BIOLOGY OF REPRODUCTION **77,** 312–319 (2007)
Published online before print 9 May 2007.
DOI 10.1095/biolreprod.107.060459

Microarray Analyses of Newborn Mouse Ovaries Lacking *Nobox*[1]

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ABSTRACT

***Nobox* is a homeobox gene expressed in oocytes and critical in oogenesis. *Nobox* deficiency leads to rapid loss of postnatal oocytes. Early oocyte differentiation is poorly understood. We hypothesized that lack of *Nobox* perturbs global expression of genes preferentially expressed in oocytes as well as microRNAs. We compared *Nobox* knockout and wild-type ovaries using Affymetrix 430 2.0 microarray platform. We discovered that 28 (74%) of 38 of the genes downregulated more than 5-fold in the absence of *Nobox* were preferentially expressed in oocytes, whereas only 5 (15%) of 33 genes upregulated more than 5-fold in the absence of *Nobox* were preferentially expressed in oocytes. Protein-binding microarray helped identify nucleotide motifs that NOBOX binds and that several downregulated genes contain within putative promoter regions. MicroRNA population in newborn ovaries deficient of *Nobox* was largely unaffected. Genes whose proteins are predicted to be secreted but were previously unknown to be significantly expressed in early oogenesis were downregulated in *Nobox* knockouts and included astacin-like metalloendopeptidase (*Astl*), Jagged 1 (*Jag1*), oocyte-secreted protein 1 (*Oosp1*), fetuin beta (*Fetub*), and R-spondin 2 (*Rspo2*). In addition, pluripotency-associated genes *Pou5f1* and *Sall4* are drastically downregulated in *Nobox*-deficient ovaries, whereas testes-determining gene *Dmrt1* is overexpressed. Our findings indicate that *Nobox* is likely an activator of oocyte-specific gene expression and suggest that the oocyte plays an important role in suppressing expression of male-determining genes, such as *Dmrt1*.**

follicular development, folliculogenesis, gamete biology, microarray, microRNA, Nobox, *oocyte, oocyte development, oogenesis, ovarian failure, ovary, transcription factor*

INTRODUCTION

Oocyte differentiation begins in the mouse embryonic gonad after oogonia enter meiosis around 13.5 days postcoitum (dpc). In the mouse embryonic gonad, oocytes exist as clusters also known as *cysts* [1, 2]. Within a few days of birth, oocyte clusters break down, and individual oocytes become enveloped by flat somatic pregranulosa cells. The early germ cell-somatic units are called *primordial follicles*, and oocytes in these units are usually less than 20 μm in diameter. Several signaling molecules, such as *Kitl*, *Fgf2*, *Fgf7*, neurotrophins, *Lif*, and steroids, have been implicated in the primordial to primary and growing follicle transition [3–9]. Oocyte-somatic cell communications are paramount for the successful differentiation of the follicular unit, but little is known about the molecules that initiate germ cell cluster breakdown [10]. Factor in the germline alpha (*Figla*) was the first oocyte-specific basic helix-loop-helix transcription factor that was shown to affect primordial follicle formation in mammals [11]. However, downstream targets of *Figla* that are critical for the formation of primordial follicles are largely unknown.

We identified *Nobox*, a homeobox gene preferentially expressed in the germline, as critical in the early oocyte differentiation [12, 13]. *Nobox* knockout females are infertile and lose oocytes quickly, so that by 2 weeks of postnatal life the ovary is depleted of oocytes. At the time of birth, *Nobox* knockout females contain oocytes that grossly do not differ from the wild-type ovaries. We therefore used whole-mouse genome expression arrays and microRNA arrays to identify genes and microRNAs perturbed significantly by the lack of *Nobox*. In addition, we used protein binding microarray (PBM) technology [14] to identify NOBOX binding preferences and identified a subset of genes that contain such elements in their promoter regions.

MATERIALS AND METHODS

Microarray Analysis

Newborn ovaries were pooled separately from wild-type and $Nobox^{-/-}$ animals, and total RNA was isolated using RNeasy mini kit (Qiagen). Newborn ovaries were collected within 12 h of delivery. Animal experimentation was approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine. Three independently pooled RNA samples from wild-type and $Nobox^{-/-}$ newborn ovaries were used to generate biotinylated cRNA. Biotinylated cRNA was hybridized to GeneChip Mouse Expression Set 430 2.0 (Affymetrix Inc.). Since three independent experiments were performed from three independent pools of wild-type and $Nobox^{-/-}$ RNA, signal intensities for particular genes were averaged between the three chips, and ratio of the wild-type over the knockout signal was calculated. Signal less than 100 was considered background. The raw data in the Affymetrix CEL files were normalized by the RMA method (robust multiarray analysis) [15, 16]. Then, the null hypothesis that there is no significant change in gene expression between the treatment pairs was tested. This was done by LIMMA [17] and the pooled local error (LPE) method [18]. The raw *P* values were adjusted by the Benjamini-Hochberg method for the false-discovery rate of 5% [19]. We used the DAVID database [20] to aid in functional annotation of genes affected by *Nobox* deficiency.

Total RNA was isolated for microRNA microarray, size fractionated (<200 nt) using *mir*Vana RNA isolation kit (Ambion), and labeled with Cy3 (wild-type) and Cy5 (*Nobox* knockout). Microarray microRNA assays were performed on a μParaFlo microfluidics chip (LC Sciences LLC) containing 373 distinct microRNAs based on Sanger miRBase Release 9.0. The melting temperature of the detection probes was balanced by the incorporation of a

[1]Supported by grant HD044858, and a March of Dimes grant (6-FY05-70) to A.R.
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Received: 1 February 2007.
First decision: 5 March 2007.
Accepted: 30 April 2007.

ISSN: 0006-3363. http://www.biolreprod.org

varying number of modified nucleotides with increased binding affinities. The maximal signal level of background probes was less than 250.

