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Dicer is required for female reproductive tract development and fertility in the mouse

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Abstract

Dicer encodes a riboendonuclease required for microRNA biosynthesis. *Dicer* was inactivated in Müllerian duct mesenchyme-derived tissues of the reproductive tract of the mouse, using an *Amhr2-Cre* allele. Although *Amhr2-Cre; Dicer* conditional mutant males appeared normal and were fertile, mutant females were infertile. In adult mutant females, there was a reduction in the size of the oviducts and uterine horns. The oviducts were less coiled compared to controls and cysts formed at the isthmus near the uterotubal junction. Unfertilized, degenerate oocytes were commonly found within these cysts, indicating a defect in embryo transit. Beads transferred into the mutant oviduct failed to migrate into the uterus. In addition, blastocysts transferred directly into the mutant uterus did not result in pregnancy. Histological analysis demonstrated that the mutant uterus contained less glandular tissue and often the few glands that remained were found within the myometrium, an abnormal condition known as adenomyosis. In adult mutants, there was ectopic expression of *Wnt4* and *Wnt5a* in the luminal epithelium (LE) and glandular epithelium (GE) of the uterus, and *Wnt11* was ectopically expressed in GE. These results demonstrate that *Dicer* is necessary for postnatal differentiation of Müllerian duct mesenchyme-derived tissues of the female reproductive tract, suggesting that microRNAs are important regulators of female reproductive tract development and fertility.

Keywords

uterus; oviduct; microRNA; tissue-specific knockout; *Wnt*; adenomyosis

Introduction

Small silencing ribonucleic acids are short single-stranded RNAs that bind to complementary nucleotide sequences found in the 3′ untranslated regions (3′ UTR) of messenger RNA (mRNA) (Lee et al. 1993; Wightman et al. 1993). This binding can lead to repression of mRNA translation and/or degradation of target transcripts. One major type of small silencing RNAs is the microRNA (miRNA). MicroRNAs form when RNA polymerase II transcribes a primary transcript containing a hairpin-loop structure (pri-miRNA). The pri-miRNA is processed inside the nucleus by Drosha into a double-stranded RNA (dsRNA) stem-loop of ~70 nucleotides (pre-miRNA) (Lee et al. 2003). The pre-miRNA is transported out of the nucleus by Exportin-5 in a Ran-GTP-dependent manner, where it is furthered cleaved in the cytoplasm by the riboendonuclease Dicer into 19-25 nucleotide dsRNA fragments (Yi et al. 2003). In the final step of miRNA maturation, the

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dsRNAs are separated into guide and passenger strands. The guide strand is bound to the RNA-induced silencing complex (RISC) while the passenger strand is degraded. Once incorporated into the RISC complex, the guide strand binds to a complementary sequence found in the 3′ UTR of target mRNA, inhibiting translation (Bernstein et al. 2001).

Dicer is found in nearly all organisms, suggesting that it has been conserved throughout evolution. Its essential role in animal development has been well established. *Dicer*-null mice die at embryonic day (E) 7.5, demonstrating that *Dicer* is essential for early mouse embryo viability (Bernstein et al. 2003). In addition, target-selected inactivation of the *Dicer* in zebrafish resulted in developmental arrest around 8 days post-fertilization, demonstrating an essential role in fish development (Wienholds et al. 2003). It is known that microRNAs are required to inhibit gene expression in a tissue-specific manner. Therefore, the inactivation of *Dicer* in specific tissues can be used to indirectly assess the role of microRNAs during tissue and organ development and differentiation.

The female reproductive tract, composed of the oviducts, uterus, cervix, and vagina, is essential for the propagation of all mammalian species. These organs develop from the Müllerian duct, a tube formed by specification of surface epithelial cells of the rostral mesonephros around embryonic day (E) 11.5. These cells invaginate towards the Wolfian duct and elongate adjacent to it until reaching the urogenital sinus by ~E13.5 (Orvis and Behringer 2007). In females, the Müllerian duct differentiates into the female reproductive tract, however in males the Müllerian duct is eliminated due to the action of anti-Müllerian Hormone (AMH) secreted by the Sertoli cells of the fetal testis (Josso 2008). AMH, a member of the Transforming Growth Factor-β (TGF-β) superfamily, signals through type I receptors (Bone morphogenetic protein receptor type 1A and activin A receptor type 1), a type II receptor (AMH receptor type 2, AMHR2), and Smad1, Smad5, and Smad8 to induce Müllerian duct regression (Jamin et al. 2002; Mishina et al. 1996; Orvis et al. 2008; Visser et al. 2001).

The forming Müllerian duct consists of two types of cells, an inner mesoepithelial tube with a surrounding mesenchyme. The mesopithelial layer most likely differentiates into the luminal epithelium (LE) and the glandular epithelium (GE) while the mesenchyme likely differentiates into the endometrial stroma, and the inner circular and outer longitudinal myometrium of the uterus. At birth, the mouse female reproductive tract is not fully developed but requires further patterning and cytodifferentiation to complete development by postnatal day 14 (P14). The oviducts lengthen and coil and differentiate into three regions: the infundibulum, the ampulla and the isthmus. The uterotubal junction connects the oviducts to the uterine horns. Embryo implantation in the uterus and subsequent development requires nutrients from the mother. Uterine glands deliver necessary factors and nutrients to the developing embryo (Bazer 1975). The formation of uterine glands in mammals is referred as adenogenesis, a process believed to originate when a single cell of the LE is specified to undertake a GE fate. Once specified, these monoclonal epithelial cells will bud out from the LE and invaginate as a tubular extension into the stroma and later coil and branch (Gray et al. 2001a). In mice, uterine adenogenesis initiates around postnatal day 4 and completed by day 14 (Hu et al. 2004).

