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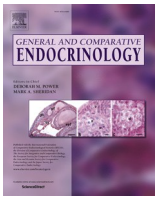

Using transcriptome analysis to investigate the induction of vitellogenesis in female Japanese eels (*Anguilla japonica*)

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ABSTRACT

Oogenesis, encompassing folliculogenesis, development, and maturation, is a complex physiological process that is not solely regulated by gonadotropins but is also actively influenced by multiple growth factors produced by the oocyte and its surrounding follicular cells. The Japanese eel (*Anguilla japonica*) has a complex life history, resulting in many uncertainties regarding its growth, development, and reproduction. Under artificial culture conditions, oocyte development in the Japanese eel is arrested and can only progress to the vitellogenic stage through artificial induction. In the present study, we observed that, despite receiving the same hormone treatment as normally developing individuals, a small proportion of female eels exhibited oocytes arrested at the perinucleolar stage. Transcriptome analysis revealed that differentially expressed genes are involved in multiple reproductive-related physiological processes and functional pathways, such as tachykinin system, MAPK signaling pathway, steroid-related pathways, oocyte meiosis, Wnt signaling pathway and GnRH signaling pathway. The abnormal expression of the two follicle-stimulating hormone (FSH) subunit genes may be a key factor contributing to this phenomenon. This study reveals the underlying causes of ovarian developmental arrest in hormonally induced female Japanese eels from the perspective of the brain–pituitary–gonad (BPG) axis, providing a research foundation for the artificial reproduction of Japanese eels.

1. Introduction

Oogenesis is a complex biological process that involves several physiological stages. The initial phase includes the formation of primordial germ cells (PGCs) and their transformation into oogonia (Patiño and Sullivan, 2002). Subsequently, oogonia develop into oocytes and form follicles, which are accompanied by surrounding follicular layer cells (Selman et al., 1993). As meiosis begins, the follicles enter the primary growth phase, during which they can develop independently of pituitary gonadotropic hormones (Gth), a phase known as the GTH-independent phase (Billard, 1992). Follicular development then progresses to the secondary growth phase, which also marks the onset of puberty, characterized by the appearance of cortical vesicles (Chen et al., 2022). In fish, puberty is the period during which an individual becomes capable of sexual reproduction, signifying the functional competence of the brain-pituitary-gonad (BPG) axis (Jalabert, 2005; Schulz and Goos, 1999; Weltzien et al., 2004). After cortical vesicle formation, the follicle enters the vitellogenic phase, marked by the accumulation of lipids and vitellogenin. During this phase, levels of follicle-stimulating hormone (FSH) and estradiol in the blood increase, along with FSH receptor expression in the ovaries (Ge, 2005; Santos et al., 2001). At this stage, the oocyte accumulates essential nutrients, including maternal messenger RNA, proteins, lipids, carbohydrates, vitamins, and hormones, all of which are critical for embryo development (Lubzens et al., 2010). After nutrient accumulation, the follicles enter the maturation phase, characterized by the resumption of oocyte meiosis. This phase includes the breakdown of the germinal vesicle (GVBD), the cleavage of vitellogenin into yolk proteins, and the hydration of the oocyte (Clelland and Peng, 2009). Following the extrusion of the first polar body, the oocyte enters meiotic metaphase II and is expelled from the follicle (Nagahama et al., 1995). At this point, the egg is capable of fertilization (Brooks et al., 1997).

The Japanese eel (*Anguilla japonica*) is a significant aquatic species with considerable economic value in the aquaculture industries of East

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Asia. As a catadromous migration fish, the Japanese eel exhibits a complex life history, which presents challenges in understanding various aspects of its growth, development, and reproduction. The artificial reproduction of the Japanese eel remains a major unresolved issue globally. Despite significant advances over the past century through continuous research, successful industrial-scale reproduction has yet to be achieved (Ijiri et al., 2011; Tanaka et al., 2001; Tanaka et al., 2003).

