CMLS, Cell. Mol. Life Sci. 62 (2005) 171–178
1420-682X/05/020171-8
DOI 10.1007/s00018-004-4349-8

CMLS Cellular and Molecular Life Sciences

Research Article

α-1,3-Fucosyltransferase-VII stimulates the growth of hepatocarcinoma cells via the cyclin-dependent kinase inhibitor p27^{Kip1}

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Received 5 August 2004; received after revision 25 October 2004; accepted 11 November 2005

Abstract. After the transfection of α-1,3-fucosyltransferase (FucT)-VII cDNA into H7721 human hepatocarcinoma cells, the protein expression of some cyclins, cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CDIs) p16^{INK4} and p21$^{waf1/Cip1}$ were unchanged. However, CDI p27^{Kip1} protein, both the total amount and the amount that bound to CDK2, but not its mRNA, was significantly reduced. The de-inhibited CDK2 stimulated the phosphorylation of retinoblastoma (Rb) protein and facilitated the G1/S transition and growth rate of the cells. The decrease of p27^{Kip1} protein, the increase of CDK2 activity and Rb phosphorylation, as well as the cell growth and percentage of S phase cells were correlated to the increased amount of cell surface sialyl Lewis X (SLe^x) antigen in cells with different α-1,3-FucT-VII expression. The reduction in p27^{Kip1} and the difference in its expression among different transfected cells were blocked by the SLe^x antibody KM93 in a dose-dependent manner, indicating that p27^{Kip1} expression was influenced by α-1,3-FucT-VII and its product SLe^x. The MEK/MAPK signaling pathway was more important than the PI-3K pathway in the regulation of p27^{Kip1} expression.

Key words. α-1,3-Fucosyltransferase (FucT)-VII; sialyl Lewis X; cyclin-dependent kinase (CDK); cyclin-dependent kinase inhibitor (CDI); p27^{Kip1}; retinoblastoma (Rb) protein.

Human fucosyltransferase (FucT) is a family of glycosyltransferases responsible for the synthesis of fucosyl-containing compounds, including the A, B and O blood groups and Lewis antigens. It catalyzes the transfer of a fucosyl residue from GDP-α-L-fucose to a sugar acceptor, usually galactose or N-acetylglucosamine, in the sugar chains of glycoproteins or glycolipids. Human FucT is divided into three subfamilies, α-1,2-FucT, α-1,3-FucT and α-1,6-FucT [1]. Among these, the α-1,3-FucT subfamily is the main glycosyltransferase participating in the synthesis of Lewis antigens, which are a series of fucosylated galactose-β-1,3-/β-1,4-N-acetylglucosamine (sialylated or not sialylated) sugar epitopes on the cell surface, mainly located at the termini of sugar chains in glycolipids and Ser/Thr-linked glycans of glycoproteins [2]. To date, six α-1,3-FucTs have been cloned [3–8]. Four, α-1,3-FucT-III, -V, -VI and -VII, efficiently fucosylate sialylated acceptors and produce sialyl Lewis antigens, while α-1,3-FucT-IV and -IX prefer neutral acceptors and usually form non-sialyl Lewis antigens as their products. α-1,3-FucT-III is the only α-FucT which can synthesize both α-1,3-fucosyl-containing Le^x or SLe^x and α-1,4-fucosyl-containing SLe^a, while α-1,3- FucT-VII catalyzes the synthesis of SLe^x only [1, 7].

Lewis antigens are known to participate in the processes of leukocyte infiltration during inflammation and cell ad-

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hesion during metastasis. The interaction of sialyl Lewis antigens on the tumor cell surface and E-/P-selectin expressed on vascular endothelial cells was reported to mediate the adhesion of malignant cells to vascular endothelia [2, 9]. Our group has demonstrated that surface SLe^x is a metastasis-related sugar structure. It was increased on H7721 human hepatocarcinoma cells after transfection of the metastasis-promoting gene c-erbB2/neu [10], or treatment with epidermal growth factor [11], whereas it was decreased by the metastasis-suppressive gene nm23H1 [12] or all-trans retinoic acid [11]. The metastatic potential, including cell adhesion to laminin, cell migration through a transwell and cell invasion through a matrigel, was always positively correlated to SLe^x expression on the cell surface [10–12]. In addition, transfection of α-1,3- FucT-VII cDNA into H7721 cells to increase the synthesis of SLe^x antigen led to an enhancement of the metastatic potential [10]. Clinical studies indicated that cancer cells with strong SLe^x expression had a high risk of developing hematogenous metastasis, and this factor statistically affected the overall prognosis of the patients [13–15].

