In silico analysis-based identification of the target residue of integrin α6 for metastasis inhibition of basal-like breast cancer

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24

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27

Abstract

Metastasis causes death in breast cancer patients. To inhibit breast cancer metastasis, we focused on integrin α6, a membrane protein that contributes to cell migration and metastasis. According to *in silico* analysis, we identified Asp-358 as an integrin α6-specific vertebrate-conserved residue, and consequently as a potential therapeutic target. Because Asp-358 is located on the surface of the β propeller domain that interacts with other molecules for integrin α6 function, we hypothesized that a peptide with the sequence around Asp-358 competitively inhibits integrin α6 complex formation. We treated basal-like breast cancer cells with the peptide and observed reductions in cell migration and metastasis. The result of the immunoprecipitation assay showed that the peptide inhibited integrin α6 complex formation. Our immunofluorescence for phosphorylated paxillin, a marker of integrin-regulated focal adhesion, showed that the peptide reduced the number of focal adhesions. These results indicate that the peptide inhibits integrin α6 function. This study identified the target residue of integrin α6 and designed the inhibitory peptide. For breast cancer patients, metastasis inhibition therapy may be developed in the future based on this study.

Introduction

Breast cancer is the most common cancer in women worldwide (Ferlay et al., 2015). Although various procedures have been developed, many breast cancer patients die from metastasis (Gupta & Massagué, 2006). Therefore, the development of metastasis inhibition therapy is in high demanded.

During the process of metastasis, cancer cells move from the primary site to other parts of a body (Nguyen, Bos, & Massagué, 2009). This metastatic process includes multiple steps, such as local invasion, intravasation, anoikis resistance, extravasation and formation of metastatic focus. Because the migratory ability promotes some of these steps, an acquisition mechanism for this high migratory ability may be a therapeutic target. In metastatic basal-like breast cancer (Yehiely, Moyano, Evans, Nielsen, & Cryns, 2006), we previously reported that integrin α6β1 promotes cell migration and metastasis through activation of focal adhesion dynamics (Itou et al., 2017). This suggests that an inhibitor for integrin α6β1 may result in inhibiting breast cancer metastasis.

Integrins are membrane proteins and form a complex of α and β subunits (Shattil, Kim, & Ginsberg, 2010). The extracellular region of the integrin complex binds

60 to the extracellular matrix, and the intracellular domain recruits focal adhesion molecules,
61 such as paxillin and vinculin (Huttenlocher & Horwitz, 2011; C. Lawson & Schlaepfer,
62 2012). In focal adhesions, paxillin is phosphorylated by focal adhesion kinase (Turner,
63 2000). The function of focal adhesion is the activation of intracellular signaling and
64 organization of the actin cytoskeleton (Huttenlocher & Horwitz, 2011), which contributes
65 to the promotion of cell migration. In addition to cell migration, integrin is involved in
66 cell proliferation, survival and stemness in various cancers (Chang et al., 2015;
67 Desgrosellier & Cheresh, 2010; Groulx et al., 2014).

68 In the human genome, there are 18 α and 8 β integrin subunit genes. Previously,
69 various integrin inhibitors have been developed. An inhibitor of integrin α2β1 was
70 designed for cardiovascular disease (Miller et al., 2009). Cilengitide, an inhibitor of αvβ3
71 and αvβ5, is being evaluated in clinical trials for glioblastoma therapy (Scaringi, Minniti,
72 Caporello, & Enrici, 2012). Ley *et al*. reviewed inhibitors of integrin αIIbβ3, α4β7 and
73 αEβ7 (Ley, Rivera-Nieves, Sandborn, & Shattil, 2016). Several of these inhibitors are in
74 clinical trials or clinically used. For example, antagonists against αIIbβ3, such as
75 abciximab, eptifibatide and tirofiban, were developed for percutaneous coronary

interventions (Bledzka, Smyth, & Plow, 2013). Treatment with natalizumab, an antibody for the α4 subunit, is in a clinical trial for multiple sclerosis (Singer, 2017) and is approved for Crohn's disease (McLean & Cross, 2016). A small molecule inhibitor of αLβ2, lifitegrast, was shown to reduce inflammation for dry eye disease (Perez, Pflugfelder, Zhang, Shojaei, & Haque, 2016).

For cancer, a cyclic integrin-binding peptide reduced cell migration in prostate and pancreatic cancer cells in a neuropilin-1 dependent manner (Sugahara et al., 2015). In ovarian cancer cell lines, neutralizing antibodies for α3, α6, αv and β1 reduce cell proliferation, and the antibodies for α3, α6 and β1 inhibits migration (Ahmed, Riley, Rice, & Quinn, 2005). An anti-integrin α6 antibody inhibited angiogenesis and tumor growth in xenograft experiments with a breast cancer cell line (T. H. Lee et al., 2006). Another antibody for integrin α6 reduces cell proliferation and invasion in esophageal squamous cell carcinoma (Kwon et al., 2013). In addition, anti-integrin β1 antibody inhibited metastasis of prostate cancer (Y. C. Lee et al., 2013). However, although it is considered that complex formation of integrin α and β subunits is required for their function, no inhibitor of integrin complex formation has been designed for cancer therapy.

92 Integrin α6 (also known as VLA-6 and CD49f, coded by *ITGA6*) is expressed
93 in normal and cancer cells (Ahmed et al., 2005; Chen et al., 2016; Ding et al., 2013; Kwon
94 et al., 2013; Pontier & Muller, 2009). It forms a heterodimer with integrin β1 or β4, which
95 binds to a component of the extracellular matrix, laminin (Hynes, 1992). The mouse null
96 mutation of integrin α6 causes early postnatal death with aberration in the skin tissue
97 (Georges-Labouesse et al., 1996). In human, homozygous mutation of the integrin α6
98 gene causes a skin disease with blisters (Pulkkinen et al., 1997). Integrin α6 contributes
99 to radiotherapy resistance in breast cancer cells through activation of Akt and Erk
100 signaling (Hu, Zhou, Zhao, & Wu, 2016). In malignant breast cancer cell lines, integrin
101 α6 is involved in migration and stemness (Chang et al., 2015; Itou et al., 2017). However,
102 amino acid residue(s) required for integrin α6 functions has not been fully understood.
103 This study focused on integrin α6 for the inhibition of cell migration in breast
104 cancer cells. Our *in silico* analyses identified Asp-358 as a target residue for metastasis
105 inhibition in the integrin α6 amino acid sequence. A peptide with the same sequence
106 around Asp-358 inhibits cell migration. Zebrafish metastasis assays and mouse tail vein

injection show that the peptide has a potential to inhibit metastasis. Our findings may contribute to establishing a therapy for breast cancer metastasis.

