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Alteration in folate carrier expression via histone deacetylase inhibition in BeWo human placental choriocarcinoma cells

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Abbreviations

FRα, folate receptor α; HDAC, histone deacetylase; NRF-1, nuclear respiratory factor 1; PCFT, proton-coupled folate transporter; RFC, reduced folate carrier; TSA, trichostatin A; VPA, valproic acid

Abstract

Folates are essential nutrients for fetal development during pregnancy. Valproic acid (VPA), an inhibitor of histone deacetylases (HDACs), alters the expression of folate carriers in placental cells; however, the underlying mechanisms remain unclear. Here, we aimed to determine the profiles of folate carriers (folate receptor alpha [*FOLR1*], solute carrier [*SLC*]-*19A1*, and *SLC46A1*) after inhibition of HDACs, especially class I and IIa HDACs, using different inhibitors and gene knockdown tests. Quantitative polymerase chain reaction revealed that BeWo cells (a trophoblast model) expressed HDACs and folate carriers, similar to human placental villi. *FOLR1* expression was upregulated by VPA, apicidin, and trichostatin A, but downregulated by MS-275 after 24 h treatment. VPA and apicidin upregulated the expression of *SLC46A1*. These inhibitors downregulated *SLC19A1* expression. TMP269 (a class IIa inhibitor) did not affect folate carrier levels. *HDAC1/2* knockdown upregulated *FOLR1* and *SLC46A1* levels, whereas *HDAC1/3* knockdown downregulated *FOLR1* levels. Our findings suggest that the pharmacological inhibition of class I HDACs alters the expression of folate carriers in BeWo cells. By contrast, HDAC inhibitors exert different regulatory effects on folate carriers. Moreover, HDAC1/2 inhibition may be a potential mechanism involved in altering *FOLR1* and *SLC46A1* levels.

Keywords: FRα/*FOLR1*, HDAC, PCFT/*SLC46A1*, Placenta, RFC/*SLC19A1*, Valproic acid

1. Introduction

Folates are necessary for adequate fetal development, and folate status during pregnancy affects the health outcomes of both the fetus and mother (De-Regil et al., 2015; Feng et al., 2015; Hua et al., 2016; Zhang et al., 2017). The placenta is important for nutrient and folate transport from the mother to the fetus (Radziejewska and Chmurzynska, 2019). In the placenta, syncytiotrophoblasts, which differentiate from cytotrophoblasts, are important constituents of the blood–placenta barrier (Tetro et al., 2018). Folate carriers, including proton-coupled folate transporter (PCFT/*SLC46A1*), reduced folate carrier (RFC/*SLC19A1*), and folate receptor α (FRα/*FOLR1*), contribute to the influx of folates into the placenta at the apical side (Solanky et al., 2010). RFC is expressed on the basolateral side and affects folate transport from the placenta to the fetus.

The expression of folate carriers is altered by different maternal physiological and pathological conditions (Chen et al., 2018; Williams et al., 2012). Therefore, regulating the expression and function of folate carriers in the placenta may be important in preventing fetal and pregnancy complications. Folate carriers are regulated by several transcriptional, translational, and post-translational mechanisms. FRα/*FOLR1* is regulated by several factors, such as capicua (CIC) (Cao et al., 2021) and glucocorticoid receptor (GR) (Tran et al., 2005). RFC/*SLC19A1* is regulated by the nuclear respiratory factor 1 (NRF-1) (Alam et al., 2020), vitamin D receptor (VDR) (Alam et al., 2017), and aryl hydrocarbon receptor (AhR) (Halwachs et al., 2010). PCFT expression is

regulated by NRF-1 (Gonen and Assaraf, 2010), VDR (Eloranta et al., 2009), and Kruppel-like factors (Furumiya et al., 2013). Rosario et al. (2016) have reported that the mechanistic target of rapamycin signaling regulates trophoblast folate uptake by modulating the cell surface expression of FRα and RFC. Furthermore, the epigenetic regulation of folate carriers in the placenta has recently been reported. Piñuñuri et al. (2020) demonstrated the epigenetic regulation (methylation) of *FOLR1* in the human placenta of preterm newborns. However, the specific mechanisms underlying folate carrier regulation in the placenta remain unclear.

FRα levels are upregulated and RFC levels are downregulated in human placental BeWo cells after exposure to valproic acid (VPA) for 2 and 5 d (Rubinchik-Stern et al., 2015). We previously reported that exposure to VPA (24 h) alters the FRα and PCFT mRNA expression and transport activity of folic acid in BeWo cells (Kurosawa et al., 2018). In the previous study, we demonstrated that 1 mM VPA exposure increased FRα and PCFT mRNA levels to 171 and 175% of control, respectively, and increased the uptake of [^{3}H]-folic acid into BeWo cells. VPA use during pregnancy is associated with increased fetal risks, such as malformations and autism, after birth (Christensen et al., 2013; Tomson et al., 2011). Therefore, meticulous consideration is required when administering VPA to women of childbearing age. In some cases, VPA has been used to control seizures. However, the mechanisms underlying the adverse effects of VPA on fetuses have not yet been fully elucidated. One hypothesis is that the folate dynamics are affected (Reynolds and Green, 2020). These findings led us to investigate the regulatory mechanisms of folate carriers in

trophoblasts. Therefore, the effects of VPA on the regulatory mechanisms of folate carriers in the placenta require further exploration to understand the reproductive toxicity of VPA and estimate its risk to the fetus.

VPA exerted inhibitory effects on histone deacetylases (HDACs). HDACs regulate several genes via histone deacetylation. In mammals, the HDAC isoforms are divided into four classes: class I (HDAC1–3 and HDAC8), class II (class IIa: HDAC4, HDAC5, HDAC7, and HDAC9; class IIb: HDAC6 and HDAC10), class III (SIRT1–7), and class IV (HDAC11) (Li and Seto, 2016). Based on their chemical structures, HDAC inhibitors are classified into short-chain fatty acids (e.g., VPA and butyrate), cyclic peptides (e.g., apicidin and romidepsin), hydroxamates (e.g., trichostatin A [TSA] and suberoylanilide hydroxamic acid [SAHA]), and benzamides (e.g., MS-275). VPA act on class I and IIa HDACs (Gurvich et al., 2004).

HDACs are involved in regulating transporter expression in different cell types. Green et al. (2017) reported that VPA increases the expression of the dopamine transporter (DAT/*Slc6a3*) via HDAC in a rat dopaminergic cell line. HDAC inhibitors upregulate the expression of P-glycoprotein (P-gp/*ABCB1*) by increasing histone acetylation in the human brain capillary endothelial cells (hCMEC/D3) (You et al., 2019). Furthermore, P-gp is regulated by HDAC2-Foxo3α-mediated signaling in the liver (Xun et al., 2022). Teafatiller et al. (2022) have reported that VPA upregulates sodium-dependent vitamin C transporter-2 (SVCT2/*SLC23A2*) levels via HDAC2 in neuronal cells. As regulation in placental cells, HDAC inhibition upregulates placental P-gp expression in

trophoblast cell lines (BeWo and JAR) (Duan et al., 2017a), and HDAC2 is involved in this process (Duan et al., 2017b). HDAC inhibition upregulates MRP2 expression in BeWo cells and HDAC1 is primarily involved in this process (Duan et al., 2017c).

To date, only a few studies have investigated the ability of HDAC inhibitors to regulate folate carrier levels in placental cells. Therefore, in this study, we evaluated the effects of HDAC inhibition on the expression of folate carriers in human placental cells using different inhibitors and gene knockdown tests.

2. Materials and methods

2.1. Chemicals

VPA (Sigma-Aldrich; St. Louis, MO, USA), apicidin (Sigma-Aldrich), MS-275 (Cayman Chemical; Ann Arbor, MI, USA), TMP269 (Cayman Chemical), and TSA (Wako; Osaka, Japan) were used to investigate the effects of HDAC inhibition on folate carrier levels. Valpromide (VPM) was purchased from Santa Cruz Biotechnology (Santa Cruz; CA, USA). Forskolin was obtained from Wako to induce cell differentiation.

