國家科學及技術委員會補助專題研究計畫報告

研究Serine/threonine-protein kinase 24調節骨髓來源抑制性細胞和上皮細胞-間質細胞轉換在胃癌轉移中的作用及其機制

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計 畫 主 持 人： 陳怡伶
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計畫參與人員： 此計畫無其他參與人員

本研究具有政策應用參考價值：■否 □是，建議提供機關
（勾選「是」者，請列舉建議可提供施政參考之業務主管機關）
本研究具影響公共利益之重大發現：□否 □是

中 華 民 國 111 年 09 月 24 日

中 文 摘 要 ： 胃癌 (Gastric cancer, GC) 是最常見的癌症，大多數患者存在遠處轉移(distant metastasis)和晚期。已發現絲氨酸/蘇氨酸蛋白激酶 24 (Serine/threonine-protein kinase 24)可促進胃癌發生。我們進一步研究了 STK24 在胃轉移(gastric metastasis)中的潛在作用。在這項研究中，我們探討了絲氨酸/蘇氨酸蛋白激酶 24 (STK24) 在癌症轉移中的作用。 CRISPR（常間回文重複序列叢集）/Cas9 技術用於在人類 MKN45 和小鼠 M12 胃癌細胞的基因體 DNA 誘導 STK24 基因剔除。還使用了針對 STK24 穩定轉染到 MKN45 和 M12 細胞中的方法、西方墨點法(western blot)、體外細胞遷移(cell migration)和傷口癒合測定(wound healing assays)以及體內轉移。 降低STK24 基因表現，增加了胃癌肝轉移模型(liver metastatic model)中的腫瘤轉移。腫瘤中的 STK24 基因沉默減少了 CD4 T 細胞浸潤，並誘導了脾臟中 CD11b+Ly6C+ 細胞和 F4/80+ 巨噬細胞的增加。 降低MKN45 細胞中的 STK24 基因表現，誘導 E-cadherin（CDH1、Cadherin-1 或上皮鈣粘蛋白）表現量的下降。我們採用西方墨點法分析檢查了 38 個配對的胃腺癌和正常組織標本中 STK24 和 CDH1 的表達量。值得注意的是，STK24和CDH1的表達量呈顯著正相關（r=0.5507，P<9.72×10-8）。 Oncomine 和 KM 圖分析胃癌患者 E-cadherin 缺失和預後不良(poor prognosis)有關。結果表明，STK24表現量的降低增加了癌細胞的遷移、轉移潛力，並且與胃癌轉移中的CDH1表現量呈正相關性。我們已經在近親品系小鼠(syngeneic inbred mice)中建立了胃癌的實驗性轉移模型，並證明 STK24 在胃癌細胞轉移過程中對免疫調節和調節 CDH1 表現量具有重要性。

中 文 關 鍵 詞 ： 胃癌, 絲氨酸/蘇氨酸蛋白激酶 24, 轉移, 髓源性抑制細胞, 免疫抑制

英 文 摘 要 ： Gastric cancer (GC) is a most common cancer and the majority of patients present with distant metastasis and advanced stages. Serine/threonine-protein kinase 24 has been discovered to promote gastric tumorigenesis. We further investigated the potential role of STK24 in gastric metastasis. In this study, we explored the effect of serine/threonine-protein kinase 24 (STK24) in cancer metastasis. CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 technology was used to induce a STK24 genetic knockout at the genomic DNA level in human MKN45 and mouse M12 gastric cancer cells. The stably transfected against STK24 into MKN45 and M12 cells, western blot, cell migration and wound healing assays in vitro and metastasis in vivo were also employed. The knockdown of the STK24 gene increased the tumor metastasis in an liver metastatic model of gastric cancer. The STK24 gene silencing in tumors decreased CD4 T cells and induced the expansion of CD11b+Ly6C+ cells and F4/80+ macrophages in spleen. The STK24 gene silencing in MKN45 cells caused the downexpression of E-cadherin (CDH1, Cadherin-1 or

epithelial cadherin). We examined the levels of STK24 and CDH1 expression in 38 matched specimens of gastric adenocarcinomas and normal tissues by a western blot analysis. Remarkably, a significant positive correlation was found between the expression levels of STK24 and CDH1 ($r=0.5507$, $P<9.72 \times 10-8$). Loss of E-cadherin and poor prognosis in gastric cancer patients by Oncomine and KM plot analysis. The results demonstrated that knockdown of STK24 expression was increased migration, metastastic potential and positively correlated with CDH1 expression in gastric cancer metastasis. To the best of our knowledge, we have developed the experimental metastatic model of gastric cancer in syngeneic inbred mice and suggested that STK24 is important for immune regulation and regulate CDH1 expression during the gastric metastasis.

