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Functional analysis of β1 integrin and its *N*-glycosylation on cell proliferation and migration

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Contents

Summary

Integrins are a large family of heterodimeric glycoprotein that function as adhesion molecules and play key roles in many biological processes. β1 integrin, which constitutes the largest subgroup of integrins, is aberrantly expressed in human breast carcinoma and contributes to diverse malignant phenotypes, including epithelial-to-mesenchymal transition (EMT), metastasis, and angiogenesis. Due to its multiple important roles in cancer, a better understanding of β1 is critical for the development of efficacious treatments for cancer. So firstly, we compared cell behaviors between normal and deletion of β1 gene MDA-MB-231 cells. As the result shown in part 1, we found β1 exhibited opposite effects on cell proliferation dependent on cell densities, and β1 was essential for the abilities of cell survival and migration.

As an *N*-glycosylated protein, the *N*-glycosylation of integrin β1 is involved in diverse biological functions including cell adhesion, cell migration and trans-membrane signaling. However, most studies have focused mainly on the entire alteration in *N*-glycosylation and little is known about the function of individual *N*-glycans on integrin β1. Given the fact that β1 contains 12 potential *N*-glycosylation sites, during which our laboratory have previously identified that three potential *N*-glycosylation sites on the I-like domain of β1, No. 4, 5, and 6, are essential for the α5β1 heterodimer formation, cell surface expression and cellular function. So we hypothesized that the other *N*-glycosylation sites on β1 subunit may also have unique functions. As shown in part 2, we found that activation of β1, β1-mediated complex formation and cellular signaling were strictly regulated by *N*-glycosylation on the membrane-proximal region of β1. In addition, the structures of *N*-glycans were also important for the functions, since the silence of α2,6-sialylation significantly decreased the β1 activation and cell membrane complex formation.

Part 1: Distinct effects of β1 integrin on cell proliferation and cellular signaling in MDA-MB-231 breast cancer cells.

In order to elucidate the biological functions of β1, we compared cell behaviors between wild type (WT) and knockout of β1 gene (KO) MDA-MB-231 cells. We found that the expression of β1 exhibited opposite effects on cell proliferation. These effects were dependent on cell densities, and they showed an up-regulation of cell proliferation when cells were cultured under sparse conditions, and a down-regulation of cell growth under dense conditions. By comparison with WT cells, the phosphorylation levels of ERK in

KO cells were consistently suppressed under sparse culture conditions, but consistently up-regulated under dense culture conditions. The phosphorylation levels of epidermal growth factor receptor (EGFR) were increased in the KO cells. By contrast, the phosphorylation levels of AKT were decreased in the KO cells. The abilities for both colony and tumor formation were significantly suppressed in the KO cells, suggesting that β1 plays an important role in cell survival signaling for tumorigenesis. In addition, we also found that loss of β1 resulted in cell-cell adhesion, decreased cell migration. These phenotypes in the KO cells were restored in the rescue cells (Res). Furthermore, the inhibitory effectiveness of AG1478, an EGFR inhibitor, for cell proliferation was greatly increased in the KO cells as well as in the WT cells pretreated with anti-β1 antibody. These results clearly showed the distinct roles of β1 in cancer cells — the inhibition of cell growth, the promotion of cell survival and migration, which may shed light on cancer therapies.

Part 2: The importance of membrane-proximal *N*-glycosylation on integrin β1 in its activation and complex formation.

Considering that the *N*-glycosylation of integrin α5β1 plays important roles in its dimer formation, cell adhesion and migration, we further investigate the function of WT cells and the S4-6 mutant, which expresses mutant integrin β1 bearing only three *N*-glycosylation sites within I-like domain essential for β1 heterodimer formation with integrin α5. We showed that despite the little difference in the expression levels of α5β1 on the cell surface between WT cells and the S4-6 mutant, the cell spreading and migration ability were significantly decreased in S4-6 mutant cells. Consistent with these phenotypes, the integrin β1-mediated cellular signaling and its activation were shown to be clearly suppressed in the S4-6 mutant cells. These observations, interestingly, could be only rescued by restoration of the *N*-glycosylation sites in membrane-proximal domain (S9-12), but not others. Further study on the regulatory mechanisms suggested that the membrane-proximal *N*-glycosylation was critical for intermolecular interactions between integrin β1 and other cell membrane proteins. Moreover, the expression level of active β1 was also rescued in the S4–6+7-8 cells, although the cell migration could not be rescued as described above. Considering the fact that β-galactoside α2,6-sialyltranferase 1 (ST6GAL1)-mediated sialylation plays vital roles in β1 function, we checked the α2,6-sialylation levels in these mutants. The results showed the levels of the sialylated β1 and α5 were clearly higher in the S4–6+7-8 and S4–6+9-12 mutant cells than that in other mutant cells. These results demonstrated that the terminal α2,6-sialic acids on the membrane-proximal *N*-glycans

catalyzed by ST6GAL1 was also required for the integrin β1 activation.

Overall, the results of the two parts suggest integrin β1 plays important roles in regulating cell proliferation and migration, and the *N*-glycosylation closed to the cell membrane on integrin β1 may serve as a platform, which facilitates its complex formation on the cell membrane, thereby affecting integrin-mediated functions.

Reference

1. Hou S., Isaji T., Hang Q., Im S., Fukuda T., Gu J.. Distinct effects of β1 integrin on cell proliferation and cellular signaling in MDA-MB-231 breast cancer cells. ***Sci Rep.*** 2016 Jan 5;6:18430.
2. Hou S., Hang Q., Isaji T., Lu J., Fukuda T., Gu J.. The importance of membrane-proximal *N*-glycosylation on integrin β1 in its activation and complex formation. ***FASEB J.*** 2016 Dec;30(12):4120-4131.

Part 1

Distinct effects of β1 integrin on cell proliferation and cellular signaling in MDA-MB-231 breast cancer cells

1.1 Introduction

Integrins comprise a group of transmembrane heterodimeric proteins consisting of α and β subunits (1) that drive most of the interactions between cells and the extracellular matrix (ECM). β1 integrin, which constitutes the largest subgroup of integrins, is aberrantly expressed in human breast carcinoma and contributes to diverse malignant phenotypes, including EMT, metastasis, and angiogenesis (2-4). In addition to the roles of β1 integrin in cancer progression, growing evidence has highlighted its relationship with tumor resistance to therapeutic modalities (5, 6). Due to its multiple important roles in breast cancer, the targeting of β1 is a promising strategy that can enhance therapeutic outcomes.

Several experimental models have shown that targeting β1 could partly attenuate aggressive tumor phenotypes in three-dimensional cell cultures and human breast cancer xenografts (7-9). However, the effects of β1 on cell proliferation and cell survival in breast cancer cells are controversial, and the underlying mechanisms remain unclear. As a positive regulator, treatment with a functional blocking antibody against β1 is known to decrease cell proliferation and induce cell apoptosis (8). In contrast, at least one study found that the functional blocking antibody had no inhibitory effects on cell growth, cell survival or capacity to form colonies in several breast tumor cell lines (10). Therefore, a better understanding of the molecular mechanisms responsible for these differences is critical for the development of efficacious treatments for breast cancer.

The multiple downstream signaling pathways of β1, including focal adhesion kinase (FAK), phosphatidylinositol-3 kinase (PI3K) and mitogen-activated protein kinase (MAPK), coordinating signaling through receptor tyrosine kinases (RTKs), are involved in the modulation of tumor initiation, progression, and ultimately metastasis (4, 11-13). Although ample evidence has demonstrated that β1 plays critical roles in breast cancer, the targeting of β1 by using a monotherapy approach has not shown much benefit. Some possible mechanisms are involved in this phenomenon, such as the activation of intracellular protein kinase signaling pathways (e.g. PI3K and MAPK) and cross-talk between β1 and RTKs (14, 15). These mechanisms provide evidence that the biological events mediated by β1 are not limited to one signaling pathway, which highlights the fact that these signaling networks act dynamically and intersect with each other to control the physiological and pathological responses (14). In addition, the dynamics of β1 signaling is further complicated by the cross-talk with RTKs, which is a crucial event in breast cancer progression (6). Until just recently, the integrin-mediated dynamics of the regulation between different signal pathways have remained largely unknown.

Notably, the correct integration of signals from cell–ECM, cell–cell, and growth factor pathways is pivotal for a wide range of cellular biological functions, while deregulation of these signaling pathways results in a loss of tissue organization and contributes to tumorigenesis and progression (16, 17). β1 integrin integrates signals that maintain a balance of the biological functions in mammary tumor development primarily by appropriate interactions between cell-ECM and cross-talk with EGFR (6). These signal integrations can also be achieved even when other signaling pathways are constitutively deregulated (15, 18). However, the roles of β1 in these processes remain unclear.

To solve these issues, here we investigated the biological functions of β1 in WT, KO and Res MDA-MB-231 cells, and found that β1 exhibited opposite effects on cell proliferation that were dependent on cell densities: up-regulation of cell proliferation when cells were cultured under sparse conditions, and down-regulation of cell growth when cells were cultured under dense conditions. The abilities for cell survival were clearly decreased in KO cells, compared with those in WT and Res cells. Additionally, a treatment with AG1478, an inhibitor of EGFR, could more efficiently inhibit cell proliferation in KO cells than in WT cells. Thus, our study clearly showed the dynamic regulation by β1 for cell behavior, which may provide an underlying mechanism for the possibility of drug resistance due to β1 presence, and highlights the importance of combination treatment including β1 integrin and EGFR.

1.2 Materials and Methods

1.2.1 Antibodies and reagents

The experiments were performed using the following antibodies: Antibody against human integrin β1 subunit (P5D2) was from Developmental Studies Hybridoma Bank, University of Iowa. Mouse mAb against Smad2, rabbit mAbs against EGFR, p-EGFR, ERK1/2, p-ERK1/2, AKT, p-AKT, p-Src, and p-SMAD2 were from Cell Signaling Technology. Rabbit pAb to β4 integrin and goat antibody against α3 integrin were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse mAbs against β1 integrin, α5 integrin, β4 integrin, αv integrin, FAK, pFAK, and rat antibody against α6 integrin were from BD Biosciences. MAb against α-tubulin and α-SMA were from Sigma. Mouse mAbs against Src was from upstate biotechnology. Alexa Fluor® 488 and 647 goat anti-mouse IgG was obtained from Invitrogen (Life Technologies). The peroxidase-conjugate goat antibody against mouse, rabbit and goat IgG were obtained from Promega and Cell Signaling Technology. The TO-PRO-3 was from Molecular Probes; the selective EGFR blocker Tyrphostin AG1478 was obtained from sigma; the Sulfo-EGS was from Thermo Scientific; and, the Quantikine Human EGF Immunoassay kit was from R&D Systems.

1.2.2 Cell culture

The 293T and MDA-MB-231 cell lines were purchased from ATCC. Cells were maintained at 37 °C in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), under a humidified atmosphere containing 5% CO_2, except for the virus production. β1-KO and rescue cells**,** as generated by the process described below, were also maintained in DMEM.

1.2.3 Generation of CRISPR/Cas9-based β1-KO MDA-MB-231 cells

The CRISPR/Cas9-based β1-KO MDA-MB-231 cells were established as described previously (19). Briefly, the sgRNA-specifying oligo sequences spanning human β1 integrin exon 2 (CACCGGAGGAATGTTACACGGCTGC-forward; AAACGCAGCCGTGTAACATTCCTCC-reverse), which were chosen from the human KO library sgRNAs (20) were cloned into the pSpCas9 (BB)-2A-GFP (Addgene plasmid ID: 48138) vector. The plasmid was electroincorporated into the MDA-MB-231 cells according to the manufacturer's instructions. After 72 h of transfection, GFP-positive cells were sorted using the FACSAria II (BD Bioscience). Following about 10 days culture, the β1-positive and GFP-negative cells were sorted another five

times. The β1-KO cells were defined by FACS and western blot analyses as described below.

1.2.4 Establishment of β1 rescued MDA-MB-231 cells

The vector of pENTR-D-Topo-β1 was previously established in our laboratory (21). We then used a Gateway™ cloning System kit (Invitrogen) for getting the expression vectors. Briefly, a LR clonase reaction (Invitrogen) was used to transfer the cDNAs of β1 from the entry vectors into CSII-EF-Rfa. The CSII-EF-Rfa-β1 was cotransfected with pCAG-HIVgp and pCMV-VSV-G-RSV-Rev into 293T cells. After infection for 48 hours, the virus media was collected. The KO cells were infected with the resultant virus for 72 h, and the β1 positive cells were selected using the FACSAria II (BD Biosciences) twice. The stable cell line was used in subsequent studies.

1.2.5 Western blot (WB)

Cells of high, middle and low densities were seeded in 60-mm dishes overnight, then washed with ice-cold PBS and lysed in lysis buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100) with protease inhibitors and phosphatase inhibitors (Nacalai Tesque, Kyoto, Japan) for 30 min. After centrifugation, the supernatants were collected and protein concentrations were determined using a BCA protein assay kit (Pierce). The protein lysates were subjected to SDS-PAGE. After electrophoresis, the proteins were transferred to a PVDF membrane (Millipore). The membrane was detected with primary and secondary antibodies, and the proteins were visualized by Immobilon Western Chemiluminescent HRP Substrate (Millipore) according to the manufacturer's instructions.

1.2.6 Flow Cytometric Analysis

Cells were grown to about 90% confluency and then detached from the culture dishes using trypsin containing 1 mM EDTA, washed with ice cold PBS and stained with the primary antibodies, followed by incubation with Alexa Fluor 647 goat anti-mouse IgG (Invitrogen) for 60 min on ice, respectively. Finally, the cells were washed three times with PBS and analyzed via FACSCalibur flow cytometry (BD Biosciences).

1.2.7 Immunofluorescence Staining

Cells were cultured on a glass-bottom dish, washed with PBS and fixed with ice-cold methanol and permeabilized with 0.2% Triton-X-100. Antibodies against β1 (P5D2), pFAK and β4 were used followed by incubation with anti-mouse Alexa Fluor 488

secondary antibodies (Invitrogen) and Alexa Fluor 546 phalloidin (Invitrogen). Fluorescence images were observed via confocal microscopy using a FluoView FV1000 (Olympus, Tokyo, Japan).

