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Mechanisms of reduced fertility in *Hoxa-10* mutant mice: uterine homeosis and loss of maternal *Hoxa-10* expression

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SUMMARY

The establishment of a receptive uterine environment is critical for embryonic survival and implantation. One gene that is expressed in the uterus during the peri-implantation period in mice and is required for female fertility is the homeobox gene *Hoxa-10*. Here we characterize the peri-implantation defects in *Hoxa-10* mutant females and investigate functions of *Hoxa-10* in the uterine anlage during morphogenesis and in the adult uterus during pregnancy. Examination of pregnancy in *Hoxa-10* mutant females has revealed failure of implantation as well as resorption of embryos in the early postimplantation period. Morphologic analysis of the mutant uterus has demonstrated homeotic transformation of the proximal 25% into oviduct. Histology and molecular markers confirm this anterior transformation. Furthermore, in situ hybridization shows that this region coincides with the anterior limit of embryonic *Hoxa-10* expression in the urogenital ducts and a parallel transformation is observed in *Hoxa-10* mutant males at the junction of the epididymis and ductus deferens. Female fertility could be compromised by either the homeotic transformation or the absence of *Hoxa-10* function in the adult during pregnancy. To distinguish between these two potential mechanisms of infertility, wild-type blastocysts were transferred into mutant uteri distal to the transformed region on day 2.5 of pseudopregnancy. This procedure did not rescue the phenotype, suggesting that adult uterine expression of *Hoxa-10* is required during pregnancy. Moreover, when implantation was experimentally delayed, homozygous uteri were able to support survival of blastocysts comparable to wild-type controls, indicating that the requirement for *Hoxa-10* is intrinsic to implantation. While expression of LIF and HB-EGF appears unaffected in the mutant uteri, a decrease is observed in the intensity and number of blue dye reactions, an indicator of increased vascular permeability in response to implantation. In addition, mutant uteri exhibited decreased decidualization in response to artificial stimuli. These results show that *Hoxa-10* is required during morphogenesis for proper patterning of the reproductive tract and in the adult uterus for peri-implantation events.

Key words: homeobox, pattern formation, uterus, fertility, implantation, mouse

INTRODUCTION

Implantation is a complex series of processes which establishes the connection between maternal and embryonic tissues. Implantation requires both development of the embryo to the blastocyst stage and an intricate program of uterine preparation (Abrahamson and Zorn, 1993; Psychoyos, 1973). Prior to implantation, ovarian hormones stimulate proliferation and differentiation of the epithelium and stroma. The epithelium becomes a suitable surface for attachment and the stromal fibroblasts become competent to undergo transformation into specialized decidual cells. The attachment of the blastocyst to the luminal epithelium induces this transformation to produce a decidual reaction localized around the implanting embryo. Subsequent apoptosis of the luminal epithelium brings the embryonic trophoblast into direct contact with the maternal decidua. The coordination of embryonic development with uterine preparation yields a narrow window of implantation. Blastocysts transferred to pseudopregnant recipients during the pre-receptive stage become dormant until the uterus becomes receptive, while blastocysts which are transferred to recipients past the receptive stage fail to implant and degenerate (Dickmann and Noyes, 1960). While the ability of the stroma to undergo decidualization parallels the receptivity of the uterus to implantation, the mechanisms by which uterine receptivity is achieved at the molecular level are largely unknown. The advent of targeted mutagenesis has allowed the definition of some of the maternal factors which regulate these peri-implantation processes.

Recently, maternal expression of the transcription factor Hoxa-10 was determined to be required for female fertility (Satokata et al., 1995). Females that are homozygous for a mutation in *Hoxa-10* are either sterile or give birth to small litters. Furthermore, embryos from mutant females could be rescued by transfer to pseudopregnant wild-type oviducts on day 0.5, demonstrating that the homozygous maternal environment was deficient.

Hoxa-10 is an *Abdominal B* (*AbdB*) class homeobox gene (Benson et al., 1995). These genes are located at the 5′ end of the *Hox* clusters and are expressed in posterior domains of the developing mouse embryo including the intermediate mesoderm, which gives rise to the gonads and the genitourinary tract (Benson et al., 1995; Dollé et al., 1991). The *AbdB* genes of the *Hoxd* cluster exhibit nested patterns of expression along the genitourinary tract, suggesting that these structures represent a developmental axis patterned by *Hox* genes (Dollé et al., 1991). In support of this model, the targeted disruption of *Hoxa-11* resulted in a homeotic transformation of the ductus deferens towards an epididymis-like fate (Hsieh-Li et al., 1995). Therefore, *Hoxa-10* mutant females could exhibit a homeotic transformation of the female reproductive tract altering the adult uterine environment.

Interestingly, the adult uterus also expresses *Hoxa-10* and *Hoxa-11*, as well as several other *Hox* genes (Satokata et al., 1995; Hsieh-Li et al., 1995; Redline et al., 1992; Vogels et al., 1990). While morphogenesis of most organs is complete by adulthood, the uterus undergoes a remarkable program of cellular proliferation and differentiation during pregnancy (Abrahamson and Zorn, 1993). Because embryonically expressed *Hox* genes regulate these functions during morphogenesis, *Hox* genes expressed in the adult uterus may also be involved in similar processes during implantation and placentation. A detailed study of *Hoxa-10* expression during early pregnancy (Satokata et al., 1995) suggests that this gene is expressed in cells proliferating under steroidal regulation (Huet-Hudson et al., 1989). *Hoxa-10* is expressed in the luminal and glandular epithelium on days 0.5 and 1.5 post coitum (pc), expands in the stroma on day 2.5, and becomes restricted to the stroma on day 3.5. Following implantation on day 4.5, expression continues during transformation of stromal cells into the decidua but is downregulated as cells achieve full differentiation. This pattern of expression is also closely correlated with the expression of several cytokines and growth factors (Das et al., 1995, 1994). Thus, it is possible that adult expression of *Hox* genes could mediate aspects of endometrial cell proliferation and/or secretion necessary for pregnancy to proceed.