英文關鍵詞：gastric cancer, serine/threonine-protein kinase 24, metastasis, myeloid-derived suppressor cells, immune-suppression

研究 Serine/threonine-protein kinase 24 調節骨髓來源抑制性細胞和上皮細胞-間質細胞轉換在胃癌轉移中的作用及其機制

To study the effect and mechanism of STK24 regulate myeloid-derived suppressor cells and epithelial–mesenchymal transition in gastric metastasis

The Salient and Novel Findings: this is the first study to demonstrate the effects of STK24 on migration and metastasis in human and mouse gastric cancer cells. Then, we demonstrate that the silencing of STK24 in tumors induced the expansion of F4/80^{+} macrophages and Ly6 high (Ly6Chi) and low (Ly6Clo) monocytes *in vivo* and suggest that the increased macrophages/monocytes may have an important role in gastric metastasis. Most importantly, decreased expression of STK24 is associated with CDH1, beta catenin and CD44 expression in human gastric cancer. Both E-cadherin and beta catenin expression were suppressed by STK24 silencing in MKN45 cells. In this study, we aimed to explore the association between the STK24 and CDH1 in patients with gastric adenocarcinoma. Remarkably, a significant positive correlation was identified between the expression levels of STK24 and CDH1. Analysis of the Oncomine cancer microarray database revealed that *CDH1* gene expression was significantly decreased in gastric diffuse gastric adenocarcinoma. The downregulation of *CDH1* in DGA and GITA was associated with a poor prognosis by the Kaplan-Meier Plotter. MKN45 sgSTK24-expressing cells exhibited a relatively high CD44 expression. Analysis of the Oncomine cancer microarray database revealed that *CD44* gene expression was significantly increased in diffuse gastric adenocarcinoma. These results suggest that declined STK24 expression promoted gastric metastasis and suggesting that STK24 provided a potential therapeutic target for gastric cancer.

The key findings of the study

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 determined the changes in the cell motility of MKN45 cell clones and M12 cell clones *in vitvo*. STK24 suppression of MKN45 cell clones reduced the number of migrating cells in the wound healing assay at 24 h (**Figure 1 A and C**). STK24 suppression of M12 cell clones reduced the number of migrating cells in the wound healing assay at 12 h (**Figure 1 B and D**). MKN45 and M12 sgSTK24-expressing cells exhibited a relatively high cell migration potential. On the other hand, the motility of cell clones was assessed by incubating cells in Boyden chambers for 8 h and using 10% FBS as a chemoattractant. Therefore, the correlation between metastatic potential and STK24 expression in the gastric cancer cell line suggests an important role of STK24 in mediating metastasis.

