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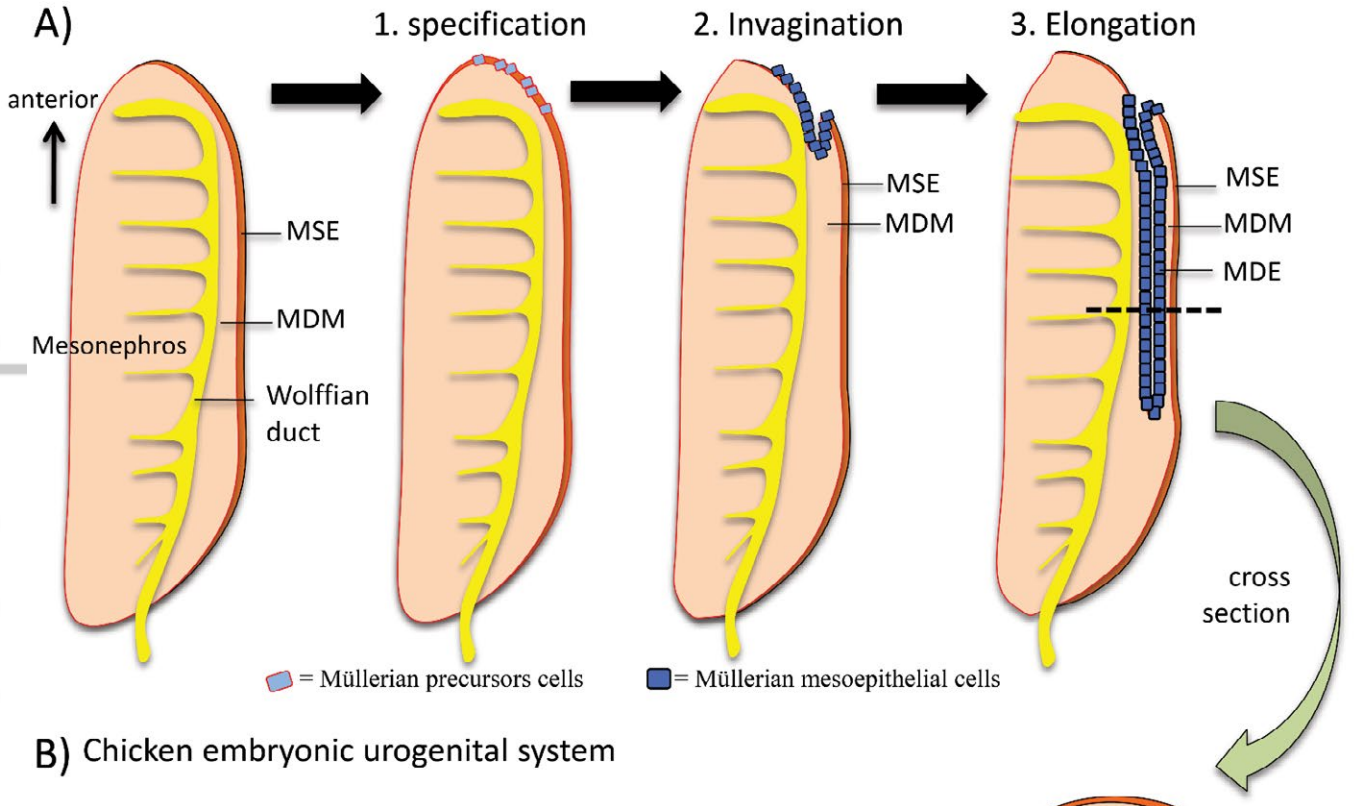
Abstract

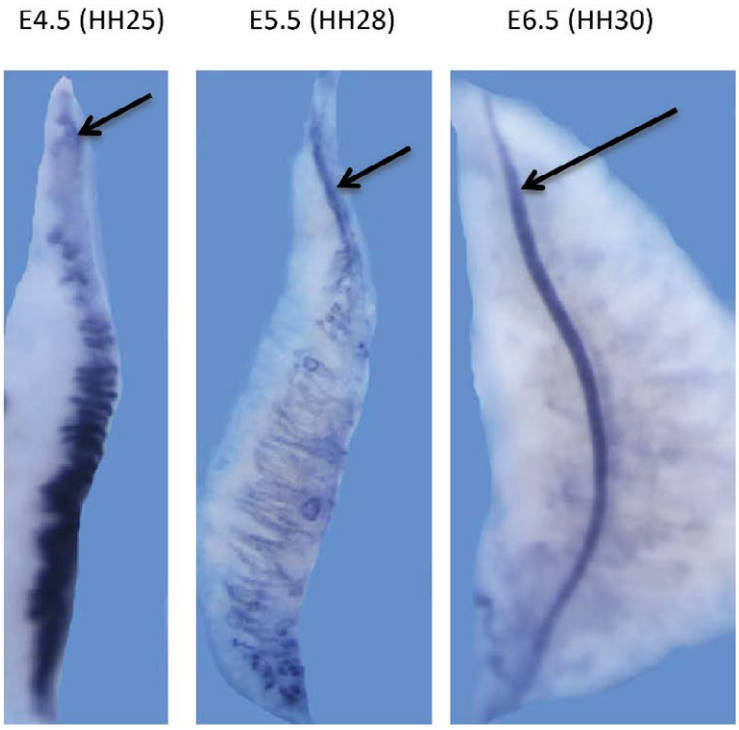
The Müllerian ducts are part of the embryonic urogenital system. They give rise to mature structures that serve a critical function in the transport and development of the oocyte and/or embryo. In most vertebrates, both sexes initially develop Müllerian ducts during embryogenesis, but they regress in males under the influence of testis-derived Anti-Müllerian Hormone (AMH). A number of regulatory factors have been shown to be essential for proper duct development, including Bmp and Wnt signalling molecules, together with homeodomain transcription factors such as PAX2 and LIM1. Later in development, the fate of the ducts diverges between males and females and is regulated by AMH and Wnt signalling molecules (duct regression in males) and Hox genes (duct patterning in females). Most of the genes and molecular pathways known to be involved in Müllerian duct development have been elucidated through animal models, namely, the mouse and chicken. In addition, genetic analysis of humans with reproductive tract disorders has further defined molecular mechanisms of duct formation and differentiation. However, despite our current understanding of Müllerian duct development, some questions remain to be answered at the molecular genetic level.

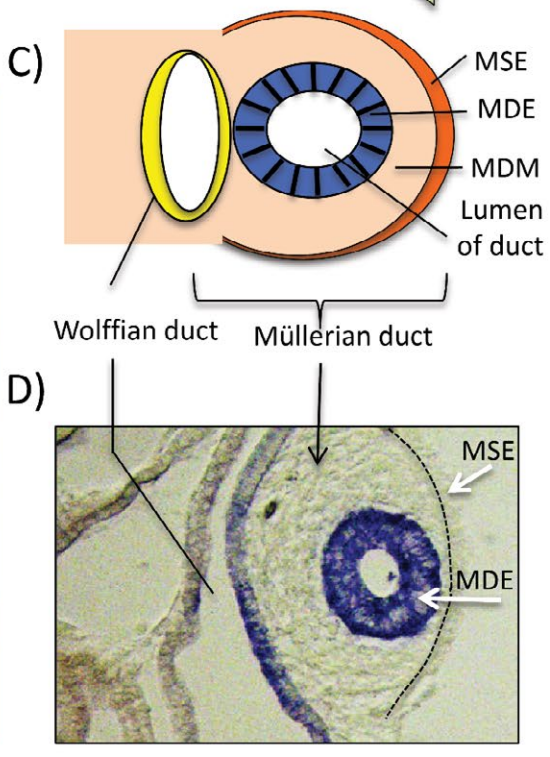
Key words: Müllerian duct; AMH; chicken embryo; Wnt4; PAX2; Lim1; Wnt9b; Wnt7a

Graphical/Visual Abstract and Caption: Morphogenesis of the Müllerian duct



B) Chicken embryonic urogenital system





INTRODUCTION

In 1830, German physiologist Johannes Peter Müller described the paired ducts running beside the embryonic kidneys that develop into the fallopian tubes, uterus and upper part of the vagina. At that time, it was recognised that these ducts played a role in female reproduction, and they became known as the Müllerian ducts. These embryonic tubes are indeed essential structures of the female reproductive system (Minami et al., 1993). When differentiated, they are the site of fertilization in many vertebrates, they transport and nourish the egg and/or facilitate development of the embryo (Pulkkinen, 1995). The Müllerian (paramesonephric) ducts develop from mesoderm in close association with the Wolffian (mesonephric) ducts. Both sets of ducts develop on the surface of the mesonephric kidneys (Fig. 1) and indeed the Wolffian duct (WD) is required for proper Müllerian duct (MD) development (Ayers et al., 2015; Gruenwald, 1941; Kobayashi et al., 2003; Orvis et al., 2007). In human males, the WD differentiates into the epididymis, vas deferens and seminal vesicle, mediated by testis-derived androgens (Fig. 1), while it regresses in females (George et al., 1994). Testis-derived Anti-Müllerian Hormone (AMH) directs regression of the Müllerian ducts in males (Behringer et al., 1994; Jamin et al., 2003; Josso, Cate, et al., 1993; Josso et al., 2001). Lack of both testosterone and AMH in female embryos allows regression of the Wolffian ducts and differentiation of the Müllerian ducts into the female reproductive tract: fallopian tubes, uterus, cervix and upper third of the vagina (Fig. 1). Key genes involved in Müllerian duct formation have been identified through the analysis of animal models (mouse and chicken) and humans with dysfunctional reproductive organs (Ayers et al., 2015; Kobayashi et al., 2003; Orvis et al., 2007).

