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**Interplay of cAMP and MAPK pathways in hCG secretion and fusogenic gene expression in a trophoblast cell line**

**Short Title:** Signalling network of cAMP and MAPK in BeWo cells

**M. Delidaki, M. Gu, A. Hein, M. Vatish, and D.K. Grammatopoulos**

Laboratory of GPCR Pathophysiology Research, Endocrinology and Metabolism, Clinical Sciences Research Institute, Warwick Medical School, University of Warwick, Gibbet Hill Road, Coventry, CV4 7AL, UK

**Correspondence to :** Prof. D.K Grammatopoulos  
Warwick Medical School  
The University of Warwick  
Gibbet Hill Road, Coventry, CV4 7AL, UK  
Tel. No: +44-2476-524206  
Fax No: +44-2476-574637  
e-mail: [d.grammatopoulos@warwick.ac.uk](mailto:d.grammatopoulos@warwick.ac.uk)

**Key words:** placenta, BeWo, cAMP, MAPK, syncytin, hCG

**ABSTRACT**

Differentiation of human placental mononuclear trophoblasts into a multinucleate syncytium involves up-regulation of key proteins promoting cell fusion and increased capacity for placental hormonogenesis. It is well established that activation of adenylyl cyclase leads to increased expression of trophoblast fusogenic gene machinery and human chorionic gonadotropin (hCG) secretion. We used the forskolin-induced syncytialisation of BeWo choriocarcinoma cells as a model to characterise in detail the signalling pathway downstream of adenylyl cyclase. Forskolin treatment induced a rapid and potent ERK1/2 and p38MAPK phosphorylation; this cascade required PKA-AKAP interactions and led to downstream CREB-1/ATF-1 phosphorylation via ERK1/2-dependent but p38MAPK-independent mechanisms. Interestingly both p38MAPK and ERK1/2 were involved in forskolin-induced hCG-secretion, suggesting presence of additional p38MAPK-dependent but CREB-1/ATF-1-independent pathways. Forskolin treatment of BeWo cells significantly up-regulated expression of various fusogenic gene mRNAs, including syncytin-1 and -2 (by 3 and 10 fold, respectively) the transcription factors old astrocyte specifically-induced substance (OASIS) and glial cells missing a (GCMa) (by 3 and 6 fold, respectively) and the syncytin-2 receptor, major facilitator superfamily domain containing 2 (MFSD2) (by 2 fold). Up-regulation of AKAP79 and AKAP250, (by 2.5 and 4 fold, respectively) was also identified in forskolin-treated BeWo cells. Forskolin effects on all these genes were suppressed by chemical inhibition of p38MAPK whereas only specific genes were sensitive to ERK1/2 inhibition. This data provide novel insights into the signalling molecules and mechanisms regulating fusogenic gene expression by the adenylyl cyclase pathway.

**Key words:** placenta, BeWo, cAMP, MAPK, syncytin, hCG

## ***1. INTRODUCTION***

One of the fundamental processes involved in the establishment of successful human pregnancy is the formation and maintenance of the syncytial layer during placentation (Red-Horse et al., 2004), achieved by continuous incorporation and cell fusion of trophoblast cells into the overlying syncytiotrophoblast. Disturbed trophoblast fusion and syncytiotrophoblast formation has been linked to the pathogenesis of pregnancy-related complications such as pre-eclampsia, intrauterine growth restriction (IUGR), and haemolysis elevated liver enzymes and low platelets (HELLP)-syndrome (Gauster et al., 2009; Langbein et al., 2008)

A number of proteins have been identified as crucial regulators of trophoblast cell fusion. Syncytin-1, a protein encoded by a human endogenous retrovirus-W envelope protein gene (Mi et al., 2000), is abundantly expressed in placental trophoblasts and appears to be critical for trophoblast fusion. Syncytin-1 effects might be mediated through binding to the amino acid transporters ASCT1 and ASCT2 (Blond et al., 2000). In the trophoblast, its expression levels are dynamically regulated by transcription factors such as glial cells missing a (GCMa) (Yu et al., 2002) and the bZIP-Type transcription factors cAMP response element-binding protein (CREB) as well as upstream regulators of GCMa expression such as old astrocyte specifically-induced substance (OASIS) and the transducer of regulated CREB activity (TORC1), the human co-activator of CREB (Schubert et al., 2008). In addition, syncytin-2 and its transmembrane receptor-namely major facilitator superfamily domain containing 2 (MFSD2), which belongs to a large family of presumptive carbohydrate transporters with 10–12 membrane-spanning domains, also appears to contribute to placental morphogenesis (Esnault et al., 2008; Malassiné et al., 2007). Differentiation of trophoblasts into syncytia is associated with increased production of human chorionic gonadotropin (hCG), which can act in an autocrine manner to increase syncytium formation (Shi et al., 1993).

Regulation of trophoblast fusogenic machinery and hormonogenesis is achieved by coordinated action of a number of signalling molecules. In both primary human trophoblast cells and choriocarcinoma-derived cell lines the cAMP/PKA cascade appears to play a major role in regulating GCMa and syncytin-1 expression and hCG production through phosphorylation of CREB-1 and ATF-1 transcription factors (Keryer et al., 1998; Knerr et al., 2005; Knöfler et al., 1999). Other

kinases such as protein kinase C (PKC) might also interact synergistically with cAMP/PKA to induce trophoblast morphological differentiation and hCG production (Andersen et al., 1988). Moreover, two members of the mitogen-activated protein kinases (MAPK), ERK1/2 and p38MAPK are suggested to play important roles in trophoblast differentiation (Daoud et al., 2005; Vaillancourt et al., 2009); ERK1/2 appears to be involved in phorbol ester induced trophoblast differentiation through PKC-dependent pathways (Suzuki et al., 2002). The activity of this pathway appears to be negatively regulated by Src kinases (Daoud et al., 2006).

The molecular actions of ERK1/2 and p38MAPK and mechanisms coordinating its activity in trophoblasts are not fully elucidated. To investigate potential signalling cross-talk between the cAMP/PKA and MAPK pathways involved in trophoblast differentiation and hCG secretion, we employed the BeWo choriocarcinoma cell line, as these trophoblast-like cells have the capacity to fuse and undergo extensive morphological and biochemical differentiation to yield syncytia in the presence of the adenylyl cyclase activator, forskolin (Ringler et al., 1990; Wice et al., 1990). In this cellular model we examined signalling pathways leading to ERK1/2 and p38MAPK phosphorylation as well as characteristics of activated ERK1/2 and p38MAPK. The role of these molecules on forskolin-induced expression of fusogenic genes mRNA such as syncytin-1 and -2, ASCT2, MFSD2 and transcription factors such as GCMa, OASIS and TORC1 was determined by quantitative RT-PCR whereas the hormone-secreting capacity of differentiated BeWo cells was determined by measurement of hCG levels. Finally, specific chemical inhibitors were employed to delineate the signalling pathways and mechanisms regulating these effects.

