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Expression pattern of Wif 1 during development of anorectum in fetal rats with anorectal malformations

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Purpose: This study was performed to investigate the expression pattern of Wnt inhibitory factor 1 (Wif1) during anorectal development in normal and anorectal malformation (ARM) embryos and the possible role of Wif1 in the pathogenesis of ARM. **Methods:** ARM was induced with ethylenethiourea on the 10th gestational day in rat embryos. Cesarean deliveries were performed to harvest the embryos. The expression pattern of Wif1 protein and mRNA was evaluated in normal rat embryos (n=288) and ARM rat embryos (n=306) from GD13 to GD16 using immunohistochemical staining, Western blot, and real time RT-PCR. **Results:** Immunohistochemical staining revealed that in normal embryos Wif1 was constantly expressed in the cloaca from GD13 to GD16. On GD13 and GD14, Wif1-immunopositive cells were extensively expressed in the cloaca. On GD15, the expression of Wif1 were mainly detected on the very thin anal membrane. In ARM embryos, the epithelium of the hindgut and urorectal septum demonstrated faint immunostaining for Wif1 from GD14 to GD16. Western blot and real time RT-PCR revealed that Wif1 protein and mRNA expression level was significantly decreased in the ARM groups compared with the normal group on GD14 and GD15 ($p<0.05$).**Conclusions:** This study demonstrated that the expression pattern of Wif1 was disrupted in ARM embryos during anorectal morphogenesis, which demonstrated that downregulation of Wif1 at the time of cloacal separation into the primitive rectum and urogenital septum might related to the development of ARM.

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Abstract

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during anorectal development in normal and anorectal malformation (ARM) embryos and the possible role of Wif1 in the pathogenesis of ARM.

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Keywords Anorectal malformation · Development · Embryogenesis · Wif1

Introduction

Anorectal malformations (ARM) are very common surgical disorders frequently encountered in pediatric surgery practice. The incidence is approximately 1 in 5000 live births. There is a wide spectrum of ARM phenotypes, ranging from stenotic anus to cloacal malformation (Endo **et al. 1999**). Surgical operation is the main modality of treatment. Although the level of ARM surgical treatment has improved, there are still different degrees of complications, which seriously affect the quality of life (Peña **et al. 1998;** Peña **et al. 2000;** Bai **et al. 2000;** Levitt **et al. 2005;** Rintala**. 2016**). Up to now, the etiology of ARMs is unknown. Genetic factors are important contributing factors in the pathogenesis of ARMs. Genetic signaling must be precisely regulated in any stage of the hindgut development and its dysregulation contributes to ARMs. Wif1 is a member of the families of secreted molecules known to inhibit Wnt signalling activity. Wif1 was first identified as an expressed sequence tag from the human retina, and highly conserved orthologues have been isolated from mouse, Xenopus and zebrafish (Hsieh **et al. 1999**). So far reports on the regulatory functions of Wif1 in

embryonic development are limited. Previous study has detected that Wif1 expressed in the midline cloaca endoderm, and dysregulated Wif1 expression caused septation defects (**Ng et al. 2014**). These results suggest that Wif1 is required for urorectal development. However, the expression pattern of Wif1 has not been described previously in the embryogenesis of rat ARMs. To provide an insight into the role of Wif1 in anorectal morphogenesis, we have analyzed the expression of Wif1 protein and mRNA in normal and ethylenethiourea (ETU)-induced ARM rat embryos on embryonic stages GD13 to GD16, a critical time in anorectal development.

Materials and methods

Animal model and tissue collection

Mature Wistar rats (body weights, 250-300g) were provided by the Medical Animal Center, Shengjing Hospital of the China Medical University (Shenyang, PR China). Ethical approval was obtained from the China Medical University Animal Ethics (no. 200(7) PS14) prior to the study. Procedures for generating ARMs in fetal rats are described in earlier study (**Bai et al. 2004**). 70 time-mated pregnant Wistar rats were randomly divided into two groups: ETU-treated group and control group. In the ETU-treated group, 40 pregnant rats were gavage-fed a single dose of 125 mg/kg of 1% ETU (2-imidazolidinethione; Aldrich Chemical, Penzberg, Germany) on GD10 (GD0=sperm in vaginal smear after overnight mating). 30 control rats received corresponding doses of ETU-free saline on GD10. Embryos were harvested by cesarean delivery from GD13 to GD16. One third of the embryos were fixed in 4% paraformaldehyde for 12 to 24 hours 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 ARMs was determined by light microscope. Then, the embryos were divided into normal and ARM groups. Under magnification, the cloaca/hindgut of other specimens was dissected and removed from surrounding tissues. The cloaca/hindgut was immediately frozen in liquid nitrogen for Western blot analysis and real-time RT-PCR.

Immunohistochemical staining

The slides were treated and incubated with primary Anti-Wif1 (1:200 dilution, Rabbit polyclonal, abCam, UK) and horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Bio-technology). Antibody incubations were performed in phosphate-buffered saline (PBS) supplemented with 10% goat serum. Incubation with the secondary antibody was performed for 20min at room temperature, and signals were visualized by using 3 ’3Pdiaminobenzidine (DAB; Sigma, UK). Two pathologists independently reviewed the

immunohistochemical stained slides and agreed on results by consensus.

