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Acid-sensing receptor GPR4 plays a crucial role in lymphatic cancer metastasis

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Abstract

Cancer tissues exhibit an acidic microenvironment owing to the accumulation of protons and lactic acid produced by cancer and inflammatory cells. To examine the role of an acidic microenvironment in lymphatic cancer metastasis, gene expression profiling was conducted using human dermal lymphatic endothelial cells (HDLECs) treated with a low pH medium. Microarray and gene set enrichment analysis revealed that acid treatment induced the expression of inflammation-related genes in HDLECs, including genes encoding chemokines and adhesion molecules. Acid treatment-induced chemokines C-X3-C motif chemokine ligand 1 (CX3CL1) and C-X-C motif chemokine ligand 6 (CXCL6) autocrinally promoted the growth and tube formation of HDLECs. The expression of vascular cell adhesion molecule 1 (VCAM-1) increased in HDLECs after acid treatment in a time-dependent manner, which, in turn, enhanced their adhesion to melanoma cells. Among various acid-sensing receptors, HDLECs basally expressed G protein-coupled receptor 4 (GPR4), which was augmented under the acidic microenvironment. The induction of chemokines or VCAM-1 under acidic conditions was attenuated by *GPR4* knockdown in HDLECs. In addition, lymph node metastases in a mouse melanoma model were suppressed by administering an anti-VCAM-1 antibody or a GPR4 antagonist. These results suggest that an acidic microenvironment modifies the function of lymphatic endothelial cells via GPR4, thereby promoting lymphatic cancer metastasis. Acid-sensing receptors and their downstream molecules might serve as preventive or therapeutic targets in cancer.

KEYWORDS
acidic microenvironment, GPR4, lymph nodes, lymphatic endothelial cells, metastasis

Abbreviations: ASIC, acid-sensing ion channel; CX3CL1, C-X3-C motif chemokine ligand 1; CXCL6, C-X-C motif chemokine ligand 6; GPCR, G protein-coupled receptor; GPR4, G protein-coupled receptor 4; HDLECs, human dermal lymphatic endothelial cells; HUVECs, human umbilical vein endothelial cells; LECs, lymphatic endothelial cells; LNs, lymph nodes; TRPV1, transient receptor potential vanilloid subtype 1; VCAM-1, vascular cell adhesion molecule 1; VECs, vascular endothelial cells; VEGF, vascular endothelial growth factor.

1 | INTRODUCTION

Metastasis is a major risk to survival in patients with advanced cancers. Several biological steps, such as angiogenesis/lymphangiogenesis, local invasion, adhesion, and transmigration into vessels, and colonization of cancer cells in distant organs or lymph nodes (LNs) are involved in the development of hematogenous/lymphatic metastasis.[1–7] These processes are regulated by various growth factors.

Acidic conditions are characteristic features of the tumor microenvironment (TME) and are accelerated by the accumulation of protons and lactic acid produced by cancer cells and inflammatory cells.[8–11] A monocarboxylate transporter that excretes lactic acid is co-expressed with a member of the vascular endothelial growth factor (VEGF) family in cancer cells, suggesting crosstalk between metabolic reprogramming and angiogenesis.[12] Lactic acid promotes angiogenesis by producing interleukin (IL)-8 by activating nuclear factor kappa B (NF-κB) in vascular endothelial cells (VECs).[13] An acidic condition promotes VEGF-C expression in human melanoma cells by activating NF-κB signaling, leading to angiogenesis/lymphangiogenesis.[14]

Acidic conditions evoke many cellular responses via ion channels, such as transient receptor potential vanilloid subtype 1 (TRPV1), acid-sensing ion channel (ASIC) 1–4, and proton-sensing G protein-coupled receptors, including G protein-coupled receptor (GPR) 4, GPR65 (also known as TDAG8), GPR68 (OGR1), and GPR132 (G2A).[15,16] These acid-sensing receptors are expressed in various cancer cells and are involved in cell growth, migration, and metastasis.[8,10,17–19] ASIC1 expression in human breast cancer cells is upregulated under acidic conditions and is responsible for inducing IL-8-mediated cancer progression.[20]

Acid-sensing receptors are expressed in other cellular components of the TME. Activation of GPR4 in human umbilical vein endothelial cells (HUVECs) induces the expression of adhesion molecules, resulting in their adhesion to monocytes.[21] Acid-induced GPR4 activation in VECs provokes inflammatory responses,[22] increases paracellular gap formation, and enhances vascular permeability.[23] These GPR4-mediated functional alterations of VECs have been implicated in cancer metastasis.

Similarly, various acid-sensing receptors are expressed in lymphatic endothelial cells (LECs).[24] We have previously reported that acidic conditions induce IL-8 production in LECs via TRPV1 and promote lymphangiogenesis.[24] Rhythmic contractile responses of the thoracic ducts decline markedly under acidic conditions, suggesting functional alterations such as dilatation or permeability.[25] These findings implicate the contribution of an acidic microenvironment in lymphangiogenesis and lymphatic metastasis of cancers. However, the precise roles of acid-sensing receptors in lymphatic cancer metastasis have not been elucidated comprehensively.

In the present study, the functional alterations in human dermal lymphatic endothelial cells (HDLECs) in an acidic microenvironment were investigated, in addition to the role of GPR4 in inducing inflammation-related molecules, such as chemokines and adhesion molecules, under acidic conditions. Furthermore, we assessed the effect of blocking GPR4 activation on lymphangiogenesis using a mouse melanoma model.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Primary HDLECs were purchased from PromoCell (Heidelberg, Germany). HDLECs were cultured as previously described.[26] Cells were treated with a medium with pH 6.4, prepared by the addition of lactic acid (Fujifilm Wako Pure Chemical, Osaka, Japan).[24] Mouse malignant melanoma B16F10 cells (Riken BRC, Ibaraki, Japan) were cultured in DMEM (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS and 1% penicillin–streptomycin solution (Life Technologies, Grand Island, NY, USA). To establish a highly metastatic subclone, parental B16F10 cells were stably transfected with Venus (improved GFP, kindly provided by Professor Atsushi Miyawaki, Riken, Saitama, Japan) and inoculated into the plantar of mouse. Venus–positive cells were isolated from metastatic tumors in the LNs and reinoculated onto the plantar of another mouse. After six serial transplantations, subclones were established as B16F10-LM6.

2.2 | Adhesion assay

Monolayers of HDLECs cultured in 24-well plates were starved in 1% FBS-containing MV2 medium for 16 h and treated with control or pH 6.4-conditioned media for another 24 h. B16F10-LM6 cells (1×10^5/mL/well) were seeded onto HDLEC monolayers. After 1 h of coculture, non-adherent B16F10-LM6 cells were removed by washing with PBS. Adherent B16F10-LM6 cells were fixed in 4% paraformaldehyde (PFA). The number of adherent B16F10-LM6 cells was counted in five independent fields. For the experiment of vascular cell adhesion molecule 1 (VCAM-1) blockade, HDLEC monolayers were treated with mouse anti-VCAM-1 antibody (1.G11B1; Novus Biologicals, Centennial, CO, USA) or a mouse IgG_1 isotype control (11711; R&D Systems, Minneapolis, MN, USA) for 1 h before coculturing with B16F10-LM6 cells. B16F10-LM6 cells were suspended in control or pH 6.4-conditioned medium in the presence of control IgG or anti-VCAM-1 antibody. To analyze the adhesion of *GPR4*-knocked down HDLECs, they were transfected with small interfering RNA (siRNA) targeting *GPR4* (siGPR4) or negative control siRNA (siNC) by electroporation and immediately seeded in 24-well plates. After the formation of HDLEC monolayers, HDLECs were starved for 10 h and then cultured in control or pH 6.4-conditioned media for another 24 h. Adhesion of B16F10-LM6 cells was analyzed as described above.

