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Transgenic zebrafish with fluorescent germ cell: a useful tool to visualize germ cell proliferation and juvenile hermaphroditism in vivo

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Abstract

Juvenile zebrafish are hermaphroditic; undifferentiated gonads first develop into ovary-like tissues, which then either become ovaries and produce oocytes (female) or degenerate and develop into testes (male). In order to fully capture the dynamic processes of germ cells' proliferation and juvenile hermaphroditism in zebrafish, we established transgenic lines TG(β-actin:EGFP), harboring an enhanced green fluorescent protein (EGFP) gene driven by a medaka β-actin promoter. In TG(β-actin:EGFP), proliferating germ cells and female gonads strongly expressed EGFP, but fluorescence was only dimly detected in male gonads. Based on the fluorescent (+) or nonfluorescent (−) appearance of germ cells seen in living animals, three distinct groups were evident among TG(β-actin:EGFP). Transgenics in ++ group (44%) were females, had fluorescent germ cells as juveniles, and female gonads continuously fluoresced throughout sexual maturation. Transgenics in +− (23%) and −− (33%) groups were males. Fluorescent germ cells were transiently detected in +− transgenics from 14 to 34 days postfertilization (dpf), but were not detected in −− transgenics throughout their life span. Histological analyses showed that 26-dpf-old transgenics in ++, +−, and −− groups all developed ovary-like tissues: Germ cells in −− group juveniles arrested at the gonocyte stage and accumulated low quantities of EGFP, while those in ++ group juveniles highly proliferated into diplotene to perinucleolar stages and accumulated high quantities of EGFP. In +− group juveniles, degenerating oocytes, gonocytes, and spermatogonia were coexistent in transiently fluorescent gonads. Therefore, the fluorescent appearance of gonads in this study was synchronous with the differentiation of ovary-like tissues. Thus, TG(β-actin:EGFP) can be used to visualize germ cells' proliferation and juvenile hermaphroditism in living zebrafish for the first time.

Keywords: Enhanced green fluorescent protein; Germ cell; Juvenile hermaphroditism; Sex differentiation; Transgenic zebrafish

Introduction

Sex differentiation in teleosts is labile and sensitive to the external environment. Fluctuation of external factors, such as temperature (Goto et al., 1999; Baras et al., 2000; Pavlidis et al., 2000), population density (Roncarati et al., 1997), and pH (Zelennikov, 1997), or administration of exogenous sex steroids (Yamazaki, 1983; Francis, 1992; Baroiller et al., 1999; Pandian and Koteeswaran, 1999) during sex differentiation can significantly alter the adult sex ratio. Sex differentiation patterns are also divergent and versatile in teleosts. In differentiated gonochoristic species, such as gray mullet (Chang et al., 1995), channel catfish (Patino et al., 1996), pikeperch (Zakes et al., 1996), and medaka (Tanaka et al., 2001), the undifferentiated gonads differentiate into ovaries in the genetic female and, much later, into testes in the genetic male. However, in undifferentiated gonochoristic species, such as European eel (Colombo and Grandi, 1996), masu salmon (Nakamura, 1984), Sumatra barb (Takahashi and Shimizu, 1983), and zebrafish (Takahashi, 1977; Uchida et al., 2002), undifferentiated gonads first develop into ovary-like gonads. In half of these fish, the ovary-like gonads become ovaries and produce oocytes. In the other half, they develop into testes and the fish become males. Thus, the appearance of premeiotic oocytes is not a reliable indicator of sex differentiation in species with hermaphroditic juveniles (Nakamura et al., 1998).

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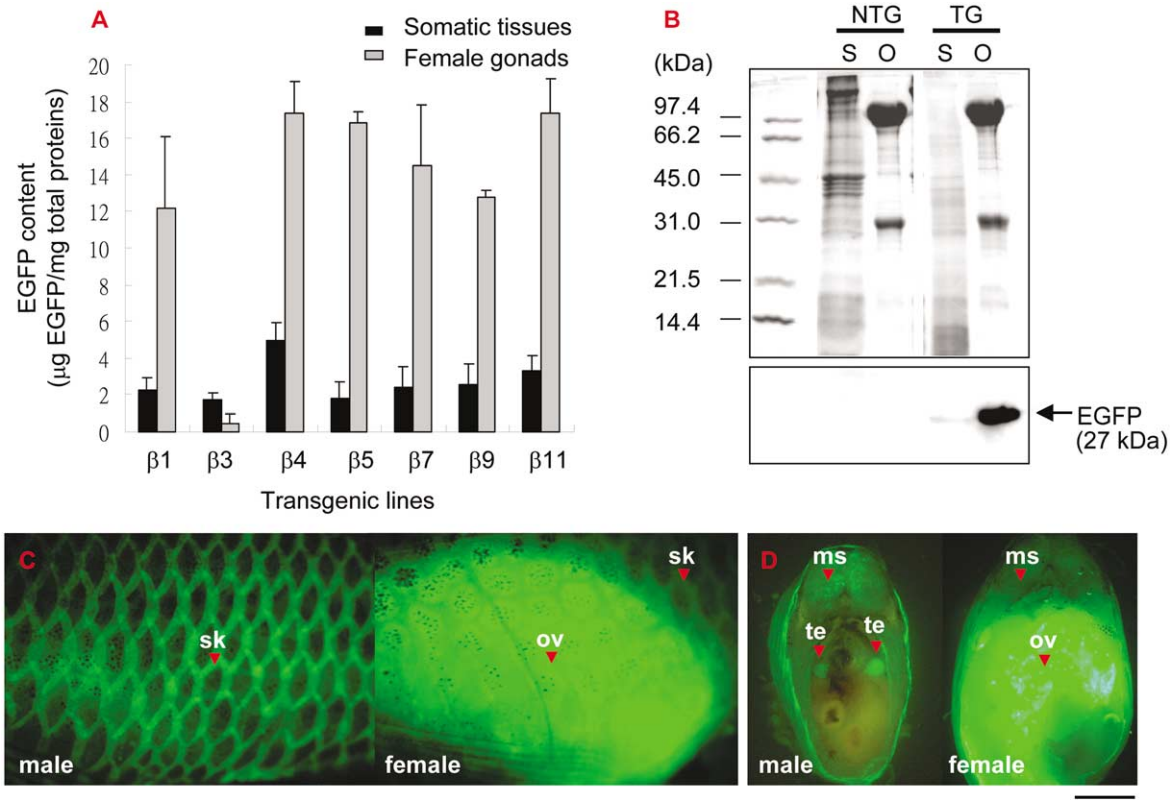

