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Original Article

The BMP antagonist, SOSTDC1, restrains gastric cancer progression via inactivation of c-Jun signaling

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Abstract: Gastric cancer is commonly diagnosed at an advanced stage when metastasis is almost inevitable. Despite numerous novel regulators have been identified in driving gastric cancer progression, much remains unclear due to the complex nature of cancer. Comparison of the transcriptome profiles of gastric primary tumor tissue, with its matched non-tumor and lymph node metastasis revealed frequent stepwise down-regulation of sclerostin domain containing 1 (SOSTDC1) related with tumor progression. Clinically, deficiency of this gene is associated with shortened survival of patients. Our results suggest that SOSTDC1 confers tumor-suppressive features in gastric cancer and silencing of it accelerates tumor growth and promotes the formation of lung metastasis. Although SOSTDC1 displayed limited inhibition of canonical SMAD-dependent bone morphogenetic proteins (BMP) pathway, it remarkably restrained the c-Jun activation and transcription of c-Jun downstream targets in the noncanonical BMP signaling pathway. Furthermore, c-Jun N-terminal kinase (JNK) blockage attenuated cell proliferative and migrative advantages of SOSTDC1 knockdown cell lines. Our study comprehensively elucidated the role of SOSTDC1 in gastric cancer progression and the results translate into potential therapy for gastric cancer.

Keywords: SOSTDC1, gastric cancer progression, JNK inhibition, BMP antagonist

Introduction

Gastric cancer (GC) is the third leading cause of cancer death and remains a major global health care burden. It exhibits great geographical variation: while a steady decline in Europe and America is observed recently, incidence rates are still markedly rising in Eastern Asia [1]. As early disease is often asymptomatic, gastric cancer is frequently diagnosed late with metastasis in the liver, lung, peritoneum and lymph nodes [2]. Once distant metastatic lesions are formed, the 5-year survival drastically drops to less than 10% with a median survival of 9-10 months [3]. Although improved understanding of the genomic landscape involving novel regulators and numerous cellular pathways have been seen in the past decade, continual efforts to unmask the underlying mechanism of tumor initiation and progression is urgently needed to inform effective and personalized treatment for this deadly disease [4].

Bone morphogenetic proteins (BMPs) refer to a group of extracellular molecules with classically defined roles in embryonic and postnatal development [5]. In recent years, extensive research suggests that BMP signaling pathway is also involved in the progression of human disease such as cancer [6]. BMP signaling transduction is triggered by binding of BMP to their receptors, resulting in activation of canonical Smad pathway as well as other noncanonical effectors. This pathway is tightly controlled by BMP antagonists, which are typically small proteins secreted extracellularly [7]. Maintaining a sophisticated balance of expression of BMPs and their antagonists is fundamental in the normal developmental process and dysregulated BMP antagonists are often reported contributing to tumor formation [8-10]. Noggin, as a well-known BMP antagonist, has been suggested that it could inhibit the expansion of osteolytic bone metastasis in prostate cancer [11, 12]. It is reported that levels of epithelial GREM1,

another BMP antagonist, could aid the progenitor cells in reacquisition of stem-cell like properties and initiate colonic tumorigenesis [13]. Furthermore, Gao et al. [8] reveled that Coco reactivates the metastasis -initiating cells from dormant status at secondary site by blocking BMP pathway in the study of breast cancer, which provides clues in the understanding of metastatic quiescence and reactivation. However, there are conflicting reports on the role of BMP antagonists and whether they are pro- or anti-tumorigenic remains unclear [7, 9, 14].

There is emerging evidence showing the prevalence of tumor heterogeneity and clonal evolution of cancer cells [15, 16]. Not all cancer cells are equally malignant and only a fraction of tumor cells could eventually form metastatic lesions in distant organs. It is believed that alterations in some crucial genes may be responsible for driving the progression. Therefore, we hypothesized that by comparing the differential expression patterns of tissues at different stages of tumor progression, we might reveal new novel genes that regulated the aggressiveness of tumor cells. Here, we report that the downregulation of BMP antagonist, sclerostin domain containing 1 (SOSTDC1) promotes gastric cancer progression via activation of c-Jun pathway and its expression is associated with prognosis. Treatment with c-Jun N-terminal kinase (JNK) blockage could attenuate proliferative and invasive advantages of cancer cells.

Results

Identification of SOSTDC1 from transcriptome profiles of gastric cancer cases

To identify the key regulatory genes during gastric cancer progression, RNA sequencing of tissues from four cases of clinical gastric cancer was performed. From each patient, primary tumor tissue, its matched adjacent non-tumor tissue and lymph node metastasis tissue were excised for sequencing (**Figure 1A**). Comparison of the transcriptome profiles of different tissues allowed identification of critical genes responsible for the metastasis. By focusing on candidate genes being down-regulated in stepwise manner along with tumor progression, genes were selected according to the following standards. First, their expression levels in non-tumor tissues were at least 2-fold higher than primary tumor tissues. Second, there should be a further 1.7-fold higher expression in tumors than in the lymph node metastases. After obtaining the gene list that met these criteria in each clinical case, we performed an overlap of all 4 cases (**Figure 1B**) and screened for 16 down-regulated candidate genes that were commonly found in at least 3 cases (**Figure 1C**). Among these genes, we focused on SOSTDC1 since it encoded a BMP antagonist and its role as a potential biomarker in multiple types of cancer had been suggested [17-20]. However, there is only a few reports demonstrating SOSTDC1 down-regulation in gastric cancer and the molecular mechanism remains unclear. Therefore, we selected this gene for further studies.

SOSTDC1 depletion is frequently observed and associated with poor outcome in human gastric cancer

To validate the expression level alteration of SOSTDC1 in a larger scale, we stained a tissue microarray (TMA) with selective anti-SOSTDC1 antibodies. The TMA is comprised of 200 primary GC tumor tissues, paired non-tumor tissues as well as matched lymph node metastases. Informative results were obtained from 183 primary tumor tissues, 164 non-tumor tissues and 73 lymph node metastatic tissues. Non-informative samples included lost samples and unrepresentative samples, which were not included in data complication. Then, a score was given to each tissue according to the staining intensity (**Figure 1D**). It was found that the distribution of scores varied in different tissues. Whereas almost all non-tumor tissues expressed SOSTDC1 and more than 50% of samples were highly positive, a considerable fraction of primary tumors and lymph node metastases had no detectable levels (**Figure 1E**). Collectively, the average expression levels in non-tumor tissues were significantly higher than in the remaining two groups (**Figure 1F**), implying the potential of SOSTDC1 expression as a predictive biomarker of gastric cancer progression. Further Kaplan-Meier survival analysis revealed that patients with primary tumors exhibiting low SOSTDC1 expression (Score ≤1.5) had shorter survival time than patients with high expression (log-rank test, P=0.0123; **Figure 1G**). Similar survival disadvantages were

