**INHIBITION OF THE RAS ONCOPROTEIN REDUCES PROLIFERATION OF HEPATOCYTES
IN VITRO AND IN VIVO IN RATS**

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Short title: Ras inhibition and hepatocytes proliferation

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Abstract

Ras oncoproteins are likely implicated in normal and malignant cell growth in various organs. Inhibition of Ras interferes with cell proliferation of non-hepatic cells *in vitro* and *in vivo*.

A potential role for Ras in normal and malignant hepatocytes proliferation prompted us to evaluate the impact of Ras inhibition by S-Farnesylthiosalicylic acid (FTS) on hepatocyte proliferation *in vitro* on the human hepatic tumour cell line Hep G2 and *in vivo* after partial hepatectomy (PH) in rats. Rats were administered FTS intraperitoneally (1,8, 16h after PH) and sacrificed 12, 24 and 48 hours after PH. Cell proliferation, the MAPkinase pathway and cell cycle effectors (cyclin D, E, Cdk2, Cdk4) were assessed in FTS treated rats compared to controls.

FTS significantly decreased overall cell count, PCNA expression and BrdU incorporation of HepG2 cells after 7 days of culture. FTS treatment significantly reduced BrdU incorporation and PCNA expression in hepatocytes after PH. Unlike control rats, cell membrane expression of ras was reduced in FTS-treated animals after PH resulting in decreased Raf membrane recruitment and phosphorylation and in diminished phosphorylation of Erk1/2. The anti-proliferative effect of FTS was linked to a reduced expression and activity of the cyclin E/Cdk2 complex without affecting cyclin D and Cdk4.

Ras inhibition by FTS significantly reduced proliferation of HepG2 cells and of normal hepatocytes after a strong and highly synchronized proliferation stimulus elicited by PH. The inhibitory effect was at least partially mediated by inhibition of Ras-Raf-MAPkinase signalling. It seems worthwhile to evaluate the impact of Ras inhibition on the development of hepatocarcinomas *in vivo* in adequate animal models.

Introduction

There is a general consensus that the Ras oncoprotein may play an important role, not only in the regulation of normal cellular proliferation and differentiation but also in carcinogenesis [1,2]. The Ras oncoprotein activates several intra-cellular signalling pathways including the MAPkinase pathway that plays an important role in promoting DNA synthesis and cell proliferation [3]. *In vitro*, studies have shown that expression of the Ras oncoprotein increased between the G1 and S phases of the cell cycle [4,5] and its inhibition resulted in a cell cycle block in G1 phase. [6,7]. *In vivo* in the liver, it remains, however, ill defined whether interfering with ras activation could lead to reduced liver cell proliferation. An interesting model to study the impact of targeted inhibition of intracellular pathways in the liver is liver cell proliferation following partial hepatectomy (PH). A cascade of events moves cells from their resting state into cell cycle progression in order to restore the liver mass [8]. Several intra-cellular signalling pathways are involved including activation of the small GTPase Ras in response to mitogens [8,9]. Ras mRNA expression increases in the regenerating liver in close relationship to the peak of DNA synthesis [10] and seems to be specifically related to cellular proliferation and cell cycle progression in various animal models of liver cell proliferation [11,12]. In addition, modulation of ras expression in the liver influences the course of liver regeneration in mice [13]. Very recently, it has also been shown that ras and the MAPkinase pathway might play a role in the development of hepatocellular carcinomas in humans [14,55], a cancer with poor survival and scarce therapeutic options. However, additional and unequivocal data are needed before targeting of the ras oncoprotein as a future approach in preventing cellular transformation and malignant liver cell growth can be recommended.

Farnesylation of the Ras oncoprotein is important for its membrane anchorage and activation [16] and inhibition of this process arrests cell growth [17,18]. The Ras antagonist S-Farnesylthiosalicylic acid (FTS) acts over the three isoforms of Ras and seems to achieve inhibition of their membrane anchorage and activation by two distinct mechanisms: the first one involves inhibition of the enzyme prenylated protein methyltransferase (PPMTase) [19,20], the second one, the antagonism of the mature form of the Ras oncoprotein [20,21]. Since FTS exhibits anti-tumoral effects in non liver cell tumours *in vivo* in nude mice and prevents cirrhosis development in rats [22,23], we were prompted to evaluate the impact of inhibition of Ras activation through FTS on normal hepatocyte proliferation in rats after partial hepatectomy (PH) *in vivo* as well as on a hepatic tumour cell line *in vitro*. The

principal aim of the study is to determine whether targeted inhibition of the ras oncoprotein in vivo by such small molecules as FTS does impact on hepatocytes proliferation and therefore could be a potential target for cancer chemoprevention in the liver.

