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## ***Lin28a* regulates germ cell pool size and fertility**

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### **Abstract**

Overexpression of *LIN28A* is associated with human germ cell tumors and promotes primordial germ cell (PGC) development from embryonic stem cells *in vitro* and in chimeric mice. Knockdown of *Lin28a* inhibits PGC development *in vitro*, but how constitutional *Lin28a* deficiency affects the mammalian reproductive system *in vivo* remains unknown. Here, we generated *Lin28a* knockout (KO) mice and found that *Lin28a* deficiency compromises the size of the germ cell pool in both males and females by affecting PGC proliferation during embryogenesis. Interestingly however, in *Lin28a* KO males the germ cell pool partially recovers during postnatal expansion, while fertility remains impaired in both males and females mated to wild type mice. Embryonic overexpression of *let-7*, a microRNA negatively regulated by *Lin28a*, reduces the germ cell pool, corroborating the role of the *Lin28a/let-7* axis in regulating the germ lineage.

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#### Author Contribution

G.S.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing and final approval of manuscript. T.Y.S. and M.T.S.: collection and/or assembly of data. A.Y., J.P.H., and R.I.G.: provision of study materials. Y.F. and P.Y.H.: collection and/or assembly of data, and data analysis and interpretation. E.G.M.: provision of study material or patients. G.Q.D.: conception and design, data analysis and interpretation, manuscript writing and final approval of manuscript.

#### DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors declare no competing financial interests.

## Keywords

Germ Cells; Lin28a; miR-let7; Fertility

## INTRODUCTION

The RNA-binding protein *lin-28* was originally identified in *C. elegans* as a heterochronic gene that regulates developmental timing [1, 2]. We and others have shown that mammalian *Lin28a/b* regulates the biogenesis of the *let-7* family of tumor-suppressor microRNAs [3–7]. *LIN28* additionally functions as a cellular reprogramming factor, as an oncogene, and in the regulation of organismal growth and glucose metabolism [8–10]. By loss-of-function analysis with shRNA, we previously defined a molecular pathway whereby *Lin28a* influences primordial germ cell (PGC) development *in vitro* via the *let-7* target *Blimp1*, a key regulator of PGC specification [11], but to date there has been no assessment of the lifelong effects of *Lin28a*-deficiency on the germ lineage. Here we generated *Lin28a* knockout mice and found that *Lin28a* deficiency reduces the size of the germ cell pool during embryogenesis, leading to impaired fertility in adults. Interestingly, in males the germ cell pool size recovers with age due to postnatal expansion of germ cells, indicating compensatory mechanisms regulating spermatogonial stem cells in adults, though fertility remains reduced. Although Lin28 protein can exert effects on protein translation independently of *let-7* through mRNA binding, we demonstrate that overexpression of *let-7* phenocopies *Lin28a* deficiency, confirming a role for the Lin28/*let-7* axis in the regulation of the germ lineage.

## MATERIALS AND METHODS

### Mice

All animal procedures were approved by the Institutional Animal Care and Use Committee. The conditional *Lin28a* knockout mouse was reported previously [9]. We used a *Ddx4-Cre* strain (The Jackson Laboratory) to delete the floxed allele and generated conventional *Lin28a* KO mice. In parallel, we developed another *Lin28a* knockout strain (Fig. S1). A targeting construct with loxP sites flanking exon 2 (of 4) of *Lin28a* was electroporated into 129/SvEv Embryonic Stem (ES) cells. Southern analysis confirmed that clone H7 had the predicted hybridization pattern for both wild type and targeted alleles using 5-prime, 3-prime, and Neo<sup>R</sup> probes. Transient cre expression and subsequent clone selection was used to derive a targeted allele where the floxed exon and Neo<sup>R</sup> cassette was deleted. This deletion ES line was verified by PCR and used for blastocyst injection. Heterozygous mice were obtained by breeding with C57BL/6NTac female mice. The allele *Lin28a<sup>tm1Egm</sup>* lacks exon 2 which encodes the conserved CSD domain of Lin28a. Exons 3 and 4 are out of frame with exon 1. The phenotypes observed were consistent between these strains. *iLet-7* mice were previously reported [9]. Doxycycline (1g/mL) was given to mice in water. For all experiments, littermate controls were used.

### Sperm analysis

Epididymides were harvested. Cauda epididymis and vas deferens were cut with a razor blade in HTF media (Millipore) and the number of cells released was counted after 1 hour. Sperm motility and morphology was determined according to the criteria from The Jackson Laboratory web site (<http://reproductivegenomics.jax.org/maleprotocol.html>).

## Fertility test and superovulation

One 2–3 month-old female of either *Lin28a* +/- or -/- was placed into a cage with a fertility-proven wild-type male. Cages were monitored daily and the number of pups and litters were recorded for 10 weeks. One 7–10 week-old or 7–8 month-old male of either *Lin28a* +/+, +/-, or -/- was placed into a cage with 2 fertility-proven wild-type females. Cages were monitored daily and the number of pups and litters were recorded for 13 weeks. For the female plugging test, one 5 month-old male of either *Lin28a* +/+, +/-, or -/- was placed into a cage with two wild-type females. Cages were monitored daily and the number of plugs was recorded for 4 weeks. Females were replaced every 7 days. Superovulation was performed by successive injections of pregnant mare serum gonadotropin (10 units/mouse, Calbiochem) and human chorionic gonadotropin (10 units/mouse, Sigma) with a 48 hour interval. Oocytes were harvested and counted on the following day.

## *In vitro* fertilization

*In vitro* fertilization was performed as described previously [12]. Briefly, cumulus masses were collected from the ampullae of 3–4 week-old superovulated 57 female mice. Sperm from cauda epididymis of 4–6 month-old *Lin28a* +/+, +/-, and -/- males (N=2–3) were collected and counted after 10 minutes of capacitation.  $5 \times 10^5$  sperm/ml concentration was used. Embryos were cultured with 5% CO<sub>2</sub>. Developing embryos were counted on the following day (day 1) for two-cell embryos, and on days 3 and 4 for expanded blastocysts.

