Biochemical and Biophysical Research Communications 434 (2013) 460–465

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

The human leukocyte antigen G promotes trophoblast fusion and β-hCG production through the Erk1/2 pathway in human choriocarcinoma cell lines

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ARTICLE INFO

Article history:
Received 1 April 2013
Available online 10 April 2013

Keywords:
HLA-G
β-hCG
Trophoblast fusion
Placenta
Syncytiotrophoblast
Erk1/2

ABSTRACT

The human leukocyte antigen G (HLA-G) is expressed on the fetal–maternal interface and plays a role in protecting fetal-derived trophoblasts from the maternal immune response, allowing trophoblasts to invade the uterus. However, HLA-G also possesses immune suppressing-independent functions. We found that HLA-G expressing BeWo choriocarcinoma cells increased cell–cell fusion compared to control BeWo cells under forskolin treatment. Regardless of forskolin treatment, the expression of fusogenic gene mRNAs, including syncytin-1, the transcription factor glial cell missing 1 (Gcm1), and beta human chorionic gonadotropin (β-hCG) were elevated. HLA-G up-regulates β-hCG production in human choriocarcinoma cells because HLA-G knockdown in JEG-3 cells induces a dramatic decrease in β-hCG compared with control cells. The defect in β-hCG production in HLA-G knocked-down cells could not be completely overcome by stimulating hCG production through increasing intracellular cAMP levels. HLA-G expressing cells have increased phosphorylation levels for extracellular signal-regulated kinase1/2 (Erk1/2) in BeWo cells. The Erk1/2 pathway is inactivated after the inhibition of HLA-G expression in JEG-3 cells. Finally, Erk1/2 inhibition was able to suppress the increased hCG production induced by HLA-G expression. Together, these data suggest novel roles for HLA-G in regulating β-hCG production via the modulation of the Erk1/2 pathway and by inducing trophoblast cell fusion.

1. Introduction

The placenta is a critical organ in implantation and fetal development [1]. Trophoblast fusion and the consequential formation of the multinucleated syncytiotrophoblast layer are essential determinants of placentation [2]. During implantation, intercellular fusion of trophoblast cells surrounding the inner cell mass leads to the first generation of syncytiotrophoblast, which is capable of penetrating the uterine epithelium [3]. Without the formation of this multinucleated layer, invasion and implantation of blastocyst would not occur [4]. Syncytiotrophoblasts secrete reproductive hormones, including human placental lactogen (hPL) and human chorionic gonadotrophin (hCG) [3]. hCG, a hormone highly associated with human reproduction, plays important roles in placental, uterine and fetal development [5]. hCG is composed of an α subunit and a unique β subunit. The concentration of β-hCG is commonly used to determine levels of total hCG. The embryo first produces β-hCG at the eight-cell stage [6]. In the first week of pregnancy, hCG is an autocrine factor, acting on promoting the implantation of pregnancy [7]. Abnormally low levels of β-hCG clearly mark failing pregnancies, including early pregnancy loss, spontaneous abortion [5].The cyclic AMP (cAMP)-dependent protein kinase A (PKA) pathway was known to stimulate β-hCG biosynthesis in choriocarcinoma cell lines [8]. Two mitogen-activated protein kinase (MAPK) family members, Erk1/2 and p38, are involved in cAMP/PKA-induced β-hCG secretion [9,10].

The human leukocyte antigen G (HLA-G) belongs to the HLA class Ib of molecules, which are specifically expressed at the maternal–fetal interface [11]. The mRNA and protein of HLA-G is expressed in human embryos and especially on the trophectoderm, which differentiates into cytotrophoblasts and syncytiotrophoblasts [12]. HLA-G in soluble form is also expressed in the cytotrophoblasts which undergo the syncytial fusion with the syncytiotrophoblast [13]. HLA-G has been characterized as an immunosuppressor [14] and is associated with several common pregnancy complications, including unexplained miscarriage and pre-eclampsia [13,15]. Several studies have investigated the expression of soluble HLA-G (sHLA-G), which exists in early embryonic culture supernatants, and its correlation with pregnancy outcome [16,17]. However, the non-immune function that

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http://dx.doi.org/10.1016/j.bbrc.2013.04.004

HLA-G plays in trophoblast differentiation along with the fusion pathway and secretion of β-hCG has not been well addressed.

