The origin of the Mullerian duct in chick and mouse

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Received for publication 24 July 2006; revised 25 September 2006; accepted 27 September 2006
Available online 3 October 2006

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.

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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 mice 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).
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 LmNot1 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 where Mullerian duct growth is affected by the absence of the Wolffian duct (Bishop-Calame, 1966; Didier, 1971, 1973; Hashimoto, 2003; Inomata et al., 1989; Jacob et al., 1999; Wrobel, 2003; Wrobel and Sub, 2000).

To begin understanding the mechanisms of early development of the Mullerian duct, 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 Tammann, 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% CO2 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 pCSC2+ 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 540/ 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
embedded in OCT. Transverse sections were cut with a Leica cryostat. Mouse samples were treated for whole-mount immunohistochemistry. After a 30’ fixation in 4% PFA at 4°C, they were rinsed in PBS and incubated in blocking solution (PBS:2% BSA, 0.5% Triton) for at least 4 h at 4°C. The incubation with the antibody was carried out overnight at 4°C in blocking solution. After three 1 h rinses in PBS:0.1% Tween, samples were incubated with secondary antibodies in PBS:0.1% Tween overnight at 4°C. Samples were then rinsed few hours in PBS:0.1% Tween before visualisation under the fluorescent stereo-microscope. Selected samples were transferred to 30% sucrose overnight at 4°C and then embedded in OCT for sectioning.

Primary antibodies: rabbit polyclonal anti-PAX2 (1:1200 dilution) (PRB-276P from Covance). Secondary antibodies: goat anti-rabbit Alexa-555 or Alexa-488 (1:400 dilution) from Molecular Probes. Fluorescence images were captured on an Olympus IX70 inverted microscope using a Deltavision cooled CCD imaging system (Photometrics CH350L liquid cooled CCD camera; Softworx image acquisition software) (Applied Precision), with the exception of images in Fig. 3B: confocal Z-stack of images captured with a Leica TCS-SP1 system and processed using the volume rendering software Volocity (Improvision).

12.5 dpf mouse embryos were dissected from pregnant females heterozygous for the I.9LacZ transgene, fixed 30’ in 4% PFA, rinsed in PBS and stained in X-Gal solution overnight at 37°C. Positive samples were embedded in paraffin, sectioned with a microtome and eosin counterstained.

5-bromo-2′-deoxyuridine (BrdU) labelling

Pregnant mice received a single intra-peritoneal injection of BrdU (Sigma B-5002) at 50 mg/kg of body weight 2 h before dissection.

Following the removal of 2.5 ml of albumen from chicken eggs incubated at 37.5°C for 7 days, 100 µg of BrdU dissolved in 100 µl of PBS was dropped on the vitelline membrane. The eggs were sealed with tape and incubated for another 2 h.

Chicken and mouse embryo urogenital ridges were fixed in 4% PFA at 4°C for 2 h, rinsed in PBS and put in 30% Sucrose overnight at 4°C before embedding in OCT. 12 µm sections cut with a Leica cryostat were washed three times 5’ in PBS, fixed again for 5’ in 4% PFA at RT, rinsed in PBS and incubated in 2 M HCl at 37°C for 30’. Following three 5’ washes in 0.1 M Borate buffer pH8.5 and one wash in PBS, the sections were incubated in blocking buffer (PBS:0.5% BSA, 2% sheep serum, 0.1% triton) for 1 h. Primary antibodies were added to the blocking solution overnight at 4°C (mouse monoclonal anti-BrdU 1:300 from Chemicon and rabbit polyclonal anti-PAX2 1:1200). After three washes in PBS:0.1% Tween, the sections were incubated with secondary antibodies (goat anti-rabbit Alexa 555 and goat anti-mouse Alexa 488 1:400, from Molecular Probes) for 2 h and finally washed in PBS:0.1% Tween.

Results and discussion

MDE and MDM of chick embryos derive from different populations of coelomic epithelial cells

Lineage tracing experiments were performed in ovo, beginning with chick embryos at HH14–17. At these stages the Wolffian duct has already reached the cloaca, while the Mullerian duct is not yet apparent. In order to label the right or left dorsal coelomic epithelium, including the cells overlying the mesonephros, a plasmid expressing GFP under control of an ubiquitous promoter was injected into the right or left coelomic cavity and electroporated towards the dorsal/medial side. The embryos were screened for GFP expression 3–4 days after manipulation, at HH30–32.

