Original Article

Disruption of Planar Cell Polarity Pathway Attributable to Valproic Acid-Induced Congenital Heart Disease through Hdac3 Participation in Mice

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Abstract

Background: Valproic acid (VPA) exposure during pregnancy has been proven to contribute to congenital heart disease (CHD). Our previous findings implied that disruption of planar cell polarity (PCP) signaling pathway in cardiomyocytes might be a factor for the cardiac teratogenesis of VPA. In addition, the teratogenic ability of VPA is positively correlated to its histone deacetylase (HDAC) inhibition activity. This study aimed to investigate the effect of the VPA on cardiac morphogenesis, HDAC1/2/3, and PCP key genes (Vangl2/Scrib/Rac1), subsequently screening out the specific HDACs regulating PCP pathway.

Methods: VPA was administered to pregnant C57BL mice at 700 mg/kg intraperitoneally on embryonic day 10.5. Dams were sacrificed on E15.5, and death/absorption rates of embryos were evaluated. Embryonic hearts were observed by hematoxylin-eosin staining to identify cardiac abnormalities. H9C2 cells (undifferentiated rat cardiomyoblasts) were transfected with Hdac1/2/3 specific small interfering RNA (siRNA). Based on the results of siRNA transfection, cells were transfected with Hdac3 expression plasmid and subsequently mock-treated or treated with 8.0 mmol/L VPA. Hdac1/2/3 as well as Vangl2/Scrib/Rac1 mRNA and protein levels were determined by real-time quantitative polymerase chain reaction and Western blotting, respectively. Total HDAC activity was detected by colorimetric assay.

Results: VPA could induce CHD ($P < 0.001$) and inhibit mRNA or protein expression of Hdac1/2/3 as well as Vangl2/Scrib in fetal hearts, in association with total Hdac activity repression (all $P < 0.05$). *In vitro*, Hdac3 inhibition could significantly decrease Vangl2/Scrib expression ($P < 0.01$), while knockdown of Hdac1/2 had no influence ($P > 0.05$); VPA exposure dramatically decreased the expression of Vanlg2/Scrib together with Hdac activity ($P < 0.01$), while overexpression of Hdac3 could rescue the VPA-induced inhibition ($P > 0.05$).

Conclusion: VPA could inhibit Hdac1/2/3, Vangl2/Scrib, or total Hdac activity both *in vitro* and *in vivo* and Hdac3 might participate in the process of VPA-induced cardiac developmental anomalies.

Key words: Congenital Heart Disease; Hdacs; Planar Cell Polarity; Valproic Acid

Introduction

Accumulating evidence has strongly suggested that adverse environmental factors contribute to congenital heart disease (CHD) by interfering with regulatory pathways directing embryonic development early in gestation.[1] Maternal exposure to teratogenic drugs has been identified as potential environmental factors for CHD. Valproic acid (VPA) is one of the most frequently prescribed

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antiepileptic drugs (AEDs) during pregnancy. Although VPA possesses a good side effect profile with little sedation and few behavioral effects, it warrants particular attention owing to more teratogenic than other AEDs.[2] Compelling body of studies and our previous studies have confirmed that *in utero* exposure to VPA was associated with cardiac anomalies.[3,4] Yet, on the whole, the teratogenic mechanism of VPA is still largely unexplained.[5] Therefore, exploration of the molecular mechanisms by which regulatory pathways VPA-induced cardiac abnormalities is crucial for prevention of CHD and development of safer drugs.

For further unraveling the teratogenic effect of VPA on the cardiomyocytes, we used H9C2 cells (undifferentiated rat cardiomyoblasts) to be treated with VPA, which demonstrated that VPA exposure resulted in defective cell polarity, migration, and lamellipodia formation (data unpublished). These findings indicated that disruption of cardiomyocyte polarity might be a factor for the cardiac teratogenesis of VPA. It has been well recognized that cardiomyocyte polarity is strictly controlled by planar cell polarity (PCP) signaling pathway.[6,7] PCP pathway is required for transformation of cardiomyocytes from initially spherically shaped to ultimately rod-shaped polarized cells through modification of cytoskeleton, playing indispensable roles in cellular organization and directed migration within chamber walls.[7,8] Vangl2, Scrib, and Rac1 are pivotal molecules in the PCP pathway to govern apical-basal polarity and tissue development. Convincing evidence has demonstrated that genetic knockout or mutation of *Vangl2*, *Scrib*, and *Rac1* in mouse all resulted in disruption of proliferation, differentiation, and migration of embryonic cardiomyocytes, ultimately contributing to CHD.[9-13] Therefore, we postulated that VPA might induce CHD by interfering with these key genes of PCP pathway.

Apart from its antiepileptic property, VPA is a well-recognized histone deacetylase (HDAC) inhibitor. As a pivotal enzyme involved in histone modifications, HDACs play a major role in removing acetyl groups from histone tails, stabilizing nucleosome structure, and then compacting chromatin, leading to blockage of DNA accessibility for transcriptional activators and repress gene transcription.[14] Recent studies have provided convincing evidence that the teratogenic ability of VPA was positively correlated with its HDAC inhibition activity, by which VPA could disrupt gene expression through affecting the status of histone acetylation/deacetylation, resulting in congenital anomalies.[3,15-18] VPA preferentially inhibits Class I (1, 2, 3, and 8) HDACs.[19] Among them, HDAC1, 2, and 3, core members of Class I HDACs, are ubiquitously expressed, proven to have substantial roles in cell differentiation, proliferation, and morphogenesis.[20] Recent studies in transgenic mice showed that HDAC1/2/3 were required for normal growth and morphogenesis of the heart, while specific deletions of these enzymes would result in various cardiac abnormalities.[21-23] Given the pivotal molecular switches of downstream gene regulation, however, little is known about the mechanism by which pathway HDAC1/2/3 affect cardiac development. In this regard, we hypothesized that CHD caused by VPA might be related to interruption of PCP key genes through HDAC1/2/3 participation. Therefore, the aim of the study was to investigate the effect of the VPA on cardiac morphogenesis, aforementioned HDACs, and PCP molecules, subsequently screening out the specific HDACs regulating PCP pathway, which might preliminarily illuminate molecular mechanism of VPA-induced CHD.