RNA Isolation, RT-PCR, Quantitative PCR, and In Situ Hybridization

Multitissue RT-PCR was performed as previously described [21]. Oligonucleotides corresponding to *Nlrp14* (also known as *Nalp14*), *Nlrp4a* (also known as *Nalp4a*), *Nlrp4f* (also known as *Nalp4f*), *Nlrp4c* (also known as *Nalp4c*), *Oas1c, Oas1d, Oas1e, Oas1a, Bax, Bcl2, Bcl2l2,* and *Casp2* were selected using Primer 3 software (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) to generate an approximately 500-bp nucleotide fragment that is interrupted by an intron within the mouse genome. The sequences of these primers are available on request from A.R. Mouse actin-specific primers were used to verify cDNA synthesis from RNA isolated from each tissue. Polymerase chain reaction was carried out for 28 cycles. For each cycle, the template was denatured at 94°C for 30 seconds, annealed at 55°C for 30 seconds, and extended at 72°C for 30 seconds, and PCR products were resolved on 2% agarose gels. RT-PCR results were verified on three independently collected pools of newborn ovaries.

Quantitative real-time PCR was performed on the ABI Prism 7500 Sequence Detection System (ABI, Foster City, CA) using Assays-On-Demand (ABI) PCR primer and probe sets for each gene and mouse *Gapdh* (VIC-labeled MGD probe; primer limited; ABI) as the endogenous control. RT-PCR was performed using the TaqMan Universal PCR Master Mix (ABI) in 20 μl. Each sample was analyzed in duplicate from at least three independent newborn wild-type and $Nobox^{-/-}$ cDNA samples. Two nontemplate controls (RNase-free water) samples were included on each plate for each primer-probe set. The relative amount of transcripts was calculated by the ΔΔCT method as described by Applied Biosystems using the ABI 7500 System Software (V.1.2.3) and was normalized to the endogenous reference (*Gapdh*). One wild-type sample was randomly chosen to serve as the reference sample to which all other samples were normalized. The average and standard error were calculated for the triplicate measurements, and the relative amount of target gene expression for each sample was plotted. Significance was performed using Student *t*-test using Excel (Microsoft).

Mouse cDNA fragments corresponding to *Oas1d, Nlrp14, Nlrp4f,* and *AU016322* were subcloned into the pGEM-T Easy vectors (Promega) and used to generate antisense and sense strands by labeling with [α-^{35}S]UTP using the Riboprobe T7/SP6 Combination System (Promega) [22]. In situ hybridization was carried out as previously described [21]. In situ hybridizations were performed on ovarian sections derived from at least two different animals.

Protein Binding Microarray

We fused NOBOX amino acids 124–205 (containing the homeodomain region) with the glutathionine *S* transferase protein in the pet-41B vector (Novagen) to create a GST-NxHD protein. GST-NxHD protein fusion was purified on the GST bind resin column (Novage) and dialyzed against 1× PBS. Protein-binding microarrays were performed and enrichment scores calculated essentially as described previously [14, 23–25].

RESULTS

Nobox *Deficiency Disproportionately Affects Expression of Genes Preferentially Expressed in the Oocytes*

We showed previously that several genes preferentially expressed in oocytes, such as *Gdf9*, *Pou5f1* (also known as *Oct4*), and *Mos,* are drastically downregulated in ovaries deficient in NOBOX [13]. In addition, our data suggest that NOBOX directly binds *Gdf9* and *Pou5f1* promoters, and may therefore directly regulate transcription of *Gdf9* and *Pou5f1* [26]. In order to better characterize effects of *Nobox* deficiency on the global newborn ovary transcriptome, we analyzed *Nobox* knockout and wild-type newborn ovaries with the whole-genome mouse expression arrays. We chose to compare newborn ovary transcriptomes due to the grossly similar histology and oocyte numbers in both wild-type and *Nobox*-deficient mouse ovaries. We used Affymertrix 430 2.0 mouse microarray because it is the most comprehensive array system that allows expression inquiry for more than 34 000 well-substantiated mouse genes and expressed sequence tags (ESTs). Analysis of three independent experiments comparing *Nobox*-deficient and wild-type newborn ovaries revealed that a total of 1432 genes showed significantly different expression levels (Supplementary Tables 1–4, available online at www.biolreprod.org). Among these 1432 genes were genes that we previously identified as downregulated [13], except for *Dnmt1o* and *Fgf8*, which were not detected in our microarray analysis. The oocyte-specific isoform of *Dnmt1* (*Dnmt1o*) is not represented on the Affymetrix 430 chip, and as such will not be detected. *Fgf8* is not abundantly expressed and therefore is detectable only by RT-PCR but not by microarrays in the newborn ovaries.

Genes that showed greater than 2-fold expression differences in *Nobox*-deficient ovaries are involved in many different biologic processes (Supplementary Table 2). We chose to further analyze a 5-fold difference arbitrarily, thinking that such genes are more likely to be directly regulated by *Nobox*. A total of 38 genes were downregulated more than 5-fold in the *Nobox*-deficient newborn ovaries (Table 1 and Supplementary Tables 3 and 4). We used Unigene EST database and GNF Sym Atlas resources to determine which genes are preferentially expressed in germ cells [27]. A total of 28 (74%) of 38 genes that were downregulated more than 5-fold showed preferential expression in oocytes (Table 1), whereas only 5 (15%) of 33 genes that were upregulated more than 5-fold showed preferential expression in oocytes (Table 2).