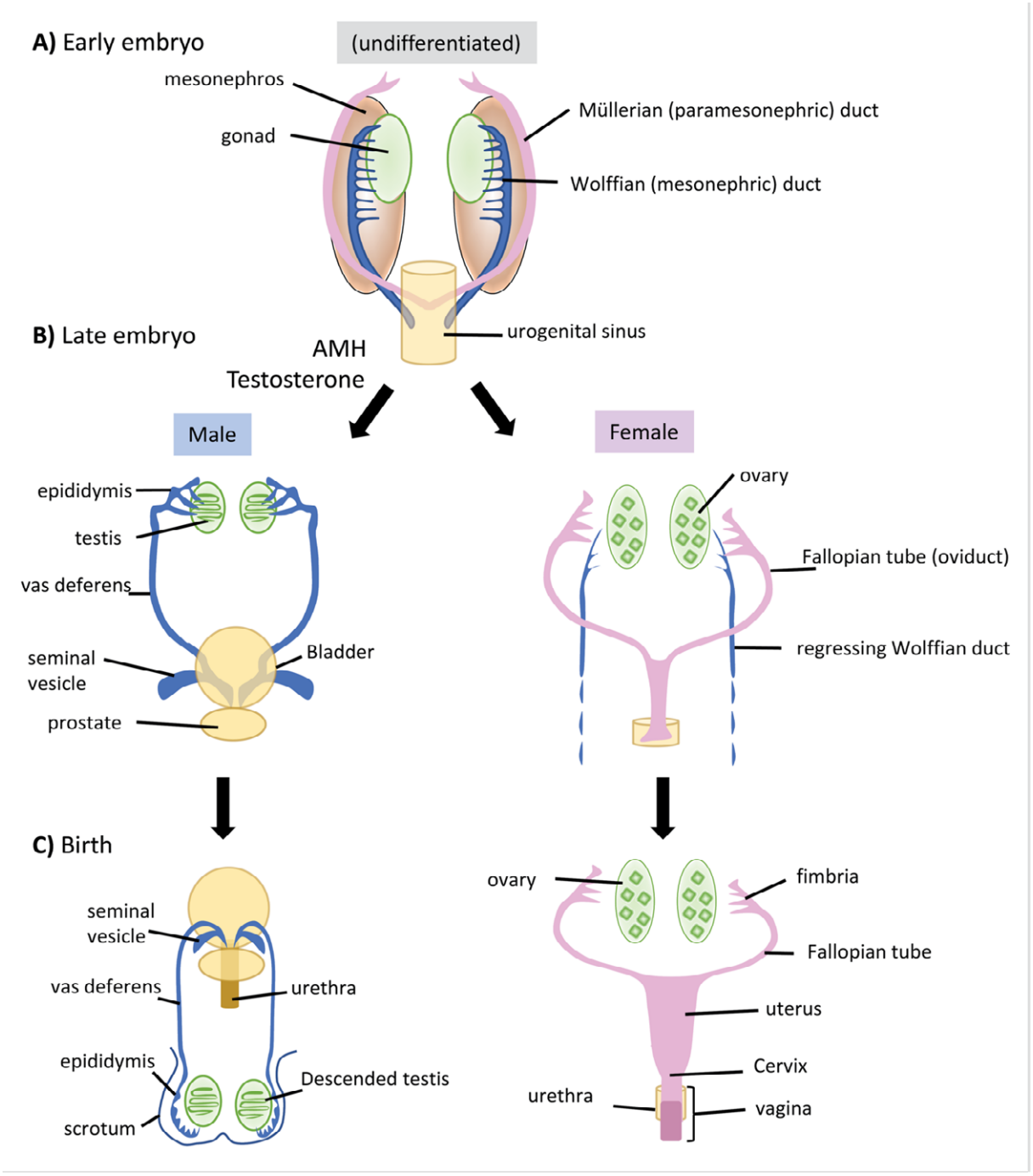


Figure 1: Schematic view of urogenital system development in humans. (**A**) At the undifferentiated phase, the gonads develop on the ventromedial surface of the mesonephric kidneys. Two pairs of ducts form, the Wolffian (mesonephric) ducts and the Müllerian (paramesonephric) ducts. (**B**) Later in development, the action of AMH in males leads to Müllerian duct regression, while testosterone stimulates differentiation of the Wolffian ducts into male structures (vas deferens, epididymis and seminal vesicles). In the female, lack of these hormones leads to Müllerian duct differentiation into female structures (fallopian tubes) and regression of the Wolffian ducts. The mesonephros regresses in both sexes. (**C**) By birth, the paired testes and associated male ducts descend into the scrotum. In females, the fallopian tubes fuse caudally, giving rise to the uterus and upper portion of the vagina. (Bladder omitted in the female for clarity).

EMRYONIC DEVELOPMENT OF THE MÜLLERIAN DUCTS

The paired Müllerian ducts are of mesodermal origin and develop in an anterior to posterior direction within the dorsolateral edge of the mesonephric kidneys. Three phases of duct formation are recognised; specification, invagination and elongation (Atsuta et al., 2016; Guioli et al., 2007; Kobayashi et al., 2003; Mullen et al., 2014; Orvis et al., 2007; Song et al., 2011) (Fig. 2A). These stages are exemplified in the chicken embryo (Fig. 2B). Specification occurs at the cranial pole of the mesonephros, adjacent to the Wolffian duct. A number of cells within a specific region of the coelomic epithelium overlying the mesonephros (the Müllerian Surface Epithelia or MSE), are specified as Müllerian duct (MD) precursors. This region is a placode-like thickening that has been called the Müllerian ridge by some authors (Jacob et al., 1999). The precursor cells express the marker, Lim1 (Lhx1), a homeodomain transcription factor (Fig. 2B). The MSE cells then invaginate, and proliferate caudally, moving through mesenchyme that exists between the coelomic epithelium and Wolffian duct (Müllerian duct mesenchyme, MDM) (Fig. 2A). The invaginating cells then come into physical contract with the Wolffian duct, and begin to form a canalised tube, the Müllerian Duct Epithelium (MDE) (Guioli et al., 2007; Mullen et al., 2014). In the final phase of development, the MDE cells proliferate and continue to migrate in a cranio-caudal direction, elongating and expanding the tube alongside the Wolffian duct until it ultimately fuses with the urogenital sinus (Fig. 2A and 2B). The developing duct hence comprises three tissues in cross section; the outer Müllerian Surface epithelium (MSE), underlying Müllerian Duct Mesenchyme (MDM) and the inner Müllerian Duct Epithelium (MDE), marked by *Lim1* expression (Fig. 2C and 2D). Studies employing *Lim1-LacZ* reporter mouse embryos indicate that specification and invagination occur from embryonic day (E) 11.5-11.75 and elongation

is complete at E13.5 (about 48 hours) (Orvis et al., 2007). Ultrastructural, histochemical and cell labelling studies have shown that essentially the same process occurs in the chicken embryo over three days; E3.5 (Hamilton and Hamburger (HH) stage 20/21) through E6.5-6.7 (HH30) (Atsuta et al., 2016; Guioli et al., 2007; Hamburger et al., 1951; Jacob et al., 1999) (Fig. 2B). The elongating ducts migrate through an extracellular matrix rich in laminin and entactin (Jacob et al., 1999). After the duct has formed, it regresses in males, but in females it differentiates into the components of the reproductive tract during later stages of development.

