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ORIGINAL ARTICLE

Spatiotemporal pattern analysis of transcription factor 4 in the developing anorectum of the rat embryo with anorectal malformations

Tao Zhang · Yu Zuo Bai · Da Jia Wang · Hui Min Jia · Zheng Wei Yuan · Wei Lin Wang

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Abstract
Purpose As a member of the transcription factors family, transcription factor 4(Tcf4) is known to influence gene expression in endodermally derived tissues including lung, liver, pancreas, stomach, and intestine. However, it remained unknown if this capability is active during anorectal development in the normal and anorectal malformations (ARM) rat embryos.
Materials and methods In this study, ethylenethiourea (ETU)-induced ARM model was introduced to investigate the expression pattern of Tcf4 during anorectal development using immunohistochemical staining, reverse transcriptase polymerase chain reaction (RT-PCR), and Western blot analysis.
Results Immunostaining revealed that Tcf4 expression showed space-dependent changes in the developing anorectum: in normal embryos, Tcf4 protein is initially expressed in the dorsal endoderm of urorectal septum (URS) and hindgut on embryonic day 13 (E13). Additionally, separate expression domain develops intensively on the dorsal CM on E14. On E15, positive cells are then detected in the fused tissue of URS, and prominently in the anal membrane. In the ARM embryos, however, the epithelium of the cloaca, URS, and anorectum was negative or faint for Tcf4. In Western blot and RT-PCR, time-dependent changes of Tcf4 protein and mRNA expression were remarkable during the anorectal development: on E14, E14.5, and E15, the expression level reached the peak; after E16, Tcf4 expression gradually decreased. In contrast, in ARM embryos, spatiotemporal expression of Tcf4 was imbalanced during the anorectal morphogenesis from E13 to E16.
Conclusions These data implied that the downregulation of Tcf4 at the time of cloacal separation into rectum and urethra might be related to the development of ARM.

Keywords Anorectal malformations · Tcf4 · Embryogenesis · Rat

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Introduction

Anorectal malformations are among the most common congenital anomalies, occurring in approximately 1/5,000 to 1/1,500 live births [1], which influenced the quality of life of the patients severely [2–4]. However, as ARM is a multigenerational complex disease, the etiology, embryology, and pathogenesis of ARM were poorly understood and controversial; it is impetuous to research into the developmental bases of normal and abnormal anorectal organogenesis. In previous studies, ARM is frequently associated with mutations of genes involved in Wnt signal transduction cascades. Wnt signal pathways play a critical role in both initiating and patterning morphological adaptations of anorectum [5]. Wnts exert their biological effects by activating the target genes that preserve the consensus motif (A/TA/TCAAAG) for Tcf4 binding in the promoter region [6, 7]. Therefore, Tcf4 may play an important role in activation of target genes to exert the biochemical functions of its downstream genes.

In addition, phenotypic analyses of Tcf4 mutant mice have shown that Tcf4 signaling influences the development

of many organ systems, especially of the terminal colon. $Tcf4^{-/-}$ mice exhibit severe caudal truncations of the embryonic axis and various defects of the urogenital system and the hindgut, indicating that Tcf4 may be an endodermal signal essential for the development of these hindgut derivatives. Whereas more severe abnormalities include a complete absence of the genital tubercle, hindgut, as well as signs of aberrant branching of neural tube [8]. These observations illustrate the importance of gene dosage in the Tcf4 signaling pathway during anorectal development and perhaps indicate that defective Tcf4 signaling can lead to the dysplasia of anorectum. Moreover, the ETU-induced ARM rat model has been used for the study of the ARM and the related abnormalities. The spectrum of ARM induced by this model has a striking similarity to those seen in $Tcf4^{-/-}$ mutant mice and in humans with anorectal abnormalities. However, expression patterns of Tcf4 have not been described in the embryogenesis of ARM ever before. To provide insights into the role of Tcf4 in anorectal embryogenesis, in the current study, we analyzed the distribution of Tcf4 mRNA and protein in the rat anorectum at different developmental stages to determine its temporospatial distribution and the relationship of expression with the developmental stage E13–E16, which are the critical time points in the anorectal development.

Materials and methods

Animal model and tissue collection

The study conformed to the principles of laboratory animal care of China Medical University, which were consistent with the World Medical Association Declaration of Helsinki, and were approved by the Experimental Animal Committee and Ethics Committee at China Medical University (no. 200 (7) PS14). Mature Wistar rats (body weights from 250 to 300 g) were provided by the Medical Animal Center, Shengjing Hospital of the China Medical University. The procedures for creating ARM in fetal rats were described previously [9, 10]. Eighty-five time-mated pregnant Wistar rats were randomly divided into two groups: ETU-treated group and control group. In the ETU-treated group, 55 pregnant rats were gavage-fed a single dose of 125 mg/kg of 1% ETU (2-imidazolidinethione, Aldrich Chemical Co., Germany) on E10 (E0 sperm in vaginal smear after overnight mating). Thirty control rats received corresponding doses of ETU-free saline on E10. Embryos were harvested by cesarean section on E13 to E16 and E18, E21, respectively. Approximately one-third of the embryos were fixed in 4% paraformaldehyde for 12 to 24 h depending on their size. Then the embryos from each age group were dehydrated, embedded in paraffin, and sectioned serially sagittally at 4-μm thickness for immunohistochemical staining.

