



Vasoactive intestinal peptide (VIP) induces proliferation of human hepatocytes

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Complete List of Authors:	<p>Khedr, Mogibelrahman; University of Southampton, Clinical & Experimental Sciences; Suez Canal University, Faculty of Medicine, Department of Clinical Pharmacology</p> <p>Abdelmotelb, Ahmed; University of Southampton, Clinical & Experimental Sciences; Tanta University, Faculty of Medicine, Department of Pharmacology</p> <p>Bedwell, Thomas Andrew; University of Southampton, Clinical & Experimental Sciences</p> <p>Shtaya, Anan; St George's University of London</p> <p>Alzoubi , Mohammad ; University of Southampton, Clinical & Experimental Sciences; University of Jordan</p> <p>Abu Hilal, Mohammed ; University of Southampton, Clinical & Experimental Sciences</p> <p>Khakoo, Salim; University of Southampton, Clinical & Experimental Sciences</p>
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3 **1 Vasoactive intestinal peptide (VIP) induces proliferation of human hepatocytes**

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5 **2 Running title:** VIP stimulates proliferation of hepatocytes.
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10 4 Mogibelrahman M.S. Khedr^{1,2}, Ahmed M. Abdelmotelb^{1,3}, Thomas A. Bedwell¹, Anan
11 5 Shtaya⁴, Mohammad N. Alzoubi^{5,6}, Mohammed Abu Hilal^{1,6}, and Salim I. Khakoo^{1,6}.
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15
16 7 ¹Clinical and Experimental Sciences Academic Unit, Faculty of Medicine, University of
17 8 Southampton, Southampton, United Kingdom. ²Faculty of Medicine, Suez Canal University,
18 9 Ismailia, Egypt. ³Faculty of Medicine, Tanta University, Tanta, Egypt. ⁴St George's
19 10 University of London, London, United Kingdom. ⁵University of Jordan, Amman, Jordan.
20 11 ⁶Southampton University Hospitals NHS Trust, Southampton, United Kingdom.
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29 **13 Corresponding authors:**

30
31 14 Mogibelrahman M.S. Khedr

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33 15 MBChB, MSc, PhD

34
35 16 Clinical & Experimental Sciences Academic Unit,

36
37 17 Faculty of Medicine, University of Southampton,

38
39 18 Southampton University Hospital,

40
41 19 Tremona Road, Level F, MP837, SO16 6YD

42
43 20 Tel/ 02381206153, Mob/ 07961762098

44
45 21 E-mail: M.E.Khedr@soton.ac.uk
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3 **26 Author contributions:**

4
5 27 MMS Khedr, AM Abdelmotelb, TA Bedwell an MN Alzoubi were responsible for data
6
7 28 acquisition and analysis. AM Abdelmotelb and A Shtaya were concerned with ethical
8
9 29 considerations. M Abu Hilal and SI Khakoo contributed to the conception, design of the work
10
11 30 or of parts of it, and to its interpretation. MMS Khedr and SI Khakoo drafted and revised the
12
13 31 manuscript, AM Abdelmotelb and M Abu Hilal revised it critically for intellectual content,
14
15
16 32 and T Bedwell proofread the manuscript.
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20 **34 Abbreviations used in this article:**

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22 35 ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PH,
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24 36 Partial Hepatectomy; VPAC1, VIP and pituitary adenylate cyclase-activating polypeptide
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27 37 receptor-1.
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3 38 **Abstract: 225 words**

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5 39 **Objectives:** Proliferation of hepatocytes *in vitro* can be stimulated by growth factors such as
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7 40 epidermal growth factor (EGF), but **the** role of vasoactive intestinal peptide (VIP) remains
8
9 41 unclear. We have investigated the effect of VIP on **maintenance and** proliferation of human
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11 42 hepatocytes.
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15
16 44 **Materials and Methods:** Human hepatocytes were isolated from liver specimens obtained
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18 45 from patients undergoing liver surgery. Treatment with VIP or EGF was started 24 hours
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20 46 after plating and continued for three or five days. DNA replication was investigated **by**
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22 47 Bromodeoxyuridine (BrdU) incorporation and cell viability **detected** by MTT assay. Cell
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24 48 lysate was analysed by western blotting and RT-PCR. Urea and albumin secretion **into** the
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26 49 culture supernatants were measured.
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31 51 **Results:** VIP increased DNA replication in hepatocytes in a dose dependant manner, with a
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33 52 peak response at day three of treatment. VIP **treatment** was associated with an increase in
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35 53 mRNA expression of antigen identified by monoclonal antibody Ki-67 (MKI-67) and Histone
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37 54 Cluster 3 (H3) genes. Western blotting analysis showed that VIP can induce **a PKA/B-Raf**
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39 55 dependant phosphorylation of extracellular signal-regulated kinases (ERK). Although EGF
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41 56 can maintain hepatocyte functions up to day five, no marked effect was found with VIP.
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46 58 **Conclusions:** VIP induces proliferation of human hepatocytes with little or no effect on
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48 59 hepatocyte differentiation. **Further investigation of the role of VIP is required to determine if**
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50 60 **it may ultimately support therapeutic approaches of liver disease.**
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3 61 **Introduction:**

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5 62 Hepatocyte transfusions have shown promise as an alternative to conventional liver
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7 63 transplantation in treatment of some genetic disorders and acute liver failure^{1,2}. These
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9 64 potential therapies are compromised by poor viability, rapid de-differentiation, the low
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11 65 proliferative capacity of primary hepatocytes *in vitro*³ and the need for high numbers of
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13 66 hepatocytes. In addition, there is often poor liver cell viability after cryopreservation⁴.

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15 67 Improving hepatocyte *in vitro* viability and growth is crucial for progress in their use as a
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17 68 replacement therapy and in drug screening.

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22 70 VIP is a 28-amino acid neuropeptide found largely in the brain, gastrointestinal tract and
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24 71 liver⁵. Moreover, VIP receptors have been characterised and purified from the liver^{6,7}.

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26 72 Reports have shown that VIP can change the metabolic functions of rat hepatocytes, and can
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28 73 stimulate gluconeogenesis, ureagenesis, and inhibit glyconeogenesis^{8,9}. VIP has been found

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31 74 to be involved in regulation of hepatic blood flow, and modulation of both innate and
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33 75 adaptive immune functions¹⁰⁻¹². Interestingly, VIP mRNA expression is present in rat liver

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35 76 following partial hepatectomy (PH)¹³. Unlike Hepatic Growth Factor (HGF) and Epidermal
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37 77 Growth Factor (EGF), the role of VIP in liver regeneration is under-investigated. Previous

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39 78 reports have shown that VIP may exert bi-directional inhibitory or stimulatory effect on cell
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41 79 proliferation of a number of cell types. Kar *et al.* (1996) described a stimulatory effect of VIP

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43 80 alone on hepatocytes obtained from regenerated liver of rats¹³. In addition, it has been
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45 81 reported that VIP may have a mitogenic effect on HT29 and H9 cell lines^{14,15}, while it can

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47 82 cause an inhibition of proliferation of human HepG2 cells¹⁶.

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52 84 The mitogen-activated protein kinase (MAPK) pathway has been reported to play a crucial
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54 85 role in hepatocyte replication¹⁷. Moreover, EGF induced proliferation of rat hepatocytes is

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3 86 mainly dependant on the p44 and p42 isoenzymes (extracellular signal-regulated kinases,
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5 87 ERK1 and ERK2) of the MAPK pathway¹⁸. VIP stimulates intracellular production of cyclic
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7 88 adenosine 3':5'-monophosphate (cAMP) in various cell types, including hepatocytes⁸.
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9 89 Activation of cAMP-dependant Rap1 GTPase may be associated with either activation or
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11 90 inhibition of the (MAPK/ERK kinase) MEK/ERK cascade. This effect relies on the presence
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13 91 or absence of the serine/threonine-protein kinase B-Raf, respectively in cells¹⁹. Of relevance
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15 92 is that B-Raf kinase has been detected in liver²⁰. These findings support that hypothesis that
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17 93 VIP may contribute in hepatocytes proliferation.
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22 95 In the present study, we have investigated the effects of VIP on cell proliferation, gene
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24 96 expression, cell signalling and function in human hepatocytes.
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3 97 **Materials and Methods:**

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5 98 **Isolation of human hepatocytes:**

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7 99 Tissue samples (2-10gm) were obtained from fresh surgical macroscopically normal liver
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9 100 tissue resections from patients undergoing hepatectomies with informed consent (Research
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11 101 Ethics Committee, REC North East - Newcastle & North Tyneside 2, REC ref. 13/NE/0070).

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13 102 A total of 46 human liver cell preparations derived from the unaffected resection margins of
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15 103 the livers from 39 different donors with primary or metastatic liver tumors (24 men and 15
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17 104 women) were used. Patients' ages ranged from 29 to 83 years. Hepatocytes were isolated
18
19 105 using a two-step perfusion procedure as described previously²¹ with some modifications.
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21 106 Cells were plated on mouse collagen type IV gel layer 1 - 2.5µg cm⁻² (Corning Ltd.,
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23 107 Flintshire, UK) in William's E medium (Thermo Fisher, Inchinnan, UK) and incubated at
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25 108 37°C in a humidified incubator with 5% CO₂.

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31 110 **5-Bromo-2'-deoxyuridine (BrdU) DNA incorporation assay:**

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33 111 EGF (Sigma, Gillingham, UK) at 5, 10 and 20ng ml⁻¹ or VIP (Sigma) at 10⁻⁸, 10⁻⁷ or 10⁻⁶M
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35 112 was added 24 hours following cell seeding. Hepatocytes were incubated with BrdU (10µg ml⁻¹,
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37 113 Sigma) for 2 hours at 37°C. DNA-integrated BrdU was detected by rat anti-BrdU antibody
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39 114 (Bio-Rad, Hertfordshire, UK) and subsequently donkey anti-rat IgG-Alexa 488 (Thermo
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41 115 Fisher). Nuclei were stained with 4'-6-diamidino-2-phenylindole, DAPI (Sigma). Using
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43 116 fluorescence microscopy, numbers of BrdU⁺ and DAPI⁺ cells were determined in 6 different
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45 117 high power fields per well.

