Integrin β1 is an essential factor in vasculogenic mimicry of human cancer cells

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Vasculogenic mimicry (VM) formation by cancer cells is known to play a crucial role in tumor progression, but its detailed mechanism is unclear. In the present study, we focused on integrin β1 (ITGB1) and assessed the role of ITGB1 in VM formation. We used in vitro methods to seed cancer cells on Matrigel to evaluate the capability of VM formation. We carried out ITGB1 gene deletion using the CRISPR/Cas9 system, and these ITGB1-knockout cells did not show a VM-like network formation. Further, reintroduction of ITGB1 rescued VM-like network formation in ITGB1-knockout cells. In conclusion, ITGB1 is a critical factor in VM of human cancer cells, and inhibition of ITGB1 may be a novel therapeutic approach for malignant cancer.

KEYWORDS
CRISPR/Cas9, focal adhesion kinase, integrin β1, metastasis, vasculogenic mimicry

1 INTRODUCTION

Tumor growth and metastasis are major causes of death by cancer. As cancer progresses, the tumor requires oxygen and nutrients through blood vessels for their growth. Angiogenesis is a well-known blood supply system, and the tumor recruits blood vessels through vascular endothelial cell stimulation.1 Recent studies indicated another mechanism, VM, which promotes cancer progression by the formation of blood vessel-like structures with cancer cells only, without vascular endothelial cells, and the phenomenon has attracted attention as a novel blood supply system for tumors.2 VM has been characterized in various cancer types, such as melanoma, lung, ovary, and breast,2–5 and correlates with tumor metastasis and poor clinical outcome.6 Thus, targeting VM may be an ideal cancer therapy. VM is observed in the ECM-rich region, and it has been reported that interactions between tumor cells and their surroundings are necessary for VM formation.7 The role of VM in tumor progression has gradually been reported, but the detailed mechanism of VM formation is unclear.

Integrins are heterodimeric transmembrane cell surface receptors that mediate cell-cell and cell-ECM binding. The integrin receptor family consists of 18 α-subunits and 8 β-subunits, giving rise to 24 different integrin heterodimers in mammals.8 Binding affinities to ECM components of each integrin are different.9 Integrin β1 (ITGB1) is the representative member of the integrin subfamily, and it has 12 α-subunits that can be heterodimerized. ITGB1 has multiple functions in cell adhesion, migration, and proliferation; thus, the phenotype of ITGB1-KO mice is embryonic lethal.9–12 ITGB1 is also overexpressed in cancer cells and contributes to various malignant phenotypes, such as EMT and tumor metastasis.13,14 Some reports demonstrated that integrins were related to VM formation;15–17 however, it is suggested that ITGB1 is not associated with VM formation in melanoma cells.15 In this report, the authors used only neutralizing antibody to block ITGB1 functions and assessed the capability of VM formation;15 however, results of whether the neutralizing antibody actually blocked ITGB1 functions were not shown. Thus, we could not determine whether or not ITGB1 is necessary in VM formation.

In the present study, we assessed the capability of VM formation by general and widely used in vitro methods to seed cancer cells on Matrigel, which contains abundant ECM components.18–20 We

Abbreviations: CINP, collagen-induced network phenotype; EMT, epithelial-mesenchymal transition; FAK, focal adhesion kinase; ITGB1, integrin β1; RGD, arginine-glycine-aspartic acid; VM, vasculogenic mimicry.
established ITGB1-KO cells using the CRISPR/Cas9 system and showed that VM-like network formation on Matrigel was completely abolished in ITGB1-KO cells. Further, we rescued ITGB1 expression in KO cells, and network formation was recovered in ITGB1-rescued cells. These results will promote mechanistic understanding of VM formation and enhance the development of novel anticancer reagents targeting integrins.

2 | MATERIALS AND METHODS

2.1 | Cell culture

HT1080 human fibrosarcoma, MDA-MB-231 human breast adenocarcinoma, CHL-1 human skin melanoma, and HEK293T human embryonic kidney cell lines were cultured in DMEM (Nissui Pharmaceutical Co., Ltd, Tokyo, Japan), supplemented with 5% (v/v) (HT1080, MDA-MB-231, and HEK293T) or 10% (v/v) (CHL-1) FBS, 100 units/ml penicillin G, 100 mg/L kanamycin, 600 mg/L l-glutamine, and 2.25 g/L NaHCO₃ at 37°C in a humidified incubator with 5% CO₂.

