Requirement of *Lim1* for female reproductive tract development

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Summary

Lim1 **encodes a LIM-class homeodomain transcription factor that is essential for head and kidney development. In the developing urogenital system,** ***Lim1*** **expression has been documented in the Wolffian (mesonephric) duct, the mesonephros, metanephros and fetal gonads. Using, a** ***Lim1 lacZ*** **knock-in allele in mice, we identified a previously unreported urogenital tissue for** ***Lim1*** **expression, the epithelium of the developing Müllerian duct that gives rise to the oviduct, uterus and upper region of the vagina of the female reproductive tract.** ***Lim1*** **expression in the Müllerian duct is dynamic, corresponding to its formation and differentiation in females and regression in males. Although female** ***Lim1*****-null neonates had ovaries they lacked a uterus and oviducts. A novel female mouse chimera assay was developed and revealed that** ***Lim1*** **is required cell autonomously for Müllerian duct epithelium formation. These studies demonstrate an essential role for** ***Lim1*** **in female reproductive tract development.**

Key words: Müllerian duct, Uterus, Chimera, Lim1 (Lhx1), MIS (AMH), Wnt4, Mouse

Introduction

The mammalian female reproductive tract is an organ system that is composed of the oviducts, uterus, cervix and vagina. The primary role of this organ system is reproduction, i.e. the continuation of the species. The female reproductive tract organs are also a significant concern for women's health and disease, notably infertility and cancer. Some diseases of the female reproductive tract are caused by abnormalities that occur in the female fetus during embryogenesis. These include Müllerian agenesis and vaginal septum, which can prevent normal reproduction (Gidwani and Falcone, 1999). Agenesis of the female reproductive tract has been estimated to occur in one in 4000 to 20,000 women (Kim and Laufer, 1994). It is also suggested that deregulation of embryonic genes is observed in some types of cancers in several organs (Peifer and Polakis, 2000; Chi and Epstein, 2002; Ruiz i Altaba et al., 2002). Most studies of the female reproductive tract are focused upon its biology in adults. Thus, the molecular mechanisms that regulate female reproductive tract development are largely unknown (Kobayashi and Behringer, 2003).

During vertebrate embryogenesis, the urogenital system derives from the intermediate mesoderm of the gastrula. The female reproductive tract system develops primarily from the Müllerian (paramesonephric) duct and the male reproductive tract forms from the Wolffian (mesonephric) duct. In the mouse, the Wolffian duct is first formed from the intermediate mesoderm by embryonic day 9 (E9). Subsequently, the Müllerian duct starts to form by invagination of the surface epithelium of the anterior mesonephros around E11.5 in the developing urogenital ridge. This epithelial invagination extends caudally along the Wolffian duct laterally and then medially towards the cloaca to form the primordium of the female reproductive tract (Gruenwald, 1941; Kaufman and Bard, 1999). Thus, embryos have both male and female reproductive tract primordia regardless of their genetic sex before sexual differentiation occurs. The Müllerian duct can differentiate into the oviduct, uterus, cervix and upper part of the vagina of the female reproductive tract. The Wolffian duct can differentiate into the epididymis, vas deferens and seminal vesicle of the male reproductive tract.

Mammalian sex determination depends on the genetic sex in the gonad (Swain and Lovell-Badge, 1999; Capel, 2001). XY embryos usually become males and XX embryos usually become females. It is also known that, XX↔XY chimeric mice mostly develop into males (Tarkowski, 1998) and Sertoli cells of the testis from these chimeric animals are predominantly XY (Burgoyne et al., 1988; Palmer and Burgoyne, 1991; Patek et al., 1991). This indicates that the testis-determining gene acts cell-autonomously in this cell lineage and that high contribution of XY cells in this cell lineage in the gonads of XX↔XY chimeric mice can result in the male phenotype. The testis-determining gene on the Y chromosome, *Sry*, is both essential and sufficient for triggering testis differentiation to cause male differentiation (Gubbay et al., 1990; Koopman et al., 1991). *Sry* is expressed transiently and dynamically in the bipotential gonad of XY males (Bullejos and Koopman, 2001). It has been suggested that *Sry* is expressed in precursor cells of Sertoli cells (Albrecht and Eicher, 2001). In XX females, the absence of the Y chromosome permits the bipotential gonad to differentiate into an ovary leading to the female

phenotype. Although the loss of Y chromosome is known to cause Turner's syndrome in humans, XO mice are phenotypically normal females except for a transient developmental delay until early mid-gestation stage and early loss of oocytes after birth (Morris, 1968; Lyon and Hawker, 1973; Burgoyne and Baker, 1981; Burgoyne et al., 1983).

After gonadal sex is determined, the differentiating gonads secrete sexual hormones to promote sexual differentiation of the body. In males, the fetal testis secretes hormones including Müllerian inhibiting substance (MIS; AMH – Mouse Genome Informatics), testosterone and insulin-like 3 (Insl3) (Nef and Parada, 2000). MIS causes the elimination of the Müllerian duct and testosterone promotes the differentiation of the Wolffian duct. In the mouse, regression of the Müllerian duct system is observed cytologically from E13.5 (Dyche, 1979). All three hormones are involved in testicular descent. In humans and mice, males deficient for MIS or its type II receptor (MISRII; AMHR2 – Mouse Genome Informatics) are normally virilized and possess a male reproductive tract but fail to regress the Müllerian duct and retain ectopic female reproductive tract organs (Behringer et al., 1994; Mishina et al., 1996; Belville et al., 1999). In female fetuses, the differentiating ovaries do not produce MIS, testosterone or Isl3, which allows the Müllerian duct to differentiate into the female reproductive tract, the Wolffian duct to degenerate and the ovaries to remain in an intra-abdominal position (Kobayashi and Behringer, 2003).

Lim1 (also known as *Lhx1*) encodes a transcription factor with a DNA-binding homeodomain and two cysteine-rich LIM domains that are thought to be involved in protein-protein interactions (Dawid et al., 1998; Bach, 2000; Hobert and Westphal, 2000). During mouse urogenital system development, *Lim1* is expressed in the intermediate mesoderm at E7.5 and this expression is subsequently restricted to the nephric duct that differentiates into the Wolffian duct (Barnes et al., 1994; Tsang et al., 2000). *Lim1* is also expressed in the developing mesonephros in embryos and in the definitive kidney (metanephros) in both embryos and adults (Barnes et al., 1994; Fujii et al., 1994; Karavanov et al., 1996; Karavanov et al., 1998). *Lim1* expression is also detected in the fetal gonad (Nagamine and Carlisle, 1996; Nagamine et al., 1999; Birk et al., 2000; Bouchard et al., 2002). These expression data indicate that *Lim1* may play important roles in multiple processes of urogenital system development, including the reproductive organs. The *Lim1* gene was previously mutated in the mouse and was found to be required for head and kidney formation (Shawlot and Behringer, 1995). However, except for very rare neonates, most *Lim1*-null mutants die around E10 probably owing to the failure of chorio-allantoic fusion to form the placenta. Therefore, the roles of *Lim1* at later stages of development have remained unclear.

In this study, we show that *Lim1* is also expressed in the developing Müllerian duct during female reproductive tract development. This expression for the first time allows for the visualization of Müllerian duct formation and regression in embryos. In the absence of *Lim1* function, the female reproductive tract is absent in female neonates. We also show, using a novel female mouse chimera assay, that *Lim1* activity is required cell-autonomously in the epithelium of the developing Müllerian duct. These data establish a new and essential role for *Lim1* in female reproductive tract development.

