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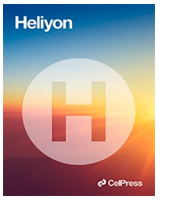

Research article

Pcgf5: An important regulatory factor in early embryonic neural induction

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A R T I C L E I N F O

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A B S T R A C T

Polycomb group RING finger (PCGF) proteins, a crucial subunits of the Polycomb complex, plays an important role in regulating gene expression, embryonic development, and cell fate determination. In our research, we investigated Pcgf5, one of the six PCGF homologs, and its impact on the differentiation of P19 cells into neural stem cells. Our findings revealed that knockdown of Pcgf5 resulted in a significant decrease in the expression levels of the neuronal markers Sox2, Zfp521, and Pax6, while the expression levels of the pluripotent markers Oct4 and Nanog increased. Conversely, Pcgf5 overexpression upregulated the expression of Sox2 and Pax6, while downregulating the expression of Oct4 and Nanog. Additionally, our analysis revealed that Pcgf5 suppresses Wnt3 expression via the activation of Notch1/Hes1, and ultimately governs the differentiation fate of neural stem cells. To further validate our findings, we conducted *in vivo* experiments in zebrafish. We found that knockdown of pcgf5a using morpholino resulted in the downregulated expression of neurodevelopmental genes such as sox2, sox3, and foxg1 in zebrafish embryos. Consequently, these changes led to neurodevelopmental defects. In conclusion, our study highlights the important role of Pcgf5 in neural induction and the determination of neural cell fate.

1. Introduction

Embryonic neural induction is a pivotal stage in the formation of the entire nervous system. This process involves the transformation of primitive ectodermal cells through the action of distinct signalling and regulatory factors, resulting in their differentiation into neuroectodermal cells [1–5]. As a result, neural stem cells establish the neuroepithelium of the neural plate and neural tube, giving rise to diverse cell types. Through subsequent differentiation processes, various types of neurons and glial cells develop, ultimately shaping the distinctive regions of the nervous system [6,7]. The process of neural induction encompasses a sophisticated network of

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molecular regulatory mechanisms that play vital roles in the establishment of the nervous system, including the formation of neural tissue structures and the determination of neural cell fates. Disruptions in this intricate process can result in neurodevelopmental abnormalities and a range of neurological disorders, significantly impacting the well-being and survival prospects of the developing foetus [8,9]. Hence, elucidating the molecular regulatory mechanisms underlying neural induction holds great potential for gaining profound insights into the pathogenesis of neurological diseases. Moreover, such studies can serve as a foundation for the diagnosis and treatment of various neurological conditions, thereby enhancing the quality of care in the field of neurology.

Polycomb group proteins (PcGs) constitute a crucial group of proteins involved in the regulation of gene expression. Initially

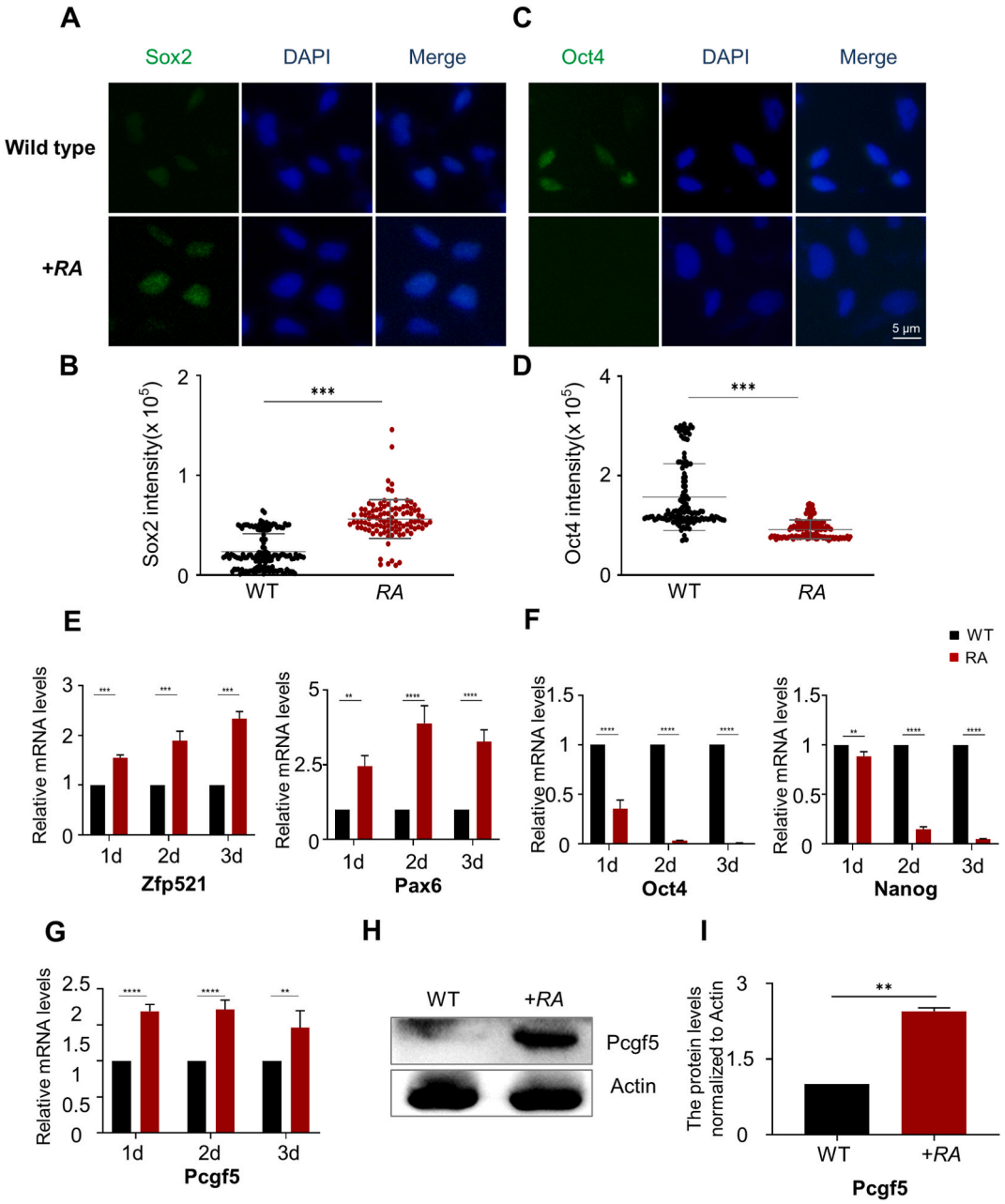

Fig. 1. Pcgf5 was upregulated during RA-induced neural induction in P19 cells.
(**A,C**) Representative images show immunofluorescence of Sox2 and Oct4 from P19 cells with DMSO (Wild Type) or 0.5 μM RA (RA) induction. Scale bar, 5 μm. (**B,D**) Quantification of (A) and (C). More than 100 cells were counted in each experiment. (**E,F,G**) qPCR was used to analyse the expression of Zfp521, Pax6 and Oct4, Nanog and Pcgf5 during RA induction. (**H,I**) Western Blot was used to detect the level of Pcgf5 during RA induction. Full-length gels before cropping are noted in Fig. S4. Data are presented as means ± SEM of at least three independent experiments. (E-G, I), ** ($p < 0.01$),*** ($p < 0.001$), **** ($p < 0.0001$), ns: no significance.

