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ARTICLES

α1,3 Fucosyltransferase-VII Up-Regulates the mRNA of α5 Integrin and its Biological Function

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Abstract After transfection of α1,3fucosyltransferase (FucT)-VII cDNA into H7721 human hepatocarcinoma cells, the expression of α5, but not β1 integrin was significantly up-regulated. This was evidenced by the increase of α5 integrin on cell surface as well as the increase of α5 mRNA and protein in the cells. However, the expressions of sialyl Lewis X (SLe^x, the product of α1,3FucT-VII) on both α5 and β1 integrin subunits were unchanged. Concomitantly, the tyrosine autophosphorylated FAK and dephosphorylated Src (FAK and Src involve in the signal transduction of integrin α5β1) were up-regulated, while the Tyr-527 phosphorylated Src was down-regulated. The above-mentioned alterations were correlated to the expressions of α1,3FucT-VII in different α1,3FucT-VII transfected H7721 cell lines. In addition, after α1,3FucT-VII transfection, cell adhesion to fibronectin (Fn) and chemotaxic cell migration were obviously promoted. The cell adhesion could be blocked by α5 integrin antibody, and cell migration was obviously attenuated by the antibodies to both α5 integrin and SLe^x. These findings suggest that the increased surface α5 integrin caused by the up-regulation of α5 mRNA promotes the cell adhesion to Fn, cell migratiom, and Fn-induced signaling of α5β1 integrin. The up-regulation of surface SLe^x originated from the over expression of α1,3FucT-VII also led to the stimulation of cell migration. This is the first time to report that α1,3FucT-VII can regulate the mRNA expression of integrin. J. Cell. Biochem. 104: 2078–2090, 2008. © 2008 Wiley-Liss, Inc.

Key words: α1,3fucosyltransferase-VII; integrin; cell adhesion and migration; fibronectin; FAK; Src

Human fucosyltransferase (FucT) is a family of glycosyltransferases responsible for the synthesis of fucosyl-containing compounds. It catalyzes the transference of fucosyl residue (Fuc) from GDP-α-L-fucose to a sugar acceptor, usually galactose (Gal) or *N*-acetylglucosamine (GlcNAc or Gn), in the sugar chains of glycoconjugates. Human FucT is divided into three main subfamilies, α1,2FucT, α1,3FucT, and α1,6 FucT [Narimatsu, 1998; de Vries et al., 2001]. α1,2FucT (FucT-I, -II) participates in the synthesis of ABO blood group antigens and Lewis antigen Y and B (Le^y, Le^b), both Le^y and Le^b contain two fucosyl residues with α1,2 linkage on Gal and α1,3 (or α1,4) linkage on GlcNAc. α1,6 FucT (FucT-VIII) is responsible for the synthesis of core α1,6 fucose in Asn-linked sugar chains (*N*-glycans). α1,3FucT subfamily is the main glycosyltransferase participating in the synthesis of Lewis antigen, which is a series of sugar chains derived from Gal-β1,3/β1,4 GlcNac-backbone. The Gal residue in Lewis antigens can be α2,3-sialylated, α1,2-fucosylated or un-substituted, and α1,3 or α1,4 fucose is attached to β1,4GlcNAc or β1,3GlcNAc residue respectively. Lewis antigens are mainly located at the outer chains of glycolipids as well as the *O*- and *N*-linked sugar chains on cell surface glycoproteins [Varki, 1994]. Uptodate, six α1,3FucTs have been identified [Narimatsu,

Abbreviations used: FucT, fucosyltransferase; Gal; galactose; GlcNAc, *N*-acetylglucosamine; SA, sialic acid; FAK, focal adhesion kinase; FITC, fluorescein isothiocynate; RT-PCR, reverse transcriptase-polymerase chain reaction; HEPES, N-(2-hydroxyethyl) piperazine-N-(2-ethylsulfonic acid); MES, 2 (*N*-morpholino) ethansulfonic acid; PMSF, phenylmethylsulfonyl fluoride; HRP, horseradish peroxidase; ECL, enhanced chemiluminescence; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; FCS, fetal calf serum; BSA, bovine serum albumin; PBS, phosphate buffered saline.

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1998]. Each enzyme has a unique acceptor substrate binding pattern, and each generates a unique range of fucosylated products. Four of them, α1,3 FucT-III, -V, -VI, -VII efficiently fucosylate sialylated acceptors and produce sialyl Lewis antigens, while α1,3FucT-IV and -IX prefer neutral acceptors and usually form non-sialyl Lewis antigens as their products. α 1,3FucT-III is the only α FucT which can synthesis both α1,3 fucosyl containing Le^x, SLe^x, and Le^y as well as α1,4 fucosyl containing Le^a, SLe^a, and Le^b [Narimatsu, 1998]. α1,3-FucT-VII which mainly expresses in leukocyte catalyses sialylated substrates, and SLe^x [SAα2,3Galβ1,4 (Fuc α1,3) GlcNAc-] is its only product. The gene of α1,3-FucT-VII is located at chromosome 9q34.3, and its cDNA is 1.7 kb, which codes a 39.2 kDa protein containing 342 amino acid residues [Sasaki et al., 1994]. α1,3-FucT-VII is increased in some malignant cells, especially certain leukemia, and is responsible for the increased SLe^x on leukemia cell surface. However, α1,3-FucT-VII was reported to be also involved in the expression of SLe^x in cancer cells of epithelial origin [Kannagi, 2004].

SLe^x and some other sialylated Lewis antigens serve as the ligands for E- or P-selectin expressed on the surface of vascular endothelial cells, and mediate the adhesion of malignant cells to vascular endothelium [Kannagi, 2004; Kannagi et al., 2004]. Clinical studies indicate that cancer cells with strong SLe^x expression have the high risk of developing hematogenous metastasis, and this factor statistically affects the overall prognosis of the patients [Nakamori et al., 1993; Ogawa et al., 1994; Jorgensen et al., 1995]. In canine mammary carcinoma, it was found that a significant relationship between the levels of SLe^x expression and the presence of lymph node metastases [Pinho et al., 2007].

Our lab reported that after transfection of metastasis-stimulating gene (*c-erb*B2/*neu*) [Liu et al., 2001a] or treatment with proliferation-inducer (EGF or phobol ester) [Liu et al., 2001b], the expression of α1,3-FucT-VII in H7721 human hepatocarcinoma cells was up-regulated which was correlated to the increased cell surface SLe^x and metastatic potential in vitro. The metastatic potential includes cell adhesion to human umbilical vein epithelial cells, as well as chemotaxic cell migration and invasion as determined with transwell method. Oppositely, transfection of metastasis-suppressive gene (*nm23*-H1) [Liu et al., 2002] or treatment with differentiation-inducer (ATRA or 8-bromo-cAMP) [Liu et al., 2001b] led to the concomitantly decrease of the expressions α1,3-FucT-VII and surface SLe^x as well as metastatic potential. On the other hand, transfection of α1,3FucT-VII also resulted in the stimulation of cell growth [Wang et al., 2005a] and the up-regulation of some growth factor-signaling molecules [Wang et al., 2007b] as well as the modification of cell susceptibility to apoptosis [Wang et al., 2007a]. Therefore, α1,3-FucT-VII can be seemed as a growth and metastasis-related enzyme. The mechanism of the enhanced cancer metastasis by α1,3FucT-VII, however, is still poorly understood.

