Diabetologia (2011) 54:954–964
DOI 10.1007/s00125-010-2032-1

ARTICLE

Oleate-mediated activation of phospholipase D and mammalian target of rapamycin (mTOR) regulates proliferation and rapamycin sensitivity of hepatocarcinoma cells

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Received: 27 September 2010 / Accepted: 3 December 2010 / Published online: 15 January 2011

Abstract
Aims/hypothesis A high-fat diet and obesity are associated with increased risk of liver cancer. Because increased delivery of NEFA to the liver occurs in these conditions, we investigated the involvement of the unsaturated fatty acid oleate in hepatocarcinoma cell proliferation using human-derived hepatocarcinoma cell lines as model systems.
Methods Western blotting, FACS analysis and [^{3}H]thymidine incorporation were used to study the signalling pathways and the proliferation of cells cultured for up to 72 h with or without a concentration of oleate considered to be relevant to human pathophysiology (50 μmol/l) or a concentration considered elevated (1 mmol/l).
Results In HepG2 cells, proliferation was increased in the presence of 50 μmol/l oleate, but was decreased at 1 mmol/l. This differential effect was correlated with the activation of the mammalian target of rapamycin complex 1 (mTORC1) and with increased translation of cell cycle regulators. Oleate-mediated mTORC1 activation required phospholipase D activation, which produces phosphatidic acid and is known to render mTORC1 rapamycin resistant. Remarkably, rapamycin resistance was found to affect specifically the mTORC1/eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) branch of the mTORC1 pathway in the presence of 50 μmol/l oleate. Furthermore, inhibition of phosphatidic acid production abolished oleate-induced increases in mTORC1 activity and cyclin A production. Importantly, the same differential effects of oleate on mTORC1 activation, cell cycle regulators and rapamycin resistance were found in SK-Hep1 cells.
Conclusions/interpretation Oleate stimulates mTORC1 activation and rapamycin resistance. We propose that rapamycin-derived mTOR inhibitors are likely to be of limited therapeutic use to restrain hepatic tumour growth, particularly in the context of associated obesity.

Keywords Liver cancer · mTORC1 · Oleate · Phospholipase D · Proliferation · Rapamycin resistance

Abbreviations

4E-BP1	Eukaryotic initiation factor 4E-binding protein 1
HCC	Hepatocellular carcinoma
mTORC1	Mammalian target of rapamycin complex 1
PA	Phosphatidic acid
PKB	Protein kinase B
PLD	Phospholipase D
PTEN	Phosphatase and tensin homologue
S6	Ribosomal protein S6
S6K	p70 S6 kinase

Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death worldwide [1] and the most frequent

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form of liver cancer. HCC is thought to occur in response to chronic liver injury, which may drive compensatory hyperproliferation of transformed hepatocytes [2]. Chronic liver injury can result from alcohol consumption, viral infection or the progression of non-alcoholic fatty liver disease (NAFLD) to non-alcoholic hepatosteatitis (NASH) [3]. In agreement with this, epidemiological observations have revealed a link between high-fat diet feeding/obesity and the risk of HCC. Indeed, high-fat feeding and obesity-associated insulin resistance increase the delivery of NEFA to the liver, which, together with increased liver lipogenesis, contributes to NAFLD development and liver regeneration [4]. Furthermore, obesity-associated low-grade chronic inflammation favours NAFLD progression to NASH [5].

NEFA have the intrinsic potential of inducing both liver damage and compensatory hyperproliferation. The saturated NEFA palmitate is known to promote liver damage and to exert lipotoxic effects on hepatocytes [6]. Conversely, the unsaturated NEFA oleate has recently been shown to increase HepG2 hepatoma proliferation through an mTOR-dependent mechanism [7]. According to the authors, the subsequent increase in cell proliferation could involve protein kinase B (PKB) activation. However, another study does not support PKB activation as the mechanism promoting hepatoma proliferation in obese mice [8]. Indeed, PKB activation in the liver is blunted in the context of obesity and insulin resistance.

Alternative means used by oleate to stimulate hepatoma proliferation could be directly related to the activation of the mammalian target of rapamycin complex 1 (mTORC1). First, this pathway is known to be crucial for cell growth and proliferation [9]. Furthermore, its activation was shown to be increased in the liver of HCC-bearing obese mice, whereas PKB activation was shown to be decreased [8]. While the ability of NEFA to activate the mTOR pathway was described several years ago, the underlying mechanism remains poorly defined [10]. The mTOR kinase responds to growth factors, amino acids, energy or stress signals to control cell growth, proliferation, survival and metabolism [11]. Depending on its partners, mTOR forms two distinct multiprotein complexes, mTORC1 and mTORC2, which exhibit different sensitivities to the immunosuppressant drug rapamycin and display different substrate specificities. The two main substrates of mTORC1 are the translational regulators p70 S6 kinase (S6K) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), whereas mTORC2 phosphorylates PKB on serine 473. In addition, regulation of the mTORC1 pathway through the binding of the lipid messenger phosphatidic acid (PA) to mTOR [12, 13] and S6K [14] has been described. PA is produced through the activity of phospholipase D (PLD) isozymes, among which PLD2 has been shown to be concentration-dependently regulated by oleate [15]. Thus, one would expect that oleate-mediated activation of mTORC1 may, at least in part, rely on PLD activity. Interestingly, the activity of PLDs is increased in numerous cancers, and has been suggested to favour the survival/metastatic potential of cancer cells and to render them rapamycin resistant [16]. Moreover, elevated PLD-mTOR signalling has been suggested to mediate the Warburg effect [17], a metabolic shift characteristic of cancer cells that provides them with the energetic fuels needed for cell growth [18].

To sum up, a large amount of information on the implication of NEFA in cancer biology prompted us to revisit the mechanisms by which oleate may influence the proliferation and related growth signalling pathways in two human hepatocarcinoma cell lines, HepG2 and Sk-Hep1, which we used as model systems.

Methods

Antibodies and other reagents DMEM and Eagle's minimum essential medium (EMEM) were purchased from Gibco Laboratories (Invitrogen, Cergy Pontoise, France) and ATCC (Manassas, VA, USA), respectively. Bovine Serum Albumin fraction V, fatty acid free (catalogue no. 04-100-814) was from Euromedex (Souffelweyersheim, France). Oleic acid conjugated to fatty acid-free BSA (O3008), rapamycin and hepatocyte growth factor were purchased from Sigma-Aldrich (St Quentin-Fallavier, France). [Methyl-^{3}H]thymidine was purchased from Perkin-Elmer (Courtaboeuf, France). Antibodies directed to cyclin A and E were from Santa Cruz Biotechnology (Heidelberg, Germany). Antibody to α-tubulin was from Sigma-Aldrich (Lyon, France). All other antibodies were purchased from Cell Signaling Technology (Ozyme, Montigny le Bretonneux, France).

