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Original Article

Molecular mechanism of Hoxd13-mediated congenital malformations in rat embryos

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Abstract: Objective: To investigate the molecular mechanism of Hoxd13-mediated congenital malformations in rat embryos. Methods: SD female rats were mated with male rats in a 1:1 mating scheme. Thirty pregnant female rats were randomly divided into three groups: the control group receiving a normal diet, the model group receiving a vitamin A-deficient diet, and the treatment group receiving a vitamin A-deficient diet supplemented with pcDNA-Hoxd13. The expression of Hoxd13 mRNA and protein in normal embryonic tissue and congenital malformations was determined by RT-PCR and Western blot analysis. At day 20, rats were dissected, and the fetal weight, body and tail length, and the number of live births, absorbed fetus, and stillbirth in each group were recorded. Wnt and Slim1 expression was detected by RT-PCR and Western blot analysis. β-catenin and c-myc expression was also quantified by Western blot analysis. Results: The expression of Hoxd13 mRNA and protein in congenital malformations was significantly lower compared with normal embryonic tissue (P<0.01). The administration of exogenous Hoxd13 in the treatment group markedly increased the fetal weight, body and tail length (P<0.05), improved the embryonic survival rate, and reduced the embryonic resorption rate and stillbirth rate (P<0.05). Exogenous Hoxd13 markedly promoted the expression of Wnt2, Wnt5a, Wnt7b and Slim1 protein and mRNA (P<0.01), and the expression of β-catenin and c-myc protein in congenital malformations (P<0.01). Conclusion: Hoxd13 expression was decreased in rat embryos with congenital malformations. The administration of exogenous Hoxd13 alleviated fetal malformation probably through stimulation of Slim1 expression and Wnt/β-catenin signaling pathway.

Keywords: Congenital malformations, Hoxd13, Wnt/β-catenin signaling pathway

Introduction

Embryonic development involves the tightly coordinated activities of a large number of genes, each switched on at a specific time and place in the growing organism under the strict control of promoters. Hox genes, also known as homeotic genes, are a group of 180-base-pair highly conserved DNA sequences that are crucial for the embryonic development. Each Hox gene encodes a 61-amino acid protein domain containing a helix-turn-helix (HTH) structure that specifically binds to the TAAT box within the DNA, and regulates the expression of downstream genes [1]. Hox genes are primarily expressed in the embryonic ectoderm such as the central nervous system, and the mesoderm such as the lungs, intestines, kidneys, etc. While Hox genes present at the 3' end are expressed during early embryonic stages, thereby promoting the development of the proximal end of body axis by stimulating the proliferation and migration of cells, Hox genes at the 5' end are expressed during late embryonic stages, and regulate the development of the distal end of body axis by promoting the differentiation and apoptosis of cells. During embryonic development, abnormalities in the expression or structure of Hox gene often cause fetal malformations in central nervous system, lungs, kidneys, heart, limbs, urinary and reproductive system [2-6].

The Hox gene family is composed of 39 genes that are divided into four clusters, namely Hoxa, Hoxb, Hoxc and Hoxd. It has been previously reported that abnormalities in Hoxd-13 expression are closely associated with fetal malforma-

Table 1. Primers used in RT-PCR

Gene	Primer (5'-3')	bp
Hoxd13	For: GTACCCAGGGACCTCTGAGC	384
	Rev: ACGTGCTGATAGGGACTCGT	
Slim1	For: ATTGCGTGAAGTGCAACAAG	244
	Rev: CCTTCATAGGCCACCACACT	
Wnt3	For: CCATCCTCTGCCTCAAATTC	74
	Rev: TGGACAGTGGATATAGCAGCA	
Wnt5a	For: CATTGGAGAAGGCGCGAAGAC	644
	Rev: AGTCCGGACTTGGGTCGATGT	
Wnt7b	For: AGAAGCAAGGCTACTACAACCA	155
	Rev: TGCCTCATTGTTGTGAAGGT	
β-actin	For: GTCGTACCACTGGCATTGTG	291
	Rev: CTCTCAGCTGTGGTGGTGAA	

tions. The expression of Hoxd13 is down-regulated in ethylene thiourea-induced anorectal malformations in fetal rats, especially in the rectal epithelial cells [7]. Hoxd13 expression is also reduced in 12.5-day fetuses with congenital clubfoot [8, 9]. Additionally, Hoxd13 mutations can cause varying degrees of hand-foot-genital syndrome during embryogenesis [10].

