Inhibition of Fatty Acid Synthase Attenuates CD44-Associated Signaling and Reduces Metastasis in Colorectal Cancer

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Abstract

Fatty acid synthase (FASN) and ATP-citrate lyase, key enzymes of de novo lipogenesis, are significantly upregulated and activated in many cancers and portend poor prognosis. Even though the role of lipogenesis in providing proliferative and survival advantages to cancer cells has been described, the impact of aberrant activation of lipogenic enzymes on cancer progression remains unknown. In this study, we found that elevated expression of FASN is associated with advanced stages of colorectal cancer (CRC) and liver metastasis, suggesting that it may play a role in progression of CRC to metastatic disease. Targeted inhibition of lipogenic enzymes abolished expression of CD44, a transmembrane protein associated with metastases in several cancers including CRC. In addition, inhibition of lipogenic enzymes and reduced expression of CD44 attenuated the activation of MET, Akt, FAK, and paxillin, which are known to regulate adhesion, migration, and invasion. These changes were consistent with an observed decrease in migration and adhesion of CRC cells in functional assays and with reorganization of actin cytoskeleton upon FASN inhibition. Despite the modest effect of FASN inhibition on tumor growth in xenografts, attenuation of lipogenesis completely abolished establishment of hepatic metastasis and formation of secondary metastasis. Together, our findings suggest that targeting de novo lipogenesis may be a potential treatment strategy for advanced CRC.

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Introduction

Colorectal cancer (CRC) is the second leading cause of cancer-related deaths in the United States (1). The prognosis and life expectancy of patients with CRC are primarily determined by presence or absence of metastases, not by growth of the primary tumor (2). Treatment options for metastatic CRC are limited and more selective therapeutic targets are needed to improve survival.

The signaling networks that determine progression of cancer to a metastatic phenotype include multiple alterations in the oncogenic pathways and significant changes in cellular metabolism. The fact that, in contrast to normal cells, the majority of fatty acids in malignant cells are derived from de novo lipogenesis regardless of the availability of extracellular lipids, suggests the importance of upregulation of endogenous lipid biosynthesis in malignant transformation (3). ATP-citrate lyase (ACLY) and fatty acid synthase (FASN), the key enzymes of de novo lipogenesis, are significantly upregulated in many cancers including CRC (3). Indeed, expression of FASN was increased in 86% of aberrant crypt foci (ACF) compared with that of adjacent normal colonic mucosa (4). Furthermore, metabolic profiling of CRC has shown an overall increase in the lipid content of polyps and tumors (5).

Neoplastic lipogenesis provides a selective proliferative and survival advantage and contributes to drug resistance in cancer cells (6–8). However, the impact of aberrant activation of lipogenic enzymes on metastases remains unknown. Expression of FASN is highest in metastatic tumors and correlates with decreased survival and disease recurrence in several tumor types (9, 10). Interestingly, proteomic characterization of CRC cell lines indicates that an increased expression of lipogenic enzymes is associated with a more aggressive metastatic phenotype (11). Furthermore, pharmacologic inhibition of FASN provides indirect evidence of a possible connection between activation of lipogenesis and metastatic behavior of cancer cells (12–14).

Progression to a metastatic phenotype is associated with differential expression of proteins on the cell surface (11, 15). CD44, a transmembrane glycoprotein with multiple isoforms, is implicated in tumor progression and metastasis (16). Expression of CD44 is increased in CRC and correlates with poor clinical outcome (17, 18). The role of CD44 in
metastases might be linked to its interaction with receptor tyrosine kinases such as c-MET, a proto-oncogene involved in tumor growth, invasion, and metastasis (19). Association of c-MET with CD44 isoforms at the plasma membrane seems to be essential for activation of c-MET and downstream signaling in CRC (20).

In this study, we determined the role of lipogenic enzymes in metastatic CRC. We show that in human tissue arrays, FASN is progressively increased with advancing stages of CRC. For the first time, this study establishes the link between expression of lipogenic enzymes and CD44. We further show that suppressed expression of FASN decreases the tumorigenic and metastatic potential of CRC cells in vitro and in vivo. Collectively, our data suggest that upregulation of de novo lipogenesis is a critical step in CRC progression to metastases and that a better understanding of the link between metabolic changes in cancer cells and development of metastasis may lead to novel strategies to prevent and/or control advanced CRC.