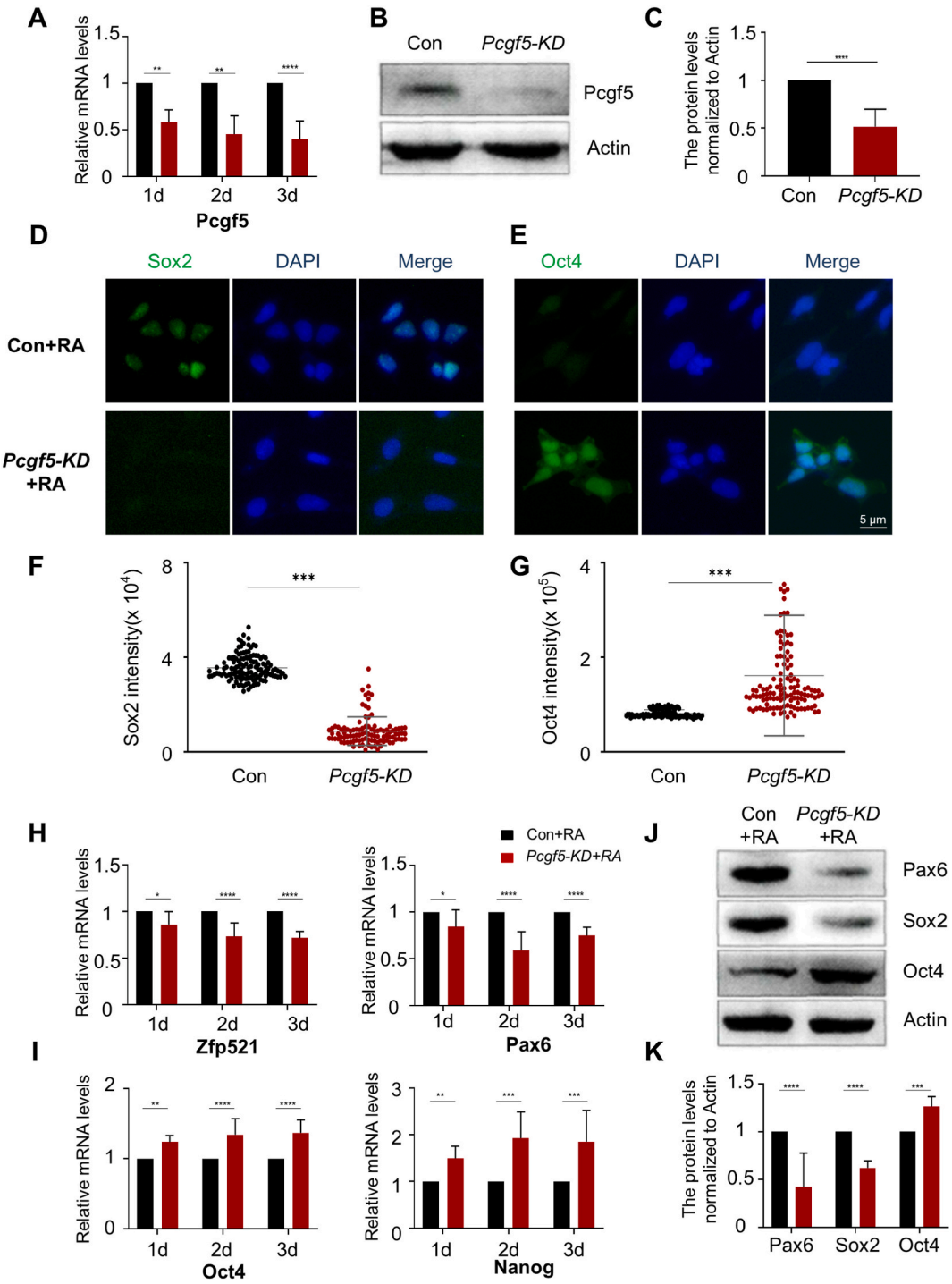

Fig. 2. Pcgf5 is involved in the regulation of RA-induced neural induction in P19 cells.
(**A-C**) qPCR and Western Blot was used to analyse the interference efficiency of Pcgf5. (**D,E**) Representative images show immunofluorescence of Sox2 and Oct4 from Con and Pcgf5-KD after 0.5 μM RA induction. Scale bar, 5 μm. (**F,G**) Quantification of (D) and (E). More than 100 cells were counted in each experiment. (**H,I**) Following the knockdown of Pcgf5, qPCR was utilized to evaluate the expression of Zfp521, Pax6 and Oct4, Nanog. (**J,K**) Western Blot was used to detect the expression of Pax6, Sox2 and Oct4 after Pcgf5 knockdown. Full-length gels before cropping are noted in Fig. S5. Data are presented as means ± SEM of at least three independent experiments (A,C,H–I,K), * ($p < 0.05$), ** ($p < 0.01$),*** ($p < 0.001$), **** ($p < 0.0001$).

discovered in Drosophila, these proteins primarily exert control over cell development and differentiation by repressing the transcription of specific genes [10,11]. PcG proteins typically organized into complexes, the most important of which are polycomb repressive complexes (PRCs), which are composed of two main isoforms known as PRC1 and PRC2 [12]. PRC2 can facilitate the addition of a methyl group to histone H3 at position K27 (H3K27me3) [13,14]. PRC1 is divided into classical PRC1 and nonclassical PRC1 [15,16]. Classical PRC1 consists of Cbx, Pcgf, Ring, and Phc proteins, while nonclassical PRC1 incorporates Rybp/Yaf2 in place of Cbx [16]. Within the complex, the Ring1a/b proteins, which act as E3 ubiquitin ligases, bind to the Pcgf protein and catalyses the addition of a ubiquitin molecule to histone H2A at position K119 (H2AK119ub1). This ubiquitination process represents a vital mechanism employed by PcG proteins to silence gene expression [16–18]. PRC1 plays a critical role in determining the fate of embryonic stem cells and regulates the trajectory of embryonic stem cell differentiation [19–21]. The PCGF proteins serve as a core component of PRC1, and consists of six members (Pcgf1- Pcgf6) in both humans and mice. These members play diverse roles in regulating biological development [12,22]. While PRC1 is typically associated with gene inhibition, Pcgf3/5, in particular, act as a transcriptional activators and primarily participate in regulating the differentiation of the three germ layers [23]. In mouse embryonic stem cells (mESCs), Pcgf3/5 collaborate with Tex10 to stimulate the expression of numerous genes involved in mesodermal differentiation [23]. Moreover, Pcgf3/5 contributes to the epigenetic silencing of the X chromosome in male mammalian cells [24]. In addition to its cooperative role with Pcgf3, Pcgf5 also play an important role in negatively regulating the TGF-β pathway during the mESC neural differentiation process by maintaining appropriate levels of H2AK119ub1 and H3K27me3 in key genes of the TGF-β/SMAD2 signalling pathway [14]. However, there is a limited understanding of the timing and underlying mechanisms of Pcgf5 in regulating early vertebrate development.