Results

Integrin α6 contributes to cell migration in basal-like breast cancer cells.

Breast cancer is classified into various subtypes. To identify the subtype that has high integrin α6 expression, we immunostained a tissue microarray with anti-integrin α6 antibody. The results showed that integrin α6 expression was observed in the cytoplasm and membrane and tends to be higher in basal-like breast cancer than other subtypes (Fig. 1A). The results of immunoblotting also showed high integrin α6 expression in a basal-like breast cancer cell line, MDA-MB-231, whereas the expression was low in luminal ones, MCF-7 and T-47D (Fig. 1B). Integrin β1 is a binding partner of Integrin α6 (Hynes, 1992), and integrin α6β1 promotes cell migration in basal-like breast cancer (Itou et al., 2017). Under our conditions, integrin β1 expression was observed in both luminal and basal-like breast cancer cell lines. MDA-MB-231 cells showed higher integrin β1 expression than Luminal breast cancer cell lines (Fig. 1B).

123 To analyze the migratory ability of breast cancer cell lines, we performed
124 Boyden chamber assays, and observed that basal-like breast cancer cells have a higher
125 migratory ability than luminal breast cancer cells (Fig. 1C). To determine the role of
126 integrin α6 in cell migration, we performed shRNA-mediated integrin α6 knockdown and
127 Boyden chamber assays. We used two shRNA constructs (Itou et al., 2017), and both
128 reduced the integrin α6 level in MDA-MB-231 cells (Fig. 1D). In the Boyden chamber
129 assays, integrin α6 knockdown reduced the number of migrated cells (Fig. 1E). These
130 results indicate that high expression of integrin α6 promotes cell migration in basal-like
131 breast cancer cells.
132
133 *Integrin α6 has the potential to promote the cell migration of luminal breast cancer cells.*
134 Integrin α6 has two isoforms, named α6A and α6B, which share the extracellular region
135 and have different cytoplasmic domains (Hogervorst, Kuikman, van Kessel, &
136 Sonnenberg, 1991). We designed primers for each isoform and total α6. Primers for each
137 isoform specifically amplified the target (Supporting information Fig. S1). We calculated
138 the mRNA copy numbers of the isoforms and total α6 in the samples from basal-like

breast cancer cell lines. The sum of the copy numbers of α6A and α6B were comparable with that of total α6, supporting the reliability of our quantification (Fig. 2A). The results of quantitative RT-PCR revealed that α6A expression is higher than α6B in basal-like breast cancer cells (Fig. 2A).

Luminal breast cancer cells have weak integrin α6 expression (Fig. 1B) and a low migratory ability (Fig. 1C). We overexpressed integrin α6A in these cells (Fig. 2B). In a 2-dimensional culture, luminal breast cancer cell lines showed compacted colonies. We observed that integrin α6A overexpression disperse cells (Fig. 2C). Because this change is indicative of an increase in cell migration, we performed Boyden chamber assays to analyze the migratory ability. The results showed that integrin α6A overexpression significantly enhanced the cell migration of luminal breast cancer cells (Fig. 2D), suggesting that integrin α6 has the potential to promote the cell migration of breast cancer cells.

A peptide with the sequence around Asp-358 inhibits cell migration.

Our data suggest that inhibition of integrin α6 reduces cell migration and metastasis in breast cancer cells. To design an inhibitory peptide for integrin α6, target amino acid residue(s) should be identified. Because functionally important amino acid residues are conserved among species, we performed *in silico* analyses to identify an integrin α6-specific vertebrate-conserved amino acid residue. First, we compared the amino acid sequences of the integrin α family in the human genome to extract integrin α6-specific residues (Supporting information Doc. S1). The results showed that integrin α6 has 276 specific residues. Next, to determine whether these residues are conserved among species, we aligned the amino acid sequences of integrin α6 for 65 vertebrates, such as human, mouse, chicken, frog and zebrafish (Supporting information Doc. S2). The amino acid analysis identified Asp-358, which is located at the β propeller domain of integrin α6 (Fig. 3A). The integrin α6 structure was predicted based on the crystal structures of integrin α5 and αV (Nagae et al., 2012; Xiong et al., 2009) with a protein structure prediction platform (Guex, Peitsch, & Schwede, 2009), and the location of Asp-358 was analyzed. The results showed that Asp-358 is located at the surface of the β propeller domain (Fig. 3B).

Because the β propeller domain integrin α subunits interact with other molecules, such as integrin β subunits, to function (Hynes, 1992; Shattil et al., 2010), it is possible that a peptide with the sequence around Asp-358 reduces cell migration through competitive inhibition of integrin α6 complex formation. We designed an 8 amino acid peptide with the sequence of FGYDVAVV (Fig. 3A and 3C, the peptide structure was depicted by software (Lamiable et al., 2016)). We treated the luminal breast cancer cell line, MCF-7, with the peptide and analyzed cell migration. The peptide significantly reduced cell migration in integrin α6A overexpressing cells, whereas no change was observed in the control cells (Fig. 3D). In the basal-like breast cancer cell line MDA-MB-231, peptide treatment significantly reduced cell migration. However, there was no reduction effect in cell migration in the peptide-treated integrin α6-knocked-down cells compared with the no-peptide control (Fig. 3E). These results suggest that the peptide inhibits integrin α6-mediated cell migration.

The peptide reduced cell migration in two basal-like breast cancer cell lines, MDA-MB-231 and SUM159, in a dose-dependent manner (Fig. 3F). No change was

185 observed in the cells treated with mutant peptide, the sequence of which was
186 FGYAVAVV.

187 Zebrafish metastasis assay enables us easily to identify a metastasized cell by
188 live imaging (Itou et al., 2017; Teng et al., 2013). Because this is an advantage of
189 analyzing *in vivo* metastatic ability at the single-cell level compared to mammalian
190 models and appropriate for the purpose of this study, we performed zebrafish metastasis
191 assays with or without the peptide. Tg[*fli1a:egfp*]^{y1} fish that express green fluorescent
192 protein in their endothelial cells (N. D. Lawson & Weinstein, 2002) were used. We
193 injected cells into the abdominal cavity of a 2 days post-fertilization embryo. After 3 days
194 of culturing, we performed fluorescence microscopy, and identified an extravasated cell
195 at a distal part from the primary site as a metastasized cell (Fig. 3G). We observed a
196 reduction in metastasis rate in the peptide-treated group, compared with the no-peptide
197 control (Fig. 3GH). We did not observe remarkable effects on fish growth and behavior
198 in the peptide-treated group, although it was not determined whether the peptide affects
199 zebrafish integrins. These results indicate that the peptide have a potential to reduce
200 metastasis through the inhibition of cell migration.

