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# Crucial contribution of GPR56/ADGRG1, expressed by breast cancer cells, to bone metastasis formation

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## Abstract

From a mouse triple-negative breast cancer cell line, 4T1, we previously established 4T1.3 clone with a high capacity to metastasize to bone after its orthotopic injection into mammary fat pad of immunocompetent mice. Subsequent analysis demonstrated that the interaction between cancer cells and fibroblasts in a bone cavity was crucial for bone metastasis focus formation arising from orthotopic injection of 4T1.3 cells. Here, we demonstrated that a member of the adhesion G-protein–coupled receptor (ADGR) family, G-protein–coupled receptor 56 (GPR56)/adhesion G-protein–coupled receptor G1 (ADGRG1), was expressed selectively in 4T1.3 grown in a bone cavity but not under in vitro conditions. Moreover, fibroblasts present in bone metastasis sites expressed type III collagen, a ligand for GPR56/ADGRG1. Consistently, GPR56/ADGRG1 proteins were detected in tumor cells in bone metastasis foci of human breast cancer patients. Deletion of GPR56/ADGRG1 from 4T1.3 cells reduced markedly intraosseous tumor formation upon their intraosseous injection. Conversely, intraosseous injection of GPR56/ADGRG1-transduced 4T1, TS/A (mouse breast cancer cell line), or MDA-MB-231 (human breast cancer cell line) exhibited enhanced intraosseous tumor formation. Furthermore, we proved that the cleavage at the extracellular region was indispensable for GPR56/ADGRG1-induced increase in breast cancer cell growth upon its intraosseous injection. Finally, inducible suppression of *Gpr56/Adgrg1* gene expression in 4T1.3 cells attenuated bone metastasis formation with few effects on primary tumor formation in the spontaneous breast cancer bone metastasis model. Altogether, GPR56/ADGRG1 can be a novel target molecule to develop a strategy to prevent and/or treat breast cancer metastasis to bone.

**KEYWORDS**

bone metastasis, fibroblast, GPR56/ADGRG1, mouse, triple-negative breast cancer

## 1 | INTRODUCTION

More than half of patients with breast cancer at an advanced stage are complicated by bone metastasis, which can cause fractures, bone pain, and altered calcium homeostasis, thereby severely impairing the patients' life expectancy and quality of life.[1] Bone metastasis proceeds through multiple steps: cancer cell colonization in bone marrow, its adaptation to bone marrow environment, and its growth therein.[2] During these steps, cancer cells deregulate the bone remodeling process, an indispensable process to maintain bone homeostasis, which proceeds by cooperative actions of osteoclasts and osteoblasts.[3] Osteoblasts regulate bone matrix formation and mineralization and control osteoclast formation and activity by providing receptor activator of nuclear factor-κB ligand (RANKL). Cancer cells in bone marrow acquire properties mimicking osteoblasts and eventually induce an exaggerated formation and activity of osteoclasts, thereby promoting cancer cell growth.[4] However, several lines of evidence suggest that bone metastasis can also advance under the influence of other types of cells present in bone marrow, including hematopoietic stem cells, immune cells, endothelial cells, nerves, and fibroblasts.[5]

We previously established a mouse triple-negative breast cancer (TNBC) cell line (4T1.0)-derived clone, 4T1.3, which can metastasize with a high incidence to bone upon its orthotopic injection into mammary fat pad (MFP) in immune-competent mice.[6] Subsequent analysis revealed that a higher capacity of 4T1.3 clone to metastasize to bone was ascribed neither to its accelerated growth at the primary sites nor to its enhanced migration to the bone but arose from its higher ability to grow in the bone marrow. We further unraveled that 4T1.3 clone exhibited constitutively enhanced expression of a chemokine, CCL4, and that intraosseous injection of 4T1.3 increased type I collagen–positive fibroblasts which expressed CCR5, a specific receptor for CCL4, but neither osteoclasts nor osteoblasts in the bone cavity.[6] Moreover, we demonstrated that CCR5 blockade inhibited fibroblast accumulation and reduced tumor formation in bone arising from intraosseous injection of 4T1.3 cells, suggesting that intraosseous tumor formation crucially depends on the interaction between cancer cells and fibroblasts.

We assumed that fibroblasts accumulated in the bone should provide a growth factor for cancer cells expressing a corresponding receptor. With this hypothesis in mind, we searched for a receptor whose gene expression was enhanced in 4T1.3 cells grown in a bone cavity, compared with 4T1.3 cells under in vitro culture conditions or parental 4T1.0 cells grown in a bone cavity, as well as a growth factor whose gene expression was increased in intraosseous fibroblasts of 4T1.3-bearing mice, compared with those of 4T1.0-bearing mice. We uncovered that a member of the adhesion G-protein–coupled receptor (ADGR) family, G-protein–coupled receptor 56 (GPR56)/adhesion G-protein–coupled receptor G1 (ADGRG1), and its ligand, type III collagen were expressed in 4T1.3 cells in a bone cavity and intraosseous fibroblasts of 4T1.3-bearing mice, respectively. Inspired by controversies on the pathological relevance of GPR56/ADGRG1 and its activation mechanism in carcinogenesis, we extensively investigated breast cancer bone metastasis processes, focusing on the type III collagen–GPR56/ADGRG1 axis. We proved the crucial involvement of the type III collagen–GPR56/ADGRG1 axis and GPR56/ADGRG1 autoproteolysis in breast cancer cell growth in a bone cavity. Thus, GPR56/ADGRG1 can be a novel target to prevent and/or treat bone metastasis.

## 2 | MATERIALS AND METHODS

Eight-week-old specific pathogen–free BALB/c mice and SCID Beige mice were obtained from Charles River Laboratories and were kept under specific pathogen–free conditions. All the animal experiments in this study complied with the Guideline for the Care and Use of Laboratory Animals of Kanazawa University with the approval of the Committee on Animal Experimentation of Kanazawa University. Other detailed experimental procedures were described in Supplementary Methods.

