European Journal of Pharmacology 654 (2011) 173–180

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Pulmonary, Gastrointestinal and Urogenital Pharmacology

Endosomal signalling of epidermal growth factor receptors contributes to EGF-stimulated cell cycle progression in primary hepatocytes

Yi Luo [1], Zhong Cheng, C. Jane Dixon, John F. Hall, Emma Taylor [2], Michael R. Boarder *

The Cell Signalling Laboratory, Leicester School of Pharmacy, De Montfort University, Leicester, LE1 9BH, England, United Kingdom

ARTICLE INFO

Article history:
Received 15 June 2010
Received in revised form 9 November 2010
Accepted 15 November 2010
Available online 21 December 2010

Keywords:
Endosomal signalling
Cell proliferation
EGF receptors
Akt
ERK
Hepatocytes

ABSTRACT

Agonist-induced internalisation of receptors may lead to the formation of signalling endosomes. There is little evidence relating to whether this occurs to native receptors in non-transformed cells, and no previous studies asking whether this endosomal signalling can promote cell cycle progression in non-transformed cells. We investigated the hypothesis that in primary hepatocytes clathrin-dependent epidermal growth factor (EGF)-induced internalisation of the EGF receptor leads to signalling from endosomal EGF–EGF receptor complexes which may support EGF-stimulated cell cycle progression. We used EGF-stimulation of rat hepatocytes followed by confocal microscopy, and Western blots for phosphoproteins. [^{3}H]thymidine incorporation into DNA was used as a indicator of progression to S-phase. Confocal microscopy demonstrated co-internalisation of EGF, EGF receptors and transferrin into endosomes. Internalisation of EGF/EGF receptor/transferrin was blocked by expression of dominant-negative dynamin, but not by the tyrosine kinase inhibitor AG 1478. Dominant-negative dynamin expression reduced EGF-stimulated extracellular signal-related kinase and Akt signalling, but increased tyrosine phosphorylated EGF receptor. EGF-stimulated cell cycle progression requires stimulation of EGF receptors during an initial period (e.g. 1 h) and also later during a 24 h incubation. EGF receptor internalisation in the presence of AG 1478 followed by removal of the inhibitor resulted in signalling from internalised EGF receptors that is sufficient for the initial stimulation to provide progression to S-phase of the cell cycle. These observations on hepatocytes characterise, for the first time in non-transformed cells, endosomal signalling from internalised EGF receptors, and provide evidence that this endosomal signalling may support the early phase of EGF-stimulated cell cycle progression.

1. Introduction

Agonist-induced internalisation of cell surface receptors has been associated with loss of response to agonist and receptor degradation/recycling. However, there is evidence that receptors which have internalised to endosomes may continue to signal; some elements of signalling may be switched on, or amplified, as a result of internalisation, creating endosomal-specific signal selection (Kermorgant and Parker, 2008; Murphy et al., 2009; Sadowski et al., 2009; Sorkin and von Zastrow, 2009; von Zastrow and Sorkin, 2007).

A number of studies e.g. (Madshus and Stang, 2009) have investigated intracellular trafficking of epidermal growth factor (EGF) receptors. In many cells EGF promotes cell cycle progression. However, little is known about whether endosomal receptors play a significant role in delivering signals to the nucleus which control the cell cycle. Support for this hypothesis relies on studies on MDCK and BT-20 cell lines (Pennock and Wang, 2003). It is known that EGF receptors may be internalised by both clathrin-dependent and clathrin-independent mechanisms; it has been suggested that the clathrin route preferentially supports cell cycle progression, while the clathrin-independent route leads to receptor degradation (Sigismund et al., 2008). However, it has recently been reported that cell surface EGF receptors in MBA-MD-468 breast cancer cells support cell proliferation, while endosomal localisation of activated receptors leads to apoptosis (Hyatt and Ceresa, 2008), indicating cell-type variation in the role of endosomal signalling. These previous studies have been on cancer-derived cell lines: it is not known whether endosomal signalling is involved in cell cycle progression in non-transformed cells.

One initial observation came from the liver, when it was shown that early endosomes contain EGF receptors in complex with downstream signalling partners SOS, Grb2 and SHC (Di Guglielmo et al., 1994). Understanding hepatocyte proliferation has significant clinical consequences, including restoration of liver mass following resection surgery and provision of hepatocytes for cell therapy

* Corresponding author. The Cell Signalling Laboratory, Leicester School of Pharmacy, De Montfort University, Leicester, LE2 9BH, England, United Kingdom. Tel.: +44 116 257 7110; fax: +44 116 257 7135.
E-mail address: mboarder@dmu.ac.uk (M.R. Boarder).
[1] Current address: Department of Biochemistry and Molecular Biology, Jefferson Medical College and the Kimmel Cancer Center, Thomas Jefferson University, 233 S. 10th St., BLSB/915, Philadelphia, PA 19107, United States.
[2] Current address: Systems Toxicology Group; MRC Toxicology Unit, Leicester, LE1 9HN, England, United Kingdom.

0014-2999/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2010.11.038

procedures. Accordingly, previous studies have looked at the role and mechanisms of action of EGF in hepatocyte proliferation, both *in vivo* and in culture e.g. (Fausto and Riehle, 2005; Loyer et al., 1996). In hepatocytes it has been shown that EGF and hepatocyte growth factor activate the Raf/extracellular signal-related kinase (ERK) and phosphatidylinositol 3-kinase (PI3K)/Akt pathways (Coutant et al., 2002; Dixon et al., 2005; Luo et al., 2007; Ribaux et al., 2002; Roberts et al., 2000; Scheving et al., 2008; Schulze-Bergkamen et al., 2004), leading to growth factor-stimulated entry into the cell cycle and inhibition of apoptosis. However, despite the early role played by liver studies in developing the idea of endosomal signalling, and recent confirmation of the presence of activated EGF receptors in early endosomes prepared from EGF-treated rats (Balbis et al., 2007), little is known of the role of signalling from internalised EGF receptors in hepatocyte proliferation. In the present study we have asked whether signalling from endosomal EGF receptors can support cell cycle progression in primary cultures of hepatocytes. We provide the first evidence in non-transformed cells that EGF receptors internalised by the clathrin-dependent pathway can support signalling that leads to cell cycle progression.

2. Materials and methods

2.1. Cell preparation and culture

Hepatocytes were isolated from male Wistar-strain rats (200–300 g) as described previously (Dixon et al., 2003) and seeded at a density of 1×10^5 cells/well in collagen-coated 24-well plates, and 6×10^5 cells/well in collagen-coated 6-well plates. Cells were cultured in William's medium E (WME) supplemented with 10% foetal calf serum and insulin (as the Sigma ITS cell culture supplement: 10 μg/ml insulin; 5.5 μg/ml transferrin; and 6.7 ng/ml sodium selenite). After 4 h medium was replaced with serum-free WME without ITS. Cells were used within 48 h.

