

# Premature ovarian failure in nobox-deficient mice is caused by defects in somatic cell invasion and germ cell cyst breakdown

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## Abstract

**Purpose** To understand the mechanism of premature ovarian failure (POF).

**Methods** The ultrastructural (electron microscopy) analysis of primordial ovarian follicles in Nobox deficient mice.

**Results** We studied, for the first time, the fate of oogonia in embryonic (prenatal) mouse ovaries and showed that the abolishment of the transition from germ cell cysts to primordial follicles in the ovaries of Nobox deficient mice is caused by defects in germ cell cyst breakdown, leading to the formation of syncytial follicles instead of primordial follicles.

**Conclusions** These results indicate that POF syndrome in Nobox deficient mice results from the faulty signaling

between somatic and germ line components during embryonic development. In addition, the extremely unusual and abnormal presence of adherens junctions between unseparated oocytes within syncytial follicles indicates that faulty communication between somatic and germ cells is involved in, or leads to, abnormalities in the cell adhesion program.

**Keywords** Premature ovarian failure · Nobox · Homeobox · Ovary · Germ cells

## Introduction

The newborn ovary homeobox gene (*nobox*) is an oocyte-specific homeobox gene expressed in germ cell cysts, as well as primordial and growing oocytes. The proper expression of NOBOX is crucial for ovarian development in both mice and humans [1–10]. *Nobox* deficiency in mice and perturbation of NOBOX homeodomain in women result in the premature ovarian failure (POF) syndrome characterized by postnatal oocyte loss and the replacement of follicles by fibrous tissue [1, 2, 6, 8, 10]. In mice, as in the majority of other animal species, mitotic divisions of primary oogonia, followed by incomplete cytokinesis, lead to the formation of germ cell cysts (nests, clusters) containing several oocytes connected by intercellular bridges [11–13]. Subsequently, the germ cell cysts break apart: somatic cells invade the cysts and separate and surround individual oocytes, resulting in the formation of individual ovarian follicles, which, in mammals, are called primordial follicles [11–13]. Histological studies of oocyte development in mice between the newborn stage and day 14 after birth showed that ovaries of *Nobox*<sup>+/-</sup> and *Nobox*<sup>-/-</sup> newborns have similar overall numbers of germ cell cysts

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**Capsule** Premature ovarian failure (POF) syndrome in Nobox deficient mice results from the faulty signaling between somatic and germ line components during embryonic development.

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and oocytes, indicating no overall germ cell loss [7]. However, *Nobox*<sup>-/-</sup> ovaries had much more oocytes in the form of germ cell cysts than those in the form of primordial follicles [7]. At day 7 after birth, *Nobox*<sup>-/-</sup> ovaries still had the majority of oocytes in the form of cysts, with only few primordial follicles and no secondary follicles; by day 14, most oocytes had been degenerating [7]. Although these light microscopy (histological) analyses indicate that primordial follicle formation and primordial follicle pool are highly deficient in *Nobox*<sup>-/-</sup> ovaries, there are no studies on the events taking place in embryonic (prenatal) ovaries and the underlying mechanism of this deficiency remains elusive. The ultrastructural analysis of developing embryonic ovaries of *Nobox*<sup>+/-</sup> and *Nobox*<sup>-/-</sup> mice performed in our study indicates that the primordial follicle deficiency in *Nobox*<sup>-/-</sup> mice originates during embryonic development of the ovary from the inability of somatic cells to invade the cysts and to separate and surround individual oocytes, thus resulting in defects in the formation of individual primordial follicles.

## Material and methods

### Animals

All murine experiments were carried out on the C57BL/6 × 129S6/SvEv hybrid background. Litters were weaned at 3 wk of age, and breeding pairs were set up at 6 wk of age. All procedures described within were reviewed and approved by the Baylor College of Medicine Institutional Animal Care and Use Committee, and all experiments were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals.

### Fixation

Embryonic gonads isolated from *Nobox*<sup>+/-</sup> and *Nobox*<sup>-/-</sup> mice embryos at embryonic day 16.5–17.5 (E16.5–E17.5) were fixed in 2% formaldehyde, 3% glutaraldehyde (both EM grade from Ted Pella Inc., Redding, CA, USA; glutaraldehyde: 8% stock, cat# 18421; formaldehyde: 16% stock, cat# 18505) in 1× phosphate buffered saline (PBS) in molecular-grade water, for 2 h at room temperature.

