Benchmarks

A lens-specific co-injection marker for medaka transgenesis

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Recent advances in generating transgenic fish have improved the efficiency of germline transmission and enabled the generation of large numbers of transgenic animals. A suitable co-injection marker may help facilitate the preselection of transgenic embryos. For this purpose, a lens-specific marker appears to be a suitable candidate since the lens is a well-defined tissue that is easily accessible for examination of reporter gene expression. We constructed reporter vectors including the mouse gamma-F crystallin (mγF-Cry) promoter, which drives high levels of lens-specific heterologous expression of the reporter gene and thereby enables easy sorting of transgenic fish.

Recently, several novel approaches based on I-*Sce*I–mediated and transposon-mediated transgenesis have been reported for generating transgenic medaka (1–3). Since the co-injection of a fluorescent marker substantially facilitates the preselection of transgenic animals (4), we searched for a suitable driver of reporter genes to develop a robust co-injection marker. We focused on crystallins, which are known to be expressed at high levels in the lens (5), and constructed and tested mγF-Cry–based fluorescent reporters for application in the transgenesis procedure.

For injections, Cab and Heino strains (6) of medaka were used with no observed differences in mγF-Cry reporter expression. Reporter vector p817-Rx3-EGFP (7) and empty vector p817 were provided by Jochen Wittbrodt (European Molecular Biology Laboratory, Heidelberg, Germany). Bovine rhodopsin promoter (-225 ± 73) (8) was PCR-amplified using *Pfu* DNA polymerase (Stratagene, La Holla, CA, USA) from bovine genomic DNA using primers 91-PV (forward): 5′-GCGGTACCAG-GCCTCTGCTCTTTCCCAG-3′ and 93-PV (reverse): 5′-CTAGCTAGCCG-GCGGCGCGAACCCGGGGAT-3′, and blunt-cloned into p817 (*Bam*HI, blunted). The p817-mCherry plasmid was constructed from p817 by replacing a region encoding enchanced GFP (EGFP) with mCherry (9) sequences. To generate mγF-Cry-EGFP and mγF-Cry-mCherry reporter constructs, mγF-Cry promoter (-226 ± 45 bp) (10) was inserted into p817 or p817-mCherry, respectively. These co-injection reporter plasmids are available from AddGene (Deposit no. 23154 for mCherry and 23156 for EGFP; Cambridge, MA, USA). I-*Sce*I–mediated transgenesis was performed as described (4). If not otherwise stated, the concentration of all plasmids in the injection mix was 20 ng/μL. Developing embryos were screened for reporter expression under the fluorescent binocular microscopes Leica MZ16FA/DFC480 (Leica, Wetzlar, Germany) and Olympus SZX7/SZX9 (Olympus, Hamburg, Germany).

A 271-bp mouse mγF-Cry promoter drives the expression of the reporter specifically in the lens (Figure 1, A–D). The onset of the fluorescent signal from both EGFP and mCherry was observed from stage 22–23 (11), beginning as a small fluorescent patch in the middle of the developing lens (Figure 1E). Later on, the signal was observed throughout the whole lens and the intensity of the fluorescent signal became extremely high. The signal persisted in hatched fish and was still observable in adults (Figure 1F). The strength of the signal was demonstrated by the fact that mCherry fluorescence could be observed and documented (Figure 1, G and H) with a GFP filter set (460–490 nm excitation, 510 nm–IR emission) that is not optimal for this fluorescent protein, with preferable excitation and emission maxima at 587 and 610 nm, respectively.

