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# LIN28 Is Selectively Expressed by Primordial and Pre-Meiotic Germ Cells in the Human Fetal Ovary

Andrew J. Childs, Hazel L. Kinnell, Jing He, and Richard A. Anderson

Germ cell development requires timely transition from primordial germ cell (PGC) self-renewal to meiotic differentiation. This is associated with widespread changes in gene expression, including downregulation of stem cell-associated genes, such as *OCT4* and *KIT*, and upregulation of markers of germ cell differentiation and meiosis, such as *VASA*, *STRA8*, and *SYCP3*. The stem cell-expressed RNA-binding protein Lin28 has recently been demonstrated to be essential for PGC specification in mice, and LIN28 is expressed in human germ cell tumors with phenotypic similarities to human fetal germ cells. We have therefore examined the expression of LIN28 during normal germ cell development in the human fetal ovary, from the PGC stage, through meiosis to the initiation of follicle formation. *LIN28* transcript levels were highest when the gonad contained only PGCs, and decreased significantly with increasing gestation, coincident with the onset of germ cell differentiation. Immunohistochemistry revealed LIN28 protein expression to be germ cell-specific at all stages examined. All PGCs expressed LIN28, but at later gestations expression was restricted to a subpopulation of germ cells, which we demonstrate to be primordial and premeiotic germ cells based on immunofluorescent colocalization of LIN28 and OCT4, and absence of overlap with the meiosis marker SYCP3. We also demonstrate the expression of the LIN28 target precursor pri-microRNA transcripts pri-*LET7a/f/d* and pri-*LET-7g* in the human fetal ovary, and that expression of these is highest at the PGC stage, mirroring that of *LIN28*. The spatial and temporal restriction of LIN28 expression and coincident peaks of expression of *LIN28* and target pri-microRNAs suggest important roles for this protein in the maintenance of the germline stem cell state and the regulation of microRNA activity in the developing human ovary.

## Introduction

THE RNA-BINDING PROTEIN LIN28 was identified as a regulator of developmental timing in *Caenorhabditis elegans* [1], and homologous genes exist in diverse species, including mammals [2]. LIN28 is expressed in pluripotent stem cells [3,4] and widely during mammalian embryogenesis [5,6], but displays restricted expression in adult tissues [5]. LIN28 inhibits the production of mature microRNAs (miRNAs) of the *LET-7* (*Mirlet7*) family from precursor pri-miRNA transcripts [4,7]. LIN28 can also promote mRNA translation through direct association with target mRNAs [8–11], including that encoding the pluripotency-associated transcription factor OCT4 [12].

Lin28 is required for normal development of the primordial germ cell (PGC) population in vivo, and for derivation of PGC-like cells from embryonic stem (ES) cells in vitro [13]. In the absence of Lin28, *Let-7*-mediated translational repression inhibits the production of Blimp1, a key factor in PGC specification [14,15]. Lin28 expression is detectable in mouse PGCs from embryonic day (e) 7.5, with Lin28-negative germ

cells first observed at e12.5, corresponding to the time at which sex determination occurs and immediately before entry into meiosis in the female [13]. In the postnatal mouse testis, *Lin28* is repressed by retinoic acid (RA), resulting in increased *Let-7* miRNA expression and possibly spermatogonial differentiation [16].

LIN28 is expressed by germ cells in the human fetal testis [17], and in some human gonadal tumors [13,17–19] characterized by retention of a fetal germ cell-like phenotype [20]. The expression of LIN28 in the developing human fetal ovary has not been previously reported. Here, we characterize the expression of LIN28 during human ovarian development, from the postmigratory PGC stage, through the onset of meiotic differentiation, to primordial follicle formation and meiotic arrest.

