Study on the Function and Mechanism of Lin28B in the Formation of Chicken Primordial Germ Cells

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Simple Summary: In this study, we explored the function and molecular mechanism of Lin28B in the formation of chicken primordial germ cells (PGCs) in detail. Our results indicate that Lin28B participates in the formation of PGCs through let-7a-3p, which set a theoretical foundation for improving the function and mechanism of the Lin28 family in the formation of PGCs.

Abstract: Lin28A and Lin28B are two homologues of the same family of RNA binding proteins (RBPs). The function and molecular mechanism of Lin28A in the formation of primordial germ cells (PGCs) are very clear, but the related research on Lin28B is rarely reported. Here, we found that the overexpression of Lin28B can promote the formation of PGC in vivo. Furthermore, the overexpression of Lin28B also resulted in the inhibition of totipotency gene expression and upregulated the PGCs marker genes, and a significant increase in the number of PGCs in genital ridge, as detected by Periodic Acid-Schiff (PAS) staining. However, the inhibited Lin28B expression showed completely opposite results, which were confirmed on the PGC induction model in vitro. Mechanistically, we found that the overexpression of Lin28B can inhibit the maturation of let-7a-3p, and the results of high-throughput sequencing indicated that let-7a-3p was a negative regulator of the formation process of PGCs. Therefore, we conclude that our results determine that Lin28B participates in the formation of PGCs through let-7a-3p, which set a theoretical foundation for improving the function and mechanism of Lin28 family in the formation of PGCs.

Keywords: primordial germ cells; Lin28B; gene function; let-7a; miRNA

1. Introduction

The function of Lin28 is primarily concentrated on embryo development [1,2] and cell reprogramming [3,4], which could code two RNA-binding proteins, Lin28A and Lin28B [5]. As early as 2009, it was shown that Lin28A played an important role in the cytogenesis of primary germ cells (PGCs) [6]. Particularly, Lin28A inhibits the maturation of let-7, thereby activating the expression of the Blimp1 gene (key gene for the formation of PGCs) [7,8]. However, the function of Lin28B in the formation of PGCs remains unknown. Recently, several research groups have found that the SNP locus in Lin28B is closely related to the age of female menarche [9–11], indicating the close correlation of Lin28B in animal reproduction.

Subsequently, increasing studies observed the function of Lin28A and Lin28B in the PGCs development of mammals. The proliferation of PGCs in mice with Lin28A knockout was impaired, leading to decreased number of germ cells during embryo development [12]. Lin28A knockout during the differentiation of E7Cs into PGCs could significantly reduce the expression of PGCs-formation related genes, such as Blimp1, Prdm14, and Stella [13]. Similar to Lin28A, the knockdown of Lin28B could impair the formation of PGCs that are...
derived from Embryoid Bodies (EBs). The knockdown of Lin28B in ESCs could inhibit the differentiation of ESCs into PGCs [8]. Contrastingly, the expression pattern of Lin28A and Lin28B in the reproductive system is not always consistent. When there are only PGCs in genital ridge, the expression level of Lin28A is the highest. With the progress of pregnancy, the expression level of Lin28 decreased significantly, but that of Lin28B did not change markedly [14,15], which makes the function of Lin28B in PGCs uncertain.

Although Lin28A and Lin28B are homologies, they bind to target miRNA and play a major role in post-transcriptional control [16,17]. Lin28A avoids Dicer processing by inducing the 3′ end of pre-let-7 into uracil, and, finally, degrades it [18]. Contrastingly, Lin28B is a posttranscriptional inhibitor of pre-let-7, it binds to the end of pre-let-7 through its cold shock domain (CSD) and to the GGAG motif of pre-let-7 through its zinc finger domains (ZFD) [19,20]. This raises a question regarding whether totally different mechanism will lead to totally different function. For this reason, the function and underlying molecular mechanisms of Lin28B in the formation of PGCs were comprehensively studied while using chicken PGCs as the study objects. The results of the study may lay foundations for analyzing the function and mechanism of Lin28B in the formation of chicken PGCs.