The peptide-mediated integrin α6 inhibition reduces metastatic focus formation.

Integrin α6 contributes stemness in breast cancer cells (Chang et al., 2015; Goel et al., 2014). Stemness and anoikis resistance is required for formation of a metastatic focus. To analyze stemness and anoikis resistance, we performed sphere formation assay. We observed reduced sphere formation rate in the cells treated with the peptide (Fig. 4A), indicating that the peptide reduces stemness and/or anoikis resistance. In breast cancer cells, peptide treatment did not change cell growth in basal-like breast cancer cells (Fig. 4B).

Although zebrafish metastasis assay can evaluate *in vivo* cell migration, intravasation and extravasation, it is not able to analyze anoikis resistance and metastatic focus formation. To determine whether the peptide also inhibits anoikis resistance and metastatic focus formation, we conducted mouse tail vein injection of MDA-MB-231 cells with or without the peptide treatment. We counted the number of metastatic foci in the lungs of the injected mice. The peptide treatment significantly reduced the number of foci (Fig. 4C). These results indicate that the peptide reduces metastasis through

217 inhibition of anoikis resistance, extravasation and metastatic focus formation, in addition
218 to inhibition of cell migration.
219
220 *The peptide blocks the interaction of integrin α6 with β1, and reduces the number of focal*
221 *adhesions*.
222 To determine whether the peptide disrupts the interaction of integrin α6 with β1, we
223 conducted immunoprecipitation assay with anti-FLAG antibody in MDA-MB-231 cells
224 overexpressing integrin α6-FLAG. In the control sample, we observed integrin β1 band
225 (Fig. 5A). The band was not observed in the peptide treated cells (Fig. 5A), indicating
226 that the peptide inhibits the complex formation of integrin α6 and β1.
227 Integrins regulate focal adhesion formation (Huttenlocher & Horwitz, 2011).
228 To analyze the effect of the peptide with respect to focal adhesion, we treated basal-like
229 breast cancer cells with the peptide for 2 h, and then performed immunostaining with an
230 antibody for phosphorylated paxillin, a marker of focal adhesion. Focal adhesion signals
231 were detected primarily at the peripheral region of a cell, and the number of focal
232 adhesions per a cell was reduced in the peptide-treated groups (Fig. 5B). Treatment with

various concentrations of the peptide showed a reduction in the number of focal adhesions in a dose-dependent manner. The reduction was observed in the cells treated with more than 3.125 μM (MDA-MB-231 cells) and 6.25 μM peptide (SUM159 cells) (Fig. 5C). At higher concentrations, the numbers of focal adhesions were reduced to approximately 50% of the no-peptide controls. The half maximum effective concentrations (EC50) were 2.865 μM (MDA-MB-231 cells) and 3.377 μM (SUM159 cells).

Integrin α6β1 binds to laminin 511 (Nishiuchi et al., 2006). To determine whether the peptide-mediated inhibition of integrin α6β1 dimerization reduces laminin binding ability, we performed laminin binding assay in MDA-MB-231 cells with or without the peptide. The results showed reduction in laminin binding ability in the peptide treated cells (Fig. 5D). Taken together, these results indicate that the peptide inhibits integrin function through disruption of the integrin α6β1 complex formation.

Discussion

In this study, we observed high integrin α6 expression in basal-like breast cancer cells. Integrin α6 expression enhanced cell migration. We identified the integrin α6-specific

249 vertebrate-conserved residue, Asp-358. A peptide with the same sequence around Asp-
250 358 reduced cell migration in basal-like breast cancer cells. Zebrafish metastasis assays
251 and mouse tail vein injections of breast cancer cells showed the reduced metastatic ability
252 in the peptide-treated group. Moreover, our immunoprecipitation showed that the peptide
253 disrupts integrin α6 complex formation.

254 Under our conditions, integrin α6 and β1 expressions were high in MDA-MB-
255 231 cells. In luminal cell lines, integrin β1 expression was detected (Fig. 1). Integrin α6
256 has two isoforms α6A and α6B. We previously reported that both isoforms promote cell
257 migration in basal-like breast cancer cells (Itou et al., 2017). This study showed that α6A
258 is the major isoform in basal-like breast cancer cells. We overexpressed integrin α6A in
259 luminal cell lines and observed the promotion of cell migration (Fig. 2). These results
260 suggest that integrin α6Aβ1 can enhance cell migration in luminal breast cancer cells.

261 *In silico* analyses can identify functional residues in a protein. In this study, we
262 found Asp-358 to be a candidate for the functional residue of integrin α6. The result of
263 the prediction of the integrin α6 structure showed that Asp-358 is located at the surface
264 of the β propeller domain. The β propeller domain of the integrin α family is located at

265 the N-terminal region and has 7 β sheets (Springer, 1997). Each sheet consists of 4
266 antiparallel β strands and loops. *In silico* tools for protein structure (Guex et al., 2009)
267 predicted that Asp-358 is at a boundary between a β strand and a loop. Because the β
268 propeller domain of the integrin α subunit interacts with the integrin β subunit (Shattil et
269 al., 2010), it is possible that Asp-358 is involved in integrin complex formation. To
270 support this, the peptide with the sequence around Asp-358 of integrin α6, inhibited the
271 dimerization of integrin α6β1 (Fig. 5).

272 Our peptide, inhibited cell migration in Boyden chamber assays (Fig. 3). Our
273 zebrafish metastasis assay and mouse tail vain injection of breast cancer cells showed that
274 the peptide inhibited metastasis (Fig. 3,4). These results suggest that the peptide has the
275 potential to contribute to development of a metastasis inhibition therapy for breast cancer
276 patients.

277 Our immunoprecipitation assay showed that the peptide inhibits the
278 heterodimer formation of integrin α6 and β1 (Fig. 5). Integrins regulate focal adhesion
279 formation (Huttenlocher & Horwitz, 2011; C. Lawson & Schlaepfer, 2012). We observed
280 a reduction in the number of focal adhesions in the peptide-treated groups (Fig. 5). In

281 laminin binding assay, the peptide-treatment reduced the laminin binding ability (Fig. 5).
282 These results suggest that the peptide may impair integrin α6β1 function through
283 inhibition of their complex formation.

