Placental leucine aminopeptidase/oxytocinase gene regulation by activator protein-2 in BeWo cell model of human trophoblast differentiation

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Received 19 June 2003; accepted 16 July 2003

First published online 27 August 2003

Edited by Julio Celis

Abstract Placental leucine aminopeptidase (P-LAP) is located preferentially in syncytiotrophoblasts in human placenta. Here we investigated P-LAP expression and the regulatory mechanisms in BeWo choriocarcinoma cells with forskolin (FSK)-induced differentiation. Morphologically differentiated cells revealed enhanced P-LAP staining. FSK significantly increased P-LAP activity and mRNA. Deletion or mutation of activator protein-2 (AP-2) binding site in the footprint-3 (−216 to −172) of P-LAP promoter abrogated the stimulatory effects of FSK on luciferase activity of the construct −216/+49. In AP-2-deficient Hep-G2 cells, FSK failed to stimulate luciferase activity of the construct −216/+49. Among the isoforms, BeWo expressed AP-2α and AP-2γ, while FSK increased only AP-2α. These results suggest differentiation-dependent P-LAP expression in trophoblasts, which involves increased AP-2α binding.

Key words: Aminopeptidase; Cell differentiation; Gene regulation; Oxytocin; Placenta

1. Introduction

During placental development, cytotrophoblasts differentiate and fuse to syncytiotrophoblasts. Biochemical differentiation including the synthesis of trophoblast-specific hormones occurs in parallel with this morphological differentiation. Several lines of evidence suggest that cAMP plays a significant role in stimulating both morphological and biochemical differentiation [1,2]. Evidence that intracellular cAMP levels rise prior to the fusion of cytotrophoblasts [3] and that cAMP or its analogues stimulate human chorionic gonadotrophin (hCG) production, a good marker of trophoblast differentiation [4,5], supports this notion.

Placental leucine aminopeptidase (P-LAP) is a predominant oxytocinase in the placenta and maternal serum [6,7], which is involved in the maintenance of pregnancy via regulating oxytocin levels. Immunohistochemistry shows predominant localization of P-LAP to syncytiotrophoblasts, but little or no positive staining in cytotrophoblasts [8,9], suggesting that P-LAP expression depends on trophoblast differentiation. However, no in vitro studies have been performed to confirm this observation. We isolated genomic clones containing the 5′-upstream region of the P-LAP gene [10] and found that transcription factor activator protein-2 (AP-2) and Ikaros cooperatively enhance P-LAP transcription in trophoblastic cells [11,12]. In addition to the association with development and differentiation of adipocytes and keratinocytes [13,14], AP-2 also plays a critical role in spontaneous and cAMP-stimulated trophoblast differentiation, resulting in the increase of hCG and human placental lactogen (hPL) expression [15–17]. The AP-2 family consists of at least three different genes referred to as AP-2α, AP-2β, and AP-2γ [18–20], among which AP-2α and AP-2γ are expressed in human placenta [21]. Our previous study also suggested an association of AP-2α and AP-2γ with the basal expression of P-LAP [12]. AP-2α mRNA has been shown to increase during the differentiation of human trophoblasts [21,22], but to date, changes in AP-2 isoforms at the protein level have not been confirmed.

These findings led us to hypothesize that P-LAP expression is regulated in a differentiation-dependent manner involving AP-2 binding in trophoblastic cells. To test this hypothesis, we investigated the changes in P-LAP expression and its accompanying regulatory mechanisms using BeWo choriocarcinoma cells exposed to adenylate cyclase activator forskolin (FSK) as a model for trophoblast differentiation [1].

2. Materials and methods

2.1. Cell culture

BeWo (ATCC CCL-98) and Hep-G2 (ATCC HB 8065) cells were grown in RPMI 1640 (Sigma) and Dulbecco's modified Eagle's medium (Sigma), respectively, supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in an atmosphere with 5% CO_2.

2.2. FSK treatment and P-LAP enzymatic activity

BeWo cells were treated with water-soluble FSK (FSK-NKH477, a gift from Nippon Kayaku Co, Ltd.) at 50 μM as described [23]. The amount of hCG secreted in the supernatant of BeWo cell cultures was determined using an hCG-CTP-ELISA kit (Wako). P-LAP activity was measured as previously described [6].

