Developmental Biology 409 (2016) 106–113

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Clarification of mammalian cloacal morphogenesis using high-resolution episcopic microscopy

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ARTICLE INFO

Article history:
Received 23 June 2015
Received in revised form
7 October 2015
Accepted 15 October 2015
Available online 17 October 2015

Keywords:
Cloaca
Bladder
Shh
Dkk1
HREM

ABSTRACT

The developmental process through which the cloaca transforms from one hollow structure to two separated urinary and digestive outlets remains controversial and speculative. Here, we use high-resolution episcopic microscopy to examine a comprehensive series of normal and mutant mouse cloaca in which the detailed 3-dimensional (3-D) morphological features are illuminated throughout the development. We provide evidence that the dorsal peri-cloacal mesenchyme (dPCM) remains stationary while other surrounding tissues grow towards it. This causes dramatic changes of spatial relationship among caudal structures and morphological transformation of the cloaca. The 3-D characterizations of *Dkk1* mutants reveal a hyperplastic defect of dPCM, which leads to a significant anterior shift of the caudal boundary of the cloaca, premature occlusion of the cloaca and, imperforate anus phenotype. Conversely, *Shh* knockout causes a severe hypoplastic defect of cloaca mesenchyme including dPCM and persistent cloaca. Collectively, these findings suggest that formation of the dPCM is critical for cloacal morphogenesis and furthermore, growth and movement of the mesenchymal tissues towards the dPCM lead to the cloaca occlusion and separation of the urinary and digestive outlets.

1. Introduction

The cloaca is a transient widening of the hindgut. It is divided during embryogenesis so that the digestive and urinary tracts of placental mammals exit the body separately. Abnormal cloaca development causes urogenital and anorectal malformations, which are among the most common forms of human birth defects. Despite extensive investigation, however, the process of mammalian cloacal morphogenesis remains a subject of controversy and speculation.

The century-old urorectal septum (URS)-based septation model offers the simplest interpretations. The model was primarily based on images of the midline sagittal sections, which give an impression of a septal structure, *i.e.* the URS, between the primitive hindgut and bladder. Rathke (1832) posited that the putative bilateral plicae fuse to form the septum and divide the cloaca through a "zipper-like" process. Tourneux (1888), on the other hand, suggested that the septum descends like a "theater curtain" to separate the cloaca in the rostral-to-caudal direction. Many have attempted to experimentally test the septum-based hypotheses. For instance, Hynes and Fraher have performed a serial histological section analysis together with the computer-assisted three-dimensional (3-D) reconstruction and concluded that the presumptive URS is derived from the progressive median fusion of longitudinal folds, *i.e.* Rathke's plicae, on the lateral walls of the cloaca (Hynes and Fraher, 2004). However, using the similar approaches and scanning electron micrograph (SEM), several other investigators suggested that there is no evidence of the Rathke plicae nor the Tourneux fold in mouse (Penington and Hutson, 2003), rat (Kluth et al., 1995), pig or human embryos (Nievelstein et al., 1998; Paidas et al., 1999; van der Putte, 1986). The reason for the discrepancy is not entirely clear but the curved shape of the cloaca may render unambiguous description difficult in the absent of a complete set of 3-D images.

Closely related to cloaca morphogenesis is the caudal development during the secondary axis formation of the vertebrate body plan. After gastrulation, which establishes the primary body axis, vertebrate embryos grow substantially in length at the caudal end and at the same time undergo dramatic morphological

Abbreviations: A, anus; Bl, bladder; CD, cloacal duct; Cl, cloaca; CM, cloaca membrane; CND, common nephric duct; dPCM, dorsal pericloacal mesenchyme; GT, genital tubercle; GTM, genital tubercle mesenchyme; HG, hindgut; ICM, intracloacal mesenchyme; LB, limbbud; MM, metanephric mesenchyme; PC, peritoneal cavity; R, rectum; T, tail; TB, tail bud; TG, tailgut; UA, umbilical artery; UG, urethral groove; UGS, urogenital sinus; vPCM, ventral pericloacal mesenchyme

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http://dx.doi.org/10.1016/j.ydbio.2015.10.018
0012-1606/

changes including rotation and repositioning of caudal and ventral structures (Hassoun et al., 2010; Muller and O'Rahilly, 2004; Tam, 1981; Wilson and Beddington, 1996). Characterization of a series of human embryos ranging from 25 to 54 days' gestation suggested that transformation of the cloaca is a passive process secondary to caudal development (Nievelstein et al., 1998; Paidas et al., 1999). This theory rejects the notion that the URS grows caudally in the direction of and fuses with the cloaca membrane (CM). It further suggests that the apparent decrease in distance between the URS and the CM is due to the caudal unfolding process, which passively changes the spatial relationship between the involved structures.

Because of the rapidly changing size, shape and spatial relationships among multiple structures during cloaca development, high quality serial 3-D images are required to fully appreciate the morphological complexity. Here, we use high-resolution episcopic microscope (HREM) technology (Mohun and Weninger, 2012a), which preserves and digitizes fine 3-D morphological features. This allows us to generate a series of 3-D "snapshots" of mouse cloaca, to create *de facto* live imaging of the entire process of cloacal morphogenesis and, to quantitative analyze size and dimensions of all structures involved. We provide evidence that the dorsal pericloacal mesenchyme (dPCM) demarcates the caudal boundary of the cloaca. During cloaca development, the dPCM remains stationary while other mesenchymal tissues surrounding the cloaca grow towards it. In this way, the cloaca is occluded and the remnant of the cloaca is displaced to the epithelial surface of the perineum. Results from this study also suggest that defects of the dPCM likely contribute to the pathogenesis of urogenital and anorectal anomalies. We believe that the serial high-resolution 3-D images generated herein will serve as a useful resource for studying human cloaca defects using mouse models.

