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Original Article

Analysis of BMP4 expression during development of the striated muscle complex in rat embryos with anorectal malformations

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Abstract: This study aimed to investigate the spatiotemporal expression pattern of BMP4 in the striated muscle complex (SMC) in ethylenethiourea (ETU)-exposed rat embryos with anorectal malformations (ARMs), and to explore the possible role of BMP4 during morphogenesis of the SMC. The ETU-induced ARM model- on embryonic day 10 (E10)- was used to investigate the expression pattern of BMP4 during development of the SMC. Immunohistochemistry staining, western blotting and quantitative real-time PCR (qRT-PCR) were applied to confirm the expression levels of protein and mRNA of BMP4 between normal rat embryos and embryos with ARMs. Immunostaining revealed that BMP4 expression showed space-time dependent changes in the developing SMC of embryos with ARMs. With the growth of rat embryos, the BMP4 positive stained cells gradually increased from E17. The same changes in expression of BMP4 were detected in embryos with ARMs. However, compared to embryos with ARMs, BMP4 expression was significantly higher in normal tissues. In western blotting and qRT-PCR, BMP4 expression in the SMC of rat embryos with ARMs was lower at both the mRNA level and the protein level compared with normal rat embryos. This study demonstrated that BMP4 expression in embryos with ARMs was remarkably reduced, which indicated that BMP4 could play an important role in the pathogenesis of the SMC in embryos with ARMs.

Keywords: Anorectal malformations, BMP4, gene expression, rat embryos, striated muscle complex, development

Introduction

Anorectal malformations (ARMs) are very common surgical problems, with a clinical spectrum that ranges from anal stenosis to imperforate anus to persistent cloaca, and affects approximately 1/5000 to 1/1500 live births [1]. In spite of numerous technical advances in the surgical treatment of ARMs, some patients continued to suffer from fecal incontinence and constipation after procedures [1-5]. The etiology of postoperative defecation problems are multifactorial, and maldeveloped pelvic floor musculature (PFM) has been identified as one of the most important underlying factors [6-10]. The striated muscle complex (SMC) in rats resembles the gross anatomy of the puborectalis muscle in humans, and contributes substantially to micturition, defecation, continence, and support of the pelvic organs [11].

Previous studies have demonstrated that bone morphogenetic proteins (BMPs) are important for activation and proliferation of muscle satellite cells, and in the prevention of premature myogenic differentiation [12, 13]. BMPs are members of the transforming growth factor-β (TGF-β) super family and comprise a highly conserved and expanding family of 15 genes that are critical to various developmental events [14-16]. BMP signals are mediated by serine/threonine kinase receptors, which are classified into types I and II. Upon ligand binding, transphosphorylation of type-1 serine/threonine kinase receptors by type-2 receptors activates the receptor-regulated Smad transcription factors-Smad1, Smad5 and Smad8-for assembly with Smad4 and translocation to the nucleus [15, 17, 18]. These Smad complexes control transcription of several regulatory elements, including some of the inhibitor of differentiation (Id) proteins. Binding of Id proteins to ubiquitously expressed E-proteins prevents activation of the muscle-specific transcription factors myogenin and MyoD, and impedes myo-

genic differentiation [19]. Furthermore, Ono Y *et al.* found that the stimulation of BMP signaling- by adding BMP4- resulted in an increased number of activated satellite cells and a decreased number of differentiating myoblasts [13]. In addition, it was revealed that BMP4 played an important role in the development of the hindgut, and downregulation of BMP4 could be partly related to the maldevelopment of the terminal hindgut in ARMs [20, 21]. However, it is unknown whether BMP4 continues to play a role in the development of the SMC after the occurrence of ARMs. To gain an insight into the pattern of BMP4 expression and the possible role of BMP4 during development of the SMC in embryos with ARMs, we conducted a systematic study on the spatiotemporal expression of BMP4 in normal rat embryos compared to rat embryos with ARMs.

