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Original Article

Inhibition of SALL4 suppresses carcinogenesis of colorectal cancer via regulating Gli1 expression

Ji Cheng, Rui Deng, Chuanqing Wu, Peng Zhang, Ke Wu, Liang Shi, Xinghua Liu, Jie Bai, Meizhou Deng, Jinbo Gao, Xiaoming Shuai, Guobin Wang, Kaixiong Tao

Department of Gastrointestinal Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology 1277 Jiefang Avenue, Wuhan, Hubei Province, China

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Abstract: Background: SALL4 is a novel oncogene mediating tumorigenesis in multiple carcinomas. However, its actual role and mechanisms participating in the development of colorectal cancer remains unclear. Methods: Immunohistochemical staining and Western blot were conducted to detect the expression of SALL4 and other molecules. siRNA of SALL4 was transfected to silence SALL4 expression in Caco-2 cell line. Flow cytometry was used for cell cycle and apoptosis analysis. Wound healing and transwell assay were used for invasion test. CCK-8 test was employed for cell proliferation and drug sensitivity assessment. Results: By inhibition of SALL4 expression, the proliferation, invasiveness and drug resistance were dramatically reduced while apoptosis rate was up-regulated. Gli1 was found to decrease its expression in SALL4 silencing cells. Moreover, the inhibition on tumorigenesis of Caco-2 by SALL4 silencing was antagonized by Gli1 up-regulation, suggesting Gli1 as a downstream target of SALL4 in cancer development. Conclusion: SALL4 inhibition limited oncogenesis on colorectal cancer by reducing Gli1 expression.

Keywords: SALL4, Gli1, colorectal cancer, carcinogenesis

Introduction

Colorectal cancer is a lethal malignancy worldwide without effective measures to cure. Its incidence rate gets higher year by year due to the unhealthy lifestyle and worsening environment especially in developing countries such as China [1]. Although current multi-modality treatments including primary surgical resection and adjuvant therapies promote the overall survival rate obviously, a specific targeted therapy is still urgently needed for further improvement on life quality of cancer patients. To figure out an available targeted molecule is a current research hot spot [2, 3].

SALL4 is recognized recently as a zinc finger transcriptional factor by regulating multiple downstream targeted genes, functioning as an oncogene in diverse tumors [4]. It has been confirmed that SALL4 is aberrantly elevated and participate in oncogenesis in leukemia [5], germ cell tumors [6], liver cancer [7] and gastric cancer [8], interacting with several classical cancer signaling, such as Wnt/β-catenin pathway [9]. Silencing of SALL4 can drastically alleviate the malignancy of such tumors, revealing its potentials as a targeted molecule for cancer therapy. Although SALL4 has been verified to associate with metastasis and early diagnosis in colorectal cancer based on tissue specimens analysis [10, 11], the actual role of SALL4 on carcinogenesis on colorectal cancer cells and its internal mechanisms have not been clarified yet. Thus we intend to perform experiments unveiling the actual effect and internal mechanisms of SALL4 on proliferation, cell cycle arrest, invasiveness, apoptosis and drug resistance of colorectal cancer cells, offering theoretical basis for future clinical practice of SALL4 targeted therapy.

Materials and methods

Tissue specimens

All procedures under tissue specimens collection was approved by the Ethics Committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology. We performed all actions in accordance with the ethical standards of Declaration of Helsinki. Written informed consents have been gathered from all persons involved.The tissue specimens

