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13. ABSTRACT ***(Maximum 200 Words)***

The overall goal of this project is to determine the regulatory mechanisms that activate fatty acid (FA) synthetic metabolism during malignant progression of prostate cancer, and to evaluate FA synthesis as a therapeutic target. The biosynthetic enzyme, fatty acid synthase (FAS), is expressed at elevated levels in PCa cells, and is a marker of tumors with activated FA synthesis. While FAS expression is androgen responsive, it persists or is reactivated in tumors after androgen ablation, and is high in 83% of lethal tumors examined at autopsy. Experimental model systems of PCa (*in vitro* and *in vivo*) show similar patterns of FAS activation, with increased FAS during progression to androgen independence. Tumor cell changes in fatty acid metabolism are insensitive to nutritional regulation, and tumor cells produce predominantly membrane lipids. Our experiments have led to the observation that growth factor signals mediated by activated kinases in PCa cells and transformation models activate the fatty acid synthesis pathway via modulation of SREBP-1c transcription factor levels, resulting in the tumor phenotype of activated FA metabolism that is insensitive to nutritional regulation. Small molecules that inhibit FAS are cytotoxic to tumors, and the mechanism of tumor cell killing is under investigation.

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INTRODUCTION: Prostate cancer represents the second leading cause of death from cancer in American men. Elevated expression of the biosynthetic enzyme, fatty acid synthase (FAS), occurs in most biologically aggressive prostate cancers, and corresponds to increased fatty acid (FA) synthetic activity. While FAS expression is androgen responsive, it persists or is reactivated in tumors after androgen ablation. This malignancy-associated cellular function represents a novel therapeutic target. The dependence of prostate cancer on FAS activity is demonstrated in both cell culture and xenograft tumor models: selective inhibition of FAS by anti-metabolite drugs significantly reduces cell growth and survival. These observations lead to the hypothesis that FAS expression/FA synthesis is functionally critical in prostate carcinogenesis and malignant progression, probably through support of cell growth and survival. FAS expression may be upregulated by multiple alternate signaling pathways important for prostate cancer growth. The activity observed in model systems predicts that FA synthesis inhibition will be cytotoxic to all prostate carcinomas that have activated the pathway.

Specific Aim 1: To determine 1] the FAS expression/activity of prostate carcinoma model systems under androgen stimulated, acutely androgen deprived, chronically androgen deprived and androgen independent conditions, 2] the relative contributions of androgen mediated versus androgen independent mechanisms for FA pathway activation under androgen deprived conditions, and the functional significance of insulin-like growth factor stimulation of fatty acid synthase.
Specific Aim 2: To 1] characterize the susceptibility of prostate cancer models to FAS inhibition and 2] optimize the use of FAS inhibitors in preclinical xenograft models for translation to clinical trials.
Specific Aim 3: To characterize the FAS expression/activity of ex-vivo surgically resected primary prostate carcinomas correlated with Gleason grade, stage, and serum levels of androgen, PSA, insulin-like growth factors I and II, and insulin-like growth factor binding protein-3.

BODY: Research Accomplishments of DAMD17-99-1-9580:

Much of our progress has been submitted for publication in 3 papers provided as Appendices A, B and C, and referenced in the body of the report (1-3).

Specific Aim 1:

Task 1. Characterization of hormonal responsiveness of FAS gene and pathway: FAS expression and activity of a panel of androgen independent prostate carcinoma cells are shown in Figure 2 A and D of Appendix A. Changes in FAS enzyme content and pathway activity in response to manipulation androgen levels in LNCaP and LAPC4 cell culture systems are presented in Figure 2 B,C and D of Appendix A.
Basic characterization of FAS expression in hormone responsive xenografted tumors is shown in Figure 2 E,F and G of Appendix A.

Characterization of FAS gene expression and pathway activity under androgen independent conditions:

Our recent data suggest that, in contrast to nutritional regulation of lipogenesis in liver or adipose tissue, tumor cell changes in fatty acid metabolism are driven by increases in growth factor signaling, acting in major part through the MAP kinase and PI 3-kinase signaling cascades. This result provides a likely basis for the linkage of up-regulated fatty acid metabolism with acquisition of androgen independence, and with tumor virulence, since MAP kinase and PI 3-kinase signaling contribute to both (4-9). Considered together with the information in the literature, our recent data suggest a model, that tumors coordinately regulate growth, including metabolic functions like lipogenesis, and the associated functions of membrane synthesis, through growth factor signaling altered by malignant transformation.

These results are presented in Appendices B and C. Appendix B demonstrates that EGF signaling can be altered via introduction of a mutant ras oncogene into non-transformed epithelial cells, resulting in cell transformation, increased MAP kinase and PI 3-kinase signaling, increased SREBP-1 protein levels, and increased lipogenesis. Similar effects occur in cancers of many different organs. In prostate cancer, loss of the PI 3-kinase signaling regulator, PTEN, provides a major source of PI 3-kinase signaling upregulation leading to increased lipogenesis (10).

In Appendix C, the regulatory effects of increased MAP kinase and PI 3-kinase signaling on SREBP-1 transcription factors were confirmed, and the analysis was extended to show that the 1c isoform of SREBP is regulated at the mRNA level by MAP kinase and PI 3-kinase signaling, and coordinately modulates with FAS mRNA levels both in cultured tumor cells and in surgically resected tumor tissues.

Specific Aim 2:

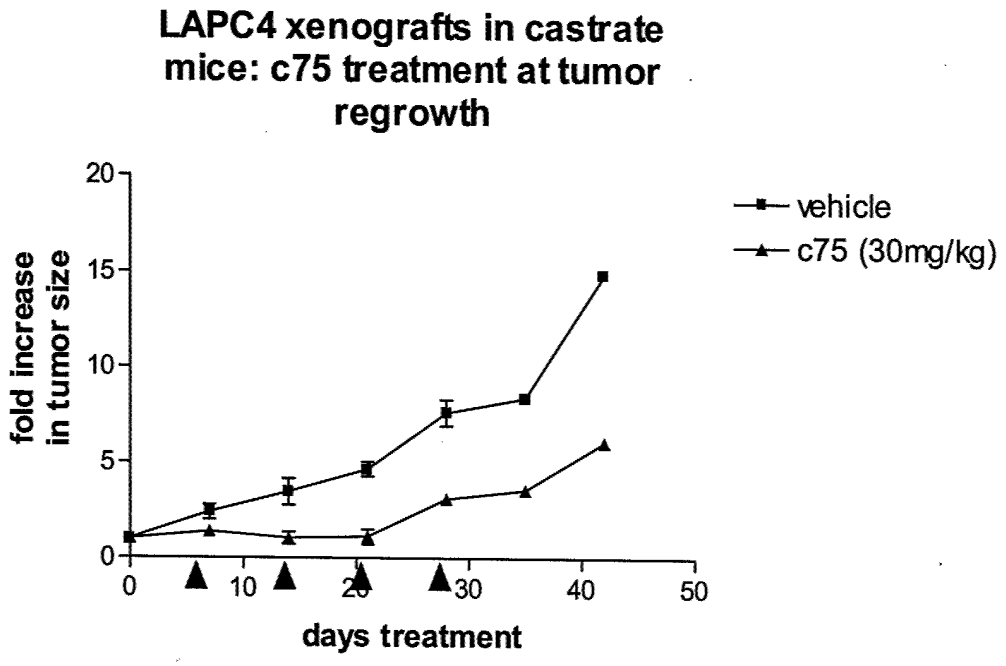
Task 1. Evaluation of FAS inhibitors, cerulenin and c75 against *in vitro* prostate cancer models.

Characterization of cytotoxicity of c75 in *in vitro* systems in parallel with hormonally manipulated changes in FAS activation is shown in Figure 3 C and D of Appendix A.

Task 2. Evaluation of FAS inhibitor c75 against xenograft prostate cancer models.

C75 treatment of hormone independent prostate carcinoma lines PPC-1 and TSU-Pr1 as xenografts (11) is shown in Figure 4 A and B of Appendix A.

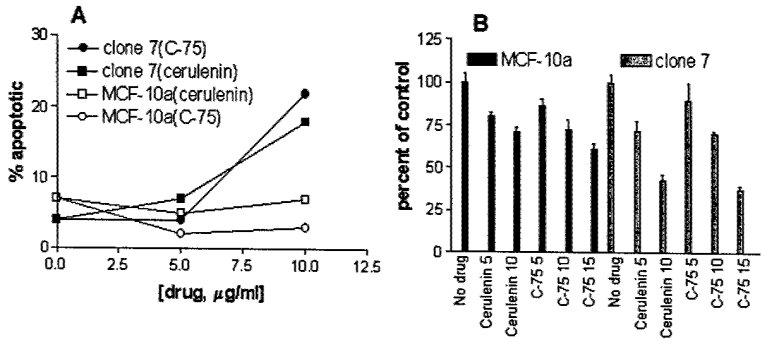
C75 was tested against the LAPC4 xenograft with castration of host mice and treatment of individual arms at castration or regrowth. Treatment with c75 at castration failed due to decreased tolerance of drug toxicity. The result of treatment at regrowth is shown in the figure below. Drug toxicity was higher in these mice than is seen in many other populations. The trend for c75 treatment was positive, however the fraction of tumors that grew after castration did not provide a statistically significant data set.



C75 inhibits the growth of LAPC4 prostate carcinoma flank xenografts in nude mice during emergent androgen independent regrowth after castration. 20 xenografted mice were castrated after establishment of flank tumors, and monitored for increases in tumor size. At regrowth, mice were randomized to treatment with weekly intra-peritoneal injections of c75 [30 mg/kg] or vehicle (arrows). Results are for 6 control and 3 c75 treated animals. There were 3 deaths in the treatment arm.

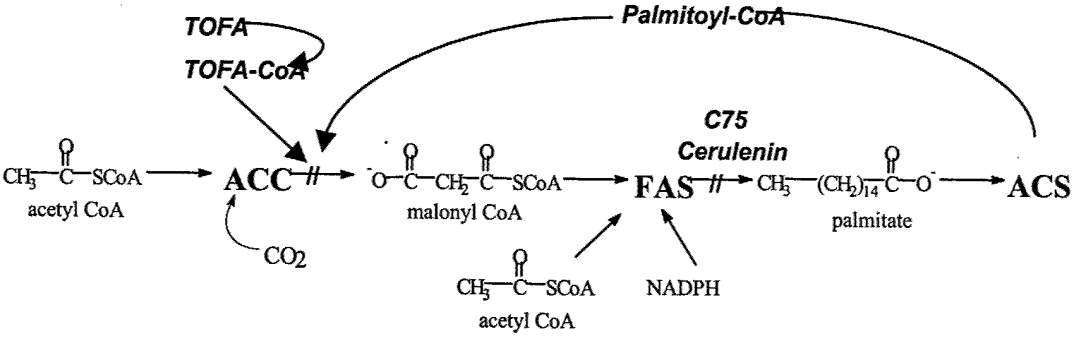
The data support continued investigation of FAS inhibitors for treatment of androgen independent prostate cancer.

A potential mechanism for emerging FAS inhibitor sensitivity was demonstrated in the in vitro transformation model described in Appendix B. H-*ras* transformation sensitizes MCF-10a epithelial cells to the induction of apoptosis by FAS inhibitors, cerulenin and C-75. Since earlier comparisons of unrelated cells with different levels of FA synthesis pathway activity had demonstrated increased FAS inhibitor cytotoxicity in cells with high FA synthesis pathway activity, the effects of FAS inhibitors were compared in the closely related MCF-10a and subclone 7, which differed only in the phenotype produced by H-*ras* transformation, including elevated FA synthesis. MCF-10a and subclone 7 had very similar growth rates, and both were growth inhibited by the FAS inhibitors cerulenin and C-75. A detailed analysis of the growth inhibition using a recently developed flow cytometric assay (12) demonstrated a characteristic apoptotic response in clone 7 cells, with parallel loss of clonogenic activity, while MCF-10a cells had a cytostatic response as measured by flow cytometry (not shown) and retained more clonogenic activity.



H-ras transformation sensitizes MCF-10a epithelial cells to the induction of apoptosis by FAS inhibitors, cerulenin and C-75. A. MCF-10a cells and *H-ras* transformed clone 7 were subjected to multi-parameter flow cytometry after 24 h of exposure to cerulenin or C-75 at the indicated doses. Clone 7 cells enter the apoptotic pathway in greater numbers than the parental line after inhibition of FAS. **B.** Clonogenic activity of clone 7 cells is lower than that of MCF-10a cells after 6 hour exposure to cerulenin or C-75 at the indicated doses. Determinations in triplicate, Bars:SEM.

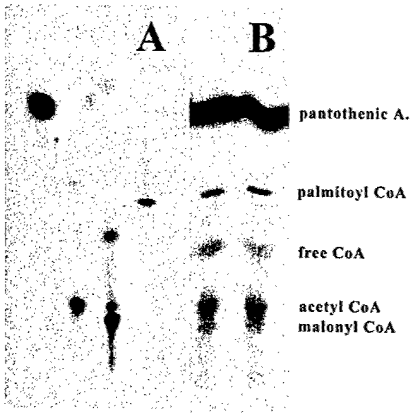
Cytotoxic mechanisms of FAS inhibitors. Cultured cells treated with inhibitors of FAS, including the fungal product, cerulenin, and our novel compound, C-75, demonstrated a rapid reduction in FA synthesis, and a complex cellular stress response. Our data suggest two components to this stress response: a cytotoxic effect resulting from accumulation of the committed substrate for FA synthesis, malonyl-CoA, which triggers apoptosis in tumor cells but occurs minimally in non-transformed cells, and a cytostatic effect resulting from limitation of FA production (12). The selective toxicity of C-75 for tumor cells was sufficient to produce significant growth inhibition of human prostate cancer xenografts in immunodeficient mice (1), with only mild, reversible systemic toxicity (13).



Inhibitors of the FA synthesis pathway. Schematic representation of the FA synthesis pathway showing the specificity of cerulenin and C-75 for fatty acid synthase (FAS) and of TOFA for acetyl-CoA carboxylase (ACC). The three FA synthesis inhibitors (2 FAS inhibitors and 1 ACC inhibitor) are all capable of reducing FA synthesis activity (incorporation of [$U^{14}C$]-acetate into extractable lipids) by comparable amounts. ACS=acyl-CoA synthase.

Our experiments have shown that pharmacologic inhibition of fatty acid synthesis at the physiologically regulated enzyme, acetyl-CoA carboxylase (ACC), was not cytotoxic to cancer cells. Inhibitors of the downstream enzyme, FAS, induced a rapid increase of its

substrate, malonyl-CoA, while the ACC inhibitor reduced malonyl-CoA levels. Prevention of malonyl-CoA accumulation resulted in significantly reduced cytotoxicity and apoptosis, implicating malonyl-CoA accumulation in the cytotoxic mechanism. Malonyl-CoA comprises a substantial fraction of the total cellular CoA pool during normal growth of tumor cells with active FA synthesis. The several fold increased malonyl-CoA levels produced by FAS inhibition may be toxic to tumor cells by an as yet undetermined mechanism. Alternatively, malonyl-CoA accumulation may sufficiently deplete the free CoA pool in tumor cells to inhibit other critical metabolic functions that use CoA, like the Krebs cycle, mevalonate synthesis or protein acetylation. We have recently developed an assay for metabolic labeling and quantitation of total cellular CoA to facilitate further evaluation of these alternative mechanisms.

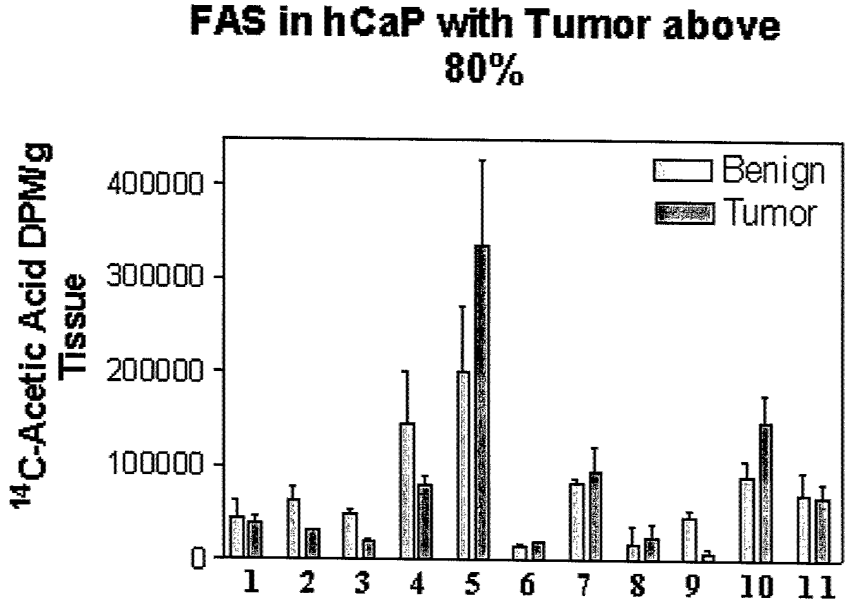


Constitutively elevated FA synthesis activity in cancer cells results in high steady state levels of malonyl-CoA. The total cellular CoA pool in MCF-7 carcinoma cells was labeled with ^{14}C-pantothenic acid, which is incorporated stoichiometrically into CoA, and cell extracts were separated by thin layer chromatography. **A.** Standards. **B.** Cell extracts from duplicate cultures.

