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# Trophoblast apoptosis is inhibited by hepatocyte growth factor through the Akt and β-catenin mediated up-regulation of inducible nitric oxide synthase

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**Abstract**

Excessive apoptosis of trophoblast cells is thought to be a contributing factor in complications of pregnancy such as pre-eclampsia. Hepatocyte growth factor (HGF) inhibits apoptosis in trophoblasts and we have investigated the signalling pathways through which this anti-apoptotic effect is mediated. Treatment of cells with HGF led to rapid phosphorylation of Akt while an Akt inhibitor blocked the protective effect of HGF. Glycogen synthase kinase-3β (GSK-3β) was found to be one of the downstream targets of Akt. HGF treatment inactivated GSK-3β which in turn led to the activation of the transcription factor β-catenin. Pharmacological inhibition of GSK-3β, independently of HGF treatment, strongly increased both β-catenin activity and cell survival, suggesting that β-catenin alone has a pronounced anti-apoptotic effect. We also found that both HGF treatment and pharmacological activation of β-catenin leads to increased expression of inducible nitric oxide synthase (iNOS). We suggest that the Akt mediated activation of β-catenin leads to inhibition of trophoblast apoptosis following increased expression of iNOS.


*Keywords:* Trophoblast apoptosis; Hepatocyte; Akt; β-catenin; Nitric oxide synthase

## 1. Introduction

The multifunctional cytokine hepatocyte growth factor (HGF) is known to regulate a number of cellular processes including motility, cell division, apoptosis and morphogenesis in a wide range of cell types [1–5]. The effects of HGF are mediated through its interaction with the tyrosine kinase receptor c-Met which triggers autophosphorylation of the receptor and initiates a diverse range of signalling pathways including activation of phosphatidylinositol-3-kinase (PI3-kinase), phospholipase C, Src, STAT, NF-κB and MAP kinase pathways [6].

During early pregnancy trophoblast cells from the fetus differentiate, migrate and invade into the uterine stroma. The extravillous trophoblast sub-population invades the uterine wall and its blood vessels, remodeling the maternal spiral arteries. The extravillous trophoblasts displace smooth muscle and endothelial cells, in order to produce a blood vessel with a larger diameter, increased blood flow and reduced resistance. This is an essential step in establishing and maintaining a normal pregnancy and is necessary for the higher blood requirement of the fetus later in pregnancy.

HGF plays an important role in pregnancy, although its precise function is poorly defined [7–10]. Mouse knock-out models have demonstrated that the homozygous HGF knock-out phenotype is lethal to developing embryos [11]. Embryos from these mice are characterised by small placentae and a lack of trophoblast growth. Similarly embryos lacking c-Met die from placental insufficiency

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caused by abnormal placental morphogenesis [12]. Both HGF and c-Met have been detected in the placenta during human pregnancies [7,13], with HGF concentrations increasing during pregnancy but declining after birth [9,10].

Extravillous trophoblast apoptosis occurs during normal pregnancies [14,15] but is significantly elevated in pregnancies complicated with pre-eclampsia [16–20]. The mechanisms regulating extravillous trophoblast apoptosis are still poorly understood. An understanding of the mechanisms involved in regulating apoptosis in these cells is important for our understanding of the early events of pregnancy and may provide insights into the processes that occur in complications of pregnancy such as pre-eclampsia.

We have previously demonstrated that HGF has a number of important effects on extravillous trophoblasts such as promoting invasion [2], increasing motility [21] and inhibiting apoptosis [1]. HGF has been shown to inhibit apoptosis in a range of cell types such as endothelial cells [22], keratinocytes [23], hepatocytes [24] and epithelial cells [25]. In many cases the mechanism of action is thought to be PI3 kinase/Akt dependent [23,24,26,27], although the downstream consequences of this signalling are often unknown. However the signalling pathways triggered by HGF in extravillous trophoblasts are not well defined and we have therefore investigated the role of HGF in these cells in order to determine how apoptosis is regulated.

## 2. Materials and methods

### 2.1. Materials

Caspase substrates were purchased from Alexis Biochemicals (UK). TNF-α was obtained from Serotec (UK) and HGF was purchased from R&D Systems (UK). The antibodies against Akt, phospho-Akt, phospho-GSK-3β, and IκBα were obtained from Cell Signalling (New England Biolabs, UK). Mouse IgG1 was from Sigma (UK). The Akt inhibitor (1L-6-Hydroxymethyl-chiro-inositol 2-(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate) was obtained from Calbiochem (UK), while the GSK-3β inhibitor kenpaullone and the CDK inhibitor purvalanol were from Sigma. The luciferase assay kit was obtained from BD Biosciences (UK) and the iNOS antibody was from Santa Cruz Biotechnology.

### 2.2. Cell lines

SGHPL-4 cells are derived from primary first trimester extravillous trophoblasts transfected with the early region of SV40. Characterization of these cells has confirmed an extravillous trophoblast-like phenotype including expression of HLA-G, cytokeratin-7, CD9, hPL and hCG [2,28,29]. SGHPL-4 cells were cultured in Hams F10 media supplemented with glutamine (2 mM), penicillin (100 units/ml), streptomycin (0.1 mg/ml) and 10% (v/v) fetal calf serum.

### 2.3. Primary cell culture

Primary human extravillous trophoblast cells were isolated from first trimester placentae obtained at therapeutic termination of apparently healthy pregnancies. All ethical approval was in place and informed consent obtained. Chorionic villi were plated onto collagen gels in a serum-free mixture (50:50) of DMEM and Hams F12 media supplemented with glutamine (2 mM), penicillin (100 units/ml) and streptomycin (0.1 mg/ml). Extravillous trophoblasts differentiate and migrate out of the villi at high purity [30] over the next seven days, and apoptosis experiments were performed on day seven in Hams F10 media containing 10% FCS.

