RESEARCH ARTICLE

Loss of smooth muscle myosin heavy chain results in the bladder and stomach developing lesion during mouse fetal development in mice

Running title. Bladder and stomach lesion without SM-MHC

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Abstract

Smooth muscle myosin heavy chain (SM-MHC) exclusively expresses in smooth muscle, which takes part in smooth muscle cell contraction. Here we have an insertional mutation mouse whose heavy polypeptide 11 (*Myh11*) gene has been disrupted and no SM-MHC protein has been detected. Compared to wild-type and SM-MHC$^{+/-}$ mice, the SM-MHC$^{-/-}$ neonates had large round bellies, thin-walled giant bladders, and large stomachs with huge gas bubbles. Most died within 10 hours and the rest within 20 hours after birth. Further analysis of developing fetuses from 16.5 days post coitum (dpc) stage to newborn showed no significant ($P < 0.05$) difference in the ratio of Mendelian inheritance and average body weight among SM-MHC$^{+/+}$, SM-MHC$^{+/-}$, and SM-MHC$^{-/-}$ mice whereas the abnormal exterior appearance was observed in each SM-MHC$^{-/-}$ bladders from 16.5 dpc. Histological analysis showed no difference in stomach tissues but evidently thin-walled smooth muscle layer and a giant cavity in bladders of SM-MHC$^{-/-}$ fetuses at various stages from 15.5 dpc to newborn. The results indicated that the SM-MHC defect lead to the bladder developing lesions initially at 15.5 dpc stage in mouse and also implied that the SM-MHC loss might result in the gas bubbles in stomach. The study should facilitate further detailed analyses of the potential role of SM-MHC in bladder and stomach development.

Keywords. *Myh11*; smooth muscle myosin heavy chain; bladder; stomach; insertional mutation; fetal development.

Introduction

Smooth muscle myosin heavy chain (SM-MHC) isoforms are the products of alternative splicing of a

single gene (Babij and Periasamy 1989; Babij 1993; Babu *et al*. 2000), which exclusively expressed in smooth muscle (Nagai *et al*. 1988; Madsen et al. 1998). Alternative splicing generates four smooth muscle myosin heavy chain isoforms SM-1A, SM-1B, SM-2A, and SM-2B (Loukianov *et al*. 1997; Babu *et al*. 2000). SM1 mRNA encodes 43 unique amino acids at the carboxyl terminus, and has a molecular mass of 204 kDa whereas SM2 mRNA carries a 39-nucleotide insertion with an internal stop codon, and has a shorter carboxyl terminus and a molecular mass of 200 kDa (Kelley *et al*. 1992; Kelley *et al*. 1993; Loukianov *et al*. 1997; Rovner *et al*. 2002). SM1 has a relative lower critical concentration for myosin filament assembly and the filaments formed by SM1 are more stable than those formed by SM2 (Rovner *et al*. 2002). The SM-B isoform contains an additional seven-amino acid insertion at the junction of the 25/50 kDa tryptic peptides (Babu *et al*. 1991; Kelley *et al*. 1993; White *et al*. 1993), which is necessary and sufficient in producing a higher Mg^{2+}-adenosine triphosphatase activity (Kelley *et al*. 1993; Rovner *et al*. 1997; White *et al*. 1998) and a higher shortening velocity than without this insertion (Kelley *et al*. 1993; Rovner *et al*. 1997; Karagiannis and Brozovich 2004).

In situ hybridization of staged mouse embryos revealed that SM-MHC transcripts first appear at 10.5 dpc in the developing aortas. At 12.5-13.5 dpc, the SM-MHC mRNA appears in smooth muscle cells of the developing gut and lungs as well as peripheral blood vessels. At 17.5 dpc, SM-MHC transcripts have accumulated in esophagus, bladder, and ureters (Miano *et al*. 1994; Loukianov *et al*. 1997; Babu *et al*. 2000). SM1 myosin predominantly expresses during early stages of smooth muscle development whereas SM2 myosin appears only during the postnatal period (Aikawa *et al*. 1993; Loukianov *et al*. 1997; Babu *et al*. 2000). SM-A myosin accumulates more postpartum whereas the ratio of SM-B myosin rises gradually in accompany with fetal development (Low and White 1998; White *et al*. 1998; Babu *et al*. 2000). SM-A mRNA expresses predominantly in vascular smooth muscle tissues, and SM-B mRNA is found primarily in visceral smooth muscle tissues (Loukianov *et al*. 1997). SM-B mRNA expression is relatively robust in bladders of all embryonic, neonatal, and adult mice (Haase and Morano 1996; Loukianov *et al*. 1997; Low and White 1998; Wetzel *et al*. 1998; White *et al*. 1998; Arafat *et al*. 2001), and it contributes to the greater rate of force generation, which is necessary for rapid voiding (Low *et al*. 2006).

