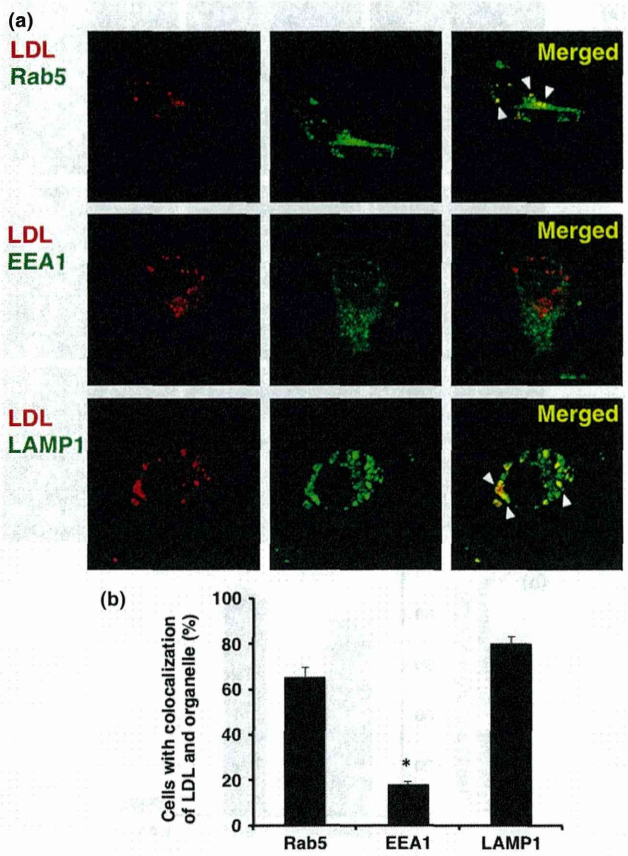

Fig. 5. Low-density lipoprotein (LDL) was targeted to the dynamic early endosome followed by translocation to the late endosome/lysosome for degradation. (a) Human monocyte-derived dendritic cells were incubated at 37°C with Alexa 594-labeled LDL. Organelles were stained with an anti-EEA1 mAb, anti-Rab5 polyclonal antibody, and anti-LAMP-1 polyclonal antibody, followed by Alexa 488-conjugated goat anti-rabbit IgG or anti-mouse IgG. Arrowheads indicate colocalization of internalized LDL and each organelle. (b) To quantify the percentage of colocalization, a single z-plane of one cell was evaluated. For each protein and organelle combination, a total of 150 cells (50 cells from three independent experiments) were analyzed. Data are shown as means + SEM of three independent experiments. $^*P < 0.01$.

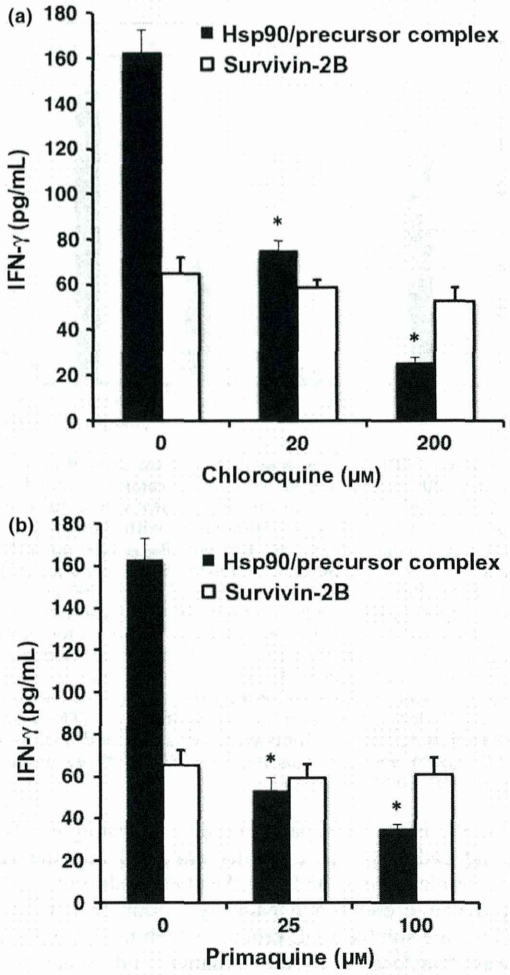

Fig. 6. Heat shock protein 90 (Hsp90)–peptide complex is cross-presented through an endosome-recycling pathway. Human monocyte-derived dendritic cells (Mo-DCs) were pre-incubated with chloroquine (a) or primaquine (b) at 37°C for 2 h and then loaded with survivin-$2B_{80\text{-}88}$ peptide alone or Hsp90–survivin-$2B_{75\text{-}93}$ precursor peptide complex for 2 h. The Mo-DCs were then fixed, washed, and cultured overnight with survivin-$2B_{80\text{-}88}$-specific CTL clone. Activation of CTL was measured as γ-interferon (IFN-γ) production using ELISA.

