Schlafen-3 decreases cancer stem cell marker expression and autocrine/juxtacrine signaling in FOLFOX-resistant colon cancer cells

Phil-Sun Oh,1,3 Vaishali B. Patel,1,3 Matthew A. Sanders,1,3 Shailender S. Kanwar,1,3 Yingjie Yu,1,3 Jyoti Nautiyal,1,3 Bhaumik B. Patel,1,2,3 and Adhip P. N. Majumdar1,2,3

1Veterans Affairs Medical Center, 2Karmanos Cancer Institute, and 3Department of Internal Medicine, Wayne State University, Detroit, Michigan

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Colorectal cancer is the most common visceral malignant disease and remains a major cause of cancer mortality worldwide. Epithelial cells derived from stem cells located near the base of the colonic crypt migrate to the luminal surface. During migration, the cells lose their proliferative capability and differentiate, thereby acquiring the definitive features of mature colonocytes. Terminally differentiated cells are then shed into the intestinal lumen from the surface epithelium (2, 19, 35). Most colorectal cancers are thought to originate from stem cells, which have acquired mutations leading to aberrant activation of the Wnt/β-catenin pathway (11). Despite the development of targeted molecular therapies, most patients who present with metastatic disease relapse after chemotherapy, probably due to cancer cell resistance to chemotherapeutic agents (21, 22). Increasing evidence suggests that colon cancer metastasis may be driven by a small subpopulation of self-renewing cells termed cancer stem cells (CSCs) within the heterogeneous cell population of the primary lesion. CSCs for colon and other cancers have been identified based on the enhanced ability of a subpopulation of tumor cells expressing a particular protein to initiate tumors when injected in immunodeficient mice. For colon tumors, CSC markers include the surface epitopes, CD44, CD133, CD166, and the enzyme aldehyde dehydrogenase 1 (ALDH1) (5, 28). Additionally, CSCs from several cancers display an increased ability to form spherical colonies in stem cell medium (SCM) and increased ability to efflux the dye Hoechst 33342, an indicator of chemoresistance (7, 22, 39, 40).

Multiple mechanisms have been identified as mediators of CSC chemoresistance, including active DNA repair capacity, activation of ATP-binding cassette (ABC) transporters, which efflux various anti-cancer drugs, and resistance to apoptosis (9, 12, 20). Currently, the combination of 5-fluorouracil (5-FU) and oxaliplatin (FOLFOX) is the most widely used chemotherapy for metastatic colorectal cancer. 5-FU inhibits activity of the enzyme thymidylate synthase during DNA replication, whereas oxaliplatin covalently binds DNA, thereby forming platinum-DNA adducts that cause prolonged G2 arrest and inhibition of growth, leading to apoptotic cell death (27, 42). These drugs, however, have limited effectiveness, and such chemotherapy often results in the survival of cells with an enhanced capacity for initiating metastasis or causing cancer recurrence, indicating the existence of multipotent CSCs. Thus these chemoresistant cells show enrichment of CSC markers and have properties consistent with the CSC phenotype, including the ability to form spheroids. Additionally, CSCs isolated from tumor tissue are able to form spheroids that contain cells with elevated expression of CSC markers compared with adherent tumor cells. Moreover, our recent in vitro studies have demonstrated that the expression of several CSC markers is greatly elevated in chemoresistant cells compared with parental cells (26, 44). Since current chemotherapeutic agents generally target rapidly proliferating cells, effective treatment of metastatic colon cancer will require the development of therapies that target CSCs, in addition to the more differentiated cells that form the bulk of the tumor.

