

***BMP4* knockdown of NCSCs leads to aganglionosis in the middle embryonic stage**

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Abstract. Transplacental bone morphogenetic protein (BMP)4 RNA interference (RNAi) is a technique used to knockdown genes in embryos. BMP4 are essential for the development of nervous system in the differentiation of neural crest stem cells (NCSCs). The failure of differentiation and migration of NCSCs may lead to aganglionosis. In the present study, pregnant mice were divided into three groups: Ringer's group, pSES group and RNAi-*BMP4* group. In order to silence the *BMP4* gene in the first generation (F1), 11.5 day pregnant mice were injected with the small interfering RNA *BMP4* plasmid, pSES or Ringer's solution via the tail vein. Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) and western blotting were employed to ensure the downregulation of *BMP4*. Finally, X-rays were performed following a barium enema. Aganglionosis was diagnosed by general anatomy and immunohistochemistry. Compared with the control group, transplacental RNAi was able to downregulate the *BMP4-Smad4* of 11.5 day embryos, as determined by semi-quantitative RT-PCR and western blotting. The megacolon of the mice were demonstrated by X-ray and confirmed by general anatomy. Aganglionosis of colonic mucosa and submucosa were diagnosed by pathology, and immunohistochemistry. Knockdown of *BMP4* in pregnant mice at the middle embryonic stage led to aganglionosis. It was therefore demonstrated that *BMP-Smad* was essential to the NCSCs of middle stage embryos. *BMP-Smad* served important roles in the generation of aganglionosis. This technique of knockdown

BMP4 gene may be used to establish an aganglionosis mouse model.

Introduction

Aganglionosis or Hirschsprung's disease (HD) is a congenital intestinal dynamic disorder characterized by intestinal submucosal and myenteric plexus parasympathetic ganglion cell loss, leading to persistent diseased colonic convulsions, and contractions in addition to the obstruction of intestinal contents (1-4). This disease is a colonic motor disorder with an incidence of 1:5,000 live births worldwide (5-7). In previous studies, the failure of neural crest stem cells (NCSCs) to differentiate and migrate was demonstrated to be associated with the poor development of the enteric nervous system, and identified as the main cause of aganglionosis (8,9).

Bone morphogenetic proteins (BMPs) are among the most important proteins involved in the development of the enteric nervous system (8-12). BMP/Smad signalling serve a key role in NCSC migration at an early embryonic stage, while NCSC differentiation occurs during the middle embryonic stages (8,11). Eventually, BMP/Smad signalling determines the function of the intestinal nervous system (10-12). The current studies were designed to investigate the effects of the downregulation of *BMP4* gene expression in NCSCs using a transgenic mouse model in which low expression level of the *BMP4* gene was governed by RNAi-*BMP* genomic sequences (13-16). A series of pregnant mice at 6.5-14.5 days post coitum (dpc) with post-implantation staged mouse embryos were injected via the tail vein with the pSES-small interfering RNA (si)*BMP4* plasmid to silence the *BMP4* gene, and these procedures were applied to establish an animal model (14). One of the objectives of the present study was to expand current knowledge on the effect of *BMP4* on NCSCs during the middle stage of embryo development. The current study also aimed to assess the neurodevelopmental abnormalities associated with the knockdown of *BMP4*.

Materials and methods

Animals and experimental groups. Balb/c mice (male 12, female 36; 8-12 weeks old; weight 14-18 g) were purchased

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from the Animal Center of Chongqing Medical University (Chongqing, China). The mice were kept in a specific pathogen-free facility room at the Chongqing Children's Hospital Animal Center (Chongqing, China), with 50±5% humidity and a temperature of 25±2°C. Food and water were provided *ad libitum*. Male and female mice were kept in single cages at a 1:2-1:4 ratio. A female mouse in which a vaginal plug was identified the next dawn was marked as 0.5 dpc and housed separately. The present study was approved by The Ethics Committee of Chongqing Medical University.