Encouraged by the specificity and signal strength, we next explored the possibility of lowering the concentration of mγF-Cry reporters, which would bring several advantages. First, lower DNA concentrations reduce post-injection mortality. Higher ratios between the transgene of interest and co-injection marker also increase the

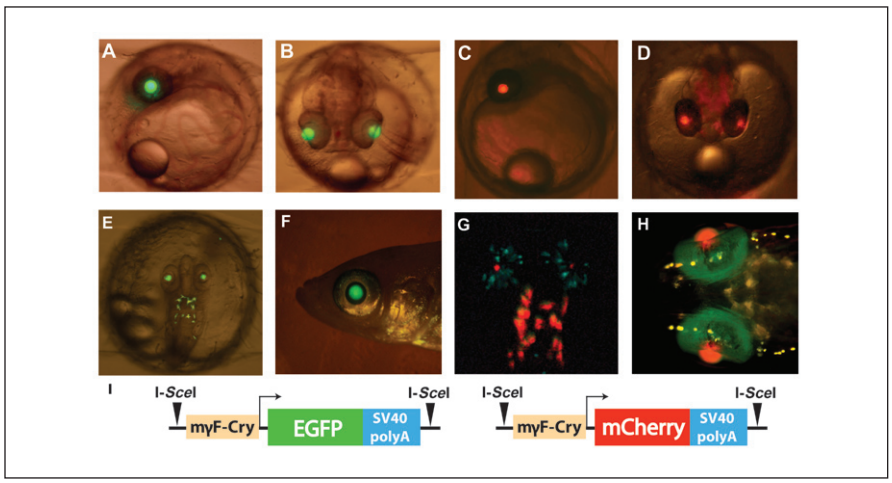

Figure 1. Lens-specific expression of mγF-Cry reporters in medaka. (A,B) Expression of EGFP (stage 33) and (C,D) mCherry (stage 30). (E) The onset of EGFP reporter expression at stage 23; (F) the expression of EGFP driven by mγF-Cry in a 15-month adult animal. (G) The onset of mCherry reporter expression correlated with Rx3-EGFP reporter in developing retina (7). (H) Expression of mγF-Cry-mCherry reporter co-injected with bovine rhodopsin promoter–EGFP construct 4 days after hatching, ventral view; (I) Schematic diagram of the mγF-Cry reporter constructs. All fish shown are Heino strain, except for panels C, D, and F showing the expression of mγF-Cry reporters in Cab strain. The green signal in Figure 1E, orange in Figure 1G, and yellow in Figure 1H outside the lenses is caused by non-specific autofluorescence of guanophores visualized by different sets of emmision filters used for capturing the images.

Table 1. The efficiency of transgenesis in F_0 with decreasing concentrations of mγF-Cry-EGFP

mγF-Cry-EGFP (ng/μL)	Eggs injected	Eggs survived	EGFP positive	% EGFP positive
20	148	100	88	88.0
5	148	110	74	67.3
3	91	54	23	42.6
1	52	52	9	17.3

proportion of fish bearing the transgene of interest in all marker-positive animals. At lower concentrations of mγF-Cry reporter (5 and 3 ng/μL), the percentage of fluorescent fish decreased (Table 1) without affecting the robustness of the signal. Decreasing the concentration of mγF-Cry reporter to 1 ng/μL was not suitable for further application, since it led to a lower intensity of fluorescent signal, a low frequency of transgenic animals, and a higher occurrence of expression in only a single eye. The concentration of the marker plasmid (5 and 3 ng/μL) is 4–10× lower than the standard concentration used for medaka injection (20–30 ng/μL) in the I-*Sce*I approach. To test the feasibility of using mγF-Cry–driven reporters as co-injection markers at low concentration, we performed co-injection experiments with Rx3:EGFP reporter constructs. In co-injection experiments with 5 ng/μL mγF-Cry-mCherry and 20 ng/μL Rx3-EGFP (1:4 ratio, n = 262), 95% of mCherry-positive animals were also positive for EGFP in the F_0 generation. The shh-GFP marker reported in a previous study does not produce observable signals at lower concentrations (Thomas Czerny, personal communication), and its co-injection at a 1:1 ratio with the transgene of interest resulted in just 50% of marker-positive F_0 animals to also be positive for the transgene of interest (4).

Together, the mγF-Cry–based reporters generated in this study represent useful co-injection markers for medaka transgenesis that save time and effort.

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Competing interests

The authors declare no competing interests.

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