However, the relationship between α-1,3-FucT-VII and its product, SLe^x in the proliferation of cells has not been clarified. In the present investigation, the cell growth rate and the cell cycle were studied in the H7721 cell line with or without transfection of α-1,3-FucT-VII cDNA. Furthermore, the expression of some cell cycle-related protein families, such as cyclin, cyclin-dependent kinase (CDK) and CDK inhibitor (CDI) as well as retinoblastoma protein (Rb) was determined, to elucidate the molecular mechanism of α-1,3-FucT-VII effects on cell growth.

Materials and methods

Materials

The H7721 cell line was obtained from the Institute of Cell Biology, Academic Sinica. RPMI 1640 and DMEM media were purchased from GIBCO/BRL. The monoclonal antibodies (mAbs) mouse anti-human $p16^{INK4}$ and $p21^{waf1/Cip1}$ were from Neomarkers Company and mAbs to human cyclin D1, Rb (catalogue no. sc-102), β-actin as well as rabbit polyclonal antibodies against human cyclin A, E, CDK2, CDK4 and CDK6 were from Santa Cruz. Rabbit polyclonal antibodies to human $p27^{Kip1}$ and mAb to SLe^x (KM93) were the products of Cell Signaling and Seikagaku, respectively. Horseradish peroxidase (HRP)-labeled secondary antibodies (goat anti-rabbit IgG and anti-mouse IgG) were purchased from Dako. LY294002 and PD98059, the specific inhibitors of phosphotidylinositide-3-kinase (PI-3K) and mitogen-activated protein kinase kinase (or MAPK kinase, MEK), respectively, histone H1 and Protein G PLUS agarose were from Sigma. Polyvinylidene difluoride (PVDF) membrane was from Bio-Rad. TRIzol and AMV reverse transcriptase were from Promega. Other reagents, including dNTP, oligo (dT)-18, enhanced chemiluminescence (ECL) reagent and γ-^{32}P-ATP (5 µCi/pmol) were commercially available in China.

The H7721 cell line transfected with α-1,3-FucT-VII was established in our laboratory as previously reported [10]. Two transfectants were selected: one expressed a high level of α-1,3-FucT-VII mRNA and was named as FucTVII-H, another expressed moderate mRNA of α-1,3-FucT-VII and was designated FucTVII-M. H7721 cells mock-transfected with the empty vector pcDNA3.1 were used as the control, which expressed a low level of α-1,3-FucT-VII mRNA.

Cell culture and treatment

The method for cell culture has been described previously [10–12]. For the treatment with specific inhibitors of cell signaling or mAb of SLe^x (KM93), the final concentration of LY294002, PD98059 and KM93 were 20 µM, 50 µM and 10–30 µg/ml, respectively. The duration of treatment was 24 h.

Determination of cell growth

Cells growing at the logarithmic stage were digested to a single-cell suspension with 0.25% trypsin, and 0.5×10^3 cells were inoculated into each well of a 96-well plate. The medium was changed every 48 h. After 24–96 h incubation, the cells were treated with trypsin, stained with trypan blue, and counted under the microscopy.

Analysis of the cell cycle

Cells were synchronized using the serum starvation (2% FCS in RPMI-1640) method for 48 h, collected, washed and treated with EDTA and 75% ethanol at –20 °C for 2 h, then subjected to FACScan according to our previously reported method [16]. The percentage of cells at each phase of the cell cycle was analyzed and normalized with the Modfit program.

Analysis of the expression of cyclins, CDKs, CDIs and Rb proteins using Western immunoblot

Briefly, the cells were homogenized in 0.1 M MES buffer (pH 6.5)/150 mM NaCl/2% TritonX-100/25% glycerol/0.1 mg% leupeptin and pepstatin, then centrifuged at 1000 g at 4 °C for 15 min. After determination of the protein concentration, aliquots of 50 µg protein samples were subjected to 10% SDS-PAGE and Western blot according to the modified method of Knudsen et al. [17]. The membranes were blocked with 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) overnight and treated with 1:500 diluted primary antibodies of cyclins (D1, E, A), CDKs (CDK4, CDK6, CDK2), CDIs ($p16^{INK4}$, $p21^{waf1/Cip1}$, $p27^{Kip1}$) or Rb for 3 h (the antibody to Rb can react with both unphosphorylated and

phosphorylated Rb), followed by washing with 0.05% Tween-20/PBS three times and incubation with 1:500 diluted HRP-labeled secondary antibody for a further 3 h. Then the membrane was washed again and stained with ECL reagent. β-Actin was used as loading control and stained with 1:800 diluted primary antibody and 1:500 HRP-labeled secondary antibody. The protein bands were quantified with densitometric analysis. The expression of each protein was calculated by the ratio of the intensity of this protein to that of β-actin.