2.3 | Mouse model of lymph node metastasis

All experiments were approved by the Institutional Animal Use Committee of Wakayama Medical University and conducted according to the guidelines. Five-week-old male C57BL/6J mice were obtained from SLC Japan (Hamamatsu, Japan).

For the lymphatic metastasis model, Venus-expressing B16F10-LM6 cells (5×10^5 in 50 μL PBS) were subcutaneously injected into the plantar under a combination anesthesia with 0.3 mg/kg medetomidine, 4.0 mg/kg midazolam, and 5.0 mg/kg butorphanol. For VCAM-1 blockade, mice were intraperitoneally injected (thrice per week) with 200 μg VCAM-1-blocking antibody (M/K-2.7; Bio X Cell, Lebanon, NH, USA; $n=8$) or control rat IgG (BE0088; Bio X Cell; $n=7$). For the administration of GPR4 antagonist (NE 52-QQ57; MedChemExpress, Monmouth Junction, NJ, USA), mice were treated orally with reagents suspended in 0.5% methylcellulose, 0.5% Tween 80, and 99% water at a dosage of 20 mg/kg ($n=6$). A methylcellulose solution was administered orally to the control group ($n=6$). Four weeks after tumor injection, popliteal and inguinal LNs and plantar tissue were surgically removed from euthanized mice. Extracted LNs were weighed, fixed with 4% PFA for 24 h, and then sliced into 10-μm frozen sections. The slices were covered with VECTASHIELD mounting medium with DAPI (Vector Laboratories, Newark, CA, USA). The metastatic tumor area was calculated by measuring the area of Venus-positive cancer cells in the LNs using a BZ-X800 microscope (Keyence, Osaka, Japan). Dissected plantar tissues were fixed with 4% PFA and decalcified using 15% EDTA. The tissues were embedded in paraffin, and sections were used for histological examinations.

2.4 | Statistical analysis

Data are presented as mean ± SD. Student's *t*-test was used to compare data between two groups. For comparison of data of more than three groups, one-way ANOVA followed by the Tukey–Kramer or Dunnett test was used (JMP Pro software v.14.1, SAS Institute Inc., NC, USA). Statistical significance was set at $p<0.05$. significant.

Additional information on materials and methods are provided in Data S1 and Table S1.

3 | RESULTS

3.1 | Genes of inflammation-related chemokines are upregulated in LECs under an acidic microenvironment

To identify the molecules in HDLECs induced by an acidic microenvironment, cells were treated with control (pH 7.4) or acidic (pH 6.4) media for 24 h and used for gene expression profiling. Microarray analysis demonstrated that 656 genes were upregulated by more than 2-fold under the acidic condition (Figure 1A,B). IL-8 (*CXCL8*), which was reportedly increased by acidic stimulation,[24] was also detected as an upregulated gene (Figure 1B), suggesting that acidic microenvironment-induced genes were successfully extracted. Among the top 50 upregulated genes, those encoding several cytokines or chemokines were included (Table S2). Gene set enrichment analysis (GSEA) revealed that the upregulated genes were associated with inflammatory responses (Figure 1C,D).

Based on the results, we focused on the role of acid-induced chemokines in lymphatic metastasis. RT-qPCR analysis showed significant upregulation in the mRNA expression of C-X3-C motif chemokine ligand 1 (*CX3CL1*) and C-X-C motif chemokine ligand 6 (*CXCL6*) by the acidic treatment (Figure 2A). Acidic conditions increased chemokine secretion in HDLECs in a time-dependent manner (Figure 2B). Interestingly, increased expression of the chemokines was also detected in HDLECs under an acidic condition induced by HCl (Figure S1), suggesting that these gene alterations are a general response in HDLECs under the acidic microenvironment. CX3CL1 and CXCL6 bind to specific receptors CX3CR1 and CXCR1/2, respectively. We noticed that HDLECs expressed mRNA for these receptors and that acidic conditions augmented their expression (Figure S1). When HDLECs were cultured with exogenous CX3CL1 and CXCL6, proliferation was significantly enhanced (Figure 2C). In contrast, treatment with CX3CL1 or CXCL6 did not clearly influence the in vitro migratory activity of HDLECs (Figure 2D). Next, we assessed whether these chemokines affected lymphangiogenesis in HDLECs. Notably, HDLECs treated with CX3CL1 showed accelerated formation of tube-like networks compared with that by the control cells (Figure 2E). These results suggest that acidic conditions induce the production of several chemokines by LECs. These chemokines might mainly act on LECs themselves in an autocrine manner and promote lymphangiogenesis in the TME.

3.2 | Vascular cell adhesion molecule 1 is induced by acidic conditions and promotes cancer cell adhesion to lymphatic endothelial cells

During lymphatic metastasis, adhesion of cancer cells to LECs is necessary following lymphangiogenesis. Therefore, cell adhesion-related molecules were extracted from the 656 genes upregulated by acidic stimulation in HDLECs (Figure 3A). E-selectin (*SELE*), *VCAM1*, and intracellular adhesion molecule 1 (*ICAM1*) were among the top-ranking genes (Figure 3A, Table S3). As VCAM-1 mediates leukocyte-endothelial adhesion during inflammation, its role in adhering cancer cells and LECs in an acidic microenvironment was further investigated.

Immunocytochemistry revealed co-expression of podoplanin (a marker for LECs) and VCAM-1 in HDLECs, even in the absence of acidic treatment (Figure 3B). VCAM-1 expression in mRNA and protein levels in HDLECs was further increased by acidic stimulation (Figures 3C,D). Upregulation of *VCAM1* mRNA was reproduced in HDLECs after the treatment with HCl (Figure S2). Next, we examined the adhesion of HDLECs to mouse melanoma cells B16F10-LM6 that express α4β1 integrin, a receptor for VCAM-1. Adherence of B16F10-LM6 cells to HDLECs increased under acidic conditions, which was attenuated by treatment with an anti-VCAM-1 antibody (Figure 3E), suggesting that acidic conditions