Materials and Methods:

Cell culture

HepG2 cells (2.25×10^5 cells per 100 mm of diameter dish), a human hepatoblastoma derived cell line were cultured in DMEN (Gibco, Harelbeek, Belgium) supplemented with 10% fetal bovine serum, 1% streptomycin and penicillin (Gibco), 1% sodium pyruvate (Gibco) and 1% minimal essential medium (MEM, Gibco) over 7 days. DMSO (solvent for FTS) or FTS at a final concentration of 100 μ M were included into the culture medium from the beginning of the experiment. BrdU was added to the culture medium at a final concentration of 10 μ M from 48 hours of culture onwards. Culture medium was changed every other day. Cells were harvested by trypsinisation at the end of the experiment for flow cytometry analysis and cell count using a Burkert cell. In a subset of dishes, cells were directly lysed and the supernatant was recovered for Western blot analysis. Cell viability was checked using trypan blue staining. At least, three independent experiments were performed for each analysis.

Animals

Male Wistar rats (body weight 180–250 g) were obtained from the rat breeding facilities of the Catholic University of Louvain Medical School, Brussels, Belgium. Animals were kept in a temperature and humidity-controlled environment in a 12 h light–dark cycle. They were allowed free access to water and standard food pellet diet (Usine d’Alimentation Rationnelle, Villemoisson-sur-Orge, France). The animals were handled according to the guidelines established by the Catholic University of Louvain.

Surgical procedures and experimental design

All operations were carried out under light ether anaesthesia at room atmosphere using a clean but a not sterile technique. Partial hepatectomy (PH) was performed according to Higgins and Anderson [24]. Rats were injected intra-peritoneally (i.p.) with 50mg/kg b.w. of BrdU 2 hours before sacrifice. Animals were sacrificed at 12, 24 and 48h after PH. Livers were removed, weighed and snap-frozen or conserved in formol.

The initial approach consisted in testing the toxicity of FTS in our animal model by injecting a dose of 75mg/kg b.w. per injection, the inferior limit of the reported LD50 in rats, initially to non-hepatectomised rats and then to rats subjected to two-third PH. Three intra-peritoneal injections were administered to each animal during a 24 hours period respecting an interval of 8 hours between each injection. Death of the animals was considered as the primary end-point for this part of the study. According to the results obtained during assessment of FTS toxicity, the highest dose of FTS that does not produce any death in hepatectomized rats during the

first 24 hours after PH was chosen for the final experiments. Consequently, the scheme that was used for the final experimental procedure comprised 3 i.p. injections of 50mg/kg b.w. of FTS at 1, 8 and 16 hours after PH. Controls received a similar volume of DMSO (vehicle for FTS) at similar time points. At least three and maximum six animals per experimental group were used.

Synthesis of farnesylthiosalicylic acid (FTS)

FTS was prepared as described previously [19]. Briefly, thiosalicylic acid (2.65 g, 6 mmol), guanidine carbonate (3.82 g, 7 mmol), and *trans,-trans*-farnesyl bromide (5 g, 6 mmol) were mixed overnight in 220.6 ml of acetone at room temperature. After the acetone had evaporated, chloroform was added together with a few drops of 2 N HCl. The mixture was washed with water, and the organic phase was separated and dried on magnesium sulfate and evaporated. FTS was purified on silica gel with mixtures of chloroform and ethyl acetate. FTS was dissolved in DMSO (20mg FTS / 500 μ L DMSO) for injection.

Preparation of liver homogenates, cell lysates and nuclear extracts

Livers were homogenized and cells lysed (HepG2) in ice-cold buffer (0,1% Triton X-100, 50mM HEPES, 150mM NaCl, 10% glycerol, 1.5mM MgCl₂, 1mM DTT, 1mM NaF, 1mM PMSF, 100mg/ml Leupeptin, 10mg/ml Aprotinin and 0.1mM Na₃VO₄). Nuclear extracts were prepared from frozen liver using a commercially available nuclear extract kit according to the instructions of the manufacturer (Active Motif, Rixensart, Belgium).

