Polycomb group proteins are essential for spinal cord development

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1. ABSTRACT

Birth defects are the leading cause of infantile mortality, followed by neural tube defects (NTD) and congenital heart defects. Spina bifida and anencephaly are among the most common forms of NTD. NTD etiologies are complex, and are associated with both genetic and environmental factors. Polycomb group proteins are essential for vertebrate development; therefore, the purpose of this study was to determine the role of PcGs in spinal cord morphogenesis in normal and all-trans-retinoic acid (RA)-treated fetal rat models of spina bifida. Pregnant rats were gavage-fed RA, resulting in fetal NTD, and embryos were obtained on day 15.5, 17.5, and 19.5. Western blot and immunohistochemistry were used to reveal PcGs expression in the normal and RA-treated E15.5-19.5 rat sacral cords. Western blot and immunohistochemistry revealed decreased EED, RNF2, SUZ12, and H3K27me3 expression in the normal, E15.5-19.5, rat sacral cords. In addition, the spinal cord of RA-treated rats during embryonic development exhibited altered PcGs protein expression. Administration of excess RA results in NTD. Our results suggest that the Polycomb proteins may be involved in spinal cord development.

2. INTRODUCTION

Nervous system formation during embryonic vertebrate development is a complex process which requires precise coordination between cellular programs that control determination of proliferating neuronal progenitors and those that govern terminal differentiation of functional neurons (1,2).

Closure of the neural tube is one of the important early events in vertebrate central nervous system development. Defects in this closure process lead to a number of malformations, resulting in developmental problems for the fetus. Neural tube defects (NTD) are severe congenital malformations, often resulting in perinatal morbidity and mortality, because the neural tube fails to correctly form during embryogenesis. Many factors can induce NTD, including genetic, environmental, and nutritional factors (3).

If pregnant mothers come into contact with heavy metals, such as arsenic and copper, the risk of neural tube defects significantly increase. Evidence also suggests that many metabolic pathways, e.g., the folate pathway,

play a key role in influencing the risk of neural tube defects (4).

Retinoic acid (RA), known as the active metabolite of vitamin A, is an important signaling molecule for vertebrate embryos, in particular for central nervous system development. Furthermore, it has been demonstrated that RA induces differentiation in a variety of cells. RA also is a transcription factor, binding to the RA receptors RARS. However, administration of excess RA results in dramatic teratogenic effects to early central nervous system development, including neural tube defects and a multitude of abnormalities, such as spina bifida (5).

It has been demonstrated that many genes contribute to the risk of NTD (6). The polycomb group proteins (PcGs) have been shown to be part of the histone code for the histone modification (7). These proteins are considered to be transcriptional repressors, and Hox genes are their classical targets (8,9). PcGs consist of two multiprotein complexes known as 'Polycomb-Repressive Complexes' (PRCs): PRC1 and PRC2. These complexes contain histone H3 lysine (K) 27/9 and histone H1 K26 methyltransferase activity (10). PRC2 comprises EZH2, SUZ12, and EED, whereby EZH2 is a histone lysine methyltransferase (HKMT) targeting lysine residues on histone H3. The remaining PRCs are also essential for lysine residue targeting (11). In addition, EZH2 is essential for early mouse development (12). As a component of the PRC2 complex, EED is essential for proper anterior-posterior patterning of the primitive streak during early murine gastrulation (13). Mice lacking Suz12, Ezh2, or Eed are not viable and die during early post-implantation stages, displaying severe developmental and proliferative defects. Moreover, PRC2 is required for the regulation of proliferation and embryogenesis.

PRC1 comprises BMI1 and RNF2. Previous studies suggest that without PRC1 recruitment, PRC2 is not capable of inducing HKMT activity. Recently, studies have demonstrated that PRC1 exhibits ubiquitin E3 ligase activity, which can catalyze monoubiquitylation of histone H2A, and can also transcriptionally repress genes of PcG targets (14). Moreover, Rnf2 (Ring1b) deficiency results in gastrulation arrest.

Through genome-wide location analysis, the target genes of PRC1\PRC2\H3K27me3 have been determined to be embryonic growth and cell determining genes. Because PcG genes are essential for vertebrate development, we hypothesize that a relationship may exist between spinal cord development and PcGs.

