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# Cell cycle-related genes *p57kip2*, *Cdk5* and *Spin* in the pathogenesis of neural tube defects

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## Research Highlights

(1) Genes that were differentially expressed in normal embryos and embryos with developmental neural tube defects at embryonic days 9.5 and 10.5 were compared using gene chip analysis, providing the first functional classification of differentially-expressed genes in neural tube defects.
(2) Genes related to apoptosis, signal transduction, transcription and translation regulation, protein synthesis and regulation, matrix and cytoskeletal proteins, energy and metabolism, and especially the cell cycle, were all involved in the pathogenesis of neural tube defects.
(3) Cell cycle-related genes including *p57kip2*, *Cdk5* and *Spin* were downregulated by retinoic acid, but upregulated in the normal neural tube.
(4) This study provides the basis for further research into the mechanisms underlying developmental neural tube defects, and for the prenatal screening and diagnosis of these defects.

## Abstract

In the field of developmental neurobiology, accurate and ordered regulation of the cell cycle and apoptosis are crucial factors contributing to the normal formation of the neural tube. Preliminary studies identified several genes involved in the development of neural tube defects. In this study, we established a model of developmental neural tube defects by administration of retinoic acid to pregnant rats. Gene chip hybridization analysis showed that genes related to the cell cycle and apoptosis, signal transduction, transcription and translation regulation, energy and metabolism, heat shock, and matrix and cytoskeletal proteins were all involved in the formation of developmental neural tube defects. Among these, cell cycle-related genes were predominant. Retinoic acid ment caused differential expression of three cell cycle-related genes *p57kip2*, *Cdk5* and *Spin*, the expression levels of which were downregulated by retinoic acid and upregulated during normal neural tube formation. The results of this study indicate that cell cycle-related genes play an important role in the formation of neural tube defects. *P57kip2*, *Cdk5* and *Spin* may be critical genes in the pathogenesis of neural tube defects.

## Key Words

neural tube defects; neurulation; gene chip; cell cycle; retinoic acid; regulatory factor; neural development; regeneration; neural regeneration

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**Conflicts of interest:** None declared.

**Ethical approval:** Experimental projects were approved by Animal Ethics Committee of Deyang People's Hospital in China.

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**Supplementary information:** Supplementary data associated with this article can be found in the online version by visiting www.nrronline.org.

## INTRODUCTION

Neural tube defects are a pathological outcome of developmental neural tube anomalies, and can occur at any stage from the formation of the neural tube to birth. Neurulation defects contribute to the formation of neural tube defects[1-2]. Gene expression and regulation are complex processes in the pathogenesis of neural tube defects, and existing studies of these defects have focused mainly on the key genes inducing neural tube defects[3-4] and the signal transduction pathways of the genes and their products[5]. Preliminary studies used gene sequence analysis to compare gene expression profiles in normal and retinoic acid-induced defective neural tubes before and after closure of the embryonic neural tube (embryonic-9.5 and 10.5 days in rats). The current study aimed to further classify the differentially- expressed genes and to identify key functional genes associated with neural tube defects and neurulation. The results will provide the basis for further studies of the genetic mechanisms involved in embryonic cell growth regulation during retinoic acid-induced neural tube defects.

## RESULTS

### Quantitative analysis of experimental animals

Sixty pairs of adult Kunming rats were used in this study. Sixty pregnant female rats were divided randomly into a retinoic acid group and a normal control group, with 30 rats in each group. Two pregnant rats in the retinoic acid group died of starvation after treatment, and the remainder were further assigned to either an embryonic 9.5 days (E9.5 days; $n$ = 16) or an embryonic 10.5 days (E10.5 days; $n$ = 12) group. Two rats in the normal control group were also excluded. A total of 56 pregnant rats were therefore suitable for final analysis. The effects of retinoic acid on rat embryos are shown in Table 1. The effect of retinoic acid on rat embryo malformation is shown in supplementary data 1 and supplementary Table 1 online, and rat embryo morphology is shown in supplementary Figure 1 online).

