Developmental Biology ■ (■■■■) ■■■–■■■

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

DMRT1 is required for Müllerian duct formation in the chicken embryo

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ARTICLE INFO

Article history:
Received 23 December 2014
Received in revised form
29 January 2015
Accepted 4 February 2015

Keywords:
DMRT1
Müllerian ducts
Chicken
Female reproductive tract
Sex development

ABSTRACT

DMRT1 is a conserved transcription factor with a central role in gonadal sex differentiation. In all vertebrates studied, DMRT1 plays an essential function in testis development and/or maintenance. No studies have reported a role for DMRT1 outside the gonads. Here, we show that DMRT1 is expressed in the paired Müllerian ducts in the chicken embryo, where it is required for duct formation. *DMRT1* mRNA and protein are expressed in the early forming Müllerian ridge, and in cells undergoing an epithelial to mesenchyme transition during duct morphogenesis. RNAi-mediated knockdown of *DMRT1 in ovo* causes a greatly reduced mesenchymal layer, which blocks caudal extension of the duct luminal epithelium. Critical markers of Müllerian duct formation in mammals, *Pax2* in the duct epithelium and *Wnt4* in the mesenchyme, are conserved in chicken and their expression disrupted in *DMRT1* knockdown ducts. We conclude that DMRT1 is required for the early steps of Müllerian duct development. DMRT1 regulates Müllerian ridge and mesenchyme formation and its loss blocks caudal extension of the duct. While *DMRT1* plays an important role during testis development and maintenance in many vertebrate species, this is the first report showing a requirement for DMRT1 in Müllerian duct development.

Introduction

In vertebrates, the female reproductive tract derives from the Müllerian ducts, paired tubular structures that arise as part of the embryonic urogenital system. The Müllerian ducts develop in both sexes during embryo development. Following sexual differentiation of the gonads, the ducts are then modified as part of the female or male sexual development programme. In males, the Müllerian ducts degenerates in a process controlled by the Anti-Müllerian Hormone (AMH), produced by the developing testes. Females do not produce AMH during embryonic life, and Müllerian ducts are retained for further differentiation into the oviducts, uterus, cervix and upper vagina. Proper development of the Müllerian ducts is vital to female reproduction. In humans, up to 3% of births have a female reproductive tract-related disorder. Many of these stem from abnormal Müllerian duct development in the embryo, which can result in several developmental disorders or infertility (reviewed in Epelman et al. (2013), Layman (2013)). As Müllerian ducts initially form in both sexes then regress in males, developmental defects can be observed in both females and males. In male embryos, disruption in AMH production or mutations in the AMH receptor result in Persistent Müllerian Duct Syndrome (PMDS) (Josso et al., 2005), which can lead to ectopic development of female reproductive tract organs (Mullen and Behringer, 2014).

The Müllerian duct consists of a canalised epithelial tube (the Müllerian Duct Epithelium; MDE), which is surrounded by a mesenchyme (Müllerian duct mesenchyme; MDM). Externally these tissues are covered by the coelomic epithelium (MCE). These ducts form along the anterior–posterior axis of the embryo in close proximity to the Wolffian (mesonephric) ducts. Both the MDM and MDE layers of the Müllerian duct originate from MCE cells (Guioli et al., 2007; Orvis and Behringer, 2007). Development of the Müllerian duct can be divided into three distinct stages—initiation, invagination and extension. The initiation phase is marked by thickening of the coelomic epithelium to form the Müllerian ridge, which occurs concurrently along the entire length of the mesonephros directly adjacent to the Wolffian duct. The ridge provides the source of cells and signalling factors required for subsequent duct formation. The thickened MCE gives rise to mesenchymal cells (MDM), through an epithelial to mesenchymal transition (EMT). This process primarily occurs at the cranial pole, but may also occur all along the length of the duct (Guioli et al., 2007; Orvis and Behringer, 2007). Following this process, a subset of progenitor MCE cells differentiate to form the founding cells of the duct. These cells invaginate into the MDM to form the opening of the duct lumen at the most anterior pole (reviewed in Mullen and Behringer (2014), Jacob et al. (1999), Kobayashi et al. (2004)).

