Control of Cell Growth and Survival by Enzymes of the Fatty Acid Synthesis Pathway in HCT-116 Colon Cancer Cells

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Abstract

Purpose: For many tumor cells, *de novo* lipogenesis is a requirement for growth and survival. A considerable body of work suggests that inhibition of this pathway may be a powerful approach to antineoplastic therapy. It has recently been shown that inhibition of various steps in the lipogenic pathway individually can induce apoptosis or loss of viability in tumor cells. However, it is not clear whether quantitative differences exist in the ability of lipogenic enzymes to control tumor cell survival. We present a systematic approach that allows for a direct comparison of the control of lipogenic pathway enzymes over tumor cell growth and apoptosis using different cancer cells.

Experimental Design: RNA interference-mediated, graded down-regulation of fatty acid synthase (FAS) pathway enzymes was employed in combination with measurements of lipogenesis, apoptosis, and cell growth.

Results: In applying RNA interference titrations to two lipogenic enzymes, acetyl-CoA carboxylase 1 (ACC1) and FAS, we show that ACC1 and FAS both significantly control cell growth and apoptosis in HCT-116 cells. These results also extend to PC-3 and A2780 cancer cells.

Conclusions: Control of tumor cell survival by different steps in *de novo* lipogenesis can be quantified. Because ACC1 and FAS both significantly control tumor cell growth and apoptosis, we propose that pharmacologic inhibitors of either enzyme might be useful agents in targeting cancer cells that critically rely on fatty acid synthesis. The experimental approach described here may be extended to other targets or disease-relevant pathways to identify steps suitable for therapeutic intervention.

Fatty acid synthase (FAS) is a multidomain protein that catalyzes the reductive biosynthesis of saturated long-chain fatty acids from acetyl-CoA and malonyl-CoA in the cytosol (1, 2). Many solid tumors and cell lines derived from these tumors overexpress FAS (3, 4) and have been shown to rely on the enzyme for survival and proliferation. Thus, inhibition of FAS by pharmacologic agents or by genetic means in tumor cells is cytotoxic, causing reduced proliferation rates and induction of apoptosis (5–14). These characteristics extend to tumor cells grown in cell culture. In contrast, FAS expression is usually low in normal cells *in vivo* due to transcriptional control exerted by the presence of fatty acids in the circulation derived from the diet. Nontransformed cells are generally unaffected by FAS knockdown or pharmacologic inhibition and are not dependent on *de novo* lipogenesis for growth and proliferation (15–19). Importantly, animals generally tolerate systemically administered FAS inhibitors (11, 20, 21). Taken together, these findings raise the possibility that FAS inhibitors may provide well-tolerated novel cancer therapies (3).

The fatty acid synthesis pathway has multiple entry and exit points for various metabolites and cofactors such as malonyl-CoA or NADPH. Therefore, interfering with the pathway at a particular step will change the balance of pathway intermediates (which might affect cell growth and survival) in a way unique to that step. Based on experiments with pharmacologic inhibitors of the FAS pathway enzymes acetyl-CoA carboxylase 1 (ACC1) and FAS, it has been proposed that inhibition at the level of FAS is an effective way of inducing apoptosis in cancer cells, whereas inhibition of ACC1 would be ineffective. It was suggested that buildup of malonyl-CoA following FAS inhibition is critical for inducing apoptosis in tumor cells (22–24). However, experiments in cell culture have since shown that apoptosis can also be induced when other enzymes of the lipogenic pathway are knocked down using small interfering RNA (siRNA; ref. 4), such as cytosolic ACC1 (19, 25) or ATP citrate lyase (26, 27). The most straightforward interpretation for these findings is that one or several pathway intermediates

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or products downstream to all these reactions (such as the NADPH/NADP ratio, or palmitate, the predominant product of the lipogenic pathway) are critical to maintaining tumor cell survival. Of interest, palmitate supplementation has been used in several studies that examine the role of the FAS pathway in cancer cells by inhibiting the pathway (19, 25, 28). However, palmitate supplementation is not able to rescue all cell types from FAS pathway inhibition-mediated toxicity (25). This finding points to a potentially complicated picture as to how the enzymes of the lipogenic pathway, as well as the various pathway intermediates, are involved in maintaining viability and survival in cancer cells.

A key question from the standpoint of targeting the pathway for antineoplastic therapy is how much the different pathway enzymes (including ATP citrate lyase, ACC1, and FAS) each control tumor cell survival. These enzymes are in principle druggable and several classes of inhibitors have been published in particular for ACC1 and FAS (3, 29, 30). Here, we did a direct comparison of the two pathway enzymes, ACC1 and FAS, with respect to their ability to control tumor cell growth and survival in HCT-116 colon cancer cells. Using RNA interference-mediated graded knockdown, we derive quantitative measures of control of FAS and ACC1 over lipogenesis, apoptosis, and cell growth. Our results show that the two enzymes both have significant control over these variables, suggesting that pharmacologic inhibitors of either enzyme might be useful agents in targeting cancer cells that critically rely on fatty acid synthesis.

