Protein kinases orchestrate cell cycle regulators in differentiating BeWo choriocarcinoma cells

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Abstract

Choriocarcinoma, a trophoblastic neoplasia, occurs in women as an incidence of abnormal pregnancy. BeWo choriocarcinoma cells derived from the abnormal placentation are a suitable model system to study the factors associated with differentiation, invasion and other cellular events as an alternative to clinical samples. Many protein kinases orchestrate the complex events of cell cycle and in case of malignancy such regulators are found to be mutated. In the present study, BeWo cells treated with forskolin (Fo) and phorbol 12-myristate 13-acetate (PMA) were used to study the role of PKA (protein kinase A) and PKC (protein kinase C), respectively, on the expression pattern of differentiation-related genes, membrane markers, PKC isoforms and cell cycle regulators. The effect of Fo and PMA on the cell proliferation was assessed. Progressive induction of alkaline phosphatase level and formation of multinucleated differentiated cells were observed in the cells treated with Fo. Exposure of cells to Fo and PMA induced the mRNA transcripts of α -hCG, β -hCG and endoglin and down-regulates E-cadherin at mRNA and protein levels. Synergistic levels of both up- and down-regulated genes/proteins were observed when cells were treated with the combination of Fo and PMA. The mRNA levels of cyclin D1, cyclin E1, p21, Rb, p53, caspase-3 and caspase-8 decreased gradually during differentiation. Fo significantly inhibited the protein levels of PCNA, Rb, PKC- α and PMA stimulated mRNA expression of PKC- ϵ and PKC- δ . Further, failure in the activation of essential components of the cell cycle machinery caused G2/M phase arrest in differentiating BeWo cells.

Keywords BeWo choriocarcinoma cells · Cell cycle · Differentiation · Forskolin · Phorbol esters · Protein kinases

Introduction

Choriocarcinoma, a gestational trophoblastic neoplasia, occurs in women as a placental disorder. Gestational trophoblastic disease (GTD) develops with failure in common regulatory mechanisms of cells which leads to benign hydatidiform mole, malignant choriocarcinoma, invasive mole or placental site trophoblastic tumour [1, 2]. GTD causes adverse pregnancy outcome including placental abruption, preeclampsia, preterm birth and intrauterine growth restriction [3]. Unlike other forms of GTD, choriocarcinoma initially localized in the uterus and aggressive neoplasm gradually spreads to the other organs, viz. lung, liver and brain, and remain fatal if untreated [4, 5]. Gestational choriocarcinoma produces high levels of β -hCG (human chorionic gonadotropin) even in the absence of embryo. Gestational choriocarcinoma responds to chemotherapy with a cure rate of 80% [6, 7]. Despite a markedly improved prognosis because of chemotherapy, a substantial proportion of patients with

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choriocarcinoma continue to succumb to the disease. The relative rarity of the incidence and clinical background make it difficult to obtain sufficient samples for investigation of the disease [8]. Hence, choriocarcinoma cell lines derived from human spontaneous choriocarcinoma are widely used to study molecular mechanisms underlying syncytialization, adhesion and migration due to constraint use of *in vivo* samples [9].

Trophoblast is considered as a specialized structure having the characteristics of an epithelial cell, involved in the exchange of gases, nutrients and waste across the maternal–foetal interface [10]. The human trophoblast is characterized by a tightly controlled programme of cell proliferation, differentiation, invasion, angiogenesis and apoptosis [11]. During the initial phase of human trophoblast differentiation, the cytotrophoblast cells fuse to form multinucleated syncytiotrophoblasts and largely express human placental lactogen, alkaline phosphatase, hCG, endoglin and syncytin following the repression of E-cadherin in differentiated cells [12–16]. The formation of syncytium, characterized as multiple nuclei sharing one cytoplasm, is the most important hallmark of cytotrophoblast differentiation [17, 18]. However, the mechanism by which villous cytotrophoblast undergoes intercellular fusion and functional differentiation is not clear, although there is unsurpassed evidence that supports the involvement of hormones, growth factors, cytokines, membrane proteins, protein kinases, intracellular proteases and transcription factors [19]. Many protein kinases orchestrate the complex events of cell cycle and differentiation, but in malignancy most of the regulator genes are mutated and exhibit altered levels of mRNA and protein expression [20]. Such changes contribute to deregulation of cell cycle and altered activity of kinases or phosphatases associated with epithelial–mesenchymal transition, aberrant cell division and proliferation [21]. Different phases of cell division (G1, S, G2 and M) function as an intricate system that safeguard as cell cycle checkpoints. The phases are mediated mainly by cyclins, cyclin-dependent kinases (CDK), Rb, p53 and E2F proteins. CDKs are also controlled by the family of CDK inhibitory proteins (INK4) and kinase inhibitory proteins (KIP) [22, 23].