To assess the role of small silencing RNAs in the development of the mouse female reproductive tract, we utilized a *Dicer* conditional null allele (Harfe et al. 2005). In the *Dicer* conditional null allele, exon 23 that encodes the active site of an RNaseIII domain, is flanked by *lox*P sites (*fx*) for Cre-mediated deletion. Deletion of exon 23 appears to completely inactivate Dicer processing activity of pre-miRNAs (Harfe et al., 2005). *Dicer fx/fx* mice are normal and fertile. *Amhr2-Cre* knock-in mice express Cre recombinase along the entire length of the mesenchyme of the forming Müllerian duct (Jamin et al. 2002).

Therefore, we examined the consequences of inactivating *Dicer* in cells derived from the mesenchyme of the Müllerian duct for female reproductive tract development.

Results

Amhr2-Cre; Dicer conditional mutant females are infertile

To establish the breeding pairs to produce *Amhr2-Cre; Dicer fx/fx* females, we also generated *Amhr2-Cre; Dicer fx/fx* males (see Materials and Methods). *Amhr2-Cre; Dicer fx/fx* males appeared normal and proved to be fertile. Therefore, we used these males in crosses with *Dicer fx/fx* females to generate *Amhr2-Cre; Dicer fx/fx* females and *Dicer fx/fx* littermate controls. *Amhr2-Cre; Dicer fx/fx* females were obtained at the predicted Mendelian ratios, appeared normal, but when bred with wild-type males (n = 6) and vaginal plugs were found, they did not become pregnant. In addition, when these mutant females were left with wild-type males for 4-6 months, they produced no progeny. Another group of mutant females (n=4) was mated with wild-type males, vaginal plugs obtained, and then sacrificed on E14.5. No embryos were observed inside the uterus. These results suggest that *Dicer* expression in Müllerian duct mesenchyme-derived tissues of the female reproductive tract is required for fertility.

Currently, there is no antibody that is available for immunohistochemistry against Dicer to demonstrate that Dicer is lost only in Müllerian duct mesenchyme-derived tissues of the conditional mutants. Therefore, to confirm that *Amhr2-cre* was able to delete exon 23 in the *Dicer fx* allele, PCR amplification of was performed (Fig. 1A). DNA was extracted from the uterus and oviducts of control and mutant females and amplified using *Dicer*-specific primers. PCR amplification of the DNA resulted in ~600-bp product, confirming that exon 23 of *Dicer* had been deleted in cells of the female reproductive tract (Fig. 1B). The same DNA was also amplified using *Dicer* primers specific for the *fx* allele, resulting in a 420-bp product from tissue without the deletion. (i.e. epithelial-derived). As controls, DNA was extracted from *Dicer fx/fx* females that did not inherit the Cre allele. *Dicer* deletion-specific primer amplification could not be detected while *Dicer fx*-specific amplification resulted in the predicted 420-bp product.

Morphological and histological abnormalities in *Amhr2-Cre; Dicer* conditional mutant female reproductive tract

The reproductive organs of adult control and mutant females and males were dissected. No gross abnormalities were observed in the mutant male reproductive organs. Mutant males were fertile and bred with *Dicer fx/fx* females to increase the probability of generating *Amhr2-cre; Dicer fx/fx* mice. In mutant males, no Müllerian remnants (uterus and oviducts) were observed, indicating that the Müllerian ducts regressed normally. However, abnormal phenotypes were observed in the mutant female reproductive tract. In adult mutant females, all the components of the female reproductive tract were present, including ovaries, oviducts, uterine horns, a cervix and the vagina. No gross morphological abnormalities were observed in the ovary. However, the uterine horns appeared shorter and thinner when compared to control littermates (Fig. 2A, B). The oviducts also had several abnormalities; they were shorter in length and less coiled (Fig. 2C, D). In the majority of the mutant females, the oviducts had spheroidal expansions near the uterotubal junction, resembling cysts (Fig. 3A). These cysts were found unilaterally (n=9, 11%) and bilaterally (n=9, 89%). The cysts were found in the isthmus, the region of the oviduct that connects the ampulla to the uterus. These were observed to develop from a transparent fluid-filled cyst into a large cyst filled with a yellowish soft mass that filled the entire oviduct (Fig. 3A, B). Histologically, the mass inside the oviduct contained immune/inflammatory cells (Fig. 3E-H). The mutant oviducts also showed a deficiency of smooth muscle (Fig. 3C, D, and F)

perhaps causing a weakening in the oviductal wall, leading to cyst formation. In many unmated mutant females, oocytes were found inside the cysts (Fig. 3I). The oocytes were isolated and observed under the microscope. Most of the oocytes appear to retain the zona pellucida, the acellular surrounding membrane of the oocyte, however the cytoplasm appeared fragmented and crystalline structures where found inside the zona (Fig. 3J). These findings demonstrate that the mutant ovaries can ovulate.

The female reproductive tract of control and mutant females were dissected at various times after birth to determine when the phenotypic differences were first apparent in the mutant females. On postnatal day 4 there were no significant differences observed in the length of the uterus of mutant females when compared to control females (Fig. 2E, F). However, the mutant uterus appeared thinner and the oviducts were less coiled than the control littermates (Fig. 2G, H). Similar phenotypes were observed on postnatal day 8 (data not shown). On postnatal day 21, there was a marked difference in the size of mutant reproductive tract when compared to control littermates. Expansion of the isthmus, forming oviductal cysts was first observed around postnatal day 28 (Fig. 3A). At this stage, small yellow masses, apparently the onset of an inflammatory response were observed (Fig. 3A). To determine the progression of the cysts and the extent of their growth, 8 week (8 control, 8 mutant), 4 month (3 control, 4 mutant), and 8 month-old (2 control, 2 mutant) females were sacrificed. The cysts were present at all three time points in the mutants. However, at 8 weeks the inflammation appeared to be more prominent than at 4 and 8 months because the cysts were generally smaller. Interestingly, in two 8 week-old mutant females with cysts, the uterus was apparently fused to the abdominal wall and intestines.