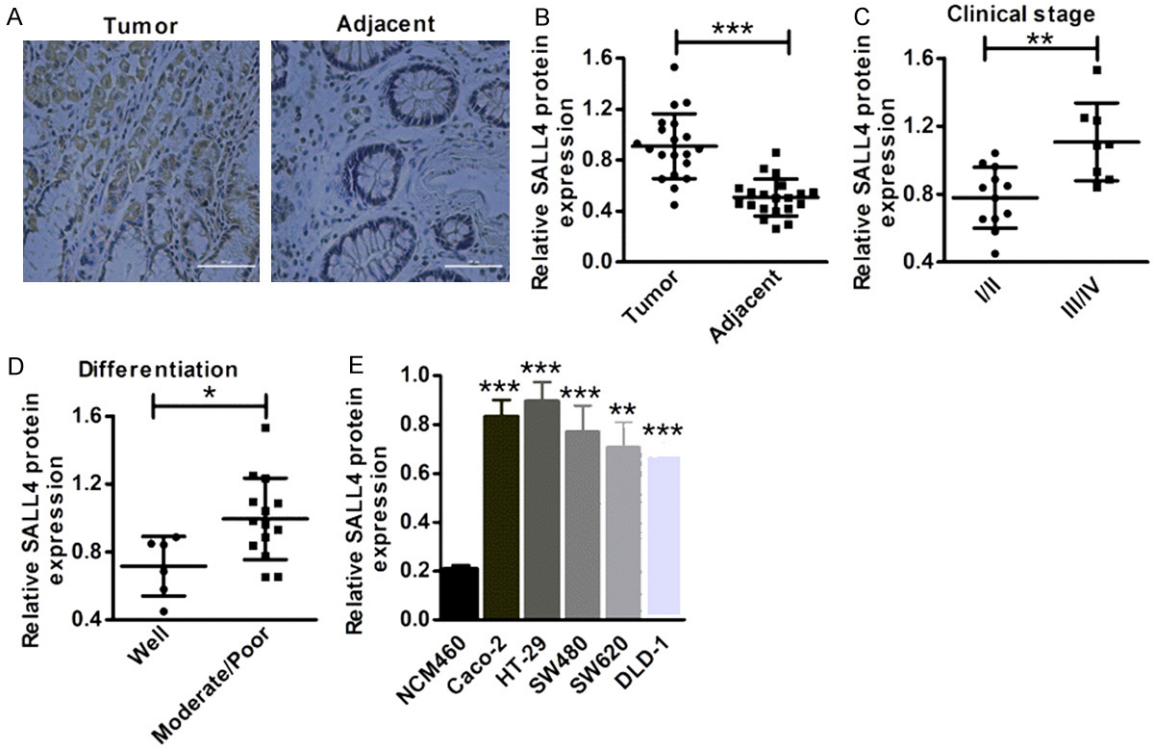

Figure 1. SALL4 expression in specimens and cell lines. A. Representative figures of immunohistochemistry staining of SALL4 on tumor and adjacent normal tissues (400 ×); B. Relative SALL4 protein expression (SALL4/GAPDH) in tumor and adjacent normal tissues among 20 patients; C. Relative SALL4 protein expression (SALL4/GAPDH) in different clinical stages among 20 patients (the stage classification was based on AJCC/UICC 2010 version); D. Relative SALL4 protein expression (SALL4/GAPDH) in different pathological differentiation among 20 patients; E. Relative SALL4 protein expression (SALL4/GAPDH) in five colorectal cancer cell lines compared to normal NCM460 cell line. Column: means; Bar: SD. Statistical labels: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

were collected from 20 patients who had surgical operations in Department of Gastrointestinal Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology in 2014. All patients received surgical procedures ahead of any adjuvant therapies. All specimens were confirmed by post-surgical pathology reports. Adjacent normal tissues were used as controls.

Immunohistochemistry

Envision two-step method was applied for immunohistochemistry staining. All procedures were conducted under recommended instruction. Cells displaying with obvious yellowish staining was regarded as positive cells.

Cell culture

NCM460, a normal human colon epithelial cell line, was donated by our laboratory instructor as control to colorectal cancer cell lines Caco-2, HT-29, SW480, SW620 and DLD-1, which were purchased from ATCC (Manassas, VA, USA). DMEM medium (Life Technologies, USA) supplemented with 10% fetal bovine serum (SJQ, Zhejiang Tianhang Biotechnology, Hangzhou, China) was used for cell culture and the cells were incubated at 37°C with 5% CO_2.

Western blot

All primary antibodies in our experiments: anti-SALL4 (ab29112, Abcam, USA); anti-GAPDH (ab37168, Abcam, USA); anti-p21 (sc-397, Santa Cruz, USA); anti-cyclin D1 (sc-753, Santa Cruz, USA); anti-MMP-9 (sc-10737, Santa Cruz, USA); anti-E-cadherin (sc-7870, Santa Cruz, USA); anti-N-cadherin (sc-7939, Santa Cruz, USA); anti-BAX (sc-493, Santa Cruz, USA); anti-cleaved caspase-3 (sc-22171-R, Santa Cruz, USA); anti-cleaved caspase-9 (#7237, Cell Signaling Technology, USA); anti-P-gp (ab129450, Abcam, USA); anti-MRP1 (sc-13960, Santa Cruz, USA).

Total proteins were isolated from specimens or cultured cells by RIPA buffer. Pierce™ BCA

