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The origin of the Mullerian duct in chick and mouse

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Abstract

In vertebrates the female reproductive tracts derive from a pair of tubular structures called Mullerian ducts, which are composed of three elements: a canalised epithelial tube, mesenchymal cells surrounding the tube and, most externally, coelomic epithelial cells. Since the first description by Johannes Peter Muller in 1830, the origin of the cells making up the Mullerian duct has remained controversial. We report the results from lineage-tracing experiments in chicken and mouse embryos aimed to provide information of the dynamics of Mullerian duct formation. We show that all Mullerian duct components derive from the coelomic epithelium in both species. Our data support a model of a Mullerian epithelial tube derived from an epithelial anlage at the mesonephros anterior end, which then segregates from the epithelium and extends caudal of its own accord, via a process involving rapid cell proliferation. This tube is surrounded by mesenchymal cells derived from local delamination of coelomic epithelium. We exclude any significant influx of cells from the Wolffian duct and also the view of a tube forming by coelomic epithelium invagination along the mesonephros. Our data provide clues of the underlying mechanism of tubulogenesis relevant to both normal and abnormal development of the female reproductive tract.

Keywords: Mullerian duct; Wolffian duct; Coelomic epithelium; Tubulogenesis; Endometriosis

Introduction

Tubulogenesis is a process vital to the development and function of many tissues and organs. Despite the apparent simplicity of a tube, there are many different ways that tubes can form during embryogenesis including folding, branching, mesenchymal to epithelial transitions and formation of a lumen in an initial solid cord (Myat, 2005). Moreover, tubulogenesis is a process requiring precise control over cellular events, including changes in cell shape and adhesion, cell division and apoptosis, as well as active alteration of the cellular environment. How these different processes are coordinated in space and time to form a tube is not sufficiently understood for any system.

The female reproductive tract represents a tubular structure of obvious importance to the continuation of a species. Its anatomy varies markedly among vertebrates, depending on the type of fertilisation, mode of reproduction, type of placentation and other factors. Anatomical differences can even be observed within a species. Despite the extremely high degree of specialisation of the adult structures, the internal genital tracts derive, with few exceptions such as the Teleost fish (Suzuki and Shibata, 2004), from a pair of tubular structures called Mullerian ducts or paramesonephric ducts.

The Mullerian ducts arise during embryogenesis as part of the urogenital system in both sexes. They develop along the anterior–posterior (A–P) axis of the embryos in close proximity and lateral to the mesonephric (or Wolffian) ducts. It is possible to distinguish three cellular components in the Mullerian duct: the epithelial cells forming the inner tube (Mullerian duct epithelium: MDE), the mesenchymal cells surrounding the tube (Mullerian duct mesenchyme: MDM) and the coelomic epithelial cells defining the external borders of the duct (Mullerian coelomic epithelium: MCE).

Molecular genetic studies in mouse have contributed to the identification of a number of proteins essential for the formation of the Mullerian duct. These include the homeodomain transcription factors Pax2, Pax8, Lim1, Emx2, Hoxa13 and the signalling molecule Wnt4, defining a genetic cascade for early Mullerian development (Kobayashi and Behringer, 2003; Kobayashi et al., 2004).

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Classical embryological studies of extirpation or blockage of the Wolffian duct in chick embryos have shown that the Wolffian duct is required for the formation and caudal extension of the Mullerian duct (Bishop-Calame, 1966; Didier, 1971, 1973; Grunwald, 1941). Further evidence for this requirement comes from mouse and human studies. Mice with a conditional deletion of *Lim1* in the Wolffian duct have a truncated Mullerian duct due to the inability of maintaining the mesonephric duct (Kobayashi et al., 2005; Pedersen et al., 2005), while in humans, several syndromes have been identified where Mullerian duct growth is affected by the absence of the Wolffian duct. One such a condition is a form of kidney aplasia associated with the absence of a fallopian tube on one side and uterus unicornus on the other side (Grunwald, 1941). How the dependence of the Mullerian duct on the Wolffian duct is achieved is not yet known.

To begin understanding the mechanisms of early development of the Mullerian tube, it will be necessary to integrate the molecular data with a cellular understanding of the process, which is still poor. One of the key issues is the origin of the cells contributing to the duct during its formation and during its growth. Most of our current knowledge derives from light and electron microscopy observations of Mullerian duct sections at different development time-points in different organisms (Abdel-Malek, 1950; Del Vecchio, 1982; Dohr and Tarmann, 1984; Frutiger, 1969; Furbringer, 1878; Grunwald, 1941; Hall, 1904; Hashimoto, 2003; Inomata et al., 1989; Jacob et al., 1999; Wrobel, 2003; Wrobel and Sub, 2000).

