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Gene Expression Profiling Reveals Progesterone-Mediated Cell Cycle and Immunoregulatory Roles of *Hoxa-10* in the Preimplantation Uterus

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Human infertility and recurrent pregnancy loss caused by implantation defects are poorly understood. *Hoxa-10*-deficient female mice have severe infertility and recurrent pregnancy loss due to defective uterine implantation. Gene expression profiling experiments reveal that *Hoxa-10* is an important regulator of two critical events in implantation: stromal cell proliferation and local immunosuppression. At the time of implantation, *Hoxa-10* mediates the progesterone-stimulated proliferation of uterine stromal cells. *Hoxa-10* mutants express a stromal cell proliferation defect that is accompanied by quantitative or spatial alterations in the expression of two cyclin-dependent kinase inhibitor genes, *p57* and *p15. Hoxa-10* deficiency also leads to a severe local immunological disturbance, characterized by a polyclonal proliferation of T cells, that occurs in place of the normal progesterone-mediated immunosuppression in the periimplantation uterus. (*Molecular Endocrinology* 17: 610–627, 2003)

INFERTILITY AFFECTS MORE than 6.1 million women and their partners in the United States (1). Implantation failure due to intrinsic uterine defects is thought to span a wide clinical spectrum including spontaneous abortion, unexplained infertility, and recurrent pregnancy loss. Gene targeting in mouse models has identified several genes that are critical for implantation (reviewed in Refs. 2 and 3). A complex series of synchronized molecular interactions occur within the uterus before implantation, and between the uterus and the implanting blastocyst, both of which are required for successful implantation.

At the same time, gene targeting studies show that implantation of a healthy embryo can fail due to a defective uterine environment. Priming of the uterine stroma by progesterone (P4) is essential for the establishment of an appropriate uterine environment for implantation. Although knowledge of the molecular pathways that act downstream of P4 in implantation is limited, one gene that is both P4 responsive and required for the establishment of an appropriate environment for embryo implantation in the mouse uterus is *Hoxa-10*, a member of the *AbdB* subclass of *Hox* genes (4–6). Although *Hox* genes are well known as regulators of patterning in both vertebrate and invertebrate embryonic development, *Hoxa-10*-deficient adult female mice exhibit a severe failure of implantation and defective decidualization that lead to recurrent pregnancy loss and infertility (6).

The uterus is comprised of three major cellular compartments: epithelium, stroma, and myometrium, which are under differential hormonal regulation by ovarian estrogens and P4 (2, 3). In murine reproductive physiology, the major preovulatory ovarian estrogen, estradiol (E2), stimulates the uterine epithelium on d 0.5 to 1.5 post coitum (p.c.), whereas P4 stimulation of the uterine stroma is first evident by d 2.5 p.c. On d 3.5 p.c., estrogen levels rise again so that the uterine stroma has been sequentially primed by estrogen and P4 and is now regulated by both hormones simultaneously (2).

Hoxa-10 is up-regulated by P4 in the uterine stroma at d 3.5 p.c., 24 h before implantation, and its expression persists in the developing decidua (5, 7). Moreover, previous studies have shown that *Hoxa-10* is required for P4 responsiveness in the uterine stroma (8). For example, in a model in which *Hoxa-10* mutant females are ovariectomized to eliminate variability in hormonal cycling between animals, and then treated with estrogen and P4, stromal cell proliferation is impaired, whereas epithelial cell proliferation is unaf-

Abbreviations: BrdU, Bromodeoxyuridine; cdk, cyclin-dependent kinase; CKI, cyclin-dependent kinase inhibitor; DRG, differentially regulated gene; E2, estradiol; EST, expressed sequence tag; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; P4, progesterone; p.c., post coital; PR, progesterone receptor; PRKO, PR knockout; RT, room temperature; SAM, statistical analysis of microarrays; SOM, self-organizing map; USCs, uterine stromal cells.

fected. In this assay, exogenous P4 and estrogen greatly amplify the magnitude of the basal uterine stromal proliferation defect in ovariectomized *Hoxa-10* mutants relative to wild type (8).

Hox genes have been proposed to act as local regulators of cell proliferation during development (9, 10), but molecular effectors for their proposed cell cycle-regulatory function have not been identified. Based on the observed proliferation defect in the *Hoxa-10* mutant stroma, we reasoned that P4 administration to ovariectomized wild-type and *Hoxa-10*-deficient females should amplify differences in gene expression that underlie the *Hoxa-10* mutant stromal proliferation defect. We therefore employed global gene expression profiling to identify *Hoxa-10* downstream genes that mediate the stimulatory effect of P4 on cell cycle progression in the uterine stroma. At the same time, we sought to gain insight into other P4-dependent downstream events that contribute to the defective implantation phenotype in *Hoxa-10* mutant females.

Although implantation depends upon the precisely coordinated effects of estrogen and P4, to study the effects of one steroid hormone at a time, we initially chose to focus only on the gene expression changes produced by P4. However, to establish physiological relevance of findings derived from an ovariectomy model to normal implantation, we then further evaluated the results in a combined E2 + P4 context, d 3.5 p.c. of natural pregnancy.

RESULTS

Experimental Design: General Considerations

To identify genes that are differentially expressed between wild-type and *Hoxa-10* mutants, RNA was collected from uteri of ovariectomized female mice 6 h after P4 injection (Fig. 1A). In the ovariectomy model, denoted OVX/P4/6 h, in which ovarian production of endogenous P4 and E2 is absent, P4 injection permits the study of differential gene expression specific to the mediation of the P4 response by *Hoxa-10*. To extend the previous finding that *Hoxa-10* mRNA expression is significantly up-regulated in the uterine stroma 6 h after P4 injection (6), we performed Western blot analyses for Hoxa-10 protein expression at 0, 1, 3, 6, 9, 12, 18, and 24 h after P4 injection. Hoxa-10 protein was up-regulated in response to P4 and reached its peak expression 6 h after injection (Fig. 1B). Hoxa-10 protein levels are stable at the later time points, declining by 24 h (data not shown). The 6-h time point was therefore chosen to optimize the identification of immediate or near-immediate targets of *Hoxa-10*. Compared with the physiological periimplantation uterus, the proportion of stroma to epithelium is greater in the OVX/P4/6 h model. Thus, the strategy chosen was designed to maximize the yield of *Hoxa-10* downstream genes in the uterine stroma.

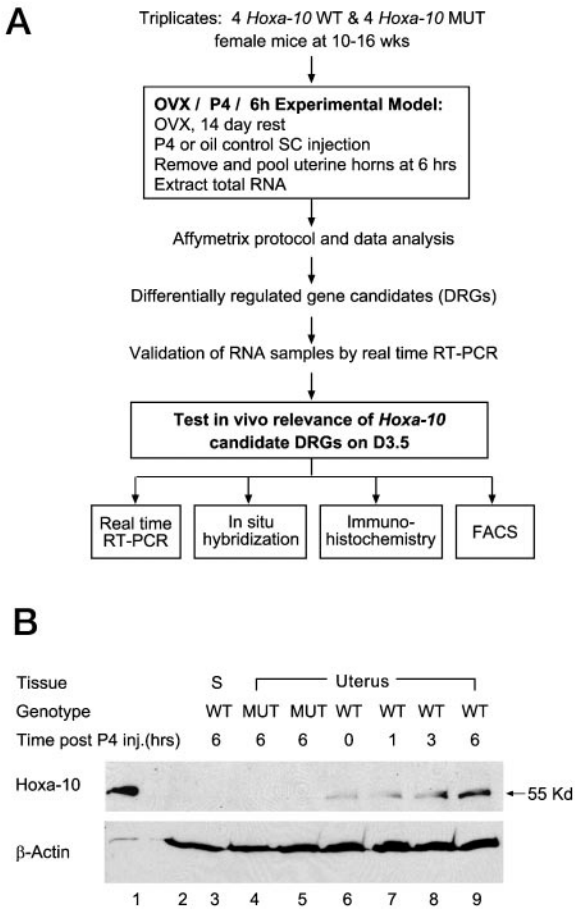

Fig. 1. Experimental Strategy

A, Protocol for gene expression profiling and experimental evaluation of DRGs in the *Hoxa-10* mutant uterus. B, Total uterine protein extracts collected at 0, 1, 3, and 6 h after P4 injection in ovariectomized wild-type mice show significant up-regulation of Hoxa-10 protein (~55 kDa) by 6 h. Subsequent levels were constant, declining by 24 h (not shown). Lane 1, Positive control (10 μg of protein extract from U2OS cells transfected with *Hoxa-10* expression plasmid driven by cytomegalovirus promoter); lane 2, no sample loaded; lanes 3–9 were each loaded with 25 μg of protein extract except for lane 5 (40 μg). WT, Wild type; MUT, *Hoxa-10* mutant; S, spleen.

Because we sought to study the function of *Hoxa-10* in the preimplantation uterus, we then further tested the *in vivo* relevance of select candidate downstream genes derived from the OVX/P4/6 h model in the context of normal pregnancy. This was done in d 3.5 p.c. pregnant mice by using quantitative real time RT-PCR, *in situ* hybridization, flow cytometry, and bromodeoxyuridine (BrdU) incorporation experiments (Fig. 1A). We chose d 3.5 p.c. as it represents an early physiological time point in the response of the uterus to P4 and coincides with the onset of *Hoxa-10* expression in the uterine stroma. Furthermore, it precedes the earliest observable implantation defect, the failure of embryo attachment to the uterus, which normally occurs late on d 3 p.c. Therefore, to a first approximation,

differential gene expression occurring at d 3.5 p.c. is less likely to reflect a failure of implantation and more likely to reflect changes in gene expression that contribute to the implantation defect in the *Hoxa-10* mutant.

Gene Expression Profiling Detects Differentially Regulated Genes (DRGs) in the *Hoxa-10* Mutant Uterine Stroma

Gene expression profiles of more than 12,000 genes (6,000 known genes and 6,000 expressed sequence tags) represented on the U74Av.2 oligonucleotide array (Affymetrix, Santa Clara, CA) were first analyzed by dChip (11, 12). All subsequent analyses were performed on dChip model-based expression indices. The fold difference cutoff in all analyses was 1.5-fold unless otherwise specified. Independent analyses using Statistical Analysis of Microarrays (SAM) and conventional *t* test (13, 14) yielded 92 and 81 genes, respectively, that were differentially expressed between wild-type and *Hoxa-10* mutant uteri in the OVX/6 h/P4 model (Table 1). A total of 57 genes were identified by both SAM and *t* test. The high degree of overlap (>50%) between these two analytical methods, which are based on very different statistical assumptions, points to the robustness of the data analysis. Combining the gene lists obtained by SAM and *t* test yielded a total of 116 differentially regulated genes, or DRGs, and all subsequent data analyses and experiments were based on these 116 DRGs (Table 1).

