Effects of valproic acid on syncytialization in human placental trophoblast cell lines.

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Abbreviations: ASCT2, alanine serine cysteine transporter 2; CBZ, carbamazepine; CLB, clobazam; Col, collagen; CT, cytotorophoblast; ELISA, enzyme-linked immunosorbent assay; FK, forskolin, GBP, gabapentin; GCM1, glial cells missing transcription factor 1; HDAC, histone deacetylase: hCG, human chorionic gonadotropin; hPL, human placental lactogen; LCM, lacosamide; LEV, levetiracetam; LTG, lamotrigine; MFSD2, major facilitator superfamily domain-containing protein 2; MRP4, multidrug resistance protein 4; ST, syncytiotrophoblast; TPM, topiramate; TS, trophoblast stem; VPA, valproic acid; YWHAZ, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta

Abstract

The placenta is a critical organ for fetal development and a healthy pregnancy, and has multifaceted functions (e.g., substance exchange and hormone secretion). Syncytialization of trophoblasts is important for maintaining placental functions. Epilepsy is one of the most common neurological conditions worldwide. Therefore, this study aimed to reveal the influence of antiepileptic drugs, including valproic acid (VPA), carbamazepine, lamotrigine, gabapentin, levetiracetam, topiramate, lacosamide, and clobazam, at clinically relevant concentrations on syncytialization using *in vitro* models of trophoblasts. To induce differentiation into syncytiotrophoblast-like cells, BeWo cells were treated with forskolin. Exposure to VPA was found to dose-dependently influence syncytialization-associated genes (*ERVW-1*, *ERVFRD-1*, *GJA1*, *CGB*, *CSH*, *SLC1A5*, and *ABCC4*) in differentiated BeWo cells. Herein, the biomarkers between differentiated BeWo cells and the human trophoblast stem model (TS^{CT}) were compared. In particular, *MFSD2A* levels were low in BeWo cells but abundant in TS^{CT} cells. VPA exposure affected the expression of *ERVW-1*, *ERVFRD-1*, *GJA1*, *CSH*, *MFSD2A*, and *ABCC4* in differentiated cells (ST-TS^{CT}). Furthermore, VPA exposure attenuated BeWo and TS^{CT} cell fusion. Finally, the relationships between neonatal/placental parameters and the expression of syncytialization markers in human term placentas were analyzed. *MFSD2A* expression was positively correlated with neonatal body weight, head circumference, chest circumference, and placental weight. Our findings have important implications for better understanding the mechanisms of toxicity of antiepileptic drugs and predicting the risks to placental and fetal development.

Key words: antiepileptic drug, differentiation, placenta, syncytialization, trophoblast, valproic acid

1. **Introduction**

The placenta is a temporal and essential organ for a healthy pregnancy and proper fetal development. In particular, the placenta has multifaceted functions, such as nutrient transport to the fetus, metabolism and elimination of products, gas exchange, and hormone secretion. Placental trophoblasts play an important role in these functions. Human cytotrophoblast progenitor cells differentiate via two pathways: villous trophoblasts and extravillous trophoblasts (Ji et al., 2013). In the villous pathway, mononucleated cytotrophoblasts (CTs) fuse to form multinucleated syncytiotrophoblasts (ST), resulting in a syncytial layer that covers the placental villi. The syncytialization of trophoblast cells, including differentiation and fusion, is important for adequate placental development and pregnancy (Gupta et al., 2016). Both biochemical and morphological alterations occur during the differentiation of CT cells into ST cells (Handwerger, 2010). The human endogenous retrovirus envelope proteins, syncytin-1 (*ERVW-1*) and syncytin-2 (*ERVFRD-1*), are involved in the fusion of trophoblast cells (Ji et al., 2013). Syncitin-1 exists in ST cells, and its expression is maintained throughout gestation. In contrast, the expression of syncytin-2 slowly decreases throughout gestation (Okahara al., 2004). Human chorionic gonadotropin (hCG/*CGB*) and human placental lactogens (hPL/*CSH*) are hormones produced during pregnancy. hCG increases progesterone production and contributes to syncytialization (Ji et al., 2013). hPL is a polypeptide hormone synthesized by trophoblasts and secreted into maternal blood. hPL is associated with the regulation of lipid and carbohydrate metabolism in the mother and is essential for energy homeostasis (Costa, 2016). These proteins have been employed as syncytialization markers. The alanine serine

cysteine transporter 2 (ASCT2/*SLC1A5*) acts as a receptor of syncytin-1 for cell fusion (Lavillette et al., 2002), and the major facilitator superfamily domain-containing protein 2 (MFSD2/*MFSD2A*), is a receptor for syncytin-2 (Esnault et al., 2008). Glial cells lacking transcription factor 1 (GCM1/*GCM1*) is a transcription factor that regulate syncytin-1 expression (Huang et al., 2014). Multidrug resistance protein 4 (MRP4/*ABCC4*) is a transporter involved in cAMP efflux (Copsel et al., 2011). As cAMP is a key regulator of trophoblast differentiation (Handwerger, 2010), we monitored the expression of MRP4 as a potential marker in this study. The gap junction protein, Connexin-43 (*GJA1*), is involved in the fusion of trophoblasts (Frendo et al., 2003).

Anomalies in the expression of hormones and syncytialization-associated genes have been associated with obstetric complications. Studies have reported the downregulation of syncytin-1 in placentas with fetal growth restriction and preeclampsia (Lee et al., 2001; Knerr et al., 2002; Chen et al., 2006; Langbein et al., 2008; Ruebner et al., 2010). Furthermore, alterations in syncytin-2, MFSD2, hCG, and connexin-43 in the placenta are associated with these complications (Langbein et al., 2008; Ruebner et al., 2010; Otto et al., 2015). The levels of syncytin-2 and MFSD2 have been reported to decrease in gestational diabetic placentas (Soygur et al., 2016). An evaluation of alterations in these placental markers is thus important for predicting fetal and maternal health.

The expression of syncytialization-associated proteins and hormones can be altered by maternal conditions, such as exposure to substances. In fact, environmental contaminants have been reported to disrupt hormone secretion (Le et al., 2014; Drwal et al., 2020). Exposure to benzo(a)pyrene

induced the differentiation and production of hCG in human choriocarcinoma BeWo cells (Le et al., 2014). Further, polycyclic aromatic hydrocarbons were found to modulate hCG, hPL, and placental growth factor levels in the human choriocarcinoma cell lines, BeWo and JEG-3 (Drwal et al., 2020). High concentrations of high-density lipoprotein cholesterol (HDL) and oxidized HDL reduce hCG secretion, GCM1 expression, and cell fusion in BeWo cells (Wang et al., 2021). Delta-9-tetrahydrocannabinol attenuates syncytialization in BeWo cells (Walker et al., 2020). Selective serotonin reuptake inhibitors have been reported to affect the fusion of primary villous trophoblasts and modify hCG secretion from BeWo cells (Clabault et al., 2018).

Pharmacotherapy during pregnancy requires consideration of the benefits to the mother and risks to the fetus. Epilepsy is one of the most common neurological conditions (Avachat et al., 2018). Recently, a variety of antiepileptic drugs have been approved and have contributed to the treatment of seizures in epileptic patients (Avachat et al., 2018). Most pregnant women with epilepsy require continued antiepileptic drugs as uncontrolled seizures may have adverse effects on the mother and fetus (Tomson et al., 2011). Furthermore, unintended pregnancy commonly occurs in women with epilepsy (Herzog et al., 2017). Therefore, a toxicological assessment of antiepileptic drugs for placental function could provide meaningful information for estimating risks to the fetus.

Exposure to certain antiepileptic drugs increases the risks to the fetus. Especially, the use of valproic acid (VPA) during the periconceptional period dose-dependently increased the risk of fetal malformations (Tomson et al., 2011). Besides, exposure to high-dose VPA during pregnancy increases

the risk of impaired cognitive function, compared with that associated with the exposure to other commonly used antiepileptic drugs (Meador et al., 2013). *In utero* exposure of VPA was reported to be associated with an increased risk of autism spectrum disorders in the offspring (Christensen et al., 2013). Therefore, VPA should be avoided in pregnant women with epilepsy, except in cases where other medications are ineffective or not tolerated (Kim et al., 2019). Furthermore, the use of carbamazepine (CBZ) at a high dose, phenobarbital, and phenytoin increased the risk of teratogenicity (Meador and Loring, 2016; Tomson et al., 2018). Newer antiepileptic drugs are more commonly used during pregnancy (Vajda et al., 2014). Among these, the use of topiramate (TPM) in early pregnancy increased the risk of teratogenicity (Hernandez-Diaz et al., 2018). Clobazam (CLB) has been reported to have a higher risk profile (Thomas et al., 2017). Risk of major malformations associated with lamotrigine (LTG) and LEV have been reported to be within the range of risk in individuals who are unexposed to antiepileptic drugs (Tomson et al., 2018). LTG and LEV are recommended for women of reproductive age with epilepsy. Based on these observations, consideration should be given to the type and dose of antiepileptic drugs when used for women of reproductive age (Pennell, 2018). However, in a population-based longitudinal cohort study, a noticeable proportion of women were treated with VPA and TPM despite known teratogenicity risks (Kim et al., 2019). Increases in the prescription of gabapentin (GBP) and pregabalin were observed in the U.K. after 2012, although data on GBP use during pregnancy are insufficient to make a conclusion about its potential risk to the fetus (Hurault-Delarue et al., 2019). Lacosamide (LCM) is a third-generation antiepileptic drug. Although

133 data on the use of LCM during pregnancy is limited, in a recent study, increased occurrences of
134 pregnancies in women administered newer antiepileptic drugs, including LCM, were reported
135 (Hoeltzenbein et al., 2023). To date, valproic acid VPA and LEV have been reported to reduce the
136 secretion of hormones, such as hCG, progesterone, and 17β-estradiol, from BeWo cells (Kwiecińska
137 et al., 2011). However, the assay was conducted under undifferentiated conditions and the detailed
138 effects of antiepileptic drugs on trophoblast differentiation remain unclear.

139 Histone deacetylases (HDACs) play critical roles in trophoblast syncytialization (Maltepe et
140 al., 2005; Pan et al., 2008; Jaju Bhattad et al., 2020). The mammalian HDAC isoforms are classified
141 into four classes: class I (HDAC1–HDAC3 and HDAC8), class II (class IIa: HDAC4, HDAC5,
142 HDAC7, and HDAC9; class IIb: HDAC6 and HDAC10); class III (SIRT1–SIRT7); and class IV
143 (HDAC11) (Milazzo et al., 2020). HDACs are important for differentiation of trophoblasts and
144 trophoblast stem cells in mouse (Maltepe et al., 2005; Pan et al., 2008). Recently, HDAC inhibitors,
145 such as trichostatin A (a pan-HDAC inhibitor) and FK228 (an HDAC1/2 inhibitor) were reported to
146 inhibit human trophoblast fusion and the activity of HDAC1 and HDAC2 was shown to be important
147 for differentiation (Jaju Bhattad et al., 2020). VPA is widely known to exhibit inhibitory effects on
148 HDACs (Gurvich et al., 2004). In fact, as an HDAC inhibitor, VPA, in addition to the activators of
149 Wingless/Integrated (Wnt) and EGF and inhibitors of TGF-β and Rho-associated protein kinase
150 (ROCK), is used to maintain the stem cell-state of human trophoblast stem cells (Okae et al., 2018).
151 However, there are various isoforms of HDACs and the affinity of HDAC inhibitors for each isoform

is different (Milazzo et al., 2020). The pharmacological effects of VPA on syncytialization have not been fully elucidated. Furthermore, TPM and major metabolite of LEV can inhibit HDACs, although with lower potencies than VPA (Eyal et al., 2004). CBZ induced acetylation of histone H4 in HepG2 cells and inhibited HDAC3 and HDAC7 (Beutler et al., 2005). LCM reduced HDAC1 levels in the cerebral cortex of rats (Bang et al., 2015). LCM was reported to enhance histone hyperacetylation in BeWo cells (Granit et al., 2018). In this context, we focused on the effects of antiepileptic drugs, especially VPA, on trophoblast syncytialization.