Cell culture conditions and treatments HepG2 and SK-Hep1 cells (HB-8065 and HTB-52; ATCC) were, respectively, cultured in DMEM or EMEM supplemented with 10% (vol./vol.) FCS, 40 U/ml penicillin and 40 μg/ml streptomycin in a 5% CO_2 atmosphere at 37°C. For most experiments, 4×10^5 cells were grown in six-well plates in medium containing 10% (vol./vol.) FCS for 16 h. Thereafter, the medium was replaced with DMEM or EMEM containing fatty acid-free BSA (control condition), or conjugated to 50 μmol/l or 1 mmol/l oleic acid for the indicated times (medium was replaced every 24 h). The effects of 50 μmol/l or 1 mmol/l oleate were compared with the corresponding BSA conditions, $BSA_{50\mu mol/l}$ (0.025 mmol/l BSA) or $BSA_{1mmol/l}$ (0.5 mmol/l BSA), respectively, as different amounts of BSA were required to conjugate 50 μmol/l vs 1 mmol/l oleate. However, only the

$BSA_{1mmol/l}$ control condition is presented, as no differences were found between $BSA_{50\mu mol/l}$ and $BSA_{1mmol/l}$. When indicated, rapamycin or hepatocyte growth factor (HGF) was used at 50 nmol/l or 50 μg/ml, respectively. 1-Butanol and *tert*-butanol were used at 0.6% (vol./vol.). When indicated, cells cultured in medium containing 10% (vol./vol.) FCS were used as a positive control.

Oil Red O staining and triacylglycerol content determination Cells cultured for 24 h were washed twice with PBS and fixed in 4% (vol./vol.) paraformaldehyde in PBS for 20 min. After washing, cells were stained with Oil Red O for 20 min at room temperature and images were captured under a microscope. For quantitative analysis of cellular triacylglycerol, Oil Red O-stained cells were rinsed exhaustively with PBS before addition of 200 μl isopropyl alcohol. The extracted dye was removed and its absorbance was monitored at 510 nm as described [19].

Cell counting and FACS analysis of cell viability Cells cultured for 24, 48 and 72 h were trypsinised and counted with a haemocytometer. For FACS analysis, both floating and adherent cells were washed and stained with 25 μg/ml propidium iodide in PBS, 2 mmol/l EDTA, 1% (wt/vol.) BSA. Propidium iodide-positive cells were counted by analysis of 1×10^4 cells with a FACScan flow cytometer using Cell Quest Pro software (Becton Dickinson, Mountain View, CA, USA). Results were normalised with respect to the control condition.

[^{3}H]thymidine incorporation Cells were cultured for 72 h as described except that 37 kBq (1 μCi) [^{3}H]thymidine was added 4 h before the medium was removed. Incorporated [^{3}H]thymidine was precipitated by the addition of 10% (vol./vol.) trichloroacetic acid (TCA) for 30 min on ice. Cells rinsed with 5% (vol./vol.) TCA were lysed with 400 μl of 0.2 mol/l NaOH and neutralised with an equal volume of HCl. Lysates were transferred in scintillation buffer and assayed for radioactivity in a scintillation counter.

PLD activity measurement and suppression of PA production by alcohol trap assay Cells cultured for 72 h were washed in PBS and lysed by three freeze–thaw cycles in a solution of 150 μl ice-cold 250 mmol/l Tris, 25 mmol/l $CaCl_2$, pH 8. Then, 50 μl of lysate was incubated for 1 h at 37°C with Amplex Red reaction buffer (Amplex Red Phospholipase D Assay Kit; Molecular Probes, Invitrogen) and PLD activity was estimated with a Synergy 4 fluorometer (Biotek, Colmar, France). A standard curve was generated with purified PLD from *Streptomyces chromofuscus* (Sigma-Aldrich). All results were normalised with respect to the protein concentration of the lysate (BCA Assay; Interchim, Montluçon, France). For PLD inhibition experiments, cells were cultured for 72 h with DMEM supplemented with 0.6% (vol./vol.) of 1-butanol or *tert*-butanol; cell extracts were analysed by western blotting.

Western blot analysis Cells were scraped and incubated for 20 min in ice-cold lysis buffer [20]. The lysates were centrifuged at 13,000×*g* for 20 min at 4°C, and protein concentrations were determined by the BCA colorimetric assay. Lysates were subjected to SDS-PAGE gel electrophoresis and transferred to polyvinylidene fluoride membranes (Bedford, MA, USA). Membranes were presoaked in blocking buffer and incubated with antibodies, according to the manufacturer's instructions. Results were normalised with respect to α-tubulin levels and the BSA condition used as control was arbitrarily assigned a value of 1.

Statistical analysis Experiments were performed at least three times and results are presented as mean ± SEM, and *p* values were determined by the unpaired Student's *t* test. Results were considered significant when $p<0.05$.

Results

Concentration-dependent effect of oleate on HepG2 steatosis and proliferation We first compared the effect of BSA vs BSA-conjugated oleic acid on triacylglycerol accumulation in HepG2 cells (Fig. 1a, b). Treatment of HepG2 cells with oleate for 24 h induced concentration-dependent triacylglycerol accumulation compared with the control condition, with up to a 2.7-fold increase observed at 1 mmol/l oleate. To evaluate the effect of oleate on cell proliferation (Fig. 2a), fetal FCS and HGF were used as controls to increase and decrease HepG2 proliferation, respectively [21]. Compared with the number of cells initially plated, all treatments except 1 mmol/l oleate and HGF led to a significantly increased cell number at the 72 h time point. When comparing the effect of BSA with that of BSA-conjugated oleate, divergent results were observed between 50 μmol/l and 1 mmol/l oleate. Indeed, exposure to 50 μmol/l oleate significantly increased the cell number after 72 h treatment, whereas 1 mmol/l oleate had no significant effect. We next analysed [^{3}H]thymidine incorporation and the number of propidium iodine-positive cells after 72 h of treatment with BSA, 50 μmol/l or 1 mmol/l oleate, FCS or HGF. As seen in Fig. 2b, [^{3}H]thymidine incorporation was increased by 50 μmol/l oleate and FCS but decreased by 1 mmol/l oleate and by HGF, albeit not significantly for the latter. Conversely, the number of propidium iodine-positive cells was unchanged by FCS or HGF but significantly decreased by 50 μmol/l and further decreased by 1 mmol/l oleate (Fig. 2c), indicating that

