibitory effects in integrin β1-overexpressing cells were observed at 48 and 72 h, and the inhibitory rates in β1-7721 cells were near 45 and 53%, respectively, compared with mock-7721 cells. In colony-forming ability assay, β1-7721 cells formed about 50% fewer colonies than mock-7721 (Fig. 1A, the bottom panel). These results indicated that overexpression of integrin β1 inhibited cell proliferation in SMMC-7721.

Cell cycle progression of the eukaryotic cells is regulated by a series of protein complexes composed of cyclins and CDKs, which activity is in turn controlled by a group of CKIs [Sherr, 1996]. Among these CKIs, p27 plays a pivotal role in cell proliferation control. To elucidate the mechanism of cell proliferation–inhibition induced by integrin β1 overexpression, we detected the expression of p27 in mock-7721 and β1-7721 cells. As shown in Figure 1B, integrin β1 protein level was increased about 2.5-fold in β1-7721 cells compared with that in mock-7721 cells. Meanwhile, the protein level of p27 had more than 2.5-fold amount in β1-7721 than mock-7721 cells. However, semi-quantitative RT-PCR results showed that the mRNA

Fig. 1. Overexpression of integrin β1 inhibits cell proliferation by upregulation of p27 protein. **A**: MTT assay and colony-forming ability assay for cell proliferation. All results shown are representative of at least three independent experiments. The histogram shows the mean ± SD (* $P<0.01$, compared with mock-7721 cells). **B**: Overexpression of integrin β1 increased p27 protein level. The β1 subunits appeared as two bands because of variable post-translational modification (mainly N-glycosylation). The bands of 115 and 130 kDa represent hypoglycosylated and hyperglycosylated forms, respectively. The protein levels of β-actin were detected to determine the loading amount in each well in the SDS–PAGE gel. The histogram shows the mean ± SD (*$P<0.01$, compared with mock-7721 cells). Message RNA levels of p27 were assessed by semi-quantitative RT-PCR (**C**) and real-time RT-PCR (**D**). **E**: Knock-down of p27 protein expression blocked the cell growth inhibition induced by integrin β1 overexpression in SMMC-7721 cells. Confluent integrin β1 overexpressing cells were transiently transfected with pSi–p27-401, pSi–p27-469, and pSi-nonsense (pSi-NS) plasmids, respectively. Then MTT assay and colony-forming ability assay were performed. All results shown are representative of at least three independent experiments. The histogram shows the mean ± SD (*$P<0.01$, $^{\#}P<0.05$, compared with the cells transfected with pSi-NS).

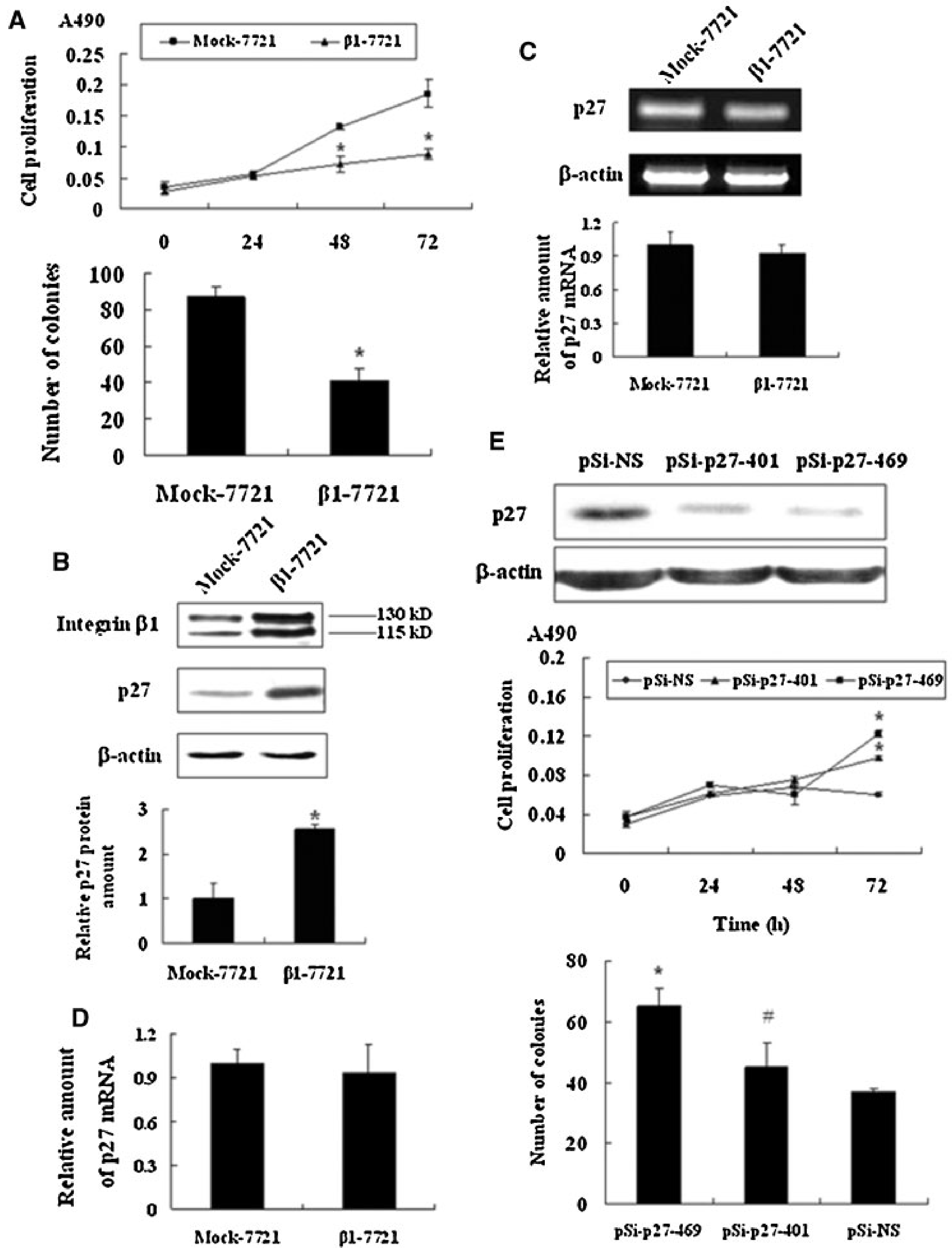

Fig. 1.

