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Transient and permanent gene transfer into the brain of the teleost fish medaka (*Oryzias latipes*) using human adenovirus and the Cre-loxP system

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ABSTRACT

In this study, we demonstrated that human type-5 adenovirus infected the brain of the teleost fish, medaka (*Oryzias latipes*), in vivo. Injection of adenoviral vector into the mesencephalic ventricle of medaka larvae induced the expression of reporter genes in some parts of the telencephalon, the periventricular area of the mesencephalon and diencephalon, and the cerebellum. Additionally, the Cre-loxP system works in medaka brains using transgenic medaka carrying a vector containing DsRed2, flanked by loxP sites under control of the β-actin promoter and downstream promoterless enhanced green fluorescent protein (EGFP). We demonstrated that the presence of green fluorescence depended on injection of adenoviral vector expressing the Cre gene and confirmed that EGFP mRNA was transcribed in the virus-injected larvae.
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1. Introduction

The adenoviral (Ad) vector is an attractive method of effectively delivering foreign genes to cells in vivo [1–4] and in vitro [5], which allows for direct genetic modulation of cells [4,5] or indirect genetic modulation mediated by the Cre-loxP system [1]. In the field of neuroscience, Ad vectors are used for various purposes, such as labeling a subset of neurons [1,2], tracing a neural cell lineage [1,3], and modulating neuronal function [4]. Some species of teleost fish (zebrafish and medaka) are used as model animals for molecular genetics in various fields such as developmental biology [6] and neuroscience [7,8]. Therefore, the use of viral vectors in teleost fish will contribute to further genetic analysis of neural function using small teleost fish. To date, however, there have been no studies demonstrating that human Ad can infect neural cells in teleosts in vivo. In the present study, we evaluated the ability of infecting medaka brain with Ad vectors and whether the Cre-loxP system could be applied in vivo.

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2. Materials and methods

2.1. Preparation of medaka larvae

Adult medaka (d-rR strain) were reared in fresh water at a temperature of 25 °C on a 14:10 h light–dark photoperiod. Fertilized eggs were collected in the morning and incubated in modified Yamamoto's Ringer's solution (0.75% NaCl, 0.02% KCl, 0.02% $CaCl_2$, 0.02% $NaHCO_4$) at 25 °C until injection of the virus. For all experiments, we used 14-days post-fertilization (14 dpf) larvae. Adult transgenic medaka was reared under the same conditions as the d-rR strain. Fertilized eggs were prepared by crossbreeding heterozygotic transgenic medaka, and heterozygotic or homozygotic transgenic larvae were selected by body color.

2.2. Generation of transgenic medaka

The pβ-actin-loxP-DsRed2-loxP-enhanced green fluorescent protein (EGFP) vector (pbact-RG, Genbank Accession number AB557594) was prepared by modifying pDsRed2-1 (Clontech, Palo Alto, CA, USA) (Supplementary Fig. 1). The transgenic medaka strain, d-rR-Tg (beta-actin-loxP-DsRed2-loxP-GFP), was generated

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by introducing pbact-RG with the previously described microinjection method [9].

2.3. Preparation of adenoviral vector

The Ad vectors, AdexCAG-NL-LacZ [1–3] and AdexCAG-NL-Cre [1], express nuclear-targeted β-galactosidase (NL-LacZ) and nuclear-targeted Cre recombinase (NL-Cre), respectively, under the control of the CAG promoter [10]. AdexCAG-mCherry expresses red fluorescent protein (mCherry) under the control of the CAG promoter. AdexCAG-mCherry was constructed as described in Supplementary Fig. 1. Ad vectors were purified and concentrated by double cesium step gradient centrifugation [1]. A high-titer viral stock (1×10^{11} plaque-forming units [pfu]/mL in 10% [v/v] glycerol/PBS) was stored at −80 °C. The viral stock titers were determined by plaque assay with HEK293 cells.