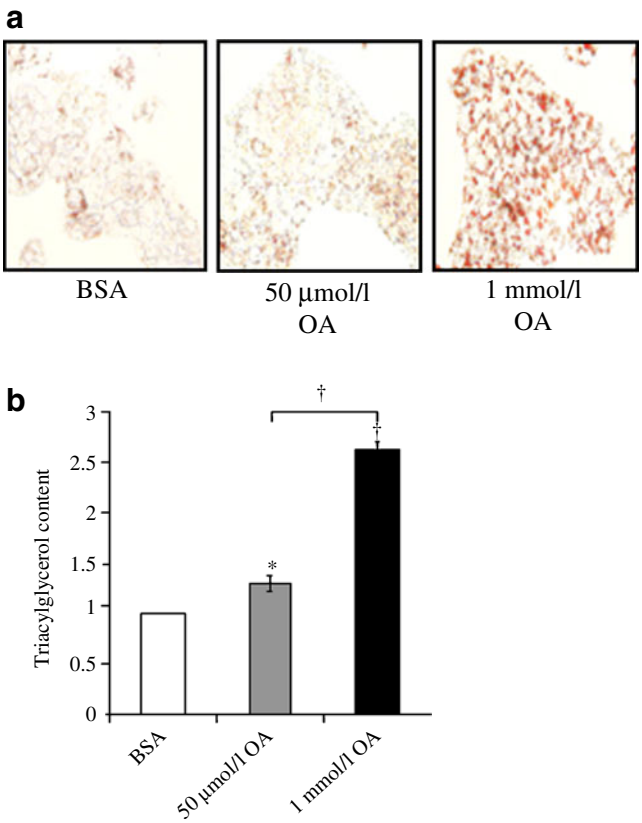

Fig. 1 Concentration-dependent effect of oleic acid (OA) on HepG2 steatosis. **a** Oil Red O staining of HepG2 cells treated with BSA or 50 µmol/l or 1 mmol/l oleate for 24 h. **b** Triacylglycerol content of HepG2 cells treated with BSA or 50 µmol/l or 1 mmol/l OA for 24 h determined by an Oil Red O-based colorimetric assay at 510 nm (n=3). Results were normalised with respect to the BSA condition. *p<0.05, †p<0.001

increasing the oleate concentration indeed protects against cell death.

In summary, our results show that 50 µmol/l oleate increases HepG2 cell number by concomitantly increasing cell proliferation and decreasing cell death.

Oleate-mediated proliferation of HepG2 cells involves the regulation of cell cycle components The differential effects on cell proliferation induced by the low vs high concentration of oleate prompted us to search for a differential modulation of known cell cycle regulators produced by these treatments. Notably, hyperphosphorylation of the cell-cycle regulator retinoblastoma protein (RB) and increased production of cyclin A and E have been implicated in HCC initiation and progression [22, 23]. As seen in Fig. 3a–d, a 24 h treatment with the low concentration of oleate increased the level of phosphorylated RB and the protein levels of cyclin A and E. In contrast, the high concentration of oleate decreased phosphorylated RB and had no significant effect on cyclin A and E protein levels. Similar results were observed with a 72 h treatment (data not shown). Furthermore, exposure to oleate had no effect on cyclin A and E transcript levels, which suggests a translational effect of oleate on their respective mRNAs (data not shown).

Thus, these early effects of oleate on cell-cycle components are likely to explain its proliferative action.

Oleate drives mTORC1-dependent HepG2 proliferation and induces resistance to rapamycin We took advantage of the concentration-dependent effects of oleate treatment to search for the signalling pathways potentially involved in oleate-induced proliferation. We reasoned that the relevant pathway would be differentially regulated by the low vs

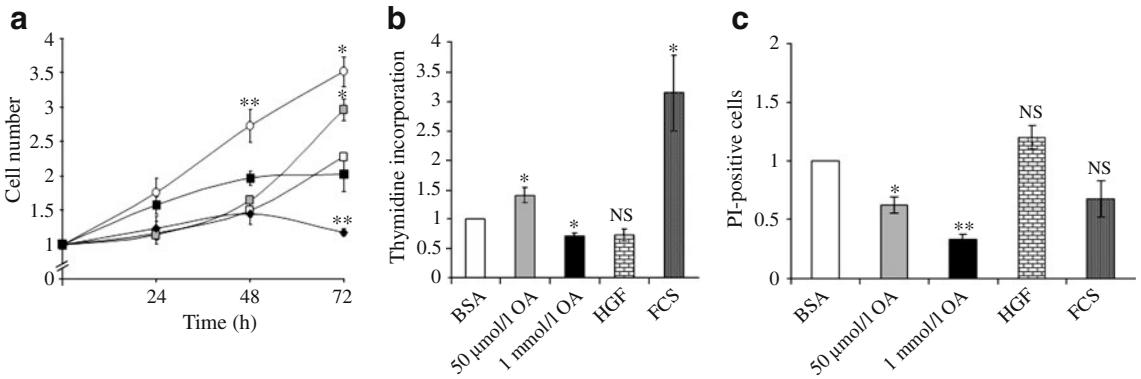

Fig. 2 Concentration-dependent effect of oleic acid (OA) on HepG2 proliferation. **a** Proliferation of HepG2 cells exposed to BSA, 50 µmol/l or 1 mmol/l OA, 50 ng/µl HGF or 10% (vol./vol.) FCS for 24, 48 or 72 h assessed by counting viable cells with a haemocytometer at the corresponding time points (white squares, BSA; grey squares, 50 µmol/l OA; black squares, 1 mmol/l OA; white circles, FCS; black diamonds, HGF 50 nmol/l, n=3). **b** Proliferation of HepG2 cells exposed to BSA, 50 µmol/l or 1 mmol/l OA, 50 ng/µl HGF or 10% (vol./vol.) FCS for 72 h assessed by [^{3}H]thymidine incorporation (n=4). **c** Viability of HepG2 cells exposed to BSA, 50 µmol/l or 1 mmol/l OA, 50 ng/µl HGF or 10% (vol./vol.) FCS for 72 h assessed by FACS analysis of propidium iodine (PI)-positive cells (n=3). Results were normalised with respect to the BSA condition. *p<0.05, **p<0.01