The current study aimed to reveal the effects of eight antiepileptic drugs, VPA, CBZ, LTG, GBP, LEV, TPM, LCM, and CLB, at clinically relevant concentrations on trophoblast syncytialization. These antiepileptic drugs are generally used for epilepsy, and are also prescribed for reproductive-aged and pregnant women, as described above. We included various characteristic drugs considering clinical situations known to be associated with risks to fetus (e.g., VPA, CBZ, and TPM), drugs that are recommended for pregnant women (e.g., LTG and LEV), and drugs for which limited information on usage among pregnant women is available (e.g., CLB, GBP, and LCM). Furthermore, we considered the biochemical properties of antiepileptic drugs (e.g., VPA, CBZ, TPM, and LCM) that may affect histone acetylation as selection criteria. BeWo cells were used as a trophoblast model to screen the effects of these antiepileptic drugs. This cell line has the characteristics of human trophoblasts and has been widely used to investigate syncytialization (Ji et al., 2013). BeWo cells can differentiate into ST-like cells upon treatment with forskolin (FK), an adenylate cyclase activator (Handwerger, 2010).

Recently, Okae et al. established human trophoblast stem (TS) cells from CT cells isolated from first-trimester placentas (Okae et al., 2018). This model is expected to be a useful tool for research on human placental development and function. As an additional model, TS^{CT} cells were employed in this study.

2. Materials and methods

2.1. Chemicals

VPA and CLB were purchased from Sigma-Aldrich (St. Louis, MO, USA). LTG, GBP, LEV, and TPM were purchased from Tokyo Chemical Industry (Tokyo, Japan). LCM was purchased from Toronto Research Chemicals (North York, ON, Canada). CBZ and FK were purchased from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Cell culture

Human placental choriocarcinoma BeWo cells were cultured in Ham's F-12K (Kaighn's) medium (Wako) supplemented with fetal bovine serum (15%) and penicillin-streptomycin (1%) at 37 °C with 5% CO_2, as described previously (Furugen et al., 2017). Human trophoblast stem (TS^{CT}) cells derived from human cytotrophoblasts (CT) were purchased from Riken Cell Bank (Saitama, Japan) (CT29, RCB4937). TS^{CT} cells were cultured according to a previously reported protocol (Okae et al., 2018). Briefly, TS^{CT} cells were seeded on collagen (Col IV)-coated dishes or plates, and cultured in TS medium. The TS medium consisted of DMEM/F12 (Wako) supplemented with 0.1 mM 2-mercaptoethanol (GIBCO), 0.2% FBS, 0.5% penicillin-streptomycin, 0.3% BSA (Wako), 1% ITS-X supplement (Wako), 1.5 mg/ml L-ascorbic acid (Wako), 50 ng/mL EGF (Wako), 2 mM CHIR99021 (Wako), 0.5 mM A83-01 (Wako), 1 mM SB431542 (Wako), 0.8 mM VPA (Wako), and 5 mM Y27632 (Wako). Cells were cultured at 37 °C with 5% CO_2.

196 **2.3. Differentiation of cells and exposure to antiepileptic drugs**

197 To induce differentiation, BeWo cells were treated with medium containing 20 μM FK or
198 0.1% dimethyl sulfoxide (DMSO) (as control) for 48 h. Antiepileptic drugs, including VPA, CBZ,
199 LTG, GBP, LEV, TPM, LCM, and CLB, were dissolved as previously described (Kurosawa et al.,
200 2018). Stock solutions of VPA, GBP, LEV, TPM, and LCM were dissolved in methanol. Stock
201 solutions of CBZ, LTG, and CLB were dissolved in DMSO. They were further diluted with the cell
202 culture medium (solvent final concentration ≤0.27%). After 2 days of culture, BeWo cells were
203 exposed to antiepileptic drugs in the presence or absence of FK for 48 h. Equal volumes of the
204 respective solvents were added to the cells to serve as a control. The concentrations of the antiepileptic
205 drugs were 1 mM (VPA), 50 μM (CBZ), 50 μM (LTG), 100 μM (GBP), 270 μM (LEV), 50 μM (TPM),
206 40 μM (LCM), and 1 μM (CLB). Antiepileptic drugs were exposed at high concentrations within the
207 therapeutic range (Jacob and Nair, 2016). To assess the concentration dependence of the effects of
208 VPA, we used clinically relevant range (250–1,000 μM) of the drug. In plasma, the reference
209 concentration range of VPA is between 50 and 100 μg/mL (347–693 μM) for epilepsy (Patsalos et al.,
210 2018). The serum concentrations range from 50 μg/mL to 100 or 150 μg/mL (1040 μM) (Sztajnkrycer
211 et al., 2002).

212 TS^{CT} cells (1.0×10^5 cells/well) were seeded on Col IV-coated 6-well plastic plates and
213 cultured for three days in TS-medium. For induction of ST cells, the TS medium was changed to ST
214 medium and the cells were cultured for 72 h. The ST-medium consisted of DMEM/F12 supplemented

with 0.1 mM 2-mercaptoethanol, 0.5% Penicillin-Streptomycin, 0.3% BSA, 1% ITS-X supplement, 2.5 mM Y27632, 2 mM FK, and 4% KnockOut™ Serum Replacement (Thermo Fisher Scientific). To determine the influence of VPA on the expression of syncytialization markers, the TS-medium was removed, and the cells were washed twice with DMEM/F12 to eliminate the TS-medium. The cells were then treated with ST medium containing VPA (0 or 1 mM).

2.4. Collection of placental tissue

Human term placentas were donated by 11 women who delivered via a cesarean section. The study participants were pregnant women who were admitted to the Obstetrics Department of Hokkaido University Hospital and met the following criteria: (1) older than or 20 years of age at the time of obtaining consent; (2) delivery by planned cesarean section; and (3) freely gave their consent to participate after receiving sufficient explanation of the study and confirming that they understand the study. Placenta was obtained within 1 h of delivery and used for the experiments. The placental samples obtained from five patients and used for gene expression analyses were the same as those used in a previous study (Hasegawa et al., 2020). The donated placentas were collected at the following gestation times: from 37 weeks 2 days to 39 weeks 1 day. The study comprising human placentas was approved by the Ethics Committee of Hokkaido University Hospital (018-0138).

2.5. Real-time PCR

BeWo cells (2.0×10^5 cells/well) were seeded on 12-well plastic plates and cultured as described in sections 2.2 and 2.3. Total RNA was isolated from BeWo cells and placental villous tissues using ISOGEN II (Nippon Gene, Japan) as previously described (Furugen et al., 2017; Hasegawa et al., 2020). TS^{CT} cells were seeded on Col IV-coated 6-well plastic plates and cultured as described in sections 2.2 and 2.3. Total RNA was extracted using an RNeasy Mini Kit (QIAGEN) in accordance with the manufacturer's protocol. cDNA was synthesized from total RNA (1 μg) via reverse transcription using ReverTra Ace® (TOYOBO, Osaka, Japan).

Real-time PCR analysis was conducted using the Mx3000P™ or Mx3005™ Real-time PCR System (STRATAGENE) with the KAPA SYBR® Fast qPCR kit (KAPA Biosystems, Boston). The primers used in this study are listed in Supplemental Table 1. The specificity of primers was assessed using the melting curve analysis and agarose gel electrophoresis (Supplemental Figure 1). The cycling conditions were as follows: 40 cycles at 95 °C for 3 s and 60 °C for 20 s (except for ASCT2/*SLC1A5*), 40 cycles at 95 °C for 3 s, and 65 °C for 20 s for *SLC1A5*. The levels of the target genes were normalized to tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*). As shown in Supplemental Figure 2, no substantial changes were noted in Ct values during differentiation. We confirmed that YWHAZ was suitable for use as a reference gene, although we could not evaluate all the candidate housekeeping genes in the present study. Data were analyzed by the relative standard curve method (Figure 1, 2, 4A, and Supplemental Figure 3). To investigate the

252 expression levels in BeWo (FK), ST-TS^{CT}, and placenta (Supplemental Figure 5), data were analyzed
253 by the comparative cycle time (Ct) method.

254 **2.6. Enzyme-linked immunosorbent assay**

255 BeWo cells (1.0×10^5 cells/well) were seeded on 24-well plastic plates and cultured as
256 described in sections 2.2 and 2.3. After treatment with FK (20 μM) and VPA (1 mM) or LTG (50 μM),
257 the cell culture medium was collected. hCG secretion was measured using an hCG enzyme-linked
258 immunosorbent assay (ELISA) kit (Abcam, Cambridge, UK) as previously described (Koishikawa et
259 al., 2022).

260

261 **2.7. Western blotting**

262 BeWo cells (4.0×10^5 cells/well) were seeded on 6-well plastic plates and cultured as described
263 in sections 2.2 and 2.3. After treatment with FK (20 μM) and VPA (1 mM), the cells were lysed in ice-
264 cold lysis buffer supplemented with cOmplete™ Mini protease inhibitor cocktail tablets with 1 mM
265 phenylmethylsulfonyl fluoride. Western blotting was performed as previously described (Furugen et
266 al., 2017). The primary antibodies used were rabbit anti-SLC1A5 (1:1,000, Abcam, #ab237704) and
267 mouse anti-actin (1:1000, Chemicon, Temecula, CA, #MAB1501) monoclonal antibodies. The bound
268 proteins were detected using horseradish peroxidase-conjugated secondary antibodies and ECL
269 Prime™ western blotting Detection Reagent or ECL Western Blotting Detection Reagents (GE
270 Healthcare, Buckinghamshire, England) . The bands were detected using Image Quant LAS 4000 (GE

Healthcare). Band intensities were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.8. Cell fusion assay

BeWo cells (5.0×10^4 cells/dish) were seeded on collagen-coated glass-bottom dishes and cultured. Thereafter, the cells were treated with FK (20 μM) and VPA (1 mM) as described in section 2.3. TS^{CT} cells (1.0×10^5 cells/dish) were seeded on Col IV-coated glass-bottom dishes and cultured as described in sections 2.2 and 2.3. After treatment, the cells were washed with phosphate buffer saline (PBS) and fixed in 4% formaldehyde (Thermo Fisher Scientific) for 15 min at room temperature. To prevent non-specific antigen binding, the cells were blocked with 5% goat serum (Abcam) in PBS for 1 h at room temperature. Thereafter, the samples were incubated with rabbit monoclonal anti-E-cadherin antibody (1:100, #3195, Cell Signaling Technology) diluted in antibody dilution buffer (PBS, 1% BSA, 0.3% Triton X-100) overnight at 4 °C, followed by Alexa Fluor® 488-conjugated goat anti-rabbit IgG diluted in an antibody dilution buffer (1:1000, #4412, Cell Signaling Technology) for 1 h in the dark at room temperature. The nuclei were stained with DAPI (Wako) in PBS for 10 min at room temperature in the dark. After staining, the cells were covered with Prolong® Gold Antifade Reagent (Cell Signaling Technology) and stored at 4 °C in the dark until microscopic assessment using FluoView FV10i (Olympus) or BZ-9000 (KEYENCE). Four fields were selected for the analysis. The results were analyzed as previously described (Clabault et al., 2018; Morosin et al., 2021). A syncytium is defined as a cell containing two or more nuclei in the cytoplasm without E-cadherin-stained

membranes. The fusion index is the total number of nuclei in the syncytium divided by the total number of nuclei present; the resulting value was converted to a percentage by multiplying by 100.