Methods

Animals and drug treatment

The healthy and adult SPF class C57BL mice about 6–8 weeks old (weigh 21 to 26 g) were purchased from Sichuan University Animal Institution. Animals were housed in groups of five, had free access to food and water, and maintained on a 12 h light/dark cycle (light phase: 08:00–20:00) in a temperature-controlled environment (21 ± 2°C). All the procedures were performed in accordance with the National Institutes of Health Guide and with the approval of the Sichuan University Committee for the Care and Use of Laboratory Animals. Female mice were mated with the male at 5:00 PM and inspected for the vaginal plug in the next morning. Pregnancy was defined after the presence of a vaginal plug and designated as embryonic day 0.5 (E0.5). Pregnant mice were randomly allotted to treatment group, vehicle group, or blank group: the treatment group received a single intraperitoneal injection of VPA (P4543, Sigma-Aldrich, USA) at 700 mg/kg on embryonic day 10.5 (E10.5), the vehicle group received an equivalent volume of saline, and the blank received no interventions. The time point and dose of the treatment were selected as several studies and our previous studies, which demonstrated that E10.5–15.5 was a pivotal stage of cardiac development in fetal mice, in association with abundant expression of PCP pivotal molecules in the heart,[9-13,24] and the one time injection of VPA at 700 mg/kg showed the highest rate of cardiac abnormality with acceptable fetal death/reabsorption rates.[4] The pregnant mice were sacrificed through cervical dislocation on E15.5. The fetuses were euthanized by decapitation. The embryonic hearts were quickly removed and stored at −80°C for further mRNA/protein expression analysis, total HDAC activity assay, or fixed in 10% formalin solution for hematoxylin-eosin staining.

Hematoxylin-eosin staining of embryonic hearts

Embryonic hearts were fixed in 10% formalin solution for at least 12 h. The hearts were dehydrated progressively in 60%, 70%, 80%, and 100% ethanol for 1 h, respectively. After xylene treatment, 100% paraffin was used for tissue embedding. Then, embryonic hearts were serially sectioned at 5 μm from the top of the aortic arch to the apex. The slides were viewed and photographed by the microscope (Nikon, Tokyo, Japan).

Cell line and culture conditions

H9C2 cells (undifferentiated rat cardiomyoblasts) obtained from the Cell Bank of Chinese Academy of Science were

cultured in 10% fetal bovine serum-DMEM/F-12 (Thermo Fisher Scientific, USA) supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco, USA) at 37°C in a humidified atmosphere with 5% CO_2.

Transfection of Hdac1/2/3 small interfering RNA *in vitro*

An amount of 1×10^5 or 4×10^5 H9C2 cells were seeded initially in 24-well or 6-well plates for real-time quantitative polymerase chain reaction (PCR) or Western blot analysis, respectively. After cells were grown to 30–50% confluence, 80 nmol/L small interfering RNA (siRNA) specific for Hdac1 (siG1379144052, GuangZhou RiboBio. Co., USA)/Hdac2 (siB160628043037, GuangZhou RiboBio. Co.)/Hdac3 (siG160824094833, GuangZhou RiboBio.Co.) or control siRNA (siN05815122147, GuangZhou RiboBio. Co.) was transfected into H9C2 cells using Lipofectamine® RNAiMAX Reagent (13778-150, Invitrogen, USA) according to the manufacturer's instruction. Cells were harvested after 48 h of transfection for next step tests. All experiments were repeated three times.

The siRNA sequences used were as follows:
Hdac1 Sense: 5'-GCAAGUACUACGCC GUUAAdTdT-3'
Hdac1 Anti-sense: 3'-dTdT CGUUCAUGAUGCGGCAA UU-5'
Hdac2 Sense: 5'-GAUGGACUCUUUGAGUU UU dTdT-3'
Hdac2 Anti-sense: 3'-dTdT CUACCUGAGAAACUC AAAA-5'
Hdac3 Sense: 5'-GCCAGAAGCACCCAAU GAA-3'
Hdac3 Anti-sense: 3'-dTdT CGGUCUUCGUGGGUUAC UU-5'.

Cell transfection of Hdac3 expression plasmid and valproic acid treatment

Based on the results of siRNA transfection *in vitro* experiments, demonstrating only Hdac3 was involved in *Vanlg2* and *Scrib* regulation, Hdac3 expression plasmid or control plasmid was transfected into H9C2 cells using Lipofectamine™ 3000 Transfection Reagent (L3000015, Invitrogen) according to the manufacturer's instruction. The plasmids were kindly provided by Liu lab of Sichuan University (Chengdu, China). Following 48 h posttransfection, cells were mock-treated or treated with 8.0 mmol/L VPA for another 48 h. Subsequently, cells were collected and subjected to real-time quantitative PCR or Western blotting analysis.

The Hdac3 plasmid sequences used were as follows:
Sense: TTGGATCCACTATCGGCCAAGACCGTGGC
Anti-sense: AAGCTCGAGTTATTGCTCCA CATCGCTTTC.

Real-time quantitative polymerase chain reaction analysis

Total RNA was isolated and purified using the Trizol reagent (Invitrogen). The concentration of purified RNA samples was assessed spectrophotometrically using the Nanodrop 2000 instrument (Thermo Scientific). RNA (1 μg) was reverse transcribed using PrimeScript™ RT Reagent Kit with gDNA eraser (RR047A, Takara, Japan) according to the manufacturer's instructions.