Preparation of rat liver cytosolic and membrane fractions

Liver membrane fractions were prepared as described by Wheeler et al. [25] with minor modifications. Livers were homogenized in a buffer containing 10 mM Tris HCl (pH 7.4), 1 mM EDTA (pH 7.6), 0.25 M sucrose, 2 μ g/ml PMSF, 2 μ g/ml Aprotinin, 2 μ g/ml Leupeptin, 1mM DTT and 1mM Benzamidin and centrifuged at 10,000 g for 10 min at 4°C. The supernatant was centrifuged at 100,000 g for 1 h at 4°C to prepare cleared cytosol. The resulting pellet was solubilized in 1ml of a lysis buffer containing 20mM Tris HCl (pH 7.4), 2.5mM EDTA, 1% Triton X-100, 1% NaDOC, 0.1% SDS, 100mM NaCl, 100mM NaF and 1mM Na₃VO₄ and centrifuged at 100,000 g for 1 h at 4°C and was used as the membrane fraction.

Western Blotting

Proteins were separated by SDS-PAGE and transferred to PVDF Transfer membranes according to standard techniques. Membranes were incubated with primary and secondary antibodies as specified in table 1. The antigen–antibody reaction was visualized using the Western Lightning Chemiluminescence Reagent Plus (PerkinElmer) detection system.

Membranes were stripped using Restore Western blot stripping buffer (Pierce Chemical, Rockford, IL, USA) and reprobed whenever possible following the instructions of the manufacturer. Quantification of immune-reactive proteins was obtained by densitometry using the Gel Doc 2000 device and software (Bio-Rad, Nazareth, Belgium).

Immunoprecipitation and Cdk kinase assays

Immunoprecipitation and Cdk kinase assays were performed as previously described [11]. The histone H1 kinase assay was used to assess Cdk2 activity and the Rb (769) fusion protein (Santa Cruz Biotechnology) functioned as a substrate for Cdk4.

Histology and immunohistochemistry

Hepatic sections were stained with hematoxylin–eosin for conventional histology or incubated with a mouse anti-BrdU antibody (Dako; 1/100; overnight) and a secondary anti-mouse Envision System Labelled Polymer-HRP antibody (Dako; 1h) to visualise cell proliferation.

Flow cytometry

Incorporation of BrdU into DNA was quantified by flow cytometry essentially as described by Borbath et al. [26].

Reverse transcription and quantitative polymerase chain reaction (PCR)

cDNA was prepared by reverse transcription of total hepatic RNA as previously described by Stärkel et al. [27]. Quantitative PCR analysis was performed with the GeneAmp 5700 Sequence Detection System and software (Applied Biosystems, Den Ijssel, Netherlands) according to the standardized thermal profile of the system previously set by the manufacturer with SYBR Green for detection. All primers (table 2) were designed using the Primer Express design software (Applied Biosystems). Ribosomal protein L19 (RPL19) RNA was chosen as an internal standard. Expression of mRNA was quantified using the $\Delta\Delta\text{CT}$ method as specified by the manufacturer.

Statistical Analysis

Results are expressed as means $\pm$ SEM. The statistical differences between the groups were tested using the Student's *t*-test. Statistical significance was assumed for *P* values < 0.05 .

Results:

FTS inhibits liver tumour cell proliferation *in vitro*

First, we examined whether FTS is able to inhibit proliferation of the hepatic tumour cell line HepG2 *in vitro*. After 3 days of culture in the presence of FTS, a slight non significant reduction in cell proliferation was observed compared to untreated cell cultures (not shown). However after 7 days of culture, FTS treated cultures showed a marked reduction in overall cell density (figure 1a) and a total cell count using a Burker cell confirmed a significant reduction in the number of cells per dish (figure 1b). Trypan blue staining of the cells showed viability of more than 98% of the recovered cells. Furthermore, a significant decrease in BrdU incorporation into the cells (figure 1b) as well as a reduced expression of PCNA by Western blot (figure 2) was found in the FTS treated cultures compared to controls. These observations clearly show that FTS inhibits proliferation of tumour derived hepatocytes *in vitro*.

FTS significantly reduces hepatocytes proliferation *in vivo* after PH

Since FTS effectively inhibited hepatocytes proliferation *in vitro*, we next examined whether FTS is also able to inhibit hepatocytes proliferation *in vivo*. To study the *in vivo* effect of FTS on hepatocytes, the partial hepatectomy model was chosen as it induces a synchronous, vigorous and reproducible proliferation of hepatocytes in rats.