2.9. MTT assay

Cell viability was investigated using the MTT assay. BeWo cells (2.0×10^4 cells/well) were seeded in 96-well plastic plates. TS^{CT} cells (5.0×10^3 cells/well) were seeded in Col IV-coated 96-well plastic plates. After growth of the cells, VPA was added as described in Section 2.3. Before the end of treatment, 10 μL of the MTT solution (5 mg/mL in phosphate-buffered saline) was added to the cells. The cells were then incubated for 1 h, and the culture medium was subsequently aspirated. The MTT formazan was dissolved in 200 μL of DMSO, and the absorbance of solution in each well was read at 570 nm.

2.10. Statistical analysis

The experiments were repeated at least three times. The data are expressed as mean ± standard error (S.E.) of independent experiments. Student's t-test was used for comparisons between the two groups while Tukey–Kramer or Dunnett's test was used for multiple comparisons. Correlations between the expression levels of syncytialization markers and neonatal/placental parameters were analyzed using the Spearman's correlation test. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Screening investigation of the effects of antiepileptic drugs on gene expression associated with syncytialization

First, we screened the effects of eight antiepileptic drugs, VPA, CBZ, LTG, GBP, LEV, TPM, LCM, and CLB, on the gene expression of syncytialization markers in FK-induced differentiated BeWo cells. We previously tested the effects of 24-hour exposure of antiepileptic drugs (VPA, CBZ, LTG, GBP, LEV, TPM, LCM, and CLB) on the viability of undifferentiated BeWo cells (Kurosawa et al., 2018). Because these antiepileptic drugs did not affect the viability of undifferentiated BeWo cells, we chose the concentration close to that used in the previous study. In this study, we opted to evaluate syncytin-1 (*ERVW-1*), syncytin-2 (*ERVFRD-1*), connexin-43 (*GJA1*), β-hCG (*CGB*), hPL (*CSH*), ASCT2 (*SLC1A5*), MFSD2 (*MFSD2A*), and MRP4 (*ABCC4*). FK (20 μM) upregulated the expression of *ERVW-1*, *ERVFRD-1*, and *CGB* (Figure 1A, 1B, and 1E), and downregulated the expression of *GJA1*, *SLC1A5*, *CSH*, and *ABCC4* (Figure 1C, 1D, 1F, and 1G). Exposure to VPA (1 mM) reduced the expression of *ERVW-1*, *ERVFRD-1*, and *CGB* under FK-induced conditions (Figure 1A, 1B, and 1E). *SLC1A5* and *CSH* mRNAs levels were reduced by VPA in both undifferentiated and differentiated cells (Figure 1D and 1F). Exposure to VPA increased the expression of *ABCC4* in both undifferentiated and differentiated conditions (Figure 1G). The decreased *GJA1* expression in differentiated cells tended to be restored by exposure to VPA (Figure 1C). In contrast, exposure to other antiepileptic drugs, such as

CBZ, LTG, GBP, LEV, TPM, LCM, and CLB, had no noticeable effect on the expression of these genes. As *MFSD2A* levels were very low in undifferentiated BeWo cells, we did not investigate the effects of the antiepileptic drugs on MFSD2 expression in this cell line. We also investigated the effects of VPA and LTG on several genes using another reference gene (succinate dehydrogenase complex flavoprotein subunit A, *SDHA*) (Supplemental Figure 3). The trend was similar when the expression levels were normalized against *YWHAZ* or *SDHA*.

3.2. Concentration-dependent effect of VPA on gene expression associated with syncytialization

VPA poses several risks to the fetus. Notably, the harmful effects of VPA on fetus, such as teratogenicity and reduced cognitive abilities, have been reported to be dose-dependent (Tomson et al., 2011; Meador et al., 2013). In this study, we investigated the concentration-dependent effect of VPA on gene expression in differentiated BeWo cells. As shown in Figure 2, the effects of VPA on the levels of *ERVW-1*, *ERVFRD-1*, *GJA1*, *CGB*, *CSH*, *SLC1A5*, and *ABCC4* tended to increase with increasing VPA concentration (Figure 2). GCM1/*GCM1*, a transcription factor that regulates the expression of *ERVW-1*, was also investigated in this assessment. *GCM1* was slightly upregulated by FK and suppressed by exposure to VPA (Figure 2D).

3.3. Effect of exposure to VPA on protein expression associated with syncytialization

As VPA exposure considerably affected the expression of genes associated with

syncytialization in BeWo cells, protein expression after VPA exposure was investigated. Briefly, the protein levels of hCG and ASCT were assessed. Based on ELISA, hCG secretion increased after FK stimulation and was suppressed by exposure to VPA (1 mM) (Figure 3A). We also assessed the effects of LTG on hCG secretion. LTG did not have any significant effect on the mRNA levels of syncytialization markers (Figure 1). As described in the Introduction section, LTG is recommended for women of reproductive-aged with epilepsy. LTG had no significant effect on hCG secretion (Figure 3A). Before the effect of VPA on ASCT2 protein levels was determined, the reactivity of the antibody was confirmed by western blotting. The ASCT2 antibody detected two bands, and siRNAs of ASCT2 reduced the band detected between 120 and 210 kDa (Supplemental Figure 4). The predicted size of ASCT2 is 75 kDa. Further, ASCT2 forms dimer and trimer (Pingitore et al., 2013). Based on the result, ASCT2 exists as a multimer. The bands were further analyzed. ASCT2 expression was reduced by VPA under differentiated condition (Figure 3B).

3.4. Effect of VPA exposure on cell fusion by BeWo cells

The effect of VPA on cell fusion was evaluated using a fusion assay. In this study, E-cadherin, which contributes to cell-cell adhesion, was immunostained (green) and the nuclei were stained with DAPI (blue). The degree of cell fusion was analyzed using the fusion index. As shown in Figure 3C, cells were predominantly mononucleated under unstimulated conditions (control). FK (20 μM) treatment induced cell fusion, as indicated by the presence of multinucleated syncytia lacking distinct

cell boundaries; the fusion index was significantly higher than that of the control. Exposure to VPA significantly suppressed cell fusion under FK treatment. Overall, VPA induces morphological changes in BeWo cells in addition to altering syncytialization-associated genes.

3.5. Effects of VPA in human trophoblast stem TS^{CT} cells

In the present study, we determined the effects of VPA on gene expression associated with syncytialization using another trophoblast model, human trophoblast stem cells (TS^{CT} cells). Prior to the assay, we investigated the expression levels of genes associated with syncytialization in human term placentas, FK-induced differentiated BeWo cells (BeWo (FK)), TS^{CT} cells, and TS^{CT}-derived ST cells (ST-TS^{CT}) (Supplemental Figure 5). In the present study, we used the villous part of term placenta, which is abundant in syncytiotrophoblasts. The expression levels were normalized against *YWHAZ*. *TBP*, *SDHA*, and *YWHAZ* have been reported to be stable and appropriate for comparative expression studies in placenta (Meller et al., 2005). As shown in Supplemental Table 2, average Ct values were not much different among BeWo cells, TS cells, and term placenta. All markers were detected in both BeWo (FK) and ST-TS^{CT} cells. *ERVW-1* level in ST-TS^{CT} cells was significantly higher than in BeWo (FK) cells. *ERVFRD-1* levels in the placenta and BeWo (FK) cells were comparable; however, its expression was higher in the ST-TS^{CT} cells. *GJA1* expression in the placenta was higher than in the cell lines (BeWo (FK) and ST-TS^{CT} cell). The levels of *GCM1* were comparable between the placental and BeWo (FK) cells. *SLC1A5* levels in BeWo (FK) and ST-TS^{CT} cells were higher than in the placenta.

SLC1A5 levels in BeWo (FK) cells were higher than those in ST-TS^{CT} cells. The expression of *MFSD2A* was high in the placenta and sufficient in ST-TS^{CT} cells. Furthermore, the expression of *CGB* was prominent in ST-TS^{CT} cells, whereas that of *CSH* was comparable between BeWo (FK) and ST-TS^{CT} cells. *ABCC4* levels were comparable among the placenta, BeWo (FK) cells, and ST-TS^{CT} cells. Of note, we could not accurately analyze the expression of *CSH* in the placenta using the current method due to non-specific amplification.

As shown in Supplemental Figure 5, cell culture in ST-medium markedly induced the expression of *CGB*, which has been reported to be upregulated in TS^{CT}-derived ST cells (ST-TS^{CT} cells) (Okae et al., 2018), confirming differentiation. The expression of *ERVW-1* and *ERVFRD-1* increased, whereas that of *ABCC4* decreased in ST-TS^{CT} cells. These results were consistent with those observed in BeWo cells (Figure 1 and 2). The expression of *MFSD2A* in undifferentiated BeWo cells was low (Ct value were approximately 35). Although *MFSD2A* was detected in BeWo (FK) cells, the Ct values were > 30. Meanwhile, the expression of *MFSD2A* was sufficiently detected in ST-TS^{CT} cells (Mean Ct value = 26.2; n = 3). Although the difference was not statistically significant by multiple comparison analysis (p = 0.1439), the expression of *CSH* in TS^{CT} cells tended to increase with differentiation, which contradicted the results in BeWo cells (Figure 1F). We proceeded to determine the effects of VPA-containing ST medium on gene expression associated with syncytialization (Figure 4A). VPA exposure decreased the expression of *ERVW-1*, *ERVFRD-1*, and *CSH*, and increased that of *GJA1* and *ABCC4*. Although not significant (p = 0.059), *CGB* expression tended to decrease after VPA

exposure. The effects of VPA on TS^{CT} cells were similar to those observed in BeWo cells. As shown in Figure 4B, the fusion index was significantly increased in ST-TS^{CT} cells compared with that in TS^{CT} cells. Exposure to VPA significantly suppressed cell fusion. The results suggest that VPA also affected syncytialization of TS^{CT}-derived cells. *SLC1A5* and *GCM1* expression was not altered by VPA-containing ST medium. The expression of *MFSD2A*, which was not analyzed in BeWo cells, was decreased by exposure to VPA. Overall, VPA affects the differentiation in human ST cells.