Determination of $p27^{Kip1}$ in immuno-coprecipitated CDK2- $p27^{Kip1}$complex

After the cells had been washed with ice-cold PBS, the cell lysis buffer (50 mM pH 7.5 Tris-HCl/150 mM NaCl/15 mM $MgCl_2$/5 mM EDTA/5 mM EGTA/1% Nonidet P-40/60 mM β-glycerol phosphate/0.1 mM sodium orthovanadate/0.1 mM NaF/0.1 mM benzamide/ 1 mg% leupeptin/1 mg% aprotinin/1 mM PMSF) was added and the protein content was determined. After 500 µg cell lysate had been pre-incubated with 25 µl protein G PLUS agarose at 40 °C for 2 h and centrifuged, the supernatant was incubated with 2 µg CDK2 antibody at 4 °C for 1 h, followed by the addition of 40 µl protein G PLUS agarose and incubated again at 4 °C for 3 h. After centrifugation, the precipitate was washed three times with 1 ml lysis buffer, and subjected to SDS-PAGE and Western blot as described above.

Determination of the expression of $p27^{Kip1}$ mRNAs with RT-PCR

The routine method of RT-PCR in our laboratory was adopted [18]. The cDNAs were synthesized from 3 µg total cell RNA. The RT-PCR mixture contained 5 µl cDNA, 0.2 µM of the primer pair of $p27^{Kip1}$ or β-actin (internal standard) and subjected to denaturation, followed by 28 cycles and final elongation. Then, 10 µl amplified DNA was applied for agarose gel electrophoresis. After the DNA bands had been scanned, the photographs were analyzed with NIH Image software. The semiquantitative data were obtained by the intensity ratios of $p27^{Kip1}$ bands to the β-actin. The primers of $p27^{Kip1}$ and β-actin were synthesized by Sangon Company according to the reported sequences [19, 20] as follows. $P27^{Kip1}$, F: 5′-AACCTCTTCGGCCCGGTGGACCAC-3′; R: 5′-GTCTGCTCCACAGAACCGGCATTT-3′ (product 471 bp); β-actin, F: 5′-GATATCGCCGCGCTCGTCGTCGAC-3′; R: 5′-CAGGAAGGAAGGCTGGAAGAGTGC-3′ (product 789 bp).

Determination of CDK2 kinase activity

CDK2 was immunoprecipitated as described above. The CDK2-protein G PLUS agarose complex was incubated with 40 µl CDK2 assay mixture (50 mM Tris-HCl pH7.5/15 mM $MgCl_2$/1 mM DTT/50 µM ATP/γ-^{32}P-ATP 10 µCi/histone H1 1.5 µg) at 30 °C for 30 min. Then, 10 µl SDS-PAGE loading buffer was added and the reaction mixture was boiled for 10 min. After SDS-PAGE, the product of CDK2, ^{32}P-histone H1, was measured by radioautography.

Results

Effect of transfection of α-1,3-FucT-VII cDNA on H7721 cell proliferation

Figure 1A shows that the growth rate of α-1,3-FucT-VII cDNA-transfected cells was higher than that of mock-transfected cells, especially after 48 h incubation. The cell numbers of α-1,3-FucTVII-M and α-1,3-FucTVII-H cells at 48 h were 272% and 580% of the 'Mock' levels (both $p < 0.01$), while those at 96 h were 197% and 263% of the 'Mock' value, respectively (both $p < 0.01$).

The cell cycle of transfected H7721 cells with different α-1,3-FucT-VII expression was analyzed using the flow-cytometric method. The results in figure 1B and C show that most of the 'Mock' cells were in the G1 (including G0) phase. After transfection with α-1,3-FucT-VII cDNA, the percentages of G1 cells decreased: they were 76.6%, 65.1% and 54.2% for 'Mock,' FucTVII-M and FucTVII-H, respectively. In contrast, the percentages of S-phase cells were increased in α-1,3-FucT-VII transfected cells, being 13.4%, 20.0% and 29.4% for 'Mock', FucTVII-M and FucTVII-H, respectively. However, the percentages of G2/M cells were not significantly changed. These findings indicated that the transfection of α-1,3-FucT-VII cDNA facilitated cell progression from G1 to the S phase. In other words, the elevation of α-1,3-FucT-VII mRNA resulted in a greater transition of cells from G1 to the S phase in the cell cycle.

Effect of α-1,3-FucT-VII transfection on the expression of cyclins and cyclin dependent kinases

There is a 'checkpoint' at the G1/S transition in the cell cycle controlled by three families of cell cycle-related molecules, including cyclins [21, 22], CDKs (2, 4, 6) and CDIs, such as $p16^{INK4a}$, $p21^{Waf1/Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ [23–25]. Cyclins are positively regulated by CDK activities via phosphorylation, and CDKs are negatively regulated by CDIs via protein-protein interactions. The expression of cyclin D1, E and A and CDK4, 6 and 2 was detected by Western blot. Their expression was not obviously changed in α-1,3-FucT-VII-transfected cells compared with the 'Mock' cells (fig. 2A, B).