In placental choriocarcinoma cells, syncytiotrophoblast formation is a downstream fusogenic event and the cascade is believed to begin with increased cyclic AMP (cAMP) levels, which occurs via the activation of adenylyl cyclase and the consequent activation of PKA (protein kinase A) that resembles the *in vivo* trophoblast cell differentiation. Forskolin (Fo), an activator of adenylyl cyclase, is used to induce the differentiation of *in vitro* cell models [24]. The role of cAMP in cell cycle growth and cancer development is well established with various cancer cell types [25]. The effects of gonadotropins on ovarian cell proliferation and differentiation are mediated by changes

in intracellular cAMP that activates PKA [26]. Hormonal cAMP regulation of ERK cascade provides an important crosstalk between hormones and growth factor signaling [27]. The cAMP appears to act as both an activator as well as an inhibitor of cell proliferation [28] and effects the production of a variety of proteins including hormones, enzymes, cellular oncogenes and extracellular matrix proteins. Researchers from various laboratories reported that cAMP inhibits the cell growth of adipocytes [29], endothelial cells [30], NIH 3T3 cells [31] and hepatocytes [32] by blocking the activation of ERKs and cascades. The cAMP is also shown to activate multiple intracellular signalling cascades independent of its activation of PKA [28]; however, most of the studies examining cAMP inhibition of ERKs show the requirement for PKA [29–31].

Protein kinase C (PKC), a superfamily of serine/threonine kinases and its isoenzymes, has been shown to display variable expression profiles during cancer progression. PKC isoenzymes are classified into conventional (α , β and γ), novel (δ , ϵ , η and θ) and typical (μ , ξ and τ) forms based on the differences in their N-terminal regulatory domain [33]. PKCs have been traditionally viewed as lipid-sensitive enzymes activated by growth factor and its receptors. PKCs are pharmacologically activated by phorbol esters such as phorbol 12-myristate 13-acetate (PMA), which anchors PKC in their active conformation. Thus, the activation of different types of PKCs depends on the different cellular responses and cross-talks between PKC isoenzymes are specific for particular cell types [34]. PMA stimulates the differentiation of leukaemia cells via activation of MAPK kinase/ERK signaling pathway [35, 36] and used as an agent in ‘differentiation therapy’, a promising strategy for cancer treatment [37]. In intestinal epithelial cells, PKC- α exerts anti-proliferative action in association with ERK activation by inhibiting p21 and p27 during the G1 phase of cell cycle [38]. PKC- δ down-regulates the expression of cyclins causing G1 and G2 phase of cell cycle arrest in lung adenocarcinoma cells [39, 40]. Earlier reports demonstrate the PMA-induced differentiation and production of hCG with the involvement of signal transduction pathways [41] and the activation of both PKA and PKC results in a synergistic increase in the transcription of α and β subunits of hCG in choriocarcinoma cell lines [42, 43]. Although several reports on the role of PKA and PKC in choriocarcinoma are available, the multiple signal transduction pathways involving different types of protein kinases during the differentiation of cytotrophoblast to syncytiotrophoblast are less understood. Hence, in the present study Fo is used to induce the differentiation of human choriocarcinoma cells. Further, the role of PKA activated by Fo and PKC by PMA on the expression pattern of differentiation-related genes/proteins, membrane markers and PKC isoforms and their effects on different cell cycle regulators in BeWo cells are investigated.

Materials and methods

Materials

Nutrient mixture F-12 Ham Kaighn's medium (Ham's F-12K), fetal bovine serum (FBS), penicillin, streptomycin, glutamine, dimethyl sulfoxide (DMSO), trypsin, trypan blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), phenol, DEPC (diethyl pyrocarbonate), sodium acetate, chloroform, isopropyl alcohol, Tris base, EDTA, glacial acetic acid, formaldehyde, Tris-HCl, glycerol and ethidium bromide were purchased from HiMedia (Mumbai, India). Bromophenol blue, acrylamide and bis-acrylamide were procured from Sisco Research Laboratories Pvt. Ltd. (Mumbai, India). T-25 and T-75 culture flasks, 6-well and 96-well culture plates and PCR tubes were purchased from Tarsons (Bengaluru, India). TRIzol reagent, secondary anti-rabbit/

mouse antibodies and oligo forward and reverse primers (Table 1) were designed and were purchased from Sigma-Aldrich (St Louis, USA). Oligo dTs and Superscript III First-Strand cDNA Synthesis kit were obtained from Invitrogen BioServices India Pvt. Ltd. (Bengaluru, India). Taq DNA polymerase (1 U/ μ L) and Luminata Forte Western HRP substrate were procured from Merck-Millipore (Mumbai, India).

Methods

Culturing of BeWo cells

BeWo cells (human choriocarcinoma cell line) was a kind gift by Prof. Satish Kumar Gupta, Reproductive Cell Biology Laboratory, National Institute of Immunology, New Delhi, India and was originally procured from American Type Culture Collection, VA, USA) were cultured with or without

Table 1 Sequence of primers (F: forward and R: reverse) used for the amplification of genes