### 2.4. Time-lapse digital image microscopy

Apoptosis was observed over time using an Olympus IX70 inverted microscope equipped with a Hamamatsu C4742-95 digital camera. The microscope and stage were enclosed within a heated (37 °C) chamber (Solent Scientific, UK) and cells were cultured in 5% $CO_2$ in air. Images were captured every 15 minutes and analysed using Image Pro Plus software (Media Cybernetics, USA). In each treatment, 40 cells per field of view were randomly chosen at the beginning of the time-lapse sequence and were scored according to whether they became apoptotic over the time-course of the experiment. Apoptotic cells were also scored according to the time at which clear apoptotic morphology was first observed. Apoptotic morphology was considered as cytoplasmic and nuclear shrinkage and a change to a phase bright appearance, often with the formation of membrane blebs/blisters [1,31].

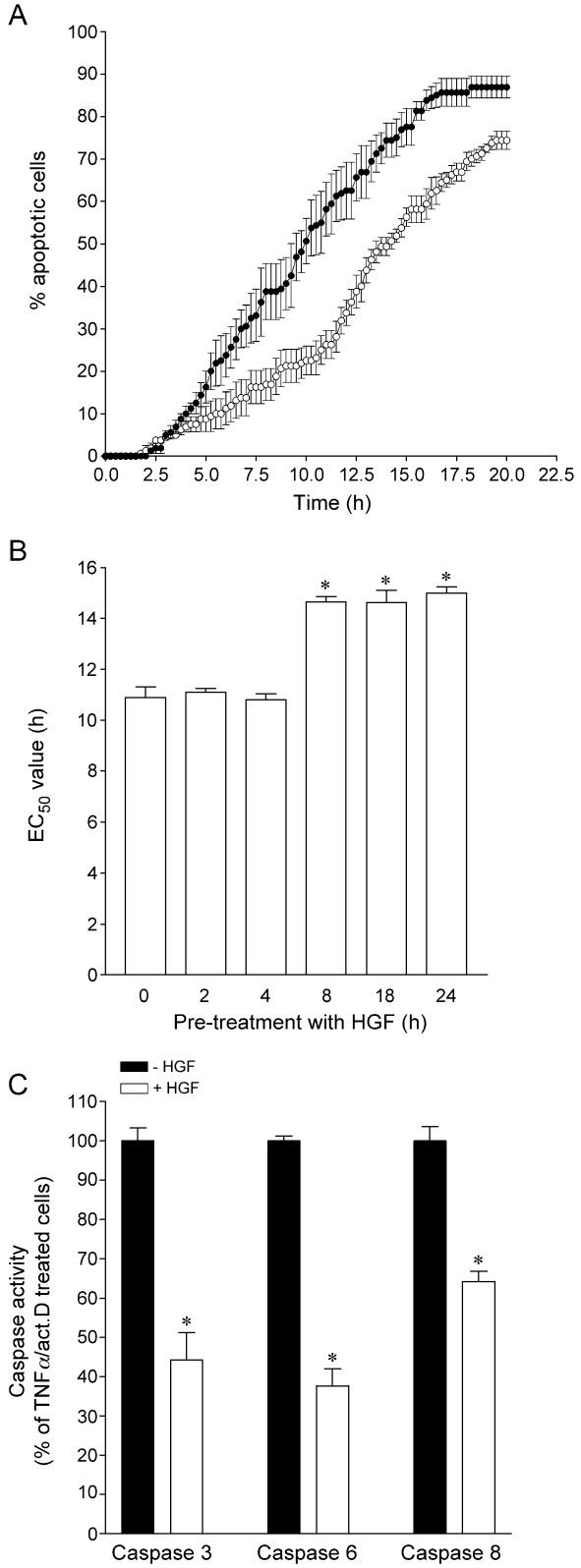
### 2.5. Caspase activity assays

Cells ($5\times10^6$) were pelleted, resuspended in lysis buffer (10 mM Hepes, 2 mM EDTA, 0.1% NP40, 5 mM DTT, 1 mM PMSF, 10 μg/ml pepstatin A, 20 μg/ml leupeptin and 10 μg/ml aprotinin) and incubated on ice for 15 min. The lysates were centrifuged at 10,000×*g* for 2 min, the supernatant removed and protein content quantified by the Bradford assay (BioRad, UK). To determine caspase activity, 178 μl of reaction buffer (100 mM Hepes, 20% (v/v) glycerol, 0.5 mM EDTA and 5 mM DTT) was mixed with up to 20 μl of cell lysate (containing 100–200 μg total protein) and 2 μl of a colorimetric caspase 3 substrate (Z-DEVD-pNA, 5 mM stock in DMSO). Volumes were adjusted to 200 μl where necessary and plates incubated at 37 °C. Absorbance was measured at 405 nm every 30 min to demonstrate enzyme activity. Caspase activity was determined after 2 h and represented as the percentage change in absorbance of treated cells compared to untreated cells.

### 2.6. Western blot analysis

Following SDS-PAGE, proteins were transferred onto Hybond P membrane (Amersham, UK). Unless otherwise

stated, membranes were blocked for 1 h in 5% (w/v) non-fat milk at room temperature. Primary antibody incubations were performed according to the manufacturer's instructions and are described in the appropriate figure legends. Detection was performed using ECL Plus (Amersham) according to the manufacturer's instructions. Western blots shown are representative of at least three separate experiments.




### 2.7. Transfections and luciferase assay

Transient transfections of SGHPL-4 cells were performed in 6-well plates in the presence of 5% FCS using poly-L-ornithine (15 000 Mw) from Sigma mixed with DNA at a ratio of 0.9:1 (w/w). Cells were incubated with the poly-L-ornithine/DNA mixture at a DNA concentration of 2.5 μg/ml at 37 °C for 5 h. DMSO (30% in RPMI media) was added for 1 min, washed twice with PBS and fresh media (Hams F10, 10% FCS) added. Analysis of the cells was carried out 24 h post-transfection. Luciferase assays were performed according to the manufacturer's instructions (BD Bioscience).