Crabp2 (cellular retinoic acid binding protein 2) and *Stra8* (stimulated by retinoic acid gene 8) are both involved in the retinoic acid metabolism and are among the few upregulated genes expressed in oocytes (Fig. 1G and Table 2). *Crabp2* is not essential for oogenesis, because *Crabp2*-deficient mice are fertile and grossly normal [28]. *Stra8* is essential for fertility and is expressed in premeiotic germ cells [29]. *Stra8* may be essential for the onset of meiosis, but it disappears as oocytes become arrested in meiosis in wild-type ovaries, and it is not detectable at the newborn ovary stage. *Stra8* persistent expression in the *Nobox* knockout newborn ovary (Fig. 1G) signifies that *Nobox*-deficient oocytes are immature at the molecular level, despite forming primordial-like follicles.

Genes Essential for Pluripotency Are Downregulated in Nobox *Knockout Ovaries*

Pou5f1 and *Sall4* transcription factors are downregulated in *Nobox* knockout ovaries more than 30- and 20-fold, respectively (Fig. 1, A and D) [13]. *Pou5f1* is a POU domain-encoding homeobox gene that is essential for pluripotency and critical in primordial germ cell biology [30]. *Pou5f1* expression diminishes around Embryonic Day 15.5 (E15.5), when oocytes enter meiosis, and reappears after birth [13, 31]. The role of *Pou5f1* in folliculogenesis is unknown, but its reexpression in the newborn ovary may be essential in establishing reprogramming potential to the egg. Indeed, another transcription factor, *Sall4*, is a zinc finger transcriptional regulator known to be essential for pluripotency [32], expressed in oocytes [27], and to regulate *Pou5f1* expression [32]. *Sall4* is drastically downregulated in *Nobox* knockout ovaries (Fig. 1D) and may also play an important role in conferring oocytes the ability to reprogram. Other transcriptional regulators implicated in pluripotency, such as *Sox2*, *Nanog,* and *Nr6a1*, were not detectable in the wild-type mouse newborn ovary microarrays (data not shown). *Lin28* is a developmental regulator preferentially expressed in oocytes, early embryos, and embryonic stem cells [27, 33]. *Lin28* is expressed in wild-type newborn ovaries, but its expression is not affected by the

TABLE 1. Genes down-regulated at least five-fold in *Nobox* knockout ovaries.

Gene	Fold change	Germ cell specific	Function
Mos	109.2	Y	Protein kinase
H1foo	77.8	Y	Histone, oocyte-specific
Rfpl4	72.3	Y	Ring finger protein
C86187	69.3	Y	Unknown
D5Ertd577e	63.2	Y	Unknown
BC052883	48.7	Y	Unknown
Ttid	39.6	N	Actin binding
Nlrp4f	39.2	Y	Unknown
Nlrp14	37.2	Y	Unknown
Oog1	36.9	Y	Unknown
Pou5f1	30.5	Y	Transcription
BG071013	29.6	N	Unknown
1700008J08Rik	29.3	Y	Transcription
D9Ertd414e	25.1	N	Unknown
Sall4	24.1	N	Transcription
Oas1e	23.7	Y	Oligoadenylate synthetase
BM229829	19.5	Y	Unknown
Oas1d	18.9	Y	Oligoadenylate synthetase
D11Ertd636e	18.6	Y	Unknown
2610028F08Rik	17.9	Y	Protein kinase
Oas1c	16.6	Y	Oligoadenylate synthetase
Slc6a20	15.7	N	Solute carrier
Rims1	14.9	N	Exocytosis
Padi6	14.7	Y	Arginine deiminase
Astl	13.9	Y	Secretory factor
AK005675	13.2	Y	Unknown
Gdf9	12.5	Y	Secretory factor
E130009J12Rik	10.8	Y	Unknown
Oosp1	8.9	Y	Secretory factor
E330034G19Rik	8.9	Y	Aldehyde dehydrogenase
Oas1h	7.7	Y	Oligoadenylate synthetase
Aldrl6	7.4	N	Aldehyde reductase
Dnahc8	7.4	Y	Microtubule movement
Fetub	7.1	N	Secreted factor
Nlrp4c	5.6	Y	Unknown
Jag1	5.3	N	Notch signaling pathway
Olfr976	5.2	Y	Olfactory receptor

TABLE 2. Genes up-regulated at least five-fold in *Nobox* knockout ovaries.

Gene	Fold change	Germ cell specific	Function
Krt34	5.4	N	Keratin complex, cytoskeleton
A930031D07Rik	5.4	N	Unknown
Hsd17b2	5.4	N	Estrogen biosynthesis
Plac1	5.5	N	Unknown, early embryo and placenta
Trib3	5.7	N	Cellular signalling
Tekt2	5.8	Y	Cytoskeleton, testes
9530026P05Rik	6.2	N	Unknown
4933405K07Rik	6.5	Y	Transcription factor, zinc finger, testes
Btn1a1	7.0	N	Unknown, butyrophilin
Slc13a4	7.8	N	Ion transporter
Krt77	7.8	N	Cytoskeleton, keratin
Ehf	8.1	N	DNA binding, transcription
Mat1a	8.3	N	Methionine adenosyltransferase
LOC433110	8.5	N	Unknown
4921517J23Rik	8.5	N	Unknown
1110017I16Rik	8.5	N	Unknown
Gad1	8.7	N	Glutamate decarboxylase
AK136780	9.0	N	Unknown
BB769641	10.0	N	Ion channel
1700019D03Rik	10.4	Y	Unknown, testes specific
Pte2a	10.5	N	Peroxisomal thioesterase
Dmrt1	10.6	Y	Transcription, sexual differentiation
Smyd1	10.8	N	Muscle transcription factor
Stra8	13.7	Y	Retinoic acid induced, meiosis
Grb7	16.9	N	Growth factor receptor bound protein
Asb9	17.6	N	Unknown function
Crabp2	18.9	N	Retinoic acid binding protein
Gjb5	19.9	N	Gap junction protein
Fgf21	31.3	N	Fibroblast growth factor 21
Fgfbp1	35.2	N	Fibroblast growth factor binding protein
Gypa	45.5	N	Glycophorin A, red blood cell antigen
Sftpd	55.2	N	Surfactant associated protein D
2210409E12Rik	66.2	N	Unknown, blastocyst specific

lack of *Nobox* (Fig. 1F). The role of *Lin28* in the pluripotency of the stem cell biology is not clear and awaits further investigations. The expression of *Pou5f1*, *Sall4*, and *Lin28* in the newborn oocytes as well as the downregulation of *Pou5f1* and *Sall4* in the *Nobox*-deficient ovaries signifies that these essential pluripotency networks lie downstream of *Nobox*.

