CD44v3,8-10 is essential for Slug-dependent vimentin gene expression to acquire TGF-β1-induced tumor cell motility

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CD44v3,8-10 is essential for Slug-dependent *vimentin* gene expression to acquire TGF-β1-induced tumor cell motility

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Abstract

CD44 is a widely expressed polymorphic adhesion molecule that has pleiotropic functions in development and tumor progression. Its mRNA undergoes alternative splicing to generate multiple variant (CD44v) isoforms, although the function of each CD44v isoform is not fully elucidated. Here, we show that CD44v plays an important role in the induction of vimentin expression upon transforming growth factor-β1 (TGF-β1)-induced epithelial–mesenchymal transition (EMT). Among multiple CD44v isoforms expressed in NUGC3 gastric cancer cells, CD44v8-10 and CD44v3,8-10 are involved in the acquisition of migratory and invasive properties associated with TGF-β1-induced EMT, and only CD44v3,8-10 induces the transcription of *vimentin* mediated by the EMT transcription factor Slug. In primary tumor specimens obtained from patients with gastric cancer, CD44-containing variant exon 9 (CD44v9) expression and EMT features [E-cadherin(−)vimentin(+)] were significantly correlated, and EMT features in the cells expressing CD44v9 were associated with tumor invasion depth, lymph node metastasis, and pStage, which indicate invasive and metastatic properties, and poor prognosis. These results indicate that certain CD44v isoforms promote tumor cell motility and metastasis in gastric cancer in association with EMT features, and CD44v3,8-10 may contribute to these clinical characteristics.

KEYWORDS
CD44v3,8-10, epithelial–mesenchymal transition, slug, TGF-β1, vimentin

1 | INTRODUCTION

The single-pass transmembrane glycoprotein CD44 is a widely expressed polymorphic adhesion molecule that binds to hyaluronic acid and contributes to cell–cell and cell–extracellular matrix adhesion, cell growth, trafficking, and tumor progression.[1] Most tumor cells express CD44, and its expression in combination with that of other antigens is a surrogate marker of cancer-initiating cells.[2] *CD44* undergoes extensive alternative splicing to generate two groups of isoforms: CD44 variant (CD44v) isoforms, which contain variant exons, and the CD44 standard isoform (CD44s), which is the smallest and devoid of variant exons.[3] In several types of cancer, for instance, breast cancer

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and hepatocellular carcinoma, the CD44s isoform promotes tumor cell survival, the invasive phenotype, and metastasis.[4–7] Conversely, multiple CD44v isoforms are expressed mainly in gastrointestinal cancer, and each of these isoforms promotes cancer stem cell activities, tumor initiation, and metastasis.[8–14] Among them, CD44v8-10 interacts with and stabilizes xCT, a glutamate–cystine transporter, and contributes to ROS defense by enhancing the capacity for synthesizing reduced glutathione (GSH).[12,14] The CD44v protein markedly accumulates in tumor cells with EMT features induced by TGF-β1.[9,15]

TGF-β1 is an extracellular signaling molecule that plays key roles in EMT. During EMT, carcinoma cells dissociate from primary tumors and acquire increased motility, thereby developing an invasive phenotype at the initial step of metastasis.[16,17] The cellular hallmarks of EMT include the loss of epithelial markers, for instance, E-cadherin and claudins, and the upregulation of mesenchymal markers, for instance, vimentin, fibronectin, and N-cadherin. A group of core EMT transcription factors (EMT-TFs), including Snail, Slug, Twist, and Zeb1, reprogram the transcriptional network that mediates the downstream biological effects.[18] Although EMT induced by TGF-β1 is often associated with the expression of CD44v, the effect of CD44v expression during EMT has not been fully elucidated.

In this study, we analyzed the role of CD44v in TGF-β1-induced EMT. In NUGC3 gastric cancer cells, CD44v8-10 and CD44v3,8-10 contributed to the acquisition of migratory and invasive properties in response to TGF-β1-induced EMT. We provide the first evidence that CD44v3,8-10 plays an essential role in TGF-β1-induced Slug-dependent transcriptional activation of the *vimentin* (*VIM*) gene, which confers the migratory and invasive properties. By contrast, CD44v8-10, which also contributes to the acquisition of migratory and invasive properties, does not possess the ability to induce *VIM* gene expression upon TGF-β1-induced EMT. In primary gastric tumors, the combination of CD44-containing variant exon 9 (CD44v9) expression and EMT features [E-cadherin(−) vimentin(+)] was significantly associated with invasive and metastatic activities, indicating that CD44v is critically involved in tumor progression via EMT.

2 | MATERIALS AND METHODS

2.1 | Cell culture and reagents

NUGC3 cells were purchased from the Japanese Cancer Research Resources Bank (JCRB0822). MKN1 cells were purchased from RIKEN (RCB1003). A549 cells were kindly provided by M. Takeshita (Kyushu University). These cells were confirmed to be negative for mycoplasma by MyoAlert (Lonza). NUGC3 and MKN1 cells were cultured in RPMI 1640 (Thermo Fisher).; A549 cells were cultured in DMEM (Thermo Fisher). All media were supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. All cell lines were incubated at 37°C in a humidified cell culture incubator containing 5% CO_2. Recombinant human TGF-β1 was purchased from R&D Systems. siRNAs were purchased from Ambion and transfected using Lipofectamine RNAiMAX (Thermo Fisher). The siRNAs are listed in Table S4.

2.2 | Generation of CD44-knockout NUGC3 (CD44KO) cells using CRISPR/Cas9 gene-editing

The annealed oligonucleotide targeting the sequence in exon 1 of the *CD44* gene (5′-CGTGCCGCTGAGCCTGGCGC-3′) was cloned into pX330-U6-Chimeric_BB-CBh-hSpCas9 (42230; Addgene), which was kindly provided by Feng Zhang.[19] NUGC3 cells were co-transfected with this plasmid and a puromycin-resistant gene cassette (631626; Takara) using a 4D Nucleofector (Lonza). CD44KO clones were selected from puromycin-resistant clones by immunoblotting, and the indels of the target gene locus were confirmed by direct sequencing.
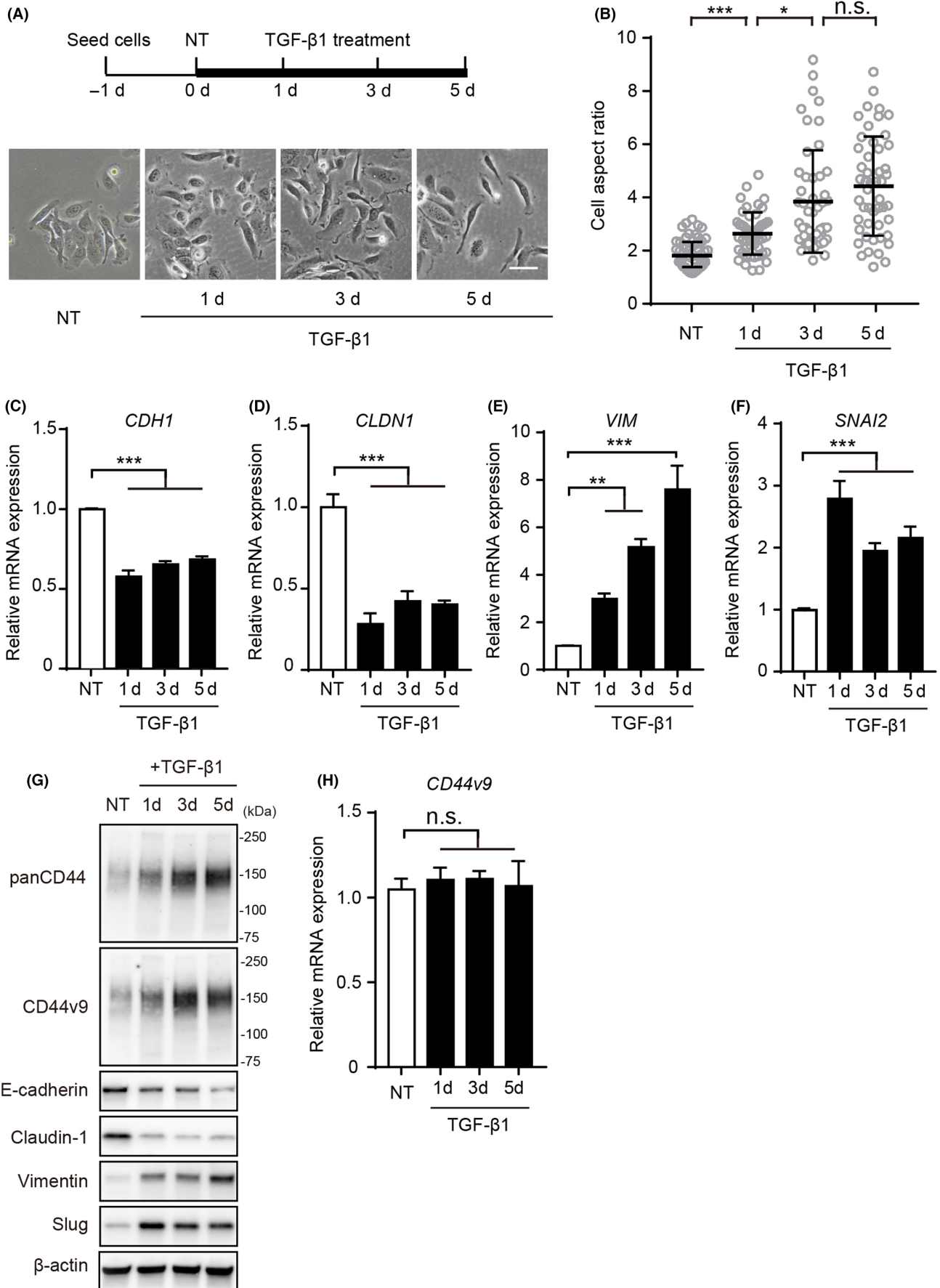
2.3 | Generation of CD44KO cells stably expressing CD44 isoforms

