c-Fos overexpression increases the proliferation of human hepatocytes by stabilizing nuclear Cyclin D1

Meryem Güller, Kahina Toualbi-Abed, Agnès Legrand, Laurence Michel, Alain Mauviel, Dominique Bernuau, Fanny Daniel

Abstract

AIM: To investigate the effect of stable c-Fos overexpression on immortalized human hepatocyte (IHH) proliferation.

METHODS: IHHs stably transfected with c-Fos (IHH-Fos) or an empty vector (IHH-C) were grown in medium supplemented with 1% serum or stimulated with 10% serum. Cell proliferation was assessed by cell counts, 3H-thymidine uptake and flow cytometry analyses. The levels of cell cycle regulatory proteins (Cyclin D1, E, A) cyclin dependent kinases (cdk) cdk2, cdk4, cdk6, and their inhibitors p15, p16, p21, p27, total and phosphorylated GSK-3β and epidermal growth factor receptor (EGF-R) were assayed by Western blotting. Analysis of Cyclin D1 mRNA levels was performed by reverse transcription-polymerase chain reaction and real-time polymerase chain reaction (PCR) analysis. Stability of Cyclin D1 was studied by cycloheximide blockade experiments.

RESULTS: Stable c-Fos overexpression increased cell proliferation under low serum conditions and resulted in a two-fold increase in [³H]-thymidine incorporation following serum addition. Cell cycle analysis by flow cytometry showed that c-Fos accelerated the cell cycle kinetics. Following serum stimulation, Cyclin D1 was more abundantly expressed in c-Fos overexpressing cells. Cyclin D1 accumulation did not result from increased transcriptional activation, but from nuclear stabilization. Overexpression of c-Fos correlated with higher nuclear levels of inactive phosphorylated GSK-3β, a kinase involved in Cyclin D1 degradation and higher levels of EGF-R mRNA, and EGF-R protein compared to IHH-C both in serum starved, and in serum stimulated cells. Abrogation of EGF-R signalling in IHH-Fos by treatment with AG1478, a specific EGF-R tyrosine kinase inhibitor, prevented the phosphorylation of GSK-3β induced by serum stimulation and decreased Cyclin D1 stability in the nucleus.

CONCLUSION: Our results clearly indicate a positive role for c-Fos in cell cycle regulation in hepatocytes. Importantly, we delineate a new mechanism by which c-Fos could contribute to hepatocarcinogenesis through stabilization of Cyclin D1 within the nucleus, evoking a new feature to c-Fos implication in hepatocellular carcinoma.

© 2008 The WJG Press. All rights reserved.

Key words: c-Fos; Cyclin D1; GSK-3; Cell growth; Cell cycle; Hepatoma; Epidermal growth factor

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world, with an increasing number of new cases emerging each year. Etiologically it is linked to chronic viral infections (hepatitis B and C viruses), alcohol-related cirrhosis or aflatoxin B1 exposure, which
all cause disruptions in signal transduction cascades leading to abnormalities in gene expression.

The proto-oncogene *c-fos* is an important member of the activating protein 1 (AP-1) transcription factor involved in major cellular functions such as transformation, proliferation, differentiation and apoptosis\(^{6-8}\). Such a large variety of functions has been summarized by the combination of different Jun (c-Jun, JunB or JunD) and Fos (c-Fos, FosB, ΔfosB, Fra-1, Fra-2) family members forming various AP-1 homo and heterodimers. *c-fos* is an immediate early gene whose expression is rapidly and transiently induced after mitogenic stimulation\(^7\). The role of c-Fos in cell proliferation and transformation remains controversial. c-Fos is required during all phases of the cell cycle in exponentially growing cells and is a potent inducer of cell proliferation\(^{10}\). However, some studies have suggested that c-Fos poorly contributes to proliferation\(^{11}\), was totally dispensable for\(^{12}\), or even down-regulated, cell growth\(^{13}\). Overexpression of c-Fos leads to morphological transformation of fibroblasts to a cancerous form, and to osteosarcoma formation in transgenic mice\(^{10,11,12}\). Apart from one study describing a negative role for c-Fos in hepatocellular tumorigenesis\(^8\), several reports rather support a potential positive role for c-Fos in this process. High expression levels of c-Fos were determined in tumour tissue compared to the adjacent non-tumour liver in human HCC\(^{13,15}\), as well as in several models of HCC in rodents\(^{16-18}\). A recent study in humans identified a subtype of HCC sharing gene expression patterns with foetal hepatoblasts which can be distinguished from another HCC subtype closer to adult hepatocytes\(^9\). Interestingly, c-Fos, but not c-Jun expression was higher in the foetal subtype which displayed a poorer prognosis and a greater tendency to invasion than the adult subtype. In addition, the expression of DNA 5-methylcytosine transferase, a c-Fos target gene involved in DNA methylation\(^{25}\) is increased in human tumour cells and in HCCs\(^{20}\). Despite these studies showing that c-Fos overexpression might be an important step towards the development of liver cancer, its precise role in hepatocarcinogenesis remains ill-defined.