2.2 | Vasculogenic mimicry-like network formation assay

HT1080, MDA-MB-231, and CHL-1 cells suspended in culture medium were seeded at 1.6 × 10⁵ cells/well into a 96-well plate, which was precoated with 40 µL/well Matrigel (Corning Inc., Corning, NY, USA), and cultured at 37°C. These cells were photographed at 2 weeks. After selection, clonal cell lines were isolated by limiting dilution method, and KO of ITGB1 was confirmed by western blot and sequence analysis.

2.3 | WST assay

WST assay was carried out to measure living cell number with CCK-8 (Wako Pure Chemical Industries, Ltd, Osaka, Japan). CCK-8 was added to cells and incubated for 2 hours at 37°C and then absorbance was measured at 450 nm.

2.4 | Establishment of ITGB1-KO cell lines using the CRISPR/Cas9 system

Integrin β1-KO cell lines were established using the CRISPR/Cas9 system as previously described. The oligos to generate single-guide RNA (sgRNA) were inserted into the BbsI sites of the pSpCas9n(BB)-2A-Puro (PX462) V2.0 plasmid, which was a gift from Feng Zhang (#62987; Addgene, Cambridge, MA, USA). This plasmid was modified to express Cas9 nickase (D10A mutant), which generates a single-strand break. Thus, we used 2 close pairs of sgRNAs, and target sequences were designed in exon 4 of human ITGB1. The primers used to clone the guide sequence were as follows: forward 1, 5'-CA CGAAATGTAACACACGCTAGCAA-3' and reverse 1, 5'-AAACTTGCTACGGTTGTTACATT-3'; forward 2, 5'-CCAGCTTATATCCTTGG AGGCCTC-3' and reverse 2, 5'-AACAGGCTTCAAAAGATATACAG C-3'. Each pair of primers was annealed and then inserted into the plasmid. These 2 plasmids were cotransfected into HT1080, MDA-MB-231, and CHL-1 cells, followed by selection with 2 µg/mL puromycin dihydrochloride (Merck KGaA, Darmstadt, Germany) for about 2 weeks. After selection, clonal cell lines were isolated by limiting dilution method, and KO of ITGB1 was confirmed by western blot and sequence analysis.

2.5 | Western blot

We carried out western blot using a slightly modified version of previously described methods. Cells were seeded on Matrigel precoated dishes (20 µg/cm² Matrigel) as necessary and cultured at 37°C. Collected cells were lysed in lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.1% (w/v) SDS, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, and 1 mmol/L PMSF [to detect protein phosphorylation only, phosphatase inhibitor cocktail PhosSTOP (Roche, Basel, Switzerland) was then added]) at 4°C with sonication. The lysates were centrifuged at 17 800 g for 10 minutes, and the amount of protein in each lysate was measured by Coomassie Brilliant Blue G-250 staining (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Then, 6× loading buffer (350 mmol/L Tris-HCl, pH 6.8, 30% [w/v] glycerol, 0.012% [w/v] bromophenol blue, 6% [w/v] SDS, and 30% [v/v] 2-mercaptoethanol) was added to each lysate. Samples were subsequently boiled for 3 minutes and electrophoresed on SDS-PAGE. Proteins were transferred to PVDF membranes and immunoblotted with anti-ITGB1 (#ab52971; Abcam, Cambridge, UK), anti-phospho-FAK (Y397) (#ab81298; Abcam), or anti-α-tubulin (#T5168; Merck KGaA). Signals were detected with ECL using Western Lightning Plus-ECL (PerkinElmer, Inc., Waltham, MA, USA) or Immobilon Western Chemiluminescent HR substrates (Merck KGaA).

2.6 | Establishment of ITGB1-rescued cell lines

Human ITGB1 cDNA was amplified from an HT1080 cell cDNA library and cloned into the XhoI/EcoRI restriction sites of the CSII-CMV-MCS plasmid (RIKEN BioResource Center, Tsukuba, Japan). Sequences of the primers to amplify ITGB1 cDNA were as follows: forward, 5'-TTTTTCTCGAGATGAATTTACAACCTTTTTCTCG-3' and reverse, 5'-TTTTTCCGGCCGCCTATTTTTTTTTTCTCG-3'. To prevent Cas9 recognition and deletion of exogenously introduced ITGB1 gene, we generated a Cas9-resistant ITGB1 gene by codon optimization without any amino acid substitutions. Sequences of the primers to amplify the Cas9-resistant ITGB1 gene were as follows: forward, 5'-GGGAAAGTACGGATCGAAAAATTATTATTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
with the lentivirus media for 72 hours. After infection, clonal cell lines were isolated by limiting dilution method, and reintroduction of ITGB1 was confirmed by western blot.