Of the 116 DRGs, 26 genes were more highly expressed in the wild-type uterus, whereas 90 genes were more highly expressed in the *Hoxa-10* mutant uterus [Tables 2 and 3, Refs. 15 and 16, and see the supplemental data for complete list of data and their corresponding GEO accession numbers (Ref. 12 and published on The Endocrine Society's Journals Online web site, http://mend.endojournals.org)]. The preponderance of up-regulated DRGs in the *Hoxa-10* mutant uterus in response to P4 was robust and pertained regardless of the threshold fold difference. This result suggests that the predominant downstream effect of *Hoxa-10* in this tissue is repressive, or that *Hoxa-10* up-regulates transcriptional repressors. None of the DRGs were significantly differentially regulated in control experiments in which oil vehicle rather than P4 was injected (data not shown; Refs. 15 and 17). The functional categories represented by the DRGs and the number of DRGs in each category are listed in Table 4. The five predominant functional categories of DRGs relate to: 1) immune, complement, and chemokine functions; 2) enzyme function or metabolism; 3) adipocyte function, fat metabolism, and energy balance; 4) transcriptional regulation; and 5) cell cycle control and cell proliferation. A panel of eight DRGs representing different functional categories was quantitatively validated by real-time RT-PCR. Consistent with the microarray data, *Hoxa-11*, *Follistatin,* and *Slap* were more highly expressed in wild type relative to *Hoxa-10* mutant uterus, whereas *Adipsin*, *Inhibitor of DNA binding-1*, *Lactotransferrin*, *Gas6,* and *p57* were more highly expressed in the *Hoxa-10* mutant (12). In addition, the stromal expression of 14 DRGs, including five of the above-mentioned genes, was either demonstrated by *in situ* hybridization experiments or ascertained from the literature (12). Thus, the experimental strategy was successful in enriching for stromally expressed *Hoxa-10*-dependent DRGs in the preimplantation uterus.

Hoxa-11 Is Coregulated by *Hoxa-10* and P4

As *Hoxa-11* is known to be up-regulated by P4 (7), we independently analyzed all RNA samples by real-time RT-PCR analysis for *Hoxa-11* expression to determine whether its expression is altered in the *Hoxa-10* mutant uterus. Compared with wild-type mice injected with oil, mice injected with P4 showed significantly higher *Hoxa-11* expression levels at each time point analyzed, with peak expression occurring at 6 h (Fig. 2A). Notably, however, the induction of *Hoxa-11* expression was markedly attenuated in *Hoxa-10* mutant uteri compared with wild type. In four independent real-time RT-PCR experiments, each conducted in triplicate and consisting of pooled uterine RNA from four wild-type and four mutant mice, the mean normalized *Hoxa-11* expression level was 2.5 ± 0.2 (mean ± SEM) in wild type, compared with 1.5 ± 0.1 in *Hoxa-10* mutants (Fig. 2B). This attenuation in *Hoxa-11* expression in the *Hoxa-10* mutant 6 h after P4 injection was highly significant ($P = 0.01$; two-tailed *t* test). Therefore, as suggested by the microarray analysis (Table 2) and confirmed by real-time RT-PCR, the

Table 1. Summary of DRG Identification by SAM and *t* Test

	No. of Genes Identified by			
	SAM	*t* Test	Both SAM and *t* Test	SAM or *t* Test
Higher expression in wild type	15	23	12	26
Higher expression in mutant	77	58	45	90
Total no. DRGs[a]	92	81	57	116

[a] The total number of 116 DRGs was represented by 119 probe sets because in three cases, two probe sets represented the same DRG.

Table 2. Genes that Have Significantly Higher Uterine Expression Levels in the Wild Type Compared with *Hoxa-10* Mutant at 6 h after P4 Injection in Ovariectomized Mice (15, 16)

Probe Set ID	Gene Name	Statistical Tests	
		Fold Change by SAM	Paired *t* Test
98817_at	Follistatin	[a]4.91	[a]
92970_at	Homeobox A10	[a]3.49	[a]
99926_at	Polymeric Ig receptor	[a]2.68	
98577_f_at	Endogenous retroviral sequence 4 (with leucine tRNA primer)	[a]2.54	[a]
93860_i_at	Mouse endogenous murine leukemia virus modified polytropic provirus[b]	[a]2.48	[a]
93861_f_at	Mouse endogenous murine leukemia virus modified polytropic provirus[b]	[a]2.22	[a]
100381_at	Actin, α1, skeletal muscle	[a]2.11	[a]
92796_at	Alkaline phosphatase 2, liver	[a]2.04	[a]
92852_at	Fibronectin 1	1.82	[a]
99637_at	Procollagen, type XV	1.81	[a]
101900_at	Cdk inhibitor 2B (p15, inhibits Cdk4)	[a]1.75	[a]
161670_f_at	EST *Mus musculus* cDNA, GB no. AV140884	[a]1.73	[a]
100064_f_at	Gap junction membrane channel protein α1	[a]1.72	[a]
96602_g_at	EST *M. musculus* cDNA, 3 end/clone = UI-M-BH1-akt-a-08-0-UI	1.70	[a]
161595_at	EST *M. musculus* cDNA, GB No. AV292586	[a]1.66	[a]
104480_at	Cathepsin L	1.65	[a]
93873_s_at	Homeobox A11, opposite strand transcript	[a]1.63	[a]
102259_at	3-Monooxygenase/tryptophan 5-monooxygenase activation protein, γ	[a]1.63	
103049_at	*M. musculus* N-myc gene, 3 end; MoMuLV-like endogenous provirus	1.63	[a]
101294_g_at	Glucose-6-phosphate dehydrogenase 2	1.62	[a]
104021_at	Homeobox A11	[a]1.61	[a]
160899_at	Purkinje cell protein 4	1.59	[a]
101707_at	Alcohol dehydrogenase family 1, subfamily A7	1.56	[a]
160376_at	EST *M. musculus* cDNA, clone = UI-M-BH2.2-aqm-e-10-0-UI	1.56	[a]
95246_at	EST *M. musculus* cDNA, GB no. AA516958	[a]1.51	
102637_at	TGFβ receptor III	1.48	[a]
160923_at	EST *M. musculus* cDNA, clone = IMAGE-1478803	1.45	[a]

These genes have significantly different uterine expression levels in *Hoxa-10* wild type compared to mutant in the OVX/6h/P4 model by paired analysis in SAM, conventional *t* test, or both. EST, Expressed sequence tag.

[a] Statistical significance based on the following criteria: *t* test, fold change of at least 1.5, $P \leq 0.1$, an absolute difference in expression of at least 75 units; SAM, fold change of at least 1.5 in paired testing and median false detection rate of 10%.

[b] Two probe sets representing the same gene were identified.

uterine stromal expression of *Hoxa-11* is positively regulated, directly or indirectly, by *Hoxa-10* as well as by P4.

Identification of Additional Genes that Are Coregulated by *Hoxa-10* and P4

We next tested the DRGs for further evidence of coregulation by using a time series model, in which uteri were collected from ovariectomized wild-type mice at 0, 1, 3, 6, 9, 12, 15, 18, and 24 h after P4 injection (15, 18, 18a). In contrast to the OVX/P4/6 h model in which genotype (wild type *vs.* mutant) was the variable, in the time series model, genotype was constant (wild type only), whereas the time after P4 injection exposure varied. A self-organizing map (SOM) algorithm (19) was used to cluster genes that showed sufficient variation in expression during the time series. The criteria for sufficient variation were: 1) the minimum fold change in expression level between at least two time points is greater than 2; and 2) the minimum difference in expression level between at least two time points is greater than 75 U; 1675 genes fulfilled the inclusion criteria and were grouped into 18 clusters. The SOM algorithm arranged the clusters as nodes in a 3×6 grid configuration, with the physical distance between two clusters on the grid reflecting the degree of similarity of their expression profiles. As a result, adjacent clusters are more similar than nonadjacent clusters. The 3×6 arrangement of these 18 SOM clusters and their respective gene lists can be viewed in the supplemental data (Ref. 12 and published on The Endocrine Soceity's Journals Online web site, http://mend.endojournals.org).

Four such clusters are shown in Fig. 3. Cluster 3 demonstrates the consistency of the data and the power of this analytical method in clustering coregulated genes. Of the genes in cluster 3, 78% (40/51) consisted of *Ig* genes, which are B lymphocyte specific (Fig. 3A). Interestingly, there was no apparent difference in the regulation of these *Ig* genes between wild type and mutant. Nonetheless, the striking coordinate down-regulation of *Ig* genes in response to P4 indicates that major changes in the dynamics of intra-

Table 3. Genes that Have Significantly Higher Uterine Expression Levels in the *Hoxa-10* Mutant Compared with Wild Type at 6 h after P4 Injection in Ovariectomized Mice (15, 16)