## 3 | RESULTS

### 3.1 | Identification of an adhesion G-protein–coupled receptor, GPR56/ADGRG1, in breast cancer metastasis to bone

To identify the molecules involved crucially in the interaction between cancer cells and the fibroblasts accumulated in the bone marrow, we selected receptor genes that were expressed more abundantly in tumor cells in the 4T1.3-injected bone marrow cavity, compared with those in the 4T1.0-injected bone cavity or in vitro–cultured tumor cells (Figure 1A). We concomitantly purified a fibroblast-enriched fraction from bone metastasis sites, which was not contaminated with endothelial cells, hematopoietic cells, and immune cells as previously described[6] and did not contain adipocytes as evidenced by undetected adipose cell–specific gene expression (Figure S1). We then examined the gene expression of cognate ligands in this obtained fibroblast-enriched fraction. We finally identified five receptor genes, which were selectively expressed in 4T1.3 cells at tumor foci and whose ligands were expressed more abundantly in the fibroblast-enriched fraction at the 4T1.3-injected bone cavity, compared with those at the 4T1.0-injected bone cavity: epidermal growth factor receptor (EGF-R) and EGF, GPR56/ADGRG1 and type III collagen, type 1 insulin-like growth factor receptor (IGF-1R) and IGF, platelet-derived growth factor receptor (PDGF-R) α, PDGF-Rβ, and PDGF (Figure 1A and Figure S2). Among these receptor-ligand pairs, EGF-R,[7] IGF-1R,[8] and PDGF-R[9,10] were previously reported to be involved in bone metastasis, suggesting the authenticity of the analysis. Flow cytometric analysis detected the expression of these five receptors on 4T1.3 at tumor sites in the bone cavity but not under in vitro culture conditions (Figure 1B). Deletion of *Gpr56/Adgrg1*, but not that of other receptor genes, significantly reduced tumor formation upon intraosseous injection (Figure 1C and Figure S3). In order to validate the reproducibility from another mouse TNBC cell line, TS/A, we additionally obtained TS/A.3 clone, which exhibited no differences in the growth rates at

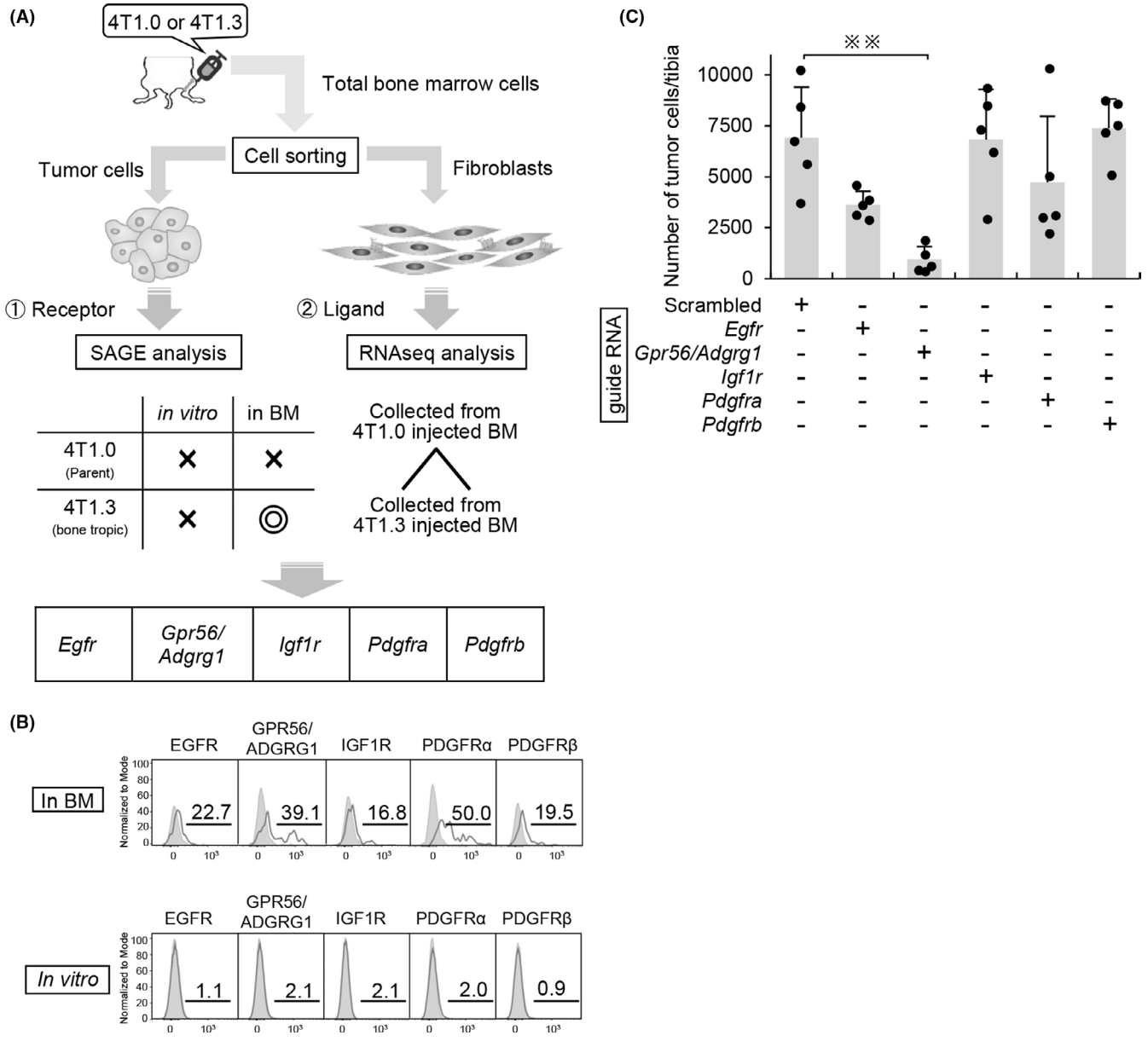


**FIGURE 1** Identification of GPR56/ADGRG1 as a receptor expressed by tumor cells at bone metastasis sites and its involvement in breast cancer metastasis to bone. A, Schematic representation of the bioinformatics analysis to search for candidate receptor genes. A total of five receptors were identified, which fulfilled the conditions. B, Flow cytometric analysis was conducted to determine the protein expression levels of five candidate receptors in 4T1.3 cells, which grew in bone marrow cavity or were cultured in vitro. Isotype controls are shown by the gray-filled histograms. Representative results are shown from three independent experiments. C, Effects of candidate receptor gene deletion on intraosseous tumor formation of the gene-modified 4T1.3 cells. Target gene–deleted 4T1.3 cells were injected into bone marrow cavity of tibiae. Symbols indicate the numbers of individual animals, while the histograms indicate the means and 1 SD of each group (n = 5). Statistical significance was calculated by Tukey-Kramer post hoc test. $^{※※}P < .01$