Effect of α-1,3-FucT-VII transfection on the expression of CDIs

At least two subfamilies of CDI have been reported. One is the INK4 (inhibitor of CDK4) family, including $p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$ and $p19^{INK4d}$, which inhibits the con-

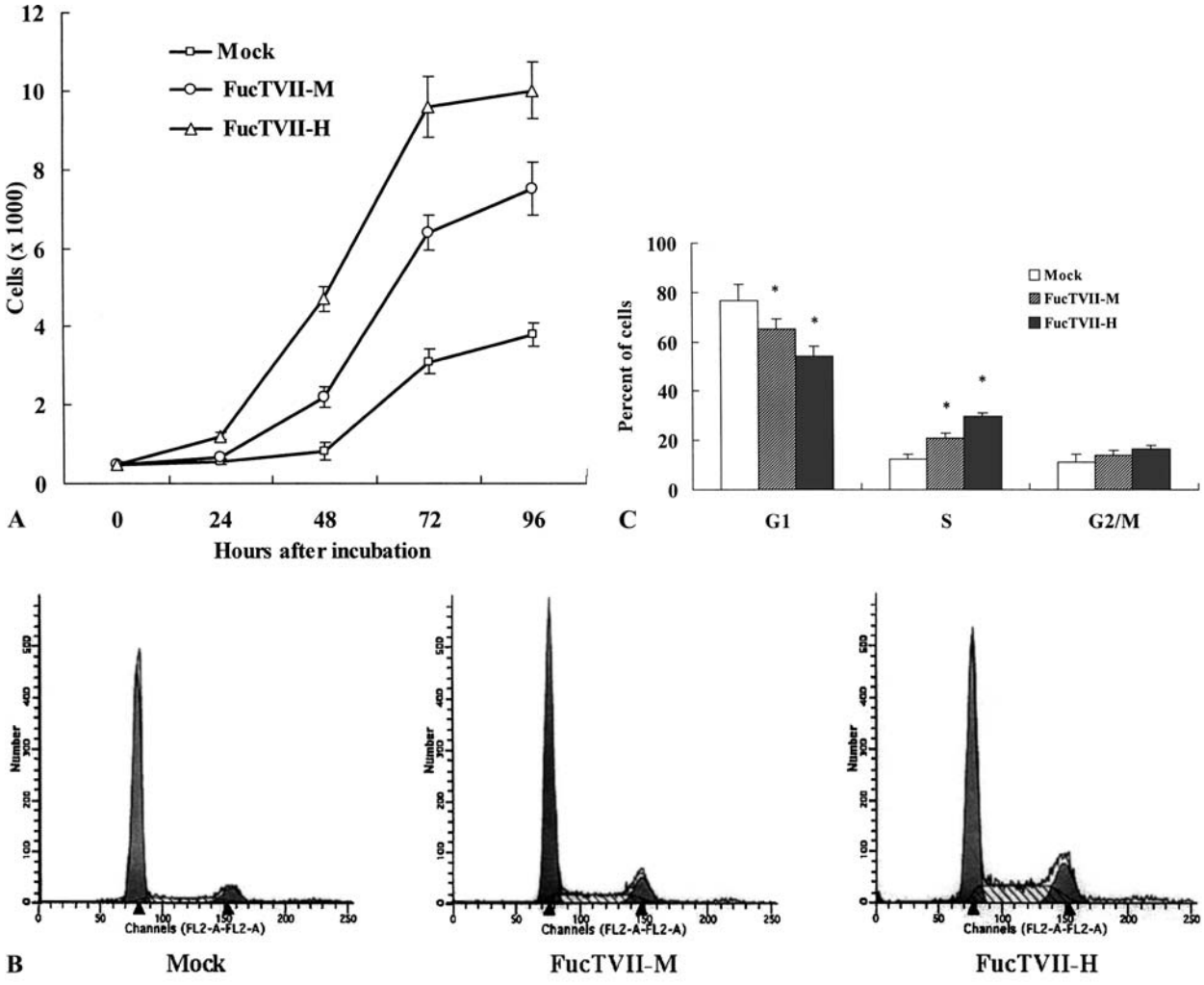

Figure 1. Effects of α-1,3-FucT-VII cDNA on cell growth and cell percentage at different stages of the H7721 cell cycle. (*A*) Mock, H7721 cells transfected with pcDNA3.1 vector; FucTVII-M, H7721 cells with moderate expression of the transfected pcDNA3.1/FucT-VII; FucTVII-H, H7721 cells with high expression of the transfected pcDNA3.1/FucT-VII. Cell growth curves of the mock and α-1,3-FucT-VII cDNA-transfected cells. (*B*) Cell cycle profiles of the mock- and α-1,3-FucT-VII cDNA-transfected cells. (*C*) Percentage of cells at G1, S and M phases of the mock- and α-1,3-FucT-VII cDNA-transfected cells. Data were expressed as the mean ± SD from three independent experiments. *$p < 0.01$ (FucTVII-M vs Mock and FucTVII-H vs FucTVII-M).