levels of p27 in these two cells had no significantly different (Fig. 1C). Similar results were obtained in real-time RT-PCR (Fig. 1D). These results suggested that overexpression of integrin β1 upregulated p27 only at the protein level, but not the mRNA level.

To further explore whether upregulation of p27 protein expression is really involved in cell growth inhibition in integrin β1-overexpressing cells, we employed p27 SiRNA to reduce the expression of p27, then investigated the effect of p27 on cell proliferation. Both the silencing plasmids of p27 (pSi–p27-401 and pSi–p27-469) were capable of effectively silencing p27 expression in integrin β1-overexpressing cells after 48 h of transfection (Fig. 1E). And protein levels of p27 were reduced to 30 and 14% in integrin β1-overexpressing cells transfected with pSi–p27-401 and pSi–p27-469 plasmids, respectively. Meanwhile, inhibition of cell proliferation induced by overexpression of integrin β1 was also significantly blocked in cells treated with pSi–p27-401 and pSi–p27-469. At 72 h, the growth rates of cells transfected with pSi–p27-401 and pSi–p27-469 were increased 1.6- and 2.0-fold, respectively, compared with cells transfected with pSi-NS. Colony-forming ability assay showed the similar results. The colonies were increased 75 and 22%, respectively, in the cells transfected with pSi–p27-401 and pSi–p27-469, compared with pSi-NS. These results suggested that upregulation of p27 could be responsible for cell growth inhibition induced by integrin β1 overexpression in SMMC-7721 cells.

Overexpression of Integrin β1 Upregulates p27 Protein Level by Preventing Skp2-Dependent Proteasome Degradation

Since integrin β1 overexpression upregulated the p27 protein level, but not its mRNA level, we postulated that p27 might be controlled at the level of protein stability. To test this hypothesis, we compared stabilities of p27 in β1-7721 and mock-7721 cells using Chx (50 μg/ml), which inhibits protein synthesis. As shown in Figure 2A, the half-life of p27 was prolonged in β1-7721 cells (more than 6 h), while it was less than 4 h in mock-7721 cells. To further investigate the mechanism by which p27 protein level was increased in β1-7721 cells, we also exploited pulse-chase experiment to monitor protein degradation of p27 (Fig. 2B). In mock-7721 cells, p27 levels were markedly decreased with time, and its half-life was less than 2 h. On the contrary, the alleviating degradation of p27 was observed in β1-7721g cells, where the half-life of p27 was about 6 h. Taken together, these findings suggested that overexpression of integrin β1 increased the half-life of p27 in SMMC-7721 cells through inhibiting its degradation.

Proteasome-dependent proteolysis represents a cellular pathway for rapid downregulation of specific proteins for which distinct temporal expression is required. Importantly, many cell cycle components are targets for proteasomes, including cyclins (A, B, D, and E), and the Cdk2 inhibitors p21 and p27 [Pagano et al., 1995; Blagosklonny et al., 1996; King et al., 1996]. However, whether integrins could regulate the proteasome-dependent proteolysis remains obscure. To investigate how integrin β1 overexpression inhibited the degradation of p27, cells were treated with a specific proteasome inhibitor MG132, and p27 levels were observed. Figure 2C showed that the p27 level was increased 1.9-fold in mock-7721 but remained stable in β1-7721 in the presence of 10 μM MG132. Because it has been known that MG132 inhibits not only the proteasome but also cathepsins in the lysosome [Marques et al., 2004], we used the lysosome inhibitor chloroquine (20 μM) as a control. The protein levels of p27 did not change in the presence of chloroquine, both in mock-7721 and β1-7721 cells. These results indicated that proteasome-mediated proteolysis pathways were involved in degradation of p27 in SMMC-7721 cells. It also implicated that overexpression of integrin β1 suppressed the proteasome-mediated p27 degradation, so no inhibition of p27 degradation by MG132 could be observed in β1-7721 cells.

In fact, there are two proteasome-dependent pathways mediating the degradation of p27: Skp2-dependent and Kip1ubiquitination-promoting complex (KPC)-dependent degradation. Skp2, an F-box protein, belongs to the ubiquitin E3 ligase SCF (Skp1-Cul1-F-box proteins) complex, recognizes the phosphorylated p27 at Thr187, and then mediates the degradation of nuclear p27 [Carrano et al., 1999; Sutterluty et al., 1999]. KPC is a different ubiquitin E3 ligase from SCF, which mediates the degradation of cytoplasmic p27 [Kamura et al., 2004]. To identify which degradation pathway of p27 was inhibited by integrin β1 overexpression, cells were treated with MG132, fractionated, and then p27 levels were detected. As shown

