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ORIGINAL ARTICLE

Normal development of hindgut and anorectum in human embryo

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Abstract
Purpose The aim of the present analysis is to examine the morphological changes, the spatiotemporal distribution of apoptosis/proliferation in the human embryonic anorectum, to reveal the normal development of human anorectum, and investigate the possible roles of apoptosis/proliferation during anorectal development.
Materials and methods The embryos were sectioned serially and sagittally, stained with hematoxylin and eosin (H & E) between the third and eighth week of gestation, TdT-mediated dUTP-digoxigenin nick end-labeling (TUNEL) and proliferative cell-specific nuclear antigen (PCNA) immunohistochemical staining from the sixth to the eighth week.
Results From the fourth to the seventh week, with the growth of the mesenchyme around the cloaca, the cloaca was remolded, subsequently, the cloacal membrane (CM) moved perpendicularly then horizontally. The dorsal cloaca gradually descended to the tail groove, the urorectal septum (URS) and the CM approximated; however, the fusion of URS with the dorsal CM was never observed. During the eighth week, the URS shifted ventrally and finally fused with the ventral CM. Moreover, from the sixth to the eighth week, the apoptotic cells were concentrated in the CM, the mesenchyme of terminal rectum, and the dorsal rectum. Meanwhile, the proliferative cells could be observed in the ventral mesenchyme around the cloaca, the CM, the fused tissue between the URS, and the ventral CM.
Conclusions During the development of human anorectum, it was intriguing to reveal that the URS never fused with the dorsal CM before dorsal CM disintegration, the normal anorectal development may depend on the dorsal cloaca and the dorsal CM; furthermore, the distribution of apoptosis and proliferation in the anorectum and ventral cloacal mesenchyme played a pivotal role in the formation of the anorectum.

Keywords Anorectal malformations · Urorectal septum · Cloacal membrane · Human · Embryology · Apoptosis · Proliferation

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Introduction

Despite a long history of embryological research, the developmental process of the hindgut/anorectum remained unclear [1]. Traditionally, it was considered that the primitive cloaca began to be divided into the urogenital system (UGS) anteriorly and hindgut posteriorly by the urorectal septum (URS) in the fourth week. The division of the cloaca has been completed during the sixth week when the URS fused with cloacal membrane (CM). By the seventh week of human gestation, the UGS and the

anorectum would have been separate entities [2–7]; however, in recent studies, the mechanism of cloacal septation was debated. It was considered that the major events were: ① a change in the relative position of the dorsal cloaca or ② the process of rotation of the CM and configuration transformation of the cloaca [4, 5]. It was the fact that the previous studies provided further developmental progress while it seemed conspicuous that the process of cloacal septation was still controversial and advanced studies are needed to obtain more discriminative data.

The dramatic changes in the morphology and configuration of embryonic anorectum were considered to be the result of embryonic cell differentiation, cell proliferation, and apoptosis [8, 9]. Previous studies stated the important role of cellular proliferation/apoptosis during the cloacal separation in murine, suggesting that proliferation/apoptosis occurs in a specific temporo-spatial sequence in the cloacal area and appears to be an important mechanism in urorectal separation and rupture of the anal membrane [9–12]; however, in humans, there was no general consensus on the spatiotemporal distribution of apoptosis and proliferation during anorectal embryogenesis.

For these reasons, we were encouraged to produce the present work to examine the morphological changes and the spatiotemporal distribution of apoptosis/proliferation in the human embryonic hindgut/anorectum to reveal the normal development of hindgut/anorectum and investigate the possible roles of apoptosis/proliferation during anorectal development. The more precise information of each in the hindgut/anorectum development will enhance our understanding of ARM.