Amplification of cDNA was performed with SsoFast EvaGreen Supermixture (Bio-Rad Laboratories, Hercules, CA, USA) using 5 μl reaction mixture, 0.5 μl forward primer, 0.5 μl reverse primer, 3 μl nuclease-free H_2O, and 1 μl cDNA in a total volume of 10 μl. Preceded by an initial denaturation of 3 min at 95°C, and followed by a continuous melt curve from 65–95°C, the PCR conditions for mice samples were 39 cycles of 30 s at 95°C, 10 s at 58°C for *Hdac1*/*Hdac2*/*Hdac3*/*Vangl2*/*Scrib*/*Rac1*/*Gapdh*; for H9C2 samples were 39 cycles of 30 s at 95°C, 10 s at 55°C for *Hdac1/Hdac2/Hdac3/Vangl2/Scrib/Rac1/Gapdh*. A validation experiment had been undertaken in which equal quantities of cDNA were used. Similar amplification efficiencies and cycle threshold (CT) values of *Gapdh* were obtained among the controls and treatment group. The stability of *Gapdh* expression among the controls and treatment group guaranteed its use as an appropriate endogenous control for normalization. In addition, we have ascertained the efficiencies of amplifications for all genes involved in our study (*Hdac1/Hdac2/Hdac3/Vangl2/Scrib/Rac1/Gapdh*), which were consistent across a range of template concentrations. All the slopes of the amplification efficiency curves were more than 95% and efficiencies for the target genes (*Hdac1/Hdac2/Hdac3/Vangl2/Scrib/Rac1*) and the endogenous control (*Gapdh*) were approximately equal (0.956–0.989). All samples were amplified in triplicates. Gene expression was represented for the CT value by the mean of triple tests. Relative expressions of the target genes in each sample were normalized to expression of *Gapdh* using $2^{-\Delta\Delta Ct}$.

The primer sequences specific for mouse target genes were as follows:
Hdac1: 5'-GAACTACCCACTGCGAGACG-3' (forward)
Hdac1: 5'-ACAGGGAATCTGAGCCACAC-3' (reverse)
Hdac2: 5'-GAAGGTGAAGGAGGTCGTAGG-3' (forward)
Hdac2: 5'-AGGGTTGCTGAGTTGTTCTGA-3' (reverse)
Hdac3: 5'-TGGTGGGAAGGA AAGTATGG-3' (forward)
Hdac3: 5'-TGAGAGGGACAATCATCAGG-3'(reverse)
Vangl2: 5'-GTACCTTCGCACCACCAA AC-3' (forward)
Vangl2: 5'-GCTCACCAAGGTCCACTGTT-3' (reverse)
Scrib: 5'-ACGTGGAGTCGGTGGATA A-3' (forward)
Scrib: 5'-TGTCACTGAGGCCCA ACTTT-3' (reverse)
Rac1: 5'-CGACACCACTGTCCCAATAC-3' (forward)
Rac1: 5'-CTTGAGTCCTCGCTGTGTGA-3'(reverse)
Gapdh: 5'-TTCACCACCATGGAGAAGGC-3' (forward)
Gapdh: 5'-GGCATGGACTGTGGTCATGA-3' (reverse)

The primer sequences specific for H9C2 target genes were as follows:
Hdac1: 5'-CACAGCGACGACTACATCAAG-3' (forward)
Hdac1: 5'-ACAGAACTCAAACAAGCCATCA-3' (reverse)
Hdac2: 5'-CAGTTGCCCTTGATTGTGAAAT-3' (forward)
Hdac2: 5'-TCTGGAGTGTTCTGGTTTGTCA-3' (reverse)
Hdac3: 5'-CGTCCGAAATGT TGC-3' (forward)
Hdac3: 5'-GAAGTTCCTCACTAATGG-3' (reverse)
Vangl2: 5'-CGA AACAGCAGCCTTACCAC-3' (forward)
Vangl2: 5'-CTCGCTCACCAATGTCCAC-3' (reverse)

Scrib: 5'-ACCGCATCCTAGCAGTGAAT-3' (forward)
Scrib: 5'-CCTCCGTACAAGCAGACACA-3' (reverse)
Rac1: 5'-TGCTGTCAAATACCTGGAGTGCT-3' (forward)
Rac1: 5'-TTGGCATCAGACGCGAAGACTCw-3' (reverse)
Gapdh: 5'-CAGGGTCGACCTTGCCCACAGCCTT-3' (forward)
Gapdh: 5'-GCCAAGGATATCCATGACAACT-3' (reverse).

Western blotting analysis

Samples were lysed by RIPA (P0013B, Beyotime, China) with complete protease inhibitor cocktail (P8340, Sigma-Aldrich) for 20 min at 4°C, centrifuged at 12,000 g for 5 min at 4°C. *Supernatants* were collected and the protein concentration was determined by enhanced Bicinchoninic Acid protein assay kit (P0010S, Beyotime, China) according to the manufacturer's protocol. Cell lysates were boiled in ×4 sample buffer for 5 min and 50 μg protein/lane was subjected to 8% sodium dodecyl sulfate-polyacrylamide gel. Proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked for 60 min with Tris base buffer containing 0.1% Tween 20 (TBST) and 5% nonfat milk and incubated overnight at 4°C with primary antibodies' against Hdac1 (10197-1-AP, Proteintech, USA, 1:1000)/Hdac2 (12922-3-AP, Proteintech, 1:1000)/Hdac3 (10255-1-AP, Proteintech, 1:500)/Vangl2 (21492-1-AP, Proteintech, 1:1000)/Scrib (C0613, Santa Cruz, USA, 1:100)/Rac1 (D2115, Santa Cruz, 1:200) and Gapdh (CW0100A, CWBIO, 1:500). Following extensive washing with TBST, the membranes were immunoblotted with horseradish peroxidase-conjugated goat antirabbit/goat antimouse/rabbit antigoat immunoglobulin G secondary antibodies (1:2500 dilution) for 2 h at room temperature. Washed several times in TBST, membranes were exposed to enhanced chemiluminescence detection system. The protein expression levels were quantified by software Gelpro32 (Media Cybernetics, USA) and normalized against the Gapdh as an endogenous control. We have confirmed the specificity of antibodies (Hdac1/Hdac2/Hdac3/Vangl2/Scrib/Rac1) through preincubation with the peptide epitope and prevention of binding at 60,000/55,000/49,000/60,000/220,000/22,000, respectively.

