BMP4 Signaling Activates the Wnt-Lin28A-Blimp1-Wnt Positive Feedback Pathway to Promote Primordial Germ Cell Formation via Altering H3K4me2

CURRENT STATUS: Posted

Qisheng Zuo, Yani Zhang, Guohong Chen, Bichun Li

Qisheng Zuo
Yangzhou University

Yani Zhang
Yangzhou University

Guohong Chen
Yangzhou University

Bichun Li
744366503@qq.com Corresponding Author
ORCiD: https://orcid.org/0000-0003-0209-0479

Prescreen

10.21203/rs.3.rs-26853/v1

Subject Areas

Cell Communication and Signaling

Keywords
Primordial germ cells, Wnt signals, Lin28A, H3K4me2, Blimp1
Abstract

Background) The unique developmental characteristics of bird primordial germ cells (PGC) have enabled genetic engineering–based breeding and restoration of endangered birds via transplantation in vitro. However, the limited number of PGC has limited their application. Thus, there is an urgent need to elucidate the mechanism of PGC formation in vitro to enhance its efficiency.

Results) Here, we confirmed that activation of BMP4 and Wnt signaling (Wnt5A/β-catenin/TCF7L2) is critical for PGC formation via RNA-seq (ESCs, PGC and SSCs) and in vitro induction models. Wnt signaling activated via BMP4 in turn activates transcription of Lin28A by inducing β-catenin to compete with LSD1 for binding to the transcription factor TCF7L2, causing LSD1 to dissociate from the Lin28A promoter and enhanced H3K4me2 methylation in this region. Lin28A promotes PGC formation by inhibiting gga-let7a-3p maturation to initiate Blimp1 expression. Interestingly, expression of Blimp1 helped sustain Wnt5A expression by preventing LSD1 binding to the Wnt5A promoter. We thus elucidated a positive feedback pathway involving Wnt-Lin28-Blimp1-Wnt, with BMP4 functioning as an activator that ensures PGC formation.

Conclusion) In summary, our study clarified the molecular mechanism by which BMP4 and Wnt signaling regulate PGC formation via a positive feedback system. Our data provide both a theoretical and technical basis for studies aimed at enhancing the generation of PGC in vitro.

Background

Mammalian somatic cell cloning and gene editing techniques are not applicable to oviparous birds, thus greatly limiting the application of biotechnology in birds1,2. Transplantation of exogenous primordial germ cells (PGC) into blood vessels of recipient chickens can migrate to the genital ridge via the blood and produce offspring3,4, suggesting that PGC transplantation could be used to aid the recovery of endangered bird species, as well as in gene editing and the generation of transgenic birds5. However, relatively few PGC can be isolated in vivo (1000–5000/embryo), which is insufficient for transplantation (2000–5000/embryo)6. Producing offspring from donor PGC when there is less than 5000/embryo is difficult7,8. We generated offspring from PGC induced allogeneically in vitro, but the PGC induction efficiency was low, in part due to poor understanding of the molecular mechanism of PGC formation.

Other studies9,10 reported induction of PGC (Fraglis+ Blimp1+) formation from mouse precursor cells in the proximal epidermis mediated via BMP4 signaling. Inhibition of BMP4 signaling markedly decreases the number of PGC in mouse gonads11–13. However, other studies14–16 confirmed that Wnt signaling also plays an important role in PGC formation in mice. BMP4 and Wnt signaling interact during PGC formation15,19. Both BMP4 and Wnt signaling regulate PGC formation in mice by activating Blimp1 via the mesoderm-specific transcription factor T15,17,18. Wnt signaling also induces formation of Prdm1+ germ cells along with BMP419. Saitou20 characterized the relationship between BMP4 and Wnt signaling in detail. In mouse epidermis, activation of BMP4 signaling and suppression of Wnt signaling leads to partial or complete loss of marker gene (such as Blimp1) expression in early PGC, whereas activation of Wnt signaling enhances the epidermal response to BMP4 signaling. Although the function of BMP4 and Wnt signaling in the formation of PGC has been characterized in mammals, how BMP4 and Wnt signaling activates Blimp1 expression to regulate PGC formation and how these signaling pathways interact remain to be elucidated.

In a previous study using RNA-seq of chicken ESCs (Embryonic stem cells) and PGC21, we found that BMP4 and Wnt signaling are significantly upregulated during chicken PGC formation. Functional verification analyses demonstrated that BMP4 signaling promotes the formation of chicken PGC22. However, the role of Wnt signaling in PGC formation in chickens and whether Wnt and BMP4 interact remain to be determined. Using both in vivo
and in vitro approaches, in the present study, we confirmed that Wnt signaling positively regulates PGC formation. Our results also indicate that PGC formation is controlled by a Wnt-Lin28A-Blimp1-Wnt positive feedback loop regulated by upstream BMP4 signaling and H3K4me2. Our research thus highlight a new strategy useful for optimizing both the culture and induction conditions for in vitro PGC formation.

Results

Wnt5a-β-catenin-TCF7L2 Positively Regulates the Formation of PGC

The molecular mechanism regulating PGC formation was evaluated using KEGG pathway analysis of development-related DEGs (Differentially expressed genes) in ESCs and PGC (SRR3720923 and SRR3720924) (Supplementary Fig. 1A,B). Wnt signaling was also activated during PGC formation (Fig. 1A; Supplementary Fig. 1C), in addition to enrichment of TGF signaling genes. Wnt signaling–related genes were also significantly activated in an in vitro PGC induction model (Supplementary Fig. 1D). These results indicate that Wnt signaling is involved in PGC formation.

We determined that Wnt5a, β-catenin, and TCF7L2 are the key Wnt signaling molecules in the formation of chicken germ stem cells (Supplementary Fig. 1C,D). To further demonstrate the involvement of Wnt5a/β-catenin/TCF7L2 signaling, we conducted Wnt5a overexpression/interference during the process of inducing ESCs to form PGC with BMP4. The expression of signaling molecules in 4d-induced cells was assessed by qRT-PCR, which indicated significant down-regulation of β-Catenin and TCF7L2 expression (P < 0.01) following Wnt5A interference, whereas overexpression of Wnt5A significantly up-regulated β-Catenin and TCF7L2 expression (P < 0.01) (Fig. 1B). In vitro experiments produced similar results (Fig. 1C). Co-immunoprecipitation (co-IP) of lysates of DF1 cells co-transfected with pcDNA3.1-Myc-TCF7L2 and pcDNA3.1-β-Catenin revealed that TCF7L2 and β-catenin interact (Fig. 1D). These data indicate that Wnt5A mediates Wnt5A/β-catenin/TCF7L2 signaling during the differentiation of ESCs into PGC. We previously confirmed that Wnt5A/β-catenin/TCF7L2 signaling promotes PGC formation (Fig. 1E). Collectively, our data indicate that Wnt5a-β-catenin-TCF7L2 positively regulates PGC formation.

Lin28A is a Specific Target of Wnt5a Signaling in PGC

To identify the specific target genes regulated by Wnt during PGC formation, we examined enrichment of target genes of TCF7L2 in humans (15727), rat (15652), and mouse (15374) in the GTRD (no bird database available) (Fig. 2A). Lin28A was the only common gene in GO and Venny analyses of 7473 target genes identified in these species (Fig. 2B; Supplementary Fig. 2), suggesting that Lin28A, a highly conserved gene targeted by Wnt, is involved in the generation of reproductive stem cells. These results are consistent with other reports.