the primary sites but enhanced tumor formation upon intraosseous injection, compared with its parental clone[11,12] (Figure S4A). TS/A.3 exhibited enhanced GPR56/ADGRG1 expression at tumor foci arising from the intraosseous injection, but not under in vitro culture conditions (Figure S4B). Moreover, the deletion of the *Gpr56/Adgrg1* gene attenuated tumor formation upon intraosseous injection of TS/A.3 clone (Figure S4C). Thus, GPR56/ADGRG1 can provide these mouse breast cancer cell lines with a growth advantage in a bone cavity. Consistently, its ligand, *type III collagen (Col3a1)*, was expressed more copiously at mRNA levels in the fibroblast-enriched fraction of 4T1.3-injected mice compared with that of 4T1.0-injected mice (Figure S2).

### 3.2 | Effects of *Gpr56/Adgrg1* gene deletion on a spontaneous bone metastasis model

We next examined the effects of *Gpr56/Adgrg1* gene deletion on bone metastasis arising from an orthotopic injection of 4T1.3

clone into MFP (Figure 2A). Primary tumors efficiently grew when mice received an orthotopic injection of nontargeted CRISPR-Cas–treated control 4T1.3 clone, and subsequent removal of primary tumors caused bone metastasis development (Figure 2B–D and Figure S5). GPR56/ADGRG1-deleted 4T1.3 clone displayed primary tumor growth at similar rates as the control clone did (Figure 2B) but gave rise to little bone metastasis formation as evidenced by immunohistochemical and flow cytometric analyses (Figure 2C,D and Figure S5). Thus, GPR56/ADGRG1 can have roles in breast cancer growth at bone metastasis sites but not at primary sites.

### 3.3 | Enhanced tumor cell proliferation in bone cavity by the interaction of GPR56/ADGRG1 with its ligand, type III collagen

The lack of GPR56/ADGRG1 expression in 4T1.3 and TS/A.3 clone under in vitro culture conditions prompted us to establish 4T1.0, TS/A, or a human breast cancer cell line, MDA-MB-231, expressing GPR56/ADGRG1 constitutively, in order to elucidate the roles of GPR56/ADGRG1 in breast cancer biology. *Gpr56/Adgrg1* gene transduction enhanced its mRNA expression and protein expression in these breast cancer cell lines (Figure 3A,B, and Figure S6A,B). Intraosseous injection of GPR56/ADGRG1-expressing clones caused larger tumor formation compared with that of control clones (Figure 3C and Figure S6C). Moreover, GPR56/ADGRG1 expression increased Ki67-positive cell–proliferating tumor cell numbers with few effects on ssDNA-positive apoptotic cell numbers in metastatic foci (Figure S7). However, GPR56/ADGRG1-expressing clones did not exhibit a significant increase in in vitro proliferation rates (Figure 3D and Figure S6D). We presumed that the presence of a ligand(s) could enhance in vitro proliferation of GPR56/ADGRG1-expressing cells. Accumulating evidence indicates that GPR56/ADGRG1 can use two distinct molecules, type III collagen and tissue transglutaminase (TG2), as its ligands.[13,14] *Col3a1* expression was enhanced in bone marrow of 4T1.3- or TS/A.3-injected mice but not that of 4T1.0- or TS/A-injected mice, whereas *Tg2* gene (*Tgm2*) expression was barely detected in the bone marrow of mice injected with all clones (Figure 4A and Figure S8A). Consistently, type III collagen–expressing cells were detected more abundantly in the tumor foci arising from intraosseous injection of 4T1.3 or TS/A.3 compared with those of 4T1.0 and TS/A (Figure 4B and Figure S8B). These observations would indicate that type III collagen might be a ligand for GPR56/ADGRG1 expressed by breast cancer cells at bone metastasis sites to support tumor growth in bone microenvironment. Consistently, the presence of type III collagen enhanced in vitro cell proliferation of GPR56/ADGRG1-expressing 4T1.0 clone (Figure 4C). Enhanced cell proliferation can be ascribed to either cell cycle progression or decrease of cell death. Indeed, type III collagen promoted cell cycle progression (Figure 4D) and reduced apoptosis of GPR56/ADGRG1-expressing clone (Figure 4E). Similar observations were obtained on GPR56/ADGRG1-expressing TS/A and MDA-MB-231 clones (Figures S9 and S10). To elucidate further the effects of the type III collagen–GPR56/ADGRG1 axis on breast