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50 119 **Measurement of lactic dehydrogenase (LDH):**

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52 120 Equal volumes of 200mM Tris (hydroxymethyl) aminomethane (Tris) pH 8, 50mM Lithium
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54 121 lactate, freshly prepared substrate solution [100µl P-Iodonitrotetrazolium Violet, INT (33mg

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3 122 ml⁻¹ in dimethyl sulfoxide (DMSO) + 100µl, Phenazine methosulfate, PMS (9 mg ml⁻¹) + 2.3
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5 123 ml β-nicotinamide adenine dinucleotide (NAD) hydrate (3.74 mg ml⁻¹)] and samples or
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7 124 positive control (5µg ml⁻¹ L-Lactic Dehydrogenase from bovine heart, Sigma) were loaded
8
9 125 into an assay plate. The V_{max} was measured at 490nm for 10min in a SpectraMax® Plus 384
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11 126 Microplate Reader (Molecular Devices, Wokingham, UK) and LDH activity (U ml⁻¹) was
12
13 127 calculated.
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18 129 **Viability and Proliferation Assays:**

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20 130 Viability was determined using a colourimetric MTT assay (Sigma) and Quick Cell
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22 131 Proliferation Assay kit II (Abcam, Cambridge, UK) were used according to manufacturers'
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24 132 instructions.
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29 134 **Polymerase Chain Reaction (PCR) and Real time PCR (rt-PCR):**

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31 135 RNA was extracted using a RNeasy® kit (Qiagen, Crawley, West Sussex, UK) following the
32
33 136 manufacturer's instructions. Complementary DNA (cDNA) was synthesised using a Primer
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35 137 Design Precision nanoScript 2 reverse transcriptase kit (Millbrook, Southampton, UK)
36
37 138 according to the manufacturer's instructions in a MasterCycler® 480 thermocycler
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39 139 (Eppendorf, Hamburg, Germany). The rt-PCR Primers were designed using the ProbeFinder
40
41 140 software version 2.5 (Lifescience.roche.com) and oligonucleotide primers **for albumin,**
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43 141 **Antigen Identified By Monoclonal Antibody Ki-67 (MKI-67), Histone Cluster 3 (H3) were**
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45 142 **obtained from Eurofins MWG/operon (Ebersberg, Germany) (Supplementary data 1). VIP**
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47 143 **and pituitary adenylate cyclase-activating polypeptide receptor-1 (VPAC1)** and EGF receptor
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49 144 (EGFR) mRNA expression was assessed using GoTaq® Hot Start Polymerase (Promega UK
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51 145 Ltd, Southampton, UK) according to manufacturer's instructions. PCR products were
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53 146 visualized on 2% agarose gel, band **densities** were measured and normalised to that of
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3 147 Glycerinadehyde-3-Phosphate-Dehydrogenase, GAPDH using a ChemiDoc™ imaging
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5 148 system (Bio-Rad). The qPCR was performed using a SYBR green Mastermix buffer (Primer
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7 149 Design) in an A&B 7900HT Fast Real-Time PCR System thermocycler (Applied Biosystems,
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9 150 CA, USA). The Ct values were normalized to the GAPDH and calibrated to untreated cells.
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11 151 The fold change of mRNA expression was calculated according to the $\Delta\Delta C_t$ method.
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16 153 **Detection of Phospho-p44/42 MAPK (Erk1/2) and VPAC1 in hepatocytes using Western**

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18 154 **Blotting:**

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20 155 Hepatocytes were serum starved for 24 hours prior to incubation with EGF (20 ng ml⁻¹) or
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22 156 VIP (10⁻⁶ M). The B-RAF inhibitor, SB-590885 and the PKA inhibitor, Rp-cAMP
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24 157 triethylammonium salt (Rp-cAMPS) were used. Cells were lysed using TruPAGE™ LDS
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26 158 Sample Buffer (Sigma) with phosphatase and protease inhibitors. Protein concentrations were
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28 159 measured and separated in a TruPAGE® 10% precast gels (Sigma) under reducing
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30 160 conditions, then transferred to nitrocellulose membranes. The membranes were probed with
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32 161 rabbit anti-human Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody or rabbit anti-
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34 162 human p44/42 MAPK (Erk1/2) antibody (New England Biolabs, Hertfordshire, UK),
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36 163 followed by goat anti-rabbit-horseradish peroxidase (HRP) (DakoCytomation,
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38 164 Cambridgeshire, UK). Reactive bands were detected using the Luminata Forte Western HRP
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40 165 substrate (Millipore UK Ltd., Hertfordshire, UK). In another experiment, the level of VPAC1

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42 166 protein expression in untreated or VIP (10⁻⁶M) treated hepatocytes, was investigated using a
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44 167 rabbit polyclonal anti VPAC1 (Abcam) and followed by goat anti-rabbit-horseradish
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46 168 peroxidase (HRP) (DakoCytomation).
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52 170 **cAMP Direct Immunoassay:**
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3 171 Levels of cAMP in hepatocytes 24 h following cell seeding and at day 3 or 5 following
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5 172 stimulation with 10^{-6} M VIP treatment were detected using a cAMP direct immunoassay
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7 173 (Abcam) according to the manufacturer's instructions. cAMP concentrations (μ M) were
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9 174 determined and corrected to total proteins concentrations in samples (μ g).
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14 176 **Albumin ELISA and Urea concentration assay:**

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16 177 Albumin and urea concentrations in the supernatant of hepatocytes cultures were determined
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18 178 using the ELISA DuoSET[®] kit for human albumin (R&D Systems, Oxfordshire, UK) and the
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20 179 QuantiChrom[™] urea assay kit (QuantiChrom, BioAssay Systems, Hayward, CA)
21
22 180 respectively, according to the manufacturer's instructions.
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27 182 **Statistics:**

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29 183 Two-way analysis of variants (ANOVA) followed by Fisher's least significant difference
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31 184 (LSD) multiple comparisons tests were performed using GraphPad Prism version 7.7.1 for
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33 185 Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. Data has been
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35 186 represented by Mean \pm standard error of the mean (SEM) or standard deviation (SD) as
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37 187 indicated. $P < 0.05$ was taken as significant.
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42 189 For further details regarding the materials and methods, please refer to the supplementary
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44 190 data 1.
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3 191 **Results:**

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5 192 **Stimulation of DNA replication in hepatocytes by VIP:**

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7 193 EGF at high concentrations such as 50ng ml^{-1} , has been reported to be responsible for an
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9 194 increase in $[^3\text{H}]$ methylthymidine incorporation in rat hepatocytes. The response to EGF
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11 195 maximised at 24 hour and continued with persistent exposure²². In the current work,
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13 196 proliferation of human hepatocytes was investigated by detecting BrdU incorporation (Figure
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15 197 1A). Herein, EGF resulted in an increase of BrdU positive cells at concentrations of 10ng ml^{-1}
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17 198 ¹ (a mean of $1.3 \pm \text{SD } 0.9$ fold) and 20ng ml^{-1} (a mean of $1.8 \pm \text{SD } 1.4$ fold) at day 3 (Figure
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19 199 1B), and this effect was continued at day 5 of treatment, 10 ng ml^{-1} (a mean of $1.5 \pm \text{SD } 0.5$
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21 200 fold) and 20 ng ml^{-1} (a mean of $1.7 \pm \text{SD } 0.5$ fold) (Figure 1C). Interestingly, VIP stimulated
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23 201 proliferation of human hepatocytes in a dose dependant manner at day 3 up to a mean of 3.2
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25 202 $\pm \text{SD}1.1$ fold at 10^{-6}M (Figure 1D). However, a decline of hepatocyte response to VIP was
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27 203 observed at day 5 (a mean of $1.2 \pm \text{SD } 0.6$ fold up to 10^{-6}M) (Figure 1E). Similarly, EGF
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29 204 addition was associated with a rise in total cell numbers at day 3; 10ng ml^{-1} (a mean of $1.4 \pm$
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31 205 $\text{SD } 1.1$ fold) and 20ng ml^{-1} (a mean of $1.9 \pm \text{SD } 1.7$ fold) and day 5; 10ng ml^{-1} (a mean of 1.7
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33 206 $\pm \text{SD } 0.4$ fold) and 20 ng ml^{-1} (a mean of $1.7 \pm \text{SD } 0.3$ fold). VIP at day 3 resulted in an
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35 207 increase of total cells by a mean of 2.2 ± 0.9 fold at 10^{-7}M and $3.4 \pm \text{SD } 1.4$ fold at 10^{-6}M .
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37 208 The drastic decrease in hepatocyte response to VIP at day 5 raised a concern about changes in
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39 209 cell viability and status, and was investigated further.
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46 211 **VIP treatment has a limited effect on hepatocyte survival *in vitro*:**

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48 212 Effects of EGF or VIP on hepatocyte integrity was tested by measuring LDH release in the
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50 213 cell culture supernatants. In the first 24 hours following cell extraction, LDH activity was
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52 214 high (a mean of $0.90 \pm \text{SD } 0.29\text{ U ml}^{-1}$) (Figure 2A), which may be a result of the isolation
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54 215 process or spontaneous activation of hepatocyte apoptosis^{23,24}. A dramatic decrease in LDH
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3 216 levels was observed in the following 24 hours (a mean of $0.14 \pm \text{SD } 0.16$ U/ml). This may
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5 217 have been caused by washout of old medium containing dead and apoptotic cells. No further
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7 218 change in LDH activity was observed up to day 5. Treatment of hepatocytes with EGF
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9 219 resulted in a minimal change in LDH activity in the supernatants at day 1 compared to
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11 220 untreated cells. A decrease in LDH activity was observed at days 3 and 5 at various
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13 221 concentrations of EGF (Figure 2B). When VIP was added to the medium, no change in LDH
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15 222 activity was observed at day 1 or 3 of treatment (Figure 2C). At day 5, cells treated with VIP
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17 223 showed a marked increase in LDH levels, with approximately 2, 8 and 10 fold changes at 10^{-8}
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19 224 M, 10^{-7} M and 10^{-6} M of VIP respectively. There was also a rise in LDH activity when both
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21 225 agents were added together to the hepatocyte culture medium (data not shown).
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27 227 The metabolic activity of the cell was assessed using the MTT assay. At day 3, EGF showed
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29 228 a marked improvement in cell viability (Figure 2D) and VIP treatment was associated with a
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31 229 concentration dependant increase in hepatocyte metabolic activity, peaking at a concentration
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33 230 of 10^{-6} M (Figure 2E). Results showed low metabolic activity of primary human hepatocytes
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35 231 after day 5 of cell seeding, irrespective of the addition of EGF or VIP. This result may reflect
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37 232 cell loss.
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42 234 Previous results have shown that the support of hepatocyte survival was lacking when VIP
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44 235 was used alone and cells have entered a late phase of death or apoptosis. In order to address
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46 236 this, we have tested DMSO as an agent which may prevent this deterioration of cell viability
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48 237 and as reported, can maintain hepatocyte differentiation and improve liver-specific
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50 238 functionality²⁵. DMSO alone induced cell death as compared to medium alone, however
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52 239 addition of 2% DMSO to culture medium was associated with the restoration of the
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54 240 hepatocyte response to EGF and the VIP mitogenic effect at day 5 of treatment (Figure 3A
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3 241 and 3B). In addition to hepatocytes loss, the noticeable decrease in the effect of VIP by day 5
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5 242 and a change in expression of VIP receptors may contribute to hepatocyte resistance VIP. To
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7 243 test this possibility mRNA expression of VPAC1, the most abundant VIP receptor in the
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9 244 liver, was investigated using a semi-quantitative RT-PCR technique²⁶. In untreated
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11 245 hepatocytes, level of mRNA expression of VPAC1 or EGFR did not change significantly at
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13 246 day 3 (Figure 3C). However, at day 5 cells expressed lower levels of EGFR mRNA which is
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15 247 a phenomenon that has been reported previously²⁷ but VPAC1 mRNA expression did not
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17 248 show any change. Western blotting revealed several forms of VPAC1 in human hepatocytes
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19 249 at molecular weights of ~250, ~100 and ~52 kDa (Figure 3D), as described previously²⁸.
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21 250 During hepatocytes culture, VPAC1 protein expression did not show marked changes, but
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23 251 VIP treatment was associated with a marked decrease in VPAC1 gene mRNA expression at
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25 252 day 5 of cell culture (Supplementary Figure 1). The level of VPAC1 activation has previously
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27 253 been assessed by measuring intracellular cAMP concentrations. Interestingly, exposure of
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29 254 VPAC1 to VIP at a concentration of 10^{-6} M at 24 hours following cell seeding was found to
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31 255 stimulate production of cAMP by hepatocytes as compared to untreated cells (mean
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33 256 concentration $5.96 \mu\text{M}/\mu\text{g}$ of protein \pm SEM 0.64 versus 4.18 ± 0.60 respectively, $P =$
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35 257 0.0029) (Figure 3E). Production of cAMP as a response to VIP continued but to a lesser
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37 258 extent until day 3 of hepatocyte culture (mean of $5.90 \mu\text{M}/\mu\text{g}$ of protein ± 0.77 and $4.95 \pm$
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39 259 0.97 respectively, $P = 0.0761$). Notably, constitutive cAMP showed a lower concentration at
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41 260 day 5 of cell culture in untreated cells (a mean of $3.85 \pm$ SEM $0.84 \mu\text{M}/\mu\text{g}$ of protein) and
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43 261 VPAC1 receptors did not show as clear a response to VIP as that seen at early time points
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45 262 ($4.52 \pm 0.69 \mu\text{M}/\mu\text{g}$ of protein). Taken together, these finding may suggest a change in
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47 263 receptor functionality over time.
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3 264 **Expression of proliferation-associated genes was induced by VIP treatment:**

