



Original Article

Blockade of Src signaling prevented stemness gene expression and proliferation of patient-derived gastric cancer stem cells

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ABSTRACT

Objectives: Gastric cancer (GC) is one of the most malignant tumors. Mounting studies highlighted gastric cancer stem cells (GCSCs) were responsible for the failure of treatment due to recurrence and drug resistance of advanced GC. However, targeted therapy against GCSC for improving GC prognosis suffered from lack of suitable models and molecular targets in terms of personalized medicine. To address this issue, two patient-derived GC cell lines SD209 and SD292 with cancer stem cells (CSCs) such as phenotype were isolated for establishing targeted therapy aiming at critical metastatic signaling in GC. **Materials and Methods:** The primary patient-derived GCSCs were established from parts of GC tissues for characterization of stem cells (SCs) phenotype at both cellular and molecular levels. Western blot and Immunohistochemistry (IHC) were performed for identifying the deregulated signaling in GC tissue. Immunofluorescence was used for analyzing proliferating and SC markers in GCSC attached on fibroblast. Acridine orange and propidium iodide analyses were performed for the survival of GCSC in suspensions. **Results:** In the culture environments of both SD209 and SD292, a lot of mesenchymal fibroblasts spread and crowd together on which a lot of cell clumps, suspected as GCSC, were firmly attached. In the IHC analysis, the GCSC stemness genes CD44 and Ep-CAM increased in tumor tissues of SD209, whereas Nanog-1 and octamer-binding transcription factor 3 (OCT-3) increased in that of SD292. By immunofluorescent analysis of a proliferation marker Ki67, the growth of SD209 and SD292 on mesenchymal fibroblasts was found to be reduced by dasatinib, the inhibitor of the Src kinase whose activity was upregulated in tumor tissues of both GCs. Dasatinib also suppressed the expression of Nanog-1 and OCT-3 in SD292 attached on mesenchymal fibroblasts. **Conclusion:** This study may provide a base for targeted therapy against GCSCs/GCs progression in future preclinical/clinical settings.

KEYWORDS: Cancer stem cells, Gastric cancer, Src, Targeted therapy

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INTRODUCTION

Gastric cancer (GC) is one of the most malignant tumors ranking the fourth cause of cancer death [1]. Numerous factors are associated with the progression of GCs, including *Helicobacter pylori* infection, alcohol consumption, smoking, and high salt intake [2]. Currently, surgical resection is still one of the curative treatments of GCs. For patients with unresectable GCs, conventional chemotherapy using drugs such as 5-fluorouracil is the only option. However, these drugs are prone to cause severe side effects such as renal dysfunction [3]. The other challenge in GC management is the high recurrence rate (60%) of GC patients who undergo curative surgery [4]. This was ascribed to the highly metastatic potential of GC, particularly toward the liver and peritoneum [5]. There are a lot of molecular

and cellular factors involved in triggering GC metastasis, among which cancer stem cells (CSCs) were highlighted recently.

It is well known that CSCs represent a very small subset of cancer cells which exhibit characteristics of both stem cells

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(SCs) and cancer cells. CSCs possess the capacity for self-renewal and producing the heterogeneous lineages of cancer cells [6]. Importantly, CSCs are the initiating cells of malignant tumors playing a key role in cancer progression [7-9]. CSCs are capable of educating neighboring cells to provide nutrients and survival factors, creating a favorable environment for tumor growth. Moreover, CSCs give rise to heterogeneous cell populations responsible for its plasticity required for immune dormancy. It is also worthy of noting that mesenchymal SCs, also known as cancer-associated fibroblast (CAF), contribute to the maintenance of stemness through providing support to the niche for CSC survival [10].

Under normal physiological conditions, gastric epithelial cells renew once every 2–7 days, with higher frequency when there is injury. During this process, the gastric stem cells (GSCs) are highly active [11]. Many studies suggested that gastric cancer stem cells (GCSCs) might be derived from GSCs [12]. GSCs, mainly located in the isthmus and bottom of the gastric pit, may migrate to various sites within the stomach. These GSCs may be transformed into GCSCs by environmental stimulation or infection [13], resulting in atypical hyperplasia of the gastric mucosa and increasing the formation of GC [14].