(https://www.protocols.io/view/immunohistochemical-staining-kujcwun)

Protein preparation and Western blot

Protein preparation was performed as described previously (Mandhan **et al. 2006**): the cloaca/hindgut per condition were pooled and sonicated in ddH2O containing protease inhibitors. Protein extracts were seperated on SDS-PAGE electrophoresis, and transferred to PVDF membranes, blocked with 5% fat-free milk in Tris-buffered saline (2hr, room temperature). Membrane were incubated in primary antibody against Wif1 (diluted 1:500, Rabbit polyclonal, abCam, UK) or anti-β-Actin rabbit monoclonal antibody (1:2000 dilution; Sigma, St Louis, MO, USA), and incubated with the secondary antibody (diluted 1:3,000, goat anti-rabbit HRP conjugate; Jackson Immunoresearch, West Grove, Pa., USA). Membranes were developed by using a chemiluminescent substrate kit (Pierce, Pierce, Rockford, Ill., USA) and densitometric values were analyzed by using the ECL Plus detection system (Millipore, Billerica, Mass., USA).

(https://www.protocols.io/view/western-blot-analysis-kumcwu6)

RNA Isolation and Real-Time RT-PCR

Total RNA was isolated with the TRIzol reagent (Invitrogen) according to the manufacturer's protocol. RNA (1 μg) was reverse transcribed by using the Prime Script RT reagent kit (TaKaRa) following the manufacturer's instructions. Quantitative real-time RT-PCR was accomplished with SYBR Premix Ex Tap (TaKaRa) on the 7900HT fast real-time PCR system (Applied Biosystems) under the following conditions: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, 60°C for 60 s. A dissociation procedure was performed to generate a melting curve for confirmation of amplification specificity. GAPDH was used as the reference gene. The relative levels of gene expression were represented as ΔCt= Ct gene–Ct reference, and the fold-change of gene expression were calculated with the $2^{-\Delta\Delta Ct}$ method. Experiments were repeated in triplicate. The primer sequences spanning the intron-exon junction were as follows:

Wif1 forward: 5'-AGCCATTCCCGTCAATATCCAC-3';

reverse: 5'-TGCCATGATGCCTTTATCCAG-3'.

GAPDH forward: 5'-GCTGGTCATCAACGGGAAA-3';

reverse:5′ -CGCCAGTAGACTCCACGACAT-3 ' .

Statistical Analysis

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

analysis. The 2-sample Student's t test was used to compare the Wif1 levels between the ARM and normal groups. All numerical data were presented a mean ± standard deviation. A value of $p<0.05$ was considered statistical significance.

Results

General observations

In this study, no malformations were observed in the 288 embryos of the normal rats. Among the ETU-treated embryos, all 378 embryos had short or no tail and 19 of embryos died in utero. The incidence of ARMs in ETU-treated embryos was 81.0% (306/378). The embryos for immunohistochemistry staining, Western blot, and real time RT-PCR in each group are shown in Table 1. The type of ARMs was persistent cloaca or rectourethral fistula.X

⊏ Immunohistochemical s**taining**

Normal group

On GD13, the cloaca was divided into urogenital sinus (UGS) ventrally and primitive hindgut dorsally by the L-shaped urorectal septum (URS). Wif1-immunopositive cells were extensively expressed on the epithelium and mesenchyme of the cloaca (Fig. 1a, b).

On GD14, a potential cana located between the tip of the URS and the cloacal membrane (CM). Wif1-immunopositive cells were detected on the hindgut, URS, urethra and CM (Fig. 2a, b).

On GD15, the epithelium on the tip of the URS fused with the dorsal CM, leading to separation of the hindgut and UGS. The anal membrane (AM) was nearly ruptured. Wif1-immunopositive cells were mainly detected on the very thin AM (Fig. 3a, b).

On GD16, the AM ruptured and the rectum separated from the UGS completely. The anorectum communicated with the outside. Wif1-immunolabeled cells were observed on the epithelium of the distal anorectum (Fig. 4a, b).

ARM group

On GD13, comparing with normal embryos, the distance between the URS and the CM was long, and the CM was shorter and thicker. Wif1-labeled cells were extensively expressed on the epithelium and mesenchyme of the cloaca (Fig. 1c, d).

On GD14, the URS was high in the cloacal cavity, and the distance between URS and CM was relatively long. Wif1 was faintly expressed on the epithelium of the hindgut, URS and the urethra (Fig. 2c, d).

On GD15, the distance between the URS and CM shortened, but the URS did not fused with the CM. The fistula between the rectum and urethra was evident, and the hindgut did not separate from UGS. Positive cells were sparsely located on the epithelium of the hindgut, fistula and the urethra (Fig. 3c, d).

On GD16, the fistula between the rectum and urethra was existing, and rectal terminus was still not opened to the outside. Wif1 demonstrated low expression on the epithelium of the rectum, fistula and the urethra (Fig. 4c, d).