Materials and methods

Sample preparation and H & E staining

This study was approved by the China Medical University Ethics Committee (No. 200(7) PS14). After giving informed consent, women selected for this project were those who were having therapeutic termination of unplanned pregnancy without hereditary disease. One hundred eight phenotypically normal human embryos from 3 to 8 weeks were obtained from chemically induced/gentle curettage terminations of pregnancy (Table 1). Immediately, the embryos were washed by cold phosphate buffered saline (PBS, pH 7.4), then fixed in 4% buffered paraformaldehyde at 4°C for 24 h. Samples were dehydrated, embedded in paraffin, and sectioned sagittally at 4-μm thickness. Every fifth section of a series was stained with H & E for morphological examination by independent embryologists; the age of the embryos was assessed according to the Carnegie stages, which is the standard reference for staged human embryos [13]. The morphological changes of the URS, CM, rectum, and UGS during each stage were examined serially with special attention. Part of the sections from the sixth to the eighth was employed for TUNEL labeling and PCNA immunohistochemical staining.

Table 1 The distribution of embryos at different ages

Gestational age (week)	3	4	5	6	7	8	>8	Total
Number of embryos	9	12	13	25	26	15	8	108

TUNEL labeling

For the TUNEL method, the In Situ Cell Death Detection Kit, POD (Roche, Germany) was used. Briefly, dewaxed and rehydrated sections were incubated with 20 μg/ml of proteinase K for 15 min at room temperature, endogenous peroxidase activity was quenched by incubation in 3% H_2O_2 for 10 min. To block the nonspecific reaction, sections were incubated with normal goat IgG for 20 min. Incubated in TUNEL reaction mixture overnight at 4°C, followed by incubation in Converter-POD at 37°C for 30 min. Visualization of the reaction products were performed with 3,3P-diaminobenzidine (DAB) (Sigma, UK) chromogen reaction, and slightly counter-stained with haematoxylin.

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-PCNA (1:200, goat polyclonal, Santa Cruz Biotechnology, CA) and HRP-conjugated secondary antibody (Santa Cruz, CA). Antibody incubations were performed in PBS, 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. Signals were visualized using DAB (Sigma, UK). Sections were counterstained with hematoxylin.

Results

Morphological observations

According to the morphological changes, the development of the hindgut/anorectum could be described in three phases: (1) cloacal period (third to fifth week); (2)

Table 2 The developmental characteristics of the anorectum from the third to the seventh week

Gestational age (week)	Cloaca (anorectum/UGS)	CM	URS
3 (Fig. 1a, b)	The cloaca was visibly recognizable. The cloaca was continuous with the hindgut dorsocranially, and the allantois, ventrocranially, was bounded ventrally and separated from the amniotic cavity (AC) by the CM.	The CM itself assumed the characteristics of a multilayered epithelial plate orientated in the sagittal plane and extended to the umbilical cord.	The URS started to expand and elongated ventrocaudally and turned into a V shape, dividing cloaca into UGS ventrally and primitive anorectum dorsally.
4 (Fig. 1c)	As the mesenchyme grew the around the cloaca, the genital tubercle elongated, and the cloaca was remolded and its shape altered. The ventral outline of the UGS appeared triangular.	The CM moved from a vertical to a horizontal position; the ventral CM was significantly thicker than the dorsal part.	The URS descended and acutely changed into a U shape.
5 (Fig. 1d)	The cloaca with the above described features remained visible; however, the mesenchyme surrounding the various structures associated with the cloaca grew, and the cloaca became relatively smaller.	The dorsal cloaca gradually descended to the tail groove, the URS and the CM approximated, and the URS divided the cloaca clearly into a primitive rectum and UGS.	The disproportionate growth of the UGS over the anorectal part, the dorsal CM became further thinner while the ventral CM extended to the tip of the developing tubercle.
6 (Fig. 2a)	The cloaca continuously developed.	The dorsal part of the CM became rapidly thinner and reverted to 1–4 layers of squamous epithelial cell membranes and later disaggregated.	The URS was more closely related to the dorsal CM than before, with a narrow communication between the UGS and anorectum; however, the fusion of the URS with the dorsal CM was never observed.
7 (Fig. 2b)	The anorectum and UGS opened to the amniotic cavity.	The dorsal CM ruptured; the URS shifted ventrally and caudally, its tip was near to the ventral CM and the distance between these two structures became narrower.	The URS came to lie on the surface of the embryo where it formed the central margin of the perineum.

the development of a UGS and anorectum (fifth to seventh week); and (3) the establishment of the anus and perineum (eighth week and later). The developmental characteristics of the first two phases were summarized in Table 2.