Probe Set ID	Gene Name	Statistical Test	
		Fold Change by SAM	Paired *t* Test
160375_at	EST *M. musculus* cDNA, GB no. AJ006474	[a]3.09	[a]
103238_at	Wingless-related MMTV integration site 4	[a]3.00	[a]
94057_g_at	Cluster Incl M21285:Stearoyl-coenzyme A desaturase 1[b]	[a]2.67	[a]
99671_at	Adipsin	[a]2.61	[a]
101115_at	Lactotransferrin	[a]2.46	[a]
99104_at	Adipocyte complement related protein of 30 kDa	[a]2.42	[a]
102798_at	Adrenomedullin	[a]2.41	[a]
94056_at	Cluster Incl M21285:Stearoyl-coenzyme A desaturase 1[b]	[a]2.38	[a]
160852_at	Keratin complex 1, acidic, gene 15	[a]2.37	[a]
97402_at	Thioether *S*-methyltransferase	[a]2.08	[a]
102823_at	Ig heavy chain 3 (serum IgG2b)[b]	[a]2.08	
93996_at	Cytochrome P450, 2e1, ethanol inducible	[a]2.07	[a]
161354_f_at	EST *M. musculus* cDNA, GB no. AV161034	[a]2.00	[a]
99369_f_at	Ig kappa light chain variable region precursor (Vk10c) gene	[a]1.97	
103538_at	DNA segment, Chr 5, ERATO Doi 189, expressed	[a]1.92	[a]
92805_s_at	ADP-ribosylation-like 4	[a]1.86	
102824_g_at	Ig heavy chain 3 (serum IgG2b)[b]	[a]1.86	
160173_at	EST *M. musculus* cDNA, GB no. AI852838	[a]1.86	
93855_at	Rad and gem related GTP-binding protein	[a]1.82	[a]
100334_f_at	Kallikrein 13	[a]1.81	[a]
93515_at	Cadherin 16	[a]1.78	
96088_at	N-myc downstream regulated 2	[a]1.78	[a]
101947_at	Neighbor of A-kinase-anchoring protein 95	[a]1.72	[a]
102725_at	Potassium voltage-gated channel, shaker-related subfamily, β-member 1	[a]1.72	
104469_at	Glycoprotein 38	[a]1.72	[a]
93198_at	Colony-stimulating factor 3 receptor (granulocyte)	[a]1.67	
98792_at	EST *M. musculus* cDNA, GB no. W51672	[a]1.67	[a]
93486_at	Solute carrier family 27 (fatty acid transporter), member 1	[a]1.66	
104242_f_at	EST *M. musculus* cDNA, GB no. AI835622	[a]1.65	
96765_at	Paternally expressed gene 3	[a]1.65	
98453_at	FMS-like tyrosine kinase 1	[a]1.64	
101578_f_at	Actin, β, cytoplasmic	[a]1.64	
95356_at	Apolipoprotein E	[a]1.63	[a]
103819_at	Src homology 2 domain-containing transforming protein D	[a]1.63	
97950_at	Xanthine dehydrogenase	[a]1.62	[a]
100050_at	Inhibitor of DNA binding 1	[a]1.61	[a]
100998_at	Histocompatibility 2, class II antigen A, β1	[a]1.61	[a]
95471_at	cdk inhibitor 1C (P57)	[a]1.60	[a]
95759_at	Stearoyl-coenzyme A desaturase 2	[a]1.60	[a]
93615_at	Pre-B-cell leukemia transcription factor 3	[a]1.60	
96825_at	EST *M. musculus* cDNA, GB no. AI854794	[a]1.60	[a]
99481_at	ATPase, Na^+/K^+ transporting, α2 polypeptide	[a]1.60	
101969_at	Neuroblastoma, suppression of tumorigenicity 1	[a]1.60	[a]
98960_s_at	UDP-Gal:betaGlcNAcβ1,3-galactosyltransferase, polypeptide 3	[a]1.59	[a]
100069_at	Cytochrome P450, 2f2	[a]1.59	[a]
99187_f_at	EST *M. musculus* cDNA, GB no. AI835662	[a]1.59	[a]
103954_at	Rat generating islet-derived, mouse homolog 3α	[a]1.59	[a]
160373_i_at	EST *M. musculus* cDNA, GB no. AI839175	[a]1.59	[a]
95082_at	IGF binding protein 3	[a]1.58	[a]
96907_at	DNA segment, Chr 8, Wayne State University 96, expressed	[a]1.58	
161610_at	N-myc downstream regulated 2	[a]1.57	[a]
102157_f_at	Ig V-κ 10-Ars-A kappa chain gene, complete cds	[a]1.57	
102371_at	Nuclear receptor subfamily 4, group A, member 1	[a]1.57	[a]
92836_at	EST *M. musculus* cDNA, 5 end/clone = IMAGE-1316437	1.56	[a]
93015_at	Glutathione *S*-transferase, α3	[a]1.56	
99828_at	EST *M. musculus* cDNA, GB no. AA673252	[a]1.56	

Table 3. Continued

Probe Set ID	Gene Name	Statistical Test	
		Fold Change by SAM	Paired *t* Test
95019_at	Glutathione-*S*-transferase, θ1	[a]1.56	[a]
101082_at	Malic enzyme, supernatant	[a]1.55	
100153_at	Neural cell adhesion molecule	[a]1.55	
160905_s_at	Hypothetical protein MNCb-2875	[a]1.55	
97548_at	FK506-binding protein 3 (25kD)	[a]1.55	
96592_at	Phosphatidylinositol 3-kinase, p85α regulatory subunit	[a]1.53	[a]
98790_s_at	Myeloid ecotropic viral integration site 1	[a]1.53	[a]
100431_at	Leptin receptor	[a]1.53	[a]
161310_at	EST *M. musculus* cDNA, GB no. AV079187	[a]1.53	
92245_at	EST *M. musculus* cDNA, GB no. AI642262	[a]1.52	
96038_at	Ribonuclease, RNase A family 4	[a]1.52	[a]
97835_at	EST AI303516	[a]1.52	[a]
101593_at	Nudix (nucleotide diphosphate linked moiety X)-type motif 3	[a]1.52	[a]
98868_at	Cluster Incl L31532:B-cell leukemia/lymphoma 2	[a]1.51	
99067_at	Growth arrest specific 6	[a]1.51	[a]
102744_at	T cell receptor γ, variable 4	[a]1.51	
160306_at	Thyroid hormone responsive SPOT14 homolog (Rattus)	[a]1.51	
160841_at	D site albumin promoter binding protein	[a]1.51	
97317_at	Ectonucleotide pyrophosphatase/phosphodiesterase 2	[a]1.50	[a]
104000_at	EST *M. musculus* cDNA, GB no. AI181346	[a]1.50	[a]
104285_at	3-Hydroxy-3-methylglutaryl-Coenzyme A reductase	[a]1.50	
104601_at	Thrombomodulin	[a]1.50	
92366_at	Laminin, α2	[a]1.50	
160564_at	Lipocalin 2	[a]1.49	[a]
92534_at	GTP binding protein (gene overexpressed in skeletal muscle)	[a]1.48	[a]
99603_g_at	TGFβ inducible early growth response	1.48	[a]
104516_at	Claudin 5	1.48	[a]
104716_at	Retinol binding protein 1, cellular	1.48	[a]
160834_at	EST *M. musculus* cDNA, clone = UI-M-AP1-agn-a-04-0-UI	1.48	[a]
161907_s_at	Tenascin X	1.47	[a]
93527_at	Kruppel-like factor 9	1.47	[a]
104217_at	EST *M. musculus* cDNA, clone = UI-M-BH1-akt-a-10-0-UI	1.47	[a]
93330_at	Aquaporin 1	1.46	[a]
95693_at	Isocitrate dehydrogenase 2 (NADP+), mitochondrial	1.46	[a]
96148_at	EST *M. musculus* cDNA, clone = UI-M-AL1-ahk-f-10-0-UI	1.46	[a]
104728_at	Protein S (α)	1.44	[a]

[a] Statistical significance based on the following criteria: *t* test, fold change of at least 1.5, $P \leq 0.1$, an absolute difference in expression of at least 75 units; SAM, fold change of at least 1.5 in paired testing and median false detection rate of 10%.
[b] Two probe sets representing the same gene were identified.

uterine B cell trafficking, *Ig* gene expression, or both, occur in response to P4.

Of the 18 time series SOM clusters, 3 contain a significantly large number of DRGs, based on calculations using a hypergeometric distribution, followed by Bonferroni correction for multiple tests (Table 5, Fig. 3B, and Refs. 12 and 20). Clusters 1, 5, and 6 are significantly enriched with 12, 7, and 15 DRGs, respectively, ($P < 0.003$; Fig. 3B). The *P* values calculated for the enrichment of each cluster are listed in Table 5. Interestingly, whereas cluster 3 consists predominantly of genes of the *Ig* family, clusters 1, 5, and 6 contain genes with diverse functions. The enrichment of DRGs in clusters derived from the P4 time series model reflects their coregulation by Hoxa-10 and P4 and further supports the premise that the DRGs identified in the OVX/P4/6 h model are relevant to implantation.

Hoxa-10 Alters Expression of the Cyclin-Dependent Kinase Inhibitor (CKI) Genes *p57(Kip2)* and *p15(Ink4b)*

To further evaluate the *in vivo* relevance of DRGs identified in the OVX/P4/6 h model, we tested specific DRGs for their differential regulation in wild-type and *Hoxa-10* mutant uteri at d 3.5 p.c. of pregnancy. Interestingly, the expression of two CKI genes, *p57(Kip2)* and *p15(Ink4b)* was altered, either quantitatively or qualitatively, in the d 3.5 p.c. *Hoxa-10* mutant uterus. Although *in situ* hybridization revealed that *p57* was diffusely expressed throughout both d 3.5 wild-type and mutant stroma (data not shown), quantitative real-time RT-PCR indicated that *p57* transcripts were increased by 6.6 ± 1.8-fold (mean ± SEM; $P < 0.08$) in

Table 4. Functional Categories of DRGs

DRGs (n)	Functional Category
16	Immune function/complement system/cytokine-related
16	Enzyme/metabolism
13	Adipocyte function/fat metabolism/energy balance
9	Transcription regulation/transcription factors
8	Cell cycle/cell proliferation
7	TGFβ signaling pathway
6	Ras-related small GTP-binding proteins/GTPase superfamily
2	Cell-cell junction
4	Cell adhesion/integrin
6	Signaling protein/mesenchymal-epithelial signaling
3	Cytoskeletal/structural
3	Ion or water channels/regulation of intracellular osmotic content
1	Golgi function/regulation of intracellular transport/vesicular transport
1	Nuclear/DNA-binding proteins
1	Iron utilization
1	Protease
2	Murine virus
1	Novel gene
1	Apoptosis inhibitor
1	Blood clotting factor
44	Unknown genes/ESTs

DRGs were classified according to their described functions based on literature search on PubMed. A DRG was placed in more than one category if multiple functions have been described. See supplemental data (Ref. 12 and http://mend.endojournals.org/) for complete list of DRGs in each functional category.

the mutant (Fig. 4A), consistent with microarray results of the OVX/P4/6 h model.

On the other hand, although microarray analysis suggested that *p15* expression in the OVX/P4/6 h model was decreased in the mutant, this result was not confirmed by real-time RT-PCR analysis of d 3.5 p.c. uterine RNA. Instead, these analyses showed only a nonsignificant increase in *p15* expression in mutant relative to wild type (Fig. 4A). Nonetheless, to our surprise, *in situ* hybridization showed that the spatial expression pattern of *p15* was clearly altered in the *Hoxa-10* mutant at d 3.5 p.c. (Fig. 4B). In contrast to the restricted wild-type expression of *p15* in the submyometrial stroma and myometrium, in the *Hoxa-10* mutant uterus, *p15* was expressed throughout the entire stroma (Fig. 4B). Similar results were observed in the OVX/P4/6 h model (data not shown). Collectively, these results provide a link between *Hox* gene function and the expression of cell cycle-regulatory genes and provide a potential molecular correlate to the cell proliferation defect previously observed in the *Hoxa-10* ovariectomy model after combined E2 plus P4 treatment (8).