Hdac activity assay

The total Hdac activity was determined by Hdac activity colorimetric Assay Kits (K331-100, BioVision, USA) according to the manufacturer's protocol. The assay was based on a two-step colorimetric reaction. The first step of reaction was deacetylation of the acetylated lysine side chain of Hdac colorimetric substrate by a sample containing Hdac activity. Deacetylation of the substrate sensitized the substrate so that, in the second step, treatment with the lysine developer produced a chromophore, analyzed by the microplate reader, which was proportional to the deacetylation activity of the sample. Briefly, the sample was lysed and protein was extracted using the protocol mentioned above. Following measurement of protein concentration, 100 μg of protein was incubated with Hdac colorimetric substrate for 1 h at 37°C. The lysine developer was added to above reaction mixture and incubated for another 30 min at 37°C. Total Hdac activity was determined by assessing the absorbance at 405 nm using a microplate reader (Varioskan Flash, Thermo scientific), expressed as the relative O.D value per μg protein. Background readings were subtracted from all the samples. Then, based on the prepared standard curve using the known amount of deacetylated standard in the kit, the absolute amount of deacetylated lysine generated in the sample could be determined (μmol/L/μg protein).

Statistical analysis

Mean ± standard error was calculated for quantitative data, while qualitative data were presented as *n*/%. All analyses were conducted with SPSS 17.0 version (SPSS, Chicago, IL, USA). Shapiro–Wilk test and homogeneity test of variance were used to confirm that quantitative data from different groups came from a normal distribution and met the homogeneity of variance. The differences between two groups were determined by the independent sample *t*-test. The differences among different groups were determined by one-way analysis of variance followed by Tukey's honestly significant difference multiple range test. Chi-square test was used to compare proportions and Fisher's exact Test was used if not matched. A two-tailed $P < 0.05$ was considered statistically significant.

Results

Examination of litters at E15.5 revealed that there were no significant differences in the average number of dams per pregnant mouse among VPA-treated, vehicle, or blank group (VPA-treated vs. blank: $q = 2.172$, $P > 0.05$; VPA-treated vs. Vehicle: $q = 2.764$, $P > 0.05$; blank vs. Vehicle: $q = 1.812$, $P > 0.05$). However, the death and resorption rates of embryos were significantly higher in VPA-treated group ($\chi^2 = 64.21$, $P < 0.001$). Histological examination of cardiac tissues showed VPA-induced dominant increase of cardiac abnormalities compared with the controls ($\chi^2 = 35.90$, $P < 0.001$) [Table 1]. Cardiac malformations, most commonly affecting the interventricular septum and the ventricular myocardium, were seen in the mice treated with VPA. Among them, in contrast to the normal closure of interventricular communication [Figure 1a and 1b], one--fifth (29/145) had ventricular septum defects, accounting for 74.4% of all types of cardiac abnormalities [Table 1 and Figure 1e, 1f]. In addition, by E15.5, the normal right and left ventricle had a thick compact myocardium [Figure 1c and 1d]. In contrast, the VPA-treated embryo had a thinned ventricular wall with little compact layers, the marked trabeculae with deep intratrabecular recesses being seen [Figure 1g and 1h]. These anomalies in the disorganization of the ventricular myocardium resembled those seen in the human cardiomyopathy – noncompaction of ventricular myocardium.[24] In comparison with the treated group, cardiac abnormalities were sparse in control groups, only two cases of atrial septal dysmorphogenesis being observed [Table 1].

As shown in Figure 2, after exposure to VPA, Hdac1/2/3 mRNA and protein levels in E15.5 embryonic hearts were significantly decreased in comparison with the blank and vehicle groups (mRNA: $q = 4.523/4.762$ and $P = 0.027/0.014$ for *Hdac1*, $q = 4.018/4.655$ and $P = 0.034/0.021$ for *Hdac2*, and $q = 4.502/4.711$ and $P = 0.028/0.016$ for *Hdac3*, in comparison with the blank and the vehicle, respectively; protein: $q = 5.483/4.806$ and $P = 0.018/0.017$ for Hdac1, $q = 7.414/6.052$ and $P = 0.002/0.008$ for Hdac2, and $q = 7.499/9.589$ and $P = 0.002/0.001$ for Hdac3, in comparison with the blank and the vehicle, respectively) [Figure 2a and 2b]. Referring to PCP genes, VPA dominantly ablated Vangl2/Scrib mRNA and protein expressions (mRNA: $q = 4.296/3.583$ and $P = 0.011/0.027$ for *Vangl2* and $q = 5.123/4.459$ and $P = 0.002/0.006$ for *Scrib*, in comparison with the blank and the vehicle, respectively; protein: $q = 7.717/6.265$ and $P = 0.002/0.004$ for Vangl2 and $q = 6.090/4.574$ and $P = 0.005/0.018$ for Scrib, in comparison with the blank and the vehicle, respectively), whereas no significant differences in Rac1 expression were noted after VPA treatment (mRNA: $q = 1.920/1.221$ and $P > 0.05$; protein: $q = 0.829/0.945$ and $P > 0.05$) [Figure 2b and 2d]. In addition, VPA treatment significantly repressed total Hdac activity of embryonic hearts ($q = 4.885/5.051$ and $P = 0.018/0.012$, in comparison with the blank and vehicle, respectively) [Figure 2e].

As revealed in Figure 3, after transfection of Hdac1/2/3 siRNA into H9C2 cells, endogenous expressions of Hdac1/2/3 were successfully inhibited, respectively, as evidenced by qRT-PCR [Figure 3a] and Western blotting [Figure 3c and 3e] in comparison with the control group (mRNA: $t = 5.237$, $P = 0.006$ for *Hdac1*; $t = 6.933$, $P = 0.002$ for *Hdac2*; and $t = 6.771$, $P = 0.002$ for *Hdac3*; protein: $t = 4.682$, $P = 0.009$ for Hdac1; $t = 4.858$, $P = 0.008$ for Hdac2; and $t = 5.976$, $P = 0.002$ for Hdac3). Compared with the control, the inhibition of Hdac3 could significantly result in repression of either *Vangl2*/*Scrib* mRNA [Figure 3b] or protein [Figure 3d and 3e] expressions (mRNA: $q = 9.947$, $P < 0.001$ for *Vanlg2*; $q = 7.081$, $P = 0.005$ for *Scrib*; protein: $q = 13.502$, $P < 0.001$ for Vangl2; $q = 8.896$, $P = 0.001$ for