2.7 Genetic analysis and neutralizing antibodies

The protocol used for genetic analysis and information on neutralizing antibodies are indicated in supplementary materials and methods (Doc. S1).

3 RESULTS

3.1 Vasculogenic mimicry-like network formation is regulated by Ca²⁺

In previous studies, it was reported that divalent cations, such as Ca²⁺ and Mg²⁺, regulate integrin activities and that calcium signaling affects VM formation in melanoma cells. Therefore, we assessed the role of Ca²⁺ for VM-like network formation by treatment with EGTA in HT1080 cells. As a result, the network formation on Matrigel was inhibited by treatment with EGTA (Figure 1A,B). Furthermore, we cotreated with EGTA and CaCl₂ at equal molar ratios to counteract the chelation of Ca²⁺. As expected, the VM-like network formation was rescued by Ca²⁺ recovery (Figure 1A,B). As depletion of Ca²⁺ had a toxic effect on cells, we confirmed living cell numbers by WST assay. Although living cell numbers in EGTA-treated cells and EGTA/CaCl₂-cotreated cells were decreased compared with control cells, the difference between treatment with EGTA and cotreatment with EGTA/CaCl₂ was not significant (Figure 1C). Therefore, the resultant phenotypes of both EGTA-treated cells were not the cause of cell death, and these results implied that the functions of integrins are important for VM formation in cancer cells.

3.2 Integrin β1 is associated with VM-like network formation in various cancer cell lines

Integrin β1 is a representative member of the integrin subfamily, and it has multiple functions in cell adhesion, migration, and proliferation. In addition, ITGB1 contributes to tumor malignancy, so we focused on the association between ITGB1 and VM. To explore the role of
ITGB1 for VM formation, we established ITGB1-KO HT1080 cells using the CRISPR/Cas9 system. To minimize off-target risks, we used a Cas9 nickase mutant (D10A) expression vector, and guide RNA sequences were designed at 2 close positions in exon 4 of ITGB1, as shown in Figure 2A. The constructed ITGB1-KO vectors were cotransfected into human fibrosarcoma HT1080 cells and ITGB1-KO clonal cells were selected. We carried out western blot and analyzed genetic alterations to confirm the complete KO of ITGB1 in the cell line (Figures 2B; S1A). Cell morphology of HT1080 cells was altered by KO of ITGB1 (Figure S1B). Furthermore, ITGB1-KO cells showed high-level growth ability compared with parental cells (Figure S1C) as previously reported.11 Using the established cell line, we assessed the capability of the VM-like network formation on Matrigel in ITGB1-KO HT1080 cells. Surprisingly, network formation on Matrigel was absolutely abolished by the KO of ITGB1 for 3 hours (Figure 2C,D), and this phenotype was maintained for 24 hours (Figure 2E), suggesting that ITGB1 is necessary for VM-like network formation in HT1080 cells.

We next confirmed whether or not VM-like network formation is dependent on ITGB1 in other human cancer cells. A previous study showed that melanoma cells treated with a neutralizing antibody for ITGB1 did not affect VM formation.15 To validate whether or not ITGB1 is required for VM formation in melanoma cells, we selected the human skin melanoma CHL-1 cell line. Furthermore, we also examined the human breast adenocarcinoma MDA-MB-231 cell line, which is known to form VM.17 We also used the CRISPR/Cas9 system and established ITGB1-KO clonal MDA-MB-231 and CHL-1 cell lines (Figure 3A). As a result, the VM-like network formation was also inhibited by the KO of ITGB1 in both cell lines (Figure 3B). Notably, in our study, ITGB1 was associated with VM-like network formation in melanoma cells, which is a distinct result of a previous report. Taken together, these results strongly suggested that ITGB1 is essential for VM-like network formation in various cancer cell lines.

### 3.3 Integrin β1 is an essential factor for VM-like network formation

To confirm whether ITGB1 is necessary for VM formation, we carried out ITGB1 rescue experiments. As the ITGB1-KO clonal cells sustainably express sgRNAs and Cas9 nickase, we paid attention not to edit the reintroduced ITGB1 gene by Cas9 nickase. Therefore, target sequences of sgRNAs in ITGB1 were optimized to resist Cas9 nickase without any amino acid substitutions (see Materials and Methods). We introduced Cas9-resistant ITGB1 into ITGB1-KO HT1080 cells, and a
clonal cell line, ITGB1-rescued HT1080, was established. Re-expression of ITGB1 in ITGB1-rescued HT1080 cells was confirmed by western blot (Figure 4A). Indeed, VM-like network formation was recovered in ITGB1-rescued HT1080 cells within a short period as in parental HT1080 cells (Figure 4B,C), demonstrating that VM-like network formation was dependent on the expression of ITGB1.

