Received: 20 January 2020 | Revised: 20 May 2020 | Accepted: 25 May 2020

DOI: 10.1002/dvdy.213

PATTERNS & PHENOTYPES

Developmental Dynamics WILEY

GFP expression pattern in pituitary and gonads under the control of nuclear progesterone receptor promoter in transgenic zebrafish

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Funding information
Program for New Century Excellent Talents in Fujian Province University; National Natural Science Foundation of China, Grant/Award Numbers: 41976092, 31672628

Abstract

Background: The nuclear progesterone receptor (Pgr) is a ligand-dependent transcription factor primarily responsible for mediating progesterone actions relevant for reproduction across vertebrates. Information on the cellular localization of Pgr expression in the reproductive system is required for developing a comprehensive approach to elucidate the role of Pgr in reproduction.

Results: We generated transgenic zebrafish *Tg(pgr:eGFP)* that express enhanced green fluorescent protein (eGFP) driven by promoter sequence of *pgr* gene. The tissue distribution pattern of *egfp* mRNA is consistent with the *pgr* mRNA expression in *Tg(pgr:eGFP)*. In the pituitary, GFP signals are found in the proximal pars distalis. In order to better discern the cellular localization of GFP signals in gonads, *Tg(pgr:eGFP)* line was crossed with *Tg(gsdf:nfsB-mCherry)* line, specifically expressing nitroreductase-mCherry fusion protein in granulosa and Sertoli cells in ovary and testis, respectively. Imaging of testis tissue showed that GFP expression was confined to Leydig cells. In the ovary, GFP expression colocalized with the mCherry signal in granulosa cells. Intriguingly, we also identified some non-granulosa cells close to where blood vessels branched, expressing stronger GFP signals than granulosa cells.

Conclusions: Analyzing *Tg(pgr:eGFP)* expression in zebrafish provided leads toward new routes to study the role of Pgr in reproduction.

KEYWORDS

gonad, nuclear progesterone receptor, pituitary, promoter, transgenic GFP expression, zebrafish

1 | INTRODUCTION

Progestagenic sex steroid hormones play critical roles in reproduction across vertebrates. The biological activity of progestin is mediated via specific receptors. The classic nuclear progesterone receptor (Pgr), a member of the nuclear receptor superfamily,[1] functions primarily as a ligand-dependent transcription factor to regulate target gene expression,[2] while membrane-associated Pgrs are involved in nongenomic mechanisms.[3] To date, only a single *pgr* gene has been reported in the majority of vertebrates, except for *Xenopus laevis*,[4] Japanese eel (*Anguilla japonica*),[5,6] and European eel (*Anguilla anguilla*),[7] in which two distinct paralogous copies of *pgr* genes have been described. In humans, rodents, and chicken, two isoforms (A and B), encoded by a single *PGR* gene but originating from different translational initiation sites at two in-phase ATG codons, have been reported.[8,9] In teleost fish species

studied to date, only gilthead seabream (*Sparus aurata*) exhibit alternative splicing of the *pgr*.[10]

Studies using *Pgr* knockout (KO) mice, which was generated using CRE-loxP gene-targeting approach, indicated that only PGR-A is both necessary and sufficient to elicit the progesterone-dependent reproductive responses required for female fertility, while PGR-B is needed to elicit the normal proliferative responses of the mammary gland to progesterone.[2] Considering the data in mice and the predominant expression of *pgr* in the gonads of different species, we assume that the major function of Pgr is to mediate the effect of the teleost progestins, 17,20β-dihydroxypregn-4-en-3-one (DHP)[11] and 17,20β,21-trihydroxy-pregn-4-en-3-one (20β-S),[12] on reproductive physiology. Indeed, in zebrafish (*Danio rerio*), *pgr* KO females, which was generated using TALEN-mediated gene-editing technology, was infertile due to ovulation defects.[13] The *pgr* KO males were fertile, but *fshb* transcripts levels no longer responded to DHP.[14] In tilapia (*Oreochromis niloticus*), *pgr* KO males, which was generated using CRISPR/Cas9 technology, showed subfertility, reflected by significantly lower gonadosomatic indices, sperm counts and motility, reduction of all stages of germ cells.[15] Although results from these *pgr* KO models provide clear evidence that the Pgr plays important roles in fish reproduction, the molecular mechanisms underlying these phenotypes remain to be further elucidated.

The identification of Pgr-expressing cell types in reproductive organs is a critical first step to investigate molecular mechanism regulated by Pgr. So far, research on the cellular localization of Pgr in vertebrate gonads often provided controversial results. In human testis, PGR mRNA and protein have been detected in spermatogenic, Sertoli, and Leydig cells,[16,17] or PGR protein expression was reported in a few peritubular and interstitial cells, but not in germ cells.[18] In a murine Leydig cell, tumor line PGR was not detected,[19] while *Pgr* promoter-driven LacZ expression was induced by intratesticular hormonal deprivation in mouse Leydig cells.[20] In zebrafish testis, the *pgr* mRNA was detected in Leydig and Sertoli cells,[21] while Pgr protein was detected in spermatogonia and spermatocytes.[22] So far, most of these studies on the cellular localization of PGR were mainly conducted by immunohistochemistry (IHC) and/or in situ hybridization (ISH). IHC and ISH may show inconsistent results due to methodological issues, such as the fixation used, the specificity of antibodies or cRNA probes, or techniques used to detect the primary antibodies or cRNA probes. Therefore, the cellular localization of PGR in the reproductive system requires further studies using other approaches.

In the present study, in order to investigate spatial and temporal expression patterns of the *pgr* gene in zebrafish, we have used the Tol2 transposon system to generate a transgenic zebrafish strain *Tg(pgr:eGFP)*, in which a ~2.8-kb proximal promoter region is fused to the coding region of enhanced green fluorescent protein (eGFP). The cloned promoter region mimicked the expression profile of endogenous *pgr* transcripts in zebrafish. To better identify the gonadal cell types expressing GFP driven by the *pgr* promoter, the *Tg(pgr:eGFP)* strain was crossed with another transgenic zebrafish strain, *Tg(gsdf:nfsB-mCherry)*, which expresses mCherry protein in gonads under the control of the promoter of zebrafish *gonadal soma-derived factor* (*gsdf*) gene, resulting in transgene expression in Sertoli and granulosa cells.[23] The results of the cellular localization of *pgr* gene expression in pituitary and gonads give information on the identity of the Pgr target cells of these central tissues of the reproductive system, providing the required basis for future, targeted studies on progestogen functions in these tissues.