## **2. MATERIALS AND METHODS**

### **2.1 Chemicals**

Forskolin, U0126 (MEK12 inhibitor), SB202190 (p38MAPK inhibitor), bisindomaleimide I, PKA inhibitors 14-22 amide (cell-permeable, myristoylated) were from Calbiochem/Merck Biosciences (Nottingham, UK). PKI(5-24) was from BIOMOL Intl (Enzo Life Sciences UK Ltd, Matford Court, Exeter, UK). Phospho(Ser133)-CREB-1, phospho-ERK1/2 (Thr202/Tyr204), total ERK2, total Akt, phospho (Thr180/Tyr182) and total p38MAPK antibodies were from Cell Signalling

(Chandlers Ford, Hampshire, UK). Beta-actin antibody was purchased from Abcam (Cambridge, UK). Secondary antibodies Alexa Fluor<sup>®</sup>488, Alexa Fluor<sup>®</sup>680 and *Slowfade*<sup>®</sup> gold antifade reagent with DAPI were from Invitrogen, Molecular Probes (Paisley, UK), while IRDye 800-conjugated goat antirabbit IgG was from Rockland Immunochemicals (Gilbertsville, PA). Cell culture media and *Taq* polymerase was from Gibco/Invitrogen (Paisley, UK). dNTPs and DNA ladder were purchased from Fermentas Life Sciences (York, UK). GeneElute Mammalian Total RNA Kit was from Qiagen UK (Crawley, West Sussex, UK), Ht-31 peptide, random hexamers, AMV reverse transcriptase, RNasin were from Promega (Madison, WI, USA). Primers were purchased from VH Bio Ltd (Gateshead, UK). ELISA Direct Cyclic AMP kit was purchased from Assay Designs Inc. (Ann Arbor, MI, USA). All other chemicals were purchased from Sigma Aldrich Company Ltd (Gillingham, UK).

## 2.2 Subjects

Placental biopsies were obtained from women undergoing normal uncomplicated elective cesarean sections at term (no=5). Informed consent was obtained from each woman, and ethical approval was granted from the local ethical authority. All subjects studied were matched for stage of gestation and age. All five uncomplicated elective cesarean sections at term were following maternal request. Immediately after delivery, the maternal and fetal surfaces of the placenta were dissected off, and fetal membranes were peeled away from the placenta. Placental samples were washed in PBS and immediately snap-frozen in liquid nitrogen.

## 2.3 Cell culture of BeWo cells and human chorionic gonadotropin (hCG) secretion assay

BeWo cells were obtained from American Type Culture Collection (Rockville, MD, USA) and maintained at 37 °C in F-12 Nutrient Mixture (Ham) Kaighn's Modification (F12K) containing 10%(v/v) FBS heat-inactivated (Biosera UK, East Sussex, UK), 100 U/ml penicillin, and 100 µg/ml streptomycin, under a humidified atmosphere of 5% CO<sub>2</sub> in air. Cells were grown to confluence and subcultured (4:1) every 3–4 days.

For the hCG secretion assays, forskolin was prepared in DMSO and was added to the medium (final concentration 100 µM) of cells grown in 6-well plates with confluency of about 70%, Cells were

incubated for 24h. Control cultures received the DMSO vehicle in the same concentration as the forskolin-treated cultures. The medium was collected after 24h of incubation. Supernatants were aspirated, centrifuged at 500g for 5 min at 4°C to remove cell debris, and stored at -80°C until analysis. The amount of secreted hCG was determined by the Dept of Biochemistry (University Hospitals Coventry and Warwickshire NHS Trust) using the Elecsys® Intact hCG+b electrochemiluminescence immunoassay (ECLIA) and the fully automated modular analytics E170 testing system from Roche Diagnostics (Mannheim, Germany). Results were expressed as IU/ml per  $1 \times 10^5$  cells.

In some experiments BeWo cells were pretreated with various signalling molecule inhibitors (U0126, SB202190, bisindolylmaleimide I (Bis I), Ht-31, PKI 5-24 or PKA inhibitor peptide myristoylated), for 30min-4h prior to forskolin addition.

#### **2.4 Signalling assays**

Cells were grown in 12-well plates to 80% confluency. Cyclic AMP levels were determined by a commercially available ELISA Direct Cyclic AMP kit (Assay Designs Inc., Ann Arbor, MI, USA) as previously described (Markovic et al. 2008). For MAPK and CREB-1 phosphorylation assays, 18h prior to treatment cells were washed and serum-starved and incubated overnight with plain medium. The following day, cells were washed with plain F12K medium and pre-treated with various chemical inhibitors (U0126 - 10 $\mu$ M, Bis I - 2 $\mu$ M, PKI 5-24 - 100 $\mu$ M, PKA 14-22 amide myristoylated - 100 $\mu$ M, SB202190 - 2.5 $\mu$ M, St-Ht31 - 25 $\mu$ M), for 20-60min prior to forskolin addition for the indicated time points. The selection of inhibitor concentration used was based on manufacturer's data, comparable published studies and validation in preliminary experiments. ERK1/2, p38MAPK and CREB-1 phosphorylation was determined as previously described (Markovic et al. 2008), using immunoblotting with specific antibodies and the Odyssey Infrared Imaging System (LI-COR Biosciences, Cambridge, UK). Fluorescent signal intensity of both phosphorylated and total ERK1/2, p38MAPK and CREB-1 was quantified using the Odyssey software Version 2.1, and results were normalized for total protein (ERK1/2 or p38MAPK or CREB-1) expression by determining the phospho-/total protein ratio.

#### **2.5 Confocal microscopy studies**

BeWo cells were grown on aminopropylethoxysilane (APES)-treated glass cover slips. Phospho-ERK1/2 and p38MAPK was monitored in serum-deprived cells treated with 10 $\mu$ M forskolin for 10-30min, as previously described (Markovic et al. 2008). The scan speed was set at 400 Hz, and the format was 1024x1024 pixels. Optical sections (0.5  $\mu$ m) were taken, and representative sections corresponding to the middle of the cells are presented. For each treatment, between 20 and 30 individual cells in five random fields of view were randomly selected and examined.

## 2.6 RNA extraction and RT-PCR

Cells were washed with cold PBS and total RNA was extracted using RNeasy Plus mini kit (Qiagen, Crawley West Sussex), according to the manufacturer's instructions. Complementary DNA synthesis was performed as previously described (Markovic et al. 2007). Placental total RNA was extracted as previously described (Karteris et al., 2005). Fusogenic gene expression levels were assessed by real-time PCR using the Light Cycler thermal cycler (Roche Molecular Biochemicals, Mannheim, Germany). The reaction was performed in glass capillary tubes in a 10  $\mu$ l reaction mixture containing 5  $\mu$ l of PCR 2x Mastermix with 2 mM MgCl<sub>2</sub> (Bio/Gene, Kimbolton, UK), 0.5  $\mu$ l of SYBR Green I (Biomole), 1  $\mu$ l of each primer in a concentration varying from 5  $\mu$ M to 10 $\mu$ M and 2.5  $\mu$ l of cDNA. The PCR protocol consisted of initial denaturation at 95 °C for 60s, followed by 40 cycles of amplification at 95°C for 1s, 55°C - 60°C (annealing temperature appropriate to each primer) for 5-10 s, 72 °C for 15 s, and one final cycle of cooling and holding at 40 °C for 30 seconds. Melting curve analysis was performed to confirm specificity of amplification. Reaction mixtures without reverse transcriptase or RNA were run in parallel to ensure absence of sample contamination. Each treatment was tested in duplicate in three at least independent experiments. To confirm the identity of the amplified DNA fragment, the PCR products were sequenced in an automated DNA sequencer (Department of Biological Sciences, The University of Warwick, Warwick, UK) and the data were analyzed using Blast Nucleic Acid Database Searches from the National Center for Biotechnology Information. The mRNA levels were expressed as a ratio, using the DDC<sub>T</sub> method for comparing relative expression between treatments. The sequence of primers used were as follows: Syncytin-1: *Forward* 5'-TCATATCTAAGCCCCGCAAC-3', *Reverse* 5'-TGATCTTGCAAGGTGACCAG-3' (product size: 187 bp); ASCT2: *Forward* 5'-ACATCCTG GGCTTGGTAGTG-3', *Reverse* 5'-