2. Materials and methods

2.1. Animals

All animal studies were approved by the Institutional Animal Care and Use Committee at Boston Children's Hospital. C57Bl6 wild type mice are obtained from Charles River. *DKK1* and *Shh* mutant mice have been described previously (Guo et al., 2014). Image acquisition and processing protocols are included as supplementary data (Fig. S1).

2.2. High-resolution episcopic microscopy imaging

Block face images were captured using a procedure similar to that described (Mohun and Weninger, 2012b). Briefly, tissue blocks were mounted on a rotary microtome (Leica, RM2265). The stop position of after each rotation was kept constant. A stereo zoom microscope (Zeiss, Axio Zoom.V16) was mounted perpendicularly to the block surface. A digital camera (Hamamatsu Photonics K.K., C11440-10C) equipped with green fluorescent protein (GFP) filter cube set (excitation 470 +/−20 nm, dichroic 495 nm, emission 525 +/−25 nm) was used to capture images of the block face. Images (1920 × 1440 pixels) were scaled automatically from 1.5 × 1.5 μm/pixel to 3.6 × 3.6 μm/pixel (Zen pro 2012, Zeiss) depending on the size of embryos. Section thickness was set at 1.5 μm (e9.5 and e10.0), 2.0 μm (e10.5–e12.0) and 2.5 μm (e12.5–e13.5). Images were captured in grayscale mode. The exposure time was adjusted from 120 ms to 320 ms accordingly.

2.3. Image processing and data analysis

All images were inverted using Photoshop (Adobe, V13.0) and further converted to volume data using the 3-D visualization software (Amira V5.4.5). When loading the images, we included resolution and thickness into the text fields of the Voxel Size port to define the size of voxels, so that the scale of the reconstructed image accurately reflected the size of each specimen. In order to optimize the quality of the 3-D reconstructed images, we used the AlignSlices module to align the original 2-D images automatically and adjusted these manually where necessary. During this procedure, the Least-Squares alignment mode with default settings was applied. Subsequently, the 3-D views were generated using the Volren module. Using this whole mount image as a reference, we generated virtual sections through the embryo by the ObliqueSlice module. In this module, the section plane could be freely rotated and shifted. The Scale module was used to show the 2-D coordinates. A snapshot was taken in the Orthogonal Projection mode.

The somites were visualized both in sagittal and whole mount images. To determine the relative location (somite number) of the leading edges of each structure, we first established the midline sagittal view. We then created another planes perpendicular to the sagittal image to determine the corresponding somite. In order to visualize the cloacal lumen, we labeled the images manually in the Segmentation Editor using the LabelField module. We used the lasso and brush tools to label the lumen every three original 2-D slices. The Interpolate command was applied to label the intervening slices automatically. The labeling was inspected manually and adjusted if needed. Surface views of the cloacal lumen were visualized using the SurfaceGen module. To create video animations, we connected the CameraRotate module to the cloacal lumen, added the ObjectTranslate module to the sectional images, and generated flythrough videos by CameraPath module. These actions were edited with the DemoMaker Module. All videos were recorded into movie files using the MovieMaker module.

3. Results

3.1. 3-D overviews of mouse cloaca during morphogenesis

Mouse embryos were collected from a total of 9 separate developmental stages between day 9.5 (e9.5) or somite stage 25 (s25) and e13.5/s65, the period during which mouse cloaca is formed and transformed into the urogenital and the anorectal outflow tracts (Wang et al., 2013). To directly visualize the morphogenetic process, we generated 3-D HREM images from these embryos at all developmental stages (Figs. 1, S1 and S2, $n \geq 3$). Both vaginal plug date and somite number were used to better estimate embryo age. Important morphological features of the cloaca began to emerge from the 3-D images. At e9.5, an enlarged segment of the hindgut or the cloaca ended bluntly into the tail bud or the caudal eminence (Fig. 1A and a). The CM was detected at the ventrocaudal midline region of the hindgut (Fig. 1, yellow line and between two white arrowheads). There were no significant morphological changes of the hindgut during the next 12 hours with the exception of the tail gut formation (Fig. 1B and b). However, the cloaca began to distinguish itself morphologically starting at e10.5 (Fig. 1C and c). During the next 48 h, the large cloaca cavity was reduced to a thin tube at e12.5, the cloaca duct (CD) (Fig. 1C–G and c–g). At the same time, the primitive ventral urogenital and dorsal anorectal tracts emerged and were physically connected via the CD (Fig. 1G and g). Twelve hours later at e13.0 (Fig. 1H, h, I and i), the lumen of the CD narrowed significantly and the epithelial tube began to disintegrate. As the result, the urogenital and the anorectal tracts appeared to be separated completely. The actual fate of the CD is likely the ventral midline epithelial seam, *i.e.* the perineal and penile raphe (Seifert et al., 2008). This is the end stage of cloacal morphogenesis and