SM-MHC transcripts can be altered in different pathophysiological conditions. Balloon injury as well as atherosclerosis leads to neointimal smooth muscle cells expressing SM1 but not or very little SM2 isoforms (Zanellato *et al*. 1990; Kuro-o *et al*. 1991). Bladder-outlet obstruction leads to the ratio of SM1/SM2 changing (Cher *et al*. 1996). Hirschsprung's disease as well as hypertrophied urinary bladder leads to down regulation of SM-B isoform (Sjuve *et al*. 1996; Siegman *et al*. 1997). It is to be noted that the loss of one or the whole isoforms would affect the contractile function in some smooth muscle especially in the bladder and could even lead to death within various days of birth based on different extent of the isoforms loss (Babu *et al*. 2000; Morano *et al*. 2000; Babu *et al*. 2001; Low *et al*. 2006; Chi *et al*. 2008).

We obtained a *Myh11* insertional mutation mouse from another transgenic mice study in our lab. The insertion of the exogenous DNA made it unable to produce *Myh11* mRNA and express the SM-MHC protein. For the previous reports mainly revealed the mutational lesion in newborn and infant mice (Morano *et al*. 2000; Chi *et al*. 2008), we further investigated the changes of SM-MHC$^{-/-}$ fetuses during mouse development in the current study. Besides the mutation leading to death soon after birth with the pathological symptom of a huge gas bubble in the stomach as well as a thin-walled giant bladder full of urine, the results mainly revealed that the smooth muscle layer lead to dysplasia of embryonic bladder was impaired from 15.5 dpc initially.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma-Aldrich unless otherwise stated.

DNA analysis

Genomic DNA from the mouse tails was extracted utilizing the standard phenol-chloroform method and dissolved in TE buffer (pH 7.4) for gene analysis. Thermal asymmetric interlaced PCR (TAIL-PCR) was used to define the chromosomal boundaries of the insertional site of exogenous DNA as described in our previous studies (Yan *et al*. 2010; Yan *et al*. 2013). In brief, TAIL-PCR contains three cycles and each amplification used an arbitrary degenerate primer (5´-STTGNTASTNCTNTGC-3´) and the other is orderly one of the nested primers specific for poly A in 3´of exogenous DNA (F1: 5´-CCTTGAGCATCTGACTTCT-3´; F2: 5´-GTTGGCTATAAAGAGGTCATCAG-3´; F3: 5´-CCCTGCTGTCCATTCCTT-3´). The detail of amplification programs as described in our previous study (Yan *et al*. 2013). Amplified PCR products in the tertiary cycling was sequenced and BLAST analyzed. After the 3´ exactly position of exogenous DNA was obtained, the template genomic DNA was amplified with a primer (5´-TATGGTGCCCACTCCCAGTA-3´) in mouse *Myh11* gene and a primer (5´-GCCAAGTGGGCAGTTTA-3´) in CMV enhancer of exogenous DNA. The resulting fragment was sequenced for the 5´insertion site determination.

Animals and tissue preparation

Mice were maintained in a light-controlled room (14L:10D, lights on at 0500 h) at a temperature of 22°C. All animal procedures in the present study were approved by the Committee for Experimental Animals of China Agricultural University. Each mouse embryos at 14.5, 15.5, 16.5, 17.5, 18.5, or 19.5 dpc were respectively isolated and timely weighted and then samples of tail for DNA extraction for genotyping was carried out. The bladder and stomach were dissected from each embryo under anatomical lens and placed in PBS (pH 7.4) buffer. Half of the bladder and stomach tissues were stored in -80°C for RNA and protein analysis. The other immersed in PBS containing 4% formaldehyde at 4°C for histological analysis.

Genotyping

Genomic DNA of embryos or neonates generated from SM-MHC$^{+/-}$ parents mating were amplified with the primer pairs (forward: 5´-TAACCATGTTCATGCCTTCTTC-3´; reverse: 5´-CACCTTGTTGTAGTGTCCGTTT-3´) for a 514 bp fragment that spanned the partial exogenous DNA or with the primer pairs (forward: 5´-TATGGTGCCCACTCCCAGTA-3´; reverse: 5´-GGCGTGTGGTTACTGACTGA-3´) for a 448 bp murine genomic *Myh11* fragment. The above two fragments were amplified at the same conditions and the PCR amplification was performed under the following conditions: 95°C for 5 min; 32 cycles of 94°C for 30 sec, 62°C for 30 sec, and 72°C for 1 min; and a final extension at 72°C for 10 min.

