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# Fusion Gene Vectors Allowing for Simultaneous Drug Selection, Cell Labeling, and Reporter Assay in Vitro and in Vivo

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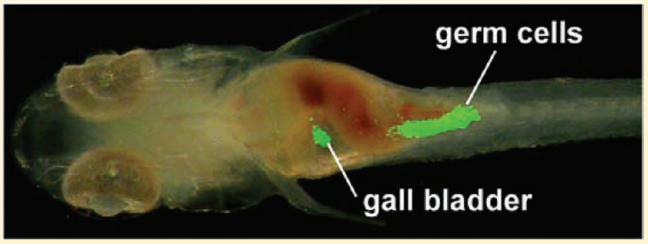
Supporting Information

**ABSTRACT:** Vector systems allowing simultaneously for rapid drug selection, cell labeling, and reporter assay are highly desirable in biomedical research including stem cell biology. Here, we present such a vector system including pCVpf or pCVpr, plasmids that express *pf* or *pr*, a fusion protein between puromycin acetyltransferase and green or red fluorescent protein from CV, the human cytomegalovirus enhancer/promoter. Transfection with pCVpf or pCVpr produced a ~10% efficiency of gene transfer. A 2-day pulse puromycin selection resulted in ~13-fold enrichment for transgenic cells, and continuous puromycin selection produced stable transgenic stem cell clones with retained pluripotency. Furthermore, we developed a PAC assay protocol for quantification of transgene expression. To test the usefulness for cell labeling and PAC assay in vivo, we constructed pVASpf containing *pf* linked to the regulatory sequence of medaka germ gene *vasa* and generated transgenic fish with visible GFP expression in germ cells. PAC assay revealed the highest expression in the testis. Interestingly, PAC activity was also detectable in somatic organs including the eye, which was validated by fluorescence in situ hybridization. Therefore, the *pf* and *pr* vectors provide a useful system for simultaneous drug selection, live labeling, and reporter assay in vitro and in vivo.




A vector system is highly desirable if it enables rapid drug selection for cell labeling and reporter assay for quantification of transgene expression. A dozen of natural genes can be used to measure transcriptional activity of regulatory DNA elements, to localize temporospatial gene expression in particular cells/tissues, and to enrich for transgenic cells by drug selection. For enzymatic quantification of reporter gene expression, there are three sensitive reporter assay systems, which are based on the bacterial chloramphenicol acetyltransferase (CAT assay[1]), $\beta$-galactosidase as the protein product of gene LacZ (LAC assay), and the firefly luciferase (LUC assay[2]), respectively. Of these, only LacZ ($\beta$-galactosidase) allows for localization of reporter gene expression by histochemical staining. None confers drug resistance on eukaryotic cells. Green (GFP) and red fluorescent protein (RFP) offer a unique opportunity to trace temporospatial gene expression in living cells and organisms. They do not allow for drug selection and quantitative reporter assays. Several genes allowing for drug selection of transgenic cells are available. Positive selectable markers include neomycin phosphotransferase (NEO), hygromycin phosphotransferase (HYG), and puromycin acetyltransferase (PAC) that confer resistance to G418, hygromycin, and puromycin, respectively. Reporter assay procedures for these three genes have also been reported but have not been widely used. Of these three antibiotics, puromycin selection is the quickest. With puromycin, mammalian cells are killed after 1 day,[3,4] compared to weeks needed for G418 and hygromycin selection.

Plasmid constructs expressing bifunctional fusion genes have been generated that combine a reporter gene and a selectable marker. These include *geo* between $\beta$-galactosidase and *neo*,[5] *pac:tk* between *pac* and herpes simplex virus thymidine kinase,[3] *neo-tk* between *neo* and *tk*,[6] *hyg-gfp* between *hyg* and *gfp*,[7] *gfp-tk* between *gfp* and *tk*,[8] EGFP-puro between GFP and *pac*,[9] neo-EGFP between *neo* and *gfp* (Clontech). Here *gfp* is the gene encoding GFP. *pac* is the gene encoding PAC, and *tk* is the gene encoding the herpes simplex virus TK. Retroviral vectors have also been generated that express triple fusion genes *hyg/gfp/tk* and *puro/gfp/tk*.[10] Development of these fusion genes has increased the utility of the reporter genes and selectable markers. However, none of these vectors has been used for simultaneous drug selection, cell labeling, and quantitative reporter assay.

This study was aimed at the development of plasmid vectors for simultaneous cell labeling, rapid drug selection, and reporter assay. These vectors express fusion genes *pf* or *pr* that combine PAC and GFP or RFP. This vector system provides a powerful tool for obtaining pure population of fluorescently labeled cells within two days of drug selection. We show that *pf* or *pr* expression and puromycin selection do not compromise the pluripotency of medaka stem cells. More importantly, PAC assay

