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The Effect of Receptor Protein Tyrosine Phosphatase Kappa on the Change of Cell Adhesion and Proliferation Induced by *N*-Acetylglucosaminyltransferase V

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ABSTRACT

N-acetylglucosaminyltransferase V (GnT-V) has been reported to be positively associated with tumor progression, but its mechanism still remains unknown. In the present study, we found that GnT-V overexpression not only changed the glycosylation of receptor protein tyrosine phosphatase kappa (RPTPκ) but also decreased its protein level. Moreover, GnT-V overexpression decreased cell calcium-independent adhesion and increased the tyrosine phosphorylation level of β-catenin, in which RPTPκ played an important role. Since RPTPκ has an RXKR motif, which is a favored cleavage site for furin, we used furin inhibitor to further explore the effect of RPTPκ on the change of cell adhesion and β-catenin signaling induced by GnT-V. Our results showed that preventing RPTPκ cleavage rescued the above effects of GnT-V, suggesting that furin cleavage could be one of the factors for RPTPκ to regulate cell adhesion and β-catenin signaling in GnT-V overexpression cell lines. In addition, the increased tyrosine phosphorylation level of β-catenin was associated with the increased nuclear level of β-catenin and downstream signaling molecules such as c-myc and cyclin D1 that were associated with cell proliferation. Our results suggest that GnT-V could decrease human hepatoma SMMC-7721 cell adhesion and promote cell proliferation partially through RPTPκ. J. Cell. Biochem. 109: 113–123, 2010.

KEY WORDS: *N*-ACETYLGLUCOSAMINYLTRANSFERASE V; RECEPTOR PROTEIN TYROSINE PHOSPHATASE KAPPA; β-CATENIN; ADHESION; PROLIFERATION

N-acetylglucosaminyltransferase V (GnT-V) catalyzes the transfer of GlcNAc from UDP-GlcNAc to an α-mannoside to synthesize the GlcNAc-β1,6 Man branch, then forms tri- or tetra-antennary N-linked oligosaccharide chains [Arango and Pierce, 1988; Brockhausen et al., 1988; Pierce et al., 1997; Chen et al., 1998; Ko et al., 1999; Guo et al., 2000]. Previous studies demonstrated that the increased β1,6 branch was associated positively with invasion and migration in various tumors [Dennis and Laferte, 1989; Fernandes et al., 1991; Seelentag et al., 1998; Yao et al., 1998; Yamamoto et al., 2000]. GnT-V gene transfected into the lung epithelioid cell, for example, the transfectant showed an increased tumorigenicity [Demetriou et al., 1995]. The mice that lack GnT-V expression due to targeted deletion showed diminished progression and metastasis [Granovsky et al., 2000]. Moreover, the patients suffering from colorectal or breast cancer with increased GnT-V glycan product expression lowered survival rates by 5 years

Abbreviations: GnT-V, *N*-acetylglucosaminyltransferase V; RPTPκ, receptor protein tyrosine phosphatase kappa; EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; FAK, focal adhesion kinase; ERK, extracellular signal-regulated kinase; PHA-L, phytohemagglutinin-leukoagglutinin; ConA, concanavalin A; GlcNAc, *N*-acetyl-glucosamine.

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[Seelentag et al., 1998; Handerson et al., 2005]. These data provided strong evidence that GnT-V itself could be the key biological factor in carcinoma progression. Therefore, it is important to explore the mechanism that GnT-V was linked to carcinoma progression.

The target molecules of GnT-V play an important role in GnT-V-related biological events. The receptor protein tyrosine phosphatase kappa (RPTPκ), identified as a substrate of GnT-V by Kim et al. [2006], is composed of extracellular adhesion molecule-like domains, a single transmembrane domain, and a cytoplasmic phosphatase domain [Yang et al., 1997]. The extracellular domains of RPTPκ consist of one MAM domain, one Ig-like domain, and four fibronectin type III-like repeats [Yang et al., 1997]. It has been proved that RPTPκ is able to support homophilic cell–cell calcium-independent adhesion [Zondag et al., 1995; Cheng et al., 1997]. To be precise, the MAM motif of RPTPκ has been previously shown to be involved with cell–cell recognition [Takagi et al., 1991; Jiang et al., 1992; Hirata et al., 1993]. In addition, RPTPκ can mediate homophilic cell–cell interaction when expressed in nonadherent insect cells [Brady-Kalnay et al., 1993; Gebbink et al., 1993; Sap et al., 1994], suggesting that it serves as a normal physiological function in cell-to-cell signaling. These data show that RPTPκ plays a vital role in cell–cell adhesion.

In the cytoplasmic domain, RPTPκ contains two tandemly repeated conserved phosphatase domain: one catalytic domain is close to the cell membrane and responsible for the main enzymic activity; the other is located in the C-terminal, almost without phosphatase function [Eswaran et al., 2006]. It has been reported that RPTPκ can dephosphorylate β-catenin tyrosine [Fuchs et al., 1996; Novellino et al., 2008]. Tyrosine phosphorylation of β-catenin can lead to a decrease of membrane-bound β-catenin and, concomitantly, to an increase in the cytoplasmic and nuclear pool [Gujral et al., 2008]. Moreover, β-catenin in the nuclear pool has been found to serve as a key component in Wnt signaling [Peifer, 1997; Moon et al., 2002], where it interacts with transcription factors of T-cell factor/lymphoid enhancer factor (Tcf/Lef) family to activate target genes such as c-myc and cyclin D1 involved in cell growth control [He et al., 1998; Tetsu and McCormick, 1999]. Therefore, the tyrosine phosphorylation of β-catenin is an important culprit in tumor progression [Jean et al., 2009; Zhang et al., 2009].

It is reported that the minimal catalytic domain of GnT-V exists in its carboxyl terminus, and the deletion of as few as 4–8 amino acids from its carboxyl terminus can destroy its catalytic activity [Korczak et al., 2000]. Accordingly, we previously constructed the mutation Δc-GnT-V, the wild-type GnT-V plasmid with the deletion of the C-terminal six amino acid residues, which could be associated with endogenous wild-type GnT-V and made the wild-type GnT-V inactivated [Korczak et al., 2000; Wang et al., 2007, 2009]. In our study, we introduced three stable cell lines (Mock, wt-7721, and Δc-7721) established previously to address the question whether the change of cellular adhesion and proliferation induced by GnT-V was associated with RPTPκ. The wt-7721, human hepatoma SMMC-7721 cell overexpressed wild-type GnT-V plasmid; the Δc-7721 cells did the mutant ΔcGnT-V plasmid; Mock did pcDNA3.0 as control. We found that GnT-V decreased cell–cell calcium-independent aggregation and promoted cell proliferation by RPTPκ, and RPTPκ was an important factor to the change of cell adhesion and proliferation induced by GnT-V.

MATERIALS AND METHODS

CELL LINES, CULTURE, AND TREATMENT

A human hepatocellular carcinoma cell line (SMMC-7721) was obtained from the Liver Cancer Institute in Zhongshan Hospital of Fudan University (Shanghai, China); human colon carcinoma Lovo cell line, from Tumor Hospital of Fudan University (Shanghai, China). Stable transfection cell lines (Mock, wt-7721, and Δc-7721) were established previously by ourselves [Wang et al., 2007, 2009]. All above cell lines were cultured in RPMI 1640 (Gibco, Carlsbad, CA) supplemented with 10% calf bovine serum at 37°C with 5% CO_2. Mock and wt-7721 cells were treated with 100 μmol Dec-RVKR-CMK (Calbiochem). Incubated with Dec-RVKR-CMK for 24 h, the cells were assayed and lysed.