### 2.8. Antisense oligonucleotide transfection

Cells were transfected with antisense phosphorothioate derivatives of oligodeoxynucleotides according to the method of Noiri et al. [32]. Oligonucleotides were synthesized by MWG-Biotech AG (Milton Keynes, UK) and were either antisense to iNOS mRNA (5′ -acaggccatctctatggcttaca-3′ ) or a scrambled oligonucleotide sequence as a control.

### 2.9. Statistics

All experiments were performed in triplicate unless otherwise stated. The time taken to induce apoptosis in 50% of cells, the $EC_{50}$ value, was determined by non-linear regression using GraphPad Prism (GraphPad Software, USA). Unless otherwise stated triplicate $EC_{50}$ values were then analysed for their statistical significance using either a Mann–Whitney or a paired *t*-test where appropriate and error values expressed as standard errors of the mean. Statistical significance was assumed at $p<0.05$.

Fig. 1. (A) Survival curves of SGHPL-4 cells treated with TNFα (30 ng/ml) in combination with actinomycin D (800 ng/ml) as determined by time-lapse microscopy. Closed circles=absence of HGF; open circles=cells pre-treated with 10 ng/ml HGF 24 h prior to the addition of apoptotic stimulus. Values shown are means and SEM of triplicate experiments. (B) Effect of different HGF pre-treatment times on the inhibition of apoptosis. Survival times ($EC_{50}$) determined by time-lapse microscopy. Values shown are means and SEM of triplicate experiments. *Significantly different from control ($p<0.01$) as determined by repeated measures ANOVA with Tukey's post-analysis. (C) Caspase activity assays demonstrating the effect of HGF treatment (24 h) on the activity of caspase 3, caspase 6 and caspase 8 following an 18-h exposure to TNFα and actinomycin D. Values shown are means and SEM of triplicate experiments. *Significantly different from control ($p<0.01$).

## 3. Results

### 3.1. HGF inhibits extravillous trophoblast apoptosis and caspase 3, 6 and 8 activity

HGF (10 ng/ml) delayed the onset of apoptosis in SGHPL-4 trophoblast cells induced by a combination of TNFα (30 ng/ml) and actinomycin D (800 ng/ml) and also reduced the percentage of apoptotic cells observed over the course of the experiment (Fig. 1A) as determined by time-lapse microscopy. This anti-apoptotic effect was observed following a 24-h pre-treatment of the cells with HGF. A time-course of HGF pre-treatment (Fig. 1B) revealed that a minimum of 8 h pre-treatment was required to inhibit apoptosis, with shorter treatments proving ineffective. To confirm the results of the time-lapse analysis the effect of HGF on caspase activity was examined (Fig. 1C). HGF (24 h pre-treatment) was found to significantly reduce the activity of caspase 3 (55.7% inhibition±16.8, $p$=0.001), caspase 6 (62.3% inhibition±7.5, $p$=0.0002) and caspase 8 (35.8% inhibition±4.57, $p$=0.0013).

### 3.2. HGF inhibits apoptosis through an Akt dependent mechanism

HGF was found to stimulate the phosphorylation of Akt on Serine 473 (Fig. 2A and B). Phosphorylation was detected after 5 min treatment with HGF, with the degree of phosphorylation maintained for at least 60 min. An extended time-course (Fig. 2C and D) showed that phosphorylation had decreased to basal levels by 4 h. To determine whether Akt phosphorylation was necessary for the anti-apoptotic action of HGF, cells were treated with the Akt inhibitor 1L-6-Hydroxymethyl-chiro-inositol 2-(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate (10 μM) 15 min prior to the addition of HGF (Fig. 2E). Treatment of extravillous trophoblast cells with HGF increased the $EC_{50}$ value from 10.37±1.72 to 14.51±0.79 h ($p$=0.02). However in the presence of an Akt inhibitor the effect of HGF was significantly inhibited ($p$=0.03) producing an $EC_{50}$ value of 10.49±1.736 which was not significantly different from control cells ($p$=0.93) treated with TNF and actinomycin D alone.