2.2. [^{3}H]-thymidine incorporation into DNA

Cells were cultured under the conditions indicated, in 24-well plates for 24 h. They were then incubated for a further 24 h with 3 nM EGF present as indicated. 1 μl/ml methyl-[^{3}H]-thymidine (37 MBq/ml: Amersham, Bucks, UK) was added for the final 4 h. AG 1478 (4-(3-Chloroanillino)-6,7-dimethoxyquinazoline), an inhibitor of the EGF receptor tyrosine kinase activity, was used at 300 nM except when otherwise stated. To investigate recovery of responses following removal of AG-1478, cells were stimulated twice with EGF. The first stimulation with 3 nM EGF was started at 0 h for either 15 or 60 min with AG-1478 present where indicated. This was terminated by washout of both EGF and AG-1478 as described below, followed by incubation with WME and no EGF. The second 18 h stimulation with 3 nM EGF was started at 6 h, when the medium was replaced with fresh medium ± EGF. At 20 h [^{3}H]thymidine was added as above, and after 4 h DNA was extracted and counted. We found that a full [^{3}H] thymidine response to the second long incubation required the presence of EGF for the first short incubation (Fig. 5); the purpose of the double stimulation procedure was to ask whether, for the first stimulation, signalling from endosomes could elicit this subsequent increase in DNA synthesis.

2.3. Confocal imaging of Alexa 488-coupled EGF and immunofluorescence

Cells were cultured for 24 h on collagen-coated 16 mm diameter glass coverslips as described above, after which cells were made serum-free and cultured for a further 24 h. After 30 min incubation with, where indicated, Alexa 488-coupled EGF (488-EGF: final concentration 0.4 μg/ml), cells were rinsed with phosphate buffered saline and fixed in 4% formalin for 15 min at 4 °C before washing 3 times in phosphate buffered saline. For immunohistochemistry cells were immediately fixed in 4% formaldehyde, then permeabilised with 1% Triton X-100, blocked with 10% donkey serum and labelled with 1:50 anti-EGF receptor overnight, followed by incubation with 1:200 Cy5 tagged donkey anti-rabbit. To see internalisation by the clathrin-dependent pathway we used Alexa 546-coupled transferrin. Cells were visualised with a Leica TCS SM2 confocal imaging system, using a 63×/1.20 objective and an acoustic-optical beam splitter system in place of a conventional filter system for simultaneous data acquisition for each of the fluorophore pairs used. The 2 fluorophore pairs used in Fig. 1 are Alexa-488/Cy5 and Alexa-488/Alexa-546; separate image capture for each fluorophore confirmed that there was no significant spectral bleed across these pairs with the biological samples used here. The same protocol and confocal settings were used for all images collected within an experiment. For Fig. 1 all images in Panel A were collected at the same time, and processed identically. Similarly for the images in Panels B and C.

2.4. Western blots

Hepatocytes were cultured in 6-well plates under the conditions described above. Cells were pre-incubated with inhibitors (AG 1478, Calbiochem; nystatin and concanavalin A, Sigma) for 15 min before addition of UTP or EGF. Following stimulation, 100 μl/well lysis buffer was added (20 mM Tris–HCl; 250 mM NaCl; 3 mM EDTA; 3 mM EGTA; 0.5% Triton X100; 1 mM phenylmethylsulfonylfluoride; 2 mM sodium orthovanadate; 1 mM β-mercaptoethanol; 20 μg/ml aprotinin; 5 μg/ml leupeptin; pH 7.6). Samples were equalised for protein content, then separated on 10% polyacrylamide gels. Blots were probed with anti-phospho-ERK and anti-pan ERK (Promega, Southampton, UK), anti-phospho-Akt (Ser 473) and pan-Akt, anti-phospho-EGF receptor (tyrosine 1173) and anti-pan-EGF receptor (from Cell Signalling Laboratories, Herts, UK), and visualised using ECL$^+$ Plus (Amersham).

2.5. Incubations with dominant-negative dynamin expressing adenovirus

Hepatocytes were cultured for 4 h in WME supplemented with ITS and 10% foetal calf serum, which were then replaced with serum-free WME containing encoding kinase-dead Dn-dynamin adenovirus (kind gift of Dr. V. Shah, Mayo Clinic). Batches of adenovirus were diluted to give consistent protein expression, as determined by western blots for dynamin II. In parallel cells were incubated with green fluorescent protein-encoding adenovirus to monitor adenoviral infection rates and as a control. Incubation with virus was for 16 h followed by further incubation for 32 h without adenovirus to allow protein expression. Cells were then stimulated with 3 nM EGF prior to confocal microscopy or western blot.

2.6. Washout of AG 1478 and EGF

To remove AG 1478 and extracellular EGF, cells were placed on ice, washed with cold serum-free medium, incubated on ice with cold 0.5 M NaCl/0.2 M acetic acid for 5 min, washed twice with cold serum-free medium, removed from ice and washed once with serum-free medium at 37 °C. Cells were then incubated at 37 °C incubator as indicated. To confirm that cell surface-bound EGF was removed by the acid wash we showed that when cells were incubated with EGF in the cold an acid wash prevented a subsequent EGF receptor tyrosine phosphorylation response when the temperature was raised to 37° for 10 min (data not shown).

2.7. Statistical analysis

Statistical analysis using Graph-Pad Prism was by ANOVA followed by Dunnett's post test to compare selected pairs of data and Bonferonni's post test to compare data sets with control values.