2. Materials and Methods

2.1. Ethics Statement

All of the procedures involving the care and use of animals conformed to the U.S. National Institute of Health guidelines (NIH Pub. No. 85-23, revised 1996) and they were approved by the Laboratory Animal Management and Experimental Animal Ethics Committee of Yangzhou University.

2.2. Reagents

BMP4 (cyt-361) was from PROSPEC (Beijing, China). Dulbecco’s modified eagle medium (DMEM, 41965062) and fetal bovine serum (FBS, 10100-147) were supplied by Gibco (Carlsbad, CA, USA). The transfection reagents FuGENE® HD (E2311) and Dual-Luciferase® reporter assay system were from Promega (Madison, WI, USA). The PrimeSTAR® Max DNA polymerase, the reverse-transcription kit (RR036A), the quantification kit for qRT-PCR (RR820A), and the restriction endonucleases SnaB I, Kpn I, and Xho I were supplied by Takara (Takara, Dianlian, China). The CVH (DDX4) antibody (ab27591) and the CKIT antibody (ab5634) were from abcam (San Francisco, CA, USA).

2.3. Cell Treatments and Grouping

We studied the role of Lin28B in the formation of PGCs in vitro while using the BMP4 induction model that was established previously. The isolation and cultivation of ESCs are based on previous study [21]. Well-grown ESCs were transferred to 24-well plates and then treated/grouped, as follows. The routinely induced BMP4 was used as a control. The ESCs that were transfected with oeLin28B and siLin28B vector then induced by BMP4 were the treatment group. During induction, the culture media were replaced every two days. Cell morphology was checked with a fluorescence inversion microscope system. The zero-day-old, two-day-old, four-day-old, and six-day-old cells were collected for later analysis. The in vivo experiment was performed, as follows. The in vivo experiment was performed, as follows: the vectors of oeLin28B and siLin28B were mixed with PEI (M:V = 1:1), respectively, and then the mixture was injected into the blood vessels of 2.5 day-old chicken embryos with 1µg. The oeLin28B group and siLin28B group were the treatment group, and chicken embryos were checked every two days. The genital ridges of 4.5-day-old chicken embryos were collected for later analysis.

2.4. Construction of Lin28B Overexpression Vector

The forward and reverse primers were designed while using the Primer 5.0 software according to the sequence of chicken Lin28B in GenBank (accession no.: NM_001034818.1). The forward primer was (F):5′-cgggatccATGGCCGAAGGGCG-3′ and the reverse
primer was (R):5′-cgggaattcCGCACA TGACACA-3′. The enzyme digestion sites were BamH I and EcoR I. The total RNA of 4.5-day-old reproductive ridge was extracted and then reverse-transcribed to cDNA. With the cDNA as a template, clonal fragments of the target gene *Lin28B* were obtained through the use of PCR. The conditions for PCR were three min. at 98 °C, 25 s at 98 °C, 30 s at 64 °C, and 1 min. at 72 °C. The PCR cycles numbered 35 with an extension time for 7 min. at 72 °C. The linear plasmid pcDNA3.1 after dual-enzyme digestion and amplified target gene fragments of *Lin28B* were ligated in order to construct the *Lin28B* overexpression vector, oe*Lin28B*.

### 2.5. Construction of the *Lin28B* Interference Vector

We designed three targets according to the sequence of *Lin28B* and synthesized a single-strand RNA (Supplementary Table S1). After being annealed, the RNA was ligated to the lentivirus interference vector skeleton of pLTNT-siRNA-GFP to construct four overexpression interference vectors of *Lin28B*, called si*Lin28B*-1, si*Lin28B*-2, si*Lin28B*-3, and si*Lin28B*-4. These four vectors were then transfected to confluent DF-1 cells, according to the mixture of plasmids and FuGENE HD at a ratio of 1:3 (M/V). Forty-eight hours later, the transfected DF-1 cells were selected with 10 ng/µL of puromycin for 24 h, after which the expression of green fluorescence proteins was observed with a fluorescence microscope. The DF-1 cells were collected from every group, and their total RNA was extracted with the Trizol method. The cDNA was synthesized through the use of a reverse-transcription kit, and the relative expression level of *Lin28B* was assayed with qRT-PCR. The amount of expression was calculated according to the $2^{-\Delta\Delta Ct}$ method, and β-actin was the internal reference gene.