Materials and Methods

Cell lines, lentiviral transduction, siRNA
Human CRC lines KM20 and HCT116 were used as described previously and their identity was authenticated at the Johns Hopkins Genetic Resources Core Facility in October 2010, as previously reported (21). HT29 cells were purchased from American Type Culture Collection. For generation of stable knockdown KM20, HT29, and HCT116 cell lines, the lentiviral transduction particles containing short hairpin RNA (shRNA) for ACLY (SHCLNV-NM_001096), FASN (SHCLNV-NM_004104), or nontarget shRNA (CHC002V) in pKO.1-puro plasmid were purchased from Sigma. Cells were transduced with virus in the presence of polybrene (10 µg/mL) for 24 hours and then selected on puromycin (10 µg/mL). ON-TARGET plus CD44 siRNAs (LU-00999907 and LU-00999908) and control siRNA (D-001810-10) were purchased from Dharmacon and used in a concentration of 100 µM/L.

Antibodies
Antibodies for Western blot and immunofluorescent staining were purchased from Cell Signaling: FASN (#3180), ACLY (#4332), pACLY (#4331), pCD44 (#3570), pMET (#3129), MET (#3148), pSrc (#2101), Src (#2109), pAkt (#4058L), Akt (#4691L), p-paxillin (#2541), paxillin (#2542), pFAK (#3283), FAK (#3285), and RhoA (#2117).

Human tissue arrays
The CO702 tissue array (US Biomax), A203 (IV) tissue array (AccuMax), and FASN antibody (Cell Signaling) were purchased. Scoring was carried out blindly by a pathologist according to a semiquantitative method (21). The extent score was assessed on a scale of 0 to 3 (no positive cells = 0, <10% = 1, 10%–50% = 2; positive staining of >50% = 3); the intensity score was also measured on a scale of 0 to 3 (negative = 0, weak = 1, moderate = 2, strong = 3). The immunoreactive score was obtained by summing these 2 scores.

Analysis of de novo lipogenesis
De novo palmitate synthesis was analyzed by stable isotope labeling. Cells were plated in normal growth medium. On day 2, medium was replaced with medium containing sodium acetate (2–13C, 99%, Cambridge Isotope Laboratories) for 20 hours at a concentration of 10 mmol/L. Extraction of the lipids was carried out as described previously (22) in the presence of an internal standard. Fatty acids were converted to 3-acyloxyethyl-1-methylpyridinium iodide (AMPP) derivatives and the AMPP derivative of palmitic acid was quantified using a Shimadzu HPLC coupled to ABI 4000 Q-Trap hybrid linear ion trap triple quadrupole mass spectrometer operated in positive electrospray ionization (ESI) mode as previously described (23). The instrument was operated in multiple reaction monitoring mode and the precursor product ion pairs monitored were 362.2/124.5 which represent the [M+H]+ mass of the AMPP derivative of palmitic acid and a fragment ion containing the N-pyridyl moiety. Enrichment of 13C was determined from the ratio of integrated peak areas corresponding to the mz/363.2/124.5 and the M+1 isotopeomer 362.2/124.5 precursor/product ion pairs.

Quantitative real-time PCR
Total RNA was isolated using an RNeasy mini kit (QIAGEN). cDNA was synthesized using a high capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative real-time PCR (qRT-PCR) was carried out using a TaqMan Gene Expression Master Mix (#4369016) according to manufacture protocol and TaqMan probes for human CD44 (ID Hs01078622_m1) and human GAPDH (# 4333764F; Applied Biosystems).

Flow cytometry analysis and cell sorting
Cells were labeled with 2 µg/mL CD44–FITC antibody (Abcam) per 5 × 10^6 cells. For cell sorting, cells were washed and resuspended in basic sorting buffer. For analysis of CD44 expression on cell surface, cells were resuspended in PBS, 10% FCS, 1% sodium azide. Cell analysis and cell sorting were conducted by the UK Flow Cytometry Service Facility.

Transwell migration assay
A Boyden chamber migration assay with collagen-coated Transwells was conducted with KM20 and HT29 cells for 20 hours as described previously (21). FBS (10%) was used as a chemoattractant. Cells were counted in 4 different fields with an inverted microscope.