In order to clarify c-Fos implication in hepatocarcinogenesis, we examined the effect on proliferation of stable c-Fos overexpression in immortalized human hepatocytes (IHH). We show, for the first time, that a positive role for c-Fos on hepatocyte proliferation can be attained by stabilization of Cyclin D1 in the nuclear compartment, a mechanism which has not been described as a c-Fos related process in any cell type to date.

**MATERIALS AND METHODS**

**Cell culture and reagents**

IHH were cultured in Williams’ medium E (Invitrogen, Cergy Pontoise, France) supplemented with 100 mL/L fetal calf serum (FCS) (Biochrom AG, Cambridge, UK), 1% penicillin-streptomycin, 1% Glutamax and 1% DMEM sodium pyruvate (Invitrogen). Specific reagents were AG1478 (Calbiochem, San Diego, CA) and cycloheximide (CHX) (Euromedex, Souffelweyersheim, France).

**Generation of stably transfected cells**

The human *c-fos* cDNA was inserted into the cytomegalovirus driven pcDNA expression vector (Promega, Charbonnières, France) containing a neomycin resistance gene to obtain the pcDNA-*c-fos* vector. Cells were stably transfected by electroporation (230V, 960 μF) in PBS-Hepes Buffer 10 mmol/L, pH 7.4 with the empty vector (pcDNA) or with pcDNA-*c-fos*. Two days post-transfection, stable clones were selected in media containing 500 μg/mL of G418 (Invitrogen). The resistant clones were pooled after 3 wk of selection, and maintained with G418. c-Fos overexpression was verified by Western blot analysis as shown in Figure 1A.

**Flow cytometry**

DNA cell cycle analysis was measured by 5-bromodeoxyuridine (BrdU) incorporation and propidium iodide staining of the nuclei by flow cytometry (FACScanLibur, BD Biosciences, Mansfield, MA) and analyzed with the ProCellQuest software provided by the manufacturer. Cell cycle progression was measured by pulse/chase experiments. Cells plated at a density of 5 × 10⁴ per 6-cm dish were serum starved for 24 h, serum stimulated for 12 h and stained with BrdU (30 μg/mL) for 1 h. Cells were then chased with BrdU free medium for 0, 3, 6, 9, 12 h, stained with propidium iodide and harvested in 70% ethanol. Cells were then treated with 2 N HCl and pepsin (0.2 mg/mL) for 30 min. BrdU content was determined in culture media containing 1.5 μg/mL of tritiated thymidine ([³H]dT) (specific activity of 740 GBq/mmol) (Perkin-Elmer, Waltham, Ma) for 4 h. Cells were fixed and washed in ice-cold 10% trichloroacetic acid. DNA was solubilized in 0.1 mol/L NaOH for 1 h at 37°C. [³H]dT incorporated into the DNA was measured using liquid scintillation counting.

**Western blot analysis**

Nuclear proteins were extracted as described\(^{12}\). Total proteins were extracted with lysis buffer [1% (v/v) SDS, 1 mmol/L NaVO₄, 10 mmol/L Tris pH 7.4, 1% benzamone] for 10 min at room temperature and heated for

---

www.wjgnet.com
5 min at 100°C. Equal quantities of nuclear proteins were fractionated on a SDS-polyacrylamide gel and transferred onto nitrocellulose membranes by electroblotting. The antibodies used in this study were as follows: c-Fos, Cyclin D1, Cyclin E, Cyclin A, cdk2, cdk4, cdk6, p15, p16, p21, p27 and EGF-R (Santa Cruz Biotechnology, Santa Cruz, CA), GSK3β (Affinity BioReagents, Golden, CO), Phospho-GSK3β (Serine9) (Abcam, Paris, France). Immunoreactive bands were visualized using the ECL kit (Amersham BioSciences, Saclay, France) according to the manufacturer’s instructions.

Reverse transcription-polymerase chain reaction (RT-PCR) and real-time quantitative PCR analysis
mRNA was isolated from cells by Nucleospin RNA II kit (Macherey-Nagel, Hoerdt, France) following the manufacturer’s instructions. RNA (1 μg) was reverse transcribed using ThermoScript™ RT-PCR System (Invitrogen, Cergy-Pontoise, France). Real-time quantitative PCR was performed using the following primers: Cyclin D1: forward 5’-GCATGTTCGTGGCCTCAAGA-3’ and reverse 5’-CGGTGTAGATGCACAGTTCTC-3’, EGF-R: forward 5’-GCGTCTCTGGCCGAATGT-3’ and reverse 5’-GGCTCACCCTCCAGAAGGT-3’. Real-time quantitative PCR was performed with an ABI PRISM 7700 instrument (Applied Biosystems, Foster City, CA), using SYBRGreen PCR core reagents (Applied Biosystems). Fold changes in mRNA were calculated by the ∆∆Ct method using cyclophilin A (forward: 5’-CAAATGCTGGACCCAACACA-3’; reverse: 5’-TGCCATCCAACCACTCAGT-3’) as a standard. All PCR reactions were done in triplicate.