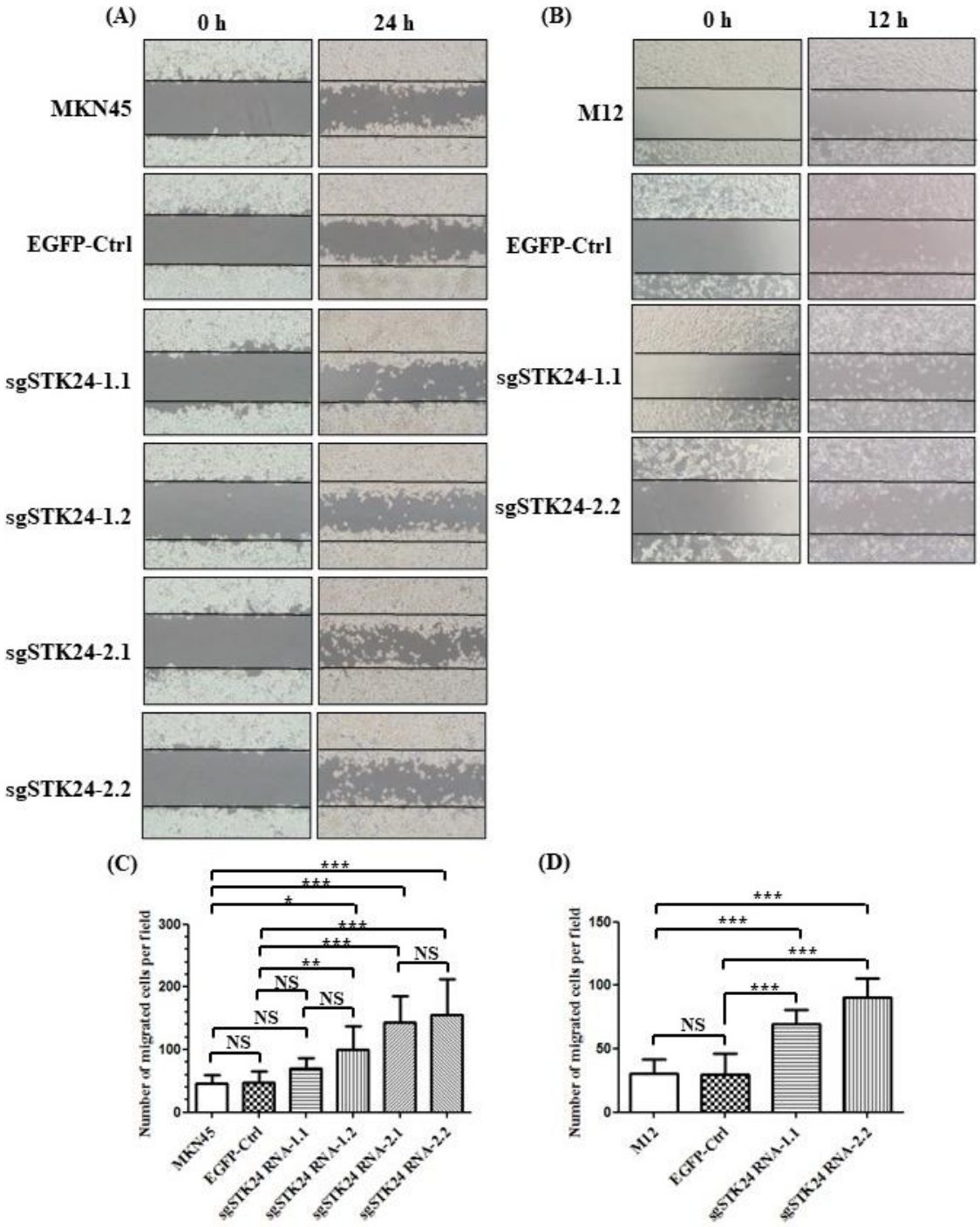

Figure 1. STK24 silencing in MKN45 and M12 cell clones suppressed cell migration in vitro. STK24-knockdown (A) MKN45 cell clones and (B) M12 cell clones were determined in the wound healing migration assay. STK24-knockdown cell clones were grown in culture medium containing 10% FCS. (A) and (C) The quantitative results of the in vitro wound-healing assay at 24 h. (B) and (D) The quantitative results of the in vitro wound-healing assay at 12 h. Data were obtained from three independent experiments. The data exhibited the number of cells/field of view, and mean SD showed the average of three independent experiments. NS, not significant; *P < 0.01; **P < 0.001; ***P < 0.0001.

Effect of STK24 suppression on liver metastasis in murine gastric cancer cells

To test the hypothesis that STK24 plays an important role in tumor metastasis, we examined the changes in metastatic ability of the cell clones *in vivo*. M12 parental cells metastasized to the liver when cells were injected into the spleen. We found that there were macroscopic metastatic nodules indicative of liver metastasis in cell clones (**Figure 2A**). The weights of the livers (**Figure 2B**) and spleens (**Figure 2C**) in C57BL/6 mice injected with sgSTK24-1.1 and sgSTK24-2.1 cells were significantly higher than mice injected with EGFP-Ctrl cells. Moreover, histopathologic analyses of liver sections were performed, and tumor nodules were observed after M12 EGFP-Ctrl, sgSTK24-1.1 and sgSTK24-2.1 cell clone injection (**Figure 2D**). We used M12 murine model to demonstrate that the metastatic burden was increased in STK24 knockdown. The results of the *in vitro* and *in vivo* assays demonstrated that STK24 plays a significant role in the metastasis of mouse gastric cancer cells.