2 | RESULTS

2.1 | Establishment of transgenic zebrafish lines

In order to compare the tissue expression profile of the *Tg(pgr:eGFP)* transgene with the endogenous *pgr* gene, the levels of the *egfp* and endogenous *pgr* transcripts were determined by reverse transcription PCR of seven tissues. Similar to our previous observations,[21] *pgr* mRNA was detected in several organs (testis, ovary, brain, pituitary, and kidney), but not in the heart and liver (Figure 1A). *Egfp* mRNA showed a similar tissue expression pattern (Figure 1B), suggesting that the ~2.8 kb promoter fragment contained the regulatory elements required to reliably convey the *pgr*'s tissue-specific expression pattern.

2.2 | Cellular localization of GFP under the control of *pgr* promoter in the pituitary

The pituitary of zebrafish is below the brain and lay within a hypophyseal fossa. After removing the brain, the pituitary can be found by using a fluorescent dissecting microscope, the GFP signals being in the middle of the pituitary (Figure 2A-C). The dorsal view of the pituitary showed that the GFP-positive cells was located on the proximal pars distalis (PPD), occupying the major part of PPD (Figure 2D). Most of the GFP-positive cells were associated in large aggregates, whereas only a few were scattered as single cells (Figure 2D). A lateral view of the pituitary confirmed that the GFP-positive cells located to the PPD (Figure 2E), in particular to its central and ventral parts (Figure 2E).

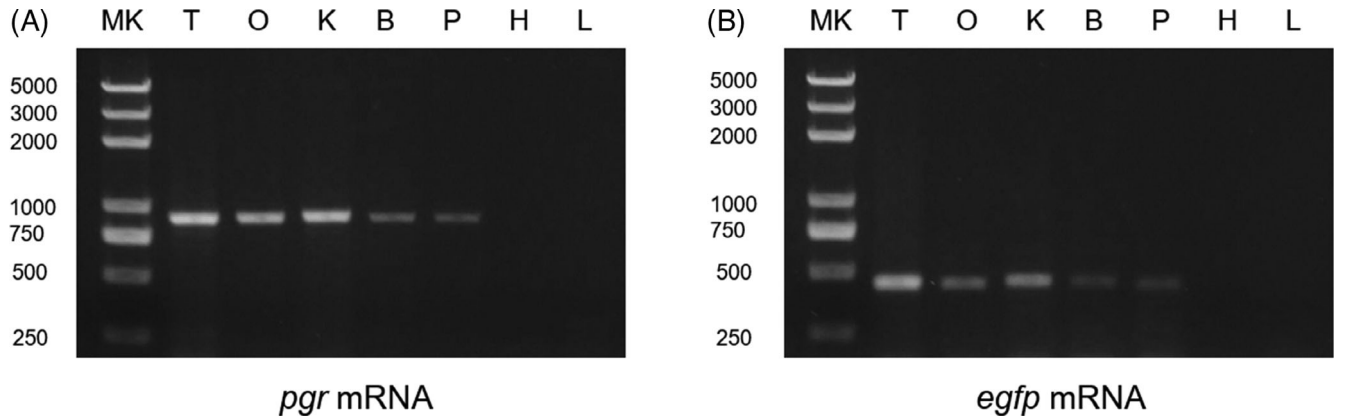

FIGURE 1 The gene expression of *pgr* and *egfp* in the *Tg(pgr:eGFP)* zebrafish. The tissue expression pattern of the endogenous *pgr* gene (A) and *egfp* gene (B) was assessed on reverse-transcribed total RNA by PCR on zebrafish transgenic lines. The pituitary sample was pooled from 20 fish, and except the ovary, other tissues were collected from the same adult fish. B, brain; eGFP, enhanced green fluorescent protein; H, heart; K, kidney; L, liver; MK, marker; O, ovary; P, pituitary; pgr, progesterone receptor; T, testis

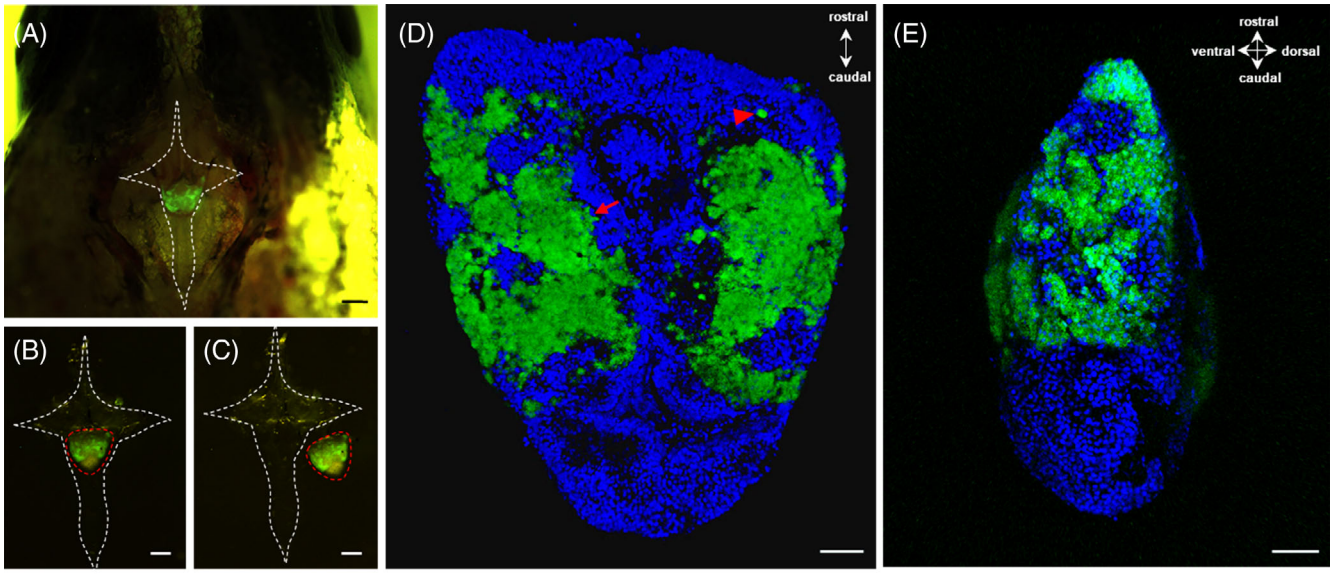

FIGURE 2 Cellular localization of green fluorescent protein (GFP) under the control of *pgr* promoter in the pituitary. A, The pituitary expressing GFP signals laid within a hypophyseal fossa. The white dash line indicated a cross-shaped bone containing the pituitary. B, Dissected cross-shaped bone (white dash line) containing the pituitary (red dash line). C, The intact pituitary (red dash line) was collected by breaking the bone (white dash line). D, Dorsal and, E, side view of the pituitary indicated the expression of GFP in the proximal pars distalis (PPD), and most of the GFP-positive cells normally formed aggregates (red arrow), whereas a few were scattered as single cells (red arrowhead). The blue fluorescence represented the nucleus which was stained by Hoechst33342. Scale bars = 200 μm (A, B, C) and 50 μm (D, E). Pgr, progesterone receptor