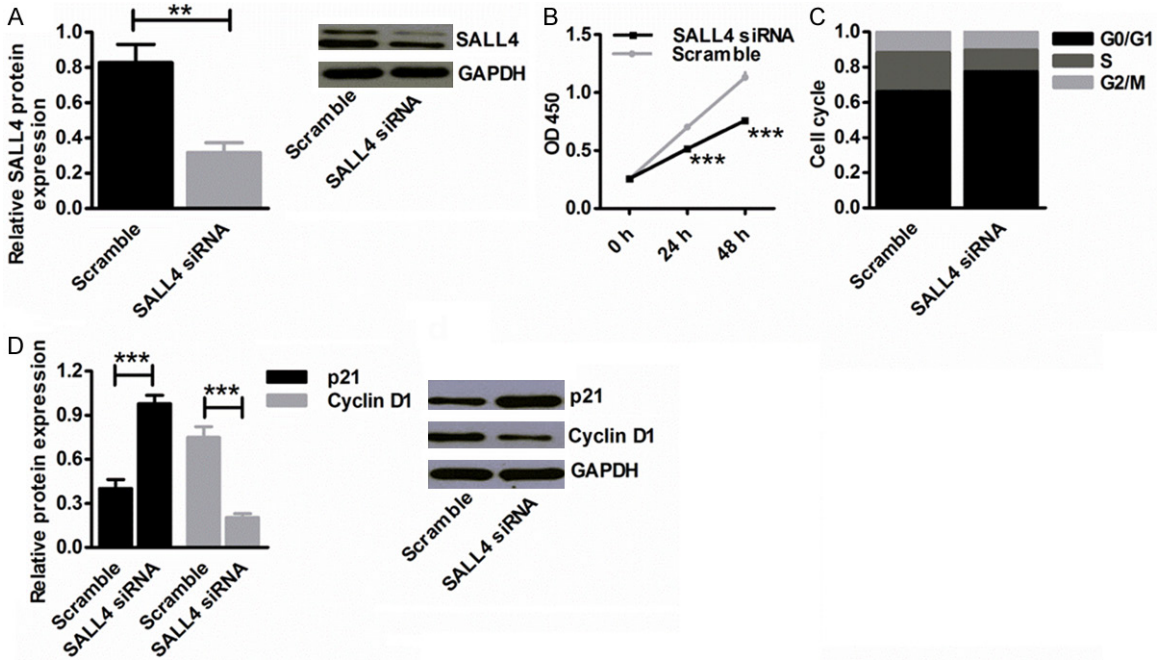

Figure 2. Inhibition of SALL4 expression reduced cell proliferation and induced cell cycle arrest in Caco-2 cell line. A. Left: Diagram of relative SALL4 protein expression (SALL4/GAPDH) in scramble group and SALL4 siRNA group. Right: Representative figure of Western blot; B. The OD450 absorbance in scramble group and SALL4 siRNA group after 24 h and 48 h incubation; C. Diagram of cell cycle arrest in scramble group and SALL4 siRNA group; D. Left: Diagram of relative protein expression (GAPDH) of p21 and Cyclin D1 in scramble group and SALL4 siRNA group. Right: Representative figure of Western blot. Column: means; Bar: SD. Statistical labels: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Protein Assay Kit (23225, Life Technologies, USA) was used to measure protein concentration by manufacturer's instructions. Proteins lysates (40 μg/lane) were separated on 10% SDS polyacrylamide gels and then transferred onto PVDF membranes. 5% fat-free milk was used for blockage. The membranes were then incubated at 4°C overnight with the above primary antibodies. Then anti-rabbit IgG-HRP secondary antibody (074-1506, KPL, USA) was co-incubated for 2 h. At last, protein bands were displayed and visualized by X-ray film using Pierce™ ECL Western Blotting Substrate (32209, Life Technologies, USA). All experiments had done in triplicate.

Cell transfection

Scramble and siRNA of SALL4 were designed and purchased in RiboBio Company (Guangzhou, China). Cells were incubated in 6-well plates at a density of 10^5. Scramble and siRNA of SALL4 were transfected into cultured cells respectively via Lipofectamine® 2000 Transfection Reagent (11668-019, Life Technologies, USA) following manufacturer's procedures while the cell confluence reaches 70%. Transfection efficiency was evaluated by qRT-PCR and Western blot assays. Subsequent cell assays were conducted 48 h post-transfection.

Cell proliferation assay

We used CCK-8 test for proliferation analysis. Cells were incubated in 96-well plates in a concentration of 10^4 per well, with 5 parallel wells each group. CCK-8 kit (Dojindo, Japan) was adopted for detecting the proliferation of cells after 24 h and 48 h incubation. 10 μl of CCK-8 reagent was added into each well in different time points before 2 h incubation. Then the absorbance was measured at 450 nm. Three independent experiments were performed.

Cell migration and invasion assays

Wound healing assay: A 200 μl pipette tip scratched on the well surface to make artificial wounds. We supplied pure DMEM medium without fetal bovine serum to incubate cells for 24 h before assessments.

Transwell assay: A 24-well Matrigel Invasion Chamber with pore size 8 μm (Corning, USA) was applied for invasion assay. 10^5 cells were