Materials and methods

Animal model and tissue collection

Ethical approval was obtained from the China Medical University Animal Ethics Committee before the study. A total of 80 time-mated pregnant Wistar rats were divided into normal and ARMs groups. In the ARMs group, pregnant rats were gavage fed a single dose of 125 mg/kg of 1% ethylenethiourea (ETU; 2-imidazolidinethione, Aldrich Chemical Co., Germany); In the normal group, pregnant rats received an equal dose of saline on E10 (E0-sperm in vaginal smear after overnight mating). Embryos were collected via cesarean delivery on E17, E19 and E21. For immunohistochemical studies, normal embryos and embryos with ARMs were fixed in 4% paraformaldehyde for 12 to 24 h depending on their size. Embryos were then embedded in paraffin in a routine manner and sectioned sagittally at a thickness of 4 μm. For western blot and qRT-PCR analysis, the SMC tissues were collected under magnification and immediately stored at -80°C until required for use. Only male fetuses were used in this study, because the SMCs are thinner in female fetal rats. We were able to observe the gonads under the light microscope to determine the sex of the rats; Specifically, the testis has a characteristic "striped" appearance, while the ovary has a characteristic "spotty" appearance.

Immunohistochemical staining

Immunohistochemical staining was performed according to the previously described method [22]. For antigen retrieval, slides were incubated in boiling 0.01 mol/L citrate buffer (pH 6.0) for 10 min, followed by cooling at room temperature, and endogenous peroxidase activity was then blocked with 3% H_2O_2. After incubation in 10% normal goat serum for 30 min to block nonspecific binding sites, sections were incubated with BMP4 primary antibody at a dilution of 1:200 (rabbit polyclonal, ab39973, Abcam, Cambridge, UK) overnight at 4°C. Sections were then incubated with biotinylated goat anti-rabbit IgG (Maixin, Fuzhou, China) for 30 min at room temperature after the primary antibody was washed off. Immunoreactions were visualized by DAB (Maixin, Fuzhou, China) and sections were counterstained with hematoxylin. Specimens were photographed using a digitized microscope camera (Nikon E800, Japan). In all experiments, negative controls were performed by either omitting the primary or secondary antibody step.

Protein preparation and western blotting

SMC tissue collected from the normal and ARMs groups were pooled and sonicated in ddH_2O containing protease inhibitors. Protein extracts (50 μg) were heated at 100°C for 5 min and size fractionated on Bis-Tris SDS-PAGE gels (Invitrogen, Carlsbad, CA, USA). Protein samples were denatured, separated by SDS/PAGE, and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA); This was followed by blocking with 5% fat-free milk in Tris-buffered saline (1.5 h, room temperature). PVDF membranes with proteins were incubated overnight at 4°C in primary antibody against BMP4 (dilution 1:1000), and then incubated with secondary antibody (dilution 1:2000) for 2.5 h at room temperature. The immunostained bands were detected with a Proto Blot II AP System using a stabilized substrate (Promega Biological Products, Ltd., Shanghai, China). GAPDH was used as an internal standard to normalize protein levels in each lane.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated with TRIZOL RNA extraction reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. We used 100 mg of tissues from normal and ARMs specimens. The harvested RNA was

