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Original Article

Serine/threonine-protein kinase 24 is an inhibitor of gastric cancer metastasis through suppressing *CDH1* gene and enhancing stemness

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Abstract: Gastric cancer patients often present with distant metastasis and advanced stages. Suppressing serine/threonine-protein kinase 24 (STK24, also known as MST3) is known to promote gastric tumorigenesis. Here, we investigated the effects from STK24 on the metastasis of gastric cancer. We used CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 technology for genetic knockout of *STK24* at the genomic DNA level in human MKN45 and mouse M12 gastric cancer cells. To assess the consequences of *STK24* knockdown, western blot, cell migration, and wound healing assays were conducted *in vitro*. An *in vivo* mouse model of liver metastasis was established and tested, and bioinformatics analyses were performed. The knockdown of the *STK24* gene enhanced cell migration and increased liver metastasis in the mouse model of gastric cancer. *STK24*-silenced tumors suppressed $CD4^+$ T cells and enhanced the expansion of $CD11b^+Ly6C^+$ myeloid-derived suppressor cells (MDSCs) and $F4/80^+$ macrophages in the spleen of the mice. In MKN45 cells, *STK24* silencing resulted in downregulation of E-cadherin (gene CDH1, Cadherin-1, or epithelial cadherin). In 38 paired specimens of gastric adenocarcinomas and normal tissues, we examined STK24 and CDH1 expression levels via western blot; a positive correlation between the expression levels of STK24 and CDH1 was found ($R^2 = 0.5507$, $P = 9.72 \times 10^{-8}$). Furthermore, in Oncomine database and Kaplan-Meier plotter analysis, the loss of *CDH1*, increase in *CCL2*, and upregulation of *CD44* were correlated with poor prognosis of gastric cancer patients. Our results demonstrate that knockdown of *STK24* increases cell migration through suppressing CDH1 and enhancing CD44. In experimental model of metastatic gastric cancer in syngeneic inbred mice, STK24 is important for immune suppression through expansion of $CD11b^+Ly6C^+$ MDSCs and $F4/80^+$ macrophages. We confirmed that STK24 is an inhibitor of gastric cancer metastasis.

Keywords: Gastric cancer, serine/threonine-protein kinase 24, STK24, metastasis, myeloid-derived suppressor cells, MDSC, E-cadherin, CDH1, stemness, CD44

Introduction

Serine/threonine-protein kinase 24 (STK24) belongs to the germinal center kinase III (GCKIII) subfamily and is expressed in normal and gastric cancer tissues [1, 2]. In a previous study, the STK24 protein was found in normal tissues at significantly greater levels than in gastric cancer samples and, according to bioinformatics analyses of Kaplan-Meier Plotter and Oncomine data, the *STK24* gene was correlated with the prognosis of gastric cancer patients; importantly, *STK24* knockdown was shown to promote the tumorigenicity of gastric cancer [2]. In a cell-based study, suppression of endogenous STK24 by small interference RNA was found to enhance cellular migration in MCF-7 breast cancer cells, whereas overexpression of STK24 inhibited the migration of these cells [3]. To date, however, the effects of STK24 on gastric cancer metastasis are yet to be studied in detail; therefore, we investigate the association between the two in the present study.

Escape from immune surveillance is a critical element of metastasis. One of the tumor eva-

sion mechanisms is the expansion of immunosuppressive myeloid-derived suppressor cells (MDSCs) in the tumor microenvironment [4]. MDSCs are a collection of heterogeneous myeloid cells. They consist of two major groups of cells: mononuclear and polymorphonuclear MDSCs (M-MDSCs and PMN-MDSCs, respectively) [5-7]. Mouse M-MDSCs and PMN-MDSCs are respectively defined as $CD11b^{+}Ly6C^{high}$ $Ly6G^{-}$ and $CD11b^{+}Ly6C^{low}Ly6G^{+}$ inflammatory monocytes [6]. MDSCs play an important role in immune suppression during tumor growth and in the formation of the premetastatic niche [7]. The accumulation of circulating MDSCs is associated with the advanced stage of gastric cancer in patients [8]. Tumor metastasis and poor response to chemotherapy in advanced non-small cell lung cancer are correlated with the accumulation of M-MDSCs [9]. Both M-MDSCs and PMN-MDSCs are associated with the development of metastases and poor survival in melanoma cases [10]. Moreover, in an orthotopic immunocompetent gastric cancer model, *STK24* silencing in tumors induces an expansion of $CD11b^{+}Ly6C^{+}$ cells and $F4/80^{+}$ macrophages cells [2].

In the present study, we hypothesized that STK24 plays an important role in tumor metastasis. To test this hypothesis, we investigated the metastatic abilities of gastric cancer cell lines after knockdown of *STK24*. In addition, a mouse model of liver metastasis was used to explore the effect of STK24 in gastric cancer and tumor-infiltrating MDSCs.

Material and methods

Antibodies

The following antibodies used in this study were purchased from BD PharMingen (San Diego, CA): mouse anti-CD4 PE (H129.19), anti-CD8a PE (53-6.7), anti-CD11b PE (M1/70), anti-F4/80 PE (BM8), and anti-Ly6C FITC (AL-21) monoclonal antibodies. In addition, PE-conjugated anti-mouse CD44, PE rat IgG1, and FITC rat IgG2a isotype control antibodies were purchased from eBioscience (San Diego, CA). The remaining antibodies used were as follows: anti-MST3/STK24 (EP1468Y; Abcam, UK), mouse anti-CDH1 (BD Transduction Laboratories, San Jose, CA), rabbit anti-beta catenin (GeneTex, Inc., San Antonio, TX), rabbit anti-AKT1 and peroxidase-conjugated goat anti-rabbit IgG (Cell Signaling, Boston, MA), mouse anti-β-actin (GeneTex, Inc.), and peroxidase-conjugated sheep anti-mouse IgG (Chemica, San Diego, CA).

Cell culture

The M12 cell lines were authenticated in 2013 by DNA short tandem repeat profiling at Bioresource Collection and Research Center. The MKN45 cells were kindly provided by Professor Ming-Derg Lai (National Cheng Kung University, Tainan, Taiwan). These cell lines were maintained in RPMI 1640 medium containing 10% fetal bovine serum (FBS) (Gibco, Life Technologies, Grand Island, NY) and 1% penicillin/streptomycin. M12 cells were maintained in Dulbecco's modified Eagle's medium/high glucose supplemented with 10% FBS and 1% penicillin/streptomycin.

Mice and ethics statement

For use in all animal experiments, 8-week-old C57BL/6 mice were purchased from the Laboratory Animal Center of National Cheng Kung University (Tainan, Taiwan) and maintained under pathogen-free conditions. The animal study was approved by the institutional animal care and use committee of National Cheng Kung University with approval No. NCKU-IACUC-106-288. The methods were carried out in accordance to the approved guidelines.

Metastatic model of gastric cancer

The metastatic abilities of M12 cells were evaluated *in vivo* using a hepatic metastasis model in immunocompetent C57BL/6 mice [11]. To establish this model, mice were first anesthetized by an intraperitoneal injection of Zoletil (50 mg/kg; Parnell Laboratories, Alexandria, Australia) and xylazine (10 mg/kg; Troy Laboratories, Glendenning, Australia). A small midline incision was then made in the abdomen. The spleen was exteriorized and 5×10^5 tumor cells in 0.05 mL of PBS were injected into the spleen using a 1-cm^3 U-100 disposable insulin syringe (Becton-Dickinson, Franklin Lakes, NJ). Fourteen days after this injection, the mice were sacrificed. Hepatic and splenic masses were examined macroscopically and histologically. Formalin-fixed/paraffin-embedded sections of the stomach, liver, and spleen were subjected to hematoxylin and eosin stain-

ing. Fresh tissues were also collected for western blotting or flow cytometry. Number of animals was at least 5 mice in each group.

Construction of CRISPR knockout STK24 plasmid and generation of stable cancer cell clones

Single guide RNAs (sgRNAs) targeting mouse *Stk24* and human *STK24* were purchased from the National RNAi Core Facility (Academia Sinica, Taiwan; http://rnai.genmed.sinica.edu.tw). The DNA sequences for generating mouse sgRNA were prepared as previously described [2]. The DNA sequences of human sgSTK24-RNA1 and sgSTK24-RNA2 were as follows: sgSTK24-RNA1 forward: 5'-CAC CGC GCC AAA GTC CGC CAG CTT C-3'; sgSTK24-RNA1 reverse: 5'-AAA CGA AGC TGG CGG ACT TTG GCG C-3'; sgSTK24-RNA2 forward: 5'-CAC CGT AGT TTC CTT CCA ACG TCG G-3'; and sgSTK24-RNA2 reverse: 5'-AAA CCC GAC GTT GGA AGG AAA CTA C-3'. A Cas9/gRNA vector construct was introduced into the MKN45 and M12 cells by transfection with Lipofectamine 3000 (Invitrogen, Waltham, MA) for 48 h. To create stable clones, selection was performed with puromycin (1 μg/mL) (Sigma-Aldrich, St. Louis, MO) for 2 weeks. Single-cell clones of the transfectants were selected using the limiting dilution method. Expression of STK24 protein in the stable transfectants was analyzed by western blotting for monitoring the efficacy of *STK24* silencing.

Patients

Fresh specimens were collected from 38 patients with gastric adenocarcinoma who underwent radical resection at the National Cheng Kung University Hospital between August 2003 and August 2008. Pairs of cancerous tissues and matched adjacent normal gastric mucosa were collected and analyzed by Western blotting.

Western blot analysis

Total cell lysates and fresh samples from patients were prepared by administrating in lysis buffer at 4°C for 30 minutes, then centrifuged for 5 minutes at 12,000 g to remove the debris. The protein concentration of the supernatants was measured by BCS™ protein assay kit according to manufacturer's protocol. Twenty mg protein was separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes as previously described [11]. Following blocking with 5% nonfat skim milk, the PVDF membranes were incubated with primary antibody overnight at 4°C. For quantification, the bands were measured using the AlphaImager 2200 system (Alpha Innotech, San Leandro, CA) and normalized using the density of β-actin. The expression of STK24 was quantified and given as the STK24-to-β-actin ratio. These experiments were repeated three times using independent batches of cell clones or cell lysates. Quantitative data are presented as values relative to those in control cells.

Cell migration and wound healing assays

Cell migration was evaluated in modified Boyden chambers (NeuroProbe, Inc., Gaithersburg, MD) for 8 h. MKN45 cells (70 μL of 1 × 10^6 cells per mL) or M12 cells (70 μL of 2 × 10^5 cells per mL) were seeded in an ibidi culture insert (Applied BioPhysics, Inc., Martinsried, Germany) on top of a 24-well plate. After overnight incubation, the insert was carefully removed to form a cell-free gap in the attached cells. The time of incubation was dependent on the tumor cells used. The number of migrating cells was calculated and analyzed. Six fields were randomly selected for analysis.

Flow cytometry analysis

Spleens were harvested from M12 liver metastasis-bearing mice, minced into small pieces, then treated with lysis buffer on ice for 10 minutes to remove the red blood cells. The single cell suspension of splenocytes were washed by flow staining buffer (BD, Pharmingen, San Diego, CA), then filtered through a 0.35-μm cell strainer. The cells were stained with Fc blocker for 15 minutes on ice. After Fc blocking, suspension of splenocytes were stained with mouse anti-CD4 PE (H129.19), anti-CD8a PE (53-6.7), anti-CD11b PE (M1/70), anti-Ly6C FITC (AL-21), anti-F4/80 PE (BM8), or anti-CD44 PE (IM7) monoclonal antibodies. After staining, BD Cytofix/Cytoperm™ Plus Fixation/Permeabilization Kit was used according to manufacturer's protocols. A FACScan (Becton Dickinson, CA) flow cytometer was used to characterize the immune cells as previously described [12].

