

Modulation of *Dishevelled* and *Vangl2* by All-*trans*-retinoic Acid in the Developing Mouse Central Nervous System and its Relationship to Teratogenesis

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Abstract The response to exposure to all-*trans*-retinoic acid (RA) during embryogenesis varies from physiologic to severe teratogenic effects and is dependent upon the dose and the stage of development in all species. *Vangl2* and *Dishevelled* genes play key roles in establishing planar cell polarity and regulating convergent extension movements during the neurula period. The effects of RA-mediated teratogenesis might be due to its misregulation of *Vangl2* and *Dishevelled* genes. The aim of this study is to monitor the modulation of *Vangl2* and *Dishevelled* in Kunming mouse embryos following maternal treatment with a single oral dose of 30 mg/(kg body weight) of RA during the neurula period. Exposure of 7.75 d embryos to RA induced characteristic morphological changes. The most obvious external effect was the failure of neural tube closure in the midbrain and forebrain regions in 10 d embryos, resulting in exencephaly in later embryos. RA treatment also led to a pronounced decrease of *Vangl2* mRNA at 4 and 18 h and a pronounced increase at 66 h after maternal treatment, as detected by reverse transcription-polymerase chain reaction. Western blot analysis showed a marked decrease of Vangl2 protein at 18 and 42 h and a marked increase at 66 and 90 h after maternal treatment. *Dishevelled1/2/3* mRNA was significantly down-regulated at 4 and 18 h and up-regulated at 42 h in the fetus after RA treatment, except for an up-regulation of *Dishevelled3* at 66 h. The *Dishevelled2* mRNA and its protein matched each other. These results hinted that *Vangl2* and *Dishevelled* genes might take part in RA teratogenesis of mouse embryos.

Keywords teratogenesis; retinoic acid; *Vangl2*; *Dishevelled*

Retinoic acid (RA) is a small (300 Da) lipophilic signaling molecule that acts to mediate gene expression by binding to at least two classes of ligand-activated transcription factors: the retinoic acid receptor (RAR), which binds all-*trans*-RA [1], and retinoid X receptor, which binds 9-*cis*-RA [2]. RA mediates multiple biological events during cell growth, differentiation and apoptosis, and plays important roles in vision, reproduction and embryonic patterning of a wide range of species at its physiological concentration. However, it is well known that both excess and deficiency of RA can cause a spectrum of dose- and stage-specific birth defects during embryogenesis [3–5], and the central nervous system (CNS) represents a major site of the teratogenic action of RA. Inappropriate gene expression

has been supposed as the mechanistic basis of retinoid teratogenicity. Morphological changes after retinoid modulation in embryos could be explained by the alteration of the spatial and temporal pattern of gene expression that controls proliferation, differentiation, apoptosis and morphogenesis.

The planar cell polarity (PCP) pathway is referred to as the non-canonical Wnt pathway. In contrast to the canonical Wnt pathway that acts through stabilization and nuclear translocation of β -catenin in vertebrates to regulate transcription of target genes, the PCP pathway is not directly involved in stabilization of β -catenin but acts through various effectors of cell polarity such as small GTPases and kinases, potentially including the c-jun N-terminal kinase cascade, to mediate cytoskeletal changes [6]. The PCP pathway plays a key role in regulating convergent extension (CE) movements and establishing PCP

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during the neurula period in vertebrate. CE is the process by which the presumptive notochord and neural plate lengthens and narrows due to mediolateral intercalation of cells. PCP is the coordinate organization of cells within the plane of a single layered sheet of cells, perpendicular to the apicobasal axis of the cells. Extensive studies have shown that both PCP and CE movement rely on the formation of a multiprotein membrane complex consisting of core PCP proteins *Vangl1*, *Prickle*, *Dishevelled*, and *Frizzled*. PCP proteins are initially arranged symmetrically at the apical membrane in individual cells, but become asymmetrically redistributed during the establishment of PCP and CE movement. At the molecular level, *Vangl2* has been shown to interact directly with cytoplasmic *Dishevelled*, leading to their recruitment to the membrane [7–11]. In vertebrates, the inactivation of *Vangl2* [12–16] or *Dishevelled* [12,15–19] can induce the misorientation of the normally highly organized neural epithelial structures and a failure of CE in mesenchyme, which produces animals with shorter, wider trunks and a significantly widened neural plate in the midline and, as a result, neural tube defects (NTDs).

