STAT3 Is Necessary for Proliferation and Survival in Colon Cancer–Initiating Cells

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Abstract

STAT3 is constitutively activated in colon cancer but its contributions in cancer-initiating cells have not been explored. In this study, we characterized STAT3 in aldehyde dehydrogenase (ALDH)-positive (ALDH+) and CD133-positive (CD133+) subpopulations of human colon tumor cells that exhibited more potent tumor-initiating ability than ALDH+/CD133− cells in tumor xenograft assays in mice. We found that ALDH+/CD133+ cells expressed higher levels of the active phosphorylated form of STAT3 than either ALDH+/CD133− or unfractionated colon cancer cells. STAT3 inhibition by RNA interference–mediated knockdown or small-molecule inhibitors LLL12 or Static blocked downstream target gene expression, cell viability, and tumor-sphere-forming capacity in cancer-initiating cells. Similarly, treatment of mouse tumor xenografts with STAT3 short hairpin RNA (shRNA), interleukin 6 shRNA, or LLL12 inhibited tumor growth. Our results establish that STAT3 is constitutively activated in colon cancer–initiating cells and that these cells are sensitive to STAT3 inhibition. These findings establish a powerful rationale to develop STAT3 inhibitory strategies for treating advanced colorectal cancers. Cancer Res; 71(23); 7226–37. ©2011 AACR.

Introduction

Colorectal cancer is a tumor caused by abnormal division of the cells lining the large intestine. According to the American Cancer Society, there were an estimated 102,900 new cases and 51,370 deaths due to colorectal cancer in the United States in 2010. As such, there is a need for better treatment approaches for colorectal cancer. The cellular mechanisms contributing to colorectal cancer are still not well understood but involve signaling protein dysregulation which includes the constitutive activation of STAT3 (1–3). The constitutive activation of STAT3 is frequently detected in primary human colorectal carcinoma cells and established human colorectal cancer cell lines (1–3), and elevated levels of STAT3 phosphorylation were correlated with the tumor invasion, nodal metastasis, and the stage (P < 0.05; refs. 1, 3). Constitutive STAT3 activation in colorectal cancer cells is associated with invasion, survival, and growth of colorectal cancer cells and colorectal tumor model in mice in vivo (2, 4–6). These reports indicate that STAT3 is one of the major oncogenic pathways activated in colorectal cancer and can serve as an attractive therapeutic target for colorectal carcinoma. To date, however, whether STAT3 is activated in colorectal cancer stem cells is unknown.

The concept of the cancer stem cells or cancer-initiating cells holds that only a minority of cells within a tumor have the ability to generate a new tumor. Cancer stem cells were reported to show pluripotency and self-renewal (7). Cancer stem cells were first identified in leukemias and more recently in solid tumors. Increasing evidence suggests that the cancer stem cells concept is also relevant to colorectal cancer (8). CD133, a transmembrane protein (Prominin-1 or AC133) was used to isolate stem cells from a host of other normal and cancerous tissues, including colorectal cancer. However, the specificity of CD133 alone as a marker for colonic stem cells is uncertain (9–11).

A promising new marker for cancer stem cells is aldehyde dehydrogenase 1 (ALDH1). ALDH is a detoxifying enzyme that oxidizes intracellular aldehydes and thereby confers resistance to alkylating agents (12). Corti and colleagues (13) showed that ALDH+ cells isolated from murine brain were capable of self-renewal and of differentiating into multiple lineages. Further studies showed that ALDH1 is a specific marker for breast cancer stem cells (14, 15). ALDH was also investigated as a specific marker for identifying and isolating normal and malignant human colonic stem cells and as a way to quantify the number of stem cells over the course of colon cancer
development (16). Xenograft tumors were successfully generated using ALDH+ cells from 7 primary colon cancer cells and ALDH− cells did not generate tumor xenografts (16). When using ALDH and CD133 together to form tumor xenografts, ALDH+/CD133+ cells showed an increased ability to generate tumor xenografts compared with ALDH+/CD133− or ALDH− alone (16). Taken together, these data suggest that ALDH is a better marker than CD133 for colorectal cancer stem cells. However, using both ALDH and CD133 seem to be better than to enrich the cancer stem cell population using ALDH or CD133 alone.

This study extends that work by using both ALDH and CD133 together as markers for cancer-initiating cells or colorectal stem cells and examines the STAT3 phosphorylation and interleukin 6 (IL)-6 expression in these cancer-initiating cells. Our results showed that colorectal cancer-initiating cells, characterized by ALDH+/CD133− subpopulation of colorectal cancer cells expressing higher levels of STAT3 phosphorylation and IL-6, compared with unseparated and ALDH+/CD133− subpopulations. These results suggest that STAT3 is a novel therapeutic target in colorectal cancer-initiating cells.