Histological analysis of *Amhr2-Cre; Dicer* conditional mutant female reproductive tract

To assess the cytological differences between control and mutant female reproductive tracts, a histological analysis initially using hematoxylin and eosin (H&E) staining was performed. Tissue sections of the control and mutant female reproductive tracts collected from postnatal day 4 and day 8 mice, showed no differences in uterine cytoarchitecture or in the amount of endometrial gland tissue. However, on postnatal day 21, less endometrial gland tissue was observed in the mutant female uterus when compared to controls (Fig. 4). The reduced amount of uterine gland tissue in the mutants was found at all later time points examined (Fig. 5).

On postnatal day 28, in the oviduct of mutant females, epithelial cells in the ampulla region appeared morphologically normal compared to controls. However, the epithelial cells lining the lumen of the cysts that developed in the isthmus region of mutant females appeared morphologically abnormal (Fig. 3D). Epithelial cells of the expanded isthmus contained a large number of vacuoles inside the cytoplasm, known as intracellular edema, there was a loss of typical columnar epithelium morphology, and epithelial folds were absent (Fig. 3D). Furthermore, inside the lumen of the isthmus a large number of white blood cells clumps were observed.

Tissue sections collected after 6 weeks of age showed the same major differences between control and mutant female reproductive tracts. There was an expanded isthmus with intracellular edema, loss of mucosal folding of the epithelial cells, an immune/inflammatory response inside the oviduct, and lower amount of glandular tissue in the uterus. Interestingly, in older females (> 4 month of age), many times the glands that were present in the mutant uterus were found within the myometrium (Fig. 4G, H).

E-cadherin is a convenient marker expressed in both the LE and GE of the uterus and epithelial component of the oviduct, therefore control and mutant female reproductive tracts were immunostained for E-cadherin and analyzed by fluorescent microscopy. This analysis

confirmed the H&E results, showing that mutant females had significantly lower amount of endometrial gland tissue in the uterus compared to controls (Fig. 4C-F). No differences in LE morphology were detected between controls and mutants.

The *Dicer* mutant females should also express Cre in granulosa cells of the postnatal ovary (Jorgez et al. 2004), thus hormone produced by granulosa cells could potentially be altered, affecting the female reproductive tract. Serum estradiol and progesterone levels were measured. The data showed a slight difference between control and mutant females but was not significantly different between the two groups (Fig. 6). Although there was a high degree of variability in these hormone measurements, we can conclude that estradiol and progesterone levels in the mutants were not dramatically different from controls.

Abnormal function of the conditional mutant oviduct, uterotubal junction, and uterus

In most of the mutant females, oocytes were found inside the cystoid expansions of the oviduct. Normally, oocytes are fertilized inside the ampulla, then peristaltic contractions and the cilia of the epithelial folds move the oocytes into the uterus, where they implant in the endometrium. If this transport mechanism is disrupted or the path is obstructed the embryos will be unable to reach the uterus. In most of the mutant oviducts collected at 6 week, 8 week and 4 months, there was obvious inflammation blocking the path of the oocytes (and embryos if sperm could reach the ampulla). However, mutant females sometimes also had an oviduct that was free of inflammation. Therefore, to test if the oviduct transport mechanism was disrupted in the mutants, known numbers of Affi-Gel Blue affinity chromatography beads that are similar in size to mouse preimplantation embryos (75-150 mm), were transferred into oviducts. For these experiments, 5 week-old females (2 control, 3 mutant) were selected because at this age the inflammatory response is at an early stage that might not interfere with embryo movements. 1-4 days after bead transfer the reproductive tracts were examined. Most of the beads transferred inside control oviducts had moved into the uterus or were located at the uterotubal junction, whereas beads transferred into mutant females remained in a clump at about the same position of the initial transfer inside the oviduct near the ampulla (Fig. 7A-D).

Impaired ciliogenesis or unidirectional flow created by cilia pulsation was also examined. The oviducts of 5 week-old females, both control and mutant, were dissected and placed in tissue culture medium. A longitudinal incision was made along the wall of the oviduct, and the oviducts were placed in a glass bottomed Petri dish. Both control and mutant oviducts contained cilia, thus ciliogenesis was not impaired (data not shown). To test for unidirectional flow by cilia, a small drop of concentrated dye solution was added and the direction of the flow of dye was recorded using time-lapse imaging. Control and mutant cilia showed unidirectional flow as indicated by the dye, thus cilia of the oviductal epithelium appear to be functional in both control and mutant females (data not shown).

The uterotubal junction regulates embryo movement into the uterus and retrograde flow from the uterus into the oviduct (Newbold et al. 1983).To examine the uterotubal junction, the rostral reproductive tract separated from the cervix of 2 week and 4 month-old females was isolated and a blue dye was injected into the caudal uterine lumen. In control reproductive tracts, the injected dye moved up to the most rostral end of the uterine horn but did not enter the oviduct (Fig. 7E). In mutant reproductive tracts, the dye also flowed up to the most posterior part of the uterine horn, however, it was able to move past the uterotubal junction and enter the oviduct (Fig. 7F). Thus there is a defect in the uterotubal junction of the mutant females such that retrograde flow into the oviduct is not regulated.

Although the infertility of the mutant females is likely due to abnormal embryo transit in the oviduct, we also tested the function of the uterus to support embryo development by embryo

transfer. Wild-type blastocyst stage (E3.5) embryos were collected from Swiss matings. Approximately 10-12 embryos were transferred into the most rostral part of the uterus of pseudopregnant (E2.5) surrogate mutant females (n=3). The reproductive tracts of the surrogate mice were dissected 11 days after embryo transfer but none were observed to be pregnant nor were there embryo reabsorption sites.