The presence of ARM can be determined under the light microscope prior to staining.Then embryos can be divided into normal and ARM group. Other specimens were frozen in liquid nitrogen. Sequentially sagittal sections were cut on a cryostat and mounted on coated glass slides, examined under the microscope to confirm the occurrence of ARM in ETU-treated embryos. Thus, embryos with or without ARM can be distinguished by light microscopy prior to the microdissections. Then the cloaca from E13 to E14 was dissected and removed free from surrounding tissues under magnification and immediately frozen in liquid nitrogen to prepare for RT-PCR and Western blot analysis, and the full-thickness rectum was dissected on E15 to E21. Micro-dissections were performed on the same areas in normal embryos.

Immunohistochemical staining

The endogenous peroxidase activity was blocked by incubation in 3% H_2O_2 for 10 min. Antigen retrieval was performed by heating the slides in 10 mM citrate buffer (pH 6.0) at 98°C for 10 min. The sections were treated and incubated with primary Anti-Tcf4 (1:200, goat polyclonal, Santa Cruz Biotechnology, CA, USA) and HRP-conjugated secondary antibody (Santa Cruz). Antibody incubations were performed in PBST (PBS+0.1% Triton X), supplemented with 10% rabbit serum. Primary antibody was incubated with sections at 4°C for 16 h. Incubation of secondary antibody was performed for 10 min at room temperature (RT). The immunoreactions were visualized using DAB (Sigma, UK) as chromogen. Sections were counterstained with hematoxylin. In all experiments, the extent of non-specific binding of the antisera was evaluated by omitting the primary antibody step. HeLa were used for the positive control. Two pathologists independently reviewed the immunohistochemical-stained slides and agreed on diagnoses by consensus.

Table 1 The distribution of embryos in different age and treatment groups

Age group	Normal			ARMs		
	IHC	WB	RT-PCR	IHC	WB	RT-PCR
GD13	23	27	24	29	28	20
GD13.5	22	25	23	25	29	21
GD14	23	24	22	23	25	19
GD15	21	19	20	20	19	17
GD16	23	18	20	17	21	15
Total	112	113	109	114	122	92

Protein preparation and Western blot

Protein preparation was performed as described previously [11]: the cloaca/hindgut per condition were pooled and sonicated in ddH_2O containing protease inhibitors. Protein extracts (50 μg) were heated at 90°C for 10 min and size fractionated on Bis–Tris SDS-PAGE gels (Invitrogen, CA, USA). Protein samples were denatured, separated by SDS-PAGE, and transferred to PVDF membranes (Millipore, Billerica, MA, USA), blocked with 5% fat-free milk in Tris-buffered saline (1 h, RT), and incubated overnight at 4°C in primary antibody against Tcf4 (diluted 1:500). The membrane was incubated with secondary antibody (diluted 1:3,000) and immunostained bands were detected with a ProtoBlot II AP System with a stabilized substrate (Promega). β-Actin protein was used as internal control.

RT-PCR

Specimens from cloaca and anorectal tissues were submerged in RLT buffer (Qiagen, Shanghai, China). After disruption with a mortar and pestle, samples were shredded