Materials and methods

Mice

Lim1$^{tlz/+}$ mice (Kania et al., 2000) were maintained on a 129/SvEv×C57BL/6J mixed genetic background, *Lim1*$^{+/-}$ (Shawlot and Behringer, 1995), *Mis*$^{+/-}$ (Behringer et al., 1994), *Misr2*$^{+/-}$ (Mishina et al., 1996) and *MT-hMIS*$^{tg/+}$ (Behringer et al., 1990) mice on a C57BL/6J congenic background, *Wnt4*$^{+/-}$ and *Wnt7a*$^{+/-}$ mice on a 129/Sv×C57BL/6J mixed background (Parr and McMahon, 1998; Vainio et al., 1999), *Pax2*$^{+/-}$ mice on a 129/Sv×C3H×C57BL/6J mixed genetic background (Torres et al., 1995), and the X-linked GFP mice (Hadjantonakis et al., 1998) on a Swiss Webster outbred background. To examine *Lim1* expression in the urogenital system, Swiss Webster female mice (Taconic, Germantown, NY) were crossed with *Lim1*$^{tlz/+}$ male mice. To examine *Lim1* expression in *Mis* and *Misr2* deficient males, *Mis*$^{+/-}$*; Lim1*$^{tlz/+}$ and *Misr2*$^{+/-}$*; Lim1*$^{tlz/+}$ males were crossed with *Mis*$^{+/-}$ and *Misr2*$^{+/-}$ females, respectively. To examine *Lim1* expression in *MT-hMIS*$^{tg/+}$ transgenic embryos, Swiss Webster female mice were crossed with *MT-hMIS*$^{tg/+}$*; Lim1*$^{tlz/+}$ males. To examine *Lim1* expression in *Pax2*, *Wnt4* and *Wnt7a* deficient embryos, *Pax2*$^{+/-}$*; Lim1*$^{tlz/+}$ *Wnt4*$^{+/-}$*; Lim1*$^{tlz/+}$ and *Wnt7a*$^{+/-}$*; Lim1*$^{tlz/+}$ males were crossed with *Pax2*$^{+/-}$, *Wnt4*$^{+/-}$, and *Wnt7a*$^{+/-}$ females, respectively.

PCR genotyping

Mice were genotyped by PCR using the following primers (5′ to 3′). For *Lim1*$^{tlz/+}$ mice: mLim1-Fw8, GGCTACCTAAGCAACAACTACA; mLim1-Rv9, AGGAGTGAAGGTCACCGTGA; lacZ-A, GCATCGAGCTGGGTAATAAGGGTTGGCAAT; and lacZ-B, GACACCAGACCAACTGGTAATGGTAGCGAC. The wild-type and *lacZ* bands are 305 bp and 822 bp, respectively. For *Lim1*$^{+/-}$ mice: mLim1-Fw8, GGCTACCTAAGCAACAACTACA; mLim1-Rv9, AGGAGTGAAGGTCACCGTGAG; and PGK-FX3, AGACTGCCTTGGGAAAAGCGC. The wild-type and mutant bands are 405 bp and ~230 bp, respectively. For *Mis* mice: mMIS-oIMR501, GGAACACAAGCAGAGCTTCC; mMIS-oIMR502, GAGACAGAGTCCATCACGTACC; and mMIS-oIMR029, TCGTGCTTTACGGTATCGC. The wild-type and mutant bands are 243 bp and ~520 bp, respectively. For *Misr2* mice: mMISR2-FW1, CCTCATACTTTCCTTAGAATGA; mMISR2-Rv2, TATGACCCGTTAGTCTATGACA; PGK-FX3, AGACTGCCTTGGGAAAAGCGC. The wild-type and mutant bands are 394 bp and ~170 bp, respectively. For *MT-hMIS*$^{tg/+}$ mice: hMIS-Fw1, CCCTAGTGCTGTCTGCCCT; hMIS-Rv2, GGAGCTGCTGCCATTGCTG; Rap-A, AGGACTGGGTGGCTTCCAACTCCCAGACAC; and Rap-B, AGCTTCTCATTGCTGCGCGCCAGGTTCAGG. The transgene and control bands are 176 bp and 590 bp, respectively. For sex genotyping by *Sry*: mSry-Fw6, TGACTGGGATGCAGTAGTTC; mSry-Rv6, TGTGCTAGAGAGAAACCCTG; and Rap-A and RapB primers described above. The *Sry* and control bands are ~230 bp and 590 bp, respectively. For *Pax2* mice: mPax2-Fw1, CCCACCGTCCCTTCCTTTTCTCCTCA; mPax2-Rv2, GAAAGGCCAGTGTGGCCTCTAGGGTG; PGK-FX3, AGACTGCCTTGGGAAAAGCGC. The wild-type and mutant bands are 245 bp and ~150 bp, respectively. For *Wnt7a* mice: mWnt7a-#553, TCACGTCCTGCACGACGCGAGCTG; mWnt7a-#1143, CTCTTCGGTGGTAGCTCTGG; mWnt7a-#1144, CCTTCCCGAAGACAGTACGC. The wild-type and mutant bands are 208 bp and ~330 bp, respectively. All PCR protocols were performed using a DNA Thermal Cycler 480 (Perkin-Elmer, Wellesley, MA) with 35 cycles at 94°C for 30 seconds, 65°C for 30 seconds, 72°C for 45 seconds (except for *Mis* and *Misr2* mice; 35 cycles at 94°C for 30 seconds, 56°C for 30 seconds, 72°C for 45 seconds). A PCR genotyping method for *Wnt4* mice was described previously (Stark et al., 1994).

Generation of XO ES cell lines and female chimeras

To generate XO ES cell lines, *Lim1*$^{-/-}$ and *Lim1*$^{+/+}$ *Rosa26*$^{tg/+}$ XY ES cell lines (Shawlot et al., 1999) were plated at clonal density on culture dishes coated with feeder cells. Colonies were recovered and

prescreened by dot blot hybridization and subsequently by Southern hybridization after *Eco*RI digestion using a Y chromosome-specific repeat probe Y353/B (Bishop and Hatat, 1987). After expansion of the XO ES cells, their genotypes were reconfirmed by Southern blot hybridization with Y353/B and a 5′ *Lim1* probe (Shawlot and Behringer, 1995). The loss of the Y chromosome in the ES cell lines was confirmed by karyotype analysis (Nagy et al., 2003). Chimeras were generated by injection of ES cells into blastocysts derived from X-linked GFP males bred with Swiss Webster female mice (Bradley, 1987). Yolk sacs of chimeric embryos were collected for sex genotyping using the *Sry* gene.

X-gal staining of embryos

X-gal staining for β-gal activity was performed as described (Nagy et al., 2003). After overnight post-fixation with 4% paraformaldehyde in PBS, photographs were taken. For histological analysis, paraffin wax-embedded tissues were sectioned at 7 μm and counterstained with 0.33% eosin-Y.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed as described (Shawlot and Behringer, 1995)

Results

Dynamic expression of *Lim1* during Müllerian duct development

To further understand *Lim1* function during urogenital organogenesis, we examined *Lim1* expression in developing urogenital tissues using mice that were heterozygous for a *Lim1-tau-lacZ* (*Lim1*^{tlz}) knock-in allele (Shawlot et al., 1999; Kania et al., 2000). β-galactosidase (β-gal) activity was observed in the Wolffian ducts and mesonephric tubules (Fig. 1A-F), consistent with previous reports of *Lim1* expression in these tissues (Barnes et al., 1994; Fujii et al., 1994; Karavanov et al., 1998). Surprisingly, we also detected β-gal activity in the Müllerian duct, which was very dynamic during embryogenesis (Fig. 1). The onset of *Lim1*^{tlz} expression in the Müllerian duct was detected at embryonic day 11.5 (E11.5) in the most anterior region of the urogenital ridge (Fig. 1A). At this stage, the Müllerian duct has just formed as a small invagination of the surface epithelium of the mesonephros, located adjacent to the cranial end of the Wolffian duct. Subsequently, *Lim1*^{tlz} expression in the Müllerian duct extends caudally parallel to the Wolffian duct (Fig. 1B). At E12.5, *Lim1*^{tlz} is expressed in the Müllerian duct along the cranial two-thirds of the urogenital ridge (Fig. 1C). *Lim1*^{tlz} expression was also detected in the developing metanephros, the definitive kidney in mammals (Fig. 1C,E,F). At E13.5, *Lim1*^{tlz} expression in the Müllerian duct further extended caudally towards the cloaca and was observed in both Müllerian and Wolffian ducts along the entire urogenital ridge in both sexes (Fig. 1E,F). No obvious sexual dimorphic pattern of *Lim1*^{tlz} expression in the reproductive ducts was observed up to this stage.

