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

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Research

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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 birds^{1,2}. Transplantation of exogenous primordial germ cells (PGC) into blood vessels of recipient chickens can migrate to the genital ridge via the blood and produce offspring^{3,4}, 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 difficult^{7,8}. 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 studies^{9,10} reported induction of PGC (*Fragilis*⁺ *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 gonads^{11–13}. However, other studies^{14–16} confirmed that Wnt signaling also plays an important role in PGC formation in mice. BMP4 and Wnt signaling interact during PGC formation^{15,19}. Both BMP4 and Wnt signaling regulate PGC formation in mice by activating *Blimp1* via the mesoderm-specific transcription factor T^{15,17,18}. 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 genes²¹. Wnt signaling-related genes were also significantly activated in an *in vitro* PGC induction model²⁴ (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)²⁵. 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 formation²⁵ (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^{26,27}.

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 2 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 *Mll2* 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 Mll2 is relatively fixed^{28,29}, 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 TCF7L2 and β -catenin³⁰, we hypothesize that in ESCs, TCF7L2 binding in the *Lin28A* promoter recruits LSD1, which reduces the level of H3K4me2 enrichment, inhibiting *Lin28A* transcription; during PGC formation, β -catenin enters the nucleus ectopically and competes with LSD1 for binding to TCF7L2, which increases the level of H3K4me2 enrichment and promotes *Lin28A* transcription. To test this hypothesis, DF1 cells were co-transfected with LSD1-Flag, TCF7L2-Myc, and β -catenin vectors. Co-IP indicated that LSD1 did not bind to TCF7L2, whereas β -catenin did bind to TCF7L2 (Fig. 5D). Collectively, these results indicate that β -catenin competes with LSD1 for binding to TCF7L2, which de-methylates H3K4me2 in the *Lin28A* promoter via LSD1 and activates *Lin28A* 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 micRNA-let7 maturation²⁶. However, the micRNA-let7 that interacts with Lin28A during chicken PGC formation remained to be identified. To determine the key micRNAlet7s targeted by Lin28A, 17 gga-let7s in the chicken micRNAlet7 family were screened using miRDB (Fig. 6A,B). 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^{31,32}. 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 *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 H3K4me2³³, 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 sh*LSD1* 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 sh*LSD1* ($P < 0.01$), 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 preliminary 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^{34,35}. The Wnt/β-catenin pathway promotes induction of the ectoderm

response to BMP4 signaling and participates in determining PGC specialization in mammals^{15,17}, with Wnt directly regulating the expression of *Blimp1* and *Prdm14* via mesoderm and notochord transcription factor *T*^{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 *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 *c-myc* and *Jun*^{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^{38,39}. Although many studies have examined the interaction between Wnt and *Lin28A* in mammals, primarily as it relates to cancer^{40–42}, a role for this interaction in PGC formation has not been reported. This study confirmed for the first time that *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^{43–45} and regulates the chromatin status of target gene promoters by recruiting epigenetic factors that also regulate gene expression⁴⁶. 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^{47,48}. In *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 transition⁴⁹. In addition to β -catenin, the transcription factor TCF also recruits histone methylation–modifying enzymes⁵⁰. TCF4 regulates the transcription of classical Wnt signaling target genes by recruiting the histone-modification enzyme spindlin⁵¹. 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 *Lin28A* promoter and activating *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 chickens²⁰, 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, *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. Saitou²⁰ 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 *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 oe*TCF7L2-Myc* vector; and one group was transfected with the oeβ-*Catenin* and oe*TCF7L2-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 sh*LSD1*; one group was transfected with sh*MLL2*; 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 p*Lin28A*-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 Eugene 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 μ l of cell lysate was added to each tube and mixed gently. Next, 70 μ l of fluorescent solution was added to each well and gently mixed. Renilla fluorescence was measured using a fluorescent plate reader after addition of 70 μ l 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 $\pm$ 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 sh β -Catenin; one group was transfected with oe β -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 si*Lin28A*; one group was transfected with oe*Lin28A*; 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 *micRNA-let7s*. The *micRNA-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 × miRcute Plus miRNA 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. Based 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 × 10⁵ 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 FuGENEHD was 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 blowing, 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 ($|\log_2|$ values) was performed using Heml. Relative gene expression was calculated from PCR data using the $2^{-\Delta\Delta C_t}$ 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 $\pm$ 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.

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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.

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Availability of data and materials

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

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Figures

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:200 μ m , Down Scale bar:40 μ m.

Figure 2

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.

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.

Figure 5

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.

Figure 6

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 micRNAlet7s 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.

Figure 7

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.

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:60 μ m. E. Flow cytometry analysis of PGC formation efficiency after BMP4 removal.

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