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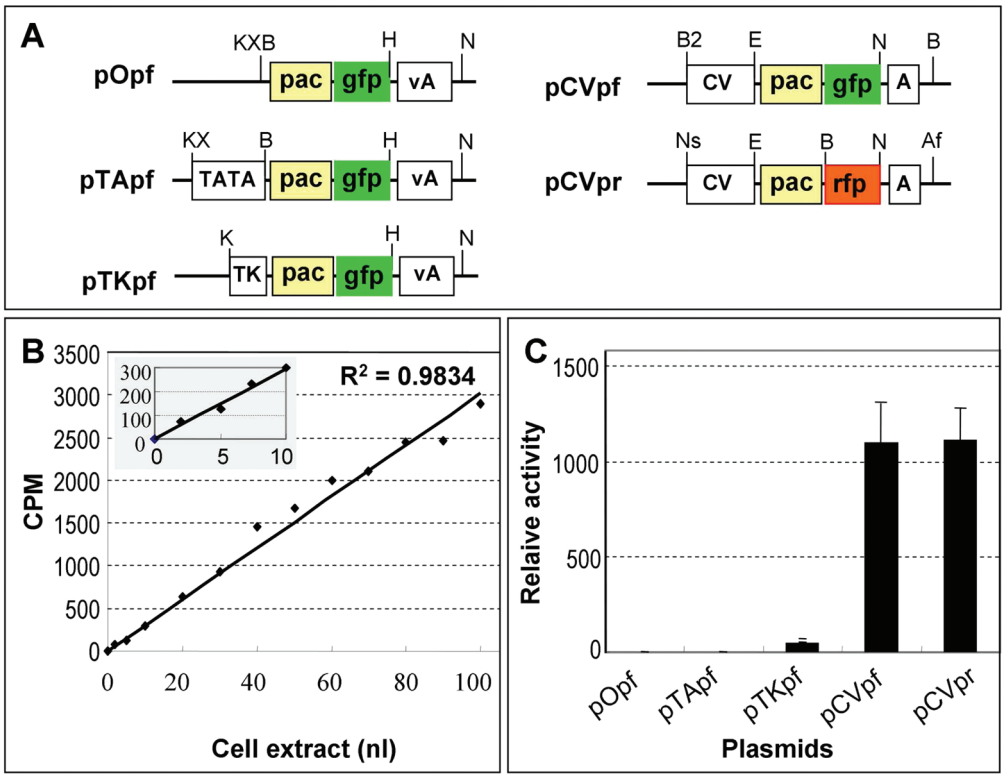


**Figure 1.** Vectors and PAC assay in vitro. (A) Schematic structures of *pf* and *pr* plasmid vectors. Thin line, plasmid backbone; cytomegalovirus, CV, early gene enhancer/promoter; O, promoterless; TA, a 81-bp TATA box-containing minimal promoter of the human herpes simplex virus (HSV) thymidine kinase (TK); TK, a 150-bp HSV TK promoter; *pac*, gene encoding puromycin acetyltransferase; *pf* and *pr*, fusion between *pac* and *gfp* or *rfp*; A, SV40 polyA signal; vA, 3′-untranslated region including the polyA signal of the medaka *vasa* gene. Also shown are important restriction sites: Af, *Afl*II; B, *Bam*HI; B2, *Bgl*II; H, *Hind*III; K, *Kpn*I; N, *Not*I; Ns, *Nsi*I; X, *Xho*I. (B) PAC assay using stable transgenic MES1 cells expressing the *pf*, showing the linearity within a wide range from 1 to 100 nL of cellular extract. PAC activity is expressed as counts per minute (CPM). (C) Relative activities by PAC assay after transient gene transfer in MES1 cells. Values are means ± standard errors (bars) from three independent experiments in triplicates. The promoterless plasmid pOpf serves as a negative control whose value is considered as the background and subtracted from the values obtained with other constructs. As a basal reference, pTApf provides unit activity for comparison. Relative activities of different samples are calibrated for transfection efficiency using LacZ assay as the internal control after cotransfection with pSVTKlacZ.

in vivo in combination with validation by fluorescence in situ hybridization (FISH) reveals the promoter activity of medaka germ gene *vasa* not only in germ cells but also in several somatic organs.

## MATERIALS AND METHODS

**Fish.** Work with fish followed the guidelines on the Care and Use of Animals for Scientific Purposes of the National Advisory Committee for Laboratory Animal Research in Singapore (permit number 27/09). Medaka strains Orange, *i1*, *i3*, af, and transgenic line Vg were maintained as described.[11−15]

**Construction of Plasmids.** Five plasmid vectors were generated, and their schematic structures are shown in Figure 1. Briefly, the *pac* was released with *Bam*HI and *Spe*I (blunted) from pCMVpac:tk[3] and was inserted between *Bgl*II and *Nco*I (blunted) in pNeoEGFP (Clontech,); in this construct, the *pac* replaces *neo* by fusion 5′ to *gfp*, generating pCVpf in which a fusion gene (*pf*) between *pac* and *egfp* is under the control of the cytomegalovirus early gene enhancer/promoter (CV). For pOpf, the fusion gene *pf* was amplified by PCR using primers Pa (AAGGATCCGATGACCG) and GF (TTTGCGGCCGCTTAAGCTTTACTTGTACAGCTCGTCCAT) from pCVpf, cloned between *Bam*HI and *Not*I sites of pBluescript KS II+. A 648-bp fragment of the polyA signal sequence of the medaka *vasa* gene was amplified by PCR using primers VE23 (AACAAGCTTCAGCAGGA; underlined letters in primers are restriction sites for cloning) and V3 (AAAGCGGCCGCGAAAAAAAGAATCTAGCAGTCTGGG) from medaka genomic DNA and cloned between *Hind*III and *Not*I sites downstream of the *pf*. To construct pTKpf, the tk promoter (TK) from pBLcat5 (Boshart et al.[1]) was cut by *Hind*III (blunted) and *Bgl*II and inserted between *Xho*I (blunted) and *Bam*HI sites upstream of the *pf* in pOpf. Similarly, an 81-bp fragment of the minimal TK containing a TATA box was amplified by PCR using primers TK5′ (ACTCGAGCGCATATTAAGGTG) and TK3′ (ATGGATCCGCGGCACGCTGTTG) and cloned into pOpf between *Xho*I and *Bam*HI sites upstream of the *pf*. To construct pCVpr, the *pac* gene was PCR-amplified using primers P1 (TTTGAATTCGCCACCATGACCGAGTACAAGCCCAC) and P2 (CGGTGGATCCCGACTAGTGGCACCGGGCTTGCG) from pCMVpac:tk,[3] a gift from Dr. Karreman. The PCR product was digested with *Eco*RI and *Bam*HI and cloned into pDsRed1-1 (Clontech) in front of *rfp* (gene encoding red fluorescent protein), forming a fusion gene (*pr*) between the *pac* and *rfp* driven by the CV enhancer/promoter.