Abnormal *Wnt* gene expression in *Amhr2-Cre; Dicer* conditional mutants

Wnt4, *Wnt5a*, and *Wnt7a* are expressed in the developing Müllerian duct and adult female reproductive tract (Miller et al. 1998b). *Wnt5a* and *Wnt7a* knockout mice lack endometrial glands and show and array of defects in the reproductive tract (Mericskay et al. 2004; Miller and Sassoon 1998). In sheep, downregulation of *Wnt11* by estrogens appears to inhibit adenogenesis (Hayashi and Spencer 2006). Therefore, we performed in situ hybridization using probes against the mRNA of *Wnt4, Wnt5a, Wnt7a*, and *Wnt11*. On postnatal day 4, both control and mutant females expressed *Wnt4* and *Wnt5a* in the uterine and oviduct mesenchyme and uterine LE, whereas *Wnt7a* was expressed in the LE (Suppl. Fig. 1). On postnatal day 14, in both control and mutant females, expression of *Wnt5a* was restricted to the uterine stroma and oviduct mesenchyme, while *Wnt7a* was expressed in the LE (Fig. 8A-D). However, by 8 weeks of age, mutant females exhibited abnormal spatial expression of *Wnt* genes in the uterus. Normally, *Wnt4* is expressed in the stratified epithelium of the cervix and the vagina, however, in mutant females, *Wnt4* was ectopically expressed in the LE and GE (Fig. 8E, F). In the control uterus, *Wnt5a* was restricted to the stroma, whereas in the mutant uterus, *Wnt5a* was ectopically expressed in the LE and GE (Fig. 8G, H). In the control uterus, *Wnt11* expression was restricted to the LE and was absent in the GE, however in the mutant uterus, *Wnt11* was expressed in the LE and ectopically expressed in the GE (Fig. 8I, J).

Hoxa genes are involved in patterning the female reproductive tract (Taylor et al. 1997). *Hoxa9* is expressed in the oviduct and anterior part of the uterine horns, *Hoxa10* and *Hoxa11* are expressed in the uterine horn, and *Hoxa13* is expressed in the cervix and vagina (Taylor et al. 1997). *Hoxa* gene expression was examined in tissue sections, from both control and mutant females, collected on postnatal day 14. In controls and mutants, *Hoxa9* gene expression was detected in the oviduct and anterior stroma of the uterine horns (Suppl. Fig. 2A, B). In controls and mutants, *Hoxa10* expression was detected in the uterine stroma adjacent to the LE (Suppl. Fig. 2C, D). Thus, on postnatal day 14, no difference *Hoxa9 and Hoxa10* gene expression was detected between control and mutant females.

Discussion

Dicer is essential for processing pre-miRNA into miRNAs that regulate gene expression and tissue differentiation (Alvarez-Garcia and Miska 2005; Stefani and Slack 2008). Therefore, loss of *Dicer* should lead to a deficiency of miRNA production (Harfe et al. 2005). Epithelial-mesenchymal interactions are required for proper formation and development of the female reproductive tract in the mouse (Kurita et al. 2001). By taking advantage of the Cre/*lox*P system, *Dicer* was conditionally inactivated in the mesenchyme of the Müllerian duct and its tissue derivatives of the female reproductive tract by Cre recombinase expressed from the endogenous *Amhr2* locus (Jamin et al. 2002). Tissue-specific inactivation of *Dicer* resulted in a wide range of female reproductive tract abnormalities not limited to mesenchyme-derived tissues (i.e. epithelial tissues). Thus, the abnormalities in the epithelial compartments of the mutant female reproductive tracts must be an indirect consequence of *Dicer* inactivation in mesenchymal tissues. These abnormal phenotypes suggest that small silencing RNAs acting within tissues derived from the Müllerian duct mesenchyme are necessary for mesenchymal and epithelial development of the female reproductve tract.

Female infertility in *Dicer* mutants

Fertilization, embryo transit, implantation and development require an elaborate synchrony of physiological processes (Guzeloglu-Kayisli et al. 2007). Synchronized epithelial-mesenchymal, epithelial-epithelial interactions, in conjunction with endocrine factors are all essential for a successful pregnancy. Reduction of *Dicer* expression using a hypomorphic gene trap allele resulted in female mouse infertility, showing impaired corpus luteum angiogenesis that led to low progesterone production (Otsuka et al. 2008). Alterations in systemic hormone levels could lead to the abnormal phenotypes observed in the *Amhr2-Cre*, *Dicer* mutant female reproductive tract. However, serum estradiol and progesterone levels in the *Dicer* mutants although quite variable were not significantly different from controls, suggesting that systemic hormones may not be the cause of the infertility.

The infertility of the *Dicer* conditional mutant females appears to be due to several oviduct and uterine abnormalities. Cilia in the oviduct are important for the movement of preimplantation embryos to the uterus (Halbert et al. 1976). Ciliogenesis and cilia function in the oviducts of the mutant females did not seem to be impaired, however blue beads transferred inside the ampulla failed to move into the uterus, suggesting a defect in the transport mechanism for oviductal movement of preimplantation embryos. Peristaltic contractions caused by oviductal smooth muscle also contribute to the movements of preimplantation embryos (Maistrello 1971). Interestingly, we observed regions of the mutant oviducts that were deficient in smooth muscle. The Müllerian duct mesenchyme is likely the progenitor tissue of the oviduct smooth muscle and uterine myometrium (Arango et al. 2008). Therefore, *Dicer* may be important for the generation of miRNAs that regulate smooth muscle differentiation or maintenance. Interestingly, Müllerian duct mesenchyme-specific knockout of beta-catenin results in smaller uteri and a deficiency of myometrium that is replaced by adipose tissue (Arango et al. 2005). These findings are consistent with the idea that miRNAs may be important for maintaining correct Wnt signaling for myometrial differentiation. The abnormalities in oviduct smooth muscle could also explain the development of the cysts because a weakness in the oviduct wall caused by smooth muscle abnormalities could lead to a weakness in the oviduct wall. Although the *Dicer* mutant females were successfully mated as judged by the presence of a vaginal plug, no pregnancies occurred. This could be due in part to the oviductal inflammation perhaps blocking access of the sperm to the oocytes. Finally, the mutant females had defects in the uterotubal junction. This structure functions to regulate embryo transit into the uterus and retrograde flow from the uterus into the oviduct (Newbold et al. 1983). Abnormalities in these uterotubal junction functions may also contribute to the infertility.