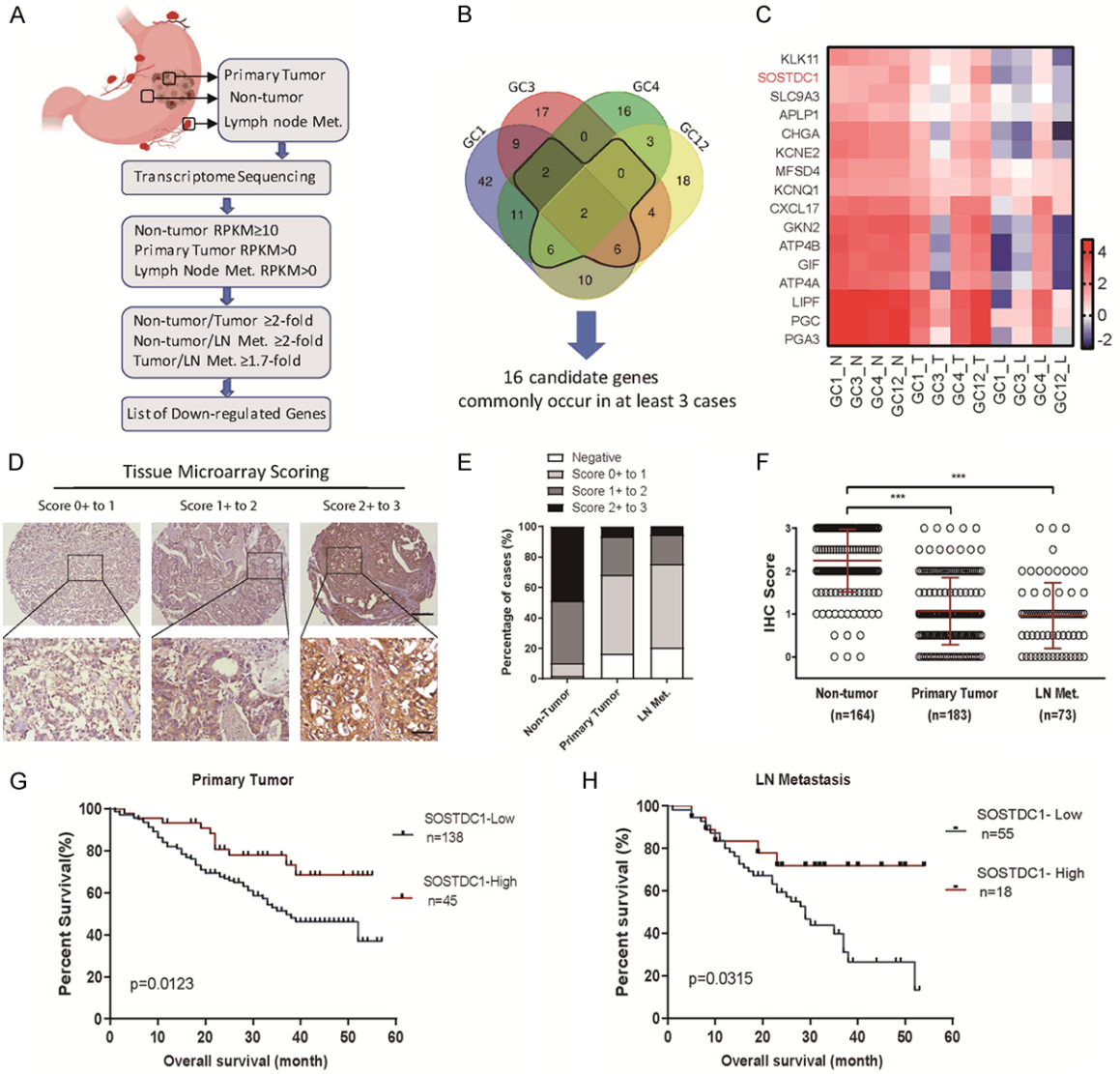


Figure 1. Down-regulation of SOSTDC1 is associated with poor outcome in gastric cancer patients. (A) Screening of down-regulated candidates from transcriptome sequencing. In each clinical case, primary gastric tumor tissue together with matched non-tumor tissue and lymph node metastasis were used for RNA-seq and down-regulated genes that met the selection criteria were chosen for further screening. (B) Venn diagram showing number of down-regulated genes that were commonly found in 4 gastric cancer cases. (C) List of 16 genes that occurred in at least 3 cases and heat map displays the expression level in transcriptome sequencing (N: Non-tumor; T: primary tumor; L: lymph node metastasis). (D-F) Results of TMAs comprising of 183 primary gastric tumors, 164 paired non-tumor tissues as well as 73 matched lymph node metastases stained with anti-SOSTDC1. (D) Representative scoring of TMAs based on their intensity of positivity. Scale bars, up: 200 μm; down: 50 μm. (E) Distribution of staining scores across 3 types of tissues in TMA. (F) Mean SOSTDC1 scores in non-tumor, tumor and lymph node tissues. Student *t*-test. Bar graphs are shown as mean ± SD. ***P<0.001. (G and H) Overall survival analysis of TMA patients according to their SOSTDC1 expression levels in primary tumors (G) and in lymph node metastases (H). Kaplan-Meier survival analysis.

also illustrated in patients with low levels of SOSTDC1 in the lymph node metastases (log-rank test, *P*=0.0315; **Figure 1H**). These observations suggest that depletion of SOSTDC1 is frequent in gastric cancer progression and it is associated with poor outcomes of patients.

SOSTDC1 has tumor suppressive function in gastric cancer

With the observation of decreased level of SOSTDC1 in gastric tumors, we hypothesized that depletion of this gene might confer advan-

tages in the tumorigenesis process. To examine the biological function of SOSTDC1, we first stably over-expressed it in two gastric cancer cell lines, SGC7901 and NUGC4 (**Figure 2A**). Proliferative assay showed that high levels of SOSTDC1 inhibited cell growth *in vitro* (**Figure 2B** and **2C**). In addition, the capacity for single cell to survive and form a colony was attenuated in SOSTDC1-overexpressing cells (**Figure 2D**). In contrast, knockdown of SOSTDC1 by two distinct short hairpin RNAs (shRNAs) consistently enhanced tumor cell proliferation and colony formation (**Figure 2F-I**). To better scrutinize the tumorigenic potential, cancer cells with overexpression or silencing of SOSTDC1 were subcutaneously injected into the immunocompromised mice. Xenograft tumor were isolated and compared after 40 days of inoculation. It was found that high levels of SOSTDC1 retarded the tumor growth, resulting in smaller size of subcutaneous tumors compared to controls (**Figure 2E**). SOSTDC1 depleted cells, however, could profoundly facilitate the tumor expansion and mice had heavier tumor burdens (**Figure 2J**, **2K**). These results suggested that SOSTDC1 plays an inhibitory role in the tumorigenesis and downregulation of it enhanced favorable features such as cell proliferation, colony formation and tumor expansion.

SOSTDC1 deficiency promotes cell motility and lung metastasis

As revealed in the transcriptome profiles of 4 gastric cancer specimens, there was a further reduction in the levels of SOSTDC1 in the lymph node metastases relative to the primary tumors. Unfortunately, we could not demonstrate a significant decrease in the analysis of TMA cohort, possibly due to the paucity of paired lymph node tissues. However, it was shown that high levels of SOSTDC1 protected patients from distant metastasis (**Figure 1F**) and prolonged patients' survival time (**Figure 1G**, **1H**). These observations imply that SOSTDC1 may not only suppress oncogenic capacities but also regulate the cancer metastasis. To explore the effect of SOSTDC1 in cell motility, we first characterized overexpressing cells and their controls with Transwell migration assay and Matrigel invasion assay. Restoration of SOSTDC1 impaired the cell migrative abilities in penetrating the porous membrane and fewer cells were invaded through Matrigel in SOSTDC1-High cells (**Figure 3A**, **3B**). On the contrary, silencing of SOSTDC1 enhanced cell motility in both cell lines (**Figure 3C**).

