STAT3 is necessary for proliferation and survival in colon cancer-initiating cells

Running Title: STAT3 in colorectal 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 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 RNAi-mediated knockdown or small molecule inhibitors LLL12 or Stattic blocked downstream target gene expression, cell viability and tumorsphere-forming capacity in cancer-initiating cells. Similarly, treatment of mouse tumor xenografts with STAT3 shRNA, IL-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.

Precis:

By demonstrating the importance of STAT3 function in cancer-initiating stem-like cells in colon cancer, these findings establish a powerful rationale to develop IL-6 and STAT3 inhibitory strategies to treat advanced colorectal cancers.
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 Signal Transducer and Activator of Transcription 3 (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) (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 seven 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 appear to be better than to enrich the cancer stem cell population using ALDH or CD133 alone.

The current 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 IL-6 expression in these cancer-initiating cells. Our results demonstrated that colorectal cancer-initiating cells, characterized by ALDH\(^+\)/CD133\(^+\) subpopulation of colorectal cancer cells expression higher levels of STAT3 phosphorylation and IL-6 comparing to un-separated and with ALDH\(^+\)/CD133\(^-\) subpopulations suggesting 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 (Manassas, VA) and maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS (Invitrogen, Carlsbad, CA). These cancer cell lines have been routinely tested and authenticated by the American Type Culture Collection and Asterand respectively. The known genotype relative to adenomatous polyposis coli (APC), beta-catenin (CTNNB1) and DNA mismatch repair enzymes (MLH1, MSH2) genes were shown in Supplemental Table 1. ALDH+/CD133+ cancer-initiating cells were grown in a serum-free mammary epithelial basal medium (MEBM) (Clonetics division of Cambrex BioScience, Walkerville, MD) 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 μM beta-mercaptoethanol (Sigma-Aldrich).

STAT3 inhibitors, LLL12, and Stattic

The laboratory of Dr. Pui-Kai Li’s at the Ohio State University College of Pharmacy synthesized small molecule LLL12 that selectively targets STAT3 (17). Stattic, a previously reported STAT3 inhibitor (18), was purchased from Calbiochem (San Diego, CA).

Isolation of colon cancer-initiating cells

The population with high ALDH enzymatic activity was used to isolated by using the ALDEFLUOR kit (StemCell Technologies, Durham, NC, USA) as previously described (14). Briefly, cells were trypsinized to single cells and subsequently suspended in ALDEFLUOR assay buffer containing ALDH substrate (BAAA) and then incubated for 40 minutes at 37°C. In all
experiments, the ALDEFLUOR-stained cells treated with diethylaminobenzaldehyde (DEAB), a specific ALDH inhibitor, served as ALDH-negative controls. Anti-human PE-CD133 antibody were purchased from Miltenyi Biotec (Auburn, CA). ALDH+/CD133+ and ALDH-/CD133- subpopulations were separated from SW480, HCT116, DLD-1, and HT29 colon cancer cells by a FACS Wantage SE (Becton Dickinion, Palo Alto, CA, USA) Flow Cytometry. After sorting, ALDH+/CD133+ cells were cultured in serum-free stem cell medium (MEBM) to maintain cancer stem cell characteristics. ALDH-/CD133- cells and un-separated cells were cultured in regular medium and replaced with stem cell medium (MEBM) for three days before harvesting.

**Tissue microarray slides, Immunohistochemistry, and Immufluorecence 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 baked and deparaffinized, the slides were boiled in a pressure cooker filled with 10mM Sodium Citrate (PH6.0), and then subjected to immunohistochemistry or immufluorecence staining. Phospho-STAT3 (Tyr705) antibody (1:25; Signaling Technology, Beverly, MA) and or ALDH1 (1:100; BD Pharmingen, San Diego, CA), CD133 (1:50, Milteny Biotec, Auburn, CA, USA) were used. Alexa Fluor® 488 conjugated anti-rabbit IgG and Alexa Fluor® 594 conjugated anti-mouse IgG (Cell Signaling Technology, Beverly, MA) and the Histostain-Plus Kits (Invitrogen, Carlsbad, CA) were used in immufluorecence or immunohistochemistry staining as described by manufacturer. Immuno-stained 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 two-sided Pearson Chi-square ($\chi^2$) test. $P <0.05$ was considered as statistical significance.
Cell Viability Assay

Colon cancer-initiating cells maintained serum-free mammary epithelial basal medium (3,000/well in 96-well plates) were incubated with desired concentrations of compounds in triplicate at 37°C for 72 hours. 3-(4,5-Dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT) viability assay was done according to manufacturer’s protocol (Roche Diagnostics, Mannheim, Germany). The absorbance was read at 595 nm.