Signaling Proteins

Kit, *Kitl,* and multiple other growth factors have been implicated in the promotion of primordial to primary follicles, but little is understood about oocyte-specific secretory factors that are critical in the formation of germ cell cysts and primordial follicles [9]. We therefore examined whether proteins predicted to be involved in signaling were affected by *Nobox* deficiency. Transcripts corresponding to astacin-like metalloendopeptidase (*Astl*), jagged 1 (*Jag1*), growth differentiation factor 9 (*Gdf9*), fetuin beta (*Fetub*), oocyte-secreted protein 1 (*Oosp1*), and R-spondin 2 homolog (*Rspo2*) were downregulated at least 3-fold (Fig. 1, B and E). *Gdf9* plays a central role in oocyte signaling to the somatic cells and is downregulated in *Nobox*-deficient ovaries [13]. *Gdf9* knockout experiments show primary follicle arrest [34], and *Gdf9* does not appear to be essential in the formation of primordial follicles.

Little is known about the roles of *Astl*, *Oosp1*, *Rspo2*, *Jag1,* and *Fetub* in oogenesis. *Astl* is a member of the astacin family of metalloproteinases and is preferentially expressed in human and mouse oocytes [35]. Although its function has not been determined by knockout, its location and structure suggest that *Astl* is similar to hatching proteases in arthropods and fish. It is therefore likely that *Astl* is a maternal effect gene and does not play significant function in early folliculogenesis. *Oosp1* is exclusively expressed in oocytes, but its function is unknown [36]. Functions of *Fetub*, *Jag1*, and *Rspo2* in oogenesis are currently unknown, and future studies are necessary to determine the role of these genes in early folliculogenesis. Therefore, it is clear that transcripts encoding several secreted proteins are downregulated in *Nobox*-deficient ovaries, and some of these proteins may be critical in oocyte-somatic communication required for the breakdown of germ cell cysts and the formation of primordial follicles. Future studies on these proteins may improve the efficiency of in vitro maturation of oocytes.

Nlrp *and* Oas *Family of Gene Expression in* Nobox *Knockout Ovaries*

Nlrp genes belong to a large family of genes that encode proteins involved in apoptotic and inflammatory signaling pathways. Several *Nlrp* genes play critical functions in humans and mice. *Nlrp5* (also known as *Nalp5* and *Mater*) is a maternal effect gene essential for embryonic development past the two-