Full-length cDNAs encoding *CD44* isoforms expressed in NUGC3 cells were amplified using primers (CD44_FL_F and CD44_FL_R), cloned into pENTR-D-TOPO (Thermo Fisher) and confirmed by sequencing. cDNAs encoding CD44s, CD44v8-10, CD44v3,8, CD44v3,8-10, and CD44v6-10 were transferred to a pcDNA3.1(+)-based destination vector using a Gateway LR Clonase II Enzyme Mix (Thermo Fisher). The S301A point mutation was introduced into CD44v8-10 using a QuikChange Site-Directed Mutagenesis Kit (Stratagene) and primers (CD44_S301A_F and CD44_S301A_R). CD44KO cells were transfected with plasmids encoding each CD44 isoform using a 4D nucleofector (Lonza) and selected with G418. The sequences of primers are listed in Table S4.

2.4 | Cloning of human *VIM* and *SNAI2* cDNAs

The cDNAs encoding full-length *VIM* and *SNAI2* expressed in NUGC3 cells were amplified using the primers (*VIM*: VIM_FL_F and VIM_FL_R;

FIGURE 1 TGF-β1-induced EMT is associated with CD44v protein accumulation in NUGC3 cells. (A) NUGC3 cells cultured in the presence of 10 ng/ml TGF-β1 for the indicated days. Phase contrast images are shown. NT, nontreated. Scale bar, 50 μm. (B) Cell aspect ratios of NUGC3 cells in (A). The medians and interquartile ranges (*n* = 50 per sample) are shown by horizontal long and short bars, respectively. Kruskal–Wallis ANOVA test. *$p < 0.05$, ***$p < 0.001$, n.s., not significant. (C–F) Relative mRNA expression of *CDH1* (C), *CLDN1* (D), *VIM* (E), and *SNAI2* (F). Expression of each gene was normalized by that of β-actin, and expression in the NT sample was set to 1. Bars and error bars represent means and standard deviations, respectively, of three independent experiments. Student's *t* test. **$p < 0.01$, ***$p < 0.001$. (G) Western blot. NUGC3 cells were cultured in the presence of 10 ng/ml TGF-β1 for the indicated days. The molecular weight markers (kDa) are shown in panCD44 and CD44v9 immunoblots. (H) Relative *CD44v9* mRNA expression. Student's *t* test. n.s., not significant

(A) Seed cells NT TGF-β1 treatment
−1 d 0 d 1 d 3 d 5 d

NT 1 d 3 d 5 d
TGF-β1

(B) Cell aspect ratio
NT 1 d 3 d 5 d
TGF-β1

(C) CDH1

(D) CLDN1

(E) VIM

(F) SNAI2

(G) +TGF-β1
NT 1d 3d 5d (kDa)
panCD44 −250 −150 −100 −75
CD44v9 −250 −150 −100 −75
E-cadherin
Claudin-1
Vimentin
Slug
β-actin

(H) CD44v9
NT 1d 3d 5d
TGF-β1

SNAI2: SNAI2_FL_F and SNAI2_FL_R), cloned into pENTR/D-TOPO (Thermo Fisher), and confirmed by sequencing. The full-length *VIM* and *SNAI2* cDNAs were transferred into the pcDNA3.1(+)-based destination vector using Gateway LR Clonase II Enzyme Mix (Thermo Fisher). The sequences of primers are listed in Table S4.

2.5 | Migration and invasion assays

A total number of 1×10^5 cells were suspended in culture medium containing 2% FBS and loaded in the upper well, migration and invasion assays were performed according to the manufacturer's instructions (354480; Corning). After 48 h incubation, crystal violet-stained cells on the lower surface were imaged using a BZ-9000 microscope (Keyence) and counted in three randomly selected microscopic fields per well.

2.6 | Immunoblotting

Cells were directly lysed in 1× SDS sample buffer, sonicated, and boiled at 95°C. Protein concentration was determined by XL-Bradford (Integrale Co. Ltd.), and equal amounts of protein were loaded on an SDS-PAGE gel. Immunoblotting was performed using antibodies listed in Table S5. Signals were detected using the ImageQuant LAS4000 system (GE Healthcare).

2.7 | Quantitative RT-PCR

Total RNA extraction and cDNA synthesis were performed using a Maxwell RSC simplyRNA Cells Kit (Promega) and SuperScript III First-strand Synthesis Super Mix (Thermo Fisher), respectively. Quantitative RT-PCR was performed using the THUNDERBIRD SYBR qPCR Mix (QPS-201; TOYOBO). The primer sequences are listed in Table S4.

2.8 | Dual-luciferase reporter assay

A total number of 1×10^4 cells were transfected with 500 ng of each reporter plasmid in 24-well plates using FuGENE6 Transfection Reagent (E2693; Promega). Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (E1910; Promega). Relative luciferase activity was determined by normalizing fire-fly luciferase activity to Renilla luciferase activity.

2.9 | Quantification of intracellular ROS and glutathione

Intracellular ROS were measured using a Cellular ROS Assay Kit (ab113851; Abcam). The fluorescence intensity of 2′,7′-dichlorofluorescein (DCF) was measured using FACSVerse (BD Biosciences). Intracellular glutathione was measured using a GSH-Glo Glutathione Assay Kit (V6911; Promega).

2.10 | Patients and tissue microarray (TMA) of primary gastric cancer specimens

This study enrolled 102 patients with primary gastric cancer who underwent gastrectomy from 1994 to 2002, in Kyushu University Hospital. All patients provided full written informed consent. The study was approved by the Ethics Committee of Kyushu University (ID#: 30-505). The basic characteristics of the patients are shown in Table S1. All tumors were staged according to the TNM classification of the Japanese Classification of Gastric Carcinoma 15th edition, and the degree of differentiation was determined according to the grade classification of the Union for International Cancer Control. TMA blocks assembling representative core areas of primary tumors from each patient were produced by Pathology Institute Corp.