In this paper we describe homeotic transformations of the male and female reproductive tracts in mice homozygous for a mutation in *Hoxa-10* and investigate the requirement for the expression of *Hoxa-10* in the adult uterus.

MATERIALS AND METHODS

Mice

Disruption of the *Hoxa-10* gene was performed by insertion of a neomycin resistance cassette into an *Xho*I site within the homeobox by homologous recombination in 129/SvJ ES cells and generation of chimeric mice (Satokata et al., 1995). Three different genetic backgrounds have been generated: 129/SvJ, mixed BALB/c × 129/SvJ, and mixed C57BL/6 × 129/SvJ. The percentage of females that failed to produce a litter during a two month period and the average litter size for each background are: 129/SvJ: 60%, 2.3; mixed BALB/c × 129/SvJ: 45%, 2.3; mixed C57BL/6 × 129/SvJ: 33%, 1.8. While there was a difference in penetrance between the backgrounds, no difference in phenotype was observed. Age matched females of 2-7 months of age were used in the experiments described. In all experiments, the day of the vaginal plug is considered day 0.5 pc and mice were killed between 1000 and 1600 hours on days 3.5-8.5 pc.

X-gal histochemistry

Endogenous β-galactosidase activity was detected in the epididymis and ductus deferens of 7-month old wild type and mutant males (Chapman and Killian, 1984) by fixation in 4% paraformaldehyde, washing in PBS, and incubation in 16 mM potassium ferrocyanide, 16 mM potassium ferricyanide, 2 mM magnesium chloride, and 0.1% X-gal in PBS (Sanes et al., 1986).

Histology and in situ hybridization

Tissues from various days pc were fixed in 4% paraformaldehyde. For histology, 7 μm sections of paraffin embedded tissues were stained with hematoxylin and eosin. Section and whole-mount in situ hybridization was performed as described by Sassoon and Rosenthal (1993); Izpisúa-Belmonte et al. (1993). Antisense riboprobes were synthesized from Hoxa-10 (Satokata et al., 1995), Msx-1 (Pavlova et al., 1994), c-myc (Morgenbesser et al., 1995), amphiregulin (AR; Das et al., 1995), or heparin binding epidermal growth factor (HB-EGF; Das et al., 1994) templates. The leukemia inhibitory factor (LIF) template contained sequence from bp 3429-3708 (Stahl et al., 1990). Sense riboprobes did not demonstrate any specific hybridization signals.

Blue dye reaction

For blue dye reactions (Paria et al., 1993; Psychoyos, 1973), 0.1 ml Evan's blue dye (1% in saline) was injected into the tail vein at 1200-1600 hours on day 4.5. Mice were killed 5 minutes later, and uteri inspected for the presence of blue bands. Uteri were then fixed and serially sectioned to determine total blastocyst number.

Embryo transfer

Embryos were flushed with M2 medium from day 3.5 pc uteri of 3- to 6-week old wild-type donors which had been superovulated (Hogan et al., 1986). Healthy blastocysts were selected and six were transferred to each uterine horn of mutant mixed BALB/c × 129/SvJ day 2.5 pseudopregnant recipients. The transfer was performed as described by Hogan et al. (1986) except that embryos were transferred to the distal uterus to avoid the transformed region. Age matched wild-type or heterozygous females were used as controls. Recipients were either killed on day 7.5 pc or allowed to proceed to term.

Delayed implantation

Homozygous 129/SvJ females or age matched controls were mated to fertile males. On the morning of day 1.5, they were ovariectomized and maintained on daily injections of 2 mg of progesterone from days 1.5 to 4.5 pc (Das et al., 1994). On day 5.5, they were killed and the uteri were flushed with saline to recover embryos.

Decidual response

To examine decidualization in response to an artificial stimulus, 25 μl of sesame oil was injected into the lumen of one uterine horn of day 3.5 pseudopregnant homozygous or wild-type females (BALB/c-129/SvJ or 129/SvJ) (Hetherington, 1968). The contralateral horn was not injected with oil and served as an internal control. Mice were killed on day 7.5 and the wet weight of each uterine horn recorded. Fold weight increase was calculated as the ratio of the weight of the injected horn to the weight of the noninjected horn. In three cases where oil had diffused into the noninjected horn and caused its decid-

ualization, fold weight increase was estimated by dividing the weight of the injected horn by an average weight of the nondecidualized horns. To exclude decreased progesterone as a source of decidual failure, a second group of mutants and controls were given 1 mg of progesterone on day 1.5 and 2.5 of pseudopregnancy and 2 mg of progesterone from day 3.5 to 6.5 and tested for their ability to decidualize as above.

RESULTS

Peri-implantation failure in *Hoxa-10* mutant females

Expanded analysis of day 1.5 post coitum mice reveals that there is a failure of fertilization in approximately 30% (4/15) of *Hoxa-10* mutant females (Table 1). This probably accounts for some of the preimplantation failure previously observed (Satokata et al., 1995). However, the remaining 70% had near normal numbers of embryos prior to implantation (Table 1). To identify when failure of pregnancy occurs in *Hoxa-10* mutant females, the number and morphology of embryos from mutant and wild-type uteri were determined on days 4.5 to 8.5 pc (Table 1). On day 4.5, in wild-type females, 56 of 56 (100%) embryos had formed a distinct outer trophectoderm and a well formed inner cell mass (Fig. 1A), whereas nine of 42 (21%) embryos in mutant females were small and degenerating (Fig. 1B) or had collapsed (Fig. 1C). Additional embryos appeared developmentally retarded: while only 4 of 56 (7%) embryos in wild-type uteri had not yet undergone implantation, 18 of 42 (43%) embryos in homozygous uteri were still free in the uterine lumen.

Further analysis of homozygous uteri on days 6.5-8.5 pc revealed embryos in 16 of 21 (76%): 14 in which embryos had implanted and two in which embryos were present but had not implanted. By gross analysis 56 of 84 (67%) implantation sites were abnormal as determined by increased blood in the sites or in the uterine lumen adjacent to the sites. In 42 of 48 sites examined histologically, the uterine lumen contained red blood cells and polymorphonuclear leukocytes. Empty decidua were found in seven of the sites examined, suggesting that embryos had died early during implantation, but after they had initiated a decidual response. In 12 other implantation sites embryos were present, but were significantly smaller or had a disorganized embryonic component compared to embryos in wild-type controls (Fig. 1D,E). An additional eight implantation sites contained embryos which were abnormally positioned within the decidua. In general, the size of the decidual swellings were reduced in the mutant compared to wild-type uteri.