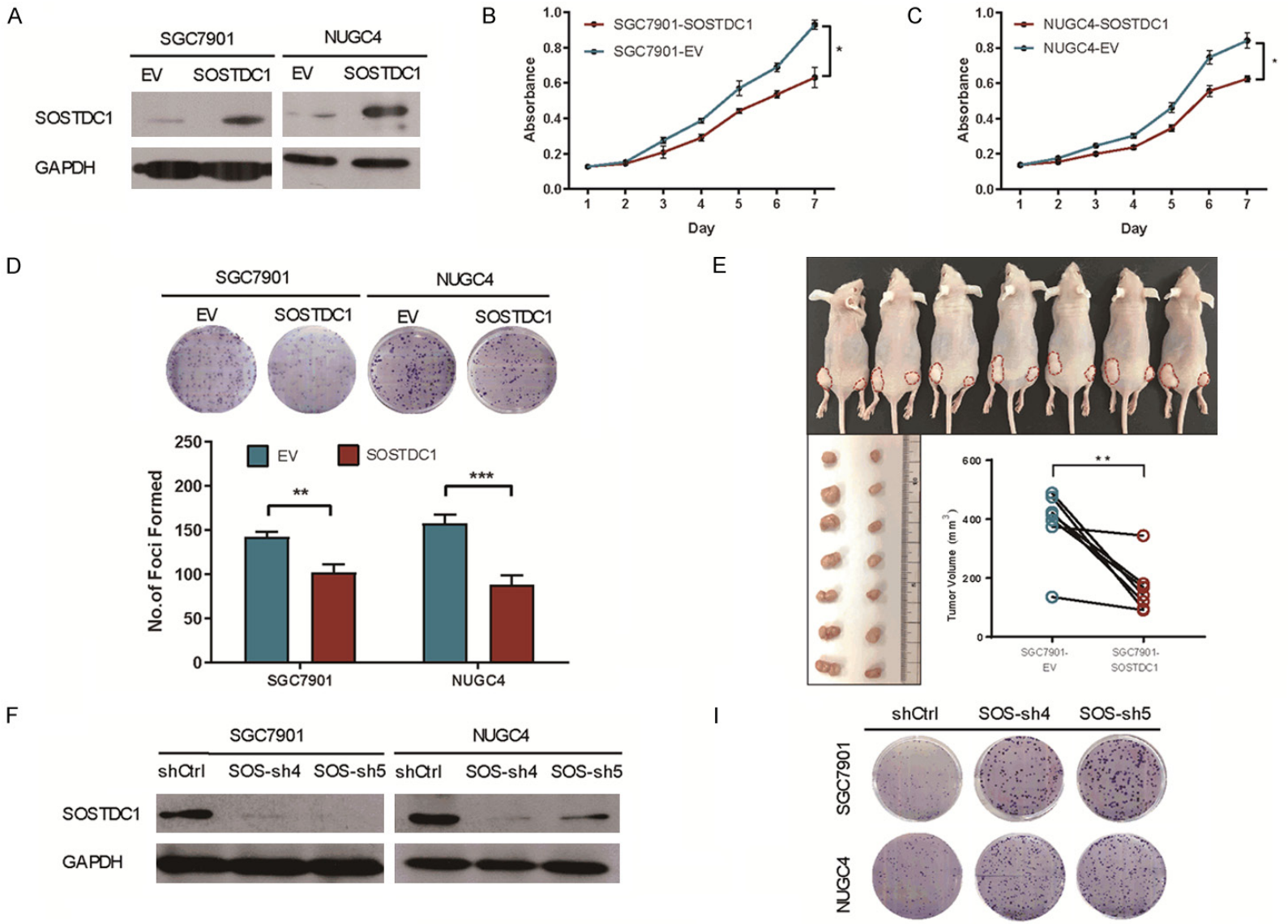
Aiming to mimic the metastasis formation in vivo, tail vein injection mouse model was used. Briefly, Luciferase-labelled cells were administrated through mouse tail vein and the formation of lung metastasis was regularly monitored via bioluminescence imaging. Compared to control group, restoration of SOSTDC1 delayed the onset of lung metastasis in mice and significantly abrogated the colonization in lungs after 3 months of injection (**Figure 3D**, **3E**). At the endpoint, whole lungs were isolated for processing and sectioning. IHC staining with anti-cytokeratin 7 (CK7) antibodies confirmed the presence of malignant lesions in lung tissues (**Figure 3D**, **3E**). On the other hand, depletion of SOSTDC1 accelerated the development of lung metastasis and several lesions were observed in the mice of SOSTDC1-silencing group (**Figure 3F**). Collectively, these findings suggest that SOSTDC1 deficiency could enhance the cancer cell movement and promote the formation of lung metastatic relapse *in vivo*.

Treatment with exogenous SOSTDC1 suppresses gastric cancer oncogenic potential

In addition to modulating the endogenous expression levels of SOSTDC1 in gastric cancer cells, we also used human recombinant SOSTDC1 (rhSOSTDC1) to treat naïve SGC7901 and NUGC4 cancer cells and studied their phenotypic changes. Similar inhibitory effects on cell proliferation (**Figure 4A**, **4B**) were observed when rhSOSTDC1 was added into the culture medium of cells. Treatment with rhSOSTDC1 abolished the ability of individual cells to survive and form a colony (**Figure 4C**, **4D**). Furthermore, administration rhSOSTDC1 also caused profound suppression in migrative and invasive abilities (**Figure 4E**, **4F**). These results suggest that treatment of exogenous SOSTDC1 induces unfavorable proliferative and migrative features of gastric cancer cells.

SOSTDC1 antagonizes BMP signaling pathway predominantly by regulating the activation of c-Jun

With the observations of phenotypic changes caused by SOSTDC1 level, we then questioned the mechanism through which SOSTDC1 exert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NUGC4-SOSTDC1
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EV
SOSTDC1
EV
SOSTDC1
No.of Foci Formed
SGC7901
NUGC4
E
Tumor Volume (mm3)
SGC7901-EV
SGC7901-SOSTDC1
F
SGC7901
NUGC4
shCtrl
SOS-sh4
SOS-sh5
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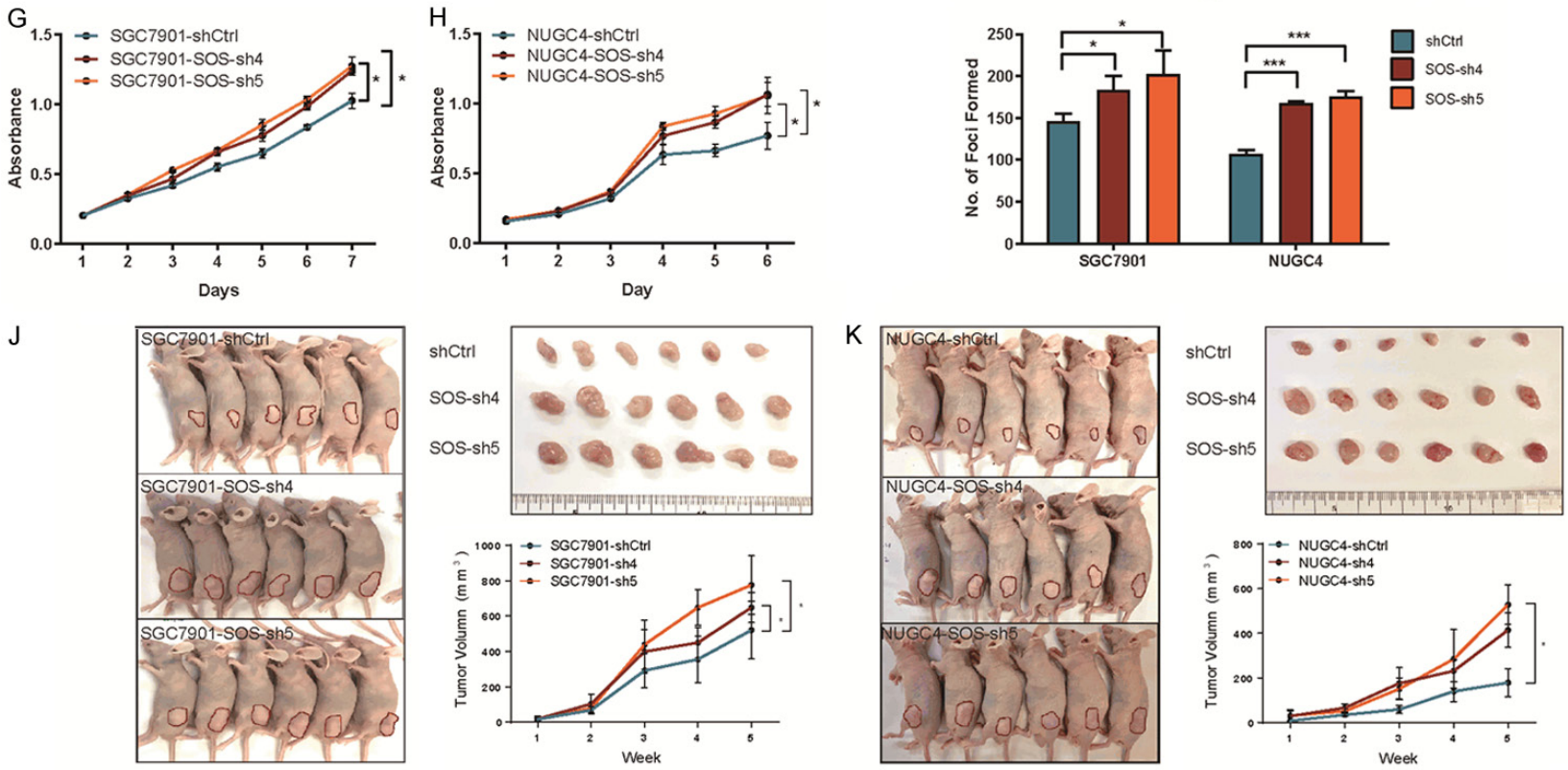
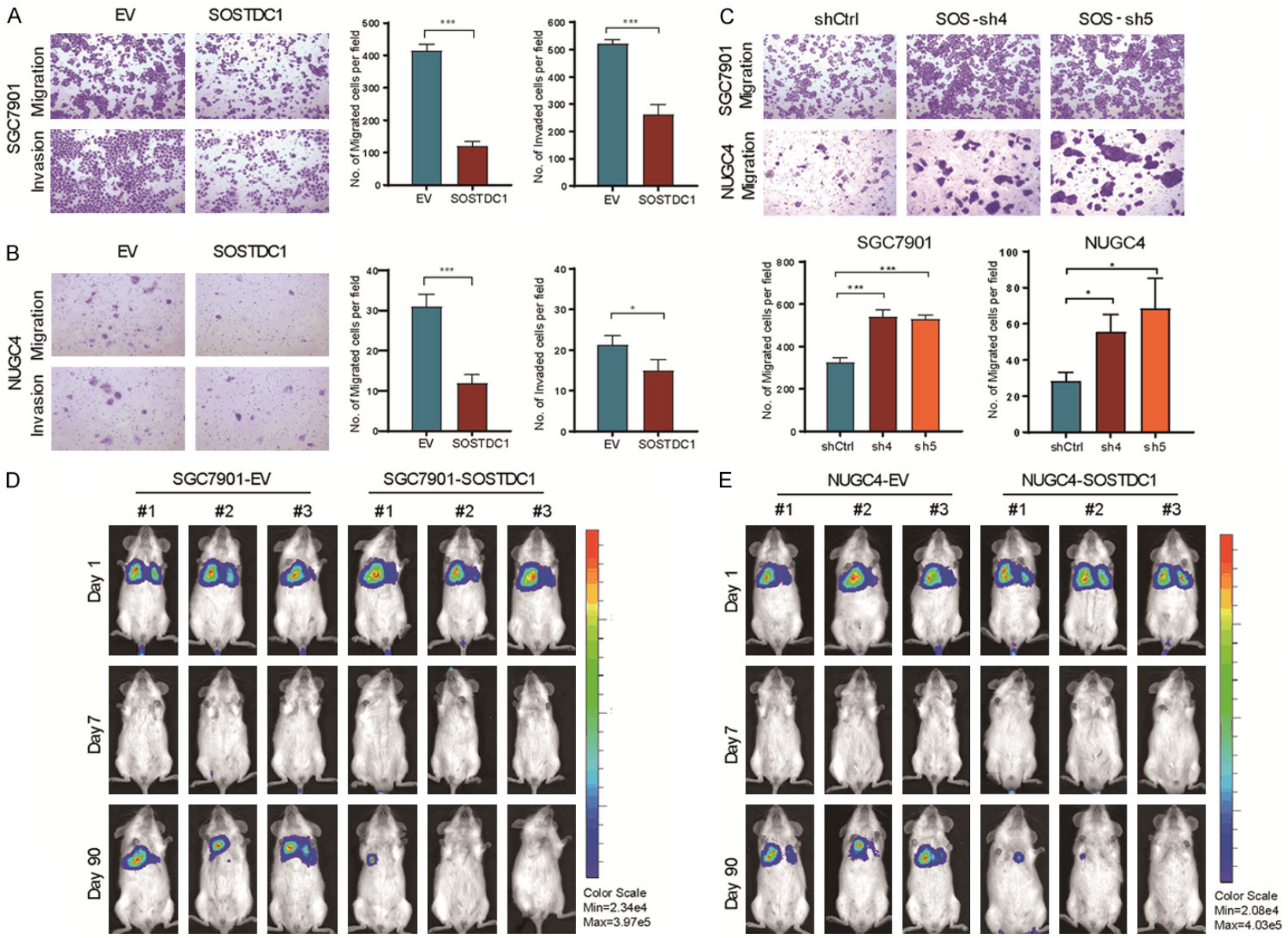