cling MHC class I molecules occurs within early endosomal compartments.[15] We have shown that Hsp90–peptide complex-mediated[4] and ORP150–peptide complex-mediated[16] cross-presentation was independent of TAP and was sensitive to primaquine, indicating that sorting of peptides onto MHC class I occurs through an endosome-recycling pathway. Lakadamyali *et al.*[17] have shown that early endosomes are comprised of two distinct populations: a dynamic population that is highly mobile on microtubules and matures rapidly toward the late endosome, and a static population that matures much more slowly. Cargos destined for degradation, including LDL, epidermal growth factor, and influenza virus, are internalized and targeted to the $Rab5^+$, $EEA1^-$-dynamic population of early endosomes as we have observed using LDL, thereafter trafficking to $Rab7^+$-late endosomes. In contrast, the recycling ligand transferrin is delivered to $Rab5^+$, $EEA1^+$-static early endosomes, followed by translocation to $Rab11^+$-recycling endosomes. Furthermore, Burgdorf *et al.*[18] clearly indicated that a mannose receptor introduced OVA specifically into an $EEA\text{-}1^+$, $Rab5^+$-stable early endosomal compartment for subsequent cross-presentation. In contrast, pinocytosis conveyed OVA to lysosomes for class II presentation. Of interest, OVA endocytosed by a scavenger receptor did not colocalize with EEA1 but colocalized with LAMP-1 in lysosomes, leading to presentation in the context of MHC class II molecules. We showed that the human Hsp90–peptide complex is targeted into $Rab5^+$, $EEA1^+$-early endosomes after internalization by Mo-DCs, suggesting that preferential sorting to the "static" endosome is necessary for cross-presentation of Hsp90–peptide complexes. In contrast, soluble LDL protein was targeted to the $EEA1^-$ and $LAMP\text{-}1^+$-dynamic early endosome–late endosome/lysosome pathway, leading to degradation and presentation in the context of MHC class II molecules. These findings suggested that Hsp90 shuttled the chaperoned precursor peptide into the static endosome-recycling pathway, preventing further degradation, followed by transfer of the peptide onto recycling MHC class I molecules. Together, our findings indicate that the role

of Hsp90 in cross-presentation is to navigate the associated Ag into static early endosomes within human Mo-DCs. Thus, Hsp90 appears to be a promising natural immunoactivator for use of cancer vaccine development due to its excellent ability to target human DCs and to induce specific CTLs.

Disclosure Statement

The authors have no conflict of interest.

Abbreviations

Ag	antigen
DC	dendritic cell
GM-CSF	granulocyte/macrophage colony-stimulating factor
HSP	heat shock protein
Hsp90	heat shock protein 90
IFN	interferon
IL	interleukin
LDL	low-density lipoprotein
Mo-DC	monocyte-derived dendritic cells
OVA	ovalbumin
pAb	polyclonal antibody
PE	phycoerythrin
PHA	phytohemagglutinin
TAP	transporter associated with antigen processing

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J Mol Med (2014) 92:1271–1281
DOI 10.1007/s00109-014-1183-9

JMolMed

ORIGINAL ARTICLE

Tumor-α9β1 integrin-mediated signaling induces breast cancer growth and lymphatic metastasis via the recruitment of cancer-associated fibroblasts

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Received: 19 March 2014 / Revised: 27 May 2014 / Accepted: 12 June 2014 / Published online: 8 August 2014

Abstract
Tumor-derived matricellular proteins such as osteopontin (OPN) and tenascin-C (TN-C) have been implicated in tumor growth and metastasis. However, the molecular basis of how these proteins contribute to tumor progression remains to be elucidated. Importantly, these matricellular proteins are known to interact with α9β1 integrin. Therefore, we hypothesized that tumor-derived α9β1 integrin may contribute to tumor progression. To clarify the roles of α9β1 integrin in tumor growth and lymphatic metastasis, we used an inhibitory anti-human α9β1 integrin antibody (anti-hα9β1 antibody) and a α9β1 integrin-positive human breast cancer cell line, MDA-MB-231 luc-D3H2LN (D3H2LN), in vitro functional assays, and an in vivo orthotopic xenotransplantation model. In this study, we demonstrated that tumor, but not host α9β1 integrin, contributes to tumor growth, lymphatic metastasis, recruitment of cancer-associated fibroblasts (CAFs), and host-derived OPN production. We also found that CAFs contributed to tumor growth, lymphatic metastasis, and host-derived OPN levels. Consistent with those findings, tumor volume was well-correlated with numbers of CAFs and levels of host-derived OPN. Furthermore, it was shown that the inoculation of D3H2LN cells into mammary fat pads with mouse embryonic fibroblasts (MEFs), obtained from wild type, but not OPN knock-out mice, resulted in enhancement of tumor growth, thus indicating that CAF-derived OPN enhanced tumor growth. These results suggested that tumor α9β1-mediated signaling plays a pivotal role in generating unique

Electronic supplementary material The online version of this article (doi:10.1007/s00109-014-1183-9) contains supplementary material, which is available to authorized users.

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primary tumor tissue microenvironments, which favor lymphatic metastasis and tumor growth.

Key messages

- Tumor α9β1 integrin promotes lymphatic metastasis through enhancing invasion.
- Tumor α9β1 integrin promotes tumor growth through CAFs.
- Tumor α9β1 integrin enhances the recruitment of CAFs into the primary tumor.
- Tumor cells induce the production of OPN by CAFs in the primary tumor.
- CAF-derived OPN promotes tumor growth.

Keywords Osteopontin · α9β1 integrin · Tumor microenvironment · Cancer-associated fibroblasts · Breast cancer

Introduction

Metastasis is a multistage process that includes tumor invasion and colonization in the metastatic site [1]. These steps require a receptive microenvironment constructed by surrounding stromal cells [2]. The most common reactive stromal cells are cancer-associated fibroblasts (CAFs), which enhance tumor growth and metastasis by production of cytokines, growth factors, and extracellular matrix (ECM) proteins [3]. ECM proteins support stable cell adhesion, and transmit signals that are critical for cell growth, migration, and survival. These ECM proteins include collagen, laminin, and fibronectin, which are regarded as classical ECM proteins.

