Pcgf1 regulates early neural tube development through histone methylation in zebrafish

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Abstract

Objective

Early neural tube development in the embryo includes neural induction and self-renewal of neural stem cells (NSCs). The abnormal of neural tube development could lead to neural tube defects. The research on the mechanism of neural induction is the key to reveal the pathogenesis of the abnormal of neural tube. Though studies have confirmed a genetic component, the responsible mechanisms for the abnormal of neural tube are still largely unknown. Polycomb repressive complex 1 (PRC1) plays an important role in regulating early embryonic development, and has been sub-classified into six major complexes based on the presence of a Pcgf subunit. Pcgf1, as one of six Pcgf paralogs, is an important requirement in early embryonic brain development. Here, we intended to investigate the role and mechanism of Pcgf1 in early neural tube development of zebrafish embryos.

Material and methods

Morpholino (MO) antisense oligonucleotides were used to construct a Pcgf1 loss-of function zebrafish model. We analyzed the phenotype of zebrafish embryos and the expression of related genes in the process of neural induction by in situ hybridization, immunolabelling and RNA-sEq. The regulation of histone modifications on gene was detected by western blot and chromatin immunoprecipitation.

Results

In this study, we found that zebrafish embryos exhibited small head and reduced or even absence of telencephalon after inhibiting the expression of Pcgf1. Moreover, the neural induction process of zebrafish embryos was abnormal, and the subsequent NSCs self-renewal was inhibited under the inhibition of Pcgf1. RNA-seq and gene ontology (GO) analysis identified that the differentially expressed genes were enriched in many functional categories which related to the development phenotype. Finally, our results showed that Pcgf1 regulated the trimethylation of histone H3K27 in the Ngn1 and Otx2 promoter regions, and the levels of H3K4me3 at the promoters of Pou5f3 and Nanog.

Conclusion

Together, our data for the first time demonstrate that Pcgf1 plays an essential role in early neural induction phase through histone methylation in neural tube development. Our findings reveal a critical context-specific function for Pcgf1 in directing PRC1 to control cell fate.

Background
Early neural tube development in the embryo includes two important processes: neural induction and self-renewal of neural stem cells (NSCs). Neural induction refers to the process in which the mesoderm induces the ectoderm to develop into a neural plate, and then closes to form neural tube in the early stage of embryonic development. Later in development, the self-renewal of NSCs is the cytological basis of early neural tube development\(^1\). The neural induction constitutes the initial step in the generation of the vertebrate nervous system. It played a decisive role during early neural tube development\(^1\). The failure of the morphogenetic process of neural tube closure could lead to neural tube defects\(^3\)–\(^5\). The abnormal of neural tube development causes serious damage to the child survival and the quality of life\(^6\),\(^7\). Therefore, research on the mechanism of neural induction is the key to reveal the pathogenesis of the abnormal neural tube development, which will provide new ideas for its clinical treatment.

The PcG protein family was originally discovered in Drosophila\(^8\), and has always been considered as a class of proteins that inhibit the transcription of target genes at the level of chromatin, which has a stable role in inhibiting transcription through epigenetic modification of histones during stem cell biology and development\(^9\)–\(^12\). It includes two protein complexes PRC1 and PRC2\(^13\). PRC1 complexes can be grouped as canonical PRC1 and non-canonical PRC1\(^14\). PRC1 complex plays an important role in regulating early embryonic development. It can regulate the differentiation of embryonic stem cells (ESCs), and is closely related to the differentiation process of the three germ layers\(^15\)–\(^20\). There is a type of protein in PRC1 that can form a dimer complex with RING1A/B, called Pcgf protein (including 6 homologous proteins, Pcgf1-Pcgf6)\(^13\). It had been reported that Pcgf2/Mel18 is essential for ESCs differentiation into early cardiac-mesoderm precursors\(^16\); Pcgf3/5 mainly function as transcriptional activators to drive expression of many genes involved in mesoderm differentiation\(^18\); Pcgf4/Bmi1 is a key regulator of self-renewal of embryonic and adult central nervous system stem cells\(^21\); Pcgf6 directly regulates Oct4, Nanog, Sox2, and Lin28 expression to maintain ESCs identity\(^19\). It is well documented that Pcgf protein are implicated in early embryonic development. Here, we focus on
Pcgf1, which mainly expressed in nervous system\textsuperscript{22}. In vitro studies showed that ESCs deficient in Pcgf1 displayed severe defects in ectoderm and mesoderm differentiation\textsuperscript{15}; Hui Li et al reported that Pcgf1 has a positive role in maintaining the pluripotency of P19 cells by directly regulating Oct4\textsuperscript{23}; The growth of zebrafish was reduced in early developmental stages due to the absence of Pcgf1, and about 35% of Pcgf1\textsuperscript{−/−} fish showed signs of premature aging. However, these studies did not explore the role and mechanism of Pcgf1 in early neural tube development\textsuperscript{24}.