During the eighth week, the URS progressively grew ventrally. The slit-like orifice became inconspicuous, and the endodermal URS epithelium has fused with the ventral part of the endodermal layer of the CM with persistent epithelium between the mesenchymes of the two structures. The mesenchyme in the urogenital tubercle insert into the two layers of the CM to establish the urethra leaving a median epithelial plate between the urethra and epithelial skin ventrally (Fig. 3a). The confluence of URS with the endodermal and ectodermal epithelia of CM was the location of the future central tendon of the perineum (Fig. 3b).

Spatiotemporal distribution of apoptosis/proliferation in human embryonic anorectum

During the sixth week, the apoptotic cells could be noted in the epithelium of the anorectum, URS, and UGS. In particular, abundant apoptotic cells started to appear within the epithelium at the level of the anal orifice (Fig. 4a). During the seventh week, quantities of apoptotic cells could be detected in the mesenchyme of the terminal rectum and dorsal rectum (Fig. 4b). During the eighth week, the apoptotic cells were not detected in the epithelium of the anorectum; remarkably, the fused tissue between the URS and the ventral CM were strong and constant (Fig. 4c).

Furthermore, during the sixth week, the proliferative cell was faintly observed in the epithelium of the anorectum, URS, and UGS. Sporadic proliferative cell could be observed in the dorsal CM (Fig. 5a). During the seventh week, the proliferative cells in the URS mesenchyme continuously extended, most of them were found in the anorectum and the ventral mesenchyme of the URS (Fig. 5b). During the eighth week, the proliferative cells were observed in the epithelium of the anorectum and urethra, remarkably detected on the fused tissue between the URS and the ventral CM, strongly and constantly (Fig. 5c). The regular pattern of apoptotic/proliferative cells from sixth to the eighth week was thoroughly compared in Table 3.