### 2.6. qRT-PCR

Zero-day-old, two-day-old, four-day-old, and six-day-old cells during in vitro induction as well as the zero-day and 4.5-day-old in vivo induction cells from each group were collected. The total RNA was extracted with the Trizol method and it was transcribed to cDNA. The expression of the *NANOG* and the marker genes of PGCs genesis, such as DEAD-box helicase 4 (*DDX4*, also called *Cvh*), Chicken tyrosine kinase receptor (*C-kit*), and PR domain 1 (*Prdm1*, also called *Blimp1*), was determined with β-actin as the internal reference gene. qRT-PCR assay was based on the reverse-transcription kit from Tiangen (production no.: FP215). The 20 µl of reaction system includes 2 µl of cDNA (50 ng), 10 µl of 2 ×SuperReal Color PreMix, 0.6 µl of the forward and reverse primers (10 µM), and ddH2O. The reaction procedure of PCR is 15 min. at 95 °C, 10 s at 95 °C, and 32 s at 62 °C for 40 cycles. The expression was analyzed with the Ct values, and the primes are supplied in Supplementary Table S2.

### 2.7. PAS Staining

4.5-day-old chicken embryos from each group were collected and fixed for 24 h. The fixed chicken embryos were then treated with 79% alcohol overnight, 70% alcohol for 1 h, 80% alcohol for 1 h, 90% alcohol for 1 h, 100% alcohol for 1 h, xylene for 10 min., and then xylene for an additional period of 10 min. The resulting chicken embryos were then immersed into 65 °C wax for 1 h and they were cooled down. Subsequently, the chicken embryos were sectioned at 8 µm, dewaxed, hydrated, and then subjected to glycogen staining with the PAS kit (Solarbio, Beijing, Chinas; product no.: G1281).

### 2.8. Screening and Testing of *Lin28B*-Binding micRNA

The online software (http://mirdb.org/cgi-bin/search.cgi) is used in order to predict chicken micRNA *Let7s*. Well-grown DF-1 cells were transfected with oe*Lin28B* and si*Lin28B*, respectively. 48 h later, the transfected cells were collected and the total RNAs were extracted according to the miRNA isolation kits (thermofisher, Shanghai, Chinas) and then transcribed into cDNA, according to the manufacturer’s recommendations. The relative expression level of micRNA-*let7s* was assayed while using *U6* as an internal reference.
Supplementary Table S3 lists the primers for qRT-PCR of miRNA-let7s. The condition for qRT-PCR was, as follows, according to the reagent kit. The reaction system includes 50 ng cDNA, 10 µL of 2 × miRucte Plus miRNA PreMix, 0.4 µL of forward primer, 0.4 µL of reverse primer (10 µM), and ddH2O. The entire reaction volume is 20 µL. The procedure for PCR is 15 min. at 95 °C, 20 s at 94 °C, 30 s at 63 °C, and 34 s at 72 °C for five cycles, followed by 20 s at 94 °C and an extension at 60 °C for 34 s. The total number of cycles was 40. The expression was analyzed with the Ct values.

2.9. Immunocytochemical Detection of Reproductive Marker Protein

All collected groups of ESCs were cultured for six days, washed twice with PBS, fixed with 4% paraformaldehyde for 30 min., washed three times with PBS, treated with 0.1% Triton for 15 min., washed three times with PBS, and then added 10% FBS-PBS. After blocking for 2 h, added primary antibody CVH, CKIT, incubated for 2 h at 37 °C and overnight at 4 °C; washed primary antibody with PBS, added secondary antibody, incubated for 2 h at 37 °C in the dark; after that, washed secondary antibody with PBS, incubate for 15 min., stained with 5 ng/µL DAPI, blocked with glycerol (50% glycerol, 50% PBS).

2.10. Data Analysis

qRT-PCR were replicated three times. The data were analyzed by ANOVA with SPSS 19.0 software package (SPSS, Chicago, IL, USA). The means were compared by the least significant difference (LSD) test. Each replication was an experiment unit. p < 0.05 was considered to be significant, and p < 0.01 was highly significant. The charts were prepared in GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA).