2.11 | Immunohistochemistry (IHC)

Paraffin-embedded TMA sections were deparaffinized with xylene and rehydrated in a graded series of ethanol. Antigens were retrieved in 10 mM citrate buffer (pH 6.0) by heating specimens in an autoclave. Endogenous nonspecific peroxidases were quenched with 3% H_2O_2 (E-cadherin and vimentin) or 0.3% H_2O_2 (CD44v9) in absolute methanol for 30 min. After blocking with 10% goat normal serum for 10 min, each TMA section was incubated overnight at 4°C with primary antibodies (Table S5). Sections were subsequently

FIGURE 2 Loss of CD44v reduces the migration and invasion capacity. (A–D) NUGC3 cells treated with control (si*GL3*) or CD44v-targeting (si*CD44v*) siRNA were cultured with vehicle (−) or in the presence of 10 ng/ml TGF-β1 (+) for 3 days. (A) Western blot. (B) Relative DCF fluorescence. DCF fluorescence of NUGC3 cells treated with si*GL3* and cultured with vehicle was set to 1. (C) Migration. The number of cells on the lower surface of transwell chamber was counted. HPF: high power field. (D) Invasion. The number of cells on the lower surface of transwell chamber was counted. (E–H) NUGC3 and CD44KO cells were cultured with vehicle (−) or in the presence of 10 ng/ml TGF-β1 (+) for 3 days. (E) Western blot. (F) Relative DCF fluorescence. DCF fluorescence of nontreated NUGC3 cells was set to 1. (G) Migration. (H) Invasion. (I–N) NUGC3 (WT) and CD44KO cells stably expressing CD44v8-10 or CD44v8-10 (S301A) were cultured with vehicle (−) or in the presence of 10 ng/mL TGF-β1 (+) for 3 days. (I) Schematic of the CD44v8-10–xCT interaction and its effect on the intracellular ROS level. (J) Western blot. (K) Intracellular GSH level. (L) Relative DCF fluorescence. DCF fluorescence of CD44KO/Mock cells cultured with vehicle was set to 1. (M) Migration. (N) Invasion. All bars and error bars represent means and standard deviations, respectively, of three independent experiments. Student's *t* test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. n.s., not significant
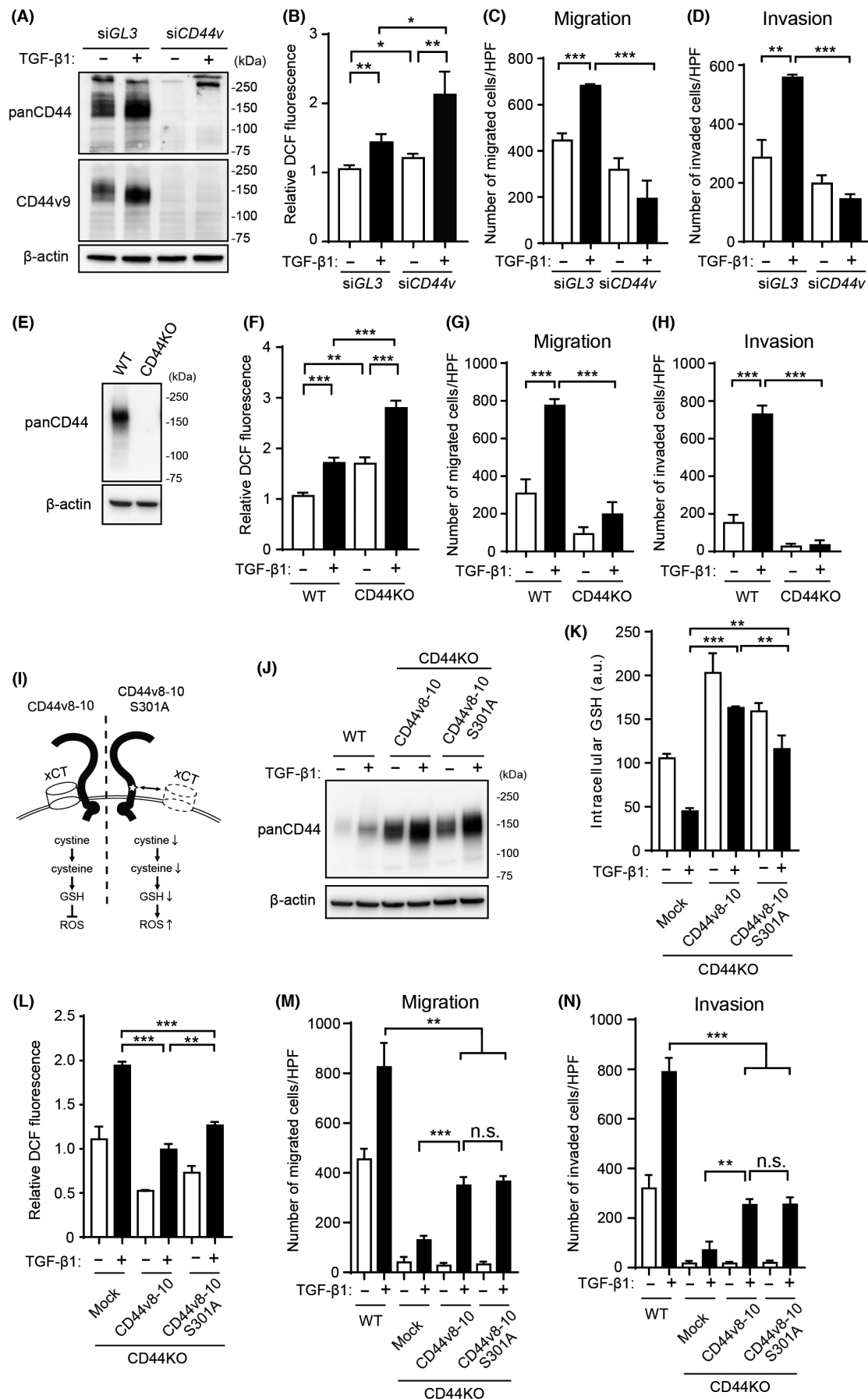
color-developed with 3,3′-diaminobenzidine, counterstained with 10% Mayer's hematoxylin, dehydrated, and mounted. Images were captured using a Nano-Zoomer (Hamamatsu Photonics).

2.12 | Statistical analysis

Statistical analysis was performed using GraphPad Prism.

3 | RESULTS

3.1 | TGF-β1 induces Slug and vimentin, suppresses epithelial markers, and accumulates CD44v9 in NUGC3 cells

To investigate the role of CD44v in EMT, we explored cell lines that express CD44v9 and undergo EMT upon TGF-β1 treatment, and chose NUGC3 cells, a human diffuse-type gastric cancer cell line.[20] Treatment with TGF-β1 caused a gradual change in cell morphology from epithelial island-forming to a front-to-back polarized spindle shape (Figure 1A,B). These morphological changes were accompanied by the downregulation of epithelial marker genes [*E-cadherin/CDH1* (Figure 1C) and *Claudin-1/CLDN1* (Figure 1D)] and upregulation of the mesenchymal marker gene *vimentin* (*VIM*) (Figure 1E) and the EMT-TF gene *Slug/SNAI2*) (Figure 1F). Although the downregulation of *CDH1* and *CLDN1* and upregulation of *SNAI2* peaked on day 1 (Figure 1C,D,F), *VIM* gene expression gradually increased until day 5 (Figure 1E), suggesting that a different mechanism is involved in the regulation of *VIM* gene expression. These characteristic fluctuations were also observed at the protein level (Figure 1G). By contrast, expression of Snail and Zeb1 was not detected (Figure S1). During EMT, total CD44 protein (panCD44) and CD44v9 protein accumulated gradually (Figure 1G), whereas the expression of *CD44v9* mRNA remained unchanged (Figure 1H). Collectively, the results indicate that TGF-β1-induced EMT is associated with the induction of Slug, suppression of epithelial markers, and gradual accumulation of CD44v9 and vimentin in NUGC3 cells.

3.2 | NUGC3 cells predominantly express CD44v, which suppresses intracellular ROS and supports migration and invasion

To evaluate the functional relevance of CD44v protein accumulation, we performed siRNA experiments. Total CD44 protein (Figure 2A) and *CD44* mRNA expression (Figure S2A) were efficiently suppressed by siRNA targeting a *CD44*v region (exon 15) (si*CD44v*), which specifically downregulated the expression of *CD44v* (Figure S2B) but not that of *CD44s* mRNA (Figure S2C), both in the absence and presence of TGF-β1, indicating that CD44v was predominantly expressed and accumulated during EMT in NUGC3 cells.