3.6. Association between neonatal/placental parameters and expression of genes associated with syncytialization in human term placenta

As described in the Introduction section, previous studies have indicated that anomalies in the expression of hormones and syncytialization markers are associated with obstetric complications. However, systematical analyses of relationships between syncytialization-associated genes in placenta and neonatal/placental parameters have not been fully evaluated. Finally, we assessed the correlation between neonatal/placental parameters and expression associated with syncytialization in human term placentas (Figure 5). *MFSD2A* expression level in the placenta was positively correlated with neonatal weight, head circumference, chest circumference, and placental weight (Figure 5F). *ABCC4* levels were negatively correlated with placental weight (Figure 5G). *CGB* levels in the placenta had a negative correlation with neonatal head circumference (Figure 5H). No significant correlation was found between *ERVW-1*, *ERVFRD-1*, *GJA1*, *GCM1*, and *SLC1A5* expression.

4. Discussion

Most pregnant women with epilepsy must continue taking their medication for seizure management (Tomson et al., 2011). In addition to older antiepileptic drugs, newer antiepileptic drugs, which have more favorable pharmacokinetic profiles and fewer drug interactions, have become available for clinical use (Avachat et al., 2022). Notably, prescription patterns of antiepileptic drugs during pregnancy have changed globally over the past decade (Avachat et al., 2022). For example, the Australian pregnancy register reported a declining trend in the use of VPA and CBZ, which are older antiepileptic drugs (Vajda et al., 2014; Vajda et al., 2019). Based on the outcomes and neurodevelopmental effects of antiepileptic drugs in the USA, LTG and LEV, which are newer antiepileptic drugs, were reported as the most commonly prescribed monotherapies between 2012 and 2016 (Meador et al., 2018). In contrast, VPA was the most commonly prescribed antiepileptic drug in Japan between 2005–2016 (Ishikawa et al., 2019). Furthermore, unplanned pregnancy commonly occurs in women with epilepsy (Herzog et al., 2017). To better understand the mechanism of reproductive toxicity induced by antiepileptic drugs and predict the possible risks to the fetus, the effects of multiple drugs, including older and newer ones, must be comprehensively analyzed. Recently, trophoblast functions were reported to be altered by maternal conditions (Le et al., 2014; Clabault et al., 2018; Drwal et al., 2020; Walker et al., 2020; Wang et al., 2021). Therefore, the current study aimed to determine the effects of antiepileptic drugs on the differentiation of human placental trophoblasts.

Screening using the BeWo cell line, a trophoblast model, revealed that VPA altered

syncytialization-associated genes and cell fusion. In addition, changes in hCG and ASCT2 protein levels were observed. In contrast, exposure to other antiepileptic drugs at clinically relevant concentrations, namely CBZ, LTG, GBP, LEV, TPM, LCM, and CLB, had no significant effect on the expression of syncytialization-associated genes. We confirmed that LTG did not affect the secretion of hCG from BeWo cells. Although we did not evaluate the effects of all antiepileptic drugs in the current study, the results support the notion that gene expression analysis may be useful in predicting the effects of drugs on syncytialization. Further studies are required to evaluate whether antiepileptic drugs other than VPA affect syncytiotrophoblast formation. The risks associated with VPA use during pregnancy, such as teratogenicity, neurodevelopmental delay, and autism spectrum disorder, are widely recognized. However, information on other obstetric complications associated with antiepileptic drug use during pregnancy, particularly fetal growth and placentation, has not been fully elucidated. Previously, we revealed that repeated VPA administration decreased placental weight in Wistar rats (Jinno et al., 2020). VPA concentrations in maternal plasma and umbilical blood have been reported to have an inverse relationship with neonatal body height and weight (Kacirova et al., 2015). The adverse effects of *in utero* exposure to VPA, reduced cognitive abilities, and malformations are dose-dependent (Tomson et al., 2011; Meador et al., 2013). In the present study, the effects of VPA on syncytialization-associated genes were demonstrated to be dose-dependent.

Generally used *in vitro* models are isolated primary cultures, placental explants, and trophoblast cell lines (Ji et al., 2013). BeWo is a continuous cell line derived from human placental

choriocarcinoma that is widely used for toxicological assessment and investigation of syncytialization (Ji et al., 2013). However, differences in gene expression and responses to various stimuli between cancer cell lines and normal trophoblasts should be considered (Myllynen et al., 2013). The derivation of human trophoblast stem cells from CT cells isolated from human placentas (TS^{CT} cells) has been reported (Okae et al., 2018). In this study, we utilized this cell model for the analyses. The expression of several genes, including *ERVW-1*, *ERVFRD-1*, *CGB*, and *MFSD2A*, was predominant in ST-TS^{CT} compared with BeWo (FK) cells. In particular, *MFSD2A* levels were very low in BeWo; therefore, we could not investigate the effects of antiepileptic drugs on *MFSD2A* expression in cells. Using ST-TS^{CT} cells, we found that VPA downregulated *MFSD2A* levels. When BeWo cells and TS^{CT} cells were compared, the expression patterns of markers during differentiation were found to display similar trends, except for *CSH*. The expression of *CSH* in BeWo cells decreased after differentiation, whereas its expression tended to increase in ST-TS^{CT} cells. The phenomenon observed in BeWo cells was consistent with that found in a previous study (Clabault et al., 2018). *CSH* expression increases during the differentiation of human primary trophoblasts (Handwerger, 2010). Overall, the TS^{CT} model is more suitable for investigating hPL/*CSH* expression and function. With regard to sensitivity to antiepileptic drugs, alteration of the expression of markers by exposure to VPA displayed similar trends, except for *SLC1A5* and *GCM1*. The expression levels of *SLC1A5* and *GCM1* in differentiated BeWo cells were decreased by VPA exposure; however, these effects were not observed in ST-TS^{CT} cells. The fusion assay revealed the effects of VPA exposure on syncytialization in ST-TS^{CT} cells. Because we

480 could not measure protein concentration in the present study, further studies are needed to assess the
481 alterations in protein levels of syncytialization markers. To compare the expression levels of genes
482 associated with syncytialization in placenta and cell lines, we used the villous part of term placenta.
483 Because placental tissue is heterogeneous, trophoblasts isolated from placenta villous need to be
484 analyzed for accurate comparison. Further investigations are required to reveal the effects of VPA on
485 primary trophoblasts and the causes of these differences.

486 VPA affects the biochemistry of folate, which is a key component in nucleotide synthesis and
487 thereby proliferation (Fathe K et al., 2014; Reynolds and Green, 2020). Moreover, VPA, at relatively
488 higher concentrations, exhibits antiproliferative activity in cancer cells (Du et al., 2022). In the study,
489 we chose the concentrations without significant effect on the viability of undifferentiated BeWo cells
490 based on a previous study (Kurosawa et al., 2018). In addition, no effect of VPA doses ranging from
491 0.45 to 1.5 mM on undifferentiated BeWo cells was observed after 24–48 h of exposure (Kwiecińska
492 et al., 2011). However, the effects of antiepileptic drugs on the viability of differentiated cells have not
493 been examined. We investigated the effects of VPA on the viability of differentiated BeWo or TS cells
494 using the MTT assay. As shown in Supplemental Figure 6, the viability of differentiated BeWo cells
495 was not significantly affected upon treatment with 1 mM VPA. However, the proliferation of ST-TS^{CT}
496 cells was reduced compared with that of TS^{CT} cells. Exposure to VPA restored the proliferation of ST-
497 TS^{CT} cells. These differences may be due to the cell type (cancerous or normal cells). On the contrary,
498 alterations in the expression of differentiation markers upon VPA exposure displayed similar trends in

differentiated BeWo cells and ST-TS^{CT} cells. Although we could not completely exclude the possibility that cell proliferation subsequently affects differentiation markers, these observations suggest that the effects of VPA on these markers are independent of alterations in cell proliferation. In the present and previous study (Kurosawa et al., 2018), we assessed cell viability by only MTT assay at single time point. Although the MTT assay is widely used for the assessment of cytotoxicity, cell viability, and proliferation, the data obtained by this assay should be interpreted with caution (Stockert et al., 2012). Furthermore, the present study could not address whether cell proliferation (doubling and growth) was affected by exposure to antiepileptic drugs. Future studies are required to evaluate the detailed effects of VPA and other antiepileptic drugs on cytotoxicity and cell proliferation using various approaches (e.g., LDH assay, thymidine incorporation assay, and real-time cell analysis).

Placental microenvironment has been reported to exhibit severe hypoxic conditions, especially in the first trimester (Zhao et al., 2021). Furthermore, O_2 levels affect the differentiation of cytotrophoblast into syncytiotrophoblast (Zhao et al., 2021). In the present study, cells were cultured under normal O_2 conditions. Future studies should investigate the effects of antiepileptic drugs on differentiation under hypoxic conditions.

In the present study, we analyzed the relationship between neonatal/placental parameters and gene expression associated with syncytialization in human term placentas. *MFSD2A* was found to be positively correlated with several parameters (neonatal body weight, head circumference, chest circumference, and placental weight). MFSD2/*MFSD2A* is the receptor for syncytin-2 and has been

518 reported to play an important role in ST development in the human placenta (Esnault et al., 2008).
519 Further, MFSD2 expression in the placenta decreases obstetric complications, such as fetal growth
520 restriction and gestational diabetic (Ruebner et al., 2010; Soygur et al., 2016). In this study, VPA
521 downregulated *MFSD2A* mRNA in ST-TS^{CT} cells. Placenta-derived nucleic acids in maternal plasma
522 can be used to predict pregnancy-related complications (Manokhina et al., 2005). These observations
523 suggest that *MFSD2A* can be used as a biomarker to predict fetal and placental toxicity of drugs.
524 Because we obtained RNA samples for gene analyses in the present study, protein levels of
525 syncytialization markers, including MFSD2, were not evaluated. Further studies are required to
526 evaluate the association between MFSD2 protein levels and neonatal/placental parameters, and
527 MFSD2/*MFSD2A* expression in placenta donated by women treated with antiepileptic drugs.
528 In summary, the current study was conducted to comprehensively investigate the effects of
529 eight antiepileptic drugs on syncytialization-associated genes in the BeWo human trophoblast cell line.
530 VPA was found to have a dose-dependent effect on alterations in gene expression accompanied by
531 differentiation. VPA also suppressed the fusion of BeWo and TS^{CT} cells. The present findings suggest
532 that TS^{CT} cells are useful as trophoblast models for toxicological research to investigate drug-induced
533 alterations of syncytialization. In addition, our study implied that *MFSD2A* expression in term
534 placentas is positively correlated with several neonatal/placental parameters. This study had some
535 limitations. First, we did not analyze the effects of VPA on primary trophoblasts or *in vivo*. The
536 differences between the experimental models and the extrapolation of results obtained using the cell

lines to real trophoblasts must be performed with caution. Second, for the analysis of syncytialization markers in the placenta, we could not precisely unify patient characteristics. Further, the sample number of the placenta was relatively low for accurate analysis. Third, we did not explore the underlying mechanisms of reduced syncytialization following VPA exposure. As mentioned in the Introduction section, VPA exhibits inhibitory effects on HDACs (Gurvich et al., 2004), which are involved in the regulation of gene expression. Because HDAC inhibitors impair human syncytiotrophoblast formation (Jaju Bhattad et al., 2020), the present results of VPA affecting the differentiation of BeWo and TS^{CT} cells are reasonable. However, other antiepileptic drugs, namely CBZ, TPM, and LCM, that altered the HDAC activity did not have significant effect on syncytialization-associated genes in BeWo cells. Further studies are required to evaluate the involvement of HDACs in syncytialization of cells exposed to antiepileptic drugs. Furthermore, various signaling pathways are reported to be involved in the syncytialization process (Gupta et al., 2016). Future studies should address these issues to enable a more robust examination of the mechanism of VPA toxicity and estimate the risk of obstetric complications.