Tumor metastasis involves adhesive and migratory events in addition to proteolytic degradation of ECM, all of which require the continuous and coordinated formation and disassembly of adhesive structures [Liotta, 1992; Hakomori, 1996]. It involves stable attachment of cells to the extracellular matrix which requires transmembrane receptors of the integrin family. Integrin is a super-family, and each of its members is a heterodimer composed of two noncovalently associated different subunits, α and β, both of them are glycoproteins. The sizes of the α subunits are varied between 120 and 180 kDa, and those of β subunits are 90–130 kDa. At least 18 α and 8 β subunits were discovered. Different combinations of α and β subunits produce more than 24 different receptors [Brakebusch and Fasslar, 2005]. Most integrins are expressed on the surface of a wide variety of cells, and most cells express several integrins [Hynes, 1992]. Each αβ dimer has its own binding specificity and signaling property. Most integrins recognize several extracellular matrix (ECM) proteins, and each ECM protein binds to several integrins. For example, α1β1, α2β1, α10β1 and α11β1 are binding to collagen, α1β1, α2β1, α3β1, α6β1 and α7β1 to laminin, α4β1, α5β1, α8β1 and αVβ1 to fibronectin (Fn), α9β1 to tenascin C and αVβ1 to vitronectin [Brakebusch and Fasslar, 2005]. Among them, α5β1 integrin specifically binds to fibronectin (Fn) and is seemed as a typical receptor of Fn [Zheng et al., 1994]. ECM-integrin interaction generates intracellular signaling, which regulates the activities of growth factor receptors, cytoplasmic protein kinases and ion channels, resulting in the induction of focal adhesion, cell spreading and migration, as well as cell cytoskeleton formation. These processes are

implicated in cell growth, differentiation, apoptosis and cancer metastasis, and nearly all of the other complex physiological or pathological processes in vertebrate organisms [Giancotti and Ruoslahti, 1999; Schwartz, 2001; Brakebusch and Fasslar, 2005].

In the present investigation, we studied the expression of α5 and β1 integrin in α1,3FucT-VII transfected H7721 cells at both protein and mRNA levels. The tyrosine-phosphorylation of FAK and Src (these two molecules involve in the signal transduction of integrin α5β1) was also studied to confirm the change of integrin expression. The results suggest that α1,3FucT-VII up-regulates the expression of integrin α5 at both protein and mRNA level. The enhanced interaction of integrin α5β1 with fibronectin (Fn) up-regulated the intracellular signaling through FAK/Src and subsequently accelerates the cell adhesion to Fn and chemotaxic cell migration.

MATERIALS AND METHODS

Materials

The human hepatocarcinoma H7721 cell line was obtained from the Institute of Cell Biology, Academic Sinica. RPMI 1640 and DMEM were purchased from GIBCO/BRL (Rocville, MD). Monoclonal antibodies against human integrin α5, β1, β-actin and rabbit polyclonal antibodies to human FAK, as well as Protein G PLUS agarose were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal antibodies against human Src and phospho-Src (Tyr527) were from Cell Signaling Technology. HEPES, MES, fibronectin, phosphotyrosine antibody (PT66), FITC-conjugated second antibodies (goat anti-mouse IgG) and HRP-labeled second antibody (goat against rabbit IgG) were purchased from Sigma (St. Louis, MO) Geneticin (G418) was the product of Invitrogen. PVDF membrane was from Bio-Rad (Hercules, CA). TRIzol and AMV reverse transcriptase were from Promega (Midison, WI). Other reagents, including Taq enzyme, RNAase inhibitor, dNTP, oligo (dT)-18, ECL reagent were commercially available in China.

The α1,3FucT-VII transfected H7721 cell line was established in our laboratory as previously reported [Wang et al., 2005a, 2007b]. Two transfectants were selected; one expressed high level α1,3FucT-VII mRNA designated as FucTVII-H, and the other expressed moderate α1,3FucT-VII mRNA entitled FucTVII-M. H7721 cells (expressed very low level of α1,3FucT-VII) mock-transfected with the vector pcDNA3.1 was used as the control and named "Mock". The expression ratio of α1,3FucT-VII mRNA in "Mock", FucTVII-M, and FucTVII-H was 1:3.73:6.13. The expression ratio of SLe^x, the product of α1,3-FucT-VII, on cell surface was 1:1.71:2.84 [Wang et al., 2007b].

Cell Culture

Cells were cultured at 37°C, 5% CO_2 in RPMI-1640 medium containing 10% FCS, penicillin and streptomycin as previously described by our laboratory [Wang et al., 2005a, 2007b]. When antibodies were used to block the surface α5 integrin and SLe^x, the final concentrations of α5 antibody and SLe^x monoclonal antibody (KM93) in the culture medium were 10 and 30 μg/ml respectively.

Detection of Integrin Subunits on Cell Surface with Flow Cytometry

Detection of cell surface integrin subunits was performed according to the method reported by our lab [Guo et al., 2003]. The cultured cells were dispersed in 2 mM EDTA in PBS and washed twice with PBS, then 1×10^6 cells were incubated with 1:100 diluted monoclonal antibody against human α5 or β1 integrin subunit in blocking buffer (1% BSA in PBS) for 45 min at 4°C. In "(−) Control" samples the primary antibody was omitted. The cells were washed twice with blocking buffer, mixed with a 1:100 diluted FITC-labeled second antibody, and incubated for 30 min at 4°C. Then the cells were washed again with PBS, suspended in 0.5 ml PBS and subjected to flow cytometry. Fluorescence activated cell spectra (FACS) was drawn automatically, and the left- or right-shift of the curve indicated the decrease or increase of the mean fluorescence intensity (MFI) respectively.