### Embedding and sectioning

Samples were contrasted with 0.5% uranyl acetate and osmium tetroxide, dehydrated in ethanol at increasing concentrations, infiltrated, and embedded in LX-112 medium (Epon substitute; Ladd Research Industries, Burlington, VT, USA). The samples were polymerized in an oven at 70°C for 2 days. The blocks were cut with a Reichert Ultracut (Reichert

Jung, Wien, Austria). Semithin sections (0.7–1.0 μm) were stained with 1% methylene blue in 1% borax and examined with a Leica DMR (Leica, Wetzlar, Germany) microscope. Ultrathin sections (70–100 nm) were contrasted with uranyl acetate and lead citrate according to standard protocols [14]; the sections were then examined in a JEOL 100SX transmission electron microscope (JEM, Japan) at an accelerating voltage of 80 kV.

### Tex14 immunostaining

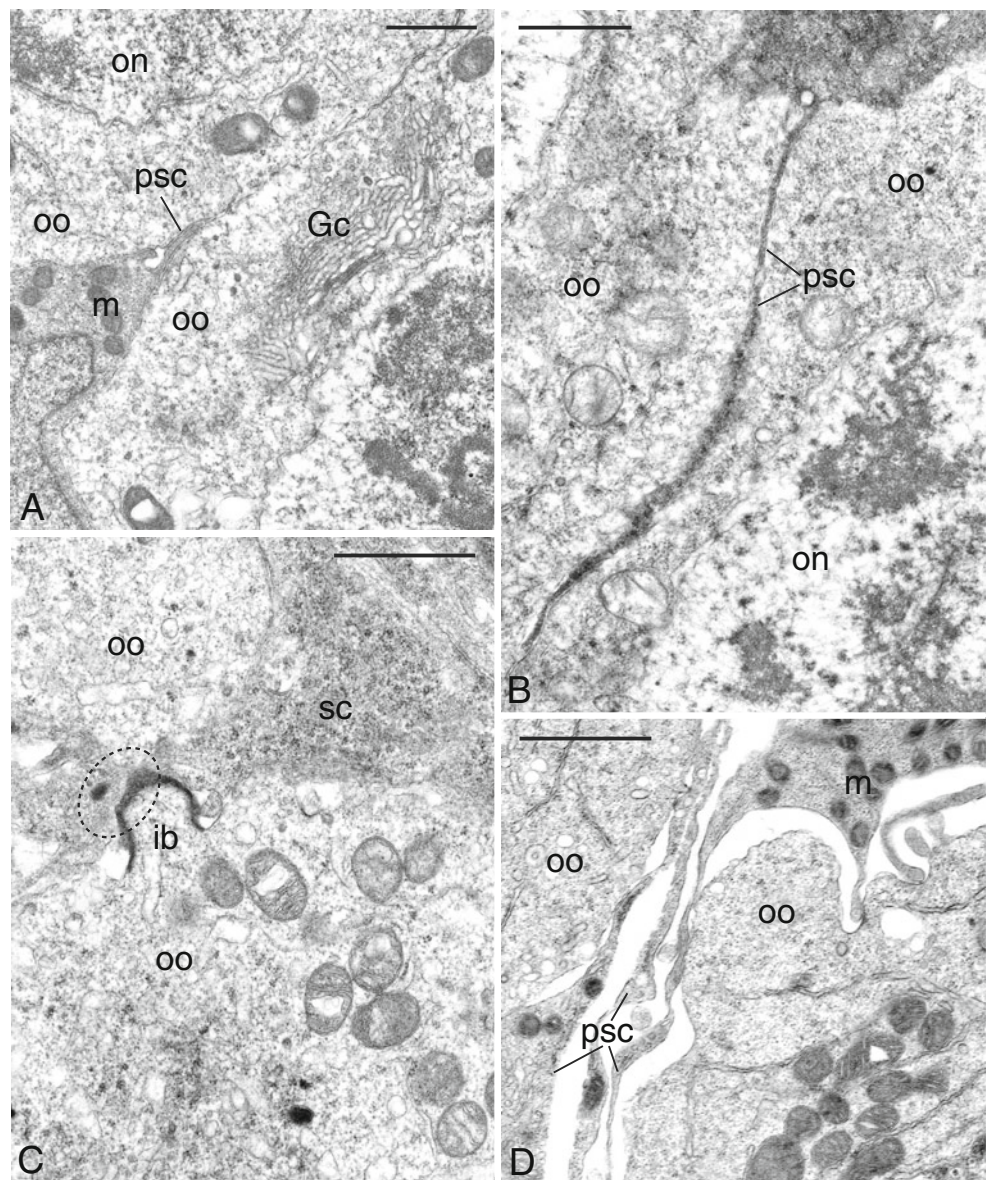
Ovaries were fixed in 4% paraformaldehyde in Tris-buffered saline (TBS), processed, and stained as previously described [15]. Five sections from five mice for each genotype were examined; the sections were obtained from E16.5–17.5 and P0 and P2. Representative images were selected. Tissue samples were circled with a PAP pen (Zymed, South San Francisco, CA) and blocked in TBS plus 3% bovine serum albumin (BSA) for 1 h at room temperature. Antibodies were diluted in TBS plus 3% BSA and used for overnight incubation at 48°C at the following dilution: goat anti-TEX14 1:300 (obtained from Dr. Greenbaum at Baylor College of Medicine). Alexa-555 secondary antibodies were purchased from Molecular Probes (Eugene, OR). Samples were mounted using the Prolong Gold mounting media with 40, 60-diamidino-2-phenylindole (DAPI) (Invitrogen, San Diego, CA) and examined on a Zeiss microscope (Carl Zeiss MicroImaging, Thornwood, NY).