2.2. Cell culture

BeWo cells were derived from human placental choriocarcinomas; these are commonly used as placental trophoblast models. BeWo cells were cultured in Ham's F-12 K (Kaighn's

77 modification) medium (Wako) with 15% fetal bovine serum and 1% penicillin–streptomycin (Sigma
78 Aldrich) at 37°C in an incubator with 5% CO_2 as described previously (Ohyama et al., 2023).
79 Next, the cells were cultured in a medium containing an HDAC inhibitor or 0.1% dimethyl
80 sulfoxide (DMSO) as a control for 24 h. In the study, we investigated the effects of TSA at 0.1 and
81 0.25 μM, apicidin at 0.1, 0.5, and 1 μM, MS-275 at 0.5, 1, and 5 μM, and TMP269 at 10 μM. The
82 concentration settings were based on previous reports that inhibited HDACs or led to histone
83 acetylation (Bhattad et al. 2020; Lobera et al., 2013; You et al., 2019). Because VPA was dissolved in
84 methanol, the control cells were treated with 0.2% methanol. VPA was used at a concentration of 1
85 mM. We selected the concentration that can induce FRα and PCFT mRNA levels based on our
86 previous study (Kurosawa et al., 2018). Furthermore, the concentration was within the clinically
87 relevant range (Sztajnkrycer, 2002). For differentiation, BeWo cells were cultured for 2 days. Next,
88 the medium was changed to another containing 20 μM forskolin or 0.1% DMSO (control). After
89 treatment with forskolin for 48 h, the cells were grown in a medium containing VPA for 24 h.
90

91 **2.3. Collection of placenta**

92 Term placentas were collected from pregnant women within 1 h of delivery via cesarean
93 section at the Obstetrics Department of Hokkaido University Hospital (Sapporo, Japan). The
94 placental villi were collected and subjected to total RNA extraction. This study was performed in
95 accordance with the Japan Ethical Guidelines for Medical and Health Research involving Human

Subjects. This study was approved by the Ethics Committee of Hokkaido University Hospital (approval number: 018-0138) and written informed consent was obtained from all participants. Placental samples used for gene expression analyses were the same as those used in a previous study (Ohyama et al., 2023).

2.4. mRNA expression analyses

BeWo cells were seeded into a 12-well plate (200,000 cells/well) and cultured in a medium containing each HDAC inhibitor, as described in Section 2.2. The total RNA was isolated using ISOGEN II (Nippon Gene, Japan) following the manufacturer's protocol. RNA purity and concentration were determined using NanoDrop2000c. Next, 1 μg of RNA was reverse-transcribed into cDNA using ReverTraAce (TOYOBO, Osaka, Japan).

Quantitative polymerase chain reaction (qPCR) was performed using the Mx3000 Real-Time PCR system (STRATAGENE) with the KAPA SYBR Fast qPCR kit (Kapa Biosystems; Wilmington, MA, USA) to analyze mRNA expression. PCR (2-step cycling) was conducted for 40 cycles as follows: 95°C for 3 s, 57°C (HDAC4, 5, 7, 8, and 9) or 60°C (folate transporters and HDAC1 and 3) or 65°C (HDAC2) for 20 s. mRNA expression of the target genes was normalized to that of β-actin (*ACTB*). The expression of reference genes in BeWo cells was not significantly altered after exposure to the inhibitors. Data were analyzed using the relative standard curve method. All

primers used are listed in Table S1. Primer specificity was assessed by melting curve analysis and agarose gel electrophoresis.

To compare the mRNA levels between BeWo cells and placental villi, the comparative cycle time (Ct) method was used to calculate the relative mRNA expression using the following equation: relative mRNA expression = $2^{-(\text{Ct target gene - Ct reference gene})}$. Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*) was used as the reference gene (Ohyama et al., 2023).

2.5. Western blotting

BeWo cells were seeded into a 6-well plate (400,000 cells/well) and cultured in a medium containing each HDAC inhibitor, as described in Section 2.2. BeWo cells were lysed in a buffer containing 1.0% Triton X-100, 0.1% sodium dodecyl sulfate, 4.5 M urea, and a Complete Mini protease inhibitor cocktail (Roche; Mannheim, Germany). The suspension was left on ice for 5 min, sonicated for 30 s, centrifuged at 14,000 × *g* for 10 min at 4°C, and the clear supernatant was collected. Protein concentration was determined using bicinchoninic acid (BCA) (Thermo Fisher Scientific; Waltham, MA, USA) with 0–2000 μg/mL bovine serum albumin (BSA) as a concentration standard. A total of 5 μg of protein was denatured at 100°C for 3 min in a blue loading buffer containing dithiothreitol (DTT) (Cell Signaling Technology; MA, USA). The samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on a 15%

polyacrylamide gel and transferred electrophoretically onto nitrocellulose membranes. Membranes were blocked with tris-buffered saline with Tween 20 (TBS-T) containing 5% skim milk for 1 h at room temperature, washed with TBS-T, and incubated overnight with mouse anti-actin (Chemicon; Temecula, CA, USA) or anti-acetyl-histone H3 (Lys9/Lys14; Cell Signaling Technology #9677) primary antibodies. After that, the membranes were washed with TBS-T and incubated with horseradish peroxidase-conjugated (1:4000) anti-mouse IgG or anti-rabbit secondary antibodies for 1 h at room temperature. After washing with TBS/T, the protein bands were visualized using enhanced chemiluminescence with an Image Quant LAS 4000 (GE Healthcare; Buckinghamshire, UK). Band intensities were analyzed using ImageJ software (National Institutes of Health; Bethesda, MD, USA).

2.6. Knockdown using small interfering RNA (siRNA)

Negative controls (NC; Silencer Select Negative Control #1) and four siRNAs (HDAC1 siRNA: s75, HDAC2 siRNA: s6493, HDAC3 siRNA: s16876, and HDAC8 siRNA: s31698) were purchased from Thermo Fisher Scientific. According to the manufacturer's instructions, BeWo cells were transfected with siRNAs using Lipofectamine RNAiMAX (Invitrogen; Carlsbad, CA, USA). The concentration of the RNAi duplex was 10 nM. The cells were incubated for 72 h at 37°C under 5% CO_2 and subsequently used for experiments.

152 **2.7. Chromatin immunoprecipitation (ChIP)-qPCR assay**

153 BeWo cells were seeded in a 150-mm dish (1,000,000 cells/dish) and cultured in a medium
154 containing VPA (1 mM). ChIP assay was conducted according to the manufacturer's instructions
155 using the SimpleChIP Enzymatic Chromatin IP Kit (Magnetic Beads; Cell Signaling Technology). To
156 crosslink proteins to DNA, BeWo cells were treated with 1% formaldehyde (Sigma-Aldrich) for 10
157 min, and the reaction was stopped by adding 0.125 M glycine. After removing the medium, cells
158 were washed with ice-cold phosphate-buffered saline (PBS), scraped into ice-cold PBS-containing
159 protease inhibitors, and centrifuged at 2,000 × g for 5 min at 4°C. The pellets were used for nuclear
160 preparation and chromatin digestion. Chromatin digestion was performed using a micrococcal
161 nuclease to obtain DNA fragments of 150–900 bp, which were confirmed by agarose gel
162 electrophoresis. ChIP was performed using the following antibodies: acetylated histone H3K9/K14
163 (1:50; Cell Signaling Technology, #9677), histone H3 (1:50; D2B12, 4620; Cell Signaling
164 Technology; positive control), and normal rabbit IgG (1:50; 2729S; Cell Signaling Technology;
165 negative control). Immunoprecipitated samples were incubated overnight at 4°C with rotation.
166 Protein G magnetic beads were used to precipitate chromatin–protein–antibody complexes. The
167 DNA was purified using spin columns after elution of chromatin from the antibody/magnetic beads
168 and reversal of the crosslinks. The DNA was quantified using the Mx3000 Real-Time PCR system
169 with SimpleChIP Universal qPCR Master Mix (Cell Signaling Technology) and specific primers for
170 proximal regions of the FRα/*FOLR1* promoter (accession no. NM_016725.3). The primer sequences

are listed in Table S2. The data were analyzed using the percentage input method with the following equation: Percent input = $2\% \times 2^{(Ct\ 2\%Input\ Sample\ -\ Ct\ IP\ Sample)}$

2.8. Statistical analyses

Data from at least three independent experiments are presented as the mean with standard error. Student's *t*-test was used to analyze the differences in the means of the two groups. One-way analysis of variance (ANOVA) followed by Dunnett's test (Fig. 3, Fig. 4B, and Fig. 5A) or the Tukey–Kramer test (Fig. 1A) was used to evaluate the statistical significance of the differences among the means of more than two groups. All statistical analyses were conducted using JMP Pro 16 software (SAS Institute; Cary, NC, USA). Statistical significance was set at $p < 0.05$.