Previously, GCSCs were identified using the cell surface markers CD44 [15] and EpCAM [16] through fluorescence-activated cell sorting. Subsequently, a lot of other GCSC-specific cell surface markers including CD71, CD90, CD133, leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5), aldehyde dehydrogenase 1 (ALDH1), and C-X-C chemokine receptor type 4 were identified [17-20]. Meanwhile, a lot of intracellular GCSC markers were found to overexpress in the CSC-rich population of GC, including Nanog homeobox (Nanog), octamer-binding transcription factor 3 / 4 (OCT3 / 4), muscle/intestine/stomach expression 1 (Mist1), and ATP-binding cassette subfamily B member 1/multidrug resistance protein 1 (ABCB1/MDR1) [21].

The role of GCSCs in GC progression has been well established in the past decades [22]. For example, Zhu *et al.* showed the effect of GCSCs on GC invasion, migration, and angiogenesis [23]. Furthermore, yes-associated protein is thought to enhance the expression of GCSC surface markers and self-renewal GCSCs through transforming growth factor- β -activated kinase 1, which in turn promotes the progression of GC [24]. Recently, mounting studies highlighted GCSCs to be responsible for the failure of treatment due to recurrence [25] and drug resistance of advanced GC [21,22,26]. Therefore, targeting the molecular pathways associated with the biological properties of GCSCs become a promising therapeutic strategy for preventing GC progression [22].

Another issue for GCSC therapy regards the heterogeneity of GCSCs. It is well known that intra-tumor heterogeneity of CSCs can be generated by installing a differentiation hierarchy, leading to a range of distinct cell types presenting within the tumor microenvironment (TME) [27]. Specifically, the categories of GCSC markers may be distinct between individual GCs. Thus, the underlying molecular and cellular mechanisms responsible for GCSC progression [21] need to

be delineated in each specific GC patient for identifying more suitable targets.

In this study, two patient-derived GC cell lines with CSC-like phenotypes were isolated from clinical GC tissues. Both GCSCs showed differential overexpression of CSC markers.

Moreover, prevention of the proliferation of GCSC can be achieved by suppressing the metastatic Src signal pathway upregulated in surgical GC tumor tissues.

MATERIALS AND METHODS

Ethics Approval

The study was conducted in accordance with the Declaration of Helsinki and was approved by Tzu Chi Hospital with (approval number: IRB 112-041 A). Informed consents were obtained from all patients.

Primary culture of gastric cancer stem cell

For personalized targeted therapy of GC, the patient-derived GCs were established in 2023 from parts of GC tissues obtained from surgery. Tissues were pretreated with collagenase and cultivated on 6-well Petri dishes followed by identification and characterization of GCSC as described in the result section. Some of the tissues were harvested for lysates used in western blots whereas the others were kept in formalin for immunohistochemistry (IHC) analysis.

Abs and chemicals

Antibodies for phosphorylated JNK (p-JNK), Src (p-Src), AKT (p-AKT), Ki67, GAPDH, and CSC markers including CD44, EpCAM, CD71, CD90, CD133, Lgr5, ALDH1, CXCR4, NANOG, OCT3 / 4, Mist1, and ABCB1/MDR1 were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Dasatinib for inhibiting Src was from Sigma Aldrich (St. Louis, MO, USA).

Western blot

Western blots were performed according to previous studies [24]. The band intensities on the blots were quantified using Image J software (version 1.50 i).

Immunohistochemistry

IHC was performed by the EnVision + Dual Link System-HRP (DAKO, Carpinteria, CA, USA). Briefly, the tissue section was incubated with Dual Endogenous Enzyme Block to remove any endogenous peroxidase activity. Subsequently, the sample was incubated with primary Ab, followed by the 2nd Ab-HRP labeled polymer. Staining was completed by incubation with 3,3'-diaminobenzidine (DAB+) substrate chromogen, which resulted in a brown-colored precipitate at the antigen site. Quantitation of the staining intensity was performed using the Image J. software (version 1.50 i).

Staining of the indicated proteins was confirmed by that no signal appears in the negative control without adding the primary Ab in the aforementioned IHC procedure (data not shown).

