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Expression of GFP in transgenic tilapia under the control of the medaka β-actin promoter: establishment of a model system for germ cell transplantation

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Abstract

The Nile tilapia (*Oreochromis niloticus*) stands out as one of the most important fresh water edible fish, possessing remarkable characteristics that make it desirable for both commercial culture and as a laboratory model. For the utilization of tilapia in germ cell transplantation experiments, appropriate cell markers are required to evaluate the colonization behavior of donor-derived germ cells in recipient gonads. Here we report the production of a medaka β-actin/EGFP transgenic tilapia strain expressing the green fluorescent protein (GFP) reporter gene in several tissues including germ cells in testis and ovary. Fluorescent observations in F2 generation transgenic individuals showed GFP positive cells along the body axis in pre-hatched embryos, while in hatching embryos the GFP gene was strongly expressed in the area surrounding the gills, operculum and in the cephalic region. In early larvae, fluorescent cells were scattered throughout the body, forming aggregations around the dorsal-cephalic and mouth areas. At 38 days post-fertilization, juvenile fish expressed the GFP homogeneously in the whole body. GFP fluorescence was also observed in caudal fins, muscle, and in several internal organs (gills, heart, testes, and ovaries) in 140 and 240 day F_2 and F_3 individuals. Immunohistochemistry using a monoclonal anti-GFP antibody in juvenile and adult gonads showed that both mitotic and meiotic germ cells were labeled with GFP. The utilization of this transgenic line in a germ cell transplantation system could offer a fast and reliable screening of donor-derived transgenic offspring, as well as accurate tracing of donor-derived cell colonization in the recipient gonad by means of immunohistochemistry using GFP antibodies. In the future, germ cell transplantation using Nile tilapia also could help to preserve the genetic resources of threatened cichlids, through cryopreservation and interspecies transplantation of germ cells from endangered cichlids into *O. niloticus* recipients.

Keywords: Nile tilapia, *Oreochromis niloticus*, transgenic fish, green fluorescent protein (GFP), germ cell transplantation.

Introduction

The establishment of germ cell transplantation techniques (Brinster, 2002) has had an enormous impact on the study of spermatogenesis and germ-line stem cell biology. Since the first reports in 1994 by Brinster and colleagues (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994), spermatogonial transplantation has become the unequivocal assay to evaluate stem cell *activity* (long-term self-renewal and differentiation). Germ cell transplantation techniques have already been utilized as a novel tool to generate transgenic progeny (Nagano *et al*., 2001; Wistuba and Schlatt, 2002), and may have applications in the treatment of male infertility (Ogawa *et al*., 2000) and the preservation of genetic stocks of endangered or valuable species (Yoshizaki *et al*., 2003; Hill and Dobrinski, 2006; Okutsu *et al*., 2006b).

Germ cell transplantation has been applied to several animal species, including goats, pig, cattle, primates, and chicken (Schlatt, 2002; Dobrinski, 2005a, b; Lee *et al*., 2006; Trefil *et al*., 2006). In 2003, our laboratory first reported an intraspecies primordial germ cell (PGC) transplantation system for fish (Takeuchi *et al*., 2003), and we latter applied this technique to interspecies transplantation (Takeuchi *et al*., 2004). More recently, we described a novel system for intra-and interspecies transplantation of spermatogonia (Okutsu *et al*., 2006a, 2007). Such a system is currently only available for salmonids, but it could be applied to other valuable fish species.

Cichlids (Cichlidae; Perciformes) are one of the most successful families among teleosts, with more than 1350 species (Nelson, 2006) and presumably many more yet undiscovered. Two major tribes of cichlids are the Haplochromini, accounting for the greater part of the family's richness, covering almost every modality of teleosts feeding specializations (van Alphen *et al*., 2004), and the tilapiini, with fewer species which are mainly herbivorous (Trewavas, 1983). The majority of haplochromine cichlids occur only in three East African lakes: Victoria, Malawi and Tanganyika, and are one of the most conspicuous examples of explosive speciation and adaptive radiation in nature (Turner *et al*., 2001).

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Unfortunately, many haplochromine cichlids are facing conservation problems and several species have become extinct during the last decades (Witte *et al*., 1992). Eutrophication and introduction of foreign predator species have been described as the main threats (Seehausen *et al*., 1997). The tilapiine cichlids include some genera with high importance in fisheries and aquaculture, including *Tilapia, Sarotherodon*, and *Oreochromis*. Among them, the Nile tilapia (*Oreochromis niloticus*) stands out as one of the most important freshwater edible fish species. The species exhibits remarkable characteristics that make it a desirable fish for aquaculture, such as rapid growth, fast attainment of sexual maturity, and tolerance for both crowding and relatively poor water conditions (Stickney, 2000).

The use of tilapia as a model system for germ cell transplantation offers remarkable potential applications. Germ cells can be used as a vehicle for foreign genes for the mass production of transgenic fish carrying beneficial genetic traits. As mentioned above, germ cell transplantation could also help preserve genetic resources in combination with cryopreservation of germ cells (Kobayashi *et al*., 2003, 2006; Okutsu *et al.*, 2006b). In previous work, we demonstrated that adult male donor germ cells can colonize undifferentiated recipient testes and, upon recipient maturation, further differentiate into functional spermatozoa capable of yielding normal offspring (Okutsu *et al*., 2006a). Moreover, the adult male germ cells proliferated in undifferentiated recipient ovaries and differentiated later into fully functional oocytes, capable of producing normal live offspring possessing the donor derived haplotype (Okutsu *et al*., 2006a). Therefore, by using our germ cell transplantation approach, only male-derived spermatogonia could be used to produce both eggs and sperm. Considering the endangered status of many species of haplochromine cichlids, the cryopreservation and inter-specific transplantation of their germ cells into *O. niloticus* recipients could make it possible to regenerate the haplochromine target species, even in cases of extinction. As a first step to establish such a surrogate fish system in tilapia, an intraspecific transplantation method has to be developed.