1.2.8 In vitro wound-healing assay

Cells were seeded in a 6-well plate and grown to a confluent monolayer. A "scratch" with a p200 pipet tip was made through the cell layer. After washing with PBS, DMEM containing 2% FBS was added in each well. Wounded areas were photographed under a light microscope at 10× objective after 24 h. All experiments were repeated three times.

1.2.9 RT-PCR for mRNA expression analysis

Total RNA was treated with TRIzol reagent (Invitrogen), and 1 μg of total RNA was reverse-transcribed using a SuperScript III first-strand synthesis system (Invitrogen) according to the manufacturer's instructions. The sequences of the primers used for the PCR (sense and antisense, respectively) were as follows: E-cadherin, 5'-ACGCATTGCCACATACA-3' and 3'- CGTTAGCCTCGTTCTCA-5'; α-SMA, 5'-CCAGCGACCCTAAAGCTTCC-3' and 3'-ACCATCACCCCCTGATGTCTG-5'. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as a control.

1.2.10 Cell growth assay

Cells (3×10^4) were seeded into 60-mm dishes overnight and then serum-starved for 24 h. After starvation, the cells were released with complete media with or without tyrphostin AG1478 (3 μM), an inhibitor of EGFR tyrosine kinase activity, or P5D2 (3 μg), an inhibitory anti-β1 antibody for 48 h. Cells in the same area were photographed in phase contrast at the indicated times, then the numbers of living cells were counted. Cell numbers were normalized to those at 0 h and statistically analyzed.

1.2.11 Soft agar assay

Cells were mixed in 0.33% agarose and layered on top of prepared 0.5% base agar plates in DMEM containing 10% FBS. The agar plates were incubated at 37°C and the cultured media were changed twice weekly. After culture for 20 days, the plates were stained with 0.005% crystal violet, and then colonies were counted from three independent dishes of each sample.

1.2.12 Clonogenic assay

Cells (500/per well) were seeded in a 6-well plate in complete media, incubated at 37°C

and the cultured media were changed twice weekly. After culture for 18 days, the foci were stained with 0.005% crystal violet, and colonies containing more than 50 cells were counted from three independent wells of each sample.

1.2.13 Cell survival in suspension

A 1% base agar was prepared in complete media on the bottom of a 60 mm dish. Cells were collected, washed and plated on top of the agar dishes, and then cultured for 3 days in serum-free media. Cells were collected by centrifugation, stained with trypan blue, and the living cells were counted.

1.2.14 Xenograft assay

The bilateral flanks of the five-week-old female nude mice (Charles River Laboratories, Japan) were injected subcutaneously with indicated cells (1×10^6). Tumor growth was monitored every 3 days. The tumor tissues were harvested after 18 days and their volumes and weights were measured. All experiments involving animals were performed according to protocols approved by the Tohoku Pharmaceutical University Research Ethics Board.

1.2.15 ELISA

WT and KO cells were seeded on uncoated 6-well plates and after 24 h the cells were incubated with serum-free media. After 72 h, the media was collected, and stored at −80 °C until assay. EGF in the media was assayed using a Quantikine® Human EGF Immunoassay kit (R&D Systems), according to a procedure described by the manufacturer.

1.2.16 Chemical cross-linking of EGFR in intact cells

Cells were incubated with 5 mM Sulfo-EGS dissolved in PBS on ice for 2 h and then stopped using 10 mM Tris for 15 min. Cells were then solubilized with lysis buffer and subjected to anti-EGFR to detect both EGFR monomers and dimers.

1.2.17 Statistical analysis

Statistical analyses were performed via a Student's *t* test, using GraphPad Prism5. Results are presented as the mean±s.e.m. Statistical significance was defined as $p < 0.05$ ($^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

1.3 Results

1.3.1 Knockout of the β1 integrin gene altered cell morphology.

To explore the functional significance of β1 integrin, we established β1-KO MDA-MB-231 cells via a CRISPR/Cas9-based approach. Western blot and FACS analysis were performed to confirm the effective knockout of β1 in the cell line (Fig. 1A). As expected, the expression levels of β1-associated major α subunits were significantly decreased, but β4 integrin demonstrated a compensatory up-regulation in KO cells (Fig. 1B). During cell culture, we noticed that the KO cells lost the typical epithelioid morphology of WT cells, and showed a cell aggregation that appeared as a colony with a round shape (Fig. 1C upper panel). This phenomenon was also observed in the breast cancer cell lines treated with a blocking antibody against β1 antibody (15). Consistently, immunofluorescent staining showed that KO cells exhibited cortical actin around the cell surface and decreased the formation of filopodia and lamellipodia (Fig. 1C middle and lower panel). Furthermore, the phosphorylation levels of focal adhesion kinase (FAK), a focal adhesion marker, were significantly decreased, while β4 integrin, a hemi-desmosome marker, showed an increase in the KO cells compared with the results seen in WT cells (Fig. 1D). These results indicated that β1-KO cells induced a disruption of focal adhesions and instead enhanced the cell-cell adherens junctions, which inhibited cell mobility.

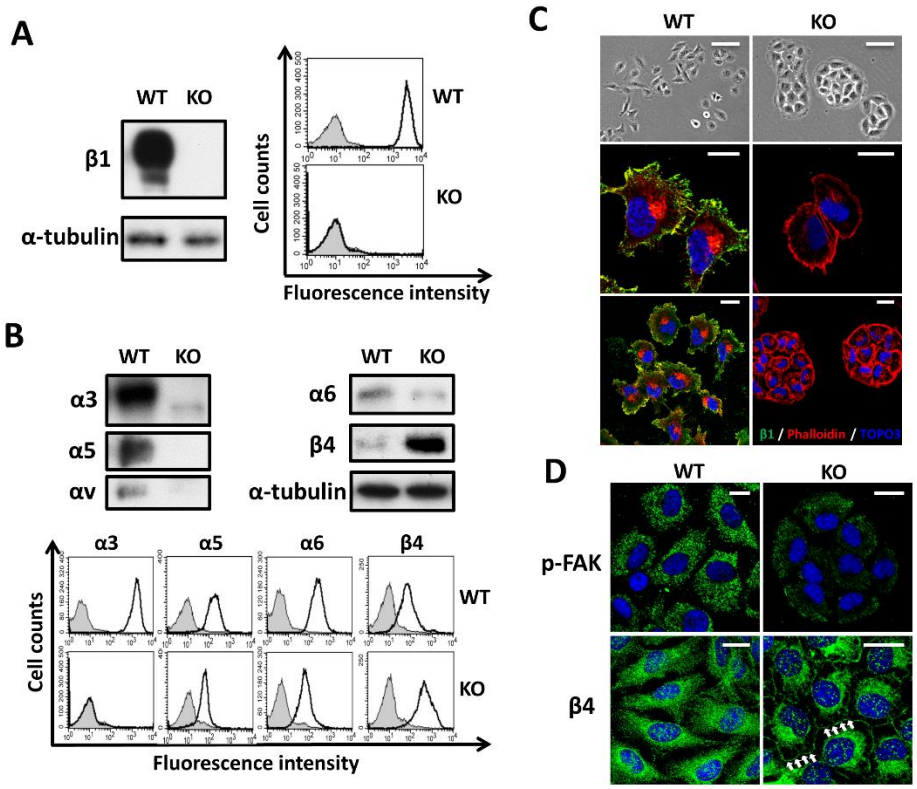

Figure 1. Knockout of β1 in MDA-MB-231 cells altered cell morphology and the expressions of other integrin subunits. (A) WT and KO cells were immunoblotted by anti-β1, α-tubulin was used as a loading control (left panel). WT and KO cells were collected and incubated with (bold line) or without (grey shadow) anti-β1, followed by incubation with Alexa Fluor 647 goat anti-mouse IgG subjected to FACS analysis (right panel). (B) Cell lysates from WT and KO cells were immunoblotted with anti-α3, anti-α5, anti-α6, anti-αV and anti-β4. α-Tubulin was used as a loading control (upper panel). Cells were collected and incubated with (bold line) or without (grey shadow) anti-α3, anti-α5, anti-α6 and anti-β4, followed by incubation with Alexa Fluor 647 goat anti-mouse IgG subjected to FACS analysis (lower panel). (C) Bright field pictures were taken to show the representative cell morphology. Scale bar, 50 μm (upper panel). WT and KO cells were stained with anti-β1, followed by incubation with fluorescent secondary antibody. Localization of F-actin was examined by staining with Alexa Fluor 546 phalloidin, the bar denotes 20 μm (middle and low panel). (D) The indicated cells were stained with anti-pFAK and anti-β4 antibody, followed by the incubation with fluorescent secondary antibody. Scale bar, 20 μm. The arrows indicate β4 integrin, the hemidesmosome maker, expressed in the cell-cell contact.

1.3.2 β1 integrin was essential for cell migration and the maintenance of mesenchymal phenotypes by stabilizing focal adhesions.

It is well known that β1 plays crucial roles in the regulation of cell migration and in intracellular cell adhesion signaling such as in the phosphorylation of FAK (22). Using a standard wound healing assay, as expected, KO cells showed a significant decrease in cell migration, compared with the results seen in WT cells (Fig. 2A). Consistently, the phosphorylated levels of FAK and AKT were also decreased in KO cells, compared with the results seen in WT cells (Fig. 2B). As shown in Fig. 2C, WT cells showed high levels of α-smooth muscle actin (α-SMA) and marginal expression levels of E-cadherin, which were mesenchymal and epithelial makers, respectively. Interestingly, in agreement with the changes in morphology, KO cells showed greatly enhanced expression levels of E-cadherin and reduced levels of α-SMA. The phenotypes of KO cells, such as increased cell-cell adhesions, typical changes in mesenchymal and epithelial makers, and suppression of cell migration are reminiscent of a re-differentiation towards an epithelial phenotype, resembling mesenchymal-epithelial transition (MET), which is the reverse process of epithelial-mesenchymal transition (EMT). We next investigated whether transforming growth factor-β (TGF-β), a well-established physiological inducer of EMT, could overcome the requirements for β1 integrin. As shown in Fig. 2D, treatment with TGF-β normally induced the phosphorylation of Smad2, a specific signaling pathway of TGF-β, promoted the loss of E-cadherin and accelerated the induction of α-SMA. Although EMT was induced in the KO cells, it did not trigger morphological changes. These results suggested that β1 was crucial for the maintenance of mesenchymal phenotypes by stabilizing focal adhesions, which may also be required for cell survival.

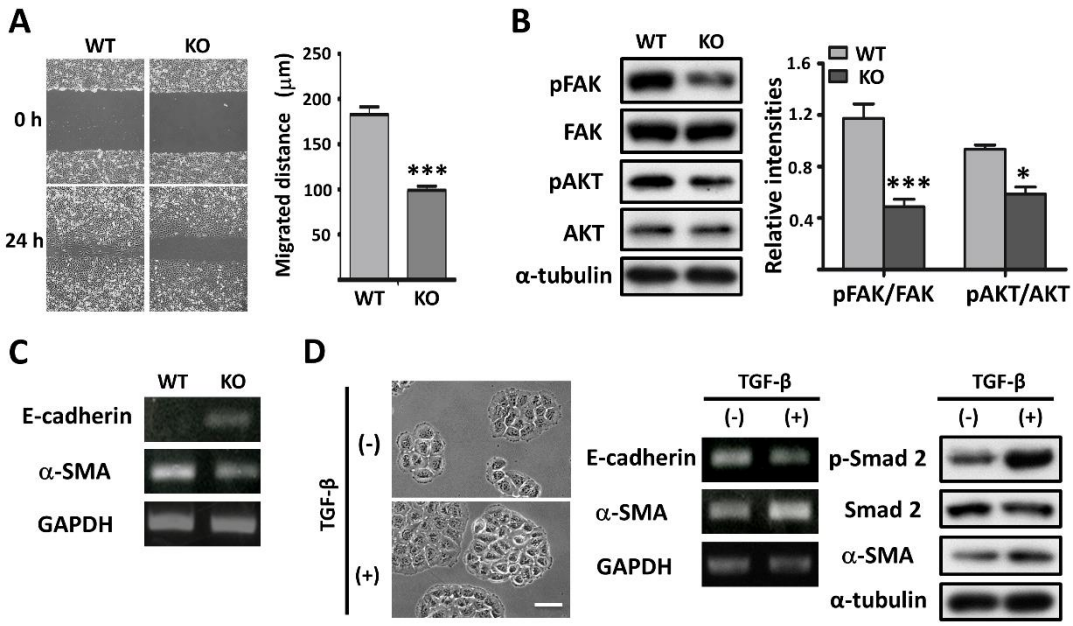

Figure 2. β1 was essential for malignant phenotype reversion and FAK/AKT signaling. (A) Cells were cultured until reaching more than 90% confluence. A scratch was made with a pipet in each well, and was photographed at 0 h and 24 h (left panel). Quantification of cell migrated distances are expressed as the means±s.e.m from three independent experiments (***, $p<0.001$ by two-tail unpaired t-test) (right panel). (B) Cell lysates from WT and KO cells were immunoblotted by anti-pFAK, anti-FAK, anti-pAKT and anti-AKT antibodies. α-Tubulin was used as a loading control. The quantitative data are presented as the means±s.e.m from three independent experiments (*, $p<0.05$, ***, $p<0.001$ by two-tail unpaired t-test). (C) RT-PCR analysis using total RNA extracted from WT and KO cells was carried out to examine the expression levels of E-cadherin and α-SMA. The expression level of GAPDH was used as a loading control. (D) Images of β1-KO cells that were untreated or stimulated with TGF-β for 48 hr. Scale bar represents 50 μm (left panel). RT-PCR using total RNA extracted from indicated cells was carried out to detect the expression levels of E-cadherin and α-SMA. GAPDH was used as a loading control (middle panel). Representative western blots corresponding to the expression of pSmad2, Smad2 and α-SMA in TGF-β treated and untreated β1-KO cells, α-tubulin was used as a loading control (right panel).