Immunofluorescence for Ki67, p-Src and cancer stem cell markers

GCSC cultured systems were treated with indicated drugs followed by fixation with 4% formalin for 15 min. After

blocking with 1% BSA, the cell was incubated with the indicated primary Abs, followed by suitable 2nd Abs including Goat anti-Rabbit IgG (H+L) Alexa Fluor™ 488 and Goat anti-Mouse IgG (H+L) Alexa Fluor™ 594. Fluorescence of the cells was then detected under an inverse microscope (Nikon Ti2) at the indicated wavelength. Quantitation of the intensity of staining was performed using the NIS-Elements ARAnalysis software.

Acridine orange and propidium iodide analyses for the survival of cells in suspension

After appropriate treatments of suspended SD292, the cells were pelleted down and incubated with acridine orange (AO) and propidium iodide (PI) dyes for staining nucleic acid in live and dead cells, respectively, using Nexcelom Cellometer K2 (Lawrence, MA, USA). The cell survival rates were calculated as the ratio (%) of AO- versus PI-stained cells.

Statistical analysis

Analysis of variance was conducted to evaluate the quantitative differences between samples in western blotting, IHC, and immunofluorescence (IF) analyses. Quantitative data were expressed as the mean \pm coefficient of variation (%).

RESULTS

Enhanced activation of Src, AKT, and JNK in GC tissues

Recently, a lot of GC tissues were collected in Tzu Chi Hospital, Hualien, Taiwan, for screening the metastatic signaling molecules known to be involved in GC progression. These included the well-known oncogenic receptor tyrosine kinase (RTKs), EGFR and c-Met, and a non-receptor tyrosine kinase, Src involved in GC progression [28]. The activity of AKT and JNK, and the oncogenic signal kinases downstream of RTKs and Src were also detected. Interestingly, a lot of the tumors with high metastatic potential exhibited simultaneous elevation of activity of Src (phosphorylated Src, p-Src), AKT (phosphorylated AKT, p-AKT), and JNK (phosphorylated JNK, p-JNK). The data of two of the GC patients, SD209 and SD292, were demonstrated. IHC in Figure 1a and b showed the prominent increase of p-Src in tissue sections of SD209 and SD292 whereas Western blot in Figure 1c demonstrated the simultaneous elevation of p-Src, p-AKT, and p-JNK in SD292 tumor tissues, compared with those in the normal counterparts by 3.2–4.1 fold using GAPDH as an internal control. The signal intensities of p-Src, p-AKT, and p-JNK were also normalized with those of total (including both unphosphorylated and phosphorylated) Src, AKT, and JNK, respectively. However, by normalization with GAPDH, total Src, AKT, and JNK also significantly increased in tumor tissues, compared with those in the normal part by 2.1, 2.0, and 2.3 fold, respectively [Figure 1c]. Thus, after normalization of p-Src, p-AKT, and p-JNK with Src, AKT, and JNK, respectively, the increases of p-Src, p-AKT, and p-JNK in tumor tissues compared with those in the normal part were only about 1.5–1.8 fold. The detailed mechanisms for how total Src, AKT, and JNK increase in GC tissue are awaiting further investigation. Interestingly, the increase of total Src [29] and AKT [30] in pancreatic and bone tumor tissues, respectively, have also been reported previously, consistent with the phenomenon observed in our results.

Isolation and characterization of SD209/SD292 gastric cancer stem cells

During the isolation of patient-derived GC cell lines from GC tissues with upregulated Src signaling, the primary cultures from patients SD209 and SD292 were obtained. Interestingly, both cultures displayed a variety of cellular populations. In the SD209 culture, a lot of small round cells gradually multiplied in the TME, some of which were clumped and attached to the culture dish [indicated by red circles in Figure 2a, the upper two panels]. This is reminiscent of the “spheroid colony formation” that was the hallmark of candidate CSCs [16,31]. Notably, a lot of cells detached from cell clumps which can be isolated and maintained as cell suspensions [Figure 2a, lower left panel]. Moreover, a lot of fibroblast cells were found to spread from the bottom of GCSC clumps [indicated by yellow arrows in Figure 2a, upper right panel]. These are supposed to be mesenchymal fibroblasts or CAF capable of modulating immune responses, angiogenesis, and supporting the growth and metastasis of CSC [10]. In addition, some adipocyte-like cells with deposited lipid fats, known to be associated with GC [32], were also observed [indicated by orange arrows in Figure 2a, lower right panel].