The present study was undertaken to investigate the modulation of *Vangl2* and *Dishevelled* in developing Kunming mouse embryos after maternal exposure to RA. Results indicated that *Vangl2* and *Dishevelled* were both initially down-regulated, followed by up-regulation during RA-induced teratogenesis. This might provide further insight into the mechanistic basis of RA-induced developmental defects.

Materials and Methods

Animal housing and RA treatment

One hundred time-pregnant Kunming mice were purchased from the Laboratory Animal Center of Shandong University (Jinan, China). Noon of the day of vaginal plug was considered 0.5 days post coitum (d.p.c.). The dams were housed at 23 °C and 50% relative humidity with a 12 h light/dark cycle and offered pelleted food and fresh water ad libitum. The animal-use protocols complied with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and the guide of the Institutional Animal Care and Use Committee. To investigate the effect of RA on *Vangl2* and *Dishevelled2* genes during organogenesis, dams (7.75 d.p.c.) were given a single oral dose of 30 mg/(kg body weight) of all-*trans*-RA [20] (Sigma, St. Louis, USA) dissolved in sesame oil. The control

dams were treated only with an equal volume of sesame oil (vehicle). All-*trans*-RA was formulated and dispensed under yellow light to prevent photoisomerization. The females were killed by cervical dislocation at 4, 18, 42, 66 or 90 h after RA or vehicle treatment (five animals per group). The fetuses from RA- and vehicle-treated dams were collected surgically by abdominal incision and freed from extraembryonic membranes. The fetuses were stripped of their neural tube, and these were quickly frozen in liquid nitrogen and stored at –80 °C for future analysis. Some fetuses were collected at 10 or 16 d.p.c., weighed, measured for the crown-rump length and tail length, and examined for external malformations after RA or vehicle treatment.

Semiquantitative assessment of *Vangl2* and *Dishevelled* gene expression by reverse transcription-polymerase chain reaction (RT-PCR)

The RNA was isolated from the fetuses and converted to cDNA by RT-PCR using a similar protocol described previously [21]. For semiquantitative measurement, the number of cycles within an exponential phase was determined by initial trials to ensure gene amplification in the linear exponential phase. Samples were amplified for 25 cycles for *β-actin* and 30 cycles for *Vangl2* and *Dishevelled* in a GeneAmp 2400 thermal cycler (Perkin Elmer, Norwalk, USA). The annealing temperature was optimized using a gradient. An annealing temperature of 55 °C was used for *β-actin*, *Vangl2* and *Dishevelled1* and 50 °C for *Dishevelled2* and *Dishevelled3*. The primers for *β-actin*, *Vangl2* and *Dishevelled1/2/3*, chosen by the Primer 5 program, are shown in **Table 1**. Negative controls with the same primers without cDNA were used in the PCR reactions. After amplification, 10 µl of PCR products was mixed with DNA dye and run on 2.0% agarose gel stained with ethidium bromide for electrophoretic separation. The gels showed single bands of the correct size and were photographed in a backlit ultraviolet transilluminator and quantified with Image-Pro Plus 5.0 (Media Cybernetics, Silver Spring, USA).

Total pixel counts for *Vangl2* and *Dishevelled* were normalized with *β-actin*.

Determination of *Vangl2* and *Dishevelled2* protein in neural tissue extracts by Western blot

The proteins from tissue were isolated using a similar protocol described previously [22]. All the subsequent steps were carried out on ice and centrifuged at 4 °C. Briefly, 5–18 fetuses were ground in liquid nitrogen and the powdered embryos were mixed with 1 ml of solution A

Table 1 Primers used for reverse transcription-polymerase chain reaction

| Gene | Sequence (5'→3') | Size of product (bp) |
|---------------------|------------------------------|----------------------|
| <i>β-actin</i> | CGCGGGCGACGATGCTC (F) | 289 |
| | TTCACGGTTGGCCTTGGGGTTCAG (R) | |
| <i>Vangl2</i> | TGAGGGCCTTTCATCTCC (F) | 528 |
| | GCCCGTGGAGTTAATTGGT (R) | |
| <i>Dishevelled1</i> | CTGAGTCTGTGCTCCTGCTG (F) | 542 |
| | AACTGCCCTTTGGGTAAGT (R) | |
| <i>Dishevelled2</i> | ACCTGGCTGGCTACGAGAG (F) | 538 |
| | TCGAGGGAGGGTGAAGTAG (R) | |
| <i>Dishevelled3</i> | TCGTCTTTCAGCAGCATCAC (F) | 541 |
| | CCATGTCACTGTGGATGGAG (R) | |

F, forward; R, reverse.