Germ cell transplantation experiments in adult *O. niloticus* have been performed before by Lacerda *et al*. (2006), using spermatogonia-enriched germ cell suspensions labeled with a cell surface marker (PKH26; Sigma Chemical Co., St. Louis, Missouri, USA). Upon transplantation, labeled cells were detected after two and four weeks in the seminiferous tubules of recipient testes. Unfortunately, labile cell markers can only be distinguished for short or moderate periods, depending on the marker and the targeted cells, before they vanish. Conversely, the use of GFP gene (a permanent cell marker) permits long-term tracing of donor-derived germ cells in recipient gonads. Additionally, GFP can be inherited, allowing an easy screening of donor derived F_1 embryos by fluorescence observation.

Here, we report the production of an *Oreochromis niloticus* transgenic fish line carrying the GFP gene driven by a medaka β-actin promoter. GFP expression was characterized by fluorescence observation in F_2 and F_3 generations, at different developmental stages and in several organs. Detailed immunohistochemistry in male and female gonads is further reported. The utilization of this transgenic line in a germ cell transplantation system could offer fast and reliable screening of donor-derived transgenic offspring, as well as accurate tracing of donor-derived cell colonization in the recipient gonad by means of immunohistochemistry.

Materials and Methods

Parental fish rearing conditions

O. niloticus parental fish were maintained in the Tokyo University of Marine Science and Technology, Laboratory of Aquaculture (Tokyo, Japan). They were reared under a 14-h light/10-h dark photoperiod at 28 ± 1°C in aerated and de-chlorinated tap water. The fish were fed commercial pellets for eels (Nippon Formula Feed MFG, Tokyo, Japan) two times per day until they reached satiation.

Establishment and maintenance of transgenic Nile tilapia strains

The medaka β-actin/EGFP transgenic tilapia were produced by microinjecting embryos at the one-cell stage, as described by Kobayashi *et al*. (2007). Gamete collection and artificial fertilization procedures are described elsewhere (Biswas *et al*., 2005). The gene construct introduced was a plasmid containing the promoter, enhancer regions, and polyadenylation signal of the medaka β-actin gene, and the coding region of EGFP with a human codon usage, described previously by Hamada *et al*. (1998).

Newly hatched *O. niloticus* were reared in the laboratory under the same conditions described above for parental fish. GFP-positive founder (G_0) were crossed with wild-type female for the production of the F_1 generation. The resultant F_1 transgenic fishes were identified by fluorescence observation. To determine the inheritance of the transferred DNA fragments from F_1 to F_2 generation, an F_1 GFP-positive male (screened by both fluorescence observation and PCR) derived from β-actin/EGFP-injected embryos was crossed with a wild-type female. The resultant F_2 progeny embryos were reared and their green fluorescent signals were observed at 140 days post-fertilization by direct observation of the caudal fin under a fluorescence stereomicroscope. Fish were segregated into two groups according to their GFP fluorescence expression: GFP(+)

= fluorescence detected; GFP(-) = fluorescence not detected. The number of fish in each group was registered. Additionally, to corroborate the fluorescence observation sorting, PCR analysis was performed on genomic DNA extracted from randomly selected individuals belonging to each group (8 samples per group).

DNA extraction and screening of transgenic parental fish by PCR

Genomic DNA was extracted from caudal fin clips of first (F_1), second (F_2) and third (F_3) generation progeny fish using a Puregene Core Kit A (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The PCR reactions were carried out in a total volume of 10 µl consisting of 1 µl 10× Ex Taq buffer, 2.5 µM dNTPs, 0.25 U Ex Taq polymerase (Takara Bio Inc., Shiga, Japan), 1 µl cDNA template, and 0.1 µM of each of the forward (5′-TCTTCAAGGACGACGGCAACTACA-3′) and reverse (5′-TGCTGCCGTCCTCGATGTTGTGGC-3′) primers, both based on sequences within the EGFP gene. These primers yielded a PCR product of 229 bp. PCR amplification was performed for 3 min at 94ºC, followed by 35 cycles of 30 s at 94°C, 30 s at 58°C, 30 s at 72°C, and 3 min at 72°C. A 1-µl sample from the reaction was electrophoretically separated on a 2% agarose gel, stained with ethidium bromide and photographed under ultraviolet (UV) light.

Fluorescence observation

Embryos, fry, and juveniles were observed with an Olympus BX50 microscope with a BX-FLA attachment and GFP filter set, and an Olympus SZX12 stereomicroscope with a SZX-RFL attachment and GFP filter set. For observation of GFP expression in muscle and internal organs, 140 and 240 days post-fertilization individuals were euthanized by an over-dose of 10% benzocaine (ethyl 4-aminobenzoate, Wako Pure Chemical Industries, Osaka, Japan), and the internal organs were removed and kept in an isotonic physiological solution for freshwater teleosts (NaCl, 7.5 g; KCL, 0.2 g; $CaCl_2$, 0.2 g; $NaHCO_3$, 0.02 g /1000 ml) until use.

