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ORIGINAL ARTICLE

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# Vasoactive intestinal peptide induces proliferation of human hepatocytes

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## Abstract

**Objectives**: Proliferation of hepatocytes in vitro can be stimulated by growth factors such as epidermal growth factor (EGF), but the role of vasoactive intestinal peptide (VIP) remains unclear. We have investigated the effect of VIP on maintenance and proliferation of human hepatocytes.

**Materials and methods**: Human hepatocytes were isolated from liver specimens obtained from patients undergoing liver surgery. Treatment with VIP or EGF was started 24 h after plating and continued for 3 or 5 d. DNA replication was investigated by Bromodeoxyuridine (BrdU) incorporation and cell viability detected by MTT assay. Cell lysate was analysed by western blotting and RT-PCR. Urea and albumin secretion into the culture supernatants were measured.

**Results**: VIP increased DNA replication in hepatocytes in a dose-dependant manner, with a peak response at day 3 of treatment. VIP treatment was associated with an increase in mRNA expression of antigen identified by monoclonal antibody Ki-67 (MKI-67) and Histone Cluster 3 (H3) genes. Western blotting analysis showed that VIP can induce a PKA/B-Raf dependant phosphorylation of extracellular signal-regulated kinases (ERK). Although EGF can maintain hepatocyte functions up to day 5, no marked efffect was found with VIP.

**Conclusions**: VIP induces proliferation of human hepatocytes with little or no effect on hepatocyte differentiation. Further investigation of the role of VIP is required to determine if it may ultimately support therapeutic approaches of liver disease.

## 1 | INTRODUCTION

Hepatocyte transfusions have shown promise as an alternative to conventional liver transplantation in treatment of some genetic disorders and acute liver failure.[1,2] These potential therapies are compromised by poor viability, rapid de-differentiation, the low proliferative capacity of primary hepatocytes in vitro[3] and the need for high numbers of hepatocytes. In addition, there is often poor liver cell viability after cryopreservation.[4] Improving hepatocyte in vitro viability and growth is crucial for progress in their use as a replacement therapy and in drug screening.

VIP is a 28-amino acid neuropeptide found largely in the brain, gastrointestinal tract and liver.[5] Moreover, VIP receptors have been characterized and purified from the liver.[6,7] Reports have shown that VIP can change the metabolic functions of rat hepatocytes, and can stimulate gluconeogenesis, ureagenesis and inhibit glyconeogenesis.[8,9] VIP has been found to be involved in regulation of hepatic blood flow, and modulation of both innate and adaptive immune functions.[10-12] Interestingly, VIP mRNA expression is present in rat liver following partial hepatectomy (PH).[13] Unlike hepatic growth factor (HGF) and epidermal growth factor (EGF), the role of VIP in liver regeneration is under-investigated. Previous reports have shown that VIP may exert bi-directional inhibitory or stimulatory effect on cell proliferation of a number of cell types. Kar et al. (1996) described a stimulatory effect of VIP alone on hepatocytes obtained from regenerated liver of rats.[13] In addition, it has been reported that VIP may have a mitogenic effect on HT29 and H9 cell lines,[14,15] while it can cause an inhibition of proliferation of human HepG2 cells.[16]

The mitogen-activated protein kinase (MAPK) pathway has been reported to play a crucial role in hepatocyte replication.[17] Moreover, EGF-induced proliferation of rat hepatocytes is mainly dependant on the p44 and p42 isoenzymes (extracellular signal-regulated kinases, ERK1 and ERK2) of the MAPK pathway.[18] VIP stimulates intracellular production of cyclic adenosine 3′:5′-monophosphate (cAMP) in various cell types, including hepatocytes.[8] Activation of cAMP-dependant Rap1 GTPase may be associated with either activation or inhibition of the (MAPK/ERK kinase) MEK/ERK cascade. This effect relies on the presence or absence of the serine/threonine-protein kinase B-Raf, respectively in cells.[19] Of relevance is that B-Raf kinase has been detected in liver.[20] These findings support that hypothesis that VIP may contribute in hepatocytes proliferation.

In the present study, we have investigated the effects of VIP on cell proliferation, gene expression, cell signalling and function in human hepatocytes.

## 2 | MATERIALS AND METHODS

### 2.1 | Isolation of human hepatocytes

Tissue samples (2-10 g) were obtained from fresh surgical macroscopically normal liver tissue resections from patients undergoing hepatectomies with informed consent (Research Ethics Committee, REC North East–Newcastle & North Tyneside 2, REC ref. 13/NE/0070). A total of 46 human liver cell preparations derived from the unaffected resection margins of the livers from 39 different donors with primary or metastatic liver tumours (24 men and 15 women) were used. Patients' ages ranged from 29 to 83 y. Hepatocytes were isolated using a 2-step perfusion procedure as described previously[21] with some modifications. Cells were plated on mouse collagen type IV gel layer 1-2.5 μg $cm^{-2}$ (Corning Ltd., Flintshire, UK) in William's E medium (Thermo Fisher, Inchinnan, UK) and incubated at 37°C in a humidified incubator with 5% $CO_2$.

### 2.2 | 5-Bromo-2′-deoxyuridine (BrdU) DNA incorporation assay

EGF (Sigma, Gillingham, UK) at 5, 10 and 20 ng $mL^{-1}$ or VIP (Sigma) at $10^{-8}$, $10^{-7}$ or $10^{-6}$M was added 24 h following cell seeding. Hepatocytes were incubated with BrdU (10 μg $ml^{-1}$, Sigma) for 2 h at 37°C. DNA-integrated BrdU was detected by rat anti-BrdU antibody (Bio-Rad, Hertfordshire, UK) and subsequently donkey anti-rat IgG-Alexa 488 (Thermo Fisher). Nuclei were stained with 4′-6-diamidino-2-phenylindole, DAPI (Sigma). Using fluorescence microscopy, numbers of $BrdU^+$ and $DAPI^+$ cells were determined in 6 different high-power fields per well.

### 2.3 | Measurement of lactate dehydrogenase (LDH) activity

Equal volumes of 200 mM Tris (hydroxymethyl) aminomethane (Tris) pH 8, 50 mM lithium lactate, freshly prepared substrate solution (100 μL p-iodonitrotetrazolium violet, INT [33 mg $mL^{-1}$ in dimethyl sulfoxide (DMSO) + 100 μL, phenazine methosulfate, PMS [9 mg $mL^{-1}$] + 2.3 ml β-nicotinamide adenine dinucleotide (NAD) hydrate [3.74 mg $mL^{-1}$]) and samples or positive control (5 μg $mL^{-1}$ L-lactic dehydrogenase from bovine heart, Sigma) were loaded into an assay plate. The $V_{max}$ was measured at 490 nm for 10 min in a SpectraMax® Plus 384 Microplate Reader (Molecular Devices, Wokingham, UK) and LDH activity (U $mL^{-1}$) was calculated.

### 2.4 | Viability and proliferation assays

Viability was determined using a colourimetric MTT assay (Sigma) and Quick Cell Proliferation Assay kit II (Abcam, Cambridge, UK) were used according to manufacturers' instructions.

### 2.5 | Polymerase chain reaction and real-time PCR

RNA was extracted using a RNeasy® kit (Qiagen, Crawley, West Sussex, UK) following the manufacturer's instructions. Complementary DNA (cDNA) was synthesised using a Primer Design Precision nanoScript 2 reverse transcription kit (Millbrook, Southampton, UK) according to the manufacturer's instructions in a MasterCycler® 480 thermocycler (Eppendorf, Hamburg, Germany). The RT-PCR Primers were designed using the ProbeFinder software version 2.5 (Lifescience.roche.com) and oligonucleotide primers for albumin, antigen identified by monoclonal antibody Ki-67 (MKI-67), histone cluster 3 (H3) were obtained from Eurofins MWG/operon (Ebersberg, Germany) (Data S1). VIP and pituitary adenylate cyclase-activating polypeptide receptor-1 (VPAC1) and EGF receptor (EGFR) mRNA expression was assessed using GoTaq® Hot Start Polymerase (Promega UK Ltd, Southampton, UK) according to manufacturer's instructions. PCR products were visualized on 2% agarose gel, band densities were measured and normalized to that of glyceraldehyde-3-phosphate dehydrogenase, GAPDH using a ChemiDoc™ imaging system (Bio-Rad). The qPCR was performed using a SYBR Green Mastermix buffer (Primer Design) in an A&B 7900HT Fast Real-Time PCR System thermocycler (Applied Biosystems, CA, USA). The Ct values were normalized to the GAPDH and calibrated to untreated cells. The fold change of mRNA expression was calculated according to the ΔΔCt method.