(0.6% NP-40, 150 mM sodium chloride, 10 mM HEPES, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 ml of dithiothreitol, 5 μg/ml aprotinin, and 5 μg/ml leupeptin) and homogenized in a 2 ml Dounce tissue homogenizer (Botong, Shanghai, China) with pestle B. After five strokes, the homogenate was transferred to a 1.5 ml tube and centrifuged at 2000 *g* for 30 s to get rid of the unbroken tissues. The supernatant was incubated on ice for 5 min and centrifuged for an additional 5 min at 12,000 *g*. The supernatant was stored at -80 °C. Protein concentration from neural tissue extracts was quantified by the external absorbent method and corrected by the formula ($1.45 \times A_{280} - 0.74 \times A_{260}$). An equal amount of protein was loaded in each well. An aliquot of 15 μg of the supernatant protein from each sample was heated with 4×sodium dodecyl sulfate buffer at 95 °C for 5 min, and separated electrophoretically on 10% sodium dodecyl sulfate-polyacrylamide gel for Vangl2. The proteins were transferred onto 0.45 μm pore size polyvinylidene difluoride membranes and blocked overnight with phosphate buffered saline containing 5% milk and 0.05% Tween-20. Membranes were exposed to Vangl2 primary polyclonal antibody (developed in goat; Santa Cruz Biotechnology, Santa Cruz, USA) and Dishevelled2 primary polyclonal antibody (developed in goat; Santa Cruz Biotechnology) in the blocking buffer and later incubated with horseradish peroxidase-conjugated polyclonal anti-goat rabbit immunoglobulin G (Sigma). Proteins were visualized with diaminobenzidine at room temperature, which showed single bands of the correct size, photographed in a backlit ultraviolet transilluminator and digitized with Image-Pro Plus 5.0. Negative controls (vehicle or RA-treated group) were also used in the Western blot analysis with the blocking buffer but without primary antibody. Total pixel counts

for Vangl2 and Dishevelled2 were normalized with β-actin as described for PCR above.

Statistical analysis

For analysis of the fetal weight, crown-rump length and tail length between vehicle- and RA-treated groups, Student's *t*-test assuming equal variances was used. $P < 0.05$ was considered significant. For analysis of gene expression at all the time points between vehicle- and RA-treated groups, ANOVA was used. The error bars represent standard error of the mean. $P < 0.05$ was considered significant.

Results

RA toxicity

A known teratogenic dose of RA was given as a single oral treatment at 7.75 d.p.c.. No overt sign of retinoid toxicity was detected among the RA-treated dams. In the vehicle-treated group ($n=5$), 3 resorptions out of 60 implantation sites were observed at 10 d.p.c.; in the RA-treated group ($n=5$), 2 resorptions out of 57 implantation sites were observed. There was no significant difference between the RA- and vehicle-treated groups. Counting the numbers of implantation sites and resorptions at 16 d.p.c., we found 1 resorption out of 43 implantation sites in the vehicle-treated group ($n=5$) and 38 resorptions out of 53 implantation sites in the RA-treated group ($n=5$). There was a statistically significant difference between the two groups ($P < 0.05$).

All-*trans*-RA given at a dose of 30 mg/(kg body weight) at 7.75 d.p.c. of embryogenesis induced characteristic

morphological changes in the embryos. The most obvious external effect of the RA treatment was the failure of neural tube closure in the midbrain and forebrain regions (**Fig. 1**), resulting in exencephaly in the later developed embryos (**Fig. 2**). This was in agreement with the findings of Leonard *et al.* [20]. In addition, some embryos displayed craniorachischisis or a failure of neural tube closure in the posterior neuropore. In the vehicle-treated group ($n=5$), 1 embryo with a failure of neural tube closure (EF) out of 55 live embryos (LEs) was observed at 10 d.p.c.; in the RA-treated group ($n=5$), 50 EFs out of 53 LEs were seen. There was a significant difference between the RA- and vehicle-treated groups ($P<0.05$). Counting the number of EFs and LFs at 16 d.p.c., we found 0 EFs out of 42 LEs in the vehicle-treated group ($n=5$) and 10 EFs out of 13