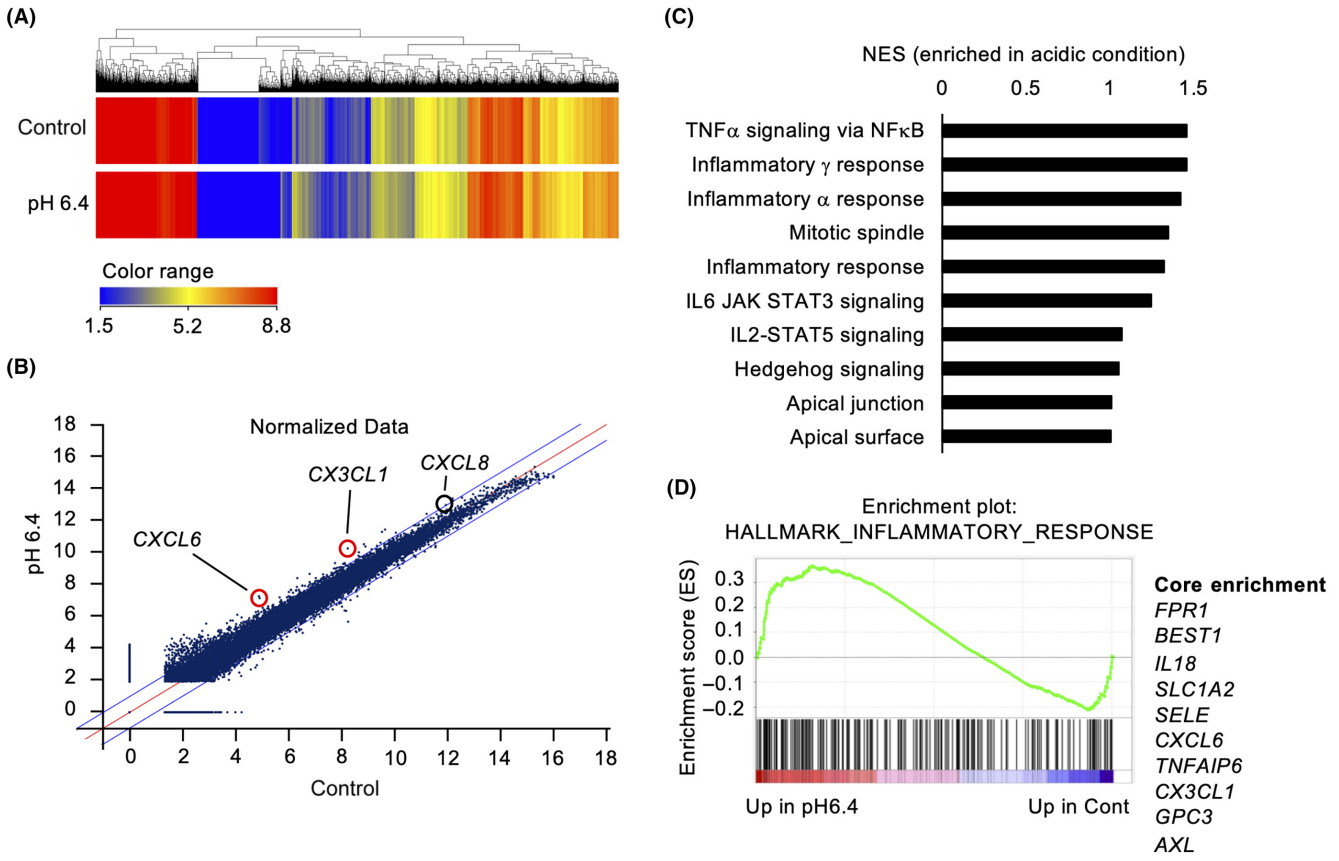


FIGURE 1 (A) Microarray analysis of human dermal lymphatic endothelial cells (HDLECs) treated with an acidic medium (pH 6.4) compared to that treated with the control medium (Cont). (B) Scatter plot of normalized data. (C) Gene set enrichment analysis (GSEA) of upregulated genes in HDLECs cultured in an acidic condition. (D) Representative gene sets enriched in HDLECs treated with an acidic condition (left). Represented genes are listed (right).

promote mutual adhesion between cancer cells and LECs by inducing VCAM-1.

3.3 | Vascular cell adhesion molecule 1-mediated adhesion of cancer cells to lymphatic endothelial cells is involved in lymph node metastasis

To verify whether VCAM-1 is involved in lymphatic metastasis of cancer cells, C57BL/6J mice bearing melanoma were administered an anti-VCAM-1 antibody or control IgG thrice a week for 3 weeks, starting 1 week after inoculating cancer cells (Figure 4A). When tumor tissues were stained with a marker for cell proliferation, no significant differences were observed in the growth of cancer cells between the two groups (Figure 4B). However, regarding the formation of metastatic tumors, the weight of ipsilateral inguinal LNs was significantly higher than the contralateral LNs in the control IgG-treated group, which was not significant in the anti-VCAM-1 antibody-treated group (Figure 4C). Direct comparison between the control IgG- or anti-VCAM-1 antibody-treated group revealed that the weight of the ipsilateral inguinal LNs was relatively low in the anti-VCAM-1 antibody-treated group, although not significantly (Figure 4C). Histological examination revealed that the area of metastatic tumor in inguinal LNs decreased significantly in mice treated with anti-VCAM-1 antibody when compared with that in mice treated with the control IgG (Figure 4D). Considering that the expression of VCAM-1 in LECs increased in an acidic microenvironment, lymphatic metastasis of cancer cells might be enhanced via VCAM-1-mediated cell adhesion under acidic conditions.

3.4 | G protein-coupled receptor 4 is critical for inducing chemokines and vascular cell adhesion molecule 1 in acidic conditions

To clarify the regulatory mechanisms of acid-induced chemokines and VCAM-1, the expression of acid-sensing receptors was analyzed in HDLECs. Consistent with our previous report,[24] RT-PCR analysis showed that HDLECs expressed several acid-sensing receptors (Figure 5A). Among these, we focused on the function of GPR4. Immunocytochemistry showed that HDLECs expressed GPR4 in the cytoplasm and plasma membrane (Figure 5B). Interestingly, acidic conditions induced the expression of several acid-sensing receptors, particularly GPR4 (Figure 5C). In

