β-Catenin is essential for Müllerian duct regression during male sexual differentiation

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SUMMARY

During male sexual differentiation, the transforming growth factor-β (TGF-β) signaling molecule anti-Müllerian hormone (AMH; also known as Müllerian inhibiting substance, MIS) is secreted by the fetal testes and induces regression of the Müllerian ducts, the primordia of the female reproductive tract organs. Currently, the molecular identity of downstream events regulated by the AMH signaling pathway remains unclear. We found that male-specific *Wnt4* expression in mouse Müllerian duct mesenchyme depends upon AMH signaling, implicating the WNT pathway as a downstream mediator of Müllerian duct regression. Inactivation of β-catenin, a mediator of the canonical WNT pathway, did not affect AMH signaling activation in the Müllerian duct mesenchyme, but did block Müllerian duct regression. These data suggest that β-catenin mediates AMH signaling for Müllerian duct regression during male sexual differentiation.

KEY WORDS: β-Catenin, AMH, MIS, Müllerian duct regression, *Wnt4*, Mouse

INTRODUCTION

Prior to sexual differentiation in amniotes, all embryos form the anlagen for both male and female reproductive tract organs, the Wolffian (mesonephric) and Müllerian (paramesonephric) ducts, respectively (Kobayashi and Behringer, 2003; Masse et al., 2009). At embryonic day (E) 11.5 in mice, the Müllerian duct forms as an invagination of the mesonephric (coelomic) epithelium of the anterior urogenital ridge, which subsequently elongates posteriorly lateral to the Wolffian duct and reaches the cloaca at E13.5 (Kobayashi and Behringer, 2003; Kobayashi et al., 2004; Guioli et al., 2007; Orvis and Behringer, 2007; Ma, 2009). Delaminating mesonephric (coelomic) epithelial cells adjacent to the extending Müllerian duct epithelium contribute to the surrounding Müllerian duct mesenchyme (Zhan et al., 2006; Guioli et al., 2007).

In mammals, sex is determined genetically (Waters et al., 2007; DeFalco and Capel, 2009; Sekido and Lovell-Badge, 2009): XY individuals become males, whereas XX individuals become females. The sex-determining gene on the Y chromosome *Sry* directs differentiation of the XY fetal gonad into a testis (Polanco and Koopman, 2007; DiNapoli and Capel, 2008; Sekido and Lovell-Badge, 2009). Subsequently, the testis secretes male-specific hormones that virilize the rest of the body (Nef and Parada, 2000). One of these male fetal hormones is anti-Müllerian hormone (AMH; also known as Müllerian inhibiting substance, MIS), a member of the transforming growth factor-β (TGF-β) family (Munsterberg and Lovell-Badge, 1991; Teixeira et al., 2001; Josso et al., 2006). AMH signaling is both essential and sufficient for

Müllerian duct regression (Behringer et al., 1990; Tsuji et al., 1992; Behringer et al., 1994; Belville et al., 1999). Loss of *Amh* function in the mouse results in the differentiation of ectopic female reproductive tracts in males, including oviducts, uterus and vagina (Behringer et al., 1994; Belville et al., 1999). The presence of these female organs in an otherwise normal male causes infertility in *Amh*-null males because they disrupt the passage of spermatozoa out of the body. Ectopic expression of human *AMH* (*hAMH*) under the widely expressed mouse metallothionein1 promoter (*Mt-hAMH*) causes regression of the Müllerian ducts in female mice (Behringer et al., 1990). Thus, male-specific expression of AMH ensures elimination of the female reproductive tract organs in males for normal fertility.

Molecular components of the AMH signaling pathway have been identified (di Clemente et al., 2003). TGF-β family signaling is mediated by two receptors, type I and II, which phosphorylate receptor SMAD proteins (Massague et al., 2005; Kitisin et al., 2007; Moustakas and Heldin, 2009). The anti-Müllerian hormone type 2 receptor gene (*Amhr2*, also known as *MISIIR*) is expressed in the Müllerian duct mesenchyme and adjacent mesonephric (coelomic) epithelium (Baarends et al., 1994; di Clemente et al., 1994; Zhan et al., 2006; Arango et al., 2008). Inactivation of *Amhr2* also causes complete retention of ectopic female reproductive tract organs in males, identical to *Amh*-null males (Mishina et al., 1996; Belville et al., 1999). *Mt-hAMH*[tg/+]; *Amhr2*^{−/−} males are identical in phenotype to *Amh*- and *Amhr2*-null males, indicating that the AMH type II receptor is the only type II receptor that transduces the AMH signal (Mishina et al., 1999). Two type I receptors, ALK3 (BMPR1A – Mouse Genome Informatics) and ALK2 (ACVR1 – Mouse Genome Informatics), and three receptor SMADs, SMAD1, SMAD5 and SMAD8 (also known as SMAD9 – Mouse Genome Informatics), redundantly mediate AMH signaling (Jamin et al., 2002; Zhan et al., 2006; Orvis et al., 2008). Upon activation of AMH signaling, the receptor SMADs enter the nucleus and regulate transcription of downstream target genes. However, little is known about the downstream molecular regulation of AMH signaling. Although it was shown that matrix

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metalloproteinase 2 (*Mmp2*) mediates Müllerian duct regression ex vivo, the Müllerian duct regresses normally in male mice that lack *Mmp2* activity in vivo (Roberts et al., 2002).

Previous studies indicate that the AMH and WNT signaling pathways might interact mutually during Müllerian duct regression. In both sexes, *Wnt7a* expression in the Müllerian duct epithelium is required for *Amhr2* expression in the adjacent Müllerian duct mesenchyme (Parr and McMahon, 1998). *Wnt7a*-null males do not regress the Müllerian duct system, leading to the development of female reproductive tract organs. These findings indicate that *Wnt7a* induces the Müllerian duct mesenchyme to become competent to respond to AMH. AMH signaling also regulates the expression of WNT pathway components. Sexually dimorphic expression patterns of two secreted Frizzled-related genes, *Sfrp2* and *Sfrp5*, are regulated by AMH signaling (Cox et al., 2006). Activation of the canonical WNT pathway induces accumulation of β-catenin (CTNNB1 – Mouse Genome Informatics) in the nucleus, where β-catenin regulates transcription of downstream target genes with other cofactors, including LEF1 (van Amerongen and Nusse, 2009; Verheyen and Gottardi, 2010). The canonical WNT pathway can directly regulate *Lef1* transcription, which is independent of LEF1 function (Filali et al., 2002). AMH signaling induces the accumulation of β-catenin and LEF1 in the nucleus of Müllerian duct mesenchyme cells (Allard et al., 2000). However, Müllerian duct regression occurs normally in compound *Sfrp2*- and *Sfrp5*-null mutant males (Cox et al., 2006), and the role of β-catenin and LEF1 during Müllerian duct regression has not been studied. Therefore, the functions of the WNT/β-catenin pathway for Müllerian duct regression remain unclear.