Gene	Primer sequence (5' → 3')	Annealing temp. (°C)	Product size (bp)
α -hCG	F: GAGAAAGGAGCGCCATGGAT R: ACAAGTACTGCAGTGGCACG	61.5	347
β -hCG	F: GTTCCAGGGGCTGCTGCTGTT R: CGGGAGTCGGGATGGGCTTGG	64	457
E-cadherin	F: CGCATTGCCACATACA R: CGTTAGCCTCGTTCTCA	56	502
Endoglin	F: CGTGGACTGGTTAGTTGA R: AGGCTGTCCATGTTGAGGCAGT	55	416
PKC ϵ	F: CTTCTCGACCCCTACATTGCC R: GCAGGTGCAGACTTGACACTG	61	449
PKC δ	F: CTGCAAGAAGAACAATGGCAAG R: ATCCACGTCCTCCAGGAAATACT	62	96
p53	F: GAGCCCCCTCTGAGTCAG R: GCAAAAACATCTTGTTGAG	56	375
Rb	F: AAGTACCCATCTAGTACT R: AAGTTACAGCATCTCTAAA	58	547
p21	F: GATCACAAGCAGTGGGGTGA R: CTGAGTGACTGCACGACCTT	58	160
CDK-4	F: AGTGGCGGATCCATGGCTACCTCTCGATAT R: TCTCGGAAGCTTTCACTCCGGATTACCTTCA	60	912
Cyclin D1	F: AGACCTGCGCGCCCTCGGTG R: GTAGTAGGACAGGAAGTTGTTG	58	574
Cyclin E1	F: GTCCTGGCTGAATGTATACATGC R: CCCTATTTTGTTCAGACAACAT	60	415
Caspase-8	F: GATATTGGGGAACAACCTGGAC R: CATGTCATCATCCAGTTTGCA	58	366
Caspase-3	F: TTAATAAAGGTATCCATGGAGAACAAC R: TTAGTGATAAAAATAGAGTTCTTTTGAG	59	848
β -actin	F: TACCACTGGCATCGTGATGGACT R: TCCTTCTGCATCCTGTGGCAAT	62	516

Columns 3 and 4 show the different annealing temperatures used and the size of the amplified products, respectively

serum in Ham's F-12K medium supplemented with 10% FBS, penicillin (100 u/mL) and streptomycin (100 u/mL) under the atmosphere of 5% CO₂ and 95% air in a humidified incubator at 37 °C. The 25 cm² culture flask containing 80–90% confluent cells were subcultured into a 96-well plate (3 × 10³ cells/well) or a 6-well plate (3 × 10⁵ cells/well) for the experimental studies.

Differentiation of BeWo cells

BeWo cells were cultured in Ham's F-12K medium supplemented with 10% FBS for 24 h in a CO₂ incubator. For induction of differentiation, the cells were serum starved for 4 h. Further, cells were treated with Fo (50 µM) in serum-free Ham's F-12K medium supplemented with 1X ITS solution containing final concentrations of insulin (10 µg/mL), transferrin (5.5 µg/mL), selenium (0.005 µg/mL) and BSA (500 µg/mL) for different time periods (24, 48 and 72 h).

Assessment of cell viability

The cell viability assay was carried out using MTT as per the protocol described earlier [44] with modifications. BeWo cells (3 × 10³ cells/well) were cultured in a 96-well plate in Ham's F-12K medium and incubated for 24 h in CO₂ incubator prior to exposure for experimental conditions. At the end of experiment 50 µL of MTT solution (2 mg/mL) was added to each well and incubated further for 4 h at 37 °C in CO₂ incubator. The formazan product formed in cells was dissolved using DMSO (100 µL) and the absorbance was measured at 540 nm using PerkinElmer Multimode plate reader (MA, USA).

Alkaline phosphatase assay

BeWo cells (3 × 10⁵ cells/well) were cultured in a 6-well culture plate in Ham's F-12K medium and incubated for 24 h in a CO₂ incubator. Cells were treated with Fo (50 µM) for 24, 48 and 72 h in serum-free Ham's F-12K medium supplemented with 1X ITS solution. After each time point, the conditioned medium was removed, cells were washed with PBS, cell lysate was prepared using lysis buffer [Tris-HCl (50 mM, pH 8.0), NaCl (150 mM), Triton X-100 (0.1%) and PMSF (100 µM)] and total protein concentration was measured by Bradford method [45]. Alkaline phosphatase (ALP) expressed in the control and treated cells was analysed using p-nitrophenylphosphate (p-NPP) according to the method described earlier [46] with modifications and the activity was expressed per mg protein. The sample containing ALP which results in the appearance of yellow colour was measured spectrophotometrically at 405 nm using a PerkinElmer Multimode plate reader (MA, USA).

RNA isolation and RT-PCR

BeWo cells were treated with Fo (50 µM) for 24, 48 and 72 h and in another set of experiments Fo with or without PMA (10 nM) was treated for 72 h by keeping appropriate vehicle control. After each time point, the conditioned medium was removed and total RNA was isolated using TRI Reagent as per the manufacturer's protocol (Sigma-Aldrich). The quality and concentration of RNA isolated were assessed on 1% MOPS-formaldehyde gel. Further, RT-PCR analysis was carried out as per the protocol described earlier [47]. In brief, equal concentrations of total RNA (2 µg) from the control and treated samples were reverse transcribed. The cDNA obtained was subjected to PCR using forward and reverse primers of respective genes (Table 1). Amplified PCR products were analysed by agarose gel electrophoresis. Relative mRNA levels were quantified using image analysis software (ImageJ—<https://imagej.nih.gov/ij/>).