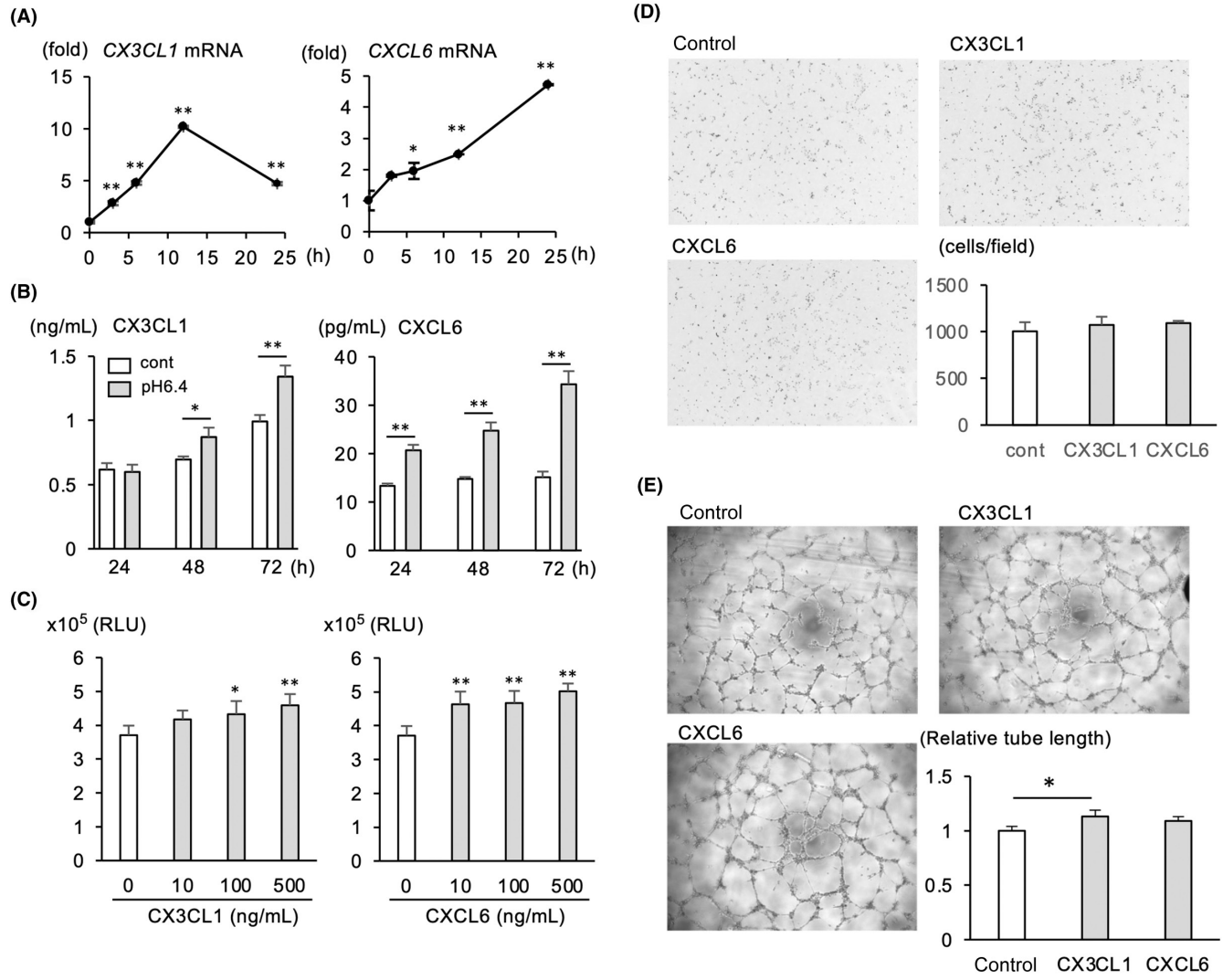


FIGURE 2 Promotion of chemokine expression and lymphangiogenesis in human dermal lymphatic endothelial cells (HDLECs) under acidic stimulation. (A) HDLECs were treated with an acidic medium. *CX3CL1* and *CXCL6* expressions were evaluated by RT-qPCR analysis. Data are shown as fold expression normalized to that at 0 h (mean ± SD; $n = 3$). $^{*}p < 0.05$; $^{**}p < 0.01$. (B) CX3CL1 and CXCL6 levels were measured by ELISA. Data are shown as mean ± SD ($n = 3$). $^{*}p < 0.05$; $^{**}p < 0.01$. (C) Growth assay of HDLECs stimulated with CX3CL1 or CXCL6 for 3 days. Relative light units (RLU) are shown as mean ± SD ($n = 6$). $^{*}p < 0.05$; $^{**}p < 0.01$ vs. control (untreated). (D) The effects of CX3CL1 (200 ng/mL) and CXCL6 (200 ng/mL) on migration of HDLECs were evaluated by Transwell assay. Representative images and the number of migrated cells are indicated. Data are shown as mean ± SD ($n = 3$). (E) HDLECs seeded on Matrigel were incubated in control or chemokine-containing media for 6 h. Representative images and tube lengths relative to those of the control group are indicated. Data are shown as mean ± SD ($n = 4$). $^{*}p < 0.05$.

addition, induced GPR4 expression was detected by immunoblotting (Figure 5D).

According to a public database, GPR4 expression is observed in various types of human cancers and might serve as a poor prognostic marker in renal and lung cancers (Figure S3). Because GPR4 might have various functions in cancer progression, its specific role was determined using a knockdown experiment in HDLECs. In HDLECs transfected with siNC, acidic stimulation increased the expression of *GPR4* mRNA but not in siGPR4-transfected cells (Figure 6A). Similar to the results shown in Figure 2A, acidic stimulation induced the mRNA expression of *CX3CL1* and *CXCL6* in HDLECs. However, *GPR4* knockdown attenuated these effects (Figure 6A, Figure S4). The induction of CX3CL1 and CXCL6 by acid treatment was significantly inhibited by *GPR4* knockdown (Figure 6B). In addition, *GPR4* knockdown inhibited acid-induced tube formation in HDLECs (Figure 6C). Acid-induced expression of VCAM-1 decreased in HDLECs transfected with siGPR4 (Figure 6D,E). Moreover, augmentation of melanoma cell adhesion to HDLECs under acidic conditions was not observed in *GPR4*-knocked-down HDLECs (Figure 6F). These data suggest that acidic conditions induce the expression of chemokines and VCAM-1 via GPR4 activation, which might contribute to the adhesion of cancer cells and LECs and lymphangiogenesis.