Materials and Methods

Cell culture. Cell lines were obtained from the American Type Culture Collection and cultured in either McCoy's 5A medium w/L-glut (HCT-116 colon cancer cells), high-glucose DMEM (A2780 ovarian cancer cells) or DMEM/Ham's F-12 mix (PC-3 prostate cancer cells) essentially as described by the American Type Culture Collection.

Small interfering RNA. siRNAs were either designed at Rosetta Inpharmatics or as published previously (25) and purchased from Sigma Proligo. Sequences were as follows: FAS 5′-GCAUCAAUGUCCUGCUGAATT-3′ and 5′-GCAUGGCUAUCUUCCUGAATT-3′, ACC1 5′-CAAUGGCAUUGCAGCAGUGTT-3′ and 5′-AGAAUUAAUUCCACAUUUGTT-3′, and luciferase 5′-CGUACGCGGAAUACUUCGATT-3′. All sequences were submitted to a BLAST search to ensure specificity. Transfection efficiency was >99% of cells as estimated by using a FITC-labeled version of the luciferase control siRNA (Proligo). For knockdown experiments, cells were seeded onto six-well plates at a density of 65,000 per well and allowed to adhere overnight. The next day, the cells were transfected with either siRNA oligonucleotides for FAS, ACC1, or luciferase as a control using Xtreme gene transfection reagent (Roche). Cells were re-fed with growth medium 18 h after transfection. To obtain a titration of FAS and ACC1 knockdown, two sequences per gene at two concentrations (30 and 60 nmol/L) were used for transfections. One sequence for luciferase was used at both 30 and 60 nmol/L as controls. Within this concentration range, no effects were seen on cell growth (number of viable cells assessed at 72 h after transfection with the luciferase control siRNA). Cells were processed for mRNA expression 24 h after transfection. Protein expression, lipid biosynthesis, tumor cell growth, apoptosis, and cell cycle analysis was evaluated 72 h after transfection.

Transient knockdown of FAS and ACC1 in HCT-116, PC-3, and A2780 cells. Three short hairpin RNA (shRNA) vector constructs for each gene (FAS and ACC1) were used. The 19mer shRNA stems were chosen by use of a standard siRNA selection algorithm. The shRNA sequences were expressed from a mouse U6 promoter engineered into the pDONR backbone (Invitrogen). Sequences were as follows:

FAS_shRNA1:
GCTGAGGAAGGAGGGTGTGTTCAAGAGACACACCCTCCTTCCTCAGC (19mer target sequence: GCTGAGGAAGGAGGGTGTG);
FAS_shRNA2:
GCCCAACACCTCCGTGCAGTTCAAGAGACTGCACGGAGGTGTTGGGC (19mer target sequence: GCCCAACACCTCCGTGCAG);
FAS_shRNA3:
GGGAAGCACATTGGCAAAGTTCAAGAGACTTTGCCAATGTGCTTCCC (19mer target sequence: GGGAAGCACATTGGCAAAG);
ACC1_shRNA1:
GTCCTTCCTGCTCATACACTTCAAGAGAGTGTATGAGCAGGAAGGAC (19mer target sequence: GTCCTTCCTGCTCATACAC);