1.3.3 Effects of β1 on cell proliferation via a cell density-dependent manner.

As described above, β1 had a strong impact on cell behaviors such as morphology and migration, so we further investigated the effect that the deletion of β1 had on these biological functions. Surprisingly, when compared with WT cells, the KO cells tended to reach a confluence under normal culture conditions (Fig. 3A). Considering the lower level of phosphorylated AKT in the KO cells, as shown in Fig.2B, we hypothesized that changes in cell density might be critical for the regulation of cell proliferation. As shown in Fig. 3A, when cells were cultured at a lower density level in the first 24 h of incubation, the KO cells showed a significant decrease in cell proliferation, by comparison with the WT cells. However, once the KO cells reached a certain density, their cell proliferation ability was significantly increased, compared with the WT cells. We questioned whether the extracellular matrix could affect the anchorage-dependent cell growth, which would suggest that the dependence on cell densities could be the result of the concentration of ECM secreted from the cells, particularly under low-density conditions. Therefore, we checked the cell growth following a coating with ECMs. Interestingly, we found that an accumulation of laminin-332, rather than fibronectin, eliminated the difference between WT and KO cells under sparse conditions (Fig. 3B), which indicated that the up-regulation of β4 integrin can partially rescue the cell proliferation of β1 KO cells. These data also suggested that cell survival signaling from β1 integrin might play an important role in cell proliferation when cells are under strict conditions. To confirm this notion, we then conducted a clonogenic assay to test the single-cell potential for survival and ‘unlimited’ cell division (23). The KO cells consistently formed smaller and fewer foci compared with the WT cells (Fig. 3C). Furthermore, we compared the anchorage-independent cell growth of WT and KO cells via soft-agar assays. Anchorage-independent cell growth is thought to be closely related to cell survival signaling (24). As expected, the KO cells formed 50% fewer colonies than the WT cells (Fig. 3D). The abilities for cell survival were further examined under serum-free conditions after being cultured for 3 days, and we found that the deletion of β1 dramatically suppressed cell survival (Fig. 3E). Since the soft-agar and clonogenic assays were performed under low cell density culture conditions, these results strongly supported the results in Fig. 3A, which show that β1 affected cell proliferation in a cell density-dependent manner.

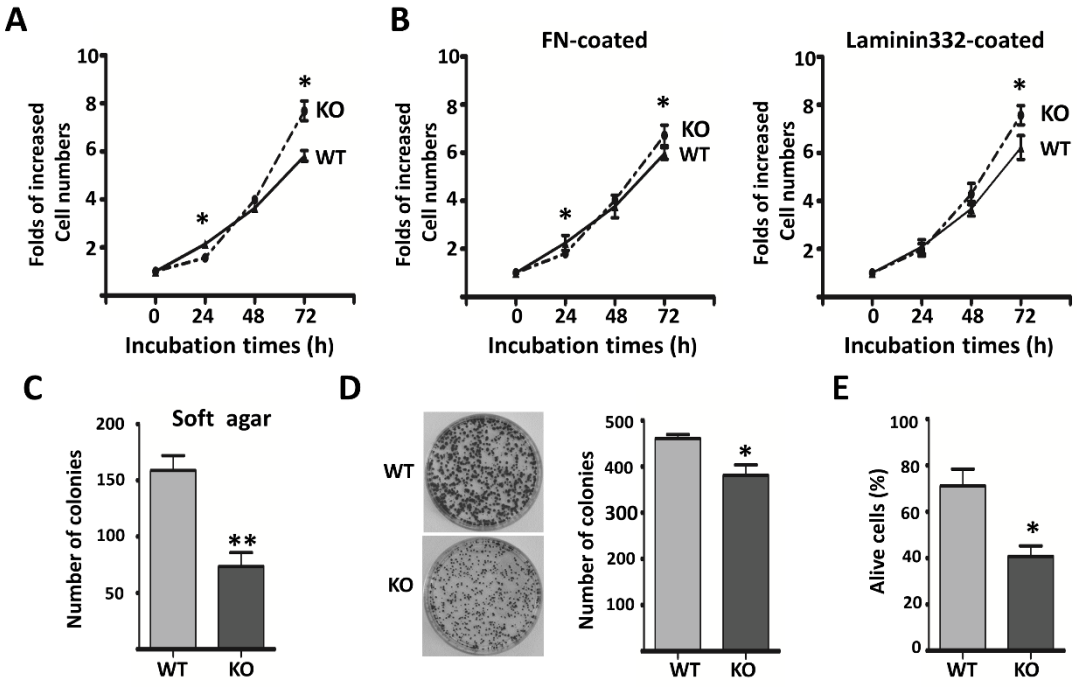

Figure 3. Effects of β1 on the proliferation and survival in MDA-MB-231 cells. (A-B) WT and KO cells were cultured on the none-coated (A) or *FN*, Laminin 332-coated (B) diashes, starved with serum-free DMEM for 24 h and then released with DMEM containing 10% FBS, the number of live cells were counted at the indicated times. Cell numbers were normalized to those at 0 h. Data are represented as the means±s.e.m (n=3). (C) WT and KO cells (500/per well) were grown for 16 days, then stained with crystal violet and the foci in each well were counted. Two representative wells are shown (*, p<0.05 by two-tail unpaired t-test). (D) WT and KO cells (500/per well) were cultured in the soft-agar plates for 18 days, the colonies were stained with crystal violet and counted, the quantitative data are presented as the means±s.e.m from three independent experiments (**, p<0.01 by two-tail unpaired t-test). (E) WT and KO cells were cultured over a layer of 1% agarose in serum-free media for 72 h. Cells were then collected by centrifugation and stained with trypan blue. The percentage of live cells was calculated. The quantitative data are presented as the means±s.e.m from three independent experiments (*, p<0.05 by two-tail unpaired t-test).

1.3.4 Deletion of β1 suppressed tumorigenesis in vivo.

To further evaluate the impact of β1 on tumor growth, we examined tumorigenesis in vivo using a well-established xenograft tumor model. After subcutaneous injection of the WT and KO cells ($1x10^6$) into the right and left flanks of nude mice, respectively, to allow tumor formation, we monitored tumor growth for 18 days. Compared with the WT group, the xenograft tumor formation in the KO group was significantly suppressed

in terms of both weight and volume (Fig. 4A-D), which indicated that β1 played important roles in tumorigenesis in vivo.

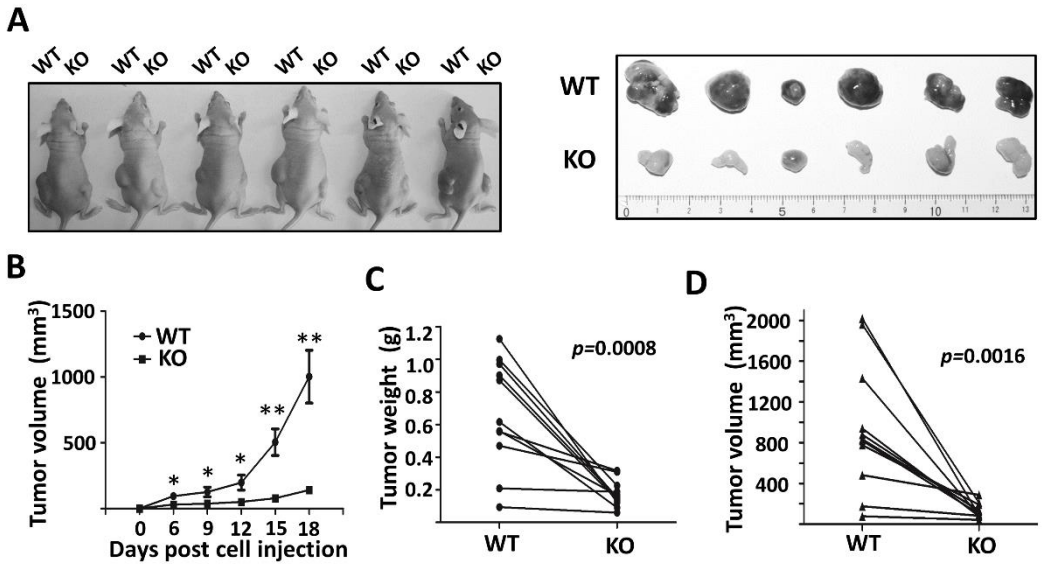

Figure 4. Deletion of β1 suppressed xenograft tumor growth in vivo. (A) WT cells (1×10^6) were injected subcutaneously into the left side of the flank and KO cells (1×10^6) were injected into the opposite side of the same mice. Tumor volume was measured every 3 days after injection for 6 days until the tumor had grown to the approved size (B). Tumors were dissected, weights (C) and volumes (D) were measured. Values are presented as the means±s.e.m (n =11, *, $p<0.05$, **, $p<0.01$ by two-tail unpaired t-test).

1.3.5 Activation of EGFR and cellular signaling were altered in the KO cells via a cell density-dependent manner.

As described above, the effects of β1 on cell proliferation were dependent on cell densities, we then examined the signaling events triggered by the cell-ECM interactions of WT and KO cells under different cell density conditions. Most of the studies regarding EGFR have focused on lung cancer, and the role of EGFR in breast carcinogenesis is still poorly established (25, 26). In recent studies, however, EGFR has assumed a relevant place in breast cancer etiology after its association with aggressive clinical behavior showing a characteristic pattern of metastatic dissemination to the lungs and brain (27) and poor responses to conventional chemotherapies (28). Src-family kinases are controlled by many cell surface receptors, including integrin receptors and protein-tyrosine kinases, which participate in pathways regulating cell proliferation and survival (29). It was particularly interesting that under normal culture

conditions the levels of phosphorylated EGFR and Src were significantly up-regulated in KO cells by comparison with WT cells (Fig. 5A), which suggested that expression of β1 suppressed EGFR activation and Src was involved in EGFR signaling in this cell line. By contrast, the activation of ERK appeared to happen in a cell density-dependent manner where decreased levels of phosphorylated ERK occurred at lower cell densities, and increased levels of phosphorylated ERK were found in higher cell densities in the KO cells, compared with those in WT cells. However, the levels of phosphorylated AKT were decreased in all cell culture densities in the KO cells, compared with the WT cells, which further strongly suggested that the expression of β1 played an important role in cell survival signaling. As the level of phosphorylated EGFR was increased in KO cells, we next investigated whether the increased levels of phosphorylated EGFR were due to excessive EGF secretion. As shown in Fig. 5C, media-secreted EGF was not affected by β1, thus confirming that activation of the EGFR in KO cells is a ligand-independent event. Dimerization is certainly a critical part of the mechanism during EGFR activation and EGF-induced signal transduction (30). To study the mechanism for the deletion of β1-mediated EGFR activation, we compared EGFR dimerization in both types of cells. Cells were chemically cross-linked and then extracts were subjected to western blot testing to detect both EGFR monomers and dimers. During this testing, fewer EGFR dimmers were visualized in WT cells (Fig. 5D), suggesting that β1-KO cells facilitated EGFR activation.

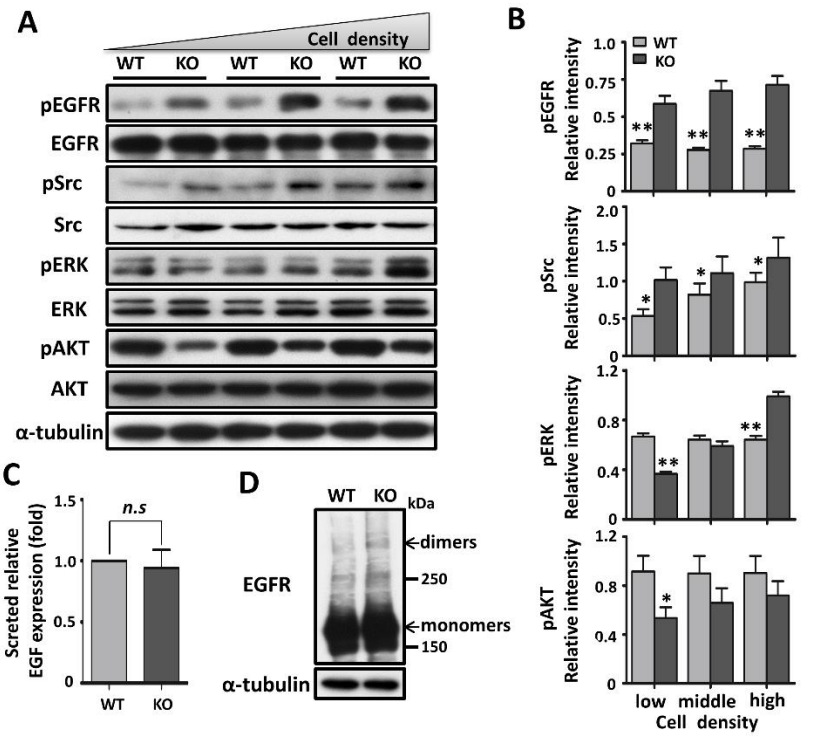

Figure 5. β1 regulated cell signaling with a cell density-dependent manner and was dispensable for EGFR dimer formation. (A) Low, middle and high densities of cell lysates were immunoblotted by anti-pEGFR, anti-EGFR, anti-pSrc, anti-Src, anti-pERK, anti-ERK, anti-pAKT and anti-AKT antibodies. α-Tubulin was used as a loading control. (B) Graphical representation of relative level of pEGFR, pSrc, pERK and pAKT in low, middle and high densities of MDA-MB-231 cells, respectively. Data are represented as the means±s.e.m of three independent experiments (*, $p<0.05$, **, $p<0.01$ by two-tail unpaired t-test). (C) EGF concentration in the secreted media was analyzed by EGF ELISA. EGF levels of KO cells were normalized to WT cells. Data are represented as the means±s.e.m (n=3). (D) Cells were subsequently cross-linked using 5 mM Sulfo-EGS as chemical cross-linker on ice for 2h and stopped with 10mM Tris for 15 min. Cell lysates from those cells were subjected to anti-EGFR to detect EGFR monomers and dimers. α-Tubulin was used as a loading control.

1.3.6 The aberrant phenotypes in the KO cells were restored in the β1-rescue cells.

Given the observation that the KO cells exhibited both aberrant cell morphology and cell growth ability, we restored β1 expression in the KO cells (Res) to examine whether it would rescue these phenotypes. The efficiencies of Res cells were confirmed by western blot and FACS analysis (Fig. 6A). As expected, the EGFR activation was greatly suppressed in the Res cells, similar to the WT cells, by comparison with the KO

cells (Fig. 6A). The aggregated cell morphology of the KO cells was reversed to a mesenchymal morphology, including filopodia formation and loss of cortical actin formation in the Res cells (Fig. 6B). In addition, cell proliferation was suppressed, while the ability of colony formation, both in soft agar and in culture dishes, was increased in the Res cells, compared with those in the KO cells (Fig. 6C-E).