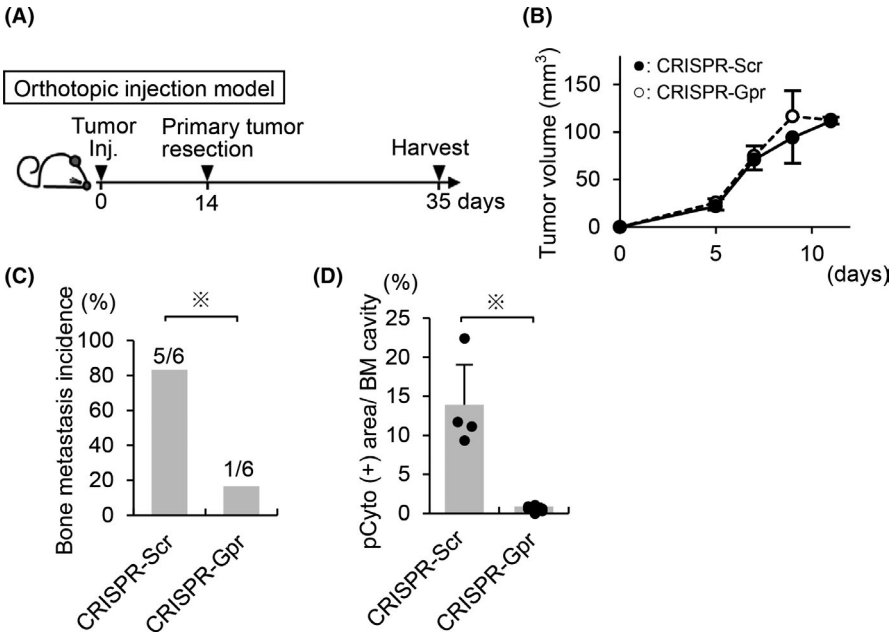


**FIGURE 2** Effects of constitutive suppression of GPR56/ADGRG1 expression on spontaneous breast cancer bone metastasis model. A, Schematic representation of experimental procedures. B, Tumor growth rates at the primary tumor sites. Nontargeted scrambled control (CRISPR-Scr) or *Gpr56/Adgrg1*-deleted (CRISPR-Gpr) 4T1.3 cells were injected into mammary fat pad (MFP) of mice. All values represent mean + SD (n = 5). C and D, The incidence of bone metastasis focus formation (C; n = 6) and the ratios of pan-Cytokeratin (pCyto)-positive to bone marrow (BM) whole areas (D) were calculated. Symbols indicate the numbers of individual animals, while the histograms indicate the means and 1 SD of each group (n = 4 to 5). Representative results are shown from two independent experiments. Statistical significance was calculated by Fisher's exact test (C) or Mann-Whitney's U test (D). ※$P < .05$

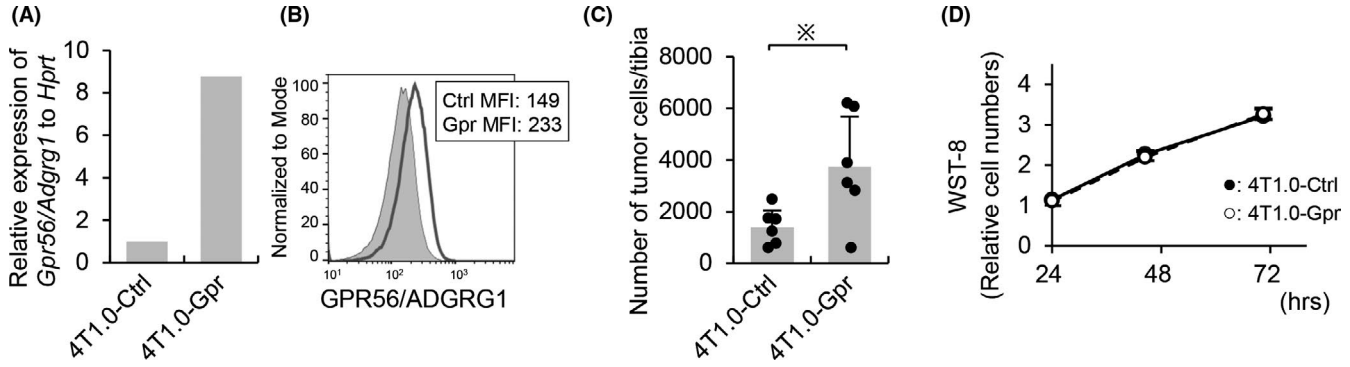


**FIGURE 3** Effects of *Gpr56/Adgrg1* gene transduction on intraosseous tumor formation and in vitro cell proliferation. A, *Gpr56/Adgrg1* mRNA expression in control (Ctrl) or *Gpr56/Adgrg1* (Gpr)-transduced 4T1 cells. Representative results from three independent experiments are shown. B, GPR56/ADGRG1 protein expression in control or *Gpr56/Adgrg1*-transduced 4T1 cells. Control cells and *Gpr56/Adgrg1*-transduced cells are shown by the gray-filled histograms and lines, respectively. The mean fluorescence intensity (MFI) of each population is indicated. Representative results from three independent experiments are shown. C, Intraosseous tumor formation arising from intraosseous injection of control or *Gpr56/Adgrg1*-transduced 4T1 cells. Symbols indicate the numbers of individual animals, while the histograms indicate the means and 1 SD of each group (n = 6). Representative results are shown from two independent experiments. Statistical significance was calculated by Mann-Whitney's U test. ※$P < .05$. D, In vitro cell proliferation of control or *Gpr56/Adgrg1*-transduced 4T1 cells. The means and SD values were calculated and are shown. All values represent mean + SD (n = 6)

cancer cells, we conducted RNAseq analysis on GPR56/ADGRG1-expressing 4T1.0 and TS/A clones, which were cultured in the presence or absence of type III collagen. According to the classification of process category,[15] the gene sets associated with cell proliferation were enriched in GPR56/ADGRG1-expressing 4T1.0 and TS/A clones, compared with a control clone in the presence of type III collagen (Figure S11). Altogether, GPR56/ADGRG1 can promote bone metastasis by enhancing cell proliferation and suppressing the apoptosis of tumor cells in a bone cavity through the interaction with its cognate ligand, type III collagen, present in bone metastasis sites.

## 3.4 | Role of cleavage of GPR56/ADGRG1 in its signal pathway

A G protein–coupled receptor (GPCR) autoproteolysis-inducing (GAIN) domain exists between a long extracellular domain with adhesive functions and the trimeric G protein–coupled 7 transmembrane domain of ADGRs.[16] The cleavage at this site was assumed to expose their tethered peptide agonist (termed the stachel sequence), thereby delivering intracellular signals.[17] Controversies on the roles of the autoproteolysis[18-20] prompted us to determine the role of GAIN domain–mediated autoproteolysis in GPR56/ADGRG1 signaling in bone metastasis. We prepared two distinct vectors which can drive the expression of murine cleavage-insensitive mutants, T383A and F385A, by introducing a single-point amino acid mutation in the domain[21,22] (Figure S12). The resultant mutant proteins are presumed to lack a capacity of GAIN-mediated autoproteolysis and subsequent stachel exposure. When these mutant- and wild-type GPR56/ADGRG1-expressing vectors were transfected into 4T1.0 clones, all transfectants expressed GPR56/ADGRG1 proteins at similar levels, as revealed by flow cytometric analysis (Figure 5A). Western blotting analysis demonstrated that wild-type clone ($Gpr56^{WT}$) exhibited two bands, each with a molecular weight of 75 and 25 kDa, representing uncleaved full-length and cleaved forms of GPR56/ADGRG1 proteins, respectively (Figure 5B). On the contrary, under the same conditions, mutant clones ($Gpr56^{T383A}$ or $Gpr56^{F385A}$) exhibited only a band with a molecular weight of 75 kDa (Figure 5B), suggesting that mutant GPR56/ADGRG1 proteins cannot be cleaved. Moreover, in contrast to wild-type GPR56/ADGRG1-expressing clones, type III collagen failed to induce enhancement of cell proliferation in mutant GPR56/ADGRG1-expressing clones (Figure 5C). Furthermore, intraosseous injection of mutant GPR56/ADGRG1-expressing clones gave rise to reduced tumor formation, compared with that of wild-type GPR56/ADGRG1-expressing clones (Figure 5D). On the contrary, there was no significant difference in type III collagen–expressing cell numbers at tumor sites among these 4T1.0-derived clones (Figure S13). Thus, GAIN domain cleavage can be indispensable for type III collagen–mediated activation of GPR56/ADGRG1 signaling and subsequent tumor cell growth in a bone cavity.