2.4. Injection of the adenoviral vector

Medaka larvae were immobilized by mounting in 1% agarose LGT (Nacalai Tesque, Kyoto, Japan). The larvae were then randomly divided into three groups, which were used for injection of Ad vectors (Ad-vector-injected group), 10% glycerol in PBS (vehicle-injected group), or nothing (intact group). Glass capillaries (Harvard Apparatus, Cambridge, MA, USA) were inserted into the mesencephalic ventricle using a micromanipulator (Narishige Scientific Instruments Laboratories, Tokyo, Japan). Approximately 20–30 nl vehicle or Ad vector was injected into each larval head. After injection, medaka larvae were released from the agarose and reared in modified Yamamoto's Ringer's solution at 25 °C for 1 week.

2.5. β-Gal histochemistry

One week after injection of the Ad vector, medaka larvae were fixed with 0.2% glutaraldehyde and 2% formaldehyde for 30 min at 4 °C. The fixed larvae were then rinsed with PBS and stained with 5 mM $K_4[Fe(CN)_6]$, 5 mM $K_3[Fe(CN)_6]$, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% Nonidet P-40, and 1% X-gal in PBS overnight at 37 °C. After several rinses with PBS, the larvae were fixed and embedded in paraffin by the method reported previously [11]. Paraffin-embedded larvae were sectioned in the cortical plane at a 5 μm thickness and mounted on MAS-coated glass slides. Coronal sections were deparaffinized, rehydrated in CLEAR PLUS and degraded ethanol, and counterstained in 0.5% cresyl violet.

2.6. Microscopic observation

All larvae were observed after embedding in 0.2% agarose LGT. For observation of the whole head, we used a fluorescence stereomicroscope MZ16F (Leica) and camera system DFC300FX (Leica) to obtain images. To obtain optical sections, we used a confocal laser scanning microscope, LSM 710 (Carl Zeiss, Oberkochen, Germany). Images were merged using Photoshop (Adobe, San Jose, CA) and ZEN (Carl Zeiss).