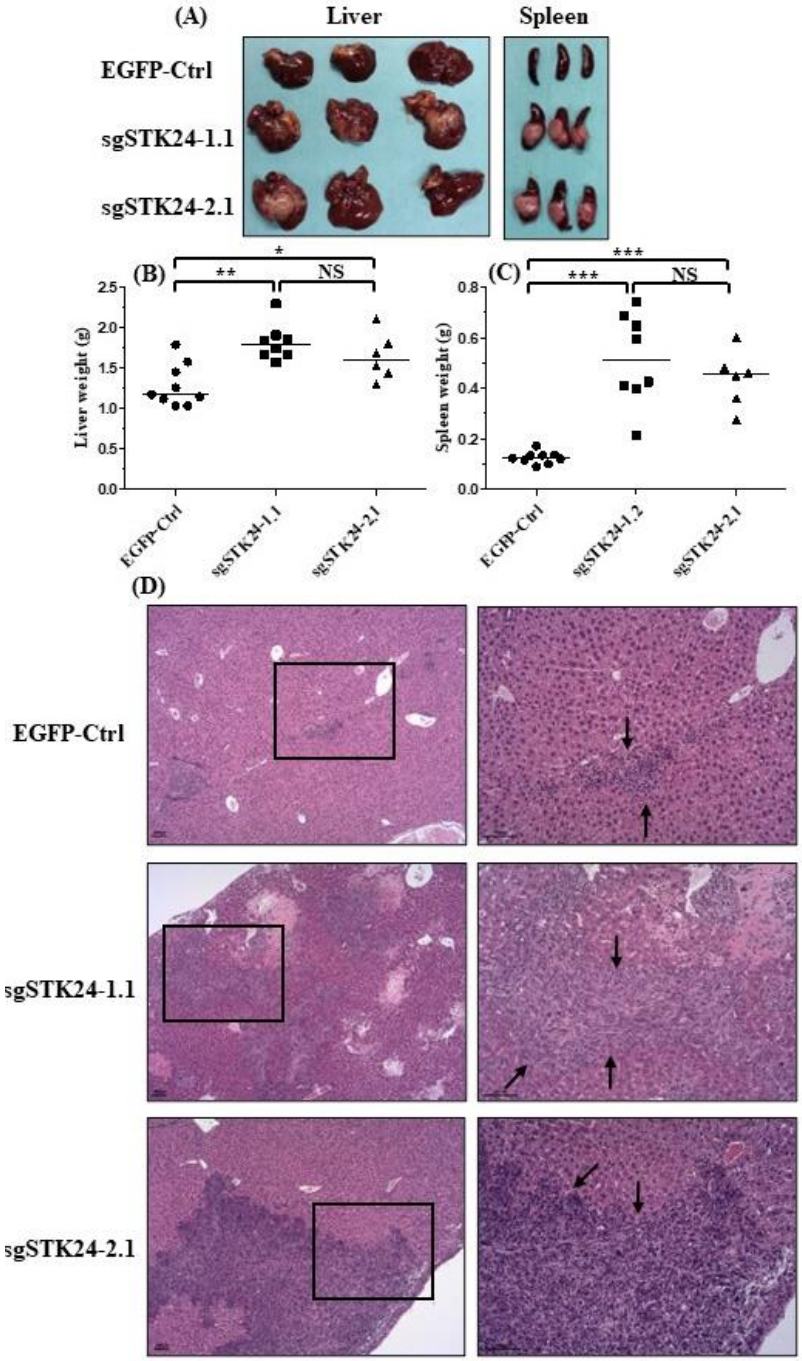

Figure 2. Knockout of STK24 expression in M12 cell clones promoted tumor metastatic ability in vivo. (A) The macroscopic appearance of the liver and spleen tumor masses occurred after the intrasplenic injection of the EGFP-Ctrl and STK24-suppressed cell clones. The weights of livers (B) and spleens (C) in C57BL/6 mice injected with M12 cell clones on day 14. The results are expressed as the mean values ± s.d. in two independent experiments (n = 6–9 per group). EGFP-Ctrl, EGFP control; sgSTK24-1.1 and sgSTK24-2.1, STK24-specific sgRNAs 1 and 2. (D) Histologic image shows part of a mouse liver lobule with tumor nodules. H&E stain to determine the distribution of tumor cells of liver was performed on paraffin-embedded specimens from EGFP-Ctrl, sgSTK24-1.1 and sgSTK24-2.1 cell clones. The boxed area in left (×100) is shown at higher magnification in right (×400). Tumor cells are indicated by arrows. NS, not significant, **P < 0.001, ***P < 0.0001.

The proportion of $CD4^+$ cells, $CD8^+$ cells, $F4/80^+$ macrophages and $CD11b^+Ly6C^+$ cells in the spleens of tumor-bearing mice

To study STK24-mediated immunity in gastric metastasis, we investigated the splenocyte subtypes in the metastatic animal model of gastric cancer. The proportion of $CD4^+$ cells was significantly higher in the spleens of EGFP-Ctrl-bearing mice than in sgSTK24-1.2-bearing and sgSTK24-2.1-bearing mice (**Figure 3A and 3B**). Then, the proportion of the $CD8^+$ T cells of splenocytes significantly decreased in the sgSTK24-1.1-bearing mice but did not decrease in the sgSTK24-2.1-bearing mice (**Figure 3A and 3C**). The proportion of $F4/80^+$ macrophages was significantly increased in the spleens of sgSTK24-1.1-bearing and sgSTK24-2.1-bearing mice (**Figure 3A and 3D**). MDSC consists of two major subsets of $CD11b^+Ly6C^+$ or the $CD11b^+Ly6G^+$ phenotype. The $CD11b^+Ly6C^+$ subtype was significantly increased in the spleens of sgSTK24-1.1-bearing and sgSTK24-2.1-bearing mice (**Figure 3E and 3F**). In addition, the subpopulations of infiltrating monocytes were examined. The accumulation of $CD11b^+$Ly6C high (Ly6Chi) cells (inflammatory monocytes) and $CD11b^+$Ly6C low (Ly6Clo) cells (reparative monocytes) was confirmed by gating on $CD11b^+Ly6C^+$ cells (**Figure 3E**). Inflammatory $CD11b^+$Ly6Chi and reparative $CD11b^+$Ly6Clo cells were markedly increased in the spleens of sgSTK24-1.1-bearing and sgSTK24-2.1-bearing mice (**Figure 3E and 3F**). These results indicate that the silencing of STK24 in tumors induced the expansion of $F4/80^+$ macrophages and Ly6 high (Ly6Chi) and low (Ly6Clo) monocytes *in vivo* and suggest that the increased macrophages/monocytes may have an important role in gastric metastasis.