## Results

### *Nobox*<sup>+/-</sup> ovaries

The electron microscopy analysis showed that the ultrastructure of ovaries from E.5–17.5 and P0 heterozygous (*Nobox*<sup>+/-</sup>) mice does not differ from that of ovaries from wild-type mice. As in the wild-type mice, the ovaries of *Nobox*<sup>+/-</sup> E16.5–17.5 contained numerous germ cell cysts containing early oocytes connected by intercellular bridges. The cysts were surrounded by elongated somatic cells whose slender projections penetrated between plasma membranes of neighboring oocytes belonging to the same cyst (Fig. 1a, b). Very often, the basal part of somatic cell projections contained groups of mitochondria (Fig. 1a). The *Nobox*<sup>+/-</sup> ovaries from P0 mice contained not only oocyte cysts but also individual oocytes surrounded by somatic cells (i.e., primordial ovarian follicles). In the cysts of these ovaries, somatic cell projections were often observed in direct contact with the intercellular bridges interconnecting the oocytes (Fig. 1c). Careful examination of the sections tangential to the bridge surface showed that the projections contained parallel arrays of microfilaments (Fig. 1c, circle).

**Fig. 1** *Nobox*<sup>+/-</sup> ovaries. (**a**, **b**) E17.5. Projections of somatic cells (psc) penetrating between plasma membranes of connected oocytes (oo); Golgi complex (Gc); mitochondria (m); oocyte nucleus (on). (**c**) P0. An intercellular bridge (ib) surrounded by broad projections of somatic cells (sc); the projections containing arrays of microfilaments (encircled); the section tangential to the bridge surface. (**d**) P0. Two neighboring primordial ovarian follicles; the oocytes (oo) completely separated by somatic cell projections (psc); mitochondria (m). Scale bar is equal to 1  $\mu$ m



The ultrastructural analysis of the events occurring during germ cell cyst breakdown and the formation of primordial ovarian follicles showed that the progressing penetration of somatic cell projections between the oocytes within the cysts led to the constriction of the intercellular bridges, which correlated with the breakdown of the cysts, the separation of individual oocytes, and eventual formation of the primordial ovarian follicles (Fig. 1d). All these observations clearly indicate that the process of oocyte separation leading to the breakdown of the cysts and the formation of primordial follicles in *Nobox*<sup>+/-</sup> mice does not differ from the events occurring in wild-type mice.