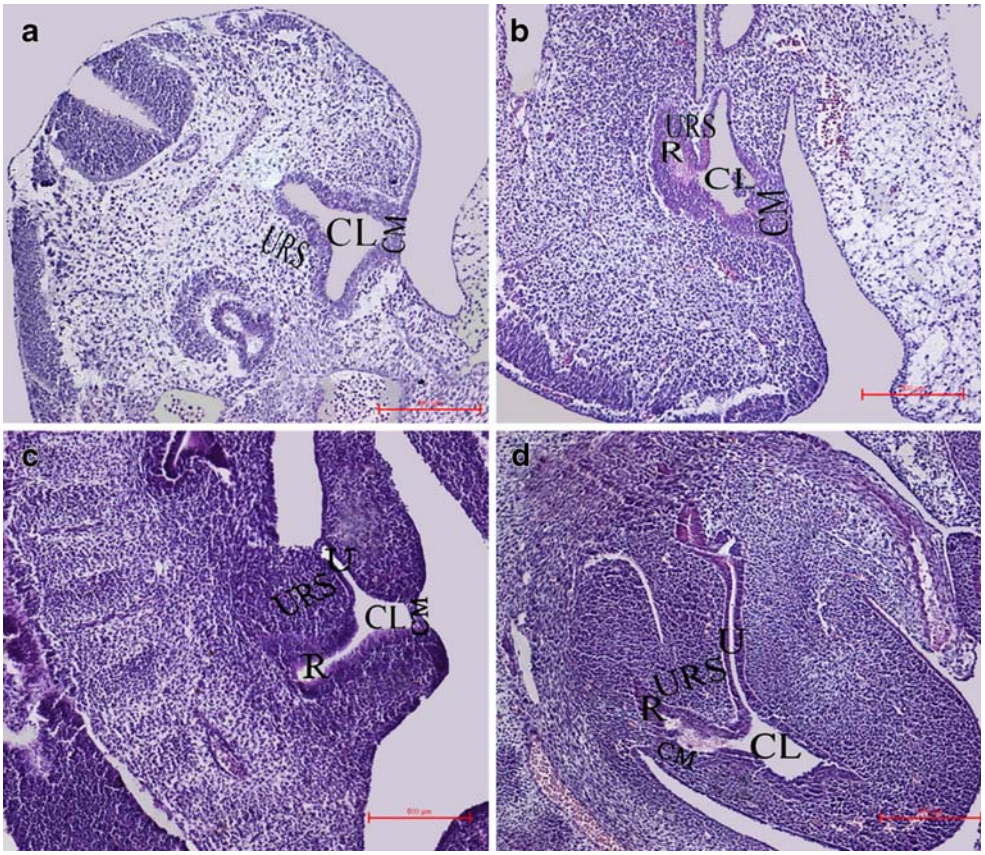

Fig. 1 Sagittal section through human embryo from the third to the fifth week. **a** Indicates the beginning of the third week, the cloaca was defined dorsally, separated from AC by the CM. **b** Indicates the third week, the cloacal mesenchyme multiplied, the bending V-shaped cloaca divided into UGS and anorectum; **c** during the fourth week, the cloacal shape altered, the CM moved from a vertical to a horizontal position, the ventral CM was significantly thicker than the dorsal part. **d** During the fifth week, the dorsal cloaca descended to the tail groove, the URS and the CM approximated, and a clearly short and narrowing communication of the primitive rectum and UGS. *AC* amniotic cavity, *CL* cloaca, *URS* urorectal septum, *CM* cloacal membrane, *R* rectum, *U* urethra; H & E, ×100

Discussion

Generally, the embryological principles about anorectal development could be constructed as follows: the URS descended constantly, fused with the CM, divided the cloaca into separate anal and urogenital membranes, and finally formed the perineum [13–17]. Recent improvement in the comprehension of the mechanisms by which embryos grow and develop, the roles of local signals [2], and molecular defect from adjacent epithelia, mesenchyme [18], and the aberrant nerve supply [19] have sparked updated interest in reviewing the normal development of the

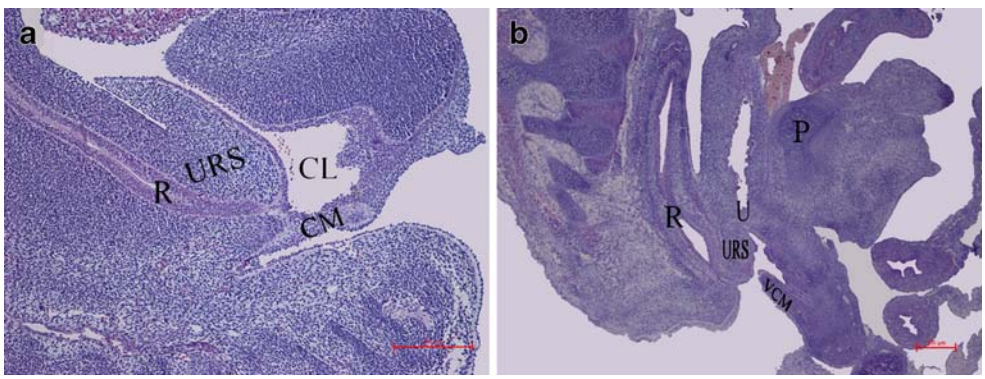

Fig. 2 Sagittal section through human embryo from the sixth to the seventh week. **a** During the sixth week, there was a constrictive canal between the UGS and anorectum. The dorsal CM became much thinner; however, the fusion of the URS with the dorsal CM was never observed. **b** Showed during the seventh week, as the result of CM rupturing, the urethra and anorectum communicated with the amniotic cavity. *CL* cloaca, *URS* urorectal septum, *VCM* ventral cloacal membrane, *R* rectum, *U* urethra; H & E, ×100 and ×40 for **a** and **b**, respectively