## Results and Discussion

Expression of the *LIN28* gene was investigated at 3 gestational ages representing key stages in human fetal ovarian germ cell differentiation. At 8–11 weeks of gestational age

(wga), the fetal ovary contains only undifferentiated, proliferating PGCs. By 14–16 wga widespread germ cell differentiation has initiated, with germ cells forming syncytial clusters and some germ cells initiating meiosis. At 17–20 wga, germ cell clusters begin to break down, forming primordial follicles containing oocytes arrested at the diplotene stage of meiosis [21]. At later gestations (beyond 12–13 wga), germ cells at various stages of differentiation are seen in the same ovary, arising from considerable asynchrony in the progression of human fetal ovarian germ cell development [22,23]. Mitotic PGC-like cells are restricted to the periphery of the ovary, with progressively more differentiated germ cells found in a gradient toward the center [22–24].

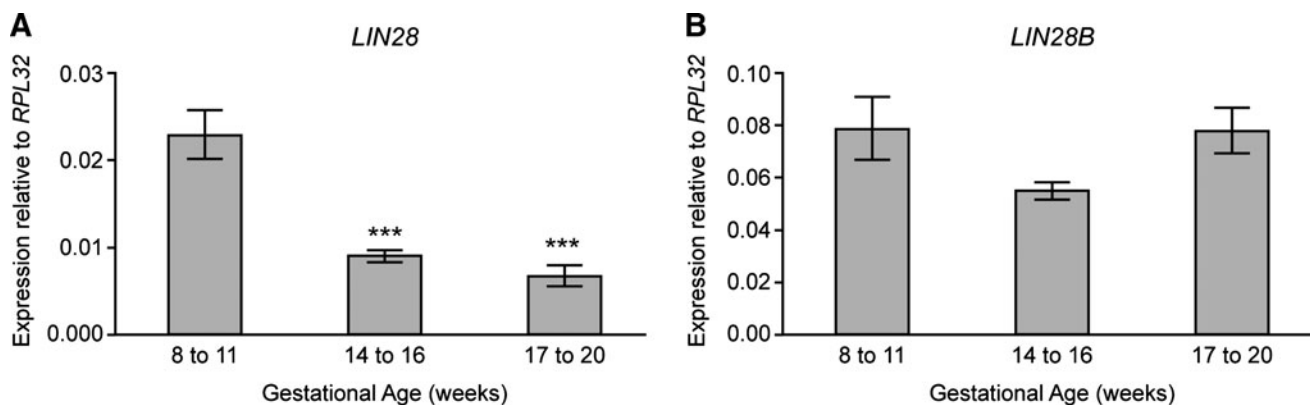
*LIN28* gene expression decreased significantly between 8–11 wga and 14–16 wga (Fig. 1A) coincident with the onset of meiotic germ cell differentiation, and remained low at 17–20 wga. This pattern of expression is strikingly similar to that we have reported previously for *POU5F1* (encoding OCT4) [22], and is the opposite to that observed for markers of more mature germ cells such as *DAZL* and *VASA* [22,23], and genes encoding proteins involved in meiosis, such as *STRA8*, *SYCP3*, and *SPO11*, that are upregulated from 11 to 12 wga [25–27]. This suggests that *LIN28* expression is associated with PGCs in the human fetal ovary and downregulated at the onset of germ cell differentiation. The paralogous gene *LIN28B* has been implicated in the timing of human puberty and menopause [28], but its expression was unchanged (Fig. 1B).

To establish whether the pattern of *LIN28* gene expression was conserved at the protein level, we performed immunohistochemistry for LIN28 on sections of human fetal ovaries. At 9 wga, intense LIN28 immunostaining was detected exclusively in PGCs, in both the nucleus and the cytoplasm (Fig. 2A), and was present in all germ cells at this stage. By 14 wga expression was restricted to a population of germ cells at the periphery of the fetal ovary, with some immunopositive germ cells detected deeper in the ovarian cortex (Fig. 2B). By 18 wga, almost all immunopositive germ cells were restricted to a thin margin at the periphery of the ovary (Fig. 2C, D). Large germ cells with cartwheel chromatin indicative of the leptotene stage of meiosis [29] displayed no staining (Fig. 2D), supporting the hypothesis that LIN28 expression is downregulated as germ cells differenti-