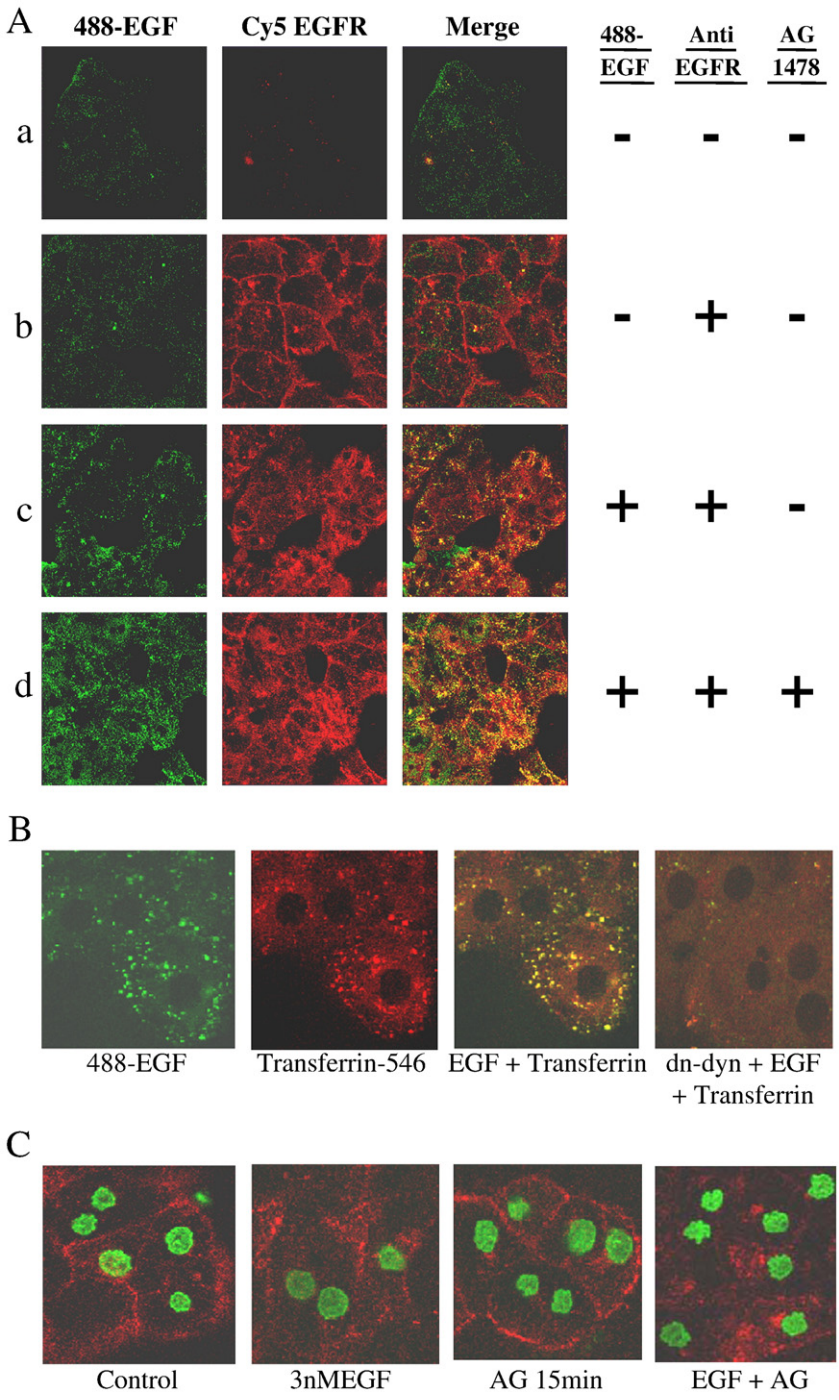

Fig. 1. Confocal images of hepatocytes. A. Cells were incubated as indicated with Alexa 488-coupled EGF (488-EGF; 0.4 μg/ml for 30 min) in the presence or absence of AG 1478 (300 nM; 15 min pre-incubation). Following fixing cells were exposed to anti-EGF receptor (EGFR) primary antibody with a cy5-tagged second antibody. The left column was the 488 channel, the middle was Cy5, and the right column was these 2 merged. Lane a, control (no 488-EGF, no primary antibody); lane b, the anti-EGF receptor in the absence of agonist; lane c, incubation with the 488-labelled agonist followed by anti-EGF receptor immunohistochemistry; lane d, pre-incubation with AG 1478 followed by 488-EGF and anti-EGF receptor. B. Left 3 panels, cells were incubated for 30 min with Alexa 488-EGF and Alexa 546-transferrin (20 μg/ml for 30 min) showing the 488 channel, the 546 channel and merge in sequence. The right hand panel shows the 488/546 merged result when the cells had been pre-exposed overnight with dominant-negative dynamin (dn-dyn). C. Cells were incubated as indicated with 3 nM EGF for 30 min and/or AG 1478, followed by immunohistochemistry with anti-EGF receptor and nuclei visualised with SYTOX (100 nM for 30 s).

3. Results

3.1. EGF–EGF receptor internalisation is clathrin-dependent but independent of receptor tyrosine phosphorylation

To determine whether internalisation of EGF–EGF receptor complexes can be visualised in cultured hepatocytes we incubated cells on coverslips with Alexa-488-coupled EGF (488-EGF) for 30 min followed by a primary anti-EGF receptor antibody and visualised with a Cy5-labelled secondary antibody. Typical images in Fig. 1A show that 30 min incubation with 488-EGF led to the accumulation of punctate fluorescence (left hand column, lane c; also Fig. 1B). When EGF receptor immunostaining was in the absence of EGF, the immunofluorescence illuminated the cell periphery (middle column, b). When the EGF receptor was visualised in cells pre-incubated with 488-EGF the receptors redistributed into the cells (middle column, c)

exhibiting co-localisation with the punctate 488-EGF staining (right hand column, c). To investigate whether receptor tyrosine phosphorylation was required for this internalisation, the procedure was carried out in parallel in the presence of AG-1478, shown in Fig. 2 to inhibit EGF receptor tyrosine phosphorylation. The drug alone had no effect on the confocal images (not shown). There was no inhibition by AG-1478 of the EGF-stimulated redistribution of EGF receptor immunofluorescence from the periphery into the cell (Fig. 1A, middle column, d); if anything there was an increase in accumulation of 488-EGF punctate fluorescence (left column, d), again mainly co-localised with EGF receptor immunofluorescence (right column, d). These results show co-internalisation of EGF and EGF receptor during the first 30 min of stimulation in a manner not requiring EGF receptor tyrosine phosphorylation.

Pre-incubation of cells with the non-selective endocytosis inhibitor concanavalin A (250 μg/ml) prevented 488-EGF accumulation into endosomes (not shown). To establish whether hepatocyte EGF–EGF receptor internalisation is clathrin-dependent we co-incubated cells with 488-EGF and Alexa-546-coupled transferrin (transferrin-546); internalisation of transferrin is known to be clathrin-dependent. This was done both with and without pre-exposure of cells to an adenoviral dominant-negative dynamin construct. (Dynamin is required for clathrin-dependent endocytosis). Fig. 1B shows that internalised 488-EGF and transferrin co-localised into the same fluorescent bodies. When cells had previously been incubated with the dominant-negative dynamin adenovirus neither label was internalised. The results indicate that under the conditions of this experiment most of the internalisation of EGF–EGF receptor is clathrin-dependent.

These studies required using 60 nM 488-EGF to get clear fluorescent images of the labelled EGF. It is notable that even with this high concentration, labelling of cell surface receptors with 488-EGF under conditions in which internalisation did not take place (e.g. very short incubations or in the presence of concanavalin A or dominant-negative dynamin as in Fig. 1B) was too diffuse to clearly visualise. The concentration of receptors, as occurs with internalisation, was necessary before sufficiently bright fluorescence could be seen.

To ask whether lower concentrations of EGF also lead to receptor internalisation we stimulated cells with 3 nM EGF and immunostained the EGF receptor. Fig. 1C shows that in response to 3 nM EGF the EGF receptor leaves the cell surface to settle within the cell, and furthermore that this is not inhibited by AG 1478. This confirms that agonist-stimulated receptor internalisation does not require EGF receptor phosphorylation.

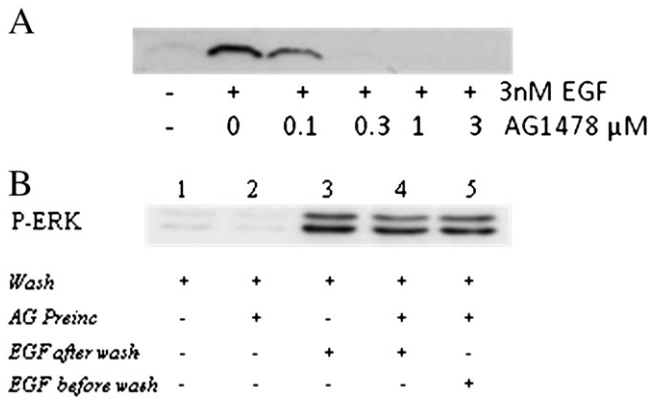

Fig. 2. Inhibition by AG 1478 and signalling following its removal. A. Pre-incubation (15 min) with the concentration of AG 1478 shown, followed by stimulation with 3 nM EGF for 20 min, and western blot for tyrosine phosphorylated EGF receptor. B. All cells were acid washed as indicated in Materials and methods; lane 1, control; lane 2, AG 1478 pre-incubation (300 nM, 15 min); lane 3, EGF (3 nM, 5 min) added after acid wash; lane 4, AG 1478 pre-incubation with EGF added after acid wash; lane 5, AG 1478 pre-incubation, EGF (25 min) added before acid wash.