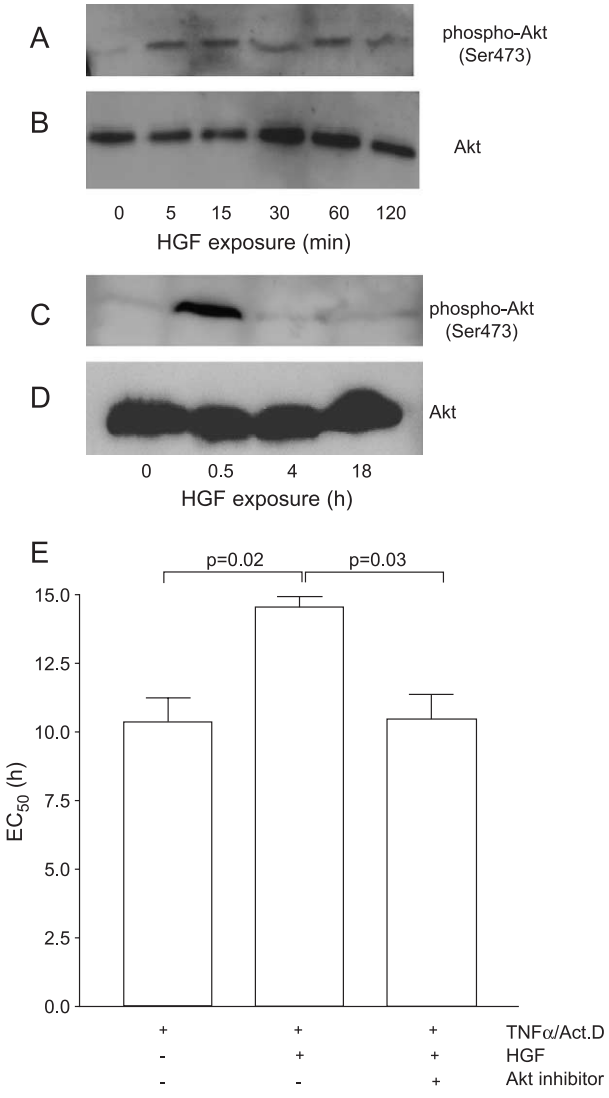



Fig. 2. (A–B) Western blot analysis of the effect of HGF (10 ng/ml) on the phosphorylation of Akt on Serine473 in SGHPL-4 cells (A), with total Akt as a loading control (B). Figures shown are representative of three separate experiments. (C–D) An extended time-course of HGF stimulated Akt phosphorylation (C), with total Akt (D) as a loading control. Figures shown are representative of three separate experiments. (E) Survival times ($EC_{50}$ values), as determined by time-lapse microscopy, of SGHPL-4 cells treated with TNFα (30 ng/ml)/actinomycin D (800 ng/ml). Cells were treated with a 16-h exposure to HGF (10 ng/ml) in the presence or absence of an Akt inhibitor (10 μM). Values shown are means and SEM of triplicate experiments.

### 3.3. Degradation of IκBα and activation of NF-κB are necessary for the anti-apoptotic effect of HGF

One of the known substrates of Akt is IκBα, an inhibitor of the transcription factor NF-κB. Phosphorylation of IκBα by Akt normally leads to its degradation, which releases NF-κB and allows its translocation to the nucleus. It was found that IκBα is degraded within 30 min following the addition of HGF (Fig. 3A and B), with expression increasing again over the following 90 minutes. The role of NF-κB in the HGF mediated inhibition of apoptosis was investigated by time-lapse microscopy in the presence of the NF-κB inhibitor TLCK (50 μM). It was found that TLCK was able to partially, but not completely inhibit the anti-apoptotic effect of HGF (Fig. 3C) reducing the $EC_{50}$ from 13.57±2.99 to 11.51±2.02 h ($p$=0.043).

### 3.4. Glycogen Synthase Kinase-3β (GSK-3β) and its substrate β-catenin are downstream targets of HGF-stimulated Akt activation

Other downstream targets of Akt were investigated by western blot analysis. It was found that glycogen synthase

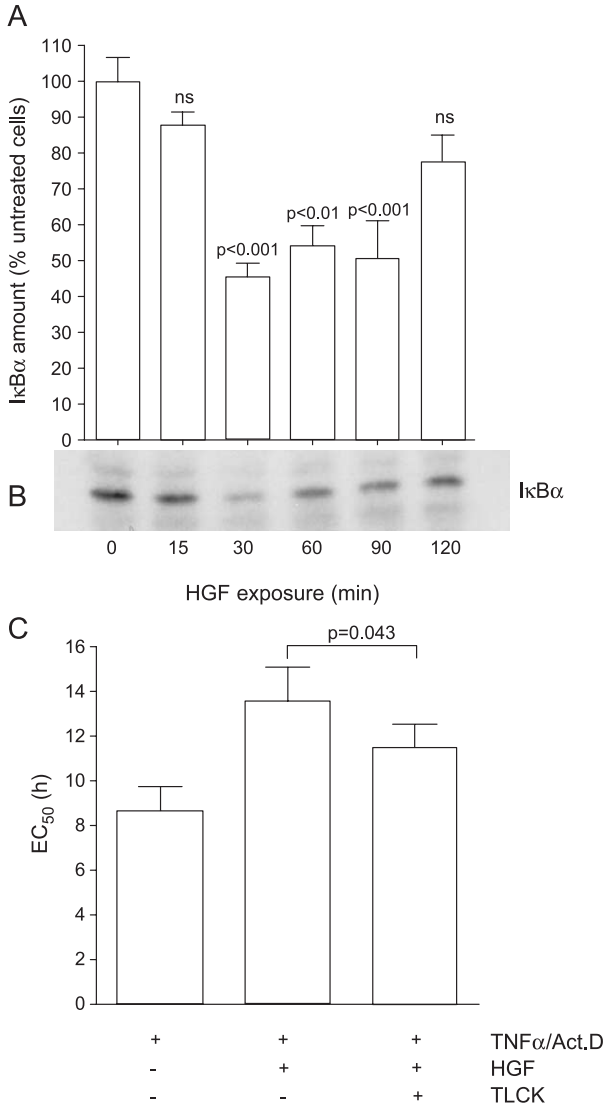



Fig. 3. (A) Degradation of IκBα by treatment with HGF as determined by western blot analysis and quantified by densitometry. Values are from three separate experiments and statistical analysis was by repeated measures ANOVA and Tukey's post-analysis. Timepoints for HGF treatment are those shown in panel B. (B) Western blot analysis of the effect of HGF on the degradation of IκBα. Figure shown is representative of three separate experiments. (C) Survival times ($EC_{50}$ values), as determined by time-lapse microscopy, of SGHPL-4 cells treated with TNFα (30 ng/ml)/actinomycin D (800 ng/ml). Cells were treated with a 16 h exposure to HGF (10 ng/ml) in the presence or absence of the NF-κB inhibitor TLCK (50 μM). Values shown are means and SEM of triplicate experiments.

kinase-3β (GSK-3β) is phosphorylated, and therefore inactivated, following HGF treatment (Fig. 4A). It is known that GSK-3β phosphorylates a number of downstream targets including the transcription factor β-catenin. Phosphorylation of β-catenin by GSK-3β leads to its inactivation and degradation; phosphorylation of GSK-3β therefore prevents the phosphorylation of β-catenin and leads to the activation of this transcription factor.

