

***In silico* analysis-based identification of the target residue of integrin $\alpha 6$ for
metastasis inhibition of basal-like breast cancer**

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24

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27

28 **Abstract**

29 Metastasis causes death in breast cancer patients. To inhibit breast cancer metastasis, we
30 focused on integrin $\alpha 6$, a membrane protein that contributes to cell migration and
31 metastasis. According to *in silico* analysis, we identified Asp-358 as an integrin $\alpha 6$ -
32 specific vertebrate-conserved residue, and consequently as a potential therapeutic target.
33 Because Asp-358 is located on the surface of the β propeller domain that interacts with
34 other molecules for integrin $\alpha 6$ function, we hypothesized that a peptide with the
35 sequence around Asp-358 competitively inhibits integrin $\alpha 6$ complex formation. We
36 treated basal-like breast cancer cells with the peptide and observed reductions in cell
37 migration and metastasis. The result of the immunoprecipitation assay showed that the
38 peptide inhibited integrin $\alpha 6$ complex formation. Our immunofluorescence for
39 phosphorylated paxillin, a marker of integrin-regulated focal adhesion, showed that the
40 peptide reduced the number of focal adhesions. These results indicate that the peptide
41 inhibits integrin $\alpha 6$ function. This study identified the target residue of integrin $\alpha 6$ and
42 designed the inhibitory peptide. For breast cancer patients, metastasis inhibition therapy
43 may be developed in the future based on this study.

44 **Introduction**

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

49 During the process of metastasis, cancer cells move from the primary site to
50 other parts of a body (Nguyen, Bos, & Massagué, 2009). This metastatic process includes
51 multiple steps, such as local invasion, intravasation, anoikis resistance, extravasation and
52 formation of metastatic focus. Because the migratory ability promotes some of these steps,
53 an acquisition mechanism for this high migratory ability may be a therapeutic target. In
54 metastatic basal-like breast cancer (Yehiely, Moyano, Evans, Nielsen, & Cryns, 2006),
55 we previously reported that integrin $\alpha 6 \beta 1$ promotes cell migration and metastasis through
56 activation of focal adhesion dynamics (Itou et al., 2017). This suggests that an inhibitor
57 for integrin $\alpha 6 \beta 1$ may result in inhibiting breast cancer metastasis.

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

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

In the human genome, there are 18 α and 8 β integrin subunit genes. Previously, various integrin inhibitors have been developed. An inhibitor of integrin $\alpha 2\beta 1$ was designed for cardiovascular disease (Miller et al., 2009). Cilengitide, an inhibitor of $\alpha v\beta 3$ and $\alpha v\beta 5$, is being evaluated in clinical trials for glioblastoma therapy (Scaringi, Minniti, Caporello, & Enrici, 2012). Ley *et al.* reviewed inhibitors of integrin $\alpha I\text{Ib}\beta 3$, $\alpha 4\beta 7$ and $\alpha E\beta 7$ (Ley, Rivera-Nieves, Sandborn, & Shattil, 2016). Several of these inhibitors are in clinical trials or clinically used. For example, antagonists against $\alpha I\text{Ib}\beta 3$, such as abciximab, eptifibatide and tirofiban, were developed for percutaneous coronary

interventions (Bledzka, Smyth, & Plow, 2013). Treatment with natalizumab, an antibody for the $\alpha 4$ 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 $\alpha L\beta 2$, 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 $\alpha 3$, $\alpha 6$, αv and $\beta 1$ reduce cell proliferation, and the antibodies for $\alpha 3$, $\alpha 6$ and $\beta 1$ inhibits migration (Ahmed, Riley, Rice, & Quinn, 2005). An anti-integrin $\alpha 6$ 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 $\alpha 6$ reduces cell proliferation and invasion in esophageal squamous cell carcinoma (Kwon et al., 2013). In addition, anti-integrin $\beta 1$ 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.

Integrin $\alpha 6$ (also known as VLA-6 and CD49f, coded by *ITGA6*) is expressed in normal and cancer cells (Ahmed et al., 2005; Chen et al., 2016; Ding et al., 2013; Kwon et al., 2013; Pontier & Muller, 2009). It forms a heterodimer with integrin $\beta 1$ or $\beta 4$, which binds to a component of the extracellular matrix, laminin (Hynes, 1992). The mouse null mutation of integrin $\alpha 6$ causes early postnatal death with aberration in the skin tissue (Georges-Labouesse et al., 1996). In human, homozygous mutation of the integrin $\alpha 6$ gene causes a skin disease with blisters (Pulkkinen et al., 1997). Integrin $\alpha 6$ contributes to radiotherapy resistance in breast cancer cells through activation of Akt and Erk signaling (Hu, Zhou, Zhao, & Wu, 2016). In malignant breast cancer cell lines, integrin $\alpha 6$ is involved in migration and stemness (Chang et al., 2015; Itou et al., 2017). However, amino acid residue(s) required for integrin $\alpha 6$ functions has not been fully understood.