## 3.5 | Clinical relevance of GPR56/ADGRG1 expression in breast cancer metastasis to bone

In order to prove the clinical relevance of GPR56/ADGRG1 expression in breast cancer bone metastasis, we examined its expression on bone metastasis sites of human breast cancer patients. Immunohistochemical analysis detected GPR56/ADGRG1 proteins in three out of six bone metastasis foci of human breast cancer patients (Figure 6A). These observations were further strengthened by the study on additional human breast cancer bone metastasis samples, which detected GPR56/ADGRG1 and type III collagen proteins irrespective of breast cancer subtype (Figure S14). From the PrognoScan database, we next selected the data, consisting of

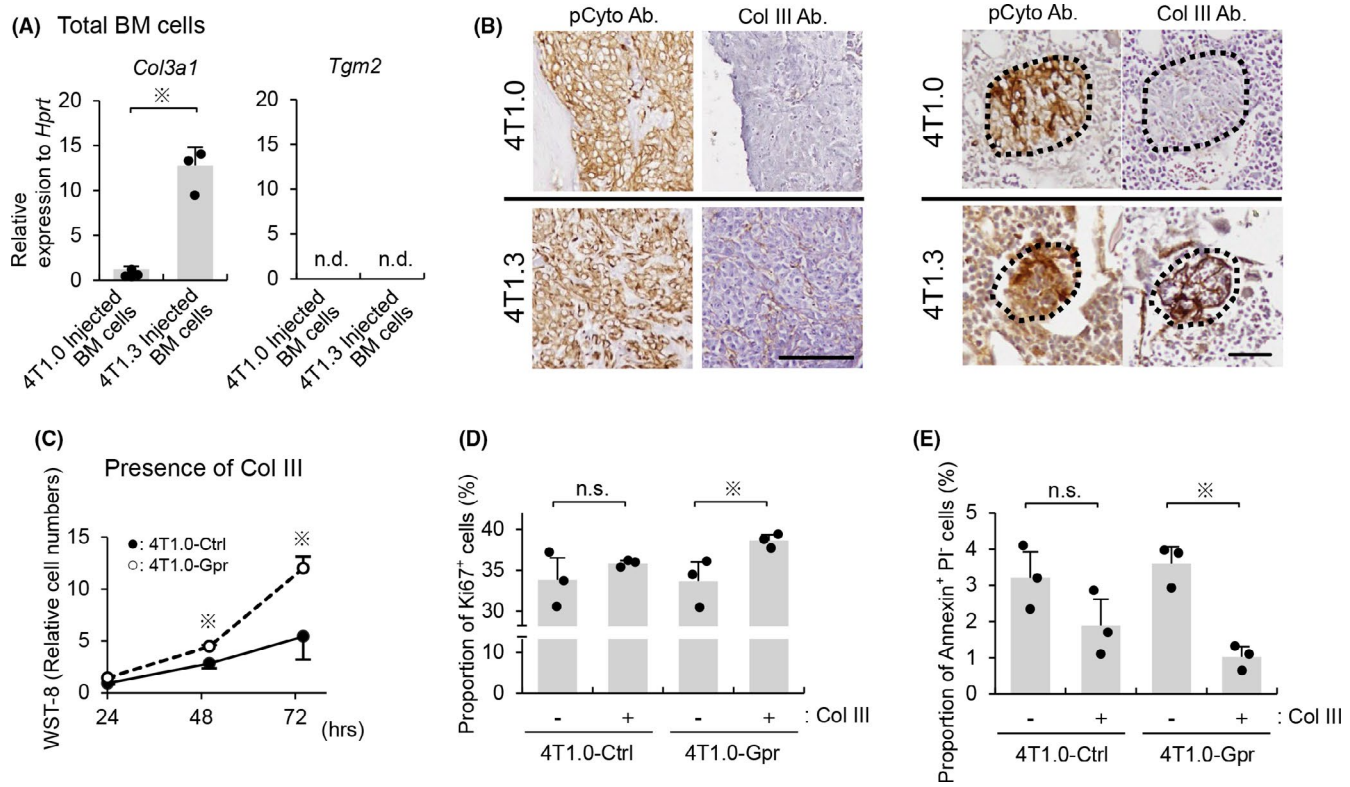


**FIGURE 4** Effects of type III collagen on in vitro cell proliferation of *Gpr56/Adgrg1*-transduced 4T1.0 cells. A, *Type III collagen* (*Col3a1*) or *Tg2* (*Tgm2*) mRNA expression in bone marrow (BM) after intraosseous injection of 4T1.0 or 4T1.3 cells. All values represent mean + SD (n = 3). Representative results are shown from two independent experiments. Statistical significance was calculated by Mann-Whitney's U test. ※$P < .05$; n.d., not detected. B, Immunohistochemical staining of tibial bones with pan-Cytokeratin (pCyto) and anti-type III collagen (Col III) antibodies. 4T1.3 cells were injected into the BM. Representative results from five independent animals are shown with a bar of 100 μm (left panels, ×100). Right panels indicate tumor focus with a higher magnification (×400). Bar = 40 μm. C, In vitro cell proliferation of control (Ctrl) or *Gpr56/Adgrg1*-transduced (Gpr) 4T1.0 cells in the presence of type III collagen. All values represent mean + SD (n = 6). Statistical significance was calculated by Mann-Whitney's U test. ※$P < .05$. D and E, Proportion of Ki67 highly expressing cells (D) and Annexin$^+$ PI$^-$ cells (E) of control (Ctrl) or *Gpr56/Adgrg1*-transduced (Gpr) 4T1.0 cells in the presence of type III collagen. Symbols indicate the numbers of individual animals, while the histograms indicate the means and 1 SD of each group (n = 3), respectively. Statistical significance was calculated by Mann-Whitney's U test. ※$P < .05$, n.s., not significant