Fig. 1. High accumulation of EGFP in female gonads of TG(β-actin:EGFP). (A) Comparison of EGFP accumulated in somatic tissues (black filled boxes) and female gonads (gray filled boxes) among seven distinct transgenic lines (β1, β3, β4, β5, β7, β9, and β11). EGFP content was evaluated with a fluorescence spectrophotometer and normalized against the same amount of a total soluble protein. Values are the means ± SE of five replicates. (B) SDS-PAGE (upper panel) and Western blot (lower panel) analyses of EGFP accumulated in gonads of β9 TG(β-actin:EGFP). S, soluble proteins extracted from somatic tissues; O, soluble proteins extracted from female gonads; NT, non-transgenics; TG, transgenics. Arrow indicates the positive signal of the 27-kDa EGFP protein. Protein markers are shown on the left; sizes are in kDa. (C) Sexually dimorphic expression of EGFP in the male (left) and female (right) gonads of β9 TG(β-actin:EGFP) at 60 dpf. TG(β-actin:EGFP) are viewed laterally; anterior to the left of the figure. (D) Transverse sections showing the relative intensity of fluorescent gonads in male (left) and female (right) TG(β-actin:EGFP). ms, muscle; ov, ovary; sk, skin; te, testis. Scale bar = 250 μm in (C) and 200 μm in (D).

Zebrafish (*Danio rerio*) have transparent embryos, a short generation time, year-round breeding, and thousands of mutant strains. These traits make them a popular vertebrate model for studying early development, genetics, and disease mechanisms (Kimmel, 1989; Ingham, 1997; Vascotto et al., 1997; Briggs, 2002; Ward and Lieschke, 2002). However, little is known regarding the mechanism controlling sex determination in zebrafish (Horstgen-Schwark, 1993; Martin and McGowan, 1995; Corley-Smith et al., 1996). Until now, neither sex chromosomes nor sex-linked phenotypic markers have been characterized in zebrafish (Schreeb et al., 1993; Pijnacker and Ferwerda, 1995; Amores and Postlethwait, 1999; Traut and Winking, 2001). Based on morphological criteria, the pattern of sex differentiation in juvenile zebrafish has been described as hermaphroditic (Takahashi, 1977). Using the terminal-deoxynucleotidyl-transferase-mediated dUTP nick-end labeling (TUNEL) assay, Uchida et al. (2002) confirmed Takahashi's previous observation and demonstrated that the transition from ovary-like tissues to testes in male fish is mediated by apoptosis. However, due to the shortage of sex-linked phenotypic markers, researchers must perform tedious tissue sections and TUNEL assays, comparing the detailed morphological difference between genetic all-females and wild-type males, in order to fully capture the dynamic processes of sex differentiation in zebrafish (Uchida et al., 2002). Therefore, establishment of an in vivo model for visualizing the transition from ovary-like tissues to testes and for exploring the molecular mechanism controlling sex differentiation in zebrafish is considered necessary.

Transgenic fish lines harboring a germ cell-specific, vasa-EGFP construct were successfully established in rainbow trout (Yoshizaki et al., 2000), medaka (Tanaka et al., 2001; Wakamatsu et al., 2001), and also in zebrafish (Krovel and Olsen, 2002). However, in zebrafish, due to the complicated regulatory elements required for fully recapitulating the endogenous expression pattern of the vasa gene, vasa-EGFP transgenes were silent in transgenic males. Although maternally deposited EGFP in primordial germ cells (PGC) was be detected at a juvenile stage, it gradually became undetectable at late developmental stages. In a previous