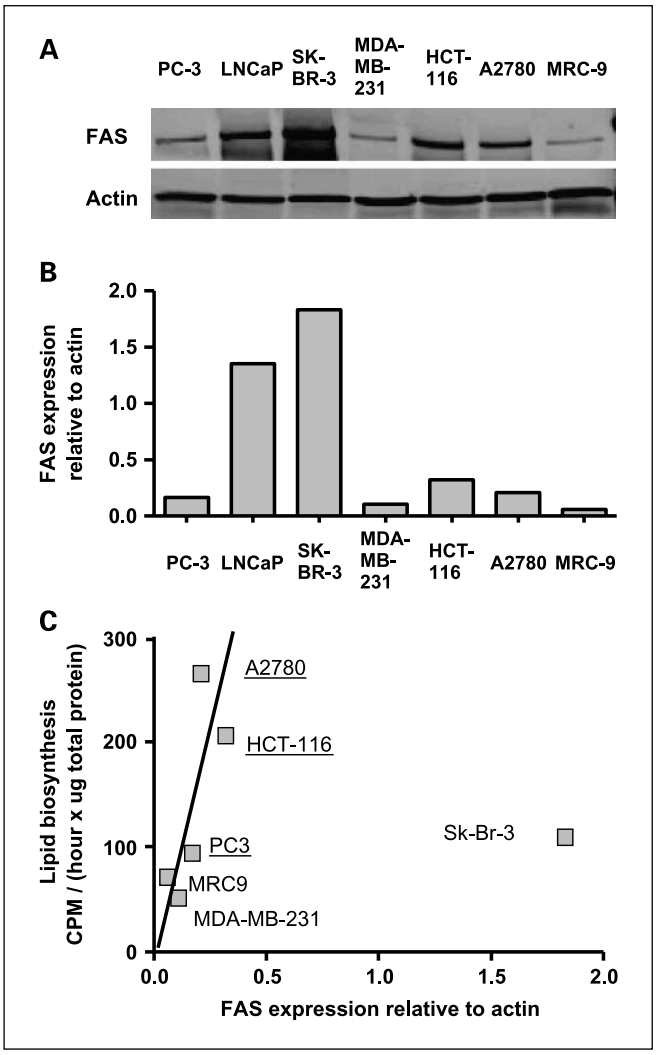

Fig. 1. Cell line selection for siRNA experiments. *A,* representative Western blot showing FAS protein expression (20 μg protein/lane) in various tumor cell lines [prostate (PC-3 and LNCaP), breast (SK-BR-3 and MDA-MB-231), colon (HCT-116), and ovary (A2780)] and a nontransformed fibroblast line (MRC-9). *B*, FAS content was quantified relative to actin expression levels in the panel of cell lines shown in *A. C,* lipogenic activity was measured using a [^{14}C]acetate incorporation assay, and lipogenic activity of tumor cells was plotted versus FAS expression levels. For most cells, FAS pathway activity correlates well with FAS expression levels, indicating that graded knockdown of FAS protein is likely to yield corresponding decreases in lipid biosynthesis.

Table 1. Cell line selection criteria

Cell line	Tissue of origin	FAS expression levels	Gradual FAS knockdown predicted to affect FAS pathway activity	Context of FAS expression and pathway activity
HCT-116	Colon	Medium	Yes; very amenable to transfection	Mutant ras; PI3K/mitogen-activated protein kinase inhibitors reduce FAS expression and lipid biosynthesis
A2780	Ovary	Medium	Yes; very amenable to transfection	Mutant ras, mitogen-activated protein kinase family members, and PTEN; sensitive to Akt inhibition
PC-3	Prostate	Medium-low	Yes	PTEN (-/-); sensitive to Akt inhibition or PTEN expression
LNCaP	Prostate	High	Possibly, but difficult to transfect and slow growing	PTEN (-/-); PI3K pathway activated
Sk-Br-3	Breast	Very high	No	High HER-2 expression; sensitive to pharmacologic FAS inhibition (12)
MDA-MB-231	Breast	Low	Yes	Not very sensitive to FAS inhibition (low HER-2/*neu* expression)

ACC1_shRNA2:
GTTCCTGGATCTCCCATATTTCAAGAGAATATGGGAGATCCA-GGAAC (19mer target sequence: GTTCCTGGATCTCCCATAT), and ACC1_shRNA3:
GTGACAGACTACAGGTTCTTTCAAGAGAAGAACCTGTAGTCT-GTCAC (19mer target sequence: GTGACAGACTACAGGTTCT).

Cells were seeded into 12-well plates and incubated overnight to reach about 50% confluence the next day. Transient transfection was done on day 2 using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions (two to three transfections per hairpin plus scramble control). Cells were then incubated overnight and the medium was changed to standard medium as follows: DMEM/Ham's F-12 (PC-3), DMEM high-glucose (A2780), and McCoy's 5A w/L-glut (HCT-116). After 48 - 72 h, proliferation was assessed and Western blotting was done on cell extracts to quantitatively determine FAS and ACC1 protein levels (see sections below).

RNA expression. HCT-116 cells were processed for mRNA expression 24 h after transfection with siRNA oligonucleotides. Cells were washed with PBS and spun through a QiaShredder (Qiagen) for disruption. RNA was extracted using RNeasy kits (Qiagen) including DNase treatment and converted to cDNA with a high-capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative reverse transcription-PCR for FAS and ACC mRNA expression was done using TaqMan gene expression probes Hs_00188012_m1 and Hs_201046076_m1, respectively (Applied Biosystems). β-Actin was used as an endogenous control for normalization.

Lipid biosynthesis. Seventy-two hours after transfection of cells with siRNA, 1 μCi of 55 mCi/mmol [2-^{14}C]acetic acid (GE Healthcare) was added per six wells for 2 h. Cells were washed with PBS, scraped into 2% KOH, and incubated overnight at 80°C to saponify lipids. Sterols were extracted by adding hexanes and spinning for 5 min at 600 × *g*. The organic phase containing sterols was discarded. To the remaining aqueous phase 2 N H_2SO_4 was added to acidify lipids. The acidified fatty acids were extracted with hexanes and spun for 5 min at 600 × *g*. The organic phase containing fatty acids was dried at room temperature and counted on a scintillation counter. The data was normalized to total cell number.

Western blot analysis. Following siRNA transfections as described above, cells were washed with cold PBS and lysed with radioimmunoprecipitation buffer (Upstate) containing a protease inhibitor cocktail (Roche). Protein concentrations were determined using BCA Kit (Pierce) and 30 μg total protein separated on 4% to 20% SDS-PAGE gels (Bio-Rad criterion system). After transferring to nitrocellulose, the membrane was probed for FAS (Transduction Labs) or ACC1 (using a custom-made polyclonal anti-ACC1 antibody) and actin (Sigma), incubated with Cy5-labeled secondary antibodies (Zymed), and imaged using the Typhoon imaging system (GE Healthcare). To measure protein expression following shRNA-mediated knockdown, a different secondary antibody (anti-mouse IgG, horseradish peroxidase secondary antibody; GE Healthcare) and detection kit (Amersham ECL Plus) were used, and protein bands were quantitated using Image Quant software (GE Healthcare).