Specific Aim 3:

Evaluation of FAS activity in *ex vivo* prostate cancers.

Viable tumor and benign prostate tissue was harvested from a series of radical prostatectomy specimens in an ongoing collaboration with Drs Nelson and Pflug, Dept. of Urology, University of Pittsburgh. The histologically verified tissues were metabolically labeled with ^{14}C-acetic acid followed by Folch extraction of lipids (14). Previous data had demonstrated that fatty acid synthetic activity was consistently higher in *ex vivo* primary prostate carcinoma than in benign prostate tissue, however the current data show greater variability in pathway activity (Figure 8). Efforts are currently in progress to determine whether variations in labeling protocol introduced at individual research sites, or true biological differences between clinical samples account for these results.



FA synthesis in *Ex vivo* tissues from radical prostatectomies. Tumor and control tissues were transferred to labeling medium after weighing. Fatty acid synthesis was assayed with a 2 hour pulse of [U-^{14}C]-acetic acid, 1μCi/ml, followed by Folch extraction and scintillation counting. All determinations were in duplicate, except when insufficient tissue was available for replicate samples. Data are presented as mean values with bars showing the standard error.

KEY RESEARCH ACCOMPLISHMENTS, DAMD17-99-1-9580:

- Characterization of hormonal responsiveness of FAS gene and pathway in Pca in vitro models: FAS expression and activity of a panel of androgen independent prostate carcinoma cells detailed in Appendix A.
- Mutagenesis of FAS promoter demonstrates importance of SREBP transcription factor binding site in FAS transcription in tumor cells.
- Analysis of FAS expression in hormone responsive xenografts.
- Characterization of cytotoxicity of c75 in *in vitro* systems in parallel with hormonally manipulated changes in FAS activation.
- Identification of MAP kinase and PI 3-kinase signaling as major regulators of FAS expression in advanced malignancy.
- Development of an *in vitro* model for epithelial cell transformation that recapitulates neoplastic changes in lipogenic function that are observed during PCa carcinogenesis.
- Identification of SREBP-1 transcription factors as key regulatory elements that link tumor growth signals and lipogenesis.
- Demonstration that the 1c isoform of SREBP coordinately modulates with FAS *in vitro* and *in vivo*.
- Characterization of cytotoxicity of C-75 in *in vitro* systems in parallel with oncogene mediated changes in FAS activation.

- Development of biochemical assays to study the cytotoxic mechanism of FAS inhibitors.
- Significant growth inhibition with c75 treatment of hormone independent prostate carcinoma lines, PPC-1 and TSU-Pr1, as xenografts.
- C75 treatment of LAPC4 xenograft with castration of host mice and treatment of individual arms at castration or at regrowth showed a trend toward growth inhibition with treatment at regrowth.
- Metabolic labeling of primary human prostate carcinoma tissue demonstrates functional activation of the fatty acid synthetic pathway in clinical disease.

REPORTABLE OUTCOMES, DAMD17-99-1-9580:

Three manuscripts have been produced: two are published, one is in press, provided as Appendices A, B, C.

Three patent applications have been filed:

PCT/US00/31067, filed 11/20/2000,The United States Patent and Trademark Office: Increased Malonyl CoA Levels or other Manipulation of the Fatty Acid Synthesis Pathways as a Means to Selectively Kill Cancer Cells. The Johns Hopkins University School of Medicine.

PCT/US00/31068, filed 11/20/2000,The United States Patent and Trademark Office: Depletion of Cellular Coenzyme-A Levels as a Means to Selectively Kill Cancer Cells. The Johns Hopkins University School of Medicine.

PCT/US02/04408, filed 2/15/2002,The United States Patent and Trademark Office: Cytostatic Effects of Fatty Acid Synthase Inhibition.

CONCLUSIONS:

The data generated under this project support the following conclusions: Elevated FAS expression occurs with prostate cancer progression after androgen withdrawal. As androgen independence emerges, FAS expression may be upregulated by alternate signaling pathways important for prostate cancer growth, including the MAP kinase and PI 3-kinase signaling cascades. The SREBP-1 transcription factors are important components of this regulatory pathway, with SREBP-1c the modulated component. The cytotoxicity of C-75 and other FAS inhibitors increases in parallel with oncogenic changes in FAS activation. The mechanism of FAS inhibitor cytotoxicity involves fluxes in levels of intermediary metabolites derivatized to CoA . The activity observed in model systems predicts that FA synthesis inhibition will be cytotoxic to prostate carcinomas that have activated this metabolic pathway.

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PUBLICATIONS RESULTING FROM DAMD17-99-1-9580:

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3. Yang, Y., Morin, P., Han, W., Chen, T., Bornman, D., Gabrielson, E., and Pizer, E. Regulation of Fatty Acid Synthase Expression in Breast Cancer by Sterol Regulatory Element Binding Protein-1c. Exp Cell Res, *In Press*, 2002.

PERSONNEL FROM DAMD17-99-1-9580:

NAME	Role on Project
Pizer, Ellen	Prin. Invest.
Nelson, Joel	Co-Invest
Pflug, Beth	Fellow /Co-Invest
Li, Ji Nong	Fellow
Yang, Yu-An	Fellow
Wan Fang Han	Technician
TingHua Chen	Technician

The Prostate 47:102–110 (2001)

Appendix A

Increased Fatty Acid Synthase as a Therapeutic Target in Androgen-Independent Prostate Cancer Progression

Ellen S. Pizer,[1]* Beth R. Pflug,[2] G. Steven Bova,[1,3,4] Wan Fang Han,[1] Michael S. Udan,[3] and Joel B. Nelson[2]

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BACKGROUND. Fatty acid synthase (FAS) performs the anabolic conversion of dietary carbohydrate or protein to fat. FAS expression is low in most normal tissues, but is elevated in many human cancers, including androgen-sensitive and androgen-independent prostate cancer.
METHODS. Immunohistochemical evaluation of FAS expression was performed in human prostate cancer specimens under various states of androgen ablation. In vitro and in vivo prostate cancer models were evaluated for FAS expression and activity under androgenic and androgen-depleted conditions, and were tested for sensitivity to antimetabolite drugs that target fatty acid synthesis.
RESULTS. While FAS expression in the prostate was androgen responsive, it persisted or was reactivated in human prostate carcinoma after androgen ablation, and was high in 82% of lethal tumors examined at autopsy. Similar patterns of FAS expression and fatty acid synthesis were seen in cell culture and xenograft models of human prostate cancer. Pharmacologic inhibition of FAS resulted in a dose-dependent reduction of tumor growth in these models, including fourfold inhibition of an androgen-independent human prostate cancer xenograft with little associated toxicity.
CONCLUSIONS. The data suggest that FAS expression/FA synthesis provides an important functional aspect of the malignant phenotype in prostate cancer, perhaps supporting cell growth or survival. FAS expression may be upregulated by alternate signaling pathways important for prostate cancer growth under androgen withdrawal. The re-emergence of FAS expression and activity during the development of androgen independence demonstrate that FAS may serve as a novel target for antimetabolite therapy in prostate cancer. *Prostate 47:102–110, 2001.* © 2001 Wiley-Liss, Inc.

KEY WORDS: **fatty acid synthase; prostate cancer; androgen; tumor progression; chemotherapy**

INTRODUCTION

In prostate cancer, treatment with androgen ablation often produces dramatic objective responses, yet from this initial hormone sensitivity emerges lethal prostate cancer, an androgen independent disease that kills almost 40,000 men a year in the United States. Certain androgen-regulated gene products, such as the biomarker prostate-specific antigen (PSA), will

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predictably decline after androgen ablation, but a subsequent rise in PSA heralds the advent of androgen-independent disease progression. Identifying and targeting other gene products uncoupled from androgen regulation is an attractive therapeutic approach. One such candidate gene product is the biosynthetic enzyme, fatty acid synthase (FAS), which, like PSA, shows increased gene expression and enzyme activity during androgen stimulation of androgen-responsive prostate carcinoma cells [1,2]. FAS is the major enzyme required for the anabolic conversion of dietary carbohydrate to fatty acids. It functions normally in the liver to make lipids for export to metabolically active tissues or storage in adipose tissue [3]. FAS has specialized physiological functions producing milk lipids in lactating breast tissue, surfactant in lung, and may contribute to the production of ejaculate lipids by the prostate [4–7]. However, FAS expression is low in most normal adult tissues, which appear to utilize circulating lipids preferentially for the synthesis of new structural lipids [8]. FAS is expressed at markedly elevated levels in biologically aggressive subsets of human prostate cancers, as well as breast and other cancers [9–13], and cell lines derived from these tumors display concordant elevation of FAS enzyme content and fatty acid synthetic activity [14]. Similarly, ex vivo colorectal carcinoma tissue displays elevated FA synthesis that parallels FAS expression levels compared to adjacent non-neoplastic mucosa [15]. While the malignancy-associated function of FAS is not yet determined, the major lipid product in most tumors is phospholipid. Likewise, the mechanism(s) for upregulation of FAS in tumor cells is unknown. FAS expression likely provides an important function for tumor cells, since FAS upregulation is common and linked to tumor virulence in studies of human tissues. Furthermore, FAS inhibitors are selectively cytotoxic to tumor cells in experimental systems, apparently due to acute intracellular accumulation of the committed substrate, malonyl-CoA, that only occurs when fatty acid synthesis is active [16–18].

These observations prompted the present study of FAS expression and activity in human prostate cancer, with the hypothesis that the initial sensitivity of FA synthesis to androgen withdrawal in prostate cancer is lost in parallel with the emergence of the androgen-independent phenotype, and that specific inhibition of FAS thus provides a therapeutic target for prostate cancer. We therefore studied the expression of FAS in matched benign and malignant prostatic tissues and cell lines under different hormonal conditions, and in metastatic lesions obtained at autopsy from men dying of androgen-independent disease, and established the sensitivity of prostate cancer cell lines to FAS inhibition in vitro and in vivo.

MATERIALS AND METHODS

Prostate Carcinomas

Radical prostatectomy specimens with tumors of intermediate or high grade (Gleason score 6 or higher) were obtained from the surgical pathology files of The Johns Hopkins Hospital and The Johns Hopkins Bayview Medical Center. Autopsy tissues were obtained as part of the Project to ELIminate lethal CANcer (PELICAN) at Johns Hopkins University School of Medicine.

Immunohistochemistry

Four or five-μm sections were subjected to heat induced epitope retrieval, followed by monoclonal anti-human-FAS antibody at a concentration of 0.1–0.5 μg/ml (US patent number 5,864,011), detected with the LSAB2 system from DAKO. Analysis of immunostaining for FAS was performed using a scoring system based on summation of negative, weakly positive, and strongly positive staining in each tissue, with FAS expression values assigned on a scale of 0–8, as described in [11]. Graphics were prepared and statistical analysis were performed in SigmaPlot 5.0 (SPSS).

Cell Lines and Culture Conditions

With the following exceptions, cell lines were obtained from the American Type Culture Collection (Rockville, MD), and were cultured in RPMI-1640 with 10% fetal bovine serum (Hyclone, Logan, UT). TSU-Pr1 [19], PPC-1 [20], and LAPC-4 cells [21] were provided by the originating laboratories. The LAPC-4 cell line was grown in Iscove's Medium with 10% fetal bovine serum. The androgen deprived clones of LNCaP cells were derived as previously described in [22] and were grown continuously in RPMI-1640 (without phenol red) with 10% charcoal stripped fetal bovine serum (Hyclone, Logan, UT). The LAPC-4 AD cells were androgen deprived for 4–6 months in Iscove's medium (without phenol red) with 10% charcoal stripped FBS. Normal prostate epithelial cells (PrEC) and stromal cells (hPS) were purchased from Biowhittaker (Walkersville, MD). The hPS cells were grown in RPMI-1640 with 10% FBS and the PrEC cells were grown in PrEGM (Biowhittaker, Walkersville, MD) supplemented according to the manufacturers recommendations. Cells were plated in 24-well culture plates at 5×10^4 cells/well 24 hr before treatment. Cerulenin (Sigma Chemical Co., St. Louis, MO) and c75 were added as stock 5 mg/ml solutions in dimethyl sulfoxide (DMSO). The final concentration of DMSO in cultures was at or below 0.2%.

Immunoblot Analysis

Cells were lysed in 10 mM Tris buffer (pH 8.0) containing 135 mM NaCl, 1% TritonX-100, 10% glycerol with antipain (5 μg/ml), aprotinin (10 μg/ml), leupeptin (10 μg/ml), pepstatin (1 μg/ml), phenylmethylsulfonyl fluoride (1 mM) added fresh. The lysates were centrifuged at 12,000*g* for 10 min and the supernatants used for immunoblot analysis. Equal amounts of protein (20 μg of cell line and xenograft, 80 μg of mouse liver) were separated on 4% SDS-PAGE under reducing conditions and electrotransferred onto PVDF membranes. Immunodetection was with rabbit anti-FAS antibody (1:4,000) [14] followed by secondary antibody (goat anti-rabbit HRP; 1:20,000, Boehringer Mannheim, Mannheim, Germany). After washing, immunoreactivity was visualized using ECL (Amersham Life Sciences, Arlington Heights, IL) and XAR film (Kodak, Rochester, NY).

Metabolic Labeling

Cultured cells were plated at 5×10^4/well in 1 ml in 24-well plates and incubated overnight prior to labeling. Replicate cultures were trypsin treated and counted to correct for plating efficiency and growth. Ex vivo tissues from xenografted mice were transferred rapidly to labeling medium after weighing. Fatty acid synthesis was assayed with a 2-hr pulse of [U-^{14}C]-acetic acid, 1 μCi/ml, followed by Folch extraction and thin layer chromatography with standards, as described [16]. Quantitation of lipid classes was performed on a Storm (Molecular Dynamics, Sunnyvale, CA). All determinations were in duplicate, except when insufficient xenograft tumor was available for replicate samples. Data are presented as mean values with bars showing the standard error. Calculations and graphing were performed in Prism 2.0 (GraphPad, San Diego, CA).

Xenograft Studies

Subcutaneous flank xenografts of the human prostate cancer cell lines, LAPC-4 and PPC-1 were established from cells in culture in 4–6 week old, male nu/nu mice (National Cancer Institute, Bethesda, MD). All animal experiments complied with institutional animal care guidelines. Mice received subcutaneous flank injections of 4×10^6 LAPC-4 cells in Iscove's medium, and after two months, the animals with tumors were randomized to castration ($n = 10$) or left intact ($n = 3$). Upon regrowth of the tumors, the animals were sacrificed and FAS expression and activity of each xenograft was analyzed as described above.

Other mice were injected with 2×10^6 PPC-1 or TSU-Pr1 cells in RPMI-1640. Tumors were measured with calipers in three dimensions twice weekly. Tumor volumes were calculated using the formula: length × width × height × 0.5. When the tumors became palpable, animals with measurable tumors were randomized to treatment with weekly doses of c75 at 30 mg/kg intraperitoneal (ip), or 3 mg/kg ip or vehicle (20% DMSO/80% RPMI ip) ($n = 10$ animals/arm). The experiments were terminated when the controls reached the surrogate endpoint.

RESULTS

FAS Expression in Prostate Cancer Tissues

Thirty-one intermediate- and high-grade primary prostate cancer specimens (Gleason score 6 or higher) were evaluated by FAS immunohistochemistry, and expression was scored by a combined assessment of FAS levels and distribution [11] (Fig. 1). Twenty-one were from intact and 10 were from androgen-ablated patients. The benign epithelium from hormonally intact patients displayed widely variable FAS immunoreactivity, both within and among individual samples. In all but two cases, the tumors in these prostates expressed more FAS than the benign tissue. In the 10 cases studied after 3–6 months of androgen deprivation therapy, FAS expression was significantly reduced in the benign epithelium relative to the intact group (Mean FAS score 2.4 vs 4.8, Student *t* test, $T = 5.84$, $P = 0.000002$). However, FAS expression levels in the tumors after androgen ablation remained comparable to tumors from intact patients (Mean FAS scores 7.1 and 7.0). Similarly, when 77 samples of primary and metastatic prostate carcinoma were evaluated from 18 autopsy cases, all of which had been treated with prolonged androgen ablation, elevated FAS immunoreactivity (FAS score < 4) was found in 82%, while the residual benign prostatic epithelium had very low FAS. Thus, while FAS expression was androgen responsive in benign prostatic epithelium, it persisted following androgen ablation in the remaining viable tumor, and showed high expression in lethal, androgen-independent disease.