Immunohistochemistry

Gonads from four transgenic tilapias in maturation (including one ovotestis), and six transgenic juveniles were analyzed by means of immunohistochemistry utilizing a monoclonal antibody anti-GFP (Covance Research Products Inc, Richmond, California, USA), at 1:3500 dilution in PBS (0.57 µg/ml final concentration). As negative controls, the primary antibody was omitted in the GFP positive samples. Moreover, immunohistochemistry for GFP in testis samples from wild adult Wistar rat and gonad samples from wild tilapia embryos were also utilized as negative controls. The immunohistochemistry reaction was performed in gonadal fragments immersed into Bouin`s fixative solution. The samples were dehydrated in ethanol, and embedded in Paraplast. Sections were processed according to the ABC technique (Hsu *et al*., 1981). After deparaffinization and dehydration the sections were pre-treated with 0.03% H_2O_2 for 30 min, at room temperature, to block endogenous peroxidase activity. Samples were then washed in PBS, pH 7.4, two times for 5 min each, and immersed into a 1:1 solution containing 6% normal goat serum and super blocking solution (Pierce Chemical Co., Rockford, Illinois, USA) for 30 min to block non-specific staining. The sections were then incubated overnight at 4°C with the primary antisera diluted in PBS with 1% bovine serum albumin (BSA). They were washed in PBS three times for 5 min and incubated with the diluted biotinylated anti-mouse IgG for 30 min, washed in PBS three times for 5 min and incubated for 30 min with avidin–biotin complex. Immunoreactive sites were revealed using buffered solution of 3,3-diaminobenzidine–tetrahydrochloride (DAB) (Substrate–chromogen System, Dako Ltd., Carpinteria, California, USA).

In order to confirm the reliability of the GFP immunoreaction in our medaka β-actin/EGFP transgenic tilapia, we performed epifluorescence observation followed by immunohistochemistry for GFP, using muscle tissue dissected from beneath the dorsal fin in the area surrounding the pterygiophore.

Results

Establishment of the transgenic strain

In order to generate lines of transgenic tilapia containing the exogenous GFP gene, we introduced the medaka β-actin construct into 75 tilapia eggs. Twelve individuals survived until one month old and they were subjected to fluorescence screening for GFP expression. As a result, we identified ten fish expressing the GFP, one of which was found to transmit the transgene to his progeny. This germline transmitter was used to produce the F_1 generation by crossing it with wild-type fish. The transmission rate of the transgene to the F_1 generation was 28.5% (4 GFP(+) offspring/14 examined offspring), as detected by fluorescence observation. In agreement with the heterozygous condition of the F_1 male fish, the transgene transmission measured in the F_2 generation showed Mendelian inheritance with about 50% (136 GFP(+) offspring/291 total offspring) of the offspring expressing the GFP fluorescent signal. The genomic PCR analysis validated the results of the fluorescence observation, meaning that no GFP(-) individuals analyzed (n = 8) carried the GFP gene in their genomes, while the GFP(+) fishes (n = 8) did carry the transgene (Fig. 1).

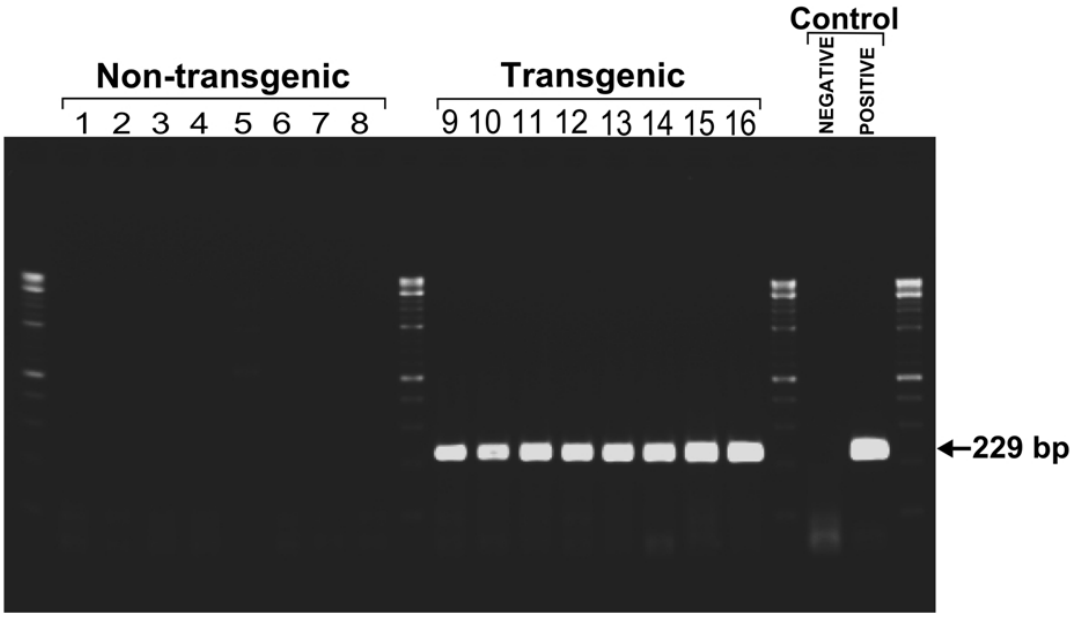

Figure 1. Presence of the medaka beta-actin/EGFP transgene in fluorescence-sorted F_2 generation Nile tilapia individuals, as detected by PCR of genomic DNA extracted from caudal fin clips. Lanes 1-8 contain DNA of GFP(-) fluorescence sorted fish. Lanes 9-16 contain DNA from GFP(+) fish. Negative control: PCR product without template DNA; positive control: beta-actin/EGFP plasmid DNA (10 pg) + wild-type genomic DNA (10 ng). Arrow: 229 bp PCR product.