ANTIBODY AND REAGENTS

Phytohemagglutinin-leukoagglutinin (PHA-L), concanavalin A (ConA) purchased from Sigma (Saint Louis, MI) were labeled with horseradish peroxidase as described previously [Osaw and Tsuji, 1987]; polyclonal antibody against RPTPκ extracelluar domain and monoclonal antibody against cyclin D1 from Santa Cruz Biotechnology (Santa Cruz, CA); monoclonal antibody against β-catenin and PY20, c-myc, from BD Biosciences Pharmingen; monoclonal antibody to GAPDH and secondary antibodies conjugated with HRP from Kang-Chen Biotech (Shanghai, China); polyclonal antibody against GnT-V from Abcam; the protein A/G plus-Agarose from Santa Cruz Biotechnology; and the furin inhibitor (Dec-RVKR-CMK) from Calbiochem.

RNA INTERFERENCE ASSAY

RNA interference was undertaken using the pSilencer2.0 vector (Ambion, Inc.). RNA interference target sequences were 5′-ATGCCAACTCGATCATTGG-3′ which was chosen according to the reference [Kim et al., 2006]. Target oligonucleotides were synthesized, annealed, and cloned into pSilencer vector between the *Bam*I and *Hin*dIIIλ sites. Recombinant plasmid DNA was prepared and tested for silencing activity against RPTPκ mRNA expression. A negative control vector comprising a scrambled sequence was also prepared. The recombinant siRPTPκ plasmid was transfected into SMMC-7721 and wt-7721 cells.

SEMI-QUANTITATIVE AND REAL-TIME REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION

Total RNA was extracted with RNA Rose reagent (Invitrogen) according to the manufacturer's manual. Semi-quantitative RT-PCR was basically performed as described elsewhere [Wang et al., 2007]. The primer pair for RPTPκ was used according to the reference [Kim et al., 2006]. β-actin was used as an internal control for RT-PCR, the primers designed according to the reference [Takano et al., 2000]. Real-time PCR was performed using an iCycler thermal cycler

(Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Reactions were performed with 0.2 mM primers. Nucleotides, Taq DNA polymerase, and buffer were included in SYBR Green JumpStart Taq ReadyMix for quantitative PCR. cDNA amplification consisted of one cycle at 95°C for 90 s, 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 by iCycler software (Bio-Rad), and the quantification data were analyzed following the DDCT method using GAPDH.

MEMBRANE ISOLATION USING THE MEM-PER KIT

About 5×10^5 cells in microcentrifuge tubes were pelleted down, washed once with phosphate-buffered saline (PBS), and then supplemented with 150 μl reagent A (lysis buffer) from the Mem-Per kit (Pierce, Rockford, IL). The mixture was incubated at room temperature for 10 min and then 450 μl of reagent B/C was added to the mixture, followed by incubation for 30 min on ice. The mixture was then centrifuged at 10,000*g* for 3 min. The supernatant, containing solubilized proteins, was separated and then incubated at 37°C for 10 min to separate phases. Following this, the mixture was centrifuged at 10,000*g* for 2 min. The top layer, containing the hydrophilic proteins, and the bottom layer, containing the hydrophobic membrane proteins, were separated. The fractions separated were assayed for protein and then appropriate aliquots containing 25–50 μg protein were removed, mixed with SDS–PAGE treatment buffer, placed in boiling water for 5 min, and then subjected to SDS–PAGE followed by Western blotting.

PREPARATION OF NUCLEAR EXTRACTS

Nuclear proteins were isolated according to the method of Schreiber et al. [1989]. Briefly, cell pellets were resuspended in 400 μl of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM PMSF) on ice for 15 min, before 25 μl of 10% Nonidet P-40 was added. After centrifugation, the nuclear pellets were resuspended in 50 μl of ice-cold buffer C (20 mM HEPES, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM PMSF) for an hour and vortexed at 4°C for 15 min. Upon centrifugation, the supernatants were collected.

WESTERN BLOTTING

The cells were washed with TBS (0.1 M Tris–HCl, pH 7.4, 0.15 M NaCl) and lysed in a buffer containing 50 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, phosphatase inhibitors (100 mM Na_3VO_4, 10 mM NaF), and protease inhibitor (1 mM PMSF), with the samples quantitated using the Lowry protein assay. For the total proteins, the cells were lysed in 1× SDS lysis buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 1 mM PMSF, and 1 mM Na_3VO_4). The equal amount of lysate (50 μg) was loaded on 10% SDS–PAGE and blotted onto PVDF membranes (Millipore, Corp.). The samples were blocked in TBS–Tween (TBST; 0.1 M Tris–HCl, pH 7.4, 0.15 M NaCl, 0.1% Tween-20) with 5% nonfat dry milk, and the membranes, incubated with primary antibodies at appropriate dilutions in TBST with 5% milk overnight at 4°C. After that, the membranes were washed three times with TBST solution, followed by incubation with horseradish peroxidase-linked secondary antibody (1:3,000) in TBST with 5% milk. The results were visualized by fluorography using an enhanced chemiluminescence system (Perfect Biotech, Shanghai, China).

LECTIN BLOTTING

For lectin blotting assay, the membranes were blocked with 3% BSA in TBST overnight at 4°C and incubated with 0.5 μg/ml of HRP–PHA-L and HRP–ConA in TBST with 3% BSA for 2 h at room temperature. The membrane washed with TBST three times, the protein bands were developed with ECL reagents, and exposed on X-ray film.

IMMUNOPRECIPITATION

As described elsewhere [Guo et al., 2002], the cells were washed with ice-cold PBS, and lysed in lysis buffer (50 mM Tris–HCl, pH 7.5, 200 mM NaCl, 15 mM EGTA, 0.5% (w/v) Nonidet P-40, 1 mM PMSF, 1 mM DTT, 1 mM Na_2VO_3, 100 mM NaF, 5 mg/ml of leupeptin, and 5 mg/ml of aprotinin), the cell lysates centrifuged at 12,000 rpm for 15 min at 4°C, the supernatants collected, and protein concentrations determined by means of Lowry protein assay. The equal amount of protein samples (1 mg) was incubated with 2 μg antibody for 3 h at 4°C, followed by an addition of pre-equilibrated protein A/G PLUS-agarose beads (20 μl) and incubation overnight. The immunoprecipitates were washed four times with lysis buffer, and the bound proteins, boiled in 15 μl of 2× SDS sample buffer, were eluted and resolved on a 10% SDS–PAGE gel. The proteins were transferred onto PVDF membrane and probed with primary antibodies at appropriate dilutions in TBST with 5% milk overnight at 4°C. After that, the membranes were washed three times with TBST solution, followed by an incubation with secondary antibody (1:3,000) in TBST with 5% milk. The results were visualized by fluorography using an enhanced chemiluminescence system (Perfect Biotech).

IMMUNOFLUORESCENCE MICROSCOPY

The cells were washed once with PBS and fixed in 4% paraformaldehyde in PBS for 20 min at room temperature. When the cells had been washed three times with PBS, the nonspecific binding sites were blocked by 1 h incubation with 3% bovine serum albumin at room temperature before being incubated with antibody at 4°C overnight, and then stained with FITC-conjugated secondary antibody for 2 h at 4°C, and the nuclei were stained with DAPI for 6 min at room temperature. The fluorescence of the cells was visualized under a photomicroscope with epifluorescence (IIRS, Olympus, Japan).