Early development of zebrafish can be easily observed because of external fertilization and optical transparency of the embryos, which makes it a good model for studying morphogenesis and development of neural tube\textsuperscript{25–28}. In our study, we found that Pcgf1 was maternally expressed, and the zebrafish embryos exhibited telencephalic malformations after knocked down Pcgf1. In the neural induction stage, the expression of markers related to early neurodevelopment was increased and activated in advance. As a result, the proliferation of NSCs decreased and cells exited the cell cycle early, which eventually led to the abnormal development of neural tube. At the same time, it was initially confirmed that Pcgf1 may participate in the determination of early neurodevelopmental fate by dynamically regulating the network of neural development-related transcription factors through epigenetic modification.

**Materials And Methods**

**Zebrafish maintenance**

Zebrafish maintenance was performed as described previously\textsuperscript{29}. Adult zebrafish are reared in a constant-temperature circulating aquaculture system at 28.5°C. The cycle of 14 h light and 10 h dark is maintained, and the hatched eggs are fed once a day in the morning and evening. The fertilized zebrafish embryos were placed in artificial configured water and were cultured in a constant temperature incubator at 28.5°C. The development of embryo was observed with stereo microscope.

**Cell culture**

P19 cells were cultured in MEM medium (DMEM) (HyClone) that was supplemented with 3% fetal bovine serum, 7% calf bovine serum (FBS; ExCell Bio) and 1% penicillin-streptomycin-amphotericin B
(macgene). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂.

For RA induced differentiation, P19 cells were digested with 0.25% trypsin and resuspended with MEM medium that was supplemented with 0.5 μM all-trans-RA (Sigma-Aldrich Co. LLC,St. Louis, MO,USA), then replaced with N2 medium after 3-4 days.³⁰

**Bromodeoxyuridine (BrdU) assay**

Zebrafish embryos were collected at stages of 16 hpf and 24 hpf. Embryos were immersed in 10 mM BrdU solution at 4°C for 20 min after peeled off the membrane, then embryos were fixed with 4% paraformaldehyde overnight. The next day, embryos were incubated with anti-BrdU antibody overnight and washed with PBST. After incubation with HRP secondary antibody for 1 h, DAB staining was performed to observe BrdU-positive cells.

**Acridine orange stain**

Zebrafish embryos were collected at stages of 16 hpf and 24 hpf. Embryos were incubated in 2 μg/ml acridine orange solution at 28°C for 30 min in dark after peeled off the membrane. The embryos were analyzed by fluorescence microscope after washing embryos with PBS.

**Microinjection of morpholino (MO) antisense oligonucleotides**

Morpholine ring modified antisense oligonucleotides for Pcgf1 was designed in this study to target the translation initiation region of Pcgf1 mRNA. The sequence is 5′-CCTTGCTCCGCCATCTTTGGGAATT-3′. The standard control for experiments was the sequence 5′-CCTTCCTCCCCATGTTAGCGAATT-3′. MO oligonucleotides were injected into zebrafish embryos at the one-cell stage with 5-9 ng/embryo, and the injection volume was generally 2-3 nl.