284 This study proposed the peptide-mediated inhibition of breast cancer metastasis.
285 In contrast from previous integrin inhibitors, which targets the protein itself, we first
286 determined the target residue of integrin α6, Asp-358, by *in silico* analyses and designed
287 the peptide. The peptide showed an inhibitory effect on cell migration and metastasis in
288 integrin α6-expressing cells. This suggests that drug design with *in silico* analyses can be
289 a useful approach to identify target residues and can be applied for various target
290 molecules, which are involved in not only for metastasis, but also proliferation,
291 angiogenesis, stemness and drug resistance. Because a previous study has reported that
292 exosomal integrin α6 is involved in breast cancer metastasis (Hoshino et al., 2015), the
293 peptide may reduce metastasis via inhibition of integrin α6 positive exosomes. Although
294 integrin α6 is also expressed in normal cells (Pontier & Muller, 2009), this study may
295 contribute to the establishment of metastasis inhibition therapy for breast cancer patients
296 in the future.

297

298 **Experimental procedures**

299 *Cell culture*

300 MCF-7 cells were obtained from the American Type Culture Collection (Manassas, VA,
301 USA) and maintained in RPMI-1640 containing 10% heat-inactivated FBS and 1nM β-
302 estradiol (Sigma, E2758, St. Louis, MO, USA). T-47D and MDA-MB-231 cells were also
303 obtained from the American Type Culture Collection and maintained in RPMI-1640
304 containing 10% FBS. SUM159 cells were obtained from Asterand (Detroit, MI, USA)
305 and maintained in Ham's F-12 nutrient mixture containing 5% FBS, 5 μg/mL insulin, 1
306 μg/mL hydrocortisone and 10 mM HEPES. Short tandem repeat analyses were performed
307 for cell authentication in July 2017 (MCF-7), June 2018 (T-47D) and May 2017 (MDA-
308 MB-231 and SUM159), and the results showed no contamination and no alterations of
309 these cells. Mycoplasma contamination was checked every 3 months by staining with
310 Hoechst 33342 (Dojindo, 346-07951, Kamimashiki, Japan, 1:1000 dilution) and no
311 contamination was observed. For drug selection to obtain infectants, 1 μg/mL puromycin
312 or 10 μg/mL blasticidin S was used. The peptide was synthesized with 90.0% HPLC

purity by GenScript (Piscataway, NJ, USA). Sterilized water was used to dissolve the peptide powder. For cell growth assays, cells were counted manually.

Immunohistochemistry

A tissue microarray obtained with the patient's informed consent was purchased from US BioMax (BR1921b, Rockville, MD, USA). After deparaffinization, the sample was incubated in boiled citrate buffer (pH6.0) for 40 min for heat induced epitope retrieval. Blocking solution with normal goat serum and BSA was used. To detect integrin α6 expression, the sample was incubated with anti-CD49f/ITGA6 antibody (Acris, SM038PT, Rockville, MD, USA, 1:100 dilution) overnight at 4°C. Subsequently, the sample was treated with the Vectastain elite ABC-HRP kit for rabbit IgG (Vector, PK-6101, Burlingame, CA, USA). Signals were detected with the NovaRED substrate kit (Vector, SK-4800). Hematoxylin was used for counterstaining. The dehydrated sample was mounted with Malinol (Muto pure chemical, 2009-1, Tokyo, Japan). Investigations were performed according to the principles expressed in the Declaration of Helsinki.

Immunoblotting

The primary antibodies used were anti-integrin α6 antibody (Cell Signaling Technology, 3750, Danvers, MA, USA, 1:1000 dilution), anti-integrin β1 antibody (Cell Signaling Technology, D2E5, 9699, 1:1000 dilution) and anti-DYKDDDDK antibody (Wako, 018-22386, Osaka, Japan, 1:1000 dilution, for FLAG tag detection). For the secondary reaction, the Easy-Western-II detection system (Beacle, BCL-EZS21, Kyoto, Japan) was used. For an internal control, an anti-β-actin antibody (Abcam, ab6276, Cambridge, UK, 1:10000 dilution) and anti-mouse IgG antibody conjugated to a peroxidase (Pierce biotechnology, 31340, Rockford, IL, USA, 1:50000 dilution) were used. Signals were developed with ECL select reagent (GE Healthcare, RPN2235, Buckinghamshire, UK). Images were collected with Ez-Capture II (ATTO, Tokyo, Japan) and Image Server 5 software (ATTO).

Loss- and gain-of-function studies

For integrin α6 knockdown, the shRNA-mediated gene silencing system with a lentiviral vector, pLKO.1 (Addgene, 8453, Cambridge, MA, USA) was used. The target sequences

were #2: 5'-CGAGAAGGAAATCAAGACAAA-3' and #3: 5'-CGGATCGAGTTTGATAACGAT-3'. The shRNA control sequence was 5'-CCTAAGGTTAAGTCGCCCTCG-3'. For integrin α6A overexpression, integrin α6A-FLAG gene was constructed and inserted it between the *Eco*RI and the *Xho*I sites of a lentiviral vector, pLenti-6.3 (Life Technologies, V533-06, Carlsbad, CA, USA). The FLAG (DYKDDDDK) expression vector was used for the control. A previously established lentiviral system was used (Dull et al., 1998). Virus particles were produced in Lenti-X 293T cells (Takara, 632180, Otsu, Japan).

Boyden chamber assay

Eight-μm pore culture inserts for 24-well plates (Greiner, 662638, Kremsmünster, Austria) were used. Twelve thousand and five hundred cells were plated with serum-free medium in the upper compartment and incubated for 1 h at 37°C. Then, medium containing 5% FBS was added to the lower compartment. After 6 or 24 h, cells were fixed and stained with crystal violet. The numbers of migrated cells were counted manually by using ImageJ software (Schneider, Rasband, & Eliceiri, 2012).

361

362 *Quantification of the copy number of mRNAs*

363 Integrin α6A and α6B cDNAs were cloned into the pCR-II vector (Life Technologies,
364 45-0245) and the pENTR vector (Life Technologies, K2400-20), respectively. They were
365 used as the reference for the calculation of mRNA copy numbers. First strand cDNAs
366 were reverse-transcribed from 1 μg of total RNA samples with an oligo dT18 primer.
367 Real-time PCR was performed with primer sets for α6A: forward 5'-
368 GCCACATATCACAAGGCTGAG-3' and reverse 5'-GCGTTTAAAGAATCCACACA
369 -3', α6B: forward 5'-CAAATGCAGGCACTCAGGTTC-3' and reverse 5'-
370 GCGTTTAAAGAATCCACACT-3', and total α6: forward 5'-
371 GGACAGCAAGGCGTCTCTTATT-3' and reverse 5'-
372 CGGCAGCAGCAGTCACATCAA-3'. Primers were validated by checking whether
373 specific amplification was observed with the α6A or α6B vector and whether the
374 amplicons have a single melting temperature in the melting curve analysis (Supporting
375 information Fig. S1).