In addition, five uteri with no visible implantation sites on days 6.5-8.5 pc were flushed to detect nonimplanted embryos. Two of these yielded four hatched blastocysts (Fig. 1F) which had the appearance of embryos isolated from uteri in cases of delayed implantation (Yoshinaga and Adams, 1966). Overall, these results demonstrate that the embryonic failure in *Hoxa-10* mutant uteri is a combination of implantation failure and embryonic degeneration and resorption during the early postimplantation period.

Homeosis of male and female reproductive tracts

Analyses of the male and female reproductive tracts of *Hoxa-10* mutants have revealed homeotic transformations affecting these structures. In wild-type males, the epididymis comprises a single highly coiled tubule and is subdivided into three distinct regions: the caput, the corpora and the cauda. From the cauda, the tubule straightens and widens to form the ductus deferens (Fig. 2A). In homozygous males however, the distal epididymis and the proximal ductus deferens have acquired some morphologic features of more anterior segments (Fig. 2B). The corpora of the epididymis is wider and the coils of the tubule are more densely packed, resembling the caput. In addition, the cauda is enlarged and the proximal segment of the ductus deferens remains tortuous as it exits the cauda. Overall the length of the ductus deferens is reduced by approximately 25%, suggesting that the proximal segment has adopted an epididymal-type fate and has been partially incorporated into the cauda. These findings are consistent with a partial anterior transformation of the distal epididymis and proximal ductus deferens.

To examine the expression of region specific markers in the transformation, X-gal histochemistry was performed. Strong endogenous β-galactosidase activity is normally restricted to the caput and the corpora of the epididymis (Fig. 2C). In the mutant, the cauda and proximal ductus deferens also demonstrate strong β-galactosidase activity (Fig. 2D), confirming

Table 1. Peri-implantation death and failure of implantation in *Hoxa-10* mutant females

Day p.c. and genotype	Females with fertilized embryos/total females (%)	No. nonimplanted embryos/total embryos (%)	No. morphologically abnormal embryos/ total embryos (%)	Avg. no. normal embryos/ pregnant female*
1.5				
+/+	15/15 (100)	n.a.	0/94 (0)	6.3
−/−	11/15 (73)	n.a.	0/58 (0)	5.3
4.5				
+/+	9/9 (100)	4/56 (7)	0/56 (0)	6.2
−/−	8/13 (62)	18/42 (43)	9/42 (21)	4.1
6.5-8.5				
+/+	10/11 (91)	0/91 (0)	3/91 (3)	9.1
−/−†	16/21 (76)	4/84 (5)	56/84 (67)	1.8

Mice were killed at the given day pc and embryos were detected by flushing oviducts with PBS or by examining serial sections of uteri. Embryos that were degenerating, collapsed, or resorbing were scored as abnormal. Data were obtained from 129/SvJ, mixed BALB/c × 129/SvJ, and mixed C57BL/6 × 129/SvJ backgrounds. No differences between backgrounds were observed (see methods). n.a., not applicable.

*Total number of morphologically normal embryos divided by the number of females with fertilized embryos.

†These include nine day-6.5, ten day-7.5, and two day-8.5 females.