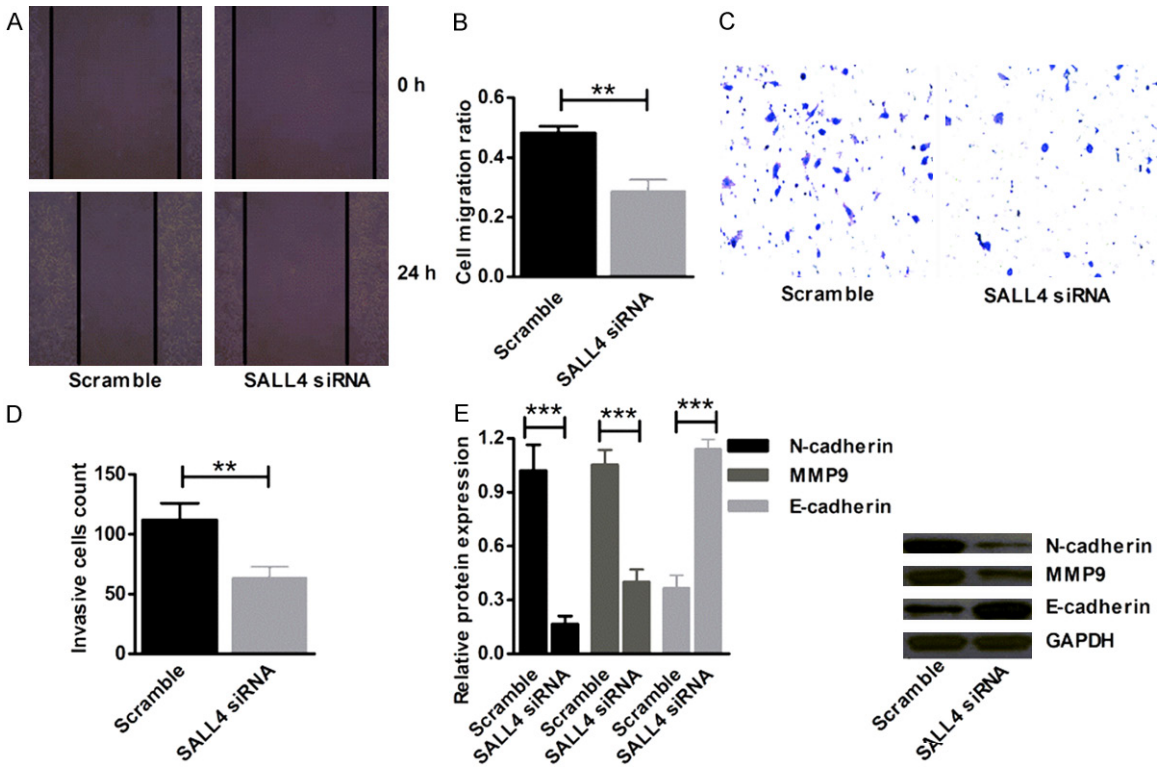

Figure 3. Inhibition of SALL4 expression reduced cell migration and invasion in Caco-2 cell line. A. Diagrams of wound healing assay in scramble group and SALL4 siRNA group; B. Cell migration ratio analysis in scramble group and SALL4 siRNA group; C. Diagrams of transwell assay in scramble group and SALL4 siRNA group; D. Invasive cells count based on transwell assay in scramble group and SALL4 siRNA group; E. Left: Diagram of relative protein expression (GAPDH) of N-cadherin, MMP9 and E-cadherin in scramble group and SALL4 siRNA group. Right: Representative figure of Western blot. Column: means; Bar: SD. Statistical labels: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

seeded into the upper compartments of the chamber with serum-free medium, and complete medium with 10% fetal bovine serum filled in the lower compartments. After 48 h incubation, non-invading cells on the upper surface were eliminated by cotton swabs. 95% ethanol and 0.1% crystal violet solution were used to fixate and stain the invasive cells. Numbers of invasive cells were counted from four randomly selected fields in high magnification view (200 ×). All experiments were conducted in triplicate.

Cell cycle and apoptosis analysis

Cell cycle: 70% ethanol was used to fix cells overnight at-20°C. Then cells were treated with DNA staining solution containing 3.4 mM Tris-Cl (PH = 7.4), propodium iodide, 0.1% triton X-100 buffer and 100 mg/ml RNase A. Cell cycle was then analyzed in flow cytometry (BD, USA).

Apoptosis: Annexin V-FITC/PI dual staining kit (KGA108, KeyGEN BioTECH, Nanjing, China) was adopted for apoptosis staining. Approximate 3×10^5 cells were collected each well for analyzing after 24 h incubation in flow cytometry (BD, USA) following standard procedures. All experiments had done in triplicate.

Drug resistance analysis

Fluorouracil (Yabao Pharmaceuticals, China) and Oxaliplatin (Sanofi-Aventis, Hangzhou, China) were used to testify the drug sensitivity of cells in different groups. CCK-8 test was applied for cell viability assessment after 24 h drug exposure.

Special chemicals: Human Sonic hedgehog ligand recombinant protein, SHH (14-8679-80, eBioscience, USA). The concentration we used in the experiments was 5 μg/ml.

Statistics analysis

Student t test was applied for analyzing significant difference between quantitative data. $P < 0.05$ was considered significant statistically.