Fluorescence observation

In the F_2 generation, some fluorescent cells were first observed at 2 days after fertilization in gastrula stage embryos (results not shown), but they became conspicuously visible along the body axis at 6 days post-fertilization in pre-hatched embryos (Fig. 2A). In hatching embryos (8 days post-fertilization), the GFP gene was strongly expressed in the area surrounding the gills and operculum, as well as in the cephalic region (Fig. 2B). In early larvae (10 days post-fertilization), fluorescent cells were scattered throughout the body, forming aggregations around the dorsal-cephalic and mouth areas (Fig. 2C).

Fluorescence observation of 38 days post-fertilization juvenile fish showed a uniformly expressed pattern of the GFP gene (Fig. 3A). This green fluorescence contrasts with the yellow autofluorescence of the abdominal region between the lateral and anal fins containing the internal organs. Control non-transgenic fish belonging to the same batch did not show any green fluorescence (Fig. 3B).

We also analyzed the GFP fluorescence in the caudal fins, muscle, and in several internal organs (gills, heart, liver, testes, and ovaries) using 140 and 240 days post-fertilization individuals (Fig. 4). In the caudal fin area, a patch of GFP(+) cells was observed around the basis of the fin rays, and along the posterior part of the lateral line (Fig. 4A). In muscle, GFP was expressed ubiquitously, but we found a stronger fluorescence in the cells beneath the dorsal fin, in the area surrounding the pterygiophore (Fig. 4C and 5A). In the gills, a cluster of GFP(+) cells was observed near the area of the arch's cranial-insertion (Fig. 4E). A group of GFP(+) cells was also observed near the proximal region of the aorta in the heart (Fig. 4G). No noticeable fluorescence was detected in liver from transgenic individuals when compared with non-transgenic controls (Fig. 4I and J). In testis, we observed high autofluorescence in the posterior part of the gonad, near the genital opening (Fig. 4K and L). Spot-like GFP(+) cell clusters were also observed in this posterior area (Fig. 4K; inset arrow heads), but in the anterior and medium areas, GFP expression was weak (Fig. 4K) and not clearly distinguishable from non-transgenic control testes (Fig. 4L). In ovaries dissected from transgenic females, GFP was conspicuously expressed throughout the organ, in a variegated fashion (Fig. 4M). In non-transgenic females, GFP fluorescence was not observed in the ovaries (Fig. 4N).

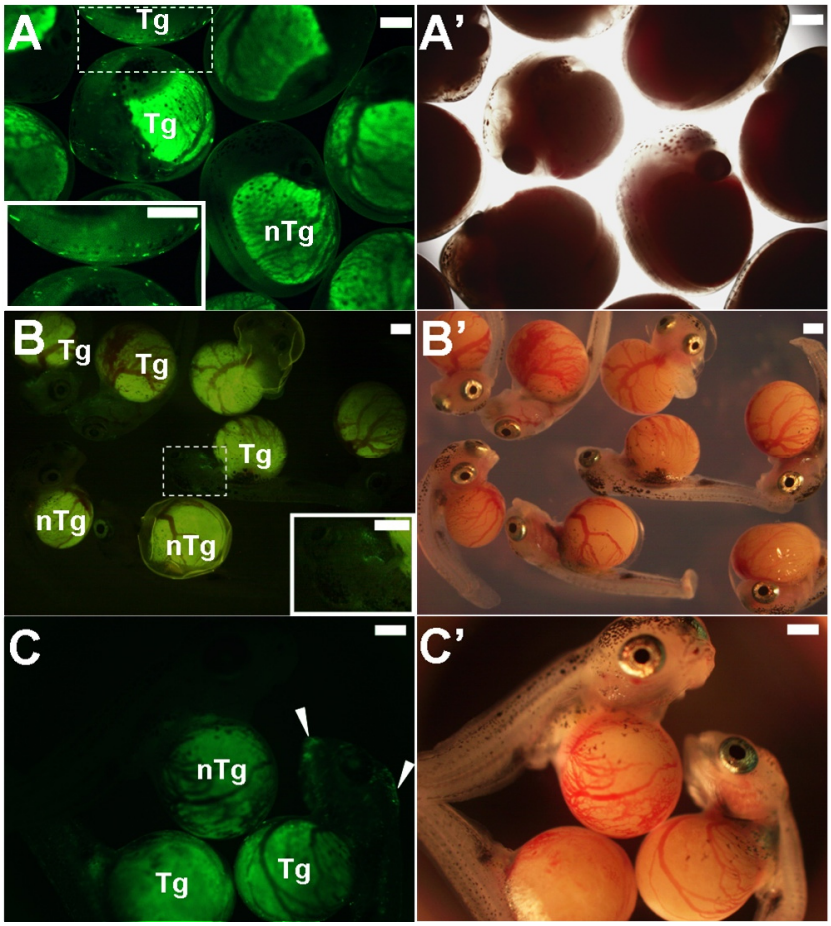

Figure 2. Medaka beta-actin/EGFP transgenic Nile tilapia embryos at 6 **(A and A')**, 8 **(B and B')**, and 10 days post-fertilization **(C and C')**, showing GFP expression. Left side: Fluorescent view. Right side: Bright field. **(A)** In 6 days post-fertilization transgenic (**Tg**) embryos, GFP is expressed along the body axis in a spot-like pattern (inset figure), while no GFP expression is observed in non-transgenic (**nTg**) embryos. **(B)** In 8 days post-fertilization hatching transgenic embryos, GFP is mainly visualized around the operculum and head (inset figure). No GFP expression was observed in non-transgenic embryos. **(C)** In 10 days post-fertilization larvae GFP fluorescent cells are scattered throughout the body, with aggregations around the cephalic region and mouth areas (arrow heads). No GFP fluorescence was observed in non-transgenic embryos. Bar: 5 mm for **A**, **A'**, **C** and **C'**; bar: 1 mm for **B** and **B'**.