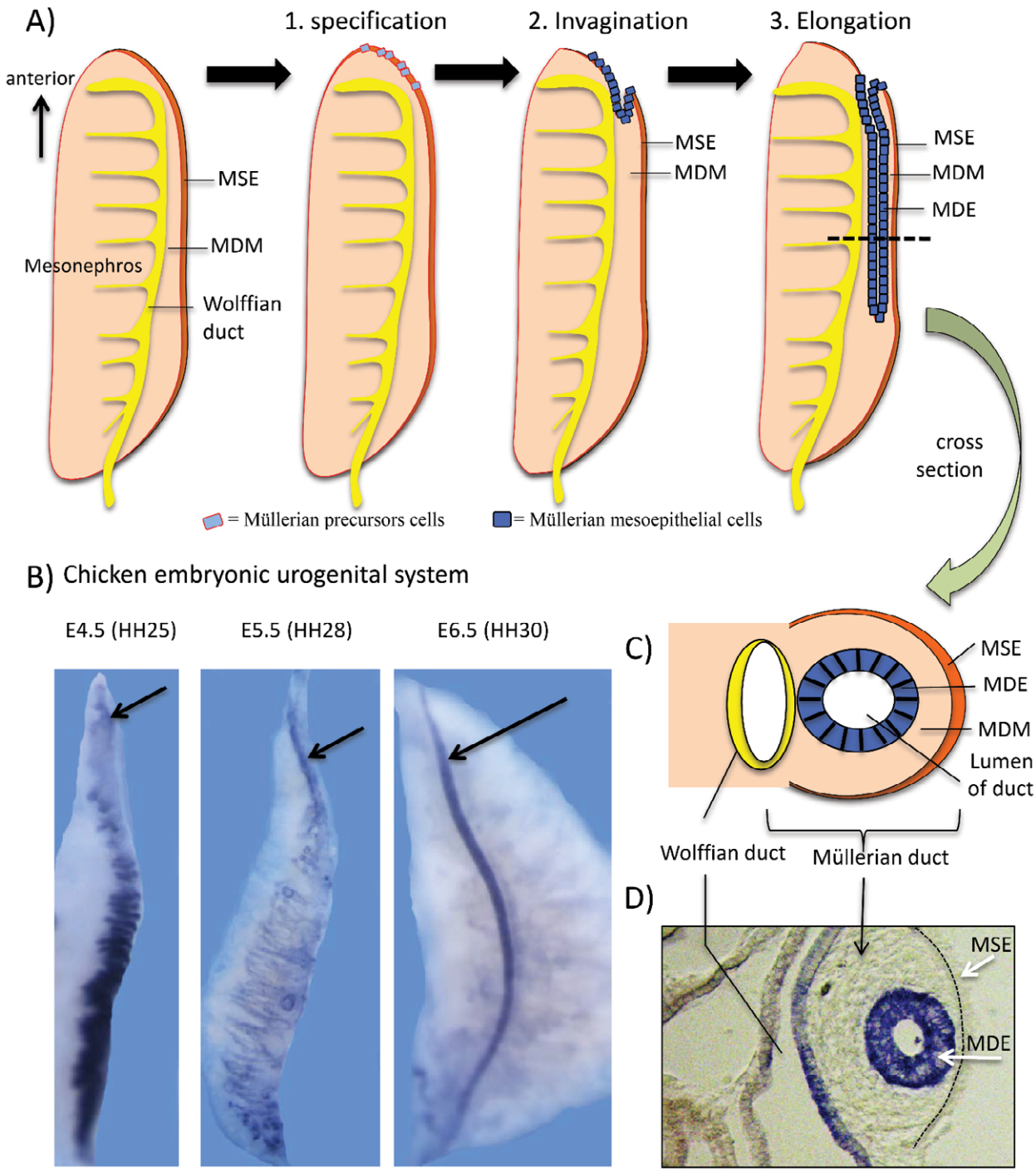


Figure 2: The widely accepted three phase model of Müllerian duct development in vertebrate embryos, based on data from mouse and chicken. Only one side of the urogenital system is shown (dorsal view). (**A**) The Müllerian duct forms in a cranio-caudal direction. During phase 1 (specification), Müllerian precursor cells are specified in the surface epithelium (Müllerian surface epithelium, **MSE**) at the cranial pole of the mesonephros (embryonic kidney). During phase 2 (invagination), these cells become "mesoepithelial" and invaginate in a caudal direction, moving between mesenchyme (the Müllerian duct mesenchyme, **MDM**). During phase 3, invaginating cells form the Müllerian Duct Epithelium (**MDE**) make contract with the Wolffian duct and the duct elongates caudally, eventually fusing with the urogenital sinus. (**B**) Müllerian invagination and elongation in the chicken embryo, stained for expression of the duct marker, *Lim1*. (**C**) Schematic cross-section shows Wolffian and Müllerian ducts in the transverse plane. The Müllerian duct has three components, from the outer to inner surface: the MSE, the MDM and the MDE. (**D**) Cross section of *Lim1*-expressing chicken stage 28 Müllerian duct.

The cells of the MDE are of coelomic epithelial origin, having derived from the surface epithelium overlaying the cranial aspect of the mesonephros. These cells proliferate and invaginate through the underlying mesenchyme, forming a tube that grows caudally. Both cells at the tip and those more cranial undergo proliferation (Guioli et al., 2007). These cells appear to have characteristics of both epithelial and mesenchymal cells. In mammalian and avian embryos, the MDE cells express mesenchymal markers such as vimentin, but not epithelial markers, such as cytokeratin (Jacob et al., 1999; Magro et al., 1995; Orvis et al., 2007). However, the cells are arranged together on a basement membrane, typical of epithelia. It has been concluded that the Müllerian duct is not a true epithelial tube, but is "mesoepithelial" in nature. In the mouse, the MD becomes a true epithelial tube after birth (Orvis et al., 2007). In mouse and chicken embryos, the Müllerian duct elongates through cell proliferation and active migration of the tip of the caudally progressing MDE (Fujino et al., 2009; Guioli et al., 2007; Jacob et al., 1999; Orvis et al., 2007) (Fig. 2). The MDE cells at the caudal tip have a high proliferation index. Caudal growth of the duct occurs through proliferation and migration of MDE cells, with little apoptosis, and this does not require intact cells at the cranial pole of the duct (Fujino et al., 2009; Orvis et al., 2007). The use of inhibitors in organ culture has shown that caudal extension of the Müllerian duct in rat embryos involves the phosphatidylinositol 3-kinase signalling (PI3K/AKT) pathway (Fujino et al., 2009). This pathway is linked to cell growth, apoptosis and migration in other systems (Park et al., 2007), and is typically activated by growth factor tyrosine kinase receptors, or by basement membrane components. There may be a chemoattractant (morphogen) present along the cranio-caudal axis that promotes migration via a concentration gradient. WNT9B derived

from the neighbouring Wolffian duct is required for Müllerian duct elongation and could serve as this diffusible signal (see below) (Carroll et al., 2005), although it is not clear if this growth factor has a graded pattern of expression.

There is likely to be a role for the surface epithelium after establishment of the invaginating Müllerian luminal epithelium at the cranial pole. Many modern studies have not considered a role for the surface epithelium beyond the provision of cranial Müllerian precursors during the initiation and invagination phases of duct development. We have previously found that the sex-related transcription factor, *DMRT1*, is expressed in the MSE and in the underlying MDM in the early chicken embryo, and is indeed required for duct development (Ayers et al., 2015). The expression pattern of *DMRT1* extends in a restricted region along the entire craniocaudal length of the surface epithelium, both anteriorly and posteriorly, before the elongating duct has extended caudally. Expression is more diffuse caudally. This suggests that the surface epithelium is pre-patterned to play a role in duct morphogenesis along this region prior to duct elongation. Targeted knockdown of *DMRT1* expression along the length of this region in the chicken embryo results in truncation of duct development where *DMRT1* mRNA is lost, even just in posterior regions. This points to a role for the surface epithelium in duct elongation. Expression of *DMRT1* in these surface epithelial cells could regulate a specific field of cells that undergo an epithelial to mesenchymal transition (EMT), contributing mesenchyme to the developing duct. To investigate the origin of the MDM, researchers have labeled cells with GFP-expressing plasmids or MitoTracker dyes, and followed the fate of cells either in organ culture or *in ovo* (in living chicken embryos). In mouse and chicken, these studies indicate that the MSE cells can contribute to the MDM along the length of the mesonephros in the vicinity of the Wolffian duct. Hence, the expression of genes such as *DMRT1* along this same region may signal the MSE to undergo an EMT, generating the underlying mesenchyme (Guioli et al., 2007). Studies in rat embryos suggest that the caudally migrating MDE induces migration of MDM around the forming duct (Fujino et al., 2009). The MDM may also come from the mesonephros (Ayers et al., 2015).

Early anatomists noted the close association between the developing Müllerian duct and the neighbouring Wolffian duct (Dohr, 1984; Gruenwald, 1941). It has been shown through

physical or genetic ablation studies that an intact Wolffian duct is required for normal elongation of the developing Müllerian duct in chicken and rodent embryos (Bishop-Calame, 1966; Carroll et al., 2005; Chiga et al., 2014; Didier, 1971; Kobayashi et al., 2003). Some studies suggested that the Wolffian duct contributes cells to the developing Müllerian duct, but genetic and cell fate mapping studies have ruled this out (Guioli et al., 2007; Orvis et al., 2007). It is therefore most likely that the Wolffian duct secretes a local signalling molecule or morphogen that induces cell proliferation and/or caudal migration of the Müllerian duct. This factor (or one of these factors) is Wolffian duct-derived secreted factor, WNT9B. In *Wnt9b* null mouse embryos, duct specification and invagination still occur, and key genes such as *Pax2* and *Lim1* are still expressed, but the duct fails to elongate (Carroll et al., 2005). The POU homeodomain transcription factor, HNF1B, activates *Wnt9b* expression in the WD (Lokmane et al., 2010). Although the WD is thought not to be required for the earlier stages of Müllerian duct specification and invagination in mouse, it may be required for *LIM1* expression during MD specification in chicken (Atsuta et al., 2016; Orvis et al., 2007). Ablation of the WD in the early chicken embryo leads to loss of *LIM1* expression in MD precursor cells and failure of duct formation (Atsuta et al., 2016). Furthermore, while MD elongation requires the release of WNT9B from the WD, at least in mouse (Carroll et al., 2005), it remains unclear whether this is the primary extrinsic signal for cranial-caudal MD growth. Other factors could be involved, such as a graded chemoattractant (Fujino et al., 2009). It seems likely that a caudally-derived morphogen or a cranial repulsive factor must signal the MDE to elongate in that direction. The identity of this factor remains unknown.

GENETIC REGULATION OF MÜLLERIAN DUCT FORMATION

Much of our current understanding of Müllerian duct formation comes from studies on mouse and chicken embryos. The mouse is readily amenable to genetic analysis, while the *in ovo* development of the chicken embryo allows access and experimental manipulation of the developing ducts. These models have shown that morphogenesis of the Müllerian ducts is regulated by the coordinated action of transcription factors and signalling molecules. This process involves a close interaction between epithelial cells and underlying mesenchyme. (Fig. 2 and 3). Important players in duct formation include the homeodomain transcription

factors, EMX2, HOXA13, PAX2 and LIM1, and members of the Wnt family of secreted growth factors (namely, WNT4, WNT9B and WNT7A) (Bouchard et al., 2002; Kobayashi et al., 2003; Kobayashi et al., 2004; Prunskaite-Hyyrylainen et al., 2016; Torres et al., 1995) (Fig. 3). Although required for Müllerian duct elongation, the first phases of development (specification and invagination) are thought to be independent of the Wolffian duct (Carroll et al., 2005; Mullen et al., 2014). Targeted deletion in mouse show that the homeodomain transcription factors, *Pax2* and *Lim1*, are required for Müllerian duct formation (Bouchard et al., 2002; Huang et al., 2014; Kobayashi et al., 2004; Torres et al., 1995). Although these two genes are also expressed in the neighbouring Wolffian duct, *Lim1* and probably *Pax2* are required cell-autonomously in cranial coelomic epithelial cells, leading to specification of the Müllerian duct precursor cells (Kobayashi et al., 2004). Using the chicken model, Atsuta et al. (2016) recently demonstrated that the initiation phase of duct formation is regulated by sequential actions of Bmp/PAX2 and Fgf/LIM1 signalling (Fig. 3). These pathways are required for specification of the MSE (Atsuta et al., 2016). Firstly, Bmp signalling in the region of the cranial mesonephros initiates expression of PAX2 in the coelomic epithelial cells. The BMPs (BMP2/4/7) are thought to derive from the epithelial cells themselves, but the specific trigger is not known (Atsuta et al., 2016). The WD is also a robust source of BMP, but it is thought not to be required for this early phase of MD development (at least in mouse). The PAX2 positive cells are considered to be pre-specified Müllerian precursors.