1. Injection Scheme of FTS and mortality

As a first step we determined the potential toxic/lethal dose of FTS in our experimental setting. Given the lethal dose 50 (LD50) of FTS of 75 to 100 mg/kg b.w. reported in rats [22], we started with intraperitoneal injections of 75 mg/kg of FTS, the inferior limit of the LD50 range. Considering death as the primary end-point, we treated rats, subjected or not to a PH, with up to three consecutive injections of 75 mg/kg of FTS at 1, 8 and 16 hours. No mortality was observed in the non-hepatectomised rats (n=6/group) receiving either FTS or DMSO (vehicle for FTS). By contrast, three injections of 75 mg/kg of FTS caused death within 24 hours in 57% of the rats subjected to the PH (4 out of 7 rats) but not in rats receiving the vehicle (DMSO) only (n=8). Therefore, we decreased the dose of FTS to 50 mg/kg b.w. and repeated the experiment in partial hepatectomised rats. At that time, three consecutive injections of FTS at 1, 8 and 16 hours after PH did not induce any mortality in the animals (n=8). These results suggest that, in our experimental conditions, only the association of three injections of 75 mg/kg of FTS with PH elicits death in rats. Consequently, the scheme of 3 injections of the lower dose of FTS (50 mg/kg b.w.) at 1, 8, and 16 hours after PH was used

for further studies assessing the impact of FTS on ras inhibition, cell proliferation and the cell cycle.

2. PCNA Expression

The proliferating Cell Nuclear Antigen (PCNA) is widely used as a cell proliferation marker [28]. Therefore, homogenates from DMSO and FTS-treated rats, sacrificed at 12, 24 and 48 hours after PH, were subjected to Western blot analysis with a PCNA specific antibody. Low PCNA expression was found in DMSO-treated animals at 12h. PCNA peaked at 24 h and decreased at 48h (figure 3a). FTS treatment significantly reduced PCNA expression at 12 and 24h after PH compared to the DMSO group. At 48h, PCNA expression was similar in FTS and DMSO treated animals. However and by contrast to the DMSO group, no prominent peak of PCNA expression was observed during the first 48h after PH in the FTS-treated animals (figure 3b).

3. BrdU incorporation

To confirm the impact of FTS treatment on DNA synthesis, we determined the incorporation of BrdU into DNA of hepatocytes during S-phase of the cell cycle by immunohistochemistry and quantified this incorporation by flow cytometry analysis (FACS). BrdU incorporation was negligible at 12h after PH. BrdU incorporation peaked in DMSO-treated animals at 24h and decreased thereafter (figure 4a). As with PCNA, FTS strongly reduced BrdU incorporation at 24h after PH predominantly in hepatocytes (figure 4a,b) and returned thereafter to levels observed in DMSO-treated animals (figure 4a). In parallel to PCNA expression, BrdU incorporation was also blunted in FTS-treated animals over the first 48h after PH compared to their DMSO-treated littermates that exhibited a clear peak at 24h.

Taken together, the data obtained for PCNA expression and BrdU incorporation strongly suggest that FTS impairs hepatocytes proliferation.

4. liver to body weight index

The liver to body weight index reflects recovery of the liver mass proportional to the weight of the animal. In accordance with reduced cellular proliferation in FTS-treated animals at 24 hours after PH, the liver to body mass index was significantly lower in FTS-treated rats compared to DMSO controls ($1.5\% \pm 0.08$ vs $1.7\% \pm 0.03$, respectively; $p < 0.001$). The differences between both groups were attenuated with partial recovery of cellular proliferation at 48h after PH ($2.4\% \pm 0.01$ vs $2.5\% \pm 0.08$ in FTS and DMSO-treated animals, respectively).

FTS dislodges ras from the cell membrane and interferes with downstream signaling through the MAPkinase pathway

Since FTS reduced cell proliferation, we next verified that this effect was indeed due to functional inhibition of the ras protein.

1. Sub-cellular ras localization

Cytosol and membrane fractions from DMSO and FTS-treated animals were analysed for ras expression by Western Blotting. During the first 48 hours after PH, Ras expression was principally confined to the cell membranes in DMSO animals (figure 5). No ras immunoreactive protein was detected at 12 hours (not shown) and only small amounts of ras protein were found in the cytosol beyond the 12th hour after PH (figure 5). By contrast, Ras membrane expression decreased progressively in FTS- treated animals and substantial amounts of ras protein could be demonstrated in the cytosol fractions from the 12th hour onwards after PH (figure 5). In addition, FTS did not affect ras mRNA expression which significantly and similarly increased in both groups after PH (not shown).

These results confirm that FTS principally dislodged the ras oncoprotein from the cell membranes without affecting the induction of its mRNA levels.

2. expression of membrane bound p-raf

Activation of the ras oncoprotein recruits cytosolic raf to the plasma membrane where it becomes activated by phosphorylation [29]. Therefore, we subjected membrane fractions of DMSO and FTS-treated animals to Western blot analysis for p-raf expression. P-raf was selectively detected at the membranes of DMSO but not of FTS treated animals at 24 hours after PH (figure 6a). No p-raf expression could be detected at any time during the first 48 hours after PH in FTS-treated rats (not shown) suggesting FTS treatment interferes with activation of raf by ras.

