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Hoxa11 Regulates Stromal Cell Death and Proliferation during Neonatal Uterine Development

KENNETH H. H. WONG, HEATHER D. WINTCH, AND MARIO R. CAPECCHI

Department of Human Genetics and Howard Hughes Medical Institute, University of Utah School of Medicine, Salt Lake City, Utah 84112-5331

Increasing evidence indicates that the *Hoxa11* gene plays a critical role in the proper development of the uterus. In this report, we describe potential altered cellular processes in the developing uterus of *Hoxa11* mutants. Histologic analysis demonstrates normal uterine morphology in *Hoxa11* mutants as compared with controls at the newborn stage and d 7 after birth. Stromal tissue was moderately reduced in the *Hoxa11* mutant uterus by d 14 after birth and was absent by d 21 after birth. There is decreased cellular proliferation in the *Hoxa11* mutant uterus both at 7 and 14 d after birth. Terminal deoxyribonucleotide transferase-mediated deoxyuridine triphosphate nick-end labeling analysis demonstrates that apoptosis was markedly increased in the *Hoxa11* mutant uterus at d 14 after birth. *p27* is decreased in the *Hoxa11* mutant as evidenced by real-time PCR. Epidermal growth factor receptor expression is dramatically decreased as evidenced by both real-time PCR and immunohistochemistry results. These findings suggest that *Hoxa11* is required for proper cellular proliferation and apoptotic responses in the developing neonatal uterus and that the regulation of epidermal growth factor receptor is critical to these processes. (*Molecular Endocrinology* 18: 184–193, 2004)

NORMAL UTERINE GROWTH in mice occurs during the neonatal stages after birth. From the day of birth to d 3, the uterine lumen becomes complex in shape surrounded by several layers of mesenchymal cells (1). The mesenchyme segregates into distinct stromal and myometrial layers by 5 d after birth (1). By d 10, the luminal epithelium becomes more complex in shape and uterine glands extend from the luminal epithelium into the stroma. The basic adult configuration of the uterus is established by d 15 with complex luminal epithelial folding and a random orientation of the endometrial stromal nuclei (1).

Although the morphologic and histologic appearance of the murine uterus throughout development has been well established, the profile of genes that is critical to uterine proliferation and differentiation during the neonatal time period is unknown. Expression studies have revealed several factors present in the developing uterus including epidermal growth factor (EGF), EGF receptor (EGFR), keratinocyte growth factor (KGF), KGF receptor, and estrogen receptors (2–6). The importance of these genes in the proper development of the female reproductive tract, however, has not been established. Furthermore, estrogen receptors are prime examples of protein being present in uterine stroma throughout neonatal development (5–7), but not affecting uterine proliferation as evidenced by ovariectomy/adrenalectomy studies (8). This nonreliance of neonatal uterine growth upon estrogen is further evidenced by the normally developed reproductive tissues of both the αERKO (estrogen receptor α null) and βERKO (estrogen receptor β null) mice (9).

Gene targeting studies have identified several genes with possible roles in uterine development. However, the exact role that some of these genes (*Pax-2, Emx-2, Wnt-4*) play in the growth and differentiation of the uterus cannot be ascertained due to the complete absence of the uterus in the mutant animals (10–12). Similarly, the exact role of *Sp4*, a transcription factor, in uterine differentiation and proliferation has not been established. Despite the findings of a reduced uterine size in the adult *Sp4* knockout animal, neonatal uterine tissue was not examined (13).

In contrast, abnormal adult uterine morphology resulting from targeted gene disruptions has implicated the potential role of homeobox-containing genes in the development of the female reproductive tract. One of these homeobox genes, *Hoxa11*, is part of a family of genes that provides cells with specific positional identities, which result in the proper development and spatial arrangement of organs and structures along the anterior-posterior axis (14, 15). This genetic regulation consists of a set of structurally similar genes that were originally discovered in *Drosophila* through homeotic transformations (16). These genes possess a sequence of 180 bp (the homeobox) encoding a 60-

Abbreviations: BrdU, Bromodeoxyuridine; cdk, cyclin-dependent kinase; EGF, epidermal growth factor; EGFR, EGF receptor; *G3PDH*, glyceraldehyde-3-phosphate dehydrogenase; KGF, keratinocyte growth factor; QRT-PCR, quantitative RT-PCR; TUNEL, terminal deoxyribonucleotide transferase-mediated deoxyuridine triphosphate nick-end labeling.

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amino acid DNA-binding motif (the homeodomain). In turn, the homeodomains are part of larger proteins that function as sequence-specific transcription factors (17). Currently, it is believed that the *Hox* genes, acting individually or in concert with each other, coordinate a multitude of downstream target genes that assemble functioning, region-specific tissue and organs (15, 18–21).

Hoxa11 homozygous mutant adult females mice are sterile, producing no offspring when mated with their wild-type counterparts (22). The female *Hoxa11* deficient mice produced a defective uterine environment with high embryo absorption rates in the homozygotes. Further investigations into the role of *Hoxa11* gene in the differentiation and function of the female reproductive tract have revealed abnormal stromal and glandular cell development in adult mutant uteri (23). These same *Hoxa11* adult female mutants also failed to display a normal decidual reaction secondary to natural or artificial stimuli (23). Taken together, *Hoxa11* may be critical for normal uterine stromal cell and glandular differentiation and implantation.

