

Serine/threonine-protein kinase 24 is an inhibitor of gastric cancer metastasis

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Research Article

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

Posted Date: March 17th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-217140/v1>

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Abstract

Background: 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 association between STK24 and the *metastasis of gastric cancer*.

Methods: CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 technology was used for genetic knockout of *STK24* at the genomic DNA level in human MKN45 and mouse M12 gastric cancer cells. To assess the effects 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.

Results: 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 induced the expansion of CD11b⁺Ly6C⁺ myeloid-derived suppressor cells and F4/80⁺ macrophages in the spleen of the mice. In MKN45 cells, *STK24* silencing resulted in downregulation of E-cadherin (CDH1, Cadherin-1, or epithelial cadherin). In 38 matched specimens of gastric adenocarcinomas and normal tissues, we examined STK24 and CDH1 expression levels via western blot; a significant positive correlation was found between the expression levels of STK24 and CDH1 ($R^2 = 0.5507$, $P = 9.72 \times 10^{-8}$). 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 in gastric cancer patients.

Conclusions: Our results demonstrate that knockdown of STK24 increases cell migration and metastasis. STK24 suppression is also positively correlated with CDH1 expression in gastric cancer metastasis. Having developed an experimental metastatic model of gastric cancer in syngeneic inbred mice, we have shown that STK24 is important for immune regulation and regulates CDH1 expression during gastric metastasis.

Background

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 associated with the poor 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. Tumor evasion mechanisms include the expansion of immunosuppressive myeloid-derived suppressor cells (MDSCs) in the tumor microenvironment [4]. MDSCs represent a heterogeneous population of 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 correlated with the advanced stage of gastric cancer in patients [8]. The accumulation of M-MDSCs is associated with tumor metastasis and poor response to chemotherapy in advanced non-small cell lung cancer [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 examined the changes in 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.

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

MKN45 cells were kindly provided by Professor Ming-Derg Lai (National Cheng Kung University, Tainan, Taiwan). The MKN45 cell lines were authenticated in 2013 by DNA short tandem repeat profiling at Bioresource Collection and Research Center. 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

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.

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-cm³ 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 staining. Each animal experiment was performed at least twice.

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://rna.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. To monitor the efficacy of *STK24* silencing, *STK24* expression in the stable transfectants was analyzed by western blotting.

Western blot analysis

Total cell lysates were prepared and analyzed by SDS-PAGE as previously described [11]. For quantification, the bands were measured using the Alphamager 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, as previously described [11]. MKN45 cells (70 μ L of 1×10^6 cells per mL) or M12 cells (70 μ L 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

To characterize the immune cells from the spleens of tumor-bearing mice, individual spleens were isolated and subjected to flow cytometry as previously described [12].

Patients

For patient studies, 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. In total, 38 pairs of cancerous tissues and matched adjacent normal gastric mucosa were collected and analyzed as previously described [13].

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-b-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 \pm 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

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 (Fig. 1a) and M12 (Fig. 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 (Fig. 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 (Fig. 2a and c) and 12 h (Fig. 2b and d), 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 Fig. S1 in Additional file 1). This association between cell migration and STK24 expression in gastric cancer cell lines suggests STK24 plays important role in mediating metastasis.

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 (Fig. 3a and c) and spleens (Fig. 3b and d) 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 (Fig. 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 (Fig. 4a and b). The proportion of the CD8⁺ T cells in splenocytes significantly decreased in sgSTK24-1.1-tumor-bearing mice but not in sgSTK24-2.1-bearing mice (Fig. 4a and c). The proportion of F4/80⁺ macrophages significantly increased in the spleens of sgSTK24-tumor-bearing mice (Fig. 4a and d). 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 (Fig. 4e and f). 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 CD11b⁺Ly6C⁺ cells (Fig. 4e). Inflammatory CD11b⁺Ly6C^{hi} and reparative CD11b⁺Ly6C^{lo} cells were markedly increased in the spleens of sgSTK24-tumor-bearing mice (Fig. 4e and f). These results indicate that STK24 silencing in tumors 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. Because CCL2 is associated with metastatic behavior in cancer cells [18], 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 Fig. S2a–c in Additional file 1). Compared to the other subtypes of gastric cancer, the expression of *CCL2* was significantly increased in DGA (see Fig. S2d–f in Additional file 1). Therefore, analysis 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 STK24 expression did not affect the AKT1 protein of MKN45 cells (Fig. 5a). E-cadherin and b-catenin are key proteins in EMT; expression of each protein was suppressed by STK24 silencing in MKN45 cells (Fig. 5b and c). 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 in Additional file 1). Fig. 5d shows the relative expression of STK24 and CDH1 in these tissues. The relative ratio of CDH1-to-b-actin was higher in normal gastric tissues than in gastric cancer tissues (Fig. 5e). Furthermore, a significant positive correlation was identified between the expression levels of STK24 and CDH1 ($R^2 = 0.5507$; Fig. 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 (Fig. S3a in Additional file 1).