## Flow cytometry

Genital ridges were isolated from E12.5 or E13.5 embryos and dissociated in the “EB dissociation enzyme mix” described previously [13]. Cells were then washed once and resuspended in staining buffer (2% serum/PBS, SB) with primary antibodies (FITC mouse anti-SSEA1 [BD Pharmingen] and APC rat anti-c-Kit [BD Pharmingen]) and incubated for 20 minutes on ice. Cells were washed twice and resuspended in 500 ul SB, followed by addition of PI (1:1000) or DAPI (1:1000). Samples were sorted and analyzed on a FACS Calibur system or a FACS Aria system and via BD CellQuest or BD FACS Diva software (BD Biosciences).

## Quantitative RT-PCR

RNA was collected using Trizol reagent (Invitrogen). Total RNA was reverse-transcribed and subjected to Taqman miRNA assay (Applied Biosystems) followed by pre-amplification of cDNA using PreAmp master mix (Applied Biosystems) following the manufacturer’s instructions. miRNA expression was measured by quantitative PCR using the delta-delta CT method.

## Histology

Tissue samples were fixed in 10% buffered formalin or Bouin’s solution and embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E).

## Immunohistochemistry and immunofluorescence

Immunohistochemistry was performed as described [10]. Immunofluorescence was performed in the same manner except using Alexa488 or Alexa568-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies. The primary antibodies used in this study include: rabbit anti-Lin28 (1:300, Cell Signaling Technology), rabbit anti-Mvh (1:1000, abcam), and mouse anti-Ki67 (1:50, Dako).

## Follicle counting

Adult ovaries were collected during early estrus and estrus stages of the estrous cycle determined by analysis of vaginal smears. Follicle count was performed using ovary sections with H&E staining or immunostained by an anti-Mvh antibody for easier detection of oocytes. Follicles were classified as primordial (flattened or at least one cuboidal granulosa cells (GC)), primary (one complete layer of cuboidal GC), secondary (two or more complete layers of GC), and antral (antrum formation).

## Plasma hormone measurement

Testosterone was measured by an enzyme immunoassay kit (Assay Designs). TSH and LH were measured by The University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core.

## Statistical analysis

Data is presented as mean  $\pm$  SEM, and Student's t test (two-tailed distribution, two-sample unequal variance) was used to calculate p values. Statistical significance is displayed as \* p < 0.05 or \*\* p < 0.01.

## RESULTS

### Lin28a expression in juvenile and adult mice

The localization of Lin28a in adult mouse testis was reported previously [14]. However its expression and localization during development remained to be characterized. We evaluated Lin28a expression patterns in the germ lineage from midgestation to adult stages in both males and females. Lin28a was prominently expressed in SSEA1+ PGCs of the developing embryo at day 13.5 (Fig. 1A and data not shown). In the ovary, oocytes expressed Lin28a in newborns and adults alike (Fig. 1B, C). In the testis, nearly all gonocytes defined as Mvh<sup>+</sup> cells expressed Lin28a in newborns (Fig. 1D and data not shown), expression of which was limited to undifferentiated spermatogonia in adults (Fig. 1E). Lin28a localization remained cytoplasmic at all developmental stages analyzed.

### Lin28a deficiency compromises germ cell pool size

To evaluate *Lin28a* in the germ cell lineage and reproductive system function *in vivo*, we generated two independent *Lin28a* knockout (KO) mouse strains (Supplementary Fig. 1 and [9]). We confirmed that the second exon of *Lin28a* was deleted in both strains and observed that the phenotypes such as higher *let-7* levels in embryos, lower number of germ cells in neonates, and reduced fertility described below were consistent between these strains. The data presented in this article are mainly taken from the strain in Supplemental Fig. 1.

We examined PGC development in midgestation embryos, as our previous experiments using an *in vitro* embryonic stem cell differentiation system indicated that knockdown of *Lin28a* significantly diminished the numbers of PGCs *in vitro* [11]. We found markedly fewer SSEA-1<sup>+</sup> and SSEA-1<sup>+</sup>c-Kit<sup>+</sup> PGCs in the genital ridges of embryonic day 13.5 *Lin28a* KO embryos compared to controls (Fig. 2A). qPCR revealed that *Lin28a* KO PGCs at this stage express higher *let-7* miRNAs, consistent with the loss of Lin28a suppression (Fig. 2B). Correspondingly, neonatal mouse testes and ovaries also contained fewer gametes positive for the germ cell-specific marker Mvh (Fig. 2C–F). Ki-67 staining revealed fewer proliferating germ cells in the newborn KO testes compared to wild type controls (Fig. 2E, G). Together, these results indicate a role for *Lin28a* in regulating germ cell pool size in embryonic and neonatal stages of both sexes.

### ***Lin28a* KO females have reduced fertility**

Next, we examined female *Lin28a* KO mice to determine whether reduced germ cell pool size impacts fertility. *Lin28a* KO mice were runted compared to controls (30–50% smaller body size) whereas average ovary weight of *Lin28a* KO mice was approximately one-third that of controls; thus, relative to body weight, the *Lin28a* KO ovary is disproportionately small (Fig. 3A–C). *Lin28a* KO ovaries harbored reduced numbers of primordial ovarian follicles per given area (Fig. 3D), whereas the number of maturing follicles and corpora lutea was indistinguishable from controls (Fig. 3E), indicating that despite a reduced pool of primordial follicles, morphological oocyte maturation remained intact in *Lin28a* KO animals. However, we noted a reduction in the numbers of live oocytes collected from KO animals following hormone-induced superovulation (Fig. 3F). Ultimately, fertility tests yielded lower total numbers of pups and smaller litter sizes in spite of comparable frequencies of delivery, suggesting fewer functionally mature oocytes in the KO females (Fig. 3G–I). These data indicate that in *Lin28a* KO females, the germ cell pool size is reduced at embryonic and adult stages, resulting in reduced numbers of mature oocytes and decreased fertility.