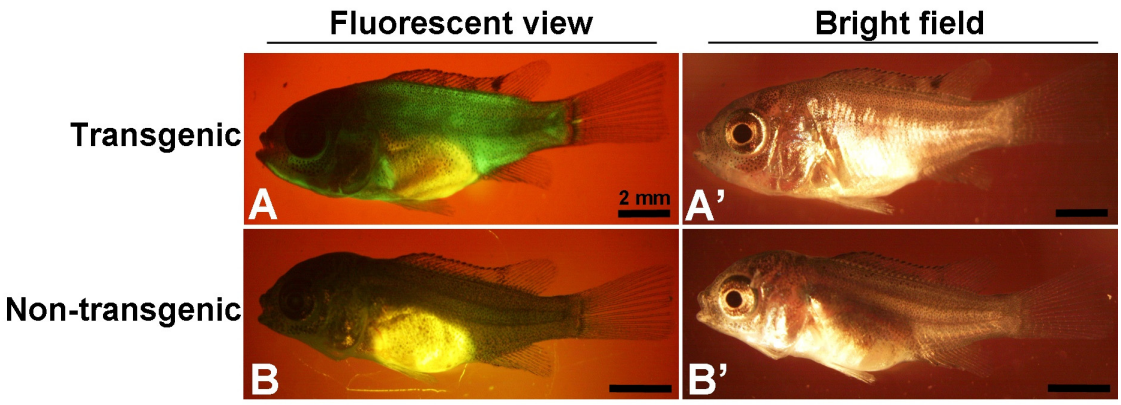

Figure 3. Epifluorescence and bright-field pictures from 38 days post-fertilization medaka beta-actin/EGFP transgenic and non-transgenic Nile tilapia individuals. **(A and A')** GFP is ubiquitously expressed in the whole body, with strong autofluorescence in the abdominal area. (**B and B'**) No GFP expression was detected in non-transgenic juveniles. **(A')** Bright field view of the transgenic individual. **(B')** Bright field view of the non-transgenic individual.