### Differential gene expression between normal rat embryos and embryos with neural tube defects

Gene sequencing scanning results identified 114 genes differentially expressed between normal embryos at E9.5 days and normal embryos at E10.5 days; 20 genes differentially expressed between normal embryos and embryos with neural tube defects at E9.5 days; and 28 genes differentially expressed between normal embryos and embryos with neural tube defects at E10.5 days (Figure 1).

Genes can be classified according to their functions into genes related to the cell cycle and apoptosis, signal transduction, transcription- and translation regulation, protein synthesis and regulation gene, matrix and cytoskeletal proteins, energy and metabolism, heat shock, and other functions. Among the differentially expressed genes in this study, cell cycle- and apoptosis- related genes predominated, including 32 genes in normal E9.5–10.5 days of embryos, nine in the neural tube defect group at E9.5 days and eight in the neural tube-defect group at E9.5 days. Cell cycle- and apoptosis-related genes accounted for 28% (32/114), 45% (9/20) and 29% (8/28) of all the differentially-expressed genes. Among these, *p57kip2*, *Cdk5* and *Spin* showed significantly different expression levels in normal and defective neural tube tissues; their expressional levels were upregulated during the formation of the normal neural tube (Cy5/Cy3 > 2.0), but downregulated in retinoic acid-induced neural tube defects (Cy5/Cy3 < 0.5; Tables 2–5, Figures 2–4).

### Northern hybridization

Northern hybridization analysis confirmed the microarray results. APC4 and Cdc25a expression levels were decreased in normal neural tube compared with defective neural tube, while *p57kip2* and *Cdk5* expression levels were upregulated. There were no significant changes in expression levels of Hsp60 and Rac1 (Figure 5, supplementary

Figure 2 online).

Table 1 Incidence [*n*(%)] of retinoic acid-induced neural tube defects in rat embryos

| Embryonic age (day) | *n* | Surviving embryos | Dead embryos | Absorbed embryos | Fetal deformity |
|---|---|---|---|---|---|
| 9.5 | 16 | 186(67.1) | 30(10.8) | 61(22.0) | 159(87.5) |
| 10.5 | 12 | 123(78.3) | 16(10.2) | 18(11.5) | 92(77.7) |

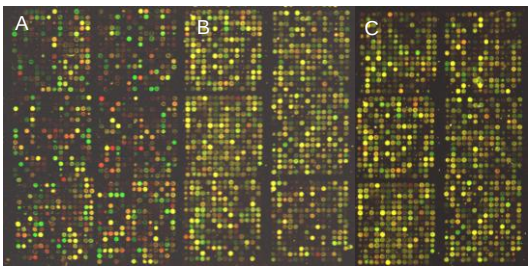


Figure 1 Gene sequence scanning image of the rat embryonic neural tissue.

Normal control group labeled with Cy3 markers (green); retinoic acid group labeled with Cy5 (red). If the Cy5 signal was stronger than the Cy3 signal, the two fluorescent signals were superimposed and stained red, otherwise they were stained green.

(A) Differentially-expressed genes in normal embryos at embryonic 9.5 and 10.5 days.

(B) Differentially-expressed genes in normal and neural tube defect embryos at embryonic 9.5 days.

(C) Differentially-expressed genes in normal and neural tube defect embryos at embryonic 10.5 days.

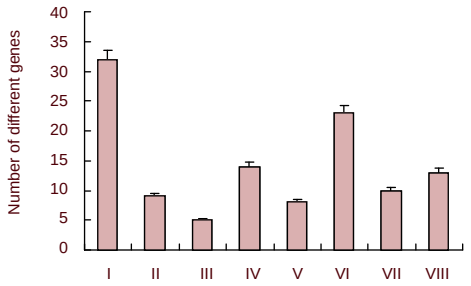


Figure 2 Functional distribution of differentially-expressed genes in neural tube tissues at embryonic days 9.5–10.5.