Our laboratory has previously reported that schlafen-3 (Slfn-3) expression increases during cellular differentiation in intestinal mucosa, is downregulated in the colon of aging Fisher 344 rats, and also mediates stimulation of intestinal epithelial cell differentiation by cyclic strain (31, 32, 45).
Slfn-3 belongs to a murine multigene family, consisting of 10 genes, which contain several highly conserved SWADL domains defined by a five-amino acid (Ser-Trp-Ala-Asp-Leu) signature, which is thought to be slafen protein specific (16, 30). The slafen protein family has been classified into three groups based on overall sequence homology and size of the encoded proteins: the shortest proteins are in group I (Slfn-1, -2, and slafen-like-1), while slafen proteins of intermediate size (Slfn-3, -4, -6, and -7) are in group II. The group III proteins (Slfn-5, -8, -9, -10, and 1–4) possess large COOH-terminal extensions. All slafen proteins have a common “slafen box”, which lies adjacent to a GTP/ATP binding AAA domain that functions similarly to the classical AAA domains in GTP/ATP binding protein. Classical AAA domain-containing proteins perform a variety of functions, including protein degradation and folding, vesicle transport/fusion, and transcription (16, 30). Among slafen family of proteins, Slfn-3 has been shown to regulate cell growth and differentiation in vitro, presumably through the inhibition of cyclin D1 (4, 31, 32). However, the role of Slfn-3 in regulating chemoresistant colorectal cancer cells that are highly enriched in CSCs is unknown. In the present study, we examined whether Slfn-3 can regulate CSC characteristics in colon cancer stemlike cells. To determine the role of Slfn-3 in colon CSCs, we evaluated tumorosphere/colonosphere formation, drug transporter activation, and autocrine growth factor expression using FOLFOX-resistant colon cancer HCT-116 and HT-29 cells that are highly enriched in CSCs (25).

METHODS AND MATERIALS

Chemicals

Dulbecco’s modified Eagle’s medium (DMEM), DMEM/F-12, FBS, and antibiotic-antimycotic were obtained from Gibco BRL (Bethesda, MD). Protease inhibitor cocktail and all other chemicals were obtained from Sigma (St. Louis, MO). Anti-CD44 and anti-Aldh1 were purchased from Cell Signaling (Beverley, MA) and BD Bioscience (San Jose, CA), respectively. CD166, ABCG2, EGF receptor (EGFR), and transforming growth factor (TGF)-α antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Cultures and Transfection

Human colon cancer HCT-116 (p53 positive; K-ras mutant) and HT-29 (p53 mutant and K-ras wild type) cells were obtained from American Type Culture Collection (Rockville, MD). They were maintained in tissue culture flasks in a humidified incubator at 37°C in an atmosphere of 95% air and 5% CO2. The HCT-116 and HT-29 cells maintained in DMEM were supplemented with 10% FBS and 1% antibiotic-antimycotic. The FOLFOX (chemotherapy)-resistant cells maintained in DMEM were supplemented with 10% FBS and antibiotic-antimycotic. The FOLFOX-resistant cells were transfected with Lipofectamine 2000, according to the manufacturer’s instructions. Approximately 0.5–1 × 10^6 cells in 2 ml of DMEM/10% FBS were plated in six-well tissue culture plates. After 24 h, the cells were transfected using 4 μg/well of plasmid DNA. Experiments were performed 48 h after the start of transfection.

Measurement of Alkaline Phosphatase Activity

Status of differentiation was determined by measuring the alkaline phosphatase (ALP) activity using SensoLyte, p-nitrophenylphosphate ALP colorimetric assay kit (Anaspec, San Jose, CA), according to the manufacturer’s instructions.

Hoechst 33342 Dye Exclusion

The protocol was based on the study by Goodell et al. (18), with slight modifications. Briefly, cells (1 × 10^6 cells/ml) were incubated in prewarmed DMEM/2% FBS containing freshly added Hoechst 33342 (5 μg/ml final concentration) for 90 min at 37°C with intermittent mixing. In positive control experiments, cells were incubated with the Hoechst dye in the presence of verapamil (50 μM). At the end of incubation, cells were spun down in the cold and resuspended in ice-cold PBS. An appropriate aliquot of cells was mounted on glass slides and then immediately examined and photographed using an AX70 fluorescence microscope (Olympus, Orangeburg, NY).