Materials and Methods

Colorectal cancer cell lines

Human colorectal cancer cell lines (SW480, HCT116, DLD-1, and HT29) were purchased from the American Type Culture Collection (ATCC) and maintained in Dulbecco’s Modified Eagle Medium supplemented with 10% FBS (Invitrogen). These cancer cell lines have been routinely tested and authenticated by the ATCC and Asterand, respectively. The known genotype relative to adenomatous polyposis coli, beta-catenin, and DNA mismatch repair enzymes (MLH1, MSH2) genes were shown in Supplementary Table S1. ALDH−/CD133− initiating cells maintained serum-free MEBM (MEBM; Clonetics division of Cambrex BioScience), supplemented with B27 (Invitrogen), 20 ng/mL EGF (BD Biosciences), 4 μg/mL Gentamycin (Invitrogen), 1 ng/mL hydrocortisone (Sigma-Aldrich), 5 μg/mL Insulin, and 100 μmol/L beta-mercaptoethanol (Sigma-Aldrich).

STAT3 inhibitors, LLI12, and Stattic

The laboratory of Dr. Pui-Kai Li’s at the Ohio State University College of Pharmacy synthesized small-molecule LLI12 that selectively targets STAT3 (17). Stattic, a previously reported inhibitor, served as ALDH-negative controls. Anti-human PE-CD133 antibody were purchased from Miltenyi Biotec. ALDH+/CD133+ and ALDH−/CD133− subpopulations were separated from SW480, HCT116, DLD-1, and HT29 colon cancer cells by a fluorescence-activated cell sorting Wantage SE (Becton Dickinson) Flow Cytometry. After sorting, ALDH+/CD133− cells were cultured in serum-free stem cell medium (MEBM) to maintain cancer stem cell characteristics. ALDH+/CD133− and unseparated cells were cultured in regular medium and replaced with stem cell medium (MEBM) for 3 days before harvesting.

Tissue microarray slides, immunohistochemistry, and immunofluorescence staining

Human colon cancer tissue microarray slides were obtained from the Biochain Institute, Inc. and AccuMax ISU ABXIS Co. containing 109 colon cancer cases. After baking and deparaffinization, the slides were boiled in a pressure cooker filled with 10 mmol/L sodium citrate (pH 6.0) and then subjected to immunohistochemistry or immunofluorescence staining. Phospho-STAT3 (Tyr705) antibody (1:25; Signaling Technology) and or ALDH1 (1:100; BD Pharmingen), CD133 (1:50; Miltenyi Biotec) were used. Alexa Fluor 488–conjugated anti-rabbit IgG and Alexa Fluor 594–conjugated anti-mouse IgG (Cell Signaling Technology) and the Histostain-Plus Kits (Invitrogen) were used in immunofluorescence or immunohistochemistry staining as described by the manufacturer. Immunostained slides were scored under microscope by using the criteria of percentage and intensity positive as described previously by Ginestier (14). Significance of correlation between phospho-STAT3 and ALDH1 or CD133 was determined, respectively, using 2-sided Pearson χ2 test. P < 0.05 was considered as statistical significance.

Cell viability assay

Colon cancer–initiating cells maintained serum-free MEBM (3,000 per well in 96-well plates) were incubated with desired concentrations of compounds in triplicate at 37°C for 72 hours. MTT viability assay was done according to manufacturer's protocol (Roche Diagnostics). The absorbance was read at 595 nm.

Western blot analysis

Cells were lysed in cold radioimmunoprecipitation assay lysis buffer containing protease inhibitors and subjected to SDS-PAGE. Proteins were transferred on to polyvinylidene difluoride membrane and were probed with a 1:1,000 dilution of antibodies (Cell Signaling Technology) against phospho-specific STAT3 (Tyrosome 705; P-STAT3), phospho-independent STAT3, phospho-specific ERK1/2 (Threonine 202/Tyro-sine 204), cleaved PARP, cleaved caspase-3, cyclin D, survivin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The degree of changes in P-STAT3 was determined using densitometry and normalized to GAPDH.

Reverse transcriptase PCR

Total cell RNA was collected from cells by using RNeasy Kits (Qiagen). cDNA was generated from 500 ng sample RNA using Omniscript RT (Qiagen). Two microliter of cDNA was subsequently used for PCR using Taq PCR Master Mix Kit (Qiagen) according to the manufacturer’s instruction. Primer sequences were designed using Primer3.
and source information can be found in Supplementary Table S2.