This study focused on integrin $\alpha 6$ for the inhibition of cell migration in breast cancer cells. Our *in silico* analyses identified Asp-358 as a target residue for metastasis inhibition in the integrin $\alpha 6$ amino acid sequence. A peptide with the same sequence 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 $\alpha 6$ 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 $\alpha 6$ expression, we immunostained a tissue microarray with anti-integrin $\alpha 6$ antibody. The results showed that integrin $\alpha 6$ 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 $\alpha 6$ 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 $\beta 1$ is a binding partner of Integrin $\alpha 6$ (Hynes, 1992), and integrin $\alpha 6\beta 1$ promotes cell migration in basal-like breast cancer (Itou et al., 2017). Under our conditions, integrin $\beta 1$ expression was observed in both luminal and basal-like breast cancer cell lines. MDA-MB-231 cells showed higher integrin $\beta 1$ expression than Luminal breast cancer cell lines (Fig. 1B).

To analyze the migratory ability of breast cancer cell lines, we performed Boyden chamber assays, and observed that basal-like breast cancer cells have a higher migratory ability than luminal breast cancer cells (Fig. 1C). To determine the role of integrin $\alpha 6$ in cell migration, we performed shRNA-mediated integrin $\alpha 6$ knockdown and Boyden chamber assays. We used two shRNA constructs (Itou et al., 2017), and both reduced the integrin $\alpha 6$ level in MDA-MB-231 cells (Fig. 1D). In the Boyden chamber assays, integrin $\alpha 6$ knockdown reduced the number of migrated cells (Fig. 1E). These results indicate that high expression of integrin $\alpha 6$ promotes cell migration in basal-like breast cancer cells.

Integrin $\alpha 6$ has the potential to promote the cell migration of luminal breast cancer cells.

Integrin $\alpha 6$ has two isoforms, named $\alpha 6A$ and $\alpha 6B$, which share the extracellular region and have different cytoplasmic domains (Hogervorst, Kuikman, van Kessel, & Sonnenberg, 1991). We designed primers for each isoform and total $\alpha 6$. Primers for each isoform specifically amplified the target (Supporting information Fig. S1). We calculated the mRNA copy numbers of the isoforms and total $\alpha 6$ in the samples from basal-like

breast cancer cell lines. The sum of the copy numbers of $\alpha 6A$ and $\alpha 6B$ were comparable with that of total $\alpha 6$, supporting the reliability of our quantification (Fig. 2A). The results of quantitative RT-PCR revealed that $\alpha 6A$ expression is higher than $\alpha 6B$ in basal-like breast cancer cells (Fig. 2A).

Luminal breast cancer cells have weak integrin $\alpha 6$ expression (Fig. 1B) and a low migratory ability (Fig. 1C). We overexpressed integrin $\alpha 6A$ in these cells (Fig. 2B). In a 2-dimensional culture, luminal breast cancer cell lines showed compacted colonies. We observed that integrin $\alpha 6A$ 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 $\alpha 6A$ overexpression significantly enhanced the cell migration of luminal breast cancer cells (Fig. 2D), suggesting that integrin $\alpha 6$ has the potential to promote the cell migration of breast cancer cells.

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

154 Our data suggest that inhibition of integrin $\alpha 6$ reduces cell migration and metastasis in
155 breast cancer cells. To design an inhibitory peptide for integrin $\alpha 6$, target amino acid
156 residue(s) should be identified. Because functionally important amino acid residues are
157 conserved among species, we performed *in silico* analyses to identify an integrin $\alpha 6$ -
158 specific vertebrate-conserved amino acid residue. First, we compared the amino acid
159 sequences of the integrin α family in the human genome to extract integrin $\alpha 6$ -specific
160 residues (Supporting information Doc. S1). The results showed that integrin $\alpha 6$ has 276
161 specific residues. Next, to determine whether these residues are conserved among species,
162 we aligned the amino acid sequences of integrin $\alpha 6$ for 65 vertebrates, such as human,
163 mouse, chicken, frog and zebrafish (Supporting information Doc. S2). The amino acid
164 analysis identified Asp-358, which is located at the β propeller domain of integrin $\alpha 6$
165 (Fig. 3A). The integrin $\alpha 6$ structure was predicted based on the crystal structures of
166 integrin $\alpha 5$ and αV (Nagae et al., 2012; Xiong et al., 2009) with a protein structure
167 prediction platform (Guex, Peitsch, & Schwede, 2009), and the location of Asp-358 was
168 analyzed. The results showed that Asp-358 is located at the surface of the β propeller
169 domain (Fig. 3B).

170 Because the β propeller domain integrin α subunits interact with other
171 molecules, such as integrin β subunits, to function (Hynes, 1992; Shattil et al., 2010), it
172 is possible that a peptide with the sequence around Asp-358 reduces cell migration
173 through competitive inhibition of integrin $\alpha 6$ complex formation. We designed an 8
174 amino acid peptide with the sequence of FGYDVAVV (Fig. 3A and 3C, the peptide
175 structure was depicted by software (Lamiable et al., 2016)). We treated the luminal breast
176 cancer cell line, MCF-7, with the peptide and analyzed cell migration. The peptide
177 significantly reduced cell migration in integrin $\alpha 6$ A overexpressing cells, whereas no
178 change was observed in the control cells (Fig. 3D). In the basal-like breast cancer cell line
179 MDA-MB-231, peptide treatment significantly reduced cell migration. However, there
180 was no reduction effect in cell migration in the peptide-treated integrin $\alpha 6$ -knocked-down
181 cells compared with the no-peptide control (Fig. 3E). These results suggest that the
182 peptide inhibits integrin $\alpha 6$ -mediated cell migration.