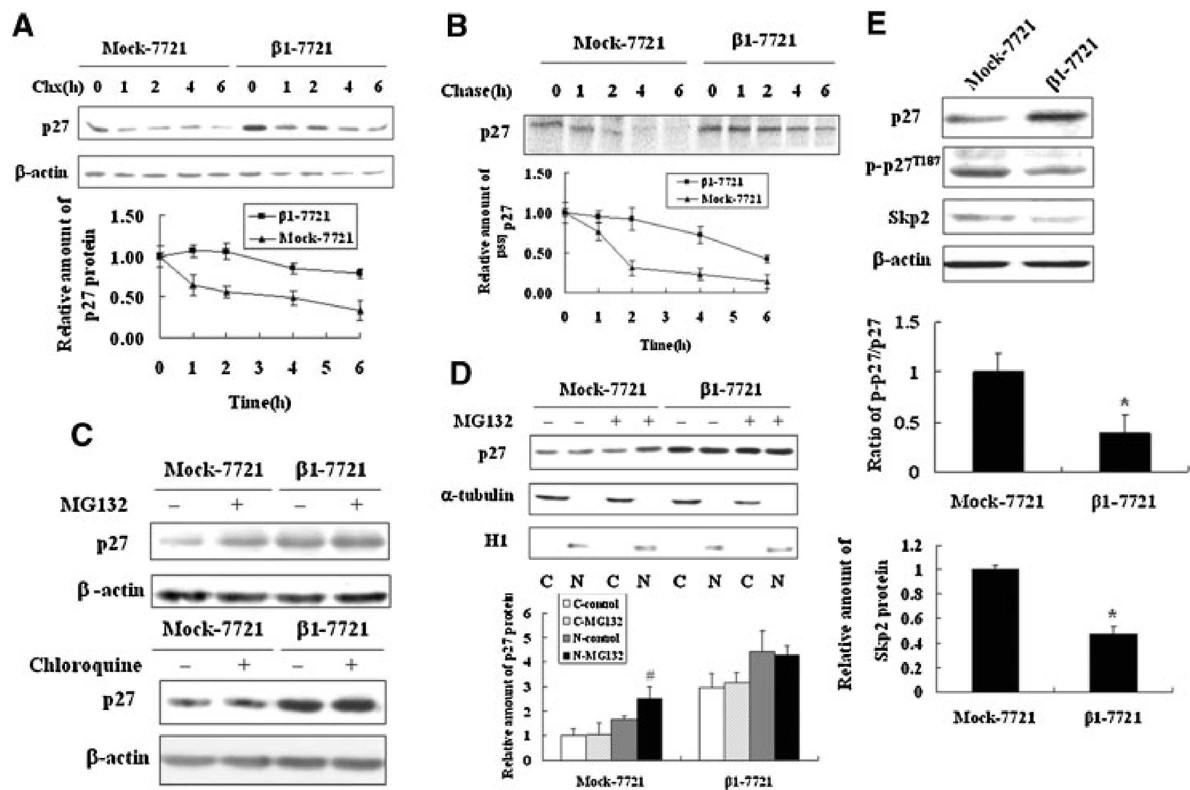

Fig. 2. Overexpression of integrin β1 upregulates p27 protein level by preventing Skp2-dependent proteasome degradation. **A**: Mock-7721 and β1-7721 cells were trypsinized and then re-plated. At 12 h after re-plating, cells were treated with Chx (50 μg/ml) for the indicated intervals. Cells were collected at 0, 1, 2, 4, and 6 h after Chx treatment, and p27 protein levels were detected by western blotting. **B**: The in vivo stabilities of p27 protein in mock-7721 and β1-7721 cells were assessed by pulse-chase and immunoprecipitation assays as described in ''Materials and Methods''. Quantitative analysis based on densitometry is shown as a graph. **C**: Degradation of p27 protein was proteasome-dependent in SMMC-7721 cells. The mock-7721 and β1-7721 cells were treated with 10 μM MG132 (a specific proteasome inhibitor) and 20 μM chloroquine (an inhibitor of lysosome) for 6 h, respectively, then the level of p27 was detected with immunoblotting assay. **D**: The mock-7721 and β1-7721 cells were treated with MG132 (10 μM) for 6 h. According to the method as ''Materials and Methods'' described, we then collected the cytoplasmic and nuclear portions from mock-7721 and β1-7721 cells, and the p27 protein levels in the cytoplasm (C) and nucleus (N) were detected. The α-tubulin and histone H1 were used as cytoplasmic and nuclear protein controls, respectively. The levels of cytoplasmic and nuclear p27 either without (C-control or N-control) or with MG132 (C-MG132, N-MG132) were plotted to each control protein ($^{\#}P < 0.05$, compared with the cells without MG132 treatment). **E**: Overexpression of integrin β1 reduced the ratio of phosphorylated p27 at Thr187 and the Skp2 protein expression. The mock-7721 and β1-7721 cell lysates were detected by immunoblotting for p27, p-p27-Thr187, Skp2, and β-actin. The data represent the mean ± SD of three independent analyses ($^{*}P < 0.01$, compared with the mock-7721 cells).

in Figure 2D, only the p27 in nucleus was increased (1.5-fold) in mock-7721 cells treated with MG132, the level of p27 in cytoplasm was not influenced in the presence of MG132. Moreover, the levels of p27 in both cytoplasm and nucleus did not significantly change in β1-7721 cells treated with MG132. These results suggested that overexpression of integrin β1 might mainly suppress the proteasome-mediated p27 degradation in nucleus. Therefore, we subsequently tested the levels of phosphorylation of p27 at Thr187 and Skp2. We found that the ratio of phosphorylated p27 at Thr187 reduced nearly 50% in β1-7721 cells compared with mock-7721 cells, and the level of Skp2 was also decreased (Fig. 2E). These results suggested that overexpression of integrin β1 not only reduced the amount of phosphorylated of p27 at Thr187, but also inhibited the expression of Skp2, both of which might be result in the upregulation of p27.