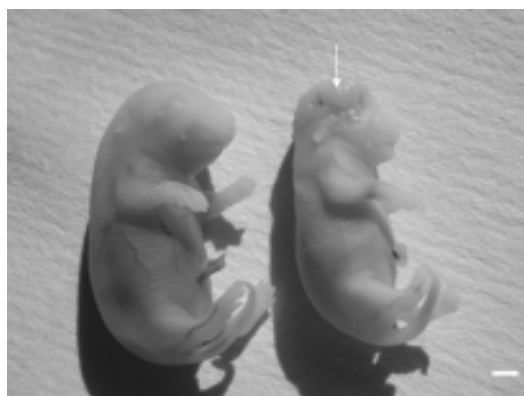


Fig. 2 External appearance of vehicle- and retinoic acid (RA)-treated mouse fetuses at 16 d post coitum

On the left, the lateral view of a vehicle-treated fetus. On the right, the lateral view of an RA-treated exencephalous fetus with an exposed cerebrum (white arrow). Scale bar=3 mm.

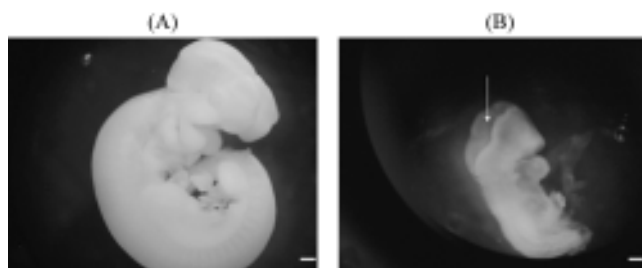


Fig. 1 External appearance of vehicle- and retinoic acid (RA)-treated mouse embryos at 10 d post coitum

(A) Lateral view of a vehicle-treated embryo. (B) Lateral view of an RA-treated embryo. The RA-treated embryo in which neural tube closure failed in the mid-brain and forebrain regions shows an extroverted neural tube (white arrow). Scale bar=500 μ m.

LEs in the RA-treated group ($n=5$). There was a significant difference between the two groups ($P<0.05$).

The average weight, crown-rump length and tail length of the fetuses were decreased significantly in the RA-treated group compared with the vehicle-treated group at 10 d.p.c. ($P<0.05$) (**Table 2**), but no significant difference was found at 16 d.p.c. (**Table 3**).

Semiquantitative assessment of gene expression of *Vangl2* and *Dishevelled* by RT-PCR

Fetuses (5–18) from the independent dams ($n=5$) were used for *Vangl2* and *Dishevelled* mRNA analysis by

Table 2 Fetal weight, crown-rump length and tail length at 10 d post coitum after an oral dose of retinoic acid in 7.75 d post coitum Kunming mice

| Litter (n) | Fetus (n) | Group | Body weight (mg) | Crown-rump length (mm) | Tail length (mm) |
|----------------|---------------|---------------|------------------|------------------------|------------------|
| 5 | 53 | Vehicle | 12.60±0.41 | 7.41±0.84 | 1.57±0.11 |
| 5 | 47 | Retinoic acid | 6.10±1.03* | 5.10±1.34* | 0.93±0.13* |

In vehicle group, sesame oil without containing retinoic acid is used. Results were analyzed by ANOVA. Values represent mean±SD. * $P\leq 0.05$.

Table 3 Fetal weight, crown-rump length and tail length at 16 d post coitum after an oral dose of retinoic acid in 7.75 d post coitum Kunming mice

| Litter (n) | Fetus (n) | Group | Body weight (g) | Crown-rump length (cm) | Tail length (cm) |
|----------------|---------------|---------------|-----------------|------------------------|------------------|
| 5 | 43 | Vehicle | 1.32±0.19 | 2.33±0.17 | 1.07±0.08 |
| 5 | 21 | Retinoic acid | 1.23±0.26 | 2.23±0.29 | 1.04±0.13 |

In vehicle group, sesame oil without containing retinoic acid is used. Results were analyzed by ANOVA. Values represent mean±SD.