Activation of β-catenin was determined using the β-catenin reporter construct, TOPFLASH. HGF treatment (4 h) led to a significant (47.2±19.1%, $p$=0.002) increase in luciferase activity (Fig. 4B). The HGF stimulated activity of β-catenin was blocked by the addition of an Akt inhibitor. The increase in luciferase activity fell from 47.2% to 21.6±13.5% ($p$=0.041) with a 10-μM dose of the Akt inhibitor and to 6.9±3.9% ($p$=0.009) with a higher dose (30 μM) of the inhibitor (Fig. 4B). These results confirm

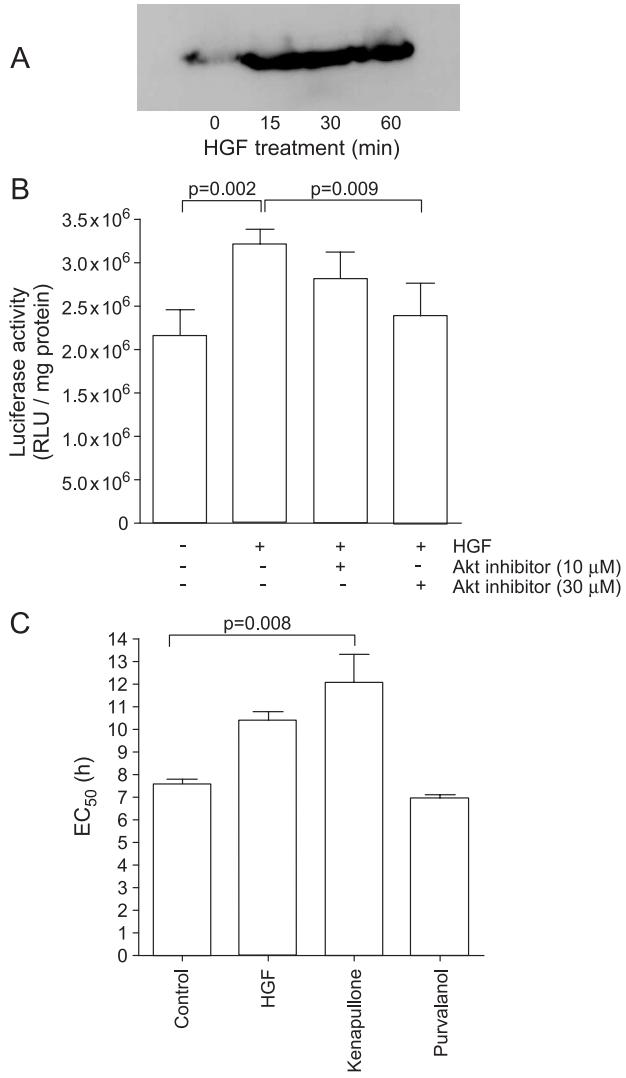



Fig. 4. (A) Western blot analysis of the phosphorylation of glycogen synthase kinase-3β following HGF treatment (10 ng/ml) in SGHPL-4 cells. Figure shown is representative of three separate experiments. (B) Luciferase assay following transient transfection with the β-catenin reporter plasmid TOPFLASH. Twenty-four hour post-transfection cells were treated with HGF (4 h) in the presence or absence of the Akt inhibitor (10 and 30 μM). Treatment of cells with a GSK-3β inhibitor (Kenpaullone 10 μM) produced much higher levels of luciferase activity with RLU/mg of $6.42\times10^6\pm1.4\times10^6$ (data not shown). Values shown are means and SEM of triplicate experiments. (C) Survival times ($EC_{50}$ values), as determined by time-lapse microscopy, of SGHPL-4 cells treated with TNFα (30 ng/ml)/actinomycin D (800 ng/ml). Cells were treated with a 16-h exposure to HGF (10 ng/ml) or to the GSK-3β inhibitor kenpaullone (10 μM) prior to the addition of the apoptotic stimulus. Purvalanol (10 μM) is a cdk-1 inhibitor and served as a control for the non-specific effects of kenpaullone.

that the activation of β-catenin is downstream of Akt activity.

A construct with a mutated TCF binding site (FOPFLASH) was used as a negative control. This construct showed very low basal levels of luciferase activity ($6.5\times10^4$ RLU/mg protein for FOPFLASH compared with around $2.5\times10^6$ for TOPFLASH, representing an approximately 40-fold increase in luciferase activity with TOPFLASH compared to FOPFLASH. Furthermore, no increase in FOPFLASH activity following HGF treatment was detected (data not shown).

Treatment of cells with the GSK-3β inhibitor kenpaullone (10 μM, 4 h) also increased β-catenin activity, in this case by 113.2±40.1% ($p$=0.043). This is greater than was achieved with HGF alone suggesting that, at this dose, HGF does not completely inactivate all of the cellular GSK-3β. Since the GSK-3β inhibitor was effective at stimulating β-catenin transcription factor activity, its effect on trophoblast apoptosis was investigated. Inhibition of GSK-3β, and subsequent activation of β-catenin was found to significantly inhibit apoptosis induced by TNFα and actinomycin D (Fig. 4C). The time taken to induce apoptosis in 50% of the cells rose from 6.9±1.8 to 14.4±3.8 h ($p$=0.0008) following a 16-h treatment with kenpaullone (10 μM). Since kenpaullone is also known to function as an inhibitor of cyclin dependent kinases (cdk) it was necessary to control for the effects of cdk inhibition using the cdk-1 inhibitor purvalanol (10 μM). There was no significant effect of this control inhibitor on either luciferase activity (17.97±2.15% increase in activity over control, $p$=0.162) or apoptosis ($EC_{50}$ value of 6.4 h±1.1, $p$=0.662).

