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Lin28a Is Dormant, Functional, and Dispensable During Mouse Oocyte-to-Embryo Transition[1]

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ABSTRACT

The oocyte-to-embryo transition (OET) denotes transformation of a highly differentiated oocyte into totipotent blastomeres of the early mammalian embryo. OET depends exclusively on maternal RNAs and proteins accumulated during oocyte growth, which implies importance of post-transcriptional control of gene expression. OET includes replacement of abundant maternal microRNAs (miRNAs), enriched also in differentiated cells and exemplified by the Let-7 family, with embryonic miRNAs common in pluripotent stem cells (the miR-290 family in the mouse). *Lin28a* and its homolog *Lin28b* encode RNA-binding proteins, which interfere with Let-7 maturation and facilitate reprogramming of induced pluripotent stem cells. Both *Lin28a* and *Lin28b* transcripts are abundant in mouse oocytes. To test the role of maternal expression of *Lin28a* and *Lin28b* during oocyte-to-zygote reprogramming, we generated mice with oocyte-specific knockdown of both genes by using transgenic RNA interference. *Lin28a* and *Lin28b* are dispensable during oocyte growth because their knockdown has no effect on Let-7a levels in fully grown germinal vesicle (GV)-intact oocytes. Furthermore, transgenic females were fertile and produced healthy offspring, and their overall breeding performance was comparable to that of wild-type mice. At the same time, 2-cell embryos derived from transgenic females showed up-regulation of mature Let-7, suggesting that maternally provided LIN28A and LIN28B function during zygotic genome activation. Consistent with this conclusion is increased translation of *Lin28a* transcripts upon resumption of meiosis. Our data imply dual repression of Let-7 during OET in the mouse model, the selective suppression of Let-7 biogenesis by *Lin28* homologs superimposed on previously reported global suppression of miRNA activity.

early development, *genome activation*, *guinea pigs*, *Let-7*, LIN28, *mice*, *microRNA*, *oocyte*, *rats*, *RNAi*, *rodents*, *transgenic/knockout model*, *voles*, *zygote*

INTRODUCTION

Successful zygotic genome activation is entirely dependent on cytoplasmic factors, as demonstrated by cloning using somatic nuclear transfer [1]. Post-transcriptional mechanisms engaging mRNAs and proteins produced in the oocyte during oogenesis are thus essential for establishment of correct zygotic gene expression. Oocyte maturation and fertilization induce major changes in translation and stability of cohorts of maternal transcripts. The selection of maternal mRNAs for different post-transcriptional events has been shown to be driven by *cis*-acting elements located in 3′-untranslated regions (3′UTRs), and *trans*-acting factors that bind to them [2–4]. The number, position, and combination of *cis*-acting elements and the presence of *trans*-acting factors offer a complex combinatorial system controlling mRNA localization, stability, and translation [3].

Dormancy is a classical example of post-transcriptional regulation of maternal mRNAs, where certain deadenylated and translationally inactive mRNAs are recruited for translation in response to developmental cues. Translation of dormant mRNAs is induced through cytoplasmic polyadenylation, which is mediated by a *cis*-acting 3′UTR element called cytoplasmic polyadenylation element (CPE) and its binding protein (CPEB) (reviewed in [5, 6]). CPEB is one of many RNA binding proteins which recognize either sequence motifs or secondary structures within 3′UTRs and regulate mRNA metabolism.

Another class of *trans*-acting factors binding *cis*-acting 3′UTR elements is represented by microRNAs (miRNAs), which act as sequence-specific guides, usually imperfectly basepairing with 3′UTR binding sites. MicroRNAs are the dominant small RNA class in mammalian cells. More than 1000 miRNAs have been identified and implicated in the regulation of a large fraction of mammalian genes and in many cellular processes [7]. MicroRNAs are essential also for pluripotency [8–10]. Two antagonistic miRNA families strongly contribute to control of developmental capacity in mammals. Highly conserved Let-7 miRNAs are typically found in differentiated animal tissues and are associated with negative regulation of pluripotency and tumor suppression in mammals [11]. Remarkably, Let-7 miRNAs are the most abundant maternal miRNAs although their expression is minimal in early embryos [12, 13]. In contrast, miR-290 family miRNAs are virtually absent in oocytes and become expressed during zygotic genome activation and early development [13, 14]. Notably, the miR-290 family is the most abundant and most active miRNA family in embryonic stem cells [14, 15]. Opposing roles of Let-7 and miR-290 miRNA families were demonstrated in pluripotent stem cells where Let-7 promotes differentiation [16], whereas miR-290-related miRNAs suppress senescence, promote rapid proliferation, and facilitate formation of induced pluripotent stem cells [14, 17, 18]. Interestingly, the ability of miRNAs to repress translation during oocyte-to-embryo transition (OET) is strongly reduced despite intact miRNA biogenesis [12, 19–21]. This unique observation is consistent with the impressive tolerance of mouse oocytes to loss of miRNA, which extends far into the early development [20] where the first functional requirement