To construct pVASpf, the 5007-bp regulatory sequence including exon 1 and part of exon 2 of the medaka *vasa* gene was


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obtained by PCR with primers VP (TAGCTCGAGTAATGGTTCTCTATTTCTCTT) and VE2 (CAGGGATCCATCGTCT GATCTGAAGACACAAGA) and were subcloned to *Xho*I and *Bam*HI upstream of the *pf* in pOpf. Finally, the V2pf cassette was released from pVASpf and subcloned into Isce-pBluescript II SK+,[16] leading to pIsceV2pf. In pIsceV2pf, there are two meganuclease *I-Sce*I recognition sites (TAGGGATAACAGGGTAAT) flanking the multiple cloning sites.

**Cell Transfection, Drug Selection, and Chimera Formation.** Plasmid DNA was prepared using Qiagen Midi plasmid kits. Medaka diploid ES cell line MES1,[12] medaka spermatogonial cell line SG3,[17] and medaka haploid ES cell line HX1[15] have been described and were maintained in ESM4 medium. Cell transfection was performed using the GeneJuice reagent (Novagen) as described.[18] For drug selection, puromycin was added at 1 $\mu$g/mL to cell cultures at $\geq$18 h post-transfection (hpt; after medium change). Cell transplantation was performed as described.[11] Briefly, MES1 stably expressing *pr* and HX1 stably expressing *pf* were mixed at a 1:1 ratio, and 200 cells were transplanted into blastula recipients of Orange medaka.

**Production of Transgenic Medaka.** Embryo microinjection was performed essentially as described.[19] Briefly, plasmid pVASpf was linearized by *Kpn*I digestion, dissolved at 50 ng/$\mu$L in Yamamoto Ringer's solution containing 0.1% phenol red, and approximately 500 pL was microinjected into medaka embryos at the 1-cell stage. GFP-positive fry were screened by fluorescent microscopy and reared to the adulthood. Founders (F0) were crossed to wildtype fish to produce F1 progeny, which were self-crossed to generate F2 animals. Germline transmission was fluorescently scored during embryonic development. Homozygous adults in F2 were used for PAC assay.

**Fluorescence in Situ Hybridization (FISH).** FISH was performed using an antisense *vasa* riboprobe as described.[20]

**PAC Assay.** For the reporter assay, cells in 24-well plates were transfected with each of the *pf* vectors plus pSVTKlacZ that expresses the *lacZ* gene from the SV40 early gene enhancer linked to the TK promoter (Winkler et al.[19]). At 48 hpt, cellular extract was prepared using the $\beta$-galactosidase enzyme assay system (Promega, #E2000). Briefly, cells were washed with PBS, incubated in 100 $\mu$L of lysis buffer for 15 min at room temperature, scraped, and transferred into 1.5 mL tubes. After centrifugation, the clear supernatant was collected into 1.5 mL tubes. Protein concentration was determined using the BioRad protein assay (#500-0006). For PAC assay, we adopted the protocol by de la Luna and Ortin[21] with modifications. Briefly, 40 $\mu$L of crude cellular extract was mixed with 10 $\mu$L of PAC assay cocktail [0.1 M Tris−HCl, pH 8.0, 0.2 mM puromycin, and 0.5 $\mu$L of [$^3$H]-acetyl-CoA (Amersham)]. After incubation for 4 h at 37 °C, the reaction was terminated by adding 200 $\mu$L of 5 M NaCl−0.1 M borate, pH 9.0. The reaction was transferred to a scintillation vial containing 3 mL of scintillation fluid (BetMax ES; ICN), and content was gently mixed. The activity was measured on a liquid scintillation counter (Winspectral 1414, Wallac).

For PAC assay in vivo, various organs were dissected from adult control medaka and F2 transgenics produced by microinjection of pCVpf (see above). Organs were frozen at −80 °C in 1.5 mL tubes and homogenized with Eppendorf pestles. The homogenates were subjected to three freezing−thawing cycles. After centrifugation with maximal speed for 15 min at 4 °C (Micro 22R, Hettich), the clear supernatant was transferred to new 1.5 mL tubes. Protein concentration was estimated as above, and an equal amount of crude proteins was used for PAC assay.

