Significance of expression of CD109 in osteosarcoma and its involvement in tumor progression via BMP signaling

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Abstract

Osteosarcoma, the most common primary malignant bone tumor, is defined by the formation of neoplastic osteoid and/or bone. This sarcoma is a highly heterogeneous disease with a wide range of patient outcomes. CD109 is a glycosylphosphatidylinositol-anchored glycoprotein that is highly expressed in various types of malignant tumors. We previously reported that CD109 is expressed in osteoblasts and osteoclasts in normal human tissues and plays a role in bone metabolism *in vivo*. While CD109 has been shown to promote various carcinomas through the downregulation of TGF-β signaling, the role and mechanism of CD109 in sarcomas remain largely unknown. In this study, we investigated the molecular function of CD109 in sarcomas using osteosarcoma cell lines and tissue. Semi-quantitative immunohistochemical analysis using human osteosarcoma tissue revealed a significantly worse prognosis in the CD109-high group compared with the CD109-low group. We found no association between CD109 expression and TGF-β signaling in osteosarcoma cells. However, enhancement of SMAD1/5/9 phosphorylation was observed in CD109 knockdown cells under bone morphogenetic protein-2 (BMP-2) stimulation. We also performed immunohistochemical analysis for phospho-SMAD1/5/9 using human osteosarcoma tissue and found a negative correlation between CD109 expression and SMAD1/5/9 phosphorylation. *In vitro* wound healing assay showed that osteosarcoma cell migration was significantly attenuated in CD109-knockdown cells compared with

control cells in the presence of BMP. These results suggest that CD109 is a poor prognostic factor in osteosarcoma and affects tumor cell migration via BMP signaling.

Keywords

CD109, osteosarcoma, prognostic marker, BMP, SMAD

Abbreviations

TGF-β, transforming growth factor-β; BMP, bone morphogenetic protein.

Introduction

Sarcomas have not been well studied because of their variation and rarity. Furthermore, sarcoma is a complex disease that includes many different tumor types; therefore, it is more difficult to carry out molecular research in sarcomas than in carcinomas [1,2]. Osteosarcoma, the most common primary malignant bone tumor, is characterized by the highest level of heterogeneity affecting patient outcome [3]. This is partly because osteosarcoma is defined phenotypically rather than molecularly as a sarcoma that forms neoplastic osteoid and/or bone. Thus, the identification of a prognostic factor for osteosarcoma is critical.

CD109, a glycosylphosphatidylinositol-anchored cell surface glycoprotein, is a member of the α_2-macroglobulin/C3, C4, C5 family of thioester-containing proteins [4,5]. CD109 is expressed in various cells and tissues, such as myoepithelial cells of mammary, lacrimal, salivary and bronchial glands, basal cells of epidermis and bronchial and prostate epithelia, seminiferous tubules of testis, osteoblasts and osteoclasts in bone, and platelets in blood [4,6-8]. CD109 primarily functions by negatively regulating transforming growth factor (TGF)-β signaling through its binding to TGF-β receptor I, TGF-β, ALK1 and 78-kDa glucose-regulated protein [9-14]. High levels of CD109 expression have been detected in various tumor cell lines and tumor tissues, including squamous cell carcinomas (SCCs) of the lung, esophagus, uterus and oral cavity, adenocarcinomas of the lung and pancreas, breast cancer, glioblastoma and urothelial carcinoma [15]. Previous studies reported CD109 expression in malignant

tumors and its correlation with the prognosis of patients with malignant tumors such as SCC, adenocarcinoma and glioma [15-17]. In SCCs, CD109 promotes tumor initiation by suppressing the TGF-β/SMAD/nuclear factor erythroid 2–related factor-2 pathway [18]. Other studies reported that CD109 is not associated with TGF-β signaling in non-epithelial tumors such as glioma [16], suggesting that the function of CD109 in TGF-β signaling is dependent upon cell type. While we previously reported that CD109 is expressed in osteoblasts and osteoclasts in normal human tissues and plays a role in bone metabolism *in vivo* [8], the function of CD109 in sarcomas remains largely unclear.

In this study, we investigated the molecular function of CD109 in sarcomas using osteosarcoma cell lines and tissue. We demonstrated that CD109 regulates signaling of the BMP-2 pathway, but not that of TGF-β, in osteosarcoma cells. Additionally, we revealed that CD109 expression level negatively correlated with BMP signaling activity in human osteosarcoma tissue and the overall survival of osteosarcoma patients. These data suggested that CD109 may be involved in tumor progression via the control of BMP signaling in osteosarcoma.

Materials and methods

Cell culture and RNA interference

The human adipose derived mesenchymal cell line ASC52telo was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The human bone marrow-derived mesenchymal cell

line UE7T-13 was purchased from the JCRB Cell Bank (Osaka, Japan). The human fibrosarcoma cell line HT-1080 was kindly provided by Kenji Kadomatsu (Nagoya University, Nagoya, Japan). Human osteosarcoma MG-63 and SaOS-2 cells were purchased from RIKEN BioResource Research Center (Tsukuba, Japan) and ATCC, respectively. ASC52telo and UE7T-13 cells were cultured in mesenchymal stem cell growth medium (MSCGM BulletKit; Lonza, Basel, Switzerland). HT-1080 and MG-63 cells were cultured in DMEM supplemented with 8% fetal bovine serum. SaOS-2 cells were cultured in McCoy's 5A medium with 10% fetal bovine serum. Cells were maintained at 37 °C in 5% CO_2.

For transient silencing of CD109, osteosarcoma cell lines were transfected with Stealth RNAi siRNA targeting human CD109 or Stealth RNAi siRNA Negative Control (Thermo Fisher Scientific, Waltham, MA, USA) at a final concentration of 20 nM using Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific) following the manufacturer's protocols. CD109 siRNAs were designed using BLOCK-iT RNAi Designer (Thermo Fisher Scientific) [19]. Two siRNA sequences were used for targeting CD109 expression, #1: 5′-UGGAGGAUUCCAGUGAGCUACAGUU-3′ and #2: 5′-UGGGUGUCAUCAGAGUCCAAACUUU-3′ [17].

In some experiments, cells at 70%–80% confluency were starved for 3 h in growth factor-free medium. Cells were washed and treated with 100 pM TGF-β1 (PeproTech, Cranbury, NJ, USA) or 50 ng/mL BMP-2 (Bio-Techne, Minneapolis, MN, USA) for indicated times before use in experiments. Note that 100 pM TGF-β1 and 50 ng/mL BMP-2 are frequently used doses *in vitro* [20].

Western blot analysis

Western blot analysis was performed by a conventional protocol as previously described [7]. Briefly, cell lysates were sonicated, boiled for 2 min with 2% 2-mercaptoethanol, subjected to SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). Membranes were blocked and incubated with primary antibody for 1 h at room temperature (RT). After washing, the membranes were incubated with secondary antibody conjugated to HRP for 1 h at RT. The membranes were washed, and bands were visualized using the ECL Detection Kit (GE Healthcare, Buckinghamshire, UK) following the manufacturer's instructions. Densitometric analysis was performed using ImageQuant TL (GE Healthcare, Chicago, IL, USA). The primary and secondary antibodies used in this study are listed in Supplementary Table S1.

Cell proliferation and migration assays

Cell proliferation was measured using the water-soluble tetrazolium (WST)-1 assay (Roche, Indianapolis, IN, USA). Briefly, MG-63 cells transfected with either control or CD109 siRNA were plated in 96-well plates 48 h after transfection. WST-1 reagent was added and cells were incubated for 2 h. The absorbance of each well was measured at 450 nm using a microplate reader (Powerscan4, DS Pharma Biomedical, Osaka, Japan).

Directional cell migration of human cancer cells was stimulated in a monolayer using an *in vitro* scratch wound assay, as previously described [21]. Briefly, MG-63 cells were seeded on 35-mm dishes and transfected with either control or CD109 siRNA. Confluent cells were scratched with a 200-μL disposable plastic pipette tip and then plates were cultured for 5 days. Digital images were taken with a microscope (IX70, Olympus). The percentage of the unfilled wound area to the initial wound area was calculated.

Human osteosarcoma samples

Bone biopsy samples were obtained from 55 high-grade (i.e., grade 3) osteosarcoma patients that visited Nagoya University Hospital from 2000 to 2015. All samples used in this study were obtained before treatment, including neoadjuvant chemotherapy. All patients underwent margin-negative surgical resection. Fifty-three patients were treated with neoadjuvant and adjuvant chemotherapy and two patients were treated with surgery alone. Tumor stages were determined on the basis of the Enneking staging system [22, 23]. Pelvic osteosarcomas were excluded in this study because most pelvic osteosarcomas are unresectable. This study was approved by the Ethics Committee of Nagoya University Graduate School of Medicine. All patients provided informed consent.