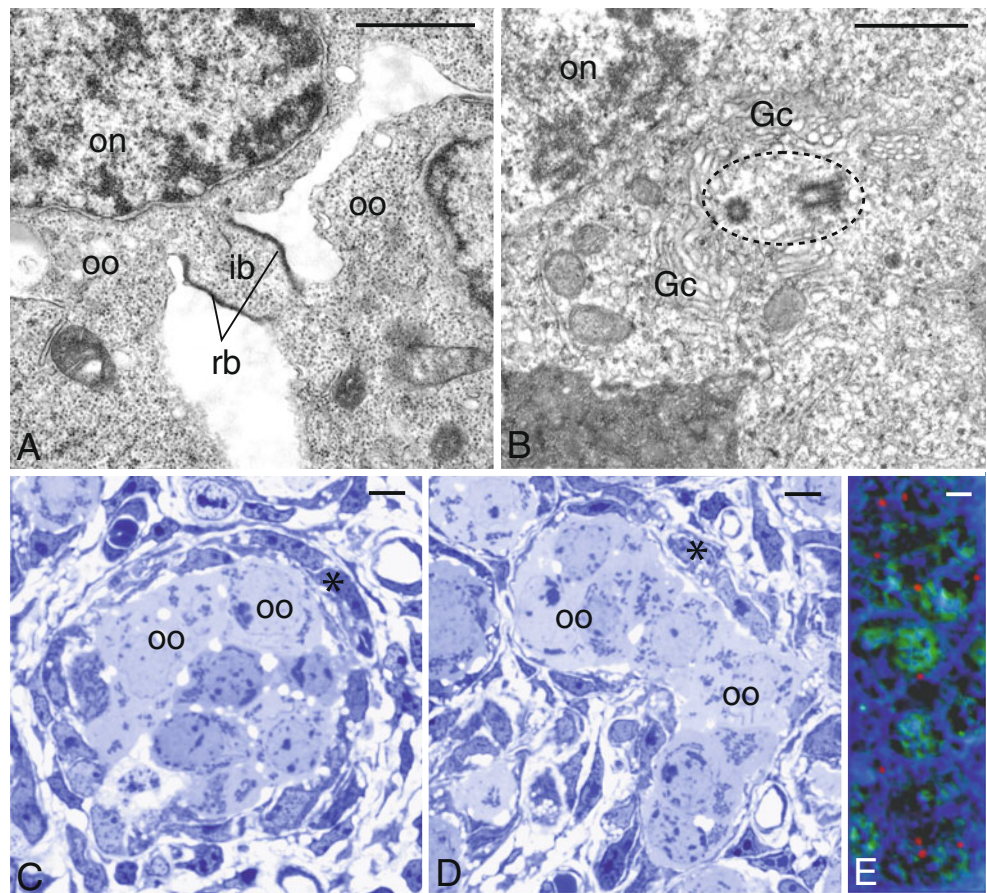
#### *Nobox*<sup>-/-</sup> ovaries

The ultrastructural analysis of the ovaries from E 16.5–17.5 *Nobox*<sup>-/-</sup> mice showed that they contained germ cell cysts

composed of early oocytes connected by intercellular bridges with characteristic electron-dense rims (Fig. 2a). We also found that the oocytes present in the cysts contained the organelles typical for wild-type oocytes, including Balbiani bodies that are composed of several Golgi complexes surrounding a more or less centrally located pair of centrioles (i.e., the centrosome) (Fig. 2b). Immunostaining with an antibody against TEX14, a novel protein that localizes to germ cell intercellular bridges and is indispensable for the formation of the bridges [16], showed that similar to those of wild-type mice, the bridges of *Nobox*<sup>-/-</sup> mice contain TEX14 protein (Fig. 2e). The analysis of P0 and P2 mice showed that, in contrast to wild-type and *Nobox*<sup>+/-</sup> mice, the ovaries of *Nobox*<sup>-/-</sup> mice contained, instead of broken-down cysts and primordial follicles, the unbroken germ cell cysts in which neighboring oocytes remained unseparated (Fig. 2c, d). As in the wild-type and *Nobox*<sup>+/-</sup> fetal ovaries,