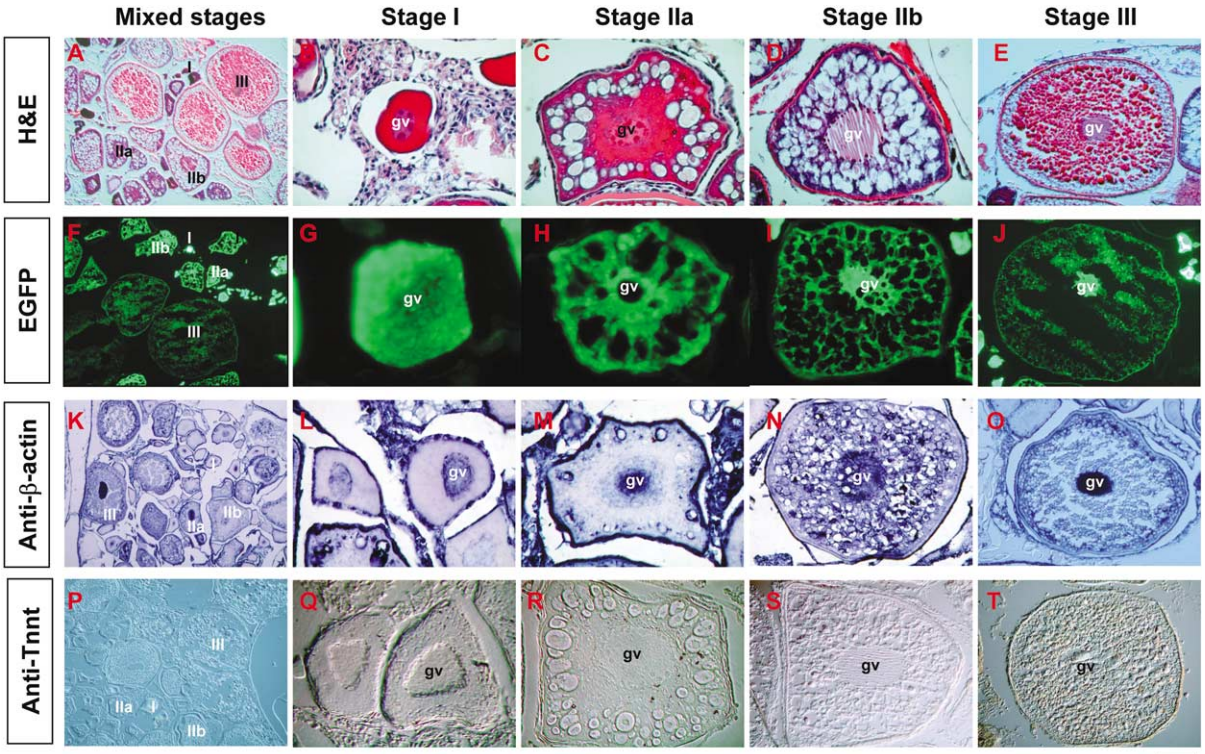

Fig. 2. Subcellular distribution of EGFP and β-actin in female gonads of β9 TG(β-actin:EGFP) detected by cryosection or immunohistochemistry. (A–E) Paraffin sections of female gonads. Sections are stained with H&E. (F–J) Cryosections of female gonads. (K–O) Immunohistochemistry of female gonads with β-actin monoclonal antibody. (P–T) Immunohistochemistry of female gonads with troponin T monoclonal antibody. Overviews of gonadal sections at low magnification (A, F, K, P). High magnification of oocytes at the primary growth stage (B, G, L, Q), early (C, H, M, R), middle (D, I, N, S), and late (E, J, O, T) vitellogenic stages. gv, germinal vesicle; I, stage I (primary growth stage) oocyte; IIa, stage IIa (early vitellogenic stage) oocyte; IIb, stage IIB (middle vitellogenic stage) oocyte; III, stage III (late vitellogenic stage) oocyte. Scale bar = 250 μm in (A, F, K, P), 100 μm in (E, J, O, T), 60 μm in (D, I, N, S), and 30 μm in (B, C, G, H, L, M, Q, R).

study, we established TG(β-actin:EGFP) expressing EGFP ubiquitously in the entire body (Hsiao et al., 2001). Unexpectedly, we also noticed sexually dimorphic expression of EGFP in gonads of TG(β-actin:EGFP) at sexual maturation. These interesting findings have prompted us, in this study, to document the expression of this phenotypic marker in living zebrafish from juveniles to sexually mature males and females, in order to visualize the transitional process from ovary-like tissues to testes and sex differentiation.

Materials and methods

Quantification of EGFP accumulated in TG(β-actin:EGFP)

Female gonads, male gonads, and somatic tissues (whole fish excluding gonads) dissected from sexually mature fish of transgenic lines β1, β3, β4, β5, β7, β9, and β11 were homogenized in protein extraction buffer (0.5 M Tris–HCl at pH 7, 0.5 N NaCl, 5 mM EDTA, and 1 mM phenylmethyl sulfonyl fluoride) and centrifuged for 10 min at 10,000*g* and 4°C to remove the insoluble fraction. The total soluble protein concentration in the supernatant was measured with a protein assay kit (BioRad), using bovine serum albumin as a standard solution. Equal amounts of soluble proteins (100 μg) from gonads and somatic tissues were measured with a fluorescence spectrophotometer (5 replicates for each transgenic line). Soluble proteins (10 μg) were separated by 10% SDS-PAGE then transferred onto polyvinylidene difluoride membrane (Amersham). Alkaline phosphatase-conjugated EGFP-specific polyclonal antibody (Clontech) was used to perform Western blots. The membrane was then developed in alkaline phosphatase buffer, with nitro-blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as the substrates.

Establishing homozygous lines with the EGFP transgene

Transgenic lines harboring the β-actin-EGFP-ITR transgene were described previously (Hsiao et al., 2001). In order to obtain a more homogenous and stronger expression of EGFP, hemizygous F2 of transgenic line β9 was crossed to produce homozygous F3. Male homozygous F3 were screened based on whether all of the F4 progeny exhibited green fluorescence before the mid-blastula transition. Since EGFP is maternally deposited in oocytes, 100% transgene-positive F4 progeny were used to screen homozygous fe-