RT-PCR

Total RNA was extracted from each bladder or stomach using RNAiso Plus (TaKaRa) and treated with DNase I (TaKaRa). The purified RNA was used for first-strand cDNA synthesis, and reverse transcription was performed using an M-MLV reverse transcriptase with oligo-dT primers (Promega). To avoid genomic contamination, reactions were also performed in the absence of reverse transcriptase for each RNA sample tested. The resulting cDNA was used for PCR amplification with the *Myh11* specific primers (forward: 5´-CAGGAGCAGTTGCAGGCAGAG-3´; reverse: 5´-CAAGATGTCATCCTCCAGTTTC-3´) that produced a 288 bp fragment. The heavy polypeptide 9 (*Myh9*) specific primers (forward: 5´-GGGAGTCTGGAGCAGGGAA-3´; reverse: 5´-CGTTGGACAGGAAGCGGTA-3´) that produced a 396 bp fragment. PCR amplification was performed as follows: 95°C for 5 min; 32 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 1 min; and a final extension at 72°C for 10 min. The calponin 1 (*Cnn1*) specific primers (forward: 5´-ACCAACCATACACAAGTTCAGTCCA-3´; reverse: 5´-TCCAATGATGTTCCTGCCTTCTCTC-3´) that produced a 153 bp fragment. The alpha smooth muscle actin (*Acta2*) specific primers (forward: 5´-TCAGGGAGTAATGGTTGGAATGGGC-3´; reverse: 5´-ACAATCTCACGCTCGGCAGTAGTCA-3´) that produced a 507 bp fragment. PCR amplification was performed as follows: 95°C for 5 min; 32 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min; and a final extension at 72°C for 10 min.

Western blot

Proteins extracted from bladder or stomach tissues with 2 × SDS loading buffer were transferred onto nitrocellulose membranes (Millipore, USA) after SDS-PAGE separation (8% separated gel for SM-MHC and non-muscle myosin IIA (NMHC IIA); 12% separated gel for calponin 1 and alpha smooth muscle actin (α-SMA)). Each of the four nitrocellulose membrane was blocked with 5% skimmed milk and incubated with the rabbit polyclonal anti-SM-MHC antibodies (Abcam, 1:3000), rabbit polyclonal anti-NMHC IIA antibodies (Abcam, 1:1000), rabbit monoclonal anti-calponin 1 antibodies (Abcam, 1:7000), or rabbit monoclonal anti-α-SMA antibodies (Abcam, 1:3000) to detect

the respective antigen followed by the secondary goat anti-rabbit antibodies (Abcam, 1:2000). Proteins were visualized using enhanced chemiluminescence (Thermo) on X-ray film (Kodak).

Histologic analysis

Histologic analysis was performed following the basic methods described in our previous study (Li *et al*. 2015). In brief, bladder and stomach tissues were fixed by immersion in 4% formaldehyde in PBS at 4°C and then dehydrated before embedding in paraffin. Tissue sections were dewaxed in xylene followed by rehydration, and histological details were observed after staining with hematoxylin-eosin.

Statistical analysis

All values are presented as the means ± SD. The statistical significance was carried out using the Student's *T*-test. Differences of $P < 0.05$ were considered to be significant.

Results

The identification of SM-MHC$^{-/-}$ mice

TAIL-PCR and BLAST analysis displayed that the exogenous DNA was integrated within the intron between exon 22 and 23 of murine *Myh11* gene. The insertion site was 864 bp away from the end of exon 22 and 15 bp away from the start of exon 23 (Fig 1a). The insertional mutation resulted in no *Myh11* transcript was detected by RT-PCR (Fig 1b) and no SM-MHC protein was detected by western blot in bladder and stomach tissues from the mutated fetuses at 15.5 or 19.5 dpc by comparison with the samples of SM-MHC$^{+/-}$ and SM-MHC$^{+/+}$ mice (Fig 1c). Additionally, the results indicated that the *Myh11* mRNA and its encoded protein expressed early at 15.5 dpc in bladders and stomachs of SM-MHC$^{+/-}$ or wild-type fetuses. On the other hand, the RT-PCR results revealed that the *Myh11* insertional mutation had no effect on normal transcription of the other muscle contraction genes such as *Myh9*, *Cnn1*, and *Acta2*. Western blot analysis found theirs encoded NMHC IIA, calponin 1, α-SMA proteins expressed normally in bladder and stomach tissues (Fig 1d).

Survival time of SM-MHC$^{-/-}$ neonatal mice

All SM-MHC$^{-/-}$ neonatal mice died within 20 h after birth with a higher mortality rate within the front 10 h whereas the lifetime of SM-MHC$^{+/-}$ mice appeared to be normal. Either after breast-feeding (Fig 2a, 2b) or in the state of hunger (Fig 2c, 2d), the SM-MHC$^{-/-}$ neonatal mice had large round bellies (Fig 2a, 2c), thin-walled giant bladders, and large stomachs with huge gas bubbles (Fig 2b, 2d) compared to the SM-MHC$^{+/-}$ and SM-MHC$^{+/+}$ neonatal mice.

Genotype ratio and average body weight of SM-MHC$^{-/-}$ mice

Genotyping and average body weight of the SM-MHC$^{-/-}$ fetuses were investigated from 16.5 dpc to birth. Embryos at all stages were of the approximately expected 1:2:1 ratio for Mendelian inheritance (Table 1) and no significant ($p > 0.05$) difference in average body weight were detected among SM-MHC$^{-/-}$, SM-MHC$^{+/-}$, and SM-MHC$^{+/+}$ fetuses (Table 2).