Tumorosphere/Colonosphere Formation Assay

To examine the effects of Slfn-3 on tumorosphere/colonosphere formation, FOLFOX-resistant HCT-116 and HT-29 cells transfected with pEGFPN1 (vector) or pEGFPN1/Slfn-3 were suspended in serum-free SCM containing DMEM/F12 (1:1) supplemented with B27 (50× dilution; Life Technologies, Gaithersburg, MD), 10 ng/ml fibroblast growth factor (Sigma), and antibiotic-antimycotic. The cells (100 cells/well) were plated in an ultra-low-attachment 96-well plate (Corning, Lowell, MA). Once the tumorospheres/colonospheres were formed after 7 days, they were evaluated for the number and size by light microscopy.

Extreme Limiting Dilution Analysis

Extreme limiting dilution analysis (ELDA) was performed essentially according to Hu and Smyth (23), as described by Kanwar et al. (25). Briefly, single-cell suspensions obtained from FOLFOX-resistant HT-29 cells were plated at a concentration of 100, 10, and 1 cell/100 μl SCM in 96-well ultra-low-attachment plates and incubated for 5 days. At the end of 5 days, the number of wells showing formation of tumorospheres/colonosphere was counted. The frequency of sphere-forming cells in a particular cell type was determined using the ELDA webtool at http://bioinf.wehi.edu.au/software/elda.

Western Blot Analysis

Protein extracts were prepared as previously described (32). Briefly, the cells were solubilized in lysis buffer (50 mM Tris, 100 mM NaCl, 2.5 mM EDTA, 1% Triton X-100, 1% Nonidet P-40, 2.5 mM Na3VO4, 25 μg/ml aprotinin, 25 μg/ml leupeptin, 25 μg/ml pepstatin A, and 1 mM phenylmethylsulfonyl fluoride) containing a protease inhibitor cocktail solution. Following centrifugation at 14,000 g for 15 min, the supernatant was used for Western blot analysis. In all analyses, protein concentration was determined by the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA). Cell lysates containing 50 μg of proteins were separated by SDS-PAGE and transferred to immobilin-P nylon membranes (Millipore, Bedford, MA). After incubation with primary and secondary antibodies, protein bands were visualized using the enhanced chemiluminescence detection system (GE Healthcare, Buckinghamshire, UK). All experiments were performed at least three times for each experiment. The results of immunoblot assays were calculated as relative intensity using Scion imaging software (Scion Image Beta 4.02, Frederick, MD).
**Immunoprecipitation**

Immunoprecipitation for TGF-α-associated EGFR was performed according to our standard protocol (43). Briefly, aliquots of cell lysates containing 1 μg protein were incubated with polyclonal anti-EGFR and Sepharose-protein G beads overnight at 4°C. The immunoprecipitates were washed three times with Tris-buffered saline buffer, and boiled at 95°C for 5 min in an equal volume of 2× Laemmli sample buffer (125 mM Tris, pH 6.8, 4% SDS, 10% glycerol, 4% β-mercaptoethanol, and 0.02% bromophenol blue). The samples were subjected to 18% SDS-PAGE, and electrophoresed proteins were transferred to Immobilon-P nylon membranes, as described above.

**Apoptosis Assay and Caspase-3 Activity**

**Apoptosis assay.** Acridine orange/ethidium bromide (AO/EtBr) staining methodology was used to quantitatively assess apoptosis (26). Apoptosis assay.

**Immunoprecipitation for TGF-α-associated EGFR.**

Immunoprecipitation for TGF-α-associated EGFR was performed according to our standard protocol (43). Briefly, aliquots of cell lysates containing 1 μg protein were incubated with polyclonal anti-EGFR and Sepharose-protein G beads overnight at 4°C. The immunoprecipitates were washed three times with Tris-buffered saline buffer, and boiled at 95°C for 5 min in an equal volume of 2× Laemmli sample buffer (125 mM Tris, pH 6.8, 4% SDS, 10% glycerol, 4% β-mercaptoethanol, and 0.02% bromophenol blue). The samples were subjected to 18% SDS-PAGE, and electrophoresed proteins were transferred to Immobilon-P nylon membranes, as described above.