### 2.6 | Detection of Phospho-p44/42 MAPK (Erk1/2) and VPAC1 in hepatocytes using western Blotting

Hepatocytes were serum starved for 24 h prior to incubation with EGF (20 ng $mL^{-1}$) or VIP ($10^{-6}$ M). The B-RAF inhibitor, SB-590885 and the PKA inhibitor, *Rp*-cAMP triethylammonium salt (Rp-cAMPS) were used. Cells were lysed using TruPAGE™ LDS Sample Buffer (Sigma) with phosphatase and protease inhibitors. Protein concentrations were measured and separated in a TruPAGE® 10% precast gels (Sigma) under reducing conditions, then transferred to nitrocellulose membranes. The membranes were probed with rabbit anti-human phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody or rabbit anti-human p44/42 MAPK (Erk1/2) antibody (New England Biolabs, Hertfordshire, UK), followed by goat anti-rabbit horseradish peroxidase (HRP) (DakoCytomation, Cambridgeshire, UK). Reactive bands were detected using the Luminata Forte Western HRP substrate (Millipore UK Ltd., Hertfordshire, UK). In another experiment, the level of VPAC1 protein expression in untreated or VIP ($10^{-6}$M) treated hepatocytes, was investigated using a rabbit polyclonal anti-VPAC1 (Abcam) and followed by goat anti-rabbit horseradish peroxidase (HRP) (DakoCytomation).

### 2.7 | cAMP direct immunoassay

Levels of cAMP in hepatocytes 24 h following cell seeding and at day 3 or 5 following stimulation with $10^{-6}$ M VIP treatment were

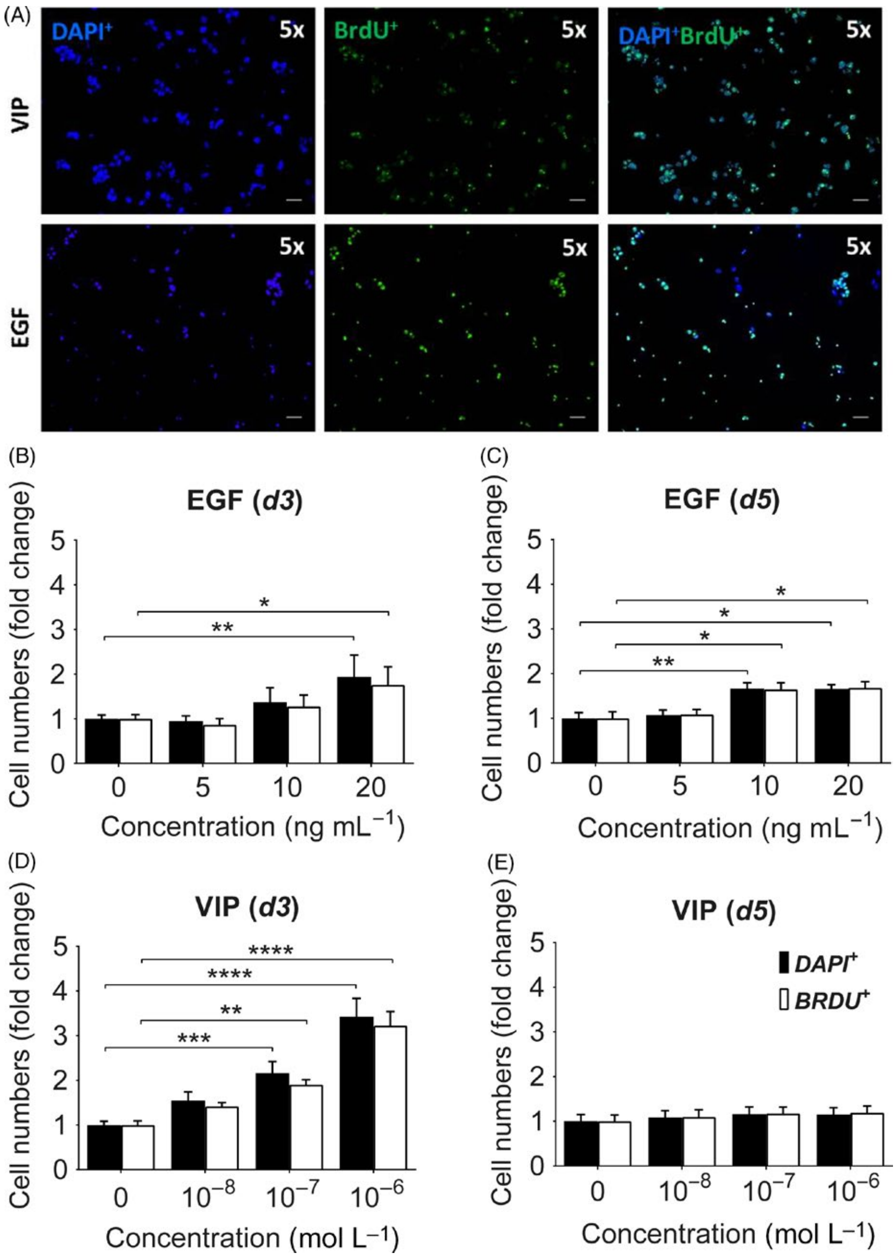


**FIGURE 1** Hepatocyte proliferation was stimulated by EGF or VIP. A, Representative images of hepatocytes treated with either EGF (20 ng $mL^{-1}$) or VIP ($10^{-6}$ M) for 3 d. DNA incorporation of BrdU was determined (Green) and DAPI (Blue) was used as a nuclear counter stain. B-E, The effects of EGF or VIP were demonstrated on total and proliferating cell numbers. n = 3 different donors per condition. *P* values shown in the graph are for comparison to hepatocytes maintained on medium alone. **P* < .05, ***P* < .005, ****P* < .0005, *****P* < .0001. Mean ± SEM. Two-way ANOVA followed by Fisher's least significant difference (LSD)

detected using a cAMP direct immunoassay (Abcam) according to the manufacturer's instructions. cAMP concentrations (μM) were determined and corrected to total proteins concentrations in samples (μg).

### 2.8 | Albumin ELISA and Urea concentration assay

Albumin and urea concentrations in the supernatant of hepatocytes cultures were determined using the ELISA DuoSET® kit for human albumin (R&D Systems, Oxfordshire, UK) and the QuantiChrom™ urea assay kit (QuantiChrom, BioAssay Systems, Hayward, CA, USA) respectively, according to the manufacturer's instructions.

### 2.9 | Statistics

Two-way analysis of variants (ANOVA) followed by Fisher's least significant difference (LSD) multiple comparisons tests were performed using GraphPad Prism version 7.7.1 for Windows, GraphPad Software, La Jolla, CA, USA, www.graphpad.com. Data has been represented by Mean ± SE of the mean (SEM) or standard deviation (SD) as indicated. $P < .05$ was taken as significant.

For further details regarding the materials and methods, please refer to the Data S1.

## 3 | RESULTS

### 3.1 | Stimulation of DNA replication in hepatocytes by VIP

EGF at high concentrations such as 50 ng mL$^{-1}$, has been reported to be responsible for an increase in [3H] methylthymidine incorporation in rat hepatocytes. The response to EGF maximized at 24 h and continued with persistent exposure.[22] In the current work, proliferation of human hepatocytes was investigated by detecting BrdU incorporation (Figure 1A). Herein, EGF resulted in an increase of BrdU-positive cells at concentrations of 10 ng mL$^{-1}$ (a mean of 1.3 ± SD 0.9-fold) and 20 ng mL$^{-1}$ (a mean of 1.8 ± SD 1.4-fold) at day 3 (Figure 1B), and this effect was continued at