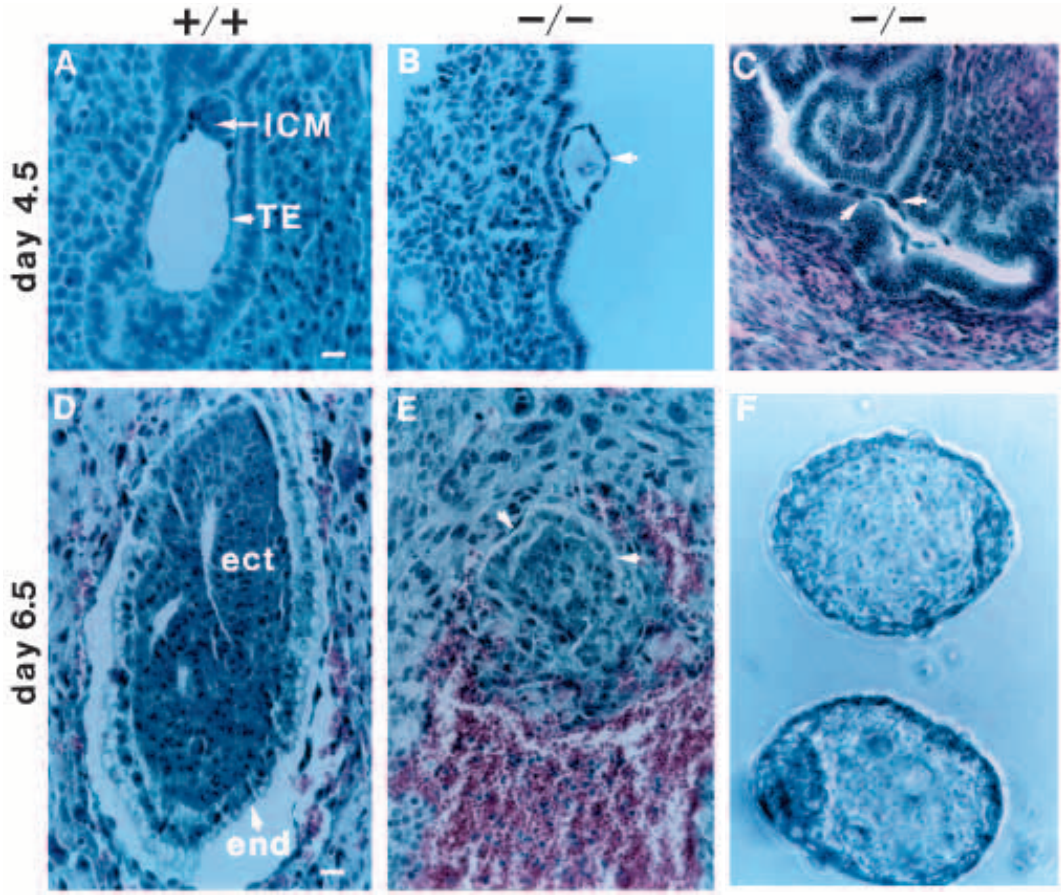

Fig. 1. Peri-implantation phenotype in *Hoxa-10* mutant uteri. (A) H&E stained section through an embryo implanted in a wild-type uterus on day 4.5 pc, showing a well formed inner cell mass (ICM) and trophectoderm (TE). Scale bar, 20 μm. (B) Section through an abnormal embryo in a mutant uterus on day 4.5. The embryo (arrow) has attached, but is reduced in size and shows degeneration of the ICM. The separation of the uterine walls is an artifact of the fixation procedure. (C) Section through a collapsed embryo (between the arrows) in the transformed region of a mutant uterus. Implantations into this region were observed in only one of fourteen mutant females. (D) Section through an egg cylinder stage embryo in a wild-type uterus on day 6.5. Scale bar, 40 μm. (E) Section through a resorption site in a day 6.5 mutant uterus. A few embryonic cells are present but disorganized (arrows). Maternal blood cells have infiltrated the site. (F) Phase contrast microscopy of two blastocysts flushed from a day 6.5 mutant uterus with no apparent implantation sites. Embryos have hatched from their zona pellucidae, similar to blastocysts isolated under conditions of delayed implantation.

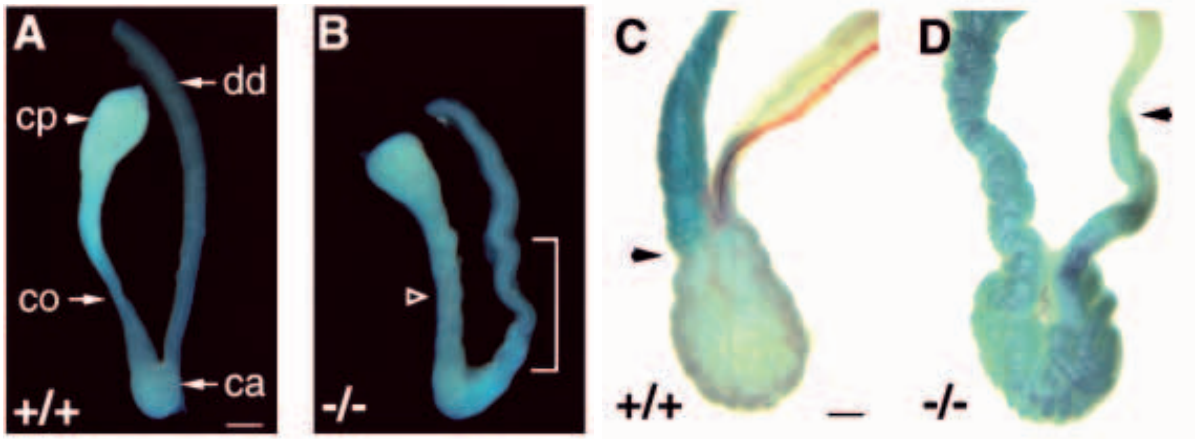

Fig. 2. Anterior homeotic transformation of the male reproductive tract. (A,B) Comparison of gross morphology of the caput (cp), corpora (co), and cauda (ca) of the epididymis and ductus deferens (dd) of wild-type (A) and mutant (B) 4-week old males. The mutant shows increased width of the corpora (open arrowhead), expansion of the cauda, and continued tortuousity of the ductus deferens exiting the cauda (bracketed). Scale bar, 1 mm. (C,D) Endogenous β-galactosidase activity in the epididymis of wild-type (C) and mutant (D) males at seven months of age. A sharp decrease in activity is seen between the corpora and the cauda of the wild-type epididymis (arrow). The activity was weaker in the mutant (D) but had the same intensity throughout the corpora and cauda, with a distal limit in the ductus deferens (arrow). Scale bar, 1.5 mm.

transformation to a more anterior fate at the molecular level. In addition to the homeotic transformation of the epididymis and ductus deferens, morphologic changes were observed in the coagulating gland and the dorsal prostate (R. Seo and W. Bushman, personal communication).

In *Hoxa-10* mutant females, a parallel homeotic transformation is observed at the junction between the oviduct and the uterus. In wild-type mice, the oviduct is coiled and narrow, and there is an abrupt transition in diameter at the uterotubal junction where the oviduct enters the wider, straighter uterus (see arrows, Fig. 3A). In homozygous females, a distinct uterotubal junction is absent. Instead, the proximal 25% of uterus has acquired the narrow, coiled morphologic appearance of the oviduct (ut* in Fig. 3B), and it gradually straightens and widens to the full diameter of the uterus. The normal valve-like projection of the oviduct into the uterus is absent (data not shown).

Histologically, the wild-type uterus and oviduct are clearly distinguishable (Fig. 3C,D). A transverse section of the affected region shows that while the outer longitudinal layer of the myometrium is present, in the inner cell layers histologic characteristics of the oviduct rather than the uterus predominate (Fig. 3E, see also Fig. 1C). The epithelium and stroma are raised in angular mucosal folds; the epithelium does not penetrate the stroma to form glands; the stroma is only a few cell layers thick; and there is a close investiture of circular muscle. As the tube continues distally, the lumen widens, the thickness of the stromal layer increases and glands appear until it is indistinguishable from wild-type uterus. Thus both the gross appearance and the histology demonstrate an anterior transformation of the proximal uterus to oviduct.