3. MATERIALS AND METHODS

3.1. Establishment of a spina bifida rat model
Adult male and virgin, female, Sprague–Dawley rats (Experimental Animal Center, National Research Institute for Family Planning) were maintained on a 12-h light/dark cycle in environmentally controlled rooms. Timed-pregnant Sprague–Dawley rats were obtained by housing one female rat with a single male for 4 h.

Subsequent presence of a vaginal plug was regarded as impregnation, and this day was designated 0 dpc. At 10.5 dpc, the female rats received a single, oral dose of all-trans-RA (Sigma; 135 mg/kg .) in the morning, and were sacrificed at 5, 7, and 9 days after treatment. Surgical procedures were approved by the European Communities Council Directive of 24 November 1986 (86/609/EEC).

RA was dissolved in ethanol (10 mg/ml). However, fresh dilutions were prepared in olive oil (Sigma) on the day of use. Control animals received an appropriate volume of vehicle only. Subsequent to macroscopic and microscopic examination of the malformations, the embryos were resected in cold, phosphate-buffer saline, and the sacral cord was dissected. The sacral cords were separately pooled from each litter, rapidly frozen, and stored in -80°C until further use. Simultaneously, several embryos were fixed in formalin, and subsequently processed and embedded in paraffin.

3.2. Protein extraction and Western blot analysis
Protein extracts were prepared with lysis buffer containing 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 2 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Immunoblotting was performed with the following antibodies: goat anti-Suz12 (Santa Cruz Biotechnology), goat anti-Eed (Santa Cruz Biotechnology), mouse anti-Rnf2 (MBL), and rabbit anti-H3K27me3 (Upstate). A total of 50 μg tissue lysate was used for SDS-PAGE. The expression levels of ®-actin (Sigma) served as the loading control for Western blots. The blots were developed with a chemiluminescent substrate (GE). Primary antibodies were detected with anti mouse-HRP, anti goat-HRP, or anti-rabbit HRP (ZSGB), followed by ECL detection.

3.3. Immunohistochemical detection of H3K27me3 expression
Subsequent to deparaffinization, endogenous peroxidase was inhibited by incubating tissue sections for 30 minutes at room temperature in 0.3% H_2O_2, diluted in methanol. Antigens were retrieved by boiling for 10 minutes in citrate buffer (pH=6), followed by successive rinses in phosphate-buffered saline (PBS) containing 0.5% Triton (30 minutes), and in PBS only (3-5 minutes). H3K27me3 expression was detected using H3K27me3 rabbit polyclonal antibody (Upstate). Samples were incubated with biotinylated anti-rabbit goat IgG at room temperature for 30 minutes, followed by avidin-biotin horseradish peroxidase complexes for 30 minutes at 37°C. Diaminobenzidine served as the chromogen, and hematoxylin as the nuclear counterstain. Photographs were taken with a Nikon microscope.

4. RESULTS

4.1 PcG gene expression in the normal spinal cord
Because PcG genes are essential for early development, PcG gene expression was measured in the normal spinal cord at 15.5, 17.5, and 19.5 dpc. Western blot analysis revealed down-regulated levels of Eed, Rnf2, and Suz12 expression during embryonic development. The
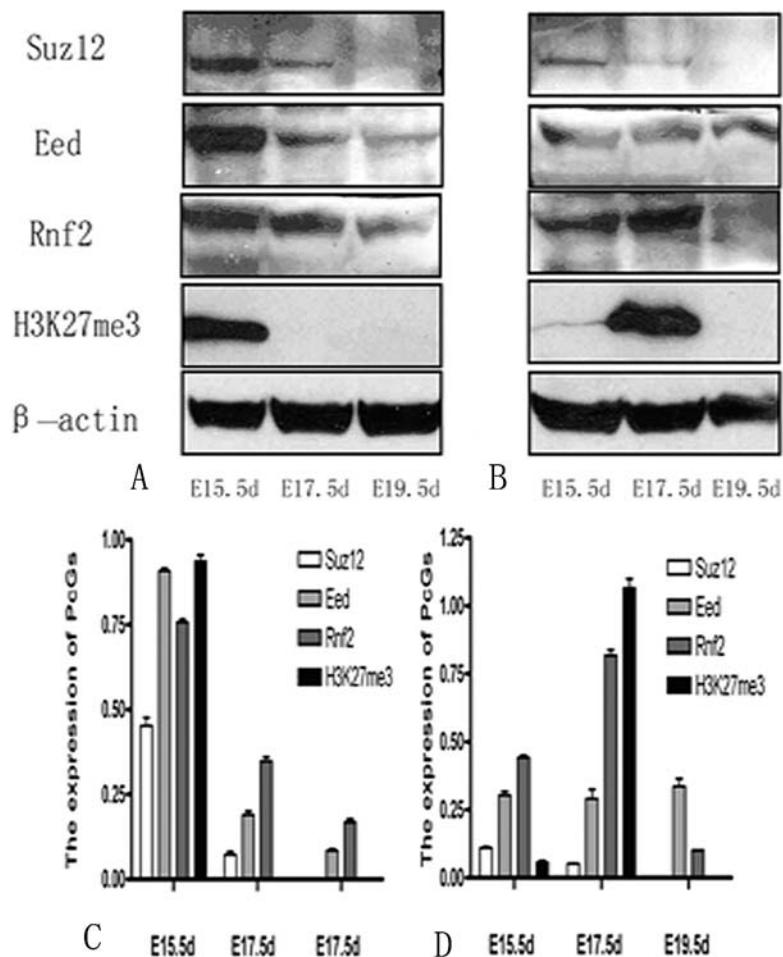
Polycomb group proteins are involved in the spinal cord development.