2.3 | Cellular localization of GFP under the control of *pgr* promoter in testis

Direct observation of the intact testis of *Tg(pgr:eGFP)* showed strong GFP signals in a mesh or network topology (Figure 3A, B). Cryosections showed germ cells at different stages presented in the spermatogenic tubules. GFP-positive cells were present in the intertubular compartment (Figure 3C, D). Direct observation of the whole testis of double transgenic fish *Tg(pgr:egfp/gsdf:nfsB-mCherry)* showed that there was no colocalization of GFP and mCherry signals (Figure 3E, F). The mCherry signal was present within spermatogenic tubules, delineating the borders of the spermatogenic cysts (Figure 3F). Analyzing cryosections

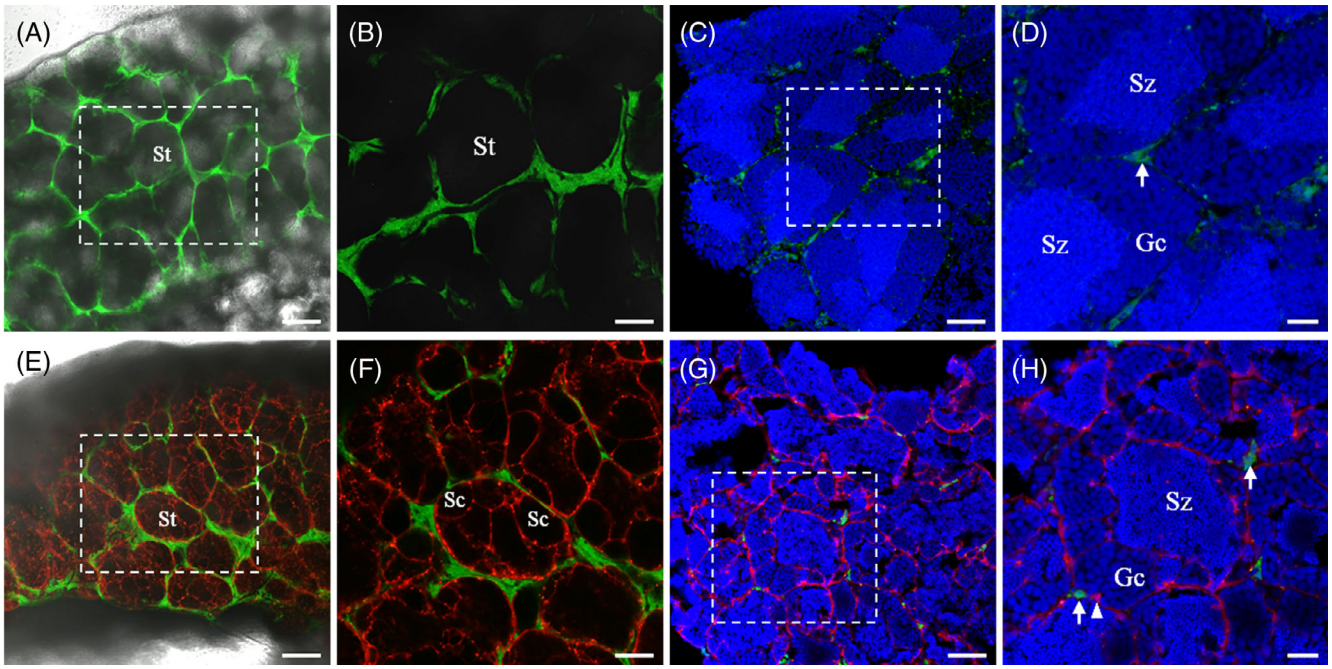

FIGURE 3 Cellular localization of green fluorescent protein (GFP) under the control of *pgr* promoter in testis. Intact (A, B) and cryosection (C, D) of testis of the *Tg(pgr:eGFP)* showed GFP signals at intertubular compartments. In Intact (E, F) and cryosection (G, H) of testis of the *Tg(pgr:egfp/gsdf:nfsB-mCherry)* showed GFP signals and mCherry signals presented at intertubular compartments and spermatogenic cysts, respectively. Note that there was no colocalization of GFP and mCherry signals in testis. B, D, F, and H correspond to a higher magnification of the quadrangle area indicated by the dash line in A, C, E, and G, respectively. The blue fluorescence represented the nucleus which was stained by Hoechst33342. Gc, germ cell; eGFP, enhanced green fluorescent protein; Pgr, progesterone receptor; Sc, spermatogenic cysts; St, spermatogenic tubules; Sz, spermatozoa; white arrow, Leydig cell; white arrowhead, Sertoli cells. Scale bars = 100 μm (A, E), 50 μm (B, C, F, G), and 20 μm (D, H).

similarly showed distinctly localized GFP and mCherry signals in the intertubular and intratubular compartments, respectively (Figure 3G, H). In the cystic type of spermatogenesis typically found in fish, the epithelium of the spermatogenic tubules is composed of spermatogenic cysts, where thin cytoplasmic extensions of Sertoli cells (being mCherry-positive in our model) envelope a developing germ cell clone. The GFP-positive cells, on the other hand, were located in the intertubular compartment.

2.4 | Cellular localization of GFP under the control of *pgr* promoter in the ovary

The zebrafish ovarian follicle is completely surrounded by a follicle cell layer. Direct observation of the surface of intact ovarian follicles of *Tg(pgr:eGFP)* showed that some of the follicle cells expressed GFP signals in the follicular layer (Figure 4). In primary growth follicle, the GFP signals were rarely found. During follicle maturation, the number of GFP-positive cells gradually increased, reaching the maximum when the oocytes attained the fully grown stage (Figure 4).