Histological analysis and scoring

Tissues were fixed in 10% neutral-buffered formalin, dehydrated and embedded in paraffin. Immunohistochemical staining was performed as previously described [7]. Briefly, paraffin sections were deparaffinized, dehydrated, subjected to antigen retrieval with Target Retrieval Solution, pH 9 (Agilent), blocked with 10% normal goat serum (Nichirei Bioscience, Tokyo, Japan) and incubated with primary antibodies for 1 h at RT. Endogenous peroxidase was inhibited by incubation in 3% hydrogen peroxide in PBS for 15 min. The slides were incubated with secondary antibody conjugated to HRP-labeled polymer (EnVision+; Agilent) for 15 min at RT. Reaction products were visualized with diaminobenzidine (Agilent); nuclear counterstaining was performed with hematoxylin. The primary and secondary antibodies used in this study are listed in Supplementary Table S2.

Two independent pathologists evaluated the immunohistochemical and hematoxylin and eosin (H&E)–stained sections, as previously described [16,17]. The immunostaining score for CD109 was calculated as the sum of the proportion score (PS) and intensity score (IS). The proportion score was determined by the estimated fraction of positively stained tumor cells (score 0, < 33%; score 1, 33%–66%; and score 2, 66%–100%). The intensity score was determined as the estimated staining intensity (score 0, negative; score 1, minimal; score 2, moderate; and score 3, strong). The total score ranged from 0 to 5. We defined samples with a total score >3 as CD109-high. This study was approved by the Ethics Committee of Nagoya University Graduate School of Medicine (approval number: 2017-0127-5).

Statistical analysis

For comparison of the differences between two groups, a two-tailed Student's t test, Bonferroni post hoc analysis or one-way analysis of variance (ANOVA) was performed. In Kaplan–Meier analysis, the significance of differences between groups was evaluated by the log-rank test using GraphPad Prism 6 software (GraphPad, San Diego, CA, USA). Spearman's rank correlation coefficient (r_s) was evaluated using SPSS Statistics version 28.0 (IBM Corp., Armonk, NY, USA). Clinicopathological features were evaluated with Fisher's exact test. Multivariate analysis was performed using a logistic regression model in EZR software version 1.60 (Saitama Medical Center, Jichi Medical University, Saitama, Japan). A P value of ≤ 0.05 was considered significant. Data are expressed as the mean ± standard deviation.

Results

CD109 is highly expressed in osteosarcoma cells and its expression correlates with prognosis in osteosarcoma patients

CD109 expression in tumor stroma was recently reported [24], which prompted us to examine CD109 expression in various stromal cell lines. Western blot analysis revealed that the expression level of CD109 is extremely high in mesenchymal cell lines including osteosarcoma cell lines (Fig. 1a).

To examine the significance of CD109 *in vivo*, we analyzed CD109 expression in human osteosarcoma tissues by histological and immunohistochemical methods. The results revealed CD109 expression in

osteosarcoma tissue (Fig. 1b). Additionally, the expression level of CD109 in tumor cells varied among cases, while CD109 expression in non-tumor cells including macrophages was relatively constant.

We then examined the expression of CD109 protein in tumor cells in osteosarcoma samples from 55 patients using immunohistochemistry. The patient age ranged from 6 to 94 years, with a mean of 22.0 years. The clinicopathological features of the 55 patients are summarized in Table 1a. CD109 staining in tumor cells was scored and the cases were categorized into two groups as described in the Methods: CD109-low and CD109-high (Fig. 1c). This quantitative scoring system, which combined the proportion and intensity scores, revealed that CD109 expression was heterogeneous in osteosarcoma tumor cells, and that the higher the scores, the larger the number of patients was, except for IS3 and TS5 (Supplementary Fig. S1a). Neoplastic osteoids or bones in the representative cases are shown in Supplementary Fig. S1b. Kaplan–Meier analysis revealed that patients with CD109-high osteosarcomas had a significantly shorter overall survival compared with those with CD109-low osteosarcomas (P = 0.041), indicating that high CD109 expression correlated with a poor prognosis (Fig. 1d). Multivariate analysis revealed that CD109 expression was an independent prognostic factor for death within 5 years from diagnosis (P = 0.0309, Table 1b). Interestingly, we found no significant difference in the survival rate between the *CD109*-high and -low groups by analyzing publicly available RNA-seq data (Supplementary Fig. S2a). This is partly because RNA-seq data reflect *CD109* expression in tumor and non-tumor cells. Additionally, we performed *in silico* analysis using a publicly available single-cell

RNA-seq dataset from osteosarcoma patients [25]. This analysis revealed that *CD109* expression in osteosarcoma cells was more heterogeneous than *RUNX2* expression (Supplementary Fig. S2b). *CD109* expression was also confirmed in osteoclasts as in a previous report [8].

CD109 does not regulate TGF-β signaling, but it suppresses BMP-2 signaling in osteosarcoma cells

To investigate whether CD109 regulates TGF-β signaling in osteosarcoma cells, we performed CD109 knockdown by RNA interference. We used two different siRNAs targeting CD109 (siCD109), #1 and #2, and two human osteosarcoma cell lines, MG-63 and SaOS-2. Both siRNAs effectively knocked down CD109 protein expression in the two cell lines at a concentration of 20 nM (Fig. 2a). We examined SMAD2/3 phosphorylation in cells treated with 100 pM TGF-β1. The results showed that CD109 did not regulate TGF-β signaling in osteosarcoma cells (Fig. 2b), which is different from previously reported results observed in epithelial cells [9,17,18]. We also performed semi-quantitative analysis by densitometry and found no significant difference in SMAD phosphorylation between CD109-knockdown MG-63 or SaOS-2 cells and controls (Fig. 2c). The same results were obtained in experiments using siCD109#1 in MG-63 cells and siCD109#2 in SaOS-2 cells (data not shown).

BMP is one of the TGF-β superfamily members and plays an important role in bone metabolism. Therefore, we next examined whether CD109 influenced BMP-2 signaling in osteosarcoma cells. Notably, BMP-2-induced SMAD1/5/9 phosphorylation was enhanced in CD109-knockdown cells

compared with the control cells (Fig. 3a, b). The same results were obtained in experiments using two different cell lines and two different siRNAs. Semi-quantitative analysis of phosphorylated SMAD1/5/9 relative to total SMAD1 confirmed that BMP-2-induced SMAD1/5/9 phosphorylation in MG-63 and SaOS-2 cells was significantly enhanced in CD109-knockdown cells compared with control cells at 1 h after stimulation (Fig. 3c, d). SMAD1/5/9 phosphorylation was also significantly enhanced at 0.5 h after stimulation in CD109-knockdown MG-63 cells (Fig. 3c). BMP-2-induced ERK1/2 phosphorylation [26, 27] was slightly enhanced in CD109-knockdown MG-63 cells using siCD109#1 but not in the experiment using siCD109#2 (Supplementary Fig. S3a, b). We also examined STAT3 phosphorylation that was previously reported to be associated with CD109 [7, 28-31]. No apparent differences were observed in IL-6- or TGF-β-induced STAT3 phosphorylation between CD109-knockdown and control osteosarcoma cells (Supplementary Fig. S3c-e).

SMAD1/5/9 phosphorylation was attenuated in CD109-high osteosarcoma tissue

Our *in vitro* results indicated that CD109 suppresses BMP-2 signaling in osteosarcoma cells. Thus, we investigated the association between CD109 expression and BMP signaling *in vivo* by evaluating SMAD1/5/9 phosphorylation in human osteosarcoma tissue. We performed immunohistochemical analysis for phospho-SMAD1/5/9 in serial sections of two cases and found that CD109 expression level negatively correlated with SMAD1/5/9 phosphorylation (Fig. 4a). Furthermore, we calculated the total

scores of phospho-SMAD1/5/9 staining in 10 randomly selected sequential cases of human osteosarcomas and examined the correlation between CD109 expression and SMAD1/5/9 phosphorylation. One-tailed Spearman's correlation analysis revealed a significantly negative correlation between the expression level of CD109 and the phosphorylation of SMAD1/5/9 in human osteosarcoma tissues ($r_s = -0.63$, $P = 0.0254$; Fig. 4b). These results suggest that CD109 suppresses BMP-2 signaling in osteosarcoma *in vitro* and *in vivo*.

CD109 promotes osteosarcoma cell migration but not cell proliferation in the presence of BMP-2

We next investigated the role of CD109 in BMP-2-mediated effects on osteosarcoma proliferation and migration. WST-1 assay revealed no significant difference in cell proliferation between CD109-knockdown and control MG-63 cells in the presence of BMP-2 (Fig. 5a). To investigate the role of CD109 in BMP-2-mediated effects on cell migration, we performed *in vitro* wound healing assays. We found that cell migration was decreased in BMP-treated CD109-knockdown MG-63 cells compared with BMP-treated control cells (Fig. 5b). Quantification of the migration results revealed that cell migration was significantly decreased in CD109-knockdown MG-63 cells compared with the control cells (Fig. 5c). To clarify the role of CD109 in migration, we performed *in vitro* wound healing assays in the absence of BMP-2. We observed no significant difference in cell migration between CD109-knockdown MG-63 cells and control cells in the absence of BMP-2 (Supplementary Fig. S4). These

results show that CD109 suppresses BMP-2 signaling and promotes BMP-2-mediated cell migration in osteosarcoma cells *in vitro*.