FAS in Model Systems of Prostate Cancer

In vitro and in vivo models of human prostate cancer provide the opportunity to characterize FA synthesis pathway function in parallel with FAS expression, and to manipulate tumor growth conditions in a controlled manner. The steady-state expression levels of FAS and FA synthesis were characterized in 11 prostate carcinoma cell lines, including 4 androgen-independent lines, 2 androgen-dependent lines, and their 5 androgen-independent subclones, selected after chronic growth in androgen-depleted media

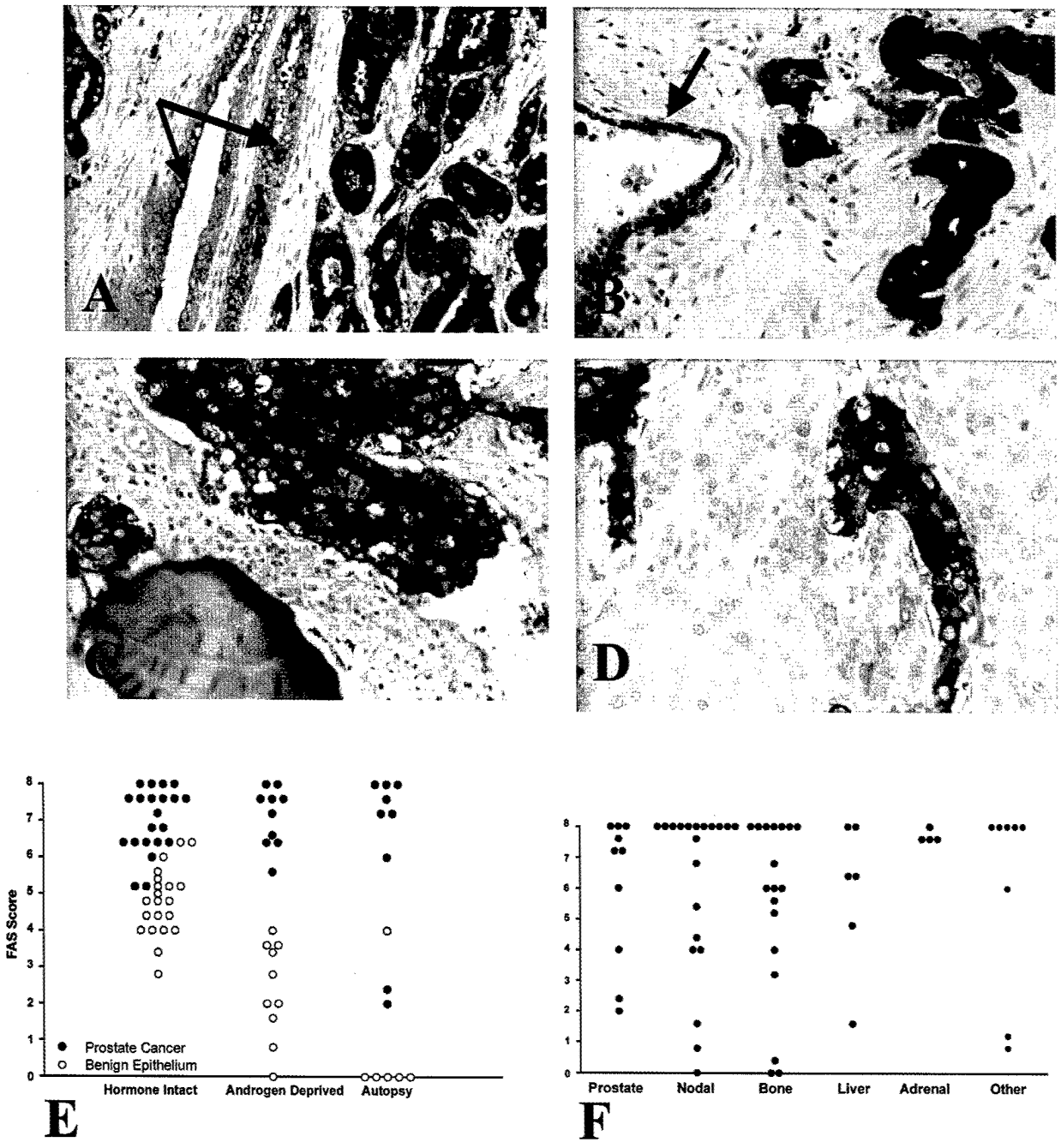

Fig. 1. FAS immunoreactivity of prostate cancer tissues. **A** and **B:** Sections from radical prostatectomies from patients without and with prior androgen ablation therapy. Benign glands (arrows) show moderate (A), or very low (B) FAS expression. Tumor (all unlabeled glands) has uniformly high FAS. **C** and **D:** Sections from bony and liver metastasis of prostate cancer sampled at autopsy. Tumor cells show high FAS expression in contrast to negative expression in bone marrow and low expression in liver. **E:** Scatter plot of FAS immunoreactivity scores of benign glands (○) and tumor (•) for radical prostatectomies from 21 intact and 10 androgen-ablated patients, and from prostates of 9 patients with lethal prostate cancer sampled at autopsy after prolonged androgen ablation. Significant reduction in mean FAS expression score resulted for benign epithelium after androgen ablation (Student *t* test, $P = 0.000002$), while tumor scores were not significantly different. **F:** Scatter plot of FAS immunoreactivity scores of 68 metastatic deposits of prostate cancer from 18 autopsies.

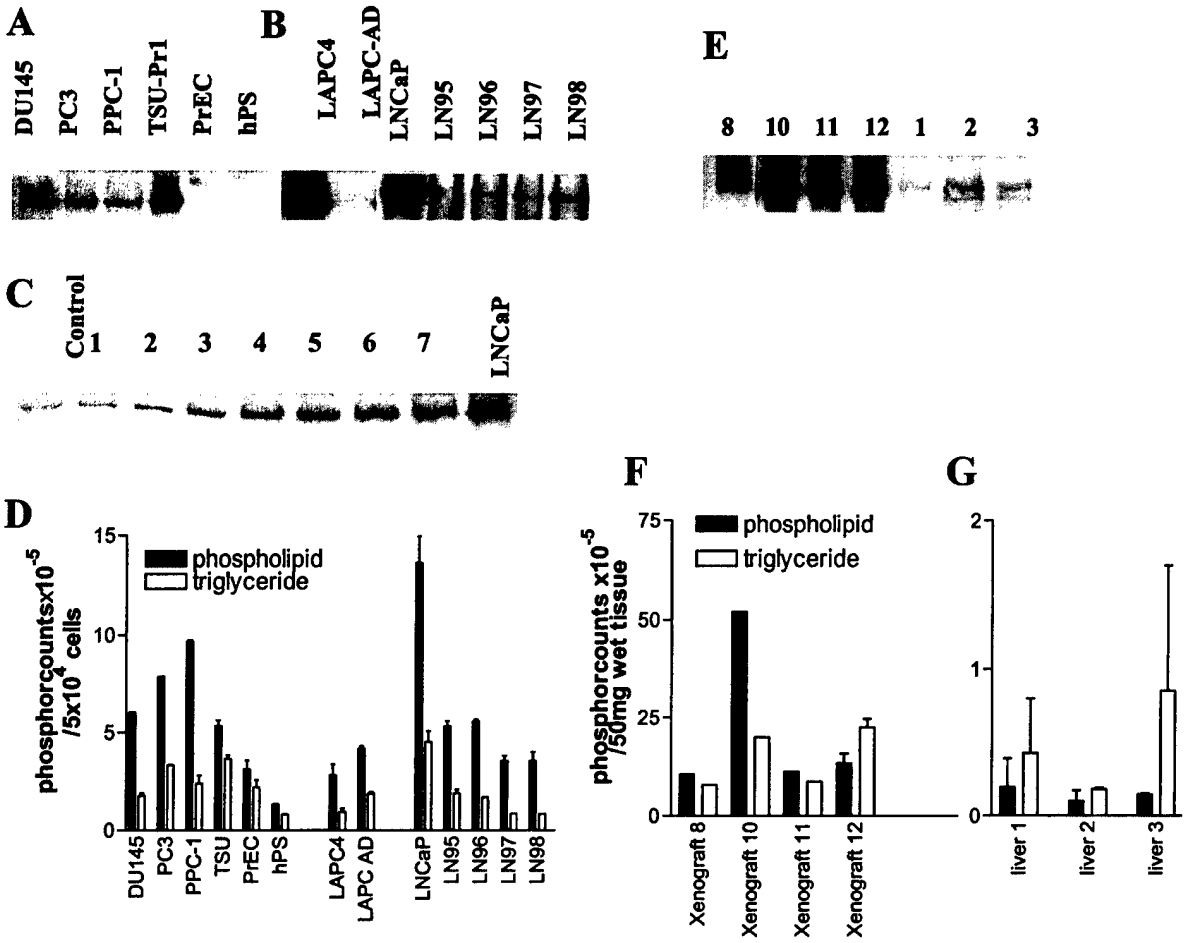

Fig. 2. Fatty acid synthase activation in prostate carcinoma cell lines and a prostate carcinoma xenograft under emergent androgen independence. **A** and **B:** FAS enzyme levels in prostate cancer cell lines detected by immunoblot. Twenty micrograms of protein were loaded in each lane. A: DUI45, PC3, PPC-I, and TSU are androgen-independent tumor lines. PrEC and hPS are benign epithelial and stromal cells, respectively. B: LAPC4 and LNCaP are androgen dependent tumor lines. LAPC-AD is a subclone of LAPC4 selected in androgen depleted culture. LN95–98 are subclones of LNCaP selected in androgen depleted culture. These clones, though androgen independent, remain androgen sensitive. C: LN96 cells were cultured under control conditions, or were exposed to 5α-dihydrotestosterone [10^{-9} M] for I to 7 days before immunoblot analysis of FAS content. Twenty micrograms of protein were loaded in each lane. The parental line, LNCaP is shown for comparison. **D:** Fatty acid synthesis activity and products of these tumor lines. Samples were pulse labeled with ^{14}C-acetate, lipids were extracted and separated by thin layer chromatography into major classes. Minor lipids representing less than 10% of total not shown. **E:** FAS enzyme levels in tissues from xenografted mice detected by immunoblot. LAPC4 xenografts; animals 8 and 10 were castrated, animals 11 and 12 were left intact. Tumor lysates, 20 mg; liver lysates, 80 μg. Immunohistochemical evaluation of the xenografts demonsrated similar staining to the human tissue samples in Figure I (not shown). **F** and **G:** Fatty acid synthesis activity and products of these tissues. Fatty acid synthesis was an order of magnitude more active in the tumors than in liver (scale is different for F and G). Tumor lines and xenografts show elevation of both FAS expression and FA synthesis over controls. Xenografts studied during periods of active growth have elevated FAS expression and FA synthesis without or with androgen ablation.

(Figs. 2A,B). FAS enzyme content was higher in the androgen-independent tumor lines than in benign prostatic epithelial and stromal cells (Fig. 2A), and was very high in the two androgen-dependent lines, LNCaP and LAPC4 (Fig. 2B). Comparison of LNCaP and LAPC4 with their subclones grown in androgen-depleted conditions demonstrated substantial reduction in FAS protein content after androgen withdrawal, consistent with androgen stimulation of FAS expression. These androgen-deprived clones retain the ability to respond to androgen, and reintroduction of dihydrotestosterone to one of these lines partially restored FAS expression (Fig. 2C). Most of the tumor lines also had elevated FA synthetic pathway activity compared to benign prostatic epithelial and stromal cells, although LAPC4 did not (Fig. 2D). Although the major product of the fatty acid synthesis pathway in these cell lines was membrane phospholipid, substan-

tial amounts of triglyceride were also produced. Other lipid classes represented less than 10% of the total extractable lipids, and are not shown. The very high FA synthetic activity of LNCaP was substantially reduced in the androgen-deprived subclones, LN95–98 (Fig. 2D), similar to enzyme content (Fig. 2B).

FAS Phenotype in an Androgen-Dependent Prostate Carcinoma Xenograft Model

The LAPC4 prostate carcinoma line produces androgen-sensitive subcutaneous flank tumors in male nu/nu mice: following initial regression after castration and prolonged latency, the tumors begin to grow again, providing a model of chronic androgen deprivation, and ultimately androgen independence [21, 23]. We determined the expression levels of FAS and measured FA synthesis ex vivo in a cohort of LAPC4 xenografts that had regrown six months after castration of the host animals. FAS expression and function were both elevated in all xenografts examined during regrowth (Figs. 2E, F), by comparison with liver (Figs. 2E, G). The cases illustrated are representative of the cohort. While some xenografts from castrated animals showed reduced FAS content relative to tumors from intact animals (xenograft 8), others showed no reduction in FAS content (xenograft 10). Tumor FA synthesis activity was comparable in castrated and intact animals, and an order of magnitude higher than in liver. Thus, during emergent androgen-independent growth, FAS expression was high, generating a phenotype similar to the androgen-independent cell lines, DU145, PC3, PPC-1, and TSU-Pr1. Indeed, ex vivo pulse labeling of PPC-1 xenografts demonstrated similar FA synthetic activity (data not shown).

Sensitivity of Prostate Cancer Models to FAS Inhibitors

Studies of experimental models of other cancers with high FAS have shown sensitivity to FAS inhibitors [14,16–18]. Most of these utilized the antimetabolite cerulenin, a natural product of the fungus *Cephalosporium caerulens*. Cerulenin, (2R, 3S)-2,3-epoxy-4-oxo-7,10-*trans,trans*-dodecadienamide, has been known since the 1960s as a specific inhibitor of FA synthesis across a broad phylogenetic spectrum [24]. Cerulenin irreversibly inhibits FAS by forming a covalent adduct with the active site cysteine of the beta-keto-acyl synthase moiety, which performs the condensation reaction between the elongating fatty acid chain and each successive malonyl residue. We have recently developed a novel small molecule inhibitor of FAS called c75, with comparable inhibitory effects on FA synthesis and a chemical stability greater than cerulenin [25]. Exposure of human cancer cells to cerulenin or c75 triggers apoptosis [17,18]. To evaluate the sensitivity of prostate cancer cells to FAS inhibitors, and to determine whether androgenic modulation of FAS in tumor cells would alter their sensitivity to these drugs, we evaluated the growth inhibition in response to the antimetabolites cerulenin or c75 of four androgen-independent human prostate cancer cell lines, two androgen-dependent lines, and their five androgen-depleted subclones (Fig. 3). Both cerulenin and c75 demonstrated selective, dose-dependent cytotoxicity for tumor cells, with IC_{50}s in the dose range of 2–8 mg/ml and 1–4 mg/ml, respectively. Benign prostatic epithelial and stromal cells (PrEC and hPS in Fig. 3) were significantly less sensitive to these agents at several doses levels. Although the androgen-sensitive lines LNCaP and LAPC4 had comparable sensitivities, their androgen-independent subclones were significantly less sensitive to cerulenin, and the LNCaP subclones showed modestly decreased sensitivity to c75, in parallel with the decreased FAS expression and FA synthesis demonstrated in Figure 2. In separate experiments, FAS inhibitors induced apoptosis in these cell lines (data not shown).

Sensitivity of Prostate Cancer Xenografts to FAS Inhibition

Given the potency of the FAS inhibitors against prostate cancer cell lines in vitro, the therapeutic potential of c75 was studied using subcutaneous xenografts of the androgen independent, human prostate cancer cell lines PPC-1 and TSU-Pr1 in nu/nu mice. Cerulenin has limited utility for in vivo FAS inhibition studies because it is chemically unstable, and does not produce systemic activity. The pharmacological properties of c75 are substantially improved in these regards. Cohorts of 10 male nude mice carrying established, palpable flank xenografts of PPC-1 were treated with four intraperitoneal doses of c75 on a weekly schedule (Fig. 4A). Mice treated with high-dose c75 (30 mg/kg/week) had a greater than four-fold reduction in tumor growth, relative to low-dose c75 (3 mg/kg/week) and vehicle-treated control mice. A similar treatment protocol (omitting the low-dose c75 arm) produced 2.7-fold growth inhibition of TSU-Pr1 flank xenografts (Fig. 4B).