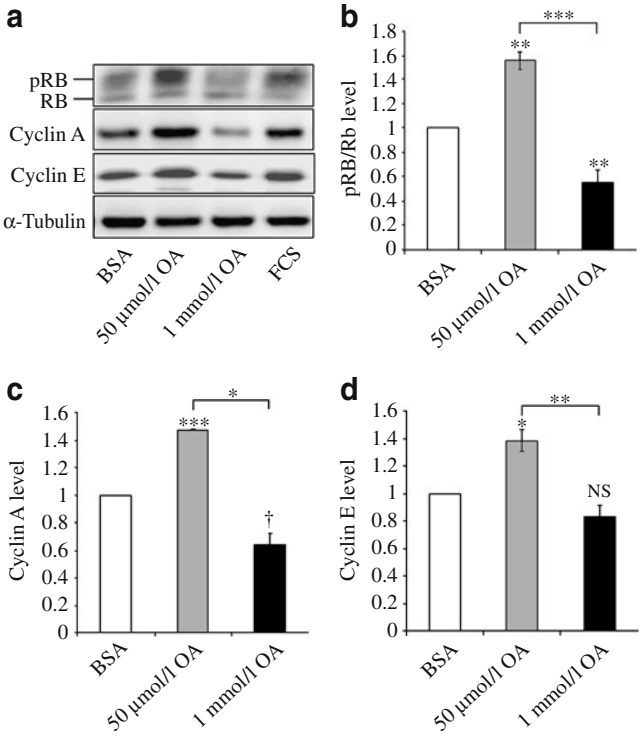

Fig. 3 Oleic acid (OA) regulates the level and activation of cell cycle components. **a** Representative blots showing the level of RB phosphorylation (pRB) and levels of cyclin A and E in HepG2 cells exposed to BSA, 50 μmol/l or 1 mmol/l OA or 10% (vol./vol.) FCS for 24 h. **b–d** Quantification of RB phosphorylation (**b**), cyclin A level (**c**) and cyclin E (**d**) level in HepG2 cells exposed to BSA, 50 μmol/l or 1 mmol/l OA for 24 h (n=3). Results in **b–d** were normalised with respect to the BSA condition. *p<0.05, **p<0.01, ***p<0.001, $^{\dagger}p$=0.052

high concentration of oleate. Among the potentially relevant candidate cascades, we examined the extracellular signal-regulated kinase (ERK)1/2, PKB and mTORC1 phosphorylation levels, and phosphatase and tensin homologue (PTEN) level by western blot analysis of HepG2 cells treated with 50 μmol/l or 1 mmol/l oleate (Fig. 4a–e). A 24 h treatment with the low and high oleate concentrations had a similar effect on ERK and PKB activation, i.e. it reduced ERK and increased PKB. Both concentrations were without effect on PTEN level. Interestingly, we observed a differential effect of oleate on markers of the activity of the mTORC1 pathway (Fig. 5a–d). Indeed, at a low concentration, oleate increased the phosphorylation of mTOR, that of its target 4E-BP1 and that of the ribosomal S6 protein, the downstream target of S6K. Importantly, the high concentration of oleate had the opposite effect on the phosphorylation state of these molecules. Similar results were observed with a 72 h treatment (data not shown).

To further illustrate the concentration-dependent effects of oleate on mTORC1 activation and cyclin A level, HepG2 cells were exposed for 72 h to different concentrations of oleate (Fig. 5e). Oleate increased mTORC1/4E-BP1 phosphorylation and cyclin A level at low concentrations with a maximal increase seen at 50 μmol/l oleate. Starting from 100 μmol/l, these effects decreased. Hence, the two concentrations chosen for our experiments seem to be appropriate because at 50 μmol/l the mTOR pathway is fully activated while at 1 mmol/l it is severely inhibited.

To confirm that mTORC1 activation supported oleate-induced proliferation of HepG2 cells, we analysed the effect of rapamycin on the proliferation induced by a 72 h treatment with 50 μmol/l oleate. Surprisingly, rapamycin treatment alone efficiently inhibited the proliferation of HepG2 cells and reduced the level of cyclin A, but was without effect in the presence of oleate (Fig. 6a, b). Hence, we reasoned that oleate rendered a component of the mTORC1 pathway insensitive to rapamycin. Indeed, it has been shown recently that cap-dependent translation could occur despite rapamycin treatment and S6K inhibition, and that rapamycin resistance may be specific to the mTORC1/4E-BPs arm of the mTORC1 pathway [24]. As seen in Fig. 6c and d, in the presence of oleate, rapamycin reduced the phosphorylation of the ribosomal S6 protein to control levels, but not that of 4E-BP1.

Oleate increases cyclin A level and mTORC1 activity through PLD activity The existence of an oleate-sensitive PLD that is only stimulated by low concentrations of oleate [15] and the notion that PA renders mTORC1 resistant to rapamycin prompted us to examine whether oleate-induced mTORC1 activation may involve activation of PLDs. As seen in Fig. 7a, PLD activity was increased in HepG2 cells treated for 72 h with 50 μmol/l, but it was decreased by 1 mmol/l oleate. Oleate had no effect on PLD1 or PLD2 mRNAs (data not shown). Moreover, inhibition of PLD-mediated PA production by 1-butanol abolished mTORC1 activation assessed by mTOR, S6 and 4E-BP1 phosphorylation (Fig. 7b–d). Furthermore, treatment with 1-butanol blunted the increase in cyclin A protein levels induced by 50 μmol/l oleate compared with the control condition (Fig. 7e).

Oleate activates mTORC1 and favours rapamycin resistance in SK-Hep1 hepatoma cells To exclude the possibility that oleate's main effects could be unique to HepG2 cells, we performed similar experiments on SK-Hep1 cells. Indeed, compared with the basal condition, 50 μmol/l oleate addition for 48 h robustly activated the mTORC1 pathway and increased the cyclin A level (Fig. 8a–e). However, 1 mmol/l oleate also induced a modest, albeit not significant, increase in mTOR phosphorylation and cyclin A level, and a significant increase in S6 and 4E-BP1 phosphorylation, compared with the basal condition. These differences in the effect of 1 mmol/l oleate between HepG2 and SK-