Gene expression in gastric cancer from TCGA was hierarchically clustered as EMT-related and EMT-unrelated (see Fig. S3b in Additional file 1). The upper half was correlated with mesenchymal phenotypes and the lower half with epithelial phenotypes, 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 Fig. S3c in Additional file 1). 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 (Fig. 6a and b). *CDH1* expression significantly decreased in DGA than other subtypes of gastric cancer (Fig. 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 (Fig. 7), a significant relationship existed between *CDH1* mRNA expression and patient survival: **low expression of *CDH1* was correlated with worse OS, PFS, and PPS (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 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 Fig. S4 in Additional file 1). Western blotting showed that MKN45-sgSTK24-expressing cells exhibited relatively high CD44 expression (see Fig. S5 in Additional file 1). 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 Fig. S6a–i in Additional file 1), with CD44 expression being significantly increasing in DGA relative to the other subtypes (see Fig. S6j).**

A scheme of STK24 signaling in gastric cancer is shown in Fig. 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 effects of STK24 on migration and metastasis in human and mouse 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 human MKN45 and mouse M12 gastric cancer cell lines *in vitro* and *in vivo*. Our data suggest that STK24 suppression is associated with 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 is associated with decreased responsiveness to chemotherapy and chemoradiation [30–32]. The possible mechanism of progression 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.* [30] 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. 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⁻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 [2]; in particular, downregulation of the *STK24* gene is associated with the poor prognosis of gastric cancer patients. 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 b-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 b-catenin and activation of CD44 cancer stemness in metastasis of gastric cancer.

Conclusion

In conclusion, STK24 silencing induces overexpression of CD44 and stemness in gastric cancer, while suppression of CDH1 (E-cadherin) promotes gastric cancer metastasis. Furthermore, reduced expression of STK24 induces CCL2 secretion and the recruitment of M-MDSCs to promote metastasis. Overall, decreased STK24 expression apparently promotes gastric metastasis through stemness and immunosuppression. 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.

Declarations

Ethics approval and consent to participate

The animal study was approved by the institutional animal care and use committee of National Cheng Kung University (approval number: NCKU-IACUC-106-288). All procedures were conducted in accordance with approved guidelines. All patients involved in this study provided written informed consent, and the patient study was approved by the Institutional Review Board of National Cheng Kung University Hospital (IRB number: ER-97-148).

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this article.

Competing interests

The authors declare that they have no competing interests.

Funding

The present study was supported by grants from the Ministry of Science and Technology, Taiwan (MOST 107-2314-B-006-037, 108-2314-B-006-082, and 109-2314-B-006-018-MY3), and National Cheng Kung University Hospital (grant NCKUH-11002013). This research was supported in part by Higher Education Sprout Project, Ministry of Education to the Headquarters of University Advancement at National Cheng Kung University.

Author contributions

YLC performed most of the experiments and wrote the first draft. HPH contributed to the experimental design and edited the manuscript. CYW contributed to the experimental design and bioinformatics analysis. JHF assisted in the animal experiments. All authors read and approved the final manuscript.

Acknowledgements

We are grateful for support from the Human Biobank, the Research Center of Clinical Medicine, and the National Cheng Kung University Hospital.

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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Figures