I: Cell cycle and apoptosis; II: energy and metabolism; III: heat shock and stress; IV: matrix and structural proteins; V: protein synthesis/regulation; VI: signal transduction; VII: transcription and chromosome; VIII: others.

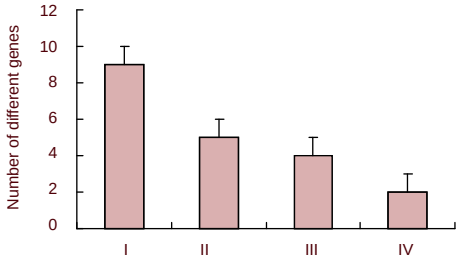


Figure 3 Functional distribution of differentially-expressed genes in normal and retinoic acid-treated embryos at embryonic day 9.5.

I: Cell cycle/apoptosis; II: energy/metabolism; III: signal transduction; IV: transcription/chromosome.

Table 2 Functional classification of differentially-expressed genes in normal neural tube tissues at embryonic days 9.5 and 10.5

| Functional classification | Expression | Differentially-expressed genes |
|---|---|---|
| Cell cycle and apoptosis | ↑ | Perp, Cdk5, p18, GADD45A, CCNB1, CCNB2, Spin, E2f6, Ywhag, p57, p57Kip2 |
| | ↓ | APC4, Api5, CDC5L, Ccnd2, MAP2K4, Cdc25a, Ccna2, PAL31, CCNE1, KNSL5, Cdc6, Casp8ap2, Ccnh, TACE, Cdk4, Rbbp7, Cenph, Mm.22417, Mm.27598, Mm.155228, M.Ccnf |
| Energy and metabolism | ↑ | Ldh1, Eno1, Cpt1a |
| | ↓ | Aldo1, Gdm1, Gstp2, FACL4, Abcc1b, Fth |
| Heat shock stress | ↑ | HSP60 |
| | ↓ | HSP86-1, HSP105, HSP84-1, Mt1 |
| Matrix and cytoskeleton protein | ↑ | Fasn, Anxa2 |
| | ↓ | CLTC, Nrf1, Timm8a, Add1, Vldlr, Fabp3, Dcn, Ero1l-pending, C1qb, Cryab, Cbg, Lrp1 |
| Protein synthesis and regulation | ↑ | — |
| | ↓ | PSMD1, Psma4, Poh1-pending, Nedd4a, Psmb2, Psmb4, Psma6, 4632426K06Rik |
| Signal transduction | ↑ | Pgk1, igf2r, Igfbp5 |
| | ↓ | Pl1, Macs, Ctsl, MAP4K5, SMAP, Ncor1, TAK1, Pld1, MAPK13, Igfbp3, Hint, Jun, Ctsz, Efs, Ctsd, MAP4K4, Gas1, Pla2g4, Nr2f1, Fyn |
| Chromosome and transcription | ↑ | Smarcc1, Nr2f2 |
| | ↓ | Hdac1, LOC63437, Mzf31, Tebp-pending, ldb1, Nfkbia, Bmp1, zac1 |
| Other | ↑ | Fosb, E2f6, Sox4, Hba-a1, COL1A1, Mglap, v-erb-a, Osf2-pending, PTH |
| | ↓ | Adfp, Dnaja1, Ptn, Zfp36 |

↑: Upregulation; ↓: downregulation; —: none.

Table 3 Functional classification of differentially-expressed genes in normal and retinoic acid-treated embryos at embryonic day 9.5

| Functional classification | Expression | Differentially-expressed gene |
|---|---|---|
| Cell cycle and apoptosis | ↑ | E2f6 |
| | ↓ | Son, Akt, TACE, Nek7, Rb1 ZW10, Ccnd2, Spin |
| Energy and metabolism | ↑ | apoa-4, Gpx1, Cpt1a, Hba-a1 |
| | ↓ | Csf3r |
| Signal transduction | ↑ | igf2r |
| | ↓ | Csf3r, Il1rak, IGFBP5 |
| Chromosome and transcription | ↑ | Fosb, Nr2f2 |
| | ↓ | — |

↑: Upregulation; ↓: downregulation; —: none.

