Reproductive System and Sexual Disorders: Current Research

Yin et al. Reproductive Sys Sexual Disord 2011, S:1
DOI: 10.4172/2161-038X.S1-001

Research Article Open Access

Molecular Analysis of Implantation Defects in Homeobox Gene *Hoxa10*-*Deficient* Mice

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Abstract

The success of embryo implantation depends on the receptivity of the uterus. Abdominal B class homeobox gene *Hoxa10* is dynamically expressed around implantation, and is indispensable for establishing uterine receptivity. However, the exact mechanism through which HOXA10 exerts its function remains elusive. In this report, we investigated the molecular basis for the implantation failure caused by *Hoxa10* deficiency using both knock-out mouse models and global gene expression profiling approaches. We demonstrated that augmented local immune response through T/B cells alone is not sufficient to explain the implantation defects observed in the *Hoxa10*-null uterus. We also uncovered a group of potential genes and signaling pathways that may participate in the downstream events mediated by HOXA10.

Keywords: Hoxa10; Implantation; Uterine receptivity; Immunosuppression

Introduction

The embryonic development of all eutherian mammals occurs in the uterus, where they acquire continuous nutrition support from their mothers. The physical connection between the embryos and the mother is first established during implantation, where the blastocysts attach and invade into the uterine wall as a result of comprehensive molecular communication between the two parts. In order for this to occur, the uterus must be properly prepared to become transiently receptive to the approaching blastocysts. This so called window of receptivity [1,2] or uterine receptivity [3], is preceded by a pre-receptive phase when the uterus is yet ready to allow implantation, and is followed by a refractory phase. The receptivity of the uterus is subject to the regulation by genetic and environmental factors, and is a major etiology for human infertility (for review, see [4]). Although substantial effort has been invested, more work is required to understand the molecular pathways involved in establishing uterine receptivity.

The uterus undergoes major morphological and physiological changes in a relative short period of time to prepare for implantation which has long been known to be dependent on the actions of progesterone and estrogen and their downstream signaling cascade on the uterus [5,6]. Components of major signaling pathways involved are mostly controlled by these steroids. Further, complex interaction between the stroma and epithelia, as well as that between the endometrial epithelium and the implanting blastocyst are very important. As an example, it has been recently shown that heart and neural crest derivatives expressed transcript 2 (*Hand2*) expression occurs in the uterine stroma underlying the luminal epithelium as the mouse uterus enters the receptive phase [7,8]. Uterine specific deletion of *Hand2* in mice resulted in uteri unable to show adequate expression of certain fibroblast growth factors in the endometrial epithlium resulting in the inability of their uteri to become receptive and support implantation [8]. Examples of other key local factors involved in obtaining uterine receptivity include: indian hedgehog (*Ihh*) and leukemia inhibitory factor (*Lif*) plus downstream genes they regulate such as nuclear receptor subfamily 2 group F member 2 (*Nr2f2*), bone morphogenic protein 2 (*Bmp2*) and wingless-related MMTV integration site 4 (*Wnt4*) [9-13]. Their functions in the implantation process have been demonstrated by studies on knockout or conditional knockout mouse models.

One of the key regulators of uterine sensitization is also believed to be the transcription factor encoded by the homeobox *Hoxa10* gene. *Hoxa10* gene is an *Abdominal B* (*AbdB*) class homeobox gene. In mammals, it is located in the 5' end of the *Hoxa* cluster and is expressed in the urogenital system along with *Hoxa9*, *a11* and *a13* in a nested fashion from anterior to posterior [14-16]. *Hoxa10* expression has been characterized in the mouse uterus during the peri-implantation period [17]. It is initially expressed in the luminal and glandular epithelia of the endometrium at 0.5 and 1.5 dpc. Later its expression shifts to the stroma and decidua as the uterus enters its receptive period on 3.5 dpc. This stroma-specific expression is regulated by progesterone, and maintains through implantation process until approximately 5.5 dpc after the onset of implantation [16]. Such a dynamic expression pattern suggests a role for *Hoxa10* in regulating uterine receptivity in mice [18] and a similar role for HOXA10 has been speculated in humans [19].