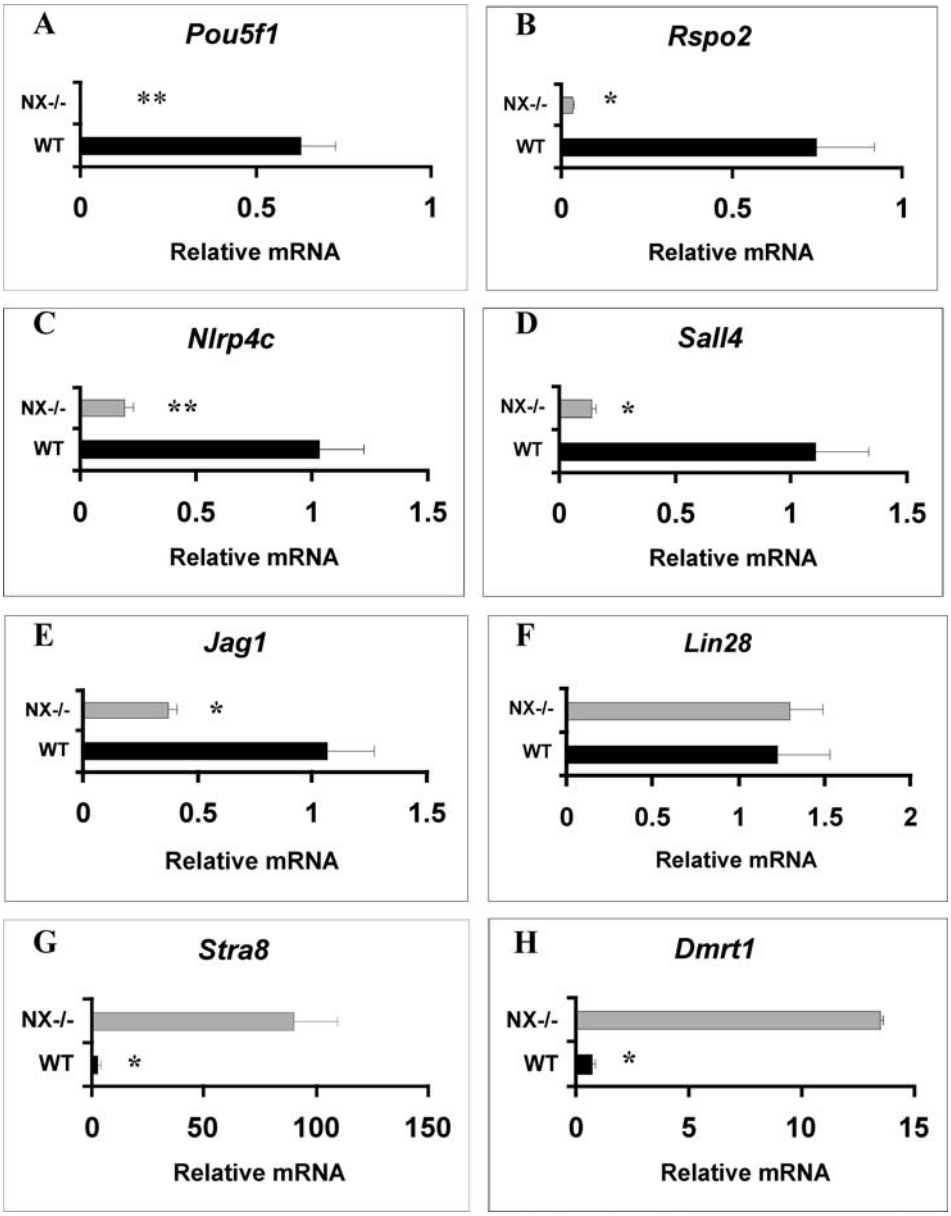

FIG. 1. Expression of *Pou5f1* (**A**), *Rspo2* (**B**), *Nlrp4c* (**C**), *Sall4* (**D**), *Jag1* (**E**), *Lin28* (**F**), *Stra8* (**G**), and *Dmrt1* (**H**) transcripts in the newborn wild-type (WT) and *Nobox*-deficient (Nx−/−) ovaries was quantitated by qPCR. *Pou5f1, Rspo2, Nlrp4c, Sall4,* and *Jag1* show statistically significant downregulation in mutant ovaries, whereas *Stra8* showes up regulated in $Nobox^{-/-}$ ovaries. Expression of *Lin28* showed no statistically significant difference between WT and $Nobox^{-/-}$ ovaries ($P > 0.05$). Data are normalized to *Gapdh* expression and presented as the mean relative quantity (compared to the wildtype). Student *t*-test was used to calculate *P* values. $^{*}P < 0.05$; $^{**}P < 0.01$.

cell stage [37]. *NLRP7* (also known as *NALP7*) is a human maternal effect gene important in recurrent spontaneous abortions and molar pregnancies [38], whereas *Nlrp3* (also known as *Nalp3*) dysfunction causes multisystemic inflammation. Several *Nlrp* genes are expressed preferentially in germ cells and include *Nlrp14*, *Nlrp4f*, and *Nlrp4c* [39]. *Nlrp14* is preferentially expressed in oocytes and spermatogonia, and mutations in *Nlrp14* can be found in men with spermatogenic failure [40, 41]. However, little is known about the in vivo roles for *Nlrp14*, *Nlrp4f,* and *Nlrp4c* in germ cell biology, as knockout mouse models have not been reported. *Nlrp14* is drastically downregulated in *Nobox* newborn ovaries (Fig. 2, A and B) as well as *Nlrp4f* and *Nlrp4c* (Figs. 2A and 1C). Whether *Nlrp14*, *Nlrp4f,* or *Nlrp4c* is critical in early folliculogenesis or, like *Nlrp5,* acts as a maternal effect gene, awaits generation of animals that lack these genes.

Oas1d, *Oas1c*, *Oas1e*, and *Oas1h* belong to a 2′,5′-oligoadenylate synthetase (OASs) family of genes. OAS family expression is highly induced by alpha/beta interferons as a response to viral infection. OAS proteins bind double-stranded RNA produced by virally infected cells, which ultimately leads to global inhibition of protein synthesis that blocks viral proliferation [42]. The unique feature of *Oas1d, Oas1c, Oas1e,* and *Oas1h* genes is their restricted expression to oocytes [27, 43]. In addition, *Oas1d*, *Oas1c*, *Oas1e*, and *Oas1h* comprise a subfamily of OAS proteins that can bind double-stranded RNA but lack OAS activity [43]. It therefore appears that these oocyte-specific OASs act by blocking OASs involved in interferon response and protect oocyte depletion from interferon-induced cell death, a property that oocytes share with embryonic stem cells [44]. *Oas1d*, *Oas1c*, *Oas1e*, and *Oas1h* proteins may therefore act to blunt oocytes' responses to an inflammatory milieu that characterizes folliculogenesis and ovulation, and therefore to stem oocyte loss to interferon-like responses. All four oocyte-specific OASs are downregulated in *Nobox* knockout newborn ovaries (Fig. 1, A and B), and it is tempting to speculate that such downregulation predisposes *Nobox*-deficient oocytes to premature death. Our findings that oocyte-specific OASs and NLRP proteins are downregulated in *Nobox*-deficient mice may signify that OAS and NLRP proteins play critical functions in oocyte survival. *Bax*, *Bcl2l2*, and *Casp2* expressions do not significantly differ between