The follicle cell layer consists of the inner granulosa cell and the outer theca cell layer. To identify whether the GFP signals were present in the granulosa cell layer and/or theca cell layer, we generated double transgenic fish *Tg(pgr:egfp/gsdf:nf3sB-mCherry)* that express mCherry in granulosa cells.[23] As expected, mCherry-positive granulosa cells formed a ring surrounding the oocyte (Figure 5A). The GFP-positive cells also form a ring, and almost all the GFP signals colocalized with mCherry (Figure 5B-D), suggesting that, under the control of *pgr* promoter, GFP is expressed in granulosa cells. Intriguingly, a few GFP-positive cells displayed a much stronger signal than others (Figure 5B). Those strongly GFP-positive cells were only found in the outer theca cell layer and did not express mCherry (Figure 5E-H). The cells expressing both, GFP and mCherry, localized exclusively to the inner granulosa cell layer (Figure 5E-H).

Direct observation of the surface of intact ovarian follicles also showed a few GFP-positive cells with an irregular shape expressing a stronger GFP signal than the more round granulosa cells that expressed both GFP and mCherry (Figure 6A). Remarkably, the mCherry signal was found in granular spots in the granulosa cells

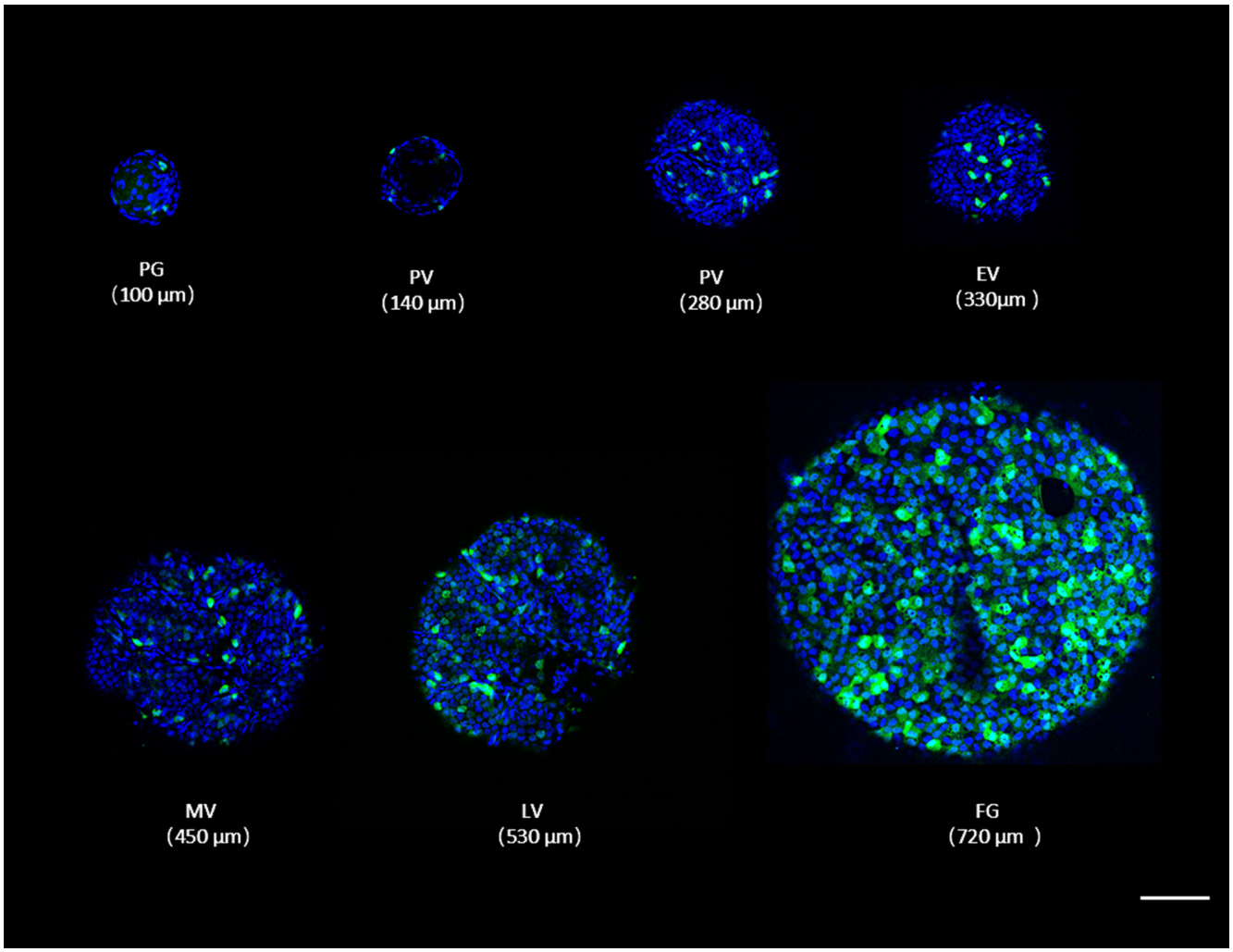

FIGURE 4 Cellular localization of green fluorescent protein (GFP) under the control of *pgr* promoter in ovarian follicles during folliculogenesis in *Tg(pgr:eGFP)*. The number of GFP expressing cells increases during folliculogenesis. The diameter of the ovarian follicle was indicated within brackets. The blue fluorescence represented the nucleus which is stained by Hoechst33342. Scale bars = 100 μm. eGFP, enhanced green fluorescent protein; EV, early-vitellogenic; FG, full-grown stage; LV, late-vitellogenic; MV, mid-vitellogenic; PG, primary growth; Pgr, progesterone receptor; PV, pre-vitellogenic

(Figure 6B), which may reflect aggregations often seen with mCherry as part of a fusion protein.[24] Higher magnifications showed that mCherry signals were absent in the cells expressing strong GFP signals, again suggesting that these cells are not granulosa cells (Figure 6C). On the surface of the ovarian follicle, a network of interconnected, unstained (dark) channels was visible in samples from double transgenic fish (Figure 6A). The cells closely adjacent to the dark channels displayed neither GFP nor mCherry; the shape of their cell nuclei is different from cells expressing GFP and/or mCherry signals. The channel network was reminiscent of the blood vessel system on the surface of ovarian follicles, as suggested by the fli promoter-driven GFP expression in endothelial cells (Figure 6D). Moreover, the shape of cell nuclei without fluorescence signals lining the dark channels is similar to that of vascular endothelial cells (Figure 6D). Therefore, it seems likely that the channels are blood vessels (Figure 6D). The cells exhibiting strong GFP signals were often located close to branching points of the assumed blood vessels (Figure 6A).