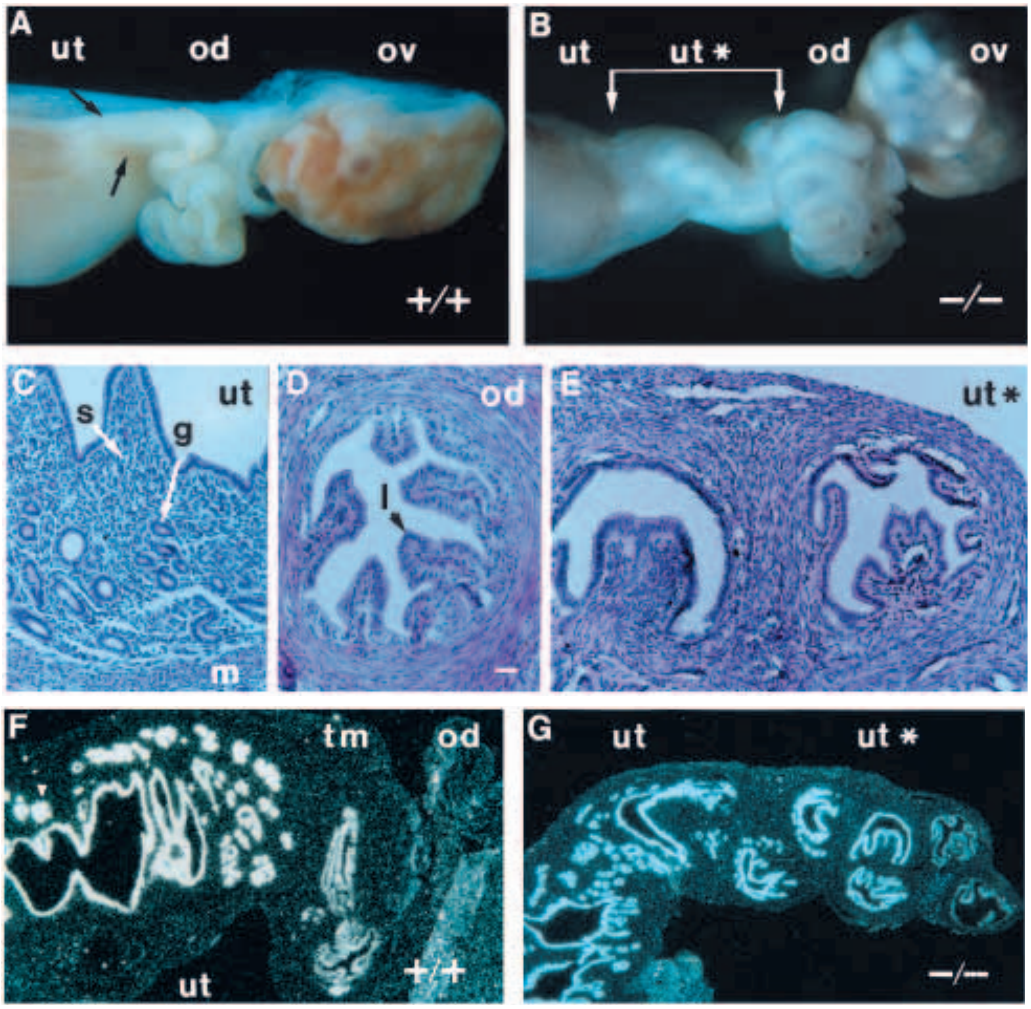

Fig. 3. Anterior homeosis of the female reproductive tract. (A) Wild type reproductive tract showing the ovary (ov), the oviduct (od), and the uterus (ut). Note the sharp transition in diameter as the oviduct enters the uterus (arrows). (B) Homozygous reproductive tract showing the transformation of the proximal uterus to oviduct (ut*). (C) Transverse section of wild-type uterus showing the epithelium which has invaginated to form glands (g), the thick stromal layer (s), and the myometrial wall (m). (D) Histology of the wild-type transmural oviduct showing the luminal epithelium (l) with no gland formation, the thin stromal layer, and the thick circular muscle layer. Scale bar, 40μm. (E) In the mutant, the transformed region also lacks glands and has little stroma. (F,G) Molecular characterization of the transformed region. (F) In the wild type, *Msx-1* is strongly expressed in the luminal and glandular epithelium of the uterus (ut), weakly expressed in the luminal epithelium of the transmural portion of the oviduct (tm), and absent from the remaining oviduct (od). (G) In the mutant, while strongly expressed in the luminal and glandular epithelium of the uterus, *Msx-1* decreases proximally to a level similar to the transmural oviduct. The oviduct of the mutant (not shown) lacks expression.

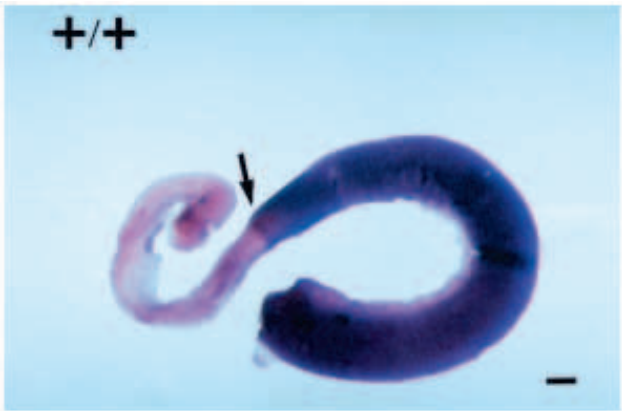

Fig. 4. Whole-mount in situ hybridization showing the expression of *Hoxa-10* in the developing female reproductive tract on embryonic day 17.5. The anterior boundary of expression coincides with the narrowing of the duct at the future uterotubal junction (arrow). Scale bar, 200 μm.

Table 2. Embryonic survival following blastocyst transfer to the distal horn of day 2.5 wild type or *Hoxa-10* mutant pseudopregnant females

Recipient genotype	No. with embryos	Implantations*		Resorptions	
		Total	Avg.	Total	Avg.
+/+	7/13	46	6.6	0	0
−/−	3/13	8	2.7	4	1.3

Twelve day-3.5 embryos from superovulated wild-type females were transferred to the distal uterine horns of day-2.5 pseudopregnant recipients. Females were killed at day 7.5-8.5 (n=10) or allowed to progress to term (n=3).
*Includes implantations observed at day 7.5-8.5 and pups delivered at term.