To confirm that Wnt-associated regulation of Lin28A also plays a role in the formation of chicken PGC, we compared the structure of Lin28A across species and found it is highly conserved (Supplementary Fig. 3). Chicken and mammalian Lin28A were exactly the same (Supplementary Fig. 3). Most importantly, the binding site for the transcription factor TCF7L2 was also present in the chicken Lin28A promoter (Supplementary Fig. 4A), which further suggests that Lin28A is a target of Wnt during the formation of chicken PGC.

To confirm that Wnt signaling regulates Lin28A, we examined the expression of Lin28A after activation/inhibition of Wnt signaling during PGC formation in vivo. Overexpression of Wnt5A significantly increased Lin28A expression (Fig. 2C), whereas inhibition of Wnt5A expression significantly inhibited Lin28A expression (Fig. 2D). Lin28A expression also decreased/increased significantly following inhibition/overexpression of β-catenin (Fig. 2C,D). Similar results were observed in induction experiments in vitro (Fig. 2C,D). Collectively, these results indicate that Lin28A responds to Wnt signaling. To examine this response further, we identified the core promoter of Lin28A (~ 584 ~ + 100 bp) using the dual luciferase detection system (Fig. 2E; Supplementary Fig. 4A–C). Activation of Wnt signaling (overexpression of β-catenin) significantly increased the Lin28A promoter activity (P < 0.01) (Fig. 2F). However, mutation of the TCF7L2 binding site significantly reduced the activity of the
Lin28A promoter (P < 0.01) (Fig. 2E,F; Supplementary Fig. 4D); Activation of Wnt signaling could not rescue Lin28A promoter activity following introduction of point mutations (Fig. 2E – G), indicating that Lin28A responds to Wnt signaling via the TCF7L2 binding site in the promoter.

Previous analyses indicated that there are two TCF7L2 binding sites in the Lin28A promoter. Therefore, we examined the binding of TCF7L2 to the Lin28A promoter using ChIP-qPCR and found enrichment of β-catenin/TCF7L2 complexes in the Lin28A promoter (Fig. 2H). Activation of Wnt signaling significantly increased binding of the β-catenin/TCF7L2 complex to Lin28A (P < 0.01), whereas inhibition of Wnt signaling significantly reduced this binding (P < 0.01) (Fig. 2H). These results indicate that Lin28A is a downstream target of Wnt signaling.

Lin28A Positively Regulates PGC formation in vitro and in vivo

Next, we investigated the function of Lin28A in PGC formation (Fig. 3A). Lin28A was inhibited/overexpressed during BMP4-induced differentiation of ESCs into PGC in vitro (Fig. 3A; Supplementary Fig. 5A). Morphologic observations on day 2 after BMP4 induction indicated that the cells had begun to grow larger. A few embryoid bodies (EBs) appeared on day 4, and the number of EBs increased on day 6; however, no EBs appeared between days 6 and 6 after Lin28A inhibition. In contrast, small EBs began to appear on day 2 after Lin28A overexpression, and on day 4 these EBs became larger and began to break. The number of EBs increased on day 6, the cell edges began to rupture, and a few cells were released from the EBs (Fig. 3B; Supplementary Fig. 5B).

Lin28A overexpression significantly decreased expression of the pluripotency marker gene Nanog and increased Cvh, C-kit, and Blimp1 expression. Flow cytometry analyses demonstrated that Lin28A overexpression promoted PGC formation in the BMP4 model (Fig. 3C; Supplementary Fig. 5D,E). Similar results were observed in in vivo experiments (Fig. 3D,E; Supplementary Fig. 5E). Periodic acid-Schiff staining was used to monitor changes in the number of PGC formed in the genital ridge after Lin28A overexpression/interference. Compared with the number of PGC in the genital ridge during the normal in vivo hatching process (38 ± 1.53), the number of PGC in the genital ridge significantly increased following Lin28A overexpression (46 ± 2.10; P < 0.01) and significantly decreased (20 ± 1.64; P < 0.01) following Lin28A interference (Fig. 3D; Supplementary Fig. 5C). Collectively, these results indicate that Wnt/β-catenin signaling promotes PGC formation by activating Lin28A expression.

Lin28A is Regulated by H3K4me2

In a previous study examining H3K4me2 regulation of SSC formation, we performed RNA-seq analysis of LSD1-treated SSCs (LSD1: H3K4me2 demethylation modifying enzyme) and identified Lin28A as a DEG (Supplementary Fig. 6A). qRT-PCR analyses indicated significant up-regulation of Lin28A expression in SSCs following LSD1-mediated interference (Supplementary Fig. 6B). ChIP-qPCR analyses indicated that LSD1 regulates H3K4me2 enrichment in the Lin28A promoter (Supplementary Fig. 6C). To examine the effect of H3K4me2 on Lin28A regulation during PGC formation in the present study, we interfered with LSD1 and MLL2 expression in the in vitro BMP4 induction model. Lin28A expression was significantly higher than that induced by BMP4 after interfering with LSD1 expression, and the opposite trend was observed after interfering with MLL2 (Fig. 4A), indicated that H3K4me2 positively regulates Lin28A transcription in vitro. Lin28A expression in PGC on day 4.5 PGC was examined using qRT-PCR. Compared with the normal hatching process, H3K4me2 demethylation (interfering with MLL2) significantly decreased Lin28A expression, whereas H3K4me2 methylation (interfering with LSD1) significantly increased Lin28A expression (Fig. 4B). These results suggest that Lin28A is regulated by H3K4me2. To confirm that Lin28A is a target of H3K4me2, we examined the level of H3K4me2 enrichment in the Lin28A promoter in PGC using ChIP-qPCR. Compared with the control, H3K4me2 in the Lin28A promoter was significantly down-regulated following MLL2 interference and significantly up-regulated following LSD1 interference (Fig. 4C). Further results confirmed that changes in H3K4me2 regulate the Lin28A promoter (Fig. 5A). Collectively, these results indicate that in addition to Wnt signaling, H3K4me2 also regulates Lin28A expression during PGC formation.

Competition Between β-Catenin and LSD1 in Combination with TCF7L2 Regulates Lin28A Expression during PGC Formation

To further elucidate the molecular mechanism regulating Lin28A expression, we investigated interactions
between Wnt and H3K4me2 using the dual luciferase system. Interference with MiR2 expression suppressed the response of Lin28A to Wnt signaling, whereas interference with LSD1 significantly enhanced the response (Fig. 5A). The position of H3K4me2 enrichment in the Lin28A promoter is near the TCF7L2 binding site. It is reasonable to speculate that β-catenin/TCF7L2 complexes affect the level of H3K4me2 enrichment to regulate Lin28A expression by altering the binding of LSD1 or MLL2 to the Lin28A promoter. Considering that the complex involving MiR2 is relatively fixed, we used Co-IP to assess interactions between β-catenin, TCF7L2, and LSD1. Co-IP performed after co-transfection of DF1 cells with LSD1-Flag and β-catenin vectors indicated no interaction between LSD1 and β-catenin (Fig. 5B). However, in cells co-transfected with LSD1-Flag and TCF7L2-Myc, interaction between Flag and TCF7L2 was observed (Fig. 5C). Considering the correlation between H3K4me2 and Wnt signaling, we examined whether Blimp1 regulates PGC formation by inhibiting gga-let-7a-2-3p expression during PGC formation.