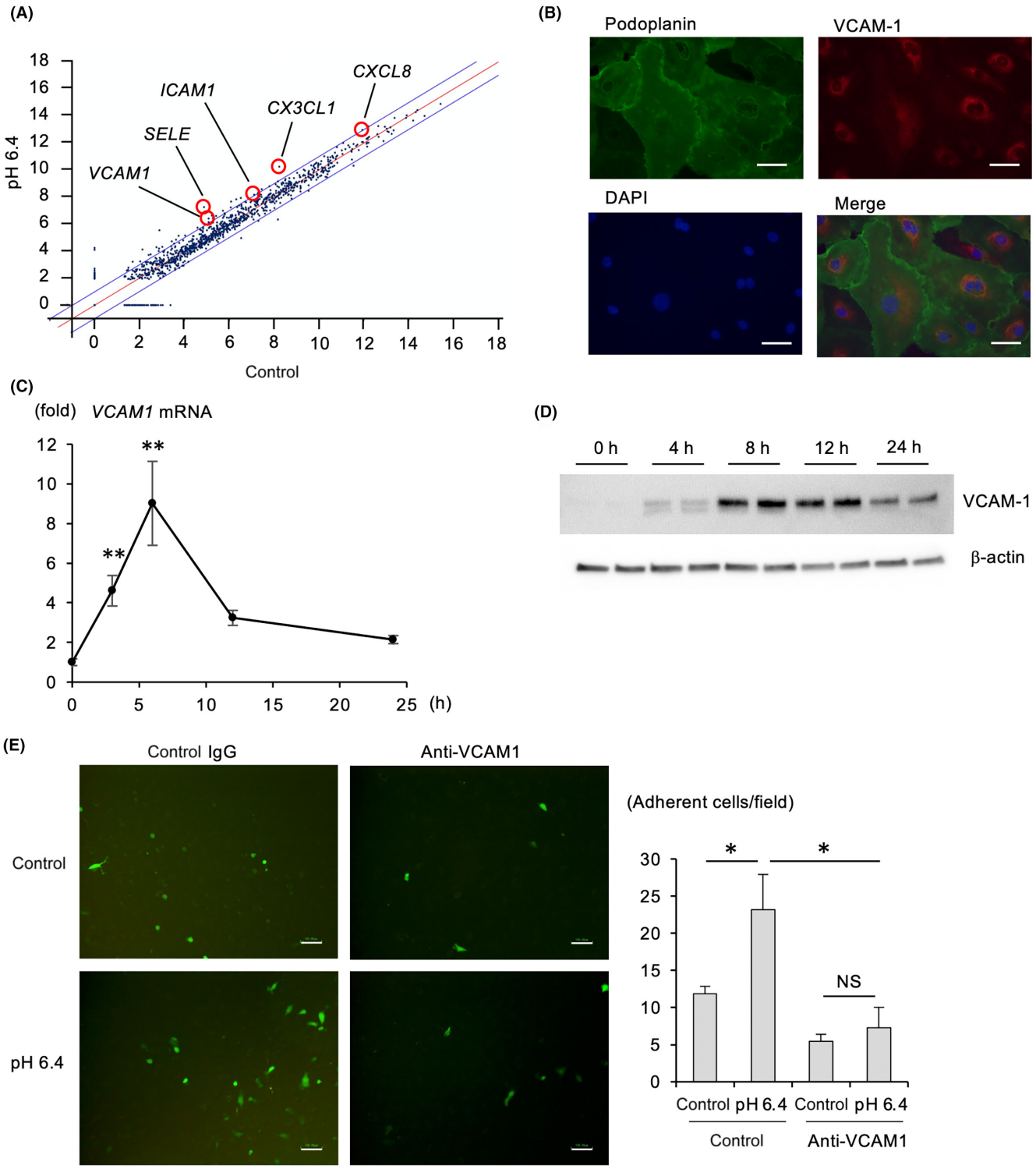


FIGURE 3 The effect of acid-induced vascular cell adhesion molecule 1 (VCAM-1) expression in human dermal lymphatic endothelial cells (HDLECs) on their adhesion with cancer cells. (A) Reanalysis of gene expression data from microarray analysis in Figure 1, which was filtered with "function of cellular adhesion." (B) Immunocytochemistry for podoplanin and VCAM-1 in HDLECs. Bar, 50 μm. (C) *VCAM1* mRNA expression was evaluated by RT-qPCR analysis. Data are shown as fold change normalized with respect to that of the control (0 h) (mean ± SD; $n = 3$). $^{**}p < 0.01$. (D) Immunoblotting of VCAM-1 in HDLECs treated with an acidic medium. (E) Representative images (left panels) and quantification (right panel) of adherent Venus-positive B16F10-LM6 cells to HDLECs. Bar, 100 μm. The number of adherent B16F10-LM6 cells is shown as mean ± SD (five fields/well, three wells/condition). $^{*}p < 0.05$.

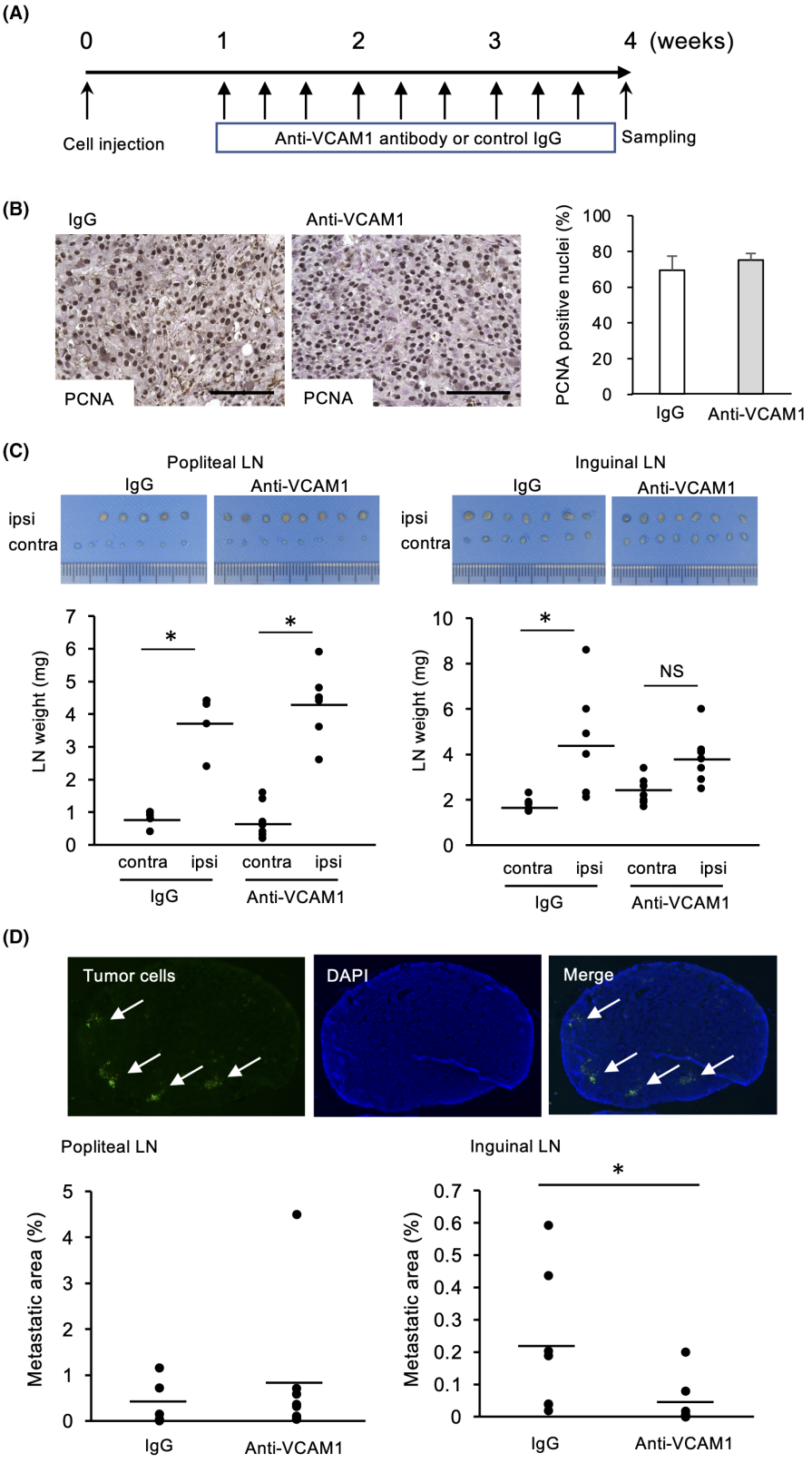


FIGURE 4 The effects of vascular cell adhesion molecule 1 (VCAM-1) blockade on lymph node (LN) metastasis in mouse melanoma models in vivo. (A) Experimental protocol. (B) Immunohistochemistry for proliferating cell nuclear antigen (PCNA) in plantar tumors. Bar, 100 μm. Representative images (left panels) and quantification (right panel). (C) Macroscopic images (upper panels) and weights of LNs (lower panels) in mice treated with control IgG or anti-VCAM-1 antibody are shown. (D) Representative images of popliteal LN in IgG-treated mice (upper panels). Metastatic melanoma cells were detected by fluorescence of Venus (arrows). The percentage of metastatic tumor area as ratio per LN (lower panels). $^*p < 0.05$.

3.5 | G protein-coupled receptor 4 inhibition reduces lymphangiogenesis and lymph node metastases in vivo

Finally, the effect of a selective GPR4 antagonist NE 52-QQ57 on lymphatic cancer metastasis was examined using mouse tumor models (Figure 7A). No significant differences were noticed in primary tumor growth (Figure 7B) or body weight between the control IgG- and antagonist-treated mice. The frequency of LN metastasis was similar in the control IgG-and GPR4 antagonist-treated groups (81.8% and 75.0% of popliteal LNs and 33.3% and 41.7% of inguinal LNs, respectively). Although no significant difference was noticed

in the metastatic tumor area between control IgG- or antagonist-treated LNs, the weight of popliteal LNs was relatively low in antagonist-treated mice (Figure 7C). The weight of inguinal LNs significantly decreased in the antagonist-treated group (Figure 7C).

Additionally, we investigated the density of lymphatic vessels in LNs and primary tumors. Although no differences were observed in the lymphatic vessels of popliteal LNs, a significant decrease in vessel density was noticed in inguinal LNs and primary tumors in the GPR4 antagonist-treated group (Figure 7D). Taken together, these data suggest that an acidic microenvironment upregulates the expression of chemokines and adhesion molecules via the acid-sensing receptor GPR4 in LECs. These alterations might promote adhesion of cancer cells to LECs and lymphangiogenesis, resulting in the progression of lymphatic cancer metastasis (Figure 7E).