RT-PCR at 4, 18, 42 and 66 h after the vehicle and RA treatment. Results showed that the level of *Dishevelled1/2/3* mRNA was low at 4 h, increased at 18 h, then decreased at 42 and 66 h after maternal treatment. RA treatment resulted in a significant decrease ($P<0.05$) at 4 and 18 h and a significant increase ($P<0.05$) at 42 h in *Dishevelled1/2/3* mRNA level in fetuses after maternal treatment. No significant difference was found in *Dishevelled1/2* mRNA at 66 h after maternal treatment except an increase in *Dishevelled3* mRNA (Figs. 3–5). In the vehicle groups, the level of *Vangl2* mRNA was low at 4 h, increased significantly at 18 h and reached its peak value at 42 h, then decreased rapidly at 66 h. RA treatment resulted in a significant decrease ($P<0.05$) at 4 and 18 h and a significant increase ($P<0.05$) at 66 h in *Vangl2* mRNA level in fetuses after maternal treatment, and no significant difference was found at 42 h after maternal treatment (Fig. 6).

Vangl2 and Dishevelled2 protein expression by Western blot analysis

Fetuses (5–18) from the independent dams ($n=5$) were used for protein analysis by Western blot at 18, 42, 66 and 90 h after vehicle and RA treatment. Results showed that the level of Vangl2 protein was low at 18 h and increased rapidly at 42 h after vehicle treatment, then decreased continuously at 66 and 90 h. RA treatment resulted in a

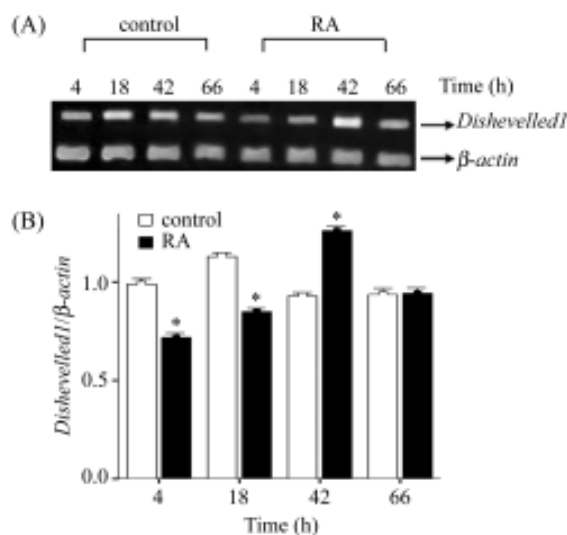


Fig. 3 *Dishevelled1* mRNA expression in neural tube tissue of vehicle- and retinoic acid (RA)-treated mouse embryos

Neural tube tissues were assayed by reverse transcription-polymerase chain reaction at 4, 18, 42 and 66 h after maternal treatment with a single oral dose of 30 mg/(kg body weight) of RA, or vehicle only. Representative gel (A) is shown over respective bar graph (B). Results are expressed as mean \pm SEM ($n=5$). * $P\leq 0.05$.

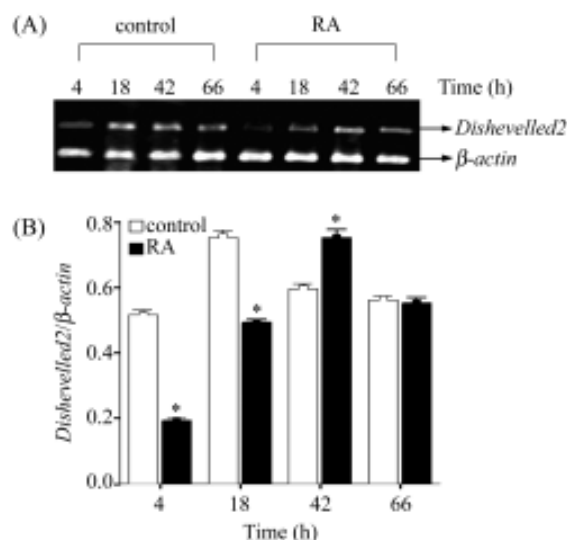


Fig. 4 *Dishevelled2* mRNA expression in the neural tube tissue of vehicle- and retinoic acid (RA)-treated mouse embryos

Neural tube tissues were assayed by reverse transcription-polymerase chain reaction at 4, 18, 42 and 66 h after maternal treatment with a single oral dose of 30 mg/(kg body weight) of RA, or vehicle only. Representative gel (A) is shown over respective bar graph (B). Results are expressed as mean \pm SEM ($n=5$). * $P<0.05$.