Table 4 Functional classification of differentially- expressed genes in normal and retinoic acid-treated embryos at embryonic day 10.5

| Functional classification | Expression | Differentially-expressed gene |
|---|---|---|
| Cell cycle and apoptosis | ↑ | Rbbp7, kininogen |
| | ↓ | Ccng, Rb1, p57Kip2, ZW10, Nek7, Cdk5 |
| Energy and metabolism | ↑ | apoa-4, Psma1, Ldh1, Eno1 |
| | ↓ | — |
| Heat shock stress | ↑ | MT-II, Hsp60 |
| | ↓ | HSPF1 |
| Matrix and skeleton protein | ↑ | Pgk1-ps1 |
| | ↓ | Mdm2, Fasn |
| Signal transduction | ↑ | — |
| | ↓ | Ifngr2, Plcb3, PSMC6, IGFBP5, Csf3r |
| Chromosome and transcription | ↑ | Tpi |
| | ↓ | Smarcc1 |
| Others | ↑ | — |
| | ↓ | Sox4, Cnn2, Syt11 |

↑: Upregulation; ↓: downregulation; —: none.

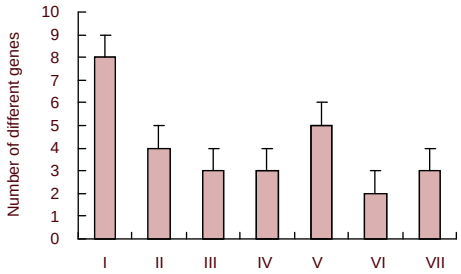


Figure 4 Functional distribution of differentially-expressed genes in normal and retinoic acid-treated embryos at embryonic day 10.5.

I: Cell cycle/apoptosis; II: energy/metabolism; III: heat shock/stress; IV: matrix/structural proteins; V: signal transduction; VI: transcription/chromosome; VII: others.

## DISCUSSION

There is currently a lack of evidence regarding the expression of genes regulating embryonic development in neural tube defects induced by retinoic acid. This study compared differentially-expressed genes in normal and affected embryos with neural tube defects at E9.5 and E10.5 days. Many genes were found to be involved in the development of neural tube defects, including genes with functions in the cell cycle and apoptosis, signal transduction, transcription and translation regulation, protein synthesis and regulation, matrix and cytoskeletal proteins, and energy and metabolism. Among these, cell cycle-related genes accounted for the largest proportion. These results provide the first evidence for the differential expression of *p57kip2*, *Cdk5* and *Spin*, the expression levels of which were down regulated after treatment with retinoic acid, but increased in normal neural tube.

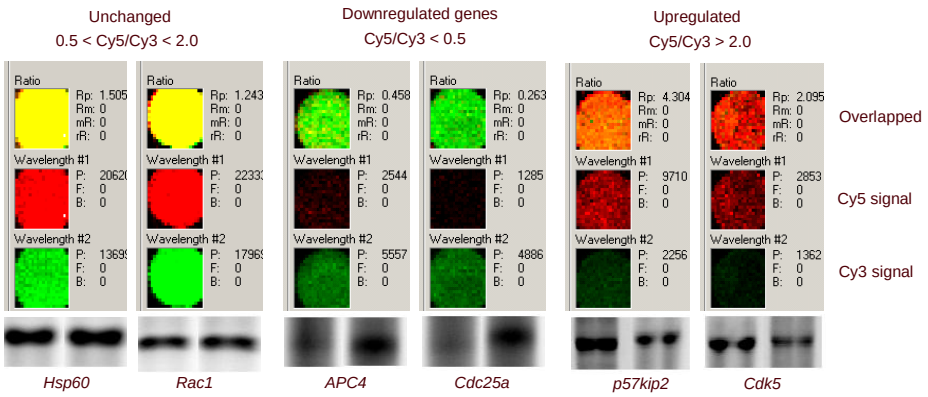


Figure 5 Northern blot hybridization verification.