Further evidence of the importance of *Hoxa11* in the developing reproductive tract is suggested by the localization of *Hoxa11* transcripts in the immature stromal cells of the Mullerian ducts of E14 embryos (22). *Hoxa11* continues to be expressed in the stromal cells of the developing female reproductive tract and is also expressed in the uterine epithelial cells by 2 wk of age (24).

Despite the growing evidence of *Hox* gene involvement in the female reproductive tract, the exact role of *Hoxa11* in uterine development is not known. More specifically, what cellular mechanisms and genes are altered in *Hoxa11* mutants in the proper development of the murine uterus have not been established.

In this report, we investigate the role of *Hoxa11* gene in the development of the female reproductive tract. At the histologic level, the *Hoxa11* mutant uterus was observed to be identical to the wild-type uterus at the newborn stage and 7 d after birth. The amount of stromal tissue was moderately decreased in the *Hoxa11* mutant uterus by d 14 after birth and was absent by d 21 after birth. Bromodeoxyuridine (BrdU) cellular proliferation studies demonstrated decreased cellular proliferation in the *Hoxa11* mutant uterus both at 7 and 14 d after birth. Terminal deoxyribonucleotide transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) analysis demonstrated that apoptosis was markedly increased in the *Hoxa11* mutant uterus at d 14 after birth. The expression of many genes involved in cell cycle regulation and apoptosis at both 7 and 14 d after birth was normal except for a marked decrease in *p27*. EGFR expression is dramatically decreased as evidenced by both real-time quantitative RT-PCR (QRT-PCR) and immunohistochemistry results. These findings suggest that *Hoxa11* is involved in controlling cellular proliferation and apoptotic responses in the neonatal uterus and that the regulation of EGFR is critical to these processes.

RESULTS

Abnormal Adult and Neonatal Uterine Morphology in Homozygous *Hoxa11* Mutant Mice

The generation of the *Hoxa11* mutant has been previously described (15, 25). Examination of uteri obtained from adult *Hoxa11* mutants shows a reduction in size compared with wild-type adults. The uterine diameter is dramatically smaller as well as the overall uterine length (not shown). Histologically, *Hoxa11* adult mutant uteri displayed little to no stromal tissue (labeled S) as well as no endometrial glands (*arrows*) compared with wild-type controls (Fig. 1). The luminal epithelium (labeled E) and the surrounding muscular layers (labeled M) appear to be normal, histologically.

The abnormal histologic appearance of the uterus in *Hoxa11* mutant adults, especially the minimal amount of stromal tissue, does not originate from the lack of stroma in early developmental stages. Examination during neonatal time points has revealed the uteri from *Hoxa11* mutants as having normal appearing stromal tissue in the newborn stage (Fig. 2, A and B) and d 7 after birth (Fig. 2, C and D) as compared with wild-type controls. However, the amount of stromal tissue appears to decrease as development progresses (Fig. 2, E–H). Stromal tissue is decreased in the mutant in comparison to wild-type control mice by d 14 after birth (Fig. 2, E and F). By 21 d after birth, the mutant uteri display a histologic architecture similar to an adult mutant configuration of minimal to no stromal tissue and absent endometrial glands (Fig. 2, G and H).

Decreased Neonatal Uterine Cellular Proliferation and Increased Apoptosis in Homozygous *Hoxa11* Mutant Mice

To determine the basis of the abnormal stromal tissue, we examined cellular proliferation and apoptosis in the uteri of *Hoxa11* mutants. Based upon our findings of little to no stromal tissue remaining in the d 21 *Hoxa11* mutants, we concentrated our investigations on d 7 and 14 after birth time points. Cellular proliferation, as evidenced by BrdU incorporation, was altered in both the stromal and the epithelial components of the mutants (Fig. 3). *Hoxa11* mutants exhibited decreased BrdU incorporation in the uterine stroma at both 7 (3.43 *vs.* 6.38% in wild-type, Fig. 3C) and 14 d (4.24 *vs.* 10.95%, Fig. 3E) of age. In contrast, the epithelial components of *Hoxa11* mutants exhibited decreased proliferation only at d 7 after birth (11.73 *vs.* 16.62%, Fig. 3D). BrdU incorporation at d 7 after birth in the *Hoxa11* mutants was similar to wild-type controls (10.58 *vs.* 9.02%, Fig. 3F).

In addition to decreased cellular proliferation, *Hoxa11* mutants also exhibited increased apoptosis in the uterus. TUNEL analysis was performed on uteri obtained from both wild-type and *Hoxa11* mutants at d 7 and 14 after birth. At d 7, both wild-type and *Hoxa11* mutant mice exhibited minimal to no apopto-