Endothelial cell adhesion assay
CRC cells were labeled with Calcein AM (2.5 µg/mL final concentration) at 37°C, added atop a monolayer of human microvascular endothelial cells from lungs (HMVEC-L) for 30 minutes. Before the experiment, HMVEC-L cells were activated with 10 ng/mL of TGFβ for 4 hours. Unattached cells were removed by washing with PBS (5×). Three images per well were taken to count attached cells.
**In vivo studies**

Male athymic nude

5–6 weeks old; Charles River Laboratories) were housed in the Markey Cancer Center Small Animal Facility. All procedures were carried out with protocols approved by the U.K. Animal Care and Use Committee. To establish CRC xenografts, mice were injected with 1 \times 10^6 cells per 100 \mu L of KM20 or HT29 with nontargeting control (NTC) shRNA and FASN shRNA (5 mice per group). To assess establishment of hepatic metastasis, HT29-GFP-Luc cells (2 \times 10^6 cells per 100 \mu L) were injected intrasplenically as previously described (24). For experimental metastasis models, control and stable knockdown HT29-GFP-Luc cells or KM20-GFP-Luc (2 \times 10^6 cells per 300 \mu L) were injected into the tail vein of athymic nude mice (n = 4 and 5 per group, respectively). To monitor metastasis, mice were anesthetized with isoflurane, given a single i.p. dose of 150 mg/kg \( \beta \)-luciferin in PBS and imaged 8 minutes after injection (IVIS Spectrum; Caliper Sciences). Results were analyzed by Living Image 3.0 software.

**Statistical methods**

ANOVA with test for linear trend and pairwise comparisons was carried out to compare immunoreactivity scores in normal mucosa and CRC of various stages. ANOVA was also employed for multiple group comparison of shRNA (ACLY and FASN) with NTC for CD44 levels, whereas 2-sample t test or Wilcoxon rank-sum test was used for 2 group comparisons of migration, number of colonies, and tumor weight between FASN versus NTC. Comparison of log values of tumor volume measured over time was conducted using a linear mixed model. Analyses were conducted separately for each cell line.

**Results**

**FASN is highly expressed in primary CRCs and liver metastases**

Evaluations of FASN in human ACF and primary CRCs have revealed elevated expression of this enzyme in early stages of colorectal tumorigenesis as compared with normal mucosa (4, 25–27). To extend these studies, we analyzed expression of FASN in CRC clinical samples by immunohistochemistry (Fig. 1A). Expression of FASN was detected in the cytoplasm in all stages of primary CRC, though some variability was observed within different regions of individual tumor cores. Statistical evaluation of immunoreactivity scores showed increased expression of FASN in stages II to IV CRC as compared with normal mucosa. Analysis of primary tumors, matched liver metastases, and normal colonic mucosa from stage IV patients showed significantly higher expression of FASN in both the primary CRC and liver metastasis as compared with normal colon mucosa (Supplementary Fig. S1).

We also tested expression of ACLY and FASN in a panel of CRC cell lines. Consistent with results obtained from human tissue arrays, Western blot analysis showed high expression/activation of lipogenic enzymes (Fig. 1B). These data suggest that enhanced de novo lipid biosynthesis may play a role in progression of primary CRC to metastatic disease.

Figure 1. Expression of FASN in CRC tissues and cell lines. A, expression of FASN in normal colon mucosa and stage I to IV primary CRC (US Biomax tissue array CO702, total 69 cores, \( \times \)200 magnification). Immunoreactivity score was determined according to a semiquantitative method as described in Materials and Methods. B, cell lyses were prepared from a panel of human CRC cell lines and immunoblot analysis was conducted for FASN, ACLY, and pACLY expression. \( \beta \)-actin was used as a loading control.
Inhibition of lipogenic enzymes decreases expression of CD44

CD44 and c-Met have been implicated in CRC metastasis via regulation of tumor cell growth, adhesion, migration, and invasion (16, 28, 29). Considering that tumor-produced fatty acids preferentially partition into lipid rafts (30), we hypothesized that altered expression of lipogenic enzymes may affect CD44 and c-Met because their functions have been attributed to these rafts (31, 32). First, we analyzed expression of CD44 and c-Met in a panel of CRC cell lines. CD44 variant isoforms (CD44v), which are primarily implicated in regulation of metastasis, are highly expressed in most cell lines (Fig. 2A). In contrast with other cell lines, the nonmetastatic SW480 cell line expresses a low level of CD44v and a high level of CD44 standard isoform (CD44s). Activation of c-Met was observed in KM20, HT29, and HCT116 cell lines, and we used these 3 cell lines to establish a stable shRNA-mediated knockdown of either ACLY or FASN to investigate the role of de novo lipogenesis in metastatic CRC.