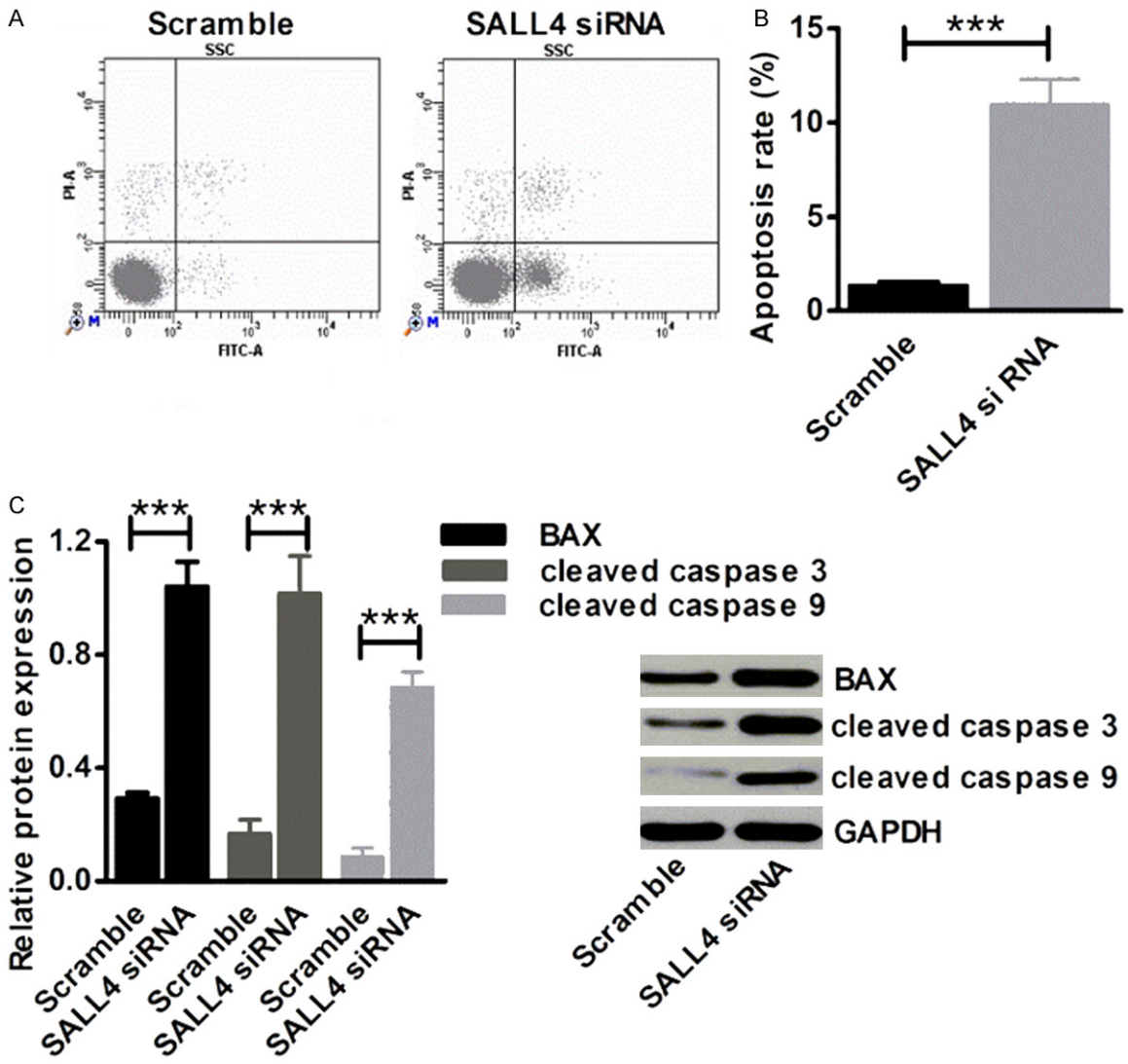

Figure 4. Inhibition of SALL4 expression induced apoptosis in Caco-2 cell line. A. Diagrams of flow cytometry apoptosis analysis in scramble group and SALL4 siRNA group; B. Apoptosis rate in scramble group and SALL4 siRNA group; C. Left: Diagram of relative protein expression (GAPDH) of BAX, cleaved caspase 3 and cleaved caspase 9 in scramble group and SALL4 siRNA group. Right: Representative figure of western blot. Column: means; Bar: SD. Statistical labels: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Results SALL4 expression was aberrantly elevated in tumor specimens and cancer cell lines compared to normal controls

By using immunohistochemistry and western blot assay, we analyzed the expression of SALL4 in 20 tumor specimens compared to adjacent normal ones. We figured out that SALL4 was up-regulated in cancer tissues dramatically ($P < 0.001$, **Figure 1A** and **1B**). Moreover, advanced clinical stage and poorer differentiation cases displayed higher SALL4 expression compared to less malignant cases ($P < 0.05$, **Figure 1C** and **1D**). Additionally, SALL4 expression was remarkably increased in cancer cell lines in contrast to normal colon epithelial cell line NCM460 ($P < 0.01$, **Figure 1E**), which accorded with the results in specimen analysis. Thus, the aberrantly elevated expression of SALL4 in colorectal cancer hinted that it might function as an important participant in cancer origination and development.