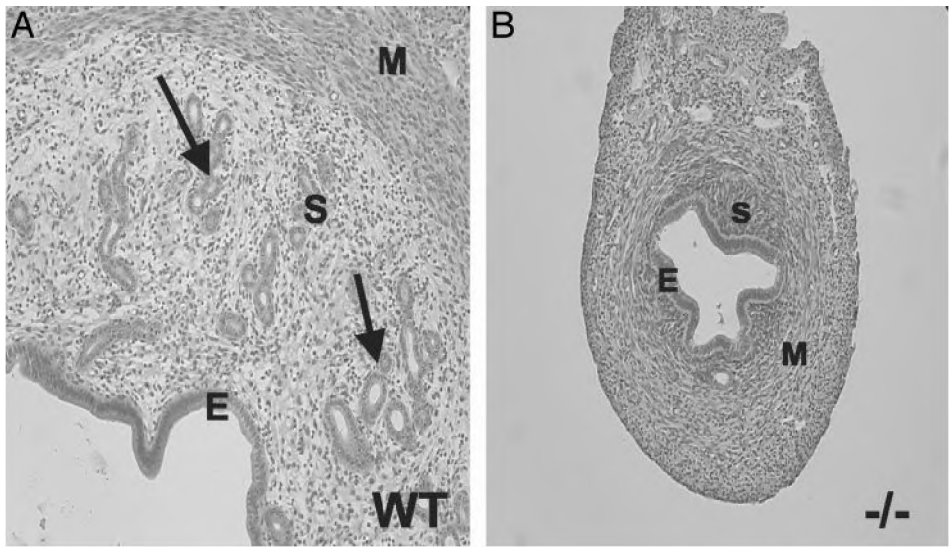

Fig. 1. Representative Hematoxoylin and Eosin Uterine Sections (×100) from Wild-Type (WT) (A) and *Hoxa11* Mutant (−/−) (B) Adult Mice (>8 wk)

The stromal tissue (S) and endometrial glands (*black arrows*) in the wild-type uterine section are absent in the Hoxa11 mutant. The endometrial luminal lining (E) and outer muscular layers (M) appear to be similar in the wild-type and mutant uteri.

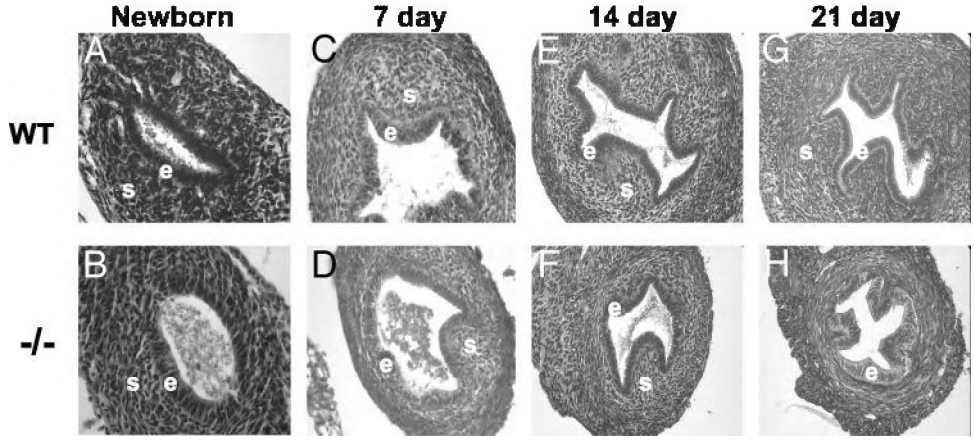

Fig. 2. Uterine Sections (A and B, ×400, C–H, ×250) Stained with Hematoxylin and Eosin from Wild-Type (WT) and *Hoxa11* Mutant (−/−) Mice at Newborn, 7, 14, and 21 d after Birth

The amount of stromal tissue (s) between the luminal epithelium (e) and the muscular layers and the number of endometrial glands in the wild-type uterine section is similar to the *Hoxa11* mutant at the newborn stage (A and B) and d 7 of life (C and D). By d 14 (E and F), there is diminished stromal tissue in the *Hoxa11* mutant compared with control. There is an absence of uterine stromal tissue by 21 d after birth in the Hoxa11 mutant (G and H). In addition, there are minimal to absent endometrial glands in the *Hoxa11* mutant at 21 d after birth.

sis in the uterus (Fig. 4, A and B). By d 14, there is markedly increased apoptosis in the mutant uterus as evidenced by greatly increased fluorescence as compared with control (Fig. 4, C and D). The apoptosis as evidenced by TUNEL appears throughout the uterus including the stromal area.

Altered Neonatal Uterine Gene Expression in *Hoxa11* Mutants

To characterize the alteration in neonatal uterine gene expression between wild-type and *Hoxa11* mutants, we used QRT-PCR analysis on cDNA samples generated from uterine extracted RNA. Based on the BrdU and TUNEL data, examination was focused upon genes important in cellular proliferation and apoptosis. Important regulators of the cell cycle and proliferation include the cyclin proteins. These proteins regulate the passage through the various phases of the cell cycle including cyclin D1 and E in the G_1 phase, cyclin A in the S phase, and cyclin B in the G_2 phase (26). Regulators of apoptosis include BAX as a promoter of apoptosis and Bcl-2 as a suppressor of apoptosis (27).