To confirm inhibition of lipid biosynthesis when ACLY and FASN are inhibited, cells were exposed to stable isotope-labeled acetate and its incorporation into palmitic acid was measured by mass spectrometry. Targeted inhibition of ACLY and FASN led to a 60% to 100% decrease in de novo lipogenesis (Fig. 2B). Inhibition of ACLY and FASN attenuates expression of CD44. A, immunoblot analysis for CD44, p-MET, and total MET in a panel of CRC cell lines. β-Actin was used as a loading control. B, de novo lipogenesis was analyzed by stable isotope labeling. Abundance of 13C acetate in palmitic acid was determined in CRC cell lines with NTC shRNA, ACLY shRNA, and FASN shRNA by mass spectrometry. Data shown as the ratio of integrated peak areas (means ± SD of triplicate determinations). No 13C is an abundance of 13C in unlabeled cells (a baseline isotope ratio). * p < 0.05 versus control. C, expression of CD44 in KM20, HT29, and HCT116 CRC cell lines with NTC shRNA, ACLY shRNA, and FASN shRNA. D, expression of CD44 in HT29 cell line treated with various concentrations of C-75 (μmol/L), an inhibitor of FASN, for 24 hours. E, expression of FASN and CD44 in HT29 cell line transiently transfected with myr-Akt1, myr-Akt2, or a control plasmid. F, immunoblot analysis for FASN and CD44 in HT29 FASN shRNA cells labeled with CD44-FITC antibody and sorted for expression of CD44. Cells with the lowest (10%) and the highest (10%) expression of CD44 were used for analysis. G, relative CD44 mRNA levels in KM20, HT29, and HCT116 cell lines with stable knockdown of ACLY or FASN assessed by real-time PCR. CD44 mRNA expression was calculated by the ΔΔCt method (means ± SD of triplicate determinations; * p < 0.05 vs. control).
palmitate synthesis (Fig. 2B). Interestingly, reduced expression of ACLY and FASN was also associated with changes in cellular morphology (Supplementary Fig. S2). We show that inhibition of ACLY and FASN significantly decreases expression of the CD44 protein. A more pronounced inhibition of CD44 was observed in cells with knockdown of FASN compared with that of ACLY (Fig. 2C). Treatment of HT29 cells with C-75, a synthetic inhibitor of enzymatic activity of FASN, also led to a dose-dependent decrease in expression of CD44 (Fig. 2D). Interestingly, upregulation of FASN expression by transient transfection of myristoylated, constitutively active form of Akt1 (myr-Akt1) or Akt2 (myr-Akt2) had an opposite effect and increased expression of CD44 in HT29 cells (Fig. 2E).

To further evaluate the correlation of CD44 expression and FASN, we carried out direct labeling of FASN knockdown HT29 cells with the CD44-FITC antibody. These cells were cultured for 3 weeks before the experiment and showed a mixed
population of cells with differential expression of FASN as confirmed by immunofluorescent staining (data not shown). CD44-FITC–labeled cells were sorted based on the level of CD44 expression. Cells with low expression of CD44 showed concomitant reduction of FASN expression (Fig. 2F).

To elucidate the mechanism for the downregulation of CD44 in ACLY and FASN knockdown cells, we assessed the level of CD44 mRNA in KM20, HT29, and HCT116 cell lines each with stable knockdown of either ACLY or FASN. We observed a slight decrease in CD44 mRNA in HCT116 and KM20, but not in the HT29 cell line (Fig. 2G). It has been previously shown that expression of CD44 can be regulated by posttranscriptional modifications such as palmitoylation (32). To test the possible involvement of this mechanism in the regulation of CD44 expression in CRC cells, we used 2-bromopalmitate (2-BP), a nonmetabolizable palmitate analog, to block the incorporation of palmitate into proteins. HT29 NTC and FASN knockdown cells were treated with either dimethyl sulfoxide (DMSO) or 200 μmol/L of 2-BP, labeled with CD44-FITC antibody, and analyzed by flow cytometry. The analysis conducted a 2-fold decrease in signal intensity on the cell surface in FASN knockdown cells and 2-BP–treated cells versus control HT29 cells (Supplementary Fig. S3A). Consistent with these data, subcellular fractionation revealed that expression of CD44 in the membrane fraction is significantly lower in FASN knockdown and 2-BP–treated cells compared with control HT29 cells (Supplementary Fig. S3B). Together, these data suggest that de novo lipogenesis regulates expression of CD44 at a posttranscriptional level, possibly by altering its palmitoylation.