Bioinformatics

A search was conducted in the Oncomine database (http://www.oncomine.com) [14] to systematically assess the expression level of *CDH1* genes in gastric cancer. For differential analyses, we compared normal tissues and cancer tissues, specifically via analysis of *P* values, fold changes, and cancer subtypes. The prognostic value of *CDH1* genes in gastric cancer was also analyzed using the Kaplan-Meier Plotter (http://kmplot.com/analysis/) as previously described [15]. The overall survival (OS), progression-free survival (PFS), and postprogression survival (PPS) were recorded, and the cut-off points for gene expression were automatically selected using the default setting. The probe of *CDH1* gene was "201131_s_at". The hazard ratio (HR), 95% confidence intervals, and log rank *P* values were displayed. Data from the Oncomine database and Kaplan-Meier Plotter were extracted between July and August 2020. Finally, the association between CDH1 protein expression (CDH1-to-β-actin ratio) and the Lauren classification (intestinal, diffuse, and mixed) of patients with gastric adenocarcinoma was assessed in the fresh specimens. The statistical differences between each two groups were analyzed.

Epithelial mesenchymal transition (EMT)-related genes were defined according to a meta-analysis of 14 gene expression studies [16]. The gene lists were applied to the raw data of gastric cancer in The Cancer Genome Atlas (TCGA). Hierarchical clustering was performed in R to produce a heatmap. Gene expression data were also obtained from GSE112369, a dataset of gastric organoids for which the raw data was publicly available [17]. Expression levels of *STK24* and *CDH1* were extracted. Gastric organoids forming from *CDH1*-single-knockout and parental cells were selected for further comparison.

Statistical analysis

Data were expressed as means ± standard deviations (SDs). Statistical analyses were performed in Prism (Graphpad Software, San Diego, CA). Student's *t*-test was used for two-group comparisons, whereas one-way ANOVA followed by Tukey's test was used for multiple-group comparisons. *P* values < 0.05 were considered statistically significant.

Results

Suppression of STK24 expression in the gastric cancer cells

Expression of STK24 proteins was examined in human and mouse cancer cell lines. Three gastric cancer cell lines expressed lower level of STK24 protein than colon cancer cell lines (Figure S1). To examine the effect of STK24 in cancer metastasis, we knocked down *STK24* gene expression using two different sgRNAs in gastric cancer cell lines (human MKN45 and mouse M12). We established four clones of *STK24*-sgRNA constructs (sgSTK24-1.1, sgSTK24-1.2, sgSTK24-2.1, and sgSTK24-2.2) and one clone of a pEGFP (enhanced green fluorescent protein) control in each cell line. The successful suppression of the STK24 protein in MKN45 (**Figure 1A**) and M12 (**Figure 1B**) cells was validated by western blotting. The cell proliferation rates of the pEGFP control (EGFP-Ctrl) and sgSTK24-expressing cells were similar in MKN45 cells (**Figure 1C**). In a previous study, the knockout of *STK24* expression did not affect the cell growth rates of mouse M12 cancer cells [2]. Therefore, the suppression of STK24 did not affect the cell growth rates of gastric cancer.

The effect of STK24 suppression on cell migration in gastric cancer cells

To test the hypothesis that STK24 plays an important role in tumor migration, we examined the cell motility of MKN45 and M12 cells *in vitro*. STK24 suppression of MKN45 cells and M12 cells increased the number of migrating cells in a wound healing assay at 24 h (**Figure 2A**, **2C**) and 12 h (**Figure 2B**, **2D**), respectively. MKN45- and M12-sgSTK24 cells each exhibited stronger potential for cell migration. In addition, M12-sgSTK24 cells exhibited a relatively higher potential for migration in a Transwell migration assay performed for 8 h using 10% FBS as a chemoattractant (see Figure S2). This association between cell migration and STK24 expression in gastric cancer cell lines suggests STK24 plays important role in mediating metastasis.

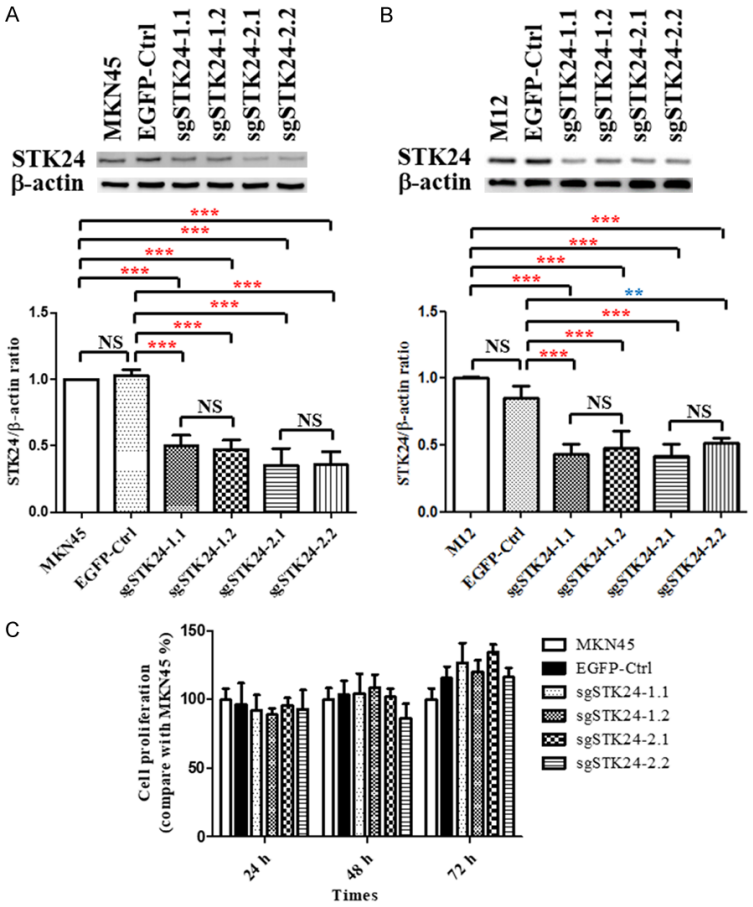

Figure 1. STK24 silencing in gastric cancer cell lines does not influence cell proliferation. A. Expression of STK24 protein in human MKN45 cells and stable transfectants. B. Expression of STK24 protein in mouse M12 cells and stable transfectants. Western blotting results were obtained from three independent experiments. C. The proliferation rates of MKN45 cells and STK24 sgRNA stable transfectants determined at 24, 48, and 72 h. Data in all graphs represent the mean ± SD. EGFP-Ctrl: EGFP control; sgSTK24-1.1, sgSTK24-1.2, sgSTK24-2.1, and sgSTK24-2.2: STK24-specific sgRNAs 1 and 2; NS: not significant; $^{*}P < 0.01$; $^{**}P < 0.001$; and $^{***}P < 0.0001$.

Effect of STK24 suppression on liver metastasis in a mouse model of gastric cancer

To test the hypothesis that STK24 plays an important role in tumor metastasis, we examined the changes of metastatic ability in the *in vivo* orthotopic intrasplenic implantation model of gastric cancer established in C57BL/6 mice [11]. Injection of M12 parental cells resulted in macroscopic nodules in the liver (**Figure 3A**). The weights of the livers (**Figure 3A**, **3C**) and spleens (**Figure 3B**, **3D**) of mice injected with sgSTK24-1.1 and sgSTK24-2.1 cells were significantly higher than the weights of equivalent organs in mice injected with EGFP-Ctrl cells. Moreover, the nodules were confirmed as liver metastasis by histopathologic analyses of liver sections (**Figure 3E**). In the M12 mouse model, we demonstrated that the metastatic burden was increased in STK24-knockdown cells. Thus, *in vitro* and *in vivo* results showed that STK24 plays a significant role in the metastasis of mouse gastric cancer.

Immune regulation in tumor-bearing mice

M12 mouse gastric cancer cells were transfected with EGFP-Ctrl or two types of STK24-sgRNA. Liver metastases developed after intrasplenic injection of cancer cells in immunocompetent mice; subtypes of splenocytes were then investigated to assess STK24-mediated immunity in liver metastasis of gastric cancer. The proportion of $CD4^+$ cells was significantly higher in the spleens of EGFP-Ctrl-tumor-bearing mice than in those of both types of sgSTK24-tumor-bearing mice (**Figures 4A**, **4B** and S3A; Table S2). The proportion of the $CD8^+$ T cells in splenocytes decreased in sgSTK24-2.1-tumor-bearing mice. The knockdown efficacy of sgSTK24-1.1 was not strong enough to induce alteration of intrasplenic $CD8^+$ T cells (**Figures 4A**, **4C** and S3B; Table S2). The proportion of $F4/80^+$ macrophages significantly increased in the spleens of sgSTK24-tumor-bearing mice (**Figures 4A**, **4D** and S3C; Table S2). Considering the two major MDSC subtypes, i.e., the $CD11b^+Ly6C^+$ or $CD11b^+Ly6G^+$ phenotypes, the $CD11b^+Ly6C^+$ subtype significantly increased in the spleens of sgSTK24-tumor-bearing mice (**Figures 4E**, **4F** and S3D; Table S2). In addition, the subpopulations of infiltrating monocytes were assessed: accumulations of $CD11b^+Ly6C^{high}$ ($CD11b^+Ly6C^{hi}$) cells (inflammatory monocytes) and $CD11b^+Ly6C^{low}$ ($CD11b^+Ly6C^{lo}$) cells (reparative monocytes) were confirmed by gating on

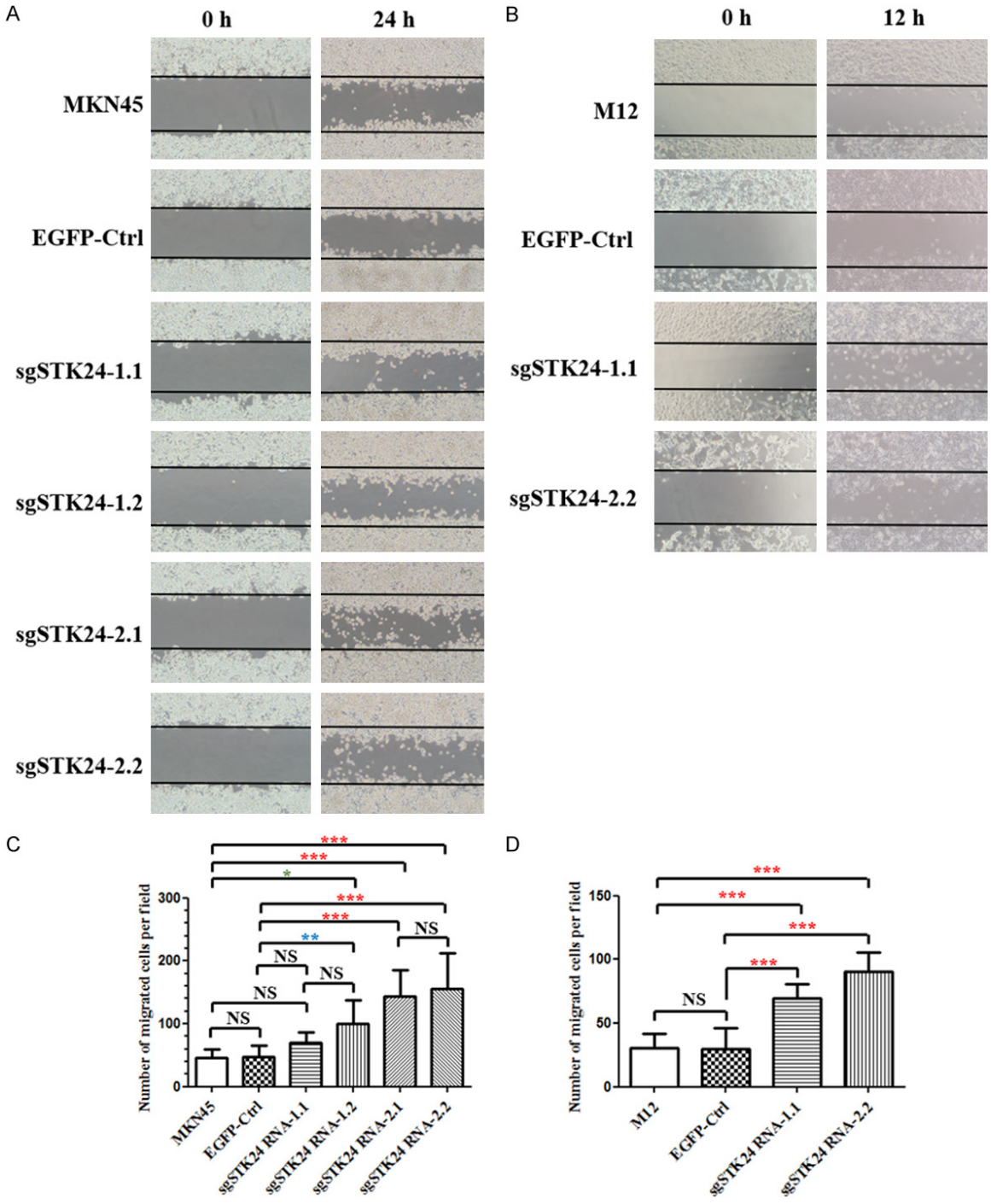

Figure 2. Cell migration is enhanced in *STK24*-silenced gastric cancer cells. Wound healing assays of (A) human MKN45 parental and *STK24*-knockdown cells and (B) mouse M12 parental and *STK24*-knockdown cells. *STK24*-knockdown cells were grown in culture medium containing 10% FBS. Quantitative results from (C) the wound healing assay with human MKN45 cells at 24 h and (D) the wound healing assay with mouse M12 cells at 12 h. Data represent the mean number of cells per field of view ± SD from three independent experiments. NS: not significant; $^{*}P < 0.01$; $^{**}P < 0.001$; and $^{***}P < 0.0001$.