3 | DISCUSSION

In order to identify cell types expressing *pgr* in pituitary and gonadal tissue, we generated *Tg(pgr:eGFP)* zebrafish, in which GFP was expressed under the control of a ~2.8-kb fragment of the *pgr* promoter. Our first trials with a shorter fragment (~1.9-kb; data not shown) failed to drive GFP expression. Our tissue screening experiments suggested that the promoter fragment reflects the

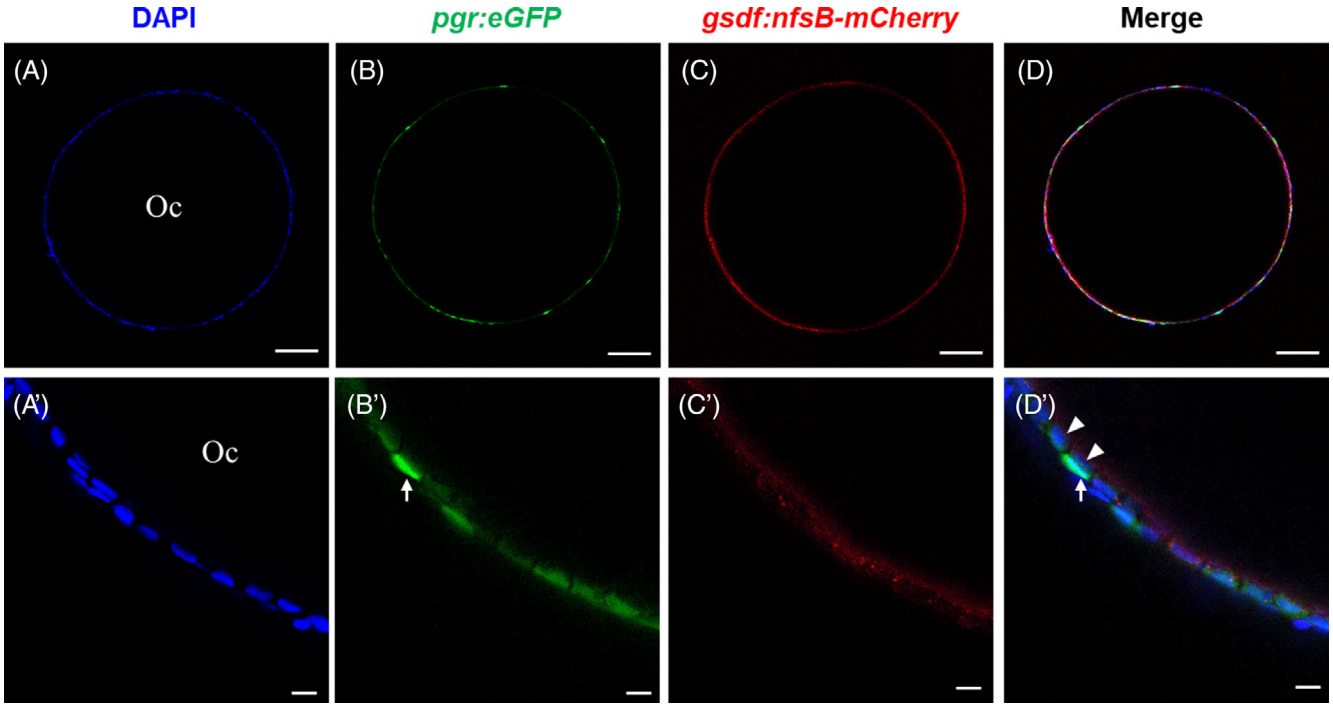

FIGURE 5 Cellular localization of green fluorescent protein (GFP) under the control of *pgr* promoter in ovarian follicles layer in *Tg (pgr:egfp/gsdf:nfsB-mCherry)*. The ovarian follicle consisted of an oocyte and a follicle cell layer (A, A′) which showed both GFP (B, B′) and mCherry (C, C′) signals. Most of GFP-positive cells expressed mCherry signals (D), and located at inner follicle layer (D′). A few of GFP-positive cells without mCherry signals showed strong GFP signals (B′, D′) and located at outer follicle layer (D′). The blue fluorescence represented the nucleus which is stained by Hoechst33342. Oc, oocyte; white arrow, thecal cell; white arrowhead, granulosa cell. Scale bars = 10 μm. Pgr, progesterone receptor

normal *pgr* expression pattern in adults. However, future experiments will have to show if also temporal aspects during ontogenesis are similar.

The adenohypophysis in fish is divided into three distinct zones: rostral pars distalis (RPD), PPD, and pars intermedia. In the present study, only cells in the PPD region of the zebrafish pituitary gland expressed GFP. Following a general pattern in teleost fish, gonadotropes (Fsh and Lh-producing cells), somatotropes (growth hormone-producing cells), and thyrotropes (Tsh-producing cells) are localized in the PPD.[25] Previous studies in zebrafish demonstrated that both Fsh cells and Lh cells are localized in the PPD.[26,27] Our previous work demonstrated that DHP exerted a Pgr-mediated, direct stimulatory effect on *fshb* mRNA, and that *pgr* mRNA was expressed only in *fshb*-expressing cells.[14] PGR protein was mainly localized to gonadotrophs also in rodent and primate pituitaries.[28,29] Therefore, it is very likely that Pgr is expressed in Fsh-producing gonadotrophs in the PPD of the zebrafish pituitary. There is little information on the localization of Pgr in vertebrate somatotropes and thyrotropes. A recent study demonstrated that ewes with elevated serum levels of progesterone exhibited greater levels of CXCR4, a receptor for chemokine ligand 12, in somatotropes.[30] Further studies using single-cell sequencing-based technologies would be necessary to identify other cell types potentially expressing Pgr in zebrafish pituitary, although expression levels are expected to be lower than in Fsh-producing gonadotrophs.

Since the cellular sites of PGR expression in the testis are controversial, the physiological functions of progestins mediated via PGR remain an interesting and underexplored question. In the present study, the GFP signals were exclusively observed in the Leydig cells. A previous study in zebrafish also reported that *pgr* mRNA was expressed in Leydig cells, and DHP stimulated 11beta-hydroxysteroid dehydrogenase activity via a Pgr-dependent manner in adult zebrafish testis.[21] The expression of PGR in Leydig cells has been reported in human, nonhuman primates, mouse, Japanese eel, and gilthead seabream.[31] Therefore, the effects of progestins on testis seem to be mediated through PGR in Leydig cells, and Leydig cell products, in turn, may affect spermatogenesis. In mammals, progestins inhibit spermatogenesis and sperm vitality,[32-34] and accordingly, the KO of the *PGR* increases testicular development and sperm production.[20] However, in teleosts, progestins seem to support, instead of inhibiting, spermatogenesis (see reviews by[11,35]). This is also supported by genetic evidence, considering that