Human choriocarcinoma cell lines have served as useful models for investigating the factors that regulate trophoblast fusion and the synthesis and secretion of β-hCG. Among those cell lines, HLA-G is expressed on JEG-3 but not on BeWo cells [11]. BeWo cells exhibit a low spontaneous fusion efficiency, which can be significantly increased by up-regulating the intracellular cAMP concentration [8].

The aim of this study was to investigate the potential role of HLA-G in the processes of trophoblast cell–cell fusion and synthesis of β-hCG. The role of HLA-G in modulating β-hCG production was investigated by loss- and gain-of-function studies on two human choriocarcinoma cell lines. Finally, we showed that β-hCG upregulation was attenuated by an inhibitor to the Erk1/2 MAPK pathway, which was activated on HLA-G expressing cells.

2. Materials and methods

2.1. Reagents

Reagents were purchased from the following sources: monoclonal antibodies to HLA-G (4H84, Abcam), actin (C4, Abcam), E-cadherin (H-108, Santa Cruz Biotechnology); polyclonal antibody to β-hCG (ab9376, Abcam), MEK1/2 inhibitor U0126 (9903), MEK1 inhibitor PD98059 (9900), antibodies to phospho-MAPK family pathway (9910, Cell Signaling Technology), p38 inhibitor SB202190 (559388, Calbiochem), hygromycin B (H7772) and forskolin (F6886, Sigma), DAPI and secondary antibodies (Invitrogen). The following siRNA oligonucleotides were obtained from Ambion: HLA-G siRNA (siHLA-G), GGUAUGAACAGUAUGCCUATT; control siRNA (siControl), CCCGUAUACGACACCGAGUAGUCUU.

2.2. Cell lines and transfection

The human choriocarcinoma cell line JEG-3 (ATCC) was cultured in DMEM: Ham's F12 (1:1, Gibco) supplemented with 10% FBS (Gibco) and 20 mM HEPES (Gibco). BeWo cells (ATCC) were cultured in Ham's F12K medium (Gibco) supplemented with 10% FBS. For the inhibitor treatments, the cells were starved for 24 h and pretreated with inhibitors or DMSO (Sigma) as a vehicle control for 1 h. Thereafter, 10% FBS was added into the indicated medium. For the siRNA transient transfection, the cells were transfected using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.

2.3. Lentiviruses

In our culture system, BeWo cells were resistant to plasmid transfection using the Lipofectamine 2000 reagent. Consequently, a lentiviral system was employed to generate stable control and HLA-G expressing cells. Lentiviruses were generated by co-transfecting the packaging plasmid (CMVΔ8.92 and pMDG) and iDuet-101a or iDuet-101a-HLA-G-FLAG (NCBI Reference Sequence: NM_002127.5) constructs into the HEK293FT cell line. The BeWo cells were infected with the control and HLA-G expressing lentivirus. Hygromycin B (100 μg/ml; Sigma) was used to select for infected cells at 48 h post-infection, and the resistant cells were pooled and expanded for following studies.

2.4. RNA isolation and quantitative real-time RT-PCR

Total RNA was extracted using the TRIzol reagent (Invitrogen). Five micrograms of total RNA was reverse transcribed to cDNA using Superscript II reverse transcriptase regents (Invitrogen). The sequencing real-time PCR reaction contained 2 μL cDNA template, each specific primer at a concentration of 0.4 μM, and 10 μL of two times SYBR® Premix Ex Taq™ II (TaKaRa). The sequences of the forward and reverse primers were 5′-TGGAACAACTTCAGCACAGA-3′ and 5′-GCCATTCAAACAACGATAGG-3′ for syncytin-1, 5′-CTGACA AGGCTTTTTTCTTCACA-3′ and 5′-CCAGACGGGACAGGTTT-3′ for Gcm1, 5′-GCTACTGCCCCACCATGACC-3′ and 5′-ATGGACTCGAAGCG CACATC-3′ for β-hCG, 5′-GCCATCAATGACCCCTTCATT-3′ and 5′-TTGACGGTGCCATGGAATTT-3′ for GAPDH. GAPDH was employed as the internal control. Each reaction was performed in triplicate.

2.5. Immunofluorescence and cell fusion analysis

After the appropriate treatment, cells cultured on glass coverslips were fixed with 3.7% paraformaldehyde in PBS. Following a 30-min incubation period with 10% donkey serum to block the nonspecific binding of the antibodies, the cells were incubated with primary antibodies overnight (4 °C). The secondary antibody incubation was performed at room temperature for 1 h. Confocal microscopy was performed using a Zeiss LSM 780 microscope (Carl Zeiss) with a 20× objective. BeWo cells were fixed and immunostained. Intercellular boundaries were stained with an E-cadherin antibody, and the nuclei were stained with DAPI. Ten random fields were photographed for a cell fusion analysis. The total number of nuclei, the number of nuclei within multinucleated (more than two nuclei) syncytia and the number of syncytia were counted. Ratios of the fused cell number to the total number of nuclei were calculated as the cell fusion index to quantify the cell fusion ratio as described previously [18].