**Caspase-3 activity.** The enzyme activity was measured using the colorimetric assay kit from BioVision Research Products, which detects the chromophore p-nitroanilide after cleavage from the labeled substrate DEVDP-pNA. After treating vector or Slfn-3 transfected cells with FOLFOX or vehicle for 48 h, they were washed with PBS and lysed in lysis buffer for 10 min on ice. After clarification by centrifugation for 1 min at 10,000 × g, the supernatant was used for analysis.

**Expression of Schlafen-3 (Slfn-3) in 5-fluorouracil + oxaliplatin (FOLFOX)-resistant HCT-116 cells following transfection of pEGFP-N1/Slfn-3 and stimulation of differentiation of FOLFOX-resistant HCT-116 cells.** A: photomicrograph showing green fluorescent protein (GFP)-tagged Slfn-3 in FOLFOX-resistant colon cancer HCT-116 cells and Western blot, demonstrating expression of 82-kDa GFP-Slfn-3 fusion protein in them following transfection of pEGFP-N1/Slfn-3 and 27-kDa GFP band for the vector transfected control. B: induction of alkaline phosphatase (ALP) activity in FOLFOX-resistant colon cancer HCT-116 cells 48 h after Slfn-3 transfection (n = 3). Values are means ± SD. *P < 0.05, compared with vector transfected control. Magnification, ×20.
FOLFOX-resistant HCT-116 and HT-29 cells. Thus the number represents the relative decrease compared with control vector transfected cells after normalization to β-actin.

Statistical Analysis

All data are expressed as means ± SD. The results were analyzed using the unpaired, two-tailed Student’s t-test. *P < 0.05 was designated as the level of significance.

RESULTS

Slfn-3 Inhibits Expression of CSC Markers in FOLFOX-resistant HCT-116 and HT-29 Cells

Since Slfn-3 has been shown to regulate cell growth and differentiation in vitro (32), we measured the brush-border enzyme ALP activity as a marker of differentiation in FOLFOX-resistant HCT-116 cells. Transfection of pEGFPN1/Slfn-3 cDNA in FOLFOX-resistant HCT-116 cells, which is devoid of Slfn-3, led to the expression of green fluorescent protein (GFP)-Slfn-3 fusion protein (Fig. 1A). Forty-eight hours after transfection of Slfn-3 in FOLFOX-resistant HCT-116 cells, ALP activity was increased by 102%, compared with vector transfected control cells (Fig. 1B). To examine whether Slfn-3 affects colon CSC marker expression in FOLFOX-resistant colon cancer cells, we measured CD44, CD133, CD166, and ALDH1 mRNA and protein levels in FOLFOX-resistant HCT-116 and HT-29 cells (Fig. 2). As expected, the constitutive levels of several CSC specific markers, including CD44, CD166, and ALDH1, were found to be higher in FOLFOX-resistant cells, compared with parental HCT-116 and HT-29 cells, indicating that the FOLFOX-resistant colon cancer cells are highly enriched CSCs. Slfn-3 transfection of FOLFOX-resistant HCT-116 cells, however, decreased CD44, CD166,
and ALDH1 protein levels by 20, 48, and 71%, respectively, compared with the vector-transfected control cells. Additionally, Slfn-3 transfection reduced CD44, CD133, CD166, and ALDH1 mRNA expression by 34.8, 73.7, 53.5, and 43.9%, respectively, in FOLFOX-treated HCT-116 cells. In FOLFOX-resistant HT-29 cells, transfection of Slfn-3 decreased CD44, CD166, and ALDH1 protein levels by 24, 32, and 56%, respectively, and reduced CD44, CD133, CD166, and ALDH1 mRNA expression by 52.5, 69.3, 57.6, and 58.5%, respectively, compared with the vector-transfected controls. These results clearly indicate that Slfn-3 downregulates CSC markers in chemoresistant colon cancer cells.