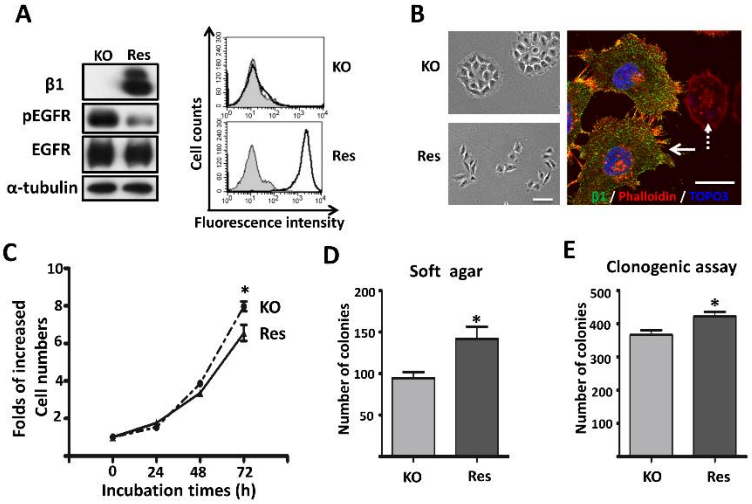

Figure 6. Forced expression of β1 rescued cell morphology and proliferation ability. (A) KO and KO-overexpressing β1 (Res) cells were immunoblotted by anti-β1, anti-pEGFR and anti-EGFR. α-tubulin was used as a loading control (left panel). KO and Res cells were collected and incubated with (bold line) or without (grey shadow) anti-β1, followed by incubation with Alexa Fluor 647 goat anti-mouse IgG subjected to FACS analysis (right panel). (B) Bright field pictures were taken to show representative cell morphology of KO and Res cells (left panel). Scale bar represents 50 μm. Res cells were stained with anti-β1, followed by incubation with fluorescent secondary antibody. Localization of F-actin was examined by staining with Alexa Fluor 546 phalloidin. The bar denotes 20 μm. (C) KO and Res cells were starved with serum-free DMEM for 24 h and then released with DMEM containing 10% FBS, and the number of cells were counted at indicated time points. Cell numbers were normalized to those at 0 h. Values are the means±s.e.m (n=3, *, $p<0.05$ by two-tail unpaired t-test). (D) KO and Res cells (500/per well) were cultured in the soft-agar plates. After 18 days, the colonies were stained with crystal violet and the numbers of colonies in each plate were counted. The quantitative data are presented as the means±s.e.m from three independent experiments (*, $p<0.05$ by two-tail unpaired t-test). (E) KO and Res cells (500/per well) were grown for 16 days and the foci in each well were counted. The quantitative data are presented as the means±s.e.m from three independent experiments (*, $p<0.05$ by two-tail unpaired t-test).

1.3.7 EGFR tyrosine kinase inhibitor, AG1478, efficiently suppressed cell proliferation in KO cells or WT cells in the presence of anti-β1 antibody.

Different anti-EGFR agents, which include the EGFR tyrosine kinase inhibitor gefitinib, have shown an inhibition of the growth of human breast carcinoma cells (31, 32). However, clinical studies of gefitinib in breast cancer have resulted in few clinical responses and in a disease control rate of approximately 10% (33). This observation indicates that resistance to gefitinib is a common phenomenon in breast cancer. AG1478 shares the same structural quinazoline backbone with gefitinib, and it has also shown an ability to inhibit the function of EGFR (34). As described above, the differential activation of EGFR and its downstream pathways including Src, ERK and AKT were observed in the WT and KO cells. In the present study, we examined the influence of AG1478 on cell proliferation, which was inhibited in both types of cells, but the effect clearly was more effective in the KO cells than in the WT cells (Fig. 7A). Consistently, MDA-MB-231 cells treated with AG1478 combined with the inhibitory anti-β1 antibody (P5D2) and exhibited a significant decrease in cell proliferation, compared with a single treatment with either P5D2 or AG1478 alone (Fig. 7B). These results show that a combination treatment with anti-EGFR and anti-β1 agents exerts a synergistic inhibitory effect on cell growth, which may also solve the resistance problem for a single inhibitor in breast cancer treatment.

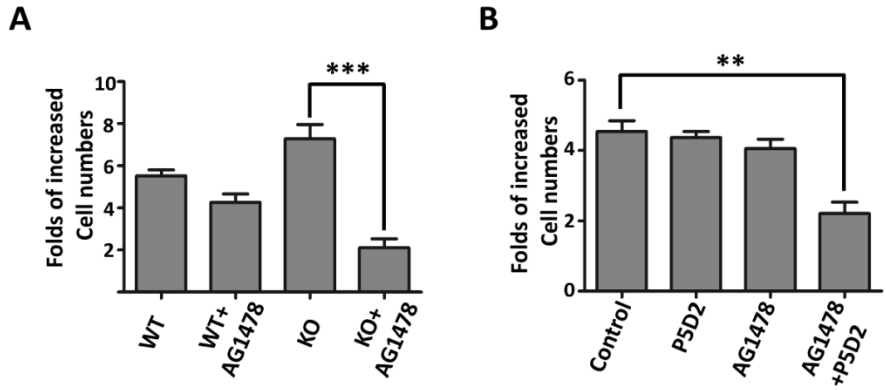

Figure 7. Treatment with AG1478 efficiently suppressed cell proliferation in KO cells. (A) After attachment for 24 h, WT and KO cells treated with 3 μM of AG1478 for 48 hr, the number of live cells were counted. Cell numbers were normalized to those at 0 h. Data are represented as the means±s.e.m (***, $p<0.001$ by two-tail unpaired t-test). (B) MDA-MB-231 cells were untreated, or treated with P5D2, AG1478, or P5D2 plus AG1478 for 48 hr. Cell proliferation was evaluated by the number of live cells. Cell numbers were normalized to those at 0 h as 1. Data are represented as the means±s.e.m (**, $p<0.01$ by two-tail unpaired t-test).

1.4 Discussion

There is an increasing body of evidence implicating members of the integrin family as important signaling components involved in mammary tumorigenesis and progression (15), since integrins play important roles in cell migration, cell proliferation and cell survival. In the present study, we performed a combined biochemical technique via the knockout and restoration of the β1 gene in MDA-MB-231 cells, a highly invasive breast cancer cell line, and found that β1 is not only important for cell migration and survival, it also regulates cell proliferation in a novel cell density-dependent manner. When cells are cultured under a relatively low degree of density, they exhibit β1-dependent growth. However, once cells reach a certain density, the effects of β1 are reversed, and then they suppress cell proliferation and EGFR signaling. Although the underlying mechanism remains unclear, at least two possible explanations could be concluded from the present study. One is the up-regulation of β4 integrin in KO cells, since previous researchers have already found that α6β4 integrin in MDA-MB-231 cells induces EGFR clustering (35). The other possibility is that culturing under low density exposes cells to strict conditions, in which cell survival signaling from the β1-mediated cell-ECM interaction is essential for cell growth. When cells then reach a certain density, the cell survival signaling, which can be obtained from both cell-cell and cell-ECM interactions, is not important for cell proliferation (Figure 8). The significance of β1 in cell proliferation and survival remains controversial. Some studies have reported that the blocking of β1 inhibited cell proliferation and induced cell apoptosis (8, 15). However, other studies have shown that a down regulation of β1 expression promotes cell growth (10, 36). These discrepancies might be partly explained by the observations described above and other possibilities cannot be excluded, such as different approaches for blocking β1 and different cell lines, which express differential associations of β1 with divergent α subunits.

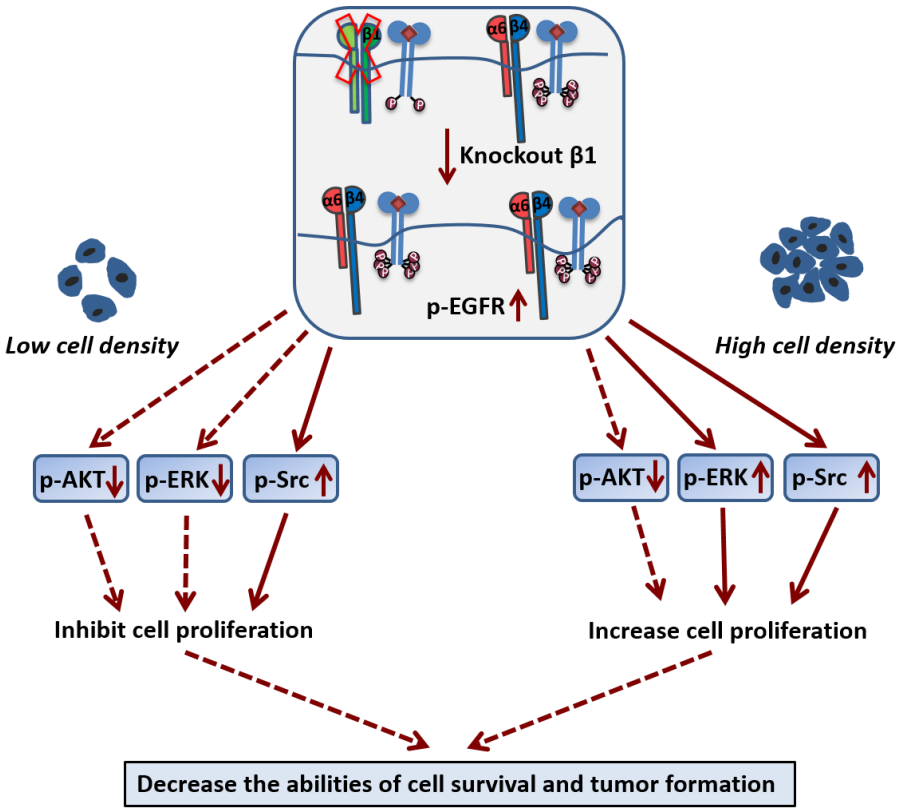

Figure 8. Proposed molecular mechanism for the regulation of cell behaviors and cellular signaling by integrin β1.

Our data clearly demonstrated that the expression of β1 suppressed cell proliferation and down-regulated the phosphorylation of EGFR, Src and ERK, but that the phosphorylation of Akt was up-regulated (Fig. 5). These results suggested that EGFR-mediated signaling, rather than integrin β1-mediated FAK phosporylation, plays a dominant effect on Src and ERK signaling, and that it regulates cell proliferation in KO cells. Because both integrin and EGFR can activate the PI3K/AKT signaling pathway, we can speculate that integrin β1-mediated activation could be a dominant PI3K/AKT signaling pathway in the cell. In fact, the inactivation of Akt in the KO cells was demonstrated by decreases in cell survival signaling and cell migration. In line with our observations, Mebratu, et al. reported that the blockage of β1 stimulated the MEK1/2-ERK1/2 signal pathway to promote cell proliferation (37). These results suggested that an increase in cell proliferation ability could be a consequence of β1 inhibition. The restoration of the β1 gene in the β1-KO cells consistently and significantly suppressed EGFR activation (Fig. 6), which suggested the existence of a negative feedback loop between EGFR and β1. In fact, treatment with trastuzumab, a targeted therapy for HER2, is known to have resulted in an activation of β1-related

signal pathways such as PI3K/AKT and ERK by circumventing the anti-cell proliferative activity of trastuzumab (38, 39). Taken together, these results indicate that the signaling from integrin and EGFR contributes to therapy resistance, a phenomenon that has also been observed in other cancer cells (40). It is intriguing that the inhibitory effects of EGFR tyrosine kinase inhibitor AG1478 on cell proliferation were more effective in β1-KO cells than in WT cells, which suggested that a treatment combining anti-EGFR therapy with anti-integrin drugs wound be important for cancer therapy. Considering that the blockage of β1 may inhibit cell survival while simultaneously promoting cell proliferation, a combination treatment may be suitable for those post-treated with anti-EGFR drugs where cell survival signals are more essential than cell-growth signals for the treatment of cancer cells. The inhibitory effects of integrins on cell growth have also been confirmed by other studies; a deletion of α1β1 integrin enhanced EGFR cellular signaling through a clustering of EGFR (18); additionally, the targeting of α2β1 promoted cell growth in some breast cancer cells (41). Here, however, we could not exclude the possible involvement of β4 integrin, since its expression level was significantly increased in KO cells (Fig.1). By cooperation with RTKs, α6β4 is known to positively regulate cell proliferation. Specifically, α6β4 can induce both EGFR and ErbB-2 clustering, which then promotes the proliferation of MDA-MB-231 cells (35, 42). Further investigation is obviously needed to elucidate the mechanistic roles of the reciprocal regulation between integrin and EGFR.

It is well known that EMT is an important malignant phenotype characterized by a loss of the cell–cell junction and the acquisition of cell migratory and invasive behavior in breast cancer (6, 43). In the present study, KO cells exhibited disruption of focal adhesions, stabilized cell-cell adhesion and typical changes in EMT markers to show a malignant phenotype reversion, even after TGF-β stimulation, which indicated that the malignant phenotype exhibited by MDA-MB-231 cells was primarily mediated by β1 integrin. β1 integrin is also necessary for induction into the murine 4 T1 cell, which is another invasive mammary cell line (6). This is reasonable since β1 plays a crucial role in the changing of cell shape and in the forming of invasive protrusions during the cell migration process (44-46). However, the GE11 cell, a β1-deficient normal cell line, is responsible for TGF-β stimulation, which can induce EMT-like changes (46, 47). These discrepancies could be explained by the fact that the requirement for β1 during malignancy progression is dependent on cell types, particularly in mammary cells. In fact, the balanced interactions between cell-ECM, cell-cell, and their dynamic regulations are vital for physiologic and pathologic events, and altered interactions with ECM have been observed in mammary tumor development (17).

In conclusion, the current study clearly showed that integrin β1 dynamically regulates cell proliferation and cellular signaling. Basically, the expression of β1 negatively regulates EGFR activation. Considering the importance of β1 in cell survival and cell migration, a combination treatment with anti-EGFR and anti-β1 drugs is recommended as a very useful therapy for breast cancer.