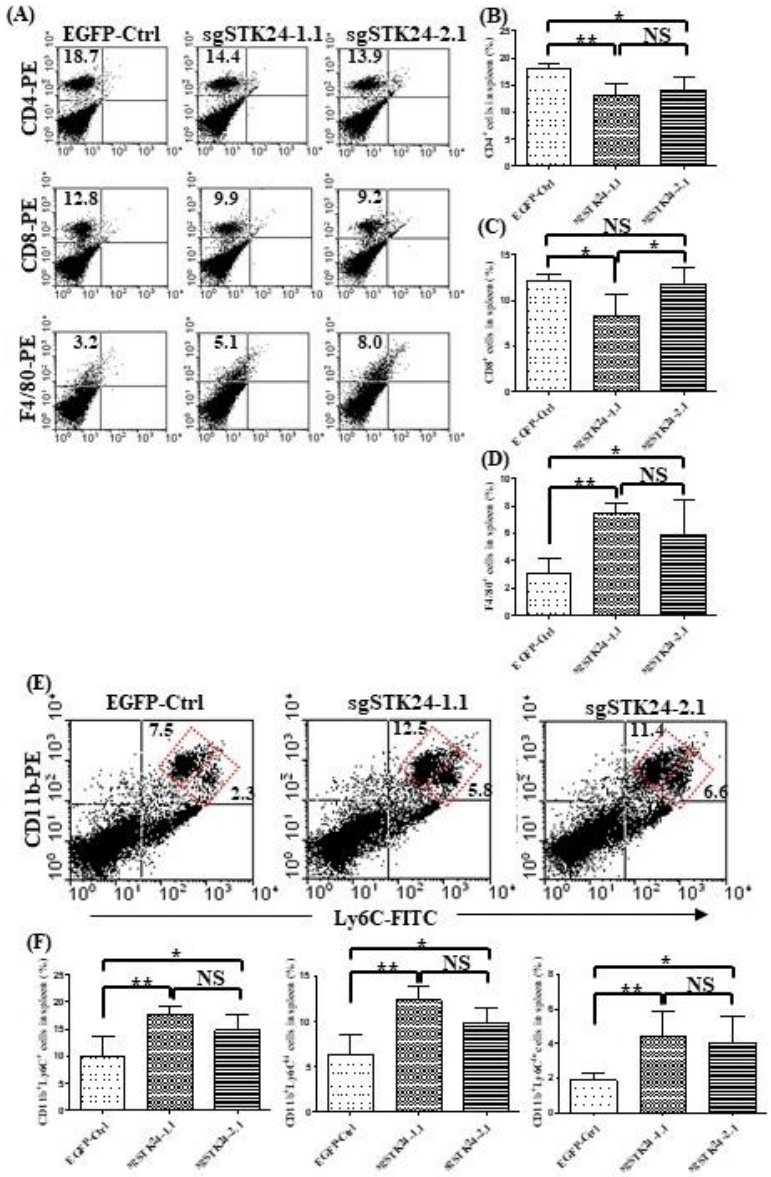

Figure 3. Accumulation of F4/80$^+$ macrophages and CD11b$^+$Ly6C$^+$ cells in spleens of tumor-bearing metastatic mice. Flow cytometry was performed on the spleens of EGFP-Ctrl, sgSTK24-1.1 and sgSTK24-2.1 mice after M12 tumor cell implantation into spleen on Day 14. Splenocytes from EGFP-Ctrl and STK24-suppressed cell clones were stained with anti-CD4, anti-CD8, F4/80, CD11b and anti-Ly6C antibodies. (A) Typical example of flow cytometry analysis. The numbers shown are the percentage of total cells. The percentage of (B) CD4$^+$ (C) CD8$^+$ (D) F4/80$^+$ cells in spleen from EGFP-Ctrl, sgSTK24-1.1 and sgSTK24-2.1 bearing mice. (E) Cells were double stained with anti-CD11b and anti-Ly6C Ab. Flow cytometric analysis of inflammatory monocytes (CD11b$^+$ Ly6C high) and reparative monocytes (CD11b$^+$ Ly6C low) in spleen. (F) The percentage of CD11b$^+$Ly6C$^+$ (left), CD11b$^+$ Ly6C high (middle) and CD11b$^+$ Ly6C low (right) cells in spleen from EGFP-Ctrl, sgSTK24-1.1 and sgSTK24-2.1 bearing mice. The rectangle area in

bottom left is shown $CD11b^+$ Ly6C low (Ly6Clo) population represents reparative monocytes. The rectangle area in bottom right is shown $CD11b^+$ Ly6C high (Ly6Chi) subset represents inflammatory monocytes. The results are expressed as the mean cell population ± SD, and the data are averaged from two independent experiments. NS, not significant, *P < 0.01, **P < 0.001. EGFP-Ctrl, EGFP control; sgSTK24-1.1 and sgSTK24-2.1, STK24-specific sgRNAs 1 and 2.

