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The novel α4B murine α4 integrin protein splicing variant inhibits α4 protein-dependent cell adhesion

Hitomi Kouro[1,§], Shigeyuki Kon[1,§,*], Naoki Matsumoto[1], Tomoe Miyashita[1], Ayaka Kakuchi[1], Dai Ashitomi[1], Kodai Saitoh[1], Takuya Nakatsuru[1], Sumihito Togi[1], Ryuta Muromoto[1], and Tadashi Matsuda[1,*]

[1]Department of Immunology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-12 Nishi-6, Kita-Ku, Sapporo, 060-0812, Japan

*****Running title: *α4B is a novel endogenous inhibitor of α4 integrin*

[§]These authors contributed equally to this work
[*]To whom all correspondence should be addressed:
Dr. S. Kon, Department of Immunology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-12 Nishi-6, Kita-Ku, Sapporo, 060-0812, Japan. E-mail: kon@pharm.hokudai.ac.jp
Phone: +81-11-706-3920 Fax: +81-11-706-4990
Dr. T. Matsuda, Department of Immunology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-12 Nishi-6, Kita-Ku, Sapporo, 060-0812, Japan. E-mail: tmatsuda@pharm.hokudai.ac.jp
Phone: +81-11-706-3243 Fax: +81-11-706-4990

Key words: Integrin, Extracellular matrix, Cell adhesion, Alternative splicing, Tumor metastases,

Background: α4 integrin participates in tumor metastasis and autoimmune diseases.
Results: α4B markedly inhibited α4 integrin-dependent cell adhesion through cytoplasmic region of α4B.
Conclusion: α4B is a novel endogenous inhibitor of α4 integrin.
Significance: This study provides a possible role of α4B as an inhibitor of α4 integrin-mediated metastasis.

ABSTRACT

Integrins affect the motility of multiple cell types to control cell survival, growth, or differentiation, which are mediated by cell-cell and cell-extracellular matrix interactions. We previously reported that the α9 integrin splicing variant, SFα9, promotes wild-type (WT) α9 integrin-dependent adhesion. In this study, we introduced a new murine α4 integrin splicing variant, α4B, which has a novel short cytoplasmic tail. In inflamed tissues, the expression of α4B, as well as WT α4 integrin, was up-regulated. Cells expressing α4B specifically bound to VCAM-1, but not other α4 integrin ligands such as fibronectin CS1 or osteopontin. The binding of cells expressing WT α4 integrin to α4 integrin ligands is inhibited by co-expression of α4B. Knockdown of α4B in metastatic melanoma cell lines results in a significant increase of lung metastasis. Expression levels of WT α4 integrin are unaltered by α4B; with α4B acting as a regulatory subunit for WT α4 integrin by dominant-negative effect or inhibiting α4 integrin activation.

Integrin adhesion receptors are large

heterodimeric cell surface receptors that mediate the adhesion of cells to the extracellular matrix (ECM), and to other cells (1). They participate in embryonic development and in the maintenance of homeostatic balance (2). They also function in a range of pathological processes, including wound repair, inflammation, leukocyte trafficking, and tumor metastasis (3). These integrin adhesion receptors comprise large extracellular, transmembrane, and small cytoplasmic domains. The extracellular domain is responsible for ligand binding, while the cytoplasmic domains play a crucial role in the attachment of cells to ECM ligands (4). Activation of integrins is accompanied by a conformational shift from a low to a high binding affinity state courtesy of ligand stimulation (5).

The α4 integrin (CD49d) is expressed on several types of leukocytes and tumor cells, and is involved in autoimmune diseases and tumor metastases by mediating cell attachment to: vascular cell adhesion molecule-1 (VCAM-1); the CS1 domain within alternative splicing forms of fibronectin; the pro-polypeptide of von Willebrand factor (pp-vWF); and osteopontin (OPN) (6-9). The α4 integrin on $CD4^+$ T cells is required for the development of experimental autoimmune encephalomyelitis (EAE) (10). On tumor cells, α4 integrin promotes dissemination to distal organs by increased adhesion to vasculature endothelium, and by facilitating the extravasation of tumor cells (6). *In vivo* blocking studies demonstrated that autoimmune diseases including EAE, and tumor metastases are inhibited by antibodies against α4 integrin (11,12). Analyzing the regulatory mechanisms of α4 integrin is important for understanding extravasation of autoimmune diseases and tumor metastasis.

In this study, we attempted to clone a novel murine α4 integrin splicing variant (α4B), which contained a short cytoplasmic tail. This variant α4B is endogenously translated and expressed on the cell surface with the β1 integrin. The α4B variant is able to bind to VCAM-1, but is dependent upon KVIL cytoplasmic sequences.

EXPERIMENTAL PROCEDURES

Mice - Mice were kept under specific pathogen-free (SPF) conditions, and provided food and water *ad libitum*. All experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of Hokkaido University.

Cell culture and reagents - NIH3T3, Plat-E cells, CHO cells, and B16 mouse melanoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Anti-mouse α4 integrin antibodies, (R1-2), (9C10) (Biolegend) and (PS/2) (Millipore) were used in flow cytometry and immunoprecipitation. An anti-β1 integrin antibody (KMI6) was obtained from eBiosciences. For western blotting analyses, the following antibodies were used: p-ERK (E-4) (Santa Cruz Biotechnology); ERK1 (K-23) (Santa Cruz Biotechnology); p-AKT (S473) (193H12) (Cell signaling); AKT1/PKB (paired 473) (Anaspec); p-FAK (Y397) (Cell Signaling); FAK (clone 4.47) (upstate); Paxillin (clone 349) (BD Transduction Laboratories); p-Paxillin (PY118) (BD Biosciences Pharmingen); and α4 integrin (C-20) (Santa cruz).

α4B cDNA cloning - The cDNAs encoding α4B were cloned from B16-BL6 cells using a 3′ rapid amplification of cDNA ends (RACE) method. Total RNA isolated from B16-BL6 cells was reverse transcribed using an oligo-dT primer containing an anchor sequence. Amplification using polymerase chain reaction (PCR) was carried out using the anchor primer and an α4 integrin-specific primer (5′-AGC GAT AAC AAA CTC CCC ACT-3′). To increase the specificity of the reaction, a nested PCR was conducted with a second α4 integrin-specific primer (5′-TAG AGG CCA CAT ACC ACC TTG-3′ or 5′-TGG ATC TAG CGA AGA AAA CGA-3′). Amplicons were

then cloned into the TOPO-TA vector (Invitrogen) and sequenced. The coding region of α4B was amplified by PCR and cloned into pcDNA3.1 (Invitrogen) or pBabepuro and pWZL-blast2 using the infusion system (Takara). FLAG-tagged deletion mutants of α4B (α4BΔVIL) cDNAs were amplified using specific primers (5′-GGC GCC GGC CGG ATC CGC CAC CAT GGC TGC GGA AGC GAG GTG-3′ and 5′-ATT CCA CAG GGT CGA CTT ACT TGT CAT CGT CAT CCT TGT AGT CCT TCC ACA TAA CAC ATG AAA T-3′) then cloned into pBabepuro.

EAE induction – EAE was induced in SJL/J mouse by immunization with an emulsion of 100 μg PLP 139-151 peptide in a mixture containing 100 μl complete Freund's adjuvant (Difco). Each mouse received an intravenous injection of 400 ng of pertussis toxin on days 0 and 2 (13).