Statistical analysis
Data were expressed as means ± SE. Student’s t-test was performed and statistical significance was considered as P < 0.05.

RESULTS
Growth rate and cell cycle regulation by c-Fos overexpression
To determine whether c-Fos could modulate hepatocyte growth, we carried out growth curve assays. While

Figure 1 Overexpression of c-Fos accelerates the cell cycle. A: IHH-C and IHH-Fos were grown in 1% FCS, cultured for 5 d and counted daily. Cell growth was determined by counting the number of attached cells every day. Results are the mean ± SE of three independent experiments; B: [3H] thymidine incorporation into DNA. Non-synchronized IHH-C or IHH-Fos serum starved for 24 h then serum stimulated for 4 h were incubated with [3H] thymidine for 4 h. DNA was extracted as described in materials and methods, and [3H] thymidine incorporation into DNA was assessed by scintillation counting. Results are expressed as percentage of increase of [3H] thymidine incorporation in serum-stimulated cells over that of quiescent cells for each cell population. Results are the mean ± SE of six independent experiments; C: Flow cytometry analysis for quantification of cell cycle phase distribution and progression through cell cycle. IHH-C or IHH-Fos serum starved for 24 h were incubated with BrdU for 1 h and stained with propidium iodide 0, 4, 8 and 24 h after serum stimulation. The percentage of cells in each phase is plotted against time. Results of a representative experiment are shown (out of 3); D: IHH-C or IHH-Fos serum starved for 24 h were serum stimulated for 12 h, BrdU pulsed for 1 h, chased with fresh medium for 0, 3, 6, 9, 12 h, and then stained with propidium iodide. The percentage of BrdU-negative cells in the G1 phase (G1 exit) (left panel) and of the BrdU-positive cells in the G1 phase (G1 entry) (right panel) of the cell cycle is plotted against time. Results are representative of four independent experiments.

www.wjgnet.com
cell growth was similar in the presence of 10% FCS in IHH-C and IHH-Fos (data not shown), we observed that the growth pattern of the two cell lines differed in low serum conditions (1% FCS). While the number of IHH-Fos increased exponentially over 5 d in culture, IHH-C number increased slowly during the first 3 d of culture, and then reached a plateau, due to the induction of cell death by serum deprivation (Figure 1A and data not shown). Thus, c-Fos overexpression correlated with a more rapid growth in low serum conditions. The effect of c-Fos overexpression on cell proliferation was further established by measuring \[^3H\]dT incorporation following 4 h serum stimulation of cells deprived of serum for 24 h. The increase of \[^3H\]dT incorporation induced by serum was 2.2 times higher in IHH-Fos than in IHH-C (231% and 107%, respectively), and the difference was statistically significant (\(P < 0.001\)) (Figure 1B). To further analyze the role of c-Fos on the cell cycle, cell cycle phase distribution and cell cycle kinetics were analyzed by flow cytometry. Following serum stimulation, the percentage of cells in the G1 phase decreased, while the percentage of cells in the S-phase increased 24 h after serum stimulation. However, the percentage of cells in the different stages of the cell cycle was comparable in IHH-Fos and IHH-C (Figure 1C). Cell cycle progression was measured by BrdU pulse/chase experiments. The rate at which BrdU positive cells progress into G1 indicates the rate of transit through S, G2 and M phases. Similarly, the rate at which BrdU negative cells become depleted from the G1 pool indicates the transit rate through G1. We show that IHH-Fos quit (Figure 1D, left panel) and enter (Figure 1D, right panel) G1 faster than IHH-C, which reflects a global increase in cell cycle kinetics. The fact that the cell cycle profile was not altered by c-Fos indicates that the acceleration is proportional in all phases of the cycle. These data taken together indicate that c-Fos overexpression increases the growth of exponentially growing cells cultured in low serum medium as well as the proliferation response induced by serum refeeding.