Cell counts, apoptosis, and sub-G_1 analysis. Cell counts and apoptosis were assessed 72 h after transfection. Viable cell counts were determined by trypan blue exclusion using a Vi-Cell analyzer (Beckman Coulter). Results were expressed as percentage of matched luciferase controls. DNA fragmentation and cell cycle analysis were carried out on trypsinized cells, fixed in 1% paraformaldehyde on ice for 1 h, which were subsequently washed in PBS, and resuspended in 70% ethanol. Fixed cells were first stained for DNA fragmentation by incubating with FITC-dUTP and the enzyme terminal deoxynucleotidyl transferase for 2 h at 37°C followed by a 15 min incubation with propidium iodide at room temperature for cell cycle analysis. Fluorescence-activated cell sorting analysis was done for both DNA fragmentation and cell cycle status simultaneously (BD Biosciences, Apo-direct kit and FACSCalibur flow cytometer).

Regression analysis. Curve-fitting and linear regression analysis (including determination of 95% confidence intervals and SE of slopes) was done using the GraphPad Prism software package.

Results

Cell line selection for siRNA titrations. To select an appropriate cell line for our siRNA studies, we applied the following criteria: (*a*) the cell line should express significantly elevated FAS protein levels relative to nontransformed cells. (*b*) FAS protein expression should approximately correlate with activity; in other words, gradual knockdown of FAS (or other FAS pathway enzymes) should be expected to yield an appreciable and correlated decrease in lipogenesis. (*c*) There is evidence of an oncogenic pathway driving FAS expression (FAS expression is not an artifact of merely culturing the cells at the high-glucose concentrations usually present in cell culture medium). (*d*) Transfection efficiency should be high (see Materials and Methods).

As shown in Fig. 1A to C and Table 1, several cell lines that we studied fit these criteria. In particular, the HCT-116 colon cancer cell line has significantly elevated levels of both FAS expression and FAS activity compared with nontransformed MRC-9 fibroblasts (Fig. 1A-C). HCT-116 cells also cluster among cell lines that show lipid biosynthesis approximately commensurate

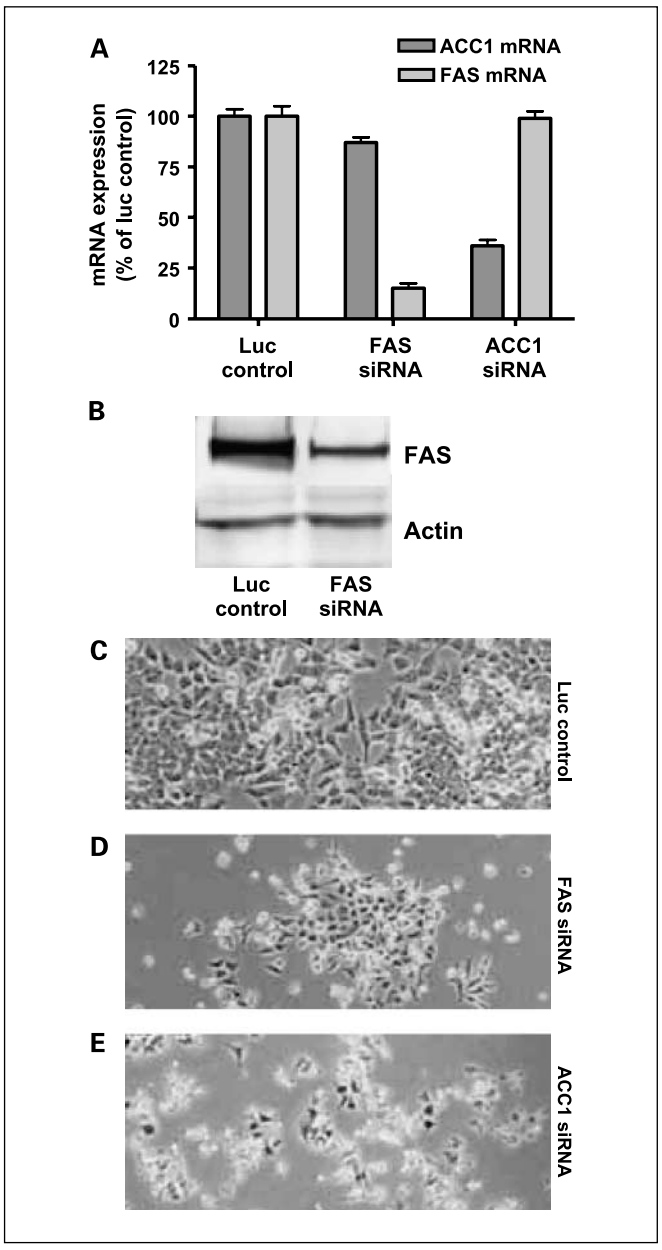

Fig. 2. Knockdown of FAS or ACC1 in HCT-116 cells using a single concentration of siRNA oligonucleotides (60 nmol/L). Representative results. *A,* effect of FAS or ACC1 knockdown on mRNA expression. *Bars,* range ($n = 2$). *B,* Western blot analysis for FAS siRNA shows a corresponding decrease in protein expression. FAS or ACC1 siRNAs, but not luciferase control siRNA, result in reduced cell growth, significant cell death, and detachment from the well by visual inspection (*C-E*). mRNA expression was measured at 24 h post-transfection. Protein expression and images were analyzed at 72 h post-transfection.