Western Blot analysis

Cells were lysed in cold RIPA lysis buffer containing protease inhibitors and subjected to SDS polyacrylamide gel electrophoresis. Proteins were transferred on to PVDF membrane and were probed with a 1:1000 dilution of antibodies (Cell Signaling Technology, Beverly, MA) against phospho-specific STAT3 (Tyrosine 705) (P-STAT3), phospho-independent STAT3, phospho-specific ERK1/2 (Threonine 202/Tyrosine 204), cleaved Poly (ADP-ribose) polymerase (PARP), cleaved caspase-3, cyclin D, survivin, and GAPDH. The degree of changes in P-STAT3 was determined using densitometry and normalized to GAPDH.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total cell RNA was collected from cells by using RNeasy Kits (Qiagen, Valencia, CA). cDNA was generated from 500ng sample RNA using Omniscript RT (Qiagen). Two µl of cDNA was subsequently used for PCR using Taq PCR Master Mix kit (Qiagen) according to the manufacturer’s instruction. Primer sequences and source information can be found in Supplemental Table 2.
Tumorsphere culture

The ALDH+/CD133+ cells was plated as single cells in ultra-low attachment six-well plates (Corning, Lowell, MA, USA) and plated at a density of 25,000 viable cells /well. Cells were grown in a serum-free MEBM 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 2.5-5 μM of LLL12 or 5-10 μM of Stattic. 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, Rocky Hill. NJ) as described by the manufacturer.

Lentiviral infections

Lentivirus short hairpin RNA (ShRNA) that specific 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. (Santa Cruz, CA). 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 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 x 10^2, 1 x 10^3, or 1 x 10^4) from SW480, HCT116, DLD-1, and HT29 were mixed with 50% matrigel (Invitrogen, Carlsbad, CA) in a total of 100 μl and were injected subcutaneously into the right flank area of 4- to 5-week-old female non-obese diabetic/severe combined immunodeficiency (NOD/SCID) which were purchased from Jackson Laboratory (Bar Harbor, ME). 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× 10^5) 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) LW^2.

To detected the effects of STAT3 inhibitor LLL12 in vivo, ALDH+/CD133+ SW480 and HCT116 colon cancer-initiating cells (1 x 10^5) were mixed with 50% matrigel (Invitrogen, Carlsbad, CA) in a total of 100 μl and were injected subcutaneously into the flank area of female NOD/SCID. After 17 and 19 days, SW480 and HCT116 mice were divided into two treatment groups consisting of 5 and 6 mice/group respectively: (a) DMSO control vehicle and (b) 5mg/kg of LLL12 (dissolved in 10% DMSO, 18% Cremophor EL and 72% sterile 5% Dextrose). Tumor growth and bodyweights 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 three independent colon cancer cell lines using phospho-specific STAT3 antibody. Our results demonstrated that LLL12 significantly inhibited STAT3 phosphorylation at Tyrosine residue 705 (Y705, P-STAT3) in SW480, HCT116, and DLD-1 human colon cancer cell lines (Supplemental Figure 1). Phosphorylation at Y705 is important to activate STAT3 (23-25). The inhibition of P-STAT3 by LLL12 is consistent with the decrease of STAT3 down-stream target genes and the induction of apoptosis, as evidenced by the cleavages of caspase-3 (Supplemental Figure 1).

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 (Supplemental Figure 2A). The percentages of ALDH+/CD133+ in the four colon cancer cell lines were shown in Supplemental Figure 2B. Our results showed that while 5-8 out of 7-8 mice of $10^2$ to $10^4$ ALDH+/CD133+ cells injected formed tumors, only 1-2 out of 7-8 mice of $10^3$ ALDH-/CD133- cells form tumor and none of the $10^2$ ALDH-/CD133- cells injected formed tumor (Table 1).
Tumors formed by ALDH+/CD133+ are also larger than tumors formed by ALDH-/CD133- cells (Supplemental Figure 3). 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 to 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 (Figure 1A), and HT29 (Supplemental Figure 4A) colorectal cancer cells expressed higher levels of P-STAT3 compared to un-separated 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 four colon cancer cell lines (Figure 1A, Supplemental Figure 4A). These results suggested the STAT3 pathway appears 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 four colorectal cancer cell lines. These results demonstrates 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 express elevated levels of phosphorylated STAT3 also associated with CD133 (P<0.01) (Table 2). In the 109 cases,
there are 32 samples (29.36%) in which P-STAT3 (Y705), ALDH1 and CD133 are all positive, and 38 samples (34.86%) with negative P-STAT3 (Y705), ALDH1 and CD133. Taken together, there are 64.22% samples have the similar expression, much more than the samples with different expression of these three 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 were shown in Figure 1B and 1C. These results from human colon cancer tissue further demonstrate that the elevated levels of P-STAT3 is expressed in colon cancer initiating cells. These results indicate that constitutive STAT3 signaling may be a novel therapeutic target in colorectal cancer-initiating cells.