The FAS inhibitors produce a transient weight loss resulting from appetite suppression related to malonyl Co-A accumulation in the brain [14, 26]. c75 toxicity was further evaluated in mice without tumors using the same high dose and schedule (30 mg/kg/week $\times$ 4 injections), followed by necropsy with gross and microscopic pathological survey of organs and tissues. In parallel with the observed weight loss, c75-treated mice demonstrated lipid depletion from adipose tissue

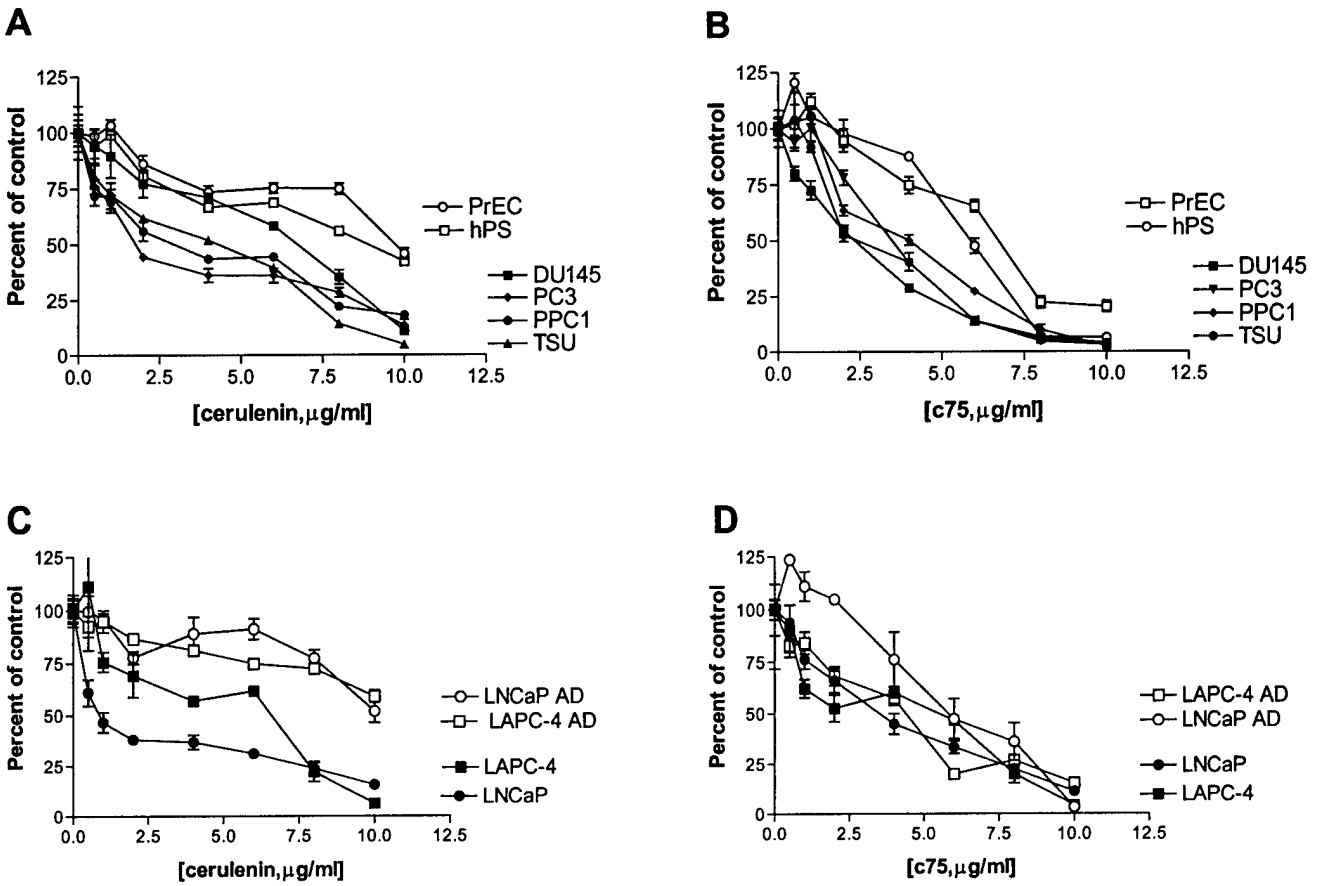

Fig. 3. Growth inhibition of prostate carcinoma lines by FAS anti-metabolites. **A and B:** FAS antimetabolites, cerulenin, and c75 inhibit the growth of androgen-independent prostate carcinoma lines DU145, PC3, PPC-1, and TSU with IC_{50}s in the range of 2–8 μg/ml and 1–4 μg/ml, respectively. Nontransformed lines, PrEC and hPS are less sensitive. **C and D:** Prostate carcinoma lines LAPC4 and LNCaP, with high FAS under androgen stimulation, show greater growth inhibition by cerulenin than their low FAS expressing subclones selected in androgen depleted culture. Changes in sensitivity to c75 were minimal.

(Figs. 4C, D). No other consistent organ damage was observed.

DISCUSSION

Elevated FAS Expression Indicates Progression After Androgen Withdrawal

In clinical practice, the progression of androgen-dependent to androgen-independent prostate cancer is defined by re-expression of certain gene products, most notably PSA. In this study, FAS has been identified as another potentially important gene product in prostate cancer progression. In benign prostatic epithelial cells, FAS expression was always lower than the coexistent prostate cancer and, following androgen deprivation, FAS expression in benign cells was persistently reduced. In prostate cancer, however, FAS expression was elevated, both in human tissues, cells lines, and xenograft models, and escaped the effects of androgen withdrawal. Therefore, like PSA, although FAS initially responded to the androgenic environment, the re-expression of FAS was also characteristic of the ultimately fatal, androgen-independent disease.

Previous studies of FAS expression in primary prostate carcinomas demonstrated variable FAS expression levels in intermediate-grade tumors that were strongly predictive of pathologic stage or recurrence [11, 12]. In these studies, benign prostatic glands were either negative or only weakly immunoreactive with antibody to FAS. Recent advances in immunohistochemistry, including availability of a monoclonal antibody against human FAS, have allowed increased sensitivity of detection of FAS in tissue sections. Similar FAS immunoreactivity of benign prostatic glands has recently been reported by another group, using an antipeptide antibody to FAS [27].

Elevated FAS Expression Marks a Cellular Function

As supported by the [U ^{14}C] acetate labeling data, elevated FAS expression serves as a marker for a

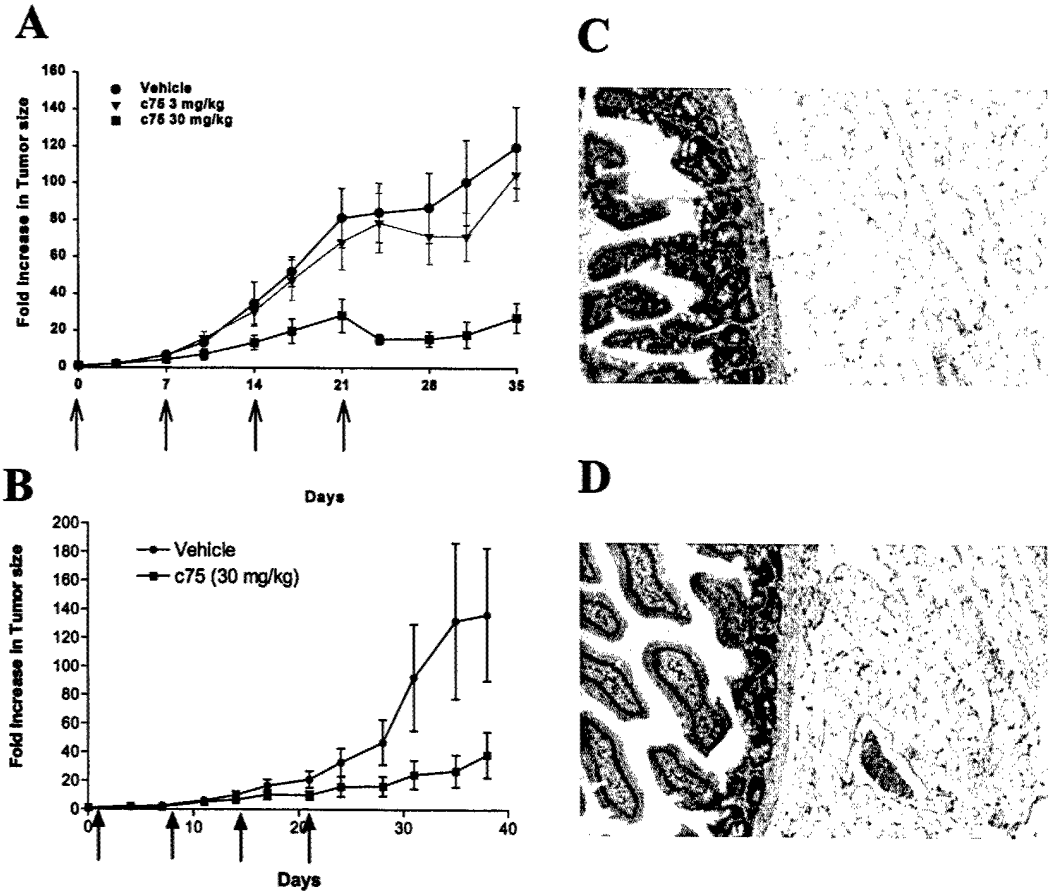

Fig. 4. c75 inhibits the growth of prostate carcinoma flank xenografts in nude mice. PPC-1 flank xenografts **(A)**. TSU-Prl flank xenografts **(B)**. 10 xenografted mice per cohort were treated with weekly intraperitoneal injections of c75 or vehicle (arrows). c75 dosed at 30 mg/kg produced fourfold inhibition of PPC-1 tumor growth relative to controls and low dose c75, and 2.7-fold inhibition of TSU-Prl tumor growth. **C:** control. **D:** c75 treated mouse bowel. In a parallel experiment, c75 dosed at 30 mg/kg produced marked depletion of lipid from adipose tissues, but other organs and tissues showed no histologic changes after c75.

highly active metabolic pathway in cells. Prostate carcinoma tumor lines and xenografts with high FAS had elevated fatty acid synthesis relative to control lines and tissues. Phospholipids and triglycerides represented the bulk of the lipid products of the pathway. Expression of this phenotype requires coordinate regulation of numerous cellular functions to produce the substrates for fatty acid synthesis, and to maintain an anabolic state. The frequent emergence of this complex phenotype from androgenic regulation during tumor progression suggests a functional role for active fatty acid synthesis in the growth or survival of prostate carcinoma cells. This idea is also supported by the association of FAS expression with poor prognosis in other studies of prostate cancer, and in cancers of other organ systems [9–13].

Inhibition of vital cellular functions often produces cytotoxicity, as demonstrated by targeted blockade of fatty acid synthesis in prostate cancer models. The FAS inhibitors, cerulenin and c75, were cytotoxic to prostate carcinoma lines, with greater sensitivity in tumor lines that had high FAS and fatty acid synthesis relative to controls, or tumor lines with downregulated FAS.

FAS Inhibition Is Therapeutic In Vivo Against Prostate Carcinoma Xenografts

The identification of fatty acid synthesis reactivation during tumor progression raises new questions about the cell biology of prostate cancer. Perhaps more important is the identification of a novel therapeutic target for androgen independent prostate cancer. In this study, we demonstrated a significant growth inhibition of two human, androgen independent tumors following systemic administration of the FAS inhibitor, c75, in doses that were well tolerated by the host animals. This result indicates that the FAS

activation typical of late stage, androgen independent prostate cancer may confer clinically useful sensitivity to FAS antimetabolites.

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Appendix B

Activation of Fatty Acid Synthesis during Neoplastic Transformation: Role of Mitogen-Activated Protein Kinase and Phosphatidylinositol 3-Kinase[1]

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Activation of fatty acid synthase (FAS) expression and fatty acid synthesis is a common event in tumor cells from a variety of human cancers and is closely linked to malignant transformation and to tumor virulence in population studies of human cancer. We now show that, in contrast to nutritional regulation of lipogenesis in liver or adipose tissue, changes in fatty acid metabolism during *in vitro* transformation of the human mammary epithelial cell line MCF-10a are driven by increases in epidermal growth factor signaling, acting in major part through the mitogen-activated protein (MAP) kinase and phosphatidylinositol (PI) 3-kinase signaling cascades. H-*ras* transformation of MCF-10a cells resulted in upregulation of MAP kinase and PI 3-kinase signals, upregulation of sterol regulatory element binding protein 1 (SREBP-1) transcription factor levels, and upregulation of FAS expression and FA synthesis. Deletion of the major SREBP binding site from the FAS promoter abrogated transcription in transformed MCF-10a cells. Inhibitors of MAP and PI 3-kinases downregulated SREBP-1 levels and decreased transcription from the FAS promoter, reducing FAS expression and fatty acid synthesis in transformed MCF-10a cells and in MCF-7 and HCT116 carcinoma cells. H-*ras* transformation sensitized MCF-10a cells to the FAS inhibitors cerulenin and C-75. These results confirm an important role for SREBP-1 in neoplastic lipogenesis, and provide a likely basis for the linkage of upregulated fatty acid metabolism with neoplastic transformation and with tumor virulence, since MAP and PI 3-kinase signaling contributes to both. © 2002 Elsevier Science (USA)

Key Words: **fatty acid synthase; mitogen-activated protein kinase; phosphatidylinositol 3-kinase; sterol regulatory element-binding proteins; neoplasia.**

INTRODUCTION

Regulation of the lipogenic capacity of tissues is mediated by modulation of the concentrations of the lipogenic enzymes. Fatty acid synthase (FAS, EC 2.3.1.85) is the major biosynthetic enzyme for synthesis of fatty acids from small carbon units, and its expression reflects the regulation of lipogenesis [1, 2]. Most studies of FAS expression have focused on liver or adipose tissues where the bulk of physiological lipogenesis occurs. In these tissues FAS expression is regulated by nutritional and hormonal conditions, primarily at the transcriptional level. Feeding previously fasted animals a high-carbohydrate, low-fat diet causes a brisk induction of FAS expression, coordinately regulated by glucose, insulin, glucagon, glucocorticoids, and thyroid hormone [3]. Recent studies of nutritional regulation of FAS expression have demonstrated that phosphatidylinositol (PI) 3-kinase-Akt signaling is downstream of insulin in the adipocyte lipogenic response [4]. Certain rapidly proliferating nonneoplastic cells and tissues, including some fetal tissues, have high FAS expression in cycling cells [5]. It appears that one of the major function(s) that fatty acid synthesis provides for proliferating cells is to support membrane synthesis, since the bulk of endogenously synthesized fatty acids is incorporated into membrane lipid, and inhibition of endogenous fatty acid synthesis causes secondary inhibition of phospholipid synthesis in experimental systems [6].

Several recent studies have demonstrated elevated expression of FAS in tumor cells from a variety of human cancers [7–13]. Elevated serum FAS levels were also found in breast cancer patients, similar to the elevated expression in tumor tissue [14]. Activation of FAS expression and fatty acid synthesis is closely linked to malignant transformation, since upregulation of fatty acid synthesis is a very common event in tumors and their precursor lesions, and is linked to tumor virulence in population studies of human cancer. Surgically resected carcinoma tissues also display

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elevated fatty acid synthesis compared with adjacent nonneoplastic tissues [10]. Similarly, cell lines derived from such tumors also display elevation of fatty acid synthetic activity relative to nontransformed human cell lines [13, 15]. Pharmacological inhibition of FAS is selectively cytotoxic to tumor cells in culture and *in vivo* [13, 15–17], and thus represents an experimental strategy for cancer therapy.

Important mediators of FAS transcriptional regulation in liver and adipose tissue include sterol regulatory element-binding proteins (SREBPs), a family of transcription factors that activate genes involved in the synthesis of cholesterol and fatty acids and their uptake from plasma lipoproteins [18, 19]. SREBPs are synthesized as membrane-bound precursors and cleaved in the Golgi complex, releasing the amino-terminal domain to travel to the nucleus, where it binds to sterol regulatory elements located in the promoters of target genes, including FAS. In the FAS promoter, there are two independent SREBP binding sites that flank an E-box between bases −73 and −54 that confers SREBP responsiveness [20]. There are three isoforms of SREBP. Two of these, SREBP-1a and SREBP-1c, are derived from a single gene through alternate splicing, and exert greater effects on lipogenesis [21]. Indeed, the fasting/refeeding induction of lipogenesis described above is abolished in SREBP-1 knockout mice [22]. The third isoform, SREBP-2, is derived from a separate gene and acts more strongly on the cholesterol biosynthetic pathway.

The control mechanisms regulating fatty acid synthesis and FAS expression in tumors must be different from those in liver or adipose tissue, since tumors are proliferative, and are relatively insensitive to nutritional signals [23]. However, while the governing signals must be different between tumors and normal metabolically active tissues, the signal transduction pathways share some downstream elements. We and others have observed that sterol regulatory element binding protein 1 (SREBP-1) proteins are also involved in the regulation of FAS in tumor cells [11, 24]. In this study, we have used *in vitro* transformation of the immortalized but nontransformed human mammary epithelial cell line, MCF-10a, to model neoplastic activation of FAS expression, to explore the upstream regulation of lipogenesis in tumors. MCF-10a cells modestly upregulated FAS expression in response to EGF stimulation. Many investigators have shown that MCF-10a cells exhibit altered growth and morphology consistent with transformation after transfection with mutant H-*ras*. H-*ras* transformation of MCF-10a cells was EGF-dependent [25, 26]. Some H-*ras* transformed MCF-10a clones exhibited anchorage-independent growth and invasiveness *in vitro* and became tumorigenic in irradiated nude mice [25, 27, 28]. Our experiments demonstrated that H-*ras* transformation of MCF-10a cells resulted in brisk activation of lipogenesis. Neoplastic activation of FAS expression in H-*ras* transformed MCF-10a cells occurred primarily via activation of PI 3-kinase and mitogen-activated protein (MAP) kinase pathways, which in turn increased SREBP-1 levels and transcription from the FAS promoter. Two carcinoma cell lines gave similar results, indicating a broadly relevant regulatory mechanism. This result provides a likely basis for the linkage of upregulated fatty acid metabolism with cell proliferation, and with tumor virulence, since PI 3-kinase signaling and MAP kinase signaling contribute to both [29–33].