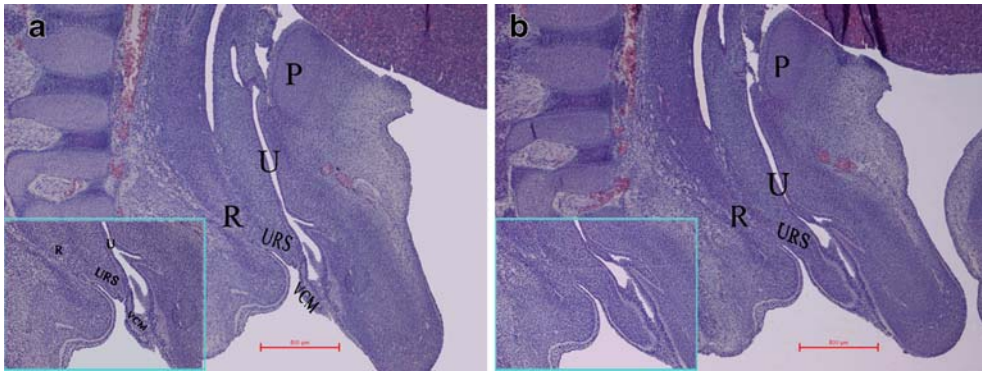

Fig. 3 Sagittal section through the eighth week human embryo. The endodermal URS epithelium has fused with the ventral part of the endodermal layer of the CM (**a**). The *inset* showed the URS and ventral CM under high-power magnification (original magnification, ×200).The confluence of the URS with the endodermal and ectodermal epithelia of the CM is the location of the future central tendon of the perineum. At this stage, the anorectum was clearly open, and the epithelial bordered between the columnar and squamous epithelium was clear. Smooth muscular layers could be discerned, which ended distally on a level with the pectinate line (**b**). The *inset* showed the URS and ventral CM under high-power magnification (original magnification, ×200). *URS* urorectal septum, *VCM* ventral cloacal membrane, *R* rectum, *U* urethra, *P* pubis; H & E, ×100

anorectum even though the development of the anorectum in human embryo has been and is being extensively disputed. Anyhow, until normal development is not better understood, it is difficult to conclusively determine the pathogenesis of anogenital malformation. In this study, histological analyses introduced several concepts that conflicted with current ideas about the anatomical changes during anorectal embryogenesis, namely the derivation of the anorectum, the nature of the URS between the anorectum and UGS or the perineum, and the spatiotemporal distribution of apoptosis/proliferation in human embryonic anorectum. Moreover, there was an apparent agreement that the cloacal development plays a significant role in the embryogenesis of the anorectal and urogenital system.

Up to now, the description of the cloacal development is still contradicted. Whether the URS fused with the CM or not has being chattered both recently and in the past. It is traditionally considered that when the embryo grew, the caudal curvature decreased and the distance between the