Determination of the Expression of Integrin mRNA With RT-PCR

Total RNA was extracted from cells using TRIzol according to the protocol provided by Promega. Complementary DNAs (cDNAs) were synthesized with oligo (dT) primers and AMV reverse transcriptase from 3 μg of the total RNA in 30 μl reaction mixture. The RT-PCR was performed in a volume of 50 μl containing 5 μl cDNA, 0.2 μM specific pair of primers for

integrins or β-actin (loading control), PCR buffer (10 mM Tris–HCl pH 8.3/50 mM KCl/ 5 mM $MgCl_2$), 0.2 μM of each dNTP and 1 IU Taq polymerase. The cDNA was subjected to denaturation at 95°C for 5 min, followed by 28 cycles of PCR. Each cycle included denaturation at 94°C for 1 min, annealing at 61.5°C for 1 min and elongation at 72°C for 1 min. Finally, the sample was further incubated for elongation at 72°C for 10 min and 4°C for 5 min. After completion of the RT-PCR, 10 μl products were applied to 2% agarose gel containing ethidium bromide for electrophoresis. The intensities of the amplified DNA bands were scanned by ImageMaster System and the screened photos were analyzed with NIH Image software. The semi-quantitative data were obtained by the intensity ratios of integrin bands to the β-actin band. The primers used for α5 and β1 integrins were α5 F: 5′-ACCAAGGCCCCAGCTCCATTAG-3′; R: 5′-GCCTCACACTGCAGGCTAAATG-3′, β1 F: 5′-AACTTGATCCCTAAGTCAGC AGTAG-3′; R: 5′-ATCAGCAGTAATGCAAGGCC-3′. The primers used for β-actin were F: 5′-GATATCGCCGTCGTCGAC-3′; R: 5′-CAGGAAGGAAGGCTGGAAGAGTGC-3′ [Guo et al., 2003].

Immunoprecipitation of Integrins, Determination of Integrin With Western Blot and Analysis of SLe^x Expression on Integrins

The cultured cells were washed with cold PBS, and lysed by 200 μl lysis buffer (50 mM pH 7.4 HEPES/150 mM NaCl/100 mM NaF/1 mM $MgCl_2$/1.5 mM EGTA/1% Nonidet P-40/10 μg/ml leupeptin and pepstatin, 1 mM PMSF). Cell lysate containing 500 μg protein (determined by Lowry's method) was incubated with 2 μg monoclonal antibody to α5 or β1 integrin at 4°C for 1 h. Then 20 μl Protein G PLUS agarose suspension was added, and the sample was further incubated at 4°C for 3 h to immunoprecipitate the integrin, followed by centrifugation and washing of the pellet. The protein concentrations of the precipitated α5 and β1 samples were adjusted to the same concentration (30 μg/ml) and subjected to SDS–PAGE, then transferred to PVDF membranes and treated with 1:1,000 diluted antibody to α5 or β1 integrin and KM93 (antibody to SLe^x). The color was developed by 1:500 HRP-labeled second antibody and ECL reagents, and the membranes were put under X-ray film for exposure, followed by densitometric scanning of the film for quantification. The relative expressions of α5 and β1 integrins were calculated from the intensity-ratios of integrin proteins (α5 and β1) to β-actin, and the SLe^x on α5 and β1 integrin subunits was calculated from the ratio of SLe^x intensity to the protein intensity of α5 or β1 integrin.

Determination of FAK Autophosphorylation

Cells (1×10^5) were added onto culture dishes coated with 10 μg/ml Fn for 3 h. Then the cells were washed twice with ice cold PBS, and the monolayer cells were lysed with 200 μl lysis buffer. FAK was immunoprecipitated with polyclonal antibody using the method as described in the section of "Immunoprecipitation of Integrins, Determination of Integrin With Western Blot and Analysis of SLe^x Expression on Integrins". The precipitated FAK was divided into two parts and subjected to 8% SDS–PAGE and Western blot as described above, then the membranes were probed with 1:1,000 monoclonal phosphotyrosine antibody (PT66), or 1:500 FAK antibody, followed by incubation with 1:500 diluted HRP labeled second antibody. The color was also developed with ECL reagent. The tyrosine phosphorylation (Tyr-p) of FAK was calculated from the ratio of staining intensity of PT66 treated band to that of FAK antibody treated band after normalized with β-actin.

Determination of Src and Tyr-527-Phospho-Src

The cells cultured in Fn coated dish for 3 h as described above were washed and homogenized in 0.1 M MES buffer (pH 6.5)/150 mM NaCl/2% Triton X-100/25% glycerol/0.1 mg% leupeptin and pepstatin, then centrifuged with 1,000*g* at 4°C for15 min. Aliquots of 50 μg of protein samples were subjected to 10% SDS–PAGE and Western blot. Then the membranes were probed with 1:1,000 Src or phospho-Src (Tyr527) antibody, followed by incubation with 1:500 diluted HRP labeled second antibody and ECL reagent. The relative expression of Src and phospho-Src was calculated from the ratio of staining intensity of Src or phospho-Src to β-actin.

Assay of Cell Adhesion to Fn

Cell adhesion experiment was according to the methods described by Busk et al. [1992]. In brief, the wells of the culture plate were coated with 0.1 ml of different concentration of Fn. Additional 1 mg/ml poly-L-lysine and 1% BSA were coated onto two wells as maximal

and minimal adhesion controls respectively. The plate was incubated at 37°C for 1 h, and blocked by 1% BSA at 37°C for 0.5 h after washing. Cells (1×10^5) were added to each coated well and incubated for 2 h at 37°C. The wells were then washed twice and stained with crystal violet, and the absorbance (Abs) at 595 nm was measured. The relative adhesion of cells to the coated wells was calculated from a formula described in our previous paper [Liu et al., 2000]. The data were expressed as the mean value of triplicate experiments.

Determination of Cell Migration

The chemotaxic cell-migration assay was performed using 24-well transwell units with polycarbonate filter of 8 μm pore size according to the method of Mensing et al. [1983] Each lower compartment of the transwell contained 600 μl 0.5% FBS in DMEM as chemoattractant or 0.5% BSA as the negative control. Cells (2×10^5) in 0.1 DMEM/0.1% BSA were added to the upper compartment of the transwell unit and incubated for 6 h at 37°C in a humidified atmosphere containing 5% CO_2. The cells were then fixed with glutaraldehyde and stained with crystal violet. The number of cells that migrated to the lower side of polycarbonate filter was counted in eight high-power fields (200×). The data were expressed by the mean value of cells per high-power field in triplicate wells.

Statistical Analysis

Values were expressed as mean ± SD. Statistical significance was determined with SPSS 10.0. Results were evaluated by Student's t tests. $P < 0.05$ and $P < 0.01$ were considered statistically significant and very significant respectively.

RESULTS

Expression of Integrin Subunits on Cell Surface After Transfection With 1,3FucT-VII cDNA

Firstly, the level of integrin α5 and β1 on the cell surface was determined. Figure 1A,B showed that the expression of α5 subunit was up-regulated on FucTVII-M and FucTVII-H cells, up to 1.47 and 2.11 times respectively of the value in mock-transfected cells (both were $P < 0.01$). However, the expression of cell surface β1 subunit was slightly decreased in 1,3FucT-VII transfected cells but not statistically significant as compared with the "Mock" cells.

Expression of the mRNAs of Integrin Subunits After Transfected With 1,3FucT-VII cDNA

The mRNAs of α5 and β1 integrin subunits were determined by means of RT-PCR to elucidate the mechanism of the increased surface α5 integrin. As indicated in Figure 2A, The RT-PCR products of α5 and β1 integrins were 375 and 1,200 bp respectively, which were in accordance with the predicted lengths. The product of β-actin was 789 bp, also compatible with the predicted value. In mock-transfected cells, the mRNA expression of β1 integrin was far higher (approximately 3.3 times) than that of α5 integrin (Fig. 2B). The mRNA of α5 subunit was increased to 2.06 and 3.11 times in FucTVII-M and FucTVII-H cells respectively as compared with the "Mock" cells (both $P < 0.01$), while the mRNA of β1 subunit was unchanged in 1,3FucT-VII transfected cells (Fig. 2A,B). This finding suggests that the surface expression of integrin subunits is mainly regulated at the transcription level, and the elevated cell surface α5 integrin is originated from the increased mRNA of α5 integrin.