Generation of stable cell lines - The α4 integrin-pBabepuro or α4B-pBabepuro vectors were transfected into Plat-E packaging cells (14) using Lipofectamine 2000 (Invitrogen). At 2 and 3 days post-transfection, virus-containing supernatants were harvested and filtered through a 0.45-μm filter and added to 20% confluent NIH3T3 cells in the presence of 8 μg/ml polybrene, then cultured for 18–20 h. The virus-containing medium was aspirated and cells were cultured in DMEM containing 10% FCS and 10 μg/ml puromycin (Sigma-Aldrich). For generation of NIH3T3 cells expressing both α4 integrin and α4B, retroviruses were generated by transfecting α4B-pWZLblast2 into Plat-E cells, added to NIH3T3 cells expressing α4 integrin. Transduced NIH3T3 cells were cultured in DMEM containing 10% FCS, 10 μg/ml puromycin, and 2.5 μg/ml blasticidin (Invitrogen). NIH3T3 cells expressing the α4 integrin and/or α4B were identified by flow cytometry using the anti-α4 antibody. For generation of CHO cells co-expressing α4B and α4 integrin, CHO cells transfected with α4B-pcDNA3.1neo were cultured in DMEM containing 10% FCS, 800 μg/ml G418 (Invitrogen), analyzed for expression of α4B by flow cytometry with the anti-α4 antibody (R1-2). CHO cells differentially expressing α4B on cell surface were transfected with α4 integrin-pcDNA3.1Hygro, then cultured in DMEM containing 10% FCS, 250 μg/ml Hygromycin B (Invitrogen). Cells expressing WT α4 integrin at comparable level were selected by flow cytometry with the anti-α4 antibody (19E4).

Integrin ligands for cell adhesion tests - Plasma fibronectin (FN) (Sigma) was used for assessing Arg-Gly-Asp (RGD)-dependent cell adhesion. The integrin ligands used in the adhesion tests were: SVVYGLR (15); CQDHSFSIVIETVQ (8); and VTLPHPNLHGPEILDVPST (16). These encode the binding sequences of osteopontin (OPN), the pro-polypeptide of von Willebrand factor (pp-vWF), and fibronectin-CS1 (FN-CS1), respectively. VCAM-1 (Peprotech) was used for α4 integrin-dependent cell adhesion. These molecules were used at the following concentrations in the cell adhesion assays: 2 μg/ml (FN); 10 μg/ml (OPN); 10 μg/ml (pp-vWF); 10 μg/ml (FN-CS1); and 2 μg/ml (VCAM-1).

Cell adhesion assays - The wells of 96-well plates were coated with the various integrin ligands overnight at 4ºC, followed by blocking with 0.5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 h at room temperature. Cells were suspended in DMEM containing 0.25% BSA and 200 μl of cell suspension (at a cell density of 2.5×10^5 cells/well), seeded into the 96-well plates, and incubated for 1 h at 37ºC. The medium was then removed and all wells were washed twice. Adherent cells were fixed and stained with 0.5% crystal violet in 20% methanol for 30 min. Wells were rinsed three times with water, and adherent cells lysed with 20% acetic acid. The resulting supernatants from each well were analyzed using a plate reader (Bio-Rad Laboratories), with the absorbance at 595 nm measured to determine the relative number of adherent cells.

Generation of antibodies – Anti-WT α4 integrin antibody (clone #19E4) or anti-α4 integrin antibody (clone #5X2) was generated in Sprague-Dawley rats or Syrian hamsters, respectively, immunized with cells expressing WT α4 integrin. Their splenocytes were fused with X63-Ag8-653 mouse myelomas as previously described (17). Hybridoma cells were screened using enzyme-linked immunosorbent assays (ELISAs). Anti-α4B antibody was generated in rabbit immunized with a peptide derived from the C-terminal domain of α4B, CVMWKVIL as previously described (17).

Immunoprecipitation and western blot analysis - NIH3T3 cells expressing α4 integrin, α4B, or B16 cells were lysed on ice for 30 min in lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, and 1× protease inhibitors (1× Complete Mini Protease Inhibitor Cocktail; Roche Molecular Biochemicals)). Lysates were clarified by centrifugation at 16,000 × g for 10 min at 4ºC, and incubated with protein G-Sepharose beads coated with anti-α4 integrin antibody at 4ºC for 1 h. The beads were washed with the same buffer five times, and precipitated polypeptides were extracted in Laemmli sample buffer. Protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions, probed with the antibody against FLAG, HA, β1 integrin, α4 integrin, or α4B and positive signals detected by Plus-ECL (PerkinElmer).

Flow cytometry - For α4 integrin or α4B expression, cells were blocked with normal goat serum then incubated with a phycoerythrin (PE)-labeled anti-mouse α4 integrin antibody. For WT α4 integrin specific expression, cells were incubated with the #19E4 antibody and PE-labeled goat anti-rat IgG antibody after blocking. All analyses were conducted on a FACSCalibur flow cytometer (BD Biosciences).

Analysis of mRNA expression - Total RNA from mouse tissues, and from the spinal cords of EAE mice at day 14 were extracted with Trizol (Invitrogen). Specific primers were used for reverse transcription PCR (RT–PCR) and quantitative PCR (qPCR) assays to amplify G3PDH (5′-ACC ACA GTC CAT GCC ATC AC-3′ and 5′-TCC ACC ACC CTG TTG CTG TA-3′), α4 integrin (5′-AAG GAA GCC AGC GTT CAT ATT-3′ and 5′-TCA TCA TTG CTT TTG CTG TTG-3′), α4B integrin (5′-AAG GAA GCC AGC GTT CAT ATT-3′ and 5′-AAA GGC ATG GTG TCC ATG TAA-3′), and α9 integrin (5′-GTC TGG GAG GAG GCT AAA CC-3′ and 5′-CAC TGA GGT GCT GTG ATG TTG-3′). The qPCR assays were conducted on an Mx3005P (Stratagene). Amplified cDNAs was detected using SYBR Green (Invitrogen) and standardized to ROX dye levels. The cDNA concentrations were expressed as the number of cycles to threshold (Ct), and Ct values were normalized to G3PDH cDNA levels in the same samples. The absolute copy numbers of particular transcripts in B16 cells were calculated from standard curves generated with a 10-fold dilution series of a quantified template DNA.

Tumor metastasis - B16-BL6 cells were transfected with 50 nM siRNA (0.1 ml/cm^2) and complexed with LipofectAMINE 2000 (Invitrogen). Mice were inoculated intravenously (i.v.) into the lateral tail vein with 2×10^5 B16 cells that had been transfected with siRNA in 0.2 ml of PBS. Mice were sacrificed at 21 days post-inoculation. Lungs were removed and weighed immediately, and lung metastatic foci counted.

Statistical analysis - Data are presented as means ± the standard error of the mean (SEM), and are representative of at least three independent experiments. The statistical significance of differences between groups was calculated using a two-tailed Student's t-test. Differences were considered to be significant when $P < 0.05$ (*) or 0.005 (**).

RESULTS

α4B is an alternative splicing variant of α4 integrin - We used a 3′ RACE method involving mouse melanoma B16 cell cDNAs, and identified a novel mouse α4 integrin splicing variant, which we designated α4B (Genbank accession number: AB850880). The α4B variant consisted of identical extracellular and transmembrane domains as the WT α4 integrin, and contained the novel short amino acid sequence, KVIL (Fig. 1A). Truncation occurred after Lys100, the last amino acid of exon 27. The α4B cDNA that encoded the novel VIL amino acid sequence was from an intron (designated region Ex27b) occurring after exon 27.

Expression of α4B mRNAs in normal and inflamed mouse tissues - The WT α4 integrin is expressed in various normal tissues, especially immune tissues such as the spleen and lymph nodes. Using normal mouse tissues, RT-PCRs were conducted to determine the expression levels of α4B mRNAs; these transcripts were detected in cells expressing WT α4 integrin (Fig. 1B). The WT α4 integrin is involved in the development of EAE by $CD4^+$ T cells infiltrating the spinal cord. We found that α4B mRNAs, as well as WT α4 integrin levels, in the spinal cords of EAE mice were increased (Fig. 1C).

α4B is expressed on the cell surface with β1 integrin – Cell lysate from NIH3T3 cells expressing HA-tagged α4 integrin or FLAG-tagged α4B were analyzed by western blot. 150 kDa and 140 kDa bands of α4 integrin, which are mature and precursor form (18), were detected by anti-HA antibody. A 130 kDa of α4B was detected by anti-FLAG antibody. Three bands were detected in lysate from NIH3T3 cells co-expressing HA-tagged α4 integrin and FLAG-tagged α4B (α4 integrin/α4B/NIH3T3) by antibody mixture against HA and FLAG (Fig.1D). Next, we used flow cytometry to analyze surface expression of α4B on NIH3T3 (Fig.1E). We tested whether α4B associates with the β1 integrin subunit. Immunoprecipitation analysis using α4B/NIH3T3 lysates and an anti-α4 integrin antibody revealed that the β1 subunit was co-immunoprecipitated with α4B (Fig. 1F).

