Marine Biotechnology (2025) 27:126
https://doi.org/10.1007/s10126-025-10491-4

RESEARCH

Biological Functions of Growth Differentiation Factor 9 in Early Ovarian Development of Japanese Eel (*Anguilla japonica*)

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Received: 27 May 2025 / Accepted: 7 July 2025 / Published online: 20 August 2025

Abstract

Growth differentiation factor 9 (GDF9) is a member of the transforming growth factor-β (TGF-β) superfamily and is expressed in an oocyte-specific manner. It plays a crucial role in the early stage of ovarian development. Japanese eel (*Anguilla Japonica*), a spawning migration teleost, has artificial reproduction still under investigation. Aimed at developing a novel method for the successful artificial reproduction of Japanese eel, in the present study, the role of GDF9 in the regulation of early ovarian development was investigated. The *Gdf9* gene in Japanese eel was 1293 bp in length, coding for 430 amino acids. Expression analysis in different tissues showed that *gdf9* is highly expressed in the ovary, and the *gdf9* mRNAs are localized in early developmental oocytes. Injection experiments showed that GDF9 significantly increased the gonadosomatic index in Japanese eel. However, histological observations indicated that GDF9 injection alone was insufficient to overcome the cortical alveolus stage and initiate vitellogenesis in Japanese eel, which is consistent with the lack of significant changes in serum estradiol and vitellogenin levels. Transcriptomic analysis revealed that GDF9 is involved in various molecular functions and physiological processes within the ovary. Overall, our findings suggest that GDF9 plays a regulatory role in the early ovarian development of Japanese eel, possibly by promoting the formation and activation of primordial follicles. These findings offer novel evidence for understanding the regulation of early ovarian development in Japanese eel and provide a valuable foundation for advancing artificial breeding in this species.

Keywords Growth differentiation factor 9 · Japanese eel · Ovarian development

Introduction

Growth differentiation factor 9 (GDF9), a member of the transforming growth factor-β (TGF-β) superfamily, is an oocyte-specific factor that coordinates folliculogenesis by signaling to surrounding follicle cells (McGrath et al. 1995).

As a crucial paracrine regulatory factor, GDF9 plays a significant role throughout ovarian development. During these key stages, GDF9 regulates granulosa cell proliferation, promotes the formation of a functional follicular structure, and facilitates the growth and maturation of oocytes, thus ensuring successful ovarian development (Elvin et al. 2000; Vitt and Hsueh 2001; Monniaux 2016; Sanfins et al. 2018). Studies have shown that GDF9 is essential for the transition from primordial follicles to primary follicles, as well as the progression to the cortical alveolus stage (Cook-Andersen et al. 2016). Moreover, GDF9 has been reported to function synergistically with other hormones and growth factors, such as bone morphogenetic protein 15 (BMP15) and follicle-stimulating hormone (FSH) (Wu and Matzuk 2002; Otsuka et al. 2011). These interactions contribute not only to the coordination of follicular growth and oocyte maturation, but also to the modulation of granulosa cell proliferation and differentiation, thereby playing a multifaceted role in female reproductive function (Yan et al. 2001; McNatty et al. 2005; Peng et al. 2013). In addition, studies have shown that in male mammals, GDF9 can regulate spermatogenesis via action on germ cells or somatic cells (Guo et al. 2013) and modulate key functions in Sertoli cells (Nicholls et al. 2009).

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Japanese eel is one of the most important species of the aquaculture industry of East Asia. However, reduction in eel resources and catches of glass eels as seedlings for aquaculture have been a serious problem in recent years (Kagawa et al. 2013). Although great progress in artificial reproduction studies of elopomorphes has been made, the shortage of the seedings still limits this industry to the new horizon. Therefore, it is an urgent problem to realize the artificial breeding of Japanese eel, and ovarian development is particularly important as a key link. However, as an important regulator in promoting follicle growth beyond the primary stage, the potential role of GDF9 in ovarian development of Japanese eel is still unknown. This study focuses on the functional role of GDF9 in early ovarian development of Japanese eel, offering insights for the artificial breeding of this species.

Materials and Methods

Animals

All the female Japanese eels with body weight of 1.0 ± 0.2 kg (Supplementary material 1) were obtained from an aquafarm in Jiangmen, China. All animal experiments were approved and performed in accordance with the respective Animal Research and Ethics Committees of Ocean University of China (Permit Number: 20141201). The fish were anesthetized with tricaine methane sulfonate (MS-222) (Tian et al. 2019). Tissue samples were collected immediately and frozen in liquid nitrogen.

RNA Extraction and cDNA Preparation

Total RNA was extracted from Japanese eel ($n = 3$) tissues (heart, liver, spleen, kidney, intestine, brain, ovary, pituitary, head kidney, and gill) using SparkZol Reagent (Sparkjade, China). The quantity and quality of the RNA were estimated using a biophotometer (OSTC, China) and agarose gel electrophoresis, respectively. The RNA was reverse-transcribed to complementary DNA (cDNA) using 1 μg of total RNA per 20 μL reaction with an Evo M-MLV RT Kit with gDNA Clean for qPCR (Accurate Biology, China) according to the manufacturer's instructions.

Gene Clone and Molecular Characterization

According to the genome, the open reading frame (ORF) of *gdf9* in Japanese eel was predicted, and gene-specific primers were designed by Primer Premier 5 software (Premier, Canada). To amplify cDNA fragments of Japanese eel *gdf9*, PCR was performed using specific primers corresponding to Japanese eel *gdf9* cDNA sequence.

All PCR reactions were performed using 2 × Phanta Max Master Mix (Dye Plus) (Sparkjade, China) for cloning, following the manufacturer's instructions. The amplified products were purified and inserted into a TA cloning vector (Vazyme, China) for transformation. Positive clones containing inserts of the expected size were selected and subjected to sequencing to confirm their identity.

The signal peptide in GDF9 was predicted using SignalP 5.0 Server (http://www.cbs.dtu.dk/services/SignalP-5.0/). Multiple sequences were aligned and compared using Clustal X (Thompson et al. 1994), and a phylogenetic tree was constructed by MEGA7 using the neighbor-joining method (Li et al. 2012b). ExPASy Compute pI/MW tool (http://web.expasy.org/compute_pi/) was used to calculate the molecular weight and isoelectric point of GDF9. The 3D structure of GDF9 in Japanese eel was modeled using SWISS-Model (https://swissmodel.expasy.org/).