**Induction of cell cycle regulatory proteins by c-Fos**

The levels of various cell cycle regulatory proteins before and after serum stimulation of IHH-C and IHH-Fos were analyzed by Western blotting experiments. In both cell lines, serum addition induced an increase in the nuclear levels of Cyclin A, cdk2 and cdk4, but no change in Cyclin E. Of interest, the nuclear levels of Cyclin D1 were increased after 8 h of stimulation in IHH-Fos, but not in IHH-C (Figure 2A). In addition, the levels of p27 were higher in the absence of serum stimulation or following serum stimulation in IHH-Fos than in IHH-C (Figure 2B).

Quantitative RT-PCR analysis was performed to determine whether the increase of Cyclin D1 at 8 h of serum stimulation in IHH-Fos was controlled transcriptionally. Interestingly, a similar 2-fold increase in Cyclin D1 mRNA 2 h following serum stimulation was observed in IHH-Fos and IHH-C, without any significant differences at any of the time points (Figure 2C), indicating that the higher levels of Cyclin D1 in the nucleus in IHH-Fos are not due to transcriptional mechanisms.

**Cyclin D1 stabilization in the nucleus**

We next determined whether post-translational regulations could explain the increase of nuclear Cyclin D1 in serum-stimulated IHH-Fos. CHX, a translational inhibitor, was used to block protein synthesis. While in IHH-C, nuclear Cyclin D1 protein levels started to decline as from 1 h, and decreased by 85% after 2 h of CHX treatment, Cyclin D1 nuclear levels were decreased by only 20% upon 2 h of CHX treatment in IHH-Fos (Figure 3), indicating that c-Fos overexpression correlates with increased stability of nuclear Cyclin D1.

**Inactivation of GSK-3β in IHH-Fos contributes to Cyclin D1 stabilization**

Previous studies have indicated that Cyclin D1 degradation is triggered by GSK-3β-induced phosphorylation on a single threonine residue (Thr-286)\(^{[23]}\). Of note, phosphorylated GSK-3β is the inactive form of the protein\(^{[24]}\). We, therefore, compared the level of
phosphorylated GSK-3β between the two cell lines by Western blot analysis. As shown in Figure 4A, the levels of GSK3β phosphorylation were much higher in the nucleus of IHH-Fos than in IHH-C, both in unstimulated and in serum stimulated conditions, indicating higher basal, and induced levels of inactive GSK-3β in IHH-Fos (Figure 4A). Therefore, a decrease in active GSK-3β in IHH-Fos could be responsible for the increased stability of nuclear Cyclin D1 after serum stimulation.

Several signaling pathways are able to induce GSK-3β phosphorylation, including the two main cascades targeted by tyrosine kinase receptors: the phosphatidylinositol 3-kinase (PI3K) and the Ras/mitogen activated protein kinase pathways. Since the epidermal growth factor receptor (EGF-R) is a known transcriptional target of AP-1, we tested the hypothesis that overexpression of EGF-R might contribute to high levels of GSK-3β phosphorylation in IHH-Fos. Quantitative RT-PCR analysis revealed a 2.2-fold increase in the basal level of EGF-R mRNA in IHH-Fos compared to IHH-C (Figure 4B). Higher levels of EGF-R protein were also observed in serum starved and serum-stimulated IHH-Fos compared to IHH-C (Figure 4B), strongly indicative of increased EGF-R signaling in c-Fos-overexpressing cells. Altogether, these data suggest that increased EGF-R signaling might contribute, at least partly, to increased levels of GSK-3β phosphorylation in IHH-Fos cells.

To demonstrate the implication of EGF-R in GSK-3β-mediated Cyclin D1 stabilization, IHH-Fos cells were treated with AG1478, a specific inhibitor of the EGF-R tyrosine kinase before serum stimulation. Western blot analysis indicated that AG1478 treatment did block the phosphorylation of GSK-3β induced by serum (Figure 4C). Interestingly, the decrease in the nuclear level of Cyclin D1 protein observed after a 2 h-CHX treatment of IHH-Fos cells stimulated by serum was more important in cells treated with AG1478 (50% decrease) than in untreated cells (10% decrease), and the difference was statistically significant (P < 0.05, Figure 4D), confirming that blockade of EGF-R induced signaling in IHH-Fos leads to a more rapid nuclear Cyclin D1 degradation.

**DISCUSSION**

Our results indicate that c-Fos overexpression accelerates
cell growth under reduced serum concentration suggesting that hepatocytes overexpressing c-Fos become relatively independent of the presence of growth factors. We show that c-Fos enhances DNA synthesis after serum stimulation, and accelerates hepatocyte cell cycle progression without altering the overall distribution of cells in each phase due to a proportional acceleration of cell cycle kinetics in all phases.