struction and activity of the cyclinD1-CDK4/6 complex [23]. Another is the Cip/Kip (CDK inhibitory protein/kinase inhibitory protein) family, containing mainly $p21^{Waf1/Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$, which inhibits the construction and activity of cyclinE-CDK2 and cyclinA-CDK2 complexes [24, 25]. $P16^{INK4}$, $p21^{waf1/Cip1}$ and $p27^{Kip1}$ were selected as three representatives of these two families, and their expression studied in α-1,3-FucT-VII cDNA-transfected H7721 cells. Figure 3A shows that the expression of $p27^{Kip1}$, but not that of $p16^{INK4}$ and $p21^{waf1/Cip1}$, was decreased in the α-1,3-FucT-VII-transfected cells, and the levels in FucTVII-M and FucTVII-H cells were 76.0% and 45.1% of the 'Mock' value, respectively (both $p < 0.01$). Using immuno-coprecipitation, we found that not only was the total cellular $p27^{Kip1}$ decreased, but the CDK2-bound $p27^{Kip1}$ was also apparently reduced (fig. 3B). The amounts of $p27^{Kip1}$ bound to CDK2 in FucTVII-M and FucTVII-H cells were 55.1% and 20.9% of the 'Mock' level, respectively (both $p < 0.01$). As a consequence of $p27^{Kip1}$ de-inhibition, CDK2 activity was obviously elevated in FucTVII-M and FucTVII-H cells (fig. 3C), being 2.52 and 6.01 times the 'Mock' cell value (both $p < 0.01$). In an RT-PCR experiment, however, we found that the mRNA level of $p27^{Kip1}$ was not altered (fig. 3D). This suggested that the reduction in $p27^{Kip1}$ in α-1,3-FucT-VII cDNA-transfected cells was probably at the post-transcriptional level.

Effect of α-1,3-FucT-VII transfection on the expression of Rb protein

One of the important target proteins of CDKs is the Rb protein, known as a tumor suppressor and a dominant in-

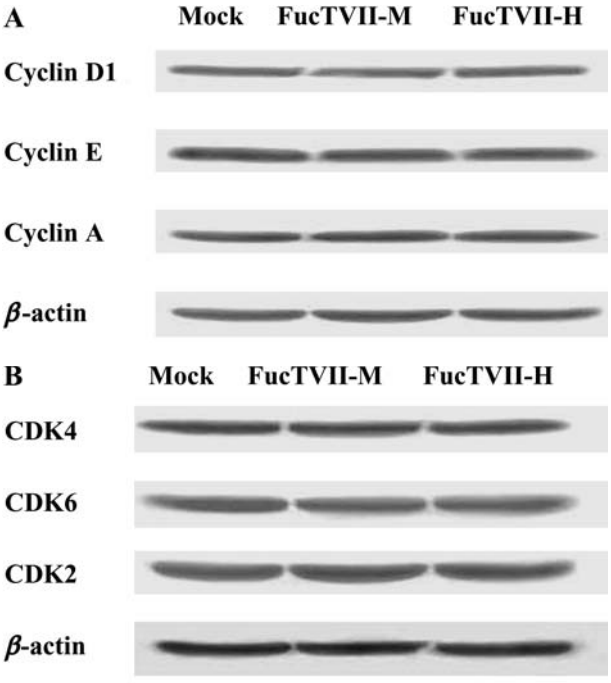

Figure 2. Western blot profiles of cyclins and CDKs in mock- and α-1,3-FucT-VII cDNA-transfected cells. Mock, FucTVII-H, FucTVII-M: same as figure 1. Three independent experiments were performed and the results were reproducible. (*A*) Expression of cyclins D1, E and A. (*B*) Expression of CDK4, CDK6 and CDK2.

