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Lhx1 is required in Müllerian duct epithelium for uterine development

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ABSTRACT

The female reproductive tract organs of mammals, including the oviducts, uterus, cervix and upper vagina, are derived from the Müllerian ducts, a pair of epithelial tubes that form within the mesonephroi. The Müllerian ducts form in a rostral to caudal manner, guided by and dependent on the Wolffian ducts that have already formed. Experimental embryological studies indicate that caudal elongation of the Müllerian duct towards the urogenital sinus occurs in part by proliferation at the ductal tip. The molecular mechanisms that regulate the elongation of the Müllerian duct are currently unclear. *Lhx1* encodes a LIM-homeodomain transcription factor that is essential for male and female reproductive tract development. *Lhx1* is expressed in both the Wolffian and Müllerian ducts. Wolffian duct-specific knockout of *Lhx1* results in degeneration of the Wolffian duct and consequently the non-cell-autonomous loss of the Müllerian duct. To determine the role of *Lhx1* specifically in the Müllerian duct epithelium, we performed a Müllerian duct-specific knockout study using *Wnt7a-Cre* mice. Loss of *Lhx1* in the Müllerian duct epithelium led to a block in Müllerian duct elongation and uterine hypoplasia characterized by loss of the entire endometrium (luminal and glandular epithelium and stroma) and inner circular but not the outer longitudinal muscle layer. Time-lapse imaging and molecular analyses indicate that *Lhx1* acts cell autonomously to maintain ductal progenitor cells for Müllerian duct elongation. These studies identify LHX1 as the first transcription factor that is essential in the Müllerian duct epithelial progenitor cells for female reproductive tract development. Furthermore, these genetic studies demonstrate the requirement of epithelial–mesenchymal interactions for uterine tissue compartment differentiation.

Introduction

Two pairs of urogenital ducts, the Wolffian and Müllerian ducts, form during embryonic development in mammals, reptiles and birds (Massé et al., 2009). In male mammals, the Wolffian duct differentiates under the influence of testosterone into the vas deferens, epididymis and seminal vesicle of the reproductive system. In females, due to the lack of androgen support, the Wolffian duct degenerates during late embryonic development (Josso, 2008; Welsh et al., 2009). In female mammals, the Müllerian duct differentiates into the oviducts, uterus, cervix and upper portion of the vagina of the reproductive tract. In males, the Müllerian ducts are actively eliminated by the anti-Müllerian hormone signaling pathway (Behringer et al., 1994; Mishina et al., 1996; Jamin et al., 2003; Orvis et al., 2008). Thus, amniotes develop through an ambisexual stage relative to the urogenital ducts that subsequently requires differentiation toward the male or female reproductive tract phenotypes.

The Wolffian duct develops from the intermediate mesoderm (Jacob et al., 1991; Obara-Ishihara et al., 1999) at embryonic day (E) 9 in the mouse and its formation is complete by E10.5. Müllerian duct epithelial cells are initially specified in the rostral mesonephric epithelium around E11.5 in the mouse and subsequently invaginate and elongate caudally along the Wolffian duct (Guioli et al., 2007; Orvis and Behringer, 2007). Elongation of the Müllerian duct is complete when its caudal tip connects to the urogenital sinus at E13.5. Previous studies have demonstrated that the elongation of the Müllerian duct requires the presence of the Wolffian duct (Gruenwald, 1941; Kobayashi et al., 2005; Orvis and Behringer, 2007). The Wolffian duct expresses *Wnt9b* and genetic studies suggest that WNT9B secreted by the Wolffian duct is a trophic factor required for Müllerian duct elongation (Carroll et al., 2005). *Hnf1b* encodes a POU homeodomain transcription factor

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that is expressed in the Wolffian duct and directly activates *Wnt9b* transcription. *Hnf1b* knockout mice have defects in Wolffian duct development and consequently a lack of Müllerian duct formation (Lokmane et al., 2010).