In the SD292 TME, most of the cells attached to Petri dishes showed a fibroblastic morphology (indicated by yellow arrows in Figure 2b, upper and lower panels at $\times 100$ and $\times 200$ magnification, respectively). These are suspected of mesenchymal fibroblasts or CAFs as observed in SD209. Remarkably, most fibroblasts crowded together on which a lot of cell clumps developed [indicated by the red circles in Figure 2b, upper and lower panels]. Moreover, the number and size of cell clumps expanded very rapidly, with an accompanied release of floating cells, possibly detached from the cell clumps [indicated by blue arrows in Figure 2b lower panel, at $\times 200$ magnification]. After the isolation of these floating cells, they are still alive in suspension [Figure 2c]. These suspended cells can be maintained for more than 1 month without significant changes of total cell number (data not shown). It is worthy of noting that in the suspension, some larger cells filled with granules were gradually breakdown upon reaching their maximal size [Figure 2c, indicated by red arrow heads], while some newly produced small round cells appeared [Figure 2c, indicated by orange arrows]. In summary, the development and enlargement of SD292 cell clumps can be promoted by the mesenchymal fibroblasts on which the cell clump is attached whereas some of the SD292 cells in the cell clump may be released as a suspension of single cells with self-renewal ability.

Gastric cancer stem cell marker analysis in gastric cancer tissues

To investigate whether the GC tissues (from which both SD292 and SD209 cultures derived) acquire the characteristics of GCSCs, several GCSC markers known to be associated with GCSC stemness [25] were screened. These include the surface markers: CD44, EpCAM, CD71, CD90, CD133, Lgr5, ALDH1, and CXCR4 and the intracellular markers: Nanog-1, OCT3 / 4, Mist1, and ABCB1/MDR1. As shown in the IHC results [Figure 3a and b] at the $\times 40$ magnification, the staining intensity for each of the CD44 and Ep-CAM in SD209 and