Part 2

The importance of membrane-proximal *N*-glycosylation on integrin β1 in its activation and complex formation

2.1 Introduction

In part 1, we have compared cell behaviors between normal and β1-KO MDA-MB-231 cells and found loss of β1 resulted in cell-cell adhesion, decreased cell migration. Considering the fact that our group have previously identified three potential *N*-glycosylation sites on the I-like domain of β1 are essential for the α5β1 function, so we hypothesized that if the importance of other *N*-glycosylation sites can be identified, this will facilitate an understanding of the underlying molecular mechanisms as to how and why *N*-glycans alter integrin functions. Therefore, the purpose of the part 2 is to elucidate the mechanisms by which *N*-glycans regulate the integrin functions.

We reconstituted with wild-type (WT), and a *N*-glycosylation mutant on the β1 I-like domain (S4–6), which expresses mutant integrin β1 bearing only three *N*-glycosylation sites within I-like domain essential for β1 heterodimer formation with integrin α5. We showed that despite the little difference in the expression levels of α5β1 on the cell surface between WT cells and the S4-6 mutant, the cell spreading and migration of the S4-6 mutant was significantly decreased. Consistent with these phenotypes, the integrin β1-mediated cellular signaling and its activation were shown to be clearly suppressed in the S4-6 mutant cells. These observations, interestingly, could be rescued by restoration of the *N*-glycosylation sites in membrane-proximal domain (S9-12), but not others. Further study on the regulatory mechanisms suggested that the membrane-proximal *N*-glycosylation was critical for intermolecular interactions between integrin β1 and other cell membrane proteins. Moreover, the terminal α2,6-sialic acids catalyzed by ST6GAL1 was required for the integrin β1 activation. Overall, our data suggest a novel regulatory mechanism that *N*-glycosylation closed to the cell membrane on integrin β1 may serve as a platform, which facilitates its complex formation on the cell membrane, thereby affecting integrin-mediated functions.

2.2 Materials and Methods

2.2.1 Antibodies and reagents.

The experiments were performed using the following antibodies: antibody against human integrin β1 subunit (P5D2) was from Developmental Studies Hybridoma Bank, University of Iowa; rabbit mAbs against AKT, p-AKT and EGFR were from Cell Signaling Technology; goat antibody against α3 integrin was from Santa Cruz Biotechnology; integrin α5, FAK and p-FAK were from BD Biosciences; mouse mAbs against human integrin α5β1, rabbit mAbs against integrin β1 and mouse antibody against active integrin β1 (HUTS-4) were from Millipore; rabbit mAbs against syndecan 4 was from novus biologicals; mAb against α-tubulin was from Sigma; Alexa Fluor® 488 and 647 goat anti-mouse IgG was obtained from Invitrogen (Life Technologies). The peroxidase-conjugate goat antibody against mouse, rabbit and goat were obtained from Promega, Cell Signaling Technology and Santa Cruz Biotechnology, respectively. Sambucus sieboldiana agglutinin (SSA)-agarose was from J-OILMILLS; fibronectin (FN) was obtained from sigma; TO-PRO-3 was from Molecular Probes; Ab-Capcher ExTra was obtained from Protenova; Sulfo-NHS-SS Biotin for cell surface biotinylation and Streptavidin-conjugated agarose were from Thermo Scientific and Millipore, respectively.

2.2.2 Cell lines and cell culture.

The MDA-MB-231, a human breast cancer cell line, was obtained from the American Type Culture Collection (ATCC). The 293T and HeLa cell lines were provided from RIKEN cell bank (Japan). The β1-KO MDA-MB-231 cells were previously established in our laboratory (48). All the cell lines were maintained at 37 °C in Dulbecco's modified Eagle's medium (DMEM, Invitrogen), supplemented with 10% fetal bovine serum (FBS), under a humidified atmosphere containing 5% CO_2, except for the virus production.

2.2.3 Generation of CRISPR/Cas9-based integrin β1-knockout (β1-KO) HeLa cells and integrin β1/ST6GAL1-double-knockout (DKO) MDA-MB-231 cells.

The CRISPR/Cas9-based integrin β1-KO HeLa and DKO MDA-MB-231 cells were established as described previously (19). Briefly, the integrin β1-KO- and ST6GAL1-KO- specifying pSpCas9 (BB)-2A-GFP vector, which were established previously in our laboratory (48, 49), were transfected into the parent HeLa cells and

β1-KO MDA-MB-231 cells, respectively, using Cell Line Nucleofector TM kits (Lonza) according to the manufacturer's instructions. After 72 h of transfection, GFP highly expressed cells were sorted using the FACSAria II (BD Bioscience). Following about 10 days culture, the β1- and GFP- negative Hela cells or the ST6GAL1- and GFP-negative MDA-MB-231 cells were sorted another 7 or 2 times, respectively. The β1-KO HeLa and the DKO MDA-MB-231 cells were defined via flow cytometry and western blot analyses as described below.

2.2.4 ST6GAL1 and integrin β1 expression vectors.

The WT or S4-6 mutant integrin β1- and ST6GAL1-overexpressing lentiviral vectors (CSII-EF-Rfa-WT-β1, CSII-EF-Rfa-S4-6-β1, CSII-EF-Rfa-D4-6-β1, CSII-EF-Rfa-D9-12-β1, and CSIV-TRE-CMV-3xFLAG-ST6GAL1) were previously established in our laboratory (21, 50). Other integrin β1 mutation vectors (S4-6+1-3, S4-6+7-8, and S4-6+9-12) were constructed using the in-fusion kit (Takara Bio) according to the manufacturer's instructions. The resultant cDNAs were sequenced to confirm the presence of the desired mutations, and then cloned into the CSII-EF-Rfa using a Gateway^{TM} cloning System kit (Thermo Fisher Scientific) to acquire all the expression vectors.

2.2.5 Virus production and infection.

The virus production and infection was performed as described previously (21). In brief, the CSII-EF-Rfa-related-β1- or CSIV-TRE-CMV-3xFLAG-ST6GAL1- overexpressing lentiviral vectors were cotransfected with pCAG-HIVgp and pCMV-VSV-G-RSV-Rev into 293T cells. After transfection for 48 hours, the lentivirus supernatants were collected. The β1-KO MDA-MB-231 or HeLa cells and the DKO MDA-MB-231 cells were infected with the indicated lentiviruses. The β1- or ST6GAL1-positive cells were sorted 3 times using FACSAria II after infection for 72 h. The stable cell lines were used in subsequent studies.

2.2.6 Western Blot (WB) and Immunoprecipitation (IP)

WB was shown in Chapter **1.2.5**

For IP, cells were lysed with lysis buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 % Triton X-100) with protease inhibitors and phosphatase inhibitors (Nacalai Tesque, Kyoto, Japan) for 30 min. After centrifugation at 13,000rpm for 10 min, the supernatant was collected and protein concentrations were determined using a BCA protein assay kit.

Equivalent amounts (600 μg) of the supernatants were immunoprecipitated by Ab-Capcher ExTra (ProteNova) with anti-P5D2 antibody, or Streptavidin-conjugated agarose for 1h at 4°C with rotation and then the immunoprecipitates were washed twice with TBS buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl) and subjected to SDS-PAGE. The lectin precipitations were performed using Sambucus sieboldiana agglutinin (SSA)-agarose (J-OILMILLS, J318), which specifically recognized α2,6-sialylation. The precipitated glycoproteins were subjected to SDS-PAGE.

2.2.7 Cell surface biotinylation

Cells were gently washed with PBS, then incubated with ice-cold PBS containing 0.2 mg/ml Sulfo-NHS-SS Biotin for 1 h at 4 °C. After incubation, cells were washed three times with ice-cold PBS, and then harvested by lysis buffer. The biotinylated proteins were precipitated with Streptavidin-conjugated agarose, and then subjected to SDS-PAGE as described above.

2.2.8 Flow cytometric analysis

Shown in Chapter **1.2.6**

2.2.9 Immunofluorescence staining

Shown in Chapter **1.2.7**

2.2.10 Cell migration

Wound-healing assay was shown in Chapter **1.2.8**

For transwell migration assay, each Transwell (BD BioCoat TM control inserts, 8.0-mm inserts; BD Biosciences) was coated on the bottom side with 10 μg/ml fibronectin (FN) at 4 °C overnight. Cells were starved in serum-free medium overnight, trypsinized, and suspended in DMEM containing 10% FBS. The suspended cells were centrifuged, and the supernatants were removed. Cells were resuspended with serum-free DMEM and diluted to 0.8×10^5 cells/ml. To each FN-coated transwell, 500 μl aliquots of the cell suspension were added; then the cells were incubated at 37 °C for 4 h for HeLa cells and 3 h for MDA-MB-231 cells. After incubation, cells on the upper side were removed by scraping with a cotton swab. The membranes of each transwell were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet for 2 h. Cells that had migrated to the lower side were counted using a phase-contrast microscope.

2.2.11 Cell spreading assays

The cell spreading assays were performed as described previously (51). Briefly, 6-well plates were coated with FN (10 μg/ml) in PBS at 4 °C overnight and then blocked with 1% bovine serum albumin (BSA) in DMEM for 1 h at 37 °C. The indicated cells were detached and suspended in serum-free DMEM with 0.1% BSA at 4×10^4 cells/ml. After 20-min incubation, non-adherent cells were removed by washing with PBS, and the attached cells were fixed with 4% paraformaldehyde (PFA) in PBS, and representative photos were then taken by phase contrast microscopy.

2.2.12 Video microscope

A glass-bottom dish (Asahi Techno Glass, Japan) was precoated with FN (10 μg/ml) in PBS at 4 °C overnight and then blocked with 1% BSA in DMEM for 1 h at 37 °C. 4×10^4 cells were suspended in 2 ml growth medium and then added to each FN-coated glass-bottom dish, which was followed by monitoring for 16 h using time-lapse video equipment (Carl Zeiss, Germany). Images were acquired using inverted microscopes (Axio Observer.D1, Carl Zeiss) every 10 min with 5% CO_2 at 37 °C in a heated chamber with temperature and CO_2 controller (Onpu-4 & CO2, Air Brown, Japan) during time-lapse imaging. Cell motility was evaluated using Chemotaxis and Migration Tool 2.0.

2.2.13 Statistical analysis

Statistical analyses were performed via a Student's t test using GraphPad Prism5. The results are presented as the mean±s.e.m. Statistical significance was defined as $p < 0.05$ (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

2.3 Results

2.3.1 Effects of β1 underglycosylation on its heterodimer formation and FN-mediated cell spreading.

Given the important role of *N*-glycosylation sites on the I-like domain of β1 subunit in α5β1 heterodimer formation and cell adhesion (21), we hypothesized that the other *N*-glycosylation sites on β1 subunit may also have unique functions. After establishing the β1-null MDA-MB-231 cells, we reconstituted with wild-type (WT), a *N*-glycosylation mutant on the β1 I-like domain (S4–6) which represents the minimal *N*-glycosylation required for α5β1 function in GE11 cells (21), or with *N*-glycosylation sites on the I-like domain deleted mutant (D4-6) (Fig. 1A). To determine the effects of *N*-glycosylation on β1 expression on the cell surface, we performed flow cytometric analysis and biotinylation assay. As shown in Figs. 1B and C, those three cells exhibited similar expression levels of β1 on the cell surface and in the whole cell lysates, suggesting that the *N*-glycosylation of β1 had little influence in its expression in MDA-MB-231 cells. It seems to be inconsistent with previous study, which showed that D4-6 reduced the expression levels of β1 in GE11 cells (21). In fact, the α5 subunit is a main constituent in β1 integrin-containing dimers in GE11 cells (52), and, therefore, the inhibition of α5β1 heterodimer formation in the D4-6 cells greatly reduced the expression levels of β1. However, the MDA-MB-231 cells highly expressed either α5 or α3, α2 and α6 integrin subunits (53). Therefore, we wondered if D4-6 β1 forms heterodimers with other α subunits such as α3. As shown in Fig. 1D, the α5 subunits were consistently clearly detected in the immunocomplexes of WT or S4–6 mutant, but not the D4-6 mutant. It was interesting that the α3β1 heterodimer was observed in all three types of cells, indicating that α3 integrin is different from α5 and requires different *N*-glycosylation for αβ dimer formation. It is noteworthy that the expression level of integrin α5β1 on the cell surface in S4-6 cells was comparable to that in the WT cells, but the cell spreading ability was significantly decreased in S4-6 cells compared with WT cells (Fig. 1E). Collectively, these results suggest that *N*-glycosylation on other domains, rather than the I-like domain of the β1 subunit may be involved in the regulation of its various cellular functions.