Under artificial culture conditions, the ovary of the Japanese eel can only develop to the cortical vesicle stage and fails to progress to the vitellogenic stage. As a result, current research on the reproduction of the Japanese eel relies on the artificial induction of parent fish. In a previous experiment, we observed that oocytes from a small proportion of female individuals exhibited undeveloped ovaries, arrested at the perinucleolar stage, despite receiving the same hormone treatment as the fully developed individuals. In this study, we conducted a transcriptome analysis of brain (including pituitary) and ovary tissues from both normally developed and undeveloped Japanese eels. Our aim was to investigate the underlying causes from the perspective of the brain-pituitary-gonad axis, thereby providing practical insights and theoretical support for the artificial reproduction of Japanese eels.

2. Materials and methods

2.1. Animals

All fish experiments were approved by the respective Animal Research and Ethics Committees of Ocean University of China (Permit Number: 20141201). The present study did not involve endangered or protected species.

All female Japanese eels weighed between 0.8 and 1.0 kg and were sourced from a wholesale aquatic market in Qingdao, China. After a one-month seawater acclimatization period, the eels were administered injections of human chorionic gonadotropin (hCG) (NSHF, Ningbo, China) and luteinizing hormone-releasing hormone analogues (LHRH-A2) (NSHF, Ningbo, China). Injections were administered once a week for a total of 10 weeks. Prior to sampling, all eels were anesthetized using an appropriate anesthetic, and the samples were immediately stored under proper conditions.

2.2. Haematoxylin-eosin (H&E) staining

Ovarian tissues were fixed in 4 % paraformaldehyde, embedded, and sectioned into 7-μm slices. The sections were stained with hematoxylin and eosin and subsequently observed and photographed under an Olympus bright-field light microscope (Olympus, Tokyo, Japan).

2.3. RNA extraction

Total RNA was extracted using SparkZol Reagent (Sparkjade, Jinan, China). RNA quantity and quality were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA).

2.4. RNA isolation and library construction

RNA was extracted from the brain (including pituitary) and ovarian tissues of six Japanese eels (n = 3 per group) for transcriptional analysis. The NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, United States) was employed to generate nine sequencing libraries according to the manufacturer, and index codes were added to attribute sequences to each sample. The samples were sequenced on an Illumina HiSeq X Ten platform, and 150-bppaired-end reads were generated.

2.5. Differentially expressed genes (DEGs) identification and enrichment analysis

The clean data obtained by removing low-quality sequences and adaptors of raw read were aligned to the reference *Anguilla japonica* genome (PRJNA852364) with HISAT2 (Kim et al., 2015). The quantification analyses were accomplished using the StringTie software (Pertea et al., 2016). Specifically, the multiple mapped reads were removed, and the count numbers of unique mapped reads and FPKM (Fragments Per Kilobase Per Million) were assessed and normalized with previous references (Anders et al., 2015). Principal component analysis (PCA), referred to investigate the relationship between groups, was performed by the ggplot2 package.

With a cutoff "padj" < 0.05 and absolute foldchange values greater than 1, statistical analysis of transcripts was marked as significantly differentially expressed genes (DEGs) using the DESeq2 package. Additionally, the DEGs were assigned to Gene Ontology (GO) classification by the aid of the Blast2GO program with the p-value threshold < 0.05 (Götz et al., 2008), meanwhile Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis (KEGG, a database of biological systems, https://www.genome.jp/kegg/) was performed to significant pathway enrichment analysis. Cluster Profiler R package was employed to test the statistical enrichment of DEGs in KEGG pathways with the threshold of p-value threshold < 0.05 (Kanehisa et al., 2017).

2.6. Protein-protein interaction (PPI) analysis and hub gene identification

The protein sequences of DEGs in the BP and O groups were extracted separately using TBtools software (Chen et al., 2020), and the PPI network was predicted using the STRING website (https://cn.string-db.org/). Only interaction relationships with confidence greater than 0.4 were considered reliable and imported into Cytoscape software for hub gene identification and visualization (Yu et al., 2024). The hub genes were identified using the degree algorithm in the cytoHubba plugin (Zhang and Feng, 2023).

3. Results

3.1. Morphological and histological observation of Japanese eels with normal and undeveloped ovaries

As shown in Fig. 1, the pectoral fin of Japanese eels with developed ovaries appeared black, and a large ovary was visible upon dissection of the abdominal cavity. Histological examination of ovary tissue sections revealed that the follicles were in the vitellogenic stage. In contrast, the pectoral fin of Japanese eels with undeveloped ovaries was gray and transparent, and the ovaries were noticeably small. Histological observations revealed that the follicles were at the perinucleolar stage.