$CD11b^{+}Ly6C^{+}$ cells (**Figures 4E** and S3D). Inflammatory $CD11b^{+}Ly6C^{hi}$ and reparative $CD11b^{+}Ly6C^{lo}$ cells were markedly increased in the spleens of sgSTK24-tumor-bearing mice (**Figures 4E**, **4G**, **4H** and S3D; Table S2). These results indicate that *STK24* silencing in tumors

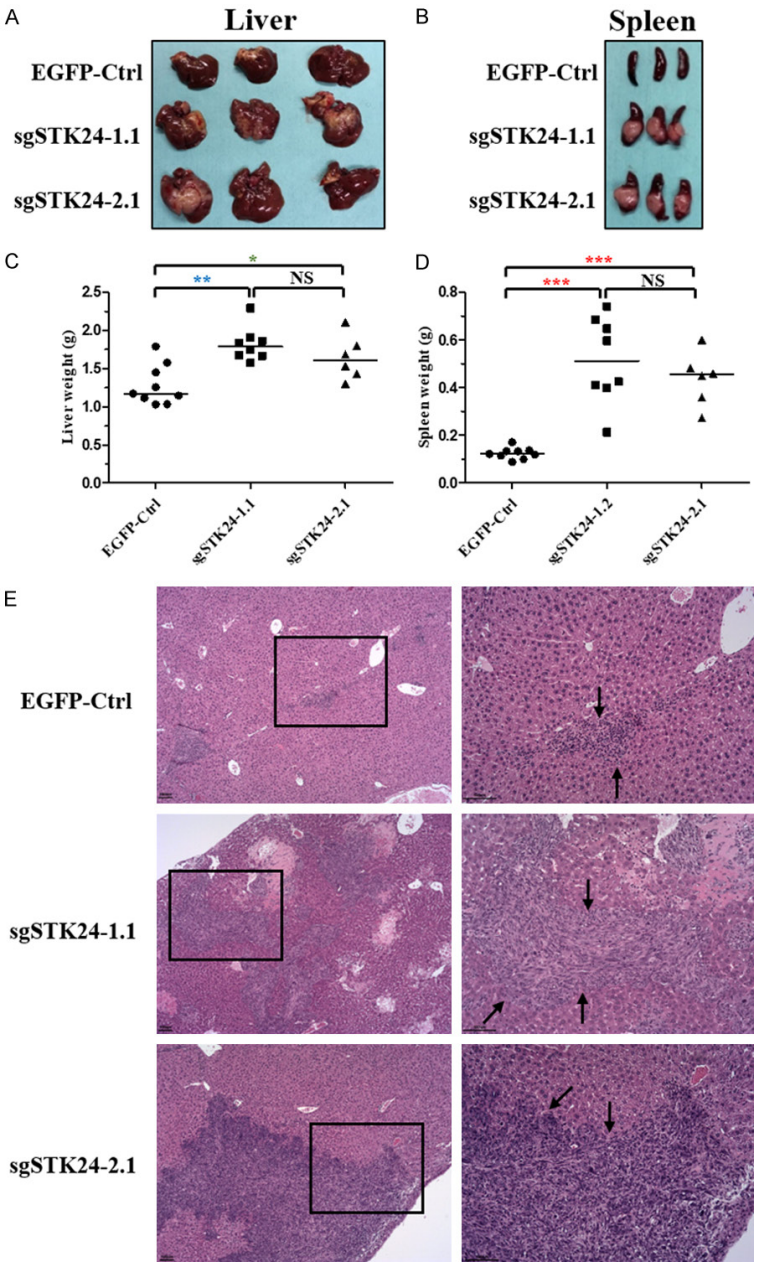

Figure 3. Knockout of *STK24* expression in mouse M12 gastric cancer cells promotes metastatic ability. (A) The gross appearance of the liver metastasis and (B) spleen implants after intrasplenic injections of EGFP-Ctrl and *STK24*-silencing cancer cells. The weights of (C) the livers and (D) the spleens of C57BL/6 mice 14 days postinjection. Data represent the means ± SDs of two independent experiments (n = 6-9 per group). (E) Hematoxylin and eosin staining of formalin-fixed, paraffin-embedded sections from liver metastasis samples. Metastatic lesions are larger in *STK24*-silenced tumors. EGFP-Ctrl: EGFP control; sgSTK24-1.1 and sgSTK24-2.1: STK24-specific sgRNAs 1 and 2. The boxed area in the left panels (× 100) is shown at a higher magnification (× 400) in the right panels. Tumor cells are indicated by arrows. NS: not significant; $^{*}P < 0.01$; $^{**}P < 0.001$; and $^{***}P < 0.0001$.

induces the expansion of F4/80^{+} macrophages, CD11b^{+}Ly6C^{hi}, and CD11b^{+}Ly6C^{lo} monocytes *in vivo*; thus, an increase in these types of monocytes/macrophages may play an important role in gastric metastasis.

The recruitment of immune cells relies on cancer-secreted cytokines and CCL2-assocated cytokine network involves in cancer metastasis [18]. Therefore, we explored the transcript expression of *CCL2* genes in gastric cancer patients using the Oncomine database. We focused on datasets in which cancer patients and normal patients were compared [19-22]. The histological type of gastric adenocarcinoma was divided into gastric intestinal adenocarcinoma (GITA), diffuse gastric adenocarcinoma (DGA), and gastric mixed adenocarcinoma (GMA), all of which showed upregulation of *CCL2* (see Figure S4A-C). Compared to the other subtypes of gastric cancer, the expression of *CCL2* was significantly increased in DGA (see Figure S4D-F). Our analyses of the Oncomine cancer microarray database revealed that *CCL2* gene expression was significantly increased in gastric cancer, especially in DGA.

Regulation of the EMT process by STK24

We hypothesized that *STK24* silencing induced migration and metastasis of gastric cancer through the EMT process; hence, we examined the EMT proteins of MKN45 parental cells and knockdown clones *in vitro*. The knockout of STK-24 expression did not affect the AKT1 or phospho-AKT Ser^{473} protein of MKN45 cells (**Figures 5A** and S5). E-cadherin and β-catenin are key proteins in EMT; expression of each protein was suppressed by

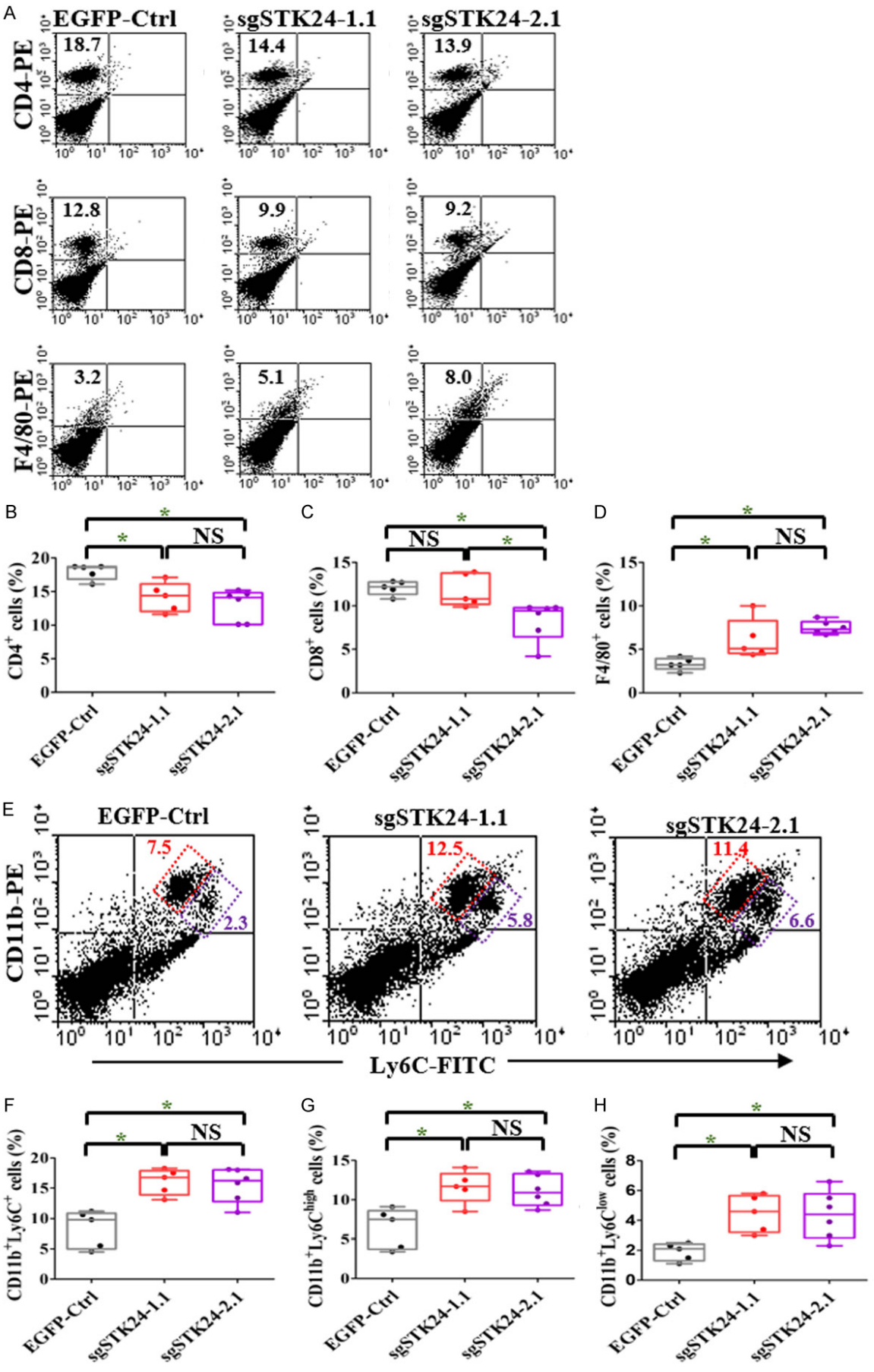
A
EGFP-Ctrl
sgSTK24-1.1
sgSTK24-2.1
CD4-PE
18.7
14.4
13.9
CD8-PE
12.8
9.9
9.2
F4/80-PE
3.2
5.1
8.0
B
CD4+ cells (%)
C
CD8+ cells (%)
D
F4/80+ cells (%)
NS
EGFP-Ctrl
sgSTK24-1.1
sgSTK24-2.1
E
CD11b-PE
7.5
2.3
12.5
5.8
11.4
6.6
Ly6C-FITC
F
CD11b+Ly6C+ cells (%)
G
CD11b+Ly6Chigh cells (%)
H
CD11b+Ly6Clow cells (%)

Figure 4. Populations of immune cells in the spleens of liver metastasis-bearing mice. Accumulations of F4/80$^+$ macrophages and CD11b$^+$Ly6C$^+$ cells were detected by flow cytometry in mice with STK24-silenced liver metastasis. (A) Typical example of flow cytometry analysis. The numbers shown indicate the percentage of total cells. The percentage of (B) CD4$^+$ cells, (C) CD8$^+$ cells, and (D) F4/80$^+$ cells in the spleens of liver metastasis-bearing mice. (E) Cells were double-stained with anti-CD11b and anti-Ly6C antibodies. Flow cytometric analysis of inflammatory monocytes (CD11b$^+$Ly6C^{high}: violet square) and reparative monocytes (CD11b$^+$Ly6C^{low}: red square) in the spleens of liver metastasis-bearing mice are shown. The percentage of (F) CD11b$^+$Ly6C$^+$ cells, (G) CD11b$^+$Ly6C^{high} cells, and (H) CD11b$^+$Ly6C^{low} cells in the spleens of liver metastasis-bearing mice. All data represent the value of each mouse, median, 1st and 3rd quartiles, minimum and maximum from Table S2. EGFP-Ctrl: EGFP control; sgSTK24-1.1 and sgSTK24-2.1: STK24-specific sgRNAs 1 and 2; NS: not significant; $^*P < 0.05$.