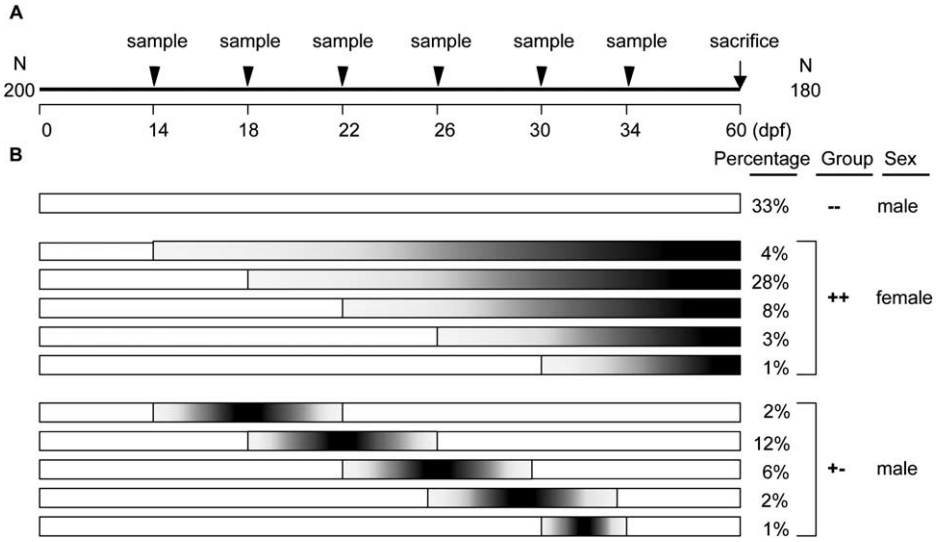

Fig. 3. Three distinct groups of TG(β-actin:EGFP) were identified, based on the fluorescent appearance of their gonads. (A) Schematic diagram showing the experimental design to measure the onset and duration of fluorescent gonads among a zebrafish population. Arrowheads indicate sampling days (14, 18, 22, 26, 30, and 34 dpf), and the arrows show when fish were sacrificed (60 dpf). The number of fish (*N*) at the beginning of the experiment is shown on the left, and the number at the end is shown on the right. (B) Within the entire transgenic zebrafish population, three distinct groups of TG(β-actin:EGFP) were identified based on if and when their gonads fluoresced. Gonads of the fish in the $--$ group never fluoresced and developed into testes at sexual maturation. Fish in the $++$ group had fluorescent gonads from juveniles to adults and became females at sexual maturation. Fish in the $+-$ group exhibited transient fluorescence in their gonads as juveniles and became males at sexual maturation. Filled black boxes indicate the time of appearance of fluorescent gonads. The levels of green fluorescence in gonads are indicated by the darkness of the shading: the darker the shading, the higher the level of green fluorescence expression. The unshaded boxes indicate no fluorescent appearance of gonads.

male F3 by polymerase chain reaction (PCR). Except for the internal control primers, the PCR conditions used to amplify the transgene were the same as those described previously (Hsiao et al., 2001). The primers Tnnt1-F (5′-AGAAGTAGCACCATGTGCGACAC-3′) and Tnnt1-R (5′-TTCAATTCGGTTCTTCAACGCTAC-3′) were designed to amplify the zebrafish slow troponin T (Tnnt1) gene (Hsiao et al., 2003) as DNA-quality control. After both sexes of homozygous F3 were identified, they were crossed to amplify the population. EGFP expression in transgenic fish was detected with a fluorescence stereoscopic microscope (MZFLIII, Leica) equipped with a 480-nm excitation filter and a 510-nm barrier filter (filter set GFP2). Fluorescent images were recorded with a color digital camera (COOLPIX 996, Nikon) mounted on the fluorescence stereoscopic microscope.

Gonadal histology and immunohistochemistry

For cryosection, matured gonads dissected from transgenic line β9 were fixed in 4% paraformaldehyde with 0.1 M phosphate buffer at pH 7.4, 15 mM $CaCl_2$, and 5% sucrose (PFA) for 2 days, then embedded in OCT. Sections (10 μm) were prepared on a Leica cryomicrotome and placed on gelatin-coated slides. For paraffin sections, β9 transgenics, harvested at specific stages, were fixed in 4% PFA for 2 days; then they were decalcified (Moore et al., 2002), dehydrated in a graded series of ethanol, and embedded in paraffin. Sections (5 μm) were prepared and placed on gelatin-coated slides. The sections were cleared by 100% xylene then rehydrated by successive immersions in descending concentrations of ethanol. Finally, the sections were rinsed in distilled water, washed with 0.01 M PBS, and stained with hematoxylin and eosin (Shandon). For the immunohistochemical detection of endogenous β-actin and troponin T, sections were placed on gelatin-coated slides, deparaffinized, and rehydrated. The immunoreactions were performed overnight in alkaline phosphatase buffer with NBT and BCIP as the substrates. β-Actin (1:200 dilution, Sigma) and troponin T (1:200 dilution, Sigma) monoclonal antibodies were used as primary antibodies for detecting endogenous β-actin or to serve as a negative control, respectively. Alkaline phosphatase-conjugated goat anti-mouse IgGs (DAKO) were used at a final dilution of 1:2000 as the secondary antibodies. Subsequently, the slides were mounted with Histomount (Shandon) and evaluated with a differential interference contrast microscope (DMR, Leica) with a color digital camera (COOLPIX 996, Nikon) attached.

Ontogeny of fluorescent gonads in living zebrafish

Homozygous F5, β9 transgenics were randomly divided into 4 groups of 200 fish each. All transgenics in each group were placed in 30 L of oxygenated fresh water in a 40-L plastic container, and reared at 28.5°C on a 14-h light, 10-h dark cycle. Transgenics younger than 10 dpf were fed excess artificial rotifer suspension (OSI, USA) twice daily.

Older transgenics were fed excess Artemia larvae (OSI, USA) twice daily, until sexual maturation. All transgenics in all four groups were examined once at 14, 18, 22, and 32 days postfertilization (dpf), respectively, to calculate the percentage of juveniles with fluorescent gonads. Juveniles with fluorescent gonads were reared to sexual maturation separate from those whose gonads did not fluoresce. The sex of each fish was confirmed by dissection at the end of the experiment with a survival rate of around 80% (78–81%).

Detection of juvenile hermaphroditism in living zebrafish

To determine the duration of juvenile hermaphroditism, 200 homozygous F5 β9 transgenics were reared in a single tank under the same conditions described above. All the fish were examined at 14 dpf to calculate the percentage of juveniles with fluorescent gonads. After examination, transgenics with fluorescent gonads were reared separately from those whose gonads did not fluoresce. From 14 to 34 dpf, we examined all transgenics every 4 days, separating those with fluorescent gonads from those without fluorescent gonads each time (procedures were summarized in Fig. 3A). Using this experimental design, germ cell proliferation was followed in each individual in the entire population. The sex of each fish was confirmed by dissection at the end of the experiment with a survival rate of 90% (180/200).