Tail vein injections. Pregnant mice were randomly divided into the following groups: Ringer's group (n=12), pSES group (n=12) and RNAi-*BMP4* group (n=12); these were injected with 10 µl/g Ringer's solution, 50 ng/µl pSES empty vector and 50 ng/µl pSES-Si*BMP4* vector, respectively. The pSES vectors bore a copy of the entire DsRed coding region, allowing fluorescent detection of the delivered plasmids. At 11.5 dpc, the solution (at a volume of 10 µl/g) was injected into the tail vein. A 31G needle was used and the injection usually performed within 5±1 sec. Plasmids for siRNA were purchased from the Oncogene Laboratory, Biological Sciences Division, University of Chicago and contained the following 4 sites of RNAi to silence the *BMP4* gene, lowercase is the gene match to the cutting site): (5'aGGTCCAGGAAGAAGAATAAttt3', mouse *BMP4* sim*BMP4*-site 1, sense strand; 3'aTTATTCTTCTTCCTGGACCTttt5', mouse *BMP4* sim*BMP4*-site 1, antisense strand. 5'aGAGCCATGCTAGTTTGATAttt3', mouse *BMP4* sim*BMP4*-site 2, sense strand; 3'aTATCAAAC TAGCATGGCTCttt5', mouse *BMP4* sim*BMP4*-site 2, antisense strand. 5'aGGGAAAAGCAACCCAATTAttt3', mouse *BMP4* sim*BMP4*-site 3, sense strand; 3'aTAATTGGGTTGCTTTTCCCTttt5', mouse *BMP4* sim*BMP4*-site 3, antisense strand. 5'aGGGAAAAGCAACCCAATTAttt3', mouse *BMP4* sim*BMP4*-site 4, sense strand; 3'aTAATTGGGTTGCTTTTCCCTttt5', mouse *BMP4* sim*BMP4*-site 4, antisense strand). At 1 week [Ringer's group F1 mice (n=16), pSES group (n=18) and RNAi-*BMP4* group (n=10)], 2 weeks [Ringer's group (n=15), pSES group (n=15) and RNAi-*BMP4* group (n=12)] and 4 weeks [Ringer's group (n=21), pSES group (n=15) and RNAi-*BMP4* group (n=7)] following birth, the F1 mice were sacrificed by cervical dislocation. Then, the target tissues were removed and rinsed in PBS at 4°C. Parts of the tissues were stored at -80°C and used for western blotting. The remaining samples were stored at 4-20°C in 4% paraformaldehyde.

Reverse transcriptase-semi-quantitative polymerase chain reaction (RT-sqPCR) detection of *BMP4* and *Smad4* genes. Total RNA was extracted from the colon using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. To generate cDNA, RT was performed with Prime Script RT Enzyme mix reverse transcriptase at 4°C (Invitrogen; Thermo Fisher Scientific, Inc.). Then, the cDNA samples were amplified by PCR using the following cycling conditions: 94°C for 5 min; followed by 39 cycles at 94°C for 30 sec; 58°C for 30 sec; 72°C for 30 sec; and a final step at 72°C for 5 min. Oligonucleotide primers were purchased from Invitrogen (Thermo Fisher Scientific, Inc.) as follows: *BMP4* forward (F), GACTTCGAGGCG ACACTTCT and reverse (R), CCTGGGATGTTCTCCAGA