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

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

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

201

202 *The peptide-mediated integrin $\alpha 6$ inhibition reduces metastatic focus formation.*

203 Integrin $\alpha 6$ contributes stemness in breast cancer cells (Chang et al., 2015; Goel et al.,
204 2014). Stemness and anoikis resistance is required for formation of a metastatic focus. To
205 analyze stemness and anoikis resistance, we performed sphere formation assay. We
206 observed reduced sphere formation rate in the cells treated with the peptide (Fig. 4A),
207 indicating that the peptide reduces stemness and/or anoikis resistance. In breast cancer
208 cells, peptide treatment did not change cell growth in basal-like breast cancer cells (Fig.
209 4B).

210 Although zebrafish metastasis assay can evaluate *in vivo* cell migration,
211 intravasation and extravasation, it is not able to analyze anoikis resistance and metastatic
212 focus formation. To determine whether the peptide also inhibits anoikis resistance and
213 metastatic focus formation, we conducted mouse tail vein injection of MDA-MB-231
214 cells with or without the peptide treatment. We counted the number of metastatic foci in
215 the lungs of the injected mice. The peptide treatment significantly reduced the number of
216 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 $\alpha 6$ with $\beta 1$, and reduces the number of focal*
221 *adhesions.*

222 To determine whether the peptide disrupts the interaction of integrin $\alpha 6$ with $\beta 1$, we
223 conducted immunoprecipitation assay with anti-FLAG antibody in MDA-MB-231 cells
224 overexpressing integrin $\alpha 6$ -FLAG. In the control sample, we observed integrin $\beta 1$ 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 $\alpha 6$ and $\beta 1$.

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 μ M (MDA-MB-231 cells) and 6.25 μ M 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 μ M (MDA-MB-231 cells) and 3.377 μ M (SUM159 cells).

Integrin α 6 β 1 binds to laminin 511 (Nishiuchi et al., 2006). To determine whether the peptide-mediated inhibition of integrin α 6 β 1 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 α 6 β 1 complex formation.

Discussion

In this study, we observed high integrin α 6 expression in basal-like breast cancer cells. Integrin α 6 expression enhanced cell migration. We identified the integrin α 6-specific

vertebrate-conserved residue, Asp-358. A peptide with the same sequence around Asp-358 reduced cell migration in basal-like breast cancer cells. Zebrafish metastasis assays and mouse tail vein injections of breast cancer cells showed the reduced metastatic ability in the peptide-treated group. Moreover, our immunoprecipitation showed that the peptide disrupts integrin $\alpha 6$ complex formation.

Under our conditions, integrin $\alpha 6$ and $\beta 1$ expressions were high in MDA-MB-231 cells. In luminal cell lines, integrin $\beta 1$ expression was detected (Fig. 1). Integrin $\alpha 6$ has two isoforms $\alpha 6A$ and $\alpha 6B$. We previously reported that both isoforms promote cell migration in basal-like breast cancer cells (Itou et al., 2017). This study showed that $\alpha 6A$ is the major isoform in basal-like breast cancer cells. We overexpressed integrin $\alpha 6A$ in luminal cell lines and observed the promotion of cell migration (Fig. 2). These results suggest that integrin $\alpha 6A\beta 1$ can enhance cell migration in luminal breast cancer cells.

In silico analyses can identify functional residues in a protein. In this study, we found Asp-358 to be a candidate for the functional residue of integrin $\alpha 6$. The result of the prediction of the integrin $\alpha 6$ structure showed that Asp-358 is located at the surface of the β propeller domain. The β propeller domain of the integrin α family is located at

the N-terminal region and has 7 β sheets (Springer, 1997). Each sheet consists of 4 antiparallel β strands and loops. *In silico* tools for protein structure (Guex et al., 2009) predicted that Asp-358 is at a boundary between a β strand and a loop. Because the β propeller domain of the integrin α subunit interacts with the integrin β subunit (Shattil et al., 2010), it is possible that Asp-358 is involved in integrin complex formation. To support this, the peptide with the sequence around Asp-358 of integrin $\alpha 6$, inhibited the dimerization of integrin $\alpha 6\beta 1$ (Fig. 5).

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

Our immunoprecipitation assay showed that the peptide inhibits the heterodimer formation of integrin $\alpha 6$ and $\beta 1$ (Fig. 5). Integrins regulate focal adhesion formation (Huttenlocher & Horwitz, 2011; C. Lawson & Schlaepfer, 2012). We observed 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 $\alpha 6 \beta 1$ 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 $\alpha 6$, 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 $\alpha 6$ -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 $\alpha 6$ is involved in breast cancer metastasis (Hoshino et al., 2015), the
293 peptide may reduce metastasis via inhibition of integrin $\alpha 6$ positive exosomes. Although
294 integrin $\alpha 6$ 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 μ g/mL insulin, 1
306 μ g/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 μ g/mL puromycin
312 or 10 μ g/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 $\alpha 6$ 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.