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5 265 Expression of the active cell cycle marker, MKI-67²⁹ and the mitotic marker, H3³⁰ genes
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7 266 were studied using quantitative rt-PCR. EGF alone induced a six-fold increase in mRNA
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9 267 expression of MKI-67, most significantly at day 3 of treatment at concentrations up to 10ng
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11 268 ml⁻¹ (Figure 4A). In addition, EGF treatment resulted in up to a four-fold increase in
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13 269 expression of H3 mRNA by day 3 of treatment, most noticeably at 20ng ml⁻¹ EGF (Figure
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15 270 4B). Addition of VIP to cultured hepatocytes were associated with a two-fold increase in
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17 271 MKI-67 gene expression at day 3, rising to four-fold at day 5 of treatment at a concentration
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19 272 of 10⁻⁶M (Figure 4C). Similarly, VIP induced a concentration dependant increase in
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21 273 expression of H3 at days 3 and 5 (Figure 4D). Although the combination of EGF and VIP
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23 274 was associated with a considerable increase in expression of MKI-67 at day 5, there was no
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25 275 difference compared to either EGF or VIP alone (Figure 4E). The presence of EGF and VIP
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27 276 together in the culture medium had little effect on expression of H3 at day 3 (Figure 4F).
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33 278 **Production of Phospho-p44/42 MAPK (Erk1/2) in VIP treated hepatocytes:**

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35 279 Binding of VIP to its receptors initiates cAMP production and subsequent protein kinase A
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37 280 (PKA)³¹. A PKA-dependent phosphorylation of the GTPase Rap1 resulted in stimulation of
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39 281 ERKs in the presence of B-Raf in cells such as hepatocytes¹⁹. EGF at 20ng ml⁻¹ stimulated
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41 282 phosphorylation of ERK as early as 10 minutes, after which activation declined with time
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43 283 (Figure 5A). Interestingly, VIP was found to increase pERK following 10 minutes incubation
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45 284 with hepatocytes. However, ERK activation increased further up to 60 minutes (Figure 5B).
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47 285 In addition, VIP stimulation of freshly isolated hepatocytes failed to elicit phosphorylation of
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49 286 ERK (data not shown). Both agents did not preferentially activate either pERK 1 or 2. Pre-
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51 287 incubation of human hepatocytes with 5µM of SB-590885 (SB), a B-RAF inhibitor prior to
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53 288 treatment or Rp-cAMPS (cAMP inhibitor) at 500µM was associated with inhibition of VIP
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3 289 induced pERK (Figure 5C and 5D). Interestingly, SB was found to preferentially block ERK2
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5 290 phosphorylation to a greater extent than ERK1. Whereas inhibition of cAMP mobilization
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7 291 with Rp-CAMP inhibitor blocked both ERK1 and ERK2.
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11 293 **VIP treatment does not support human hepatocytes specific functions:**

13 294 Albumin gene expression was suppressed initially, but recovered by day 3 of incubation with
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15 295 EGF at a concentration of 5 ng ml⁻¹ and markedly increased at day 5 with concentrations up
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17 296 to 5 to 20 ng ml⁻¹ (Figure 6A). Conversely, VIP had no marked effect on albumin gene
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19 297 expression in human hepatocytes in this model (Figure 6B). When EGF and VIP were
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21 298 combined together, the stimulatory effect of EGF on albumin gene expression was
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23 299 significantly lower than that of EGF alone (Figure 6C).
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28 301 Albumin levels in the supernatants dropped from a mean of 75.14 ± SD 22.13ng ml⁻¹ in the
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30 302 first 24 hours following hepatocyte seeding to a mean of a mean of 40.24 ± SD 16.82 ng ml⁻¹
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32 303 at day 2 and no marked change was observed subsequently. EGF stimulated production of
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34 304 albumin from liver cells in a concentration dependent manner as compared to the untreated
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36 305 control at day 1 of treatment yielded a mean of 120.91± SD 79.91 ng ml⁻¹ which continued up
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38 306 to day 5 of treatment to reach a mean of 152.80 ± SD 87.20 ng ml⁻¹ with 20ng ml⁻¹ EGF
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40 307 (Figure 6D). At day 3, there was an increase in albumin production up to a mean of 66.9 ±
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42 308 SD 76.83 ng ml⁻¹ from hepatocytes cultured in the presence of 10⁻⁶M VIP (Figure 6E). When
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44 309 both agents were added together, the stimulatory effect of EGF was inhibited (Figure 6F).
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46 310 When both agents were added sequentially, an inhibitory effect of VIP on EGF stimulated
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48 311 albumin production was observed (Supplementary Figure 2). Urea production from
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50 312 hepatocytes was dramatically decreased during the 24 hours following cell plating from a
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52 313 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
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3 314 was observed at day 3 and 5 (a mean of $1.26 \pm \text{SD } 0.37 \text{ mg dL}^{-1}$ and $1.10 \pm \text{SD } 0.36 \text{ mg dL}^{-1}$
4
5 315 respectively). EGF increased urea production on the first day of hepatocyte culture compared
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7 316 to untreated cells (a mean of $1.31 \pm \text{SD } 0.23 \text{ mg dL}^{-1}$ at 10 ng ml^{-1} EGF), but this effect
8
9 317 disappeared with time (Figure 6G). However, 10^{-7}M VIP resulted in a limited increase (a
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11 318 mean of $1.53 \pm \text{SD } 0.51 \text{ mg dL}^{-1}$) in urea production at day 3 as compared to control (Figure
12
13 319 6H) and adding VIP to EGF abolished the effect of EGF on urea production in cultures
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15 320 hepatocytes (Figure 6I).

For Review Only

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3 321 **Discussion:**

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5 322 Our findings have shown that EGF or VIP alone has the ability to induce DNA synthesis in
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7 323 cultured human hepatocytes and to stimulate expression of genes that may be involved in cell
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9 324 proliferation. Interestingly, EGF was able to maintain hepatocyte proliferation further up to
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11 325 day 5 whilst VIP did not. In addition, VIP was found to stimulate phosphorylation of ERK1
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13 326 and 2 protein kinases. However, unlike EGF, VIP has a limited effect on hepatocyte function
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15 327 *in vitro*.

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20 329 Hepatocytes move from G0 to G1 phase of cell cycle spontaneously during isolation
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22 330 process³² and progress further towards and stop at a restriction point in mid-late G1 phase
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24 331 usually 24 and 48 h after plating²². Onward movement to S phase is dependent on growth
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26 332 factors such as EGF³³. In agreement with that, we have demonstrated that EGF stimulated
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28 333 DNA synthesis when added 24 h following hepatocytes seeding. Strikingly, we observed a
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30 334 comparable effect with VIP which disagree to that previously reported by Kar *et al*¹³. The
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32 335 outcome of proliferative stimuli is related to the cell cycle. A few hours following isolation,
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34 336 VIP can facilitate entry of cells into G1 phase but it did not encourage them to pass the
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36 337 restriction point³⁴. This effect could increase the number of cells at susceptible to the
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38 338 mitogenic effect of EGF. These findings might explain why VIP alone failed to stimulate
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40 339 DNA synthesis in hepatocytes but may potentiate the effect of EGF on cell proliferation at
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42 340 this early time point^{13,22,35}. We found that VIP did activate MAPK at this early time which
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44 341 consistent with that has been reported³⁵. The underling mechanism could involve activation
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46 342 of p70 ribosomal S6 protein kinase (p70S6k) activity, cyclin D3-cyclin-dependent kinase
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48 343 (CDK)-4 assembly or a CDK2/cyclin C-dependent inhibitory phosphorylation of the
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50 344 transcription factor LSF (late simian virus 40 factor) at serine 309³⁶⁻³⁸.

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3 345 As we have shown, later in culture VIP or EGF stimulated formation of *p*ERK which has
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5 346 been described previously^{18,39}. This effect was found to be closely related to induction of
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7 347 hepatocyte proliferation¹⁷ and may involve an MAPK-dependent reactivation
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9 348 phosphorylation of LSF at serine 291 which could be essential for cell cycle progression to S
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11 349 phase⁴⁰. Dependence of VIP induced ERK activation on B-Raf kinase could support our
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13 350 hypothesis that VIP alone is able to induce hepatocyte proliferation, but VIP exerted an
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15 351 inhibitory effect on EGF (Figure 7). In accordance with these results, it has been reported that
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17 352 high levels of cAMP could result in a decrease in EGF-dependent MAPK production and loss
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19 353 of its DNA stimulatory effect³⁵. In addition, several reports have shown that cAMP-
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21 354 dependent PKA is able to phosphorylate EGFR on serine residues which results in decrease
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23 355 in tyrosine kinase activity and EGFR auto-phosphorylation induced by EGF^{41,42}. Moreover,
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25 356 cAMP-GEFs can directly inhibit Raf-1 by phosphorylation at ser259 or indirectly by a PKA-
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27 357 dependent activation of the Raf-1 inhibitor, Akt (protein kinase B, PKB)^{43,44}. This interaction
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29 358 could explain the reported VIP inhibitory effect on HepG2 proliferation. HepG2 survival and
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31 359 proliferation is depending on the presence of FBS in medium^{45,46}. VIP has been shown to
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33 360 inhibit HepG2 proliferation through a cAMP – dependent signal transducers and activators
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35 361 of transcription-3 (STAT-3) pathway inhibition¹⁶, the pathway that can be stimulated by
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37 362 growth factors which present in FBS.
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44 364 The DNA synthesis in primary hepatocytes started early in culture and maximised at day 3,
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46 365 with expression of activated transcriptional regulators for EGF and ERK pathway⁴⁷, but
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48 366 decreased afterwards even in the presence of EGF^{13,48}. Following day 3 of culture, substantial
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50 367 hepatocyte death has been reported and the remaining cells may become flattened and
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52 368 polykaryotic or smaller and apoptotic³. We have noticed that, at day 5 of EGF treatment,
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54 369 there was a lower number of living hepatocytes, and that the remaining cells replicated, but to
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3 370 a lower extent. This is in agreement with previous findings to that has been reported
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5 371 before^{33,49}.