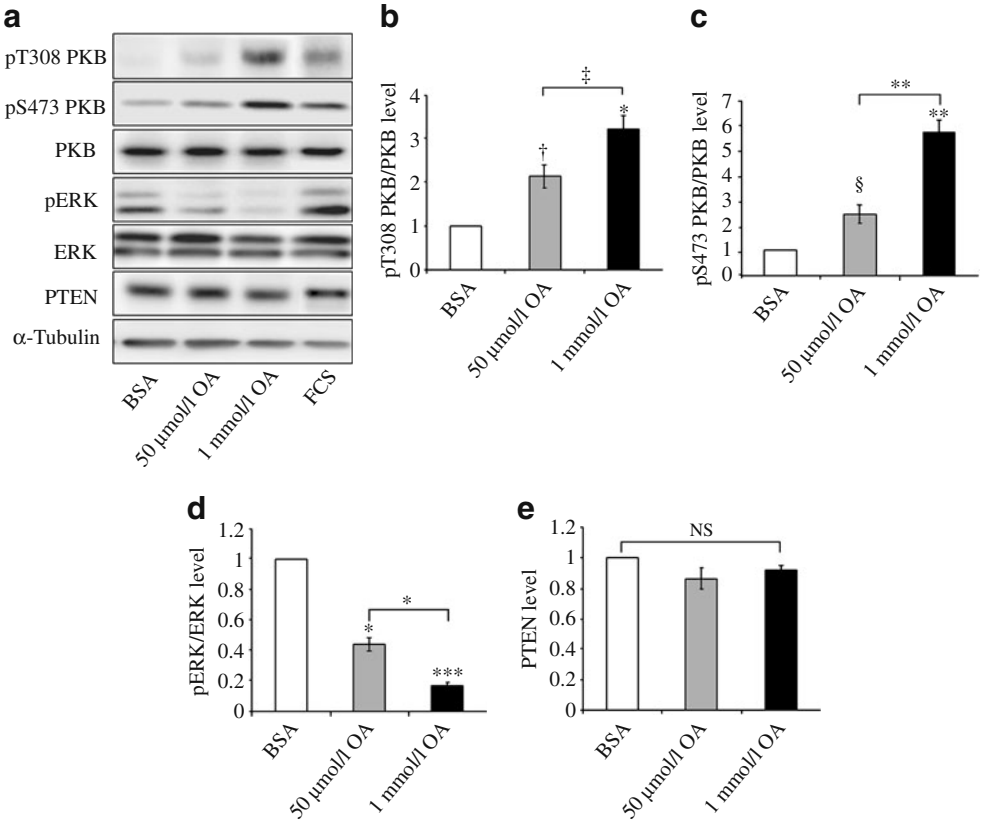

Fig. 4 Oleic acid (OA) regulates the PKB and ERK signalling pathways. **a** Representative blots showing the levels of PKB and ERK phosphorylation and of PTEN in HepG2 cells exposed to BSA, 50 μmol/l or 1 mmol/l OA or 10% (vol./vol.) FCS for 24 h. **b**–**e** Quantification of PKB phosphorylation at T308 (**b**) and S473 (**c**), ERK phosphorylation (**d**) and PTEN (**e**) level in HepG2 cells exposed to BSA, 50 μmol/l or 1 mmol/l OA for 24 h (n=3). Results in **b**–**e** were normalised with respect to the BSA condition. *p<0.05, **p<0.01, ***p<0.001, $^{\dagger}p$=0.051, $^{\ddagger}p$=0.052, $^{\S}p$=0.059

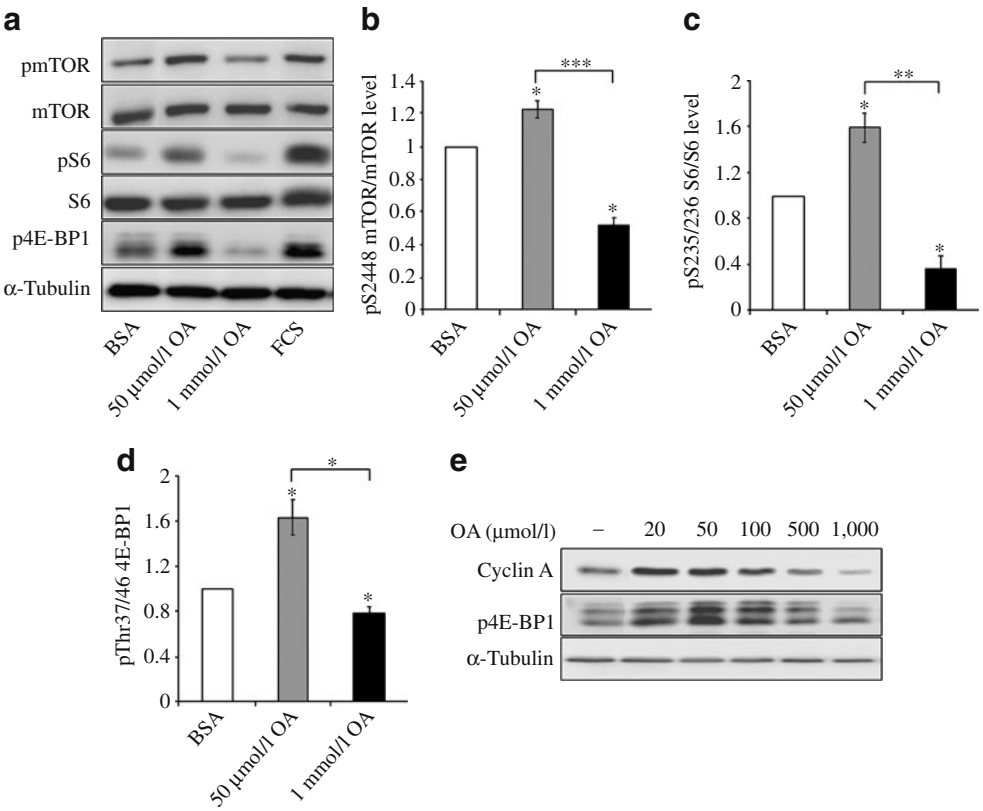

Fig. 5 Concentration-dependent effect of oleic acid (OA) on the mTOR signalling pathway. **a** Representative blots showing the level of pS2448 mTOR (pmTOR), mTOR, phosphorylated S6 (pS6), S6 and pThr37/46 4E-BP1 in HepG2 cells exposed to BSA, 50 μmol/l or 1 mmol/l OA or 10% (vol./vol.) FCS for 24 h. **b**–**d** Quantification of pS2448 mTOR (**b**), pS6 (**c**) and pThr37/46 4E-BP1 (**d**) in HepG2 cells exposed to BSA, 50 μmol/l or 1 mmol/l OA for 24 h (n=3). Results in **b**–**d** were normalised with respect to the BSA condition. **e** Representative blots showing the levels of cyclin A and pThr37/46 4E-BP1 in HepG2 cells exposed to BSA or 20, 50, 100, 500 or 1000 μmol/l OA for 72 h (n=3). *p<0.05, **p<0.01, ***p<0.001