Fig. 1 shows examples of urogenital ridges labelled with GFP on their dorsal side. Along the Mullerian duct, GFP is in coelomic epithelial cells, but also in the subjacent region. Three distinct GFP patterns were observed:

Type 1 GFP was in the MCE and subjacent MDM only at the most anterior tip of the duct, which forms the funnel, but not along the mesonephros; it was also found within the entire length of the MDE (Figs. 1A–B).

Type 2 GFP was in the MCE and subjacent MDM at the most anterior tip of the duct, and along the mesonephros; it was also found within the entire length of the MDE (Figs. 1C and 2A).

Type 3 GFP was in the MCE and subjacent MDM, along the mesonephros but not at the most anterior tip of the duct; it was never found in the MDE (Figs. 1D–F and 2B).

In all type 1 and type 2 mosaics, GFP was observed within cells of the MDE from the anterior opening of the duct to an endpoint located in the posterior half of the mesonephros. This posterior ending coincided with the tip of the Mullerian duct, as established by analysing cross sections of the urogenital ridges at and below this point. Moreover GFP was also always present within MCE and MDM cells of the funnel region: the most anterior tip of the duct at the edge of the mesonephros. In contrast, in type 3 samples, that were negative within the MDE, GFP was never observed in MCE and MDM at the anterior tip regardless of how extensive the labelling of MCE and MDM along the mesonephros was (Fig. 1D).

The localisation of GFP in MCE and MDM along the mesonephros of type 2 and type 3 samples was equally variable. In some samples the entire length of the mesonephros contained green cells, as shown in Fig. 1E, while in others, patches of GFP positive cells were scattered along the A–P axis, as shown in Fig. 1F. In samples with relatively few GFP positive cells, it was possible to see discrete rows of GFP cells in the coelomic epithelium overlaying the mesonephros. These rows always ran along the dorsal–ventral (D–V) axis, never along the A–P axis. In the Mullerian field, these rows included both MCE and MDM cells. So, while the MDE cells move from anterior to posterior through the mesonephros, the mesonephric coelomic epithelial cells and their derivatives do not move along the A–P axis, within the time-frame analysed. In the Mullerian field, these cells contribute to both MCE and MDM, maintaining a movement perpendicular to the A–P axis of the embryo.

These data show that the dorsal coelomic epithelium contributes to all the components of the Mullerian duct: the duct epithelium, the mesenchymal layers around it and the coelomic epithelial cells at the borders of the entire structure. Nevertheless the presence of GFP in the MDE along the mesonephric region is independent of the presence of GFP in the overlaying MCE and MDM, which indicate that the coelomic cells contributing to the MDE reside in a specific, restricted position along the A–P axis of the embryo.

It is well established that Mullerian duct development proceeds from anterior to posterior. Therefore, the GFP positive cells found in the MDE have to derive from a discrete population of coelomic epithelial cells of the HH14 stage embryos localised rostrally. This area is likely to correspond to the pronephro-mesonephro transition region. The founder cells then extend the tube posteriorly. None of the type 3 mosaics (e.g. Figs. 1E and 2B) showed any GFP positive cells within the
MDE, even in areas along the mesonephros where the MCE was heavily targeted with GFP. This indicates that the MDE does not intermingle with coelomic epithelial cells localised more posteriorly along the mesonephros, during its caudal extension. This confirms that the coelomic epithelial cells of the MDE anlage segregate from the epithelium before extending through the mesonephros. Cell recruitment from the MCE does not occur at all below the point of segregation.

In some type 1 and 2 samples, most MDE cells were found to be GFP positive, indicating that the coelomic epithelium must be at least the major contributor to the Mullerian duct. So despite not being possible to test directly any potential contribution from the Wolffian duct, it is clear that, if there is any contribution at all during MDE migration, it has to be minor. Typically, in samples where the duct was heavily labelled, such as the example in Fig. 2A, less than 20% of the duct cells were GFP negative and, while these cells could be of Wolffian duct origin, they could also be from untargeted coelomic epithelial cells or from targeted cells that have proliferated and diluted out or lost the marker.

In our hands, the stage of the chick embryos used for the electroporation was critical for successful targeting of GFP to the MDE. Jacob et al. (1999) report the appearance of a MDE anlage at HH24 in chick, but we were only successful in targeting GFP to the MDE when we used HH14–15 embryos. Embryos slightly older (e.g. HH17) never showed GFP within the MDE, suggesting two possibilities: the MDE anlage is already segregating from the surface epithelium immediately after these stages, or the target area is difficult to reach at later stages (HH17), using our technique.