Quantitative Real-Time PCR

According to the reagent instructions, SYBR Green Premix *Pro Taq* HS qPCR kit (Accurate Biology, China) was used for qPCR. The concentration of the template in the sample was determined by relating the Ct value to a standard curve. The transcript levels were normalized against *18 s* transcript levels. The qPCR primers were designed using Primer Premier 5 software (Premier, Canada) and are shown in Table 1.

In Situ Hybridization (ISH)

The ovaries of Japanese eel were removed and fixed in buffered 4% paraformaldehyde for 24 h and then embedded in paraffin. Seven-micron-thick sections were cut for ISH. The sections were pasted onto aminopropylsilane-treated glass slides and dried in an oven at 37℃. Sense and antisense digoxigenin (DIG)-labeled riboprobes about 500 bp in length were synthesized from the open reading frames (ORF) of Japanese eel *gdf9* gene using a DIG RNA Labeling Kit (Roche Diagnostics, Mannheim, Germany). The in situ hybridization was performed as we previously reported (Qi et al. 2015; Zhou et al. 2019).

Prokaryotic Expression of Recombinant GDF9

Primers (Table 1) were designed to amplify the mature peptide sequence of Japanese eel GDF9 by PCR. The amplified fragment was recombined into the N-His-SUMO-pET expression vector and subsequently transformed into *Escherichia coli* Rosetta-gami B (DE3). The procedures for plasmid construction and the subsequent induction of recombinant protein expression were performed as previously described (Yan et al. 2022).

Table 1 Primer sequences used for ORF cloning, qPCR, ISH, and vector construction

Primers	Sequence (5′−3′)
Primers for ORF clone	
gdf9-clone-F	ATGGAGACTCTAGTGAAAATAATTATTGTACT
gdf9-clone-R	TCATCGACATGTACACTTTGTGGC
Primer for qPCR	
gdf9-F	GGGTTTTCGTTGGCTTTCCC
gdf9-R	CAGGGATTAGTTTCGGGGCA
18 s-F	GAAACGGCTACCACATCC
18 s-R	CACCAGACTTGCCCTCCA
Primers for ISH probe preparation	
gdf9-ish-F	CGCATTTAGGTGACACTATAGAAGCGTCGACTCCACTGTTTACGGC
gdf9-ish-R	CCGTAATACGACTCACTATAGGGAGACATACGGCGACTCCTTTCTGTG
Primers for N-His-SUMO-pET vector construction	
gdf9-pET-F	ACAGAGAACAGATTGGTGGTCGCTGGAGACGCAACTTAC
gdf9-pET-R	GCTTTGTTAGCAGCCGGATCGTTAGCGACAGGTACATTTTGTGGC

The harvested *E. coli* cells were lysed and centrifuged. Due to misfolding, recombinant GDF9 (rGDF9) was present in the sediment as inclusion bodies. The sediment was washed several times and then dissolved in denaturing solution (100 mM NH_4Cl, 50 mM Tris–HCl, 8 M urea, 10% (V/V) glycerin, pH = 8.0) and centrifuged to remove the precipitated impurities. Add the dissolved inclusion bodies to the renaturation solution several times for dilution and refolding, and kept at 4 °C overnight to ensure that they could remodel their conformation. Finally, rGDF9 was dialyzed into 1 × PBS and concentrated using an ultrafiltration tube.

The concentration of rGDF9 protein was determined by Bradford Protein Assay Kit (Beyotime Biotechnology, China). The protein was mixed with the SDS-PAGE Sample Loading Buffer (epizyme, China) and incubated at 95℃ for 5 min to denature. After SDS-PAGE, staining was performed using Coomassie Blue Fast Staining Solution (epizyme, China). The collected rGDF9 protein was snap-frozen in liquid nitrogen and stored in an ultra-low temperature freezer.

Recombinant GDF9 Injection Experiment in Japanese Eels

Healthy adult female Japanese eels were selected and cultured in seawater for one month prior to the experiment. The control experiment was designed to investigate the biological function of rGDF9 in female Japanese eels. The treatment group was injected with rGDF9 (1 mg/kg) once a week for 6 weeks, and the control group was injected with the same volume of saline ($n = 8$). Blood samples from the two groups of Japanese eels were collected, and after separation of components, the serum was retained for the determination of relevant substance concentrations. Gonadosomatic index (GSI) was counted for each group of Japanese eels, and ovarian tissues were stored in liquid nitrogen for RNA extraction.

Estradiol and Vitellogenin Concentration Measurement by ELISA

The concentrations of estradiol (E2) and vitellogenin (VTG) in serum were measured by ELISA kit (Runyu, China). The serum was collected from the two groups of Japanese eels in the aforementioned injection experiment ($n = 8$), and the concentrations of E2 and VTG were measured according to the instructions provided in the reagent kit.

Hematoxylin–Eosin (H&E) Staining

Ovary tissues were fixed with 4% paraformaldehyde, cut into 7-micron sections, and stained with haematoxylin–eosin. The sections were observed by a bright-field light microscope (Olympus, Japan) and photographed.

RNA-Seq Library Construction and Sequencing

Ovaries RNA from the two groups of the injection experiment was used for transcriptome sequencing ($n = 4$). Eight RNA libraries (GDF9_C1, GDF9_C2, GDF9_C3, GDF9_C4, GDF9_T1, GDF9_T2, GDF9_T3, GDF9_T4) were constructed, and 150 bp paired-end reads were generated (Zuo et al. 2025). Clean reads were obtained by removing low-quality sequences and adapter contamination from the raw data. These clean reads were then aligned to the Japanese eel reference genome (PRJNA852364) using HISAT2 (Kim et al. 2015), and quantification analysis was performed with StringTie (Pertea et al. 2016). Moreover, multi-mapped reads were removed, and uniquely mapped

reads were used to calculate expression levels as FPKM (fragments per kilobase of transcript per million mapped reads), which were normalized based on previous references (Anders et al. 2015). Differentially expressed genes (DEGs) were identified using DESeq2 package with a threshold of p-value < 0.05 and an absolute fold change greater than 1. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were conducted as described (Götz et al. 2008; Kanehisa et al. 2017).