Figure 1. Expression of PcG genes and H3K27me3 in the spinal cord. (A) Western blot analysis of Suz12, Eed, Rnf2, and H3K27me3 expression in the normal embryonic spinal cord at 15.5, 17.5, and 19.5 dpc. Beta -actin served as a control. (B) Western blot analysis of Suz12, Eed, Rnf2, and H3K27me3 expression in the RA-treated spinal cord at 15.5, 17.5, and 19.5 dpc. Beta -actin served as a control. (C) The densitometric scanning result of Suz12, Eed, Rnf2, and H3K27me3 expression in the normal embryonic spinal cord at 15.5, 17.5, and 19.5 dpc. (D) The densitometric scanning result of Suz12, Eed, Rnf2, and H3K27me3 expression in the RA-treated spinal cord at 15.5, 17.5, and 19.5 dpc.

downstream co-target, H3K27me3, was also down-regulated. To verify these results, immunohistochemical analysis was performed, leading to similar results. In addition, H3K27me3 expression was higher in the dorsal part of sacral cord, compared with the ventral spinal cord and DRG. These results suggest that PcG genes have an influential role during normal spinal cord development.

4.2 PcG genes expression changes in the RA–treated spinal cord.

Western blot methodology was utilized to examine the expression of PcG genes in the spinal cord following RA-treatment. Compared to their expression in the normal spinal cord in different stages, the expression of PcG genes in 15.5 dpc is reduced, however, their expression in 17.5dpc is up-regulated. Finally, in the last stage, 19.5 dpc, it seemed that there had no change. Results also revealed that expression patterns of Eed, Rnf2, Suz12, and H3K27me3 during the spinal cord development were

altered, to view transversally, most genes exhibited a pattern firstly step up then step down. Consistent with these results, immunohistochemistry also demonstrated altered H3K27me3 protein expression. Taken together, these results reveal that PcG genes play an essential role in spinal cord development. In addition, we speculate that neural tube defects are associated with PcG gene expression.

5. DISCUSSION

NTD are the second most common form of severely disabling birth defects in the world. The incidence varies from 1/100 live births in certain regions of China to about 1/5000 live births in Scandinavian countries. This failure to properly neurulate comprises three principal forms: anencephaly, encephalocele, and spina bifida cystica. To date, many factors have been determined to result in NTD. Most NTD cases are thought to be a result of coordinated genetic predisposition and environmental