**LacZ Assay.** For calibration of transfection efficiency between different samples cotransfected with pSVTKlacZ, the LacZ assay was performed using 40 $\mu$L of crude cellular extract plus 10 $\mu$L of lysis buffer, which was combined with 50 $\mu$L of 2× assay buffer in 96 well-plates. After incubation for 4 h at 37 °C, the reaction was terminated by adding 150 $\mu$L of 1 M sodium carbonate and measured at a wavelength of 420 nm on a plate reader (Spectra Max 340, Molecular Devices, Sunnyvale, CA).

**Microscopy and Photography.** Living cells and embryos were observed and photographed as described.[15,20] Micrographs were taken using a Zeiss AxioCam MRc digital camera equipped with AxioVision 4 software. Cells were counted on a hemocytometer and/or on micrographs merged from phase contrast and fluorescent optics. Embryos and fry were visualized using a Leica MZFLIII stereo microscope equipped with a Fluo III UV-light system and a GFP2 filter and photographed using a Nikon E4500 digital camera (Nikon Corp).

**Statistics.** Statistical analyses were calculated using Graphad Prism v4.0. Data consolidated were presented as mean ± s.d.[11]

## RESULTS AND DISCUSSION

**Construction of Vectors.** Simultaneous rapid drug selection, cell labeling, and reporter assay of transgene expression after transient and stable transfer of the same plasmid vector(s) in vitro and in vivo is highly desirable in many cases. We generated five vectors expressing fusion genes between the *pac* and green (*pf*) or red fluorescent protein (*pr*). The *pac* gene encodes puromycin acetyltransferase and confers on eukaryotic cells resistance to antibiotic puromycin. Selection by puromycin is the most rapid system in mammalian cells.[3,4] The PAC enzyme can also be used for reporter assay.[21] Fluorescent proteins are popularly used living markers for cell labeling. Except pOpf that is promoterless, the fusion genes are driven by a minimal promoter containing a TATA-box (pTApf), the basal TK promoter (pTKpf), or the strong CV enhancer/promoter (pCVpf and pCVpr). The TATA sequence and TK promoter have been widely used as basal references in reporter assay in many organisms including fish.[2] The CV sequence is widely used for transgene expression at high levels after transient and stable gene transfer.

**Gene Transfer and PAC Assay in Vitro.** Acetyltransferase has widely been used for reporter assay. In fact, CAT assay based on the chloramphenicol acetyltransferase has been a standard of a first generation of reporter assay (Boshart et al.[1]). PAC activity can also be quantitatively determined in an enzymatic reaction called PAC assay (de la Luna & Ortin[21]). The fact that pCVpf and pCVpr were able to confer the puromycin resistance indicated the presence of PAC activity. To quantify PAC activity, we established a PAC assay procedure. The radioactive donor substrate [$^3$H]-acetyl-CoA is transferred to puromycin as the recipient, thus producing [$^3$H]-acetyl-puromycin that is water-insoluble and partitioned to the organic phase allowing for measurement by scintillation. When different amounts of cellular extract from pCVpf-expressing MES1 cells (see below) were used for PAC assay, linearity ($R^2$ = 0.983) was obtained within the range of input protein extract tested from 1 to 100 nL (Figure 1B).

To show the suitability of our PAC assay in combination with the *pf* or *pr* cassettes for determining relative activities of different regulatory sequences, three additional plasmids were generated

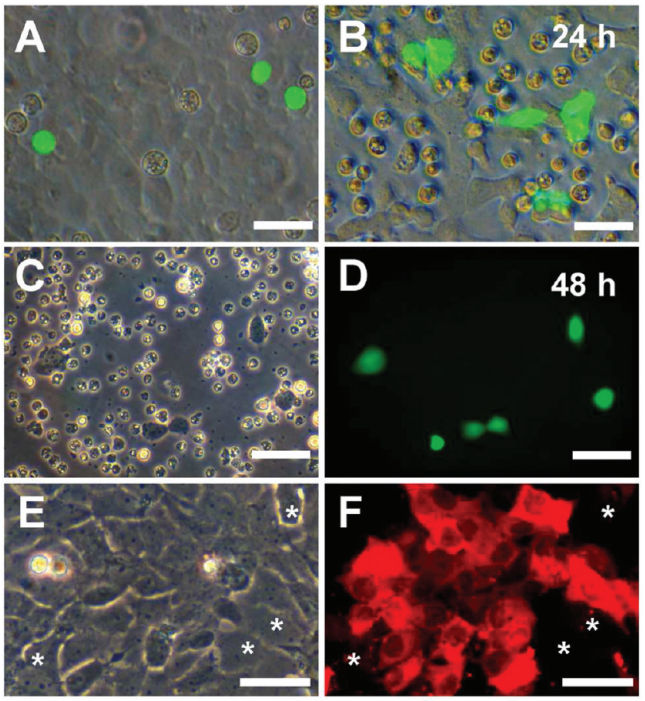


**Figure 2.** Cell labeling and drug selection in medaka ES cells. (A and B) pCVpf transfection in the medaka diploid ES cell line MES1. (A) Micrograph of cells at 42 hpt without drug selection. (B) Micrograph of cells at 42 hpt with drug selection for 18 h. (C and D) pCVpf transfection in the medaka haploid ES cell line HX1. Cells were photographed at 90 hpt with drug selection for 48 h under bright field (C) and green fluorescence optics (D). (E and F) pCVpr transfection in the medaka male germ stem cell line SG3, showing the majority of cells are RFP positive and the minority are RFP negative (asterisks). pCVpr-transfected cells were subjected to a 48 h pulse drug selection until 90 hpt and then grown in the absence of drug for 14 days when photography was made under bright field (E) and red fluorescence optics (F). For drug selection, puromycin was added at 1 $\mu$g/mL. Scale bars, 50 $\mu$m.