Because the *lacZ* reporter gene that was introduced into the mouse *Lim1* locus replaces the entire *Lim1*-coding region including all four introns (Shawlot et al., 1999; Kania et al., 2000), there was concern that the inserted *lacZ* transgene might disturb the transcriptional regulation of *Lim1* gene expression and thus the *lacZ* reporter mice may not reflect endogenous *Lim1* expression patterns. To exclude this possibility, we performed whole-mount in situ hybridization of urogenital tissues from wild-type embryos. The distribution of *Lim1*

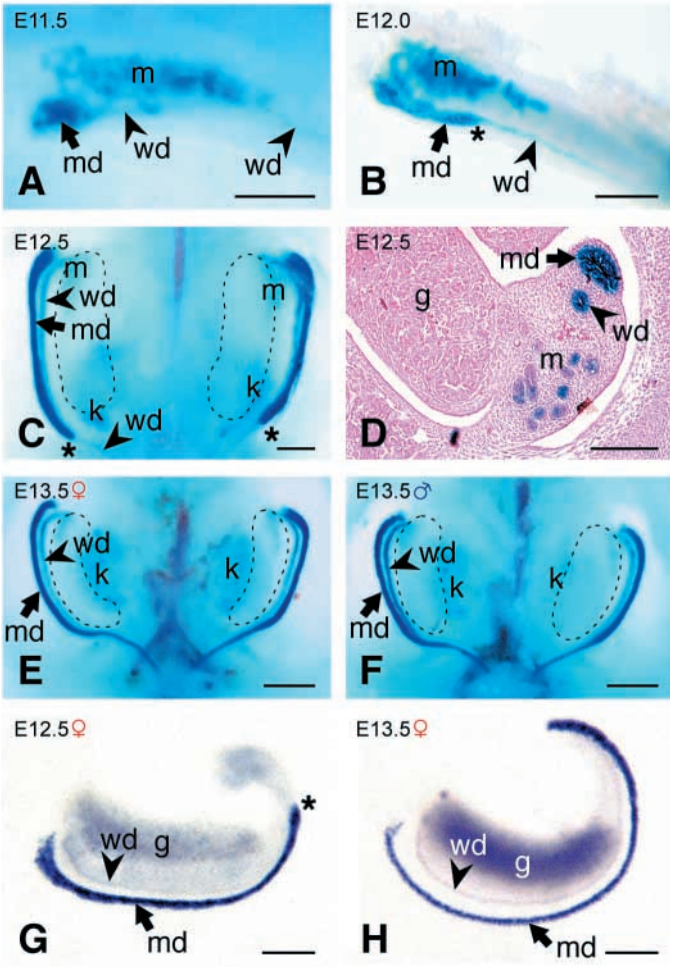

Fig. 1. Dynamic expression of *Lim1* during Müllerian duct formation. (A-F) *Lim1*^{tlz} expression in the developing urogenital system. Cranial mesonephric region at E11.5 (A) and E12.0 (B). Ventral view at E12.5 (C) and at E13.5 in females (E) and males (F). Cross-section of the anterior mesonephric region at E12.5 counterstained with Eosin (D). (G,H) *Lim1* expression detected using whole-mount in situ hybridization at E12.5 (G) and E13.5 (H) in the female genital system. Asterisks point to the posterior end of the Müllerian duct and broken lines indicate the gonad. g, gonad; k, kidney (metanephros); m, mesonephros; md, Müllerian duct; wd, Wolffian duct. Scale bars: 250 μm in A-C,G,H; 100 μm in D; 500 μm in E,F.

transcripts in the Wolffian and Müllerian ducts of wild-type embryos was identical to the β-gal expression pattern in *Lim1*^{tlz} heterozygotes. *Lim1* is expressed in the Wolffian duct along the entire urogenital ridge, and *Lim1* expression in the Müllerian duct dynamically extends caudally between E11.5 and E13.5 (Fig. 1G,H, data not shown). These data indicate that the β-gal expression pattern in *Lim1*^{tlz} mice faithfully reflects endogenous *Lim1* expression in the developing genital ducts and validate our expression data using the *Lim1*^{tlz} reporter.

To determine which tissues specifically express *Lim1* in the urogenital tract, we performed a histological analysis. We found that *Lim1*^{tlz} is specifically expressed in the epithelium of the Müllerian and Wolffian ducts and the mesonephric tubules (Fig. 1D), but not in the surrounding mesenchyme of these

tissues. At this stage (E12.5), the mesonephric tubules begin to regress. Histological analysis of serial sections along the longitudinal axis of the urogenital ridge revealed that no epithelial structure of the Müllerian duct was detected posteriorly to the distal tip of *Lim1*^{tlz} expression in the Müllerian duct (data not shown). These data indicate that *Lim1* expression is coincident with Müllerian duct formation and suggests a role in female reproductive organ formation.

Although *Lim1* expression has been detected in the fetal gonads by RT-PCR and in situ hybridization (Fig. 1G,H) (Nagamine and Carlisle, 1996; Nagamine et al., 1999; Birk et al., 2000; Bouchard et al., 2002) (A.K. and R.R.B., unpublished), no β-gal staining was observed in the gonads of *Lim1*^{tlz} heterozygous knock-in embryos (Fig. 1A-F).

Sexual dimorphic expression of *Lim1* in the developing reproductive tract

We further examined *Lim1* expression at later stages of urogenital development using the *Lim1*^{tlz} reporter. Sexual dimorphic patterns of *Lim1*^{tlz} expression were observed in the reproductive tract beginning at E14.5. In females, strong *Lim1*^{tlz} expression was observed in the Müllerian duct at E14.5 (Fig. 2A) and this strong expression persists until E15.5 (Fig. 2C,E). *Lim1*^{tlz} expression becomes weaker throughout the Müllerian duct at E16.5 (Fig. 2G). By E17.5, *Lim1*^{tlz} expression in the Müllerian duct becomes restricted anteriorly, to the prospective oviduct. *Lim1*^{tlz} expression in the posterior presumptive uterus region is downregulated and becomes undetectable (Fig. 2I). *Lim1*^{tlz} expression in the Müllerian duct was not observed at later stages (data not shown). In the Wolffian duct of females, *Lim1*^{tlz} expression is lost from the anterior gonadal region around E15.25 and becomes undetectable at later stages (Fig. 2E,G,I, data not shown).

In males, *Lim1*^{tlz} expression in the Müllerian duct appears thinner at E14.5 (Fig. 2B) compared with the expression in females at the same stage (Fig. 2A). At E15.25, *Lim1*^{tlz} expression in the Müllerian duct becomes discontinuous from its cranial region (open arrows in Fig. 2D). Subsequently, this discontinuity of *Lim1*^{tlz} expression spreads caudally and *Lim1*^{tlz} expression in the Müllerian duct shows a fragmented pattern throughout the entire reproductive tract region at E15.5 (Fig. 2F). At E16.5, *Lim1*^{tlz} expression in the Müllerian duct becomes undetectable except for expression in a few vesicular structures (arrows in Fig. 2H). Histological analysis of serial sections of the male urogenital system at E15.5 showed that there is no epithelium tissue of the Müllerian duct where *Lim1*^{tlz} expression is absent (data not shown). This indicates that the *Lim1*^{tlz} expression fragmentation is likely caused by loss of the epithelial tissue during the process of Müllerian duct regression. *Lim1*^{tlz} expression in the Wolffian duct and its derivatives such as the epididymis and vas deferens persists in males during these stages (Fig. 2B,D,F,H,J) and becomes upregulated around E17.5 (Fig. 2J).