CELL AGGREGATION ASSAY

Subconfluent cells were washed with PBS and then detached with HCMF buffer containing 2 mM EGTA. Single cell suspensions were prepared, washed, and resuspended in HCMF buffer at a concentration of 10^5 cells/ml. Afterwards, 300 μl aliquots of cell suspension were added to the wells of 24-well plates pre-coated with 1% BSA overnight at 4°C and incubated for different periods of time at 37°C with 80 rpm agitation. The cell aggregation was measured using an inverted phase-contrast microscope by counting the single cell number in each field over a total of six randomly chosen fields. The aggregation index was calculated by $(N_0 - N_t)/N_0$, where N_0 is

the number of single cells before the experiment started and N_t is the number of single cells at the final time. The assay was repeated three times, and the means and SDs were determined [Vleminckx et al., 1994].

MTT ASSAY

The cells were seeded onto 96-well plates at a density of 2×10^4 cells/well in 200 μl culture medium, and grown for 0, 1, 2, 3, 4, 5, and 6 days, respectively. For each assay, 20 μl of MTT (1 mg/ml) added, these plates continued to be cultured for 4 h. Following incubation, the medium was discarded before 150 μl of DMSO was added to each well. The plates were vibrated gently for 10 min, followed by detection in the universal microplate reader at 490 nm. The assay was repeated three times, and the means and SDs were determined.

COLONY FORMING ASSAY

The cells (2×10^3) were replanted in 100 mm dishes and undisturbedly cultured for 15 days. The plates were fixed and stained with crystal violet, and then the number of colonies >1 mm in diameter was counted. The assay was done in triplicate.

RESULTS

GnT-V OVEREXPRESSION ALTERED THE GLYCOSYLATION AND PROTEIN LEVEL OF RPTPκ

To explore the effect of GnT-V on the oligosaccharides of RPTPκ, we introduced three stable cell lines (Mock, wt-7721, and Δc-7721) established previously [Wang et al., 2007, 2009]. The overexpression of wtGnT-V or ΔcGnT-V was detected via Western blotting and immunofluorescence microscopy assay. The level of GnT-V was higher in wt-7721 and Δc-7721 cells than that in Mock (Fig. 1A). Moreover, the overexpression and distribution of exogenous GnT-V plasmid also were proved by immunofluorescence microscopy (Fig. 1B). As shown in Figure 1B, the green dots representing the GnT-V were mainly expressed in cell cytoplasm and overexpressed in both wt-7721 and Δc-7721 cell lines. It was reported that RPTPκ was the substrate of GnT-V in colonal carcinoma [Kim et al., 2006]. To identify the relationship between RPTPκ and GnT-V, we

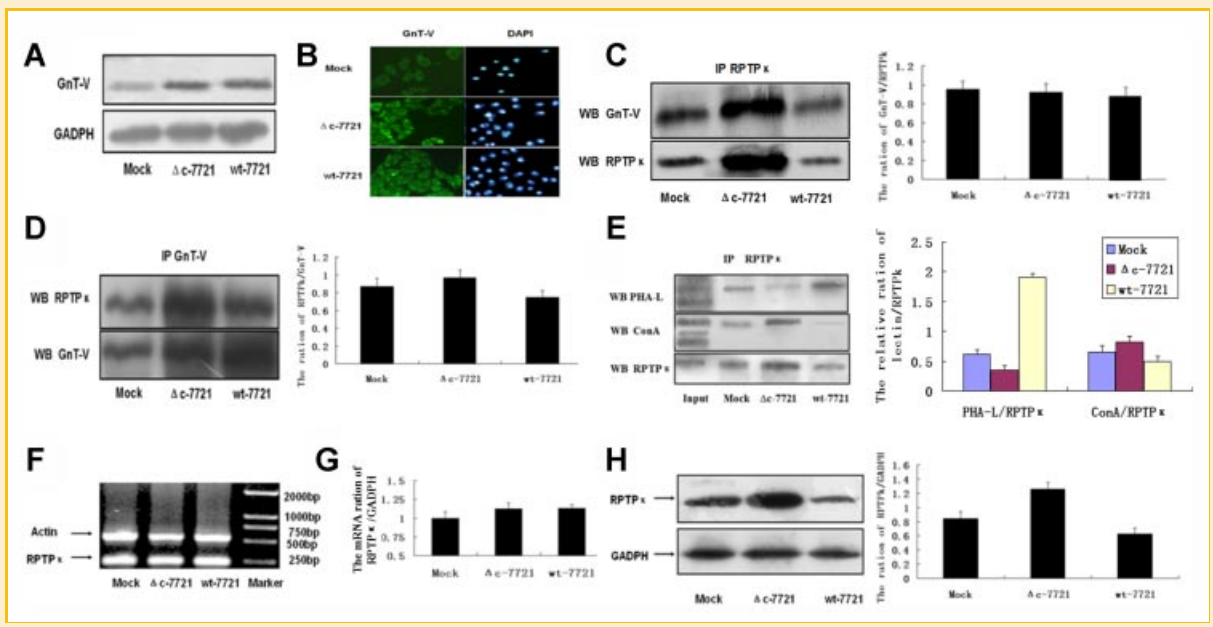

Fig. 1. GnT-V overexpression altered the glycosylation and protein level of RPTPκ. A: The overexpression of exogenous wtGnT-V and ΔcGnT-V was detected by Western blotting assay using anti-GnT-V antibody. The band of GADPH was used as the loading control. B: The transfection of exogenous wtGnT-V and ΔcGnT-V plasmids was detected by immunofluorescence microscopy. Stable transfectants were immunostained by using anti-GnT-V antibody followed by FITC-anti-rabbit secondary antibody (left panels). Nuclear staining with 4,6-diamidino-2-phenylindole (DAPI) was shown in the right panels. C: Immunoprecipitation (IP) was performed to detect the association of GnT-V with RPTPκ. The protein was immunoprecipitated with anti-RPTPκ antibody, and then was detected using the anti-GnT-V antibody. D: The protein was immunoprecipitated with anti-GnT-V antibody, and then was detected using the anti-RPTPκ antibody. E: Effect of GnT-V overexpression on the glycan structure of RPTPκ. The glycan structure of RPTPκ in Mock, Δc-7721, and wt-7721 cells was detected using immunoprecipitation as described under the Materials and Methods Section. ConA specifically binds to high-mannose type glycans, and PHA-L strongly recognize tri- and tetra-antennary complex type *N*-glycans, specific for β1,6 GlcNAc branching. IP, immunoprecipitation; WB, Western blotting. The graph of right panel represents the PHA-L and ConA activity of RPTPκ. The assays were performed in triplicate measurements of the same sample. The data represent the mean ± SD of three independent analyses. F: GnT-V did not alter the mRNA level of RPTPκ. Total RNA was isolated from three stable cells, and transcripts for the RPTPκ were quantified by RT-PCR. Sense primer is 5′-gcgagtcaagttatcaaacc-3′ and anti-sense primer is 5′-taagcaggcttctaaaatgg-3′. β-actin was used as a loading control. G: The mRNA level of RPTPκ was determined by real-time PCR. The levels of the target genes were standardized against those of the GAPDH gene detected in the identical cDNA samples. The results were expressed as averages of duplicate assays of each transfectant; error bars indicated variations from the mean. H: GnT-V decreased the protein level of RPTPκ. The protein level of RPTPκ in three stable transfectants was measured by Western blotting. The band of GADPH was used as the loading control. The graph (right panel) represented the quantitative analyses of the protein expression of RPTPκ based on densitometry in the three cell lines. The results (mean ± SD) of three independent experiments are shown. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