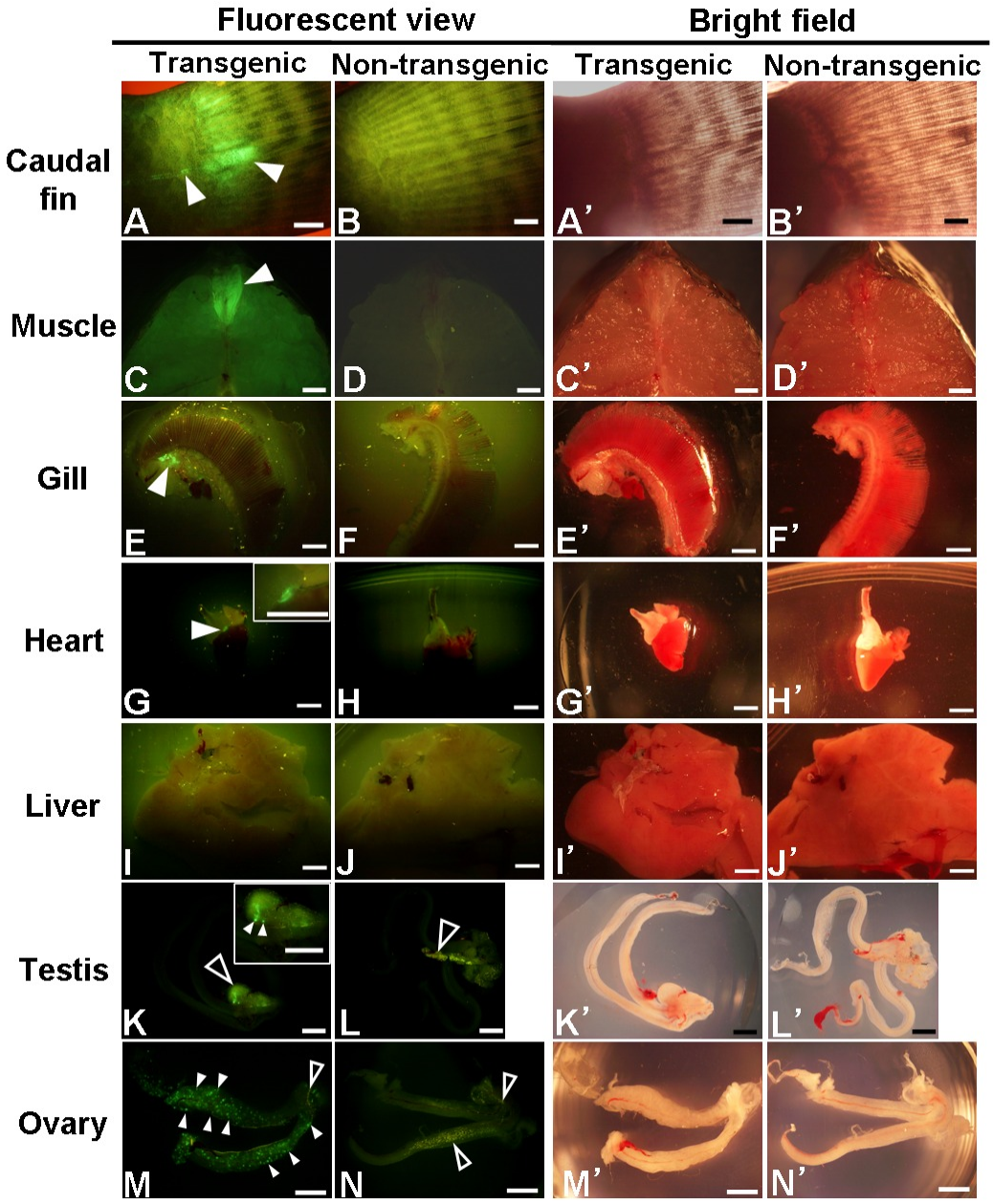

Figure 4. Epifluorescence and bright-field pictures from caudal fins, muscle, and dissected internal organs in medaka beta-actin/EGFP transgenic and non-transgenic Nile tilapia individuals, showing the F_2 generation at 140 days post-fertilization (except for figures **C**, **D**, **C'**, and **D'**, taken from F_3 generation individuals at 240 days post-fertilization; bars: 2 mm). **(A)** GFP(+) cells in the mid-anterior region of the caudal fin and along the posterior part of the lateral line (arrow heads). **(B)** No GFP(+) cells were observed in non-transgenic individuals. **(C)** GFP was expressed ubiquitously in the muscle, with a patch of stronger fluorescence in the cells located beneath the dorsal fin (arrow head). **(D)** No GFP expression was observed in non-transgenic individuals. **(E)** Hemibranch showing a cluster of GFP(+) cells near the cranial insertion of the gill's arch (arrow head). **(F)** No GFP(+) cells were observed in non-transgenic individuals. **(G)** A group of GFP(+) cells was visualized near the proximal region of the aorta (arrow head; inset figure). **(H)** No GFP expression was detected in non-transgenic individuals. **(I and J)** In liver GFP(+) cells were not observed in transgenic or non-transgenic individuals. **(K)** Spot-like GFP(+) cell clusters were observed in the posterior area of the testis (inset arrow heads), and autofluorescence was visualized in both transgenic **(K)** and non-transgenic **(L)** testes (black arrow heads). **(M)** In ovaries, GFP(+) cells were scattered in the whole organ (arrow heads) in a mosaic-like pattern of expression; weak autofluorescence was observed in the posterior zone (black arrow heads). **(N)** No GFP expression was observed in non-transgenic samples, but only strong autofluorescence in the posterior and mid-sections (black arrow heads).

Immunohistochemistry

As expected, we observed a corresponding distribution/intensity of GFP immunostaining in the muscle tissue, when we compared it with the original GFP expression detected by epifluorescence (Fig. 5B and 5A). The area showing stronger GFP fluorescence (Fig. 5A, red dotted line) was also strongly labeled by the antibody after the immunohistochemistry processing (Fig. 5B, red dotted line), while the region with weaker fluorescence (Fig. 5A, white broken line) showed weak immunostaining in the histological section (Fig. 5B, white broken line). No specific staining was detected when the primary antibody was omitted (Fig. 5B').

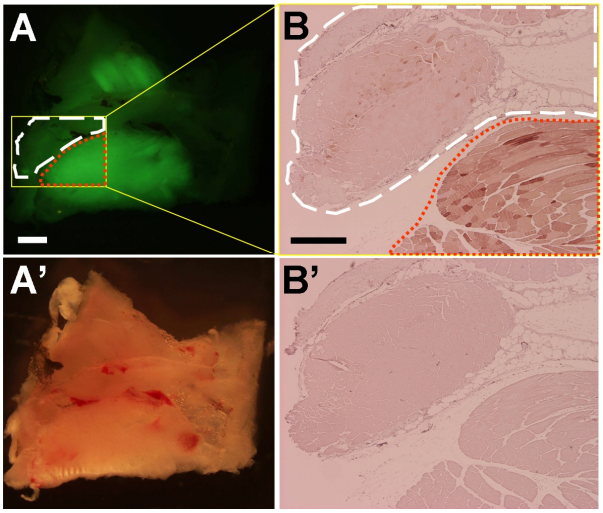

Figure 5. Epifluorescence **(A)** and bright-field **(A')** pictures of muscle tissue surrounding the pterygiophore showing strong GFP expression, dissected beneath the dorsal fin from a GFP(+) Nile tilapia (white bar: 1 mm). **(B)** Immunohistochemistry for GFP from the same tissue sample, showing differential expression in the intensity of the immunoreaction, which relates to the original fluorescence pattern showed in **(A)** (black bar: 0.5 mm). The region enclosed by a yellow solid line square in **(A)** shows the approximate area used for immunohistochemistry in **(B)**. The white broken lines enclose the areas showing weak fluorescence **(A)** and weak immunostaining **(B)**. The red dotted lines enclose the areas possessing strong fluorescence **(A)** and strong immunostaining **(B)**. **(B')** Negative control for GFP immunohistochemistry in the GFP(+) tilapia muscle, in which the primary antibody was omitted.

The germinal epithelium and interstitial tissue from gonads of juveniles and sexually mature Nile tilapia individuals were in general broadly labeled (Fig. 6A, 6D, 6E and 6F). In the testes of GFP adult (Fig. 6A, 6D and 6E) and juvenile tilapias (Fig. 6F) germ cells were strongly labeled. Primary spermatogonia cytoplasm was slightly labeled (6D), however a more intensive labeling was found in the area around the nuclear and cell membrane. Primary spermatocytes, inside cysts, were homogeneously and slightly labeled, while spermatids were the most intense germ cell labeled (Fig. 6D). Concerning somatic cells, Sertoli and Leydig cells were also broadly labeled (Fig. 6D). Female germ cells were also broadly labeled (Fig. 6F). No specific staining was detected in any negative controls utilized (Fig. 6B, 6C, 6G and 6H).

