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 (InsI3) (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 InsI3, 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-allaantoic 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+/- mice (Kania et al., 2000) were maintained on a 129/SvEvxC57BL/6J mixed genetic background. Lim1+/- (Shawlot and Behringer, 1995), Mis+/- (Behringer et al., 1994), Misr2+/- (Mishina et al., 1996) and MT-MISR+/- (Behringer et al., 1990) mice on a C57BL/6J congenic background, Wnt4+/- and Wnt7a+/- mice on a 129/SvxC57BL/6J mixed background (Parr and McMahon, 1998; Vainio et al., 1999), Pax2+/- mice on a 129/SvC3HxC57BL/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+/- male mice. To examine Lim1 expression in Mis and Misr2 deficient mice. For Lim1 expression in Mis and Misr2 deficient male mice. To examine Lim1 expression in MT-hMIS+/- transgenic embryos, Swiss Webster female mice were crossed with MT-hMIS+/-; Lim1+/- mice. To examine Lim1 expression in Pax2, Wnt4 and Wnt7a deficient embryos, Pax2+/-; Lim1+/-/ Wnt4+/-; Lim1+/-/ Wnt7a+/-; Lim1+/-/ 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+/- mice: mLim1-Fw8, GGCTACCTAAGACAAACCTACA; mLim1-Rv9, AGGAGTGAGGTGAGCTGGTT; lacZ-A, GCATCGAGCTGGTAATAAGGTTGGCAAT; and lacZ-B, GAGACACAGGCTGAATGTTAGCAGAC. The wild-type and mutant bands are 305 bp and ~822 bp, respectively. For Lim1+/- mice: mLim1-Fw8, GGCTACCTAAGACAAACCTACA; mLim1-Rv9, AGGAGTGAGGTGAGCTGGTT; and control bands are ~230 bp and 590 bp, respectively. For sex genotyping by PCR, males, females and control bands are 540 bp and ~230 bp, respectively. For sex genotyping by PCR, males, females and control bands are 540 bp and ~230 bp, respectively. For sex genotyping by PCR, males, females and control bands are 540 bp and ~230 bp, respectively. For sex genotyping by PCR, males, females and control bands are 540 bp and ~230 bp, respectively. For sex genotyping by PCR, males, females and control bands are 540 bp and ~230 bp, respectively. For sex genotyping by PCR, males, females and control bands are 540 bp and ~230 bp, respectively. For sex genotyping by PCR, males, females and control bands are 540 bp and ~230 bp, respectively. For sex genotyping by PCR, males, females and control bands are 540 bp and ~230 bp, respectively. For sex genotyping by PCR, males, females and control bands are 540 bp and ~230 bp, respectively.

Generation of XO ES cell lines and female chimeras
To generate XO ES cell lines, Lim1+/- and Lim1+/- Rosa26+/- 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 EcoRI digestion using a Y chromosome-specific repeat probe Y353/B (Bishop and Hattat, 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 (Lim1lacZ) 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 Lim1lacZ 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 third of the urogenital ridge (Fig. 1C). At E12.5, Lim1lacZ is expressed in the Müllerian duct along the cranial two-thirds of the urogenital ridge (Fig. 1C). Lim1lacZ expression was also detected in the developing metanephros, the definitive kidney in mammals (Fig. 1C,E,F). At E13.5, Lim1lacZ 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 Lim1lacZ 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 transcripts in the Wolffian and Müllerian ducts of wild-type embryos was identical to the β-gal expression pattern in Lim1lacZ 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 Lim1lacZ mice faithfully reflects endogenous Lim1 expression in the developing genital ducts and validate our expression data using the Lim1lacZ reporter.