GGGCAAAGAGTAAACCCACA-3' (product size: 213 bp); GCMA: *Forward* 5'-GTGGACCCCATGAAGCTCTA-3', *Reverse* 5'-GATGATTTCTCCCC AAAGCA-3' (product size: 154 bp); Syncytin-2: *Forward* 5'-AGCAGCCGTAGTCCTTCAAA-3', *Reverse* 5'-AGGGGAAGAACCCAAGAGAA-3' (product size: 231 bp); MFSD2: *Forward* 5'-CCTTGTTTCCAGGACCTCAA-3', *Reverse* 5'-GAAGTAGGCGATTGGCTCAG-3' (product size: 216 bp); TORC1: *Forward* 5'-GACACCAGCTGGAGAAGGAC-3', *Reverse* 5'-TGCTTGCT TGGAAAGGT TTT-3' (product size: 183 bp); OASIS: *Forward* 5'-AGGTGGAGACCCTGGAGAAT-3', *Reverse* 5'-AGGGGGTCTTCCTTCACAGT-3' (product size: 223 bp); AKAP75: *Forward* 5'-TCAC GGAAAGATGGTGATGA-3', *Reverse* 5'-GCTGGGGTTGAACATCT TGT-3' (product size: 178bp bp); AKAP95: *Forward* 5'-GAAAGAAACCGCAAAACCAA-3', *Reverse* 5'-AACTGTTTCAGCA GCCAACCT-3' (product size: 186bp); AKAP250: *Forward* 5'-GAAACAGCCACCGAAATGTT-3', *Reverse* 5'-GCGGTTGACTCTGACTCCTC-3' (product size: 170bp); GAPDH: *Forward* 5'-GAGT CAACGGATTTGGTCGT-3', *Reverse* 5'-GACAAGCTTCCCGTTCTCAG-3' (product size: 185bp).

Expression of AKAP 75, 95, and 250 mRNA in human term placenta was assessed by RT-PCR in an Eppendorf Mastercycler (Eppendorf UK Ltd, Histon Cambridge, UK). A final volume of 50 µl per reaction was prepared, using 5 µl 10X PCR Buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl], 1.5 µl 50 mM MgCl<sub>2</sub>, 1 µl 10 mM dNTP Mix, 1 µl (10 µM) of each primer, 0.4 µl *Taq* DNA polymerase (5 U/µl) and 2 µl (200ng) of cDNA. PCR grade water was added up to 50 µl. The PCR amplification conditions were as follows: for AKAP75 and AKAP95 96°C for 15s, 62°C for 30s, 72°C for 1 min whereas for AKAP250, 95°C for 30s, 58°C for 30s, 72°C for 1 min, in a total of 35 cycles and finally 72°C for 5 min. Ten microliters of the reaction mixture were subsequently electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide, using a 1-kilobase (kb) DNA ladder (Fermentas, York UK) to estimate the band sizes. As a negative control for all reactions, PCR grade water was used in place of cDNA. The PCR products were sequenced in an automated DNA sequencer (Department of Biological Sciences, The University of Warwick) and the sequence data were analyzed using Blast Nucleic Acid Database Searches from the National Center for Biotechnology Information.

## 2.7 Data analysis and statistical methods

Results from triplicate experiments were pooled and analyzed by one-way ANOVA with Student-Newman-Keuls multiple comparison methods, using SigmaStat version 3.0 (SPSS, Chicago, IL).

## 3. RESULTS

### 3.1 Signalling pathways activated by forskolin

Forskolin exerts its effects in cells, primarily, but not exclusively, through stimulation of the catalytic domain of adenylyl cyclase that leads to increased intracellular cAMP levels and activation of downstream effectors such as protein kinase A (PKA). Indeed, in placental trophoblasts, PKA has been shown to be an important mediator of fusogenic gene expression as well as hCG induction (Keryer et al., 1998; Knerr et al., 2005; Knöfler et al., 1999). In BeWo trophoblasts, the signalling molecules activated in response to adenylyl cyclase activation were investigated. In agreement with previous studies, forskolin exerted a potent stimulatory effect and rapidly increased cAMP levels by 150 to 200 fold within 15min (Fig.1a). Interestingly, forskolin also induced a time-dependent increase in phosphorylation of ERK1/2 and p38MAPK (Fig. 1b). Both ERK1/2 and p38MAPK maximal stimulation ( $10\pm 4$  and  $4\pm 2$  fold respectively) were observed after 5-10min and substantial MAPK activation was evident after 30 min of treatment (Fig. 1c). Forskolin effects on ATF-1 and CREB-1 phosphorylation was also investigated in BeWo cells using a phospho-CREB (Ser133) specific antibody that also detects phosphorylated ATF-1. Within 10min forskolin treatment induced a 10x fold increase in ATF-1/CREB-1 phosphorylation and this stimulation was sustained for at least 30min (Fig.1d).

To examine the spatial characteristics of ERK1/2 and p38MAPK activation, indirect immunofluorescence confocal microscopy was employed with phospho-specific antibodies, to monitor the relative subcellular distribution of activated MAPK after agonist stimulation. In unstimulated BeWo cells, very low levels of activated (phosphorylated) ERK1/2 were detected. Within 10min of forskolin treatment the amount of fluorescent signal corresponding to phospho-ERK1/2 was significantly increased and localized primarily in the nucleus although substantial localization in the plasma membrane was also evident (Fig. 2). Treatment of BeWo cells with

forskolin for longer periods (20-30min) did not significantly alter phospho-ERK1/2 nuclear localization, although the cytoplasmic distribution was significantly increased. Similar results were obtained for phospho-p38MAPK (*data not shown*).

Interestingly, prolonged treatment of BeWo cells for 24h, which was associated with increased hCG secretion, significantly altered ERK1/2 and p38MAPK (total) expression levels. The total amount of ERK1, ERK2 and p38MAPK were reduced by 30-50%, 70-90% and 40-50% respectively (Fig.3). In contrast, Akt protein levels were not affected during the differentiation process.

We investigated in greater detail the functional components of the cAMP pathway involved in activation of MAPK signalling. BeWo cells were pretreated with the selective PKA inhibitor (PKA inhibitor 14-22 amide myristoylated-PKAI) or the Ht-31 peptide, which prevents binding between PKA regulatory subunits and A-kinase anchoring proteins (AKAP). The results showed that forskolin-induced ERK1/2 and p38MAPK phosphorylation requires intact PKA-AKAP interactions since cell pre-treatment with either myr-PKAI or Ht-31 significantly attenuated phospho-ERK1/2 and p38MAPK levels by 50-70% (Fig.4a). At least 3 AKAP subtype mRNAs, AKAP79, 95 and 250 (gravin), were detected in both BeWo cells and term human placenta by conventional RT-PCR (Fig.4b). In BeWo cells the mRNA expression of these signalling effectors was sensitive to the actions of forskolin (Fig.4c); stimulation of cells for 24h was associated with a significant up-regulation of AKAP79 and 250 (by 2.5- and 4-fold, respectively).