Genetic studies in mice suggest that *Wnt4* in mesonephric mesenchyme is essential for Müllerian duct invagination and elongation (Vainio et al., 1999). As the Müllerian duct elongates mesenchyme cells surround the Müllerian duct epithelial cells and separate the epithelium from the Wolffian duct and mesonephric epithelium in a rostral to caudal manner. Studies suggest that the Müllerian duct mesenchyme that initially surrounds the Müllerian duct epithelium is derived from the mesonephric mesenchyme and lateral mesonephric epithelium (Zhan et al., 2006; Guioli et al., 2007). In females, differentiation of the Müllerian duct into the adult female reproductive tract is highly dependent on the interactions between the Müllerian duct epithelium and mesenchyme (Kurita et al., 2001). Signaling molecules such as WNT4 (Bernard and Harley, 2007), WNT5A (Mericskay et al., 2004), WNT7A (Miller and Sassoon, 1998; Carta and Sassoon, 2004) and several HOX transcription factors (Hsieh-Li et al., 1995; Warot et al., 1997; Du and Taylor, 2004) all participate in the differentiation of the female reproductive tract during late gestation and early postnatal stages.

Lhx1 (also known as *Lim1*) encodes a LIM-homeodomain transcription factor (Jurata et al., 1998). *Lhx1* is essential for the formation of the head organizer, kidneys, and retinal layers (Shawlot and Behringer, 1995; Kobayashi et al., 2005; Poché et al., 2007). It is also required redundantly with *Lhx5* for cerebellar cell survival (Zhao et al., 2007). Previous studies demonstrated that *Lhx1* is expressed in the Wolffian and Müllerian ducts and is critical for female reproductive tract formation and/or maintenance (Kobayashi et al., 2004; Pedersen et al., 2005; Orvis and Behringer, 2007). In addition, heterozygous *LHX1* missense mutations have been found in patients with Müllerian aplasia, the congenital loss of the uterus and vagina (Sandbacka et al., 2013). Wolffian duct-specific knockout of *Lhx1* results in Wolffian duct degeneration but also secondary loss of the Müllerian duct (Kobayashi et al., 2005). Thus, the role of *Lhx1* specifically in the Müllerian duct is currently unknown. *Emx2* and *Pax2* also encode transcription factors that are expressed in both the Wolffian and Müllerian ducts that are required for female reproductive tract development (Torres et al., 1995; Miyamoto et al., 1997). However, because mutants for these genes also have Wolffian duct defects these studies need to be interpreted with caution because of the potential non-cell-autonomous effect of defective Wolffian duct development on subsequent Müllerian duct development.

In the present study, we generated Müllerian duct epithelium-specific *Lhx1* conditional knockout mice (*Lhx1 cKO*), using a *Wnt7a-Cre* transgene (Winuthayanon et al., 2010). Unlike *Lhx1* null mutant mice (Shawlot and Behringer, 1995), *Lhx1* conditional knockouts are viable. *Lhx1 cKO* males are healthy and fertile; however *Lhx1 cKO* females are sterile because they have a shortened oviduct and lack a uterus, cervix and upper vagina. Interestingly, residual uterine tissue in the *Lhx1 cKO* females appears to be the longitudinal muscle layer. Developmental studies demonstrate that Müllerian duct elongation is blocked, resulting in an absence of caudal Müllerian duct. Increased cell death and decreased cell proliferation in the Müllerian duct epithelium provide two cellular mechanisms responsible for the block in ductal elongation. Time-lapse imaging in organ culture revealed high motility and cell protrusions of the Müllerian duct leading tip during elongation. This phenotype is compromised upon conditional loss of *Lhx1*. These data suggest a cell-autonomous requirement of *Lhx1* for Müllerian duct cell survival, proliferation and elongation. In addition, these genetic studies indicate complex epithelial–mesenchymal interactions for the development of distinct uterine tissue compartments.