2.6. Western blotting

To detect non-phosphorylated protein, cells were lysed in RIPA buffer supplemented with a protease inhibitor cocktail (Roche). The BCA assay (Pierce) was employed to determine protein concentrations. For phosphorylated protein detection, cells were lysed in two times sample buffer. Membranes were incubated with primary antibodies overnight (4 °C) and secondary antibodies (Pierce) for 1 h at room temperature. ECL kits (Pierce) were used for chemiluminescence detection.

2.7. β-hCG quantification in the conditional medium

The culture medium was collected and centrifuged at 12,000 rpm for 5 min to remove cell debris. Solid phase sandwich ELISA was performed to estimate the secreted β-hCG level using a β-hCG ELISA kit following the manufacturer's instructions (SUNBIO).

2.8. Statistical analysis

For a comparison of two treatments, statistical analyzes were performed using SPSS 17.0 (SPSS) with Student's *t* test. At least three replicates were performed for each experiment. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. HLA-G promotes BeWo cell fusion and fusogenic gene expression

The BeWo cells are characterized as an *in vitro* model for mimicking trophoblast differentiation from cytotrophoblasts to the syncytiotrophoblasts phenotype [8]. To estimate the role of HLA-G in trophoblast fusion, BeWo cells stably expressing HLA-G and control BeWo cells generated using HLA-G expressing and control lentiviruses, were treated with 50 μM DMSO or forskolin for 48 h,

in which case DMSO was used as the vehicle control. Fusion events were monitored using E-cadherin immunofluorescence (Fig. 1A) and quantified using the cell fusion index (Fig. 1B), as described in the Section 2. The BeWo cells exhibited a scarcely observable spontaneous cell–cell fusion; whereas a 50 μM forskolin treatment could induce cell–cell fusion with morphological changes (Fig. 1A). Both the control and HLA-G expressing BeWo cells exhibited a low efficiency of spontaneous cell–cell fusion (1.003% vs. 1.152%, Fig. 1B). In contrast, HLA-G expressing BeWo cells showed a significant increase in cell fusion index under forskolin treatment compared with control cells (28.25% vs. 10.90%, $P < 0.001$, Fig. 1B). We further studied the role of HLA-G regulation of trophoblast fusogenic and fusion-related gene expression under DMSO or forskolin treatment. It has been reported that up-regulation of Gcm1 and syncytin-1 are required for forskolin-induced BeWo cell fusion [18,19]. Real-time PCR was employed to analyze the transcriptional levels of Gcm1 and syncytin-1. HLA-G stimulated the endogenous expression of Gcm1 (1.504-fold, $P < 0.001$, Fig. 1C) and syncytin-1 transcripts (1.250-fold, $P < 0.001$, Fig. 1D). During the forskolin treatment, the HLA-G expressing cells showed a significant increase in Gcm1 (1.165-fold, $P < 0.001$, Fig. 1C) and syncytin-1 (1.247-fold, $P < 0.01$, Fig. 1D) mRNA expression level compared with control cells. We then determined whether HLA-G affected the expression level of β-hCG, a product of syncytiotrophoblasts, using real-time PCR, ELISA and immunoblotting. The HLA-G expression in BeWo cells transfected with HLA-G expressing lentiviruses was concomitant with an increase of β-hCG mRNA (6.391-fold, Fig. 1E) and secreted protein (12.29-fold, Fig. 1F) levels as compared with the control BeWo cells. When cultured in the presence of forskolin, a significant increase in β-hCG mRNA (2.229-fold, Fig. 1E) and secreted protein (4.387-fold, Fig. 1F) levels was detectable in the HLA-G expressing BeWo cells, as compared with control BeWo cells. Protein immunoblotting confirmed these differences in β-hCG induction (Fig. 1G). Without