Previous studies in BeWo cells identified PKC as an important mediator of phorbol ester-induced ERK1/2 activator (Suzuki et al., 2002). The potential involvement of PKC in forskolin-induced ERK1/2 and p38MAPK activation was investigated by using Bisindolylmaleimide I (Bis I), a specific PKC inhibitor. Pretreatment of cells with Bis I had no effect on effect on forskolin-induced ERK1/2 and p38MAPK activation (Fig. 5), thus excluding PKC involvement in forskolin-MAPK interactions in BeWo cells.

### **3.2 Contribution of MAPK cascades in BeWo hormonogenesis**

The crucial role of the placental cAMP/PKA cascade in up-regulated fusogenic gene expression and differentiation-associated hCG production is well established (Keryer et al., 1998; Knerr et al., 2005; Knöfler et al., 1999). The potential contribution of ERK1/2 and p38MAPK to these processes was

also evaluated. Results showed that incubation of BeWo cells with forskolin for 24h in the presence of SB202190 or U0126 significantly attenuated hCG secretion by 40-55% (Fig.6a). Simultaneous inhibition of ERK1/2 and p38MAPK inhibited almost completely forskolin-dependent hCG production, suggesting a synergistic action of ERK1/2 and p38MAPK in the control of hCG production.

Previous studies in primary villous trophoblasts used as models of *in-vitro* trophoblast differentiation demonstrated cAMP/PKA-dependent hCG promoter transcriptional activity that requires functional cAMP-response elements (CREs) which interact with phosphorylated activating transcription factor-1/cAMP response element-binding protein-1 (ATF-1/CREB-1) DNA-binding proteins (Knöfler et al., 1999). The role of ERK1/2 and p38MAPK in forskolin-induced ATF-1 and CREB-1 phosphorylation was also investigated. In the presence of a selective PKA inhibitor [PKI(5-24)] or Ht-31, the forskolin effect was significantly attenuated (Fig.6b). Interestingly, U0126 significantly attenuated forskolin-induced ATF-1/CREB-1 phosphorylation by 70-80%, whereas SB202190 had no effect (Fig.6c), suggesting that p38MAPK actions on hCG secretion do not involve regulation of ATF-1/CREB-1 phosphorylation.

### **3.3 Contribution of MAPK cascades in BeWo fusogenic gene up-regulation**

In agreement with previously published results (Esnault et al., 2008; Knerr et al., 2005; Kudo and Boyd, 2002; Vargas et al., 2009) forskolin treatment of BeWo cells for 24h induced substantial mRNA up-regulation of a cassette of genes encoding fusogenic proteins such as syncytin-1 and -2, MFSD2, OASIS and GCMA (by 3-, 10-, 2-, 3- and 6-fold, respectively), without affecting TORC1 mRNA levels. The mRNA levels of the syncytin-1 receptor, ASCT2, were slightly reduced by 15-20%, (*data not shown*). The relative contribution of the ERK1/2 and p38MAPK signalling cascades in this process was investigated by pre-treatment of BeWo cells with SB202190 or U0126. Depletion of p38MAPK activity dramatically impaired by 80-90% forskolin-driven up-regulation of both syncytin-1 and -2 mRNA whereas regulation of GCMA, OASIS and MFSD2 mRNA were less dependent on intact p38MAPK activity (50%, 40%, and 30% inhibition of forskolin effect, respectively) (Fig.7). In contrast, inhibition of ERK1/2 activity reduced forskolin effects only on syncytin-1 mRNA levels, albeit to a lesser degree compared to p38MAPK inhibition (Fig.7).

#### 4. DISCUSSION

The ability of trophoblasts to differentiate and fuse into a multinucleate syncytium and acquire an active endocrine phenotype is under the control of a plethora of hormonal signals. These signals finely tune specific mechanisms that stimulate expression of a specific cassette of important fusogenic genes such as syncytin-1 and -2 and increase production of hormones such as human chorionic gonadotrophin (hCG) and human placental lactogen (hPL) (Knerr et al., 2005; Peters et al., 2000). Studies in choriocarcinoma cellular models as well as primary villous trophoblasts and placental explants, established the important role of the cAMP/PKA and MAPK cascades in these processes. For example, regulation of trophoblast differentiation by hormones such as hCG and calcitonin-gene related peptide (CGRP), involve cAMP/PKA-dependent pathways (Green, et al., 2006; Shi et al., 1993), whereas growth factors such as EGF induce villous trophoblast differentiation through ERK1/2 and p38MAPK pathways (Johnstone et al., 2005; Yang et al., 2003).

In the BeWo choriocarcinoma cells, we demonstrated that both ERK1/2 and p38 MAPK pathways are activated downstream of adenylyl cyclase and play discrete roles in hCG secretion and upregulation of specific fusogenic genes. The pattern and spatio-temporal characteristics of cAMP-induced MAPK activation (sustained activation with substantial nuclear as well as cytoplasmic localization) suggest potential to target multiple substrates and influence distinct intracellular processes involved in trophoblast differentiation. This is supported by studies in other biological examples such as rat PC12 cells, which demonstrated that this pattern of sustained MAPK activation associated with nuclear translocation, leads to cell cycle arrest and differentiation (Marshall, 1995). Given that in BeWo cells the MAPK activation primarily involves the cAMP/PKA/AKAP pathway, it is possible that cAMP signaling compartmentalization regulated by AKAPs, is important for spatial topology of activated MAPK. Interestingly, the expression levels of both ERK1/2 and p38MAPK appeared to be under the negative control of the cAMP system, indicating the presence of complex regulatory mechanisms that tightly control MAPK expression and activity during differentiation. This is in agreement to previous *in vitro* studies investigating primary trophoblast differentiation (Daoud et al., 2005) as well as studies employing placental biopsies to examine activated and total ERK1/2

expression pattern throughout gestation (Kita et al., 2003). Furthermore, these mechanisms appear to specifically regulate MAPK expression since the expression levels of Akt/PKB, the downstream target of PI3-K which also plays an important role in trophoblast differentiation (Kamei et al., 2002) remained intact.

