Female Reproductive Tract Development & Function

Primary Papers:

Discussion

Student 10: Contemporary Paper-Ref #1 above
- What are the functions of the oviduct?
- What methods were used?
- Are secretions important?

Student 11: Contemporary Paper-Ref #2 above
- What are HOX genes and role in development?
- What are endocrine disruptors and mechanism?
- How do they alter female reproductive tract?

Student 12: Contemporary Paper-Ref #3 above
- What evo-devo approach for female reproductive tract was used?
- What transcription genes involved were discussed?
- What conserved processes are observed in female reproductive tract development?
The Oviduct: Functional Genomic and Proteomic Approach

1 Mondéjar1*, OS Acuña2*, MJ Izquierdo-Rico2, P Coy1 and M Avilés2
1Department of Physiology, Veterinary Faculty and 2Department of Cell Biology and Histology, Faculty of Medicine, University of Murcia, Murcia, Spain

Contents
The mammalian oviduct is an anatomical part of the female reproductive tract, which plays several important roles in the events related to fertilization and embryo development. This review examines and compares several studies related to the proteomic and transcriptomic profile of the oviduct in different domestic animals. This information could be important for clarifying the role of oviductal factors in different events regulating fertilization and early embryo development, as well as for improving synthetic media for in vitro maturation/in vitro fertilization/embryo culture techniques (IVM/IVF/EC).

Introduction
The concept of the oviduct as a passive structure involved in the transport of gametes has been substituted by that of a dynamic structure actively involved in several functions. The oviduct, also called the Fallopian tube in primates, is the organ in which fertilization takes place. Moreover, numerous studies have indicated that the oviduct and, especially, oviductal secretions play a key role in aspects related to gamete maturation, sperm capacitation and the development of the preimplantation embryo (Hunter 1998; Avilés et al. 2010).

Anatomically, the oviduct consists of four regions designated infundibulum, ampulla, isthmus and uterine-tubal junction. The mucosa of the oviduct shows primary and secondary folds of different height and orientation with a typical tree branch-like structure of varying degrees of complexity. Interspersed among these mucosal projections is a complex system of crypts, pockets and grooves (Hunter et al. 1991; Yániz et al. 2000). This complex anatomical structure contributes to sperm selection and probably participates in the regulation of the number of sperm that reach the site of fertilization, thus controlling polyspermy and providing different oviduct microenvironments (Hunter 2012). The epithelium is mainly formed by two different cell types: ciliated cells and non-ciliated cells or secretory cells (Fig. 1). The distribution and morphology of both cell types changes during the oestrous cycle, the anatomical region of the oviduct and even the specific region of the mucosa fold (apical or basal regions). Thus, it has been reported that ciliated cells are more abundant along lateral walls and in the apical region of longitudinal folds than in the basal regions among the mucosa folds (Abe 1996; Yániz et al. 2000; Yániz et al. 2006). In pig, a morphometric analysis even showed differences in the epithelial cells between two breeds (Abe and Hoshi 2008). In addition to these anatomical and histological differences, there are species-specific differences in the physiology of the oviduct. For example, in some species, ovulation is restricted to one of the two oviducts. It was previously reported that the concentration of different hormones (e.g. progesterone, prostaglandins) and the gene expression pattern in the ipsilateral oviduct differs from that observed in the contralateral oviduct (Wisajyungunawardane et al. 1998; Bauersachs et al. 2003).

Nowadays, the production and development of embryos until the blastocyst stage in most of mammalian species can be achieved under in vitro conditions, with limitations that depend on the species (Table 1 and Data S1). In pig, for example, the developmental competence of in vitro-produced embryos is low compared with their in vivo counterparts (Kikuchi et al. 1999). An insufficient cytoplasmic ability for the development and polyspermy of in vitro matured oocytes and improper culture conditions for IVF embryos are thought to be responsible for this low efficacy (reviewed in Nagai et al. 2006). In cattle, although polyspermy is not a real problem in in vitro embryo production, the process is considered inefficient; while maturation and fertilization may appear to proceed normally, the proportion of embryos reaching the transferable stage is rarely over 40% and those that do reach this stage are often compromised in quality and competence. In equine species, IVF and development rates remain low (Hinrichs et al. 2002; Goudet 2011). The technology of in vitro oocyte maturation followed by the application of ICSI has been established to achieve fertilization in vitro. In this way, the rates of embryo development ranged from 5% to 10% in early studies but can reach 40% in later ones.

As mentioned above, fertilization and early embryonic development occurs in the oviduct. The quality of
the in vitro-produced embryos does not reflect the quality of their in vivo counterparts. Results of many studies suggest that culture conditions during in vitro embryo production may influence the developmental potential of the early embryo and its quality (Lonergan et al. 2007). Strategies developed to improve embryo development include the use of coculture media with epithelial cells, culture media supplemented with different proteins or growth factors and embryo culture in a foreign oviduct. The trans-species transfer of embryo to oviducts has been used to optimize early embryo development in different species, with the oviduct proving to be the best environment (Gandolfi and Moor 1987; Gutiérrez-Adán et al. 2004).

The Oviduct is Involved in Regulation of Sperm, Oocyte and Embryo Physiology

The efficiency of fertilization is lower in vitro than in vivo for most species. However, it is unknown whether this is the result of (i) failures in final gamete maturation, (ii) deficient sperm-oocyte interaction or (iii) the lower ability of the recently formed zygote to develop. All these steps, which probably jointly affect the final outcome of in vitro procedures, take place in the oviduct under physiological conditions and, consequently, a study of the factors affecting them is of great importance for further advances in the reproductive biology field.

Sperm

The role of the oviduct in male gamete capacitation mediated by binding of the spermatozoa to oviductal epithelial cells has been described in several species (Suarez 1998; Hunter 2012; Goudet 2011). In most cases, bound spermatozoa in the isthmus have been shown to decrease their movement and to prolong their survival, delaying the capacitation process (Suarez 2008; Fazeli et al. 2003). Partial identification of different proteins and carbohydrates involved in sperm binding and release from the oviduct (Suarez 2001; Talevi and Gualtieri 2010; Gualtieri et al. 2010; Talevi et al. 2010), as well as of the relationship between ovulation and the release of capacitated spermatozoa, has also been made (Gualtieri et al. 2005; Suarez 2007). However, a complete description of all the molecular pathways involved in these processes remains under research (Hunter 2012), and better understanding of these pathways will offer new tools for improving in vitro reproduction in domestic animals and also in humans.

Other possible roles of the oviduct as regards the male gamete have been related to the selection and guidance of spermatozoa towards the egg (Holt and Fazeli 2010). Moreover, the arrival of spermatozoa within the oviduct regulates gene expression in oviductal epithelial cells (Thomas et al. 1995; Fazeli et al. 2004; Georgiou et al. 2005, 2007). The oviduct may also be involved in a sperm selection process (Rodríguez-Martínez et al. 2005). After mating, where a large number of sperm are deposited in the vagina or uterus, very few are able to reach the site of fertilization. Severely deformed sperm cannot enter the oviduct (Styrna et al. 2002); however, sperm with a normal morphology or with few anomalies and a progressive linear movement can penetrate the uterotubal junction and enter the isthmus (Shalgi et al. 1992; Holt and Van Look 2004; Nakanishi...
et al. 2004). Then, sperm with appropriate receptors on their surface may bind to the epithelial cells for a period up to 30 h and form a preovulatory sperm reservoir (Rodríguez-Martínez et al. 2005; Hunter 2012). A seminal plasma protein called, BSP1 (or PDC-109), and annexin present in the apical membrane of the epithelial cells play a key role in this process (reviewed in Hung and Suarez 2010).

It has been reported that the female genital tract has a positive effect on the fertilization potential of spermatozoa that have been genetically altered (Kawano et al. 2010; Turunen et al. 2012). In mouse, Turunen et al. pointed to an 80% decrease in in vitro fertilization with sperm that lack CRISP4 compared with the wild type. These data indicated that, even if the physiology of the sperm is seriously compromised, the genetically modified mice were fertile, as wild-type animals, in normal mating. In our opinion, these results provide a new view of the uterine/oviductal contribution to sperm maturation in the genital tract.

The above studies could contribute to the development of sperm treatments with uterine and/or oviductal secretions (or uterine/oviductal tissue explants) to improve the sperm quality of animals with a low seminal quality, or in the case of damaged sperm after cryopreservation. We consider that this finding in mouse is worth investigating in other domestic animals and also in humans.

Oocyte
With regard to the oocyte, the role of the oviduct on its final maturation, especially at the zona pellucida (ZP) level, has not been deeply studied, although it was suggested more than 20 years ago that oviductal glycoproteins may act to enhance the various functions of the ZP (Yang and Yanagimachi 1989). Recently, this role has been partially clarified when OVGP1 and heparin-like glycosaminoglycans from the oviducal fluid were seen to bind to the ZP and make it resilient to enzymatic digestion and to sperm binding and penetration (Coy et al. 2008). This mechanism represents a novel view of the so-called ‘ZP hardening’, which has been considered until now as a post-fertilization event associated with cortical granule exocytosis (cortical reaction). Now, it is known that the ZP undergoes those maturational changes in the oviduct before the arrival of spermatozoa and that these modifications may be crucial for any further oocyte response to sperm entry. As an example, polyspermy levels in the pig and cow are significantly affected by the contact of the ZP with oviductal secretions and, as a consequence, the final rate of fertilization is modified (Coy et al. 2008). A similar effect of the oviducal fluid on ZP maturation has recently been shown in the sheep and goat (Mondéjar 2011).

Embryo
Finally, it cannot be forgotten that the recently formed zygote remains in the oviduct for a variable period of time, depending on the species but never <48 h. During this time, oviductal secretions are subjected to important changes derived from hormonal transition from an oestrogen-dependent to a progesterone-dependent environment. As we reported previously, the oviducal fluid protects the embryo against adverse impacts on mtDNA transcription/replication and apoptosis (Lloyd et al. 2009a). Moreover, a number of embryotrophic factors from the oviduct have been described (Lee and Yeung 2006; revised by Avilés et al. 2010), although functional experiments to clarify the specific role of each one and their potential use for improving in vitro culture systems remain to be performed. In the following paragraphs the identification and role of some of these factors will be discussed.

Functional Genomic and Proteomic Analysis of the Oviducal Cells and Secretions
Oviducal fluid is a complex fluid formed by different metabolic and macromolecular components from blood plasma and epithelial cell secretions (reviewed in Buhi et al. 2000; Aguilar and Reyler 2005; Georgiou et al. 2005; Leese et al. 2008; Avilés et al. 2010). Most studies of the oviducal fluid have identified one, or a low number of, protein(s) in the oviduct by means of conventional analytical methods (Avilés et al. 2010). Other studies have tried to identify more components using complex technologies that include two dimensional electrophoresis (Gandolfi et al. 1989; Buhi et al. 2000). However, until now, it has been possible to identify only some of the proteins detected in the 2D gel by preparing specific antibodies. Fortunately, thanks to the development of the mass spectrometry instruments and the deciphering of the genome of different species, it is nowadays possible to identify a large number of proteins contained in complex body fluids and to study gene expression patterns in different tissues. Here, we describe the results previously reported in the literature and by our group obtained by using transcriptomic and proteomic analysis.

Transcriptomic Analysis
A transcriptomic analysis of the oviduct has been performed in bovine, human and mouse species (Bauersachs et al. 2003; Fazeli et al. 2004; Bauersachs et al. 2004; Tone et al. 2008; George et al. 2011).

In cattle, the epithelial cells were obtained by scraping the mucosal epithelial layer of the complete oviduct using a glass slide from heifers in oestrous and dioestrous phases (Bauersachs et al. 2004) and in the postovulatory period (Bauersachs et al. 2003). During the postovulatory period, authors found differences for 35 genes when comparing gene expression in the ipsilateral and contralateral oviduct. Twenty-seven genes were up-regulated in the ipsilateral oviduct, and eight were down-regulated (Bauersachs et al. 2003). The comparative analysis of the gene expression between oestrous and dioestrous phase showed that 77 genes were differentially expressed; 37 and 40 genes were up-regulated in the oestrous and dioestrous phases, respectively (Bauersachs et al. 2004). These genes have been related to the immune response, protein secretion and
modification, endocytosis, signalling and the regulation of transcription.