### **Spermatogenesis partially recovers in adult *Lin28a* KO testes**

Like *Lin28a* KO females, males are runted. The testes of *Lin28a* KO adult males were approximately half the size of controls but proportional to their lower total body weight (Fig. 4A–C). Sperm counts were lower in KO males, whereas motility and morphology of the KO sperm were comparable to those of controls (Fig. 4D–F). The histology of KO testes was comparable to controls, showing normal maturation during spermatogenesis and comparable numbers of Ki-67<sup>+</sup> proliferating germ cells and Cyclin D1<sup>+</sup> proliferating spermatogonia (Fig. 4G–I). These data indicate a homeostatic recovery to near normal sperm production in adult *Lin28a* KO males.

### ***Lin28a* KO males have reduced fertility**

We then evaluated the fertility of *Lin28a* KO males. Given that the adult *Lin28a* KO testes showed a recovery of testis histology and sperm differentiation relative to neonates, we compared fertility in young post-weanling (7–10 weeks old) and older (7–8 months old) males. Both age groups sired fewer litters and produced lower overall numbers of pups during the experimental period relative to controls (Fig. 5A–E). We performed an *in vitro* fertilization experiment and confirmed that the lower numbers of pups did not result from less fertilizing capacity of *Lin28a* KO sperm (Table S1). Interestingly, while younger *Lin28a* KO males produced smaller litter sizes in two distinct strain backgrounds (Fig. 5C), older males were comparable to controls (Fig. 5F). The older *Lin28a* KO males had less frequent mating behavior as evidenced by reduced plugging in females, which could explain less frequent female deliveries and lower total numbers of pups in spite of similar litter sizes (Fig. 5G). In considering what may account for less frequent mating behavior in relatively older males, we found the plasma testosterone (T) levels were lower in *Lin28a* KO mice than in controls (Fig. 5H). Luteinizing hormone (LH) secreted from the pituitary gland positively controls T production from Leydig cells in testes [15]. *Lin28a* KO males had similar plasma LH levels to controls, indicating that the physiological origin of lower T levels was distal to pituitary function and likely originated from the Leydig cells themselves (Fig. 5I). The other gonadotropin, follicle-stimulating hormone (FSH), which stimulates sperm production through Sertoli cells in the testis, was higher in *Lin28a* KO plasma (Fig. 5J), consistent with the lower sperm count seen in the *Lin28a* KO mice (Fig. 4D), and the known feedback role of FSH in sperm production [15]. Thus, although *Lin28a* KO males recovered near normal spermatogenesis, they nonetheless manifest reduced fertility in the setting of lower sex hormone levels.

## Embryonic overexpression of *let-7* miRNA reduces germ cell pool size

*Let-7* miRNAs are a major downstream target of *Lin28a* and known to regulate cell cycle and cell proliferation. We hypothesized that overexpression of *let-7* during embryogenesis might compromise germ cell pool development similar to *Lin28a* KO mice. *iLet-7* mice [9], which overexpress *let-7g* upon induction by doxycycline (dox), were fed dox in their drinking water throughout pregnancy, and we confirmed that SSEA-1<sup>+</sup> PGCs from E13.5 embryos overexpressed *let-7g* 3-fold (Fig. 6A). Neonatal testes and ovaries of *iLet-7* mice exposed to doxycycline during gestation showed a reduced frequency of Mvh<sup>+</sup> germ cells compared to littermate controls (Fig. 6B–D), suggesting that *Lin28a* regulates the germ cell pool size in both males and females at least in part via modulation of the *let-7* miRNA pathway.

## DISCUSSION

In previous work, we performed an inhibitory shRNA screen during the *in vitro* differentiation of mouse embryonic stem cells into the germ lineage to implicate *Lin28a* as a regulator of PGC numbers via effects on the *let-7* target gene *Blimp1*, a known master regulator of germ cell development [11]. Here we analyze *Lin28a* KO mice and reciprocal transgenic strains that overexpress *let-7* to unequivocally establish a role for the *Lin28a/let-7* axis in establishing germ cell pools during embryogenesis and influencing reproductive function throughout adult life. While a marked reduction in the size of the germ cell pool is evident at birth in both males and females, our analysis of germ cell numbers and fertility in adults revealed interesting sex-specific differences in the mechanisms of lifelong reduced fertility. Coupled to our prior studies, which established that transgenic *Lin28a* induced marked delays in puberty [10], our work enriches our understanding of this pathway as one of the critical regulators of reproductive function through effects on germ cell development, timing of sexual maturation, and reproductive behavior throughout adult life.

PGCs, which emerge in the epiblast-derived extraembryonic mesoderm, migrate to the genital ridge and proliferate by E13.5 [16]. Our *Lin28a* KO and *iLet-7* mice data indicate that repression of *let-7* at this stage is necessary for establishing proper numbers of germ cells to populate the developing gonad in both males and females. Our prior *in vitro* study indicated that *Lin28a* and *let-7* mediate opposing effects on PGC numbers, and that *Lin28a* functions in large part via regulation of the *let-7* microRNAs and *Blimp-1* [11]. We cannot exclude the possibility that other *let-7* targets are involved or that *Lin28a* might bind to mRNAs that affect PGC development independently of *let-7*. These alternatives notwithstanding, our results illustrate the importance of the *Lin28a/let-7* axis regulating the germ cell pool size as early as E13.5, and the effects are apparent throughout post-natal life.