P19 cells are derived from mouse embryonic germinoma and are an embryonic cancer cell line that can differentiate into each of the three germ layers under specific induction conditions [25,26]. In the absence of specific inducers, P19 cells can maintain an undifferentiated state and possess totipotency as primitive ectodermal cells [27,28]. In our study, we utilized P19 cells as a model to investigate the role of Pcgf5 in neural induction. Our findings revealed that Pcgf5 plays a crucial role in determining the neural fate of P19 cells and facilitating their differentiation into neural stem cells. During this process, Pcgf5 regulates the expression of Notch1 and its downstream gene Hes1, while also promoting glial cell differentiation through its antagonistic interaction with the Wnt3 gene in the Wnt pathway. Through *in vivo* experiments conducted in zebrafish, we observed that knockdown of *pcgf5a* resulted in defective neuroectoderm development, characterized by a reduced brain area and delayed eye development. These results highlight the essential role of *pcgf5a* in the early neurodevelopment of zebrafish.

2. Results

2.1. Pcgf5 was upregulated during RA-induced neural induction in P19 cells

Mouse teratoma cells (P19 cells) can differentiate into neuroectodermal cells and give rise to neural stem cells when exposed to 0.5 μM retinoic acid (RA) induction [29]. In the present study, P19 cells were treated with 0.5 μM RA, and the expression of Sox2, a marker of neural stem cells, was significantly upregulated after RA induction (Fig. 1AB), whereas the expression of the pluripotent marker Oct4 was notably downregulated (Fig. 1CD). Additionally, the qPCR results demonstrated substantial increases in the expression levels of the neural markers Zfp521, Pax6 (Fig. 1E) and Nestin (Fig. S1A) following RA induction. Conversely, the expression of the pluripotent markers Oct4 and Nanog was significantly reduced (Fig. 1F). These findings confirmed the successful establishment of an early neural induction model. The expression of the Pcgf5 gene in cultured P19 cells was assessed through qPCR and Western blot analysis following RA induction. The results revealed that Pcgf5 was significantly upregulated in the RA-treated group compared to the control group (Fig. 1G–I), indicating that Pcgf5 might play a crucial role in the early stages of the neural differentiation process.

2.2. Pcgf5 is involved in the regulation of RA-induced neural induction in P19 cells

In this study, we utilized siRNA to interfere with the Pcgf5 gene expression, effectively generating P19 cells with reduced levels of Pcgf5. These modified cells were then cultured in medium supplemented with 0.5 μM RA for induction. The efficiency of Pcgf5 knockdown was assessed using qPCR and Western blot, which confirmed a reduction of more than 50% in Pcgf5 gene expression (Fig. 2ABC). Suppression of the Pcgf5 gene resulted in a substantial reduction in Sox2 immunofluorescence intensity (Fig. 2DF), accompanied by a notable increase in Oct4 immunofluorescence intensity (Fig. 2EG). These findings suggest that inhibiting the expression of the Pcgf5 gene promotes the retention of an undifferentiated state in P19 cells, impeding the process of neural induction. Furthermore, through qPCR analysis, we observed decreased expression levels of Zfp521 and Pax6 upon Pcgf5 knockdown (Fig. 2H), accompanied by increased expression levels of Oct4 and Nanog (Fig. 2I). These findings were corroborated by the Western blot results, which confirmed the decreases in Pax6 and Sox2 protein levels and the increase in the Oct4 protein expression level upon Pcgf5 inhibition (Fig. 2JK). Collectively, our data strongly indicate that Pcgf5 plays a critical role in the early neural induction stage. Inhibition of Pcgf5 expression maintains cell pluripotency and restrains cell differentiation towards neural stem cells. These findings highlight the importance of Pcgf5 as a key regulator of cell fate determination during neural development.

2.3. Overexpression of Pcgf5 promotes the neural induction of P19 cells

To further validate the experimental findings following Pcgf5 gene knockdown, we generated a Pcgf5-Pultra overexpression plasmid and transfected it into P19 cells. Through qPCR and Western blot analyses, we assessed the efficiency of Pcgf5 overexpression