376

377 In silico *analyses*

378 Amino acid sequences were obtained from GenBank. Alignment was performed by using

379 ClustalW software (Larkin et al., 2007). The three-dimensional structure of integrin α6

380 was predicted by using a platform SWISS-MODEL (Guex et al., 2009). The structure of

381 the peptide was depicted with the software PEP-FOLD3 (Lamiable et al., 2016).

382

383 *Zebrafish metastasis assay*

384 The mCherry-expressing MDA-MB-231 cells (Itou et al., 2017) were injected into the

385 abdominal cavity of an anesthetized 2 days post-fertilization Tg[*fli1a:egfp*]^{y1} zebrafish (N.

386 D. Lawson & Weinstein, 2002). After injection, the fish was recovered in water and

387 incubated for 3 days at 32°C with or without 25 μM peptide. The animal experiments in

388 this study were approved by the Ethics Review Board for Animal Experiments of Kyoto

389 University. The approval number is J-13-15-2.

390

391 *Sphere formation assay*

392 The procedure of sphere formation assay has been described previously (Matsumoto, Itou,
393 Sato, & Toi, 2018). A thousand cells were cultured in a well of an ultralow attachment
394 24-well plate (Corning, 3473, Kennebunk, ME, USA) with 500 μL of DMEM/F-12
395 medium containing 10 ng/mL EGF, 10 ng/mL basic FGF, 1% B-27, 5 μg/mL insulin and
396 0.03% LA717 (Wako, 381-09041) for 16 days. The number of spheres larger than 100
397 μm was counted manually by using ImageJ software.
398
399 *Mouse tail veil injection*
400 Six-week old nude mice were used. Two hundred thousand of MDA-MB-231 cells were
401 suspended in serum-free RPMI-1640 medium with or without 100 μg peptide and injected
402 into a tail vein. After 10 weeks, lung was harvested and the number of metastatic foci
403 were counted. The animal experiments in this study were approved by the Animal
404 Research Committee of Kyoto University, number MedKyo18321. All animals were
405 maintained according to the Guide for the Care and Use of Laboratory Animals (National
406 Institute of Health Publication).
407

Immunoprecipitation

Integrin α6A overexpression was introduced to MDA-MB-231 cells. Cells were treated with or without 25 μM peptide for 24 h. Immunoprecipitation was performed with a Dynabeads Co-Immunoprecipitation Kit (Veritas, DB14321, Tokyo, Japan) and anti-FLAG M2 antibody (Sigma, F1804). Additionally, 0.5% NP-40 was added to the extraction buffer of the kit used.

Immunofluorescence

Three thousand cells were plated in the well of a chamber slide (Matsunami glass, SCS-008, Osaka, Japan). After 2-day of culturing, cells were treated with various concentrations of the peptide for 1 h. Fixed cells were permeabilized with 0.1% TritonX-100 and then blocked with a blocking solution containing 5% goat serum and 1% BSA. The primary antibody was anti-phospho-paxillin (Tyr118) antibody (Cell Signaling Technology, 2541, 1:20 dilution). The secondary antibody was anti-rabbit IgG antibody conjugated to Alexa 546 (Life Technologies, A11010, 1:1000 dilution). Cells were counterstained with Hoechst 33342 (Dojindo, 1:1000 dilution). Fluoromount-G reagent

(SouthernBiotech, 0100-01, Birmingham, AL, USA) was used for mounting. The number of phosphorylated-paxillin signals was counted manually using ImageJ software (Schneider et al., 2012).

Laminin binding assay

For laminin binding assay, wells of a 24-well plate were coated with 400 μL of phosphate buffered saline containing 1 μg of laminin-511 E8 fragment (Nippi, 892013, Tokyo, Japan) for 1h at 37°C. After washing, the wells were blocked with 1% BSA for 1h at 37°C and washed. Then, 1,000 cells were plated with medium and incubated for 30 min at 37°C. The wells were washed, and cells were stained with crystal violet. Bound cell number was counted manually.

Microscopy

The images of cultured cells, migrated cells and immunostainings were collected with an all-in-one microscope, BZ-9000 (Keyence, Osaka, Japan) and the BZ-II Viewer software

(Keyence). Images of zebrafish embryos were collected with a fluorescent stereoscope, MZ16 FA (Leica, Mannheim, Germany) and analyzed with LAS AF software ver 2.6.0.

Statistics

Student's *t*-test was used for Boyden chamber assays, real-time PCR, sphere formation assays, cell growth assays and laminin binding assay (Fig. 2AD, 3E, 4AB, 5D). For multiple comparisons, Dunnett's test was used (Fig. 1CE, 3DF, 4C). Fisher's exact test was used in the zebrafish metastasis assay (Fig. 3G). $P<0.05$ was considered statistically significant. Error bars indicate standard deviations.

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Conflicts of interest

455 ST, NS, AI, ASF, TI and FS declare no conflict of interest. MT received research funding
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Figures