Western blot analysis

Western blot specific for Wif1 was performed to quantify protein expression in the anorectal development (**Table. 2 and Fig. 5**). Wif1 was detected as an approximately 41 kDa band among the proteins extracted from normal and ARM tissue. Each protein band was normalized to a corresponding β-Actin band. Wif1 protein expression was decreased in the ARM hindgut compared with normal hindgut from GD13 and GD15. On GD14 and GD15, the key periods of anus formation, Wif1 expression reached optimal levels in the normal group but was relatively low in the ARM group ($p<0.05$).

Real-time RT-PCR

Wif1 mRNA expression was calculated in the normal and ARM groups (**Table. 3 and Fig. 6**). On GD14 and GD15, *Wif1* mRNA expression reached the estimated optimum levels in the normal group. In the ARM group, *Wif1* mRNA was minimally expressed from GD13 to GD15. *Wif1* mRNA expression was significantly decreased in the ARM hindgut compared with normal hindgut on GD14 and GD15 ($p<0.05$).

Discussion

WIF1 is one member of Wnt antagonists, which bind to Wnt directly and inhibit the link with their receptors, and as a result, the accumulation of β-catenin is reduced and canonal and noncanonal pathway are inhibited (Malinauskas **et al. 2011**). Previous study has detected that Wif1 expressed in the midline cloaca endoderm, and dysregulated Wif1 expression caused septation defects (Ng **et al. 2014**). Thus, it is important to carry out future studies to examine the important role of Wif1 in the normal development of the cloaca. ETU-induced ARMs in rat embryos has been previously employed to study the morphological changes of ARMs by several groups, including our laboratory (Qi **et al. 2002;** Bai **et al. 2004;** Mandhan **et al. 2006;** Zhang **et al. 2009;** Wang **et al. 2009;** Tang **et al. 2014a;** Tang **et al. 2014b;** Zhang **et al. 2015**)⊑. In this study, we investigated the expression pattern of Wif1 during anorectal development by immunohistochemical staining, Western blot, and real time RT-PCR. We found that in normal rat embryos, the Wif1 expression reached estimated highest level

on GD14 and GD15, but decreased after the anus was formed. However, in ARM embryos, the Wif1 expression level were significantly lower on GD14 and GD15, suggesting that Wif1 might play an essential role not only in the embryogenesis of the anorectum, but also the development of ARMs.

In this study, expression of Wif1 gene in the anorectum showed differences in spatial distribution between normal and ARM embryos. On GD15, Wif1-immunopositive cells were mainly detected on the very thin AM in normal embryos and sparsely located on the epithelium of the hindgut, fistula and the urethra in ARM embryos. On GD16, Wif1-immunolabeled cells were observed on the epithelium of the distal anorectum in normal embryos and faintly expressed on the epithelium of the rectum, fistula and the urethra in ARM embryos. Therefore, relative spatial imbalance exist between the normal and ARM embryos during embryogenesis of the anorectum. This results suggest that morphogenic events in the anorectum depend on Wif1 signal induction. Wif1 protein located in an unusual region might contribute to disturbances in proliferation or differentiation in local microenvironment, inducing further maldevelopment of the anorectum.

Wif1 expression shows time-dependent changes during anorectal development. Western blot analysis and real time RT-PCR shown that, in the normal embryos, Wif1 expression was at its highest level at the key time-point of anorectal development (GD14 and GD15), suggesting that it may play an role in the development of the anorectum. However, Wif1 expression levels on GD14 and GD15 were significantly lower in the ARM group compared with the normal group, implying that downregulation of Wif1 expression may influence signal transduction from endoderm to mesoderm during the critical period of anorectal development, and affect the differentiation from endoderm to intestinal epithelium, thus contributing to the ARMs. Additionally, when the anus opened on GD16, the expression of Wif1 protein decreased. This suggest that Wif1 may play an essential role during initial morphogenesis of the anorectum, but its role during subsequent development of the anorectum may be less important.

Fusion of URS with CM is a traditional theory in the development process of anorectum (de Vries **et al. 1974; Qi et al. 2002; Bai et al. 2004**). Expression of Wif1 gene in the anorectum showed differences in spatial distribution between normal and ARM embryos. On GD15, Wif1-immunopositive cells were mainly detected on the very thin AM in normal embryos. In contrast, only sporadic Wif1 immunostaining located on the epithelium of the hindgut, fistula and the urethra in ARM embryos. Therefore, Wif1 might be important for the development of the CM during embryogenesis of the anorectum. The results suggest that morphogenic events in the anorectum depend on Wif1 signal induction.

ETU is known to disturb the expression of the shh signaling pathway during the development of the hindgut (Mandhan**et al. 2006**). RC-L Ng had reported that endoderm Shh-Wif1-Wnt-b-catenin signaling must be precisely regulated and its dysregulation contributes to ARMs (Ng **et al. 2014**). Wif1 levels were reduced in ETU exposed embryos during hindgut development. This downregulation of Wif1 may provide a molecular explanation for the incomplete division of the cloaca which results in a variety of hindgut malformations.

Conclusions

In summary, the expression pattern of Wif1 was impaired during development of anorectum in fetal rats with ETU-induced ARMs. This indicates that Wif1 might play an important role in morphogenesis of the anorectum. Decreased Wif1 expression might be related to the development of ARMs. Further studies are needed to define the specific roles of Wif1 during anorectal development, and thus improve our understanding of the pathogenesis of ARMs.