STAT3 inhibitors, LLL12 and Stattic 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 Stattic in SW480, HCT116, DLD-1, and HT29 colorectal cancer-initiating cells. The results showed that LLL12 and Stattic inhibited P-STAT3 (Y705) but not ERK1/2 phosphorylation (Figure 2A, 2B and Supplemental Figure 4B) in ALDH+/CD133+ subpopulation of colorectal cancer cell lines. In Figure 2B, Stattic appears 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 to ShRNA control (ShRNA CTL) (Figure 2C). The inhibition of STAT3 by LLL12 also down-regulates 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), Notch-1 and Noth-3 (28, 29) (Figure 2D-a, Supplemental Figure 4C). These genes are related to cancer cell proliferation, survival, and angiogenesis. Moreover, the Notch pathway was reported to be involved...
in self-renew of human cancer stem cells and tumorigenicity (28, 30). These results indicate the LLL12 is also potent in terms of inhibiting P-STAT3, down-regulating STAT3-downstream genes, and induces apoptosis in these colorectal cancer-initiating cells. Furthermore, the expression of STAT3 down-stream genes, such as Cyclin D1, survivin, Bcl-XL, Notch-1 and Noth-3 were also reduced by STAT3 ShRNA (Figure 2D-b).

**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 (Figure 3A,3B, 3C), and HT29 (Supplemental Figure 4D) colorectal cancer cells, further supporting the idea that colorectal cancer-initiating cells is sensitive to the inhibition of STAT3. LLL12 and Stattic also inhibited the cell viability of ALDH-/CD133- subpopulation (Supplemental Figure 5A, B) and the bulk tumor cells (Supplemental Figure 5C, D). These results may be explained by at least two possibilities: Some (HCT116) or low (SW480 and DLD-1) levels of P-STAT3 are expressed in ALDH+/CD133- cells; Stattic and LLL12 may also inhibit both P-STAT3 (Tyr705) and un-phosphorylated STAT3 or non-nuclear function of STAT3(31). The inhibition of un-phosphorylated STAT3 or non-nuclear function of STAT3 in ALDH+/CD133- cells might contribute to the reduction of cell viability.

Further, 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 demonstrated that LLL12 and Stattic can inhibit tumorsphere forming capacity in the ALDH+/CD133+ cells of SW480, HCT116, DLD-1 (Figure 3D), and HT29
(Supplemental Figure 4E) 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-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 5μM of LLL12, 10 μM of Stattic, 10μM of Doxorubicin and 10μM 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 (Figure 4A, Supplemental Figure 6A), suggesting that this subpopulation of colon cancer initiating cells is sensitive to STAT3-mediated inhibition. We found that 10 μM of Stattic also decreased the percentage of ALDH+/CD133+ subpopulation (Figure 4A, Supplemental Figure 6A). However, 10 μM of Doxorubicin or 5-Fu increased the percentage of ALDH+/CD133+ colorectal cancer-initiating cells ($P<0.05$) (Figure 4A), which might indicate that colon cancer initiating cells are resistant to these chemotherapy. We also detected the effects of Dox or 5-FU (10 μM) in STAT3 activation in the bulk tumor cell population (Supplemental Figure 6B). The results showed different responses to both drugs on P-STAT3 in two 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 (Figure 4B). Interestingly, the expression of IL-6, IL-6R, GP130, IL-8 were higher in ALDH+/CD133+ cells than ALDH-/CD133- cells (Figure 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 (Figure 4C-b). In addition, LLL12 inhibited the expression of IL-6, GP130, and IL-8 (Figure 4C-b). However, the expression of IL-6R was not changed consistently in all four 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 (Supplemental Figure 6C). 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 dependence on upstream ligand, IL6, we treated ALDH+/CD133+ cells with lentiviral IL-6 ShRNA versus control lentivirus without encoding IL-6 ShRNA. Our results in Figure 4D-a demonstrated that lentiviral IL-6 ShRNA but not control lentivirus down-regulated 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 down-regulates phosphorylated STAT3.