Unchanged genes (*Hsp60* and *Rac1*) (left), downregulated genes (*APC4* and *Cdc25a*) (center) and upregulated genes (*p57kip2* and *Cdk5*) (right). Normal control group was labeled with Cy3 markers (green); retinoic acid group was labeled with Cy5 (red). If the Cy5 signal was stronger than the Cy3 signal, the two fluorescent signals were superimposed and stained red, otherwise they were stained green.

Table 5 Percentage of [*n*(%)] genes with different functions among the total differentially-expressed genes

| Functional classification | Normal embryonic 9.5 days *vs*. normal embryonic 10.5 days | Normal *vs*. neural tube defect | |
|---|---|---|---|
| | | Embryonic 9.5 days | Embryonic 10.5 days |
| Cell cycle and apoptosis | 32(28) | 9(45) | 8(29) |
| Energy and metabolism | 9(8) | 5(25) | 4(14) |
| Heat shock stress | 5(4) | 0 | 3(11) |
| Matrix and skeleton protein | 14(12) | 0 | 3(11) |
| Protein synthesis and regulation | 8(7) | 0 | 0 |
| Signal transduction | 23(20) | 4(20) | 5(18) |
| Chromosome and transcription | 10(9) | 2(10) | 2(7) |
| Other | 13(11) | 0 | 3(11) |
| Total | 114(100) | 20(1) | 28(100) |

Developmental neurobiology studies have demonstrated that accurate and ordered regulation of the cell cycle and apoptosis during neural tube formation are key factors influencing the formation of a normal neural tube. Eukaryotic cells must pass through two important restriction sites during the cell cycle: $G_1$-S phase to enter DNA synthesis, and $G_2$-M phase to enter mitosis. Cyclin combines with cyclin-dependent protein kinase (CDK) complexes[6], and its activation and inactivation contribute to the normal cell cycle. In addition, various internal and external environmental factors acting during the four phases of the cell cycle ($G_1$, S, $G_2$, M) may have an impact on related molecules and accordingly participate in the regulation of the cell cycle[7]. The main CDKs expressed at $G_1$ phase are CDK2, CDK4 and CDK6, and the expression of their binding cyclins (including cyclins D1, D2, D3 and C, E) is mediated by various mitogens. Type D cyclin is considered to be the most important regulator at $G_1$ phase, and its combination with CDK4 and CDK6 can promote the initial phosphorylation of retinoblastoma protein and suppress the disengagement of cyclin E, which combines with CDJ2 compound to achieve a transition from $G_1$ to S phase. Rb is thus an important substrate for cyclin/CDKs[8]. Activation of a variety of E2F transcriptional factors is required during this process, and these factors can induce the gene expression necessary for the S phase. The majority of E2F transcriptional factors are inactivated in early $G_1$ phase as a result of binding phosphorylation of retinoblastoma protein, and CDK phosphorylation of retinoblastoma protein can suppress the inhibition of E2F[9]. The transition from $G_2$ phase to M phase is mediated mainly by CDK1 and CDK2, and the substrates include cyclins A, B1, and B2. In addition, two CDK inhibitors are essential for the regulation of the cell cycle. CDK inhibitors can prevent CDK phosphorylation of phosphorylation of retinoblastoma protein, retard cells at $G_1$ phase, and even transform cells to $G_0$ phase. CDK inhibitors can be divided functionally into CDK4 inhibitors (including p17lnk4a, p15lnk4b, p18lnk4c, p19lnk4d) and KIP/CIP (including p21cip1, p27kip1, p57kip2)[10].