4 | DISCUSSION

In the TME, LECs interact with tumor cells by secreting various growth factors, cytokines, chemokines, and adhesion molecules,

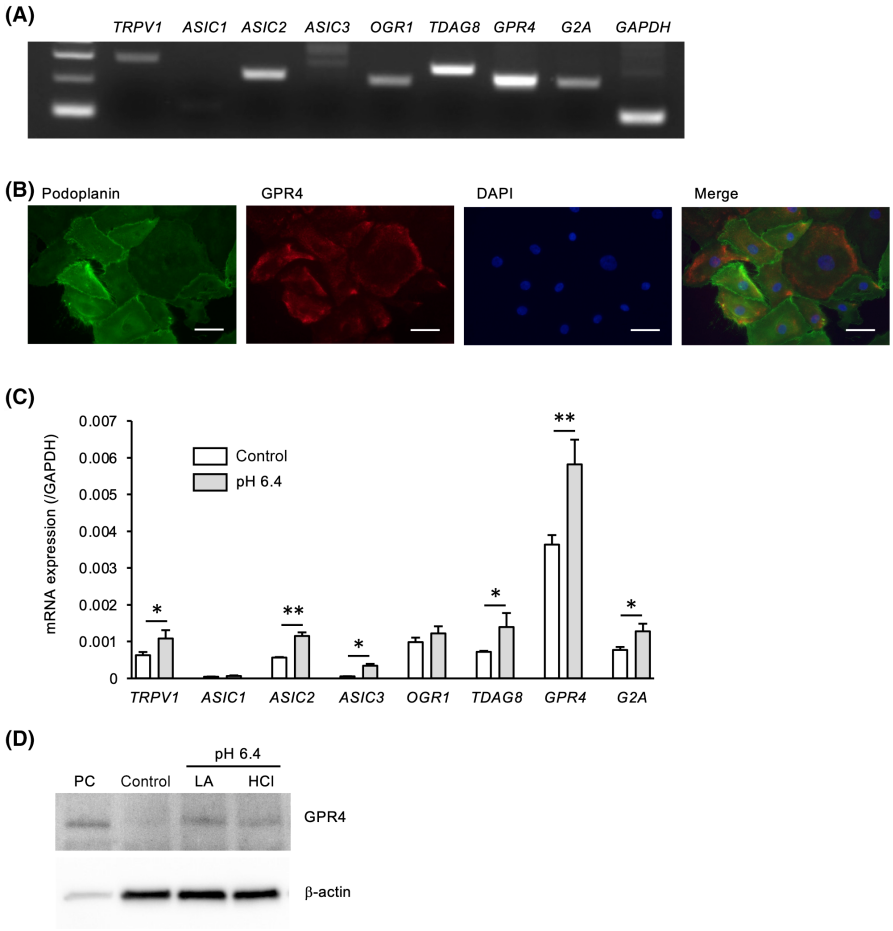
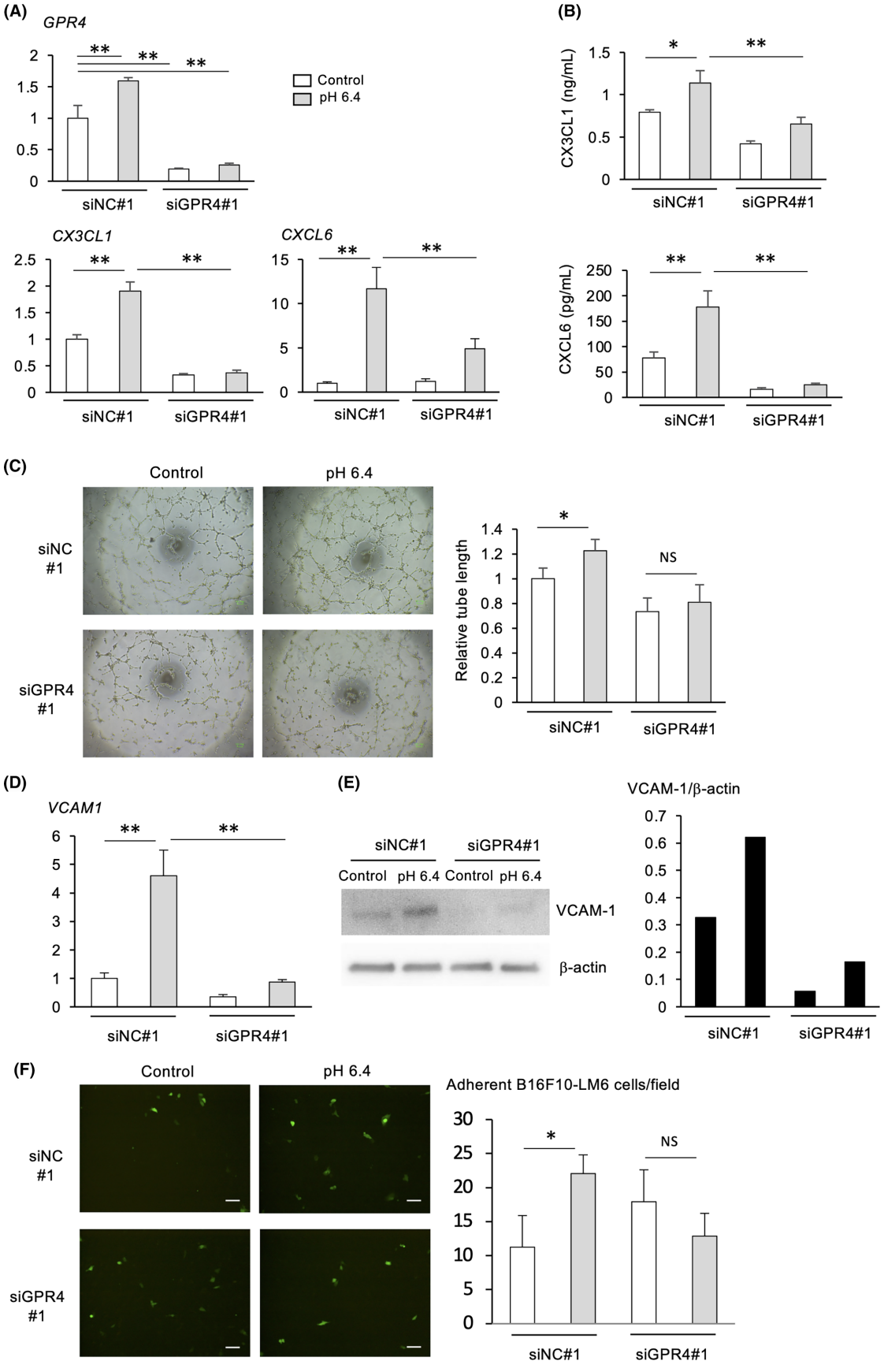


FIGURE 5 The expression of acid-sensing receptors in human dermal lymphatic endothelial cells (HDLECs). (A) RT-PCR analysis of genes encoding acid-sensing receptors. (B) Immunocytochemistry for podoplanin and GPR4 in HDLECs. Bar, 50 μm. (C) HDLECs were treated with control or acidic media for 24 h. Gene expression of acid-sensing receptors was evaluated by RT-qPCR analysis. mRNA expression was normalized with respect to *GAPDH* expression (mean ± SD) ($n = 3$). $^{*}p < 0.05$; $^{**}p < 0.01$. (D) Immunoblotting of GPR4 in HDLECs. HEK293T cells overexpressing GPR4 were used as positive control (PC). Acidic condition was created by addition of lactic acid (LA) or HCl. The expression of β-actin was analyzed as the loading control.