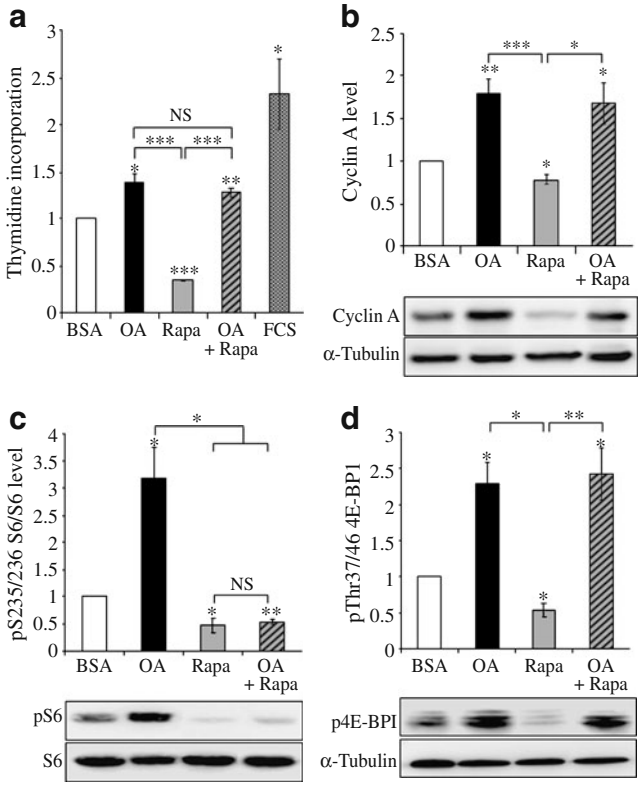

Fig. 6 Selective rapamycin resistance in the presence of 50 μmol/l oleic acid (OA) in HepG2 cells. **a** Proliferation of HepG2 cells exposed to BSA, 50 μmol/l OA, 50 nmol/l rapamycin (Rapa), 50 μmol/l OA plus 50 nmol/l rapamycin or 10% (vol./vol.) FCS for 72 h assessed by [^{3}H]thymidine incorporation (n=3). **b** Representative blots and quantification of cyclin A level in HepG2 cells exposed to BSA, 50 μmol/l OA, 50 nmol/l rapamycin or 50 μmol/l OA plus 50 nmol/l rapamycin for 72 h (n=4). **c**, **d** Representative blots and quantification of phosphorylated S6 (pS6) (**c**) and pThr37/46 4E-BP1 (**d**) in HepG2 cells exposed to BSA, 50 μmol/l OA, 50 nmol/l rapamycin or 50 μmol/l OA plus 50 nmol/l rapamycin for 72 h (n=3). Results were normalised with respect to the BSA condition. *p<0.05, **p<0.01, ***p<0.001

Hep1 cells can be attributed to the low basal level of mTOR activation in the latter cell line.

To investigate whether oleate could also induce rapamycin resistance in this model, SK-Hep1 cells were treated concomitantly with 50 μmol/l oleate and 50 nmol/l rapamycin for 48 h (Fig. 9a–c). Interestingly, 4E-BP1 phosphorylation and cyclin A level were quite insensitive to rapamycin in the presence of oleate, whereas S6 phosphorylation was not. Overall, these findings are similar to the observations made in HepG2 cells.

Discussion

Although the link between overweight, obesity and cancer is well established, the underlying mechanisms remain obscure. Among other hypotheses [25], the fact that most obese patients are insulin resistant led to the idea that their compensatory hyperinsulinaemia may act as a cell growth stimulus. Another explanation could involve the direct effects of NEFA on the liver and potentially on other sensitive organs. Indeed, in the context of obesity-associated insulin resistance, there is both an increase in the systemic release of NEFA stored by adipose tissue and an increase in hepatic lipogenesis. Concerning the release of NEFA by adipose tissue, a metabolomic study has revealed an increase in the circulating levels of oleate from 150 μmol/l in lean humans to 200 μmol/l in obese ones [26]. Although the local tissue concentration of oleate in

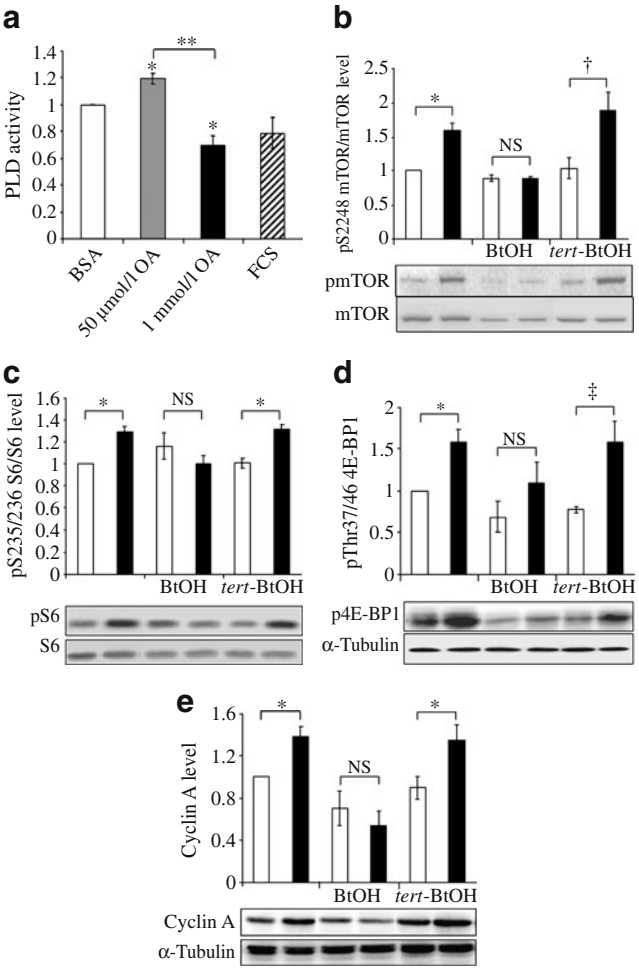

Fig. 7 Oleic acid (OA)-mediated PLD activation regulates mTOR sensitivity to rapamycin, and cyclin A level. **a** PLD activity in HepG2 cells exposed to BSA, 50 μmol/l or 1 mmol/l OA or 10% (vol./vol.) FCS for 72 h (n=3). Results were normalised to the protein content of each sample. **b**–**e** Representative blots and quantification of phosphorylated S2448 mTOR (pS2448 mTOR) (**b**), pS235/233 S6 (**c**), pThr37/46 4E-BP1 (**d**) and cyclin A (**e**) level in HepG2 cells exposed to BSA or 50 μmol/l OA, and with or without 1-butanol or *tert*-butanol for 72 h. Results were normalised with respect to the BSA condition. White bars, BSA; black bars, 50 μmol/l OA) (n=3). Bt-OH, 1-butanol; *tert*-BtOH, *tert*-butanol. *p<0.05, **p<0.01, $^{\dagger}p$=0.06, $^{\ddagger}p$=0.051