CD44v8-10 (containing variant exons 8, 9, and 10) associates with xCT and confers antioxidant potential in cells.[12] Moreover, TGF-β1 can stimulate ROS production in cancer cells.[21] We first measured the intracellular ROS level using 2′,7′-dichlorofluorescin diacetate (DCFDA) upon TGF-β1 treatment. The intracellular ROS level was significantly higher in si*CD44v*-treated cells than in control siRNA (si*GL3*)-treated cells (Figure 2B). Next, we performed transwell migration and invasion assays to evaluate the effect of CD44v on cell motility in the presence of TGF-β1. TGF-β1 significantly increased migration and invasion of si*GL3*-treated cells, whereas si*CD44v* abolished this effect (Figures 2C,D, and S2D,E). These data indicated that CD44v functionally contributes to ROS defense and to the migratory/invasive properties acquired in response to TGF-β1 treatment.

To further evaluate the correlation between ROS defense and the migratory/invasive properties promoted by CD44v, we first generated CD44KO cells by CRISPR-Cas9-mediated genome editing (Figures 2E and S2F). CD44KO cells displayed similar phenotypes to si*CD44v*-treated cells. Specifically, the intracellular ROS level was significantly elevated (Figure 2F), and migration and invasion in response to TGF-β1 were severely compromised (Figures 2G,H, and S2G,H). Next, we established CD44KO cells stably expressing wild-type CD44v8-10 (CD44KO/CD44v8-10) (Figure 2I,J). Consistently, CD44KO/CD44v8-10 cells accumulated more intracellular GSH (Figure 2K) and exhibited a lower level of intracellular ROS (Figure 2L) than CD44KO/Mock cells. We also established CD44KO cells stably expressing CD44v8-10 S301A (CD44KO/CD44v8-10 S301A), which compromises the interaction of CD44v8-10 with xCT and its antioxidant potential[12] (Figure 2I). CD44KO/CD44v8-10 S301A cells also accumulated more GSH and exhibited a lower level of ROS than CD44KO/Mock cells, but not to the same extent as CD44KO/CD44v8-10 cells (Figure 2K,L). This result may reflect the significance of this mutation because CD44KO/CD44v8-10 S301A and CD44KO/CD44v8-10 cells expressed an equivalent level of protein (Figure 2J). The defects in migration and invasion induced by TGF-β1 were rescued to an equivalent extent in CD44KO/CD44v8-10 and CD44KO/CD44v8-10 S301A cells (Figures 2M,N, and S2I,J). These results indicated that CD44v8-10 supports migration and invasion of NUGC3 cells that have undergone EMT, whereas the antioxidant potential mediated by the Ser 301 residue of CD44v8-10 has limited effects. Migration and invasion were lower in CD44KO/CD44v8-10 cells than in NUGC3 cells (Figure 2M,N) despite showing higher expression levels of CD44v8-10 than that of NUGC3 cells (Figure 2J). These results suggest that CD44v8-10 does not completely rescue the defects in the migratory and invasive properties of CD44KO cells.

3.3 | CD44v is required for TGF-β1-induced vimentin expression, which is critical for migration and invasion

The above data suggest that the elevated intracellular ROS level was not the sole reason for the impaired motility of CD44v-depleted cells
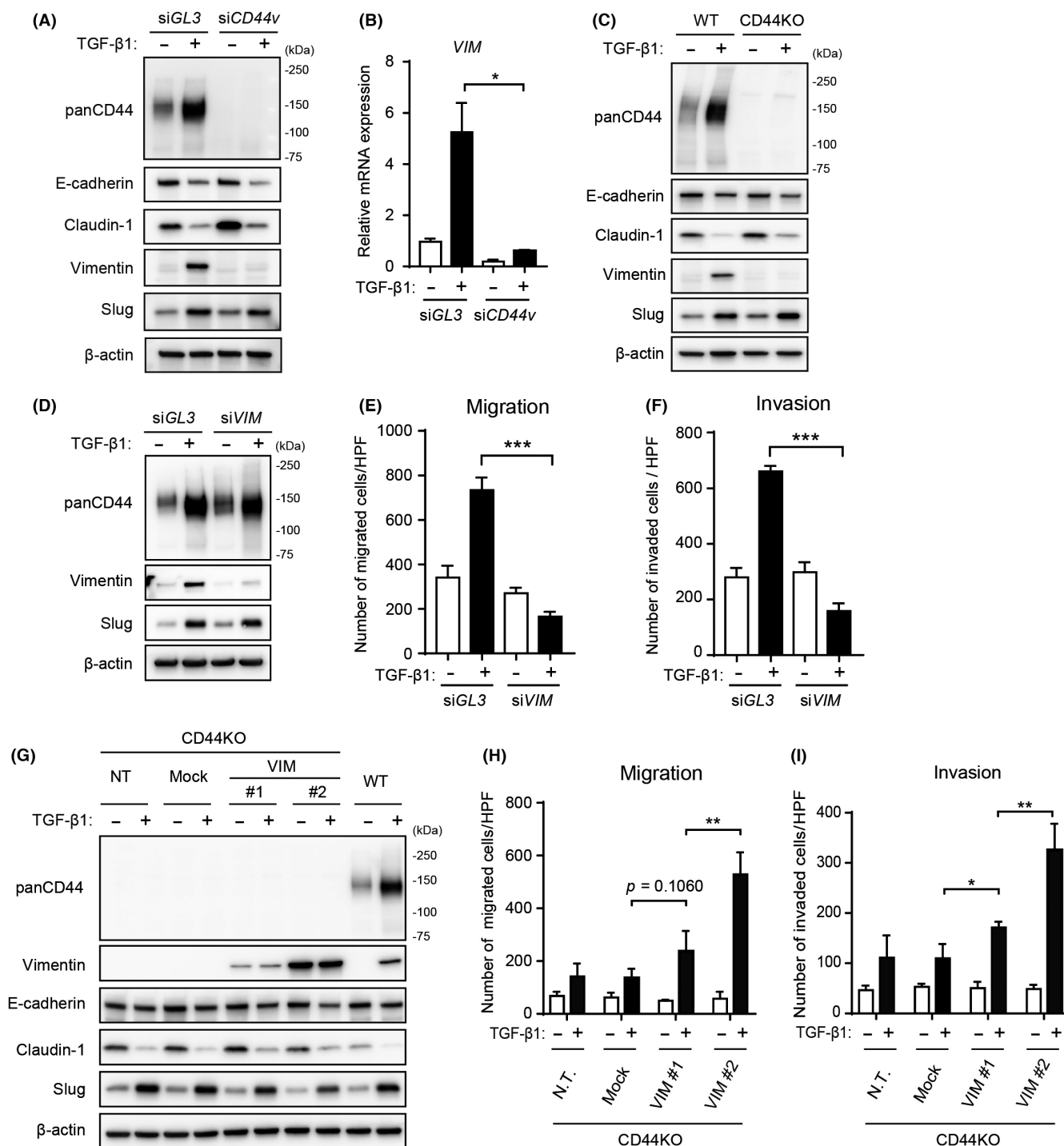
FIGURE 3 CD44v depletion is accompanied by suppression of vimentin expression, which is critical for the migration and invasion capacity. (A) Western blot. NUGC3 cells treated with control (si*GL3*) or CD44v-targeting (si*CD44v*) siRNAs were cultured with vehicle (−) or in the presence of 10 ng/ml TGF-β1 (+) for 3 days. (B) Relative *VIM* mRNA expression in (A). (C) Western blot. NUGC3 (WT) and CD44KO cells were cultured with vehicle (−) or in the presence of 10 ng/ml TGF-β1 (+) for 3 days. (D–F) NUGC3 cells treated with control (si*GL3*) or vimentin-targeting (si*VIM*) siRNAs were cultured with vehicle (−) or in the presence of 10 ng/ml TGF-β1 (+) for 3 days. (D) Western blot. (E) Migration. (F) Invasion. (G–I) CD44KO cells stably expressing vimentin (CD44KO/VIM #1 and #2) were cultured with vehicle (−) or in the presence of 10 ng/ml TGF-β1 (+) for 3 days. (G) Western blot analysis. (H) Migration. (I) Invasion. All bars and error bars represent the mean and standard deviation, respectively, of three independent experiments. Student's *t* test. *p < 0.05, **p < 0.01, ***p < 0.001. n.s., not significant