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7 372 In our model, VIP did not show any change in hepatocyte proliferation, consistent with
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9 373 previous work¹³. Notably, the cells which proliferated under the effect of VIP mostly died by
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11 374 day 5 of treatment and VIP did not markedly increase DNA synthesis in the remaining cells.
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13 375 The lack of support of the differentiated state of hepatocytes with VIP treatment was
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15 376 observed from the albumin production and urea secretion at day 5, a finding that has been
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17 377 previously reported⁹. Interestingly, MKI-67 and H3 mRNA expression in hepatocytes showed
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19 378 a tendency to increase at day 5 of treatment while albumin expression decreased with time,
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21 379 which may be an indication of a loss of differentiation.
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27 381 The dramatic change in hepatocyte response to VIP could be a consequence of changes in
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29 382 VIP receptors expression. We found that hepatocytes did not show such a change in
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31 383 expression of VPAC1 during culture time course. However, VIP failed to induce cAMP
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33 384 production in hepatocytes at day 5 of cell culture, which suggests an alteration of receptor
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35 385 signaling response. Indeed, the interaction between VIP and its receptors in proliferating
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37 386 hepatocytes is not completely understood. In rat liver 3 days after PH, the maximal response
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39 387 of VIP was reduced as a result of low number of receptors and changes in the receptor
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41 388 structure⁵⁰. In addition, the decrease in VIP receptors sensitivity could be a result of high
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43 389 expression of VIP in proliferating liver¹³. Moreover, VPAC1 harbours several potential *N*-
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45 390 glycosylation sites which are critical for VIP binding⁵¹ and receptor delivery to plasma
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47 391 membrane⁵². An alteration in *N*-glycosylation of proteins has been reported in de-
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49 392 differentiated rat hepatocytes⁵³, and could explain the decreased in VPAC1 response to VIP,
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51 393 but this possibility needs further investigations. In addition, we have demonstrated that
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3 394 addition of high concentration of VIP was associated with downregulation of VPAC1, the
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5 395 phenomenon that has been reported with VIP with other cell types^{54,55}.
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7 396 Our findings have demonstrated that VIP alone was able to induce proliferation of adult
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9 397 human hepatocytes when added 24 hours following hepatocyte plating and this effect may be
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11 398 PKA/B-Raf-ERK dependent. VIP exerts an inhibitory effect on EGF signaling pathway at
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13 399 this time point of cell cycle. Stimulation of the VIP pathway may aid hepatocyte proliferation
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16 400 *in vitro*.

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21
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27
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35 409 **Conflict of interest:**

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37 410 All authors declare no conflict of interest.
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3 554 **Figures' Legends:**

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5 555 **Figure 1 Hepatocyte proliferation was stimulated by EGF or VIP.** (A) **Representative**
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7 556 **images of hepatocytes treated with either EGF (20 ng ml⁻¹) or VIP (10⁻⁶ M) for 3 days.** DNA
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9 557 incorporation of BrdU was determined (Green) and DAPI (Blue) was used as a nuclear
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11 558 counter stain. (B - E) The effects of EGF or VIP were demonstrated on total and proliferating
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13 559 cell numbers. n = 3 different donors per condition. *P* values shown in the graph are for
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15 560 comparison to hepatocytes maintained on medium alone. * *P*<0.05, ** *P*<0.005, ***
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17 561 *P*<0.0005, **** *P* <0.0001. Mean ± SEM. Two-way ANOVA followed by Fisher's least
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19 562 significant difference (LSD).
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25 564 **Figure 2 Hepatocyte viability with EGF or VIP.** (A) LDH activity (U ml⁻¹) in supernatants
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27 565 of untreated human hepatocytes with time course. (B) LDH release (expressed as percentage
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29 566 of total LDH activity) in supernatants of human hepatocytes treated with EGF or (C) VIP at
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31 567 previous concentrations following day 1, 3 and 5 of treatments. n = 3 different donors per
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33 568 condition. *P* values shown in the graph are for overall comparison with hepatocytes at day 0
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35 569 (A) or untreated control (B and C). * *P*<0.05, ** *P*<0.005, *** *P*<0.0005, **** *P*<0.0001.
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37 570 (D) Viable cells were detected following addition of EGF (5, 10 or 20 ng ml⁻¹) or (E) VIP
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39 571 (10⁻⁸, 10⁻⁷ or 10⁻⁶ M) treatment for 3 or 5 days by MTT assay. *A*, Absorbance. n = 3 different
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41 572 donors per condition. *P* values shown in the graph are for overall comparison between
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43 573 hepatocytes at day 3 and 5. Mean ± SEM. Two-way ANOVA followed by Fisher's LSD.
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49 575 **Figure 3 The effect of DMSO on cell response to VIP and VIP and pituitary adenylate**
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51 576 **cyclase-activating polypeptide receptor-1 (VPAC1) expression and activation in**
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53 577 **hepatocytes.** (A and B) Hepatocytes were treated with either EGF (5, 10 or 20 ng ml⁻¹) or
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55 578 VIP (10⁻⁸, 10⁻⁷ or 10⁻⁶ M), 2 % DMSO was added at day 3 and cell proliferation was
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3 579 investigated at day 5 using the WST-1 Quick Cell Proliferation Assay kit II (Abcam). n = 3
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5 580 different donors per condition. *P* values shown in the graph are for comparison at individual
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7 581 concentrations and overall comparison with hepatocytes maintained in medium without
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9 582 DMSO. (C) Band density analysis (fold change) of VPAC1 and Epidermal Growth Factor
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11 583 Receptor (EGFR) mRNA of gene expression on 2% agarose gel in non-treated cells
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13 584 following 1, 3 or 5 days of hepatocyte culture (6 donors), (D) VPAC1 protein expression as
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15 585 detected in hepatocytes by western blotting techniques at day 3 and 5 of hepatocyte culture, a
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17 586 representative blot of 3 independent experiments. Molecular weights were indicated for
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19 587 VPAC1 isoforms. (E) Effect of VIP (10^{-6} M) on cAMP concentrations ($\mu\text{M } \mu\text{g}^{-1}$ of protein) in
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21 588 hepatocytes with time course control (ctrl.). n = 3. * $P < 0.05$, ** $P < 0.005$. Mean \pm SEM.
22
23
24 589 Two-way ANOVA followed by Fisher's LSD.
25
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29 591 **Figure 4 Expression of Monoclonal Antibody Ki-67 (MKI-67) and Histone Cluster 3**
30
31 592 **(H3) genes in human hepatocytes cultured in the presence of EGF or VIP.** Expression of
32
33 593 mRNA was quantified by qPCR at days 1, 3 and 5 of EGF (5, 10 or 20 ng ml⁻¹) or VIP (10^{-8} ,
34
35 594 10^{-7} or 10^{-6} M). (A – D) Concentration dependant effects of EGF or VIP, and (E and F) the
36
37 595 effect of EGF (20 ng ml⁻¹) or VIP (10^{-6} M) or a combination of both. n = 3 different donors
38
39 596 per condition. *P* values shown in the graph are for comparison at individual concentrations
40
41 597 and overall comparison with hepatocytes at day 1. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$.
42
43 598 Mean \pm SEM. Two-way ANOVA followed by Fisher's LSD.
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48
49 600 **Figure 5 Phosphorylation of ERK in EGF or VIP-treated human hepatocytes analysis**
50
51 601 **using western blotting.** (A) Hepatocytes treated with either EGF (20 ng ml⁻¹) or (B) VIP (10^{-6}
52
53 602 M) and analysed by western blotting at indicated time points. (C) The effects of downstream
54
55 603 pathway inhibitors was investigated using 2.5 - 10 μM of SB-590885 (SB) or (D) 500 μM of
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3 604 *Rp-cAMPS*. Hepatocytes were incubated with inhibitors for 1 h prior to addition of EGF or
4
5 605 VIP for another 1 h. n = 4 with different donors.

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10 607 **Figure 6 Expression and production of albumin, and urea from human hepatocytes**

11 608 **cultured with EGF and VIP.** Albumin gene mRNA expression at days 0, 1, 3 and 5 of (A)

12 609 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

13 610 either EGF (20 ng ml⁻¹), VIP (10⁻⁶ M) or combination of both were determined. *P* values

14 611 shown in the graph are for comparison at individual concentrations and overall comparison

15 612 with hepatocytes at day 1. (D – F) Albumin (ng ml⁻¹) and (G – I) urea (mg dL⁻¹)

16 613 concentrations in supernatants of cultured hepatocytes with EGF, VIP or both were

17 614 determined. n = 3 different donors per condition. *P* values shown in the graph are for

18 615 comparison with hepatocytes at day 0 or with untreated cells. * *P*<0.05, ** *P*<0.005, ***

19 616 *P*<0.0005, **** *P*<0.0001. Mean ± SEM. Two-way ANOVA followed by Fisher's LSD.

20 617

21 618 **Figure 7 A schematic diagram for VIP and EGF signaling in hepatocytes. Late in culture,**

22 619 **binding of VIP with the G-protein coupled VIP receptor type 1 (VPAC1) activates**

23 620 **intracellular adenylyl cyclase (AC) resulting in cAMP production and the following protein**

24 621 **kinase A (PKA) activation. Subsequently, phosphorylated Rap-1 can activate B-Raf and**

25 622 **thereby, stimulate the mitogen-activated protein kinase (MAPK) / extracellular signal-**

26 623 **regulated kinase (ERK) kinase, MEK/ERK cascade. Phosphorylation of ERK1/2 results in**

27 624 **stimulation of cell proliferation and induces mRNA expression the proliferation-associated**

28 625 **genes, the monoclonal antibody Ki-67 (MKI-67) and Histone Cluster-3 (H3) genes. EGF**

29 626 **interaction with its receptors, EGFR results in a Ras/Raf dependent activation of MEK,**

30 627 **induction of cell proliferation and improvement of cell functions. VIP-activated Rap-1 may**

31 628 **block EGF signaling through inhibition of Ras/Raf activation. VIP signaling can be inhibited**

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629 by the B-RAF inhibitor, SB-590885 and PKA inhibitor, *Rp*-cAMP triethylammonium salt
630 (*Rp*-cAMPS).