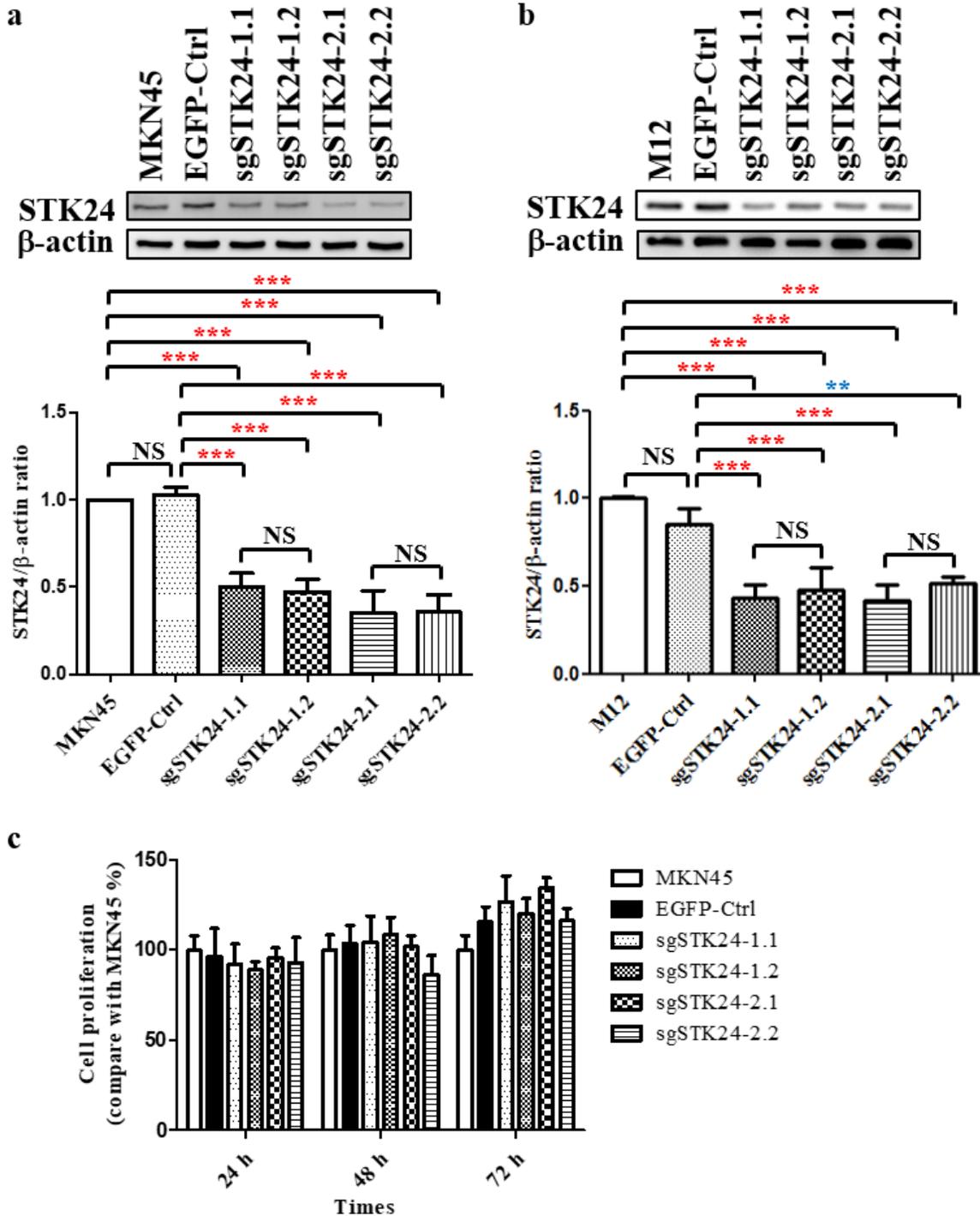


Figure 1

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 \pm 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.