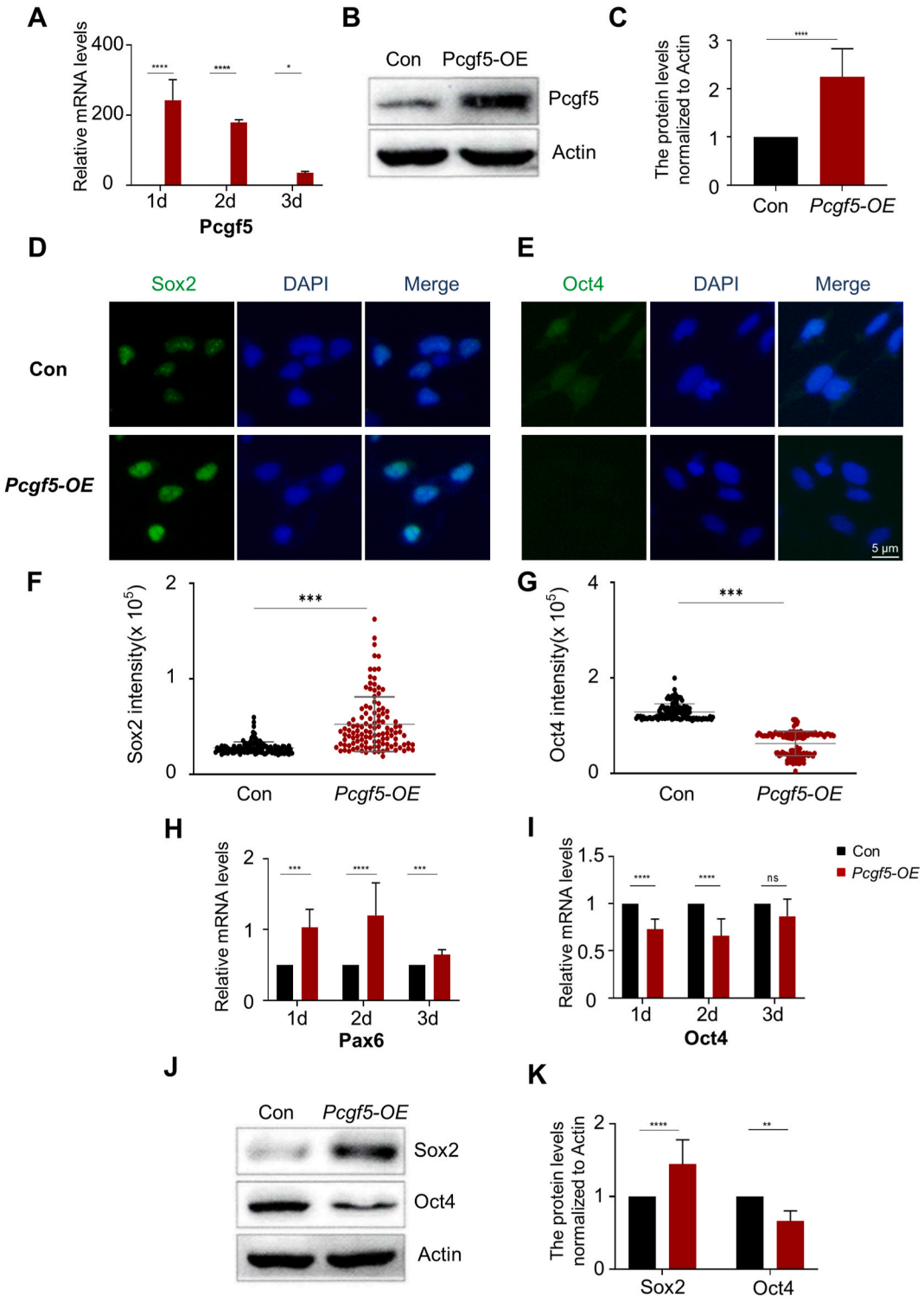

Fig. 3. Overexpression of Pcgf5 gene promotes the neural induction process in P19 cells.
(**A-C**) qPCR and Western Blot was used to analyse the overexpression level of Pcgf5. Full-length gels before cropping are noted in Fig. S6. (**D,E**) Representative images show immunofluorescence of Sox2 and Oct4 from WT and Pcgf5-OE without RA induction. Scale bar, 5 μm. (**F,G**) Quantification of (D) and (E). More than 100 cells were counted in each experiment. (**H,I**) Following the overexpression of Pcgf5, qPCR was utilized to evaluate the expression of Pax6 and Oct4. (**J,K**) Western Blot was used to detect the expression of Sox2 and Oct4 after Pcgf5 overexpression. Full-length gels before cropping are noted in Fig. S6. Data are presented as means ± SEM of at least three independent experiments (A,C,H -I,K), * ($p < 0.05$), ** ($p < 0.01$),*** ($p < 0.001$), **** ($p < 0.0001$), ns: no significance.

and observed significant increases in both the mRNA and protein levels of Pcgf5 (Fig. 3ABC). Immunofluorescence analysis revealed that Pcgf5 overexpression led to a substantial increase in the immunofluorescence intensity of Sox2 (Fig. 3DF), even without RA induction. Conversely, the immunofluorescence intensity of Oct4 decreased significantly (Fig. 3EG). These findings suggest that Pcgf5 overexpression promotes the differentiation of P19 cells into neural stem cells. Additionally, the qPCR results demonstrated significant upregulation of Pax6 expression following Pcgf5 overexpression (Fig. 3H), while the expression of Oct4 significantly decreased (Fig. 3I). Consistent with these findings, Western blot analysis confirmed an increase in Sox2 protein levels and a decrease in Oct4 protein levels upon Pcgf5 overexpression (Fig. 3JK). Collectively, these results indicate that Pcgf5 overexpression facilitates the process of neural differentiation in P19 cells.

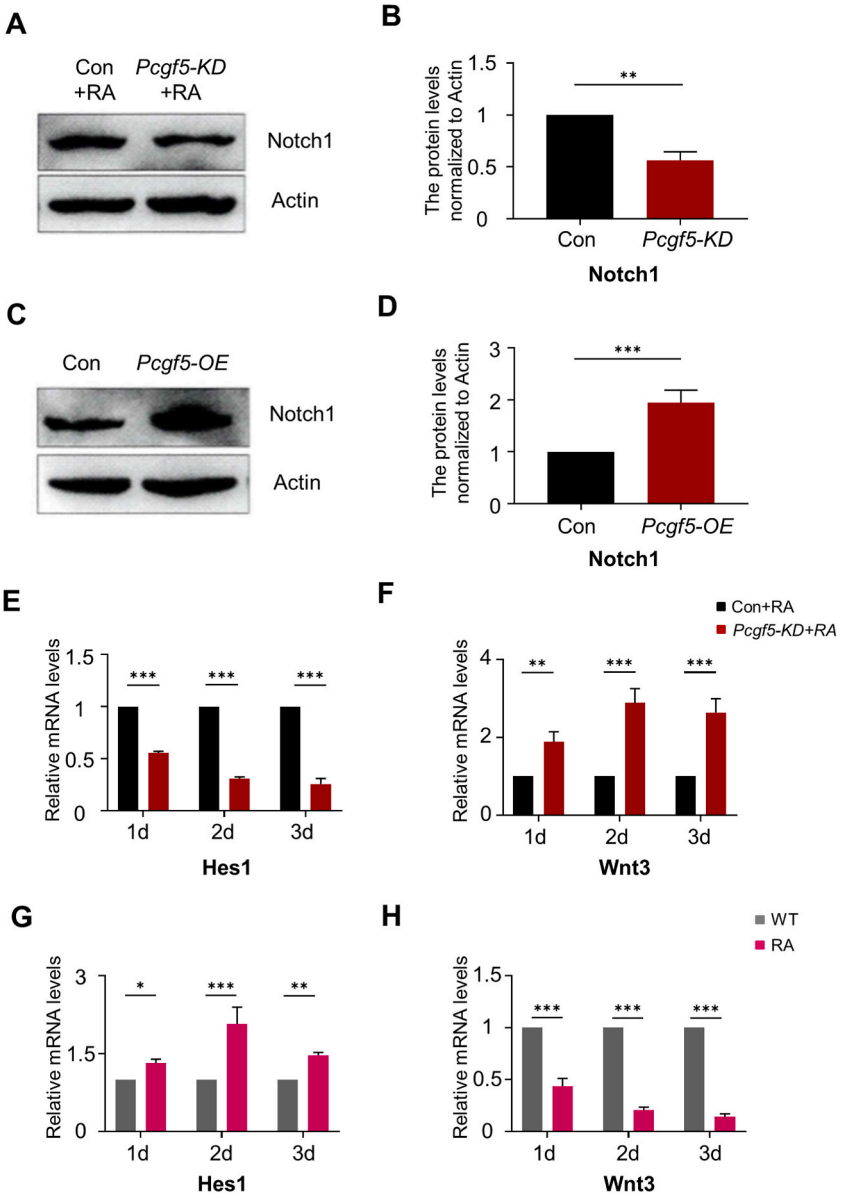

Fig. 4. Pcgf5 participates in the neural induction process by regulating Notch1.
(**A-D**) Western Blot was used to detect the expression of Notch1 after Pcgf5 knockdown or overexpression. Full-length gels before cropping are noted in Fig. S7. (**E,F**) Following the knockdown of Pcgf5, qPCR was utilized to evaluate the expression of Hes1 and Wnt3. (**G,H**) qPCR was used to analyse the expression of Hes1 and Wnt3 during RA induction in P19 cells with DMSO (Wild Type) or 0.5 μM RA (RA) induction. Data are presented as means ± SEM of at least three independent experiments (B,D,E-H), ** ($p < 0.01$),*** ($p < 0.001$).

2.4. Pcgf5 participates in the neural induction process by regulating Notch1

The Notch pathway plays a crucial role in various physiological processes, including cell proliferation, cell fate determination, stem cell property maintenance and cell death, throughout the development of multiple tissues [30,31]. We observed that Pcgf5 knockdown resulted in a significant decrease in the Notch1 protein level (Fig. 4AB), while Pcgf5 gene overexpression led to a significant increase in the Notch1 protein level (Fig. 4CD). These findings suggest that Pcgf5 can activate Notch1 expression during neural induction. Furthermore, qPCR analysis revealed that the transcript level of Hes1, a downstream transcription factor of the Notch1 pathway, significantly decreased following Pcgf5 knockdown (Fig. 4E). Conversely, the transcript level of Wnt3, a gene in the Wnt pathway, significantly increased (Fig. 4F). Notably, in RA-induced P19 neural differentiation, there is a typically increase in the expression of Hes1 (Fig. 4G), and a corresponding decrease in the expression of Wnt3 (Fig. 4H). These results prompted us to investigate the interaction between Notch and the Wnt pathway in neural induction.