In the current study, we analyzed the differentially- expressed genes before and after the formation of normal neural tube and after retinoic acid-induced neural tube defects. The majority of differentially-expressed genes were associated with the cell cycle and apoptosis. The expression of a variety of positive regulators at $G_1$ phase was significantly reduced before and after the formation of the neural tube, including CCND2, CCNE1, CCNH, Rb1, CDK4 and CDC25A, while the expression levels of two cell proliferation inhibition factors, P18 (CDK4 inhibitor)[11] and *p57/kip2*, were increased. This suggests that the numbers of neural epithelial cells retarding at the $G_1$ phase were increased, and the proliferative capacity was decreased, as neural tube formation proceeded. In addition, the expression levels of cell cycle regulators of $G_2$-M transition, CDC5L, cdc25A, cyclinA, were also downregulated, indicating that the $G_2$-M phase transition was attenuated after neural tube formation. Cell cycle-related genes were shown to be the most differentially-expressed genes in embryos with retinoic acid-induced neural tube defects at E9.5 and E10.5 days. Downregulation of Rb1[12], Ccnd2[13], Nek7[14] and ZW10[15] suggest retardation at $G_1$ phase. In contrast to gene expression in the process of normal neural tube formation, some cell cycle-related genes, including *p57kip2*, *CDK5*, and *Spin*, which were upregulated in the normal neural tube, were downregulated after treatment with retinoic acid.

P57kip2 is an important inhibitor of $G_1$ phase regulators. Its combination with cyclinE-CDK2, cyclinD2-CDK4 and cyclinA-CDK2 can make it function as a negative regulator of cell proliferation to inhibit the cell cycle, as well as

a key regulator maintaining the non-proliferative status[16]. *p57kip2* expression is closely associated with cell apoptosis[17]. However, the role of *p57kip2* in the development of the nervous system remains unclear. When the normal neural tube is closed, CDK inhibitors such as *p57kip2* begin to be expressed, thus inhibiting the transition from $G_1$ to S phase, and DNA synthesis. This mechanism ensures the appropriate assembly of protein complexes with the right enzymatic activity in the right place at the right time, thereby regulating cell differentiation[18]. Although the mitotic division of neural progenitor cells was inhibited, their differentiation was promoted. After closure of the normal neural tube, some nerve cells exit from the mitotic cycle or escape from the cell cycle and become post-mitotic neurons; cell cycle inhibitory factors play an important role in maintaining the post-mitotic state. As the neural tube forms, the number of neural epithelial cells retarding at $G_1$ phase is increased significantly, while the proliferative capacity is decreased. Accordingly, *p57kip2* regulates the transcription and translation processes, thus regulating the cell cycle and controlling cell gene expression and protein structure[19]. The *p57kip2* gene was expressed in embryos with retinoic acid- induced neural tube defects at E9.5 and E10.5 days, but was down regulated. This suggests that retinoic acid may decrease the expression of *p57kip2* and promote cell transition from $G_1$ to S phase during the period of neural tube closure, thus interfering with the differentiation of neural progenitor cells and leading to neural tube defects. This is consistent with the findings of Ye *et al* [20-21], who found that *p57kip2* could modulate the differentiation of precursor cells and escape from the cell cycle, thus serving an important role in the cell proliferation and differentiation processes. However, the issue of whether the *p57kip2* mechanism during neurulation is changed under the influence of retinoic acid needs further investigations.

*CDK5* is a member of the cell cycle family, which is expressed specifically during the processes of nervous system development and differentiation. However, its role in cell division is poorly understood. Growing numbers of studies have focused on its regulation of post-mitotic cell function; for example, *CDK5*/*P35* can regulate microtubule-associated protein phosphorylation, promote migration, axonal growth and adhesion, and support cytoskeletal morphology during nerve cell development. In addition, *CDK5* gene knock-out mice showed significant nervous system disorders and embryonic defects[22], all of which indicate the significance of *CDK5* in central nervous system development. May previous studies have indicated a role for *CDK5* in the developing nervous system[23-26]. *CDK5* is involved mainly in post-mitotic neural cell migration and axonal growth, and its expression is gradually increased as cells escape from the cell cycle.