**Tumorsphere culture**

The ALDH+/CD133+ cells were plated as single cells in ultra-low attachment 6-well plates (Corning) and plated at a density of 25,000 viable cells per well. Cells were grown in a serum-free MEPM as described above in a humidified incubator (5% CO2) at 37°C. At the second day after seeding, the ALDH+/CD133+ cells were treated with 2.5 to 5 μmol/L of LLL12 or 5 to 10 μmol/L of Static. Tumorspheres were observed under microscope 15 days later.

**IL-6 ELISA assay**

After sorting, ALDH+/CD133+ and ALDH−/CD133− cells were cultured in 96-well plates at a density of 12,000 viable cells per well. Twenty-four hours later, the medium was collected and the IL-6 concentrations were detected by using the Human IL-6 ELISA Development Kit (Peprotech) as described by the manufacturer.

**Lentiviral infections**

Lentivirus short hairpin RNA (shRNA) that specifically targets human STAT3 (19) and control lentivirus that expresses green fluorescent protein (GFP) were provided by Dr. Antonio Iavarone at the Columbia University. IL-6 shRNA lentivirus was purchased from Santa Cruz Biotechnology, Inc. STAT3, IL-6, or control GFP shRNA lentivirus (CTL shRNA) was introduced into SW480 and HCT116 colon cancer-initiating cells for 48 hours, followed by selection with puromycin (0.2 μg/mL) for 72 hours. Western blot assay was used to detect the expression of P-STAT3 and STAT3 in colon cancer-initiating cells. MTT cell viability and reverse transcriptase PCR (RT-PCR) assay were conducted and in vivo cancer-initiating cell growth was determined.

**Mouse xenograft tumor model**

All animal studies were conducted in accordance with the principles and standard procedures approved by IACUC at the Research Institute at Nationwide Children’s Hospital. For tumor-initiation study, the ALDH+/CD133+ or ALDH−/CD133− cells (1 × 10⁶, 1 × 10⁵, or 1 × 10⁴) from SW480, HCT116, DLD-1, and HT29 were mixed with 50% Matrigel (Invitrogen) in a total of 100 μL and were injected subcutaneously into the right flank area of 4- to 5-week-old female nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice, which were purchased from Jackson Laboratory. The tumor incidence ALDH+/CD133+ and ALDH−/CD133− cells are the numbers of tumor detected/numbers of mice inoculated and were determined 50 days after the inoculation of cells in mice.

For shRNA lentivirus study, after sorting ALDH+/CD133+ HCT116, colon cancer-initiating cells (1 × 10⁶) were infected with STAT3, IL-6, or GFP shRNA lentivirus (CTL shRNA) for 48 hours. After 72 hours of selection with puromycin, cells were mixed with an equal volume of Matrigel and injected subcutaneously into the flanks area of 4- to 5-week-old female NOD/SCID mice. Tumor growth was determined by measured the length (L) and width (W) of the tumor every week with a caliper and tumor volume was calculated on the basis of the following formula: volume = (π/6) LW².

To detect the effects of STAT3 inhibitor LLL12 in vivo, ALDH+/CD133+ SW480 and HCT116 colon cancer-initiating cells (1 × 10⁵) were mixed with 50% Matrigel (Invitrogen) in a total of 100 μL and were injected subcutaneously into the flank area of female NOD/SCID mice. After 17 and 19 days, SW480 and HCT116 mice were divided into 2 treatment groups consisting of 5 and 6 mice per group, respectively: (a) dimethyl sulfoxide (DMSO) control vehicle and (b) 5 mg/kg of LLL12 (dissolved in 10% DMSO, 18% Cremophor EL and 72% sterile 5% dextrose). Tumor growth and body weights of mice were measured every other day during 14 days period treatments. At the end of treatments, tumors were harvested from euthanized mice. A portion of tumor tissues was snap-frozen in liquid nitrogen and stored in −80°C to examine the expression of STAT3 phosphorylation by Western blot. The rest of tumors tissues were dissociated mechanically and enzymatically to obtain a single-cell suspension. The single-cell suspension was used for ALDEFLUOR and CD133-PE staining and followed flow cytometry assay as previously described (20).