α4B inhibits cell adhesion mediated by WT α4 integrin - We assessed an ability of α4B to support cell adhesion to α4 ligands since α4B is expressed on cell surface. Plasma FN was used as positive control. NIH3T3 cells transfected with an empty vector (Mock/NIH3T3) bound to FN but not to other ligands, indicating that α4 and α9 integrins are not expressed on NIH3T3 cells. In NIH3T3 cells expressing α4 integrin (α4 integrin/NIH3T3), appropriate binding to OPN, pp-vWF, FN-CS1 and VCAM-1 was observed. The α4B/NIH3T3 cells specifically bound to the VCAM-1 protein. Divalent cations such as Mn^{2+} can stimulate integrin interactions with ligand (19). Mn^{2+} leads to enhanced cell adhesion of α4 integrin/NIH3T3, whereas does not influence the adhesion manner of α4B/NIH3T3 (Fig. 2A).

Cytoplasmic region of integrin is shown to elicit signals from outside of cells and regulates integrin-mediated cell functions (20,21). Therefore, we next examined cellular signal transductions involved in cell adhesion after cell attachment using phospho-specific antibodies to evaluate the activation status of signaling molecules. Cell lysates from α4 integrin/NIH3T3, α4B/NIH3T3, or α4 integrin/α4B/NIH3T3 cells after attachment to FN or VCAM-1 were analyzed by western blot. The expression of α4B resulted in weak ERK activation compared with that induced by WT α4 integrin; no altered activation of AKT, Paxillin, or FAK was observed (Fig. 2B).

We next examined whether α4B modulates the function of WT α4 integrin. For this experiment, α4 integrin/α4B/NIH3T3 cells were used for cell adhesion assay. This assay revealed that α4 integrin/α4B/NIH3T3 cells exhibited decreased cell adhesion to α4 ligands compared with α4 integrin/NIH3T3 (Fig. 2C). The negative effect of α4B is partially overcome by Mn^{2+} treatment. To

confirm the possibility that α4B down-regulates expression of WT α4 integrin on the cell surface, we conducted flow cytometry to determine surface expression level of the WT α4 integrin on α4 integrin/α4B/NIH3T3 cells. We found that three commercially available antibodies against α4 integrin cross-reacted with WT α4 integrin and α4B (Fig. 3A). We attempted to generate novel antibodies specific for α4 integrin and successfully established the #19E4 antibody, which recognized the WT α4 integrin (Fig. 3B). Using the #19E4 antibody in flow cytometry, we found that the surface expression level of WT α4 integrin was unaltered by α4B (Fig. 3C). Thus, decreased cell adhesion of α4 integrin/α4B /NIH3T3 was not due to down-regulation of WT α4 integrin expression on the cell surface.

The KVIL α4B cytoplasmic sequence plays an important role in activation of WT α4 integrin - Our successful generation of #19E4 antibody indicates that the conformation between α4 integrin and α4B differs. In comparison with WT α4 and α4B, the VIL sequence in the α4B cytoplasmic region is derived from Ex27b. This conformational change in α4B might be caused by the VIL sequence in the cytoplasmic region. We examined whether the VIL sequence in α4B is critical for intrinsic conformation, functions during cell adhesion in the same way as VCAM-1. A VIL sequence-deleted α4B mutant, α4BΔVIL, was generated (Fig. 4A). We observed that α4BΔVIL was expressed on the cell surface (Fig. 4B). In cell adhesion assays using α4BΔVIL, binding to the VCAM-1 protein was completely inhibited, although no significant effects on cell adhesion to an irrelevant substrate (plasma FN) were observed (Fig. 4C). To confirm the importance of the VIL sequences, NIH3T3 cells expressing both WT α4 integrin and α4BΔVIL (α4 integrin/α4BΔVIL/NIH3T3) were established. α4 integrin/NIH3T3, α4 integrin/α4B/NIH3T3, and α4 integrin/α4BΔVIL/NIH3T3 cells showed comparable expression level of WT α4 integrin. α4B and α4BΔVIL were expressed at the comparable level in α4 integrin/α4B/NIH3T3 and α4 integrin/α4BΔVIL/NIH3T3 cells (Fig.4D). Cell adhesion assays demonstrated that α4 integrin/α4BΔVIL/NIH3T3 cells bound to α4 integrin ligands, while α4 integrin/α4B/NIH3T3 cells exhibited decreased binding as shown in Fig 2C (Fig. 4E). These results suggest that WT α4 integrin receives negative signal elicited by cytoplasmic sequence of α4B.

Inhibitory effect of α4B is due to a dominant-negative effect for VCAM-1 adhesion, but not for OPN, pp-vWF, FN-CS1 – We next asked whether α4B exerts dominant-negative effect. For this experiment, we established CHO cells co-expressing WT α4 integrin and differentially expression level of α4B (Fig.5A). Then, we found that cells having lowest α4B expression (clone # 10L) did not inhibit cell adhesion to VCAM-1, whereas all three cells exhibited reduced cell adhesion to OPN, pp-vWF, and FN-CS1 (Fig.5B). This result suggests that inhibition of α4 integrin-dependent cell adhesion by α4B is caused by a dominant-negative effect for VCAM-1 binding, but not for OPN, pp-vWF, FN-CS1.

α4B knockdown in B16 melanoma cells promotes lung metastasis - Expression of endogenous α4B in B16 melanoma cells was confirmed by RT-PCR (Fig.6A). We also found that α9 integrin is expressed on B16 cells. Endogenous α4B protein was evaluated by first determining that, of the 3 monoclonal antibodies against α4 integrin, 5X2 was most efficient for WT α4 integrin and α4B immunoprecipitation (Fig. 6B). 5X2 was then used to immunoprecipitate lysates of B16 cells. Two bands (150 kDa and 140 kDa) of WT α4 integrin, or a band (130 kDa) of α4B were detected in B16 cells by antibody against α4 integrin (C-20) or α4B, respectively (Fig. 6C). To determine the role of α4B expression during B16 lung metastasis, we knocked down endogenous α4B in B16 cells using

specific short interfering RNAs (siRNAs; si-α4B) based on the unique 3′ sequence of α4B derived from exon 27b. Results from the qPCR assays showed that si-α4B treatment of B16 melanoma cells resulted in more than 70% reduction in α4B mRNA levels. No effect on WT α4 integrin mRNA expression was observed (Fig. 6D). Western blotting confirmed that the expression of α4B protein was substantially reduced by si-α4B treatment. No effect on cell surface expression of WT α4 integrin was observed by flow cytometory (Fig. 6E). Thus, si-α4B treatment specifically reduced expression of α4B on B16 melanoma cells. We transfected si-α4B or control siRNAs into B16 melanoma cells and then injected these cells intravenously into C57BL/6 mice. A significant increase in lung metastasis and lung weight was observed in the group of mice inoculated with B16 melanoma cells transfected with si-α4B (Fig. 6F). Collectively, these findings demonstrate that α4B acts as a novel endogenous inhibitor protein of WT α4 integrin function.

DISCUSSION

Cytoplasmic domains of integrin are highly conserved during evolution, whereas extracellular domains are not as highly conserved. This would suggest that integrin signaling is indispensable for the maintenance of cellular functions (4). Alternative splicing variants of several integrins in extracellular or cytoplasmic regions have been identified (22). Among them, at least six integrin subunits (α3, α6, α7, β1, β3, and β5) have alternative splicing variants in their cytoplasmic domains (23).

Recently, we reported that an alternative splicing variant in the extracellular domain of α9 integrin (SFα9) promotes WT α9 integrin cell adhesion by inside-out signaling (24). Both α4 and α9 integrin, which are in the same integrin family because of structural and functional similarity, are involved in autoimmune diseases (25-27). We have demonstrated the expression and function of a novel murine α4 integrin splicing variant, α4B in this study. It contains a unique cytoplasmic amino acid sequence, KVIL. The VIL residues of the KVIL cytoplasmic sequence are derived from exon27b. We evaluated expression patterns of WT α4 integrin and α4B in mouse tissues and B16 melanoma cells by PCR. We found that α4B is expressed in all tested cells expressing the WT α4 integrin. The α4B variant is expressed on cell surfaces with the β1 integrin. These results suggest that α4B modulates the function of WT α4 integrin by co-expressing on the cell surface.