**RNA extraction and quantitative real-time PCR assays**

Total RNA was extracted using TRIzol reagent (TRANS) and reversed transcribed. cDNAs were amplified by quantitative real-time PCR (qPCR). Real time PCR was performed with SYBR Green Realtime PCR Master Mix (TOYOBO CO., Ltd., Japan) following the manufacturer’s protocol. Cycle thresholds were normalized to an internal control: actin for mRNA assays. The relative expression of mRNA was calculated with the 2⁻ΔΔCT method. The primer sequences were as follows: Pcgf1 (forward,
5′-GCGATAGCTATGCGGCTAAG-3′; reverse, 5′-CATTCTGTGATGGTGGTTGC-3′), wnt3a (forward, 5′-TCACTGACCACATGTACCTGAA-3′; reverse, 5′-TTCTCAACCACATTCCGATG-3′), Pou3f1 (forward, 5′-TCGAGGTGGGTGTCGCAAAGG-3′; reverse, 5′-GGCGCATAAACGTCGTCCA-3′), Pax6 (forward, 5′-GGCGGTTACACTCTTAA-3′), Nanog (forward, 5′-TTGCTTACAAGGGTGCTGCTTTACTG-3′; reverse, 5′-ACTGTTAGAGAATCAGGGCT-3′), Oct4 (forward, 5′-CACAACGAGAAGAGTATGA-3′; reverse, 5′-GCCCTCTTTTCATCTCCTTTCC-3′), Sox2 (forward, 5′-TCGAGGTGGGTGTCGCAAAGG-3′; reverse, 5′-GGCGCATAAACGTCGTCCA-3′), Sox3 (forward, 5′-CTCGATGGGTGTTTCGTT-3′; reverse, 5′-CTCTTTGAGGCAAGATGC-3′), Pou3f1 (forward, 5′-TCACTGACCACATGTACCTGAA-3′; reverse, 5′-GCCCGTTACACTCTTAA-3′), Otx2 (forward, 5′-CTCGATGGGTGTTTCGTT-3′; reverse, 5′-CTCTTTGAGGCAAGATGC-3′), Ngn1 (forward, 5′-ATGTCGTATCTCAAGGGAGACCAT-3′; reverse, 5′-GGCGCATAAACGTCGTCCA-3′), P21 (forward, 5′-TAAAGTACCACCCCTCGAAG-3′; reverse, 5′-GGCGCGAAGAAGGCTGATAG-3′), TGFβ (forward, 5′-TCGAGGTGGGTGTCGCAAAGG-3′; reverse, 5′-GGCGCATAAACGTCGTCCA-3′), Ngn1 (forward, 5′-ATGTCGTATCTCAAGGGAGACCAT-3′; reverse, 5′-GGCGCATAAACGTCGTCCA-3′), P57 (forward, 5′-TAAAGTACCACCCCTCGAAG-3′; reverse, 5′-GGCGCGAAGAAGGCTGATAG-3′), Zfp521 (forward, 5′-TCGAGGTGGGTGTTTCGTT-3′; reverse, 5′-CTCTTTGAGGCAAGATGC-3′), Hes1 (forward, 5′-TGGCTTATCCTCCACCC-3′; reverse, 5′-CTCTTTGAGGGAAGAGGCTGATAG-3′), Smad1 (forward, 5′-GCTAAACTCTCACCAGCTCCTG-3′; reverse, 5′-GGAGCAGTGGGAGAGCAGCAC-3′), Smad2 (forward, 5′-GTCCATCTCAGATTACAGGATAACTACCC-3′; reverse, 5′-GGAGCAGTGGGAGAGCAGCAC-3′), Smad5 (forward, 5′-TGAGTCACAAGCAGCTCCTG-3′; reverse, 5′-GTCCATCTCAGATTACAGGATAACTACCC-3′; reverse, 5′-GGAGCAGTGGGAGAGCAGCAC-3′), Wnt8a (forward, 5′-TTTGCAGTGGGTATGTCGTCTTTATG-3′; reverse, 5′-CTGCTCTGTTAGATGTCGTCTTTATG-3′), β-catenin (forward, 5′-GCAGATACCTTCCACCC-3′; reverse, 5′-CTGCTCTGTTAGATGTCGTCTTTATG-3′).

**In situ hybridization**

4% paraformaldehyde was used to fix embryos at different developmental stages (shield stage, 75% outsourcing stage, 10 hpf, 16 hpf, 24 hpf). The fixed embryos were pre-treated with proteinase K, washed with PBST, prehybridized in hybridization buffer for 4 hours at 65 °C, and then incubated with antisense RNA probes (Pcgf1, Otx2, Ngn1, Sox3, Sox2) and shaken at 60°C water bath overnight. The next day, the embryos were washed in washing liquid, and blocking solution was used to block the nonspecific binding sites at room temperature. Then, the embryos were incubated with anti-digoxigenin-AP at 4°C in the dark overnight. On the third day, NBT/BCIP was used for color detection,
and images were acquired with a stereomicroscope (Olympus SZX16).

**Western blotting**

Cells and zebrafish embryos were lysed in RIPA buffer with protease inhibitors. The lysates were separated on 10% SDS-polyacrylamide gel and transferred to PVDF membranes. The membranes were incubated with 5% skim milk for 2 h and probed overnight at 4°C with the indicated primary antibodies, and then with secondary antibodies for 1 h at room temperature after washing. Protein bands were analyzed by Immobilon™ Western Chemiluminescent HRP Substrate (MILLIPORE). The primary antibodies were used as follows: Pcgf1 (ab84108), Pax6 (ab5790), H3K4me3 (ab8580), H3K27me3 (ab6002) and H3 (ab1791) from Abcam; Oct4 (#2750) from Cell Signaling Technology and anti-β-actin from TransGen Biotech (HC201-01).

**Chromatin immunoprecipitation assay (ChIP)**

The ChIP assay was performed as reported earlier\(^2^9\). Briefly, protein-DNA complexes of zebrafish embryos were cross-linked by treatment with 1% formaldehyde for 15 min, and then glycine was used to stop cross-linking. Chromatin was sonicated to shear into 20-500 bp. Equal aliquots of isolated chromatin were immunoprecipitated with H3K27me3 and H3K4me3 antibody (CST) or a control antibody (anti-IgG) overnight at 4 °C with rotation, followed by incubation with protein G agarose for 1 h. The DNA fragments associated with specific immunoprecipitates was purified and used as templates for real-time PCR. The ChIP qPCR primer sequences were as follows: Ngn1 (forward, 5’- TCTCCCAGCCCCACCAAATAAG-3’; reverse, 5’-TCACAGCTTGAGGTTCATCCAT-3’), Otx2 (forward, 5’- ATGTCGATCTCAAGCAACCAC-3’; reverse, 5’- GTCCCTTTCTCGTCTGTTTC-3’), Pou5f3 (forward, 5’- TGAACGAGGCGAAAACTC-3’; reverse, 5’-ACGACTCTAGACAGACCAACGG -3’) and Nanog (forward, 5’- ACCAGCAGTGATACACCCAG-3’; reverse, 5’- CCAGGATGGCAGATTGAGGT-3’).