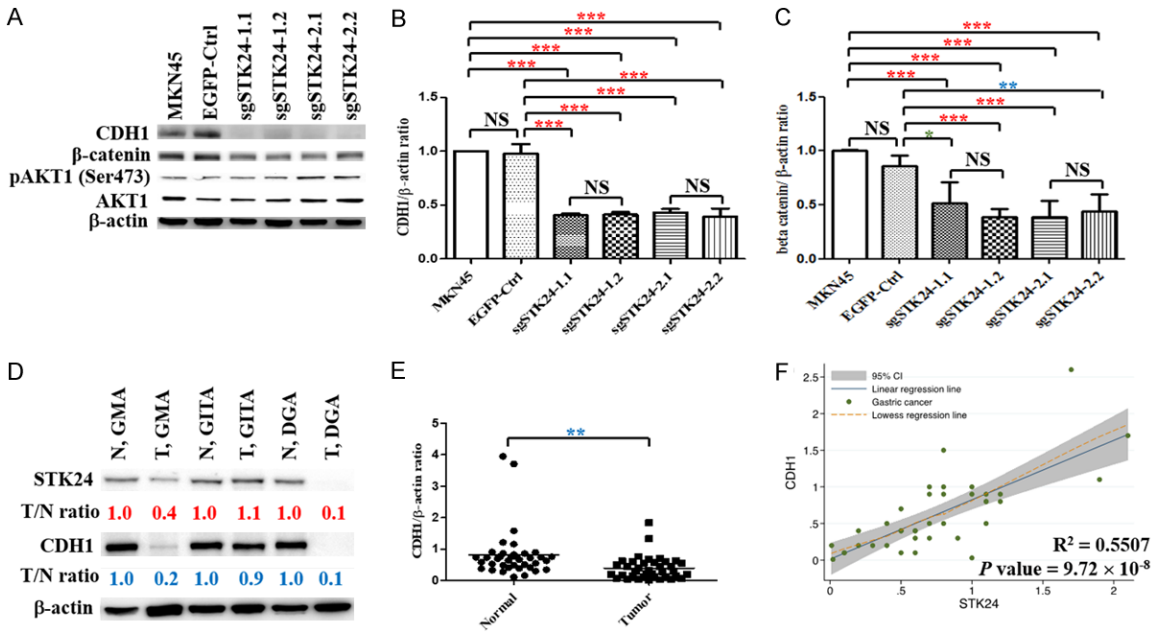

Figure 5. Knockdown of *STK24* expression suppresses CDH1 protein in human gastric cancer cells. (A) *STK24*-specific sgRNAs inhibit expression of CDH1, β-catenin protein in human MKN45 gastric cancer cells, but not pATK1 Ser473 and AKT1 proteins. Quantitative results of (B) the CDH1/β-actin ratio and (C) the β-catenin/β-actin ratio obtained by western blot analysis. Data were collected in three independent experiments. β-actin was used as the internal controls. (D) STK24 and CDH1 expression measured in 38 specimens of gastric cancer and paired normal stomach tissues by western blot. (E) Quantitative results of the CDH1/β-actin ratio compared between normal stomach and gastric cancer tissues. Normal gastric tissue had a higher CDH1/β-actin ratio. (F) Positive correlation of STK24 and CDH1 protein levels in gastric cancer ($R^2 = 0.5507$, $P < 0.001$, Pearson's correlation coefficient). EGFP-Ctrl: EGFP control; sgSTK24-1.1 and sgSTK24-2.1: STK24-specific sgRNAs 1 and 2; NS: not significant; $^*P < 0.01$; $^{**}P < 0.001$; and $^{***}P < 0.0001$.

STK24 silencing in MKN45 cells (**Figure 5B**, **5C**). To further investigate the relationship between STK24 and CDH1 in gastric cancer, we compared the expression of STK24 and CDH1 in 38 matched specimens of gastric adenocarcinoma (which included 11 DGA, 22 GITA, and 5 GMA) and normal tissues by western blot analysis (see Table S1). **Figure 5D** shows the relative expression of STK24 and CDH1 in these tissues. The relative ratio of CDH1-to-β-actin was higher in normal gastric tissues than in gastric cancer tissues (**Figure 5E**). Furthermore, a significant positive correlation was identified between the expression levels of STK24 and CDH1 ($R^2 = 0.5507$; **Figure 5F**). This positive correlation was validated using TCGA database. The mRNA expression levels of *STK24* and *CDH1* showed a significant positive correlation in clinical data of stomach adenocarcinoma (Figure S6A).

Gene expression in gastric cancer from TCGA was hierarchically clustered as EMT-related and EMT-unrelated (see Figure S6B). The upper half was correlated with mesenchymal phenotypes and the lower half with epithelial pheno-

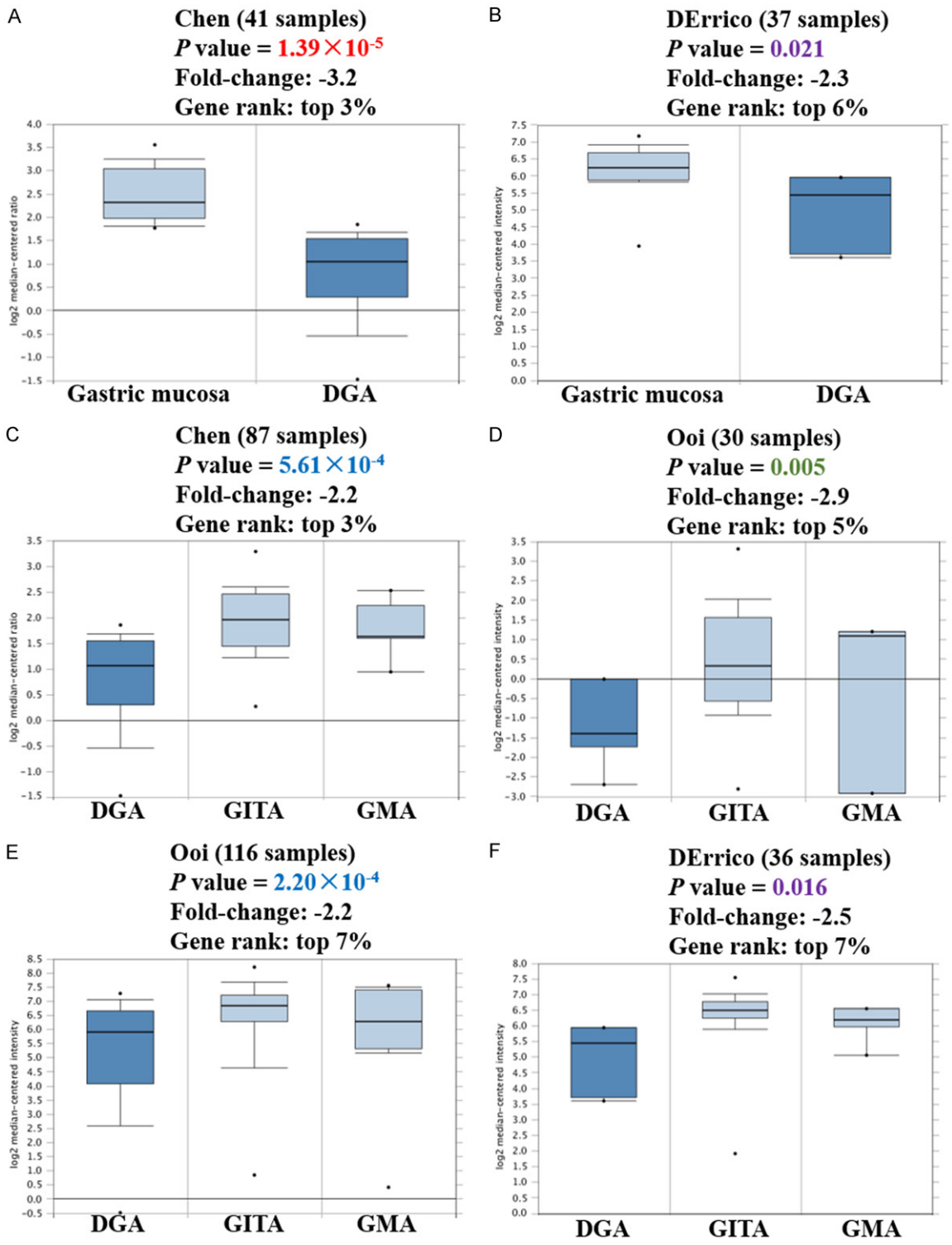

Figure 6. *CDH1* gene expression analysis of gastric cancer data acquired from the Oncomine database. A. Chen dataset. B. DErrico dataset. Expression of *CDH1* in diffuse gastric adenocarcinoma (DGA) is lower than in normal gastric mucosa. C. Chen dataset. D, E. Ooi dataset. F. DErrico dataset. The expression of *CDH1* is significantly decreased in DGA compared with its expression in other subtypes of gastric cancer. GITA: gastric intestinal type adenocarcinoma; GMA: gastric mixed adenocarcinoma; CDH1: Cadherin-1 or epithelial cadherin (E-cadherin).

types, while *CDH1* and *STK24* were strongly related. These correlations from our patients' cancer specimens and TCGA confirmed the positive association between CDH1 and STK24;

however, such correlations do not prove causal relationships. Currently, high-throughput datasets including *STK24*-knockout cells are unavailable; however, we identified a dataset of gastric organoids for which the raw data was publicly available (i.e., GSE112369) [17]. The expression level of *STK24* was similar in *CDH1*-single-knockout and parental cells (see Figure S6C). Thus, knockdown of *CDH1* was directly shown to have no effect on the expression of *STK24*; furthermore, *STK24* was indirectly shown to be upstream of CDH1.

Association of CDH1 expression with the survival of gastric cancer patients

Because E-cadherin is associated with metastatic behavior in cancer cells, we explored the expression of *CDH1* genes in gastric cancer patients using the Oncomine database to compare data from cancer and normal patients [19, 20, 22]. The statistical significance and fold change of *CDH1* expression were comparatively analyzed in normal and cancer tissues. Downregulation of *CDH1* genes occurred in DGA than normal gastric mucosa (**Figure 6A**, **6B**). *CDH1* expression significantly decreased in DGA than other subtypes of gastric cancer (**Figure 6C-F**). Thus, analysis of the Oncomine cancer microarray database revealed that *CDH1* gene expression was significantly downregulated in DGA.