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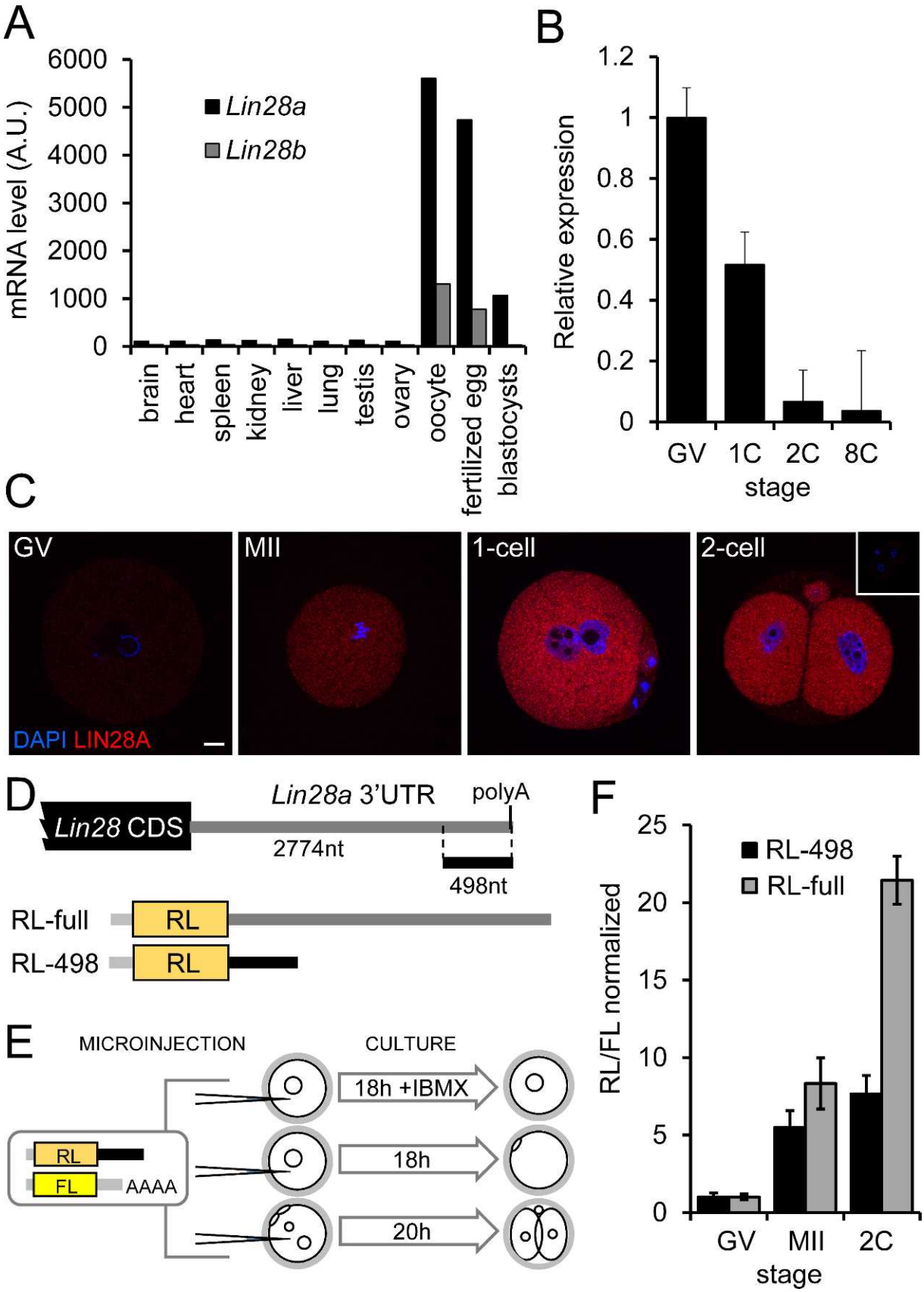


FIG. 1. *Lin28a* and *Lin28b* expression analysis. **A**) Microarray data from the BioGPS database (NCBI accession number GSE1133 [36, 37]) show that *Lin28a* and *Lin28b* are highly expressed in the oocyte but not in somatic tissues. Data for each tissue represent relative average (n = 2) fluorescence intensity of gene-detecting probes on the GNF1M platform (Affymetrix). Data from microarrays were normalized using the GC content adjusted Robust Multi-Array (gcRMA) algorithm and global median scaling, where the median was set to 200. **B**) Temporal expression profile of *Lin28a* transcripts during early development. Relative abundance of *Lin28a* transcripts was determined by real-time PCR. Expression data (CtΔΔ values) obtained from individual

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for miRNAs was observed during implantation [22–24]. MicroRNA inactivity in mouse oocytes likely prevents maternal Let-7 miRNAs from suppressing transcripts necessary for maternal genome reprogramming and facilitates exchange of the two antagonistic miRNA families [25].

Global suppression of mature miRNA activity during OET might be aided by suppression of Let-7 biogenesis by LIN28A and LIN28B, which bind Let-7 precursors and block their processing into mature miRNAs [26–30]. LIN28A (also known as LIN28) and LIN28B are highly conserved mammalian homologs of *Lin28*, which was first identified as a regulator of developmental timing in *Caenorhabditis elegans* [31]. LIN28 proteins bind Let-7 pre-miRNAs as well as various mRNAs and have multiple roles in pluripotency, development, growth, and metabolism [32–34]. Remarkably, LIN28 was used together with OCT4, SOX2, and NANOG as a pluripotency reprogramming factor to reprogram human fibroblasts into induced pluripotent stem cells [35]. Both *Lin28a* and *Lin28b* mRNAs are highly expressed in oocytes, embryos, and embryonic stem cells [36, 37]. *Lin28a*$^{-/-}$mice have reduced germ cell pool sizes at embryonic and adult stages. Consequently, *Lin28a*$^{-/-}$ female mice have smaller ovaries, produce smaller numbers of mature oocytes, and have reduced fertility (~30% smaller litter size) [38]. In contrast, postzygotic morpholino-based knockdown of *Lin28a* resulted in most early embryos arresting between 2- and 4-cell stages, and LIN28A has been implicated in nucleogenesis during early development [39]. Here we report results of LIN28A expression analysis during OET and functional analysis of *Lin28a* and *Lin28b*, using oocyte-specific transgenic RNA interference (RNAi) [40].

MATERIALS AND METHODS

Mouse Oocyte/Egg/Embryo Collection, Cell Culture, and Microinjection

Fully grown, germinal vesicle (GV)-intact oocytes, metaphase II (MII) eggs, and 1-cell and 2-cell embryos were collected as previously described [41, 42]. GV oocytes were cultured in Chatot-Ziomek-Bavister (CZB) medium containing 0.2 mM isobutylmethylxanthine (IBMX; Sigma) to inhibit GV breakdown. MII eggs were cultured in CZB medium [43] and fertilized eggs in simplex optimization method derived medium with increased K+ concentration (KSOM) [44]. Two-cell embryos were isolated by flushing oviducts of superovulated and mated mice 42–44 h after human chorionic gonadotropin (hCG) injection. All animal experiments were approved by the Institutional Animal Use and Care Committee and were performed consistent with legal guidelines.

DNA Constructs

The following cloning strategy was used to generate *Lin28a/Lin28b* (*Lin28a/b*) inverted repeat for transgenic maternal *Lin28a* and *Lin28b* knockdown. Two fragments of different length were amplified from *Lin28a* transcript by RT-PCR, using forward primers GCGGATCCAGGGAGGAA GAGGAAGAGAT (long fragment) and GCGGATCCAGGAGTTTAAG GAAAGAGGCA (short fragment) with a reverse primer GCAAGCTTGGTTTGACACTTGTTTCGCT. *Lin28b* fragment was cloned with the forward primer GCAAGCTTCCTTTGATTCAGAAACGGAA and the reverse primer GCTCTAGAGTGCCTGTACTCTTATGTGA. PCR products were digested with *Hind*III and ligated to form *Lin28a/b* hybrid arms. These were further digested with *BamH*I and ligated together, and the resulting inverted repeat was cloned into the pZP3EGFP vector [45] by using a unique *Xba*I site to produce pZP3EGFP-Lin28IR for transgenic RNAi. Positions of *Lin28a* and *Lin28b* sequences used for transgenic RNAi are shown in Supplemental Figure S1 (all supplemental data are available online at www.biolreprod.org).