Studies in the chicken embryo indicate that PAX2 works with BMP to activate the homeodomain transcription factor, LIM1, in MD precursor cells, marking their specification (Fig. 3) (Atsuta et al., 2016). *Lim1* is essential for MD specification and hence development. In the mouse, targeted deletion of *Liml* leads to a complete loss of MD-derived structures in females (Kobayashi et al., 2004). In the chicken, ablation of the WD prevents *LIM1* expression and invagination, while *PAX2* is still expressed. This would mean that the WD is required for early specification of the duct in chicken, but perhaps not in mouse. Fibroblast growth factor signalling in the MSE, triggered by PAX2 and mediated by FGFR2, has been shown to initiate LIM1 expression in the chicken (Atsuta et al., 2016) (Fig. 3). The Müllerian duct precursor cells are considered specified when they become LIM1-positive. Invagination

of the MSE gives rise to the MDE, during which the specified cells undergo a significant change in morphology, with polarisation of actin-myosin filaments, apical constriction of cells and breakdown of the basement membrane. This process involves Fgf signalling though Ras/ERK and probably also involves RHOA signal transduction (Atsuta et al., 2016). Invagination also requires WNT4 signalling (Fig. 3). WNT4 is produced and released from the MDM cells, which lie between the Wolffian duct and MSE. These mesenchymal cells likely derive from the MSE through an epithelial to mesenchyme transition (EMT), and/or directly from the mesonephric region. Targeted deletion of *Wnt4* in mouse does not affect specification but blocks proper invagination and hence mice lack MD-derivatives (Prunskaite-Hyyrylainen et al., 2016; Vainio et al., 1999).

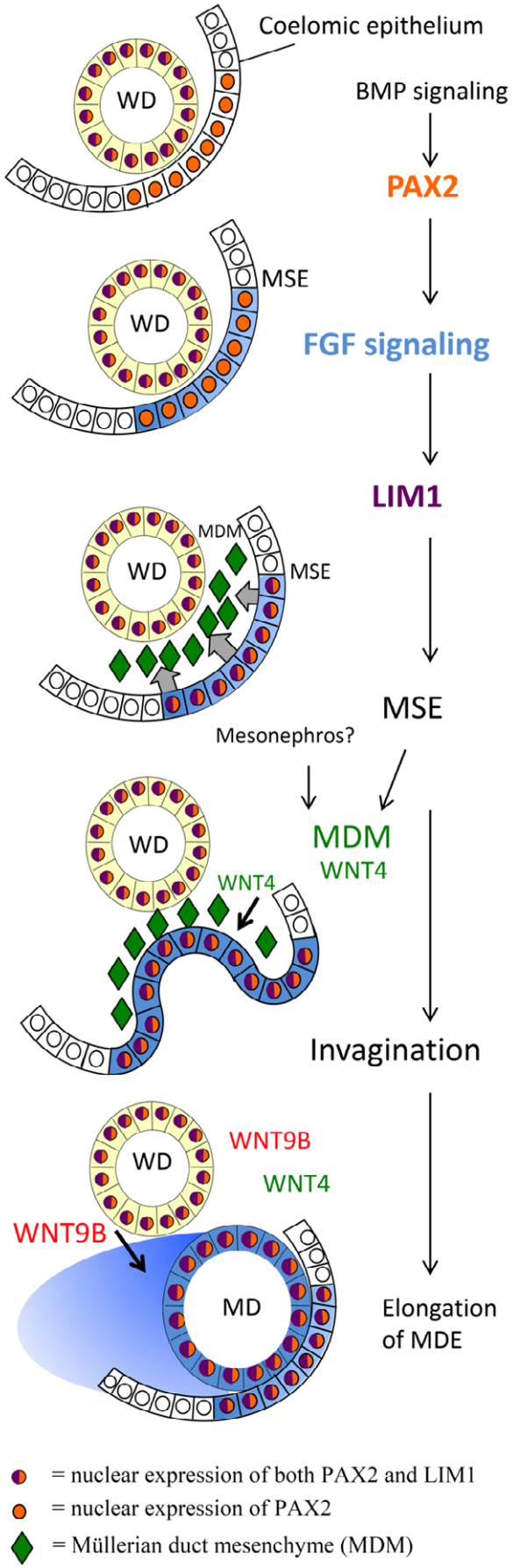


Figure 3: Genetic regulation of Müllerian duct (MD) specification, invagination and elongation, based on data from chicken and mouse embryos. Re-drawn and modified from Atsuta et al. (2016) with permissions from The Company of Biologists Ltd. Bmp signalling induces PAX2 expression in the cranial coelomic epithelium (orange) adjacent to the Wolffian duct (**WD**). BMP/PAX2 then activate Fgf signalling (blue) in the Müllerian surface epithelium (**MSE**). This then triggers LIM1 expression in the MSE (purple). Cells from the MSE (and possibly the mesonephros) give rise to the underlying Müllerian duct mesenchyme (**MDM**) which requires WNT4 expression (green). PAX2/LIM1-positive MSE cells undergo invagination to from the Müllerian duct epithelium (**MDE**). WNT9B derived from the WD is required for duct elongation (red).

In the chicken embryo, WNT4 also is expressed in the MDM, and hence this compartment is essential for normal duct development (Ayers et al., 2015). The effects of Wnt molecules on duct development are likely to operate via the canonical ² -catenin signalling pathway, as targeted deletion of the beta-catenin gene (*Ctnnb1*) in the MDM causes a failure of proper duct morphogenesis (Deutscher et al., 2007). The Wnt receptor, *Frizzled*, is expressed in the MDM and MDE (Deutscher et al., 2007). Recent fate mapping and transgenic mouse studies have shown that WNT4 is also responsible for coordinating the migration of MDE cells during duct elongation. Indeed, in mouse, the leading MDE tip cells appear to derive from *Wnt4*-expressing cells (Prunskaite-Hyyrylainen et al., 2016). Downstream of *Wnt4*, other Wnt molecules also play a role in MD elongation. These include WNT7a, a marker of elongation; *Wnt7a*$^{-/-}$ mice have abnormal MD-derivatives (Miller et al., 1998). Similarly, *Wnt5a* plays a role (Mericskay et al., 2004). Other genes implicated in the formation and/or maintenance of the embryonic Müllerian ducts are listed in Table 1. In addition to the pervasive role of Wnt signalling, retinoic acid (RA), for example, also plays a role in MD development and differentiation. Double mutant mice lacking both retinoic acid receptors ± and ² 2 lack MDs (Kastner et al., 1997; Mendelsohn et al., 1994). However, the exact role for RA signalling during duct formation is unclear.

FATE OF THE MÜLLERIAN DUCTS IN MALES AND FEMALES

Sex-specific interactions between the MDE and the MDM determine the fate of the Müllerian ducts. Regression of the ducts occurs in males. In the mouse embryo, the critical period for regression is E13-E14, while the first histological signs are evident from E14.5 (duct thinning) to E15.5 (apparently random regression along the length of the duct) (Mullen et al., 2014). In the chicken embryo, the right duct also regresses in females, concomitant with the regressing right gonad (Hutson et al., 1985). Müllerian duct regression has been reasonably well-characterised genetically. Male-specific regression is driven by Anti-Müllerian Hormone (AMH), a member of the TGFB growth factor family (Josso, 1991; Josso, Lamarre, et al., 1993). *Amh*$^{-/-}$ male mice retain MD-derived structures, while female mice transgenically mis-expressing AMH lack MD-derived structures (Behringer et al., 1990; Behringer et al., 1994; Mishina et al., 1999). Mutations in *AMH* cause Persistent Müllerian

Duct Syndrome (PMDS) in humans (see Table 1) (Belville et al., 1999). During embryogenesis, AMH binds to its type II and I receptors in the MDM, leading to signal transduction via SMAD phosphorylation. *Amhr2* is expressed in the MDM, together with type I receptors. In mouse, the type II receptor (AMHR2) is specific to AMH, while there is some functional redundancy among the type I receptors and intracellular Smad proteins used by AMH (ALK2/3 and SMADS1, 5 and 8) (Orvis et al., 2008; Visser et al., 2001) (Fig. 4). AMH can use the ALK2 type I receptor. However, conditional deletion of *Alk2* does not block duct regression, while targeted deletion of mouse of *Alk3* (*Bmpr1a*) results in 50% of mutants failing to show duct regression, similar to *Amh* null mutants (Clarke et al., 2001; Gouedard et al., 2000; Jamin et al., 2002).