Results

Sexual dimorphic expression of EGFP in TG(β-actin: EGFP)

In TG(β-actin:EGFP), EGFP was constitutively and ubiquitously detected in the entire body. High expression of EGFP was detected in the olfactory epithelium, gill, and urogenital ducts in both genders of TG(β-actin:EGFP) (data not shown). Interestingly, we also noticed very strong EGFP expression in female gonads at sexual maturation. To compare the relative amount of EGFP accumulated in the gonads and somatic tissues, tissues were dissected from seven sexually matured lines of TG(β-actin:EGFP) (β1, β3, β4, β5, β7, β9, and β11), and the EGFP in each was quantified. Much more EGFP was detected in female gonads than in somatic tissues in all lines, except β3 (Fig. 1A). Western blots confirmed that the intensity of the EGFP-positive signal was much stronger in female gonads than in somatic tissues (Fig. 1B). Thus, high levels of EGFP detected in female gonads were common to our TG(β-actin:EGFP). On the contrary, the intensity of EGFP detected in male gonads was similar to that in skin and muscle but much less than found in female gonads (Fig. 1C and D). Therefore, the fluorescent appearance of gonads proved a reliable phenotypic marker to sex zebrafish at sexual maturation, i.e., all fish with fluorescent gonads were females, and all fish with nonfluorescent gonads were males.

EGFP is highly accumulated in primary growth stage oocytes

Oocyte development is asynchronous in zebrafish (Selman et al., 1993). Histological analysis revealed that oocytes at different developmental stages (from primary growth to vitellogenic stages) could be detected in the sexually matured females (Fig. 2A–E). In cryosections of oocytes, EGFP was detected in both cytosolic and nucleolar compartments, and also in the surrounding follicular layers (Fig. 2F). The relative intensity of EGFP was strongest in primary growth stage oocytes (stage I, Fig. 2G) and then gradually declined from early/middle vitellogenic oocytes (stage II, Fig. 2H and I) to late vitellogenic oocytes (stage III, Fig. 2J). This observation raised the possibility that the strong green fluorescence detected in primary growth stage oocytes might reflect the endogenous β-actin promoter activity during mitotic and meiotic divisions. We tested this hypothesis by immunohistochemical staining female gonads of β9 TG(β-actin:EGFP) with β-actin monoclonal antibody. Since germ cells and oocytes have high endogenous alkaline phosphatase activity (Dutt et al., 1975; Mester and Scripcariu, 1979; Ginsburg et al., 1990), we also performed troponin T staining, to serve as a negative control. Results demonstrated that subcellular localization of endogenous β-actin is quite similar to that of exogenous EGFP, i.e., β-actin was detected in oocytes of both cytosolic and nucleolar compartments, and also in the surrounding follicular layers (Fig. 2K–O). However, unlike EGFP (Fig. 2F–J), β-actin showed no predominant expression in primary growth stage oocytes but showed uniform expression in different stage oocytes (Fig. 2K–O). The negative control staining of troponin T did not give any background signal (Fig. 2P–T). Therefore, we believed the high EGFP detected in primary growth stage oocytes was not related to the endogenous β-actin promoter activity, but maybe related to the position where the transgenes integrated.

The onset of fluorescent gonads in TG(β-actin:EGFP)

To determine when the gonads began fluorescing in each transgenic line, TG(β-actin:EGFP) were examined noninvasively with light excitation during development. We found that the onset of fluorescent gonads varied from line to line. In lines β1, β3, β4, β5, β7, and β11, the gonads began fluorescing at 16 to 22 dpf. In line β9, however, the gonads became fluorescent as early as 14 dpf in 6% of the individuals examined. However, the onset of fluorescent gonads is temperature-dependent. Increasing the rearing temperature from 28.5 to 30°C increased the percentage of fish with fluorescent gonads at 14 dpf from 6 to 21% (data not shown). Interestingly, oocyte and sperm development is not morphologically distinguishable until 22 dpf in females and 27 dpf in males, respectively (Takahashi, 1977; Uchida, et al., 2002). Therefore, TG(β-actin:EGFP) strongly expressing EGFP in their gonads provided an excellent op-

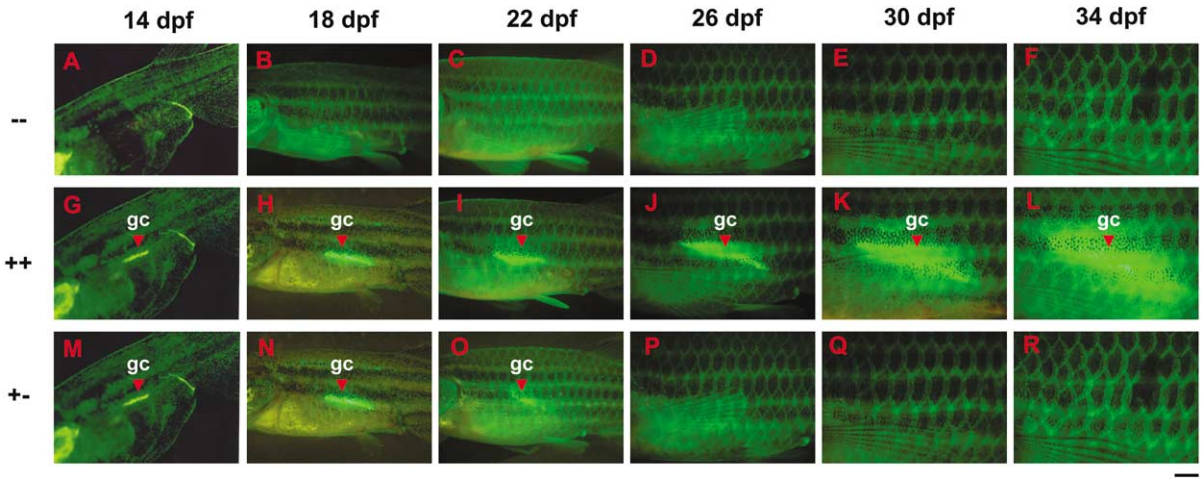

Fig. 4. Visualization of juvenile hermaphroditism in living TG(β-actin:EGFP) by following the fluorescent appearance of gonads. (A–F) In $--$ group juveniles (33%), no fluorescent appearance of gonads could be detected from juveniles throughout sexual maturation. (G–L) In $++$ group juveniles (44%), gonads were fluorescent from juveniles and continued to fluoresce throughout sexual maturation. (M–R) In $+-$ group juveniles (23%), gonads transiently fluoresced at the juvenile stage but gradually lost their fluorescence in later development stages. Fish in all pictures are viewed laterally and the anterior is to the left. All pictures were recorded at the same exposure time (2 s). Development stages are indicated in each panel. dpf, day postfertilization; gc, germ cells. Scale bar = 500 μm in (A, G, M) and 1 mm in all other pictures.

portunity to monitor germ cell proliferation much earlier than at the onset of morphological sex determination.