Acknowledgments

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Figure 1

Image of IHC result on GD13

a, b Normal group. On GD13, Wif1-immunopositive cells were extensively expressed on the epithelium and mesenchyme of the cloaca. **c, d** ARM group. On GD13, Wif1-labeled cells were extensively expressed on the epithelium and mesenchyme of the cloaca. (CM cloacal membrane, H hindgut, U urethra, URS urorectal septum). Scale bar = 100μm in a,c; = 50μm in b,d. Yellow rectangles in **a, c** are shown at higher magnification in **b, d.** Original magnification: ×100 (**a, c**), ×200 (**b, d**).

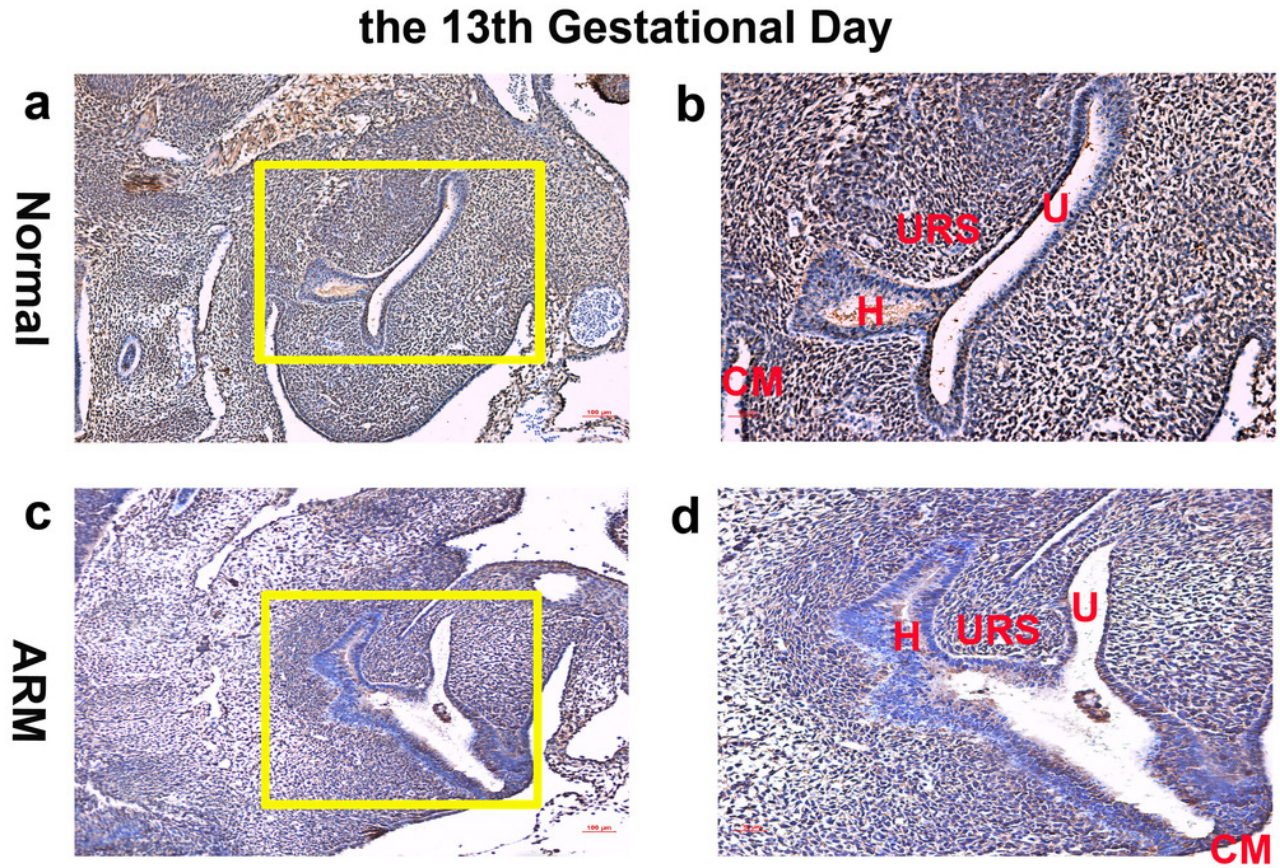

Figure 2

Image of IHC result on GD14

a, b Normal group. On GD14, Wif1-immunopositive cells were detected on the hindgut, urorectal septum, urethra and cloacal membrane. **c, d** ARM group. On GD14, Wif1 was faintly expressed on the epithelium of the hindgut, urorectal septum and the urethra. (CM cloacal membrane, H hindgut, U urethra, URS urorectal septum). Scale bar = 100μm in a,c; = 25μm in b,d. Yellow rectangles in **a, c** are shown at higher magnification in **b, d.** Original magnification: ×100 (**a, c**), ×400 (**b, d**).