Western blot analysis

BeWo cells were treated with Fo (50 µM) for 24, 48 and 72 h and in another set of experiments Fo with or without PMA (10 nM) was treated for 72 h by keeping appropriate vehicle control. After each time point, the conditioned medium was removed and total protein was isolated using cell lysis buffer. Protein concentration was measured by Bradford's method [45] and subjected to western blot analysis as described earlier [48]. Equal concentrations of protein (40 µg/lane) samples were electrophoresed on SDS-PAGE and transferred onto a PVDF membrane. Membrane was blocked for 2 h and incubated with primary antibody (1:500 dilutions of rabbit anti-β-hCG/ALP/PKC-α/Rb and 1:1000 dilution of mouse anti-E-cadherin/PCNA/GAPDH) overnight at 4 °C. Further, the membrane was washed using TBST, incubated with anti-rabbit/mouse secondary antibody-HRP (1:1000) for 2 h and washed 3–4 times. Protein bands were visualized using a Luminata Forte Western HRP substrate in a Syn-gene Gel Documentation system (MD, USA) and quantified using image analysis software (ImageJ—<https://imagej.nih.gov/ij/>) [β-hCG, ALP and PKC-α antibodies were procured from neobiolab (MA, USA) and Rb was procured from Santa Cruz Biotech (CA, USA). PCNA and GAPDH antibodies are kind gift by Prof. A. J. Rao, Dept. of Biochemistry and E-cadherin by Prof. Annapoorni Rangarajan, Dept. of MRDG, IISc, Bengaluru].

Cell cycle analysis

BeWo cells were treated with or without Fo and PMA for 72 h. Flow cytometry analysis was performed as previously described [49]. Cells were harvested, washed with PBS and fixed in 70% cold ethanol for 1 h. Further, cells were washed

with PBS and suspended in RNase solution (200 µg/mL) for 30 min incubation at 37 °C. The samples were stained with propidium iodide (PI) (50 µg/mL) and analyzed using a Becton Dickinson FACS Accuri 6 plus flow Cytometry system.

Statistical analysis

The experimental data are shown as mean $\pm$ standard deviation from three independent experiments. Statistical analyses were done by Student's *t* test and one-way ANOVA followed by Tukey's post hoc test. Values were considered statistically significant at $*P < 0.05$ and $**P < 0.005$ compared to control, $^{\#}P < 0.05$ compared to Fo-treated sample and $^{\$}P < 0.05$ when compared to PMA-treated sample.

Results

Forskolin decreases the proliferation of BeWo cells with increase in time intervals

To study the effect of Fo on cell proliferation, BeWo cells were treated with or without Fo and PKA inhibitor. Cells treated with 1 and 10 µM Fo showed a maximum increase in the proliferation of about 12 and 5%, respectively, at 24 and 48 h. The cell number gradually decreased and attained the level of control at 24 h and a significant decrease of 10% in the cell number was observed at 100 µM at the time point of 48 h. In cells treated for 72 h, proliferation was decreased in a dose-dependent manner and a statistically significant decrease of 30%

was observed at 100 µM concentration of Fo (Fig. 1a). PKA inhibitor alone was found to have no effect on the cell growth. However, an increase in the proliferation was observed in the cells treated with Fo + PKA inhibitor compared to the cells treated with Fo alone (Fig. 1b).

PMA decreases the proliferation of BeWo cells

To study the effect of PMA on cell growth, BeWo cells were treated with different concentrations of PMA. Cell proliferation was decreased with increase in the concentration and statistically a significant decrease of 10% at 10 nM and an almost 20% decrease in cell proliferation were observed at higher PMA concentrations (100 nM and 1 µM) (Fig. 2).

Forskolin induces alkaline phosphatase in BeWo cells

To confirm the action of Fo-mediated differentiation in BeWo cells, the alkaline phosphatase activity was measured. In the presence of Fo (50 µM), cellular alkaline phosphatase level increased significantly with an increase in the time interval compared to control. The cells treated with Fo alone induce the ALP level by 18 and 62% at 24 and 48 h, respectively. Progressively, the exposure of cells to Fo for 72 h show time-dependent induction of ALP enzyme level (Fig. 3a) and a twofold increase in ALP protein expression was also observed at 72 h (Fig. 3b).

Fig. 1 Effect of Fo on the proliferation of BeWo cells. BeWo cells (3×10^3 cells/well) were treated with or without different concentrations of **a** Fo (1–100 µM) for different time intervals (24, 48 and 72 h) and in another group; **b** cells were treated with Fo alone (50 µM), PKA inhibitor (0.1 µM) alone and Fo + PKA inhibitor for 72 h and

cell growth was measured by MTT assay. Results were expressed as % cell viability compared to control (mean $\pm$ SD, $n = 4$). Values are significantly different from control at $*P < 0.05$ as analysed using Student's *t* test

Fig. 2 Effect of PMA on the proliferation of BeWo cells. BeWo cells (3×10^3 cells/well) were treated with or without different concentrations of PMA (1 nM–1 μ M) in a 96-well plate for 72 h and cell growth was measured by MTT assay. Results were expressed as % cell viability compared to control (mean $\pm$ SD, $n=4$). Values are significantly different from control at $*P<0.05$ as analysed using Student's t test