Overexpression of Integrin β1 Downregulates Skp2 mRNA Level by Inhibiting Cell Adhesion

To investigate the detailed mechanism of Skp2 downregulation in integrin β1—overexpressing cells, we measured the mRNA level and stability of Skp2. The RT-PCR results

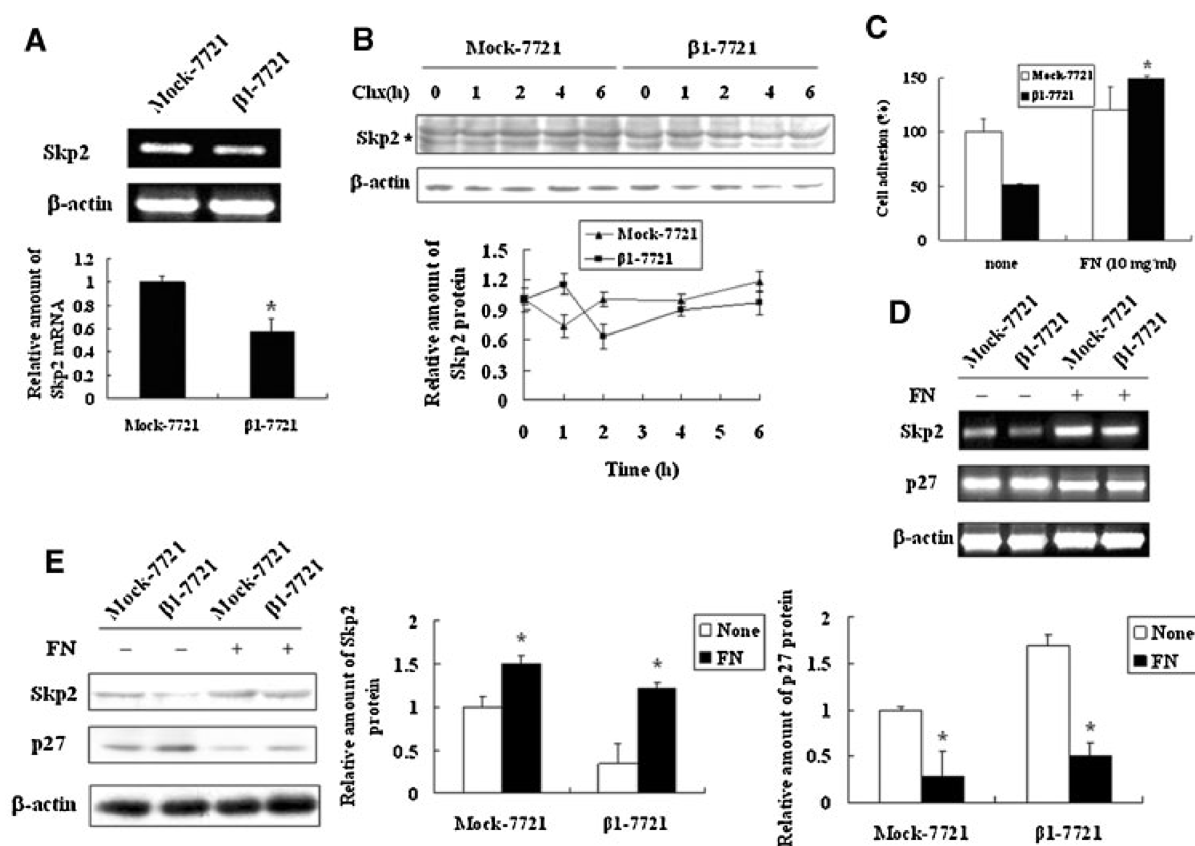

Fig. 3. Overexpression of integrin β1 downregulates Skp2 mRNA level by inhibiting cell adhesion. **A**: Message RNA levels of Skp2 were assessed by semi-quantitative RT-PCR, and normalized by that of β-actin. The data represent the mean ± SD SD of three independent analyses (*$P < 0.01$, compared with the mock-7721 cells). **B**: Overexpression of integrin β1 did not affect the half-life of Skp2 protein. Mock-7721 and β1-7721 cells were trypsinized and then replated. Then cells were treated with Chx (50 μg/ml) for the indicated intervals. The decay of Skp2 protein was detected. Asterisk represents the band of Skp2 protein. **C**: Overexpression of integrin β1 inhibited the capacity of cell adhesion. The mock-7721 and β1-7721 cells were seeded onto 96-well culture plates coated with or without FN (10 μg/ml), and the capacity of cell adhesion was assayed. Meanwhile, the 96-well culture plates coated with poly-lysine (100 μg/ml) or 1% BSA(1%) as controls. The data represent the mean ± SD of at least three independent analyses (*$P < 0.01$, compared with the cells seeded onto FN-uncoated plates). Cells were seeded onto 6-well culture plates coated with or without FN (10 μg/ml) for 60 min, then the mRNA levels (**D**) and protein levels (**E**) of Skp2 and p27 were determined. The data represent the mean ± SD of three independent analyses (*$P < 0.01$, compared with the cells seeded onto FN-uncoated plates).

showed that overexpression of integrin β1 inhibited 42% mRNA level of Skp2 compared with that in mock-7721 cells (Fig. 3A). However, integrin β1 overexpression did not influence the stability of Skp2 (Fig. 3B).