*Lin28a* influences both male and female fertility in some common and some distinct ways. In fertility tests, young *Lin28a* KO males and females both yielded smaller litter sizes, reflecting fewer mature gametes, consistent with germ cell pool size reductions seen in the embryonic and neonatal gonads in both sexes. In male gametogenesis, spermatogonial stem cells proliferate and differentiate to form sperm throughout life. Our results showed that older (7–8 month-old) *Lin28a* KO mice had similar testis histology and litter sizes relative to control mice, although their sperm counts remained lower. This indicates that the size of the male germ cell pool at least partially recovers via postnatal expansion and that *Lin28a* is dispensable for the postnatal germ cell expansion. In *Lin28a* KO females, the germ cell pool does not change postnatally, consistent with the fact that female germ cells enter meiosis and then arrest midway through meiosis I during embryogenesis and do not produce new gametes postnatally. Although this traditional concept has been challenged recently [17], if females continue to produce oocytes in adults from germ line stem cells, we might have anticipated a similar recovery of the germ cell pool in females, as we did in males.

Another factor affecting fertility is regulation by sex hormones. Our results show that plasma testosterone levels in *Lin28a* KO males are lower despite comparable or even higher gonadotropin (LH, FSH) levels. The mechanism of the testosterone deficiency remains unknown. As Leydig cells do not express *Lin28a* (Fig. 1), other factor(s) outside of the pituitary/gonadal axis may regulate plasma testosterone levels. Genetic variation in *LIN28B*, a paralog of human *LIN28A*, has been linked to the timing of menarche in human genome-wide association studies (GWAS) [18–21], it will be interesting to explore endocrinological roles of *Lin28a* in the reproductive system.

Our current data reveal that the *Lin28a/let-7* pathway is critical for proper germ cell pool development *in vivo*, primarily through effects at the PGC stage. Future studies may include analysis of human *LIN28A* levels that correlates with infertility, especially in patients with primary ovarian failure and short stature.

## CONCLUSIONS

In this study we generated and analyzed *Lin28a* knock-out mice and found that *Lin28a* deficiency compromises the size of the germ cell pool during embryogenesis in both males and females, leading to reduced fertility in adults. Interestingly, the germ cell pool size partially recovers in adult males due to postnatal expansion of germ cells. Embryonic *let-7* overexpression reduces the germ cell pool, reinforcing the role of the *Lin28a/let-7* axis in regulating the germ lineage.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