Forskolin induces α -hCG, β -hCG and endoglin and down-regulates E-cadherin mRNA expression in BeWo cells

To assess the effect of Fo on the differentiation of BeWo cells, the mRNA levels of α -hCG, β -hCG, endoglin and E-cadherin were analysed by semi-quantitative RT-PCR. BeWo cell differentiation is associated with increased synthesis and secretion of hCG. Choriocarcinoma cells show increasing amounts of hCG in response to Fo. The cells treated with Fo (50 μ M) induced α -hCG mRNA expression 2.1-fold at 24 h and remain almost similar to the 72-h treatment. However, a time-dependent increase in the mRNA levels of β -hCG and endoglin was observed and a maximum

increase of 4.3- and 1.3-fold was observed at 72 h when compared to control (Fig. 4a). E-cadherin, a cell adhesion molecule, was down-regulated by 15 and 42% at 24 and 72 h, respectively, in the fusing cells (Fig. 4a). Further, microscopic observations of multinucleated cells confirm the differentiation of BeWo cells (Fig. 4b).

Down-regulation of PKC- ϵ and PKC- δ transcripts in differentiating BeWo cells

BeWo cells treated with Fo to confirm its action on the mRNA expressions of PKC- ϵ and δ . Cells treated with Fo progressively inhibit the expression of both PKC- ϵ and δ mRNAs. Initially, at the 24-h time point PKC- ϵ shows marginal induction and decline in expression at the 72-h time point by 60% compared to the 0-h treatment. In contrast, Fo down-regulates PKC- δ mRNA levels in a time-dependent manner with a maximum decrease of 32% at 72 h (Fig. 5).

Forskolin regulates mRNA expression of cell cycle regulators in BeWo cells

To study the effect of Fo on apoptotic and cell cycle regulators in BeWo cells, the mRNA levels were analysed by semi-quantitative RT-PCR. Cells treated with Fo for different time intervals progressively down-regulates the mRNA expressions of CDK-4, p21, p53, Rb and caspase-3 in a time-dependent manner. However, cyclin D1, cyclin E1 and caspase-8 were induced initially at the 24-h time point and the increase in treatment time gradually reduced their expression compared to the control cells (Fig. 6). Cyclin D1 and cyclin E1 mRNA levels were induced by 22 and 16% at 24 h and 16 and 10% at 48 h, respectively. Further, at 72 h, the mRNA levels of cyclin D1 and cyclin E1 decreased to 12 and 6%, respectively, compared to control.

Fig. 3 Effect of forskolin on alkaline phosphatase **a** enzyme activity and **b** protein expression in BeWo cells. BeWo cells (3×10^5 cells/well) were treated with or without Fo (50 μ M) for different time intervals (24, 48 and 72 h). Results were expressed as alkaline phosphatase activity/mg protein and as relative expression compared to control (mean $\pm$ SD, $n=3$). Values are significantly different from control at $**P<0.05$ as analysed using one-way ANOVA followed by Tukey's post hoc test

Fig. 4 Effect of Forskolin on **a** the expression of α -hCG, β -hCG, endoglin and E-cadherin and **b** the morphology of differentiating BeWo cells. BeWo cells (3×10^5 cells/well) were treated with Fo (50 μ M) for different time intervals. The expressions of amplified genes were analysed on 1% agarose gel and β -actin was used as a positive control and for normalization. The bar graph represents the densitometric analysis of mRNA levels (mean $\pm$ SD, $n=3$). Differences in mRNA levels are statistically significant at $*P<0.05$ compared with 0-h values as analysed using one-way ANOVA followed by Tukey's post hoc test

Fig. 5 Effect of forskolin on the expression of PKC- ϵ and PKC- δ in BeWo cells. BeWo cells (3×10^5 cells/well) were treated with Fo (50 μ M) for different time intervals. The expressions of amplified genes were analysed on 1% agarose gel and β -actin was used as a positive control and for normalization. The bar graph represents the den-

sitometric analysis of mRNA levels (mean $\pm$ SD, $n=3$). Differences in mRNA levels are statistically significant if $*P<0.05$ compared with the 0-h values as analysed using one-way ANOVA followed by Tukey's post hoc test

CDK4 mRNA levels were down-regulated by 10, 22 and 30% at the 24-, 48- and 72-h time points, respectively. The major tumour suppressor genes were found to be down-regulated with an increase in time of exposure to Fo. p21 and p53 genes were down-regulated time dependently almost 10 and 25% at 48 h and showed a more than 50% decrease at 72 h. While Rb mRNA expression decreased 10% up to 48 h and recorded a 60% reduction in its level compared to control. Fo induced the mRNA levels of caspase-8 by 20% at the 24-h time point and drastically inhibited the same at the 48- and 72-h time points by 10

and 45%, respectively. However, caspase-3 clearly showed 15, 40 and 65% time-dependent inhibition of mRNA levels with the 24-, 48- and 72-h treatments, respectively.