**Results**

**LLL12 is a potent agent to inhibit the STAT3 phosphorylation in colorectal cancer cells**

STAT3 is frequently activated in many types of human solid and blood cancers and contributes to progression of those cancers (21, 22). The STAT3 pathway is also frequently constitutively activated in colorectal cancer and is considered to play an important role in colorectal cancer carcinogenesis (1–6). To confirm the important role of STAT3 in colon cancer cells, the novel STAT3 inhibitor LLL12 (17) was used to target STAT3 in 3 independent colon cancer cell lines using phosphospecific STAT3 antibody. Our results showed that LLL12 significantly inhibited STAT3 phosphorylation at tyrosine residue 705 (Y705, P-STAT3) in SW480, HCT116, and DLD-1 human colon cancer cell lines (Supplementary Fig. S1). Phosphorylation at Y705 is important to activate STAT3 (23–25). The inhibition of P-STAT3 by LLL12 is consistent with the decrease of STAT3 downstream target genes and the induction of apoptosis, as evidenced by the cleavages of caspase-3 (Supplementary Fig. S1).

**ALDH+/CD133+ cells exhibit more potent tumor-initiating ability than ALDH−/CD133− cells in mouse tumor xenografts**

It has been shown that ALDH+ and CD133+ subpopulations in colorectal cancer cells exhibit colorectal cancer-initiating cells properties in vitro and in vivo (16). To verify whether ALDH+/CD133+ cells contain tumor-initiating ability, we separated ALDH+/CD133+ and ALDH−/CD133− subpopulations from SW480, HCT116, DLD-1, and HT29 colorectal cancer cells (Supplementary Fig. S2A). The percentages of ALDH+/CD133+ in the 4 colon cancer cell lines were shown in Supplementary Fig. S2B. Our results showed that although 5 to 8 of 7 to 8 mice of 10² to 10⁵ ALDH+/CD133+ cells injected formed tumors,
only 1 to 2 of 7 to 8 mice of \(10^3\) ALDH\(^+/\)CD133\(^-\) cells form tumor, and none of the \(10^2\) ALDH\(^+/\)CD133\(^-\) cells injected formed tumor (Table 1). The volume of tumor formed by ALDH\(^+/\)CD133\(^+\) are also larger than tumors formed by ALDH\(^-/\)CD133\(^+\) cells (Supplementary Fig. S3). Taking together, these results suggest that using both ALDH\(^+/\) and CD133\(^+\) markers can enrich colorectal cancer-initiating cells. These results increase our confidence to isolate colorectal cancer-initiating cells using both markers to examine the STAT3 phosphorylation.

### The ALDH\(^+/\)CD133\(^+\) subpopulation of colorectal cancer cells express higher levels of STAT3 phosphorylation compared with the ALDH\(^-/\)CD133\(^+\) subpopulation

The STAT3 phosphorylation of ALDH\(^+/\)/CD133\(^+\) and ALDH\(^-/\)/CD133\(^+\) subpopulations in SW480, HCT116, DLD-1, and HT29 colorectal cancer cell lines were examined. Interestingly, our results showed that the ALDH\(^+/\)/CD133\(^+\) subpopulation of SW480, HCT116, DLD-1 (Fig. 1A), and HT29 (Supplementary Fig. S4A) colorectal cancer cells expressed higher levels of P-STAT3 compared with unseparated cells and the ALDH\(^-/\)/CD133\(^+\) subpopulation. ERK1/2 phosphorylation (Threonine 202/Tyrosine 204) was not at consistently high levels in the ALDH\(^+/\)/CD133\(^+\) cells in all 4 colon cancer cell lines (Fig. 1A; Supplementary Fig. S4A). These results suggested that STAT3 pathway seems to serve a more important purpose in colorectal cancer-initiating cells, but ERK may not play a key role in colorectal cancer-initiating cells, at least in these 4 colorectal cancer cell lines. These results show that colorectal cancer-initiating cells in the ALDH\(^+/\)/CD133\(^+\) subpopulation expresses phosphorylated or activated STAT3.

To further investigate the STAT3 activation in clinical colon cancer samples, P-STAT3 and ALDH1, CD133 protein expression in human colon cancer tissues were also examined using tissue microarray slides. We observed that there was a significant association \((P < 0.01)\) between staining of P-STAT3 and staining of ALDH1 (Table 2). In addition, the tumor samples expressed elevated levels of phosphorylated STAT3 also associated with CD133 \((P < 0.01;\) Table 2). In the 109 cases, there were 32 samples (29.36%) in which P-STAT3 (Y705), ALDH1, and CD133 were all positive, and 38 samples (34.86%) with negative P-STAT3 (Y705), ALDH1, and CD133. Taken together, there were 64.22% samples which had the similar expression, much more than the samples with different expression of these 3 proteins \((P < 0.05;\) Table 2). So there was a significant association between P-STAT3, ALDH1 and CD133. The representative examples of immunohistochemistry/immunofluorescence staining of P-STAT3 and ALDH1/CD133 are shown in Fig. 1A and C. These results from human colon cancer tissue further showed that the elevated levels of P-STAT3 is expressed in colon cancer-initiating cells. These results indicated that constitutive STAT3 signaling may be a novel therapeutic target in colorectal cancer-initiating cells.