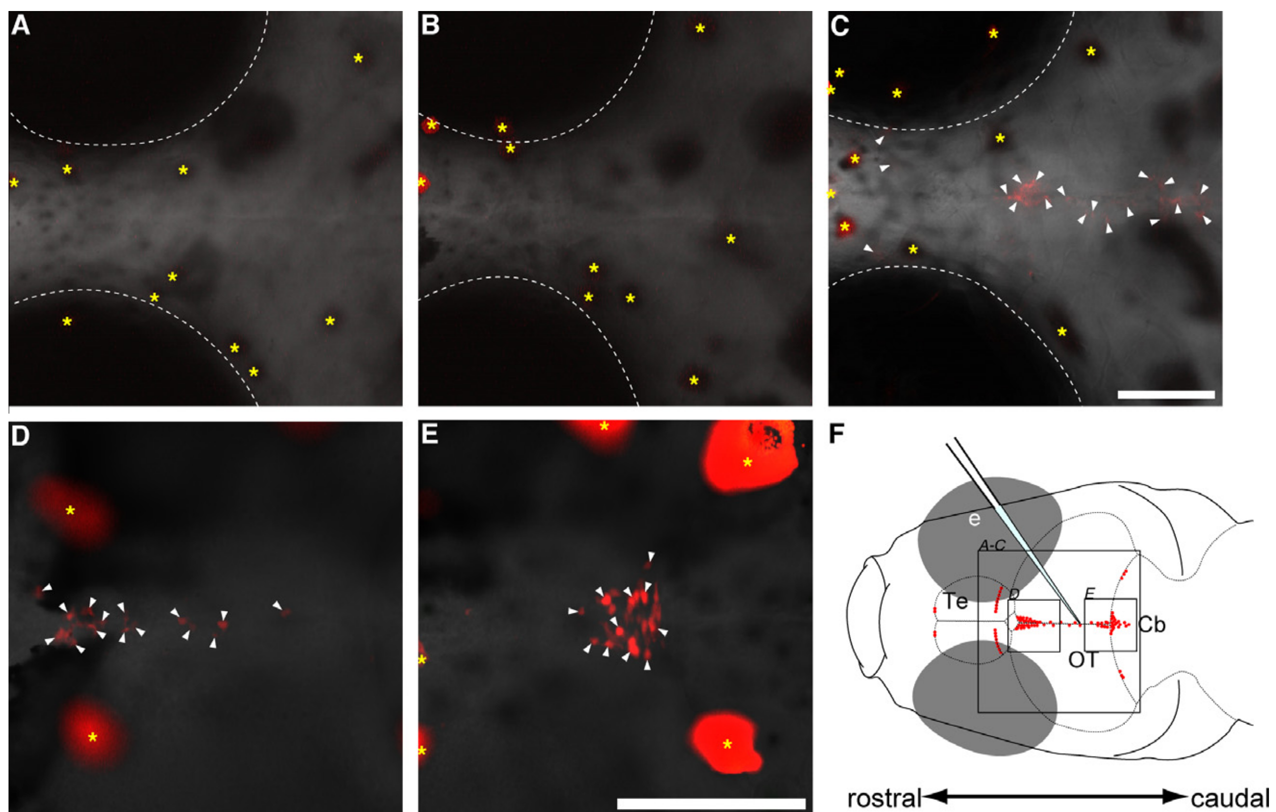

Fig. 1. Detection of red fluorescence after AdexCAG-mCherry injection in optical sections. (A–C) Optical sections showed red fluorescence at the periventricular area of the mesencephalon, the boundary area between the optic tectum and cerebellum, and the cerebellum after the Ad vector injection (C). Vehicle-injected and intact larvae showed only intrinsic fluorescence (asterisks in A and B). (D and E) Magnified images in a limited area (displayed in F) of the larval head. Several cells in the boundary area between the telencephalon and optic tectum; the periventricular area of the mesencephalon (D), and the boundary areas between the optic tectum and cerebellum (E) showed red fluorescence. (F) A schematic illustration of the medaka larval head. Fluorescence signals detected in only Ad-vector-injected larvae are indicated by red filled circles. Injection site was shown by capillary. E, eye; Te, telencephalon; OT, optic tectum; Cb, cerebellum. Scale bar, 100 μm. Asterisks show intrinsic fluorescence.

3. Results

3.1. Injection of Ad vector into the heads of medaka larvae induced expression of a reporter gene in vivo

To examine the feasibility of infecting the medaka brain with Ad vectors in vivo, we injected AdexCAG-mCherry into the mesencephalic ventricle in 14-dpf medaka larval brains (Fig. 1F). We prepared vehicle-injected larvae and intact larvae simultaneously as negative controls. When the solution was injected, we confirmed that the injected solution spread to the telencephalon, cerebellum, and spinal cord through the ventricles by co-injecting blue dextran (data not shown). One week after the injection, in the Ad-vector-injected larvae, red fluorescent signals were detected at the rostral end of the telencephalon; the sulcus between medial areas 2 and 3 of the dorsal telencephalon [12]; the periventricular area of the mesencephalon, especially the boundaries between the telencephalon and optic tectum; and between the optic tectum and cerebellum (Fig. 1C–E and Supplementary Fig. 2). Fluorescent signals in these regions were first detected 3 days after the Ad injection (Supplementary Fig. 2G and H). The majority (19/25; 76%) of larvae in the Ad-vector-injected group had red fluorescence in all or parts of those regions, whereas none of the larvae in the vehicle-injected or intact group (0/21 and 0/26) had red fluorescent signals in these regions (Fig. 1A and B, Supplementary Fig. 2). The size of the area in which fluorescent signals were detected in each region was different among the Ad-vector-injected larvae. Although Ad-vector-injected larvae also showed fluorescent signals in the chromatophores, we concluded that these signals were due to intrinsic fluorescence, because similar fluorescence was observed