Table 1: Fetal loss and cardiac abnormalities in VPA exposed dams

Treatment	Number of dams	Total number of fetuses	Number of death/resorption, *n* (%)	Number of cardiac abnormalities in live fetuses, *n* (%)	Types of cardiac abnormalities, *n* (%)		
					VSD	NVM	ASD
VPA-treated	26	209	64 (30.6)*	39 (18.6)*	29 (74.4)	10 (25.6)	0
Vehicle	17	105	2 (1.9)	1 (1.0)	0	0	1
Blank	15	98	1 (1.0)	1 (1.0)	0	0	1
χ^2			64.21	35.90			
P			<0.001	<0.001			

Pregnant mice received a one-time intraperitoneal injection of VPA (700 mg/kg) at E10.5. The fetuses were examined at E15.5 after execution of the dam as described in materials and methods. Chi-square test was performed to compare proportions and Fisher's exact test was used if not matched. A two-tailed $P<0.05$ was considered statistically significant. *$P<0.001$. VSD: Ventricular septum defect; NVM: Noncompaction of ventricular myocardium; ASD: Atrial septal dysmorphogenesis, VPA: Valproic acid.

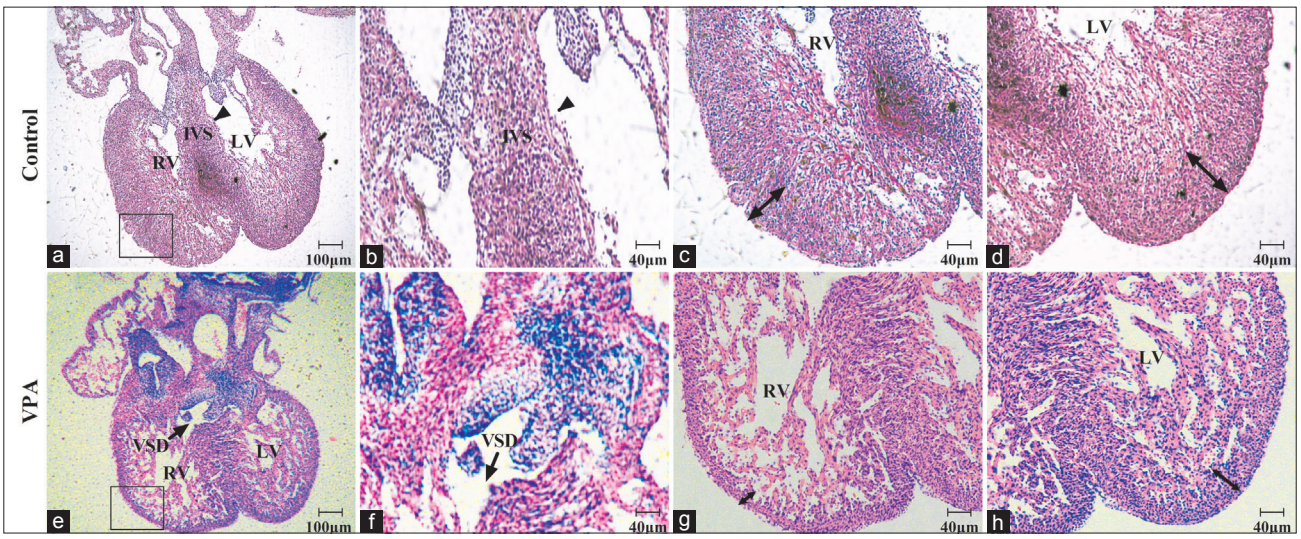

Figure 1: Effect of VPA on embryonic hearts. H and E staining was performed on E15.5 paraffin sections. (a-d) Normal development of interventricular septum with formation between the ventricles (arrowhead in a and b) and thick compact ventricular myocardium in control groups (box in a and double arrows in c and d). (e-h) Ventricular septum defect was found in VPA-treated group (arrow in e and f). In contrast to a, c, and d, the compact walls of right and left ventricles were significantly thinner and the trabeculae were prominent (box in e and double arrow in g, h) in VPA-treated group. RV: Right ventricle; LV: Left ventricle; IVS: Interventricular septum; VSD: Ventricular septum defect; VPA: Valproic acid. E15.5: Embryonic day 15.5.

Scrib); however, knockdown of Hdac1/2 was not (mRNA: q = 1.567/2.062 for *Vangl2*; q = 3.372/2.709 for *Scrib*; protein: q = 1.110/2.039 for Vangl2; and q = 1.599/0.795 for Scrib; all P > 005). No significant differences in Rac1 expression among different groups were found after Hdac1/2/3 silencing (mRNA: q = 3.094/3.818/1.056; protein: q = 0.539/0.733/0.795; all P > 0.05) [Figure 3b, 3d and 3e].

To further determine the role of Hdac3 on VPA target gene expression, the total RNAs/proteins were extracted from the control or Hdac3-overexpression H9C2 cells in the presence or absence of VPA treatment and subjected to qRT-PCR and Western blotting analysis. As shown in Figure 4a and 4b, VPA exposure significantly decreased the mRNA [Figure 4a] and protein levels [Figure 4b] of Vanlg2