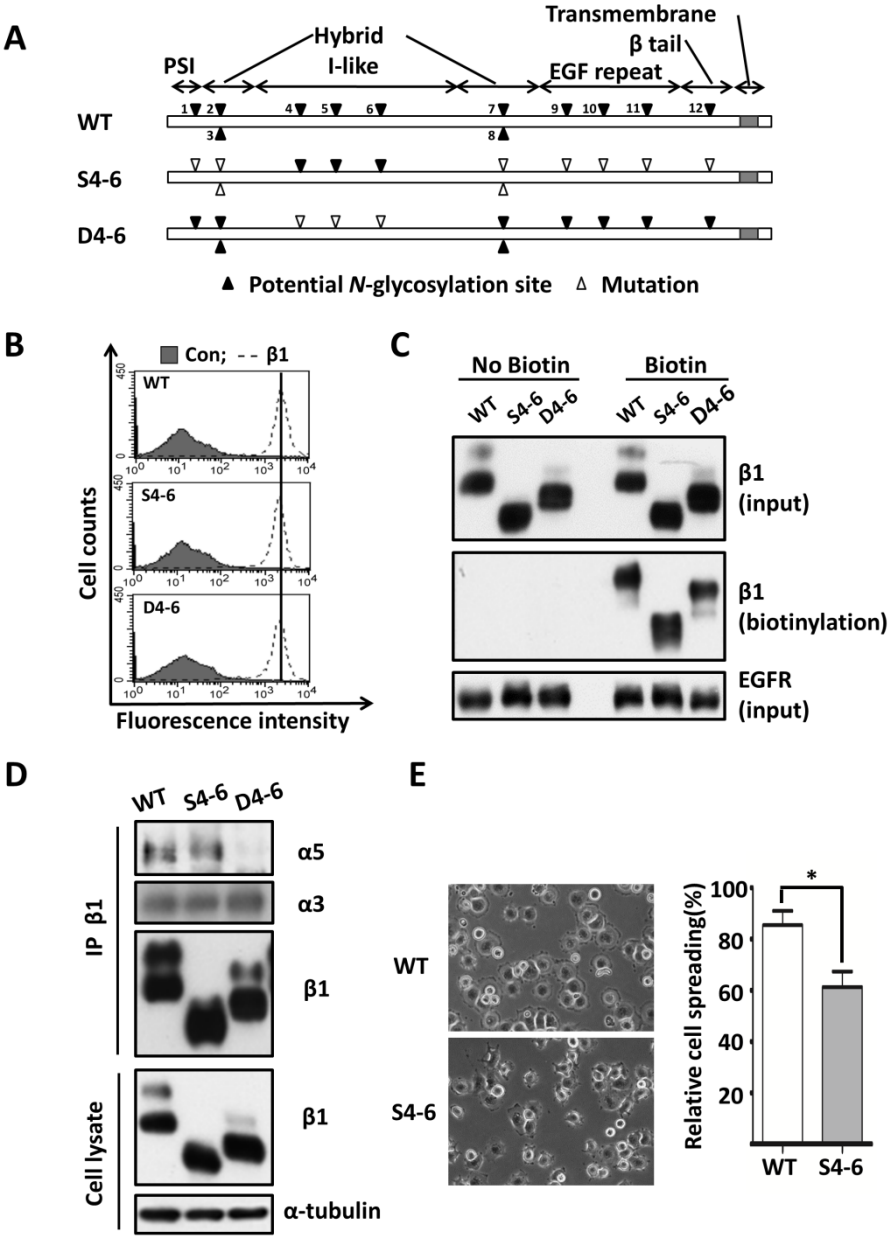

Figure 1. Comparison of β1 expression, its association with α subunits and FN-mediated cell spreading among WT and *N*-glycosylation mutants of integrin β1. (A) Schematic diagram of potential *N*-glycosylation sites on integrin β1 subunit (Asn 50, Asn 94, Asn 97, Asn 212, Asn 269, Asn 363, Asn 406, Asn 417, Asn 481, Asn 520, Asn 584, and Asn 669). *N*-Glycosylation sites are indicated by closed triangles, and point mutations are indicated by open triangles. (B) The WT, S4–6 and D4-6 cells exhibited the same expression levels as β1 on the cell surface. Cells were collected and incubated with (dotted line) or without (grey shadow) anti-β1 antibody, followed by incubation with Alexa Fluor 647 goat anti-mouse IgG, and then were subjected for flow cytometric analysis. (C) The expression levels of β1 on the cell surfaces (biotinylation) and in total cell lysates were

immunoblotted with anti-β1 antibody. EGFR was used as a loading control. (D) Cell lysates from the WT, S4–6 and D4-6 cells were immunoprecipitated (IP) with anti-β1 antibody (P5D2) and blotted with anti-α3, anti-α5 and anti-β1 antibodies. (E) WT and S4–6 cells were detached and then replated on the FN-coated (10 μg/ml) dishes. After incubation for 20 min, cells were fixed with PFA, and the images were taken. The percentages of spread cells were statistically analyzed as the mean±s.e.m of three independent experiments (*, $p<0.05$ by two-tail unpaired t-test).

2.3.2 Removal of *N*-glycosylation sites on β1 suppressed FN-mediated cell migration.

N-glycans on β1 integrin play important roles in the regulation of its biological functions. Our wound healing assay and boyden chamber analysis showed that S4-6 cells exhibited a significant decrease in cell migration compared with WT cells (Figs. 2, A and B). To check whether underglycosylation of β1 also suppresses the FN-mediated migration of other cells, integrin β1 was knocked out in Hela cells using a CRISPR/Cas9 system and WT or S4-6 of β1 that was restored in the KO cells. In similar manner, the decreased cell migration was also observed in S4-6 mutant HeLa cells (Figs. 2, A and B). To further confirm the phenotype of the S4-6 cells, the cell motilities of WT and S4-6 mutant cells were examined and compared using in vitro time-lapse imaging. Consistently, WT cells apparently moved faster than the underglycosylated S4-6 mutant cells (Fig. 2C). Taken together, these results demonstrate that *N*-glycosylation on the β1 subunit is capable of promoting cell migration.

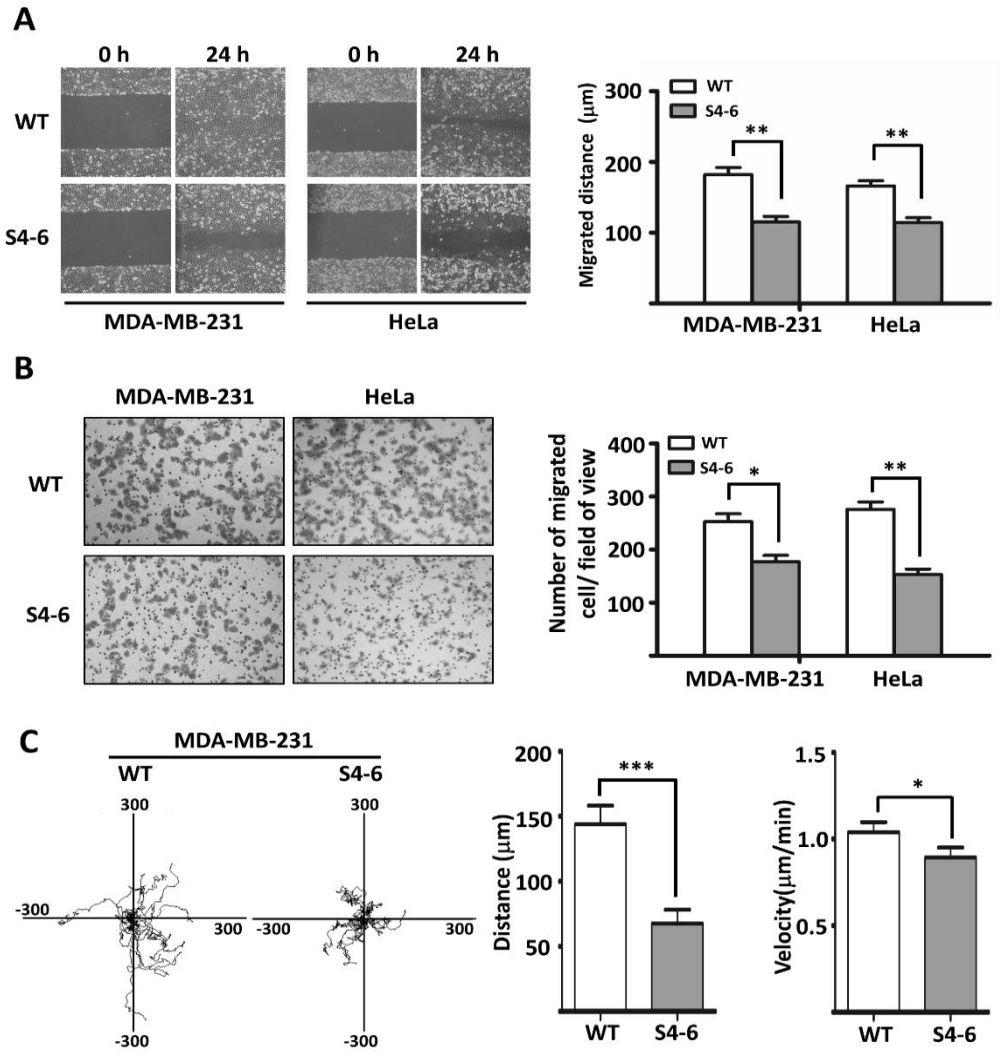

Figure 2. Removal of *N*-Glycosylation Sites on β1 suppressed FN-mediated cell migration in MDA-MB-231 and HeLa cells. (A) The indicated cells were cultured until more than 90% confluence. A scratch was made with a p200 pipet in each well, and photographs were taken at 0 h and 24 h (left panel). Quantitative data of migrated distances were expressed as the mean±s.e.m from three independent experiments (**, p<0.01 by two-tail unpaired t-test) (right panel). (B) The migration ability was also analyzed by transwell assay. Briefly, cells which migrated through the Transwell membrane were stained with 0.5% crystal violet. The representative photos were recorded by phase contrast microscopy (left panel). The migrated cells were counted and the quantitative data were obtained from three independent experiments (*, p<0.05, **, p<0.01 by two-tail unpaired t-test) (right panel). (C) Individual migration tracks of WT and S4-6 cells were monitored by time-lapse microscopy as described in the "EXPERIMENTAL PROCEDURES" section (left panel). Values for the distance and velocity of the cell migration were analyzed and each bar represents the mean±s.e.m of 16 cells (*, p<0.05, ***, p<0.001 by two-tail unpaired t-test) (right panel).

2.3.3 Depletion of *N*-glycosylation on β1 affects integrin-mediated classic signaling on FN and its activation.

Activation of FAK is an important step for β1-mediated intracellular signaling and cell migration (54, 55). To determine whether *N*-glycosylation of the β1 subunit could also affect integrin signaling, the phosphorylation level of FAK was assessed using cell lysates collected at indicated times. As shown in Fig. 3A, the response of the FN-induced activation of FAK was attenuated in S4-6 cells, compared with WT cells. In addition, there were more lamellipodia formation and membrane protrusions in WT than in S4-6 cells, as detected by phalloidin staining. These observations provided molecular support for the decreased motility of S4-6 cells (Fig. 3B). Consistent with the phosphorylated levels of FAK, the phospho-AKT levels were also decreased in S4-6 cells compared with WT cells (Fig. 3C). These data indicate that *N*-glycosylation on the β1 subunit might affect the status of integrin activation. To test this idea, the β1 activation was examined by flow cytometry analyses and immunofluorescence staining with anti-active β1 antibodies. As expected, loss of *N*-glycosylation resulted in a significant decrease in the expression level of active β1 both in the intracellular domain and on the cell surface (Figs. 3D and E).

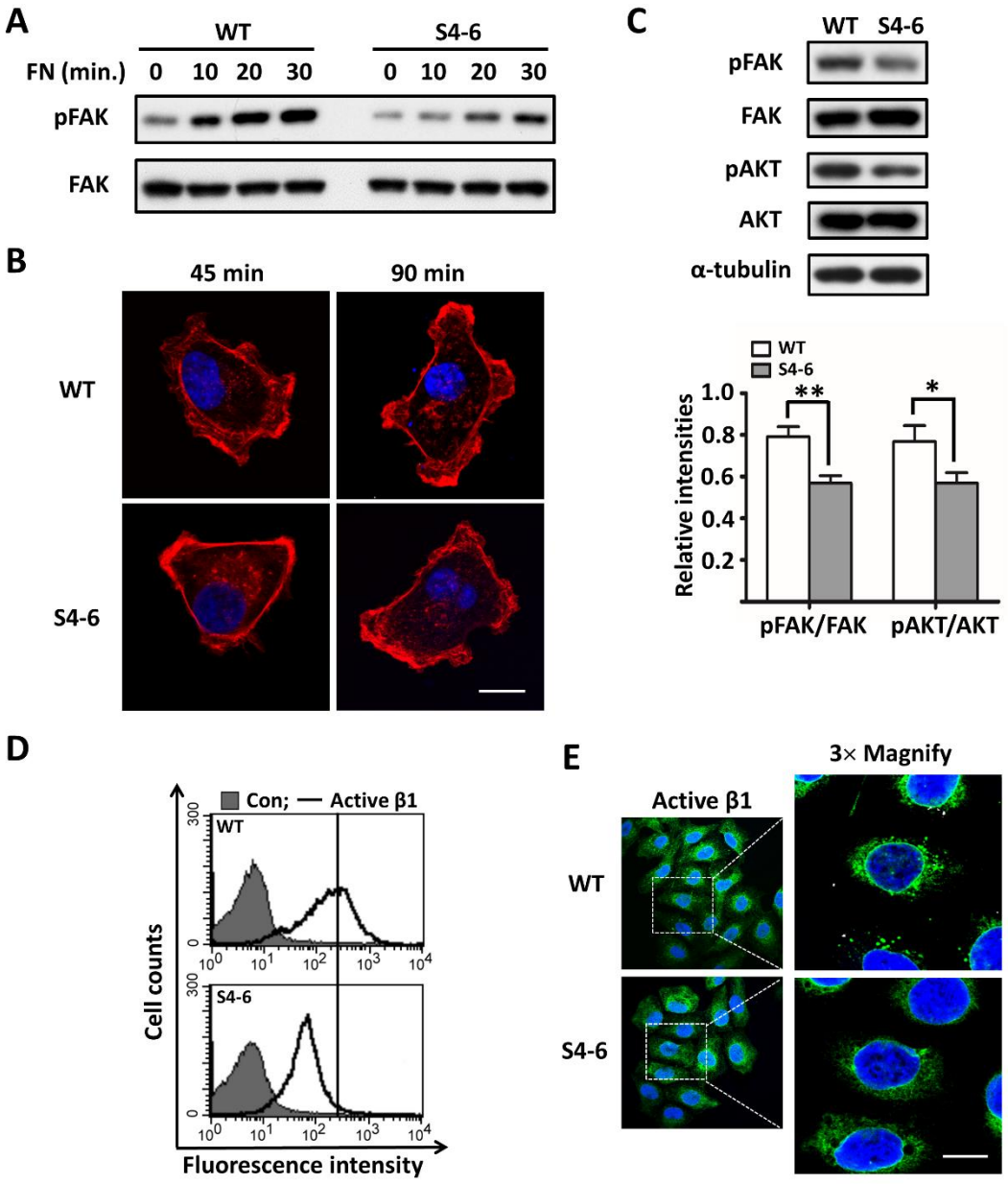

Figure 3. *N*-Glycosylation on β1 was essential for integrin-mediated cellular signaling and its activation. (A) WT and S4-6 cells were detached and then replated on the FN-coated (10 μg/ml) dishes for the indicated times. Cell lysates were collected and then immunoblotted by anti-pFAK, anti-FAK antibodies. (B) The indicated cells were detached and kept in suspension for 30 minutes prior to seeding. Then the cells were plated on FN-coated (10 μg/ml) glass bottom dishes for 45 min (left) or 90 min (right) in serum-free media. Localization of F-actin was examined by staining with Alexa Fluor 546 phalloidin, Scale bar, 20 μm. (C) Cell lysates from WT and S4-6 cells were immunoblotted with anti-pFAK, anti-FAK, anti-pAKT and anti-AKT antibodies. α-Tubulin was used as a loading control (upper panel). The quantitative data are presented as the mean±s.e.m from three

independent experiments (low panel) (*, $p<0.05$, **, $p<0.01$ by two-tail unpaired t-test). (D) WT and S4-6 cells were collected and incubated with (bold line) or without (grey shadow) anti-active β1 antibody (HUTS-4), followed by Alexa Fluor 647 goat anti-mouse IgG, and then subjected to flow cytometry. (E) I Immunofluorescence staining patterns of active integrin β1 in WT and S4-6 cells. The cells were stained with anti-active β1antibody (HUTS-4), followed by incubation with fluorescent secondary antibody. Scale bar, 20 μm.