As reported previously, the mRNA level of Skp2 can be regulated by cell adhesion [Carrano and Pagano, 2001]. Integrins are the major cell surface receptors mediating cell adhesion. Thus, to elucidate the relationship between integrin β1 overexpression and reduced Skp2 mRNA level, we assayed the capacity of cell adhesion in β1-7721 cells. As shown in Figure 3C, the capacity of cell adhesion in β1-7721 cells was reduced about 50% when cells were seeded onto FN-uncoated plates as compared with mock-7721 cells. However, when cells were plated onto FN-coated plates, the capacities of cell adhesion in two cell lines were increased, especially in β1-7721 cells, the capacity of cell adhesion was increased about threefold compared with that onto FN-uncoated plates. These results suggested that the inhibitory effects of cell adhesion induced by integrin β1 overexpression might be attributed to the relative lack of ECM, such as FN. Next, we analyzed the effects of cell adhesion on the mRNA and protein levels of Skp2 and p27. Both the mRNA and protein levels of Skp2 were increased when mock-7721 and β1-7721 cells were plated onto FN. However, the mRNA levels of p27 did not change and the protein

levels of p27 were decreased when mock-7721 and β1-7721 cells were plated onto FN (Fig. 3D,E). These findings suggested that the increased capacity of cell adhesion upregulated Skp2 at mRNA level, while only influenced p27 at protein level. Together, these data implicated that overexpression of integrin β1 inhibited the capacity of cell adhesion, which resulted in the downregulation of Skp2 at mRNA level.

Reduced Activity of PI3K Pathway Increases the Stability of P27 Protein

By adhesion to ECM, integrins can activate a series of signaling pathways, promoting cell proliferation. FAK, PI3K/PKB, and MAPK pathways are main signaling pathways mediated by integrins [Giancotti, 2003]. To explore which signaling pathways might participate in the inhibition of protein degradation of p27 by overexpression of integrin β1, the activities of FAK, PI3K/PKB, and MAPK pathways were detected. In integrin β1-overexpressing cells, the total amounts of PKB protein remained the same in β1-7721 cells as in mock-7721 cells. But the level of phosphorylated PKB at Ser473 was significantly decreased in β1-7721 cells compared with mock-7721 cells, and the phosphorylated GSK-3β at Ser9 (the downstream substrate of PKB) was also repressed in β1-7721 cells, indicating that the PI3K/PKB pathway was inactive in integrin β1-overexpressing cells (Fig. 4A, the left panel). On the other hand, neither total protein of FAK nor its tyrosine phosphorylated form was affected by integrin β1 overexpression. The level of phosphorylated ERK was also unchanged in β1-7721 cells compared with mock-7721 cells (Fig. 4A, the right panel). Furthermore, we found that the levels of p-PKB^{S473} and p-GSK-3β^{S9} were increased when cells were seeded onto FN-coated plates, especially in β1-7721 cells (Fig. 4B), indicating overexpression of integrin β1 reduced the activity of PI3K pathway through inhibiting cell adhesion.

Next we used PI3K inhibitor LY294002 to investigate the role of this signaling pathway on the expression of p27. The results showed that LY294002 (25 μM) increased the protein level of p27 in both mock-7721 and β1-7721 cells (Fig. 4C). LY294002 treatment could mimic the effect of integrin β1 overexpression on the expression of p27 protein, implicating that overexpression of integrin β1 might regulate the protein degradation of p27 through PI3K pathway. To verify this phenomenon, cells were treated with 25 μM LY294002 for 20 h, then p27 mRNA level and its protein stability was observed. As shown in Figure 4D, both in mock-7721 and β1-7721 cells, the mRNA levels of p27 were not significantly different in the presence of LY294002. However, the half-life of p27 protein was dramatically prolonged in mock-7721 cells in the presence of LY294002, but it was not significantly different in β1-7721 cells in the presence of LY294002 (Fig. 4E). These results suggested that PI3K pathway upregulated p27 at post-translational level, and inhibition of PI3K could increase stability of p27 protein. Together with inhibition of PI3K/PKB pathway in integrin β1-overexpressing cells, these results indicated that overexpression of integrin β1 inhibited the protein degradation of p27 through inactive PI3K pathway.

Reduced Activity of PI3K Pathway Downregulates the Expression of Skp2

To further investigate whether PI3K pathway increased p27 protein level through regulation of the expression of Skp2, we detected the protein level of Skp2 in the presence of 25 μM LY294002. As shown in Figure 5A, treatment with 25 μM LY294002 decreased the levels of Skp2 protein both in mock-7721 and β1-7721 cells. Meanwhile, the Skp2 mRNA level was apparently decreased in the presence of LY294002, either in mock-7721 or β1-7721 cells (Fig. 5B). These results were similar to Skp2 expression changes in integrin β1-overexpressing cells, suggesting that inactive PI3K pathway was involved in the downregulation of Skp2 induced by overexpression of integrin β1. Taken together, these data indicated that overexpression of integrin β1 prevented the Skp2-dependent protein degradation of p27 by inactivating PI3K pathway.