We have examined the relationship between AMH signaling and the WNT/β-catenin pathway during Müllerian duct regression. Male-specific expression of *Wnt4* was regulated by AMH signaling in the Müllerian duct mesenchyme. Inactivation of β-catenin in the Müllerian duct mesenchyme did not alter the sexually dimorphic expression pattern of *Wnt4*, suggesting that β-catenin is dispensable for activation of the AMH signaling pathway in the Müllerian duct mesenchyme. However, loss of β-catenin in the Müllerian duct mesenchyme caused complete retention of ectopic female reproductive tract organs in males, indicating a requirement of β-catenin for Müllerian duct regression during male sexual differentiation. Thus, the WNT/β-catenin pathway mediates Müllerian duct regression downstream of the AMH signaling pathway.

MATERIALS AND METHODS
Mice
Amhr2^{+/–} (Mishina et al., 1996), *Mt-hAMH*[tg/+] (Behringer et al., 1990), *R26R*[lacZ/+] (Soriano, 1999), *Mox2*[Cre/+] (*More-Cre*) (Tallquist and Soriano, 2000), *Wnt4*^{+/–} (Stark et al., 1994) and *Wnt7a*^{+/–} (Parr and McMahon, 1995) mice were maintained on a C57BL/6J congenic background. *Amhr2*[Cre/+] mice were maintained on a C57BL/6J × 129/SvEv mixed background (Jamin et al., 2002). *Ctnnb1*[flox/flox] mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and maintained on a C57BL/6J × 129X1/SvJ × 129S1/Sv mixed background (Brault et al., 2001). Using *Amhr2-Cre* expression in the male germ-line, *Amhr2*[Cre/+]; *Ctnnb1*[del/+] mice were generated by mating *Amhr2*[Cre/+]; *Ctnnb1*[flox/+] males with C57BL/6J females. *Amhr2*[Cre/+]; *Ctnnb1*[flox/del] mice were generated by mating *Ctnnb1*[flox/flox] females with *Amhr2*[Cre/+]; *Ctnnb1*[del/+] males. Littermate males were used for controls.

Wnt4 conditional null mice were generated as follows. Genomic fragments at the *Wnt4* locus were PCR subcloned into pBlueScript KS(–) (Stratagene) using 129/SvEv genomic DNA as a template. The primers used for the 5′ arm were Sal-5arm-Fw1, ATCGGTCGACATCTCC-CTGTTACATTCAGAGCCTGAGGGA and Cla-loxP-BglII-5arm-Rv2,

ATCGATCGATATAACTTCGTATAGCATACATTATACGAAGTTATAG-ATCTGGGAACTGGGTTCACTCCAACAAAGCCTGC; for the floxed exon2 were Cla-exon2-Fw1, ATCGATCGATAGCTGCTTTGCCCAA-ACTGTCAATCTCTAA and RI-loxP-exon2-Rv2, ATCGGAATTCATAA-CTTCGTATAGCATACATTATACGAAGTTATGCCGCATACAGACCC-TAGATGCCCAGAGTT; and for the 3′ arm were RI-3arm-Fw1, ATCGGAATTCCCTGGGTGTCTGGGAAAATGATATTTCTAG and Not-3arm-Rv2, ATCGGCGGCCGCTGGCTGCACAGGTCTGAGTC-CTATGTGTCCT. The primers used for the 5′ Southern blot probe were RI-5probe-Fw1, ATCGGAATTCTTGGTTTGCTTTGTTCATCTGTG-AAATGAG and Xba-5probe-Rv2, ATCGTCTAGACGGCCGGAA-CGACGGTGGCAGAGACACTGC; and for the 3′ Southern probe were Xba-3probe-Fw1, ATCGTCTAGAAGCCTTGGGGTACTCTGGGAA-GAAAACCAG and 3probe-Rv2-RI, ATCGGAATTCGAACTCAG-TCTTTTCTCACTGACT. After combining sequence-confirmed PCR products of the 5′ arm, floxed exon 2 and 3′ arm, FRT-PGK-neo-bpA-FRT and MC1-TK cassettes were inserted at an *Eco*RI site and between *Xho*I and *Sal*I sites, respectively. Gene targeting was performed using AB1 ES cells as previously described (Behringer et al., 1994; Shawlot and Behringer, 1995). After screening 142 ES cell clones, five correctly targeted clones were obtained. Three clones were injected into C57BL/6J blastocysts and germ line transmission was obtained from one clone. The resulting *Wnt4*[flox-neo/+] mice were crossed with *Rosa26-Flpe* mice (Farley et al., 2000) to remove the FRT-PGK-neo-bpA-FRT selection cassette and subsequently intercrossed to maintain homozygous *Wnt4*[flox/flox] mice on a C57BL/6J × 129/SvEv mixed background.

To inactivate *Wnt4* in the Müllerian duct mesenchyme, *Wnt4*^{+/–} mice were crossed with *Amhr2*[Cre/+] mice to generate *Amhr2*[Cre/+]; *Wnt4*^{+/–} males, which were subsequently mated with *Wnt4*[flox/flox] females to obtain *Amhr2*[Cre/+]; *Wnt4*[flox/–] males. Littermate males were used for controls.

PCR genotyping
Amhr2^{+/–}, *Mt-hAMH*[tg/+], *Wnt4*^{+/–} and *Wnt7a*^{+/–} mice were genotyped as described previously (Kobayashi et al., 2004). *Mox2*[Cre/+] and *Amhr2*[Cre/+]; *Ctnnb1*[flox/del] mice were genotyped using the following primers: mCtnnb1-Fw12, CACAGGGACTTCCATACCAGTTGACATGGTA; mCtnnb1-Fw13, GAGAGCAAGGTAGAGTGATGAAAGTTGTT; mCtnnb1-Rv15, GGCATCCTCTCACTGCCTCCTCCTAGGTA; Cre-Fw1, GGACATG-TTCAGGGATCGCCAGGC; and Cre-Rv2, CGACGATGAAGCATG-TTTAGCTG, which give a 299-bp band for the *Ctnnb1* wild-type allele (mCtnnb1-Fw13 and mCtnnb1-Rv15), a 402-bp band for the *Ctnnb1* floxed allele (mCtnnb1-Fw13 and mCtnnb1-Rv15), a 557-bp band for the *Ctnnb1* deleted allele (mCtnnb1-Fw13 and mCtnnb1-Rv15) and a 219-bp band for the *Amhr2-Cre* allele (Cre-Fw1 and Cre-Rv2). *Wnt4* alleles were genotyped using the following primers: mWnt4-Fw10, GCAGAGAGGCCCAGCCTGCCCCTCA; and mWnt4-Rv12, CAT-GTGCCTGGCCCTAGAAATATCAT, which give a 239-bp band for the *Wnt4* deleted allele, a 642-bp band for the *Wnt4* wild-type and null allele and a 819-bp band for the *Wnt4* floxed allele.