upon TGF-β1 treatment. Therefore, to investigate whether CD44v expression supported TGF-β1-induced migration and invasion, we monitored the expression of EMT factors upon TGF-β1 treatment

when CD44 was depleted. In si*CD44v*-treated cells, TGF-β1-induced vimentin expression was completely abrogated, whereas the repression of E-cadherin and claudin-1, and the induction of Slug, were not

affected (Figure 3A), suggesting that CD44v is required for vimentin expression upon EMT but not for the induction of EMT itself. The induction of *VIM* gene expression by TGF-β1 was also abrogated in si*CD44v*-treated cells (Figure 3B), indicating that the defect occurred at the transcriptional level. The abrogation of *VIM* mRNA induction upon TGF-β1 treatment was confirmed when NUGC3 cells were treated with another si*CD44* targeting the 5′UTR (si*CD44*#1), a constant region in exon 2 (si*CD44*#2), or the *CD44* variant region in exon 14 (si*CD44v*#2) (Figure S3A–C). Furthermore, vimentin was not induced in CD44KO cells treated with TGF-β1 (Figure 3C), confirming that CD44 depletion caused the defect in TGF-β1-induced vimentin expression.

Vimentin is a type III intermediate filament protein required for migration of cancer cells that have undergone EMT.[22] Consistently, treatment with siRNA against *vimentin* (si*VIM*) significantly suppressed cell migration and invasion upon TGF-β1 treatment (Figures 3D–F, and S3D,E). To explore whether the absence of vimentin induction upon TGF-β1 treatment in CD44-depleted cells was primarily responsible for the impaired cell motility, we generated CD44KO cells ectopically expressing vimentin (CD44KO/VIM #1 and #2; Figure 3G), and measured their migration and invasion. No significant difference in migration or invasion was observed in the absence of TGF-β1; however, in the presence of TGF-β1, ectopic vimentin expression significantly increased cell migration (Figures 3H and S3F) and invasion (Figures 3I and S3G) in a dose-dependent manner. These results indicated that CD44v is required for TGF-β1-induced expression of vimentin, which plays a critical role in migration and invasion of NUGC3 cells that have undergone EMT.

3.4 | CD44v3,8-10 is specifically required for TGF-β1-induced vimentin expression

Our data suggest that CD44v is required for vimentin expression upon TGF-β1 treatment. However, ectopic expression of CD44v8-10 did not restore TGF-β1-induced vimentin expression (Figure S4A), indicating that CD44v8-10 is not responsible for vimentin expression. We suspected that NUGC3 cells express CD44v isoforms other than

CD44v8-10 that are required for vimentin expression. To explore this possibility, we amplified full-length *CD44* cDNAs by RT-PCR from NUGC3 cells in the absence (vehicle) or presence of TGF-β1 treatment (Figure 4A). TGF-β1 treatment did not change the sizes of amplified fragments encoding *CD44* cDNA (Figure 4A). Extensive sequencing of *CD44* cDNA cloned into a plasmid vector revealed that NUGC3 cells expressed multiple types of CD44v isoforms, including CD44v3,8, CD44v8-10, CD44v3,8-10, and CD44v6-10, as well as CD44s. Among these, CD44v8-10, CD44v3,8-10, and CD44v6-10 contain variant exon 9 (Figure 4B).

Next, we established CD44KO cell lines ectopically expressing each CD44v isoform or CD44s (Figure S4B) and examined whether vimentin expression was restored upon TGF-β1 treatment (Figure 4C). Among these transfectants, only CD44v3,8-10-expressing CD44KO cells (CD44KO/CD44v3,8-10) exhibited the restoration of vimentin protein (Figure 4C) and *VIM* mRNA (Figure 4D) expression upon TGF-β1 treatment. Of note, *VIM* mRNA expression was rather suppressed in CD44s- and other CD44v-expressing CD44KO cells (Figure 4D). To verify that CD44v3,8-10 was involved in the transcription of the *VIM* gene, we performed a luciferase assay using the promoter of the *VIM* gene (VIMpro: −840 bp to +99 bp from the transcription start site (TSS) of *VIM* mRNA; Figure S4C). The dual-luciferase reporter detected TGF-β1-induced activation of VIMpro in NUGC3 cells (Figure S4D). TGF-β1-induced activation of VIMpro was significantly reduced in CD44KO/Mock cells, and significantly restored in CD44KO/CD44v3,8-10 cells (Figure 4E), indicating that CD44v3,8-10 activates TGF-β1-induced *VIM* gene expression. Finally, we analyzed migration and invasion of these CD44 transfectants. Migration and invasion of CD44KO/CD44v8-10 and CD44KO/CD44v3,8-10 cells were significantly restored in the presence of TGF-β1 (Figures 4F,G, and S4E,F). CD44KO/CD44v3,8-10 cells exhibited an equivalent level of intracellular ROS to CD44KO/Mock cells upon TGF-β1 treatment, whereas the intracellular ROS level was significantly suppressed in CD44KO/CD44v8-10 cells (Figure S4G), indicating that CD44v3,8-10 cannot suppress intracellular ROS. Collectively, these data indicated that CD44v3,8-10 is specifically required for TGF-β1-induced vimentin expression and supports cellular migration and invasion in response to TGF-β1 treatment.