It is now known that there also are nonclassical ECM proteins. These nonclassical ECM proteins are termed "matricellular proteins". Matricellular proteins: (1) induce cell motility, rather than provide scaffolds for stable cell adhesion; (2) are transiently upregulated in pathological conditions rather than constitutively expressed; and (3) can be present as soluble proteins rather than as structural components [4]. Among these matricellular proteins, osteopontin (OPN) and tenascin-C (TN-C) are well characterized and known to be involved in the tumor progression. Breast cancer-derived TN-C enhances the survival of cancer stem cells in metastatic sites [5]. Malignant breast cancer-derived OPN enhances the migration of bone marrow-derived stromal cells into the distant indolent tumor tissue leading to acquisition of malignancy of the indolent tumor cells [6]. Several groups have demonstrated that plasma OPN levels are potential diagnostic and prognostic markers for several human malignancies [7, 8]. Recently, a role of not only tumor-derived but also host-derived OPN and TN-C in tumor biology has been reported. OPN from CAFs enhances tumor growth [9, 10], and TN-C from host fibroblasts can contribute to tumor progression including cancer cell colonization at metastatic sites [11]. However, the molecular basis for the contribution of these matricellular proteins in tumor progression is yet to be determined. Both OPN and TN-C interact with integrins. OPN binds to αvβ1, αvβ3, αvβ5, and α5β1 integrins through its RGD (Arg-Gly-Asp) sequence, or α4β1 and α9β1 integrins through the SVVYGLR sequence, which can be exposed by thrombin cleavage [4]. TN-C also binds to αvβ1, αvβ3, αvβ6, and α8β1 integrins through its RGD sequence, or to α9β1 integrin through the third FN typeIII repeat (Tnfn3) [12]. Importantly, α9β1 integrin is a common receptor of OPN and TN-C. Elevated levels of α9β1 integrin in the primary tumor significantly correlate with poor breast cancer patient survival [13]. Interestingly, a human breast cancer cell line, MDA-MB-468LN with an aggressive lymph node metastatic ability, was shown to express high levels of both α9β1 integrin and OPN, as compared to its parental cell line, MDA-MB-468 [14].

Here, we hypothesized that α9β1 integrin expressed by tumor cells might be involved in tumor progression. We demonstrated that inhibition of human (tumor) α9β1 integrin-mediated signaling reduced cell motility, lymph node metastasis, and in vivo tumor growth through the reduction of recruitment of CAFs into the primary tumor tissue. Furthermore, upon co-inoculation with tumor cells, MEFs produced OPN, which significantly contributed to tumor growth. Thus, our data suggested that tumor α9β1 integrin might be a possible therapeutic target for the treatment of breast cancer.

Materials and methods

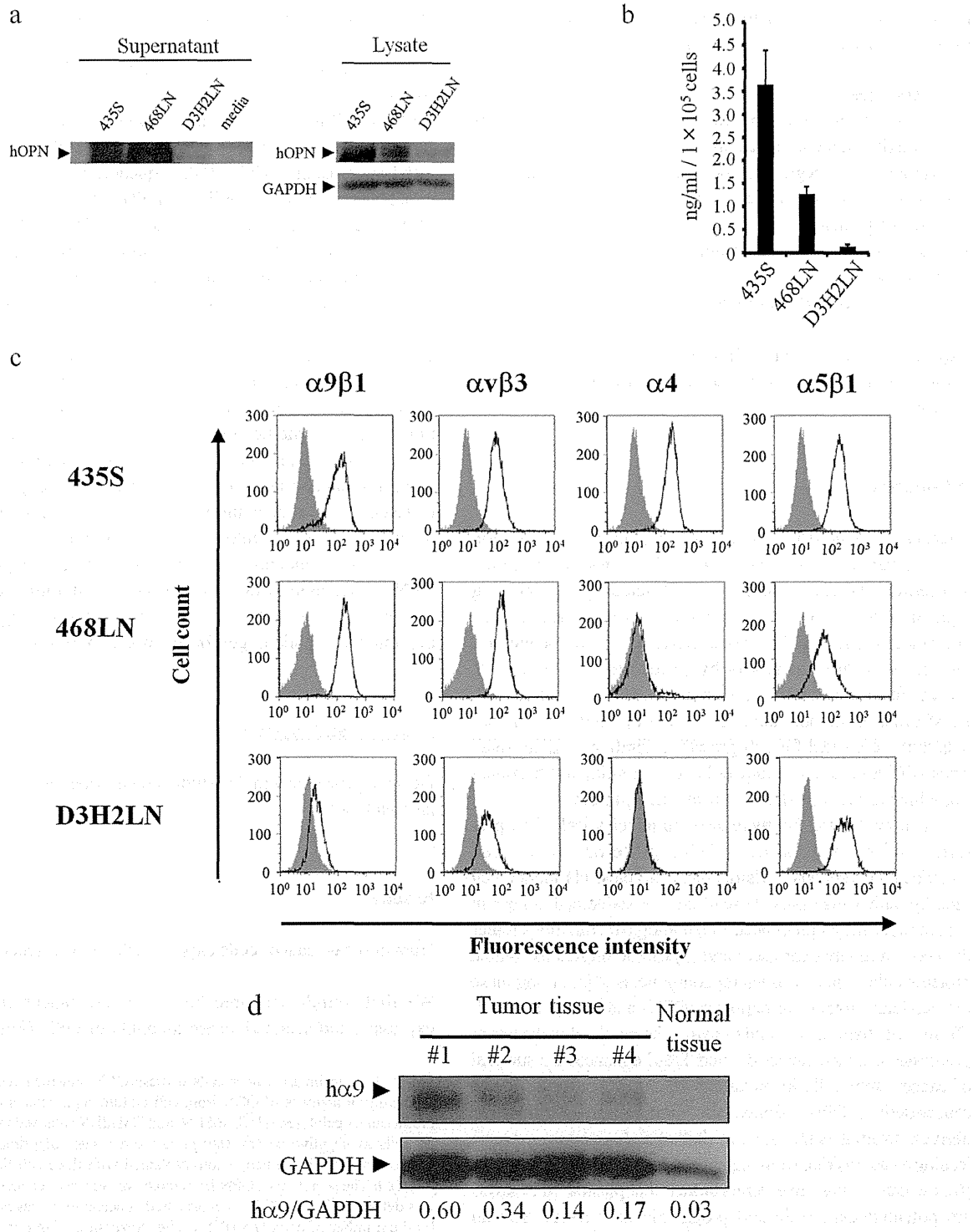
Detailed procedures in this study are described in Supplementary Methods.

Results

Human breast cancer cells express OPN and its receptors

We first found that three human breast cancer cell lines expressed and secreted varied amounts of OPN (Fig. 1a, b).