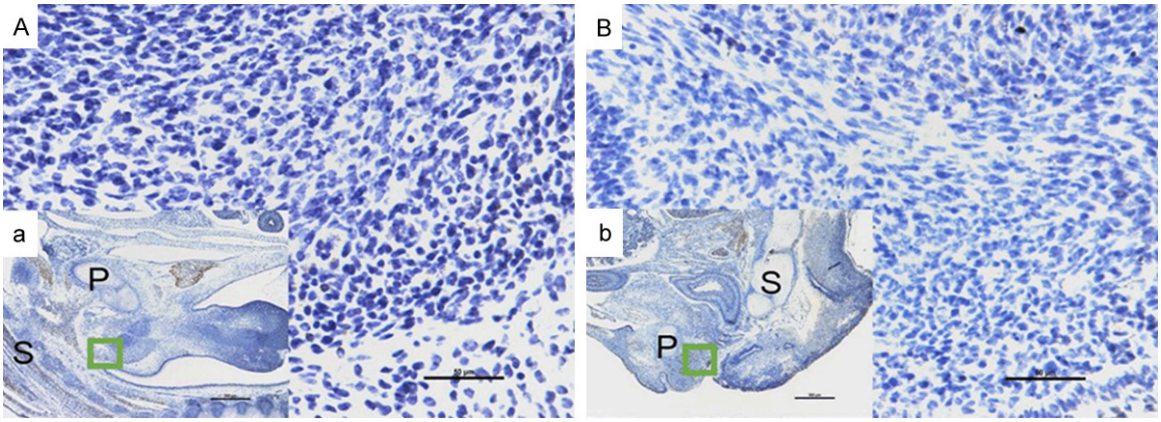

Figure 1. Immunohistochemical staining of BMP4 on E17. (A and a) Represent the normal group; On E17, sporadic BMP4 positive stained cells were detected in the SMC. (B and b) Represent the ARMs group; No obvious immunoreactivity specific to BMP4 was detected in the SMC on E17. (A and B: Original magnification ×400; a and b: original magnification ×40). P, pubis; S, sacrum. The region marked with a square in (a and b) is magnified in (A and B).

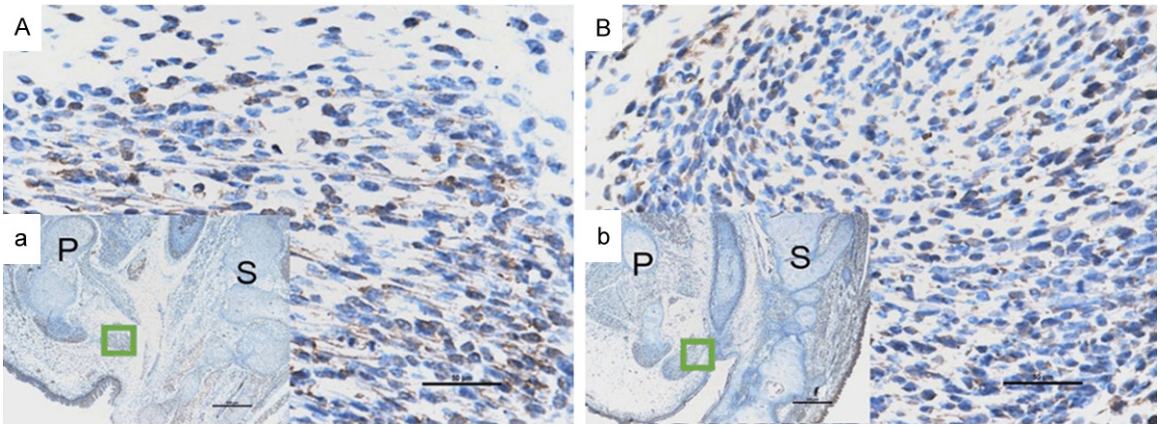

Figure 2. Immunohistochemical staining of BMP4 on E19. (A and a) Represent the normal group; On E19, the SMC was verydistinct. BMP4-labeled cells were mainly localized to the SMC. (B and b) Represent the ARMs group; The SMC developed poorly, and very little immunoreactivity specific to BMP4 was detected in the SMC on E19. (A and B: Original magnification ×400; a and b: original magnification ×40). R, rectum; U, urethra; P, pubis; S, sacrum. The region marked with a square in (a and b) is magnified in (A and B).

diluted to a concentration of 1 μg/μl and stored at -80°C. The total RNA was reverse-transcribed into cDNA using a SYBR Prime Script RT-PCR kit (Takara, Dalian, China), according to the manufacturers' instructions. The BMP4 primers used for qRT-PCR were as follows: Forward, 5'-TGCCATTGTGCAGACCCT-3' and reverse, 5'-CACCACCTTGTCGTACTCGTC-3'. The housekeeping gene β-actin (Takara, code D3783) was used as an internal control. qRT-PCR was performed with a 20 μl reaction system in triplicate for each specimen in the presence of SYBR® Green PCR Master Mix (TaKaRa Biotechnology Co., Dalian, China) in a Lightcycler® (Roche Molecular Biochemicals, Co., Mannheim, Germany). The reaction program was: 3 min pre-denaturation at 95°C and 45 cycles of 10 sec of denaturation at 95°C and 30 sec of annealing at 60°C. After the termination of qRT-PCR, the production was automatically analyzed by the Lightcycler system. A dissociation procedure was performed to generate a melting curve to confirm amplification specificity. The relative levels of gene expression were determined as ΔCt=Ct gene -Ct reference, and the fold change in gene expression was calculated using the 2-ΔΔCt method.