Bladder developing lesion of SM-MHC$^{-/-}$ fetuses

We further observed the exterior appearance of bladders from 14.5 dpc to newborn and discovered no different physical appearance at 14.5 and 15.5 dpc among SM-MHC$^{+/+}$, SM-MHC$^{+/-}$, and SM-MHC$^{-/-}$ bladders whereas an abnormal bladder was observed in each SM-MHC$^{-/-}$ fetus from the stages of 16.5 dpc to newborn (Fig 3). At 16.5 dpc, the volume of bladders among SM-MHC$^{+/+}$, SM-MHC$^{+/-}$, and SM-MHC$^{-/-}$ groups were not significantly different but the exterior appearance of SM-MHC$^{-/-}$ bladders were transparent and filled with urine. Moreover, along with the continuous accumulation of urine in the SM-MHC$^{-/-}$ bladders from the stage of 17.5 dpc, theirs cavity volume went up by multiple times in contract to the normal SM-MHC$^{+/-}$ and SM-MHC$^{+/+}$ bladders.

Histological analysis

Histological analysis of bladders showed that no different pathological changes at 14.5 dpc among SM-MHC$^{+/+}$, SM-MHC$^{+/-}$, and SM-MHC$^{-/-}$ bladders. However, in each SM-MHC$^{-/-}$ bladders, a thin layer of smooth muscle and a giant cavity surfaced at the beginning of 15.5 dpc (Fig 4). At 15.5 dpc, the SM-MHC$^{-/-}$ bladders had the same cross sectional area as similar size to the normal SM-MHC$^{+/-}$ and SM-MHC$^{+/+}$ bladders but apparently theirs cavity volume became larger.

On the other hand, no distinct difference was observed in the smooth muscle layer among SM-MHC$^{+/+}$, SM-MHC$^{+/-}$, and SM-MHC$^{-/-}$ stomachs of mice from the stage of 14.5 dpc to newborn.

Discussion

During the study of the insertional site of transgenic mice in our lab, we obtained the insertional mutated mouse model whose *Myh11* gene on chromosome 16 has been disrupted completely. Analysis of the insertional site of the exogenous DNA revealed that microhomology was 3 bp long in the 5′ and 2 bp long meanwhile 4 bp deletion in the 3′ , it follows the mechanism of DNA repair via homologous illegitimate random integration in our previous studies (Yan *et al*. 2010; Yan *et al*. 2013). Consequently, the exogenous DNA integrated in the intron of *Myh11* resulted in the loss of SM-MHC protein in bladder and stomach at 15.5 dpc, although the SM-MHC proteins were normally existed in the tissues of bladder and stomach from wild-type fetus. The previous study also suggested that the SM-MHC transcripts had accumulated in the bladder at 17.5 dpc (Miano *et al*. 1994).

It was reported that the loss of SM-B had no effect on the survival and reproduction of mice (Babu *et al*. 2001), however, loss of SM2 could lead to the death of juvenile mice within 30 days after birth

(Chi *et al*. 2008) or the whole SM-MHC could lead to the death of neonatal mice within 12-24 h after birth respectively (Morano *et al*. 2000). Furthermore, the contractile function of bladders were abnormal and even produced some pathological changes such as urinary retention in the juvenile mice which lacked SM2 (Chi *et al*. 2008) and a giant, thin-walled bladder in the neonatal SM-MHC$^{-/-}$ mice (Morano *et al*. 2000). In the present study, the SM-MHC$^{-/-}$ neonates died within 20 h of birth and showed a higher mortality rate within the front 10 h. They had large round bellies, thin-walled giant bladders filled with urine although the lifetime and appearance of SM-MHC$^{+/-}$ mice were fully recovered and showed no different from that of wild type. Moreover, we also observed that the SM-MHC deficient neonates had large stomachs with huge gas bubbles. It might be caused by the subdued wriggle of gastrointestinal tract with no SM-MHC.

In the previous study, the SM-MHC$^{-/-}$ neonates delivered at the expected ratio of Mendelian inheritance and had normal body weight, it suggested that the loss of SM-MHC proteins did not affect the normal fetal development (Morano *et al*. 2000). For the expression of SM-MHC was as early as from the stage of 10.5 dpc, we further investigated the potential changes from 16.5 dpc to birth and no any difference in the ratio of Mendelian inheritance and average body weight was detected during the later fetal development. It indicated that the whole SM-MHC deficiency during development was not harmful to fetal survival and its growth exhibited normally.

In the present study, the insertional site of exogenous DNA was located in the *Myh11* gene that it spanned from 14.1 Mb to 14.3 Mb on chromosome 16. Besides larger stomach with huge gas bubbles, this mutation mainly resulted in the thinner layer of smooth muscle in bladder wall from the beginning of 15.5 dpc and the bladder distension from 17.5 dpc and all died within 20 h of birth. Importantly, the above deficiency could be fully restored in SM-MHC$^{+/-}$ mice. Interestingly, in another insertional mutation mouse exhibited megabladder, whose genome sequence spanning from 26.6 and 27.5 Mb on chromosome 16 was interrupted by an exogenous DNA (Singh *et al*. 2007) and its *Myh11* gene was intact apparently, but the thin bladder at 15 dpc and the severe bladder distension from 18 dpc were also observed. Additional, rarely mgb$^{-/-}$ mice survived beyond 4 to 6 week of age and the mgb$^{+/-}$ bladders displayed an intermediate smooth muscle phenotype (Singh *et al*. 2007). This results suggest that more factors from 14.1 to 27.5 Mb on chromosome 16 might involve in the normal bladder development. At least, the respective roles of *Myh11* gene and sequence from 26.6 to 27.5 Mb in developing bladder need to be clarified.