Statistical Analysis

All data were expressed as the mean values ± SEM. Data analyses were performed by *t*-test, and significance was considered at $p < 0.05$. All statistics were performed using SPSS 19.0 (SPSS, Chicago, IL, USA).

Results

Identification and Synteny Analysis of the gdf9 Gene in Japanese Eel

Gene cloning results showed that the length of *gdf9* (accession number: OK376498) cDNA fragment was 129 base pairs (bp), and the open reading frame (ORF) of *gdf9* was 1293 bp, encoding 430 amino acids (aa) precursor. The predicted signal peptide at the front of the peptide contains 23 aa, and its function is to guide the precursor protein into the endoplasmic reticulum as shown in Fig. 1A.

As shown in Fig. 1B, sequence comparative analysis and domain prediction of GDF9 in Japanese eel, European eel (*Anguilla Anguilla*), zebrafish (*Danio rerio*), rainbow trout (*Oncorhynchus mykiss*), Nile tilapia (*Oreochromis niloticus*), human (*Homo sapiens*), mouse (*Mus musculus*), and chicken (*Gallus gallus*) revealed a signal peptide and a highly conserved domain of transforming growth factor β (TGF-β) superfamily.

To further investigate the evolutionary relationships of *gdf9*, a phylogenetic tree was constructed (Fig. 1C). The *gdf9* genes from fish and other vertebrates formed two distinct clades. Within teleost, *gdf9* is relatively conserved, particularly in the Japanese eel and the European eel. The result indicates that *gdf9* has strong evolutionary conservation, especially within fish lineages. This high degree of sequence conservation suggests that *gdf9* may play a conserved role in reproductive regulation across teleost species.

Tissue Expression and Localization of gdf9 in Japanese Eel

The expression patterns of the *gdf9* gene are shown in Fig. 2A. *Gdf9* mRNA was widely expressed in the selected tissues. Among them, *gdf9* mRNA of the Japanese eel was mainly significantly expressed in ovaries, but extremely low in the heart, liver, spleen, kidney, intestine, brain, pituitary, head kidney, and gill.

The spatial distribution of *gdf9* mRNA in the ovary was examined by ISH. Ovarian tissue sections from Japanese eel were subjected to ISH analysis. The results revealed that *gdf9* signals were specifically detected in oocytes, where they were prominently expressed (Fig. 2B).

Prokaryotic Expression, Renaturation, and Functional Verification of rGDF9

The mature peptide of GDF9 was inserted into the N-His-SUMO-pET plasmid to yield pET-His-SUMO-GDF9 expression vector (Fig. 3B). Subsequently, the rGDF9 of Japanese eel with a molecular weight of 29.2 kDa was obtained by prokaryotic expression and renaturation of inclusion bodies (Fig. 3C). The nucleotide and amino acid correspondences are shown in Fig. 3D. The biological activity of the rGDF9 was verified by western blot analysis (Supplementary material 2).

rGDF9 Increases Gonadosomatic Index During Early Ovarian Development in Japanese Eel

To investigate the role of GDF9 in early ovarian development of Japanese eel, an injection experiment was conducted on females with undeveloped ovaries. The result showed that females injected with rGDF9 exhibited a significantly higher GSI compared to the control group (Fig. 4A). As shown in Fig. 4B, C, E2 and VTG concentrations in the serum did not differ significantly between the two groups of Japanese eels. In addition, histological observation showed that the follicles of the Japanese eels in the two groups did not enter the vitellogenic stage; most of them were at the cortical vesicle stage, while a minority were at the perinucleolar stage (Fig. 5). Taken together, our results indicate that GDF9 can enhance the GSI during the early stages of ovarian development in Japanese eel. However, it does not appear to be involved in the accumulation of yolk substances.

RNA-seq Reveals the Function of rGDF9 in Early Ovarian Development of Japanese Eel

In order to investigate the functional mechanism of GDF9 in early ovarian development of Japanese eel, eight cDNA libraries ($n = 4$) were constructed and sequenced on Illumina platform (accession number: PRJNA1180505). A total of 296,891,984 raw reads were obtained by high-throughput sequencing, and 279,943,529 clean reads were filtered. The average clean reads of the control group and treatment group were 33,673,766 and 36312116, with Q30 average