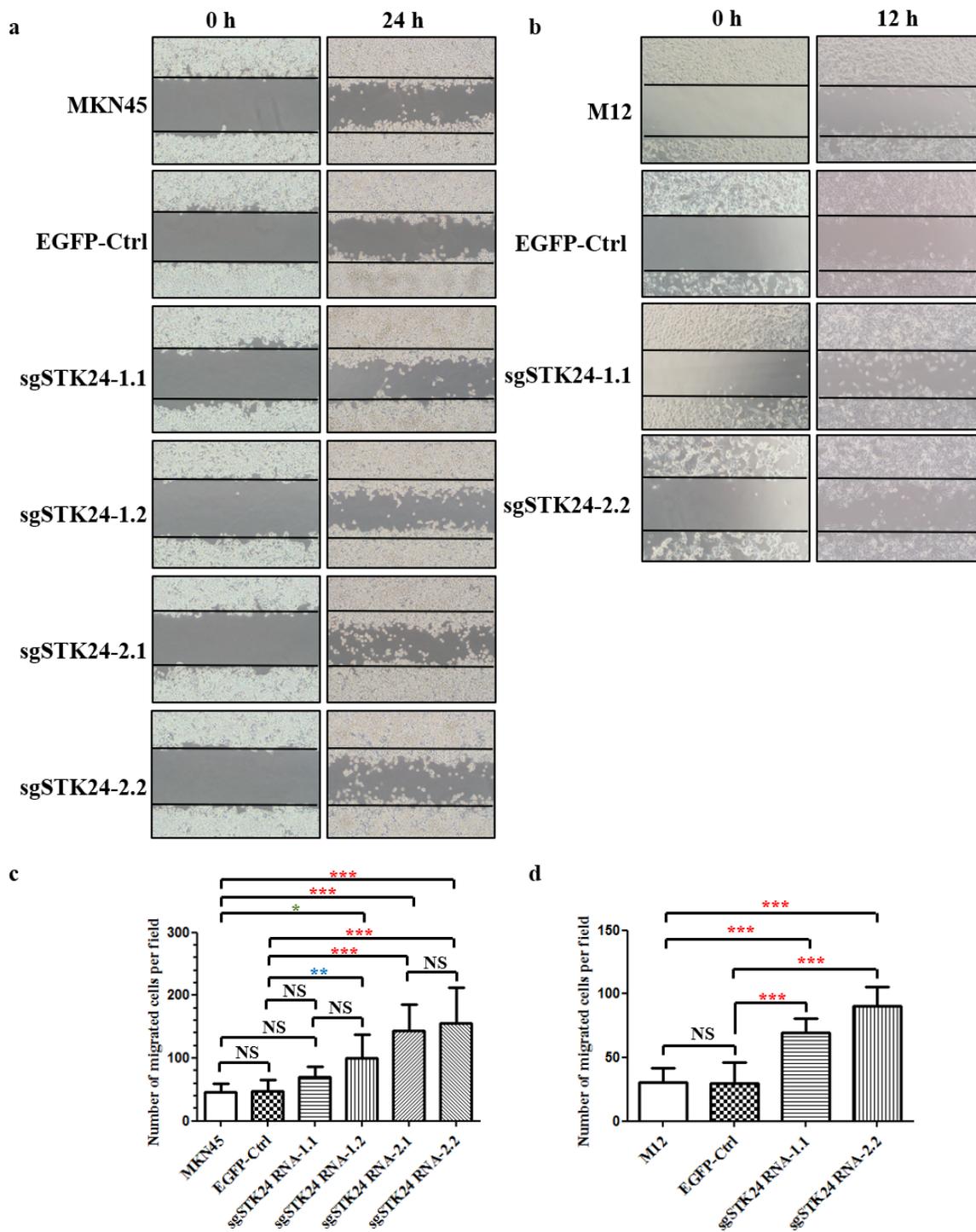


Figure 2

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 \pm SD from three independent experiments. NS: not significant; * $P < 0.01$; ** $P < 0.001$; and *** $P < 0.0001$.

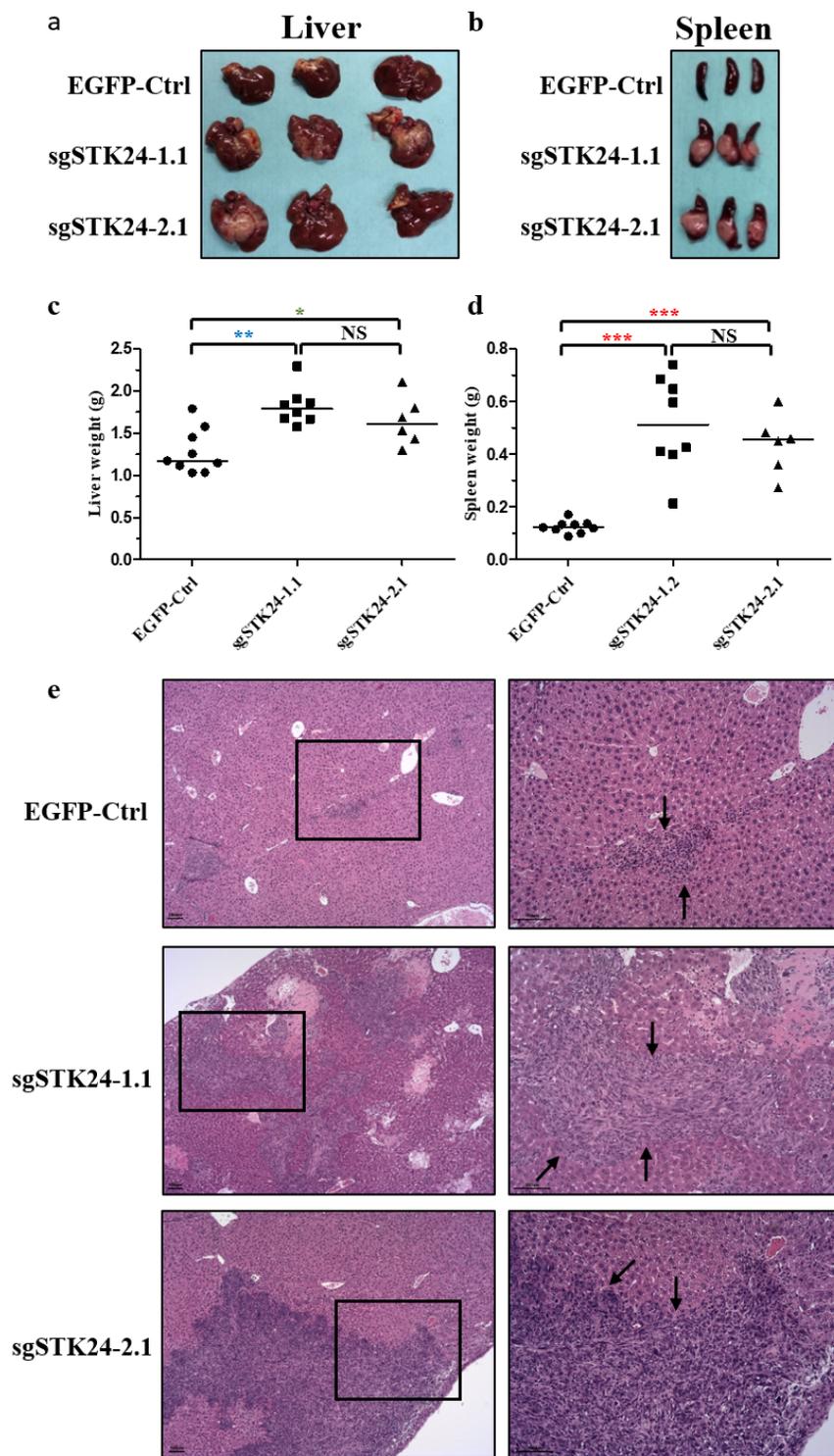


Figure 3

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 \pm 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 ($\times 100$) is shown at a higher magnification ($\times 400$) in the right panels. Tumor cells are indicated by arrows. NS: not significant; * $P < 0.01$; ** $P < 0.001$, and *** $P < 0.0001$.