### STAT3 inhibitors, LLL12 and Static, inhibited STAT3 phosphorylation, and STAT3 downstream targets in ALDH\(^+/\)/CD133\(^+\) cells

To confirm the important role of STAT3 in colon cancer-initiating cells, we next examined the effect of STAT3 inhibitors, LLL12 and Static in SW480, HCT116, DLD-1, and HT29 colorectal cancer–initiating cells. The results showed that LLL12 and Static inhibited P-STAT3 (Y705) but not ERK1/2 phosphorylation (Fig. 2A and B and Supplementary Fig. S4B) in ALDH\(^+/\)/CD133\(^+\) subpopulation of colorectal cancer cell lines. In Fig. 2B, Static seems to decrease STAT3 expression, which could explain, in part, the observed decrease in level of P-STAT3. In addition, STAT3 shRNA also inhibited P-STAT3 in ALDH\(^+/\)/CD133\(^+\) subpopulation of colorectal cancer cell lines compared with shRNA control (shRNA CTL; Fig. 2C). The inhibition of STAT3 by LLL12 also downregulates the expression of many known or putative STAT3-regulated genes in colorectal cancer–initiating cells such as cyclin D1 (26), survivin (27), Bcl-2, Bcl-XL (26), Notch1, and Notch3 (28, 29; Fig. 2D-a, Supplementary Fig. S4C). These genes are related to cancer cell proliferation, survival, and angiogenesis. Moreover, the Notch pathway was reported to be involved in self-renewal of human cancer stem cells and tumorigenicity (28, 30). These results indicate that the LLL12 is also potent in terms of inhibiting P-STAT3, downregulating STAT3 downstream genes, and induces apoptosis in these colorectal cancer–initiating cells. Furthermore, the expression of STAT3 downstream genes, such as cyclin D1, survivin, Bcl-2, Bcl-XL, Notch1, and Notch3 were also reduced by STAT3 shRNA (Fig. 2D-b).

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<th>(1 \times 10^3)</th>
<th>(1 \times 10^2)</th>
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<td>5/7</td>
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<td>5/8</td>
<td>1/7</td>
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Table 1. The tumor-initiating ability (tumor incidence: the numbers of tumor detected/numbers of mice inoculated) of ALDH\(^+/\)/CD133\(^+\) and ALDH\(^-/\)/CD133\(^-\) cells in NOD/SCID mice for 50 days
LLL12 and Stattic inhibit cell viability and tumorsphere-forming capacity of ALDH+/CD133+ subpopulation of colorectal cancer cells

We next examined the inhibitory effects of LLL12, Stattic, and STAT3 shRNA on cell viability in colorectal cancer–initiating cells. Our results showed that LLL12, Stattic, and STAT3 shRNA could inhibit cell viability of the ALDH+/CD133+ cells from SW480, HCT116, DLD-1 (Fig. 3A–C), and HT29 (Supplementary Fig. S4D) colorectal cancer cells, further supporting the idea that colorectal cancer–initiating cells are sensitive to the inhibition of STAT3. LLL12 and Stattic also inhibited the cell viability of ALDH-/CD133- subpopulations (Supplementary Fig. S4E) and ALDH-/CD133- subpopulations were separated from SW480, HCT116, and DLD-1 colon cancer cells by flow cytometry. Phosphorylation of STAT3 (Y705), ERK 1/2 (T202/Y204), phospho-independent STAT3, and GAPDH were detected by Western blot. B, representative examples of the expression of P-STAT3, ALDH1, and CD133 were shown by immunohistochemistry (IHC) using colon cancer tissue microarray slides. Negative/weak staining of P-STAT3 (Y705)/ALDH1/CD133 (a) and positive staining of P-STAT3 (Y705)/ALDH1/CD133 (b and c) tumor tissues were shown. The spots for P-STAT3 (Y705), ALDH1 and CD133 were from the matched tissues section from the same patient. The negative controls are stained with no antibody. C, colon cancer tissue microarray slides were double-stained with P-STAT3 (Y705) and ALDH1 using immunofluorescence (IF). ALDH1 high expression tumor cells (cytoplasm, green) also expressed phosphorylated-STAT3 in nuclei (red). Scale bar: 10 μm.
inhibition of unphosphorylated STAT3 or nonnuclear function of STAT3 in ALDH+/CD133− cells might contribute to the reduction of cell viability.