To generate the *Lin28a Renilla* luciferase reporters, the full-length sequence and the last 500 bp of *Lin28a* 3′UTR were amplified using the forward primer GCTCTAGAGGCCCAGGAGTCAGGGTTATTC (full-length) and GCTCTAGATCAAACCAATGTAATCTGTCAC (500 bp) with a common reverse primer, AGCGGCCGCAGTACCAACTCTGGAGTACCA. PCR products were digested with *Xba*I and *Not*I and cloned into *Xba*I and *Not*I opened phRL-SV40 plasmid (Promega). The control firefly luciferase reporter (pFL-SV40) was created by exchanging the *Renilla* luciferase-encoding sequence in phRL-SV40 with the firefly luciferase-encoding sequence.

In Vitro Transcription

The phRL-SV40-Lin28a 3′UTR variants and the control pFL-SV40 were linearized by NotI digestion. Capped RNAs were synthesized by in vitro transcription using a T7 mMessage mMachine kit (Ambion) according to the manufacturer's instruction. Following in vitro transcription, template DNA was removed by Turbo DNase (Ambion), and the control firefly luciferase RNA was polyadenylated using poly(A) tailing kit (Ambion). RNAs were purified with RNeasy mini-kit columns (Qiagen) and eluted in RNase-free water.

Immunocytochemistry and Immunoblotting

Oocyte or embryo samples were fixed in 4% paraformaldehyde for 1 h at room temperature as described previously [46]. The cells were then permeabilized for 15 min in PBS containing 0.1% Triton X-100, blocked in PBS containing 0.2% immunoglobulin G (IgG)-free BSA and 0.01% Tween-20 for 30 min (blocking solution), and then incubated with rabbit anti-LIN28 antibody (clone D1A1A; diluted 1:400 in blocking solution; Cell Signaling Technology) at 4°C overnight. After 4 10-min washes in blocking solution, samples were incubated for 1 h with Alexa 594-conjugated donkey-anti-rabbit secondary antibody (diluted 1:500 in blocking solution; Invitrogen). After an additional 4 10-min washes in blocking solution, the samples were mounted in mounting medium (Vectashield) containing DAPI (4′,6-diamidino-2-phenylindole; Vector Laboratories). Images were captured with a TCS SP5 model laser-scanning confocal microscope (Leica) as described previously [47]. Antibody specificity for LIN28A was confirmed by transgenic RNAi (see Fig. 3).

RNA Isolation, Reverse Transcription, and Real-Time PCR

Total RNA was isolated from 50 oocytes/zygotes/2-cell embryos (48 h post-hCG injection) and from 30 8-cell embryos/blastocysts (72 and 96 h post-hCG, respectively) using RNAzol and polyacryl carrier (Molecular Research Center, Inc.) and reverse-transcribed with Superscript III reverse transcriptase (Invitrogen) using random hexamers as primers. The cDNA equivalent of 0.4 oocyte/zygote/2-cell embryo and 0.15 8-cell embryo/blastocyst per reaction was subjected to quantitative real-time PCR (qPCR) using Maxima SYBR Green qPCR Master Mix (Thermo Scientific) with a Mx3000P system (Stratagene). PCR primers are listed in Supplemental Table S1. Obtained real-

GV-intact oocytes (GV, n = 11) and from 1-cell (1C, n = 6), 2-cell (2C, n = 9), and 8-cell (8C, n = 4) embryos were averaged, and *Lin28a* expression was calculated relative to that of the GV stage. Error bars are SEM. **C**) Immunofluorescence analysis shows accumulation of LIN28A during meiotic maturation and after fertilization. GV = fully grown oocytes; MII = metaphase II egg; 1-cell = 1-cell embryo; 2-cell = 2-cell embryo. In MII, the confocal plane was placed to visualize chromosomes. LIN28A signal is shown in red. DNA staining with DAPI is shown in blue. Bar = 10 μm. Inset in the 2-cell stage panel shows the absence of LIN28A signal in a 2-cell stage stained with the secondary antibody only. **D**) Schematic representation of *Lin28a* 3′UTR and *Renilla* luciferase (RL) reporter constructs. At the top is shown the 5′ end of the *Lin28a* coding sequence (CDS) and 3′UTR fragments (full-length 3′UTR [2997 nucleotides, represented by the dark gray line] and 3′ end [498-nucleotide fragment, represented by the black line]) used to make RL reporter constructs (shown below). **E**) Schema of the experimental design: a tested, capped, and deadenylated sample of *Renilla* reporter RNA was co-injected with capped and adenylated firefly luciferase (FL) into GV oocytes or 1-cell embryos. Luciferase activities were measured at the stages shown after 18–20 h in culture. **F**) Activity of luciferase reporters in GV oocytes, MII eggs, and 2-cell embryos. *Luc* reporter RNAs were injected into GV oocytes or 1-cell embryos. Luciferase activity was analyzed in MII eggs or embryos at 18 and 20 h after microinjection, respectively. Injected GV oocytes cultured in medium containing IBMX for 18 h served as controls. The experiment was performed three times, and data are means ± SEM.