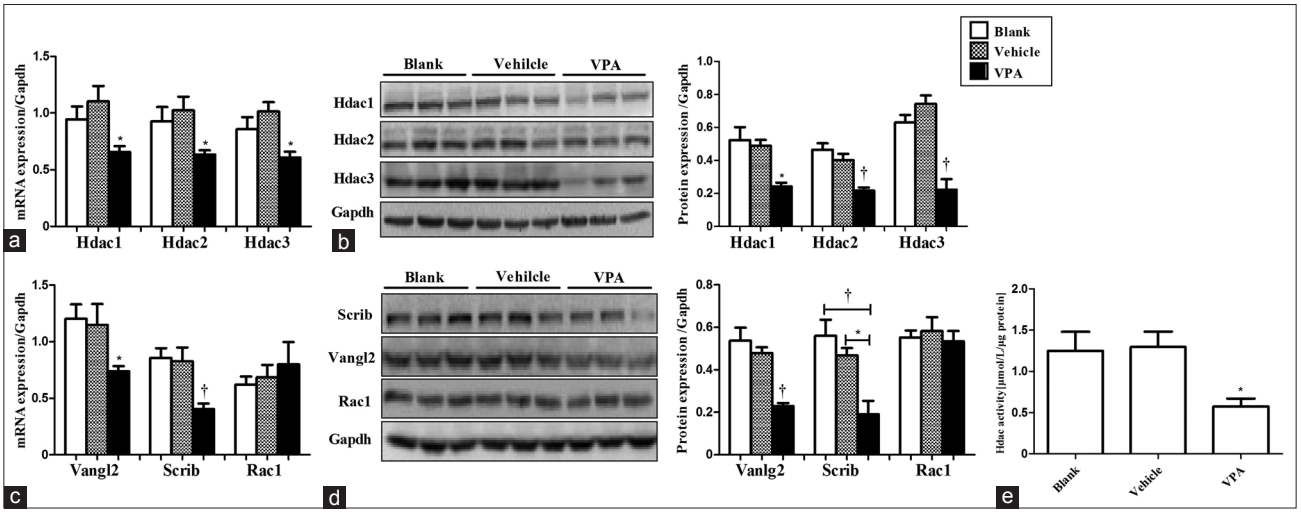

Figure 2: Hdac and PCP genes expression of embryonic hearts after VPA exposure. The mRNA (a and c) or protein levels (b and d) of Hdac1/Hdac2Hdac3/Vanlg2/Scrib/Rac1 together with Hdac activity (e) of embryonic hearts were measured at E15.5. Differences among different groups were determined by ANOVA followed by Tukey's honestly significant difference multiple range test. N = 6 to 8 for each group. Data were expressed as mean ± SE. *P < 0.05, †P < 0.01. ANOVA: Analysis of variance; PCP: Planar cell polarity; VPA: Valproic acid; E15.5: Embryonic day 15.5; SE: Standard error.

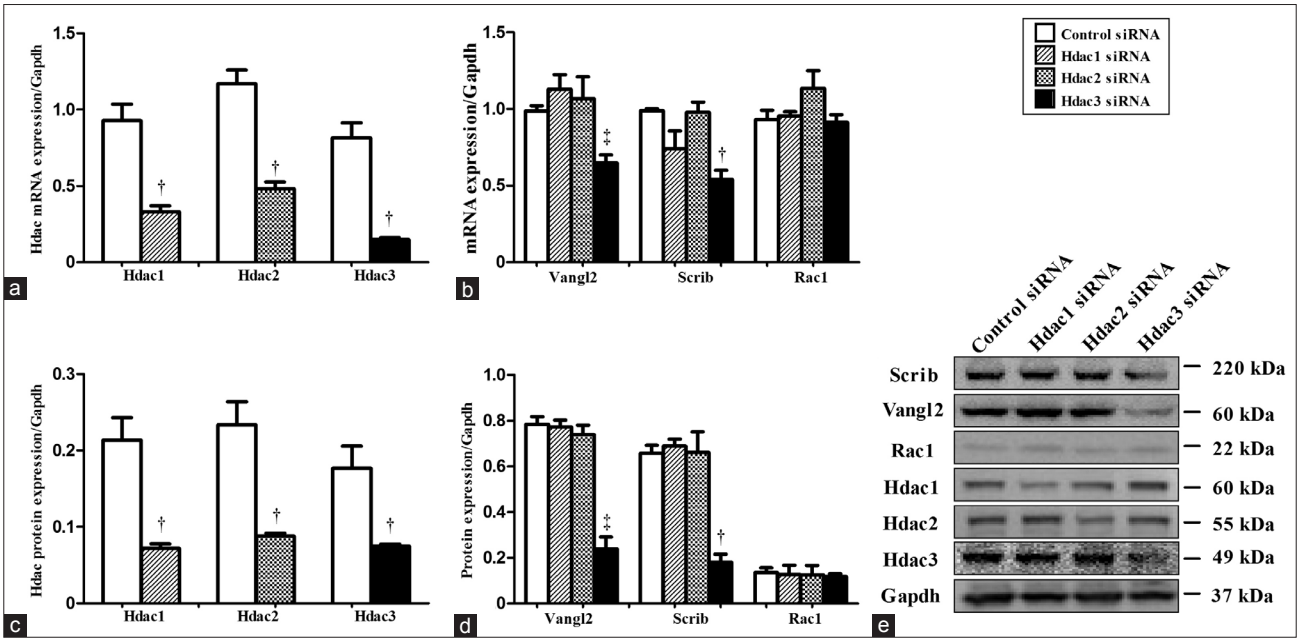

Figure 3: Involvement of Hdacs in the regulation of PCP genes in H9C2 cells. H9C2 cells were transfected with control or specific siRNA for Hdac1, Hdac2, or Hdac3. After 48 h of transfection, the levels of Hdac1/Hdac2/Hdac3 and Vangl2/Scrib/Rac1 mRNA (a and b) and protein expressions (c-e) were analyzed by qRT-PCR and Western blotting, respectively. Differences of Hdac expression between the control and transfected group were determined by two-tailed Student's t-test; differences of PCP gene expressions among different specifically transfected groups were determined by ANOVA followed by Tukey's honestly significant difference multiple range test. N = 3 for each group. Data were expressed as mean ± SE. †P < 0.01, ‡P < 0.001. PCP: Planar cell polarity; siRNA: Small interfering RNA; qRT-PCR: Quantitative real-time polymerase chain reaction; ANOVA: Analysis of variance; SE: Standard error.