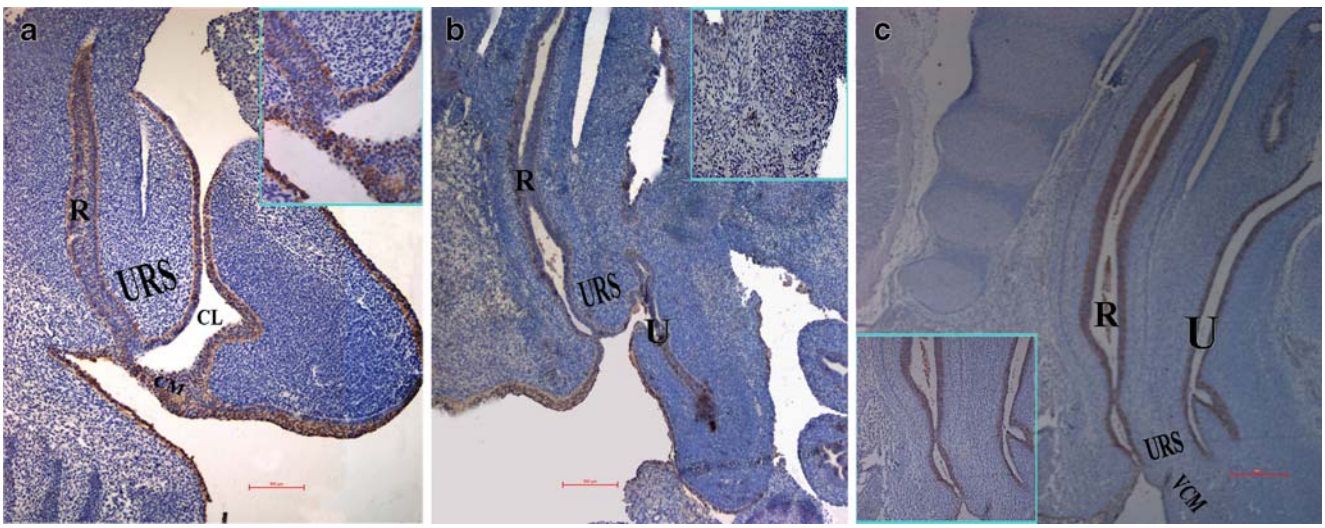

Fig. 4 TUNEL staining of the human anorectum from the sixth to the eighth week. **a** During the sixth week, apoptotic cells were focused in the URS and CM, **b** during the seventh week, large quantities of apoptotic cells were detected in the anal canal, **c** during the eighth week, the apoptotic cells were remarkably detected in the anorectum and the ventral mesenchyme of the URS. *URS* urorectal septum, *VCM* ventral cloacal membrane, *R* rectum, *U* urethra (original magnification, ×100). The *inset* showed the details under high-power magnification (original magnification, ×400)