Fig. 1 Human breast cancer cells express OPN and their receptors. **a** Western blot analysis of OPN from cell culture supernatants of human breast cancer cell lines, 435S, 468LN, and D3H2LN cells and media with no cells as negative control (*left panel*) and lysates of primary tumor tissues obtained from mice xenotransplanted with three cell lines (*right panel*). **b** The secretion of OPN from three human breast cancer cell lines was determined by ELISA. The measured concentration was normalized by the number of cells (1×10^5). **c** The expression of integrins α9β1, αvβ3, α4, and α5β1 in human breast cancer cell lines (435S, 468LN, and D3H2LN cells) was determined by flow cytometry. **d** The expression of α9 integrin protein in breast tumor tissues from four distinct patients ($n=4$) and noncancer breast tissue ($n=1$) was determined by western blot analysis. *Numbers at bottom of figure* indicate the normalized densitometric band densities as determined human α9 integrin/GAPDH



All three cell lines expressed αvβ3, α9β1, and α5β1 integrins (Fig. 1c), to which OPN can bind. We also demonstrated that human breast cancer tissues expressed elevated levels of α9β1 integrin proteins as compared to normal human breast tissue (Fig. 1d). It should be pointed out that MDA-MB-231 luc-D3H2LN (D3H2LN) cells secrete very little if any human OPN as compared to other two cell lines (Fig. 1b), allowing us to examine the role of tumor-derived

versus host-derived OPN in various aspects of tumor biology. Therefore, we used D3H2LN cells in the following experiments.

In vitro human breast cancer cell invasion is regulated by tumor α9β1 integrin

To evaluate the role of tumor α9β1 integrin in progression of human breast cancer, we generated an inhibitory monoclonal antibody that specifically reacts to the human α9β1 integrin (anti-hα9β1 antibody). Anti-hα9β1 antibody reacts with CHO cells transfected with human α9 integrin (hα9/CHO), but not with control CHO cells, CHO cells transfected with the human α4 integrin (hα4/CHO), or murine fibroblasts transfected with murine α9 integrin (mα9/NIH). In addition, this antibody could detect endogenous α9β1 integrin expressed by D3H2LN cells (Supplementary Fig. S1a). Anti-hα9β1 antibody could inhibit the binding of hα9/CHO cells to the synthetic peptides SVVYGLR and AEIDGIEL (which are internal sequences within OPN and TN-C, respectively, and are ligands for α9 integrin), but not to the GRGDS peptide (Supplementary Fig. S1b). We analyzed whether tumor α9β1 integrin-mediated signaling had any effect on tumor cell invasion and proliferation in vitro. Invasion was significantly reduced by anti-hα9β1 antibody (Fig. 2a), indicating that tumor α9β1 integrins are involved in the invasion process. In sharp contrast to cell invasion, proliferation of D3H2LN cells was not α9β1 integrin-dependent (Fig. 2b).

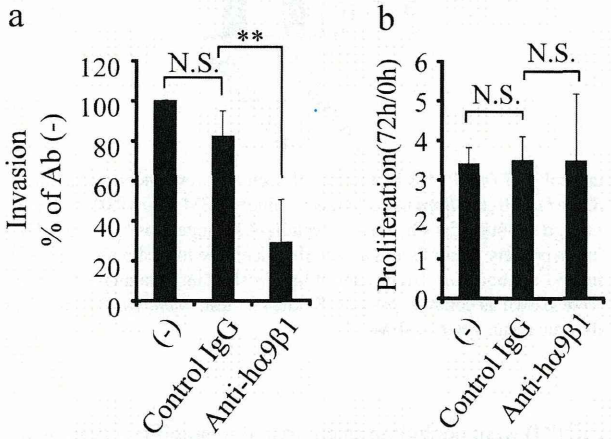


Fig. 2 Human breast cancer cell migration and invasion in vitro are regulated by tumor α9β1 integrin. **a** Matrigel invasion assay using D3H2LN cells treated with 20 μg/ml of anti-hα9β1 or control IgG, was performed. *Bars* indicate mean values±S.D. of three independent experiments. *N.S.* No significant difference, $^{**}p<0.01$, ANOVA with post test analysis (PLSD). **b** The proliferation of D3H2LN cells was examined in the presence or absence of 20 μg/ml of anti-hα9β1 antibody or control IgG. *Column* indicates the fold increase of OD.450 nm at 72 h as compared to that at the time of culture initiation (0 h). *Bars* indicate mean values±S.D. of three independent experiments. *N.S.* No significant difference, ANOVA with post test analysis (PLSD)

Thus, our in vitro study demonstrated that invasion, but not proliferation, is regulated by tumor α9β1 integrin.

In vivo human breast cancer cell growth and metastasis are regulated by tumor α9β1 integrin

We next analyzed the in vivo role of α9β1 integrin on tumor growth and metastasis using orthotopically xenografted human breast cancer cells in nude mice as shown in Fig. 3a. Primary tumor growth of D3H2LN cells implanted into the mammary fat pad was significantly reduced by anti-hα9β1 antibody treatment (Fig. 3b), which was well-correlated with the reduction of Ki-67 positive cells in primary tumor tissues (Fig. 3c). It is well known that angiogenesis is important for tumor growth and metastasis [15]. However, here, we found that angiogenesis as judged by CD31 staining in primary tumor was not significantly affected by the anti-hα9β1 antibody treatment (Fig. 3d). The incidence of lymph node metastasis at day 30 after inoculation was significantly inhibited by anti-hα9β1 antibody treatment as assessed by two different assays, in vivo imaging of luciferase and counting numbers of metastasis positive lymph nodes in histological sections (Fig. 4a, Supplementary Table). It should be pointed out that the inhibition of tumor metastasis incidence was not significant after day 40. However, importantly, the volumes of metastatic foci at lymph nodes in the anti-hα9β1 antibody-treated group were significantly smaller than those of the control IgG group at day 50 (Fig. 4b). The reduction of metastatic lymph node number and metastatic burden per lymph node may not be due to the inhibition of tumor cell proliferation at lymph nodes since Ki-67 positive cell numbers within human vimentin-positive breast cancer cells were not significantly different between control IgG and anti-hα9β1 antibody-treated group (Supplementary Fig. S2). Thus, it is likely that tumor metastasis was reduced due to the inhibition of cell invasion. Thus, these results suggest that tumor α9β1 integrin contributed to lymphatic metastasis in this model. It has been reported that α9β1 integrin expressed by lymph vessels is a receptor for VEGF-C and -D and involved in lymphangiogenesis, thus lymph node metastasis [16]. Therefore, we asked whether endogenous (murine) α9β1 integrin plays a role in xenografted human breast cancer cell growth and metastasis. To explore this aim, we injected a blocking monoclonal antibody against murine α9β1 (anti-mα9β1 antibody) in tumor-bearing mice (Supplementary Fig. S3a). We found that murine α9β1 integrin plays a little role if any on tumor growth and metastasis (Supplementary Fig. S3b, c). Consistently, anti-mα9β1 antibody did not reduce lymphangiogenesis, induced by tumor inoculation as judged by LYVE-1 staining in the primary tumor tissue and axillary lymph node (Supplementary Fig. S3d). Thus, our data demonstrated that in vivo primary tumor growth and metastasis were regulated by tumor α9β1 integrin.