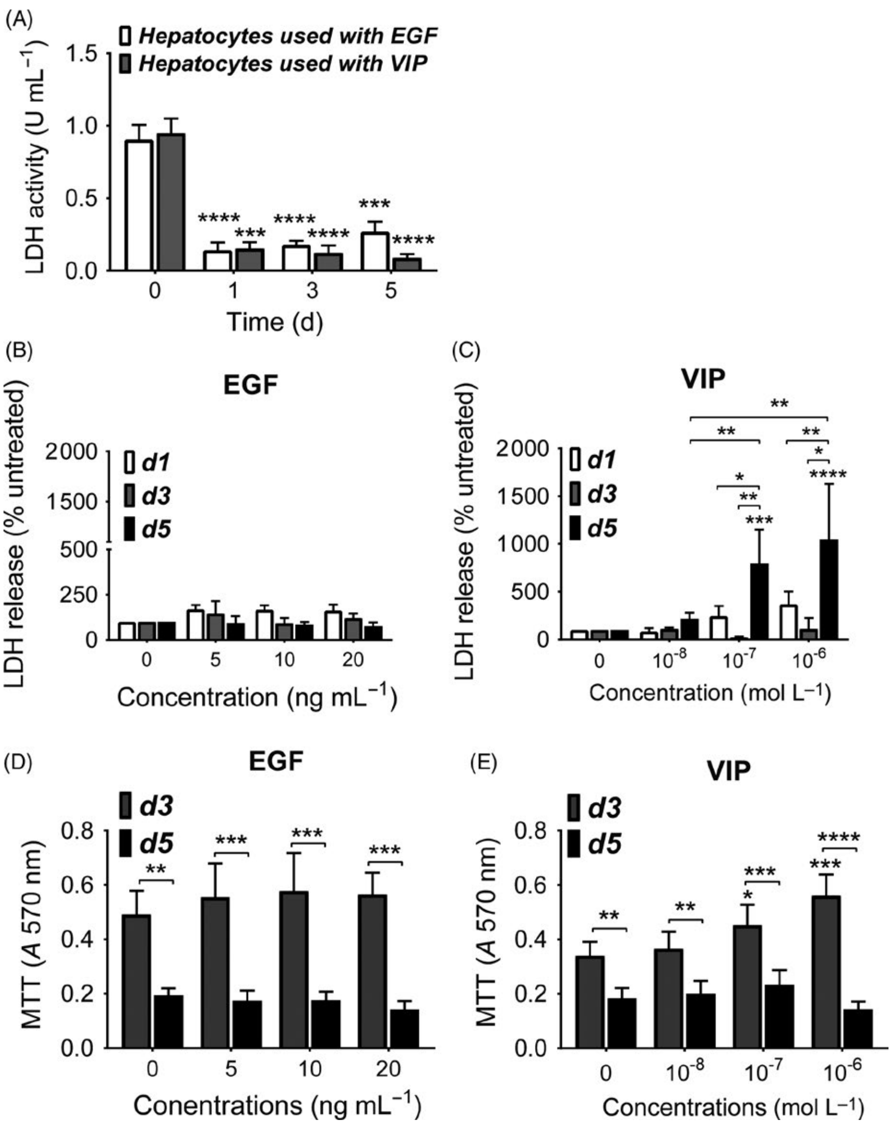


**FIGURE 2** Hepatocyte viability with EGF or VIP. A, LDH activity (U mL$^{-1}$) in supernatants of untreated human hepatocytes with time course. B, LDH release (expressed as percentage of total LDH activity) in supernatants of human hepatocytes treated with EGF or C, VIP at previous concentrations following day 1, 3 and 5 of treatments. n = 3 different donors per condition. *P* values shown in the graph are for overall comparison with hepatocytes at day 0 (A) or untreated control (B and C). $^{*}P < .05$, $^{**}P < .005$, $^{***}P < .0005$, $^{****}P < .0001$. D, Viable cells were detected following addition of EGF (5, 10 or 20 ng mL$^{-1}$) or (E) VIP ($10^{-8}$, $10^{-7}$ or $10^{-6}$ M) treatment for 3 or 5 d by MTT assay. *A*, Absorbance. n = 3 different donors per condition. *P* values shown in the graph are for overall comparison between hepatocytes at day 3 and 5. Mean ± SEM. Two-way ANOVA followed by Fisher's LSD

day 5 of treatment, 10 ng $mL^{-1}$ (a mean of 1.5 ± SD 0.5-fold) and 20 ng $mL^{-1}$ (a mean of 1.7 ± SD 0.5-fold) (Figure 1C). Interestingly, VIP-stimulated proliferation of human hepatocytes in a dose-dependant manner at day 3 up to a mean of 3.2 ± SD 1.1-fold at $10^{-6}$ M (Figure 1D). However, a decline of hepatocyte response to VIP was observed at day 5 (a mean of 1.2 ± SD 0.6-fold up to $10^{-6}$ M) (Figure 1E). Similarly, EGF addition was associated with a rise in total cell numbers at day 3; 10 ng $mL^{-1}$ (a mean of 1.4 ± SD 1.1-fold) and 20 ng $mL^{-1}$ (a mean of 1.9 ± SD 1.7-fold) and day 5; 10 ng $mL^{-1}$ (a mean of 1.7 ± SD 0.4-fold) and 20 ng $mL^{-1}$ (a mean of 1.7 ± SD 0.3-fold). VIP at day 3 resulted in an increase in total cells by a mean of 2.2 ± 0.9-fold at $10^{-7}$M and 3.4 ± SD 1.4-fold at $10^{-6}$M. The drastic decrease in hepatocyte response to VIP at day 5 raised a concern about changes in cell viability and status, and was investigated further.

## 3.2 | VIP treatment has a limited effect on hepatocyte survival in vitro

Effects of EGF or VIP on hepatocyte integrity was tested by measuring LDH release in the cell culture supernatants. In the first 24 h following cell extraction, LDH activity was high (a mean of

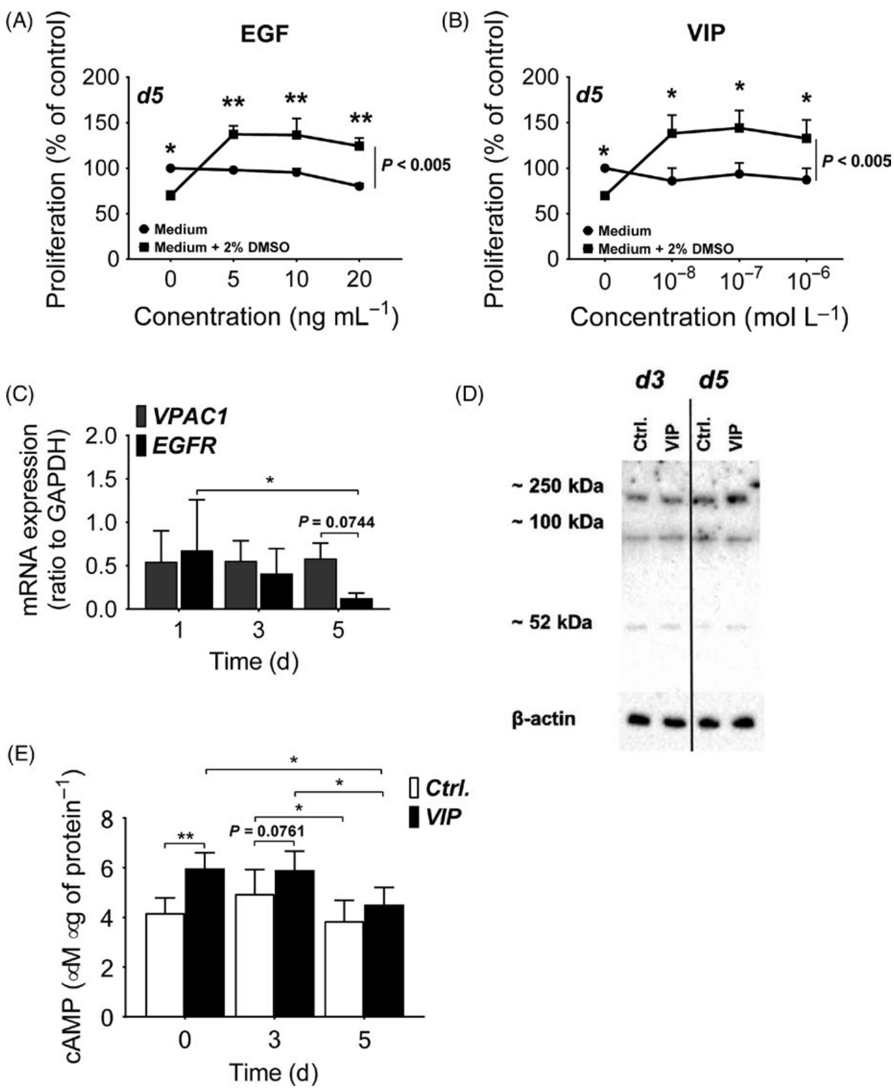


**FIGURE 3** The effect of DMSO on cell response to VIP and VIP and pituitary adenylate cyclase-activating polypeptide receptor-1 (VPAC1) expression and activation in hepatocytes. (A and B) Hepatocytes were treated with either EGF (5, 10 or 20 ng $mL^{-1}$) or VIP ($10^{-8}$, $10^{-7}$ or $10^{-6}$ M), 2% DMSO was added at day 3 and cell proliferation was investigated at day 5 using the WST-1 Quick Cell Proliferation Assay kit II (Abcam). n = 3 different donors per condition. *P* values shown in the graph are for comparison at individual concentrations and overall comparison with hepatocytes maintained in medium without DMSO. C, Band density analysis (fold change) of VPAC1 and epidermal growth factor receptor (EGFR) mRNA of gene expression on 2% agarose gel in non-treated cells following 1, 3 or 5 d of hepatocyte culture (6 donors), D, VPAC1 protein expression as detected in hepatocytes by western blotting techniques at day 3 and 5 of hepatocyte culture, a representative blot of 3 independent experiments. Molecular weights were indicated for VPAC1 isoforms. E, Effect of VIP ($10^{-6}$ M) on cAMP concentrations (μM $\mu g^{-1}$ of protein) in hepatocytes with time course control (ctrl.). n = 3. **P* < .05, ***P* < .005. Mean ± SEM. Two-way ANOVA followed by Fisher's LSD