3. The Results

3.1. Subsection Construction of Overexpression and Interference Vector of Lin28B

The CDS region (979 bp long) of Lin28B(NM_001034818.1) was successfully amplified while using PCR. The results of agar gel electrophoresis showed there were specific bands at approximately 1000 bp (Figure 1A, Left). The amplified fragment was ligated to pcDNA3.1 in order to construct an overexpression vector of Lin28B, and the results of dual-enzyme digestion showed that there were two bands at 979 bp and 5400 bp (Figure 1A, Right). The sequencing results showed that the amplified product of 979 bp had a 99% similarity to the coding area of Lin28B, thus indicating that the Lin28B vector was successfully constructed, and the vector was named oeLin28B (Figure 1B). The interference vector of Lin28B was constructed while using piLenti-siRNA-GFP as the skeleton (Figure 1C). The sequencing results confirmed that the expression interference vector of Lin28B was successfully constructed (Figure 1C), and the vectors were named siLin28B-1, siLin28B-2, siLin28B-3, and siLin28B-4. The expression interference vectors were transfected into DF1 cells (Figure 2A). The results showed that the transfection efficiency of these expression interference vectors in DF1 was greater than 70%. The results of qRT-PCR showed that siLin28B-1, siLin28B-2, and siLin28B-3 could significantly reduce the expression of Lin28B (p < 0.01), and the expression levels were reduced by 88%, 62%, and 31%, respectively (Figure 2B). Moreover, siLin28B-1 had the highest efficiency in interfering with the expression of Lin28B, whereby it was named siLin28B. Meanwhile, the rescue experiment further showed that oeLin28B overexpression in DF-1 markedly rescued the expression of Lin28B following siLin28B interference (Figure 2C). The results suggest that oeLin28B and siLin28B could both overexpress and interfere with the activity of Lin28B.
Figure 1. Construction of overexpression and interference vector of Lin28B. (A) Left: cloning results of Lin28B, 1: Lin28B amplification bands, M: DL5000 marker; Right: double digestion results of Lin28B overexpression vector, M: DL 5000 marker; 1: no enzyme digestion; 2: single enzyme digestion; 3: double enzyme; 4: ddH2O. (B,C) Construction schematic diagram of Lin28B overexpression and interference vector.

Figure 2. Activity detection of overexpression and interference vector of Lin28B. (A) Lin28B interference vector transfected into DF-1 cells, non-transfected cells were blank control, Scale bar: 50 μm. (B) qRT-PCR was used to detect the relative Lin28B gene expression following siLin28B1-4 vector transfection. (C) Activity detection of Lin28B overexpression and interference vector by the rescue experiment. Different uppercase letters represent highly significant, and the same letters represent no significant.

3.2. Lin28B Overexpression/Interference Could Promote/Inhibit PGCs Formation In Vivo

In order to investigate the role of Lin28B in the formation of PGCs, oeLin28B and siLin28B were injected into chicken embryos incubated for 2.5 days (E2.5, HH14) through embryo blood vessels, and were then incubated for 4.5 days after injection. qRT-PCR detected the expression of PGCs related genes in the genital ridges of chicken embryos that were incubated for 4.5 days. The results showed that, when oeLin28B was injected into the...
genital ridge of the chicken embryo, Lin28B gene expression was significantly up-regulated. Contrastingly, when siLin28B was injected into the genital ridge of the chicken embryo, Lin28B gene expression was significantly down-regulated. This suggests that oeLin28B and siLin28B could both be expressed in chicken embryo (Figure 3A). Further gene expression analysis showed that, when Lin28B was overexpressed, the PGC makers Cvh, C-kit, and Blimp1 were significantly up-regulated, while the totipotency marker gene NANOG was significantly down-regulated (Figure 3B,C). Contrastingly, a contrary result was observed when Lin28B was inhibited. In order to further confirm the regulating role of Lin28B in the formation of PGCs, we collected 4.5-day-old chicken embryos with differentLin28B treatments, prepared paraffin slices, and quantified the change in numbers of PGCs in the genital ridges through PAS staining. The results showed that the number of PGCs in the genital ridge of the siLin28B group was significantly reduced when compared to the control group (25 ± 1.25 vs. 38 ± 1.53, p < 0.01). Contrastingly, the number of PGCs in the genital ridges of the oeLin28B group was significantly increased (44 ± 2.56, p < 0.01) (Figure 4). In conclusion, during the formation of PGCs in the chicken embryos, PGC proliferation within the germinal ridge during chicken embryo development is significantly altered when overexpressing or inhibiting Lin28B expression.