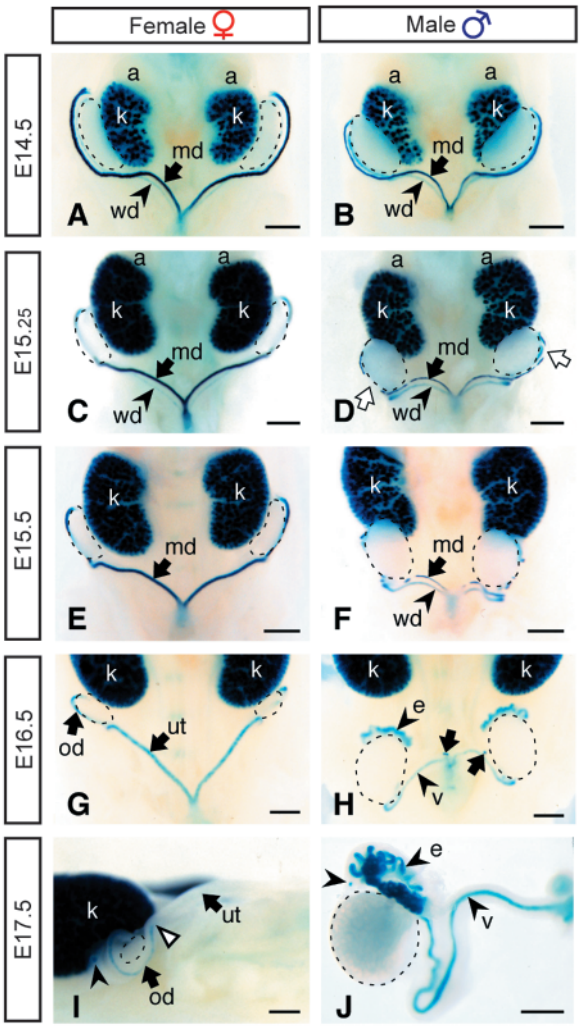

Fig. 2. Sexual dimorphic expression pattern of *Lim1* in the developing reproductive tract. *Lim1*^{tlz} expression in the developing urogenital system in females (A,C,E,G,I) and males (B,D,F,H,J). (A,B) Ventral view at E14.5. Note that *Lim1*^{tlz} expression in the Müllerian duct is thinner in males compared to its expression in females. (C,D) Ventral view at E15.25. *Lim1*^{tlz} expression in the Müllerian duct is discontinuous in the anterior region of males (open arrow in D). (E,F) Ventral view at E15.5. *Lim1*^{tlz} expression in the Müllerian duct is fragmented in males (arrow in F). (G,H) Ventral view at E16.5. *Lim1*^{tlz} expression in the Müllerian duct is restricted to a few vesicles in males (arrow in H). (I,J) Lateral view at E17.5. *Lim1*^{tlz} is expressed in the presumptive oviduct, but not in the uterus in females (I). The open arrowhead indicates the boundary of the presumptive oviduct and uterus. *Lim1*^{tlz} expression is upregulated in the male reproductive tract (J). a, adrenal; e, epididymis; k, kidney (metanephros); md, Müllerian duct; od, oviduct; ut, uterus; v, vas deferens; wd, Wolffian duct. Scale bar: 500 μm.

Visualization of Müllerian duct regression by *Lim1*^{tlz} expression

MIS signaling is both essential and sufficient for Müllerian duct regression. MIS ligand is expressed by Sertoli cells in the testis from E11.5 (Hacker et al., 1995; Swain and Lovell-Badge, 1999) and the MIS type II receptor, *Misr2*, is expressed in the mesenchyme of the Müllerian duct from E13.0 in an anterior to posterior manner (A.K. and R.B., unpublished) in the mouse. We observed sexual dimorphic expression patterns of *Lim1*^{tlz} expression in the Müllerian duct from E14.5 (Fig. 2), 1 day after *Misr2* is expressed in the mesenchyme along the entire length of the Müllerian duct. The timing of this differential expression pattern suggests that the fragmentation of *Lim1*^{tlz} expression reflects MIS-induced Müllerian duct

regression in males during embryogenesis. Therefore, we examined *Lim1*^{tlz} expression in MIS signaling mutant male mice. *Lim1*^{tlz} mice were bred with mice with mutations in *Mis*, *Misr2* or *Wnt7a*. We found that the fragmentation of *Lim1*^{tlz} expression (i.e. loss of Müllerian duct epithelium) is completely inhibited and *Lim1*^{tlz} is expressed continuously along the persistent Müllerian duct in *Mis*, *Misr2* and *Wnt7a* mutant males (Fig. 3A-D). *Lim1*^{tlz} expression in the Wolffian duct and kidney was not affected in these mutant males. These data indicate that the fragmentation of *Lim1*^{tlz} expression in the Müllerian duct depends on MIS signaling.

We also examined *Lim1*^{tlz} expression in female transgenic mice, in which human *MIS* is ectopically expressed (Behringer et al., 1990). This ectopic human *MIS* expression in females causes Müllerian duct regression. We detected no difference in *Lim1*^{tlz} expression patterns until E13.5 between wild-type and transgenic females (data not shown). However, *Lim1*^{tlz} expression in the Müllerian duct of *Mis* transgenic female mice (Fig. 3E) becomes thinner than the *Lim1*^{tlz} expression of wild-type littermate females at E14.5 (Fig. 3F). At E15.5, *Lim1*^{tlz} is still strongly expressed in the Müllerian duct of wild-type females (Fig. 3G). At the same stage, the *Lim1*^{tlz} expression in the Müllerian duct becomes discontinuous in *Mis* transgenic female mice (Fig. 3H). At E16.5, *Lim1*^{tlz} is weakly expressed along the entire Müllerian duct in wild-type females (Fig. 3I). At the same stage, *Lim1*^{tlz} expression in the Müllerian duct is restricted to the cranial end and within a few vesicles in *Mis* transgenic female mice (Fig. 3J). Ectopic expression of *Mis* causes thinner and flattened ovaries (Fig. 3E-J) (Lyet et al., 1995) but did not affect *Lim1*^{tlz} expression in the Wolffian duct and kidney (Fig. 3E-L). These data suggest that MIS signaling is sufficient to cause fragmentation of *Lim1*^{tlz} expression in the Müllerian duct. We also examined *Lim1*^{tlz} expression in *Mis* transgenic males. The fragmentation of *Lim1*^{tlz} expression is enhanced in *Mis* transgenic males compared with wild-type males at E15.5 (Fig. 3K,L).

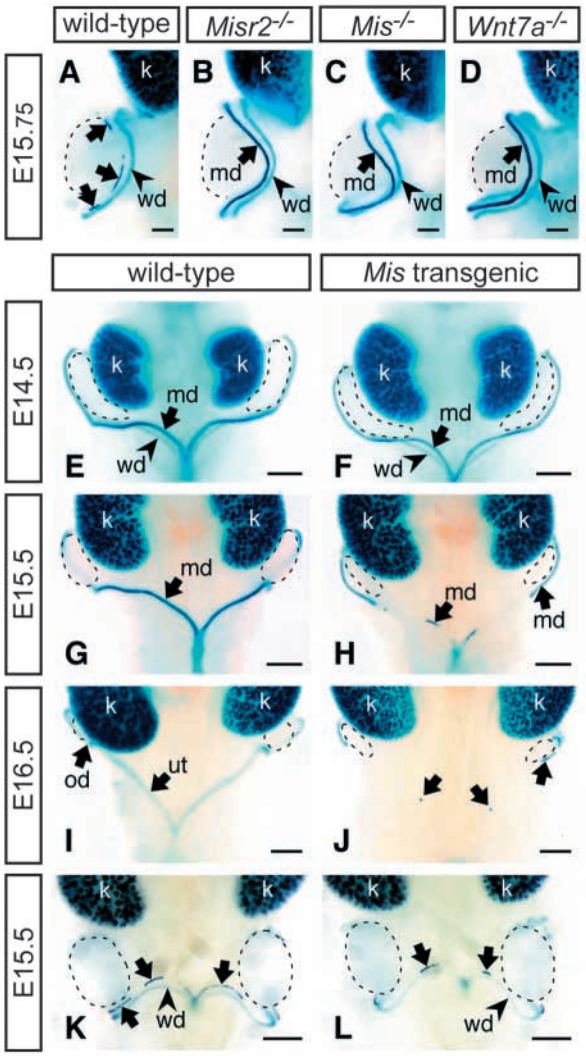

Fig. 3. *Lim1* expression in MIS signaling mutant mice. (A-D) Lateral view of the testicular region in wild-type (A), *Misr2*$^{-/-}$ (B), *Mis*$^{-/-}$ (C) and *Wnt7a*$^{-/-}$ (D) male embryos at E15.75. Fragmentation of *Lim1*^{tlz} expression in the Müllerian duct is observed in wild-type embryos (arrows in A), which is suppressed in MIS signaling-deficient mutants (B-D). (E-J) Ventral view of the urogenital system in wild-type (E,G,I) and *MT-hMIS*$^{tg/+}$ transgenic (F,H,J) female embryos. (K,L) Ventral view of the testicular region in wild-type (K) and *MT-hMIS*$^{tg/+}$ transgenic (L) male embryos. Note that fragmentation of *Lim1*^{tlz} expression in the Müllerian duct is enhanced in MIS transgenic males. k, kidney (metanephros); md, Müllerian duct; od, oviduct; ut, uterus; wd, Wolffian duct. Scale bars: 250 μm in A-D; 500 μm in E-L.