**Fig. 2** *Nobox*<sup>-/-</sup> ovaries. (a) E16.5. A longitudinal section through intercellular bridge (ib); the absence of somatic cell projections between connected oocytes (oo); electron-dense rims of the bridge (rb); oocyte nucleus (on). (b) E17.5. A Balbiani body in the oocyte; a pair of centrioles (the centrosome, encircled) surrounded by large Golgi complexes (Gc); oocyte nucleus (on). (c, d) P0. Syncytial follicles composed of unseparated oocytes (oo) and somatic cells (asterisks). Semithin section, methylene blue. (e) E16.5. Ovaries showing the presence of intercellular bridges (Tex14, red fluorescence) between oocytes. Scale bar is equal to 1  $\mu\text{m}$  in a and b, 10  $\mu\text{m}$  in c and d, and 15  $\mu\text{m}$  in e



the *Nobox*<sup>-/-</sup> oocytes contained typical organelles: characteristic aggregates of mitochondria and rough endoplasmic reticulum (RER) cisternae (Fig. 3a) and well-developed Balbiani bodies (Fig. 3e). However, in some oocytes, the centrosomes, normally positioned in the center of a Balbiani body (see Figs. 4, 5 and 8 in Kloc et al., [12]), were located outside of the Balbiani body, often in the vicinity of oocyte plasma membrane (Fig. 3e and f). Further analysis of *Nobox*<sup>-/-</sup> ovaries showed that their cysts, composed of unseparated oocytes, were surrounded by two to three layers of somatic cells (Fig. 2c and d). Such conglomerates of germ and somatic cells, which were never observed in wild-type or *Nobox*<sup>+/-</sup> ovaries, were named “syncytial follicles”. The somatic cells of syncytial follicles were equipped with few short projections (Fig. 3b). In contrast to the wild-type and *Nobox*<sup>+/-</sup> somatic cell projections, these projections did not penetrate between the connected oocytes but remained in contact with the surface of unbroken cysts (Fig. 3b, d and f). The contact zones between the cellular membranes of somatic cells and unseparated oocytes showed prominent adherens junctions (Fig. 3b, d and f, arrows). Surprisingly, similar adherens junctions were also present between the cellular membranes of neighboring oocytes (Fig. 3c, arrows). Although the adherens junctions were also present in wild-