**Slfn-3 Inhibits Sphere Formation in FOLFOX-resistant HCT-116 and HT-29 Cells**

Since our previous study showed that chemoresistant cells may contain a subpopulation of CSCs that have the ability to form tumorospheres/colonosphere in SCM (26, 44), we assessed the effect of Slfn-3 on tumorosphere/colonosphere formation by FOLFOX-resistant HCT-116 and HT-29 cells. Slfn-3 transfection formed significantly fewer tumorospheres/colonosphere than vector-transfected cells (Fig. 3A). The size of tumorospheres/colonosphere was reduced by 39.4 and 48.7%, respectively, in Slfn-3-transfected FOLFOX-resistant HCT-116 and HT-29 cells compared with cells with vector-transfected controls (Fig. 3B). The number of tumorospheres/colonosphere formed from HCT-116 and HT-29 cells was also reduced by 55.3 and 45% after Slfn-3 transfection, respectively (Fig. 3C). ELDA indicated that the tumorosphere/colonosphere formation frequency was inhibited by fourfold in Slfn-3-transfected FOLFOX-resistant HT-29 cells compared with vector-transfected control cells (Table 1).

**Slfn-3 Inhibits Hoechst 33342 Dye Exclusion and ABCG2 Expression in FOLFOX-resistant HCT-116 and HT-29 Cells**

Because CSCs are thought to be more resistant to chemotherapy than the differentiated tumor cells that form the bulk of the tumor, we determined the role of Slfn-3 on live-cell DNA-binding dye Hoechst 33342 exclusion and ABCG2 expression in FOLFOX-resistant HT-29 cells. Slfn-3 transfection showed a significant decrease in Hoechst 33342 dye exclusion and ABCG2 expression (Fig. 3D).

**Table 1. Extreme limiting dilution analysis of tumorospheres/colonosphere forming frequency of FOLFOX-resistant HT-29 transfected (Vector or Slfn-3) cells**

<table>
<thead>
<tr>
<th>No. of Cells/Well</th>
<th>Vector transfected</th>
<th>Slfn-3 transfected</th>
</tr>
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<tbody>
<tr>
<td>100</td>
<td>24/24</td>
<td>15/16</td>
</tr>
<tr>
<td>10</td>
<td>14/16</td>
<td>8/16</td>
</tr>
<tr>
<td>1</td>
<td>1/8</td>
<td>0</td>
</tr>
</tbody>
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Sphere-forming frequency (95% CI) 1/6 (1/10–1/4) 1/26 (1/47–1/15) $P$ value < 0.001

Values are pooled from three independent experiments for each. CI, confidence interval; Slfn-3, schlafen-3.
Infection in FOLFOX-resistant HCT-116 and HT-29 cells significantly reduced the Hoechst 33342 dye exclusion by 59 and 40%, respectively, compared with vector-transfected control cells (Fig. 4A). Since Hoechst 33342 dye exclusion by cancer cells is associated with the ABC membrane transporter superfamily member ABCG2, we further determined ABCG2 protein expression in Slfn-3-transfected FOLFOX-resistant HCT-116 and HT-29 cells. ABCG2 expression was decreased by 24 and 50%, respectively, in FOLFOX-resistant HCT-116 and HT-29 cells transfected with Slfn-3 (Fig. 4B).

Slfn-3 Inhibits TGF-α-induced Cancer Cell Autoactivation in FOLFOX-resistant HCT-116 and HT-29 Cells

Since the TGF-α/EGFR pathway appears to play a critical role in colon cancer progression, we determined the level of TGF-α bound to EGFR in Slfn-3-transfected FOLFOX-resistant HCT-116 and HT-29 cells (Fig. 5) as an indicator of TGF-α/EGF autocrine signaling. We observed that the amount of TGF-α bound to EGFR was found to be decreased by 18 and 43% after Slfn-3 transfection in FOLFOX-resistant HCT-116 and HT-29 cells, respectively. However, the levels of total

Fig. 4. Slfn-3 inhibits Hoechst 33342 dye exclusion and ABCG2 expression in FOLFOX-resistant HCT-116 and HT-29 cells. A: photomicrograph showing bright field and fluorescence of Hoechst 33342 dye excluded FOLFOX-resistant HCT-116 and HT-29 cells following Slfn-3 transfection. B: ABCG2 expression after Slfn-3 transfection was determined using Western blot, and the relative intensities of bands were calculated using Scion Imaging Software (Scion Image Beta 4.02) (n = 3). Values are means ± SD. *P < 0.05, compared with vector-transfected control.