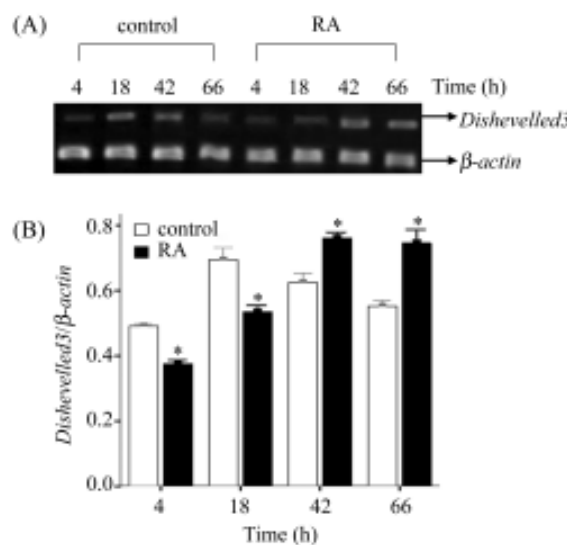


Fig. 5 *Dishevelled3* mRNA expression in the neural tube tissue of vehicle- and retinoic acid (RA)-treated mouse embryos

Neural tube tissues were assayed by reverse transcription-polymerase chain reaction at 4, 18, 42 and 66 h after maternal treatment with a single oral dose of 30 mg/(kg body weight) of RA, or vehicle only. Representative gel (A) is shown over respective bar graph (B). Results are expressed as mean \pm SEM ($n=5$). * $P<0.05$.

significant decrease ($P<0.05$) at 18 and 42 h and a significant increase ($P<0.05$) at 66 and 90 h in Vangl2 protein in

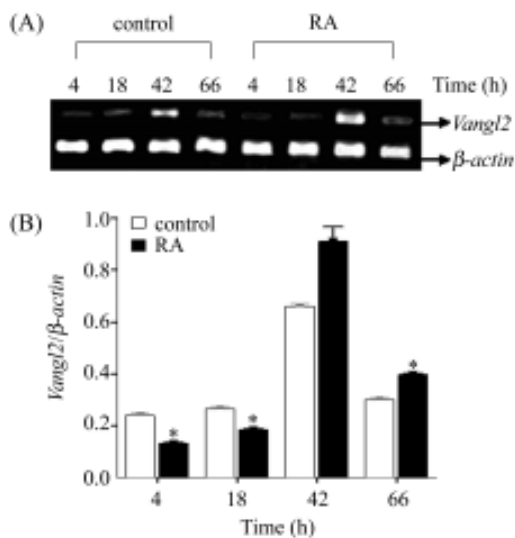


Fig. 6 Vangl2 mRNA expression in the neural tube tissue of vehicle- and retinoic acid (RA)-treated mouse embryos

Neural tube tissues were assayed by reverse transcription-polymerase chain reaction at 4, 18, 42 and 66 h after maternal treatment with a single oral dose of 30 mg/(kg body weight) of RA, or vehicle only. Representative gel (A) is shown over respective bar graph (B). Results are expressed as mean \pm SEM (n=5). *P<0.05.

fetuses after maternal treatment (Fig. 7). The pattern of Vangl2 protein in vehicle- and RA-treated groups was supported by their RT-PCR analysis. The level of Dishevelled2

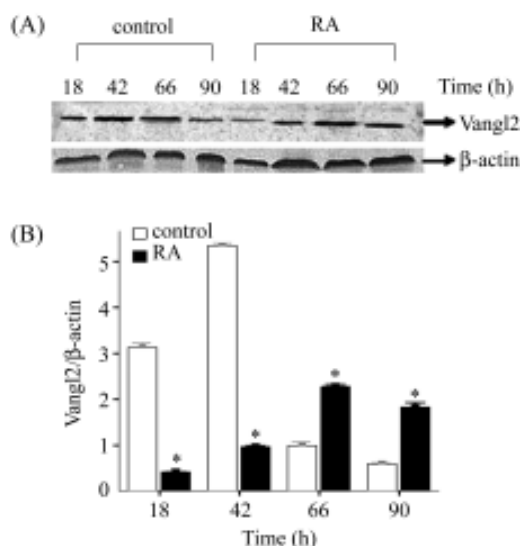


Fig. 7 Vangl2 protein expression in the neural tube tissue of vehicle- and retinoic acid (RA)-treated mouse embryos