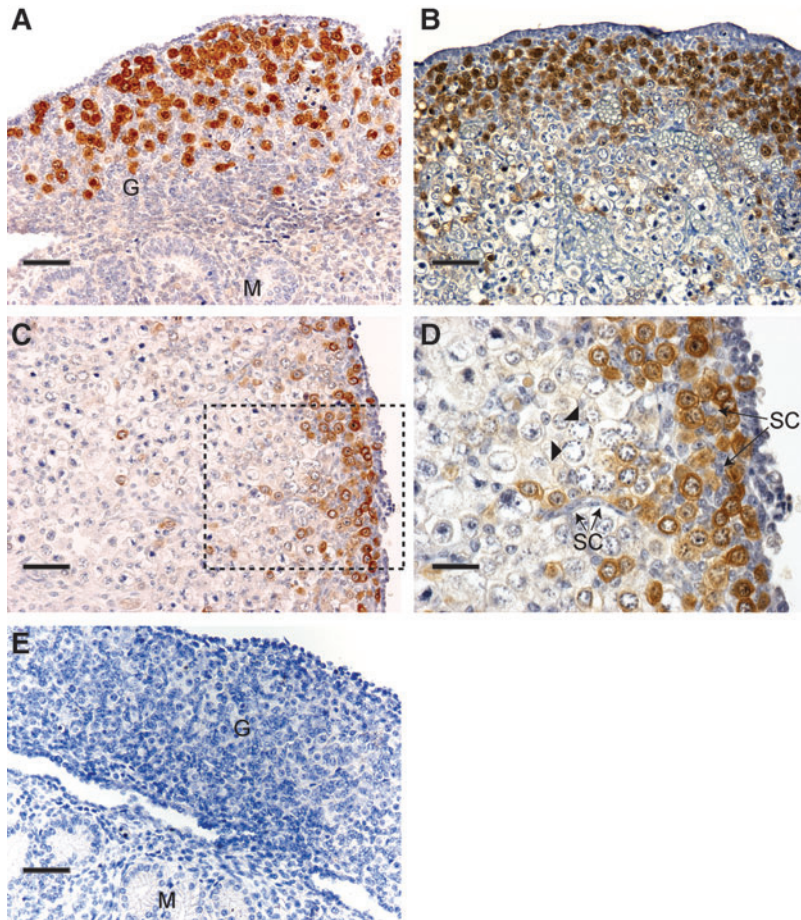
ate. Additionally, we noted a possible change in the predominant subcellular localization of the LIN28 protein with gestation; at 9 wga all germ cells displayed both nuclear and cytoplasmic staining, while most LIN28-positive germ cells at 18 wga appeared to have immunonegative nuclei (compare Fig. 2A and Fig. C, D). Ovarian (Fig. 2D) and mesonephric (Fig. 2A) somatic cells were immunonegative at all stages examined, as was the ovarian surface epithelium, despite recent reports of the isolation of LIN28-positive germ cell-like cells from the adult ovarian surface epithelium [30], and the expression of LIN28 by a subpopulation of epithelial ovarian cancer cells [31]. Negative controls also displayed no staining (Fig. 2E).

As human germ cell development is highly asynchronous, we performed double immunofluorescence to compare the distribution of LIN28 with stage-specific markers of germ cell maturation, namely, the PGC marker OCT4 [22], and the meiosis-associated synaptonemal complex protein SYCP3 [32]. We detected extensive overlap between the expression of LIN28 and OCT4 at 14 wga (Fig. 3A). All OCT4-positive germ cells appeared to express LIN28, however, OCT4-negative/LIN28-positive germ cells could also be detected, revealing persistence of LIN28 expression beyond the stage at which germ cells downregulate OCT4. High-magnification confocal images revealed that in germ cells in which LIN28 localized to the nucleus, expression was restricted to a distinct subnuclear compartment from which OCT4 appeared to be excluded (Fig. 3B), a finding consistent with previous reports of nucleolar localization of LIN28 [8]. No coexpression of LIN28 and SYCP3 was detected at 15 wga at either the periphery (Fig. 3C) or deeper within the cortex (Fig. 3D) of the fetal ovary, confirming that LIN28 is downregulated before extensive germ cell meiotic differentiation. Negative controls also displayed no staining (insets, Fig. 3A, C). It appears therefore that in the human fetal ovary LIN28 marks OCT4-positive PGC-like germ cells and, additionally, a population of germ cells at a slightly later yet still premeiotic stage of differentiation.