Hoxa10-null female mice are severely subfertile and exhibit anatomical defects in their reproductive tracts. The anterior part of the uteri in *Hoxa10* mutants undergoes a homeotic transformation to an oviduct-like structure, whereas the posterior uteri remain largely unaffected [17,20]. However, the mutant uterus fails to support embryo implantation even when embryos were transferred directly to the uterine lumen, suggesting that the anterior structural malformation is not likely to be the main reason for the infertility phenotype [9,16].

Several previous studies have implicated *Hoxa10* in the regulation of implantation processes. One of the most attractive hypotheses is that a marked increase in leukocyte infiltration and proliferation observed in *Hoxa10* mutant uterine stroma, could potentially cause implantation failure given the cytolytic and inflammatory activities

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Received October 17, 2011; **Accepted** November 16, 2011; **Published** November 18, 2011

Citation: Yin Y, Lin C, Sawalha D, Bany BM, Ma L (2011) Molecular Analysis of Implantation Defects in Homeobox Gene *Hoxa10-Deficient* Mice. Reproductive Sys Sexual Disord S1:001. doi:10.4172/2161-038X.S1-001

of these lymphocytes [21]. In addition, it is believed that HOXA10 plays a physiological role in implantation and decidualization through regulating uterine stromal cell responsiveness to progesterone [10]. This regulation is mediated, at least in part, through PGE_2 receptor subtypes EP_3 and EP_4 and subsequent PG signaling [10]. These hypotheses, unfortunately, have yet to be tested by definitive genetic means.

In the present study, we tested the hypothesis that the failure of *Hoxa10*-null uterus to support implantation is due to T-cell over-reactivity. We generated *Hoxa10*-null mice on an immune-deficient *Rag1*-null mouse genetic background and found that complete removal of mature B and T lymphocytes could not rescue the infertility phenotype of *Hoxa10* mutants. We also performed global expression profiling experiment using samples from *Hoxa10* mutant and control uteri around the onset of implantation and uncovered novel HOXA10 downstream regulated genes and pathways that may be involved in early implantation regulation.

Materials and Methods

Mice

All mice were housed in the animal facility at Washington University with controlled light/dark cycles and handled in accordance with National Institutes of Health guidelines. All procedures were approved by the Washington University Institutional Animal and Use Committee. Mice were mated and the morning a vaginal plug was identified was defined as 0.5 days post coitus (dpc). Mice carrying disrupted *Hoxa10* gene (in C57BL/6-129/Sv hybrid genetic background) were generated by gene-targeting and were described previously [17]. *Rag1*-deficient (*Rag1*$^{-/-}$) mice (in C57BL/6J genetic background) were purchased from the Jackson Laboratory (Bar Harbor, MN). *Hoxa10*$^{+/-}$; *Rag1*$^{+/-}$ males and females were bred to generate single-mutants (*Hoxa10*$^{-/-}$), double-mutants (*Hoxa10*$^{-/-}$*Rag1*$^{-/-}$) and control animals with a variety of other genotypes. Two to six month-old age-matched female mice were used for each experiment.

Embryo transfer

Embryo transfer was performed according to a slightly modified procedure by Hogan et al. [22]. Briefly, 3.5 dpc blastocysts were collected by flushing the uteri of naturally-pregnant females and examined under a microscope. Healthy blastocysts were selected and transferred to fresh 37°C M2 medium (Chemicon, Temecula, CA) with a pulled Pasteur pipette. Day 2.5 p.c. pseudo-pregnant recipients were anesthetized, the uteri exposed. A hole was made on the uterine wall 2-4 mm posterior to the utero-oviductal junction with a 30-gauge needle then the blastocysts were transferred to the uterine horn exactly as described previously [22].