Figure 2. Rescue of SOSTDC1 displays inhibitory tumorigenic features in gastric cancer. (A) SOSTDC1 protein levels in SGC7901 and NUGC4 cell lines transduced with SOSTDC1-expressing or empty vector (EV). (B and C) SGC7901 (B) and NUGC4 (C) cells overexpressing SOSTDC1 or empty vector were subjected to proliferation assay for continuous 7 days. (D) Foci formation assay using overexpressing cells. 500 cells were seeded and cultured for 2 weeks. (E) SGC7901 cells expressing SOSTDC1 or EV were inoculated subcutaneously into the left and right dorsal flank of nude mice respectively and tumor volume were measured after 5 weeks growth (n=7). (F) Knockdown level of 2 shRNAs targeting SOSTDC1 (sh4 and sh5) in SGC7901 and NUGC4 gastric cancer cell lines. (G and H) proliferation assay of SOSTDC1-silenced SGC7901 (G) and NUGC4 (H) cells. (I) Foci formation assay of SOSTDC1 knockdown cells. (J and K) Subcutaneous tumor growth assay using SOSTDC1-silenced SGC7901 (J) and NUGC4 (K) cells. Tumor volume was monitored once a week. n=6 for each group. Student *t*-test. Bar graphs are shown as mean ± SD. $^{*}P<0.05$. $^{**}P<0.01$. $^{***}P<0.001$.


A
EV
SOSTDC1
SGC7901
Migration
Invasion
No. of Migrated cells per field
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B
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C
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shCtrl
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sh5
D
SGC7901-EV
SGC7901-SOSTDC1
#1
#2
#3
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Day 7
Day 90
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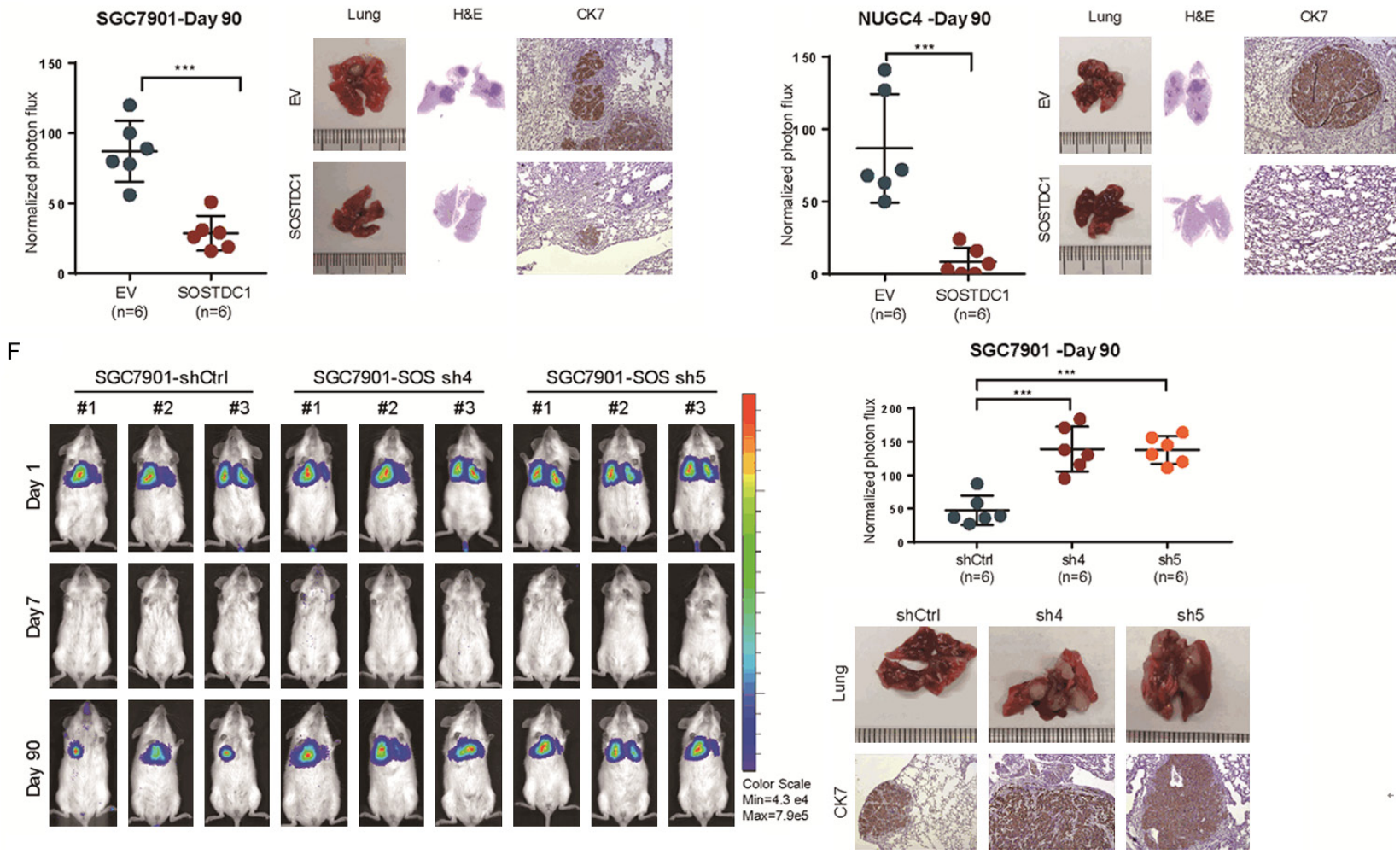