329 *Immunoblotting*

330 The primary antibodies used were anti-integrin $\alpha 6$ antibody (Cell Signaling Technology,
331 3750, Danvers, MA, USA, 1:1000 dilution), anti-integrin $\beta 1$ antibody (Cell Signaling
332 Technology, D2E5, 9699, 1:1000 dilution) and anti-DYKDDDDK antibody (Wako, 018-
333 22386, Osaka, Japan, 1:1000 dilution, for FLAG tag detection). For the secondary
334 reaction, the Easy-Western-II detection system (Beacle, BCL-EZS21, Kyoto, Japan) was
335 used. For an internal control, an anti- β -actin antibody (Abcam, ab6276, Cambridge, UK,
336 1:10000 dilution) and anti-mouse IgG antibody conjugated to a peroxidase (Pierce
337 biotechnology, 31340, Rockford, IL, USA, 1:50000 dilution) were used. Signals were
338 developed with ECL select reagent (GE Healthcare, RPN2235, Buckinghamshire, UK).
339 Images were collected with Ez-Capture II (ATTO, Tokyo, Japan) and Image Server 5
340 software (ATTO).

341

342 *Loss- and gain-of-function studies*

343 For integrin $\alpha 6$ knockdown, the shRNA-mediated gene silencing system with a lentiviral
344 vector, pLKO.1 (Addgene, 8453, Cambridge, MA, USA) was used. The target sequences

345 were #2: 5'-CGAGAAGGAAATCAAGACAAA-3' and #3: 5'-
346 CGGATCGAGTTTGATAACGAT-3'. The shRNA control sequence was 5'-
347 CCTAAGGTTAAGTCGCCCTCG-3'. For integrin α 6A overexpression, integrin α 6A-
348 FLAG gene was constructed and inserted it between the *Eco*RI and the *Xho*I sites of a
349 lentiviral vector, pLenti-6.3 (Life Technologies, V533-06, Carlsbad, CA, USA). The
350 FLAG (DYKDDDDK) expression vector was used for the control. A previously
351 established lentiviral system was used (Dull et al., 1998). Virus particles were produced
352 in Lenti-X 293T cells (Takara, 632180, Otsu, Japan).

353

354 *Boyden chamber assay*

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

361

362 *Quantification of the copy number of mRNAs*

363 Integrin α 6A and α 6B 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 μ g of total RNA samples with an oligo dT18 primer.
367 Real-time PCR was performed with primer sets for α 6A: forward 5'-
368 GCCACATATCACAAGGCTGAG-3' and reverse 5'-GCGTTTAAAGAATCCACACA
369 -3', α 6B: forward 5'-CAAATGCAGGCACTCAGGTTC-3' and reverse 5'-
370 GCGTTTAAAGAATCCACACT-3', and total α 6: forward 5'-
371 GGACAGCAAGGCGTCTCTTATT-3' and reverse 5'-
372 CGGCAGCAGCAGTCACATCAA-3'. Primers were validated by checking whether
373 specific amplification was observed with the α 6A or α 6B 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 $\alpha 6$
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[*flila: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 μ M 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*

The procedure of sphere formation assay has been described previously (Matsumoto, Itou, Sato, & Toi, 2018). A thousand cells were cultured in a well of an ultralow attachment 24-well plate (Corning, 3473, Kennebunk, ME, USA) with 500 μ L of DMEM/F-12 medium containing 10 ng/mL EGF, 10 ng/mL basic FGF, 1% B-27, 5 μ g/mL insulin and 0.03% LA717 (Wako, 381-09041) for 16 days. The number of spheres larger than 100 μ m was counted manually by using ImageJ software.

Mouse tail vein injection

Six-week old nude mice were used. Two hundred thousand of MDA-MB-231 cells were suspended in serum-free RPMI-1640 medium with or without 100 μ g peptide and injected into a tail vein. After 10 weeks, lung was harvested and the number of metastatic foci were counted. The animal experiments in this study were approved by the Animal Research Committee of Kyoto University, number MedKyo18321. All animals were maintained according to the Guide for the Care and Use of Laboratory Animals (National Institute of Health Publication).

408 *Immunoprecipitation*

409 Integrin $\alpha 6A$ overexpression was introduced to MDA-MB-231 cells. Cells were treated
410 with or without 25 μM peptide for 24 h. Immunoprecipitation was performed with a
411 Dynabeads Co-Immunoprecipitation Kit (Veritas, DB14321, Tokyo, Japan) and anti-
412 FLAG M2 antibody (Sigma, F1804). Additionally, 0.5% NP-40 was added to the
413 extraction buffer of the kit used.

414

415 *Immunofluorescence*

416 Three thousand cells were plated in the well of a chamber slide (Matsunami glass, SCS-
417 008, Osaka, Japan). After 2-day of culturing, cells were treated with various
418 concentrations of the peptide for 1 h. Fixed cells were permeabilized with 0.1% TritonX-
419 100 and then blocked with a blocking solution containing 5% goat serum and 1% BSA.
420 The primary antibody was anti-phospho-paxillin (Tyr118) antibody (Cell Signaling
421 Technology, 2541, 1:20 dilution). The secondary antibody was anti-rabbit IgG antibody
422 conjugated to Alexa 546 (Life Technologies, A11010, 1:1000 dilution). Cells were
423 counterstained with Hoechst 33342 (Dojindo, 1:1000 dilution). Fluoromount-G reagent

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

427

428 Laminin binding assay

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

435

436 *Microscopy*

437 The images of cultured cells, migrated cells and immunostainings were collected with an
438 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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provided by Taiho Pharmaceutical Co., Ltd.