and included for transfection: promoterless pOpf, pTApf using the 81-bp minimal TK promoter, and pTKpf containing the 195-bp TK promoter. The relative activities of these five constructs were examined by PAC assay using cellular extract prepared at 48 hpt. Compared to pTApf that served as a reference, TK and CV enhanced the PAC activity by ~40 and more than ~1100-fold (Figure 1C). Therefore, PAC assay is quantitative to determine relative activities after stable and transient gene transfer in vitro.

**Rapid Drug Selection in Vitro.** We then examined whether the fusion genes retained the ability to confer fluorescence and puromycin resistance. When pCVpf was transfected into the medaka ES cell line MES1, the GFP signal appeared from 18 hpt onward (Figure 2A). Transfection efficiency was found to be 10% (see below). A similar observation was obtained also with pCVpr. Thus, the fusion does not compromise the fluorescing property of both GFP and RFP.

Puromycin is an aminonucleoside antibiotic and blocks protein synthesis by interacting with prokaryotic and eukaryotic ribosomes. This interaction is followed by peptide bond formation, which releases the nascent polypeptide in the form of polypetidyl puromycin. PAC activity can act as a dominant selectable marker gene, because it confers puromycin resistance by inactivating the antibiotic by N-acetylation of the tyrosinyl

**Table 1. Enrichment for Transgenic Cells by Pulse Puromycin Selection[a]**

| | fluorescing cells at 42 hpt (%)[b] | | | fluorescing cells at 90 hpt (%)[b] | | |
|---|---|---|---|---|---|---|
| vector | no drug | drug, 24 h | enrichment[c] | no drug | drug, 48 h | enrichment[c] |
| pOpf | 0 | 0 | 0 | 0 | 0 | 0 |
| pCVpf | 8.8 | 71.2 | 8.1 | 5.8 | 90.9 | 15.7 |
| pCVpr | 10.1 | 73.9 | 7.3 | 7.3 | 94.9 | 13.0 |

[a] MES1 cells were used for transfection with pCVpf or pCVpr using the GeneJuice reagent. More than 1000 living cells (attached) were counted on micrographs to determine fluorescing and nonfluorescing cells. [b] Percentages of fluorescing cells were derived by comparing GFP- or RFP-positive cells to the total number of cells. [c] Enrichment factor was derived by comparing the frequency of fluorescing cells in the presence of puromycin at 1 $\mu$g/mL for indicated periods (drug) to that in the absence of puromycin (no drug).

group, rendering a product that cannot accept peptidyl moieties. We tested the puromycin selectability of the fusion genes. To this end, medaka stem cell lines MES1 and SG3 were transfected with pCVpf or pCVpr and subjected to puromycin selection from 18 hpt onward. In MES1, gene transfection and *pf* expression had little adverse effect on cell growth (Figure 2A). Massive cell death took place after 24 h of growth in the presence of puromycin at 1 $\mu$g/mL (Figure 2B). After 48 h of puromycin selection, non-transgenic cells were all dead (Figure 2C,D). Puromycin led to enrichment for GFP-positive cells by 8- and 16-fold after 24 and 48 h of drug selection (Table 1). Similar results were obtained also with pCVpr (Table 1). In SG3, when pCVpr transfectants were drug selected for 2 days and then expanded in the absence of drug for 2 weeks, the majority of cells were RFP positive, with the minority being RFP negative (Figure 2E,F), consistent with transient expression in certain cells. Therefore, the fusion proteins PF and PR retain the PAC activity for rapid drug selection.

The fusion genes *pf* and *pr* were further examined in stable gene transfer. MES1 cells were transfected with pCVpf or pCVpr and subcultured at 3 days post-transfection (dpt) into 10 cm dishes. After 15−21 days of growth in the presence of puromycin at 1 $\mu$g/mL, 48 and 44 cell colonies were formed that showed varying levels of the GFP or RFP signal. Representative colonies were expanded to stable transgenic populations. The efficiency of pCVpf and pCVpr for stable gene transfer is comparable to that obtained by G418 and hygromycin selection after transfection with pSTneo and pHygEGFP.[18] The advantage for pCVpf and pCVpr consists in the rapid speed of puromycin selection for transgenic cells within 2 days rather than 2 weeks of selection by G418 and hygromycin.