In women, a recent study compared the gene expression profile of epithelial cells of the Fallopian tube between the follicular and luteal phases (George et al. 2011). The authors identified five genes up-regulated and 15 down-regulated in the luteal phase (supplementary file in George et al. 2011). Some of these genes are of potential interest for different aspects related to fertilization and embryo development. For example, mRNA for heparanase was detected in the human oviductal mucosa. In a previous study, our group provide strong evidence to support a role for OVGP1 and heparin in blocking polyspermy (Coy et al. 2008). It was observed that heparin contributes to the stabilization of the OVGP1 effect. Our proteomic analysis also identified the existence of heparanase in the porcine oviductal fluid, agreeing with the results mentioned previously for the human oviduct. Heparin molecules have also been related to the release of bovine sperm bound to the oviductal epithelia (Gualtieri et al. 2010). Therefore, it seems possible that the heparanase present in the oviduct contributes to the regulation of these processes. Future experiments are necessary to confirm this hypothesis.

GPX3 mRNA, a glutathione peroxidase, is another gene differentially expressed in the human oviduct. This enzyme is involved in the redox balance and could make an important contribution to the control of DNA damage that affects gametes and the embryo (Aitken and De Iuliis 2010) and also to the sperm binding to the oviduct through the reduction of SS to SH (Gualtieri et al. 2009).

We have performed a detailed analysis of human specimens from the microarray experiment stored in the Gene Expression Omnibus (GEO) accessible through GEO Series accession number GSE10971 (Tone et al. 2008; Data S2). A total of 5703 genes of the original 54 675 probes present in the microarray were detected. This number of expressed genes is in accordance with a previous study performed in other human tissues using microarray analysis (Su et al. 2004). The list of genes was analysed and classified using the DAVID Bioinformatics Resource 6.7 (DAVID). Genes were classified according to the cellular localization, and genes encoding secreted proteins were also included (Fig. 2). It can be observed that 394 (8%) correspond to genes that codify for plasma membrane proteins and 245 (5%) of the expressed genes correspond to secreted proteins that can be classified into different groups (Fig. 3).
Some of the mRNA detected in this study has previously been identified in the oviductal epithelium in other species (Avilés et al. 2010); however, future studies are needed to confirm these data. The study by Tone et al. (2008) of gene expression analysis was performed using the oviductal mucosa and laser microdissection. Therefore, other cells (fibroblast, endothelial cells, lymphocyte, mast cells, etc.) present in the laminae propria are included in this analysis, and, consequently, some of the genes detected may not correspond to the epithelial cells covering the oviductal lumen. Other techniques like immunocytochemical and in situ hybridization analysis can be used to demonstrate the direct relationship between the expressed genes and the oviductal epithelium.

A gene expression study of the porcine oviduct in different phases of the oestrous cycle is currently in progress in our laboratories. In this review, we will provide unpublished information about the gene expression in the oviduct in the preovulatory phase of the cycle. For the analysis, the ampullary-isthmic junction region of the oviduct was selected because fertilization takes place in this region. The hybridization was performed using a microarray of 43 803 probes (Porcine (V2) 4x44K) from Agilent (Agilent Technologies, Madrid, Spain). Further details of the methodology employed is described in Data S2. Then, a total of 2968 genes were detected; this number is low compared with the human samples owing to incomplete annotation of the porcine genome. More than 480 genes are shared between the human and the porcine oviduct. Similarities between the porcine and bovine gene expression are even lower owing to the low number of gene available for comparative purposes (Bauersachs et al. 2007) (Fig. 4).

The known genes expressed in the bovine oviduct epithelial cells are reduced owing to the fact that only differentially expressed genes were analysed using a subtracted library. In mice, a comparison of the only 82 of the genes are shared with the human oviduct, 17 are shared with the pig, and only two genes are expressed in the four species studied. In general, these results indicate that some genes are expressed in all four species, suggesting that basic components play a similar function and are evolutionarily conserved. These results are in agreement with functional events observed in a heterologous situation. Thus, embryo development from bovine species can be produced in the ewe oviduct (Rizos et al. 2007). Moreover, a pre-fertilization hardening of the ZP was observed when oocytes and oviductal fluids from different species are used (Mondéjar 2011); but this process is not always produced on the same scale or in all species. This finding is probably due to the existence of expressed genes that are not shared among the species.

**Proteomic Analysis**

More than 160 proteins have been seen to be expressed or secreted by the oviduct of different species (Avilés et al. 2010). In pig, numerous proteins have been identified in the epithelial cells (Buhi et al. 2000; Georgiou et al. 2005; Sostaric et al. 2006; Seytanoglu et al. 2008) and also in the oviductal secretions (Georgiou et al. 2005, 2007; Mondéjar et al. 2009; Mondéjar 2011). At least 32 proteins from the oviductal fluid are affected by the presence of gametes, most of which are affected by the presence of the male gamete (Georgiou et al. 2007). In our laboratory, we performed an analysis of the porcine oviductal fluid from preovulatory phase. The oviductal fluid was obtained by luminal aspiration of previously dissected oviducts as previously reported (Carrasco et al. 2008). After centrifugation (5200 × g for 10 min) to remove the mucus and cellular debris, the samples were analysed by one dimensional SDS-PAGE electrophoresis under reducing conditions. The different bands were visualized by silver staining and were cut and digested with trypsin for subsequent proteomic analysis. The data were analysed by LC/MSD Trap Data Analysis Version 3.2 (Bruker Daltonik, GmbH, Germany), and the search for matches was conducted with the Spectrum Mill MS Proteomics Workbench (Agilent Technologies, Santa Clara, CA, USA) against the most recent version of the NCBInr database.

A total of 291 proteins were identified in the porcine oviductal secretions (Mondéjar 2011); however, only 35 of these proteins were detected in our microarray analysis (Mondéjar 2011), probably due to the incomplete array annotation. The different proteins can be classified into different groups as performed previously (Avilés et al. 2010) (Fig. 5). In this analysis, only 27 secreted proteins (9.3%) were identified; however, most of the proteins correspond to cellular components (90.7%). It is striking that a low number of the proteins identified correspond to typically secreted proteins compared with the total number of proteins. In the oviductal fluid, other proteins that are not typically secreted by the epithelial cells can be detected. These proteins may be divided into two main groups: proteins that come from the transudate of blood (for example, albumin and plasminogen) and other non-secreted cellular proteins. Some of them are proteins present in cellular organelles such as the nucleus, mitochondria,
endoplasmic reticulum and lysosomes. The origin of these proteins is probably related to different processes such as epithelial cell renewal or the secretory activity by apocrine and holocrine processes (Crow et al. 1994; Steffl et al. 2008); however, blood or oviductal cells contamination cannot be excluded.

The most abundant peptides identified correspond to albumin and the OVGP1 as expected owing to the fact that these proteins are abundant in oviductal fluid. Owing to space restriction, only three of the identified proteins are discussed in more detail.

Several heat shock proteins (HSP) were detected in our proteomic analysis, confirming their existence, as reported previously (Bauersachs et al. 2004; Georgiou et al. 2005). These proteins are usually considered to be intracellular proteins, although they have also been detected in the human serum and plasma (Molvarec et al. 2010). It was observed that HSP70 can be secreted by a non-classical pathway (Mambula and Calderwood 2006). A similar process, which remains to be confirmed, could exist in the oviduct. It was reported that HSPA8 and HSP60 are able to associate with spermatozoa, thus improving their survival in several species (Elliott et al. 2009; Lloyd et al. 2009b).

Another interesting protein identified in our study is annexin, which is considered a cytosolic protein; however, it was also reported that this protein can be secreted by a non-classical pathway (Christmas et al. 1991). It was previously reported that the bovine sperm interaction with the oviductal epithelium is mediated by BSP1 associated with the sperm membrane and with annexin molecules in the oviductal epithelium (Ignotz et al. 2007). Annexin in the oviductal fluid could regulate this type of interaction.

In our study, we detected, by both gene expression and proteomic analysis, the deleted in malignant brain tumours 1 (DMBT1) protein, confirming its presence at mRNA level in the bovine (Bauersachs et al. 2004) and human (Tone et al. 2008) oviduct. Very recently, it was reported that the DMBT1, previously called SPG, is expressed in the porcine oviduct (Teijeiro et al. 2012). In this study, the authors demonstrated the presence of this protein at the apical surface of the epithelial cells.

Concluding Remarks

A large body of evidence strongly supports the complexity of and the important role played by the oviduct and its secretion in different aspects of gamete maturation, fertilization and embryo development. The oviduct undergoes important changes in several aspects, including its anatomy in different regions, changes in the histology and physiology of the mucosa during the ovarian cycle and a complex gene expression pattern that is modified according to the ovarian cycle status and also to the presence of gametes and embryos. More precise information is needed about the different genes expressed and proteins synthesized and secreted by the oviduct in its different regions, hormonal and other physiological conditions to clarify the role played by the oviduct. However, for this, knowledge of the complete genome annotation of different animals is necessary. The information obtained for different animals will also contribute to understanding the mechanisms conserved in the different species and also others that are responsible for species specificity. This information will contribute to improving different aspects of the methodology used in ARTs in domestic animals, endangered wildlife species and even human beings.

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Conflict of interest

None of the authors have any conflicts of interest to declare.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Data S1. References.
Data S2. Methods.

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Author's address (for correspondence): Manuel Avilés, Department of Cell Biology and Histology, Faculty of Medicine, Campus de Espinardo, E-30100, University of Murcia, Murcia, Spain. E-mail: maviles@um.es

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The Role of Hox Genes in Female Reproductive Tract Development, Adult Function, and Fertility

Hongling Du and Hugh S. Taylor

Department of Obstetrics, Gynecology and Reproductive Sciences, Yale University School of Medicine, New Haven, Connecticut 06520

Correspondence: hugh.taylor@yale.edu

Hox genes convey positional identity that leads to the proper partitioning and adult identity of the female reproductive track. Abnormalities in reproductive tract development can be caused by Hox gene mutations or altered Hox gene expression. Diethylstilbestrol (DES) and other endocrine disruptors cause Mullerian defects by changing Hox gene expression. Hox genes are also essential regulators of adult endometrial development. Regulated HOXA10 and HOXA11 expression is necessary for endometrial receptivity; decreased HOXA10 or HOXA11 expression leads to decreased implantation rates. Alteration of HOXA10 and HOXA11 expression has been identified as a mechanism of the decreased implantation associated with endometriosis, polycystic ovarian syndrome, leiomyoma, polyps, adenomyosis, and hydrosalpinx. Alteration of Hox gene expression causes both uterine developmental abnormalities and impaired adult endometrial development that prevent implantation and lead to female infertility.

Hox genes comprise a family of regulatory molecules that encode highly conserved transcription factors. In the past several decades, molecular and genetic evidence indicates that Hox genes are expressed along anterior–posterior axes and control morphogenesis and cell differentiation during normal embryonic axial development; this mechanism for assigning differential identity along previously uniform axes is used in species as diverse as Drosophila and humans (McGinnis and Krumlauf 1992). Hox genes have a similar role in the specification of the developmental fate in individual regions of the female reproductive tract, where they regulate developmental axis in the embryonic period. Hox genes also give specific identity to the developing endometrium during the menstrual cycle in adults. The cyclic growth of endometrium is dependent on the ordered production of estrogen and progesterone. Hox gene expression is regulated by sex steroids, and this regulated expression plays an important role in endometrial development and endometrial receptivity (Taylor et al. 1997, 1998, 1999b). Here, we review the role of Hox genes, specifically the HOXA/Hoxa genes, in reproductive tract development, endometrial cyclic growth and embryo implantation, and the alterations in HOXA/Hoxa gene expression that can lead to infertility.
H. Du and H.S. Taylor

**HOX GENES AND THEIR ROLE IN THE BODY PLAN**

**HOX Genes**

Homeobox genes (as known as HOX genes) comprise a group of highly conserved genes that are essential regulators of anterior–posterior (A–P) axial pattern development. In 1978, the relationship between the location of a homeotic gene and positional development identity was first recognized in *Drosophila* (Lewis 1978). Six years later, the HOX genes were cloned and sequenced in the fruit fly *Drosophila melanogaster* (McGinnis et al. 1984a,b; Scott and Weiner 1984). Since then, multiple HOX genes have been identified in many species, including humans. HOX genes encode proteins that act as transcription factors. In each of the HOX genes, a 183-bp highly conserved sequence was identified, which encodes a 61-amino acid region, called the homeodomain (HD). Structural analyses have shown that the HD can self-fold, and form a structural motif called a “helix-turn-helix motif.” Through this motif, the HD, a DNA binding domain, recognizes a typical core DNA sequence, typically TAA TTA T, and regulates the expression of target genes, many of which play a role in axial development (Gehring et al. 1994; Krumlauf 1994; Gruschus et al. 1999; Passner et al. 1999).