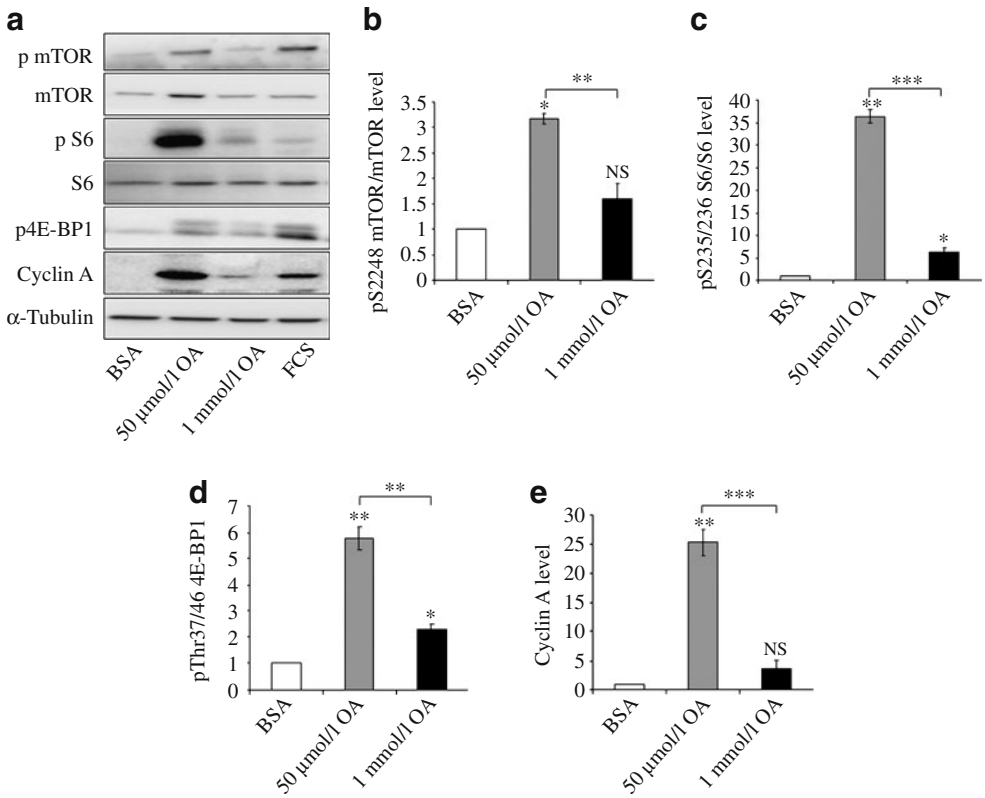

Fig. 8 Oleic acid (OA)-mediated mTORC1 activation and cyclin A level in SK-Hep1 cells. **a** Representative blots showing the level of phosphorylated S2448 mTOR (pS2448 mTOR), mTOR, pS6, S6 and pThr37/46 4E-BP1 and cyclin A level in SK-HEP1 cells exposed to BSA, 50 μmol/l or 1 mmol/l OA or 10% (vol./vol.) FCS for 48 h. **b**–**e** Quantification of pS2448 mTOR (**b**), pS6 (**c**), pThr37/46 4E-BP1 (**d**) and cyclin A (**e**) in SK-HEP1 cells exposed to BSA, 50 μmol/l or 1 mmol/l OA for 48 h (n=3). Results in **b**–**e** were normalised with respect to the BSA condition. *p<0.05, **p<0.01, ***p<0.001

lean and obese people is unknown, this 50 μmol/l increase may in part explain the increased risk of cancer found in obese people. This fact is highly relevant to our present work, which shows a specific proliferative effect of oleate at this concentration on the HepG2 hepatocarcinoma cell line.

The increase in liver lipogenesis observed in the context of obesity has remained puzzling until recently because it was thought to rely on PKB activation, which is blunted in the context of insulin resistance [27]. A recent study showing that mTORC1 drives lipogenesis in the presence of insulin resistance helps to explain the paradox [28]. Again, this is relevant to our work and that of others [7] showing that oleate, one of the major triacylglycerol building blocks, is able to activate mTORC1. It suggests that excess NEFA taken up by the liver may favour lipogenesis and hepatic steatosis by activating mTORC1. Hence, oleate should be added to the list of nutrients whose availability is sensed by mTOR to adapt cell metabolism accordingly, notably in lipid biosynthesis [29].

Concerning the exact mechanism by which oleate increases hepatoma proliferation, Vinciguerra et al. [7]

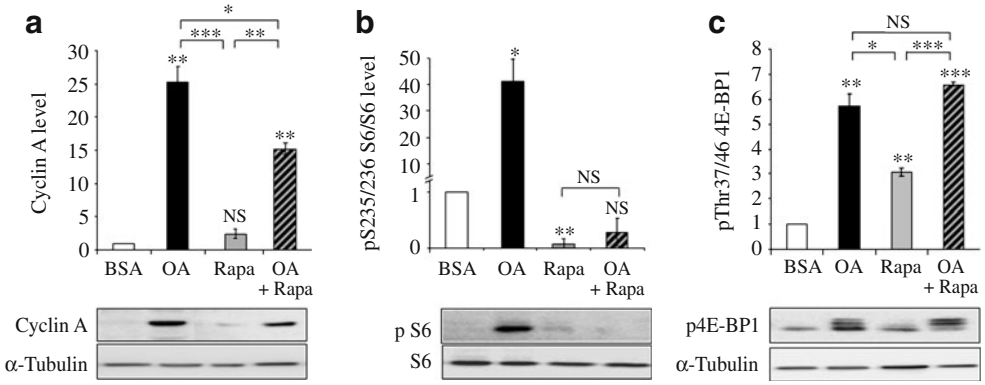

Fig. 9 Selective rapamycin resistance in the presence of 50 μmol/l oleic acid (OA) in Sk-Hep1 cells. **a**–**c** Representative blot and quantification of cyclin A level (**a**), phosphorylation of S6 (**b**) and phosphorylation of 4E-BP1 (**c**) in SK-Hep1 cells exposed to BSA, 50 μmol/l OA, 50 nmol/l rapamycin (Rapa) or 50 μmol/l OA plus 50 nmol/l rapamycin for 48 h (n=3). Results were normalised with respect to the BSA condition. *p<0.05, **p<0.01, ***p<0.001