Discussion

This medaka β-actin/EGFP transgenic tilapia strain expressing GFP in several tissues, including germ cells in testis and ovary, would constitute a good material for germ cell transplantation experiments either utilizing juvenile or adult transgenic specimens as donors. In order to evaluate the colonization behavior of donor-derived germ cells in recipient gonads, appropriate cell markers are required. Chemical dyes can only be recognized in target germ cells for relatively short periods before they vanish, making it impossible to assess the long-term colonization dynamics of donor cells in recipient gonads. Additionally, these markers cannot be transmitted to successive generations, making the tracing of donor-derived offspring impossible. By using the transgenic strain established in this study, donor-derived germ cells could be identified in the recipient gonads by immunohistochemistry and their behavior can be traced for long periods of time. In addition, donor-derived offspring could be readily identified and sorted by simply raising the produced embryos until 10 days post-fertilization, when GFP expression becomes strong enough for accurate screening of transgenic embryos. At around 38 days post-fertilization, GFP is widely expressed and intense, and is easily detectable by fluorescence stereomicroscopy. In adult individuals, expression of GFP becomes patchy, but is still detectable in the mid-anterior region of the caudal fin, and screening for transgenic individuals is still possible.

Another potential strategy for tagging donor-derived embryos is the utilization of color mutants, such as albino strains (Boonanuntanasarn *et al.*, 2004). One limitation of this approach is that color is a single-locus marker, and spontaneous color mutations could occur randomly during the gametogenic process, reducing the reliability of the method. On the contrary, green fluorescence will never be produced by spontaneous mutations, making it a very reliable marker of donor-derived offspring.

According to the immunohistochemical analysis, GFP driven by the medaka β-actin promoter was expressed in germ cells from transgenic tilapias, but the antibody against GFP did not react with any antigen in wild-type germ cells. These results indicate that by using immunohistochemistry it would be possible to trace the transgenic donor-derived germ cells in the wild-type recipient gonads. Thus, the GFP tilapia males