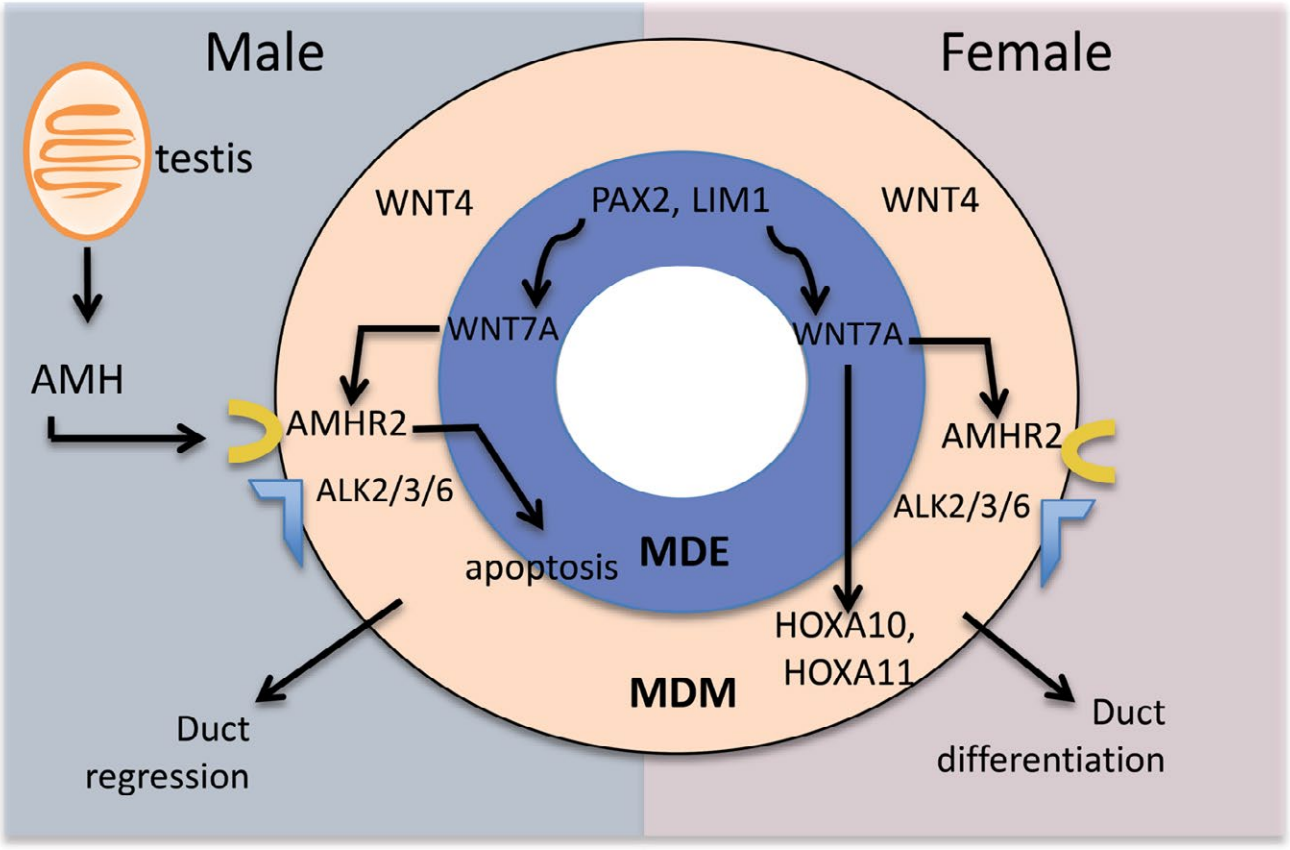


Figure 4: Fate of the Müllerian duct in males and females. In both sexes, PAX2 and LIM1 are expressed during duct specification and elongation. These genes directly or indirectly regulate the expression of WNT7A in the Müllerian duct epithelium (**MDE**). WNT7A can activate expression of AMHR2 (AMH type II receptor) in the Müllerian duct mesenchyme (**MDM**). WNT4 is also expressed in the MDM. The AMH type I receptors (ALK2/3/6) also play a role in AMH signalling. Only in males, AMH secreted by the developing testis binds to AMHR2 to recruit type I receptors, and initiate intracellular Smad signalling, leading to activation of metalloproteases, apoptosis and duct regression. In females, lack of foetal AMH allows further duct development rather than regression. WNT7A can activate Hox genes involved in duct differentiation.

Binding of AMH to its receptors induces a change in MDM morphology and apoptosis of the MDE in males, probably via release of matrix metalloproteinase, among others (Fig. 4) (Roberts et al., 2002). The process of duct regression also involves a thickening of the MSE, breakdown of the basement membrane, an EMT, and invasion of cells around the MDE (Klattig & Englert, 2007; Kobayashi et al., 2003; Roberts et al., 2002). As is the case for duct formation, there is also a role for Wnt signalling in Müllerian duct regression. *Wnt7a* is required for this process; *Wnt7a* null mice do not exhibit MD regression (Parr et al., 1998). This Wnt molecule is expressed in the MDE and is linked to activation of *Amhr2* in the

adjacent MDM (Fig. 4). The transcription factor WT1 (Wilm's tumour 1) is also a key regulator of *Amhr2* expression (Klattig, Sierig, et al., 2007). It has been shown that mis-regulation of ² -catenin signal transduction in the MDM (independently of AMH expression) can prevent MD regression, pointing to a key role for canonical Wnt signalling in duct regression in males (Tanwar et al., 2010). In the chicken model, regression of the MDs in male embryos involves the expression of two homeobox genes, *MSX1* and *MSX2*, although a functional role for these factors has yet to be established (Ha et al., 2008).

In the chicken embryo, both sexes express AMH, although always more highly in males (Cutting et al., 2014; Smith et al., 2007). This is consistent with the fact that the right MD regresses in most female birds, coincident with regression of the right gonad (Guioli et al., 2014; Vaillant et al., 2001). The left duct of female chicken embryos is likely to be protected from AMH by local estrogens (Doi et al., 1988; Hutson et al., 1982; Song et al., 2011). Estrogen Receptor-± (ESR1) is expressed in the avian MD (Andrews et al., 1997) and application of exogenous estrogen to chicken embryos during duct formation can block regression in both sexes (Cutting et al., 2014; Hutson et al., 1985; Hutson et al., 1982; Mattsson et al., 2011; Stoll et al., 1990). Under natural conditions, estrogen stimulates development and function of the avian Müllerian ducts, regulating formation of tubular glands and differentiation of the oviductal epithelium into ciliated and goblet cells (Dougherty et al., 2005; Oka et al., 1969a, 1969b; Palmiter et al., 1971). In reptiles, the MD develops in both sexes and then regresses in males, as in other vertebrates (Wibbels et al., 1999). MD development is also estrogen sensitive in reptiles (Austin, 1989; Dodd et al., 2008). Interestingly, the effects of estrogen on embryonic duct development are stage dependent in birds and reptiles. Hypertrophy can be induced if exogenous estrogen is applied after the time of duct differentiation, but the effects can be inhibitory if applied earlier, causing disrupted caudal elongation (Dodd et al., 2008; Stoll et al., 1993a). These effects are mediated by ESR1, but not Estrogen Receptor-² (ESR2) (Mattsson et al., 2011). Similarly, duct development can also be perturbed in mammalian embryos by exposure to exogenous estrogens (Block et al., 2000). However, this effect in mammals does not inhibit duct elongation, but appears to involve the induction of morphological abnormalities due to disturbed Hox gene expression

(Block et al., 2000). Hox genes are important for regional differentiation of the MDs in female mammals (see below). The expression of Hox genes in the developing MDs has not been reported in non-mammals.

In female mammals, the MDs differentiate into fallopian tubes, uterus, cervix and the upper portion of the vagina (Fig. 1). At embryonic stages, the MDE, previously of mesoepithelial nature, beings to express markers of true epithelia, such as cadherin 1 (CDH1) (Orvis et al., 2007). The fate of the ducts in both males and females requires interactions between the MDE and MDM (Fig. 4). Duct patterning in the female involves Wnt and Hox gene expression. This again involves *Wnt7a: Wnt7a*$^{-/-}$ female mice have abnormal female reproductive tracts, characterised by a small and thin uterus lacking uterine glands (Miller et al., 1998; Parr et al., 1998). One of the functions of WNT7A during female duct development is the maintenance of *Hoxa10* and *Hoxa11* expression, which are needed for proper duct development in females (probably redundantly) (reviewed in (Kobayashi et al., 2003)). Indeed, a subset of the Hoxa gene cluster (*Hoxa9*, *Hoxa10*, *Hoxa11* and *Hoxa13*) is required for the correct patterning and differentiation of the female MD (Fig. 5). In the mouse and human, differentiation of the duct into fallopian tube, uterus, cervix and vagina is regulated by segmental expression along the MD of *Hoxa9*, *Hoxa10*, *Hoxa11* and *Hoxa13* (Fig. 5) (Lynch et al., 2004; Taylor et al., 1997). Loss-of-function mutations in these genes can cause homeotic transformations within the developing female MD. For example, *Hoxa10* null mice develop fallopian tube morphology instead of a uterus in a proportion of the developing female reproductive tract (Benson et al., 1996). It has recently been shown that a cranial-caudal gradient of retinoic acid production along the prenatal mouse duct directs differentiation of the duct epithelium into uterus versus vagina via a mechanism that involves indirect activation of *Hoxa10* (Nakajima et al., 2016). Sex steroid hormones also play a role in modulating Hox gene expression in the female (L. Ma et al., 1998). Mammalian foetuses exposed to the non-steroidal estrogen analogue, DES (diethylstilbestrol) have partial homeotic transformations of the MD (Block et al., 2000; Cermik et al., 2003), with altered Hox gene expression (Cermik et al., 2003). In the chicken embryo, estrogen plays a key role in triggering cell proliferation and differentiation of the MD in females, and it likely protects the

left MD from AMH-induced regression. Later in development, *Wnt4* and *Wnt5a* are required for further duct differentiation, at least in mammals. Conditional deletion of *Wnt4* in mice causes abnormalities in uterine morphology and function, while *Wnt5a* is needed for caudal differentiation of the MD, epithelial-mesenchyme interactions and also Hox regulation (Du et al., 2015; Mericskay et al., 2004; Mullen et al., 2014). Wnt signalling in the female must be tightly regulated. Constitutive activation of ² -catenin signal transduction in the mouse MDM results in loss of Fallopian tubes, smaller uteri, endometrial gland defects and infertility (Stewart et al., 2013).