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Conflicts of Interest

The authors report no conflicts of interest.

References

Avachat, C., Barry, J. M., Lyu, X., Sherwin, C. M., Birnbaum, A. K., 2022. Management of Anti-Seizure Medications during Pregnancy: Advancements in The Past Decade. Pharmaceutics. 14, 2733. https://doi.org/10.3390/pharmaceutics14122733

Bang, S.R., Ambavade, S.D., Jagdale, P.G., Adkar, P.P., Waghmare, A.B., Ambavade, P.D., 2015. Lacosamide reduces HDAC levels in the brain and improves memory: Potential for treatment of Alzheimer's disease. *Pharmacol. Biochem. Behav.* 134:65-69. https://doi.org/ 10.1016/j.pbb.2015.04.011.

Beutler, A.S., Li, S., Nicol, R., Walsh, M.J. , 2005. Carbamazepine is an inhibitor of histone deacetylases. *Life Sci.* 76:3107-3115. https://doi.org/10.1016/j.lfs.2005.01.003.

Chen, C. P., Wang, K. G., Chen, C. Y., Yu, C., Chuang, H. C., Chen, H., 2006. Altered placental syncytin and its receptor ASCT2 expression in placental development and pre-eclampsia. *BJOG*. 113:152-158. https://doi.org/10.1111/j.1471-0528.2005.00843.x

Christensen, J., Grønborg, T.K., Sørensen, M.J., Schendel, D., Partner, E.T., Pedersen, L.H., 2013. Vestergaard M. Prenatal valproate exposure and risk of autism spectrum disorders and childhood autism. *JAMA*. 309:1696-1703. https://doi.org/10.1001/jama.2013.2270.

Clabault, H., Flipo, D., Guibourdenche, J., Fournier, T., Sanderson, J. T., Vaillancourt, C., 2018. Effects of selective serotonin-reuptake inhibitors (SSRIs) on human villous trophoblasts syncytialization. *Toxicol. Appl. Pharmacol*. 349:8-20. https://doi.org/10.1016/j.taap.2018.04.018

Copsel, S., Garcia, C., Diez, F., Vermeulem, M., Baldi, A., Bianciotti, L. G., Russel, F. G., Shayo, C., & Davio, C., 2011. Multidrug resistance protein 4 (MRP4/ABCC4) regulates cAMP cellular levels and controls human leukemia cell proliferation and differentiation. *J. Biol. Chem*. 286:6979-6988. https://doi.org/10.1074/jbc.M110.166868

Costa, M.A., 2016. The endocrine function of human placenta: an overview. *Reprod. Biomed. Online*. 32:14-43. https://doi.org/10.1016/j.rbmo.2015.10.005

Drwal, E., Rak, A., Tworzydło, W., Gregoraszczuk, E. Ł., 2020. "Real life" polycyclic aromatic hydrocarbon (PAH) mixtures modulate hCG, hPL and hPLGF levels and disrupt the physiological ratio of MMP-2 to MMP-9 and VEGF expression in human placenta cell lines. *Reprod. Toxicol*. 95:1-10. https://doi.org/10.1016/j.reprotox.2020.02.006

Du, S., Wang, X., Hu, Y., Zhang, S., Wang, D., Zhang, Q., Liu, S., 2022. Valproic acid regulates MIEF1 through MST2-HIPPO to suppress breast cancer growth. *Life Sci.* 15;309:120976. https://doi.org/10.1016/j.lfs.2022.120976.

Esnault, C., Priet, S., Ribet, D., Vernochet, C., Bruls, T., Lavialle, C., Weissenbach, J., Heidmann, T., 2008. A placenta-specific receptor for the fusogenic, endogenous retrovirus-derived, human syncytin-2. *Proc. Natl. Acad. Sci. U S A.105*: 17532–17537. https://doi.org/10.1073/pnas.0807413105

Eyal, S., Yagen, B., Sobol, E., Altschuler, Y., Shmuel, M., Bialer, M., 2004. The activity of antiepileptic drugs as histone deacetylase inhibitors. *Epilepsia*. 45:737-744. https://doi.org/10.1111/j.0013-9580.2004.00104.x.

Fathe, K., Palacios, A., Finnell, R.H.., 2014. Brief report novel mechanism for valproate-induced teratogenicity. *Birth Defects Res. A Clin. Mol. Teratol*. 100:592-597. https://doi.org/10.1002/bdra.23277.

Frendo, J. L., Cronier, L., Bertin, G., Guibourdenche, J., Vidaud, M., Evain-Brion, D., Malassine, A., 2003. Involvement of connexin 43 in human trophoblast cell fusion and differentiation. *J. Cell Sci*. 116:3413-3421. https://doi.org/10.1242/jcs.00648

Furugen, A., Ishiguro, Y., Kobayashi, M., Narumi, K., Nishimura, A., Hirano, T., Iseki, K., 2017. Involvement of l-type amino acid transporter 1 in the transport of gabapentin into human placental choriocarcinoma cells. *Reprod. Toxicol*. 67:48-55. https://doi.org/10.1016/j.reprotox.2016.11.002

Granit, A., Tetro, N., Shmuel, M., Peretz, T., Eyal, S., 2018. Lacosamide at therapeutic concentrations induces histone hyperacetylation in vitro. *Epilepsia Open*. 3:535-539. https://doi.org/10.1002/epi4.12269.

Gupta, S.K., Malhotra, S.S., Malik, A., Verma, S., Chaudhary, P., 2016. Cell Signaling Pathways Involved During Invasion and Syncytialization of Trophoblast Cells. *Am. J. Reprod. Immunol.* 75:361-371. https://doi.org/10.1111/aji.12436

Handwerger S., 2010. New insights into the regulation of human cytotrophoblast cell differentiation. *Mol. Cell Endocrinol*. 323:94-104. https://doi.org/10.1016/j.mce.2009.12.015

Hasegawa, N., Furugen, A., Ono, K., Koishikawa, M., Miyazawa, Y., Nishimura, A., Umazume, T., Narumi, K., Kobayashi, M., Iseki, K., 2020. Cellular uptake properties of lamotrigine in human

placental cell lines: Investigation of involvement of organic cation transporters (SLC22A1-5). *Drug Metab. Pharmacokinet*. 35:266-273. https://doi.org/10.1016/j.dmpk.2020.01.005

Hernandez-Diaz, S., Huybrechts, K.F., Desai, R.J., Cohen, J.M., Mogun, H., Pennell, P.B, Bateman, B.T., Patorno, E., 2018. Topiramate use early in pregnancy and the risk of oral clefts: A pregnancy cohort study. *Neurology*. 90:e342-e351. https://doi.org/ 10.1212/WNL.0000000000004857.

Herzog, A. G., Mandle, H. B., Cahill, K. E., Fowler, K. M., Hauser, W. A., 2017. Predictors of unintended pregnancy in women with epilepsy. *Neurology*.;88:728-733. https://doi.org/10.1212/WNL.0000000000003637

Hoeltzenbein, M., Slimi, S., Fietz, A.K., Stegherr, R., Onken, M., Beyersmann, J., Dathe, K., Schaefer, C., 2023. Increasing use of newer antiseizure medication during pregnancy: An observational study with special focus on lacosamide. *Seizure*. 107:107-113. https://doi.org/10.1016/j.seizure.2023.02.015.

Huang, Q., Chen, H., Li, J., Oliver, M., Ma, X., Byck, D., Gao, Y., Jiang, S. W., 2014. Epigenetic and non-epigenetic regulation of syncytin-1 expression in human placenta and cancer tissues. *Cell Signal*. 26:648-656. https://doi.org/10.1016/j.cellsig.2013.11.002

Hurault-Delarue, C., Morris, J.K., Charlton, R., Gini, R., Loane, M., Pierini, A., Puccini, A., Neville, A., Snowball, J., Damase-Michel, C.; EUROmediSAFE consortium., 2019. Prescription of antiepileptic medicines including valproate in pregnant women: A study in three European countries. *Pharmacoepidemiol. Drug Saf*. 28:1510-1518. https://doi.org/ 10.1002/pds.4897.

Ishikawa, T., Obara, T., Jin, K., Nishigori, H., Miyakoda, K., Suzuka, M., Ikeda-Sakai, Y., Akazawa, M., Nakasato, N., Yaegashi, N., Kuriyama, S., Mano, N., 2019. Examination of the prescription of antiepileptic drugs to prenatal and postpartum women in Japan from a health administrative database. *Pharmacoepidemiol. Drug Saf*. 28:804-811. https://doi.org/10.1002/pds.4749

Jacob, S., Nair, A. B., 2016. An Updated Overview on Therapeutic Drug Monitoring of Recent Antiepileptic Drugs. *Drugs R. D.* 16:303-316. https://doi.org/10.1007/s40268-016-0148-6

Jaju, Bhattad, G., Jeyarajah, M.J., McGill, M.G., Dumeaux, V., Okae, H., Arima, T., Lajoie, P., Bérubé, N.G., Renaud, S.J., 2020. Histone deacetylase 1 and 2 drive differentiation and fusion of progenitor cells in human placental trophoblasts. *Cell Death Dis*. 11:311. https://doi.org/10.1038/s41419-020-2500-6.

Ji, L., Brkić, J., Liu, M., Fu, G., Peng, C., Wang, Y. L., 2013. Placental trophoblast cell differentiation: physiological regulation and pathological relevance to preeclampsia. *Mol. Aspects Med. 34*, 981–1023. https://doi.org/10.1016/j.mam.2012.12.008

Jinno, N., Furugen, A., Kurosawa, Y., Kanno, Y., Narumi, K., Kobayashi, M., Iseki, K., 2020. Effects of single and repetitive valproic acid administration on the gene expression of placental transporters in pregnant rats: An analysis by gestational period. *Reprod. Toxicol*. 96:47-56. https://doi.org/10.1016/j.reprotox.2020.04.077

Kacirova, I., Grundmann, M., & Brozmanova, H., 2015. Serum levels of valproic acid during delivery in mothers and in umbilical cord - correlation with birth length and weight. *Biomed. Pap. Med. Fac. Univ. Palacky. Olomouc. Czech. Repub*. 159:569-575. https://doi.org/10.5507/bp.2015.055

Kim, H., Faught, E., Thurman, D.J., Fishman, J., Kalilani, L., 2019. Antiepileptic Drug Treatment Patterns in Women of Childbearing Age With Epilepsy. *JAMA Neurol*. 76:783-790. https://doi.org/10.1001/jamaneurol.2019.0447.