We wanted to determine whether *pf* expression and puromycin selection would affect the cell property, an essential issue for use of puromycin selection and our vectors in highly demanding systems such as stem cells. MES1 is well-suited to address this issue. Its key property is the pluripotency, and the conditions for analyzing its pluripotency have been well established by chimera formation.[13,22] Its pluripotency remains after transient gene transfer[13] and stable gene transfer via long-term hygromycin selection.[18] We took the advantage of chimera formation to analyze the retention of pluripotency.[11] To this end, pCVpf- and pCVpr-transgenic MES1 cells were cotransplanted into recipient blastulae, and chimera formation and the donor cell distribution during embryogenesis were monitored by fluorescent microscopy. These stable transgenic


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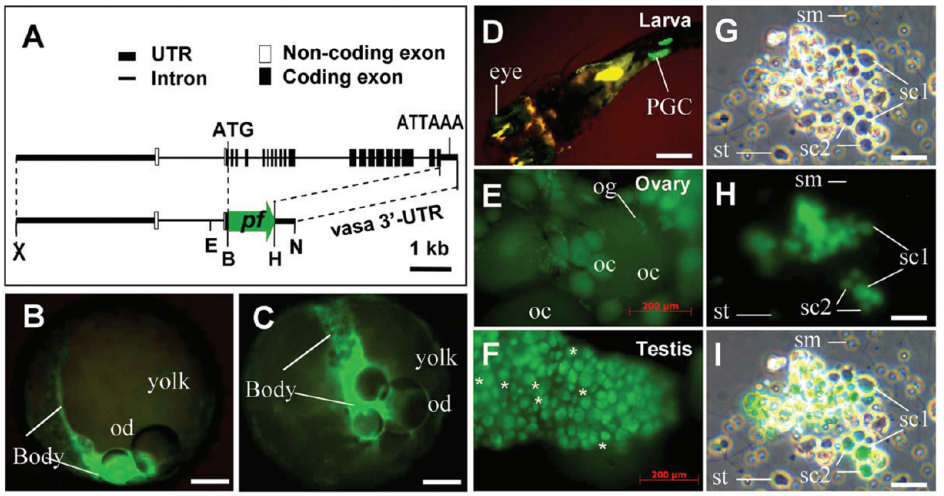


**Figure 3.** Transgenic production. (A) Expression vector pVASpf, which contains the fusion gene *pf* under the control of *vasa* promoter and the 3′-untranslated region (3′-UTR). Shown are restriction sites: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; N, *Not*I; X, *Xho*I. (B) 2-day-old medaka embryo microinjected with pVASpf, showing wide GFP expression in the embryo body. The location of oil droplets (od) delineates the vegetal pole. (C) 2-day-old embryo from a stable VASpf-transgenic medaka, showing similarly wide GFP expression in the embryo body. (D) Larva of stable transgenic medaka, showing transgene expression in primordial germ cells (PGCs). (E) Adult ovary of stable transgenic medaka, showing transgenic expression in oogonia (og) and oocytes (oc). (F) Adult testis of stable transgenic medaka, showing transgenic expression in cysts of male germ cells (asterisks). (G–I) Dissociated testicular cells from a stable VASpf-transgenic male medaka, showing transgene expression during stages of spermatogenesis. sc1, primary spermatocyte; sc2, secondary spermatocyte; st, spermatid; sm, sperm. Scale bars, 200 $\mu$m in (B–F) and 20 $\mu$m in (G–I).

cells gave rise to 100% of chimera formation and contributed widely to many embryonic structures (Figure S1, Supporting Information). Therefore, *pf* or *pr* expression and puromycin selection have no detectable adverse effect on the ES cell pluripotency in vitro and in vivo.

**Gene Transfer and PAC Assay in Vivo.** We used the *pf* fusion gene to label a particular cell lineage and to quantify reporter expression by PAC assay. We chose the medaka *vasa* regulatory sequence to drive the *pf* expression. Vasa is an RNA-binding protein that is highly conserved in sequence and germ cell-specific expression across animal phyla. The *vasa* regulatory sequence has been shown to be able to drive GFP expression exclusively in germ cells of transgenic rainbow trout,[23] medaka,[24] and zebrafish.[25] In medaka, a 5-kb region of the *vasa* gene in combination with the *vasa* 3′ untranslated region (3′-UTR) is sufficient to drive GFP expression in germ cells.[24] We used this 5 kb region and the 3′-UTR to construct pVASpf (Figure 3A). Of 1290 embryos microinjected with linearized pVASpf at the 1 cell stage, 364 (28%) displayed wide GFP expression at 2 dpf (Figure 3B) and developed into 154 adults. Progeny testing revealed four founders (1 male and 3 female) capable of ≥10% germline transmission into four independent transgenic lines upon three generations of mating. The four lines exhibited a similar GFP expression pattern. The GFP signal was widely distributed at day 2 of development (Figure 3C) and became restricted to germ cells of larvae (Figure 3D) and adult gonads of both sexes (Figure 3E,F). When testicular cells from adult transgenic fish were dissociated and seeded in culture, GFP expression was easily seen in spermatocytes but absent in spermatids and sperm (Figure 3G–I). Hence, visible GFP expression in transgenic animals appears to be specific to germ cells.