Fig. 6 Effect of forskolin on the mRNA expression of apoptotic and cell cycle regulators in BeWo cells. BeWo cells (3×10^5 cells/well) were treated with Fo (50 μ M) for different time intervals. The expressions of amplified genes were analysed on 1% agarose gel and β -actin was used as a positive control and for normalization. The bar graph represents the densitometric analysis of mRNA levels (mean $\pm$ SD, $n = 3$). Differences in mRNA levels are statistically significant if $*P < 0.05$ compared with the 0-h values as analysed using one-way ANOVA followed by Tukey's post hoc test

Forskolin and PMA synergistically induce the hCG mRNA levels and down-regulates E-cadherin mRNA expression; and PMA induces and Fo inhibits the mRNA expression of PKC- ϵ and PKC- δ in BeWo cells

To study the combinatorial effect of Fo and PMA on BeWo cells, cells were treated with Fo alone, PMA alone and Fo+PMA for 72 h and the mRNA levels of α -hCG, β -hCG, endoglin, E-cadherin, PKC- ϵ and PKC- δ are analysed by RT-PCR. The cells treated with Fo alone and PMA alone showed an increase in the expression of the mRNA levels of α -hCG and β -hCG and down-regulated E-cadherin mRNA expression compared to control. However, cells treated with Fo+PMA synergistically induced the expression of both α -hCG and β -hCG and reduced E-cadherin expression compared to the Fo- and PMA-alone treatment (Fig. 7a). PKC- ϵ and PKC- δ show induced mRNA expression only in PMA treatment and Fo significantly decreases the above mRNA transcripts compared to control (Fig. 7b).

Regulation of cell cycle regulator transcripts by Fo and PMA in BeWo cells

To study the individual and combinatory action of PKA and PKC on mRNA expressions of apoptotic and cell cycle regulators in BeWo cells, Fo and PMA alone and in combination were treated for 72 h and the mRNA levels were analysed by RT-PCR. Cells treated with Fo alone decreased the cyclin D1 mRNA by 40% and the cells treated with PMA alone showed a marginal increase in cyclin D1. And the cells treated with Fo+PMA down-regulated the cyclin D1 mRNA level by more than 35% when compared to control and the expression was similar to the Fo-treated sample. However, the CDK-4 level recorded a 10% decrease in Fo- and PMA-alone treatments and the combination treatment showed 20% inhibition of CDK-4 mRNA expression compared to control. The expression of tumour suppressor genes studied here exhibited decreased expression in all the three treatments, in which Fo and PMA alone inhibit p21 expression by 33 and 38%, respectively, and with the combination

Fig. 7 Effect of forskolin and PMA on the mRNA expression of **a** α -hCG, β -hCG, endoglin, E-cadherin, **b** PKC- ϵ and PKC- δ in BeWo cells. BeWo cells (3×10^5 cells/well) were treated with Fo (50 μ M), PMA (10 nM) and Fo + PMA for 72 h. The expressions of amplified genes were analysed on 1% agarose gel and β -actin was used as a positive control and for normalization. The bar graph represents the densitometric analysis of mRNA levels (mean $\pm$ SD, $n = 3$). Differences in mRNA levels are statistically significant if * $P < 0.05$ compared to control, # $P < 0.05$ compared to Fo and $^S P < 0.05$ compared to PMA as analysed using one-way ANOVA followed by Tukey's post hoc test

treatment a 55% decrease is observed compared to control. Also the level of p53 mRNA was decreased by 38, 15 and 37%, respectively, in the cells treated with Fo alone, PMA alone and Fo + PMA. Further, Fo decreases Rb mRNA levels by 53% and Fo + PMA showed only 30% inhibition; however, a marginal decrease by 10% was observed in the cells treated with PMA alone when compared to control. The mRNA level of caspase-8 was reduced by 30% in the cells treated with Fo alone and PMA alone, and an 18% decrease was observed in the cells treated with Fo + PMA when compared to control (Fig. 8).

Fo and PMA regulate protein expressions of β -hCG, PKC- α , E-cadherin, PCNA and Rb in BeWo cells

Functional levels of any genes after being transcribed into protein are analysed by western blotting. The protein levels of β -hCG, PKC- α , E-cadherin, PCNA and Rb were analysed following the treatment with Fo and PMA in BeWo cells. Cells treated with Fo for different time intervals showed decreased levels of E-cadherin, PCNA and Rb proteins with the increase in time (Fig. 9a). The cells treated with Fo + PMA induced the expression of β -hCG twofold compared to Fo alone and PMA increased β -hCG levels marginally compared to control. However, PKC- α decreased marginally in Fo treatment and a significant decrease was observed in the cells treated with PMA alone and Fo + PMA. Further, E-cadherin and Rb proteins'

expressions were down-regulated significantly in the cells treated with Fo alone and PMA alone, while in the cells treated with Fo + PMA both proteins are inhibited with negligible expressions. The expression of PCNA protein was reduced in the cells treated with Fo alone, while there was no change observed in the PCNA protein level in the cells treated with PMA alone and Fo + PMA (Fig. 9b).