According to the Kaplan-Meier Plotter (**Figure 7**), a significant relationship existed between *CDH1* mRNA expression and patient survival: low expression of *CDH1* was correlated with worse OS, PFS, and PPS (**Figure 7A-C**, respectively). Analysis of the Kaplan-Meier survival curves revealed that *CDH1* gene expression significantly reduced OS, PFS, and PPS in DGA and GITA patients specifically (**Figure 7A-C**, respectively). In summary, the downregulation of *CDH1* in DGA and GITA was associated with poor patient prognosis.

Prediction of protein-protein interactions in gastric cancer

The Search Tool for the Retrieval of Interacting Genes (STRING) database was used to identify relevant protein-protein interactions (**Figure 8A**). A network including STK24, CDH1, AKT1, and CTNNB1 was constructed and linked to CCND1, IL6, STAT3, and CD44 via this database. As *STK24* knockdown was associated with enhanced cell migration (**Figure 2**), we considered the upregulation of other migration-associated molecules. Specifically, we analyzed the expression of stem cell marker CD44 in MKN45 cells. Flow cytometry revealed that increased membranous expression of CD44 occurred in MKN45-STK24-sgRNA cells (see Figure S7). Western blotting showed that MKN45-sgSTK24-expressing cells exhibited relatively high CD44 expression (see Figure S8). We also extracted expression data of *CD44* transcripts from the Oncomine database for gastric cancer, focusing on comparisons between cancer and patient datasets [19, 20, 23]. Our analysis included comparisons of statistical significance and fold change of *CD44* expression between normal and cancer tissues. Upregulation of *CD44* was determined in DGA, GITA, and GMA than normal gastric mucosa (see Figure S9A-I), with *CD44* expression being significantly increasing in DGA relative to the other subtypes (see Figure S9J).

A scheme of STK24 signaling in gastric cancer is shown in **Figure 8B**. *STK24* suppression effectively enhances the migration and metastatic potential of human MKN45 and mouse M12 gastric cancer cells *in vitro* and *in vivo*. Our data suggest that the STK24 mediates stemness and immunosuppression in gastric cancer through CD44 and via interactions with macrophages and $CD11b^{+}Ly6C^{+}$ MDSCs.

Discussion

In our previous studies, we demonstrate that suppression of *STK24* in M12 gastric cancer cells promotes tumorigenesis in an animal model [2] and is a predictor of poor prognosis in gastric cancer patients [13]. In the present study, we demonstrate the suppression of *STK24* promotes migration and metastasis in gastric cancer cells. STK24 is constitutively expressed in MKN45, AGS, and NCI-N87 cells, and the relative expression of the STK24 protein in normal tissues is significantly greater than that in gastric cancer samples [2]. We also find that the suppression of *STK24* does not affect the proliferation of MKN45 cancer cells *in vitro*; however, *STK24* suppression effectively enhances the migration and metastatic potential of gastric cancer cells *in vitro* and *in vivo* with overexpression of membranous CD44.

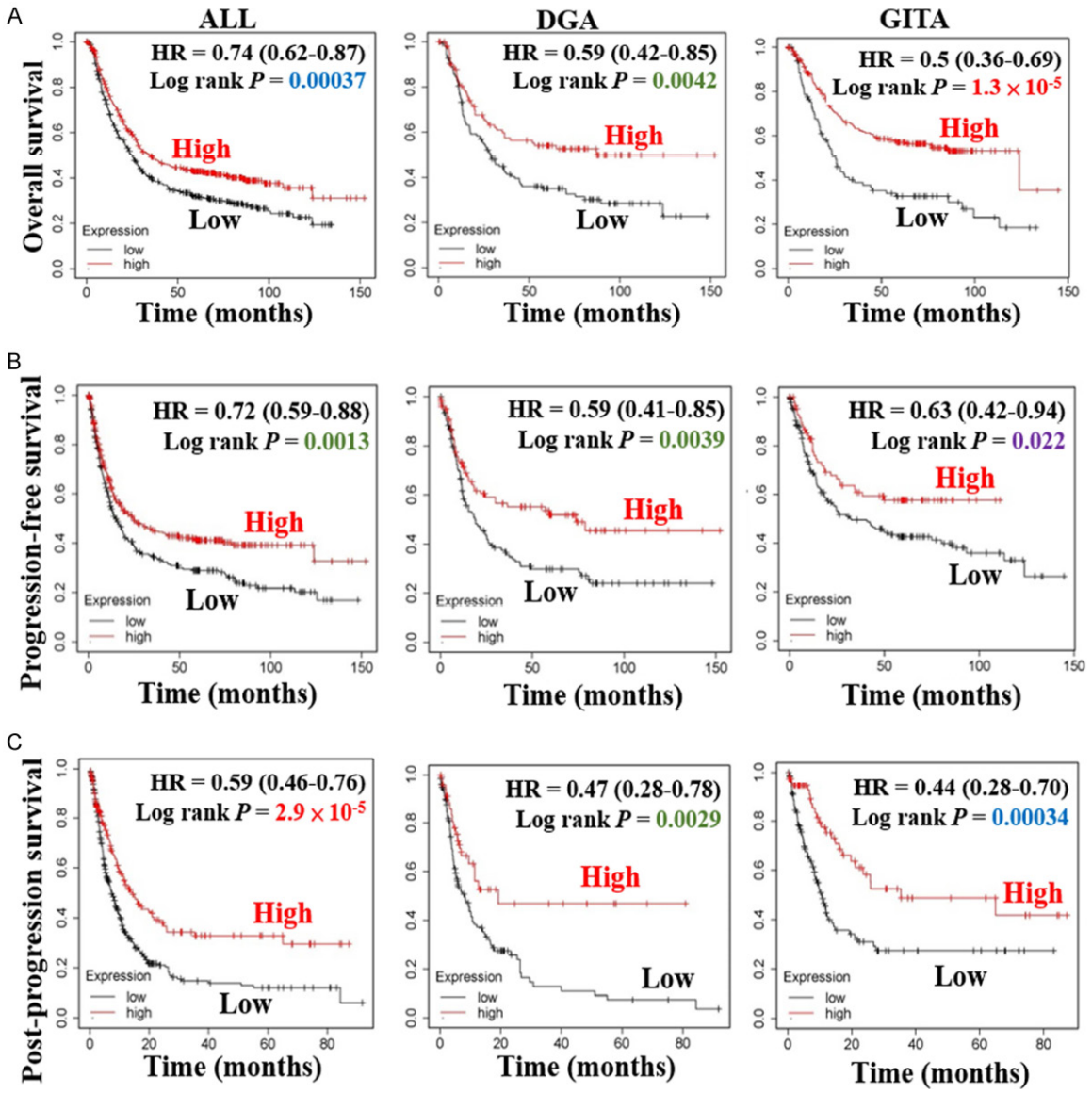

Figure 7. Kaplan-Meier Plotter survival curves of patients with gastric cancer relative to their *CDH1* expression levels. A. Overall survival. B. Progression-free survival. C. Post-progression survival. Data for patients in the low and high *CDH1* gene expression groups are shown along with hazard ratios (HR) and *P* values (log rank *P*). DGA: diffuse gastric adenocarcinoma; GITA: gastric intestinal type adenocarcinoma.

$CD11b^+Ly6C^+$ MDSCs and $F4/80^+$ macrophages were expanded in spleen of mice with *STK24*-silenced tumors. In human gastric cancer, positive correlation of STK24 and CDH1 expression was shown. Downregulation of CDH1, upregulation of CCL2 and CD44 were correlated with a worse prognosis of gastric cancer patients. Our data suggest that STK24 suppression takes the responsibility of the high metastatic potential of gastric cancer through stemness and immunosuppression.

The human MKN45 gastric cancer cell line is derived from a metastatic lesion in the liver; these cells exhibit a poorly differentiated primary gastric cancer of diffuse histology [24]. Gastric adenocarcinomas can be approximately subgrouped into 50% GITA, 30% DGA, and 15%-20% GMA [25]. DGA is an aggressive, infiltrating carcinoma with poor prognosis [26-29]. DGA patients have a decreased responsiveness to chemotherapy and chemoradiation [30-32]. The possible mechanism of progres-

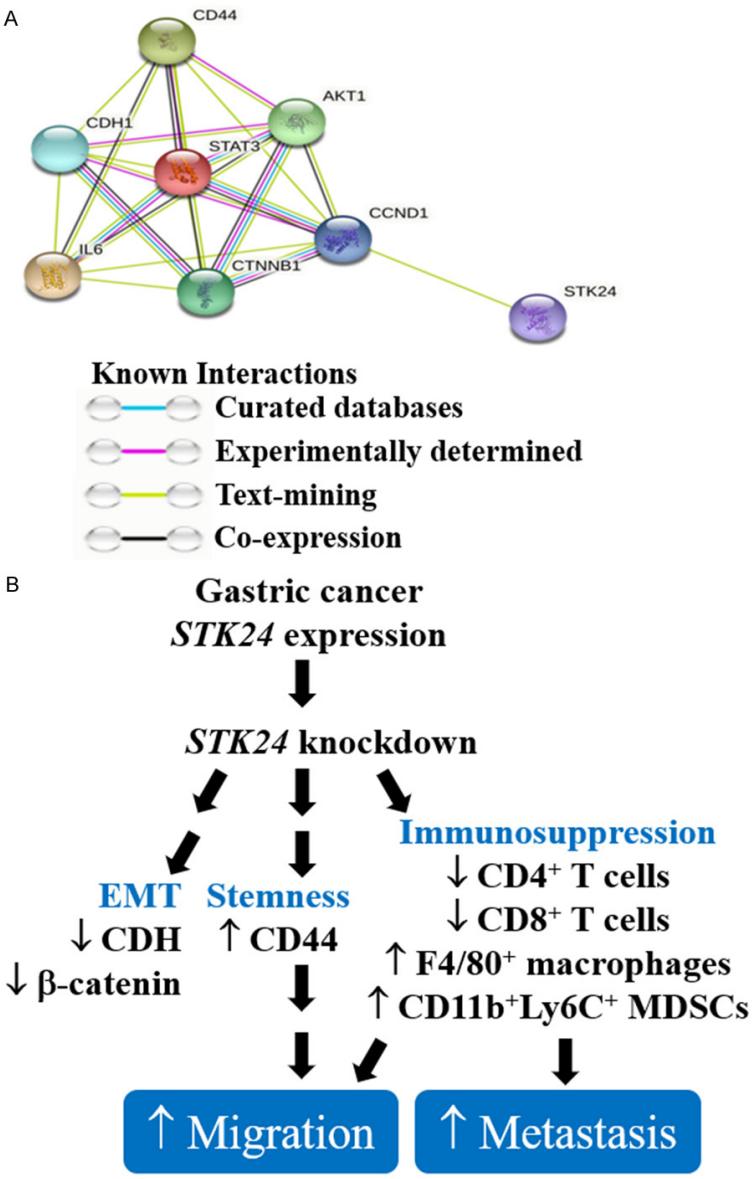

Figure 8. Function of STK24 in gastric cancer. A. Protein-protein interaction network including STK24, CDH1, AKT1, and CTNNB1, with links to CCND1, IL6, STAT3, and CD44 (constructed with STRING software). Colored lines between proteins indicate the various types of evidence demonstrating the interactions: experimental (purple), curated database (blue), co-expression (black), and text-mining (green) evidence. B. A proposed model for the effects of *STK24* on cell migration and metastasis in gastric cancer cells. The suppression of *STK24* promotes cell migration, which is associated with the loss of CDH1 and β-catenin and with an increase in CD44 (a stemness protein). *STK24* silencing increases intrasplenic F4/80$^+$ macrophages and CD11b$^+$Ly6C$^+$ MDSCs. STK24: Serine/threonine-protein kinase 24; CCND1: G1/S-specific cyclin-D1; IL6: Interleukin-6; CDH1: Cadherin-1; AKT1: RAC-alpha serine/threonine-protein kinase; STAT3: Signal transducer and activator of transcription 3; and CTNNB1: β-catenin.

sion in gastric adenocarcinomas has been studied. For example, tumor suppressor p53 (TP53) is known to be altered in ~50% of gastric cancer [33], and DGA is characterized by the loss of E-cadherin due to mutation or hypermethylation [34]. The expression levels of the STK24/MST3 protein are examined in the tumor and adjacent normal gastric tissues of DGA, GITA, and GMA [2]. Low *STK24* gene expression is correlated with poor OS and first progression in both GITA and DGA. We successfully establish an orthotopic gastric cancer model and mutated p53 in C57BL/6 mice. Further knockdown of *STK24* in this model enhances tumorigenesis [2]. Another study shows that the loss of *CDH1* and *TP53* promotes gastric tumorigenesis and metastases [35]. Our present data indicate that high STK24 and CDH1 expression in gastric cancer are protective factors; thus, they are apparently advantageous to survival. We also find that upregulation of CD44 and proliferation of F4/80$^+$ macrophages or CD11b$^+$Ly6C$^+$ MDSCs occur after *STK24* knockdown. Therefore, targeting CD44 or immunotherapy against macrophages/MDSCs could be used as potential treatments for selected gastric cancer patients with low STK24.