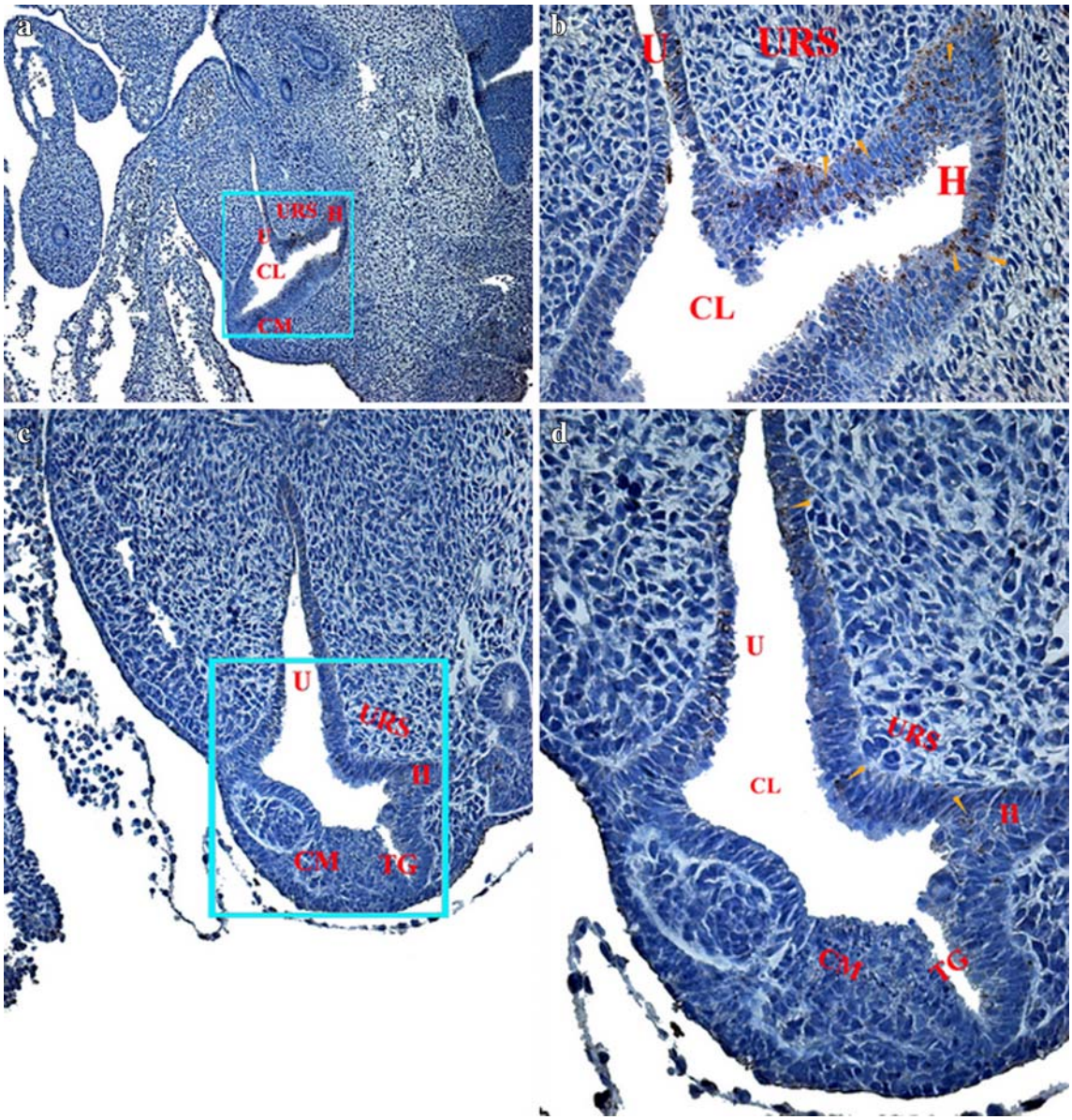

Fig. 1 (**a**) and (**b**) indicated the normal group; on E13, immunoreactivity specific to Tcf4 was detected most abundantly in the epithelium of the URS, the hindgut, and on the CM. The tailgut did not degenerate, and the CM was shorter and thicker than normal; few positive cells could be detected on the dorsal URS and the hindgut ((**a**) and (**c**), original magnification ×100; (**b**) and (**d**), original magnification ×400). *Orange arrowheads*: positive cells. *H* hindgut, *URS* urorectal septum, *CM* cloacal membrane, *CL* cloaca, *TG* tailgut

in a Qiagen shredder column for homogenization, and RNA was isolated according to the protocol of the RNeasy Mini Kit (Qiagen). Purity of extracted total RNA was determined as the 260/280-nm ratio with expected values between 1.8 and 2 and the RNA was then stored at −80°C. The total RNA was reverse transcribed into complementary DNA (cDNA) using SuperScript III First-Strand Synthesis System (Invitrogen Corporation) as per manufacturers' instructions. The primers used for Tcf4 RT-PCR were as follows: sense, 5′-ATGGAGCAATGGGCAGTC-3′; antisense, 5′-GGGTG GGTTCAAGTCAGG-3′ (191 bp). The primers used for β-actin RT-PCR were as follows: sense, 5′-CACCCT GTGCTGCTCACCGAGGCC-3′; antisense, 5′-CCACACA GATGACTTGCGCTCAGG-3′ (690 bp). The amplification protocol consisted of one cycle at 95°C for 2 min followed by 35 cycles at 94°C for 20 s, 59°C for 20 s, 72°C for 40 s, and final extension at 72°C for 5 min. All PCR products were separated by electrophoresis in 3.5% agarose gels and visualized by ethidium bromide staining. The densitometry of Tcf4 mRNA levels was normalized to those of β-actin, a housekeeping gene, and was expressed as a ratio.

Statistical analysis

All numerical data were presented as mean±SD. Statistical analysis was performed using the two-sample *t* test. *P* values of 0.05 or less were considered significant.

Results

General observations

In this study, malformations were not observed in the 335 embryos of the normal rats. Among the ETU-treated embryos, all 506 embryos had short or absent tail; 15 of the embryos died in utero. In this study, the incidence of ARM in ETU-treated embryos was 65.8% (333/506). The embryos for IHC,