Expressions of Cell α5 and β1 Integrin and SLe^x on Integrin Subunits After Transfected With 1,3FucT-VII cDNA

By using immunoprecipitation and Western immunoblotting, it was found that the protein expression of β1 integrin in cells was also far higher than that of α5 integrin, the ratio of β1:α5 was 3.85. The α5 integrin was also up-regulated to 202% and 308% of the "Mock" level in FucTVII-M and FucTVII-H cells respectively (both $P < 0.01$), while the protein of β1 integrin was also unchanged. (Fig. 3A,B) This result was compatible with the increased mRNA of α5 subunit and the unaltered mRNA of β1 subunit as shown in Figure 2. The expressions of SLe^x on integrin α5 and β1 subunits were observed after immunoprecipitation of these two subunits and Western immunoblot with KM93, the monoclonal antibody against SLe^x. It was interesting to find that the total amount of SLe^x on α5 subunit was increased in 1,3FucT-VII transfected cells, up to 218.2% and 291.4% of the "Mock" level in FucTVII-M and FucTVII-H cells respectively (both $P < 0.01$). However, their relative amount, calculated from the ratio of

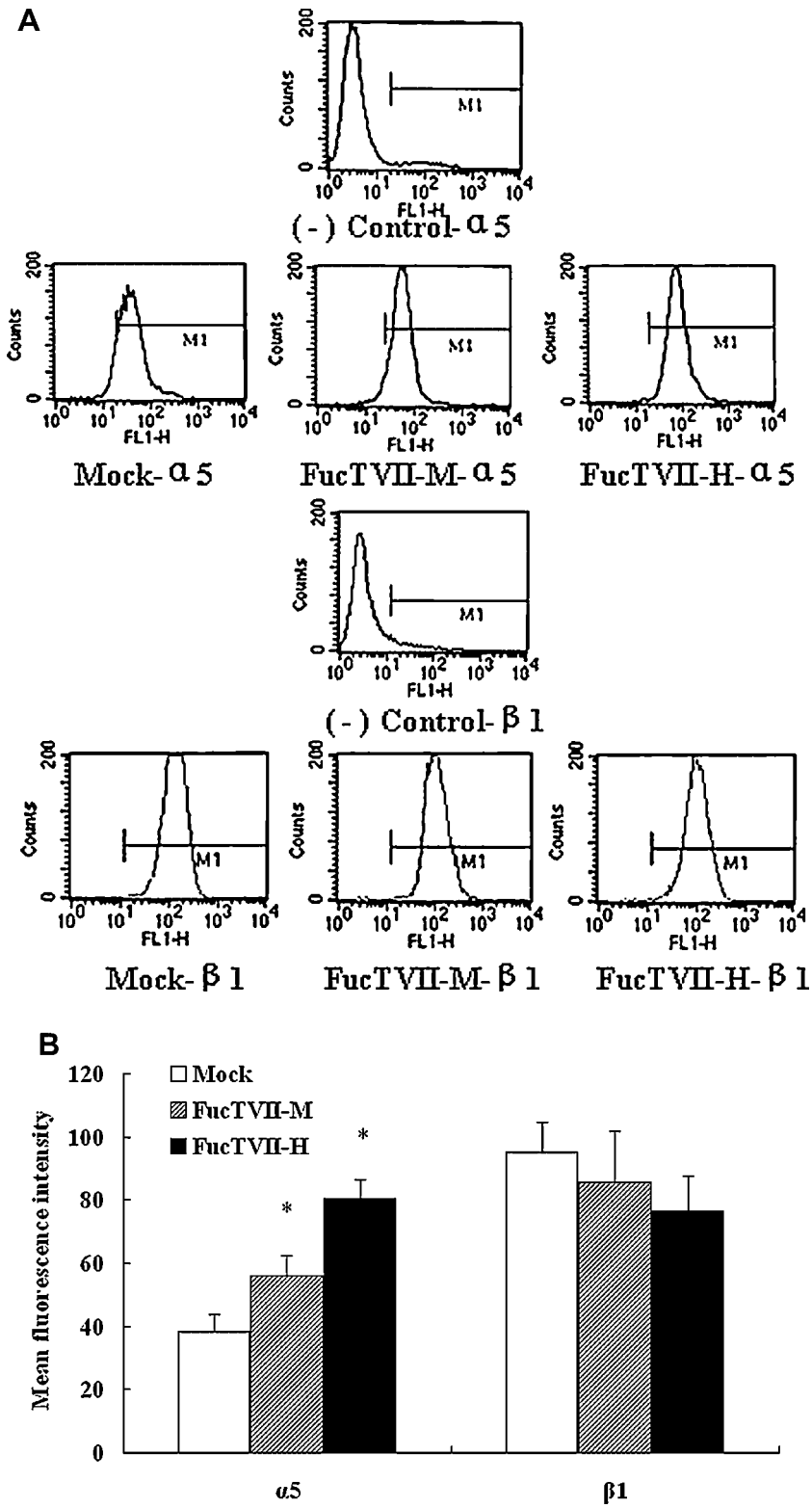

Fig. 1. Flow-cytometric analysis of α5 and β1 integrin subunits on cell surface after transfection with 1,3FucT-VII cDNA. **A**: Fluorescence activated cell spectra (FACS) of surface α5 and β1 integrin subunits. (−) Control: Sample without addition of intrgrin antibody. **B**: Quantification of surface α5 and β1 integrin subunits. The data were expressed as the mean fluorescence intensity (MFI) ± SD. $^*P < 0.01$ compared to ''Mock'' (n = 3). Mock: H7721 cells transfected with pcDNA3.1 vector; FucTVII-M: H7721 cells with moderate expression of transfected α1,3FucT-VII cDNA; FucTVII-H: H7721 cells with high expression of transfected α1,3FucT-VII. α5:α5 integrin, β1:β1 integrin. ''A'' is the representative of three independent and reproducible experiments. The experiment procedure for flow-cytometric analysis was described in the ''Materials and Methods'' Section.