3.2. Analysis report of brain and pituitary transcriptome

Six groups of cDNA libraries from six Japanese eel brain and pituitary tissues were performed to make transcriptional analysis (n = 3). In total, 271,506,286 raw reads were obtained by sequencing, and the raw reads were filtered to 261,988,718 clean reads. The average clean reads of undeveloped group (BP_UD) and developed group (BP_D) were 44,216,015 and 43113558, with Q30 average percentages of 92.77 and 92.98, respectively (Supplementary material 3). The BioProject accession number of raw sequence is PRJNA1212544 on the Short Read Archive (SRA) of the National Center for Biotechnology Information (NCBI).

As shown in Fig. 2**A**, the PCA demonstrated significant differences between the BP_UD and BP_D groups. A total of 624 DEGs (Supplementary material 1) were identified in BP_UD vs BP_D group, of which 220 genes were up-regulated and 404 genes were down-regulated (Fig. 2**B**). In this study, we focused on down-regulated genes in BP_UD vs

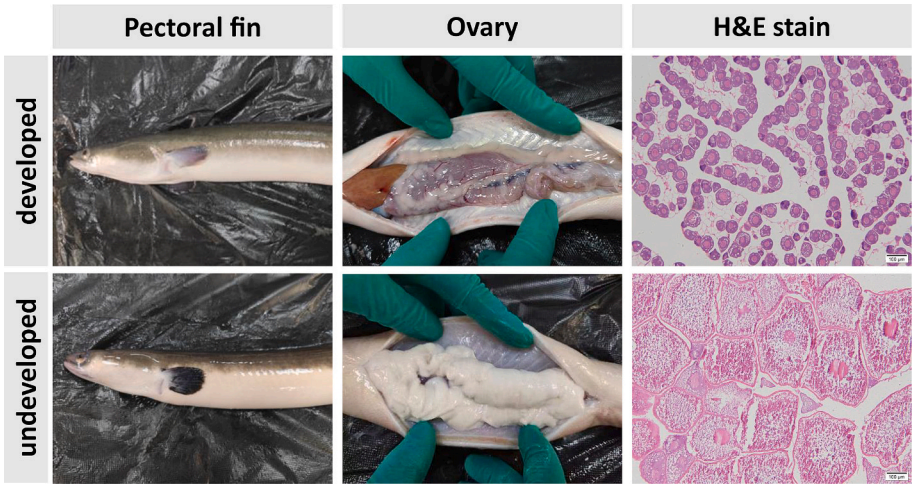

Fig. 1. Comparison of Japanese eel pectoral fins, ovaries, and ovarian histological observations with normal and abnormal ovary development, respectively.