Neural tube tissues were assayed by Western blot at 18, 42, 66 and 90 h after maternal treatment with a single oral dose of 30 mg/(kg body weight) of RA, or vehicle only. Representative gel (A) is shown over respective bar in graph (B). Results are expressed as mean \pm SEM (n=5). *P<0.05.

protein was high at 18 h and decreased continuously at 42, 66 and 90 h after vehicle treatment. RA treatment resulted in a significant decrease (P<0.05) at 18 h and a significant increase (P<0.05) at 42 h in Dishevelled2 protein in fetuses, and no significant difference was found at 66 or 90 h (Fig. 8).

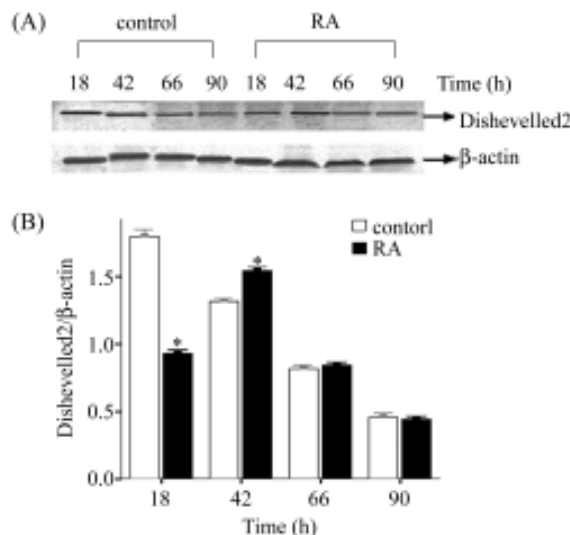


Fig. 8 Dishevelled2 protein expression in the neural tube tissue of vehicle- and retinoic acid (RA)-treated mouse embryos

Neural tube tissues were assayed by Western blot at 18, 42, 66 and 90 h after maternal treatment with a single oral dose of 30 mg/(kg body weight) of RA, or vehicle only. Representative gel (A) is shown over respective bar graph (B). Results are expressed as mean \pm SEM (n=5). *P<0.05.

Discussion

Our results indicated that all-trans-RA given at a dose of 30 mg/(kg body weight) at 7.75 d.p.c. resulted in NTDs of Kunming mouse embryos. The most common NTD was exencephaly. This was in accordance with the findings of Leonard *et al.* [20]. Many reports have revealed that the forebrain neuroepithelium and its derivatives can be morphologically and functionally affected by an excess of retinoids [23–27]. However, not all RA-exposed fetuses had NTDs in our study. This phenomenon occurred in another study [28], and it might be due to the disparity of conception time and the individual difference in sensitivity to RA. Earlier reports [29,30] revealed that small differences in retinoid disposition in mice might have significant effects on their teratogenic potential. In addition, we found that many RA-treated embryos were absorbed

between 10 and 16 d.p.c. The CNS, serving all organs, is the first to develop and its maldevelopment can cause damage to other emerging structures. Gardner, Seller and Kalousek reported that the particular specific disturbance of the neural tube could induce additional abnormalities [31,32]. In addition, the anatomic defect in the CNS influenced the commencement of breathing movements and fetal heart rate in defected fetuses [33,34]. These might play important roles in the resorption or death of the NTD fetuses during development at 10–16 d.p.c. The average weight, crown-rump length and tail length of the RA-treated fetuses were decreased significantly at 10 d.p.c., and had no significant change at 16 d.p.c. compared with that of the vehicle-treated fetuses. The lack of significant change might be due to the death of severely affected embryos at this time, whereas more mildly affected embryos were able to compensate for their earlier growth.