Results

Wnt7a-Cre activity is specific to the Müllerian duct epithelium during urogenital organogenesis

WNT7A is a member of the WNT glycoprotein family. *Wnt7a* is expressed in the Müllerian duct epithelium and is required for sexual dimorphic differentiation of the reproductive tract (Parr and McMahon, 1998). *Wnt7a-Cre* transgenic mice were generated by modifying a bacterial artificial chromosome that contains the *Wnt7a* locus (Winuthayanon et al., 2010). Matings between *Wnt7a-Cre* mice and *Rosa26 lacZ* Cre reporter (*R26R-lacZ*) mice resulted in β-gal expression in the Müllerian duct. The β-gal expression pattern in these mice was similar to the endogenous expression pattern of *Wnt7a* in the Müllerian duct epithelium (Huang et al., 2012). β-gal activity was also detected in regions of the developing central nervous system, limb buds and hair follicles (data not shown).

Further characterization of β-gal expression mediated by the *Wnt7a-Cre* transgene during urogenital development showed that transgene activity was detected in the Müllerian duct beginning at tail somite stage 22 (TS 22) (Fig. 1A and B). Subsequently, the *Wnt7a-Cre* transgene faithfully recombines the *R26R-lacZ* transgene in the Müllerian duct during its elongation. β-gal activity was specifically detected throughout the Müllerian duct epithelium but was not detected in the Wolffian ducts. The *Wnt7a*-Cre transgene also labels the caudal tips of the elongating Müllerian ducts (Fig. 1C–F). These results demonstrate that the *Wnt7a-Cre* transgenic mouse line can be used for Müllerian duct epithelium-specific gene modifications.

Generation and characterization of Müllerian duct-specific Lhx1 conditional knockout mice

Given the overlapping temporal expression pattern of *Lhx1* and *Wnt7a* (Parr and McMahon, 1998; Kobayashi et al., 2004) in the developing Müllerian duct, it should be feasible to generate viable, Müllerian duct-specific *Lhx1* conditional knockout (*Lhx1 cKO*) mice using the *Wnt7a-Cre* transgene. $Lhx1^{lacZ/+}$; *Wnt7a-Cre Tg/+* bigenic males were bred with females homozygous for an *Lhx1* flox allele ($Lhx1^{fx/fx}$). The $Lhx1^{lacZ}$ allele serves as a null allele but also marks *Lhx1*-expressing cells by simple X-gal staining (Kania et al., 2000). We found that both control and *Lhx1 cKO* neonates were born at the expected Mendelian ratio. Analysis of the female reproductive tract from control and *Lhx1 cKO* neonates revealed several structural differences. Control female neonates (postnatal day 0, P0) developed normal female reproductive tracts (Fig. 2A), including oviducts, uterus, cervix and vagina. However, *Lhx1 cKO* female neonates were found to have short oviducts relative to controls but their uterus, cervix and upper vagina were replaced by a thin membrane-like tissue (Fig. 2B). *Lhx1 cKO* females can survive to adulthood but are infertile. In contrast, *Lhx1 cKO* adult males were normal and fertile. When compared to control animals (Fig. 2C), it was apparent that the uterine horns, cervix and upper vagina did not develop in *Lhx1 cKO* female adults (Fig. 2D). Oviducts from control adult females were highly coiled (Fig. 2E) but in the *Lhx1 cKO* adult females the oviducts were shorter, although they did form a coiled structure and had normal cytoarchitecture (Fig. 2E, F).

Hematoxylin and eosin staining showed the well-organized luminal epithelium (Fig. 2G) surrounded by condensed mesenchymal cells in the control uterus at P0. In *Lhx1 cKO* neonates, there were no epithelial cells in the membranous tissue and the surrounding mesenchymal cells were loose and showed histological indications of cell death (Fig. 2H). In the uteri of adult control mice, the two muscle layers and the entire endometrium including