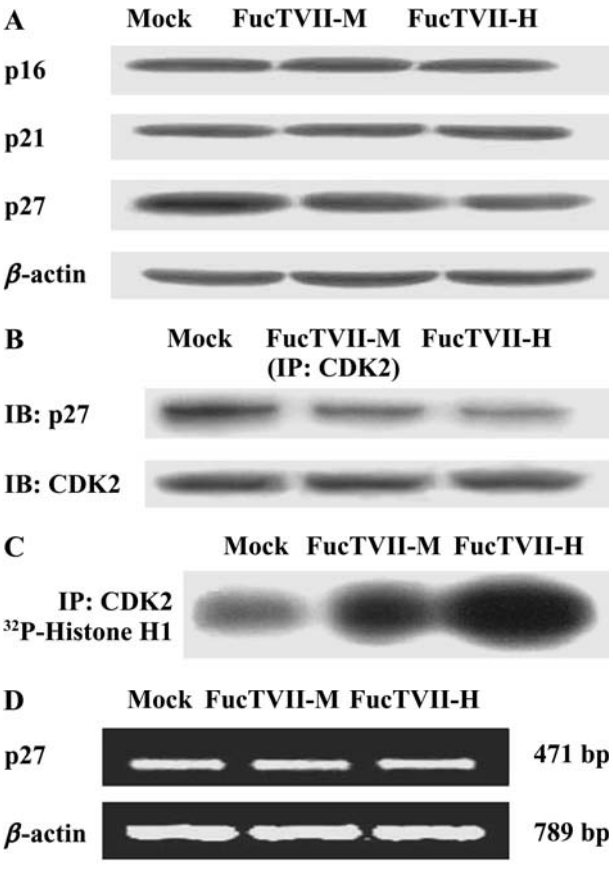

Figure 3. Effects of transfection of α-1,3-FucT-VII cDNA on the expression of CDI and CDK2 activity in H7721 cells. Mock, FucTVII-H, FucTVII-M: as in figure 1. IP, immunoprecipitation; IB, immunoblot. Three independent experiments were performed and the results were reproducible. (*A*) Expression of $p16^{INK4}$, $p21^{waf1/Cip1}$ and $p27^{Kip1}$ in mock- and α-1,3-FucT-VII cDNA-transfected cells determined with Western blot. (*B*) CDK2-bound $p27^{Kip1}$ in mock- and α-1,3-FucT-VII-transfected cells. $P27^{Kip1}$ was immuno-coprecipitated with CDK2 and detected after Western blot using $p27^{Kip1}$ antibody. (*C*) Activity of CDK2 in mock- and α-1,3-FucT-VII-transfected cells, γ-^{32}P-ATP and histone H1 protein were used as substrates. (*D*) RT-PCR determination of $p27^{Kip1}$ mRNA in mock- and α-1,3-FucT-VII-transfected cells.

hibitor of G1/S cell cycle progression [22, 26]. Rb is regulated by cyclinD1-CDK4/6, cyclinE-CDK2 and cyclinA-CDK2 complexes via phosphorylation at multiple sites [22, 26]. Our findings obtained from Western blot experiments revealed that the phosphorylated Rb (p-Rb) was almost undetectable in 'Mock' cells, but significantly elevated in FucTVII-M and FucTVII-H cells, especially in the latter. In contrast, the unphophorylated Rb protein was decreased in α-1,3-FucT-VII-transfected cells (fig. 4).

Effect of KM93 on the expressions of $p27^{Kip1}$ proteins

To study the relationship between the expression of cellular $p27^{Kip1}$, p-Rb and that of cell surface SLe^x (the product of α-1,3-FucT-VII), the expression of $p27^{Kip1}$ and p-Rb was determined after cell surface SLe^x had been blocked by KM93, the monoclonal antibody of SLe^x, at different concentrations (10 µg/ml and 30 µg/ml). $p27^{Kip1}$ was apparently increased in all the KM93-treated cell lines, including 'Mock,' FucTVII-M and FucTVII-H cells (fig. 5A), when compared with the findings from the experiments without KM93 treatment, as indicated in figure 3A. In contrast, the p-Rb level was obviously decreased in all cell lines when compared with the results from the experiments in the absence of KM93 as shown in figure 4. The blocking effect of 30 µg/ml KM93 was greater than that of 10 µg/ml, as shown by the fact that the color intensities of $p27^{Kip1}$ in cells treated with 30 µg/ml KM93 were higher than those in cells treated with 10 µg/ml KM93, and the intensities of p-Rb were lower in cells treated with 30 µg/ml KM93 than those in cells treated with 10 µg/ml KM93. In addition, the intensity differences of both $p27^{Kip1}$ and p-Rb among the 'Mock,' FucTVII-M and FucTVII-H cells (shown in figs. 3A, 4) were obviously attenuated after blockage of surface SLe^x by 10 and 30 µg/ml KM93. At the latter concentration, the intensity difference of $p27^{Kip1}$ among three different cell lines and the difference of p-Rb between FucTVII-M and FucTVII-H cells ('Mock' cells hardly express p-Rb) almost disappeared (fig. 5B).