with their endogenous FAS expression level (Fig. 1C), unlike, for example, SK-BR-3 breast cancer cells, which have very high FAS levels but only moderate FAS pathway activity compared with some of the other cells tested. Importantly, FAS expression and activity in HCT-116 cells are driven by abnormal activation of both phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase signaling as a consequence of a mutation in codon 13 of the K-ras protooncogene (31, 32). In this context, inhibitors of PI3K and mitogen-activated protein kinase signaling reduce both SREBP-1 transcription factor levels and FAS transcription (33).

PC-3 and A2780 cells similarly fit the above mentioned criteria. There is strong experimental evidence for oncogenic activation of pathways that directly up-regulate FAS expression and activity. In PC-3 cells, FAS levels are abnormally elevated due to a loss of function of the tumor suppressor PTEN, which in turn activates PI3K/Akt signaling and, consequently, increases FAS expression (34). A2780 cells carry mutations in ras and mitogen-activated protein kinase family members (35, 36) and feature PTEN mutations as well as loss of one PTEN allele (37). They are also sensitive to inhibition of the PI3K/Akt pathway (38), a known regulator FAS expression and activity. Thus, to further extend and validate the results achieved with siRNA titrations, we also studied HCT-116, A2780, and PC-3 cells using shRNA-mediated knockdown as an alternative RNA interference approach.

Qualitative effects of FAS and ACC1 knockdown in HCT-116 cells. We first confirmed that transfection of cells with FAS and ACC1 siRNA reduced both expression of FAS and ACC1 and *de novo* lipogenesis. Dose-response experiments using targeting and nontargeting (luciferase) siRNA sequences showed that siRNA concentrations up to 60 nmol/L were well tolerated with

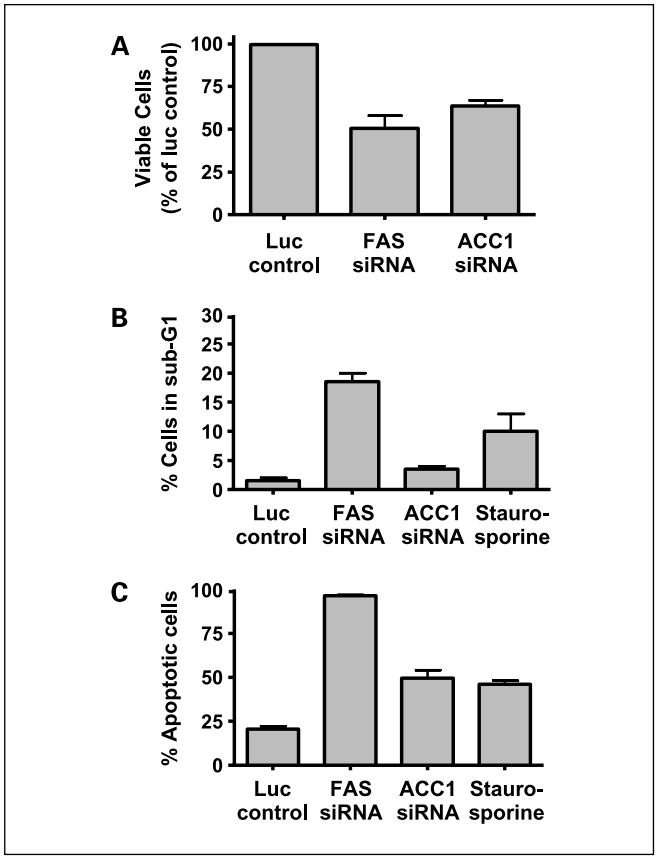

Fig. 3. Quantitation of functional effects of FAS or ACC1 knockdown in HCT-116 cells using a single siRNA concentration of oligonucleotides (60 nmol/L). FAS or ACC1 knockdown affects cell growth (assessed by counting viable cells using trypan blue exclusion; *A*), number of cells in sub-G_1 phase (measured by propidium iodide staining; *B*), and levels of apoptosis (determined by flow cytometric analysis of DNA fragmentation; *C*). All data points were collected at 72 h post-transfection. Staurosporine was used as a positive control. Representative results from experiments done in duplicate. *Bars,* range.