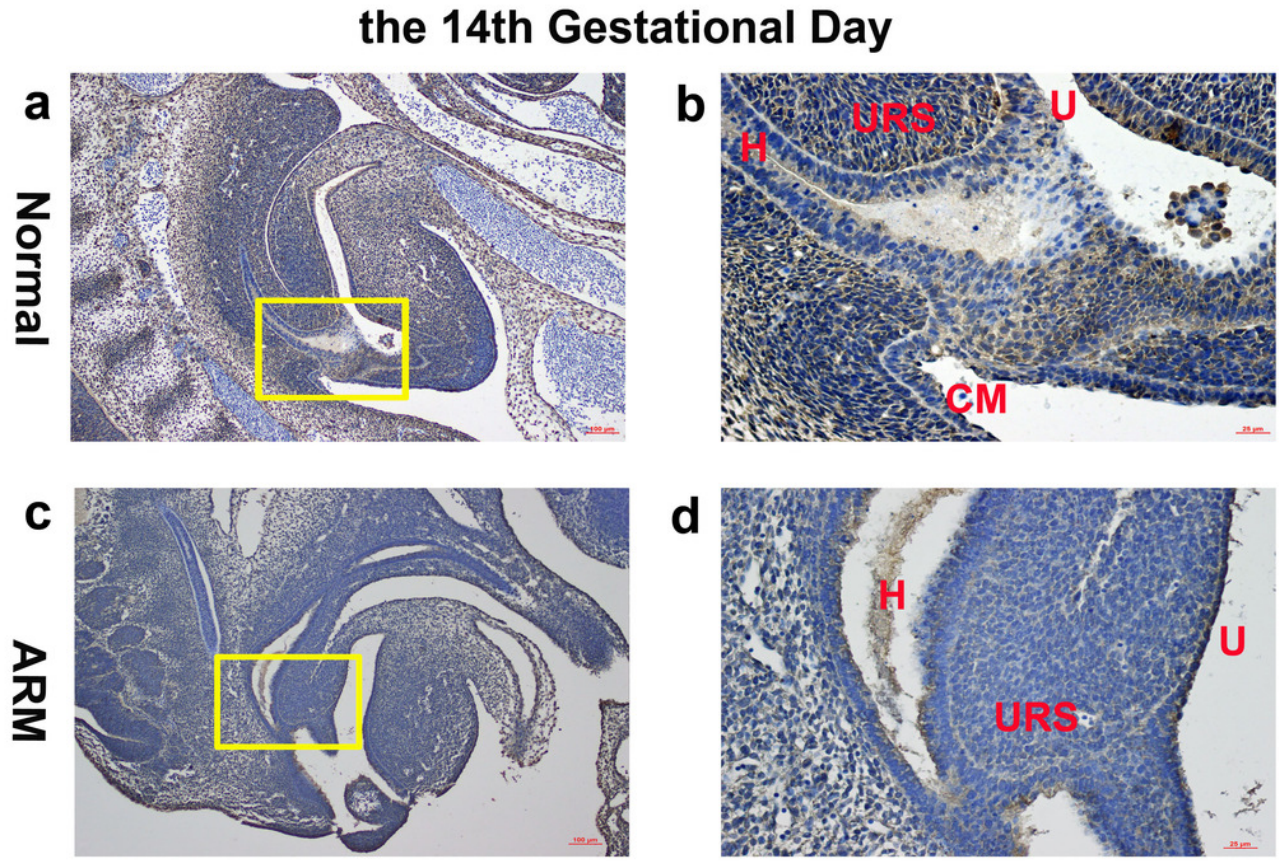

Figure 3

Image of IHC result on GD15

a, b Normal group. On GD15, Wif1-immunopositive cells were mainly detected on the very thin anal membrane. **c, d** ARM group. On GD15, Wif1-positive cells were sparsely located on the epithelium of the hindgut, fistula and the urethra. (AM anal membrane, F fistula, H hindgut, U urethra, URS urorectal septum). Scale bar = 100μm in a,c; = 25μm in b,d. Yellow rectangles in **a, c** are shown at higher magnification in **b, d**. Original magnification: ×100(**a, c**), ×400 (**b, d**).