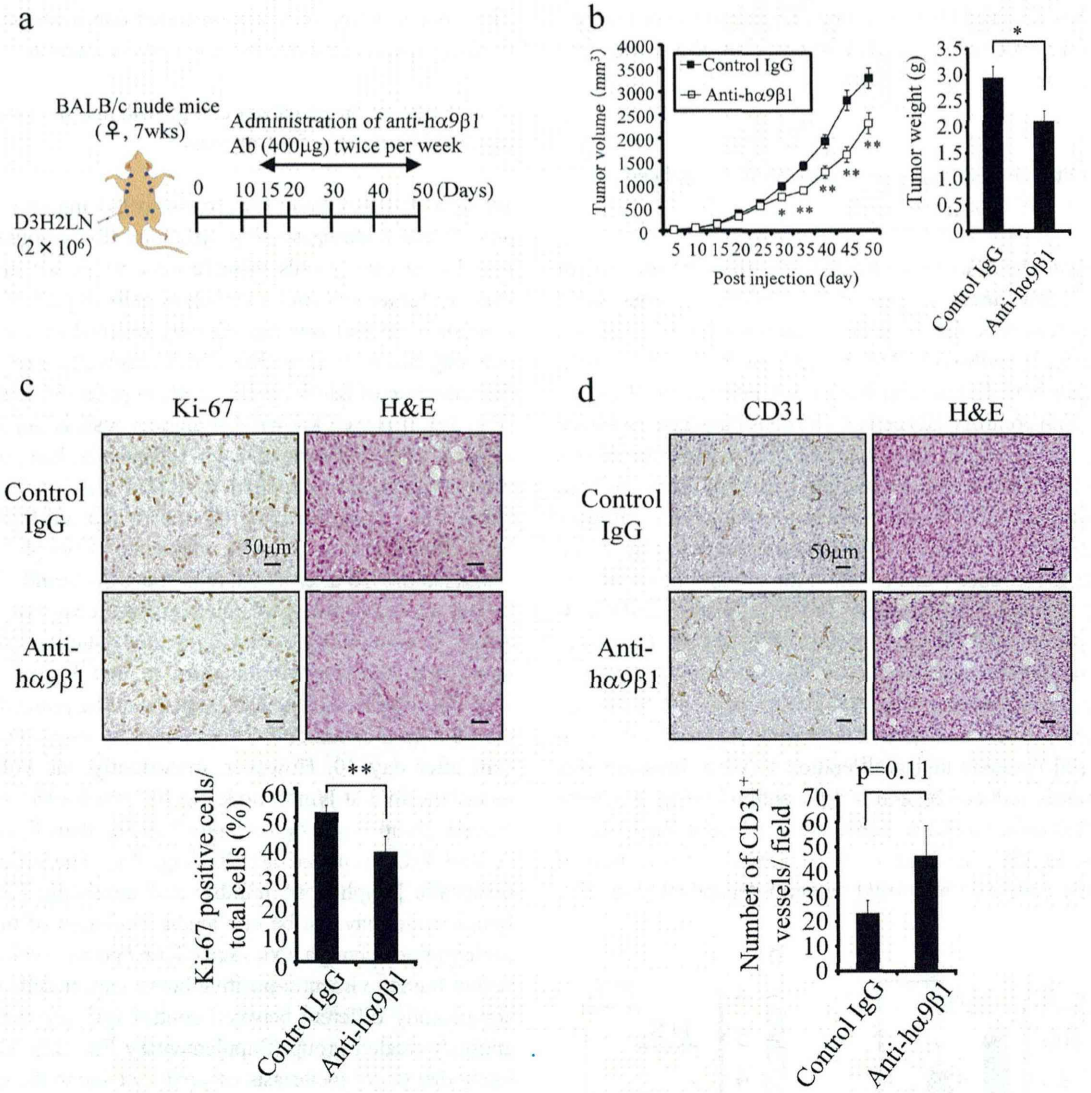


Fig. 3 Human breast cancer cell growth is regulated by tumor α9β1 integrin in vivo. **a** The protocol of in vivo experiments. Details are described in Supplementary Methods. **b** Tumor volume (*left panel*) and the tumor weight (*right panel*) were measured on day 50. *$p<0.05$, **$p<0.01$. Mice were treated with either anti-hα9β1 antibody ($n=10$) or control IgG ($n=8$). Student's *t* test. *Bars* indicate mean values±SEM. **c** Immunohistochemical detection of tumor proliferation by Ki-67 staining at day 30. Mice were treated with either anti-hα9β1 antibody ($n=10$) or control IgG ($n=8$). H&E staining of sections were shown as controls. *Scale bar* 30 μm. *Bars* indicate mean values±SEM. **$p<0.01$, Student's *t* test. **d** Immunohistochemical detection of angiogenesis by CD31 staining in primary tumor tissues at day 30. Mice were treated with either anti-hα9β1 antibody ($n=10$) or control IgG ($n=8$). H&E staining of sections were shown as controls. $p=0.11$, Student's *t* test. *Scale bar* 50 μm. *Bars* indicate mean values±SEM