Conflicts of interest

ST, NS, AI, ASF, TI and FS declare no conflict of interest. MT received research funding from Taiho Pharmaceutical Co., Ltd. JI is an employee of Kyoto University's Sponsored Research Program funded by Taiho Pharmaceutical Co., Ltd. The funding source had no role in the study design, experiment, analysis, interpretation or writing the manuscript.

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Figures

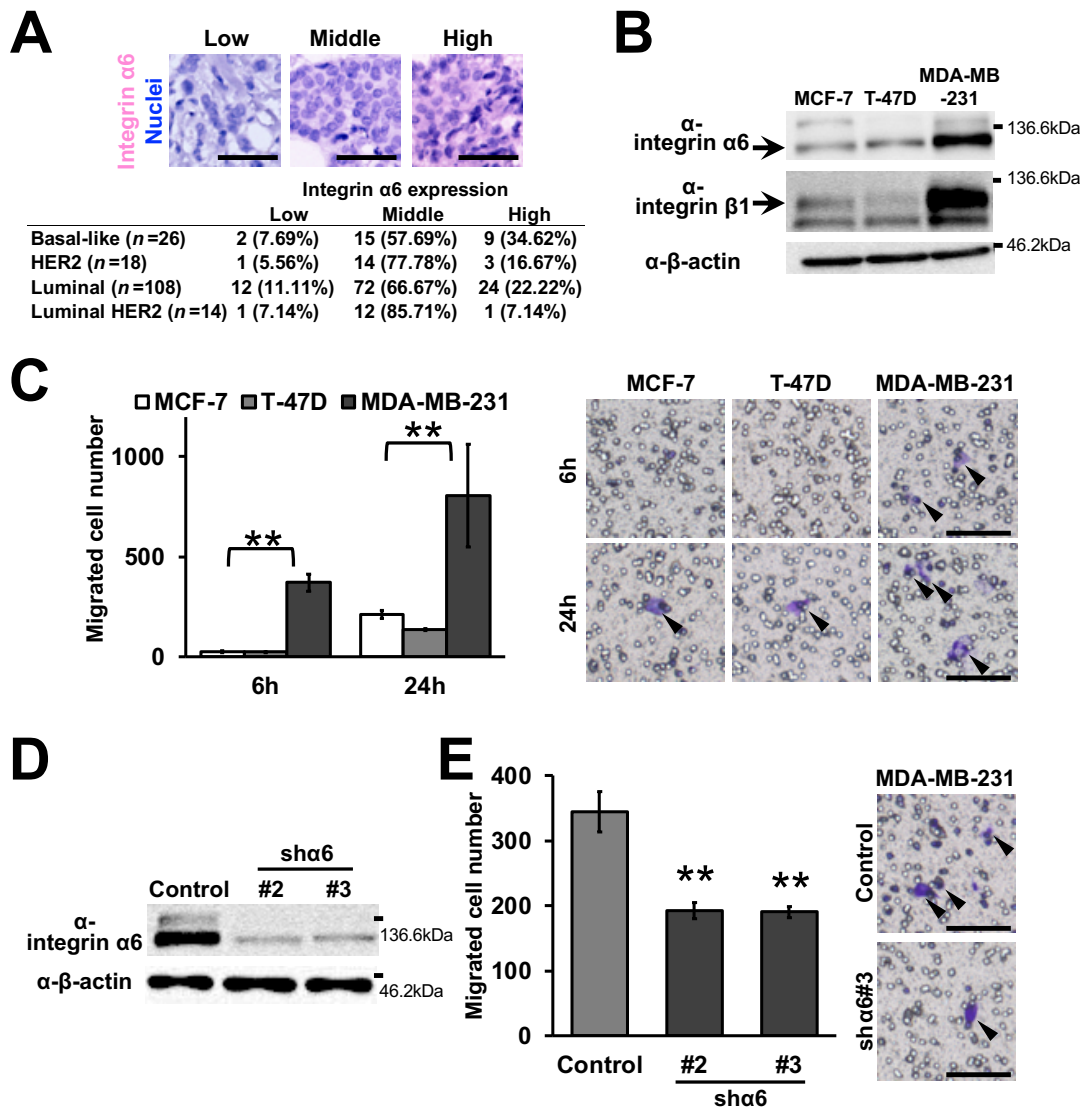


Figure 1. Integrin α6 expressing cells have high migratory ability in breast cancer. (A) Immunohistochemistry was performed in a tissue microarray with breast cancer tissues. Integrin α6 immunoreaction was visualized as pink staining. Classification of integrin α6 staining intensities was determined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

$\alpha 6$ and $\beta 1$ expressions were analyzed in breast cancer cell lines, MCF-7, T-47D and MDA-MB-231. β -actin was detected as the internal control. (C) The migratory ability of MCF-7, T-47D and MDA-MB-231 cells were analyzed by Boyden chamber assays ($n = 3$). Cell migration was analyzed at 6 h and 24 h. Arrowheads indicate migrated cells. Bars indicate 100 μ m. (D) The efficiency of integrin $\alpha 6$ knockdown constructs were investigated in MDA-MB-231 cells. Immunoblotting images were shown. (E) Migrated cell numbers of integrin $\alpha 6$ knockdown cells are shown (6h, $n = 3$). Arrowheads indicate migrated cells. Bars indicate 100 μ m. **: $P < 0.01$.