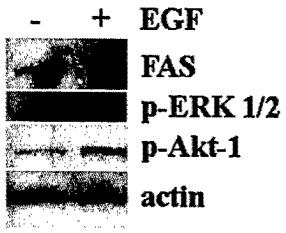

FIG. 1. EGF increases FAS expression in MCF-10a cells. MCF-10a cells were cultured in medium supplemented with 10% serum, insulin, glucocorticoids and without or with EGF (10 ng/ml). FAS enzyme level and the active forms of MAP kinases p44/42 ERK 1/2 and Akt-1 (Phospho-ERK 1/2 Thr202/Tyr204, phospho-Akt-1 Ser473) were determined in parallel by immunoblot.

MATERIALS AND METHODS

Cell lines and culture conditions. MCF-10a, MCF-7, and HCT116 cells were obtained from the American Type Culture Collection, and were cultured as recommended. Culture medium for MCF-10a cells and sublines was Ham's nutrient mixture F12:DMEM 1:1 containing 10% fetal bovine serum, epidermal growth factor (EGF) (10 ng/ml), insulin (20 μg/ml), and hydrocortisone (500 ng/ml) except where indicated. U0126 (Promega), PD98059, LY290042, and wortmannin (Calbiochem) were added from stock solutions prepared as recommended.

FAS promoter deletion mutant. The FAS promoter deletion mutant, pGL3-P1d20, was constructed using pGL3-P1 [11] as a template. pGL3-P1 contains base pairs −798 to +12 of the human FAS promoter (Genbank Accession No. HSU52428) cloned into pGL3 (Promega). The upstream and downstream promoter segments flanking the SRE/E box element at −73 to −54 were polymerase chain reaction (PCR) amplified separately using the following primers and primers based in pGL3: 5′ AACTGCAGCTTGGCTGCGCCGCCCAG 3′ and 5′ AACTGCAGCCCGGGGATGGCCGCG 3′. The PCR products were digested with *Pst*I and ligated together to generate a 20-base deletion of the SRE/E box element containing a *Pst*I site, and were reinserted into pGL3. The deletion was sequence verified prior to use in biological assays. In separate studies, this mutant has been further modified by reinsertion of the deleted segment to reconstitute wild type levels of transcriptional activity.

MCF-10a transformation assay. Subconfluent MCF-10a cells were transfected using 6 μg H-*ras* mutant, pHo6T1 [34], or 6 μg of constitutively active mutants of MEK-1 (Stratagene, pFC-MEK1) or Akt-1 (myr-HA-Akt-1, generously provided by J. Testa [35]) in FuGENE 6 (Boehringer-Mannheim) using transfection conditions specified by the manufacturer. Plates were fixed in neutral buffered

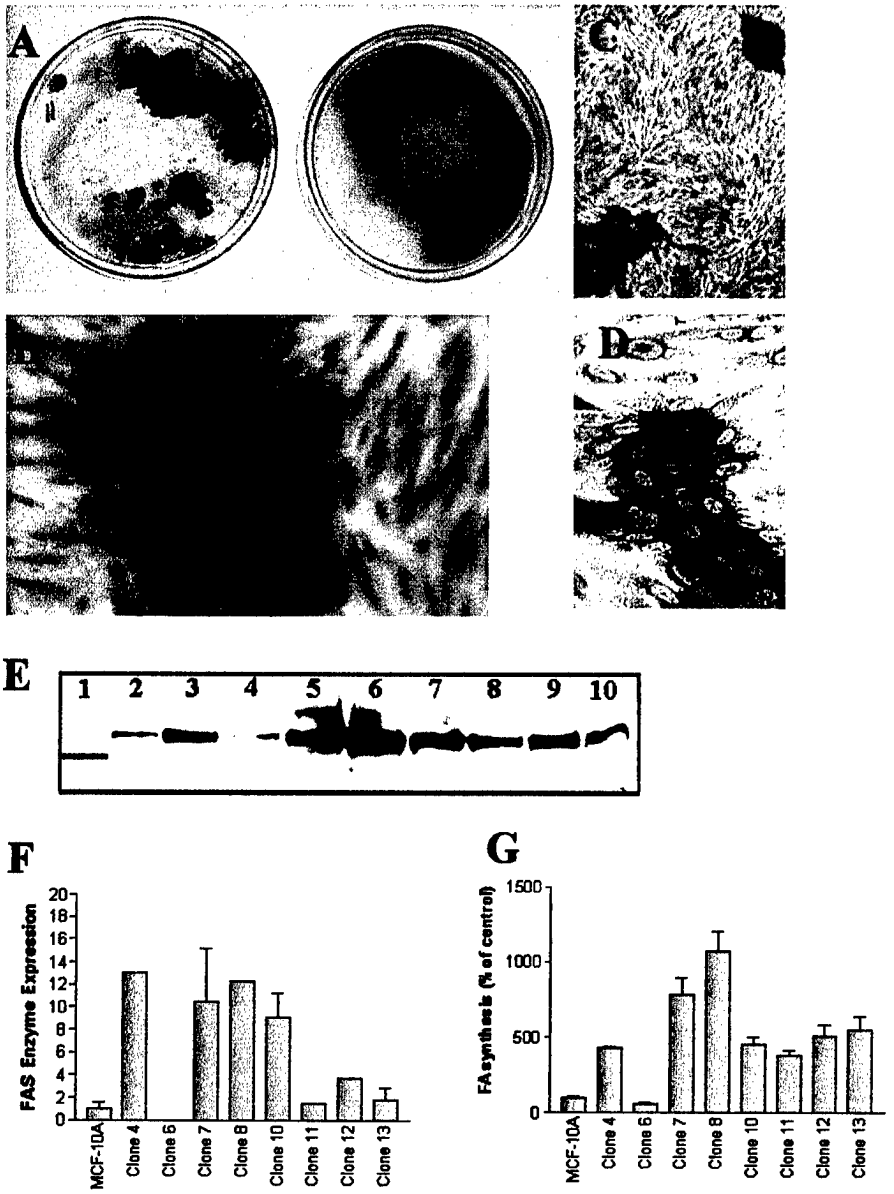

FIG. 2. Mutant H-*ras* induces EGF-dependent transformation of MCF-10a cells characterized by elevated FAS expression and fatty acid synthesis. (A) Transformation by *ras* is EGF dependent. *ras* transfected cells were plated in culture medium supplemented with 10% serum, insulin, glucocorticoids and with (plate 1) or without (plate 2) EGF. Plates were stained with crystal violet after 3 weeks. Large, dense foci of cells that have lost contact-mediated growth inhibition are present on plate 1 only. (B) High magnification shows nuclear atypia in *ras* transformed foci. (C, D) Transformation by *ras* produces elevated FAS expression. Immunocytochemical detection of FAS enzyme in monolayer cultures of MCF-10a cells 14 days after *ras* transfection. Low magnification (C) and high magnification (D). (E–G) A number of cloned H-*ras* transformed sublines were established. Transformed clones showed coordinate elevation of FAS enzyme content and fatty acid (FA) synthesis. (E) Immunoblot analysis of FAS enzyme content in cloned sublines after *ras* transfection. Lane 1: 218-kDa marker, lane 2: MCF-10a, lanes 3–10: clones 4, 6, 7, 8, 10, 11, 12, 13. (F) Quantitation of immunoblot in (E), normalized to internal control actin immunoblot. *Y* values are fold elevation over MCF-10a. (G) Quantitation of steady-state FA synthesis levels in MCF-10a cells (control) and *ras* transformed clones, determined by metabolic labeling with [U-^{14}C]acetic acid followed by organic extraction of total cellular lipids. Bars: SEM.

formalin after 2 weeks and FAS was detected by immunocytochemistry using monoclonal anti-human-FAS antibody (described in U.S. Patent 5,864,011) detected with the LSAB2 system from DAKO. Transformed foci were counted macrosocpically. Stably transformed subclones were obtained by limiting dilution cloning after transfection, and selection based on morphological transformation. A pool population of H-*ras* transformed MCF-10a cells was generated by selection of transfected cells in G418 (200 μg/ml).

Immunoblot analysis. One million cells per 60-mm plate were lysed into 100 μl Laemmli sample buffer with β-mercaptoethanol and protease inhibitors. Fifteen microliters of each lysate per lane was separated by SDS–PAGE, transferred to nitrocellulose, and exposed to primary antibodies, including anti-human-FAS [15], anti-human-SREBP-1 (Santa Cruz), anti-phospho-ERK 1/2 Thr202/Tyr204, anti-phospho-Akt-1 Ser473 (New England Biolabs), with actin (Santa Cruz) as loading control, followed by

horseradish peroxidase-conjugated secondary antibody (Pierce), and enhanced chemiluminescence plus (Amersham). Protein levels were quantified directly using a STORM imaging system (Molecular Dynamics). Calculations and graphing were performed in Prism 2.0 (GraphPad).

Determination of fatty acid synthesis pathway activity. Triplicate cultures were plated at 5×10^4/well in 1 ml in 24-well plates and incubated overnight. Fatty acid synthesis was assayed with a 2-h pulse of [U-^{14}C]acetic acid, 1 μCi/ well, followed by Folch extraction and scintillation counting [17]. Calculation, graphing, and statistical analysis were performed in Prism 2.0 (GraphPad).

Transient transfection assay for FAS promoter-luciferase constructs. Subconfluent cultures (10^5 MCF-10a cells, or 5×10^4 carcinoma cells) were transfected using 0.5 μg pGL3-P1 or pGL3-P1d20 and 0.2 μg pCMV-β (Clontech) in FuGENE 6 (Boehringer-Mannheim) using transfection conditions specified by the manufacturer. In some experiments, 0.5 μg pHo6T1, pFC-MEK1, myr-HA-Akt-1, a constitutively active N-terminal truncation mutant of SREBP-1a (SREBP-1a-NT [36], generously provided by D. Muller-Wieland), or control plasmid was cotransfected. In some experiments, transfected cells were further treated with inhibitors of intracellular signaling as described. Luciferase and β-galactosidase activities were assayed in cell lysates, between 16 and 72 h after transfection, using kit reagents from Promega, with the FAS promoter activity in each lysate represented by the relative light units per unit of *o*-nitrophenyl produced. In some experiments, immunoblots were performed in parallel on the same lysates.

Clonogenic assays. Cerulenin (Sigma) and C-75 (generously provided by C. A. Townsend, Johns Hopkins University), dissolved in DMSO, were added from 5 mg/ml stock solutions; the final concentration of DMSO in cultures was at or below 0.2%. Subconfluent cells were exposed to the indicated drug concentrations for 6 h, then were detached from the plastic with trypsin, counted, and replated in standard medium for colony formation as described [16, 17]. Clones were fixed and stained with 0.1% crystal violet (Sigma) in 10% methanol and counted 1 week later. The loss of clonogenic survival was determined from the ratio of colonies in the treated cell group to colonies in the control group. All of the experiments were done in triplicate and performed at least twice. Calculations and graphing were performed in Prism 2.0 (GraphPad).

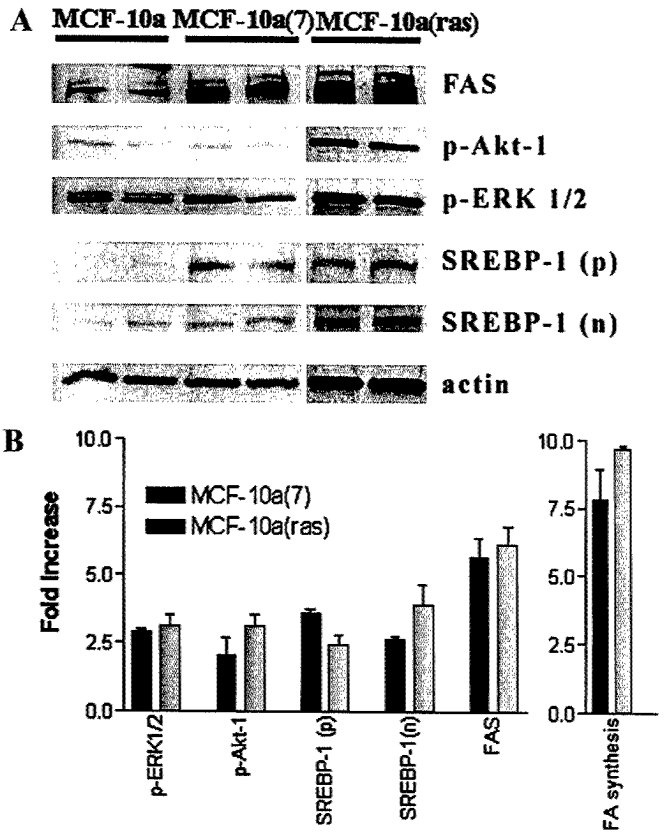

FIG. 3. *ras* transformed MCF-10a cells have increased PI 3-kinase-Akt and MAP kinase signaling and increased SREBP-1 transcription factor levels. (A) Lysates of duplicate standard cultures of MCF-10a, MCF-10a(7), and MCF-10a(ras) were analyzed in parallel by immunoblot for constitutive levels of FAS, of the active forms of MAP kinases p44/42 ERK 1/2 and Akt-1 (Phospho-ERK 1/2 Thr202/Tyr204, phospho-Akt-1 Ser473), and of precursor and mature forms of SREBP-1 transcription factor proteins. (B) Quantitation of immunoblots in (A) with actin as loading control. MCF-10a(7) and MCF-10a(ras) values are normalized to MCF-10a. Inset: Similar comparison of FA synthesis pathway activity of MCF-10a, MCF-10a(7), and MCF-10a (ras), quantitated by metabolic labeling with [U-^{14}C]acetic acid. All parameters measured showed significant increases over parental levels except phospho-Akt-1 in MCF-10a(7) (unpaired *t* test: significance defined as $P < 0.05$). Bars: SEM.

RESULTS

RAS-mediated transformation of mammary epithelial line MCF-10a produces elevated FAS expression and fatty acid synthesis. H-*ras* transformation of the immortalized but nontransformed human mammary epithelial line MCF-10a is an established mammary carcinogenesis model [25, 27, 28]. We adopted this *in vitro* transformation system to study transformation-associated changes in fatty acid synthetic metabolism. MCF-10a cells are highly responsive to EGF, which activates the PI 3-kinase-Akt and MEK-1-ERK MAP kinase pathways [26, 37]. As shown in Fig. 1, EGF alone is capable of modulating FAS expression in MCF-10a cells. MCF-10a cultures with supplementation at 10 ng/ml EGF (standard cultures) had 3-fold higher FAS expression relative to MCF-10a cultures without EGF supplementation, with similar differences in ERK 1/2 and Akt-1 phosphorylation (Fig. 1). Fatty acid synthesis activity, assayed by pulse incorporation of acetate into lipids, was also 2.6-fold higher with EGF (10 ng/ml) (not shown). As illustrated in Figs. 2A and 2B, transfection of MCF-10a cells with oncogenic H-*ras* produced foci of cells that had lost contact inhibition and had acquired transformed morphology. H-*ras* transformation was dependent on EGF supplementation of the culture medium, consistent with transformation resulting from augmentation in ligand-dependent signaling through the EGF receptor, as previously described [25]. Analysis of FAS expression in these transformed foci by immunocytochemistry demonstrated consistent upregulation with H-*ras* transformation (Figs. 2C and 2D). A number of cloned H-*ras* transformed sublines were established. Parallel determination of FAS expression by quantitative immunoblotting (Figs.

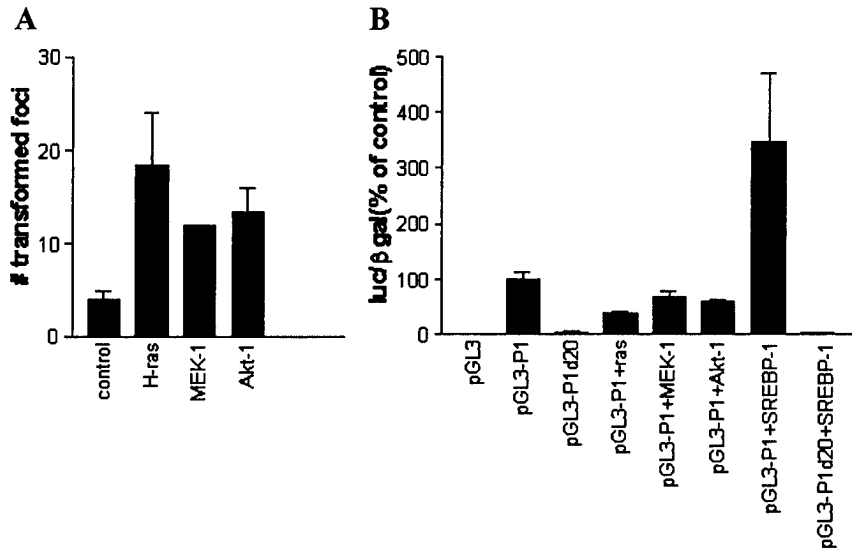

FIG. 4. H-*ras*, Akt-1, and MEK-1 upregulate FAS during oncogenic transformation but not transient transfection. (A) MCF-10a cells transfected with H-*ras*, Akt-1, or MEK-1 mutants or control vector were grown as described in Fig. 1. After 2 weeks, foci of cells with morphologically transformed phenotype were counted. Mean values of two experiments are shown. Bars: SEM. (B) Duplicate cultures of MCF-10a cells were transiently transfected with the expression plasmids listed on the *X* axis and CMV-βgal. Luciferase and β-galactosidase were measured 36 h after transfection. Results are representative of four experiments.