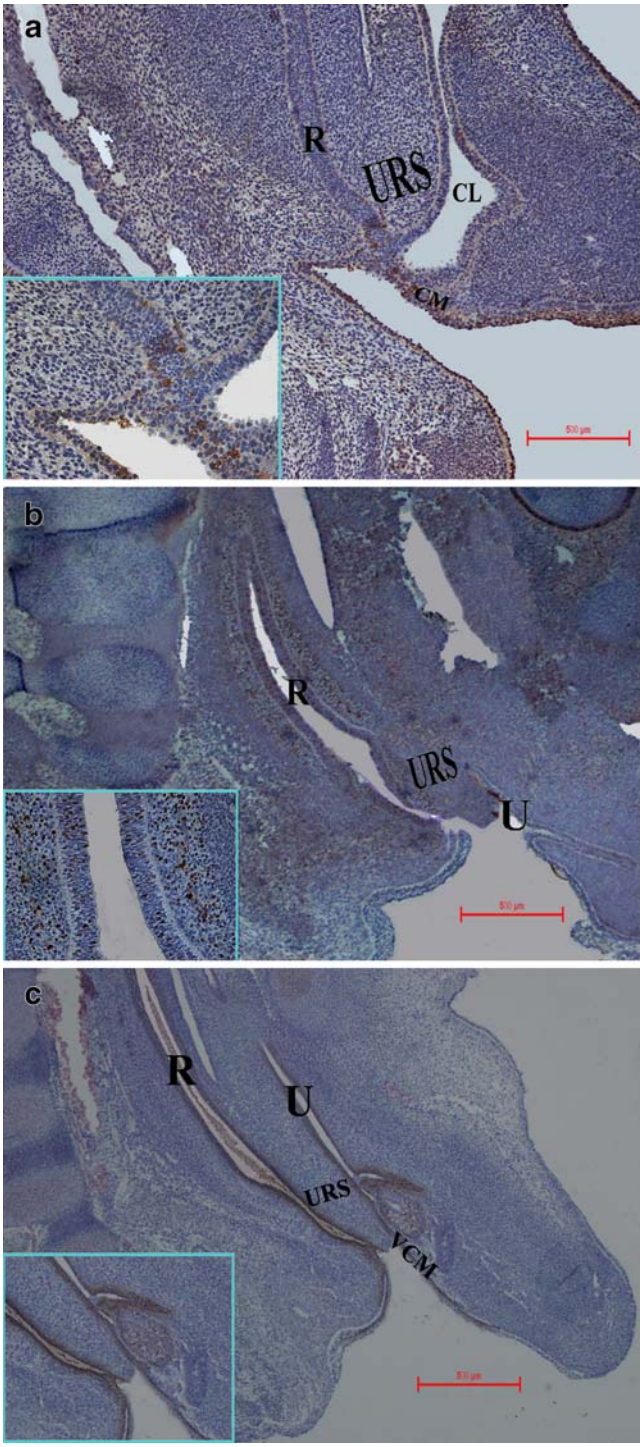

Fig. 5 PCNA staining of anorectum from the sixth to the eighth week. **a** During the sixth week, the sporadic proliferative cells could be noted in the epithelium of CM, **b** during the seventh week, major proliferative cells were found in the anorectum and the ventral mesenchyme of the URS, **c** during the eighth week, PCNA positive cells were detected on the fused tissue between the URS and the ventral CM. *URS* urorectal septum; *VCM* ventral cloacal membrane, *R* rectum, *U* urethra (original magnification, ×100). The *inset* showed the local feature (original magnification, ×400)

URS and CM decreased, rather, the two structures never fused [4–6, 20, 21]. In contrast, some authors addressed that the URS approximated and reached the CM with caudal migration of the URS [11, 16]. Nevertheless, Nebot-Cegarra J [13] and van der Putte [20, 21] contradicted the idea that the fusion of the URS with the CM did not exist. When the URS came into contact with the CM, it ruptured locally. In this study, we noted that the growth of human anorectum was associated with dramatic changes in the morphology, positioning, and relative sizes of the surrounding structures. Our observations revealed that from the fourth to the sixth week as the asymmetric growth of the mesenchyme surrounding the UGS part over the anal part, the cloaca was displaced and its shape altered. Meanwhile, the URS was more closely related to the dorsal CM. According to a previous study [17, 20, 21] and our observations, it was demonstrated that the cloaca underwent a sharp and disproportionate growth of the UGS compared with the dorsal part. It is possible that the separation of the urogenital sinus from the anorectum was caused by the process of rotation of the CM or configuration transformation of the cloaca during the unfolding of the caudal part of the embryo [4, 5]. In addition, from the fifth to the seventh week, the lumen of the cloaca gradually disappeared with the dorsal cloaca descending to the tail groove; due to the imbalanced apoptosis in the CM, the dorsal CM disaggregated. The present analysis clearly showed that the dorsal CM broke down without fusing with the URS. From the eighth week onward, the URS shifted caudally and ventrally and fused with the ventral CM. Based on these above data, it was credible to consider that the observations are complementary to explain some of the continuing controversies about the events of pristine cloacal development. According to these concepts it was suggested that:

1. The CM plays a crucial role on the cloacal embryogenesis.
2. The normal anorectal and genitourinary development depends on the dorsal and ventral CM, respectively.

During anorectal formation, lots of biological events were involved such as cell differentiation, proliferation, apoptosis, transformation, and adhesion [1, 2, 7, 9]. In this study, we intensively discussed the possible roles of apoptosis/proliferation during anorectal development. The patterns of apoptosis in the mesenchyme around the dorsal mesenchyme of the cloaca associated with transformation of the dorsal anorectum. Conversely, the line of pyknotic cells was related with the remodeling of the cloaca and disintegration of the dorsal CM in the sixth week. During the seventh week, it was shown that apoptotic cells were concentrated in the mesenchyme of the terminal rectum, implying that this phenomenon correlated with the anal canal development. From the eighth week onward, the

Table 3 Spatiotemporal distribution of apoptosis/proliferation during human embryonic anorectum

Gestational age (week)	The distribution of apoptosis	The pattern of proliferation
6	The apoptotic cells could be noted in the epithelium of anorectum, URS and UGS; in particular, abundant apoptotic cells started to appear within the epithelium at the level of the anal orifice (Fig. 4a).	The proliferative cell was faintly observed in the epithelium of the anorectum, URS, and UGS. Sporadic proliferative cell could be observed in the dorsal CM (Fig. 5a).
7	Quantities of apoptotic cells could be detected in the mesenchyme of the terminal rectum and dorsal rectum (Fig. 4b).	The proliferative cells in the URS mesenchyme continuously extended, most of them were found in the anorectum and the ventral mesenchyme of the URS (Fig. 5b).
8	The apoptotic cells were not detected in the epithelium of the anorectum, remarkably the fused tissue between the URS and the ventral CM, strongly and constantly (Fig. 4c).	The proliferative cells were observed in the epithelium of the anorectum and urethra, remarkably detected on the fused tissue between the URS and the ventral CM, strongly and constantly (Fig. 5c).