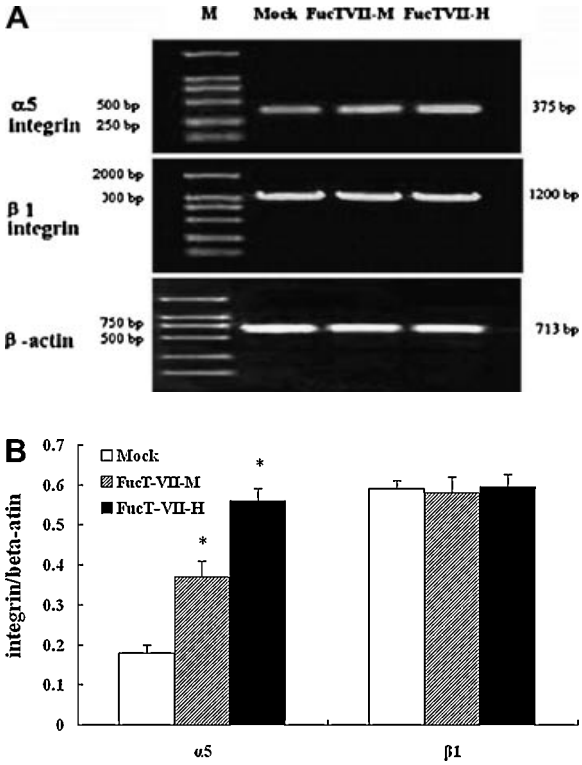

Fig. 2. RT-PCR analysis of α5 and β1 integrin subunits after cells transfected with 1,3FucT-VII cDNA. **A**: RT-PCR profiles of α5 and β1 integrin subunits. M: DNA ladder marker (100–2,000 bp). **B**: Densitometric quantification of RT-PCR profiles. The data were expressed as the intensity ratio of α5 or β1 integrin to β-actin (Mean ± SD). $*P < 0.01$ compared to ''Mock''. Mock, FucTVII-H, FucTVII-M: Same as indicated under Figure 1. α5, β1: same as Figure 1. ''A'' is the representative of three independent and reproducible experiments. The experiment procedure for RT-PCR was described in the ''Materials and Methods'' Section.

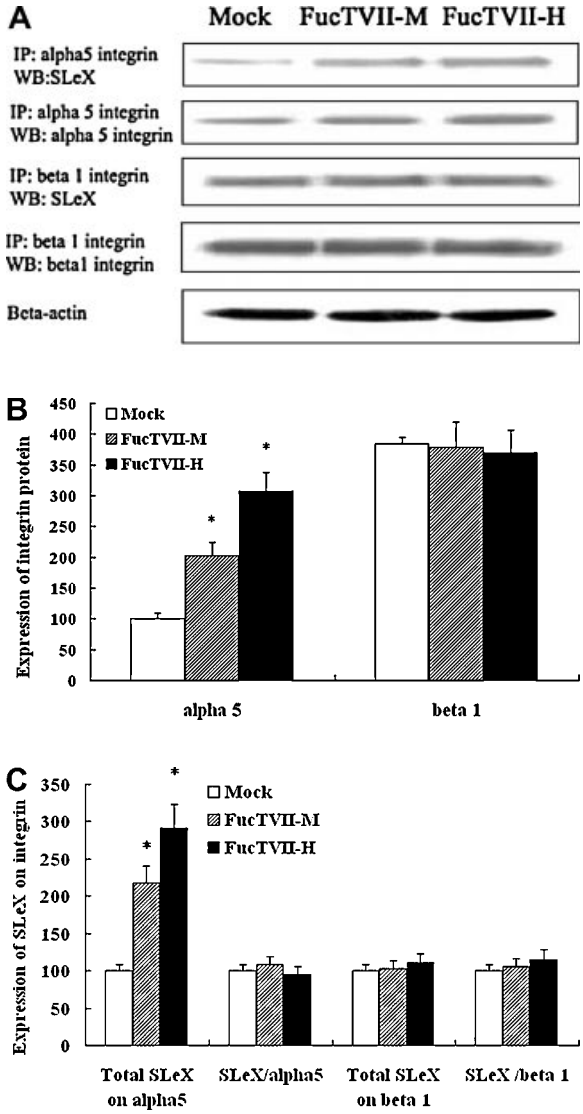

Fig. 3. Expressions of α5, β1 integrin proteins and SLe^x on integrin subunits after cells transfected with 1,3FucT-VII cDNA. **A**: Western blot profiles of immunoprecipitated α5 and β1 integrin proteins using corresponding antibodies and SLe^x antibody and HRP-labeled second antibodies. **B**: Densitometric quantification of protein expression of α5 and β1integrins in (A). **C**: Densitometric quantification of SLe^x in (A) and calculation of SLe^x expression/integrin (set the ''Mock'' cells as 100%). $*P < 0.01$ compared to ''Mock'' (n = 3). αMock, FucTVII-H, FucTVII-M: Same as indicated under Figure 1. SLe^x: sialyl Lewis X. IP: immunoprecipitation by the antibody to α5 or β1 integrin; WB: Western immunoblot with the antibodies to α5 and β1 integrins or SLe^x. ''A'' is the representative of three independent and reproducible experiments. The experiment procedure for immunoprecipitation and Western blot were described in the ''Materials and Methods'' Section.

total SLe^x on α5 integrin to α5 protein was unaltered, because the protein of α5 integrin was elevated in the same magnitude as total SLe^x. Meanwhile, both the total and relative amount of SLe^x on β1 integrin were not changed (Fig. 3A,C). These results indicated that the transfection of 1,3FucT-VII did not affect the expression of SLe^x on α5 and β1 integrin proteins.

Effect of 1,3FucT-VII Transfection on the Phosphorylation of FAK and Src

Focal adhesion kinase (FAK) is associated with the intracellular domain of the integrin β subunit, and involved in signaling transduction for cell adhesion and migration [Giancotti and Ruoslahti, 1999; Schwartz, 2001; Guo and Giancotti, 2004; Brakebusch and Fasslar, 2005]. Therefore, the effect of α1,3FucT-VII transfection on the expression and phosphorylation of FAK in Fn-induced cells was studied. Figure 4A showed that the expression of FAK