Furthermore, we examined the efficacy of LLL12 and Stattic in inhibiting colorectal cancer–initiating cells to survive and proliferate in anchorage-independent conditions and ability to form tumorspheres. Our results showed that LLL12 and Stattic can inhibit tumorsphere-forming capacity in the ALDH+/CD133− cells of SW480, HCT116, DLD-1 (Fig. 3D), and HT29 (Supplementary Fig. S4E) colorectal cancer cells. The effects of LLL12 are more potent than Stattic. These results indicate that LLL12 is an excellent drug candidate for targeting colorectal cancer cells. The effects of 5-Fu increased the percentage of ALDH+/CD133− cells (Fig. 3D), however, 10 μmol/L of Stattic also decreased the phosphorylated STAT3. These results provide evidence that elevates phosphorylated STAT3. These results provide evidence that elevates phosphorylated STAT3 in ALDH+/CD133− colorectal cancer–initiating cells for inhibiting phosphorylated or activated STAT3 in human colorectal cell lines.

**STAT3 inhibitors, LLL12 and Stattic, depleted ALDH+/CD133− subpopulation and the expression of ALDH1, CD133 in colon cancer cells**

Colon cancer stem cells are resistant to current chemotherapy and radiation regimens available (8). To examine whether STAT3 inhibition might eliminate the ALDH+/CD133− subpopulation, we treated cancer cells with 5 μmol/L of LLL12, 10 μmol/L of Stattic, 10 μmol/L of doxorubicin, and 10 μmol/L of 5-Fu for 24 hours, and sorted for the percentage of ALDH+/CD133− subpopulation by flow cytometry. Our results showed that LLL12 could decrease the ALDH+/CD133− subpopulation in HCT116 and SW480 colon cancer cells (Fig. 4A; Supplementary Fig. S6A), suggesting that this subpopulation of colon cancer–initiating cells is sensitive to STAT3-mediated inhibition. We found that 10 μmol/L of Stattic also decreased the percentage of ALDH+/CD133− subpopulation (Fig. 4A; Supplementary Fig. S6A). However, 10 μmol/L of doxorubicin or 5-Fu increased the percentage of ALDH+/CD133− colorectal cancer–initiating cells (P < 0.05; Fig. 4A), which might indicate that colon cancer–initiating cells are resistant to chemotherapy. We also detected the effects of Dox or 5-Fu (10 μmol/L) in STAT3 activation in the bulk tumor cell population (Supplementary Fig. S6B). The results showed different responses to both drugs on P-STAT3 in 2 independent colon cancer cell lines (SW480 and HCT116). To further investigate the role of IL-6/STAT3 pathway in ALDH+/CD133− colorectal cancer–initiating cells, ALDH+/CD133− and ALDH−/CD133+ colon cancer cells were collected after sorting. ELISA assay showed that ALDH+/CD133+ cells secreted higher levels of IL-6 than ALDH+/CD133− cells (Fig. 4B). Interestingly, the expression of IL-6, IL-6R, GP130, and IL-8 were higher in ALDH+/CD133− cells than ALDH+/CD133+ cells (Fig. 4C-a) as detected by RT-PCR assay. We also examined the ALDH1 and CD133 expression after the LLL12 treatment. We found that the expression of ALDH1 and CD133 was lower after treatment with LLL12 (Fig. 4C-b). In addition, LLL12 inhibited the expression of IL-6, GP130, and IL-8 (Fig. 4C-b). However, the expression of IL-6R was not changed consistently in all 4 cell lines. Our data also observed that IL-6 (25–50 ng/mL) induced the expression of IL-8 in SW480 and HCT116 colon cancer cells, which could be blocked by LLL12 (Supplementary Fig. S6C). The results confirm that the IL-6/STAT3 pathway plays a central role in the maintenance of the ALDH+/CD133− subpopulation in colon cancer cells.

**IL-6 shRNA decreased STAT3 phosphorylation of colorectal cancer–initiating cells in vitro and inhibited cancer-initiating cell growth in vivo**

To determine whether the increased levels of phosphorylated or activated STAT3 in ALDH+/CD133− cells is dependent on upstream ligand, IL-6, we treated ALDH+/CD133− cells with lentiviral IL-6 shRNA versus control lentivirus without encoding IL-6 shRNA. Our results in Fig. 4D-a shows that lentiviral IL-6 shRNA, but not control lentivirus, downregulated the phosphorylated STAT3. These results provide evidence that elevated levels of phosphorylated STAT3 in ALDH+/CD133− cells is IL-6–dependent and the inhibition of IL-6 downregulates phosphorylated STAT3.

To further show the tumor dependence on STAT3 and its upstream activation (IL-6), we used shRNA that specifically knock down STAT3 and its upstream signaling protein, IL-6. Our results in Fig. 4D-b shows that STAT3 and IL-6 shRNA significantly suppressed colon cancer stem cell tumor growth compared with lentivirus GFP (as a control). These data supported tumor dependence on STAT3 and its upstream activation by IL-6.