performed the immunoprecipitation and reciprocal immunoprecipitation assay. The results showed that RPTPκ was associated with GnT-V in three stable cell lines (Mock, wt-7721 cells, and Δc-7721 cells), and there was no significant difference in association ability among three stable transfectants (Fig. 1C,D). The reaction products of GnT-V, β1,6 branches of tri- or tetra-antennary sugar chains, were analyzed by the PHA-L lectin, which preferentially recognizes β1,6 branches of *N*-glycan in the stable transfectants. And the substrate of GnT-V was analyzed by the ConA lectin that binds to high mannose N-linked oligosaccharides [Guo et al., 2002; Ihara et al., 2002]. RPTPκ from the Δc-7721 cells showed fainter PHA-L but stronger ConA activity than did Mock. Nevertheless, the activity of PHA-L was higher, but that of ConA was lower in wt-7721 cells than that in Mock (Fig. 1E).

The RT-PCR results showed that the mRNA levels were similar among the three stable cell lines (Fig. 1F). Moreover, the results from real-time PCR also showed that GnT-V did not alter the mRNA level of RPTPκ (Fig. 1G). The protein level of RPTPκ decreased in wt-7721 cells, but increased in Δc-7721 cells compared with Mock's (Fig. 1H). The results of the quantitative analyses based on densitometry showed that the protein level of RPTPκ decreased approximately by 50% in wt-7721 cells, but increased by threefold in Δc-7721 cells compared with in Mock's (Fig. 1H, right lane).

RPTPκ WAS INVOLVED IN THE DOWN-REGULATION OF CALCIUM-INDEPENDENT CELL–CELL ADHESION INDUCED BY GnT-V

We found that the calcium-independent cell–cell adhesion decreased significantly in wt-7721 cell line, but increased in Δc-7721 cell line compared with Mock's (Fig. 2A). Then, we examined whether the decreased adhesive ability was associated with the RPTPκ. The wild-type RPTPκ plasmid transfected transiently into Mock and wt-7721 cell line, the overexpression of RPTPκ was confirmed by Western blotting (Fig. 2B). The protein level of RPTPκ increased approximately by two times in wt-7721 cell line, about onefold in Mock when RPTPκ was overexpressed (Fig. 2B, right lane). We further found that the adhesive ability of Mock and wt-7721 both increased at three periods of 30, 60, and 90 min when wild-type RPTPκ plasmid was overexpressed (Fig. 2C), suggesting that RPTPκ involved in the adhesive change in human hepatoma SMMC-7721cell lines. Then, we knocked the RPTPκ gene down and found that the RPTPκ's levels decreased upon RPTPk siRNA treatment in Mock and wt-7721 cells. The results based on densitometry showed that the RPTPκ's protein level decreased approximately by two times in wt-7721 cells, and by about three times in Mock when RPTPκ was knocked down (Fig. 2D). And the aggregation ability of the two cell lines was found to have decreased

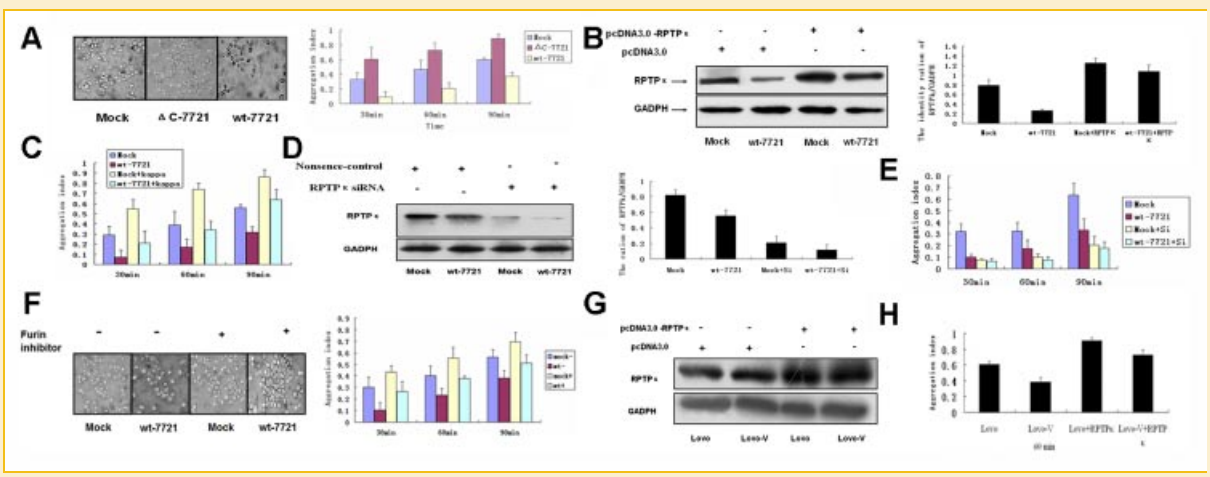

Fig. 2. RPTPκ was involved in the down-regulation of calcium-independent cell–cell adhesion induced by GnT-V. A: GnT-V overexpression decreased cell–cell calcium-independent aggregation ability. Mock, Δc-7721, and wt-7721 cells were removed from culture plates and separated into single cells, followed by constant agitation in EGTA-containing media at 37°C for various time periods (30, 60, and 90 min). An aliquot of each suspension was examined by light microscopy after agitation. The assay was repeated three times, and then the means and SDs were determined. The figures are representative of all experiments performed. B: Wild-type RPTPκ plasmid and empty vector were, respectively, transiently transfected into Mock and wt-7721 cells, and then the protein level of RPTPκ was detected by Western blotting assay using anti-RPTPκ extracellular domain antibody. The figures are representative of all experiments performed. The graph (right panel) represents the quantitative analyses of the RPTPκ protein level based on densitometry, and the data represent the mean ± SD of three independent analyses. C: RPTPκ overexpression increased the calcium-independent cell adhesion. Wild-type RPTPκ plasmid or empty vector transfected cells was allowed to aggregate in different periods of time (30, 60, and 90 min) at 37°C with constant shaking at 80 rpm. The figure was based on the data representing the mean ± SD of three independent analyses. D: RPTPκ siRNA specifically reduced RPTPκ's protein level. Mock and wt-7721 cells transfected with synthetic double-stranded control (−) or RPTPκ siRNA (+), the protein level of RPTPκ was detected by Western blotting with anti-RPTPκ antibody. The figures are representative of three experiments performed. The graph (right panel) represents the quantitative analyses of the RPTPκ protein level based on densitometry, and the data represent the mean ± SD of three independent analyses. E: RPTPκ siRNA specifically decreased calcium-independent cell adhesion. Following the silence of RPTPκ gene, calcium-independent cell adhesion was detected by aggregation assay as described under the Materials and Methods Section. The assay was repeated three times, and then the means and SDs were determined. F: Furin inhibitor increased cell adhesion. In the presence or absence of furin inhibitor, calcium-independent cell adhesion was detected by aggregation assay as described under the Materials and Methods Section. The assay was repeated three times, and then the means and SDs were determined. The figures are representative of all experiments performed. G: Wild-type RPTPκ plasmid transiently transfected into Lovo and Lovo-V cell, the level of RPTPκ was detected by Western blotting using anti-RPTPκ extracellular domain antibody. H: The overexpression of RPTPκ increased the aggregation of Lovo and Lovo-V cell. Calcium-independent cell adhesion was detected by aggregation assay as described under the Materials and Methods Section. The assay was repeated three times, and then the means and SDs were determined. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

when RPTPκ gene was knocked down (Fig. 2E), which further confirmed that RPTPκ played a critical role in regulating cell adhesive ability.