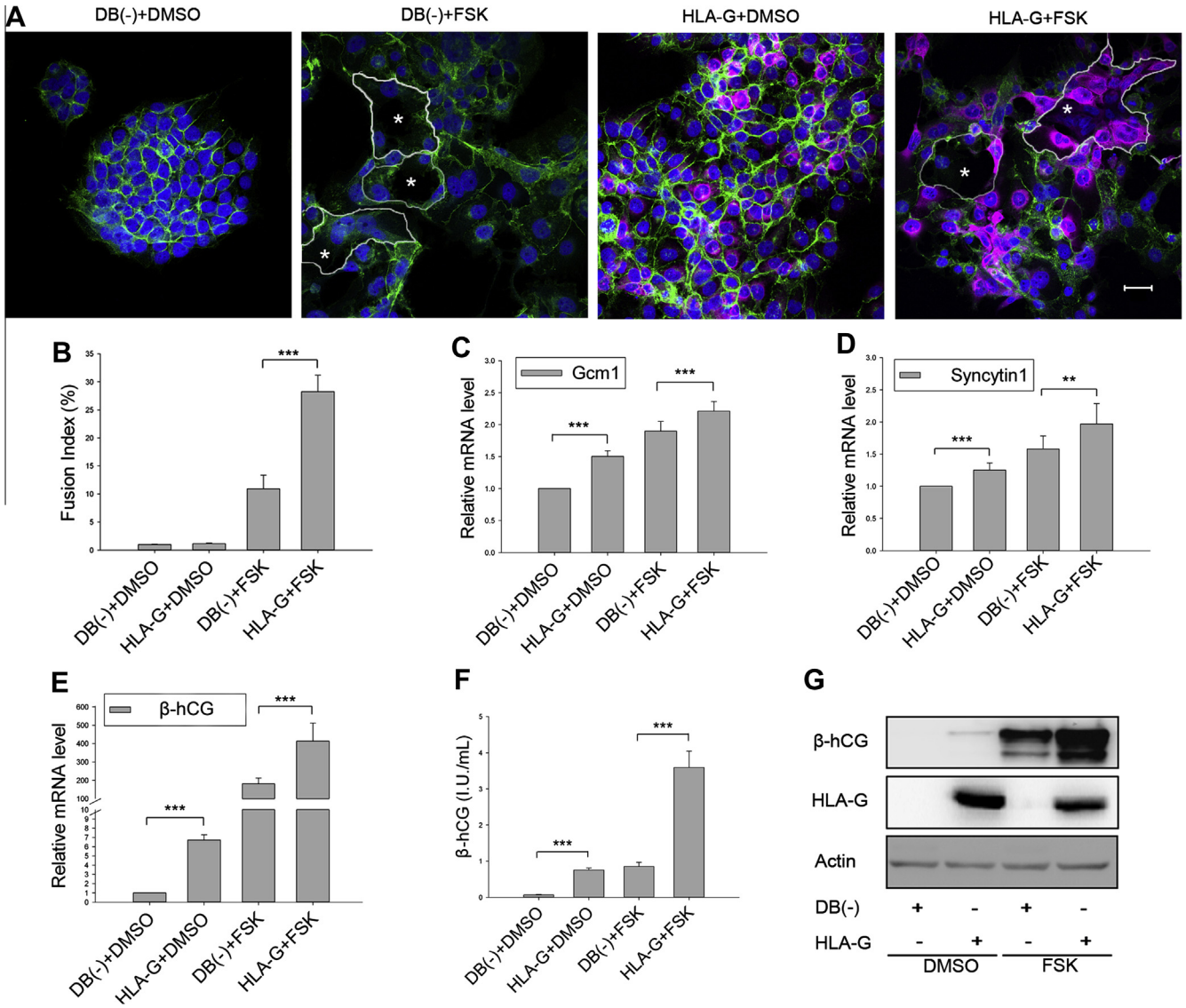

Fig. 1. HLA-G enhances the forskolin-induced BeWo cell–cell fusion mediated by Gcm1/syncytin-1 and promotes β-hCG production. (A) The effect of HLA-G expression on the extent of BeWo cell fusion after 48 h forskolin (FSK) treatment was analyzed by immunostaining with anti-E-cadherin antibody (green), anti-HLA-G antibody (magenta) and DAPI nuclear staining (blue). E-cadherin is stained to identify the intercellular boundaries. White asterisks indicate syncytium with >2 nuclei. Scale bars, 50 μm. (B) Cell fusion is quantified as fusion indexes in the presence of DMSO or forskolin ($n = 6$). Quantitative real-time RT-PCR analysis of (C) Gcm1, (D) syncytin-1, and (E) β-hCG expression of BeWo cells stably expressing HLA-G and the control cells in the presence of DMSO or FSK for 48 h ($n = 9$). β-hCG production was detected by ELISA (F) ($n = 6$) and Western blot analysis (G) ($n = 3$). Bar graphs showing the mean ± s.d. $^{**}P < 0.01$; $^{***}P < 0.001$.