Knerr, I., Beinder, E., & Rascher, W., 2002. Syncytin, a novel human endogenous retroviral gene in human placenta: evidence for its dysregulation in preeclampsia and HELLP syndrome. *Am. J. Obstet. Gynecol*. 186:210-213. https://doi.org/10.1067/mob.2002.119636

Koishikawa, M., Furugen, A., Ohyama, N., Narumi, K., Ishikawa, S., Kobayashi, M., 2022. Uptake of antiepileptic drugs in forskolin-induced differentiated BeWo cells: alteration of gabapentin transport. *Xenobiotica*. 52:405-412. https://doi.org/10.1080/00498254.2022.2085635

Kurosawa, Y., Furugen, A., Nishimura, A., Narumi, K., Kobayashi, M., Iseki, K., 2018. Evaluation of the effects of antiepileptic drugs on folic acid uptake by human placental choriocarcinoma cells. *Toxicol. In Vitro*. 48:104-110. https://doi.org/10.1016/j.tiv.2017.12.003

Kwiecińska, P., Wiśniewska, J., Gregoraszczuk, E. Ł., 2011. Effects of valproic acid (VPA) and levetiracetam (LEV) on proliferation, apoptosis and hormone secretion of the human choriocarcinoma BeWo cell line. *Pharmacol. Rep*. 63:1195-1202. https://doi.org/10.1016/s1734-1140(11)70639-9

Langbein, M., Strick, R., Strissel, P. L., Vogt, N., Parsch, H., Beckmann, M. W., Schild, R. L., 2008. Impaired cytotrophoblast cell-cell fusion is associated with reduced Syncytin and increased apoptosis in patients with placental dysfunction. *Mol. Reprod. Dev*. 75:175-183. https://doi.org/10.1002/mrd.20729

Lavillette, D., Marin, M., Ruggieri, A., Mallet, F., Cosset, F. L., Kabat, D., 2002. The envelope glycoprotein of human endogenous retrovirus type W uses a divergent family of amino acid transporters/cell surface receptors. *J. Virol*. 76:6442-6452. https://doi.org/10.1128/jvi.76.13.6442-6452.2002

Lee, X., Keith, J. C., Jr, Stumm, N., Moutsatsos, I., McCoy, J. M., Crum, C. P., Genest, D., Chin, D., Ehrenfels, C., Pijnenborg, R., van Assche, F. A., Mi, S., 2001. Downregulation of placental syncytin expression and abnormal protein localization in pre-eclampsia. *Placenta*. 22:808-812. https://doi.org/10.1053/plac.2001.0722

Le Vee, M., Kolasa, E., Jouan, E., Collet, N., Fardel, O., 2014. Differentiation of human placental BeWo cells by the environmental contaminant benzo(a)pyrene. *Chem. Biol. Interact*. 210:1-11. https://doi.org/10.1016/j.cbi.2013.12.004

Maltepe, E., Krampitz, G.W., Okazaki, K.M., Red-Horse, K., Mak, W., Simon, M.C., Fisher, S.J., 2005. Hypoxia-inducible factor-dependent histone deacetylase activity determines stem cell fate in the placenta. *Development.* 132:3393-3403. https://doi.org/10.1242/dev.01923.

Manokhina, I., Wilson, S.L., Robinson, W.P., 2015. Noninvasive nucleic acid-based approaches to monitor placental health and predict pregnancy-related complications. *Am. J. Obstet. Gynecol.* 213:S197-206. https://doi.org/10.1016/j.ajog.2015.07.010.

Meador, K. J., Baker, G. A., Browning, N., Cohen, M. J., Bromley, R. L., Clayton-Smith, J., Kalayjian, L. A., Kanner, A., Liporace, J. D., Pennell, P. B., Privitera, M., Loring, D. W., NEAD Study Group, 2013. Fetal antiepileptic drug exposure and cognitive outcomes at age 6 years (NEAD study): a prospective observational study. *Lancet Neurol*. 12:244-252. https://doi.org/10.1016/S1474-4422(12)70323-X

Meador, K.J., Loring, D.W., 2016. Developmental effects of antiepileptic drugs and the need for improved regulations. *Neurology*. 86:297-306. https://doi.org/ 10.1212/WNL.0000000000002119.

Meador, K. J., Pennell, P. B., May, R. C., Gerard, E., Kalayjian, L., Velez-Ruiz, N., Penovich, P., Cavitt, J., French, J., Hwang, S., Pack, A. M., Sam, M., Moore, E., Ippolito, D. M., & MONEAD Investigator Group., 2018. Changes in antiepileptic drug-prescribing patterns in pregnant women with epilepsy. *Epilepsy Behav*. 84:10-14. https://doi.org/10.1016/j.yebeh.2018.04.009

Meller, M., Vadachkoria, S., Luthy, D.A., Williams, M.A., 2005. Evaluation of housekeeping genes in placental comparative expression studies. *Placenta*. 26:601-607.

https://doi.org/10.1016/j.placenta.2004.09.009.

Milazzo, G., Mercatelli, D., Di Muzio, G., Triboli, L., De Rosa, P., Perini, G., Giorgi, F.M., 2020. Histone Deacetylases (HDACs): Evolution, Specificity, Role in Transcriptional Complexes, and Pharmacological Actionability. *Genes (Basel)*. 11:556. https://doi.org/10.3390/genes11050556.

Morosin, S. K., Delforce, S. J., Kahl, R. G. S., Corbisier de Meaultsart, C., Lumbers, E. R., Pringle, K. G., 2021. The (pro)renin receptor and soluble (pro)renin receptor in choriocarcinoma. *Reproduction*. 162:375-384. https://doi.org/10.1530/REP-20-0650

Myllynen, P., Vähäkangas, K., 2013. Placental transfer and metabolism: an overview of the experimental models utilizing human placental tissue. *Toxicol. In Vitro*. 27:507-512. https://doi.org/10.1016/j.tiv.2012.08.027

Okae, H., Toh, H., Sato, T., Hiura, H., Takahashi, S., Shirane, K., Kabayama, Y., Suyama, M., Sasaki, H., Arima, T., 2018. Derivation of Human Trophoblast Stem Cells. *Cell Stem. Cell*. 22:50-63.e6. https://doi.org/10.1016/j.stem.2017.11.004

Okahara, G., Matsubara, S., Oda, T., Sugimoto, J., Jinno, Y., Kanaya, F., 2004. Expression analyses of human endogenous retroviruses (HERVs): tissue-specific and developmental stage-dependent expression of HERVs. *Genomics*. 84:982-990. https://doi.org/10.1016/j.ygeno.2004.09.004

Otto, T., Gellhaus, A., Lüschen, N., Scheidler, J., Bendix, I., Dunk, C., Wolf, N., Lennartz, K., Köninger, A., Schmidt, M., Kimmig, R., Fandrey, J., Winterhager, E., 2015. Oxygen Sensitivity of Placental Trophoblast Connexins 43 and 46: A Role in Preeclampsia?. *J. Cell. Biochem*. 116:2924-2937. https://doi.org/10.1002/jcb.25240

Pan, Z., Sikandar, S., Witherspoon, M., Dizon, D., Nguyen, T., Benirschke, K., Wiley, C., Vrana, P., Lipkin, S.M., 2008. Impaired placental trophoblast lineage differentiation in Alkbh1(-/-) mice. *Dev. Dyn*. 237:316-327. https://doi.org/10.1002/dvdy.21418.

Patsalos, P.N., Spencer, E.P., Berry, D.J., 2018. Therapeutic drug monitoring of antiepileptic drugs in epilepsy: a 2018 update. *Ther. Drug Monit*. 40:526-548. https://doi.org/10.1097/FTD.0000000000000546.

Pennell, P.B., 2018. Prescribing antiepileptic drugs to women of reproductive age. *Lancet Neurol*. 17(6):485-486. https://doi.org/10.1016/S1474-4422(18)30154-6.

Pingitore, P., Pochini, L., Scalise, M., Galluccio, M., Hedfalk, K., Indiveri, C., 2013. Large scale production of the active human ASCT2 (SLC1A5) transporter in Pichia pastoris--functional and kinetic asymmetry revealed in proteoliposomes. *Biochim. Biophys. Acta*. 1828:2238-2246. https://doi.org/10.1016/j.bbamem.2013.05.034

Reynolds, E.H., Green, R., 2020. Valproate and folate: Congenital and developmental risks. *Epilepsy Behav.* 108:107068. https://doi.org/10.1016/j.yebeh.2020.107068.

Ruebner, M., Strissel, P. L., Langbein, M., Fahlbusch, F., Wachter, D. L., Faschingbauer, F., Beckmann, M. W., & Strick, R., 2010. Impaired cell fusion and differentiation in placentae from patients with intrauterine growth restriction correlate with reduced levels of HERV envelope genes. *J. Mol. Med (Berl)*. 88:1143-1156. https://doi.org/10.1007/s00109-010-0656-8

Soygur, B., Sati, L., Demir, R., 2016. Altered expression of human endogenous retroviruses syncytin-1, syncytin-2 and their receptors in human normal and gestational diabetic placenta. *Histol. Histopathol*. 31:1037-1047. https://doi.org/10.14670/HH-11-735

Stockert, J.C., Blázquez-Castro, A., Cañete, M., Horobin, R.W., Villanueva, A., 2012. MTT assay for cell viability: Intracellular localization of the formazan product is in lipid droplets. *Acta. Histochem.* 114:785-796. https://doi.org/10.1016/j.acthis.2012.01.006.

Sztajnkrycer, M.D., 2002. Valproic acid toxicity: overview and management. *J. Toxicol Clin. Toxicol.* 40:789-801. https://doi.org/10.1081/clt-120014645.

Thomas, S.V., Jose, M., Divakaran, S., Sankara, Sarma, P., 2017. Malformation risk of antiepileptic drug exposure during pregnancy in women with epilepsy: Results from a pregnancy registry in South India. *Epilepsia*. 58:274-281. https://doi.org/10.1111/epi.13632.

Tomson, T., Battino, D., Bonizzoni, E., Craig, J., Lindhout, D., Perucca, E., Sabers, A., Thomas S.V., Vajda, F, EURAP Study Group., 2018. Comparative risk of major congenital malformations with eight different antiepileptic drugs: a prospective cohort study of the EURAP registry. *Lancet Neurol.* 17:530-538. https://doi.org/10.1016/S1474-4422(18)30107-8.