Inhibition of lipogenic enzymes attenuates c-Met signaling in CRC cells

Recent studies have shown a functional link between expression of FASN and activation of c-Met (33). To test whether a
decrease in expression of lipogenic enzymes affects activity of c-Met in CRC, we first analyzed the expression and activation of c-Met in KM20 and HT29 with stable knockdown of ACLY and FASN. As shown in Fig. 3A, phosphorylation of Tyr 1234/1235 in the c-Met kinase domain is inhibited by knockdown of FASN in KM20 cells and by knockdown of both ACLY and FASN in HT29 cells.

To further confirm that attenuation of c-Met signaling is due to inhibition of lipogenic enzymes, we serum starved HT29 and KM20 cells (control, ACLY, and FASN knockdown) for 24 hours and then treated them with 10 ng/mL of hepatocyte growth factor (HGF), a c-Met receptor ligand, for 20 minutes. Consistent with data from the previous experiment, activation of c-Met by HGF was prevented by knockdown of FASN in both KM20 and HT29 cell lines (Fig. 3B).

To test whether changes in activity of c-MET are CD44 dependent, KM20 and HT29 cells were transiently transfected with 2 different CD44 siRNAs or scrambled siRNA as a control for 48 or 72 hours. F, total internal reflection fluorescence imaging of control and FASN knockdown KM20 and HT29 cells stained for F-actin (green) and either p-paxillin (red) or pFAK (red).
in Fig. 3C, inhibition of CD44 decreases c-MET activity in HT29, but not in KM20 cells.

Together, these findings suggest that upregulation of lipogenesis during cancer progression regulates c-Met signaling.

Expression of FASN regulates CRC cell growth, adhesion, and migration

An important prometastatic characteristic of cancer cells is their ability to grow in an anchorage-independent manner. KM20 and HT29 cells (control and FASN knockdown) were tested for their ability to grow without attaching to a substrate using soft agar assay. In both cell lines, we observed a significant decrease in the number of colonies formed when expression of FASN was inhibited (Fig. 4A).

Tumor cell intravasation and extravasation are important steps in metastasis and require cancer cells to interact and adhere to endothelial cells. In CRC, CD44 is implicated in regulation of adherence of cancer cells to endothelial cells and their subsequent transendothelial migration (34). To test whether inhibition of FASN can affect the ability of CRC cells to attach to endothelial cells, we used control and FASN knockdown KM20 and HT29 cells labeled with Calcein AM. Cells were added atop a monolayer of HMVEC-L preactivated with TGFβ and incubated for 30 minutes. Unattached cells

Figure 6. Inhibition of FASN suppresses primary CRC tumor growth and establishment of liver metastasis. A, KM20 and HT29 cells (1 × 10⁶; NTC or FASN shRNA) were injected subcutaneously into athymic nude mice. Immunoblot analysis for FASN and CD44 in tumor cells before injections shown with tumor volume for days 1 to 27 (5 mice per group). B, tumor weights in control and FASN shRNA groups of KM20 and HT29 xenografts. C, immunoblot analysis for FASN and CD44 in tumor tissues from KM20 and HT29 xenograft (day 27). D, images of GFP-positive cancer cells in athymic nude mice injected intrasplenically with HT29-NTC-Luc/GFP or HT29-FASNshRNA-Luc/GFP cells (abdominal view). Arrow heads indicate primary tumors. E, images of GFP-positive cells in liver. F, representative images of histologic analysis (H&E staining) of liver metastasis. L, normal liver; M, metastasis.
were removed by multiple washes. Data showed that down-regulation of FASN in KM20 and HT29 cells impairs their ability to attach to endothelial cells (Fig. 4B).

Enhanced cell migration is associated with a metastatic phenotype. The results from the Transwell migration assay showed that inhibition of FASN impairs migration in both KM20 and HT29 cell lines (Fig. 4C). Collectively, these findings suggest that de novo lipogenesis plays a significant functional role in progression of CRC.