FIGURE 6 The role of G protein-coupled receptor 4 (GPR4) in inducing chemokines and vascular cell adhesion molecule 1 (VCAM-1) in human dermal lymphatic endothelial cells (HDLECs) under acidic condition. (A) HDLECs were transfected with negative control siRNA (siNC#1) or siRNA against *GPR4* (siGPR4#1) and cultured in control or acidic media for 24 h. Gene expression was evaluated by RT-qPCR analysis, normalized with respect to *GAPDH* levels, and presented as fold expression relative to that in cells transfected with siNC#1. Data are expressed as mean ± SD ($n = 3$). $^{**}p < 0.01$. (B) HDLECs transfected with siNC#1 or siGPR4#1 were cultured in control or acidic media for 72 h. CX3CL1 and CXCL6 levels were measured by ELISA. Data are shown as mean ± SD ($n = 3$). $^{*}p < 0.05$; $^{**}p < 0.01$. (C) HDLECs transfected with siNC#1 or siGPR4#1 were seeded on Matrigel with control or acidic media for 4 h. Representative images (left panels) and relative tube lengths to siNC#1-transfected cells cultured in a normal medium (right panel). Data are shown as mean ± SD ($n = 6$). $^{*}p < 0.05$. (D) HDLECs transfected with siNC#1 or siGPR4#1 were treated with control or acidic media for 6 h. The expression of *VCAM1* mRNA was evaluated by RT-qPCR analysis, normalized with respect to *GAPDH* levels, and shown as fold expression relative to that in cells transfected with siNC#1. Data are expressed as mean ± SD ($n = 3$). $^{**}p < 0.01$. (E) Immunoblotting of VCAM-1 in HDLECs. HDLECs transfected with siNC#1 or siGPR4#1 were cultured in control or acidic media for 24 h. Images of the western blot (left panel) and densitometric intensities of the signals as the ratio of VCAM-1 to β-actin (right panel) are presented. (F) Adhesion of B16F10-LM6 melanoma cells to HDLEC monolayer transfected with siNC#1 or siGPR4#1. Representative images (left panels) and the number of adherent B16F10-LM6 cells (right panel) are presented. Bar, 100 μm. Data are shown as mean ± SD ($n = 4$). $^{*}p < 0.05$.


(A)
GPR4
Control
pH 6.4
siNC#1
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CX3CL1
CXCL6
(B)
CX3CL1 (ng/mL)
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(C)
Control
pH 6.4
siNC #1
siGPR4 #1
Relative tube length
NS
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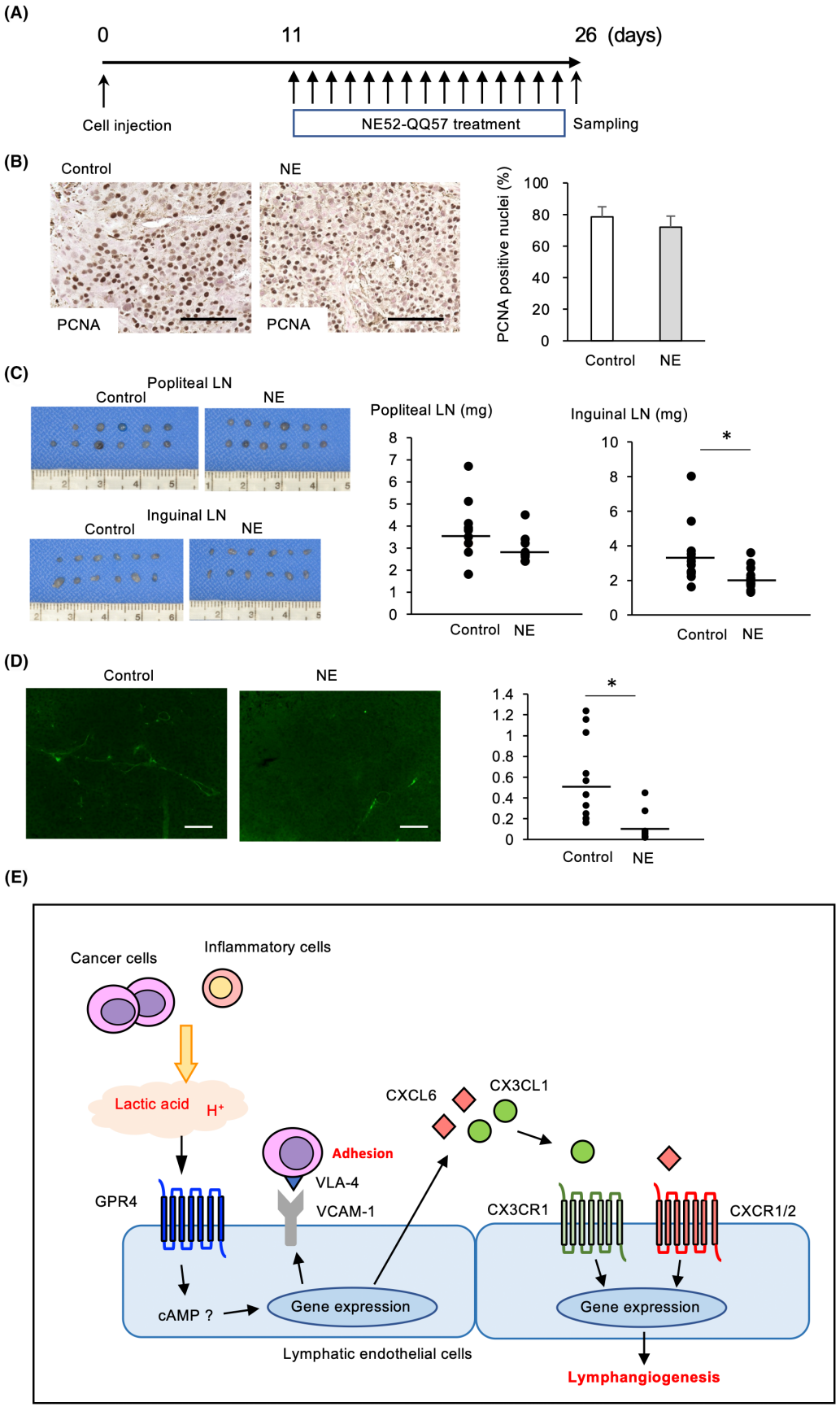
VCAM-1
β-actin
VCAM-1/β-actin
(F)
Adherent B16F10-LM6 cells/field


(A)
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Cell injection
NE52-QQ57 treatment
Sampling
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100
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60
40
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(C)
Popliteal LN
Control
NE
Inguinal LN
Control
NE
Popliteal LN (mg)
8
7
6
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4
3
2
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Inflammatory cells
Lactic acid
H+
GPR4
cAMP ?
Adhesion
VLA-4
VCAM-1
Gene expression
Lymphatic endothelial cells
CXCL6
CX3CL1
CX3CR1
CXCR1/2
Gene expression
Lymphangiogenesis

FIGURE 7 Effect of G protein-coupled receptor 4 (GPR4) inhibition on lymphatic metastasis in mouse melanoma models in vivo. (A) Experimental protocol. (B) Immunohistochemistry for proliferating cell nuclear antigen (PCNA) of plantar tumors. Bar, 100 μm. Representative images (left panels) and quantification (right panel). (C) Macroscopic images (left panels) and weights (right panels) of lymph nodes (LNs) in mice treated with GPR4 antagonist (NE) or methylcellulose solution (control). $^{*}p < 0.05$. (D) Immunohistochemistry for podoplanin in plantar tumors. Representative image (left panels) and quantification of tumor lymphatic vessels as mean area density of podoplanin-positive pixels per microscopic field (right panel) are presented. Bar, 100 μm. $^{**}p < 0.01$. (E) Model of acid-mediated regulation of lymphangiogenesis and metastasis in the tumor microenvironment. Acidic condition augments the acid-sensing receptor GPR4 in lymphatic endothelial cells (LECs), which in turn induces the expression of chemokines and vascular cell adhesion molecule 1 (VCAM-1). Chemokines autocrinally act on LECs and induce lymphangiogenesis. Simultaneously, acidic condition upregulates α4β1 integrin expression in cancer cells, resulting in their adhesion to LECs. These effects promote the invasion of cancer cells toward lymphatic vessels, leading to the development of lymphatic metastasis.

resulting in cancer progression and metastasis.[3–5,27] Our gene expression profiling identified the induction of several chemokines and adhesion molecules in HDLECs cultured under acidic conditions.