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Abbreviations: P-LAP, placental leucine aminopeptidase; FSK, forskolin; AP-2, activator protein-2

0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)00897-4

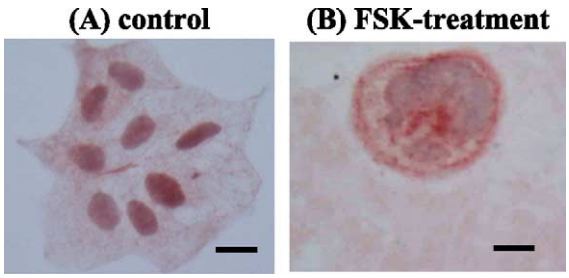

Fig. 1. Immunocytochemistry of P-LAP in (A) control and (B) FSK-treated BeWo cells. Control cells were cultured in serum-free media for 48 h and FSK-treated cells were exposed to FSK (50 μM) for 48 h. Scale bar is 15 μm.

2.3. Immunocytochemistry

BeWo cells were cultured in four-well chamber glass slides (Lab-Tek) with or without FSK. Immunocytochemical staining was performed as previously described [23] using rabbit anti-P-LAP polyclonal antibody (1:500 dilution) [24] and anti-AP-2α, AP-2β and AP-2γ polyclonal antibodies (Santa Cruz; 1:200 dilution). For negative controls, the primary antibody was replaced by a non-specific IgG at the same dilution.

2.4. RNA preparation and semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)/Southern blot procedure

Total RNA (1 μg) isolated from cells using an RNeasy kit (Qiagen) was reverse-transcribed using 2.5 μM random hexamers (Applied Biosystems) in 20 μl reactions. P-LAP was amplified by PCR in 50 μl mixtures under the conditions consisting of 94°C for 30 s, 62°C for 30 s and 72°C for 30 s using 1 μl aliquots of RT reaction products and the following primers: P-LAP sense (5′-GGGCACAGATCAGGCTTCCCACT-3′) and P-LAP anti-sense (5′-GATCTCAGCTTGTTTTTCTTGGCTTG-3′). β-actin was amplified by RT-PCR using sense (5′-AACCGCGAGAAGATGACCCAG-3′) and anti-sense (5′-CTCCTGCTTGCTGATCCACAT-3′) primers under the same PCR conditions as used for P-LAP. The PCR products (10 μl) per lane were resolved by electrophoresis on 1.0% agarose gels, then transferred to Hybond-N^+ nylon membranes (Amersham Pharmacia Biotech). Southern hybridization proceeded using [^{32}P]P-LAP cDNA and [^{32}P]β-actin cDNA as probes. P-LAP mRNA levels were normalized by β-actin expression measured with a BAS 2000 Bioimage Analyzer (Fuji Photo Film) after autoradiography.

2.5. Construction of luciferase reporter plasmids

The following P-LAP promoter-luciferase constructs were constructed by subcloning PCR-derived fragments into the pGL3-Basic vector (Promega) at the *Kpn*I site as previously described [11,12]: −216/+49 (includes Ikaros and AP-2 binding sites), −216M1 (functional for Ikaros and mutant for AP-2 binding), −216M3 (mutant for both Ikaros and AP-2 binding), and −172/+49 (a construct that excludes both sites).

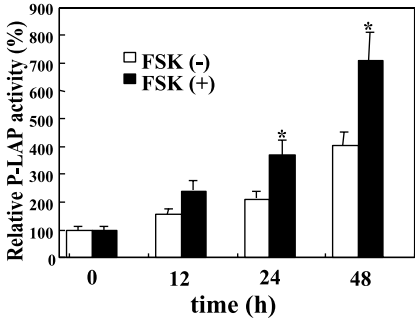

Fig. 2. Induction of P-LAP activity in BeWo cells by FSK. Results are shown as a percentage of activity present before FSK treatment. Data are expressed as means ± S.D. of triplicate samples from at least three independent experiments. * indicates comparison with control is significant at $P < 0.05$ by the Mann–Whitney *U*-test.

2.6. Transfections and luciferase assay

Transient transfections were performed using the LipofectAMINE PLUS™ Reagent (Life Technologies) in six-well plates. Firefly luciferase reporter plasmid DNA (1 μg) and 0.1 μg of pRL-TK plasmid DNA (internal control to normalize transfection efficiency) were transiently co-transfected into cultured cells. Twenty-four hours after transfection, cells were incubated with or without 50 μM of FSK for an additional 12 h and passively lysed with 500 μl of lysis buffer (Promega). Firefly and renilla luciferase activities were measured using a dual-luciferase reporter assay system (Promega). The fold increase by FSK in firefly luciferase activity, normalized by renilla luciferase activity, was calculated.