In situ hybridization
Whole-mount in situ hybridization was performed as described previously (Shawlot and Behringer, 1995). After staining, tissues were fixed in 4% paraformaldehyde at 4°C and soaked in 30% sucrose overnight at 4°C. After embedding in OCT (Sakura, 4583), cryosections were generated at 16 μm using a Microm HM560. Differential interference contrast (DIC) images were photographed using a Leica DMR fluorescence microscope.

Section in situ hybridization was performed as described previously (Wang et al., 2008). After liquid emulsion autoradiography and Hoechst staining, images were photographed using a Leica DMR fluorescence microscope.

Immunofluorescence
Immunofluorescence was performed as described previously (Chang et al., 2008; Kobayashi et al., 2008; Wang et al., 2008). Sections were incubated with primary antibodies to AMH (1:100, Santa Cruz Biotechnology, sc-6886), β-catenin (1:50, Santa Cruz Biotechnology, sc-1496; 1:1500, Thermo Scientific, RM-2101), LEF1 (1:150, Cell Signaling, 2230), cleaved

caspase-3 (1:100, Cell Signaling Technology, 9661) and E-cadherin (1:200, Invitrogen, 13-1900) and detected by Alexa 488- and Alexa 568-conjugated secondary antibodies (1:250-500, Invitrogen). After mounting with VECTASHIELD containing DAPI (Vector Laboratories, H-1200) or Immu-Mount (Thermo Scientific, 9990402) following Hoechst DAPI staining, images were photographed using a Leica DMR fluorescence microscope or a Nikon D-Eclipse C1si Confocal Microscope.

RESULTS
Male-specific *Wnt4* expression in the Müllerian duct mesenchyme during male sexual differentiation

To understand the potential interactions between the AMH and WNT pathways during Müllerian duct regression, we examined the expression of a WNT ligand gene, *Wnt4*, during Müllerian duct regression. At E12.5, the Müllerian duct mesenchyme begins to form as a thin layer of cells between the mesonephric (coelomic) epithelium and newly formed Müllerian duct epithelium in the anterior region of the urogenital system (Guioli et al., 2007; Orvis and Behringer, 2007). At this stage, *Wnt4* was not expressed in the Müllerian duct mesenchyme in both sexes (Fig. 1A,B). One day later at E13.5, the Müllerian duct mesenchyme has surrounded the lateral half of the Müllerian duct epithelium (Orvis and Behringer, 2007; Arango et al., 2008). Interestingly, *Wnt4* was expressed in the Müllerian duct mesenchyme of XY males, but not XX females (Fig. 1C,D). By E15.5, most cells of the Müllerian duct epithelium start to die by apoptosis and the Müllerian duct mesenchyme surrounds the dying cells in males, whereas the Müllerian duct continues to differentiate in females at the same stage (Fig. 1E-H) (Kobayashi and Behringer, 2003; Arango et al., 2008). The male-specific *Wnt4* expression in the Müllerian duct mesenchyme persisted during Müllerian duct regression (Fig. 1E-H). These data suggest that *Wnt4* is expressed in the Müllerian duct mesenchyme during male sexual differentiation.

AMH signaling regulates male-specific *Wnt4* expression in the Müllerian duct mesenchyme

The sexually dimorphic expression pattern of *Wnt4* during the period of Müllerian duct regression indicates the possibility of regulation of *Wnt4* expression by AMH signaling. To test this idea, we examined *Wnt4* expression in two AMH signaling mutants. In *Amhr2*-null males (Mishina et al., 1996), in which AMH signaling is absent, the male-specific activation of *Wnt4* was not observed at E13.5 (Fig. 2A,B), suggesting that AMH signaling is required to activate *Wnt4* in the Müllerian duct mesenchyme of males. Ectopic expression of human *AMH* under the widely expressed metallothionein 1 promoter (*Mt-hAMH*) activated *Wnt4* ectopically in the Müllerian duct mesenchyme of females (Fig. 2C,D), suggesting that AMH signaling is sufficient to activate *Wnt4* expression in the Müllerian duct mesenchyme. Taken together, our observations suggest that the AMH signaling is both required and sufficient for *Wnt4* expression in the Müllerian duct mesenchyme at the onset of Müllerian duct regression.

Amhr2-Cre is expressed in the Müllerian duct mesenchyme during male sexual differentiation

We previously generated an *Amhr2-Cre* knock-in mouse line, in which Cre expression was detected in the Müllerian duct mesenchyme from E12.5 using the *R26R-lacZ* reporter allele (Jamin et al., 2002). However, it was reported that the *Amhr2-Cre* activity was detected from E15.5 using the *Z/EG* Cre reporter allele
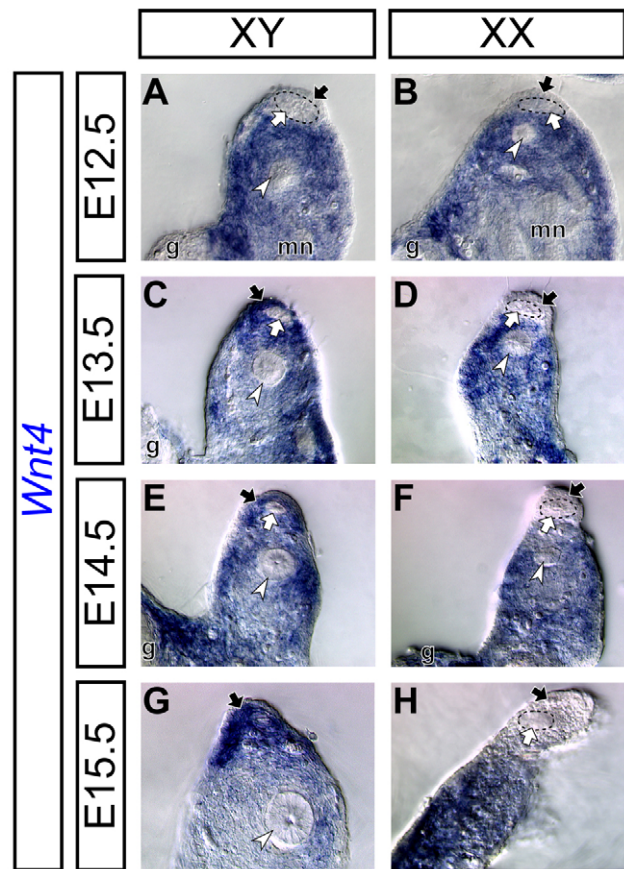

Fig. 1. Sexually dimorphic expression pattern of *Wnt4* in the mouse Müllerian duct mesenchyme during Müllerian duct regression. (**A-H**) Differential interference contrast images of *Wnt4* expression in the wild-type developing reproductive tract detected by in situ hybridization. The reproductive tract of XY males (A,C,E,G) and XX females (B,D,F,H) at E12.5 (A,B), E13.5 (C,D), E14.5 (E,F) and E15.5 (G,H). Black arrows, white arrows and white arrowheads indicate the Müllerian duct mesenchyme, Müllerian duct epithelium and Wolffian duct epithelium, respectively. g, gonad; mn, mesonephros.