Absence of the female reproductive tract in female *Lim1*-null mice

The dynamic expression pattern of *Lim1* in the developing Müllerian duct indicates that *Lim1* may have an important function during female reproductive tract organogenesis. Unfortunately, most *Lim1* mutants die at around E10 probably because of a failure of chorion-allantois fusion before the formation of the Müllerian duct that is initiated around E11.5. Previously, we found that rare *Lim1* mutants escaped this embryonic lethality and survived to the birth. These *Lim1*-null escapers lacked anterior head and kidney formation. However, only four escapers had been obtained out of more than 1000 (<0.4%) pups born (Shawlot and Behringer, 1995).

One explanation for *Lim1*-null mutant survival to birth is that the embryonic lethality is affected by genetic modifiers. Therefore, we modified the genetic background of the *Lim1* mutation to increase phenotypic variety by outcrossing *Lim1* heterozygous mice (maintained in C57BL/6J×129/SvEv mixed background) with Swiss Webster (Taconic) outbred mice. Subsequently, we intercrossed the resulting *Lim1* heterozygous offspring from different pedigrees to screen for *Lim1*-null escapers. We found one breeding pair that produced two *Lim1*-null escapers out of 76 (2.6%) pups. These escapers were genotyped and one escaper was an XX female and the other an XY male. We also intercrossed *Lim1* heterozygous offspring from the mating pair that produced the two escapers and obtained two additional XX *Lim1*-null escaper neonates.

We analyzed the three XX *Lim1*-null neonates and one of the original four escapers that was also XX for female reproductive organ development. All of four *Lim1*-null female neonates had the identical phenotype. There was no anterior head formation (Fig. 4A,B). Examination of the internal reproductive organs of these female *Lim1*-null neonates showed that they had ovaries that were morphologically normal

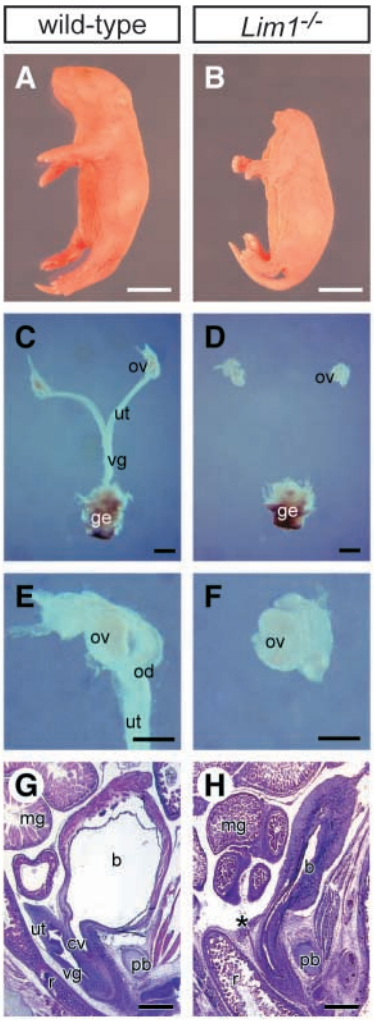

Fig. 4. Absence of the female reproductive tract in *Lim1*$^{-/-}$ female neonates. (A,B) Newborns of wild-type (A) and *Lim1*$^{-/-}$ (B). (C,D) Gross view of the female reproductive system from wild-type (C) and *Lim1*$^{-/-}$ (D) newborns. (E,F) High magnification of the anterior gonadal region in C and D, respectively. Note that *Lim1*$^{-/-}$ XX newborns have normal ovaries (ov). (G,H) Longitudinal sections of the urogenital system from wild-type (G) and *Lim1*$^{-/-}$ (H) female newborns. Note that neither epithelium nor mesenchyme of the female reproductive tract is found in *Lim1*$^{-/-}$ XX mice (asterisk in H). b, bladder; cv, cervix; ge, genitalia; mg, midgut; od, oviduct; ov, ovary; pb, pubic bone; r, rectum; ut, uterus; vg, vagina. Scale bars: 5 mm in A,B; 500 μm in C-H.

(Fig. 4D,F). The male *Lim1*-null escaper had testes that were indistinguishable from the testes of wild-type animals (data not shown). Although the female *Lim1*-null neonates had ovaries, they completely lacked derivatives of the Müllerian duct, including oviducts, uterus, cervix and the upper region of the vagina (Fig. 4C-H). Although *Lim1* is expressed only within the epithelium of the Müllerian duct (Fig. 1D), both the epithelium and the mesenchyme of the female reproductive tract were completely absent in the female *Lim1*-null neonates (Fig. 4G-H). These data suggest that *Lim1* is essential for Müllerian duct development during embryogenesis and that lack of *Lim1* activity results in Müllerian agenesis in females.

Generation of female mouse chimeras

To obtain further clues about action of *Lim1* during female reproductive tract development, we generated chimeric mice composed of *Lim1*$^{-/-}$ and wild-type cells by injection of mutant ES cells into wild-type blastocysts (Tam and Rossant, 2003). In mammals, testis determination depends on the expression of the Y chromosome-linked *Sry* gene in the gonad of embryos. Most ES cell lines commonly used are genetically XY. The incorporation of XY cells into the gonad of chimeric animals can cause female-to-male sex reversal, resulting in a male phenotype and therefore regression of the Müllerian duct system (Tarkowski, 1998). Therefore, to avoid this aspect of chimera biology and study the role of *Lim1* in female reproductive tract development, we developed a novel female mouse chimera strategy (Fig. 5A).

XY ES cells spontaneously lose the Y chromosome and XO cells are found at a ~1-2% frequency (A. Bradley, personal communication). Therefore, we exploited this phenomenon and generated *Lim1*$^{-/-}$ and *Lim1*$^{+/+}$ *Rosa26*-marked XO female ES cell lines. To establish XO ES cell lines, we plated *Lim1*$^{-/-}$ and *Lim1*$^{+/+}$ *Rosa26*-marked XY ES cells (Shawlot et al., 1999) at clonal density. 576 ES cell colonies were recovered and examined. A Y chromosome-specific repeat probe, Y353/B, was used to screen for Y-chromosome deficiency (Bishop and Hatat, 1987). Loss of the Y chromosome was observed in nine independent clones, including five *Lim1*$^{-/-}$ lines and three *Lim1*$^{+/+}$ lines (Fig. 5B). All XO ES cell lines were used to generate chimeric mice.

To generate female chimeras, *Lim1*$^{-/-}$ and *Lim1*$^{+/+}$ XO ES cells were injected into wild-type XX blastocysts (Fig. 5A). XX blastocysts were distinguished from XY blastocysts using X-linked GFP transgenic male mice (Hadjantonakis et al., 1998) for matings with wild-type females. In this strategy, only chimeric embryos derived from XX blastocysts are GFP positive. Chimeric embryos were harvested and stained with X-gal to distinguish ES cell-derived cells from blastocyst-derived cells. The sex genotype of the recipient blastocysts was further confirmed by PCR genotyping for *Sry* using the yolk sac of the chimeras.