We analyzed the functional properties of the variant α4B and showed that cells expressing α4B adhere to VCAM-1. This adhesion requires the presence of the VIL cytoplasmic amino acids, suggesting that a novel cytoplasmic sequence in α4B is critical for exertion of its function. Although the amino acid sequence of the extracellular region in WT α4 integrin and α4B is identical, the way in which they adhere α4 ligands differs. Integrin conformations and functions are regulated by the cytoplasmic region (28-30), with varying cytoplasmic sequences in each integrin inducing changes in structure and manner of binding. This issue was clarified by generating an antibody (clone #19E4) that specifically recognizes α4 integrin, but not α4B.

We also compared the activation of integrin-mediated signaling molecules (ERK, AKT, FAK, and Paxillin) after binding to VCAM-1, for WT α4 integrin, α4B. We found that activation of AKT, Paxillin, or FAK was similar. These are major molecules that undergo phosphorylation in response to adhesion via integrins. Thus, integrin-mediated signaling of α4B is similar to that for the WT α4 integrin. However, activation of ERK is markedly reduced in cells expressing α4B. These results indicate that there is a particular molecule responsible for ERK activation. A candidate molecule is caveolin-1, which interacts with some integrins through the

transmembrane domain. This functional link between integrin and caveolin-1 activates Ras-ERK signaling (31). We observed that B16 melanoma cells in which α4B was knocked down exhibited increased caveolin-1 expression levels (data not shown), suggesting that α4B may have an inhibitory function during caveolin-1 expression. It is well known that β1 integrin binds to FAK and Paxillin (32,33). Both molecules can directly activate AKT (34,35), therefore activation of AKT, FAK, and Paxillin (Fig. 2B) may be elicited by β1 integrin associating with α4B as a heterodimer subunit. Thus, α4Bβ1 integrin possesses the unique ability to modulate ERK activation.

A significant reduction of α4-dependent cell adhesion was observed in cells expressing both WT α4 integrin and α4B, irrespective of the change in expression of WT α4 integrin. There are several possible mechanisms for how α4B inhibits α4-dependent cell adhesion. Initially, we hypothesized that α4B has a predominantly negative effect on WT α4 integrins. This hypothesis is consistent with the fact that α4B has no known binding site for integrin binding proteins in its short cytoplasmic tail. However, the adhesion assay result for α4B in Fig. 2A, which indicates that α4B does not have a binding ability against OPN, pp-vWF, and FN-CS1, does not account for these dominant-negative effects. Whereas, α4B binds to VCAM-1, suggesting that α4B exerts a dominant-negative effect. This is consistent with the adhesion assay result using cells co-expressing WT α4 integrins and differential level of α4B in Fig.5, which shows lowest α4B-expressing cells (clone 10L) exhibits comparable binding against VCAM-1, but reduced binding against OPN, pp-vWF, and FN-CS1. Thus, the inhibitory mechanism of α4B may be dominant-negative for VCAM-1 binding, but not OPN, pp-vWF, and FN-CS1. The most likely possibility of negative effects on OPN, pp-vWF, and FN-CS1 by α4B is inhibition of α4 integrin activation. The α4 integrin/NIH3T3 cells expressing α4BΔVIL can bind to α4 ligands (Fig. 4D) suggesting that the cytoplasmic region of α4B elicits negative signals; however the mechanism responsible for this remains to be elucidated.

WT α4 integrin and α4B mRNA levels were increased in the spinal cords of EAE mice (Fig. 1C). This result suggests that cells expressing both molecules are infiltrated into spinal cord. It seems inconsistent to infiltrate cells expressing α4B in the spinal cords of EAE since α4B functions α4 integrin inhibition. We found that Mn^{2+} treatment overcome the negative effect of α4B (Fig. 4E), indicating that the function of α4B is reduced in the situation of α4 integrin activation states such as EAE. Thus, EAE is developed readily despite the presence of α4B.

Three different alternative splicing variants of β1 integrin have been reported; β1A is the wild-type, while β1B-D are splicing variants (23,36,37). β1B and β1C are known to have negative functions; β1B and β1C compete with β1A for the formation of the α chain or ligand binding, followed by interference with integrin signaling in cells. The expression levels of β1D integrin are similar to that for β1A localized in focal adhesions, while β1B and β1C remain diffuse on the surface and do not localize in focal adhesions. Thus, it seems that the negative effects of β1B and β1C may be due to localization and/or dominant-negative effects. The negative effects of α4B seem to be caused by two different ways, dominant-negative effect or inhibition of WT α4 integrin activation, suggesting a different mechanism compared with that for β1B and β1C. Thus, α4B is a novel endogenous inhibitor of α4 integrin through a unique mechanism.

In conclusion, our results indicate that the alternative splicing variant α4B inhibits α4 integrin-dependent cell adhesion via dominant-negative effect or inhibition of WT α4 integrin activation. This is a novel inhibitory manner for regulating α4 integrin-dependent cell adhesion.

Taken together with previous our report of SFα9, integrin splicing variants of α4 and α9 integrin family might be important for the regulation of wild type integrin functions. In this study, we introduce the expression and function of murine α4 integrin variant α4B. However, human α4 integrin variants have not been identified yet. To identify and clear the function of human α4 integrin variants deserves further investigation.

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The abbreviations used are: EAE; experimental autoimmune encephalomyelitis, Erk; extracellular signal-regulated kinases, FAK; focal adhesion kinase, FN; fibronectin, OPN; osteopontin, pp-vWF; pro-polypeptide of von Willebrand factor, VCAM-1; vascular cell adhesion molecule-1, WT; wild-type,

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FIGURE LEGENDS

Figure 1. Structure and expression of α4B. (A) Schematic representation of protein and genome structure of WT α4 integrin and α4B. α4B is identical to WT α4 integrin up to ^{100}Lys (K) in the cytoplasmic region. The cDNA sequence derived from Ex27b encodes the VIL peptide sequence. RT-PCR analysis (B) and qPCR analysis (C) of WT α4 integrin and α4B in various normal mouse tissues, and from the spinal cords of normal or EAE mice. (D) Protein expression of WT α4 integrin and α4B in NIH3T3 cells expressing each molecule or both molecules. (E) Surface expression of WT α4 integrin and α4B. Shaded peaks represent unstained cells, and unshaded peaks represent cells stained with anti-α4 integrin R1-2. (F) Association of α4B with β1 integrin. Immunoprecipitation (IP) using an antibody against α4 integrin (R1-2) and immunoblotted with anti-β1 integrin antibody. $^{*}P < 0.05$, $^{**}P < 0.005$. Data are presented as means ± SEM from three independent experiments.

Figure 2. Inhibition of α4 integrin-dependent cell adhesion by α4B. (A) Cell adhesion of NIH3T3 cells expressing vector only (Mock/NIH3T3), WT α4 integrin (α4 integrin/NIH3T3), or α4B (α4B/NIH3T3) to various integrin ligands in the presence or absence of Mn^{2+}. (B) Immunoblots of phosphorylated and total signaling molecules in lysates of α4 integrin/NIH3T3, α4B/NIH3T3 or α4 integrin/α4B/NIH3T3 cells after adhesion to FN or VCAM-1. (C) Adhesion of α4 integrin/NIH3T3 or α4 integrin/α4B/NIH3T3 cells in the presence or absence of Mn^{2+}. $^{**}P < 0.005$. Data are presented as the means ± SEM of four independent experiments.

Figure 3. Specific detection of α4 integrin by the #19E4 antibody. Flow cytometry analysis of three commercially available antibodies against α4 integrin (PS/2, R1-2 and 9C10) (A), or clone #19E4 (B, C).

Figure 4. Involvement of the cytoplasmic domain in α4B for inhibition of cell adhesion via WT α4 integrin. (A) Schematic of WT α4 integrin, α4B, and α4BΔVIL. (B) Surface expression of α4BΔVIL by flow cytometry. (C) Adhesion of α4B/NIH3T3 and α4BΔVIL/NIH3T3 cells. (D) Protein and surface expression of WT α4 integrin, α4B, or α4BΔVIL. (E) Adhesion of α4 integrin/NIH3T3, α4 integrin/α4B/NIH3T3, and α4 integrin/α4BΔVIL/NIH3T3 cells. $^{**}P < 0.005$. Data are presented as the means ± SEM of three independent experiments.