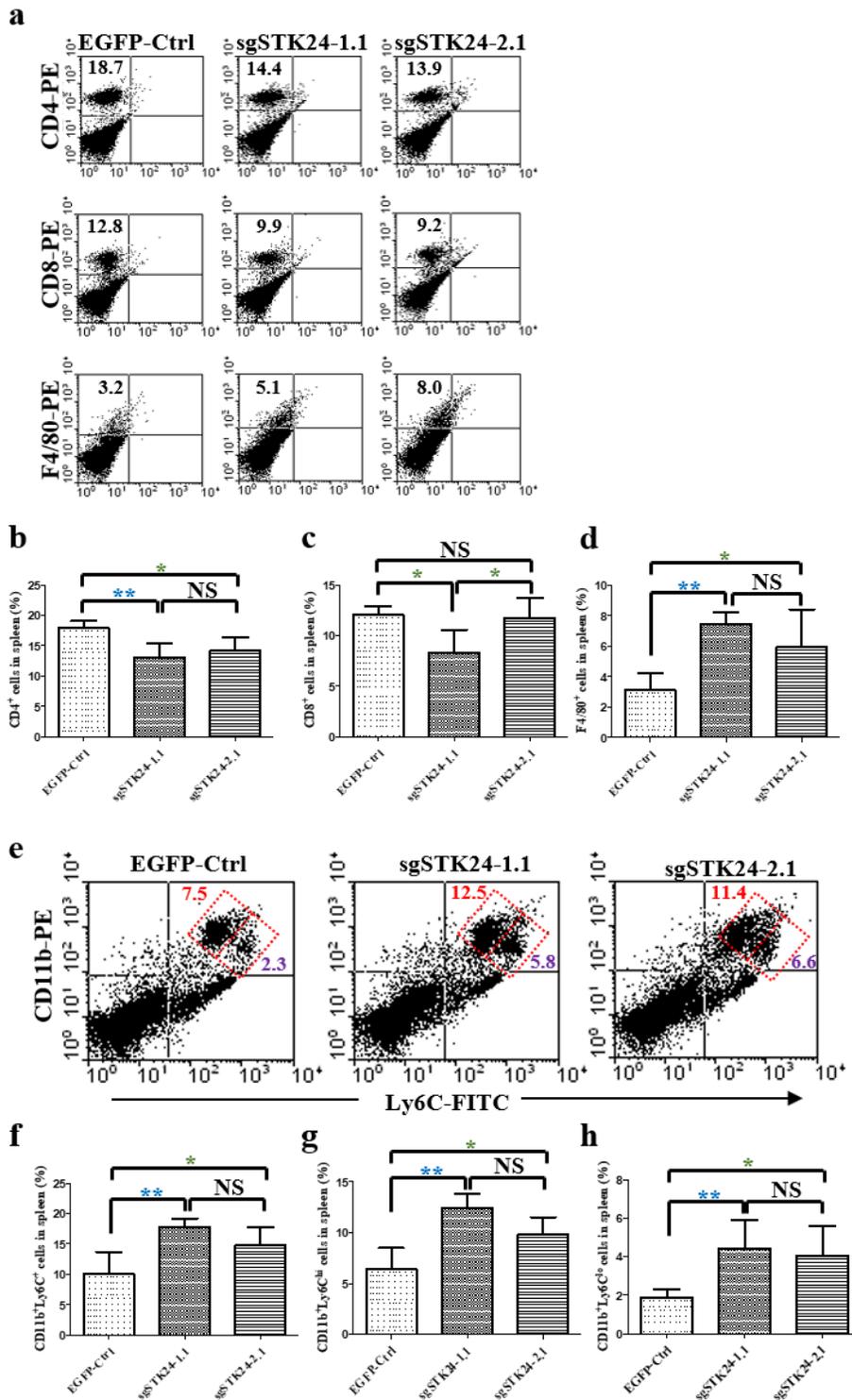


Figure 4

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+Ly6Chigh: violet square) and reparative monocytes (CD11b+Ly6Clow: red square) in the spleens of liver metastasis-bearing mice are shown. The percentage of (f) CD11b+Ly6C+ cells, (g) CD11b+Ly6Chigh cells, and (h) CD11b+Ly6Clow cells in the spleens of liver metastasis-bearing mice. All data represent the mean \pm SD from two independent experiments. EGFP-Ctrl: EGFP control; sgSTK24-1.1 and sgSTK24-2.1: STK24-specific sgRNAs 1 and 2; NS: not significant; *P < 0.01; and **P < 0.001.