Cell-autonomous requirement of *Lim1* for Müllerian duct epithelium formation

We generated 27 female chimeras and analyzed the distribution of ES-derived cells (Fig. 6A,B). Identical results were obtained for all five *Lim1*$^{-/-}$ XO ES cell lines and for all three *Lim1*$^{+/+}$ XO ES cell lines examined. High contribution of XO *Lim1*$^{-/-}$ cells in chimeric animals caused craniofacial abnormalities. These included loss of the lower jaw (data not shown) and, in more severe cases, head truncation (Fig. 6B). However, the *Lim1*-null urogenital defects were completely rescued in all chimeric animals composed of *Lim1*-null and wild-type cells that were recovered. Histological analysis was performed to understand the tissue distribution of *Lim1*$^{-/-}$ cells in chimeric female mice. In control experiments, XO wild-type cells could contribute to both the epithelium and mesenchyme of the uterus and oviduct in chimeric females at E18.5 (Fig. 6C,E). We also examined other organs and did not observe any bias of distribution in these chimeras. This indicates that XO cells can extensively contribute to the somatic tissues of chimeric mice when XO cells are mixed with XX cells. By contrast, when XO *Lim1*$^{-/-}$ cells were used to generate female chimeric mice, we found that these *Lim1*$^{-/-}$ cells

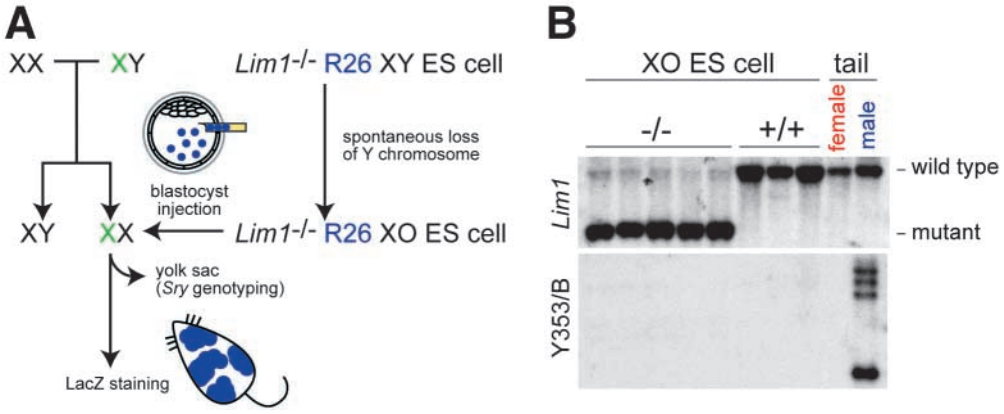

Fig. 5. Strategy to generate female mouse chimeras. (A) Schematic diagram of female chimera analysis. *Lim1*$^{-/-}$;*Rosa26*$^{tg/+}$ XO ES cells were generated from *Lim1*$^{-/-}$;*Rosa26*$^{tg/+}$ XY ES cells (Shawlot et al., 1999) by spontaneous loss of the Y chromosome by screening with an Y-chromosome-specific repeat probe, Y353/B. These XO ES cells were injected into XX blastocysts to generate chimeric female mice. XX blastocysts can be distinguished from XY blastocysts by using X-linked ubiquitous GFP male mice for breeding. Chimeric mice were recovered at different stages of embryogenesis and processed for β-gal staining to visualize *Rosa26*-marked ES cell-derived XO cells. The yolk sac was collected for reconfirming sexes of injected blastocysts by PCR genotyping for the *Sry* gene. (B) Southern blot analysis of XO ES cells. Genomic DNA of XO ES cells was blotted after *Eco*RI digestion and hybridized with a *Lim1* probe. The same blotted membrane was hybridized again with Y-chromosome-specific Y353/B probe. Genomic DNA from male and female tails was used for comparison. Note that these XO cells show the same pattern as female tail.

did not contribute to the epithelium of the uterus at E18.5, although these *Lim1*$^{-/-}$ cells could contribute extensively to the uterus mesenchyme (Fig. 6D). We also found that *Lim1*$^{-/-}$ cells were not present in the epithelium of the oviduct at the same stage (Fig. 6F). These data suggest that *Lim1* function is required cell-autonomously for epithelium development of the uterus and oviduct.

To test if *Lim1* is required for formation of the Müllerian duct, we also analyzed chimeric mice at E12.5 when the Müllerian duct is being formed prior to its most caudal extension along the urogenital ridge (Fig. 1C,G). In control experiments, XO *Lim1*$^{+/+}$ cells contribute to the both epithelium and mesenchyme of the Müllerian and Wolffian ducts of chimeric mice along with wild-type cells at this stage (Fig. 6G). However, in chimeric mice composed of XO *Lim1*$^{-/-}$ cells, no *Lim1*$^{-/-}$ cells were observed in the Müllerian duct epithelium (Fig. 6H). This indicates that *Lim1* function is required cell-autonomously during Müllerian duct epithelium formation. We also found that *Lim1*$^{-/-}$ cells were not found in the Wolffian duct epithelium at this stage. This suggests that *Lim1* function is also required cell-autonomously for epithelium development of the male reproductive tract.

Lim1 expression in Müllerian duct precursor cells is independent of *Wnt4* function

To understand interactions with other genes that are required for Müllerian duct development, we examined *Lim1*^{tlz} expression in *Pax2*, *Wnt4* and *Wnt7a* mutants (Fig. 7). *Wnt7a* is expressed in the Müllerian duct epithelium from E11.5

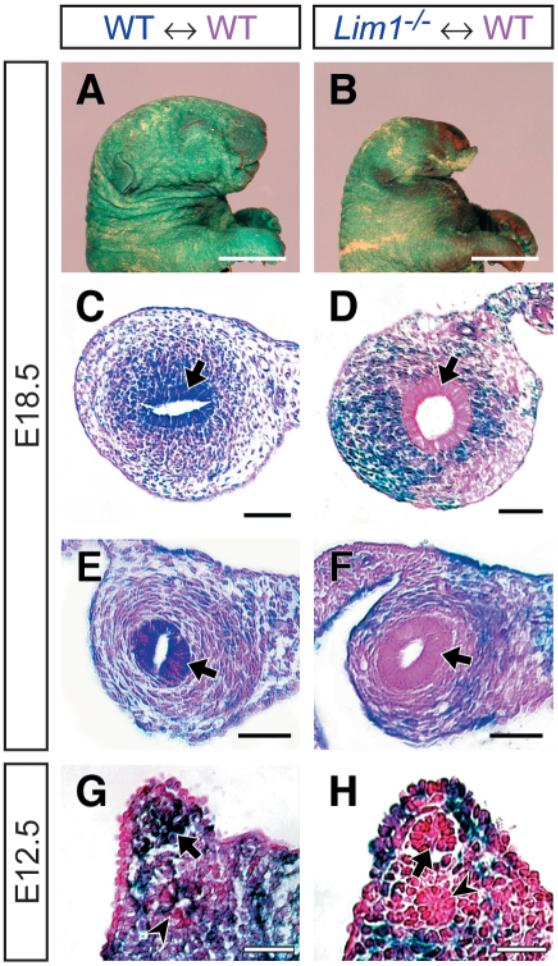

Fig. 6. Cell-autonomous requirement of *Lim1* for epithelium development of the female reproductive tract. (A-B) Craniofacial development of female chimeras at E18.5. XO cells derived from ES cells are stained blue by β-gal staining. High contribution of *Lim1*$^{-/-}$ cells in chimeras cased head truncations. (C-F) The female reproductive tract of female chimeras. XO cells derived from ES cells are stained blue by β-gal staining and wild-type XX cells derived from blastocysts are pink by eosin staining. *Lim1*$^{-/-}$ cells are not present in the epithelium in the uterus (arrow in D) and the oviduct (arrow in F) but present in the surrounding mesenchyme of the both tissues. In control experiments, wild-type XO cells can contribute to the epithelium of the uterus (arrow in C) and the oviduct (arrow in E). (G,H) The reproductive tract of female chimeras at E12.5. *Lim1*$^{-/-}$ XO cells are not present in the epithelium of the developing Müllerian duct (arrow in H). *Lim1*$^{-/-}$ XO cells are also not found in the epithelium of the Wolffian duct (arrowhead in H). Scale bars: 5 mm in A,B; 50 μm in C-F; 25 μm in G,H.