Decreased expression of STK24 is associated with CDH1 and beta catenin expression in human gastric cancer

We hypothesized that STK24 silencing induces migration and metastasis of gastric cancer through the epithelial-mesenchymal transition (EMT) process. We determined the E-cadherin and beta catenin expression of MKN45 cell clones *in vitvo*. Both E-cadherin and beta catenin expression were suppressed by STK24 silencing in MKN45 cells (**Figure 4A**). The knockout of STK24 expression did not affect the AKT1 protein of the MKN45 cells (**Figure 4A**). To further investigate the correlation between STK24 and CDH1 in gastric cancer, we compared the expression of STK24 and CDH1 in 38 matched specimens of gastric adenocarcinomas and normal tissues obtained from patients treated at the National Cheng Kung University Hospital. We also examined STK24 and CDH1 protein expression by Western blot analysis in tumor and adjacent normal gastric tissues of 38 patients. All 38 cases of gastric cancer were adenocarcinomas, including 11 diffuse gastric adenocarcinoma (DGA), 22 gastric intestinal-type adenocarcinoma (GITA) and 5 gastric mixed adenocarcinoma (GMA) **(Table 1**). The relative expression of STK24 and CDH1 in gastric mixed adenocarcinoma, gastric intestinal-type adenocarcinoma, and diffuse gastric adenocarcinoma tissues was showed (**Figure 4B**). In this study, we aimed to explore the association between the STK24 and CDH1 in patients with gastric adenocarcinoma. Remarkably, a significant positive correlation was identified between the expression levels of STK24 and CDH1 ($r=0.5507$, $P<9.72 \times 10^{-8}$) **Figure 4B-4D**).