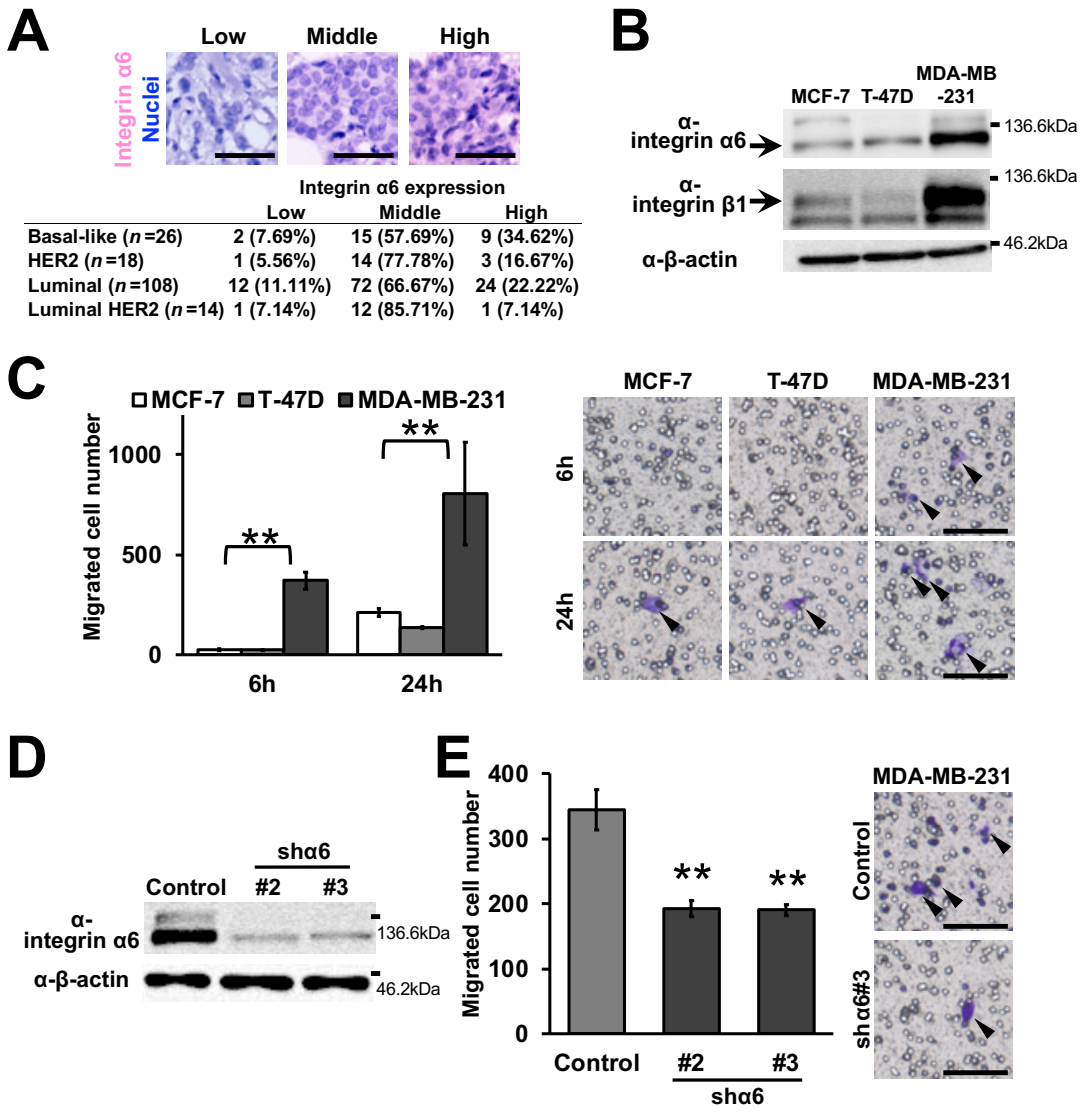

Figure 1. Integrin α6 expressing cells have high migratory ability in breast cancer. (A) Immunohistochemistry was performed in a tissue microarray with breast cancer tissues. Integrin α6 immunoreaction was visualized as pink staining. Classification of integrin α6 staining intensities was determined by 3 researchers. Bars indicate 50 μm. (B) Integrin

637 α6 and β1 expressions were analyzed in breast cancer cell lines, MCF-7, T-47D and
638 MDA-MB-231. β-actin was detected as the internal control. (C) The migratory ability of
639 MCF-7, T-47D and MDA-MB-231 cells were analyzed by Boyden chamber assays (n =
640 3). Cell migration was analyzed at 6 h and 24 h. Arrowheads indicate migrated cells. Bars
641 indicate 100 μm. (D) The efficiency of integrin α6 knockdown constructs were
642 investigated in MDA-MB-231 cells. Immunoblotting images were shown. (E) Migrated
643 cell numbers of integrin α6 knockdown cells are shown (6h, n = 3). Arrowheads indicate
644 migrated cells. Bars indicate 100 μm. **: P<0.01.
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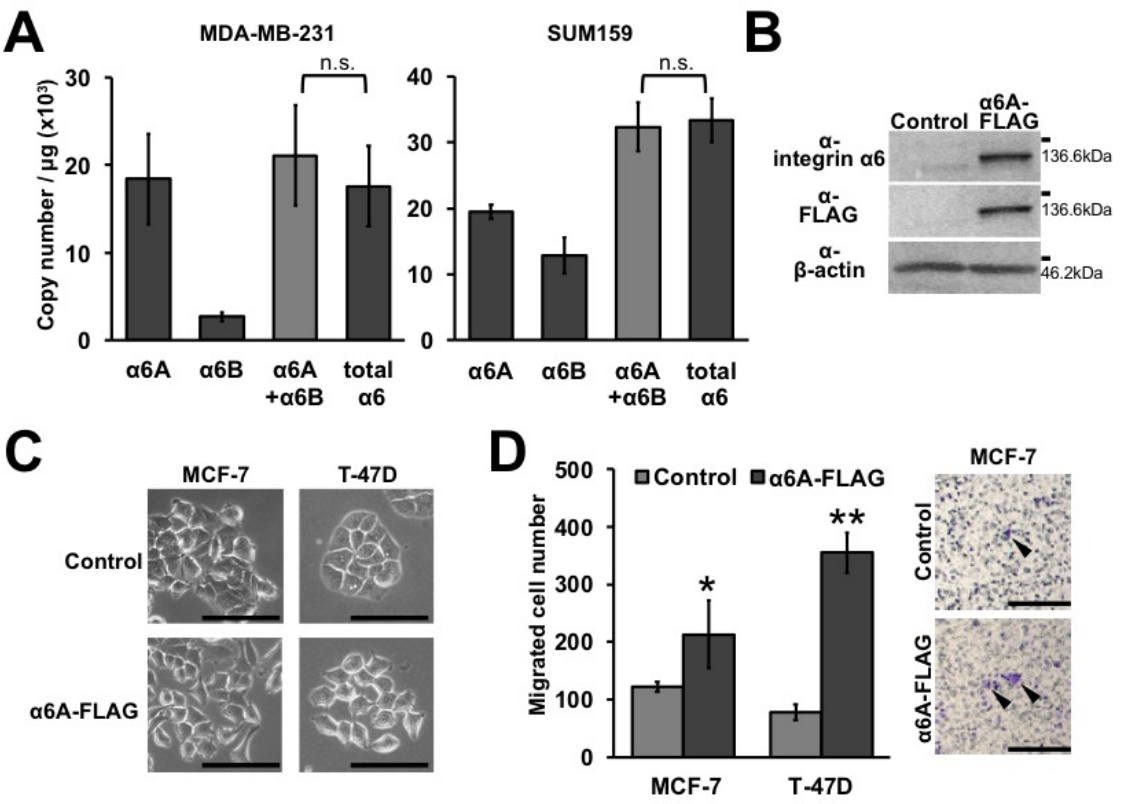

Figure 2. Integrin α6A enhances cell migration. (A) Quantification of the copy numbers of integrin α6A, α6B and total α6 was performed (n = 3). Basal-like breast cancer cell lines, MDA-MB-231 and SUM159, were used. (B) Integrin α6A overexpression was analyzed in MCF-7 cells. (C) Typical images of integrin α6 overexpressing cells are shown (n = 3 observations). MCF-7 and T-47D cells were used. Bars indicate 100 μm. (D) Cell migration was analyzed in integrin α6 overexpressing cells (24h, n = 3). Arrowheads indicate migrated cells. Bars indicate 100 μm. n.s.: not significant, *: P<0.05, **: P<0.01.