3.2. Signalling occurs from endosomal EGF receptors

The results of confocal imaging of EGF–EGF receptor internalisation raise the possibility that endosomal signalling may play an important role in the responses to EGF. Fig. 2A confirms that EGF-stimulated EGF receptor phosphorylation was abolished by AG 1478. As predicted from this, we have also shown that signalling (e.g. by Akt and ERK pathways) was effectively inhibited by AG 1478 (not shown). Despite this, the confocal studies described above also showed that blocking EGF receptor tyrosine phosphorylation with AG 1478 did not attenuate EGF–EGF receptor internalisation. To see directly whether signalling occurs from internalised receptors we stimulated cells with EGF in the presence of AG 1478 and then removed by washing both the extracellular EGF (facilitated by the high salt/acid phase of washing — see Materials and methods) and AG 1478, in an adaptation of a previously published procedure (Wang et al., 2002). Fig. 2B lane 3 shows the response when EGF was added to cells after the wash procedure in the absence of any inhibitor. Lane 4 shows the response when cells were incubated with AG 1478 followed by washout and then stimulated with EGF. The response was similar to that in lane 3, showing that the washout of inhibitor was sufficiently effective to permit a substantial EGF response. When AG 1478 and then EGF were incubated together (conditions giving internalisation in the absence of signalling), and then both removed by washing, the subsequent signalling again gave a similar level of response (compare lane 5 with lane 4). This signalling can only have occurred following washout of AG 1478, when there is no extracellular EGF — we hypothesise that this means signalling must have been from endosomal EGF–EGF receptor complexes.

3.3. Akt and ERK signalling is affected by disruption of internalisation

Having shown endosomal accumulation of EGF–EGF receptor complexes internalise into endosomes (Fig. 1) from which they continue to signal (Fig. 2), we asked whether disruption of internalisation would affect EGF-stimulated Akt and ERK phosphorylation. If signalling is in part dependent on internalisation then it will be disrupted by concanavalin A, which non-specifically blocks endocytosis. We used UTP stimulation of $P2Y_2$ receptors as a control for non-specific effects of concanavalin A on signalling ($P2Y_2$ is a G protein-coupled receptor). Stimulation by EGF or UTP with or without pre-incubation with concanavalin A was followed by western blots for phospho-Akt and phospho-ERK (with non-phospho pan-Akt and pan-ERK as controls). Fig. 3A shows that concanavalin A blocked both Akt and ERK responses to EGF, while the responses to UTP were enhanced. Since EGF-stimulated internalisation can be by caveolin- or clathrin-dependent mechanisms we pursued 2 more selective procedures of interfering with internalisation: nystatin has an effect on caveolae-mediated endocytosis while dominant-negative dynamin delivery disrupts dynamin-dependent routes, which include both clathrin-dependent and clathrin-independent endocytic processes (Mayor and Pagano, 2007). Fig. 3B shows that nystatin had no effect on EGF-stimulated Akt and ERK phosphorylation while it enhances these responses to UTP. As shown in Fig. 4, pretreatment of cells with a dominant-negative dynamin adenoviral construct inhibited EGF-stimulated Akt and ERK phosphorylations, although the inhibition was only partial. This treatment had no effect on UTP-stimulated ERK and Akt phosphorylations (not shown). Interestingly, dominant-negative dynamin expression *increased* the extent of EGF-stimulated EGF receptor tyrosine phosphorylation (Fig. 4A) while *decreasing* Akt (Fig. 4B) and ERK (Fig. 4C) phosphorylation.

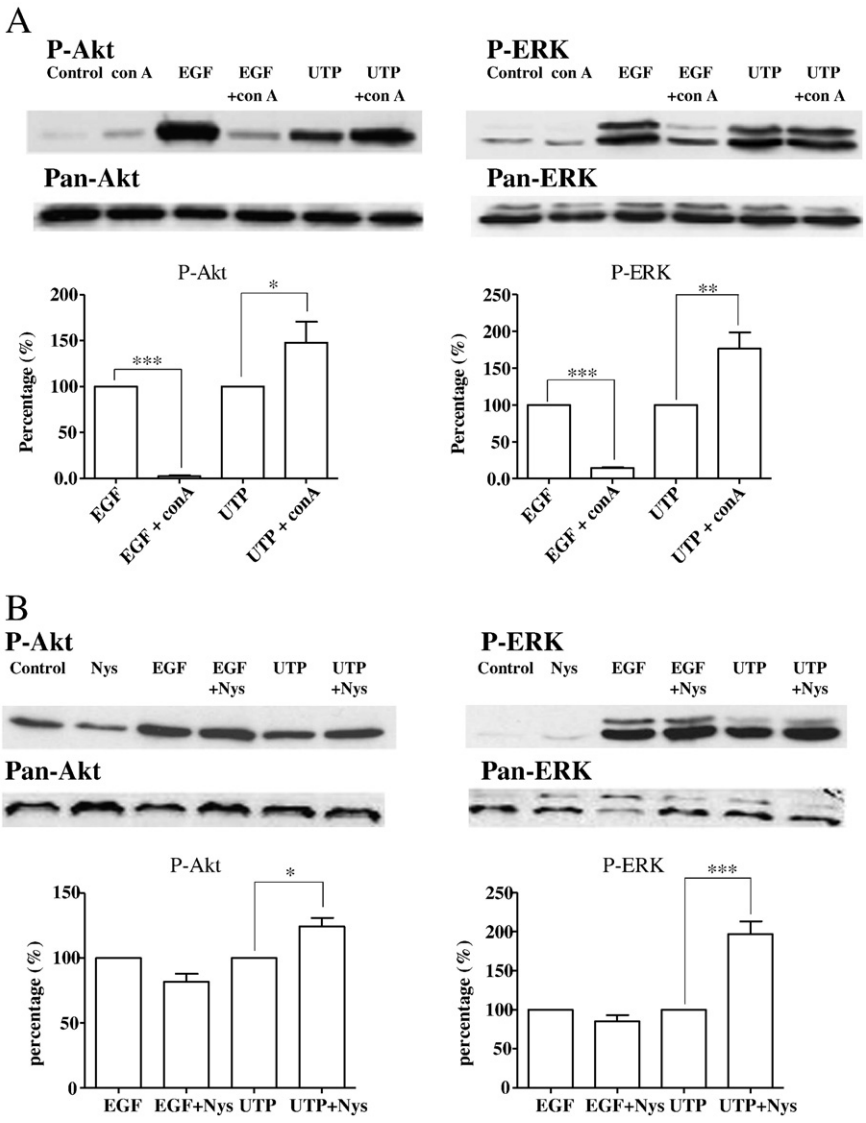

Fig. 3. Effect of concanavalin A and nystatin. Cells were pre-incubated with 250 μg/ml con A (Panel A) or 50 mg/ml nystatin (Panel B) for 15 min and then stimulated with 3 nM EGF or 100 μM UTP for 5 min (Akt) or 20 min (ERK) followed by western blots for phospho-ERK/Pan-ERK (20 min) and phospho-Akt/Pan-Akt (5 min). Western blots are representative of 3 independent experiments. Below the sample blots densitometric data from 3 blots, each normalised to the responses to EGF or UTP, are shown as mean ± S.E.M. ($n = 3$). These were analysed by one-way ANOVA and Bonferroni's post test (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