Lin28A Activates Blimp1 to Regulate PGC Formation by Inhibiting gga-let-7a-2-3p Maturation

Another study demonstrated that as an RNA-binding protein, Lin28A regulates the expression of related genes by inhibiting miRNA-let7 maturation. However, the miRNA-let7 that interacts with Lin28A during chicken PGC formation remained to be identified. To determine the key miRNAlet7s targeted by Lin28A, 17 gga-let7s in the chicken miRNAlet7 family were screened using miRDB. Expression of these mature microRNAs in chicken ESCs and PGC was evaluated by qRT-PCR after Lin28A overexpression/interference (Fig. 6A,B), which indicated that gga-let-7a-2-3p was significantly regulated by Lin28A in ESCs and PGC (Fig. 6A,B). gga-let-7a-2-3p was significantly up-regulated following Lin28A overexpression and significantly down-regulated following Lin28A interference (Fig. 6C,D), indicating that gga-let-7a-2-3p expression is regulated by Lin28A during chicken PGC formation.

Screening the miRDB identified 1143 genes targeted by gga-let-7a-2-3p. In particular, Blimp1 (PRDM1), which plays an important regulatory role in PGC formation, attracted our attention. To determine whether gga-let-7a-2-3p targets Blimp1, we synthesized a gga-let-7a-2-3p mimic and inhibitor and transfected them into DF1 cells and PGC. qRT-PCR analysis indicated that Blimp1 expression was significantly down-regulated in DF1 cells transfected with the mimic (Fig. 6E) and significantly up-regulated in cells transfected with the inhibitor, indicating that gga-let-7a-2-3p negatively regulates Blimp1 (P < 0.01 for both) (Fig. 6E, left). As Blimp1 is a PGC marker, we performed the same experiment with PGC and observed similar results (Fig. 6E, right). To further confirm that gga-let-7a-2-3p targets Blimp1, we predicted the gga-let-7a-2-3p binding site in the Blimp1 3’UTR (UUGUACA). Wild-type and mutant (complete deletion of binding site) luciferase reporter vectors of the Blimp1 3’UTR were constructed separately. DF1 cells were then co-transfected with vectors for the gga-let-7a-2-3p mimic and inhibitor with Blimp1-3’UTR-WT and Blimp1-3’UTR-Mut. The gga-let-7a-2-3p inhibitor significantly increased Blimp1-3’UTR-WT luciferase activity in the double luciferase reporter assay (P < 0.01) but had no significant effect on Blimp1-3’UTR-Mut (P > 0.05) (Fig. 6F, left). The gga-let-7a-2-3p mimic significantly reduced Blimp1-3’UTR-WT luciferase activity (P < 0.01) but had no significant effect on Blimp1-3’UTR-Mut (P > 0.05) (Fig. 6F, right). These results indicate that Blimp1 is a direct target of gga-let-7a and that gga-let-7a binds to the 3’UTR of Blimp1 to inhibit its expression.

Blimp1 Interacts with LSD1 to Regulate the Expression of Related Genes in Wnt Signaling and Participates in PGC formation

As Blimp1 is known to affect the level of H3K4me2, we examined whether Blimp1 regulates PGC formation by altering the H3K4me2 level of key genes. Therefore, the correlation between H3K4me2 and Wnt signaling was examined during PGC formation. ChIP-qPCR analyses revealed two, four, and two H3K4me2 enrichment sites in
the Wnt5A, β-Catenin, and TCF7L2 promoters, respectively. PGC exhibited significantly higher binding of H3K4me2 than ESCs (P < 0.01) (Fig. 7A). Enrichment of H3K4me2 levels in these sites in PGC was regulated by LSD1 and MLL2 (Supplementary Fig. 7), indicating that H3K4me2 regulates key Wnt signaling molecules. We then investigated in detail whether Blimp1 regulates H3K4me2 in the promoters of Wnt5A, β-Catenin, and TCF7L2. Notably, there is a Blimp1 binding site near the Wnt5A promoter H3K4me2 enrichment site (Fig. 7B). To confirm that Blimp1 binds to the Wnt5A promoter, a double luciferase reporter vector for the Wnt5A promoter was constructed and co-transfected into DF1 cells along with Blimp1 overexpression/interference vectors. The double luciferase reporter assay showed that Blimp1 overexpression significantly enhanced Wnt5A promoter activity, whereas interference with Blimp1 expression decreased promoter activity (Fig. 7C). However, Blimp1 overexpression/interference had no effect on promoter activity after mutation of the Blimp1 binding site (Fig. 7D), indicating that Blimp1 binds to the Wnt5A promoter. Expression of Wnt5A was significantly up-regulated after Blimp1 over-expression in DF1 cells (Fig. 7E), as was the level of H3K4me2 in the Wnt5A promoter (Fig. 7F). Interestingly, the level of LSD1 binding in the Wnt5A promoter was significantly down-regulated (Fig. 7G). These results indicate that Blimp1 and LSD1 interact to regulate the expression of genes related to Wnt5A signaling.

Morphologic observation after interference with LSD1 and MLL2 expression in the in vitro BMP4 induction model revealed that LSD1 interference via shLSD1 promoted PGC formation, whereas interference with MLL2 expression inhibited PGC formation (Supplementary Fig. 8A). Expression of genes that activate Wnt signaling, such as Wnt5A, β-Catenin, FZD4, and TCF7L2, increased significantly after interference using shLSD1 (P < 0.01), whereas expression of genes that suppress Wnt signaling, such as AXIN1 and APC, decreased significantly (P < 0.01) (Supplementary Fig. 8B). Completely opposite results were obtained after interference with MLL2 expression (Supplementary Fig. 8B) and in vivo (Supplementary Fig. 8C). Collectively, these data indicate that H3K4me2 regulates PGC formation by activating Wnt5A/β-catenin/TCF7L2 signaling.

**BMP4 Initiates Wnt Signaling to Ensure Normal PGC Development**

As our collective results indicated that Wnt-Lin28-Blimp-Wnt functions as a positive feedback loop during PGC formation, we sought to identify the factors that activate this feedback pathway. Previously, we confirmed that BMP4 plays an important role in PGC formation. Incubation of ESCs or PGC for 6 h in medium to which BMP4 was added led to significantly increased expression of signaling molecules such as Wnt5A, β-catenin, and TCF7L2 (P < 0.01) (Fig. 8A), whereas the AXIN1 and APC genes were significantly down-regulated (P < 0.01) (Fig. 8A). These preliminarily results indicate that BMP4 signaling activates Wnt5A signaling and that BMP4/Smads is upstream of Wnt signaling. No significant change in BMP4 expression was observed 6 h after Wnt5A overexpression/interference in ESCs or PGC (Fig. 8B), indicating that Wnt signaling is downstream of BMP4 signaling. Considering the function of both BMP4 and Wnt in PGC formation suggests that BMP4 activates downstream WNT5A/β-catenin/TCF7L2 signaling to regulate PGC formation. Therefore, we preliminarily conclude that BMP4 signaling activates the Wnt-Lin28-Blimp-Wnt feedback system. To provide additional evidence, we changed the culture medium at 6 hours (after Wnt signaling activation) during induction with BMP4 (Fig. 8C,D). Flow cytometry analysis revealed that the absence of BMP4 had no effect on formation of normal PGC (Fig. 8E). Therefore, we conclude that BMP4 signaling mediates the normal development of PGC by activating Wnt signaling.