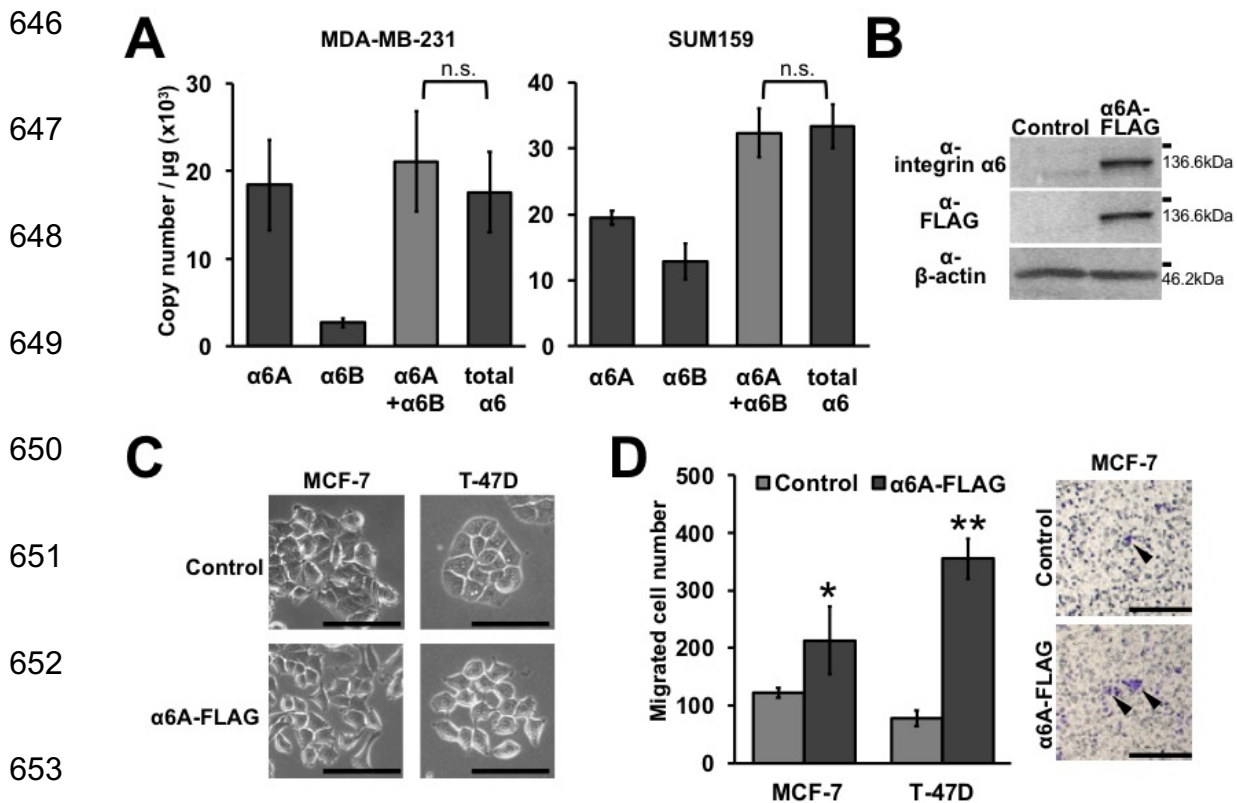


Figure 2. Integrin $\alpha 6\text{A}$ enhances cell migration. (A) Quantification of the copy numbers of integrin $\alpha 6\text{A}$, $\alpha 6\text{B}$ and total $\alpha 6$ was performed ($n = 3$). Basal-like breast cancer cell lines, MDA-MB-231 and SUM159, were used. (B) Integrin $\alpha 6\text{A}$ overexpression was analyzed in MCF-7 cells. (C) Typical images of integrin $\alpha 6$ 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 $\alpha 6$ overexpressing cells (24h, $n = 3$). Arrowheads indicate migrated cells. Bars indicate 100 μm . n.s.: not significant, *: $P < 0.05$, **: $P < 0.01$.