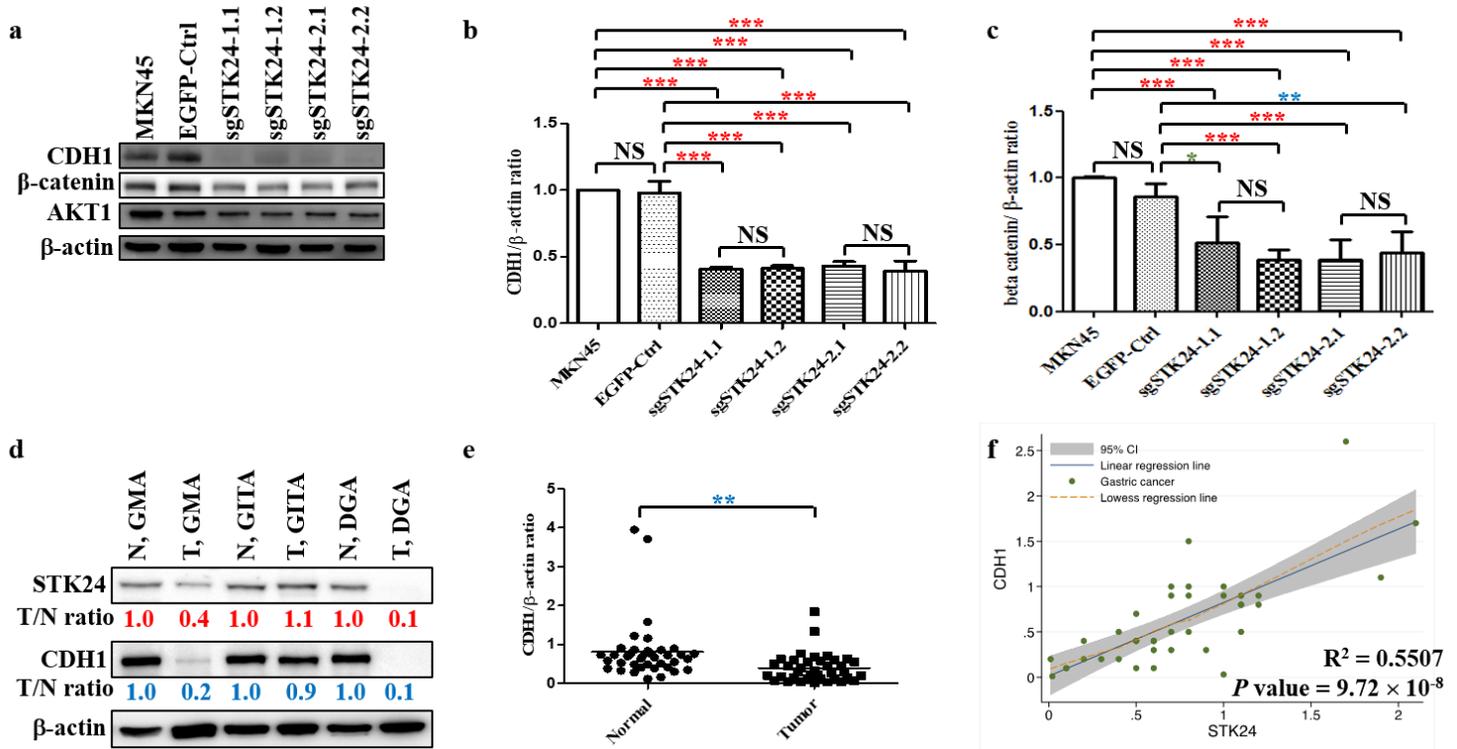


Figure 5

Knockdown of STK24 expression suppresses CDH1 protein in human gastric cancer cells. (a) STK24-specific sgRNAs inhibit expression of CDH1, β -catenin, and AKT1 protein in human MKN45 gastric cancer cells. 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; and **P < 0.001.