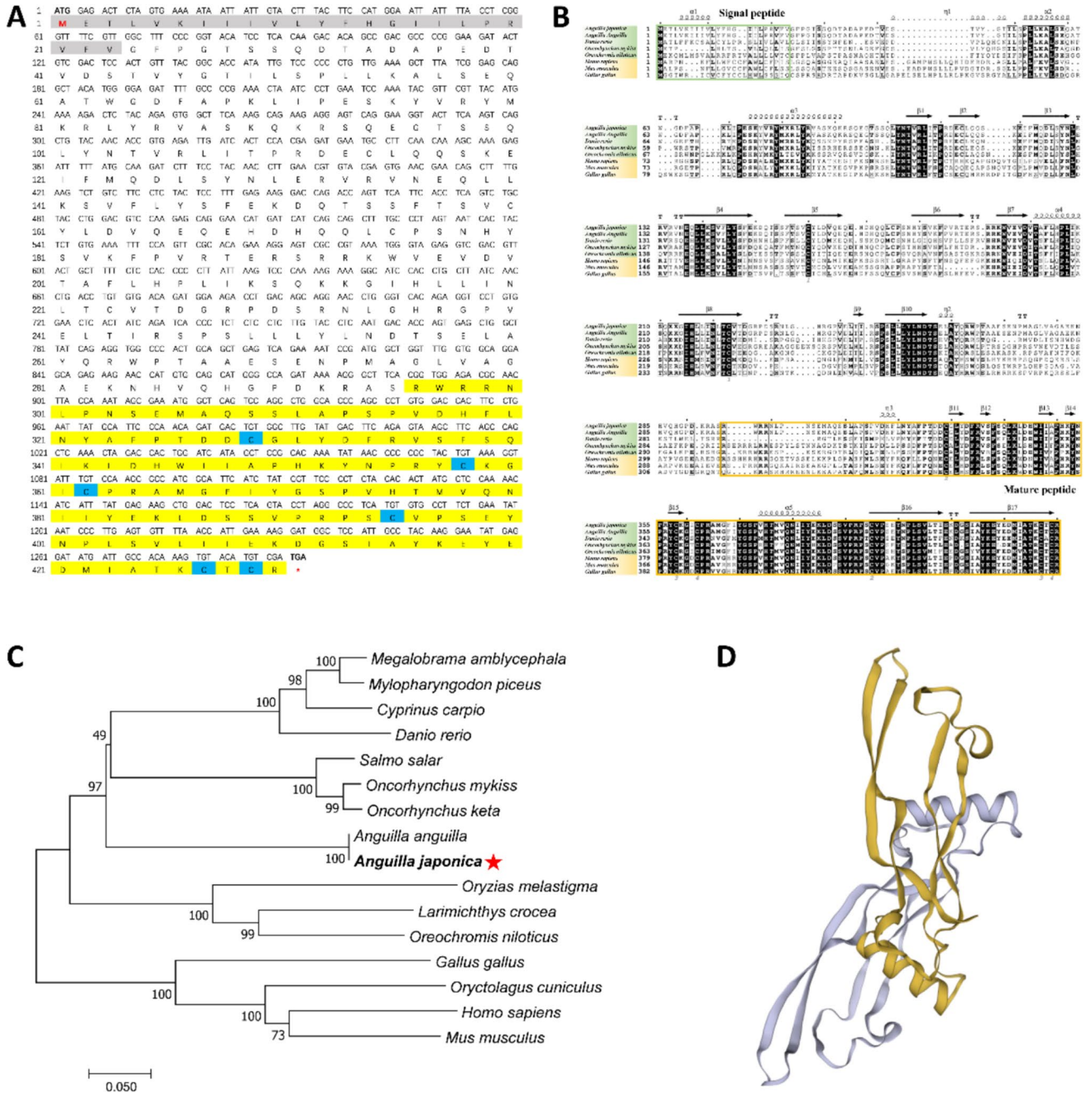

Fig. 1 **A** Nucleotide and amino acid sequences of *gdf9* in Japanese eel (gray, signal peptide; yellow, mature peptide; blue, cysteine). **B** The sequence alignment of GDF9 in Japanese eel (*Anguilla japonica*), European eel (*Anguilla anguilla*), zebrafish (*Danio rerio*), rainbow trout (*oncorhynchus mykiss*), Nile tilapia (*Oreochromis niloticus*), human (*Homo sapiens*), mouse (*Mus musculus*), and chicken (*Gallus gallus*). **C** A phylogenetic tree was constructed using the neighbour-joining method in Japanese eel and other species. Data were resampled with 1000 bootstrap replicates. Accession numbers: *Megalobrama amblycephala* (XP_048033042.1), *Mylopharyngodon piceus* (ATJ00806.1), *Cyprinus carpio* (AOW71519.1), *Danio rerio* (NP_001012383.1), *Salmo salar* (XP_014068686.2), *Oncorhynchus mykiss* (XP_021438977.2), *Oncorhynchus keta* (XP_035599787.1), *Anguilla anguilla* (XP_035263495.1), *Oryzias melastigma* (XP_024138901.1), *Larimichthys crocea* (XP_010753076.3), *Oreochromis niloticus* (XP_003455214.1), *Gallus gallus* (NP_996871.2), *Oryctolagus cuniculus* (NP_001164821.1), *Homo sapiens* (XP_011541610.1), *Mus musculus* (NP_032136.2). **D** The 3D structure of GDF9 in Japanese eel

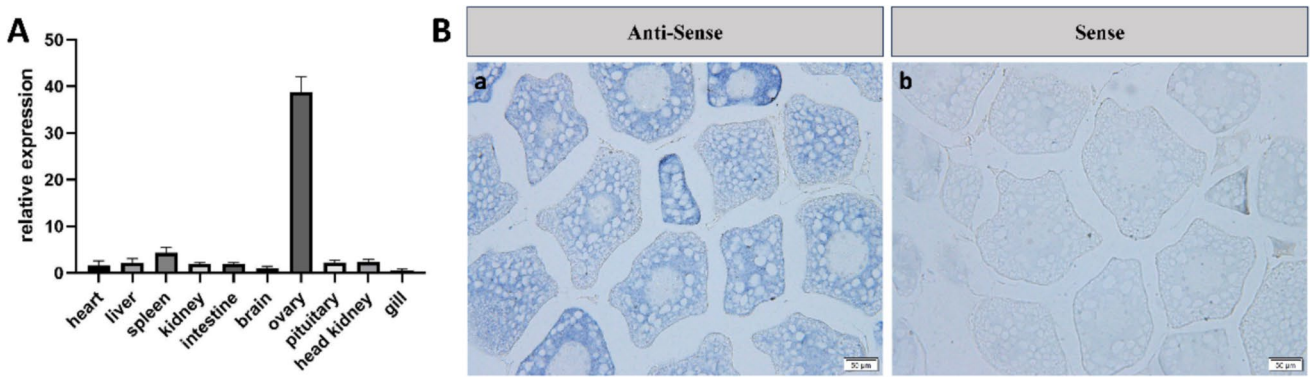

Fig. 2 **A** Relative expression levels of *gdf9* in different organs (heart, liver, spleen, kidney, intestine, brain, ovary, pituitary, head kidney and gill) ($n=3$). **B** Localization of *gdf9* mRNA in oocytes of Japanese eel via in situ hybridization. Scale bars = 50 μm