Forskolin and PMA induce G2/M phase cell cycle arrest in differentiating BeWo cells

The role of Fo and PMA in regulating cell cycle progression was studied by flow cytometry. Cells were treated with Fo alone, PMA alone and Fo + PMA for 72 h. The cells without any treatment were considered as control. Results presented in Fig. 10 showed the DNA content of control cells with a sharp peak at the G0/G1 phase corresponding to 52.3% of cells and a slight peak with 29.2% cells at the G2/M phase. Following treatment with Fo and PMA, DNA content at the G0/G1 phase decreased to 38.8 and 40%, while a gradual increase in the DNA content of 38.3 and 33.3% at the G2/M phase was observed in differentiated cells. Consistent with the syncytial markers' expression, the increase in DNA content in the G2/M phase (46.8%) and a slight peak with 28.3% at the G0/G1 phase of cell cycle demonstrate the functional regulation of differentiation by Fo and PMA in BeWo cells.

Fig. 8 Effect of forskolin and PMA on the mRNA expression of apoptotic and cell cycle regulators in BeWo cells. BeWo cells (3×10^5 cells/well) were treated with Fo (50 μ M) and PMA (10 nM) and Fo + PMA for 72 h. The expressions of amplified genes were analysed on 1% agarose gel and β -actin was used as a positive control and for normalization. The bar graph represents the densitometric analysis of mRNA levels (mean $\pm$ SD, $n = 3$). Differences in mRNA levels are statistically significant if * $P < 0.05$ compared to control, # $P < 0.05$ compared to Fo and \$ $P < 0.05$ compared to PMA as analysed using one-way ANOVA followed by Tukey's post hoc test

Discussion

Cancer is the multi process controlled phenomena and carcinoma derived cancers are highly prevalent malignancies causing a deadly disease. The survival rates vary dramatically among different type of cancer. Although development of targeted therapeutics has been improved in cancer treatment over the years, the harsh reality is that the “War on Cancer” still continues [50]. In the present study, human BeWo choriocarcinoma cells are used to investigate the action of PKA and PKC, the members of protein kinase superfamily which are involved in controlling the regulatory proteins. These regulatory proteins following the phosphorylation activate signal transduction cascades implicated in cellular functions and bring down the desired response. Choriocarcinoma cells are derived from the abnormal placentation and are an appropriate in vitro model system to study the factors associated with differentiation, invasion and other cellular events as an alternative of clinical samples. Initially, cell proliferation was monitored by treating the cells with different concentrations of Fo and PMA for specific

time periods. The cells treated with Fo and PMA showed a decrease in their proliferation with respect to time and concentration which are concurrent with the earlier findings [51, 52], where Fo significantly reduced the proliferation of BeWo cells in a time-dependent manner and a decrease in the proliferation correlates with the growth-restricted process of cellular differentiation. In parallel, the PKA inhibitor overcomes the Fo effects, which is consistent with the available reports on PKA activation by Fo in choriocarcinoma cells which induces cytotrophoblast cell differentiation into syncytiotrophoblast. PMA decreased the cell proliferation and suggested a clear indication of the differentiation of cells with loss of proliferation as reported in leukaemia HEL and K562 cells signifying the role of steady-state maintenance of mitochondrial functions in the regulation of differentiation by PMA [53]. Further, the results of the expression level and enzymatic activity of alkaline phosphatase correlate with the progression of Fo-mediated cAMP-induced cell fusion [43].

Morphological changes observed during the treatment of cells with Fo clearly reflect the differentiation process. In association with the morphological changes, expressions

Fig. 9 Effect of forskolin and PMA on the E-cadherin, PCNA, Rb, β -hCG and PKC- α protein levels in BeWo cells. BeWo cells (3×10^5 cells/well) were treated with Fo (50 μ M) for different time intervals (24, 48 and 72 h). In another treatment group, cells were treated with Fo (50 μ M), PMA (10 nM) and Fo + PMA for 72 h. GAPDH was used as a positive control and for normalization. The bar graph represents the densitometric analysis of protein levels (mean $\pm$ SD, $n = 3$). Differences in protein levels are statistically significant if * $P < 0.05$ compared to control, # $P < 0.05$ compared to Fo and \$ $P < 0.05$ compared to PMA as analysed using one-way ANOVA followed by Tukey's post hoc test

of important biochemical markers of differentiation were studied. In differentiated cells, the expression of hCG was associated with a cAMP-dependent PKA pathway [24, 54] and hCG is a glycoprotein that consists of two dissimilar subunits, α and β held together by non-covalent interactions. Biosynthesis of α subunit which is common to other glycoprotein hormones and unique subunit β -hCG in pregnant animal, placental tissue and human choriocarcinoma cell lines is extensively validated [54, 55]. In differentiating BeWo cells, the mRNA expression of α -hCG was induced early by 24 h compared to β -hCG expression which shows a clear increase in mRNA transcript over time and induced over a

period of 72 h suggesting that the production of β -hCG is strictly dependent on syncytium formation (fusion of cells) [51, 56]. Moreover, silencing of α/β -hCG in choriocarcinoma cells decreases cell fusion via down-regulation of PKA activation establishing the significance of hCG in the fusion of BeWo cells [57]. In addition, endoglin, a component of TGF- β receptor system, showed a gradual induction of its mRNA transcript in Fo-treated BeWo cells. Similarly, endoglin expressed in endothelial cells, leukemic cells and syncytiotrophoblast is validated as a distinctive differentiation marker [14, 58]. Endoglin is also found to be an active regulator of invasive pathway during human trophoblast

Fig. 10 Effect of forskolin and PMA on cell cycle progression in BeWo cells. BeWo cells (3×10^5 cells/well) were treated with Fo (50 μ M), PMA (10 nM) and Fo+PMA for 72 h. **a** Flow cytometry

analysis was performed by PI staining and **b** results were expressed as % of DNA content in different phases of cell cycle

differentiation [59] and in agreement with the statement cadherin family of transmembrane glycoprotein E-cadherin, a cell adhesion molecule involved in differentiation and invasion, down-regulated its mRNA and protein levels in Fo-treated cells progressively with an increase in time intervals. This is probably due to loss of E-cadherin on the surface of fusing cells which is mediated by cAMP in BeWo cells [16].