A heterogeneous population of MDSCs promotes tumor progression, metastasis, and resistance to immunotherapy [36]. As previously shown, high levels of MDSCs in gastric cancer patients are associated with advanced cancer stages as well as lower OS [8]. In addition, increased M-MDSCs are correlated with poor differentiation, lymph node metastasis, and lower OS in these patients [37]. Wang *et al.* detect higher levels of CD4$^+$ memory T cells and lower levels of CD8$^+$ T cells, monocytes, NK cells, myeloid dendritic cells,

and normal peritoneal fibroblasts in tumor specimens of peritoneal carcinomatosis from patients with DGA [30]. Therefore, we suggest that different subsets of MDSCs are associated with the metastases of gastric cancer types. MDSCs are increased in the spleens of human cancer patients, with M-MDSCs known to be most prominent in the spleen and peripheral blood [38]. M-MDSCs are characterized by $CD14^+CD33^+HLA\text{-}DR^{-/lo}$ expression ($CD11b^+GR1^+Ly6C^+Ly6G^-$ cells in mice) [39]. They are recruited to primary and metastatic tumor sites through chemokine secretion by tumors, primarily CCL2 and CCL5 [40, 41]. In addition, splenic M-MDSCs suppress T-cell function in an ROS-dependent manner [39, 42]. The spleen reportedly acts as the local reservoir of $Ly6C^{hi}$ monocytes, which migrate toward the tumor and differentiate into tumor-associated macrophages [43]. Thus, the targeting of MDSCs represents a promising immunotherapy for cancer patients [36]. In the present study, spleen inflammatory $CD11b^+Ly6C^{hi}$ and reparative $CD11b^+Ly6C^{lo}$ cells are markedly increased in mice with sgSTK24 tumors; thus, reduced STK24 expression in gastric cancer seems to cause an accumulation of M-MDSCs in the spleen. Moreover, inflammatory $CD11b^+Ly6C^{hi}$ and reparative $CD11b^+Ly6C^{lo}$ MDSCs may be biomarkers for liver metastasis of gastric cancer and targets for future treatment.

In our previous study, STK24 expression is significantly decreased in DGA and GITA according to bioinformatics analyses; in particular, downregulation of the *STK24* gene is associated with the poor prognosis of gastric cancer patients [2]. In the present study, STK24 is revealed as participant in cancer metastasis and immune regulation. In our constructed protein-protein interaction network, CDH1, CTNNB1, CD44, and CCND1 are all linked with STK24. Previously, decreased expression of CDH1 protein (known as E-cadherin) has been correlated with the infiltrating and metastatic abilities of gastric cancer [44]. Additionally, loss of CTNNB1 protein (known as β-catenin) has been detected in DGA and metastatic lesions of gastric cancer [45]. Patients with E-cadherin-expressing gastric cancer are known to have significantly better survival rates than those with E-cadherin-negative tumors [46]. E-cadherin is the primary mediator of cell-cell adhesion and loss of this molecule is associated with the metastatic potential of tumor cells [47, 48]. Furthermore, downregulation of E-cadherin is associated with invasion and metastasis of DGA [29, 49, 50]. Mutations of the *CDH1* gene have been reported in DGA [51, 52]. Here, we find a significant decrease in *CDH1* expression in DGA relative to *CDH1* expression in normal gastric mucosa, GITA, and GMA (according to the Oncomine database). We also show that low expression of STK24 is correlated with downregulated CDH1 protein expression in the tumors of gastric cancer patients. CD44 is a known stem cell marker in gastric cancer [53]; its expression has been positively correlated with distant metastasis [54]. Oncomine database analysis of cancer vs. normal tissues showed that CD44 mRNA was significantly upregulated in DGA, GITA, and GMA. Moreover, increased expression of CD44 was detected in sgSTK24 gastric cancer cells. Therefore, our results suggest that *STK24* suppression, which is apparently upstream of CDH1, is associated with the loss of β-catenin and activation of CD44 cancer stemness in metastasis of gastric cancer.

Conclusion

In conclusion, *STK24* silencing induces overexpression of CD44 and suppression of CDH1 (E-cadherin). Stemness transformation and EMT promotes migration of gastric cancer cells. Furthermore, reduced expression of *STK24* induces CCL2 secretion and the expansion of $CD11b^+Ly6C^+$ M-MDSCs and $F4/80^+$ macrophages to promote metastasis in mouse model of gastric cancer. Overall, decreased *STK24* expression apparently promotes gastric metastasis through stemness and immunosuppression. Interactive networks of these signaling and cell-cell interactions were important in gastric cancer metastasis, not a single signaling pathway. The findings of this study further reveal the mechanisms of gastric cancer metastasis and provide a potential therapeutic target for the development of gastric cancer treatments.

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Disclosure of conflict of interest

None.

Abbreviations

CRISPR, Clustered regularly interspaced short palindromic repeats; DGA, Diffuse gastric adenocarcinoma; EMT, Epithelial mesenchymal transition; FBS, Fetal bovine serum; GITA, Gastric intestinal adenocarcinoma; GMA, Gastric mixed adenocarcinoma; HR, Hazard ratios; MDSC, Myeloid-derived suppressor cells; OS, Overall survival; PFS, Progression-free survival; SD, Standard deviations; STRING, Search Tool for the Retrieval of Interacting Genes; TCGA, The Cancer Genome Atlas.

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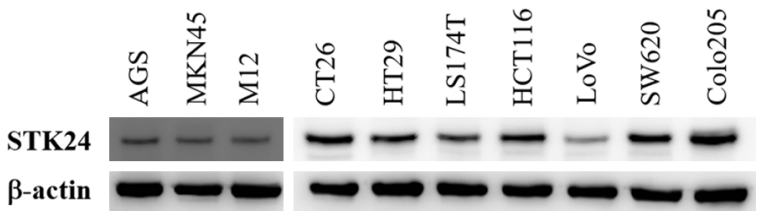

Figure S1. Expression of STK24 proteins in western blotting is slightly lower in gastric cancer cell lines than colon cancer cell lines. AGS, human gastric adenocarcinoma cell line; MKN45, human gastric cancer cell line; M12; mouse gastric cancer cell line; CT26, murine colorectal carcinoma cell line; HT29, human colon adenocarcinoma cell line; LS174T, human colon adenocarcinoma cell line; HCT116, human colon carcinoma cell line; LoVo, human colon metastatic adenocarcinoma cell line; SW620, human colon adenocarcinoma cell line; Colo205, human colon adenocarcinoma cell line.

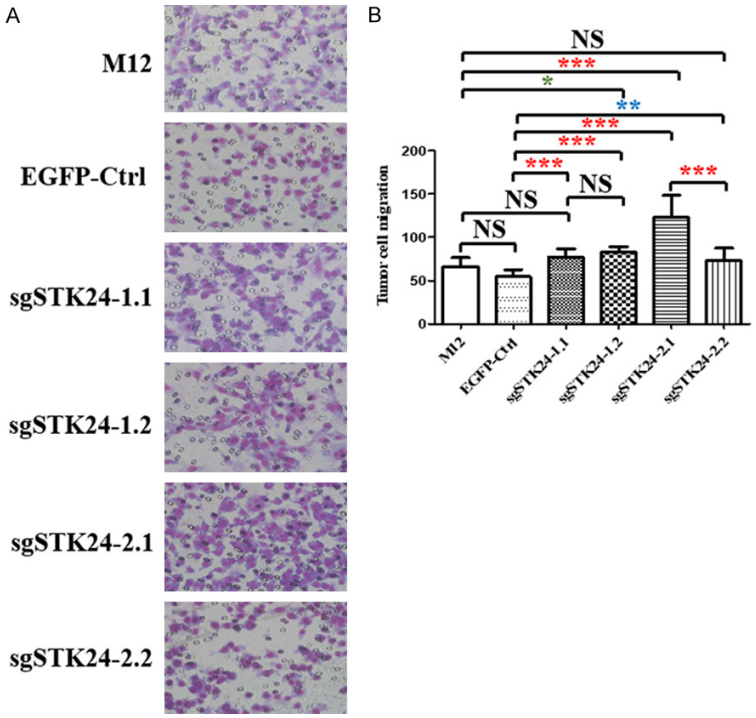

Figure S2. Cell migration is enhanced in *STK24*-silenced mouse gastric cancer cells. A. The motility of M12 cells was determined in response to 10% fetal bovine serum in a Transwell migration assay at 8 h. A polycarbonate filter with 8-μm pores is visible in the background. Magnification × 100. B. The mean number of cells per field of view ± SD of three independent experiments. NS: not significant; $^{*}P < 0.01$; $^{**}P < 0.001$; and $^{***}P < 0.0001$.