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Figure 1. Exogenous DNA is integrated in the intron between exon 22 and 23 of *Myh11* gene and the insertion site is 864 bp away from the end of exon 22 and 15 bp away from the start of exon 23 (a). RT-PCR (b) and Western blot (c) analysis of *Myh11* transcripts and its encoded SM-MHC protein in the samples of bladder and stomach from developing fetuses at 15.5 and 19.5 dpc. A specific fragment (288 bp) of the *Myh11* cDNA spanned from exon 22 to exon 23 is only amplified in SM-MHC$^{+/+}$ and SM-MHC$^{+/-}$ samples, not in SM-MHC$^{-/-}$ samples (b). Consequently, the SM-MHC antigens (228 kDa) are only detected in samples from SM-MHC$^{+/+}$ and SM-MHC$^{+/-}$, not from SM-MHC$^{-/-}$ mice (c). RT-PCR and Western blot analysis of *Myh9*, *Cnn1* and *Acta2* transcripts and theirs encoded NMHC IIA, calponin 1, α-SMA proteins in the samples of bladder and stomach from developing fetuses at 18.5 dpc (d).

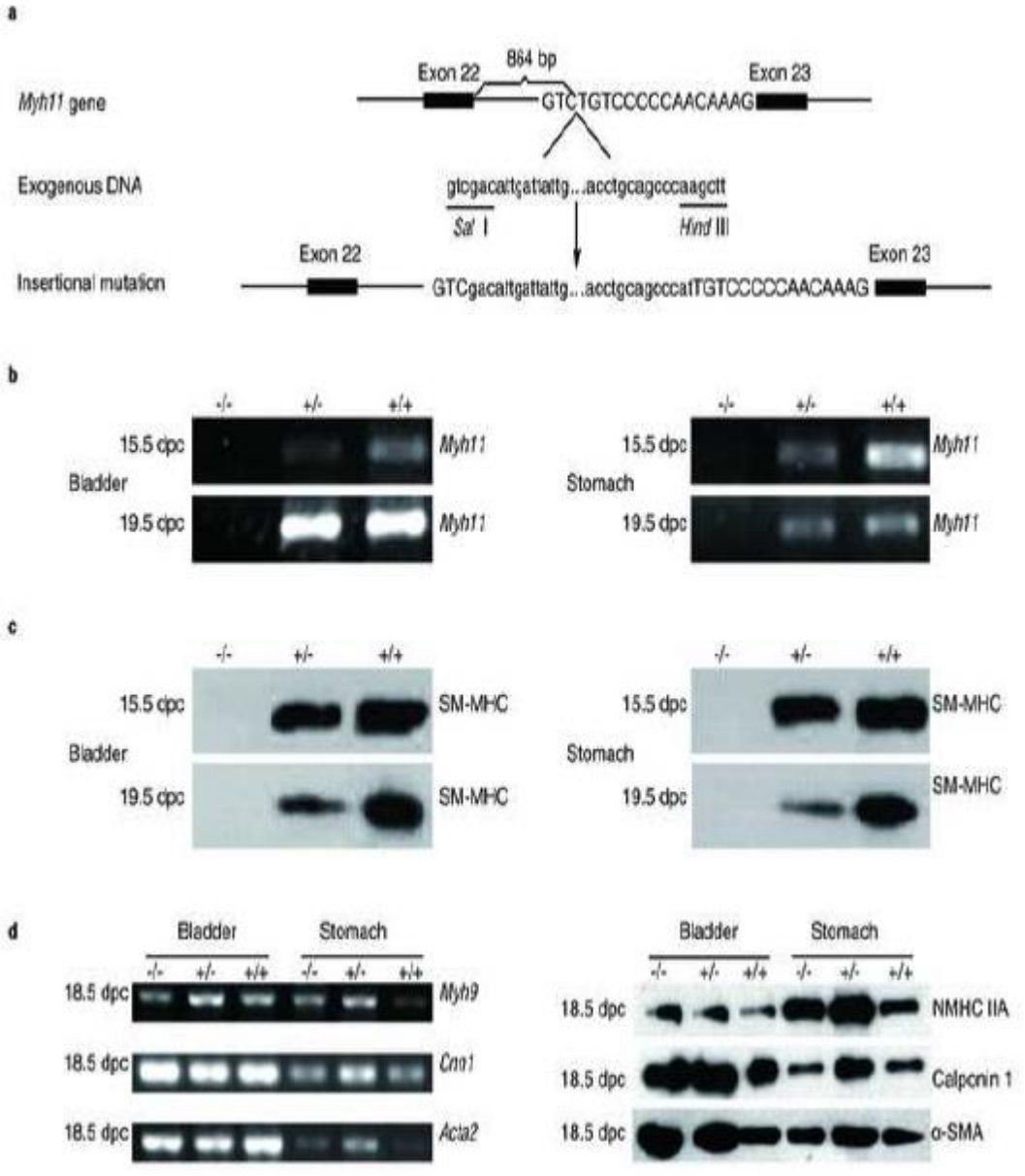

Figure 2. Photographs of SM-MHC$^{+/+}$, SM-MHC$^{+/-}$, and SM-MHC$^{-/-}$ neonatal mice at 12 h post-birth in the state of breast-fed (a, b) or of hunger (c, d). SM-MHC$^{-/-}$ mice have large round bellies (a and c), giant bladders full of urine, and large stomachs with huge gas bubbles (b, d) by comparison to the other two normal genotypes. Arrows indicate stomachs, and circles indicate bladders.

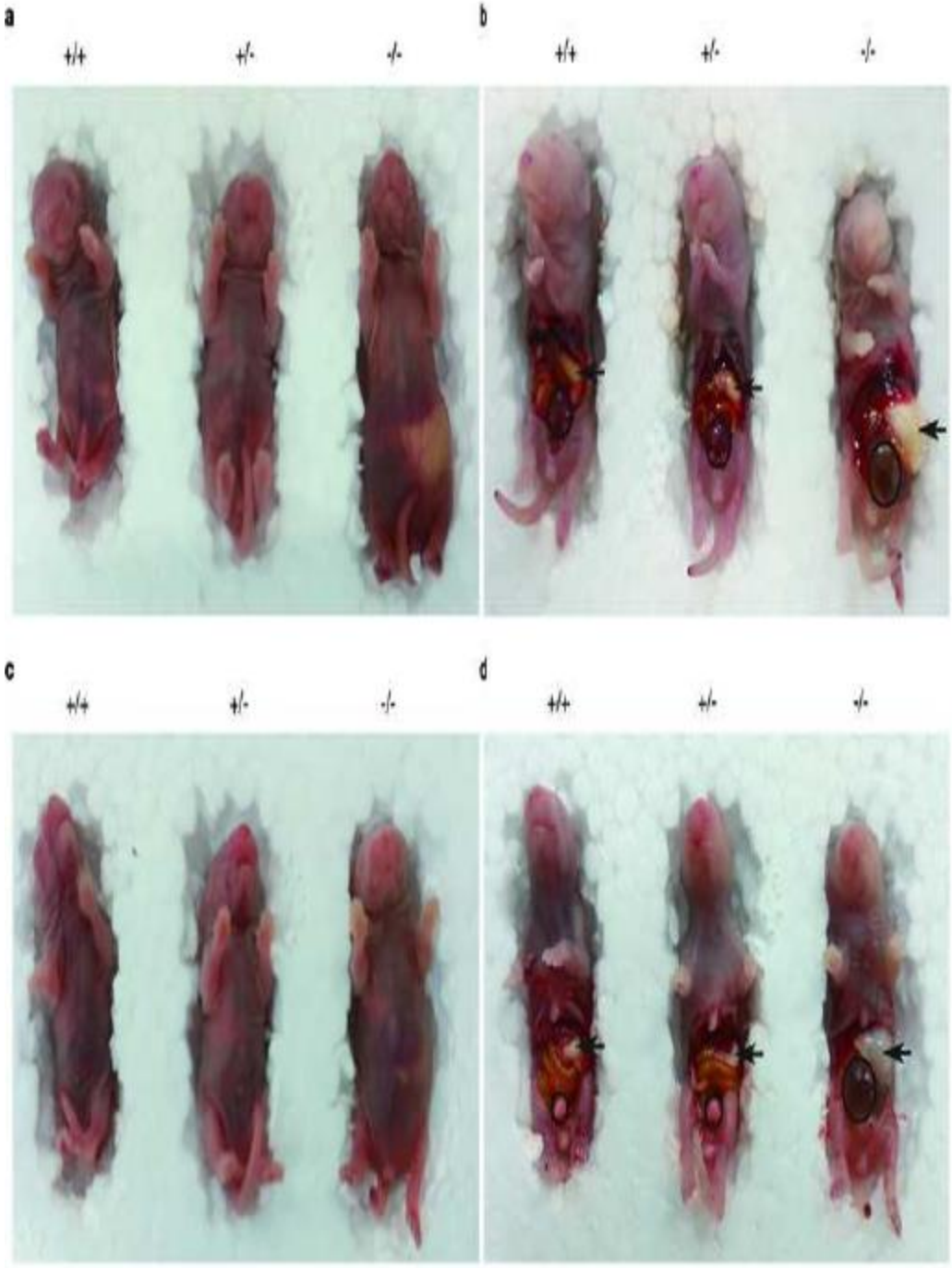

Figure 3. Abnormal development of SM-MHC$^{-/-}$ bladders. No different physical appearance shown at 14.5 and 15.5 dpc among SM-MHC$^{+/+}$, SM-MHC$^{+/-}$, and SM-MHC$^{-/-}$ bladders whereas the SM-MHC$^{-/-}$ bladder is filled with urine and becomes transparent at 16.5 dpc and its cavity volume gets up by multiple times from the stages of 17.5 dpc by comparison to the normal SM-MHC$^{+/-}$ and SM-MHC$^{+/+}$ bladders.