TG; *Smad4* F, CATTCCAGCCTCCCATTTCCTCAATC and R, CACATAGCCATCCACAGTCACAAC; β-actin F, AAG ATGACCCAGATCATGTTTGAGACC and R, GCCAGG TCCAGACGCAGGAT. The amplified products were resolved in ethidium bromide-stained 2% agarose gels. Densitometry was performed using Quantity One software version 4.6.2. (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Western blotting analysis of *BMP4*. Protein extracts were prepared from the colon. Tissue samples were homogenized in RIPA lysis buffer and phenylmethanesulfonyl fluoride (Beyotime Institute of Biotechnology, Shanghai, China), and proteins were directly extracted according to the manufacturer's protocols. Protein concentrations were determined using a Micro Bicinchoninic Acid Protein Assay reagent (Beyotime Institute of Biotechnology). Protein samples were then diluted to obtain equal (50 µg) protein amounts and heated at 100°C in an equal volume of SDS loading buffer (Beyotime Institute of Biotechnology) for 10 min. Proteins were then separated by SDS-PAGE (5% spacer gel, 40 V, 50 min; 8% separating gel, 80 V, 70 min). Proteins were then electrophoretically transferred (Bio-Rad Laboratories, Inc.) onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA) for 1.5 h at 250 mA. To block non-specific binding, the membranes were incubated with 5% bovine serum albumin (Kang Yuan Biology, Tianjing, China) in Tris-buffered saline with Tween 20 (250 µl Tween20 in 500 ml PBS) at 37°C for 1.5 h. The membranes were then incubated overnight at 4°C with rabbit anti-*BMP4* primary polyclonal antibody (GR49989-1; 1:400; Abcam, Cambridge, MA, USA) and β-actin (4AH240911; 1:150; 4A Biotech Co., Ltd., Beijing, China.). Next, the membranes were incubated at 4°C for 1 h with peroxidase-conjugated secondary anti-rabbit IgG (TA130015; 1:4,000; OriGene Technologies, Inc., Beijing, China), according to the manufacturer's protocol. The protein of interest was visualized using an enhanced chemiluminescence western blotting substrate (Boster Biological Technology, Pleasanton, CA, USA) and its relative expression was quantified using a Chemidoc XRS gel imaging system Quantity One software version 4.6.2. (Bio-Rad Laboratories, Inc.).

Immunohistochemistry. Colontissue was immediately fixed in 4% buffered formalin for 48 h, embedded in paraffin, and sectioned at 5 µm. Antigen retrieval was performed by boiling the sections in 0.01 M sodium citrate in 1L PBS (pH 7.4) followed by a 20 min incubation at room temperature, in 3% H₂O₂ for 20 min and blocked in 5% BSA (Kangyuan Biology, Tianjing, China) for 20 min at room temperature. Following incubation in 5% normal serum for 20 min at room temperature, sections were incubated with rabbit anti-*BMP4* primary polyclonal antibody overnight at 4°C (GR49989-1, ab39973; 1:500; Abcam). Slides were then stained with goat anti-rabbit secondary antibody (PV-6001, 1:1,500 dilution) from OriGene Technologies, Inc. (Beijing, China) for 1 h at room temperature. Detection was accomplished using a DAB kit (Beyotime Institute of Biotechnology, Shanghai, China). Positive staining was assessed by the degree of brown colour development. The integrated optical density of positive staining was measured by NIS-Elements Viewer version 4.0 (Nikon Corporation, Tokyo, Japan) using an Eclipse 55i microscope (x40; Nikon Corporation).