3. activation of the MAPkinase ERK1/2

The activation of Ras leads to down stream activation of the MAPkinase cascade that includes the phosphorylation and activation of the ERK 1/2 proteins [7,30]. Thus, the presence of phosphorylated ERK1/2 might serve as a marker for ras activation. Therefore, we assessed the expression of phosphorylated ERK1/2 in homogenates of DMSO and FTS-treated rats by Western blotting. Phospho-ERK 1/2 was significantly reduced in FTS treated animals during the 48h of the experiment (figure 6b). The strongest reduction was found during the first 24 hours in the FTS group. With interruption of the treatment 16 hours after PH, phospho-ERK 1/2 levels increased again but did not reach the level of expression observed in the DMSO treated animals (figure 6c).

Taken together, these data confirm the functional inhibition of ras by FTS and suggest that reduced cell proliferation might be mediated by inhibition of down-stream signalling through the MAPkinase pathway.

The effect of FTS is connected to the cell cycle principally through reduced CyclinE-Cdk2 expression and activity

The cyclinD/Cdk4 and the cyclinE/Cdk2 complexes constitute the principal factors that are involved in controlling the G0/G1 and the G1/S checkpoints of the cell cycle, respectively. It has been proposed that activation of the ras oncoprotein might directly influence both cell cycle checkpoints [31-33]. We therefore examined the expression of both components of these complexes in the nucleus as well as the activity of their kinase sub-units.

1. Nuclear cyclin D and Cdk4 expression and activity

Cyclin D expression did not change significantly during the first 48 hours after PH regardless whether the animals were administered the solvent (DMSO) or FTS, the active substance (figure 7a). Although, FTS reduced the expression of Cdk4 during the first 24 hours after PH (figure 7a,b), this reduction did not translate into a significant impact on the activity of this kinase (figure 7c).

2. Nuclear cyclin E and Cdk2 expression and activity

FTS treatment decreased Cyclin E expression during the first 48 hours after PH compared DMSO-administered animals (figure 8a). FTS treatment also reduced Cdk2 expression during the first 24 hours after PH in contrast to DMSO animals in which a steady and progressive increase in Cdk2 levels occurred throughout the experiment (figure 8a,b). As a consequence of reduced Cyclin E and Cdk2 levels, Cdk2 activity remained strikingly lower in FTS treated animals during the 48 hours after PH (figure 8c). Interestingly, Cdk2 activity at 48 hours after PH, i.e. more than 24 hours after the last dose of FTS, did not recover in the FTS group despite an increase of nuclear Cdk2 in both DMSO and FTS-treated animals (figure 8a,b,c). These observations suggest that FTS exerts an inhibitory effect on the Cdk2 kinase activity even 24 hours beyond drug withdrawal.

3. p27 and p21 mRNA expression

We next investigated whether reduced Cdk2 activity could be related to increased expression of the Cdk inhibitors p27 and p21. No significant differences in p27 mRNA expression was found at any time point in FTS-treated animals compared to their respective control (DMSO) group (data not shown).

A vigorous peak of p21 mRNA levels was observed 24 hours after PH and remained high until 48 hours after PH in DMSO-treated animals. Compared to the kinetics of DNA synthesis, the peak in p21 expression followed the up-regulation of Cdk2 activity as well as the first wave of DNA synthesis in DMSO-treated animals. By contrast, p21 levels were significantly higher in FTS-treated animals at 12 hours after PH preceding the increase in Cdk2 activity observed in DMSO-treated animals as well as the first wave of DNA synthesis, and remained elevated until 48 hours after PH (figure 9). In parallel to strong reduction in DNA synthesis in the FTS group, peak levels of p21 expression were also lower in FTS-treated animals compared to the DMSO-group. These observations suggest that p21 might contribute to down-regulate Cdk2 activity and subsequently DNA synthesis in FTS-treated animals.