Fluorescent gonads are a reliable marker for sexing zebrafish after 34 dpf

Although the fluorescent appearance of gonads proved a reliable phenotypic marker for sexing zebrafish at sexual maturation, it was now our concern as to whether it could be used as a good sex-linked marker in juveniles. To answer this question, the ontogeny of fluorescent gonads was monitored in β9 TG(β-actin:EGFP). In about 6% (9/162) of the entire population, germ cells began fluorescing at 14 dpf. The percentage of fish with fluorescent gonads increased rapidly to 48% (75/155) at 18 dpf, and reached a plateau of about 55% (92/167) by 22 dpf. However, the percentage of juveniles with fluorescent gonads at 14, 18, 22, and 32 dpf, who were female at 60 dpf, was 71 (5/7), 86 (57/66), 88 (78/89), and 99% (103/104), respectively. Thus, there were some juvenile fish with fluorescent gonads that underwent a later transition to having nonfluorescent gonads, a phenomenon that gradually decreased with time. Up until 34 dpf, fluorescent gonads did not reliably indicate a fish was female. After 34 dpf, this phenotypic marker allowed us to sex female (with fluorescent gonads) and male (without fluorescent gonads) zebrafish with 100% accuracy.

Detection of juvenile hermaphroditism in living zebrafish

Although the gonads of some juveniles clearly went from fluorescent to nonfluorescent, the reason for this transition required further investigation. From 14 to 34 dpf, juveniles of β9 TG(β-actin:EGFP) were sampled at 4-day intervals, and fish with fluorescent gonads were separated from those without fluorescent gonads (procedures are summarized in Fig. 3A). With this experimental design, the ontogeny of the fluorescent gonads in each individual could be followed in real time. Results demonstrated that there were three distinct groups within the β9 TG(β-actin:EGFP) based on fluorescent gonad duration (results are summarized in Fig. 3B). In the $--$ group, no gonadal fluorescence was ever observed (Fig. 4A–F). About 33% (59/180) of individuals among the total population were in the $--$ group, and all these fish were male at sexual maturation. In the $++$ group, the gonads were fluorescent from 14 to 30 dpf (Fig. 4G–L) and continued to fluoresce in adults. About 44% (80/180) of the individuals among population were in the $++$ group and all became females at sexual maturation. In addition, about 23% (41/180) of individuals among the total population were in the $+-$ group, in which the germ cells fluoresced transiently as juveniles (Fig. 4M–R) and upon adulthood the fish were all confirmed as males. Gonadal fluorescence ceased in this population at sexual maturation. Interestingly, there was considerable variation in the onset of fluorescence in both the $++$ or $+-$ groups (ranged from 14 to 30 dpf, Fig. 3B). Since the average body weights of fish in different samples were not significantly different (data not shown), we know variation in the onset of fluorescent germ cells did not result from differences in individual growth rates.

We also performed histological analyses to explore the correlation between the external appearance of fluorescent gonads and internal morphology of proliferating germ cells. Results demonstrated that, although germ cells indeed developed into ovary-like structures in $--$ group juveniles, the nonfluorescent gonads were largely composed of less

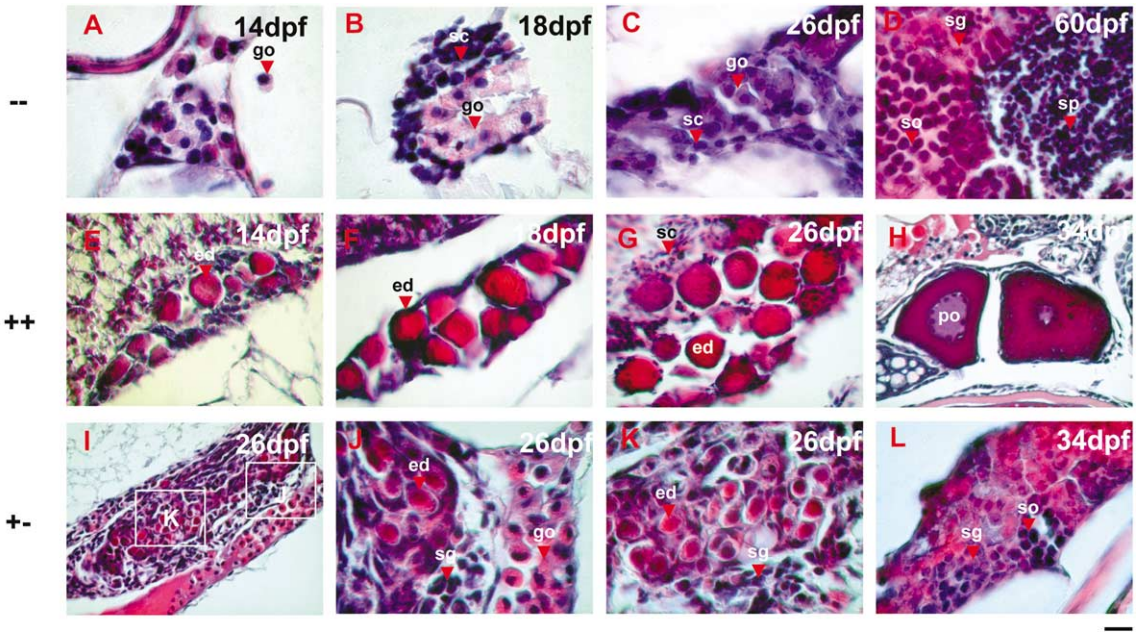

Fig. 5. Gonadal histology of TG(β-actin:EGFP) in −−, ++, and +− groups juveniles. (A–D) Transverse sections showing germ cell development in −− group juveniles. Germ cells of 14- to 26-dpf-old juveniles in −− group are solely composed of gonocytes and surrounded by somatic cells. (E–H) Transverse sections showing germ cell development in ++ group juveniles. Germ cells of 14- to 26-dpf-old juveniles in ++ group juveniles are solely composed of early diplotene oocytes. (I–L) Horizontal sections showing germ cell development in +− group juveniles. Degenerating diplotene oocytes, gonocytes, and spermatogonia coexist in the gonads of 26-dpf-old juveniles in the +− group. Developmental stages are indicated in each panel. dpf, day postfertilization; ed, early diplotene oocytes; go, gonocytes; po, perinucleolar oocytes; sc, somatic cells; sg, spermatogonia; so, spermatocytes; sp, spermatids. Scale bar = 25 μm in (E–I) and 10 μm in all other pictures.