In trophoblasts, the cAMP-dependent PKA appears to be largely responsible for mediating the biological effects of cAMP on cell differentiation and hormonogenesis. Indeed our studies suggest that PKA mediates forskolin-induced ERK1/2 and p38MAPK activation. Studies on the pattern of expression and subcellular localization of PKA subunits suggest that villous trophoblasts express the RI $\alpha$  and RII $\alpha$  regulatory subunits and the C $\alpha$  and C $\beta$  catalytic subunits, and that trophoblast differentiation is associated with RI $\alpha$  down-regulation and a change in RII $\alpha$  subcellular redistribution: in trophoblasts RII $\alpha$  is located in the Golgi-centrosomal area whereas in syncytiotrophoblast RII $\alpha$  immunoreactivity is scattered throughout the cytoplasm and accumulated underneath the apical membrane of syncytiotrophoblast in the subcortical area (Keryer et al., 1998). Unlike the RI isoforms, the RII isoforms associate with A-Kinase Anchoring Proteins (AKAPs) that facilitate PKA type II transport to organelles, cytoskeleton and membranes (Rubin, 1994) and effectively assemble upstream activators and downstream effectors. Our study provides novel evidence about the crucial role of AKAP in PKA-mediated downstream effects that lead to activation of ERK1/2 and p38 MAPK. Previous studies identified high expression in the placental syncytiotrophoblast microvilli of ezrin (AKAP78) (Berryman et al., 1995) an actin-binding protein that acts as a membrane-cytoskeleton linker (Algrain et al., 1993). We showed that the human term placenta and BeWo cells express multiple AKAP mRNA transcripts and that specific AKAPs (79 and 250) are significantly up-regulated in the BeWo cells by forskolin treatment, suggesting a self-regulatory potential of the cAMP/PKA cascade to augment its activity. Recently, the specific AC isoforms 5/6 were identified as binding partners of AKAP79 at the plasma membrane, highlighting AKAPs potential to shape the dynamics and provide added feedback regulation of of cAMP accumulation within cells (Dessauer., 2009).

In BeWo cells, ERK1/2 and p38MAPK activated downstream of the cAMP/PKA pathway appear to be major signalling determinants of hCG release during the syncytialization process by exerting distinct but synergistic actions, in agreement with observations in isolated trophoblasts (Daoud et al., 2005). Previous studies in villous trophoblast (Keryer et al., 1998) and BeWo cells (Heckert et al., 1996) identified PKA mediated activation of different DNA-binding proteins including CREM, ATF-1 and CREB-1 that interact with the cAMP-response elements (CRE) on the promoter of the alpha subunit gene that encodes a common subunit shared by all glycoprotein hormones. Our results suggest that ERK1/2 but not p38MAPK, is important for the sustained PKA-mediated phosphorylation of CREB-1/ATF-1, an important step for recruitment of the transcriptional co-activators such as the adaptor proteins CBP and p300. This raises the interesting possibility that p38MAPK role on forskolin-induced hCG release might involve direct effects in distal transcriptional activators. Alternatively, hCG release might be influenced indirectly by the pro-differentiation activity of p38MAPK, since trophoblast hCG expression is strongly correlated with the degree of cellular transformation (Graham et al., 1993).

In addition to hCG production, PKA and CREB-1 are also involved in the transcriptional up-regulation of other genes important for trophoblast differentiation such as the transcription factor GCMa (Graham et al., 1993; Knerr et al., 2005). Previous studies (Schubert et al., 2008) identified CRE-binding sites within the *GCMa* gene promoter and suggested that regulation of GCMa transcription involves CREB-1 and its specific positive co-activator, TORC1, as well as other transcription factors like OASIS. Interestingly, our results suggest that in BeWo cells, TORC1 mRNA expression levels are not affected by the pro-differentiation stimulus forskolin, whereas OASIS mRNA expression was significantly enhanced. PKA also stimulates the association of GCMa with CBP, which increases GCMa acetylation at lysine367, lysine406, and lysine409 in the transactivation domain (TAD). Acetylation of these residues is required to protect GCMa from ubiquitination and increases the TAD stability with a concomitant increase in transcriptional activity (Chang et al., 2005). Our results suggest that p38MAPK is involved in forskolin-induced GCMa mRNA up-regulation; this action might be achieved through CREB-1-independent pathways, through other transcriptional regulators. The observed p38MAPK-dependent mRNA up-regulation of the

transcriptional factor OASIS might represent one such potential mechanism. Interestingly, ERK1/2 is not required for forskolin-induced GCMA mRNA up-regulation despite its CREB-1-activating properties. This finding raises the possibility that the ERK1/2-mediated CREB-1 activation becomes an important mechanism for the transcription of only specific targets (such as hCG).

In trophoblasts, syncytin-1 represents a major downstream target of GCMA (Yu et al., 2002). Based on our previous data it is not surprising that forskolin effects on syncytin-1 mRNA regulation are extremely sensitive to inhibition of p38MAPK activity. Moreover, syncytin-1 regulation of expression appears to involve also ERK1/2, likely through GCMA-independent pathways. The transcriptional control of *syncytin-1* gene promoter involves complex mechanisms not fully understood. Demethylation of the syncytin-1 promoter in trophoblast appears to be a prerequisite for its expression and differentiation of multinucleated syncytiotrophoblast (Matoušková et al., 2006). It is possible that ERK1/2 participates in the control of these mechanisms; further studies are required to characterise this in detail.

Previous studies (Chen et al., 2008; Esnault et al., 2008; Vargas et al., 2009) demonstrated that syncytin-2 and its receptor MFSD2, which are also major determinants of the trophoblast fusogenic capacity, exhibited substantial up-regulation by forskolin at the mRNA level. We identified that this mechanism depends on p38MAPK, but not ERK1/2 activity. At present very little is known about the regulation of placental expression of these genes; trophoblast syncytin-2 expression appears to be downregulated in preeclampsia, a disease state associated with reduced activity of placental p38 MAPK (Webster et al., 2006). Thus, it is conceivable that inhibition of p38MAPK activity might contribute to attenuation of syncytin-2 expression, a hypothesis that requires further investigation.

## **5. CONCLUSIONS**

In the BeWo cells, we demonstrated that ERK1/2 and p38MAPK appear to play critical roles in hCG release and up-regulation of multiple fusogenic genes involved in syncytium formation. Activation of ERK1/2 and p38MAPK downstream of cAMP-driven pathways, involves PKA-AKAP interactions and we identified some of the molecular targets of ERK1/2 and p38MAPK important for the transcriptional control of syncytin-1 and -2 and other important signalling mediators (Fig.8). These

investigations also identified target-specific differences in the selective contribution of each MAP kinase. This data provide novel insights about the central roles of ERK1/2 and p38MAPK in the signalling networks controlling trophoblast differentiation.

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## FIGURE LEGENTS

### Figure 1

**Forskolin-activated signalling cascades in BeWo cells.** Cells were stimulated with 100 $\mu$ M forskolin for (a) 15min (for cAMP), (b) 10min or (c-d) various time intervals (for MAPK and CREB-1 assays). Cyclic AMP production was determined by ELISA as described in *Materials and Methods* and data represent the mean $\pm$ SEM. of three independent experiments. In each experiment, values were determined in duplicate. \*,  $P < 0.05$  compared with untreated cells. Time-dependent phosphorylation of ERK1/2 and p38MAPK and CREB-1/ATF-1 were determined by immunoblotting using the Odyssey detection system as described in *Materials and Methods*. Data represent the mean  $\pm$ SEM of 3 independent experiments. In each experiment, values were determined in triplicate. \*,  $P < 0.05$  compared with basal.

### Figure 2

**Time course of phospho-ERK1/2 subcellular distribution induced by forskolin in BeWo cells: visualization by confocal microscopy.** BeWo cells were stimulated with or without 100 $\mu$ M forskolin for various time intervals (10–30 min). Phospho-ERK1/2 distribution was monitored over the ensuing time period by indirect immunofluorescence using specific primary antibodies and Alexa Fluor 488 secondary antibody for phospho-ERK1/2 (*green*) as described in *Materials and Methods*. Cell nuclei were stained with the DNA-specific dye DAPI (*blue*). Identical results were obtained from four independent experiments, and at least 20 cells were examined in each experiment. *Scale bar*, 20  $\mu$ m.