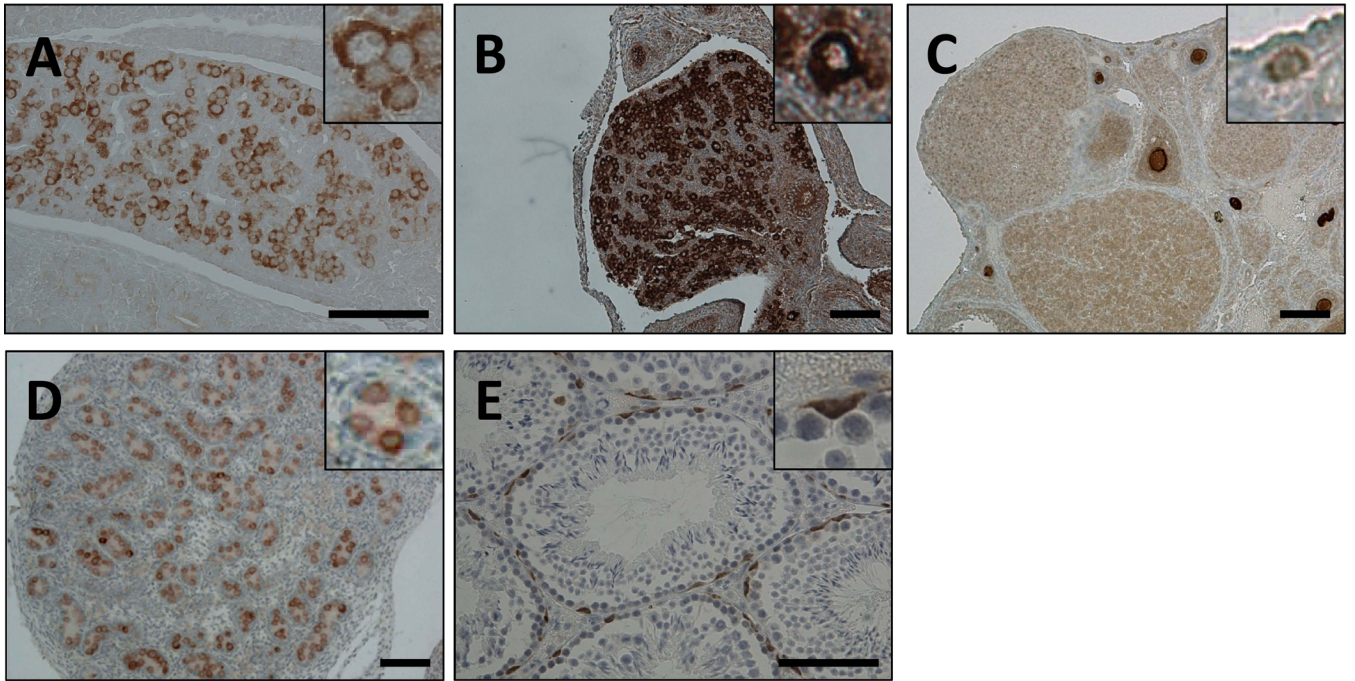
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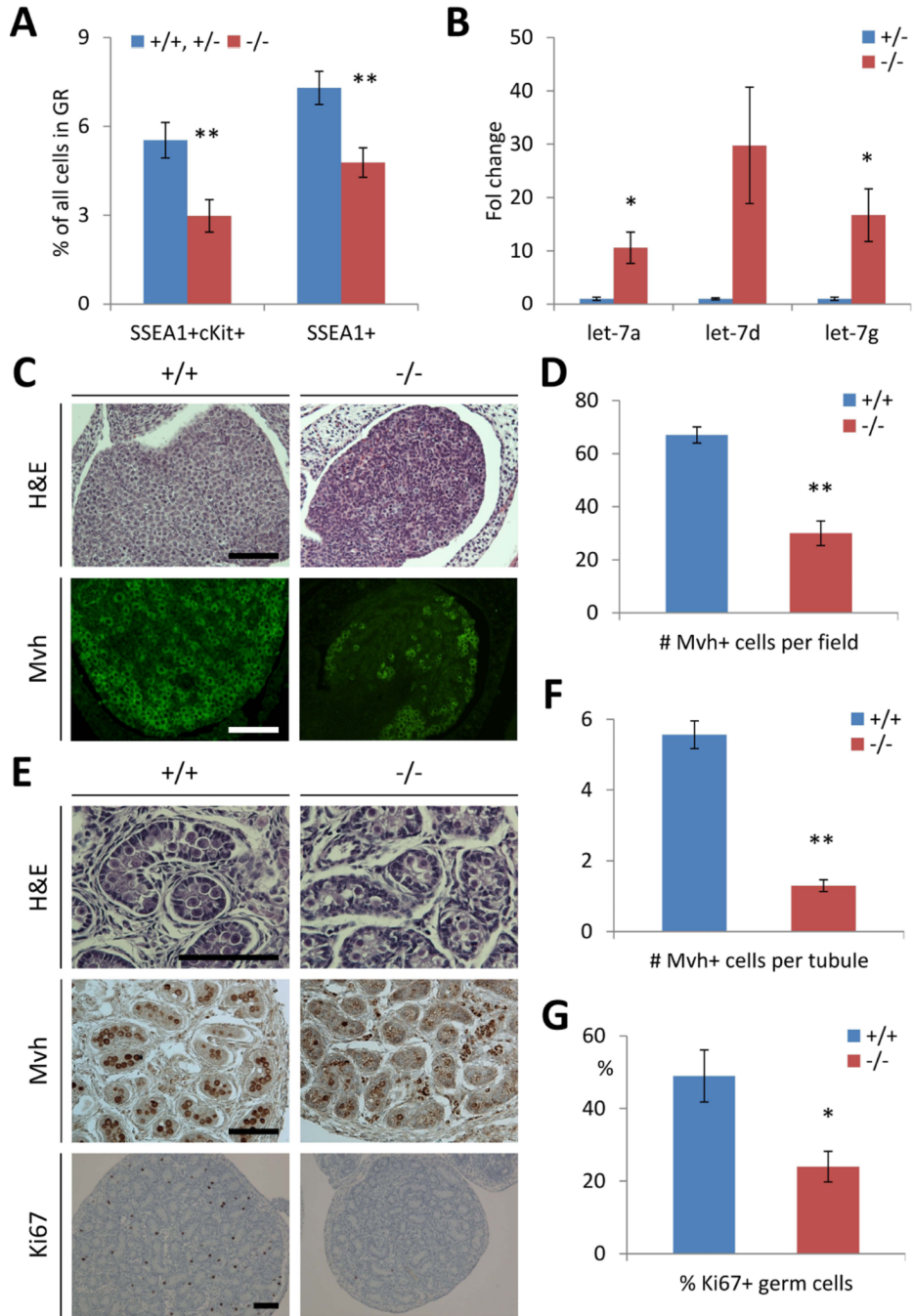
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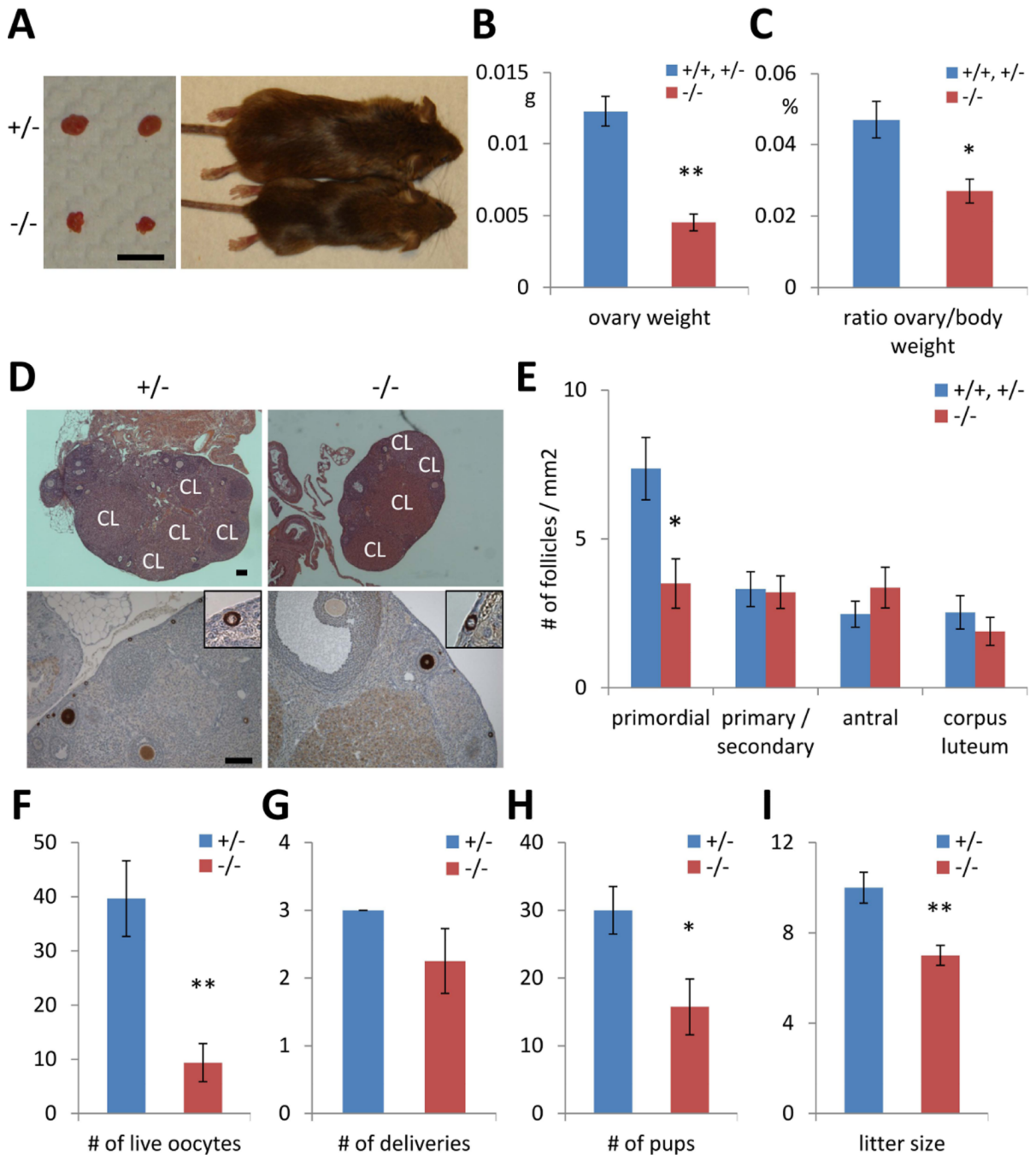