It is reported that RPTPκ has an RXKR motif, which is a favored cleavage site for furin [Campan et al., 1996]. Accordingly, furin inhibitor was used to explore the mechanism that RPTPκ regulated cell adhesion in three stable transfectants. Our previous reports had showed that the protein level of RPTPκ increased significantly in wt-7721 cells as well as in Mock following the treatment of furin inhibitor, suggesting that RPTPκ catalyzed by GnT-V was easier to be cleaved by furin, resulting in the decreased level of RPTPκ [Wang et al., 2009]. Also we found that the adhesive ability of the two cell lines increased in the presence of furin inhibitor compared with that in its absence, which could be due to the increased protein level of RPTPκ when furin was inhibited. Moreover, the furin inhibited, the adhesive ability of wt-7721 cells was found to be lower than that of Mock (Fig. 2F). Since furin can affect many proteins, including RPTPκ, we chose Lovo cell devoid of furin in our study. Wild-type GnT-V transfected into Lovo cell to be designated as Lovo-V cell. To explore the effect of RPTPκ on cell adhesion, we transfected transiently RPTPκ's plasmid into Lovo and Lovo-V cell. The overexpression of exogenous RPTPκ plasmid was confirmed by the means of Western blotting (Fig. 2G) and then we found that the cell adhesion ability was stronger when RPTPκ was overexpressed (Fig. 2H), which further confirmed that RPTPκ's cleavage could be responsible for the effect of GnT-V on cell adhesion.

RPTPκ WAS INVOLVED IN THE UP-REGULATION OF β-CATENIN'S TYROSINE PHOSPHORYLATION LEVEL INDUCED BY GnT-V

We found that the tyrosine phosphorylation of β-catenin was higher in wt-7721 cells but lower in Δc-7721 cells than in Mock, and the results of the quantitative analyses showed that the tyrosine phosphorylation level of β-catenin increased approximately two times in wt-7721 cells, but decreased by 60% in Δc-7721 cells compared with in Mock's (Fig. 3A), suggesting that GnT-V could perform an elevating function.

The previous reports that RPTPκ could interact with β-catenin prompted us to investigate the role of RPTPκ in the change of β-catenin's tyrosine phosphorylation level induced by GnT-V [Fuchs et al., 1996]. As shown in Figure 3B, RPTPκ siRNA increased the tyrosine phosphorylation of β-catenin in Mock and wt-7721 cell lines (Fig. 3B), suggesting that the gene might play a vital role in regulating the tyrosine phosphorylation of β-catenin. Moreover, we found that the overexpression of RPTPκ by gene transfer was associated with reduced β-catenin's tyrosine phosphorylation level (Fig. 3C), which further proved this crucial role of RPTPκ.

Further findings showed that the tyrosine phosphorylation of β-catenin decreased when furin was inhibited, which could be due to the inhibition of RPTPκ's cleavage (Fig. 3D). Moreover, the tyrosine phosphorylation of β-catenin was higher in Lovo cell than in Lovo-V cell (Fig. 3E), suggesting that GnT-V overexpression increased the phosphorylation level of β-catenin. With the RPTPκ's overexpression, the phosphorylation level of β-catenin decreased in Lovo and Lovo-V cell (Fig. 3E), suggesting that RPTPκ might play a critical role in the changes induced by GnT-V.

RPTPκ WAS INVOLVED IN THE REGULATION OF β-CATENIN'S DOWNSTREAM SIGNALING

And we found that the nucleus level of β-catenin increased in wt-7721 cells but decreased in Δc-7721 cells compared with Mock's; the cytoplasmatic level of β-catenin was both increased in Δc-7721 and wt-7721 cells, indicating that both nuclear and cytoplasmic β-catenin increased in wt-7721 cells (Fig. 4A). The different cytoplasmatic and nuclear distribution of β-catenin in three stable cells was also proved by immunofluorescence microscopy. The green dots, representing β-catenin, were dispersed inside the cytoplasm and detectable also in cellular area where nuclei were likely positioned in the three stable transfectants. Compared with Mock, the nuclear amount of green dots was significantly increased in wt-7721 cells but decreased in Δc-7721 cells (Fig. 4B), which was consistent with the results of Figure 4A. Reduction of endogenous RPTPκ protein by RPTPκ siRNA increased the nuclear amount of β-catenin in Mock and wt-7721 cells. Moreover, the nucleus level of β-catenin was higher in wt-7721 than in Mock. Accumulation of nuclear β-catenin was associated with increase in nuclear levels of c-myc and cyclin D1 whose genes are regulated by β-catenin/TCF/LEF transcription complex [Mazieres et al., 2005]. The changes of c-myc and cyclin D1 were consistent with those of β-catenin in cell nucleus (Fig. 4C). Upon overexpression of RPTPκ gene, the nucleus and cytoplasmic level of β-catenin decreased both in Mock and wt-7721 cells (Fig. 4D). Moreover, we found that the overexpression of RPTPκ decreased the cytoplasmic level but increased the membrane level of β-catenin in Mock and wt-7721 cells (Fig. 4E). Treated with furin inhibition, Mock and wt-7721 cells had lower nucleus amount of β-catenin protein. Moreover, the change of c-myc and cyclin D1 in the nucleus of Mock and wt-7721 was consistent with that of β-catenin in the presence or absence of furin (Fig. 4F).

In short, these results suggested that GnT-V could regulate β-catenin downstream signaling by RPTPκ.

GnT-V OVEREXPRESSION PROMOTED CELL PROLIFERATION

Since cyclin D1 and c-myc, proliferation-related genes, overexpressed in wt-7721 cells, we explored the proliferation ability of the three stable transfectants (Mock, wt-7721, and Δc-7721). The results from MTT assay indicated that the speed of cell proliferation was faster in wt-7721 but slower in Δc-7721 cell line than that in Mock (Fig. 5A); those from clone assay showed that the mass of clone from wt-7721 was larger, but that from Δc-7721 cells was smaller than that of Mock, and that the number of wt-7721 clone was more, but that of Δc-7721 was smaller than that of Mock (Fig. 5B,C), suggesting that GnT-V could promote cell proliferation due to RPTPκ, thereby activating the signaling of β-catenin and downstream.