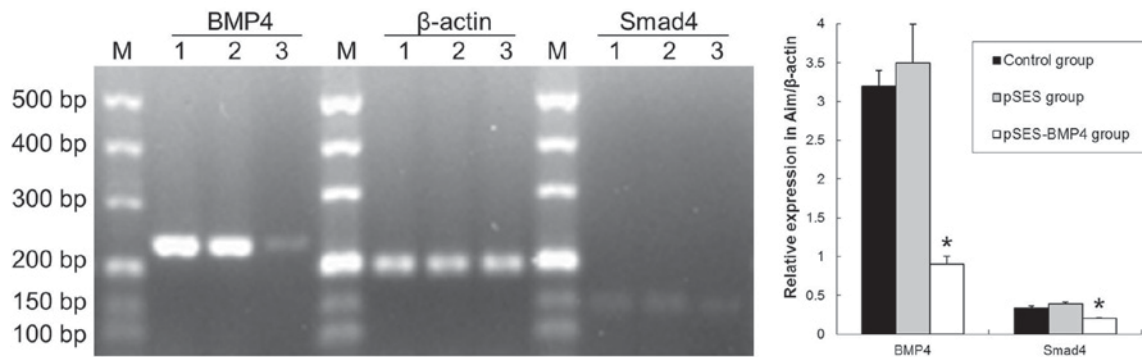


Figure 1. Semi-quantitative reverse transcriptase-polymerase chain reaction of the different groups of Balb/C mouse colons. Lane 1: Ringer's group, Lane 2: pSES group, Lane 3, RNAi-BMP4 group and M, marker. At the RNA level, the RNAi-BMP4-induced mice, in contrast with the control group, significantly downregulated the levels of *BMP4* and *Smad4* in the colon. * $P < 0.05$. RNAi, RNA interference; BMP, bone morphogenetic protein.

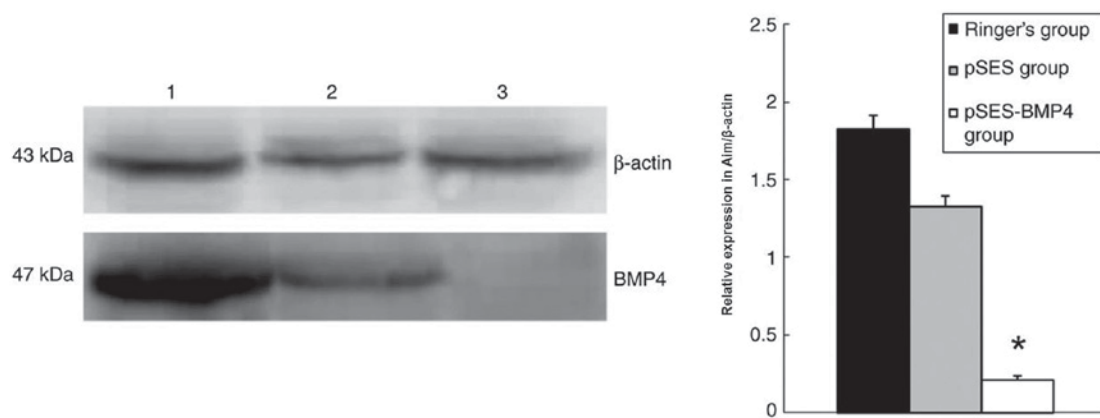


Figure 2. Western blot analysis of different groups of colon *BMP4*. The electropherogram derived from the western blot analysis of neurodevelopmental deficits associated protein *BMP4* in the colons of three mouse groups. Lane 1: Ringer's group; Lane 2: pSES group; Lane 3: RNAi-BMP4 group. At the level of protein, RNAi-BMP4-induced mice, in contrast to the control group, significantly downregulated the levels of *BMP4* in the colon. * $P < 0.05$. BMP, bone morphogenetic protein; RNAi, RNA interference.

Statistical analysis. The RT-PCR and western blotting greyscale values were expressed as the mean \pm standard deviation. The differences among groups were analysed by a one-way analysis of variance and t-tests implemented in SPSS software version 17.0 (SPSS, Inc., Chicago, IL, USA), followed by Student-Newman-Keuls-q test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

BMP4 and downstream gene downregulation in F1 mouse colon is induced by administration of the siBMP4 plasmid to pregnant mice. PCR revealed that *BMP4* and *Smad4* mRNA in the colon was significantly lower in the RNAi-BMP4 group compared with the control and pSES groups at 7 days after birth ($P < 0.05$; Fig. 1). A decrease in *BMP4*-*Smad4* in mice may lead to aganglionosis (9-11). Western blotting results revealed that colon *BMP4* in the RNAi-BMP4 group was significantly lower compared with the control and pSES groups (Fig. 2).