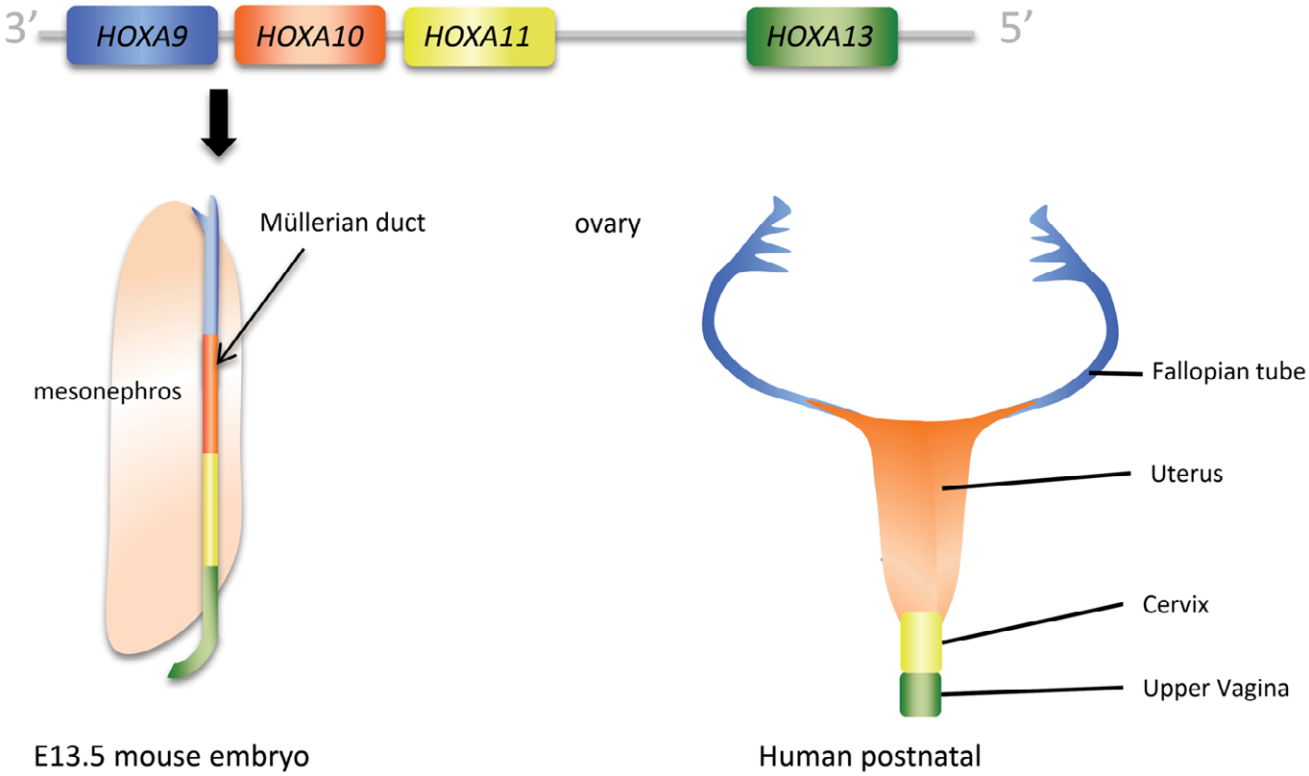


Figure 5: *HOX* gene expression domains and patterning of the mammalian female reproductive tract. A subset of the *HOXA* cluster is expressed in a linear series along the length of the embryonic Müllerian duct (mouse studies). These expression domains correlate with subsequent regional differentiation of the ducts into fallopian tubes, fused uterus, cervix and vagina. Modified from Lynch et al. (2004) with permissions from The Royal Society. Additional data from Du et al. (2004).

DISRUPTIONS OF MÜLLERIAN DUCT FORMATION IN HUMANS

Morphogenesis of the Müllerian ducts can be impaired due to effects of teratogens, or, more commonly, genetic lesions (Reviewed in (Choussein et al., 2017)). Defects include

retention of duct derivatives in males (Persistent Müllerian duct Syndrome, PMDS; OMIM 261550) and dysgenesis or duct patterning defects in females (Table 1). In males, loss-of-function mutations in the *AMH* or *AMHR2* gene can explain most cases of PMDS, in which a uterus and fallopian tube(s) are present (Belville et al., 1999); reviewed in (Mullen et al., 2014). In females, mutations that affect MD specification of subsequent differentiation can lead to disorders of the female reproductive tract such as dysplastic or absent vagina, cervix and uterus. Mayer-Rokitansky-Kuster-Hauser (MRKH) syndrome (OMIM 277000) affects the female reproductive tract of approximately 1 in 4500 females (Londra et al., 2015). It is characterised by vaginal agenesis and uterine abnormalities that range from mild to severe (Griffin et al., 1976; Londra et al., 2015; Patnaik et al., 2015). Despite these anomalies being congenital, they are usually only discovered at puberty when the patient fails to begin menstruating, ultimately resulting in reproductive complications. A broad range of associated anomalies, affecting multiple organ systems, are encountered in approximately half of the females with MRKH, which pose different issues depending on the individual's presentation. There are the two distinct phenotypes of MRKH, Type 1 (typical, restricted to the reproductive tract), and Type 2 (atypical, associated with other defects). Chromosomal deletions have been associated with MRKH. Loss-of-function mutations in early MD patterning gene such as *LIM1*, *WNT4* and *WNT9B* can cause MRKH or related abnormalities (Table 1; (Biason-Lauber et al., 2007; Biason-Lauber et al., 2004; Ledig et al., 2011; W. Ma et al., 2015; Waschk et al., 2016)). Individuals with deletions of chromosome 22qll, linked to DiGeorge syndrome, can also present with MRKH (Morcel et al., 2011). A 17q12 deletion, that affects *LIM1* but also other potential causative genes such as such as *TCF2*, has also been linked to MRKH (Bingham et al., 2002; Lindner et al., 1999). Lastly, mutations causing ectopic activation of AMH or AMHR2 in females could logically lead to MRKH, but these have not been reported (Oppelt et al., 2005). Most cases of MRKH have no known aetiology, and could be genetic or environmental.

Given the importance of Hox and Wnt genes for MD pattering in females, these molecular regulators have been scrutinised in the context of MRKH. Despite the demonstrated role of *Hoxa10*, *Hoxa11* and *Hoxa13* in mouse MD development, no HOX gene mutation has been

linked to MRKH in humans (Burel et al., 2006). However, *HOXA13* mutations are linked to HFG (Hand-Foot-Genital) (Table 1), an autosomal dominant condition characterised by hypospadias in males and shortened digits and female reproductive tract defects, such as double vagina or double uterus and cervix. These abnormalities can be attributed to failure of proper MD fusion during embryogenesis in humans. Unlike the case in rodents (Fig. 5), the ducts in humans ordinarily fuse caudally to give rise to a single (simplex) uterus, vagina and cervix in humans (Frisen et al., 2003). Duplication and polyalanine expansions of HOXA13 have also been linked to HFG (Goodman et al., 2000; Utsch et al., 2002). Most anomalies of the female reproductive tract in fact cannot be explained by mutations in known genes, pointing to the existence of other causatives genes, mutations in regulatory regions and/or a role for environmental factors such as xenoestrogens (e.g. the non-steroidal estrogen, DES) (Laronda et al., 2013; Suzuki et al., 1979).

Table 1: Major genes involved in Müllerian duct development. DES = Diethlystilbestrol; FRT = Female Reproductive Tract; HFG- Hand-Foot-Genital; MRKH = Mayer-Rokitansky-Küster-Hauser syndrome (dyplasia or absence of vagina and uterus); PMDS = Persistent Müllerian Duct Syndrome. NA = not available.

Gene name	Molecule encoded	Animal model (mouse or chicken)	Human phenotype when mutated	Reference
Formation				
Pax2	Paired box Transcription factor	Müllerian agenesis	NA	(Atsuta et al., 2016; Torres et al., 1995)
Lim1 (Lhx1)	Homeodomain Transcription factor	Müllerian agenesis	MRKH	(Kobayashi et al., 2004; Ledig et al., 2012; Ledig et al., 2011; Sandbacka et al., 2013)
Emx2	Homeodomain Transcription factor	Müllerian agenesis	NA	(Liu et al., 2015; Miyamoto et al., 1997)

Fgf signalling /Fgfr2	Fibroblast growth factor	Impaired specification (chicken)	NA	(Atsuta et al., 2016)
Bmp's (Bmp2, Bmp4)	Secreted TGF-² family member	Impaired specification (chicken)	NA	(Atsuta et al., 2016)
Wnt4	Secreted Wnt growth factor	Müllerian agenesis, reduced uterine glands	MRKH	(Biason-Lauber et al., 2007; A. B.-L. Philibert, R. Rouzier, C. Pienkowski, F. Paris, D. Konrad, E. Schoenle, C. Sultan, 2008; P. Philibert et al., 2011; Vainio et al., 1999)
Wnt9b	Secreted Wnt growth factor	Müllerian agenesis	MRKH	(Carroll et al., 2005; W. Ma et al., 2015)
Ctnnb1	Encodes β-catenin, a Wnt signal transduction factor; Cell adhesion	Hypotrophic uterine horns, coiled oviducts, uterine metaplasia	NA	(Arango et al., 2005; Deutscher et al., 2007; Jeong et al., 2009)
Retinoic acid Receptors (*Rara, Rarb)*	Nuclear receptor	Mouse compound mutants show varying degrees of Müllerian agenesis or hypoplasia	NA	(Mendelsohn et al., 1994)
Regression				

AMH (anti-Müllerian Hormone)	Hormone; Secreted TGF- β family member	Persistent Müllerian duct	PMDS; ectopic FRT in males	(Behringer et al., 1994; Belville et al., 1999; Belville C, 2009; di Clemente et al., 2003; W. Ma et al., 2015; Mishina et al., 1996; Salehi et al., 2012)
AMH-R type2 (Amhr2)	Secreted TGF-B family type II Ser/Thr transmembrane receptor	ectopic FRT in males	PMDS	(Belville et al., 1999; Belville C, 2009; di Clemente et al., 2006; Mishina et al., 1996; Salehi et al., 2012)
Wnt7a	Secreted Wnt growth factor	Ectopic FRT in males (mouse)	NA	(Parr et al., 1998)
Differentiation				
Wnt7a	Secreted Wnt growth factor	Transformation of fallopian tube to uterus and uterus to vagina in females	NA	(Miller et al., 1998; Parr et al., 1998)
Hoxa10	Homeodomain Transcription factor	Homeotic transformation of uterus to fallopian tube (mouse)	Defects in MD fusion	(Benson et al., 1996; Cheng et al., 2011; Ekici et al., 2013)
Hoxa11	Homeodomain Transcription factor	Partial homeotic transformation of uterus to Fallopian tube	NA	(Branford et al., 2000; Gendron et al., 1997)
Hoxa13	Homeodomain Transcription factor	Homeotic transformation of cervix to uterus; agenesis of caudal MD	HFG	(Post et al., 2000; Warot et al., 1997)

Estrogen receptor-alpha (ER-α)	Steroid nuclear receptor	MD development impaired (chicken)	Abnormalities of MD development (DES exposure)	(Laronda et al., 2013; Mattsson et al., 2011; Stoll et al., 1993a, 1993b)

Conclusion

The Müllerian ducts are essential components of the embryonic female reproductive system, in mammals giving rise to the fallopian tubes, uterus, cervix and upper portion of the vagina. They form early in embryogenesis as paired epithelial tubes in close association with the Wolffian ducts of the adjoining mesonephros. Cell fate mapping and genetic analyses in mouse and chicken embryos have provided details on the molecular and cellular events underlying duct specification, invagination and elongation. Future studies should now focus on establishing the exact molecular interactions governing duct morphogenesis. Identifying the direct targets of transcription factors such as PAX2, for example, or direct downstream targets of Wnt/² -catenin signalling (*Wnt4*, *Wnt7a*, *Wnt9b*). This also applies to the fate of the ducts in males and females (direct targets of AMH and WNT4 in duct regression in males, and HOX targets during duct differentiation in females). In addition, other as yet unidentified genes must exist that have yet to be implicated in duct development. These may underlie unexplained anomalies of duct development in humans.