2.7. Statistical analysis

All experiments were performed at least three times, and assays

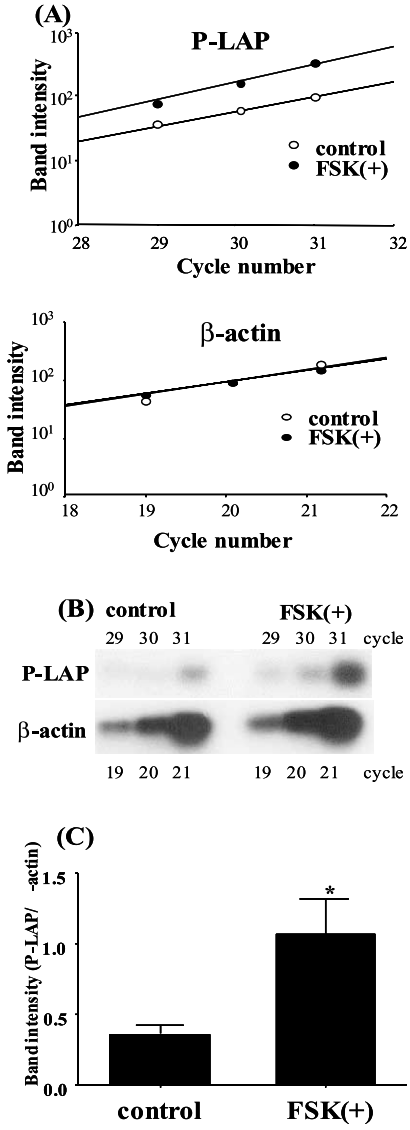

Fig. 3. Induction of P-LAP mRNA in BeWo cells by FSK. Cells were treated for 24 h in serum-free medium with 50 μM FSK. Total cellular RNA was extracted and subjected to semi-quantitative RT-PCR. A: Graphical representation of signal intensity after autoradiography of PCR products over the indicated number of cycles of RT-PCR. B: Autoradiographs of the semi-quantitative RT-PCR analysis for P-LAP and β-actin mRNA. C: Signal intensity ratio of P-LAP to β-actin mRNA. Values represent means ± S.D. of three independent experiments. * indicates comparison with control is significant at $P < 0.05$ by the Mann–Whitney *U*-test with Boneferroni correction.

were performed in triplicate. Data are expressed as means ± S.D. Since the data were not normally distributed, we employed non-parametric statistics. Comparisons between groups were made with a Mann–Whitney *U*-test for two independent samples and Boneferroni correction for multiple comparisons. Differences were considered significant when the *P* value was < 0.05.

3. Results

3.1. Induction of P-LAP expression in FSK-treated BeWo cells

The hCG concentration of the BeWo cell culture supernatant was significantly higher in FSK-treated cells than in non-treated cells (24 h, nine-fold, $P < 0.01$; 48 h, 13-fold, $P < 0.01$; data not shown), suggesting that the biochemical differentiation of BeWo cells was induced by FSK treatment. Immunocytochemical analysis showed that 48-h FSK-treated cells had enlarged and lobed nuclei and also had higher P-LAP immunoreactivity on the cell surface compared to non-treated cells, which had uniform and small nuclei (Fig. 1). FSK exposure provided a small, non-significant elevation in P-LAP activity during the first 12 h of culture, but significantly increased P-LAP activity after 24 h and 48 h compared to control cells (Fig. 2).

3.2. Effects of FSK on P-LAP mRNA

BeWo cells were incubated with FSK to examine whether P-LAP activity induction leads to an increase of P-LAP mRNA. We determined that 29–31 and 19–21 semi-quantitative RT-PCR cycles gave linear, non-saturating increases of P-LAP and β-actin products, respectively (Fig. 3A). Representative Southern blots show that FSK apparently stimulated P-LAP mRNA expression (Fig. 3B). The band density of P-LAP was 2.6-fold greater in the presence of FSK ($P < 0.01$) when normalized to the corresponding signals for β-actin by scanning densitometry (Fig. 3C).