Host-derived OPN production is regulated by tumor α9β1 integrin

Tumor-derived OPN stimulates migration of bone marrow-derived stromal cells into tumor tissues, leading to acquisition of a malignant phenotype [6]. However, the role of host-derived OPN still remains to be determined [17]. We therefore measured the levels of tumor-derived human and/or host-derived murine OPN. The samples were obtained from mice used in Fig. 3. As expected, plasma levels of human OPN (hOPN) were negligible as compared to significantly elevated levels of host-derived OPN (mOPN) in tumor-bearing mice and as compared to that in nontumor-bearing mice (Fig. 5a left and right columns). Importantly, the thrombin-cleaved form of mOPN (referred to as mOPN-N-half, hereafter) was also significantly increased in tumor-bearing mice (Fig. 5a middle column). The augmented host-derived OPN production in vivo was regulated by tumor α9β1 integrin since murine OPN levels were markedly reduced by anti-hα9β1 antibody treatment (Fig. 5a). Consistent with those observations, the

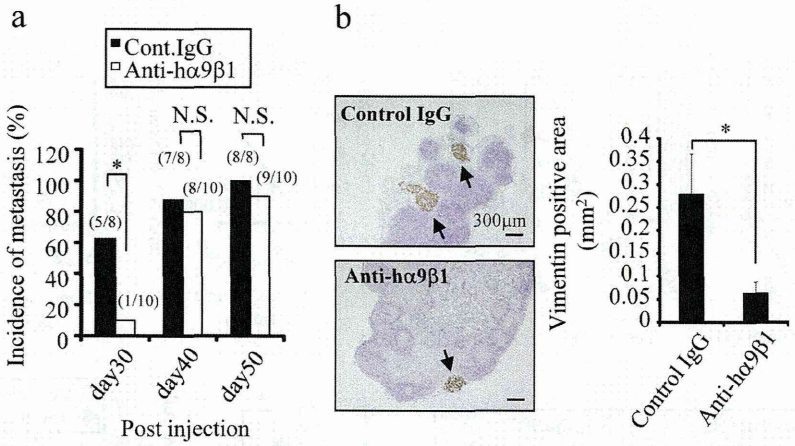


Fig. 4 Human breast cancer metastasis is regulated by tumor α9β1 integrin in vivo. **a** Metastasis to axillary lymph nodes was detected by IVIS, and incidence of lymphatic metastasis was examined on days 30, 40, and 50. Mice were treated with either control IgG treated group ($n=8$) or anti-hα9β1 antibody-treated group ($n=10$). *N.S.* No significant difference, *$p<0.05$, χ^2 test. **b** The metastatic tumor area in axillary lymph nodes was measured by immunohistochemical staining of human vimentin. Representative micrograph of vimentin-positive sites (metastatic foci indicated as *arrows*) is depicted (*left panel*) and metastatic areas of these sites were statistically evaluated (*right panel*). Lymph nodes were obtained from control IgG treated ($n=8$) and anti-hα9β1 antibody-treated ($n=10$) mice at day 50. *Scale bar*: 300 μm. *Bars* indicate mean values± SEM. *: $p<0.05$, Student's *t* test

augmented host-derived OPN production was not inhibited by anti-mα9β1 antibody, indicating that host OPN production was independent from host-derived α9β1 integrin-mediated signaling (Supplementary Fig. S3e). Of note, primary tumor volume was well-correlated with the levels of mOPN or mOPN-N-half (Fig. 5b left or right column, respectively). Taken together, we found that host-derived OPN production was regulated by tumor α9β1 integrin and that levels of host OPN correlated well with tumor volume.

The molecular basis for the recruitment of CAFs and secretion of OPN from CAFs

To search for the source of host-derived OPN, we focused on the involvement of activated CAFs (FAP-positive cells). Within xenografted tumor tissues, there were significant numbers of FAP-positive cells, and the number of FAP-positive cells was considerably reduced by anti-hα9β1 antibody treatment (Fig. 5c left and middle columns). Importantly, primary tumor volume was also well-correlated with the number of FAP-positive cells (Fig. 5c, right column). Thus, numbers of FAP-positive cells and levels of host-derived OPN correlated well with primary tumor volumes (Fig. 5b, c), which led us to examine whether FAP-positive cells produce OPN. Using immunohistochemistry, we found that FAP-positive cells within human breast cancer tissues expressed murine OPN (Fig. 6a).

To further examine how the secretion of OPN from CAFs is regulated, we co-cultured mouse embryonic fibroblasts (MEFs) (upper chamber) and human D3H2LN cells (lower chamber). The two chambers were separated by filters, thus inhibiting cell-to-cell contact between MEFs and human tumor cells. MEFs produced more murine OPN (both full length and OPN-N-half) when cultured with human tumor cells as compared to MEFs alone, indicating that human tumor cells secrete soluble factor(s), leading to the production of mOPN from MEFs (Fig. 6b). We then examined the involvement of tumor α9β1 integrins in the production and/or action of tumor-derived soluble factor(s). Anti-hα9β1 antibody did not reduce the production of OPN by MEFs when co-cultured with D3H2LN cells separately (Fig. 6c) or in the same dish (Fig. 6d).

Thus, these results suggest that (1) tumor volume correlated well with number of CAFs in primary tumors, (2) recruitment of CAFs into primary tumor tissues was regulated by tumor α9β1, and (3) primary tumors secreted soluble factor(s) that induce(s) the production of OPN by CAFs. CAFs might be an important producer of plasma OPN. OPN expression by CAFs may be indirectly induced by tumor α9β1 integrin through the recruitment.