Statistical analysis

The Statistical Program for Social Sciences, version 13.0 (SPSS, Chicago, IL), was used for

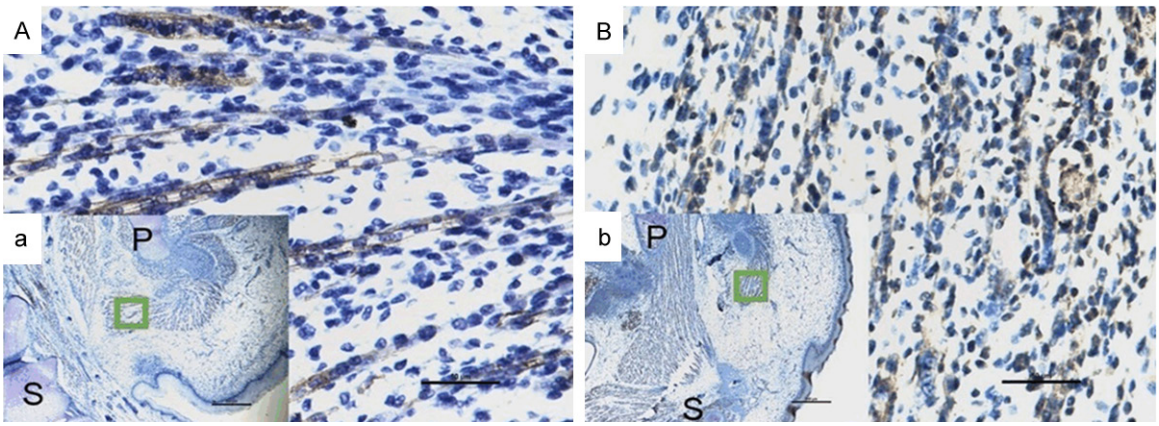

Figure 3. Immunohistochemical staining of BMP4 on E21. (A and a) Represent the normal group; On E21, increasingly more BMP4 positive stained cells were detected in the SMC and the bulbocavernosus muscle. (B and b) Represent the ARMs group; cells showing BMP4 immunoreactivity increased gradually on E21. (A and B: Original magnification ×400; a and b: Original magnification ×40). P, pubis; S, sacrum. The region marked with a square in (a and b) is magnified in (A and B).

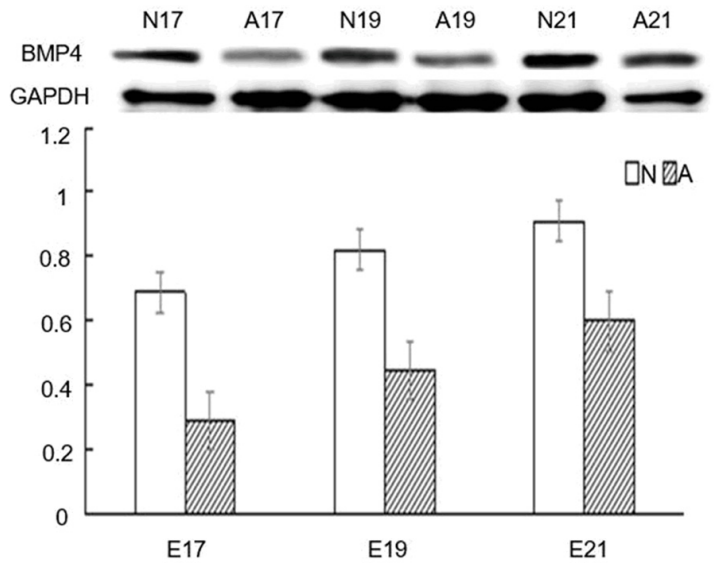

Figure 4. BMP4 protein expression relative to GAPDH expression (y-axis) in the developing SMC of normal rat embryos and rat embryos with ARMs on E17, 19 and 21 (x-axis). The immunoblot shows a remarkable BMP4 expression in the normal group, but a weak signal in the ARMs group. The trends of expression at each time point are shown. N, normal group; A, ARM group.