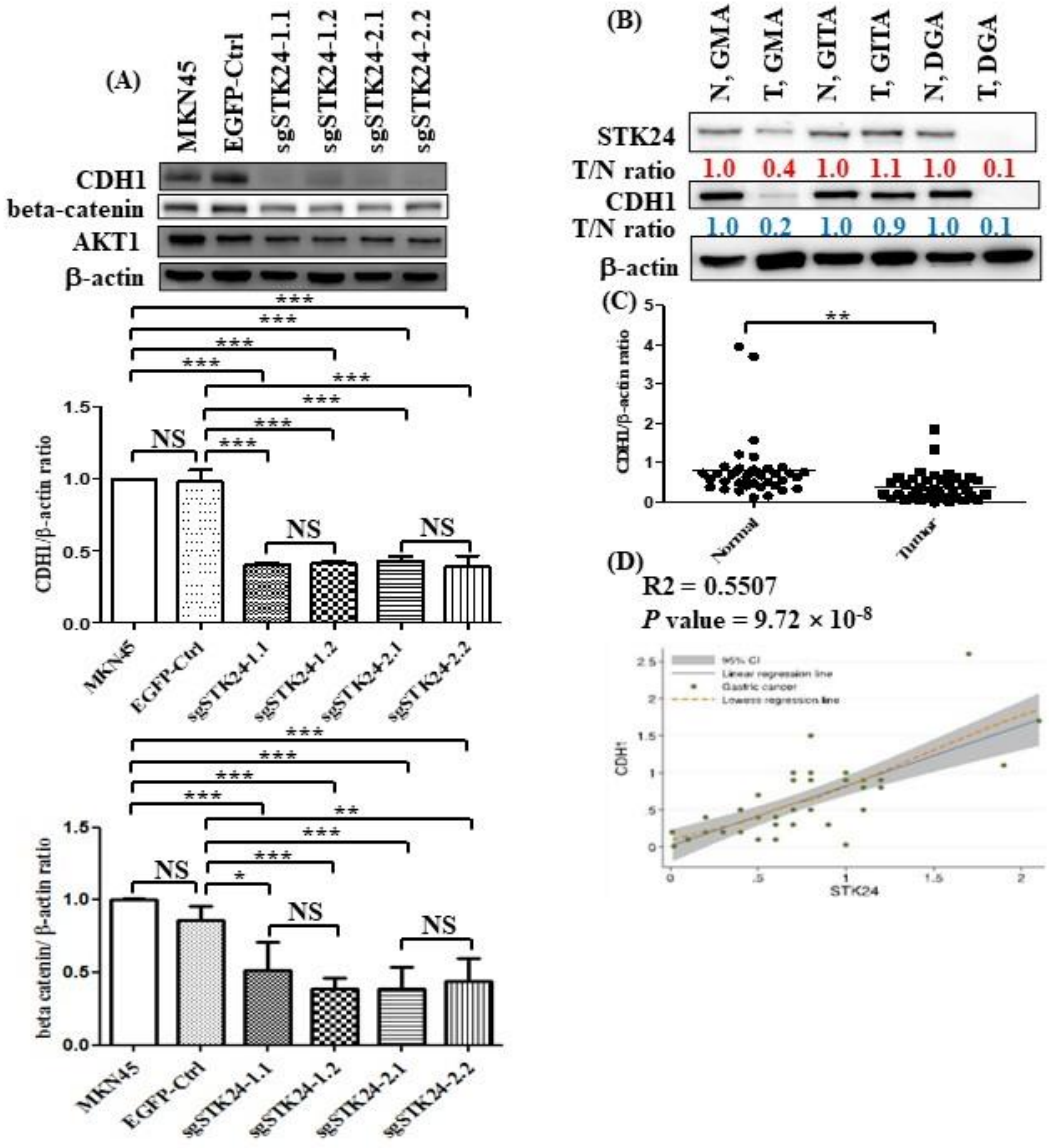

Figure 4. Knockdown of STK24 expression decreases CDH1 protein in human gastric cancer cell lines. (A) STK24-specific sgRNA decreased STK24 protein expression in MKN45 cell clones. Loss of CDH1 expression in STK24-silenced MKN45 cells. Data represent the mean values (± s.d.) of three independent experiments. β-actin was used as internal controls. (B) STK24 and CDH1 expression was measured in specimens of gastric cancer and normal stomach tissues by western blot analysis. The tumor/normal ratio of STK24 and CDH1 expression (the ratio of STK24 and CDH1expression in specimens from gastric cancer relative to that in corresponding normal gastric tissues) was determined by western blot analysis. STK24 and CDH1 expression is presented relative to that of β-actin (STK24/β-actin ratio and CDH1/β-actin ratio). STK24 and CDH1 expression was measured in GMA, GITA and DGA from gastric cancer and normal stomach tissues. N, normal tissue; T, gastric cancer tissue; DGA, diffuse gastric adenocarcinoma; GITA, gastric intestinal‑type

adenocarcinoma; GMA, gastric mixed adenocarcinoma. (C) CDH1 expression is presented relative to that of β-actin (STK24/β-actin ratio). The CDH1/β-actin ratio of 38 samples was measured in specimens of normal stomach tissues (N) and gastric cancer tissues (T) (D) Correlation of the relative protein expression levels of STK24 and CDH1 in gastric tumor tissues. ($P<0.001$ by Pearson's correlations coefficient).

Table I. Demographics and histopathological data of 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)
TNM stage	
Stage I	7 (18)
Stage II	13 (34)
Stage III	14 (37)
Stage IV	4 (11)

Decreased expression of STK24 is associated with stem cell marker CD44 expression in human gastric cancer and prediction of protein–protein interactions in gastric cancer

Moreover, we also analyzed the expression of potential stem cell marker CD44 in MKN45 cell clones. Flow cytometry analysis revealed the presence of CD44. MKN45 sgSTK24-expressing cells exhibited a relatively high CD44 expression (**Figure 5A**). We wanted to exploit protein interaction data to represent significant protein interactions in gastric cancer. Relevant protein–protein interaction information is retrieved by revealing a highly interconnected network from the Search Tool for the Retrieval of Interacting Genes (STRING) database website (**Figure 5B**). The protein-protein interactions information of STK24, CCND1, IL6, CDH1, AKT1, STAT3, CTNNB1 and CD44 proteins was obtained from the String database. An outline of the experimental results is illustrated in **Figure 5**

(C-D). STK24 suppression effectively enhanced the migration and metastatic potentials of the human MKN45 and murine M12 gastric cancer cell lines *in vitro* and *in vivo*. These data suggest that the downregulated expression of STK24 is associated with EMT, stemness and immunosuppression of gastric cancer.