Various cell functions, such as migration, invasion, and proliferation, are involved in tumor progression. FAK, which is a major downstream signal of ITGB1, contributes to these phenotypes, and the signaling is activated by autophosphorylation of the Y397 residue of FAK.28–31 We finally verified whether or not VM-like network formation regulated by ITGB1 is dependent on FAK signaling. As FAK signaling was triggered by integrin-mediated cell-ECM interactions, we assessed phosphorylation levels of FAK (Y397) in parental and ITGB1-KO HT1080 cells cultured in Matrigel-coated dishes. Phospho-FAK (Y397) was clearly downregulated in ITGB1-KO cells compared with parental cells (Figure 5A), as expected.11 In addition, reintroduction of ITGB1 upregulated phosphorylation level of FAK (Y397) in ITGB1-KO HT1080 cells (Figure 5B). These results were consistent with the phenotype of VM-like network formation, suggesting that VM-like network formation was dependent on ITGB1-mediated FAK signaling.

4 | DISCUSSION

Cancer metastasis is caused by various processes, such as EMT, invasion, migration, angiogenesis, and VM. Among these processes, VM has attracted particular attention during the latest cancer research. In the present study, we explored the essential factors for VM and discovered that ITGB1 plays a crucial role in VM formation. Although we suggested that integrins are crucial for VM formation by chelation of Ca2+ (Figure 1), there are some molecules that can be affected by the depletion of Ca2+. Therefore, we confirmed the effect of other molecules such as...
integrin αv and E-cadherin on VM formation by treatment with neutralizing antibodies. As a result, anti-ITGB1 antibody significantly inhibited VM-like network formation (Figure S2), supporting our hypothesis. Indeed, we showed that ITGB1-KO cell lines, generated by the CRISPR/Cas9 system, did not form a VM-like network structure (Figures 2,3).

VM formation is often observed in ECM-rich regions, and ITGB1 is a cell surface receptor of these proteins, such as laminin, collagen, and fibronectin.8 Notably, these proteins have RGD domains, which are necessary for cell adhesion activity, and integrin αv, which can be heterodimerized to ITGB1, is one of the RGD receptors.32 Previous studies showed that integrin αvβ3 and αvβ5 are associated with VM formation.15,16 Thus, it is considered that the interaction between ITGB1 and ECM proteins that have RGD motifs induces VM formation. Furthermore, a recent study demonstrated that the expression of ITGB1 was upregulated at the transcriptional level in high-density 3-D collagen conditions, and the expression level of ITGB1 correlated with cell network structure in this condition. The authors termed the phenomenon CINP and argued that CINP was associated with VM.33 This report supports our results, and our data demonstrated more clearly and directly the necessity of ITGB1 on VM formation not only by KO experiments, but also by rescue experiments.

FAK signaling is triggered by cell adhesion and correlates with tumor malignancy, such as metastasis and invasion.28–31 A previous study showed that chemokine (C-X-C motif) ligand 1 (CXCL1) induced ITGB1 expression and caused activation of FAK signaling, resulting in gastric cancer metastasis.34 As above, ITGB1/FAK signaling is often excessively activated in tumor cells. Indeed, our data showed that FAK signaling has a positive correlation with VM-like network formation (Figures 2,4,5). In addition, some reports suggested that FAK participates in VM formation in gallbladder cancer.35,36 Thus, FAK is probably associated with VM formation through an ITGB1-dependent mechanism(s). Further studies are necessary to elucidate the signaling cascade of ITGB1-mediated VM formation.

In the present study, we demonstrated that ITGB1 plays a crucial role in VM-like network formation and that the efficacy of ITGB1 was common in different types of VM-positive cancer cell lines. Further, ITGB1-mediated FAK signaling had a relation to VM-like network formation. Taken together, we provide new insights into the mechanistic understanding of VM formation, and it will be an ideal strategy for cancer therapeutics.

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CONFLICTS OF INTEREST
Authors declare no conflicts of interest for this article.

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SUPPORTING INFORMATION

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

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