Inhibition of SALL4 expression reduced cell proliferation and induced cell cycle arrest in Caco-2 cell line

siRNA of SALL4 was used to silence SALL4 expression in Caco-2 cell line. We examined the transfection efficiency by Western blot assay,

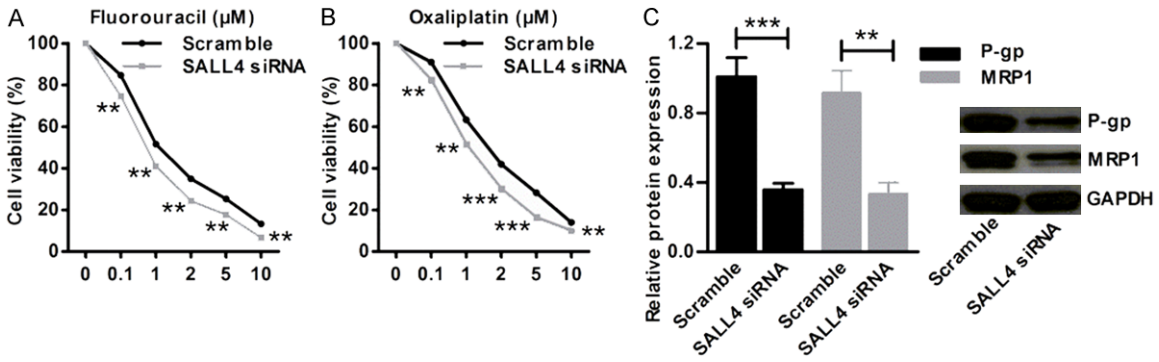

Figure 5. Inhibition of SALL4 expression reduced drug resistance in Caco-2 cell line. A. Cell viability rate after Fluorouracil exposure with different doses in scramble group and SALL4 siRNA group; B. Cell viability rate after oxaliplatin exposure with different doses in scramble group and SALL4 siRNA group; C. Left: Diagram of relative protein expression (GAPDH) of P-gp and MRP1 in scramble group and SALL4 siRNA group. Right: Representative figure of Western blot. Column: means; Bar: SD. Statistical labels: **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

and SALL4 expression was indeed reduced dramatically post-transfection (*P* < 0.01, **Figure 2A**), suggesting the effective silencing effect by siRNA of SALL4. Then we conducted CCK-8 test to check the function of SALL4 on cell proliferation. Compared to scramble group, silencing of SALL4 obviously limited cell proliferation in both 24 h and 48 h checkpoints (*P* < 0.001, **Figure 2B**), revealing a great anti-growth efficacy. Furthermore, in cell cycle analysis, we observed G0/G1 arrest after silencing of SALL4 (**Figure 2C**), which might partially explain the anti-proliferative effect. Additionally, we detected cell cycle related proteins P21 and Cyclin D1 to get further validation. P21 consisted G1 checkpoint and acted as a potent tumor suppressor, while Cyclin D1 stimulated cells to get into S phase acting as a proliferative molecule. Our experiment results showed an impressive increase on P21 expression and decrease on Cyclin D1 expression after SALL4 silencing (*P* < 0.001, **Figure 2D**), which was in accord with the cell cycle analysis. Therefore, inhibition of SALL4 could dramatically reduce cell proliferation and induce cell cycle arrest in colorectal cancer cell line.

Inhibition of SALL4 expression reduced cell migration and invasion in Caco-2 cell line

Wound healing assay was a mature approach to test cell migration ability. Our experiment results suggested that silencing of SALL4 dramatically reduced the migration ability of cells compared to scramble group cells (*P* < 0.01, **Figure 3A** and **3B**). Additionally, by conducting transwell invasion assay, we also verified that the silencing of SALL4 significantly decrease the invasive cells compared to scramble group, revealing a great anti-metastasis potential of silencing SALL4 (*P* < 0.01, **Figure 3C** and **3D**). Then we detected the expression of some classic proteins of metastasis. As expected, invasion indicators N-cadherin and MMP9 were limited due to SALL4 silencing, while E-cadherin was up-regulated instead (*P* < 0.001, **Figure 3E**). Thus our results demonstrated that SALL4 inhibition could effectively restrict the migration and invasion of colorectal cancer cells.

Inhibition of SALL4 expression induced apoptosis in Caco-2 cell line

Induction of apoptosis was an available measure to combat tumor growth. We analyzed apoptosis rate in both groups by flow cytometry and found that the apoptosis rate was elevated after SALL4 silencing (*P* < 0.001, **Figure 4A** and **4B**). Furthermore, we checked the expression of three apoptosis-related proteins: BAX, cleaved caspase 3 and cleaved caspase 9. Their expressions were up-regulated since the silencing of SALL4 (*P* < 0.001, **Figure 4C**), which meant the classical caspase3/9 pathway might be the mechanism of SALL4 induced apoptosis.

Inhibition of SALL4 expression reduced drug resistance in Caco-2 cell line

Drug resistance was a global clinical issue which decreased the survival and cure rate dramatically. Fluorouracil and oxaliplatin were two representative chemotherapy drugs used in colorectal treatment, however, certain patients showed severe resistance against these drugs