3. Results

3.1. Effects of VPA on folate carrier expression

A previous study reported that the expression of folate carriers in BeWo cells is altered by VPA treatment. BeWo cells differentiate into syncytiotrophoblast-like cells following forskolin treatment (Handwerger, 2010). Previous reports indicated that HDACs are involved in the differentiation process in trophoblasts (Bhattad et al., 2020; Ohyama et al., 2023). Therefore, in this study, the effects of VPA on the expression of folate carriers were examined after forskolin treatment to determine VPA reactivity in BeWo and differentiated cells. We have previously confirmed that

BeWo cells differentiate after 48 h of forskolin treatment (Koishikawa et al., 2022; Ohyama et al., 2023). Without forskolin, the mRNA expression of FRα and PCFT was increased by VPA exposure for 24 h (Fig. 1A). These results were consistent with those of our previous study (Kurosawa et al., 2018). Here, even in the presence of forskolin, the expression of FRα and PCFT was increased by VPA exposure. Similar changes were observed with and without forskolin. These results suggest that the differentiation stage of BeWo cells hardly influenced the regulation of folate carriers by VPA. Forskolin treatment tended to increase the expression of folate carriers such as RFC; however, the change was not statistically significant. Therefore, normal culture conditions were used for subsequent experiments.

Western blotting was performed to confirm that VPA exposure resulted in histone acetylation. It showed that exposure to 1 mM VPA increased the expression of acetyl-histone H3 (Lys9/Lys14) compared to the control (Fig. 1B).

Valpromide (VPM) is a VPA analog that lacks HDAC inhibitor activity (Balasubramanian et al., 2015). VPM (1 mM) exposure did not increase the FRα and PCFT mRNA levels (Fig. 1C). These results suggest that the inhibitory effects of HDACs are related to VPA-induced changes in the mRNA expression of folate carriers.

As shown in Fig. 1A, FRα (*FOLR1*) was considerably upregulated by VPA. To investigate whether VPA altered histone acetylation in the promoter region of *FOLR1*, a ChIP assay was performed using an anti-acetyl-histone H3K9/14 antibody. Eight different regions spanning the

209 FOLR1 transcription start site (TSS) (1180 to +1150 bp) were examined. As shown in Fig. 1D,
210 acetyl-histone H3K9/14 levels in regions 1, 2, 3, and 8 were higher than those in control samples
211 after exposure to VPA. These results suggest that VPA causes histone acetylation in the promoter
212 region of *FOLR1* and affects its transcription.
213

214 **3.2. Expression of HDACs and folate carriers in BeWo cells and human placental villi**

215 VPA inhibited class I and IIa HDACs. Therefore, we investigated the expression of HDAC
216 isoforms in both BeWo cells and human-term placental villi. As shown in Fig. 2A, all class I
217 isoforms (HDAC1–3 and 8) were detected in BeWo cells and the placenta. Among the class IIa
218 isoforms, HDAC4, 5, and 7 were detected in both BeWo cells and the placenta. However, HDAC9
219 was only slightly detected in placental villi, but not in BeWo cells. HDAC9 was not detected in 1 of
220 the 10 placentas. Among the folate carriers, FRα, RFC, and PCFT were detected in both BeWo cells
221 and placental villi (Fig. 2B).
222

223 **3.3. Effects of HDAC inhibitors on folate carrier expression**

224 HDAC inhibitors are classified according to their chemical structures as short-chain fatty
225 acids (e.g., VPA), hydroxamates (e.g., TSA), benzamides (e.g., MS-275), and cyclic peptides (e.g.,
226 apicidin). In the present study, we tested the effects of four inhibitors (TSA, apicidin, MS-275, and
227 TMP269; Table 1) on folate carrier expression in BeWo cells.

228

229 **3.3.1. Trichostatin A**

230 TSA is a pan-HDAC inhibitor that broadly inhibits both class I and II HDACs. Western
231 blotting revealed that TSA increased the levels of acetylated histone H3K9/14 (Fig. 3A). FRα mRNA
232 levels were increased by 1.51-fold at 100 nM and 1.57-fold at 250 nM. RFC expression significantly
233 decreased. PCFT expression tended to increase after TSA exposure, but was not significantly altered.

234

235 **3.3.2. Apicidin**

236 Apicidin inhibits class I HDACs. Apicidin increased the acetyl-histone H3K9/14 levels in a
237 concentration-dependent manner (Fig. 3B). Moreover, 1 μM apicidin significantly increased FRα
238 levels by 1.62-fold. Apicidin treatment decreased RFC mRNA levels in a concentration-dependent
239 manner. Similarly, 1 μM apicidin significantly increased PCFT levels by 1.5-fold. Changes in the
240 expression of FRα and PCFT by apicidin were similar to those induced by VPA.

241

242 **3.3.3. MS-275 (entinostat)**

243 MS-275 inhibits class I HDACs. The expression of acetyl-histone H3K9/14 increased after
244 MS-275 treatment (Fig. 3C). In contrast to other inhibitors (TSA and apicidin), MS-275 decreased
245 FRα mRNA levels at all concentrations (0.73-fold at 5 μM). RFC mRNA levels were significantly
246 decreased (0.21-fold at 5 μM), whereas PCFT mRNA levels were not significantly altered after MS-

275 treatment.

3.3.4. TMP269

TMP269 inhibits class IIa HDACs. Western blotting revealed that TMP269 did not affect the expression of histone H3K9/14 (Fig. 3D). qPCR analysis revealed that the mRNA levels of folate carriers did not change after TMP269 exposure. These results suggest that class IIa HDACs do not affect the mRNA expression of folate carriers.

3.4. Effects of HDAC siRNAs on folate carrier expression

Next, the effects of genetic knockdown of HDACs on folate carriers were investigated. As the class IIa inhibitor TMP269 did not affect the expression of folate carriers (Fig. 3D), we investigated the effects of knockdown of class I HDAC isoforms (HDAC1, 2, 3, and 8). As shown in Fig. 4A, siRNAs targeting HDAC1, 2, 3, and 8 significantly reduced the mRNA expression of each HDAC isoform. However, these siRNAs did not affect the expression of other HDAC isoforms.

As several reports have indicated that HDACs have the potential to compensate for each other (Jaju Bhattad et al., 2020), the effects of double HDAC knockdown were investigated in this study. As HDAC1–3 expression was prominent in class I BeWo cells (Fig. 2A), the effects of co-transfection with different combinations (HDAC1 and 2, HDAC1 and 3, and HDAC2 and 3) were investigated (Fig. 4B). Cotransfection with HDAC1 and 2 siRNAs significantly reduced HDAC1 and

HDAC2 mRNA expression. FRα and PCFT mRNA levels significantly increased by 1.36- and 1.36-fold compared to those of the negative control, respectively. Cotransfection with HDAC1 and 3 siRNAs significantly reduced the mRNA expression of HDAC1 and HDAC3. Under the same conditions, the FRα mRNA level was significantly decreased by 0.81-fold from that of the negative control. Co-transfection with HDAC2 and 3 siRNAs significantly reduced the mRNA expression of HDAC2 and HDAC3 but did not affect folate carrier expression.