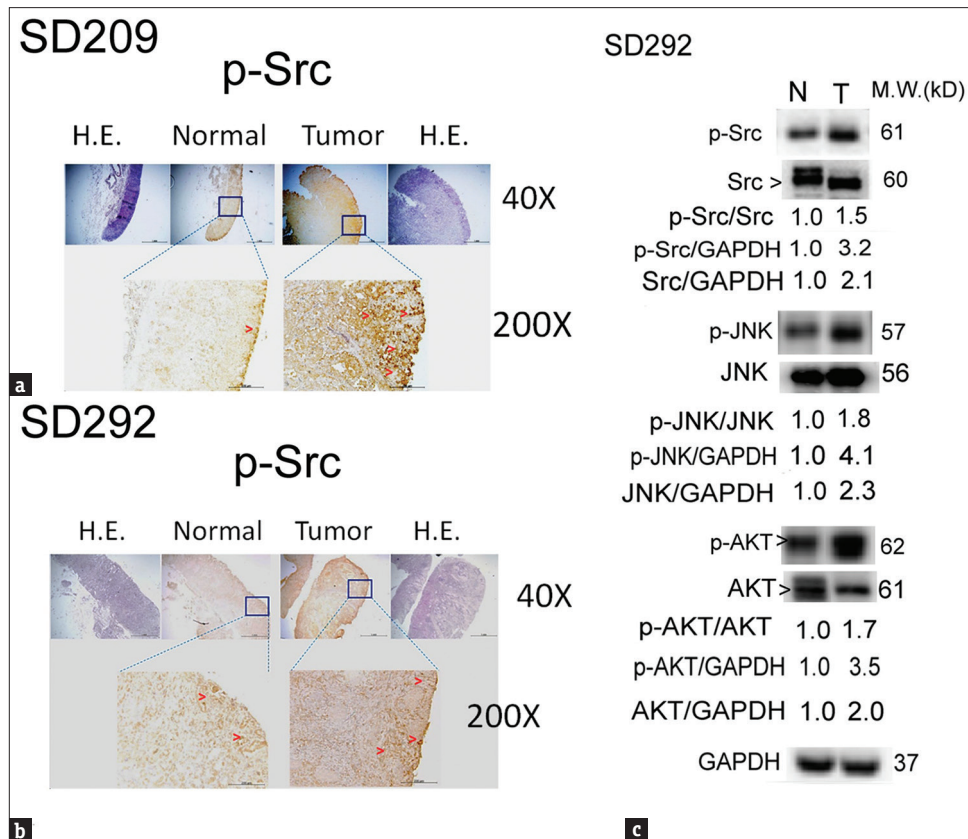


Figure 1: Elevation of activity of Src and the downstream AKT and JNK in SD292 GC tissue. Immunohistochemistry of phosphorylated Src (p-Src) (as brown color) in tissue section of SD209 (a) and SD292 (b) tumor coupled with their normal counterpart at both 40 and $\times 200$. H. E. staining of the sections were shown at $\times 40$ magnification. The $\times 200$ demonstrated the enlarged images of an area in the $\times 40$ enclosed by small blue rectangles. The red arrowheads in the $\times 200$ images indicated the dense staining regions of p-Src. (c) Western blot of p-Src, AKT (p-AKT), and JNK (p-JNK) in SD292 tumor tissues (T) and normal counterpart (N), using GAPDH or total Src, AKT and JNK as internal controls. The band intensity was quantified using Image J software. The number below each molecule represent the relative intensity of p-Src, p-AKT and p-JNK versus GAPDH; Src, AKT and JNK versus GAPDH or p-Src, p-AKT and p-JNK versus Src, AKT and JNK, respectively, taking the data of N as 1.0. The data shown are average of two reproducible results

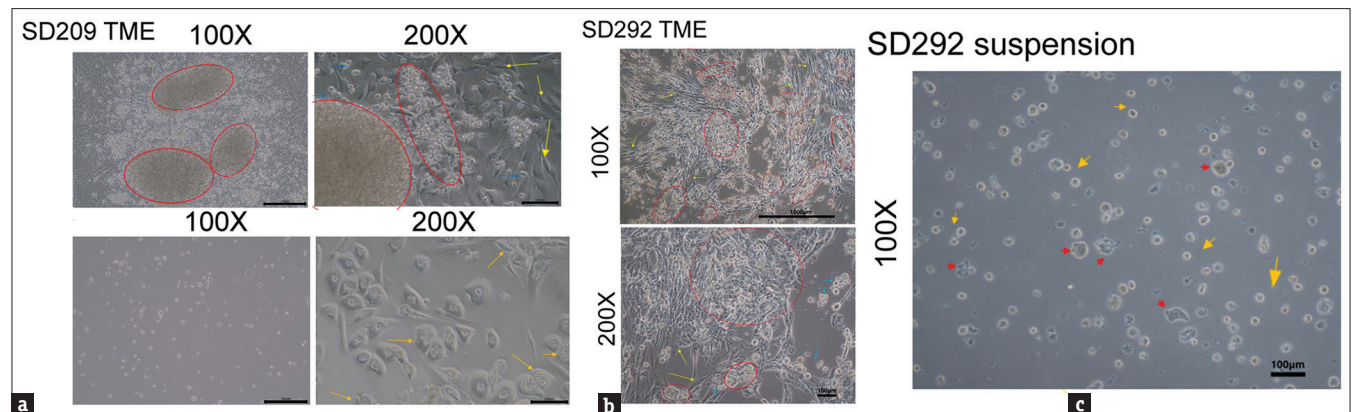


Figure 2: Heterogeneous populations in Tumor microenvironment (TME) of SD209/SD292 exhibiting GC stem cell characteristics. (a) The TME of SD209: Red circles in the upper left and right panels indicate the cells clumps (100- and 200-fold magnification, respectively); yellow arrows in upper right panel indicate the fibroblasts under the bottom of the clump (at 200-fold magnification). Orange arrows in the lower right panel indicate some of the adipocyte-like cells. The lower left panel shows the suspension cell maintained in ordinary medium for 2 weeks. (b) The TME of SD292: Red circles in upper and lower panels indicated several cell clumps on the cancer associated fibroblast at $\times 100$ and $\times 200$, respectively. Blue arrows in lower panels indicate some floating cells detached from the clumps. (c) The SD292 cells suspension: Red arrows indicate the larger cells with granules some of which are being broken; orange arrows indicate the smaller newly produced cells