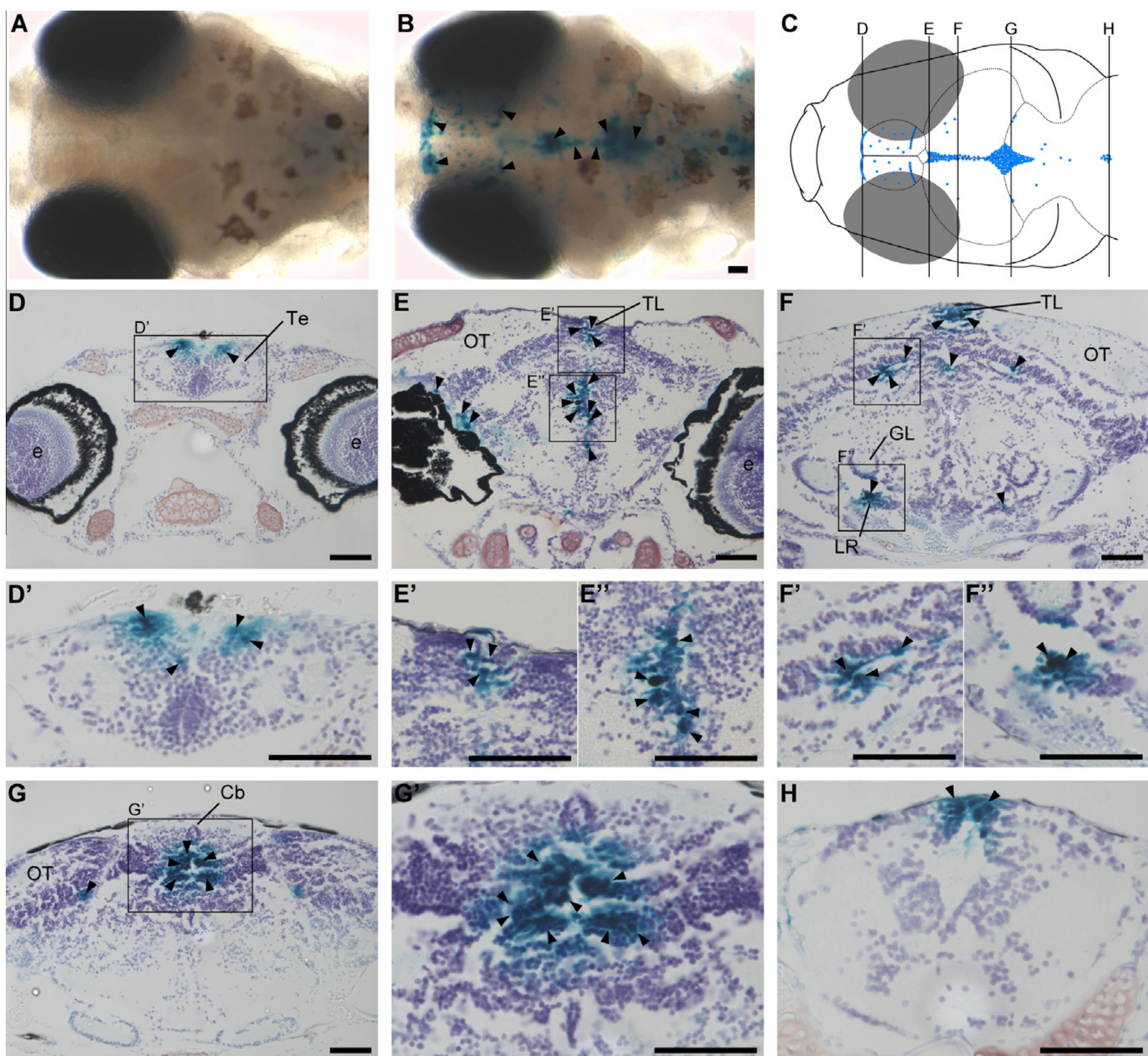

Fig. 2. The distribution of Ad-vector-infected cells after AdexCAG-NL-LacZ injection. (A and B) X-gal stained cells were detected in Ad-vector-injected larvae (B, arrowheads), but not in vehicle-injected larvae (A). (C) The position of each section in the lower panels (D–H) is shown schematically. (D–H) Coronal paraffin sections after Nissl staining using cresyl violet and magnified images of each section (D′, E′, E″, F′, F″, and G′). Surface of the telencephalon (D and D′), torus longitudinalis (E, F, and E′) and periventricular area of dorsal thalamus (E and E″), parts of medial periventricular area of the optic tectum and tegmentum (F and F′) and around lateral recessus (F and F″), granular cells of the cerebellum (G and G′), and dorsal horn of spinal cord (H) were stained. E, eye; Te, telencephalon; OT, optic tectum; TL, torus longitudinalis; GL, glomerular nucleus; LR, lateral recess; Cb, cerebellum. Scale bar, 50 μm.

in vehicle-injected and intact larvae (Supplementary Fig. 2). We also confirmed mCherry gene expression using reverse transcription (RT)-PCR (Supplementary Fig. 3A). Thus, we concluded that the medaka brain can be infected with Ad and that expression of a foreign gene delivered by Ad vector can be driven in vivo. In addition, we confirmed infection of these brain regions with AdexCAG-NL-LacZ (Fig. 2A and B).

3.2. Surface of the telencephalon, periventricular areas in the mesencephalon and diencephalon, and granular cell layer of the cerebellum were infected with Ad

To investigate the distribution of infected cells with Ad vectors in detail, the larvae infected with AdexCAG-NL-LacZ were embedded in paraffin and sectioned for Nissl staining using cresyl violet (Fig. 2C–H). Blue signals revealing X-gal stained cells overlapped with some Nissl-stained cells on the dorsal surface of the telencephalon (Fig. 2D and D′), the torus longitudinalis (Fig. 2E, F, and