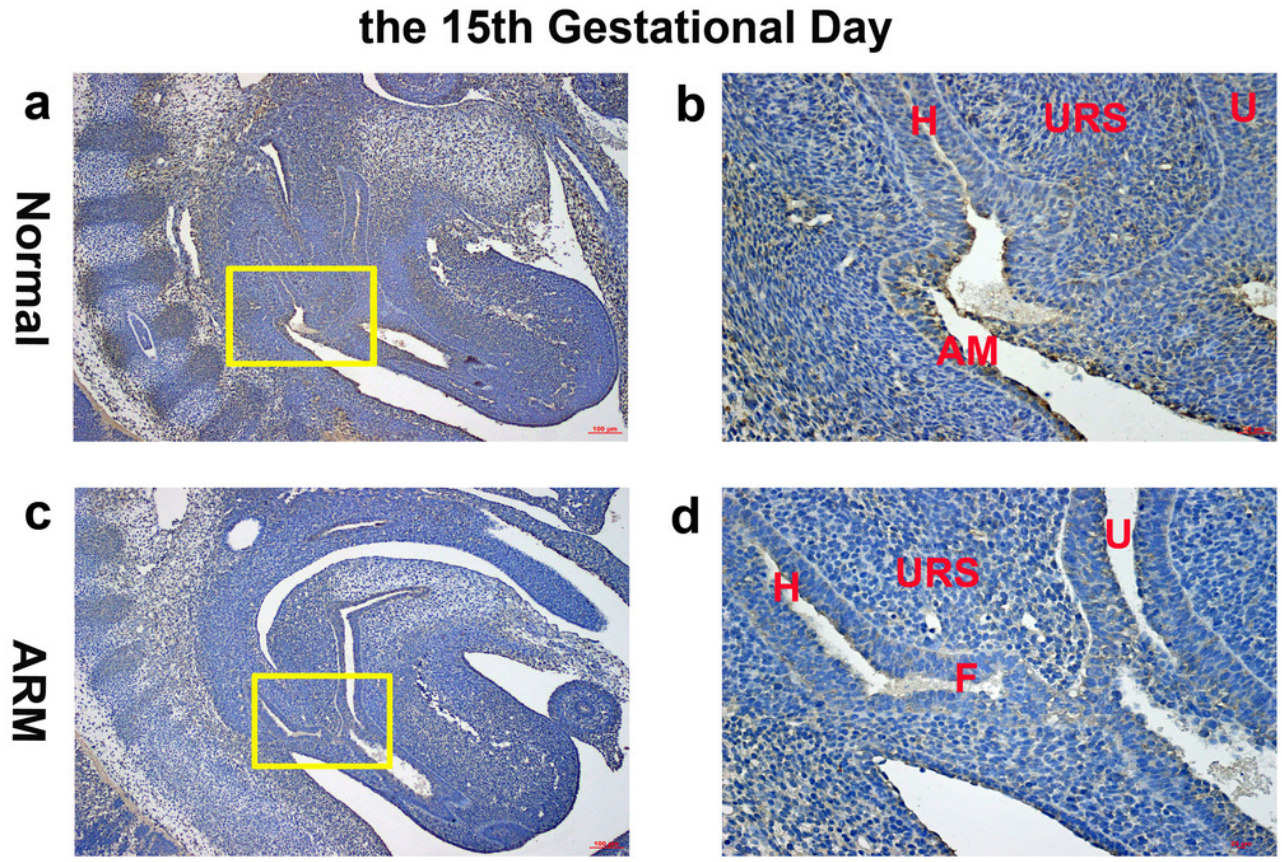

Figure 4

Image of IHC result on GD16

a, b Normal group. On GD16, Wif1-immunolabeled cells were observed on the epithelium of the distal anorectum. **c, d** ARM group. On GD16, Wif1 demonstrated low expression on the epithelium of the rectum, fistula and the urethra. (F fistula, R rectum, U urethra, URS urorectal septum). Scale bar = 250μm in a,c; = 25μm in b,d. Yellow rectangles in **a, c** are shown at higher magnification in **b, d**. Original magnification: ×40 (**a, c**), ×400 (**b, d**).