CX3CL1 is a unique chemokine, which functions as a chemoattractant and adhesion molecule.[28,29] We showed that acidic stimulation induced CX3CL1 expression in HDLECs. CX3CR1 induces the autocrine action of CX3CL1 in HDLECs, thereby enhancing their growth and tube formation. Increased expression of CX3CL1 and CX3CR1 is differentially correlated with prognosis in several types of cancer.[30] In the present study, although CX3CL1 did not alter the function of MDA-MB-231 breast cancer cells in this study (Figure S5), increased CX3CL1 expression in cancer tissues is positively associated with LN metastasis in patients with breast cancer.[31]

CXCL6 is expressed in various stromal cells, including fibroblasts and VECs, and promotes neutrophil accumulation and angiogenesis.[28,32] Our results suggest that acid-induced CXCL6 expression in LECs accelerates their growth in an autocrine manner. In contrast, CXCL6 acts as an autocrine growth factor in cancer cells. Treatment of melanoma with neutralizing antibodies against CXCL6 attenuates tumor growth and lymphatic metastasis in a mouse model.[33]

In the present study, we focused on the role of VCAM-1 as an adhesion molecule under acidic conditions. VCAM-1 in VECs mediates the adhesion and transmigration of leukocytes through the vascular endothelium under inflammatory conditions.[34,35] In addition, VCAM-1 expression has been observed in the lymphatic endothelium during dermal inflammation.[36] The inflammatory cytokine tumor necrosis factor α (TNF-α) upregulates VCAM-1 expression rapidly in LECs, resulting in leukocyte adhesion and transmigration.[37,38] Our data indicate that acidic conditions rapidly induce VCAM-1 expression in HDLECs. α4β1 integrin (VLA-4) in leukocytes and its ligand VCAM-1 in VECs are involved in their mutual interactions.[39] A similar function of α4β1 integrin might be important for the intravasation of cancer cells. Highly metastatic melanoma cells express α4β1 integrin, leading to their binding with VCAM-1 in VECs; the inhibition or silencing of α4β1 integrin within melanoma cells reduces their trans-endothelial migration in vitro.[40] Our results showed that VCAM-1 mediated the adhesion of cancer cells to LECs in vitro and lymphatic cancer metastasis in vivo. The expression of *Itga4* mRNA (encoding α4 integrin) increased by acidic stimulation in B16F10-LM6 cells (data not shown). These results indicate that the acidic microenvironment induces the adhesion of cancer cells to LECs by inducing VCAM-1 in LECs and α4 integrin in cancer cells. Usage of anti-VCAM-1 antibody in vivo could affect the cellular function of various cell types. Several studies have demonstrated that anti-VCAM-1 antibody administration attenuates inflammatory responses through the inhibition of adhesion between leukocytes and VECs.[37,41] Although we show the tumor-suppressive effect of anti-VCAM-1 antibody in the present study, immune cell function might be altered by treatment with anti-VCAM-1 antibody. In contrast, VCAM-1 has been reported to disrupt the lymphatic junction and permeability of LECs by displacing VE-cadherin, resulting in the promotion of lymphatic metastasis of cancer cells.[42] When discussing the role of acidic conditions in lymphatic metastasis, VCAM-1 might regulate the permeability of lymphatic vessels.

GPR4 is expressed mainly in VECs and plays important roles in the adhesion of leukocytes and induction of inflammatory genes.[16,21–23] Our findings clarified that GPR4 has a similar function in LECs and is involved in functional alterations of LECs under acidic conditions in the TME. ASIC3, which was induced in HDLECs under an acidic condition, was partially involved in the upregulation of CXCL6 and VCAM-1 but not CX3CL1 (data not shown). Although these acid-sensing receptors might also contribute to the regulation of chemokines and adhesion molecules, we believe that GPR4 is crucial for the response of LECs to acidic condition in the TME. Overexpression of GPR4 in head and neck squamous cell carcinoma cells increases the production of angiogenic factors within an acidic microenvironment, thereby inducing angiogenesis in a chick chorioallantoic membrane model.[43] GPR4 expression is associated with microvessel density in hepatocellular carcinoma and the overall survival of patients.[44] In addition, two orthotopic tumor models have indicated that tumor growth is significantly reduced in GPR4-deficient mice through alteration of the angiogenic response.[45] These results indicate the mechanism of tumor progression by the activation of VECs or LECs following GPR4 activation in cancer cells, VECs, and/or LECs.

In contrast, a cell-autonomous effect of GPR4 that is expressed in cancer cells remains controversial. Silencing *GPR4* in colorectal cancer cells inhibits their proliferation and migration in vitro and reduces tumor growth and liver metastasis in a mouse model.[46] GPR4 overexpression increases the migration and invasion of human melanoma cells.[47] However, GPR4 could exhibit a tumor-suppressive effect in a certain type of tumor model. GPR4 overexpression in B16F10 mouse melanoma cells reduces their migration and invasion in vitro and pulmonary metastasis following tail vein injection.[48] The

activation of GPR4 decreases cell spreading and alters the focal adhesion complex, resulting in reduced cell motility.[49]

As GPR4 is considered a target for several pathological conditions, the GPR4 antagonist 13 (NE 52-QQ57) has been examined in a wide range of animal disease models.[50–54] However, the effects of NE 52-QQ57 on cancer metastasis have not been fully elucidated. In this study, metastatic tumors in inguinal LNs, but not in popliteal LNs, were significantly reduced in mice treated with NE 52-QQ57. Considering GPR4 antagonists suppress LN metastasis by inhibiting lymphangiogenesis in plantar tumors, metastasis might be reduced in distant LNs. Whether NE 52-QQ57 directly affects B16F10-LM6 cells remains unknown. GPR4 has been reported to be tumor suppressive in B16F10 cells.[48,49] However, B16F10-LM6 cells showed a markedly lower level of GPR4 expression than B16F10 parental cells, suggesting that the involvement of NE 52-QQ57 in tumor growth was less critical in this study.

In summary, an acidic microenvironment upregulates chemokines and cell adhesion molecules in LECs by activating GPR4, leading to lymphatic metastasis of cancer cells. The results suggest that regulation of GPR4 and its downstream molecules in LECs could be a novel preventive or therapeutic target for cancer metastasis.

AUTHOR CONTRIBUTIONS

Masako Nakanishi: Conceptualization; formal analysis; funding acquisition; investigation; writing – original draft. **Akiya Ibe:** Formal analysis; investigation. **Kiyoto Morishita:** Investigation. **Kazutaka Shinagawa:** Investigation. **Yushi Yamamoto:** Investigation. **Hibiki Takahashi:** Investigation. **Kyoka Ikemori:** Investigation. **Yasuteru Muragaki:** Supervision. **Shogo Ehata:** Conceptualization; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors have no potential conflicts of interest.

ETHICS STATEMENT

Approval of the research protocol by an Institutional Reviewer Board: All experiments were approved by the Institutional Animal Use Committee of Wakayama Medical University and conducted according to the guidelines.

Informed Consent. N/A.

Registry and the Registration No. of the study/trial: N/A.

Animal Studies: Yes.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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