The Activity of PKB Only Regulates the Subcellular Distribution of P27, but not the Protein Expression of P27

PKB is an important downstream target of PI3K pathway. To investigate whether the activity of PKB was involved in the suppression of p27 protein degradation by overexpression of integrin β1, mock-7721, and β1-7721 cells were transfected with dominant negative PKB

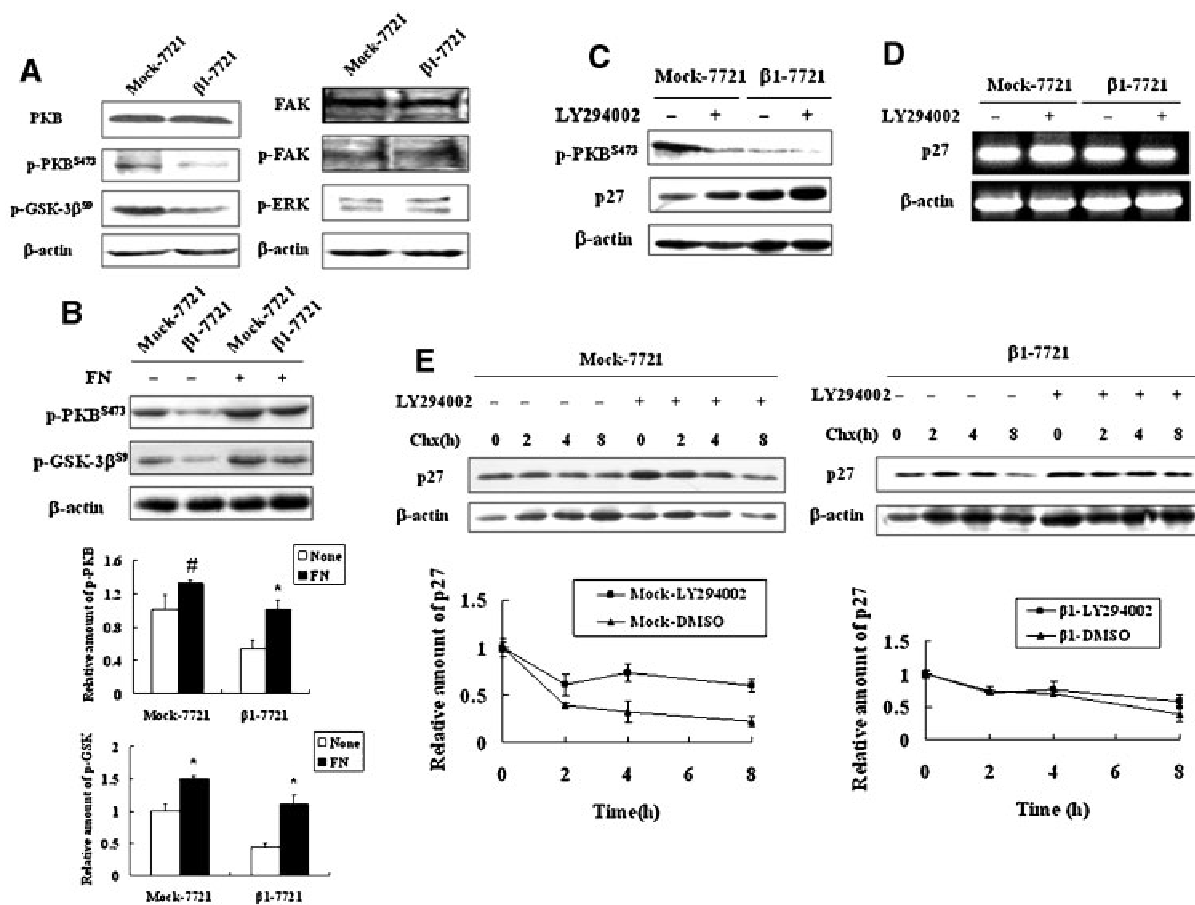

Fig. 4. Reduced activity of PI3K pathway increases the stability of p27 protein. **A**: The PI3K/PKB, but not FAK or MAPK/ERK signalling pathway was inhibited in integrin β1-overexpressing cells. Cell lysates were analyzed by western blotting for PKB, p-PKB-Ser473, p-GSK3-β-Ser9 (a substrate of PKB), FAK, p-FAK, p-ERK, and β-actin. The antibodies of p-PKB-Ser473 and p-GSK3-β-Ser9 were used to assay the activity of PKB. The antibodies of p-FAK and p-ERK represent the activation of FAK and MAPK/ERK signalling pathways. **B**: Increased capacity of cell adhesion increased the activity of PI3K pathway. Cells were seeded onto 6-well culture plates coated with or without FN (10 μg/ml) for 60 min, then the levels of p-PKB-Ser473 and p-GSK3-β-Ser9 were determined with immunoblotting. The data represent the mean ± SD of three independent analyses ($^{\#}P < 0.05$, $^{*}P < 0.01$, compared with the cells seeded onto FN-uncoated plates). **C**: The mock-7721 and β1-7721 cells were treated with 25 μM LY294002 (an inhibitor of PI3K) or DMSO (control) for 20 h, then the protein levels of p-PKB^{S473} and p27 were detected. **D**: The mock-7721 and β1-7721 cells were treated with 25 μM LY294002 or DMSO for 20 h, then the mRNA levels of p27 were assessed by semi-quantitative RT-PCR, and normalized by that of β-actin. **E**: The mock-7721 cells and β1-7721 cells were treated with 25 μM LY294002 or DMSO for 20 h. 12 h after treatment with LY294002, Chx (50 μg/ml) was added into cells for the indicated time. Then the p27 protein amounts were analyzed with immunoblotting.

plasmid (Dn-PKB) and constitutively active PKB mutant (PKB^{DD}), respectively, then the protein expression and subcellular distribution of p27 were observed. As shown in Figure 6A, the protein amount of PKB was dramatically increased, and the level of phosphorylation of GSK-3β at Ser9 was significantly decreased after mock-7721 cells were transfected with Dn-PKB. However, the reduced activity of PKB did not affect the protein level of p27, but changed subcellular distribution of p27. Transfection with Dn-PKB increased the protein amount of p27 in nucleus, which made the ratio of cytoplasmic p27 and nuclear p27 from 1:0.3 to 1:0.7. Similar results were obtained from β1-7721 cells were transfected with PKB^{DD} (Fig. 6B). After transfection with PKB^{DD} in β1-7721 cells, the increase of PKB protein level was accompanied by the increased level of phosphorylation of GSK-3β at Ser9. However, enhanced activity of PKB also could not change the protein level of p27, but increased the protein amount of p27 in cytoplasm. These findings suggested that PI3K pathway might regulate the protein degradation of p27 through other downstream target, but not PKB.