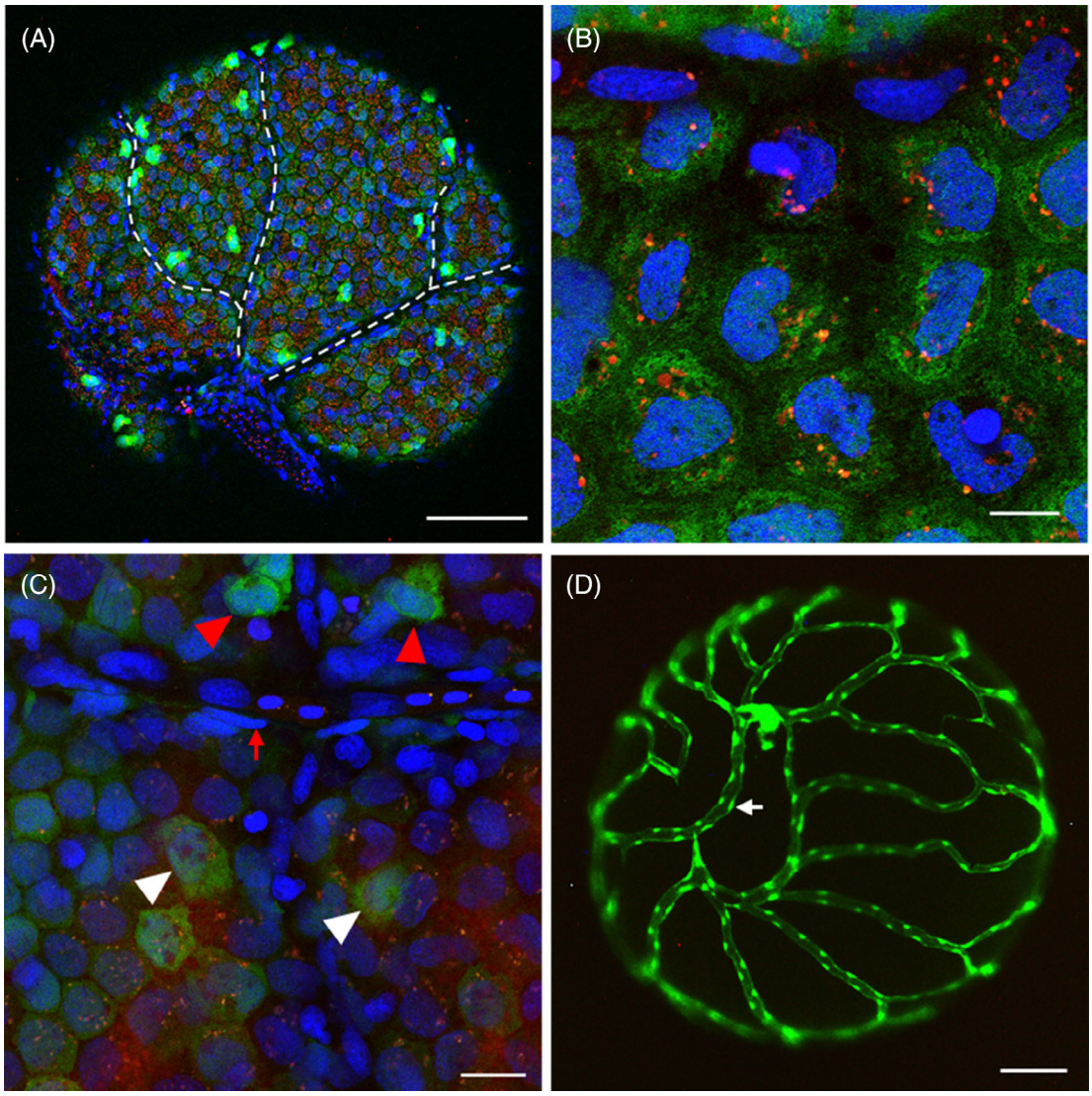

FIGURE 6 Direct observation of the surface of intact ovarian follicles in *Tg(pgr:egfp/gsdf:nfsB-mCherry)* and *Tg(fli:egfp)*. A, Direct observation of the intact ovarian follicle showed almost all granulosa cells expressing both mCherry and green fluorescent protein (GFP) signals, and a few cells exhibiting strong GFP signals. The dash line indicated a network of interconnected, unstained (dark) channels without cells presenting. B, Granulosa cells expressed both mCherry and GFP signals. Noted mCherry signals present as a few spots inside granulosa cells. C, Non-granulosa cells only exhibited strong GFP signals (red arrow head) or without GFP/mCherry signals (red arrow). White arrow head indicated granulosa cells. D, Blood vessel nest on the surface of the ovarian follicle in *Tg(fli:egfp)*. The white arrow indicated the vascular endothelial cell. The blue fluorescence represented the nucleus which was stained by Hoechst33342. Scale bars = 100 μm (A, D), 20 μm, C, and10 μm, B. Pgr, progesterone receptor

loss of Pgr function in male tilapia resulted in subfertility.[15] Interestingly, both in *Pgr* KO mice[20] and tilapia,[15] there was a significant increase in the number of Leydig cells, suggesting that PGR may regulate the number of Leydig cells. In tilapia *pgr* mutants, there was also a significant increase in the transcript levels of steroidogenesis-related genes and of serum 11-ketotestosterone levels.[15] This phenotype is difficult to understand considering that no Pgr protein was detected in tilapia Leydig cells by IHC, using antibodies raised against zebrafish Pgr.[15] Using the same antibodies, no signal was observed in zebrafish Leydig cells.[22] One possibility is that Pgr protein is only transiently expressed under specific conditions. For example, in mouse, *Pgr* promoter-driven LacZ expression was rarely detectable in Leydig cells but significantly increased when intratesticular testosterone was suppressed.[20] It would be interesting to investigate the function of Pgr in Leydig cells in the further study.

Pgr antagonist studies in humans and rodents, knockdown in a primate, as well as KO in mouse and zebrafish, have demonstrated the critical role of PGR in ovulation

(see[36] for detailed review[13,37]). In mammals, ovarian expression of PGR is restricted primarily to granulosa cells of follicles destined to ovulate (see[38] for detailed review). In zebrafish, strong Pgr immunoreactivity was observed in the nuclei of follicular layer cells surrounding all stages of oocytes.[22] Using the same antibody, a study in tilapia revealed abundant expression of Pgr in follicular cells during the early stages of ovarian differentiation.[39] In gilthead seabream (*S. aurata*), immunohistochemical localization of Pgr showed strong immunostaining in the nuclei of granulosa cells using a specific Pgr antibody against gilthead seabream Pgr.[10] In the present study, our results clearly show that the promoter of the *pgr* gene drives eGFP expression in granulosa cells as well, which indicates that PGR expression in granulosa cells is conserved among vertebrates.