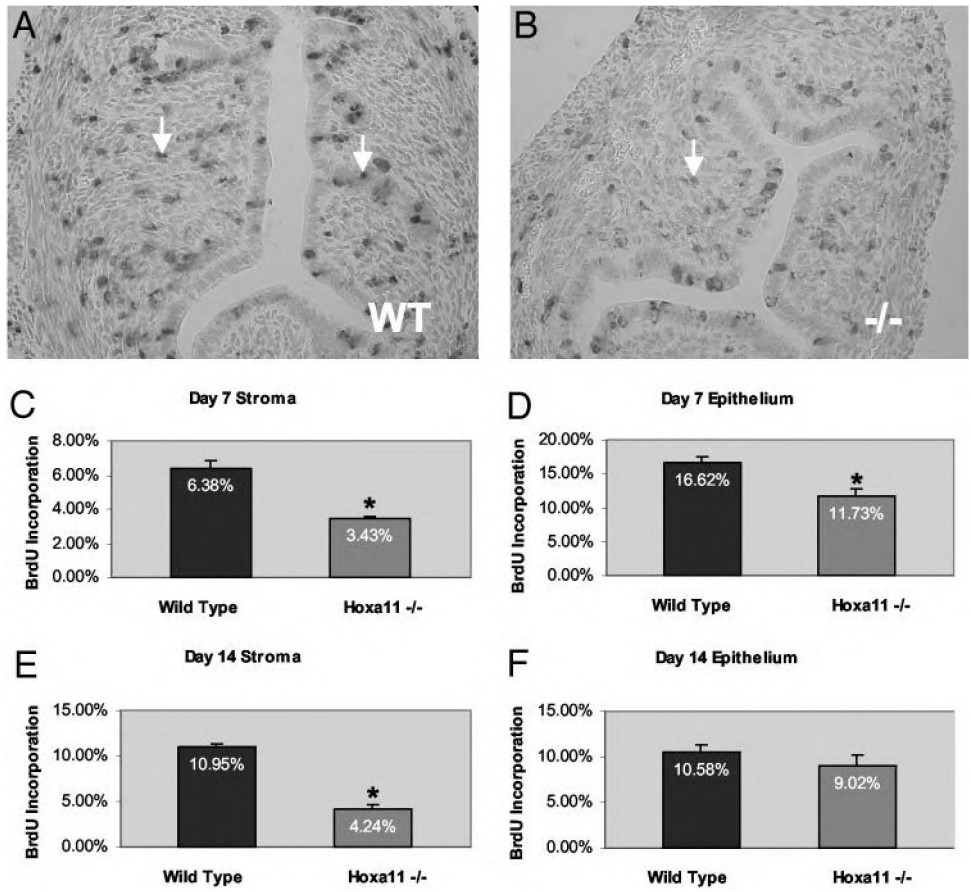

Fig. 3. BrdU Incorporation

A and B, Representative histologic sections of BrdU incorporation (×400). BrdU incorporation (*white arrows*) in representative wild-type (WT) (A) and mutant (−/−) (B) uteri at 14 d after birth. C–F, BrdU incorporation represented by mean percentage of BrdU-positive cells/total number of cells per high power field + SE (n = 4 animals). Stromal BrdU incorporation is significantly (*, $P < 0.05$) decreased in mutant tissues compared with wild-type both at 7 and 14 d of life (C and E). Epithelial BrdU incorporation is decreased significantly (*, $P < 0.05$) in d 7 mutant mice compared with wild-type (D). Epithelial BrdU incorporation is not significantly different in 14 d neonatal mutants as compared with controls (F).

Examination of the expression of several genes implicated in uterine development was also performed. Both EGF and EGFR have been implicated to play a role in the developing female reproductive tract by expression data (2) and EGFR-KO grafting data (28). Growth factors such as EGF and its receptor, EGFR, play critical roles in cellular proliferation and apoptosis (29). Alterations in the expression of either uterine EGF or EGFR in the *Hoxa11* mutant could result in the abnormal stromal development found in the adults.

Gene targeting studies have identified several genes that may have roles in the developing reproductive tract. *Wnt7a* is a member of a family of genes patterning and cell-to-cell communication. Targeted removal of *Wnt7a* leads to abnormal female reproductive development and results in diminished stromal tissue and a reduced number of endometrial glands in the adult (30). Another Hox family member, *Hoxa10*, has also been implicated in proper reproductive tract development. In contrast to *Hoxa11* mutants, *Hoxa10* mutants only display abnormal uterine morphology in the proximal horn of the uterus (31).

The QRT-PCR data are reported as the fold change between wild-type and *Hoxa11* mutants. Because we were looking for major differences in expression, which may have more biologic significance, we defined a change as greater than 2-fold difference. By QRT-PCR, there was no difference in mRNA levels of genes implicated in cell proliferation or apoptosis including *cyclin A*, *cyclin B1*, *cyclin D1*, *cyclin E*, *BAX*, or *Bcl-2* transcripts between wild-type and mutant animals at 7 d after birth (Table 1). In addition, the expression of genes implicated in uterine development, *Wnt7a*, *Hoxa10*, *EGF*, and *EGFR*, did not reveal any difference in transcript levels (Table 1).

In contrast, *Hoxa10* and *cyclin E* transcript levels were 3.5- and 3.4-fold decreased in *Hoxa11* mutants at d 14 after birth (Table 1). At 14 d of age, *EGFR* expression was decreased by 12.8-fold in *Hoxa11* mutants. There was no difference in uterine levels of