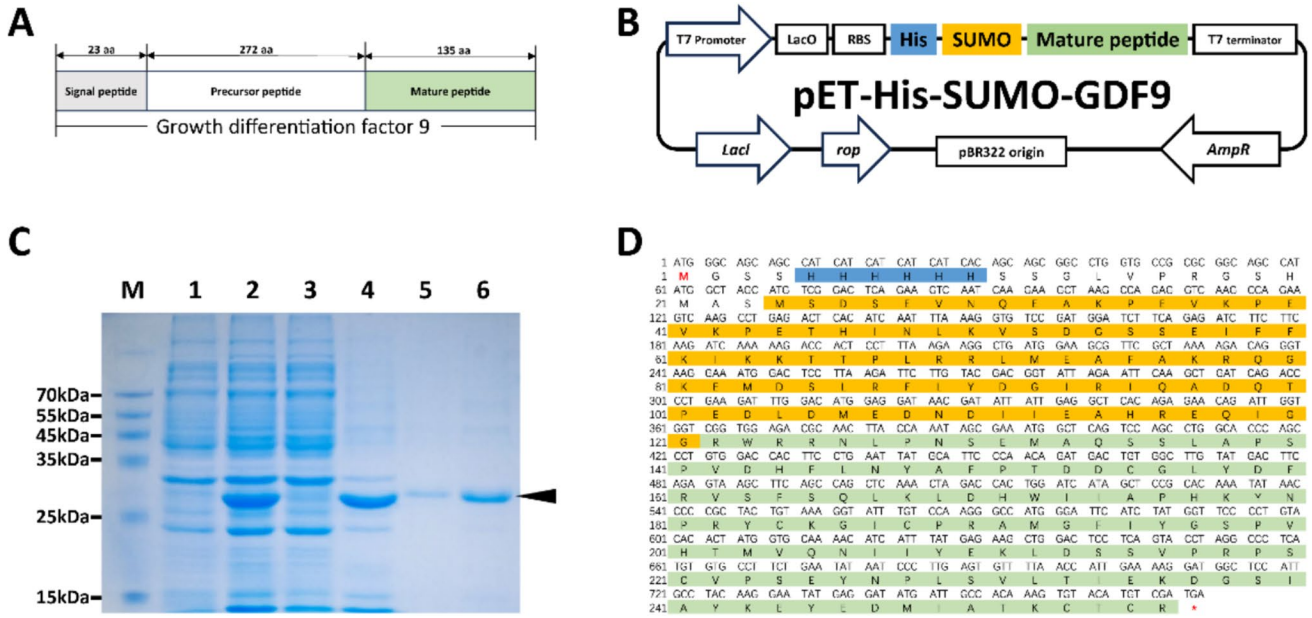

Fig. 3 **A** Structural composition of GDF9 in Japanese eel. **B** Expression plasmid of the rGDF9 of Japanese eel. **C** SDS-PAGE analysis of rGDF9 in Japanese eel (M, marker; lane 1, total protein in the thallus before induction; lane 2, total protein in thallus after induction; lane 3, protein in the precipitate after induction; lane 4, protein in the supernatant after induction; lane 5, rGDF9 after washing and refolding; lane 6, rGDF9 concentrated by ultrafiltration (29.2 kDa)). **D** Nucleotide and amino acid sequences of rGDF9 of Japanese eel (blue, Histidine tag; orange, small ubiquitin-like modifier; green, mature peptide of GDF9 in Japanese eel)

percentages of 93.78% and 93.31%, respectively (Supplementary material 3). In addition, the average total map of the control and treatment groups was obtained as 28,814,601.75 (85.57%) and 31,482,809.25 (87.00%), respectively.

The PCA showed that the eight samples of the control and treatment groups could be well typed (Fig. 6A), and the visual view of differential gene expression was performed in Fig. 6B. As shown in Fig. 6C, a total of 511 significant DEGs were identified, including 228 up-regulated genes and 283 down-regulated genes in the treatment group compared with the control group. The DEGs were classified into molecular function (*oocyte maturation*, *regulation of exocytosis*, *semaphorin-plexin signaling pathway*, *protein dephosphorylation*, *transmembrane transport*, *ion transport*, *oxidation–reduction process*, *cell differentiation*, *protein phosphorylation*, *signal transduction*), cellular component (*semaphorin receptor complex*, *cell division site*, *integral component of Golgi membrane*, *integral component of plasma membrane*, *extracellular space*, *cytoskeleton*, *membrane*, *integral component of membrane*, *plasma membrane*), and biological process (*protein kinase regulator activity*, *semaphorin receptor*

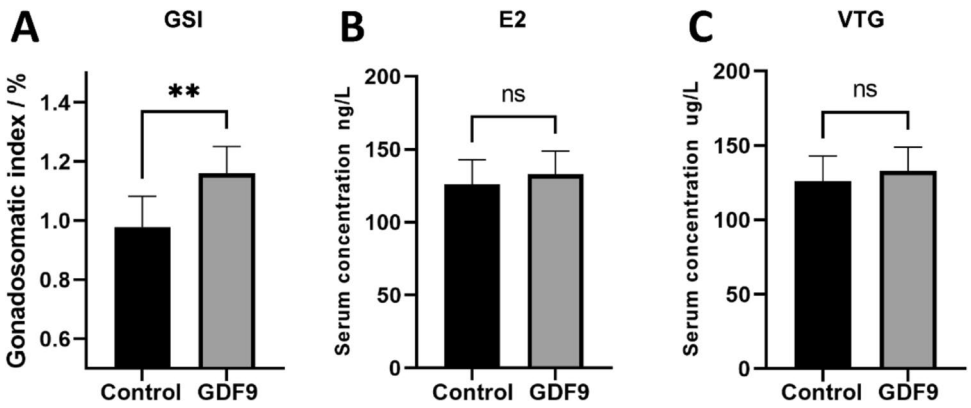

Fig. 4 **A** Gonadosomatic index of Japanese eels injected with saline and rGDF9 for 6 weeks, respectively ($n = 8$). **B** The concentration of estradiol (E2) in serum of Japanese eels injected with saline and rGDF9 for 6 weeks, respectively ($n = 8$). **C** The concentration of vitellogenin (VTG) in serum of Japanese eels injected with saline and rGDF9 for six weeks, respectively ($n = 8$). All data are presented as the mean ± SEM