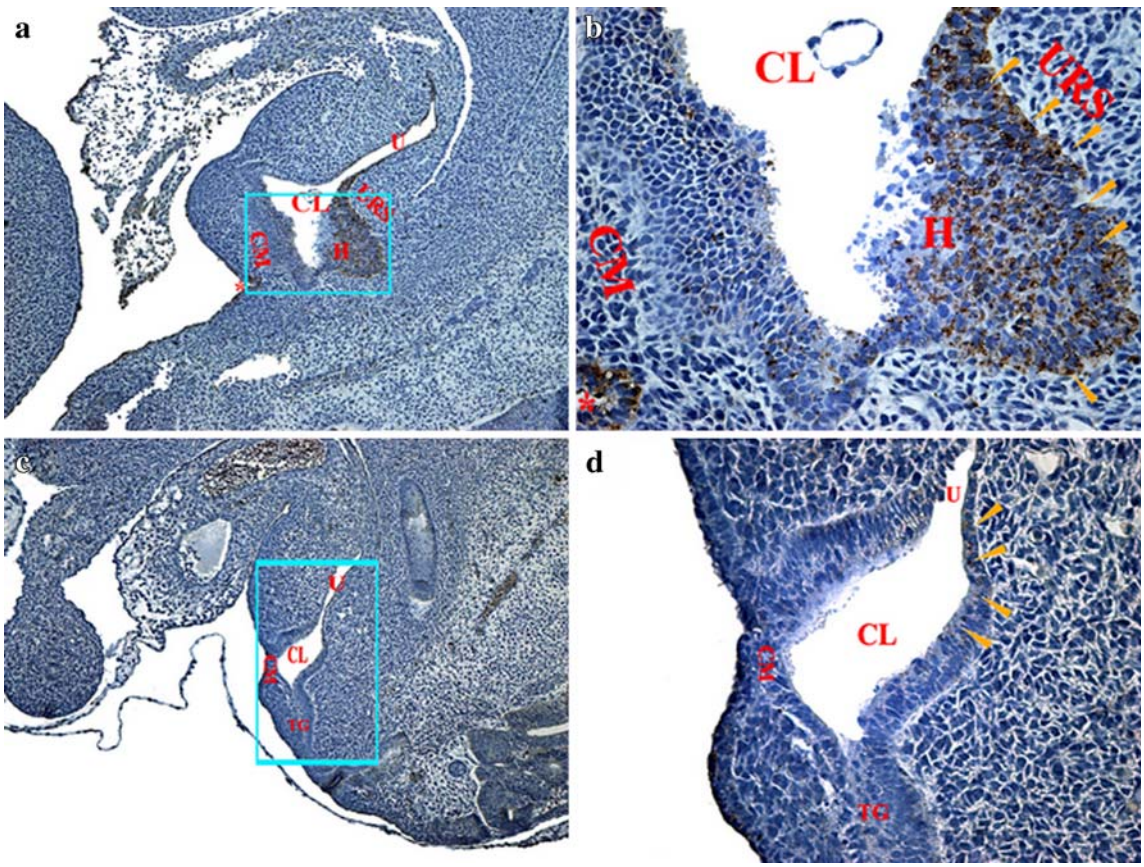

Fig. 2 (**a**) and (**b**) indicated the normal group; on E13.5, quantity of the Tcf4-labeled tissue could be detected on the epithelium of the dorsal URS and the epithelium of hindgut; (**c**) and (**d**) indicated the ARM group; on E13.5, the tailgut still existed. Cdx1 staining was abolished on the CM, the epithelium of ventral URS, and hindgut ((**a**) and (**c**), original magnification ×100; (**b**) and (**d**), original magnification ×400). *H* hindgut, *URS* urorectal septum, *CM* cloacal membrane, *CL* cloaca, *TG* tailgut

WB, and RT-PCR in each group are shown in Table 1. The type of ARM was rectourethral fistula or persistent cloaca.

Immunohistochemical results

In the normal group

On E13, the proximal tailgut has degenerated completely; L-shaped URS divided the cloaca into urogenital sinus ventrally and primitive rectum dorsally. Immunoreactivity specific to Tcf4 was detected most abundantly in the epithelium of the URS, hindgut, and CM (Fig. 1a, b). On E13.5, URS descended; cloacal canal appeared triangular. Quantity of the Tcf4-labeled tissue could be detected on the epithelium of the dorsal and the tip of URS and the hindgut. Additionally, the Tcf4 staining cells were noted in the tail groove, where it would be the location of the future anus (Fig. 2a, b). On E14, there was a potential canal between the tip of the URS and the CM. Plenty of the well-delineated Tcf4 cells were intensively detected on the dorsal CM where the URS was nearly fused with the CM. The intensity of the immunohistochemistry almost reached the peak (Fig. 3a, b).