**Statistical analysis**

Data are presented as the mean ± SE of at least three independent experiments. The statistical analyses were performed with the Student’s t-test for comparison of the two groups. One-way ANOVA was performed for three or more groups. \(P < 0.05\) was considered as statistically significant.

**Results**
Expression pattern of Pcgf1 during embryonic development in zebrafish

We first analyzed the spatiotemporal expression characteristics of the Pcgf family members at different stages of embryonic development by RT-PCR, qPCR and in situ hybridization. Our results showed that the mRNA expression of Pcgf family members in different stages was as follows: Pcgf1 is maternally expressed during zebrafish embryo development. The expression of Pcgf1 started at sphere phase and increased significantly at shield phase. At shield stage, embryonic development enters to the gastrointestinal embryo stage and begins to differentiate into the three germ layers, which is a key period of neural induction. However, other Pcgf members decreased during this time. It is suggested that Pcgf1 is specific in the early stage of neural development, especially in the neural induction stage (Fig. 1A and Fig. 1B). The results of In situ hybridization showed that Pcgf1 had certain specificity at 16 hpf, mainly concentrated in central nervous system and the entire neural tube region, especially at the head and tail. The expression of Pcgf1 increased significantly at the junction of forebrain, midbrain, cerebellum, and midbrain at 24 hpf (Fig. 1C).

Knockdown of Pcgf1 caused deficits in the development of neural tubes

To verify the role of Pcgf1 during zebrafish embryonic development, we knocked down Pcgf1 in zebrafish embryos using morpholino (MO) antisense oligonucleotides, and the efficiency of MO treatment was assessed. Western blot confirmed that the MO oligonucleotides inhibit the translation of Pcgf1 mRNA (Fig. 2A). The phenotype of zebrafish embryos showed that knockdown of Pcgf1 leaded to obvious abnormalities of telencephalon at 16 hpf and 24 hpf with small head, reduced or even absence of telencephalon and shortening of body axis (Fig. 2B and 2C).

Knockdown of Pcgf1 led to abnormal activation of neural induction

To further investigate the relationship between telencephalon deletion caused by Pcgf1 knockdown and the process of neural induction, we analyzed the expression of several neural markers during neural induction phase (shield, 75% epiboly phase and 10 hpf) of zebrafish embryonic development. The results of In situ hybridization showed the expression of neural markers Sox2, Otx2 and Ngn1 was activated abnormally compared with the control group, while the expression of Sox3 increased at shield phase and decreased from 75% epiboly phase (Fig. 3A). In order to further verify the above
conclusions, we tested the expression of neural markers by qPCR, and the results were consistent with those of in situ hybridization (Fig. 3B). These results suggested that knockdown of Pcgf1 led to abnormal activation of neural induction.

**Knockdown of Pcgf1 inhibited the proliferation of NSCs**

The proliferation, apoptosis, differentiation and migration of NSCs are also the cytological basis for the normal development of neural tubes. It is not clear whether the early activation of neural induction phase caused by knockdown of Pcgf1 could affect the subsequent self-renewal of NSCs. We first analyzed the expression of several neural markers during NSCs self-renewal phase (16 hpf and 24 hpf) of zebrafish embryonic development. The results of in situ hybridization and qPCR showed that the expression of all the neural markers Sox2, Otx2, Ngn1 and Sox3 were decreased significantly (Fig. 4A and 4B). Moreover, BrdU incorporation assay and PCNA (a well-accepted marker of proliferation) in situ hybridization were used to detect the effect of Pcgf1 on the proliferation of NSCs. The results showed that the number of BrdU positive cells and the level of PCNA both decreased in the Pcgf1 MO group. At the same time, acridine orange (AO) staining was used to detect the effect of Pcgf1 on the apoptosis of NSCs. The results showed that there was no significant change in apoptosis (Fig. 4C). Cyclin-dependent kinase (CDK) inhibitors are important for differentiation. We found that, compared with the control group, the expression of the CDK inhibitors p21 and p57 was increased after injecting of Pcgf1 MO (Fig. 4D). Taken together, these results suggested that the abnormal development of neural tube might be caused by the abnormality in the neural induction phase and the subsequent impaired proliferation and prematurely differentiation of NSCs after injecting the Pcgf1 MO.