**Inhibition of lipogenic enzymes alters the signaling network that regulates adhesion and motility in CRC**

The cooperation between CD44 and c-Met at the plasma membrane and their dynamic interactions with multiple downstream molecules leads to activation of the signaling network that regulates cell growth, adhesion, and migration (16). Activation of tyrosine kinase Src by c-Met contributes to the metastatic potential of CRC cells via regulation of the actin cytoskeleton and modulation of the formation of adhesive structures (35). Furthermore, expression of CD44 and activation of c-MET promote phosphorylation of focal adhesion kinase (FAK) and promote cell motility (36). Interestingly, cooperation of Src and FAK kinases leads to phosphorylation of paxillin and also contributes to stimulation of cell motility (37; Fig. 5A). Our data show that inhibition of expression of ACLY and FASN in KM20 and HT29 decreases phosphorylation of Src (Fig. 5B). Because phosphorylation of Src is associated with downstream activation of FAK and paxillin, we examined expression of these proteins in HT29 and KM20 with stable knockdown of either ACLY or FASN. Phosphorylation of paxillin on Tyr 118 was inhibited only by knockdown of FASN in both KM20 and HT29 cell lines (Fig. 5C). Interestingly, the effect of FASN knockdown on phosphorylation of FAK was more prominent than the effect of ACLY knockdown (Fig. 5C). Consistent with several studies showing the connection between upregulation of lipogenesis and activation of Akt (7, 8), we observed a decrease in phosphorylation of Akt in both KM20 and HT29 cell lines with stable knockdown of either ACLY or FASN (Fig. 5D). Furthermore, CD44-dependent expression of RhoA induces formation of stress fibers and is required for focal adhesion formation (38). Our data show a significant decrease in expression of RhoA in ACLY and FASN knockdown KM20 and HT29 cell lines (Fig. 5D).

To test whether these changes are CD44 dependent, KM20 and HT29 cells were transiently transfected with CD44 siRNAs or control siRNA and expression/activity of aforementioned proteins were assessed. As shown in Fig. 5E, inhibition of CD44 significantly decreases expression of RhoA and activity of Akt in both cell lines. Interestingly, knockdown of CD44 decreased activity of Src and FAK only in HT29 cells and did not affect activity of paxillin in either cell line (Fig. 5E). Actin cytoskeleton plays an important role in regulation of cell growth, adhesion, motility, and invasion. Considering that activation of CD44/c-Met and Src affects cell motility by regulating actin polymerization, depolymerization, and stress fiber formation, we carried out immunofluorescence staining for F-actin. Phalloidin staining showed significant changes in organization of actin cytoskeleton in cells with FASN knock-down compared with control cells (Fig. 5E). Furthermore, in agreement with Western blot analysis, immunofluorescent staining of control and FASN knockdown cells for phospho-FAK and phospho-paxillin showed a significant decrease in number of focal adhesions in FASN knockdown KM20 and HT29 cells. Our findings suggest that inhibition of lipogenesis in CRC cells induces molecular changes and reorganization of actin cytoskeleton, which are consistent with a less motile and invasive phenotype of cancer cells.

**Inhibition of FASN decreases tumor growth and attenuates CRC liver metastasis**

It has been previously shown that CRC xenografts treated with C-75 exhibit inhibition of tumor growth (7). To test whether molecular inhibition of FASN expression affects tumor growth *in vivo*, we injected control or FASN knockdown cells subcutaneously into athymic nude mice. Tumor growth was monitored by measurement of tumor volume (Fig. 6A). Mice were sacrificed at day 27 after the inoculation, and tumor weight was recorded (Fig. 6B). We observed a slower rate of tumor growth and smaller tumor size in the FASN-treated mice versus control (*P* = 0.002 for KM20; *P* = NS for HT29). Expression of FASN and CD44 in tumors from control and FASN knockdown groups was assessed by Western blot and showed partial recovery of expression of both proteins at the end of the experiment (Fig. 6C). The liver is the most common site for CRC metastasis, and intrasplenic injections recapitulate the process of liver metastasis. We injected GFP-labeled control or FASN knockdown HT29 cells into the spleens of athymic nude mice and assessed the presence of liver metastasis by GFP imaging and immunohistochemical analysis at 6 weeks after injection. Primary tumors were observed in 100% of mice in the control group versus 40% of mice in the FASN knockdown group (Fig. 6D). Liver metastases were observed in 80% of the mice injected with control HT29 cells, whereas complete absence of liver metastasis was observed in mice injected with FASN knockdown cells (Fig. 6D–E). Presence or absence of liver metastasis was confirmed by hematoxylin and eosin (H&E) staining (Fig. 6F). Data from these experiments provide support for the role of lipogenesis in tumor growth and potentially in the metastasis of CRC.