Formation of aganglionosis in F1 mice is induced by trans-placental administration of RNAi-BMP4 to pregnant mice at 11.5 days. Barriers to stool discharge and abdominal distention were observed in *BMP4* knockdown old mice

($n=35$; 10 mice were sacrificed at 1 week). With a barium enema, giant colons were identified in 2-week-old mice from the RNAi-BMP4 group ($n=23$; 12 mice were sacrificed at 1 week). Then, 4-week-old mice died from cerebral ischaemia and were autopsied ($n=7$, some mice succumbed naturally). Unfortunately, the mouse rectum was so small that there was no suitable pressure probe. The spastic colon, transitional colon and giant colon were exposed (Fig. 3). With immunocytochemistry, a small number of glial-like cells were positive for *BMP4* in the colon of *BMP4* knockdown mice. Additionally, absence or dysplasia of neurons was observed in RNAi-BMP4 colons.

Discussion

There are a number of ways to establish aganglionosis in animal models (17-19). The principle method is to affect the migration, differentiation and proliferation of NCSCs or artificially destroy the intestinal nervous system with drugs (20). A high folic acid (FA) diet during pregnancy leads to a gradual increase in serum FA in pregnant mice and their offspring, causing aganglionosis in the offspring (21). The level of FA in the offspring reached the highest value with 160 mg/kg FA feeding. It has also been demonstrated that a 0.1% benzalkonium

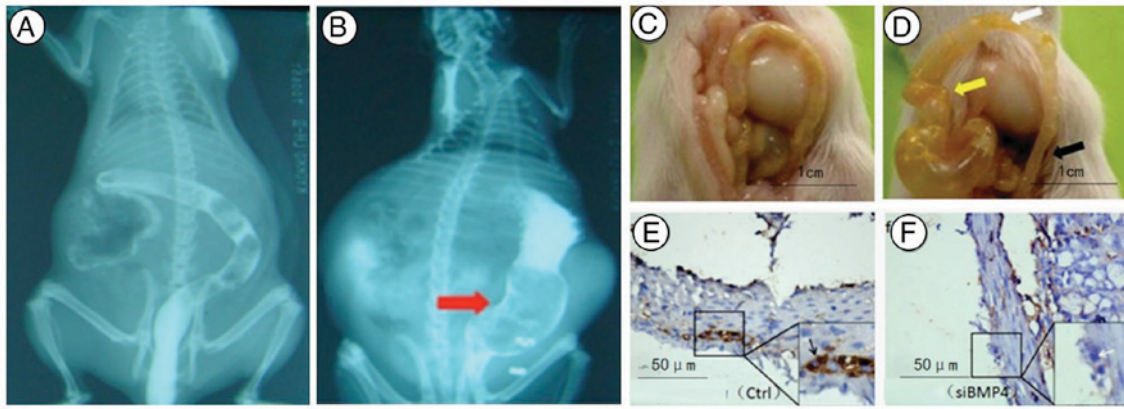


Figure 3. Colorectal shape was observed in the RNAi-BMP4 group, and immunohistochemistry staining of the colorectal region was performed in normal and RNAi-BMP4 tissues. (A) The barium enema form of a 3-week-old mouse in the control group. (B) The barium enema form of a 3-week-old mouse in the RNAi-BMP4 group; the red arrow indicates the giant colon. (C) The mouse colon of the control group. (D) The mouse colon of the RNAi-BMP4 group. The black arrow indicates the spastic colon, the white arrow indicates the transitional colon, and the yellow arrow indicates the exposed giant colon. (E) In the colon of the control group, the expression of BMP4 was assayed by immunohistochemical staining. The Figure shows the normal neurons and nerve fibres. (F) The colon of the RNAi-BMP4 group, showing agenesis and absence of nerve fibres of neurons (magnification, x200). RNAi, RNA interference; BMP, bone morphogenetic protein.