3.4. EGF-stimulated cell cycle progression requires early stimulation of signalling pathways and sustained presence of extracellular EGF

We used the incorporation of [^{3}H]thymidine into DNA as an index of S-phase. In preliminary experiments using this procedure we established that EGF-stimulated progression to S-phase was best seen in hepatocytes initially cultured for 4 h with foetal calf serum and insulin and then in the absence of foetal calf serum and insulin, with EGF added after 24 h in culture and [^{3}H]thymidine labelling 20–24 h after the onset of stimulation with EGF. We hypothesised that if progression to S-phase was supported by endosomal signalling from EGF–EGF receptor complexes within the cell, internalisation of these complexes will occur during the early part of this long incubation, leaving the later period independent of extracellular EGF. However, this does not seem to be the case: Fig. 5A shows that a 30 min or 1 h exposure to EGF followed by agonist-free incubation did not result in cell cycle progression 20–24 h later. Even after an 8 h initial pulse of EGF there was only a very partial response when [^{3}H]thymidine was added for the final 4 h, 20–24 h after beginning the stimulation.

We conducted studies on the time course of the response to addition of EGF at the level of the receptor and its signalling pathways. As shown in Fig. 5B (left panel) tyrosine phosphorylation of the receptor was very strong during the first 2 h after addition of EGF, and declined over the following hours. As expected, EGF-stimulated [^{3}H] thymidine incorporation was reduced by the EGF receptor tyrosine kinase inhibitor AG 1478 (Fig. 5C), confirming that cell cycle progression is dependent on this receptor phosphorylation. The time course of signalling was investigated by monitoring Akt and ERK phosphorylations. Both ERK and Akt phosphorylation were still apparent 2 h after EGF stimulation (Fig. 5B, middle and left panels), but by 4–8 h these signals had declined to control levels.

Since the EGF receptor tyrosine phosphorylation (and downstream signalling) had declined to a fraction of its maximal response by 4 h exposure to EGF, it seemed possible that the subsequent cell cycle response might be independent of EGF receptor tyrosine kinase activity after 4 h. To test this hypothesis we added AG 1478 4 h after stimulation with EGF, and then completed the 24 h incubation in the presence of both EGF and inhibitor. In Fig. 5C the effect of adding AG 1478 before EGF was compared to the effect of adding after 4 h exposure to EGF. In both cases the inhibitor had the same effect, indicating that a cell cycle progression response to EGF required EGF receptor tyrosine kinase activity after 4 h stimulation.

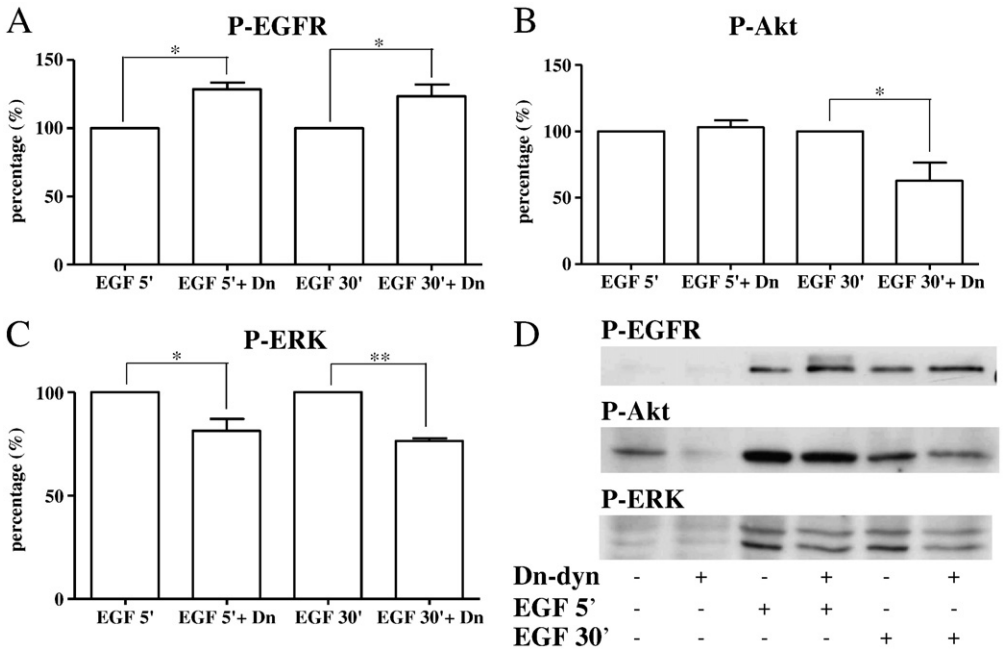

Fig. 4. Effect of dominant-negative dynamin. Hepatocytes were cultured for 4 h in WME supplemented with ITS and 10% foetal calf serum, which was then replaced with SF WME containing encoding kinase-dead Dn-dynamin adenovirus (Dn) or control (green fluorescent protein-encoding adenovirus) for 16 h and further incubated for 32 h without adenovirus before stimulation with 3 nM EGF for 5 min and 30 min as shown. Representative western blots for phospho-EGF receptor, phospho-ERK and phospho-Akt are shown; densitometric data was collected from 3 blots, normalised to the EGF responses and data presented as mean ± S.E.M. ($n = 3$), analysed by one-way ANOVA and Bonferroni's post test (*$P<0.05$, **$P<0.01$).

3.5. Signalling from endosomal EGF receptors may support progression to S-phase

The observation that EGF–EGF receptor complexes form in endosomes (Fig. 1) and evidence suggesting that signalling occurs from these endosomes (Fig. 2B) enable us to ask whether such endosomal signalling can support cell cycle progression. We used a 2-stimulation procedure, as described in Section 2.2. A short (15 min or 1 h) stimulation followed by a 6 h agonist-free incubation, with EGF then restored for a second 8 h stimulation period, gave a full [^{3}H]thymidine response (Fig. 6A and B). Using this 2-stimulation paradigm we were then able to ask whether a first short incubation with EGF in the presence of AG 1478 followed by washout, leading to endosomal signalling, is sufficient to support a subsequent cell cycle progression to S-phase. In Fig. 6A and B comparison of lanes 3 and 4 shows the effect of the first EGF stimulation (15 min in Fig. 6A, or 1 h in Fig. 6B) on the response. Comparing lanes 5 and 6 shows us the response under conditions when signalling at the first stimulation is restricted to that from internalised EGF–EGF receptor (i.e. in lane 6, EGF incubated with AG 1478 followed by washout of both). We see that signalling that is only from endosomes is sufficient to support the early events which are required for cell cycle progression in response to EGF.

