Research Article

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Inactivation of fatty acid synthase impairs hepatocarcinogenesis driven by AKT in mice and humans

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Background & Aims: Cumulating evidence underlines the crucial role of aberrant lipogenesis in human hepatocellular carcinoma (HCC). Here, we investigated the oncogenic potential of fatty acid synthase (FASN), the master regulator of *de novo* lipogenesis, in the mouse liver.

Methods: FASN was overexpressed in the mouse liver, either alone or in combination with activated N-Ras, c-Met, or SCD1, via hydrodynamic injection. Activated AKT was overexpressed via hydrodynamic injection in livers of conditional *FASN* or *Rictor* knockout mice. FASN was suppressed in human hepatoma cell lines via specific small interfering RNA.

Results: Overexpression of *FASN*, either alone or in combination with other genes associated with hepatocarcinogenesis, did not induce histological liver alterations. In contrast, genetic ablation of *FASN* resulted in the complete inhibition of hepatocarcinogenesis in *AKT*-overexpressing mice. In human HCC cell lines, FASN inactivation led to a decline in cell proliferation and a rise in apoptosis, which were paralleled by a decrease in the levels of phosphorylated/activated AKT, an event controlled by the mammalian target of rapamycin complex 2 (mTORC2). Downregulation of AKT phosphorylation/activation following FASN inactivation was associated with a strong inhibition of rapamycin-insensitive companion of mTOR (Rictor), the major component of mTORC2, at post-transcriptional level. Finally, genetic ablation of Rictor impaired AKT-driven hepatocarcinogenesis in mice.

Conclusions: FASN is not oncogenic *per se* in the mouse liver, but is necessary for AKT-driven hepatocarcinogenesis. Pharmacological blockade of FASN might be highly useful in the treatment of human HCC characterized by activation of the AKT pathway.

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Introduction

Deregulated fatty acid biosynthesis, also known as *de novo* lipogenesis, is a key aberration in cancer [1–3]. It provides rapidly proliferating cancer cells with a continuous supply of lipids and lipid precursors that are needed for membrane production, energy generation, and lipid-based post-transcriptional modifications of proteins [1–3]. At the molecular level, *de novo* lipogenesis is characterized by an upregulation in tumor cells of lipogenic enzymes, including adenosine triphosphate citrate lyase (ACLY), acetyl-CoA carboxylase (ACAC), fatty acid synthase (FASN), and stearoyl-CoA desaturase 1 (SCD1) [1–3]. FASN, the enzyme responsible for the production of long chain fatty acids from acetyl-coA and malonyl-CoA, is the most investigated lipogenic protein in cancer [1–3]. FASN levels are elevated in many tumor types, where they significantly correlate with cancer biological aggressiveness and unfavorable prognosis [1–3]. In addition, upregulation of FASN occurs in preneoplastic and pre-invasive lesions of various organs [1–3]. Also, FASN blockade triggers tumor growth restraint and massive apoptosis in numerous *in vitro* and *in vivo* models [1–3]. Furthermore, FASN overexpression induces the development of prostate intraepithelial neoplasia in transgenic mice, thus acting as a bona fide oncogene

Keywords: Hepatocellular carcinoma; Lipogenesis; Fatty acid synthase; AKT; Rictor.

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Abbreviations: ACAC, acetyl-CoA carboxylase; ACLY, adenosine triphosphate citrate lyase; AKT, v-akt murine thymoma viral oncogene homolog; ELOVL5, elongation of very long chain fatty acids protein 5; FASN, fatty acid synthase; HCC, hepatocellular carcinoma; LDH, lactate dehydrogenase; mTOR, mammalian target of rapamycin; mTORC, mTOR complex; N-Ras, neuroblastoma Ras viral oncogene homolog; PKM2, pyruvate kinase M2 isoform; Raptor, regulatory-associated protein of mTOR; Rictor, rapamycin-insensitive companion of mTOR; Rps6, ribosomal protein S6; Scd1, stearoyl-CoA desaturase 1; siRNA, small interfering RNA.

in prostate cancer [4]. Similarly, overexpression of FASN induces a cancer-like phenotype in non-tumorous breast cell lines [5].