E′) and periventricular area of the dorsal thalamus (Figs. 2E and 3E″), part of the periventricular area of the optic tectum and tegmentum (Fig. 2F and F′), the surrounding area of the lateral recess (Fig. 2F and F″), the granular cells of the cerebellum (Fig. 2G and G′), and the dorsal horn of the spinal cord (Fig. 2H) indicating that some periventricular and superficial neural tissues were infected with Ad.

3.3. Site-specific DNA recombination by injection of AdexCAG-NL-Cre

Next we examined whether Ad vector infection will function with the Cre-loxP recombination system. To use the Cre-loxP system, we generated transgenic medaka (β-actin-loxP-DsRed2-loxP-GFP line) carrying an endogenous β-actin promoter that drives *DsRed2* expression, which is flanked by loxP sites, upstream of promotorless enhanced green fluorescence protein (EGFP). The transient Cre expression in the transgenic medaka will induce loxP-site-specific recombination, resulting in *EGFP* expression instead of DsRed2. We injected AdexCAG-NL-Cre carrying the nuclear-targeted Cre gene under control of the CAG promoter into the larvae in the same manner as injection of AdexCAG-mCherry. One week after the injection, we observed green fluorescence in the Ad-vector-injected larvae (Fig. 3C and F), but we could detect only intrinsic fluorescence signals derived from the chromatophores in the intact and vehicle-injected larvae (Fig. 3A, B, D and E). Green fluorescence was detected at the rostral end of the telencephalon, in the sulcus in the dorsal telencephalon, the midline of the optic tectum, and the boundary area between the optic tectum and cerebellum (Fig. 3C and F), consistent with brain regions in which the fluorescent signals (Fig. 1) and X-gal stained cells (Fig. 2) were detected after AdexCAG-mCherry and AdexCAG-NL-LacZ infection, respectively. Confocal microscopy of the torus longitudinalis (Fig. 3G) revealed that some cells had green fluores-