3.4. Effects of HDAC inhibitors on NRF-1 expression

In the present study, treatment with class I HDAC inhibitors decreased RFC expression in BeWo cells (Fig. 3). Although VPA treatment for 24 h did not alter RFC expression (Fig. 1A), Rubinchik-Stern et al. (2015) reported that prolonged VPA treatment for 2 or 5 days decreased RFC expression. NRF-1 regulates RFC transcription in other cell lines (Alam et al., 2020). Therefore, we investigated the involvement of NRF-1 in RFC downregulation by HDAC inhibitors. First, we investigated the long-term effects of VPA (48-h exposure) on RFC (Fig. S1) expression in BeWo cells. Exposure to VPA for 48 h decreased the NRF-1 levels (Fig. 5B). Although not statistically significant, VPA (48 h) treatment decreased NRF-1 expression (0.78-fold). Furthermore, apicidin treatment significantly decreased the NRF-1 mRNA levels compared to those in control (0.69-fold at 1 μM; Fig. 5A). MS-275 significantly decreased NRF-1 mRNA levels at all concentrations (0.38-fold at 5 μM). Although not statistically significant, TSA (0.25 μM) treatment tended to decrease

NRF-1 expression. TMP269 did not affect NRF-1 expression. In BeWo cells treated with these inhibitors, a correlation was observed between changes in NRF-1 and RFC mRNA levels (Fig. 5B). In contrast, no correlation was observed between the changes in FRα/PCFT and NRF-1 levels.

4. Discussion

The placenta is an essential organ for a healthy pregnancy and proper fetal development. Folates are critical nutrients for fetal development (Radziejewska and Chmurzynska, 2019). The placenta plays an important role in nutrient transport to the fetus. HDACs are involved in the regulation of transporters in different cells, including placental villi. However, the mechanisms underlying these alterations in placental folate-carriers remain unclear. In addition to VPA, several other HDAC inhibitors have been developed (Deng et al., 2023). Therefore, this study investigated the expression profiles of folate carriers after HDAC inhibition in BeWo human placental cells. Furthermore, we compared the expression of HDACs and folate carriers between BeWo cells and term human placental villi. The expression of HDACs and folate carriers in BeWo cells and the placenta showed similar trends. This study suggests the potential of BeWo cells as a model for human trophoblasts to assess the regulatory mechanisms of transporters by HDACs.

BeWo is a continuous cell line derived from human placental choriocarcinoma and is used as an *in vitro* model for toxicological assessment. Before evaluating the role of HDACs in the regulation of folate carriers in BeWo cells, we investigated the expression of HDACs and folate

carriers in BeWo cells and human placental villi (Fig. 2). qPCR analysis detected folate carriers in both BeWo cells and placenta, and their expression was comparable. Among folate carriers, the mRNA expression of FRα was predominant. Class I and IIa isoforms have been detected in the placenta. HDAC9 was not detected in the BeWo cells. HDACs 1, 2, 3, and 5 were predominant in both BeWo cells and placenta. Bhattad et al. (2020) reported that class I isoforms (HDAC1, 2, 3, and 8) are expressed in BeWo cells. These results are consistent with those of the present study. Among class IIa isoforms, HDAC4 and 5 were detected in BeWo cells, whereas HDAC7 and 9 were undetectable. In contrast, HDAC7 was detected in both BeWo cells and the placenta in this study. However, their expression was low. Future studies should evaluate protein and functional expression in BeWo cells and the placenta. Furthermore, we used the villous part of the term placenta to compare the expression in the placenta and BeWo cells. Because the placental tissue is heterogeneous, trophoblasts isolated from the placental villi must be analyzed for an accurate comparison.

Syncytiotrophoblasts are important for the formation of the blood–placental barrier (Tetro et al., 2018). BeWo cells differentiate into syncytiotrophoblast-like cells following forskolin treatment (Handwerger, 2010). Although the expression of folate carriers tended to increase after differentiation, the changes in the expression of these carriers induced by VPA were not markedly different between undifferentiated and differentiated cells (Fig. 1A). These results suggest that the differentiation stage of BeWo cells hardly influenced the regulation of folate carriers by VPA.

323 In this study, we evaluated the effects of several HDAC inhibitors on folate carriers.
324 Inhibitors with the potential to inhibit class I isoforms, namely VPA, TSA, apicidin, and MS-275,
325 increased the expression of acetyl-histone H3K9/14 (Fig. 3). In contrast, a class IIa inhibitor
326 (TMP269) did not affect the expression of acetylated histone H3K9/14. These results suggested that
327 class I HDACs contribute to increased histone acetylation in BeWo cells. Furthermore, alterations in
328 the expression of folate carriers have been observed when chemicals that inhibit class I HDACs were
329 used. These results suggested that class I HDAC isoforms alter folate carrier expression. HDAC
330 inhibitors exhibit different patterns of folate carrier regulation. FRα mRNA increased after exposure
331 to TSA and apicidin, whereas expression decreased after exposure to MS-275. PCFT mRNA was
332 significantly increased when treated with 1 μM apicidin. HDAC inhibitors differentially regulate the
333 expression of P-gp/*ABCB1* in human brain capillary endothelial cells (hCMEC/D3) (You et al.,
334 2019). HDAC inhibitors exhibit varying affinities for each class I isoform. For example, Padige et al.
335 (2015) reported IC_{50} values of MS-275 against HDAC1, 2, and 3 as 1500, 2100, and 310 nM,
336 respectively. Therefore, the selectivity of inhibitors toward isoforms may be involved in their
337 regulatory properties. However, inconsistencies have been reported when comparing inhibition data
338 among research groups, which may be due to assay formats (mammalian cell- or baculovirus-derived
339 HDAC). Witter et al. (2008) reported that the IC_{50} values of MS-275 against HDAC1, 2, and 3 are
340 125, 340, and 370 nM, respectively. Khan et al. (2008) reported that the IC_{50} values of MS-275
341 against HDAC1, 2, and 3 were 181, 1155, and 2311 nM, respectively. Hu et al. (2003) reported that

342 the IC_{50} values of MS-275 against HDAC1 and 3 were approximately 0.3 and 8 μM, respectively. In
343 the present study, it was unclear whether each compound inhibited each isoform in BeWo cells. In
344 addition, the possibility of the involvement of alternative mechanisms (e.g., methylation and non-
345 histones) cannot be excluded from this study. In the future, the reactivity of inhibitors against each
346 isoform in placental cells should be clarified.

347 Several factors in different cells regulate FRα expression. CIC binds to the promoter region
348 of *FOLR1* and regulates FRα expression (Cao et al., 2021). Furthermore, FRα was upregulated by
349 dexamethasone (Tran et al., 2005) and androgen (Sivakumaran et al., 2010). In contrast, it is
350 downregulated by 17β-estradiol (Kelley et al., 2003). ChIP-qPCR was performed using an antibody
351 against H3K9/14 acetylation (Fig. 1D). VPA treatment statistically increased the enrichment of
352 acetylated H3K9/14 protein binding upstream of the transcription start site (TSS) (region 1: –1186 to
353 –1028, region 2: –826 to –615, and region 3: –652 to –473) and downstream of TSS (region 8: +815
354 to +1050). This suggests that VPA causes histone acetylation in the promoter region of *FOLR1* and
355 exerts a certain impact on the transcription of this gene. Region 8 contains the response elements
356 CIC and GR. Although it has not been investigated whether these factors contribute to gene
357 regulation in BeWo cells, the inhibition of HDACs by VPA promotes histone acetylation, which may
358 consequently increase the accessibility of the *FOLR1* promoter to these factors and transactivation of
359 the *FOLR1* gene.

360 Knockdown experiments using siRNAs demonstrated that each class I isoform alone did not

361 contribute to the regulation of folate carrier expression (Fig. 4A). Several reports have indicated that
362 HDACs have the potential to compensate for each other (Montgomery et al., 2007). Bhattad et al.
363 (2020) reported that HDAC1 and 2 are critical mediators of trophoblast differentiation and that the
364 knockdown of *HDAC1* or *HDAC2* was insufficient to affect the differentiation process. In this study,
365 the co-transfection of HDAC1 and 2 siRNAs increased the FRα and PCFT mRNA levels (Fig. 4B).
366 The co-transfection of HDAC1 and 3 siRNAs slightly reduced the FRα mRNA levels. These results
367 suggested that folate carriers in BeWo cells are regulated by multiple HDACs. However, changes in
368 the expression of folate carriers induced by siRNA were moderate. The knockdown effects may be
369 limited because the basal expression of HDAC1–3 is abundant in BeWo cells (Fig. 2A). To clarify
370 the detailed contribution of each isoform, other approaches such as knockout experiments may be
371 required.