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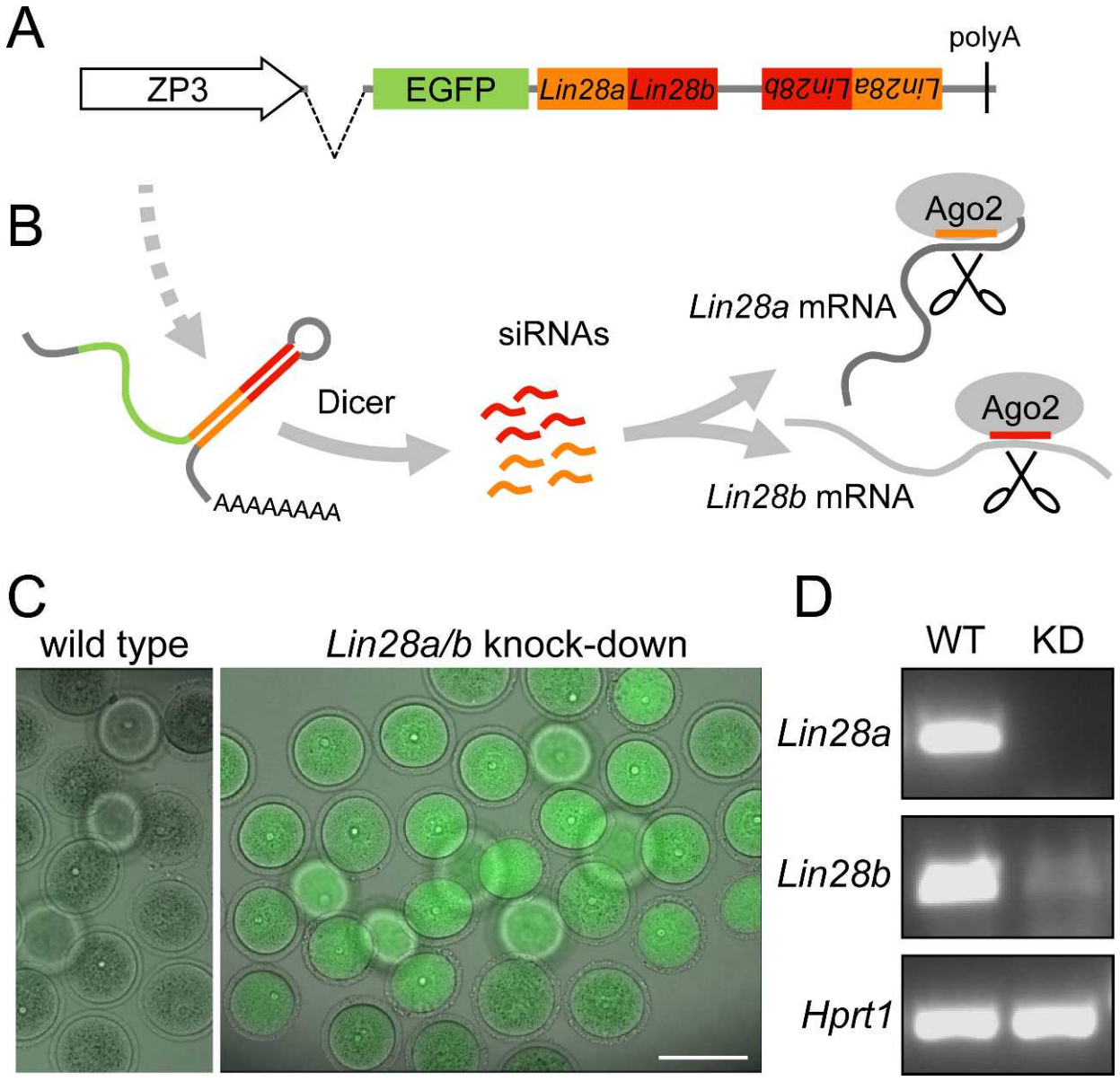


FIG. 2. Down-regulation of *Lin28a* and *Lin28b* transcripts by transgenic RNAi. **A**) Design of the transgenic RNAi vector pZP3EGFP-Lin28IR. *Lin28a* and *Lin28b* sequences used to produce the inverted repeat originated from 3′UTR regions (Supplemental Fig. S1). Detailed description of the vector construction is described in *Materials and Methods*. **B**) Schematic depiction of simultaneous targeting of *Lin28a* and *Lin28b* by transgenic RNAi. **C**) Transgenic mice carrying the pZP3EGFP-Lin28IR transgene show uniform enhanced green fluorescent green protein (EGFP) expression in oocytes. A population of fully grown GV oocytes isolated from ovaries of a transgenic animal is shown, as seen with inverted fluorescence microscopy. Bar = 100 μm. **D**) Oocytes from mice carrying the pZP3EGFP-Lin28IR transgene show strong down-regulation of *Lin28a* and *Lin28b* expression. RT-PCR analysis was carried out as described in *Materials and Methods*. PCR-amplified amplicons after 45 cycles are shown.

time PCR data were analyzed by the CtΔΔ approach, using an in-house-designed software.

Fluidigm Analysis of Lin28a *Expression*

Single oocytes or early embryos were collected in 0.5-ml tubes containing 4 μl of lysis solution (3.7 μl of water and 0.3 μl of RNAse inhibitor [Ribolock; Thermo Scientific]). Samples were immediately frozen at −80°C. Cell lysis was performed at 85°C for 10 min. Lysates were placed on ice for 2 min, and RNA was subjected to reverse transcription reaction. The reaction mixture consisted of 2 μl of dNTPs (2.5 mM each), 2 μl of 5× reverse transcriptase (RT) buffer, 0.5 μl of random hexamer primer (all Thermo Scientific), 1 μl of 1 pg of rabbit globin as an external spike control (Sigma-Aldrich) and 0.5 μl of RevertAid polymerase (Thermo Scientific). Complementary DNA was synthesized at 42°C for 50 min, followed by inactivation of the enzyme at 70°C for 10 min. Converted RNA was subjected to preamplification PCR reaction to increase the number of template molecules. Preamplification was carried out in a total volume of 40 μl, which consisted of 2 μl of cDNA, 2 μl of primer mixture (0.5 μM each primer), 30 μl of 2× SYBR Green (Thermo Scientific), and 6 μl of water. The original cDNA template was amplified by 18 cycles under the following conditions: 95°C for 10 min; denaturation of DNA strands was performed at 95°C for 15 sec, followed by annealing at 57°C for 4 min, and strand synthesis phase at 72°C for 20 sec. Two microliters of 10×-diluted preamplification mixture was used for Fluidigm 48 × 48 well dynamic array (Fluidigm), and the experiment was carried out according to the manufacturer's protocol.