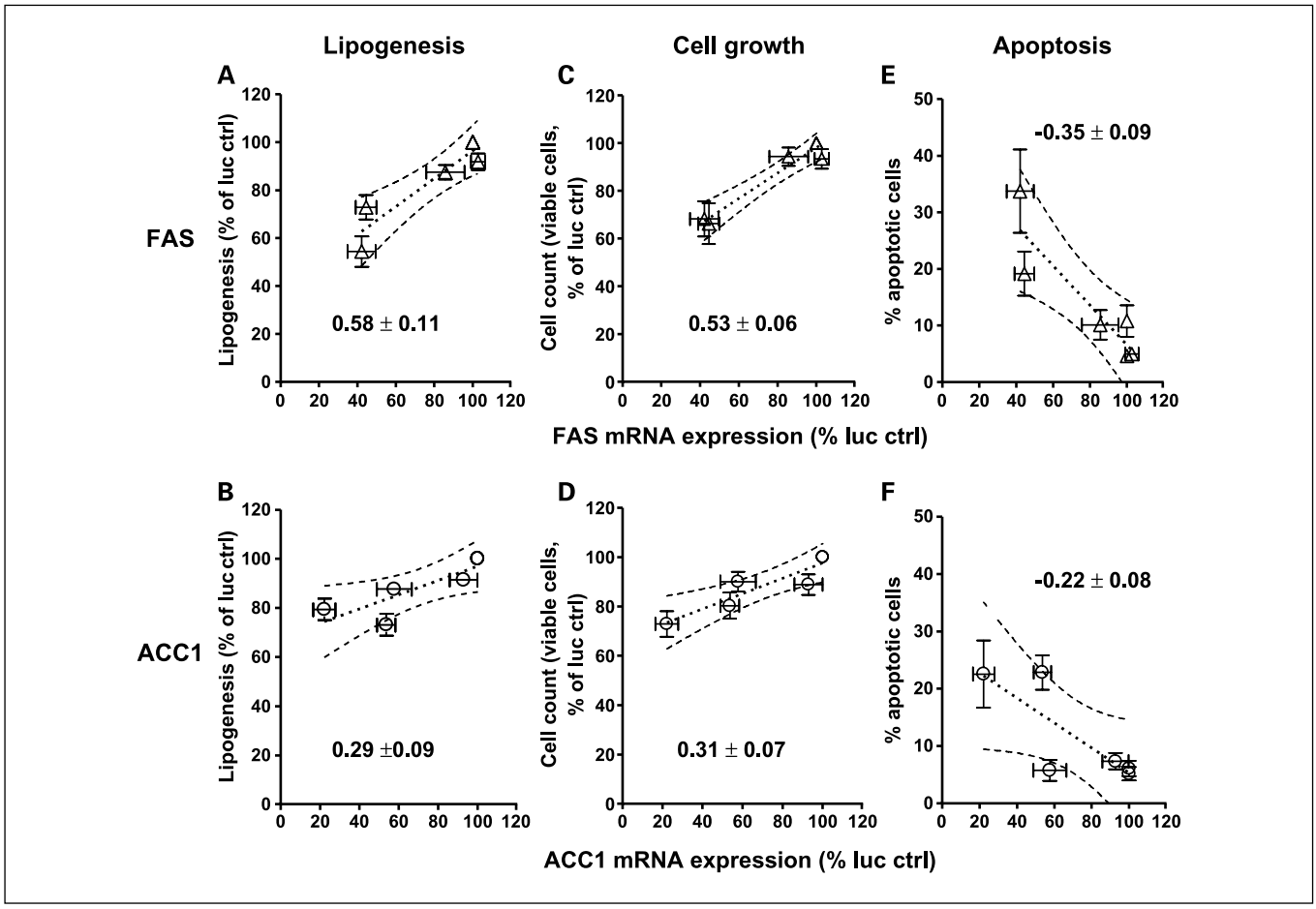

Fig. 4. Lipogenesis, cell growth, and apoptosis as a function of FAS and ACC1 expression in HCT-116 cells. Gradual knockdown of FAS (*A, C*, and *E*) or ACC1 (*B, D*, and *F*) using siRNA titrations affect lipogenesis, cell growth, and apoptosis in HCT-116 cells. Two different oligonucleotide sequences at two concentrations (30 and 60 nmol/L plus corresponding luciferase control) were used to achieve a range of mRNA expression levels. mRNA expression (relative to actin) was measured at 24 h post-transfection. Lipogenesis, number of viable cells, and apoptosis were measured at 72 h post-transfection. All data were normalized to luciferase control. Lines represent a linear curve fit to the data (n = 4-11). The 95% confidence interval for the regression is shown. Numbers indicate regression slopes ± SE.

no effect on cell growth (data not shown), and this concentration range was chosen for subsequent experiments.

As shown in Fig. 2A, transfection of cells with sequence-specific oligonucleotides targeting FAS and ACC1 at 60 nmol/L reduced mRNA expression of either gene by more than 60% after 24 h. Expression of FAS was not affected by ACC1 knockdown and vice versa (Fig. 2A), in contrast to a previous report (19). In addition, Western blotting done 72 h after transfection confirmed that FAS protein was reduced compared with control (Fig. 2B). Commercially available antibodies for ACC1 quantitatively detected ACC1 levels within a single experiment; however, in contrast to the FAS antibody used here, performance was poor and variable between experiments (data not shown).