Like all other insects, *Drosophila* has eight HOX genes, which are clustered into two complexes in close proximity, the antennapedia (Ant-C) complex and bithorax (Bx-C) complex. In mice and humans, *Hox/HOX* genes are clustered into four unlinked genomic loci, *Hox a-d* (mouse) or *HOX A-D* (human); each locus contains nine to 13 genes and all four clusters contain a total of 39 HOX genes. Those four paralogues, classified by sequence similarity, are located on chromosomes 6, 11, 15, and 2 in mice and chromosomes 7, 17, 12, and 2 in humans. The clustered HOX genes are believed to have arisen from gross duplication of a single common ancestral cluster. Presently, none of the paralogues have 13 genes, so some duplicated genes must have been lost during the course of evolution (Krumlauf 1994).

**Hox Genes and Vertebrate Axial Development**

In general, expression of the HOX genes follows a 3′ to 5′ order, which means, HOX genes at 3′ end are expressed earlier in development than their 5′ neighbors within the same cluster. The position in the cluster reflects both the timing and spatial position of developmental expression (Hunt and Krumlauf 1992; McGinnis and Krumlauf 1992). HOX genes have a well-characterized role in embryonic development, during which they determine identity along the A–P body axis. In vertebrates, gastrulation forms three germ layers: ectoderm, endoderm, and mesoderm. HOX genes are first expressed in the mesoderm during early gastrulation, and the 3′ genes are expressed first in anterior locations and then the 5′ genes are expressed later in the distal sacral regions. The role of mammalian HOX genes in regulating segmental patterns of hindbrain, skeleton axis and the limb axis is well established. In mice, gain- and loss-of-function experiments have revealed the spatiotemporal expression controlled by *Hox* genes in skeleton development (Ramirez-Solis et al. 1993; Horan et al. 1995; Fromental-Ramain et al. 1996; Favier and Dolle 1997). For instance, loss of *Hoxb4* expression leads to defects in the first and second cervical vertebrae. Targeted mutations of *Hoxa9* and *Hoxd9* result in anterior transformations of distinct lumbosacral vertebrae. There are transformations of sacral and first caudal vertebrae in *Hoxa11* knockout mice. In the vertebrate nervous system, the hindbrain or rhombencephalon develops under the regulating of such segmental patterning directed by HOX gene expression as well; regional expression of *Hox* genes in the hindbrain is thought to confer identity to rhombomeres (Carpenter et al. 1993; Mark et al. 1993; Goddard et al. 1996; Studer et al. 1996; Morrison et al. 1997; Manzanares et al. 1999; Ferretti et al. 2000; Yau et al. 2002). Mice harboring a *Hoxa1* mutation have alteration in hindbrain segmentation, deleting all or part of rhombomere5 (r5). The absence of *Hoxb1* function results in an apparent segmental transformation of r4 to an r2-like rhombomere identity. *Hox* is essential for r4 development. *Hoxa3* and *Hoxb3* genes are segmentally ex-
pressed in r4 and r6. Hoxa4, Hoxb4, and Hoxd4 have anterior limits in the hindbrain, but map to the junction between rhombomeric segments r6 and r7. Vertebrate HOX genes not only specify positional identity along the A–P axis of the body plan, but also provide positional values on the axis of the developing limb (Davis and Capecchi 1996; Nelson et al. 1996; Goff and Tabin 1997; Scott 1997). The most 5’ members of the Hoxa and Hoxd clusters (Hoxa9-13 and Hoxd9-13) are particularly important in vertebrate limb development. Hoxa9 to Hoxa10 and Hoxd9 to Hoxd10 are expressed in the developing upper arm/leg; Hoxa11 and Hoxd9 to Hoxa13 are expressed in the development of the lower part of the arm/leg. Hoxa13 and Hoxd10 to Hoxd13 are expressed during specification of the hand/foot. The first identified human limb malformation related to a defective HOX gene was synpolydactyly, which results from mutations in the HOXD13 gene (Muragaki et al. 1996). The role of HOX genes in vertebrate axial patterning is similar to but more complex than that in Drosophila. In the mice and humans, Hox/ HOX gene clusters provide a considerably overlapping expression pattern, which provides for the possibility of redundancy.

THE ROLE OF HOX GENES IN FEMALE REPRODUCTION

HOX Genes and Structure of Female Reproductive Tract

The female reproductive system is derived from the paramesonephric (Müllerian) duct, which ultimately develops into the fallopian tube (oviduct), uterus, cervix, and upper part of the vagina. The developing female reproductive tract is patterned by the differential expression of HOX genes in the Müllerian duct.

In the developing Müllerian duct, a number of posterior Abdominal B (AbdB) HOX genes were found to be expressed in partially overlapping patterns along the A–P axis. In vertebrates, HOX genes in paralogous groups Hoxa9-13 develop a characteristic spatial distribution throughout the Müllerian duct (Taylor et al. 1997; Taylor 2000; Goodman 2002). AbdB genes are expressed according to their 3’ to 5’ order in the HOX gene clusters. Hoxa9 is expressed at high levels in areas that will become the oviduct, Hoxa10 is expressed in the development of the uterus, Hoxa11 is found in the primordial lower uterus and cervix, and Hoxa13 is seen in the ectocervix and upper vagina. No gene exists in the Hoxa cluster that is a parologue of Hoxd12 or Hoxc12; hence, there is no Hoxa12 gene. This expression pattern is conserved between mice and humans (Fig. 1). Targeted mutagenesis of these genes results in region-specific defects along the female reproductive tract. Hoxa10 deficiency causes the homeotic transformation of the anterior part of the uterus into an oviduct-like structure. Hoxa13 null embryos show a hypoplastic urogenital genital sinus and agenesis of the posterior portion of the Müllerian duct. When the Hoxa11 gene is replaced by the Hoxa13 gene, posterior homeotic transformation occurs in the female reproductive tract: the uterus, in which Hoxa11, but not Hoxa13 is normally expressed, becomes similar to the more posterior cervix and vagina, in which Hoxa13 is normally expressed (Satokata et al. 1995; Benson et al. 1996; Warot et al. 1997). Although HOX genes were once considered to be expressed only during embryonic development, persistent HOX gene expression was first well characterized in the adult female reproductive tract (Benson et al. 1996; Taylor et al. 1997). The adult reproductive tract undergoes a continuing developmental process during each menstrual cycle; proliferation and differentiation of endometrium coupled with angiogenesis leads to a new endometrium in each estrus or menstrual cycle. In both mice and humans, the expression of Hoxa9-13/HOXA9-13 in the adult reproductive tract has been described as the same regions as their expression in the embryo (Dolle et al. 1991; Favier and Dolle 1997; Taylor et al. 1997; Warot et al. 1997). Specifically, Hoxa10/HOXA10 and Hoxa11/HOXA11 are expressed in the endometrium of the adult mice and humans. The expression of these two genes varies in an estrus/menstrual cycle-dependent manner (Fig. 2). Hoxa10/HOXA10 and Hoxa11/HOXA11 are expressed in the proliferative phase of the endometrium and increase during the secretory phase (Taylor et al.
Persistent HOX gene expression in the adult may be a mechanism to retain developmental plasticity in the female reproductive tract. Emx2 is a divergent Homeobox gene, which is a mammalian homolog of the Drosophila empty spiracles (ems) gene. The vertebrate Emx2 gene is located outside of the Hox cluster, and is expressed in the developing vertebrate brain as well as the urogenital system (Simeone et al. 1992a,b). In the embryo, Emx2 is expressed in the epithelial components of the pronephros, mesonephros, ureteric buds, and the Wolffian and Müllerian ducts. In mouse embryos, Emx2 expression is greatly diminished in male gonad, but strong expression remains detectable throughout the female gonad. Null mutants of Emx2 mice fail to develop kidneys, gonads or a reproductive tract (Pellegrini et al. 1997; Svingen and Koopman 2007). In adults, EMX2 has been detected in the human uterus. The expression of EMX2 displayed a dynamic pattern that varied with the developmental phase of the human reproductive cycle (Fig. 2) (Troy et al. 2003).

The Role of HOX Genes in Female Fertility

Female fertility is a broad term, which includes the ability to reproduce or become pregnant. Multiple factors influence female fertility, including normal aging and several disease processes. However, two processes are essential for normal female fertility: ovarian follicular maturation and embryo implantation. In vertebrates, HOX genes are involved in both of these processes.

Ovarian follicle development is a complex process in which many transcription factors participate. As described above, HOX genes containing the evolutionarily conserved HD sequence encode a family of DNA-binding transcription factors whose functions are crucial for embryonic development in vertebrates. In 1995, HOXA4 and HOXA7 expression was first described in the human unfertilized oocytes (Verlinsky et al. 1995). Sequence analysis of cDNA libraries generated from human unfertilized oocytes confirmed the expression of HOXA7 (Adjaye and Monk 2000). Furthermore, in human ovarian folliculogenesis, HOXA7 expression is nearly absent in primordial follicles but high in primary and mature follicles. During follicular maturation, the subcellular localization of HOXA7 changes from nuclear to predominantly cytoplasmic. This differential localization indicates that HOXA7 undergoes cell type- and stage-specific changes during the human ovarian folliculogenesis, and regulates proliferative
activities of ovarian follicles (Ota et al. 2006). Granulosa cells surround the developing oocyte, providing a critical microenvironment for follicular growth. During this process, the oocyte and the granulosa cells establish mutual interactions and their growth is regulated by coordinated paracrine mechanisms. HOXA7 modulates granulosa cell growth and proliferation not only via the regulation of the epidermal growth factor receptor (EGFR), but also forms dimers with the HOX gene cofactor pre-B-cell leukemia transcription factor 2 (PBX2) to bind the specific promoter regions in the human granulosa cells. HOXA7 plays an important role in ovarian follicular maturation (Ota et al. 2008; Zhang et al. 2010).

Embryo implantation is critical for female reproduction. This process is a complex event requiring synchronization between a developing embryo and receptive endometrium. Fundamental to this process is the dynamic and precisely ordered molecular and cellular events that drive and stabilize the interaction between the developing embryo and its host endometrium. As described above, Hoxa10/HOXA10 and Hoxa11/HOXA11 are expressed in endometrial glands and stroma throughout the estrus/menstrual cycle. These two HOX genes are essential for embryo implantation in both mice and humans (Hsieh-Li et al. 1995; Satokata et al. 1995; Benson et al. 1996; Gendron et al. 1997). Targeted mutation of either Hoxa10 or Hoxa11 in the mice leads to infertility related to defects in uterine receptivity. Embryos produced by Hoxa10 deficient mice are viable and can successfully implant in wild-type surrogates. However, those embryos are not able to implant or survive in the uteri of Hox gene knockout mice. Although the uteri of these knockout mice appear anatomically normal, they do not support the development or implantation of their own embryos, nor of embryos from the wild-type mice. Histologic abnormalities were noted in the Hoxa10 deficient mice, resulting in a homeotic transformation of the anterior part of the uterus into an oviduct-like structure. Similarly,
the mice with a homozygous mutation in the Hoxa11 gene are infertile because of implantation defects. Those mice have reduced endometrial glands and decreased leukemia inhibitory factor (LIF) secretion. Targeted mutation of orthologous Hox genes such as both Hoxd9 and Hoxd10 in mice does not result in abnormalities on uterine structure or position (De La Cruz et al. 1999). Although no human females with mutations in HOXA10 and HOXA11 have been described, it has been reported that patients with lower implantation rates have lower HOXA10 and HOXA11 expression in the secretory phase, which indicates that maternal HOX gene expression is conserved and necessary for endometrial receptivity (Taylor et al. 1999b; Bagot et al. 2000; Taylor 2000).