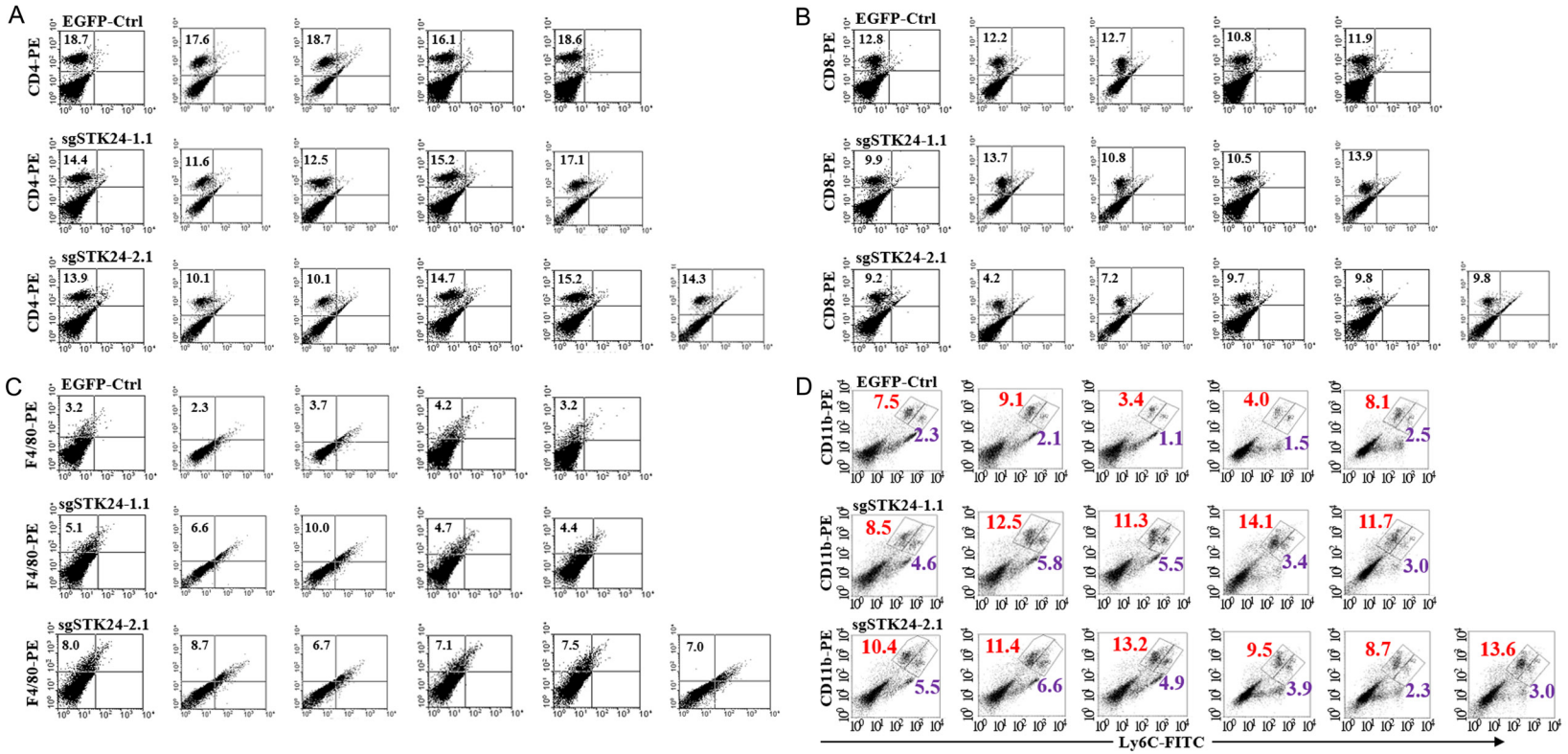

Figure S3. Populations of immune cells in the spleens of liver metastasis-bearing mice. The repeats of flow cytometry for (A) $CD4^+$ cells, (B) $CD8^+$ cells, and (C) $F4/80^+$ cells in the spleens of liver metastasis-bearing mice. The numbers shown indicate the percentage of total cells. Each graph represent data from one mouse. (D) The repeats of flow cytometry for inflammatory monocytes ($CD11b^+Ly6C^{high}$: violet square) and reparative monocytes ($CD11b^+Ly6C^{low}$: red square) in the spleens of liver metastasis-bearing mice. Statistical analyses are shown in Table S2. EGFP-Ctrl: EGFP control; sgSTK24-1.1 and sgSTK24-2.1: STK24-specific sgRNAs 1 and 2.

Table S1. Demographics and histopathological data of 38 patients with gastric cancer

Characteristic	No. of patients (%)
Patients with gastric cancer	38 (100)
Mean age ± standard deviation, years	63 ± 13
Sex	
Male	24 (63)
Female	14 (37)
Histological differentiation	
Well	1 (3)
Moderate	19 (50)
Poor	18 (47)
Lauren's classification	
Intestinal	22 (58)
Diffuse	11 (29)
Mixed	5 (13)
AJCC TNM stage	
Stage I	7 (18)
Stage II	13 (34)
Stage III	14 (37)
Stage IV	4 (11)

Table S2. Analysis of immune cells in the spleens of liver metastasis-bearing mice

Proportion of immune cells, median (range)	EGFP-Ctrl (n = 5)	sgSTK24-1.1 (n = 5)	sgSTK24-2.1 (n = 6)	*P* value			
				All three groups	EGFP-Ctrl vs. sgSTK24-1.1	EGFP-Ctrl vs. sgSTK24-2.1	sgSTK24-1.1 vs. sgSTK24-2.1
$CD4^+$ cells	18.6 (16.1-18.7)	14.4 (11.6-17.1)	14.1 (10.1-15.2)	0.009	0.013	0.006	0.429
$CD8^+$ cells	12.2 (10.8-12.8)	10.8 (9.9-13.9)	9.5 (4.2-9.8)	0.005	0.675	0.006	0.004
$F4/80^+$ cells	3.2 (2.3-4.2)	5.1 (4.4-10.0)	7.3 (6.7-8.7)	0.004	0.009	0.006	0.126
$CD11b^+Ly6C^{high}$ cells	7.5 (3.4-9.1)	11.7 (8.5-14.1)	10.9 (8.7-13.6)	0.015	0.016	0.011	0.792
$CD11b^+Ly6C^{low}$ cells	2.1 (1.1-2.5)	4.6 (3.0-5.8)	4.4 (2.3-6.6)	0.013	0.009	0.013	0.931
$CD11b^+Ly6C^+$ cells	9.8 (4.5-11.2)	16.8 (13.1-18.3)	16.3 (11-18.1)	0.011	0.008	0.009	0.792

EGFP-Ctrl: EGFP control; sgSTK24-1.1 and sgSTK24-2.1: STK24-specific sgRNAs 1 and 2.

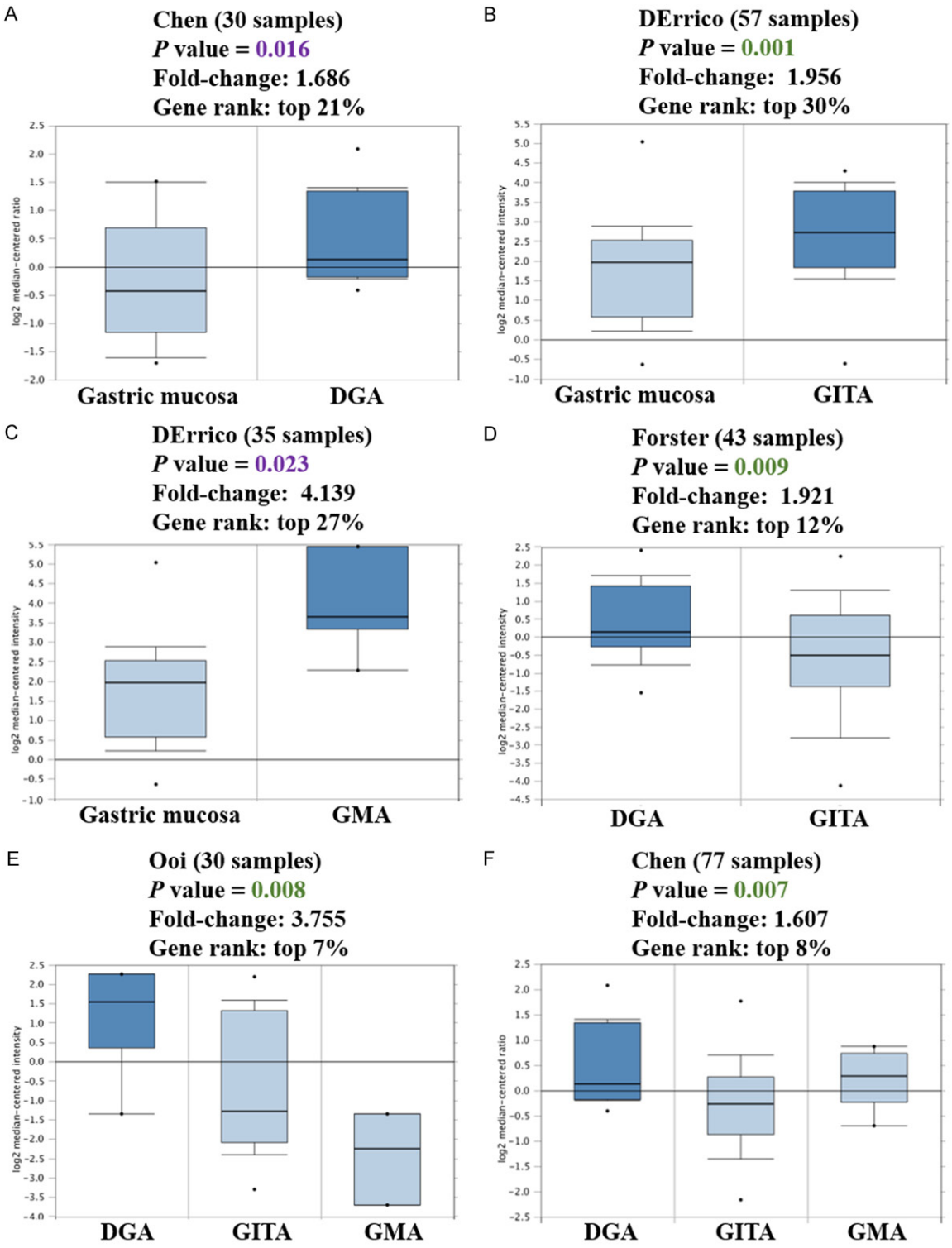

Figure S4. *CCL2* gene expression analysis of gastric cancer data from the Oncomine database. A-C. *CCL2* is over-expressed in the Chen and DErrico datasets. Expression of *CCL2* in human gastric cancer is compared with *CCL2* expression in normal tissues. D-F. Expression of *CCL2* is significantly increased in diffuse gastric adenocarcinoma (DGA). GITA: gastric intestinal type adenocarcinoma; GMA: gastric mixed adenocarcinoma.

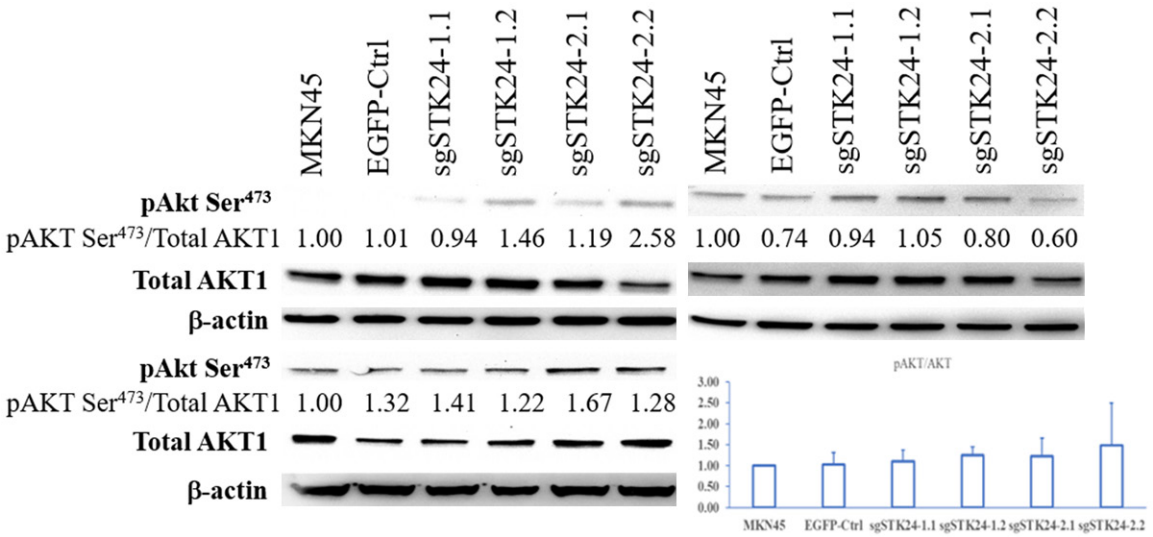

Figure S5. Western blotting of pAKT Ser^{473} and total AKT1 proteins. The knockout of STK24 expression did not affect the AKT1 or phospho-AKT Ser^{473} protein of MKN45 cells.