Figure 5. Inhibitory mechanism of α4 integrin by α4B. (A) Cell surface expression of α4B and WT α4 integrin by flow cytometry. (B) Adhesion of α4 integrin/CHO cells expressing differential cell surface level of α4B.

Figure 6. Enhancement of lung metastasis in B16 melanoma cells. (A) RT-PCR analysis of endogenous expression of WT α4 integrin, α4B, and α9 integrin in B16 cells. (B) Lysates from α4 integrin/α4B/NIH3T3 cells were immunoprecipitated by anti-α4 integrin R1-2, PS/2, or 5X2; precipitated proteins were then separated by SDS–PAGE and blotted with antibody against HA or FLAG. (C) Endogenous expression of α4B in B16 cells. Lysates from B16 cells were immunoprecipitated by 5X2; precipitated proteins were blotted with antibody against WTα4 (Santa Cruz) or α4B. (D) WT α4 integrin and α4B mRNA levels in control or α4B siRNA-transfected B16 cells at 2 days post-transduction were

quantified using qPCR assays. (E) Surface expression of WT α4 integrin using antibody #19E4 on cells transfected with control or α4B siRNAs. (F) B16 melanoma cells treated with control or α4B siRNA were injected intravenously into C57BL/6 mice via tail vain. At 21 days after injection, lungs were removed and the number of metastases counted. The weight of each lung was also determined. $^{*}P < 0.05$, $^{**}P < 0.005$. Data are presented as the means ± SEM from one representative of three independent experiments.

Fig.1

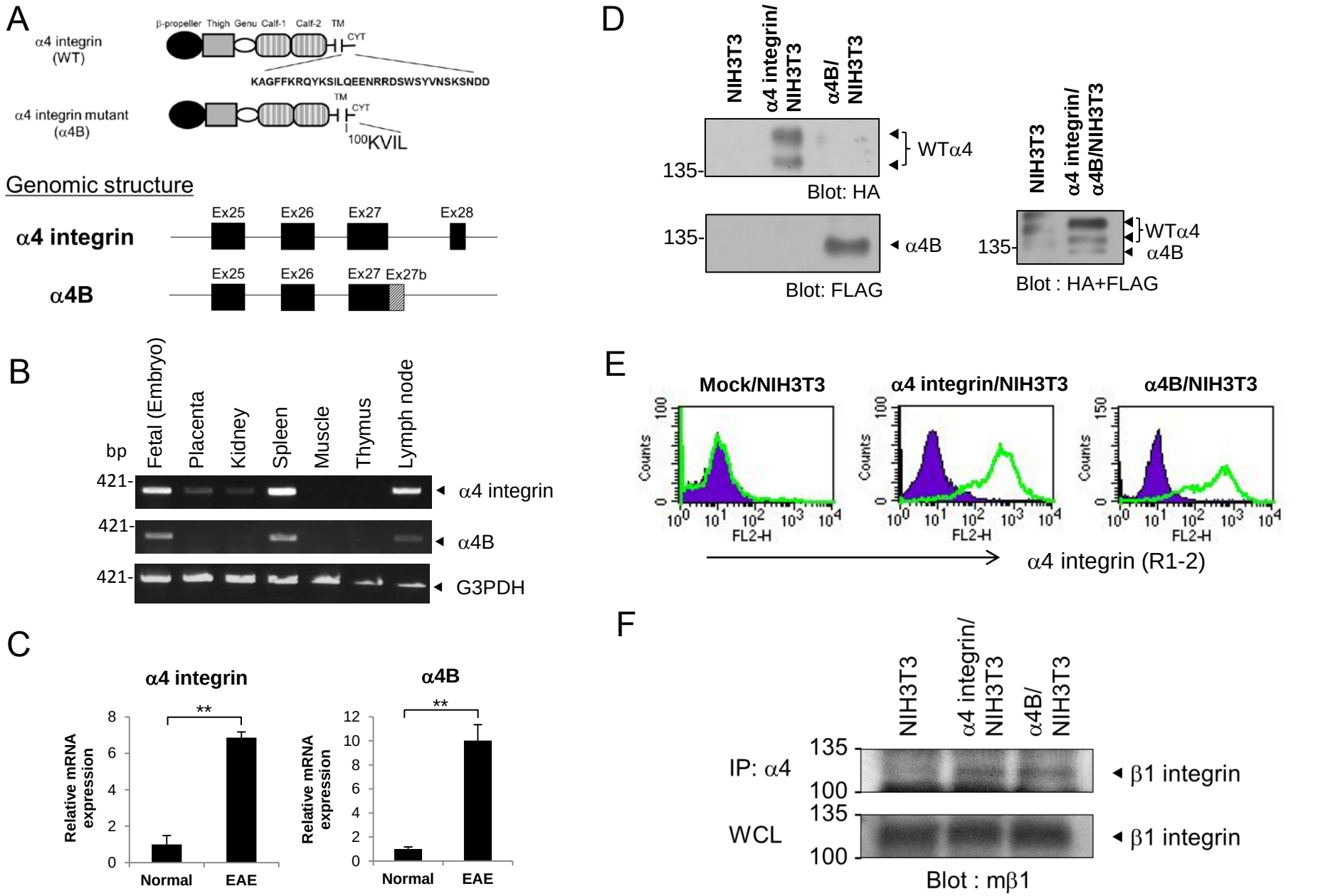
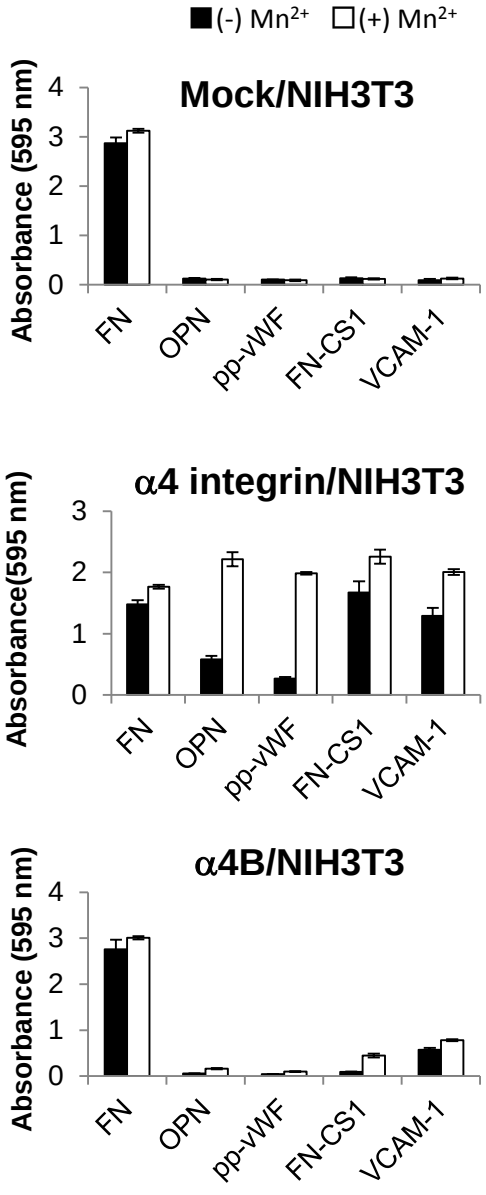
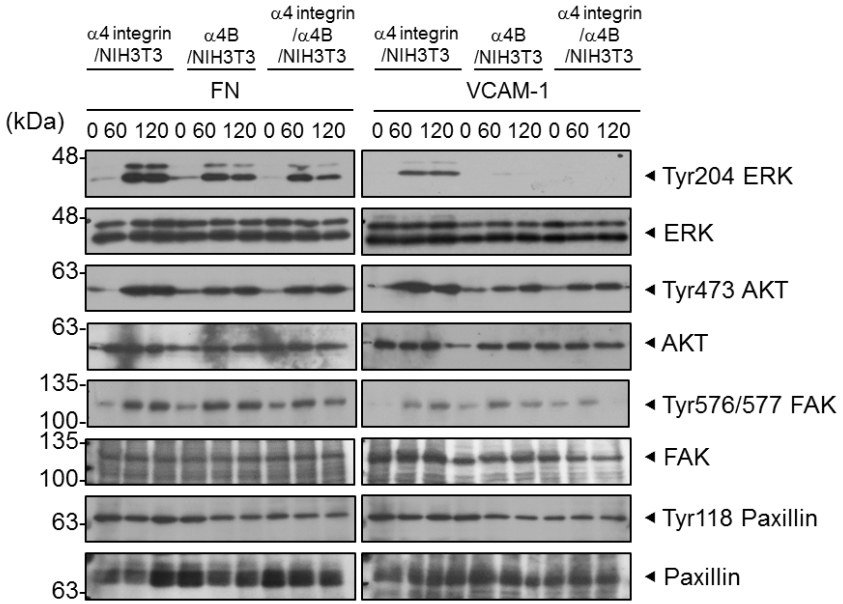