Analysis of miRNA Expression

Twenty oocytes or embryos were collected in 5 μl of water supplemented with RNase inhibitor (Ribolock; Thermo Scientific) and lysed by heating at 95°C for 5 min. Lysates were directly subjected to reverse transcription with miRCURY LNA universal cDNA synthesis kit (Exiqon). A cDNA equivalent of 0.25 oocyte or embryo was used for qPCR reaction with miRCURY LNA

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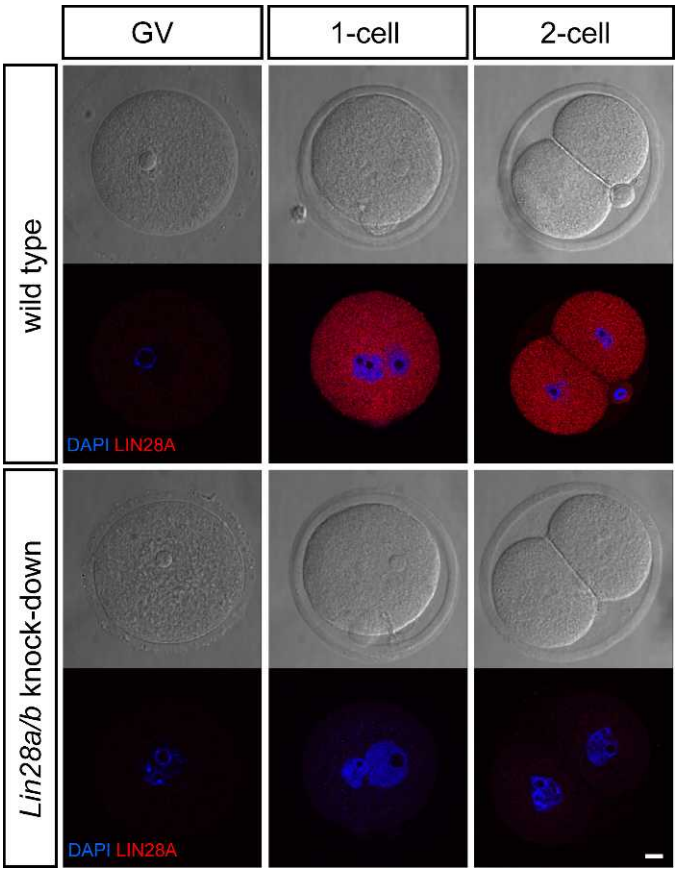


FIG. 3. Transgenic RNAi prevents accumulation of LIN28A during oocyte-to-zygote transition. GV oocytes (GV), 1-cell embryos (1-cell), and 2-cell embryos (2-cell) from wild-type and transgenic females were stained with α-LIN28A antibody (red signal). DNA was stained with DAPI (blue signal). Bar = 10 μm.

SYBR Green Master Mix and for LNA-modified primers to amplify U6 small nuclear RNA (U6-snRNA), Let-7a miRNA, and miR-30c miRNA (Exiqon). Real-time PCR was performed with an Mx3000P system (Stratagene) and analyzed by using the CtΔΔ approach, using in-house-designed software.

Luciferase Reporter Assay

Fully grown GV oocytes or zygotes (22 h post-hCG) were microinjected with ~5 pl of a mixture of control polyadenylated firefly luciferase RNA (15 ng/μl) and RNA encoding *Renilla* luciferase fused to the last 500 bp of *Lin28a* 3′UTR (16 ng/μl) or to full-length *Lin28a* 3′UTR (39 ng/μl) . Injected oocytes were cultured for 18 h in CZB medium with or without 0.2 mM IBMX to prevent or allow maturation to MII stage, respectively. Injected zygotes were cultured for 20 h in KSOM medium. Individual oocytes/eggs/embryos were lysed in 11 μl of 1× passive lysis buffer, and half of the lysate was assayed by using a dual-luciferase reporter assay system (Promega) with a Modulus microplate reader luminometer (Turner Biosystems). For signal normalization, the *Renilla* luciferase activity readout from noninjected oocytes/eggs was subtracted as background, and *Renilla* luciferase activity was then normalized to that of the co-injected control firefly luciferase.

RESULTS

Lin28a *Encodes Dormant Maternal mRNA*

We became interested in *Lin28a* because we found it among the RNA binding factors whose expression was highly enriched in mouse oocytes (Fig. 1A). Therefore, we investigated *Lin28a* expression during OET in more detail (Fig. 1, B and C). The temporal pattern of mRNA expression indicated that the maternal pool of *Lin28a* transcript is strongly depleted by the time of the zygotic genome activation at the 2-cell stage. Immunofluorescent staining of oocytes and zygotes with LIN28A-specific antibody revealed mostly cytoplasmic signal, which was low in the fully grown GV oocyte whereas the signal strongly increased during meiotic maturation and zygotic genome activation (Fig. 1C and Supplemental Fig. S2).

The apparent anticorrelation between transcript and protein levels suggested that *Lin28a* encodes a dormant maternal mRNA. To examine regulation of *Lin28a* mRNA translation, we created reporters where *Renilla* luciferase coding sequence was fused with the entire *Lin28a* 3′UTR (RL-full) or the last 498 nucleotides of *Lin28a* 3′UTR (RL-500) (Fig. 1D). Capped, but non-adenylated, recombinant *Renilla* RNAs were co-injected with a capped and polyadenylated firefly luciferase, which served as an internal control for normalization. Microinjected GV oocytes were either matured for 18 h to MII or maintained for the same time in the presence of IBMX to prevent resumption of meiosis (Fig. 1E). To examine the control of *Lin28a* translation through its 3′UTR after fertilization, we microinjected reporters into 1-cell embryos, which were cultured until the 2-cell stage for 20 h (Fig. 1E). Our results showed that both of the *Lin28a* 3′UTR fragments supported increased translation of deadenylated transcripts during meiotic maturation compared to GV oocytes (Fig. 1F). Both of the *Lin28a* 3′UTR fragments showed up-regulation of luciferase activity, comparable to our previous experiments with *Ccnb1* and *Dcp2* 3′UTRs [48]. Interestingly, RL-500 showed the same accumulation of luciferase activity during meiotic maturation and in the zygote, whereas RL-full luciferase activity was approximately three times higher in early embryos, indicating distinct post-transcriptional control of *Lin28a* in early embryos enabling further accumulation of the protein product. Taken together, *Lin28a* encodes an abundant dormant maternal transcript translated during meiotic maturation and after fertilization, thus resulting in a robust accumulation of LIN28A at the time of the zygotic genome activation.

Efficient Depletion of Lin28a *and* Lin28b *by Transgenic RNAi*

To address the role of accumulation of LIN28A during oocyte-to-zygote transition, we decided to deplete the maternal pool of *Lin28a* by transgenic RNAi, which allows studying the gene function in vivo during oocyte growth and OET. Because mouse oocytes also express *Lin28b*, a close homolog of *Lin28a*, which might potentially compensate for the loss of *Lin28a*, we decided to knock down both homologs simultaneously. In order to do that, we fused together 3′UTR fragments of *Lin28a* and *Lin28b* and created an inverted repeat, which was inserted into the transgenic RNAi vector (Fig. 2A). Upon transcription, which occurs during oocyte growth [49], this inverted repeat should be processed by RNase III Dicer into small interfering RNAs (siRNAs), which would target *Lin28a* and *Lin28b* mRNAs (Fig.2B).