![Figure 3](image_url)

**Figure 3.** Lin28B overexpression/interference could promote/inhibit the formation of PGCs in vivo. (A–C) qRT-PCR was used to detect the expression of Lin28B, primary germ cells (PGCs) marker genes (Cvh, Ckit, and Blimp1), and totipotency marker gene (NANOG) in vivo. Different uppercase letters represent highly significant, different lowercase letters represent significant, and the same letters represent not significant.

![Figure 4](image_url)

**Figure 4.** Quantity detection of PGCs by Periodic Acid-Schiff (PAS) staining; up-scale bar: 200 μm; down-scale bar: 40 μm. Left: the PGCs were marked by arrow. Right: statistical analysis of the number of PGCs in genital ridge after overexpression or interference of Lin28B. Different uppercase letters represent highly significant, and the same letters represent no significant.

3.3. Lin28B Could Promote Formation of EB in Bmp4-Induced Model In Vitro

It is well known that ESCs could be induced into PGCs in vitro [22]. In order to further confirm the function of Lin28B in the formation of PGCs, we transfected oeLin28B and
siLin28B into ESCs, respectively, and then induced by BMP4 (Figure 5A). The results of qRT-PCR showed that oeLin28B and siLin28B could overexpress/inhibit the expression of Lin28B during PGCs formation (Figure 5B). The results of morphological statistics showed that cells expanded at day 2 after BMP4 induction (control group), a small number of EBs appeared at day 4, and more EBs appeared at day 6. However, EB was not observed from day 2 to day 6 after Lin28B interference during BMP4 induction. Contrasting, small EBs were observed at day 2, more EBs were observed and began to break at day 4, and, at day 6, the edge of EBs began to break and small amounts of cells were released from the EBs (Figure 6). This suggests that Lin28 can regulate the formation of EBs.

**Figure 5.** Lin28B could promote the formation of embryoid bodies(EBs) in BMP4-induced model in vitro. (A) Schematic diagram of illustrating the Lin28B function via PGCs induction model in vitro. (B) Expression detection of Lin28B during the induction of PGCs. Different uppercase letters represent highly significant and the same letters represent no significant.

**Figure 6.** Observation (Left) and statistics (Right) of the number of EBs during induction after different treatments of Lin28B. Scale bar: 60 μm.

3.4. Lin28B Could Positively Modulate Formation of PGCs in Bmp4-Induced Model In Vitro

In order to confirm the effects of Lin28B on BMP4 induction, cells in different induction period were collected to detect the expression of NANOG, and marker genes of PGCs, such as Cdh, C-kit, and Blimp1 (Figure 7A,B). The results showed that NANOG expression
did not differ significantly from the normal BMP4 induction (control) at day 2, but it was significantly up-regulated at day 4 and day 6 ($p < 0.01$) after Lin28B inhibition. The expression of marker genes of PGCs was markedly reduced at day 4 and day 6 ($p < 0.01$). Contrastingly, although NANOG was down-regulated after Lin28B overexpression, no significant difference was observed when compared to the control. The marker genes of PGCs were not significantly expressed at day 2 after Lin28B overexpression as compared to the control, but they were markedly up-regulated at day 4 and day 6 ($p < 0.01$). The six-day-old cells were subjected to an indirect immunofluorescence test (Figure 7C). The results showed that the proportion of CVH + CKIT+ was significantly increased after Lin28B overexpression as compared to the control. Contrastingly, the proportion of CVH + CKIT+ was reduced significantly after Lin28B inhibition. In conclusion, the inhibition of Lin28B could significantly inhibit the formation of PGCs, while Lin28B overexpression could significantly promote the formation of PGCs.