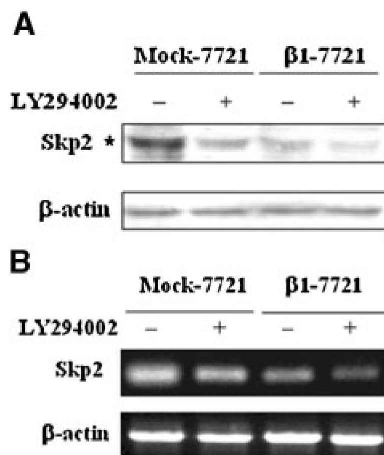

Fig. 5. Reduced activity of PI3K pathway downregulates the expression of Skp2. The mock-7721 and β1-7721 cells were treated with 25 μM LY294002 or DMSO for 20 h, then the protein levels (**A**) and mRNA levels (**B**) of Skp2 were detected. Asterisk represents the band of Skp2 protein.

DISCUSSION

Integrins binding to ECM lead to the activation of a variety of signaling pathways. These signals are important to promote cell proliferation. Many studies have demonstrated that integrins give rise to growth inhibition rather than growth stimulation [Varner et al., 1995; Meredith et al., 1999; Zhou et al., 2000]. The mutated integrin β1C strongly inhibits the growth of 10T 1/2 cells even at low expression levels [Meredith et al., 1999]. Integrin α5β1 is often lost in cancerous areas other than in its normal counterpart tissues [Su et al., 2002]. Overexpression of integrin α5β1 inhibits cell proliferation in SMMC-7721 [Zhou et al., 2000] and colon adenocarcinoma cell HT29 [Varner et al., 1995]. It is apparent from these studies that integrin signals may play a major role in negative control of cell growth, which may be lost in some cancers. However, the mechanisms of this effect are not completely known yet. In general, integrins modulate cellular functions through regulating transcription of target genes. For example, Varner et al. [1995] found that overexpression of integrin α5β1 in HT29 cells blocked the transcription of immediate early genes c-fos, c-jun, and jun B, which might be responsible for the cell proliferation–inhibition. But here, we demonstrated that overexpression of integrin β1 in SMMC-7721 inhibited cell proliferation by preventing the protein degradation of p27.

In this study, we found that overexpression of integrin β1 upregulated p27 only at the protein

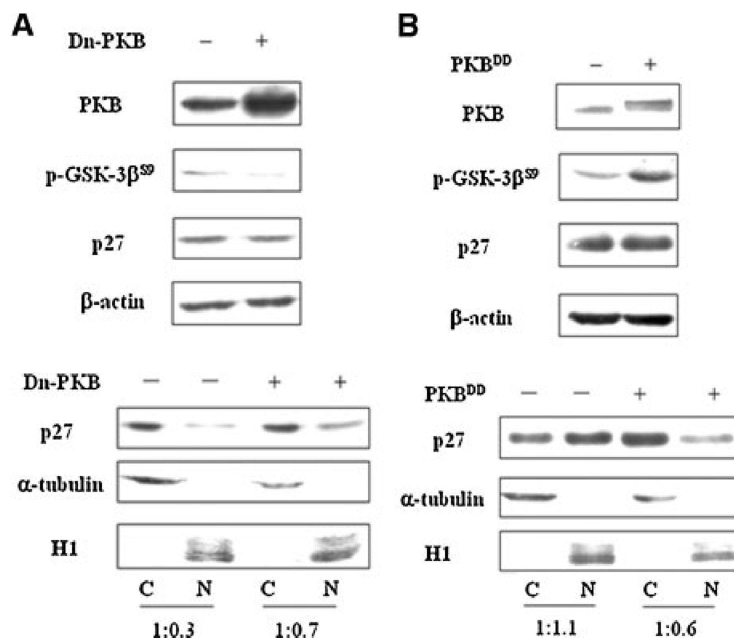

Fig. 6. The activity of PKB only regulates the subcellular distribution of p27, but not the protein expression. **A**: The mock-7721 cells were transfected with dominant negative PKB mutant (Dn-PKB) (+) and empty vector pcDNA3.0 (−) as a control. The expression of PKB was shown by western blotting and PKB activity was confirmed by blotting for phosphorylation of GSK3-β at Ser9. **B**: The β1-7721 cells were transfected with PKB constitutively active mutant (PKB^{DD}) (+), and empty vector pcDNA3.0 (−) as a control.

level. Chx treatment and pulse-chase experiments showed that overexpression of integrin β1 increased the stability of p27. Although we could not exclude the possibility of regulation of p27 at the translational level, our results at least demonstrated that overexpression of integrin β1 could upregulated p27 at the post-translational level. Accumulating evidence indicates that p27 is mainly regulated post-translationally in many cancers, especially at the protein degradation level [Slingerlands and Pagano, 2000]. Skp2 is a member of ubiquitin E3 ligase SCF complex. It is synthesized at late-G1 phase, and restricted in nucleus. Skp2 specifically recognizes p-p27^{T187} and mediates its degradation. Skp2-dependent proteasome degradation of p27 has been well characterized [Carrano et al., 1999; Sutterluty et al., 1999]. In our results, proteasome inhibitor MG132 increased the p27 protein level in mock-7721, and this increase only occurred in nucleus, suggesting that overexpression of integrin β1 might prevent the degradation of nuclear p27, which was Skp2-dependent. The downregulation of Skp2 in β1-7721 supported our view.