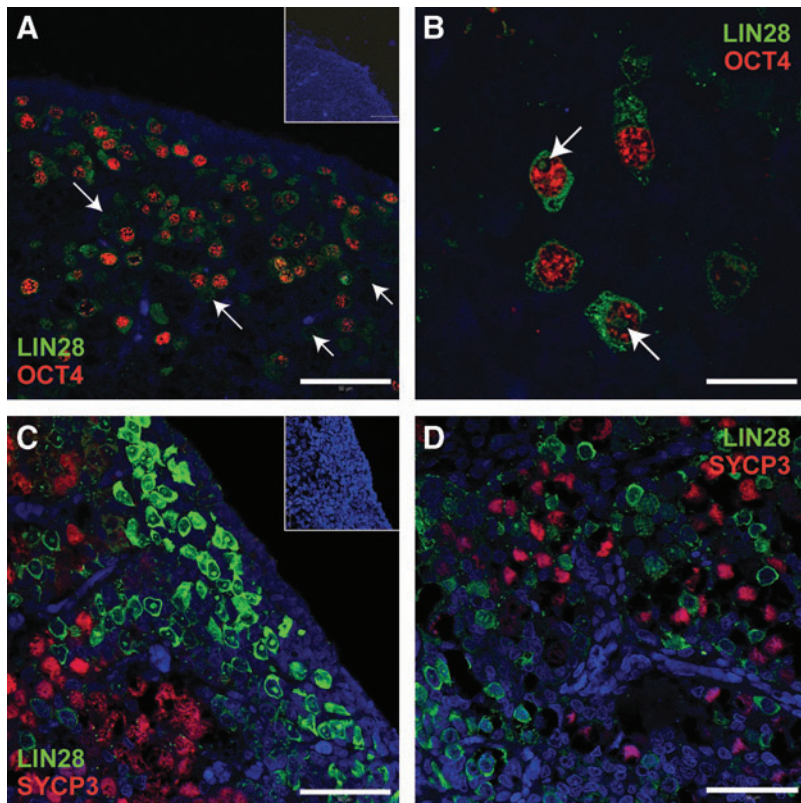
As LIN28 inhibits the production of mature *LET-7* miRNAs from precursor pri-miRNA transcripts, we examined the expression of the LIN28-target pri-*LET-7a/ff/d* and pri-*LET-7g* miRNAs during human fetal ovarian development.



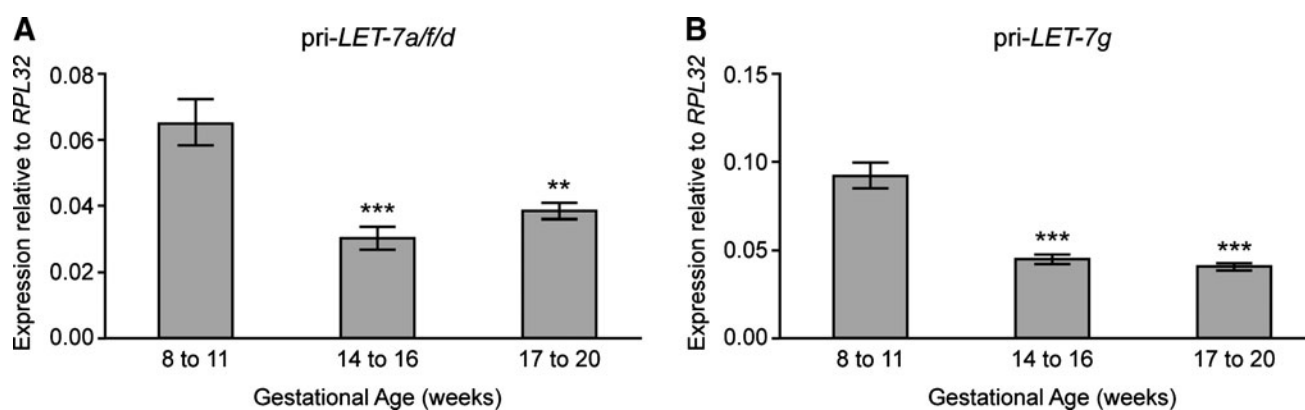
**FIG. 1.** Expression of *LIN28* and *LIN28B* during human fetal ovarian development. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis reveals expression of *LIN28* (**A**) to be highest at 8–11 wga, and significantly lower at 14–16 wga and 17–20 wga, coincident with the onset of germ cell differentiation [\*\*\* $P < 0.001$  vs. 8–11 wga (ANOVA with Neumann-Keuls post hoc testing),  $n = 6–8$  ovaries per gestational group]. *LIN28B* expression (**B**) remained unchanged across gestation. Values denote mean  $\pm$  SEM. ANOVA, analysis of variance; SEM, standard error of the mean.