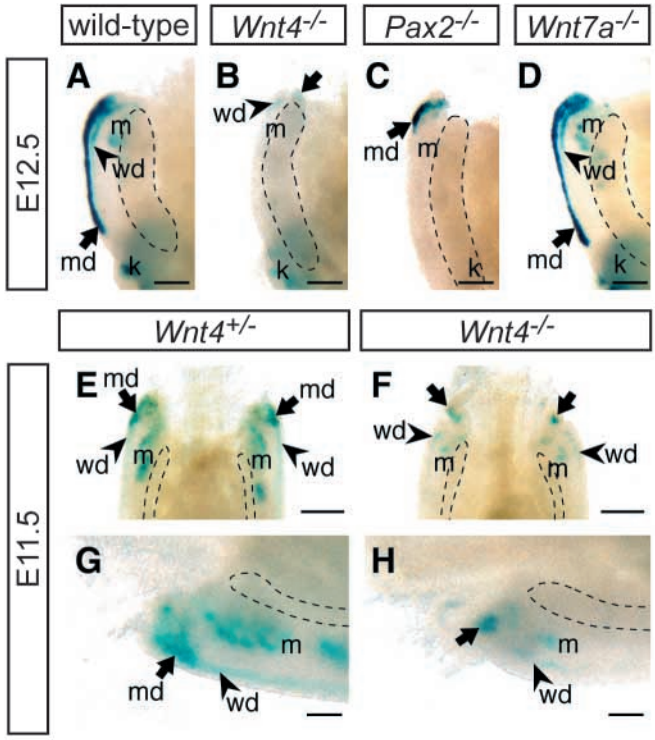

Fig. 7. *Lim1* expression in mouse mutants with Müllerian duct abnormalities. (A-D) Ventral view of the right urogenital system in wild-type (A), *Wnt4*$^{-/-}$(B), *Pax2*$^{-/-}$(C) and *Wnt7a*$^{-/-}$(D) embryos at E12.5. (E,F) Ventral view of the anterior urogenital system in *Wnt4*$^{+/-}$ (E) and *Wnt4*$^{-/-}$(F) embryos at E11.5. Arrows in F indicate *Lim1*^{tlz} expressing presumptive Müllerian duct precursor cells. (G,H) Lateral view with high magnification. Note that *Lim1*^{tlz}-positive Müllerian duct precursor cells do not show funnel-shaped invagination (arrow in H). Broken lines indicate the gonad. k, kidney (metanephros); m, mesonephros; md, Müllerian duct; wd, Wolffian duct. Scale bars: 250 μm in A-F; 100 μm in G,H.

(Vainio et al., 1999). In *Wnt7a* mutant females, the Müllerian duct is formed but tissues of the oviduct and uterus fail to form proper cytoarchitectures (Miller and Sassoon, 1998), indicating a requirement of *Wnt7a* for Müllerian duct differentiation. In *Wnt7a* mutants, *Lim1*^{tlz} expression in the urogenital system, including the Müllerian duct, was identical to wild-type at E12.5 (Fig. 7A,D). In *Pax2* mutants, the Wolffian duct is formed only anteriorly at E11.5 but starts to degenerate at E12.5 (Torres et al., 1995). *Pax2* mutants also lack mesonephric tubule formation and metanephros induction. *Pax2* is also expressed in the epithelium of the developing Müllerian duct by E13.5 and *Pax2* mutants form only the anterior region of the Müllerian duct by E13.0 but it degenerates subsequently by E16.5 (Torres et al., 1995), indicating a *Pax2* requirement for maintenance of the Müllerian duct. *Lim1*^{tlz} expression in the Wolffian duct, mesonephros and metanephros was not observed in *Pax2* mutants at E12.5 (Fig. 7C). However, the shortened Müllerian duct of *Pax2* mutants still expressed *Lim1*^{tlz} at the same stage (Fig. 7C). Taken together, these data suggest that *Lim1* expression in the Müllerian duct does not require *Wnt7a* or *Pax2* function.

It was reported that the Müllerian duct is absent in *Wnt4* mutants at E11.5 and E12.5 (Vainio et al., 1999), suggesting a *Wnt4* requirement for Müllerian duct formation. Surprisingly, we found ectopic weak staining at the anterior end of the mesonephros of *Wnt4* mutants at E12.5 (Fig. 7B). To understand the origin of these *Lim1*^{tlz} positive cells, we also examined *Lim1*^{tlz} expression in *Wnt4* mutants at E11.5, when the Müllerian duct just begins to form. We found *Lim1*^{tlz} expression at the anterior end of the mesonephros of *Wnt4* mutants (Fig. 7F), where the invagination of the Müllerian duct normally initiates (Fig. 7E). At this stage, the Müllerian duct shows a funnel shape of the invaginating surface epithelium of the mesonephros in wild-type or *Wnt4*$^{+/-}$ control embryos (Fig. 1A, Fig. 7E,G). However, the *Lim1*^{tlz} positive cells in the anterior mesonephros of *Wnt4* mutants do not invaginate for tubulogenesis of the Müllerian duct, but remain as a small cluster (Fig. 7F,H). These observations suggest that induction of *Lim1* expression in Müllerian duct precursor cells does not require *Wnt4* function. These data also suggest that *Wnt4* is required for invagination of the Müllerian duct, but not specification of its precursor cells. *Lim1*^{tlz} expression in the mesonephos of *Wnt4* mutants is greatly reduced at E11.5 and almost absent at E12.5 (Fig. 7B,F,H), suggesting a *Wnt4* requirement for mesonephros development.

Discussion

Visualization of Müllerian duct development by *Lim1*^{tlz}

Analysis of the expression of the *Lim1*^{tlz} allele in mice has revealed a previously unrecognized tissue for *Lim1* expression, the epithelium of the developing Müllerian duct and its derivatives in the uterus and oviducts. This *Lim1*^{tlz} allele provides a visual marker that reveals for the first time the dynamic morphological changes associated with the formation and differentiation of the Müllerian duct into the organs of the female reproductive tract, and the regression of the prospective female reproductive tract primordium in male fetuses. The Müllerian duct forms adjacent to the Wolffian (mesonephric) duct in an anterior to posterior manner by the invagination of the anteriolateral coelomic epithelium of the mesonephros (Gruenwald, 1941; Kaufman and Bard, 1999). *Lim1*^{tlz} expression was coincident with the initial formation of the Müllerian duct epithelium, suggesting that *Lim1* may be essential in this tissue for the formation of the Müllerian duct. Expression was maintained in the developing Müllerian duct until E16.5 but was then downregulated. However, *Lim1* expression persisted in the differentiating oviduct, indicating that *Lim1* may have later roles in the regional differentiation of the female reproductive tract perhaps regulating oviductal morphogenesis by altering cell shape or regional proliferation. Interestingly, *Hoxa10* is expressed in the developing uterus, but not in the oviduct at this stage (Benson et al., 1996; Ma et al., 1998). In *Hoxa10* mutant females, the anterior uterus is transformed into the oviduct (Benson et al., 1996), suggesting that *Hoxa10* may downregulate *Lim1* expression in the presumptive uterus to inhibit oviduct differentiation.