**Fig. 1.** *Lin28a* expression in normal gonadal tissues at different ages. (A) E13.5 genital ridge. (B) Newborn ovary. (C) 5-week-old ovary. (D) Newborn testis. (E) 8-month-old testis. Insets show higher magnification of *Lin28a* positive cells. Note that *Lin28a* expression in cytoplasm. Scale bars 100  $\mu$ m.

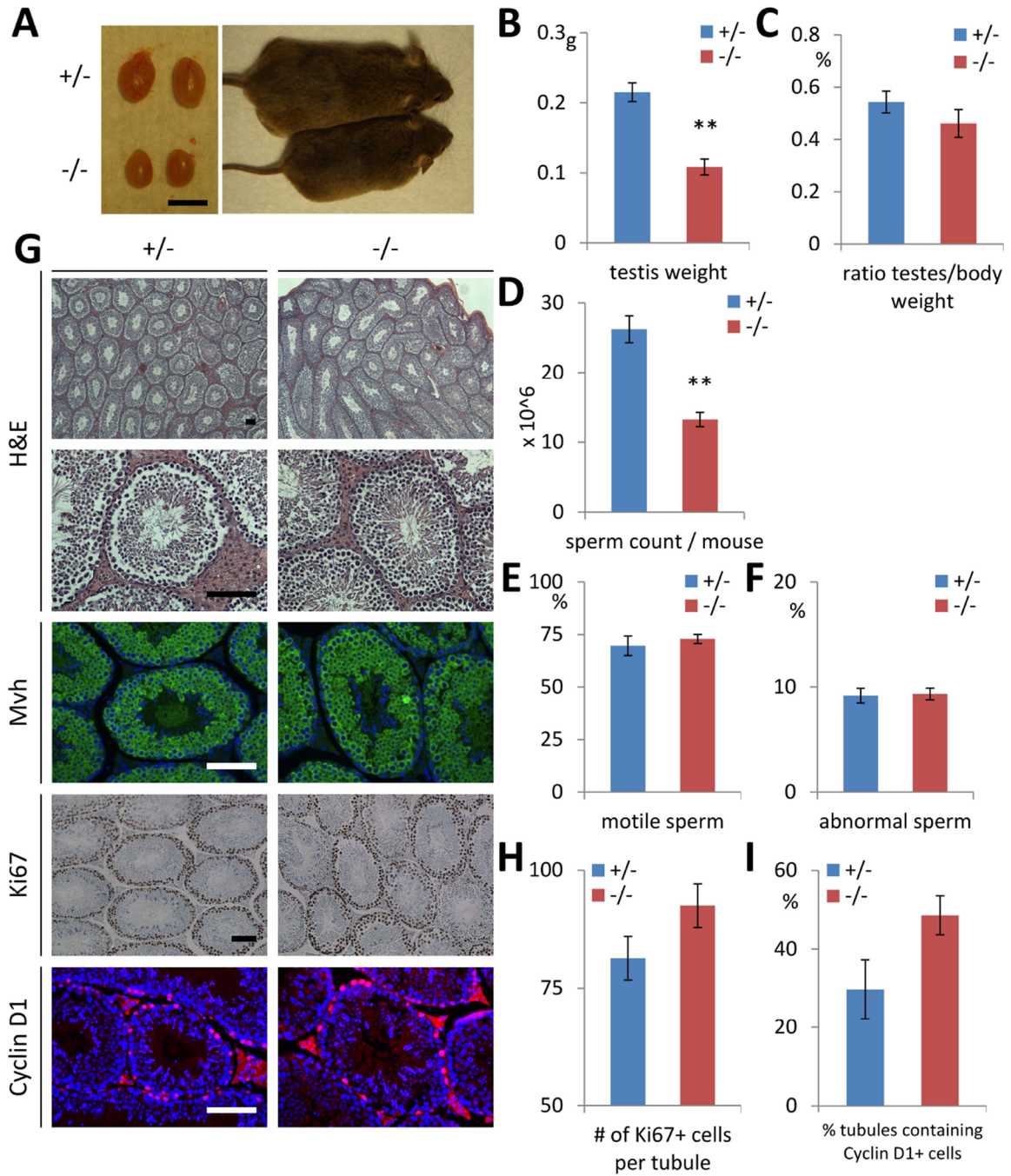


**Fig. 2.** *Lin28a* deficiency reduces germ cell pool size. (A) Percentage of primordial germ cells in *Lin28a*  $-/-$  and control genital ridges at E13.5. N=4–15. (B) *let-7* levels of primordial germ cells in E13.5 embryos. N=3. (C) *Lin28a*  $+/+$  and  $-/-$  newborn ovaries. (D) Number of Mvh+ germ cells in *Lin28a*  $+/+$  and  $-/-$  newborn ovaries. N=4–5. (E) *Lin28a*  $+/+$  and  $-/-$  newborn testes. (F–G) Number of Mvh+ germ cells per tubule (F) and % Ki67+ germ cells (G) in *Lin28a*  $+/+$  and  $-/-$  newborn testes. N=64–74 from 3–5 mice in (F) and N=3–4 in (G). \*  $p < 0.05$ , \*\*  $p < 0.01$ . Error bars represent SEM. Scale bars 100  $\mu$ m.