2E and 2F) and fatty acid synthesis activity (pulse incorporation of acetate into lipids, Fig. 2G) in the cloned sublines demonstrated upregulation of both parameters.

Ras transformed MCF-10a cells have increased PI 3-kinase-Akt and MAP kinase signaling and increased SREBP-1 transcription factor levels. We selected one of these clones, designated MCF-10a(7), for detailed comparison with the parental line, MCF-10a. FAS enzyme level and FA synthesis were elevated 5- and 8-fold in MCF-10a(7) compared with MCF-10a. FAS expression is regulated primarily at the transcriptional level in the nutritional context [3, 38] and in the limited existing studies of tumor cells [39]. Consistent with this, transient transfection of a FAS promoter-luciferase reporter construct [11] into these two cell lines demonstrated 5-fold higher transcription of the FAS promoter in MCF-10a(7) (not shown). Since EGF signaling through activated *ras* is known to upregulate a number of signal transduction pathways, including the PI 3-kinase-Akt and MEK-1-ERK MAP kinase pathways [26, 37], we compared the steady-state levels of phosphorylated (active) Akt-1 and p42/44 ERK1/2 proteins in MCF-10a and MCF-10a(7) and in a pool of H-*ras* transformed MCF-10a cells generated by G418 selection after transfection, designated MCF-10a(ras), by quantitative immunoblot analysis using phosphoprotein specific antibodies. MCF-10a(7) and MCF-10a(ras) cells demonstrated a 2- to 3-fold elevation in steady-state levels of both phospho-Akt-1 and phospho-p42/44 ERK1/2 proteins relative to MCF-10a cells (Figs. 3A and 3B). A similar analysis demonstrated 3-fold elevations in steady-state levels of both the inactive and the active precursor, nuclear forms of SREBP-1 in MCF-10a(7) and MCF-10a(ras) cells. Of note, comparison of cultures of the H-*ras* transformed lines with and without EGF supplementation, as in Fig. 1, showed less than 2-fold differences in the already elevated levels of phospho-Akt-1 and phospho-p42/44 ERK1/2 proteins, FAS, and fatty acid synthesis activity (not shown).

Akt-1 and MEK-1 can each transform MCF-10a cells and increase FAS expression. To evaluate the relative importance of PI 3-kinase-Akt and MEK-1-ERK MAP kinase pathway signaling in this transformation system, MCF-10a cells were transfected with mutant H-*ras* or with constitutively active mutants of MEK-1 (Stratagene, pFC-MEK1) or Akt-1 [35]. All three oncogenes produced similar numbers of transformed foci, which demonstrated elevated FAS expression by immunocytochemistry, similar to Fig. 2 (Fig. 4A). Transient transfection of MCF-10a cells with any of these three oncogenes together with the FAS promoter-luciferase reporter construct containing base pairs −798 to +12 of the human FAS promoter (designated pGL3-P1, Fig. 4B) did not show activation of the FAS promoter over control levels. However, cotransfection with pGL3-P1 and a constitutively active N-terminal truncation mutant of SREBP-1a [36] did show 3- to 4-fold activation of the FAS promoter over control levels, indicating that increased levels of mature SREBP-1a are sufficient to increase expression from the FAS promoter. Deletion of the SRE/E box element at −73 to −54 (the major SREBP binding site [20], designated

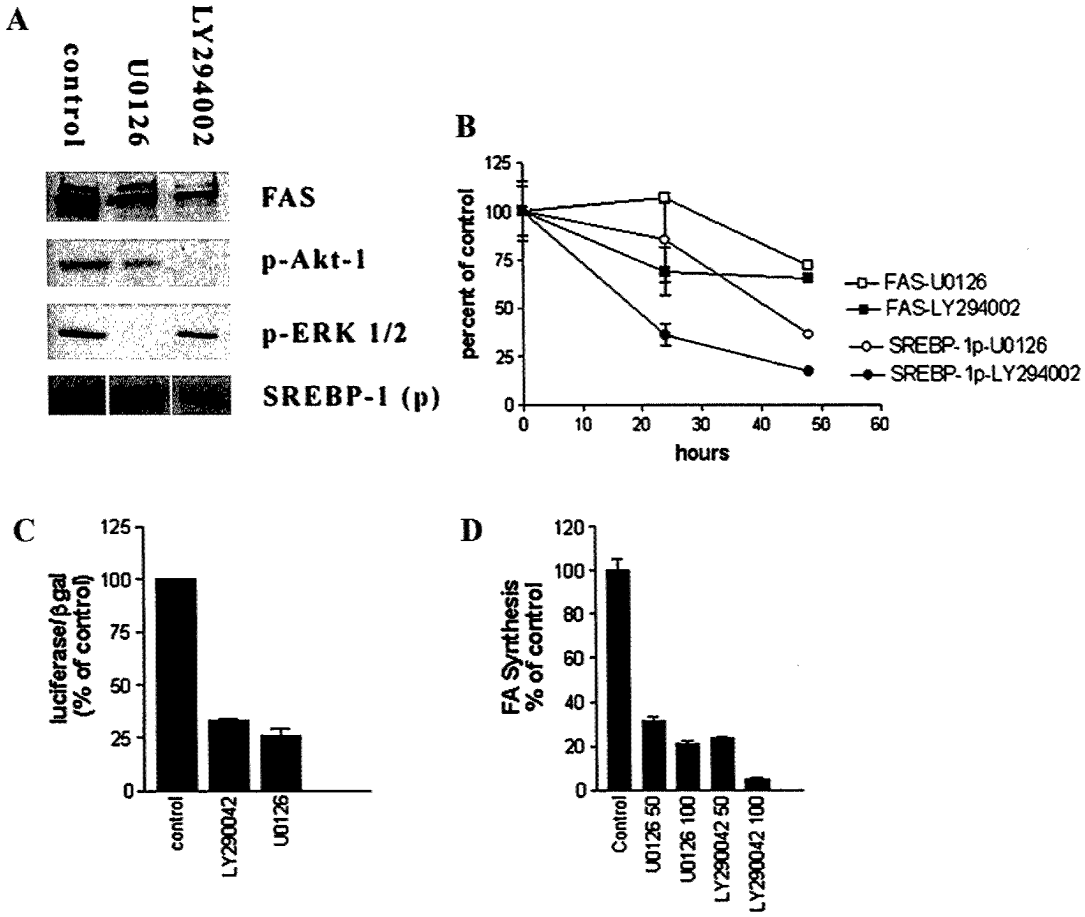

FIG. 5. Inhibition of PI 3-kinase-Akt or MEK-1-ERK MAP kinase pathway signaling in H-*ras* transformed MCF-10a cells reduces FAS and SREBP-1 levels and FAS transcription. MCF-10a(7) cells were exposed to the MEK-1 (MAPK/extra cellular signal-regulated kinase kinase) inhibitor U0126 or the PI 3-kinase inhibitor LY290042, both 100 μM, for 24 or 48 h, followed by immunoblot quantitation of FAS enzyme content, of the active forms of MAP kinases p44/42 ERK1/2 and Akt-1 (Phospho-ERK1/2 Thr202/Tyr204, phospho-Akt-1 Ser473), and of precursor and mature forms of SREBP-1 transcription factor proteins. (A) Imunoblot analysis of FAS enzyme content at 48 h, and of p-Akt-1, p-ERK1/2, and SREBP-1(p) at 24 h. (B) Quantitation of immunoblots from duplicate samples at 24 and 48 h. Bars: SEM. (C) MCF-10a(7) cells were transfected with the FAS promoter-luciferase reporter construct, pGL3-P1, and CMV-βgal, and subsequently exposed to U0126 or LY290042 (both 50 μM) for 24 h, followed by measurement of luciferase and β-galactosidase activities. (D) MCF-10a(7) cells were exposed to U0126 or LY290042 for 18 h at the indicated doses. FA synthesis pathway activity was quantitated by metabolic labeling with [U-^{14}C]acetic acid.

pGL3-P1d20) (Fig. 4B) abrogated both control and SREBP-1-stimulated expression from the FAS promoter.

Inhibition of PI 3-kinase-Akt and MEK-1-ERK MAP kinase pathway signaling in H-ras transformed MCF-10a cells reduces FAS and SREBP-1 levels and fatty acid synthesis. Next, MCF-10a(7) cells were exposed to the specific inhibitors of MEK-1 and PI 3-kinase, U0126 and LY290042, to determine the effects that reductions in PI 3-kinase-Akt or MEK-1-ERK MAP kinase pathway signaling would produce on SREBP-1 and FAS levels and on lipogenesis in these *ras* transformed cells. MCF-10a(7) cells were analyzed by immunoblot after 24 and 48 h of inhibitor exposure. Some crosstalk between MAP kinase and PI 3-kinase pathway signaling was detected. At 24 h, U0126 (100 μM) reduced phospo-ERK1/2 below detectable levels, and reduced phospho-Akt-1 to 60% of control levels. LY290042 (100 μM) reduced phospho-Akt-1 below detectable levels, and reduced phospo-ERK1/2 to 70% of control levels (Fig. 5A). FAS enzyme levels were reduced by 30 to 60% after 48 h exposure to either U0126 or LY290042 at 100 μM (Figs. 5A and 5B, and additional data not shown). In parallel, the inactive precursor form of SREBP-1 was reduced by greater than 50% within 24 h by LY290042, and within 48 h by U0126, and the active, nuclear form was reduced by about 20% within 24 h by both inhibitors, suggesting that MAP kinase and PI 3-kinase pathway signaling regulates SREBP-1 levels, directly or indirectly. Both U0126 and

LY290042 also produced substantial reductions in FAS promoter-luciferase reporter activity to 26 and 33% of control levels, respectively, within 24 h in transiently transfected MCF-10a(7) cells (Fig. 5C). Fatty acid synthesis pathway activity was also reduced to 30% or less of control levels by either U0126 or LY290042 within 18 h (Fig. 5D).

PI 3-kinase-Akt and MAP kinase signaling also regulates SREBP-1 transcription factor and FAS enzyme levels in carcinoma cells. Next, since H-*ras* transformation of MCF-10a cells is an *in vitro* model of carcinogenesis, two human carcinoma cell lines with highly active fatty acid synthesis were selected for analysis to evaluate whether MAP kinase and PI 3-kinase pathway signaling is an important regulator of lipogenesis in naturally occurring tumors. MCF-7 breast cancer cells and HCT116 colon cancer cells were first analyzed by immunoblot after 24 and 48 h of exposure to either U0126 or LY290042 at 100 μM, and FAS levels in both carcinoma lines were reduced by about half by both inhibitors (Fig. 6A). Both U0126 and LY290042 also produced substantial, dose-dependent reductions in FAS promoter activity within 24 h that were sustained at 48 h in transiently transfected carcinoma cells (Figs. 6B and 6C). Fatty acid synthesis was also substantially reduced by both U0126 and LY290042 in both carcinoma lines, similar to the results with MCF-10a(7) (not shown).

To confirm and extend these results, MCF-7 and HCT116 cells were next evaluated after exposure to either the MAP kinase inhibitor PD98059 or the PI 3-kinase inhibitor wortmannin. Low doses of inhibitor were used to allow maximal selectivity for either MAP kinase or PI 3-kinase inhibition. Tumor cells were transiently transfected with the FAS promoter-luciferase reporter construct and exposed to inhibitor for 36 h, followed by parallel determinations of phospho-Akt-1 and ERK 1/2, FAS and SREBP-1 protein levels, and FAS promoter activity (Fig. 7). PD98059 at 25 and 50 μM produced substantial and selective inhibition of MAP kinase signaling in both carcinoma lines, reduced levels of SREBP-1 precursor and mature transcription factor, and inhibited FAS promoter activity to 35% or less of control levels. Similarly, wortmannin at 1 and 3 μM also produced substantial inhibition of PI 3-kinase signaling in both carcinoma lines, although in MCF-7 cells MAP kinase signaling comodulated with PI 3-kinase signaling. Levels of SREBP-1 precursor and mature transcription factor were reduced by both inhibitors, and FAS promoter activity was inhibited to less than 15% of control levels in both carcinoma lines. FAS protein levels were reduced by 25–35% after 36 h exposure to either inhibitor in both cell lines (not shown). Similar to MCF-10a(7) cells, the FAS promoter deletion mutant, pGL3-P1d20, had transcriptional activity less than 10% that of the wild-type promoter in both cell lines (not shown).

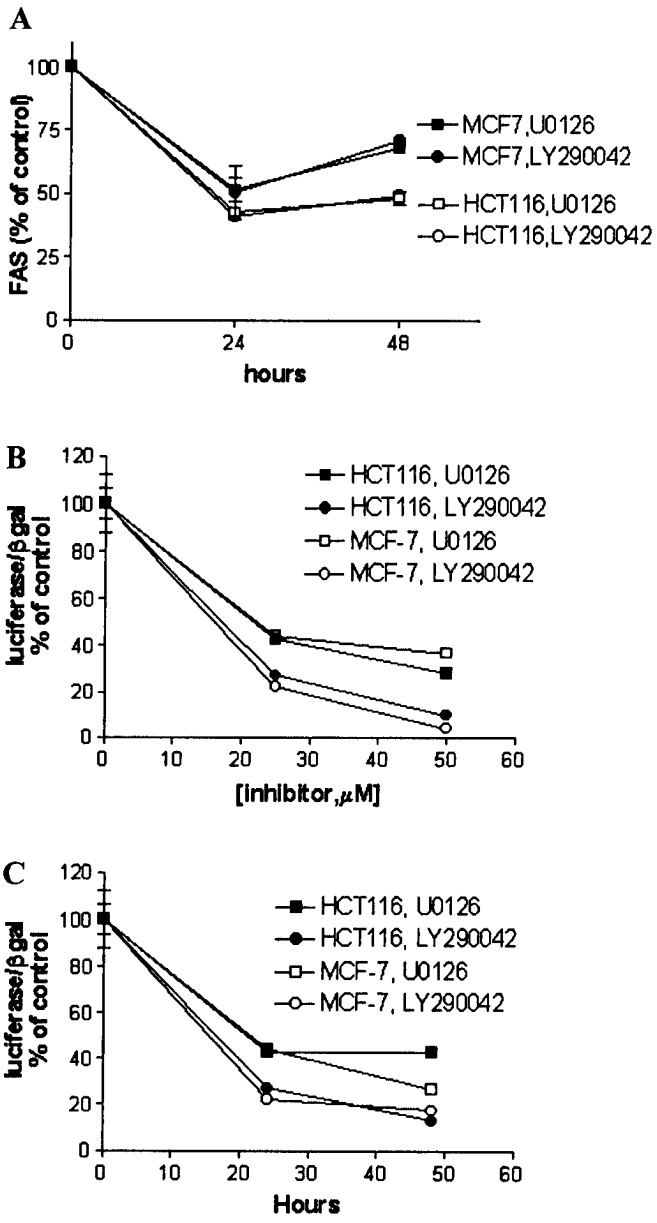

FIG. 6. Inhibition of PI 3-kinase-Akt or MEK-1-ERK MAP kinase pathway signaling reduces FAS levels and FAS transcription in carcinoma cells. (A) Immunoblot analysis of FAS enzyme content after 24 and 48 h exposure of HCT116 colon cancer cells or MCF-7 breast cancer cells to the MEK-1 inhibitor U0126 or the PI 3-kinase inhibitor LY290042, both 100 μM. (B) HCT116 or MCF-7 cells were transfected with the FAS promoter-luciferase reporter construct, pGL3-P1, and CMV-βgal, and subsequently exposed to U0126 or LY290042 at the indicated doses for 24 h, followed by measurement of luciferase and β-galactosidase activities. (C) HCT116 or MCF-7 cells were transfected as in (B), and subsequently exposed to U0126 or LY290042 (both 50 μM) for the indicated times, followed by measurement of luciferase and β-galactosidase activities. Bars: SEM.