Blue dye reaction

One hundred microliters of a Chicago Blue (Sigma-Aldrich) solution (1% w/v in sterile PBS) was injected into the tail vein of females at 0900 hours on day four of pregnancy. Five minutes later, the mice were sacrificed, and the uterine horns were exposed and the intensely blue dye-stained implantation (IS) and unstained non-implantation (NIS) segments of the uterus were separated and pooled together for each mouse. The segments were either fixed in 4% (w/v) paraformaldehyde in PBS for histological analysis or stored in RNA*later*® (Ambion, Foster City, CA) for RNA extraction at later time.

Ovariectomy and hormone treatments

Routine ovariectomy was performed on anesthetized adult females followed by recovery for two weeks before further treatments. 1 μg/ml 17β-estradiol (E2) and 10 mg/ml progesterone (P4) (Sigma-Aldrich, St. Louis, MO) in sesame oil were injected subcutanously alone or in combination into ovariectomized females (100 μl/mouse). Six hours later, the mice were sacrificed and uteri removed and stored appropriately.

RNA extraction and cDNA microarray

Whole uterine tissues from *Hoxa10*$^{-/-}$ or *Hoxa10*$^{+/-}$ mice were collected on 4.5 dpc and homogenized in RNA Stat-60 (Tel-Test, Inc., Friendswood, TX). Total RNA was extracted following the manufacturer's instructions. For microarray analysis, RNA samples were further purified by using Qiagen RNeasy kit (Qiagen, Valencia, CA) and total RNA quality was determined by Agilent 2100 bioanalyzer (Agilent Technologies) according to manufacturer's recommendations. First strand cDNA was generated by fluorophore/dendrimer specific oligo-dT primed reverse transcription (Superscript II; Invitrogen) utilizing the 3DNA Array 900 kit (Genisphere). For RNA expression level comparison, samples were paired (one *Hoxa10*$^{+/-}$, one *Hoxa10*$^{-/-}$). Two hybridizations for each sample pair were carried out in a sequential manner on the Mouse Exonic Evidence Based Oligonucleotide (MEEBO) chip following instructions from the manufacturer. Slides were scanned on a Perkin Elmer ScanArray Express HT scanner to detect Cyanine3 (Cy3) and 5 (Cy5) fluorescence. Laser power is kept constant for Cy3/Cy5 scans and photomultiplier tube (PMT) is varied for each experiment based on optimal signal intensity with lowest possible background fluorescence. A low PMT setting scan is also performed to recover signal from saturated elements. Gridding and analysis of images is performed using ScanArray v3.0 (Perkin Elmer). Three pairs of independent samples were analyzed.

Regular (RT-PCR) and quantitative (qRT-PCR) reverse transcription-polymerase chain reaction

Uterine cDNA was synthesized using SuperScriptII reverse transcriptase (Invitrogen, Carlsbad, CA). Primers used for PCR were designed using Primer3 online software [23] and are listed in Supplementary Table S1. PCR and qPCR were performed as described previously [24].

Results

Implantation failure due to *Hoxa10* deletion cannot be rescued by abolishing mature T and B cells

In a previous study, Yao and coworkers proposed that increased number of T cells in the *Hoxa10*$^{-/-}$ endometrium and aberrant uterine immunosuppression contributed to the failure of embryo implantation [21]. To test this hypothesis, we utilized the *Rag1*$^{-/-}$ mice, which do not produce any mature B or T cells due to disruption of the gene responsible for V(D)J recombination [25]. *Rag1*$^{-/-}$ mice exhibited no apparent fertility defects. *Rag1*$^{-/-}$ males were first bred to *Hoxa10*$^{+/-}$ females, and *Hoxa10*$^{+/-}$; *Rag1*$^{+/-}$ offspring were intercrossed to generate double-homozygous null and control females. Age-matched females were housed with stud males and their breeding history was documented and analyzed. As shown in Table 1, of the 11 control females studied, they gave birth to a total of 98 pups at term, averaging nearly 9 pups per litter. On the contrary, only 2 out of 8 *Hoxa10*$^{-/-}$ females gave birth, and the number of pups per litter was significantly ($P<0.0001$) lower than that of the controls (Table 1). *Hoxa10*$^{-/-}$*Rag1*$^{-/-}$ females did not exhibit significantly different birth rates and produced similar litter size as their *Hoxa10*$^{-/-}$ counterparts ($P=0.63$).