Upon pronuclear injection of the transgenic construct, we obtained one transgenic line where the transgene was expressed (Fig. 2C) and mediated highly efficient knockdown of both *Lin28* homologs (Fig. 2D). Immunostaining of transgenic and wild-type oocytes and early embryos also showed lack of LIN28A signal in 1-cell and 2-cell embryos, demonstrating that transgenic RNAi efficiently prevented accumulation of LIN28A during oocyte-to-zygote transition (Fig. 3).

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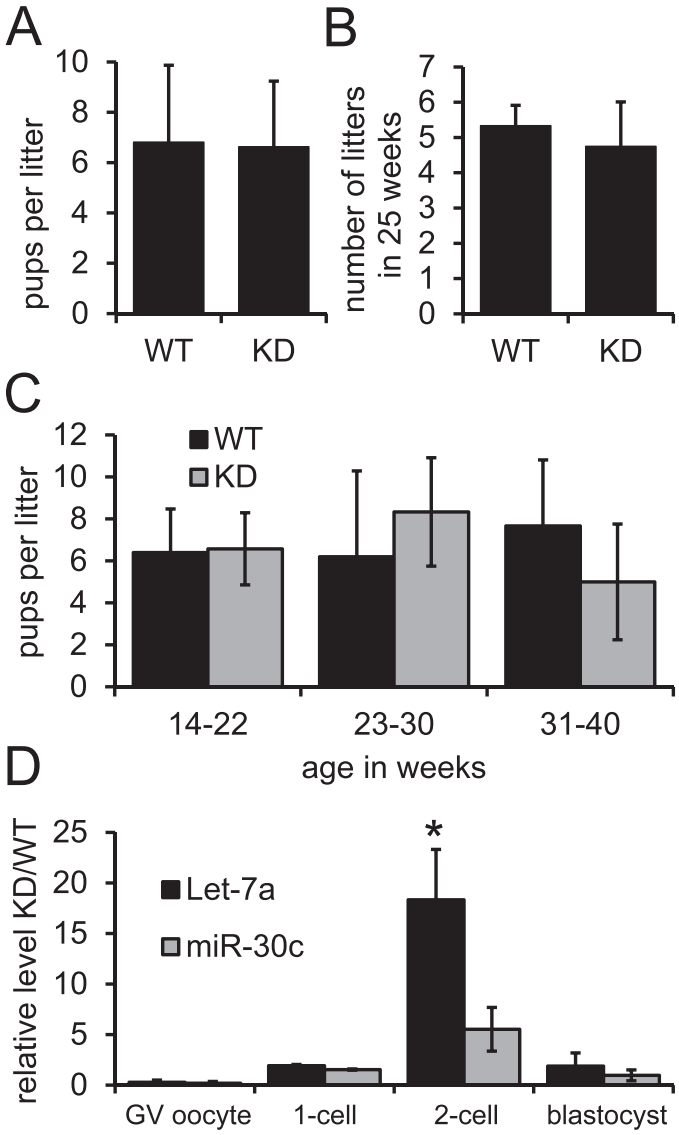


FIG. 4. Transgenic RNAi had no effect on fertility but relieved Let-7a expression during zygotic genome activation. **A**) Litter sizes of transgenic maternal *Lin28a/b* knockdown C57Bl/6 females were compared with their wild-type siblings. In total, 5–7 litters were counted from 4 knockdown and 3 wild-type females. **B**) Frequencies of productive matings in maternal *Lin28a/b* knockdown were compared with those of wild-type C57Bl/6 females over a 25-wk period. **C**) Litter sizes of maternal *Lin28a/b* knockdown females compared with those of wild-type C57Bl/6 females, classified by different ages as young (14–22 wk old), middle aged (23–30 wk old), and old (31–40 wk old). **D**) RT-qPCR analyses of Let-7a and miR-30c miRNAs in maternal *Lin28a/b* knockdown (KD) oocytes and wild-type (WT) oocytes and preimplantation embryos. MicroRNA expression values were normalized to those of U6-snRNA control. Error bars represent SEM. *Statistically significant differences (two-tailed *t*-test, $P = 0.024$) between wild-type (WT) and KD samples, which were observed only in 2-cell embryos. Data were pooled from four independent experiments performed in duplicate. Comparison with published studies of Let-7a expression in oocytes and early embryos [13, 68, 69] suggests that *Lin28a/-b* knockdown results in Let-7a levels in 2-cell embryos exceeded those in wild-type oocytes.