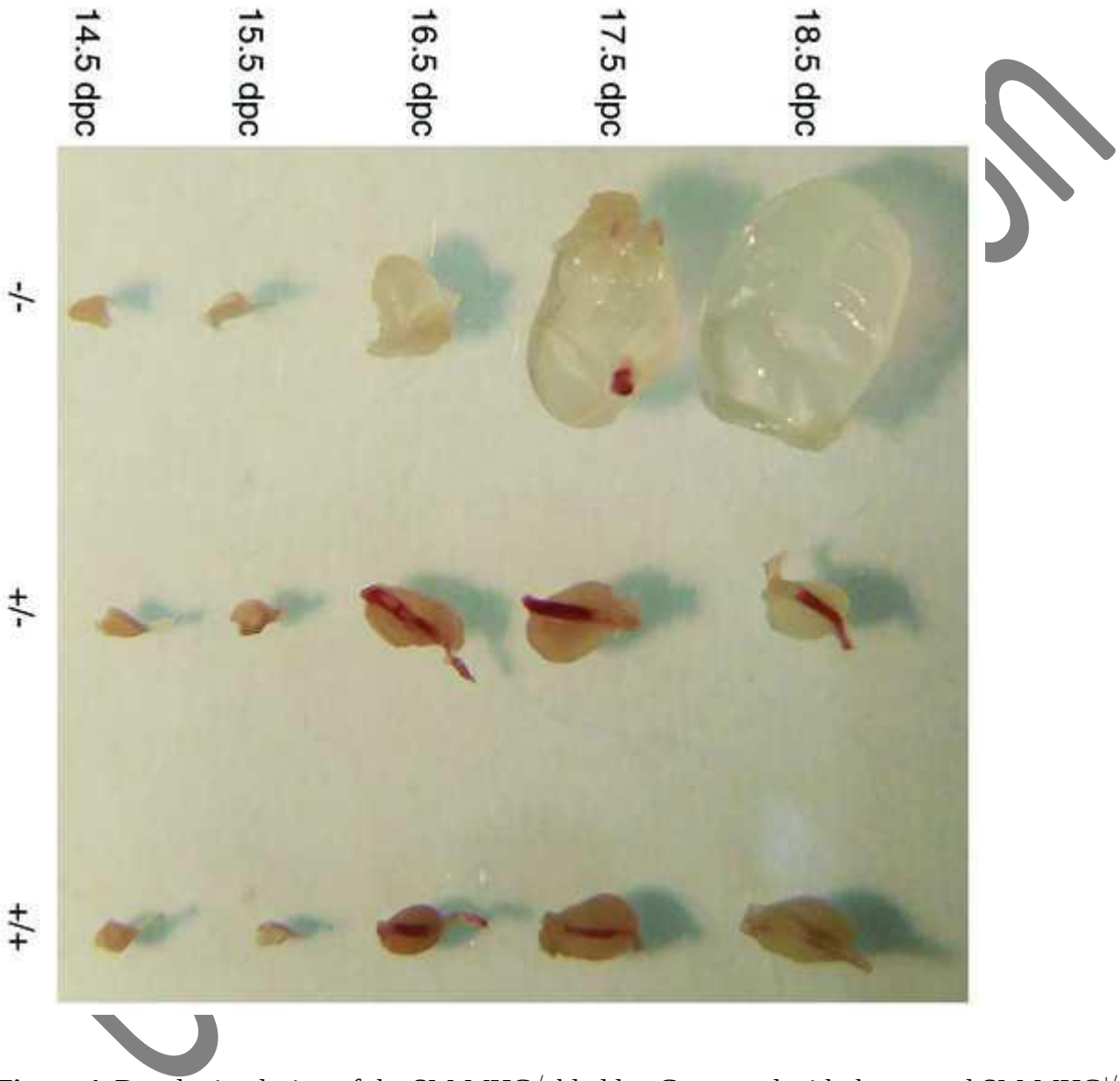

Figure 4. Developing lesion of the SM-MHC$^{-/-}$ bladder. Compared with the normal SM-MHC$^{+/+}$ as well as SM-MHC$^{+/-}$ bladders, the SM-MHC$^{-/-}$ bladders showed no any pathological changes at 14.5 dpc whereas it becomes thin-walled and giant gradually in accompany with its thinning smooth muscular layer during fetal development at the beginning of 15.5 dpc. Scale bar represents 200 μm.