In the chick, the first sign of Mullerian development has been described as the appearance of a Mullerian ridge consisting of a thickening of the coelomic epithelium adjacent to the mesonephric duct. According to Jacob et al. (1999), the primordium of the MDE becomes apparent later, at stage Hamburger and Hamilton (HH) 25, as an aggregation of cells extending caudally from a funnel (ostial funnel) formed in the cranial area of the Mullerian ridge. By HH30, the MDE has grown a considerable length. The rostral end is a luminal epithelial tube surrounded by layers of mesenchymal cells, while the caudal tip is mesenchymal and stays in close contact with the Wolffian duct. Other studies performed in Amniota (such as human, rat, bovine,) do agree that the Mullerian duct is formed by the caudally directed growth of the ostial funnel, but there are at least two important unresolved issues. The first, which is controversial, concerns the origin of the cells contributing to the duct epithelium. One model predicts that the duct anlage derives from a placode-like thickening and deepening of the coelomic epithelium, which then extends caudally, of its own accord, forming the epithelial Mullerian duct itself (Jacob et al., 1999). A second model predicts a major or sole contribution of Wolffian duct cells to the growing MDE via a budding or splitting off from the Wolffian duct itself (Del Vecchio, 1982; Frutiger, 1969; Inomata et al., 1989). The second question that has not been clearly answered in any study is the origin of the MDM along the A–P axis of the embryo.

All these issues need to be tackled with approaches that depend on following cell fate in a dynamic way. We decided first to investigate the contribution of the coelomic epithelial cells to MDE and MDM by performing lineage-tracing experiments in chick embryos. We electroporated GFP-expressing plasmids into coelomic epithelial cells *in ovo* just before the appearance of a Mullerian ridge and followed the fate of the GFP cells after 3–4 days of development. We then performed a second set of lineage tracing experiments in mouse urogenital ridges as a comparative study, to address how well the process of Mullerian duct formation is conserved.

Materials and methods

Animals

Fertilised chicken eggs were obtained from Winter Egg Farm. Mouse embryos for electroporation and MitoTracker injections were from the outbred Parkes strain maintained at the NIMR. The transgenic line *Sox9(1.9)LacZ* was maintained as heterozygote (abbreviated in the text as *1.9LacZ*/+) on a CBA/B10 background. These mice express *LacZ* under the control of a 1.9 kb regulatory region from mouse *Sox9* (Sekido and Lovell-Badge, unpublished data).

Electroporation of chick and mouse embryos

Fertilised chicken eggs were incubated at 37.5°C for 2.5 days up to stage Hamburger and Hamilton (HH) 14–17 (Hamburger and Hamilton, 1992). Following the removal of 2.5 ml of albumen with a syringe, a window was cut in the egg shell. The electroporation was performed as described in Sekido and Lovell-Badge (in press), with some modifications. In brief, DNA was injected into one side of the coelomic cavity using a glass capillary needle and an inject + matic pico-pump. Two small electrodes (4 mm length, 0.4–0.5 mm diameter) were applied in parallel, one on each side of the embryo and a difference in voltage was generated to allow directional entry of DNA into cells towards the midline. The low fixed voltage was applied with a BTX ECM-830 electroporator. The eggs were then sealed with standard tape and allowed to develop at 37.5°C. The survival rate up to stage HH30–32 was variable, ranging from one-third to two-thirds of the total number of injected embryos.

11.5 dpc wild type mouse embryos were dissected in Dulbecco's Minimal Eagle's medium (DMEM). The exact age of the embryos was then established by counting tail somites (ts) number as described in Hacker et al. (1995). Embryos were placed in a dish in PBS and the injection/electroporation was performed as for the chicken embryos. The urogenital ridges were subsequently placed into grooves of a 1% agarose support in 35 mm dishes containing DMEM with 100 units/ml penicillin, 0.05 mg/ml streptomycin, 2 mM Glutamine and 10% fetal calf serum. The samples were incubated at 37°C, in 5% CO_2 for a couple of days.

Conditions of electroporation: chicken embryos: five 50 ms pulses at 24 V; mouse embryos: five 50 ms pulses at 52 V. Electroporated DNA: plasmid $pCS2^+$ expressing EGFP under the ubiquitous promoter CMV IE94.

MitoTracker injections

MitoTracker red (CMXRos from Molecular Probes) was dissolved in DMSO at 2 mg/ml and diluted 1:4 in 10% Sucrose just before labelling the urogenital ridges of ts 13–19 wild type embryos. Using a microcapillary glass needle, the dye was delivered onto the surface of the urogenital ridge epithelium at the anterior tip or along the mesonephros. The injected samples were then cultured as described above.

Light microscopy, immunohistochemistry and β–galactosidase staining

Electroporated and mitoTracker labelled samples were screened using a Leica stereomicroscope (MZFLIII) equipped with GFP Plus Fluorescence filter (GFP2, excitation 480/40 nm) and Green Fluorescence filter (G, excitation 546/10 nm). Chick positive samples were fixed for 1 h in 4% Paraformaldehyde (PFA), rinsed in PBS, transferred to 30% Sucrose at 4°C overnight and