In hepatocellular carcinoma (HCC), aberrant expression of lipogenic enzymes including FASN has been linked both to tumor development and progression. For instance, overexpression of FASN occurs in liver preneoplastic lesions from rat models of chemically- and hormonally-induced hepatocarcinogenesis [6]. Similarly, sustained lipogenesis and FASN upregulation characterize human liver clear cell foci, whose preneoplastic nature has been hypothesized [7]. Also, levels of FASN and other lipogenic proteins as well as polymorphisms in lipogenic genes are associated with poor outcome in HCC patients [8–12]. In addition, FASN suppression has been shown to be detrimental for HCC growth *in vitro* [10,13,14]. Despite this body of evidence, key questions about FASN in HCC remain unanswered. Virtually all functional studies on FASN in HCC have been performed in HCC cell lines so far. Thus, it is unknown whether FASN contributes to liver tumor development and/or progression *in vivo*.

Here, we determined the functional contribution of FASN to liver cancer development *in vivo* by overexpressing FASN, either alone or in association with oncogenes that have been associated with hepatocarcinogenesis, in the mouse liver via hydrodynamic gene delivery. Furthermore, we assessed the importance of FASN on AKT-driven hepatocarcinogenesis by overexpressing AKT in *FASN*-depleted mice.

Materials and methods

Constructs and reagents

pT3-EF1α, pT3-EF1α-HA-myr-AKT1, pT2-Caggs-N-RasV12, pT3-EF1a-V5-c-Met, pT3-EF1α-Cre, and pCMV/sleeping beauty transposase plasmids were described previously [10,15–18]. Human(h) *FASN* (ID: 6172538) and h*SCD1* (ID: 3844850) full length cDNAs were from Open Biosystems (Lafayette, CO), and cloned into pT3-EF1a vectors via the Gateway polymerase chain reaction (PCR) cloning strategy (Invitrogen, Carlsbad, CA). Plasmids were purified using the Endotoxin free Maxi prep kit (Sigma-Aldrich, St. Louis, MO) before injecting into mice.

Hydrodynamic injection, mouse monitoring

FASN$^{fl/fl}$ mice (in C57BL/6 background) were previously described [19]. *AlbCre* mice [20], purchased from Jackson Laboratory (Bar Harbor, ME), were crossed with *FASN*$^{fl/fl}$ mice to generate liver specific FASN null mice (*AlbCre;FASN*$^{fl/fl}$ mice). Hydrodynamic injections were performed as reported previously [21]. To determine the oncogenic potential of lipogenic enzymes, 20 μg of the plasmids encoding the gene(s) of interest along with sleeping beauty transposase in a ratio of 25:1 were diluted in 2 ml saline (0.9% NaCl) for each mouse. Saline solution was filtered through a 0.22 μm filter and injected into the lateral tail vein of six- to eight-week-old mice in 5–7 s. To study the requirement of FASN for AKT-driven hepatocarcinogenesis, two approaches were employed. In the first approach, six- to eight-week-old *FASN*$^{fl/fl}$ mice were injected with AKT (8 μg) and Cre recombinase (40 μg). Additional *FASN*$^{fl/fl}$ mice were injected with AKT (8 μg) and pT3 (40 μg) as control. In the second approach, AKT (20 μg) was injected into four month old *AlbCre;FASN*$^{fl/fl}$ mice and control *FASN*$^{fl/fl}$ mice. *Rictor*$^{fl/fl}$ mice [22] were purchased from Jackson Laboratory. To determine the importance of Rictor on AKT-driven hepatocarcinogenesis, AKT (8 μg) together with Cre (40 μg) was injected into 6 to 8 weeks old *Rictor*$^{fl/fl}$ mice. AKT (8 μg) together with pT3EF1a (40 μg) was injected into *Rictor*$^{fl/fl}$ mice as control. Mice were housed, fed, and monitored in accordance with protocols approved by the Committee for Animal Research at the University of California, San Francisco.

