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Cellular mechanisms of Müllerian duct formation in the mouse

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Abstract

Regardless of their sex chromosome karyotype, amniotes develop two pairs of genital ducts, the Wolffian and Müllerian ducts. As the Müllerian duct forms, its growing tip is intimately associated with the Wolffian duct as it elongates to the urogenital sinus. Previous studies have shown that the presence of the Wolffian duct is required for the development and maintenance of the Müllerian duct. The Müllerian duct is known to form by invagination of the coelomic epithelium, but the mechanism for its elongation to the urogenital sinus remains to be defined. Using genetic fate mapping, we demonstrate that the Wolffian duct does not contribute cells to the Müllerian duct. Experimental embryological manipulations and molecular studies show that precursor cells at the caudal tip of the Müllerian duct proliferate to deposit a cord of cells along the length of the urogenital ridge. Furthermore, immunohistochemical analysis reveals that the cells of the developing Müllerian duct are mesoepithelial when deposited, and subsequently differentiate into an epithelial tube and eventually the female reproductive tract. Our studies define cellular and molecular mechanisms for Müllerian duct formation.

Keywords: Müllerian duct; Paramesonephric duct; Wolffian duct; Mesonephric duct; Nephric duct; Tubulogenesis; Cell proliferation

Introduction

Amniotes regardless of their genetic sex form two separate and distinct genital ducts, the Wolffian and Müllerian ducts, during embryonic development. In mammals, the former differentiates into the male reproductive tract, the vas deferentia, epididymides and seminal vesicles, whereas the latter develops into the female reproductive tract consisting of the oviducts, uterus and upper third of the vagina. Initially, the Wolffian ducts form from the intermediate mesoderm (Jacob et al., 1991; Obara-Ishihara et al., 1999) and in the mouse, its development is complete by embryonic day 10.5 (E10.5). Johannes Müller first described the Müllerian duct in human and chick embryos in 1830 and beginning with its first description, the mechanism for Müllerian duct growth has been controversial.

It was noted that during development, the Müllerian duct is intimately associated with the Wolffian duct. At its caudal growing tip, the forming Müllerian duct is in physical contact with the Wolffian duct and was believed to be located inside the basement membrane of the Wolffian duct. Due to this tight association, Balfour and Sedgewick (1879) believed that the cells of the Müllerian duct were derived from cells of the Wolffian duct. This belief was further supported by Gruenwald (1937) who showed that experimental interruption of the Wolffian duct in the chick resulted in incomplete formation of the Müllerian duct at the point of Wolffian duct interruption. Despite this evidence, others believed that the Wolffian duct did not contribute cells to the Müllerian duct, but simply acted as a guide. Dohr et al. (1987) demonstrated a difference in antigen expression between Wolffian duct and Müllerian duct cells, suggesting that the Wolffian duct does not contribute cells to the developing Müllerian duct. This, however, was based on the assumption that if a Wolffian duct cell transformed into a Müllerian duct cell, some antigen expression would persist and still be observed. Many studies have attempted to define the mechanism for the development of the Müllerian duct (Dohr et

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al., 1987; Gruenwald, 1941; Jacob et al., 1999; Potemkina et al., 1971), but to date; this mechanism remains to be elucidated.

Development of the Müllerian duct is considered biphasic, with the first phase consisting of an invagination of the coelomic epithelium through the mesonephros and the second phase an elongation of the Müllerian duct caudally to the urogenital sinus (Gruenwald, 1941). In the first phase, cells of the coelomic epithelium are specified to a Müllerian duct fate through an unknown mechanism. This specification can be identified by the expression of *Lim1* in cells of the coelomic epithelium (Kobayashi et al., 2004). After specification, *Wnt4* expression from the mesonephros or coelomic epithelium, induces the invagination of these specified cells (Kobayashi et al., 2004; Vainio et al., 1999). This first phase of Müllerian duct development is Wolffian duct independent (Carroll et al., 2005; Kobayashi et al., 2005). The first phase ends when the invaginating Müllerian duct extends to and contacts the Wolffian duct where the second phase begins.