Fig. 5. Slfn-3 inhibits transforming growth factor (TGF)-α activation of FOLFOX-resistant HCT-116 and HT-29 cells. EGF receptor (EGFR) was immunoprecipitated (IP), according to our standard protocol (43), as described in MATERIALS AND METHODS. The immunoprecipitates were subjected to Western blot analysis for determination of TGF-α. The relative intensities of bands were calculated using Scion Imaging Software (Scion Image Beta 4.02) (n = 3). Values are means ± SD. *P < 0.05, compared with vector-transfected control.
EGFR were found to be similar between the two groups. The results suggest that Slfn-3 may decrease autocrine growth of cancer stem-like cells through TGF-α/EGFR signaling.

Slfn-3 Increases Susceptibility to Cancer Chemotherapeutic Agents in FOLFOX-resistant HCT-116 Cells

Because Slfn-3 transfection decreased the expression of drug transporter gene ABCG2, we further examined whether Slfn-3 increases the susceptibility of resistant cells to FOLFOX treatment. In the absence of any treatment, vector-transfected chemoresistant HCT-116 cells exhibited ∼5% apoptosis, whereas FOLFOX treatment of these cells increased apoptosis (as indicated by AO/EtBr staining) to ∼14%, a 2.8-fold induction (Fig. 6A). Slfn-3 transfection of FOLFOX-treated chemoresistant HCT-116 cells caused a further twofold increase in apoptosis compared with FOLFOX-treated vector-transfected cells (Fig. 6A). Likewise, we observed a 11% increase of caspase-3 activity in vector-transfected chemoresistant HCT-116 cells treated with FOLFOX, compared with vector-transfected cells without FOLFOX. However, Slfn-3 transfection caused 22% increase (a further twofold) in caspase-3 activity of FOLFOX-treated cells (Fig. 6B).

DISCUSSION

The CSC model proposes that tumor initiation and metastasis are driven by a small subpopulation of tumor cells called CSCs, possessing self-renewal potential and multipotent differentiation capacity. The CSCs are believed to originate from normal stem cells or progenitor cells that have acquired genetic mutations, which result in aberrant activation of the Wnt/β-catation pathway (24, 25). Therefore, it is essential to understand the characteristics of CSCs as a first step in developing approaches to preventing or reversing chemoresistance in vivo. Our laboratory’s previous studies have suggested that Slfn-3 stimulates differentiation of intestinal epithelial cells, both in vitro and in vivo in rats (31, 32). In the present study, the effects of Slfn-3 on CSC marker expression, as well as tumorsphere/colonosphere formation, were evaluated in FOLFOX-resistant HCT-116 and HT-29 colon cancer cells. Our results suggest that Slfn-3 expression inhibits their expression of CSC markers and their ability to form tumorspheres/colonospheres and also reduces their expression of the membrane transporter ABCG2 that is associated with chemoresistance.

The intestinal epithelium has a morphological and functional organization that shows finger-like villi and adjacent invaginations called crypts, where cell proliferation and differentiation occur in synchronization along the crypt-villus axis. The epithelium of the villus is composed of three types of fully differentiated epithelial cells; enterocytes (the predominant cell type), enteroendocrine cells, and goblet cells. The stem cells are located in the crypts of the intestinal mucosa and are responsible for producing the immature, transient-amplifying progenitors, which, in turn, generate the differentiated cell types (2, 3). Previously, our laboratory has shown that Slfn-3 induces activity of the brush-border enzyme ALP in intestinal epithelial crypt IEC-6 cells, a widely used model cell line for normal intestinal epithelial cell function, indicating that Slfn-3 induces normal intestinal epithelial cell differentiation (32). The results of the present studies are in agreement with our earlier observation in that Slfn3 transfection in colon cancer HCT-116 cells also stimulates differentiation, as evidenced by increased ALP activity. Based on these observations, we hypothesize that Slfn-3 expression in tumors could induce differentiation of CSCs and thus make them more susceptible to chemotherapeutic agents.