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Figure 1

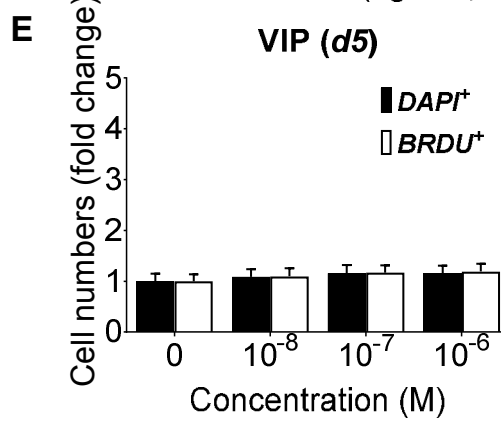
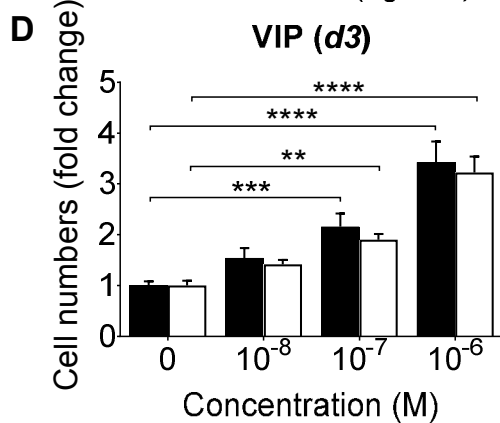
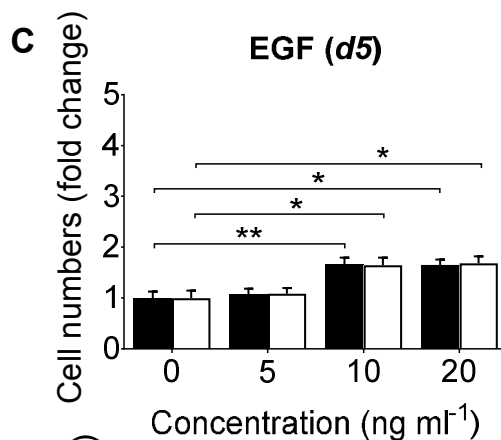
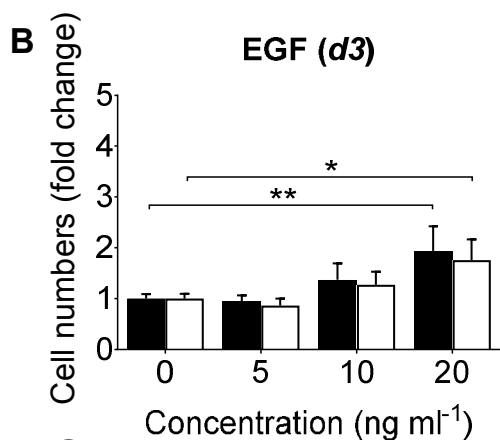
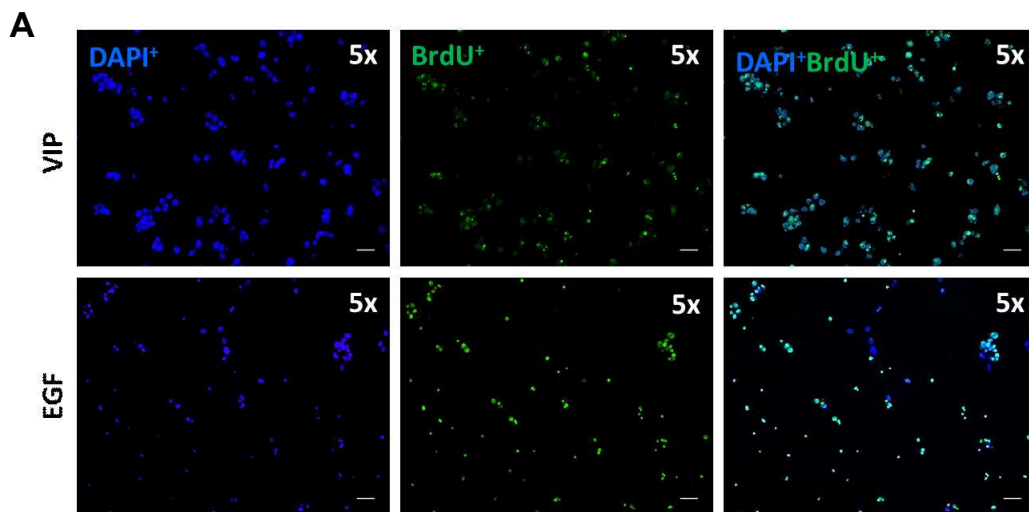


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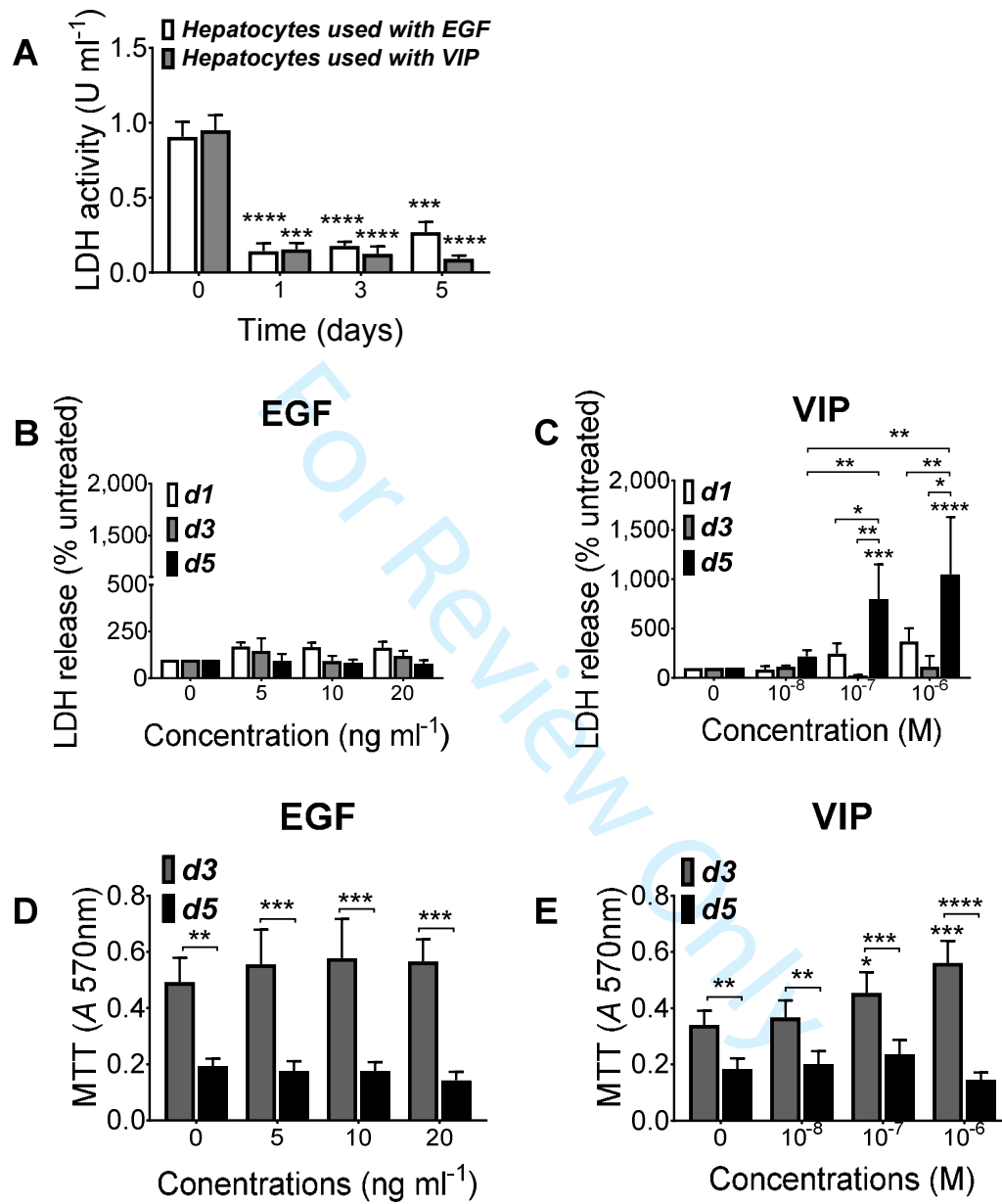


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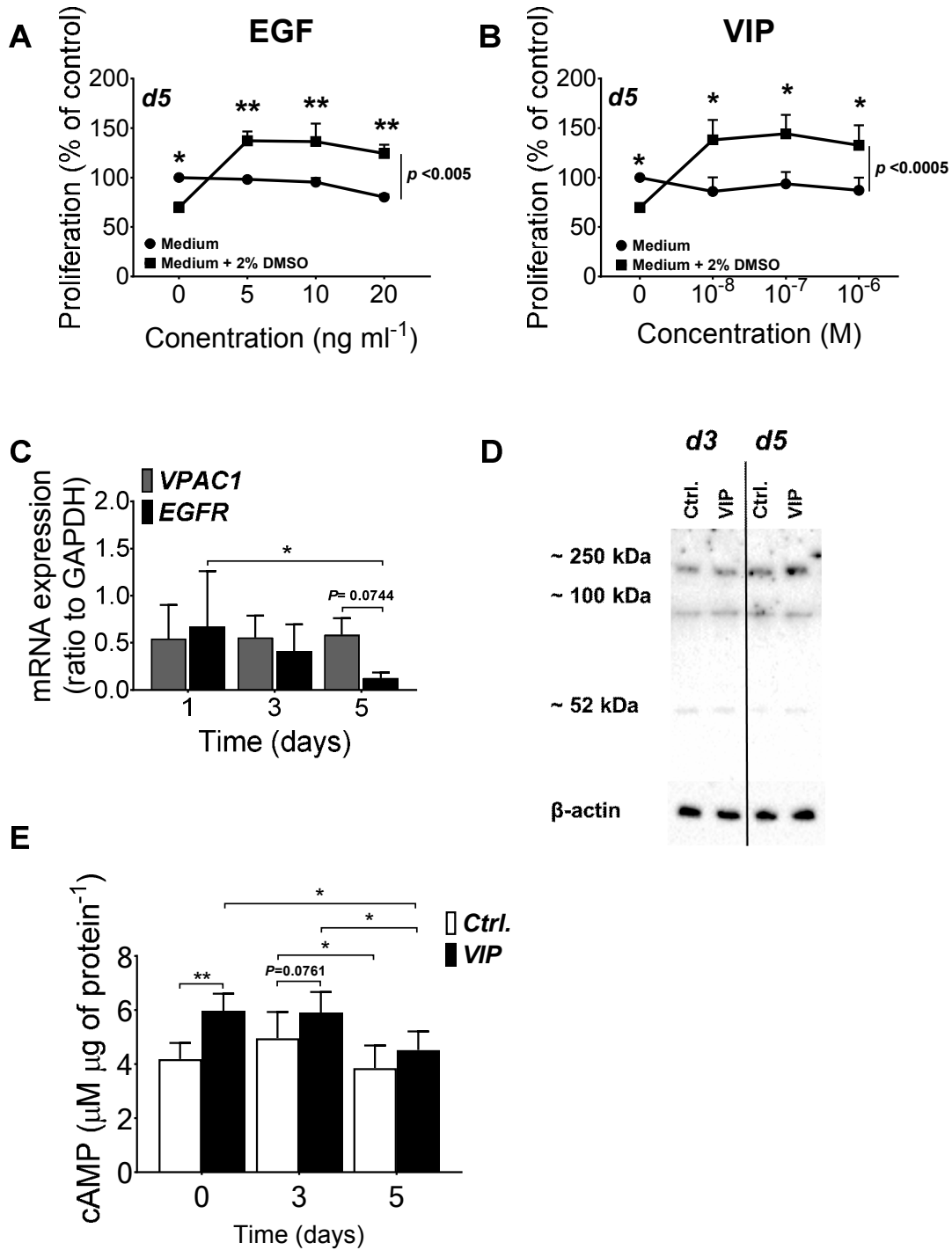