large patient numbers (159 patients) and a diversity of breast cancer subtypes. The selected data indicated the association of a higher GPR56/ADGRG1 expression with shorter relapse-free survival, irrespective of breast cancer subtypes (Figure 6B). These observations would indicate the clinical relevance of GPR56/ADGRG1 expression in breast cancer progression. We finally evaluated the adjuvant therapeutic effects of inducible GPR56/ADGRG1 blockade by using a spontaneous bone metastasis model (Figure 6C). Doxycycline administration was initiated at the time of the primary tumor resection, 14 days after the injection into MFP, at the dose which could suppress GPR56/ADGRG1 protein expression in tumor sites arising from intraosseous injection of 4T1.3 (Figure S15). There were no significant differences in primary tumor weights between both groups at the time of primary tumor resection (Figure 6D), when micrometastasis is presumed to develop in bone.[6,23] Subsequent doxycycline treatment significantly reduced bone metastasis incidence and focus areas in mice injected with the gene-modified cells, together with reduction in Ki67-expressing proliferating tumor cell numbers in metastatic foci, compared with mice injected with control cells (Figure 6E,F, and Figure S16). Thus, GPR56/ADGRG1 deletion can also have adjuvant therapeutic effects on bone micrometastasis.

## 4 | DISCUSSION

Like metastasis to other organs,[24] bone metastasis proceeds through multiple steps consisting of growth at the primary site, invasion of the basement membrane, intravasation, migration through systemic blood or lymphatic circulation, extravasation, seeding to the bone cavity, and growth therein.[25] We previously revealed that a higher capacity of 4T1.3 clone to metastasize to bone was not based on its accelerated growth at the primary site or its enhanced migration to the bone but was ascribed to its superior ability to grow in the bone microenvironment.[6] Moreover, we further unraveled the involvement of fibroblasts in intraosseous growth of 4T1.3 clone.[6] Hence, we searched for the ligand-receptor pairs that can contribute to the intercellular communication between fibroblasts and cancer cells in the bone cavity. We consequently identified the type III

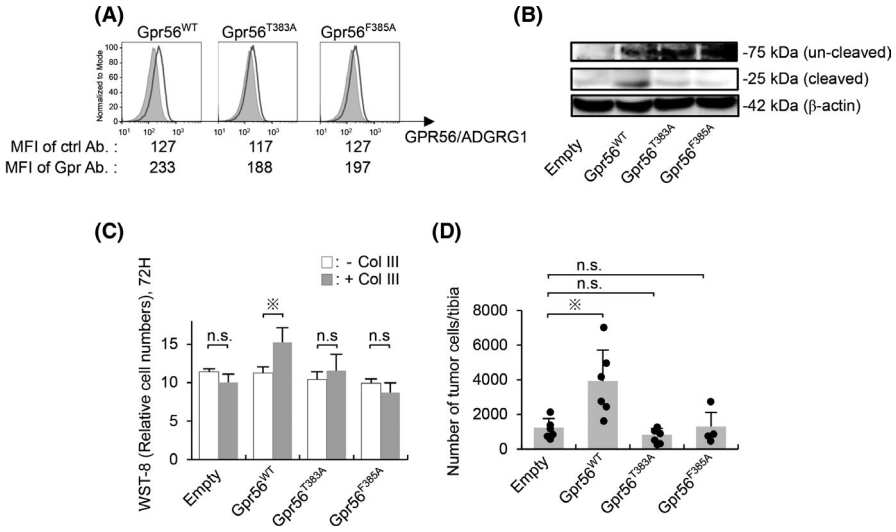


**FIGURE 5** Effects of mutations of GPR56/ADGRG1 on cell proliferation. A, GPR56/ADGRG1 protein expression in wild-type ($Gpr56^{WT}$) or mutated *Gpr56/Adgrg1*-transduced 4T1.0 clone ($Gpr56^{T383A}$, $Gpr56^{F385A}$). GPR56/ADGRG1 expression was analyzed using an antibody against the amino-terminal portion of GPR56/ADGRG1 with flow cytometry. GPR56/ADGRG1 expression and background intensities are shown by the lines and gray-filled histograms, respectively. Mean fluorescence intensity (MFI) of each population is indicated under panels. Representative experiments from three independent experiments are shown. B, Western blot of membrane fractions of cells expressing wild-type or mutated GPR56/ADGRG1 proteins. The resultant proteins were subjected to Western blotting to detect uncleaved and cleaved form of GPR56/ADGRG1 using antibody to the carboxyl-terminal portion of GPR56/ADGRG1. β-actin was used as a loading control. Empty vector–transduced 4T1.0 cells (Empty) were used as the control. Representative results from three independent experiments are shown. C, In vitro cell proliferation of wild-type or mutated *Gpr56/Adgrg1*-transduced 4T1.0 cells. The cells were cultured in the presence or absence of type III collagen, and cell proliferation was determined. All values represent mean + SD (n = 6). Statistical significance was calculated by Tukey-Kramer post hoc test. ※$P < .05$; n.s., not significant. D, Intraosseous tumor formation arising from intraosseous injection of wild-type or mutated *Gpr56/Adgrg1*-transduced breast cancer cell lines. The cells were injected into bone cavity, and the cell number in bone cavity was determined with flow cytometry. Symbols indicate the numbers of individual animals, while the histograms indicate the means and 1 SD of each group (n = 4 to 6). Representative results are shown from two independent experiments. Statistical significance was calculated by Tukey-Kramer post hoc test. ※$P < .05$; n.s., not significant

collagen–GPR56/ADGRG1 axis as the novel regulator of intraosseous growth of breast cancer cells.