To analyze the molecular nature of the transformed region, in situ hybridization was performed with a uterine specific marker, *Msx-1* (Pavlova et al., 1994). *Msx-1* is strongly expressed in the uterine luminal and glandular epithelium (Fig. 3F). Expression is decreased in the epithelium of the transmural oviduct and is absent from the rest of the oviduct. In the homozygous female, *Msx-1* is expressed strongly in the luminal epithelium of the distal transformed segment; however, expression decreases proximally to the level of that seen in the transmural oviduct (Fig. 3F). In addition, *c-myc* expression, normally present in the uterine stroma on day 4.5 pc, is present in the distal transformed segment, but absent more proximally (data not shown). These results demonstrate a change from uterine to oviductal molecular character within the region of transformation.

To determine whether the transformations observed coincide with the anterior boundary of *Hoxa-10* in the mesenchyme of the mesonephric and paramesonephric ducts, whole-mount in situ hybridization was performed. Strikingly, at embryonic day 17.5 (Fig. 4). *Hoxa-10* expression exhibits a sharp anterior boundary in the paramesonephric duct at the transition between the future uterus and oviduct. In the male, expression of *Hoxa-10* was not detected at this time in the corresponding mesonephric duct derivatives; however, expression was previously observed at embryonic day 15.5 with a distinct anterior boundary in the midepididymis (Satokata et al., 1995). Therefore, the loss of function of *Hoxa-10* results in homeotic transformations which coincide with its anterior domains of expression.

In addition to the morphologic changes attributed to the homeotic transformations which occurred in all of the homozygous females, other abnormalities were occasionally observed. First, there was often a web of tissue between the two horns of the uterus which may represent a continuation of the mesovarium. Second, paraovarian cysts were frequently detected, suggesting a possible failure of resorption of the mesonephric duct in mutant females. Third, endometrial cysts, previously reported in *Hoxa-10* deficient mice (Rijli et al., 1995) were observed in zero of ten (0%) females under 6 months of age, but were seen in six of twelve (50%) females over 6 months of age. Because reproductive failure is evidenced at the onset of reproductive life and cysts are only detected later, it is likely that the cysts are secondary and not the cause of reduced fertility. The cysts are located throughout the length of the uterus and are not localized to the area of the transformation. Ovarian cysts were also observed with greater frequency in older mice. Finally, three of 13 females examined on day 4.5 pc showed stratification of the epithelium in the distal uterine horn, possibly indicating an anterior shift in the level of the cervico-uterine junction.

Transfer of embryos into the distal uterus cannot rescue their survival

To determine the consequences of the homeotic transformation on embryonic survival, blastocysts were transferred from wild-type females directly into the distal uterus of day 2.5 pseudopregnant mutant recipients. This procedure avoids the transit of embryos through the transformed region and ensures the presence of embryos in the uterus during the receptive phase. Seven of 13 transfers to wild-type recipients resulted in pregnancy with a total of 46 successful implantations (Table 2). In contrast, only three of 13 transfers to homozygous recipients resulted in pregnancy, the outcome of which was eight implantations and four resorptions. Therefore, there is a reduction in implantation in the homozygous recipients (P<0.05, χ^2 test) and an increase in the number of postimplantation resorptions, comparable to data from days 6.5 to 8.5 pc of natural matings (Table 1). These results show that the infertility phenotype cannot be rescued by transfer of embryos to the distal uterus at day 2.5 pc and are consistent with a requirement for *Hoxa-10* during pregnancy.

Embryos survive in *Hoxa-10* deficient uteri during delayed implantation

To determine whether embryonic death was related to implantation, the ability of embryos to survive when implantation was delayed was examined. Homozygous females and wild-type controls were ovariectomized on day 1.5 pc to remove the estrogenic stimulus for implantation and then maintained on progesterone. Following sacrifice on day 5.5, the uteri were flushed and the number of delayed blastocysts counted (Table 3). 17 delayed blastocysts were isolated from six of ten mutant uteri, while eight delayed blastocysts were isolated from three of six wild-type controls. While a decrease in number of

Table 3. Embryonic survival during experimentally-induced delayed implantation

Genotype (n)	No. with embryos	No. delayed blastocysts	No. degenerated embryos	Avg. no. delayed blastocysts
+/+ (6)	3	8	0	2.7
−/− (10)	6	17	1	2.8

Mice were ovariectomized on day 1.5 p.c. to delay implantation, and pregnancy maintained by injection of 2 mg progesterone from days 1.5 to 4.5. Mice were killed on day 5.5 p.c. and the uteri were flushed with PBS to determine the number and morphology of embryos.

Table 4. Decreased decidual response in *Hoxa-10* mutant females

Genotype and treatment	No. decidualized/ total	Average weight injected horn (mg) ± s.e.m.	Average weight noninjected horn (mg) ± s.e.m	Average fold weight increase
+/+	8/13	460.4±71.5	44.8±3.6	10.7
–/–	7/13	103.9±26.1	30.0±2.8	3.5
+/+ plus progesterone	8/8	599.9±62.8	45.4±3.6	13.2
–/– plus progesterone	6/7	102.3±22.2	32.8±2.4	3.1

Hoxa-10 mutant females were tested for their ability to mount a decidualization reaction in response to an artificial stimulus both with no hormonal treatment or when given exogenous progesterone from days 1.5 to 6.5 of pregnancy. Sesame oil was injected into the lumen of one uterine horn on day 3.5 of pseudopregnancy. The contralateral horn was not injected with oil and served as a control. On day 7.5, females were killed and the wet weights of each horn were recorded.

embryos is observed in both wild-type and mutants, possibly due to trauma to the oviduct during ovariectomy, embryos can survive in a delayed state in *Hoxa-10* mutant uteri at a rate equal to wild-type controls. These results suggest that the embryonic death in the *Hoxa-10* deficient uterus is related to failure during the implantation process.