disproportionate apoptotic cells were distributed in the ventral URS mesenchyme over the dorsal part, which associated with the ventral shift of the URS. It was tempting to presume that apoptosis played a leading role in the formation of the anus and the development of the rectum.

Even though, apoptosis is commonly observed during embryogenesis, proliferation is complementary to apoptosis or differentiation in the morphogenesis and the regulation of cell populations in the embryo [10–12, 18, 20]. In agreement with Paidas CN [4] and van der Putte [20, 22], in a current study, the cellular proliferation could be observed in the epithelium of the CM, the dorsal mesenchyme of the URS, the fused tissue between the URS, and the ventral CM from the sixth to the eighth week. It must be emphasized that as a result of the ventral mesenchyme proliferation around the cloaca, the cloaca was remolded. The ventral mesenchyme proliferation also promoted the CM moving from a vertical to a horizontal position. Incorporating with the apoptosis, the development of the CM was a significant imbalance. Based on the above data, it was obvious that this proliferation was deeply involved in configuration changes; furthermore, the ventral mesenchyme of the cloaca was an important part of the cloaca and an important factor affecting ventral–caudal migration of the cloaca. The apoptosis and proliferation act in a dose-dependent and diffusible manner in organizing patterns in early vertebrate development [23, 24]. It was very likely that there was relatively a dorso-ventral pattern between apoptosis and proliferation during the anorectal embryogenesis suggesting that ectopic distribution of the apoptosis and proliferation may interfere with normal development.

During this stage, numerous reports have suggested that various developmental regulatory signaling molecules associated with down or up-regulation of apoptosis and proliferation: Hoxd-13 [23], Shh [24], Wnt5a [25, 26], Tcf4 [27], and Cdx1 [28]. In the inordinate condition, the dorsal CM was abnormal, so the inherent apoptotic/proliferative program was interrupted. The improper induction of the dorsal CM led to a hampered shift of the dorsal cloaca which gave rise to blocking normal migration of the dorsal cloaca towards the tail groove.

Taken together with the evidence from animal models [3, 6, 14, 27, 28] and human embryos [4–6, 17, 20], for clinicians, current evidence suggested the possibility that different embryonic stage and different dysplastic regions led to the variant clinical types of anorectal deformities including perineal fistula, rectourethral fistula, rectovesical fistula, vestibular fistula, common cloaca, anal stenosis, rectal atresia/stenosis and so on. Taking account of the present analysis, it was indicated strongly that the earliest morphological defect leading to ARM was a deficiency of the dorsal CM and the dorsal cloaca. The extent of the defect in the dorsal CM decided the severity of the defect in ano-urogenital system. At the early stage, an error from malfunctioned pluripotent cells in the cloaca might lead to common cloaca in the future. From the fifth to the eighth week, the defective development of the dorsal cloaca may bring the dorsal cloaca and dorsal CM into an abnormally ventral position, which resulted in various fistulas in the ano-urogenital system. The smaller defects led to slight distal defects (anal stenosis, anal atresia, and so on). The larger defects were associated with major malformations in the region as well as urogenital fistulas, even abnormalities in the development of the UGS such as urorectal septum malformation sequence (URSM) and urethral hypoplasia [29].

In conclusion, this study provided evidence that the normal anorectal development of a human may depend on the dorsal cloaca and dorsal CM. The apoptosis and proliferation pattern in the cloaca and its mesenchyme may play a pivotal role in the formation of the anorectum. Further comprehensive 3D reconstructions and related gene expression investigations may facilitate the future analysis. This may identify factors responsible for ARM and provide

more information for the development of variant clinical types of anorectal deformities.

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