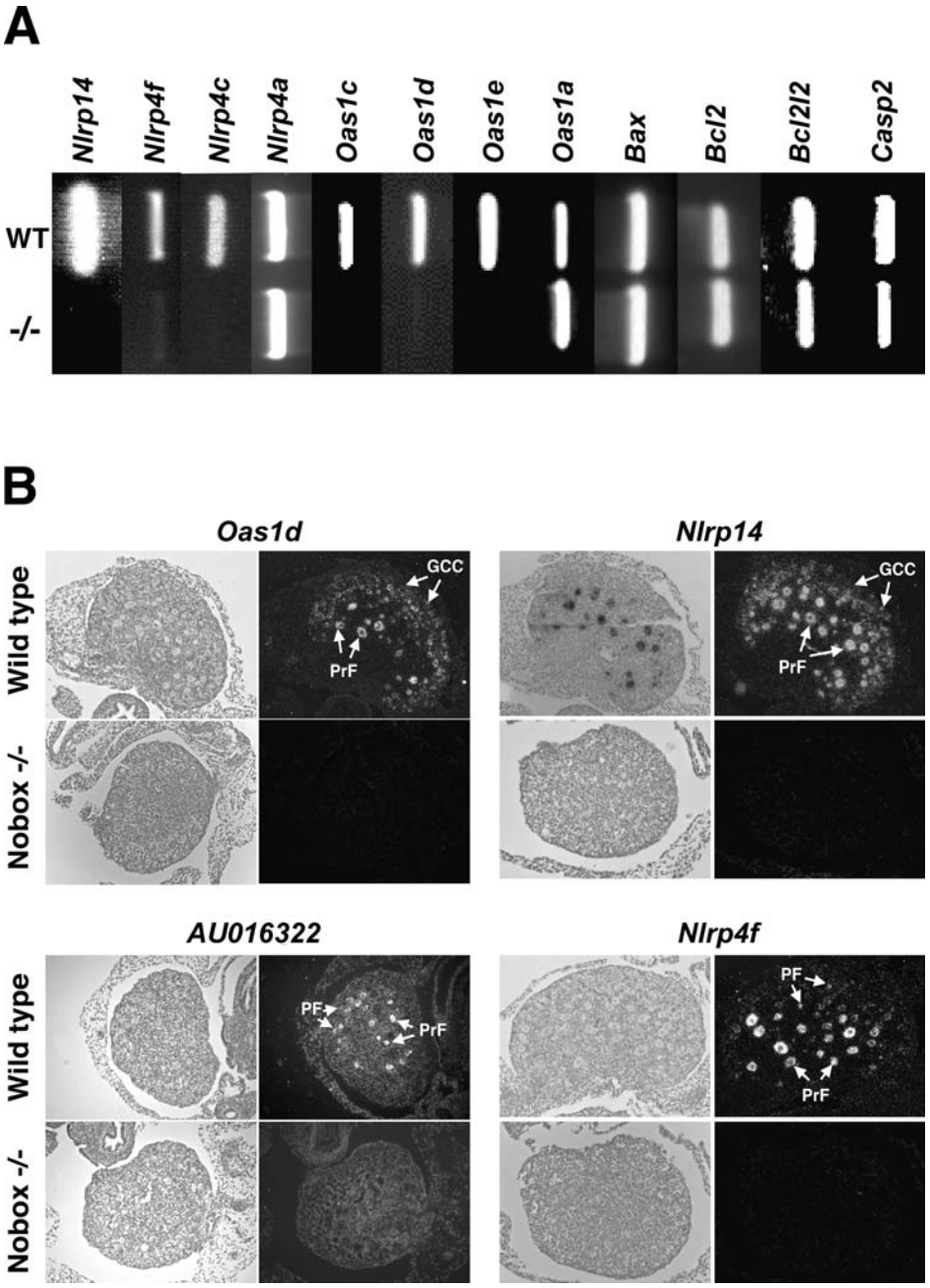

FIG. 2. Differential expression of select genes in wild-type (WT) and *Nobox* knockout ovaries. **A**) Semiquantitative RT-PCR on cDNA synthesized from WT and *Nobox* knockout (−/−) ovaries with oligonucleotides corresponding to specific genes. **B**) In situ hybridization with *Oas1d, Nlrp14, Nlrp4f,* and *AU016322* riboprobes on newborn WT and *Nobox* knockout ovaries. Bright field images are on the left, and dark field images are shown on the right. Germ cell cysts (GCC), primordial follicles (PF), and primary follicles (PrF) are indicated with arrows on the dark field images. Original magnification ×20.

Nobox knockout and wild-type ovaries (Fig. 2A), and oocyte-specific OAS proteins and NLRP proteins may be more important than other members of the classic apoptotic pathways in regulating oocyte death and survival.

Nobox *Binding Elements Discovered with Protein-Binding Microarray*

We used protein-binding microarrays to identify nucleotide motifs that NOBOX homeodomain preferentially binds [14, 23, 24]. NOBOX homeodomain was expressed as a GST fusion protein, and affinity to all possible 10-mers was assessed by protein binding microarrays. A position weight matrix shows a consensus for NOBOX-binding sequence (Fig. 3A). We analyzed 2 kb of nucleotide sequences upstream of genes downregulated more than 5-fold in the *Nobox* knockout ovaries for the presence of NOBOX DNA-binding sequences discovered by the protein-binding microarray. We have previously shown that NOBOX can bind these elements in the promoters of *Gdf9* and *Pou5f1* promoters [26]. In addition to *Gdf9* and *Pou5f1*, a number of other downregulated genes, including *Nlrp14*, *Nlrp4c*, and *Sall4*, as well as secretory molecules *Oosp1* and *Astl*, contain NOBOX DNA-binding sequences (Fig. 3C).

Among downregulated genes that contain NOBOX-binding elements is *Olfr976*. *Olfr976* is an olfactory-like receptor preferentially expressed in oocytes [27] and downregulated more than 5-fold in the *Nobox* knockout ovaries. Olfactory receptors have been described in spermatozoa [45], although their precise role in sperm chemotaxis is unknown. Unlike testicular olfactory receptors, little is known about olfactory receptors in oogenesis, and expression of *Olfr976* in the cell type specific fashion argues that it may have an important function in the biology of the oocyte. Whether *Olfr976* acts during folliculogenesis or as a maternal effect gene is not known.