3.3. Enhancement of luciferase activity by FSK

We examined the effects of FSK on luciferase activity by focusing on the AP-2 site in the FP3 (−216 to −172) region of the P-LAP gene, because the 5′ region up to −1.1 kb of the P-LAP gene contains no cAMP response elements (CREs; 5′-TGACGTCA-3′) [10]. As shown in Fig. 4A, FSK stimulated luciferase activity approximately two-fold, when construct −216/+49, which includes both Ikaros and AP-2 binding sites, was transiently transfected. By contrast, FSK did not stimulate luciferase activity over the levels obtained from the pGL3-Basic, when construct −172/+49 was used. The mutation eliminating AP-2 binding (construct −216M1) reduced the effects of FSK to levels equivalent to the construct −172/+49. Double mutations eliminating both AP-2 and Ikaros binding sites (construct −216M3) gave FSK-stimulation levels similar to construct −216M1 and the construct −172/+49. To confirm the role of AP-2 in FSK-induced P-LAP promoter activity, we also examined luciferase activity in Hep-G2 cells, which are known to lack AP-2 (Fig. 4B). In contrast to BeWo cells, the −216/+49 constructs failed to show increases in luciferase activity following FSK stimulation in Hep-G2 cells.

3.4. Induction of AP-2α protein by FSK in BeWo cells

To identify the key AP-2 family members for differentiation-dependent P-LAP gene regulation, we examined the effects of FSK on AP-2 family protein levels by immunocytochemistry. AP-2α protein increased in nuclei after 24-h FSK stimulation (Fig. 5C) compared with non-treated cells (Fig. 5A). At 48 h, FSK further induced remarkably high immunoreactivity of AP-2α, especially in the morphologically changed cells with enlarged and lobed nuclei (Fig. 5B). AP-2γ was also expressed in nuclei and cytoplasm in non-treated cells (Fig. 5F), while FSK failed to enhance the immunoreactivity after

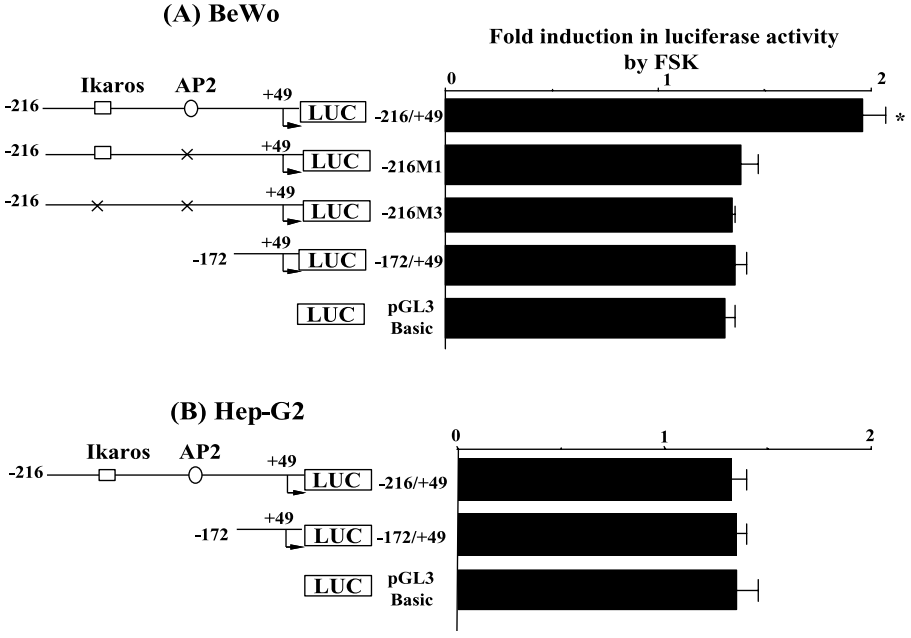

Fig. 4. The effects of FSK on luciferase activity in (A) BeWo and (B) Hep-G2 cells. After transient transfection of luciferase constructs, cells were incubated with or without 50 μM FSK for 12 h. The fold increase by FSK in firefly luciferase activity, normalized by renilla luciferase activity, is presented. An oval and a square indicate the Ikaros and AP-2 binding sites, respectively. Mutations at these sites are indicated by an x. Data are presented as means ± S.D. of three independent transfection experiments performed in triplicate. * indicates comparison with construct −172/+49 is significant at $P < 0.05$ by the Mann–Whitney *U*-test with Boneferroni correction.