**Figure 7.** Lin28B could positively modulate the formation of PGCs in BMP4-induced model in vitro. (A,B) qRT-PCR was used to detect the expression of NANOG, Cvh, Ckit, and Blimp1 during the induction of PGCs in vitro. Different uppercase letters represent highly significant, different lowercase letters represent significant, and the same letters represent no significant. (C) Indirect immunofluorescence detection of the efficiency of the formation of PGCs at day 6 in the induced model after treatment with different Lin28B in vitro. Scale bar: 60 μm.

### 3.5. Lin28B Promotes Formation of PGCs Through Inhibition of gga-Let-7a-3p

The study results showed that Lin28B functions by binding the let7 family [8]. A total of 17 gga-let7s were predicted in chicken by online software (method for details) in order to investigate the key micRNA let7s targeted by Lin28B (Figure 8A). We transfected siLin28B and oeLin28B in DF-1 cells, and then detected the expression of these micRNAs with qRT-PCR. We found that only let-7a-2-3p, let-7a-3p, let-7b, and let-7k-5p of the 17 gga-let7s were significantly regulated by Lin28B (the expression trend is opposite to Lin28B) (Figure 8A). Additionally, we collected zero-day and four-day-old cells in the PGC induction model in vitro [22] and performed transcriptome sequencing (data not published), and found that
there are five gga-let7s (let-7a-3p, let-7g-5p, let-7f-3p, let-7i, and let-7c-5p) in the process of the formation of PGCs in vivo, of which the expression of let-7a-3p, let-7g-5p, let-7f-3p, and let-7i showed a downward trend, which was consistent with the expression rule of gga-let7s during the formation of PGCs (Figure 8B). However, we found that after the overexpression of Lin28B, let-7g-5p, let-7f-3p, and let-7i showed an upward trend, which means that, in addition to let-7a-3p, the maturation process of let-7g-5p, let-7f-3p, and let-7i is not regulated by Lin28B. Moreover, we examined the expression of let-7a-3p in ESCs and PGCs, and found that let-7a-3p was significantly down-regulated, which showed a completely opposite trend to the expression of Lin28B (Figure 8C). Combining these results, we therefore conclude that Lin28B regulates the formation of chicken PGCs through let-7a-3p.

Figure 8. Lin28B promotes the formation of PGCs through the inhibition of gga-let7-3p. (A) qRT-PCR was used to detect the expression of let-7 family after overexpression and inhibition of Lin28B, only let-7a-2-3p, let-7a-3p, let-7b, and let-7k-5p showed opposite trend to Lin28B. (B) miRNA sequencing to screen let-7 (let-7a-3p, let-7g-5p, let-7f-3p, let-7i, and let-7c-5p) during the formation of PGCs in vitro. (C) qRT-PCR was used to detect the expression of let-7a-3p and Lin28B during the generation of PGCs in vivo. ** means p < 0.05, and *** means p < 0.01.

4. Discussion

The research study deeply examined the function of Lin28B in the formation of PGCs, being screened and obtained the miRNA modulated by Lin28B, and preliminarily clarified the molecular mechanism by which Lin28B participates in the process of PGC genesis by inhibiting the maturation of gga-let7-3p.

Despite that both Lin28A and Lin28B are from the same RBP family, more research has focused on Lin28A. As early as 2009 [6], it was shown that Lin28A could regulate the PGC genesis of mammals. The underlying molecular mechanism is that Lin28A could activate the expression of Blimp1 to participate in the formation of PGCs by inhibiting the maturation of let-7's precursor [18]. However, no relevant study concerning Lin28B has ever been reported. The function of Lin28B in PGC genesis was only putatively predicted by in vitro induction experiment or by addressing the association between SNP loci and menstrual cycle, because there are SNP loci in Lin28B [14,23]. However, no definite conclusion has been drawn. In the subject study, we systematically examined the function of Lin28B in...
the genesis of PGCs while using the chicken as a model animal. The results showed that, similar to Lin28A, Lin28B could also regulate PGCs genesis. Although the study object of the study is chicken, the results could serve as the foundation for understanding the specific function and mechanism of Lin28B gene family in PGCs genesis. Based on the results of this study, we can improve the regulatory role of Lin28 family in the formation of chicken PGCs, laying a foundation for the application of chicken PGCs in the production of transgenic animals, resource protection, and other fields.