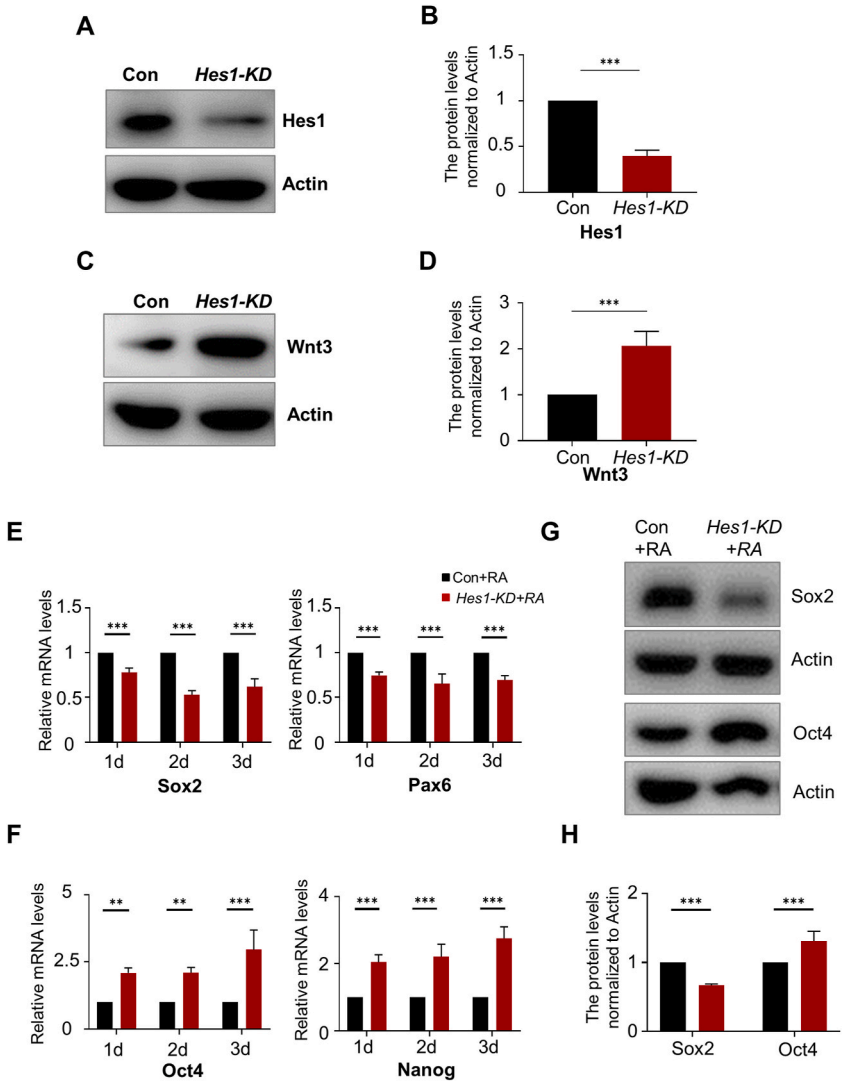

Fig. 5. Hes1 plays a crucial role in neural induction by acting as a suppressor of Wnt3 gene expression.
(**A, B**) Western Blot was used to analyse the interference efficiency of Hes1. Full-length gels before cropping are noted in Fig. S8. (**C,D**) Western Blot was used to detect the expression of Wnt3 after Hes1 knockdown. Full-length gels before cropping are noted in Fig. S8. (**E,F**) Following the knockdown of Hes1, qPCR was utilized to evaluate the expression of Sox2, Pax6 and Oct4, Nanog. (**G,H**) Western Blot was used to detect the expression of Sox2 and Oct4 after Hes1 knockdown. Full-length gels before cropping are noted in Fig. S8. Data are presented as means ± SEM of at least three independent experiments (B,D,E,F,H), ** ($p < 0.01$), *** ($p < 0.001$).

2.5. Hes1 plays a crucial role in neural induction by acting as a suppressor of Wnt3 gene expression

Utilizing siRNA we successfully knocked down the Hes1 gene, achieving a knockdown efficiency of more than 50%, as confirmed by Western blot analysis (Fig. 5AB). Following Hes1 knockdown, there was a significant decrease in the protein expression level of Wnt3 (Fig. 5CD), indicating that Hes1 plays a role in regulating Wnt3 expression. To gain further insight, we examined the expression of neural markers and pluripotency marker genes after Hes1 knockdown through qPCR and Western blot analyses. Consistent with the results observed following Pcgf5 knockdown, we observed a significant reduction in the mRNA levels of Sox2 and Pax6 (Fig. 5E). Conversely, the expression levels of Oct4 and Nanog significantly increased (Fig. 5F). These findings were corroborated at the protein level, where a decrease in the Sox2 protein level and an increase in the Oct4 protein level were observed upon Hes1 knockdown (Fig. 5GH). These findings suggest that Hes1 disrupts the undifferentiated state of P19 cells and fosters the process of neural differentiation by inhibiting the expression of Wnt3.

2.6. Knockdown of pcgf5a expression results in abnormal early embryonic development in zebrafish

We conducted *in vivo* experiments using zebrafish to validate the function of Pcgf5. Within zebrafish, there are two homologs of pcgf5, namely, pcgf5a and pcgf5b. To examine the expression of pcgf5a and pcgf5b during different developmental stages in zebrafish embryos, we employed qPCR. Our findings revealed that the pcgf5a gene exhibited maternal expression during various developmental periods, including the embryonic 1-cell stage, sphere stage, field stage, 75% outgrowth stage, 10 h post-fertilization (hpf), 16 hpf, and 24 hpf (Fig. 6A). Notably, pcgf5a levels displayed an overall increasing trend, with upregulated expression observed after 10 hpf (Fig. 6A). In contrast, the expression of the pcgf5b gene demonstrated a significant decreasing trend (Fig. 6B). These results suggest that pcgf5a, which is similar to the expression of pcgf5 in RA-induced P19 cells, likely plays a crucial role in the early embryonic development of zebrafish. Therefore, we subsequently conducted further investigations into the function of pcgf5a. After inhibiting the expression of the pcgf5a gene through microinjection of pcgf5a MO, we observed abnormal development in zebrafish embryos (Fig. 6CD). Specifically, these abnormalities manifested as defects in neural ectodermal development, including a smaller brain regions and delayed eye development (Fig. 6D). We observed the highest percentage of embryonic developmental defects with the lowest