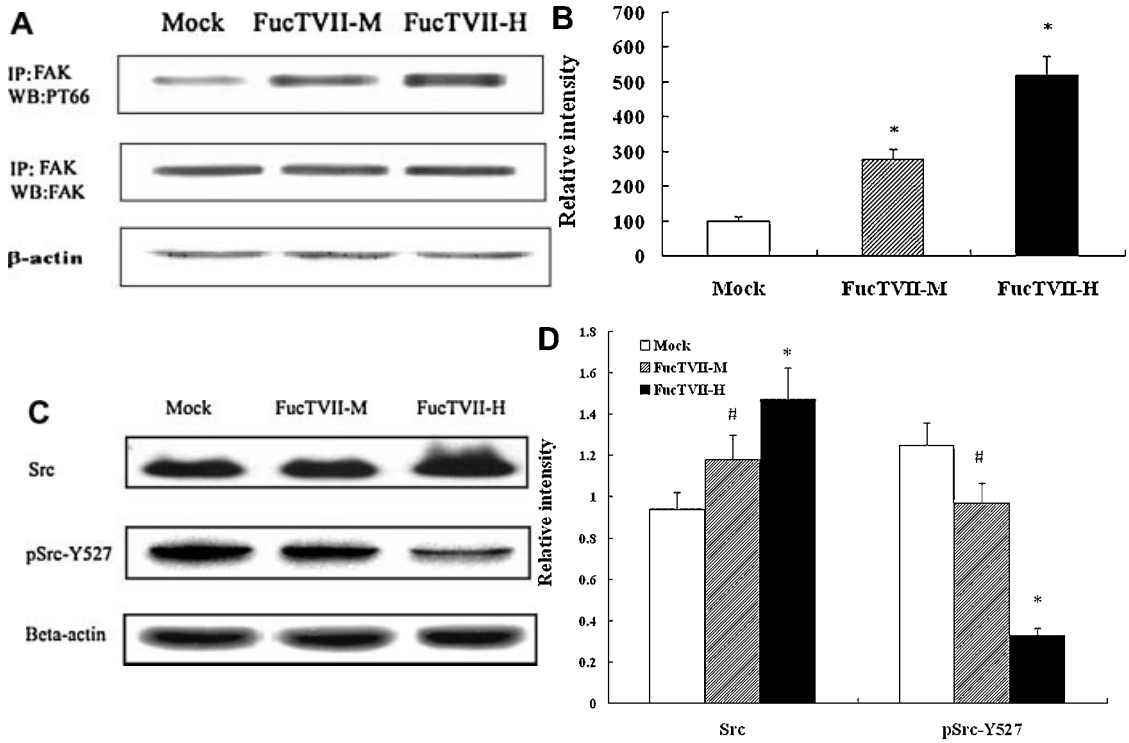

Fig. 4. Expression and phophorylation of FAK and Src after cells transfected with 1,3FucT-VII cDNA. **A**: Western blot profiles of immunoprecipitated FAK stained with phosphotyrosine antibody PT66 or FAK antibody. **B**: Densitometric quantification of FAK and Tyr-phospho-FAK expressed as relative intensity (intensity ratio of PT66/FAK, and set the ''Mock'' cells as 100%). $^{*}P < 0.01$ compared to ''Mock'' (n = 3). **C**: Western blot profiles of Src and Tyr527-phosphorylated Src stained with corresponding antibodies and HRP-labeled second antibodies. **D**: Densitometric quantification of Src and Tyr527-phosphorylated Src expressed as relative intensity (intensity ratio of Src or phospho-Src/β-actin, and set the ''Mock'' cells as 100%). $^{\#}P < 0.05$; $^{*}P < 0.01$ compared to ''Mock'' (n = 3). Mock, FucTVII-H, FucTVII-M: Same as indicated under Figure 1. pSrc-Y527: Tyr527-phosphorylated Src. IP: immunoprecipitation by the antibody to FAK; WB: Western immunoblot with the antibody to phosphotyrosine (PT66) or FAK. ''A'' and ''C'' are the representatives of three independent and reproducible experiments for each. The experiment procedure for immunoprecipitation and Western blot were described in the ''Materials and Methods'' Section.

protein was unchanged in α1,3FucT-VII transfected cells cultured on Fn coated dish, while the tyrosine autophosphorylation of FAK in FucT-VII-M and FucTVII-H cells was significantly increased to 276% and 520% of the "Mock" level respectively (both $P < 0.01$) (Fig. 4A,B).

Very recently, it has been documented that during Fn-induced activation of integrin, an enzyme on cell membrane called protein kinase phosphatase-α (PTP-α) is activated, which in turn dephospholylates Src (a member of Src family kinases, including Src, Fyn, and Yes) at Tyr-527 residue and promotes the autophosphorylation at Tyr-416, resulting in the activation of Src. Subsequently, the activated Src in Src/FAK tyrosine kinase complex phosphorylates FAK at Tyr-576 and Tyr-577 and promote the integrin-stimulated early autophosphorylation of FAK at Tyr-397 [Wang et al., 2005b; Chen et al., 2006]. It indicates that the dephosphorylation of Src at Tyr-527 is required for the activation of FAK. As shown in Figure 4C,D, the Tyr (Y)-527 phosphorylated Src was reduced to 77.6% ($P < 0.05$) and 28.0% ($P < 0.01$) of the "Mock" value respectively in FucTVII-M and FucTVII-H cells. Correspondingly, the dephosphorylated Src was increased to 125.5% ($P < 0.05$) and 156.4% ($P < 0.01$) in FucTVII-M and FucTVII-H cells respectively.

Effect of 1,3FucT-VII Transfection, α5 Integrin Antibody and SLe^x Antibody on Cell Adhesion to Fn and Cell Migration

As shown in Figure 5A, the cell adhesion to Fn was increased with the concentration of Fn both in the mock- and 1,3FucT-VII-transfected cells. Furthermore, at all concentrations of Fn, the adhesion to Fn of 1,3FucT-VII transfected cells