4. Discussion

In hepatocytes, EGF and UTP have different effects on cell cycle progression. EGF acts on the EGF receptor ErbB1 to stimulate transit through G1 into S-phase in an ERK and Akt dependent manner (Coutant et al., 2002; Dixon et al., 2005; Luo et al., 2007; Ribaux et al., 2002; Roberts et al., 2000; Schulze-Bergkamen et al., 2004; Thoresen et al., 2003). UTP acts at $P2Y_2$ receptors generating a similar ERK and Akt response, yet does not move cells into S-phase (Luo Y. and Boarder M.R., unpublished). Explanations for receptor-specific responses may invoke combinatorial differences (specific combinations of signalling events), differences in signal strength and duration, and location within the cell (Kermorgant and Parker, 2005). It is on the latter that we have concentrated in this study. Receptor internalisation may result in delivery of the signal directly to an internal site, such as the perinuclear region, and it may lead to a continuation of signalling from endosomes within the cell or the selective activation of signalling pathways (Daaka et al., 1998; Grimes and Miettinen, 2003; Hoeller et al., 2005; Kermorgant and Parker, 2008; Miaczynska et al., 2004; Murphy et al., 2009; Pennock and Wang, 2003; Sadowski et al., 2009; Sorkin and von Zastrow, 2009; Tzafriri and Edelman, 2007; Vieira et al., 1996; von Zastrow and Sorkin, 2007). For the EGF receptor it is envisaged that EGF is retained at the extracellular domain of the EGF receptor within the lumen of the endosome (Tzafriri and Edelman, 2007). This may result in persistent EGF-stimulated signalling, outlasting the presence of extracellular agonist.

This picture of internalisation as creating an environment for signalling, rather than necessarily for signal termination, is largely based on the study of transformed cells; in many cases these receptors and pathways are known to be dysregulated as part of the neoplastic transformation process. Little is known of the role of internalisation and endosomal signalling in primary cells. Here we have provided a characterisation of the co-internalisation of EGF and EGF receptors in primary hepatocytes. The formation of EGF–EGF receptor endosomal complexes was apparent when the cells were stimulated with EGF for 30 min, reflecting an event which accumulated over 30 min until it became detectable in the confocal images. When incubation was continued beyond 30 min there was no detectable further increase in EGF–EGF receptor-associated fluorescence; beyond 60 min the intracellular EGF and EGF receptor fluorescence appear to separate before diminishing in intensity (Luo Y. and Boarder M.R., unpublished). This is of interest when considering the time course of events – we show here that in hepatocytes EGF receptor phosphorylation and signalling follow a similar time dependency as the co-localisation of internalised EGF–EGF receptor, in that they are diminished from 2 h onwards.

It is more difficult to understand the observations that EGF receptor phosphorylation was largely complete with 4 h exposure to EGF, and yet the cell cycle progression response required the continuing presence of extracellular EGF. Furthermore this required continuing EGF receptor tyrosine kinase activity, as shown by the inhibitory effect of adding AG 1478 after 4 h of a 24 h stimulation. It seems likely that a sustained (i.e. 4 h and beyond) low level of EGF receptor phosphorylation is required for progression to S-phase.

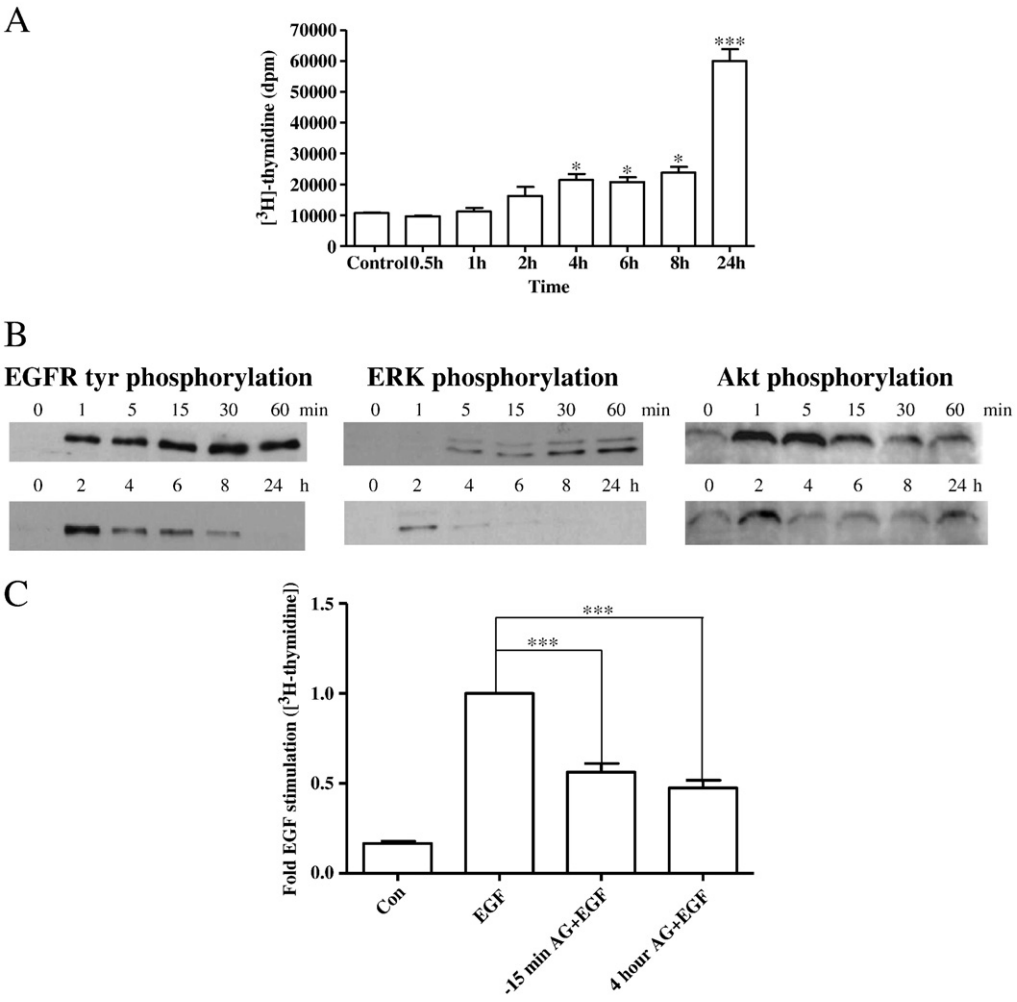

Fig. 5. Time course of responses to EGF. A. Effect of duration of EGF stimulation on [^{3}H]thymidine incorporation into DNA. After 24 h in culture 3 nM EGF was added, and then removed after the time indicated; incubation was then continued in the absence of EGF for a total of 24 h from the addition of EGF, with [^{3}H]thymidine present for the last 4 h of this 24 h incubation. Data (mean ± S.E.M., $n = 4$) were analysed by one-way ANOVA and Dunnett's post test ($^*P<0.05$, $^{***}P<0.001$ compared to unstimulated control). B. Time course of EGF-stimulated phosphorylation of EGF receptor, ERK and Akt. After 48 h in culture, cells were stimulated with 3 nM EGF for different times as indicated followed by western blots for phospho-EGF receptor, phospho-Akt and phospho-ERK. Western blots are representative of 3 independent experiments. C. Effect of addition of AG 1478 (300 nM) either 15 min before or 4 h after addition of EGF (3 nM) on [^{3}H]thymidine incorporation into DNA 20–24 h after the addition of EGF. Data (mean ± S.E.M., $n = 3$) normalised to the response to EGF without inhibitor and analysed by one-way ANOVA and Bonferonni's post test ($^{***}P<0.001$).