### Figure 3

**Protein kinase expression levels in forskolin-treated BeWo cells.** The expression of ERK1/2, p38MAPK and Akt in cells treated with or without 100 $\mu$ M forskolin for 24h, was determined by immunoblotting using the Odyssey detection system as described in *Materials and Methods*. Data represent the mean  $\pm$ SEM of 3 independent experiments. In each experiment, values were determined in triplicate. \*,  $P < 0.05$  compared with untreated cells.

**Figure 4**

**Role of PKA and AKAP in forskolin-induced ERK1/2 and p38MAPK phosphorylation and regulation of AKAP mRNA expression in BeWo cells.** (a) Cells were pretreated with or without Ht-31 or myr-PKAI prior to stimulation with 100 $\mu$ M forskolin for 10min. Activation of ERK1/2 and p38MAPK was determined as described in *Materials and Methods*. Data represent the mean  $\pm$ SEM of three independent experiments. In each experiment, values were determined in triplicate. \*,  $P < 0.05$  compared with cells without inhibitor pretreatment. (b) RT-PCR amplification of various AKAPs sequences from RNA extracted from human term placenta (PI) or BeWo trophoblast cell (BW). Specific primers able to amplify a 178-bp fragment of AKAP 79, a 186-bp fragment of AKAP 95 and a 170-bp fragment of AKAP 250 mRNA sequences were used as described in *Materials and Methods*. PCR products were resolved on 1.5% agarose gel and stained with ethidium bromide. The identities of the fragments were confirmed by direct nucleotide sequencing. (c) BeWo cells were treated with 100 $\mu$ M forskolin for 24h and expression levels of target mRNAs was determined by “real-time” quantitative RT-PCR (LightCycler; Roche Molecular Biochemicals) as described in *Materials and Methods*. Data from 4 independent experiments were analysed and are expressed as mean values  $\pm$ SEM of relative mRNA expression levels normalized against the housekeeping gene (GAPDH) and are expressed as fold increase above basal. \*,  $P < 0.05$  compared with basal.

**Figure 5**

**Role of PKC in forskolin-induced ERK1/2 and p38MAPK phosphorylation.** Cells were pretreated with or without the non-selective PKC inhibitor Bis-I, prior to stimulation with 100 $\mu$ M forskolin for 10min. Activation of ERK1/2 and p38MAPK was determined as described in *Materials and Methods*. Data represent the mean  $\pm$ SEM of 3 estimations from three independent experiments. \*,  $P < 0.05$  compared with cells without inhibitor pretreatment.

**Figure 6**

**Signalling molecules involved in forskolin-induced hCG release and CREB-1/ATF-1 phosphorylation in BeWo cells.** (a) BeWo cells were treated with 100 $\mu$ M forskolin for 24h in the presence or absence of specific signalling inhibitors (U0126 or SB202190 or both) and levels of hCG released in the medium was determined as described in *Materials and Methods*. Data represent the mean  $\pm$ SEM of 3 independent experiments. In each experiment, values were determined in triplicate. \*,  $P < 0.05$  compared with control values (no inhibitor). (b-c) Representative Western blots of phospho-CREB-1/ATF-1 activation in BeWo cells. Following pretreatment with or without specific signalling inhibitors (U0126, SB202190, Ht-31 or PKI(5-24), BeWo cells were treated with 100 $\mu$ M forskolin for 10min. After cell lysis and centrifugation, supernatants were subjected to SDS-PAGE and phospho CREB-1/ATF-1 immunoreactivity was determined by immunoblotting with specific antibodies using the Odyssey detection system as described in *Materials and Methods*.

#### Figure 7

**The role of ERK1/2 and p38MAPK on forskolin-induced up-regulation of specific mRNA transcripts in BeWo cells.** BeWo cells were treated with 100 $\mu$ M forskolin for 24h in the presence or absence of specific inhibitors (U0126 or SB202190) and expression levels of target mRNAs was determined by “real-time” quantitative RT-PCR (LightCycler; Roche Molecular Biochemicals) as described in *Materials and Methods*. Data are expressed as fold change from basal (unstimulated) cells and represent mean values  $\pm$ SEM of relative mRNA expression levels normalized against the housekeeping gene (GAPDH). \*,  $P < 0.05$  compared with control (no inhibitor) cells. The mean results of mRNA concentrations in basal (untreated) cells were arbitrarily assigned the value 1.

#### Figure 8

**Proposed signalling “cross-talk” between cAMP/PKA ERK1/2 and p38MAPK in the regulation of genes important for BeWo trophoblast differentiation and hormonogenesis.** A rise in intracellular cAMP levels through activation of the adenylyl cyclase (AC) pathway leads to PKA activation and interactions with AKAPs and downstream phosphorylation of ERK1/2 and p38MAPK. PKA and ERK1/2-dependent mechanisms activate CREB-1/ATF-1 to increase hCG production.

Human CG release is also induced through distinct p38MAPK-dependent CREB-1-independent mechanisms. Furthermore, in addition to PKA-dependent mechanisms, p38MAPK regulates syncytin-1 mRNA expression through up-regulation of the OASIS and GCMA mRNA transcripts. ERK1/2 also appears to regulate syncytin -1 mRNA expression through pathways independent of OASIS and GCMA transcriptional regulation. Messenger RNA expression levels of the other major fusogenic gene, syncytin-2 and its receptor MFSD2, are also regulated by p38MAPK, but not ERK1/2.