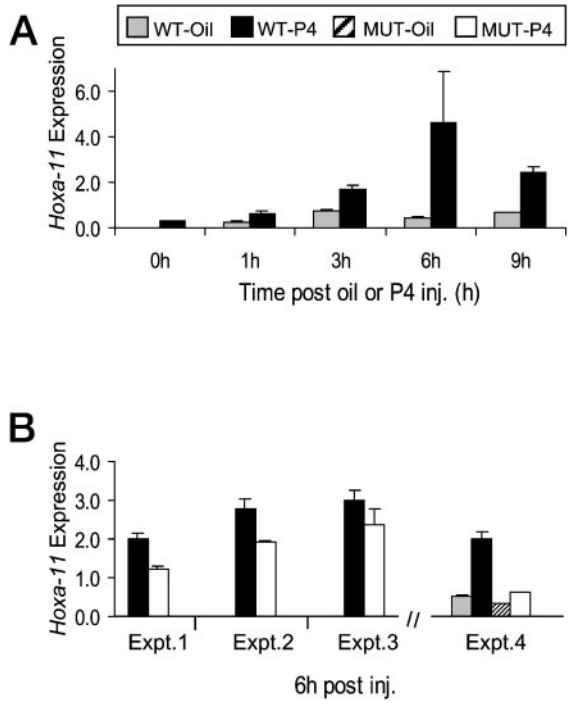

Fig. 2. Coregulation of *Hoxa-11* Expression by Hoxa-10 and P4

A, *Hoxa-11* expression in response to oil and P4 injections in wild-type mice at 0, 1, 3, 6, and 9 h. The differences in *Hoxa-11* expression in P4-injected compared with oil-injected mice are 2.3-, 2.2-, 10.5-, and 3.6-fold, respectively ($P < 0.05$ for each time point). B, Attenuation of *Hoxa-11* expression levels in the *Hoxa-10* mutant mice at 6 h after P4 injection. In four independent experiments, *Hoxa-11* was more highly expressed in the wild type than *Hoxa-10* mutant by 1.6-, 1.5-, 1.3-, and 3.1-fold, respectively. Overall, wild-type *Hoxa-11* expression was 1.9-fold higher than mutant (2.5 ± 0.2 *vs.* 1.5 ± 0.1; mean $\pm$ SEM; $P = 0.01$). In all *bar graphs*, each *bar* represents the mean of triplicate measurements of a sample comprised of pooled uterine RNA from four mice of the indicated genotype. *Error bars* for data points with mean $\pm$ SEM < 0.05 are not visible. All gene expression levels in Fig. 2 were normalized to *Rpl-7* expression levels. Student's two-tailed *t* test was used to test for statistical significance, and paired *t* test was used when appropriate.

Uterine Stromal Cell (USC) Proliferation Is Inhibited in *Hoxa-10* Mutants

To test whether stromal cell proliferation was disrupted on d 3.5 p.c. of natural pregnancy in the *Hoxa-10* mutant uterus, we injected d 3.5 p.c. pregnant wild-type and mutant mice with BrdU, and 4 h later, performed immunostaining on freshly isolated USCs with anti-BrdU antibody. The immunostained cells were then analyzed by flow cytometry. In wild-type mice, $8.9\% \pm 0.1\%$ (mean $\pm$ SEM) of USCs were $BrdU^+$, with 5% background fluorescence in unstained control cells. However, in four replicate experiments in the *Hoxa-10* mutant, only $5.7 \pm 0.2\%$ of USCs were $BrdU^+$ (Fig. 4C). The decrease in the proportion of stromal cells that incorporated BrdU

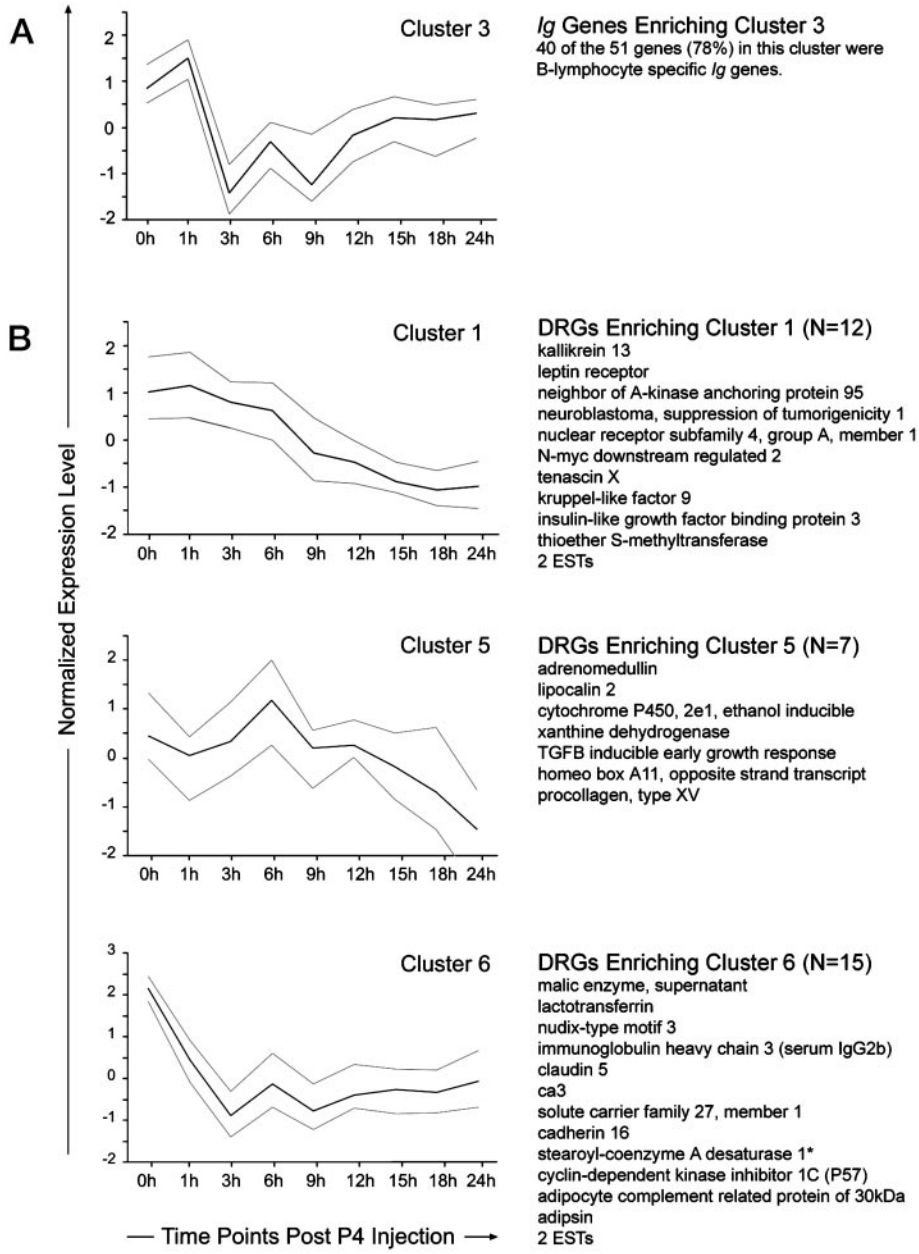

Fig. 3. Representative Clusters from SOM Analysis of P4 Time Series

A, SOM cluster 3 from the wild-type time series showed significant enrichment of Ig genes. Forty of the 51 genes (78%) in this cluster were Ig genes expressed by B lymphocytes. The results demonstrate powerful clustering of coregulated genes in the wild-type P4 time series by SOM, independent of DRGs. The *upper, middle,* and *lower lines* represent the 90th, 50th, and 10th percentile of the expression level at each time point. B, Wild-type P4 time series SOM clusters with significant enrichment of DRGs. Wild-type P4 time series clusters 1, 5, and 6 had significant enrichment with DRGs (see Table 4). Cluster 6 is especially interesting as it represents genes the repression of which was immediate and then persisted for 24 h. [See web supplement for all SOM clusters of the P4 time series (Ref. 12 and published on The Endocrine Society's Journals Online web site at http://mend.endojournals.org).]

in the *Hoxa-10* mutant was significantly lower than in wild type ($P < 0.01$). The decrease in S-phase entry by mutant USCs indicates an important cell cycle-regulatory function of *Hoxa-10* that is compatible with the altered expression of *p57* and *p15* in the *Hoxa-10* mutant periimplantation uterus.

Expression Profiling Detects Abnormal Accumulation of T Lymphocytes in the *Hoxa-10* Mutant Uterus

We next investigated the biological relevance of a second group of DRGs that was expressed at higher

Table 5. DRG Enrichment of SOM Clusters Identifies Coregulation of Genes by Hoxa-10 and P4 (15, 18)

Cluster	DRG Probe Sets (n)	Probe Sets in Cluster (n)	*P* Value
0	7	92	7.03E-02
1	12	88	1.16E-04[a]
2	4	108	6.39E-01
3	1	51	8.79E-01
4	0	23	1.00E+00
5	7	36	3.96E-04[a]
6	15	98	3.09E-06[a]
7	4	82	4.18E-01
8	5	58	7.85E-02
9	2	91	8.90E-01
10	0	77	1.00E+00
11	2	115	9.53E-01
12	4	83	4.27E-01
13	0	190	1.00E+00
14	1	162	9.99E-01
15	1	36	7.74E-01
16	1	89	9.76E-01
17	1	196	1.00E-01

A total of 1675 probe sets had a minimum relative fold difference greater than 2 and a minimum absolute difference of 75 U in expression levels between two time points and were cluster analyzed by SOM using Genecluster (20). For each cluster, *P* values based on a hypergeometric distribution were calculated using the number of probe sets representing DRGs (DRG probe sets) and the total number of probe sets in that cluster (Probe Sets in Cluster). The significant enrichment for DRGs in clusters 1, 5, and 6 (denoted by [a]) supports the coregulation of these DRGs by Hoxa-10 and P4 ($P < 0.0030$).

levels in the *Hoxa-10* mutant uterus than in wild type: those pertinent to immune function. In particular, genes expressed in T lymphocytes, including *TCRγV4, TCRδC,* and *MHC class II I-A^b*, were overexpressed in the *Hoxa-10* mutant uterine expression profile. Although this result could reflect increased expression of these genes on a per cell basis, we first chose to test the hypothesis that T lymphocytes, a distinct cell population in the uterine stroma, were increased in number in the *Hoxa-10* mutant. Indeed, flow cytometry of cells isolated from the uterine stroma of d 2.5 and 3.5 p.c. pregnant mice, with splenocytes as a control, indicated that the proportions of $CD4^+$ and $CD8^+$ T cells were significantly increased in the mutant uterine stroma (Fig. 5A). In addition, this expansion of T cells was polyclonal, as evident by increases in TCR$\gamma\delta$, TCR$\alpha\beta$, and NK lineages in the *Hoxa-10* mutant uterus on d 3.5 p.c. (Fig. 5, A and B). Consistent with microarray results showing increased expression of *TCRγV4* and *TCRδC* in the *Hoxa-10* mutant, the increase in the number of $\gamma\delta$ T cells in the physiological pregnant state occurred earliest and was the most dramatic (Fig. 5A). Microarray analysis of heterogeneous uterine tissue thus revealed alterations in gene expression that at least partly reflected changes in cell number within specific stromal T cell subpopulations. These analyses thus indicate a significant and previously unsuspected immunological phenotype in the *Hoxa-10* mutant uterus.