Vitamin A contains retinol structure, and is essential for the growth and differentiation of normal cells, as well as the maintenance of body's normal activities. Its deficiency can lead to blindness, and atrophy of testis, ovary and other organs. During embryonic development, appropriate amount of vitamin A vital for the normal growth of embryo. Animal studies have confirmed the teratogenic effects of vitamin A deficiency on embryo [11, 12]. It has also been reported that retinoid drugs taken before or during pregnancy cause fetal malformations [13], revealing that vitamin A deficiency can lead to embryo congenital malformations.

In this study, a rat model of congenital malformations was constructed by vitamin A deficiency to explore the molecular mechanism of Hoxd13-mediated congenital fetal malformation in rats.

Materials and methods

Animals

Healthy adult SD male (n=60) and female (n=60) rats weighing about 200±20 g each were purchased from Slac Laboratory Animal LLC. (Shanghai, China, the certificate No.: SCXK (Shanghai) 2012-0002). Female and male rats were raised at 23±2°C in separate animal rooms with free assess to food and water. Female rats were mated with male rats in a 1:1 mating scheme at the same animal room at 6 pm on each day, and vaginal cuff smear test was performed the next day until sperms were detected confirming the pregnancy. A total of 30 pregnant female rats were randomly divided into three groups, the control group receiving a normal diet, the model group receiving a vitamin A-deficient diet, and the treatment group receiving a vitamin A-deficient diet supplemented with pcDNA-Hoxd13.

Reagents and instruments

Rabbit anti-Wnt3, Slim1, c-myc, and GADPH antibodies were purchased from Epitomics Inc. (Burlingame, CA, USA). Rabbit anti-Wnt5a, Wnt7b, and β-catenin antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). The mini-vertical electrophoresis system and mini electrophoretic transfer cell were manufactured by Sixty-one Instrument Inc. (Beijing, China). The ChemiDocTM XRS gel imaging system was purchased from Bio-Rad Inc. (Hercules, CA, USA).

Measurement of embryonic growth

Rats were sacrificed at 20 days after pregnancy. Fetuses were carefully removed and the number of normal fetus, absorbed fetus, and stillbirth was recorded. The fetal weight, body length, and ail length was measured.

RT-PCR

Total RNA was extracted from the tissues using the Trizol extraction kit (Invitrogen, Carlsbad, CA, USA) following the manufacture's instruction. RNA was reverse transcribed into cDNA using one-step RT-PCR kit and amplified by PCR using the following primers (**Table 1**). The reaction conditions were as follows: 35 cycles of 94°C denaturing for 45 s, 59°C annealing for 45 s, and 72°C extension for 60 s. A total of 5 μL of the amplification product was subjected to electrophoresis in a 2% agarose gel. The electrophoretic bands were detected under UV spectrophotometer and photographed. The relative expression level of each mRNA was calculated as a ratio to the average value of the housekeeping gene GADPH.

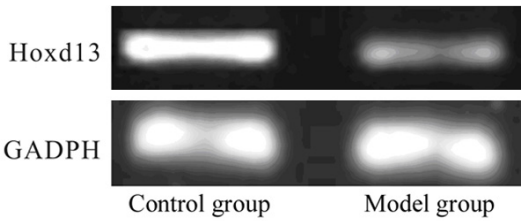

Figure 1. RT-PCR analysis of Hoxd13 mRNA expression in normal embryonic tissue and vitamin A deficient group.