To further demonstrate 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 Figure 4D-b demonstrated that STAT3 and IL-6 shRNA significantly suppressed colon
cancer stem cell tumor growth compared to 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 demonstrated that LLL12 inhibited P-STAT3, cell viability, and tumorsphere growth in colorectal cancer-initiating cells expressing elevated levels of STAT3 phosphorylatation *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 (Figure 5A-a), tumor mass (Figure 5A-b). The average of reduction in tumor weight is 49.67-61.89 % in LLL12-treated mice comparing to DMSO vehicle in xenograft mouse model (Figure 5B). LLL12 also inhibited STAT3 but not ERK1/2 phosphorylation of SW480 and HCT116 colon cancer-initiating cells (Figure 5C). We also used flow cytometry to determine the percentage of ALDH1+CD133+ subpopulation in the tumors treated with vehicle or LLL12. Our results in Figure 5D-a,b showed that LLL12 reduced the percentage of ALDH+CD133+ subpopulation in tumor. In addition, the body weights of mice have no obvious reduction in both DMSO vehicle and LLL12 treated mice (Supplemental Figure 7). These results demonstrate 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, demonstrating 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 report, 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 demonstrated 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 of 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 induce apoptosis in non-separated 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 down-regulated the expression of STAT3 phosphorylation. These results provide evidence that STAT3 activation in ALDH+/CD133+ cells is IL6-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 down-regulated 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 demonstrated 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 inflammatory microenvironment is important in promoting the oncogenesis. Ginestier et al reported that blockade of the IL-8 receptor CXCR1 selectively deplete 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 demonstrated 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 demonstrated 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 suggested that constitutive active IL-6/STAT3 in these cancer-initiating cells enhances 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 demonstrate 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 demonstrated 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, there are two literatures reported that IL-6/STAT3 pathway may be activated in Glioblastoma stem cells (41, 42). In addition, targeting STAT3 by two 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 comparing to un-separated and non-breast cancer-initiating cells (Data not shown). These results are consistent with our observation in colon cancer-initiating cells that activated STAT3 appears 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.
Acknowledgements

We thank Cynthia McAllister and Dave Dunaway at the Flow Cytometry Core of Nationwide Children’s Hospital.
References:

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.

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Table 2. The association of P-STAT3 (Y705) with the expression of ALDH1 and CD133 in Colon carcinoma

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* The correlation of P-STAT3 (Y705) with ALDH1or/and CD133 (Both positive or negative) was assessed by χ² Test. P<0.05 is considered as statistical significance. Colon cancer tissues from a total numbers of 109 cancer patients were examined.
Figure Legends

**Figure 1.** STAT3 phosphorylation of ALDH^+/CD133^+ subpopulation of colon cancer cells is higher than un-separated- and the ALDH^-/CD133^- subpopulations. (A) ALDH^+/CD133^+ 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), phosho-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 Immunofluorence (IF). ALDH1 high expression tumor cells (cytoplasm, green) also expressed phosphylated-STAT3 in nuclei (red). Scale bar: 10μm.

**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, B) ALDH^+/CD133^+ colon cancer-initiating cells were treated with DMSO, LLL12 (5μM) or Stattic (10-20μM) for 24-48 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 μM), CTL or STAT3 ShRNA. Reverse transcriptase PCR reveals decreased expression of STAT3 downstream target genes in LLL12 or STAT3 ShRNA treated cells as compared to control.

**Figure 3.** LLL12 (A), Stattic (B), and STAT3 ShRNA (C) inhibited cell viability of SW480, HCT116, and DLD-1 colon cancer-initiating cells. The ALDH+/CD133+ subpopulation of colon cancer cells was seeded in 96-well plates (3,000 cells/well) in triplicates in a serum-free MEBM. The following day, cancer-initiating cells were treated with LLL12, Stattic, CTL or STAT3 ShRNA. MTT assay was used to determine cell viability. (D) Stattic and LLL12 inhibited tumorsphere formation of ALDH+/CD133+ subpopulation of SW480, HCT116, and DLD-1 colon cancer cells. The ALDH+/CD133+ cancer-initiating cells were plated as single cells and treated with Stattic (5-20 μM) or LLL12 (2.5 and 5 μM) at the second day. Tumorspheres were observed under microscope 15 days post-treatments.