372 In the present study, chemicals that inhibit class I HDACs (TSA, Apicidin, and MS-275)
373 reduced RFC expression (Fig. 3). In addition, long-term VPA treatment (48 h) reduced RFC mRNA
374 levels (Fig. S1). Histone acetylation leads to transcriptional activation. This study suggests that class
375 I HDAC inhibitors consistently downregulate RFC in placental cells. The RFC is regulated by
376 transcription factors. Alam et al. (2020) reported that NRF-1 regulates RFC in the blood–brain
377 barrier (hCMEC/D3 cells and mouse brain). Furthermore, VDR activation upregulates RFC
378 expression and function in the blood–brain barrier (Alam et al., 2017). AhR activation downregulates
379 RFC expression and activity in rat hepatocytes (Halwachs et al., 2010). In our preliminary study, the

VDR and AhR mRNA expression was detected. However, these levels were lower in BeWo cells than in NRF-1 cells (data not shown). Therefore, in this study, we investigated the involvement of NRF-1 in RFC downregulation by HDAC inhibitors. In BeWo cells treated with these inhibitors, a correlation was observed between reduced NRF-1 and RFC mRNA levels (Fig. 5B). These results suggested that NRF-1 is involved in the downregulation of RFC by HDAC inhibitors in BeWo cells.

It has been reported that administration of VPA reduces serum folate levels (Karabiber et al., 2003). Low maternal folate levels are associated with several risks to the fetus, including fetal growth (van Uitert et al., 2013). FRα, RFC, and PCFT are involved in the transport of folates in the placenta. In the human placenta, FRα and PCFT were localized to the microvillous plasma membrane, and RFC existed in both the apical and basal membranes (Solanky et al., 2010). The alteration of these carriers by HDAC inhibition may affect folate transport to the fetus and placenta. Efflux transporters, such as multidrug and breast cancer resistance proteins, are recognized as folate carriers (Natarajan et al., 2012; Wang et al., 2021). Further studies are needed to investigate the effects of HDAC inhibition on folate transport across the placenta.

In conclusion, we observed that HDAC inhibitors, which are potent class I isoform inhibitors, altered folate carrier levels in BeWo cells. In contrast, HDAC inhibitors exerted different effects on folate-carrier levels. Our findings suggest that the inhibition of HDAC1 and 2 may be involved in the upregulation of FRα and PCFT levels. Furthermore, expression analyses revealed that folate carriers and HDACs exhibited similar expression patterns in BeWo cells and the human

placenta, suggesting the potential of BeWo cells as an *in vitro* model to investigate the histone-mediated regulation of folate carriers. However, these findings should be extrapolated to normal placentas with caution. A previous study reported that perfusion with VPA reduced FRα levels in *ex vivo* human placentas (Rubinchik-Stern et al., 2018). We previously demonstrated that repetitive VPA administration to pregnant rats decreases the expression of FRα/*Folr1* in the placenta on gestational day 20 (Furugen et al., 2021). Therefore, further investigations using normal trophoblasts are necessary to assess the safety of HDAC inhibitors and estimate their potential risks to the fetus.

Declaration of competing interest

Ayako Furugen is affiliated to the Laboratory of Healthcare Innovation Pharmacy, Faculty of Pharmacy, Keio University, Japan. This laboratory is supported by Sato Pharmaceutical Co., Ltd.

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Figure captions

Fig. 1. Effects of valproic acid (VPA) on folate carrier and histone deacetylase (HDAC) expression in BeWo cells. (A) Effects of VPA on the mRNA expression of folate carriers (folate receptor alpha [FRα/*FOLR1*], reduced folate carrier [RFC/*SLC19A1*], and proton-coupled folate transporter [PCFT/*SLC46A1*]). BeWo cells were cultured for 2 days, and the medium was replaced with another medium containing 20 μM forskolin (differentiation) or 0.1% dimethyl sulfoxide (DMSO, undifferentiation). After forskolin treatment for 48 h, the cells were cultured in a medium containing 1 mM VPA for 24 h, and the mRNA expression was determined using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Each column represents the mean with standard error (SE) of three independent experiments performed in triplicate. $^{**}p < 0.01$ compared with the control. The Tukey–Kramer test was used to evaluate the statistical significance of the differences among groups. (B) Effect of VPA on acetyl-histone H3 (Lys9/Lys14) levels in BeWo cells. BeWo cells were treated with VPA (1 mM) for 24 h. Total protein levels were assessed by western blotting. Actin was used as the loading control. Each column represents the mean with an SE of four independent experiments. (C) Effects of valpromide (VPM) on the mRNA expression of folate carriers (FRα/*FOLR1*, RFC/*SLC19A1*, and PCFT/*SLC46A1*). BeWo cells were treated with VPM (1 mM) for 24 h, and mRNA expression was determined by RT-qPCR. Data are represented as the mean with an SE of three independent experiments performed in triplicate. $^{*}p < 0.05$ compared with the control.

(D) Chromatin immunoprecipitation (ChIP) of acetyl-histone H3K9/14 around the promoter region of FRα/*FOLR1*. BeWo cells were treated with VPA (1 mM) for 24 h. Data are represented as the mean with an SE of three independent experiments. *$p < 0.05$ and **$p < 0.01$ compared with the control.

Fig. 2. mRNA levels of HDACs (class I: HDAC1, 2, 3, and 8; class IIa: HDAC4, 5, 7, and 9) (A) and folate carriers (FRα/*FOLR1*, RFC/*SLC19A1*, and PCFT/*SLC46A1*) (B) in the placenta and BeWo cells. Expression in human placental villi (closed circle; placenta, $n = 10$) and BeWo cells (open circle; $n = 3$) were determined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). "–" indicates the mean of the data. ND: not detected

Fig. 3. Effects of HDAC inhibitors on acetyl-histone H3 (Lys9/Lys14) and mRNA expression levels of folate carriers. BeWo cells were treated with (A) trichostatin A (TSA; 0.1 and 0.25 μM), (B) apicidin (0.1, 0.5, and 1 μM), (C) MS-275 (0.5, 1, and 5 μM), or (D) TMP269 (10 μM) for 24 h. Acetyl-histone H3 (Lys9/Lys14) levels were determined by western blotting analysis. Expression of folate carriers (FRα/*FOLR1*, RFC/*SLC19A1*, and PCFT/*SLC46A1*) was determined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Data are represented as the mean with an SE of three independent experiments performed in triplicate. Dunnett's test was used to evaluate the statistical significance of group differences. *$p < 0.05$ and **$p < 0.01$ compared with the

control.

Fig. 4. Effects of small interfering RNAs (siRNAs) against class I HDACs on the expression of folate carriers in BeWo cells. (A) Expression of class I HDACs (HDAC1, 2, 3, and 8) and folate carriers (FRα, RFC, and PCFT) 72 h after transfection with siRNAs against HDAC 1, 2, 3, and 8. Expression of targeted genes was determined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Data are represented as the mean with an SE of three independent experiments performed in triplicate. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the control. (B) Effects of double knockdown of HDACs on the expression of folate carriers. BeWo cells were co-transfected with negative control siRNA (NC) or siRNAs against HDACs (HDAC1 and 2, HDAC1 and 3, or HDAC2 and 3) for 72 h. Expression of targeted genes was determined by RT-qPCR. Data are represented as the mean with an SE of three independent experiments performed in duplicate. Dunnett's test was used to evaluate the statistical significance of group differences. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the control.

Fig. 5. Effects of HDAC inhibitors on the mRNA expression of nuclear respiratory factor 1 (*NRF-1*). (A) BeWo cells were treated with VPA (1 mM) for 48 h. BeWo cells were treated with trichostatin A (TSA, 0.1 and 0.25 μM), apicidin (0.1, 0.5, and 1 μM), MS-275 (0.5, 1, and 5 μM), or TMP269 (10 μM) for 24 h. Expression of targeted genes was determined by reverse transcription-quantitative

polymerase chain reaction (RT-qPCR). Student's *t*-test or Dunnett's test was used to evaluate the statistical significance of the differences between two groups or among multiple groups, respectively. $^{*}p < 0.05$ and $^{**}p < 0.01$. (B) Correlation analyses of the changes in the expression of folate carriers (FRα, RFC, and PCFT) and NRF-1. Correlations were analyzed using Pearson's correlation test.