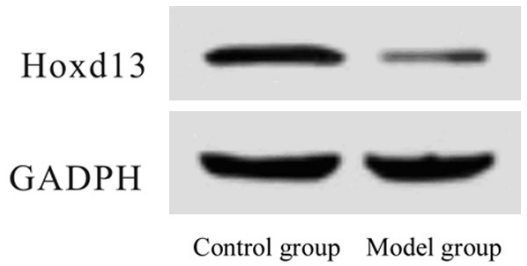

Figure 2. Western blot analysis of Hoxd13 protein in normal embryonic tissue and vitamin A deficient group.

Western blot analyses

Appropriate amount of RIPA lysis buffer was added to embryonic tissues in each group and mixed by votexing for 30 s once every 10 min. After 40 min, the mixture was centrifuged at 10600 × g for 10 min at 4°C. The supernatant was transferred to new EP tubes, and the concentration of total protein was determined using a BCA kit according to the manual. The protein sample was separated by SDS-PAGE electrophoresis and transferred to polyvinylidene difluoride membranes. The membrane was blocked in TBS buffer containing 5% skim milk and 0.1% Tween20 at room temperature for 1 h and incubated with the appropriate primary antibody overnight at pH 7.6 at 4°C with gentle shaking. The peroxidase-labeled secondary antibodies were added and the membranes were incubated at 37°C for 1 h. the membrane was rinsed, treated with ECL detection reagent. The intensity of bands was detected by a Molecular Imager® ChemiDocTM XRS System (Bio-Rad Laboratories Inc, Hercules, CA, USA). The gray value of bands was analyzed by Quantity one (Bio-Rad Laboratories Inc). The relative expression level of each protein was calculated as a ratio to the average value of the housekeeping gene GADPH.

Statistical analysis

Data were presented as mean ± standard deviation and analyzed using SPSS17.0 software. Differences between groups were compared by t tests. *P* values smaller than 0.05 were considered statistically significant.

Results

Comparison of Hoxd13 expression

As shown in **Figure 1**, the expression level of Hoxd13 mRNA in the model group receiving vitamin A deficient diet (0.21±0.04) was significantly lower compared with normal rat embryonic tissues (0.78±0.12, $P<0.05$). The expression level of Hoxd13 protein in the model group (0.20±0.03) was also significantly lower than that in normal rat embryonic tissues (0.88±0.13, $P<0.05$, **Figure 2**), revealing a reduced Hoxd13 expression in rat embryos with congenital malformations.

Effect of Hoxd13 on embryonic development

As shown in **Table 2**, the fetal weight, body length, and tail length in the model group receiving vitamin A deficient diet was significantly lower compared with normal embryos in the control group ($P<0.05$). The fetal weight, body length, and tail length in the treatment group receiving exogenous Hoxd13 was significantly increased compared with the model group ($P<0.05$), suggesting the retarded embryonic development in the model group was markedly improved by the treatment of exogenous Hoxd13.

Effect of Hoxd13 on the embryonic resorption rate and stillbirth rate

As shown in **Table 3**, the embryonic resorption rate and stillbirth rate in the model group was significantly higher than those in normal embryos in the control group ($P<0.05$), whereas the rates in the treatment group was significantly reduced compared with the model group ($P<0.05$), indicating that the live birth rate in the model group was significantly improved by the treatment of exogenous Hoxd13.

Effect of Hoxd13 on the expression of Slim1 mRNA and protein

As shown in **Figures 3** and **4**, the expression level of Slim1 mRNA and protein in the model

Table 2. Effect of Hoxd13 on embryonic development ($\bar{x}\pm s$)

Group	Number	Fetal weight (g)	Body length (cm)	Tail length (cm)
Control group	10	3.785±0.421	3.420±0.389	1.478±0.129
Model group	10	3.228±0.239[a]	3.052±0.417[a]	0.956±0.137[a]
Treatment group	10	3.567±0.209[b]	3.462±0.376[b]	1.312±0.205[b]

Note: [a]*P*<0.05, compared with the control group; [b]*P*<0.05, compared with the model group.