FIGURE 4 CD44v3,8-10 is required for TGF-β1-induced vimentin expression. (A) Nested RT-PCR of *CD44* transcripts in NUGC3 cells cultured with vehicle or in the presence of 10 ng/ml TGF-β1 for 3 days. Full-length *CD44* cDNAs were amplified using primers, CD44_FL_F and CD44_FL_R, and re-amplified using primers inside, CD44_s350_F and CD44_s700_R (Table S4). Molecular size (kbp) and the position of PCR fragment from each *CD44* isoform cDNA are indicated. (B) mRNA structure of each *CD44* isoform expressed in NUGC3 cells. Dashed box indicates CD44 variant exon 9. (C) Western blot. NUGC3 cells (WT), CD44KO NUGC3 cells (nontransfected, NT), and CD44KO cells transfected with mock vector (Mock), stably expressing CD44s, CD44v3,8, CD44v8-10, CD44v3,8-10, and CD44v6-10, were cultured in the presence of 10 ng/ml TGF-β1 for 3 days. The positions of molecular weight markers (kDa) are indicated in the panCD44 immunoblot. (D) Relative *VIM* mRNA expression. CD44KO cells stably expressing each CD44 isoform and cultured with vehicle or in the presence of 10 ng/ml TGF-β1 for 3 days. (E) Luciferase assay. NUGC3 (WT), CD44KO/Mock, and CD44KO/CD44v3,8-10 cells were transfected with dual-luciferase reporter plasmids (Basic and VIMpro; Figure S4E) and cultured with vehicle or in the presence of 10 ng/ml TGF-β1 for 48 h. (F, G) CD44KO NUGC3 cells stably expressing each CD44 isoform were cultured with vehicle or in the presence of 10 ng/ml TGF-β1 for 3 days. (F) Migration. (G) Invasion. All bars and error bars represent the mean and standard deviation, respectively, of three independent experiments. Student's *t* test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$
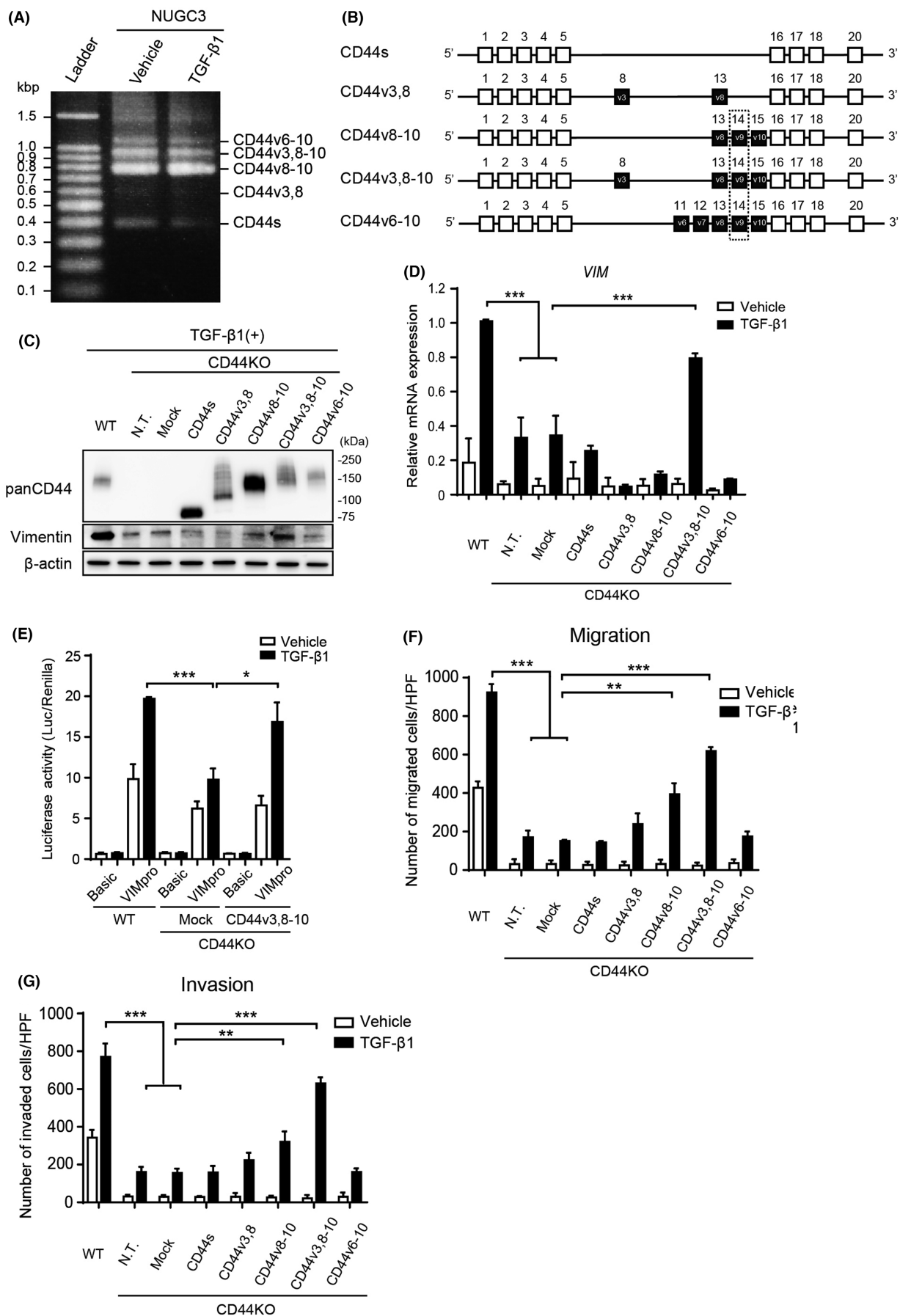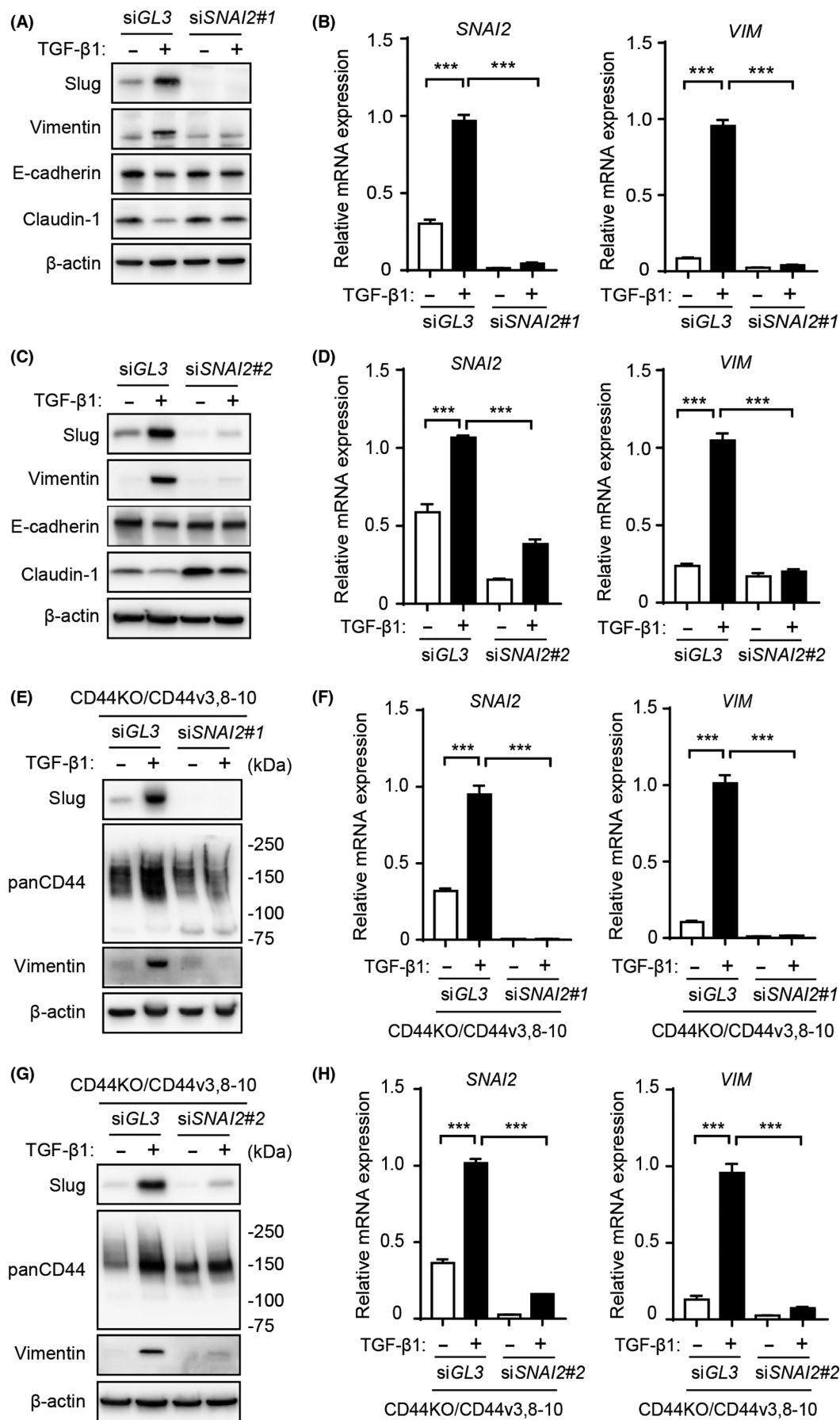
FIGURE 5 Slug is required for TGF-β1-induced downregulation of epithelial marker genes and TGF-β1-induced vimentin expression. (A–D) NUGC3 cells treated with control (si*GL3*) or Slug-targeting [si*SNAI2*#1 (A, B) or si*SNAI2*#2 (C, D)] siRNAs were cultured with vehicle (−) or in the presence of 10 ng/mL TGF-β1 (+) for 3 days. (A, C) Western blot. (B, D) Relative mRNA expression of *SNAI2* and *VIM*. (E–H) CD44KO/CD44v3,8-10 cells treated with control (si*GL3*) or Slug-targeting [si*SNAI2*#1 (E, F) or si*SNAI2*#2 (G, H)] siRNAs were cultured with vehicle (−) or in the presence of 10 ng/ml TGF-β1 (+) for 3 days. (E, G) Western blot. (F, H) Relative mRNA expression of *SNAI2* and *VIM*. All bars and error bars represent the mean and standard deviation, respectively, of three independent experiments. Student's *t* test. ***$p < 0.001$