forskolin stimulation, the HLA-G expressing cells did not demonstrate a cell fusion index difference compared to the control cells only at the basal level, i.e., approximately 1% (Fig. 1C). However, the β-hCG expression level was highly up-regulated in HLA-G expressing BeWo cells even without forskolin treatment (Fig. 1E–G). Therefore, we were unable to conclude that the up-regulation of β-hCG by HLA-G without forskolin treatment was a result of cell–cell fusion.

3.2. HLA-G depletion affects β-hCG production in JEG-3 cells

To test our hypothesis that HLA-G is a regulator of β-hCG production, we employed JEG-3 cells, which exhibit a poor cell–cell fusion activity. JEG-3 cells were transfected with either control siRNA or HLA-G siRNA, which resulted in the suppression (74.37%) of HLA-G mRNA levels (Fig. 2A). HLA-G silencing resulted in a significant decrease in β-hCG expression at the transcriptional level (53.97%) in comparison with the controls ($P < 0.001$, Fig. 2A). Secreted levels of β-hCG, measured using ELISA, exhibited a significant decrease (26.63%) in HLA-G siRNA transfected cells when compared with the control ($P < 0.05$, Fig. 2B). We then confirmed that HLA-G down-regulation impaired the expression of β-hCG using immunoblotting (Fig. 2C). These results suggest that the regulation of β-hCG by HLA-G may be a general phenomenon applicable to human choriocarcinoma cells regardless of syncytium formation. Cyclic AMP (cAMP) has been demonstrated to regulate the transcription of β-hCG genes [12]. We used forskolin to study whether the effect of HLA-G on β-hCG expression was through the regulation of the cAMP pathway. For both the control and HLA-G siRNA transfected JEG-3 cells, forskolin treatment resulted in a significant increase in β-hCG production compared with the vehicle control. During forskolin treatment, significant decreases were still observed at the β-hCG mRNA level (27.35%, $P < 0.01$, Fig. 2A) and in β-hCG secretion (14.64%, $P < 0.001$, Fig. 2B) for HLA-G-silenced cells compared with the control siRNA transfected cells. The same fold decrease was also detected using immunoblotting (Fig. 2C). The decrease in β-hCG expression for the control and HLA-G-silenced cells was weakened during forskolin treatment compared with DMSO treatment, which indicated that HLA-G may play a role in modulating β-hCG downstream of cAMP.

3.3. Up-regulation of β-hCG production by HLA-G is mediated via the Erk1/2 pathway

We have further defined the molecular mechanism by which HLA-G regulates β-hCG production. Because HLA family members have been demonstrated to be signaling molecules, we hypothesized that HLA-G might play a role in the activation of specific signaling pathways involved in β-hCG production, including the Erk1/2 and p38 MAPK pathways. The phosphorylation levels of Erk1/2 and p38 were detected using Western blotting to identify activated Erk1/2 and p38 MAPK pathways. In JEG-3 cells, Erk1/2 phosphorylation was depressed in HLA-G-silenced cells compared with the controls (Fig. 3A). To further explore the role of p38 in the up-regulation of β-hCG by HLA-G, we treated HLA-G expressing BeWo cells and control cells with the p38 MAPK inhibitor SB202190 for 24 h and assessed β-hCG expression using immunoblotting. The results indicate that the phosphorylation of p38 remains unchanged after HLA-G expression and the treatment with SB202190 did not inhibit the excess β-hCG production induced by HLA-G, this suggests the ineffectiveness of p38 MAPK in HLA-G-associated β-hCG production (Fig. 3B). To analyze the involvement of the Erk1/2 pathway, we compared the effects of the MEK1 (PD98059) and MEK1/2 (U0126) inhibitors on β-hCG production. The phosphorylation of Erk1/2 was up-regulated in HLA-G expressing BeWo cells (Fig. 3C and D). The abundant expression of β-hCG in HLA-G expressing BeWo cells was blocked by U0126 (Fig. 3D) but not PD98059 treatment (Fig. 3C). Meanwhile, the effect of the Erk1/2 pathway, which is responsible for HLA-G-induced β-hCG production, was confirmed in JEG-3 cells, as examined using ELISA. The