2.3.4 *N*-glycosylation on the membrane-proximal domain of β1 (No. 9-12) is required for integrin mediated cellular signaling and its biological function.

Then, the question arose as to which of the *N*-glycosylation site(s) on β1 were important for its function in cell migration. To address this, we established several mutant cell lines expressing β1 with the restoration of *N*-glycosylation sites on its PSI, an upstream region of the hybrid domains (S4–6+1-3), the downstream region of the hybrid domain (S4–6+7-8), EGF repeat and β-tail domains (S4–6+9-12) (Fig. 4A). All of the *N*-glycosylation-restored cells exhibited similar expression levels of α5β1 on the cell surface (Fig. 4B). However, among these, only S4–6+9-12 mutant cells showed comparable abilities of cell spreading and migration on FN to the WT cells (Figs. 4C and D). Consistently, the levels of phospho-FAK and phospho-AKT were also rescued by restoration of *N*-glycosylation on the EGF repeat and β-tail domain (No. 9-12), instead of other domains (Fig. 4E). Taken together, these results clearly suggest that the *N*-glycosylation located in the vicinity of the cell membrane domain of β1 plays a crucial role in integrin β1-mediated cell signaling and cell migration.

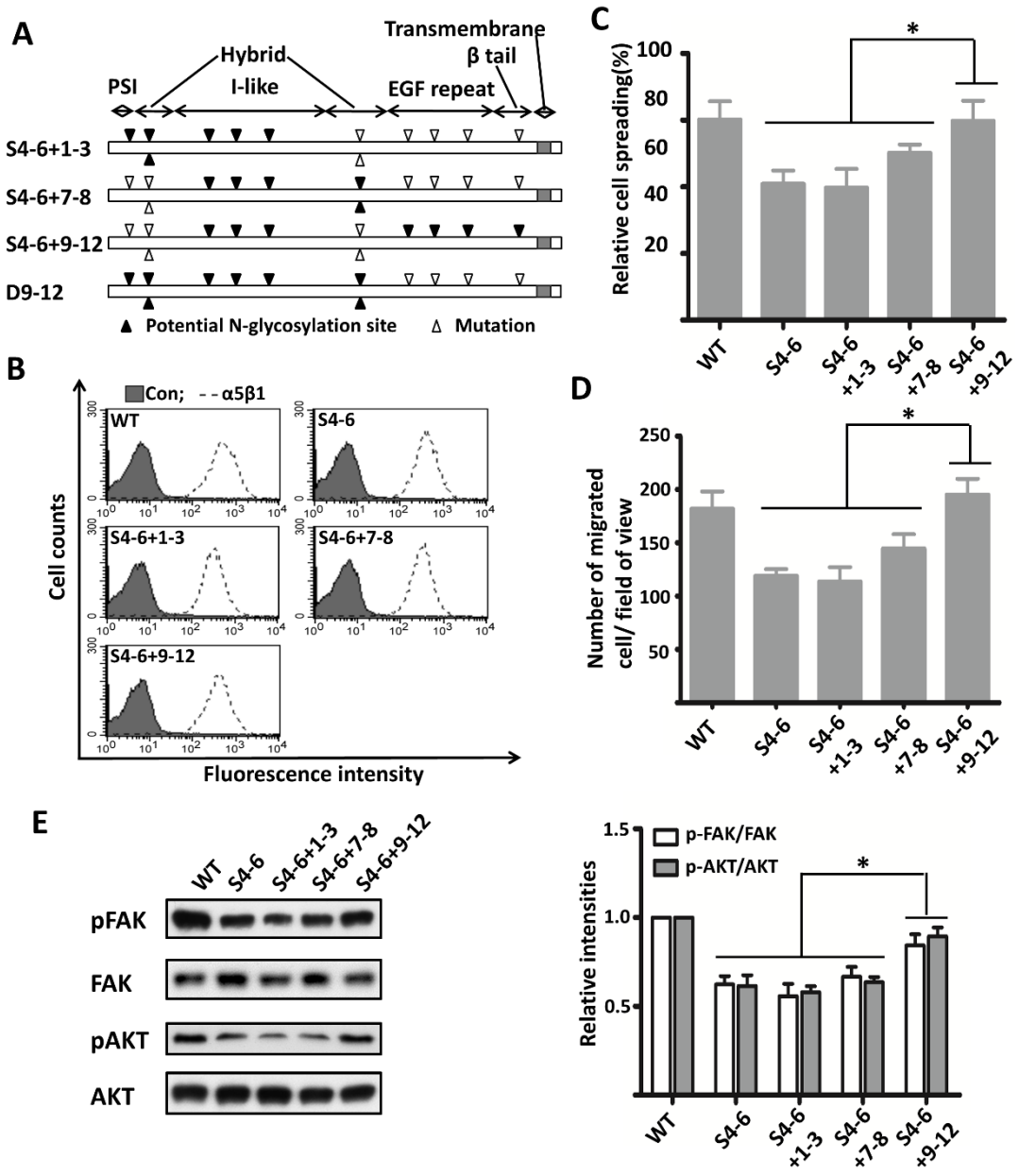

Figure 4. *N*-Glycosylation on the membrane-proximal domain played important roles in β1-mediated signal transduction and cell migration. (A) Schematic diagram of potential *N*-glycosylation mutant on β1 (S4-6+1-3, S4-6+7-8, S4-6+9-12 and D9-12). *N*-glycosylation sites are indicated by closed triangles, and point mutations are indicated by open triangles. (B) Expression levels of integrin α5β1 on cell surface analyzed by flow cytometry. Cells were collected and incubated with (dotted line) or without (grey shadow) anti-α5β1 antibody, followed by incubation with Alexa Fluor 647 goat anti-mouse IgG. (C) The indicated cells were detached and replated on the FN-coated dishes. The cell spreading was monitored as described under "EXPERIMENTAL PROCEDURES" section. The percentages of the spread cells were statistically analyzed and

presented as the mean±s.e.m of three independent experiments (*, $p<0.05$, by two-tail unpaired t-test). (D) The cell migration was determined by Transwell assay. The cells which migrated through the Transwell membrane were stained with 0.5% crystal violet. Representative photos were recorded and then the migrated cells were counted. The quantitative data were obtained from three independent experiments (*, $p<0.05$, by two-tail unpaired t-test). (E) Cell lysates from these mutant cells were immunoblotted by anti-pFAK, anti-FAK, anti-pAKT and anti-AKT antibodies. The quantitative data were presented as the mean±s.e.m from three independent experiments (*, $p<0.05$, by two-tail unpaired t-test).

2.3.5 α2,6-sialylation plays critical roles in integrin activation.

Next, to check the effect of *N*-glycosylation on β1 activation in these cells, flow cytometry analysis and immunostaining assay with anti-active β1 antibodies were carried out. The expression levels of active β1 in the intracellular and cell surface were clearly increased in the S4–6+9-12 cells, compared with S4-6 and S4-6+1-3 cells (Figs. 5A and B). Unexpectedly, the expression levels were also rescued in the S4–6+7-8 cells (Figs. 5A and B), although the cell migration could not be rescued as described above. Therefore, the question arose as to what could affect β1 activation. We considered the fact that ST6GAL1-mediated sialylation plays vital roles in β1 function and tumor progression (56-58), and a bioinformatics study estimated that sialylation on the I-like domain of β1 might regulate its activation via altering the binding of FN to β1 (59). Therefore, we checked the α2,6-sialylation levels in these mutants. As shown in Fig. 5C, the levels of the sialylated β1 and α5 were clearly higher in the S4–6+7-8 and S4–6+9-12 mutant cells than that in S4-6 cells, while the levels of sialylated α3 showed no significant difference in any of the mutant cells. In contrast, the S4–6+1-3 cells showed the lowest α2,6-sialylation levels of α5β1 among these cells, suggesting that *N*-glycosylation on PSI and on the upstream region of the hybrid (Figs. 4A) may have a negative impact on the α2,6-sialylation of α5β1 integrin. To further confirm that rescue of the integrin activation observed above was due to the increased sialylation level on β1, the ST6GAL1 gene was knocked out in WT, S4–6+7-8 or S4–6+9-12 mutant cells, and similar assays for β1 activation were performed. As expected, the ST6GAL1 knockout cells (ST6GAL1-KO) showed a clear decrease in the expression of active β1 on the cell surface, and this phenotype could be rescued in the ST6GAL1 restored cells (ST6GAL1-RES) (Fig. 5D). Collectively, these findings suggest that α2,6-sialylated *N*-glycans are crucial for integrin β1 activation.

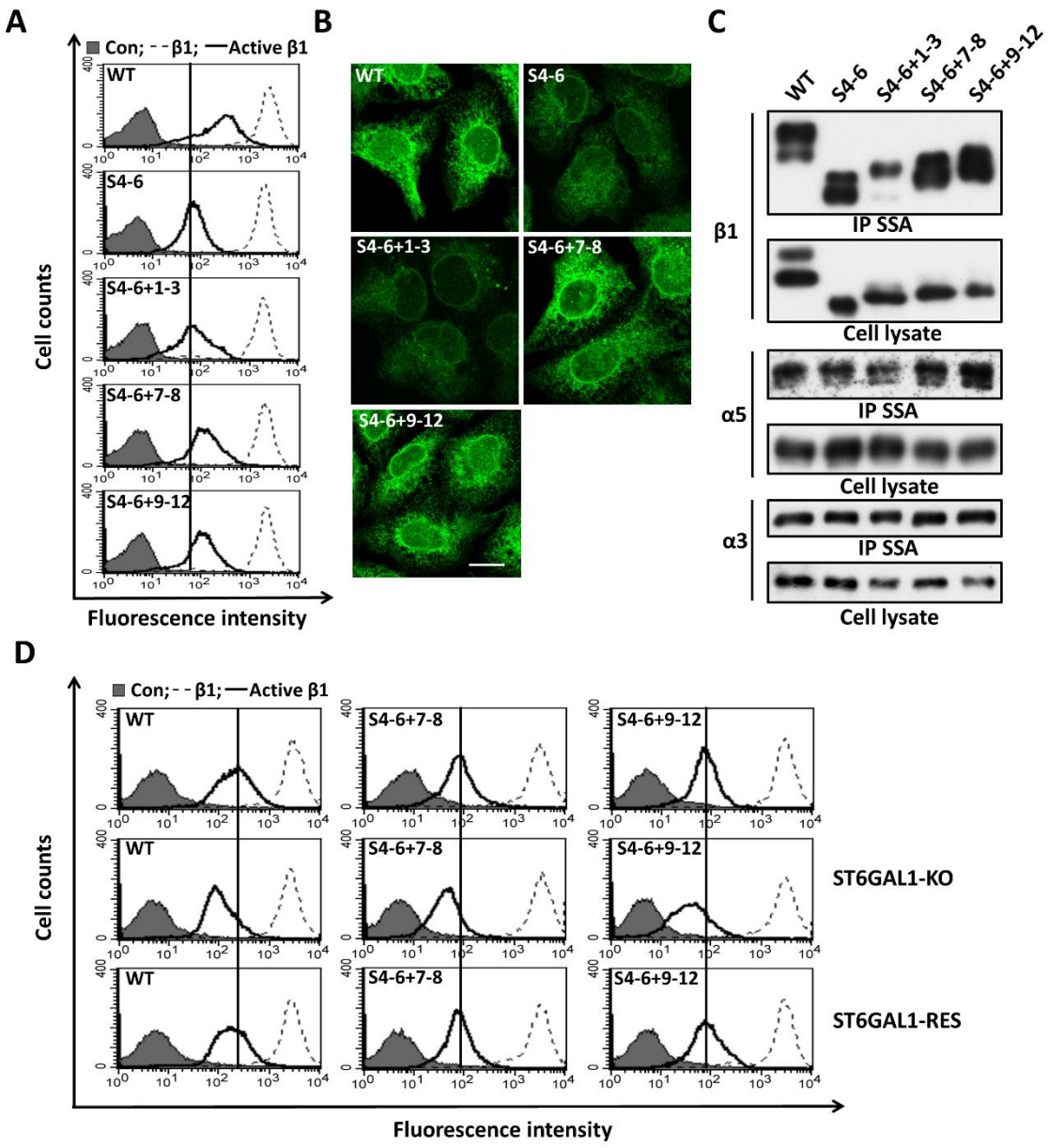

Figure 5. Effects of *N*-glycosylation on different domains on β1 activation. (A) The expression levels of total integrin β1 and active β1 on the cell surface were measured by flow cytometry. Cells were collected and incubated with anti-β1 (dotted line) and anti-active β1 (bold line) antibodies or without (grey shadow) primary antibody, followed by incubation with Alexa Fluor 647 goat anti-mouse IgG. (B) The expression levels of active β1 in different mutants, which were immunostained with antibody against active β1, followed by incubation with fluorescent secondary antibody. Scale bar, 20 μm. (C) Comparison of sialylation levels in these mutant cells. The cell lysates were immunoprecipitated by SSA-agaroses, which specifically recognized α2,6-sialylation, and probed with antibodies against integrin α3, α5, or β1. (D) Effects of sialylation on β1 activation.

The derivative cell lines as indicated were incubated with anti-β1 antibody (dotted line), anti-active β1 antibody (bold line) or without (grey shadow) primary antibody, followed by incubation with appropriate Alexa Fluor 647 goat anti-mouse IgG, and then subjected to flow cytometry. ST6GAL1-KO, ST6GAL1 knockout cells; ST6GAL1-RES, ST6GAL1-KO cells rescued by overexpression of ST6GAL1.