The in vivo role of MEF-derived OPN on xenografted tumor growth and metastasis

To investigate in vivo role of MEF-derived OPN on tumor growth and metastasis, D3H2LN cells and MEFs, obtained from either wild type mice (WT-MEFs) or OPN deficient mice (OPN KO-MEFs), were injected into mammary fat pads of nude mice. MEFs obtained from either wild type mice or OPN-deficient mice did not grow in vivo when injected in the absence of tumor cells (Fig. 7a). Furthermore, when WT-MEF or OPN KO-MEF alone were inoculated into mice, plasma levels of host-derived OPN was similar to that in normal mice (Fig. 7b), again indicating that tumor cells

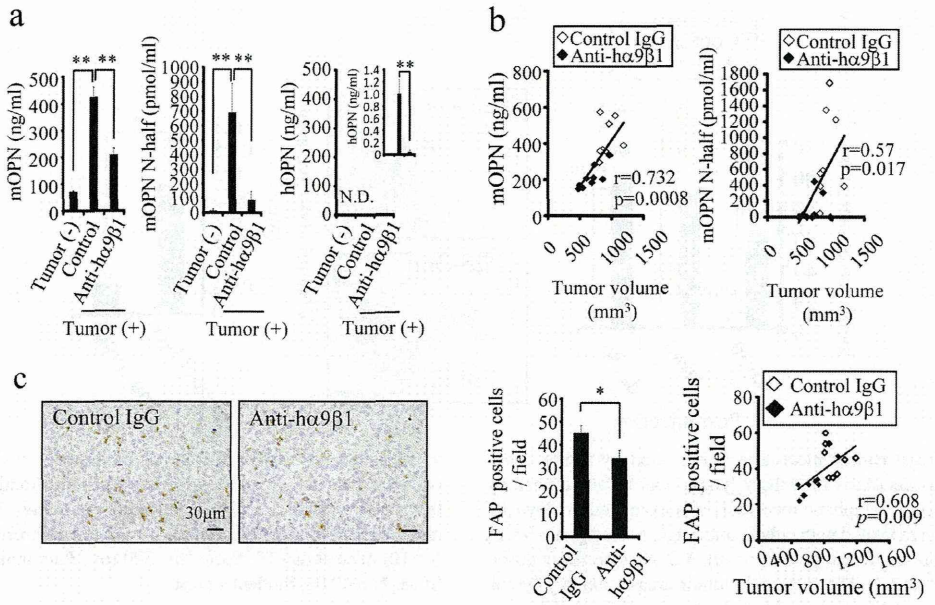


Fig. 5 Plasma levels of mOPN and numbers of FAP-positive CAFs within primary tumor are well correlated with tumor growth. **a** The concentrations of host-derived OPN (mOPN) (*left panel*), mOPN N-half (*middle panel*), and tumor-derived OPN (hOPN) (*right panel*) in plasma on day 30 after inoculation were measured by ELISA. (*N.D.* No detected, $^{**}p<0.01$. Anti-hα9β1 antibody-treated group ($n=9$), control IgG-treated group ($n=8$) or tumor (−) mice group ($n=5$), ANOVA with post test analysis (PLSD). *Bars* indicate mean values±SEM. **b** The correlation between the concentration of host-derived OPN and tumor volume on day 30. Anti-hα9β1 antibody-treated group ($n=9$) versus control IgG treated group ($n=8$). Experimental data were analyzed by using Pearson correlation. **c** The immunohistochemical detection of CAFs in primary tumor tissues on day 30. CAFs are defined as fibroblast activation protein-α (FAP) positive cells. Representative micrographs of FAP-positive cells are depicted (*left panel*) and the number of FAP-positive cells was counted (*middle panel*). *Scale bar* 30 μm. *Bars* indicate mean values ±SEM. Tumor tissues were obtained from control IgG ($n=8$) and anti-hα9β1 antibody ($n=9$) treated groups. *: $p<0.05$, Student's t test. The correlation between the number of FAP-positive cells in the primary tumor and tumor volume on day 30 was analyzed using Pearson correlation (*right panel*)

stimulated production of OPN from MEFs. D3H2LN cells co-inoculated with WT-MEFs grew better as compared to that with OPN KO-MEFs or D3H2LN cell alone (Fig. 7a). There were statistically significant differences among these three groups in terms of in vivo tumor growth (Fig. 7a), indicating that not only OPN but also other factor(s) are involved in primary tumor growth. Host-derived plasma OPN (mOPN) levels were significantly higher when WT-MEFs were co-inoculated with D3H2LN cells as compared to D3H2LN cells alone. Nevertheless, mOPN plasma levels were similar between mice injected with D3H2LN cells alone or D3H2LN with OPN KO-MEFs (Fig. 7b), indicating that the increase of host-derived OPN in tumor-bearing mice is due to both inoculated MEFs and host cells. We then asked whether MEF-derived OPN was involved in tumor metastasis. Lymph node metastasis was determined at day 25 after inoculation. A higher metastasis incidence (77 %) was detected when D3H2LN cells were co-inoculated with MEFs regardless of their source, from WT or OPN KO mice as compared to tumor cells alone (Fig. 7c), demonstrating that factor(s) other than OPN was involved in tumor metastasis at least in early stages of tumor metastasis. It should be pointed out, however, that we cannot rule out the possibility that host-derived OPN was sufficient for the induction of tumor metastasis. In contrast, OPN from exogenous MEFs contributed to the xenografted tumor growth.