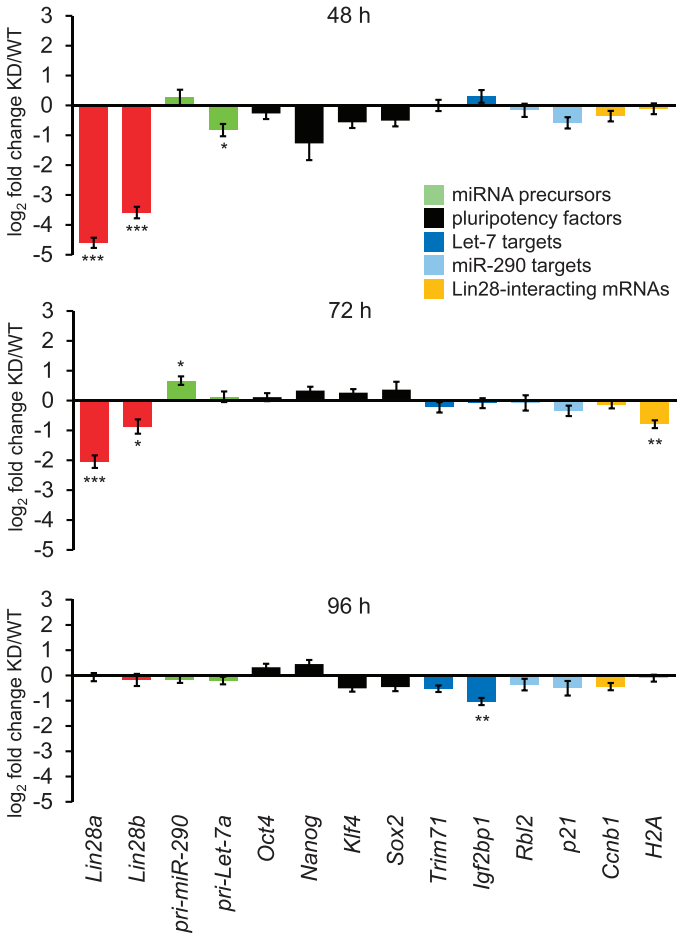


FIG. 5. Analysis of gene expression in early embryos from transgenic RNAi females. Gene expression in early embryos from transgenic females was analyzed by qPCR at 48, 72, and 96 h after hCG. Color coding indicates different categories of selected genes. Analyzed genes were (from left to right): *Lin28a* and *Lin28b*, pri-miR-290 and pri-Let-7 (primary miRNA transcripts), *Oct4*, *Nanog*, *Klf4*, and *Sox2* (core pluripotency factors), *Trim71* and *Igf2bp1* (known targets of Let-7), *Rbl2* and *p21* (known targets of miR-290 miRNAs), and *Ccnb1* and *H2A* (LIN28-bound mRNAs). Data are expressed as the relative ratio ($\log_2$-fold change) of expression in embryos from transgenic (KD) to that in wild-type females (WT). Asterisks indicate statistical significance of the change (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; n = 3). Error bars = SEM.

Effects of Lin28a *and* Lin28b *Depletion in Preimplantation Embryos*

First, we analyzed whether *Lin28a/b* knockdown had any effect on fertility. Analysis of litter sizes did not show any statistically significant difference between wild-type and transgenic mice (Fig. 4A). Likewise, mating efficiency of wild-type and transgenic mice was equal (Fig. 4B). We also tested whether there would be any age-dependent effect on fertility. Again, we did not observe any statistically significant differences in litter sizes between younger and older mice (Fig. 4C).

Next, we analyzed whether *Lin28a/b* knockdown had any effect on Let-7a miRNA levels. Real-time PCR analysis of Let-7a revealed a strong, statistically significant increase in Let-7a in 2-cell embryos isolated from transgenic females but not in GV oocytes (Fig. 4D). This result correlates with LIN28A levels and indicates that LIN28A (and presumably LIN28B) acts during the zygotic genome activation to block Let-7a biogenesis from newly transcribed precursors.

In addition to the suppression of Let-7 through binding Let-7 pre-miRNA, LIN28 proteins were shown to bind mRNAs [50–52]. In order to test whether depletion of maternally provided LIN28 proteins had any observable effects on gene expression, we analyzed gene expression of selected candidate genes during early development. We analyzed levels of miR-

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290 and Let-7 primary miRNAs (pri-miRNAs), core pluripotency factors (*Oct4*, *Sox2*, *Nanog*, and *Klf4*), known targets of Let-7 (*Trim71* and *Igf2bp1*) and miR-290 miRNAs (*Rbl2* and *p21*), and two known LIN28-bound mRNAs (*Ccnb1* and *H2A*). We also monitored *Lin28a* and *Lin28b* levels (Fig. 5). Our results showed that ~4-fold reduction of *Lin28a*/*b* transcripts persisted in embryos from transgenic mice up to the 8-cell stage (72 h post-hCG). However, 96 h after hCG injection, there were essentially no differences between embryos from wild-type and those from transgenic mice. Generally, none of the examined genes but *Lin28a* and *Lin28b* showed a strong change in gene expression. Four additional statistically significant minor expression changes did not exhibit any clear pattern, and it is not clear whether they represented direct consequences of *Lin28a*/*b* knockdown and whether they are functionally significant at all (Fig. 5). At 48 h after hCG injection, we observed a mild reduction of pri-Let-7a ($P < 0.05$), but it is unclear how LIN28 proteins could affect primary miRNA (pri-miRNA) transcript as they target the Let-7 at the next biogenesis stage, the precursor miRNA (pre-miRNA), which is released from pri-miRNA. Similarly, a mild upregulation of pri-miR-290 ($P < 0.05$) at 72 h post-hCG is of unclear origin and significance.

DISCUSSION

We report that *Lin28a* encodes a dormant maternal mRNA that is translated during oocyte-to-zygote transition in order to prevent zygotic expression of Let-7 during the zygotic genome activation. *Lin28a* mRNA expression analysis is in agreement with previous microarray analyses [53, 54] and RT-PCR profiling [39]. Altogether, these data demonstrate that *Lin28a* transcript is abundant in the oocyte and becomes degraded after fertilization, and zygotic transcripts start to accumulate after the 8-cell stage. The dormancy of LIN28 proteins is also consistent with published data, which show *Lin28a* recruitment to polysomes and increased protein abundance by immunofluorescent staining of LIN28A [2, 39]. However, there is an inconsistency concerning the localization of LIN28A. Previously, a strong nucleolar localization of LIN28A has been reported [39], whereas our immunofluorescent staining yielded a dominant cytoplasmic signal, which is consistent with other data obtained from mammalian cells, using various antibodies recognizing LIN28A [55–57]. Although we did not observe a distinct nucleolar enrichment (Supplemental Fig. S2), it is still possible that the antibodies used after the method described by Vogt et al. [39] and in our study recognize the same protein but the staining pattern differs due to variable epitope exposure. This would explain why both studies observed experimentally reduced levels of LIN28A despite a differing staining pattern (Fig. 3).

The functional study of *Lin28a* and *Lin28b* used a transgenic RNAi approach that offers several advantages over microinjection of siRNA, double-stranded RNA (dsRNA), or morpholino [58]. Transgenic RNAi allows for highly specific mRNA knockdown during oocyte growth without any off-targeting effects [59]. While insertion site effects could be a potential source of artifacts, it is extremely unlikely that the lack of developmental phenotype and post-zygotic up-regulation of Let-7 would be manifestations of insertion site effects (which would have to compensate Let-7 overexpression phenotype).

Unlike the *Lin28a* knock-out [38], transgenic RNAi knockdown of *Lin28a* and *Lin28b* did not result in reduced fertility. One explanation could be that the knockdown approach does not reduce *Lin28a* expression below the threshold level necessary for the phenotype manifestation. However, there are several arguments that this is not the case. First, maternal *Lin28a* was strongly suppressed at both mRNA and protein levels. In addition, we also down-regulated *Lin28b*, which could be partially compensating down-regulation of *Lin28a* (notably, *Lin28b* expression was not sufficient to prevent the phenotype in knock-out females [38]). Second, we detect relieved repression of Let-7 expression during zygotic genome activation demonstrating that *Lin28a* and *Lin28b* knockdown efficiently suppressed their function. Third, reduced fertility in knock-out animals might originate from events occurring at the beginning of the oocyte growth or before. *Lin28a* knock-out females have smaller ovaries and reduced numbers of primordial follicles; it was suggested that the loss of *Lin28a* affects proliferation of primordial germ cells. In contrast, transgenic RNAi knockdown is initiated at the onset of oocyte growth [49].