We performed PAC assay in adult organs from male and female medaka adults homozygous for the pVASpf transgene. A total of 16 organs were homogenized and subjected to PAC assay. We found that maximal activity was in the testis, coincident with germ cell-specific expression by fluorescent microscopy. Interestingly, PAC activity was also observed in several somatic organs including the brain and fin (Figure 4A), suggesting a low level of activity of the *vasa* promoter in certain somatic cells. Similar observations were obtained also in the transgenic female (Figure S2, Supporting Information). A closer examination of transgenic expression in the autofluorescence-free strain revealed strong GFP signal in germ cells and weak signal in the eye and others of larvae (Figure 4B,C). More importantly, when the larval eye was cryo-sectioned and subjected to fluorescence in situ hybridization (FISH), the *vasa* RNA was detected indeed in the retinal pigment epithelium, photoreceptor cells, and ganglion cells (Figure 4D,E). Taken together, PAC assay in combination with fluorescent microscopy and FISH demonstrates the *vasa* promoter activity also in certain somatic cells.

Our observation that several somatic organs of transgenic medaka containing the *pf* under the control of medaka *vasa* promoter exhibit a detectable PAC is unusual. As one of the best studied germ genes, *vasa* has been reported to show germ cell-specific expression in diverse organisms including zebrafish,[26] medaka,[27] trout,[28] and gibel carp.[29] Similarly, the ∼5 kb *vasa* promoters from these organisms can drive transgene expression in germ cells.[23−25] Evidence that vasa expression may occur outside germ cells came from the observations in medaka, where the *vasa* transcript is detectable in many cells of developing embryos during and after gastrulation,[14,20] and *vasa* knockdown affects early embryonic development.[14] In this study, we have chosen the eye to examine *vasa* expression. With the PAC assay, the *vasa* promoter activity is 10 in the eye compared to 2500 in the testis. This low level of *vasa* expression prevents detection by classical chromogenic in situ hybridization[14,20] but allows for detection by the GFP signal and sensitive FISH we used in this study. In agreement with our finding is the recent observation that, in invertebrates, *vasa* expression has recently been found in somatic stem cells of developing sea urchin embryos[30] and of adult


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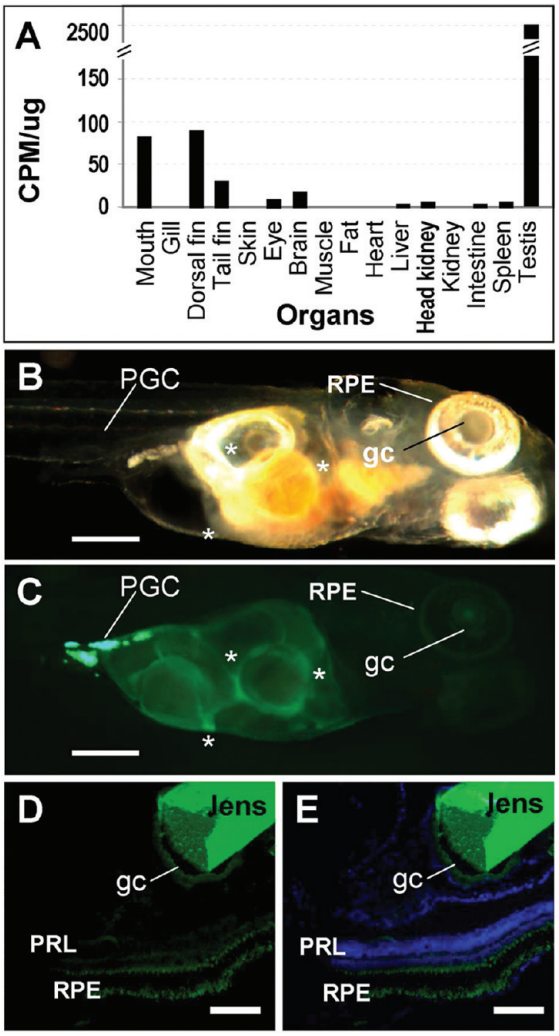


**Figure 4.** Analyses of *vasa* promoter activity and RNA expression in vivo. (A) PAC assay in vivo in male adult medaka. Tissues and organs were dissected from a pVASpf transgenic male medaka and an equal amount of their cellular extract was used for PAC assay. (B and C) GFP expression (green) driven by the medaka *vasa* promoter in transgenic larva, showing strong expression in primordial germ cells (PGC) and weak expression in ganglion cells (gc) and retinal pigment epithelium (RPE) of the eye, as well as other compartments (asterisks). (D−E) Eye section after FISH using an antisense *vasa* riboprobe, showing *vasa* RNA expression (green) in ganglion cells, photoreceptor layer (PRL), and RPE. (E) Merge between DAPI (blue) and *vasa* (green) optics. The signal in the lens is due to autofluorescence. Scale bars, 100 $\mu$m.

ctenophore.[31] In addition, ectopic expression of germ genes drives malignant brain tumor growth in *Drosophila*.[32] Our results illustrate the usefulness and sensitivity of GFP observation and PAC assay upon *pf* and *pr* fusion gene expression in vivo. Taken together, our vector system features the advantageous combination of rapid drug selection, cell labeling, and reporter assay in vitro and in vivo.

## CONCLUSIONS

In summary, we have developed a vector system on the basis of *pf* or *pr*, a fusion between *pac* and *gfp* or *rfp*. Compared to vector systems in current use, our vector system can simultaneously allows for rapid drug selection, cell labeling, and reporter assay in vitro and in vivo. The advantage of this system for rapid drug selection and cell labeling in vitro is illustrated by enrichment for transgenic ES cells that retain the pluripotency. The advantage of this system for cell labeling and reporter assay in vivo is exemplified by the microscopic observation of *vasa* promoter-driven GFP expression in germ cells and by quantitative enzymatic PAC assay of transgene expression in various adult organs, leading to the notion that medaka germ gene *vasa* is expressed also in certain somatic cells.