proliferated gonocytes (Fig. 5A and B) surrounded with somatic cells (Fig. 5B and C). When development proceeded, gonocytes were gradually transformed into spermatogonia, spermatocytes, and spermatids (Fig. 5D). The relative content of EGFP accumulated in gonocytes (data not shown) and testes (Fig. 1D) showed no difference from that in somatic tissues, thus making it impossible to detect fluorescence in gonads of −− group juveniles. Compared with −− group juveniles, germ cells were well proliferated in both ++ and +− group juveniles. In ++ group juveniles, the fluorescent gonads of 14- to 26-dpf-old transgenics were largely composed of early diplotene oocytes (Fig. 5E–G). From 26 dpf onwards, diplotene oocytes were actively proliferating into perinucleolar oocytes (Fig. 5H) and arrested at this stage until about 60 dpf (data not shown). On the contrary, we found the perinucleolar oocytes in +− group juveniles gradually degenerated inside the ovary-like tissues. At 26 dpf, coexistence of degenerating diplotene oocytes, gonocytes, and spermatogonia was evident in +− group juveniles (Fig. 5I–K), with this hermaphroditic structure finally transformed into testes at 34 dpf (Fig. 5L). Thus, the cessation of fluorescence in gonads of +− group juveniles, occurring from 22 to 34 dpf, was tightly associated with the transition from oocytes to testes. Therefore, we indeed could visualize the transitional process from ovary-like tissues to testes simply by following the external appearance of fluorescent gonads in living zebrafish.

Discussion

TG(β-actin:EGFP) allow us to visualize germ cell proliferation and juvenile hermaphroditism in living zebrafish

Transgenic lines with tissues-specific EGFP expression have been successfully utilized to study organ development in living zebrafish (reviewed by Gong et al., 2001; Udvadia and Linney, 2003). Recently, TG(vasa:EGFP) with germ cell-specific EGFP expression has been established in zebrafish by Krovel and Olsen (2002). However, due to the complicated regulatory elements required for fully recapitulating the endogenous expression pattern of the vasa gene, EGFP expression was silent in male TG(vasa:EGFP), while female TG(vasa:EGFP) failed to highly express EGFP in the germ cells. These constraints severely limited the applicability of TG(vasa:EGFP) to sex differentiation in living zebrafish. Therefore, there was a need for an in vivo model that allowed scientists to noninvasively visualize the dynamic process of sex differentiation in zebrafish. In this report, we established a novel and useful transgenic line of zebrafish in which EGFP expression in gonads was sexually dimorphic from 34 dpf to sexual maturation. This unique TG(β-actin:EGFP) allowed us to sex zebrafish reliably, based EGFP expression from 34 dpf onwards, indicating sex differentiation is already complete in zebrafish after 34

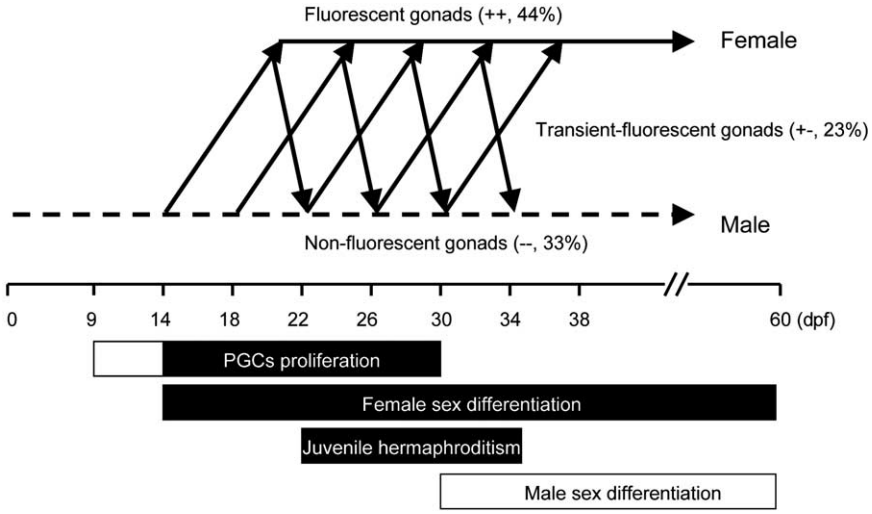

Fig. 6. Model of sex differentiation established by using TG(β-actin:EGFP). The solid arrows indicate the fluorescent appearance of gonads in ++ group juveniles as well as the transient-fluorescence appearance of gonads in +− group juveniles, while the dotted arrow indicates the nonfluorescent appearance of gonads in −− group juveniles. Cellular events that can be detected in TG(β-actin:EGFP) are highlighted with black filled boxes in the lower panel. These include germ cell proliferation from 14 to 30 dpf, juvenile hermaphroditism from 22 to 34 dpf, and female sex differentiation from 14 dpf onwards. Cellular events that cannot be detected in TG(β-actin:EGFP) are indicated by empty boxes in the lower panel. These include early germ cell proliferation, from 9 to 14 dpf, (Braat et al., 1999) and male sex differentiation from 30 dpf onwards (Takahashi, 1977; Uchida et al., 2002).