Discussion

In this study, we showed that CD109 expression was a prognostic marker of osteosarcoma by analysis of human tissue samples. We found that the expression level of CD109 negatively correlated with BMP-2 signaling in osteosarcoma cells *in vitro* and *in vivo*. We also observed that CD109 knockdown decreased cell migration in osteosarcoma cells in the presence of BMP-2. These results suggested that CD109 promotes osteosarcoma progression via BMP signaling.

A significant finding of this study is the results showing the regulation of CD109 of BMP signaling in osteosarcoma. CD109 is not associated with TGF-β signaling in non-epithelial tumors such as glioma, while it is a TGF-β-related tumor-promoting protein in various carcinomas. Although some reports suggested that soft tissue sarcomas may be associated with TGF-β signaling [19, 32], our results suggested that the function of CD109 in TGF-β signaling in non-epithelial solid tumors was different from that in carcinomas and hematopoietic cells [33, 34].

The effect of BMP-2 on cell proliferation and migration is controversial [35-39]. Gill et al. reported that BMP-2 addition did not increase the proliferation or migration of osteosarcoma cell lines [35], while Tian et al. reported that the mobility of osteosarcoma cells *in vitro* was increased via BMP signaling

[37]. The former study used BMP-2 at concentrations of 0.5, 1 and 2 μg/mL, while the latter study used BMP-2 at a concentration of 10 and 50 ng/mL. The concentration difference may explain the differences in the results. A review article also reported that BMP-2 acts in a concentration-dependent fashion *in vitro* and that BMP-2 has no effect at high concentrations such as 200 ng/mL [20].

While the molecular mechanism between CD109 and BMP-2 or its receptors is unclear, one study showed that CD109 promotes the degradation of some receptors such as TGF-β receptor 1 [11]. CD109 may also promote the degradation of BMP receptors and downregulate BMP-2 signaling. However, the detailed molecular mechanism requires further investigation.

One major limitation of this study is that we did not provide a mechanistic explanation for why CD109 does not influence TGF-β signaling in sarcomas as it does in various carcinomas [17,18,40]. One potential explanation is the difference in the cellular localization of CD109 protein between carcinomas and sarcomas; CD109 in sarcomas localizes not only on the plasma membrane but also in the cytoplasm of tumor cells, while that in carcinomas, CD109 is located at the plasma membrane [15]. The intracellular form of CD109 might function differently from the membrane and soluble forms. Cytoplasmic CD109 may have specific functions in regulating BMP signaling and the relative decreased expression of membrane CD109 may explain its lack of influence on TGF-β signaling.

Our immunohistochemical observation of osteosarcoma revealed its heterogeneity, which was similar to that observed in lung adenocarcinoma and brain tumor patients [16,17]. To evaluate such heterogeneity,

we used a quantitative scoring system by combining the proportion score and intensity score, which is the same as the Allred scoring system used in breast cancer [41], and two independent pathologists conducted an objective histological analysis (Fig. 1c). Interestingly, IHC analysis revealed significant results, whereas RNA-seq data analysis did not (Fig. 1d and Supplementary Fig. S2a). This might be because we evaluated CD109 expression in tumor cells in osteosarcoma tissues by IHC whereas the RNA-seq data reflect CD109 expression in tumor and non-tumor cells (Supplementary Fig. S2b). This suggests the importance of histological or "spatial" expression analyses.

In conclusion, our findings showed that CD109 regulates BMP signaling in osteosarcoma cells and that its expression is a significant prognostic factor in human osteosarcoma patients. Additionally, CD109 protein may be a potential therapeutic target in osteosarcoma because of its expression on the cell surface, enabling anti-CD109 antibodies or CAR-T cells to bind target tumor cells. Further analyses using sarcoma mouse models would provide further insights into the mechanism of tumor-progressing effects of CD109 in sarcomas.

Conclusions

CD109 protein is highly expressed in osteosarcoma cells and tissue and a statistically significant poor prognostic factor in human osteosarcoma. CD109 negatively regulates BMP signaling in osteosarcoma cells and negatively correlates with BMP signaling in osteosarcoma tissue. CD109 suppresses BMP-2-

mediated cell migration in osteosarcoma. These results suggest that CD109 is involved in tumor progression by regulating BMP signaling in osteosarcoma.

Statements and declarations

Competing interests

The authors have no competing interests to disclose.

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Author contributions

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References

[1] T.G. Grünewald, M. Alonso, S. Avnet, A. Banito, S. Burdach, F. Cidre-Aranaz, G. Di Pompo, M. Distel, H. Dorado-Garcia, J. Garcia-Castro, L. González-González, A.E. Grigoriadis, M. Kasan, C. Koelsche, M. Krumbholz, F. Lecanda, S. Lemma, D.L. Longo, C. Madrigal-Esquivel, Á. Morales-Molina, J. Musa, S. Ohmura, B. Ory, M. Pereira-Silva, F. Perut, R. Rodriguez, C. Seeling, N. Al Shaaili, S. Shaabani, K. Shiavone, S. Sinha, E.M. Tomazou, M. Trautmann, M. Vela, Y.M. Versleijen-Jonkers, J. Visgauss, M. Zalacain, S.J. Schober, A. Lissat, W.R. English, N. Baldini, D. Heymann, Sarcoma treatment in the era of molecular medicine, EMBO Mol. Med. 12 (2020) e11131. https://doi.org/10.15252/emmm.201911131.

[2] A.C. Gamboa, A. Gronchi, K. Cardona, Soft-tissue sarcoma in adults: An update on the current state of histiotype-specific management in an era of personalized medicine, CA Cancer J. Clin. 70 (2020) 200-229. https://doi.org/10.3322/caac.21605.

[3] K. Schiavone, D. Garnier, M.F. Heymann, D. Heymann, The Heterogeneity of Osteosarcoma: The Role Played by Cancer Stem Cells, Adv. Exp. Med. Biol. 1139 (2019) 187-200. https://doi.org/10.1007/978-3-030-14366-4_11.

[4] D.R. Sutherland, E. Yeo, A. Ryan, G.B. Mills, D. Bailey, M.A. Baker, Identification of a cell-surface antigen associated with activated T lymphoblasts and activated platelets, Blood 77 (1991) 84-93. https://doi.org/10.1182/blood.V77.1.84.84.

[5] M. Lin, D.R. Sutherland, W. Horsfall, N. Totty, E. Yeo, R. Nayar, X.F. Wu, A.C. Schuh, Cell surface antigen CD109 is a novel member of the α_2 macroglobulin/C3, C4, C5 family of thioester-containing proteins, Blood 99 (2002) 1683-1691. https://doi.org/10.1182/blood.v99.5.1683.

[6] M. Hasegawa, S. Hagiwara, T. Sato, M. Jijiwa, Y. Murakumo, M. Maeda, S. Moritani, S. Ichihara, M. Takahashi, CD109, a new marker for myoepithelial cells of mammary, salivary, and lacrimal glands and prostate basal cells, Pathol. Int. 57 (2007) 245-250. https://doi.org/10.1111/j.1440-1827.2007.02097.x.

[7] S. Mii, Y. Murakumo, N. Asai, M. Jijiwa, S. Hagiwara, T. Kato, M. Asai, A. Enomoto, K. Ushida, S. Sobue, M. Ichihara, M. Takahashi, Epidermal hyperplasia and appendage abnormalities in mice lacking CD109, Am. J. Pathol. 181 (2012) 1180-1189. https://doi.org/10.1016/j.ajpath.2012.06.021.

[8] S. Mii, A. Hoshino, A. Enomoto, Y. Murakumo, M. Ito, A. Yamaguchi, M. Takahashi, CD109 deficiency induces osteopenia with an osteoporosis-like phenotype in vivo, Genes Cells 23 (2018) 590-598. https://doi.org/10.1111/gtc.12593.

[9] K.W. Finnson, B.Y. Tam, K. Liu, A. Marcoux, P. Lepage, S. Roy, A.A. Bizet, A. Philip, Identification of CD109 as part of the TGF-β receptor system in human keratinocytes, FASEB J. 20 (2006) 1525-1527. https://doi.org/10.1096/fj.05-5229fje.

[10] S. Hagiwara, Y. Murakumo, S. Mii, T. Shigetomi, N. Yamamoto, H. Furue, M. Ueda, M. Takahashi, Processing of CD109 by furin and its role in the regulation of TGF-β signaling, Oncogene 29 (2010)

2181-2191. https://doi.org/10.1038/onc.2009.506.