The origin of MDE and MDM cells is conserved between chick and mouse

At 11.5 dpc in the mouse, the Mullerian duct has not yet formed, but a group of cells positive for Lim1 and Pax2 are present at the rostral end of the urogenital ridge in the proximity of the Wolffian duct. This patch of cells is thought to be the anlage of the Mullerian duct. By 12.5 dpc, the Mullerian duct has grown considerably along the A–P axis, running along
about one half to two thirds of the mesonephros; by 13.5 dpc the duct has run the entire length of the mesonephros (Kobayashi et al., 2004; Torres et al., 1995).

We wanted to ask if the contribution of the coelomic epithelial cells to the Mullerian duct is similar or different between chick and mouse. In order to do this comparative study, we designed strategies to trace the fate of the coelomic epithelium overlaying the urogenital ridge in culture.

It is well established that the mouse genital ridge can undergo normal differentiation into testis or ovary, when grown together with the mesonephros in culture containing serum-enriched medium. We first tested if the Mullerian duct develops normally in this organ culture, by analysing the expression of PAX2. This marker, known to be essential for the formation and maintenance of the duct, is normally expressed in Wolfian duct, Mullerian duct epithelium and mesonephric tubules (Torres et al., 1995). A proportion of 11.5 dpc urogenital ridges grown in culture for 2 days did show the expected MDE Pax2 staining, confirming the normal growth of the Mullerian duct. When the cultures were initiated from embryos at ts 14–19 (11.1–11.6 dpc), the percentage of normal growth was above 60%, while when initiated at ts11–12 (10.7–10.8 dpc), it was much lower, about 35%. The difference could reflect the presence or

Fig. 2. Transverse sections of urogenital ridges from stage HH32 chick embryos electroporated with pCS2-EGFP (in green). Nuclei are counterstained with DAPI (in blue). Three sections per sample are shown; from left to right: anterior to posterior. (A) Example of type 2 GFP pattern (corresponding to sample c in Fig. 1); at the anterior end GFP is in MCE, MDM and MDE, at the posterior end GFP is only in the MDE. (B) Example of type 3 GFP pattern (corresponding to sample e in Fig. 1); GFP is only in MCE and MDM.
absence of either the responding or inducing tissue after the
dissection, or to stage-dependent sensitivity to the culture
conditions. To follow the fate of coelomic epithelial cells
overlaying the urogenital ridge, we used two procedures:
injection/electroporation of a GFP expressing plasmid, or
labelling with MitoTracker dye (see Materials and methods).
After culture, only samples with a Mullerian duct, as assayed
by PAX2 staining, were analysed.

The electroporation of GFP was never as extensive as in the
chick, but it allowed us to clearly mark patches of cells along
the urogenital ridge, targeting the Mullerian field, as well as other
parts of the mesonephros itself and the genital ridge. Fig. 3A
shows a few examples from the collection of samples labelled at
ts17–19 (11.4–11.6 dpc). As with the chick, in the Mullerian
field GFP positive cells were found not only in the epithelial
layer but also in the inner portion (Fig. 3B). This suggests that
coelemic epithelial cells along the mesonephric portion of
the urogenital ridge either stay in the coelomic epithelium, or
contribute to the mesenchymal cells surrounding the Mullerian
duct, similar to the type 3 chick samples.

The MitoTracker labelling of discrete areas of the epithelium
overlaying the mesonephros in proximity to the Wolffian duct
confirmed that the coelomic epithelial cells along the mesone-
phros contribute to the MDM (data not shown). In several
samples labelled at their rostral tip, MitoTracker was found within the entire length of the MDE. This was independent from the presence of dye within the MDM along the mesonephros (Fig. 4). These results show that the MDE anlage originates from coelomic epithelial cells at the rostral tip of the mesonephros or in the residual pronephric region and segregate from the surface before extending caudal along the mesonephric region. Moreover the MDE does not intermingle with the coelomic epithelial cells overlaying the mesonephros following its segregation from the surface. This pattern is similar to the one found in chick. The samples labelled with MitoTracker at the anterior end also showed what appeared to be staining within the Wolffian duct (Fig. 4). At the time of injection, the Wolffian duct was already developed and some diluted dye may have become trapped by capillary action in the canal, where the staining was later observed. This was unexpected, but found in the Wolffian duct of all injected samples, whether MitoTracker positive or negative within MDE and/or MDM/MCE, indicating that the labelling of the Mullerian duct was independent of this apparent staining.