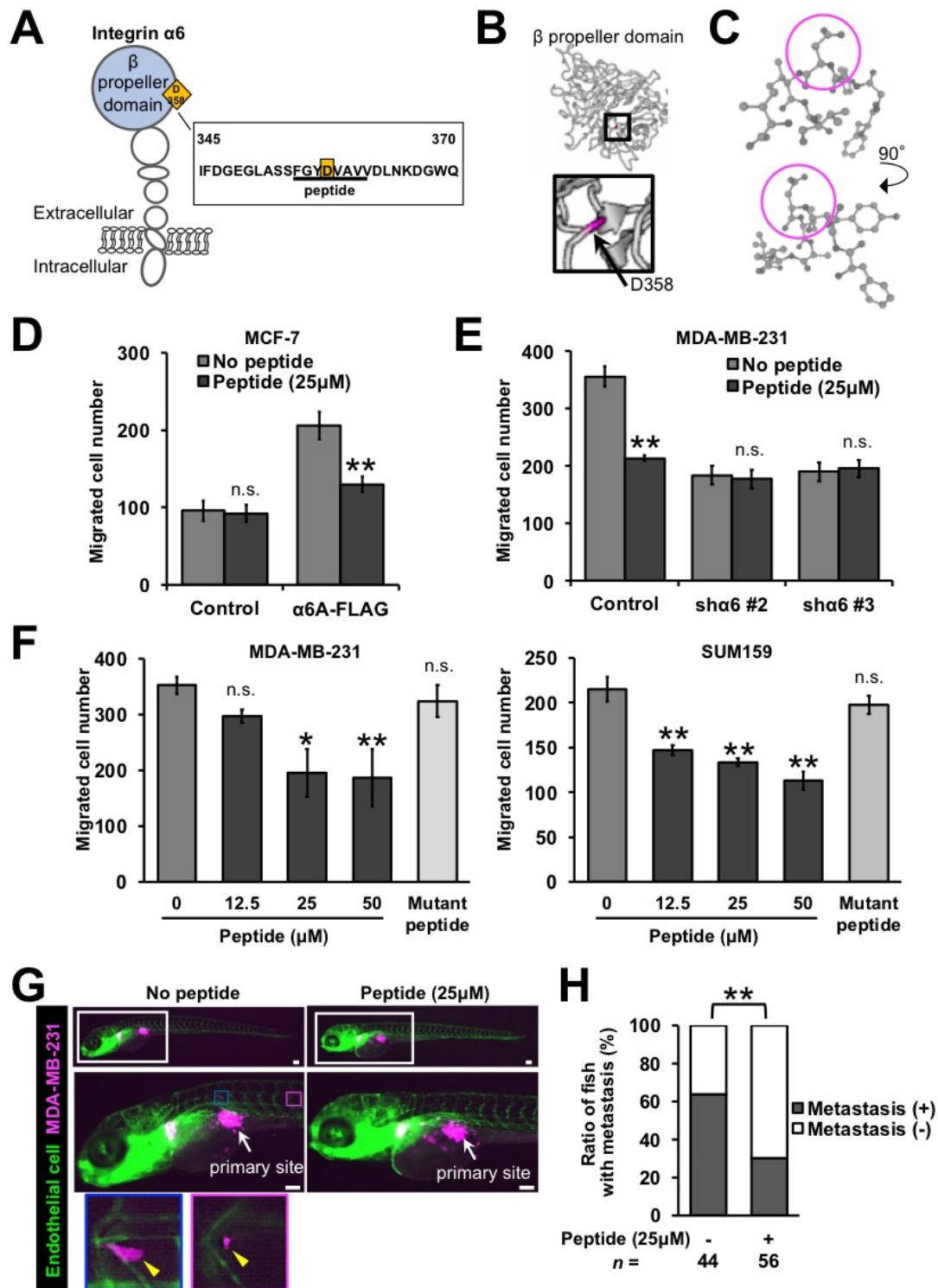


Figure 3. A peptide with the sequence around Asp-358 inhibits migration and metastasis.

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

shown. Yellow box indicates Asp-358. The underlined sequence was used for the peptide.

(B) Predicted structure of the β propeller domain of integrin $\alpha 6$ is shown. Magenta

residue indicates Asp-358 (Arrow). (C) The structure of the peptide is shown. Magenta

circles indicate the aspartic acid residues corresponding to Asp-358. (D) Boyden chamber

assays were performed in integrin $\alpha 6$ -overexpressing MCF-7 cells (24h, $n = 3$). Cells

were treated with 25 μ M peptide. (E) Cell migration was analyzed in integrin $\alpha 6$ -

knocked-down MDA-MB-231 cells (6h, $n = 3$). Cells were treated with 25 μ M peptide.

(F) The effect of the peptide on cell migration was analyzed in MDA-MB-231 and

SUM159 cells (6h, $n = 3$). The concentration of mutant peptide was 50 μ M. (G) Zebrafish

images are shown. MDA-MB-231 cells were injected. Injected fish was cultured with or

without 25 μ M peptide. Yellow arrows indicate metastasized cells. Bars indicate 100 μ m.

(H) Metastasis rates were graphed. n.s.: not significant, *: $P < 0.05$, **: $P < 0.01$.