**Inhibition of FASN attenuates establishment of CRC metastasis**

To further investigate the role *de novo* lipogenesis plays in metastasis, we examined the effect of FASN inhibition in an experimental metastasis model *in vivo*. Luciferase and GFP-labeled HT29 and KM20 cells with stable knockdown of FASN were injected i.v. into athymic nude mice, and formation of systemic metastasis was assessed by bioluminescent and fluorescent imaging. All mice in the HT29 control group showed metastatic nodules (Fig. 7A). The distribution of values for total luciferase signal per animal is shown in Fig. 7B. Luciferase signal was associated with metastatic nodules on the backs of the animals and metastasis to the mesenteric lymph nodes. In contrast, none of the metastatic nodules were formed in the HT29 FASN knockdown group. However,
histologic examination of lungs showed the presence of pulmonary micrometastasis in both the control and FASN knockdown groups (Fig. 7C).

Similar results were obtained when we used another metastatic CRC cell line, KM20. Systemic metastases were noted in 80% of mice from the control group; however, knockdown of FASN completely abolished establishment of metastasis as depicted by luciferase imaging and quantification of total luciferase signal per mouse (Fig. 7D and E). Surprisingly, in contrast to HT29-inoculated mice, histologic examination showed the presence of pulmonary micrometastasis in 60% of mice in the KM20 control group and complete absence of micrometastasis in the group injected with FASN knockdown KM20 cells (Fig. 7F). The presence of pulmonary metastasis in the control group was also visualized with GFP imaging (Supplementary Fig. S4). Taken together, these findings suggest that overexpression of FASN may play an important role in establishment of CRC metastasis in vivo.

**Discussion**

In this study, we investigated the role of de novo lipogenesis in CRC metastases. Although, multiple studies have focused on the contribution of aberrant expression of lipogenic enzymes to growth and survival of cancer cells, their role in metastasis remains unknown (39, 40). Here, we show an increase in expression of FASN with advancing stages of CRC, suggesting that FASN may play an important role in the progression of CRC to metastatic disease. Furthermore, this study shows that inhibition of lipogenic enzymes decreases expression of CD44 and attenuates the CD44-associated signaling, thus establishing the novel link between lipid biosynthesis and metastasis. Finally, we show that targeted inhibition of FASN decreases the tumorigenic potential of CRC cells in vitro and attenuates establishment of metastasis in 2 experimental metastasis models in vivo.

Increased expression of CD44 correlates with metastasis in many cancers (16). We showed that changes in CD44 in CRC cells with inhibited expression of lipogenic enzymes are not due to transcriptional regulation, but possibly to posttranscriptional modifications of protein. CD44 undergoes a sequential proteolytic cleavage and releases CD44 intracellular domain, which acts as a transcriptional factor and regulates expression of its own gene (36). This mechanism can explain a slight decrease in CD44 mRNA noted in KM20 and HCT116 cells when lipogenic enzymes are inhibited. Expression of CD44 is dramatically higher in HT29 cells, so it is possible that CD44 is not dependent on self-regulation.

C-Met is implicated in promotion of metastasis (19). Consistent with studies of diffuse large B-cell lymphoma and prostate cancer, showing the link between inhibition of FASN and decreased phosphorylation of c-Met (33, 41), our data show that inhibition of lipogenic enzymes decreases endogenous activation of c-Met and prevents its activation by HGF in CRC cells. Interestingly, CD44 is required for c-Met activation and signaling in several cancers (20, 42). Consistently, we showed that decreased phosphorylation of c-Met in FASN-depleted cells is CD44 dependent in HT29 cells. The differential effect of CD44 inhibition on c-Met activity in KM20 and HT29 cells can be explained by a significantly higher level of CD44 expression and c-MET activation in HT29 cells.