With the in-depth study in Lin28, increasing numbers of scholars have started to examine the functioning mechanism of Lin28 in totipotency, genesis of germ cells, and tumorigenesis [1,4,24]. The results showed that Lin28A and its collateral derivative Lin28B are the key participants for Let-7 processing [25], which functions by modifying its processing or stability through binding to Let-7 pre-miRNA or pri-miRNA. [26] Nie et al. [27] found the link between Blimp1 and Let7a in Hodgkin’s lymphoma cells. They found that Let-7 was highly expressed in Reed–Sternberg cells, whereas Blimp1, a primary regulator for the differentiation of B cells, was significantly inhibited. The binding of Let-7a miRNA to the target site in 3′UTR of Blimp1 was further observed, which could inhibit Blimp1 expression. This undoubtedly proven that Lin28 indirectly regulates Blimp1. It was not until 2009 when West et al. [6] confirmed that link between Lin28 and PGCs. West et al. knocked out Lin28A or Lin28B in the ESCs of Stella-GFP, which led to a small number of positive TNAP communities (an early marker of PGCs). They assumed that Lin28A and Lin28B could inhibit the expression of Blimp1 by inhibiting the expression of Let-7. In fact, the deletion of the let-7 locus in Blimp1 3′UTR can rescue the phenotypic loss of PGCs due to Lin28 knockdown. In addition, an overexpression of Lin28 could induce the expression of more marker genes of TNAP-positive PGCs. Such findings confirmed the importance of Lin28 in PGCs genesis; our results filled the gap in the study of Lin28B in the formation of PGCs in chickens. Early studies of Lin28 and Let-7 focused on mammalian PGCs formation, with few reports on chickens. Therefore, it is the first time we demonstrated the key role of Let-7 miRNA biosynthesis in PGCs. We expanded this finding from mammalian to the poultry area, studied and analyzed the function of Lin28B in PGC genesis, and targeted it to gga-let-7a-3p. Our study may provide a constructive basis for future in-depth research.

5. Conclusions
In this study, we confirmed that Lin28B promoted the formation of chicken PGCs through in vivo and in vitro experiments. The let-miRNA family that was opposite the expression of Lin28B was identified by combining the results of high-throughput sequencing, and it confirmed that Lin28B promoted the formation of chicken PGCs by inhibiting the maturation of gga-let-7a-3p.

Supplementary Materials: The following are available online at https://www.mdpi.com/2076-2615/11/1/43/s1, Table S1, Target sites sequence of Lin28B gene; Table S2 qRT-PCR primers sequence of related genes; Table S3, qRT-PCR primers sequence of related genes.

Author Contributions: B.L. conceived and designed the experiments; Q.Z. performed the experiments; Q.Z. analyzed the data; Q.Z., J.Z. wrote the manuscript; G.C., Y.Z. edited the manuscript; M.W. revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Jiangsu Science and Technology Project (Youth Fund): BK20180918; Natural Science Research Project of Jiangsu Higher Education Institutions: 18KJB230008; Key Research and Development Program; National Natural Science Foundation of China (31872341, 31572390).

Institutional Review Board Statement: All experimental procedures in the present study were reviewed and approved by the Institutional Animal Care and Use Committee of Yangzhou University (approval number: 151-2014). Procedures were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (Yangzhou University, China, 2012) and the Standards for the Administration of Experimental Practices (Jiangsu, China, 2008). We also confirm that the field studies did not involve endangered or protected species.

Informed Consent Statement: Not applicable.
Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: The authors thank the Poultry Institute of the Chinese Academy of Agricultural Sciences Experimental Poultry Farm for providing experimental materials.

Conflicts of Interest: The authors declare that there is no conflict of interest.

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