**Estrogens and Progesterone Regulate Hox Gene Expression in the Reproductive Tract**

So far, few regulators of HOX gene expression have been identified. Sex steroids have been investigated in the regulation of the HOX genes at the 5’ end of the cluster, which determine the posterior development, including the development of female reproductive tract (Taylor et al. 1997, 1998, 1999b; Ma et al. 1998; Cermik et al. 2001; Goodman 2002). During each reproductive cycle, endometrial epithelial and stromal cells display a well-defined cyclic pattern of functional differentiation under the influence of estrogen and progesterone. Menstrual cyclicity is regulated by timed expression of estrogen and progesterone, which act both independently and in concert to up-regulate Hoxa10 and Hoxa11 expression in the endometrium. In normal cycling women, Hoxa10 and Hoxa11 levels increase, reaching maximal expression during the mid-secretory phase, and remaining elevated throughout the secretory phase. In endometrial stromal cells, 17β-estradiol and progesterone significantly increase Hoxa10 and Hoxa11 expression. Hoxa9 is under the control of both estrogen and progesterone as well. The regulation of HOX gene expression in the adult uterus by ovarian steroids is related to its position within the cluster and mediated by the direct action of estrogen and progesterone receptors on these genes.

Humans are exposed to a wide variety of chemicals that have estrogenic properties. Those estrogenic compounds show profound and lasting effects on essential developmental genes in female reproductive tract. They have potential to alter the expression of estrogen responsive genes, such as HOX genes. These changes are likely to influence reproductive competence. Diethylstilbestrol (DES) is a nonsteroidal estrogen, a well-known teratogen. This chemical alters the localization of Hox gene expression along the axis of the developing murine reproductive tract, and induces developmental anomalies of female reproductive tract (Ma et al. 1998; Akbas et al. 2004). DES exposure in utero shifts Hox9 expression from the oviducts to the uterus and leads to decreases in both Hoxa10 and Hoxa11 expression in the uterus. The decreased expression of the Hoxa genes may cause a “T-shaped” uterus, a structure that is characterized by branching and narrowing of the uterus into a tube-like phenotype. This phenotype is likely caused by expression of the Hox gene that controls tubal identity (Hoxa9) ectopically in the uterus. Because the multiple HOX gene clusters provide an overlapping expression pattern in the mice and humans, the complete transformation into an oviduct is probably prevented.

Studies on xenoestrogens, such as methoxychlor (MXC) and bisphenol A (BPA), have shown that exposure to these chemicals also alters the Hoxa10 expression in female reproductive tract (Block et al. 2000; Suzuki et al. 2004; Fei et al. 2005; Markey et al. 2005; Sugiura-Okasawa et al. 2005; Daftary and Taylor 2006; Smith and Taylor 2007). MXC is a pesticide and this chemical is associated with female reproductive defects after either prenatal or postnatal exposure. MXC specifically alters Hoxa10 gene expression, specifically the Hoxa10 gene expression. This HOX gene is responsible for normal uterine development and fertility, and its expression is permanently repressed in the uterus of mice exposed to MXC in uterus. This effect is mediated through the Hoxa10 estrogen response element (ERE) in a dose-dependent pattern.
BPA, another xenoestrogen, is a common component of polycarbonate plastics, epoxies used in food storage, canned goods, and dental sealants. BPA is also associated with adverse reproductive outcomes in both animal models and humans. After exposure to BPA in utero, Hoxa10 expression is increased in female mice and this altered expression persisted in adults. The alternation of the gene expression persists long after exposure and alters the normally precise, temporal regulation of Hoxa10 in reproductive tract development. This permanently modified expression of Hoxa10 contributes to the decline in female reproductive potential. Despite its opposite effect on HOX gene expression in vivo, BPA behaves similarly to MXC in vitro by stimulating the HOX10 ERE. The difference seen after in utero exposure likely represents the unique molecular signals present in the embryo and underlies the increased risk of exposure to environmental chemicals during critical periods of development. Exposure to various xenoestrogens alters Hoxa10 gene expression in the developing reproductive tract, and these exposures may lead to permanent alteration of gene expression in the adult (Fig. 3) (Taylor 2008).

**HOX GENES AND INFERTILITY**

HOX genes are essential for endometrial development and embryo implantation in both mice and humans. As described above, the association between alteration of Hoxa gene expression and fertility is evident in animal models (Fig. 4) (Paria et al. 2002). The Hoxa10/Hoxa11 and HOXA10/11 genes act as important transcriptional moderators that either activate or repress the downstream target genes; these targets include β3-integrin and Emx2/EMX2, which are themselves important for embryo implantation. As discussed earlier, in normal cycling women, there is a surge of HOXA10 and HOXA11 expression during the mid-secretory phase; diminished HOXA10 and HOXA11 expression in the secretory phase leads to low embryo implantation rates. Impaired uterine receptivity has been studied in several gynecological diseases that lead to infertility. These include endometriosis, polycystic ovarian syndrome, leiomyoma, and hydrosalpinx. Compared with controls, there is diminished HOXA10 and HOXA11 expression in woman with each of those disorders (discussed in detail below). Although differential mechanisms may lead to decreased expression, it appears that altered HOX gene expression is so central to the process of implantation that decrease of their expression is required to diminish implantation. Alterations in the expression of HOX genes cause infertility in humans primarily by endometrial receptivity defects and impaired implantation.

**HOX Genes and Endometriosis**

Endometriosis is an estrogen-dependent benign inflammatory disease defined by the presence of viable endometrial tissue outside the uterine cavity. The prevalence of endometriosis has been estimated as up to 10% to 15% of reproductive-age women and 30%–50% of women with endometriosis have infertility (Verkauf 1987; Olive and Pritts 2001). Multiple factors are considered to contribute to endometriosis related infertility, including altered folliculogenesis, impaired fertilization, poor oocyte quality, and defective implantation. Here, we will focus on the role of diminished implantation as it is related to diminished HOX gene expression. In patients with endometriosis, implantation rates are reduced during both natural and assisted reproductive technology cycles, even in patients with minimal disease (Barnhart et al. 2002). Two of the HOXA genes, HOXA10 and HOXA11, involved in uterine embryogenesis and endometrial receptivity, have been implicated in the pathogenesis of endometriosis-associated infertility. In humans, the expression of both HOXA10 and HOXA11 rises dramatically during the implantation window and remains elevated throughout the secretory phase. However, patients with endometriosis do not show this rise in HOXA10 and HOXA11 (Taylor et al. 1999a; Kim et al. 2007; Lee et al. 2009).

HOXA10 downstream target genes are also involved in this pathologic mechanism. As discussed above, EMX2 is a divergent Hox meobox gene, cyclically expressed in the adult.
endometrium. Endometrial EMX2 expression is directly regulated by endogenous endometrial HOXA10. Normally EMX2 expression is down-regulated in the peri-implantation period; however, this regulated expression fails in women with endometriosis (Troy et al. 2003; Daftary and Taylor 2004). Further demonstrating the important role of this target gene, altering the endometrial Emx2 levels is not only associated with defective implantation, but also reduces litter size in mice (Taylor and Fei 2005). Aberrant endometrial EMX2 expression in women with endometriosis is mediated by altered HOXA10 expression.

Furthermore, another biomarker of endometrial receptivity to embryonic implantation is also found to be decreased in endometriosis. Integrins are ubiquitous cell adhesion molecules that participate in cell–cell and cell–substratum interactions. These molecules undergo dynamic alterations during the normal menstrual cycle in the human endometrium. β3-integrin is expressed in endometrium at the time of implantation, and the disruption of in-
tigrin expression is associated with decreased uterine receptivity and infertility (Lessey and Young 1997). Interestingly, β3-integrin subunit is a direct Hoxa10 downstream target gene, and directly regulated by HOXA10 in endometrial cells. Aberrant expression of both HOXA10 and integrins have been described in the endometrium of women with endometriosis (Naqvi et al. 2014). Epigenetic changes have been described in numerous studies including hypermethylation of HOXA10, progesterone receptor-β, and E-cadherin or hypomethylation of genes for estrogen receptor-β and steroidogenic factor 1 (Guo 2009; Senapati and Barnhart 2011). In both murine and baboon endometriosis models, hypermethylation of the promoter region of Hoxa10/HOXA10 and decreased expression of Hoxa10/HOXA10 genes were shown in eutopic endometrium (Kim et al. 2007; Lee et al. 2009). In humans, hypermethylation of HOXA10 was identified in the endometrium of women with endometriosis (Wu et al. 2005). The DNA methyltransferase (DNMT) is a family of enzymes, which catalyze the transfer of a methyl group to DNA. DNMT1, 3A, and 3B were found to be overexpressed in the epithelial component of endometriotic implants. However, only DNMT3A was found to be up-regulated in eutopic endometrium of women with endometriosis (Wu et al. 2007). A recently published study, using a genome-wide methylation array, shows that HOXA10 expression was repressed and methylation of HOXA10 gene was altered by 1.3-fold in human endometriosis (Naqvi et al. 2014). Other HOX genes, such as HOXD10 and HOXD11, also showed significantly altered methylation in endometriosis (Naqvi et al. 2014). Epigenetic programming of HOX gene expression in endometriosis leads to lasting alterations in endometrial receptivity.

**HOX Genes and Polycystic Ovarian Syndrome**

Polycystic ovarian syndrome (PCOS) is a common endocrine disease, afflicting 5% of women of reproductive age. It is characterized by anovulation and elevated androgen action. Infertility associated with PCOS derives from chronic anovulation. Despite the ability to correct ovulatory disorders, pregnancy rates remain paradoxically low, and spontaneous pregnancy loss rates are high. In women with PCOS, between 30% and 50% of all conceptions miscarry (Giudice 2006). Some data also suggest that poor oocyte quality, implantation failure, and higher rates of miscarriage further complicate achieving and maintaining a pregnancy in women with this disorder. Women with PCOS are also at significantly higher risk of endometrial hyperplasia (Niwa et al. 2000). PCOS may have complex effects on the endometrium, contributing to the infertility. Furthermore, increasing evidence and emerging data have shown that endometrial receptivity contributes to the infertility of PCOS even in the setting of ovulation induction (Giudice 2006). An increase in the expression of HOXA10 in the endometrium is necessary for receptivity to embryo implantation. However, endometrial biopsies obtained from women with PCOS in ovulatory cycles have shown that HOXA10 expression is decreased compared with normal fertile women during the secretory phase (Cermik et al. 2003). In vitro, HOXA10 expression is repressed by testosterone (Cermik et al. 2003). Testosterone also prevents the increased expression of HOXA10 induced by estradiol or progesterone. Dihydrotestosterone produced an effect similar to that of testosterone, whereas flutamide blocked the testosterone effect. Diminished uterine HOXA10 expression may contribute to the diminished reproduction potential of women with PCOS, illustrating a significant effect of the disease on receptivity. Elevated androgen levels may induce infertility associated with PCOS by altering HOX gene expression.

As discussed above, β3-integrin, a biomarker of endometrial receptivity to embryonic implantation, is a HOX target gene that is
directly regulated by HOXA10 in endometrial cells. The expression of this biomarker is decreased in endometrium from women with PCOS compared with fertile controls (Apparao et al. 2002). Also, as described above, after ovulation induction treatment of infertility in PCOS, implantation rates remain low. In fertile women, when ovulation is induced with clomiphene citrate, the treatment provokes the expression of endometrial integrins at the implantation window. Interestingly, integrin is decreased in endometrial biopsy specimens from women with PCOS even after clomiphene citrate treatment (Gonzalez et al. 2001; Jakubowicz et al. 2001).