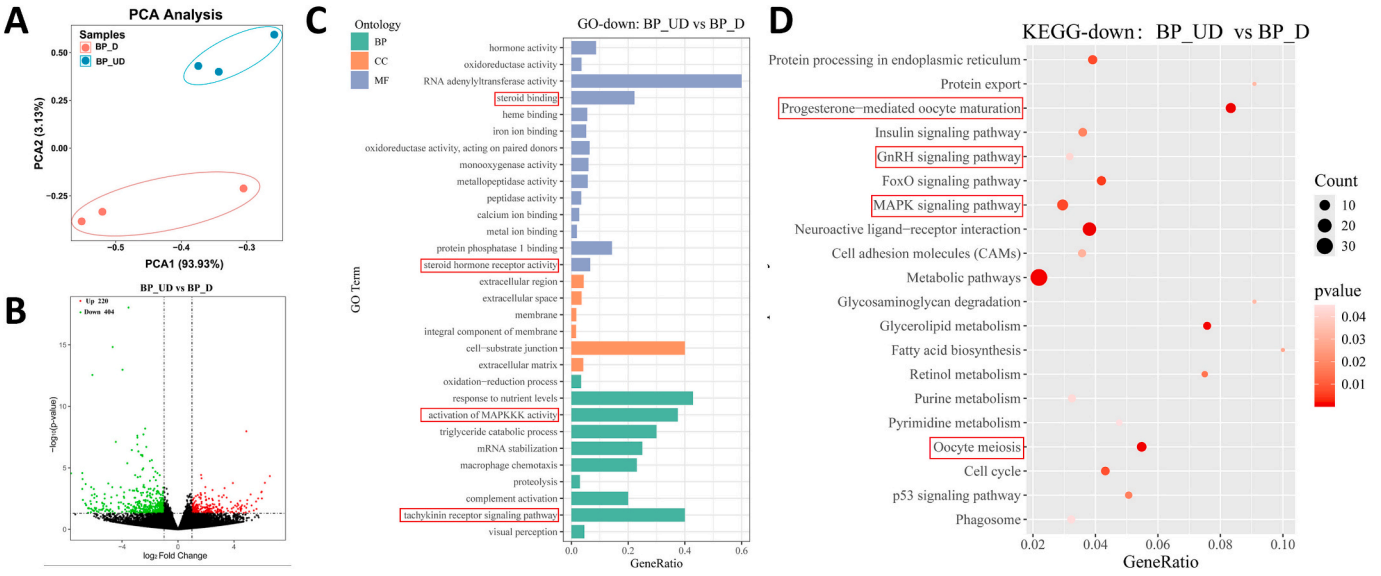

Fig. 2. (A) PCA of the six libraries in the brain and pituitary transcriptome (BP_UD1, BP_UD2, BP_UD3, BP_D1, BP_D2, BP_D3). (B) The Volcano plots for the brain and pituitary transcriptome. (C) GO terms of down-regulated DEGs in BP_UD vs BP_D. (D) KEGG analysis of down-regulated DEGs in BP_UD vs BP_D. The red square in (C, D): The key GO terms and KEGG pathways enriched by the differentially expressed genes related to reproduction.

BP_D group. GO enrichment analysis showed that down-regulated genes were enriched in terms such as *hormone activity*, *steroid binding*, *activation of MAPKKK activity*, *steroid hormone receptor activity* and *tachykinin receptor signaling pathway* (Fig. 2**C**). KEGG enrichment analysis identified 20 pathways. Among them, there were *Progesterone-mediated oocyte maturation*, *oocyte meiosis*, *GnRH signaling pathway* and *MAPK signaling pathway* related to ovary development (Fig. 2**D**).

3.3. Analysis report of ovary transcriptome

Six cDNA libraries of Japanese eel ovaries were constructed and sequenced on the Short Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) (Accession number: PRJNA1212544). A total of 252,963,514 raw reads were obtained by high-throughput sequencing, and 243,886,866 clean reads were filtered. In addition, the average clean reads of undeveloped group (O_UD) and developed group (O_D) were 37,568,232 and 43727390, with Q30 average percentages of 94.24 and 94.23, respectively (Supplementary material 3).

The PCA demonstrated significant differences between the O_UD and O_D groups (Fig. 3**A**). In total, 802 significant DEGs (Supplementary material 2) were identified, including 600 up-regulated genes and 202 down-regulated genes in O_UD group compared with O_D group (Fig. 3**B**). GO enrichment analysis showed that down-regulated genes were classified into molecular function (terms: *DNA helicase activity*, *DNA binding*, *hydrolase activity*, *acting on acid anhydrides*, etc.), cellular component (terms: *integral component of endoplasmic reticulum membrane*, *Golgi apparatus*, *nucleolus*, etc.), and biological process (terms: *rRNA base methylation*, *negative regulation of intrinsic apoptotic signaling pathway*, *regulation of transcription, DNA − templated*, *DNA replication*, etc.) (Fig. 3**C**). As shown in Fig. 3**D**, *Wnt signaling pathway*, *Metabolic pathways*, *steroid biosynthesis*, *GnRH signaling pathway*, and *Protein processing in endoplasmic reticulum* related to ovary development were identified by KEGG enrichment.