It is during this second phase that most studies have been performed. In this elongation phase, the presence of the Wolffian duct is required for development of the Müllerian duct. This dependence has been shown both *in vitro* and *in vivo*. As described above, when the Wolffian duct is disrupted at a specific point in ovo, the Müllerian duct is unable to grow past that point (Gruenwald, 1937). *Lim1* was shown to be necessary for maintenance of the Wolffian duct and conditional inactivation results in loss of the Wolffian duct epithelium. Due to the dependence of the Müllerian duct on the Wolffian duct, loss of *Lim1* in the Wolffian duct also results in incomplete development of the Müllerian duct (Kobayashi et al., 2005). The Müllerian ducts of mice mutant for the *Pax2* gene invaginate from the coelomic epithelium, but do not elongate due to degeneration of the Wolffian duct (Miyamoto et al., 1997; Torres et al., 1995). Finally, genetic evidence has shown that the Wolffian duct may not only act as a physical guide or contribute cells to the Müllerian duct, but also provides a necessary signal for its elongation. *Wnt9b* is expressed by the Wolffian duct epithelium and the loss of this gene results in incomplete development of the Müllerian duct (Carroll et al., 2005). When expression of *Wnt9b* was lost, the Müllerian duct was able to properly invaginate from the coelomic epithelium, but could not extend caudally, suggesting that the Wolffian duct is not required for the first phase of growth. Also, loss of *Wnt9b* did not affect the Wolffian duct itself, therefore, the Wolffian duct signals, through *Wnt9b*, to the developing Müllerian duct leading to the second phase of Müllerian duct development.

There are many ways in which an epithelial tube can be generated. Tubes can form through a mechanism of wrapping in which cells of an epithelium invaginate in a line and pinch off forming a separate tube, as does the vertebrate neural tube (Colas and Schoenwold, 2001; Lubarsky and Krasnow, 2003). Tubes can bud off from a larger tube forming a branching organ like the mammalian lung (Metzger and Krasnow, 1999) or the Drosophila tracheal system (Hogan and Kolodziej, 2002; Wilk et al., 1996). In these mechanisms, tubes are generated from an already polarized epithelium. Cells can change fate when subjected to certain factors, such as the mesenchymal to epithelial transition of the mammalian metanephric kidney and Wolffian duct (Jacob et al., 1991; Karavanova et al., 1996). Cavitation is a process in which a cylindrical mass of cells forms a lumen by eliminating cells in the center of the mass (Lubarsky and Krasnow, 2003). A cord of two cells can hollow forming a lumen and even one cell can form a lumen by generating an apical and basal polarity within itself (Wolff, 1972). Interestingly, in all of these cases, no tubule requires the presence of another separate and distinct epithelial tube for its morphogenesis.

In this study, we investigated the mechanisms of Müllerian duct development. We show that the Wolffian duct does not contribute cells to the developing Müllerian duct, but rather, formation of the Müllerian duct is accomplished by cell proliferation. We also examined the character of both the Wolffian and Müllerian ducts. We show that while the Wolffian duct is a true epithelial tube, the developing Müllerian duct is mesoepithelial in character and subsequently differentiates into an epithelial tube. Taken together, these data indicate that the developing Müllerian duct accomplishes its elongation predominantly by proliferation of a small group of cells located at its distal tip, guided by the Wolffian duct.

Materials and methods

Mice

Lim1$^{lz/+}$ mice (Kania et al., 2000) were maintained on a C57BL/6; 129/SvEv mixed genetic background. *Lim1*$^{lz/+}$ males were bred to Swiss females (Charles River Laboratories). Noon on the day of the vaginal plug was considered embryonic day 0.5 (E0.5) and tail somite numbers were used to stage embryos (Hacker et al., 1995). *Hoxb7-Cre*$^{tg/+}$ mice (Yu et al., 2002) were maintained on a C57BL/6 congenic background and *R26R* mice (Soriano, 1999) on a C57BL/6; 129/SvEv mixed genetic background.

Embryo sex genotyping

Embryos were sexed by Barr body staining of amnions. Amnions were placed in eppendorf tubes and fixed in 3:1 Methanol/Glacial Acetic Acid until all embryos were collected. The fixative was removed and 50 μL of 60% Glacial Acetic Acid was added. The tubes were vortexed to dissolve the amnion and the tube was filled with fixative. The cells were then spun at 5000 rpm for 3 min and inverted to remove supernatant. The cells were vortexed to resuspend and pipetted onto coverslips. 25 μL of 1% Toluidine Blue was added and the coverslips placed onto slides. The slides were then analyzed for the presence or absence of Barr bodies.

Tissue preparation and histology

X-gal staining was performed as described (Nagy et al., 2003). Tissue was fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) overnight, dehydrated through a series of ethanols and embedded in paraffin for histological sectioning. The paraffin embedded wild-type tissue was sectioned at 5 μM and processed for hematoxylin and eosin staining or immunohistochemistry/fluorescence. X-gal stained tissue was sectioned at 12 μM and counterstained with 0.33% Eosin Y. Measurements of Müllerian duct length were performed using the measure tool in Adobe Photoshop.

Immunohistochemistry/fluorescence

Sectioned urogenital ridges were deparaffinized with xylenes and rehydrated through a series of ethanols to water. Endogenous peroxidase activity was