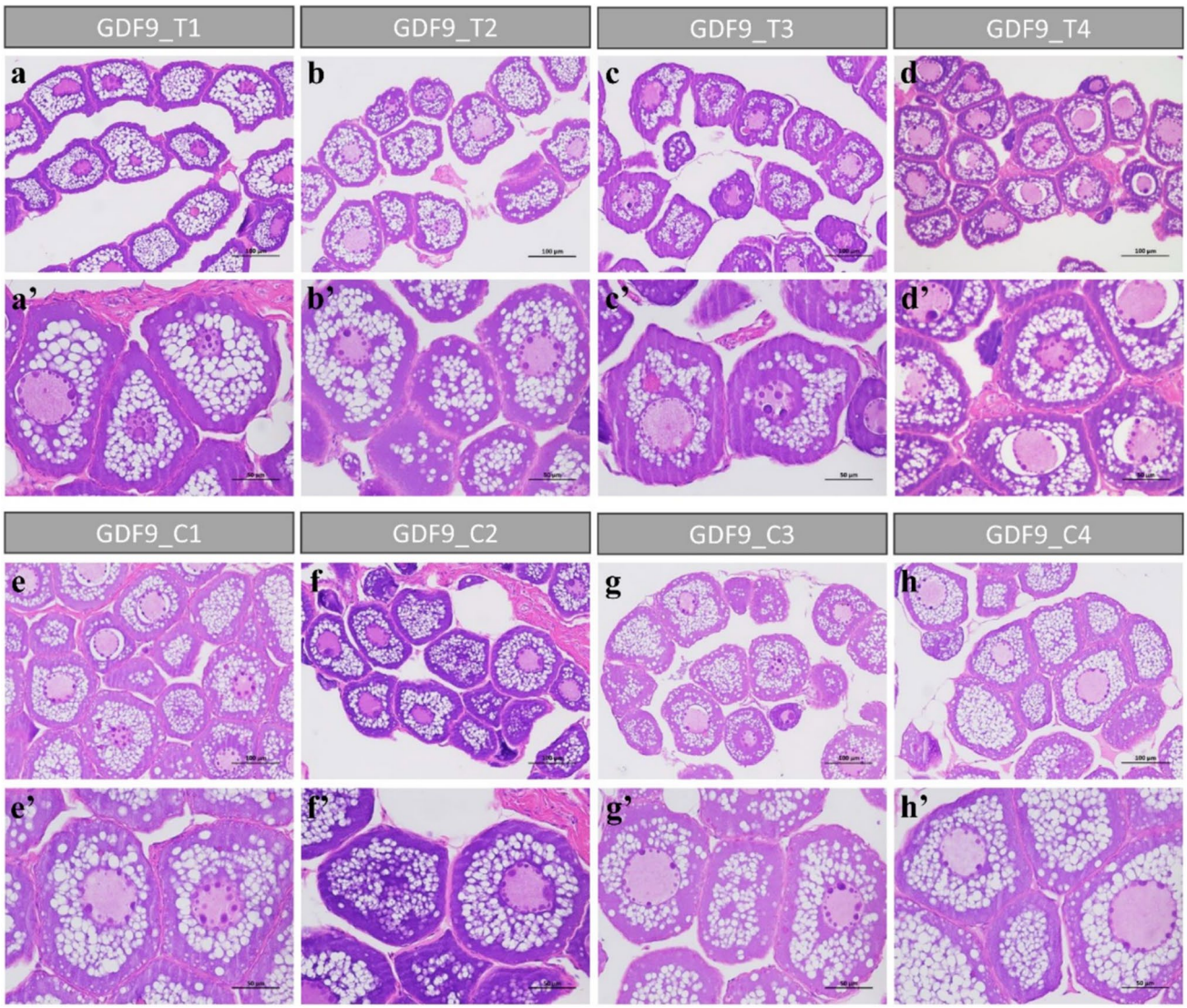

Fig. 5 Histological observation of the Japanese eel ovaries injected with saline and rGDF9 for six weeks. **a**–**h** Scale bars = 100 μm. **a'**–**h'**, Scale bars = 50 μm