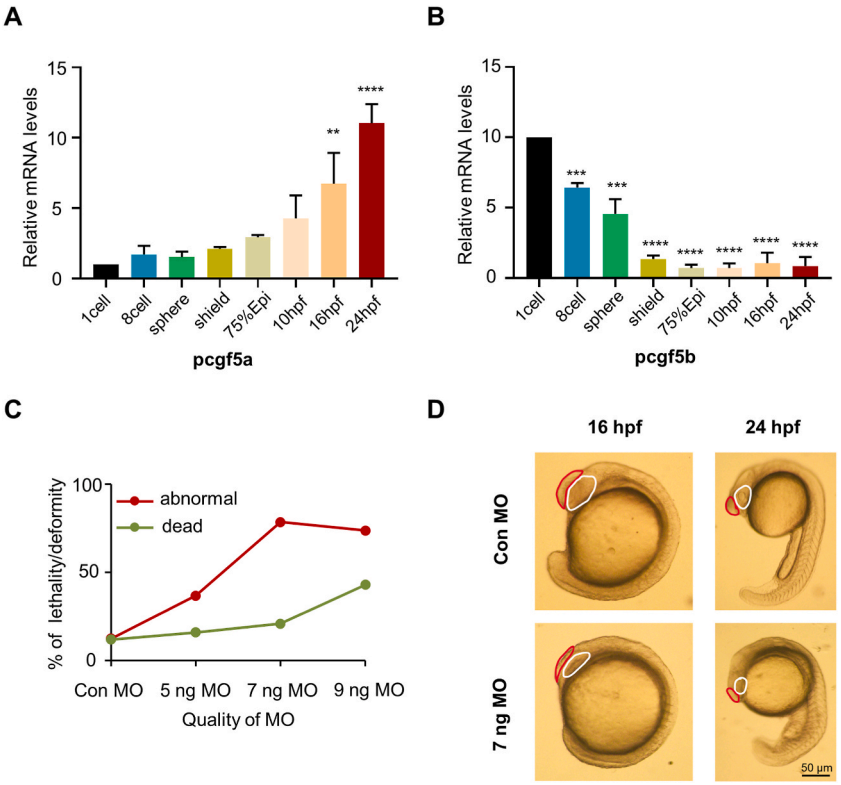

Fig. 6. Knockdown of pcgf5a expression results in abnormal early embryonic development in zebrafish.
(**A-C**) qPCR and Real-time PCR was used to detect the expression of pcgf5a and pcgf5b in zebrafish embryos at different periods, from the 1-cell stage to 24 hpf. (**D**) A dose-dependent statistical graph of the embryo phenotype, and 7 ng MO was selected as the appropriate injection dose. (**E**) The phenotypes of zebrafish embryos taken under light microscope at 16 hpf and 24 hpf stage. Red markers show that the brain size of the pcgf5a-deficient zebrafish embryos is obviously smaller, and white markers show that the eye vesicles of the pcgf5a-deficient zebrafish embryos are smaller and the eye morphology is abnormally developed. Scale bar, 50 μm.

lethality when the MO concentration was 7 ng (Fig. 6C). Consequently, subsequent experiments were carried out using a 7 ng MO injection.

2.7. *pcgf5a is involved in the regulation of early embryonic neurodevelopment in zebrafish*

To investigate the underlying cause of abnormal neurodevelopment in zebrafish embryos resulting from the inhibition of pcgf5a gene expression, we conducted *in situ* hybridization to examine the expression of marker genes associated with the neural stem cells (sox2), the neural ectoderm (sox3), and the telencephalon (foxg1) in zebrafish embryos. The findings revealed notable changes in gene expression patterns at the shield stage, 16 hpf, and 24 hpf. Specifically, in the pcgf5a MO injection group, the expression of sox2, sox3, and foxg1 was significantly lower than that in the control group, and the distribution of these proteins decreased (Fig. 7ABC). These observations indicate that the knockdown of pcgf5a led to reduced expression levels of key genes involved in neural development, consequently hindering the early-stage neural developmental process in zebrafish embryos. As a result, defective development was observed in the zebrafish embryos.

3. Discussion

The transformation of ectodermal cells into neural stem cells or neural precursor cells is termed neural induction and is a pivotal phase in ensuring proper maturation of the nervous system. The exploration of neural induction contributes significantly to our understanding of the mechanisms governing cellular fate determination and is highly useful for determining the origins of conditions linked to the nervous system. Recent reports suggest that Pcgf5, as an important member of the PcG family, is essential for determining the neural fate of embryonic stem cells [14,23,24]. However, the biological function of Pcgf5 in neural induction during the differentiation of the three germ layers and early neural tube development is still unclear. Our investigation showed that, during the neural

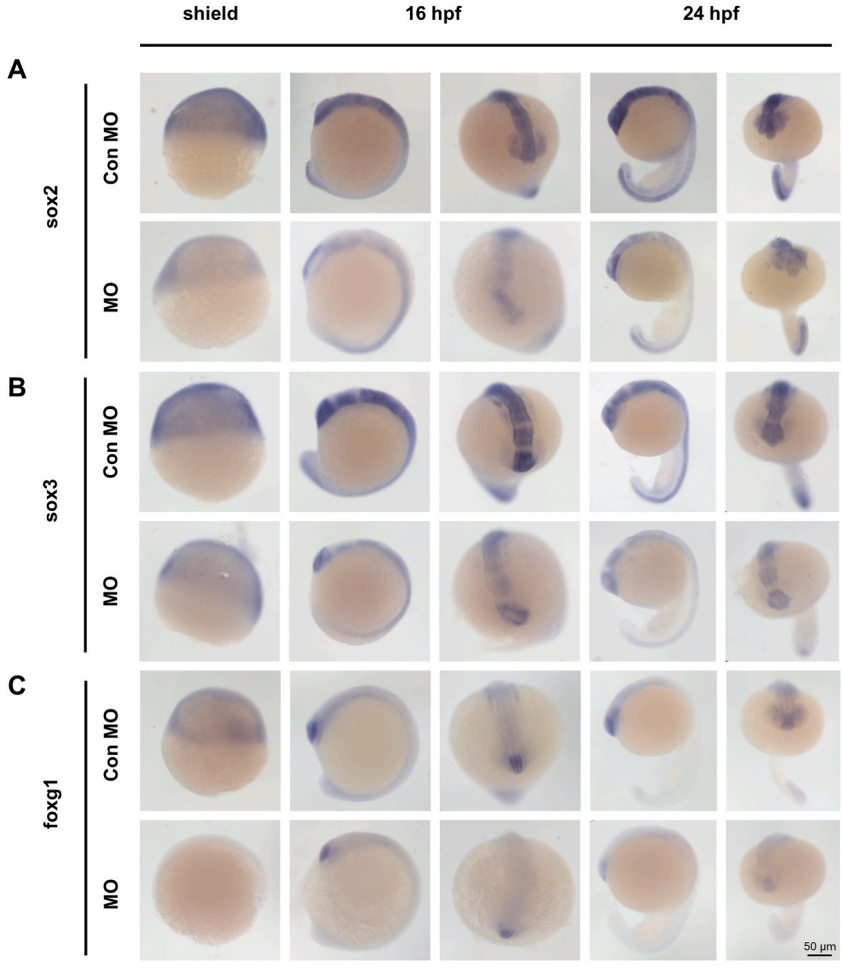

Fig. 7. pcgf5a is involved in the regulation of early embryonic neurodevelopment of zebrafish.
(**A-C**) Images show the expression of sox2, sox3 and foxg1 revealed by *in situ* hybridization. Scale bar, 50 μm.

induction of P19 cells induced by RA, Pcgf5 expression was notably upregulated. Subsequent Pcgf5 knockdown impeded the neural induction process, while conversely, upregulated Pcgf5 expression accelerated the differentiation of P19 cells into neural stem cells. This, in turn, facilitated the neural induction process, suggesting an influential regulatory role of Pcgf5. Further inquiry revealed that Pcgf5 could inhibit Wnt3 expression by activating the Notch1/Hes1 pathway, thereby accelerating the differentiation of P19 cells into neural stem cells. Next, we conducted *in vivo* experiments using zebrafish. The outcomes revealed that knockdown of pcgf5a led to impaired neurodevelopment in zebrafish embryos. Notably, our findings offer the first validation of the involvement of pcgf5a in the early embryonic development of zebrafish.