Homeotic transformation at the uterine-oviduct junction region was reported in the *Hoxa10*$^{-/-}$ females [20]. Similar morphology was

also observed in *Hoxa10*-/-; *Rag1*+/- and *Hoxa10*$^{-/-}$; *Rag1*$^{-/-}$ females (data not shown). This alteration resulted in narrowing and coiling of the anterior uteri into an oviduct-like structure, which could impede the passage of eggs, and complicated the study of implantation in these mutants. To bypass this obstacle, healthy blastocysts harvested from 3.5 dpc wild-type females were transferred directly to the anterior uterus just posterior to the transformed region, of 2.5 dpc pseudo-pregnant recipient females. All 8 control recipients were pregnant at 7.5 or 8.5 dpc, with 39 embryos successfully implanted out of a total of 49 blastocysts transferred (Table 2). However, only 2 of 5 *Hoxa10*$^{-/-}$ and 2 of 8 *Hoxa10*$^{-/-}$; *Rag1*$^{-/-}$ females exhibited implantation sites, respectively. This resulted in a significant (P<0.0001) reduction of embryos implanted in these mice compared to control mice. These results indicate that abolishing T and B cells from the endometrium cannot rescue the implantation defects observed in *Hoxa10*$^{-/-}$ mice and that failure of embryo implantation in these mutants could not be solely attribute to increased local maternal T cell abnormalities.

Differentially-Regulated Genes (DRGs) in Peri-implantation *Hoxa10*-/- Uteri Were Revealed by Microarray

To further explore the molecular basis for the implantation defects in *Hoxa10*$^{-/-}$ mice, we performed global gene profiling experiments using whole uterine tissues collected at 4.5 dpc in an effort to capture the changes in gene regulation in the implantation sites immediately after the onset of implantation. We were able to identify a total of 338 genes that are differentially regulated in the *Hoxa10* mutants. Among these genes, 101 and 237 genes were up-regulated and down-regulated in *Hoxa10*$^{-/-}$ uteri compared to controls, respectively. The DRGs were categorized according to the function of the encoded proteins, based on published literature. About 60% of genes that showed up-regulation in the *Hoxa10*$^{-/-}$ are those involved in immune and defense response (Figure 1), which is consistent with a previous report [21]. The repressed genes were mostly consisted of those encoding differentiation/structural and metabolism products. The complete list of DRGs was shown in Table S2.