0.90 ± SD 0.29 U mL$^{-1}$) (Figure 2A), which may be a result of the isolation process or spontaneous activation of hepatocyte apoptosis.[23,24] A dramatic decrease in LDH levels was observed in the following 24 h (a mean of 0.14 ± SD 0.16 U mL$^{-1}$). This may have been caused by washout of old medium containing dead and apoptotic cells. No further change in LDH activity was observed up to day 5. Treatment of hepatocytes with EGF resulted in a minimal change in LDH activity in the supernatants at day 1 compared to untreated cells. A decrease in LDH activity was observed at days 3 and 5 at various concentrations of EGF (Figure 2B). When VIP was added to the medium, no change in LDH activity was observed at day 1 or 3 of treatment (Figure 2C). At day 5, cells treated with VIP showed a marked increase in LDH levels, with approximately 2-, 8- and 10-fold changes at $10^{-8}$ M, $10^{-7}$ M and $10^{-6}$ M of VIP respectively. There was also a rise in LDH activity when both agents were added together to the hepatocyte culture medium (data not shown).

The metabolic activity of the cell was assessed using the MTT assay. At day 3, EGF showed a marked improvement in cell viability (Figure 2D) and VIP treatment was associated with a concentration dependant increase in hepatocyte metabolic activity, peaking at a concentration of $10^{-6}$ M (Figure 2E). Results showed low metabolic activity of primary human hepatocytes after day 5 of cell seeding, irrespective of the addition of EGF or VIP. This result may reflect cell loss.

Previous results have shown that the support of hepatocyte survival was lacking when VIP was used alone and cells have entered a late phase of death or apoptosis. In order to address this, we have tested DMSO as an agent which may prevent this deterioration of cell viability and as reported, can maintain hepatocyte differentiation and improve liver-specific functionality.[25] DMSO alone induced cell death as compared to medium alone, however, addition of 2% DMSO to culture medium was associated with the restoration of the hepatocyte response to EGF and the VIP mitogenic effect at day 5 of treatment (Figure 3A,B). In addition to hepatocytes loss, the noticeable decrease in the effect of VIP by day 5 and a change in expression of VIP receptors may contribute to hepatocyte resistance VIP. To test this possibility mRNA expression of VPAC1, the most abundant VIP receptor in the liver, was investigated using a semi-quantitative RT-PCR technique.[26] In untreated hepatocytes, level of mRNA expression of VPAC1 or EGFR did not change significantly at day 3 (Figure 3C). However, at day 5 cells expressed lower levels of EGFR mRNA which is a phenomenon that has been reported previously[27] but VPAC1 mRNA expression did not show any change. Western blotting revealed several forms of VPAC1 in human hepatocytes at molecular weights of ~250, ~100 and ~52 kDa (Figure 3D), as described previously.[28] During hepatocytes culture, VPAC1 protein expression

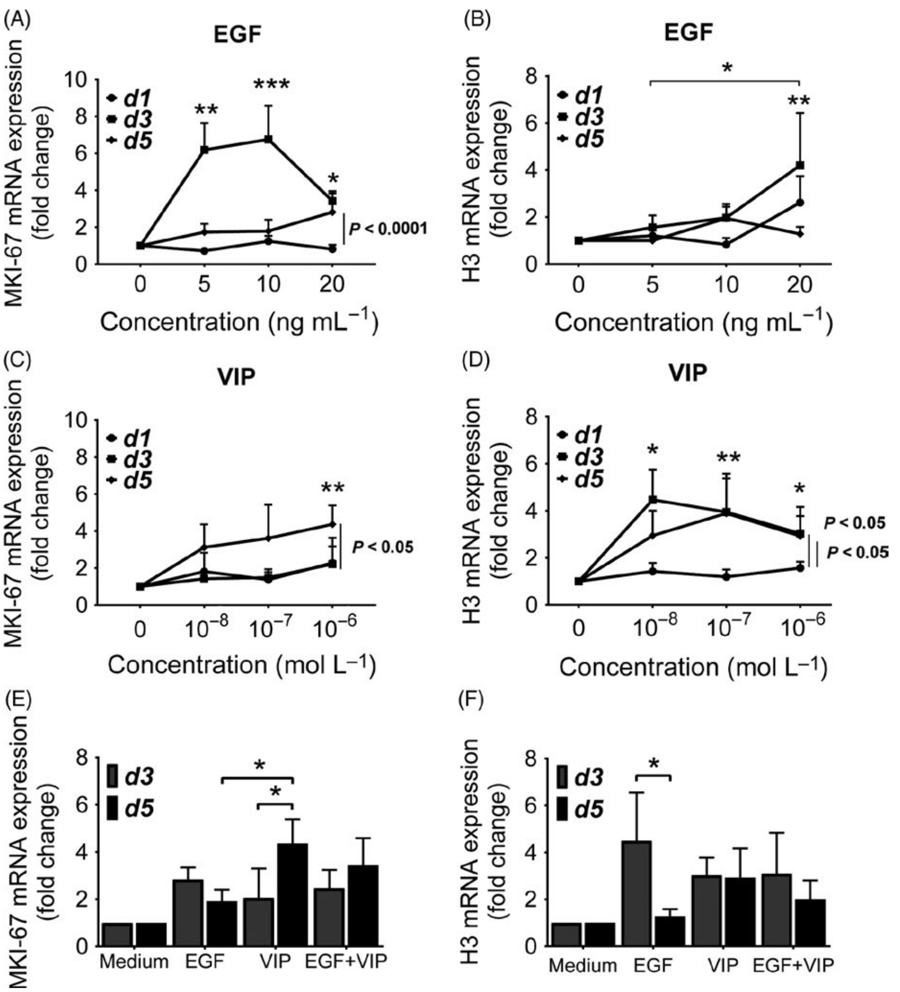


**FIGURE 4** Expression of monoclonal antibody Ki-67 (MKI-67) and histone cluster 3 (H3) genes in human hepatocytes cultured in the presence of EGF or VIP. Expression of mRNA was quantified by qPCR at days 1, 3 and 5 of EGF (5, 10 or 20 ng mL$^{-1}$) or VIP ($10^{-8}$, $10^{-7}$ or $10^{-6}$ M). (A-D) Concentration-dependant effects of EGF or VIP, and (E and F) the effect of EGF (20 ng mL$^{-1}$) or VIP ($10^{-6}$ M) or a combination of both. n = 3 different donors per condition. *P* values shown in the graph are for comparison at individual concentrations and overall comparison with hepatocytes at day 1. **P* < .05, ***P* < .005, ****P* < .0005. Mean ± SEM. Two-way ANOVA followed by Fisher's LSD