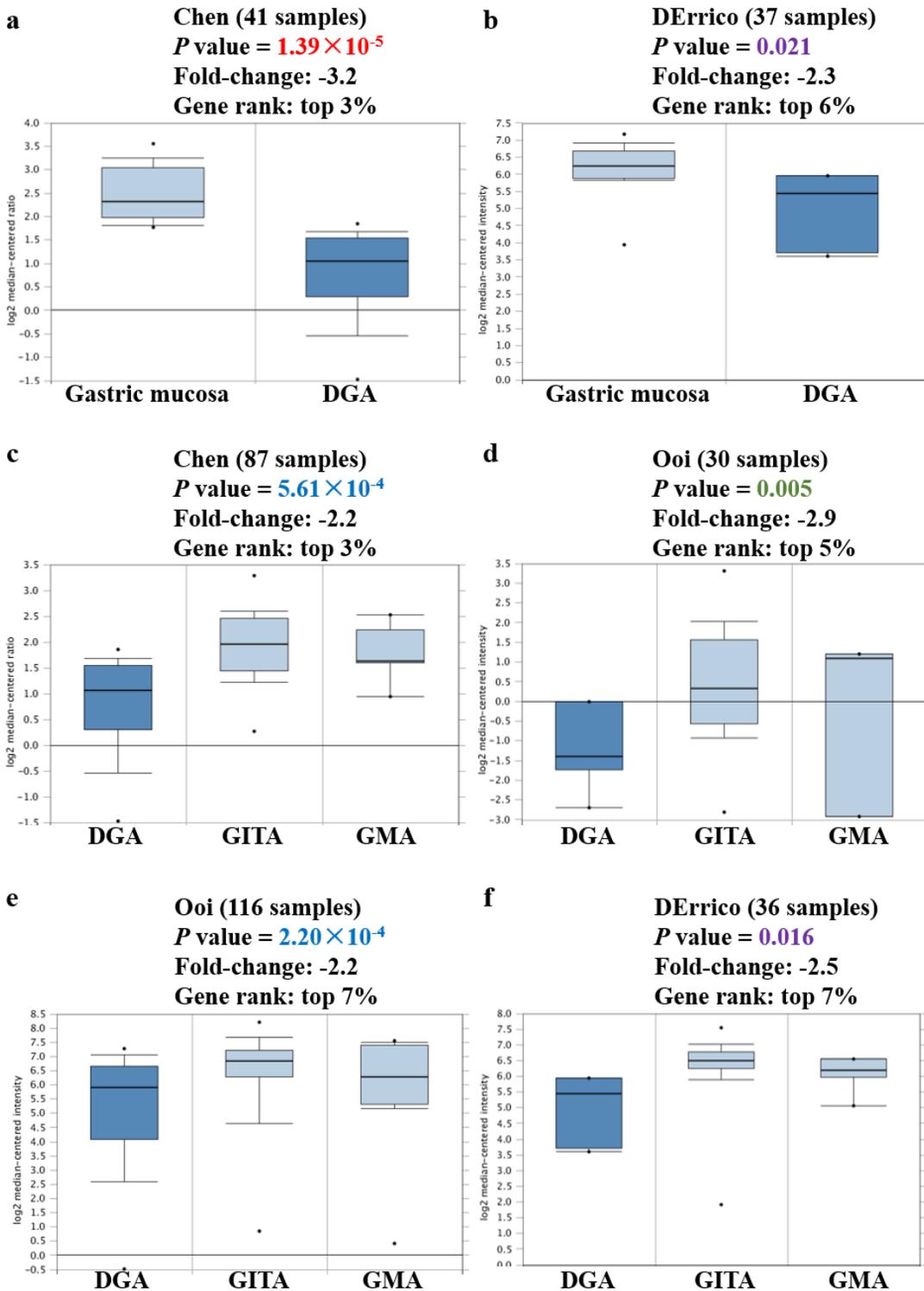


Figure 6

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).