To verify the microarray data, RT-PCR and qRT-PCR were employed to determine relative mRNA levels. RNA isolated from blue dye-stained implantation (IS) and unstained non-implantation (NIS) segments was used for 4.5 dpc control uteri, whereas whole uterine RNA had to be used for *Hoxa10*$^{-/-}$ mice, due to compromised blue dye reaction in those animals [20]. mRNA level of *Hoxa10* was examined by RT-PCR and qRT-PCR first as a control, and indeed was almost undetectable in the mutant uteri (Figure 2A). Figure 2 shows the qRT-PCR results of the relative mRNA levels of genes in uteri of 4.5 dpc mice. Complement component 1 s subcomponent (*C1s)*, which encodes a serine protease involved in the complement cascade [26], was barely detectable in the IS and NIS sites of the control *Hoxa10*$^{+/-}$ animals, but highly upregulated in the mutant *Hoxa10*$^{-/-}$ uterus, indicating an aberrant immune response in the mutant. Carboxypeptidase A3 (*Cpa3)* encodes a carboxypeptidase mostly found in mast cells [27], was also elevated in the mutant. As mast cells play critical roles in the inflammatory response, induction of *Cpa3* in *Hoxa10*-/- uteri is also consistent with an immune-associated abnormality in these mutants. Expression of *Greb1*, an estrogen-responsive gene, was low in the IS of the control uterus, high in NIS site and its expression in *Hoxa10*-/- uteri were comparable to that in NIS site. Its role during implantation was not clear. *Bmp2* was induced at 4.5dpc in control implantation sites, but not in *Hoxa10*-/-. A recent study demonstrated that *Bmp2* is critical for decidualization, as conditional knockout of the gene by PR-Cre resulted in complete infertility due to aberrant stromal cell proliferation and differentiation [12]. Canonical Wnt activity was detected in the uterus during peri-implantation period evidenced by the presence of TopGal reporter expression, along with several other components of Wnt pathway that were also found to be induced in the deciduas [12]. We uncovered that *Frzb*, a Wnt signaling antagonist, was elevated in control uteri specifically at the implantation sites, but failed to do so in *Hoxa10*-/- . Other genes that are up-regulated in the IS include *Ifi202b* (interferon activated gene 202B, previously reported to be induced by estrogen, [28]), *Mapk6* (component of the MAPKinase pathway), *Tnfaip2 (*tumor necrosis factor, alpha-induced protein 2, an angiogenic factor), *Atp12a* (an ATPase), *Dio3* (a deiodinase), and *Timp1* (inhibitor of the Metalloproteinase, Figure 2B and C). Their roles during implantation need further investigation.

Ovarian hormonal regulation of gene expression through *HOXA10*

To better understand the HOXA10-dependant gene regulation by ovarian hormones, we treated ovariectomized control (*Hoxa10*$^{+/+}$ or *Hoxa10*$^{+/-}$) and *Hoxa10*$^{-/-}$ mice with oil, 17 β-estradiol (E2), progesterone (P4) or combination of the two steroids, and collected uterine tissues 6 hours afterwards. RT-PCR results performed on whole uterine RNA extraction are shown in Figure 3. In the oil-treated control mice, the

Genotype of females mated with stud male	Total number of females examined	Number of females give birth at term	Total number of pups born	Average number of pups/ litter mean ± s.d.v.
Control*	11	11	98	8.91 ± 3.11
*Hoxa10-/-***	8	2	2	0.29 ± 0.46***
Hoxa10-/-; Rag1-/-	6	1	1	0.17 ± 0.38***

One or two adult females were housed with stud males. The date on which a vaginal plug observed was documented and considered 0.5 dpc. The number of pups born 18-20 days later was also documented. *Control group included 1 wild-type, 3 *Hoxa10*+/-, *2 Rag1*-/-, 3 *Hoxa10*+/-;*Rag1*+/- and 2 *Hoxa10*+/-;*Rag1*-/- mice. ***Hoxa10*-/- group included 6 *Hoxa10*-/- and 2 *Hoxa10*-/-;*Rag1*+/- mice. *** denotes P<0.0001 compared to control.

Table 1: Reproductive fecundity in control, *Hoxa10*-/- and *Hoxa10*-/-;*Rag1*-/- females.