(Deutscher and Hung-Chang Yao, 2007). Therefore, the onset of *Amhr2-Cre* expression might depend on the conditional allele employed.

To determine the onset of *Amhr2-Cre* expression in the Müllerian duct mesenchyme precisely, we performed Cre reporter analysis for the *Amhr2-Cre* allele at the onset of Müllerian duct mesenchyme development using the *R26R-lacZ* reporter allele (Soriano, 1999). We detected *Amhr2-Cre* reporter expression in the Müllerian duct mesenchyme and gonads in both sexes from the onset of Müllerian duct mesenchyme formation (Fig. 3). At E12.5, the Müllerian duct forms in the anterior half of the urogenital ridge (Kobayashi and Behringer, 2003; Kobayashi et al., 2004; Guioli et al., 2007; Orvis and Behringer, 2007). At this stage, *Amhr2-Cre* reporter activity was detected in the corresponding anterior region of the urogenital ridge (Fig. 3A,B). Histological analysis of the reproductive tract organs showed that *Amhr2-Cre* reporter activity was detected in the Müllerian duct mesenchyme, with some mosaicism, and some cells in the mesonephric (coelomic) epithelium (Fig. 3C,D). One day later at E13.5, when the entire Müllerian duct forms along the anterior-posterior axis of the
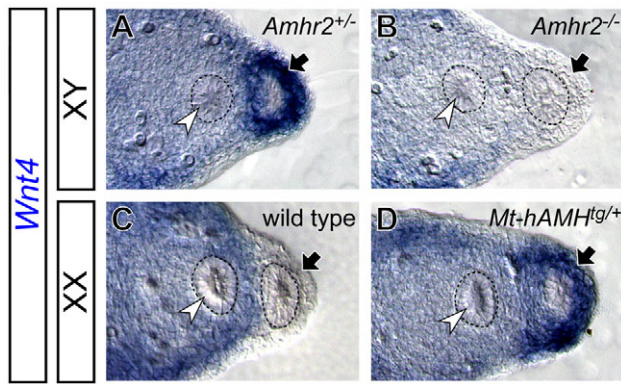
DEVELOPMENT

Fig. 2. AMH signaling regulates *Wnt4* expression in the mouse Müllerian duct mesenchyme. Differential interference contrast images of *Wnt4* expression in the developing reproductive tract from AMH signaling mutants detected by in situ hybridization at E13.5. (**A**,**B**) The reproductive tract of *Amhr2+/–* (A) and *Amhr2–/–* (B) XY males. (**C**,**D**) The reproductive tract of wild-type (C) and *Mt-hAMHtg/+* (D) XX females. Black arrows and white arrowheads indicate the Müllerian duct mesenchyme and Wolffian duct epithelium, respectively. Dashed circles indicate the epithelium of the reproductive tracts.
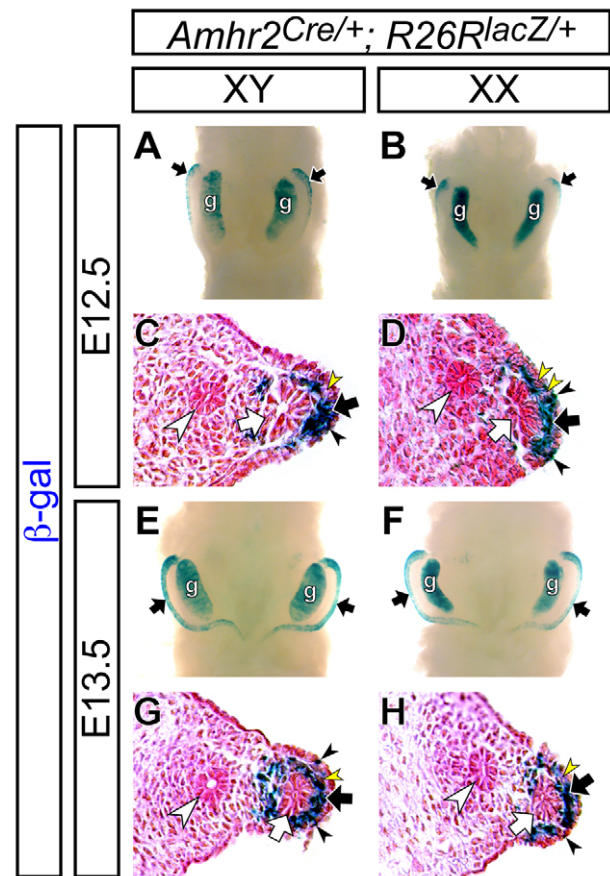

Fig. 3. Cre reporter analysis for *Amhr2-Cre* allele during Müllerian duct development. To examine *Cre* activity in the *Amhr2-Cre* mice, the *R26R-lacZ* Cre reporter allele (Soriano, 1999) was crossed with the *Amhr2-Cre* allele. (**A-H**) X-gal stained (blue) reproductive systems of *Amhr2Cre/+; R26RlacZ/+* XY males (A,C,E,G) and XX females (B,D,F,H) at E12.5 (A-D) and E13.5 (E-H). Ventral whole-mount view (A,B,E,F) and cross-sections counterstained with Eosin (pink) (C,D,G,H) are shown. β-Galactosidase (β-gal) activity was observed in the Müllerian duct mesenchyme (black arrows), gonad (g) and some cells of the coelomic epithelium (black arrowheads). Some mosaicism of β-gal activity was seen within the Müllerian duct mesenchyme (yellow arrowheads). Black arrows, white arrows and white arrowheads indicate the Müllerian duct mesenchyme, Müllerian duct epithelium and Wolffian duct epithelium, respectively.

urogenital ridge (Kobayashi and Behringer, 2003; Kobayashi et al., 2004; Orvis and Behringer, 2007), *Amhr2-Cre* reporter activity was detected in the mesenchyme along the entire length of the Müllerian duct (Fig. 3E-H). These data suggest that the *Amhr2-Cre* line provides a useful tool to manipulate the Müllerian duct mesenchyme genetically throughout Müllerian duct development, including during male sexual differentiation.