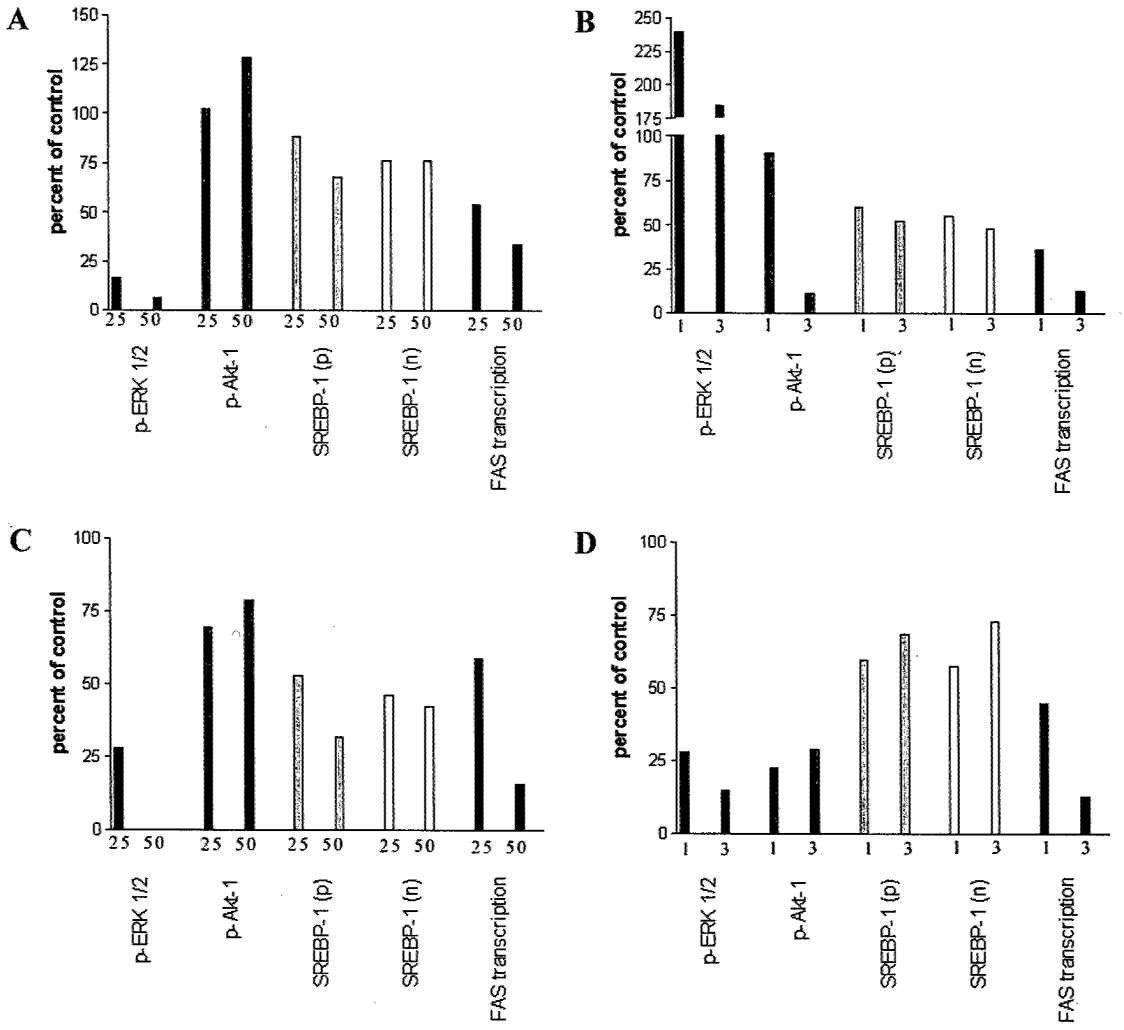

FIG. 7. PI 3-kinase-Akt and MAP kinase signaling regulate SREBP-1 transcription factor levels and FAS transcription in carcinoma cells. Panels show quantitation of phospho-Akt-1 and phospho-ERK 1/2, SREBP-1 transcription factor levels, and FAS promoter activity in: HCT116 colon cancer cells treated for 36 h with PD98059 at 25 and 50 μM (A) or wortmannin at 1 and 3 μM (B), or MCF-7 breast cancer cells treated for 36 h with PD98059 at 25 and 50 μM (C) or wortmannin at 1 and 3 μM (D). Values are given as percentages of untreated controls. Results are representative of two or more experiments.

FAS inhibitors are more potently growth inhibitory for H-ras transformed than for parental MCF-10a breast epithelial cells. Finally, since earlier comparisons of unrelated cells with different levels of FA synthesis pathway activity had demonstrated increased FAS inhibitor cytotoxicity in cells with high FA synthesis pathway activity, the effects of FAS inhibitors were compared in the closely related MCF-10a, MCF-10a(7) and MCF-10a(ras). MCF-10a and the sublines had very similar growth rates (not shown). The sensitivity of MCF-10a cells and the H-*ras* transformed sublines to the FAS inhibitors cerulenin and C-75 was compared in clonogenic assays (Fig. 8). Both MCF-10a(7) cells and MCF-10a(ras) cells demonstrated substantial, dose-dependent loss of clonogenic activity after FAS inhibitor exposure, while MCF-10a cells retained more clonogenic activity than either transformed line.

DISCUSSION

Increased MAP kinase and PI 3-kinase signaling leads to activation of lipogenesis in neoplastic cells. This study explores the biological basis for increased FAS expression and activation of fatty acid synthesis in neoplastic cells using MCF-10a human breast epithelial cells transformed by oncogenic mutant H-*ras* as a model of neoplastic transformation. Comparison of parentals with stably transformed MCF-10a cells demonstrated that *ras*-mediated activation of MAP kinase and PI 3-kinase signaling led to increased expression of FAS and increased endogenous fatty acid synthesis.

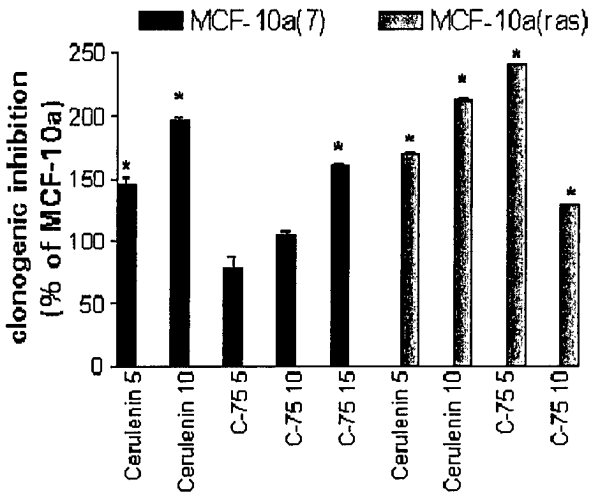

FIG. 8. FAS inhibitors cerulenin and C-75 are more potently growth inhibitory for H-*ras* transformed than for parental MCF-10a breast epithelial cells. MCF-10a, stably transfected H-*ras* transformed MCF-10a(7), and MCF-10a(ras) cells were exposed for 6 h to cerulenin or C-75 at the indicated doses, then replated in regular medium. Reduction in clonogenic activity of MCF-10a(7) or MCF-10a(ras) cells after FAS inhibition was normalized to that of MCF-10a cells. Determinations were in triplicate. Bars: SEM. *Significant differences in clonogenic inhibition (unpaired t test: $P < 0.05$). Results are representative of two experiments.

Constitutively active forms of MEK-1 and Akt-1 performed similarly to H-*ras* in the MCF-10a focus assay, suggesting that both MAP kinase signaling and PI 3-kinase signaling are capable of upregulating FAS in neoplastic cells. The ability of the three oncogenes—H-*ras,* MEK-1, and Akt-1—to induce FAS in stably transfected, but not transiently transfected cells is probably due to the indirect mechanism of their effect on FAS. Increased FAS expression was produced in major part by upregulation of SREBP-1 transcription factor levels, leading to increased FAS transcription. Deletion of the major SREBP binding site from the FAS promoter abrogated transcription in *ras* transformed MCF-10a(7) cells and in carcinoma cells. Inhibition of either MAP kinase or PI 3-kinase signaling led to reduced SREBP-1 transcription factor levels, reduced FAS transcription, and reduced FAS levels and activity.

FAS elevation in many different tumors. Activated lipogenesis is a common feature of tumor cells from a variety of human cancers that contain many different oncogenic changes [7–13]. We selected HCT116 and MCF-7, two carcinoma cell lines with high fatty acid synthesis that are typical of many that we have studied, to evaluate whether regulation of lipogenesis through MAP kinase or PI 3-kinase signaling is ubiquitous in tumor cells. HCT116 cells have activated MAP and PI 3-kinase signaling due to a mutation in codon 13 of the K-*ras* protooncogene [40]. MCF-7 cells have wild-type *Ras* genes, similar to most breast cancers, but have elevated expression of *EGFR/HER2,* also leading to activated MAP and PI 3-kinase signaling [41, 42]. Both of these cell lines demonstrated regulation of lipogenic gene expression by MAP kinase or PI 3-kinase signaling modulating SREBP-1 transcription factor levels, comparable to H-*ras* transformed MCF-10a cells. Similarly, Swinnen *et al.* have observed modulation of SREBP-1 and FAS levels by EGF stimulation of the prostate carcinoma line LNCaP [43]. And we have observed elevated expression of FAS and SREBP-1 protein in primary human colorectal carcinoma tissues relative to adjacent nonneoplastic epithelium [11]. We would predict that a variety of oncogenic changes occurring in growth factor signal transduction pathways, including growth factor receptor overexpression or mutation, kinase activation, or phosphatase inhibition or loss, might activate MAP kinase or PI 3-kinase signaling, with resulting modulation of SREBP-1 levels and activation of lipogenesis. Linkage of neoplastic lipogenesis to activated MAP and PI 3-kinase signaling provides a probable basis both for FAS upregulation in certain cancer precursor lesions [10, 44] and for its association with biologically aggressive subsets of human carcinomas [7, 12].

Neoplastic lipogenesis is unresponsive to nutritional regulation. In contrast to nutritional regulation of lipogenesis in liver or adipose tissue, tumor cell changes in fatty acid metabolism are insensitive to both anabolic and catabolic hormones [23, 45–47]. Nutritional insensitivity facilitates disease progression in tumor-bearing hosts during malnourished conditions. Recent studies of nutritional regulation of FAS expression have demonstrated that PI 3-kinase-Akt signaling is downstream of insulin in the adipocyte lipogenic response [4]. Our current results suggest that PI 3-kinase-Akt signaling may represent a common downstream element regulating lipogenesis in both nutritional and neoplastic settings. The nutritional insensitivity of the tumor cell would then be explained by the fact that PI 3-kinase signaling is activated primarily by oncogenic changes rather than insulin in neoplastic cells.

Neoplastic lipogenesis as an experimental therapeutic target. We have previously observed that pharmacological inhibition of FAS is selectively cytotoxic to tumor cells with elevated fatty acid synthesis in culture and *in vivo* [13, 15–17], apparently because elevated fatty acid synthesis rendered tumor cells susceptible to toxic fluxes in the substrate, malonyl-CoA [17]. Our current data demonstrate that H-*ras* transformation, with its associated increase in fatty acid synthesis pathway activity, sensitized MCF-10a breast epithelial cells to the FAS inhibitors, cerulenin and C-75, in clonogenic assays. This result is consistent with the previous observations, and suggests that FAS inhibi-

tors may be toxic to other neoplastic cells in which altered growth factor signaling pathways have activated MAP kinase or PI 3-kinase signaling and lipogenesis.

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Appendix C

Regulation of Fatty Acid Synthase Expression in Breast Cancer by Sterol Regulatory Element Binding Protein-1c

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Running Title: SREBP-1c Regulation of FAS in Breast Cancer

SREBP-1c Regulation of Fatty Acid Synthase in Breast Cancer

Abstract

Activation of fatty acid synthase (FAS) expression and fatty acid synthesis is a common event in human breast cancer. Sterol regulatory element binding proteins (SREBPs), are a family of transcription factors that regulate genes involved in lipid metabolism, including FAS. SREBP-1c expression is induced in liver and adipose tissue by insulin and by fasting/refeeding, and is critical for nutritional regulation of lipogenic gene expression. In contrast, upregulation of fatty acid metabolism during *in vitro* transformation of human mammary epithelial cells, and in breast cancer cells, were driven by increases MAP kinase and PI 3-kinase signaling, which increased SREBP-1 levels. SREBP-1a was more abundant than SREBP-1c in many proliferative tissues and cultured cells, and was thus a candidate to regulate lipogenesis for support of membrane synthesis during cell growth. We now show that SREBP-1c and FAS mRNA were both increased by H-*ras* transformation of MCF-10a breast epithelial cells, and were both reduced by exposure of MCF-7 breast cancer cells to the MAP kinase inhibitior, PD98059, or the PI 3-kinase inhibitior, wortmannin, while SREBP-1a and SREBP-2 showed less variation. Similarly, the mRNA levels for FAS and SREBP-1c in a panel of primary human breast cancer samples showed much greater increases than did those for SREBP-1a and SREBP-2, and were significantly correlated with each other, suggesting coordinate regulation of SREBP-1c and FAS in clinical breast cancer. We conclude that regulation of FAS expression in breast cancer is achieved through modulation of SREBP-1c, similar to the regulation in liver and adipose tissue, although the upstream regulation of liopgenesis differs in these tissues.

Key Words: Fatty Acid Synthase, EGF, MAP Kinase, PI 3-Kinase, SREBP-1c, breast cancer, real-time RT-PCR

Introduction

Fatty acid synthase (FAS, E.C. 2.3.1.85) is a multifunctional enzyme that catalyzes the synthesis of long chain fatty acids from Acetyl-CoA and Malonyl-CoA [1, 2]. The expression level of FAS reflects the lipogenic capacity of various cells and tissues. Most studies of FAS expression have focused on liver or adipose tissues where the bulk of physiological lipogenesis occurs, regulated by nutritional and hormonal conditions. Feeding a previously fasted animal a high-carbohydrate meal induces FAS expression, primarily via transcription induction, coordinately regulated by glucose, insulin, glucagon, glucocorticoids, and thyroid hormone [3-6]. Distinct from this nutritional context, certain rapidly proliferating cells and tissues, including proliferative endometrium and some fetal tissues, have high FAS expression [7, 8]. Fatty acid synthesis supports membrane synthesis in proliferating cells; most endogenously synthesized fatty acids in proliferating cultured cells are incorporated into membrane lipid, and endogenous fatty acid synthesis activity modulates phospholipid synthesis in experimental systems [9, 10].

Several studies have demonstrated elevated expression of FAS in population studies of human breast cancers, and in *in situ* ductal and lobular carcinomas. While FAS is induced during lactation by progesterone and prolactin, activation of FAS expression and fatty acid synthesis in breast cancer appeared linked to neoplastic transformation, since it was common in both pre- and postmenopausal women in tumors and their precursor lesions, was linked to tumor virulence, and was independent of the estrogen and progesterone receptor expression status of the tumors [11-15]. Elevated serum FAS levels were also found in breast cancer patients, similar to the elevated expression in tumor tissue [16].

SREBP-1c Regulation of Fatty Acid Synthase in Breast Cancer

Recent reports from a number of groups have demonstrated an important role for SREBP-1 transcription factors in the regulation of the FAS gene in liver and adipose tissue. Sterol regulatory element binding proteins (SREBPs), are a family of transcription factors of the basic helix-loop-helix-leucine zipper type that activate genes involved in the synthesis of cholesterol and fatty acids including FAS, via binding to sterol regulatory elements in their promoters [17-19]. There are three SREBP isoforms. Two of these, SREBP-1a and SREBP-1c, are derived from a single gene that has two promoters, and two alternate first exons which are alternately spliced to a common downstream open reading frame. These isoforms exert greater effects on lipogenesis *in vitro* and *in vivo* [20-22]. The third isoform, SREBP-2, is derived from a separate gene, has 50% homology to the SREBP-1 proteins, and acts more strongly on the cholesterol biosynthetic pathway [20, 23]. The SREBP-1a isoform has a longer, N-terminal activation domain, and is a more potent transcriptional activator than the SREBP-1c isoform, [22]. However, the SREBP-1c isoform mRNA is induced several fold by insulin, and after fasting/refeeding [24, 25]. And, the fasting/refeeding induction of fatty acid synthesis is abolished in SREBP-1 knockout mice, and markedly diminished in selective SREBP-1c knockouts [26, 27]. These observations suggest that nutritional lipogenic regulation is achieved primarily through modulation of SREBP-1c levels. Similarly, progesterone, which induces lipogenic gene expression in both adipose tissue and in breast tissue, appears to act via induction of SREBP-1c [28].

In our ongoing efforts to understand the regulation of lipogenesis in cancer, we have observed coordinate upregulation of SREBP-1 protein with FAS in colorectal carcinomas as well as in proliferative fetal tissues [8, 29]. More recently, we and others have shown that altered growth factor signals in neoplastic cells act via activation of phosphatidylinositol (PI) 3-kinase

and mitogen-activated protein (MAP) kinase pathways, which in turn increase SREBP-1 protein levels and transcription from the FAS promoter [30, 31]. These studies of SREBP-1 protein were performed using antibodies that do not distinguish between the SREBP-1a and SREBP-1c isoforms, since isoform specific antibodies were not available. Some investigators have suggested that SREBP-1a might be the more important SREBP isoform in lipogenic regulation for support of membrane synthesis during cell growth, since it's mRNA is much more abundant than that of SREBP-1c in proliferative tissues like spleen, and in many cultured cell lines [32, 33]. We examined this question using real-time RT-PCR to measure the relative abundance of FAS mRNA, and of the mRNAs for the three isoforms of SREBP during experimental modulation of the growth factor signals that regulate lipogenesis in tumor cells. H-*ras* transformation of MCF-10a breast epithelial cells produced coordinate increases in the mRNA levels for FAS and SREBP-1c while exposure of MCF-7 breast cancer cells to MAP or PI 3-kinase inhibitors coordinately reduced FAS and SREBP-1c mRNA levels. The analysis was extended to a panel of human breast cancer and benign breast tissue samples, and cell lines derived from these tissues. In most tumors the mRNA levels for FAS and SREBP-1c showed much greater increases relative to the reference sample than did those for SREBP-1a and SREBP-2, suggesting coordinate regulation of FAS and SREBP-1c in clinical breast cancer. The data support that modulation of SREBP-1c expression confers regulation of FAS expression in breast cancer, similar to the regulation in liver and adipose tissue.