GPR56/ADGRG1 is a member of the ADGR subfamily, which is the second-largest subfamily among GPCR families and consists of 33 members in mammals.[16] Although most ADGRs are orphan receptors with unidentified functions, some members are presumed to have roles in various physiological and pathological conditions, such as organogenesis, neurodevelopment, angiogenesis, and tumor progression.[17] ADGRs are characterized by the presence of a phylogenetically well-conserved GAIN domain, which links between the trimeric G protein–coupled 7 transmembrane domain and a long extracellular domain with adhesive functions.[16] The stachel structure is present between GAIN and transmembrane domains and is presumed to be responsible for intracellular signal transduction.[17] GPR56/ADGRG1 was originally identified as an essential regulator of human cerebral cortex development.[26] Subsequent studies revealed its diverse functions in various situations such as oligodendrocyte development,[27] muscle cell development,[28] cytotoxic T cell activation,[29] and maintenance of hematopoietic stem cells in bone marrow.[30] However, there are inconsistencies in terms of the roles of GPR56/ADGRG1 in carcinogenesis. Low GPR56/ADGRG1 expression signature predicted a better prognosis in patients with breast cancer or uterine corpus endometrial carcinoma[31] but indicated poor prognosis in patients with glioblastoma and lung squamous cell carcinoma.[31] GPR56/ADGRG1 promoted cell proliferation of breast, colon, lung, or pancreas cancer cells[32,33] but reduced that of melanoma cells.[13] Discrepant observations were also reported on the roles of GPR56/ADGRG1 in melanoma metastasis.[13,22,34] Here, we revealed that GPR56/ADGRG1 expression in breast cancer cells could promote breast cancer metastasis to bone. The observation can be consistent with the previous report[35] and the data deposited in PrognoScan.

Inconsistent roles of GPR56/ADGRG1 in carcinogenesis may be explained by distinct ligands present in tumor sites. GPR56/ADGRG1 utilizes two distinct ligands, TG2[13] and type III collagen.[14] TG2 can catalyze a calcium-dependent transamidation reaction to generate covalent crosslinks of glutamine residues with primary amino groups and is involved in various cellular processes, such as differentiation, adhesion, migration, cell death, and survival.[36] However, the interaction of GPR56/ADGRG1 with TG2 inhibited melanoma tumor growth and metastasis.[13] Consistent with this report, GPR56/ADGRG1 can internalize and degrade TG2 in melanoma cells upon binding, thereby decreasing extracellular matrix (ECM) protein deposition and attenuating focal adhesion kinase activities.[37] On the contrary, through the interaction with type III collagen, GPR56/ADGRG1 can exhibit protumorigenic functions:

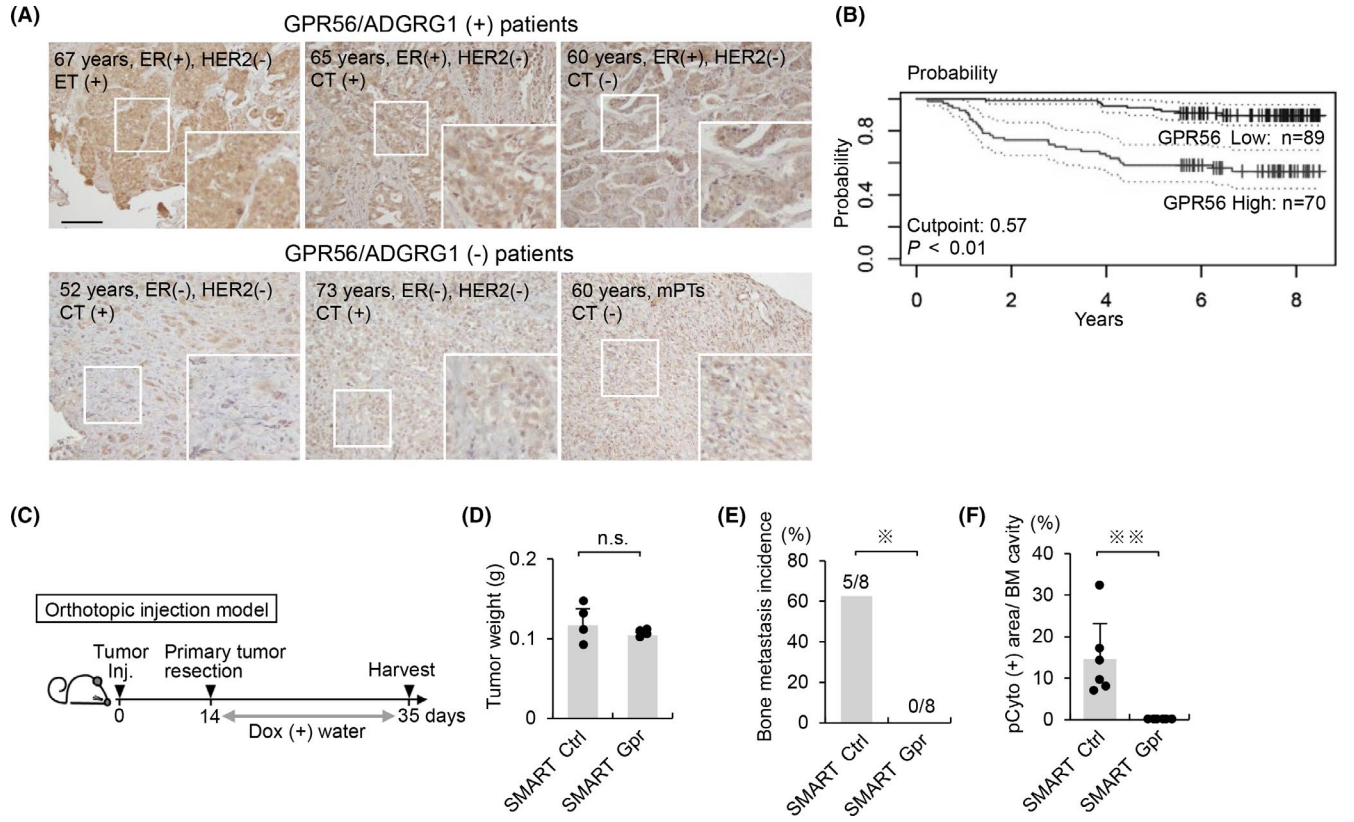


**FIGURE 6** Clinical relevance of GPR56/ADGRG1 expression in breast cancer progression. A, Immunohistochemical detection of GPR56/ADGRG1 protein in bone metastasis sites of human breast cancer patients. Results from six breast cancer patients are shown with a bar of 100 μm. B, Effects of GPR56/ADGRG1 expression on relapse-free survival among human breast cancer patients. Relapse-free survival (GSE1456-GPL96) of patients with breast cancer was analyzed based on GPR56/ADGRG1 expression by analyzing the PrognoScan database. C, Schematic representation of the experimental procedures. D, Tumor weight at the primary tumor site. Nontargeted shRNA SMART vector (SMART Ctrl)- or *Gpr56/Adgrg1* shRNA SMART vector (SMART Gpr)-transduced 4T1.3 cells were injected into mammary fat pad (MFP) of mice. Tumor weight was determined 14 days after the injection. Symbols indicate the numbers of individual animals, while the histograms indicate the means and 1 SD of each group (n = 4). Statistical significance was calculated by Mann-Whitney's U test. n.s., not significant. E and F, The incidence of metastasis focus formation (E; n = 8) and the ratios of pCyto-positive to bone marrow (BM) whole areas (F) were calculated 21 days after the initiation of doxycycline (n = 6). Symbols indicate the numbers of individual animals, while the histograms indicate the means and 1 SD of each group (n = 5 to 6). Representative results are shown from two independent experiments. Statistical significance was calculated by Fisher's exact test (E) or Mann-Whitney's U test (F). $^{※}P < .05$; $^{※※}P < .01$

activation of the RhoA pathway,[14,38,39] induction of IL-6 expression,[22] and prevention of apoptosis.[40] In the present bone metastasis model, we detected type III collagen but not *Tg2* gene expression at mRNA and protein levels in bone metastasis sites. Thus, type III collagen expression can contribute to enhanced intraosseous tumor growth.

Of interest is that the 4T1.3 clone expressed GPR56/ADGRG1 in a bone cavity but not under in vitro culture conditions. GPR56/ADGRG1 transcription may be regulated by additional transcription factors such as HOBIT[41] or heptad transcription factors including GATA2, RUNX1, and FLI1,[42] which were reported to be involved in *Gpr56/Adgrg1* gene transcription regulation in other types of cells. Alternatively, as an adherence junction transmembrane protein, vezatin, can directly induce GPR56/ADGRG1 expression in gastric cancer cells,[43] the communication with other resident cells may enhance GPR56/ADGRG1 expression in cancer cells in bone marrow.

Cancer cells seed, survive, and grow in bone marrow by interacting with various types of resident cells, including osteoclasts, osteoblasts, osteocytes, endothelial cells, hematopoietic cells.[25] Accumulating evidence indicates the crucial involvement of cancer-associated fibroblasts (CAFs) and fibrosis in cancer progression.[44,45] CAFs have diverse effects on carcinogenesis by providing cancer cells with various growth factors and immune modulators, and endothelial cells with angiogenic factors.[46] Consistently, we previously revealed that fibroblasts in a bone cavity provided cancer cells with connective tissue growth factors to promote cancer cell proliferation.[6] Moreover, fibroblasts contribute to fibrosis development by modulating the composition of ECM, which can serve as a niche for cancer cells to survive in bone marrow.[47] Indeed, we observed type III collagen expression at metastasis foci in bone marrow, which exhibits an indiscernible level of type III collagen mRNA expression under normal conditions.[48] Fibroblasts may exhibit enhanced type III collagen expression in

response to adenosine,[49] released from dying cancer cells present in bone marrow.[50] Nevertheless, abundantly expressing type III collagen interacted with GPR56/ADGRG1 on breast cancer cells to promote their growth in bone marrow. Our results may account for the association of a higher serum level of type III collagen degradation product with a poor prognosis in patients with metastatic breast cancer.[51]

Myelination in the central nervous system required removal of the GAIN domain and subsequent exposure of the stachel structure to deliver GPR56/ADGRG1 signaling.[52] A similar mechanism was responsible for GPR56/ADGRG1-mediated melanoma cell migration.[22] On the contrary, synthetic ligands can activate GPR56/ADGRG1-mediated signals without exposing the stachel structure, suggesting a dispensable role of its cleavage in signal transduction.[53] Thus, there remain controversies on the roles of the cleavage at the GAIN domain in GPR56/ADGRG1-mediated signal transduction. In order to address these controversies, we prepared two uncleavable mutant GPR56/ADGRG1-expressing vectors to establish 4T1.0-expressing uncleavable mutant GPR56/ADGRG1 proteins. Both mutant clones failed to exhibit enhanced in vitro cell proliferation and gave rise to less tumor formation upon intraosseous injection compared with wild-type GPR56/ADGRG1-expressing clone. Thus, GPR56/ADGRG1 cleavage at its GAIN domain can be indispensable for breast cancer growth in a bone cavity, irrespective of the presence of its ligand, type III collagen.

We provided the first definitive evidence to indicate the crucial involvement of GPR56/ADGRG1 activation in the intraosseous growth of breast cancer cells by using mouse models. This notion was further supported by our present clinical study on human breast cancer bone metastasis, the study that detected enhanced GPR56/ADGRG1 expression and type III collagen–expressing cell accumulation in bone metastasis sites regardless of breast cancer subtypes. Moreover, *Gpr56/Adgrg1* shRNA expression was induced to suppress GPR56/ADGRG1 expression after the resection of primary tumors arising from 4T1.3 clone injection into MFP, and at this time point, micrometastasis is presumed to develop.[23] Thus, a GPR56/ADGRG1 inhibitor can effectively treat bone micrometastasis by directly reducing cancer cell growth in bone and complement the drugs presently used to treat bone metastasis, bisphosphonates, and RANKL inhibitors, which are presumed to act mainly on osteoclast activation.[54] Two agents were already identified to inhibit GPR56/ADGRG1 expression or function. Pyrrole-imidazole polyamide was developed as an agent that can reduce the ecotropic viral integration site-1 (EVI1)-positive acute myeloid leukemia cell proliferation by inhibiting EVI1-induced GPR56/ADGRG1 expression[55] but cannot be applied to other situations where EVI1 was not involved in GPR56/ADGRG1 expression. High-throughput screening identified dihydromunduletone as a GPR56/ADGRG1 antagonist, but its high $IC_{50}$ (20 μmol/L)[56] may hinder its clinical application. Moreover, the indispensable involvement of cleavage at GAIN domain would indicate that the cleave interference can effectively inhibit GPR56/ADGRG1 activation and subsequent bone metastasis development. Nevertheless, further detailed analysis of the type III collagen–GPR56/ADGRG1 axis molecular mechanism is warranted to develop an antibone metastasis therapy targeting GPR56/ADGRG1.

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## CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

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