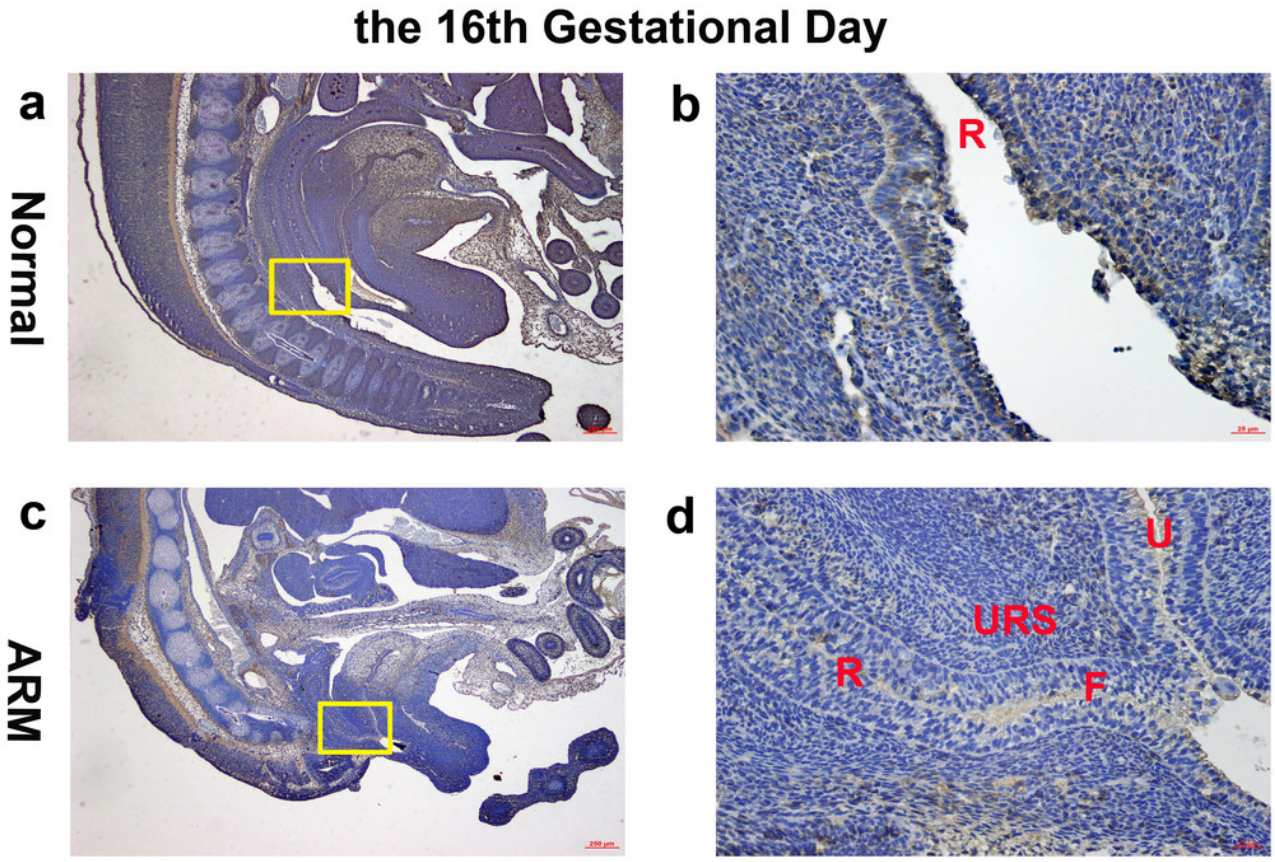

Figure 5

Western blot analysis of Wif1 protein expression levels in normal and ARM developing hindgut tissue samples.

Western blot analysis of Wif1 protein expression levels in normal and ARM developing hindgut tissue samples. Values are presented as means ± SD. *Top* Wif1 was detected as an approximately 41-kDa (kd) and on Western blots. β-Actin protein is used as an internal control. *Bottom* Histogram showing the trends of Wif1 expression at each time-point. A peak can be noted on GD14.

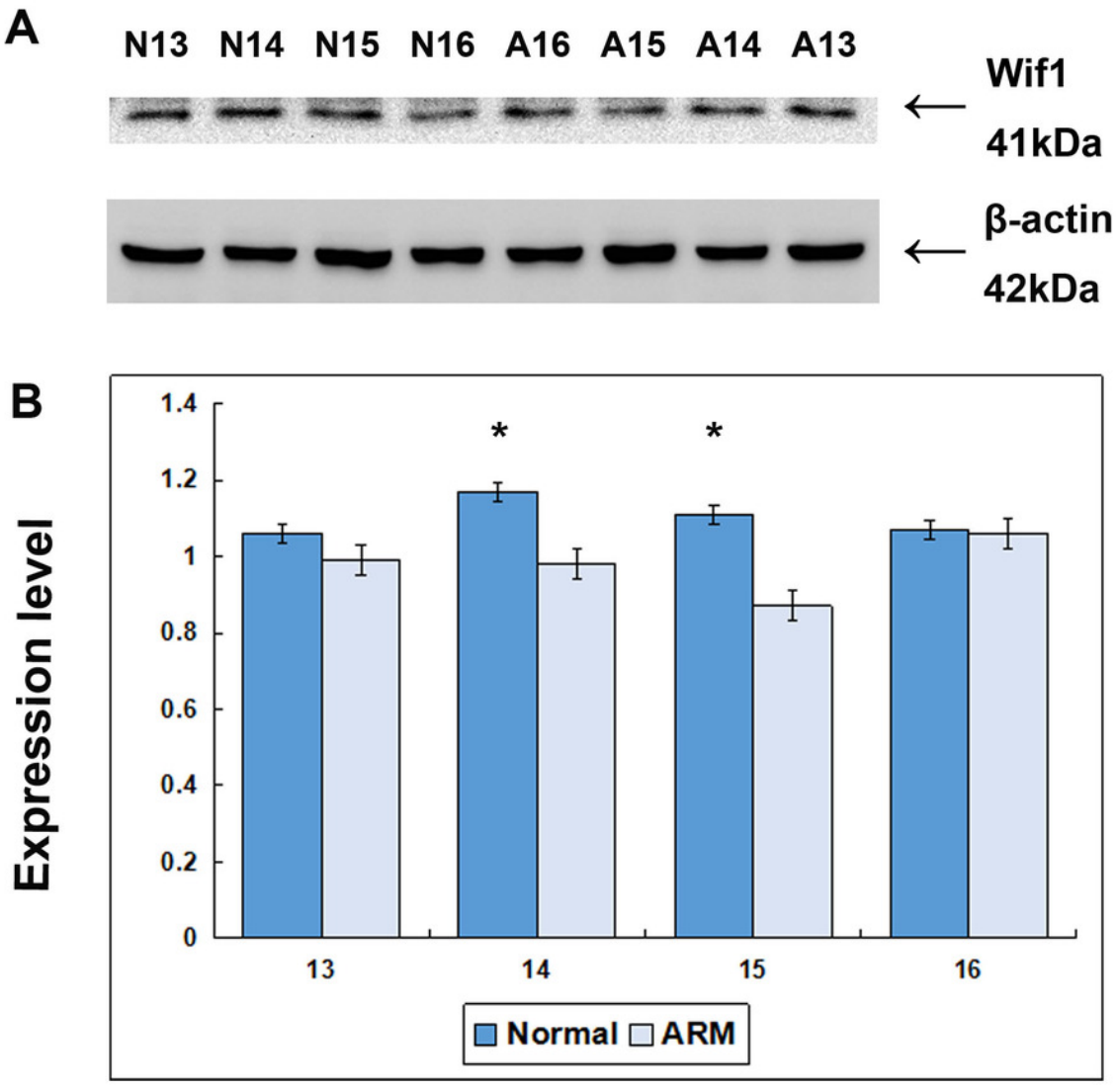

Figure 6

Real-time RT-PCR analysis of *Wif1* mRNA expression levels in normal and ARM-developing hindgut tissue samples.