Visual inspection of cells following siRNA-mediated knockdown of either FAS or ACC1 showed changes in cell morphology consistent with apoptosis as well as a significant reduction in the number of attached cells in the wells compared with the nontargeting luciferase siRNA control (luc control; Fig. 2C-E). The effect of FAS and ACC1 knockdown on cell survival was further assessed by measuring apoptosis, the number of cells in the sub-G_1 phase of the cell cycle by flow cytometry and by cell counting using trypan blue exclusion (Fig. 3). FAS knockdown tended to reduce cell growth somewhat more strongly when compared with ACC1 knockdown (Fig. 3A). FAS knockdown using a single concentration of siRNA (60 nmol/L) induced apoptosis much more potently that ACC1 knockdown or even staurosporine, a strong inducer of apoptosis through the cell-intrinsic pathway (Fig. 3B and C).

RNA interference titration experiments. The difference in apoptotic response between cells transfected with siRNA targeting either FAS or ACC1 shown in Fig. 3 could be interpreted in two ways: (*a*) either knockdown of ACC1 is per se less detrimental to cells, which would be consistent with earlier work based on pharmacologic inhibitors of FAS and ACC1 (22–24) or, more likely, (*b*) the observed difference was due to biological variation such as knockdown efficiencies of the siRNAs (Fig. 2A) and protein turnover. To facilitate a direct, quantitative comparison of the control exerted by FAS or ACC1 over tumor cell growth and survival, we designed experiments that are independent of these biological variables. The key to this approach was a series of experiments comprising siRNA-mediated knockdown of either FAS or ACC1 expression using different concentrations of siRNA oligonucleotides (much like using enzyme inhibitors). This was followed by correlating cellular response, specifically lipogenesis, number of viable

cells, and apoptosis, to mRNA expression across the range of expression levels for each target. Note that mRNA expression was used as a surrogate for protein expression, because commercially available antibodies against ACC1 in our hands showed variable performance in these particular experiments.

First, using two oligonucleotides per target at different concentrations, we showed that we were able to generate graded knockdown of FAS and ACC1 (Fig. 4A and B). As predicted from FAS protein expression studies (Fig. 1), siRNA-mediated knockdown of FAS and ACC1 produced a stepwise reduction in lipogenic activity that correlated with reduction of FAS and ACC1 gene expression. Knockdown of FAS may have a greater effect on lipogenesis than knockdown of ACC1 based on the observation that the slope for FAS is twice as steep as for ACC1 (Fig. 4A and B). However, more detailed analysis is required to confirm that this difference in slopes is statistically significant. Although the linear curve fit represents an oversimplification (the true relationship between gene expression levels and lipogenesis is likely nonlinear), the results clearly show that both FAS and ACC1 significantly control lipogenesis.

We also measured the numbers of (viable) cells and apoptosis on day 3 following transfection as a function of mRNA expression. As can be seen in Fig. 4C to F, incremental decreases in gene expression following either FAS or ACC1 knockdown led to reduced numbers of viable cells (Fig. 4C and D) and increased apoptosis (Fig. 4E and F). Similar to the lipogenesis plots in Fig. 4A and B, the data are suggestive of FAS knockdown having a greater effect on cell growth and apoptosis. This is because the slopes for FAS are about 50% steeper for ACC1 (Fig. 4C-F). However, additional experiments would be required to confirm this difference. Overall, within the error of the analysis, the data show that both FAS and ACC1 both significantly control cell growth and apoptosis.

The results presented for HCT-116 colon cancer cells may not extend to other cancer types due to known variation between cell types, alternate genetic backgrounds or genetic lesions, and also differential expression patterns of FAS and the two ACC isoforms. To gain further insight into this issue, we evaluated cell growth as a function of FAS and ACC1 protein expression in tumor cells from three different tissues (HCT-116 colon, PC-3 prostate, and A2780 ovarian cancer cells; see Table 1). In a parallel approach to the siRNA titrations, we used transient transfections with three different shRNA to target ACC1 or FAS. In contrast to the siRNA studies, we used a custom-made polyclonal antibody against ACC1 for quantitative ACC1 detection. As can be seen from Fig. 5, knockdown of both ACC1 and FAS decreased cell growth of three cell lines evaluated. In HCT-116 cells, FAS again seemed to control cell growth more strongly than ACC1, whereas in A2780 and PC-3 cells both enzymes had comparable control. In summary, these results confirm the notion that both FAS and ACC1 significantly control tumor cell growth across several tumor cell types.

Discussion

The critical role of *de novo* fatty acid synthesis in maintaining tumor cell viability has been well established in many different tumor cell types (3). However, there is little quantitative understanding to what extent different lipogenic enzymes each control lipogenesis (39, 40), and it is unknown how much lipogenic enzymes each control tumor cell survival. Understanding the distribution of control within a biochemical pathway or network is clearly important: going beyond the obvious considerations of (biochemical) druggability, it provides a rationale for selecting a particular reaction step most suitable for therapeutic intervention.