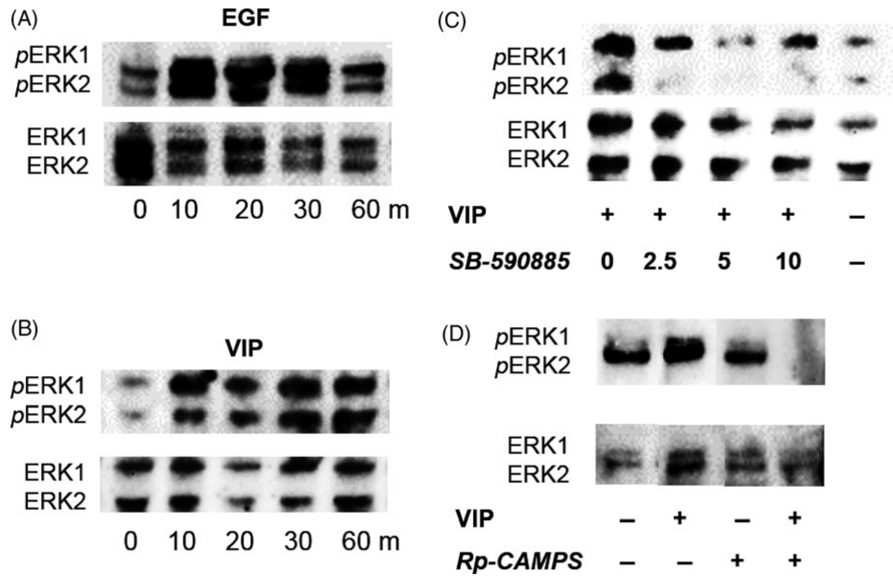


**FIGURE 5** Phosphorylation of ERK in EGF or VIP-treated human hepatocytes analysis using western blotting. A, Hepatocytes treated with either EGF (20 ng $mL^{-1}$) or B, VIP ($10^{-6}$ M) and analysed by western blotting at indicated time points. C, The effects of downstream pathway inhibitors was investigated using 2.5-10 µM of SB-590885 (SB) or (D) 500 µM of Rp-cAMPS. Hepatocytes were incubated with inhibitors for 1 h prior to addition of EGF or VIP for another 1 h. n = 4 with different donors

did not show marked changes, but VIP treatment was associated with a marked decrease in VPAC1 gene mRNA expression at day 5 of cell culture (Figure S1). The level of VPAC1 activation has previously been assessed by measuring intracellular cAMP concentrations. Interestingly, exposure of VPAC1 to VIP at a concentration of $10^{-6}$ M at 24 h following cell seeding was found to stimulate production of cAMP by hepatocytes as compared to untreated cells (mean concentration 5.96 µM $\mu g^{-1}$ of protein ± SEM 0.64 vs 4.18 ± 0.60 respectively, $P = .0029$) (Figure 3E). Production of cAMP as a response to VIP continued but to a lesser extent until day 3 of hepatocyte culture (mean of 5.90 µM $\mu g^{-1}$ of protein ± 0.77 and 4.95 ± 0.97 respectively, $P = .0761$). Notably, constitutive cAMP showed a lower concentration at day 5 of cell culture in untreated cells (a mean of 3.85 ± SEM 0.84 µM $\mu g^{-1}$ of protein) and VPAC1 receptors did not show as clear a response to VIP as that seen at early time points (4.52 ± 0.69 µM $\mu g^{-1}$ of protein). Taken together, these finding may suggest a change in receptor functionality over time.

### 3.3 | Expression of proliferation-associated genes was induced by VIP treatment

Expression of the active cell cycle marker, MKI-67[29] and the mitotic marker, H3[30] genes were studied using quantitative RT-PCR. EGF alone induced a 6-fold increase in mRNA expression of MKI-67, most significantly at day 3 of treatment at concentrations up to 10 ng $mL^{-1}$ (Figure 4A). In addition, EGF treatment resulted in up to a 4-fold increase in expression of H3 mRNA by day 3 of treatment, most noticeably at 20 ng $mL^{-1}$ EGF (Figure 4B). Addition of VIP to cultured hepatocytes were associated with a 2-fold increase in MKI-67 gene expression at day 3, rising to 4-fold at day 5 of treatment at a concentration of $10^{-6}$M (Figure 4C). Similarly, VIP induced a concentration-dependant increase in expression of H3 at days 3 and 5 (Figure 4D). Although the combination of EGF and VIP was associated with a considerable increase in expression of MKI-67 at day 5, there was no difference compared to either EGF or VIP alone (Figure 4E). The presence of EGF and VIP together in the culture medium had little effect on expression of H3 at day 3 (Figure 4F).

### 3.4 | Production of phospho-p44/42 MAPK (Erk1/2) in VIP treated hepatocytes

Binding of VIP to its receptors initiates cAMP production and subsequent protein kinase A (PKA).[31] A PKA-dependent phosphorylation of the GTPase Rap1 resulted in stimulation of ERKs in the presence of B-Raf in cells such as hepatocytes.[19] EGF at 20 ng $mL^{-1}$ stimulated phosphorylation of ERK as early as 10 min, after which activation declined with time (Figure 5A). Interestingly, VIP was found to increase *p*ERK following 10 min incubation with hepatocytes. However, ERK activation increased further up to 60 min (Figure 5B). In addition, VIP stimulation of freshly isolated hepatocytes failed to elicit phosphorylation of ERK (data not shown). Both agents did not preferentially activate either *p*ERK 1 or 2. Pre-incubation of human hepatocytes with 5 µM of SB-590885 (SB), a B-RAF inhibitor prior to treatment or Rp-cAMPS (cAMP inhibitor) at 500 µM was associated with inhibition of VIP induced *p*ERK (Figure 5C,D). Interestingly, SB was found to preferentially block ERK2 phosphorylation to a greater extent than ERK1. However, inhibition of cAMP mobilization with Rp-CAMP inhibitor blocked both ERK1 and ERK2.

### 3.5 | VIP treatment does not support human hepatocytes-specific functions

Albumin gene expression was suppressed initially, but recovered by day 3 of incubation with EGF at a concentration of 5 ng $mL^{-1}$ and markedly increased at day 5 with concentrations up to 5-20 ng $mL^{-1}$ (Figure 6A). Conversely, VIP had no marked effect on albumin gene expression in human hepatocytes in this model (Figure 6B). When EGF and VIP were combined together, the stimulatory effect of EGF