Our results are at variance with those obtained in immortalized murine hepatocytes [8]. In this model, c-Fos conditional expression for 48 h was shown to decrease cell growth and [3H]dT incorporation of cells grown in serum-supplemented medium. Besides species differences (murine vs human cells), the discrepancy in results can be explained by the use of very different cellular models which cannot be compared. The human hepatocytes used in our study were immortalized by SV40 T antigen while murine hepatocytes were immortalized using truncated c-Met [26]. Overexpression of c-Fos in our model was permanently established as the result of stable transfection, while in Mikula’s study a c-Fos-estrogen receptor fusion protein was expressed for a limited period (1-3 d) following estradiol treatment of the cultures [31]. Furthermore, the function of the conditionally expressed c-Fos protein may have been modified, since gene fusion has been shown to alter the function of Fos family proteins [29].

We aimed to determine whether the positive role of c-Fos on hepatocyte proliferation depicted in our study was mediated through changes in cell cycle regulation. Different studies have reported an effect of c-fos gene deletion or c-Fos protein overexpression on Cyclin D1 [9,30,31], Cyclin E [31] or Cyclin A [31] expression, depending on the cell type studied. In our study, while the levels of Cyclin E and A and their associated kinases varied with a similar pattern in both cell types following serum stimulation, nuclear Cyclin D1 levels were higher in IHH-Fos compared to IHH-C 8 h after serum re-feeding. Contrary to previous reports describing c-Fos as a transcriptional activator of Cyclin D1 [9], the higher levels of nuclear Cyclin D1 in IHH-Fos than in IHH-C were not due to differences in transcriptional regulation, but to increased protein stability in the nucleus. A similar lack of correlation between Cyclin D1 mRNA and protein expression has been previously described in an in vitro experimental model of HCC [31].

Our results strongly suggest a mechanism whereby c-Fos induces nuclear accumulation of Cyclin D1 without affecting the total cellular amount of the protein.

The Cyclin D1 protein is quite unstable, with a half-life of less than 30 min [34]. It accumulates in the nucleus during the G1 phase and exits into the cytoplasm during the S phase. Nuclear export of Cyclin D1, and its subsequent ubiquitination and proteolysis, are dependent on phosphorylation on a single threonine residue (Thr-286) performed mainly by GSK-3β [23], a protein kinase active only when dephosphorylated. In contrast to Cyclin D1, GSK-3β is predominantly cytoplasmic during G1 phase, but a considerable amount becomes nuclear during S phase [23]. We show herein that phosphorylated levels of nuclear GSK-3β are higher in IHH-Fos than in IHH-C. Lower levels of active GSK-3β would consequently lead to a decrease in Cyclin D1 phosphorylation, resulting in its nuclear accumulation in IHH-Fos. Since EGF-R is a known transcriptional target of AP-1 [25-27], we tested the possibility that c-Fos overexpression increases the activation of the pathways downstream to EGF signaling. EGF-R activates both the PI3K and the mitogen-activated protein kinase cascades [32], two upstream activators of GSK-3β phosphorylation [24,30]. In support of an involvement of EGF-R signaling in GSK-3β inactivation and nuclear cyclin D1 stabilization, we show that IHH-Fos display increased levels of expression of EGF-R mRNA and protein than IHH-C. Furthermore, blocking the activation of the EGF-R tyrosine kinase significantly accelerates the rate of Cyclin D1 degradation assessed in CHX experiments. Upregulated expression of EGF-R is a frequent finding in HCC [37-39], and increased EGF-R signaling has been associated with a poorer prognosis [40]. c-Fos is also frequently overexpressed in HCC tumoral tissues [13,14,41]. Our data, therefore, suggest that a causal relationship could exist between c-Fos and EGF-R overexpression in HCC.

Our finding of high levels of nuclear Cyclin D1 associated with c-Fos overexpression adds further support for a contributing effect of c-Fos on HCC development. Indeed, Cyclin D1 exit from the nucleus during S phase is essential for regulated cell division, and its retention in the nucleus is a cancer promoting or predisposing event [42]. Thus, expression of a Cyclin D1 mutant that cannot be phosphorylated by GSK-3β, and remains nuclear throughout the cell cycle is highly transforming and induces tumour growth in nude mice [43].

In accordance with previous reports [32,44], we also found that p27 protein levels were higher in c-Fos overexpressing cells. It is now well recognized that the family of p21/p27 proteins plays a dual role in cell cycle regulation. On one hand, they bind to cdk2 complexes and inhibit their kinase activities. On the other hand, they are able to promote the activation of Cyclin D1/cdk4-6 by complex stabilization, and by facilitating the nuclear import of these complexes, without inhibiting Cyclin D-associated kinase activity [45-48]. In our study, higher levels of p27 in IHH-Fos could, therefore, represent another mechanism contributing to the increase in nuclear levels of Cyclin D1, although the precise mechanisms linking c-Fos and p27 overexpression are currently unknown. Nevertheless, the mechanism is not at the level of transcription, as indicated by our quantitative PCR analysis (data not shown).