A
α4 integrin (WT)
β-propeller Thigh Genu Calf-1 Calf-2 TM CYT
KAGFFKRQYKSILQEENRRDSWSYVNSKSNDD
α4 integrin mutant (α4B)
TM CYT
100KVIL
Genomic structure
α4 integrin
Ex25 Ex26 Ex27 Ex28
α4B
Ex25 Ex26 Ex27 Ex27b
B
bp
Fetal (Embryo)
Placenta
Kidney
Spleen
Muscle
Thymus
Lymph node
421-
α4 integrin
α4B
G3PDH
C
α4 integrin
α4B
Relative mRNA expression
**
Normal
EAE
D
NIH3T3
α4 integrin/ NIH3T3
α4B/ NIH3T3
135-
WTα4
Blot: HA
α4B
Blot: FLAG
α4 integrin/ α4B/NIH3T3
Blot : HA+FLAG
E
Mock/NIH3T3
α4 integrin/NIH3T3
α4B/NIH3T3
Counts
FL2-H
α4 integrin (R1-2)
F
IP: α4
WCL
β1 integrin
Blot : mβ1

Fig.2

A


■(-) Mn2+ □(+) Mn2+
Mock/NIH3T3
Absorbance (595 nm)
α4 integrin/NIH3T3
Absorbance(595 nm)
α4B/NIH3T3
Absorbance (595 nm)
FN
OPN
pp-vWF
FN-CS1
VCAM-1

B


α4 integrin /NIH3T3
α4B /NIH3T3
α4 integrin /α4B /NIH3T3
FN
VCAM-1
(kDa)
0 60 120 0 60 120 0 60 120
0 60 120 0 60 120 0 60 120
Tyr204 ERK
ERK
Tyr473 AKT
AKT
Tyr576/577 FAK
FAK
Tyr118 Paxillin
Paxillin

C

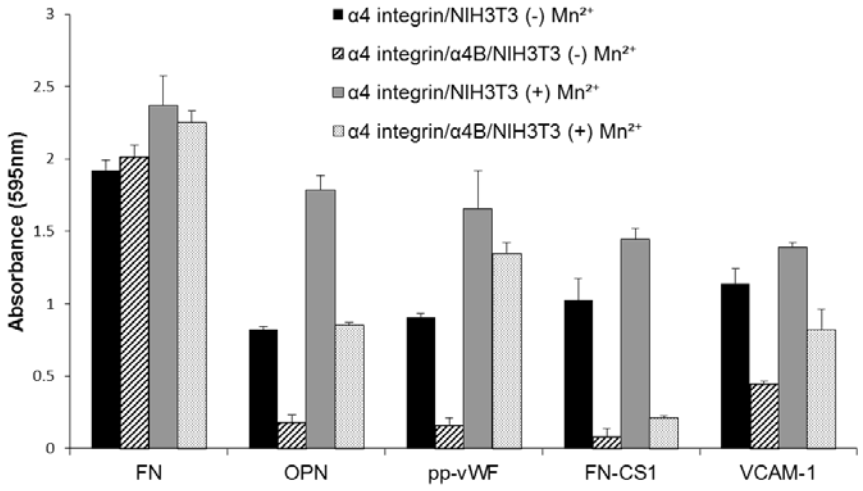

α4 integrin/NIH3T3 (-) Mn2+
α4 integrin/α4B/NIH3T3 (-) Mn2+
α4 integrin/NIH3T3 (+) Mn2+
α4 integrin/α4B/NIH3T3 (+) Mn2+
Absorbance (595nm)
FN
OPN
pp-vWF
FN-CS1
VCAM-1

Fig.3

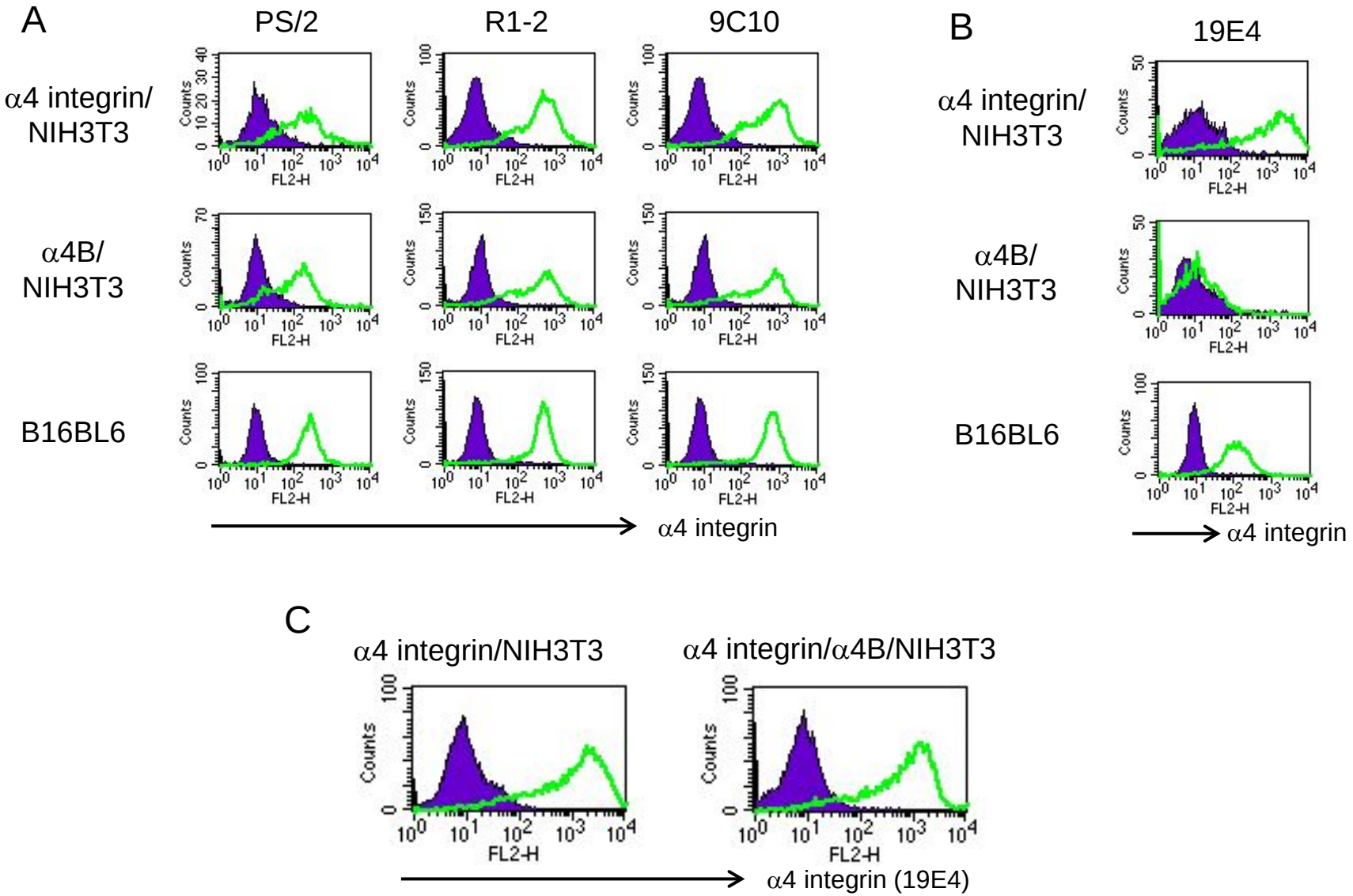

A
PS/2
R1-2
9C10
α4 integrin/
NIH3T3
α4B/
NIH3T3
B16BL6
Counts
FL2-H
α4 integrin
B
19E4
α4 integrin/
NIH3T3
α4B/
NIH3T3
B16BL6
Counts
FL2-H
α4 integrin
C
α4 integrin/NIH3T3
α4 integrin/α4B/NIH3T3
Counts
FL2-H
α4 integrin (19E4)