[11] A.A. Bizet, K. Liu, N. Tran-Khanh, A. Saksena, J. Vorstenbosch, K.W. Finnson, M.D. Buschmann, A. Philip, The TGF-β co-receptor, CD109, promotes internalization and degradation of TGF-β receptors, Biochim. Biophys. Acta 1813 (2011) 742-753. https://doi.org/10.1016/j.bbamcr.2011.01.028.

[12] C. Li, M.A. Hancock, P. Sehgal, S. Zhou, D.P. Reinhardt, A. Philip, Soluble CD109 binds TGF-β and antagonizes TGF-β signalling and responses, Biochem. J. 473 (2016) 537-547. https://doi.org/10.1042/BJ20141488.

[13] J. Vorstenbosch, C.M. Nguyen, S. Zhou, Y.J. Seo, A. Siblini, K.W. Finnson, A.A. Bizet, S.D. Tran, A. Philip, Overexpression of CD109 in the Epidermis Differentially Regulates ALK1 Versus ALK5 Signaling and Modulates Extracellular Matrix Synthesis in the Skin, J. Invest. Dermatol. 137 (2017) 641-649. https://doi.org/10.1016/j.jid.2016.09.039.

[14] Y.L. Tsai, D.P. Ha, H. Zhao, A.J. Carlos, S. Wei, T.K. Pun, K. Wu, E. Zandi, K. Kelly, A.S. Lee, Endoplasmic reticulum stress activates SRC, relocating chaperones to the cell surface where GRP78/CD109 blocks TGF-β signaling, Proc. Natl. Acad. Sci. U. S. A. 115 (2018) E4245-E4254. https://doi.org/10.1073/pnas.1714866115.

[15] S. Mii, A. Enomoto, Y. Shiraki, T. Taki, Y. Murakumo, M. Takahashi, CD109: a multifunctional GPI-anchored protein with key roles in tumor progression and physiological homeostasis, Pathol.

Int. 69 (2019) 249-259. https://doi.org/10.1111/pin.12798.

[16] Y. Shiraki, S. Mii, A. Enomoto, H. Momota, Y.P. Han, T. Kato, K. Ushida, A. Kato, N. Asai, Y. Murakumo, K. Aoki, H. Suzuki, F. Ohka, T. Wakabayashi, T. Todo, S. Ogawa, A. Natsume, M. Takahashi, Significance of perivascular tumour cells defined by CD109 expression in progression of glioma, J. Pathol. 243 (2017) 468-480. https://doi.org/10.1002/path.4981.

[17] T. Taki, Y. Shiraki, A. Enomoto, L. Weng, C. Chen, N. Asai, Y. Murakumo, K. Yokoi, M. Takahashi, S. Mii, CD109 regulates in vivo tumor invasion in lung adenocarcinoma through TGF-β signaling, Cancer Sci. 111 (2020) 4616-4628. https://doi.org/10.1111/cas.14673.

[18] M. Sunagawa, S. Mii, A. Enomoto, T. Kato, Y. Murakumo, Y. Shiraki, N. Asai, M. Asai, M. Nagino, M. Takahashi, Suppression of skin tumorigenesis in CD109-deficient mice, Oncotarget 7 (2016) 82836-82850. https://doi.org/10.18632/oncotarget.12653.

[19] M. Emori, T. Tsukahara, M. Murase, M. Kano, K. Murata, A. Takahashi, T. Kubo, H. Asanuma, K. Yasuda, V. Kochin, M. Kaya, S. Nagoya, J. Nishio, H. Iwasaki, T. Sonoda, T. Hasegawa, T. Torigoe, T. Wada, T. Yamashita, N. Sato, High expression of CD109 antigen regulates the phenotype of cancer stem-like cells/cancer-initiating cells in the novel epithelioid sarcoma cell line ESX and is related to poor prognosis of soft tissue sarcoma, PLoS One 8 (2013) e84187. https://doi.org/10.1371/journal.pone.0084187.

[20] J. Nourisa, B. Zeller-Plumhoff, H. Helmholz, B. Luthringer-Feyerabend, V. Ivannikov, R.

Willumeit-Römer, Magnesium ions regulate mesenchymal stem cells population and osteogenic differentiation: A fuzzy agent-based modeling approach, Comput. Struct. Biotechnol. J. 19 (2021) 4110-4122. https://doi.org/10.1016/j.csbj.2021.07.005.

[21] H. Miyachi, S. Mii, A. Enomoto, Y. Murakumo, T. Kato, N. Asai, K. Komori, M. Takahashi, Role of Girdin in intimal hyperplasia in vein grafts and efficacy of atelocollagen-mediated application of small interfering RNA for vein graft failure, J. Vasc. Surg. 60 (2014) 479-489.e5. https://doi.org/10.1016/j.jvs.2013.06.080.

[22] W.F. Enneking, S.S. Spanier, M.A. Goodman, A system for the surgical staging of musculoskeletal sarcoma, Clin Orthop Relat Res. 153 (1980) 106-120.

[23] A.M. Flanagan, J.Y. Blay, J.V.M.G. Bovée, M.A. Bredella, P. Cool, G.P. Nielsen, A. Yoshida, Bone tumours: Introduction, in: WHO Classification of Tumours Editorial Board (Eds.), WHO Classification of Tumours (5th ed.), Soft Tissue and Bone Tumours, IARC, Lyon, 2020, pp. 340-344.

[24] K. Adachi, Y. Sakurai, M. Ichinoe, M. Tadehara, A. Tamaki, Y. Kesen, T. Kato, S. Mii, A. Enomoto, M. Takahashi, W. Koizumi, Y. Murakumo, CD109 expression in tumor cells and stroma correlates with progression and prognosis in pancreatic cancer, Virchows Arch. 480 (2022) 819-829. https://doi.org/10.1007/s00428-021-03230-2.

[25] Y. Zhou, D. Yang, Q. Yang, X. Lv, W. Huang, Z. Zhou, Y. Wang, Z. Zhang, T. Yuan, X. Ding, L.

Tang, J. Zhang, J. Yin, Y. Huang, W. Yu, C. Zhou, Y. Su, A. He, Y. Sun, Z. Shen, B. Qian, W. Meng, J. Fei, Y. Yao, X. Pan, P. Chen, H. Hu, Single-cell RNA landscape of intratumoral heterogeneity and immunosuppressive microenvironment in advanced osteosarcoma, Nat. Commun. 11 (2020) 6322. https://doi.org/10.1038/s41467-020-20059-6.

[26] J. Lou, Y. Tu, S. Li, P.R. Manske, Involvement of ERK in BMP-2 induced osteoblastic differentiation of mesenchymal progenitor cell line C3H10T1/2, Biochem. Biophys. Res. Commun. 268 (2000) 757-762. https://doi.org/10.1006/bbrc.2000.2210.

[27] S. Zanotti, A. Smerdel-Ramoya, L. Stadmeyer, E. Canalis, Activation of the ERK pathway in osteoblastic cells, role of gremlin and BMP-2, J. Cell Biochem. 104 (2008) 1421-1426. https://doi.org/10.1002/jcb.21715.

[28] I.V. Litvinov, A.A. Bizet, Y. Binamer, D.A. Jones, D. Sasseville, A. Philip, CD109 release from the cell surface in human keratinocytes regulates TGF-β receptor expression, TGF-β signalling and STAT3 activation: relevance to psoriasis, Exp. Dermatol. 20 (2011) 627-632. https://doi.org/10.1111/j.1600-0625.2011.01288.x.

[29] C.H. Chuang, P.G. Greenside, Z.N. Rogers, J.J. Brady, D. Yang, R.K. Ma, D.R. Caswell, S.H. Chiou, A.F. Winters, B.M. Grüner, G. Ramaswami, A.L. Spencley, K.E. Kopecky, L.C. Sayles, E.A. Sweet-Cordero, J.B. Li, A. Kundaje, M.M. Winslow, Molecular definition of a metastatic lung cancer state reveals a targetable CD109-Janus kinase-Stat axis, Nat. Med. 23 (2017) 291-300.

https://doi.org/10.1038/nm.4285.

[30] X.T. Mo, T.H. Leung, H.W. Tang, M.K. Siu, P.K. Wan, K.K. Chan, A.N. Cheung, H.Y. Ngan, CD109 mediates tumorigenicity and cancer aggressiveness via regulation of EGFR and STAT3 signalling in cervical squamous cell carcinoma, Br. J. Cancer 123 (2020) 833-843. https://doi.org/10.1038/s41416-020-0922-7.

[31] P. Filppu, J. Tanjore Ramanathan, K.J. Granberg, E. Gucciardo, H. Haapasalo, K. Lehti, M. Nykter, V. Le Joncour, P. Laakkonen, CD109-GP130 interaction drives glioblastoma stem cell plasticity and chemoresistance through STAT3 activity, JCI Insight 6 (2021) e141486. https://doi.org/10.1172/jci.insight.141486.