**LLL12 suppresses tumor growth of colorectal cancer–initiating cells in mouse tumor model in vivo**

We have shown that LLL12 inhibited P-STAT3, cell viability, and tumorsphere growth in colorectal cancer–initiating cells.
expressing elevated levels of STAT3 phosphorylation in vitro. We further tested LLL12 against colorectal cancer-initiating cells isolated from the SW480 and HCT116 colon cancer cells in NOD/SCID mice xenograft model in vivo. The results showed that LLL12 significantly suppresses (P < 0.01) the tumor growth (Fig. 5A-a) and tumor mass (Fig. 5A-b). The average reduction in tumor weight is 49.67% to 61.89% in LLL12-treated mice compared with DMSO vehicle in xenograft mouse model (Fig. 5B). LLL12 also inhibited STAT3 but not ERK1/2 phosphorylation of SW480 and HCT116 colon cancer-initiating cells (Fig. 5C). We also used flow cytometry to determine the percentage of ALDH+/CD133+ subpopulation in the tumors treated with vehicle or LLL12. Our results in Fig. 5D-a, b showed that LLL12 reduced the percentage of ALDH+/CD133+

Figure 2. STAT3 inhibitors LLL12, Stattic, and STAT3 shRNA inhibited STAT3 phosphorylation and STAT3 downstream genes expression in ALDH+/CD133+ colon cancer-initiating cells. The ALDH+/CD133+ subpopulation was separated from SW480, HCT116, and DLD-1 colon cancer cells by flow cytometry, and cultured in serum-free stem cell medium (MEBM) to maintain cancer stem cell characteristics. A and B, ALDH+/CD133+ colon cancer-initiating cells were treated with DMSO, LLL12 (5 µmol/L) or Stattic (10-20 µmol/L) for 24 to 48 hours. C, STAT3 or control GFP shRNA lentivirus (CTL shRNA) was introduced into colon cancer-initiating cells for 48 hours, followed by selection with puromycin for 72 hours. Phosphorylation of STAT3 and ERK1/2, STAT3 protein, and GAPDH in colon cancer-initiating cells were detected by Western blot as described in Materials and Methods. D, ALDH+/CD133+ subpopulation of SW480, HCT116, and DLD-1 colon cancer cells were treated with DMSO, LLL12 (5 µmol/L), CTL, or STAT3 shRNA. Reverse-transcriptase PCR reveals decreased expression of STAT3 downstream target genes in LLL12 or STAT3 shRNA treated cells as compared with control.
subpopulation in tumor. In addition, the body weights of mice have no obvious reduction in both DMSO vehicle- and LLL12-treated mice (Supplementary Fig. S7). These results showed that LLL12 is potent in suppressing tumor growth from the colorectal cancer–initiating cells in vivo.

Discussion

STAT3 is frequently activated in many types of human solid and blood cancers, including colon cancer (1–3, 22). Blocking signaling to STAT3 inhibits cancer cell growth, showing that STAT3 is crucial to the survival and growth of tumor cells (21, 22, 32) and is an attractive therapeutic target for cancer. At the present time, the main effort to target constitutive STAT3 signaling is only focused on the bulk of cancer cells. No published literatures have been reported about whether STAT3 is activated in colon cancer–initiating cells, and no approach has been initiated to explore the STAT3 as a possible therapeutic target in colon cancer–initiating cells. In this article, we took a pilot study to explore
the STAT3 in colon cancer–initiating cells characterized by ALDH+/CD133+ subpopulation. Our study confirmed that ALDH+/CD133+ colon cancer cells exhibited more potent tumor-initiating ability than ALDH-/CD133- cells in mouse tumor xenografts. We showed that elevated P-STAT3 is detected in colon cancer–initiating cells cell lines and in human colon cancer tissues derived from cancer patients. These results suggest that activated STAT3 is.
indeed a novel therapeutic target in colon cancer–initiating cells.

To explore the role that STAT3 plays in colon cancer–initiating cells, we examined the effects of STAT3 inhibition. Two small molecular STAT3 inhibitors, LLL12 (17) and Stattic (18) were employed. LLL12 is a novel and more potent derivative of LLL3, a previously reported STAT3 inhibitor from our laboratories (33, 34). Our results showed that LLL12 was potent inhibitor to inhibit P-STAT3; STAT3 downstream targets expression and induces apoptosis in nonseparated colon cancer cells. Importantly, STAT3 inhibitors, LLL12 and Stattic inhibited P-STAT3, STAT3 downstream gene expression, cell viability, and the formation of tumorspheres in ALDH+/CD133+ subpopulation of colon cancer–initiating cells. In addition, STAT3 shRNA was used to inhibit the STAT3 expression and activity. Our results showed that STAT3 shRNA also inhibited STAT3 phosphorylation, cell viability, and STAT3 downstream genes.
expression in ALDH+/CD133+ subpopulation of colon cancer-initiating cells.