Nanog-1 and OCT-3 in SD292 were generally higher in the tumor tissues compared with the normal part. The $\times 200$ magnification for each of the four markers represented the enlarged images of an area in $\times 40$ magnification encloses

by small blue rectangles. For quantitation, whereas CD44 and Ep-CAM increased in tumor tissues of SD209 by 6.23 and 9.66-fold, respectively (shown at $\times 200$ magnification), Nanog-1 and OCT-3 increased in tumor tissues of SD292 by

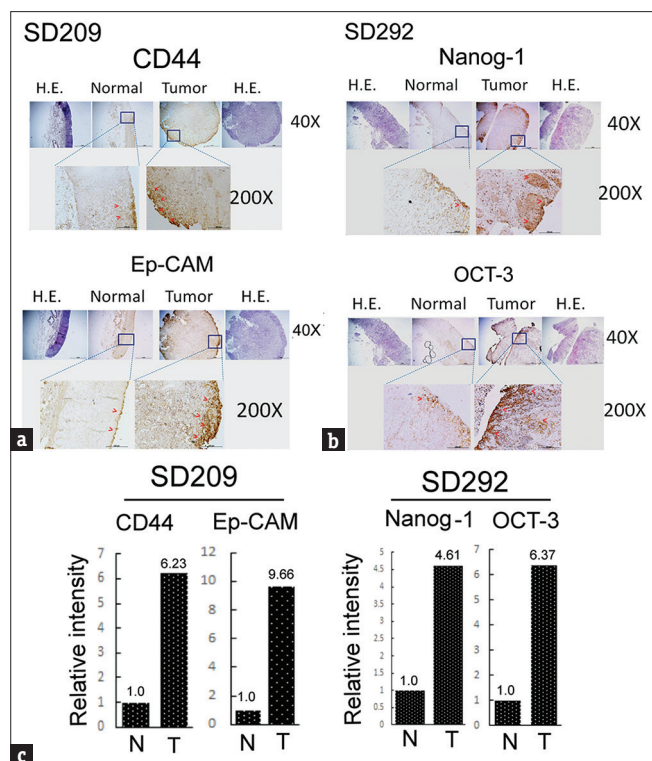


Figure 3: Differential gastric cancer stem cell (GCSC) markers overexpressed in SD209/SD292 tumor tissues. Immunohistochemistry analysis of indicated GCSC markers in tissue sections of SD209 (a) and SD292 (b) tumors coupled with their normal counterparts at both $\times 40$ and $\times 200$ H and E staining of the sections were shown at $\times 40$. The $\times 200$ demonstrated the enlarged images of an area in the $\times 40$ magnification enclosed by small blue rectangles. The markers were stained as the brown color. The red arrow heads in the $\times 200$ images indicated the dense staining regions of indicated markers. (c) demonstrated the quantitation for the staining intensity in the $\times 200$ images in (a) and (b) using Image J software. The relative intensities of each GCSC marker were calculated, taking the data of normal tissues as 1.0. The data shown were the average of two reproducible results

4.61 and 6.37-fold, respectively (shown at $\times 200$ magnification), compared with those in their normal counterparts [Figure 3c]. On the other hand, although ALDH1, Lgr5, and Mist1 are slightly expressed in normal counter tissue, they are not increased in tumors of both SD209/292 (data not shown). In addition, CD71, CD90, CD133, Lgr5 ALDH1, CXCR4, and ABCB1/MDR1 cannot be detected in both GC tissues (data not shown).