[32] A. De Vita, F. Recine, L. Mercatali, G. Miserocchi, C. Liverani, C. Spadazzi, R. Casadei, A. Bongiovanni, F. Pieri, N. Riva, D. Amadori, T. Ibrahim, Myxofibrosarcoma primary cultures: molecular and pharmacological profile, Ther. Adv. Med. Oncol. 9 (2017) 755-767. https://doi.org/10.1177/1758834017737472.

[33] M. Yokoyama, M. Ichinoe, S. Okina, Y. Sakurai, N. Nakada, N. Yanagisawa, S.X. Jiang, Y. Numata, A. Umezawa, K. Miyazaki, M. Higashihara, Y. Murakumo, CD109, a negative regulator of TGF-β signaling, is a putative risk marker in diffuse large B-cell lymphoma, Int. J. Hematol. 105 (2017) 614-622. https://doi.org/10.1007/s12185-016-2173-1.

[34] M. Tanabe, K. Hosokawa, M.A.T. Nguyen, N. Nakagawa, K. Maruyama, N. Tsuji, R. Urushihara,

L. Espinoza, M.I. Elbadry, M. Mohiuddin, T. Katagiri, M. Ono, H. Fujiwara, K. Chonabayashi, Y. Yoshida, H. Yamazaki, A. Hirao, S. Nakao, The GPI-anchored protein CD109 protects hematopoietic progenitor cells from undergoing erythroid differentiation induced by TGF-β, Leukemia 36 (2022) 847-855. https://doi.org/10.1038/s41375-021-01463-3.

[35] J. Gill, P. Connolly, M. Roth, S.H. Chung, W. Zhang, S. Piperdi, B. Hoang, R. Yang, H. Guzik, J. Morris, R. Gorlick, D.S. Geller, The effect of bone morphogenetic protein-2 on osteosarcoma metastasis, PLoS One 12 (2017) e0173322. https://doi.org/10.1371/journal.pone.0173322.

[36] Z. Lv, D. Yang, J. Li, M. Hu, M. Luo, X. Zhan, P. Song, C. Liu, H. Bai, B. Li, Y. Yang, Y. Chen, Q. Shi, Y. Weng, Bone morphogenetic protein 9 overexpression reduces osteosarcoma cell migration and invasion, Mol. Cells 36 (2013) 119-126. https://doi.org/10.1007/s10059-013-0043-8.

[37] H. Tian, T. Zhou, H. Chen, C. Li, Z. Jiang, L. Lao, S.A. Kahn, M.E.L. Duarte, J. Zhao, M.D. Daubs, Z. Buser, E.J. Brochmann, J.C. Wang, S.S. Murray, Bone morphogenetic protein-2 promotes osteosarcoma growth by promoting epithelial-mesenchymal transition (EMT) through the Wnt/β-catenin signaling pathway, J. Orthop. Res. 37 (2019) 1638-1648. https://doi.org/10.1002/jor.24244.

[38] J.K. Kendal, A. Singla, A. Affan, K. Hildebrand, A. Al-Ani, M. Ungrin, D.J. Mahoney, D. Itani, F.R. Jirik, M.J. Monument, Is Use of BMP-2 Associated with Tumor Growth and Osteoblastic Differentiation in Murine Models of Osteosarcoma?, Clin. Orthop. Relat. Res. 478 (2020) 2921-2933. https://doi.org/10.1097/CORR.0000000000001422.

[39] L. Hu, K. Li, L. Lin, F. Qian, P. Li, L. Zhu, H. Cai, L. You, J. Song, S.H.L. Kok, K.K.H. Lee, X. Yang, X. Cheng, Reversine suppresses osteosarcoma cell growth through targeting BMP-Smad1/5/8-mediated angiogenesis, Microvasc. Res. 135 (2021) 104136. https://doi.org/10.1016/j.mvr.2021.104136.

[40] S. Hagiwara, Y. Murakumo, T. Sato, T. Shigetomi, K. Mitsudo, I. Tohnai, M. Ueda, M. Takahashi, Up-regulation of CD109 expression is associated with carcinogenesis of the squamous epithelium of the oral cavity, Cancer Sci. 99 (2008) 1916-1923. https://doi.org/10.1111/j.1349-7006.2008.00949.x.

[41] D.C. Allred, J.M. Harvey, M. Berardo, G.M. Clark, Prognostic and predictive factors in breast cancer by immunohistochemical analysis, Mod. Pathol. 11 (1998) 155-168.

Figure legends

Fig. 1 Expression of CD109 correlates with prognosis in patients with osteosarcoma.

(a) CD109 expression in various stromal cell lines. MG-63 and SaOS-2 are human osteosarcoma cell lines. (b) Representative histological images of human osteosarcoma tissues. H&E staining (top panels) and immunohistochemical staining with anti-CD109 antibody (bottom panels) in the same area in the serial section. The intensity score (IS) of CD109 staining was scored from 0 to 3 (0, negative; 1+, minimal; 2+, moderate; 3+, strong) in tumor cells. Bottom panels show representative images of tissue with each IS, except for negative staining. Arrowheads indicate non-tumor cells including macrophages. (c) The proportion score was assigned as follows: 0, < 1/3 of tumor cells; 1, 1/3–2/3 of tumor cells; 2, ≥ 2/3 of tumor cells. The total score was calculated as the sum of both scores. Cases with a total score more than 3 were considered as CD109-high. (d) Overall survival of 55 patients with osteosarcoma was analyzed by the Kaplan–Meier method. H&E, hematoxylin and eosin; pSMAD1/5/9, phospho-SMAD1/5/9.

Fig. 2 CD109 expression and TGF-β signal activation in human osteosarcoma cell lines.

(a) Effects of siRNAs on CD109 expression in osteosarcoma cells. MG-63 (left panel) or SaOS-2 (right panel) cells were transfected with the indicated concentrations of two CD109 siRNAs or control siRNA. Representative images of immunoblot analysis for CD109 are shown. (b) Time course of SMAD2 and

SMAD3 phosphorylation after TGF-β1 (100 pM) stimulation in CD109 knockdown and control cells. Representative images of immunoblot analysis in MG-63 cells (left panel) using siRNA#2 targeting CD109 and in SaOS-2 cells (right panel) using siRNA#1 targeting CD109. (c) Relative densitometric intensities of immunoblot bands for SMAD2 phosphorylation (n = 3 per group). Error bars indicate standard deviation. siControl, Control siRNA; siCD109, siRNA targeting CD109; N.S., not significant.

Fig. 3 CD109 suppresses BMP-2 signaling in human osteosarcoma cell lines.

(a, b) Time course of SMAD1/5/9 phosphorylation in CD109 knockdown and control cells after BMP-2 (50 ng/mL) stimulation. Representative images of immunoblot analysis in MG-63 cells (a) and SaOS-2 cells (b). (c, d) Relative densitometric intensities of immunoblot bands for SMAD1/5/9 phosphorylation (n = 3 per group). Error bars indicate standard deviation. Control siRNA; siCD109, siRNA targeting CD109; N.S., not significant.

Fig. 4 CD109 expression correlates negatively with SMAD phosphorylation in human osteosarcoma tissue.

(a) Images of H&E staining and immunohistochemical staining for CD109 and phospho-SMAD1/5/9 in two representative cases of human osteosarcoma. The images show the same area in the serial section in each case. The total score of phospho-SMAD1/5/9 was calculated in the same way as for CD109. The

cases showed a negative correlation between CD109 expression and SMAD phosphorylation. Arrowheads indicate non-tumor cells including macrophages. (b) Total scores of CD109 and phospho-SMAD1/5/9 staining were calculated in 10 randomly selected sequential human osteosarcoma cases. The total score of each case was plotted on a graph. One-tailed Spearman's correlation analysis revealed the significant negative correlation between CD109 expression and SMAD phosphorylation in osteosarcoma ($r_s = -0.63$, $P = 0.0254$). H&E, hematoxylin and eosin; pSMAD1/5/9, phospho-SMAD1/5/9.

Fig. 5 CD109 promotes cell migration but not cell proliferation of human osteosarcoma cells in the presence of BMP-2.

(a) Cell proliferation of MG-63 cells, measured by WST-1 assay (n = 3 per group). There were no significant differences in the relative proliferation between CD109-knockdown and control cells. siRNA#1 (left panel) or #2 (right panel) targeting CD109 was used. (b) *In vitro* wound healing assay using CD109-knockdown and control MG-63 cells. Representative images taken at 12 h after wound creation are shown. Dotted lines indicate the edge of the wound area. (c) Percentage of the unfilled wound area at each time point (6, 12 and 24 h after wound creation) was calculated as described in the Materials and Methods section (n = 3 per group). siRNA#1 (left panel) or #2 (right panel) targeting CD109 was used. All experiments were performed in the presence of BMP-2 (50 ng/mL). Error bars

1 indicate standard deviation. siControl, Control siRNA; siCD109, siRNA targeting CD109; N.S., not

2 significant.