During male sexual development, the Müllerian ducts are eliminated by the actions of the MIS signaling pathway (Josso et al., 1993). Regression of the Müllerian duct system visualized by the *Lim1*^{tlz} reporter was first observed at E14.5

in male fetuses as a thinning of the Müllerian duct in comparison with females. This thinning correlates with the reduction in the diameter of the Müllerian duct as the adjacent mesenchymal cells condense during the regression process. Over the next 2 days of embryonic development, the Müllerian duct system in the male fetus is eliminated. In males that lack MIS, its type II receptor or Wnt7a, the *Lim1*^{tlz} reporter documented the persistence of the Müllerian duct in the absence of MIS signaling. Thus, the *Lim1*^{tlz} reporter provides the first opportunity to visualize Müllerian duct regression or persistence from the perspective of the ductal epithelium. In the mouse, MIS expression is first detected around E11.5 at the time of testis determination (Hacker et al., 1995; Swain and Lovell-Badge, 1999). MIS signaling requires the MIS type II receptor expressed in the mesenchyme cells adjacent to the Müllerian duct epithelium (Baarends et al., 1994; Mishina et al., 1996). This MIS type II receptor expression requires the function of Wnt7a, which is expressed in the Müllerian duct epithelium (Parr and McMahon, 1998). Using a *Misr2-lacZ* knock-in allele in mice, we have detected β-gal activity throughout the Müllerian duct mesenchyme by E13.5 (N. A. Arango, A.K. and R.R.B., unpublished). Overexpression of human MIS in female transgenic mice causes the elimination of Müllerian ducts and therefore they do not have a uterus or oviducts (Behringer et al., 1990). Interestingly, the female *Mis* transgenic fetuses had an altered spatial pattern of Müllerian duct regression in comparison with normal males. Whereas fragmentation of the *Lim1*^{tlz} β-gal pattern (i.e. loss of the Müllerian duct epithelium) in males followed an anterior to posterior pattern, in females, fragmentation was throughout the anterior-posterior limits of the Müllerian ducts. These observations indicate that at E15.5 the posterior Müllerian duct is competent for regression, consistent with the establishment of *Misr2* expression in the adjacent mesenchyme along the entire Müllerian duct at this stage. We also found that the regression of the Müllerian duct epithelium of male *Mis* transgenic fetuses was enhanced, suggesting that MIS is not at saturating levels for its signal transduction in vivo as shown previously using reconstruction from serial sections (Allard et al., 2000).

Role of *Lim1* in female reproductive tract development

Most *Lim1* mutants die at mid-gestation because of defects in allantois differentiation and the subsequent failure of chorio-allantoic fusion to establish the maternal-fetal connection (Shawlot and Behringer, 1995). *Lim1* is expressed in the primitive streak during gastrulation. Thus, these defects in allantoic development are likely to be the consequence of alterations in the posterior primitive streak directly or indirectly caused by the lack of *Lim1*. The frequency of *Lim1* mutants surviving to birth appeared to be increased when the mutation was moved onto a more diverse, outbred genetic background. The phenotypes of the *Lim1*-null neonates were consistent between animals, suggesting that there are genetic modifiers that specifically influence the expressivity of the mutation during gastrulation to affect placentation.

Analysis of four *Lim1*-null female neonates showed a complete absence of the derivatives of the Müllerian ducts, the oviducts and uterus, establishing an essential role for *Lim1* in the formation of the female reproductive tract. The uterus and oviducts derive from the Müllerian duct epithelium but also from the surrounding mesenchyme. Thus, the complete absence of the uterus in the *Lim1*-null female neonates demonstrates that the Müllerian duct epithelium has an essential role in instructing the mesenchyme to participate in uterine organogenesis. In addition, the one *Lim1*-null male neonate that was obtained did not have Wolffian duct derivatives, identifying a role for *Lim1* in male reproductive tract development. Whereas the Müllerian and Wolffian duct derivatives were absent in the *Lim1*-null neonates, there were gonads that were morphologically and histologically normal. It was previously reported that *Lim1*-null neonates lacked anterior head structures, kidneys and gonads (Shawlot and Behringer, 1995). However, it is now clear that *Lim1*-null mice can form gonads and that *Lim1* is dispensible for gonad formation. Several groups have reported *Lim1* expression in the developing gonad (Nagamine and Carlisle, 1996; Nagamine et al., 1999; Birk et al., 2000; Bouchard et al., 2002). Thus, it is still possible that *Lim1* has a role in gonad development but such a role would be compensated by other factors in its absence.

Phenotypic analysis of the female *Lim1*-null neonates established a requirement for *Lim1* in female reproductive tract development but did not provide information about when, how and in which tissues *Lim1* acted. To address these questions a chimera study was performed (Tam and Rossant, 2003). However, because there is an inherent bias towards male development when male and female cells are mixed, we devised a novel female chimera assay, exploiting the fact that genetically XO mice develop as females (Morris, 1968). Using this assay, we showed that *Lim1* activity is required cell autonomously in the epithelium of the Müllerian duct for female reproductive tract development. This was true at E18.5 and at E12.5 when the Müllerian duct is just forming. Our data indicate that *Lim1* function is required for the formation or very early steps of the differentiation of the Müllerian duct epithelium. However, because Müllerian duct formation initiates at E11.5, we cannot formally conclude that *Lim1* is essential for the initial formation of the Müllerian duct epithelium. Because *Lim1*-null cells to do not contribute to the Müllerian duct epithelium of female chimeras, we could not assess the role of *Lim1* at later stages of Müllerian duct differentiation where it might act to maintain this tissue or regulate oviductal morphogenesis. Conditional genetic strategies may be required to address this issue (Kwan and Behringer, 2002).

The role of *Lim1* has previously been investigated using mouse chimeras to understand its function in motoneuron axon trajectories in the developing limb (Kania et al., 2000). These studies identified a cell-autonomous requirement for *Lim1* in selecting a dorsal trajectory in the limb. These results and those presented in the current study that support a cell-autonomous action of *Lim1* stand in contrast to another chimera analysis of *Lim1* during gastrulation and head formation (Shawlot et al., 1999). In those studies, we showed that *Lim1* expression in primitive streak-derived tissues and the visceral endoderm acted in a cell non-autonomous manner to regulate anterior head formation. However, recent studies in frogs and mice suggest that the abnormalities in head formation may be secondary to a cell autonomous defect in cell adhesion that alters mesoderm migration (Hukriede et al., 2003). Thus, it is

possible that *Lim1* may regulate a common set of downstream targets in different tissues.

Relationship of *Lim1* with other genes that influence Müllerian duct development

We examined *Lim1* expression in mutants with abnormalities of Müllerian duct development to understand the relationship of *Lim1* with other genes in the genetic cascade of Müllerian duct development (Kobayashi and Behringer, 2003). Our chimera analysis indicated that *Lim1* is required for Müllerian duct formation. This is consistent with our observations that *Lim1* expression in the forming Müllerian duct does not require *Pax2* or *Wnt7a*, which are essential for later events of maintenance or differentiation of the Müllerian duct system, respectively (Torres et al., 1995; Miller and Sassoon, 1998). *Pax8* is also expressed in the Müllerian duct epithelium (Vainio et al., 1999) but mice lacking *Pax8* do not have defects in female reproductive tract development (Mansouri et al., 1998). It is known that Pax2 and Pax8 are functionally redundant (Carroll and Vize, 1999; Bouchard et al., 2002). In *Xenopus*, *Lim1* and *Pax8* synergistically induce pronephric tissues in kidney development (Carroll and Vize, 1999). Although the anterior region of the Müllerian duct is initially formed in *Pax2* mutants, it is possible that Lim1 and Pax2/8 also cooperate to regulate Müllerian duct formation in mice. Currently, it is not clear if *Lim1* expression in the Wolffian duct requires *Pax2* function (Fig. 7C) because the Wolffian duct starts to regress at this stage (Torres et al., 1995).

Wnt4 is one of relatively few molecules that have been shown to be required for the initial steps of Müllerian duct formation. It was reported that there is no Müllerian duct in *Wnt4* mutants by molecular expression analysis of the Müllerian duct epithelium markers, *Wnt7a* and *Pax8* (Vainio et al., 1999). Interestingly, we showed that *Lim1* is expressed in Müllerian duct precursor cells of *Wnt4* mutants and that these precursor cells do not invaginate to form the Müllerian duct. This suggests that *Lim1* expression in Müllerian duct precursor cells does not require *Wnt4* function. It is possible that *Lim1* may be required for specifying these Müllerian duct precursor cells acting genetically upstream of *Wnt4*.

It is noteworthy that *Lim1*, *Pax2*, *Emx2* and *Wnt4* are involved in Müllerian duct formation and are also required for the initial steps of kidney (metanephros) development (Stark et al., 1994; Shawlot et al., 1995; Torres et al., 1995; Miyamoto et al., 1997; Tsang et al., 2000). This infers that similar mechanisms may be functioning in Müllerian duct formation and kidney development.

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