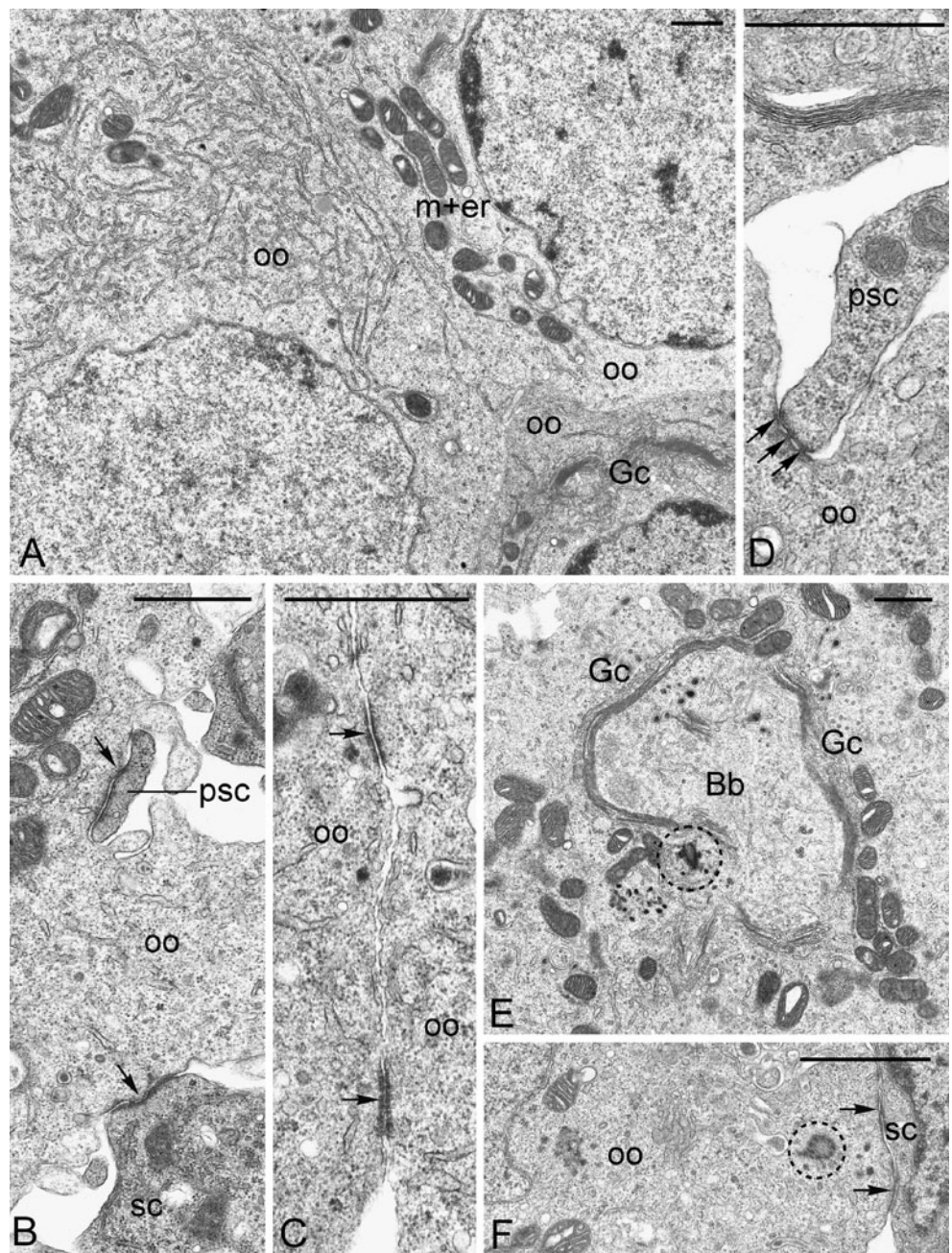
type and *Nobox*<sup>+/-</sup> ovaries, they were always much more limited in number and were never present between the oocytes. In summary, our analysis showed that in *Nobox*<sup>-/-</sup> mice, the processes of germ cell cyst breakdown and oocyte separation are severely impaired, resulting in the impairment of the formation of primordial ovarian follicles that are replaced by the complex syncytial follicles containing unseparated oocytes.

## Discussion

The POF syndrome characterized by postnatal oocyte loss and the replacement of follicles by fibrous tissue occurs in *Nobox* deficient mice and in women bearing NOBOX homeodomain mutations [1, 2, 6, 8, 10]. The light microscopy (histological) analysis in mice indicates that primordial follicle formation and primordial follicle pool are highly deficient in *Nobox*<sup>-/-</sup> ovaries [7]; however, the underlying mechanism of this deficiency remains elusive. Our present study showed that *Nobox* deficiency results in the faulty development of germ cell cysts during the embryonic development, leading to the formation of highly abnormal syncytial follicles.



**Fig. 3** *Nobox*<sup>-/-</sup> ovaries. (a–f). P0. (a). Three adjacent oocytes from the syncytial follicle shown in Fig. 2c; Golgi complex (Gc); aggregations of mitochondria and RER cisternae (m+er); the absence of somatic cell projections between plasma membranes of neighboring oocytes. (b) Adherens junctions (arrows) between plasma membranes of somatic cells (sc) or their projections (psc) and the oocyte (oo). (c) Adherens junctions (arrows) connecting plasma membranes of adjacent oocytes (oo). (d) Three adherens junctions (arrows) connecting the tip of somatic cell projection (psc) and the oocyte plasma membrane (oo). (e) A Balbiani body (Bb) in the oocyte; centriole (encircled); Golgi complex (Gc). (f) A centriole (encircled) in the vicinity of the oocyte plasma membrane; large adherens junctions (arrows) between the membrane of the oocyte (oo) and somatic cell (sc). Scale bar is equal to 1 μm