Intriguingly, we also identified some non-granulosa cells located at outer thecal cell layer, expressing GFP signals. Some of these cells locating at the branches of the interstitial blood vessels exhibited strong GFP signals. We assume that these cells are theca cells, which appear outside basement lamina associated with blood vessels, move into the interstitial region between oocytes, and finally enclose the oocytes at an early vitellogenic stage (see reference[40] for detailed review). PGR protein has been detected in theca cells from monkeys,[41] rat,[42] human,[43] and dog.[44] However, one study in mouse reported that there is no detectable immunoreactivity in theca and interstitial cells of ovary.[45] Since PGR is expressed by granulosa cells and plays a critical role in ovulation, studies have focused on granulosa cells and revealed that PGR-regulated genes unequivocally linked to the ovulatory success are the extracellular matrix proteases, for example, ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) family members.[46] Our previous study identified *adamts9*, expressed in follicle cells, as a downstream target of Pgr, which is essential for ovulation in zebrafish.[47,48] However, it is worth noting that theca cells are also vital to the mammalian ovulatory process.[49] It has been reported that *Adamts1* mRNA in the theca interna was upregulated just prior to ovulation in the horse.[50] Recently, studies in cattle indicate that ADAMTS family members produced by theca cells may play important roles in follicle rupture and the accompanying tissue remodeling in cattle.[51,52] Therefore, further studies seem warranted to examine whether the *pgr* expressed in theca cell layer participate in follicle rupture in zebrafish.

In summary, the *Tg(pgr:eGFP)* zebrafish line is a useful model to identify cellular localization of Pgr in pituitary and gonads. Further detailed analysis of the sequence between 1.9 and 2.8 kb *pgr* promoter fragment could reveal key regulatory elements involved in the regulation of *pgr* gene expression. Finally, this transgenic zebrafish line will be a valuable model for the isolation of Pgr-expressing cells to investigate molecular mechanisms mediated via Pgr in fish reproduction.

4 | EXPERIMENTAL PROCEDURES

4.1 | Animals

Tübingen strain zebrafish were propagated in our facilities (ESEN; Beijing, China). Fish were kept in recirculating aquaria at 28°C ± 0.5°C with a 14L:10D photoperiod (lights on at 08:00 AM). Fish were fed three times daily with a commercial food (Otohime B2; Marubeni Nisshin Feed, Tokyo, Japan) and supplemented with newly hatched brine shrimp. A transgenic zebrafish line *Tg(fli:egfp)*, which expresses GFP signals in vascular endothelial cells, was kindly provided by Dr. Mingyu Li (Xiamen University, Xiamen, China). All experimental protocols were approved by the Institutional Animal Care and Use Committee at the Xiamen University.

4.2 | Preparation of pTol2(pgr:eGFP) Construct

The genome sequence of the *pgr* gene was identified in the zebrafish genome using the ENSEMBL genome browser (http://www.ensembl.org/index.html). The −2798/+26 promoter fragment of the zebrafish *pgr* gene was amplified from zebrafish genomic DNA using specific primers (pgr-fw: 5′-CCATGTGATTGGACCCGATTTCCGATTACA-3′, pgr-rv: 5′-CGCAATCGTCCGAGATGCGTCCTCTTT-3′). The reaction was incubated for 7 cycles of 25 seconds at 94°C, 3.5 minutes at 72°C, and followed by 32 cycles of 25 seconds at 94°C, 3.5 minutes at 67°C. The ∼2.8-kb fragment was inserted into the upstream of the eGFP coding sequence of the pT2AL200R150G construct (kindly provided by Prof. Kawakami; National Institute of Genetics, Shizuoka, Japan) between the *NheI* and *BamHI* sites to generate the transgenic construct pTol2(pgr:eGFP) (Figure 7).

4.3 | Generation of transgenic fish lines

The transgenic zebrafish line was generated with the Tol2 Transposon System.[53] The animal pole of zebrafish one-cell-stage embryos was microinjected with a mixture containing pTol2(pgr:eGFP) vector (10 ng/μL), Tol2 transposase enzyme mRNA (10 ng/μL), and phenol red (0.25%) (P0290; Sigma-Aldrich, St. Louis, Missouri). The Tol2 transposase capped mRNA was in vitro transcribed from the pCS-zT2TP transposase vector (kindly provided

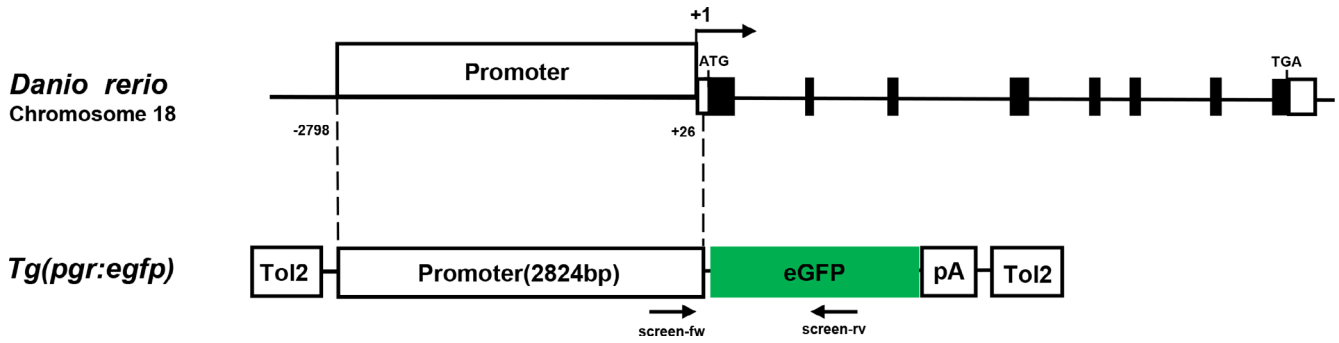

FIGURE 7 Schematic representation of the zebrafish *pgr* gene structure and transgene construct design. The zebrafish *pgr* gene is located on chromosome 18 which has eight exons. The proximal 2824 bp promoter region of the *pgr* gene was placed upstream to the open reading frame of the enhanced green fluorescent protein. In addition, the transgene was franked by Tol2 sites. The black box represents exons; pA, SV40 poly(A) signal. Pgr, progesterone receptor

by Prof. Kawakami) using the mMESSAGE mMACHINE SP6 kit (Ambion AM1340; Austin, Texas). The DNA solutions were injected using a *PV* 820 Pneumatic PicPump (World Precision Instrument; Florida) and borosilicate glass capillaries (IB100F-4; World Precision Instrument). The F0 fish were bred to maturity and screened by PCR with primers containing *pgr* promoter and *egfp* sequences (screen-fw: 5′-ACTACAGATCACAACCTCCAAAC-3′, screen-rv: 5′-TTGTAGTTGTACTCCAGCTTGTGCCC-3′). The injected embryos were raised to sexual maturity, and transgenic offspring were identified by genotyping using genomic DNA extracted from caudal fin biopsies. The positive F0 fish were mated with wild-type (WT) fish to generate F1 transgenic lines named *Tg(pgr:eGFP)*.