**Discussion**

The present study established a new regulatory model for PGC formation. After activation of Wnt5A-β-catenin-TCF7L2 signaling, β-catenin competes with LSD1 to bind to TCF7L2 in the Lin28A promoter, leading to increased H3K4me2 levels and expression of Lin28A. Lin28A then activates expression of Blimp1 by inhibiting the maturation of Let7a, thus regulating PGC formation. Notably, Blimp1 activates Wnt to initiate the Wnt-Lin28-Blimp1-Wnt positive feedback pathway.

Wnt signaling plays similar roles in the formation of mammalian and avian PGC, but the regulatory mechanisms differ markedly. The Wnt/β-catenin pathway promotes induction of the ectoderm response to BMP4.
signaling and participates in determining PGC specialization in mammals\textsuperscript{15,17}, with Wnt directly regulating the expression of Blimp1 and Prdm14 via mesoderm and notochord transcription factor T\textsuperscript{17-20}. This process differs significantly from that of chicken PGC development. Our research confirmed that Wnt signaling is involved in chicken PGC formation; the mechanism involves activation of \textit{Lin28A} expression via Wnt signaling through TCF7L2 and indirect regulation of Blimp1 expression. The entire process is also regulated by H3K4me2, indicating that although Wnt signaling plays the same biological role in formation of mammalian and chicken PGC, its regulation differs by species.

Wnt signaling primarily affects target genes such as \textit{c-myc} and \textit{Jun}\textsuperscript{36,37}. However, these genes play no obvious role in PGC formation. The present study confirmed that Wnt interacts with Lin28A, a key factor in PGC formation\textsuperscript{38,39}. Although many studies have examined the interaction between Wnt and Lin28A in mammals, primarily as it relates to cancer\textsuperscript{40-42}, a role for this interaction in PGC formation has not been reported. This study confirmed for the first time that \textit{Lin28A} expression is directly regulated by Wnt signaling in chicken ESCs and PGC, and our data provide new insights for studying the regulatory mechanism of mammalian PGC formation.

Wnt signaling activates downstream target genes through the β-catenin/TCF activation complex\textsuperscript{43-45} and regulates the chromatin status of target gene promoters by recruiting epigenetic factors that also regulate gene expression\textsuperscript{46}. In mammalian rectal cancer cells, activated Wnt signaling recruits histone methylation transferase via β-catenin, catalyzing the H3K4me3 modification in target gene promoters to regulate gene expression\textsuperscript{47,48}. In \textit{Xenopus}, the recruitment of PRMT2 by β-catenin was used to establish the target gene promoter histone H3R8me2a modification that regulates the transcription of downstream genes during mesocotyl transition\textsuperscript{49}. In addition to β-catenin, the transcription factor TCF also recruits histone methylation-modifying enzymes\textsuperscript{50}. TCF4 regulates the transcription of classical Wnt signaling target genes by recruiting the histone-modification enzyme spindlin1\textsuperscript{51}. We found that Wnt/β-catenin/TCF signaling plays a unique regulatory role: activation of Wnt signaling leads to dissociation of the TCF7L2-LSD1 complex, thereby increasing H3K4me2 modification of the \textit{Lin28A} promoter and activating \textit{Lin28A} expression, thus promoting PGC formation.

Here, we confirmed that PGC formation is regulated by the Wnt-Lin28-Blimp1-Wnt positive feedback regulation system. BMP4 induces the production of Blimp1-positive cells in early blastocysts in both mammals and chickens\textsuperscript{20}, but the underlying molecular mechanism has not been fully elucidated. We examined the mechanism of BMP4-induced Blimp1-positive cell formation. After activation of Wnt signaling by BMP4, \textit{Lin28A} expression is activated by the transcription factor TCF7L2. Lin28A then activates Blimp1 expression by inhibiting the maturation of Let7a. Finally, activated Blimp1 feeds back the signal to further activate Wnt signaling. Thus, the entire process constitutes a positive feedback regulatory system. Saitou\textsuperscript{20} studied the process of mouse PGC formation and proposed that when BMP4 signaling is activated and Wnt signaling is suppressed, the expression of early PGC marker genes such as \textit{Blimp1} is reduced or completely suppressed. Activated Wnt signaling enhances the response of epiblasts to BMP4 signaling. The Wnt-Lin28-Blimp1-Wnt positive feedback regulation system proposed in this study reasonably explains this phenomenon.

### Materials And Methods

#### Reagents

Anti-H3K4me2 (ab32356, 10 µg for chromatin immunoprecipitation [ChIP] experiments, 1:2000 for Western blotting), anti-histoneH3 (ab1791, 1:2000 dilution for Western blotting), goat anti-mouse IgG (ab6786), goat anti-rabbit IgG (ab6718), and rabbit anti-rat IgG (ab6730) were obtained from Abcam (San Francisco, CA). Anti-Myc (#14793, 1:50 dilution for Co-IP, 1:1000 dilution for Western blotting) and anti-β-catenin (9587, 1:50 dilution for Co-IP, 1:1000 dilution for Western blotting, 1:25 dilution for ChIP) were obtained from Cell Signaling (Danvers,
MA). Anti–β-actin (CW0096M, 1:1000 for Western blotting) was obtained from Kang Wei Century (Nanjing, China).

**qRT-PCR**

Total RNA was extracted from cells using Trizol reagent (Tiangen, Beijing, China) and reverse transcribed to synthesize cDNA using a Quantscript RT kit (Tiangen). Expression of Wnt-associated signaling molecules was assessed using β-actin as an internal control (Supplementary Table 1). The qPCR reaction system (total volume: 20 µl) was as follows: cDNA 2 µl; TB Green Premix Ex TaqII 10 µl; upstream and downstream primers (10 μM) 0.8 µl each; ddH₂O 6.4 µl. PCR reaction procedures were carried out according to the instructions provided with the Takara TBGreen™ PremixExTaq™ II.

**Co-IP**

DF1 cells (chicken embryo fibroblasts) with good status were selected and divided into three groups for transfection. Cells were maintained in complete medium. One group was transfected with the oeβ-Catenin vector; one group was transfected with the oeTCF7L2-Myc vector; and one group was transfected with the oeβ-Catenin and oeTCF7L2-Myc vectors. After culture at 37 °C and 5% CO₂ and saturation humidity for 48 h, the cells were collected for Co-IP experiments, as previously described²³.

**ChIP-qPCR**

ESCs and PGC with good status were selected and divided into three groups each for transfection. Cells were transfected in factor medium. One group was transfected with shLSD1; one group was transfected with shMLL2; and one group was left untreated. After incubation for 48 h at 37 °C and 5% CO₂ and saturation humidity, ChIP-qPCR was performed as follows: cell crosslinking and fragmentation; immunoprecipitation of crosslinked proteins/DNA; elution of protein/DNA complexes; and purification and recovery of DNA using centrifugal columns for ChIP-qPCR (Supplementary Table 2).