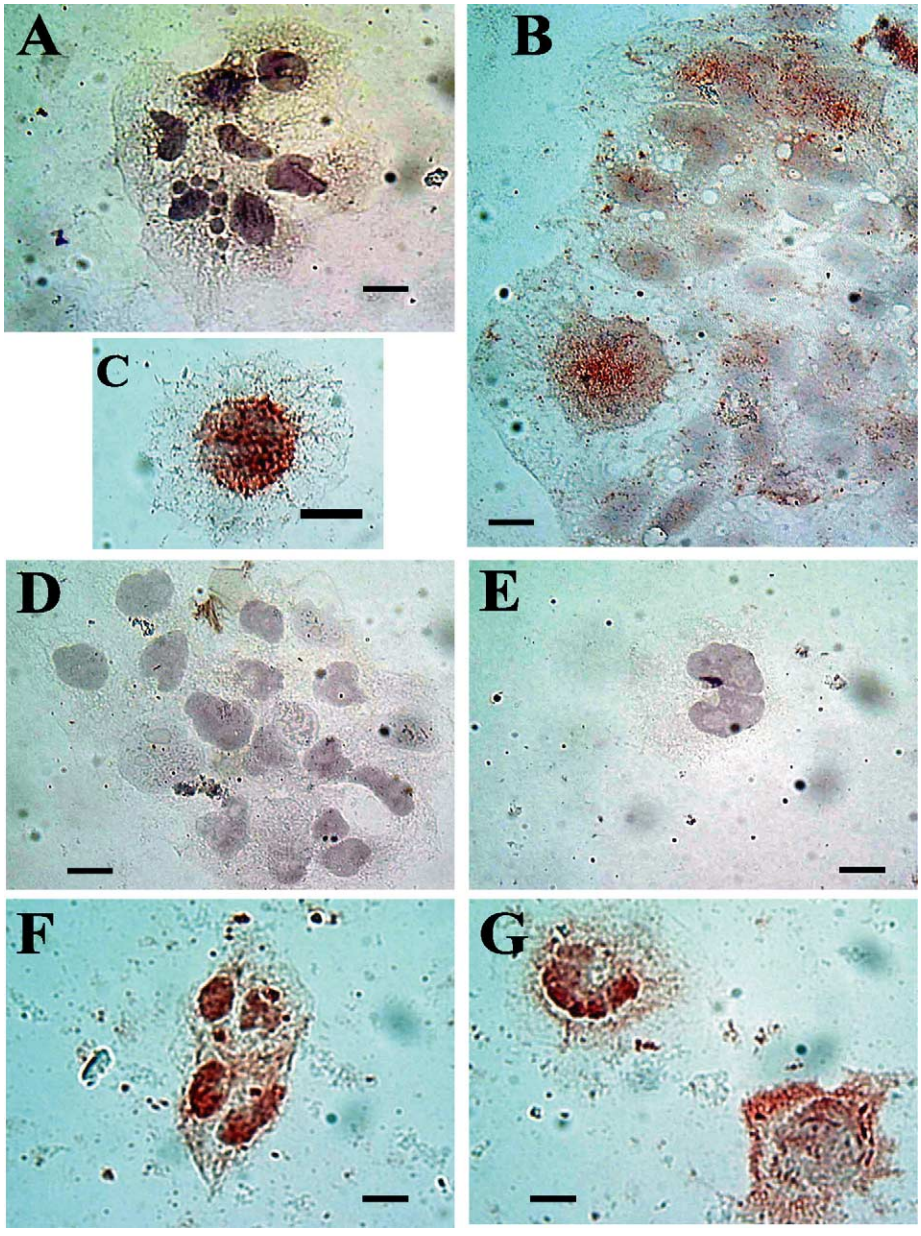

Fig. 5. Immunocytochemistry of AP-2 family members in BeWo cells. Cells were immunostained using AP-2α (A–C), AP-2β (D,E) and AP-2γ (F,G) antibody under basal state (A,D,F), 24-h FSK treatment (C) and 48-h FSK treatment (B,E,G). Control cells were cultured in serum-free media and FSK-treated cells were exposed to FSK (50 μM). Scale bar is 15 μm.