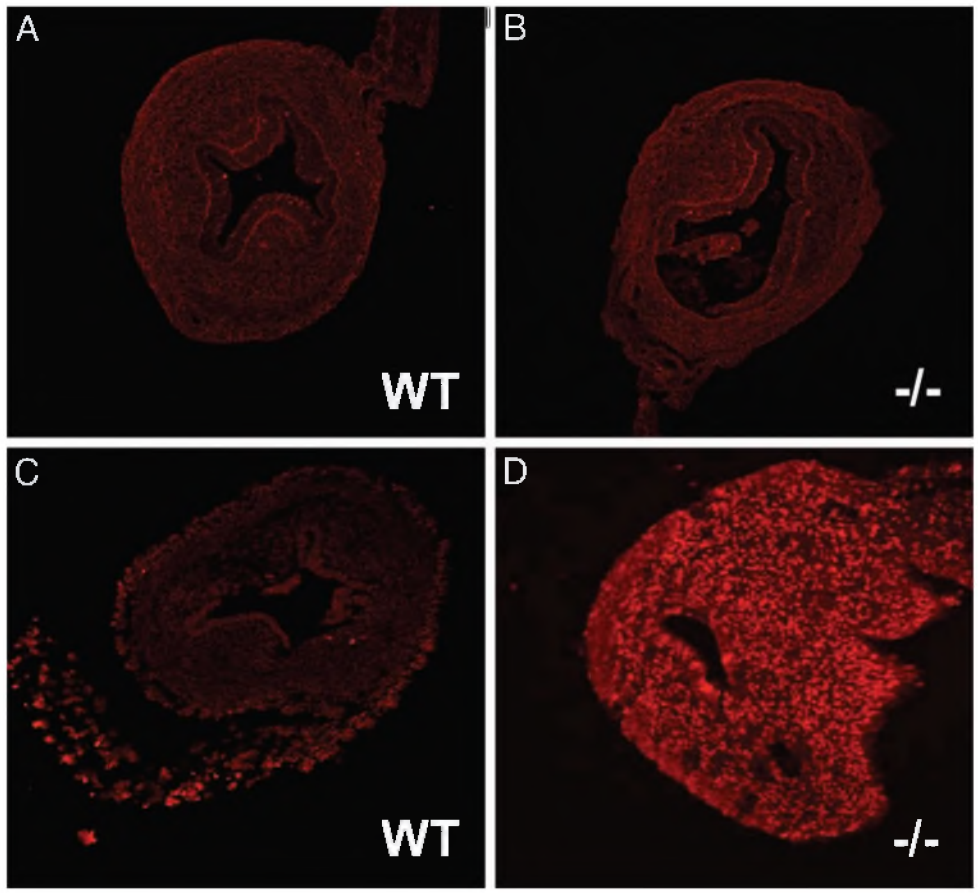

Fig. 4. TUNEL Analysis

Representative fluorescent images of TUNEL staining in uterine histologic sections from wild-type (WT) and *Hoxa11* mutant (−/−) mice at 7 and 14 d after birth. There is minimal apoptosis (*red fluorescence*) in both wild-type (A) and mutant (B) uteri at 7 d after birth. By d 14, there remains minimal apoptosis in the wild-type uterus (C). In contrast, the mutant d 14 uterus displays increased apoptosis in the stromal areas as evidenced by intense fluorescent signaling (D).

cyclin A, *cyclin B1*, *cyclin D1*, *BAX*, *Bcl-2*, *Wnt7a,* and *EGF* between mutant and wild-type mice at 14 d after birth (Table 1).

Genes implicated in the regulation of EGFR, *Sp1* and *p53*, were also examined at 7 and 14 d of age. The transcription factor, Sp1, and the tumor suppressor, p53, have both been implicated in the regulation of EGFR (32, 33). The expression of *Sp1* and *p53* were found to be no different between wild-type and *Hoxa11* mutants both at 7 and 14 d of age (Table 1).

Altered Cell Cycle Mediator Gene in *Hoxa11* Mutants

Based on the BrdU data, further examination was focused upon genes important in the cell cycle. By QRT-PCR, there was no difference in mRNA levels of genes implicated in the cell cycle including *p15*, *p18*, *p21*, *p57*, *cdk2*, *cdk4*, and *cdk6* between wild-type and mutant animals at 14 d after birth (Table 2). In contrast, *pRB* (retinoblastoma) transcript levels were slightly decreased by 2.07-fold and *p27* transcript levels were markedly decreased by 7.1-fold in *Hoxa11* mutant uteri. These data, taken together with decreased EGFR expression, suggest that *Hoxa11* regulates genes essential for normal cellular proliferation in the developing female reproductive tract.

Decreased EGFR Uterine Stromal Expression in *Hoxa11* Mutants

To further validate the finding of markedly decreased uterine expression of EGFR transcripts in the *Hoxa11* mutant, changes in the expression pattern of EGFR were examined by immunohistochemistry. The expression of EGFR protein in the uterine stroma at 7 d after birth was similar between the *Hoxa11* mutant and wild-type, which correlated with the finding of similar amounts of transcripts by QRT-PCR (not shown). At d 14 after birth, however, the expression of EGFR (*brown staining*) in the uterine stroma (labeled s) was greatly reduced in the *Hoxa11* mutant compared with wild-type (Fig. 5). In addition, expression of EGFR was reduced in the epithelial component (labeled e) of the mutant (Fig. 5).

Table 1. Gene Expression Differences in the *Hoxa11* Knockout Mouse

Genes	Fold Change	
	7-d	14-d
Cell cycle		
Cyclin A	−0.2	−0.4
Cyclin B1	1.2	0
Cyclin D1	1.9	−0.9
Cyclin E	−0.9	−3.4
Apoptosis		
Bax	−1.2	1.7
Bcl-2	−0.8	−1.3
Implicated in uterine development		
Hoxa10	1.8	−3.5
Wnt7a	−1.1	−0.9
EGF	−1.5	−1.4
EGFR	−0.7	−12.8
EGFR regulation		
SP1	−1.4	−1.5
p53	−0.8	−0.9

Negative numbers represent decreased expression in the mutant. Positive numbers represent increased expression in the mutant.