**Wnt5a/β-catenin/tcf7l2 Signaling Target-gene Prediction**

Binding-target genes of the Wnt-signaling transcription factor TCF7L2 in three different species (human, rat, and mouse) were predicted using online software (http://gtrd.biouml.org/bioumlweb/#). GO (Gene Ontology) functional annotation of the predicted target genes was carried out using DAVID (https://david.ncifcrf.gov/) and KOBAS (http://kobas.cbi.pku.edu.cn/anno_iden.php) to identify candidate genes related to germ cell development and stem cell differentiation.

**Analysis of Lin28A Promoter Activity**

The 2000-bp genome sequence upstream and downstream of the coding sequence was identified based on the Lin28A sequence obtained from the NCBI (https://www.ncbi.nlm.nih.gov/) and UCSC (http://genome.ucsc.edu/). The promoter region and transcription start site were identified based on core promoter elements (TATA box, CAAT box, and 5’-end of the coding region). Primers were designed with the transcription start site designated as + 1 for amplification of fragments. The plasmid pLin28A-EGFP was constructed and then used to transfect DF1 cells for 24–48 hours until green fluorescence was observed under a fluorescence inverted microscope. The presence/absence of green fluorescence was used to confirm that the constructed promoter fragment exhibited promoter activity. The PGL3-basic vector of different deletion fragments of the Lin28A promoter was constructed for the dual luciferase reporter gene detection system, which was used to detect promoter activity of the Lin28A target gene. The protocol was as follows: recombinant plasmids encoding different promoter fragments were co-transfected into DF1 cells with pRL-SV40 at a mass ratio of 30:1. A negative control was simultaneously prepared (co-transfection of pGL3-basic and pRL-SV40 plasmids into DF1 cells at a mass ratio of 30:1). Detailed transfection methods are available from the Fugene product manual. Three wells of cells were transfected for each group, and transfection was repeated three times. At 48 hours after initial transfection, the cells were collected and 70 µl of cell lysate was added to each tube and mixed gently. Next, 70 µl of fluorescent solution was added to each well and gently mixed. Renilla fluorescence was measured using a fluorescent plate reader after addition of 70 µl of STOP terminating reagent followed by gentle blown, and mixing.
Detection of Lin28A as a Downstream Target of Wnt5A/β-Catenin

DF1 cells were transfected with the following vectors pcDNA3.1-β-Catenin, pcDNA3.1-Myc-TCF7L2, Lin28A promoter deletion, and the corresponding TCF7L2 binding-site mutation vector (pGL3.0-Basic + pcDNA3.1 served as the negative control). The change in promoter activity was assessed using the double luciferase reporter system, and the relative fluorescence activity is reported as the mean ± standard error of three experiments. ESCs and PGC with good growth conditions were selected and divided into three groups each for transfection. Cells were transfected in factor medium. One group was transfected with shβ-Catenin; one group was transfected with oeβ-Catenin; and one group was left untreated. After incubation at 37 °C and 5% CO₂ for 48 h, ChIP-qPCR was performed.

Role of Lin28A in PGC Formation

ESCs were transfected with Lin28A overexpression and interference expression vectors and then either induced with BMP4 or injected into the blood vessels of chicken embryos. Cell samples were collected at 0, 2, 4, and 6 days after in vitro induction, and tissue samples were collected at 0 and 4.5 days during in vivo incubation. Total RNA was extracted using Trizol reagent, and cDNA was synthesized by reverse transcription. Expression of the PGC marker genes Cvh, C-kit, and Blimp1 and the totipotent marker genes Nanog and Oct4 was analyzed using β-actin as an internal reference. qRT-PCR was conducted as previously described. The efficiency of PGC formation was assessed by flow cytometry and analysis of paraffin-embedded tissue sections.

Assay of LSD1 Binding to β-Catenin and TCF7L2

DF1 cells with good status were selected, divided into six groups, and co-transfected with LSD1, β-catenin, or TCF7L2 vector at 37 °C and 5% CO₂ and saturation humidity for 48 h. Cells of each group were collected, and a lysate was prepared and subjected to IP, after which the protein concentration was determined. Subsequent IP experiments were performed using an equal volume of lysate. Binding of LSD1 to β-catenin or TCF7L2 was confirmed by monitoring the expression of target protein (co-immunoprecipitated protein) by Western blotting.

Effect of LSD1 on β-Catenin Binding to TCF7L2

DF1 cells with good status were selected and divided into two groups on the basis of co-transfection with LSD1-Flag and TCF7L2-Myc. One group was co-transfected with β-Catenin vector, and the other was co-transfected with pcDNA3.1 as a control. The cells were incubated at 37 °C and 5% CO₂ and saturation humidity for 48 h. Cells of each group were collected, and a lysate was prepared and subjected to IP, after which the protein concentration was determined. Subsequent IP experiments were carried out using an equal mass and volume of lysate, and expression of the co-precipitated target protein was evaluated by Western blotting.

Lin28A Targeted gga-let7s Screening

Online software (http://mirdb.org/cgi-bin/search.cgi) was used to predict chicken microRNA gga-Let7s. DF1 cells, ESCs, and PGC with good status were selected and divided into three groups. One group was transfected with siLin28A; one group was transfected with oeLin28A; and one group was left untreated. DF1 cells were transfected in complete medium, and ESCs and PGC were transfected in factor medium. After 48 h of incubation at 37 °C and 5% CO₂ and saturation humidity, total RNA was extracted using an miRNA extraction and isolation kit, and cDNA was synthesized by reverse transcription according to the reverse transcription kit procedure. U6 was used as an internal reference to detect changes in the relative expression of microRNA-let7s. The microRNA-let7s quantitative primer sequence is shown in Supplementary Table 3. qRT-PCR was carried out according to the instructions of the miRNA fluorescence quantitative detection kit. The reaction mixture was as follows: cDNA 50 ng; 2 x miRcute Plus microRNA PreMix 10 µl; forward primer 0.4 µl; reverse primer 0.4 µl; ddH₂O to a total volume of 20 µl. The PCR conditions were as follows: pre-denaturation at 95 °C for 15 min, 94 °C for 20 s, 63 °C for 30 s, 72 °C for 34 s (5 cycles); 94 °C for 20 s, 60 °C for 34 s annealing/extension (40 cycles); standard dissolution curve analysis.

Screening and Verification of gga-let-7a-2-3p Target Genes
Online software (http://mirdb.org/cgi-bin/search.cgi) was used to predict target genes related to reproductive differentiation. The targeting effect of gga-let-7a-2-3p on Blimp1 was assessed using the dual luciferase reporter gene detection system. DF1 cells were co-transfected with Blimp1-3'UTR-WT or Blimp1-3'UTR-Mut and PRL-TK at a mass to volume ratio of 10:1. Base on it, gga-let-7a-2-3p mimics or inhibitors were added, and negative controls were set up at the same time. The protocol was as follows: 2 × 10^5 DF1 cells/well were inoculated into a 24-well plate one day in advance of the experiment and cultured without antibiotics. When cells reached 50 to 60% confluence, mimic (or inhibitor) was added and diluted with 50 µl of Opti-MEM to a final concentration of 50 µM. Blimp1-3'UTR-WT (or the total mass of Blimp1-3'UTR-Mut cells was 1 µg) and PRL-TK were gently mixed as solution A, and 4 µl of FuGENEH were diluted with 50 µl of Opti-MEM and gently mixed for 5 min at room temperature to serve as solution B. After mixing solutions A and B, gently blew 3-5 times and set at room temperature for 20 min, and then incubated at 37 °C for 10-15 minutes. The mixture was then slowly added to the cell culture hole, mixed with 400 µl of complete medium, and incubated at 37 °C and 5% CO₂. Three wells were transfected at a time, and each transfection was repeated three times, and the cells were collected 48 h after transfection. Next, each tube was supplemented with 70 µl of cell lysate and gently mixed, after which the same volume of fluorescent solution was added to each well. Renilla fluorescence was measured using a fluorescent plate reader after addition of 70 µl of STOP terminating reagent followed by gentle blown, and mixing. Values are reported as the mean and standard error of three repeat tests. The assay was conducted according to the instruction manual of the dual luciferase reporter gene detection kit (Promega, USA).