dpf. From 14 to 34 dpf, although the cell fate of proliferating germ cells was not fixed, we were still able to distinguish the gender of zebrafish by following gonadal fluorescence. TG(β-actin:EGFP) exhibiting continuously fluorescing gonads (++ group) became females, and those with either nonfluorescent (−− group) or transiently fluorescent (+− group) gonads became males. Histological analyses confirmed the ovary-to-testis transition was solely in +− males. These results indicated that, from 22 to 34 dpf, the male-specific loss of ovary-like tissues by apoptosis (juvenile hermaphroditism) could be visualized in living +− males. The duration of the ovary-like tissues to testes transition detected here (22–34 dpf) largely over-lapped with that detected by Uchida et al. (2002) (22–29 dpf), further confirming the fidelity of our TG(β-actin:EGFP) to detect juvenile hermaphroditism in zebrafish. Due to our ability to monitor germ cell proliferation in single individuals and also in an entire population, we can further expand the temporal window of juvenile hermaphroditism as between 22 and 34 dpf (summarized in Fig. 6). We conclude the use of TG(β-actin:EGFP) can provide a more sensitive and convenient tool than histological or TUNEL assays to detect juvenile hermaphroditism in zebrafish.

Why is EGFP highly accumulated in female gonads of TG(β-actin:EGFP)?

During oogenesis, proteolytic enzymes in immature oocytes are less active than in matured oocytes and fertilized embryos (Carnevali et al., 1999). This is because many proteolytic enzymes are stored as inactive, latent proproteases in the yolk granules (Medina and Vallejo, 1989; Cho et al., 1999; Yin et al., 2001) of oocytes. Therefore, slow degradation rate of EGFP might result in large accumulation of EGFP in oocytes. However, we excluded this hypothesis since transgenic zebrafish harboring EGFP transgenes driven either by carp β-actin promoter (Gibbs and Schmale, 2000) or by endogenous β-actin promoter (Higashijima et al., 1997) showed no sexual dimorphic expression of EGFP in gonads. Immunohistochemical analyses showed that endogenous β-actin is consistently and uniformly expressed in oocytes at different growth stages and at a similar expression level in gonads and somatic tissues. Inconsistencies in the expression levels between endogenous β-actin and exogenous EGFP in oocytes therefore implies that exogenous β-actin promoter activity during meiotic and mitotic divisions was not the primary reason for the strong expression of EGFP in oocytes. Thus, the most plausible explanation for the gonadal fluorescence might relate to the inverted terminal repeat sequences (ITRs) flanking the transgene and also the position of transgene integration. The ITRs or medaka β-actin promoter might interact with some unknown cellular factors near its integration sites, thus forcing transgene to robustly express in female gonads. Therefore, strong EGFP expression was only detected in female gonads of our zebrafish TG(β-actin:EGFP).

Potential applications of TG(β-actin:EGFP)

In medaka, green fluorescence in the gonads is usually not visible in TG(vasa:EGFP) after hatching because pigment cells cover the skin and peritoneum. Thus, to see the detailed morphology of the gonads, a transparent strain of see-through medaka is needed (Wakamatsu et al., 2001).

However, due to the large accumulation of EGFP in proliferating germ cells and female gonads, it is possible to visualize germ cell proliferation and oogenesis in living zebrafish without need of a transparent strain of fish.

This trait makes TG(β-actin:EGFP) an excellent model for the large-scale screening of gonadal mutations (Bauer and Goetz, 2001), by the introduction of the transgene into mutants through crossing. Germ cell development is sensitive to environmental pollutants in fish (Rodina and Horvath, 1999; Van den Belt et al., 2001; Willey and Krone, 2001) and growth arrest in female gonads has been detected in zebrafish upon exposure to exogenous ethynylestradiol (Van den Belt et al., 2001). By employing TG(β-actin:EGFP), the toxicity of environmental pollutants on gonadal development could be tested in large samples in real time, without the need to sacrifice any fish.

Recently, numerous genes with sexually dimorphic expression have been isolated and utilized to study sex differentiation in fish (Guan et al., 2000; Kanamori, 2000; Marchand et al., 2000; Kwon et al., 2001; Trant et al., 2001; Zeng and Gong, 2002). For example, in populations of juvenile zebrafish the CYP19b gene, an extra-gonadal isoform of the cytochrome P450 gene, is expressed at two levels. However, it is not possible to determine which expression pattern is associated with juvenile males and females due to the deficiency of a sex-linked phenotypic marker (Trant et al., 2001). Thus, detection of gene transcripts in +−, −− males and in ++ females among TG(β-actin:EGFP) will allow us to explore the developmental regulation of those genes during sexual differentiation. In addition, we found that in juvenile population, germ cells in −− males were less proliferated than those in +− males and ++ females. Further studies, conducting subtractive hybridization between juvenile ++, +−, and −− groups, may identify novel genes controlling germ cell proliferation and sex differentiation or even sex determination in zebrafish.

Limitations of TG(β-actin:EGFP)

Although our TG(β-actin:EGFP) has many advantages for studying germ cell proliferation and sex differentiation in living zebrafish, the processes of PGC migration, early germ cell proliferation, and spermatogenesis remain undetectable (summarized in Fig. 6). During early somitogenesis, PGC are organized into bilateral clusters in the anterior trunk (Yoon et al., 1997; Weidinger et al., 1999, 2002) and migrate to the genital ridge at 9 dpf. From 9 to 10 dpf onwards, the PGC interact with mesenchymal cells to establish undifferentiated gonads and then undergo rapid mitosis to increase PGC number (Braat et al., 1999). However, in TG(β-actin:EGFP), fluorescent germ cells cannot be detected in juveniles younger than 14 dpf. In both ++ females and +− males, although germ cells have proliferated before 14 dpf, the EGFP accumulation inside the cell is insufficient to distinguish the low intensity fluorescence of gonads from that of the somatic tissues. In the same manner, in −− males, EGFP accumulation in testes is also insufficient to distinguish the difference between the low intensity fluorescence of gonads from that of somatic tissues. Generating transgenic lines harboring EGFP gene driven by germ cell- or male-specific promoters will overcome these limitations in the future.

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