Discussion

The ras oncoprotein is thought to play an important role in cell proliferation mechanisms including those of hepatocytes. The aim of our study was to examine whether interfering with the activation of the mature ras oncoprotein would affect liver cell proliferation, an approach that could be exploited in liver carcinogenesis. Therefore, we studied the effect of FTS on HepG2 cells, a human hepatoblastoma derived cell line *in vitro* and on normal hepatocytes *in vivo* after partial hepatectomy in rats. FTS has been shown to inhibit the activation of the ras oncoprotein *in vitro* [18-21]. This inhibiting effect is either due to inhibition of Ras farnesylation, that is essential for its membrane anchorage [16], or results from binding of FTS to the specific Ras membrane binding-site acting as a real competitive inhibitor of Ras membrane anchorage [20]. Our data clearly show that FTS does inhibit proliferation of the hepatic tumour cell line HepG2 *in vitro* which suggests that the treatment acts on transformed hepatocytes. Similarly, it has been shown that inhibition of Ras by FTS induces inhibition of the human tumor cells Panc-1 proliferation *in vitro* and *in vivo* [17,22]. Low dose chronic administration of FTS to rats did also inhibit stellate cell proliferation thereby preventing the occurrence of cirrhosis [23]. When FTS was administered to rats during the first 24 hours after PH, a delay in recovery of the liver mass as well as a highly significant reduction in hepatocytes proliferation was also observed that was confirmed by two independent proliferation markers, PCNA expression and BrdU incorporation. Although cell proliferation in FTS-treated rats remained on levels observed in the DMSO group at 48h after PH, a prominent peak of PCNA expression and BrdU incorporation that usually occurs early after PH, was not seen in FTS-treated animals. This observation suggests that a delay in hepatocytes proliferation persisted at 48 hours after PH and this despite withdrawal of the ras inhibitor at 16h after PH. Interestingly, incomplete recovery of cell proliferation at 48 hours after PH occurred in spite of an increase in cell cycle effector proteins like cyclin D, cdk4 and cdk2 to levels observed in untreated animals. However, neither phospho-ERK 1/2 levels nor Cdk2 kinase activity did fully recover after FTS withdrawal which might at least in part explain the prolonged effect of FTS on cell proliferation. In addition, the Cdk inhibitor p21 increased before initiation of DNA synthesis in FTS-treated animals and remained elevated until 48 hours after PH. These findings suggest that the block in G1/S phase transition and the prolonged down-regulation in Cdk2 activity could be related to persistently elevated p21 levels in FTS-treated animals. Since later time points have not been examined in our study, we cannot exclude that the cells might finally escape from the block leading to complete liver regeneration on the long term. Recent data confirms that FTS acts in a specific manner on the

active, GTP-bound forms of ras proteins. It principally competes with ras-GTP for binding to specific binding sites in the plasma membrane preventing active ras from activating intracellular down-stream signalling pathways [34]. Although FTS might have some effects on farnesylation of ras through interaction with the PPMTase, the physiological impact of these interactions is rather minor. Therefore, it is unlikely that effects on other farnesylated molecules besides ras account for inhibition of cellular proliferation in our study. The ras-ERK pathway is primarily involved in cell proliferation in the regenerating liver. However, we cannot exclude, that other ras-dependent downstream signalling pathways, e. g. the AKT or SAPkinase pathway, might have influenced to some extent the proliferative response elicited by partial hepatectomy.

As previously shown by our group, Cdk2 kinase activity is essential for transition from G1 to S-phase and blockage or reduction of its activity blocked hepatocytes in late G1 phase [35]. It has been shown that ras expression increases at a critical time point in the regenerating rat liver [10,36]. In addition, interference with ras gene expression also reduced liver regeneration presumably through reduction of cyclin E/Cdk2 expression [13]. Our results of strongly reduced liver cell proliferation using the Cdk2 inhibitor roscovitine [35] and the ras inhibitor FTS in this study adds further evidence for a critical role of the ras-cyclin E/Cdk2 axis in hepatocytes proliferation. It has been suggested that ras signalling via the MAPkinase pathway may be important in this process. The observations made in FTS treated animals in our study sustain an important role for the ras-raf-MAPkinase pathway in liver cell proliferation. Reduced ras membrane expression, increased cytosolic ras retention, and decreased activation of raf and Erk 1/2 in FTS treated animals suggests that the anti-proliferative effect of FTS is at least partially mediated by inhibition of the ras-raf-MAPkinase pathway. How this pathway is connected to the cell cycle still remains ill understood. It has been shown that the Ras protein activates the transcription of the cyclin D1 gene [33]. Our results are not in favour of a major effect on this cell cycle effector since cyclin D1 nuclear protein levels did not change significantly after administration of the ras inhibitor FTS. In addition, the activity of its preferred kinase partner, cdk4, was also not affected in the nucleus by the treatment making it unlikely that the inhibitory effect is a result of a reduced activation of the cyclin D/cdk4 complex. By contrast, FTS treatment reduced cyclin E and cdk2 nuclear expression with a prolonged inhibitory effect on cdk2 kinase activity in the nucleus. These results suggest that the ras-raf-MAPkinase pathway principally modulates the activity of the cyclin E/cdk2 complex in our experimental conditions. A similar observation has been made by Lüdde et al. who showed that the Ras activation, *in vivo*, has a

direct impact on the hepatocytes proliferation by modulating the activity of the cyclin E/cdk2 complex [13].