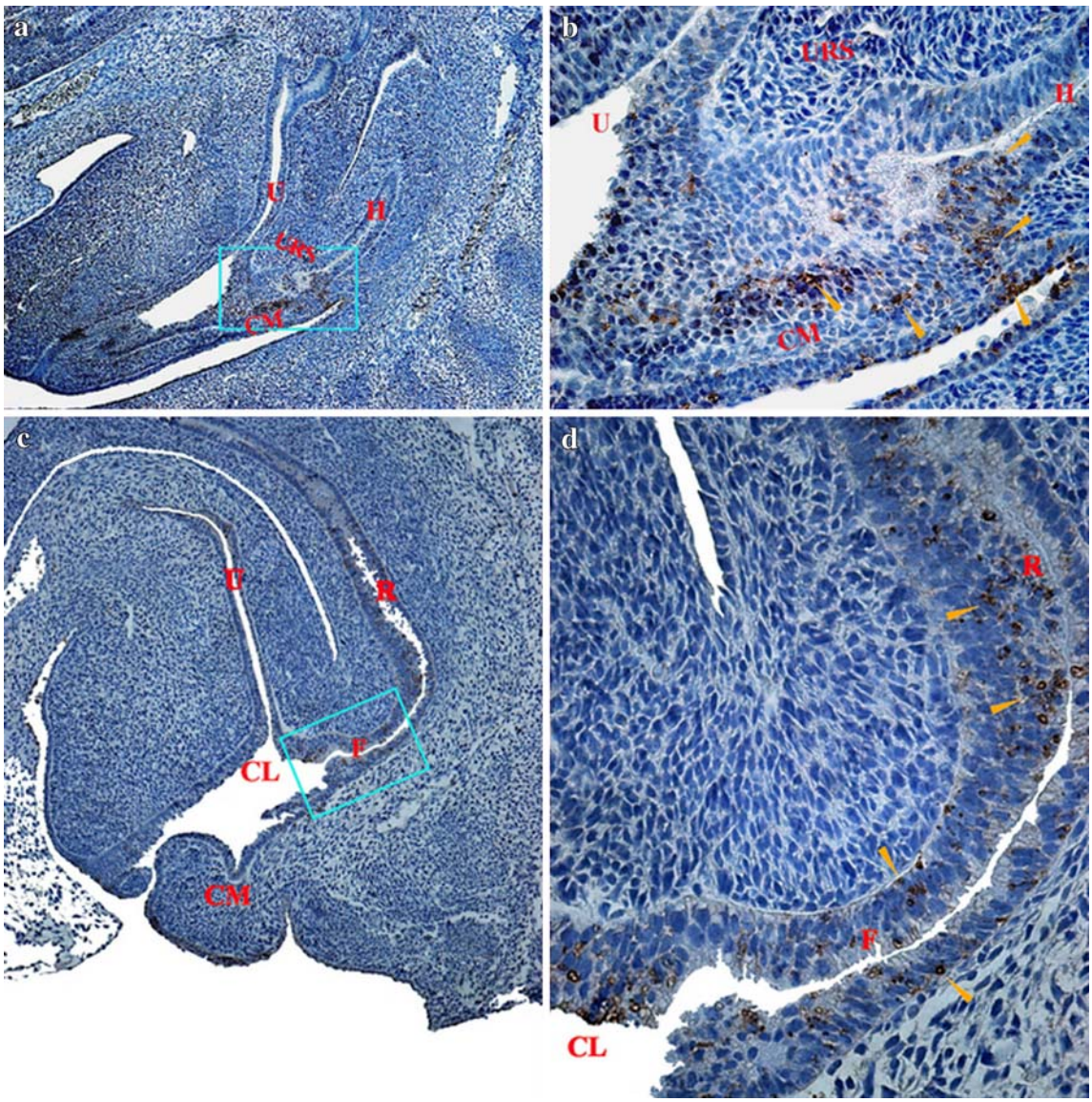

Fig. 3 (**a**) and (**b**) indicated the normal group; on E14, the distribution and quantity of the Tcf4-positive cells were intensively detected on the dorsal CM where the URS was nearly fused with the CM; moreover, Tcf4 well-delineated cells were distributed in the area between the anorectal terminus and the tail groove. (**c**) and (**d**) indicated the ARM group; on E14, sporadic staining could be noted on the epithelium of the fistula; a few staining cells could be observed on the hindgut ((**a**) and (**c**), original magnification ×100; (**b**) and (**d**), original magnification ×400). *Orange arrowheads*: positive cells. *R* rectum, *URS* urorectal septum, *CM* cloacal membrane, *CL* cloaca

On E15, as the epithelium on the tip of URS fused with that of dorsal CM, the fused tissue of the URS, especially the very thin anal membrane, were immunoreactive to Tcf4, strongly and constantly (Fig. 4a, b). On E16, the anorectum communicated with the outside; Tcf4-labeled cells were observed on the epithelium of the anorectum (Fig. 5a, b).

In the ARM group

On E13, the distance between the URS and the CM was fairly long; the CM was shorter and thicker than normal, and the sporadic Tcf4-labeled cells could be detected on the dorsal URS and hindgut (Fig. 1c, d). On E13.5, the tailgut did not degenerate; Tcf4 staining was abolished on the epithelium of the ventral URS and the hindgut (Fig. 2c, d). On E14, Tcf4 was faintly expressed on the epithelium of the fistula and the hindgut (Fig. 3c, d). On E15, the fistula between the rectum and the urethra was evident; the positive cells merely spared on the epithelium of the fistula mucous membrane of the rectum (Fig. 4c, d). On E16, the labeled cells were not remarkably detected on the epithelium of the fistula and anorectum (Fig. 5c, d).

Western blot analysis

Western blot analysis specific for Tcf4 was done to quantify the protein expression in the development of cloaca/hindgut. Tcf4 was detected as an approximately 60-kDa band on Western blots of protein extracted from both the normal and ARM tissue analyzed. Each protein band was normalized by a corresponding β-actin band (Fig. 3). On E14, E14.5, and E15, the key period of anus formation, the expressions of Tcf4 reached estimated optimal level in the normal group, while in the ARM group, the Tcf4 protein expression was faint. Tcf4 protein expression was significantly decreased in the ARM hindguts compared with the control hindguts in each age group (71.25±3.2 vs. 48.21±2.8, 70.86±2.9 vs. 47.85±2.3, and 71.02±2.7 vs. 46.21±3.6, respectively; $P<0.05$). Once the anorectum communicated with the outside on E16, the expression of Tcf4 began to decrease (Fig. 6).