Although the morphology and ultrastructure of germ cells (oocytes), as well as the ultrastructure and molecular composition [presence of *Tex14* protein, 15, 16] of intercellular bridges connecting oocytes within germ cell cysts, remains mostly unaffected by *Nobox* deficiency, the morphology and the behavior of somatic cells surrounding germ cell cysts are highly abnormal. In wild type mice and *Nobox* heterozygotes, the somatic cells surrounding the cysts produce long projections that penetrate between cyst cells (young oocytes), leading to the breakage of the cytoplasmic bridges connecting oocytes and the separation of individual oocytes. Subsequent further invasion of

somatic cells between the oocytes correlates with the breakdown of the cysts into individual oocytes surrounded by somatic cells, leading to the formation of individual primordial follicles. In *Nobox* deficient mice, somatic cells have a limited number of very short projections that are obviously unable to penetrate between the oocytes. This results in the formation of highly abnormal syncytial follicles composed of clusters of unseparated oocytes surrounded by 2–3 layers of somatic cells. It is clear that these defects in germ cell cyst breakdown are responsible for their inability to form normal primordial follicles and the eventual POF phenotype observed in *Nobox* deficient

mice. We also observed the presence of adherens junctions between unseparated oocytes within the syncytial follicles in *Nobox* deficient mice, which is extremely unusual and abnormal. In normal ovaries of all animal species, various types of cell-cell junctions (including adherens junctions) are present exclusively between somatic cell membranes or between the membranes of germ cells and somatic cells but their presence have never been observed between the membranes of neighboring germ cells [17, 18]. The presence of adherens junctions between the unseparated oocytes in *Nobox* deficient mice indicates that faulty communication between germ and somatic cells is involved in or leads to the dramatic changes in the cell adhesion program and signaling. Theoretically, the abnormal behavior of somatic cells may be caused either by defects in the somatic tissue itself or by defective signaling between somatic and germ line components of fetal ovaries. However, previous studies showing that NOBOX is exclusively expressed in oocytes [7] suggest that the faulty signal originates from oocytes but not from somatic cells. Previous studies have shown that *Nobox* deficiency results in the downregulation of a plethora of oocyte-specific genes, including *Fgf8* [7]. The protein encoded by *Fgf8* is a member of the fibroblast growth factor (FGF) family involved in a variety of biological processes, including embryonic development, cell growth, morphogenesis, tissue repair, tumor growth, and tumor invasion. The most recent studies showed that *Fgf8* is involved in beta-catenin-mediated signaling and cell adhesion in mouse embryogenesis [19]. Thus, it is possible that the downregulation of certain genes, such as *Fgf8*, results in the inability of *Nobox* deficient oocytes to properly communicate with ovarian somatic cells. Recent studies on hormonal regulation of ovarian development indicate that germ cell cyst breakdown correlates with the drop of maternal estradiol level [20], and that estradiol, progesterone and genistein inhibit cyst breakdown in vivo and in vitro [21]. There is also a study demonstrating that gonadotropin induces the changes in *Nobox* gene expression in mouse ovary [22]. Although further study is needed to understand the exact role of *Nobox* in these processes and in human POF syndrome, our present study indicates that *Nobox* is indispensable for proper development of somatic cell components and/or germline/soma communication in developing prenatal mice ovary.

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**Author contribution** Agnieszka Lechowska processed EM samples and analyzed and interpreted data, Szczepan Bilinski analyzed and interpreted EM data, Youngsok Choi maintained mouse colonies and helped with interpretation of EM, Yonghyun Shin performed immunohistochemistry with Tex14 and interpreted the data, Malgorzata Kloc wrote the manuscript, analyzed data, coordinated study Aleksandar Rajkovic, conception, design and data analysis.