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http://dx.doi.org/10.1016/j.ydbio.2015.02.001
0012-1606/

In the extension phase, the MDM extends caudally, followed closely by the MDE. In both mouse and chicken embryos, current evidence suggests that the extension of both layers occurs by a combination of elongation, proliferation and active migration (Fujino et al., 2009; Guioli et al., 2007). The Müllerian ducts extend with intimate proximity to the Wolffian ducts, requiring their presence to complete elongation. A midpoint breakage to the Wolffian duct causes a corresponding halt to Müllerian duct extension (Gruenwald, 1941; Kobayashi et al., 2005; Orvis and Behringer, 2007). It is thought that the Wolffian ducts signals to the Müllerian ducts via morphogens (Wnt ligands such as WNT9B), guiding their caudal extension (Carroll et al., 2005). The Wolffian ducts do not contribute cells to the Müllerian ducts during its formation (Guioli et al., 2007; Orvis and Behringer, 2007).

The developing Müllerian duct provides an ideal model system for studying the molecular regulation of tubulogenesis. A number of genes have been implicated in each distinct stage of Müllerian duct formation, although their exact role is yet to be determined. In the mouse, *Lim1*, *Pax2*, *Pax8*, *Emx2* and Wnt-signalling components are all highly expressed in the Müllerian ducts, and disruption of these genes results in ducts that are significantly deformed or completely absent (Deutscher and Hung-Chang Yao, 2007; Kobayashi et al., 2004; Miyamoto et al., 1997; Philibert et al., 2008; Torres et al., 1995; Vainio et al., 1999; Arango et al., 2005). *Lim1/Lhx1* is expressed in both the Müllerian and Wolffian ducts, and is essential for the proper development of the female reproductive tract (Kobayashi et al., 2004; Orvis and Behringer, 2007; Pedersen et al., 2005). Loss of this transcription factor in mice leads to a complete lack of Müllerian duct derived female reproductive structures (Kobayashi et al., 2004) which may in part be a result of loss of the Wolffian ducts. Nevertheless, conditional knockouts suggest a cell autonomous role for Lim1 in Müllerian duct cell survival, proliferation and extension (Huang et al., 2014). The Wnt4 ligand is expressed in the mesenchyme of the duct, and it is important for tubulogenesis and caudal extension (Arango et al., 2005; Cai, 2001; Klattig and Englert, 2007; Kobayashi et al., 2004; Philibert et al., 2008; Vainio et al., 1999). Several other Wnt ligands also play a role (reviewed in Christopoulos et al. (2009)), such as Wnt9 expressed in the Wolffian duct, which is itself essential for Müllerian duct extension (Carroll et al., 2005). It is thought that in mice, Wnt-signalling activates *Pax2* in the MDE (Cai, 2001; Klattig and Englert, 2007; Torres et al., 1995). *Pax2* itself is required for maintenance of the Müllerian duct, potentially through its actions in the Wolffian ducts. *Pax2* knockout mice possess only the uppermost parts of the Müllerian (and Wolffian) ducts (Torres et al., 1995).

The chicken provides an excellent model to study many aspects of urogenital system development, including Müllerian duct formation (Ayers et al., 2013; Cutting et al., 2014; Guioli et al., 2007). The cellular processes involved in Müllerian duct formation are largely conserved among mammals and chicken. In mice, Müllerian ducts begin forming at around embryonic day 11–12. In chickens, initiation phase starts with the appearance of the Müllerian ridge, which forms at stage 19 (Hamburger and Hamilton, 1951), chicken embryonic day (E) 3.0-3.5 (Jacob et al., 1999; Kobayashi et al., 2004). The invagination phase starts around stage 24 (E4.5), and the subsequent extension phase lasts until the Müllerian ducts reach the cloaca by around stage 28–30 (~E6.0-7.0). As in mammals, regression of the Müllerian ducts in males is mediated by AMH, which is expressed by developing gonads. However, unlike in mammals, the right gonad and Müllerian duct also regress in the female chicken embryo. This is mediated via asymmetric expression of the *PITX2* gene (Guioli and Lovell-Badge, 2007; Guioli et al., 2014; Hoshino et al., 2005). AMH is expressed at low levels in the gonads of female chicken embryos, where it may be required for regression of the right female duct (Oreal et al., 1998; Takada et al., 2006). We recently described the AMH type II receptor in chicken, which is expressed in gonads and developing Müllerian ducts of both sexes (Cutting et al., 2014).