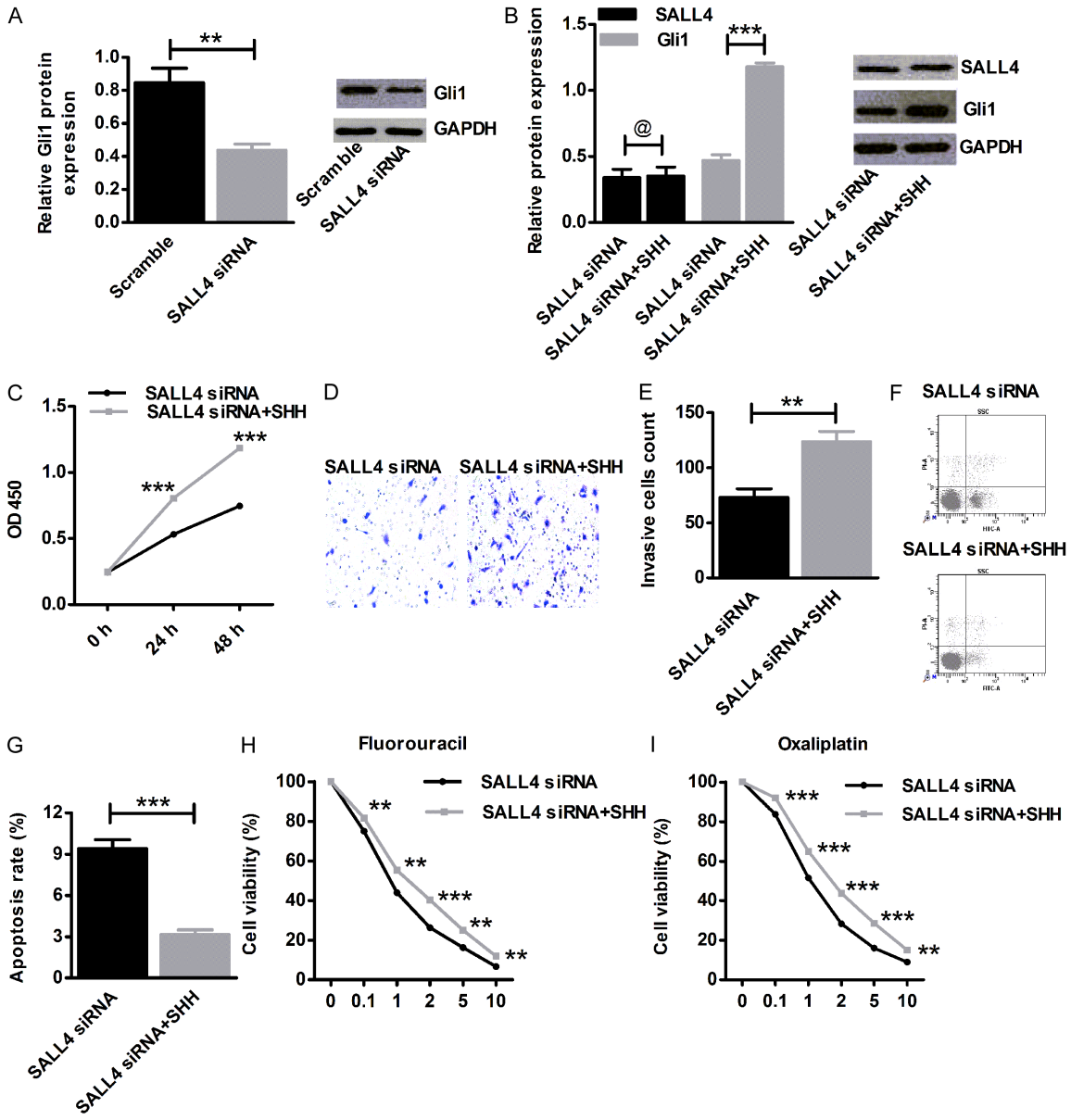

Figure 6. Gli1 was down-regulated by SALL4 inhibition and up-regulation of Gli1 could antagonize the inhibition effect of SALL4 silencing. A. Left: Relative Gli1 expression (Gli1/GAPDH) in scramble group and SALL4 siRNA group. Right: Representative figure of western blot; B. Left: Relative protein expression (GAPDH) of SALL4 and Gli1 in SALL4 siRNA group and SALL4 siRNA plus SHH group. Right: Representative figure of western blot; C. The OD450 absorbance in SALL4 siRNA group and SALL4 siRNA plus SHH group after 24 h and 48 h incubation; D. Diagrams of transwell assay in SALL4 siRNA group and SALL4 siRNA plus SHH group (200 ×); E. Invasive cells count based on transwell assay in SALL4 siRNA group and SALL4 siRNA plus SHH group; F. Diagrams of flow cytometry apoptosis analysis in SALL4 siRNA group and SALL4 siRNA plus SHH group; G. Apoptosis rate in SALL4 siRNA group and SALL4 siRNA plus SHH group; H. Cell viability rate after fluorouracil exposure with different doses in SALL4 siRNA group and SALL4 siRNA plus SHH group; I. Cell viability rate after Oxaliplatin exposure with different doses in SALL4 siRNA group and SALL4 siRNA plus SHH group. Column: means; Bar: SD. Statistical labels: @$P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

with mechanisms undefined. Our experiment results suggested that SALL4 might be a resistance targeted molecule because SALL4 silencing significantly reduced drug resistance by fluorouracil and oxaliplatin in different doses ($P < 0.05$, **Figure 5A** and **5B**). Moreover, SALL4 silencing had been connected to P-gp and MRP1, which were well-known resistance-relat-

ed proteins, revealing SALL4 might influence drug sensitivity through regulating P-gp and MRP1 expression ($P < 0.01$, **Figure 5C**).