Figure 3. SOSTDC1 deficiency promotes cell motility and lung metastasis. (A and B) Transwell migration and Matrigel invasion assay of SGC7901 (A) and NUGC4 (B) cells with ectopic overexpression of SOSTDC1. 6 random fields of view were captured and counted. Scale bars, 100 μm. (C) Transwell migration assay of SOSTDC1-silenced cells. Scale bars, 100 μm. (D and E) SOSTDC1 expression suppresses lung metastasis. SGC7901 (D) and NUGC4 (E) highly expressing SOSTDC1 or not were injected into NOD/SCID mice via tail vein and lung metastasis were measured by bioluminescent imaging (upper panel). After 90 days, lungs were isolated and sectioned for H&E staining and IHC staining using anti-CK7 antibodies (Bottom panel). n=6 mice per group. Scale bars, 200 μm. (F) SOSTDC1-silenced SGC7901 cells were intravenously inoculated into NOD/SCID mice and metastatic lesions at lung sites were indicated using anti-CK7 staining. n=6 mice per group. Scale bars, 200 μm. Student *t*-test. Bar graphs are shown as mean ± SD. $^{*}P<0.05$. $^{**}P<0.01$. $^{***}P<0.001$.

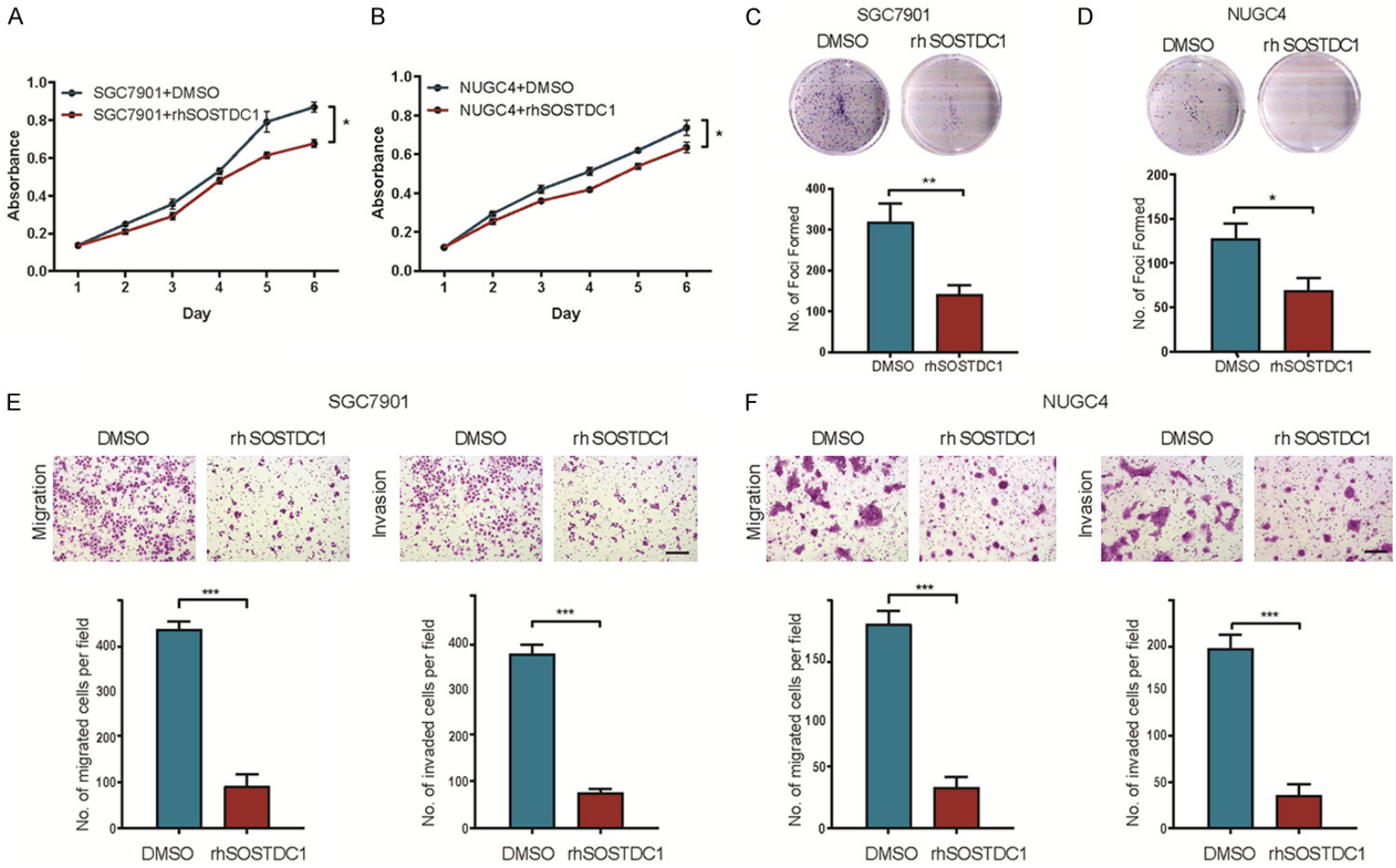


Figure 4. Treatment with recombinant SOSTDC1 suppresses oncogenic potential. (A and B) Naïve SGC7901 (A) and NUGC4 (B) cells were subjected to proliferation assay with treatment of 150 ng/mL of human recombinant SOSTDC1 protein or equal volume of DMSO. (C and D) Foci formation capacities of SGC7901 (C) and NUGC4 (D) cells with recombinant SOSTDC1 or DMSO. Culturing media were replaced every 3-4 days. (E and F) Transwell migration assay and Matrigel invasion assay of SGC7901 (E) and NUGC4 (F) cells exposed to 150 ng/mL recombinant SOSTDC1 or DMSO. Scale bars, 100 μm. Student *t*-test. Bar graphs are shown as mean ± SD. $^{*}P<0.05$. $^{**}P<0.01$. $^{***}P<0.001$.

its inhibitory functions in gastric cancer. This gene was first identified by Larurikkala and her colleagues [21] in the study of the tooth enamel knot and they named as ectodin. They also reported that it had ~37% amino acid identical to the BMP antagonist sclerostin. Subsequent studies involving SOSTDC1 indicated that it could modulate the BMP signaling [20, 22, 23]. These reviews encouraged us to start our mechanistic investigations from the canonical Smad-dependent BMP pathway, which involved the activation of receptor regulated Smads (R-Smads) and translocation into the nucleus following association of common partner Smad (Co-Smad). Western blot was performed to detect the levels of phosphorylated Smad1 and Smad1/5/9 as wells as Smad4 in the SOSTDC1-overexpression cell lines. Results show that SOSTDC1 restoration only suppresses the activation of Smad proteins in a minor way and slightly decreased levels of phospho-Smad1 and phospho-Smad1/5/9 were observed (**Figure 5A**). We next accessed the non-canonical BMP signaling. Surprisingly, significant reduced level of phospho-c-Jun and its novel transcriptional targets was observed in SOSTDC1-High cells (**Figure 5B**). In addition to overexpressed cells, we also verified in SOSTDC1 silencing cells. As expected, SOSTDC1 depletion resulted in the elevated levels of activated c-Jun (**Figure 5B**). Since the expression of c-Jun is positively autoregulated by its own product [24], it was also observed that the levels of c-Jun were modified in response to the abundancy of phospho-c-Jun. These results suggest the high possibility that SOSTDC1 might execute its function by targeting c-Jun signaling.