Fig.4

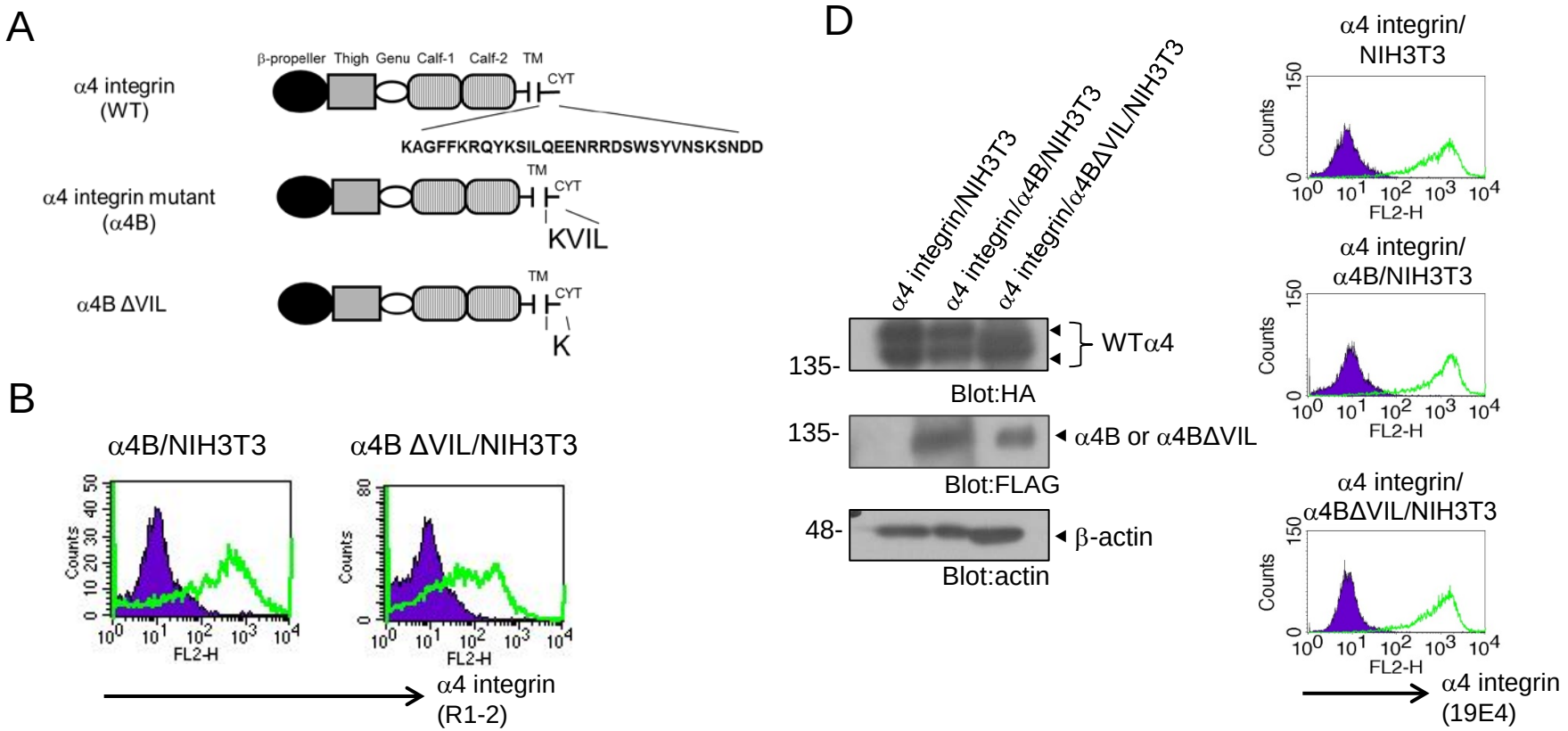
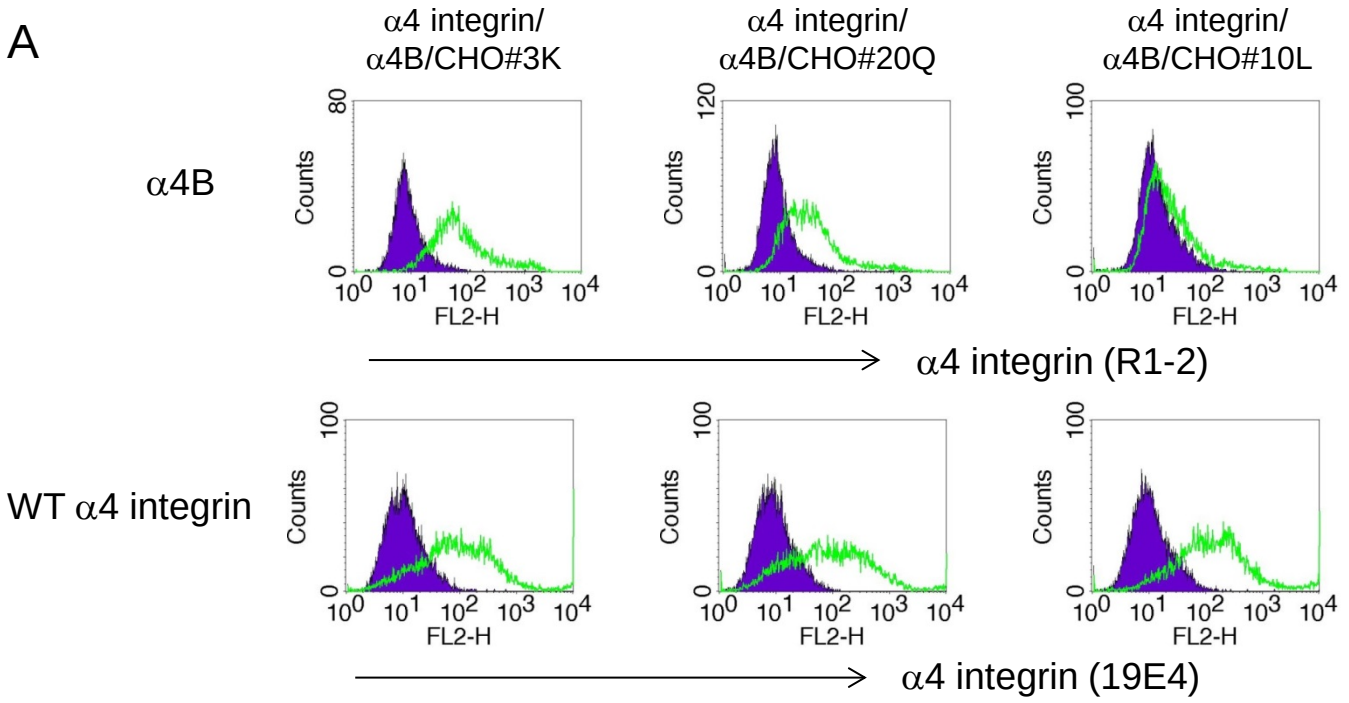