**Pcgf1 is indispensable for maintaining the pluripotency of P19 cells**

P19 cells stimulated by retinoic acid (RA) can differentiate into neural ectoderm and endoderm-derived cells, which was used to study the process of neural induction. Firstly, we found that the expression of Pcgf1 was increased with the increase of the neural markers Pou3f1 and Zfp521 and the decrease of the pluripotency markers Oct4 and Nanog (Fig. 5A-C). In order to explore the effect of Pcgf1 on cell pluripotency, we constructed stable P19 cell line that knocked down Pcgf1 or
overexpressed Pcgf1 (Fig. 5D). We observed the morphological changes of P19 cells after Pcgf1 knocked down, and found that compared with the control group, P19 cells clustered earlier at 24 h, suggesting that P19 cells entered the neural induction stage in advance (Fig. 5E). Then we examined the effect of Pcgf1 on mRNA and protein levels of cell pluripotent genes, qPCR and western blot analyses showed that the neural markers Pax6, Pou3f1 and Zfp521 increased at 24 h and 48 h, while the pluripotency markers Oct4, Hes1 and Nanog decreased after knocked down of Pcgf1 (Fig. 5F and 5G). Overexpression of Pcgf1 resulted in the decrease of the neural marker Pax6 at 24 h and 48 h, and the increase of pluripotency marker Nanog at 48 h (Fig. 5H). Western blot showed that pluripotency marker Oct4 was consistently expressed (Fig. 5I). It is suggested that Pcgf1 may maintain the pluripotency of cells.

**Pcgf1 regulated neural induction through an epigenetic mechanism**

To understand the underlying mechanism by which Pcgf1 affects neural induction and monitors the dynamic changes of gene expression during neural induction phase upon Pcgf1 loss-of-function, we performed RNA-seq analysis on wild type and Pcgf1<sup>−/−</sup> P19 cells. RNA-seq analysis identified 1745 genes with > 2-fold altered expression levels, with 1502 genes were upregulated while only 243 genes were downregulated in the absence of Pcgf1 (Fig. 6A). Heat map revealed the genes with > 2-fold expression differences in wild type and Pcgf1<sup>−/−</sup> P19 cells (Fig. 6B). Next, we used gene ontology (GO) analysis to identify the functions of the significantly downregulated genes. These genes were enriched in many functional categories which conformed to the development phenotype we observed, like the development of mesoderm, embryo and nervous system (Fig. 6C). GO analysis also revealed that the downregulated genes were mainly involved in neural induction phase related signaling pathways, like the BMP and Wnt signaling pathways (Fig. 6D). Figure 6E showed that markers related to the maintenance of pluripotency (Nanog and Pou5f1) were significantly downregulated, suggesting that the pluripotency is reduced and cells entered the neural induction stage in advance after Pcgf1 knocked down. Meanwhile, the expression levels of histone demethylases (Kdm5a, Kdm5d and Kdm7a) were upregulated significantly, which indicated that Pcgf1 may regulate neural induction
through an epigenetic mechanism in addition to regulating the signaling pathways.

**Knockdown of Pcgf1 affected H3K27me3 and H3K4me3 distributions during neural induction**

In zebrafish, the regulation of neural induction process involves the coordination of multiple signaling pathways, such as Wnt and BMP. GO analysis showed that these two signaling pathways could be affected by Pcgf1. Our results showed that the activity of the BMP signaling pathway (Smad1-Smad4-Smad5) was decreased, especially Smad4, but the activity of key molecules (wnt3a, wnt8a, β-catenin) in the Wnt signaling pathway did not change observably after knocking down Pcgf1 (Fig. 7A). However, injection with Smad4 mRNA could not rescue the most obvious phenotype caused by Pcgf1 knockdown, such as telencephalic loss (Fig. 7B). These results suggest that there may be another mechanism by which Pcgf1 regulates neural induction process.

PcG genes are identified as essential in epigenetic developmental processes. Epigenetic modification of chromatin structure results in the activation or silencing of specific genes, which has been proved to be an important molecular mechanism in development and disease. In our study, we found that the expression levels of H3K4me3 and H3K27me3 were both decreased after Pcgf1 knocked down (Fig. 7C). ChIP-qPCR results further showed that the levels of H3K27me3 at the promoters of Ngn1 and Otx2, and the levels of H3K4me3 at the promoters of Pou5f3 and Nanog were significantly decreased after injection with the Pcgf1 MO (Fig. 7D). Overall, the above results suggested that Pcgf1 may be involved in the regulation of neural development through histone modification, including transcriptional repression and transcriptional activation mechanisms: Pcgf1 may inhibit the expression of genes related to neurodevelopment through H3K27me3, and promote the expression of pluripotent genes through H3K4me3.