CSCs are thought to acquire resistance to anti-cancer agents, such as 5-FU and oxaliplatin, through multiple mechanisms, including their relative quiescence, high capacity for DNA repair, activation of ABC transporters, and resistance to apoptosis (9, 12, 29). For a colon CSC in vitro model, we have previously established two chemoresistant cell lines derived from the parental HCT-116 and HT-29 human colon cancer cell lines after exposure to the combination of 5-FU and oxaliplatin (FOLFOX) at therapeutically relevant concentrations. Earlier, we demonstrated 10-fold increased expression of the colon CSC marker CD44 and increased expression of the proposed colon CSC marker CD166 in FOLFOX-resistant HCT-116 cells compared with the parental cell line (44). Likewise, the constitutive levels of colon CSC markers CD44, CD166, and ALDH1 were found to be markedly higher in FOLFOX-resistant colon cancer cells than in the corresponding parental cells. This suggests that the FOLFOX-resistant cell line is enriched for colon CSCs. Consistent with our observations, another recent study found that colorectal cancer cell lines selected for resistance to either 5-FU or oxaliplatin showed 5- to 22-fold enrichment of cells that are double positive for expression of CD44 and the colon CSC marker CD133 (8). In addition to CD44, other proteins, such as ALDH1, have been reported as colon CSC markers (1, 17, 33). Thus we deter-
growth factor. This autocrine hypothesis has been suggested to bind only to the EGFR and plays an important role in oncogenesis and malignant progression as an autocrine stimulatory growth factor. This autocrine hypothesis has been suggested to explain the fast growth of tumor cells compared with normal cells (36, 37). Briefly, most tumor cells produce their own growth factors that are ligands for growth factor receptors on the cellular surface, resulting in the auto-stimulation of DNA synthesis and proliferation. Consequently, we examined whether Slfn-3 affected TGF-α expression in response to EGFR activation in FOLFOX-resistant HCT-116 and HT-29 cells. Our present observation that Slfn-3 decreases TGF-α expression in both FOLFOX-resistant HCT-116 and HT-29 cells suggests that Slfn-3 modulates TGF-α-mediated autocrine signaling (Fig. 5). Interestingly, TGF-α-mediated autocrine activation in FOLFOX-resistant HCT-116 cells was less affected by Slfn-3 than in FOLFOX-resistant HT-29 cells, consistent with their aggressive characteristics compared with FOLFOX-resistant HT-29 cells (6, 10). This finding is consistent with our observation that Slfn-3 transfection inhibits tumorosphere/colonosphere formation potential of both FOLFOX-resistant HCT-116 and HT-29 cells, which suggests that Slfn-3 may stimulate differentiation and inhibit ABCG2 activity and tumorosphere/colonosphere formation of colon cancer stemlike cells. Additionally, our results indicate that one of the mechanisms by which Slfn-3 may regulate tumorosphere/colonosphere formation of FOLFOX-resistant colon cancer cells is via inhibition of TGF-α autocrine signaling.

In conclusion, our present data suggest that Slfn-3 inhibits multiple CSC characteristics of FOLFOX-resistant colon cancer stem-like cells, as indicated by decreased colon CSC marker expression, tumorosphere/colonosphere formation, and ABCG2 transporter activation. Our results also suggest that these effects may be mediated by Slfn-3 inhibition of TGF-α autocrine activation of EGFR. Thus stimulation of Slfn-3 expression in colon CSCs could be an effective strategy for inhibiting progression of colon cancer by making colon CSCs more susceptible to chemotherapeutic agents. Further studies are necessary to elucidate the precise molecular mechanisms by which Slfn-3 mediates these effects in FOLFOX-resistant colon cancer stemlike cells.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

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