A
β-propeller Thigh Genu Calf-1 Calf-2 TM
α4 integrin (WT)
CYT
KAGFFKRQYKSILQEENRRDSWSYVNSKSNDD
α4 integrin mutant (α4B)
TM
CYT
KVIL
α4B ΔVIL
TM
CYT
K
B
α4B/NIH3T3
α4B ΔVIL/NIH3T3
Counts
FL2-H
α4 integrin (R1-2)
D
α4 integrin/NIH3T3
α4 integrin/α4B/NIH3T3
α4 integrin/α4BΔVIL/NIH3T3
135-
WTα4
Blot:HA
135-
α4B or α4BΔVIL
Blot:FLAG
48-
β-actin
Blot:actin
α4 integrin/ NIH3T3
α4 integrin/ α4B/NIH3T3
α4 integrin/ α4BΔVIL/NIH3T3
Counts
FL2-H
α4 integrin (19E4)
C
Absorbance (595 nm)
**
α4B integrin/NIH3T3
α4B ΔVIL/NIH3T3
FN
VCAM-1
E
α4 integrin/NIH3T3
α4 integrin/α4B/NIH3T3
α4 integrin/α4BΔVIL/NIH3T3
Absorbance (595 nm)
FN
OPN
pp-vWF
FN-CS1
VCAM-1

Fig.5


A
α4 integrin/
α4B/CHO#3K
α4 integrin/
α4B/CHO#20Q
α4 integrin/
α4B/CHO#10L
α4B
WT α4 integrin
Counts
FL2-H
α4 integrin (R1-2)
α4 integrin (19E4)

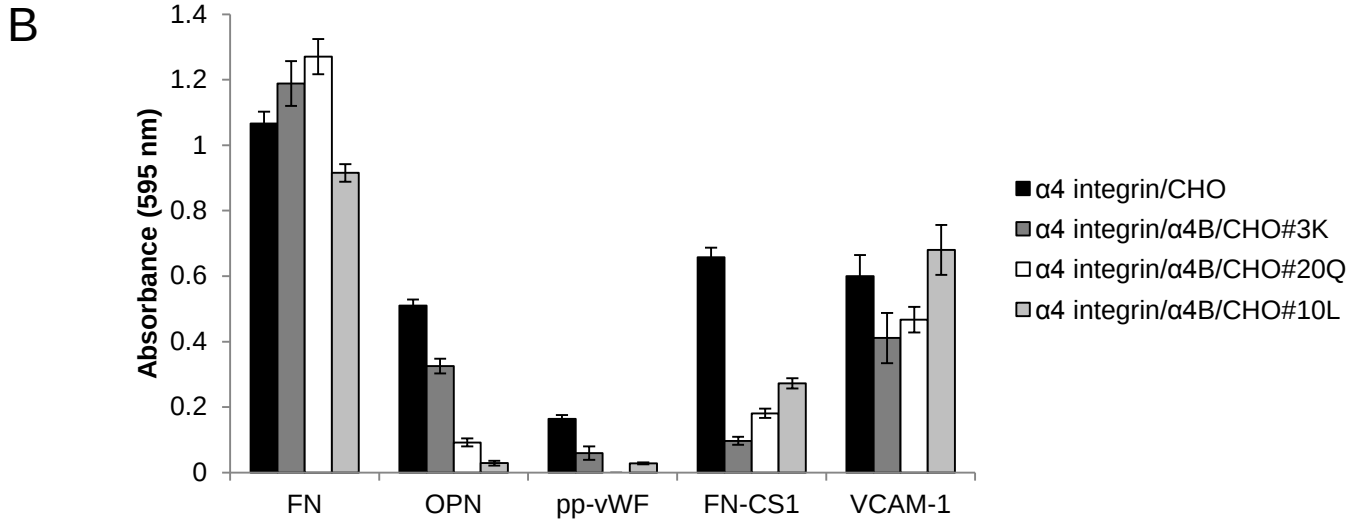
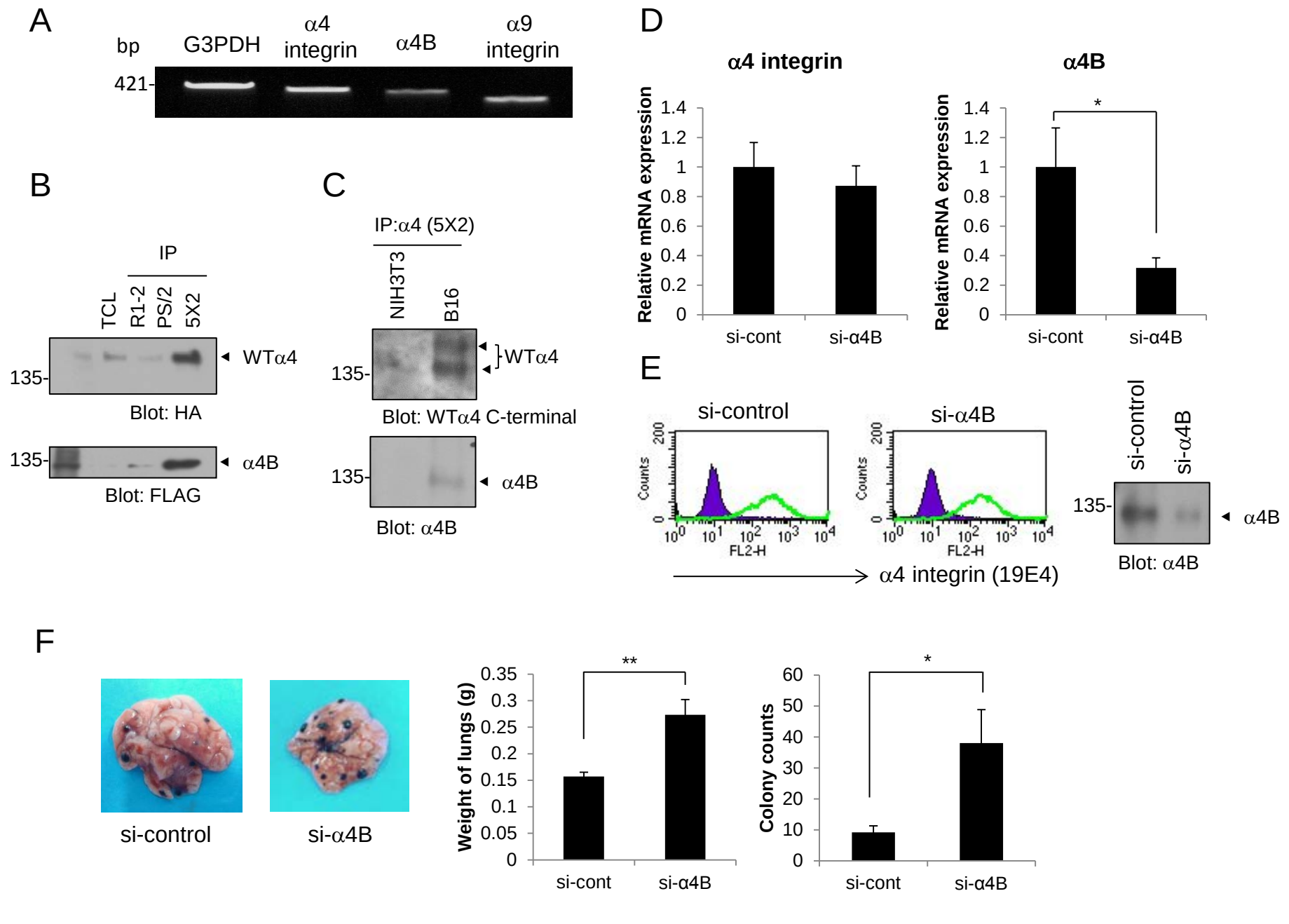

B
Absorbance (595 nm)
FN
OPN
pp-vWF
FN-CS1
VCAM-1
α4 integrin/CHO
α4 integrin/α4B/CHO#3K
α4 integrin/α4B/CHO#20Q
α4 integrin/α4B/CHO#10L

Fig.6


A
bp
G3PDH
α4 integrin
α4B
α9 integrin
421-
B
IP
TCL
R1-2
PS/2
5X2
135-
WTα4
Blot: HA
135-
α4B
Blot: FLAG
C
IP:α4 (5X2)
NIH3T3
B16
135-
WTα4
Blot: WTα4 C-terminal
135-
α4B
Blot: α4B
D
α4 integrin
α4B
Relative mRNA expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
si-cont
si-α4B
*
E
si-control
si-α4B
Counts
200
0
FL2-H
α4 integrin (19E4)
si-control
si-α4B
135-
α4B
Blot: α4B
F
si-control
si-α4B
Weight of lungs (g)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
**
si-cont
si-α4B
Colony counts
60
50
40
30
20
10
0
*
si-cont
si-α4B