### *3.5. HGF inhibits apoptosis in primary human extravillous trophoblasts in an Akt and GSK-3β mediated manner*

Primary extravillous cells derived from first trimester chorionic villous explants were treated with HGF (10 ng/ml), the GSK-3β inhibitor kenpaullone (10 μM) or the control inhibitor purvalanol (10 μM) prior to the induction of apoptosis with TNFα (32 ng/ml) and actinomycin D (200 ng/ml). The primary cells responded in a similar manner to the SGHPL-4 cell line (Fig. 5A), with HGF significantly increasing the survival time ($EC_{50}$ value increases from 4.3±0.66 to 5.87±0.98 h, $p$=0.0023, $n$=5). The GSK-3β inhibitor produced an even stronger anti-apoptotic effect with the $EC_{50}$ value increasing to 9.67±0.25 h ($p$=0.0005). No significant effect was observed with the control inhibitor purvalanol ($EC_{50}$ was 3.86±0.08 h, $p$=0.425). The involvement of Akt in the anti-apoptotic action of HGF was also investigated in primary cells (Fig. 5B). Treatment of cells with the Akt inhibitor (50 μM) significantly reduced the ability of HGF to inhibit apoptosis, with $EC_{50}$ value increasing from 3.8±0.7 h in untreated cells to 5.5±1.8 h in cells treated with HGF, and decreasing to 4.2±1.4 when cells were pre-treated with the Akt inhibitor ($p$=0.0114, $n$=4).

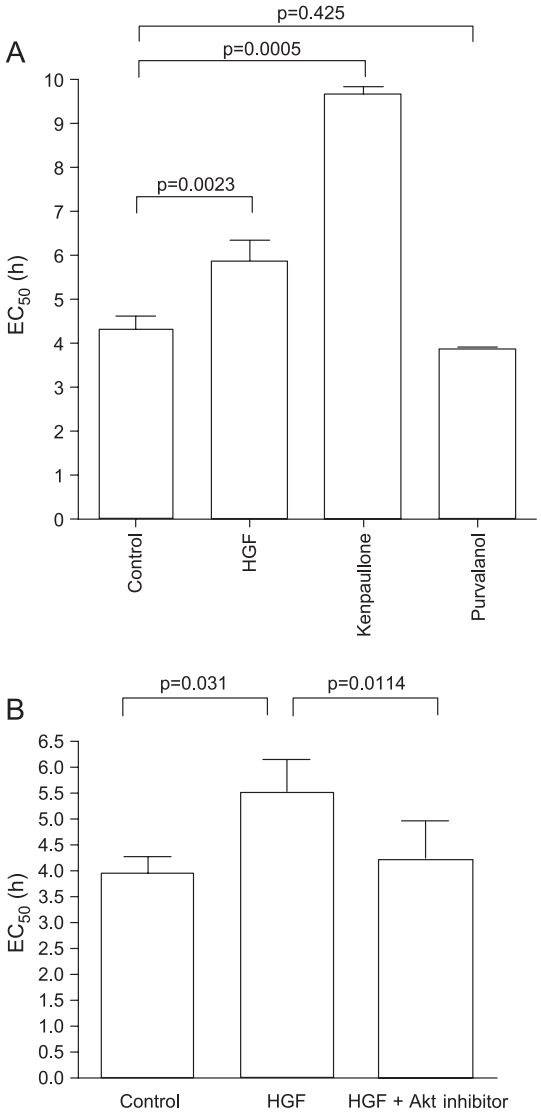



Fig. 5. Survival times ($EC_{50}$ values), as determined by time-lapse microscopy, of primary human extravillous trophoblast cells treated with TNFα (30 ng/ml)/actinomycin D (800 ng/ml). Placental explants were plated onto collagen and grown in serum free medium for 7 days. During this period extravillous trophoblast cells could be observed migrating out from the explant. (A) Primary cells were treated with a 16-h exposure to HGF (10 ng/ml) or to the GSK-3β inhibitor kenpaullone (10 μM). Purvalanol (10 μM) served as a control for the non-specific effects of kenpaullone. Values shown are means and SEM of five separate patient samples. (B) Primary cells were treated with HGF (10 ng/ml) for 16 h in the presence or absence of the Akt inhibitor (50 μM). Values shown are means and SEM of four separate patient samples.

### *3.6. Role of inducible nitric oxide synthase (iNOS) in the anti-apoptotic action of β-catenin and HGF*

We have previously shown that HGF inhibits apoptosis through the upregulation of iNOS and the production of nitric oxide [1]. The effect of HGF signalling pathways on the expression of iNOS in SGHPL-4 cells was determined by western blot analysis. It was found that pre-treatment of cells with the Akt inhibitor (50 μM) blocks the ability of

HGF to upregulate iNOS (Fig. 6A). It was also found that the GSK-3β inhibitor kenpaullone (10 μM), which leads to activation of β-catenin, was able to upregulate iNOS expression independently of the other signalling pathways activated by HGF (Fig. 6B). The western blots were quantified by densitometry and it was found that treatment with kenpaullone produced a 57% increase (±17.25, $n$=6, $p$=0.023) in iNOS expression. No effect was observed using the cdk inhibitor purvalanol (data not shown). Treatment with the GSK-3β inhibitor was able to inhibit apoptosis in SGHPL-4 cells, but this anti-apoptotic effect was blocked by transfection of the cells with antisense oligonucleotides against iNOS (Fig. 6C), reducing the $EC_{50}$ value from 17.7 h (±1.06) to 14.1 h (±0.62). No effect was observed with scrambled oligonucleotides (data not shown). This suggests that the anti-apoptotic action of GSK-3β inhibition, and therefore β-catenin activation is at least partly mediated through the upregulation of iNOS.