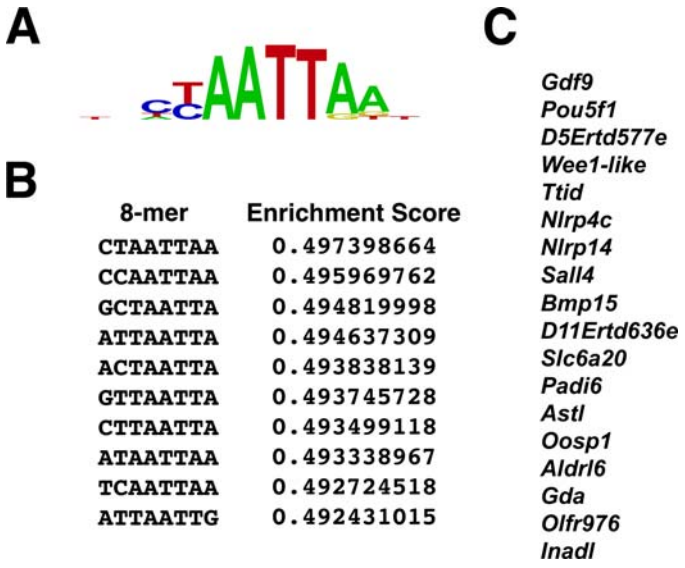

FIG. 3. *Nobox* binding elements determined by protein binding microarray and potential gene targets. **A**) NOBOX binding motif determined by position weight matrix. **B**) Top ten 8-mer sequences bound by NOBOX. **C**) List of genes that are downregulated in *Nobox* knockout ovaries and contain NOBOX binding motif within 2 kb upstream of the transcription initiation sites.

Genes Upregulated in Nobox*-Deficient Ovaries*

We analyzed genes that are significantly upregulated in the *Nobox* knockout ovaries (Table 2). The number of genes preferentially expressed in germ cells that were upregulated more than 5-fold was 15%, compared with 74% that were downregulated. A number of genes that are normally not expressed in wild-type newborn ovaries are significantly expressed in *Nobox* knockout newborn ovaries. *Dmrt1*, doublesex, and mab-3-related transcription factor 1 are not expressed in wild-type newborn ovaries. *Dmrt1* is exclusively expressed in the somatic component of the genital ridge prior to sexual differentiation and plays a critical role in male gonadal development [46, 47]. *Dmrt1* is overexpressed in *Nobox* knockout ovaries, which argues that oocyte-specific transcription factor NOBOX can repress male-determining gene expression (Fig. 1H). Other genes preferentially expressed in testes, such as *Tekt2*, 1110020C03Rik, and 1700019D03Rik [27], are upregulated more than 5-fold in the *Nobox* knockout compared with the wild-type ovary (Table 2). *Tekt2* is essential for male fertility [48], whereas the roles of 1110020C03Rik and 1700019D03Rik are unknown. Other male-determining genes such as *Wt1*, *Dhh*, *Sox9*, *Gata4,* and *Fgf9*, do not show significant differences between the wild-type and *Nobox* knockout ovaries. *Dmrt1* is expressed in germ cells, unlike other sex-determining genes such as *Sox9* and *Gata4,* whose expression is confined to the somatic cells [49]. *Dmrt1*, *Tekt2,* and *1700019D03Rik* are preferentially expressed in adult testis germ cells [27] and contain NOBOX-binding elements upstream of the transcription initiation sites.

Reexpression of male-determining genes has been observed in *Foxl2* knockout ovaries [50]. *Foxl2* is preferentially expressed in the pregranulosa and granulosa soma of the ovary and causes BPES I and II syndromes [51]. Our results indicate that transcription factors preferentially expressed in oocytes may repress male-determining gene expression.

The LH-responsive hydroxysteroid (17-beta) dehydrogenase 2 (*Hsd17b2*) is not significantly expressed in newborn mouse ovaries, but in the *Nobox* knockout ovaries *Hsd17b2* expression rises more than 5-fold. *Hsd17b2* plays an important role in the interconversion of estradiol and estrone. It therefore appears that the wild-type postnatal oocyte represses expression of *Hsd17b2* either directly or indirectly. The overexpression of mainly somatic genes in the *Nobox* knockout ovary likely represents a homeostatic response to the globally dysfunctional oocytes and disrupted signaling pathways.

TABLE 3. Most abundant miRNAs in newborn ovaries.

MicroRNA	Wild-type	*Nobox* knockout
mmu-miR-709	64820.77 ± 280	42607.40 ± 373
mmu-miR-199a	43258.45 ± 445	37270.72 ± 700
mmu-miR-26a	42654.28 ± 424	39237.99 ± 1350
mmu-let-7a	39375.93 ± 430	37097.84 ± 370
mmu-let-7c	34688.28 ± 262	33247.38 ± 361
mmu-miR-125b	34028.07 ± 377	34136.80 ± 129
mmu-let-7f	33521.84 ± 296	31219.44 ± 670
mmu-miR-214	33381.03 ± 454	31035.26 ± 840
mmu-let-7d	30847.33 ± 484	28948.56 ± 337
mmu-miR-125a	30349.96 ± 285	29061.93 ± 306
mmu-let-7b	29439.24 ± 280	28293.15 ± 798
mmu-let-7e	29407.07 ± 352	27413.03 ± 568
mmu-miR-26b	25546.49 ± 277	24086.31 ± 738
mmu-let-7g	25465.95 ± 313	23758.09 ± 165
mmu-miR-126–3p	23983.26 ± 280	23751.91 ± 146
mmu-miR-689	23526.88 ± 502	20545.65 ± 303
mmu-let-7i	23438.35 ± 198	22021.38 ± 462
mmu-miR-335	22889.06 ± 347	21242.61 ± 475
mmu-miR-23b	20165.20 ± 346	23919.92 ± 399
mmu-miR-690	19935.90 ± 362	19297.05 ± 269