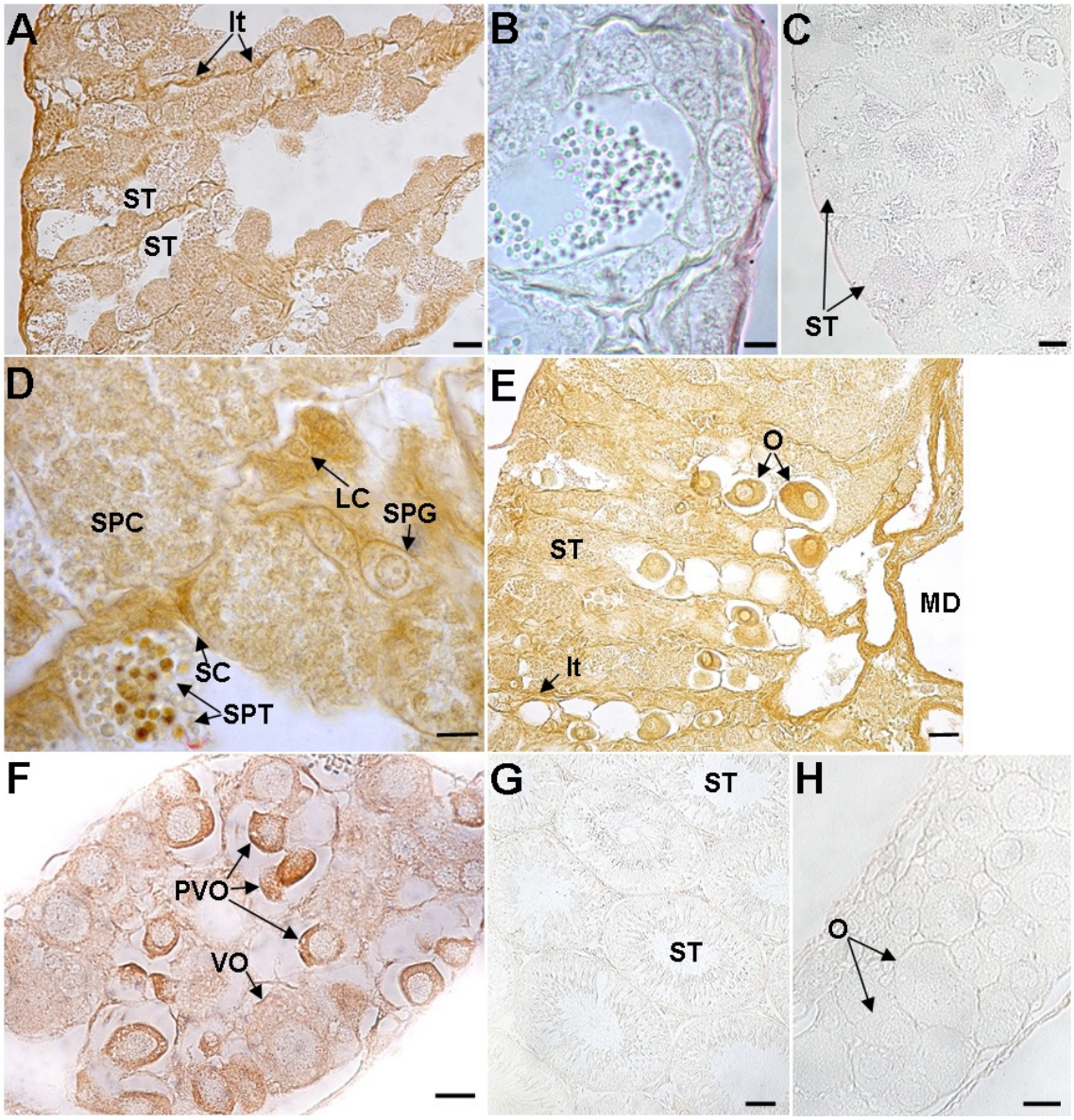

Figure 6. **(A)** Immunohistochemistry for GFP in the testis from a mature (in maturation) GFP(+) Nile tilapia, showing seminiferous tubules (**ST**) longitudinally sectioned. Observe that both interstitial tissue (**It**) and the seminiferous tubules (**ST**) are labeled by the antibody (bar: 40 µm). **(B)** Negative control for GFP immunohistochemistry in the GFP(+) tilapia testis sample, in which the primary antibody was omitted (bar: 10 µm). **(C)** Negative control for GFP immunohistochemistry reaction labeling in a wild Nile tilapia testis sample, showing seminiferous tubules (**ST**) longitudinally sectioned (bar: 40 µm). **(D)** Immunohistochemistry for GFP in the testis from a GFP(+) sexually mature Nile tilapia, showing in detail staining with GFP antibody. The primary spermatogonia (**SPG**) is labeled mainly in the regions close to the nuclear envelope and plasma membrane and lightly labeled in the nucleus and the cytoplasm. Primary spermatocytes (**SPC**) are lightly labeled, while spermatids (**SPT**) are either heavily or lightly labeled mainly around the nucleus. Sertoli (**SC**) and Leydig cells (**LC**) are also labeled (bar: 10 µm). **(E)** Immunohistochemistry for GFP in the ootestes from a GFP(+) sexually mature tilapia, showing seminiferous tubules (**ST**) longitudinally sectioned. Observe also the presence of labeled oocytes (**O**). In this GFP(+) ootestis, the pattern of labeling for germ cells was similar to that found for germ cells in GFP(+) testis (see Figs. **6A** and **6D**). **MD**: main spermatic duct (bar: 100 µm). **(F)** Immunohistochemistry for GFP in the ovary from a GFP(+) positive juvenile of Nile tilapia, showing previtelogenic (**PVO**) and vitelogenic (**VO**) oocytes. In comparison with vitelogenic oocytes, previtelogenic oocytes show heavier labeling for GFP (bar: 100 µm). **(G)** Negative control for GFP immunohistochemistry in adult Wistar rat (non-expressing GFP) testis sample. **ST**: Seminiferous tubules (bar: 50 µm). **(H)** Immunohistochemistry for GFP in a wild Nile tilapia embryo; (**O**): oocytes (bar: 100 µm).

might be used as potential germ cell donors for transplantations studies, since the presence of the GFP could be easily detected in spermatogonial cells. Another promoter used to trace germ cell behavior and colonization in fish recipient gonads is the vasa gene. The vasa gene promoter is activated specifically in the germ cell lineage (Yoshizaki *et al*., 2000a). It has been previously utilized for labeling PGCs (Yoshizaki *et al*., 2000b; Takeuchi *et al*., 2002) and spermatogonia (Okutsu *et al*., 2006a; Yano *et al*., 2008) and it has been further confirmed that these green-labeled germ cells are suitable material for germ cell transplantation (Takeuchi *et al*., 2003, 2004; Okutsu *et al*., 2006a, 2007). One disadvantage of rainbow-trout vasa/GFP is the fading of GFP expression in rainbow trout male germ lines when these germ cells enter meiosis (Yano *et al*., 2008). In contrast, medaka β-actin-mediated GFP expression is detected not only in spermatogonia, but also in spermatocytes and spermatids. These results indicate that by using this medaka β-actin/EGFP transgenic line, it would be possible to trace not only donor spermatogonia into the recipient gonad, but also more differentiated stages such as spermatocytes and spermatids. This would give us a better picture of the colonization dynamics in transplanted recipients.

Interestingly, we found considerable variations in GFP expression in the different tissues and developmental stages analyzed, as detected by fluorescence observation. There are two likely explanations. First, we used a heterologous promoter belonging originally to medaka, a non-related fish species; thus, it is possible that the promoter regulatory system did not work as faithfully as reported for the original species (Takagi *et al*., 1994; Hamada *et al*., 1998). Additionally, these variations may be the result of the position effect, in which expression of a foreign gene can be affected when it is integrated near a strong endogenous enhancer (Bonnerot *et al*., 1990). If the GFP gene was integrated near an enhancer of an endogenous gene for which expression was tissue-specific or developmentally regulated, the originally ubiquitous-permanent pattern of expression could have been altered. In this study, we could not conclude whether the promoter, the position effect, or the interaction of these phenomena was responsible for this variation. Also, since we only found one transgenic animal among the G_0 generation possessing germ cell transmission ability, it is possible that if we had injected more embryos and thus generated more transgenic lines having different integration sites in the host genome, we could had obtained lines showing a more uniform expression pattern. Further experiments will be required to elucidate this issue.

Here, we report the production of a transgenic line of Nile tilapia carrying the GFP gene driven by a medaka β-actin promoter. GFP expression was characterized by fluorescence observation in F_2 and F_3 generations at different developmental stages and in several organs and tissues. The utilization of this transgenic line in a germ cell transplantation system could offer a fast and reliable screening of donor-derived transgenic offspring, as well as an accurate and long-term tracing of donor-derived germ cells in wild-type recipient gonads. In the future, germ cell transplantation using Nile tilapia also could help to preserve the genetic resources of threatened cichlids, through cryopreservation and interspecies transplantation of germ cells from endangered cichlids germ cells into *O. niloticus* recipients.

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