3.4. PPI network and hub genes of O and BP tissue

To explore the correlations of these DEGs, the PPI networks were constructed, and the hub genes were identified. As shown in Fig. 4, there were 327 nodes and 476 edges in BP groups, 87 nodes and 94 edges in O

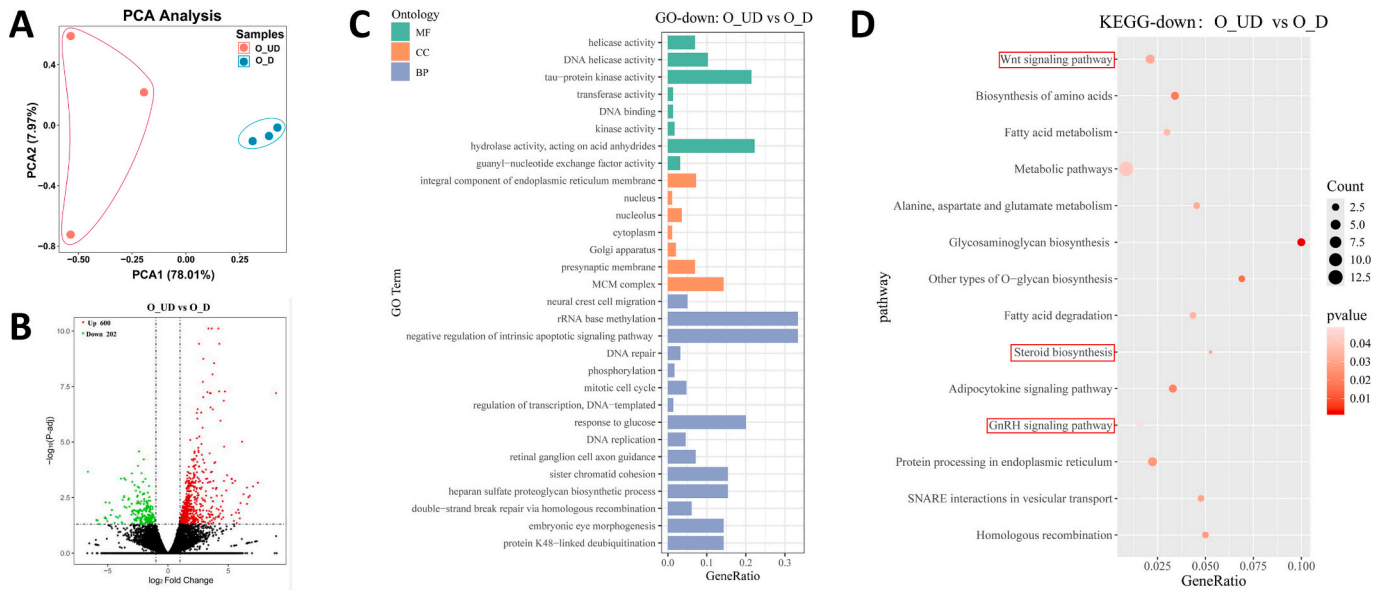

Fig. 3. (A) PCA of the six libraries in the ovary transcriptome (O_UD1, O_UD2, O_UD3, O_D1, O_D2, O_D3). (B) The Volcano plots for the ovary transcriptome. (C) GO terms of down-regulated DEGs in O_UD vs O_D. (D) KEGG analysis of down-regulated DEGs in O_UD vs O_D. The red square in (C, D): The key GO terms and KEGG pathways enriched by the differentially expressed genes related to reproduction.