**FIG. 2.** LIN28 expression is germ cell specific and becomes restricted to a subpopulation of germ cells with increasing gestation. At 9 wga (**A**), all germ cells in the gonad (G) express LIN28 (brown staining) in both the nucleus and cytoplasm. Somatic cells in the gonad (G) and mesonephros (M) display no staining. By 14 wga (**B**), LIN28-positive cells are concentrated at the periphery of the ovary, although some LIN28-positive germ cells are distributed toward the center of the ovary. At 18 wga (**C**), LIN28-positive germ cells occupy a thin margin at the edge of the ovary; no staining is detected in more mature germ cells toward the center of the ovary. (**D**) Magnified image of area identified in (**C**); meiotic germ cells (identified by their characteristic chromatin arrangement) are LIN28 negative (arrowheads). Somatic cells (SC) in streams and interspersed between LIN28-positive germ cells are also immunonegative. (**E**) No staining is detected in negative controls. Scale bars in **A**, **B**, **C**, and **E**: 50  $\mu$ m; **D**: 20  $\mu$ m.



**FIG. 3.** LIN28 is expressed in undifferentiated germ cells, but not in those undergoing meiosis. Double immunofluorescence reveals the extensive overlap between the expression domains of LIN28 (green) and OCT4 (red) (**A**). All OCT4-positive cells express LIN28, although some germ cells are LIN28-positive only (arrows). High-magnification images of the human fetal ovary at 14 wga (**B**) reveal that LIN28 occupies a distinct subnuclear compartment (arrows) from which OCT4 protein is excluded. No colocalization of LIN28 (green) with the meiosis marker SYCP3 (red) is detected at 15 wga at the periphery (**C**) or deeper within the cortex (**D**). No staining is detected on negative controls (insets, **A** and **C**). Scale bars in **A**, **C**, and **D**: 50  $\mu$ m; **B**: 10  $\mu$ m, blue staining denotes cell nuclei.



**FIG. 4.** Expression of precursor *LET-7* pri-microRNA transcripts during human fetal ovarian development. qRT-PCR analysis reveals developmentally regulated expression of (A) *pri-LET-7a/f/d* and (B) *pri-LET-7g* during human fetal ovary development. Expression of both transcripts is highest at 8–11 wga, and significantly downregulated at 14–16 and 17–20 wga. \*\*\* $P < 0.001$  versus 8–11 wga, \*\* $P < 0.01$  versus 8–11 wga (ANOVA, with Neumann-Keuls post hoc testing  $n = 6-8$  ovaries per gestational group). Values denote mean  $\pm$  SEM.

We found significant decreases in the expression of *pri-LET-7a/f/d* and *pri-LET-7g* (Fig. 4A, B) between 8–11 wga and 14–16 wga, mirroring the changes in *LIN28* expression. These data suggest that *LET-7* miRNAs may be important during early human germ cell development. Furthermore, the coincident peak expression of both *LIN28* and its target pri-miRNAs suggests that in early human germ cells *LIN28* maybe required to inhibit the high levels of *pri-LET-7* transcripts from being processed into mature miRNAs. In mouse ES cells, *pri-Let-7g* expression is directly regulated by Oct4 [33], and the pattern of *pri-LET-7* expression reported here, which reflects that of OCT4, [22] may indirectly suggest that this regulatory relationship is preserved in human fetal germ cells.