and Scrib compared with controls (mRNA: q = 6.778, P = 0.001 for *Vangl2*; q = 13.781, P < 0.001 for *Scrib*; protein: q = 4.972, P = 0.008 for Vanlg2; and q = 9.967, P < 0.001 for Scrib). Conversely, overexpression of Hdac3 induced Vanlg2 and Scrib mRNA [Figure 4a] together with protein expressions [Figure 4b] (mRNA: q = 4.574, P = 0.023 for *Vangl2*; q = 3.432, P = 0.041 for *Scrib*; protein: q = 5.236, P = 0.01 for Vanlg2; q = 8.219, P = 0.001 for Scrib). Notably, Hdac3 overexpression completely reversed VPA-induced Vanlg2 and Scrib reduction [Figure 4a and 4b] (Hdac3 plasmid + VPA vs. control: mRNA, q = 0.962/2.538 for *Vangl2*/*Scrib*, respectively, protein, q = 0.066/0.626 for Vangl2/Scrib, respectively; all P > 0.05). However, expression of Rac1 was not alter among different groups (mRNA: q = 1.356/1.161/2.225; protein: q = 1.088/1.075/1.037; all P > 0.05). Moreover, it was found that, in comparison with the control, VPA could robustly ablate Hdac activity (q = 7.477, P = 0.001), whereas Hdac3 overexpression increased that (q = 3.660, P = 0.032) and also partially rescued the inhibition caused by VPA (q = 4.711, P = 0.011) [Figure 4c].

Discussion

Emerging evidence has proven that epigenetics displays a diverse control on meticulous expression of vital genes requiring for the processes of cardiomyocyte growth and proliferation.[25] Drugs altering these epigenetic processes have been shown to play substantial roles in the development of CHD.[1] Although it is well recognized that VPA is teratogenic, few studies have looked for its effect on epigenetic status and cardiac development-related genes. To our knowledge, our study was the first one to unravel whether VPA-induced CHD was related to disruption of PCP pathway through Class I HDACs. Our results showed that *in vivo* VPA could induce cardiac malformations at the middle stage of pregnancy; VPA-induced repression of Hdac1/2/3 expression and Hdac activity were accompanied by inhibition of Vanlg2 and Scrib in embryonic hearts. In addition, *in vitro* Hdac3 knockdown by RNA silencing decreased Vangl2 and Scrib expression; Hdac3 overexpression reversed the VPA-induced inhibition of these two genes, as well as Hdac activity. These findings strongly suggested that VPA might induce CHD by inhibiting Vangl2 and Scrib through Hdac3 participation.

The vital roles of PCP signaling in cardiac development have been evidenced by gene knockout mouse models.[9-13] *Vanlg2* and *Scrib* are required for septation between ventricular chambers; moreover, *Scrib* also plays significant roles in the organization of developing cardiomyocytes and formation of the compact ventricular myocardium. Deficiency of *Vanlg2* or *Scrib* in embryonic cardiomyocytes exhibits ventricular septal defects. In addition, loss of *Scrib* displays a thinned ventricular myocardium and ventricular noncompaction as well, apart from ventricular septal defects.[9-13] The morphology of cardiac abnormalities observed after VPA exposure in our study was very similar with the types of those

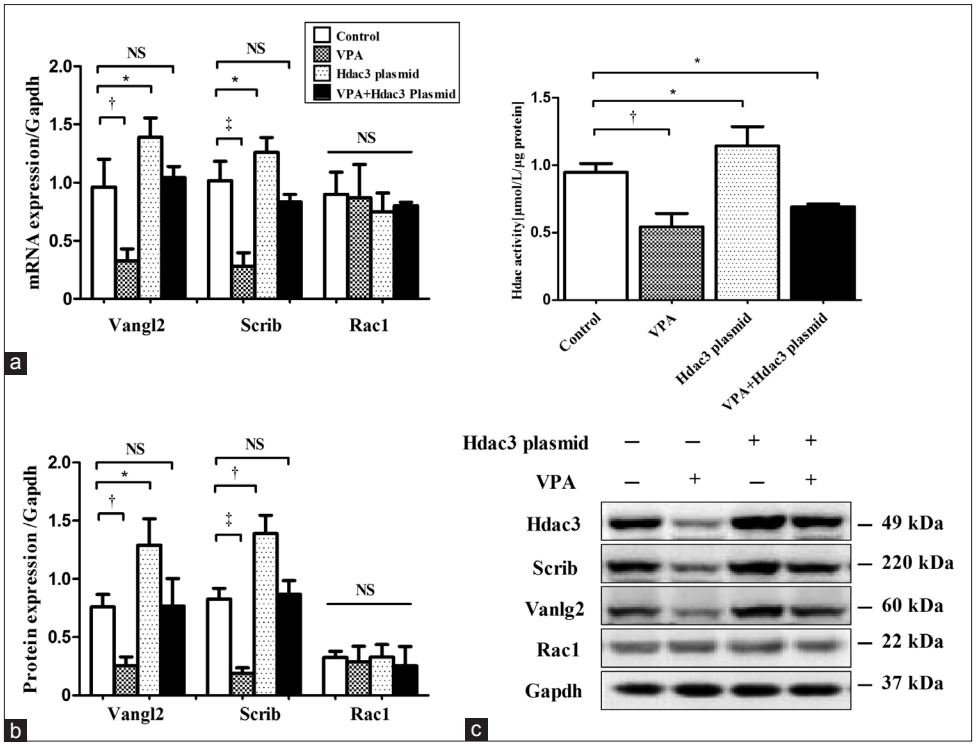

Figure 4: Hdac3 overexpression reversed VPA-mediated inhibition of PCP gene expressions in H9C2 cells. H9C2 cells were transfected with either control or Hdac3 expression plasmids for 48 h. Then, cells were treated with saline (mock) or with 8.0 mmol/L VPA for another 48 h and subjected to qRT-PCR (a), Western blotting (b), or Hdac activity assay (c), respectively. Differences among different groups were determined by ANOVA followed by Tukey's honestly significant difference multiple range test. N = 3 for each group. Data were expressed as mean ± SE. *P < 0.05, †P < 0.01, ‡P < 0.001. NS: Not significant; VPA: Valproic acid; PCP: Planar cell polarity; qRT-PCR: Quantitative real-time polymerase chain reaction; ANOVA: Analysis of variance; SE: Standard error.

found in PCP gene deletion models. Further investigation into the effect of VPA on PCP signaling revealed that VPA treatment dramatically repressed Vangl2/Scrib levels in embryonic hearts. Collectively, these results indicated that VPA-induced cardiac anomalies could be due, at least in part, to disruption of PCP pathway during cardiac development.