Loss of β-catenin activity causes retention of ectopic female reproductive tract organs in males

To understand the function of the WNT pathway during Müllerian duct regression, we inactivated WNT signaling components in the Müllerian duct mesenchyme. The AMH signaling-regulated expression of *Wnt4* indicates that *Wnt4* might be involved in Müllerian duct regression. In *Wnt4*-null mutants, the Müllerian duct fails to extend posteriorly in both sexes at E11.5 (Vainio et al., 1999; Kobayashi and Behringer, 2003), which prevents studies of *Wnt4* function during later stages of Müllerian duct regression. Therefore, we generated a conditional *Wnt4*-null (*Wnt4*-floxed) allele in the mouse (Fig. 4A,B). Using *Mox2-Cre* (*More-Cre*) mice (Tallquist and Soriano, 2000), we validated that *Wnt4del/del* mice showed identical abnormalities to the *Wnt4*-null (*Wnt4–/–*) mutants (Stark et al., 1994; Vainio et al., 1999) whereas *Wnt4flox/flox* mice were normal (data not shown). To examine *Wnt4* function in the Müllerian duct mesenchyme, we inactivated *Wnt4* function using the *Amhr2-Cre* allele. We found that Müllerian duct regression occurred normally in *Amhr2Cre/+; Wnt4flox/–* males (Fig. 5), although the *Amhr2-Cre* allele efficiently recombined the *Wnt4flox* allele in the female reproductive tract (Fig. 4C). These data indicate that *Wnt4* is not required for Müllerian duct regression.

The canonical WNT pathway is mediated by β-catenin, loss of which results in lethality during gastrulation (Haegel et al., 1995). We next inactivated β-catenin specifically in the Müllerian duct mesenchyme to examine the possible roles of the WNT/β-catenin pathway for Müllerian duct regression. When β-catenin was inactivated in the Müllerian duct mesenchyme using the *Amhr2-Cre* allele, we found that β-catenin mutant newborn males had both

male and female reproductive tract organs (Fig. 6A,B,E,F). Histological analysis revealed an ectopic female reproductive tract lateral to the male reproductive tract in the β-catenin mutant males (Fig. 6I,J). All β-catenin mutant males examined (*n*=7) retained the complete female reproductive tract. These data suggest that β-catenin function in the Müllerian duct mesenchyme is required for Müllerian duct regression.

β-Catenin function in the Müllerian duct mesenchyme is required for prenatal Müllerian duct differentiation

We also compared the ectopic female reproductive tract organs of AMH signaling mutants. The ectopic female reproductive tracts in the β-catenin mutant males were different from those in *Amhr2*-null males, in which AMH signaling was absent (Fig. 6J,K). In the absence of β-catenin, the Müllerian duct
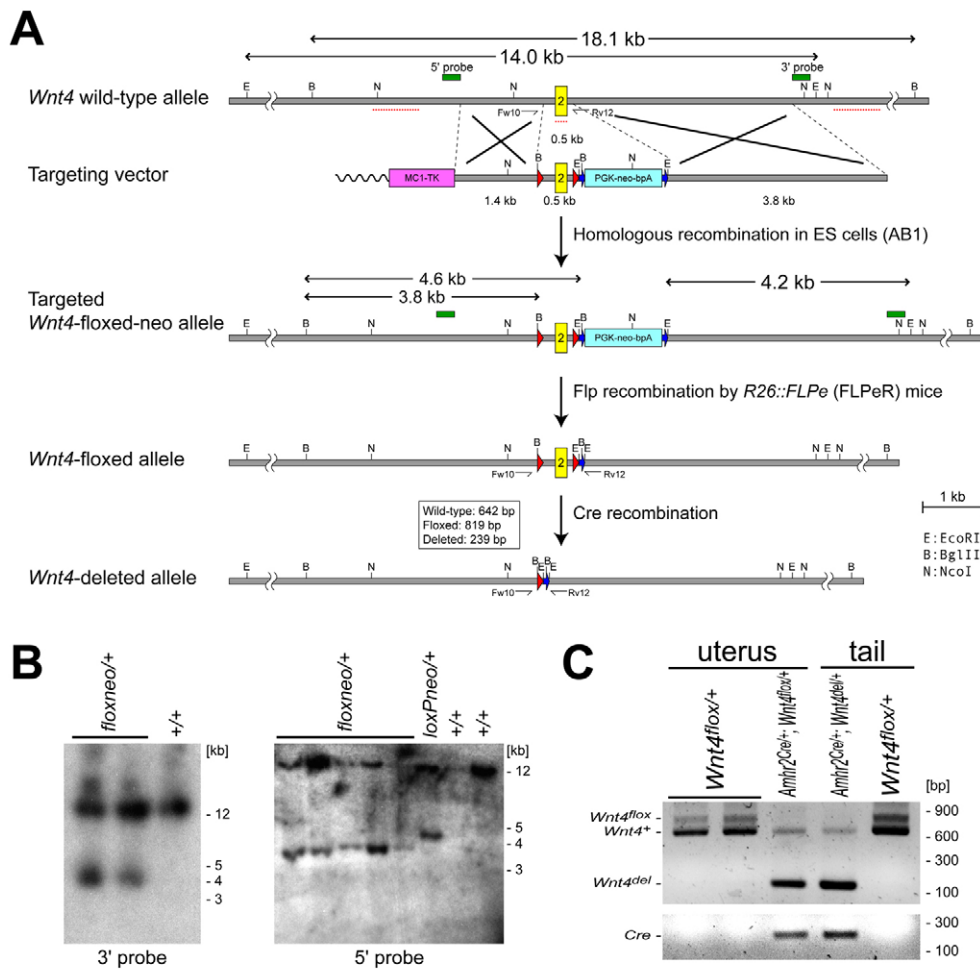
Fig. 4. Generation of a conditional null allele for *Wnt4*. (**A**) Schematic illustration of targeting strategy for a *Wnt4* conditional null (floxed) allele. The genomic loci around exon 2 (yellow box) of the *Wnt4* gene in different alleles are shown. Red triangles and green arrows indicate *loxP* and *FRT* sites, respectively. Red dashed lines indicate conserved regions between mouse and human. Fw10 and Rv12 indicate forward and reverse primers for PCR genotyping, respectively. (**B**) Confirmation of the targeted *Wnt4* floxed allele by Southern hybridization. In total, five correctly targeted ES cell clones were obtained. *Wnt4$^{flox/flox}$* mice were phenotypically normal. *Wnt4* deleted (*Wnt4^{flox}*) allele was generated from the *Wnt4^{flox}* allele using ubiquitous Cre recombination in the embryonic tissue using the *Mox2-Cre* (More) allele (Tallquist and Soriano, 2000). After intercrossing, the resulting *Wnt4$^{del/del}$* mice had identical phenotypes to those in *Wnt4*-null (*Wnt4$^{-/-}$*) mice (Stark et al., 1994; Vainio et al., 1999) in the kidney, gonad and reproductive tract at birth, confirming that the *Wnt4* floxed allele generates a null allele after Cre recombination (data not shown). (**C**) Recombination of the *Wnt4* floxed allele by the *Amhr2-Cre* allele in the female reproductive tract. PCR genotyping with genomic DNA from the uterus of *Wnt4$^{flox/+}$* and *Amhr2$^{Cre/+}$; Wnt4$^{flox/+}$* females at E18.5. Genomic DNA from the tail of *Amhr2$^{Cre/+}$;Wnt4$^{del/+}$* and *Wnt4$^{flox/+}$* mice were used as controls.

mesenchyme was less condensed, indicating an additional requirement of β-catenin for Müllerian duct differentiation during embryogenesis, as reported previously in females (Deutscher and Hung-Chang Yao, 2007; Arango et al., 2008). *Wnt7a* is required for both Müllerian duct regression and differentiation (Fig. 6D,H) (Miller and Sassoon, 1998; Parr and McMahon, 1998). The ectopic female reproductive tracts in the β-catenin mutant males were also different from those of *Wnt7a*-null males, in which the lumen of the Müllerian duct was expanded and the Müllerian duct mesenchyme was more condensed than the conditional β-catenin mutants, although the Müllerian duct mesenchyme condensation in *Wnt7a*-null mice was slightly reduced than that of *Amhr2*-null mice (Fig. 4L), indicating an involvement of other WNT ligand(s) for Müllerian duct differentiation during embryogenesis (Arango et al., 2008).