Table 2. Expression of Cell Cycle Genes in the *Hoxa11* Knockout Mouse at 14 d

Genes	Fold Change
p15	1.97
p18	1.27
p21	−1.89
p27	−7.10
p57	1.90
Cdk2	1.39
Cdk4	−1.03
Cdk6	1.05
pRb	−2.07

Negative numbers represent decreased expression in the mutant. Positive numbers represent increased expression in the mutant.

DISCUSSION

The present study provides new insights into *Hoxa11* regulation of normal uterine development. In particular, *Hoxa11* is critical to the normal nonsteroid associated uterine growth in the neonate. Utilizing the *Hoxa11* mutant, we present evidence of several mechanisms/pathways that may be involved in the proper formation of normal uterine morphology in the adult mouse. In addition, we demonstrate evidence of altered expression of a previously implicated gene in uterine development, EGFR, in *Hoxa11* mutants.

Interestingly, uteri from *Hoxa11* mutants appear histologically normal in early neonatal stages and develop abnormalities as the animals increase in age. The findings of no discernible morphologic differences in uteri between *Hoxa11* mutants and wild-type controls both at the newborn stage and d 7 after birth would suggest that *Hoxa11* is not critical in establishing the Müllerian duct. Instead, our findings would indicate that *Hoxa11* is necessary for the continued growth of the reproductive tract during the neonatal time period. The normal expression of *Hoxa11* appears to be critical for stromal tissue development but not for epithelial or muscular development in the uterus. Despite the normal morphologic appearance of the epithelial and muscular components, however, the functionality of these tissues may be altered in the mutants. This is supported by the findings of a normal histologic appearance of three fourths of the uterus in *Hoxa10* mutants but the inability to support implantation of wild-type embryos in this normal appearing area of the uterus (31).

The uterine hypoplasia seen in the *Hoxa11* mutants is caused by a combination of increased stromal cell death with decreased stromal cell proliferation. Several investigators have previously found alterations in proliferation or cell death in other *Hox* mutants. Targeted null mutations in *Hox* genes in combination with transcription factors have been associated with both elevated apoptosis and decreased cell proliferation in the thymus of $Hoxa3^{+/-}Pax^{-/-}$ mutants (34). In addition, elevated apoptosis with unaffected cellular proliferation has been detected in the hindbrains of *Hoxa1* mutants and *Hoxa1Hoxb1* double mutants (21, 35). Differential proliferation has also been proposed as a mechanism for Hox regulation of limb patterning and skeletal formation (25, 36). Thus, our data appear to implicate *Hoxa11* in promoting proper proliferation and survival of stromal cells in the developing uterus.

We were unable to detect any major alterations (greater than 2-fold difference) in the expression of the majority of cell cycle regulators or apoptotic genes except for a marked decrease in *p27*, a small decrease in *cyclin E* and *pRB*. Cyclins, cyclin-dependent kinases (cdk), and cdk inhibitors are the major regulators of the cell cycle and are implicated in multiple cellular processes including cell proliferation and differentiation (26). Cdk inhibitor *p27* is an antiproliferative mediator of the cell cycle and the *p27* knockout animals have displayed organomegaly and cellular hyperplasia (37). Some investigators, however, have found *p27* to be an activator of cdks (38), which is implicated by our findings of decreased *p27* in the *Hoxa11* mutants. Our results of altered expression of several cell cycle regulating genes may contribute to the abnormal cellular proliferation displayed in the *Hoxa11* mutants.

Similar to cell proliferation and cell cycle regulation, apoptosis is a cellular process involving many pathways and numerous factors (39). Our choice of investigating only *BAX* and *Bcl-2*, two members of the Bcl-2 family, may again have limited our study. Expanding our investigation to include other apoptotic proteins may further enhance our understanding of how *Hoxa11* regulates cell proliferation and apoptosis.

Hoxa11 does not appear to regulate *Wnt7a* in the developing uterus. Previous investigators have found that *Wnt7a* expression is necessary to maintain the

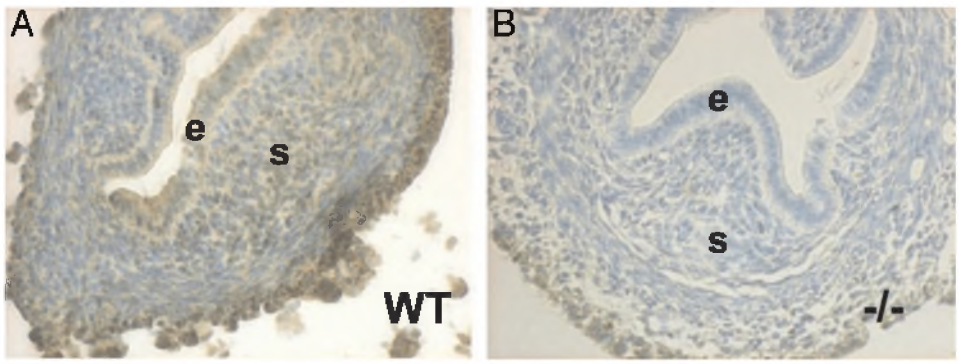

Fig. 5. EGFR Expression in the 14-d Uterus of Wild-Type (WT) and *Hoxa11* Mutant (−/−) Mice

A, In the WT animal, EGFR is expressed (*brown staining*) throughout the stromal (s) and epithelial (e) components. (B) There is minimal EGFR staining in both the stroma and luminal epithelia in the Hoxa11 mutant. Magnification, ×400.

expression of *Hoxa11* in the uterus (30). *Wnt7a* mutants eventually lose the expression of *Hoxa11* and *Hoxa10* in the stroma of the adult uterus. Our finding of possible unaltered *Wnt7a* expression in the neonatal uteri of *Hoxa11* mutants is consistent with the hypothesis that *Hoxa11* is downstream of *Wnt7a* in the developing reproductive tract.