DISCUSSION

Previous reports have shown that the level and function of glycoproteins such as β1 integrin [Wang et al., 2007], matriptase [Ihara et al., 2002], and TIMP-1 [Kim et al., 2008] can be affected by GnT-V, which accelerates tumor progression. In our study, we found

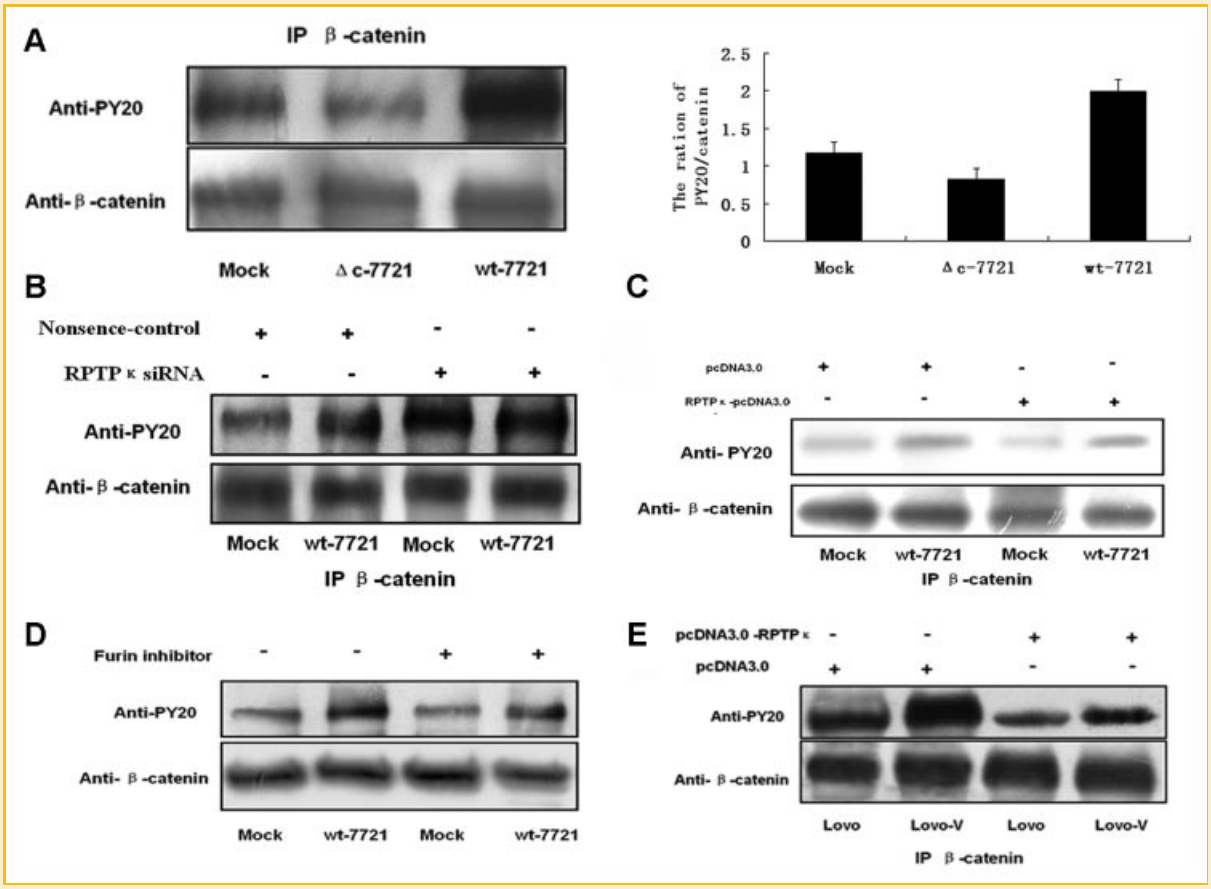

Fig. 3. RPTPκ was involved in the up-regulation of β-catenin's tyrosine phosphorylation level induced by GnT-V. A: GnT-V increased the tyrosine phosphorylation of β-catenin. The tyrosine phosphorylation of β-catenin was detected using the anti-PY20 antibody in the three stable transfectant (Mock, Δc-7721, and wt-7721 cells). The results (mean ± SD) of three independent experiments are shown (right panel). B: The knockdown of RPTPκ increased the tyrosine phosphorylation of β-catenin. Mock and wt-7721 cells transfected with synthetic double-stranded control (−) or RPTPκ siRNA (+), and then the tyrosine phosphorylation of β-catenin was detected using anti-PY20 antibody. C: The overexpression of RPTPκ decreased β-catenin tyrosine phosphorylation. Mock and wt-7721 cells transfected with pcDNA 3.0 or RPTPκ plasmid, the β-catenin tyrosine phosphorylation was detected using anti-PY20 antibody. D: In the presence or absence of furin inhibitor, the protein was immunoprecipitated with anti-β-catenin, and then the tyrosine phosphorylation of β-catenin was observed by means of Western blotting with anti-PY20 antibody. IP, immunoprecipitation. E: The overexpression of RPTPκ decreased the phosphorylation level of β-catenin in Lovo and Lovo-V cell. Wild-type RPTPκ plasmid transiently transfected into Lovo and Lovo-V cell, and the tyrosine phosphorylation of β-catenin was detected by the immunoprecipitation as above.

that GnT-V could decrease human hepatoma SMMC-7721 cell adhesion and promote cell proliferation through RPTPκ.

Kim et al. [2006] reported that RPTPκ was the substrate of GnT-V in colon cancer. In our study, we found that RPTPκ was associated with GnT-V directly in human hepatoma SMMC-7721 cell. Our previous results showed that RPTPκ's oligosaccharides had richer β1,6 branches in wt-7721 cells [Wang et al., 2009]. In this report, we chose Δc-7721 cells where the endogenous GnT-V was inactive to further explore the effect of GnT-V on the glycan structure of RPTPκ. Our results further proved that GnT-V enriched β1,6 branches onto the oligosaccharides of RPTPκ in human hepatoma SMMC-7721 cell. The addition of β1,6 branches to the oligosaccharides was one of the key factors in affecting the protein level and biological function of glycoprotein [Ihara et al., 2002; Li et al., 2007; Kariya et al., 2008; Kim et al., 2008; Yang et al., 2008]. In our study, we also found that the addition of β1,6 branches to the *N*-glycosylated RPTPκ decreased its protein level. The introduction of ΔcGnT-V into the SMMC-7721 cells caused the increased protein level, which could result from the lack of β1,6 GlcNAc branches suppressed by ΔcGnT-V. Therefore, we concluded that GnT-V could decrease the protein level of RPTPκ through the modification of its *N*-glycosylation. Next, we sought to the mechanistic explanation that the addition of β1,6 branches to the *N*-glycosylated RPTPκ decreased its protein level. The results from RT-PCR and real-time PCR showed that the mRNA level of RPTPκ was not affected by GnT-V, which might exclude the contributing factor as altered RPTPκ mRNA level expression. It is reported that RPTPκ has an RXKR motif that is a favored cleavage site for furin protease [Campan et al., 1996]. Furin is not the protease that directly induces extracellular shedding of RPTPκ. Cleaved by furin, RPTPκ undergoes proteolytic processing via ADAM10, hence its shedding from cell surface [Handerson et al., 2005]. The proteolytic processing via ADAM10 would not process without the first cleavage by furin. Therefore, we used furin inhibitor to explore this mechanistic explanation. Our previous results had gave a hint that RPTPκ with enriched β1,6 N-linked oligosaccharides by GnT-V was easier