The behavior of enzymes within networks is not necessarily obvious and in fact often very different from the kinetic behavior observed in isolation *in vitro*. This question lies at the core of Metabolic Control Analysis (see ref. 41 for a review), which provides an experimental method for determining control of enzymes over pathway fluxes, interme-

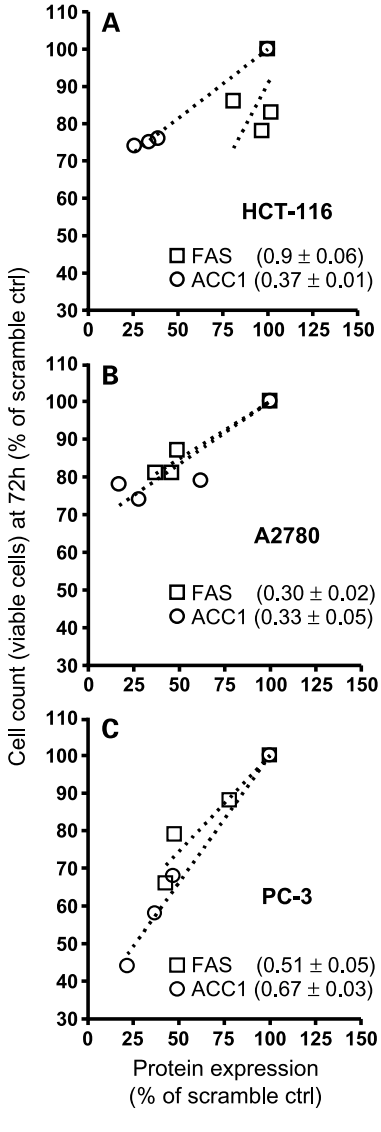

Fig. 5. Effect of FAS and ACC1 knockdown on HCT-116, A2780, and PC-3 cell growth. Gradual knockdown of FAS or ACC1 by shRNA affected cell growth in HCT-116 (*A*), A2780 (*B*), and PC-3 (*C*) cells. Lines indicate a linear curve fit to the data, with each data point representing an average of two to three measurements. Numbers indicate regression slopes ± SE.

diate concentrations, or global variables such as growth and proliferation. Quantitation of control in Metabolic Control Analysis takes the form of control coefficients; a control coefficient of 1 for a step over a variable such as pathway flux or proliferation would indicate that the step has all of the control; a value of 0 indicated no control; and a value between 0 and 1 indicates partial control, shared with other steps. The approach used here to compare two lipogenic enzymes, ACC1 and FAS, with regards to their control over lipogenesis, tumor cell growth, and apoptosis is essentially identical to determining control coefficients as defined by Metabolic Control Analysis (for general review, see ref. 41; for a practical application to the problem of control of tumor cell survival, see ref. 42). Accordingly, control coefficients of FAS and ACC1 over lipogenesis, apoptosis, or cell growth can directly and easily be derived from the slopes of the regression lines shown in Figs. 4 and 5.

From the data shown in Fig. 4A and B, control coefficients of FAS and ACC1 over lipogenesis can be determined as 0.58 $\pm$ 0.11 and 0.29 $\pm$ 0.09, respectively, with errors representing the 95% confidence interval for the slope. Within the error of the analysis, this indicates that the two enzymes actually have significant control over lipogenesis, with FAS possibly exerting even higher control than ACC1. A qualitatively similar result would be obtained regarding the control over cell growth or apoptosis (Fig. 4C-F). Thus, inhibiting either FAS or ACC1 in HCT-116 cells is very effective, suggesting that pharmacologic inhibitors of either enzyme might be useful agents to target cancer cells that critically rely on fatty acid synthesis. Our studies in prostate PC-3 and ovarian A2780 cancer cells show that these results are not limited to HCT-116 colon cancer cells, suggesting that the targets may provide a feasible tumor-targeting strategy across a wider spectrum of tumors.

Under the standard culture conditions used in the present article, HCT-116 cells are not robust against interference with the lipogenic pathway (Figs. 4 and 5): even partial inhibition of *de novo* lipogenesis is sufficient to significantly reduce tumor cell survival. The molecular mechanisms linking inhibition of the lipogenic pathway with induction of cell death in tumor cells remain unclear but are beginning to be unraveled by several groups (see ref. 43 for a succinct review of currently discussed mechanisms). Of note, apoptosis and loss of viability following FAS pathway inhibition occurs in the presence of lipids (bound to serum) in the culture medium. Indeed, it has been shown previously that tumor cells do not readily use external lipids (44), which likely is a major reason for the tumor-specific deleterious effects of FAS pathway inhibition. It is possible, however, that the ability of tumors to use external lipids is context and tissue specific. In contrast to the above, several authors reported that, in cell culture, palmitate supplementation was able to at least partially rescue cells from effects of FAS pathway inhibition (19, 25, 28), which is inconsistent with the notion that tumor cells do not import and use external lipid. In the siRNA experiments reported in this study, we were unable to reproduce consistently a rescue effect by palmitate and even observed toxicity (data not shown). Clearly, the issue of external lipid rescue still awaits full characterization. Given the complexities associated with lipid rescue experiments *in vitro*, and also given the emerging evidence of an alternative pathway of *de novo* fatty acid biosynthesis that might be operating in mitochondria of mammalian cells (45), more work is needed to conclusively show that inhibition of *de novo* lipogenesis can be efficacious in the long-term to ablate tumor growth *in vivo*.

Disclosure of Potential Conflicts of Interest

All authors are employed by Merck Research Laboratories.

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