To conclude, our results clearly indicate a positive role for c-Fos in cell cycle regulation in hepatocytes. Importantly, we delineate a new mechanism by which c-Fos could contribute to hepatocarcinogenesis through stabilization of Cyclin D1 within the nucleus, evoking a new feature to c-Fos implication in HCC.

ACKNOWLEDGMENTS

The IHH cell line was kindly provided by Dr. H Moshage (Groningen, The Netherlands). We gratefully acknowledge

www.wjgnet.com
the technical assistance of J André and C Tacheau. This project was supported by INSERM, and Meryem Güller by a doctoral fellowship from the Ministry of Research and Technologies (MRT) and a grant from the Association pour la Recherche contre le Cancer (ARC).

COMMENTS

Background
Human hepatocellular carcinoma (HCC) is the fifth most common cancer in the world. Among the numerous genes potentially implicated in hepatocarcinogenesis, the proto-oncogene c-Fos, a member of activating protein 1 (AP-1) transcription factor is a good candidate. Apart from one study reporting a negative role for c-Fos in hepatocellular tumorigensis, several papers rather support a positive role in this process. High expression levels of c-Fos were determined in tumor tissue compared to the adjacent non-tumor liver in human HCC. However, in different cell types or tissues, the role of c-Fos in cell proliferation and/or transformation remains controversial. This study was designed to determine whether c-Fos could contribute to hepatocarcinogenesis by increasing cell proliferation.

Research frontiers
The role of c-Fos on hepatocyte proliferation has never been studied in human cells, but only in murine hepatocytes. These cells had been immortalized and stably transfected by c-Fos using different techniques than those reported in the present study. The authors showed that c-Fos overexpression led to decreased hepatocyte proliferation. However, these results did not appear consistent with most studies suggesting a positive role for c-Fos in hepatocarcinogenesis.

Innovations and breakthroughs
This study shows for the first time that c-Fos deregulates hepatocyte proliferation by stabilizing Cyclin D1 in the nucleus which is a cancer promoting or predisposing event.

Applications
Strategies designed to suppress c-Fos expression in HCC could contribute reducing hepatocyte proliferation and thereby cancer development.

Terminology
Human immortalized hepatocytes are hepatocytes which have been transfected by SV40 T antigen, allowing them to proliferate when cultured contrary to normal hepatocytes. However these immortalized cells are not tumorigenic in vitro and in vivo.

Peer review
This is an interesting study. Authors investigated the effect of stable c-Fos overexpression on HHC proliferation.