Tomson, T., Battino, D., Bonizzoni, E., Craig, J., Lindhout, D., Sabers, A., Perucca, E., Vajda, F., EURAP study group, 2011. Dose-dependent risk of malformations with antiepileptic drugs: an analysis of data from the EURAP epilepsy and pregnancy registry. *Lancet Neurol*. 10:609-617. https://doi.org/10.1016/S1474-4422(11)70107-7

Vajda, F. J., O'Brien, T., Lander, C., Graham, J., Eadie, M., 2014. The efficacy of the newer antiepileptic drugs in controlling seizures in pregnancy. *Epilepsia*.55:1229-1234. https://doi.org/10.1111/epi.12711

Vajda, F. J. E., Graham, J. E., Hitchcock, A. A., Lander, C. M., O'Brien, T. J., Eadie, M. J., 2019. Antiepileptic drugs and foetal malformation: analysis of 20 years of data in a pregnancy register. *Seizure*. 65:6-11. https://doi.org/10.1016/j.seizure.2018.12.006

Walker, O. S., Ragos, R., Gurm, H., Lapierre, M., May, L. L., Raha, S., 2020. Delta-9-tetrahydrocannabinol disrupts mitochondrial function and attenuates syncytialization in human placental BeWo cells. *Physiol. Rep*. 8:e14476. https://doi.org/10.14814/phy2.14476

Wang, H. L., Liang, N., Huang, D. X., Zhao, X. Y., Dang, Q. Y., Jiang, X. Y., Xiao, R., Yu, H. L., 2021. The effects of high-density lipoprotein and oxidized high-density lipoprotein on forskolin-induced syncytialization of BeWo cells. *Placenta*. 103:199-205. https://doi.org/10.1016/j.placenta.2020.10.024

Zhao, H., Wong, R.J., Stevenson, D.K., 2021. The Impact of Hypoxia in Early Pregnancy on Placental Cells. *Int. J. Mol. Sci*. 22:9675. https://doi.org/10.3390/ijms22189675.

Figure captions

Figure 1. Effects of antiepileptic drugs on the mRNA level of *ERVW-1* (A), *ERVFRD-1* (B), *GJA1* (C), *SLC1A5* (D), *CGB* (E), *CSH* (F), and *ABCC4* (G) in BeWo cells. After 2 days of culture, BeWo cells were exposed to valproic acid (VPA, 1 mM), carbamazepine (CBZ, 50 μM), lamotrigine (LTG, 50 μM), gabapentin (GBP, 100 μM), levetiracetam (LEV, 270 μM), topiramate (TPM, 50 μM), lacosamide (LCM, 40 μM), and clobazam (CLB, 1 μM) in the presence or absence of 20 μM forskolin (FK) for 48 h. The mRNA expression levels were determined using real-time PCR. Data are presented as mean with standard error (S.E.) for three independent experiments, each performed in duplicate or triplicate. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the control. $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ compared to FK alone.

Figure 2. The concentration-dependent effects of valproic acid (VPA) on the mRNA level of *ERVW-1* (A), *ERVFRD-1* (B), *GJA1* (C), *GCM1* (D), *CGB* (E), *CSH* (F), *SLC1A5* (G), and *ABCC4* (H) in BeWo cells. After 2 days of culture, BeWo cells were exposed to VPA (0 -1000 μM) with 20 μM forskolin (FK) for 48 h. These mRNA expression levels were then determined using real-time PCR. Data are presented as mean with standard error (S.E.) for three independent experiments, each performed in duplicate or triplicate. $^{**}p < 0.01$ compared with the control. $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ compared to FK alone.

Figure 3. Effects of valproic acid (VPA) on human chorionic gonadotropin (hCG) secretion (A),

alanine serine cysteine transporter 2 (ASCT2) protein expression (B), and fusion of BeWo cells.

(A) After 2 days of culture, BeWo cells were exposed to culture medium containing VPA (1 mM) or LTG (50 μM) in the presence or absence of forskolin (FK, 20 μM) for 48 h. After treatment, hCG concentration in the culture medium was quantified using enzyme-linked immunosorbent assay (ELISA). Data are presented as mean with standard error (S.E.) for three independent experiments, each performed in triplicate. $^{**}p < 0.01$ compared with the control. $^{††}p < 0.01$ compared with the FK alone treatment.

(B) After 2 days of culture, BeWo cells were exposed to culture medium containing VPA (1 mM) in the presence or absence of forskolin (FK, 20 μM) for 48 h. ASCT expression in BeWo cells was detected using western blotting. Uncropped blots are shown in Supplemental Figure 4B. Data are presented as mean with standard error (S.E.) for three independent experiments. $^{*}p < 0.05$ compared with the control. $^{†}p < 0.05$ compared with the FK alone treatment.

(C) After 2 days of culture, BeWo cells were exposed to culture medium containing VPA (1 mM) in the presence or absence of forskolin (FK, 20 μM) for 48 h. (Left) Representative immunofluorescence images; cells were fixed in 4% formaldehyde, stained with E-cadherin (green), counterstained with DAPI for nuclei (blue), and observed under a microscope. The scale bars indicate 50 μm. (Right) The cellular fusion index was determined by counting nuclei (four images/dish); the index is expressed as the percentage of nuclei in syncytia. Mean with S.E. of three independent experiments. $^{**}p < 0.01$ compared with the control. $^{††}p < 0.01$ compared with the FK alone treatment.

Figure 4. Analyses of human trophoblast stem cells.

(A) Effects of valproic acid (VPA) on the mRNA level of *ERVW-1*, *ERVFRD-1*, *GJA1*, *GCM1*, *CGB*, *CSH*, *SLC1A5*, *MFSD2A*, and *ABCC4* in ST-TS^{CT} cells. After TS^{CT} cells were cultured for 3 days in TS-medium, the cells were exposed to differentiation medium (ST-medium) containing VPA (1 mM) for 72 h. The mRNA expression levels were determined via real-time PCR. Data are presented as mean with standard error (S.E.) for three independent experiments, each performed in duplicate or triplicate. *$p < 0.05$, **$p < 0.01$ compared with the control.

(B) Effects of valproic acid (VPA) on the fusion of TS cells. After TS^{CT} cells were cultured for 3 days in TS-medium, the cells were exposed to differentiation medium (ST-medium) containing VPA (1 mM) for 72 h. (Left) Representative immunofluorescence images; cells were fixed in 4% formaldehyde, stained with E-cadherin (green), counterstained with DAPI for nuclei (blue), and observed under a microscope. The scale bars indicate 50 μm. The cellular fusion index was determined by counting nuclei (four images/dish); the index is expressed as the percentage of nuclei in syncytia. Mean with S.E. of three independent experiments. $^{**}p < 0.01$ compared with the TS^{CT}. $^{††}p < 0.01$ compared with ST-TS^{CT}.

Figure 5. Correlations between the expression levels of the syncytialization markers (*ERVW-1* (A), *ERVFRD-1* (B), *GJA1* (C), *GCM1* (D), *SLC1A5* (E), *MFSD2A* (F), *ABCC4* (G), and *CGB* (H)) and neonatal/ placental parameters (body weight, stature, head circumference, chest circumference,

931 placental weight) were analyzed using Spearman’s correlation test.

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Figure 1

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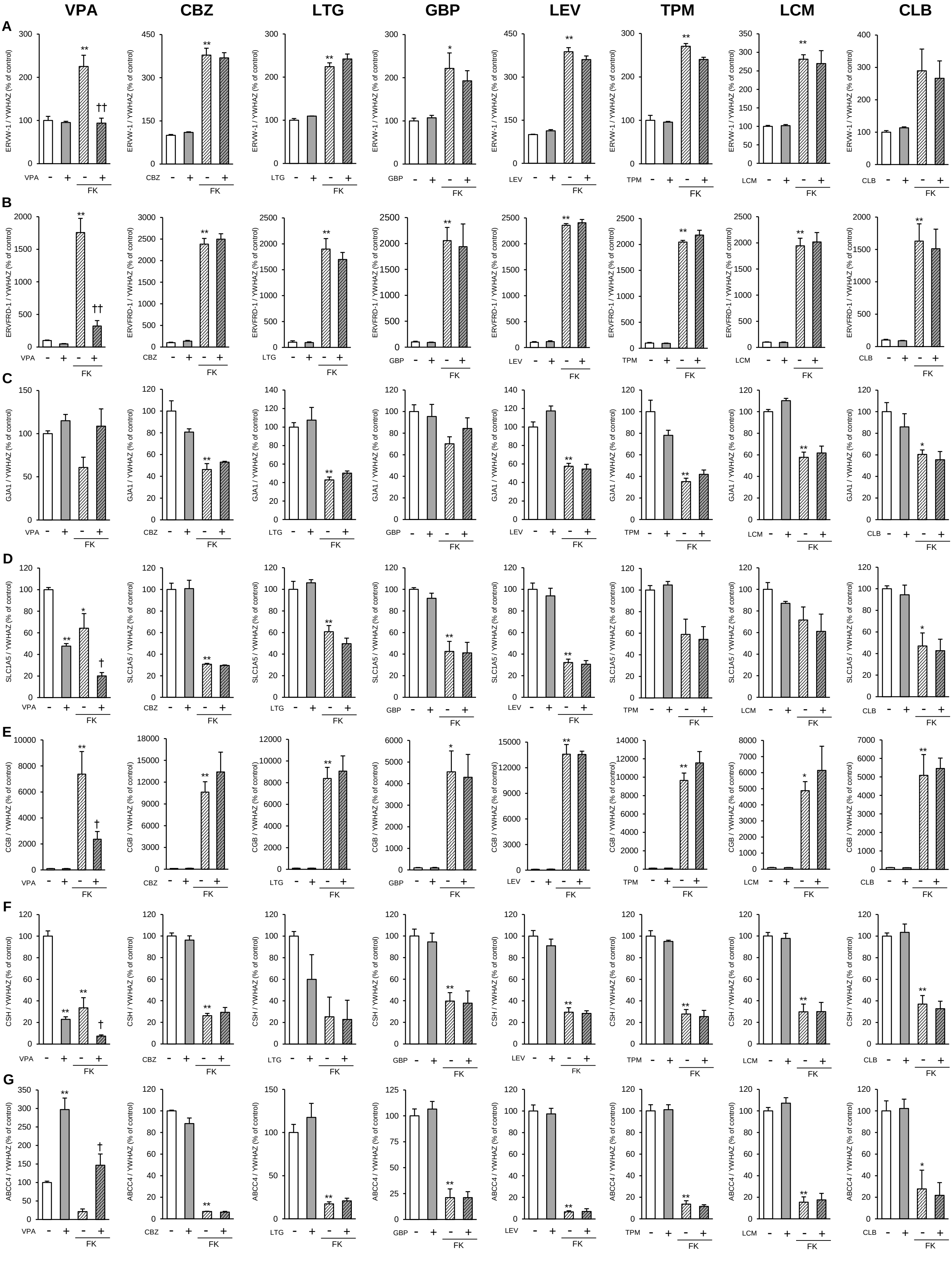