Hyperproliferation of T Lymphocytes in the *Hoxa-10* Mutant Uterine Stroma

Alterations in lymphocyte proliferation, trafficking, or both could underlie the increased number of T cells present in the *Hoxa-10* mutant uterus. To test the first possibility, we performed BrdU labeling followed by flow cytometry of uterine lymphocyte populations. These analyses showed an increased proliferation of T cells in the mutant uterine stroma. Thus, even though the overall proliferation index of cells present in d 3.5 p.c. mutant uterine stroma was decreased (Fig. 4C), the proportions of double-positive $CD4^+BrdU^+$ and TCR$\gamma\delta^+BrdU^+$ cells were actually higher in the mutant uteri by 5.3 ± 2.5- and 1.8 ± 0.1- (mean ± SEM) fold, respectively (data not shown). These results were derived from two separate experiments, each of which comprised pooled d 3.5 p.c. uteri from five mutant and five wild-type mice. One experiment was performed after a syngeneic mating, whereas the second experiment was performed after an allogeneic mating; both experiments gave similar results. The disparity between the proliferation rate of cells in the uterine stroma, analyzed *en masse*, which is depressed in the *Hoxa-10* mutant, and the proliferation rates of specific intrauterine T lymphocyte subpopulations, which are increased in the *Hoxa-10* mutant, highlights the dynamic nature of the uterine environment at the time of implantation.

We performed immunostaining experiments to further confirm the localization of T cells in the uterus. We chose the CD4 marker for these localization experiments as $CD4^+$ cells were twice as abundant as $CD8^+$ cells in all flow cytometry experiments, as seen in the representative experiment in Fig. 5B. Immunostaining experiments confirmed that the $CD4^+$ cell population resided mainly in the stromal compartment in both wild-type and *Hoxa-10* mutant uterus at both d 0.5 p.c. and d 3.5 p.c. (Fig. 5C). On d 0.5 p.c., when estrogen effects predominate, there was no overt difference in $CD4^+$ abundance between wild-type and *Hoxa-10* mutant uterus. At d 3.5 p.c., however, when P4 levels are high, $CD4^+$ immunostaining was greater in the mutant stroma than in wild type (Fig. 5C). The d 3.5 $CD4^+$ immunostaining results are thus consistent with the $CD4^+$ flow cytometry analyses (Fig. 5A).

DISCUSSION

Fidelity of Expression Profiling Applied to Mouse Implantation

We have demonstrated that gene expression profiling can be used to analyze the hormonal control of implantation by employing a gene-targeted mouse model to dissect the preimplantation events down-

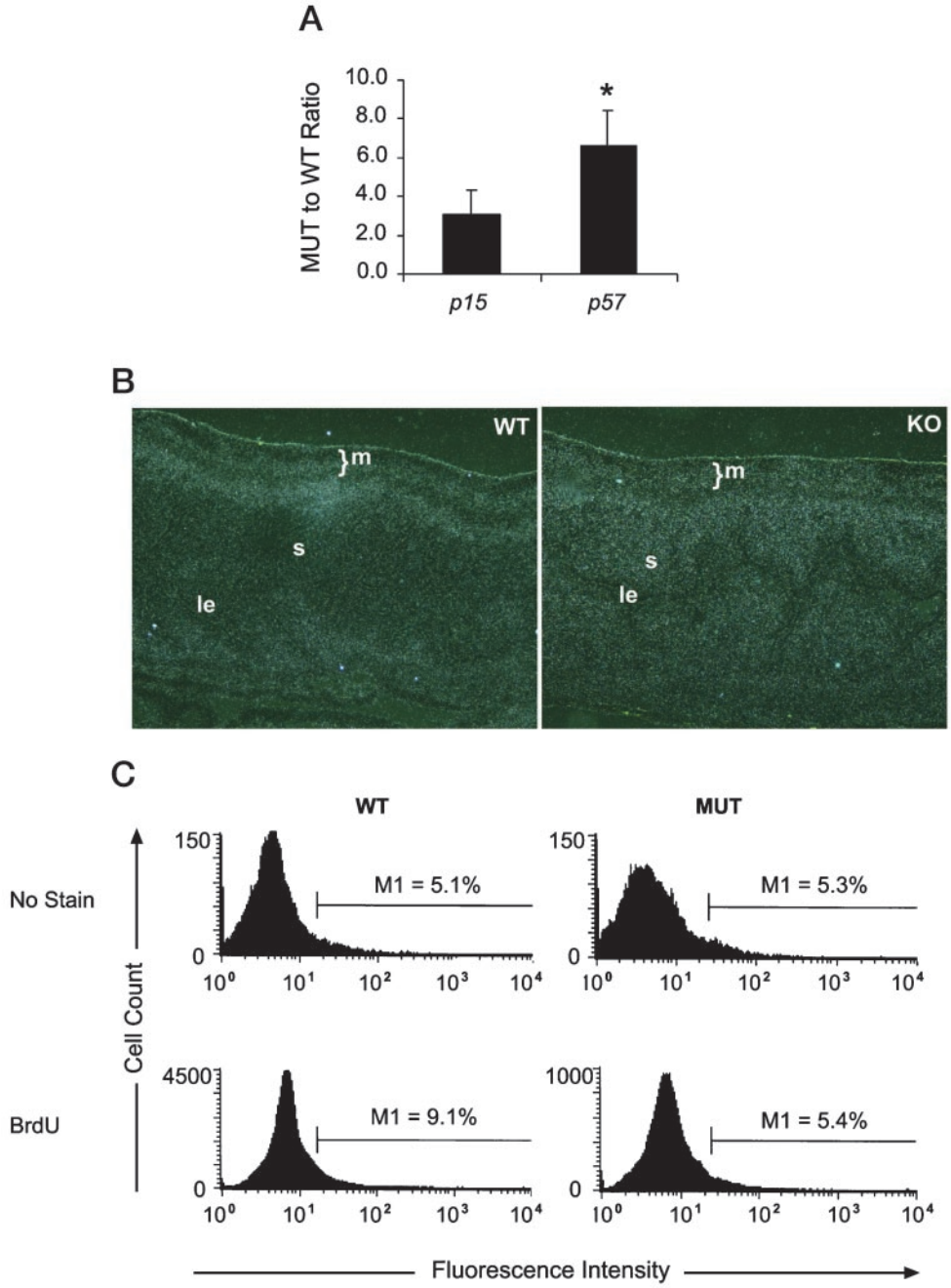

Fig. 4. Defective Regulation of CKI Genes and Stromal Cell Proliferation in the *Hoxa-10* Mutant

A, Expression of *p15* and *p57* in the d 3.5 p.c. *Hoxa-10* mutant uterus. Quantitative real time RT-PCR showed higher expression levels of *p57* (6.6 ± 1.8-fold) in the mutant compared with wild type on d 3.5 p.c. (*, $P = 0.08$ based on paired *t* test of log-transformed data). In contrast, although *p15* expression was increased by 3.1 ± 1.2-fold in the mutant, the increase was not significant. Data represent the mean ± SEM of the mutant to wild-type expression ratio from three independent experiments. All expression levels were normalized to 18S. B, *In situ* hybridization showing redistribution of *p15* expression in the *Hoxa-10* mutant uterus. On d 3.5 p.c., *p15* is expressed predominantly in the myometrium (M) and submyometrial stroma (*bright stain that is parallel to the myometrial signal*) in the wild-type uterus, with low expression in the stroma (S) underlying the luminal epithelium (LE). In contrast, in the *Hoxa-10* mutant, expression is present throughout the entire uterine stroma (×40). Similar results were observed in three wild-type and three *Hoxa-10* mutant mice, and in the OVX/P4/6 h model. C, Decreased USC proliferation in the d 3.5 p.c. *Hoxa-10* mutant. The proportion of stromal cells that have undergone cell proliferation was significantly less in the mutant (5.7 ± 0.2%; mean ± SEM) than in wild type (8.9 ± 0.1%) in four replicates; $P < 0.01$. Data are analyzed based on M1 approximately 5% $BrdU^+$ (*background*) in the no-stain control. Coordinates of M1 are set to be the same in the no-stain and BrdU histograms for each of wild type and mutant. Results shown are representative of four replicates in one experiment. The experiment was performed a total of three times with similar results.

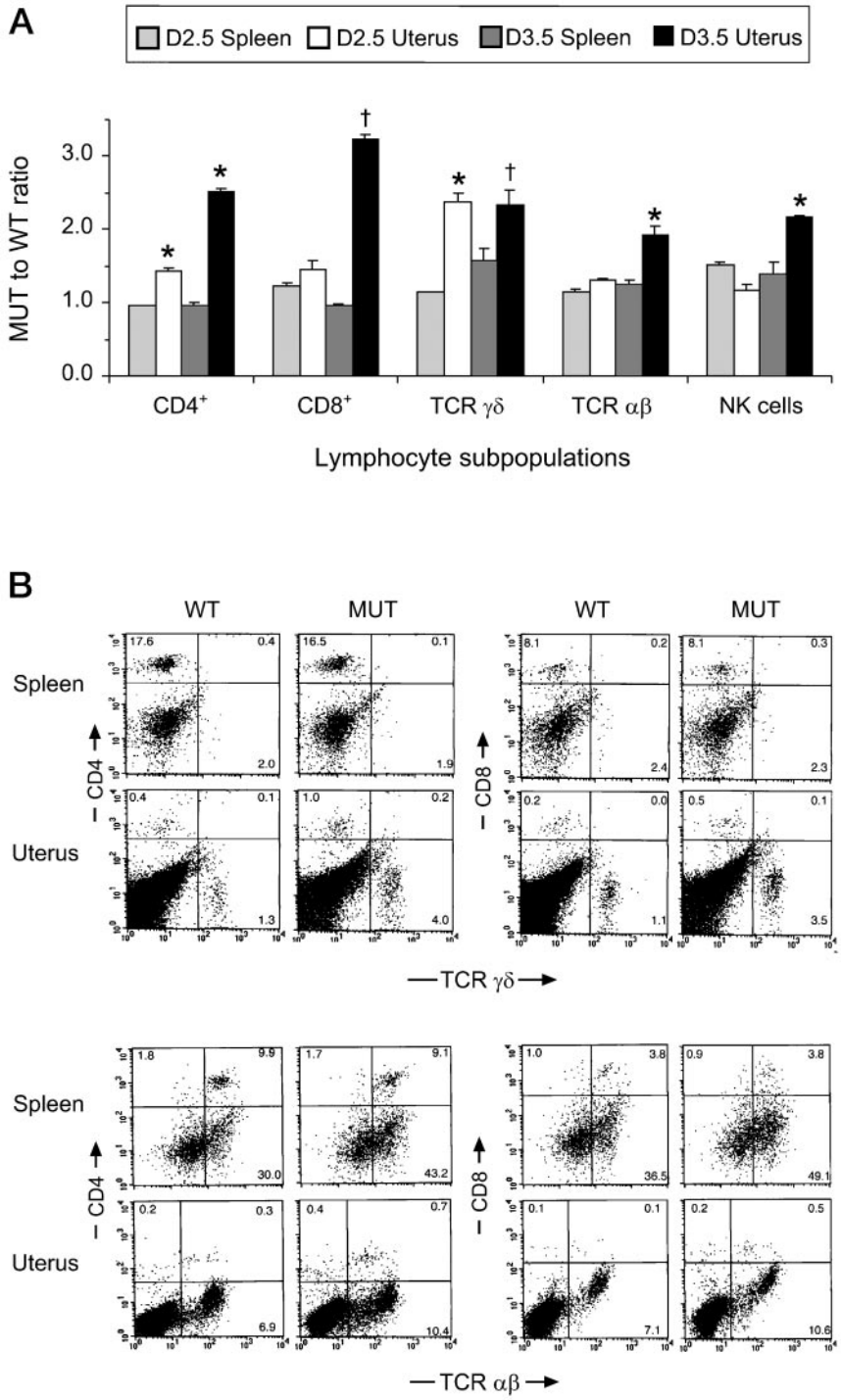

Fig. 5. Increase of Relative T Cell Number in the Periimplantation *Hoxa-10* Mutant Uterus