In vertebrate embryos, the conserved transcription factor *DMRT1* shows male-biased expression in urogenital systems of all species examined, including fishes, amphibians, birds and mammals. DMRT1 plays a central role in testis development and function. In the mouse, DMRT1 is required for the maintenance of male fate in the testis. It is also essential for germ cell differentiation in males and regulates meiosis in both males and females (Matson et al., 2011; Minkina et al., 2014; Raymond et al., 2000; Zarkower, 2013). In fish, *dmrt1* expression has been reported in the gonads of about 20 species where it consistently shows higher expression in the male (reviewed in Herpin and Schartl (2011)). Dmrt1 can also play a key role in testis development in fish. In the Medaka (*Oryzias latipes*), the Y chromosome-linked *dmrt1* homologue, *dmy*, is a male sex-determining gene (Masuyama et al., 2011; Matsuda et al., 2002, 2007; Nanda et al., 2002). Similarly, in *Xenopus laevis*, autosomal *DMRT1* plays a role in testis formation and a sex-linked dominant-negative form of *DMRT1* (*DM-W*) represses its activity in females (Yoshimoto et al., 2008). In birds, *DMRT1* is present on the Z sex chromosome, which is present in two copies in males (ZZ) and one in females (ZW). This double dose of *DMRT1* in males is linked to testis development, and knockdown of *DMRT1* induces feminisation of male gonads. Conversely, over-expression of DMRT1 in the female gonads causes masculinisation and upregulation of testis genes (Lambeth et al., 2014; Matson et al., 2011; Minkina et al., 2014; Smith et al., 2009). DMRT1 is currently thought to induce Sertoli cell differentiation in the avian embryo, and as such, likely operates as the master testis determinant.

While *DMRT1* is clearly essential for proper testis development among vertebrates, no studies have reported a role outside the gonads. In chicken embryos, *DMRT1* expression has been reported in the Müllerian ducts (Omotehara et al., 2014; Raymond et al., 2000; Smith and Sinclair, 2001). However nothing is known about its potential role in Müllerian duct formation and function. Here we show that *DMRT1* mRNA and protein are expressed in the early forming Müllerian ridge and subsequently in the MCE and MDM during development in the chicken embryo. Knockdown of *DMRT1* in these cells results in a failure of the Müllerian duct to form. MDM is blocked, and there is a loss of the duct mesenchyme marker, *WNT4*. Caudal extension of the duct also ceases, evident both histologically and by a reduction of MDE markers such as *PAX2*. These results indicate that *DMRT1* plays a role in the initial steps of duct formation, specifically the establishment of the Müllerian ridge and MDM, disruption of which subsequently cause a failure in duct extension.

Results

DMRT1 is expressed in the chicken Müllerian ducts during development

To assess the potential role of DMRT1 in the development of the Müllerian ducts, its expression was evaluated using *whole mount in situ hybridisation* (WISH) in embryonic chicken urogenital systems (UGS). *DMRT1* expression was assessed at E4.0, E5.5, E6.5 and E9.5 (HH stages 24, 27, 30 and 35) in both males and females ($n=3$ for each sex, at each stage). *DMRT1* mRNA was detected in the Müllerian ridge from E4.0, in both sexes (Fig. 1A–*d*), and in the gonads from E5.5 (Fig. 1 arrowheads). At the earliest stages assayed (E3.5 data not shown, and E4.0, Fig. 1A–*d*), *DMRT1* mRNA was expressed along the entire length of the Müllerian

Please cite this article as: Ayers, K.L., et al., DMRT1 is required for Müllerian duct formation in the chicken embryo. Dev. Biol. (2015), http://dx.doi.org/10.1016/j.ydbio.2015.02.001