The level of *Dishevelled1/2/3* mRNA increased rapidly in the time just before the period of neural tube closure, and was critical for the genesis of the neural fold and initial neural tube closure, then decreased continuously. *Dishevelled2* mRNA and its protein matched each other and *Dishevelled1/2/3* mRNA and *Dishevelled2* protein were all expressed in the developing brain and spinal cord in vehicle-treated fetuses, which was in accordance with previous reports [18, 35–37]. This suggested that *Dishevelled* genes might take part in the genesis of the neural fold and initial neural tube closure during the nervous system development. RA treatment caused a significant decrease in the level of the *Dishevelled* gene in NTDs fetuses during the early neurula period, which was followed by a significant increase. Lijam *et al.* [38] created individual disruptions for each of the three murine homologs (*Dishevelled1*, 2 and 3) and found that, although mutants had reduced social interaction, they had morphologically normal brains, except that exencephaly was rarely found in *Dishevelled2* mutant animals. When *Dishevelled* double mutants were made by breeding together individual mutants, defects in neural tube closure were seen in almost all embryos with double inactivation, emphasizing redundant developmental roles for these genes [39]. It has been reported that *Dishevelled* genes participate in the determination of neuroectodermal cell fate [40] and CE [41] in the process of neural tube closure. However, the most common NTD induced by all-*trans*-RA at a dose of 30 mg/(kg body weight) at 7.75 d.p.c. was exencephaly. Cranial neurulation is not dependent on CE, and, even in the most severe of the PCP gene mutants, such as *Vangl2* [42], the neural tube closes from the midbrain rostrally. It is plausible that *Dishevelled* might take part in the terato-

genesis of RA by disturbing the determination of neuroectodermal cell fate.

Vangl2 mRNA and its protein matched each other and all increased rapidly in the period of neural tube closure in the vehicle-treated fetuses, then decreased rapidly, suggesting *Vangl2* might play a role in the process of neural tube closure. RA treatment first resulted in a marked decrease of *Vangl2* mRNA and its protein during the early neurula period compared with the vehicle-treated groups, followed by a significant increase. However, the peak of the *Vangl2* protein expression was delayed 24 h compared with the expression of *Vangl2* mRNA in RA-treated groups. A possible explanation is that RA treatment might perturb the translation of *Vangl2* mRNA.

Recent studies in *Xenopus*, zebrafish and mouse showed that *Vangl2* played a crucial role in regulating CE and axial elongation of the cell movement during the late gastrulation and neurula periods [43,44]. The inactivation of the *Vangl2* gene resulted in NTDs through a failure of convergent extension. However, as described above, exencephaly was the most frequent NTD induced by RA in our study, and cranial neurulation is not dependent on CE. Therefore *Vangl2* likely has other mechanisms that influence RA-induced NTDs. The rigidity conferred by adherens junctions is important for neural tube closure and the disruption of the normal adherens junctions results in a failure of complete cranial neurulation [45,46]. *Vangl2* was found to regulate the cytoskeleton at the level of the adherens junctions in the neural tube of the wild-type embryos [47,48]. So the misregulation of the *Vangl2* gene in RA-treated fetuses might impair the function of neural tube adherens junctions and take part in the teratogenesis of RA.

Vangl2 and *Dishevelled* genes are all core members of the PCP signaling pathway and they have been shown to interact physically [49]. The protein of *Vangl1* or *Vangl2* could interact with all three *Dishevelled* proteins, and this interaction involved in the cytoplasmic domain of *Vangl1/2* protein and C-terminal domain of *Dishevelled* proteins such as the DEP and PDZ domains [50]. However, *Dishevelled* and *Vangl2* genes displayed different temporal patterns in vehicle-treated groups and the peak value of mRNA expression was at 8 h in *Dishevelled* and 42 h in *Vangl2* after maternal treatment, hinting that *Dishevelled* and *Vangl2* mRNA might take part in neural tube closure through different mechanisms. In RA-treated groups, the peak value of mRNA expression was at 42 h in both *Dishevelled* and *Vangl2*.

In addition, we found that RA first acted as a repressor, then inducer of gene expression of *Dishevelled* and *Vangl2*,

the phenomenon that was described in a previous study [51] in which RA first repressed the expression of *RAR α* and *RAR β* then induced their expression. A possible explanation is that RA might depress the gene expression of *Dishevelled* and *Vangl2* through its depression of *RAR α* and *RAR β* gene expression, and the excessive depression of gene expression might mediate a feedback mechanism which results in an increased gene expression as compared to the vehicle-treated groups.

Embryonic growth and development entails differentiation, growth, apoptosis and morphogenesis in a highly coordinated environment. Any alterations in the interaction between genes controlling these critical events during embryogenesis can lead to an abnormal homeostasis and concomitant developmental defects. Our current study suggests that a misregulation of the temporal pattern of *Vangl2* and *Dishevelled* genes might participate in RA-mediated teratogenesis in Kunming mouse embryos.

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