A, The proportion of each lymphocyte subpopulation in the uterine stroma was greater in mutant compared with wild type on d 3.5 p.c. by flow cytometry. On d 2.5 p.c., the proportions of $CD4^+$ and $TCR\gamma\delta^+$ populations were 1.4 ± 0.0 (mean ± SEM) and 2.4 ± 0.1 times greater in the mutant uterus, respectively. On d 3.5 p.c., the $CD4^+$, $CD8^+$, $TCR\gamma\delta^+$, $TCR\alpha\beta^+$, and NK cells ($DX5^+$) populations were 2.5 ± 0.1, 3.2 ± 0.1, 2.3 ± 0.2, 1.9 ± 0.1, and 2.2 ± 0.1 times greater in the mutant uterus compared with wild type, respectively (*, $P < 0.01$ and †, $P < 0.05$ in paired *t* tests comparing the mutant to wild-type ratio for uterus to that of spleen control). Spleen controls showed a mutant to wild-type ratio of approximately 1 for all cell populations. All data analyses for Fig. 5 were performed using a lymphocyte gate set by inclusion of more than 90% of $CD3^+$ and $CD4^+$ cells, or by using the lymphocyte gate from spleen samples within the same experiments. B, Increased percentage of single- and double-positive $CD4^+$ with $TCR\gamma\delta^+$ or $TCR\alpha\beta^+$ cells in d 3.5 p.c. mutant stroma. *Upper panel,* Flow cytometry dot plots showing the $TCR\gamma\delta^+$ *vs.* $CD4^+$ T cell markers in USCs isolated from wild type and mutant on d 3.5 p.c. with splenocytes as control. *Lower panel,* Flow cytometry

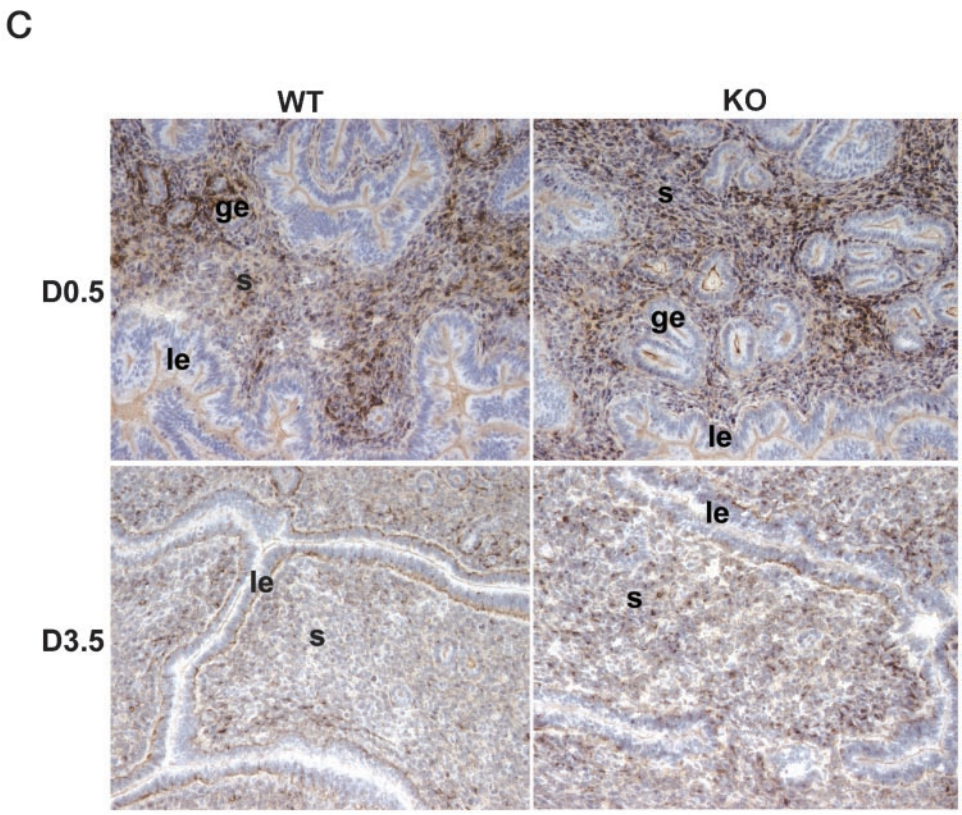

Fig. 5. *Continued.*

stream of *Hoxa-10*. A key feature of this approach was the experimental design, which proved crucial for identifying the enrichment of *Hoxa-10*-dependent DRGs expressed in the P4-primed uterine stroma. In addition, comparison of experimental data obtained under conditions of varied genotype and duration of P4 exposure permitted the identification of subsets of genes that are likely to be coregulated by *Hoxa-10* and by P4.

An attenuation of P4-induced *Hoxa-11* expression was identified in the *Hoxa-10* mutant uterus by both microarray and real-time RT-PCR. This result was previously not identified by *in situ* hybridization (8), probably because of the inherently nonquantitative nature of this technique. Cross-regulation of *Hox* genes in embryonic patterning is well known (21), as exemplified by the regulation of *Hoxb2* by *Hoxb1* in rhombomere r4 during mouse embryonic development (22). The cross-regulation of *Hoxa-11* by *Hoxa-10* also has important implications in implantation, including the possibility that the progesterone receptor (PR) and Hoxa-10 functionally cooperate to mediate the full induction of *Hoxa-11* stromal expression. The relevance of *Hoxa-10* and *Hoxa-11* regulatory interactions to the study of human infertility is further supported by the finding of up-regulated *HOXA-10* and *HOXA-11* expression in the human uterus in response to P4 during the time of implantation (23).

Comparison to Other Microarray Studies

The experimental approach described here differs from that in other microarray studies designed to identify genes involved in implantation (24–26). In one study, the same Affymetrix microarray (U74A version 2) used in our analyses was used to identify genes that are differentially expressed between implantation and nonimplantation sites in the wild-type uterus at 23–24 h of d 3 p.c. (26). In our study, we employed unopposed P4 stimulation in wild-type and *Hoxa-10* mutant females to identify *Hoxa-10*-dependent DRGs in the uterine stroma. Under these conditions, *Hoxa-10* ex-

dot plots showing the TCR$\alpha\beta^+$ *vs.* CD4$^+$ T cell markers. The *number in each quadrant* is the percentage of cells in the lymphocyte gate. Dot plots are representative of three experiments for each of d 2.5 p.c. and d 3.5 p.c. tissues. C, Immunostaining showed that CD4$^+$ T cell populations (indicated by *black staining*) in wild type and mutant were predominantly stromal at both d 0.5 and 3.5 p.c. (20×). Sections of wild-type and mutant spleens taken from the same mice were tested in parallel as positive control (not shown). ge, Glandular epithelium; le, luminal epithelium; s, stroma.

pression is strongly induced throughout the uterine stroma (7), a situation that should favor the identification of P4-inducible *Hoxa-10*-dependent stromal target genes. Importantly, however, we also tested the *in vivo* relevance of these DRGs in a physiological pregnancy model at a time point, the morning of d 3 p.c., before implantation and nonimplantation sites are distinguishable. This strategy thus permitted validation of the findings from the OVX/P4/6 h model and should have enriched for *Hoxa-10*-regulated events that precede overt morphological signs of implantation failure in the mutant.

Thus, although our OVX/P4/6 h model cannot be directly compared with the above-mentioned study, data from our OVX/P4/18 h experiments (data not shown), in which uterine RNA was extracted at 18 h after P4 injection, do detect some of the same DRGs reported by Reese *et al.* (26). For example, *BiP*, encoding a calcium-binding Hsp70 class chaperone (reviewed in Ref. 27), is expressed at higher levels in the wild-type than in the *Hoxa-10* mutant uterus. The same gene was also found by Reese *et al.* (26) to be more highly expressed in wild-type implantation sites than interimplantation sites. The expression of genes such as *BiP* may thus mark a later time point in the P4 response than that addressed by the OVX/P4/6 h model.

An especially interesting point of similarity between the present data and that of Reese *et al.* (26) relates to the striking, coordinate regulation of some 40 *Ig* genes in cluster 3 of our SOM analysis. These transcripts are markedly reduced by 3 h after P4 injection, followed by a small peak of expression at 6 h and a slow return to baseline by 24 h. In a composite list of genes showing decreased expression at implantation sites and after initiation of E2-induced implantation, Reese *et al.* identified 21 immune-related genes, of which 14 are classical *Ig* genes (Table III in Ref. 26). Strikingly, 10 of those 14 *Ig* genes are also represented in cluster 3 of our P4 time series experiments, with another three present in the next most closely related cluster, cluster 6. Analysis of sequence similarity among the probe sets representing these genes indicates that their coregulation is unlikely to be explained by cross-hybridization alone (12).

The fact that so many of the same *Ig* genes were identified in both studies, albeit in different contexts, has interesting implications concerning the role of B cells in implantation. As proposed by Medawar (28), a key requirement for establishment of an appropriate uterine implantation environment, in which the implanting blastocyst resembles an allograft, is the induction of a state of immune tolerance. Although much attention has focused on the role of T cells and cellular immunity during implantation (reviewed in Ref. 29), less is known about the regulation of B cell function. Interestingly, although the PR is expressed in B lymphocytes (30), a prior study did not identify major changes in the number of intrauterine B cells after E2 or E2 + P4 in either wild-type or PR knockout (PRKO) mice (31). Further experiments are required to determine whether P4 mediates local uterine B cell function by regulating *Ig* family gene expression rather than by controlling B cell number.