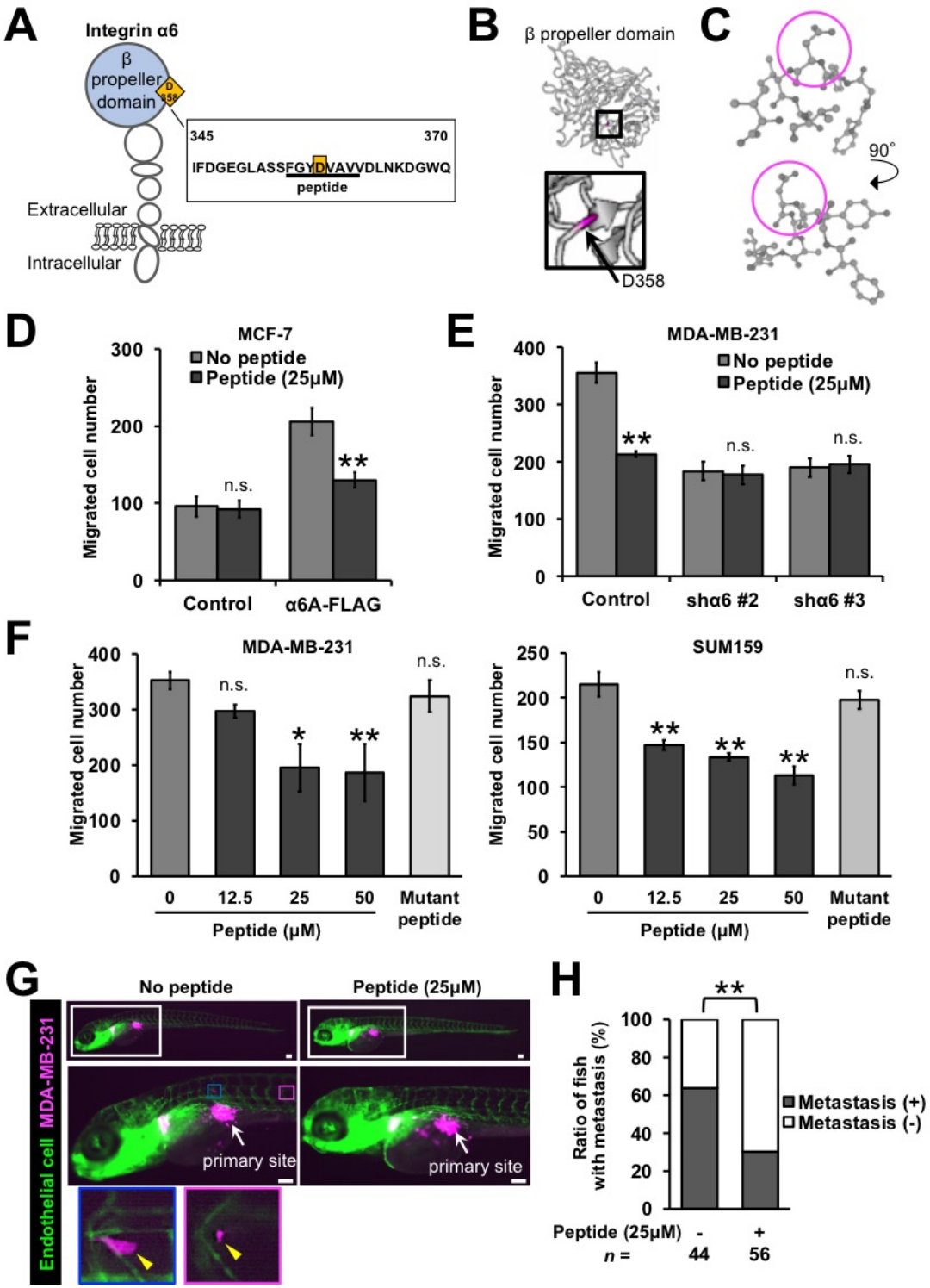

Figure 3. A peptide with the sequence around Asp-358 inhibits migration and metastasis.

(A) Location of Asp-358 (D358) is depicted. Amino acid sequence from 345 to 370 is