Materials and Methods

Tissue Samples and Cell Lines: A total of 22 human breast tumor samples, 21 infiltrating carcinomas and 1 *in situ* carcinoma, and one sample of benign breast tissue were

obtained from the Department of Pathology at Johns Hopkins University School of Medicine. The primary tissue samples were collected and frozen at the time of surgery at the Johns Hopkins Hospital or the Johns Hopkins Bayview Medical Center. Frozen sections of these tissues were placed on Rnase-Zap (Ambion)-treated slides and microdissected as described previously [34] prior to extraction of RNA. MCF-10a (immortalized, non-transformed breast epithelial cells) and MCF-7 breast cancer cells were obtained from the American Type Culture Collection. Primary breast epithelial cultures were obtained from Clonetics. All cells were cultured as recommended by the supplier. Culture media for established cell lines contained 10% fetal bovine serum (Hyclone) except for MCF-7 cell treatment with EGF, where 1% serum was used. Serum provided the only lipid supplementation in these cultures. The H-*ras* transformed MCF-10a lines have been described [30]. PD98059 and wortmannin (Calbiochem) were added to MCF-7 cell cultures from stock solutions prepared as recommended. Total RNA was isolated from the tissues and cell lines using Trizol reagent (Life Technologies).

Real-Time RT-PCR: One μg of total RNA from each sample was used to generate cDNA using the Taqman reverse transcription reagents (PE Applied Biosystems). The SYBR Green I assay and the GeneAmp 5700 Sequence Detection system were used for detecting real-time PCR products from 4 μl of the reverse-transcribed RNA samples (from 200-μl total volume). Primers for each lipogenic gene were designed to cross intron-exon boundaries to distinguish PCR products generated from genomic versus cDNA template. Each PCR reaction was optimized to ensure that a single band of the appropriate length (62–226 bp) was amplified and that no bands corresponding to genomic DNA amplification or primer-dimer pairs were present. The following primers were used: SREBP1a-F: CGCTGCTGACCGACAT; SREBP1c-F: GCCATGGATTGCACTTT; SREBP1-R: CAAGAGAGGAGCTCAATG; SREBP2-F:

CTTTGATATACCAGAATGCAG; SREBP2-R: CAGGCTTTGGACTTGAGGCTG; FAS-F: GAAACTGCAGGAGCTGTC; FAS-R: CACGGAGTTGAGCCGCAT; GAPDH-F: GAAGGTGAAGGTCGGAGTC; GAPDH-R: GAAGATGGTGATGGGATTTC. The PCR cycling conditions were as follows: 2 min at 50°C for AmpErase UNG incubation; 10 min at 95°C for AmpliTaq Gold activation; and 40 cycles for the melting (95°C, 15 s) and annealing/extension (60°C for 1 min) steps. PCR reactions for each template were performed in duplicate in one 96-well plate per gene-specific primer pair tested. For analysis of primary tumor mRNA samples, MCF-10a and MCF-7 were included in each plate as internal controls. The comparative CT method (PE Applied Biosystems) was used to determine relative quantitation of gene expression for each gene compared with the GAPDH control. The relative difference between GAPDH and each experimental value was calculated. This value was then normalized to a reference sample, to determine the relative fold elevation in mRNA level for each lipogenic gene in the experimental samples relative to the reference sample. Graphing and statistical analyses were performed in Prism 2.0 (GraphPad).

Results

SREBP-1c and FAS mRNAs are coordinately elevated in H-*ras* transformed MCF-10a breast epithelial cells. Immunohistochemical studies of breast cancer have shown that a subset of carcinomas express elevated levels of FAS enzyme relative to benign breast epithelium [11-15]. We have previously shown that *in vitro* transformation of MCF-10a cells by mutant H-*ras* results in upregulation of FAS expression and fatty acid synthesis driven by increases in EGF signaling, acting in major part through the MAP kinase and PI 3-kinase signaling cascades [30].

In vitro transformation of MCF-10a cells provides a model for lipogenic regulation in breast cancer. We used real-time RT-PCR to compare the mRNAs levels for FAS and for the three isoforms of SREBP in two H-*ras* transformed MCF-10a lines with those in the parental MCF-10a cells, to determine the changes in lipogenic gene expression produced by ras transformation. As shown in Figure 1, both FAS and SREBP-1c mRNA levels were substantially increased in the 2 H-*ras* transformed lines, while both SREBP-1a and SREBP-2 mRNA levels showed little change. MCF-7 breast carcinoma cells, shown for comparison, have similar lipogenic gene expression levels to H-*ras* transformed MCF-10a cells.

SREBP-1c and FAS mRNAs are coordinately modulated by changes in EGF signaling in breast cancer cells. To directly test whether augmentation or inhibition of EGF signaling would coordinately modulate FAS and SREBP-1c mRNA levels, MCF-7 breast carcinoma cells were exposed to EGF or to the inhibitors of MAP and PI 3-kinases, PD98059 and wortmannin, which have previously been shown to down regulate SREBP-1 levels and decrease transcription from the FAS promoter, reducing FAS expression and fatty acid synthesis in MCF-7 cells ([30], Figure 2). Exposure to EGF [10ng/ml] for 12h induced a 2-fold increase in SREBP-1c mRNA and a 3.5-fold increase in FAS mRNA, while both SREBP-1a and SREBP-2 mRNA levels showed little change. The MAP kinase inhibitior, PD98059, and the PI 3-kinase inhibitior, wortmannin each reduced FAS mRNA levels. SREBP-1c mRNA levels were profoundly reduced within 6h, followed by marked reduction in FAS mRNA levels by 12h. The sequential reduction of first SREBP-1c mRNA followed by FAS mRNA is compatible with the expected signaling cascade. Both SREBP-1a and SREBP-2 mRNA levels also showed some early reduction, particularly with PD98059 treatment.

SREBP-1c and FAS mRNAs are coordinately elevated in a subset of primary breast cancers. The coordinate regulation of SREBP-1c and FAS mRNA levels in breast cell lines suggested that these messages would also show coordinate regulation in breast cancers with elevated FAS. We therefore used real-time RT-PCR to determine the mRNA levels for FAS and for the three isoforms of SREBP in a panel of 22 human breast cancer tissues, and 3 samples of benign breast epithelium (one tissue sample, one primary breast epithelial culture, and MCF-10a cells). The breast cancer cell line, MCF-7, was also included. Figure 3 shows 3 scatter diagrams correlating the FAS mRNA levels in these samples with the mRNA levels of each of the SREBP isoforms. The FAS mRNA levels in the 3 samples of benign breast epithelium varied less than 3-fold. Using MCF-10a as the reference sample, 14 breast cancer samples had 4-fold or greater (up to 112-fold) elevation of FAS mRNA, and 9 did not. The SREBP-1c mRNA levels were low in the benign samples, showed up to 160-fold elevation in the breast cancer samples, and were significantly correlated with the FAS mRNA levels (Spearman $r = 0.66$, $p = 0.0002$). Both SREBP-1a and SREBP-2 mRNA levels showed only up to 13-fold elevation, and most samples were less than 4-fold elevated. The FAS and SREBP-2 mRNA levels were also significantly correlated (Spearman $r = 0.59$, $p = 0.001$), while the FAS and SREBP-1a mRNA levels showed no correlation.

Discussion

The data presented in this study provide evidence that FAS and SREBP-1c mRNA levels are coordinately regulated in breast cancer cells, both in primary tumors and in cell lines. The extension of our functional analysis to clinical specimens was facilitated by the use of highly quantitative real-time RT-PCR to analyze expression of several genes in a large number of

specimens. Considered together with the larger body of published data on regulation of lipogenic gene expression by SREBP transcription factors, it seems likely that SREBP-1c plays a similar role in the regulation of neoplastic lipogenesis to its role in the regulation of nutritional lipogenesis. As suggested by other investigators, SREBP-1c may provide a key control point to which several hormone signaling pathways may converge for the regulation of lipogenesis [28]. In contrast, SREBP-1a and FAS mRNA levels did not show coordinate regulation in breast cancer. Thus, although SREBP-1a is the more potent transcription factor, it is unlikely to be the dominant activator of neoplastic lipogenesis. SREBP-2 mRNA levels were also significantly correlated with FAS mRNA levels, though they were much less variable. This may reflect a contribution by SREBP-2 to lipogenic regulation in tumor cells, or may indicate modest activation of cholesterol metabolism.

If, as our observations suggest, SREBP-1c regulation of FAS expression in neoplastic and nutritional contexts is similar, then the differences in lipogenic regulation in these two contexts must lie in the upstream signals that regulate SREBP-1c. While insulin activation of PI 3-kinase signaling leads to increased transcription from the SREBP-1c promoter in liver, lipogenesis in tumors appears to result from activation of MAP and PI 3- kinase signaling related to neoplastic transformation [30, 35]. These differences in upstream signaling may be adequate to account for the ability of many tumors to sustain fatty acid synthetic activity when insulin levels and other nutritional signals in the host animal do not support hepatic fatty acid synthesis, and thus may contribute to malignant growth [36, 37].

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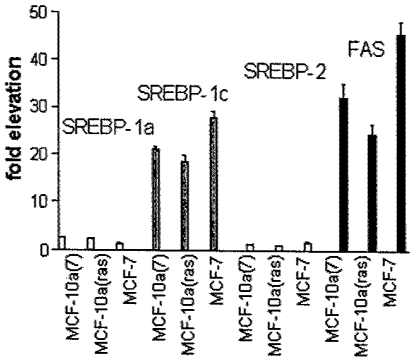
Figure Legends

Figure 1. SREBP-1c and FAS mRNAs are coordinately elevated in 2 H-*ras* transformed MCF-10a lines relative to MCF-10a. mRNA levels of FAS and the three SREBP isoforms were measured using real-time RT-PCR in breast epithelial cell line, MCF-10a, two MCF-10a sublines, each transformed by mutant H-ras (designated MCF-10a(7) and MCF-10a(ras)), and MCF-7 breast cancer cells. Relative mRNA levels were determined by first normalizing each lipogenic mRNA to GAPDH, then determining fold elevation over MCF-10a levels. Results are representative of 2 experiments.

Figure 2. SREBP-1c and FAS mRNAs are coordinately modulated by changes in EGF signaling in breast cancer cells. MCF-7 breast carcinoma cells were exposed to EGF [10ng/ml] for 12h **(A),** or to the inhibitors of MAP and PI 3-kinases, PD98059 [25 μM] **(B)** or wortmannin [3 μM] **(C)** for the indicated times. mRNA levels of FAS and the three SREBP isoforms were then measured using real-time RT-PCR. Relative mRNA levels were determined by first normalizing each lipogenic mRNA to GAPDH, then determining fractional change relative to untreated MCF-7 cells. Results are representative of 2 or more experiments.

Figure 3. SREBP-1c and FAS mRNAs are coordinately elevated in a subset of primary breast cancers. mRNA levels of FAS and the three SREBP isoforms were measured using real-time RT-PCR in a panel of 22 human breast cancer tissues and MCF-7 cells (closed symbol), and 3 samples of benign breast epithelium: one tissue sample, one primary culture, and the non-transformed cell line, MCF-10a (open symbol). MCF-10a was the reference sample.

Scatter diagrams show correlations of the FAS mRNA levels in these samples with the mRNA levels of each of the SREBP isoforms. **A.** The SREBP-1c mRNA levels were significantly correlated with the FAS mRNA levels (Spearman $r = 0.66$, $p = 0.0002$). **B.** FAS and SREBP-1a mRNA levels showed no correlation (Spearman $r = 0.22$, $p = 0.27$). **C.** The FAS and SREBP-2 mRNA levels were significantly correlated (Spearman $r = 0.59$, $p = 0.001$).



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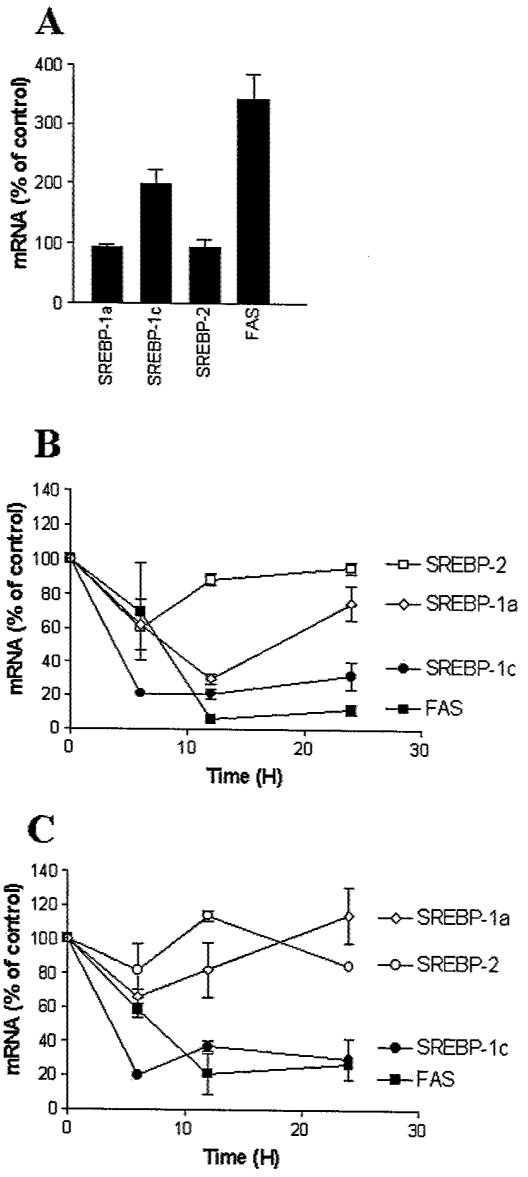
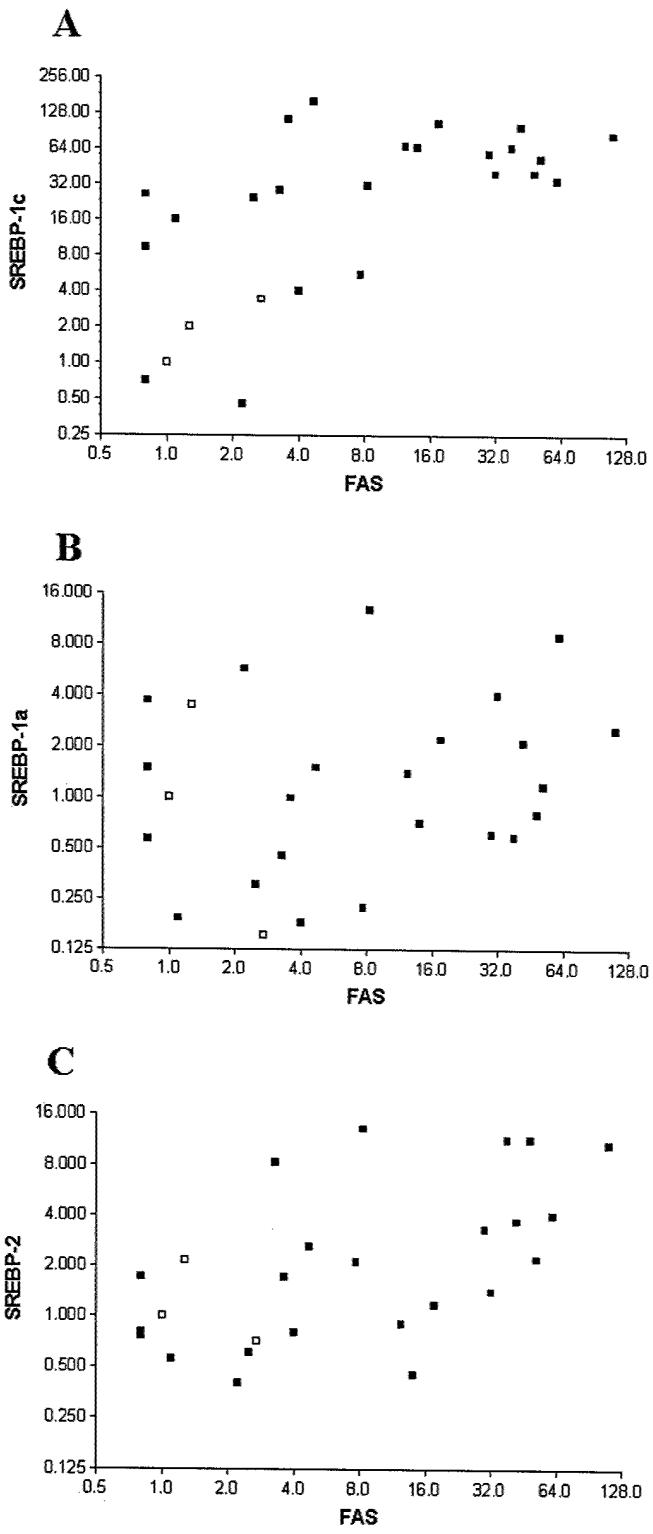


Yang et. al. Figure 2



Yang et. al. Figure 3