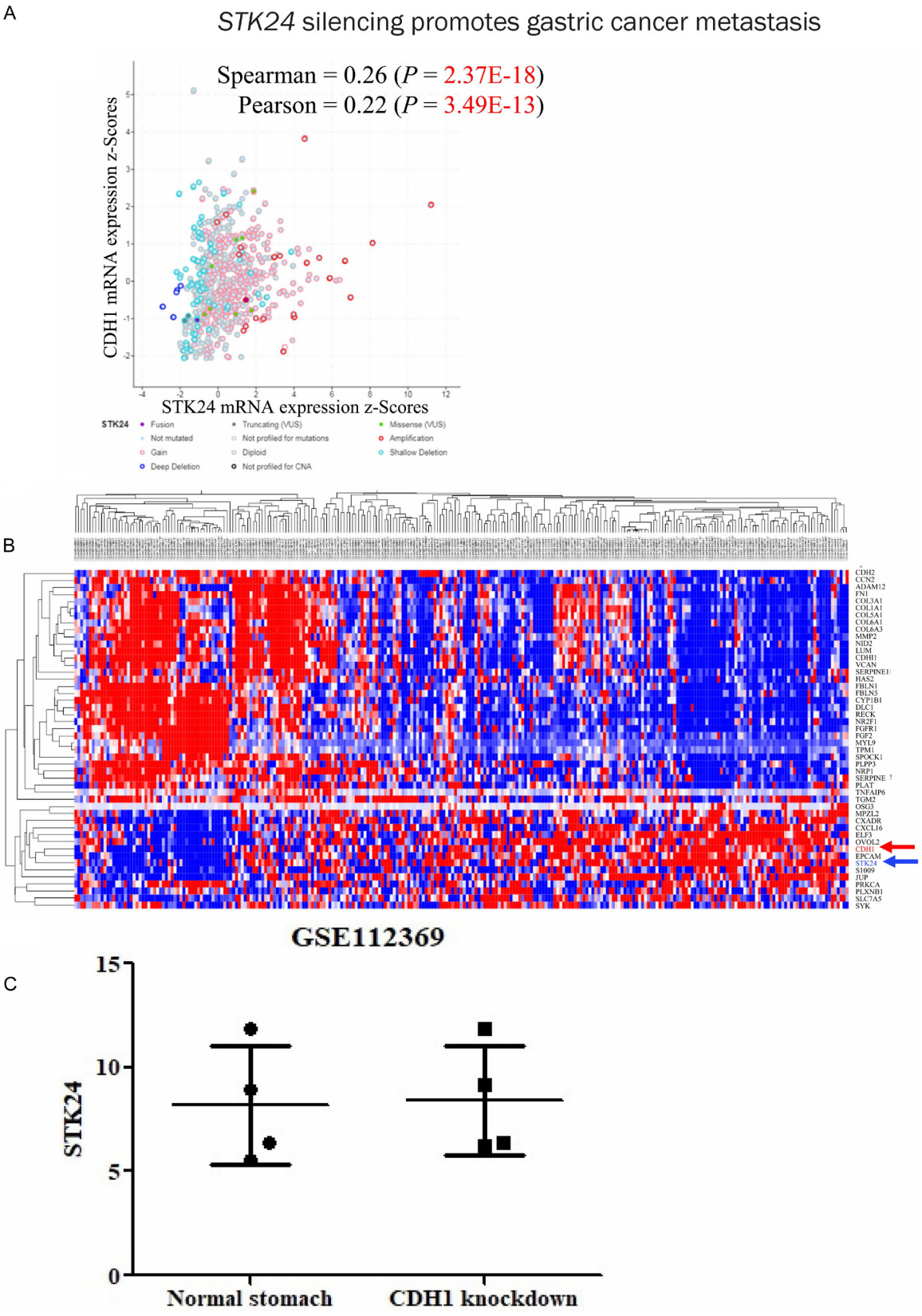

Figure S6. Correlation of *STK24* and *CDH1* expression according to data from a public database. A. Positive correlation between *STK24* and *CDH1* shown in scatter plot analysis of a dataset from The Cancer Genome Atlas (TCGA). Spearman's and Pearson's correlation coefficients are shown. B. Expression of epithelial mesenchymal transition (EMT)-related and EMT-unrelated genes in TCGA dataset is hierarchically clustered. Mesenchymal and epithelial phenotypes are shown in upper and lower halves, respectively. *CDH1* and *STK24* are indicated by the red and blue

arrows, respectively. C. Gene expression in gastric organoids from the GSE112369 dataset. The expression of *STK24* is similar in normal gastric organoids and *CDH1*-single-knockout organoids.

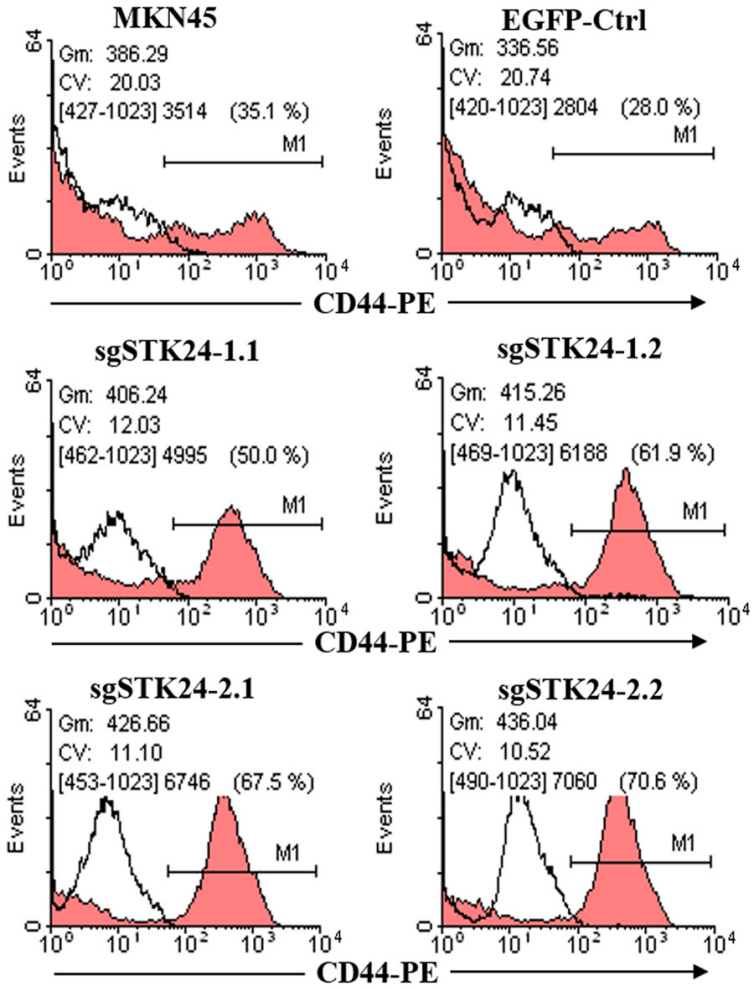

Figure S7. Expression of CD44 as detected by flow cytometry. Surface CD44 increased in *STK24*-silenced cells. EGFP-Ctrl: EGFP control; sgSTK24-1.1 and sgSTK24-2.1: STK24-specific sgRNAs 1 and 2.

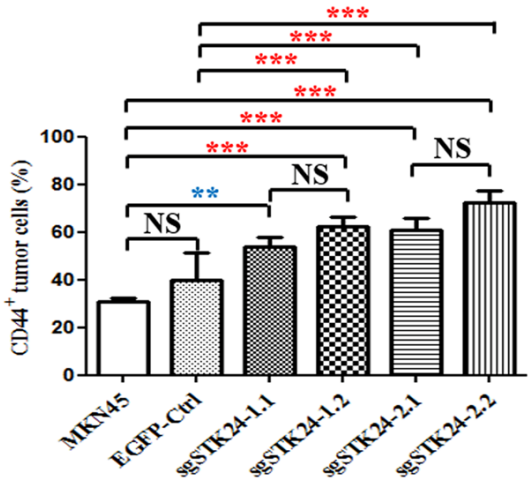

Figure S8. Quantitative results of surface CD44 detected by flow cytometry. NS: not significant; $^{*}P < 0.01$; $^{**}P < 0.001$; and $^{***}P < 0.0001$.

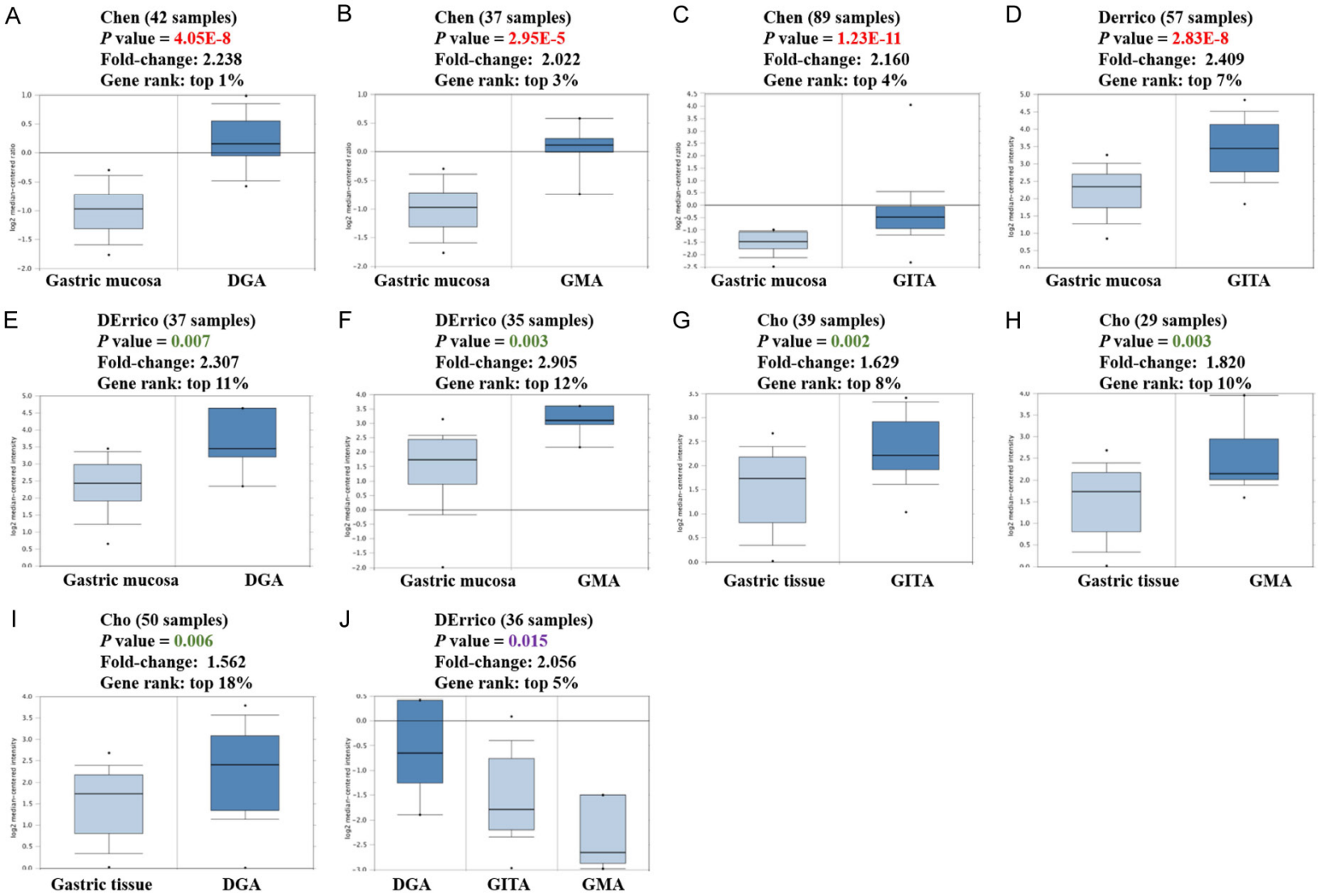

Figure S9. *CD44* gene expression analysis of gastric cancer data from the Oncomine database. A-C. *CD44* is overexpressed in diffuse gastric adenocarcinoma (DGA), gastric mixed adenocarcinoma (GMA), and gastric intestinal type adenocarcinoma (GITA) relative to *CD44* expression in normal gastric mucosa (Chen dataset). D-F. Expression of *CD44* is significantly increased in GITA, DGA, and GMA relative to *CD44* expression in normal gastric mucosa (DErrico dataset). G-I. Expression of *CD44* is significantly increased in GITA, GMA, and DGA relative to *CD44* expression in normal gastric tissue (DErrico dataset). J. Expression of *CD44* is higher in DGA than in GITA or GMA.