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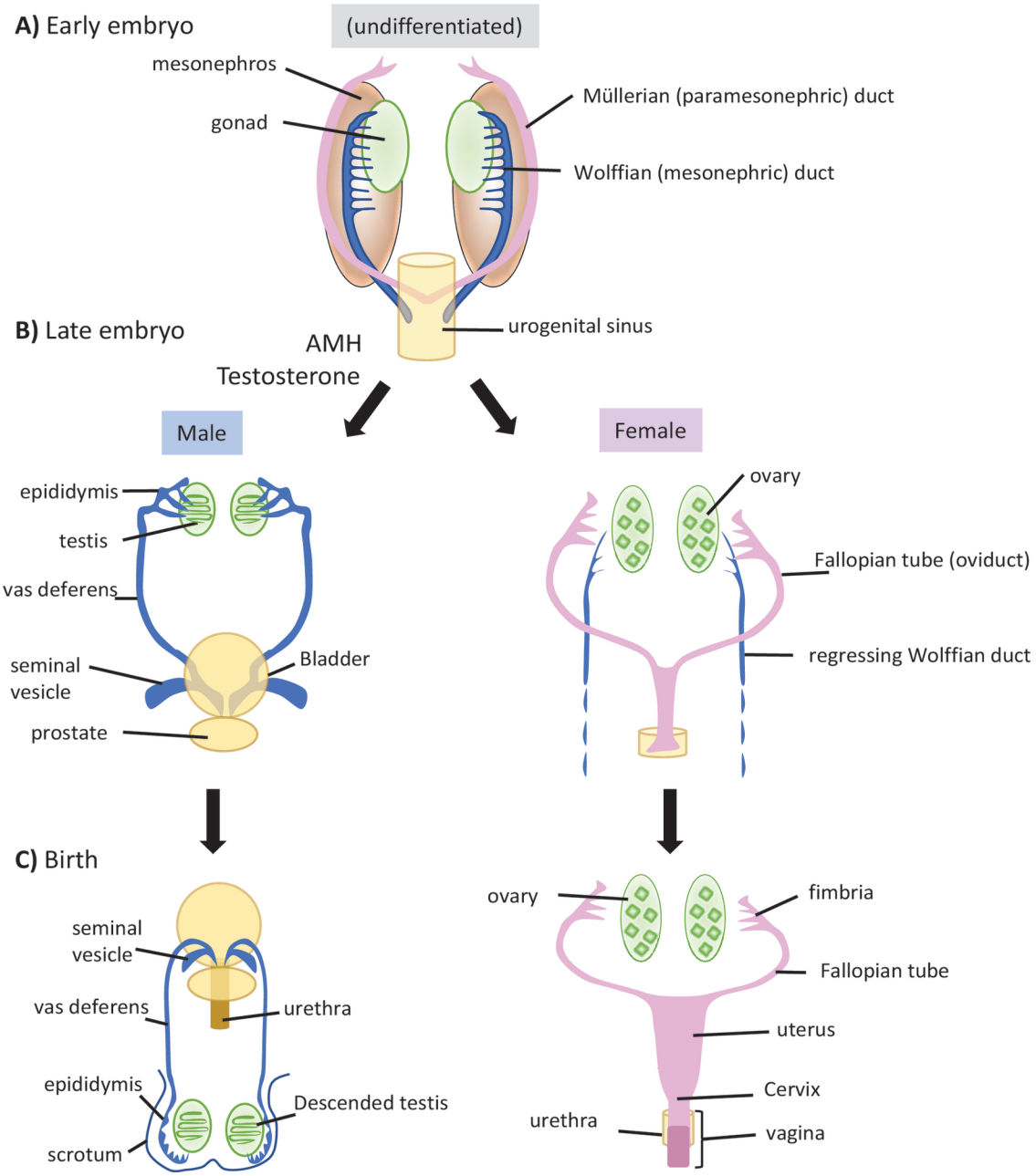

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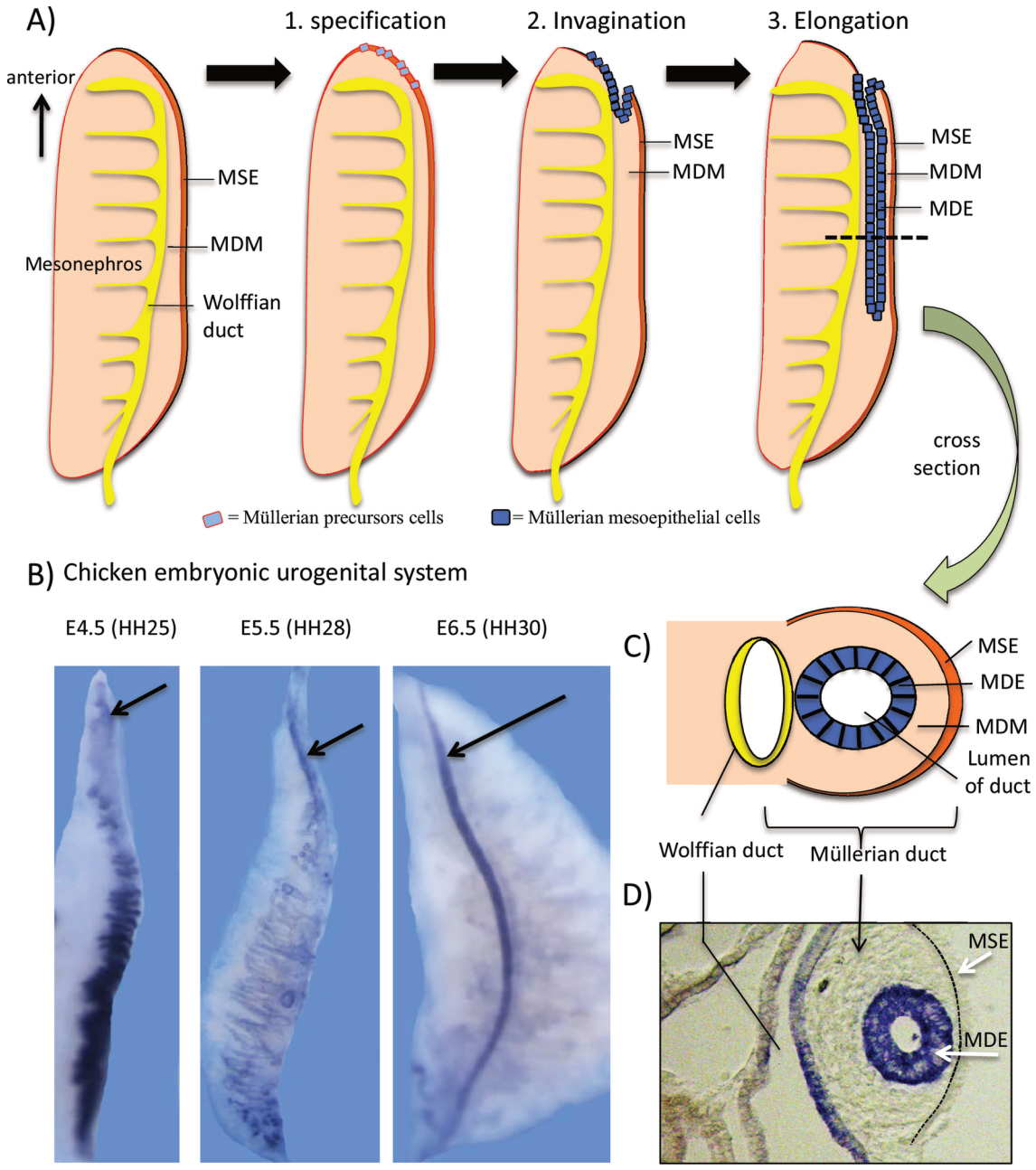

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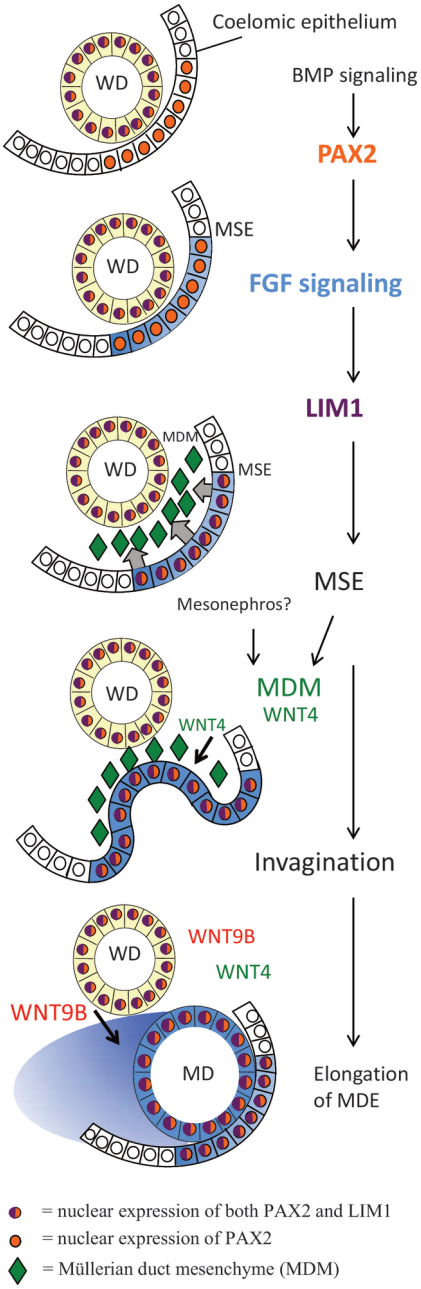