**HOX Genes and Leiomyoma**

Leiomyomas (fibroids) are the most common benign uterine tumor of reproductive age women. The growth of leiomyoma is strictly related to sex steroids and their receptors. Their presence is associated with menorrhagia and poor reproductive outcomes. The prevalence of uterine fibroids approaches 33% of women of reproductive age based on clinical assessment, and up to 50% on ultrasound scans. This disorder presents in 5%–10% of women with infertility (Payson et al. 2006; Revel 2012).

The presence of a distorted uterine cavity caused by leiomyomas significantly decreases in vitro fertilization (IVF) pregnancy rates. Fortunately, myomectomy can increase the pregnancy rates in patients with leiomyoma-related infertility (Bulletti et al. 1999; Surrey et al. 2001). However, the mechanisms by which leiomyoma cause infertility are not fully known. HOXA10 is expressed in human myometrium and its expression also has a menstrual cycle-dependent pattern. In vitro, HOXA10 expression is induced in endometrial stromal cells by progesterone, but in the primary myometrial cells, progesterone suppresses HOXA10 expression (Cermik et al. 2001; Matsuzaki et al. 2009; Rackow and Taylor 2010; Sinclair et al. 2011). It is clear that there are different factors involved in the regulation of HOXA10 by progesterone in myometrium than endometrium. Further, independent of any change in progesterone concentration, endometrial HOXA10 and HOXA11 expression are significantly decreased in uteri with submucosal myomas compared with controls. This effect is not localized to the endometrium overlying the myoma; rather the decreased HOXA10 expression is seen throughout the endometrium. This global effect of the myoma on endometrium suggests the presence of a diffusible factor that would influence endometrial receptivity remote from the myoma itself. Indeed, we have recently reported that TGFβ secreted by myomas leads to decreased BMP receptor expression and subsequent HOXA10 repression (Sinclair et al. 2011). Leiomyoma alter endometrial receptivity by secreting TGFβ and altering genes including HOXA10 that are required for implantation.

**HOX Genes and Hydrosalpinx**

Hydrosalpinx is an inflammatory disease involving the oviduct. The prevalence of hydrosalpinges in patients suffering from tubal disease is relatively common and ranges from 10% to 13% when diagnosed by ultrasound, and up to 30% when diagnosed by hysterosalpingography or at the time of surgery (Cakmak and Taylor 2011). Women with hydrosalpinges have decreased implantation rates in IVF, and their pregnancy rates can be improved with salpingectomy before IVF. The hydrosalpinx generates an inflammatory fluid that may interfere with endometrial receptivity and embryonic implantation mechanically or chemically (Zeyneloglu et al. 1998; Camus et al. 1999). Although a study has shown that culturing mice embryos in the medium containing hydrosalpinx fluid can suppress embryo maturation and promote degeneration, this toxic effect does not affect human embryos. (Mukherjee et al. 1996; Strandell et al. 1998) We performed an in vitro study demonstrating that hydrosalpinx fluid decreased endometrial HOXA10 mRNA expression in a dose-dependent pattern. Subsequently, studies on women with hydrosalpinges show that the expression of HOXA10 was significantly lower in women with hydrosalpinges compared with fertile controls. After salpingectomy, HOXA10 expression in infertile women with hydrosal-
pings was similar to that of age-matched fertile women, indicating that salpingectomy restores HOXA10 expression to physiological levels (Daftary and Taylor 2002; Daftary et al. 2007).

As described above, β3-integrin subunit is a well-characterized endometrial receptivity marker, directly regulated by HOXA10 in endometrial cells. In women with the presence of hydrosalpinges, the expression of β3-integrin is also reduced. Interestingly, two thirds of patients with hydrosalpinx who underwent salpingectomy also showed return of HOXA10 and β3-integrin back to normal levels (Bildirici et al. 2001).

**SUMMARY**

All metazoans use HOX genes to regulate embryonic patterning. HOX genes play a fundamental role in morphogenesis during embryonic development. Well-characterized examples include the role of HOX genes in the patterning of the vertebrate hindbrain, skeleton, and limbs. In reproduction, HOX genes determine positional identity during embryonic development of the female reproductive tract. Abnormalities in reproductive tract development are related to HOX gene mutations and to alterations in the normal HOX gene expression patterns. This has been clearly shown in mice with targeted Hox genes as well as in mice exposed to chemicals with estrogenic properties such as DES. In the adult, the endometrium undergoes an ordered process of differentiation leading to receptivity to implantation. HOX genes are also essential to this process. As transcription factors, HOX genes control cyclical endometrial development and receptivity by activating or repressing the expression of target genes. HOXA10 and HOXA11 expression increases drastically in the mid-secretory phase, the time of implantation, and they remain elevated throughout the secretory phase. This increased expression is necessary for embryonic implantation; decreased Hoxa10/HOXA10 and Hoxa11/HOXA11 expression at this time leads to decrease implantation rates in both mice and humans. Impaired uterine receptivity has been studied in several infertility-related gynecological diseases, such as endometriosis, polycystic ovarian syndrome, leiomyoma, and hydrosalpinx. Alternation of HOXA10 and HOXA11 expression has been identified as a mechanism of the decreased implantation associated with these disorders. Alternation of Hoxa gene expression causes both uterine developmental abnormalities and impaired adult endometrial development that prevent implantation and lead to female infertility.

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The Role of \textit{Hox} Genes in Female Reproductive Tract Development, Adult Function, and Fertility

Hongling Du and Hugh S. Taylor

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Review

An evo-devo perspective of the female reproductive tract

Andrew T. Major†, Martin A. Estermann†, Zahida Y. Roly and Craig A. Smith*

Department of Anatomy and Developmental Biology, Monash Biomedicine Discovery Institute, Monash University, Clayton, Victoria 3800, Australia

*Correspondence: Department of Anatomy and Developmental Biology, Monash Biomedicine Discovery Institute, Monash University, Clayton, Victoria 3800, Australia. Email: craig.smith@monash.edu

†These authors contributed equally to this work.

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Abstract

The vertebrate female reproductive tract has undergone considerable diversification over evolution, having become physiologically adapted to different reproductive strategies. This review considers the female reproductive tract from the perspective of evolutionary developmental biology (evo-devo). Very little is known about how the evolution of this organ system has been driven at the molecular level. In most vertebrates, the female reproductive tract develops from paired embryonic tubes, the Müllerian ducts. We propose that formation of the Müllerian duct is a conserved process that has involved co-option of genes and molecular pathways involved in tubulogenesis in the adjacent mesonephric kidney and Wolffian duct. Downstream of this conservation, genetic regulatory divergence has occurred, generating diversity in duct structure. Plasticity of the Hox gene code and wnt signaling, in particular, may underlie morphological variation of the uterus in mammals, and evolution of the vagina. This developmental plasticity in Hox and Wnt activity may also apply to other vertebrates, generating the morphological diversity of female reproductive tracts evident today.

Key words: Müllerian duct, sex determination, female reproductive tract, oviduct, Hox genes, evo-devo.

Introduction

A remarkable ability to thrive and reproduce in diverse habitats has underpinned the evolutionary success of vertebrates. This has been greatly facilitated by adaptations of the female reproductive tract. First appearing in fishes as a means of extruding gametes, the vertebrate female reproductive tract has undergone considerable modifications over evolution as lineages adopted internal fertilization, oviparity (egg-laying) and viviparity (live birth). A simple tube-like structure present at embryogenesis develops into a specialized oviduct in egg-laying species or a highly vascularised structure for supporting complete embryogenesis in therian mammals. Different reproductive strategies among vertebrates have required modifications of the female reproductive tract. Among bony fishes, a reproductive tract comparable to that seen in tetrapods (land vertebrates) is lacking in most species, while chondrichthyan (cartilaginous fishes) have well developed oviducts for oviparity or viviparity. The emergence of vertebrates onto land and freedom from water to reproduce was accompanied by differentiation of the female ducts for the development of hard-shelled eggs among reptilian and avian lineages. In therian mammals, the female reproductive tract takes on a greater role than in other lineages, being the site of fertilization, embryonic development and live birth [1].

How the diversity of female reproductive tract has been generated at the molecular genetic level is largely unknown. This review considers the female reproductive tract from a novel perspective; evolutionary developmental biology (evo-devo). We firstly summarise the comparative anatomy and physiology of the female reproductive tract among vertebrates. This sets the stage for a consideration of the molecular genetics regulating the formation and differentiation of the female reproductive tract from an evolutionary perspective.
In most vertebrates, the female reproductive tract develops during embryogenesis from paired epithelial tubes, the Müllerian ducts. We propose that morphogenesis of the Müllerian duct has involved co-option of gene regulatory pathways that play a role in tubulogenesis of the adjacent mesonephric kidney and Wolffian (pronephric) duct. Studies in mouse and chicken show that genetic regulation of early Müllerian duct formation is conserved [2]. In contrast, the subsequent duct differentiation stages have so far not been shown to be conserved between mammals and birds. This likely reflects the functional divergence of the ducts in the two clades. However, we speculate that regional differentiation of the vertebrate Müllerian duct may be under-pinned by the Hox gene code, as it is in mammals.

**Comparative anatomy of the female reproductive tract**

The female reproductive tract of tetrapods (land vertebrates) derives from a pair of embryonic epithelial tubes, the Müllerian (paramesonephric) ducts. In humans, Müllerian ducts are initially present in both sexes, together with another set of tubes, the Wolffian ducts (Fig. 1a). The ducts, gonads and associated embryonic (mesonephric) kidneys are of mesodermal origin. As both involve transfer of biological agents to the exterior, development of the reproductive tract (in both sexes) is intimately linked to the excretory system. The paired Wolffian ducts (the pronephric or mesonephric ducts) form within the body of the mesonephric kidneys. They function at embryonic stages as excretory canals, transferring nitrogenous waste from the mesonephric kidneys to allantois. At the early undifferentiated stage, (weeks 5–6 in human embryos) the Müllerian duct is a simple structure, comprising a mesenchyme, and an outer layer of surface (coelomic) epithelium [3, 4] (Fig. 1b). In therian (“placental”) mammals such as human and mouse, the epithelial and mesenchymal compartments give rise to regionalised differentiation of the duct during the late embryonic and postnatal periods, generating the Fallopian tubes, uterus and upper vagina (Fig. 1c-d). In males, Müllerian ducts typically regress during embryonic life under the influence of testis-derived Anti-Müllerian Hormone (AMH).

During vertebrate evolution, the female reproductive tract has changed anatomically to reflect a transition from the production of shelled eggs to directly supporting embryonic development (Fig. 2). In agnathans (jawless fishes such as lampreys), Müllerian duct derivatives are absent and gametes are shed directly into the coelom, then extruded. This is likely to represent the ancestral vertebrate condition. Among teleost fishes, Müllerian ducts are absent. Instead, a different structure, the gonoduct, arises from the gonad of both sexes, derived from the dorsal peritoneum. It transfers sperm in males and the ova in females. External fertilization is typical of bony fishes, but some teleosts are viviparous and the young (or eggs) develop inside simple glandular oviducts or in the ovaries themselves. These include groups such as the Poeciliidae [5, 6]. *D. rerio*, the zebrafish used widely as a developmental model, has external fertilization and the gonaduct serves purely as a vehicle to transfer gametes [7]. Interestingly, despite the lack of Mullerian ducts, teleost fishes have AMH, which has other (presumably ancestral) functions related to gonadal soma and the germ cells [8]. Most chondrichthyans (cartilaginous sharks, rays and skates) are viviparous (live-bearing) and an oviduct develops that is analogous to that of tetrapods [9–11] (Fig. 2). Embryos can develop directly in a “uterus” in such viviparous shark species [10, 12–14]. Phylogenetically, the AMH gene first appears among cartilaginous fishes, the most ancient gnathostome lineage, in line with the appearance of paired Müllerian ducts. Hence, these ducts degenerate in males, though rudiments are retained in the adult [8].