## References

- Choi Y, Rajkovic A. Genetics of early mammalian folliculogenesis. *Cell Mol Life Sci*. 2006a;63:579–90.
- Choi Y, Rajkovic A. Characterization of NOBOX DNA binding specificity and its regulation of Gdf9 and Pou5f1 promoters. *J Biol Chem*. 2006b;281:35747–56.
- Choi Y, Qin Y, Berger MF, Ballow DJ, Bulyk ML, Rajkovic A. A Microarray analyses of newborn mouse ovaries lacking *Nobox*. *Biol Reprod*. 2007;77:312–9.
- Huntriss J, Hinkins M, Picton HM. cDNA cloning and expression of the human NOBOX gene in oocytes and ovarian follicles. *Mol Hum Reprod*. 2006;12:283–9.
- Qin Y, Choi Y, Zhao H, Simpson JL, Chen ZJ, Rajkovic A. NOBOX homeobox mutation causes premature ovarian failure. *Am J Hum Genet*. 2007;81:576–81.
- Qin Y, Shi Y, Zhao Y, Carson SA, Simpson JL, Chen ZJ. Mutation analysis of NOBOX homeodomain in Chinese women with premature ovarian failure. *Fertil Steril*. 2009;91:1507–9.
- Rajkovic A, Pangas SA, Ballow D, Suzumori N, Matzuk MM. NOBOX deficiency disrupts early folliculogenesis and oocyte-specific gene expression. *Science*. 2004;305:1157–9.
- Simpson JL. Genetic and phenotypic heterogeneity in ovarian failure: overview of selected candidate genes. *Ann NY Acad Sci*. 2008;1135:146–54.
- Suzumori N, Yan C, Matzuk MM, Rajkovic A. *Nobox* is a homeobox-encoding gene preferentially expressed in primordial and growing oocytes. *Mech Dev*. 2002;111:137–41.
- Suzumori N, Pangas SA, Rajkovic A. Candidate genes for premature ovarian failure. *Curr Med Chem*. 2007;14:353–7.
- Kloc M, Bilinski S, Dougherty MT, Brey EM, Etkin LD. Formation, architecture and polarity of female germline cyst in *Xenopus*. *Dev Biol*. 2004;266:43–61.
- Kloc M, Jaglarz M, Dougherty M, Stewart MD, Nel-Themaat L, Bilinski S. Mouse early oocytes are transiently polar: three-dimensional and ultrastructural analysis. *Exp Cell Res*. 2008;314:3245–54.
- Pepling ME, Spradling AC. Female mouse germ cells form synchronously dividing cysts. *Development*. 1998;125:3323–8.
- Bilinski SM, Jaglarz MK, Dougherty MT, Kloc M. Electron microscopy, immunostaining, cytoskeleton visualization, in situ hybridization, and three-dimensional reconstruction of *Xenopus* oocytes. *Methods*. 2010;51:11–9.
- Choi Y, Yuan D, Rajkovic A. Germ cell-specific transcriptional regulator *Sohlh2* is essential for early mouse folliculogenesis and oocyte-specific gene expression. *Biol Reprod*. 2008;79:1176–82.
- Greenbaum MP, Yan W, Wu M-H, Lin Y-N, Agno JE, Sharma M, et al. *TEX14* is essential for intercellular bridges and fertility in male mice. *PNAS*. 2006;103:4982–7.
- Bogard N, Lan L, Xu J, Cohen RS. *Rab11* maintains connections between germline stem cells and niche cells in the *Drosophila* ovary. *Development*. 2007;134:3413–8.

18. Cerda J, Reidenbach S, Pratzel S, Franke WW. Cadherin-catenin complexes during zebrafish oogenesis: heterotypic junctions between oocytes and follicle cells. *Biol Reprod.* 1999;61:692–704.
19. Hierholzer A, Kempler R. Beta-catenin-mediated signaling and cell adhesion in postgastrulation mouse embryos. *Dev Dyn.* 2010;239:191–9.
20. Duta S, Pepling ME (2009) A drop in maternal estradiol levels correlates with cyst breakdown and may affect meiotic cell cycle progression. *Biol Rep.* 81, abstract 479.
21. Chen Y, Jefferson WN, Newbold RR, Padilla-Banks E, Pepling ME. Estradiol, progesterone, and genistein inhibit oocyte nest breakdown and primordial follicle assembly in the neonatal mouse ovary in vitro and in vivo. *Endocrinology.* 2007;148:3580–90.
22. Monti M, Redi C. Oogenesis specific genes (Nobox, Oct4, Bmp15, Gdf9, Oogenesis1 and Oogenesis2) are differentially expressed during natural and gonadotropin-induced mouse follicular development. *Mol Reprod Dev.* 2009;76:994–1003.