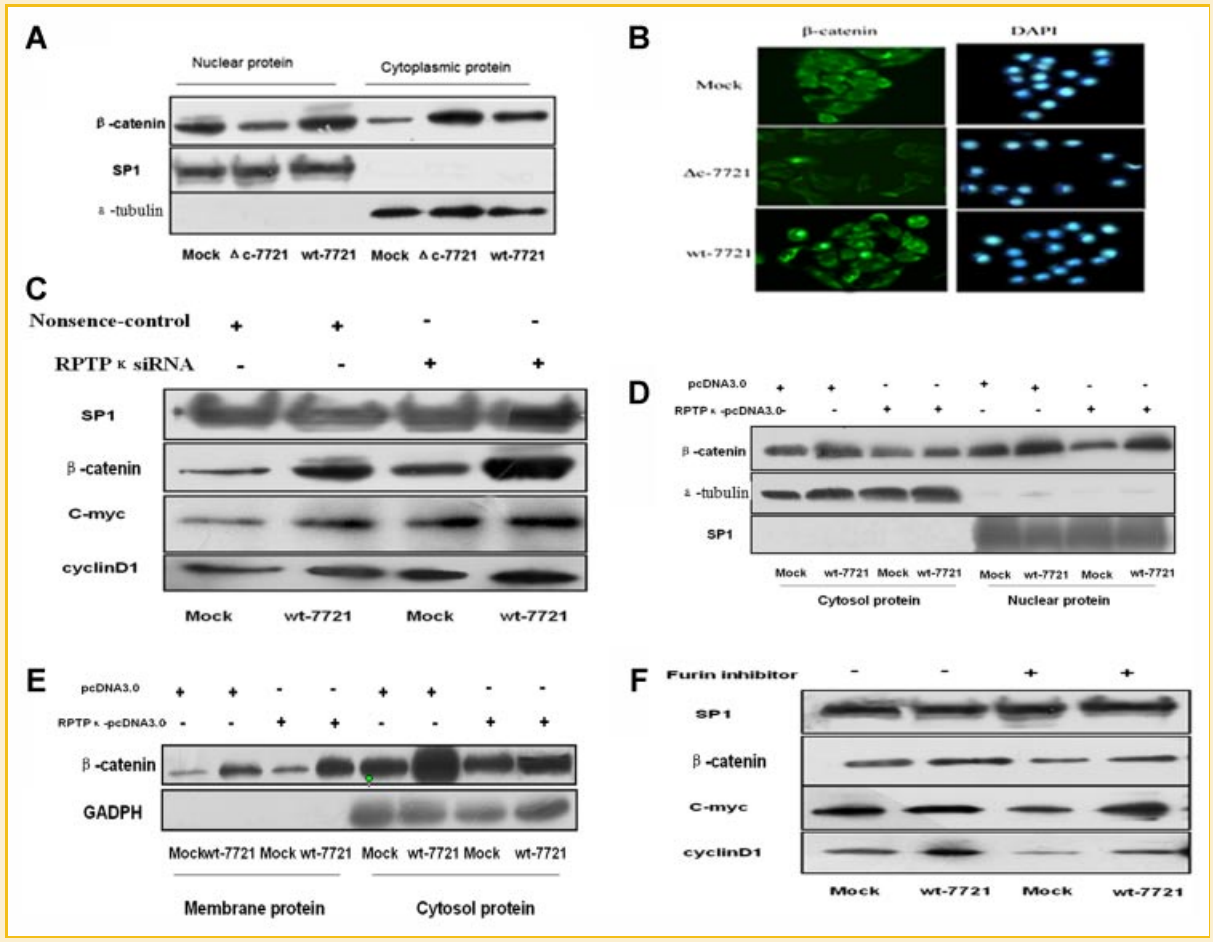

Fig. 4. RPTPκ was involved in the regulation of β-catenin's downstream signaling. A: The nucleus and cytoplasm protein of three stable transfectants were extracted, respectively, and the protein amounts of β-catenin in nucleus and cytoplasm were detected using Western blotting. The protein α-tubulin was used as the cytoplasmic control, and SP1 was used as the nucleus control. B: Stable transfectants (Mock, Δc-7721, and wt-7721 cells) were immunostained by using anti-β-catenin antibody followed by FITC-anti-mouse secondary antibody (left panels). Nuclear staining with 4,6-diamidino-2-phenylindole (DAPI) was shown in the right panels. Magnification, 250×. C: The knockdown of RPTPκ increased the nucleus level of β-catenin, cyclin D1, and c-myc. Following the silence of RPTPκ gene, the nucleus protein was extracted by the means as described under the Materials and Methods Section. Then, the nucleus level of β-catenin, c-myc, and cyclin D1 was observed using Western blotting. SP1 was used as a loading control for nucleus protein. D: The overexpression of RPTPκ decreased the nucleus and cytoplasmic level of β-catenin. Following the overexpression of RPTPκ gene, the protein amounts of β-catenin in nucleus and cytoplasm were detected using Western blotting. The protein α-tubulin was used as the cytoplasmic control, and SP1 was used as the nucleus control. E: The overexpression of RPTPκ increased the membrane protein level of β-catenin. With the overexpression of RPTPκ, the membrane and cytoplasmic protein was detected using Western blotting. F: In the presence or absence of furin inhibitor, the nucleus protein was extracted, and then the nucleus level of β-catenin, c-myc, and cyclin D1 was observed using Western blotting. SP1 was used as a loading control for nucleus protein. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

to be cleaved by furin, resulting in the decreased level of RPTPκ [Wang et al., 2009]. For Δc-7721 cells, RPTPκ protein level is increased while its mRNA level is unaltered, which could result from RPTPκ with the lack of β1,6 GlcNAc branches was difficult to be cleaved by furin. In addition, some reports showed that the addition of β1,6 branches of glycan protein affected the protein stability [Wang et al., 2007]. We performed the cycloheximide (Chx) treatment experiments, and the results showed that the overexpression of GnT-V did not affect the stability of RPTPκ (Fig. 6). Therefore, the mechanistic explanation could be that aberrant glycosylated RPTPκ with more β1,6 branches was easier to be cleaved by furin. However, why and how the aberrant glycosylated RPTPκ was prone to be cleaved by furin remains to be addressed.

Afterwards, we were employed to explore the role of RPTPκ in altered biological behavior induced by GnT-V. Our results showed that the overexpression of GnT-V decreased cell calcium-independent adhesion. Previous data have showed that RPTPκ was involved in homotypic adhesion [Zondag et al., 1995], which gave us a hint whether RPTPκ was involved in the altered adhesion induced by GnT-V. We overexpressed and knocked down RPTPκ gene to address this question. In order to exclude the effect of E-cadherin and N-cadherin on cell adhesion, we treated Mock and wt-7721 cells with EGTA, calcium chelator, and then performed calcium-independent aggregation assay. Our results showed that cell adhesive ability increased with RPTPκ overexpression but decreased with RPTPκ knockdown, suggesting that RPTPκ played an important role in regulating cell adhesion.