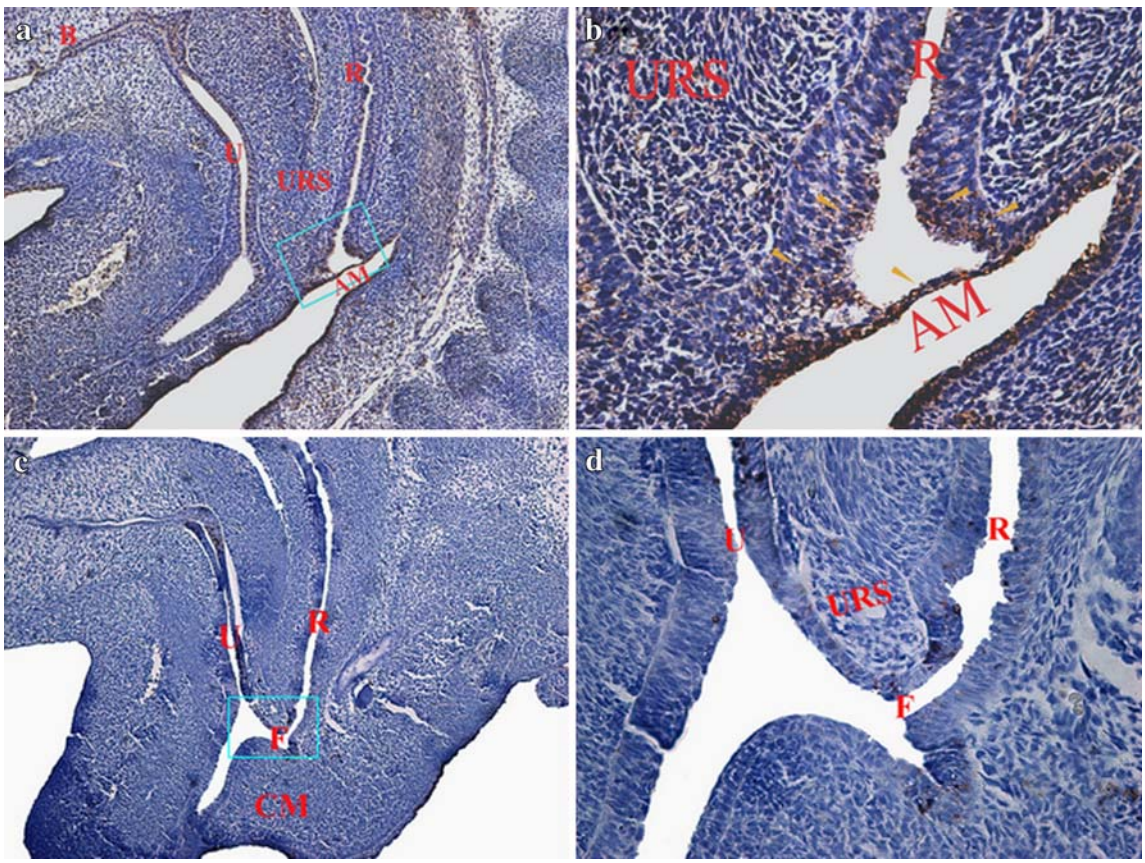

Fig. 4 (**a**) and (**b**) indicated the normal group; on E15, the epithelium on the tip of URS fused with that of dorsal cloacal membrane; the fused tissues of the URS, especially in the very thin anal membrane, were immunoreactive to TCf4. (**c**) and (**d**) indicated the ARM group; on E15, the fistula between the rectum and the urethra was evident. Tcf4 was faintly expressed on the epithelium of the fistula; the positive cells were merely spared on the anorectum ((**a**) and (**c**), original magnification ×100; (**b**) and (**d**), original magnification ×400). *Orange arrowheads*: positive cells. *R* rectum, *URS* urorectal septum, *AM* anal membrane, *F* fistula

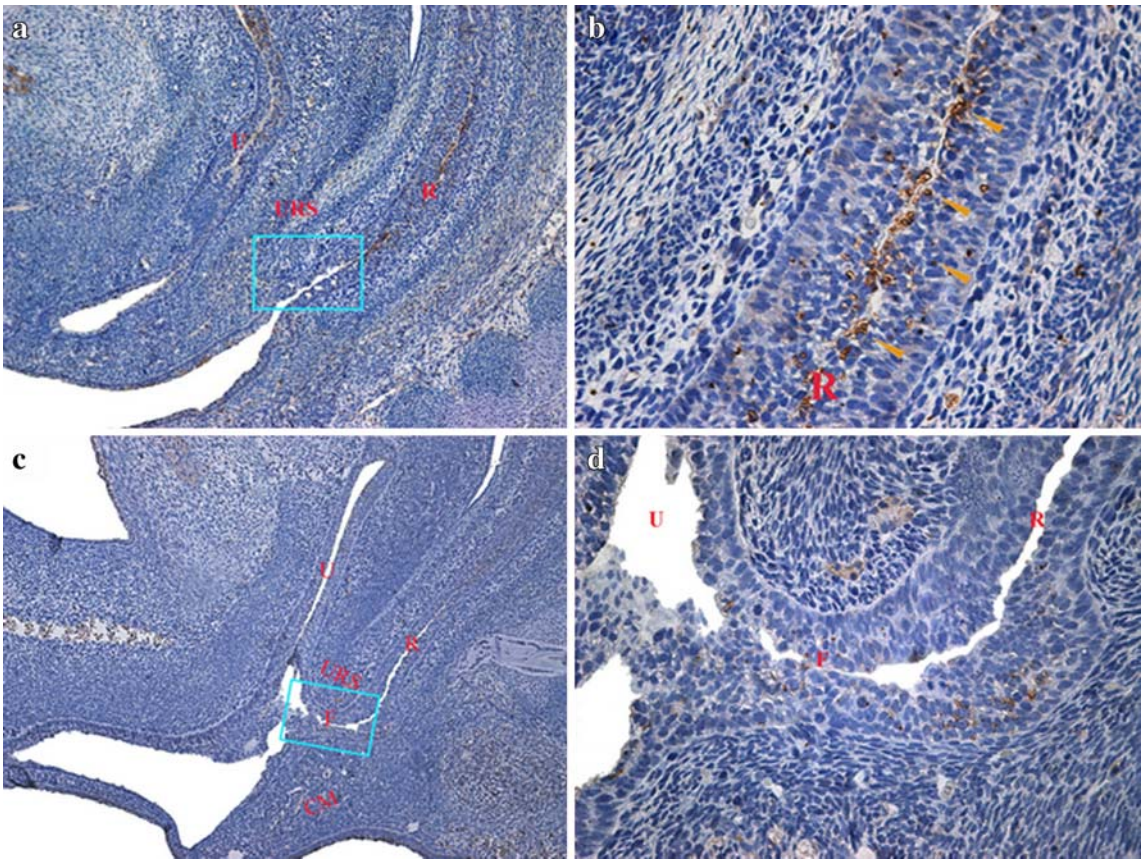

Fig. 5 (**a**) and (**b**) indicated the normal group; on E16, the anal opened; Tcf4-labeled cells were observed on the epithelium of the anorectum. (**c**) and (**d**) indicated the ARM group; on E16, the labeled cells were detected on the fistula ((**a**) and (**c**), original magnification ×100; (**b**) and (**d**), original magnification ×400). *Orange arrowheads*: positive cells. *R* rectum, *URS* urorectal septum, *F* fistula