Table 3. Effect of Hoxd13 on the embryonic resorption rate and stillbirth rate ($\bar{x}\pm s$)

Group	Number	Embryonic resorption rate (%)	Stillbirth rate (%)	Live birth (%)
Control group	10	3.6 (3/83)	0	96.4 (80/83)
Model group	10	51.3 (41/80)[a]	37.5 (30/80)[a]	11.2 (9/80)[a]
Treatment group	10	19.0 (15/79)[b]	19.0 (15/79)[b]	64.0 (49/79)[b]

Note: [a]*P*<0.05, compared with the control group; [b]*P*<0.05, compared with the model group.

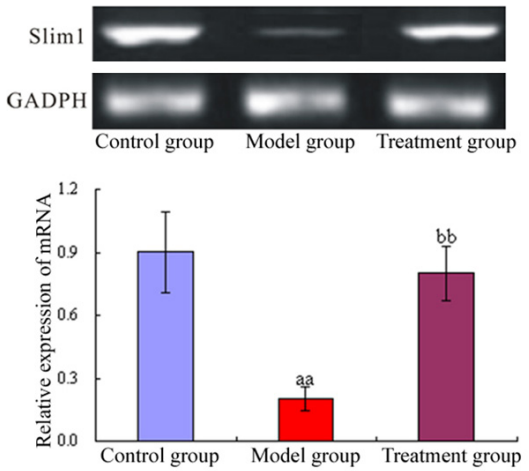

Figure 3. RT-PCR analysis of Slim1 mRNA expression in control, model and treatment groups. Note: [aa]*P*<0.01, compared with the control group; [bb]*P*<0.01, compared with the model group.

group was significantly lower than those in the control group (P<0.01), whereas the expression in the treatment group was significantly increased after the treatment of exogenous Hoxd13 (P<0.01).

Effect of Hoxd13 on the expression of Wnt mRNA and protein

The expression level of Wnt mRNA and protein in the model group was significantly lower than those in the control group (P<0.01), whereas the expression in the treatment group was significantly increased compared with the model group (P<0.01, **Figures 5** and **6**).

Effect of Hoxd13 on the expression of β-catenin and c-myc

As shown in **Figure 7**, the expression level of β-catenin and c-myc in the model group was significantly lower than those in the control group (P<0.01), whereas the expression in the treatment group was significantly increased after the treatment of exogenous Hoxd13 (P<0.01).

Discussion

Previous studies have confirmed that vitamin A is vital for the embryonic development, and its deficiency during pregnancy may induce a wide range of congenital malformations, known as vitamin A deficiency syndrome, which involve multiple organs of the body including eyes, heart, lungs, kidneys, urogenital tract, and limbs [11, 12]. Clagett-Dame et al. has identified a window period when the demand for vitamin A is substantially increased during embryonic development [14]. If vitamin A is lacking during the window period, ftal abnormalities of the central nervous system and cardiovascular system and even stillbirth may occur. In this study, a rat model with congenital malformations was successfully constructed by feeding the pregnant animals with vitamin A deficiency diet. It was found that the fetal weight, body length, and tail length in the model group was markedly reduced, whereas the embryonic resorption rate and stillbirth rate were found to be increased.

Both human and murine Hox gene family consists of 39 genes that are divided into four clusters, namely Hoxa, Hoxb, Hoxc and Hoxd. Hox genes have been known to play an important regulatory role during embryonic morphogenesis. While Hoxal knockout leads to the deletion of r4 and r5 of embryonic hindbrain [15], Hoxa2 knockout affects the development of the backside of rhombomeres r2/r3 [16]. Hoxd13 gene is expressed in late embryonic stage and in several types of tissues in adult mice including

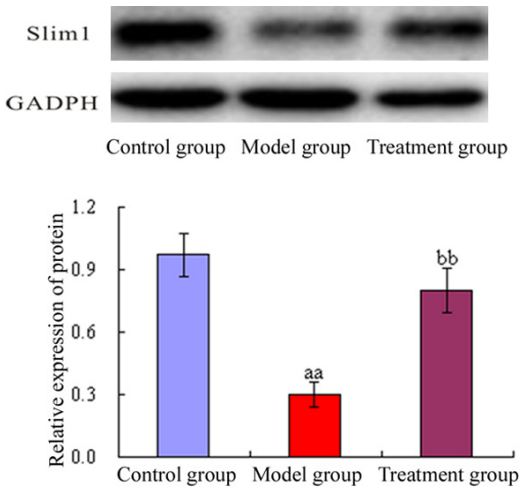

Figure 4. Western blot analysis of Slim1 mRNA expression in control, model and treatment groups. Note: $^{aa}P<0.01$, compared with the control group; $^{bb}P<0.01$, compared with the model group.