Abnormalities in the uterus of the *Dicer* mutant females also lead to female infertility. We showed that there are fewer endometrial glands in the mutant uterus. Endometrial glands are required for secretion and transport of nutrients, including leukemia inhibitory factor, necessary for embryo implantation and survival (Gray et al. 2001a). In a sheep uterine gland knockout model, embryos do not elongate and fail to implant (Gray et al. 2001b). Therefore, the reduced amount of endometrial gland tissue observed in our *Dicer* mice probably hinders embryo implantation and survival. Although the primary cause of the infertility in the mutant females remains to be determined, it is clear that *Dicer* regulates several aspects of oviduct and uterine development that are essential for fertility.

Small silencing RNA regulation of female reproductive tract development

AMH-induced Müllerian duct regression is mediated by AMH interactions with AMH receptors expressed in the Müllerian duct mesenchyme (Kobayashi and Behringer 2003; Orvis et al. 2008; Visser et al. 2001). The Müllerian ducts appeared to regress normally in the *Dicer* mutant males. Therefore, small silencing RNAs do not appear to regulate AMH

signaling in the Müllerian duct mesenchyme for Müllerian duct regression. Furthermore, mutant males did not show abnormal virilization and were fertile, suggesting that Leydig cell function necessary for virilization and spermatogenesis was normal. *Amhr2-Cre* has been shown to be active in postnatal Leydig cells (Jeyasuria et al. 2004). Our results suggest that small silencing RNAs may not have major roles in regulating gene expression in Leydig cells for virilizing hormone production. The efficacy of the *Amhr2-Cre* allele in Sertoli cells is unclear, therefore the role of small silencing RNAs in Sertoli cells remains an open question (Jamin et al. 2002; Jeyasuria et al. 2004; Pangas et al. 2008).

In females, all the components of the female reproductive derived from the Müllerian duct were present (i.e. oviduct, uterine horns, cervix and upper vagina). Histological analysis revealed abnormal cytoarchitecture in smooth muscle, luminal epithelium and glandular epithelium of mutant females when compared to controls. The phenotypic defects and the cytoarchitectural abnormalities observed in mutant females resembled previously published reports on *Wnt* gene disruption and prenatal diethylstilbestrol (DES) exposure (Mericskay et al. 2004; Miller et al. 1998a; Miller and Sassoon 1998). *Wnt5a, Wnt7a* gene knockouts and prenatally DES-treated mice lacked endometrial glands in the uterus, suggesting that improper spatial-temporal signaling during the development of the uterus leads to a complete disruption of adenogenesis (Miller et al. 1998a). In *Dicer* mutant females, endometrial glands initiate formation normally but ultimately the amount of glandular tissue was significantly lower compared to controls. In situ hybridization using probes against *Wnt5a* and *Wnt7a* demonstrated that there was proper spatial expression of *Wnt5a* and *Wnt7a* in the uterus and oviduct on postnatal day 8 and 14, the time period when endometrial glands form in the mouse (Hu et al. 2004). Therefore, the reduced amount of endometrial glandular tissue observed in the mutant uterus as early as P14 appears to be independent of *Wnt5a* and *Wnt7a. Dicer* is inactivated in Müllerian duct mesenchyme-derived cells not epithelial cells, therefore paracrine signals from the mesenchyme, other than Wnt signals, could be the cause of the reduction of endometrial glandular tissue.

In control females, after differentiation of the uterus is complete, *Wnt4* becomes restricted to the cervix and vagina, whereas *Wnt5a* is detected in the uterine stroma (Miller et al. 1998b). In situ hybridization of 8 week-old *Dicer* mutant female uteri revealed that *Wnt4* and *Wnt5a* are ectopically expressed in the uterine LE and GE, correlating with the observed abnormal cytoarchitectural differences. These results suggest that there appears to be a defect in the repression of *Wnt4, Wnt5a* and *Wnt11* transcription or message stability in endometrial glands. This ectopic expression of *Wnt* genes in the remaining glands could be the cause of the overgrowth observed, that in many cases are penetrating into the myometrium. In humans (women 35-50 years old), there is a rare medical condition similar to what we observed in our mutant mice called adenomyosis, were ectopic endometrium is found within the myometrium (Ferenczy 1998). This condition is found most commonly in multiparous women whereas the *Dicer* mutant female mice in this study were nulliparous because they were infertile. Mice treated with different selective estrogen receptor modulators developed adenomyosis, thus increased estrogen signaling could lead to the adenomyosis observed in the *Dicer* mutants (Parrott et al. 2001). However, serum levels of estradiol were not significantly different between controls and mutants.

Conditionally inactivating *Dicer* in the mesenchyme of the fetal Müllerian duct appears to recreate the malformations found when developing fetuses were exposed to DES (Haney et al. 1984). Females born from pregnant mice exposed to 100 μg/kg DES during pregnancy had a “developmentally arrested oviduct”, a complete absence of endometrial glands in adult uterus, and were infertile (Miller et al. 1998a). Furthermore, when dye was injected into the caudal uterus, it was able to flow into the oviduct, thus DES treatment causes malformation or malfunction of the uterotubal junction similar to the *Dicer* mutant females. However,

there was no statistical difference in the levels of estradiol or progesterone between adult control and *Dicer* mutant females. Thus, the reduced amount of endometrial glandular tissue found in adult mutant females appears to be hormone independent. Due to the similarity of the *Dicer* mutant phenotype and those of *Wnt7a* knockout mice and prenatal mice treated with DES, we suggest that small silencing RNAs in mesenchyme-derived tissues appear to play a role downstream of systemic hormones but upstream of Wnt signaling.