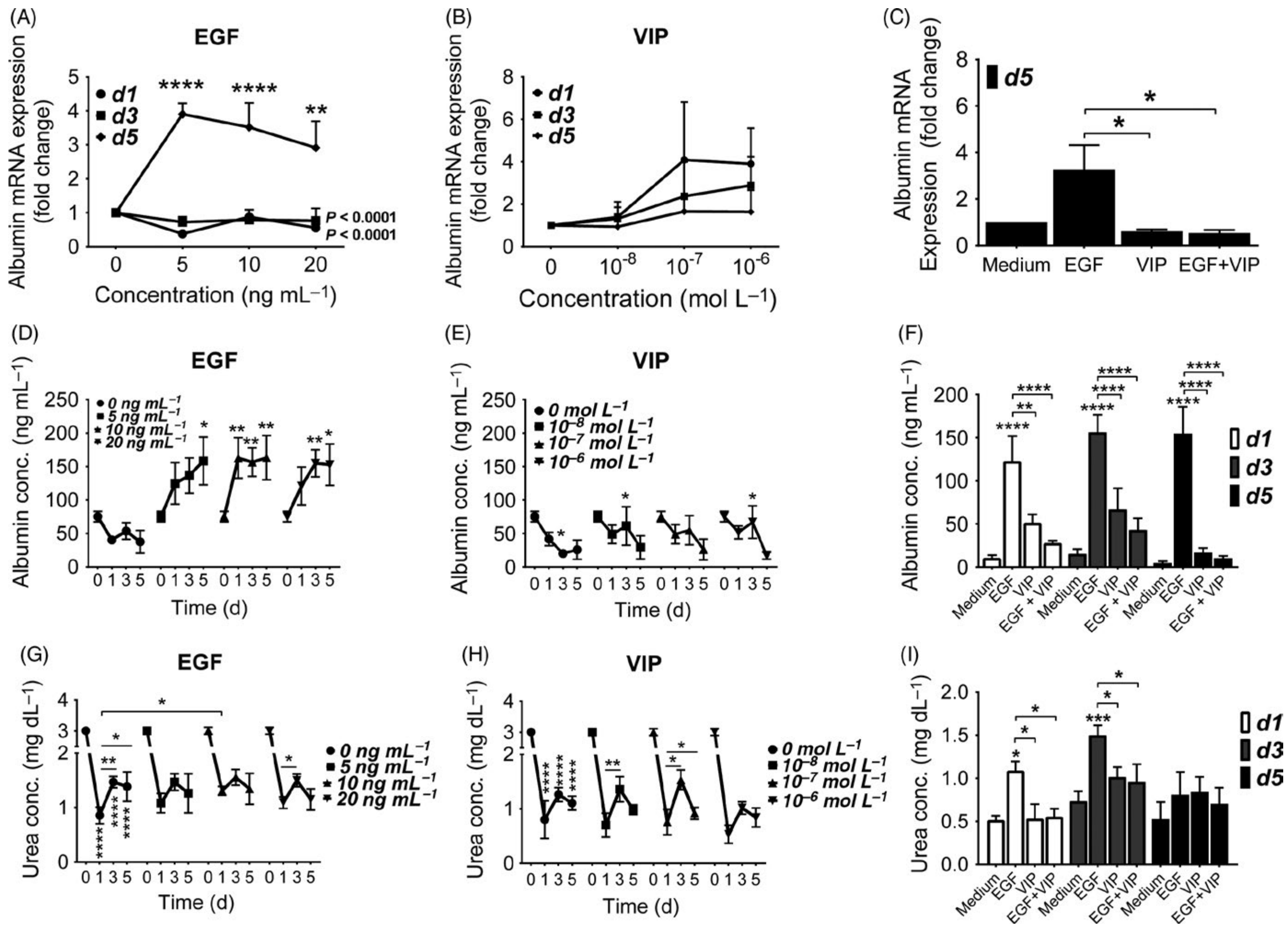


**FIGURE 6** Expression and production of albumin and urea from human hepatocytes cultured with EGF and VIP. Albumin gene mRNA expression at days 0, 1, 3 and 5 of (A) EGF (5, 10 or 20 ng $mL^{-1}$) or (B) VIP ($10^{-8}$, $10^{-7}$ or $10^{-6}$ M) treatments, and (C) the effects of either EGF (20 ng $mL^{-1}$), VIP ($10^{-6}$ M) or combination of both were determined. *P* values shown in the graph are for comparison at individual concentrations and overall comparison with hepatocytes at day 1. (D-F) Albumin (ng $mL^{-1}$) and (G-I) urea (mg $dL^{-1}$) concentrations in supernatants of cultured hepatocytes with EGF, VIP or both were determined. n = 3 different donors per condition. *P* values shown in the graph are for comparison with hepatocytes at day 0 or with untreated cells. **P* < .05, ***P* < .005, ****P* < .0005, *****P* < .0001. Mean ± SEM. Two-way ANOVA followed by Fisher's LSD

on albumin gene expression was significantly lower than that of EGF alone (Figure 6C).

Albumin levels in the supernatants dropped from a mean of 75.14 ± SD 22.13 ng $mL^{-1}$ in the first 24 h following hepatocyte seeding to a mean of a mean of 40.24 ± SD 16.82 ng $mL^{-1}$ at day 2 and no marked change was observed subsequently. EGF stimulated production of albumin from liver cells in a concentration-dependent manner as compared to the untreated control at day 1 of treatment yielded a mean of 120.91 ± SD 79.91 ng $mL^{-1}$ which continued up to day 5 of treatment to reach a mean of 152.80 ± SD 87.20 ng $mL^{-1}$ with 20 ng $mL^{-1}$ EGF (Figure 6D). At day 3, there was an increase in albumin production up to a mean of 66.9 ± SD 76.83 ng $mL^{-1}$ from hepatocytes cultured in the presence of $10^{-6}$ M VIP (Figure 6E). When both agents were added together, the stimulatory effect of EGF was inhibited (Figure 6F). When both agents were added sequentially, an inhibitory effect of VIP on EGF-stimulated albumin production was observed (Figure S2). Urea production from hepatocytes was dramatically decreased during the 24 h following cell plating from a mean of 3.01 ± SD 0.38 mg $dL^{-1}$ to a mean of 0.80 ± SD 0.98 mg $dL^{-1}$, but partial recovery was observed at day 3 and 5 (a mean of 1.26 ± SD 0.37 mg $dL^{-1}$ and 1.10 ± SD 0.36 mg $dL^{-1}$ respectively). EGF increased urea production on the first day of hepatocyte culture compared to untreated cells (a mean of 1.31 ± SD 0.23 mg $dL^{-1}$ at 10 ng $mL^{-1}$ EGF), but this effect disappeared with time (Figure 6G). However, $10^{-7}$ M VIP resulted in a limited increase (a mean of 1.53 ± SD 0.51 mg $dL^{-1}$) in urea production at day 3 as compared to control (Figure 6H) and adding VIP to EGF abolished the effect of EGF on urea production in cultures hepatocytes (Figure 6I).

## 4 | DISCUSSION

Our findings have shown that EGF or VIP alone has the ability to induce DNA synthesis in cultured human hepatocytes and to stimulate expression of genes that may be involved in cell proliferation. Interestingly, EGF was able to maintain hepatocyte proliferation further up to day 5 whilst VIP did not. In addition, VIP was found to stimulate phosphorylation of ERK1 and 2 protein kinases. However, unlike EGF, VIP has a limited effect on hepatocyte function in vitro.

Hepatocytes move from G0 to G1 phase of cell cycle spontaneously during isolation process[32] and progress further towards and stop at a restriction point in mid-late G1 phase usually 24 and 48 h after plating.[22] Onward movement to S phase is dependent on growth factors such as EGF.[33] In agreement with that, we have demonstrated that EGF stimulated DNA synthesis when added 24 h following hepatocytes seeding. Strikingly, we observed a comparable effect with VIP which disagree to that previously reported by Kar et al.[13] The outcome of proliferative stimuli is related to the cell cycle. A few hours following isolation, VIP can facilitate entry of cells into G1 phase but it did not encourage them to pass the restriction point.[34] This effect could increase the number of cells at susceptible to the mitogenic effect of EGF. These findings might explain why VIP alone failed to stimulate DNA synthesis in hepatocytes but may potentiate the effect of EGF on cell proliferation at this early time point.[13,22,35] We found that VIP did activate MAPK at this early time which consistent with that has been reported.[35] The underling mechanism could involve activation of p70 ribosomal S6 protein kinase (p70S6k) activity, cyclin D3-cyclin-dependent kinase (CDK)-4 assembly or a CDK2/cyclin C-dependent inhibitory phosphorylation of the transcription factor LSF (late simian virus 40 factor) at serine 309.[36-38]

As we have shown, later in culture VIP or EGF stimulated formation of *p*ERK which has been described previously.[18,39] This effect was found to be closely related to induction of hepatocyte proliferation[17] and may involve an MAPK-dependent reactivation phosphorylation of LSF at serine 291 which could be essential for cell cycle progression to S phase.[40] Dependence of VIP-induced ERK activation on B-Raf kinase could support our hypothesis that VIP alone is able to induce hepatocyte proliferation, but VIP exerted an inhibitory effect on EGF (Figure 7). In accordance with these results, it has been reported that high levels of cAMP could result in a decrease in EGF-dependent MAPK production and loss of its DNA stimulatory effect.[35] In addition, several reports have shown that cAMP-dependent PKA is able to phosphorylate EGFR on serine residues which results in decrease in tyrosine kinase activity and EGFR autophosphorylation induced by EGF.[41,42] Moreover, cAMP-GEFs can directly inhibit Raf-1 by phosphorylation at ser259 or indirectly by a PKA-dependent activation of the Raf-1 inhibitor, Akt (protein kinase

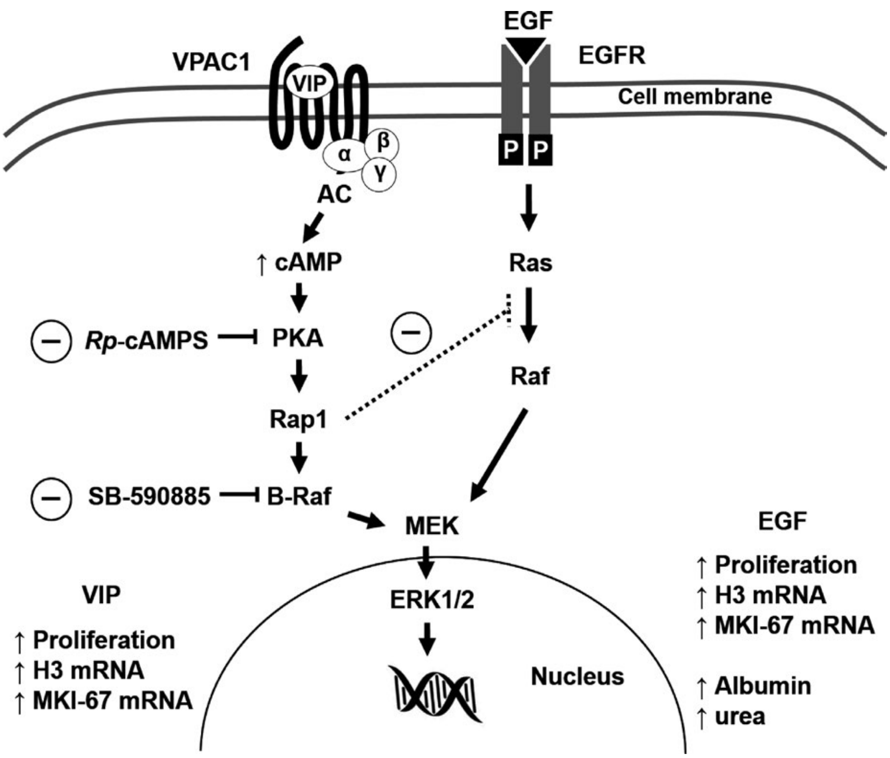


**FIGURE 7** A schematic diagram for VIP and EGF signalling in hepatocytes. Late in culture, binding of VIP with the G-protein-coupled VIP receptor type 1 (VPAC1) activates intracellular adenylyl cyclase (AC) resulting in cAMP production and the following protein kinase A (PKA) activation. Subsequently, phosphorylated Rap-1 can activate B-Raf and thereby, stimulate the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase, MEK/ERK cascade. Phosphorylation of ERK1/2 results in stimulation of cell proliferation and induces mRNA expression the proliferation-associated genes, the monoclonal antibody Ki-67 (MKI-67) and histone cluster-3 (H3) genes. EGF interaction with its receptors, EGFR results in a Ras/Raf-dependent activation of MEK, induction of cell proliferation and improvement of cell functions. VIP-activated Rap-1 may block EGF signalling through inhibition of Ras/Raf activation. VIP signalling can be inhibited by the B-RAF inhibitor, SB-590885 and PKA inhibitor, Rp-cAMP triethylammonium salt (Rp-cAMPS)