AMH signaling is activated in the Müllerian duct mesenchyme in the absence of β-catenin function

Because *Amhr2-Cre* is expressed in both the testis and Müllerian duct mesenchyme (Fig. 3) (Jamin et al., 2002), it is possible that loss of β-catenin function using the *Amhr2-Cre* allele might disrupt normal production of AMH in the testis and/or its receptor expression in the Müllerian duct mesenchyme. To exclude these possibilities, we examined the expression of AMH and *Amhr2* in β-catenin mutant males. At E13.5, AMH was normally expressed in Sertoli cells of the testis in the conditional β-catenin mutant males (Fig. 7A,B). At the same stage, the AMH type II receptor, *Amhr2*, was also normally expressed in the Müllerian duct mesenchyme and Sertoli cells in the testes of the conditional β-catenin mutant males (Fig. 7C,D).
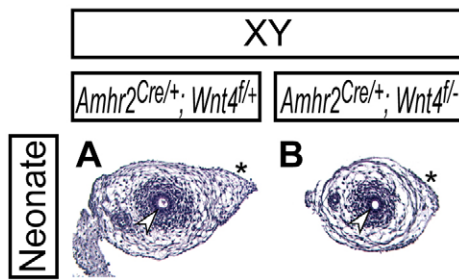
Fig. 5. Normal Müllerian duct regression in *Amhr2^Cre/+^; Wnt4^flox/del^* male mice. Histological analysis of reproductive tract organs by Hematoxylin and Eosin staining in *Amhr2^Cre/+^; Wnt4^flox/+^* (**A**) and *Amhr2^Cre/+^; Wnt4^flox/del^* (**B**) neonates. Arrowheads and asterisks indicate the Wolffian duct epithelium and the region where the Müllerian duct regressed, respectively.

Our findings regarding the AMH-dependent *Wnt4* expression indicate that *Wnt4* can be used as a molecular marker for AMH signaling activation in the Müllerian duct mesenchyme. Therefore, we examined *Wnt4* expression in the conditional β-catenin mutant males to determine whether AMH signaling is normally activated in the Müllerian duct mesenchyme. We observed normal activation of *Wnt4* expression in the Müllerian duct mesenchyme of the conditional β-catenin mutant males (Fig. 7E,F), suggesting that the AMH signaling was activated in the Müllerian duct mesenchyme in the absence of β-catenin function.

To document inactivation of β-catenin in the Müllerian duct mesenchyme by the *Amhr2-Cre* allele, we examined β-catenin expression at E14.5. At this stage, cytoplasmic accumulation of β-catenin was observed in the Müllerian duct mesenchyme of control males (Fig. 7G) (Allard et al., 2000). In the conditional β-catenin mutant males, cytoplasmic β-catenin accumulation was greatly reduced whereas β-catenin expression on the cell-surface of the Müllerian duct epithelium was intact (Fig. 7H). There were some β-catenin-expressing cells in the Müllerian duct mesenchyme of the conditional β-catenin mutant males (yellow

arrows in Fig. 7H) probably owing to incomplete recombination by the *Amhr2-Cre* allele and/or contribution from cells with a non-recombined β-catenin allele, including the coelomic epithelial cells, to the Müllerian duct mesenchyme (Guioli et al., 2007). Taken together, these data suggest that β-catenin is specifically inactivated in the Müllerian duct mesenchyme of the conditional mutants by E14.5.

We examined expression of a canonical WNT target molecule, LEF1, in the absence of β-catenin function at E14.5. At this stage, E-cadherin (CDH1) is detected strongly in the Wolffian duct epithelium and weakly in the Müllerian duct epithelium (Fig. 7I-L). We found that LEF1 upregulation in the Müllerian duct mesenchyme was not observed in the β-catenin mutant males (Fig. 7I,J), suggesting that the activation of the canonical WNT pathway in the Müllerian duct mesenchyme was abolished in the conditional β-catenin mutant males.

Lastly, we examined the expression of an apoptotic marker, cleaved caspase-3, in the Müllerian duct epithelium of the β-catenin mutant males. Because apoptosis occurs in a wave manner along the anterior-posterior axis of the Müllerian duct epithelium (Allard et al., 2000), we examined serial sections of the entire Müllerian duct and counted sections with the cleaved caspase-3 positive Müllerian duct epithelium. In control males, we observed cleaved caspase-3 positive cells in the Müllerian duct epithelium in $66.7\% \pm 15.7\%$ ($n=2$) of sections from the entire Müllerian duct (Fig. 7K; see Fig. S1 in the supplementary material). When β-catenin was inactivated by the *Amhr2-Cre* allele in males, sections with the cleaved caspase-3 positive Müllerian duct epithelium were significantly reduced ($26.1\% \pm 1.2\%$, $n=2$) (Fig. 7L), indicating suppression of apoptosis in the Müllerian duct epithelium in the absence of β-catenin activity in the Müllerian duct mesenchyme. Taken together, these observations indicate that the canonical WNT/β-catenin pathway in the Müllerian duct mesenchyme is dispensable for AMH signaling activation in the Müllerian duct mesenchyme, but required to mediate AMH signaling in the Müllerian duct mesenchyme to induce apoptosis in the Müllerian duct epithelium. Thus, AMH signaling acts upstream of β-catenin, which mediates Müllerian duct regression during male sexual differentiation (Fig. 8).
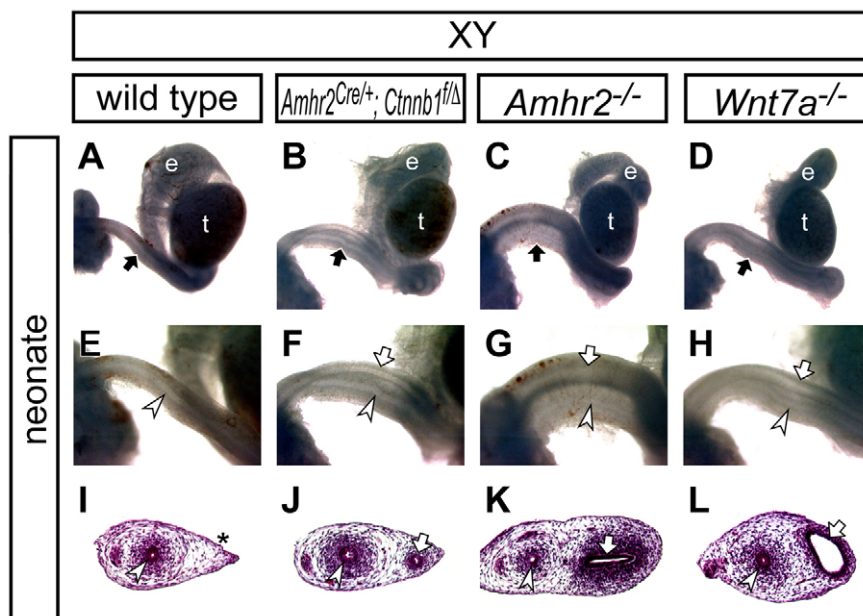