To directly investigate the contribution of cells from the Wolffian duct to the Mullerian duct, we analysed a mouse line carrying a LacZ transgene expressed under the control of a regulatory element located upstream of the Sox9 promoter (Sekido and Lovell-Badge, unpublished data). The LacZ pattern recapitulates part of the expression pattern of the endogenous Sox9 gene. At 12.5 dpc, Sox9 is weakly expressed in the Wolffian duct, mesonephric tubules and Mullerian duct epithelium of both male and female embryos (data not shown). In the transgenic line, LacZ is normally expressed in the Wolffian duct and ectopically expressed in MCE and some subjacent MDM. It is not present in the MDE at any level along the A–P axis of the embryo (Fig. 5). LacZ is quite a stable protein (Swain et al., 1998) and should be found at least in some cells of the 12.5 dpc MDE, if any of them originated from the Wolffian duct. While LacZ activity was found in all cells of the Wolffian duct along the mesonephric region and its intensity increased from anterior to posterior, no staining was present in any part of the MDE that was still in the process of extending caudally. The absence of any intermingling of blue and white cells within the MDE rules out the Wolffian duct as the source of MDE cells during Mullerian duct growth via any mechanisms including splitting or budding off from the Wolffian duct. These data strongly indicate a lack of cell contribution from the Wolffian to the Mullerian duct.

The results of the analysis of a transgenic mouse line expressing cre under the control of Pax2 enhancer elements (Pax2-cre) crossed with the ROSA26 reporter strain (Pedersen et al., 2005) are also in agreement with our conclusions. In these studies, LacZ was found in the nephric duct and its derivatives in 12.5–14.5 dpc female embryos. No blue cells were found consistently in the Mullerian duct and even when present they were few and scattered within both the MDE and MDM (Pedersen et al., 2005 and W. Shawlot, personal communication). This pattern of cre-activity indicates that the nephric duct does not contribute cells to the Mullerian duct. The few scattered cells found occasionally within MDM and MDE are most likely due to ectopic expression of cre in few coelomic epithelial cells, which later on become part of the MDE or MDM depending on their localisation along the A–P axis.

**MDE cells are proliferating during their migration along the mesonephros**

We showed that the caudal extension of the MDE through the mesonephros is not achieved via integration of new cells from the Wolffian duct or the neighbouring MDM and MCE. Cell shape changes could contribute to extension, but it seems more likely that MDE growth is sustained by proliferation. To test this hypothesis, we labelled dividing cells of chick and mouse embryos with BrdU for 2 h. The experiments were performed on HH30–31 chick embryos and 12.1 dpc mouse embryos, stages of MDE extension. Transverse sections from BrdU labelled urogenital ridges were subsequently stained with anti-BrdU and anti-Pax2 antibodies. A continuous stretch of MDE, about 500 μm from its posterior tip, was analysed, as well as scattered sections localised more anteriorly.

BrdU positive cells were identified along the length of the MDE of both chick and mouse embryos. From the count of double positive cells versus Pax2 single positive cells, the percentage of MDE cells in S-phase was found to be an average of 43.7% in mouse and 46.7% in chick. Their distribution was estimated by arbitrarily dividing the analysed MDE in 120 μm segments and comparing the mean percentage of positive cells among different segments. These values

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**Fig. 5.** Paraffin sections along the A–P axis of urogenital ridges from a 19LacZ/+ mouse embryo stained for β-galactosidase. Only the mesonephric portion containing Wolffian duct and Mullerian duct is shown. In blue LacZ, in pink eosin counterstaining. WD: Wolffian duct. The most anterior section is on the left, as indicated by the A to P arrow at the top of the panel.
were found to be quite high along the A–P axis, in the range of 39–48% in mouse and 38–52% in chick (Fig. 6). As the S-phase fraction is a measure of the tissue proliferation potential, these results indicate that the MDE cells, in both chick and mouse, were proliferating at a very high rate, suggesting that MDE extension along the mesonephros can be achieved via proliferation of the MDE itself, without a need for contribution of cells from external sources. Moreover, our data indicate that the growth is not localised to a specific MDE position, such as the posterior tip, but it is dependent on widespread proliferation along the developing duct.

Conclusions

Our lineage tracing studies in chick show that the coelomic epithelial cells overlaying the pronephros/mesonephros contribute to all components of the Mullerian duct.