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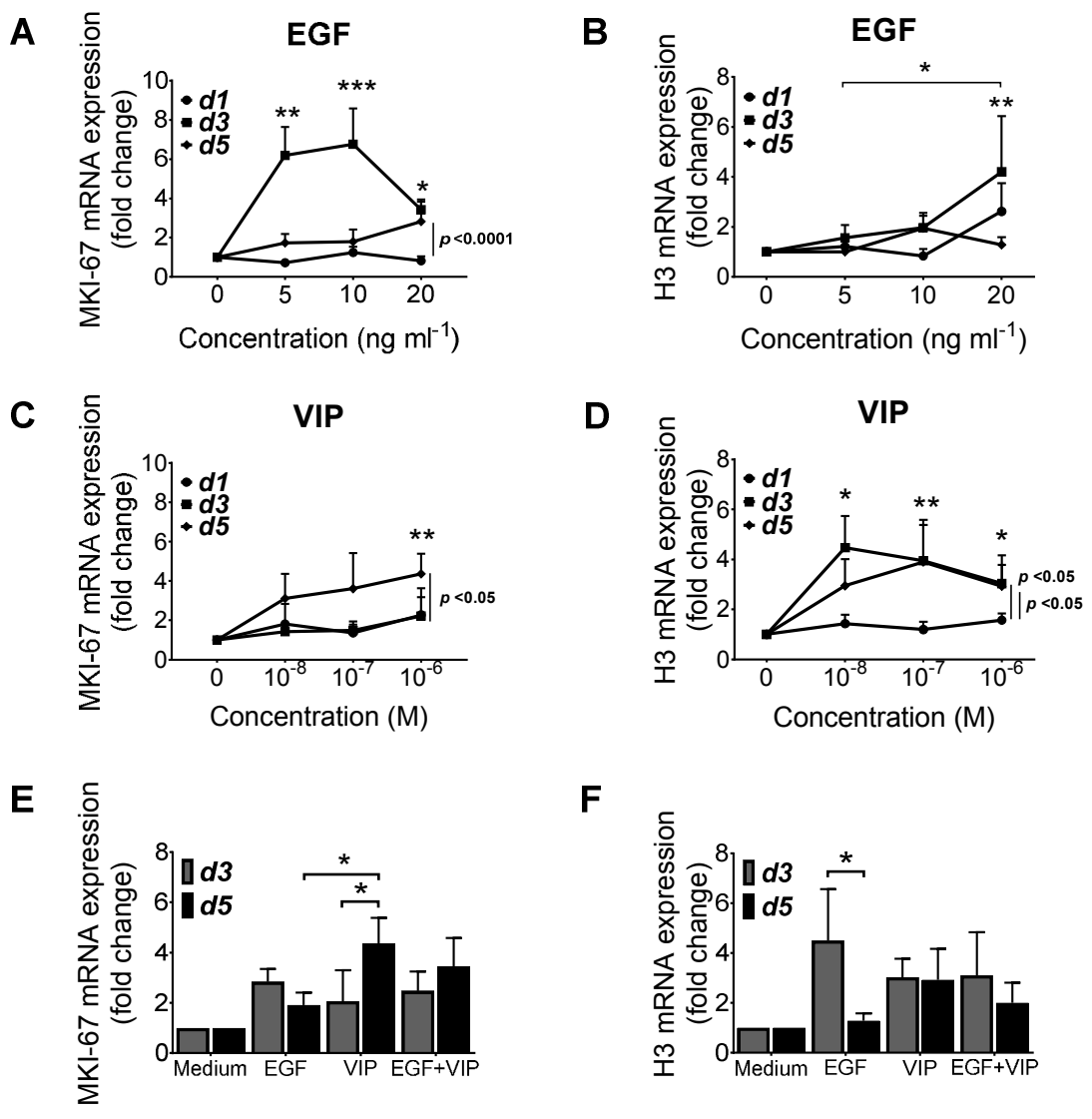


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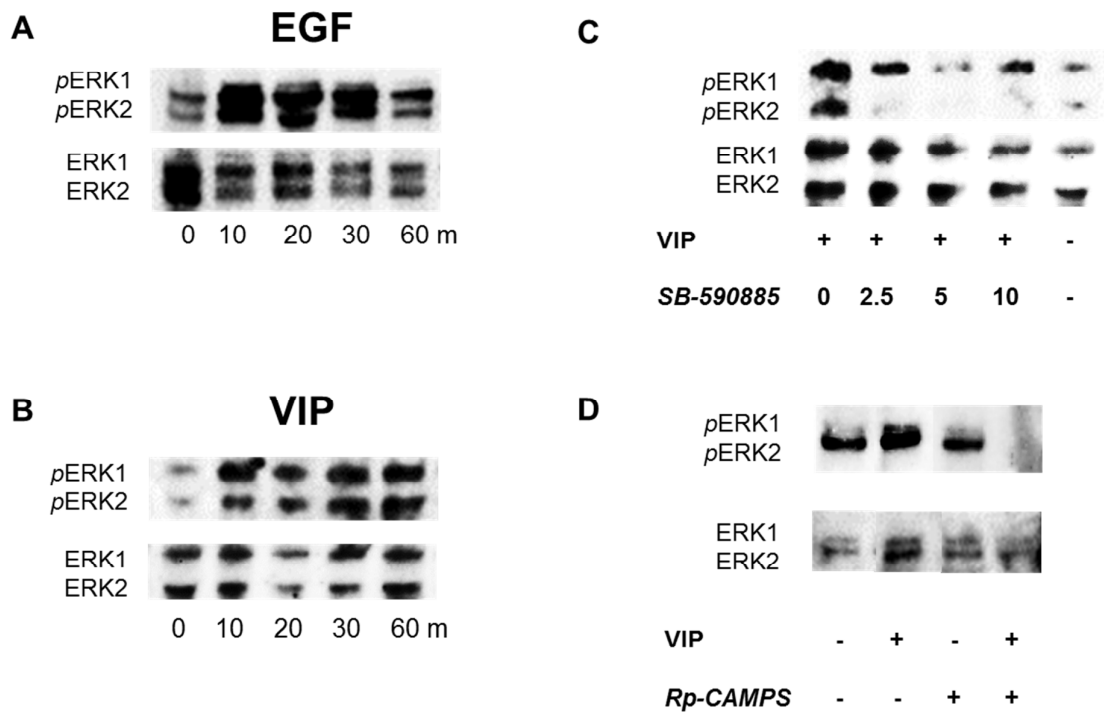


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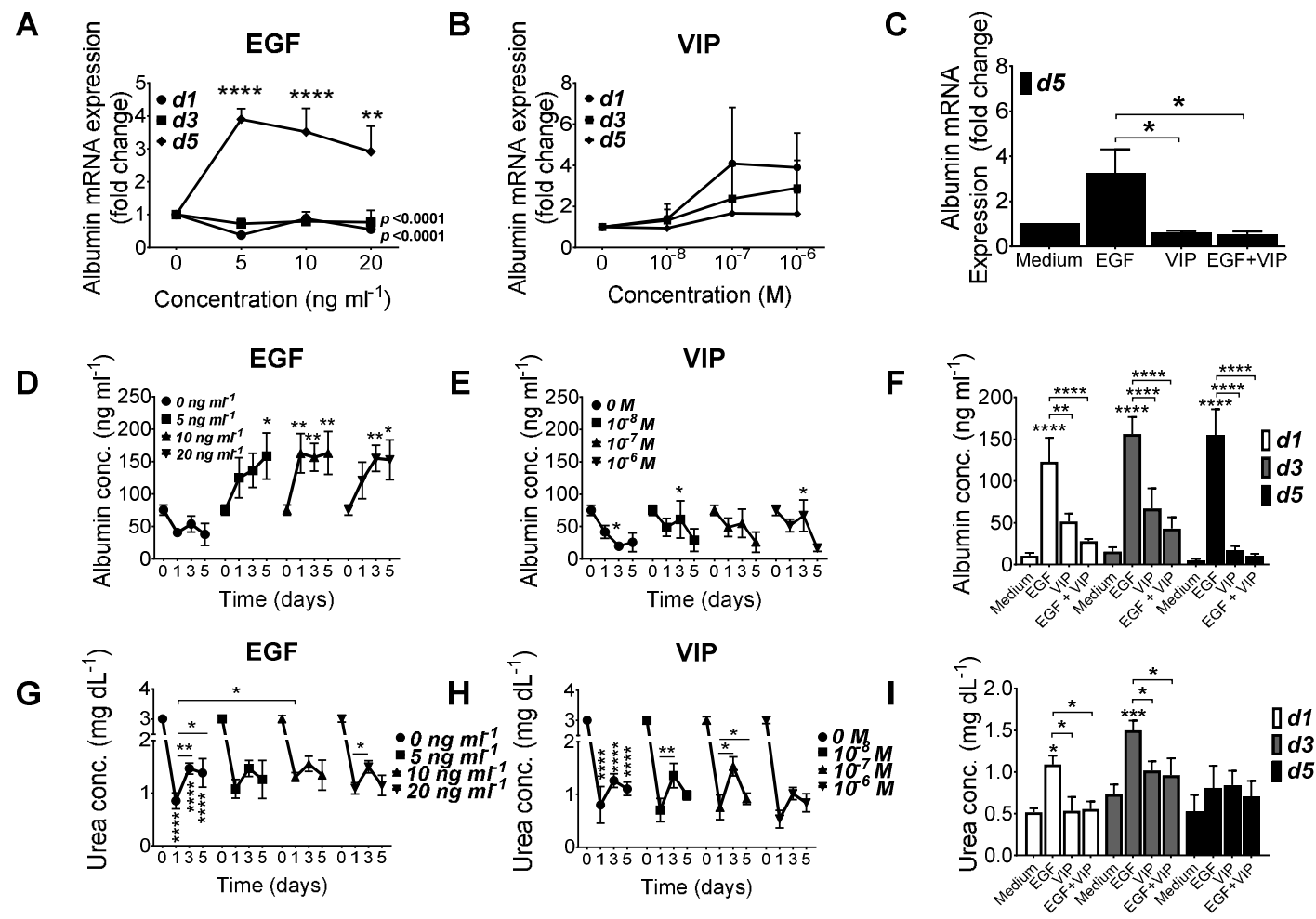
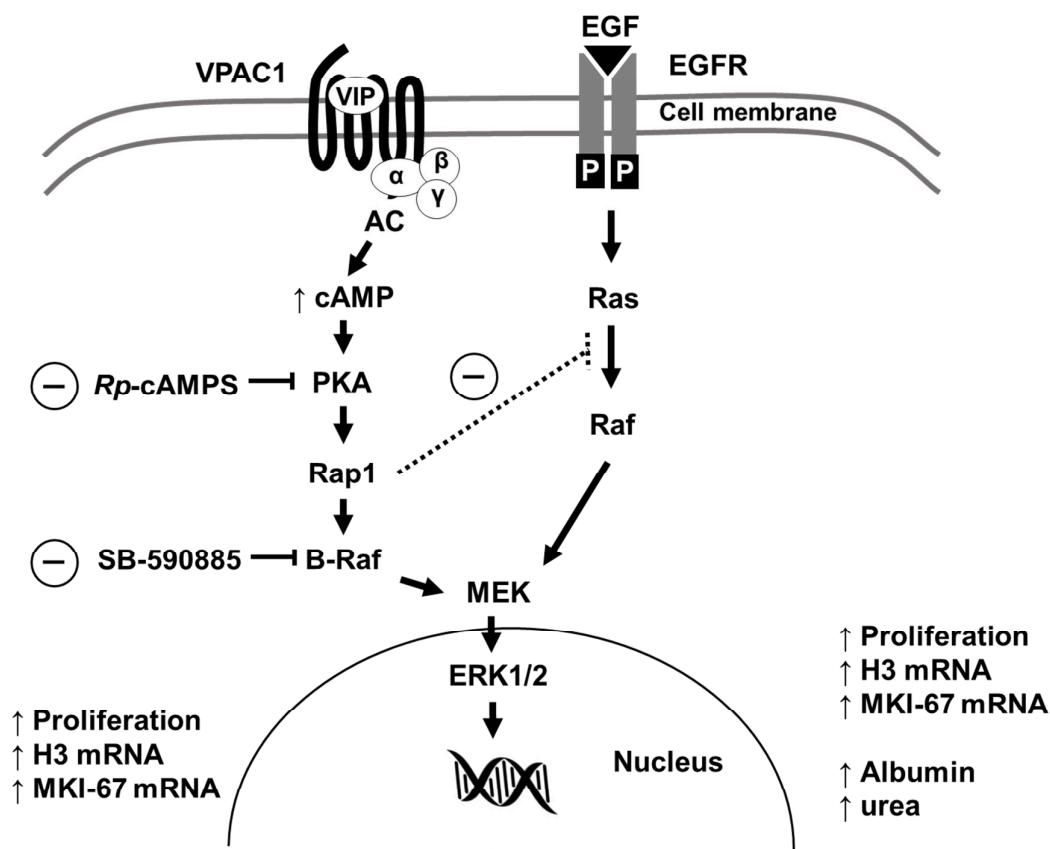