**Discussion**

Neural induction is defined as the process when ectodermal cells become specified as neural stem or precursor cells, and plays essential roles in early neural tube development. Studies uncovering the molecular regulation of neural induction are the key to reveal the pathogenesis of the abnormal neural tube development. Here, we showed that Pcgf1 is maternally expressed during zebrafish
embryo development. However, the biological function of Pcgf1 in the early neural tube development is still unclear. To study the effect of Pcgf1 on the early neural tube development, we constructed Pcgf1 knockdown zebrafish as an animal model. We found that loss of Pcgf1 resulted in telencephalic malformations in zebrafish embryos (small head, reduced or even disappeared telencephalon and shortening of body axis). Furthermore, the neural induction process was abnormal, which led to the decrease of NSCs proliferation in the zebrafish embryo with knocked down Pcgf1. Finally, our results showed that Pcgf1 regulated the levels of H3K27me3 in the Ngn1 and Otx2 promoter regions, and the levels of H3K4me3 at the promoters of Pou5f3 and Nanog, which explained the role of Pcgf1 in neural induction from the perspective of epigenetics.

The PRC1 complex functions as a transcriptional repressor in stem cell biology and development. It is divided into six groups based on the critical factors Pcgf genes. Although it had been reported that Pcgf1-6 play important roles in the self-renewal, proliferation and differentiation of ESCs\textsuperscript{16, 18, 21, 19}, the biological function of each Pcgf family member especially in early neural tube development is still unclear. Here, we first analyzed the expression of Pcgf family members at different stages of early embryonic development in zebrafish. Our results showed that Pcgf1 is expressed in all stages of zebrafish development, while the expression of other members has decreased to varying degrees in early neural induction stage, which suggested the specificity of Pcgf1 in early neurodevelopment. Meanwhile, we further analyzed the characteristics of Pcgf1 expression regions, and found that Pcgf1 was maternally expressed and concentrated in the nervous system during zebrafish embryonic development, which was consistent with the high expression of Pcgf1 in the nervous system of mouse embryos, especially in the neural tube\textsuperscript{31}. However, the biological function of Pcgf1 \textit{in vivo} is still unclear. To investigate the function of Pcgf1, we inhibited the expression of Pcgf1 in zebrafish embryos using MO oligonucleotides. We found that after the injection of Pcgf1 MO, embryos exhibited obvious morphogenesis defects with small head, reduced or even disappeared telencephalon and shortening of body axis by 24 hpf. Although previous study reported that Pcgf1 knockout leads to retarded growth and craniofacial development in zebrafish, which proves that Pcgf1 plays an
important role in the early developmental stages, it did not pay attention to the role and mechanism of Pcgf1 in early neural development\textsuperscript{24}. Therefore, this is the first time to find the role of Pcgf1 in early neurodevelopment \textit{in vivo}, which will be a breakthrough point to study the role of Pcgf1 in early neurodevelopment.

The neural induction process and NSCs self-renewal are two most important processes of neural tube development. However, most of the studies on Pcgf1 are carried out in vitro, and the role of Pcgf1 in neural development \textit{in vivo} has not been fully revealed. In the neural differentiation process of RA induced P19 cells, Pcgf1 can positively regulate the Oct4-Nanog-Sox2 axis, thus maintaining the pluripotency of P19 cells\textsuperscript{23}. In embryonic stem cells (ESCs), the absence of Pcgf1 did not affect the self-renewal of ESCs, but significantly affected the differentiation ability of ESCs, which results in the inhibition of ESCs differentiation into mesoderm and ectoderm. RNA-seq and GO analysis showed that Pcgf1 may activate genes related to mesoderm differentiation\textsuperscript{15}. In our study, we used zebrafish model to detect changes of a series of neural markers, such as Sox2, Otx2 and Ngn1, in neural induction process, and found that the expression of these markers was activated abnormally in Pcgf1 MO group. Meanwhile, the BrdU-positive cells were decreased, the expression of PCNA was reduced, and the expression of the CDK inhibitors p21 and p57 were increased during NSCs self-renewal stage after injection of the Pcgf1 MO. These results indicated that the inhibition of Pcgf1 expression during early embryonic development will affect the neural induction process, lead to abnormal activation of genes related to neural development, and then weaken the self-renewal and proliferation of NSCs, and finally cause the damage of neural tube development. It is suggested that Pcgf1 plays an important role in neural tube development and telencephalon formation, which may affect both the neural induction stage and the self-renewal of NSCs. The discovery is a breakthrough point in the study of the function of such genes in vivo.

P19 cells are derived from an embryo-derived teratocarcinoma, and these cells can differentiate into each of the three germ layers\textsuperscript{32,33}. RA induced P19 cells have been used to study the differentiation of the three germ layers\textsuperscript{34}. In our study, we used this model to study the effect and mechanism of
Pcgf1 on neural induction process. Our RNA-seq analysis showed that 1502 target genes were upregulated in Pcgf1\(^{-/-}\) P19 cells compared to wild type, whereas only 243 target genes were downregulated in all 1745 target genes with > 2-fold altered expression levels in Pcgf1\(^{-/-}\). GO analysis revealed that not only the signaling pathways but also the histone demethylase was affected by Pcgf1. Our results indicated that Pcgf1 may regulate neural induction process through dual mechanisms. Moreover, GO analysis revealed that Pcgf1 may maintain the pluripotency of cells through regulating pluripotency maintenance related genes. The results of P19 in vitro further validate those of zebrafish in vivo.