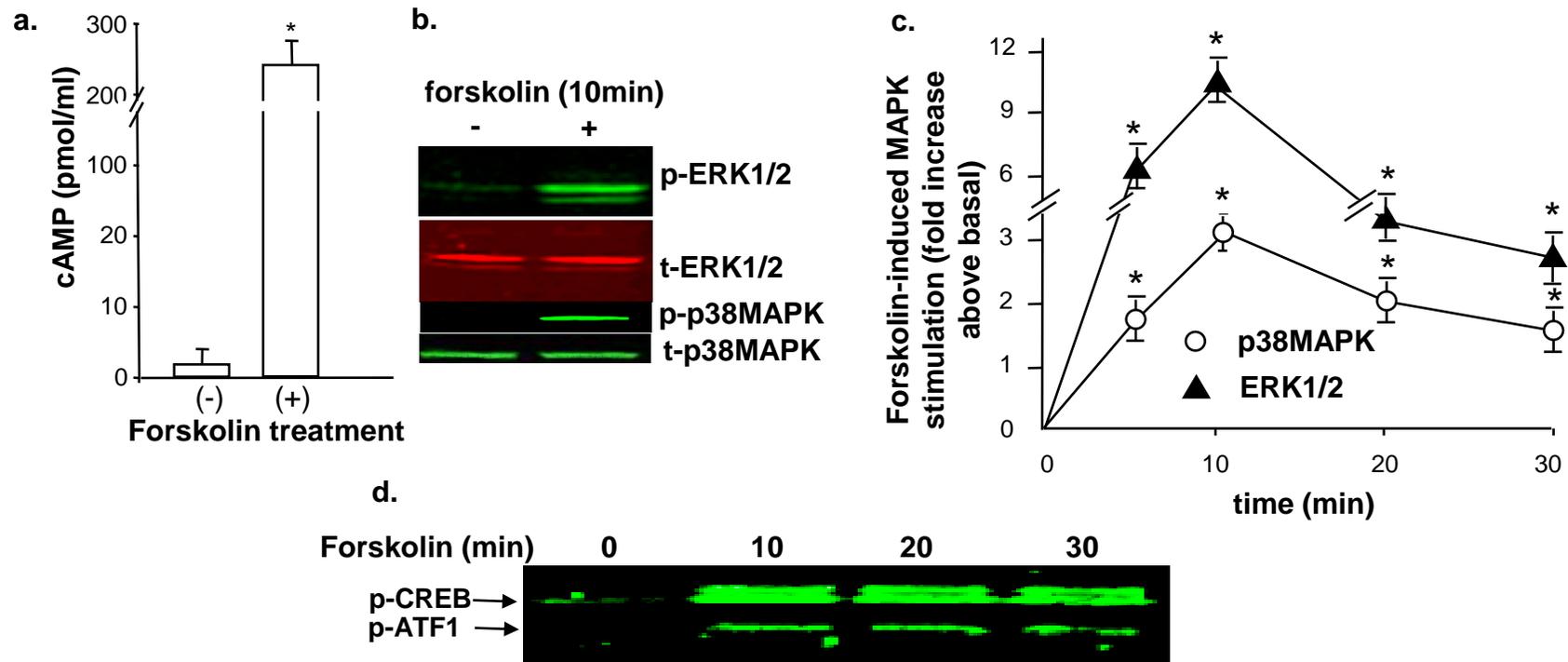


FIGURE 2

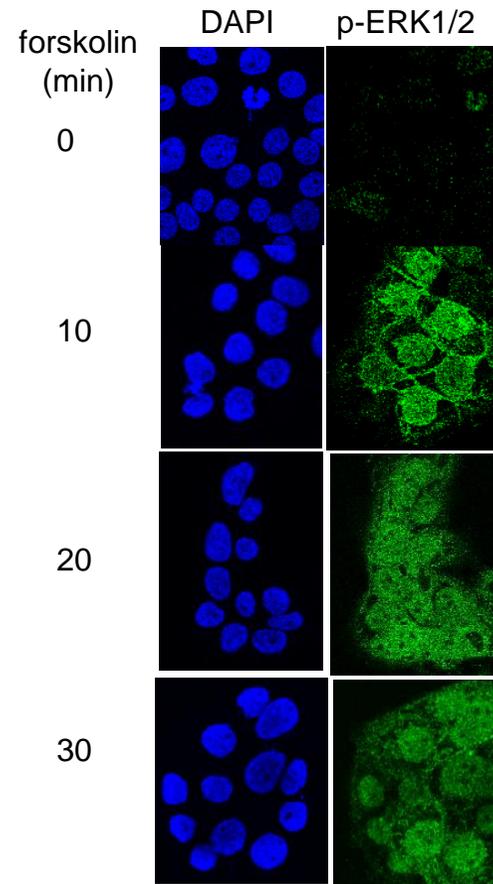


FIGURE 3

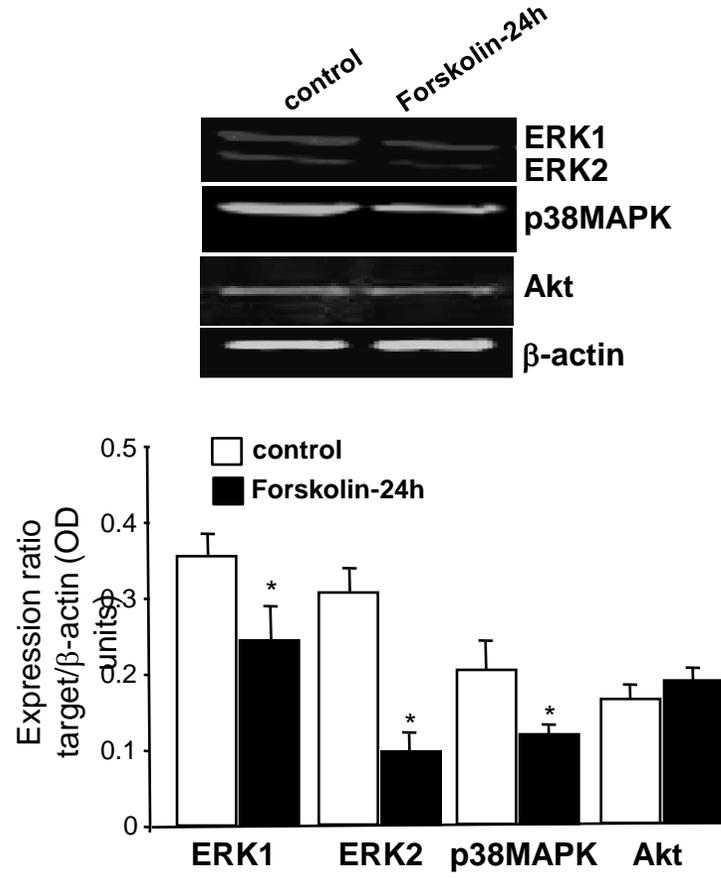


FIGURE 4

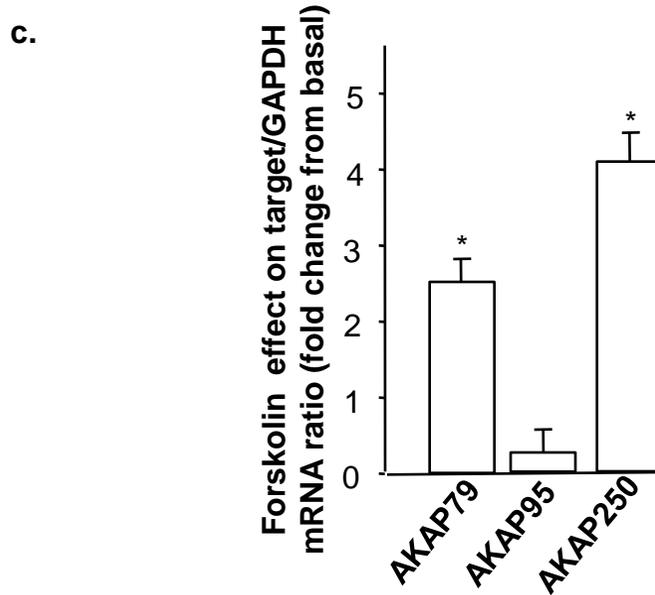
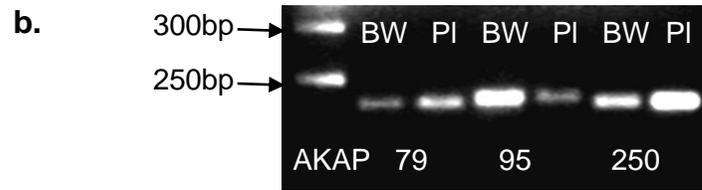
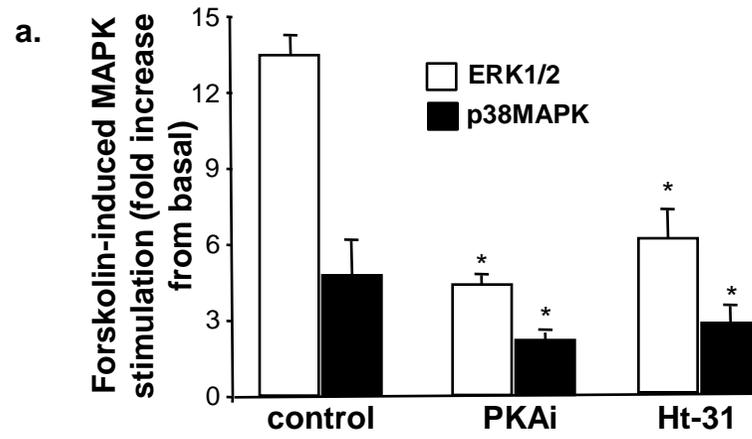