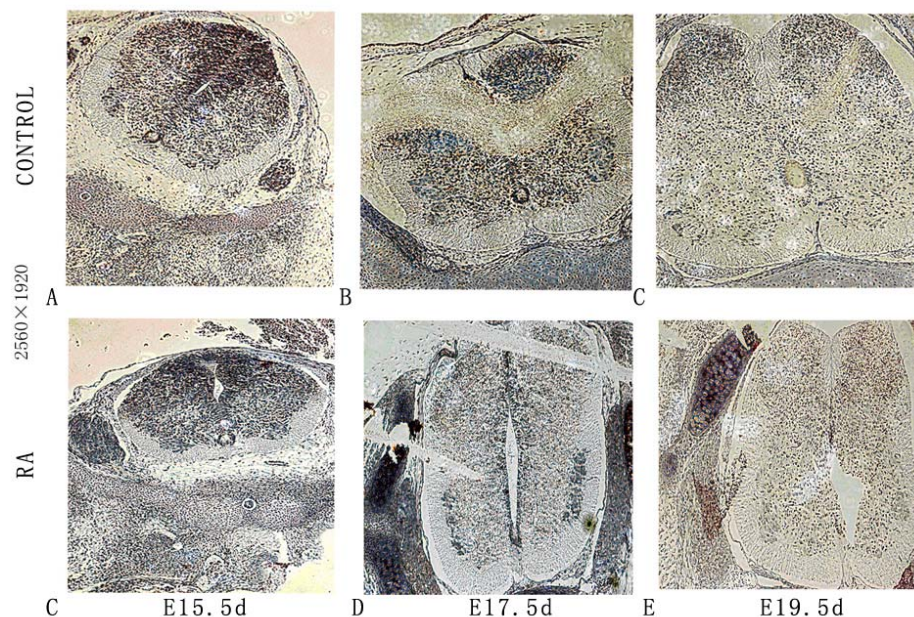**Polycomb group proteins are involved in the spinal cord development.**

Figure 2. H3K27me3 immunohistochemistry in the embryonic spinal cords of normal rats and RA-induced spina bifida rat model. (A-C) H3K27me3 down-regulation during embryonic development. H3K27me3 expression was greater in the dorsal sacral cord, compared with the ventral sacral cord and DRG. (D-F) Immunohistochemical detection of H3K27me3 expression in the RA-induced embryonic rats and not expressed in ventral sacral cord (white arrowhead) and DRG.

factors, such as heavy metals. In addition, many teratogens and nutritional deficiencies, such as folic acid deficiency, have been suggested as possible causative factors. Although research has determined many of the factors associated to NTD the underlying molecular mechanisms have not been elucidated.

PcG genes are a hot topic in the research field of vertebrate development. Studies have shown that many PcG genes, such as Suz12 and Eed, are essential during vertebrate embryonic development. The lack of PcG gene expression often results in malformation even death. Neural tube formation is considered to be a form of neural cell differentiation, with PcG genes playing a large role. Equally, PcG genes are indispensable for proper stem cell differentiation (15,16). According to results from previous studies of undifferentiated cells, PcGs bind to several genes, such as HOXA1 and ZIC1, and repress them. Upon differentiation induction, PRC1 and PRC2 are displaced by unidentified mechanism (s), which reverses the repression (17,18).

Results from the present study demonstrate a progressively decreased expression of PcG genes and H3K27me3 during normal spinal cord development. PRC1 and PRC2 become displaced from the nerve-specific genes, thereby promoting cell differentiation and brain development.

In addition, results demonstrated a possible relationship between NTD and PcGs, providing further understanding for the molecular mechanisms of NTD. Neural tube closure is a complex developmental process, as revealed by not only the original studies in human embryos, but by observations in a variety of experimental systems, including mouse, zebrafish, and chick embryos (19).

Therefore, the present study employed the use of a rat model of spina bifida, resulting from excessive RA action, to investigate PcG gene expression in the normal spinal cord, as well as the distribution of H3K27me3 expression. In normal animals, PcG expression levels, such as EED and SUZ12, decreased during normal spinal cord development. In contrast, following RA treatment, PcG expression levels were altered, they fistly increased then decreased, thus, confer to the normal spinal cord, they are higher in 17.5 dpc, lower in 15.5 dpc, as same as 19.5 dpc.

Taken together, we conclude that PcG genes are important for embryonic vertebrate development, and are closely associated with neural tube defects. Further studies are needed to understand the mechanisms involved in the regulation of PcG genes with regard to NTD.

6 ACKNOWLEDGEMENTS

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Abbreviations: NTD: neural tube defects ; RA: all-trans-retinoic acid; PcG : polycomb group ; EED: embryonic ectoderm development; SUZ12: suppressor of zester12 ; RNF2: ring finger protein 2; HKMT: histone lysine methyltransferase; PRC: polycomb-repressive complexes; EZH2: enhancer of zeste.

Key Words: Spina bifida, Neural tube defection, Retinoic acid, Polycomb group genes, Rat

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