Table 1 Clinicopathological analysis of patients with osteosarcoma

(a) Clinicopathological characteristics of osteosarcoma patients

Characteristics		Total	CD109-high n (%)	CD109-low n (%)	Fisher's exact test *P*-value
Number		55	31 (56.4)	24 (43.6)	
Age (years)	< 30	45	23 (41.8)	22 (40.0)	0.159
	≥ 30	10	8 (14.5)	2 (3.6)	
Sex	Female	23	10 (18.2)	13 (23.6)	0.168
	Male	32	21 (38.2)	11 (20.0)	
Stage (Enneking [22, 23])	IIa	9	5 (9.1)	4 (7.3)	0.999
	IIb	43	24 (43.6)	19 (34.5)	
	III	3	2 (3.6)	1 (1.8)	
Surgery with chemotherapy†		53	30 (54.5)	23 (41.8)	0.999
Surgery only		2	1 (1.8)	1 (1.8)	
Tumor site	Femur	25	15 (27.3)	10 (18.2)	0.854
	Tibia	21	11 (20.0)	10 (18.2)	
	Fibula	3	1 (1.8)	2 (3.6)	
	Humerus	3	2 (3.6)	1 (1.8)	
	Others	3	2 (3.6)	1 (1.8)	

†Chemotherapy: neoadjuvant and adjuvant chemotherapy.

(b) Multivariate analysis of prognostic parameters

	P	RR	95%CI
CD109 (high)	0.043	10.5	1.08–102.0
Age, years (≥ 30)	0.390	2.14	0.378–12.1
Sex (male)	0.136	0.288	0.056–1.48
Stage (≥ IIb)	0.795	1.37	0.130–14.4

Abbreviations: RR, relative risk; CI, confidence interval.

Figure. 1

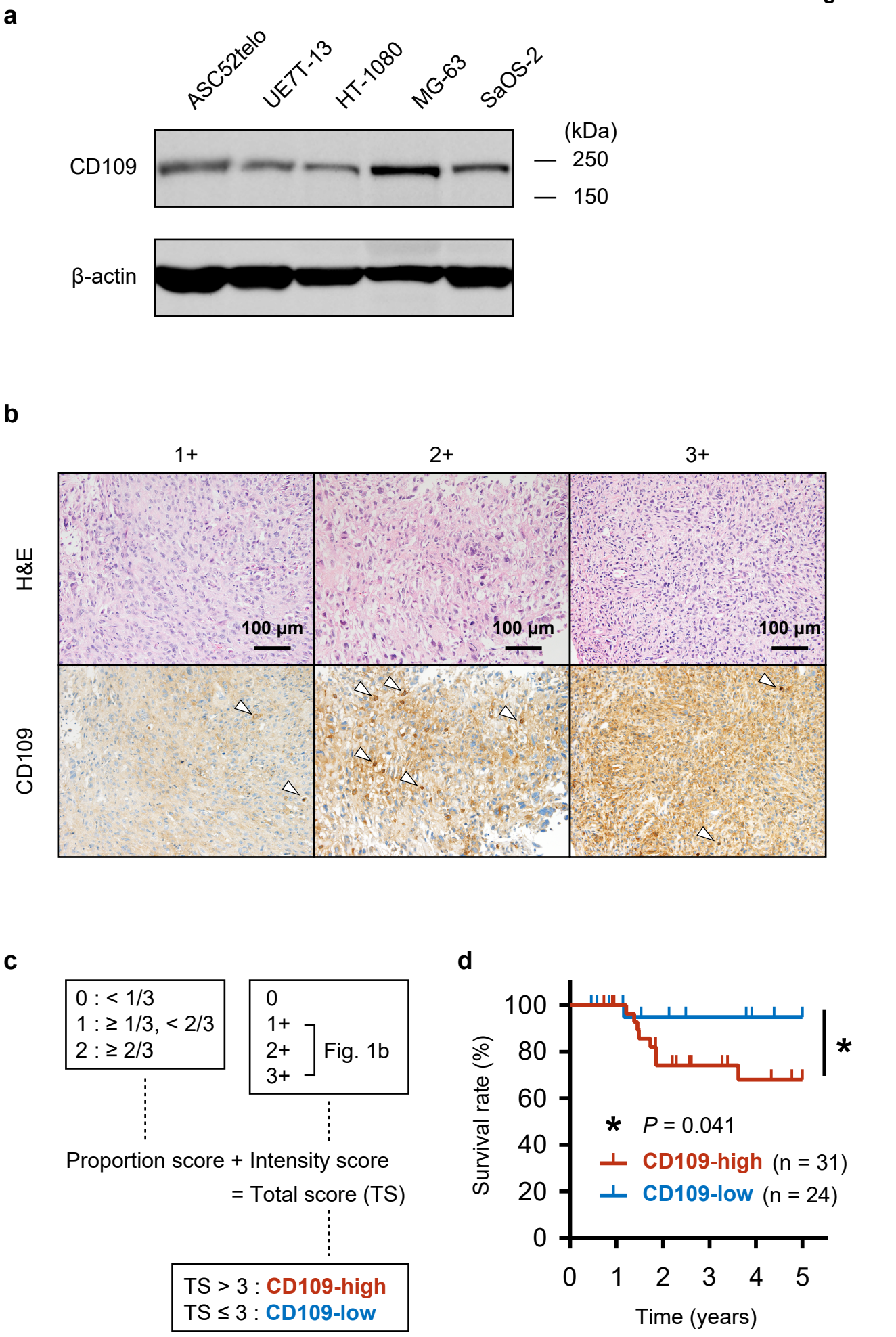

a
ASC52telo
UE7T-13
HT-1080
MG-63
SaOS-2
CD109
β-actin
(kDa)
250
150
b
1+
2+
3+
H&E
CD109
100 μm
100 μm
100 μm
c
0 : < 1/3
1 : ≥ 1/3, < 2/3
2 : ≥ 2/3
0
1+
2+
3+
Fig. 1b
Proportion score + Intensity score
= Total score (TS)
TS > 3 : CD109-high
TS ≤ 3 : CD109-low
d
Survival rate (%)
100
80
60
40
20
0
0
1
2
3
4
5
Time (years)
*
* P = 0.041
CD109-high (n = 31)
CD109-low (n = 24)