We compared the expression of IL-6/STAT3 signal pathway, such as IL-6, IL-6R, GP130, and IL-8 between ALDH+/CD133+ and ALDH-/CD133- subpopulations. IL-6 has been shown to activate STAT3 (35) and may play a role in colon cancer oncogenesis (36–38). Interestingly, our results showed that ALDH+/CD133+ cells expressed higher levels of IL-6, GP130, and IL-8 and secreted higher levels of IL-6 than those in ALDH-/CD133+ cells. In addition, introduction of the IL-6 shRNA in ALDH+/CD133+ cells downregulated the expression of STAT3 phosphorylation. These results provide evidence that STAT3 activation in ALDH+/CD133+ cells is IL-6 dependent. The expression of IL-6 and IL-8 could be reduced by STAT3 inhibitor, LLL12. It was speculated that STAT3 may regulate the expression of IL-6 (28). Our data showed that IL-6 is downregulated by STAT3 inhibitor, LLL12, supporting that IL-6 may be regulated by STAT3. Furthermore, it has been reported that activated STAT3 could selectively bind to IL-8 promoter and induce IL-8 transcription (39). Our data showed that IL-6 induced the expression of IL-8, which could be blocked by LLL12. These results suggest that IL-8 may be a downstream target of IL-6/STAT3 in colon cancer cells. Recent studies have suggested a role for interleukins, such as IL-6 and IL-8, in breast cancer stem cells (15), which imply that inflammatory microenvironment is important in promoting the oncogenesis. Ginestier and colleagues reported that blockade of the IL-8 receptor CXCR1 selectively depletes human breast cancer stem cells (40). Our data suggested that IL-6/STAT3/IL-8 activation in colon cancer-initiating cells might play an important role in the development of colon cancer.

We found that the expression of ALDH1 and CD133 was reduced after treatment with LLL12, and this may be due to the inhibition of their expression. It may also be an effect of LLL12 on cellular heterogeneity, whereby it decreases the proportion of ALDH+/CD133+ cells in the tumor cell population which was shown by our in vitro and in vivo data. In addition, our data showed that STAT3 inhibitors, but not other cancer therapeutic drugs such as doxorubicin and 5-Fu, eliminated ALDH+/CD133- subpopulation of colon cancer-initiating cells in colon cancer cell lines. These results suggested that colon cancer-initiating cells, which are more resistant to conventional drugs, might be sensitive to STAT3 inhibitors.

Furthermore, our results showed that STAT3, IL-6 shRNA and LLL12 exhibited suppressive activity on the tumor growth of human colon cancer-initiating cells derived from bulk colon cancer cells. These results suggest that constitutive active IL-6/STAT3 in these cancer-initiating cells enhanced proliferation and survival, as well as tumor growth in mice, whereas STAT3 blockade by STAT3, IL-6 shRNA and LLL12, suppressed tumor-initiating cell growth in vivo.

In summary, this study is the first report to show that IL-6/STAT3 is activated in colon cancer-initiating cells. Targeting IL-6/STAT3 may be able to eliminate the colon cancer-initiating cells and provides a promising approach to treat advanced colon cancer. Our study also showed that LLL12 is a potent STAT3 inhibitor for cancer-initiating cells and is a promising drug candidate to target constitutive STAT3 signaling in colon cancer-initiating cells. Most recently, 2 literatures reported that IL-6/STAT3 pathway may be activated in glioblastoma stem cells (41, 42). In addition, targeting STAT3 by 2 small molecules, STA-21 and S31-201 or IL-6 shRNAs, respectively, can inhibit cell viability of these glioblastoma stem cells (41, 42). Furthermore, we also observed that high levels of STAT3 phosphorylation are detected in breast cancer-initiating cells compared with unseparated and nonbreast cancer-initiating cells (data not shown). These results are consistent with our observation in colon cancer-initiating cells that activated STAT3 seems to play an important role in cancer-initiating cells. It is also of interest to investigate whether STAT3 is also activated in cancer-initiating cells in other types of human cancer. If STAT3 is constitutively activated in other types of cancer-initiating cells or cancer stem cells, it may open a new therapeutic opportunity to target STAT3 in cancer-initiating cells of those cancer types.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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