Most amphibians (frogs, newts, salamanders et al.) have external fertilization. The paired Mullerian ducts differentiate into oviducts, ciliated epithelial tubes that serve to transfer non-calciﬁed eggs via the cloaca to the exterior (Fig. 2) [15]. At the cranial pole, the oviduct differentiates into the infundibulum (ostium), a ciliated slit that receives the oocyte. In frogs, the infundibulum typically lacks fimbriae (finger-like projections). Posterior to the infundibulum is the atrium, a short segment that leads into the secretory ampulla of the oviduct, a region that becomes highly convoluted when hormonally stimulated (Fig. 2) [16, 17]. Hence, over evolution, the first signs of substantial regionalised differentiation of the oviduct (Mullerian duct) are apparent in amphibians, linked to a semi-terrestrial lifestyle and a need to physically and osmotically protect eggs laid in freshwater. Evolution of Mullerian duct derivatives beyond amphibians has involved more marked regional differentiation. The complete transition from water to land necessitated internal fertilisation, facilitating evolution of the amniote egg (the amniotic membrane, in addition to the chorion, allantois and yolk sac). This transition required the production of shelled (calcified) eggs that minimise water loss and could hold large amounts of yolk (Fig. 2). Hence, reptiles and birds have specialized regions of the oviduct that facilitate these functions [18–23]. While fertilisation takes place in the infundibular region, the development of shelled egg has led to significant structural differentiation of the oviduct. Birds and reptiles have four anatomically and histologically distinct regions of the oviduct. Adjacent to the infundibulum, the magnum is specialized to secrete albumen, a liquid medium for supporting embryonic development, containing high levels of protein. The isthmus secretes the egg shell membrane, while the most posterior region, the shell gland (“uterus”) lays down the calcified shell (Fig. 2) [24–27]. Most reptiles are oviparous (egg laying), but many squamates (lizards and snakes) are viviparous (live bearing) or ov-o-viviparous (eggs hatch in the oviducts, then live birth). In these species, the oviduct has evolved to bear live young. It has structural and physiological adaptations that facilitate formation of a chorioallantoic placenta, allowing embryonic gas exchange and delivery of nutrients [22, 28, 29]. In most (but not all) birds, the right oviduct and ovary are vestigial. This is probably due to physical constraints precluding two gravid ducts each holding fragile hard-shelled eggs [30]. Mammalian evolution has been accompanied by a reduced reliance on yolk and advanced development of the uterus as a secretory organ that supports embryonic development (Fig. 2) [31]. The Mullerian ducts of mammalian embryos differentiate into Fallopian tubes (called “oviduct” in mice), uterus and, in therians, upper portion of the vagina. Among therian mammals, the cloaca (a common urogenital and anal canal) has been lost (Fig. 2).

**Evo-devo of Müllerian duct development; conservation and divergence**

The diverse female reproductive tracts described above are all derived from a common precursor structure, the Mullerian duct. This embryonic organ is structurally very similar across groups. The Mullerian ducts are bilateral meso-epithelial tubes surrounded by mesenchyme that develop on the surface of the mesonephric kidneys at embryonic or larval stages in amphibians, reptiles, birds and mammals [2, 32–36]. Genetic regulation of duct formation
Figure 1. Development of the female reproductive tract in therian mammals. a) Schematic overview of reproductive tract formation (ventral view). In the early embryo (4–5 weeks in humans, up to 13–14.5 days postcoitum in mouse) the urogenital system is morphologically undifferentiated, characterised by paired Müllerian ducts (pink) and Wolffian ducts (blue). Later in embryogenesis, the Wolffian ducts regress in females, and the Müllerian ducts are retained. Subsequently, at postnatal stages, the Müllerian duct undergoes regionalised differentiation in Fallopian tubes, uterus, cervix / upper vagina (fused as a simplex in humans, shown here). Adapted from Roly et al. [4], with permission. b) Histology of the female reproductive tract in the mouse (transverse H&E stained sections). At the embryonic stages, the Müllerian duct is present as a tube, comprising mesenchyme and an inner Müllerian epithelium. The duct develops in close association with the Wolffian duct, adjacent to the mesonephric kidney. Image from Fujino et al [88] with permission. c) At postnatal day 3, the oviduct is not differentiated, comprising an inner epithelial layer (Müllerian epithelium), underlying mesenchyme and an outer epithelial layer, derived from the coelomic epithelium (“surface epithelium”). d) Postnatal day 28 in mouse, showing regional differentiation of the duct into Fallopian tube (ciliated columnar epithelium lining mucosal folds), uterus (glandular and columnar epithelium of the endometrium) and vagina (stratified epithelium). Images in c) and d) taken from Dunlap et al. [2011] [160], with permission. Bar = 50 μm.

is likely to be a deeply conserved process among vertebrate embryos. How, then, is the diverse comparative anatomy generated? This must involve changes to the duct differentiation process downstream of the formation stage. This may involve changes in the timing of gene regulation, or the co-option of novel genes into developmental pathways. Both of these possibilities would involve alterations to cis- and trans- regulatory regions of genes. We propose here that Müllerian duct formation has entailed co-option
for genetic programs that underlie mesonephric kidney and nephric duct formation. We further propose that the early stage of duct specification is developmentally conserved, at the cellular and genetic level, whereas duct elongation and differentiation involve divergent gene regulatory networks that reflect the different reproductive strategies across vertebrate groups. We speculate that a major point of divergence between egg-laying vertebrates and mammals may centre around the changing role of oestrogen in duct development. Lastly, we consider patterning of the duct in the context of the Hox gene code, which has only been explored in mammals, but may apply to other vertebrates. We speculate on the developmental origin of mammalian-specific Müllerian derivative such as the vagina, which may have involved plasticity of the Hox code.

Co-option of nephric regulatory genes to the Müllerian duct

The cell biology and molecular genetics of Müllerian duct formation in the mouse model has been extensively reviewed [2–4, 36, 37]. Duct regression in males under the influence of Anti-Müllerian Hormone has also been well described [38–42]. Here, we focus on genetic regulation of duct morphogenesis from a novel evolutionary development biology perspective. We find that morphogenesis of the Müllerian duct has involved co-option of genetic programs that regulate nephric duct and (mesonephric) tubule formation. All three tissues feature tubulogenesis - the formation of epithelial tubes - via inductive signalling, EMT events, invagination, cell migration and mesenchyme maturation [43, 44].

Developmental studies in mouse and chicken embryos have shown that the Müllerian duct develops from a group of precursor cells, specified in the coelomic epithelium at the cranial pole of the mesonephros [44, 45]. These cells proliferate and invaginate, forming a meso-epithelial tube that migrates caudally, similar to migration of the Wolffian duct [36]. In mouse and/or chicken models, duct progenitor cells express the transcription factors, Pax2, Lim1 and Emx2 and signalling factors such as FGF and BMP family members (BMP 2-7) [44, 46–49]. The meso-epithelial tube, called the Müllerian epithelium, is surrounded by mesenchyme (Müllerian mesenchyme), which also derives from the surface coelomic epithelium, via an EMT (Epithelial to Mesenchyme transition) [50, 51]. Müllerian duct formation is morphologically conserved among tetrapods, involving the same types of cellular events.

Müllerian duct morphogenesis is very reminiscent of tubulogenesis in the adjacent mesonephros and nephric duct. The nephric duct (i.e. the pronephric or mesonephric duct, which becomes the Wolffian duct) pre-dates evolution of the Müllerian duct. Nephric ducts are present in the most ancient vertebrate lineage, the jawless fishes, which lack Müllerian ducts [52]. Similarly, the pronephric kidney, an excretory organ derived from the nephric duct and featuring a few nephric tubules, pre-dates the Müllerian duct. Evolution of the Müllerian duct might therefore be viewed as a process that has involved recruitment of pre-existing developmental pathways that regulate the formation of tubular nephric structures (pronephric/mesonephric
tubules and Wolffian duct). Important genes expressed in these tissues are also expressed during stages of Mullerian duct development. Table 1 summarises key common genes implicated in both nephric formation (duct or tubule) and Mullerian duct morphogenesis, based on data primarily derived from mammal (rodent) and bird (chicken) studies. Several transcription factors expressed at the early stages of duct morphogenesis (specification and for invagination), are also required for Wolffian duct/mesonephric morphogenesis. These regulators include the transcription factor gene, Wt1, which is required for posterior mesonephric tubule formation [52], and for activating Amhr2 in Mullerian duct [54], and Osr1, an essential nephrogenic regulator [55-57]. The paired box transcription factor gene, Pax2, is also crucial for multiple steps in urogenital development, including both kidney and Mullerian duct formation [44, 48, 58]. Another critical transcription factor gene for mesonephric kidney, Wolffian and Mullerian duct development is Lm1 (also known as Lhx1) [46, 59]. Mice lacking Lm1 fail to develop Wolffian and Mullerian ducts [1, 46]. In fact Lm1 is specifically linked to tubular morphogenesis in the reproductive tract [59]. The requirement for Lm1 and Pax2 in vertebrate urogenital tubulogenesis would appear deeply conserved, as both are also expressed in the zebrafish mesonephros [60]. Another important homeobox gene for both nephric and Mullerian duct development is Enx2. This gene is expressed in the epithelium of mesonephric tubules, Wolffian and Mullerian ducts, and both pairs of ducts do not form in its absence [49]. All of these transcription factors regulate specification of duct/tubule precursors, EMT or mesenchyme development during tubulogenesis of both nephric structures and the Mullerian duct (Table 1).

Conserved signalling pathways for tubulogenesis are also shared between the nephric duct/tubules and the Mullerian duct. Specifically, Wnt and Fgf signalling feature prominently in both Mullerian duct formation and Wolffian and mesonephric duct development. In the Wolffian duct, FGF7 and FGF10 are expressed in duct epithelium, signalling through FGFR2, a process required for proper Wolffian duct development [61]. Similarly, FGF is required for the early specification and invagination phases of Mullerian duct development, as reveal by mouse and chicken studies (Table 1) [44]. Key Wnt growth factors include WNT4, required in mesenchyme for both mesonephric tubule differentiation and Mullerian duct development, via the canonical -catenin pathway (mouse or chicken models) [43, 62, 63]. In the mouse Mullerian duct, mesenchyme-secreted WNT4 is required for invagination of overlying epithelial Mullerian precursor cells, and coordinates cell migration and extension of the Mullerian duct [62, 64]. Wnt4 null mice lack Mullerian ducts [64]. Similarly, waves of inductive signalling that involve WNT4 are required for differentiation in the mesonephros in chicken embryos [43] and for pronephric tubulogenesis in the amphian, X. laevis [65].

**Conserved Hox gene expression in the Mullerian duct and nephric structures**

Consistent with their graded expression along the rostral-caudal body axis generally, Hox genes play a pervasive role in segmental patterning structures of the urogenital system. Patterning of the Mullerian and nephric (Wolffian) ducts involves Hox-mediated positional cues [66]. In mammals, anterior regions of both the Wolffian and Mullerian ducts express Hoxa9, while more posterior regions express Hoxa11 and Hoxa13 [67, 68]. In both tissues, loss of function mutations can cause partial homeotic transformations [69, 70]. Regional differentiation of the Mullerian duct under the influence of Hox genes again appears to reflect co-option from pre-existing nephric Hox regulatory pathways (Table 1). In mouse and/or human, Hoxa9 – Hoxa11 control regional differentiation of the Wolffian duct mesenchyme into epididymis, vas deferens and seminal vesicle [71, 72]. This Hox axis is conserved in the Mullerian duct of mouse and human, where Hoxa9-Hoxa13 regulate regional patterning into oviduct, uterus, cervix and upper vagina [73]. Hoxd13 is also implicated in both Wolffian and Mullerian duct formation, with expression being restricted to the caudal region (vas deferens and seminal vesicle in the male, and upper vagina in female). Hoxa13+/−/Hoxd13−/− compound mutant mice show a transformation of cervix at the uterus/vagina boundary to uterus in females, while the same compound mutants show disrupted seminal vesicle formation in males (Table 1) [68, 74, 75].

Nephrogenesis also involves Hox gene regulation, most notably, the requirement of Hox11 trans-paralogs form branching of the ureteric buds and interaction with metanephric mesenchyme [76]. The ontogenetic timeline of urogenital development (proephros followed by mesonephros, Wolffian and then Mullerian duct formation) is collinear with the evolutionary appearance of these structures. It is likely that a subset of Hox genes was first co-opted to nephric duct formation from the cranio-caudal body axis, and then recruited to regional segmentation of the Wolffian and Mullerian ducts.