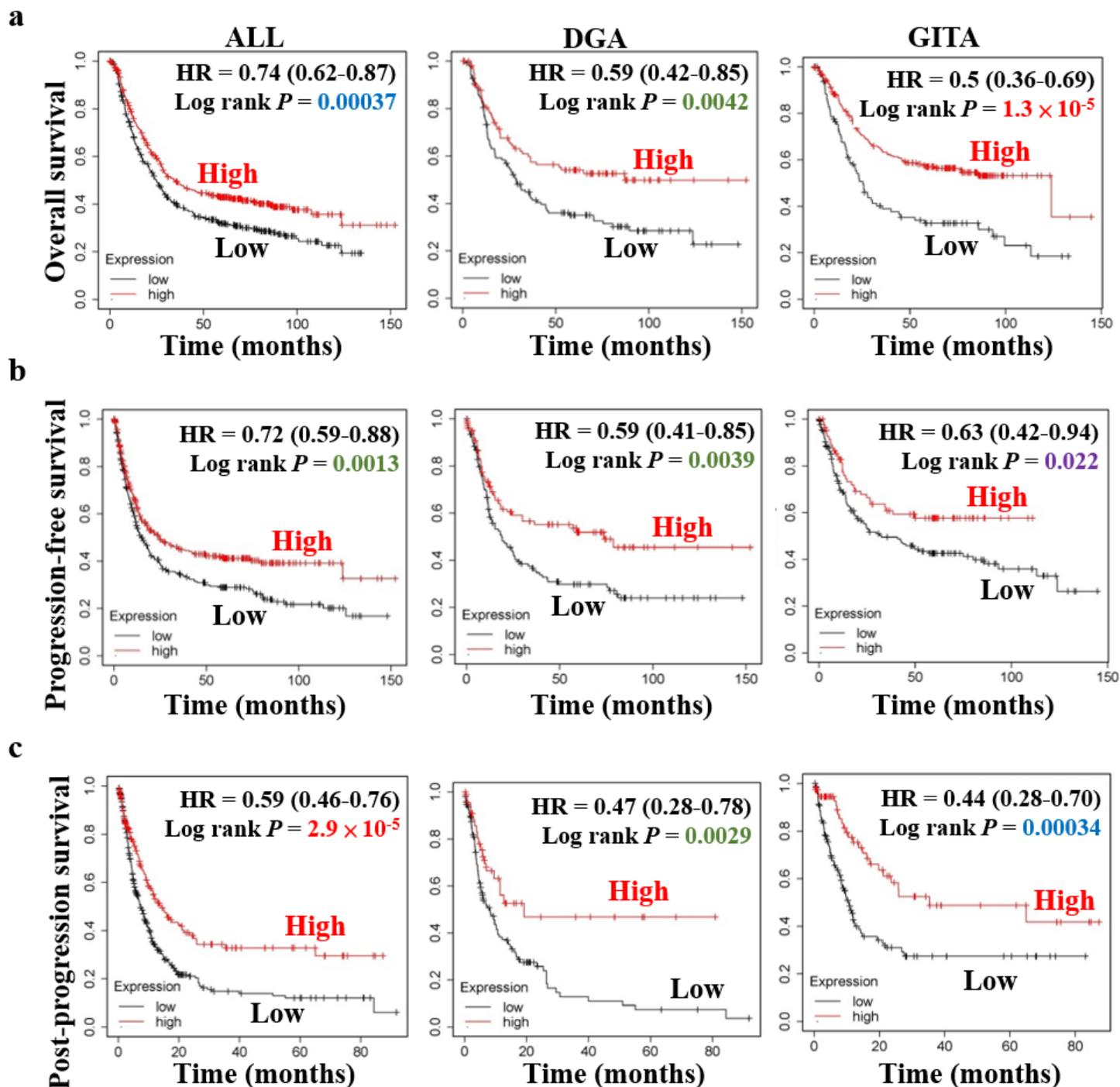


Figure 7

Kaplan-Meier Plotter survival curves of patients with gastric cancer relative to their CDH1 expression levels. (a) Overall survival. (b) Progression-free survival. (c) Postprogression 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.

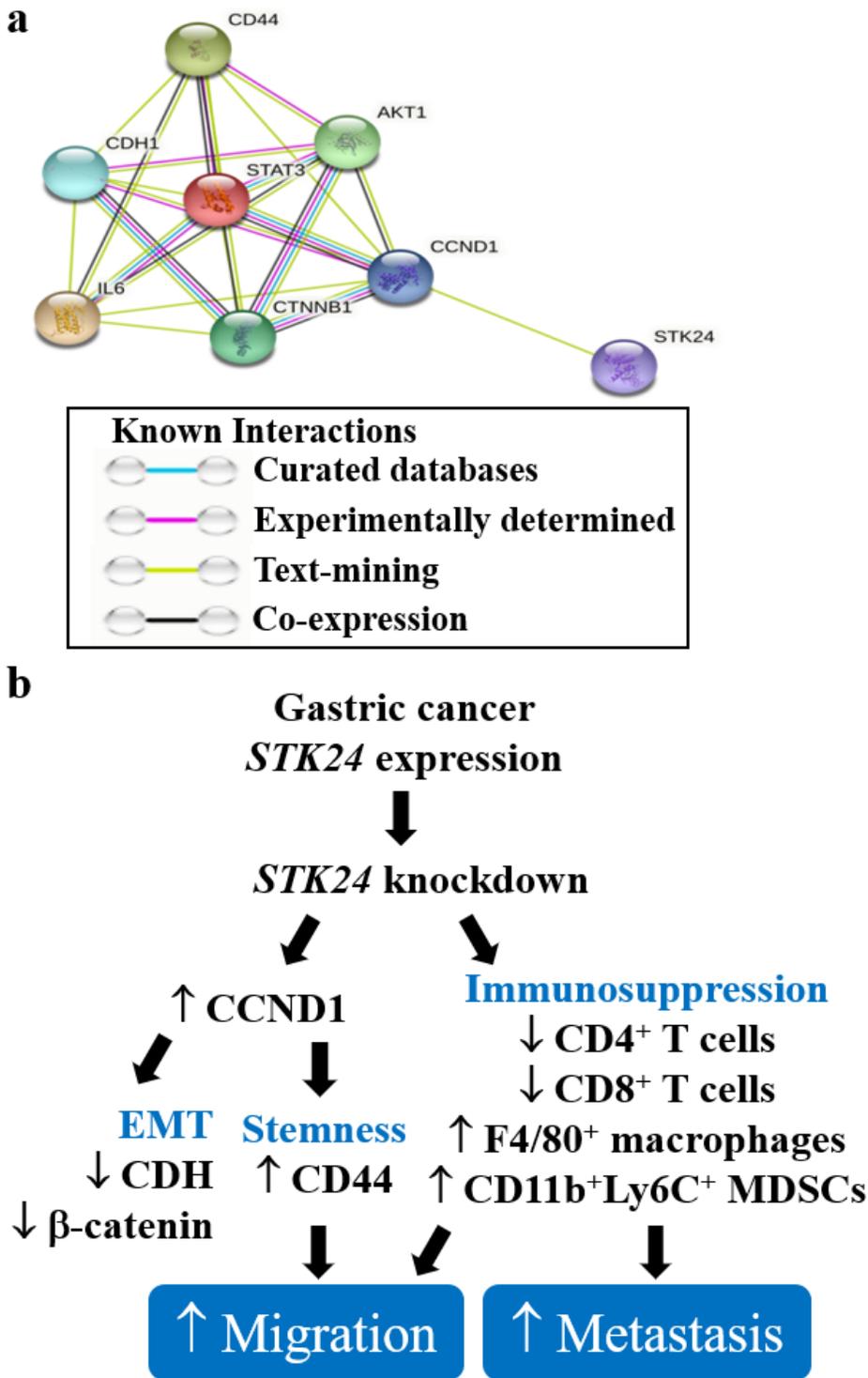


Figure 8

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), coexpression (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.

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