Hoxa-10 Is Required for P4-Mediated Stromal Cell Proliferation and CKI Repression

The proliferation of stromal cells and their subsequent differentiation into decidual cells are critical events in periimplantation uterine development. In this regard, a key finding was that two CKI genes, *p15* and *p57*, were aberrantly expressed in the *Hoxa-10* mutant uterine stroma. Interestingly, *p57* exhibits similar diffuse stromal expression in both wild type and mutant, but its expression is quantitatively more abundant in the *Hoxa-10* mutant. In contrast, *p15* undergoes a marked shift in its expression from a predominantly myometrial and submyometrial distribution in the wild-type uterus to a diffuse stromal pattern in the *Hoxa-10* mutant.

The altered expression of *p15* and *p57* in the *Hoxa-10* mutant is notable. High expression levels of these CKIs during early G_0/G_1 can induce cell cycle arrest, as p15 and its family members act as specific inhibitors of the cyclin D-dependent kinases cdk4 and cdk6 (32). p57 family members show similar interactions albeit with a broader range of cyclin-cdk complexes (32). The potential functional roles of *p15* and *p57* are especially relevant as cyclin D3 associates with cdks 4 and 6, and cyclin D3 is the major $G_1 \rightarrow S$ cell cycle regulator in the periimplantation uterine stroma (33). Interestingly, in the context of myelomonocytic cell differentiation, *Hoxa-10* directly up-regulates expression of the CKI *p21* and induces differentiation (34). In contrast, in implantation we suggest that it is the quantitative or spatially restricted repression of CKIs such as *p57* and *p15* by *Hoxa-10*, be it direct or indirect, that could explain the stromal cell proliferation defect in *Hoxa-10* mutants. *p57* null mutants die within 10 d of birth and are thus uninformative for the consequences of *p57* loss of function in the uterus; *p15* knockout mice exhibit no uterine phenotype and are even fertile. However, as these results pertain only to loss of function, the functional significance of the increased CKI expression observed in the *Hoxa-10* mutant uterus remains open.

The *Hoxa-10* Mutant Uterine Stroma Exhibits Aberrant Lymphoproliferation

A third key finding in the expression profiling experiments was that of increased transcript levels of various T cell genes. Although the absolute expression of these genes on a per cell basis may be altered, the increased number of T cells in the mutant stroma, as demonstrated by flow cytometry, is in qualitative agreement with the increased levels of T cell-related transcripts in the mutant. Moreover, flow cytometry analyses indicated an increased proliferation rate of these cells.

The lymphoproliferation observed in the *Hoxa-10* mutant uterine stroma is polyclonal and occurred after both syngeneic and allogeneic matings. These observations argue for a defect in T cell signaling and against an antigen-specific immune response as the cause for the immune phenotype. *Hoxa-10* is expressed in early myeloid progenitors but is not known to be present in mature neutrophils, monocytes, or lymphocytes (35, 36). This is consistent with our findings that splenocytes do not express Hoxa-10 (Fig. 1B). Thus, although it remains to be formally tested whether T cells in the periimplantation uterus express *Hoxa-10*, the available evidence suggests that the aberrant, intrauterine lymphoproliferation in *Hoxa-10* mutants is unlikely to result from a T cell autonomous defect. Interestingly, as exemplified by cluster 6 of the P4 time series, many chemokines, chemokine receptors, and cytokines known to be mitogenic for T cells are down-regulated in the uterine stroma in the presence of P4, and many of these same immunoregulatory genes are incompletely repressed by P4 in the *Hoxa-10* mutant stroma (12). Thus, it is attractive to propose that in the mutant uterus, *Hoxa-10*-deficient stromal cells stimulate the inappropriate proliferation of T cells by paracrine signaling mechanisms.

The distinct immunological phenotype in the *Hoxa-10* mutant uterine stroma may directly cause the implantation defect in *Hoxa-10* mutant females, as the cytolytic and inflammatory activities of T cells are well known to adversely affect the viability of implanting blastocysts. Indeed, the importance of local uterine immunosuppression to normal embryo implantation and pregnancy has been observed in several mouse models. For example, in a CBA/J $\times$ DBA/2J mouse model that exhibits a high rate of spontaneous abortion mediated by a monoclonal infiltration of $\gamma\delta$ T cells, treatment with a monoclonal antibody directed against the $\gamma\delta$ T cell clone restores fertility (29, 37). Implantation defects are also observed in mouse models with altered uterine immunosuppression (38) and aberrant T cell proliferation (39), and these defects are abolished in lymphocyte-deficient mice, which have normal fertility. Lastly, embryonic resorption and maternal leukocyte infiltration are observed at implantation sites in the *Hoxa-10* mutant (6). Thus, the aberrant lymphoproliferation observed in the *Hoxa-10*-deficient pregnant uterus can be viewed as a localized, proinflammatory response that could potentially compromise pregnancy.

It is interesting to compare the abnormal immune state present in the *Hoxa-10*-deficient uterus with the proinflammatory uterus described in PRKO mice (40). Experiments using the PRKO mice demonstrate that P4 acts via its receptor to antagonize the proinflammatory activity of estrogen, thereby decreasing the number of neutrophils and macrophages in the uterus. In contrast, the number of B lymphocytes remains unchanged (31). In the *Hoxa-10* mutant, our data suggest that P4 may act at the time of implantation through *Hoxa-10* to reduce the number of T cells in the uterus. Alternatively, Hoxa-10 may act with the PR to coregulate T cell number; moreover, these possibilities are not exclusive.

Although many possible mechanisms may underlie the preponderance of up-regulated DRGs observed in the *Hoxa-10* mutant uterus, repressive functions have been described for both PR (41–43) and Hox proteins (44–46). Thus, it is possible that Hoxa-10 may act as corepressor and be required for transcriptional regulation by PR. Repressive functions of *Hox* genes have been described in *Drosophila* development (44), and corepressor activity has been described for several mammalian *Hox* genes (45, 46), but mammalian targets of *Hox* gene repression, direct or indirect, have been difficult to ascertain *in vivo*. Based on their differential regulation in the *Hoxa-10* mutant uterus, we have identified several candidate *Hoxa-10* downstream genes, at least one of which, *Hoxa-11*, is also required for implantation (47). In addition, the analysis of specific DRGs in the d 3.5 p.c. pregnant uterus suggests a potential mechanism for how *Hoxa-10* regulates stromal cell proliferation. Lastly, our analyses at d 3.5 also reveal that *Hoxa-10* is required for the proper regulation of intrauterine T cell dynamics. Both of these latter events are critical to implantation.

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 embryonic stem cells and generation of chimeric mice (5). No differences in female infertility were observed among 129/SvJ, mixed BALB/c $\times$ 129/SvJ and mixed C57BL/6 $\times$ 129/SvJ backgrounds (6). For natural matings, virginal females between 10–16 wk of age were mated with *Hoxa-10* $^{+/-}$ 129/SvJ stud males unless otherwise specified. The day of the vaginal plug was considered d 0.5 p.c., and mice were killed between 0700 h and 0900 h on d 2–3 p.c.

Experimental Animals

All animal experimentation described was conducted in accord with accepted standards of humane animal care. Protocols for animal work were approved by the Harvard University Institutional Committee on Animal Care.

Western Blot Analysis

Uteri and spleens were pooled from groups of at least three wild-type or mutant ovariectomized mice at 0, 1, 3, and 6 h after P4 injection and placed in cold RIPA (radioimmunoprecipitation) buffer containing protease inhibitor cocktail (50 μl of 25$\times$ protease inhibitor cocktail per ml RIPA buffer, Roche, Indianapolis, IN) on ice. Tissues were then homogenized by polytron, centrifuged at 14 $\times$ *g* at 4 C for 10 min; supernatant protein was quantitated by Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA), and then stored at $-$80 C. Unless otherwise noted, 25 μg protein samples were analyzed by 10% SDS-PAGE. After nitrocellulose transfer and blocking overnight at 4 C in 5% nonfat milk, membranes

were incubated in affinity-purified rabbit polyclonal anti-Hoxa-10 antibody at 1:300 dilution for 1 h at room temperature (RT), washed with 1× Tris-buffered saline with Tween 20 (3 × 10 min), incubated with goat antirabbit (horseradish peroxidase) secondary antibody (Pharmacia Biotech, Piscataway, NJ), washed with Tris-buffered saline with Tween 20, and incubated in enhanced chemiluminescence for 1 min (Bio-Rad Laboratories, Inc.) followed by film development. Hoxa-10 polyclonal antibody was raised in rabbits against the MAP peptide H-EEAHASSSAAEELSPAPSE-8-MAP (Research Genetics, Inc., Huntsville, AL), which corresponded to a sequence in the mouse Hoxa-10 C terminus, and affinity purified using antigen-bound Affygel-10 (Bio-Rad Laboratories, Inc.).

RNA Isolation and Oligonucleotide Microarray Hybridization

Groups of four wild-type and four *Hoxa-10* mutant mice (SvJ background) at 10–16 wk of age were ovariectomized. Fourteen days later, P4 (Sigma, St. Louis, MO) dissolved in sesame oil (Sigma) was injected sc (2 mg/mouse in 100 μl), after which the animals were killed and uterine horns removed at 6 h after injection. Care was taken to exclude the region of homeotic transformation at the uterine-oviductal junction (proximal ~25% of each uterine horn) (3). Fat and mesentery were trimmed. Tissues were snap frozen in liquid N_2, pooled, and homogenized by mortar in N_2. Total RNA was extracted using Trizol (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's protocol, quantified by UV spectroscopy, and stored in diethylpyrocarbonate water (Ambion, Inc., Austin, TX) in 1-μg/μl aliquots at −80 C. A portion (0.5 μg) of each RNA sample was analyzed on a 2% agarose gel to confirm integrity. Prechip validation of RNA collected via the OVX/P4/6 h protocol was performed by assaying for up-regulation of *Hoxa-10*, *Hoxa-11* (7), and *Histidine decarboxylase* (48) as markers of P4 efficacy.

Reverse transcription used oligo-dT followed by *in vitro* transcription and biotin labeling of cRNA (Enzo Biochem, Farmingdale, NY). All cRNA samples were analyzed with a Bioanalyser 2100 (Agilent Technologies, Wilmington, DE) before chip hybridization. Fragmented, labeled cRNA (20 μg) was hybridized to Affymetrix U74A version 2 mouse oligonucleotide arrays, which were washed and scanned per Affymetrix protocol. Control samples were prepared and analyzed similarly except that sesame oil vehicle (OVX/oil/6 h) rather than P4 was injected. Triplicate pairs of wild-type and mutant samples were prepared, and *in vitro* transcription was performed in parallel for each of OVX/P4/6 h and OVX/oil/6 h RNA collections.