678 shown. Yellow box indicates Asp-358. The underlined sequence was used for the peptide.
679 (B) Predicted structure of the β propeller domain of integrin α6 is shown. Magenta
680 residue indicates Asp-358 (Arrow). (C) The structure of the peptide is shown. Magenta
681 circles indicate the aspartic acid residues corresponding to Asp-358. (D) Boyden chamber
682 assays were performed in integrin α6-overexpressing MCF-7 cells (24h, n = 3). Cells
683 were treated with 25 μM peptide. (E) Cell migration was analyzed in integrin α6-
684 knocked-down MDA-MB-231 cells (6h, n = 3). Cells were treated with 25 μM peptide.
685 (F) The effect of the peptide on cell migration was analyzed in MDA-MB-231 and
686 SUM159 cells (6h, n = 3). The concentration of mutant peptide was 50 μM. (G) Zebrafish
687 images are shown. MDA-MB-231 cells were injected. Injected fish was cultured with or
688 without 25 μM peptide. Yellow arrows indicate metastasized cells. Bars indicate 100 μm.
689 (H) Metastasis rates were graphed. n.s.: not significant, *: P<0.05, **: P<0.01.
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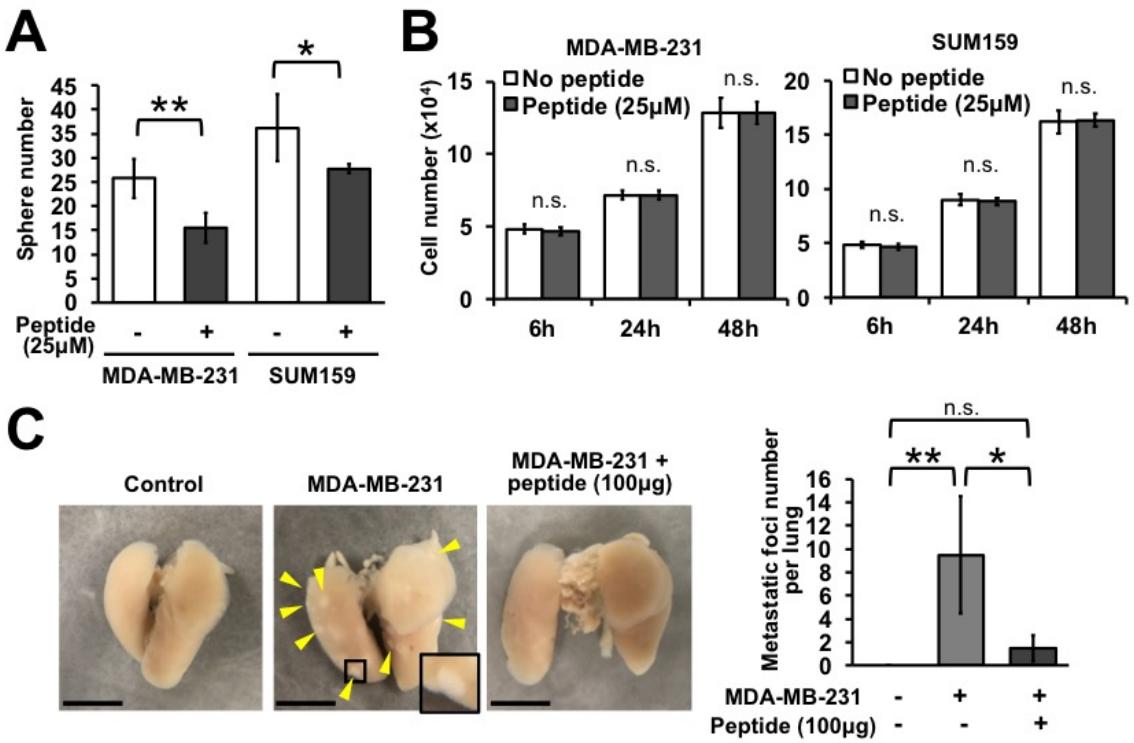

Figure 4. The peptide inhibits metastatic focus formation. (A) The results of sphere formation assays were graphed (n = 4). MDA-MB-231 cells were used. (B) MDA-MB-231 and SUM159 cells were cultured with or without the peptide (25μM). Cell number was counted at 6, 24 and 48 h (n = 3). (C) Images of the lungs of mice injected MDA-MB-231 cells are shown. Arrowheads indicate metastatic foci. For simplicity, not all foci are labeled. Bars indicate 5 mm. The numbers of metastatic foci were graphed (n = 4). n.s.: not significant, *: P<0.05, **: P<0.01.

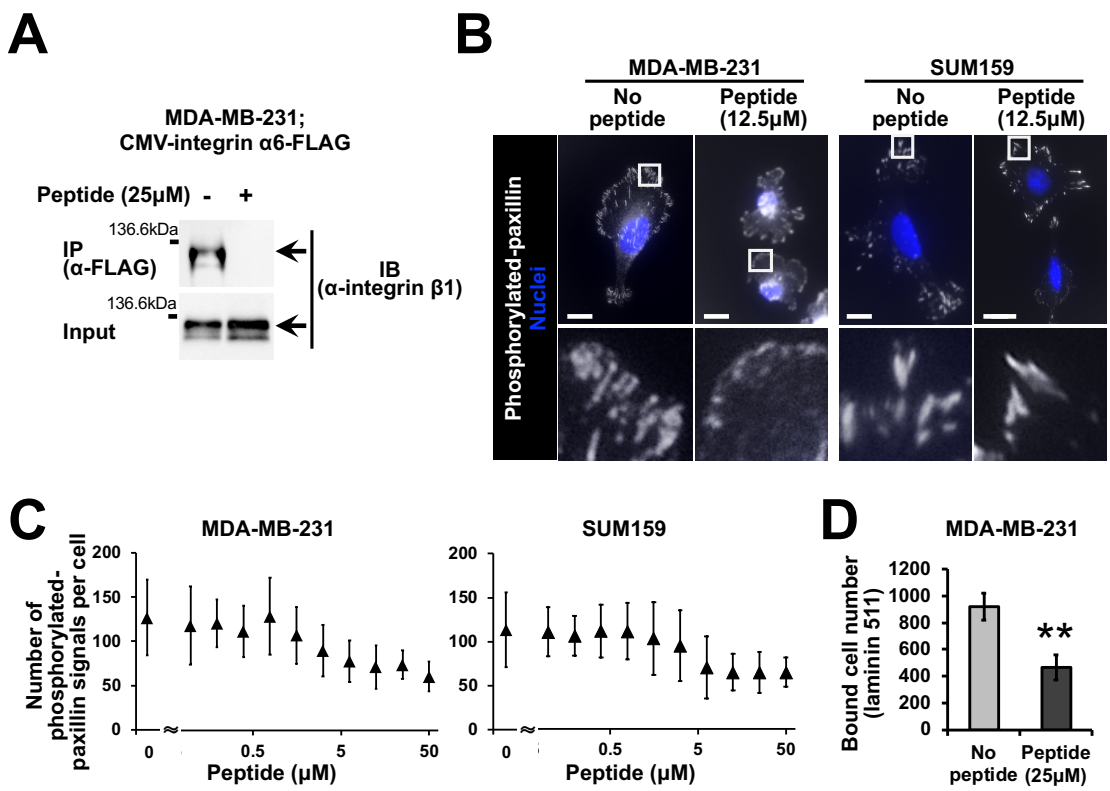

Figure 5. The peptide disrupts integrin α6 complex formation and inhibits integrin α6β1 function. (A) The result of immunoprecipitation assay is shown. Cells were treated with or without 25 μM peptide for 24 h. Immunoblotting was performed with anti-integrin β1 antibodies. (B) Phosphorylated-paxillins were immunostained. Hoechst was used for counter staining. Bars indicate 10 μm. (C) The numbers of phosphorylated-paxillin signals were graphed. More than 35 cells were analyzed in each concentration. (D) The result of laminin binding assay is graphed (n =3). MDA-MB-231 cells were used. **: P<0.01.

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728 **Supporting information**

729 **Supporting information Doc. S1** Alignment of the amino acid sequences of human

730 integrin α family

731 **Supporting information Doc. S2** Alignment of 68 integrin α6 amino acid sequences of

732 65 vertebrates

733 **Supporting information Fig. S1** Verification of primers for integrin α6 isoforms and

734 total integrin α6

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