## Conclusions

In this report we have demonstrated the expression of the RNA-binding protein *LIN28* by immature germ cells during the development of the human fetal ovary. *LIN28* is downregulated before germ cells have undergone extensive meiotic differentiation, a finding consistent with the loss of *Lin28* expression in fetal mouse gonads from e12.5 [13], just before the initiation of meiosis. In mice [34,35] and possibly humans [25,27] meiotic entry is thought to be promoted by the action of RA, which has recently been reported to repress *Lin28* expression in the postnatal mouse testis [16]. It will be important to establish therefore whether downregulation of *LIN28* in the human fetal ovary results, in part, from RA-mediated repression, thus facilitating germ cell entry into meiosis.

The expression of the *LET-7* family precursor pri-miRNAs, a target of *LIN28*, also declines with increasing germ cell differentiation, consistent with a conserved functional interaction between the two. Elucidation of the *LIN28-LET-7* relationship in human germ cells will enhance our understanding of how human PGCs switch from the stem cell state to meiotic differentiation, and provide insight into manipulating these processes for therapeutic intervention.

## Materials and Methods

### Collection of human fetal ovaries

Ovaries (8–20 weeks of gestation, calculated from the date of the last menstrual period) were obtained from morphologically normal human fetuses after elective termination of pregnancy. Informed consent was obtained, and the study was approved by the Lothian Research Ethics Committee (study code: LREC 08/S1101/1). Gestational age was determined by ultrasonography, and further confirmed (for 14–20-wga specimens) by measurement of foot length. The sex of 8–11-wga gonads was determined by polymerase chain reaction (PCR) for the *SRY* gene as described previously [36], using the following primers: *SRY-Fwd*, 5'-ACAGTAAAGGCAACGTCCAG-3' and *SRY-Rev*, 5'-ATCTGCGGGAAGCAAAGTGC-3' [37]. Gonads were dissected into Dulbecco's phosphate balanced salt solution (Life Technologies) and either snap-frozen and stored at  $-80^{\circ}\text{C}$  for extraction of total RNA for quantitative reverse transcription (qRT)-PCR analysis or fixed in Bouin's solution and processed into wax by standard methods for immunohistochemistry. All

**TABLE 1.** OLIGONUCLEOTIDE PRIMER SEQUENCES USED IN SYBR GREEN QUANTITATIVE REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION ANALYSIS

Gene	Forward primer (5'–3')	Reverse primer (5'–3')	Ref.
<i>LIN28</i>	CGGGCATCTGTAAGTGGTTC	CAGACCCTTGGCTGACTTCT	[12]
<i>LIN28B</i>	GCCCTTGGATATTCCAGTC	TGACTCAAGGCCTTTGGAAG	[35]
<i>pri-LET-7a/f/d</i>	GCGCGAGGAAACCAGGAT	GCACCACAGGAAGGCTTTTTT	[36]
<i>pri-LET-7g</i>	CAGTAAGAAATATGGTGTGGACCTCA	GATGTATTTGTTGTCATGCAAGGAC	[36]

extragonadal tissue, including mesonephroi, was dissected away from the gonad before freezing.

### RNA extraction and qRT-PCR

Total RNA was extracted from fetal ovaries using the RNeasy Micro and RNeasy Mini kits (QIAGEN) with on-column DNaseI digestion as per the manufacturer's instructions. First-strand cDNA was prepared using the Superscript VILO Mastermix kit (Life Technologies). Gene expression was assessed by qRT-PCR using SYBR green technology on an ABI7900HT Fast thermal cycler (Life Technologies) as described previously [36]. The expression level of each gene of interest was normalized to that of the housekeeping gene *RPL32* within the same sample. Negative (cDNA prepared in the absence of reverse transcriptase) and no-template (in which dH<sub>2</sub>O was substituted for template cDNA) controls were included to exclude contamination or amplification of residual genomic DNA. Primer sequences used in qRT-PCR can be found in Table 1.