SOSTDC1 modulates the expression levels of c-Jun downstream targets

Encoded by JUN gene, c-Jun is believed to be an oncogenic transcriptional factor and induces the expression of multiple cancer-related genes after double phosphorylation of JNK [25]. Previous studies revealed that it controlled various key cellular processes during cancer progression including cell proliferation, apoptosis and cell invasiveness [26-29]. Considering the possibility that tumorigenic and metastatic advantages of SOSTDC1-deficent gastric cancer cells were caused by deregulation of c-Jun activity, the expression of a set of novel c-Jun target genes was detected by quantitative PCR (**Figure 5C-F**). Our results demonstrated that the levels of EGFR, MYC, CCND1, ZEB2 and BCL2 were negatively associated with the abundance of SOSTDC1 while levels of CDKN1A and CDKN1B were positively correlated (**Figure 5C**, **5D**), implying SOSTDC1 modulates cell proliferation and apoptosis via c-Jun. Furthermore, expressions of genes regulating cell invasiveness and angiogenesis including CCL5, ITGA5, MMP2 and MMP9 were suppressed with SOSTDC1 treatment (**Figure 5C**, **5D**). Additionally, the expression at protein level of selected c-Jun key targets c-Myc and Cyclin D1 was detected and the alterations were consistent with q-PCR results. Therefore, it is suggested that SOSTDC1 triggers decreased abundance of genes that are assisting in cancer growth and invasive ability while SOSTDC1 depletion could exhibit opposite effects (**Figure 5E**, **5F**) and favor cancer progression.

JNK blockage attenuates cancer cell aggressive advantages of SOSTDC1 silencing cells

In the gastric cancer clinical specimens, depletion of SOSTDC1 was frequent observed in malignant gastric tissues, especially in metastases, implying low expression of SOSTDC1 could exert novel advantages in cancer progression. To examine whether these advantages could be abolished by c-Jun signaling inhibition, we introduced a selective JNK inhibitor SP600125 to the study. First, *in vitro* functional assays with exposure of SP600125 were performed in SOSTDC1-silencing cells. It was found that the proliferative advantages could be profoundly reversed by SP600125 (**Figure 6A**, **6B**). In the XTT proliferation assay, no more significant growth rate differences were observed with SP600125 between knockdown cells while with treatment of DMSO, shRNA-mediated SOSTDC1 depletion cells grew in a higher growth rate. Regarding the capacity of single cell survival and colony formation, the dominance of shSOSTDC1 cells were also attenuated in the presence of SP600125 (**Figure 6C**, **6D**). Meanwhile, JNK blockage decelerated the cell movement without considerable differences in SOSTDC1-slienced SGC7901 and NUGC4 cells (**Figure 6E**, **6F**). All these findings provided evidence that inhibition of c-Jun could attenuate the benefits in

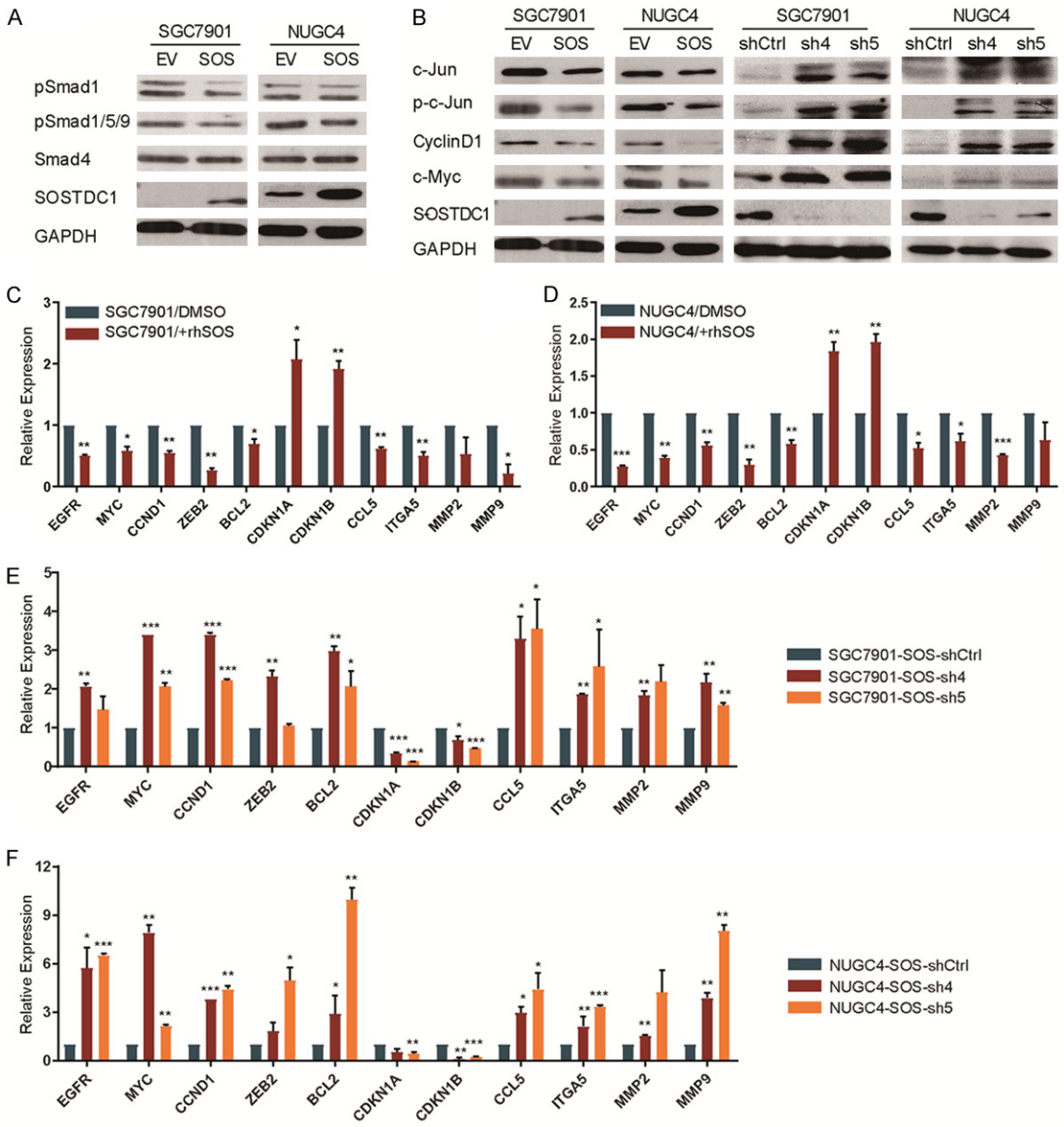
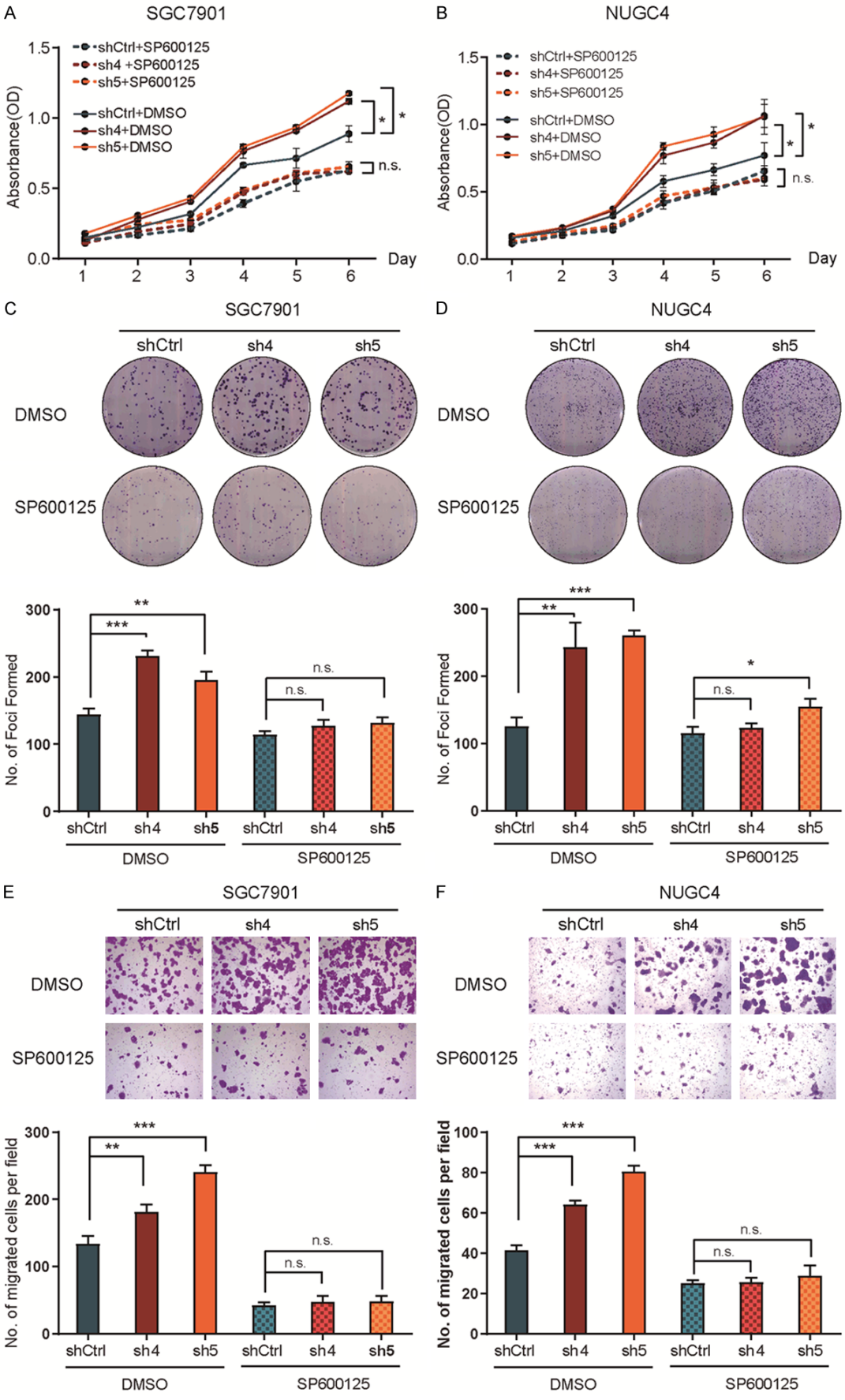