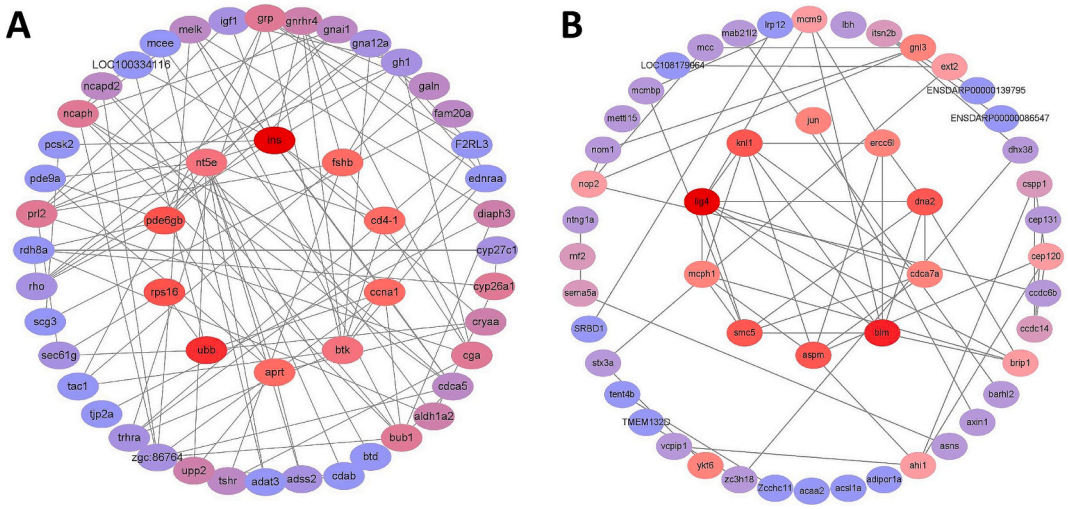

Fig. 4. Protein-Protein interactions (PPI) network of DEGs and the identified hub genes for (A) BP groups, (B) O groups, respectively. The top 50 nodes of degree were visualized. The color of the nodes represented the degree and the hub gene is located in the middle of the PPI networks.

groups, respectively. For BP groups, a total of 10 genes, including *ins*, *ubb*, *pde6gb*, *rps16*, *aprt*, *btk*, *ccna1*, *ccd4-1*, *fshb* and *nt5e* were considered as hub genes (Fig. 4**A**). Special attention should be given to *btk*, *ccna1* and *fshb*, whch related to ovary development. For O groups, a total of 10 genes, including *lig4*, *blm*, *dna2*, *smc5*, *aspm*, *knl1*, *mcph1*, *jun*, *ercc6l* and *cdca7a* were considered as hub genes (Fig. 4**B**). It is particularly noteworthy that *aspm* and *mcph1* participate in gonad development.

4. Discussion

The follicle, composed of an oocyte and surrounding follicular cells, represents the fundamental structural and functional unit of the ovary (Chen et al., 2022). Oogenesis, which encompasses folliculogenesis, growth, and maturation, is a complex physiological process involving multiple endocrine regulatory mechanisms (Lubzens et al., 2010; Nagahama et al., 1995; Xu et al., 2010). In tiger puffer (*Takifugu rubripes*), transcriptome analysis of the ovary at different developmental stages suggests that multiple genes in ovarian steroidogenesis, estrogen-mediated signaling pathways, and TGF-β signaling pathways exhibit differential expression at various stages of development, implying that these genes play important roles in ovarian development (Hu et al., 2025). In studies of the Greater Amberjack (*Seriola dumerili*), it was found that genes involved in steroid hormone biosynthesis, lipid metabolism, and the arrest and resumption of meiosis exhibit differential expression during ovarian development from stages III to V (Yang et al., 2025). Furthermore, dysfunction of certain genes can lead to reproductive disorders, as demonstrated in both female and male fish studies (Lavecchia et al., 2023, 2024). These findings suggest that oogenesis is not solely regulated by gonadotropins (GtH), but rather is actively coordinated through the influence of multiple growth factors produced by both the oocyte and its surrounding follicular cells (Ge, 2005; Li et al., 2023; Monget and Bondy, 2000; Song et al., 2022).

In this study, we observed that a small proportion of female Japanese eels subjected to artificial induction had very small ovaries, resembling

those of underdeveloped individuals. Histological examination revealed that their follicles were at the perinucleolar stage. Furthermore, differences in pectoral fin coloration further indicated the varying degrees of ovarian development between the two groups of female Japanese eels (Okamura et al., 2007). This phenomenon may be caused by the abnormal expression of certain reproduction-related genes. Therefore, to investigate the potential causes of abnormal ovarian development in some Japanese eels, this study conducted a transcriptional analysis from the perspective of the brain-pituitary-gonad axis.

In the transcriptomes of the brain and pituitary, differential genes were enriched in the tachykinin receptor signaling pathway, including the *tac1* gene. The tachykinin (TAC) family is involved in the regulation of neuropeptide secretion and plays a role in regulating GnRH synthesis and secretion (Dahl et al., 2009; Kinoshita et al., 2005), as well as the initiation of puberty during gonadal development (Bhangoo and Jacobson-Dickman, 2009; Topaloglu et al., 2009). Studies in mice (*Mus musculus*) have shown that knockout of *tac1* inhibits follicular development (Kawada et al., 2025). The low expression of the *tac1* may inhibit the development of primordial follicles in Japanese eels, potentially contributing to the ovarian developmental issues observed in this study.