To determine which tissues specifically express Lim1 in the urogenital tract, we performed a histological analysis. We found that Lim1lacZ 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 Lim1tlz 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 Lim1tlz 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 Lim1tlz reporter. Sexual dimorphic patterns of Lim1tlz expression were observed in the reproductive tract beginning at E14.5. In females, strong Lim1tlz expression was observed in the Müllerian duct at E14.5 (Fig. 2A) and this strong expression persists until E15.5 (Fig. 2C,E). Lim1tlz expression becomes weaker throughout the Müllerian duct at E16.5 (Fig. 2G). By E17.5, Lim1tlz expression in the Müllerian duct becomes restricted anteriorly, to the prospective oviduct. Lim1tlz expression in the posterior presumptive uterus region is downregulated and becomes undetectable (Fig. 2I). Lim1tlz expression in the Müllerian duct was not observed at later stages (data not shown). In the Wolffian duct of females, Lim1tlz 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, Lim1tlz 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, Lim1tlz expression in the Müllerian duct becomes discontinuous from its cranial region (open arrows in Fig. 2D). Subsequently, this discontinuity of Lim1tlz expression spreads caudally and Lim1tlz expression in the Müllerian duct shows a fragmented pattern throughout the entire reproductive tract region at E15.5 (Fig. 2F). At E16.5, Lim1tlz 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 epithelial tissue of the Müllerian duct where Lim1tlz expression is absent (data not shown). This indicates that the Lim1tlz expression fragmentation is likely caused by loss of the epithelial tissue during the process of Müllerian duct regression. Lim1tlz 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).

**Visualization of Müllerian duct regression by Lim1tlz 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 Lim1tlz 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 Lim1tlz expression reflects MIS-induced Müllerian duct
regression in males during embryogenesis. Therefore, we examined \textit{Lim1}\textsubscript{t\textsubscript{tc}} expression in MIS signaling mutant male mice. \textit{Lim1}\textsubscript{t\textsubscript{tc}} mice were bred with mice with mutations in \textit{Mis}, \textit{Misr2} or \textit{Wnt7a}. We found that the fragmentation of \textit{Lim1}\textsubscript{t\textsubscript{tc}} expression (i.e. loss of Müllerian duct epithelium) is completely inhibited and \textit{Lim1}\textsubscript{t\textsubscript{tc}} is expressed continuously along the persistent Müllerian duct in \textit{Mis}, \textit{Misr2} and \textit{Wnt7a} mutant males (Fig. 3A-D). \textit{Lim1}\textsubscript{t\textsubscript{tc}} expression in the Wolffian duct and kidney was not affected in these mutant males. These data indicate that the fragmentation of \textit{Lim1}\textsubscript{t\textsubscript{tc}} expression in the Müllerian duct depends on MIS signaling.

We also examined \textit{Lim1}\textsubscript{t\textsubscript{tc}} 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 \textit{Lim1}\textsubscript{t\textsubscript{tc}} expression patterns until E13.5 between wild-type and transgenic females (data not shown). However, \textit{Lim1}\textsubscript{t\textsubscript{tc}} expression in the Müllerian duct of \textit{Mis} transgenic female mice (Fig. 3E) becomes thinner than the \textit{Lim1}\textsubscript{t\textsubscript{tc}} expression of wild-type littermate females at E14.5 (Fig. 3F). At E15.5, \textit{Lim1}\textsubscript{t\textsubscript{tc}} is still strongly expressed in the Müllerian duct of wild-type females (Fig. 3G). At the same stage, the \textit{Lim1}\textsubscript{t\textsubscript{tc}} expression in the Müllerian duct becomes discontinuous in \textit{Mis} transgenic female mice (Fig. 3H). At E16.5, \textit{Lim1}\textsubscript{t\textsubscript{tc}} is weakly expressed along the entire Müllerian duct in wild-type females (Fig. 3I). At the same stage, \textit{Lim1}\textsubscript{t\textsubscript{tc}} expression in the Müllerian duct is restricted to the cranial end and within a few vesicles in \textit{Mis} transgenic female mice (Fig. 3J). Ectopic expression of \textit{Mis} causes thinner and flattened ovaries (Fig. 3E-J) (Lyet et al., 1995) but did not affect \textit{Lim1}\textsubscript{t\textsubscript{tc}} expression in the Wolffian duct and kidney (Fig. 3E-L). These data suggest that MIS signaling is sufficient to cause fragmentation of \textit{Lim1}\textsubscript{t\textsubscript{tc}} expression in the Müllerian duct. We also examined \textit{Lim1}\textsubscript{t\textsubscript{tc}} expression in \textit{Mis} transgenic males. The fragmentation of \textit{Lim1}\textsubscript{t\textsubscript{tc}} expression is enhanced in \textit{Mis} transgenic males compared with wild-type males at E15.5 (Fig. 3K,L).