Decrease in blue dye reaction and decidualization in *Hoxa-10* mutant females

To test individual components of the implantation process which could be downstream of *Hoxa-10*, expression patterns of c-myc, amphiregulin and LIF, and HB-EGF were examined as molecular markers of cell proliferation (Huet-Hudson et al., 1989), onset of receptivity (Das et al., 1995; Stewart et al., 1992), and initiation of implantation (Das et al., 1994) respectively. All four probes showed expression patterns similar to those seen in wild-type controls in at least two different homozygous uteri by in situ hybridization (data not shown). Next, to determine whether implantation was initiated normally in homozygous females, intravenous injection of Evan's blue dye was performed to identify areas of increased endometrial vascular permeability in response to blastocyst implantation. Discrete blue bands, or positive blue reactions, were clearly observed in all six wild-type uteri by 1200 hours on day 4.5. In contrast, only one of eight mutant uteri had strong positive blue reactions. Two other mutant uteri had very faintly visible reactions and the remaining five had none. In contrast, complete sectioning of the mutant uteri revealed the presence of embryos in the strongly positive, two weakly positive and one negative uteri, demonstrating either a delay in or failure of mutant uteri to initiate an attachment reaction in response to embryos.

Finally, to determine whether the uterine stroma was competent to respond to implantation, uteri were tested for their ability to mount a decidualization reaction following exposure to an artificial stimulus. Sesame oil was injected into one horn of the uterus at day 3.5 of pseudopregnancy (Hetherington, 1968) and the extent of decidualization was examined at day 7.5. In eight of 13 wild-type females, decidualization increased the wet weight of the injected horn by an average of 10.7 fold over the non-injected horn (Table 4). In contrast, in seven of 13 mutants, decidualization only increased the wet weight of the injected horn 3.5 fold ($P<0.01$, Student's *t*-test). To exclude decreased progesterone as a potential cause of reduced decidualization, mutants and controls given exogenous progesterone from days 1.5 to 6.5 of pseudopregnancy were tested for ability to decidualize. Wild type females demonstrated a 13.2-fold increase in weight while mutants demonstrated only a 3.1-fold increase. These results demonstrate that the decreased decidualization observed is not due to a lack of progesterone and is intrinsic to the mutant uterus.

DISCUSSION

Failure of implantation and early resorption contribute to the peri-implantation phenotype in *Hoxa-10* mutant uteri

Homeobox genes are involved in pattern formation of segmented structures during development and have been postulated to have roles in regeneration and repair in the adult (Kessel and Gruss, 1991; Simon and Tabin, 1993). Here we show that *Hoxa-10* is critical for patterning of the male and female reproductive tracts. In addition to homeotic transformations of these structures, females deficient for *Hoxa-10* exhibit reduced fertility due to peri-implantation defects. In some females, embryos develop to hatching but fail to implant and persist in a dormant state similar to delayed implantation. However, the majority of embryos commence implantation but are resorbed early in the postimplantation period. Overall, these results demonstrate that maternal *Hoxa-10* is required for morphogenesis of the reproductive tract during development and for successful implantation in the adult.

Mechanisms of infertility: homeosis versus loss of adult *Hoxa-10* expression

Hoxa-10 is expressed both in the uterine anlage during morphogenesis and in the adult uterus during pregnancy (Satokata et al., 1995). Expression at these two different time periods suggests two different models for *Hoxa-10* function in female fertility. First, loss of embryonic expression of *Hoxa-10* results in a homeotic transformation of the proximal uterus to oviduct (discussed below). The transformation may provide a morphologic cause for embryonic loss through physical or molecular changes in the uterine environment. Entry of embryos into the uterus could be delayed beyond the receptive phase. The decrease in uterine length could produce some crowding of embryos which can decrease implantation rate and reduce postimplantation survival (Hafez and Sugawara, 1978). In addition, loss of the uterotubal junction removes the valve that is formed by the projection of the oviduct into the uterus (Hafez and Black, 1969) and could expose embryos to an

altered milieu (Dickmann and Noyes, 1960). Finally, the transformation in the molecular character of the proximal uterus could adversely affect embryonic survival. Embryos retained within the oviduct experimentally are able to mature to the blastocyst stage and develop to term when transferred to the uterus (Biggers et al., 1962); however, these embryos may have a reduced potential under certain conditions (Kirby, 1962).

The second model of infertility proposes that *Hoxa-10* has a critical function in the adult uterus during pregnancy. *Hoxa-10* is expressed in the peri-implantation endometrium in a pattern which closely correlates with the action of ovarian steroid hormones (Satokata et al., 1995). This hormonal priming of the uterus is essential for implantation and embryonic survival (Psychoyos, 1973). The combined influence of estrogen and progesterone regulate uterine production of a variety of cytokines and growth factors, and receptors for these factors are expressed both by the endometrium and the blastocyst. Despite normal estradiol and progesterone levels in *Hoxa-10* mutant females (data not shown), some aspects of the *Hoxa-10* mutant phenotype are similar to that observed in hyperestrogenic states, including paraovarian cysts resulting from embryonic exposure (Haney et al., 1986) and cystic glandular hyperplasia seen following adult exposure to exogenous estrogen (Dallenbach-Hellweg, 1985). These results suggest that *Hoxa-10* may serve to modulate the effects of estrogen and progesterone in the adult uterus to produce a receptive environment.

To distinguish between these two models, wild-type embryos were transferred into mutant uteri distal to the transformation. This procedure avoids transit through the transformed segment and ensures the presence of embryos in the uterus during the receptive phase. Following transfer, mutant females had fewer pregnancies, reduced numbers of implantations, and increased numbers of resorptions compared to wild-type controls. These results are similar to the outcome of natural matings of *Hoxa-10* mutant females, indicating that the transfer does not rescue the phenotype. Thus, while we cannot formally exclude the possibility that the transformation is having some paracrine effect on transferred embryos, these results support a model in which uterine expression of *Hoxa-10* is required during pregnancy for survival and implantation of embryos.