**Data analysis**

Hierarchical clustering of differential gene expression (|log2| values) was performed using Heml. Relative gene expression was calculated from PCR data using the 2^−ΔΔCt method. The significance of between-group differences was assessed using two-sample t-tests with SPSS software, version 18.0. Data are presented as the mean ± standard deviation unless otherwise indicated. Significance was set at P < 0.05.

**Abbreviations**

Co-immunoprecipitation: co-IP; Differentially expressed genes: DEGs; Embryonic stem cells: ESCs; embryoid bodies: EBs; primordial germ cells: PGC

**Declarations**

**Ethics approval and consent to participate**

All procedures involving the care and use of animals conformed to the US National Institute of Health guidelines (NIH Pub. No. 85-23, revised 1996) and were approved by the laboratory animal management and experimental animal ethics committee of Yangzhou University.

**Consent for publication**

The study was undertaken with Yangzhou University.

**Availability of supporting data**

The supporting data used to support the findings of this study are included within the article.

**Competing interests**

The authors declare that they have no competing interests.

**Funding**
This work was supported by Jiangsu Science and Technology Project (Youth Fund): BK20180918; Natural Science Research Project of Jiangsu Higher Education Institutions: 18KJB230008; Key Research and Development Program: 2017YFE0108000; National Natural Science Foundation of China: 31872341, 31572390; and the High-Level Talent Support Program of Yangzhou University:11117.

Author Contributions

LBC and CGH conceived and designed the experiments. ZQS performed the experiments. ZQS analyzed the data. ZQS wrote the manuscript. ZYN edited the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We thank the Poultry Institute of the Chinese Academy of Agricultural Sciences Experimental Poultry Farm for providing experimental materials.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

References

1. Nakamura, Y. Avian biotechnology. Adv Exp Med Biol 1001, 187-214(2017).
2. Liu Z, Cai Y, Wang Y, Nie Y, Zhang C, Xu Y, et al. Cloning of macaque monkeys by somatic cell nuclear transfer. Cell 174, 245-251(2018).
3. Ono T, Machida Y. Immunomagnetic purification of viable primordial germ cells of Japanese quail (coturnix japonica). Comp Biochem Physiol A Mol Integr Physiol 122, 255-259(1999).
4. Zhao DF, Kuwana T. Purification of avian circulating primordial germ cells by nycodenz density gradient centrifugation. Br Poult Sci 44, 30-35(2003).
5. Lu Y, et al. Induced pluripotency in chicken embryonic fibroblast results in a germ cell fate. Stem Cells Dev, 23, 1755-1764(2014).
6. Chen D, et al. GSK-3 signaling is involved in proliferation of chicken primordial germ cells. Theriogenology 141, 62-67(2020).
7. Yasuda Y, Tajima A, Fujimoto T, Kuwana T. A method to obtain avian germ-line chimaeras using isolated primordial germ cells. J Reprod Fertil 96, 521-528(1992).
8. Tajima A, Naito M, Yasuda Y, Kuwana T. Production of germ-line chimeras by transfer of cryopreserved gonadal primordial germ cells (gpgc) in chicken. J Exp Zool 280, 265-267(1998).
9. Saitou M, Barton SC, Surani MA. A molecular programme for the specification of germ cell fate in mice. Nature 418, 293-300(2002).
10. Lawson KA, et al. BMP4 is required for the generation of primordial germ cells in the mouse embryo. Genes Dev 13, 424-436(1999).
11. Ying Y, Qi X, Zhao GQ. Induction of primordial germ cells from murine epiblasts by synergistic action of bmp4 and bmp8b signaling pathways. Proc Natl Acad Sci U S A 98, 7858-7862(2001).
12. Felici MD. The formation and migration of primordial germ cells in mouse and man. Results Probl Cell Differ 58, 23-46(2016).
13. Miyauchi H, et al. Bone morphogenetic protein and retinoic acid synergistically specify female germ-cell fate in mice. EMBO J 36, 3100-3119(2017).
14. Bialecka M, et al. Cdx2 contributes to the expansion of the early primordial germ cell population in the mouse. Developmental Biology 371, 227-234(2012).
15. Aramaki S, et al. A mesodermal factor, t, specifies mouse germ cell fate by directly activating germline determinants. Dev Cell 27, 516-529(2013).
16. Cervantes S, Yamaguchi TP, Hebrok M. Wnt5a is essential for intestinal elongation in mice. Dev Cell 326, 285-294(2009).
17. Yamaguchi TP, Takada S, Yoshikawa Y, Wu N, McMahon AP. T (brachyury) is a direct target of wnt3a
during paraxial mesoderm specification. Genes Dev **13**, 3185-3190(1999).

18. Chassot AA, Le Rolle M, Jourden M, Taketo MM, Ghyselinck NB, Chabossier MC. Constitutive wnt/ctnnb1 activation triggers spermatogonial stem cell proliferation and germ cell depletion. *Dev Biol* **426**, 17-27(2017).

19. Ohinata Y, Ohta H, Shigeta M, Yamanaka K, Wakayama T, Saitou M. A signaling principle for the specification of the germ cell lineage in mice. *Cell* **137**, 571-584(2009).

20. Saitou M, Payer B, O’Carroll D, Ohinata Y, Surani MA. Blimp1 and the emergence of the germ line during development in the mouse. *Cell Cycle* **4**, 1736-1740(2005).

21. Zhang Z, et al. Crucial genes and pathways in chicken germ stem cell differentiation. *J Biol Chem* **290**, 13605-13621(2015).

22. Zuo Q, Jin K, Zhang Y, Song J, Li B. Dynamic expression and regulatory mechanism of tgf-β signaling in chicken embryonic stem cells differentiating into spermatogonial stem cells. *Biosci Rep* **37**, BSR20170179(2017).

23. Zuo Q, Jin K, Song J, Zhang Y, Chen G, Li B. Interaction of the primordial germ cell-specific protein c2eip with ptch2 directs differentiation of embryonic stem cells via hh signaling activation. *Cell Death Dis* **9**, 497-505(2018).

24. Zuo Q, et al. Nicd-mediated notch transduction regulates the different fate of chicken primordial germ cells and spermatogonial stem cells. *Cell Biosci* **8**, 40-48(2018).

25. He N, et al. Wnt signaling pathway regulates differentiation of chicken embryonic stem cells into spermatogonial stem cells via wnt5a. *J Cell Biochem* **119**, 1689-1701(2017).