3.5 | Slug is an essential EMT-TF for TGF-β1-induced vimentin expression, which requires CD44v3,8-10

During TGF-β1-induced EMT of NUGC3 cells, Slug appeared to be the sole EMT-TF (Figure S1A). To investigate the role of Slug in *VIM* gene expression, we designed two siRNAs targeting *SNAI2* (si*SNAI2*#1: exon2; si*SNAI2*#2: exon1; Figure S5A), and monitored TGF-β1-induced EMT and vimentin expression after *SNAI2* knockdown. Consistent with previous reports showing that Slug is directly involved in the repression of epithelial marker genes upon TGF-β1 treatment as a transcriptional repressor,[23,24] both si*SNAI2*#1 and si*SNAI2*#2 alleviated the suppression of E-cadherin and claudin-1 (Figure 5A,C). Furthermore, both si*SNAI2*#1 and si*SNAI2*#2 abrogated TGF-β1-induced vimentin (Figure 5A,C) and *vimentin* gene expression (Figure 5B,D), indicating that Slug is required for TGF-β1-induced vimentin expression. si*SNAI2*#1-treated cells exhibited a decrease in the basal CD44 protein level and TGF-β1-induced accumulation of CD44 (Figure S5A) and *CD44* transcripts, both *CD44v9* and *CD44s* (Figure S5B). By contrast, si*SNAI2*#2-treated cells expressed CD44 protein and *CD44* transcripts at a level equivalent to that in si*GL3*-treated cells, although the molecular weight of the CD44 protein was smaller than that in si*GL3*-treated cells (Figure S5A). To test whether the changes in the CD44 protein observed in si*SNAI2*-treated cells contributed to the loss of TGF-β1-induced vimentin expression, we knocked down the expression of *SNAI2* in CD44KO cells stably expressing CD44v3,8-10 (CD44KO/CD44v3,8-10), which plays a critical role in TGF-β1-induced vimentin expression (Figure 4C,D). In si*SNAI2*-treated cells, TGF-β1-induced vimentin protein (Figures 5E and S5D in another clone for si*SNAI2*#1, and Figure 5G for si*SNAI2*#2) and *VIM* gene expression (Figure 5F for si*SNAI2*#1 and Figure 5H for si*SNAI2*#2) were almost completely lost even though CD44v3,8-10 protein expression was maintained, indicating that Slug itself was required for *VIM* gene expression. Consistently, ectopic expression of Slug in NUGC3 cells enhanced TGF-β1-induced vimentin expression without affecting CD44 protein levels (Figure S5E). Finally, we explored other gastric cancer cell lines in which CD44v3,8-10 and Slug played a critical role in TGF-β1-induced vimentin expression. Among the gastric cancer cell lines listed in the Cancer Cell Line Encyclopedia (CCLE), we chose five cell lines that expressed the *SNAI2* gene at a relatively high level (HGC27, GSS, LMSU, MKN1, and SH-10-TC). In these cells, CD44v9 expression was low and TGF-β1-induced vimentin expression was not observed (data not shown). Among these cell lines, only MKN1 cells showed TGF-β1-induced Slug expression (data not shown). Next, we ectopically expressed CD44v3,8-10 in MKN1 cells

and monitored TGF-β1-induced vimentin expression. In these cells, vimentin expression was clearly enhanced on day 2 (Figure S5F), indicating that the role of CD44v3,8-10 in TGF-β1-induced Slug-dependent vimentin expression is biologically relevant and is not restricted to one gastric cancer cell line, NUGC3.

3.6 | A combination of CD44v9 expression and EMT features of primary tumors correlates with the tumor invasion depth and lymphatic metastasis of gastric cancer patients

The present cellular analysis indicated that CD44v3,8-10 and CD44v8-10, both of which contain variant exon 9, are critically involved in the migration and invasion capacity upon TGF-β1-induced EMT. To assess the relationship between CD44v9 expression and EMT in the clinical setting, we prepared a tissue microarray of primary tumors of 102 gastric cancer patients (Table S1) and performed immunohistochemical staining of CD44v9, E-cadherin, and vimentin (Figure S6A). The expression of each factor was evaluated as described in the Materials and Methods section. E-cadherin(−) vimentin(+) samples were categorized as EMT(+), whereas other samples [E-cadherin(−) vimentin(−), E-cadherin(+) vimentin(−), or E-cadherin(+) vimentin(+)] were categorized as EMT(−). CD44v9 expression was significantly associated with the EMT status (Figure 6A), supporting the close correlation between CD44v expression and EMT induction (Figures 1G and 3A–D). Overall survival (OS) and recurrence-free survival (RFS) of EMT(+) or CD44v9(+) patients was shorter than that of EMT(−) or CD44v9(−) patients, respectively (Figure S6B,C). However, no clinicopathological factors were associated with EMT status (Table S2) or CD44v9 expression (Table S3). Next, we assessed a combination of EMT status and CD44v9 expression, and separated CD44v9(+)/EMT(+) patients from others. OS (Figure 6B) and RFS (Figure 6C) of CD44v9(+)/EMT(+) patients were significantly shorter than that of other patients. Furthermore, CD44v9(+)/EMT(+) expression was significantly associated with tumor invasion depth, lymph node metastasis, and pStage (Table 1). These results suggest that the acquisition of both CD44v9 expression and EMT features in tumors leads to invasive and metastatic characteristics, which cause poor clinical outcomes.

4 | DISCUSSION

CD44 is a cell adhesion molecule that plays an important role in tumor progression and metastasis. It possesses pleiotropic functions that may correlate with tumor progression and cancer stem
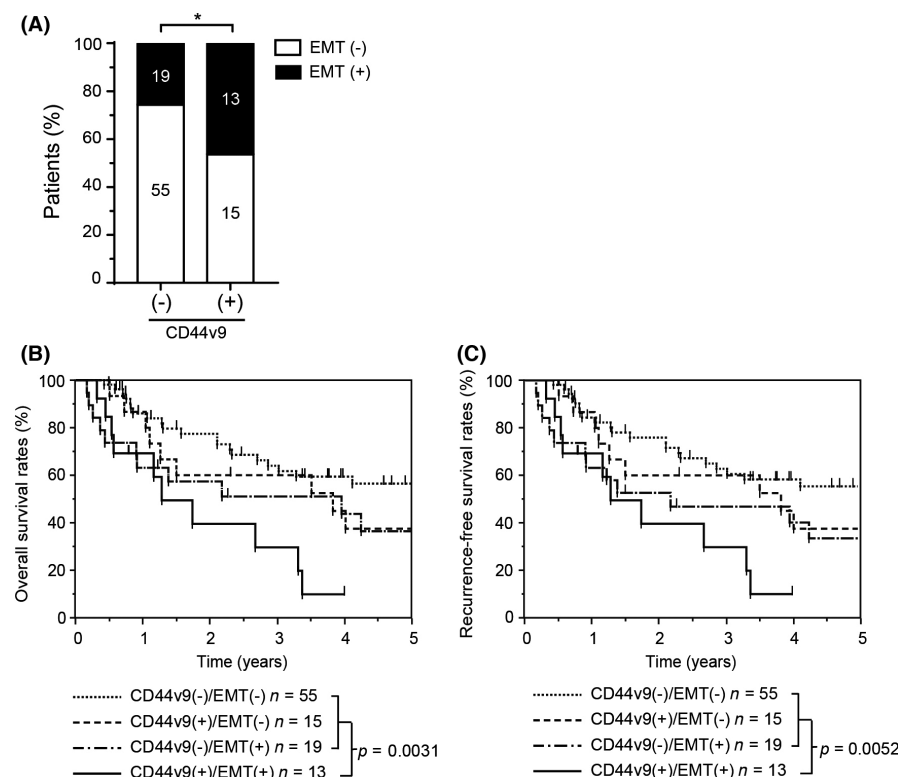
FIGURE 6 CD44v9 expression and EMT status in tumors of gastric cancer patients. (A) Correlation between CD44v9 expression and EMT status. A TMA of primary tumors from 102 gastric cancer patients was immunochemically stained with anti-CD44v9, anti-E-cadherin, and anti-vimentin antibodies. Each patient was categorized as positive or negative for each factor. The number of patients in each category is indicated inside the graph bar. Fisher's exact test. *$p < 0.05$. (B, C) Overall survival (B) and recurrence-free survival (C) of patients categorized according to CD44v9 expression and EMT status. Log-rank test. The p-value was calculated by comparing CD44v9(+)/EMT(+) patients and those in other categories