statistical analysis. We used the student t-test to compare the BMP4 levels between the normal and ARMs groups. *P* values of less than 0.05 were considered statistically significant.

Results

General observation

In our study, no malformations were observed in the 156 male normal rat embryos. We obtained a total of 196 embryos with ARMs from 413 ETU-treated male rat embryos. The incidence of ARMs in embryos of the ETU-treated group on E17 to E21 was 66.4%. However, externally visible spinal bifida and/or meningocele were also observed, and 17 embryos died *in utero*. As denervation could affect the development of SMC, specimens with neurologic defects were excluded [23]. In this study, all specimens with ARMs were determined by means of observing the fistula between the rectum and the urethra in sagittal planes under the light microscope.

Immunohistochemical analysis

In normal embryos, sporadic BMP4 positive stained cells could be detected in the SMC on E17 (**Figure 1A**). From E19, the SMC was very distinct, and increasingly more BMP4 positive stained cells were detected in the SMC and bulbocavernosus muscle (**Figures 2A** and **3A**).

However, in embryos with ARMs, on E17, no obvious immunoreactivity specific to BMP4 was detected in the SMC (**Figure 1B**). From E19, there was an increasing number of cells with BMP4 immunoreactivity in the SMC, but this

Table 1. The relative quantity of BMP4 mRNA on E17

Group	BMP4 average Ct value	β-actin average Ct value	ΔCt	ΔΔCt	Times of gene (compared to ARMs group)
A17	26.08±1.35	18.90±1.21	7.18	0	1
N17	20.52±1.17	14.21±1.62	6.31	-0.87	1.83

Table 2. The relative quantity of BMP4 mRNA on E19

Group	BMP4 average Ct value	β-actin average Ct value	ΔCt	ΔΔCt	Times of gene (compared to ARMs group)
A19	22.39±1.99	15.38±1.51	7.01	0	1
N19	21.64±1.39	16.07±1.43	5.57	-1.44	2.71

Table 3. The relative quantity of BMP4 mRNA on E21

Group	BMP4 average Ct value	β-actin average Ct value	ΔCt	ΔΔCt	Times of gene (compared to ARMs group)
A21	24.09±1.12	17.23±1.58	6.86	0	1
N21	23.70±1.61	18.64±1.01	5.06	-1.8	3.48

was significantly lower compared to normal embryos (**Figures 2B** and **3B**).

Western blot analysis

Western blot analysis specific for BMP4 was performed to quantify protein expression in the development of the SMC in the normal and ARMs groups. Accordingly, BMP4 was approximately detected as a 47 kDa band on western blots of protein extracts. The expression level of BMP4 was normalized to the protein level of GAPDH from the same specimens of embryos at E17, 19, and 21, which are the key periods of SMC development; BMP4 expression gradually increased in the normal group, while in the ARMs group, BMP4 protein expression was weak. BMP4 protein expression significantly decreased in ARMs SMC compared with the normal SMC at each time point (0.29±0.04 versus 0.69±0.06; 0.45±0.09 versus 0.82±0.09; 0.60±0.05 versus 0.91±0.13; Respectively; $P<0.05$; **Figure 4**).

QRT-PCR analysis

The OD value of total RNA calculated by A260/A280 was in the range of 1.8 to 2.0. In our study, a significant increase in the expression pattern of BMP4 was detected in the normal group compared to the ARMs group at each time point, which was consistent with the results obtained from western blot analysis. Results showed that in the normal group, the mRNA levels of BMP4 (1.83-fold, 2.71-fold and 3.48-fold) were higher than those in the ARMs group on E17, E19 and E21 ($P<0.05$), respectively (**Tables 1**-**3**).