Although our findings show that *Hoxa11* does not appear to regulate *Hoxa10* in the developing uterus, the possibility still exists that other *Hox* genes may be altered in the developing uterus of *Hoxa11* mutants. Other *Hox* genes besides *Hoxa10* and *Hoxa11* have been reported to be expressed in the uterus (24, 40, 41). In addition, the *Hoxa13* null mutant displays agenesis of the caudal portion of the Müllerian ducts (42). *Hox* genes have also been demonstrated to regulate the expression of other *Hox* genes. *Hoxa1* is required to establish the expression of *Hoxb1* in the hindbrain (35) and the expression of *Hoxb2* in rhombomere 4 of the hindbrain appears to be regulated by *Hoxb1* (43). Therefore, determining whether other *Hox* genes may be altered in the uterus of the *Hoxa11* mutant should provide further insights in the *Hoxa11* regulation of the reproductive tract.

The uterine defects in *Hoxa11* mutants may be, in part, mediated through EGFR. EGF and EGFR have both been implicated in uterine development, especially as mediators of estrogen action. The role of EGFR in the steroid-independent time period of neonatal uterine growth, however, is less well established. While the expression of both EGF and EGFR has been reported in the uterus of neonatal mice, no functional data exist (2, 3). Our findings of decreased *EGFR* transcript and protein in the stroma of *Hoxa11* mutants point, for the first time, to the potential importance of EGFR in the proper development of the uterus in neonatal mice. Although the findings of unaltered expression of *Sp1* and *p53*, both regulators of *EGFR* transcription, help point to the possibility of the direct regulation of *EGFR* by *Hoxa11*, the establishment of EGFR as a true transcriptional target of *Hoxa11* would require further studies.

The examination of the uterus in EGFR knockout animals would further substantiate the importance of EGFR in the developing uterus. The EGFR knockout animal, however, survives up to only 8 d after birth (44). Our data seem to indicate that EGFR expression is normal in *Hoxa11* mutants at d 7 after birth. Examination of EGFR knockout animals at older ages (d 14) would require a conditional knockout of EGFR.

In summary, we have demonstrated that the abnormal uterine morphology of *Hoxa11* mutants arises during neonatal development and results from a combination of decreased cellular proliferation as well as increased cell death. The uterine expression of cell cycle regulators is altered in *Hoxa11* mutants. We have also shown that EGFR expression is severely diminished in the stromal tissue of the neonatal mutants. Taken together, these findings suggest that *Hoxa11* directly or indirectly regulates cellular proliferating genes and, in turn, controls cellular growth and apoptotic responses in the developing neonatal uterus.

MATERIALS AND METHODS

Animals and Genotyping

Hoxa11 null mutant mice were generated by homologous recombination with embryonic stem cells as previously described (15, 25). All experimental procedures involving animals were performed in accordance with the University of Utah Animal Care Committee guidelines.

PCR assays, performed on genomic DNA, were used for genotyping to distinguish between wild-type and mutant alleles. Primers used to detect the *Hoxa11* mutant allele were:

5′-GCTGGCTTTTATCTGAAGCCGG-3′ (forward),
5′-CTCCCAATTCCAGTAGGCTGGA-3′ (reverse),
5′-GGTTGTTCAGACTACAATCTGACC-3′ (neo reverse).

Histologic Examination

Uterine tissues were obtained from wild-type and *Hoxa11* mutant mice (C57BL/6 background) at newborn, 7, 14, and 21 d after birth. Tissues were fixed in 4% vol/vol paraformaldehyde and then embedded in paraffin. Tissue sections (5

μm) were deparaffinized and rehydrated for histologic, immunohistochemical and immunofluorescent analyses. For light microscopic examination, tissue sections were stained with hematoxylin and eosin.

BrdU Incorporation

For the assessment of cell proliferation, neonatal wild-type and *Hoxa11* mutant mice were killed 2 h after BrdU (30 μg/g of body weight, Sigma Diagnostics, St. Louis, MO) was administered ip. Uterine sections obtained from BrdU-treated mice were fixed with 4% vol/vol paraformaldehyde and then embedded in paraffin. Tissue sections (5 μm) were deparaffinized, rehydrated and labeled with the 5-Bromo-2′-Deoxy-Uridine Labeling and Detection Kit II (Roche Molecular Biochemicals, Indianapolis, IN). The percentage of BrdU positive cells/1000 cells counted by three independent blinded evaluators was averaged. Statistical analysis was performed with the ANOVA statistical testing by using the SAS statistical package (SAS Institute, Cary, NC). Statistical significance was defined as $P < 0.05$.

TUNEL Labeling

Apoptosis was detected by using TUNEL analysis with the *In Situ* Death Detection Kit (Roche Molecular Biochemicals) on deparaffinized uterine sections. Samples were analyzed with confocal fluorescence microscopy.