Disruption of internalisation with concanavalin A or dominant-negative dynamin attenuated the signalling response to EGF; this effect was selective in that it was not seen when cells were stimulated with UTP. The selective disruption by the dominant-negative dynamin procedure was also seen in that EGF stimulation of EGF receptor tyrosine phosphorylation was not disrupted, but was increased. This could be a consequence of trapping receptors at the cell surface. The effect of dominant-negative dynamin on ERK and Akt phosphorylations was less than that of concanavalin A, perhaps reflecting that not all cells were infected. Notably, both these interventions include in their actions downregulation of clathrin-dependent internalisation. Nystatin, which more selectively disrupts calveolae formation, had no effect on responses to EGF. Taken with the co-internalisation with transferrin this work suggests that signalling in response to EGF is modulated by clathrin-dependent internalisation of EGF–EGF receptor complexes.

Attenuation of EGF receptor internalisation by inhibition of receptor kinase activity with AG 1478 is seen in some cell types (Schmidt et al., 2003) and not in others (Wang et al., 2002). It is likely that this is as a result of different internalisation processes in different cells. Our confocal images show that in hepatocytes internalisation is not dependent on EGF receptor tyrosine phosphorylation since it occurs as much, if not more, when receptor phosphorylation is blocked by AG 1478. We have observed in some cases that the subsequent separation of EGF- and EGF receptor-associated fluorescence with incubations over 60 min is delayed or reduced when AG 1478 is present (Luo Y. and Boarder M.R., unpublished), suggesting that it may alter intracellular trafficking, resulting in the retention of EGF and EGF receptor in the same compartments, explaining the increased accumulation of punctate 488-EGF fluorescence in Fig. 2A line d. It is known that diverse receptor types have complex and highly controlled trafficking that can result in trapping into intracellular compartments (Finch et al., 2010).

The presence of internalisation in the absence of signalling enables us to adapt a previously described procedure (Wang et al., 2002) in which the internalised receptors are activated by washing out the inhibitor. We show here that this washout results in a stimulation of signalling in hepatocytes. This occurs when the only EGF receptors in contact with EGF are those in endosomes, as directly visualised in Fig. 2. In the absence of any other EGF–EGF receptor complexes we conclude that signalling must therefore be endosomal in origin. When this procedure is applied to an adapted protocol for EGF stimulation of progression to S-phase, we provide evidence that this endosomal signalling can support a full cell cycle progression response.

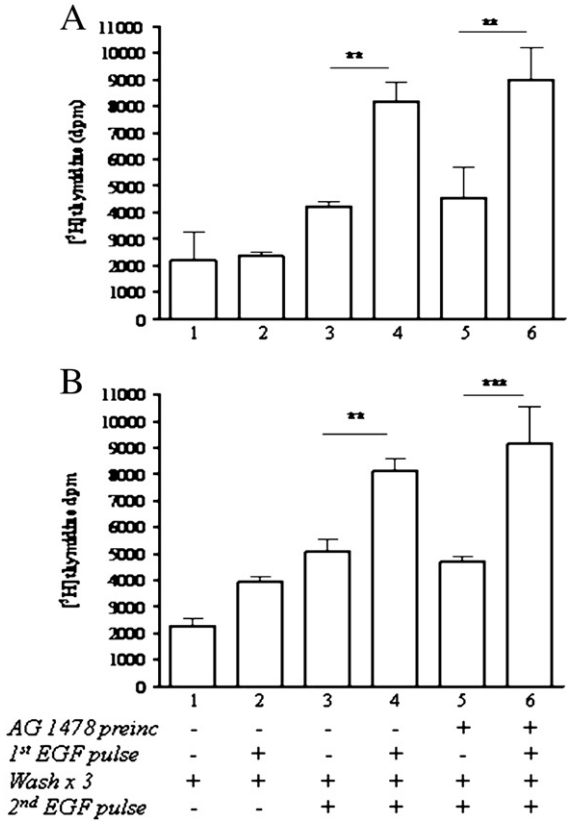

Fig. 6. [^{3}H]thymidine responses after removal of extracellular EGF. Cells were cultured serum-free for 24 h then stimulated as indicated with EGF (3 nM) twice: the 1st pulse (with 300 nM AG 1478 pre-incubation where indicated) started at 0 h and continued for 15 min (Panel A) or 1 h (Panel B), followed by washes (see Materials and methods), incubation for 6 h, followed by a 2nd pulse of EGF for 14 h with [^{3}H]thymidine labelling during the last 4 h. Lane 1, control (no AG 1478 or EGF); lane 2, EGF 1st pulse, no 2nd pulse; lane 3, no 1st pulse, EGF 2nd pulse,; lane 4, EGF both 1st and 2nd pulses; lane 5, AG 1478 pre-incubation, no 1st pulse, EGF 2nd pulse; lane 6, AG 1478 pre-incubation, EGF both 1st and 2nd pulse. Data mean ± S.E.M. $n = 4$, for both panels 1-way ANOVA $P < 0.0001$, Bonferonni's post test $^{**}P < 0.01$, $^{***}P < 0.001$.

In conclusion we have shown that the response to EGF in cultured rat hepatocytes which leads to cell cycle progression has an early phase in which occupation of the EGF receptor leads to clathrin-dependent internalisation and the formation of endosomal EGF–EGF receptor complexes. We provide evidence that signalling may occur from these endosomal receptors, and that this is sufficient to provide the early phase of stimulation required for cell cycle progression, demonstrating a role for endosomal signalling in the control of the cell cycle of primary cells. Hepatocytes are cells which *in vivo* normally sit quiescent for years, only moving through the cell cycle when liver regeneration is required. This reluctance to proliferate is also seen in culture. So it is particularly significant that endosomal signalling is necessary, and perhaps sufficient, to provide the first phase of intracellular events necessary for proliferation. However, we know from the results presented here in Fig. 5 that cell cycle progression also requires a later phase which is dependent on extracellular EGF but when there is no detectable receptor phosphorylation or PI3K/Akt or ERK signalling. The nature of events in this phase remains unknown.

References

Balbis, A., Parmar, A., Wang, Y., Baquiran, G., Posner, B.I., 2007. Compartmentalization of signaling-competent epidermal growth factor receptors in endosomes. Endocrinology 148, 2944–2954.

Coutant, A., Rescan, C., Gilot, D., Loyer, P., Guguen-Guillouzo, C., Baffet, G., 2002. PI3K-FRAP/mTOR pathway is critical for hepatocyte proliferation whereas MEK/ERK supports both proliferation and survival. Hepatology 36, 1079–1088.