Overexpression of FASN in breast cancer cells increased the size and number of colonies in soft agar (43). In agreement with this study, we show that inhibition of FASN leads to a significant decrease in the ability of CRC cells to grow in an anchorage-independent manner. In addition, we show that altered lipogenesis regulates attachment of CRC cells to endothelial cells. Suppressed migration is associated with reduced activity of FASN in ovarian carcinoma cells (14). Consistently, we also observed a significant decrease in migration of both KM20 and HT29 cell lines when expression of FASN is inhibited. Considering that suppression of lipogenic enzymes inhibits expression of CD44 and decreases activation of c-Met, these functional changes can be explained by alterations in the signaling network downstream of these 2 proteins. Our data show that KM20 and HT29 cells, with inhibited expression of FASN, exhibit significant suppression of Src, FAK, and paxillin activation, the proteins which are downstream of the CD44/c-Met complex and implicated in regulation of adhesion dynamics, migration, and invasion of cancer cells (44). Indeed, the level of FAK receptors and their phosphorylation has been shown to increase during the adenoma to carcinoma transition of colonic epithelia (45). Furthermore, activation of Src contributes to anoikis resistance in CRC cells through activation of phosphatidylinositol 3-kinase (PI3K)/Akt pathway and phosphorylation of Akt (46). In addition, overexpression of FASN has been associated with activation of Akt in human CRC tissue samples (7). Consistent with these findings, we also observed a CD44-dependent decrease in activation of Akt in HT29 and KM20 cells with inhibited expression of lipogenic enzymes. Interestingly, inhibition of CD44 attenuates activation of Src and FAK in HT29, but not in the KM20 cell line, suggesting that the effect of inhibition of lipogenic enzymes on downstream signaling may be mediated by altered composition of lipid rafts within the plasma membrane and impairment of activation/function of membrane-associated receptors or oncoproteins other than CD44 and c-MET (47).

Inhibition of lipogenic enzymes decreases cell growth in several types of cancers (40). Furthermore, FASN inhibition with C-75 slows growth of CRC xenografts (7). Our findings are consistent with these studies and show that inhibition of lipogenesis attenuates the rate of tumor growth and decreases tumor size in both KM20 and HT29 xenografts. Interestingly, we observed the partial recovery of FASN expression and a subsequent increase in expression of CD44 in xenografts, supporting the importance of lipogenesis for cancer cell survival and growth.

Implantation of cancer cells into the spleens of nude mice results in primary tumor growth and establishment of hepatic metastases (24). The liver metastasis model primarily evaluates late stages of metastasis and recapitulates the pattern of colorectal metastasis in humans (24). Using this model, we showed that despite detection of primary tumors in both control and FASN knockdown groups, inhibition of FASN prevented formation of liver metastasis. Furthermore,
results from the experimental metastasis model, carried out with both KM20 and HT29 cell lines, showed that inhibition of FASN expression blocked establishment of metastatic nodules in nude mice. The control mice developed multiple metastatic nodules, predominantly located on the upper back and mesenteric lymph nodes. This pattern of tumor formation is similar to what was reported in a previous publication by our laboratory (21). Surprisingly, gross examination of the lungs showed a different effect of the down-regulation of FASN on establishment of microscopic nodules. Knockdown of FASN in KM20 cells prevented formation of micrometastasis in the lungs; however, its inhibition in the HT29 cells did not affect formation of tumor nodules. This discrepancy can likely be explained by the different genetic backgrounds of HT29 and KM20 cell lines. It is possible that downregulation of FASN has different consequences on modulation of signaling pathways and metastatic potential in these cell lines. Furthermore, in the experimental metastasis model, colonization of cells in the lungs does not recapitulate all steps of the metastatic cascade, and formation of secondary metastatic lesions, which were depicted by the bioluminescent imaging in our experiment, provides a better model for evaluation of the metastatic potential of cancer cells. Taken together, data from our in vivo experiments suggest that downregulation of lipogenesis in cancer cells has a more prominent effect on metastasis than on the growth of primary tumors.

Due to the strong correlation between de novo lipid biosynthesis and the malignant phenotype, lipogenic enzymes have become an attractive target for therapeutic intervention. In particular, FASN, due to its structure and tumor-specific expression, represents a unique target for cancer drug discovery (48). Despite, multiple publications clearly showing the role of FASN in cancer cell growth and survival, our study is the first to provide insight into the contribution of aberrant de novo lipogenesis progression and metastasis of CRC suggesting that...
inhibition of FASN may be a potential therapeutic strategy for advanced stages of CRC. We also show the link between expression of lipogenic enzymes and expression of CD44, the protein implicated in establishing of metastasis in many cancers including CRC. Humanized CD44 antibodies have shown promising results in clinical trials, but those trials were terminated due to skin-related toxicities (49, 50). The findings presented in this study open the possibility of targeting CD44 through inhibition of lipogenesis, thus avoiding antibody-associated toxicities.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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