B, PKB).[43,44] This interaction could explain the reported VIP inhibitory effect on HepG2 proliferation. HepG2 survival and proliferation is depending on the presence of FBS in medium.[45,46] VIP has been shown to inhibit HepG2 proliferation through a cAMP-dependent signal transducers and activators of transcription-3 (STAT-3) pathway inhibition,[16] the pathway that can be stimulated by growth factors which present in FBS.

The DNA synthesis in primary hepatocytes started early in culture and maximized at day 3, with expression of activated transcriptional regulators for EGF and ERK pathway,[47] but decreased afterwards even in the presence of EGF.[13,48] Following day 3 of culture, substantial hepatocyte death has been reported and the remaining cells may become flattened and polykaryotic or smaller and apoptotic.[3] We have noticed that, at day 5 of EGF treatment, there was a lower number of living hepatocytes, and that the remaining cells replicated, but to a lower extent. This is in agreement with previous findings to that has been reported before.[33,49]

In our model, VIP did not show any change in hepatocyte proliferation, consistent with previous work.[13] Notably, the cells which proliferated under the effect of VIP mostly died by day 5 of treatment and VIP did not markedly increase DNA synthesis in the remaining cells. The lack of support of the differentiated state of hepatocytes with VIP treatment was observed from the albumin production and urea secretion at day 5, a finding that has been previously reported.[9] Interestingly, MKI-67 and H3 mRNA expression in hepatocytes showed a tendency to increase at day 5 of treatment while albumin expression decreased with time, which may be an indication of a loss of differentiation.

The dramatic change in hepatocyte response to VIP could be a consequence of changes in VIP receptors expression. We found that hepatocytes did not show such a change in expression of VPAC1 during culture time course. However, VIP failed to induce cAMP production in hepatocytes at day 5 of cell culture, which suggests an alteration of receptor signalling response. Indeed, the interaction between VIP and its receptors in proliferating hepatocytes is not completely understood. In rat liver 3 d after PH, the maximal response of VIP was reduced as a result of low number of receptors and changes in the receptor structure.[50] In addition, the decrease in VIP receptors sensitivity could be a result of high expression of VIP in proliferating liver.[13] Moreover, VPAC1 harbours several potential *N*-glycosylation sites which are critical for VIP binding[51] and receptor delivery to plasma membrane.[52] An alteration in *N*-glycosylation of proteins has been reported in dedifferentiated rat hepatocytes,[53] and could explain the decreased in VPAC1 response to VIP, but this possibility needs further investigations. In addition, we have demonstrated that addition of high concentration of VIP was associated with downregulation of VPAC1, the phenomenon that has been reported with VIP with other cell types.[54,55]

Our findings have demonstrated that VIP alone was able to induce proliferation of adult human hepatocytes when added 24 h following hepatocyte platting and this effect may be PKA/B-Raf-ERK dependent. VIP exerts an inhibitory effect on EGF signalling pathway at this time point of cell cycle. Stimulation of the VIP pathway may aid hepatocyte proliferation in vitro.

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## CONFLICT OF INTEREST

All authors declare no conflict of interest.

## AUTHOR CONTRIBUTIONS

M. M. S. Khedr, A. M. Abdelmotelb, T. A. Bedwell an M. N. Alzoubi were responsible for data acquisition and analysis. A. M. Abdelmotelb and A. Shtaya were concerned with ethical considerations. M. Abu Hilal and S. I. Khakoo contributed to the conception, design of the work or of parts of it, and to its interpretation. M. M. S. Khedr and S. I. Khakoo drafted and revised the manuscript, A. M. Abdelmotelb and M. Abu Hilal revised it critically for intellectual content, and T. Bedwell proofread the manuscript.

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## REFERENCES

1. Bilir BM, Guinette D, Karrer F, et al. Hepatocyte transplantation in acute liver failure. *Liver Transpl*. 2000;6:32-40.
2. Grossman M, Raper SE, Kozarsky K, et al. Successful ex vivo gene therapy directed to liver in a patient with familial hypercholesterolaemia. *Nat Genet*. 1994;6:335-341.
3. Chen Y, Wong PP, Sjeklocha L, et al. Mature hepatocytes exhibit unexpected plasticity by direct dedifferentiation into liver progenitor cells in culture. *Hepatology*. 2012;55:563-574.
4. Hewitt NJ. Optimisation of the Cryopreservation of primary hepatocytes. *Methods Mol Biol*. 2010;640:83-105.
5. Guijarro LG, Couvineau A, Rodriguez-Pena MS, et al. Vasoactive intestinal peptide receptors in rat liver after partial hepatectomy. *Biochem J*. 1992;285:515-520.