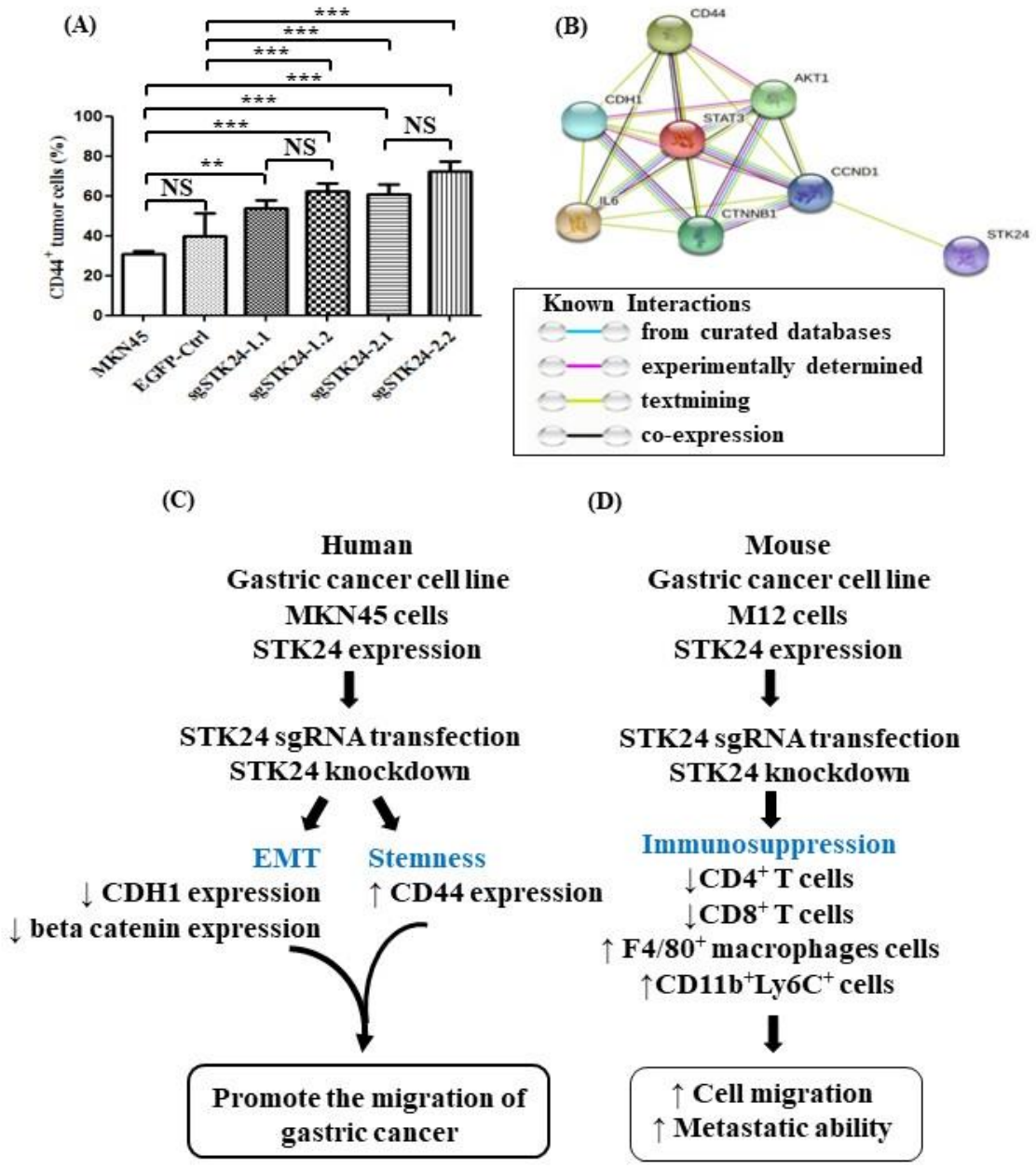

Figure 5. Analysis of cancer stem cell marker CD44 and protein−protein interaction networks. (A) Cell surface marker CD44 expression was determined by flow cytometry. (B) Protein interaction network of STK24, CCND1, IL6, CDH1, AKT1, STAT3, CTNNB1 and CD44. The colored lines between the proteins indicate the various types of evidence demonstrating the interaction. The evidence for these interactions is derived from experimental (purple lines), curated databa (blue lines), co-expression (black lines) and

text-mining evidence (green lines). 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; CTNNB1: β-catenin. The proposed model depicting the effects of STK24 on cell migration and metastasis in MKN45 and M12 gastric cancer cell lines. (C) The suppression of STK24 expression promotes cell migration in the MKN45 cell line, and this effect is associated with CDH1 and beta catenin protein loss and increased stemness. (D) STK24 silencing in the M12 gastric cancer cell line enhances cell migration and tumor metastasis, and these effects are associated with increased MDSCs.

110年度專題研究計畫成果彙整表

計畫主持人：陳怡伶	**計畫編號：**110-2635-B-041-001-
計畫名稱：研究Serine/threonine-protein kinase 24調節骨髓來源抑制性細胞和上皮細胞-間質細胞轉換在胃癌轉移中的作用及其機制	

成果項目			量化	單位	質化 （說明：各成果項目請附佐證資料或細項說明，如期刊名稱、年份、卷期、起訖頁數、證號...等）
國內	學術性論文	期刊論文	0	篇	
		研討會論文	0		
		專書	0	本	
		專書論文	0	章	
		技術報告	0	篇	
		其他	0	篇	
國外	學術性論文	期刊論文	1	篇	Am J Cancer Res 2021;11(9):4277-4293 www.ajcr.us /ISSN:2156-6976/ajcr0133231
		研討會論文	0		
		專書	0	本	
		專書論文	0	章	
		技術報告	0	篇	
		其他	0	篇	
參與計畫人力	本國籍	大專生	0	人次	
		碩士生	0		
		博士生	0		
		博士級研究人員	0		
		專任人員	0		
	非本國籍	大專生	0		
		碩士生	0		
		博士生	0		
		博士級研究人員	0		
		專任人員	0		

其他成果 （無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。）	由 YL Chen 著作 · 2021 · 被引用 5 次 — Am J Cancer Res. 2021 ;11(9):4277-4293.