Figure 5. SOSTDC1 predominately modulates activation of c-Jun and its transcriptional targets. (A) Restoration of SOSTDC1 slightly impair the activation of canonical Smad-dependent BMP pathway. (B) The effect of SOSTDC1 on the c-Jun phosphorylation and its major transcriptional downstream targets. (C and D) Q-PCR of indicated c-Jun regulated genes in SGC7901 (C) and NUGC4 (D) cells treated with 150 ng/mL recombinant SOSTDC1 for 24 hours. Among these genes, EGFR, MYC, CCND1, ZEB2, BCL2, CDKN1A and CDKN1B are associated with proliferation and apoptosis while CCL5, ITGA5, MMP2 and MMP9 are associated with invasiveness and angiogenesis. (E and F) Q-PCR results showing the levels of c-Jun transcriptional targets upon SOSTDC1 knockdown in SGC7901 (E) and NUGC4 (F) gastric cancer cell lines. Student t-test. Bar graphs are shown as mean ± SD. *P<0.05. **P<0.01. ***P<0.001.

cell growth and invasiveness resulting from SOSTDC1 depletion.

Discussion

Deciphering the genomic landscape is crucial in all cancers and this is particularly so in a highly deadly cancer like gastric cancer, tremendous efforts have been invested in identifying new molecular targets and signaling pathways involved in gastric cancer. Although previous research has highlighted the roles of numerous novel regulators such as KRAS [30], CTNNB1 [31] and TP53 [32] in gastric carcino-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DMSO
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No. of Foci Formed
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No. of migrated cells per field
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Figure 6. JNK blockage attenuates cancer cell aggressive advantages of SOSTDC1 knockdown cell lines. (A and B) SOSTDC1-silenced SGC7901 (A) and NUGC4 (B) cells were subjected to proliferation assay with addition of 10 μM SP600125 or DMSO in the culturing medium. (C and D) Attenuated colony formation advantages of SGC7901-shSOSTDC1 (C) and NUGC4-shSOSTDC1 (D) cells with treatment of SP600125. (E and F) Impaired migrative capacities of SOSTDC1-knockdown SGC7901 (E) and NUGC4 (F) cells treated with 10 μM SP600125 or DMSO. Scale bars, 100 μm. Student *t*-test. Bar graphs are shown as mean ± SD. *P<0.05. **P<0.01. ***P<0.001; n.s. not significant.

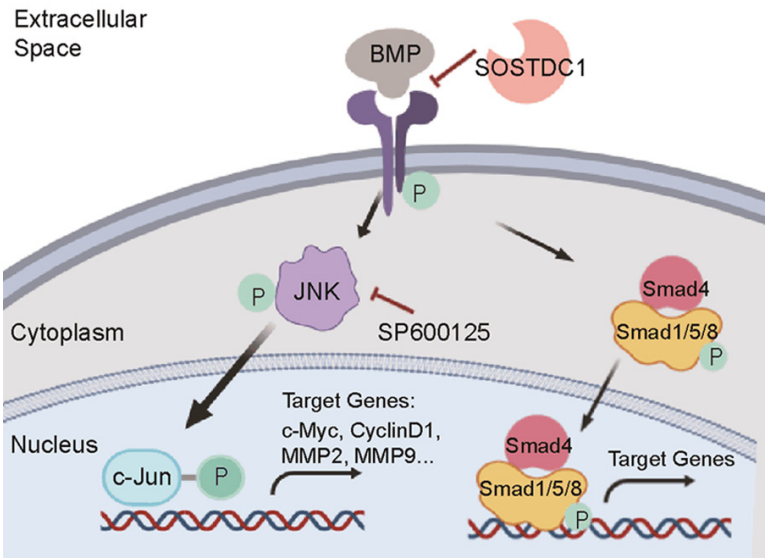

Figure 7. A schematic model of role of SOSTDC1 in gastric cancer progression.

ma, there remains a huge knowledge gap in the understanding of gastric carcinogenesis and metastasis. With the purpose of uncovering additional genes that are dysregulated in the gastric cancer, transcriptome profiling of clinical specimens was performed. Following initial screening and validation, we decided to focus on SOSTDC1 due to its frequent down-regulation in primary tumors and metastasis. Furthermore, tissue microarray revealed the unfavorable distribution of SOSTDC1 expression in malignant tissues and its down-regulation is associated with poor survival. Of note, this gene encodes a BMP antagonist and it has been demonstrated by other groups that SOSTDC1 down-regulation was corelated with tumor aggressiveness and poor prognosis in several cancer types [17-19, 33], but little is known in gastric cancer.

Transcriptomics and proteomics project revealed the special expression pattern of particular gene [34]. Interestingly, the expression of SOSTDC1 is quite tissue-specific with abundant level in normal gastric tissue, which is a prerequisite for us study the role of SOSTDC1 down-regulation in gastric cancer. Wondering whether SOSTDC1 was sufficient to modulate gastric cancer progression, we first manipulated the expression level of SOSTDC1 in gastric cancer cell lines and characterized their phenotypic changes focusing on cell proliferation and cell motility perspectives. Our results showed tumor-suppressive features of SOSTDC1 since its restoration significantly alleviated the tumor growth. The anti-proliferative function is in general agreement with the observations in other cancer types [17, 33]. In addition, the tail vein metastasis mouse model further demonstrated its protective role in the formation of lung metastasis for the first time. Phenotypic alterations caused by SOSTDC1 motivated us to ask what the underlying molecular mechanisms could be. Although it is widely reported as a BMP antagonist [21, 35], we only observed limited decrease in the activated R-SMADs and unchanged level of Co-SMADs with the high secretion of SOSTDC1 in the canonical BMP pathway. Intriguingly, restoration of SOSTDC1 could significantly suppress the JNK activity, inducing lowered level of phospho-c-Jun and preventing the c-Jun-controlled transcription (**Figure 7**). These findings may provide insights into how secreted SOSTDC1 could modulate carcinogenesis and metastasis in gastric cancer.