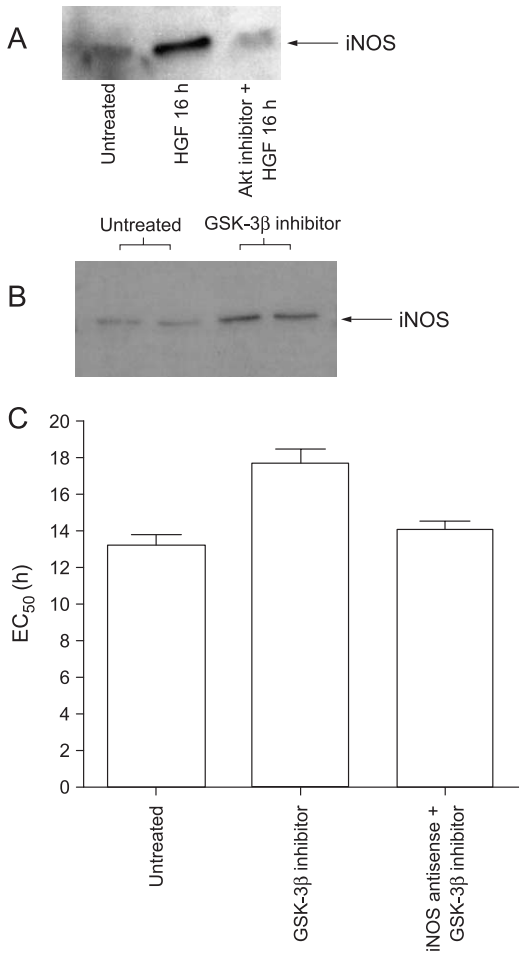



Fig. 6. Effect of HGF signalling pathways on the regulation of iNOS. (A) Western blot analysis of iNOS expression in SGHPL-4 cells following a 16-h treatment with HGF in the presence and absence of an Akt inhibitor (50 μM). Figure shown is representative of three separate experiments. (B) Western blot analysis of iNOS expression in SGHPL-4 cells treated for 16-h in the presence or absence of the GSK-3β inhibitor (Kenpaullone 10 μM). Figure shown is representative of five out of six separate experiments. In the sixth experiment basal iNOS expression was very high and no further increase was detected. (C) Effect of antisense oligonucleotides against iNOS on the anti-apoptotic effect of GSK-3β inhibitor (Kenpaullone 10 μM). Cells were treated with kenpaullone (10 μM) for 16-h prior to the induction of apoptosis with TNFα (30 ng/ml) in combination with actinomycin D (800 ng/ml). Antisense oligonucleotides were added 4 h prior to the addition of the GSK-3β inhibitor. Values shown are means and SEM of triplicate experiments.

## 4. Discussion

Hepatocyte Growth Factor (HGF) is known to inhibit apoptosis in a number of cell types and we have previously demonstrated an anti-apoptotic effect in trophoblasts [1,3,4]. The precise mechanism of HGF signalling in trophoblasts and its effects on apoptosis are still poorly understood. In this paper we have investigated HGF signalling in order to determine how it regulates apoptosis in these cells. HGF was found to lead to phosphorylation of Akt, leading to activation of the transcription factors β-catenin and NF-κB. The anti-apoptotic action of these transcription factors was found to be mediated through their role in regulating the expression of inducible nitric oxide synthase.

HGF signals through c-Met, a tyrosine kinase receptor which activates a wide range of signalling pathways including the PI-3-kinase/Akt, STAT, NF-κB and MAP kinase pathways [6]. In many cases the anti-apoptotic action of HGF has been attributed to the activation of the PI-3-kinase/Akt pathway, leading to the Akt mediated phosphorylation, and inactivation, of apoptotic proteins such as caspase 9 [33] or Bad [25,34]. However, in trophoblasts, inhibition of apoptosis by HGF occurs only following relatively prolonged treatments. At least an 8-h exposure to HGF is required to inhibit apoptosis, with shorter exposure times producing no effect. This would seem to indicate that simple anti-apoptotic signalling pathways, such as phosphorylation of Bad, are insufficient to explain its effects in extravillous trophoblasts and that longer term events such as transcriptional changes may also be required.

Analysis of Akt following HGF treatment revealed phosphorylation on Serine 473 after 5 min exposure which was persistent for at least 2 h. This indicates that Akt signalling is activated in trophoblasts following treatment with HGF. The use of an Akt inhibitor demonstrated that this activation is essential for the inhibition of apoptosis and therefore the downstream consequences of Akt activation were investigated.

There are a number of potential downstream targets for Akt, including many proteins involved in apoptosis [34–37]. We detected no phosphorylation of endothelial nitric oxide synthase (eNOS), Bad or the Forkhead transcription factor following HGF treatment (data not shown). Another known downstream target of Akt, and one which can lead to changes in gene expression, is IκBα. This is an inhibitor of the transcription factor NF-κB which is involved in the transcription of a large number of genes, including several

that might be involved in the regulation of apoptosis. In the absence of specific signals IκBα is usually bound to NF-κB, blocking its nuclear translocation motif and thereby ensuring the retention of NF-κB in the cytoplasm. Phosphorylation of IκBα by Akt leads to its ubiquitination and subsequent degradation, thus allowing NF-κB to translocate to the nucleus and initiate gene transcription. When this pathway was investigated in trophoblasts it was found that HGF stimulated the degradation of IκBα within 30 min, with the levels of IκBα gradually restored to basal over the next 90 min.