In the uterus, paracrine signals from the mesenchyme are necessary for specification and growth of endometrial glands from the luminal epithelium (Gray et al. 2001a). Small silencing RNAs appear to inhibit a signaling pathway that would be predicted to negatively regulate adenogeneis. In *Dicer* mutant females, this inhibitor(s) is not regulated, thus normal uterine adenogenesis is reduced. Perhaps small silencing RNAs are required to restrict the overgrowth of endometrial glands during their formation in prepubertal females. What are the identities of these microRNAs? Recently, there were two reports on the role of *Dicer* in *Amhr2-Cre*, *Dicer* conditional mutant mice with phenotypes similar to the ones reported here (Hong et al. 2008; Nagaraja et al. 2008). In one study, sequencing of small RNAs from the oviduct identified a set of genes that were altered in the mutants (Nagaraja et al. 2008). Interestingly, they found that *Wnt5a* expression was significantly down-regulated but *Wnt4* expression was unchanged. Our study shows that in addition to quantitative differences in *Wnt* gene expression, *Dicer* loss also causes alterations in tissue-specific expression. In summary, small silencing RNAs in the mesenchyme-derived tissues of the female reproductive tract appear to regulate genes autonomously in mesenchyme-derived tissues (oviduct smooth muscle) and non-cell autonomously in epithelial tissues (oviduct epithelium and uterine LE and GE).

Materials and Methods

Mice

Amhr2-Cre knock-in mice were maintained on a C57BL/6J × 129/SvEv mixed background (Jamin et al. 2002). *Dicer* conditional null (*fx*) mice were obtained from Dr. Cliff Tabin (Harvard Medical School). *Dicer* conditional null mice were maintained as a *fx/fx* stock on a C57BL/6J × “129” mixed genetic background (Jamin et al. 2002). *Amhr2-Cre* mice were genotyped by PCR using Cre primers, Cre forward: 5′ GGACATGTTCAGGGATCGCCAGGC 3′, Cre reverse: 5′ CGACGATGAAGCATGTTTAGCTG 3′ that yield a 219-bp DNA fragment. *Dicer* flox mice were genotyped by PCR as described (Harfe et al., 2005).

Initially, *Amhr2-Cre* heterozygotes were bred with *Dicer fx/fx* mice. The resulting *Amhr2-Cre; Dicer fx*/+ mice were bred to *Dicer fx/fx* mice to generate *Amrh2-Cre; Dicer* flox/flox males and females. *Amrh2-Cre; Dicer fx/fx* males were normal and fertile. Therefore, for the final cross, *Amrh2-Cre; Dicer fx/fx* males were bred with *Dicer fx/fx* females to generate *Amrh2-Cre; Dicer fx/fx* females. *Dicer fx/fx* female littermates served as controls.

Histology and immunofluorescence

Reproductive tracts were dissected and fixed in 4% paraformaldehyde overnight at 4°C. Paraffin-embedded tissues were sectioned at 6 μm, and stained with Harris hematoxylin (Statlab) and 0.5% eosin Y (Sigma) (H&E). For tracts at P35 and earlier, the entire tract was serially-sectioned through the oviduct to the cervix. For later ages, the entire oviduct was serially-section and ~300 μm into the anterior uterus. Histological sampling of the more posterior uterus showed phenotypes similar to the anterior uterus. Paraffin sections were immunostained for E-cadherin, using a mouse anti-E-cadherin monoclonal antibody (BD Biosciences) at a 1:200 dilution. The secondary antibody was an Alexa Fluor goat anti-

mouse IgG (Molecular Probes) used at a dilution of 1:800. Slides were mounted with Vectashield mounting media containing DAPI (Vector Laboratories). The amount of endometrial gland tissue in the uterus was estimated by counting the number of glandular duct cross sections in each uterine section. Statistical analysis was performed using a two-sample *t* test.

Oviduct transfer, dye injections, and uterine transfer

10-15 Affi-Gel Blue affinity chromatography beads (75-150 mm) were transferred into the ampulla of the oviducts of control (n=2) and mutant (n=3) females (Nagy et al., 2003). The females were sacrificed 1 to 4 days after transfer and their reproductive tracts isolated to visualize the movement of the beads through the oviduct. To examine the function of the uterotubal junction, adult reproductive tracts were dissected and 0.25% bromophenol blue was injected using a 23-gauge needle into the posterior uterine horn. Blastocysts generated from Swiss × Swiss mouse crosses were transferred into the uterine horns of E2.5 pseudopregnant control (n=3) and mutant (n=3) females (Nagy et al. 2003). 11 days after embryo transfer the females were examined for pregnancy.

Non-radioactive in situ hybridization

8 mm thick paraffin sections were hybridized with digoxygenin-labeled RNA probes as described (Xu and Wilkinson 1999). A *Wnt7a* sense probe was used as a control.

Serum estradiol and progesterone measurements

Serum was collected from individual mice, frozen, and sent to the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core (Charlottesville) for measurement of estradiol and progesterone levels by radioimmunoassay.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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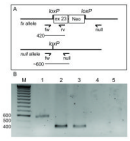

Fig. 1. Cre-mediated deletion of *Dicer* exon 23 in the mutant female reproductive tract

A, Diagram showing PCR strategy. Deletion of the exon 23 of *Dicer* was detected by using forward (fw), reverse (rv) and null primers. B, Amplification of DNA extracted from reproductive tract of *Amhr2-Cre; Dicer* mutant females using the fw/null primers generated a null band (Lane 1) and an *fx* band using the fw/rv primers (Lane 2). Tail DNA extracted from mutant females produced only an *fx* band (Lane 3). No band was observed when DNA was amplified using the fw/null primers (Lane 4). DNA extracted from control female reproductive tract, amplified using the fw/null primers (Lane 5). The wild-type segment was too large for efficient PCR amplification, using fw/null primers under the PCR conditions. M, 100 bp ladder.