Pcgf5 has been shown to be prominently expressed in neural precursor cells, and to govern neural differentiation in mouse embryonic stem cells [14]. Our research further highlights the elevated presence of Pcgf5 during the RA-induced differentiation of P19 cells into neural stem cells. Upon Pcgf5 knockdown, the expression of neural stem cell markers such as Sox2 and Pax6 decreased, while those of pluripotency markers such as Oct4 and Nanog increased. These findings suggested that Pcgf5 may promote the differentiation of P19 cells by repressing pluripotency marker genes and activating neural stem cell-related genes. Consequently, Pcgf5 emerges as a pivotal contributor to both neural induction and subsequent differentiation. In contrast, investigations involving Pcgf1 reveal its inhibitory impact on neural induction. Suppression of Pcgf1 results in anomalous neural induction activation and developmental irregularities [32]. Evidently, Pcgf1 and Pcgf5 exert completely divergent, if not opposing, functions. Additionally, Pcgf4 has been observed to facilitate neural stem cell self-renewal, thereby maintaining neural stem cell populations [33,34]. On a parallel note, Pcgf6 exerts positive control over pluripotency gene expression, thereby upholding stem cell pluripotency [35,36]. These findings collectively suggest that members of the PCGF protein family collaboratively regulate neurogenic processes, each exerting distinct functional roles.

Neurodevelopmental processes are regulated by multiple factors that interact with various signaling pathways, including Notch, Wnt, Hedgehog, and TGF-β/BMP signaling pathways [37]. Pcgf5 is also involved in the regulation of multiple signaling pathways, such as the Notch and Wnt pathways [38]. It has been found that the loss of the Pcgf5 gene inhibits the expression of Notch1, thereby suppressing neural ectoderm differentiation [38], which is consistent with our findings. However, it is unclear whether there is an interaction and co-regulation between the Notch and Wnt signaling pathways in the process of neural development.

In this study, upon knocking down Pcgf5, we observed a decrease in the expression of Notch1. Conversely, when Pcgf5 was overexpressed, Notch1 expression was significantly upregulated. These findings strongly suggest that Pcgf5 plays a crucial role in activating Notch1 expression during neural induction. Furthermore, as a downstream target of Notch1, the Hes1 gene also displayed decreased expression levels upon Pcgf5 knockdown, indicating that Pcgf5 regulates the Notch1 pathway. In the developing nervous system, the Notch1 signalling pathway is traditionally believed to impede neural differentiation [39,40]. However, numerous studies have shown that activated Notch1 signalling actually promotes the differentiation of neuroepithelial cells into neural stem cells [41,42], which is consistent with our results.

We also noted a significant increase in Wnt3 gene expression after Pcgf5 knockdown. Recent studies have demonstrated genetic interactions between the Notch and Wnt pathways, both synergistic or antagonistic, in various cell fate determination events [43–45]. Our results imply a possible antagonistic role of the Notch and Wnt pathways during neural induction. Additional experimental findings revealed that upon knockdown of Hes1, the expression of Wnt3 significantly increased. Moreover, the expression levels of neural stem cell markers, including Sox2 and Pax6, showed a decreasing trend. Conversely, the expression of pluripotency markers such as Oct4 and Nanog increased. Strongly indicate that Hes1 acts as an inhibitory factor against Wnt3 expression, thereby promoting the generation of neural stem cells. These results imply that the suppression of Hes1 removed the inhibitory controls on the Wnt3 gene, leading to increased Wnt3 transcript levels. Such activation tends to preserve stem cell traits, ensuring that P19 cells maintain their pluripotency and do not follow the typical neural induction path. Hence, we infer that Pcgf5 may inhibit Wnt3 expression by stimulating the expression of Notch1 and its downstream gene Hes1. This process possibly allows the cells to acquire the capacity for neural induction.

The above results indicate that Pcgf5 can promote the neural induction process by upregulating the expression of Notch1/Hes1 and suppressing the expression of Wnt3. However, the specific mechanism by which Pcgf5 regulates Notch1 expression remains unclear. According to the literature, Pcgf5 has the ability to directly bind to the promoter region of target genes and regulate their expression by modulating epigenetic modifications, including H2AK119ub1 and H3K27me3 [14]. We conducted an analysis of H2AK119ub1, H3K27me3, H3K18ac, and H3K4me3 levels at the cellular level, but no significant changes were observed in H2AK119ub1 and H3K27me3 levels (Fig. S2CD). However, when Pcgf5 was knocked down, it resulted in a decrease in intracellular H3K18ac level and an increase in H3K4me3 level (Fig. S2AB). Interestingly, both H3K18ac and H3K4me3 are associated with activating gene modifications [46], but they exhibited opposing trends in this case. Based on these findings, we have put forward a reasonable hypothesis suggesting that Pcgf5 may play a role in maintaining H3K18ac levels of genes involved in neural induction by activating their transcription, while simultaneously inhibiting the H3K4me3 levels of genes responsible for maintaining cell pluripotency. Currently, we are unable to provide a precise explanation for this issue as we have encountered some difficulties during the Chip experiment. In the next phase of our research, we plan to investigate the enrichment of Pcgf5 at the Notch1/Hes1 promoter and analyse the levels of histone modifications, such as H2AK119ub1, H3K27me3, H3K18ac, and H3K4me3. This will enable us to gain a deeper understanding of the mechanism by which Pcgf5 participates in the neural induction process through the regulation of the Notch pathway.

Using the P19 cell model, we discovered the crucial involvement of Pcgf5 in cellular neural induction through its regulation of the Notch1 pathway. The role of Pcgf5 *in vivo* has not been explored thus far. Zebrafish, a well-established model organism, possesses distinctive features, including a well-defined genetic background, the ability to fertilize *in vitro*, and transparent embryos. These characteristics enable researchers to easily observe and study the developmental process of embryos while conducting various experimental manipulations [47–49]. Therefore, we conducted experiments using zebrafish to validate the role of the Pcgf5 gene *in*

vivo. Our results indicated a gradual increase in pcgf5a gene expression during zebrafish embryonic development. Remarkably, a study has shown that the spatial expression pattern of the pcgf5a gene became progressively specific to the nervous system from its initial widespread expression [50], strongly suggesting that pcgf5a plays a pivotal role in the early embryonic neural development of zebrafish, which aligns with our cell modelling results. Inhibiting pcgf5a expression led to developmental defects in zebrafish embryos, which primarily manifested as smaller brain regions and delayed eye development. Furthermore, through whole-embryo *in situ* hybridization, we observed decreased expression of the neural stem cell markers sox2 and sox3, as well as the telencephalic marker foxg1. These findings indicate impaired zebrafish neural induction and neurogenesis following pcgf5a knockdown. Overall, our *in vivo* experiments provide compelling evidence for the important role of the pcgf5a gene in neural induction during early developmental stages.

In summary, our findings highlight the capacity of Pcgf5 to enhance early embryonic neural induction, and Pcgf5 may be involved in the regulation of early neural development by inhibiting the Wnt pathway through the activation of the Notch1/Hes1 pathway (Fig. S8). However, the specific mechanism by which Pcgf5 activates the Notch1 pathway requires further investigation.

4. Limitations of the study

We show Pcgf5 plays a crucial role in neural induction and the determination of neural cell fate. There are two limitations to our study. (1) If we conduct our research using mESCs at the same time, our results will be more comprehensive and persuasive. (2) The mechanism by which Pcgf5 regulates Notch1 expression has not been thoroughly investigated.