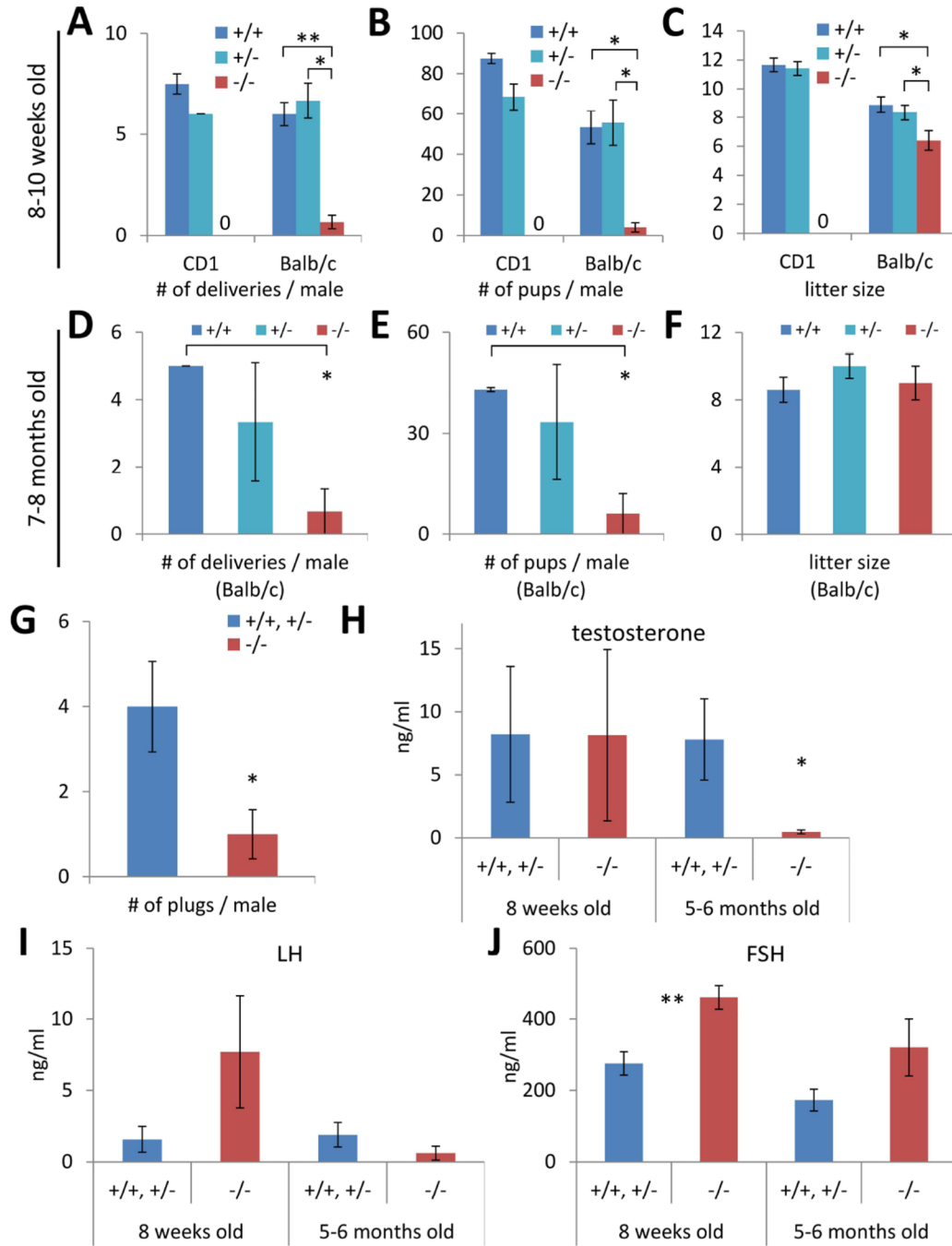


**Fig. 3.** *Lin28a* knockout females have reduced fertility. (A) Representative photographs of 5-month-old *Lin28a*  $+/-$  and  $-/-$  mice and their ovaries. Bar 5mm. (B–C) Ovary weight (B), ovary weight relative to body weight (C) in 5-month-old *Lin28a*  $+/-$  and  $-/-$  females. N=4–6. (D) H&E staining (top) and immunostaining with Mvh (bottom) of 3-month-old adult ovaries. Inset: higher magnification of primordial follicles. Note that corpus luteum (CL) are found in both *Lin28a*  $+/-$  and  $-/-$  ovaries. Scale bars 100  $\mu$ m. (E) Number of different stages of ovarian follicles per 1  $\text{mm}^2$  in 3-month-old *Lin28a*  $+/+$ ,  $+/-$  and  $-/-$  ovaries. N=8–10. (F) Number of live oocyte collected from 2-month-old *Lin28a*  $+/-$  and  $-/-$  females after superovulation. N=5–6. (G–I) Female fertility tests at 2–3 months old. Number of deliveries