**Figure 4.** (A) LLL12 (5μM) and Stattic (10μM) decreased the percentage of ALDH+/CD133+ subpopulation. However, 10μM Doxorubicin or 5-Fu increased the percentage of ALDH+/CD133+ colorectal cancer-initiating cells (*P<0.05). (B) ELISA assay showed that ALDH+/CD133+ subpopulation secreted more IL-6 than ALDH+/CD133+ subpopulation of colon cancer cells. (C) The expression of ALDH1, CD133, IL-6, IL-6R, GP130, IL-8 was detected by RT-PCR. (a) ALDH+/CD133+ and ALDH+/CD133− colon cancer cells were collected after sorting. The
expression of IL-6, IL-6R, GP130, IL-8 were higher in ALDH+/CD133+ cells than ALDH-/CD133- cells; (b) The expression of ALDH1, CD133, IL-6, GP130 and IL-8 were lower after treated with LLL12 in ALDH+/CD133+ colon cancers. (D) After sorting, ALDH+/CD133+ HCT116 and/or SW480 colon cancer-initiating cells were infected with CTL, IL-6 and/or STAT3 ShRNA. (a) IL-6 ShRNA decreased STAT3 phosphorylation of SW480 and HCT116 colorectal cancer-initiating cells in vitro; (b) IL-6 and STAT3 ShRNA inhibited ALDH+/CD133+ HCT116 cancer-initiating cell growth in vivo.

Figure 5. LLL12 suppressed tumor growth in mouse xenografts with SW480 or HCT116 colon cancer stem cells. The mice were given daily intraperitoneal dosages of 5mg/kg LLL12 or DMSO vehicle. Tumor volume (A-a), tumor mass (A-b), and tumor weight (B) were reduced in all LLL12-treated mice compared to DMSO vehicle group. (C) One representative sample from tumor tissues generated from SW480 or HCT116 colon cancer-initiating cells showing STAT3 but not ERK1/2 phosphorylation were also inhibited by LLL12 treatment. (D) At the end of treatments, tumors tissues were dissociated to obtain a single-cell suspension and analyzed by flow cytometry. LLL12 reduced the percentage of ALDH+/CD133+ subpopulation in tumor.
Figure 1

A

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Fold Changes:

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B

- P-STAT3
- ALDH1
- CD133
- Negative Control

C

- ALDH1
- P-STAT3
- DAPI

- ALDH1 / P-STAT3
- P-STAT3 / DAPI
- ALDH1 / P-STAT3 / DAPI
Figure 2

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</table>
Figure 3
Figure 4

A

B

C

D

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Figure 5

A

a

SW480 ALDH+CD133+

Tumor volume (mm³)

Days

1000

800

600

400

200

0

17 18 19 20 21 22 23 24 25 26 27 28 29 30

HCT116 ALDH+CD133+

Tumor volume (mm³)

Days

1000

800

600

400

200

0

19 20 21 22 23 24 25 26 27 28 29 30

Vehicle

5mg/kg LLL12

b

Vehicle

LLL12 5mg/kg

SW480 ALDH+CD133+

HCT116 ALDH+CD133+

B

SW480 ALDH+CD133+

Tumor weight (g)

Vehicle

LLL12 6mg/kg

HCT116 ALDH+CD133+

Tumor weight (g)

Vehicle

LLL12 6mg/kg

C

SW480 ALDH+CD133+

Vehicle

LLL12 5mg/kg

P-STAT3 (Tyr705)
P-STAT3
P-ERK1/2 (T202/Y204)
GAPDH

HCT116 ALDH+CD133+

Vehicle

LLL12 5mg/kg

P-STAT3 (Tyr705)
P-STAT3
P-ERK1/2 (T202/Y204)
GAPDH

D

ALDH+CD133+

Vehicle

LLL12 5mg/kg

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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.

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*The correlation of P-STAT3 (Y705) with ALDH1 or and CD133 (Both positive or negative) was assessed by \( \chi^2 \) Test. \( P<0.05 \) is considered as statistical significance. Colon cancer tissues from a total numbers of 109 cancer patients were examined.
STAT3 is necessary for proliferation and survival in colon cancer-initiating cells

Li Lin, Aiguo Liu, Zhengang Peng, et al.

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