Figure 7



Vasoactive intestinal peptide (VIP) induces proliferation of human hepatocytes

Mogibelrahman M.S. Khedr^{1,2}, Ahmed M. Abdelmotelb¹, Thomas A. Bedwell¹, Anan Shtaya⁴, Mohammad N. Alzoubi^{3,5}, Mohammed Abu Hilal^{1,5}, and Salim I. Khakoo^{1,5}.

¹Clinical and Experimental Sciences Academic Unit, Faculty of Medicine, University of Southampton, Southampton, United Kingdom. ²Faculty of Medicine, Suez Canal University, Ismailia, Egypt. ³St George's University of London, London, United Kingdom. ⁴University of Jordan, Amman, Jordan. ⁵Southampton University Hospitals NHS Trust, Southampton, United Kingdom.

Table of contents

Materials and Methods:	-----2
Isolation of human hepatocytes:	----- 2
5-Bromo-2'-deoxyuridine (BrdU) DNA incorporation assay:	----- 3
Measurement of lactic dehydrogenase (LDH):	----- 3
Viability assay:	----- 4
Total RNA extraction and Reverse Transcription Polymerase Chain Reaction (RT-PCR):	4
Polymerase chain reaction (PCR) and Real time PCR (rt-PCR):	----- 5
Detection of Phospho-p44/42 MAPK (Erk1/2) and VPAC1 in hepatocytes:	----- 6
Gel electrophoresis:	-----6
Western blotting:	-----6
cAMP Direct Immunoassay:	----- 7
Albumin ELISA:	----- 7
Urea concentration assay:	----- 8
References:	-----9

28 **Materials and Methods:**

29 **Isolation of human hepatocytes:**

30 Tissue samples (2-10gm) were obtained from fresh surgical macroscopically normal liver
31 tissue derived from resections from patients undergoing hepatectomies. Experimental
32 procedures were performed according to the Health Research Authority (HRA), Research
33 Ethics Committee (REC) North East - Newcastle & North Tyneside 2 (REC ref. 13/NE/0070)
34 with informed consent. A total of 46 human liver cell preparations from 39 different donors
35 have liver primary or secondary metastatic tumors (24 men and 15 women) were used.
36 Patients' ages ranged from 29 to 83 years. Hepatocytes were isolated using a two-step
37 perfusion procedure as described previously¹ with some modifications. Liver tissue was
38 washed for 10 minutes with a calcium chelating buffer [1x Hanks' balanced salt solution
39 (HBSS), 25 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) and 0.5mM
40 Ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetra acetic acid (EGTA)]. Specimens
41 were digested using 0.05% collagenase in Dulbecco's Modified Eagle Medium (DMEM) with
42 1 mM CaCl₂ for 10 to 15 minutes and collagenase activity was stopped by adding an equal
43 volume of cold medium containing 10% fetal bovine serum (FBS). Following mechanical
44 disruption, the cell suspension was filtered through a 70µm pore nylon mesh and then spun at
45 50g/5 minutes 3 times at 4°C before the cell pellets were collected. Cell number and viability
46 were determined by trypan blue exclusion using a Lecia DMIL inverted microscope (Leica
47 Microsystems, Wetzlar, Germany). Average cell yield was $1,29 \times 10^6 \pm \text{SEM } 193,540$ cell
48 per gm of liver tissue. Specimens with cell viability >85% by were chosen for subsequent
49 experiments. Cells were plated at density of $1.5 - 2.5 \times 10^5$ cell cm⁻¹ on mouse collagen type
50 IV gel layer 1 - 2.5µg cm⁻² (Corning Ltd., Flintshire, UK) in William's E medium
51 supplemented with 5% FBS (to facilitate cell adhesion) in Plating Supplement Pack (Thermo
52 Fisher, Inchinnan, UK) and incubated at 37°C in a humidified incubator with 5% CO₂. 6 – 12

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3 53 hours later, medium (containing non-attached hepatocytes) was aspirated and cells were
4
5 54 maintained in William's E medium supplemented with serum free Maintenance Supplement
6
7 55 Pack (Thermo Fisher). Medium has been changed every 3 days.
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12 57 **5-Bromo-2'-deoxyuridine (BrdU) DNA incorporation assay:**

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14 58 EGF (Sigma, Gillingham, UK) at 5, 10 and 20ng ml⁻¹ or VIP (Sigma) at 10⁻⁸, 10⁻⁷ or 10⁻⁶M
15
16 59 was added to the medium 24 hours following cell seeding. Assays were performed in
17
18 60 quadruplicates. Hepatocytes were incubated with BrdU (10µg ml⁻¹, Sigma) for 2 hours at
19
20 61 37°C. Cells were washed for 5 minutes with Phosphate buffered saline (PBS) 3 times and
21
22 62 fixed with ice-cold methanol for 30 minutes at 4°C. The nuclear membrane was
23
24 63 permeabilised by using 2M HCl for 30 min at 37°C and acid was neutralised using an equal
25
26 64 volume of 0.1M sodium borate for 2 minutes. Non-specific reactivity was blocked with 5%
27
28 65 Donkey Serum in PBS with 0.1% Triton-X (PBS-T) for 30 minutes at room temperature and
29
30 66 then washed as before with 1 x PBS-T. Rat anti-BrdU antibody (Bio-Rad, Hertfordshire, UK)
31
32 67 was used to detect DNA-integrated BrdU, 1µg ml⁻¹ in PBS-T for 1 hour at room temperature
33
34 68 and subsequently donkey anti-rat IgG-Alexa 488 (Thermo Fisher) 4µg ml⁻¹ in PBS-T. Cells
35
36 69 were incubated with 1µg ml⁻¹ 4'-6-diamidino-2-phenylindole, DAPI (Sigma) in water for 5
37
38 70 minutes in the dark and washed with PBS, then analysed by fluorescence microscopy. The
39
40 71 numbers of BrdU⁺ cells were determined in 6 different high power fields per well against
41
42 72 DAPI⁺ cells using an inverted Olympus IX81 fluorescent microscope with Olympus
43
44 73 xcellence software version 01.2 (Olympus Life Science Solutions, Tokyo, Japan).
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51 75 **Measurement of lactic dehydrogenase (LDH):**

52
53 76 Aliquots of substrate solution [100µl INT + P-Iodonitrotetrazolium Violet (33mg ml⁻¹ in
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55 77 DMSO) + 100µl PMS, Phenazine methosulfate (9 mg ml⁻¹) + 2.3 ml β-nicotinamide adenine
56
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3 78 dinucleotide (NAD) hydrate (3.74 mg ml⁻¹)] were freshly prepared. Equal volumes of 200mM
4
5 79 Tris (hydroxymethyl)aminomethane (Tris) pH 8, 50mM Lithium lactate, substrate solution
6
7 80 and samples or positive control (5µg ml⁻¹ L-Lactic Dehydrogenase from bovine heart) were
8
9 81 loaded into an assay plate. The V_{max} was measured at 490nm for 10min. LDH activity (U ml⁻¹
10
11 82 ¹) and LDH release in the supernatants (expressed as percentage of total cellular LDH
12
13 83 activity) were calculated.
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18 85 **Viability assay:**

19
20 86 Viability was determined using a colourimetric MTT assay (Sigma) according to the
21
22 87 manufacturers instruction. Cells (2x10⁵ cell/well) were seeded in duplicate into 96-well plates
23
24 88 and treated with various concentrations of EGF or VIP for 3 or 5 days. Viable cells were
25
26 89 detected by measuring the absorbance at 570nm in a SpectraMax® Plus 384 Microplate
27
28 90 Reader (Molecular Devices, Wokingham, UK). The water-soluble tetrazolium salts (WST-1)
29
30 91 Quick Cell Proliferation Assay kit II (Abcam, Cambridge, UK) were used according to
31
32 92 manufacturer instructions. Absorbance was detected at 440nm.
33
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37 94 **Total RNA extraction and Reverse Transcription Polymerase Chain Reaction (RT-
38
39 95 PCR):**

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41 96 RNA extraction was performed using a microspin column extraction kit (RNeasy® mini kit)
42
43 97 (Qiagen, Crawley, West Sussex, UK) following the manufacturer's instructions. RNA
44
45 98 quantity and purity was assessed using a NanoDrop® ND-1000 spectrophotometer (Thermo
46
47 99 Scientific). The ratio of 260nm and 280nm absorbance readings (A₂₆₀/A₂₈₀) of 1.8 to 2.0
48
49 100 was considered as an acceptable indicator of nucleic acid purity. Complementary DNA
50
51 101 (cDNA) was synthesised using a Primer Design Precision nanoScript 2 reverse transcriptase
52
53 102 kit (Millbrook, Southampton, UK) according to the manufacturer's instructions in a
54
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3 103 MasterCycler® 480 thermocycler (Eppendorf, Hamburg, Germany). The cDNA was stored at
4
5 104 -20°C until use.