The partial hepatectomy model has been chosen since it allows to study vigorous and synchronous hepatocyte proliferation under strictly controlled and reproducible conditions. The observation that the inhibitory effect of FTS on cell proliferation extended beyond drug withdrawal in this model is of particular interest from the oncology point of view. Tumour cells show less vigorous, non-synchronized proliferation patterns over a longer time period. Given the prolonged effect of FTS, these cells might therefore remain susceptible to the anti-proliferative effect of the compound. With our demonstration that FTS does inhibit proliferation of hepatic tumour cells *in vitro* as well as proliferation of normal hepatocytes *in vivo*, it is tempting to speculate that FTS might have anti-proliferative effects in hepatocellular carcinoma *in vivo*. Indeed, the ras-raf-MAPkinase signalling pathway seems to be important in liver tumour development and ras mutations or activation of the MAPkinase pathway have been demonstrated in hepatocellular carcinoma [15,37].

Taken together, our results show that interfering with the activation of the ras oncoprotein in hepatic tumour cells *in vitro* as well as in normal hepatocytes *in vivo* that have acquired a high proliferative competence dramatically reduces their ability to proliferate. This effect lasted for at least 48 hours after partial hepatectomy even though the inhibitor was withdrawn 16 hours after PH. Mutations of Ras seem to be involved in the development of many cancers including the liver [1,2]. Knowing from our data that the ras inhibitor FTS is capable to inhibit normal and malignant hepatocytes proliferation with minimal toxic manifestations, it seems worthwhile to test the preventive and/or therapeutic effects of this compound in adequate animal models of hepatocellular carcinomas. Such an approach may potentially lead to the development of new therapeutic strategies in humans.

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Legends to figures

Figure 1. A. Representative HepG2 cell density after 7 days of culture in cultures without treatment (control), exposed to the solvent DMSO only or treated with the ras inhibitor FTS (DMSO+FTS). In contrast to control and DMSO cell cultures that nearly reached confluence, a strongly reduced cell density was observed in FTS-treated cell cultures. **B.** Cell count and BrdU incorporation per culture dish in HEPG2 cells after 7 days of culture without treatment (CTL), exposed to the solvent DMSO only or treated with the ras inhibitor FTS (DMSO+FTS). FTS treatment induced a significant decrease in the number of cells per dish as well as in BrdU incorporation into cells. Represented is the mean value $\pm$ SD of 3 independent experiments. P values refer to the control (a) and DMSO (b) cultures, respectively.

Figure 2. Western blot of PCNA and densitometry analysis of the blots of HepG2 cells after 7 days of culture in cultures without treatment (control, CTL), exposed to the solvent DMSO only or treated with the ras inhibitor FTS (DMSO+FTS). FTS treatment induced a significant reduction in PCNA expression compared to control or DMSO cultures. The mean value $\pm$ SD of 3 independent experiments is represented in the densitometry analysis. P values refer to the control (a) and DMSO (b) cultures, respectively.

Figure 3. PCNA expression in DMSO (vector) and FTS (active compound) treated animals at various time points after partial hepatectomy (PH). **A.** Representative Western blot of PCNA expression in liver homogenates. **B.** Quantification of PCNA expression on Western blots by densitometry. FTS administration leads to a significant reduction of PCNA expression at 12 and 24 hours after PH.

Figure 4. BrdU incorporation into DNA in DMSO (vector) and FTS (active compound) treated animals at various time points after partial hepatectomy (PH). **A.** Quantification of BrdU positive hepatocytes by flow cytometry. **B.** Representative immunohistochemical staining of BrdU positive nuclei in hepatocytes 24 hours after PH. BrdU incorporation into hepatocytes nuclei was hardly detectable 12 hours after PH and significantly reduced at 24 hours in FTS treated animals.

Figure 5. Representative Western blot of Ras expression in the cytosol (upper panel) and in membrane fractions (lower panel) of DMSO (vector) and FTS (active compound) treated animals at various time points after partial hepatectomy (PH). FTS treatment leads to increased cytosolic localisation of ras and reduces the membrane bound fraction of the oncoprotein at all investigated time points after PH.