FIGURE 5

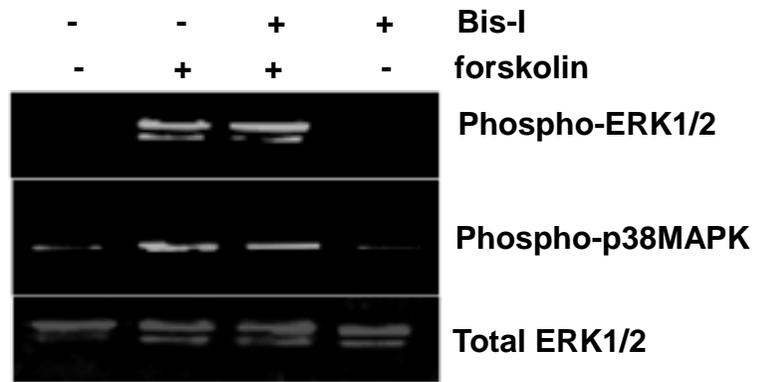


FIGURE 6

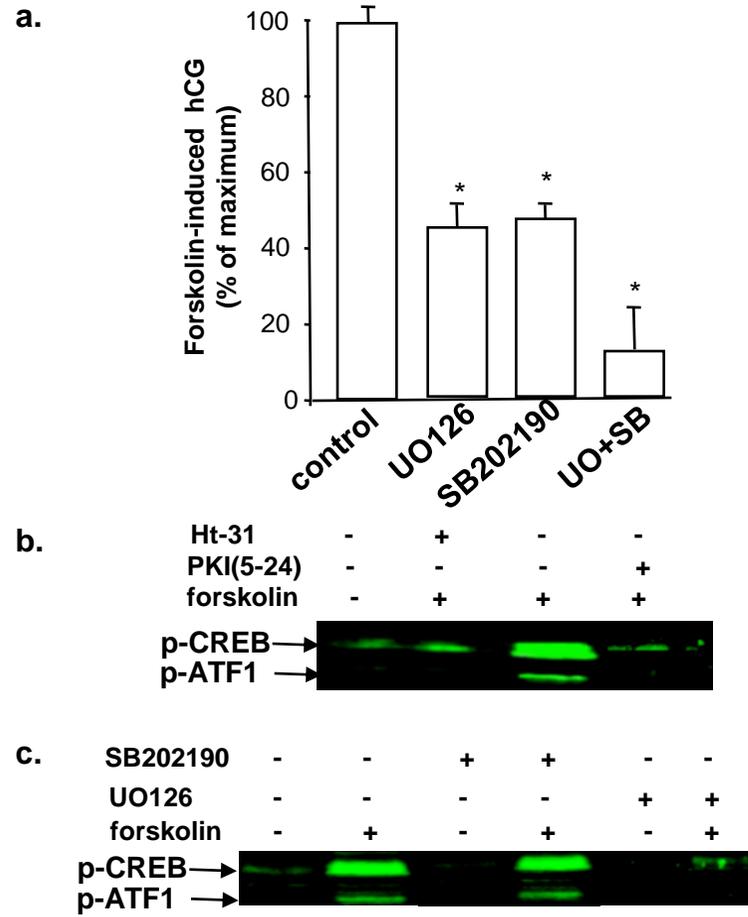


FIGURE 7

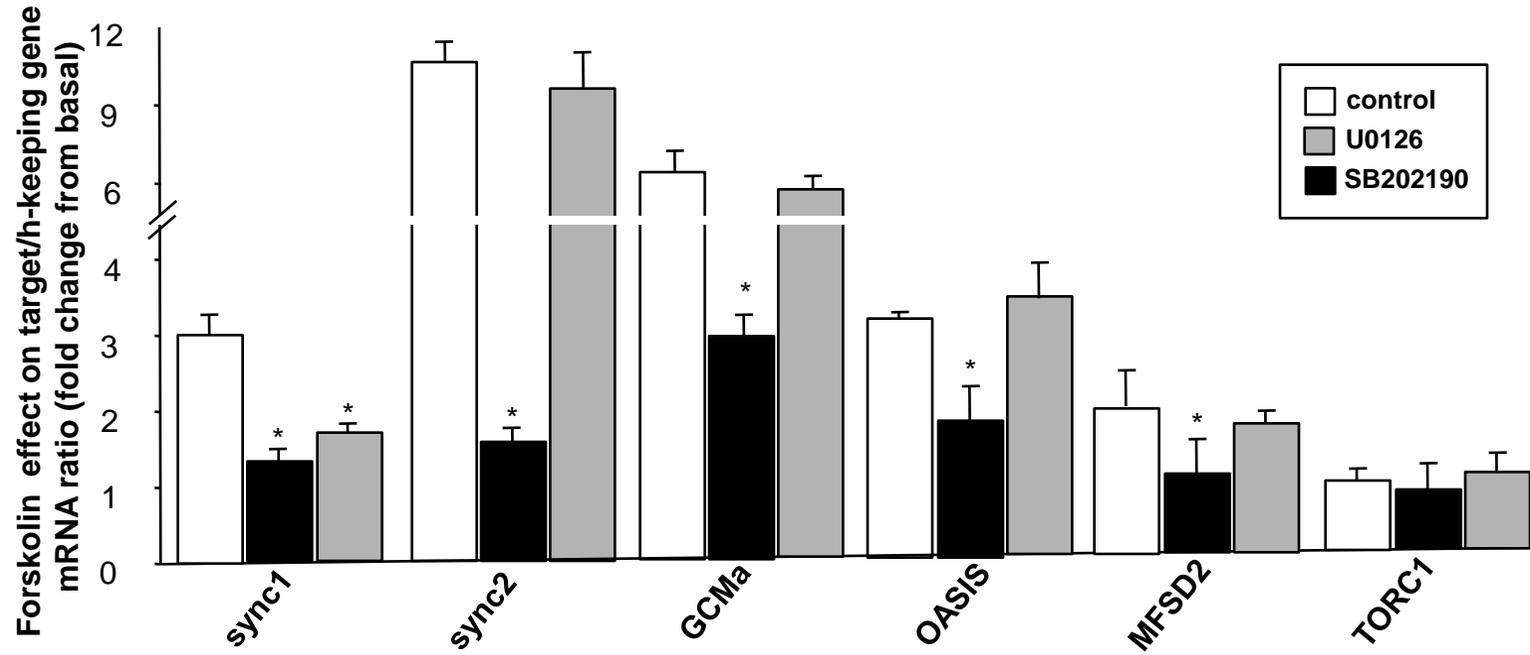


FIGURE 8