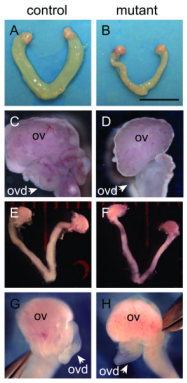

Fig. 2. Gross morphology of the female reproductive tract

A-D, Reproductive tracts from 5 week-old control (A, C) and mutant (B, D) females. E-H, Reproductive tracts from postnatal day 4 control (E, G) and mutant (F, H) females. Ov, ovary; ovd, oviduct. Scale bar, 1 cm.

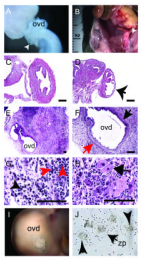

Fig. 3. Oviduct cysts and inflammation in *Dicer* mutant females

A, Inflammatory response inside the oviduct of a mutant female (white arrowhead) at postnatal day 28. B, At 8 weeks of age the inflammation response (white arrowhead) encompassed the entire oviduct. C, D, H&E stained histological section of postnatal day 28 oviducts from control (C) and mutant (D) females. The mutant showed a localized deficiency of smooth muscle (arrow). E-H, Chronic inflammation is observed in H&E stained cross sections of a 6 week-old mutant oviduct. F, There was a deficiency of smooth muscle (red arrow) in some regions of the oviduct when compared to the smooth muscle (black arrow) in other regions of the same oviduct. G, H, Higher magnification of panel E. Lymphocytes (black arrowhead), plasma cells (red arrowheads) and macrophages (black arrow) are indicative of chronic inflammation in the oviduct of mutant females. I, Oocytes are observed inside the cysts of unmated mutant females. J, Degenerate embryos (arrowheads) are found surrounded by the zona pellucida (arrow). Ovd, oviduct; zp, zona pellucida. Scale bar, 100 μm.

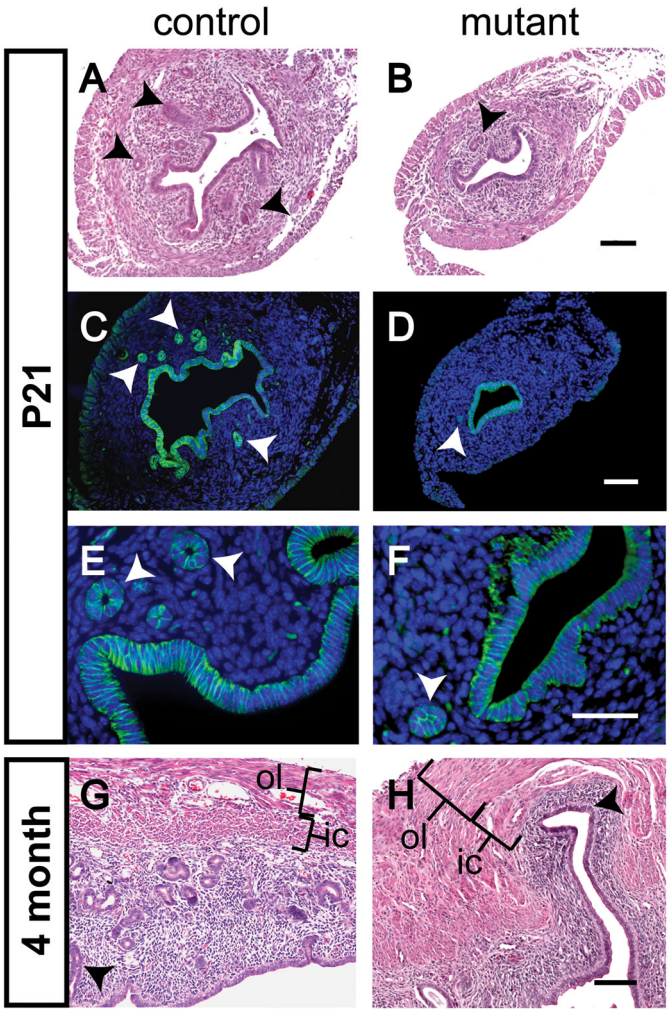

Fig. 4. Histological and immunofluorescent analysis of control and mutant females

A-F, H&E staining (A, B) and immunofluorescent staining using an E-cadherin antibody against epithelial cells and (C-F) at P21 shows less endometrial gland tissue in mutant (B, D, F) females when compared to control (A, C, E) females. G, H, H&E sections of four month-old control (G) and mutant (H) females. In the mutants, endometrial glands were observed inside the myometrium (H) and not in controls (G). Arrowheads indicate endometrial glands. Ic, inner circular; ol, outer longitudinal. Scale bar, 100 μm.

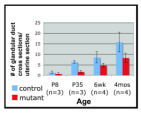

Fig. 5. Quantification of uterine glandular tissue in *Dicer* mutant females

No difference in uterine glandular tissue is observed at postnatal day 8 (P8) (n=3, p= 0.23). However, less endometrial glandular tissue is observed in the mutant uterus at P35 (n=3, p< 0.001), 6 weeks (n=4, p= 0.04), and 4 months (n=4, p= 0.04).