Although the early studies suggested that activation of BMP pathway was tumor-suppressive [36-38], recently, emerging evidence has shown that it could also perform promoting functions in cancer development and its dichotomous role is much dependent on the BMP ligand type and cancer type [6, 39]. It was reported by multiple groups that SOSTDC1 mainly interacted with BMP7 rather than other ligands [20, 40]. Of note, one study in gastric

cancer revealed that expression of BMP7 was positively correlated with tumor size and lymphatic invasion and could be one of the predictors of risk of tumor recurrence [41]. These results imply that SOSTDC1 might exert its inhibitory role by antagonizing BMP7-induced pathway in the gastric carcinomas, which remain to be defined. Our results showed that canonical Smad-dependent BMP pathway was only partially affected by the levels of SOSTDC1 because we could not exclude the possibility that there are additional secreted BMP inhibitors such as gremlin1 forming ligand traps targeting other BMPs also regulate the activation of R-Smads. Instead, it was observed that the phosphorylation of c-Jun was remarkably reduced upon SOSTDC1 restoration. Actually, one report has drawn our great interest saying that BMP7 could reverse the suppression of JNK activity in prostate cancer [42]. Although this signaling axis has not been described before in gastric cancer, BMP-mediated c-Jun pathway is an essential part of signaling networks in cancer development and elucidation of mechanistic SOSTDC1 helps us in the current understanding in BMP pathway in carcinogenesis and metastasis.

It should be pointed out that there is extensive crosstalk between different signaling pathways and c-Jun signaling would not be the sole mechanism of how SOSTDC1 modulates gastric cancer. Recently, work on signaling pathway integration is emerging and cancer initiation is believed to be the result of the accumulation of a series of, rather than a single aberrant regulation [43]. Indeed, SOSTDC1 is also a Wnt signaling antagonist, which could negatively control activity of the Wnt co-receptors Lrp5 and Lrp6 [20, 23, 44]. Taking into account that Wnt signaling controls multiple cellular processes, including cell growth, apoptosis, differentiation, stemness and cell migration in gastric cancer [45], more work along this line is required to investigate the mechanisms in detail. Further explorations on the role of SOSTDC1 with regard to integration and crosstalk between signaling pathways in gastric cancer studies shall inspire new therapeutic strategies.

Importantly, our experiments demonstrated that by targeting JNK activity, the survival and growth benefits of gastric cancer cells caused by SOSTDC1 depletion could be abolished. However, additional *in vivo* assays are required to evaluate the efficacy of JNK blockage in tumor growth and lung metastasis. Over the last decade, JNKs have been increasingly recognized as an attractive therapeutic target for human cancers as activated c-Jun triggers transcription of multiple cancer-critical genes [46, 47]. One example is that combinatory treatment with JNK inhibitor and chemotherapy could specifically induce receptor-mediated apoptosis of hepatocellular carcinoma cells, suggesting great potential of JNK targeting in the safe and effective use of chemotherapy [48]. With the rapid development of drug discovery based on JNK inhibiton, the expression of SOSTDC1 could serve as a potential predictive marker in gastric cancer.

Collectively, our findings revealed the clinical relevance, functional significance and therapeutic implication of SOSTDC1 in gastric cancer progression and metastasis. It is hoped that the work on SOSTDC1 could enhance our current understanding in cancer biology and add new pieces to the signaling pathway puzzles. Exhibiting great potential translational values in disease prognostication and therapeutic targeting, further clinical studies to full validate these findings are warranted.

Materials and methods

GC samples and cell lines

Clinical samples were collected from Linzhou Cancer Hospital (Henan, China). In the tissue microarray, there were total 200 pairs of primary GC tumors and their paired adjacent normal tissue, among which lymph node metastasis tissues are also available for 72 cases. Tissues were processed and paraffin embedded immediately upon surgical resection of gastric cancer patients. Clinical pathological information is available, which include gender, age, tumor size, type of operation, lymph node metastasis number, tumor cell differentiation, tumor-node-metastasis (pTNM) stage, overall survival time and disease-free survival time. The use of the clinical specimens was approved by the University of Hong Kong and the Committees for Ethical Review of Research.

The gastric cancer cell lines SGC7901, NUGC4 provided by the Sun Yat-Sen University Cancer Center (Guangzhou, China). The 293FT cell line

for stable lentiviral expression was purchased from Invitrogen. Gastric cancer cell lines were cultured in RPMI 1640 Complete Medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) (Gibco) and 1% Penicillin-Streptomycin (Gibco). 293FT cells were cultured in High-Glucose Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS, 6 mM L-glutamate, 0.1 mM MEMN on-Essential Amino Acids (NEAA) (Invitrogen), 1 mM sodium pyruvate (Invitrogen) and 500 μg/mL Geneticin (Roche, Mannheim, Germany). All cells were sustained at 37°C in a humidified incubator containing 5% CO_2.

In vitro functional assays

In vitro tumorigenicity and cell motility were assessed by a series of functional assays. XTT cell proliferation assay (Sigma) was used according to manufacturer's protocol. Cells were seeded into a 96-well plate, with a density of 1,000 cells per well. Cell growth rate was assessed for consecutive 7 days with the absorbance read with a scanning multi-well spectrometer (Tecan Sunrise).

For foci formation assay, 1000 cells were seeded onto a 6-well plate and cultured for 2 weeks with surviving colonies were stained with 1% crystal violet.

Cell motility was assessed by Transwell assay and Matrigel invasion assay. Transwell migration assay was conducted in 24-well Millicell hanging inserts (Millipore). Cells with a density of 5-8 × 10^4 cells per well were seeded inside the upper chamber while complete medium supplemented with 10% FBS was added below the insert. Following 48-72 hours of incubation, the penetrated cells were fixed and stained with 2% crystal violet. The number of migrated cells was counted in 6 random fields and calculated.

Procedures of Matrigel invasion assay was similar to that of migrations assay. Briefly, 5-8 × 10^4 cells were added into the BioCoat Matrigel Invasion Chambers (8 μm pore size) resuspended with serum-free medium while below the chamber complete medium was used as chemoattractant. After 48-72 hours incubation, invaded cells through the Matrigel were fixed and stained with crystal violet. The number of invaded cells was quantified by counting at 6 random fields under a microscope.

Animal studies

The experiments involving mice in this study were approved by the Committee on the Use of Live Animals in Teaching and Research (CULATR) at the University of Hong Kong. All mice were housed in cages with strictly monitored air supply, temperature (25°C) and humidity in Laboratory Animal Unit at the University of Hong Kong.

To examine the growth of xenograft tumors, 1 × 10^6 cells were subcutaneously injected into the dorsal flanks of BALB/c nude mice. Tumors induced by injection were regularly monitored with careful measurement of tumor size. Tumor volume was calculated by the formula: Volume = Length × $Width^2$ × 0.5. After observation period, typically 4-5 weeks, mice were euthanized, and xenograft tumors were isolated and fixed in 4% PFA, followed by tissue processing, paraffin embedding, sectioning.

In the tail vein injection assay, four to five-weeks of NOD/SCID mice were injected intravenously with 1 × 10^6 cells suspended in PBS through tail vein. Injected cells were Luciferase labelled so that the metastasis could be monitored by PE IVIS Spectrum in vivo imaging system. Lung metastatic burden were monitored regularly, and bioluminescence signal was measured using the ROI tool. After 90 days, mice were sacrificed at the end the experiments and lungs were isolated. Lung tissues were undergoing a standard fixation, processing, embedding and sectioning. To visualize the tumor nodules in the lungs, sections were used for H&E staining and anti-CK7staining.

Statistical analysis

SPSS version 17.0 (Chicago, IL) was used for all data analyses. An independent Student t test was used to determine the significance of data, as indicated in the figure legends. Kaplan-Meier plots and log-rank tests were applied for clinical survival analysis. Data presented as mean ± SD was obtained by three independent experiments. Results were considered statistically significant when $P<0.05$.

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Disclosure of conflict of interest

None.

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