In some cells, the activation of the cAMP pathway leads to the inhibition of receptor-mediated phospholipase C (PLC) stimulation. In contrary, stimulation of PLC may activate the wide range of PKCs that affect the magnitude of cAMP response mediated via other receptors [60]. In our report, PMA up-regulates PKC- ϵ and δ mRNA transcripts, whereas Fo alone and in combination with PMA inhibits the expression of both isoforms in differentiated cells. However, PMA fails to activate and Fo alone and in combination with PMA down-regulated PKC- α protein levels. These observations clearly suggest the involvement of cAMP-mediated PLC inhibition in the down-regulation of PKC isoform levels in BeWo cells. Omata et al. using BeWo cells demonstrated that cell fusion and differentiation are mediated by both

the PKC and PKA pathways, in which the PKC pathway activated by PMA alone can induce major differentiation markers and cell fusion as in the case of Fo-PKA-mediated syncytia. The syncytium synergistically amplified when both protein kinase C and A pathways were activated together and suggests that the two parallel pathways may interact at multiple levels in the signalling cascade [41, 61]. In compliance with the earlier work, the expression of α - and β -hCG levels induced in Fo/PMA-mediated cell differentiation and the same mRNA transcripts were synergistically increased when the cells are activated together and the β -hCG expression attains the same status in both mRNA and protein levels [42]. Synergistic mRNA and protein levels were observed not only in the up-regulated markers but also imply with E-cadherin which is down-regulated double the time when the cells are treated with Fo or PMA alone and with the combination of Fo and PMA.

The most critical step during the transition of cells from proliferating to attaining the differentiation stage is the inhibition of DNA synthesis [62]. Accordingly, proliferating cell nuclear antigen (PCNA), an essential component

of the cell cycle machinery that coordinates with cyclins to initiate DNA synthesis, showed decreased mRNA and protein levels in differentiated BeWo cells [63]. PCNA and cyclin expressions are rich in proliferating cancer cells and higher levels of PCNA were observed in the invasive hydatidiform mole placenta [64] and overexpression of cyclins in cancer cells correlates with the increase in malignancy [65]. These findings suggest that the intrinsic control could be a crucial factor that prevents the onset of malignancy and allows normal pregnancy to ensue. Increased expression of cyclins reflects the proliferating ability of the cells, whereas lower levels relate to differentiation [12, 23]. Increased cell numbers by Fo at 24-h treatment are in agreement with the induced expressions of cyclin D1 and cyclin E1 mRNA transcripts and their levels decreased over the next 48 and 72 h during which syncytialization would be occurring. PMA alone has no effect on cyclin D1 and CDK-4 mRNAs but Fo performs a similar function even in the presence of PMA [21, 40]. It was observed that there was no evidence of CDK-4 induction and its down-regulation reflects its inactivation status preceding syncytialization. Also decreased expressions of the CDK inhibitor p21 and the tumour suppressors p53 and Rb during differentiation appeared to be resistant towards apoptosis [66]. The Rb protein expression in Fo treatment reduced gradually following syncytia and only marginal levels were observed with PMA-treated BeWo cells. However, Rb protein levels completely diminished in the combinatorial activation of PKA and PKC but not with the transcript levels [37].

Syncytialization was associated with the increased levels of the anti-apoptotic and decreased expression of pro-apoptotic molecules. The present study also shows reduced levels of caspase-3 and caspase-8 transcripts in Fo-mediated differentiated cells. This is in agreement with the earlier results shown by Wei et al. [66]. Moreover, PMA exposure also decreased caspase-8 mRNA transcripts and expression reflects also in combination with Fo. Altered expressions of these transcripts or proteins bring down changes in cellular functions and failure in any of the cell cycle events disrupts the nuclear content of the cells and it happens even with the differentiated cells [62]. Overall DNA content in the G0/G1 phase reduced with progressive differentiation of cells induced by Fo/PMA and simultaneously the G2/M phase shows a dramatic increase in DNA content following syncytia in both Fo- and PMA-treated cells. Intensity of nuclear content is more in combination and suggests the G2/M phase of cell cycle arrest during differentiation. Our study provides an insight into the regulation of cell cycle players and coordinated role of protein kinases as an evidence of BeWo choriocarcinoma cell differentiation, and these agents can also be used as internal regulators in preventing cancer by differentiation therapy approach. Further, the optimization of

this approach requires an improved understanding of interactions between these classes of agents in cellular functions.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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