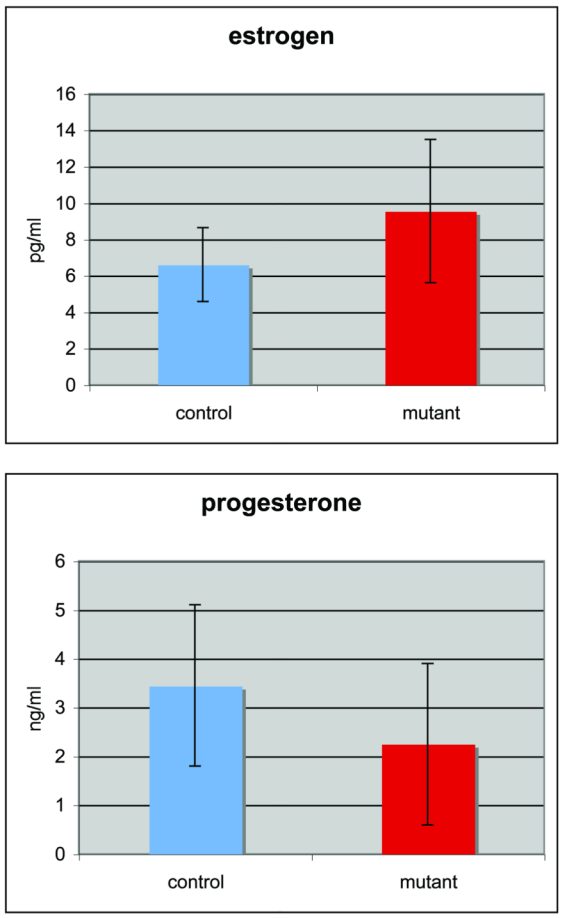

Fig. 6. Serum hormone measurements

Estradiol and progesterone levels between 6-8 week-old control (n=4) and mutant (n=7) females. The levels were not statistically different ($p > 0.05$).

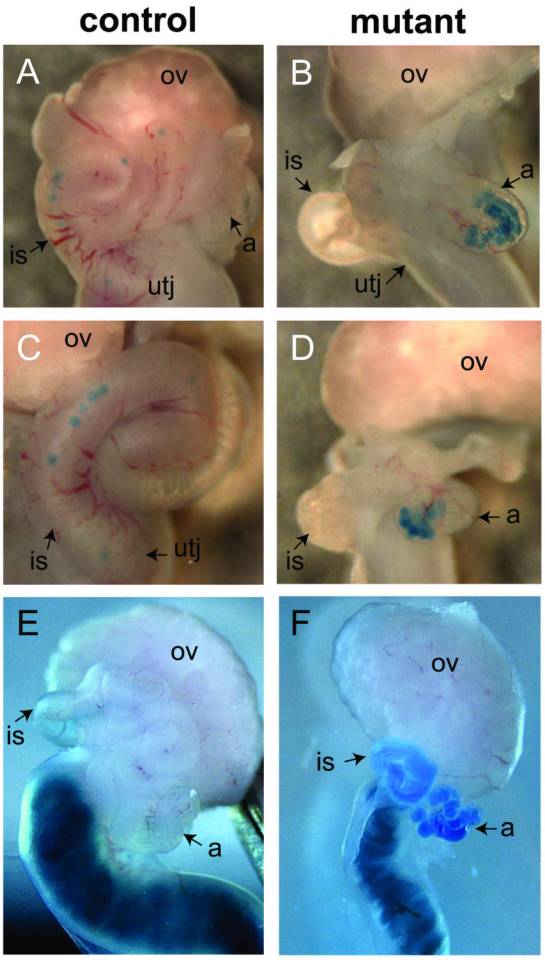

Fig. 7. Functional analysis of *Dicer* mutant female infertility

A-D, Bead transfers into the ampulla of 5 week-old control (A, C) and mutant (B, D) females. 4 days after transfer, by comparing the number of beads transferred and the number of beads observed, we estimate that most of the beads transferred into the control ampulla moved into the uterus (C), however all blue beads transferred into mutant ampulla remained as a cluster in the oviduct (D). E, F, Dye injections into the posterior lumen of the uterine horns of postnatal day 28 control (E) and mutant (F) females. The dye entered the oviduct of the mutant (F) but not the control (E). A, ampulla; is, isthmus; ov, ovary; utj, ovd, oviduct; u, uterus; utj, uterotubal junction.

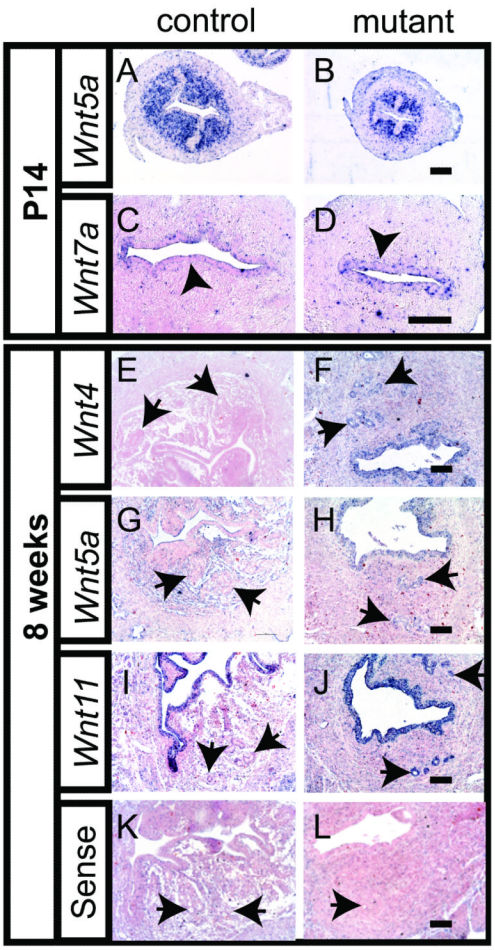

Fig. 8. *Wnt* in situ hybridization analysis of *Dicer* conditional mutant uterus

Postnatal day 14 control (A, C) and mutant (B, D) uteri. 8 week old control (E, G, I, K) and mutant (F, H, J, L) uteri. Probes for *Wnt4* (E, F), *Wnt5a* (A, B, G, H), *Wnt7a* (C, D), *Wnt11* (I, J), and a sense control (K, L) are indicated. Ectopic expression of *Wnt4*, *Wnt5a* and *Wnt11* was observed in 8 week old mutant uterus (F, H, J) compared to controls (E, G, I). Arrowheads, luminal epithelium; arrows, endometrial glands. Scale bar, 100 μm.