REFERENCES

1. Shaulian E, Karin M. AP-1 as a regulator of cell life and death. Nat Cell Biol 2002; 4: E131-E136
2. Eferl R, Wagner EF. AP-1: a double-edged sword in cell proliferation and transformation. Biochim Biophys Acta 1999; 1072: 129-157
3. Angel P, Karin M. The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. Biochim Biophys Acta 1991; 1107: 389-388
4. Pai SR, Bird RC. c-fos expression is required during all phases of the cell cycle during exponential cell proliferation. Anticancer Res 1994; 14: 985-994
5. Kovaly K, Bravo R. The jun and fos protein families are both required for cell cycle progression in fibroblasts. Mol Cell Biol 1991; 11: 4466-4472
6. Brusselbach S, Mohle-Steinlein U, Wang ZQ, Schreiber M, Lucibello FC, Muller R, Wagner EF. Cell proliferation and cell cycle progression are not impaired in fibroblasts and ES cells lacking c-Fos. Oncogene 1995; 10: 79-86
7. Balsalobre A, Jolicour P. Fos proteins can act as negative regulators of cell growth independently of the fos transforming pathway. Oncogene 1995; 11: 455-465
8. Mikula M, Gotzmann J, Fischer AN, Wolschek MF, Thallinger C, Schulte-Hermann R, Beug H, Mikulits W. The proto-oncoprotein c-fos negatively regulates hepatocellular tumorigenesis. Oncogene 2003; 22: 6725-6738
9. Miao GG, Curran T. Cell transformation by c-fos requires an extended period of expression and is independent of the fos transforming pathway. Mol Cell Biol 1994; 14: 4295-4310
10. Hennigan RF, Hawker KL, Ozanne BW. Fos-transformation activates genes associated with invasion. Oncogene 1994; 9: 3591-3600
11. Grigoriadis AE, Schellander K, Wang ZQ, Wagner EF. Osteoblasts are target cells for transformation in c-fos transgenic mice. J Cell Biol 1993; 122: 685-701
12. Wang ZQ, Grigoriadis AE, Mohle-Steinlein U, Wagner EF. A novel target cell for c-fos-induced oncogenesis: development of chondrogenic tumours in embryonic stem cell chimeras. EMBO J 1991; 10: 2437-2450
13. Feng DY, Zheng H, Tan Y, Cheng RX. Effect of phosphorylation of MAPK and Stat3 and expression of c-fos and c-jun proteins on hepatocarcinogenesis and their clinical significance. World J Gastroenterol 2001; 7: 33-36
14. Yuen MF, Wu PC, Lai VC, Lau JY, Lai CL. Expression of c-Myc, c-Fos, and c-jun in hepatocellular carcinoma. Cancer 2001; 91: 106-112
15. Taber E. Tumor suppressor genes, growth factor genes, and oncogenes in hepatitis B virus-associated hepatocellular carcinoma. J Med Virol 1994; 42: 357-365
16. Masui T, Nakanishi H, Inada K, Imai T, Mizoguchi Y, Yada H, Futakuchi M, Shirai T, Tatematsu M. Highly metastatic hepatocellular carcinomas induced in male F344 rats treated with N-nitrosomorpholine in combination with other hepatocarcinogens show a high incidence of p53 gene mutations along with altered mRNA expression of tumour-related genes. Cancer Lett 1997; 112: 33-45
17. Yao X, Hu JF, Daniels M, Yien H, Lu H, Sharan H, Zhou X, Zeng Z, Li T, Yang Y, Hoffman AR. A novel orthotopic tumor model to study growth factors and oncogenes in hepatocarcinogenesis. Clin Cancer Res 2003; 9: 2719-2726
18. Borlak J, Meier T, Halter R, Spanel R, Spanel-Borowski K. Epidermal growth factor-induced hepatocellular carcinoma: gene expression profiles in precursor lesions, early stage and solitary tumours. Oncogene 2005; 24: 1809-1819
19. Lee JS, Hao J, Libbrecht L, Chu IS, Kaposi-Novak P, Calvisi DF, Mikaelyan A, Roberts LR, Demetris AJ, Sun Z, Nevens F, Roskams T, Thorgersson SS. A novel prognostic subtype of human hepatocellular carcinoma derived from hepatic progenitor cells. Nat Med 2006; 12: 410-416
20. Bakin AV, Curran T. Role of DNA 5'-methylcytosine transferase in cell transformation by fos. Science 1999; 283: 387-390
21. Saito Y, Kanai Y, Nakagawa T, Sakamoto M, Saito H, Ishii H, Hirohashi S. Increased protein expression of DNA methyltransferase (DNMT1) is significantly correlated with the malignant potential and poor prognosis of human hepatocellular carcinomas. Int J Cancer 2003; 105: 527-532
22. Sadowski HB, Gilman MZ. Cell-free activation of a DNA-binding protein by epidermal growth factor. Nature 1993; 362: 79-83
23. Diehl JA, Cheng M, Roussel MF, Sherr CJ. Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. Genes Dev 1998; 12: 3499-3511
24. Cohen P, Frame S. The renaissance of GSK3. Nat Rev Mol Cell Biol 2001; 2: 769-776
25. Johnson AC, Murphy BA, Matelis CM, Rubinstein Y, Piebenga EC, AKers LM, Neta G, Vinson C, Birrer M. Activator protein-1 mediates induced but not basal epidermal growth factor receptor gene expression. Oncogene 2000; 9: 27-37
26. Zenz R, Scheuch H, Martin P, Frank C, Eferl R, Kenner L, Sibilia M, Scheuer EF. c-jun regulates eyelid closure and skin tumor development through EGFR signaling. Dev Cell 2003; 4: 879-889
27. Mialon A, Sankinen M, Soderstrom H, Junttila TT, Holstrom T, Koivusalo R, Papageorgiou AC, Johnson RS, Hietanen S, Elenius K, Westermark J. DNA topoisomerase I
is a cofactor for c-Jun in the regulation of epidermal growth factor receptor expression and cancer cell proliferation. Mol Cell Biol 2005; 25: 5040-5051.

28 **Amicone L**, Spagnoli PM, Spath G, Giordano S, Tommasini C, Bernardini S, De Luca V, Della Rocca C, Weiss MC, Comoglio PM, Tripodi M. Transgenic expression in the liver of truncated Met blocks apoptosis and permits immortalization of hepatocytes. EMBO J 1997; 16: 495-503.

29 **Schuemmann M**, Hennig G, Muller R. Transcriptional activation and transformation by chimaeric Fos-estrogen receptor proteins: altered properties as a consequence of gene fusion. Oncogene 1993; 8: 2781-2790.

30 **Brown JR**, Nigh E, Lee RJ, Ye H, Thompson MA, Saudou F, Pestell RG, Greenberg ME. Fos family members induce cell cycle entry by activating cyclin D1. Mol Cell Biol 1998; 18: 5609-5619.