The *Tg(pgr:eGFP)* was crossed with *Tg(gsdf:nfsB-mCherry)* zebrafish lines generated in our laboratory (will be published separately) to obtain *Tg(pgr:egfp/gsdf:nfsB-mCherry)* double transgenic fish. Driven by the proximal promoter region of the zebrafish *gsdf* gene, the *Tg(gsdf:nfsB-mCherry)* transgenic zebrafish line specifically expressed nitroreductase-mCherry fusion protein in granulosa and Sertoli cells, as described previously.[23]

4.4 | RNA extraction and reverse Transcription-PCR

Total RNA from different tissues (brain, pituitary, heart, liver, kidney, ovary, and testis) was extracted using RNAzol reagent (RN 190; Molecular Research Center, Cincinnati, Ohio) according to the manufacturer's protocol. Reverse transcription was carried out using the RevertAid First Strand cDNA Synthesis Kit (K1622; Thermo Scientific, Lithuania) with 800 ng total RNA. The PCR was conducted on the cDNA template for 32 cycles with an annealing temperature of 58°C using *pgr*-specific primers (pgr-RT-fw: 5′-AATC TCATCA TGGAGCCACCG-3′, pgr-RT-rv: 5′-CCTCTGGCT GTGTGTTGTCG-3′) and *egfp*-specific primers (egfp-RT-fw: 5′-GGACGACGGCAACTACAAGA-3′, egfp-RT-rv: 5′-GTC CATGCCGAGAGTGATCC-3′). As a negative control, an equal volume of water was added into the PCR mixture instead of cDNA. The PCR products were run on a 1.5% agarose gel and photographed in the BioDocAnalyze digital gel documentation system (Biometra GmbH, Göttingen, Germany).

4.5 | Collection of pituitary and ovarian follicles

Adult zebrafish were anesthetized in ice water. Pituitaries were isolated as described previously.[26] In brief, the pituitary glands were collected under a fluorescent dissecting microscope (Leica M165FC; Leica Microsystems, Wetzlar, Germany) with fine forceps and eye scissors. We first removed the dorsal part of the skull, followed by removing the whole brain. The pituitary gland, showing a clear GFP signal, lay within the hypophyseal fossa (Figure 2A). To collect the whole pituitary, we first dissected a cross-shaped bone containing the pituitary (Figure 2B) and further dissected the bone to collect the intact pituitary (Figure 2C). The pituitary was stained with Hoechst 33342 (C1022; Beyotime Institute of Biotechnology, Shanghai, China), mounted on a 29-mm glass-bottom dish (D29-10-1.5-N; Cellvis, China) containing 1% low-melting agarose (A9414; Sigma-Aldrich) in phosphate-buffered saline (PBS). Images were taken with an SP8 (Leica, Germany) confocal laser scanning microscope.

Isolation of ovarian follicles was conducted as described previously.[54] In brief, ovaries were pooled and quickly dispersed in a petri dish containing 60% Leibovitz L-15 medium (GIBCO BRL, Gaithersburg, Maryland).

Follicles in different stages of development were identified and manually isolated under a stereomicroscope (Leica M165FC, Germany). The follicles were classified into six stages based on the diameter, namely primary growth (diameter ≤ 100 μm, PG), pre-vitellogenic (100-300 μm, PV), early-vitellogenic (300-400 μm, EV), mid-vitellogenic (400-500 μm, MV), late-vitellogenic (500-600 μm, LV), and the full-grown stage (≥650 μm, FG). The follicles were stained with Hoechst 33342 (Beyotime Institute of Biotechnology). Images were captured using M165FC (Leica, Germany), LSM880 (Carl Zeiss MicroImaging, Oberkochen, Germany), and SP8 (Leica, Germany) confocal laser scanning microscope.

4.6 | Preparation of cryosections

The adult zebrafish were anesthetized, and testes were carefully dissected and fixed in 4% paraformaldehyde (Merck, Germany) in PBS overnight. The gonads were rinsed in PBS twice (1 hour per time) and then dehydrated in 25% sucrose at 4°C. After dehydration, the whole tissues were embedded in the Tissue Freezing Medium (Leica Biosystems, Heidelberg, Germany) and frozen by floating in liquid nitrogen. Cryosections of the embedded gonad were cut using a Leica CM1850 cryostat (Leica Microsystems, Heidelberg, Germany) at −18°C. Tissue sections of the testis (10 μm) or ovary (20 μm) were collected and then mounted onto poly-L-lysine-coated slides. The slides were air-dried for 1 hour, rinsed in PBS three times, covered with VECTASHIELD Mounting Medium with DAPI (H-1200; Vector Laboratories, Burlingame, California) and coverslipped. Pictures were taken with an LSM780 microscope (Carl Zeiss, Germany).

ACKNOWLEDGMENTS

We would like to express our great appreciation to Dr. Rüdiger Schulz at Utrecht University for his valuable comments and editing the English. This research was supported by the National Natural Science Foundation of China (No. 31672628 and 41976092) and Program for New Century Excellent Talents in Fujian Province University.

AUTHOR CONTRIBUTIONS

Jing Huang: Conceptualization; data curation; methodology; writing-original draft; writing-review and editing. **Ting Zhang:** Methodology. **Ke Jiang:** Methodology. **Wan Hong:** Supervision. **Shi Chen:** Conceptualization; funding acquisition; supervision; writing-original draft; writing-review and editing.

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How to cite this article: Huang J, Zhang TT, Jiang K, Hong WS, Chen SX. GFP expression pattern in pituitary and gonads under the control of nuclear progesterone receptor promoter in transgenic zebrafish. *Developmental Dynamics*. 2020;1–12. https://doi.org/10.1002/dvdy.213