Genotype of female recipient	Total number of females with implantation	Total number of embryos transferred	Total number of normal embryos at 7.5-8.5 dpc	Ave. percentage of successful implantation mean ± sdv %
Control*	8/8	49	39	81.48 ± 17.63
*Hoxa10-/-***	2/5	30	3	7.78 ± 10.83***
Hoxa10-/-; Rag1-/-	2/8	47	3	5.21 ± 9.90***

Blastocysts collected from 3.5dpc naturally-pregnant wild-type females were transferred to the distal uterine horn of 2.5dpc pseudo-pregnant recipient females, bypassing the homeotic-transformed region of the mutant uteri. 5 or 6 days later, recipients were sacrificed and uteri examined to determine the development of embryos. Successful implantation was defined as normal embryonic size and absence of hemorrhage at the above mentioned time points, *Control group included 1 wild-type, 5 *Hoxa10*+/-;*Rag1*+/- and 2 *Hoxa10*+/-;*Rag1*-/- mice. ***Hoxa10* -/- group included 3 *Hoxa10*-/- and 2 *Hoxa10*-/-; *Rag1*+/- mice. *** denotes P<0.0001 compared to control.

Table 2: Embryonic survival following blastocyst transfer to control, *Hoxa10*-/- and *Hoxa10*-/- ;*Rag1*-/- pseudo-pregnant females.

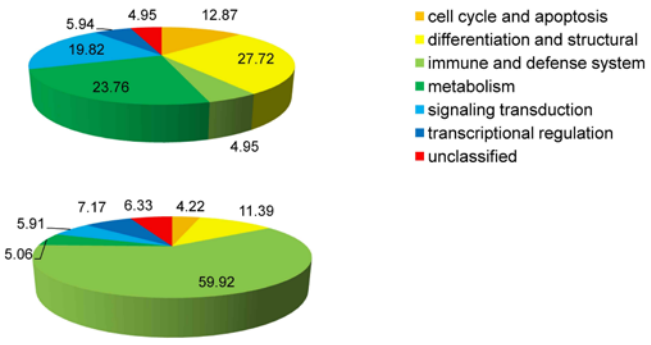

Figure 1: Pie-chart of classified differentally regulated genes (DRGs) either down- (upper panel) or up-regulated (lower panel) in *Hoxa10-/-* according to the biological functions of the gene products. 237 and 101 genes are down- or up-regulated more than 2-fold in *Hoxa10-/-* vs. control tissues at 4.5 dpc. Among the genes whose expression is repressed in *Hoxa10-/-*, about a quarter are those encoding structural proteins or involved in differentiation; another quarter encode proteins found in metabolic pathways. The majority of genes upregulated in *Hoxa10-/-* are involved in immune response.