6. Couvineau A, Voisin T, Guijarro L, et al. Purification of vasoactive-intestinal-peptide receptor from porcine liver by a newly designed one-step affinity-chromatography. *J Biol Chem*. 1990;265:13386-13390.
7. Nguyen TD, Williams JA, Gray GM. Vasoactive-intestinal-peptide receptor on liver plasma-membranes – characterization as a glycoprotein. *Biochemistry*. 1986;25:361-368.
8. Feliu JE, Mojena M, Silvestre RA, et al. Stimulatory effect of vasoactive intestinal peptide on glycogenolysis and gluconeogenesis in isolated rat hepatocytes – antagonism by insulin. *Endocrinology*. 1983;112:2120-2127.
9. Leiser J, Blum JJ. Effects of VIP and forskolin on alanine metabolism in isolated hepatocytes. *FEBS Lett*. 1984;173:407-413.
10. Richardson PD, Withrington PG. Liver blood flow. II. Effects of drugs and hormones on liver blood flow. *Gastroenterology*. 1981;81:356-375.
11. Arranz A, Juarranz Y, Leceta J, et al. VIP balances innate and adaptive immune responses induced by specific stimulation of TLR2 and TLR4. *Peptides*. 2008;29:948-956.
12. Vetrini F, Brunetti-Pierri N, Palmer DJ, et al. Vasoactive intestinal peptide increases hepatic transduction and reduces innate immune response following administration of helper-dependent Ad. *Mol Ther*. 2010;18:1339-1345.
13. Kar S, Hasegawa K, Carr BI. Comitogenic effects of vasoactive intestinal polypeptide on rat hepatocytes. *J Cell Physiol*. 1996;168:141-146.
14. Alleaume C, Eychene A, Caigneaux E, et al. Vasoactive intestinal peptide stimulates proliferation in HT29 human colonic adenocarcinoma cells: concomitant activation of Ras/Rap1-B-Raf-ERK signalling pathway. *Neuropeptides*. 2003;37:98-104.
15. Goursaud S, Pineau N, Becq-Giraudon L, et al. Human H9 cells proliferation is differently controlled by Vasoactive Intestinal Peptide or Peptide Histidine methionine: implication of a GTP-insensitive form of VPAC(1) receptor. *J Neuroimmunol*. 2005;158:94-105.
16. Absood A, Hu B, Bassily N, et al. VIP inhibits human HepG2 cell proliferation in vitro. *Regul Pept*. 2008;146:285-292.
17. Fremin C, Ezan F, Boisselier P, et al. ERK2 but not ERK1 plays a key role in hepatocyte replication: an RNAi-mediated ERK2 knockdown approach in wild-type and ERK1 null hepatocytes. *Hepatology*. 2007;45:1035-1045.
18. Talarmin H, Rescan C, Cariou S, et al. The mitogen-activated protein kinase kinase/extracellular signal-regulated kinase cascade activation is a key signalling pathway involved in the regulation of G(1) phase progression in proliferating hepatocytes. *Mol Cell Biol*. 1999;19:6003-6011.
19. Dugan LL, Kim JS, Zhang YJ, et al. Differential effects of cAMP in neurons and astrocytes - Role of B-raf. *J Biol Chem*. 1999;274:25842-25848.
20. Barnier JV, Papin C, Eychene A, et al. The Mouse B-Raf gene encodes multiple protein isoforms with tissue-specific expression. *J Biol Chem*. 1995;270:23381-23389.
21. Gomez-Lechon MJ, Lopez P, Donato T, et al. Culture of human hepatocytes from small surgical liver biopsies. Biochemical characterization and comparison with in vivo. *In Vitro Cell Dev Biol*. 1990;26:67-74.
22. Loyer P, Cariou S, Glaise D, et al. Growth factor dependence of progression through G1 and S phases of adult rat hepatocytes in vitro. Evidence of a mitogen restriction point in mid-late G1. *J Biol Chem*. 1996;271:11484-11492.
23. Vinken M, Decrock E, Doktorova T, et al. Characterization of spontaneous cell death in monolayer cultures of primary hepatocytes. *Arch Toxicol*. 2011;85:1589-1596.
24. Smets FN, Chen Y, Wang LJ, et al. Loss of cell anchorage triggers apoptosis (anoikis) in primary mouse hepatocytes. *Mol Genet Metab*. 2002;75:344-352.
25. Arterburn LM, Zurlo J, Yager JD, et al. A morphological study of differentiated hepatocytes in vitro. *Hepatology*. 1995;22:175-187.
26. Wang L, Xiao Q, Wang CH, et al. Vasoactive intestinal polypeptide suppresses proliferation of human cord blood-derived hematopoietic progenitor cells by increasing TNF-alpha and TGF-beta production in the liver. *Genet Mol Res*. 2014;13:9032-9043.
27. Block GD, Locker J, Bowen WC, et al. Population expansion, clonal growth, and specific differentiation patterns in primary cultures of hepatocytes induced by HGF/SF, EGF and TGF alpha in a chemically defined (HGM) medium. *J Cell Biol*. 1996;132:1133-1149.
28. Langer I, Leroy K, Gaspard N, et al. Cell surface targeting of VPAC1 receptors: evidence for implication of a quality control system and the proteasome. *Biochim Biophys Acta*. 2008;1783:1663-1672.
29. Endl E, Gerdes J. The Ki-67 protein: fascinating forms and an unknown function. *Exp Cell Res*. 2000;257:231-237.
30. Gurley LR, D'Anna JA, Barham SS, et al. Histone phosphorylation and chromatin structure during mitosis in Chinese hamster cells. *Eur J Biochem*. 1978;84:1-15.
31. Langer I, Robberecht P. Molecular mechanisms involved in vasoactive intestinal peptide receptor activation and regulation: current knowledge, similarities to and differences from the A family of G-protein-coupled receptors. *Biochem Soc Trans*. 2007;35:724-728.
32. Paine AJ, Andreakos E. Activation of signalling pathways during hepatocyte isolation: relevance to toxicology in vitro. *Toxicol In Vitro*. 2004;18:187-193.
33. Corlu A, Loyer P. Regulation of the g1/s transition in hepatocytes: involvement of the cyclin-dependent kinase cdk1 in the DNA replication. *Int J Hepatol*. 2012;2012:689324.
34. Anderson P, Gonzalez-Rey E. Vasoactive intestinal peptide induces cell cycle arrest and regulatory functions in human T cells at multiple levels. *Mol Cell Biol*. 2010;30:2537-2551.
35. Thoresen GH, Johansen EJ, Christoffersen T. Effects of cAMP on ERK mitogen-activated protein kinase activity in hepatocytes do not parallel the bidirectional regulation of DNA synthesis. *Cell Biol Int*. 1999;23:13-20.
36. Withers DJ. Signalling pathways involved in the mitogenic effects of cAMP. *Clin Sci (Lond)*. 1997;92:445-451.
37. Depoortere F, van Keymeulen A, Lukas J, et al. A requirement for cyclin D3-cyclin-dependent kinase (cdk)-4 assembly in the cyclic adenosine monophosphate-dependent proliferation of thyrocytes. *J Cell Biol*. 1998;140:1427-1439.
38. Hansen U, Owens L, Saxena UH. Transcription factors LSF and E2Fs: tandem cyclists driving G0 to S? *Cell Cycle*. 2009;8:2146-2151.
39. Fernandez M, Sanchez-Franco F, Palacios N, et al. IGF-I and vasoactive intestinal peptide (VIP) regulate cAMP-response element-binding protein (CREB)-dependent transcription via the mitogen-activated protein kinase (MAPK) pathway in pituitary cells: requirement of Rap1. *J Mol Endocrinol*. 2005;34:699-712.
40. Pagon Z, Volker J, Cooper GM, et al. Mammalian transcription factor LSF is a target of ERK signaling. *J Cell Biochem*. 2003;89:733-746.
41. Rackoff WR, Rubin RA, Earp HS. Phosphorylation of the hepatic EGF receptor with cAMP-dependent protein kinase. *Mol Cell Endocrinol*. 1984;34:113-119.
42. Barbier AJ, Poppleton HM, Yigzaw Y, et al. Transmodulation of epidermal growth factor receptor function by cyclic AMP-dependent protein kinase. *J Biol Chem*. 1999;274:14067-14073.
43. Zhang B, Nweze I, Lakshmanan J, et al. Activation of a cyclic amp-guanine exchange factor in hepatocytes decreases nitric oxide synthase expression. *Shock*. 2013;39:70-76.
44. Zimmermann S, Moelling K. Phosphorylation and regulation of Raf by Akt (protein kinase B). *Science*. 1999;286:1741-1744.
45. Biaggio RT, Abreu-Neto MS, Covas DT, et al. Serum-free suspension culturing of human cells: adaptation, growth, and cryopreservation. *Bioprocess Biosyst Eng*. 2015;38:1495-1507.

46. Zhuge J, Cederbaum AI. Serum deprivation-induced HepG2 cell death is potentiated by CYP2E1. *Free Radic Biol Med*. 2006;40:63-74.
47. Heslop JA, Rowe C, Walsh J, et al. Mechanistic evaluation of primary human hepatocyte culture using global proteomic analysis reveals a selective dedifferentiation profile. *Arch Toxicol*. 2017;91:439-452.
48. Enat R, Jefferson DM, Ruiz-Opazo N, et al. Hepatocyte proliferation in vitro: its dependence on the use of serum-free hormonally defined medium and substrata of extracellular matrix. *Proc Natl Acad Sci U S A*. 1984;81:1411-1415.
49. Mitaka T, Norioka K, Mochizuki Y. Redifferentiation of proliferated rat hepatocytes cultured in L15 medium supplemented with EGF and DMSO. *Vitro Cell Dev Biol Anim*. 1993;29A:714-722.
50. Guijarro LG, Couvineau A, Rodriguez-Pena MS, et al. Comitogenic effects of vasoactive intestinal polypeptide on rat hepatocytes. *Biochem J*. 1992;285:515-520.
51. Gaudin P, Couvineau A, Maoret JJ, et al. Mutational analysis of Cysteine Residues within the Extracellular domains of the human Vasoactive-Intestinal-Peptide (Vip) 1-Receptor Identifies 7 mutants that are defective in Vip binding. *Biochem Biophys Res Comm*. 1995;211:901-908.
52. Couvineau A, Fabre C, Gaudin P, et al. Mutagenesis of N-glycosylation sites in the human vasoactive intestinal peptide 1 receptor. Evidence that asparagine 58 or 69 is crucial for correct delivery of the receptor to plasma membrane. *Biochemistry*. 1996;35:1745-1752.
53. Mehta A, Comunale MA, Rawat S, et al. Intrinsic hepatocyte dedifferentiation is accompanied by upregulation of mesenchymal markers, protein sialylation and core alpha 1,6 linked fucosylation. *Sci Rep*. 2016;6:27965.
54. Boissard C, Marie JC, Hejblum G, et al. Vasoactive-intestinal-peptide receptor regulation and reversible desensitization in human colonic-carcinoma cells in culture. *Can Res*. 1986;46:4406-4413.
55. Elbattari A, Luis J, Martin JM, et al. The glycoprotein nature of the vasoactive intestinal peptide binding-site – role of carbohydrates in Vip binding on Ht-29-D4 Cells. *Ann N Y Acad Sci*. 1988;527:667-671.

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