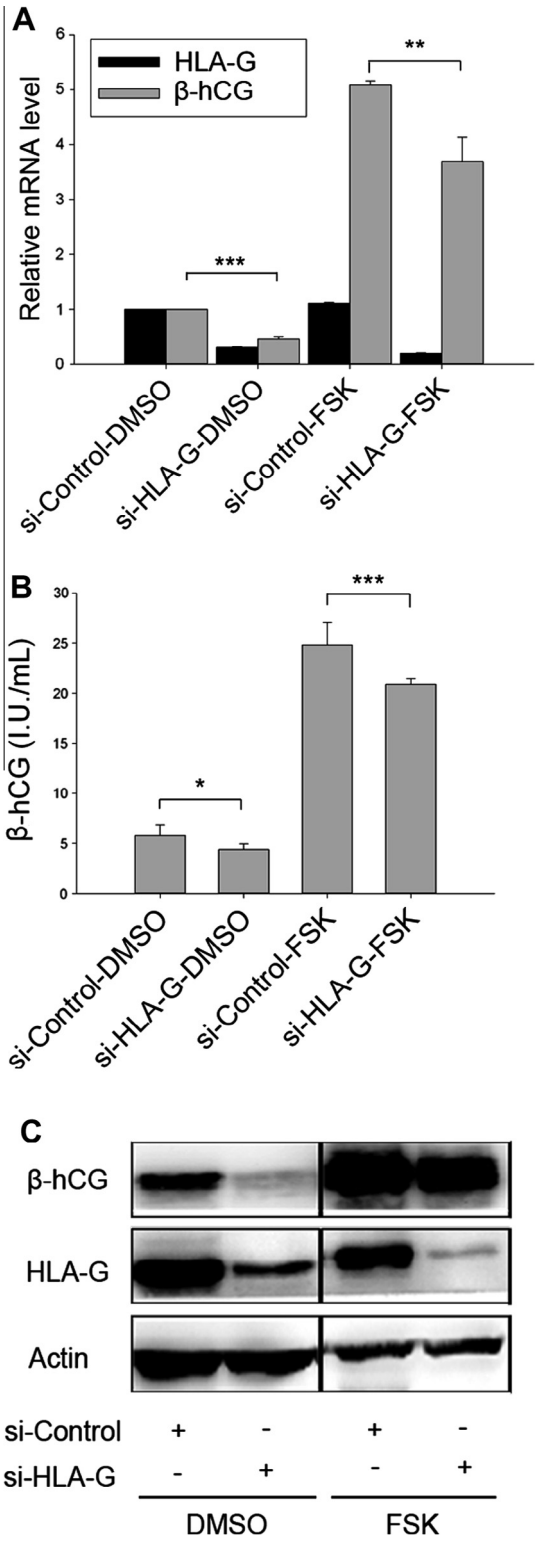

Fig. 2. HLA-G silencing inhibits the forskolin-induced increase in β-hCG in JEG-3 cells. (A) JEG-3 cells transfected with the HLA-G siRNA (si-HLA-G) and control siRNA (si-Control) were treated with 50 μM FSK or the DMSO vehicle control for 48 h, assayed for β-hCG mRNA levels using real-time PCR (A) ($n = 9$), ELISA (B) ($n = 6$), and representative Western blot analysis (C) ($n = 2$). Bar graphs showing the mean ± s.d. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