### Immunohistochemistry

Immunohistochemistry was performed on fixed sections of fetal ovary as described previously [36]. Briefly, 5- $\mu$ m-thick sections of Bouin's-fixed, paraffin-embedded tissues were mounted on electrostatically charged glass slides; dewaxed; and rehydrated through xylene (2 $\times$ 5 min) and graded alcohols [100% (2 $\times$ 30 s), 95% (30 s), and 70% (30 s)]. Antigen retrieval was performed by pressure cooking in 0.01 M sodium citrate buffer (pH 6.0) for 5 min, followed by incubation in 3% (v/v) hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity. Slides were subsequently blocked using the avidin/biotin blocking kit (Vector Laboratories, Inc.) and incubation in Tris Buffered Saline (TBS), supplemented with 5% bovine serum albumin (BSA) and 20% normal serum (NS). Polyclonal rabbit anti-LIN28 primary antibody (AB46020; Abcam) was diluted 1:500 in 5% BSA/NS/TBS and applied to the sections, and slides were incubated at 4°C overnight in a humidified chamber. Primary antibodies were detected using a goat anti-rabbit secondary antibody (DAKO; 30 min, diluted 1:500 in BSA/TBS/NS) and incubation with avidin-biotin-HRP complex (Vector Laboratories). Bound antibodies were visualized using 3,3'-diaminobenzidine tetrahydrochloride (DAB; DAKO). Negative controls in which the primary antibody was omitted were included in each experiment. Slides were counterstained with hematoxylin, dehydrated through graded alcohols and xylene, and permanently mounted using PerTex before being photographed on a Provis (Carl Zeiss Ltd.) microscope with cold-capture camera (Olympus). A minimum of 4 sections per ovary were examined.

### Immunofluorescence

Double immunofluorescence was performed as described previously [38] using either rabbit polyclonal anti-SYCP3 (AB15093; Abcam; diluted 1:50,000) or mouse monoclonal anti-OCT4 (SC-5279, which recognizes only the pluripotency-associated OCT4A isoform [39]; Santa Cruz Biotechnology; diluted 1:250) on day 1 of the procedure, and rabbit polyclonal anti-LIN28 (AB46020; Abcam; diluted

1:500) primary antibody on day 2. Anti-SYCP3/anti-OCT4 antibodies were detected using a goat anti-mouse or a goat anti-rabbit peroxidase secondary antibody (Vector Laboratories Ltd.; diluted 1:200), and then incubated with Tyramide Fluorescein Green (TSA Plus Cyanine 5 System; diluted 1:50) for 10 min at room temperature. Anti-LIN28 primary antibody was detected using a goat anti-rabbit biotinylated secondary antibody (Vector Laboratories Ltd.; diluted 1:500), followed by incubation with Streptavidin-488 (Molecular Probes, Invitrogen Life Technologies; diluted 1:200) for 1 h at room temperature. Slides were counterstained with DAPI, and mounted using PermaFluor Aqueous Mounting Medium (Beckman Coulter). Fluorescent images were captured using an LSM510 Meta confocal microscope (Carl Zeiss Ltd.). A minimum of 4 sections per ovary were examined.

### Statistical analyses

Gestational range qRT-PCR data ( $n=6-8$  ovaries from individual fetuses per gestational group) were analyzed by one-way analysis of variance with Neumann-Keuls post hoc testing in Graphpad Prism Software. *P* values of  $<0.05$  were considered statistically significant.

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### Disclosure Statement

The authors declare that they have no competing interests to disclose.

### Note Added in Proof

While this article was in peer review and production, El-Khairi et al. [40] also reported increasing expression of *LIN28* in the human fetal ovary between 6 and 9 weeks post conception (approximately equivalent to 8–11 weeks gestational age) and co-localization of *LIN28* with *OCT4* in germ cells at this stage.

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