Daaka, Y., Luttrell, L.M., Ahn, S., Della Rocca, G.J., Ferguson, S.S., Caron, M.G., Lefkowitz, R. J., 1998. Essential role for G protein-coupled receptor endocytosis in the activation of mitogen-activated protein kinase. J. Biol. Chem. 273, 685–688.

Di Guglielmo, G.M., Baass, P.C., Ou, W.J., Posner, B.I., Bergeron, J.J., 1994. Compartmentalization of SHC, GRB2 and mSOS, and hyperphosphorylation of Raf-1 by EGF but not insulin in liver parenchyma. EMBO J. 13, 4269–4277.

Dixon, C.J., Hall, J.F., Boarder, M.R., 2003. ADP stimulation of inositol phosphates in hepatocytes: role of conversion to ATP and stimulation of P2Y2 receptors. Br. J. Pharmacol. 138, 272–278.

Dixon, C.J., White, P.J., Hall, J.F., Kingston, S., Boarder, M.R., 2005. Regulation of human hepatocytes by P2Y receptors: control of glycogen phosphorylase, Ca^{2+}, and mitogen-activated protein kinases. J. Pharmacol. Exp. Ther. 313, 1305–1313.

Fausto, N., Riehle, K.J., 2005. Mechanisms of liver regeneration and their clinical implications. J. Hepatobiliary Pancreat. Surg. 12, 181–189.

Finch, A.R., S.K., Armstrong, S.P., Caunt, C.J., McArdle, C.A., 2010. Trafficking and signalling of gonadotrophin-releasing hormone receptors: an automated imaging approach.

Grimes, M.L., Miettinen, H.M., 2003. Receptor tyrosine kinase and G-protein coupled receptor signaling and sorting within endosomes. J. Neurochem. 84, 905–918.

Hoeller, D., Volarevic, S., Dikic, I., 2005. Compartmentalization of growth factor receptor signalling. Curr. Opin. Cell Biol. 17, 107–111.

Hyatt, D.C., Ceresa, B.P., 2008. Cellular localization of the activated EGFR determines its effect on cell growth in MDA-MB-468 cells. Exp. Cell Res. 314, 3415–3425.

Kermorgant, S., Parker, P.J., 2005. c-Met signalling: spatio-temporal decisions. Cell Cycle 4, 352–355.

Kermorgant, S., Parker, P.J., 2008. Receptor trafficking controls weak signal delivery: a strategy used by c-Met for STAT3 nuclear accumulation. J. Cell Biol. 182, 855–863.

Loyer, P., Cariou, S., Glaise, D., Bilodeau, M., Baffet, G., Guguen-Guillouzo, C., 1996. 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. 271, 11484–11492.

Luo, Y., Dixon, C.J., Hall, J.F., White, P.J., Boarder, M.R., 2007. A role for Akt in epidermal growth factor-stimulated cell cycle progression in cultured hepatocytes: generation of a hyperproliferative window after adenoviral expression of constitutively active Akt. J. Pharmacol. Exp. Ther. 321, 884–891.

Madshus, I.H., Stang, E., 2009. Internalization and intracellular sorting of the EGF receptor: a model for understanding the mechanisms of receptor trafficking. J. Cell Sci. 122, 3433–3439.

Mayor, S., Pagano, R.E., 2007. Pathways of clathrin-independent endocytosis. Nat. Rev. Mol. Cell Biol. 8, 603–612.

Miaczynska, M., Pelkmans, L., Zerial, M., 2004. Not just a sink: endosomes in control of signal transduction. Curr. Opin. Cell Biol. 16, 400–406.

Murphy, J.E., Padilla, B.E., Hasdemir, B., Cottrell, G.S., Bunnett, N.W., 2009. Endosomes: a legitimate platform for the signaling train. Proc. Natl Acad. Sci. USA 106, 17615–17622.

Pennock, S., Wang, Z., 2003. Stimulation of cell proliferation by endosomal epidermal growth factor receptor as revealed through two distinct phases of signaling. Mol. Cell. Biol. 23, 5803–5815.

Ribaux, P., Gjinovci, A., Sadowski, H.B., Iynedjian, P.B., 2002. Discrimination between signaling pathways in regulation of specific gene expression by insulin and growth hormone in hepatocytes. Endocrinology 143, 3766–3772.

Roberts, R.A., James, N.H., Cosulich, S.C., 2000. The role of protein kinase B and mitogen-activated protein kinase in epidermal growth factor and tumor necrosis factor alpha-mediated rat hepatocyte survival and apoptosis. Hepatology 31, 420–427.

Sadowski, L., Pilecka, I., Miaczynska, M., 2009. Signaling from endosomes: location makes a difference. Exp. Cell Res. 315, 1601–1609.

Scheving, L.A., Stevenson, M.C., Zhang, X., Russell, W.E., 2008. Cultured rat hepatocytes upregulate Akt and ERK in an ErbB-2-dependent manner. Am. J. Physiol. Gastrointest. Liver Physiol. 295, G322–G331.

Schmidt, M.H., Furnari, F.B., Cavenee, W.K., Bogler, O., 2003. Epidermal growth factor receptor signaling intensity determines intracellular protein interactions, ubiquitination, and internalization. Proc. Natl Acad. Sci. USA 100, 6505–6510.

Schulze-Bergkamen, H., Brenner, D., Krueger, A., Suess, D., Fas, S.C., Frey, C.R., Dax, A., Zink, D., Buchler, P., Muller, M., Krammer, P.H., 2004. Hepatocyte growth factor induces Mcl-1 in primary human hepatocytes and inhibits CD95-mediated apoptosis via Akt. Hepatology 39, 645–654.

Sigismund, S., Argenzio, E., Tosoni, D., Cavallaro, E., Polo, S., Di Fiore, P.P., 2008. Clathrin-mediated internalization is essential for sustained EGFR signaling but dispensable for degradation. Dev. Cell 15, 209–219.

Sorkin, A., von Zastrow, M., 2009. Endocytosis and signalling: intertwining molecular networks. Nat. Rev. Mol. Cell Biol. 10, 609–622.

Thoresen, G.H., Guren, T.K., Christoffersen, T., 2003. Role of ERK, p38 and PI3-kinase in EGF receptor mediated mitogenic signalling in cultured rat hepatocytes: requirement for sustained ERK activation. Cell. Physiol. Biochem. 13, 229–238.

Tzafriri, A.R., Edelman, E.R., 2007. Endosomal receptor kinetics determine the stability of intracellular growth factor signalling complexes. Biochem. J. 402, 537–549.

Vieira, A.V., Lamaze, C., Schmid, S.L., 1996. Control of EGF receptor signaling by clathrin-mediated endocytosis. Science 274, 2086–2089.

von Zastrow, M., Sorkin, A., 2007. Signaling on the endocytic pathway. Curr. Opin. Cell Biol. 19, 436–445.

Wang, Y., Pennock, S., Chen, X., Wang, Z., 2002. Endosomal signaling of epidermal growth factor receptor stimulates signal transduction pathways leading to cell survival. Mol. Cell. Biol. 22, 7279–7290.