Real-time RT-PCR

After genotyping, uteri were obtained from wild-type and *Hoxa11* mutant mice at 7 and 14 d after birth. Six animals with identical genotypes in the 7-d-old groups (wild-type and *Hoxa11* mutant), and four animals with identical genotypes in the 14-d-old group were pooled together to obtain sufficient total RNA for each sample. Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA). One microgram of deoxyribonuclease I-treated RNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Invitrogen, Inc.) to generate cDNA.

Gene-specific primers corresponding to the investigated genes were used for the QRT-PCR. The primer pairs used are listed in Table 3. QRT-PCR was performed with the LightCycler-based SYBR Green I detection system (Roche Molecular Biochemicals). Dilutions of cDNA were amplified at a specified annealing temperature determined by the specific primer pair. Amplification was followed by a melting curve analysis. Negative controls without cDNA were used to assess specificity. A stable housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) was used to control for input RNA. The amount of transcript was determined based on the fluorescence *vs.* cycle number plot. The cycle crossing point for each gene was determined by the LightCycler Software version 3.39 program (Roche Molecular Biochemicals). After adjusting with the *G3PDH* control, the difference in cycle crossing points were calculated for each gene between the wild-type group and mutants for a specific time point. For a theoretical efficiency of 100%, the fold difference was calculated by 2 to the power of the cycle point difference.

Immunohistochemistry

To compare the expression of EGFR in *Hoxa11* mutant uteri *vs.* wild-type uteri, fixed deparaffinized uterine sections were first treated with 2% hydrogen peroxide in methanol to quench endogenous peroxide activity. The uterine sections were then incubated with 3% normal goat serum to block endogenous nonspecific Ig binding sites. Sections were then incubated with anti-EGFR rabbit antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) at a predetermined dilution of 1:200 in blocking solution overnight at 4 C. After incubation with primary antibody, the sections were washed and immunoreactivity was assessed with the antirabbit goat biotinylated secondary antibody followed by avidin-biotin horse radish peroxidase complex (Santa Cruz Biotechnologies) and visualized with diaminobenzidene tetrahydrochloride (Santa Cruz Biotechnologies). Slides were counterstained with methyl green, then rinsed and mounted with Cytoseal (Stephens Scientific, Riverdale, NJ). Omission of primary antibody was used as a negative control.

Table 3. Primer Sequences for QRT-PCR

Gene	Forward Sequence	Reverse Sequence
G3PDH	5′-TGAAGGTCGGTGTGAACGGATTTGGC	5′-CATGTAGGCCATGAGGTCCACCAC
Cyclin A	5′-TCCAAGAGGACCAGGAGAATATCA	5′-TCCTCATGGTAGTCTGGTACTTCA
Cyclin B1	5′-CCATTATTGATCGGTTCATGCAGA	5′-CTAGTGGAGAATTCAGCTGTGGTA
Cyclin D1	5′-TGGAGCCCCTGAAGAAGAG	5′-AAGTGCGTTGTGCGGTAGC
Cyclin E	5′-CTGGCTGAATGTTTATGTCC	5′-TCTTTGCTTGGGCTTTGTCC
BAX	5′-CTGAGCTGACCTTGGAGC	5′-GACTCCAGCCACAAAGATG
Bcl-2	5′-GACAGAAGATCATGCCGTCC	5′-GGTACCAATGGCACTTCAAG
Wnt7a	5′-GACAAATACAACGAGGCCGT	5′-GGCTGTCTTATTGCAGGCTC
Hoxa10	5′-GCTGTCTCCAAGCCCCTTCAGA	5′-AAAGTTGGCTGTGAGCTCCCG
EGF	5′-AGATGAGTGTGTGCTGGCTAGATC	5′-TCCGAGTCCTGTAGTAGTAAGTCC
EGFR	5′-GTGTGAAGAAGTGCCCCCGAAAC	5′-AACGACCGCCAAAGAAAACTGACC
Sp1	5′-AGATGTTGGTGGCAATAATGGG	5′-TGAAAGTTGTGTGGCTGTGAGG
p53	5′-TGAAACGCCGACCTATCCTTA	5′-GGCACAAACACGAACCTCAAA
p15	5′-CACCGTGACATTGCGAGGTATC	5′-TGGGTAGGGTTCAAGTTTTGGAAG
p18	5′-CCAATGGGCTCACTTTTGCTG	5′-CCTTTCCTTTGCTCCTAATCAGG
p21	5′-GGATTCCCTGGTCTTACCTTAGGC	5′-AGAAGTCACTGAGAACCCCAACTG
p27	5′-AGCCTGGAGCGGATGGACGC	5′-CACCTTGCAGGCGCTCTTGG
p57	5′-GCCGGGTGATGAGCTGGGAA	5′-AGAGAGGCTGGTCCTTCAGC
Cdk2	5′-ATTCATGGATGCCTCTGCTCTCAC	5′-GGTCAATCTCAGAATCTCCAGGGAA
Cdk4	5′-ATCAGCACAGTTCGTGAGGTGGC	5′-AGCTCGGTACCAGACTGTAACAAC
Cdk6	5′-TGCACAGTGTCACGAACAGAC	5′-TGAATGAAAAGCCTGCCTGGG
pRB	5′-GAACAGATTTGTCCTTCCCGTG	5′-TCAGGTCCTTCTCCATCCTTGG

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Address all correspondence and requests for reprints to: Mario R. Capecchi, Department of Human Genetics and Howard Hughes Medical Institute, University of Utah School of Medicine, Salt Lake City, Utah 84112-5331. E-mail: mario.capecchi@genetics.utah.edu.

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