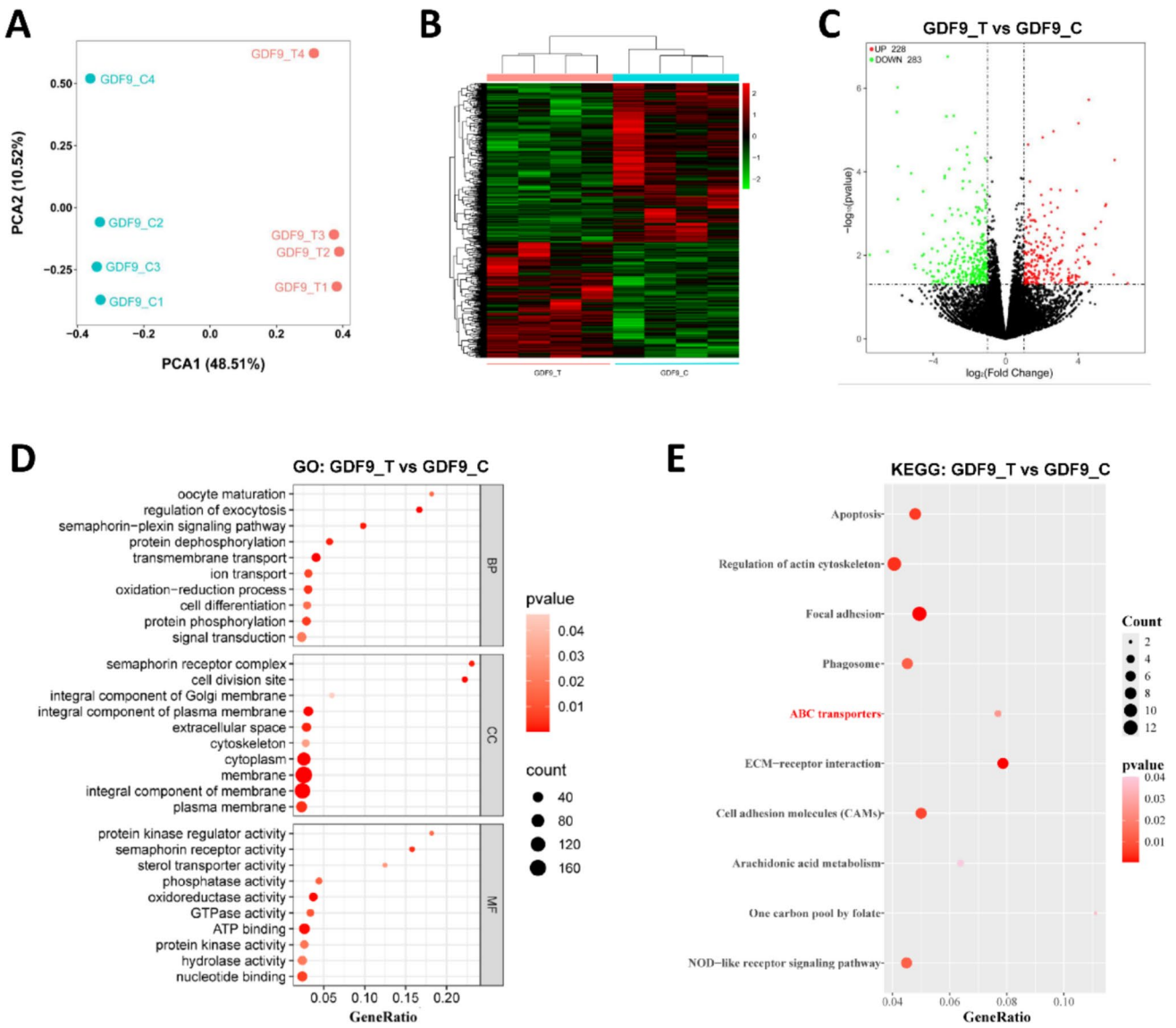

Fig. 6 **A** PCA of the eight libraries treated with saline (GDF9_C1, GDF9_C2, GDF9_C3, GDF9_C4) and rGDF9 (GDF9_T1, GDF9_T2, GDF9_T3, GDF9_T4), respectively. **B** The expression values of the DEGs in the two groups (GDF9_C and GDF9_T) are presented in a heat map. Green and red colors indicated up- and down-regulated transcripts, respectively. **C** The Volcano plots for saline (GDF9_C) vs. rGDF9 (GDF9_T) group. **D** GO terms of DEGs in rGDF9 (GDF9_T) vs. saline (GDF9_C). **E** KEGG signaling pathways of DEGs in rGDF9 (GDF9_T) vs. saline (GDF9_C)

activity, *sterol transporter activity*, *phosphatase activity*, *oxidoreductase activity*, *GTPase activity*, *ATP binding*, *protein kinase activity*, *hydrolase activity*, *nucleotide binding*) by GO enrichment analysis. Meanwhile, KEGG pathway enrichment analysis revealed several significantly enriched signaling pathways, including cellular processes (*apoptosis*, *regulation of actin cytoskeleton*, *focal adhesion*, *phagosome*), environmental information processing (*ABC transporters*, *ECM-receptor interaction*, *cell adhesion molecules (CAMs)*), metabolism (*arachidonic acid metabolism*, *one carbon pool by folate*), and organismal systems (*NOD-like receptor signaling pathway*).

Discussion

An increasing number of studies have shown that GDF9 plays an important role in growth and reproduction across vertebrates, including teleost fish (Elvin et al. 1999; Hreinsson et al. 2002; He et al. 2012; Huang et al. 2014). In Japanese flounder (*Paralichthys olivaceus*), GDF9 is secreted by oocytes and plays a key role in regulating the growth and development of ovarian follicles (Yu et al. 2020). Functional deficiency of *gdf9* in zebrafish has been shown to impair the development of primordial follicles, leading to compromised female fertility (Chen et al. 2022). Although

these mechanisms have been well characterized in some teleost species, their roles in Japanese eel remain largely unexplored.

In this study, we cloned the *gdf9* gene from the ovary of Japanese eel, then conducted multiple sequence alignment by clustal X, and constructed a phylogenetic tree of *gdf9* using the adjacent method by MEGA7. The fragment length of *gdf9* is 1293 bp coding 430 amino acids, with a 23 aa signal peptide, 272 aa precursor peptide, and 135 aa mature peptide (Fig. 3A). Phylogenetic analysis revealed that GDF9 is evolutionarily conserved, consistent with the evolutionary relationships among species. In addition, multiple sequence alignment showed a high degree of conservation in GDF9 during evolution, which is in agreement with the phylogenetic tree result. The deduced amino acid sequence of Japanese eel GDF9 showed structures typical of members of the TGF-β superfamily, such as a typical signal peptide, relatively conserved mature peptide, and cysteine (Fig. 1A, B). As shown in Fig. 1D, the two GDF9 monomers form a homodimer through covalent bonding to exert their biological functions (Yan et al. 2020). By analyzing the evolutionary relationship, we can better understand the origin and evolution of the *gdf9* gene in Japanese eel.

Real-time PCR was used to analyze the mRNA expression levels of *gdf9* in various tissues of Japanese eel. The result showed that *gdf9* was most highly expressed in the ovary. In contrast, its expression levels were extremely low in other tissues, including the heart, liver, spleen, kidney, intestine, brain, pituitary, head kidney, and gill. This tissue-specific expression pattern is consistent with findings in mammals (Yu et al. 2020) and has also been reported in several teleost species, such as zebrafish (Liu and Ge 2007), rainbow trout (Lankford and Weber 2010), gibel carp (*Carassius auratus gibelio*) (Liu et al. 2012), Japanese flounder (Yu et al. 2020), and European sea bass (*Dicentrarchus labrax*) (Halm et al. 2008). Subsequently, in situ hybridization was conducted to examine the localization of *gdf9* mRNA in the ovary of Japanese eel. The signal was detected exclusively in oocytes, which is consistent with previous findings in zebrafish (Chen et al. 2017). In conclusion, the oocyte-specific expression pattern of *gdf9* indicates that it may play a crucial physiological role in regulating ovarian development in Japanese eel.