At the cell biology level, morphogenetic mechanisms are conserved between Mullerian and Wolffian ducts. Mesenchyme-epithelium interactions are fundamental to morphogenesis of both the Mullerian and nephric ducts. Regionally specified mesenchyme regulates cell fate specification of the epithelium during duct differentiation in both the Mullerian duct and in Wolffian duct and in nephric structures [66]. This is demonstrated by tissue recombination experiments. When mouse presumptive uterine epithelium is grown with presumptive vaginal mesenchyme, for example, the epithelium adopts a squamous vaginal cell fate [77].

**Mullerian duct specification is developmentally conserved, while later stages are divergent**

The early stages of Mullerian duct formation are conserved among tetrapods, involving the same cellular processes [2, 32-35]. This implies that the underlying molecular control is likely to be conserved. Indeed, the early stages of Mullerian duct formation involve a genetic program among that appear to be conserved among vertebrates. This program drives tubulogenesis. However, later stages of duct development are developmentally divergent.

**Conservation of master regulators**

Duct formation during embryogenesis can be divided into three stages; specification/invagination, elongation and patterning. This is shown schematically in Figure 3, which also shows genes expressed at these stages across vertebrate lineages. The duct forms through specification of Mullerian precursors cells in the cranial coelomic epithelium overlaying the mesonephros, followed by delamination and invagination of these cells. The first cells to invaginate form a mesoepithelial tube (characteristics of both mesenchyme and epithelium) and has recently been called pEMT (partial Epithelium to Mesenchyme Transition) [51]. This process of mesoepithelial induction produces the duct luminal epithelium and it’s formation is deeply conserved, from fishes with ducts (sturgeon) through to...
duct formation, as revealed in rodent and chicken models. In the
As noted above, there is a key role for Fgf signaling in Mullerian
Conservation of Fgf signaling

Table 1. Known developmental genes shared between the embryonic nephric duct/or tubules and the Müllerian duct, based on mammal (rodent/human) or avian (chicken) models

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein product</th>
<th>Nephric duct/tube</th>
<th>Müllerian duct</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt1</td>
<td>Wilms' Tumor 1, a zinc finger transcription factor</td>
<td>Formation of caudal mesonephric tubules</td>
<td>Regulates AmhrII during duct regression</td>
<td>[53, 54]</td>
</tr>
<tr>
<td>Osr1</td>
<td>Odd-skipped related transcription factor (often a repressor)</td>
<td>Formation of urogenital system – kidneys and gonads</td>
<td>Expressed in ducts (conditional gene knockout not reported)</td>
<td>[55–57]</td>
</tr>
<tr>
<td>Pax2</td>
<td>Paired homeodomain Transcription Factor</td>
<td>Nephric lineage specification</td>
<td>Müllerian duct lineage progenitor specification</td>
<td>[44]</td>
</tr>
<tr>
<td>Lmx1</td>
<td>Homeodomain Transcription factor</td>
<td>Formation of nephric progenitors</td>
<td>Formation of Mullerian progenitors</td>
<td>[44, 46, 59]</td>
</tr>
<tr>
<td>Ema2</td>
<td>Homeodomain Transcription factor</td>
<td>Required in epithelial cells of mesonephric (Wolffian) duct and mesonephric tubules</td>
<td>Required in epithelial cells of Müllerian duct</td>
<td>[49]</td>
</tr>
<tr>
<td>Gata3</td>
<td>Transcription factor</td>
<td>Required in mesonephric kidney development</td>
<td>Required for mouse Mullerian duct elongation (expressed in Wolffian epithelial cells)</td>
<td>[91, 160, 161]</td>
</tr>
<tr>
<td>Fgf</td>
<td>Secreted Fibroblast growth factors</td>
<td>Fgf8 required for kidney tubulogenesis</td>
<td>Fgf/ERK signalling required for duct specification</td>
<td>[162] [44]</td>
</tr>
<tr>
<td>Wnt4</td>
<td>Wnt secreted growth factor</td>
<td>Mesonephric tubule differentiation</td>
<td>Required for duct formation and later patterning</td>
<td>[43] [64]</td>
</tr>
<tr>
<td>Hoxa9</td>
<td>Homeodomain Transcription factor</td>
<td>Patterning anterior Wolffian duct into epididymis</td>
<td>Patterning anterior Mullerian duct into oviduct</td>
<td>[71]</td>
</tr>
<tr>
<td>Hoxa10</td>
<td>Homeodomain Transcription factor</td>
<td>Patterning posterior Wolffian duct into vas deferens and seminal vesicle</td>
<td>Patterning posterior Mullerian duct into uterus</td>
<td>[72]</td>
</tr>
<tr>
<td>Hoxa11</td>
<td>Homeodomain Transcription factor</td>
<td>Patterning posterior Wolffian duct into vas deferens</td>
<td>Patterning anterior Mullerian duct into uterus</td>
<td>[163] [73]</td>
</tr>
<tr>
<td>Hoxa13</td>
<td>Homeodomain Transcription factors</td>
<td>Patterning posterior Wolffian duct into seminal vesicle</td>
<td>Patterning posterior Mullerian duct into upper vagina</td>
<td>[67, 68]</td>
</tr>
<tr>
<td>Hoxd13</td>
<td>Homeodomain transcription factor</td>
<td>Patterning posterior Wolffian duct into seminal vesicle</td>
<td>Expressed in Mullerian duct epithelia; human mutations cause abnormalities of Mullerian derivatives</td>
<td>[164, 165]</td>
</tr>
<tr>
<td>Hnf1b</td>
<td>Hepatic nuclear factor 1, a</td>
<td>Required in epithelium of Wolffian duct, and for kidney tubule development</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

tetrapods [2, 78–81]. At the genetic level, this stage has only been
examined in any detail in mouse and chicken embryos, revealing
conserved expression of master duct initiators, LIM1, PAX2 and
EMX2 transcription factors, together with FGF and BMP signalling
[46, 47], and WNT9B, derived from the mesonephros (summarised in
[4, 51](Fig. 3). In chicken, BMPs have been shown to induce PAX2
expression, while FGFs induce LIM1 expression [44].

In mouse, the POU homeodomain transcription factor, HNF1B,
is also required for Mullerian duct specification [82], and we have
also noted its expression in the chicken model [57], pointing to
a conserved role (Fig. 3). Similarly, retinoic acid (RA) signalling is
required for proper duct elongation in mouse [83], and probably
also in chicken, due to expression of RA-synthesising enzymes and
receptors in the forming duct [57]. Interestingly, the transcriptional
co-activators, Dach1 and Dach2, are redundantly required for Mul-
erian duct development in mouse, and they could have a deep
evolutionarily conserved role, as Drosophila dachshund mutant also
have a female reproductive tract phenotype [84]. These genes are
also expressed in the chicken Mullerian duct [57]. However, overall,
as highlighted on Fig. 3, very little is known about the conservation
of duct formation at the genetic level beyond the mammalian and
avian models.

Conservation of Fgf signaling

As noted above, there is a key role for Fgf signaling in Mullerian
duct formation, as revealed in rodent and chicken models. In the
chicken embryo, PAX2 expression in the coelomic epithelial duct
progenitor cells induces FGF expression, which then activates LIM1
and triggers the pEMT process [44] (reviewed in [4], [51]). FGF
action in the avian model is mediated by FGFR2 activation of
the ERK/MAPK pathway [44]. The likely ligands are FGF2, FGF8
and/or FGF9, which are expressed in chicken mesonephric tissue
[85], or in the nascent duct itself [57]. In the mouse model, an
erly role for FGF signalling has not been established, although
later Mullerian duct epithelial fate commitment (vaginal vs uter-
ine development) depends upon FGF/MAPK signaling, along with
other mesenchymal paracrine factors, such as BMP/SMAD [86,
87]. The migration stage of Mullerian duct development in rodents
involves the The phosphatidylinositol 3-kinase (PI3K)/AKT path-
way, active at the tip of the migrating duct [88]. This intracel-
lular pathway is activated by FGF, though the exact ligand is
unclear, and whether the process is conserved is also presently
unknown.

Deep conservation of WNT4 signaling

During the invagination and elongation phases, there is a deeply
conserved role for canonical WNT signalling, inferred from studies
across fishes, amphibians, birds and mammals. This applies most
notably to WNT4. WNT4 is required for Mullerian duct formation
in mouse, where it is expressed in the mesenchyme and signals
the duct progenitor cells in the coelomic epithelium to form a meso-
epithelial tube and extend caudally [62, 64]. Consequently, Wnt4
null mutant mice lack Müllerian ducts [64]. Wnt4 has a conserved expression profile in duct mesenchyme of the chicken embryo, where it is inferred to play the same role [34]. A conserved role has recently been shown in the zebrafish, which has Müllerian ducts, and expresses the osteichthyan homologue, Wnt4a. In zebrafish, as in mouse, targeted deletion of Wnt4a results in failure of duct development [89]. WNT4 would therefore appear to be a deeply conserved regulator of Müllerian duct formation, though it has not been examined during duct development in amphibians or reptiles. WNT7A is also expressed in the developing duct in both chicken and mouse (in the mesoepithelium, and later during duct differentiation) [57, 90], but, again, expression of this gene has not been examined in other species. In mouse, Wnt9b derived from the Wolffian duct, acts as a diffusible signal during the Müllerian duct elongation phase [91]. This factor has not been examined in other vertebrates, although Wnt9b mesonephric (wolffian) duct expression is conserved in chicken [92].

**Figure 3.** Conserved and divergent gene expression implicated in Müllerian duct development across vertebrates. Some genes show deep conservation of expression, while others appear novel. Conservation applies to the earlier stages of duct formation.

**Divergent gene expression**
From the duct elongation phase, molecular signals show some divergence among vertebrate groups, presumably due to divergence of function. In avians, for example, a muscular oviduct develops, adapted to deposition of a calcified shell. In eutherian mammals, the uterus develops to facilitate placentation. Interestingly, a comparison of recent RNA-seq data for chicken versus mouse shows that there is little overlap in gene expression during Müllerian duct differentiation [40, 57]. For example, the G-protein coupled receptor, GPR56, is required for duct elongation in chicken [50], though this is not the case in mouse [93]. We identified a number of transcription factors or signalling molecules that show strong expression during chicken duct development and are inferred to have an important role, such as FOXE1, SMARCA2, APCDD1, and TSHZ3 [57]. However, in mouse, targeted deletion of these genes is not accompanied by any reported Müllerian duct abnormalities [94–96]. In mouse, a role during duct cell fate commitment (vagina) has been shown for the
transcription factors Six1, Runx1 and SMAD signal transduction [86, 97]. We recently found conserved expression of Runx1 in the chicken model, earlier than in mouse, though functional data are lacking. The homeobox gene, Mx2, is required for vaginal epithelia differentiation in mouse [98]. Expression of this gene is also conserved in chicken [99], but again functional data are lacking. Overall, however, gene expression and functional analysis during Mullerian duct elongation and regional segmentation have not been characterised among other vertebrates (fishes, amphibians, reptiles) to allow any firm vertebrate-wide conclusions to be drawn.

The role of estrogen action

Estrogens have a central role in the development of the mammalian female reproductive tract, by modulating the Six1-Runx1 axis noted above [97]. Hence, while there is a clear physiological role of estrogen in the mature female reproductive tract, formation and early differentiation of the Mullerian duct is less influenced by endogenous estrogen in mammals compared to oviparous species. Altogether, the data suggest a shift away from steroid regulation of embryonic duct differentiation in the (eutherian) mammalian lineage. This might be related to chorioallantoic placentaion and requirement for the foetal duct to be refractory to maternal estrogens during its formative stages.