Our results also illustrated that VPA-induced repression of Vangl2/Scrib expression was accompanied by inhibition of Hdac1/2/3 together with Hdac activity. We further identified the specific Hdac subtype involving in Vangl2/Scrib regulation by transfection of specific siRNA into H9C2 cells. The results manifested that Hdac3 might act as an activator of Vanlg2/Scrib. Moreover, Hdac3 knockdown decreased Vangl2/Scrib mRNA as well as their protein levels, suggesting Hdac3 might activate expressions of the two genes at the transcriptional level. These findings did not correspond with the best-documented biological function of Hdac3, acting as a transcriptional repressor. Of note, this unexpected phenomenon of Hdac3 has been observed both *in vivo* and *vitro* experiments previously. It has been reported that Hdac3 was required for transcriptional activation of retinoic acid response element.[26] Cells derived from Hdac3 knockout mice displayed both downregulation and upregulation of gene expression.[27] Furthermore, gene expression profiling studies comparing the cells treated with HDAC inhibitors versus those untreated revealed that the number of genes downregulated were comparable to those upregulated.[28] One possibility is that Hdac3 may prohibit transcription of transcriptional repressors, leading to derepression of gene expression. Alternatively, Hdac3 may deacetylate and, ultimately, activate transcription activators or suppress the functions of transcription repressors independent of histone modifications. However, the process of Hdac3 regulating Vanlg2/Scrib in the cardiomyocytes still remains to be established.

The teratogenic ability of VPA is closely correlated to its Hdac inhibition activity, while VPA analogs that do not have Hdac inhibition activity are not teratogenic.[15,16] Yet, on the whole, the teratogenic mechanism of VPA is still largely unexplained. In the present study, we also found that VPA-induced cardiac abnormalities were accompanied with inhibition of Hdac activity, whereas Hdac3 overexpression not only partially reversed VPA-induced Hdac inhibition but also blocked the inhibitory effect of VPA on Vangl2/Scrib expressions, indicating that VPA probably interfered with PCP pathway through inhibition of Hdac activity, consequently producing teratogenic effects, in a Hdac3-dependent manner. Therefore, Hdac3 might be promising target for prevention of VPA-induced CHD, if these findings could be validated in animal models and human studies.

However, some limitations must be considered. First, the action mechanism of VPA on Hdacs and PCP signaling needs to be unveiled to gain a deeper insight into novel prevention for its cardiac teratogenesis and the treatment of diseases linked to the overexpression of Hdacs. Second, given the discordance between gene expression and its function, it is unknown whether the VPA and/or Hdac3 is also involved in the regulation of Vanlg2/Scrib function. In addition, we only looked into the regulation of Hdac1/2/3 on PCP pathway. However, apart from Hdac1/2/3, VPA could inhibit other Hdacs, whether other Hdacs are also involved in the regulation of this pathway or not still remain to be further clarified. Finally, a multifactorial pathogenesis with interplay between inherited and noninherited causes was involved in the development of CHD. In the present study, we merely made a preliminary exploration of the possible molecular mechanisms in VPA-induced CHD, and those findings could not be expanded indiscriminately to the complex basis of cardiac perturbations. However, our study has shown that PCP pathway was critical to cardiogenesis, and disruption of this pathway possibly resulted in the occurrence of cardiac developmental disorders. Those findings might aid in the identification of VPA-specific target genes and offer some references for preventing or ameliorating its teratogenic risk in the context of gene-environment interactions. Taken together, the findings in this study were merely the first step toward the possible molecular mechanism of VPA-induced CHD in the context of PCP pathway from the perspective of epigenetics, which could offer a clue for the prevention of its cardiac teratogenesis linked to the overexpression of Hdac3. The results need to be further validated in animal models and human studies.

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Conflicts of interest

There are no conflicts of interest.

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丙戊酸可通过组蛋白去乙酰化酶-3干扰平面细胞极性途径导致先天性心脏病

摘要

背景：妊娠期丙戊酸（VPA）暴露被证实与先天性心脏病（CHD）发生有关。本团队前期实验研究发现，干扰心肌细胞平面细胞极性（PCP）信号通路是VPA心脏致畸性的可能因素。此外，相关研究证实VPA的致畸作用与其对组蛋白去乙酰化酶（HDAC）抑制作用成正相关。本研究旨在观察VPA对心脏形态发生、HDAC1/2/3及PCP通路关键基因（Vangl2/Scrib/Rac1）的影响，进而筛选出参与PCP通路调控的特异性HDAC类型。

方法：以VPA 700mg/kg于妊娠第10.5天（E10.5）腹腔注射C57BL孕鼠。于E15.5处死孕鼠，统计胚胎死亡/流产率。采集心脏标本以苏木精-伊红染色观察心脏畸形情况。以Hdac1/2/3特异性siRNA转染H9C2细胞（大鼠未分化心肌细胞）。根据转染siRNA相关结果，以Hdac3表达质粒转染细胞，随后以假处理或VPA 8.0mmol/L干预细胞。分别采用实时定量PCR及Western-blot检测Hdac1/2/3及Vangl2/Scrib/Rac1 mRNA及蛋白表达水平。利用比色法测定总 HDAC 活性。

结果：VPA可导致CHD（$P<0.001$），并下调胚胎心脏中Hdac1/2/3、Vangl2/Scrib/Rac1 mRNA及蛋白表达水平，同时抑制总Hdac活性（$P<0.05$）。体外实验发现，抑制Hdac3可显著下调Vangl2/Scrib表达（$P<0.01$），而抑制Hdac1/2无以上作用（$P>0.05$）；VPA暴露可显著抑制Vanlg2/Scrib表达及Hdac活性 ($P<0.01$)，而过表达Hdac3可逆转VPA产生的抑制作用（$P>0.05$）。

结论：通过体外及体内实验我们均证实，VPA可抑制Hdac1/2/3、Vangl2/Scrib表达及总Hdac活性，并且Hdac3可能参与VPA所致的心脏发育异常。