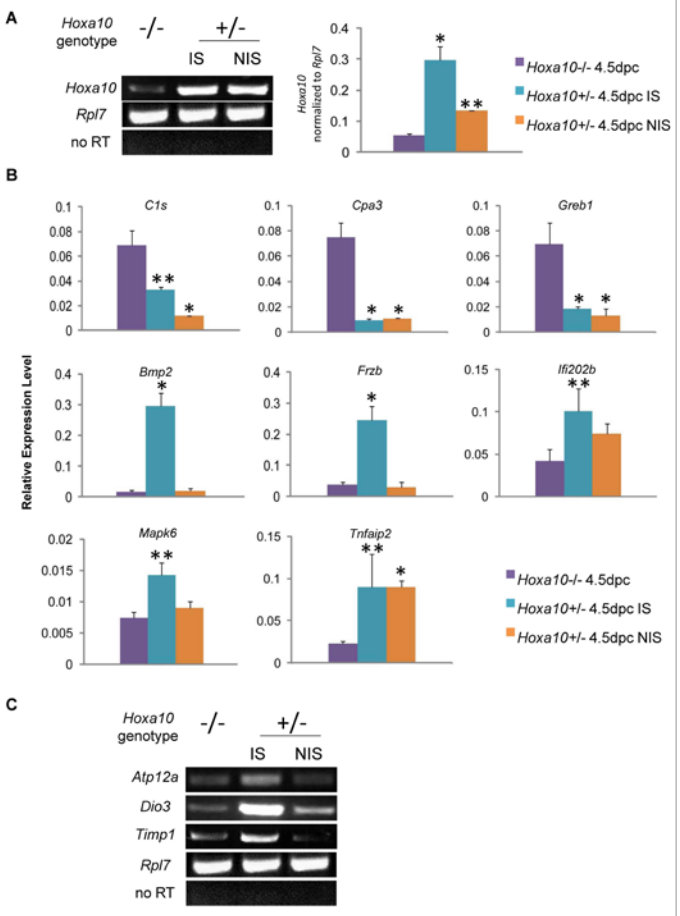

Figure 2: RT-PCR and real-time RT-PCR verification of the microarray data. A. Loss of expression of *Hoxa10* in *Hoxa10*-mutant is confirmed by RT-PCR and qRT-PCR. B. qRT-PCR of selected genes on 4.5dpc *Hoxa10-/-* and +/- uterine tissue. mRNA level was normalized to that of housekeeping gene, ribosomal protein L7 (*Rpl7*). C. RT-PCR results of some DRGs on 4.5dpc control and *Hoxa10-/-* uteri. * denotes P<0.01, and **denotes P<0.05, when compared to *Hoxa10-/-*.

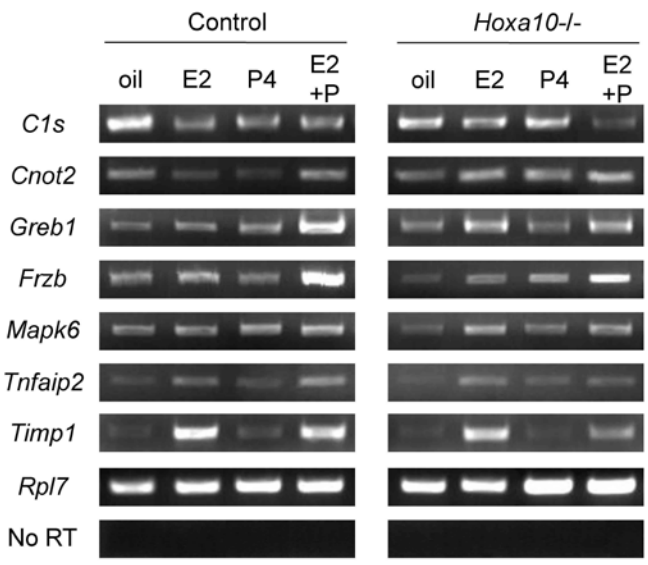

Figure 3: RT-PCR of some DRGs in ovariectomized, hormone-treated control or *Hoxa10* mutant uteri. Regulation of some DRGs by ovarian hormones is mediated through HOXA10 as expression pattern is different in *Hoxa10*-null uteri. *Rpl7* serves as a loading control and amplification with total RNA (No RT) serves as negative control.

high basal *C1s* mRNA level was repressed by any steroid treatments, whereas E2 or P4 alone but not the combination failed to repress C1s in *Hoxa10*$^{-/-}$ mutants. Similarly, *Cnot2* expression is repressed by either E2 or P4 in the controls, but not in the *Hoxa10* mutants. Expression of *Greb1* is dramatically increased in response to E2+P4 treatment which is not obvious in *Hoxa10* mutants. On the other hand, regulation of *Frzb*, *Mapk6, Tnfaip2*, and *Timp1* by E2 and P4 appears similar between control and *Hoxa10* mutant uterus. These data suggest that HOXA10 serves as an intermediate between ovary hormones and subsequent uterine cellular changes by transcriptionally regulating a subset of hormonally-regulated gene expression.