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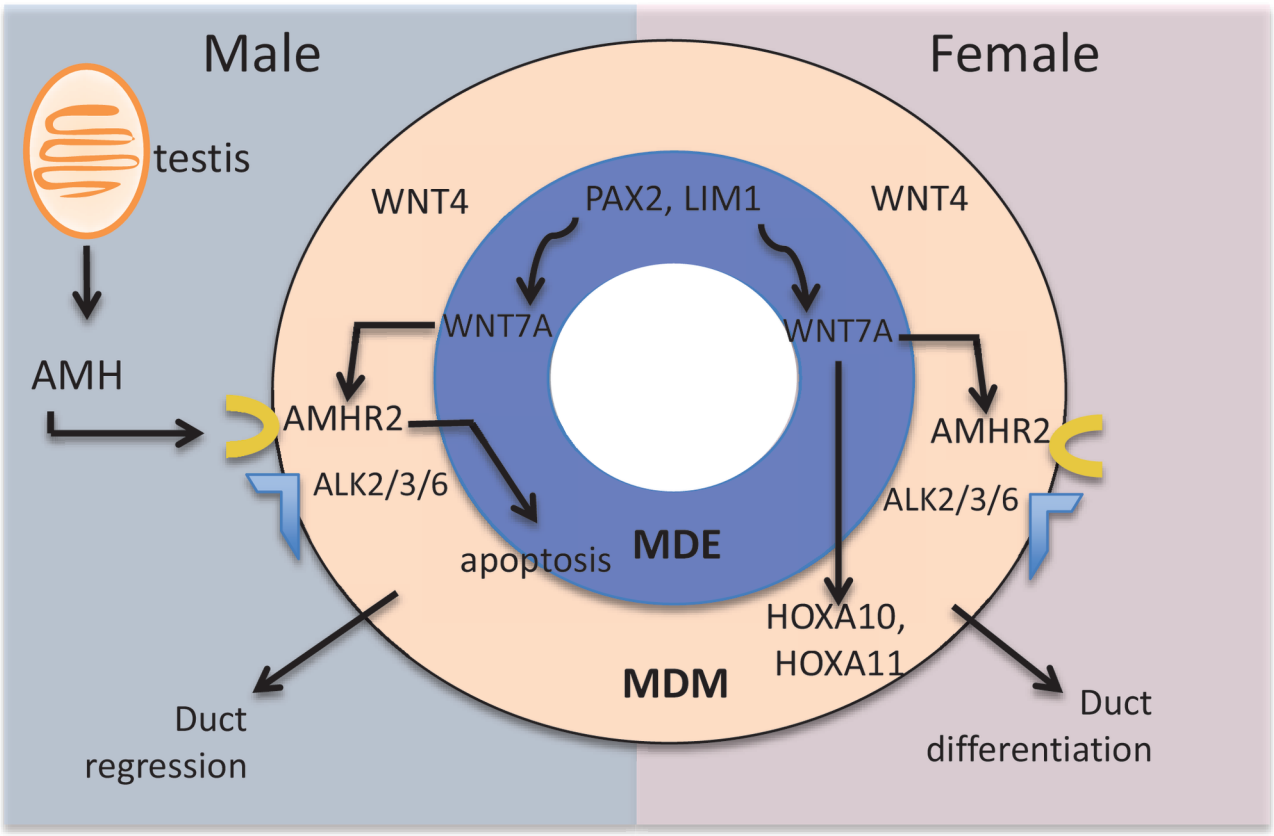

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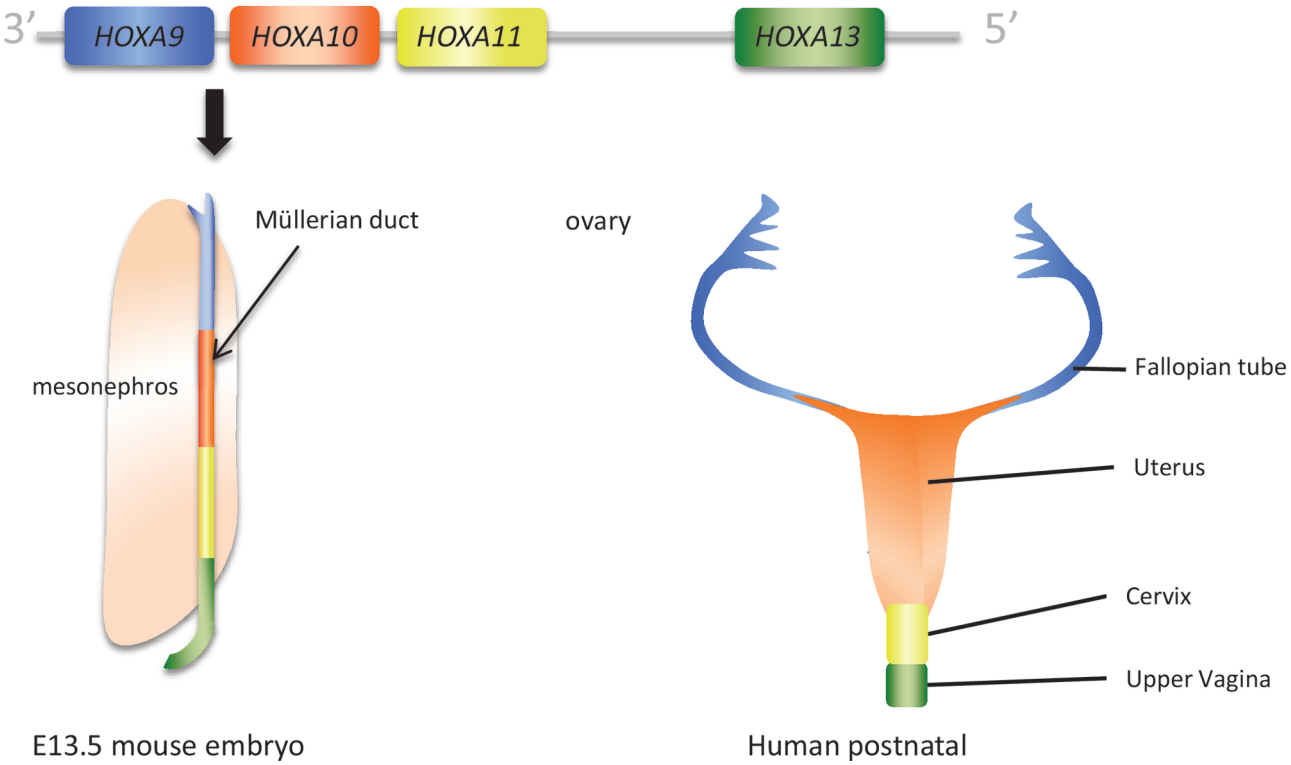

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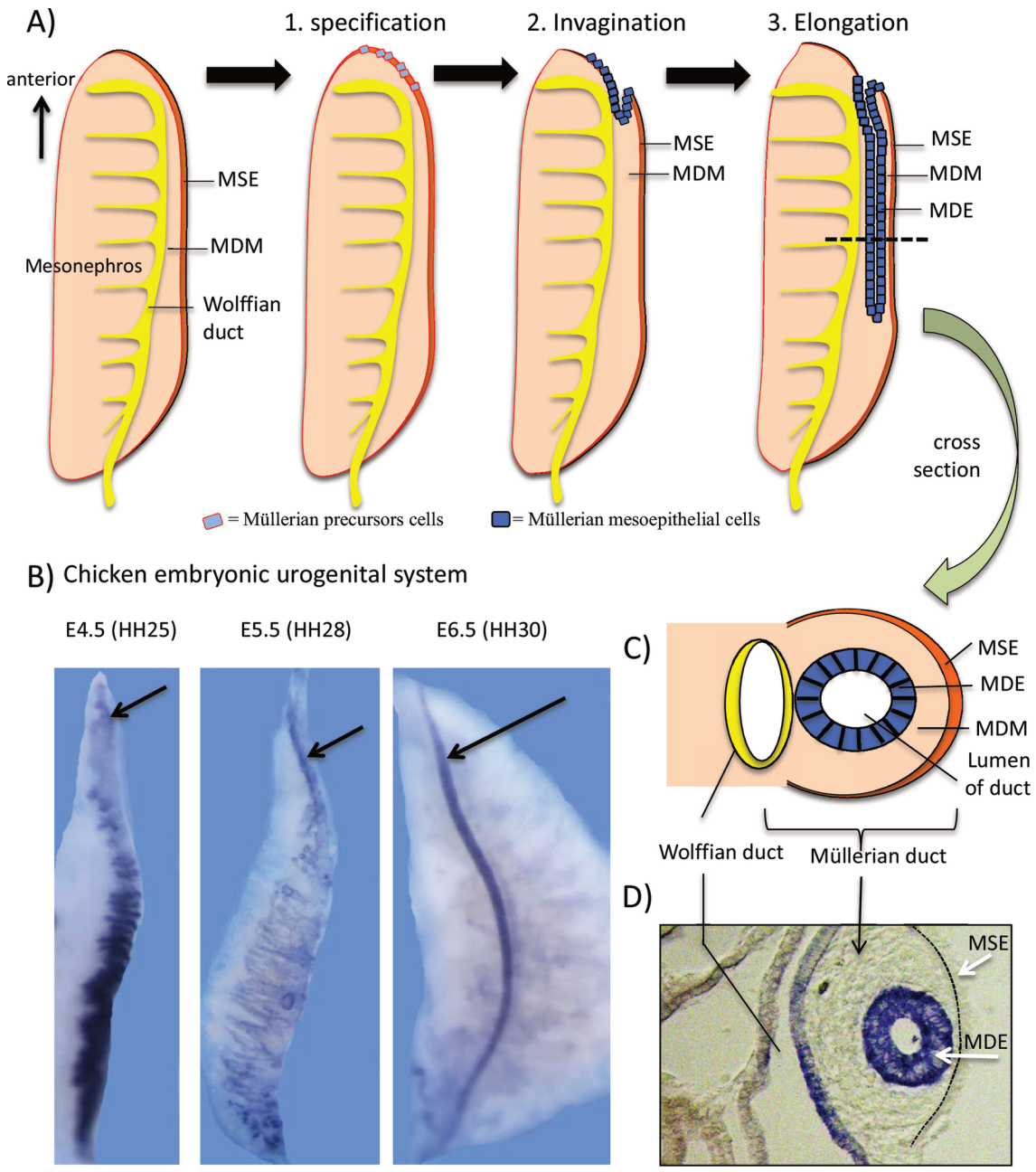

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