Gli1 was down-regulated by SALL4 inhibition and up-regulation of Gli1 could antagonize the inhibition effect of SALL4 silencing

Besides the anti-carcinogenesis effects we found by SALL4 inhibition, we intended to figure out the internal mechanism which mediated SALL4's actions. Gli1 was a downstream transcriptional factor of Sonic hedgehog signaling pathway, which was a notable developmental pathway mediating cell growth and tumor origination. By analyzing the protein expression, we found that Gli1 expression was severely reduced along with SALL4 silencing ($P < 0.01$, **Figure 6A**); suggesting SALL4 might partially influence cancer development via Gli1 regulation. Then we used a Sonic hedgehog recombinant protein SHH to stimulate this pathway. It appeared that Gli1 expression was indeed up-regulated after SHH exposure while SALL4 expression did not changed significantly with SHH intervention, eliminating the SALL4 interference effect (**Figure 6B**). Additionally, we checked the influence of SHH exposure to carcinogenesis. Compared to simple SALL4 silencing, SALL4 silencing plus SHH exposure displayed more proliferation effect ($P < 0.001$, **Figure 6C**), more invasion ability ($P < 0.01$, **Figure 6D** and **6E**), less apoptosis rate ($P < 0.001$, **Figure 6F** and **6G**) and more drug resistance ($P < 0.01$, **Figure 6H** and **6I**) in Caoco-2 cell line. Thus, we concluded that SALL4 inhibition reduced colorectal carcinogenesis via regulating Gli1 expression.

Discussion

Current chemotherapy drugs appeared with more resistance and less efficacy dealing with cancer patients including colorectal cancer. Therefore, targeted therapy is a hotspot for clinical cancer research [12, 13]. Interfering certain molecule expression by targeted therapy can efficiently alleviate the malignant behaviors and shrink the tumor volumes in various cancers, such as Trastuzumab for breast cancer [14] and Cetuximab for lung cancer [15]. However, a practical and effective targeted drug for colorectal cancer is still in deficiency. The clinical efficacy and safety of current targeted drugs used in colorectal cancer treatment are controversial. Although Cetuximab and Bevacizumab are two targeted drugs indicative for colorectal cancer, persuasive evidence based on large-scale RCTs suggested disappointing results that non-profit survival rate appeared after taking these medications [16-18]. Therefore, a targeted site and product for colorectal cancer is imminent.SALL4 is a newly discovered oncogene found in diverse tissues and organs. It is first identified in Drosophila and is rapidly confirmed as an inevitable regulatory gene for embryonic development and stem cell differentiation [19]. Expression deficiency of SALL4 results in multi-organ dysplasia and developmental disorders [20]. Due to the strongly proliferative function on cells and tissues, SALL4 is analyzed to correlate with carcinogenesis by researchers. It is verified that SALL4 is aberrantly elevated in multiple carcinomas, such as leukemia [5], germ cell tumors [6], liver cancer [7] and gastric cancer [8], acting as an oncogene and biomarker. SALL4 has wide regulatory effects by influencing proliferation, invasion, apoptosis, resistance of tumors [21], revealing its great potential as a targeted molecule for cancer treatment [4]. And our experimental results proved for the first time that SALL4 inhibition could reduce colorectal cancer malignancy via inhibiting proliferation, invasion and drug resistance. This conclusion is consistent with SALL4 function in other carcinomas, suggesting SALL4 acting as an oncogene in colorectal cancer as well. As an oncogene and zinc finger transcriptional factor as well, SALL4 achieves its regulatory goals through regulating and interacting with downstream targets and signal networks. Bmi-1 [22, 23] and PTEN [24] have been confirmed as direct targets of SALL4. SALL4 is found to stimulate Wnt/β-catenin signaling by directly binding to β-catenin [9]. In embryos, SALL4 can activate Oct-4 and Sox2 [25] to regulate embryonic development. Sonic hedgehog is a classical developmental pathway controlling tumor growth and cancer development [26]. However, whether SALL4 interacts with this pathway is undefined yet. Surprisingly, our experiment found out that in colorectal cancer cells, SALL4 could successfully regulate Gli1 expression, which was an important downstream transcriptional factor and messenger of Sonic hedgehog signaling. This result clearly confirms that SALL4 could interact with Sonic hedgehog signaling when playing its oncogene role in colorectal cancer and Gli1 is a new downstream target of SALL4. Different oncogenes are impor-

tant candidates for targeted therapy since its close interplay with oncogenesis among diverse tumors. In spite of the wide distribution and strong regulation role of SALL4 in various cancers including colorectal cancer, we need further researches to clarify its functional mechanisms more accurately. This is our goal and future research topic.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Kaixiong Tao, Department of Gastrointestinal Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology 1277 Jiefang Avenue, Wuhan, Hubei Province, China. E-mail: kaixiongtaowhuh@126.com

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