Src signaling regulated the stemness genes and proliferation of SD292

As described above, Src activity (p-Src) was greatly elevated in GC tissues of SD209 (T) and SD292 (T) [Figure 1], thus we investigated whether Src was required for the progression of SD209/SD292. Remarkably, treatment of SD209 [Figure 4a] and SD292 [Figure 4b] with one of the potent Src inhibitors dasatinib (Dasa) at 100 and 50 nM, respectively, reduced the proliferative activity of the GCSCs attached on mesenchymal fibroblasts by about 70%–80% using IF analysis of Ki67, known to be one of the proliferation markers. Note that the Ki67 signal can only be detected in cell clumps of SD209 and SD292 but not the fibroblasts on which they are attached [Figure 4a and b]. However,

Dasa did not induce cell death of SD292 cells in suspension, as evidenced by that the ratio of AO (for live cells) versus PI (for death cells) (AO/PI) staining intensity was the same between DMSO (vehicle) and Dasa-treated cell for 48 h (data not shown). On the other hand, Dasa significantly suppressed Src-activity (p-Src) and expression of Nanog-1 and OCT-3 in SD292 cells attached to mesenchymal fibroblasts by 31, 21, and 68%, respectively, using IF analysis [Figure 4c and d]. However, Dasa did not reduce the expression of Nanog-1 and OCT-3 in SD292 cell suspension by Western blot (data not shown). Collectively, these suggested that Src activity was required for the proliferation and expressions of stemness genes in SD292 attached to mesenchymal fibroblasts but not for the stemness gene expression and self-renewal of SD292 cells in suspension.

DISCUSSION

Isolation and establishment of gastric cancer stem cells as models for targeted therapy of gastric cancer

Although GCSC exists as only a small group within the GC environment, they are responsible for GC progression, recurrence, metastasis, and drug resistance due to its high proliferative/migratory activity and immune dormancy. In this regard, targeting GCSC seems to be the most promising strategy in GC prevention. However, the progress seem to be rather slow due to the lack of suitable molecular targets and cellular models for studying the cellular and molecular mechanisms relevant to GCSCs progression. In our current study, we isolated two GCSCs from SD209 and SD292 GC tissues. Both GCSCs showed differential characteristics evidenced by the variety of clumping sizes and distribution patterns of GCSC in the TME [Figure 2a-c]. Moreover, differential overexpression of CSC markers in both SD209 and SD292 (i.e. CD44/Ep-CAM in SD209 and Nanog/OCT3 in SD292) implicates diverse molecular mechanisms are responsible for regulating the biological activities in different GCSCs.

Targeting gastric cancer stem cells aiming at the critical molecular pathways

Previously, a lot of studies demonstrated the feasibility of targeted therapies for GC/GCSC prevention through inhibiting signaling pathways regulating stemness-associated genes. Some critical signaling molecules such as Wnt/ β -catenin, Notch, and Hedgehog signaling pathways have been recognized [18-21]. In our clinical tissues, we demonstrated that Src activity was upregulated in SD209/SD292 tissues [Figure 1a and b]. Moreover, inhibiting Src activity prevented the proliferation of SD209/SD292 attached to CAF [Figure 4a and b], indicating the Src-related pathway was responsible for regulating GCSC progression promoted by CAF. Interestingly, Src has been found to cross-talk with Wnt for regulating a lot of pathophysiological processes. For example, Src regulates Wnt signaling through LRP6 tyrosine phosphorylation [33]. Together, it is tempting to investigate whether Src crosses talk with Wnt or other signaling molecules in CSC for the regulation of stemness genes and progression of GCSC/GC.