Figure. 2

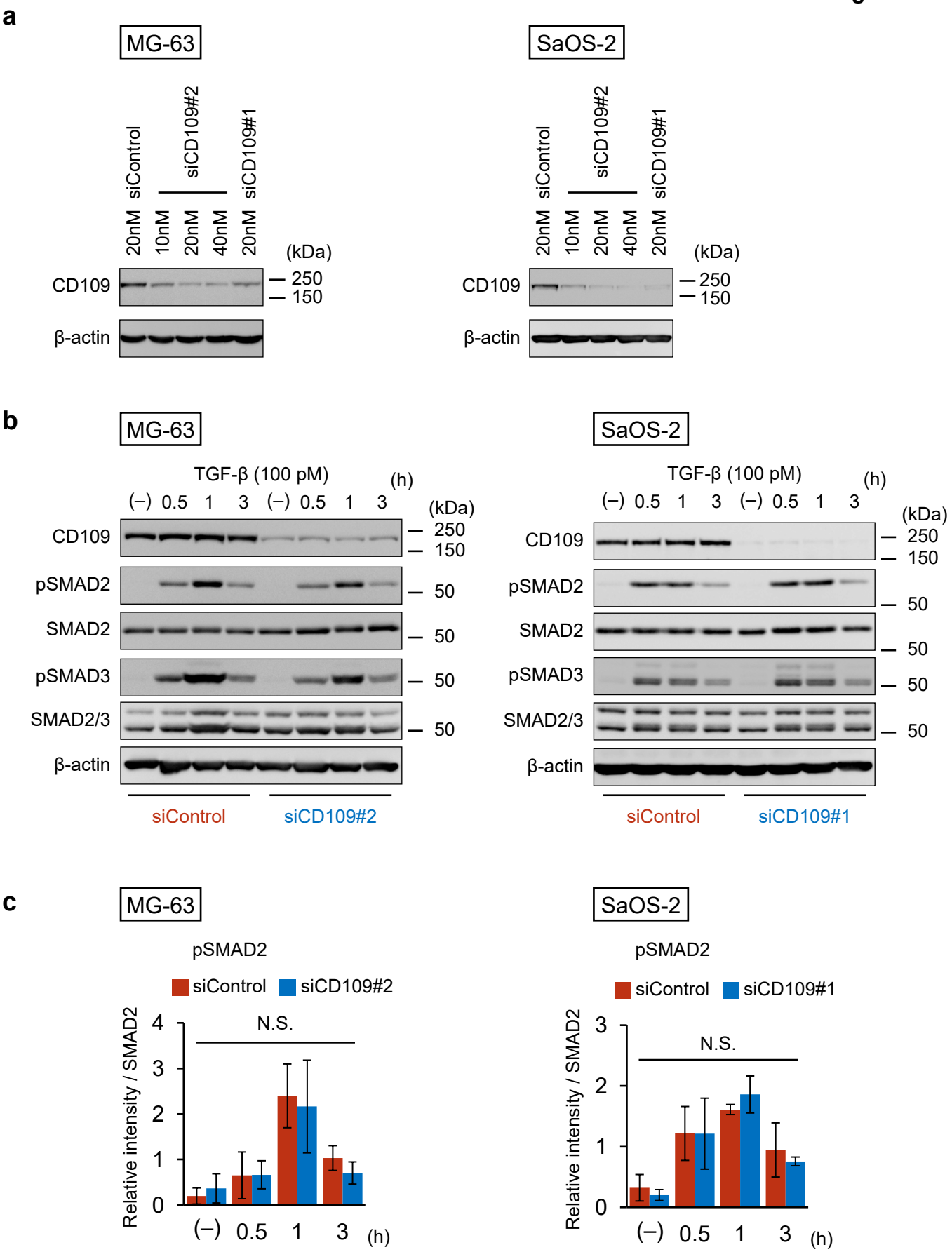

a
MG-63
SaOS-2
siControl
siCD109#2
siCD109#1
20nM
10nM
20nM
40nM
20nM
(kDa)
CD109
β-actin
250
150
b
MG-63
SaOS-2
TGF-β (100 pM)
(h)
(–) 0.5 1 3 (–) 0.5 1 3
(kDa)
CD109
pSMAD2
SMAD2
pSMAD3
SMAD2/3
β-actin
250
150
50
siControl
siCD109#2
siCD109#1
c
MG-63
SaOS-2
pSMAD2
siControl
siCD109#2
siCD109#1
N.S.
Relative intensity / SMAD2
0
1
2
3
4
(–) 0.5 1 3 (h)

Figure. 3

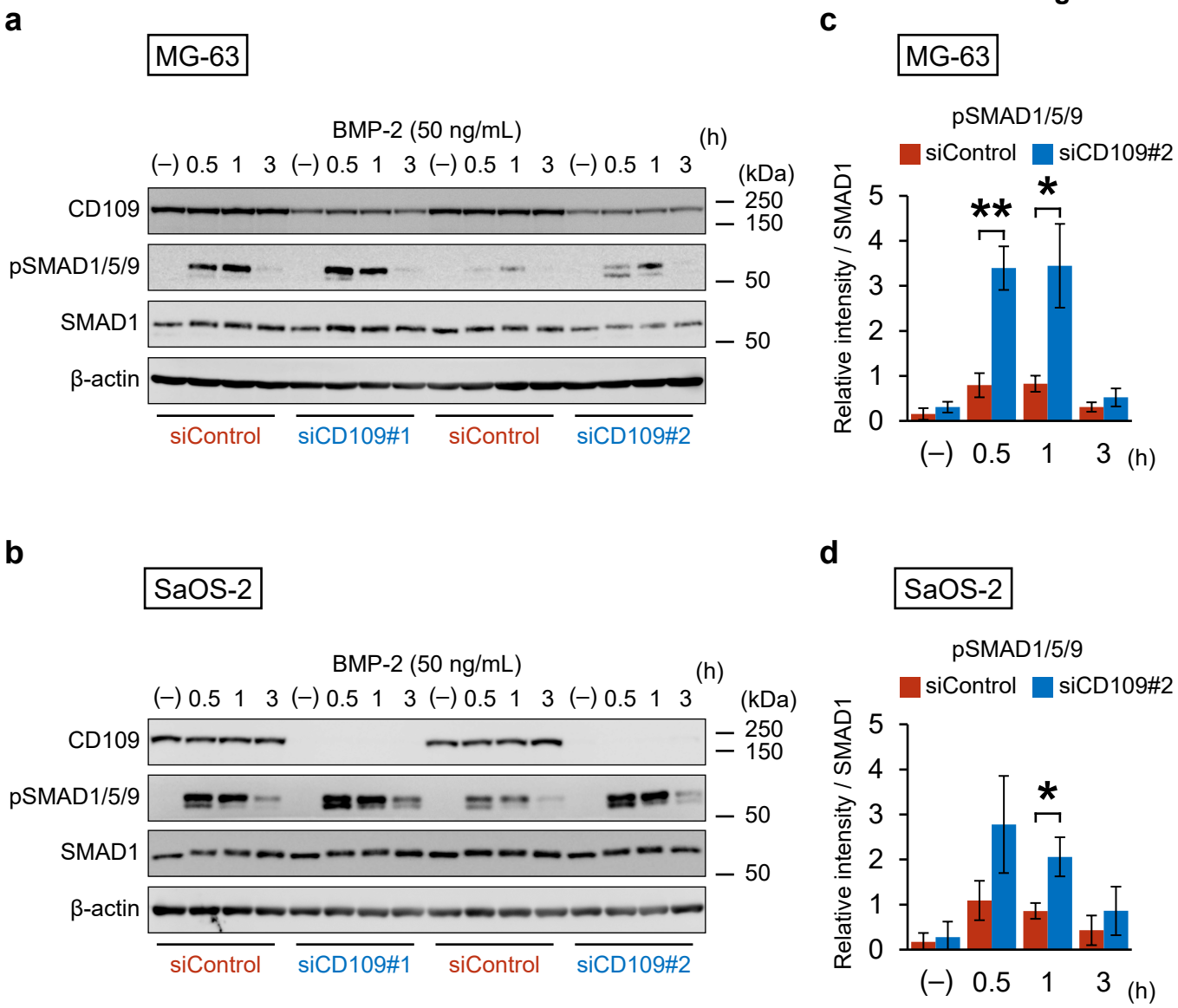
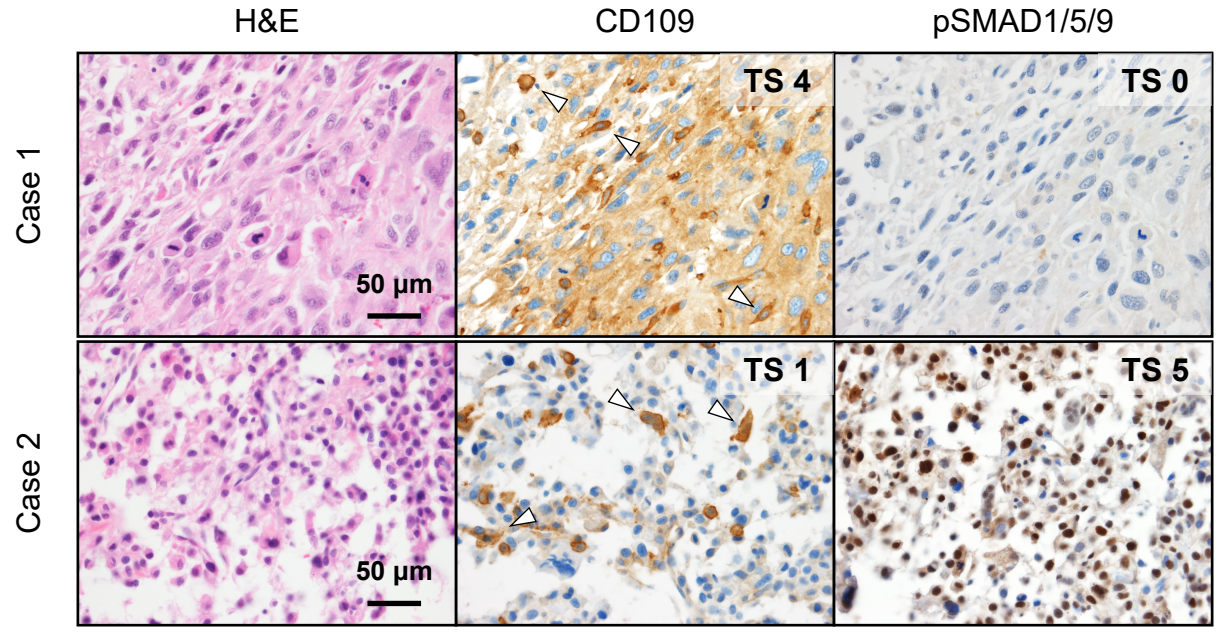

a
MG-63
BMP-2 (50 ng/mL)
(h)
(–) 0.5 1 3 (–) 0.5 1 3 (–) 0.5 1 3 (–) 0.5 1 3
(kDa)
CD109
pSMAD1/5/9
SMAD1
β-actin
250
150
50
50
siControl
siCD109#1
siControl
siCD109#2
c
MG-63
pSMAD1/5/9
siControl
siCD109#2
Relative intensity / SMAD1
**
*
(–) 0.5 1 3 (h)
b
SaOS-2
BMP-2 (50 ng/mL)
(h)
(–) 0.5 1 3 (–) 0.5 1 3 (–) 0.5 1 3 (–) 0.5 1 3
(kDa)
CD109
pSMAD1/5/9
SMAD1
β-actin
250
150
50
50
siControl
siCD109#1
siControl
siCD109#2
d
SaOS-2
pSMAD1/5/9
siControl
siCD109#2
Relative intensity / SMAD1
*
(–) 0.5 1 3 (h)