Coelomic epithelial cells localised along the length of the mesonephros give rise to MCE and MDM of the duct. At the most anterior tip of the mesonephros, probably in the transition area between pronephros and mesonephros, a discrete population of coelomic epithelial cells gives rise to the anlage of the MDE. These cells segregate from the epithelium and move along the A–P axis of the embryo to extend caudally through the mesonephric area. A similar picture has also emerged from the mouse studies, suggesting that the pattern of early Mullerian duct development is conserved in birds and mammals.

In the ongoing controversy surrounding the origin of the duct epithelial cells, both our chick and mouse results are consistent with the coelomic epithelium being the primary source of MDE cells. The mouse data derived from the analysis of the 1.9LacZ/+ line and the pattern of cre-activity observed in the Pax2-cre line (Pedersen et al., 2005) even exclude any cell contribution from the Wolffian duct, indicating the coelomic epithelial cells as the only contributors to the duct.

Our results support the deductions made by Jacob et al. (1999) in the chick, against the Wolffian duct budding model proposed by Inomata et al. (1989) from the bovine studies. The high S-phase fraction of MDE cells, a measure of the cells proliferation rate, also supports our model by showing the ability of the duct to grow of its own accord. There are a number of microscopy studies, performed in lower vertebrates, such as the work of Hall (1904) in some amphibia (Urodela, Anura) and of Wrobel (2003) in an old group of chondreostean fish (Acipenser ruthenus) that also support the view that Mullerian duct epithelial cells are not Wolffian duct derived. This suggests that the pattern of formation of the MDE is most likely conserved across vertebrates. In such a scenario, it seems likely that the Wolffian duct exerts its essential inducing role on Mullerian growth, not by providing the cells to build the tube, but via soluble signals or cell–cell interactions.

Moreover the early segregation of the duct cells from the epithelium, found in both chick and mouse, excludes the still popular view, found in some textbooks, of a duct deriving from invagination and fusion of epithelial lips proceeding from anterior to posterior along the mesonephros (Larsen, 2001).

Our findings may also be of relevance to the origins of endometriosis, which is estimated to affect approximately 10% of women. The prevailing hypothesis since its proposal by Sampson (1927) is reflux of endometrial lining cells at menstruation through the fimbria of the fallopian tubes, which then attach to peritoneal surfaces, with subsequent proliferation and invasion, with the ectopic tissue often responding to the hormonal cycle in the same way as the endometrium. While this view is still favoured by many (Al-Fozan and Tulandi, 2003; D’Hooghe, 2003), in recent years there has been increasing support for an embryonic origin at least for some cases.

This derives in part from the relative resistance to induce endometriosis in animal models by transplanting endometrium into the peritoneal cavity and from molecular comparisons of endometriosis with normal endometrium (Redwine, 2002). Moreover, the reflux model cannot explain some of the reported pathogenesis. These include thoracic endometriosis, as there is no obvious route for endometrial cells across the diaphragm (Alifano et al., 2006); endometriosis prior to menarche, or cases
of very young girls with MDE-like cells found in peritoneal pockets (known as “Mullerian cell rests”) (Batt and Mitwally, 2003; Goldstein et al., 1979). We have shown that the duct normally develops from a discrete population of coelomic epithelial cells at the border between the mesonephros and pronephros. Presumably this is in response to a local source of an inducing molecule that initiates the formation of a pocket that is then dependent on the Wolffian duct for its caudal extension. However, such pockets could be initiated at other locations in close contact with an appropriate signalling centre, in the pronephros or mesonephros. Remnants of the former could remain within the thoracic cavity and of the latter within the peritoneal cavity. It will clearly be of interest to determine the factors that initiate the MDE and the nature of their source.

Altogether our conclusions will inform future experiments to define the molecular and cellular events responsible for Mullerian duct tubulogenesis. The electroporation technique in the chick allows targeting of all the Mullerian duct components, and due to the stage-dependent targeting of the MDE even allows some control on which component to target. It is therefore a useful tool to deliver DNA and RNA molecules for the functional analysis of early Mullerian duct development, which will provide a better understanding of mechanisms of tube formation.

Acknowledgments

We thank W. Shawlot for showing us details of the expression data of the Pax2-cre transgenic line generated in his laboratory and for allowing us to refer to these; W. Hatton for assistance with histology; P. Burgoyne for help with the statistics; Kate Sullivan and Peter Laskey for help with confocal and Deltavision microscopy systems; J.M. Turner, C. Scott, C. Wise and F.W. Buas for critical reading of the manuscript. R.S. was a recipient of EMBO and HFSP long-term fellowships. This work was supported by the MRC and the Louis-Jeant Foundation.

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