proposed that it involves a mTOR driven feedback loop restraining PTEN production and hence activating PKB. As pointed out in another study [8], however, this explanation falls short in the context of obese insulin-resistant people, given that PKB activation is blunted. Indeed, we are in agreement with this view since we did not find down-regulation of the PTEN level as a potential explanation for the proliferative effects of oleate. Instead, the activation of mTORC1 by oleate per se could exert a proliferative action, given its known growth-promoting role and its function in translation regulation. Indeed, the fact that oleate regulates the levels of cyclin A and E, components involved in S-phase entry, without affecting the corresponding mRNAs is in agreement with this. Besides mTORC1, other major signal transduction pathways may participate in the proliferative effect of oleate. This could be the case for the ERK pathway, the activation of which was consistently found to be reduced by oleate in our study. Indeed, decreasing the activation of this pathway in HepG2 cells has been shown previously to increase proliferation, whereas stimulation of this pathway underlies HGF-induced inhibition of these cells [30].

We further explored the mechanism by which oleate activates mTOR and focused our efforts on PLDs, a class of enzymes previously shown to be specifically activated by low concentrations of oleate [15] and whose product, PA, regulates mTOR activation and sensitivity to rapamycin [16]. Consistent with our hypothesis, the low and high concentrations of oleate were found to increase and decrease PLD activity, respectively. Furthermore, the activation of the mTORC1 pathway and the increase in cyclin A level induced by oleate were both prevented by concomitant treatment with 1-butanol, which inhibits PLD-mediated PA production. This was not seen with *tert*-butanol used as a control. The blockade of the growth-promoting effect of oleate by 1-butanol might have clinical relevance. Indeed, of the only two amino acid changes conferring constitutive activation of mTORC1 discovered to date in human cancers, one is a mutation rendering mTOR resistant to PLD inhibition by 1-butanol [31], probably by altering the dependence of mTORC1 on PA. The fact that such a mutation occurs underscores the importance of mTOR activation by PLDs in the oncogenic process.

An additional key observation we made concerns the resistance of mTORC1 to rapamycin conferred by oleate. Despite initial high expectations, rapamycin and its derived molecules have been shown to have reduced therapeutic benefits because of the phenomenon of rapamycin resistance, whose molecular basis remains obscure. Indeed, in some cancer cells rapamycin is effective in inhibiting mTOR, but with a benefit that is limited by the concomitant relief of the Ras/mitogen-activated protein kinase (MAPK) pathway from an mTORC1-initiated negative feedback loop [32]. This suggested a promising approach to HCC therapy consisting of a combination of mTORC1 and Ras/MAPK inhibitors [33]. Additional explanations for rapamycin resistance may involve mTORC2-dependent outputs, because this complex, which is involved in PKB activation by phosphorylation of serine 473, has been shown to be less sensitive to rapamycin inhibition compared with mTORC1 [12]. However, our finding that PKB phosphorylation of S473 is increased not only by 50 μmol/l but also by 1 mmol/l oleate, which inhibits proliferation, argues against this hypothesis. Recently, another study implicated rapamycin-insensitive mTORC1 outputs in the mechanism of rapamycin resistance, specifically those of the mTORC1/4E-BP1 arm of the mTORC1 pathway [24]. Although both S6K and 4E-BP initiate translation in response to mTOR activation, they seem to act on specific mRNA populations. Although this view has been challenged recently [34], S6K is thought to specifically favour the translation of mRNA species containing an oligopyrimidine tract at the 5′ terminus (TOP-mRNAs), such as those encoding ribosomal proteins or elongation factors. The phosphorylation and dissociation of 4E-BP from eukaryotic translation initiation factor 4E (eIF-4E) allows the translation of cap-containing mRNAs and of mRNAs with 5′ secondary structures, which frequently encode proteins involved in proliferation [35]. Remarkably, our results showing that 4E-BP1 is still phosphorylated despite inhibition of S6K by rapamycin are in agreement with 4E-BP1 being a key target of rapamycin resistance. When HepG2 cells were treated concomitantly with oleate and butanol, we observed a reduction in the phosphorylation level not only of mTOR and ribosomal protein S6 but also of 4E-BP1, and in cyclin A level. These findings suggest a causal relationship since 4E-BP1 is a translational regulator and since we found that increased cyclin A production involves a post-transcriptional mechanism.

The key observations of our present work, i.e. the concentration-dependent effect of oleate on cell growth and the selective rapamycin resistance, are not unique to HepG2 cells, as we found the same general picture in the SK-Hep1 human hepatocarcinoma cell line.

Given the major challenge in treating liver cancer, it is tempting to extrapolate our findings to the human disease. Considering that physiologically relevant concentrations of oleate confer rapamycin resistance, one would expect that in obese patients suffering from liver cancer the use of this drug will have limited therapeutic success, unless specific dietary recommendations are implemented. In addition, our finding that oleate activates the mTOR pathway may be relevant to liver regeneration, given that NEFA uptake and mTOR activation are essential to this process [36, 37]. Our work begets the clinically important question of whether

the proliferative effect of oleate applies to the whole spectrum of obesity-associated cancers, notably those of epithelial origin, or only part of it. Indeed, monounsaturated fatty acids have been shown to be required for prostate cancer progression in mice [38], and recent observations suggest a rather large role of rapamycin resistance towards the mTORC1/4E-BP arm in breast, colon and cervical cancer cell lines [39], which are derived from epithelial human tumours known to belong to the gamut of obesity-associated cancers. Thus, the concurrent occurrence of oleate-induced proliferation and rapamycin resistance could be considered a novel paradigm of epithelial-derived cancers.

Acknowledgements C. Arous was supported by a PhD fellowship from the Association pour la Recherche contre le Cancer (ARC, 2009-2010) and the Ligue Nationale Contre le Cancer (LNCC, 2010-2011). Our research was supported by the Institut National de la Santé et de la Recherche Médicale, the Université de Nice-Sophia-Antipolis, the Conseil Général des Alpes-Maritimes and the Centre Hospitalier Universitaire de Nice.

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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