Data Analysis for OVX/P4/6 h Wild-Type *vs.* Mutant Comparison

Intensity and cell data were obtained using MAS 4.0.1 (Affymetrix). Data were normalized and analyzed using dChip (11) to obtain model-based expression levels for each probe set, upon which all subsequent analyses were performed. Criteria for significant differential expression levels between wild type and mutant were: a fold change in expression of at least 1.5, *P* value of at least 0.1 (based on a *t* test or other tests), and an absolute difference in expression of at least 75 U. Paired and unpaired *t* tests were performed using Excel (Office 2000, Microsoft Corp.). Expression levels from dChip were also analyzed by SAM, an Excel add-in developed by Tusher *et al.* (13), that uses a variant of the *t* statistic and a permutation analysis to estimate the false positive rate within a set of significantly up- or down-regulated genes. These false positive rates take multiple hypothesis testing into account. When using paired tests with a 1.5-fold cutoff threshold and a median false detection rate of 10%, the SAM algorithm yielded similar lists of genes to those using conventional *t* statistics. DRGs were classified according to their described functions based on a literature search of PubMed (http://www.ncbi.nlm.nih.gov/PubMed/). A DRG was placed in more than one category if multiple functions are described.

Real-Time RT-PCR

Separate aliquots of total RNA from the chip experiments were stored at −80 C. In the physiological pregnancy model, d 3.5 p.c. total uterine RNA was pooled from one or two wild-type or mutant mice. Samples were treated with deoxyribonuclease I using the DNA-free kit (Ambion, Inc.) and diluted to 20 ng/μl. Reverse transcription and quantitative PCR were performed with Superscript II reverse transcriptase (RT) and *Taq* polymerase in the One Step RT-PCR kit (Life Technologies, Inc.) on the iCycler (Bio-Rad Laboratories, Inc.). No RNA and negative RT controls (no RT, *Taq* polymerase only) were included in each set of RT-PCR experiments. All samples that would be compared were tested in the same RT-PCR run. Primer and fluorescence resonance energy transfer probe sequences were designed using Primer Express 1.0 (PE Applied Biosystems, Norwalk, CT), purchased, or extracted from published literature. All primers were from Invitrogen (San Diego, CA) and all fluorescence resonance energy transfer probes with 5′-FAM and 3′ black hole quencher labels were from BioSource Technologies, Inc. (Vacaville, CA). Sequences of primer and probe sets are listed in the supplemental data (Ref. 12 and published on The Endocrine Society's Journals Online web site, http://mend.endojournals.org). Threshold cycle numbers were obtained using iCycler software version 2.3 (Bio-Rad Laboratories, Inc.). Conditions for amplification were: RT cycle at 50 C for 15 min, 1 cycle of 95 C for 2 min, 35 cycles of 95 C for 15 sec, 59 C for 15 sec, and 72 C for 15 sec. Quantitative RT-PCR was performed in triplicate with total uterine RNA (CLONTECH Laboratories, Inc., Palo Alto, CA) as a positive control. RNA expression levels were quantitated by comparing threshold cycles of the samples against the standard curve generated by positive controls. The experiment was valid if the negative RT controls had fluorescence intensity signals that were 100-fold less than experimental samples and if the size of the PCR products was verified by gel electrophoresis. RNA expression levels from the ovariectomized/injection model were normalized to *Rpl-7* as a control (40), and samples from d 2.5 p.c. and d 3.5 p.c. were normalized to 18S.

RNA Sample Preparation for P4 Time Series

The mice used for the wild-type P4 time series were of 129/SvImJ background (The Jackson Laboratory, Bar Harbor, ME). In the wild-type P4 time series, total uterine RNA was extracted from ovariectomized mice at 0 (no injection), 1, 3, 6, 9, 12, 15, 18, and 24 h after P4 injection. Samples containing pooled total uterine RNA from at least four mice were used for each time point. The time course dataset was generated in two sets (0, 1, 3, 6, 9 h) and (0, 6, 12, 15, 18, 24 h). Samples in the same batch were processed in parallel at all steps, including ovariectomy, P4 injection, RNA collection, *in vitro* translation labeling, and chip hybridization. Time points of 0 h and 6 h were acquired in both batches to allow comparison between batches. The correlation between duplicates (at 0 h and 6 h) was much higher ($r^2 > 0.975$) than that among samples within the same batch. Therefore, all time points were merged into one dataset for analysis.

SOM Analysis of Gene Expression in P4 Response Time Series

dChip expression levels from experiments using wild-type ovariectomized mice at nine time points (0, 1, 3, 6, 9, 12, 15, 18, and 24 h) after P4 injection were used, and only genes with at least a 2-fold change and absolute signal change of at

least 75 U between any two time points were included in the SOM analysis (Genecluster, Whitehead Genome Center, Massachusetts Institute of Technology, Boston, MA) (15). Expression profiles for 1675 genes fulfilling these criteria were normalized before clustering so that the expression profile of each gene has mean 0 and variance 1 on the log scale. The number of nodes was incrementally increased until no further expression patterns emerged. A 3 × 6 geometry of nodes is depicted in the supplemental data published on the Endocrine Society's Journals Online web site, http://mend.endojournals.org (12). Clustering by SOM was also performed by using a 1.5-fold cutoff filter, with similar qualitative results.

Statistical Methods for Determining Functional Category Enrichment

A hypergeometric distribution was used to calculate the probability of observing the number of DRGs within each cluster in the time series. The probability P of observing at least k DRGs within a cluster of size (n) is:

$$P = 1 - \sum_{i=0}^{k-1} \frac{\binom{f}{i}\binom{g-f}{n-i}}{\binom{g}{n}}$$

where ($f = 119$) is the total number of probe sets representing DRGs and ($g = 12{,}386$) is the number of probe sets representing interrogated genes on the Affymetrix U74Av2 array. Alternatively, we also calculated P values based only on the subset of genes which passed the filtering for inclusion into the SOM clustering algorithm so that $f = 67$ and $g = 1675$. A Bonferonni correction for multiple tests (m = 18, the number of clusters) was used to obtain an adjusted P value cutoff (α') for a chosen target significance level ($\alpha = 0.05$) according to the following equation: $\alpha' = 1 - (1 - \alpha)^{1/(m-1)}$ (Cheung K. J., V. Badarinarayana, D. Selinger, D. Janse, and G. M. Church, submitted manuscript). Setting $\alpha = 0.05$ and m = 18; α' is 0.0030.

In Situ Hybridization and Immunohistochemistry

Uteri were cut into 4–6 mm pieces and flash frozen in Histo-Freeze (Fisher Scientific, Pittsburgh, PA). Frozen sections (11 μm) were mounted onto poly-L-lysine coated slides and fixed in cold 4% paraformaldehyde in PBS. Sections were prehybridized and hybridized at 45 C for 4 h in 50% formamide hybridization buffer containing the ^{35}S-labeled antisense cRNA probe (specific activities ~2×10^9 dpm/μg). After hybridization and washing, sections were incubated with ribonuclease A (20 μg/ml) at 37 C for 20 min. Ribonuclease A-resistant hybrids were detected by autoradiography using Kodak NTB-2 liquid emulsion (Eastman Kodak Co., Rochester, NY). Sections hybridized with the corresponding sense probe served as negative controls. Slides were poststained with hematoxylin and eosin. ^{35}S-labeled riboprobes were generated using specific RNA polymerases. An IMAGE clone of *p15* in pCMV-SPORT6 (IMAGE clone 3495097, Genbank accession no. BC002010) was purchased from Research Genetics, Inc. (Huntsville, AL) and confirmed by sequencing. Plasmid for the *p57* riboprobe was a generous gift from Dr. Stephen Elledge.

For immunohistochemistry, uteri were cut into 4–6 mm pieces and flash frozen in Histo-Freeze (Fisher Scientific, Pittsburgh, PA). Frozen sections (12 μm) were mounted onto poly-L-lysine coated slides and fixed in Bouin's fixative and washed in PBS. Immunostaining with the primary antibody (anti-CD4, H129.9, PharMingen, San Diego, CA) was performed using a Histostain kit from Zymed Laboratories, Inc. (South San Francisco, CA) following the manufacturer's protocol.

Flow Cytometric Analysis of USCs and Splenocytes

USCs were isolated from groups of four to five wild-type and *Hoxa-10* mutant mice on d 2.5 p.c. or d 3.5 p.c. as specified. In each group, uteri excluding the utero-oviductal junction were excised, trimmed of fat and mesentery, rinsed in PBS, flushed of embryos, pooled, and minced into fine fragments. Four rounds of 5-min incubation using collagenase type I (Sigma), mechanical disruption by pipetting, and 5-min sedimentation by gravity followed by removal of supernatant were performed in serum-free DMEM/F-12 (Life Technologies, Inc.) containing 1% penicillin-streptomycin (Sigma). Supernatant containing isolated USCs was passed through a 70-μm cell strainer [Falcon (BD Biosciences, Bedford, MA)] to remove cell clumps. Cell suspensions were centrifuged at 1400 rpm, 4 C, for 5 min, and USCs were resuspended in fluorescence-activated cell sorting (FACS) buffer (PBS, 0.5% BSA, 0.02% sodium azide) on ice.

Spleens were removed and pooled from the same groups of mice and kept in PBS with 5% fetal calf serum on ice. Splenocytes were prepared by homogenization of the spleen capsule using the plunger end of a syringe, passed through a 70-μm cell strainer, and suspended in PBS containing 5% fetal calf serum. Cells were pelleted by centrifugation (1800 rpm, 4 C, 5 min), resuspended in ACK buffer (0.15 M NH_4Cl, 1.0 mM $KHCO_3$, and 0.1 mM Na_2EDTA) for 5 min to lyse red blood cells, repelleted by centrifugation, and resuspended in FACS buffer. A suspension of USCs or splenocytes (100 μl) was incubated with normal rabbit serum (PharMingen) at 1:50 dilution for 5 min on ice, followed by centrifugation and aspiration of supernatant. Cell pellets were resuspended in 50 μl of directly fluorochrome-conjugated monoclonal antibodies diluted in FACS buffer to a concentration of 0.1 mg/ml for 30 min in the dark on ice. Cells were washed twice in FACS buffer and analyzed on a FACScan (Becton Dickinson and Co.) with Cellquest software within 2 h of staining. The monoclonal antibodies used were (BD PharMingen): fluorescein isothiocyanate (FITC)-anti-γ δ TCR (GL3), FITC-anti-TCR β (H57–597), FITC-anti-Pan-NK (DX5), FITC-anti-CD19 (1D3), FITC-anti-CD4 (H129.9), PE-anti-γ δ TCR (GL3), PE-anti-TCR β (H57–597), PE-anti-Pan-NK (DX5), PE-anti-CD19 (1D3), and PE-anti-CD4 (H129.9).

Cell Proliferation

Four hours before the mice were killed, 100 μl (1 mg) of BrdU (BD Biosciences) was injected ip into each mouse. Cell staining was performed as above followed by cell fixing, permeabilization, deoxyribonuclease treatment, and BrdU staining using the BrdU Flow Kit (BD Biosciences) according to the manufacturer's protocol. Fixed and stained cells were kept in FACS buffer in 4 C overnight and analyzed by FACS the next morning.

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