2.3.6 *N*-glycosylation of β1 on the membrane-proximal domain is important for the signaling complex with other receptors.

The question regarding why only the S4–6+9-12 *N*-glycosylation mutants could rescue both the cell migration and cellular signaling remains unclear. Considering that integrin-mediated cellular events are thought to be regulated by not only a conformational change caused by integrin activation, but also by coordination between integrin and other membrane proteins (60), we further investigated β1-mediated complex formation. Here, we chose two representative membrane proteins, syndecan-4 and epidermal growth factor receptor (EGFR), which have a vital influence on integrin-mediated cellular functions. Syndecan-4 is known to synergize with the FN-specific integrin α5β1 to regulate its activation, adhesion-complex formation, cell spreading and migration (61, 62). On the other hand, it is also well known that the EGFR cooperation with integrin β1 has important implications for the regulation of diverse cell events (60, 63). As shown in Fig. 6A, the immunoprecipitation assay showed little difference in the interaction of integrin β1 with EGFR or syndecan-4 between the S4–6+9-12 cells and WT cells, but those interactions were clearly inhibited in the mutant cells such as S4–6, S4–6+1-3, and even S4–6+7-8 cells. To further confirm this result, we established D9-12 cells expressing β1 without *N*-glycosylation sites on EGF repeat and β-tail domains (Fig. 4A), and found the interactions between D9-12 integrin and EGFR or syndecan-4 were clear decreased compared with those in the WT and S4-6+9-12 cells (Fig. 6B). As described above, the *N*-glycosylation on the EGF repeat and β-tail domain (No. 9-12) regulated cell membrane signaling complex formation, and we also detected an up-regulated overall level of sialylated α5β1 in this mutant. Therefore, we wondered whether α2,6-sialylation on β1 participates in its association with other membrane receptors. The attenuated interactions of β1 with EGFR or syndecan-4 were observed in ST6GAL1 KO in both WT and S4-6+9-12 cells (Fig. 6C), which indicated that the sialylation on β1 was required for its cooperation with EGFR and syndecan-4. Taken together, these results strongly suggest that the *N*-glycosylation of β1 on the membrane-proximal domain regulates cell migration and cellular signaling by promoting β1 activation and complex formation.

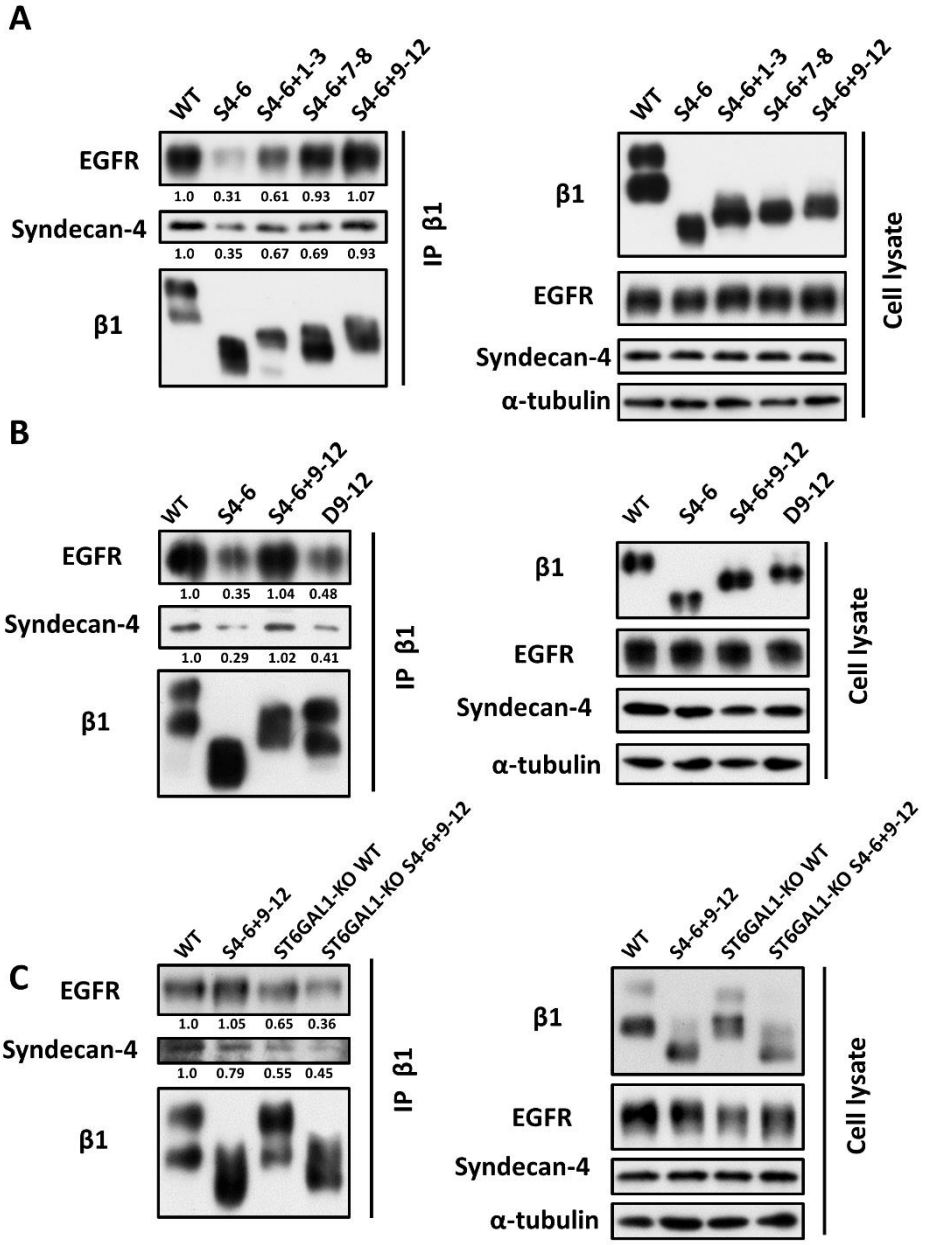

Figure 6. (A) Cell lysates obtained from WT and the 4 indicated *N*-glycosylation mutants were immunoprecipitated (IP) with anti-β1 antibody (P5D2), and these immunoprecipitants were then subjected to Western blotting with anti-EGFR, anti-syndecan-4, or anti-β1 antibodies (left panel). The whole cell lysates were also subjected to Western blotting with the indicated antibodies (right panel; as an input). (B) The cell lysates of WT and the 3 indicated mutants were immunoprecipitated with anti-β1 antibody, and these immunoprecipitants were blotted with the indicated antibodies as described above (left panel). The total expression levels of these proteins were blotted with the indicated antibodies (right panel). (C) The cell lysates of WT and the indicated 3 mutants were immunoprecipitated with anti-β1 antibody, and these immunoprecipitants were blotted with the

indicated antibodies (left panel). The total expression levels of these proteins were blotted with the indicated antibodies (right panel). The ratio of band density of EGFR or syndecan-4 vs. band density of β1 in WT cells was set as 1.0.

2.4 Discussion

The *N*-glycosylation of integrin β1 is involved in diverse biological functions including cell adhesion, cell migration and trans-membrane signaling. However, most studies have focused mainly on the entire alteration in *N*-glycosylation and little is known about the function of individual *N*-glycans on integrin β1. The present study is the first to show that different *N*-glycosylations of β1 have distinct functions and participate in different cell biological processes. In detail, although both *N*-glycosylation sites on 7-8 and 9-12 play important roles in β1 activation, only the membrane-proximal *N*-glycosylation on the EGF repeat and β-tail domain (No. 9-12) rescued cell migration and β1-mediated complex formation. Furthermore, *N*-glycosylation on the PSI and upstream region of the hybrid domain (No. 1-3) suppressed the α2,6-sialylation and β1 activation. In addition, our data clearly suggest that α2,6-sialylated *N*-glycans on the β1 membrane-proximal domain play essential roles in promoting cell migration, which provides new insights into the molecular mechanisms for aberrant *N*-glycosylation in tumors.

Structural and functional studies have shown that integrins undergo rapid conformational changes that increase the affinity for ligand binding, which is referred to as "activation". Activation is an important mechanism by which cells regulate integrin function (64, 65). Previous studies were primarily focused on the cytoplasmic domains of integrin, as Talin binding to the integrin β tail is the last common step either during "inside-out" activation or "outside-in" activation (64, 66). There are two important cytoplasmic NPxY (Asn-Pro-X-Tyr) motifs in the integrin β1 tail: one is the membrane-proximal NPxY motif, which can associate with talin and is crucial for connecting integrin to the actin cytoskeleton; the other is the membrane-distal NPxY motif, which can bind with kindlin and is responsible for the integrin α5β1 expression and degradation (46). However, recent studies have indicated that the extracellular domain is also important for its activation process by regulating ligand binding ability and the interaction with the cytoplasmic domain molecules during integrin activation (67, 68). Consistent with a molecular modeling study showing that *N*-glycosylation surrounding the RGD-binding pocket of integrin α5β1 affects its binding to FN (69), our data here also support the notion that the *N*-glycosylation of integrin is involved in its activation process. Furthermore, we showed that either the re-expression of *N*-glycans on the downstream region of the hybrid domain (No. 7-8) or the EGF repeat and β-tail domain (No. 9-12), but not S4-6 or S4-6+1-3 mutants, could rescue the β1 activation. Although the detailed mechanisms remain unclear, it is reasonable to postulate that *N*-glycosylation on these two domains may exhibit some specific *N*-glycans or/and

localizations, which contribute to integrin activation, and may also regulate the internalization of integrin α5β1.

Given the terminal location of sialic acids and their relatively strong electronegative charge, it is reasonable to assume that ST6GAL1-mediated sialylation plays crucial roles in the regulation of β1 conformation, activation and its mediated functions (56, 70). In addition to effects of the overall sialylation of integrin β1, sialylation on some specific domain, such as I-like domain, is thought to affect its binding ability to FN and α5β1-mediated cellular activities (59). Here, our results clearly showed that different *N*-glycosylation sites exhibited different patterns of α2,6-sialylation. As shown in Fig. 5, the α2,6-sialylated level in the S4–6+1-3 mutant was even lower than that in S4-6 mutants, indicating that some *N*-glycosylations might be involved in the regulation of the sialylation level of integrin β1. One possibility for its regulatory mechanism is that *N*-glycosylation on the PSI, an upstream region of the hybrid domain, regulates integrin conformation, which may block the accessibility of ST6GAL1 to modify *N*-glycosylation sites 4-6 (S4-6), as we previously demonstrated that GnT-III could modify the specific *N*-glycosylation of α5 subunit (71).

It is noteworthy that integrin activation is crucial for its functions but is insufficient for full biological functions. In the present study, we observed that although two *N*-glycosylation mutants could rescue β1 activation, only one mutant could fully rescue the β1-mediated cell migration and cellular signaling. Given the evidence that sophisticated networks of extracellular signals control diverse aspects of cell fate (63, 72), it is reasonable to think that the co-operation of integrin with other membrane proteins may serve as a possible explanation for this phenomenon. Actually, the α2,6-sialylation on the membrane-proximal domain of β1 mutant could greatly facilitate the interaction with syndecan-4 and EGFR, which plays key roles in cell migration and cell signaling. Similar to β1, we recently found that the *N*-glycosylation on the calf-1,2 domain of integrin α5 suppresses the phosphorylation of EGFR and cell signaling (73). Paszek et al. reported that a bulky glycocalyx can influence integrin clustering and activity, facilitate focal adhesion assembly and integrin-dependent growth factor signaling (74). Seales et al. described that β1 hypersialylation can promote the association with talin, which is the final step of integrin activation, and then contribute to the up-regulation of cell migration in the colon adenocarcinomas (56). Taken together, these studies strongly support the notion that integrin mediated cellular functions can be regulated by the *N*-glycans and that the importance of *N*-glycosylation is reflected in either the specific domain or the structure of *N*-glycans. Based on the results of the present study, *N*-glycosylation on the membrane-proximal domain of β1 was essential

for FN-mediated cell migration, and it is highly possible that specific *N*-glycans may exist in different conformations, which would place different influences on diverse cell behaviors. Then a question arises regarding how *N*-glycosylation on β1 mediated its association with EGFR and syndecan-4. One possibility could be that some lectins and glycosphingolipids may act as a "linker" for interaction between the *N*-glycosylation on β1 and other membrane proteins. As previously reported that galectin-3, one kind of animal lectins with affinity for β-galactosides, could associate with both β4 integrin and EGFR and thereby cross-link the two molecules (75, 76). Besides galectins, gangliosides are also involved in the interaction with integrin-EGFR complex formation (73, 77). However, we could not exclude the possibility of a direct interaction between β1 and membrane proteins, as an unknown lectin-like domain may exist on EGFR, since a lectin domain on integrin αMβ2 has been reported to mediated its association with GlcNAc on platelets (78).

Aberrant glycan structures on integrin are closely associated with malignant cell behaviors. These alterations in glycosylation are part of a long-term process, from maturation, cell surface localization to recycling until degradation (21, 73, 79). Our previous study showed that *N*-glycosylation on β1 I-like domain plays essential roles in α5β1 heterodimer formation. As part of a continuing study, the present investigation demonstrated that *N*-glycosylation on the membrane-proximal region of β1 is crucial for the regulation of cell migration and complex formation. Our sequential analysis of the *N*-glycosylation of integrin is important for elucidating the molecular mechanisms underlying integrin-mediated biological processes, which could provide insights for cancer treatment.

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Abbreviations

The abbreviations used are as follows: BSA, bovine serum albumin; DKO, double knockout; DMEM, Dulbecco's Modified Eagle's Medium; ECM, extracellular matrix; EGFR, epidermal growth factor receptor; EMT, epithelial-to-mesenchymal transition; FAK, focal adhesion kinase; FBS, fetal bovine serum; FN, fibronectin; IP, immunoprecipitation; KO, knockout; MAPK, mitogen-activated protein kinase; MET, mesenchymal-epithelial transition; TGF-β, transforming growth factor-β; PFA, paraformaldehyde; PI3K, phosphatidylinositol-3 kinase; PSI, plexin-semaphorin-integrin domain; Res, rescue; RTK, receptor tyrosine kinase; SSA, Sambucus sieboldiana agglutinin; ST6GAL1, β-galactoside 2,6-sialyltranferase 1; WB, western blot; WT, wild-type.

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