cell properties, such as enhancement of antioxidant potential.[12] In this study, we used the gastric cancer cell line NUGC3 to demonstrate a previously undescribed function of CD44v3,8-10 in the TGF-β1-induced transcriptional upregulation of the *VIM* gene, which encodes an intermediate filament protein that confers mesenchymal properties and increased migratory and invasive capacity (Figure S6D). CD44v3,8-10 is frequently expressed in colorectal adenomas and carcinomas, but not in the normal mucosa.[25] It is also preferentially expressed in primary breast tumors and axillary nodal metastases, but not in normal breast tissues.[26] The unique function of CD44v3,8-10 identified in this study indicates that the accumulation of CD44v3,8-10 may be advantageous for tumor development and progression in other types of cancer with EMT features.

Transcriptional induction of the *VIM* gene upon TGF-β1 treatment was dependent on a specific CD44v isoform, CD44v3,8-10, but not onother isoforms including CD44v8-10 and CD44v3,8 (Figure 4B,C). CD44 is a transmembrane glycoprotein that predominantly binds to hyaluronan and modulates cytoskeletal organization.[3,27] Therefore, it is unclear whether CD44v3,8-10 is directly involved in the transcriptional regulation of *VIM* gene expression as a transcription factor. CD44 interacts with other cell surface molecules through its variant exons in the stem domain.[3,9,12] CD44v3,8-10 may interact with specific molecules that contribute to TGF-β1-induced *VIM* gene expression.

In NUGC3 cells, we detected the induction of Slug, but not Snail or Zeb1, during EMT induced by TGF-β1 treatment (Figure S1). In si*SNAI2*-treated cells, we did not detect TGF-β1-induced accumulation of vimentin or suppression of E-cadherin and claudin-1 (Figure 5A), indicating that Slug is the only EMT-TF that controls the transcriptional network modulating TGF-β1-induced EMT in NUGC3

cells. Slug belongs to the Snail family of zinc finger transcriptional repressors.[28] It binds directly to E-box elements in promoters and represses the expression of the *CDH1* and *CLDN1* genes.[23,24] Consistently, TGF-β1 treatment induced Slug and *SNAI2* gene expression (Figure 1F,G) and inhibited *CDH1* and *CLDN1* gene expression (Figure 1C,D), and these effects peaked on day 1. CD44v was dispensable for this negative regulation of epithelial marker gene expression (Figure 3A,C). Furthermore, Slug was an essential EMT-TF involved in activating *VIM* gene expression in NUGC3 cells (Figure 5A,B). The induction of *VIM* gene expression increased gradually (Figure 1E,G), concomitant with the accumulation of the CD44v9 protein (Figure 1G). Although CD44v3,8-10 was required for TGF-β1-induced *VIM* gene expression (Figure 4C,D), it did not induce *VIM* expression in the absence of Slug (Figure 5D,E), suggesting that Slug is an essential transcriptional activator of the *VIM* gene upon TGF-β1 treatment with the support of CD44v3,8-10.

NUGC3 cells expressed multiple CD44 isoforms, including CD44v8-10, CD44v3,8-10, CD44v3,8, CD44v6-10, and CD44s (Figure 4A). Similar to other gastric cancer cell lines,[29] CD44v8-10 was the predominant isoform in NUGC3 cells (Figure 4A) and contributed to the increase in intracellular GSH and the suppression of ROS (Figure 2K,L), possibly resulting in tumor progression and chemoresistance.[12,30] CD44v3,8-10 may also be important for tumor malignancy because the modulation of vimentin expression determined TGF-β1-induced cell motility (Figure 3D–I). Although CD44v3,8-10 contains the v8-10 domains, which interact with xCT,[12] it had little effect on the suppression of intracellular ROS (Figure S4G), indicating that each CD44v isoform plays a distinct role in TGF-β1-induced EMT. Although CD44 isoforms other than

TABLE 1 Comparison of clinicopathological factors between CD44v9(+)/EMT(+) patients and other patients

| Factor | Others (n = 89) | | | CD44v9(+)/EMT(+)
n = 13 | p-value |
	CD44v9(−)/EMT(−) n = 55	CD44v9(+)/EMT(−) n = 15	CD44v9(−)/EMT(+) n = 19		
Sex					
Male	43 (21.8)	13 (13.3)	13 (31.6)	7 (53.8)	0.0825
Female	12 (78.2)	2 (86.7)	6 (68.4)	6 (46.2)	
Histology					
Well/Mod	23 (41.8)	6 (40.0)	4 (21.1)	6 (46.2)	0.5331
Poor/Sig	32 (58.2)	9 (60.0)	15 (78.9)	7 (53.8)	
Invasion depth					
pT1,2	20 (36.4)	6 (40.0)	7 (36.8)	1 (7.7)	0.0198*
pT3,4	35 (63.6)	9 (60.0)	12 (63.2)	12 (92.3)	
Lymph node metastasis					
Negative	19 (34.5)	7 (46.7)	7 (36.8)	1 (7.7)	0.0198*
Positive	36 (65.5)	8 (53.3)	12 (63.2)	12 (92.3)	
Ly					
Negative	17 (30.9)	5 (33.3)	5 (26.3)	1 (7.7)	0.0550
Positive	38 (69.1)	10 (66.7)	13 (68.4)	12 (92.3)	
Unknown	0 (0.0)	0 (0.0)	1 (5.3)	0 (0.0)	
V					
Negative	35 (63.6)	8 (53.3)	10 (52.6)	5 (38.5)	0.1407
Positive	20 (36.4)	7 (46.7)	8 (42.1)	8 (61.5)	
Unknown	0 (0.0)	0 (0.0)	1 (5.3)	0 (0.0)	
pStage					
I,II	24 (43.6)	7 (46.7)	9 (47.4)	2 (15.4)	0.0021*
III,IV	31 (56.4)	8 (53.3)	10 (52.6)	11 (84.6)	

Note: Data are presented as n (%).

*$p < 0.05$

CD44v8-10 and CD44v3,8-10, including CD44s, did not enhance TGF-β1-induced migration or invasion (Figure 4F,G), they may have unidentified functions that contribute to tumor progression.[4,9] In addition, it is unknown whether an individual tumor cell expresses multiple CD44 isoforms or one specific CD44 isoform. Ectopic expression of a single CD44 isoform did not fully rescue the defects in migration and invasion of CD44KO cells (Figure 4F,G). Therefore, expression of multiple CD44 isoforms in an individual cell may be necessary to acquire full migratory and invasive properties and metastatic characteristics.

CD44 contributes to tumor development and progression.[11] The identification of a novel function of a specific CD44v isoform in supporting key mesenchymal gene expression during TGF-β1-induced EMT adds a new page to the list of tumor-promoting functions of CD44. Elucidation of the precise mechanism by which CD44v3,8-10 functions in Slug-mediated transcriptional activation of the *VIM* gene will be important to understand the connection between CD44v and EMT. A considerable fraction of gastric cancer patients has the mesenchymal type with a poor prognosis, a tendency to occur at an early age, and a high frequency of recurrence.[31] In this subtype, tumors are characterized by gene expression with the EMT signature, indicating that they have been exposed to TGF-β1 supplied from cancer-associated fibroblasts, cancer cells themselves, or M2 macrophages in the microenvironment of solid tumors infiltrated into the surrounding stroma.[32] EMT is a critical event for the initiation of tumor metastasis, and CD44v9(+)/EMT(+) patients had a significantly poor prognosis (Figure 6B,C) with highly metastatic characteristics (Table 1). Therefore, targeting CD44v, especially at the early stage of cancer, may be a promising therapeutic strategy to prevent tumor metastasis.

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