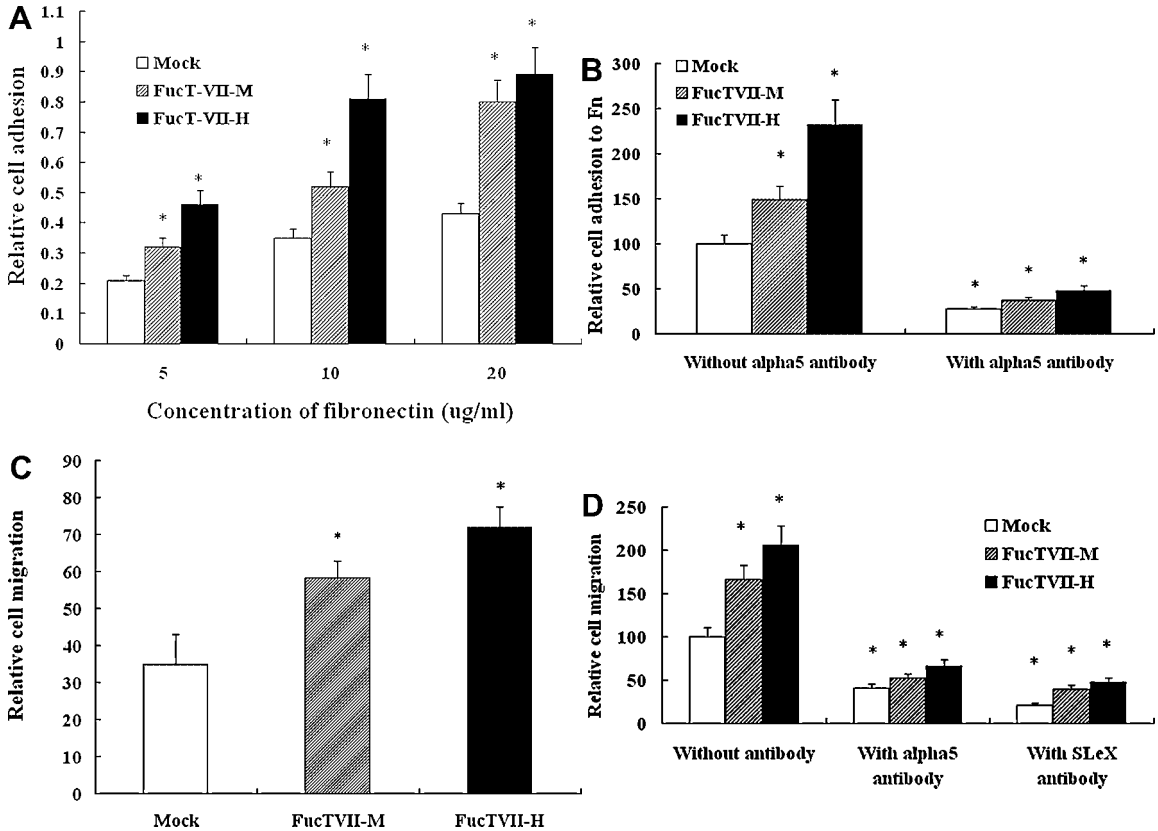

Fig. 5. Effect of α1,3FucT-VII cDNA transfection on cell adhesion and migration. **A**: Cell adhesion to fibronectin in the absence of α5 antibody. *$P < 0.01$ compared to "Mock" (n = 3). **B**: Relative cell adhesion to fibronectin (10 μg/ml) after surface α5 integrin was blocked by its antibody (set un-blocked "mock" cell as 100%). *$P < 0.01$ compared to "Mock" or corresponding un-blocked controls (n = 3). **C**: Chemotaxic cell migration through transwell in absence of α5 antibody and KM93. *$P < 0.01$ compared to "Mock" (n = 3). **D**: Relative chemotaxic cell migration after surface α5 integrin was blocked by its antibody and SLe^x was blocked by KM93 (set un-blocked "mock" cell as 100%). *$P < 0.01$ compared to "Mock" or corresponding un-blocked controls (n = 3). Mock, FucTVII-M, FucTVII-H: Same as indicated under Figure 1. All experiments were performed with triplicate in three independent and reproducible experiments. The experimental procedures for determination of cell adhesion and migration were described in the "Materials and Methods" Section.

was higher than that of "Mock" cells, especially that of FucTVII-H cells. ($P < 0.01$ compared to the "Mock" value for both FucTVII-M and FucTVII-H cells). After surface α5 integrin was block by its antibody, the cell adhesion to Fn (10 μg/ml) was dramatically declined to 20.5–27.3% of the corresponding un-blocked control values (Fig. 5B).

The chemotaxic cell migration through transwell was also increased in 1,3FucT-VII transfected cells, being 166.6% and 205.4% of the "Mock" value in FucTVII-M and FucTVII-H respectively (Fig. 5C) (both $P < 0.01$). When the antibody of α5 integrin was present in the culture medium, the chemotaxic cell migration was decreased 31.5–32.2% ($P < 0.01$) as compared with the corresponding control levels (without α5 antibody). Similarly, cell migration was significantly reduced 76.2–78.6 ($P < 0.01$) from the corresponding control values after the surface SLe^x was blocked by KM93.

DISCUSSION

In the present investigation, it was found that the expression of α5, but not β1 integrin was up-regulated in α1,3FucT-VII transfected cells. Three methods for measuring the expression of integrin α5 and β1 were performed, and the results were very compatible to each other. The increased percentage of α5 mRNA (Fig. 2B) was almost equal to that of α5 protein in both FucTVII-M and FucTVII-H cells (Fig. 3B), indicating that the mRNA of α5 integrin was translated to α5 protein very effectively, and it is possible that the degradation of α5 mRNA is not an important factor in the control of α5 protein

synthesis. However, the increased percentage of α5 protein in cells was higher than that of α5 protein on cell surface, being 202% versus 147% in FucTVII-M, and 308% versus 211% in FucTVII-H cells (Figs. 1 and 3), suggesting that only a major portion, but not all of the α5 protein is sorted to cell surface membrane. It is very likely that the up-regulation of α5 protein in α1,3 FucT-VII transfected cells is originated from the increased transcription of α5 integrin gene, and leads to the elevated expression of α5 integrin on cell surface.

In three cell lines with different α1,3 FucT-VII expression, the protein expressions of α5 integrin in cells and on cell surface were in proportional with the mRNA expression of α5 integrin. The intensity order of both mRNA and protein was "Mock" < "FucTVII-M" < "FucTVII-H". In our previous paper, it was found that the SLe^x expressed on cell surface was significantly increased after transfection with α1,3FucT-VII cDNA into H7721 cells [Wang et al., 2007b]. The present results showed that the total SLe^x content on α5 integrin was also elevated after α1,3FucT-VII transfection, and it is also positively correlated to the mRNA expression of α1,3FucT-VII in different cell lines, suggesting that the activity of α1,3FucT-VII is also "Mock" < "FucTVII-M" < "FucTVII-H", and the increased SLe^x content on α5 integrin is the result of elevated α1,3FucT-VII activity in cell lines transfected with α1,3FucT-VII cDNA. However, the increased magnitude in total SLe^x on α5 integrin and the increased magnitude in α5 protein was almost the same, so the relative content of SLe^x on each α5 molecule was unchanged.

In Figure 1B, the expression of β integrin on cell surface was slightly decreased in α1,3 FucT-VII transfected cells, we considered that this result was not important, because the decrease was not statistically significant. Moreover, the mRNA and protein of β integrin expressed in the cells were unchanged as indicated in Figures 2 and 3.

The underlying mechanism of the up-regulation of α5 mRNA in α1,3FucT-VII transfected cells is unresolved. It may be not related to the decreased degradation of α5 mRNA as discussed above, but the promoted transcription of α5 integrin gene is likely to be the main reason for the increased α5 mRNA. At least three possibilities might be implicated in the mechanism that α1,3FucT-VII up-regulates the mRNA of α5 integrin. (1) The over-expression of α1,3FucT-VII cDNA results in the elevation of SLe^x content on some surface receptors, which promotes the signaling of the receptor and finally stimulates the transcription of α5 integrin gene. In this study, the SLe^x content on both integrin α and β proteins is unchanged, (Fig. 3A,C), but we have found that the SLe^x content on insulin receptor is elevated apparently in α1,3FucT-VII transfected cells, and the signaling of insulin receptor is consequently facilitated via the increased expression and phosphorylation of some signaling molecules [Wang et al., 2007b]. However, the relation between insulin signaling and integrin expression is still elusive and remains to be clarified. (2) The increase of total SLe^x content on cell surface leads to the decrease of oxygen permeability of the cells, and the hypoxia condition can induce the up-regulation of α5 mRNA. As it was reported by Kannagi laboratory .that hypoxia of human colon cancer cells induced by hypoxia-induced factor (HIF), the expression of α5 integrin mRNA was up-regulated in a time-dependent manner [Koike et al., 2004]. (3) It is also possible that the changed expression of α5 integrin may be not related to the glycosylation activity of α1,3FucT-VII. We have used the SLe^x antibody (KM93) to block the surface SLe^x on α1,3FucT-VII transfected cells, and it was surprised to find that KM93 could not suppress the expression of α5 integrin mRNA, suggesting that the elevation of α5 mRNA might not resulted from the increase of surface SLe^x, the product of α1,3FucT-VII (to be published). This speculation was supported by Saito et al. [2002] that tumor angiogenesis induced by a secreted type of *N*-acetylglucosaminyltransferase V (GnT-V) was not mediated by the glycosylation activity of GnT-V, since the highly basic domain of GnT-V induced the release of fibroblast growth factor-2 (FGH-2) from haparan sulfate proteoglycan on the cell surface and/or extracellular matrix, leading to the FGH induced angiogenesis. In our laboratory, a plasmid containing a mutation at the catalytic domain of α1,3FucT-VII with deletion of enzyme activity is being constructed, which will be used to verify whether the up-regulation of α5 integrin mRNA is related to the glycosylation effect of α1,3FucT-VII.