Table 1. Inhibitors used in this study

Inhibitors	Chemical structure	HADC targets[a]
Valproic acid (VPA)	Short-chain fatty acids	Class I, IIa
Trichostatin A (TSA)	Hydroxamates	Class I, II, IV
Apicidin	Cyclic peptides	Class I (HDAC1 - 3)
MS-275	Benzamides	Class I (HDAC1 - 3)
TMP269		Class IIa

a: references (Deng et al., 2023; You et al., 2020)

Figure 1

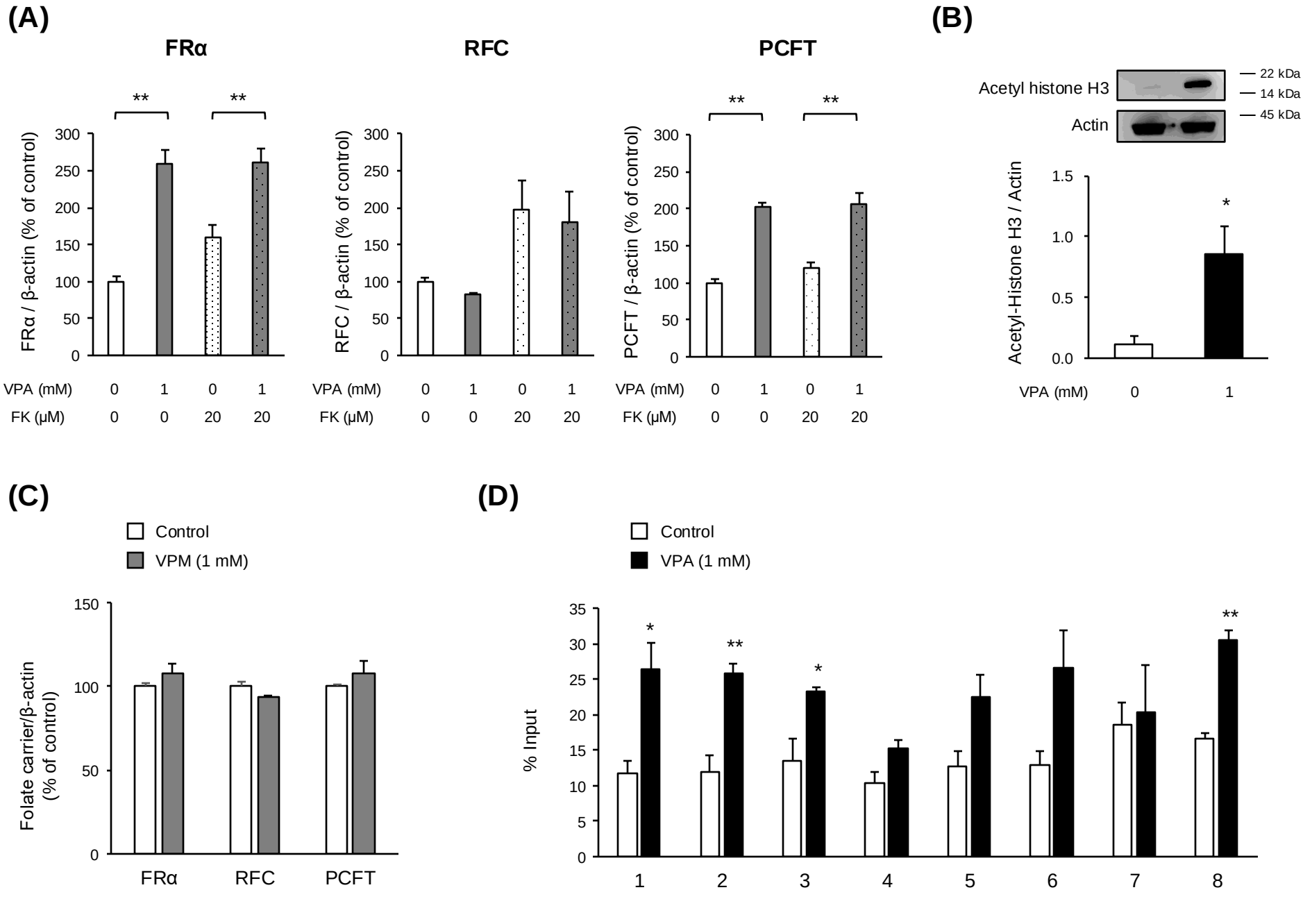

(A)
FRα
RFC
PCFT
FRα / β-actin (% of control)
RFC / β-actin (% of control)
PCFT / β-actin (% of control)
**
**
300
250
200
150
100
50
0
VPA (mM) 0 1 0 1
FK (μM) 0 0 20 20
(B)
Acetyl histone H3
Actin
22 kDa
14 kDa
45 kDa
Acetyl-Histone H3 / Actin
1.5
1.0
0.5
0.0
*
VPA (mM) 0 1
(C)
Control
VPM (1 mM)
Folate carrier/β-actin (% of control)
150
100
50
0
FRα
RFC
PCFT
(D)
Control
VPA (1 mM)
% Input
35
30
25
20
15
10
5
0
*
**
*
**
1
2
3
4
5
6
7
8