Discussion

During the embryogenesis of SMC, SMC demarcated from the bulbocarvernosus muscle, passing around the rectum, forming a sling-like structure, and converged with the contralateral muscle behind the rectum [24]. However, in rat embryos with ARMs, SMC shifted significantly cephalad, ventrally, and median ward, and considerable connective tissue was observed in the intermuscular bundles under high-power view [25]. Chen QJ *et al.* found that dysregulation of apoptosis was implicated as one of the fundamental factors in the pathogenesis of SMC maldevelopment in rats with ARMs [26]. Mi J *et al.* demonstrated that spatiotemporal expression of Wnt5a was imbalanced during SMC development in embryos with ARMs [27]. Numerous efforts have been made to understand the mechanisms of SMC development in embryos, but our current understanding of normal and abnormal development of the SMC remains incomplete.

The development of skeletal muscle proceeds through five phases, as follows [28]: Phase 1 (specification), the development of muscle progenitor cells is specified toward muscle cells in somites; Phase 2 (migration), muscle progenitor cells migrate to the presumptive places at which muscles are formed; Phase 3 (proliferation), progenitor cells proliferate and increase in number; Phase 4 (differentiation), progenitor cells finally differentiate to become myoblasts, which in turn fuse to form multinucleated myotubes; Phase 5 (maturation), the multinucleated myotubes mature into myofibers, including slow-twitch myofibers or fast-twitch myofibers. Zhang SW *et al.* found that the fibroid structure of the SMC in normal embryos and embryos with ARMs could not be identified until E16 [25]. In other words, the muscle progenitor cells

of the SMC start to migrate to the inherent location on E16, and they then participate in proliferation and differentiation processes from E17 to E21.

During development of the skeletal muscle, many signaling pathways participate in the embryogenesis and development of the SMC, namely Shh, BMP, Wnt and Notch. Previous studies have demonstrated that BMP4 plays a critical role in muscle development and regeneration. TakenaoUmemoto *et al*. reported that BMP4 expressed in myoblasts plays a positive role in myotube formation/maturation through myogenin expression, and the presence of myotubes inhibits BMP4 expression in proliferating myoblasts through transcriptional regulation, although the expression is intrinsically increased with the time of culture [29]. In this study, BMP4 expression gradually increased from E17 to E21, both in normal embryos and in embryos with ARMs, which is consistent with the previous reports mentioned. Recent studies have shown that BMPs are important in preventing premature myogenic differentiation [13]. In the current study, we found that at the same stage, the expression level of BMP4 was reduced in embryos with ARMs compared with normal embryos. These results implythat this downregulation of BMP4 expression in embryos with ARMs may accelerate premature myogenic differentiation. Considerable connective tissue infiltrated into intermuscular bundles, which resulted in the malformation of SMC in ARMs, further suggesting that optimal BMP4 levels are critical for the development of the SMC.

Furthermore, the genetics of ARMs is an extremely complex event. Morphological changes of the SMC take place after the occurrence of abnormal anorectum in rats with ARMs [25]. We suspectedthat rectum maldevelopment influenced the development of the SMC to a certain extent. Bourdelat *et al.* emphasized the importance of the rectum in the development of the sphincter, which corroborated this present study [30]. We found that spatiotemporal expression of BMP4 was imbalanced during development of the SMC in embryos with ARMs, and this trend was consistent with that in terminal hindgut development [20, 21], suggesting that the rectum could play a key role in the development of the SMC.

In conclusion, BMP4 is extremely important for the development of the terminal hindgut and the SMC in embryos with ARMs. However, our study was unable to confirm whether BMP4 was the initial event leading to malformation of the SMC, and it has been shown that numerous signaling molecules are involved in the different phases of the development of the SMC. For a precise understanding of the developmental processes and the pathogenesis in ARMs, further analysis of the molecular mechanisms should be carried out.

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Disclosure of conflict of interest

None.

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