26. Matzuk MM. Lin28 lets blimp1 take the right course. *Dev Cell* **17**, 0-161(2009).

27. Bazley FA, Liu CF, Xuan Y, Hao H, Kerr CL. Direct reprogramming of human primordial germ cells into induced pluripotent stem cells. *Stem Cells Dev* **24**, 2634-2648(2015).

28. Steward MM, Lee JS, O’Donovan A, Wyatt M, Bernstein BE, Shilatifard A. Molecular regulation of h3k4 trimethylation by ash2l, a shared subunit of mll complexes. *Nat Struct Mol Biol* **13**, 852-854(2006).

29. Glaser S, Lubitz S, Loveland KL, Ohbo K, Stewart AF. The histone 3 lysine 4 methyltransferase, mll2, is only required briefly in development and spermatogenesis. *Epigenetics Chromatin* **2**, 5-12(2009).

30. Hou N, Ye B, Li X, Margulies KB, Li F. Transcription factor 7-like 2 mediates canonical wnt/β-catenin signaling and c-myc upregulation in heart failure. *Circ Heart Fail* **9**, e003010(2016).

31. Murakami K, et al. Nanog alone induces germ cells in primed epiblast in vitro by activation of enhancers. *Nature* **529**, 403-407(2016).

32. Lin IY, et al. Suppression of the sox2 neural effector gene by prdm1 promotes human germ cell fate in embryonic stem cells. *Stem Cell Reports* **2**, 189-204(2014).

33. Minnich M, et al. Multifunctional role of the transcription factor blimp-1 in coordinating plasma cell differentiation. *Nat Immunol* **17**, 331-343(2016).

34. Cantú AV, Altshuler-Keylin S, Laird DJ. Discrete somatic niches coordinate proliferation and migration of primordial germ cells via wnt signaling. *J Cell Biol* **214**, 215-229(2016).

35. Lee HC, Lim S, Han JY. Wnt/β-catenin signaling pathway activation is required for proliferation of chicken primordial germ cells in vitro. *Sci Rep* **6**, 34510-34519(2016).

36. Tetsu O, Mccormick F. Beta-catenin regulates expression of cyclin d1 in colon carcinoma cells. *Nature* **398**, 422-426(1999).

37. He TC, et al. Identification of c-myc as a target of the apc pathway. *Science* **281**, 1509-1512(1998).

38. Childs AJ, Kinnell HL, He J, Anderson RA. Lin28 is selectively expressed by primordial and pre-meiotic germ cells in the human fetal ovary. *Stem Cells Dev* **21**, 2343-2349(2012).

39. Wang YF, et al. Expressions of lin28 and otc4 in male primordial germ cells. *Journal of Zhengzhou University* **50**, 389-392(2015).

40. Mccarty MF. Metformin may antagonize lin28 and/or lin28b activity, thereby boosting let-7 levels and antagonizing cancer progression. *Med Hypotheses* **78**, 262-269(2012).

41. Oh JS, Kim JJ, Byun JY, Kim IA. Lin28-let7 modulates radiosensitivity of human cancer cells with activation of k-ras. *Int J Radiat Oncol Biol Phys* **76**, 5-8(2010).

42. Pan P, et al. Lin28a inhibits lysosome associated membrane glycoprotein 1 protein expression in embryonic stem and bladder cancer cells. *Mol Med Rep* **18**, 399-406(2018).

43. Van Amerongen R, Nusse R. Towards an integrated view of wnt signaling in development. *Development* **136**, 3205-3214(2009).
44. Kelly KF, Ng DY, Jayakumaran G, Wood GA, Koide H, Doble BW. β-catenin enhances oct-4 activity and reinforces pluripotency through a tcf-independent mechanism. *Cell Stem Cell* **8**, 214-227(2011).
45. Schuijers J, Mokry M, Hatzis P, Cuppen E, Clevers H. Wnt-induced transcriptional activation is exclusively mediated by tcf/lef. *EMBO J* **33**, 146-156(2014).
46. Lee TI, *et al.* Control of developmental regulators by polycomb in human embryonic stem cells. *Cell* **125**, 301-313(2006).
47. Salz T, Li G, Kaye F, Zhou L, Qiu Y, Huang S. Hsetd1a regulates wnt target genes and controls tumor growth of colorectal cancer cells. *Cancer Res* **74**, 775-786(2014).
48. Willert K, Jones KA. Wnt signaling: is the party in the nucleus? *Genes Dev* **20**, 1394-1404(2006).
49. Blythe SA, Cha SW, Tadjuidje E, Heasman J, Klein PS. β-catenin primes organizer gene expression by recruiting a histone h3 arginine 8 methyltransferase, prmt2. *Dev Cell* **19**, 220-231(2010).
50. Li Z, Nie F, Wang S, Li L. Histone h4 lys 20 monomethylation by histone methylase set8 mediates wnt target gene activation. *Proc Natl Acad Sci U S A* **108**, 3116-3123(2011).
51. Su X, *et al.* Molecular basis underlying histone h3 lysine-arginine methylation pattern readout by spin/sst1 repeats of spindlin1. *Genes Dev* **28**, 622-636(2014).
Wnt5a-β-catenin-TCF7L2 positively regulates the formation of PGC. A. KEGG analysis of DEGs in ESCs and PGC. B,C. Expression detection of β-catenin and TCF7L2 by qRT-PCR after over-expression and interference of Wnt5a in vivo and in vitro. D. Co-IP results show that β-catenin and TCF7L2 can bind to each other. E. Result of IHC in genital ridge by CVH antibody, Up Scale bar:200μm, Down Scale bar:40μm.
Figure 1

Wnt5a-β-catenin-TCF7L2 positively regulates the formation of PGC. A. KEGG analysis of DEGs in ESCs and PGC.
B,C. Expression detection of β-catenin and TCF7L2 by qRT-PCR after over-expression and interference of Wnt5a in vivo and in vitro.
D. Co-IP results show that β-catenin and TCF7L2 can binding to each other.
E. Result of IHC in genital ridge by CVH antibody, Up Scale bar:200μm , Down Scale bar:40μm.
Lin28A is a specific target gene of Wnt5a signal in PGC. A. Venny analyzes of TCF7L2 target genes of Human (15727), Rat (15652), and Mouse (15374) enriched from GTRD. B. GO analysis was performed on 7473 target genes, and Lin28A was screened from GO entries of Germ cell development and Germ cell differentiation. C,D. The expression of Lin28A was detected by qRT-PCR after Wnt5A and β-catenin were overexpressed or interfered with during the formation of PGC in vitro and in vivo, respectively. E~G. The effect of TCF7L2 mutation on activity of Lin28A promoter was detected by dual luciferase system. H. CHIP-qPCR was used to detect the enrichment of TCF7L2 in the Lin28A promoter region.
Lin28A is a specific target gene of Wnt5a signal in PGC. A. Venny analyzes of TCF7L2 target genes of Human (15727), Rat (15652), and Mouse (15374) enriched from GTRD. B. GO analysis was performed on 7473 target genes, and Lin28A was screened from GO entries of Germ cell development and Germ cell differentiation. C,D. The expression of Lin28A was detected by qRT-PCR after Wnt5A and β-catenin were overexpressed or interfered with during the formation of PGC in vitro and in vivo, respectively. E~G. The effect of TCF7L2 mutation on activity of Lin28A promoter was detected by dual luciferase system. H. CHIP-qPCR was used to detect the enrichment of TCF7L2 in the Lin28A promoter region.
Figure 3