**Absence of the female reproductive tract in female \textit{Lim1}\textsubscript{null} mice**

The dynamic expression pattern of \textit{Lim1} in the developing Müllerian duct indicates that \textit{Lim1} may have an important function during female reproductive tract organogenesis. Unfortunately, most \textit{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 \textit{Lim1} mutants escaped this embryonic lethality and survived to the birth. These \textit{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 \textit{Lim1}-null mutant survival to birth is that the embryonic lethality is affected by genetic modifiers. Therefore, we modified the genetic background of the \textit{Lim1} mutation to increase phenotypic variety by outcrossing \textit{Lim1} heterozygous mice (maintained in C57BL/6Jx129/SvEv mixed background) with Swiss Webster (Taconic) outbred mice. Subsequently, we intercrossed the resulting \textit{Lim1} heterozygous offspring from different pedigrees to screen for \textit{Lim1}-null escapers. We found one breeding pair that produced two \textit{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 \textit{Lim1} heterozygous offspring from the mating pair that produced the two escapers and obtained two additional XX \textit{Lim1}-null escaper neonates.

We analyzed the three XX \textit{Lim1}-null neonates and one of the original four escapers that was also XX for female reproductive organ development. All of four \textit{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 \textit{Lim1}-null neonates showed that they had ovaries that were morphologically normal.
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−/− urogenital defects were completely rescued in all chimeric animals composed of Lim1−/− 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. 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−/− female reproductive tract is found in newborns. Note that neither epithelium nor mesenchyme of the female reproductive tract were completely absent in the female Lim1−/− newborns (Fig. 4G-H). These data suggest that Lim1 is essential for Müllerian duct epithelium formation during embryogenesis and that lack of Lim1 activity results in Müllerian agenesis in females.
**Lim1 in female reproductive tract development**

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 expression in Pax2, Wnt4 and Wnt7a mutants (Fig. 7). Wnt7a is expressed in the Müllerian duct epithelium from E11.5.
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	extsuperscript{tlz} positive cells, we also examined Lim1	extsuperscript{tlz} expression in Wnt4 mutants at E11.5, when the Müllerian duct just begins to form. We found Lim1	extsuperscript{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	extsuperscript{+/+} control embryos (Fig. 1A, Fig. 7E,G). However, the Lim1	extsuperscript{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	extsuperscript{tlz} expression in the mesonephros 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	extsuperscript{tlz}**

Analysis of the expression of the Lim1	extsuperscript{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	extsuperscript{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	extsuperscript{tlz} expression was coincident with the initial formation of the Müllerian duct just begins to form. We found Lim1 expression in the anterior region of the Müllerian duct, and differentiation of the Müllerian duct into the organs of the female reproductive tract, as well 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	extsuperscript{tlz} expression in the mesonephros 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.
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\textsuperscript{flc} reporter documented the persistence of the Müllerian duct in the absence of MIS signaling. Thus, the Lim1\textsuperscript{flc} 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 testes 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\textsuperscript{transgenic} fetuses had an altered spatial pattern of Müllerian duct regression in comparison with normal males. Whereas fragmentation of the Lim1\textsuperscript{flc} 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\textsuperscript{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 placentaion.

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 dispensable 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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