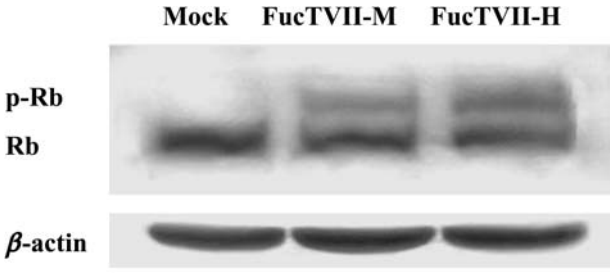

Figure 4. Expression of Rb and phosphorylated Rb in mock- and α-1,3-FucT-VII-transfected cells. Mock, FucTVII-H, FucTVII-M: same as figure 1. p-Rb, phosphorylated Rb. Three independent experiments were performed and the results were reproducible.

Signaling pathway down regulating p27 ^{Kip1}

PI-3K/protein kinase B (PKB) [27, 28] and Ras/MEK/MAPK signaling pathways [29, 30] have been reported to be involved in the regulation of $p27^{Kip1}$ expression. The role of these two pathways in reduction of $p27^{Kip1}$ in α-1,3-FucT-VII-transfected cells was also investigated using the specific inhibitors of PI-3K (LY 294002) [31] and MEK (PD98059) [32]. When the results shown in figure 6 (with inhibitor) and figure 3A (without inhibitor) were compared, $p27^{Kip1}$ expression levels were found to be increased in both LY294002- and PD98059-treated cell lines, including 'Mock,' FucTVII-M and FucTVII-H. Moreover, the intensity difference of $p27^{Kip1}$ among different cell lines (shown in fig. 3A) was still obvious in LY294002-treated cells, but almost abolished in PD98059-treated cells. Figure 6 also showed that p-Rb was reduced slightly in LY294002-treated cells and decreased very significantly in PD98059-treated cells. Furthermore, the difference in p-Rb intensity between FucTVII-M and FucTVII-H cells was still apparent in LY294002-treated cells, but attenuated significantly in PD98059-treated cells. These findings indicated that the Ras/MEK/MAPK pathway might contribute more in the down-regulation of $p27^{Kip1}$ in H7721 cells.

Discussion

The findings in the present investigation provided strong evidence that the proliferation of H7721 cells was mainly regulated by the expression of $p27^{Kip1}$, rather than other CDIs. The reduction in $p27^{Kip1}$ protein (rather than mRNA) in α-1,3-FucT-VII-transfected cells led to the de-inhibition or activation of CDK2, but the expressions of CDK4 and CDK6 proteins were not altered. The activated CDK2, in turn, stimulated the phosphorylation of Rb protein, resulting in the release of the transcription factor E2F family from Rb-E2F complexes, and then the free E2F facilitated the cells to pass through the G1/S 'checkpoint' in cell cycle [21–26], and increased the S phase

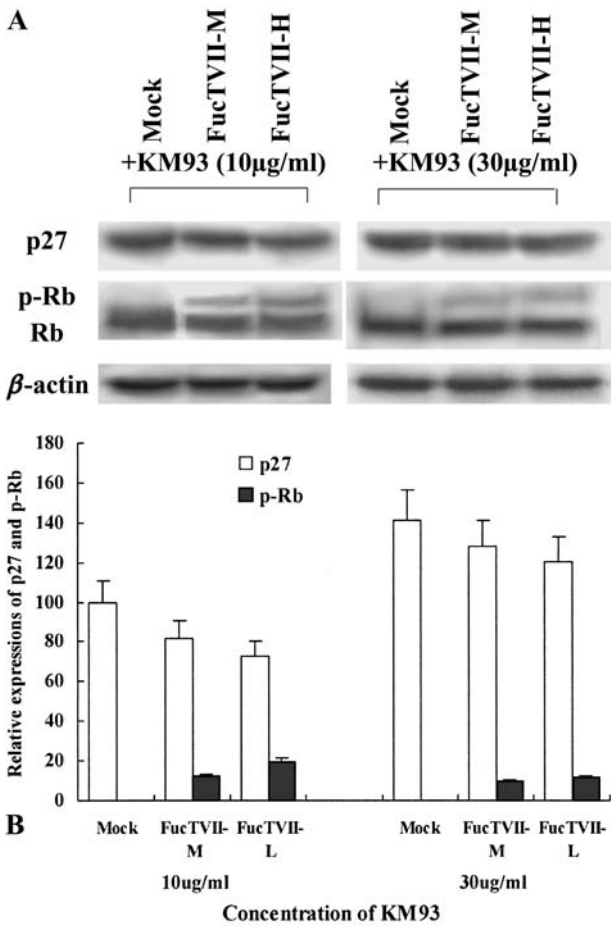

Figure 5. The expression of $p27^{Kip1}$ and p-Rb in different cells after treatment with different concentration of KM93. (*A*) Mock, FucTVII-M, FucTVII-H: same as figure 1. p-Rb, phosphorylated Rb. Three independent experiments were performed and the results were reproducible. Western blot profiles of p27 ^{Kip1}, Rb and p-Rb. (*B*) Quantification of *A*. Data were expressed as the mean ± SD from three independent experiments.