RT-PCR results

The PCR conditions described above successfully yielded the amplified fragments of expected size for Tcf4 (191 bp) in each sample. RT-PCR amplification of Tcf4 in the normal embryos showed light or strong positive bands, whereas in ARM embryos, it showed moderate or weak positive bands. In the normal embryos, Tcf4 mRNA increased on E13–E15, especially the level of Tcf4 expression was strongest on E14, E15, and then gradually decreased. At the same time, in the ARM rat embryos, Tcf4 mRNA is minimally expressed; when results were compared with normal samples, there was a significant decrease in expression in each age group (0.48±0.08 vs. 0.37±0.05, 0.49±0.08 vs. 0.39±0.04, and 0.47±0.05 vs. 0.36±0.03, respectively) ($P<0.05$). On E18 and E21, expression levels were diminished but without reaching significant levels compared with controls (Fig. 7).

Discussion

In a previous study, it was reported that the Tcf4 gene could restrict and induce the mesenchymal and epithelium cell differentiation and proliferation [12, 13]. The importance of the Tcf4 gene in gut morphogenesis, cytodifferentiation, and the maintenance of the differentiated state is now well established in many vertebrate species. In addition, Tcf4 has been shown to be involved in the development of hindgut defects by the phenotypic loss of Tcf4 function in mutant mice [8]. Given the importance of Tcf4 in maintaining epithelial stem cells in the intestine [12], Tcf4 is essential for caudal endoderm development [8]; it was intriguing to speculate that Tcf4 may play an equivalent role in the primitive gut. However, the exact role of the Tcf4 gene in the development of anorectal abnormalities has yet to be elucidated. In this study, the most important finding was that the Tcf4 expression, which occurred in the anorectum of the normal rat embryo, reached estimated optimal level on E14, E14.5, and E15 but decreased after the anus formed. In contrast, the level of Tcf4 expression in ARM embryos remained low and unchanging, suggesting that Tcf4 might play an essential role not only in the embryogenesis of the anorectum but also the development of ARM.

Despite the likely complex multifactorial etiology, ARM is generally thought to be due to the maldevelopment of URS and a failure of fusion with the CM. In this study, the observations of anorectal development were consistent with previous results [9,

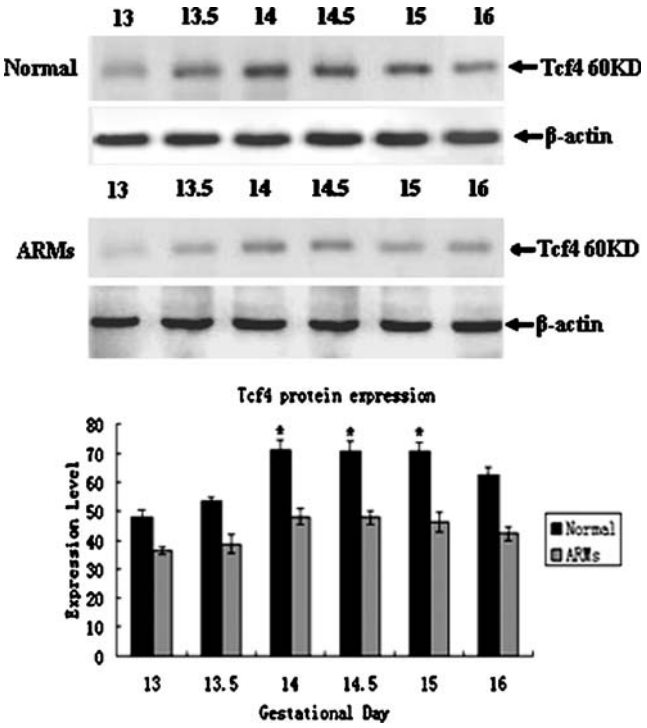

Fig. 6 Western blot analysis of Tcf4 in normal rat group and ARM group. Tcf4 was detected as an approximately 60-kDa band on Western blots of protein extracted from both the normal and ARM tissues analyzed. Immunoblot showed a remarkable Tcf4 signal protein in the normal but weak in the ARM group. It showed the trends of expression at each time point; a single and sharp peak could be noted on E14, E14.5, and E15. *Significant difference was seen from corresponding controls

10]: in normal embryos, URS descent started on E13; on E14, the tip of the URS approached to the CM and almost fused with it. On E15, the URS fused with the CM and the rectum and the urogenital sinus were completely separated. On E16, the anorectum communicated with the outside. Nevertheless, in ARM embryos, cloacal configuration was abnormal and was characterized by dorsal CM and URS maldevelopment and persistent communication between the rectum and urethra. Thus, the changes of URS and dorsal CM in the foresaid specific period were one of the key events during anorectal development.