Figure 2

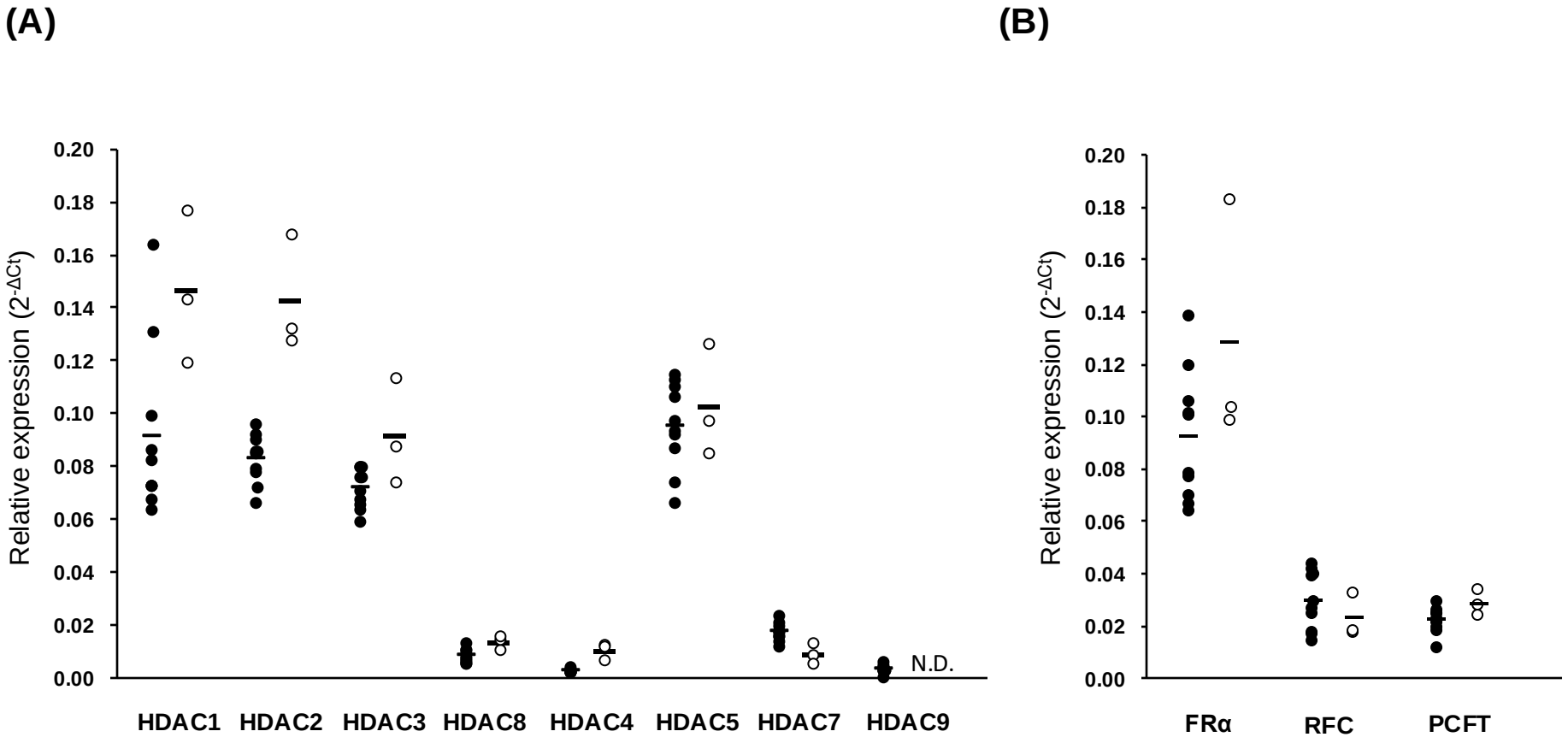

(A)
Relative expression (2-ΔCt)
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
N.D.
HDAC1
HDAC2
HDAC3
HDAC8
HDAC4
HDAC5
HDAC7
HDAC9
(B)
Relative expression (2-ΔCt)
FRα
RFC
PCFT

Figure 3

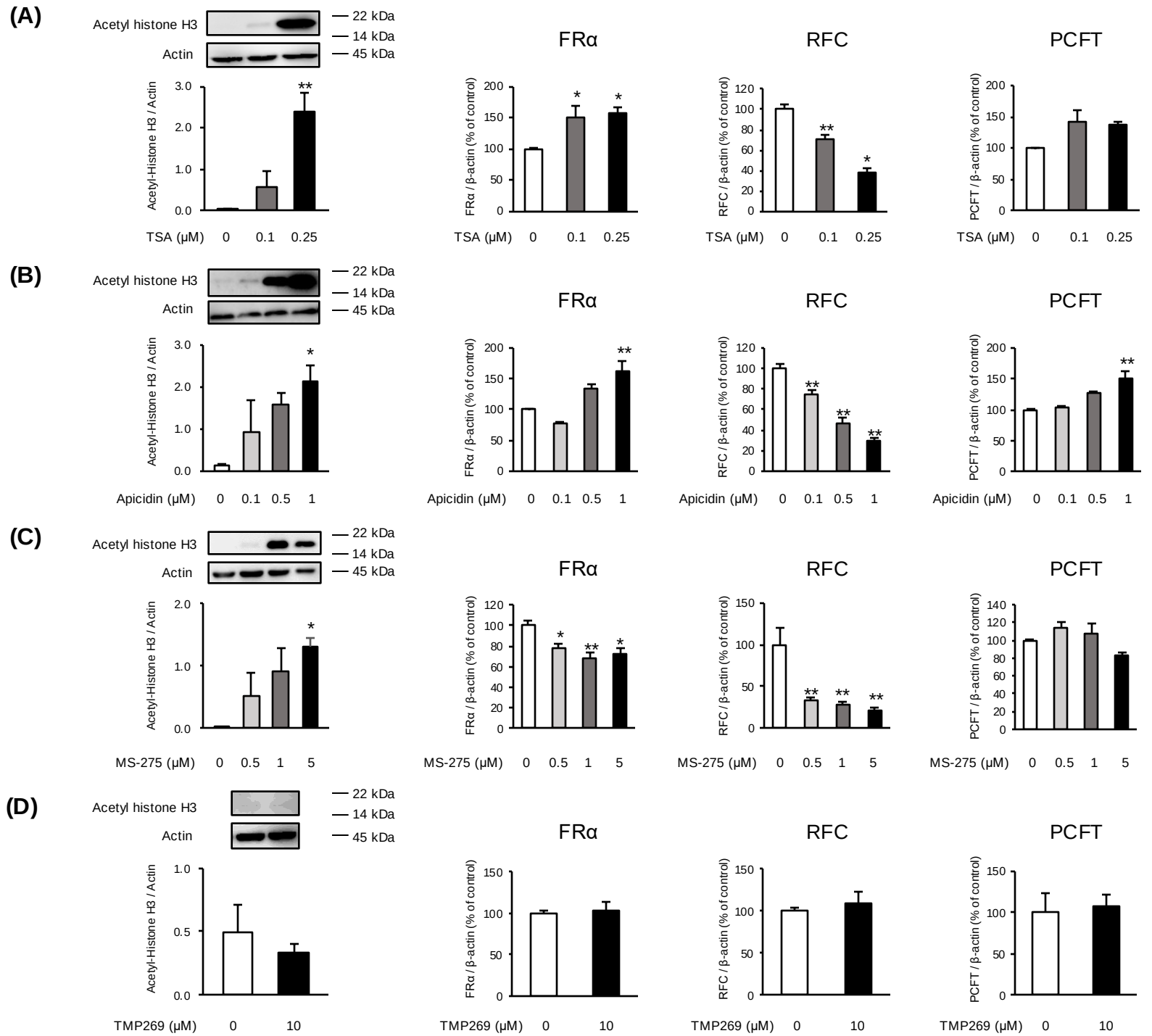
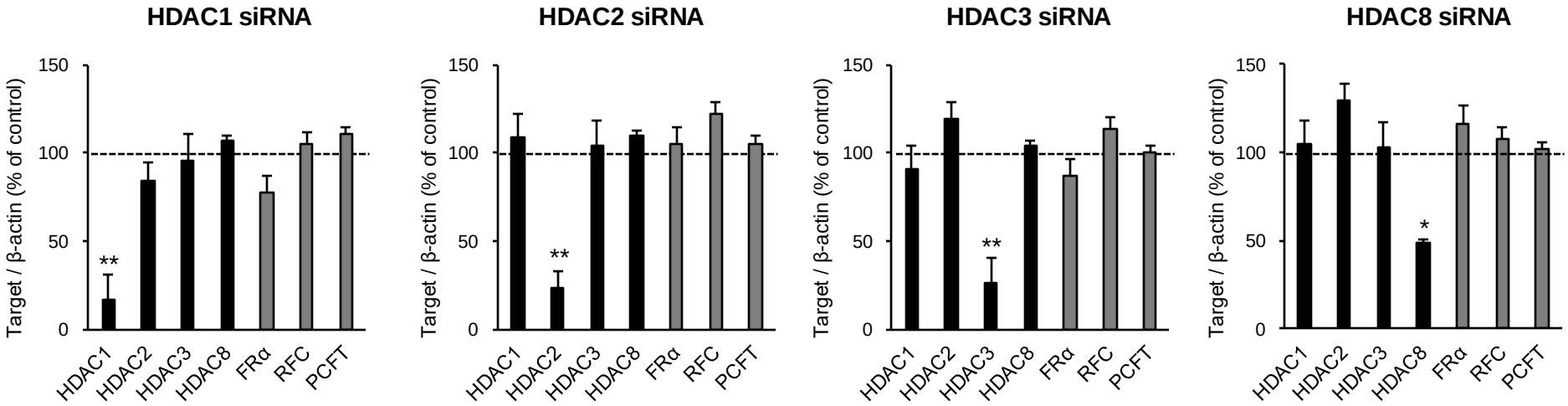

(A)
Acetyl histone H3
Actin
22 kDa
14 kDa
45 kDa
Acetyl-Histone H3 / Actin
TSA (μM) 0 0.1 0.25
FRα
RFC
PCFT
FRα / β-actin (% of control)
RFC / β-actin (% of control)
PCFT / β-actin (% of control)
(B)
Apicidin (μM) 0 0.1 0.5 1
(C)
MS-275 (μM) 0 0.5 1 5
(D)
TMP269 (μM) 0 10

Figure 4

(A)


HDAC1 siRNA
HDAC2 siRNA
HDAC3 siRNA
HDAC8 siRNA
Target / β-actin (% of control)
150
100
50
0
**
*
HDAC1
HDAC2
HDAC3
HDAC8
FRα
RFC
PCFT

(B)