Fig. 6. β-catenin is required for Müllerian duct regression. (**A-L**) Reproductive organs from wild-type (A,E,I), *Amhr2^Cre/+^; Ctnnb1^flox/del^* (B,F,J), *Amhr2^−/−^* (C,G,K) and *Wnt7a^−/−^* (D,H,L) XY males at birth. A-D show whole-mount view of male reproductive organs; E-H are higher magnification images of the reproductive tract indicated by black arrows in A-D, respectively. I-L show histological analysis of the reproductive tract by Hematoxylin and Eosin staining. The asterisk in I indicates the region where the Müllerian duct regressed. Black arrows, white arrows and white arrowheads indicate the reproductive tract, Müllerian duct epithelium and Wolffian duct epithelium, respectively. e, epididymis; t, testis.
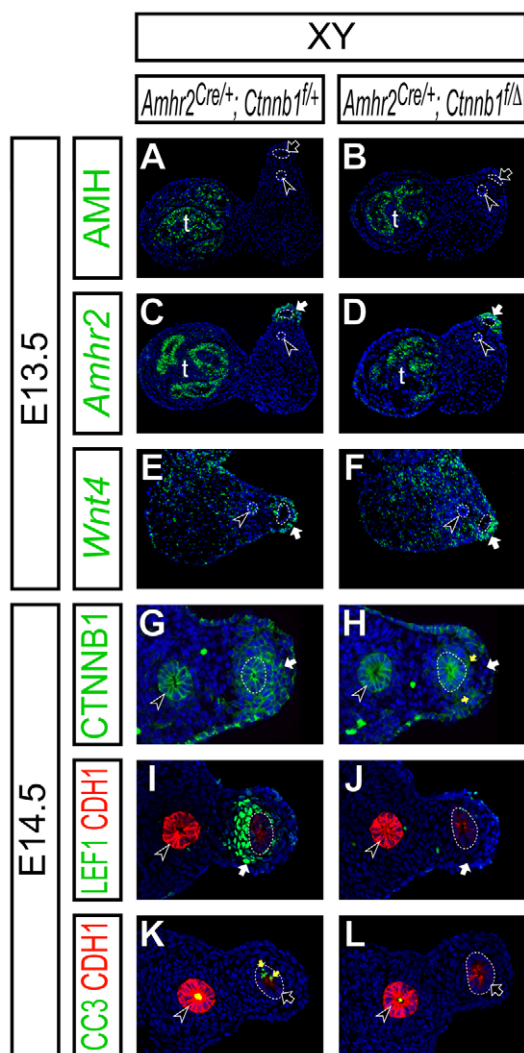
Fig. 7. AMH signaling is activated normally in the mouse Müllerian duct mesenchyme in the absence of β-catenin activity.
(**A-L**) Molecular marker analysis in the reproductive organs at E13.5 (A-F) and reproductive tracts at E14.5 (G-L) in *Amhr2*^Cre/+^; *Ctnnb1*^flox/+^ (A,C,E,G,I,K) and *Amhr2*^Cre/+^; *Ctnnb1*^flox/del^ (B,D,F,H,J,L) XY male embryos. (A,B) Epifluorescence immunostaining of AMH (green) with DAPI staining (blue). (C-F) Radioactive section in situ hybridization for *Amhr2* (green in C,D) and *Wnt4* (green in E,F) with Hoechst staining (blue). (G-L) Confocal immunofluorescence. (G,H) β-catenin (Ctnnb1, green) with DAPI (blue) staining. (I,J) LEF1 (green), E-cadherin (CDH1, red) with Hoechst (blue) staining. (K,L) Cleaved caspase-3 (CC3, green), E-cadherin (CDH1, red) with Hoechst (blue) staining. E-cadherin is highly expressed in the Wolffian duct epithelium (black arrowheads) and weakly in the Müllerian duct epithelium (dotted line) in I-L. White arrows, black arrows, black arrowheads and yellow arrows indicate the Müllerian duct mesenchyme, Müllerian duct epithelium, Wolffian duct epithelium and cleaved caspase-3 positive apoptotic cells, respectively. t, testis.

DISCUSSION
A requirement of β-catenin for Müllerian duct regression
Our data suggest that β-catenin mediates AMH signaling in the Müllerian duct mesenchyme and is required for Müllerian duct regression in male fetuses as inactivation of β-catenin in the Müllerian duct mesenchyme resulted in retention of ectopic female reproductive tract organs in males. By contrast, when β-catenin was
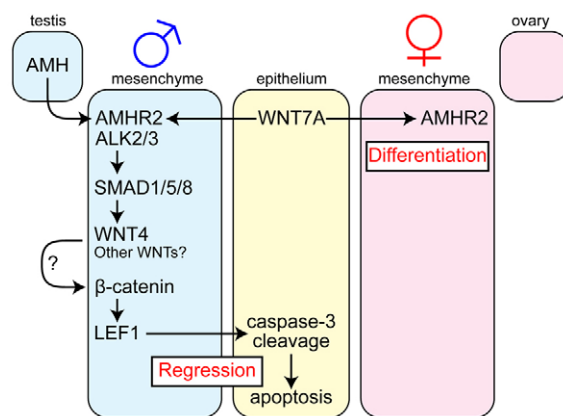

Fig. 8. A model for genetic pathways for Müllerian duct regression during male sexual differentiation. WNT7a in the Müllerian duct epithelium is required for expression of the AMH type II receptor AMHR2 in the Müllerian duct mesenchyme in both sexes. In males, the testis secretes a TGF-β signaling molecule, AMH, which binds to the type II receptor AMHR2 and type I receptors ALK2 and ALK3 in the Müllerian duct mesenchyme. The activation of the AMH signaling is mediated by three receptor SMADs, SMAD1, SMAD5 and SMAD8 (SMAD1/5/8), which directly or indirectly activate male-specific WNT4 expression in the Müllerian duct mesenchyme. WNT4, possibly with other WNT ligands, might act as an autocrine signal to stabilize β-catenin in the Müllerian duct mesenchyme. The activation of β-catenin is transmitted by unidentified molecular signal(s) to the Müllerian duct epithelium, where apoptosis is induced, at least partially, by caspase-3 cleavage, leading to Müllerian duct regression. In females, there is no production of AMH, which permits Müllerian duct differentiation into female reproductive tract organs.

inactivated in the epithelium of the Müllerian duct using the *Wnt7a-Cre* allele, the Müllerian duct regressed normally in mutant males (C.A.S. and R.R.B., unpublished data), suggesting that β-catenin in the Müllerian duct epithelium is dispensable for Müllerian duct regression. Thus, β-catenin function is required specifically in the mesenchyme of the Müllerian duct for Müllerian duct regression in males.