In this study, expression of Tcf4 gene in the anorectum showed differences in spatial distribution between normal and ARM embryos. In normal embryos, the Tcf4-positive cells were intensively focused on the key region during the dynamic and incessant progress of URS fusion with CM on E14–E16. Nevertheless, in ARM embryos, only sporadic Tcf4 staining was noted on this region, and the intensity of the immunohistochemistry of Tcf4 gene expression in the anorectum is lower than in normal embryos. Therefore, there was relative spatial imbalance between the normal and ARM embryos during the embryogenesis of the anorectum. Meanwhile, in all normal and ARM embryos, a dorso-ventral patterning of Tcf4 gene was evident. Tcf4 was expressed prominently in the dorsal parts of the cloaca that develops into hindgut, whereas in the ventral part of the cloacal epithelium, which becomes the urogenital sinus, the expression was absent or low compared with ventral parts, as previously described [5]; we speculate that Tcf4 might play a role in the DV pattern. This suggests that Tcf4 gene may play a pivotal role in the process of urorectal septation. Tcf4, as a molecular messenger, acts in a dose-dependent and diffusible manner in organizing patterns in early vertebrate development; it is very likely that the ectopic expression of Tcf4 and its physical distance from the responding tissue may interfere with normal development.

In addition, based on the results of Western blot analysis and RT-PCR, in the normal embryos, Tcf4 expression at the critical time of anorectal development (E14–E15) was at its highest level, further suggesting that it may play an important role in the development of the anorectum. However, at the same

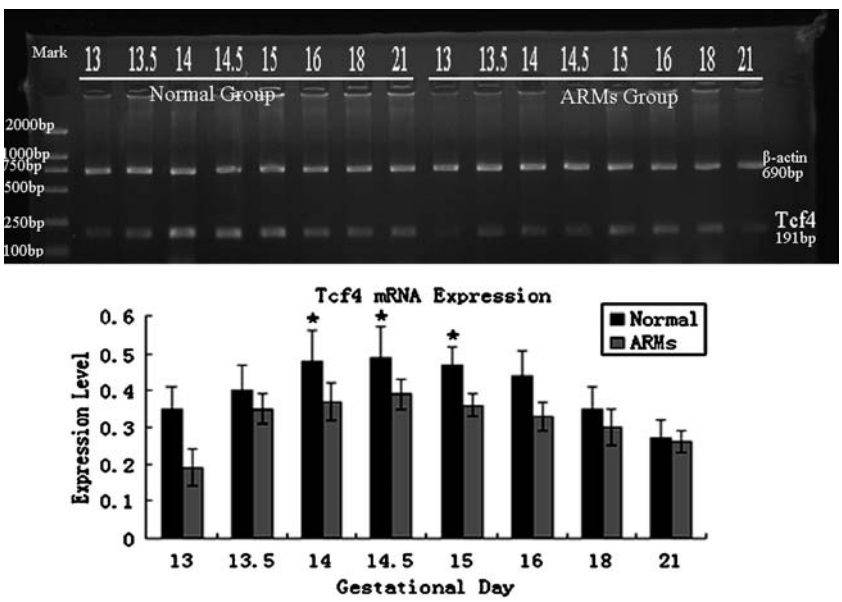

Fig. 7 Semiquantitative RT-PCR analysis of Tcf4 and control β-actin mRNA expressed in normal and ARM developing hindgut tissue samples. It showed the trends of transcription at each time point. On GD14, GD15, the key period of anus formation, the transcription of Tcf4 reached the estimated optimal level in the normal group, while the transcription in the ARM group was faint. *Significant difference was seen from corresponding controls

stage, the expression levels of Tcf4 in ARM rats were significantly lower, implying that this special downregulation of Tcf4 expression during the essential stage of anorectal development may reduce the signals from endoderm to mesoderm, and affected the transition from endoderm to intestinal epithelium, and in this way contributed to the ARM. Additionally, with the anal opening on E16, the expression of Tcf4 protein and mRNA decreased gradually, suggesting that Tcf4 may play an essential role during initial morphogenesis of the anorectum, whereas its role during subsequent development might be less important. These findings demonstrated that Tcf4 expression showed time-dependent changes in the anorectal development.

During the essential period of the anorectal formation, considerable signaling molecules are participated in the embryogenesis of the hindgut: Shh [14–17], Bmp4 [17, 18], Fgf10 [19], Hoxa-13 and Hoxd-13 [18, 20], ephrin-B2, and the ephrin receptors EphB2 [21]. Recent studies have shown that Tcf4 may play an important role in the activation of target genes to exert the biochemical functions of these genes. Moreover, crosstalk between Wnt/Tcf4 and other signaling pathways, Fgfs [22], Bmps [22], Shh [22], and EphB2 [23, 24], and integration of these multiple inputs, is likely to be as crucial for regulating normal development of the anorectum. Therefore, the impaired expression of Tcf4 in the URS during the cloacal separation may influence the mesenchymal and epithelial cells of URS proliferation and cell-cycle arrest, positive and negative regulation of apoptosis, replicative senescence, resulting in dysplasia of the URS, and disturb the inherent development, which gives rise to defective cell kinetics and disordered structure of the cloaca.

This study provides evidence that spatiotemporal expression of Tcf4 was imbalanced during the development of anorectum in ARM embryos. Furthermore, this suggests that there may be causal connection between the faulty cloacal separation and this disturbance in Tcf4; however, the disturbance could also be produced by another factor that prevents separation. Further analysis of the cascade reaction important for anorectal morphogenesis should facilitate a better understanding of the pathogenesis of ARM.

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