The expression of β1 integrin was far higher than that of α5 integrin in H7721 cells. In

"Mock" H7721 cells, the mRNA and protein levels of β1 integrin were 3.28 and 3.85 times respectively of the α5 integrin levels. If the lower molecular weight of β1 integrin (130 kDa) compared to α5 integrin (150 kDa) is considered, the molar concentration of β1 is approximately four times higher than that of α5. It is very possible that β1 subunit also combine with α subunits other than α5, since we have found that αV and α6 are present in hepatic cells. However, the possibility cannot be ruled out that a portion of β1 presents as monomer or homo-dimer in H7721 cancer cells. It has been reported that the *N*-glycan on α5 integrin is essential for the heterodimerization of α5β1 integrin [Isaji et al., 2006]. Hence, it is possible that the structural alteration of *N*-glycan on α5 integrin may prevent the α5 and β1 subunits from dimerization, resulting in the accumulation of β1 subunit.

It is an unresolved problem that transfection of α1,3FucT-VII up-regulates α5 integrin only, but not β1 integrin. Our lab has discovered that transfection of GnT-V also up-regulates α5 integrin subunits in H7721 cells at both protein and mRNA level, but β1 subunit does not increase [Guo et al., 2003]. This finding was similar to the results in the present study. The surface α5 but not β1 integrin was also increased on H7721 cells treatment with *all-trans* retinoic acid [Zhang et al., 2004]. Therefore, up-regulation of α5 integrin only, but not β1 integrin, is a common phenomenon in H7721 cells. However, transfection of antisense GnT-V led to the down-regulation of both α5 and β1 integrins [Guo et al., 2003], revealing that β1 integrin can be also regulated by exogenous factors, but the mechanism is different from that of α5. It is likely that the β1 integrin is high enough in H7721 cells, so it cannot be significantly elevated further by the transfection of α1,3FucT-VII or GnT-V. In addition. Whether α1,3FucT-VII can up-regulate other α-subunits of integrin, such as Fn-specific α4, α8, and αV, needs to be further investigated.

The cell adhesion to Fn was stimulated after α1,3FucT-VII transfection (Fig. 5A). We consider that it is mainly caused by the up-regulation of α5 integrin protein. This was evidenced by the observation that the cell adhesion was very significantly blocked by the antibody of α5 integrin (Fig. 5B). The increased α5 integrin in α1,3FucT-VII transfected cells may combine with β1 integrin (present as the hypothesized monomer, homodimer or bind with other α subunits) to form more α5β1 dimers, resulting in the promotion of cell adhesion to Fn and cell signaling to Src/FAK complex. Hence, the tyrosine-phospho-FAK and dephospho-Src are increased as indicated in Figure 4.

The increased chemotaxic migration in α1,3FucT-VII transfected cells (Fig. 5C) was also related to the elevated α5 integrin, since integrin is involved in the regulation of cell migration [Giancotti and Ruoslahti, 1999; Schwartz, 2001; Guo and Giancotti, 2004; Brakebusch and Fasslar 2005]. In the present studies, the cell migration was attenuated significantly not only after α5 integrin was blocked by its antibody, but also after the surface SLe^x was blocked by KM93, the monoclonal antibody of SLe^x (Fig. 5D). It can be assumed that both integrin and cell surface SLe^x contribute in cell migration. We have found that the glycan structure of cell surface glycans plays an important role in cell migration [Zhang et al., 2002]. Removal of sialyl or fucosyl residues on H7721 cell surface glycans by exoglycosidases resulted in the 44% and 11% decrease of cell migration respectively. This finding was similar to the present result using antibody KM93 to block the surface SLe^x. However, it was reported that integrins are not generally required for cell migration. Some cells, such as myoblasts, neurons and lymphocytes migrate normally in the absence of β1 integrin, The dependence of cell migration on integrin was different from cell to cell [Brakebusch and Fasslar, 2005].

On the other hand, the up-regulated dephosphorylation (activation) of Src and autophosphorylation of FAK in Fn stimulated cells (Fig. 4) also play important roles in the acceleration of cell migration in α1,3FucT-VII transfected cells. FAK has multiple binding sites to combine with many signaling molecules and adaptor proteins, including Src, platelet-derived growth factor receptor (PDGFR), Etk, phosphotidylinositide-3-kinase (PI-3K), growth-factor-receptor-bound protein Grb (binds to Ras), $p130^{cas}$ (CAS, binds to Rac), and GRAF (binds to Rho). Most of them, such as Src, PI-3K, Grb7, Rac, and Rho are participate in the process of cell migration [Schwartz, 2001; Guo and Giancotti, 2004]. FAK also activates extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK)

signaling pathway through the stimulation of B-Raf. Alternatively, FAK can activate ERK/MAPK by recruiting Grb2 and son-of-sevenless (SOS) complex [Guo and Giancotti, 2004], and the ERK/MAPK signaling pathway is well known as a simulator of cell migration. We had reported that Raf-MEK-MAPK pathway was stimulated after transfection of α1,3FucT-VII, and this stimulation could be attenuated after the surface SLe^x was blocked by KM3 [Wang et al., 2007b]. These observations may explain the increased cell migration by α1,3FucT-VII transfection and the abolished cell migration by KM93 treatment in this study. Moreover, upon activation of integrin by Fn, the autophosphorylation of FAK creates a binding site for the Src homology2 (SH2) domain of Src proteins. The Src kinase then phosphorylates a number of focal adhesion components, including integrin-binding cytoskeletal proteins paxillin and tensin, which are also related to cell shape and movement [Giancotti and Ruoslahti, 1999; Guo and Giancotti, 2004].

In summary, over expression of α1,3FucT-VII up-regulates the mRNA of α5 integrin, leading to the increase of α5 integrin in the cells. The increased α5 integrin subunit combines with β1 integrin to produce more α5β1 dimer on cell surface. Consequently, α5β1 dimer promotes the Fn-induced signaling of α5β1 integrin via Src/FAK and stimulates the cell adhesion to Fn as well as cell migration. In addition, the product of α1,3FucT-VII, SLe^x, also plays an important role in the promotion of cell migration.

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