Discussion

Matricellular proteins such as OPN and TN-C have important roles in tumor growth, invasion, and metastasis [18]. Recently, many reports showed that not only tumor-derived but also host-derived OPN or TN-C are implicated in various stages of breast cancer malignancy [9–11]. More recently, it was shown that the expression of α9β1 integrin, a common receptor for OPN or TN-C in human breast cancer cells was well-correlated with patient prognosis [13]. In addition, CAFs, an important component of tumor tissue microenvironments, significantly contribute to the appearance of malignant phenotype of tumors [3]. However, how those factors cooperate within in vivo tumor tissue microenvironments and contributed to tumor progression are yet to be elucidated. Our present study provides various new evidences how tumor α9β1

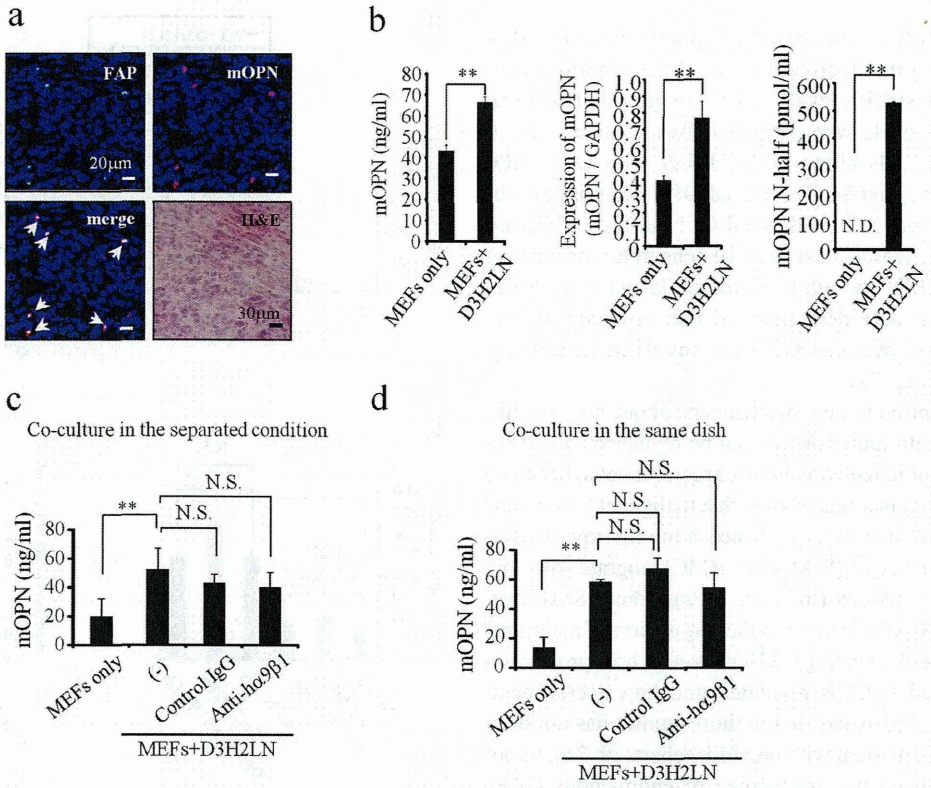


Fig. 6 Tumor cells induce the production of OPN by CAFs. **a** Double staining of FAP (as indicated by *green color*) and murine OPN (as indicated by *red color*) in primary tumor tissues on day 30. *Arrows* indicate double positive CAFs. Cell nuclei were stained with DAPI (*blue color*). *Scale bar* 20 μm (fluorescent image), 30 μm (H&E staining). **b** The production of OPN by MEFs was examined by co-culture with D3H2LN cells for 72 h under cell separated conditions. Protein levels (*left*) and mRNA levels (*middle*) of OPN and protein levels of OPN N-half (*right panel*) are shown. *Bars* indicate mean values±S.D. of three independent experiments. *N.D.* Not detected, $^{**}p<0.01$, Student's *t* test. **c** The production of OPN by MEFs was examined by co-culture with D3H2LN cells for 72 h under cell separated conditions. D3H2LN cells were treated with anti-hα9β1 antibody (20 μg/ml). *Bars* indicate mean values±S.D. of three independent experiments. *N.S.* No significant difference, $^{**}p<0.01$, ANOVA with post test analysis (PLSD). **d** The production of OPN by MEFs was examined by co-culture with D3H2LN cells for 72 h in the same dish. D3H2LN cells were treated with anti-hα9β1 antibody (20 μg/ml). *Bars* indicate mean values±S.D. of three independent experiments. *N.S.* No significant difference, $^{**}p<0.01$, ANOVA with post test analysis (PLSD)

integrin-mediated signaling can dictate various aspects of tumor biology.

Firstly, we provide molecular and cellular details how tumor α9β1 integrin contributes to primary tumor growth. It was shown that elevated levels of α9β1 integrin in the primary breast tumors correlated very well with higher incidence of lymph node metastasis and poor breast cancer patient survival [13]. However, it was not known whether α9β1 integrin was involved in tumor growth. We found that the tumor α9β1 integrin-mediated signaling regulated primary tumor growth, plasma levels of host-derived OPN, and the number of FAP-positive CAFs (Figs. 3b and 5).

Secondly, we provided new evidence about how CAFs contributed to tumor growth. It was known that CAFs contribute to the tumor growth through various distinct mechanisms [9, 19–25]. Tumor growth is promoted through CAFs-derived chemokine-induced angiogenesis [22] and connective tissue growth factor (CTGF; a member of matricellular protein)-induced angiogenesis [23]. CAFs also produce growth factors such as epiregulin [24] and hepatocyte growth factor (HGF) [26]. Production of OPN and fibroblast growth factor 2 (FGF2) from CAFs was also reported in malignant melanoma [9]. We found that primary tumor volume was well correlated with levels of host-derived mOPN and number of FAP-positive CAFs under anti-hα9β1 integrin antibody treatment (Fig. 5b, c). We demonstrated that FAP-positive CAFs within primary tumor tissues expressed OPN (Fig. 6a). We also provide data regarding how OPN production was regulated. OPN secretion/production from CAFs (MEFs) was induced by tumor-derived soluble factor(s) (Fig. 6b, c). We further analyzed the role of CAF-derived OPN in tumor growth. As shown in Fig. 7, D3H2LN cells alone induced increase of host plasma OPN levels, indicating that host stromal cells, including endogenous CAFs contributes to the increase of host OPN levels. Furthermore, co-inoculation of MEFs with D3H2LN cells further up-regulated host OPN