An inhibitor of NF-κB revealed that it plays a role in the HGF-mediated inhibition of apoptosis. The inhibitor used, TLCK, is a serine protease inhibitor that prevents the degradation of IκBα and was able to partially, but not completely, inhibit the effect of HGF. This suggests that NF-κB may help magnify the anti-apoptotic effect of HGF but may not be essential for its activity. Alternatively, since the concentration of the inhibitor used was limited by its toxicity, it is possible that NF-κB was not sufficiently inhibited to completely block its effects. The concentration used, 50 μM, is below the $IC_{50}$ (80 μM) for this inhibitor. In either case it is likely that the degradation of IκBα and the associated NF-κB activity has some role to play in the inhibition of apoptosis in extravillous trophoblasts and would also seem to play an important role in mediating the effects of HGF.

Other downstream targets of Akt were investigated and glycogen synthase kinase-3β (GSK-3β) was found to be phosphorylated following HGF treatment. GSK-3β is a multi-functional enzyme involved in glycogen synthesis and the regulation of protein synthesis and is known to be regulated by HGF in endothelial and epithelial cells [38,39]. It is a well-established component of the Wnt signalling pathway [40] and is also thought to play an important role in many other cellular processes such as proliferation, differentiation and motility.

Unlike most protein kinases GSK-3β activity is inhibited following phosphorylation. Several transcription factors are phosphorylated by GSK-3β, and in most cases their activity is inhibited by this phosphorylation [40]. The inactivation of GSK-3β following phosphoryation by Akt therefore leads to the activation of transcription factors that were previously inhibited by GSK-3β.

β-catenin is a multi-functional protein that plays an important role in cell adhesion and also functions as a component of the Wnt signalling pathway. In the absence of specific activating signals β catenin is located either at adherins junctions or bound in a high molecular weight multi-protein complex that includes GSK-3β. β-catenin is phosporylated by GSK-3β in this complex and thereby retained in the cytoplasm. Inhibition of GSK-3β kinase activity by its phosphorylation leads to dephosphorylation of β-catenin, which is then able to translocate to the nucleus and associate with transcription factors of the Lef/Tcf family to initiate gene expression [41].

The effect of HGF on β-catenin activity in trophoblasts was measured using a lucifease reporter construct containing the β-catenin/TCF binding site (TOPFLASH). Treatment of cells with HGF following transient transfection with TOPFLASH resulted in a significant increase in luciferase expression, indicating that HGF stimulates β catenin transcription factor activity. This stimulation was significantly reduced in the presence of an Akt inhibitor, suggesting that β-catenin activity is downstream of Akt activation. Pharmacological inhibition of GSK-3β with kenpaullone resulted in a much larger increase in luciferase activity in TOPFLASH transfected cells than treatment with HGF, suggesting that HGF treatment may not lead to maximal β-catenin activation and that increased β-catenin activity can be achieved with GSK-3β inhibitors. Treatment with kenpaullone was also found to strongly inhibit the induction of apoptosis by TNFα and actinomycin D. This suggests that activation of β-catenin activity alone is sufficient to protect trophoblasts from apoptosis and that activation of β-catenin may be an important survival pathway in extravillous trophoblasts. This is, to our knowledge, the first demonstration of the role of β-catenin as a transcription factor in trophoblasts, where previously its only described role was in cell adhesion. These findings in the extravillous trophoblast derived cell line were confirmed by the treatment of primary human extravillous trophoblasts derived from placental explants. HGF was also able to inhibit apoptosis in primary cells and did so in an Akt-dependent manner. Furthermore, pharmacological inhibition of GSK-3β was also able to strongly inhibit apoptosis on its own, confirming the importance of β-catenin activity.

Excessive apoptosis of extravillous trophoblasts has been implicated in the failure of trophoblasts to fully invade and modify the uterine environment, potentially leading to complications of pregnancy such as pre-eclampsia. In this study we demonstrate that HGF, an important growth factor in pregnancy, protects trophoblasts from apoptosis by the activation of β-catenin via the Akt-mediated inhibition of GSK-3β. A number of genes regulated by β-catenin have been described, but most are involved in development and cell cycle, and few anti-apoptotic genes have been identified.

We have previously demonstrated, and confirm in this study, that much of the anti-apoptotic effect of HGF in extravillous trophoblasts is dependent upon the transcription of the inducible isoform of nitric oxide synthase, iNOS [1]. Although several studies have examined the regulation of iNOS, much is still poorly understood, particularly in extravillous trophoblasts. It is clear that extravillous trophoblast cells express iNOS, even under basal conditions [1,2,42], and that HGF is able to stimulate further expression. The factors regulating iNOS expression in extravillous trophoblasts have not yet been identified, although it is possible that the pathways identified in this study may play an important role in this process. Our data

suggest that HGF stimulates the activation of the transcription factors β-catenin and NF-κB in an Akt-mediated manner. These transcription factors may then interact in the nucleus to initiate the transcription of iNOS, which in turn is able to inhibit apoptosis through the production of nitric oxide (NO). It has been well established that NF-κB is a transcription factor involved in the regulation of iNOS, but the role of β-catenin in the transcription of iNOS has not been well described. One study [43] identified putative Tcf-4/Lef-1 response elements within the iNOS promoter which would indicate a potential role for β-catenin in the activation of iNOS expression, but were unable to correlate changes in the β-catenin pathway in colorectal carcinoma cells with the increased changes in iNOS expression observed in these tumors. However, a separate study investigating colon adenocarcinomas in rats did find a link between alterations in β-catenin and the expression of iNOS [44].

To our knowledge this study provides the first evidence that β-catenin plays a role as a transcription factor involved in HGF signalling and in the regulation of iNOS expression in human extravillous trophoblasts. It is likely that other transcription factors are also involved in the regulation of iNOS in trophoblasts, although these have yet to be identified. It is also likely that both β-catenin and NF-κB are involved in the regulation of genes other than iNOS that may also play a role in protecting trophoblasts from apoptosis. Future work will seek to establish which other genes are controlled by these transcription factors in trophoblasts.

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