Developmental plasticity of the mammalian female reproductive tract

Among mammals, there is great variability with regard to the anatomy of the female reproductive tract [124]. Developmentally, this variability is due to different degrees of fusion of the paired Mullerian ducts late in embryogenesis. Four main types of uteri are recognised, based on degree of caudal duct fusion: duplex (no fusion), bipartite (some fusion), bicornate (more extensive fusion) and simplex (complete fusion). This is shown in a phylogenetic context in Figure 4. There is an overall evolutionary trend in mammals as a whole from separate uteri, through duplex to bipartite/bicornate and simplex uteri (Fig. 4). Monotremes (egg-laying mammals) have two separate uteri that open into the urogenital sinus, and they lack a vagina. The uteri secrete the egg shell around the egg, and is essentially homologous to the bird/teplant ertent. This condition can be considered ancestral among mammals. Beyond the monotremes (in therian mammals), a vagina evolved. Marsupials also have two uteri, but associated with two lateral vaginace and a medial birth canal that can be permanent (kangaroos) or transient (phalangers) (Fig. 4) [125]. (The paired vaginace in marsupilus are related to the fact that the ureters pass medially between the two vaginae, anatomiacally preventing their fusion). Among the three major clades of eutherian mammals (“placentals”) a variety of uteri is observed, without any clear phylogenetic restrictions (Fig. 4) [126–129]. This means that the various of uterine types across eutherians have arisen via a degree of convergence. Bipartite and bicornate uteri show partial duct fusion to generate uterine horns and a uterine body (more extensive in the bicornate form (Fig. 4). Most members of the archaic Xenarthra (armadillo, anteater) have a simplex uterus, although some species have less caudal duct fusion, resulting in a bicornate form [130, 131]. A bicornate uterus is typical of the Afrotherian clade (elephants, hyrax, and aquatic manatee and dugong) [132–134]. However, among the largest eutherian clade, the boreoeutherians, all four types are apparent, with no clear phylogenetic groupings of uterine types [135–139]. Among higher primates, including humans, the paired uterine progenitors have fused along their entire length into a single (simplex) uterus (Fig. 4) [1, 30, 124].

The functional morphology of these diverse uterine types is often considered to be linked to reproductive strategy. Eutherian mammals that have litters of multiple offspring, such as carnivores, have bicornate uteri with uterine horns to facilitate the development of many embryos. Higher primates, by contrast, have a simplex uterus that is adapted to the development of one or two embryos of relatively large size (due in part to advanced encephalization in utero). However, this is far from a universal trend. In fact, a reappraisal of the literature does not strongly support the notion that litter size correlates with the type of uterus. Cetaceans, elephants and aardvarks (Tubulidentata) have single offspring but bicornate uteri, while armadillos have up to 12 offspring and a simplex uterus (though armadillos have polyembryonic clones in which a single blastocyst divides into multiple embryos). Meanwhile, the panda
Figure 4. Diversity of adult female reproductive tracts across the mammalian phylogeny. Prototherians (monotremes) have separate uteri and lack a vagina, the likely ancestral state. Metatherians (marsupials) have two separate uteri and cervixes together with two lateral vaginas and a medial vagina (which can be transient, as in phalangers), and a vagina is present. Among eutherians (“placentals”) diverse uterine structures are evident, reflecting different degrees of caudal embryonic duct fusion, from duplex to bipartite, bicornate and simplex uteri. Phylogeny based upon integrated current molecular genetic data available on TimeTree (http://www.timetree.org/). Figure partly prepared using BioRender.com.

bear, sea otter and pinnipeds (seals, sea lions) have tubular bicornate or bipartite uterus but only one or two embryos [126]. Chiropterans (bats) are an interesting case. Among bats, most species have a bicornate uterus, but some have a simplex structure [140, 141]. Bat neonates are relatively large relative to maternal body weight. Overall, duplex uteri in eutherians are associated with multiple
smaller embryos while the trend towards the simplex uterus is associated with one or two larger embryos (either atrial or preccordial). However, the adaptive significance of the different mammalian uterine types remains to be fully understood.

In humans and mouse models, patterning and differentiation of the female reproductive tract at the molecular level has been correlated with nested expression domains of posterior Hox genes (Fig. 5). In both species, Hoxa genes are differentially expressed along the length of the Mullerian duct, induced, at least in part, by graded retinoic acid signalling [69, 142]. As in other tissues along the A-P axis, these genes show spatial expression domains along the Mullerian duct in postnatal mouse that are collinear with their chromosomal organisation [73, 143]. In mouse, Hoxa9, Hoxa10, Hoxa11 and Hoxa13/Hoxd13 exhibit embryonic expression domains that demarcate the future oviduct, uterus, cervix, vagina and vasa, respectively (Fig. 5) [70, 73]. Loss of function mutations in these genes can cause homeotic transformations of the female mouse reproductive tract [68, 69, 73, 143]. Segmental differentiation of the uterus requires Hoxa10 and Hoxa11. In mouse, mutations in Hoxa10 can cause partial transformation of the uterus into oviduct [74]. Disruption of Hoxa11 in mouse embryos also causes partial homeotic transformation and affects radial uterine patterning [144]. (Hoxa12 is lost in mammals). Interestingly, while these Hox genes show the spatial collinearity along the duct, they do not exhibit temporal collinearity, at least in the postnatal mouse. They are all expressed simultaneously [73]. It has been suggested that this may afford developmental plasticity as the Mullerian duct differentiates unusually late (after embryogenesis) compared to other organs [73].

Postnatally, WNT signalling also plays a role in patterning the uterus, at least in mice. Wnt7a and Wnt5a are expressed in the uterine horns of postnatal mice, in epithelium and mesenchyme, respectively [145]. Wnt expression in the female reproductive tract is conserved in the neonatal sheep [146]. In mouse, expression of these genes is highly regionalised. Wnt7a mutant mice show posteriorized female reproductive tracts. Postnatally, the posterior part of the oviduct appears uterine and the posterior part of uterus (normally simple columnar epithelium) appears vaginal (stratified squamous epithelium) [147]. Wnt5a mutant mice have shortened and coiled uterine horns [148]. The different uterine morphologies seen in different mammalian clades could be regulated by altered expression domains of Hox and/or Wnt genes, either at embryonic or postnatal stages, given that homeotic transformations or fusion defects can prevail when these factors are ablated in mice. Hoxa10, for example, which is implicated in uterine differentiation, may have temporally or spatially altered expression domains in the Mullerian ducts of diverse mammalian species that have duplex vs bicornate vs simplex uteri.

Among mammals, the vagina is a reproductive innovation not present in other vertebrates and that may have evolved through modified Hox or Wnt gene expression domains. In the mouse model, Hoxa13 and Hoxd13 are expressed in the posterior pole of the Mullerian duct and are required for differentiation of the vagina. Compound Hoxa13 and Hoxd13 mutant mice fail to complete caudal duct fusion to form the vagina [68]. Humans with HOXA13 coding region mutations exhibit hand-foot-genital syndrome, featuring a similar duct fusion defect to the mouse Hoxa13/Hoxd13 model [149, 150]. As monotreme mammals lack a vagina, while marsupials have three (Fig. 4), it would be of interest to examine Mullerian ducts (or postnatal ducts) of these animals in the context of embryonic Hoxa13 and Hoxd13 expression. In terms of WNT expression, Wnt5a mouse mutants lack clear cervical and vaginal structures [148]. Plasticity in WNT signalling may be another mechanism that has driven evolution of the vagina among the therian clade of mammals. Evolution of cis-regulatory elements controlling Hox and Wnt gene expression may be drivers of reproductive tract diversity. However, it has been shown that structural changes to the coding sequences of Hox genes themselves may have been important for female reproductive tract evolution. There has been strong directional selection of Hoxa11 and Hoxa13 in the therian lineages, which are linked to cervix and vagina development [31, 151]. It is posited that novel or expanded functions of these transcription factors has facilitated new protein–protein interactions and cellular functions that have been important for the evolution of the vagina, uterus, implantation and in utero embryonic development. More broadly, it would be informative to examine Hox and Wnt expression (and indeed global gene expression) at the time of caudal Mullerian duct fusion in accessible model species that have different uterine structures. This would be feasible in bats, which have diverse uterine structures and where embryonic development has been described in some species [141, 152].

Figure 5. Expression patterns of 5' Hoxa and Hoxd genes in the mouse Mullerian duct and the relationship to adult female reproductive tracts in mouse and human.
Most recently, unbiased global gene expression studies have been applied to the question of female reproductive tract evolution, focussing on the transition from egg laying to live birth in mammals. Lynch and colleagues used high throughput RNA-seq to characterise the transcriptome of the (adult) uterine endometrium coincident with the emergence of pregnancy. They detected thousands of genes recruited to, or lost from, the uterus during the evolution of eutherian pregnancy. Using ChIP-seq and related methods, they found that changes to cis-regulatory regions mediated via transposable elements have played a major role during uterine evolution [153]. It would be worthwhile to apply such a detailed comparative analysis to late stage vertebrate embryos during the segmental differentiation of the female reproductive tract across mammalian embryos from the major clades.

Regional differentiation of the Mullerian duct among vertebrates beyond mammals may also involve plasticity of the HOX code and/or WNT signalling outlined above. There is currently no information on the role of HOX genes or indeed other master developmental regulators in regionalised differentiation of the female reproductive tract among non-mammals. The 5′ HOXa genes are expressed in embryonic chicken Mullerian ducts [57], but their role in avian duct differentiation is unknown. Regional differentiation of the reptilian and bird female reproductive tracts (magnum, isthmus, shell gland) may be regulated by graded HOX signals as in mammals. Some previous studies have described the transcriptional landscape of the mature chicken oviduct, comparing magnum, and “uterus” or shell gland, with emphasis on genes expressed for egg production [154, 155]. However, how these compartments are genetically specified during embryogenesis remains unknown. In an evo-devo context, it would be of interest to examine the evolutionary conservation of the HOX code in the Mullerian duct including among cartilaginous fishes (sharks), where the ducts form in a fundamentally different way to those of tetrapods. While the diversity of female reproductive tract development may be underpinned by alterations in the Mullerian duct, this is at present an unexplored area of comparative reproductive anatomy.

Conclusion and outlook

Morphogenesis of the female reproductive tract has been fundamental to vertebrate development and evolution. The evolution of jawed vertebrates from agnathan ancestors has involved the development of a dedicated pair of ducts for transporting the oocyte, fertilisation, egg development and, in many cases, directly supporting embryogenesis. Developmentally, the female reproductive tract derives from paired Mullerian ducts, which appear to have arisen via co-option of gene regulatory pathways pre-existing in the pronephric (Wolfian) duct and mesonephric kidney. Over evolution, the female reproductive tract has exhibited remarkable diversification, becoming adapted to different reproductive modes. How this plasticity is genetically regulated is still largely unknown, but is ripe for detailed molecular studies. Some insights into duct evolution could actually come from genetic analysis of human females with atypical reproductive tract development. Approximately 4% of women have irregular uterine anatomy, such as didelphys (paired) or bicornate uterus, in which the caudal parts of the duct fail to fuse [156]. Genes found to be mutated in these cases would be candidate regulators of uterus evolution. In animal diverse animal models, forward and reverse genetics screens will also enhance our understanding of Mullerian duct differentiation during embryogenesis. Methods such as histone Chromatin immunoprecipitation and ATAC-seq can be combined with RNA-seq studies of whole transcriptome profiling to provide a more complete view of the gene regulatory pathways governing differentiation of the duct across different vertebrate clades [157].

Of particular value will be the application of RNA-seq and single cell RNA-seq to species beyond mammals. RNA-seq will reveal a great deal about the conserved and divergent mRNA expression patterns in the Mullerian duct during its development across animal groups. Single cell RNA-seq will also inform our understanding of cell lineage specification during duct formation across groups and whether the cell biology is conserved of divergent. Currently, single cell technology has not been applied to the Mullerian duct (not even in mouse). Most recently, fluorescent mouse reporter lines have been used to define regional segmentation of the mouse oviduct at the molecular level [158], while single cell sequencing has been conducted on the neonatal mouse uterus [159]. However, detailed single cell RNA-seq analysis during Mullerian duct morphogenesis is completely lacking. All of these approaches will yield new information regarding the evo-devo of female duct development. The female reproductive tract provides a fascinating biological model for exploring interesting questions relating to reproduction, development and evo-devo. Understanding how the female reproductive tract develops in different vertebrate groups, and the developmental mechanisms involved, will shed light on the genetics that underpin a major step in evolution.

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References