Lin28A positively regulates PGC formation in vitro and in vivo. A. Schematic diagrams of function verification of Lin28A in the formation of PGC in vitro and in vivo. B. Morphological observation of the number of embryoid bodies in PGC induction model in vitro after overexpression and interference with Lin28A. Scale bar: 60μm. C, E. Detection of PGC formation efficiency after overexpression or interference of Lin28A in vivo and in vitro by flow cytometry. D. Number of PGC in genital ridge after overexpression or interference with Lin28A in vitro were detected by PAS staining. Up Scale bar: 200μm, Down Scale bar: 40μm.
Lin28A positively regulates PGC formation in vitro and in vivo. A. Schematic diagrams of function verification of Lin28A in the formation of PGC in vitro and in vivo. B. Morphological observation of the number of embryoid bodies in PGC induction model in vitro after overexpression and interference with Lin28A. Scale bar: 60μm. C,E. Detection of PGC formation efficiency after overexpression or interference of Lin28A in vivo and in vitro by flow cytometry. D. Number of PGC in genital ridge after overexpression or interference with Lin28A in vitro were detected by PAS staining. Up Scale bar: 200μm, Down Scale bar: 40μm.
Figure 4

Lin28A is a target gene regulated by H3K4me2. A. Detection of Lin28A expression after interference with LSD1 and MLL2 in the BMP4 induction model, respectively. B. Detection of Lin28A expression after interference with LSD1 and MLL2 in PGC, respectively. C. ChIP-qPCR was used to detect the enrichment level of H3K4me2 in the Lin28A promoter region after interference with LSD1 and MLL2 in PGC, respectively.
Lin28A is a target gene regulated by H3K4me2. A. Detection of Lin28A expression after interference with LSD1 and MLL2 in the BMP4 induction model, respectively. B. Detection of Lin28A expression after interference with LSD1 and MLL2 in PGC, respectively. C. ChIP-qPCR was used to detect the enrichment level of H3K4me2 in the Lin28A promoter region after interference with LSD1 and MLL2 in PGC, respectively.
Competition of β-catenin and LSD1 in the formation of PGC in combination with TCF7L2 regulates the expression of Lin28A. A. Effects of Wnt Signaling on the Regulation of Lin28A Promoter Activity After MLL2 and LSD1 Interference were detected by by dual luciferase system. B,C. Results of Co-IP showed that LSD1 cannot bind to β-Catenin, but bind to TCF7L2. D. Results of Co-IP showed that β-Catenin competes with LSD1 binding to TCF7L2.
Competition of β-catenin and LSD1 in the formation of PGC in combination with TCF7L2 regulates the expression of Lin28A. A. Effects of Wnt Signaling on the Regulation of Lin28A Promoter Activity After MLL2 and LSD1 Interference were detected by dual luciferase system. B, C. Results of Co-IP showed that LSD1 cannot bind to β-Catenin, but bind to TCF7L2. D. Results of Co-IP showed that β-Catenin competes with LSD1 binding to TCF7L2.
Lin28A activates Blimp1 to regulate the formation of PGC by inhibiting gga-let-7a-2-3p maturation. A~D. qRT-PCR was used to detect the expression of miRNAlet7s after overexpression and interference of Lin28A. E. qRT-PCR was used to detect the expression of Blimp1 after mimic and inhibitor of gga-let-7a-2-3p were transfected into DF-1 and PGC. F. The dual luciferase system was used to detect that gga-let-7a-2-3p regulates Blimp1 3'UTR activity.
Lin28A activates Blimp1 to regulate the formation of PGC by inhibiting gga-let-7a-2-3p maturation. A~D. qRT-PCR was used to detect the expression of miRNAlet7s after overexpression and interference of Lin28A. E. qRT-PCR was used to detect the expression of Blimp1 after mimic and inhibitor of gga-let-7a-2-3p were transfected into DF-1 and PGC. F. The dual luciferase system was used to detect that gga-let-7a-2-3p regulates Blimp1 3’UTR activity.
Blimp1 interacts with LSD1 to regulate the expression of related genes in Wnt signaling and participate in the formation of PGC. A. Detection of H3K4me2 enrichment in Wnt5A, β-Catenin, and TCF7L2 promoter regions in ESCs and PGC by CHIP-qPCR. B. Schematic diagram of Blimp1 binding site and H3K4me2 enrichment site in Wnt5A promoter region. C,D. The effects of Blimp1 overexpression or interference on activity of wild-type and mutant promoter of Wnt5A were detected by dual luciferase reporter system. E. Detection of Wnt5A expression after overexpression or interference of Blimp1 by qRT-PCR. F. Detection of H3K4me2 enrichment in Wnt5A promoter regions after over-expression or interference of Blimp1 by CHIP-qPCR. G. Detection of LSD1 enrichment in Wnt5A promoter regions after over-expression or interference of Blimp1 by CHIP-qPCR.
Blimp1 interacts with LSD1 to regulate the expression of related genes in Wnt signaling and participate in the formation of PGC. A. Detection of H3K4me2 enrichment in Wnt5A, β-Catenin, and TCF7L2 promoter regions in ESCs and PGC by CHIP-qPCR. B. Schematic diagram of Blimp1 binding site and H3K4me2 enrichment site in Wnt5A promoter region. C,D. The effects of Blimp1 overexpression or interference on activity of wild-type and mutant promoter of Wnt5A were detected by dual luciferase reporter system. E. Detection of Wnt5A expression after overexpression or interference of Blimp1 by qRT-PCR. F. Detection of H3K4me2 enrichment in Wnt5A promoter regions after over-expression or interference of Blimp1 by CHIP-qPCR. G. Detection of LSD1 enrichment in Wnt5A promoter regions after over-expression or interference of Blimp1 by CHIP-qPCR.
Blimp1 interacts with LSD1 to regulate the expression of related genes in Wnt signaling and participate in the formation of PGC. A. qRT-PCR was used to detect the expression of Wnt signal related molecules after BMP signal activation. B. qRT-PCR was used to detect the expression of BMP4 signal related molecules after Wnt signal activation. C, D. Cell morphology changes after BMP4 removal in BMP4 model. Scale bar: 60μm. E. Flow cytometry analysis of PGC formation efficiency after BMP4 removal.
Figure 8

Blimp1 interacts with LSD1 to regulate the expression of related genes in Wnt signaling and participate in the formation of PGC. A. qRT-PCR was used to detect the expression of Wnt signal related molecules after BMP signal activation. B. qRT-PCR was used to detect the expression of BMP4 signal related molecules after Wnt signal activation. C, D. Cell morphology changes after BMP4 removal in BMP4 model. Scale bar:60μm. E. Flow cytometry analysis of PGC formation efficiency after BMP4 removal.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- S5.eps
- S1.eps
- S8.eps
- S5.eps
• S1.eps
• S8.eps
• S3.eps
• S2.eps
• S7.eps
• S3.eps
• S2.eps
• S7.eps
• S4.eps
• S6.eps
• STable3.docx
• STable2.docx
• S4.eps
• S6.eps
• STable1.docx
• STable3.docx
• STable2.docx
• STable1.docx