The PcG protein family has always been considered as a class of proteins that inhibit the transcription of target genes at the level of chromatin. Previous study showed that Pcgf1 promotes monoubiquitinylation of histone H2A in vivo and in vitro and knockdown of Pcgf1 reduces H2A ubiquitinylation in Hela cells\(^{35}\). In our study, the RNA-seq data showed that the expression levels of histone demethylases (Kdm5a, Kdm5d and Kdm7a) were upregulated after Pcgf1 knocked down. Meanwhile, the global levels of H3K27me3 and H3K4me3 were both decreased. This suggests that Pcgf1-mediated gene expression in RA induced P19 cells might be histone methylation-dependent and we correlated the pre-activation of neural induction in Pcgf1-deficient P19 cells with the reduced abundance of repressive H3K27me3 and active H3K4me3 marks at the neural/pluripotency markers. The results of ChIP-qPCR in vivo further confirmed that the levels of H3K27me3 at the promoters of Ngn1/Otx2, and the levels of H3K4me3 at the promoters of Pou5f3/Nanog were decreased after injected Pcgf1 MO to the embryos. There are a variety of evidences supporting the role for PcG in transcriptional activation\(^{16,36}\). Pcgf5-PRC1-AUTS2 complex is involved in gene activation by transcriptional co-activator P300\(^{37}\). Our data suggest that Pcgf1 may function as an activator and a repressor on different genes at the same time. Pcgf1 may be involved in the regulation of neural development by inhibiting the expression of neurodevelopmental-related genes through H3K27me3, and promoting the expression of pluripotent genes through H3K4me3.

Conclusions
Taken together, we first discovered the crucial role of Pcgf1 in early neural tube development especially in neural induction phase in the embryo, and established the regulatory network of transcription factors and epigenetic factors to neural induction, which laid a certain foundation for further exploring the function of other members during early neural tube development. From the perspective of disease treatment, this study provides a good basis for further revealing the role of the Pcgf family in abnormal development of neural tube and provides new ideas and new clues for the clinical treatment of abnormal neural tube closure.

Abbreviations
PRC1: Polycomb repressive complex 1; NSCs: Neural stem cells; MO: Morpholino; CDK: Cyclin-dependent kinase; RA: retinoic acid.

Declarations

Ethics approval
All animal experiments are approved by the National Institutes of Health Guide for the Care and Use of Laboratory, and the Institutional Animal Care and Use Committees of Shandong University.

Consent for publication
Not applicable

Availability of data and materials
The datasets generated and/or analysed during the current study are included within the article and are available from the corresponding authors on reasonable request.

Competing Interests
The authors declare that they have no competing interests.

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Authors’ contributions
XinYue Li performed most of the experiments and interpreted data; GuangYu Ji and Juan Zhou were
involved in the \textit{in vivo} experiments and assembly of data; Jingyi Du and Xian Li contributed to the cell
culture experiments and data analysis. WenJuan Zhou interpreted the data and co-wrote the
manuscript with Aijun Hao, and Aijun Hao provided critical input to the overall research design and
final approval of the manuscript.

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Figures

Figure 1

Expression of Pcgf1 during zebrafish embryonic development. (A and B) qPCR and RT-PCR was used to analyze the expression level of the Pcgf family genes during zebrafish developmental stages, ranging from 1 cell to 24 hpf. Pcgf1 is maternally expressed, increased at sphere phase and continued to 24 hpf. (C) The expression of Pcgf1 in zebrafish was determined by whole-mount in situ hybridization at the indicated stages (from 8 cells to 24 hpf).
Figure 2

A

![Western blot images](image)

|    | Ctrl MO | Pcgf1 MO |
|----|---------|----------|
| Pcgf1 | ![Image](image) | ![Image](image) |
| Actin | ![Image](image) | ![Image](image) |

![Bar graph](image)

**Statistical significance:**

B

|    | Ctrl MO | Pcgf1 MO |
|----|---------|----------|
| ![Image](image) | ![Image](image) |
| ![Image](image) | ![Image](image) |

![Graph](image)

**Statistical significance:**

C

|    | Ctrl MO | Pcgf1 MO |
|----|---------|----------|
| ![Image](image) | ![Image](image) |
| ![Image](image) | ![Image](image) |
| ![Image](image) | ![Image](image) |

![Graph](image)

**Statistical significance:**

21
Zebrafish embryos displayed telencephalic malformations after knockdown of Pcgf1. (A) Western blot confirmed that Pcgf1 MO could interfere Pcgf1 mRNA translation. (B) Compared with the embryos in the control group, the development of neural tube in the experimental group (MO) had obvious defects with small head and shorter body axis. Bar graphs show the statistical data for the embryo numbers. (C) The zebrafish embryos developed to 16 hpf and 24 hpf, and the Pcgf1 MO group showed that the area of the telencephalon decreased or even disappeared compared with control group.