Figure. 4

a


H&E
CD109
pSMAD1/5/9
Case 1
Case 2
TS 4
TS 0
TS 1
TS 5
50 μm
50 μm

b

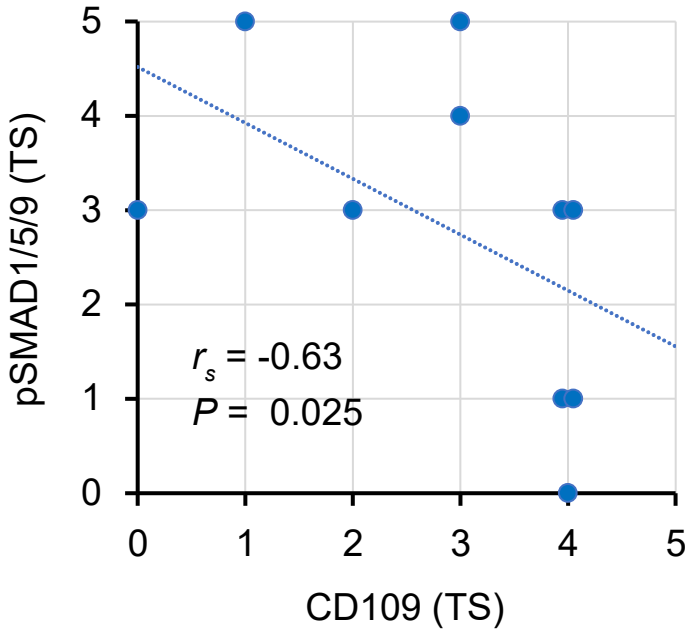
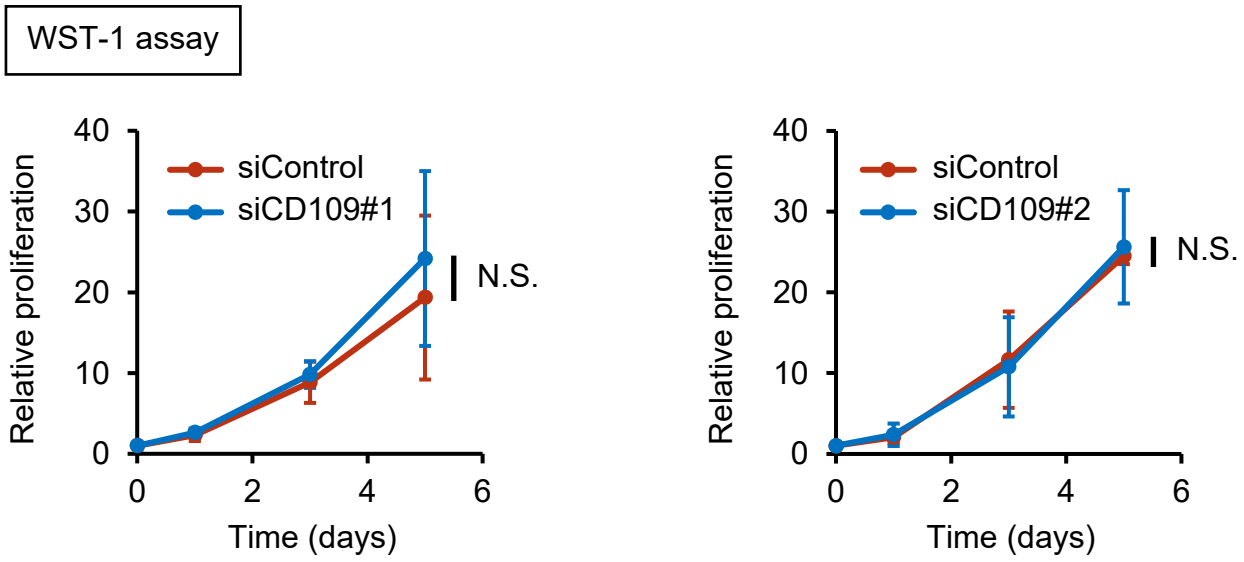
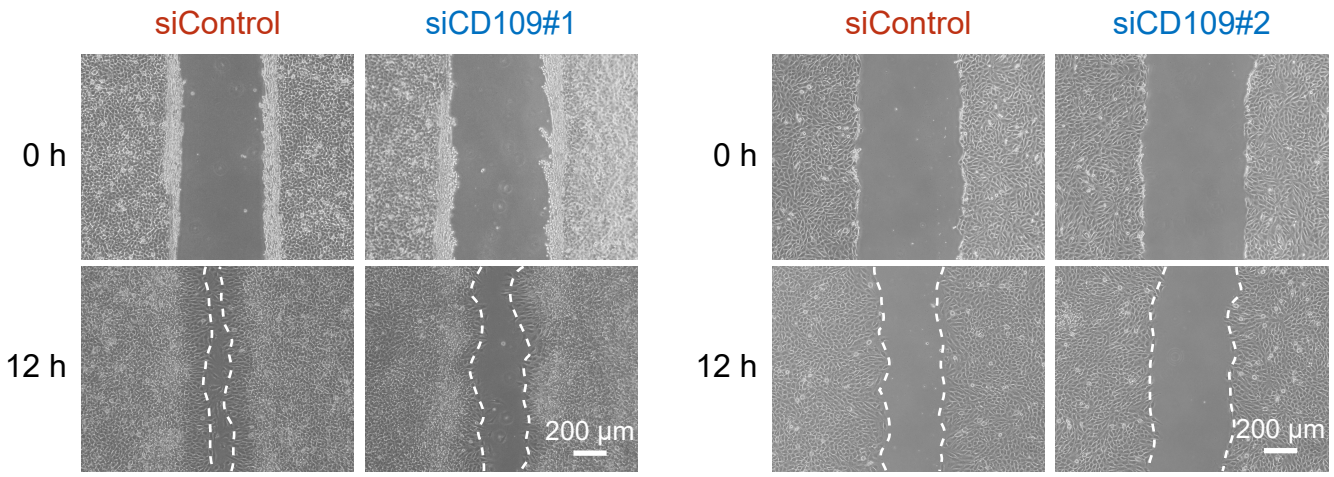

pSMAD1/5/9 (TS)
CD109 (TS)
r_s = -0.63
P = 0.025
0
1
2
3
4
5

Figure. 5

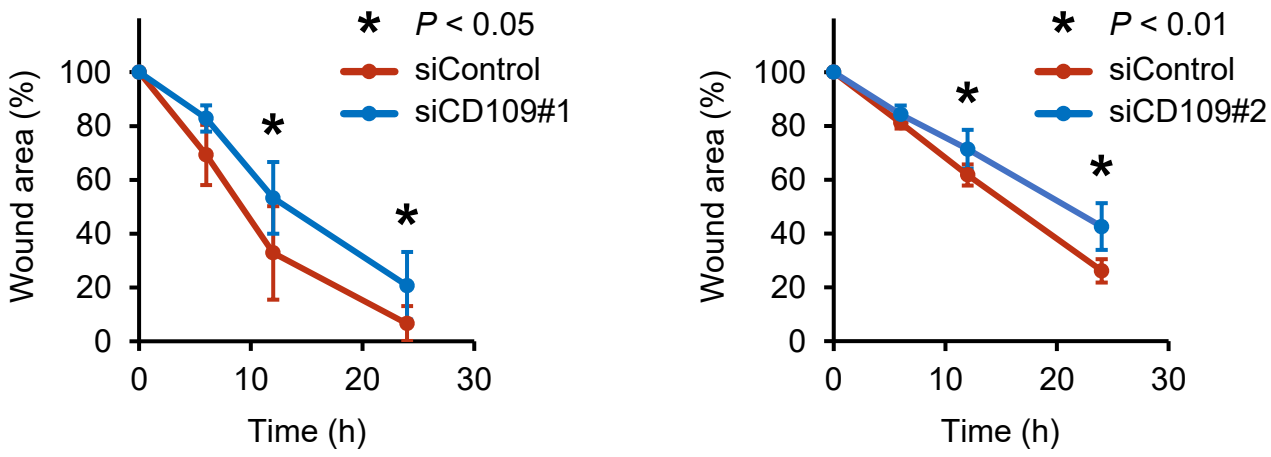
a


WST-1 assay
Relative proliferation
40
30
20
10
0
siControl
siCD109#1
N.S.
0
2
4
6
Time (days)
Relative proliferation
40
30
20
10
0
siControl
siCD109#2
N.S.
0
2
4
6
Time (days)

b


siControl
siCD109#1
0 h
12 h
200 μm
siControl
siCD109#2
0 h
12 h
200 μm

c


Wound area (%)
100
80
60
40
20
0
*
P < 0.05
siControl
siCD109#1
*
*
0
10
20
30
Time (h)
Wound area (%)
100
80
60
40
20
0
*
P < 0.01
siControl
siCD109#2
*
*
0
10
20
30
Time (h)