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7 105

8
9 106 **Polymerase chain reaction (PCR) and Real time PCR (rt-PCR):**

10 107 The rt-PCR Primers were designed using the ProbeFinder software v2.5

11 108 (Lifescience.roche.com)². Oligonucleotide primers were obtained from Eurofins

12 109 MWG/operon (Ebersberg, Germany) and their sequences as following: Human Albumin

13 110 (NM_000477.5) F: 5'-GTGAGGTTGCTCATCGGTTT -3' and R: 5'-

14 111 GAGCAAAGGCAATCAACACC -3'), Antigen Identified By Monoclonal Antibody Ki-67,

15 112 MKI-67 (NM_002417.4) F: 5'-TCAAGGAAGTTCAGGAGAAG -3' and R: 5'-

16 113 GTGCACTGAAGAACACATTTCC-3'), Histone Cluster 3, H3 (NM_003493.2) F: 5'-

17 114 GAGCTGCTAATCCGCAAGTT -3' and R: 5'-GCGCAGGTCGGTCTTAAA -3'),

18 115 Vasoactive Intestinal Peptide Receptor 1, **VIP and pituitary adenylate cyclase-activating**

19 116 **polypeptide receptor-1 (VPAC1)**³ (NM_004624) F: 5'-

20 117 CTTCTGGTCGCCACAGCTATCCTG -3' and R: 5'-

21 118 ACTGCTGTCACCTCCTGATATC-3'), Epidermal Growth Factor Receptor, Epidermal

22 119 Growth Factor Receptor (EGFR) (NM_005228.3) F: 5'-TTCCTCCCAGTGCCTGAA -3'

23 120 and R: 5'-GGGTTTCAGAGGCTGATTGTG -3') and Glycerinadehyde-3-Phosphate-

24 121 Dehydrogenase, GAPDH (NM_002046) F: 5'-GATGACATCAAGAAGGTGGTG-3' and R:

25 122 5'-GCTGTAGCCAAATTCGTTGTC-3'). Level of **VPAC1** and EGFR mRNA expression

26 123 was assessed using GoTaq® Hot Start Polymerase (Promega UK Ltd, Southampton, UK)

27 124 according to manufacturer instructions. PCR conditions were 2 minutes at 95°C, and

28 125 followed by 35 PCR cycles of 60 seconds at 95°C, 60 seconds at 60°C and 60 seconds at

29 126 72°C. PCR products were visualized on 2% agarose gel, band density were measured and

30 127 normalised to that of GAPDH using a ChemiDoc™ imaging system (Bio-Rad). The total

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3 128 reaction volume was 10 μ l of 2x qPCR SYBR green Mastermix buffer (Primer Design),
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5 129 primer pairs and cDNA template were mixed and PCR products were detected in an A&B
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7 130 7900HT Fast Real-Time PCR System thermocycler (Applied Biosystems, CA, USA). PCR
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9 131 conditions were 10 min at 95°C, and followed by 40 PCR cycles of 15 seconds at 95°C and
10
11 132 60 seconds at 60°C. All assays were performed in triplicates. The melting curve was
12
13 133 generated with a stepped temperature transition from 60 to 95°C with a rise of 1°C/5 sec for
14
15 134 each step. The Ct values were normalized to the GAPDH housekeeping gene⁴ and calibrated
16
17 135 to untreated cells. The relative quantification (RQ), expressed as fold change, was calculated
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19 136 according to the $\Delta\Delta$ Ct method.
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24 138 **Detection of Phospho-p44/42 MAPK (Erk1/2) and VPAC1 in hepatocytes:**

26 139 *Gel electrophoresis:*

28
29 140 Hepatocytes were serum starved for 24 hour following 12 to 16 hours of attachment. Then
30
31 141 incubated with EGF (20 ng ml⁻¹) or VIP (10⁻⁶ M). To block B-RAF protein or cAMP cells
32
33 142 were incubated with SB-590885 or Rp-Adenosine 3', 5'-cyclic monophosphorothioate
34
35 143 triethylammonium salt (Rp-cAMPS) inhibitors. Cells were lysed using 1x TruPAGE™ LDS
36
37 144 Sample Buffer [with 2mM Sodium orthovanadate (Na₂VO₃), 20mM Sodium Pyrophosphate
38
39 145 (Na₄P₂O₇), 1mM ethylenediaminetetraacetate (EDTA), 1mM EGTA and 0.5 μ g ml⁻¹
40
41 146 Leupeptin] and run a TruPAGE® 10% precast gels (Sigma) under reducing conditions. Total
42
43 147 protein concentrations were measured using bicinchoninic acid (BCA) colorimetric protein
44
45 148 assay kit (Sigma) in accordance with the manufacturer's instructions using a BSA standard.

48 149 *Western blotting:*

50
51 150 Proteins were transferred to nitrocellulose membranes by a wet transfer method. Membranes
52
53 151 were blocked for 1 hour in 5% non-fat blotting grade cow's milk (Bio-Rad) in 0.05 % Tris-
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55 152 buffered saline (TBS)–Tween® 20 solution. The membranes were then probed with 1:1000
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3 153 rabbit anti-human Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody (New England
4
5 154 Biolabs, Hertfordshire, UK) or 1:000 rabbit anti-human p44/42 MAPK (Erk1/2) antibody
6
7 155 (New England Biolabs) overnight at 4°C, followed by goat anti-rabbit-horseradish peroxidase
8
9 156 (HRP) (DakoCytomation, Cambridgeshire, UK) at a dilution of 1:2000 for 45 minutes.
10
11 157 Reactive bands were visualised using the Luminata Forte Western HRP substrate
12
13 158 chemiluminescent substrate (Millipore UK Ltd., Hertfordshire, UK) in a ChemiDoc™
14
15 159 imaging system (Bio-Rad). In another experiment, level of VPAC1 protein expression in
16
17 160 untreated or VIP (10⁻⁶ M) treated hepatocytes, was investigated using a rabbit polyclonal anti
18
19 161 VPAC1 (Abcam) and followed by goat anti-rabbit-horseradish peroxidase (HRP)
20
21 162 (DakoCytomation) as before.
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164 **cAMP Direct Immunoassay:**

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28 165 Levels of cAMP in hepatocytes at 24 h following cell seeding (day 0) and at day 3 or 5
29
30 166 following stimulation with 10⁻⁶ M VIP were detected using a cAMP direct immunoassay
31
32 167 (Abcam) according to the manufacturer's instructions. Absorbance reading were determined
33
34 168 at 450 nm and cAMP concentrations (μM) were calculated by plotting values against cAMP
35
36 169 standard (0-0.25 μM) following background subtraction. cAMP concentrations (μM) were
37
38 170 determined and corrected to total proteins concentrations in samples (μg).
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172 **Albumin ELISA:**

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45 173 Albumin concentrations in the supernatant of hepatocytes cultures was determined using the
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47 174 ELISA DuoSET® kit for human albumin (R&D Systems, Oxfordshire, UK) according to
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49 175 manufacturer's instructions. Absorbance values were detected at 450nm with subtraction of
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51 176 readings at 570nm to compensate for optical interference on a microplate Reader. The
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53 177 detection range was from 2.5 to 160ng ml⁻¹.
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3 178 **Urea concentration assay:**
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5 179 The QuantiChrom™ urea assay kit (QuantiChrom, BioAssay Systems, Hayward, CA) was
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7 180 used according to the manufacturer's instructions. Following 20 minutes incubation at room
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9 181 temperature, Absorbance at 430nm was measured and concentration of urea of the sample
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11 182 against 5mg dl⁻¹ standard was calculated in mg dl⁻¹.
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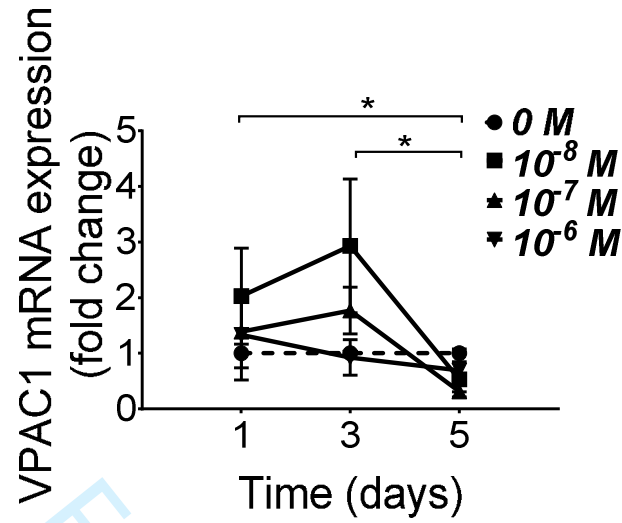
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¹Clinical and Experimental Sciences Academic Unit, Faculty of Medicine, University of Southampton, Southampton, United Kingdom. ²Faculty of Medicine, Suez Canal University, Ismailia, Egypt. ³Faculty of Medicine, Tanta University, Tanta, Egypt. ⁴St George's University of London, London, United Kingdom. ⁵University of Jordan, Amman, Jordan. ⁶Southampton University Hospitals NHS Trust, Southampton, United Kingdom.

Table of contents

Supplementary Figure 1 -----1



Supplementary Figure 1 The effect of VIP on VPAC1 mRNA gene expression in hepatocytes. $n = 3$ for each condition. P values shown in the graph are for overall comparison with hepatocytes at day 1 of treatment. * $P < 0.05$. Mean \pm SEM. Two-way ANOVA followed by Fisher's least significant difference (LSD).

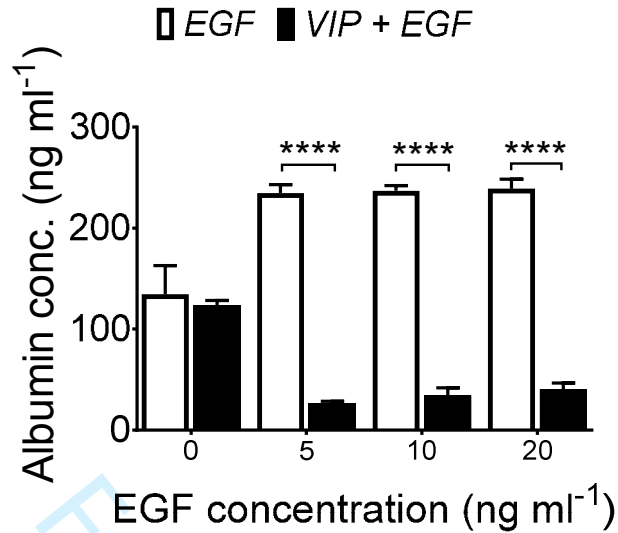
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¹Clinical and Experimental Sciences Academic Unit, Faculty of Medicine, University of Southampton, Southampton, United Kingdom. ²Faculty of Medicine, Suez Canal University, Ismailia, Egypt. ³Faculty of Medicine, Tanta University, Tanta, Egypt. ⁴St George's University of London, London, United Kingdom. ⁵University of Jordan, Amman, Jordan. ⁶Southampton University Hospitals NHS Trust, Southampton, United Kingdom.

Table of contents

Supplementary Figure 2-----1



Supplementary Figure 2 Albumin production from human hepatocytes cultured with EGF and VIP. Hepatocytes were cultured in William's E maintenance medium and EGF (5, 10 or 20 ng ml⁻¹) and VIP (10⁻⁶ M) was added one day later. n = 3 for each condition. *P* values shown in the graph are for comparison at individual concentrations. * *P*<0.05, ** *P*<0.005, *** *P*<0.0005, **** *P*<0.0001. Mean ± SEM. Two-way ANOVA followed by Fisher's least significant difference (LSD).