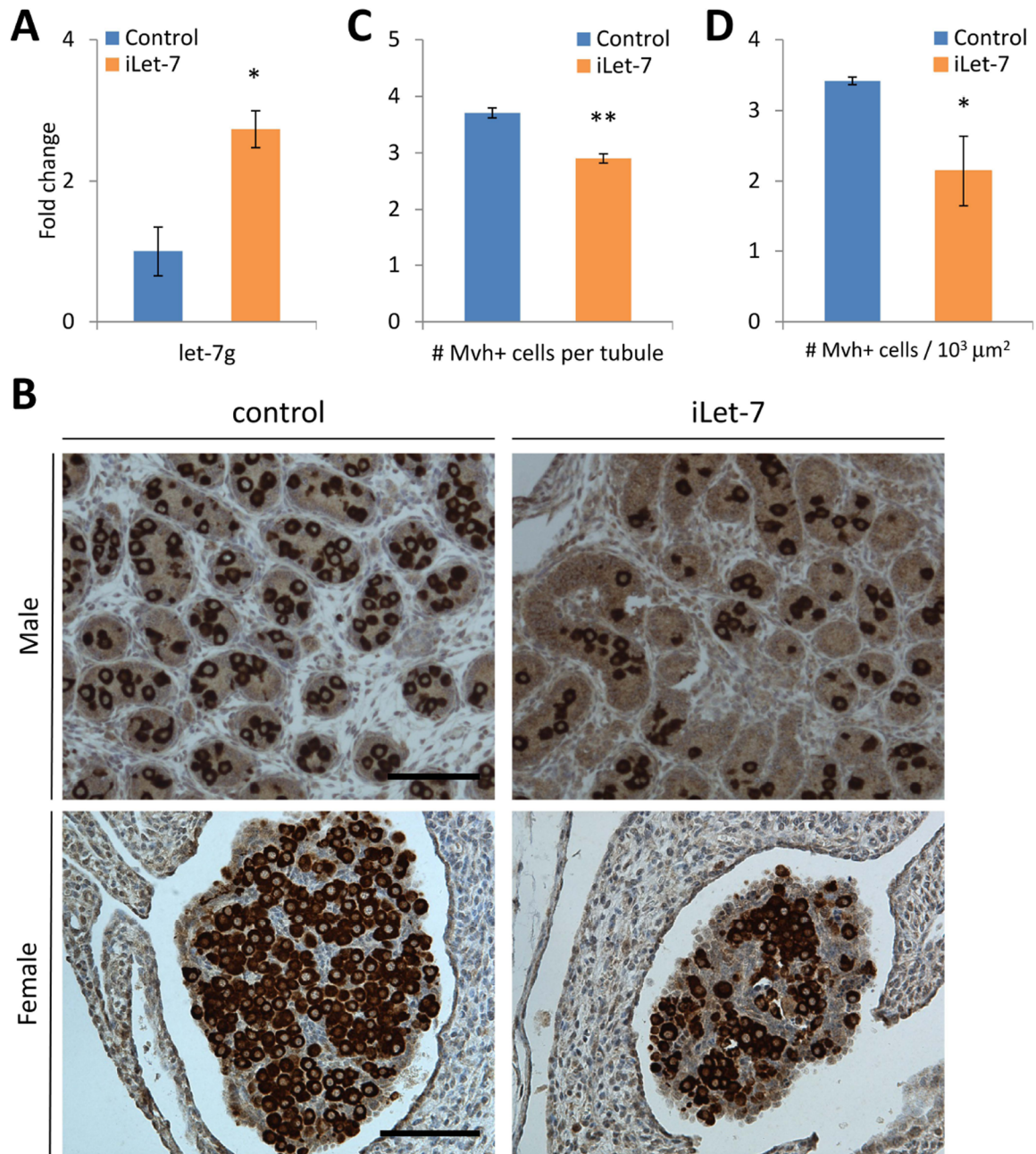
(G) and number of pups born (H) per *Lin28a* +/- and -/- female during 10-week experimental period. (I) Litter size of *Lin28a* +/- and -/- females. N=3-4. \* p<0.05, \*\* p<0.01. Error bars represent SEM.



**Fig. 4.** Partial recovery of germ cell pool size in adult *Lin28a* KO males. (A) A representative photographs of 5-month-old *Lin28a* +/- and -/- mice and their testes. Bar 5mm. (B-F) Testis weight (B), testis weight relative to body weight (C) in 5- to 6-month-old *Lin28a* +/- and -/- mice. N=6. (D-F) Total epididymal sperm count per mouse (D), percentage of motile sperms (E), and percentage of abnormal sperms (F) in 7- to 8-month-old *Lin28a* +/- and -/- mice. N=3-4. (G) H&E and immunostaining of 6-month-old *Lin28a* +/- and -/- testes. (H-I) Number of Mvh+ germ cells per tubule (H) and number of tubules containing Ki67+ cells (I) in 6- to 8-month-old *Lin28a* +/- and -/- adult testes. N=5. \*\* p<0.01. Error bars represent SEM. Scale bars 100  $\mu$ m.



**Fig. 5.** *Lin28a*<sup>-/-</sup> males are subfertile. (A–F) Male fertility tests at 7–10 weeks old (A–C) and 7–8 months old (D–F). Number of deliveries (A and D) and number of pups born (B and E) per *Lin28a*<sup>+/+</sup>, *+/–* and *–/–* male during 13-week experimental period. (C and F) Litter size of *Lin28a*<sup>+/+</sup>, *+/–* and *–/–* males. N=2–3. (G) Number of plugs per 5-month-old *Lin28a*<sup>+/+</sup>, *+/–* and *–/–* male during 4-week experimental period. N=3–6. (I–K) Plasma testosterone (I), LH (J), and FSH (K) levels in *Lin28a*<sup>+/+</sup>, *+/–* and *–/–* males. N=4–10. \* p<0.05, \*\* p<0.01. Error bars represent SEM.



**Fig. 6.** Embryonic overexpression of *let-7* miRNA reduces germ cell pool size. (A) *let-7* levels of primordial germ cells in E13.5 embryos. N=3. (B) *iLet-7* and control newborn testes and ovaries. (C) Number of Mvh+ germ cells per tubule in *iLet-7* and control newborn testes. N=286–303 from 3 mice. (D) Number of Mvh+ germ cells per 1000  $\mu\text{m}^2$  in *iLet-7* and control ovaries. N=3–5. \*  $p < 0.05$ , \*\*  $p < 0.01$ . Error bars represent SEM. Scale bars 100  $\mu\text{m}$ .