We found that overexpression of integrin β1 inhibited cell adhesion. However, when cells were attached onto FN, the inhibitory roles of cell adhesion in β1-7721 disappeared. We have reported that the β1-7721 cells show similar growth rates with mock-7721 when cells were plated onto FN [Liang et al., 2004]. These results implicated that these inhibitory effects induced by overexpression of integrin β1 might be due to the relative lake of ECM. Integrins activate a variety of adhesion-dependent signaling cascades, such as FAK, PI3K/PKB, MAPK, etc, all of which regulate cell proliferation [Giancotti, 2003]. Overexpression of integrin β1 inhibited cell adhesion. Accordingly, the activity of PI3K/PKB pathway was reduced. PI3K inhibitor LY294002 could increase the protein level of p27 in mock-7721, indicating PI3K pathway was at least partially responsible for the upregulation of p27 in β1-7721 cells, and it might be possible that overexpression of integrin β1 regulated the degradation of p27 through PI3K pathway. To this end, we used LY294002 to block the activity of PI3K, which could mimic the status of PI3K pathway in β1-7721. Not unexpectedly, LY294002 upregulated p27 at the post-translational level, and downregulated Skp2 at the mRNA level. All of these demonstrated that overexpression of integrin β1 inhibited the expression of Skp2 by inactivating PI3K pathway, prevented the degradation of p27, and finally resulted in the upregulation of p27.

Carrano and Pagano [2001] showed that cell adhesion to ECM can regulate the mRNA level of Skp2. Our findings that overexpression of integrin β1 inhibited cell adhesion, and decreased the mRNA level of Skp2, but not influenced its stability were consistent with Carrano's report. It also has been reported that ectopic expression of PTEN or treatment with LY294002 increases the expression of p27, which is closely related to the decrease of Skp2 in certain human cancer cells, such as prostate [van Duijn and Trapman, 2006], multiple myeloma [Pene et al., 2002], and glioblastoma [Mamillapalli et al., 2001]. However, it is obscure at which level PI3K pathway regulates the expression of Skp2. In addition, it is also incompletely clear whether PI3K pathway affect the degradation of p27 as a response to overexpression of integrin β1. Therefore, our finding that inactivation of PI3K induced by overexpression of integrin β1 prevented the Skp2-dependent degradation of p27 might provide a new mechanism of integrins' function on cell proliferation.

PKB is an important downstream effector of PI3K pathway. PKB regulates p27 through several mechanisms. PKB phosphorylates forkhead transcription factor FAX, resulting in the repression of p27 gene expression [Medema et al., 2000]. PKB also downregulates p27 by increasing its proteolysis [Graff et al., 2000]. Furthermore, PKB can directly phosphorylate p27 at Thr157 in breast cancer cell, preventing its nuclear import and abrogating its cyclin E/CDK2 inhibitory activity [Liang et al., 2002; Shin et al., 2002; Viglietto et al., 2002]. But it is unclear if this regulation is specific for breast cancer. Our results demonstrated that in β1-7721, PI3K pathway regulated the degradation of p27 by controlling the mRNA level of Skp2, but this regulation was PKB-independent. By transfection with dominant negative PKB plasmid or constitutively active PKB mutant, respectively, we found that decreased activity of PKB resulted in the accumulation of p27 in nucleus, and increased activity of PKB led to the accumulation of p27 in cytoplasm, implicating that reduced activity of PKB in β1-7721 cells might increase the protein level of p27 in nucleus. However, the changed activity of PKB

did not affect the total protein level of p27 in SMMC-7721. Therefore, other molecules downstream of PI3K, that is, PKC, $p70^{S6K}$, SGK might be involved in the Skp2-dependent degradation of p27 in β1-7721 cells.

In many cancers, p27 is mainly regulated post-translationally, at the levels of protein subcellular distribution and protein stability [Blain and Massague, 2002]. Moreover, these two levels are not independent. The protein subcellular distribution is closely related to the protein degradation. In this study, overexpression of integrin β1 post-translationally regulated p27 at both protein degradation and protein subcellular distribution through PI3K/PKB pathway. Overexpression of integrin β1 prevented the Skp2-dependent proteasome degradation of p27 by inactivating PI3K pathway, resulting in the upregulation of p27 protein. Overexpression of integrin β1 also increased the nuclear import of p27 by reducing the activity of PKB, leading to the accumulation of p27 in nucleus, which might affect the protein degradation of p27. p27 is not only an inhibitor of the cyclinE/CDK2 complex, but also can act as a substrate. CyclinE/CDK2 phosphorylates p27 at Thr187, which leads to recruitment of the Skp2-containing SCF complex, resulting in p27 ubiquitination and degradation by the proteasome [Sheaff et al., 1997; Slingerlands and Pagano, 2000]. In our study, the accumulation of p27 in nucleus induced by PKB in β1-7721 might inhibited the activity of cyclinE/CDK2, which subsequently reduced phosphorylation of p27 at Thr187 (Fig. 2E), and finally suppressed the degradation of p27.

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