## ASSOCIATED CONTENT

**Supporting Information.** Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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## REFERENCES

(1) Boshart, M.; Kluppel, M.; Schmidt, A.; Schutz, G.; Luckow, B. *Gene* **1992**, *110*, 129–130.
(2) Altschmied, J.; Duschl, J. *Biotechniques* **1997**, *23*, 436–438.
(3) Karreman, C. *Gene* **1998**, *218*, 57–61.
(4) Vara, J. A.; Portela, A.; Ortin, J.; Jimenez, A. *Nucleic Acids Res.* **1986**, *14*, 4617–4624.
(5) Friedrich, G.; Soriano, P. *Genes Dev.* **1991**, *5*, 1513–1523.
(6) Schwartz, F.; Maeda, N.; Smithies, O.; Hickey, R.; Edelmann, W.; Skoultchi, A.; Kucherlapati, R. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 10416–10420.
(7) Lybarger, L.; Dempsey, D.; Franek, K. J.; Chervenak, R. *Cytometry* **1996**, *25*, 211–220.
(8) Loimas, S.; Wahlfors, J.; Janne, J. *Biotechniques* **1998**, *24*, 614–618.
(9) Abbate, J.; Lacayo, J. C.; Prichard, M.; Pari, G.; McVoy, M. A. *Biotechniques* **2001**, *31*, 336–340.
(10) Oh, S. C.; Nam, S. Y.; Kwon, H. C.; Kim, C. M.; Seo, J. S.; Seong, R. H.; Jang, Y. J.; Chung, Y. H.; Chung, H. Y. *Mol. Cells* **2001**, *11*, 192–197.
(11) Hong, N.; Li, M.; Zeng, Z.; Yi, M.; Deng, J.; Gui, J.; Winkler, C.; Schartl, M.; Hong, Y. *Cell. Mol. Life Sci.* **2010**, *67*, 1189–1202.
(12) Hong, Y.; Winkler, C.; Schartl, M. *Mech. Dev.* **1996**, *60*, 33–44.
(13) Hong, Y.; Winkler, C.; Schartl, M. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 3679–3684.
(14) Li, M.; Hong, N.; Xu, H.; Yi, M.; Li, C.; Gui, J.; Hong, Y. *Mech. Dev.* **2009**, *126*, 366–381.
(15) Yi, M.; Hong, N.; Hong, Y. *Science* **2009**, *326*, 430–433.
(16) Thermes, V.; Grabher, C.; Ristoratore, F.; Bourrat, F.; Choulika, A.; Wittbrodt, J.; Joly, J. S. *Mech. Dev.* **2002**, *118*, 91–98.

(17) Hong, Y.; Liu, T.; Zhao, H.; Xu, H.; Wang, W.; Liu, R.; Chen, T.; Deng, J.; Gui, J. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 8011–8016.
(18) Hong, Y.; Chen, S.; Gui, J.; Schartl, M. *Transgenic Res.* **2004**, *13*, 41–50.
(19) Winkler, C.; Vielkind, J. R.; Schartl, M. *Mol. Gen. Genet.* **1991**, *226*, 129–140.
(20) Xu, H.; Li, Z.; Li, M.; Wang, L.; Hong, Y. *PLoS One* **2009**, *4*, e6097.
(21) de la Luna, S.; Ortín, J. *Methods Enzymol.* **1992**, *16*, 376–385.
(22) Hong, Y.; Winkler, C.; Schartl, M. *Dev. Genes Evol.* **1998**, *208*, 595–602.
(23) Yoshizaki, G.; Takeuchi, Y.; Sakatani, S.; Takeuchi, T. *Int. J. Dev. Biol.* **2000**, *44*, 323–326.
(24) Tanaka, M.; Kinoshita, M.; Kobayashi, D.; Nagahama, Y. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 2544–2549.
(25) Fan, L.; Moon, J.; Wong, T. T.; Crodian, J.; Collodi, P. *Stem Cells Dev.* **2008**, *17*, 585–597.
(26) Yoon, C.; Kawakami, K.; Hopkins, N. *Development (Cambridge, England)* **1997**, *124*, 3157–3165.
(27) Shinomiya, A.; Tanaka, M.; Kobayashi, T.; Nagahama, Y.; Hamaguchi, S. *Dev., Growth Differ.* **2000**, *42*, 317–326.
(28) Yoshizaki, G.; Sakatani, S.; Tominaga, H.; Takeuchi, T. *Mol. Reprod. Dev.* **2000**, *55*, 364–371.
(29) Xu, H.; Gui, J.; Hong, Y. *Dev. Dyn.* **2005**, *233*, 872–882.
(30) Yajima, M.; Wessel, G. M. *Development (Cambridge, England)* **2011**, *138*, 2217–2222.
(31) Alie, A.; Leclere, L.; Jager, M.; Dayraud, C.; Chang, P.; Le Guyader, H.; Queinnec, E.; Manuel, M. *Dev. Biol.* **2011**, *350*, 183–197.
(32) Janic, A.; Mendizabal, L.; Llamazares, S.; Rossell, D.; Gonzalez, C. *Science* **2011**, *330*, 1824–1827.