The requirement for *Hoxa-10* in the adult uterus is specific to implantation

The time of embryonic loss and the presence of nonimplanted blastocysts suggest that *Hoxa-10* is required for events associated with implantation. To test whether the lethality in *Hoxa-10* mutant uteri is specific to the implantation process or simply represents death due to cumulative insult, survival of embryos under conditions of delayed implantation was examined. As preimplantation ovarian estrogen secretion is required for the initiation of implantation in the mouse, elimination of this estrogen by ovariectomy blocks implantation (Huet and Dey, 1987; Yoshinaga and Adams, 1966). Under these conditions, blastocysts become quiescent and can survive for many days. In mutations which affect basic cellular processes of the embryo such as *lethal yellow* (A^y), blastocysts survival is not enhanced under conditions of delayed implantation (Papaioannou and Gardner, 1992). In contrast, in *Hoxa-10* mutant females blastocysts survive equally well as wild-type controls. These results suggest that the requirement for *Hoxa-10* is intrinsic to implantation.

Several key events associated with implantation have been defined. Implantation is normally initiated on day 3.5 when stromal edema produces luminal closure of the uterus, bringing the trophectoderm and the luminal epithelium into close apposition (Abrahamson and Zorn, 1993). At the same time, LIF expression in the glandular epithelium is upregulated and is necessary to transduce a signal to the luminal epithelium for implantation to proceed (Stewart et al., 1992). The localized expression of HB-EGF in the luminal epithelium is the earliest marker of implantation described and begins at 1600 hours on day 3.5 (Das et al., 1994). Subsequently, hatching of the blastocyst and attachment to the luminal epithelium occurs at 2200-2300 hours and coincides with a local increase in vascular permeability. Finally, the decidualization of the stromal cells occurs and the epithelium undergoes apoptosis allowing invasion of the trophectoderm. In *Hoxa-10* mutant mice, luminal closure occurs normally and, in contrast to *Hoxa-11* deficient mice (R. Gendron et al., personal communication), LIF expression was observed in *Hoxa-10* mutant mice. However, the blue dye reaction, measuring vascular permeability, was reduced or absent in three of four females with embryos. Finally, the extent of decidualization in response to artificial stimuli was decreased compared to wild type.

One other maternal mutation is known to affect peri-implantation events. In LIF deficient mice there is a complete failure of initiation of implantation resulting in nonimplanted blastocysts (Stewart et al., 1992). While the LIF receptor (LIFR) is expressed in the blastocyst and the endometrium, successful implantation of LIFR deficient embryos suggests LIF is required by the endometrium for attachment (Ware et al., 1995). LIF deficient mice also exhibit failure of decidualization in response to artificial stimuli indicating that LIF is also required in priming the stroma to undergo a decidual response (Stewart, 1994). The peri-implantation defects observed in *Hoxa-10* mutant females are less severe, but similar to, the LIF phenotype. Since *Hoxa-10* expression is restricted to the stroma during this period, it is possible that the peri-implantation defects in *Hoxa-10* deficient mice may be due to a disruption of signaling between the epithelium and the stroma.

Homeotic transformation of the reproductive tracts

Mice deficient for *Hoxa-10* show anterior homeotic transformations of the male and female reproductive tracts: the proximal uterus is transformed into oviduct, and the proximal ductus deferens is transformed into epididymis. Since the male and female reproductive tracts arise from the paired mesonephric and paramesonephric ducts respectively and *Hoxa-10* is expressed in the mesenchymal component of these ducts, these transformations probably originate from changes in gene expression in the ductal mesenchyme prior to sex differentiation. In heterotypic recombinations of stroma and epithelium from different proximodistal levels of the reproductive tract, regional specialization of the epithelium depended not upon its origin but on that of the mesenchyme (Cunha, 1976b). Subsequently, the epithelium is necessary to induce differentiation of the muscle layer; however, the regionalization of the muscle again depends on the origin of the mesenchyme (Cunha et al., 1989). The mesenchyme can also

function in induction of regional identity in epithelium from the opposite sex (Cunha, 1976a), consistent with a common mechanism of patterning in males and females.

Recently it has been demonstrated that the mesenchyme produces soluble factors which are responsible for patterning of the epithelium (Shima et al., 1995). The finding that *Hox* genes are expressed in the mesenchyme and that mutations of *Hox* genes affect the regionalization of the genitourinary tract, suggests that they control production of such region-specific factors. *Wnt5a* and *Msx-1* have been proposed to function in the epithelial mesenchymal interaction forming the uterus (Pavlova et al., 1994). *Wnt5a*, a member of the *Drosophila wingless* family of growth factors, is expressed in the uterine mesenchyme and may signal to the uterine epithelium. *Msx-1*, a homeobox gene, is expressed in the epithelium in a mesenchyme-dependent fashion and correlates with the ability of the epithelium to assume a uterine fate. We have shown that *Msx-1* expression occurs in the uterus but not in the oviduct and is reduced when the uterus is transformed to oviduct, suggesting possible regulation at the uterotubal junction by *Hoxa-10*.

From studies of homeobox gene function in *Drosophila* and in the mouse, two principles have been proposed to govern *Hox* gene function in the patterning of the vertebral column (Krumlauf, 1994). First, the Hox code posits that from anterior to posterior, vertebral segments are differentiated from one another by the sequential expression of more 5′ *Hox* genes (Kessel and Gruss, 1991). Second, posterior prevalence states that a *Hox* gene has its predominant effects in the domain limited by its anterior boundary of expression and that of the next most 5′ gene in its cluster (Duboule, 1991). From these principles, loss of function of a *Hox* gene will affect the segment at its anterior boundary of expression and will change its code to that of the next anterior most segment, leading to an anterior transformation. While the reproductive tract is not an overtly segmented structure like the vertebral column, the homeotic transformations observed here support the same paradigms. The transformations occur at the anterior boundaries of expression of *Hoxa-10* and the transformations are to more anterior identities. Furthermore, males deficient for *Hoxa-11*, the next 5′ gene in the *Hoxa* cluster, exhibit a homeotic transformation of the ductus deferens (Hsieh-Li et al., 1995) which is partially overlapping, but extends distal to the region affected in *Hoxa-10* mutant mice. Therefore, a phenotypic colinearity exists and suggests that the *Hoxa* cluster *AbdB* genes define a proximodistal axis in the reproductive tract.

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