Detailed description of materials and methods is provided as Supplementary material.

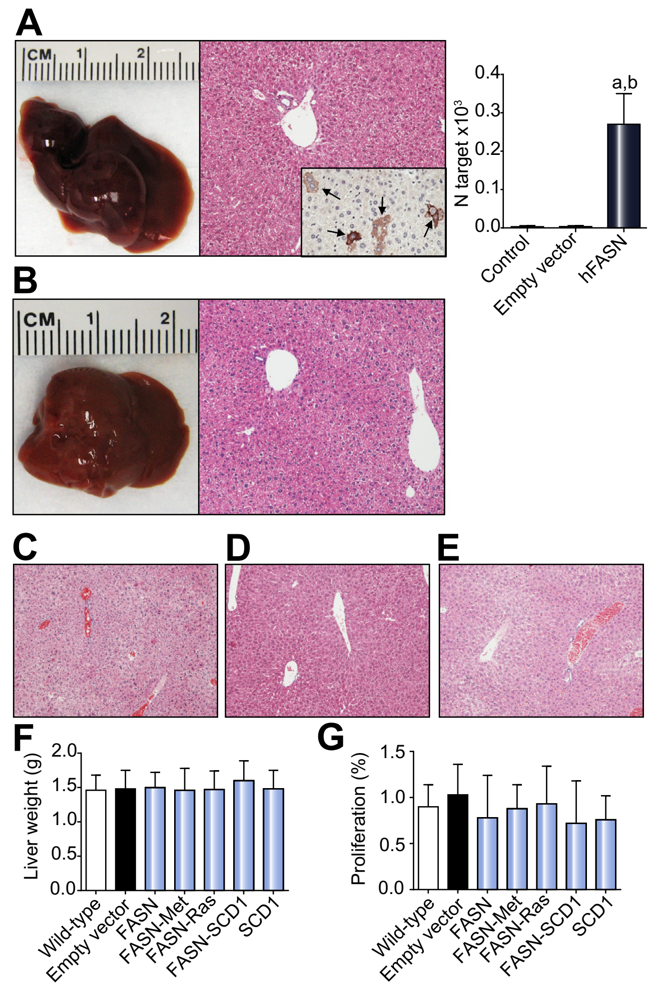

Fig. 1. FASN is not oncogenic in the mouse liver. (A) Macroscopic (left panel) and microscopic (middle panel) appearance of FASN-injected livers showing the absence of gross or histological alterations 40 weeks post hydrodynamic injection. Scattered cells positive for human (h)FASN were detected in FASN-injected livers (indicated by arrows; inset). Efficient transfection of hFASN was also detected by real-time RT-PCR in mouse livers (right panel). N target (NT) = $2^{-\Delta Ct}$, wherein ΔCt value of each sample was calculated by subtracting the average Ct value of the target gene from the average Ct value of the *β-actin* gene. Five samples per each mouse group were analyzed. Tukey-Kramer's test: *p* at least <0.001; *a*, *vs.* control livers; *b*, *vs.* livers injected with empty vector. Analogously, concomitant overexpression of *FASN* with *c-Met* (B), *N-RasV12* (C) or *SCD1* (D), or overexpression of *SCD1* (E) alone was not oncogenic in the mouse liver. (F, G) Overexpression of FASN either alone or in association with the aforementioned genes did not induce changes in liver weight (F) or hepatocyte proliferation (G) when compared with wild-type (un-injected) or empty vector injected mice. Original magnification: 100× in A-E.

Results

FASN is not oncogenic per se in the mouse liver

To determine whether FASN has oncogenic potential *in vivo*, we hydrodynamically delivered the pT3-EF1α-hFASN plasmid to the mouse liver. Overexpression of human *FASN* alone did not trigger tumor formation or histological alterations in mice up to 40 weeks post-injection (n = 6). Macroscopically and histologically, FASN-injected livers were indistinguishable from empty plasmid-injected or un-injected livers (Fig. 1A), and did not show

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