β-catenin has two different functions: one in the protein complex at adherens junctions (AJs) of epithelial cells and the other in the canonical WNT signaling pathway. Because β-catenin activity is required in the mesenchyme, but not the epithelium, of the Müllerian duct during Müllerian duct regression, it is unlikely that β-catenin functions through AJs. Furthermore, β-catenin was observed mostly in the cytoplasm and nucleus of the Müllerian duct mesenchyme only in males, whereas β-catenin was detected at the cell surface in the Müllerian duct epithelium in both sexes (Allard et al., 2000), indicating that β-catenin regulates the canonical WNT signaling pathway required for Müllerian duct regression during male sexual differentiation.

WNT ligands for Müllerian duct regression
It still remains unclear which WNT ligand(s) mediates AMH signaling during Müllerian duct regression. *Wnt7a* expressed in the Müllerian duct epithelium is essential for Müllerian duct regression during male sexual differentiation (Parr and McMahon, 1998). Because *Amhr2* expression in the Müllerian duct mesenchyme depends on *Wnt7a* activity in both sexes, the Müllerian duct lacking *Wnt7a* activity cannot respond to AMH. Because β-catenin accumulates in the nucleus of Müllerian duct mesenchyme cells in

males but not in females (Allard et al., 2000), and we showed that the AMH signaling is activated through normal *Amhr2* expression without β-catenin function in the Müllerian duct mesenchyme, we can conclude that WNT7A is not the WNT ligand that regulates β-catenin during Müllerian duct regression in males.

WNT4 is a good candidate as a WNT ligand necessary for Müllerian duct regression as AMH signaling regulates the male-specific expression of *Wnt4* in the Müllerian duct mesenchyme. Interestingly, *Wnt4* signaling is mediated by the canonical WNT/β-catenin pathway during kidney development (Park et al., 2007). However, our results of normal regression of the Müllerian duct in *Amhr2*$^{Cre/+}$; *Wnt4*$^{flox/del}$ males suggest that *Wnt4* is dispensable for Müllerian duct regression. We observed *Amhr2-Cre* reporter expression in the Müllerian duct mesenchyme from the onset of its formation at E12.5, and one day later at E13.5 detected the male-specific *Wnt4* expression in the Müllerian duct mesenchyme. Thus, timing of Cre activation is probably not the issue for *Wnt4* inactivation in the Müllerian duct mesenchyme.

Another consideration before excluding *Wnt4* involvement in Müllerian duct regression is that the *Amhr2-Cre* allele might not have sufficiently high Cre recombinase activity in the Müllerian duct mesenchyme, especially during early stages of Müllerian duct development (Deutscher and Hung-Chang Yao, 2007). We observed some mosaicism of Cre recombination in the Müllerian duct mesenchyme from the *Amhr2-Cre* allele even with the *R26R-lacZ* allele, which can be recombined more efficiently than other Cre reporter alleles (Long and Rossi, 2009). Considering that *Wnt4* encodes a secreted molecule, more complete recombination might be required for total inactivation of *Wnt4* function. Alternatively, another WNT ligand(s) might redundantly activate β-catenin with WNT4 during Müllerian duct regression.

Dual roles of β-catenin for Müllerian duct development

Our data suggest that β-catenin has dual roles in the Müllerian duct mesenchyme: one for Müllerian duct regression in males and the other for Müllerian duct differentiation in females. It was proposed that *Wnt7a* activates the canonical WNT/β-catenin pathway in the Müllerian duct (Deutscher and Hung-Chang Yao, 2007). Although our observations might support this view, we found that the ectopic female reproductive tracts in the conditional β-catenin mutants are different from those in *Wnt7a* mutants, suggesting involvement of another WNT ligand for female reproductive tract differentiation. Our observations are consistent with a previous report that loss of β-catenin in the Müllerian duct mesenchyme results in trans-differentiation of the myometrium to adipose tissues in adults, which is not observed in *Wnt7a* mutants (Arango et al., 2005). Interestingly, *Wnt5a* plays important roles for Müllerian duct differentiation during embryogenesis (Mericskay et al., 2004), suggesting that it might also act redundantly with *Wnt7a* in Müllerian duct differentiation.

The mechanism for β-catenin exerting the two distinct functions for Müllerian duct development remains to be studied. One explanation might be stage-specific functions of β-catenin during Müllerian duct regression and differentiation. It is known that the competence or regressive response of the Müllerian duct to AMH signaling occurs in a narrow window of time (Picon, 1969; Tsuji et al., 1992; Allard et al., 2000). During Müllerian duct regression (e.g. E13.5-15.5), β-catenin mediates AMH signaling pathways. Subsequently (from E15.5 to birth), β-catenin might promote Müllerian duct differentiation. Consistent with this idea, the Müllerian duct remained small and round in the neonate without β-catenin (Fig. 6J).

Relationships between the AMH and WNT/β-catenin signaling pathways

Cross talk between TGF-β/BMP and WNT/β-catenin signaling pathways has been observed during limb development, when the BMP receptor IA acts upstream of β-catenin for formation of the apical ectodermal ridge (AER) (Soshnikova et al., 2003). *Bmp2* also regulates β-catenin signaling pathway during bone formation (Chen et al., 2007; Zhang et al., 2009). Regulation of the canonical WNT/β-catenin pathway by TGF-β/BMP signaling is an important regulatory mechanism for a number of biological processes.

During kidney development, the WNT9b/β-catenin signaling pathway activates the WNT4/β-catenin signaling pathway in precursor cells of the nephron (Park et al., 2007). Inactivation of *Wnt4* alone blocks nephron formation (Stark et al., 1994; Kobayashi et al., 2005; Perantoni et al., 2005), indicating the significance of an additional signaling pathway for signaling amplification mechanisms. Thus, we propose that, like other family members, the WNT/β-catenin signaling might amplify AMH signaling to ensure complete Müllerian duct regression in males. Given these findings, mutations in WNT signaling component genes should be investigated in persistent Müllerian duct syndrome (PMDS) human patients.

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