31 **Sunters A**, McCluskey J, Grigoriadis AE. Control of cell cycle gene expression in bone development and during c-Fos-induced osteosarcoma formation. Dev Genet 1998; 22: 386-397.

32 **Sunters A**, Thomas DP, Yeudall WA, Grigoriadis AE. Accelerated cell cycle progression in osteoblasts overexpressing the c-fos proto-oncogene: induction of cyclin A and enhanced CDK2 activity. J Biol Chem 2004; 279: 9882-9891.

33 **Ramljak D**, Calvert RJ, Wiesenfeld PW, Diwan BA, Catipovic B, Marasas WF, Victor TC, Anderson LM, Gelderblom WC. A potential mechanism for fumonisin B(1)-mediated hepatocarcinogenesis: cyclin D1 stabilization associated with activation of Akt and inhibition of GSK-3beta activity. Carcinogenesis 2000; 21: 1537-1546.

34 **Diehl JA**, Zindy F, Sherr CJ. Inhibition of cyclin D1 phosphorylation on threonine-286 prevents its rapid degradation via the ubiquitin-proteasome pathway. Genes Dev 1997; 11: 957-972.

35 **Normanno N**, De Luca A, Bianco C, Strizzi L, Mancino M, Maiello MR, Carotenuto A, De Feo G, Caponigro F, Salomon DS. Epidermal growth factor receptor (EGFR) signaling in cancer. Gene 2006; 366: 2-16.

36 **Roux PP**, Shahbazian D, Vu H, Holz MK, Cohen MS, Taunton J, Sonenberg N, Blenis J. RAS/ERK signaling promotes site-specific ribosomal protein S6 phosphorylation via IRS and stimulates cap-dependent translation. J Biol Chem 2007; 282: 14056-14064.

37 **Nicholson RI**, Gee JM, Harper ME. EGFR and cancer prognosis. Eur J Cancer 2001; 37 Suppl 4: S9-S15.

38 **Ito Y**, Takeda T, Sakon M, Tsujimoto M, Higashiyama S, Noda K, Miyoshi E, Monden M, Matsuura N. Expression and clinical significance of erb-B receptor family in hepatocellular carcinoma. Br J Cancer 2001; 84: 1377-1383.

39 **Breuhahn K**, Longerich T, Schirmacher P. Dysregulation of growth factor signaling in human hepatocellular carcinoma. Oncogene 2006; 25: 3787-3800.

40 **Daveau M**, Scotte M, Francois A, Couloaur C, Ros G, Tallet Y, Hiron M, Hellot MF, Salier JP. Hepatocyte growth factor, transforming growth factor alpha, and their receptors as combined markers of prognosis in hepatocellular carcinoma. Mol Carcinog 2003; 36: 130-141.

41 **Moghaddam SJ**, Haghighi EN, Samiee S, Shahid N, Keramati AR, Dadgar S, Zali MR. Immunohistochemical analysis of p53, cyclinD1, RB1, c-fos and N-ras gene expression in hepatocellular carcinoma in Iran. World J Gastroenterol 2007; 13: 588-593.

42 **Gladden AB**, Diehl JA. Location, location, location: the role of cyclin D1 nuclear localization in cancer. J Cell Biochem 2005; 96: 906-913.

43 **Alt JR**, Cleveland JL, Hannink M, Diehl JA. Phosphorylation-dependent regulation of cyclin D1 nuclear export and cyclin D1-dependent cellular transformation. Genes Dev 2000; 14: 3102-3114.

44 **Kobayashi K**, Phuchareon J, Inada K, Tomita Y, Kozumi T, Hatano M, Miyatake S, Tokuhisa T. Overexpression of c-fos inhibits down-regulation of a cyclin-dependent kinase-2 inhibitor p27Kip1 in splenic B cells activated by surface Ig cross-linking. J Immunol 1997; 158: 2050-2056.

45 **LaBaer J**, Garrett MD, Stevenson LF, Slingerland JM, Sandhu C, Chou HS, Fattaey A, Harlow E. New functional activities for the p21 family of CDK inhibitors. Genes Dev 1997; 11: 847-862.

46 **Soos TJ**, Kiyokawa H, Yan JS, Rubin MS, Giordano A, DeBlasio A, Bottega S, Wong B, Mendelsohn J, Koff A. Formation of p27/CDK complexes during the human mitotic cell cycle. Cell Growth Differ 1996; 7: 135-146.

47 **Cheng M**, Olivier P, Diehl JA, Fero M, Roussel MF, Roberts JM, Sherr CJ. The p21(Cip1) and p27(Kip1) CDK 'inhibitors' are essential activators of cyclin D-dependent kinases in murine fibroblasts. EMBO J 1999; 18: 1571-1583.

48 **Sherr CJ**, Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev 1999; 13: 1501-1512.