MicroRNA Expression in Nobox*-Deficient Ovaries*

MicroRNAs (miRNAs) are known to play important functions in vertebrate differentiation and development. MicroRNAs are usually 21–25 nucleotides long and appear to regulate mRNA functions on various levels, including translation [52]. Little is known about miRNA expression in newborn mouse ovaries or transcriptional regulators that control miRNA expression. We therefore studied whether miRNA expression in *Nobox* knockout ovaries differs significantly from that in wild-type newborn ovaries. We performed microarray analysis on 373 known mouse miRNAs, which represent known miRNA transcripts from the Sanger miRBase Release 9.0 [53]. A total of 177 of 373 miRNAs were significantly expressed in wild-type mouse newborn ovaries (signal greater than 250). The most abundant miRNAs include mir-709, which was recently detected as a novel miRNA during microRNA profiling of mouse embryos [54]. Let-7, a founding member of the miRNA family, is critical in cell fate determination in *Caenorhabditis elegans* [55]. Let-7 miRNA family transcripts are highly expressed in the newborn ovary (Table 3). An approximately 2-fold decrease in the Nobox knockout compared with wild-type ovaries was shown in mir-801, let-7d, mir-346, and mir-699 (Table 4). Changes in miRNA are relatively small compared with *Nobox* effects on the mRNA expression. Comprehensive sequencing of miRNA transcripts in various tissues is ongoing, and mouse oocyte miRNA transcriptome is currently unknown. Tissue-specific miRNAs exist [56], and oocyte-specific miRNAs are therefore likely.

DISCUSSION

The microarray analysis of the wild-type and *Nobox* knockout newborn ovary reveals disruption in the expression of many germ cell-specific genes, with wide-ranging functions,

TABLE 4. Differentially expressed miRNAs.

MicroRNA	Fold change	Wild-type	*Nobox* knockout
mmu-miR-801	−2.2	195 ± 30	88 ± 15
mmu-let-7d	−1.9	1361 ± 211	744 ± 113
mmu-miR-346	−1.8	1703 ± 217	919 ± 107
mmu-miR-699	−1.8	238 ± 26	134 ± 18
mmu-miR-200c	1.7	383 ± 39	640 ± 75
mmu-miR-122a	−1.6	520 ± 65	304 ± 49
mmu-miR-709	−1.5	66422 ± 3635	42963 ± 3487
mmu-miR-451	1.5	4497 ± 315	6463 ± 507
mmu-miR-30e	1.4	1625 ± 171	2289 ± 240
mmu-miR-301	1.4	1239 ± 98	1671 ± 137
mmu-miR-374-5p	−1.4	4441 ± 309	3489 ± 311
mmu-miR-671	−1.4	5526 ± 312	3875 ± 297
mmu-miR-350	1.4	829 ± 114	1066 ± 131
mmu-miR-720	1.3	1187 ± 49	1584 ± 89
mmu-miR-199b	1.3	2283 ± 116	3001 ± 310
mmu-miR-98	−1.3	5266 ± 1083	4066 ± 838
mmu-miR-805	−1.3	4992 ± 405	3972 ± 411
mmu-miR-495	−1.3	2989 ± 218	2305 ± 274
mmu-miR-99a	1.3	6698 ± 502	8426 ± 582
mmu-miR-30a-5p	1.3	8287 ± 543	9496 ± 736

many novel and yet uncharacterized. The function of a large subset of these germ cell-specific genes, such as *Nlrp14*, *Nlrp4f* and *Nlrp4c*, *Olfr976*, and *Astl*, remains to be determined. Some of these genes may function as maternal effect genes and, as such, play little of a role in early folliculogenesis. Other genes, such as *Gdf9* and *Bmp15*, which are downregulated in *Nobox* knockouts, play significant roles during the growth phase of ovarian follicles [34, 57].

Pluripotency genes *Sall4* and *Pou5f1* are expressed early during oogenesis. Whether these transcription factors are necessary for folliculogenesis or postfertilization is unknown. Mammalian oocytes can differentiate into many tissue types via parthenogenesis [58], and therefore molecules such as *Sall4* and *Pou5f1* may be important mediators of oocyte's totipotency. Whether other unknown molecules are distinctly necessary for oocyte totipotency versus embryonic stem cell pluripotency remains to be determined.

Using protein-binding microarrays we have discovered 8-mer sequences that NOBOX homeodomain preferentially binds. The top ten 8-mer sequences can be found in 18 (49%) of the 37 most downregulated genes and include genes such as *Sall4*, oocyte-specific *Nlrp* genes, *Pou5f1*, and *Olfr976*. Whether these elements are functional is difficult to assess due to the lack of oocyte cell lines. We have previously reported that *Pou5f1* and *Gdf9* promoters can bind NOBOX by immunoprecipitating wild-type newborn ovary chromatin using anti-NOBOX antibodies and amplifying genomic regions corresponding to putative NOBOX binding sites in the *Pou5f1* and *Gdf9*. It is therefore likely that a number of potential NOBOX binding sites are functional. Preferential downregulation of numerous oocyte-specific genes in *Nobox*-deficient ovaries indicates that NOBOX is a transcriptional activator of oocyte-specific gene expression.

Genes essential to female determination are not well understood. Both somatic and germline components are likely to play key roles in ovarian determination. *Foxl2* is a transcription factor preferentially expressed in pregranulosa and granulosa cells in the mammalian ovary, and its deficiency causes ovarian failure as well as reexpression of genes such as *Sox9*, *Fgf9,* and *Dmrt1* [50]. It is unclear whether *Foxl2* is truly required for commitment to ovarian differentiation, or whether observed reexpression of male-determining genes in *Foxl2*-deficient ovaries results from general response to disrupted oocyte development. Unlike *Foxl2*, *Nobox* is exclusively expressed in oocytes [12]. Reexpression of Dmrt1 (>10-fold increase over wild-type newborn ovary) in the *Nobox* newborn knockout ovaries, which still contain many oocytes, indicates that oocyte-specific transcription pathways also play a key role in repressing male sexual differentiation pathways. The role of germ cell-specific transcription factors in sexual differentiation is poorly understood [59]. Identification and characterization of transcription factors that act upstream of *Nobox* will help delineate early germ cell-specific pathways that contribute to the molecular basis of ovarian determination.

ACKNOWLEDGMENT

We thank Stephanie Pangas for critical reading of the manuscript.

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