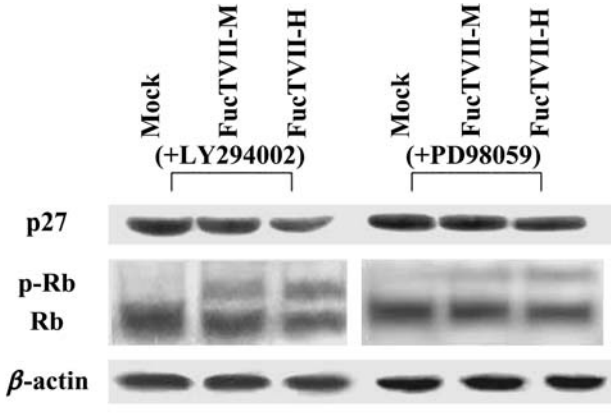

Figure 6. Expression of p27 ^{Kip1}and p-Rb in different cells in the presence of LY294002 and PD98059. Mock, FucTVII-M, FucTVII-H: same as figure 1. p-Rb, phosphorylated Rb. Three independent experiments were performed and the results were reproducible.

cells via the stimulation of DNA synthesis. As a consequence, the growth of α-1,3-FucT-VII-transfected H7721 cells was accelerated. Therefore, decreased expression of $p27^{Kip1}$ protein seems to be the key event in the stimulation of H7721 cell proliferation after transfection of α-1,3-FucT-VII cDNA.

In our previous study, we demonstrated that the mRNA of α-1,3-FucT-VII and the surface SLe^x (product of α-1,3-FucT-VII) levels were increased significantly after transfection with α-1,3-FucT-VII cDNA [10]. In this study, we re-examined these two parameters. The ratio of α-1,3-FucT-VII mRNA in 'Mock,' FucTVII-M and FucTVII-H was 1: 3.73: 6.13 and the ratio of SLe^x on the cell surface was 1:1.71:2.84. The present observations that $p27^{Kip1}$ expression in 'Mock,' FucTVII-M and FucTVII-H cells (fig. 3 A) was inversely proportional to the expression of surface SLe^x revealed that $p27^{Kip1}$was negatively associated with surface SLe^x and its synthesizing enzyme, α-1,3-FucT-VII. This conclusion was underscored by the finding that the total amount of $p27^{Kip1}$ was increased, and the difference in the magnitude of $p27^{Kip1}$ expression in the above-mentioned three cell lines was attenuated after surface SLe^x had been blocked by the antibody to SLe^x, KM93 (fig. 5).

Our results indicated that the influence of α-1,3-FucT-VII on $p27^{Kip1}$ expression was mediated by SLe^x. However, the mechanism was unknown. We have found that the total amount of surface SLe^x as well as the SLe^x content on some surface receptors were all increased, and SLe^x expression on the epidermal growth factor (EGF) receptor was very high on α-1,3-FucT-VII-transfected cells. In addition, the signal transduction of PI-3K/PKB and Ras/MEK/MAPK pathways was moderately and significantly promoted in FucTVII-M and FucTVII-H cells, respectively, when compared with the 'Mock' control (unpublished data). We speculate that the increase in SLe^x content on the sugar chains of cell surface receptors might alter the conformation of the receptors, resulting in the enhancement of the signaling of these two cell proliferation-related pathways. This speculation is supported by the increase in some signaling proteins (such as Raf, phosphatidylinositide-dependent kinase-1, protein kinase novel) and the transcription factor TCF, as well as the enhancement of phosphorylation of some signaling molecules (such as PKB and MAPK). Finally, some factor(s) regulating the synthesis and/or degradation of $p27^{Kip1}$ protein were decreased and/or increased, respectively, leading to a reduction in $p27^{Kip1}$ protein. The transcription of the $p27^{Kip1}$ gene was not altered, since the mRNA of $p27^{Kip1}$was unchanged.

We have also transfected antisense cDNA of α-1,3-FucT-VII into H7721 cells in order to demonstrate further that the change of $p27^{Kip1}$ expression is SLe^x dependent. Unfortunately, the cells died, which suggested that α-1,3-FucT-VII is a critical enzyme for the growth of H7721 cells.

However, other mechanisms of inhibiting $p27^{Kip1}$ expression by α-1,3-FucT-VII transfection unrelated to surface receptor and signal transduction cannot be ruled out. The exact mechanism by which α-1,3-FucT-VII and SLe^x affect the expression of $p27^{Kip1}$ remains to be resolved and is being investigated.

Acknowledgements. This work was supported by a grant from the National Natural Science Foundation of China (no. 30170219).

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