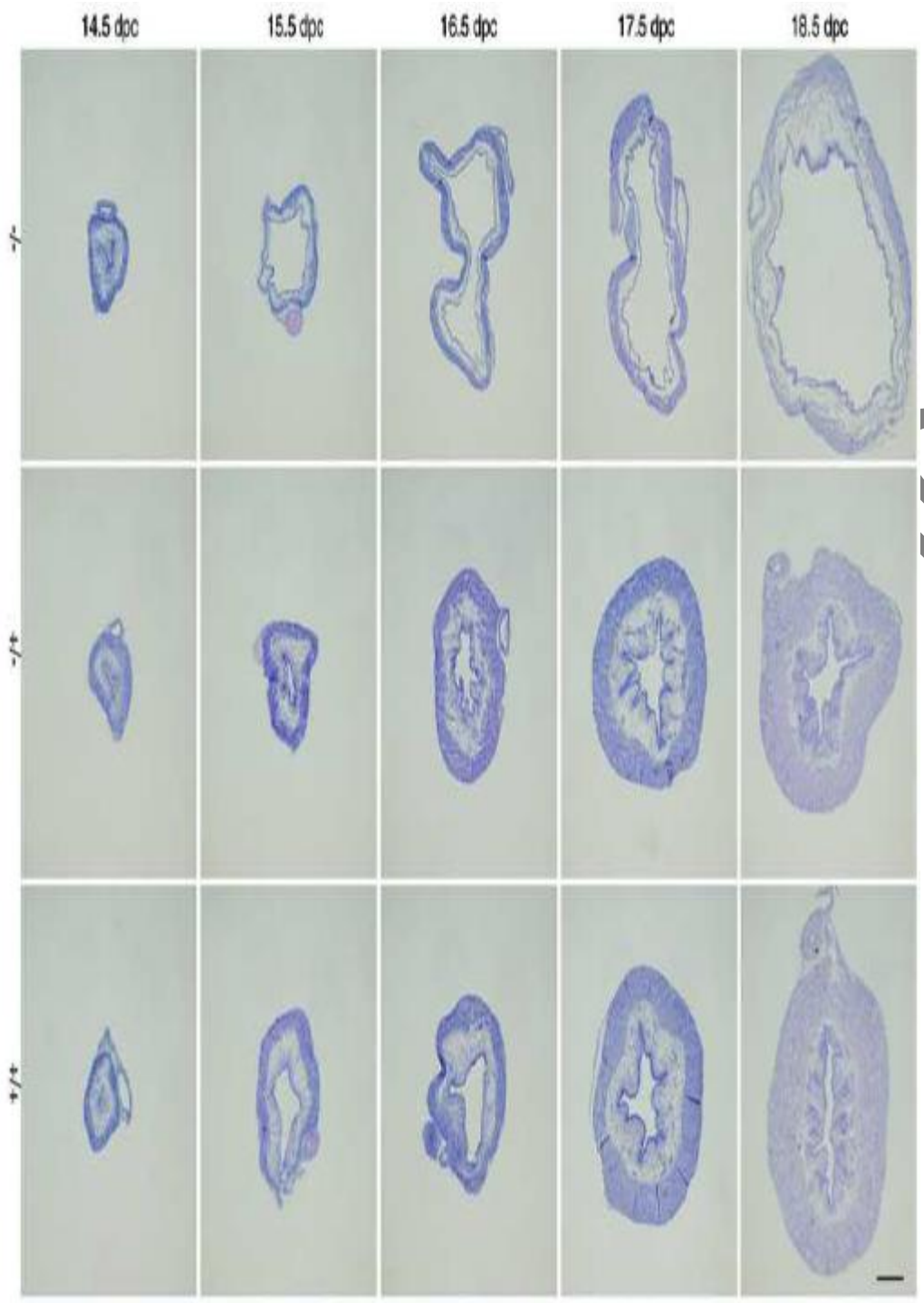

Table 1 Comparsion of Mendelian inheritance ratio of SM-MHC$^{-/-}$ mice.

	Embryonic developmental days post coitum				Birth	Total
	16.5 (6*)	17.5 (5*)	18.5 (7*)	19.5 (4*)	(9*)	(31*)
SM-MHC$^{+/+}$	23	15	20	13	25	96
SM-MHC$^{+/-}$	43	39	57	25	62	226
SM-MHC$^{-/-}$	18	19	23	12	31	103
Approximate ratio	1.3:2.4:1	0.8:2.1:1	0.9:2.5:1	1.1:2.1:1	0.8:2:1	0.9:2.2:1

Note: *, Number of litter counted.

Table 2 Comparasion of average body weight (g) of SM-MHC$^{-/-}$ mice.

	Embryonic developmental days post coitum				Birth (9*)
	16.5 (6*)	17.5 (5*)	18.5 (7*)	19.5 (4*)	
SM-MHC$^{+/+}$	0.75 ± 0.11	1.14 ± 0.10	1.38 ± 0.26	1.78 ± 0.20	1.81 ± 0.12
SM-MHC$^{+/-}$	0.81 ± 0.10	1.11 ± 0.15	1.41 ± 0.19	1.82 ± 0.13	1.76 ± 0.12
SM-MHC$^{-/-}$	0.74 ± 0.08	1.07 ± 0.14	1.38 ± 0.22	1.82 ± 0.18	1.82 ± 0.15

Note: Each value represents the mean ± SD. *, Number of litter counted.