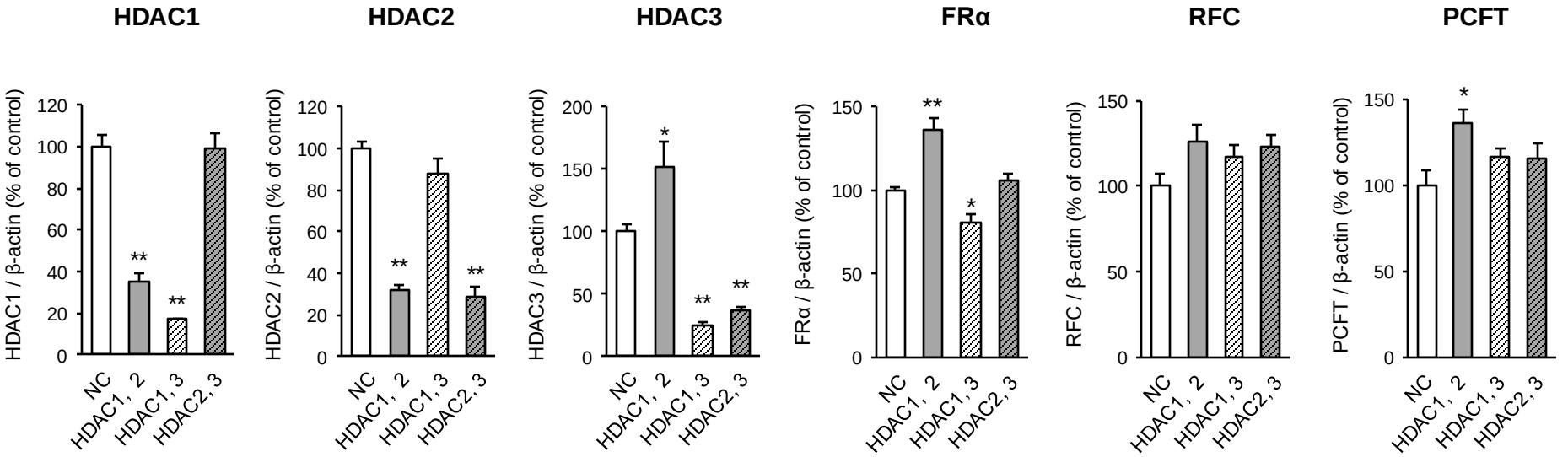
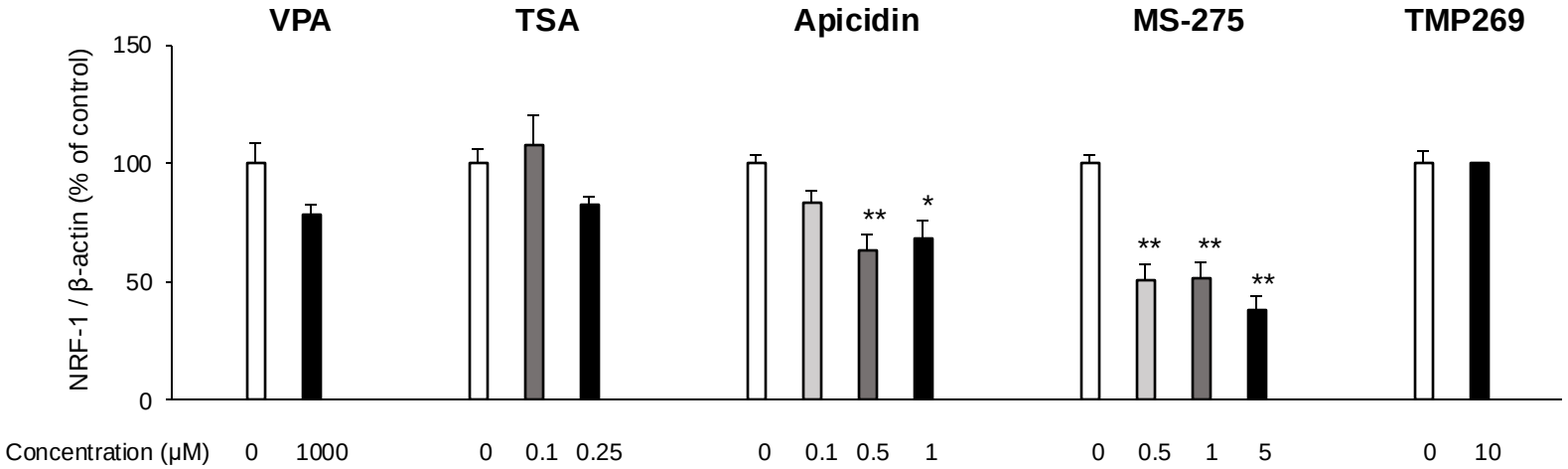
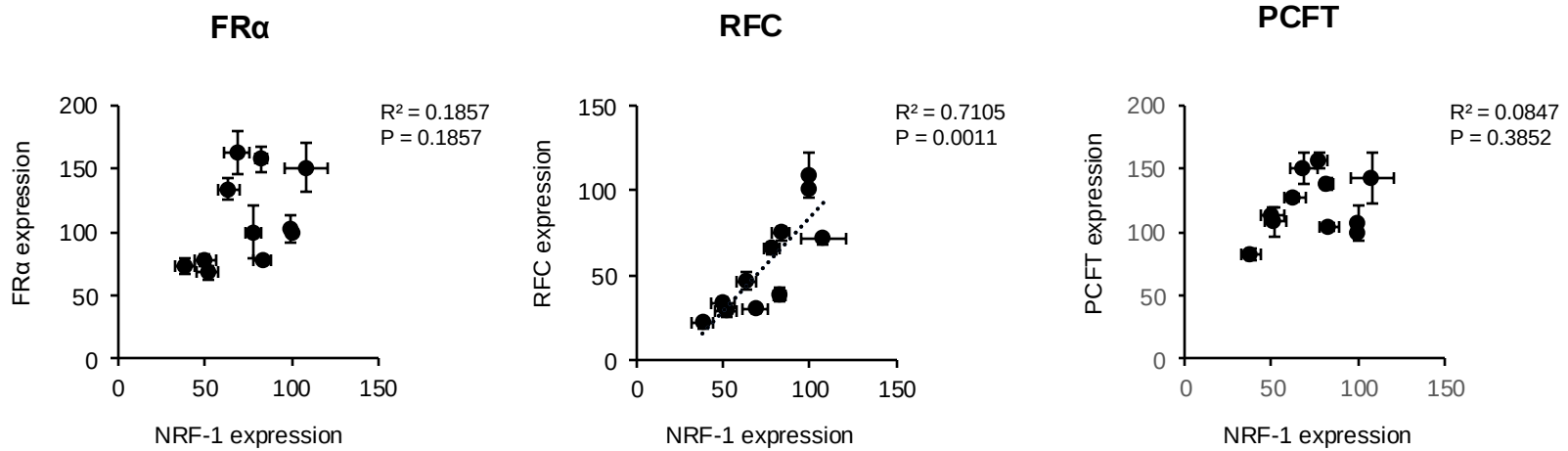

HDAC1
HDAC2
HDAC3
FRα
RFC
PCFT
HDAC1 / β-actin (% of control)
HDAC2 / β-actin (% of control)
HDAC3 / β-actin (% of control)
FRα / β-actin (% of control)
RFC / β-actin (% of control)
PCFT / β-actin (% of control)
120
100
80
60
40
20
0
200
150
50
**
*
NC
HDAC1, 2
HDAC1, 3
HDAC2, 3

Figure 5

(A)


VPA
TSA
Apicidin
MS-275
TMP269
NRF-1 / β-actin (% of control)
150
100
50
0
**
*
**
**
**
Concentration (μM)
0 1000
0 0.1 0.25
0 0.1 0.5 1
0 0.5 1 5
0 10

(B)


FRα
RFC
PCFT
FRα expression
RFC expression
PCFT expression
NRF-1 expression
R² = 0.1857
P = 0.1857
R² = 0.7105
P = 0.0011
R² = 0.0847
P = 0.3852
200
150
100
50
0
0 50 100 150

Supplemental Figure 1