48 h (Fig. 5G). We could detect AP-2β expression neither under basal state (Fig. 5D) nor after FSK treatment (Fig. 5E) in BeWo cells.

4. Discussion

P-LAP is predominantly localized in syncytiotrophoblast cells in human placenta by immunohistochemistry [8,9] and in situ hybridization [25]. This prompted us to investigate differentiation-dependent expression of P-LAP in trophoblasts. We report here that P-LAP expression was enhanced in BeWo cells in which differentiation was induced by FSK treatment. AP-2 binding to the FP3 region of the P-LAP promoter was involved in this FSK-stimulated P-LAP expression. Immunocytochemistry showed that only AP-2α was responsive to FSK treatment, which is the first to demonstrate the changes of AP-2 isoform expression with trophoblast differentiation at protein levels.

We used BeWo choriocarcinoma cells in our study. BeWo cells not only have a high degree of similarity to normal placental trophoblasts [26], but also can be transformed to syncytiotrophoblastic cells with morphological and functional differentiation by cAMP stimulation [1]. In addition, transcription factors that regulate P-LAP gene expression under basal conditions have been identified in BeWo [11,12].

The induction of BeWo differentiation, which was confirmed functionally by measuring hCG levels and morphologically by light microscopy, was associated with an increase of both P-LAP immunostaining and activity. These findings are consistent with previous observations of predominant P-LAP localization in syncytiotrophoblasts in human placenta. We have demonstrated that ultrastructurally P-LAP is expressed

on the microvilli of syncytiotrophoblasts [27]. BeWo cells, as well as normal trophoblasts, form a microvillous brush border during morphological differentiation. Therefore, increased P-LAP expression in FSK-treated BeWo may be related to the formation of the brush border.

Generally, cAMP regulates protein expression both at transcriptional and post-translational level [28]. Therefore, we examined whether FSK increased P-LAP activity via accumulation of mRNA. RT-PCR and Southern blot showed that FSK increased P-LAP mRNA, a result consistent with observed increased P-LAP activity. This suggests that the increase in P-LAP proteins during trophoblast differentiation would be regulated at the transcriptional level.

The 1.1-kb upstream region of P-LAP lacks consensus CREs as observed on the hCG-β subunit gene. cAMP increases hCG-β promoter activity via AP-2 binding sites [15]. We previously demonstrated that AP-2 binding to the FP3 region was important for high P-LAP promoter activity in BeWo under basal conditions [11,12]. We, therefore, postulated that AP-2 binding to FP3 would be, at least in part, involved in P-LAP transcript induction by FSK. We tested this hypothesis in two ways. First, we examined the effects of eliminating AP-2 binding to FP3 region on the promoter activity in FSK-treated BeWo cells. Mutation of AP-2 binding site (−216M1 construct) reduced the stimulatory effects of FSK to levels equivalent to the construct −172/+49. Secondly, we used AP-2-deficient Hep-G2 cells, since cAMP is unable to exert its effects via AP-2 in these cells. FSK failed to stimulate the luciferase activity of the construct −216/+49 over the construct −172/+49, which contrasted with the results in BeWo. These findings strongly suggest that AP-2 binding to FP3 mediates the effects of FSK on P-LAP mRNA accumulation. Ikaros did not confer induction in response to cAMP, since the double mutations of AP-2 and Ikaros gave similar cAMP-induced luciferase activity as the solo mutation of AP-2.

AP-2 is involved in regulating important genes in trophoblasts such as hCG-β [15] and hPL [17]. Interestingly, among the AP-2 family members, only AP-2α mRNA increases during trophoblast differentiation, which resulted in the enhancement of hCG-β expression [21]. This up-regulation of AP-2α is consistent with the evidence that AP-2α mRNA is predominantly expressed in human term placenta [29]. Immunocytochemistry in this study demonstrated that the nuclear AP-2α expression was up-regulated in the FSK-treated cells, particularly in morphologically changed cells, whereas AP-2γ expression remained stable after FSK treatment. To our knowledge, this is the first report to show increases in AP-2α isoform expression at protein levels during trophoblast differentiation.

In conclusion, we demonstrated that FSK treatment up-regulated P-LAP gene expression via AP-2, putatively AP-2α, in BeWo. Our results suggest that P-LAP is transcriptionally regulated in a differentiation-dependent fashion during placental development, and also support the previous finding that only AP-2α is up-regulated with trophoblast differentiation.

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