Figure 2

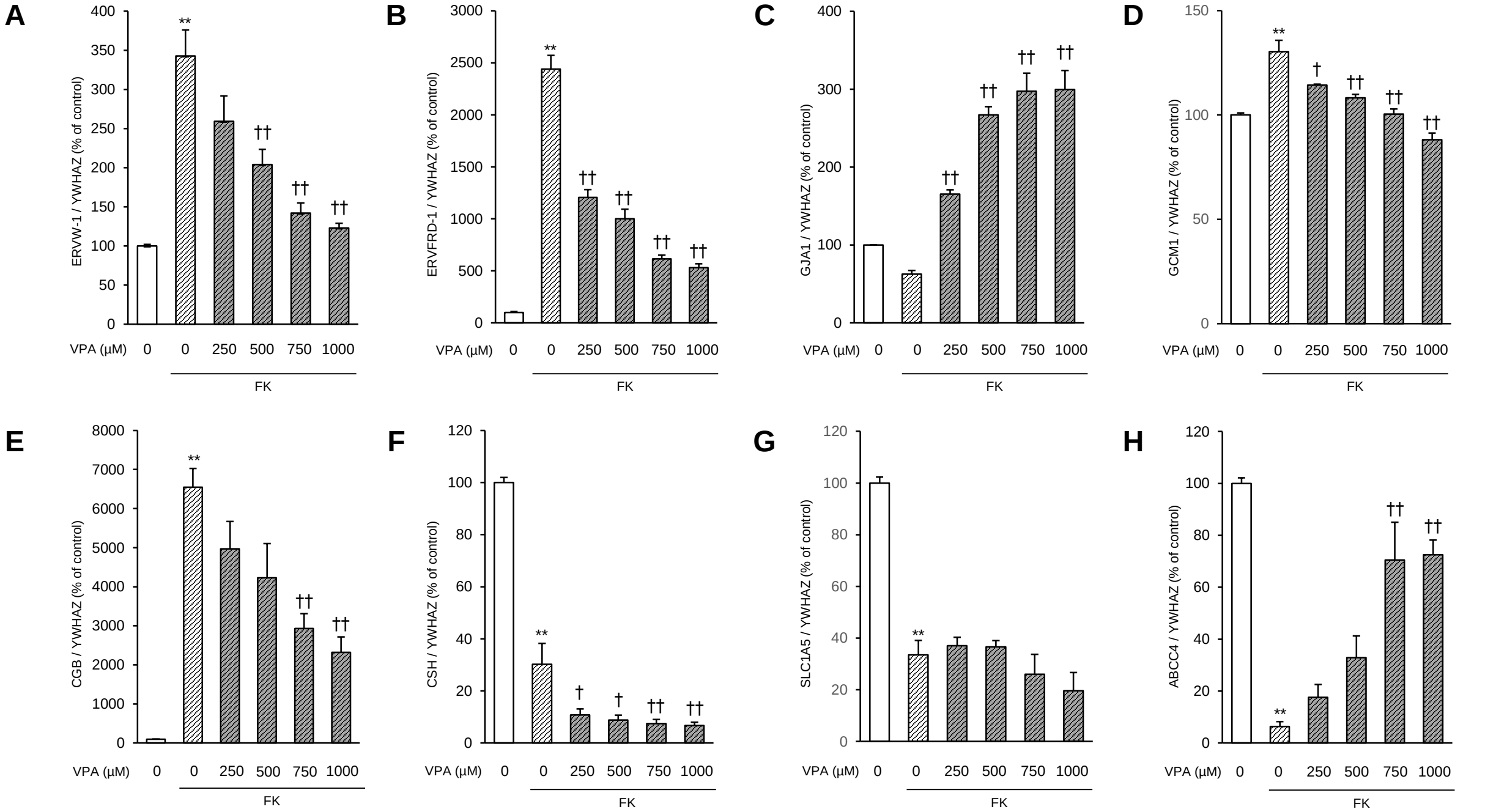

Figure 3

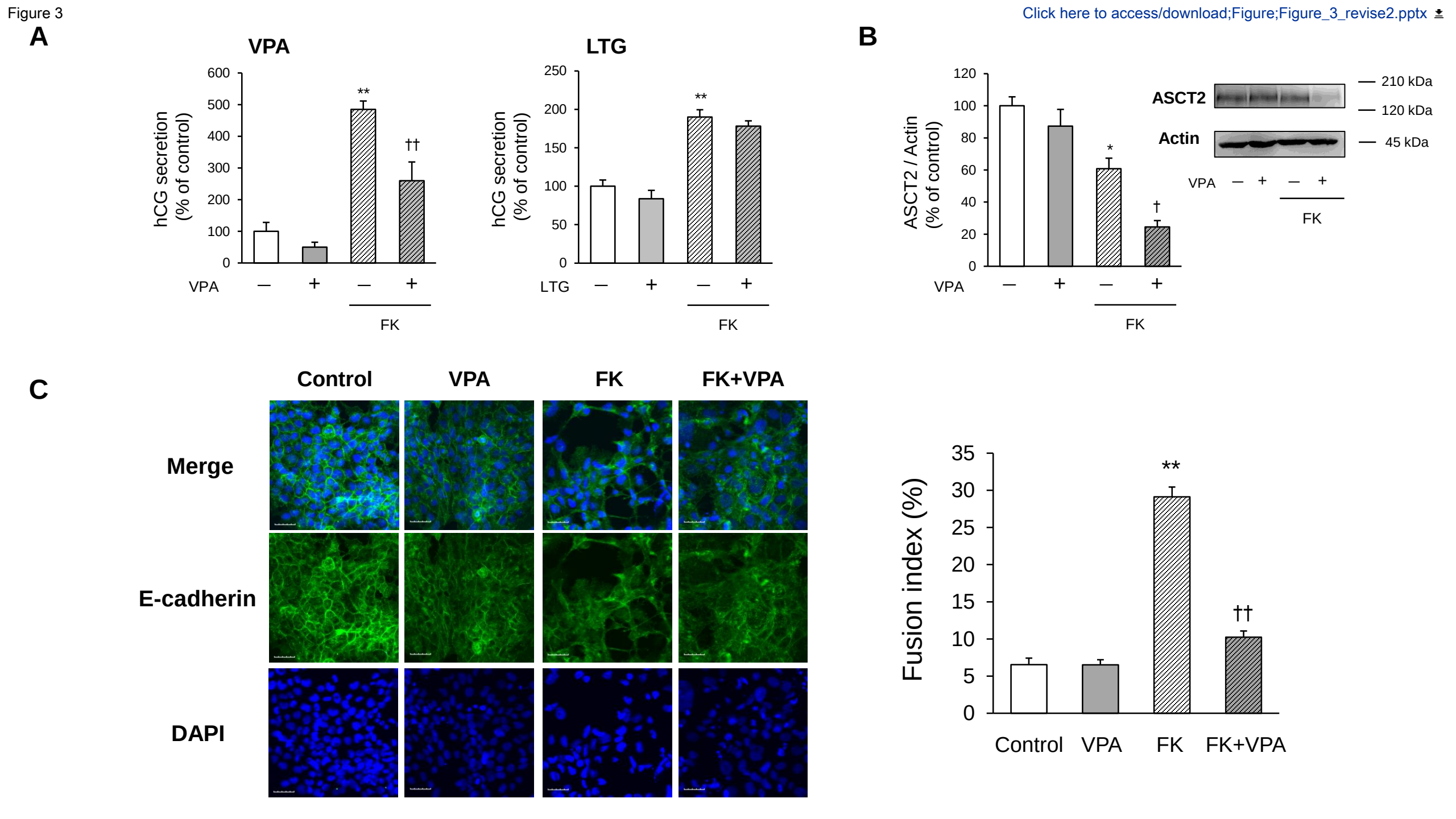

Figure 4

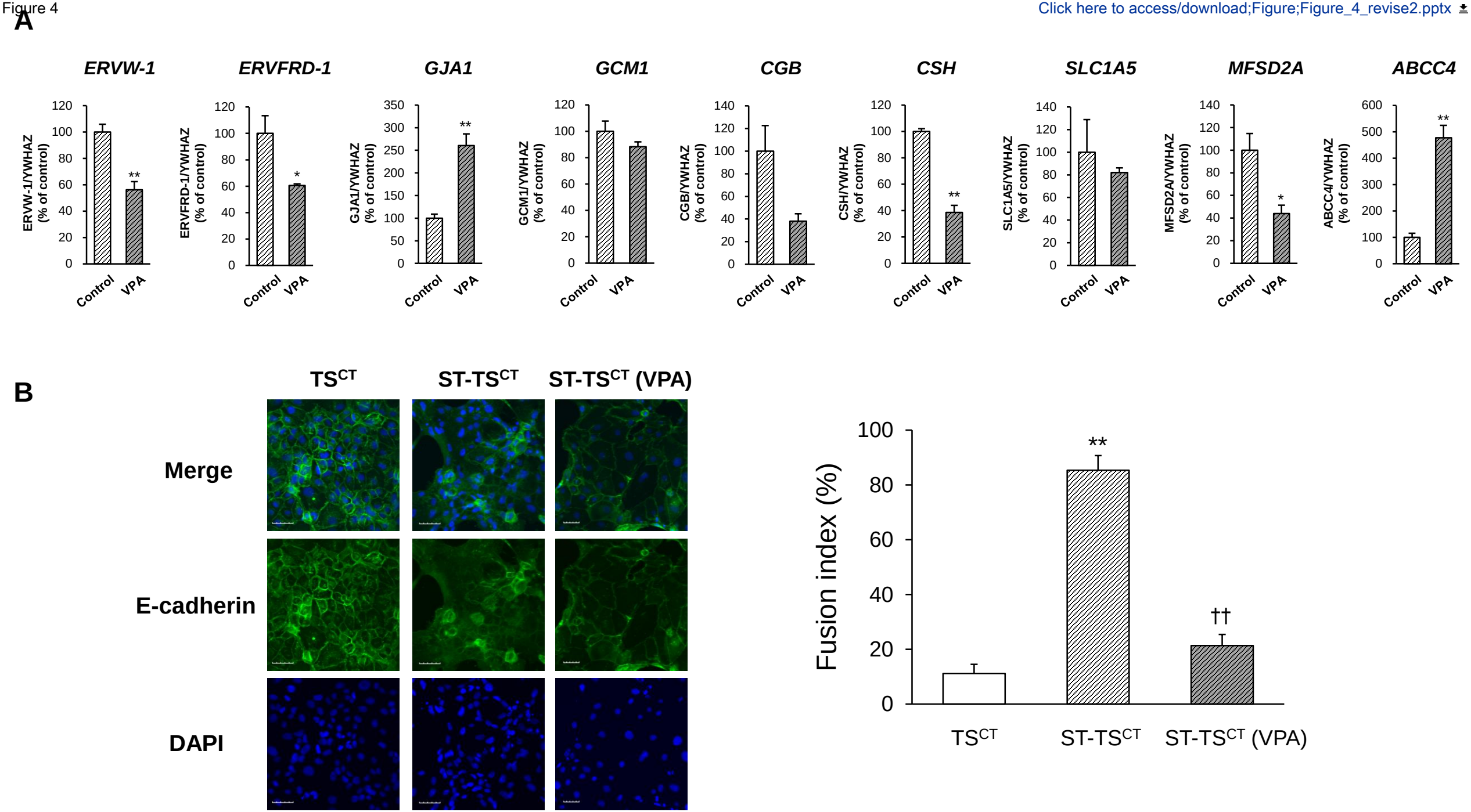

Figure 5

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