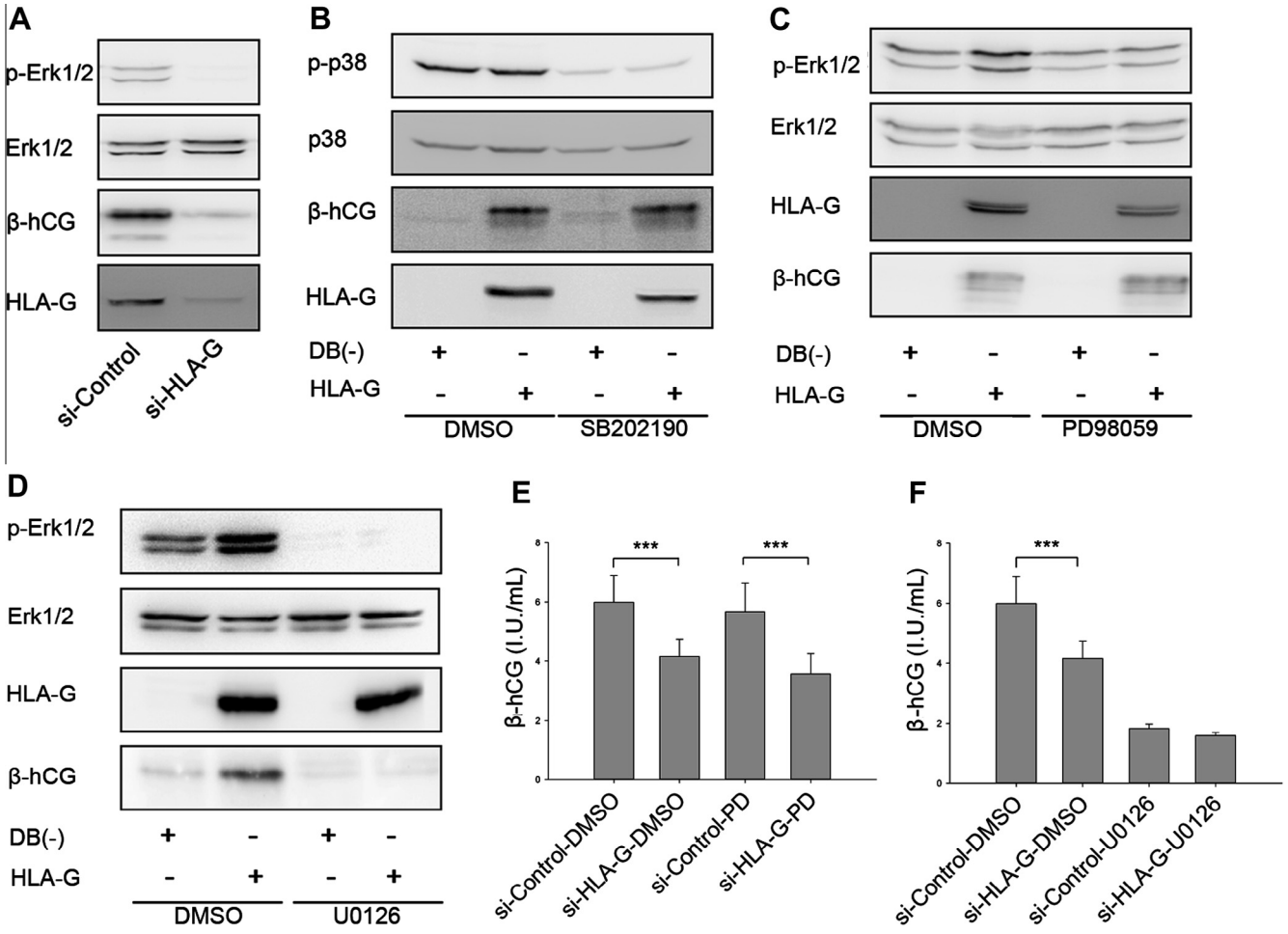

Fig. 3. β-hCG up-regulation by HLA-G is mediated via the Erk1/2 MAPK pathway. (A) The phosphorylation level of Erk1/2 upon HLA-G knockdown (si-HLA-G) was determined relative to the controls (si-Control) in JEG-3 cells by Western blot ($n = 3$). β-hCG abundance and the indicated protein phosphorylation in BeWo cells stably expressing HLA-G (HLA-G) and the control cells (DB(−)) was determined by Western blot analysis after a 24 h incubation period with the following inhibitors: SB202190 (B), PD98059 (C), U0126 (D), and DMSO as a vehicle control ($n = 3$). The JEG-3 cells were treated with PD98059 (E) or U0126 (F), and β-hCG secretion was monitored using ELISA ($n = 6$). Bar graphs show the mean ± s.d. $^{***}P < 0.001$.

treatment with U0126 inhibited β-hCG expression in the culture medium of both HLA-G-silenced and control cells (Fig. 3F). The difference in β-hCG secretion between JEG-3 cells transfected with a HLA-G siRNA or control siRNA with U0126 treatment was not significant ($P = 0.128$, Fig. 3F). These results indicate that Erk1/2 activation, through the MEK1/2 signaling pathway, results in the increase in β-hCG production in cells expressing HLA-G and that HLA-G may act upstream of Erk1/2 MAPK to regulate β-hCG production.