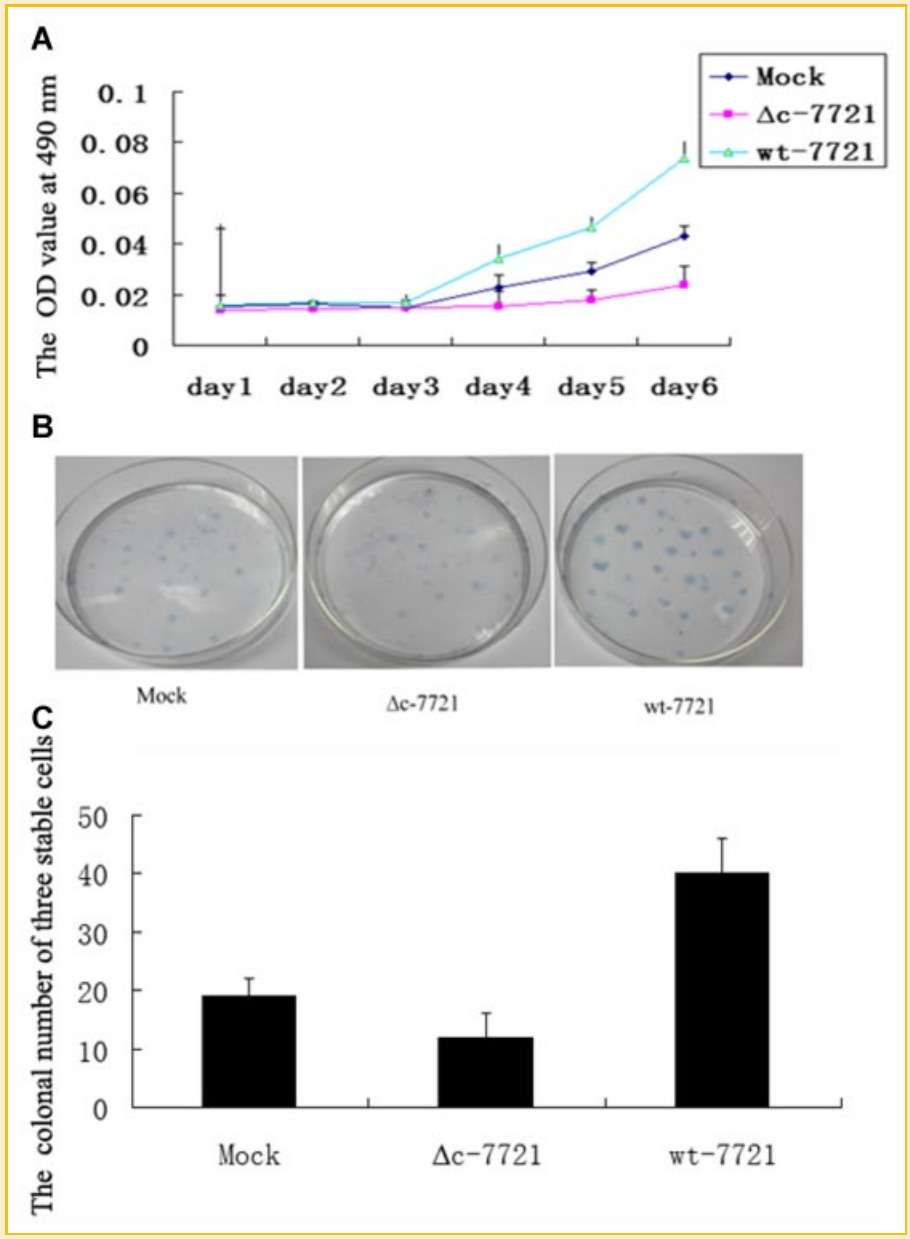

Fig. 5. GnT-V promoted cell proliferation. A: The growth curves of the three stable cells were gained by the MTT assay. Each measurement was the mean of the absorbency value at 490 nm from six wells. The assay was repeated three times, and the means and SDs were determined. B: The colony formation of the three stable cells was photographed after the culturement for 15 days. C: The number of clone was counted in the three stable cells after the culturement for 15 days. The data were expressed as the mean of three experiments separately. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

And also we found that the overexpression of GnT-V increased the tyrosine phosphorylation of β-catenin. Since RPTPκ was one of the factors to dephosphorylate β-catenin [Fuchs et al., 1996; Novellino et al., 2008], the question that whether GnT-V increased the tyrosine phosphorylation of β-catenin via RPTPκ was worth addressing. Our results showed that RPTPκ played an important role in regulating the β-catenin tyrosine phosphorylation induced by GnT-V. β-catenin transmits Wnt signals to the nucleus as a crucial role in tumorigenesis through the regulation of oncogenes including cyclin D1 and c-myc [Mazieres et al., 2005]. In our study, we found that the nucleus level of β-catenin, c-myc, and cyclin D1 increased when RPTPκ gene was knocked down. The possible mechanistic explanation was that the increased tyrosine phosphorylation of β-catenin promoted the protein into the nucleus, thereby activating the signaling of downstream.

Afterwards, we used furin inhibitor to prevent RPTPκ cleavage and found that the adhesive ability was higher and the phosphorylation level of β-catenin was lower in the presence of furin inhibitor than that in furin absence, suggesting that furin cleavage could be one of the causes for RPTPκ to regulate cell adhesion and β-catenin signaling in GnT-V overexpression cell lines. Since furin can affect other proteins, we chose Lovo cell

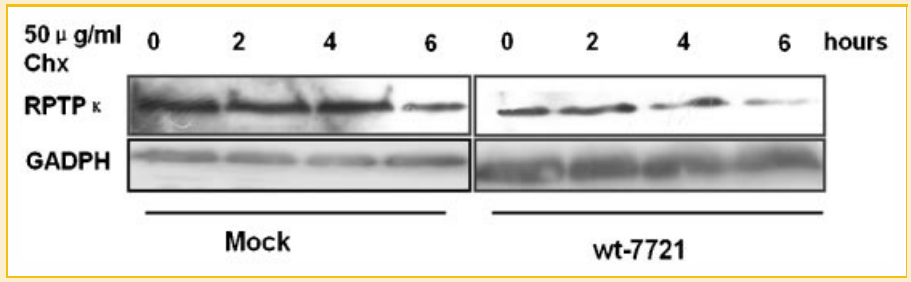

Fig. 6. GnT-V did not influence RPTPκ protein stability. Mock and wt-7721 cells were trypsinized and then replated. Twelve hours after replating, cells were treated with 50 μg/ml cycloheximide (Chx) for the indicated intervals (0, 2, 4, and 6 h). The stability of RPTPκ protein was detected by Western blotting in the two cells. The results showed that the half-life of RPTPκ protein was 4 h in Mock and wt-7721 cells.

devoid of furin to further prove our hypothesis. We first transfected wild-type GnT-V into Lovo cell and found that GnT-V over-expression did not affect mRNA and the protein level of RPTPκ, which could result from the absence of furin. Also we found that GnT-V overexpression decreased cell adhesive ability but increased the tyrosine phosphorylation level of β-catenin, which was consistence with the aforementioned results. To confirm that preventing RPTPκ's cleavage was important for RPTPκ to regulate cell adhesion and β-catenin signaling, we transfected wild-type RPTPκ plasmid into Lovo and Lovo-V cell and found that the cell adhesion ability increased and phosphorylation level of β-catenin decreased when RPTPκ was overexpressed, which further proved our hypothesis. In addition, our results indicated that the adhesive ability was lower and the phosphorylation level of β-catenin was higher in wt-7721 cells than in Mock when furin was inhibited, which brought a hypothesis whether the addition of β1,6 branches to *N*-glycosylated RPTPκ attenuated its function. We will address the question in the future.

In our study, we also found that GnT-V promoted cell proliferation, whose possible mechanistic explanation was that GnT-V increased the tyrosine phosphorylation of β-catenin via RPTPκ, thus activating the expression of oncogene such as cyclin D1 and c-myc, suggesting that RPTPκ was one of the important factors in regulating tumor progression.

In summary, we conclude that GnT-V-mediated cell adhesion and the phosphorylated level of β-catenin via RPTPκ and preventing RPTPκ's degradation could rescue these effects of GnT-V.

ACKNOWLEDGMENTS

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