5. Method details

5.1. Cell culture and transfection

Cells were cultured in RPMI Medium 1640 (Gibco, 12633012) with 10% FBS (Gibco, 26140079) and 0.2% penicillin-streptomycin (BasalMedia, S110JV). When RA was used to induce cell differentiation, the medium was changed to medium containing 0.5 μM RA (Sigma, R2625). DNA plasmids were transfected with Lipofectamine 3000 (Invitrogen, L3000015) according to the Manufacturer's instructions.

5.2. Zebrafish breeding

Zebrafish were kept in thermostatically circulating water at 28.5 °C, fed twice a day with hatchery Toyotomi shrimp, and exposed to 14 h of light and 10 h of darkness per day. Fertilized eggs produced by mating males and females were incubated in embryonic water (5 mM NaCl, 0.17 mM KCl, 0.33 mM Mg_2SO_4, 10^{-5}% Methylene Blue) at 28.5 °C. The method of staging zebrafish developmental stages for collection was performed as previously described [51].

5.3. Plasmid construction

Design of Pcgf5 siRNA interference sequences (Forward, 5'-CCCGATGGTATTGCAGTATTTCAAGAGAATACTGCAATACCATCGGGTTTTTT-3'; Reverse, 5'-AATTAAAAAACCCGATGGTATTGCAGTATTCTCTTGAAATACTGCAATACCATCGGGGGCC-3'), the interfering sequences was constructed into the Psilencer-U6 plasmid to obtain Pcgf5-Psilencer interfering plasmid. Pcgf5 overexpression primer sequences were designed and synthesized (Forward, 5'-TGCTCTAGAATGGCTACCCAAAGGAAACA-3'; Reverse, 5'-GCGGGATCCCTACCCAAAATCAATTCTCG-3'), and after PCR amplification to obtain the full-length of Pcgf5 CDS, it was constructed into Pultra plasmid to obtain Pcgf5 overexpression plasmid.

5.4. Immunofluorescence

The method of immunofluorescence was performed as previously described [52].Cells were fixed using 4% paraformaldehyde for 20 min at room temperature, permeabilized by 0.3% Triton X-100 for 10 min, blocked with 1xTBS with 3% BSA at room temperature for ~30 min, incubated with primary antibody overnight at 4 °C, followed by incubation of the appropriate secondary antibody Primary antibodies used in this study were rabbit monoclonal anti-Sox2 (abcam, ab92494; 1/100 in 1xTBS with 3% BSA), rabbit monoclonal anti-Oct4 (abcam, ab181557; 1/100 in 1xTBS with 3% BSA). Secondary antibodies were anti-rabbit IgG labeled with Alexa Fluor 488, 550 (BOSTER, Cat# BA1105 and BA1090; 1/1000 in 1xTBS with 3% BSA).

5.5. Quantitative reverse transcription PCR (RT-qPCR)

Extraction of total RNA from zebrafish embryos of all periods or P19 cells was performed following the standard procedure for TRIzol reagents. Total RNA was extracted from 30 to 40 zebrafish embryos or 1-5 X 10^7 P19 cells, roughly 1 μg of total RNA was used to reverse transcribe the mRNA to cDNA via a reverse transcription kit (TOYOBO, FSQ-101), and a 2-fold dilution of the cDNA was used for qPCR, with each reaction containing a SYBR Green mix (TOYOBO, QPK-201) performed in a LightCycle 96 Real-Time PCR system (Roche). All reactions with specific forward and reverse primers were performed in triplicate. Primers were listed in Supplementary Table S1.

5.6. Morpholino oligonucleotide microinjection

For zebrafish pcgf5a mRNA translation initiation site, the morpholino antisense oligonucleotide (MO) sequence 5'-ATCTCGACT-CAAGGGTCCACAGAGG-3' was designed and synthesized, and the sequence of the control (Con MO) was 5 '-ATgTCGAgT-CAAcGGTCgACAGAcG-3'. MO and Con MO were injected into the embryonic yolk sac before the 4-cell period of zebrafish embryos, after which the embryos were cultured in a constant temperature incubator at 28 °C.

5.7. Zebrafish embryo whole-Mount in situ hybridization

Embryos of the desired developmental period were collected, fixed using 4% paraformaldehyde overnight, rinsed by PBST and pre-hybridized at 65 °C for 4 h. 1 ng/μL digoxigenin-labeled RNA probe was added and hybridized overnight on a shaker at 65 °C, followed by the addition of Anti-DIG-AP overnight at 4 °C, and photographed under a somatic microscope for observation after NBT/BCIP color development. The RNA probes designed in this experiment included sox2, sox3 and foxg1, primers were listed in Supplementary Table S1.

5.8. Western blot

30-40 zebrafish embryos or 1-5 X 10^7 P19 cells were taken and lysed on ice using RIPA lysis solution containing protease inhibitor, after sonication and grinding, centrifugation was performed, and the precipitate was mixed with loading buffer and boiled in boiling water for 15 min. Proteins were separated using 10% SDS-PAGE gel and transferred to PVDF membranes. The Rabbit monoclonal anti-Sox2 (abcam, ab92494; 1/1000 dilution), Rabbit monoclonal anti-Oct4 (abcam, ab181557; 1/1000 dilution), Rabbit polyclonal anti-Pax6 (Gene Tex, GTX113241; 1/1000 dilution), Rabbit polyclonal anti-Wnt3 (ZENBIO, 201208; 1/1000 dilution), mouse monoclonal anti-Hes1 (ZENBIO, 381205; 1/1000 dilution), Rabbit monoclonal anti-H3K18ac (CST, 13998S; 1/1000 dilution), Rabbit polyclonal anti-H3K4me3 (CST, 9727S; 1/1000 dilution), and mouse monoclonal anti-βActin (proteintech, 66009-1-Ig; January 2000 dilution) antibodies were used for detection. Quantification was performed using ImageJ software (https://imagej.nih.gov/ij/).

6. Quantification and statistical analysis

Statistical analysis of data were performed using GraphPad Prism v8.0.2 (http://www.graphpad.com, San Diego, CA). Fig. 1BDI, Fig. 2CGF, Fig. 3CFG, Fig. 4BD and Fig. 5BD was analyzed using a *t*-test, while Fig. 1E–H, Fig. 2AHIK, Fig. 3AHIK, Fig. 4E–H and Fig. 5EFH was analyzed using a two-way ANOVA. Data in Figures 1BD, 2BD, 3BD are presented as means ± SD, and all other data are presented as means ± SEM. Sample sizes are described in figure legends. The levels of statistical significance are indicated in figures and figure legends: n.s. (no significance), $p \geq 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

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Ethics statement

This study was reviewed and approved by the Ethics Committee of Shandong Provincial Maternal and Child Health Hospital, with the approval number: NO. 2023-032.

Data and code availability

Any information required to reanalyze the data reported in this paper is available from the lead contact upon reasonable request.

CRediT authorship contribution statement

Xuan Yang: Writing – review & editing, Writing – original draft, Validation, Resources, Investigation. **Wenjuan Zhou:** Writing – review & editing, Investigation. **Juan Zhou:** Writing – review & editing, Investigation. **Anna Li:** Investigation. **Changqing Zhang:** Writing – review & editing. **Zhenya Fang:** Writing – review & editing, Investigation. **Chunying Wang:** Investigation. **Shiyu Liu:** Investigation. **Aijun Hao:** Writing – review & editing, Writing – original draft. **Meihua Zhang:** Writing – review & editing, Writing – original draft.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing

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Appendix A. Supplementary data

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