4. Discussion

Different mechanisms have been investigated to explain how HLA-G activity is involved in the pathogenesis of pregnancy complications [20,21]. Immuno-associated functions largely rely on HLA-G molecules in the β2m-linked form or the disulfide-linked homodimer form [22]. It appears that β2m-free HLA-G molecules, rather than the β2m-linked form, perform the non-immune functions [23]. HLA-G present in culture supernatants was derived not from secretion of the soluble forms but by cleavage of the full length membrane-bound HLA-G from the cell surface by metalloproteinases [24]. We attempted to determine the function of HLA-G in trophoblast fusion, which has no actual relation to the immuno-associated function. With all of these considerations, we employed a lentiviral system and a full length HLA-G sequence coding cDNA clone to replace HLA-G expression to gain a better understanding of the biology of HLA-G in trophoblast fusion.

The successful formation of syncytiotrophoblasts plays an important role in ensuring adequate nutrient and gas exchange for the developing embryo and for the synthesis of steroid and peptide hormones. The ability of HLA-G to enhance the fusion of trophoblasts to form multinucleated syncytia might be relevant to the formation of a functional placenta and the establishment of a successful pregnancy. HLA-G exists on the trophectoderm of preimplantation embryos that are capable of differentiating into several trophoblast lineages, mainly one lineage able to fuse to form syncytiotrophoblasts and another responsible for invasion into maternal decidua. HLA-G in soluble form is also expressed in the cytotrophoblasts cells destined for syncytial fusion. We hypothesize that during embryonic implantation and early pregnancy HLA-G expression on trophectoderm cells might contribute to cell–cell fusion and syncytiotrophoblast formation. We demonstrate that HLA-G expression enhances BeWo cell fusion under forskolin stimulation and that it promotes the expression of the trophoblast fusion-associated gene Gcm1 and Syncytin1. These results extend our understanding of HLA-G during trophoblast differentiation to have a hyperstimulation effect on increasing Gcm1 expression and, consequentially, up-regulating Syncytin1. Thus, HLA-G can participate in the process of trophectoderm differentiation into a syncytiotrophoblast during embryo implantation.

Our data suggest that one product of the up-regulation of HLA-G expression in BeWo cells is β-hCG production in the presence or absence of forskolin. To determine the influence of increasing cell–cell fusion on the up-regulation of β-hCG production, we used a HLA-G siRNA on JEG-3 cells, which have a poor fusion capacity. The lose-of-function study on JEG-3 cells demonstrates that β-hCG production is down-regulated after HLA-G silencing. Thus, HLA-G promotes directed β-hCG production in choriocarcinoma cells even in the absence of trophoblast fusion or defined external guidance cues. To our knowledge, this is the first report demonstrating β-hCG up-regulation by HLA-G. Because abnormally low levels of β-hCG are tightly associated with fetal loss during early pregnancy, we conclude that unexplained miscarriages and fetal loss, which correlate with the diminished levels of soluble HLA-G in early embryonic culture supernatants, might be the result of a decrease in β-hCG and impaired formation of syncytiotrophoblasts mediated by a HLA-G defect. In another respect, the up-regulation of β-hCG production by HLA-G may have a feedback effect that induces trophoblast fusion.

Erk1/2 MAPK signaling is essential for trophoblast morphological and functional differentiation in both mouse and human [25,26]. Our results suggest that HLA-G transduces signals in trophoblast cells to activate the Erk1/2 pathway; similar to the way other HLA class I and class II members activate this pathway. This signal transduction function of HLA-G has not been reported before and might provide new insights into trophoblast differentiation. The Erk1/2 pathway is highly associated with trophoblast differentiation and β-hCG production in the human placenta, which led us to speculate that Erk1/2 activation may be involved in the mechanism of HLA-G-induced β-hCG up-regulation. We demonstrate that a MEK1/2 inhibitor could suppress the increased hCG production induced by HLA-G expression. All of these results indicate that HLA-G expression activates the Erk1/2 pathway to regulate β-hCG production in human choriocarcinoma cell lines. The mechanism by which HLA-G activates the Erk1/2 pathway remains to be determined. In primary human aortic endothelial cells, HLA class I molecules interact with integrin β4 to activate the Erk pathway [27]. Some other molecules known to be involved in trophoblast fusion, including the EGFR and CD82, are associated with HLA class I molecules [28]. These reports suggest the possibility that HLA-G interacts with the molecules mentioned above to activate the Erk1/2 pathway, which will be explored in our future research.

Our observations suggest that the deregulation of HLA-G expression may contribute to abnormalities in human placental development through this novel regulatory mechanism of β-hCG production and trophoblast differentiation, in addition to its immunosuppressive function. This may provide indirect evidence that HLA-G may be a possible biomarker of embryonic developmental potential to predict human-assisted reproduction outcomes.

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