Real-time RT-PCR analysis of *Wif1* mRNA expression levels in normal and ARM-developing hindgut tissue samples. On GD14 and GD15, the key period of anus formation, *Wif1* expression reaches the estimated optimum levels in the normal group, whereas in the ARM group, *Wif1* mRNA is minimally expressed.Values are presented as means ± SD. * Significant difference from corresponding controls.

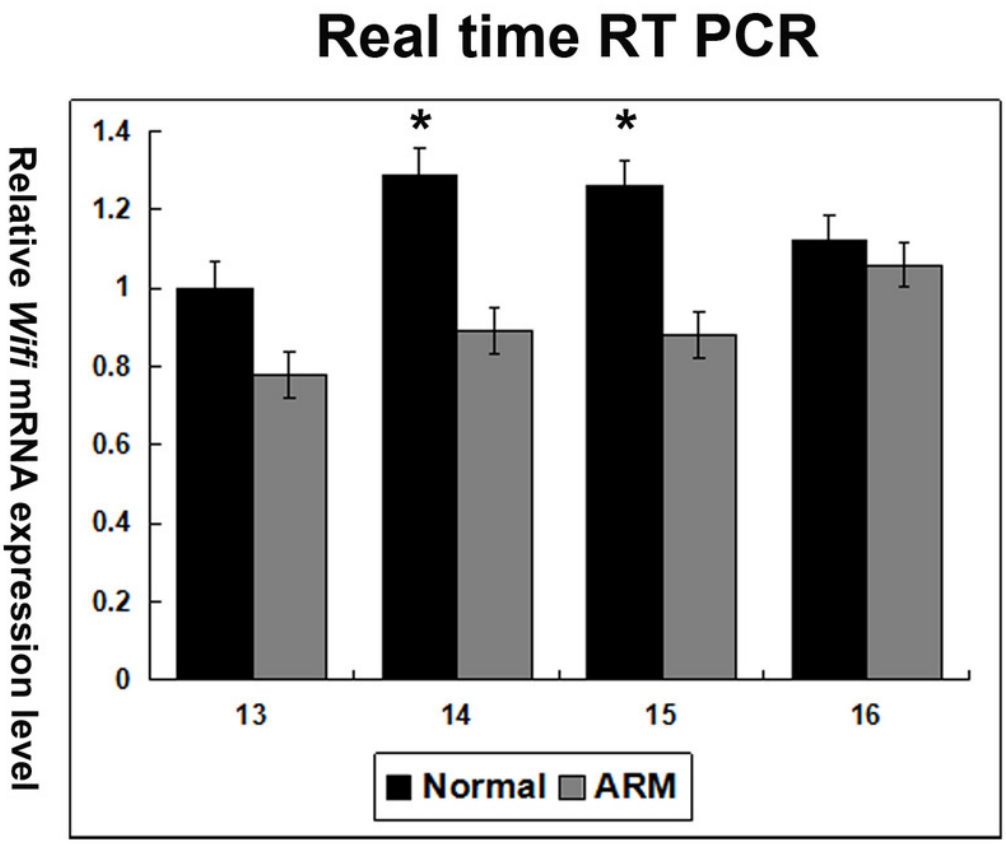

Table 1(on next page)

Distribution of embryos in the various age and treatment groups.

Table. 1 Distribution of embryos in the various age and treatment groups.

Age group	Normal			ARMs		
	IHC	WB	PCR	IHC	WB	PCR
GD13	25	26	27	30	27	28
GD14	24	25	26	29	26	27
GD15	22	25	25	24	25	25
GD16	20	22	21	21	23	21
Total	91	98	99	104	101	101

ARMs anorectal malformations, GD gestational day, IHC immunohistochemical staining, WB Western blot, PCR real time RT-PCR.

Table 2(on next page)

Wif1 protein relative expression level in the normal and ARM group.

Table. 2 Wif1 protein relative expression level in the normal and ARM group.

	GD13	GD14	GD15	GD16
Normal	1.061±0.150	1.167±0.109	1.110±0.095	1.065±0.124
ARM	0.986±0.046	0.981±0.036	0.874±0.081	1.062±0.072

Data are presented as mean ± standard deviation.

ARM, anorectal malformations; GD, gestational day.

Table 3(on next page)

Wif1 mRNA relative expression level in the normal and ARM group.

Table. 3 *Wif1* mRNA relative expression level in the normal and ARM group.

	GD13	GD14	GD15	GD16
Normal	1	1.292±0.237	1.260±0.228	1.116±0.173
ARM	0.778±0.235	0.891±0.068	0.883±0.027	1.065±0.128

Data are presented as mean ± standard deviation.

ARM, anorectal malformations; GD, gestational day.