Figure 6. Activation of the MAPkinase signalling pathway downstream of ras in DMSO (vector) and FTS (active compound) treated animals at various time points after partial hepatectomy (PH). **A.** Representative Western blot of p-raf expression in membrane fractions of DMSO and FTS treated animals 24 hours after partial hepatectomy (PH). The membrane bound phosphorylated form of raf was only detected in DMSO but not in FTS treated animals at the 24 hours time point after PH. **B.** Representative Western blot of phospo-Erk 1/2 (pErk) and total Erk (t-Erk) expression in liver homogenates (D=DMSO, F=FTS). **C.** Quantification of phospo-Erk 1/2 normalized to total Erk expression (pErk/tErk) on Western blots by densitometry. FTS administration leads to a reduction of phospo-Erk 1/2 expression at all investigated time points after PH.

Figure 7. Cyclin D1 and Cdk4 nuclear expression and activity in DMSO (vector) and FTS (active compound) treated animals at various time points after partial hepatectomy (PH). **A.** Representative Western blot of cyclin D1 and Cdk4 expression in liver nuclear extracts (D=DMSO, F=FTS, C=untreated control before PH). **B.** Quantification of Cdk4 expression on Western blots by densitometry. **C.** Immunoprecipitation of Cdk4 nuclear protein followed by a Cdk4 kinase assay using the Rb (769) fusion protein as a substrate (C=untreated control before PH). The figure is representative of 3 independent experiments. FTS treatment did not affect nuclear cyclin D1 expression at any time point of the experiment but significantly reduced nuclear Cdk4 expression 24 hours after PH. However, reduced Cdk4 levels had no impact on Cdk4 kinase activity that remained almost constant throughout the 48 hours after PH.

Figure 8. Cyclin E and Cdk2 nuclear expression and activity in DMSO (vector) and FTS (active compound) treated animals at various time points after partial hepatectomy (PH). **A.** Representative Western blot of cyclin E and Cdk2 expression in liver nuclear extracts (D=DMSO, F=FTS, C=untreated control before PH). **B.** Quantification of Cdk2 expression on Western blots by densitometry. **C.** Immunoprecipitation of Cdk2 nuclear protein followed by the histone H1 kinase assay to assess Cdk2 kinase activity (C=untreated control before PH). The figure is representative of 3 independent experiments. FTS treatment decreased nuclear cyclin E expression after PH. In addition, nuclear Cdk2 levels were significantly reduced 24 hours after PH but strongly increased thereafter. However and despite an increase in Cdk2 levels at 48 hours after PH, Cdk2 kinase activity remained depressed throughout the 48 hours after PH in FTS treated animals.

Figure 9. Quantitative PCR of p21 mRNA expression normalized to their respective Rpl19 values (internal standard) in DMSO and FTS-treated animals at various time point after partial

hepatectomy (PH). P21 increased significantly earlier in FTS-treated animals (12h after PH) and remained elevated until 48h after PH.

Table 1. Antibodies and working conditions used for Western blotting

Antibody	Reaction conditions	Source
mouse monoclonal anti-PCNA	1/750; overnight	Dako, Glostrup, Denmark
mouse monoclonal anti-Ras	1/1000; 2h	Upstate, Lake Placid, NY, USA
rabbit polyclonal anti-Phospho-Raf-1	1/500; 2h	Santa Cruz, Santa Cruz, CA, USA
Rabbit polyclonal anti Phospho-p44/42 Map kinase	1/500; overnight	Cell Signaling, Beverly, MA, USA
Mouse monoclonal anti-ERK1	1/500; overnight	BD Biosciences, Erembodegem, Belgium
mouse monoclonal anti-b-actine	1/80.000; 1h	Sigma, Saint Louis, MI, USA
secondary peroxidase-conjugated goat anti-mouse	1/20.000 to 1/40000; 1h	Jackson ImmunoResearch, West Grove, PA, USA
secondary peroxidase-conjugated goat anti-rabbit	1/20.000 to 1/100000; 1h	Jackson ImmunoResearch

Table 2. Primers used for quantitative PCR analysis

Gene	forward	reverse
p21	CCTGTTCCACACAGGAGCAA	CATGAGCGCATCGCAATC
p27	CTTC CGCCTGCAGA AAC	C TTCTCCAAGT CCCGGGTTAG
ras	AGCATAAACTGCGGAAACTGAAC	CATGAGCTGCAAGTGTGTGCT
RPL19	CAAGCGGATTCTCATGGAACA	TGGTCAGCCAGGAGCTTCTT

Figure 1

Figure 2

Figure 3

A.

B.

Figure 4

A.

B.

Figure 5

Figure 6

Figure 7

A.

B.

C.

Figure 8

A.

B.

C.