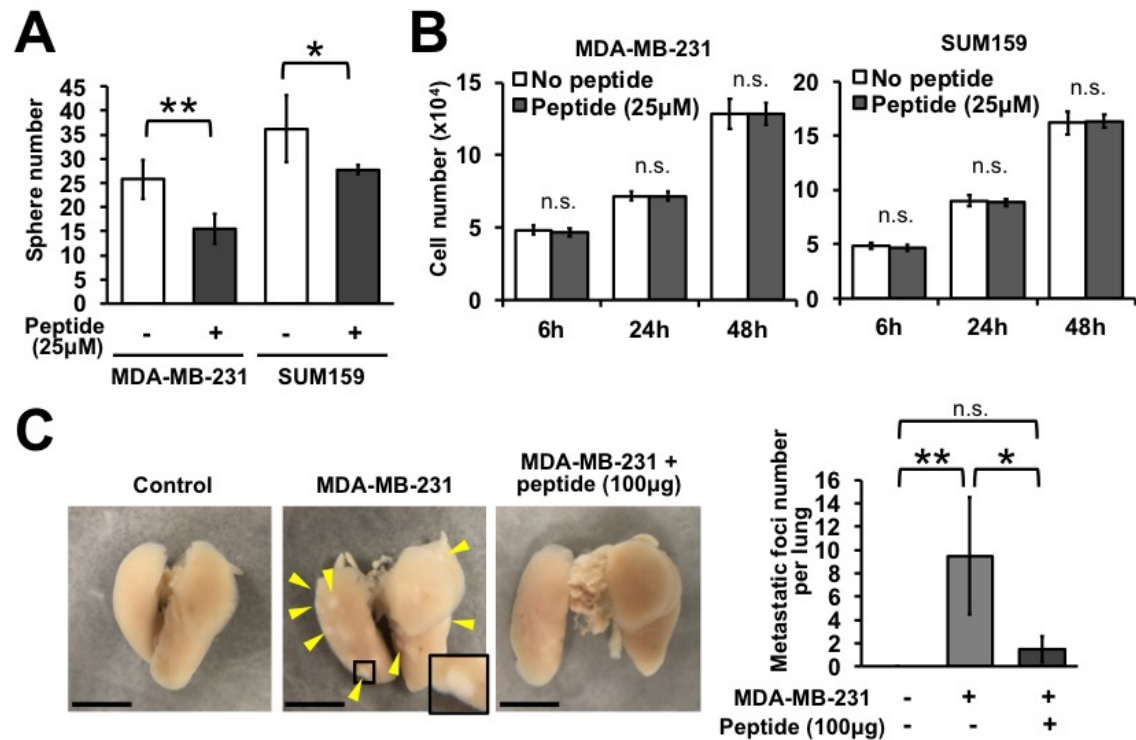


Figure 4. The peptide inhibits metastatic focus formation. (A) The results of sphere formation assays were graphed ($n = 4$). MDA-MB-231 cells were used. (B) MDA-MB-231 and SUM159 cells were cultured with or without the peptide (25μM). Cell number was counted at 6, 24 and 48 h ($n = 3$). (C) Images of 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, *: $P < 0.05$, **: $P < 0.01$.

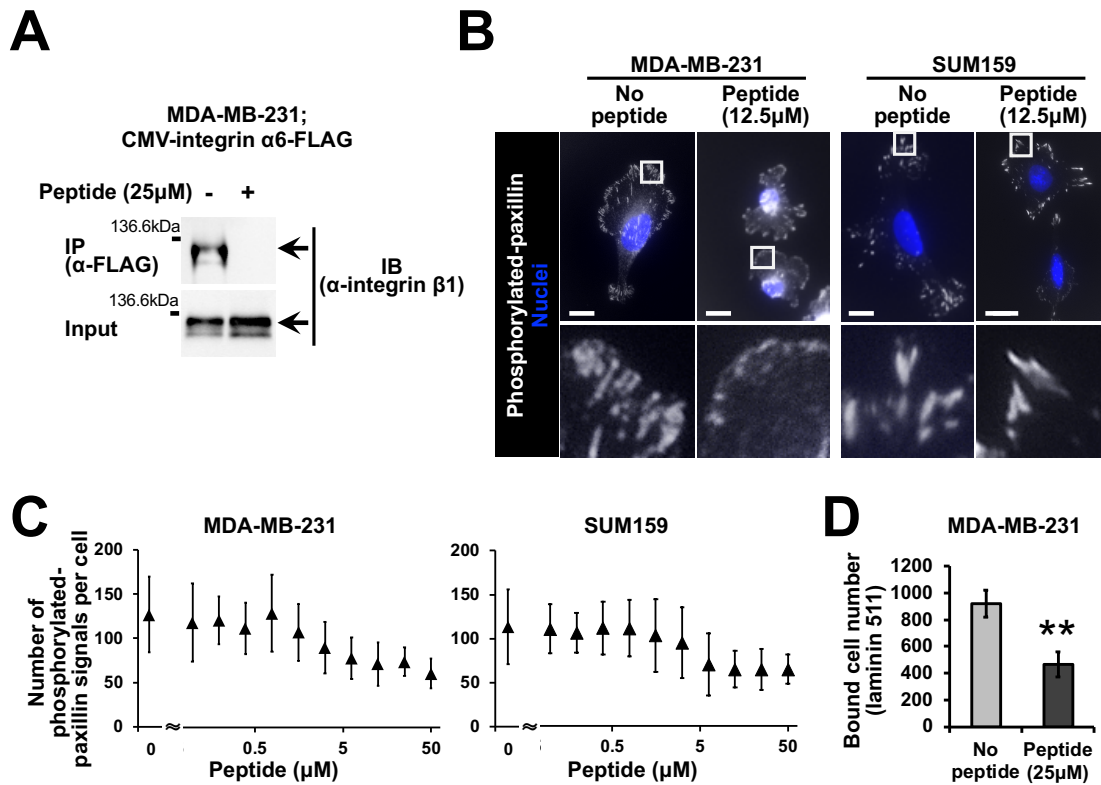


Figure 5. The peptide disrupts integrin $\alpha 6$ complex formation and inhibits integrin $\alpha 6\beta 1$ function. (A) The result of immunoprecipitation assay is shown. Cells were treated with or without 25 μ M peptide for 24 h. Immunoblotting was performed with anti-integrin $\beta 1$ antibodies. (B) Phosphorylated-paxillins were immunostained. Hoechst was used for counter staining. Bars indicate 10 μ m. (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. **: $P < 0.01$.

727

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 α 6 amino acid sequences of

732 65 vertebrates

733 **Supporting information Fig. S1** Verification of primers for integrin α 6 isoforms and

734 total integrin α 6

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