MAPK (mitogen-activated protein kinase) is a crucial mediator of signals from the cell surface to the nucleus, playing key roles in cell proliferation, differentiation, carcinogenesis, metastasis, and apoptosis (Gao and Zheng, 2024). Research has demonstrated that the MAPK signaling pathway also plays essential roles in gonadal development (Paranjpe et al., 2024; Ren et al., 2024). The MAPK signaling pathway and activation of MAPKKK activity were enriched in the brain and pituitary transcriptomes, with differential expressed genes such as *gadd45*, *ntf3*, *hgf*, and *nr4a1*. In mice, *gadd45* is involved in gonadal differentiation and development (Johnen et al., 2013). NTF3 is associated with the proliferation of ovarian cells (Chen et al., 2025). HGF regulates folliculogenesis and steroidogenesis by modulating the function of follicular membrane cells and granulosa cells in the ovary (Mi et al., 2024). In studies of the Jinhu groupers (*Epinephelus fuscoguttatus* ♀ × *Epinephelus tukula* ♂), differential expression of *nr4a1* was found to potentially influence the rate of gonadal development (Qiu et al., 2024). These findings suggest that the aforementioned genes may be involved in the abnormal ovarian development observed in Japanese eels in this study.

DEGs in oocyte meiosis pathway were significantly enriched in the brain and pituitary transcriptome. Meiosis accompanies the whole process of oogenesis, both the initiation of meiosis in the early developmental stage and the resumption of meiosis in the mature stage are essential during oogenesis (Bowles and Koopman, 2007; Fabra et al., 2006; Swain, 2006). In this study, differential genes associated with meiosis include *igf1*, *adcy3*, and *smc1b*. Studies have shown that IGF-I is closely associated with granulosa cell mitosis at the onset of vitellogenesis (Kagawa et al., 1995), while ADCY3 is crucial in the activation of primordial follicles (Zheng et al., 2023). The deletion of the *smc1b* gene leads to dysfunction of cadherin proteins and results in the loss of fertility in mice (Takabayashi et al., 2009).

Sex steroid hormones play a crucial role in yolk accumulation and oocyte maturation (Nagahama and Yamashita, 2008; Nakamura et al., 2005). During early vitellogenesis, serum estradiol levels increase significantly, promoting the synthesis and secretion of vitellogenin by the liver, which is essential for vitellogenesis (Lubzens et al., 2010; Peyon et al., 1996; Tyler et al., 1991). As the final stage of oogenesis, maturation is closely regulated by steroid hormones (Goetz, 1997; Goetz and Garczynski, 1997). At this stage, follicle cells transition from primarily producing estradiol to synthesizing maturation-inducing steroids, which activate maturation-promoting factors in the oocyte, thereby facilitating maturation (Lubzens et al., 2010; Nagahama and Yamashita, 2008).

The analysis of the two transcriptomes revealed that differential genes were enriched in steroid-related functions, such as steroid binding (*prgr*), steroid hormone receptor activity (*prgr*, *nr4a1*, *nr5a2*), steroid biosynthesis (*cyp11a1*, *cyp24a1*, *cyp1b1*, *srd5a2*), and the progesterone-mediated oocyte maturation (*ccna1*, *igf1*, *adcy3*, *gnai1*). *Prgr* is involved in the generation of 20α-hydroxysteroid dehydrogenase in the corpus luteum (Sugino et al., 1997). During steroidogenesis, *nr4a1* regulates the transcription of key steroidogenic enzymes in ovarian membrane cells, while the deletion of *nr5a2* affects follicular development and steroidogenesis (Han et al., 2025; Li et al., 2010). Among the differential genes in the steroid biosynthesis signaling pathway, *cyp11a1* and *cyp1b1* have been confirmed to play roles in steroidogenesis in multiple species (Chaube et al., 2021; Li et al., 2024; Yang et al., 2025), while *cyp24a1* is involved in the metabolism of vitamin D3, thereby regulating the reproductive process (Hrabia et al., 2023). *Srd5a2* is highly expressed in the ovary during the luteal phase, suggesting that it may be involved in progesterone synthesis (Liu et al., 2020). In the progesterone-mediated oocyte maturation signaling pathway, over expression of *ccna1* promotes the resumption of meiosis but also inhibits oocyte maturation, which may be related to its involvement in regulating the production of maturation-inducing steroids (Li et al., 2020). IGF-1 has been shown to participate in gonadal steroidogenesis in *Acipenser ruthenus* (Wuertz et al., 2007) and regulate the expression of P450 aromatase in the ovary (Nakamura et al., 2003). ADCY3 is involved in regulating the expression of estrogen receptors, while *gnai1* plays a role in regulating progesterone-mediated oocyte maturation (Redei et al., 2021; Yang et al., 2018). It can be inferred that the abnormal expression of steroid-related genes is one of the potential causes of ovarian developmental abnormalities in Japanese eels observed in this study.