In recent years, increasing attention has been given to the role of GDF9 in ovarian development, with studies across multiple species demonstrating its critical function in early folliculogenesis (Hussein et al. 2005; Kona et al. 2016; Garcia et al. 2019; Stocker et al. 2020). In addition, the underlying regulatory mechanisms by which GDF9 regulates this process have been increasingly clarified (Gilchrist et al. 2008). In the present study, injection experiments showed that the GSI of Japanese eels treated with rGDF9 was significantly higher than that of the control group, indicating that GDF9 is involved in the regulation of early ovarian development in this species. However, histological observations revealed that in both groups, ovarian follicles only developed to the cortical alveolus stage. This finding is consistent with the known phenomenon that, under artificial culture conditions, Japanese eel ovaries are unable to progress beyond the cortical alveolus stage to the vitellogenic stage without additional hormonal stimulation. Moreover, the results further demonstrate that GDF9 alone does not have the capacity to induce vitellogenesis in Japanese eel follicles. There were no significant differences in serum E2 and vitellogenin VTG levels between the treatment and control groups, which is consistent with the histological findings indicating that GDF9 treatment did not promote the transition of follicles into the vitellogenic stage. Taken together, the above results suggest that the increase in GSI observed in Japanese eels following GDF9 treatment may be attributed to its biological role in recruiting and activating primordial follicles. This is consistent with findings reported in other species. In mice, GDF9 has been shown to accelerate the formation of primordial follicles (Zhao et al. 2016). In vitro experiments revealed that GDF9 stimulates the initiation of follicle development in rat (*Rattus norvegicus*) ovaries (Vitt et al. 2000) and promotes the progression of human follicles from the primary to the secondary growth stage (Hreinsson et al. 2002). Similarly, *gdf9* deficiency in zebrafish causes a complete arrest of follicular development at the primary growth stage (Chen et al. 2022), while in pigs (*Sus scrofa*), the inactivation of GDF9 blocks folliculogenesis (Chen et al. 2023). Collectively, these results highlight the critical and evolutionarily conserved function of GDF9 in regulating the initiation and progression of early follicle development, although additional factors may be required to drive further follicular development beyond the cortical alveolus stage in species such as the Japanese eel.

Multiple functional factors of the TGF-β superfamily have important functions in ovarian development (Pang and Ge 2002; Poon et al. 2009; Li et al. 2012a; Lu et al. 2020), and their receptors are essentially serine/threonine kinases (Zhang et al. 2016). GO analysis showed that DEGs were significantly enriched in multiple terms associated with GDF9 signal transduction, such as *protein phosphorylation*, *protein dephosphorylation*, *signal transduction, protein kinase regulator activity*, *phosphatase activity*, and *protein kinase activity*. This regulatory role in signaling transduction is likely to also influence the activity of other TGF-β superfamily factors, such as BMP15, which have been shown to interact with GDF9 and jointly regulate early ovarian development (Sanfins et al. 2018). Moreover, some DEGs were enriched in *semaphorin receptor complex* and *semaphorin-plexin signaling pathway*. In mammals, semaphorins have been shown to play a crucial role in angiogenesis, with Sema 4D being capable of inducing angiogenesis both in vitro and in vivo (Conrotto et al. 2005). Based on this, we hypothesize

that GDF9 may be involved in ovarian angiogenesis, thereby regulating early ovarian development and promoting folliculogenesis in Japanese eels.

GO enrichment analysis showed that some DEGs were enriched into the biological process of oocyte maturation. Studies in pigs have shown that GDF9 participates in oocyte maturation in vitro by promoting cumulus expansion and stimulating the activity of MPF and MAPK (Lin et al. 2014). Additionally, research in humans has demonstrated that GDF9 expression levels are closely correlated with oocyte maturation, fertilization, embryo quality, and pregnancy outcomes (Li et al. 2014). These findings suggest that GDF9 not only plays a role in early ovarian development through folliculogenesis but also exerts potential functions during oocyte maturation.

KEGG analysis revealed that DEGs were enriched in several cell cycle-related pathways, which may be associated with the role of GDF9 in promoting primordial follicle formation and initiating early follicular development, consistent with previous studies in various vertebrate species (Vitt et al. 2000; Hreinsson et al. 2002; Chen et al. 2023). Additionally, several DEGs were enriched in ABC transporters. Studies have shown that ABC transporters play a crucial role in ovarian development by regulating hormone levels, maintaining cellular homeostasis, and modulating follicular development and maturation, thereby performing essential physiological functions (Bloise et al. 2016; Quiroz et al. 2020).

Transcriptomic analysis demonstrated a significant upregulation of *inhbb* in the rGDF9 group, suggesting that GDF9 can modulate *inhbb* expression. This finding is consistent with previous studies in mice (Myers et al. 2013). The activin system plays a crucial role throughout ovarian development, particularly during yolk accumulation and oocyte maturation (Wang and Ge 2004; Lu et al. 2020; Zhao et al. 2022; Zhai et al. 2023). Studies in zebrafish have also shown that the activin system is involved in GDF9-mediated regulation of ovarian development, with GDF9 activating the Smad2 signaling pathway in follicle cells to regulate the expression of activin genes (Chen et al. 2017, 2022). Taken together, the activin system was involved in ovarian development as a potential functional factor mediated by GDF9, and this conclusion was also confirmed in our study on early ovarian development of Japanese eel. In this study, GDF9 may regulate early ovarian development in Japanese eel by modulating the expression of activin genes, which is consistent with the known roles of GDF9 in folliculogenesis across species (Matzuk et al. 2002; Zhang et al. 2017).

Due to limitations in experimental conditions, this study has certain constraints in fully elucidating the role and underlying mechanisms of GDF9 in early ovarian development of Japanese eel. To gain a more comprehensive understanding of GDF9's regulatory function, future research should focus on optimizing the injection dosage and frequency, uncovering the molecular mechanisms through which GDF9 exerts its effects, and exploring its potential interactions with other regulatory factors. These efforts will be central to our future work and will contribute to a deeper understanding of the functional role and regulatory mechanisms of GDF9 in ovarian development of Japanese eel.

In summary, GDF9, as an oocyte-specific regulatory factor, plays a crucial role in ovarian development in Japanese eel. GDF9 regulates early ovarian development, likely by facilitating folliculogenesis and initiating follicle development, without involving yolk accumulation. Transcriptome analysis revealed that GDF9 exerts its effects by regulating various molecular functions and biological processes. These findings provide a foundation for further investigations into the functional role of GDF9 and the molecular mechanisms underlying gonadal development in Japanese eel.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10126-025-10491-4.

Author Contributions Xin Qi: Conceptualization, Funding acquisition, Methodology, Writing—Review & Editing; Chenpeng Zuo: Data Curation, Writing—Original Draft; Xulei Wang: Investigation, Data Curation; Xuanhan Zhang: Data Curation; Xiaojie Wang: Data Curation; Likang Lyu: Data Curation; Teng Ma: Resources; Lingming Chen: Resources; Weimin Yu: Investigation; Yun Li: Resources; Haishen Wen: Conceptualization, Resources.

Funding This research was supported by the National Natural Science Foundation of China with grant number (32270556).

Data Availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

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