Discussion

The uterus is a reproductive organ developed specifically for the purpose of accepting and supporting the embryo and is essential for species survival. To prepare for the arrival and attachment of the blastocysts, the uterus has to go through a series of hormone-dependent changes which makes it receptive. This is mediated by dynamic activation of a complex genetic network, which is coordinated by estrogen and progesterone. One of the key regulators in this process is HOXA10. HOXA10 is a downstream target of progesterone in the uterus [10] and the fertility of *Hoxa10*-deficient female mice are severely compromised. The molecular mechanisms underpinning this phenotype remain elusive. The elevated uterine immune activity, evidenced by infiltration and proliferation of the lymphocytes, is speculated to be a major mechanism causing the phenotype. Our microarray analysis also revealed that elevated immune related gene expression is the most prominent molecular signature for *Hoxa10* mutant uteri. However, whether this hyperactive immune response is the main cause or a secondary effect of implantation failure remains unknown. In this study, using *Rag1*-deficient mice, we demonstrated that immune cell-deficient *Hoxa10*$^{-/-}$ mice fail to progress past the onset of implantation indicating that this altered T/B cell response alone is not sufficient to explain the infertility phenotype. It is plausible that

the immune system is activated in the mutant in response to a failed implantation process. However, one has to keep in mind that other than T/B cells, other immune cell types including macrophage/dendritic, mast and uterine natural killer cells may also be important mediators in the implantation processes [29-31]. Early studies suggested that uterine mast cells produce histamine, a critical molecule mediating blastocyst-endometrium interaction [32]. However, the role of mast cells during implantation has been challenged as mast-cell-deficient mice was able to produce live offspring [33]. Nevertheless, we observed *Cpa3*, a gene expressed in the mast cells, is downregulated rapidly during peri-implantation in the wild-type uterus (data not shown) but remains at high levels in *Hoxa10*-deficient mice, indicating failure of mast cells depletion. Thus, it is very likely that dysregulation of cellular immune response through cells other than T/B cells may account for the infertility phenotype in the *Hoxa10*-mutants.

To further explore other mechanisms underlying the implantation deficiency in *Hoxa10* mutants, we performed mRNA microarray analysis to determine differences in uterine gene expression between control and the mutants. In contrast to previous studies which used the ovariectomized hormone-treated model, we analyzed naturally conceived control and *Hoxa10*-deficient uteri at 4.5 dpc. We adopted this strategy because we believe that other factors such as signals from embryos and influences from other ovarian hormones other than P4 may also contribute to the uterine regulation. One caveat of our approach is that we also included embryos in the microarray analysis. However, we believe that this should not impact the outcome of the experiment because of the following reasons. First, blastocysts are very small and minimally contribute to the transcriptome of the whole uterus. Second, at 4.5 dpc, similar number of blastocysts was reported to be present in the uterine lumen of *Hoxa10*$^{-/-}$ mice and the majority of them were morphologically normal [20]. Finally, we used wild type male for mating and therefore all blastocysts carried at least one wild-type *Hoxa10* allele and are thus phenotypically normal. Thus, we reasoned that including embryos should not introduce differential gene expression to samples collected from mothers of different genotypes.

The microarray analysis in this study suggests that 337 genes are differentially expressed between the control and *Hoxa10-/-* uteri at 4.5dpc. Some of these genes were previously reported to play important roles during implantation. For example, Bmp2, is critical for the decidualization process and conditional ablation of the gene in the uterus renders the females completely infertile [12]. However, de-regulation of these genes may not be a direct result of loss of *Hoxa10* but rather an outcome of implantation failure. Nonetheless, we showed that among those DRGs, some showed abnormal response to E2 and/or P4 treatments in the absence of HOXA10. These data is in support of the notion that HOXA10 is critical for mediating hormonal responsiveness of the uterine cells.

It is also noteworthy that although the transformation of the anterior uterus is the only readily distinguishable phenotype at the gross morphological level, less obvious developmental defects at cellular level may also exist and play a role in the *Hoxa10*-mutant uterus. Therefore, we feel that generation of an inducible Cre-mediated *Hoxa10* knock-out mouse model would be beneficial to address this concern.

Acknowledgements

This work was funded by NIH R01HD41492 and R01ES016597 to L. M.

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This article was originally published in a special issue, **Reproduction & Developmental Biology** handled by Editor(s). Dr. Tin-Lap Lee, National Institutes of Health, USA