In the ovary transcriptome, differentially expressed genes were significantly enriched in Wnt signaling pathway. Studies in mice have shown that Wnt signaling pathway is involved in the activation of primordial follicles (Takase et al., 2024). In *Gallus gallus*, Wnt signaling pathway participated in the proliferation of ovarian granulosa cells and could regulate the cell cycle (Ma et al., 2024; Nie et al., 2024). In this study, the enriched reproduction-related differential genes include *bambi* and *axin1*. Research has shown that *bambi* is regulated by follicle-stimulating hormone (FSH) and plays a role in oocyte development, while the deletion of *axin1* can lead to abnormalities in meiosis (Bai et al., 2014; He et al., 2016). This suggests that the Wnt signaling pathway plays a crucial role in ovarian development, and the abnormal expression of certain genes within this pathway may lead to ovarian developmental failure.

The GnRH signaling pathway is a key regulator of ovarian development and plays a crucial role in oogenesis (Laws et al., 2014; Leng et al., 2024; X. Li et al., 2022). In this study, both transcriptomes showed differential genes enrichment in the GnRH signaling pathway, including *adcy3*, *gnrhr4*, *cga*, and *fshb*. In the pituitary of grass carp (*Ctenopharyngodon idella*), the expression level of *gnrhr4* is significantly higher than that of the other three gonadotropin-releasing hormone (GnRH) receptors, which indicates its crucial role in mediating GnRH signaling (W. Li et al., 2022). *Cga* and *fshb*, as the two subunits of follicle-stimulating hormone (FSH), directly influence FSH levels, thereby further affecting ovarian development (Nagahama et al., 1995). We speculate that the abnormal expression of *cga* and *fshb* is a key factor contributing to ovarian developmental failure in Japanese eels.

PPI analysis revealed the interaction relationships among differential genes, involving several genes associated with gonadal development, including *btk*, *ccna1*, *fshb*, *aspm*, and *mcph1*. *Ccna1* and *fshb* have been discussed earlier. Additionally, studies have shown that *btk* is involved in ovarian angiogenesis (Zippo et al., 2004). In mice, mutations in the *mcph1* lead to infertility and the development of ovarian tumors in females, while mutations in the *aspm* result in a reduced number of follicles and a decrease in ovarian size (Liu et al., 2021; Mori et al., 2022; Wang et al., 2022).

In this paper, we investigated the underlying causes of ovarian developmental failure in artificially induced Japanese eels. Transcriptome analysis revealed that differentially expressed genes were

involved in multiple physiological processes and functional pathways related to gonadal development. Notably, the abnormal expression of *cga* and *fshb*, the two subunits of FSH, was identified as a key factor contributing to ovarian developmental failure. Additionally, several other reproduction-related genes were also implicated in this process. Therefore, we propose that ovarian developmental abnormalities in Japanese eels result from the combined effects of multiple genes, ultimately leading to abnormal ovarian development and follicular arrest at the perinucleolar stage.

CRediT authorship contribution statement

Chenpeng Zuo: Writing – original draft, Methodology, Investigation, Conceptualization. **Yonghang Zhang:** Software, Investigation, Data curation. **Xuanhan Zhang:** Data curation. **Jiaqi Liu:** Data curation. **Likang Lyu:** Data curation. **Teng Ma:** Resources. **Lingming Chen:** Resources. **Weimin Yu:** Resources. **Yun Li:** Resources. **Haishen Wen:** Resources, Conceptualization. **Xin Qi:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2025.114729.

Data availability

Data will be made available on request.

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