*CDK5* also participates in target-protein phosphorylation in multiple signaling pathways[27-29]. *CDK5* thus acts as a multifunctional protein. The present study detected *CDK5* gene expression both before and after the formation of normal neural tube and after retinoic acid-induced neural tube defects, and its expression levels were significantly upregulated after the formation of the neural tube, but significantly down-regulated in retinoic acid-induced neural tube defects. This evidence suggests that *CDK5* plays an important role in the occurrence of neural tube defects, and that the mechanism may involve retinoic acid-induced changes in the effects of *CDK5* on neural epithelial cells.

The Spindlin protein gene, *Spin*, is a spindle-associated protein that shows stage-specific expression at the early stage of embryogenesis[30]. *Spin* can combine with *Ssty* to form a new *Spin/Ssty* gene family. It is a member of the gametogenesis-specific gene family and is highly expressed in oocytes and early fertilized egg cells[31-32]. Spin is located in the cell nucleus and interacts with the spindle through identifying the methylation of H3K4[33] and Mos/MAPK pathway phosphorylation[30]. It thus plays an important role at metaphase, in spindle assembly and stability[34]. *Spin* over-expression may trigger mitotic disturbances and spindle defects, which can lead to chromosome instability, induce cellular senescence, multinucleation and apoptosis[35-36]. Analysis of *Spin* gene expression before and after normal neural tube formation, as well as after retinoic acid-induced neural tube defects, found that *Spin* expression was significantly increased after normal neural tube formation and decreased after retinoic acid treatment. Retinoic acid upregulated *Spin* gene expression and decreased resistance to cell apoptosis[37]. These effects lead in turn to tolerance of $G_2$-M phase cells to apoptotic signals, acceleration of cell proliferation, disruption of meiosis and fertilization, triggering of chromatid instability, disruption of embryo spindle formation, and the formation of aneuploid embryos.

In summary, numerous genes are involved in the pathogenesis of neural tube defects, among which cell cycle-related genes may play the most important role. *P57kip2*, *Cdk5* and *Spin* may be key cell cycle-related genes involved in the development of neural tube defects.

## MATERIALS AND METHODS

### Design

An open cDNA microarray analysis experiment.

### Time and setting

Experiments were performed from January 2006 to October 2007 at the Department of Neurobiology, the Third Military Medical University of Chinese PLA, China.

### Materials

A total of 120 Kunming rats (60 males and 60 females) of clean grade, aged 55–65 days, weighing 22–28 g, were provided by the Experimental Animal Center of the Third Military Medical University of Chinese PLA, China, with license number SCXK (Yu) 2007003. The experimental procedures complied with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China and the Council of the European Union (86/609/EEC) 1986-11-24.

### Methods

#### *Establishment of neural tube defect model*

Sixty pairs of adult Kunming rats were housed in cages at a ratio of one male to one female at 18:00, and were taken out at 8:00 the next day. Females were examined for vaginal plugs; appearance of a vaginal plug at 8:00 was defined as the first day of pregnancy (E0 day), while appearance at 16:00 was defined as E0.5 day.

The neural tube defect model was established according to the method of Klootwijk *et al* [38]. In brief, retinoic acid (Sigma, St. Louis, MO, USA) was dissolved in corn oil (40 g/L), and orally administered at 50 mg/kg per day to pregnant rats at E7.0–7.25 days, to produce the neural tube defect model. The normal control group was fed with corn oil alone.

#### *Specimen collection and gross morphological observations*

Pregnant rats were anesthetized by intraperitoneal injection of urethane and killed. Embryos were retrieved by caesarean section. Neural tube defects were confirmed by examination under a Leica dissecting microscope (Leica Microsystems GmbH, Wetzlar, Germany), and the placenta and fetal membranes were then isolated, and the embryo morphology was observed. The numbers of surviving and dead embryos were recorded, and the appearance and characteristics of embryonic head, face, spine and tail development were observed. Cerebral vesicle and spinal nerve tissues were obtained at each stage.