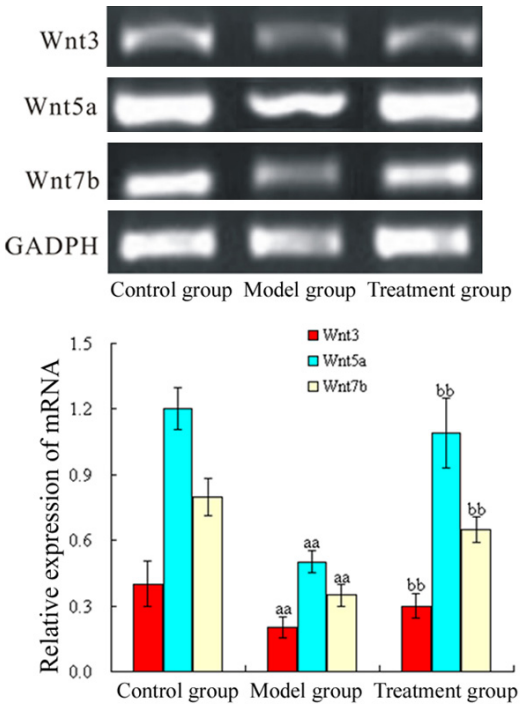

Figure 5. RT-PCR analysis of Wnt mRNA expression in control, model and treatment groups. Note: $^{aa}P<0.01$, compared with the control group; $^{bb}P<0.01$, compared with the model group.

prostate [17], urinary tract, and genital tubercle [18]. The expression of Hoxd13 is down-regulated in ethylene thiourea-induced anorectal malformations in fetal rats, especially in the rectal epithelial cells [7]. Hoxd13 expression is also

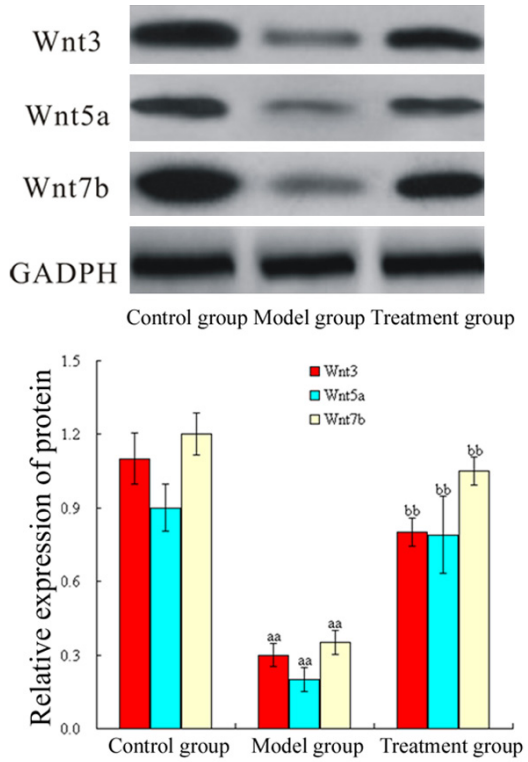

Figure 6. Western blot analysis of Wnt protein expression in control, model and treatment groups. Note: $^{aa}P<0.01$, compared with the control group; $^{bb}P<0.01$, compared with the model group.

reduced in 12.5-day fetuses with congenital clubfoot [8, 9]. In addition, Hoxd13 mutations may cause varying degrees of hand-foot-genital syndrome during embryogenesis [10]. Hoxd13 knockout may induce phenotypic changes in vascular endothelium, the loss of vascular integrity, and even placental edema [19]. Consistent with these findings, our results showed that Hoxd13 expression was decreased in embryonic tissues with congenital malformation, whereas exogenous Hoxd13 significantly improved the fetal abnormities, suggesting a strong association between Hoxd13 and congenital malformation.