Effect of Pcgf1 on neural induction phase. (A) The expression levels of Sox2, Otx2, Ngn1 and Sox3 were detected by whole-mount in situ hybridization at neural induction phase (shield phase, 75% epiboly phase and 10 hpf). (B) The expression of Sox2, Otx2, Ngn1 and Sox3 were analyzed by qPCR. Data represent the mean of at least three independent experiments ± SD, *P < 0.05 versus control.
Figure 4

Effect of Pcgf1 on NSCs in neural tubes. (A) The expression levels of Sox2, Otx2, Ngn1 and Sox3 were detected by whole-mount in situ hybridization at self-renewal phase of NSCs (16 hpf and 24 hpf). (B) The expression of Sox2, Otx2, Ngn1 and Sox3 were analyzed by qPCR. Data represent the mean of at least three independent experiments ± SD, *P < 0.05 versus control. (C) Acridine orange staining was used to detect apoptosis at 16 hpf and 24 hpf; BrdU labelling of control MO- and Pcgf1 MO-injected embryos at 16 hpf and 24 hpf; The expression of PCNA was detected by whole-mount in situ hybridization. (D) The expression of p21 and p57 were analyzed by qPCR. Data represent the mean of at least three independent experiments ± SD. *P < 0.05 versus control.
Pcgf1 had a positive role in maintaining the pluripotency of P19 cells. (A) qPCR and western blot showed that the expression of Pcgf1 increased in P19 cells induced by retinoic acid, especially 24 h later. (B and C) The expression of neural markers Pou3f1 and Zfp521 increased while pluripotency markers Oct4, Nanog expression decreased. (D) Construction of stable P19 cell line with Pcgf1 knockdown by lentivirus. (E) P19 cells clustered earlier than the control group after Pcgf1 knocked down at 24 h. Scale bar, 100 µm. (F and G) Both of the qPCR and western blot results showed that, the neural markers Pax6, Pou3f1 and Zfp521 increased at 24 h and 48 h, while the pluripotency markers Oct4, Hes1 and Nanog decreased after knocked down Pcgf1. (H) The qPCR results showed that the neural marker Pax6 decreased at 24 h and 48 h, while the pluripotency marker Nanog increased at 48 h after overexpressed Pcgf1. (I) The western blot results showed that pluripotency marker Oct4 was consistently expressed after overexpressed Pcgf1. Data represent the mean of at least three independent experiments ± SD. *P < 0.05 versus control, **P < 0.01 versus control, ***P < 0.001 versus control.
Pcgf1 regulated neural induction through an epigenetic mechanism in addition to signaling pathways. (A) Histogram and volcano plots represented differentially expressed genes in wild type and Pcgf1−/− P19 cells. Red colour indicated upregulated genes and blue colour indicated downregulated genes if they had a log2 fold change of > 1 or less than −1, respectively. The differentially expressed genes number were indicated at right. (B) A heat map of genes with > 2-fold expression differences in wild type and Pcgf1−/− P19 cells. Red indicated high expression and blue indicated low expression. (C) GO analysis of biological functions of deregulated genes in Pcgf1−/− P19 cells. (D) GO analysis of signaling pathways of deregulated genes in Pcgf1−/− P19 cells. (E) Fold changes in the expression levels of pluripotency maintenance and histone demethylase genes.
Figure 7

Pcgf1 regulated neural induction through histone methylation. (A) qPCR analysis of the expression levels of BMP signaling pathway (Smad1, Smad4, Smad5) and Wnt signaling pathway (wnt3a, wnt8a, β-catenin). Compared with the controls, the mRNA levels of Smad1, Smad4 and Smad5 in Pcgf1 MO-injected embryos decreased, but the levels of wnt3a, wnt8a and β-catenin did not change observably. Data represent the mean of at least three independent experiments ± SD. *P < 0.05 versus control. (B) Compared with embryos injected with Pcgf1 MO, the shrinking of the telencephalon has not been rescued by the addition of Smad4 mRNA. (C) Western blot showed that the expression of H3K4me3 and H3K27me3 decreased after Pcgf1 knocked down. (D) WT and Pcgf1 MO-injected group were immunoprecipitated with anti-H3K27me3, anti-H3K4me3 and IgG. The isolated DNA was analysed by gene-specific ChIP primers. The levels of H3K27me3 at the promoters of Ngn1 and Otx2, and the levels of H3K4me3 at the promoters of Pou5f3 and Nanog were significantly decreased after injection with the Pcgf1 MO. Data represent the mean of at least three independent experiments ± SD. *P < 0.05 versus control.