chloride enema may also be used to establish aganglionosis in animal models. Ethylnitrosourea (ENU) is a type of artificial synthesised compound that leads to random and single-base mutations in a variety of organisms (22,23). The offspring may end up with a severe mega colon phenotype due to ENU-induced mutations in C57BL/6 male mice. Trisomy 16 mice, which are likely to exhibit aganglionosis, are a type of genetic mouse model with clinical manifestations similar to those identified in the human trisomy 21 syndrome (24). This genetic mouse model, which does not express the endothelin 3 gene, is also known as piebald and spotted death mice and may develop defective intestinal aganglionic syndrome or congenital megacolon (25,26). However, at present, there is a lack of a genetic mouse models that exhibits HD gene knockdown with unmistakable implementation.

NCSCs originate from cells of the neural crest that migrate in chains as they colonize the embryonic gut, eventually forming the myenteric and submucosal plexus (27-29). Failure of the neural crest cells to colonize the gut leads to aganglionosis in the sigmoid colon, a pathological condition called Hirschsprung's disease, also known as congenital megacolon, in humans (28,29). At present, the mechanism associated with the signalling pathways that adjust NCSCs for migration to the intestinal tract and differentiation in the enteric nervous system remains unclear. The *BMP* signalling pathway may involve the migration process of NCSCs to the intestinal tract, in addition to the proliferation and differentiation of intestinal ganglion cells (30-32). Studies have suggested that *BMP* signalling serves an important role in differentiating NCSCs into enteric ganglia (8-12). Therefore, an improved understanding of the signalling pathways regulated by NCSCs and associated with the mechanisms of action is important to investigate the pathogenesis of aganglionosis. BMPs contribute to the largest subgroup of the TGF- β super family and were originally identified by their ability to induce bone development (27,28,31). Additionally, BMPs are expressed in the nervous system throughout its differentiation. The mechanisms by which these BMPs regulate the induction of the neuroectoderm, the CNS

primordium, and finally the neural crest, which gives rise to the NCSCs, have been reviewed (11,33). Following neural tube closure, the most dorsal aspect of the tube becomes a signalling centre for BMPs, which directs the pattern of development of the dorsal spinal cord. Additionally, certain data suggested that BMP4 was a peripherally derived factor that may regulate the survival of motor neurons (34).

The RNAi phenomenon was identified in fungi and plants (15,35). The placenta is responsible for transport between the mother and foetus and is a tissue barrier of high permeability (14,16). The present study confirmed that plasmid vector injected into the tail vein of pregnant mice was able to be transferred to foetal mice through the blood-embryo barrier. The plasmid vector transfection of tissues and organs depends on the plasmid concentration, solvent volume, injection velocity, and weight of pregnant mice. The plasmid vector achieved good results when the injection concentrations were 50 ng/ μ l and 10 μ l/g, and when the injection time was 5 sec. The majority of human aganglionosis cases have revealed an association with decreased *BMP-Smad4* (8,30,36). F1 mice that received transplacental RNAi-BMP4 at 11.5 days revealed disordered NCSCs. That is, the downregulation of *BMP4* in the middle embryo stage possibly resulted in developmental problems in the peripheral nervous system. BMP4 was also involved in the TGF- β /*BMP/Smad*-mediated signalling cascade as a transcriptional repressor of Smad proteins.

In summary, knockdown by transplacental RNAi is a powerful technique to study the effect of signalling pathways on responding tissues at the middle embryonic stage (14,37). However, different genes regulate embryonic development through specific mechanisms, and different gene plasmids possess different transfection efficiencies. Thus, deciding the dose and when to intervene should be considered when exploring the function of a novel gene (38). The results presented here suggested that downregulation of the *BMP4* transgene was an excellent prognostic factor of neurodevelopmental inactivity in mice. This approach may be used to make an aganglionosis

mouse model. As the mouse colon is relatively short, it is planned to use rabbits to research colon gene expression in different regions in future studies.

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