The Slim1 gene is a located on chromosome Xq36. As a developmentally regulated protein, Slim1 is expressed in high levels in skeletal and cardiac muscle tissue, and closely associated with the proliferation and migration of skeletal muscle cells during embryonic development. Previous studies have shown that the expression of Slim1 in mouse fibroblasts transfected with Hoxd13 expression vector is significantly increased [20]. In this study, it was also shown

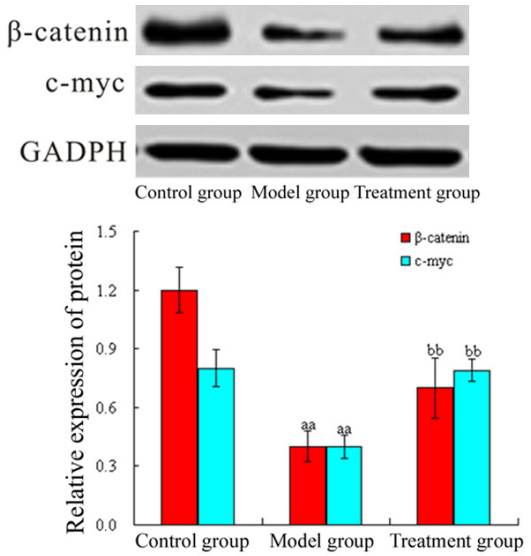

Figure 7. Western blot analysis of β-catenin and c-myc expression in control, model and treatment groups. Note: $^{aa}P<0.01$, compared with the control group; $^{bb}P<0.01$, compared with the model group.

that the expression of Slim1 in embryonic tissues was positively correlated with the level of Hoxd13.

Wnt signaling was first identified for its function in carcinogenesis, but has since been recognized for its role in embryonic development. Wnt signaling pathway is highly conserved in animals and controls a wide range of biological processes. The Wnt/β-catenin pathway is the canonical Wnt pathway that causes an accumulation of β-catenin in the cytoplasm and its eventual translocation into the nucleus to act as a regulator for the transcription of downstream genes such as c-myc, the embryonic development, as well as the proliferation, differentiation and apoptosis. Abnormal Wnt signaling during embryonic development often leads to developmental defects and even embryonic death [21]. Deletion of Wnt3 in mouse embryos results in the absence of formation of primitive streak, mesoderm and nodules, undifferentiated ectodermal cells, and the development of neural tube without front and rear axles [22, 23]. Wnt5a-null mouse embryos display maldevelopment of upper and lower jaw, limbs, tail and genitals, as well as abnormal A-P body axis extension [24]. Wnt5a and Wnt7b regulate the development of mouse placenta, and affect the development of the body of vertebrate [25-27]. The placenta of homozygous Wnt7b knockout mice develops abnormally, leading to fetal death in mid-pregnancy [28]. It has been known that the Wnt/β-catenin pathway activates the Hox gene family including Hoxd4 etc. through TCF/LEF [29]. Both Hoxd13 and Wnt5a were expressed in low levels in the uterus of infertile mutant female rats [30], suggesting a strong association between Hox gene and the Wnt/β-catenin pathway. Our results revealed a reduced expression in Wnt including Wnt 3, 5a and 7b, and β-catenin. Moreover, the Wnt/β-catenin pathway was shown to be markedly activated by the treatment of exogenous Hoxd13. Additionally, the Wnt/β-catenin pathway may affect the embryonic development through regulating the expression of the downstream gene c-Myc [31]. Consistent with previous study, our results demonstrated that the expression of c-Myc in rat embryo with congenital malformations was significantly reduced, whereas the expression was substantially increased by the treatment of exogenous Hoxd13.

In summary, the expression level of Hoxd13 was decreased in rat embryos with congenital malformations. The administration of exogenous Hoxd13 alleviated the fetal malformation probably by promoting the expression of Slim1 and the activation of Wnt/β-catenin signaling pathway.

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Disclosure of conflict of interest

None.

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