#### *Gene chip hybridization*

Total RNA was extracted and separated from normal and typical abnormal neural tube tissues using TRIzol reagent (Gibco-BRL, Gaithersburg, MD, USA). RNA sample concentrations were detected using a spectrophotometer, diluted and packaged at 3.3 μg/μL, 15 μL/tube (50 μg), and stored at –80°C. RNA 20 μg from each group was examined for purity by 1% agarose gel electrophoresis.

Total RNA (50 μg) was reverse transcribed into cDNA, which was labeled with 2 μL Cy3-dUTP (normal control group; Amersham Pharmacia, Hong Kong, China) or Cy5-dUTP (retinoic acid group; Amersham Pharmacia). The labeled cDNA was hybridized with a medium-density chip containing more than 1 100 genes (Genome Science and Technology Application Research Center, Department of Biology and Chemistry, City University of Hong Kong, China). After hybridization, microarrays were scanned with a ScanAr retinoid y4000 scanner (General Scanning, Watertown, MA, USA) and the scanned images were analyzed at Stanford University using ScanAlyze 2.51 software. The experiments were repeated three times.

The evaluation criteria for gene expression differences were as follows. (1) Ratio of gene expression intensity between the two groups (Cy5/Cy3) > 2 or < 0.5, indicating differential expression. A ratio < 0.5 indicated genes down regulated after retinoic acid treatment, while a ratio > 2 indicated upregulated genes. (2) Three experiments showing the same trend changes. (3) Two out of three experiments showing the same trend changes, and a third result that was not contradictory (Figure 6).

#### *Northern hybridization*

The reliability of the microarray hybridization results were validated by Northern hybridization for two up-regulated and two down regulated genes, and two genes with no significant difference in expression levels[39]. Total RNA 20 μg was subjected to formaldehyde denaturing gel electrophoresis, labeled using the capillary method[40], and purified using a QIAquick probe purification kit (Qiagen, Valencia, CA, USA). After the membrane was fully washed, the relative mRNA content was detected by autoradiography using photosensitive film. All sample films were again hybridized with actin cDNA probe (Incyte, Emeryville, CA, USA) as an internal reference. Finally the absorbance value was detected using an ImageScanner III scanner (GE, New York, NY, USA) and the hybridization signals were analyzed.

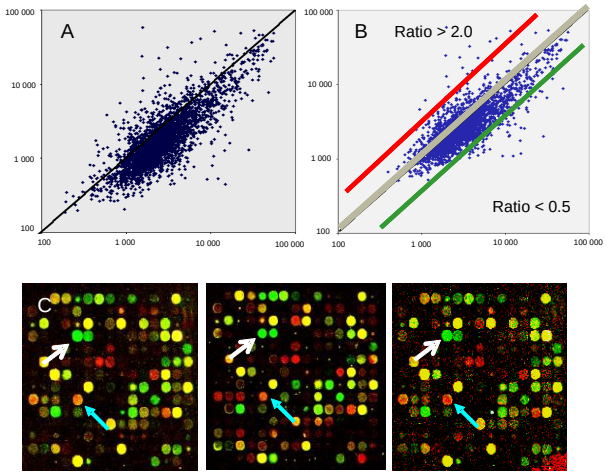


Figure 6 Evaluation of differential gene expression.

Genes demonstrating the same trend in three repeated experiments, or in two out of three experiments if the third results were not contradictory, were considered to show differential gene expression.

(A) Scatter plot of differentially-expressed genes.

(B) Ratios of gene expression intensity between the two groups. Cy5/Cy3 > 2 or < 0.5 indicated differential expression; < 0.5 indicated downregulation after retinoic acid treatment, and> 2 indicated upregulation after retinoic acid treatment.

(C) Cy3 stained green and Cy5 stained red. If the Cy5 signal intensity was stronger than the Cy3 signal, the two fluorescent signals were superimposed and stained red (blue arrows), otherwise they appeared green (white arrows).

### *Functional classification of genes*

The genes were classified and analyzed according to their known functions. The differentially-expressed genes were screened out and their functional mechanisms were investigated.

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