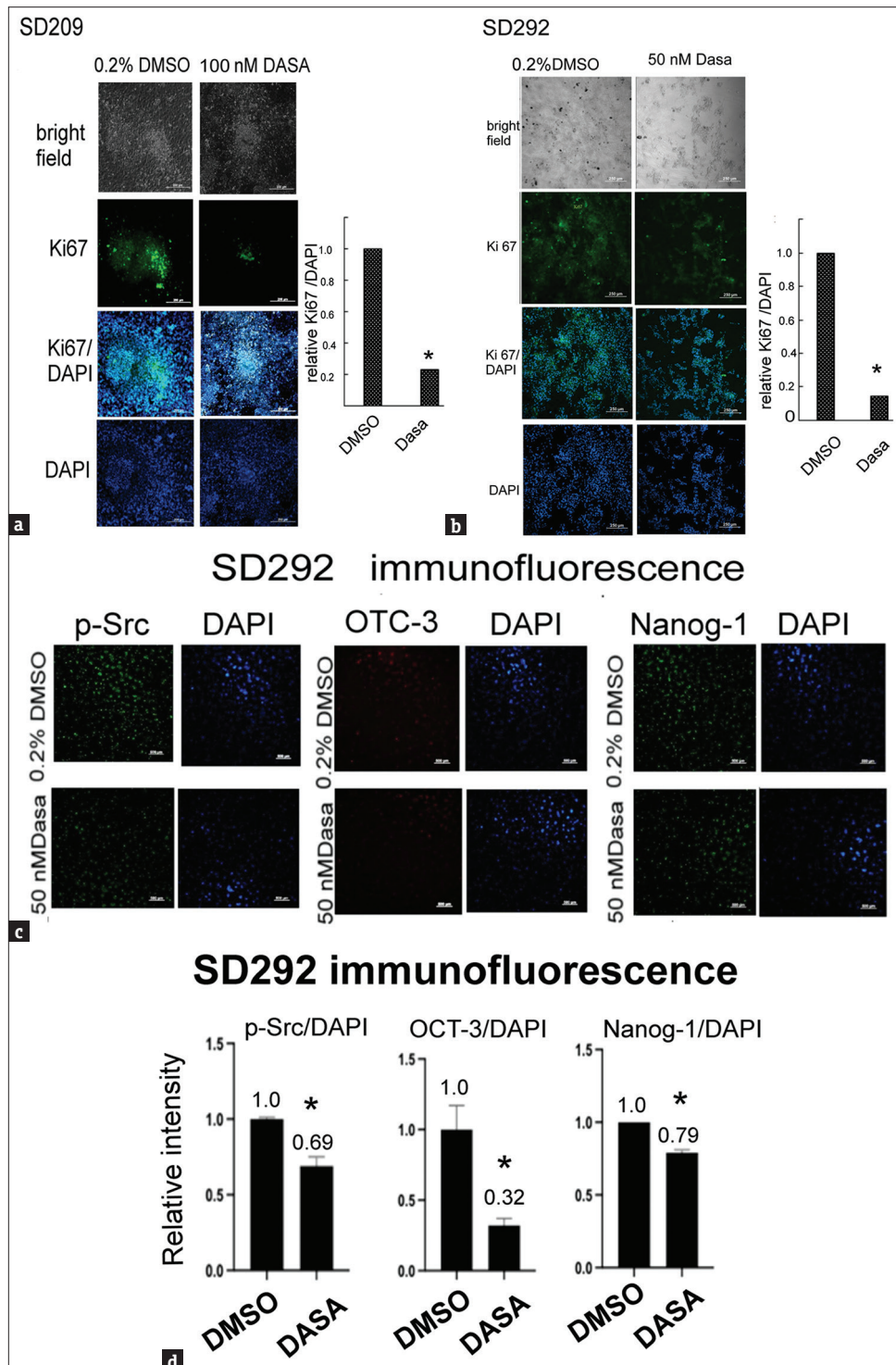


Figure 4: Dasatinib suppressed Src activation and proliferation of SD209/SD292 cells. The whole tumor environment (TME) of SD209 (a) and SD292 (b and c) were treated with Dasa at indicated concentration for 48 h. The 0.2% DMSO-treated sample was used as the vehicle control. Immunofluorescence of Ki67 (green) (a and b) and p-Src (green), Nanog (green) and octamer-binding transcription factor 3 (red) (c) were performed. DAPI was used for nuclear staining. In both (a and b), the upper panels are the bright image of the TME showing the clumping regions of GCSC attached on cancer associated fibroblast. Pictures were taken under 200- and 40-fold magnification in (a-c), respectively. The fluorescence intensity was quantified as described in Materials and Methods. (d) Is the quantitative figure for (c), showing the relative intensity of each indicated molecule versus nucleus staining intensity (DAPI), taking the data of DMSO (the vehicle) as 1.0. *represent the statistical significance ($P < 0.05$, $n = 3$) between the indicated sample and the vehicle. The mean value of each sample was shown above each bar of the graphs

CONCLUSION

Suppressing the metastatic Src signal pathway can prevent the proliferation of two patient-derived GCSCs. It is

worthy of investigating whether this model can be adopted for targeted therapy against GCSC/GC in a personalized manner.

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Data availability statement

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

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Conflicts of interest

There are no conflicts of interest.

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