A postzygotic knockdown of maternal *Lin28a* using a morpholino approach implied an essential function in preimplantation development because it caused 2- and 4-cell developmental arrest [39]. However, this result is in strong contrast with the knock-out phenotype, where maternal contribution of *Lin28a* was completely eliminated, yet it resulted in mildly reduced fertility (average litter size was reduced from ~10 to ~7 pups [38]), which might seemingly have originated in defects in oocyte development rather than in postzygotic events [38]. Similarly, our results do not provide any evidence for postzygotic developmental arrest. As mentioned above, the lack of strong postzygotic phenotype in the transgenic RNAi model could be caused by insufficient knockdown of *Lin28a* and/or *Lin28b*, where their transcript levels were not reduced below a threshold necessary for phenotype appearance. However, as discussed above, RT-PCR and immunofluorescent staining demonstrated strong knockdown that resulted in up-regulation of mature Let-7. Another explanation of the reported morpholino-induced 2- and 4-cell developmental arrest [39] is that it does not represent a *Lin28a*-specific phenotype. Although morpholinos are supposed to be highly specific, a morpholino may also inhibit a gene(s) other than the intended target [60]. Off-targeting that results in 2- and 4-cell developmental arrest was reported in morpholino experiments in early mouse embryos. For example, knockdown of *Oct4* using morpholino microinjection into zygotes caused 4-cell embryo arrest in ~60% of embryos (<10% in control) [61], whereas maternal zygotic knock-out embryos genetically lacking *Oct4* developed to the blastocyst stage [62]. Similarly, *Nanog* morpholino knockdown caused 4-cell arrest in ~50% embryos [61]; embryonic knock-out develops to the blastocyst stage [63] whereas Nanog does not seem to be maternally expressed [37, 64]. Because experimental groups were relatively small and the experiment did not include a rescue control [39], it should be considered that the observed 4-cell developmental arrest might represent an off-target effect.

Dormancy of *Lin28a* and the absence of phenotype in transgenic females, despite increased expression of Let-7 during zygotic genome activation, offers important insights into the role of LIN28 homologs in the mouse model (Fig. 6) and raises a number of interesting questions. First, LIN28 proteins have multiple roles in pluripotency, development, growth, and metabolism and, in addition to their role in Let-7 biogenesis, they bind various mRNAs [50–52]. However, none of these mRNA regulations appears to be functionally important during OET, where we observed increased cytoplasmic abundance of LIN28A.

Second, there is a strongly reduced ability of miRNAs to repress translation during OET despite generally intact miRNA

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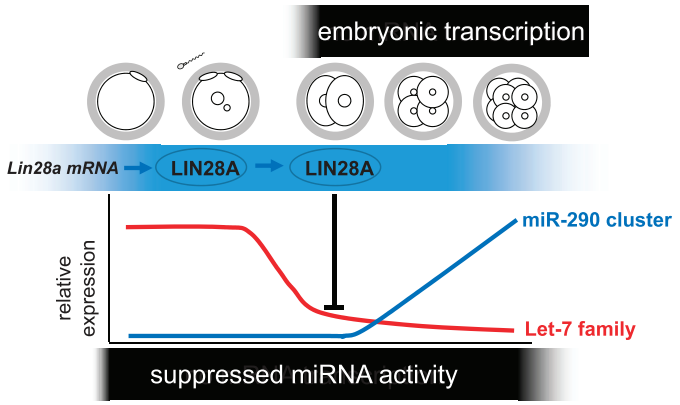


FIG. 6. Model of *Lin28* function during OET. The model integrates published data and results presented in this work. The maternal pool of *Lin28a* transcripts was translated during meiotic maturation and after fertilization (our data and those in ref. [39]). LIN28 does not target the mature Let-7 micro-RNA, but the precursor miRNA (pre-miRNA) released from the primary miRNA (pri-miRNA) transcript [26–30] suppresses zygotic expression of Let-7 (our data). The primary miRNA transcript (pri-miRNA) carrying miR-290-295 becomes expressed during the zygotic genome activation at the 2-cell stage, and miR-290-295 accumulate during early development [13, 54]. Superimposed on specific suppression of Let-7 is the global suppression of miRNA activity, which reduces the ability of mature miRNAs loaded on effector complexes to target imperfectly complementary target mRNAs [19, 20]. Likewise, oocytes and zygotes completely lacking canonical miRNAs (via maternal and maternal zygotic knock-out of *Dgcr8*, an essential miRNA biogenesis factor) develop to the blastocyst stage [20], further demonstrating that miRNAs are not important regulators of OET. Dynamics of P-bodies suggest that suppression of miRNA activity begins with the onset of oocyte growth and that miRNAs regain their function at approximately the 8-cell or morula stage [47].

biogenesis [12, 19–21]. This phenomenon should mask any phenotype caused by up-regulation of zygotic expression of Let-7. Thus, why would mouse OET use two functionally redundant but mechanistically different mechanisms to suppress Let-7 function? We propose two possible explanations for this phenomenon: first, Lin28 homologs may function as a fail-safe mechanism, which would protect the developing embryo from Let-7 activity in case global suppression of miRNA activity was relieved. We do not know the mechanism responsible for reduced ability of miRNAs to suppress translation in the oocyte and during OET. It seems that the inhibition is provided maternally by ineffective formation of the miRNA-guided effector complexes, and it is unclear how embryos acquire ability to produce fully active miRNA-guided effector complexes. Thus, maternally provided Lin28 homologs may assure Let-7 repression in the case of premature reactivation of the miRNA pathway. Second, Let-7 suppression by LIN28 during embryonic development is an evolutionarily conserved mechanism. Thus, *Lin28a* dormancy and activity during zygotic genome activation may be a vestigial mechanism that became replaced by global miRNA suppression. Whether the same global miRNA repression exists in other mammalian oocytes is not known. In fact, there is some evidence that miRNAs are active in porcine oocytes and [65] bovine early embryos [66]. Furthermore, Dicer function appears to be unique in mouse oocytes [67]. Therefore, the lack of requirement for maternally provided LIN28 homologs during OET in mice might represent another unique example of small RNA regulation rather than a common mammalian case. Consequently, there is an open possibility that the role of maternally provided LIN28 homologs is significant in other mammals during their OET.

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