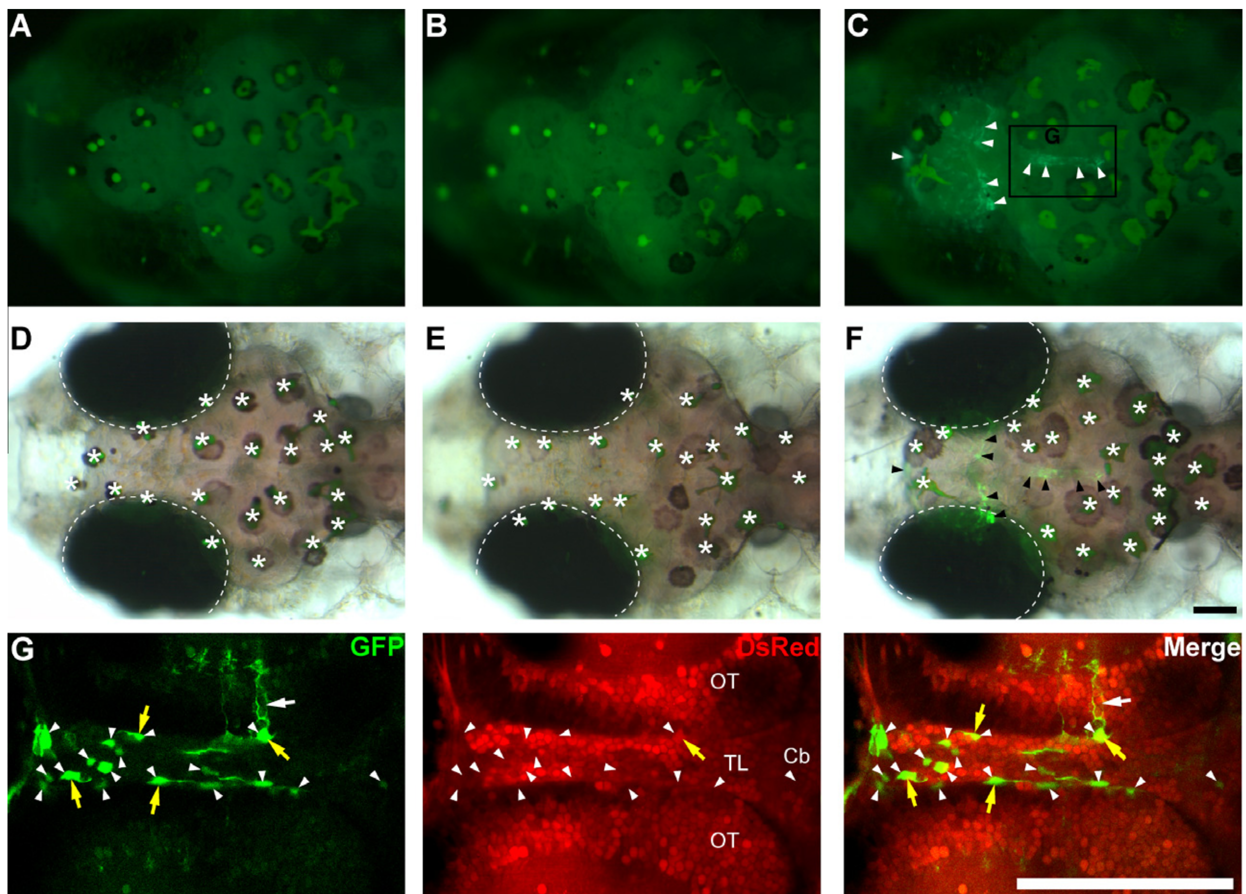

Fig. 3. Induction of loxP site-specific recombination in transgenic medaka by injection of the AdexCAG-NL-Cre. (A–F) Intrinsic fluorescence (asterisk) was detected whether Ad vector was injected (C and F) or not (A, B, D, and E). Green fluorescence at the rostral end and the sulcus of the telencephalon, midline of the optic tectum, and boundary area between the optic tectum and cerebellum were observed in larval heads depending on the injection of the Ad vector (arrowheads in C and F). Broken lines show the boundary of the eyes. (G) Optical sections of torus longitudinalis. Green fluorescent signals were shown by arrowheads. Some cells had green fluorescence instead of red fluorescence. The other cells with green fluorescence showed also red fluorescence, which may be due to insufficient degradation of DsRed. A few cells with green fluorescence (yellow and white arrows) exhibited a typical neuron-like morphology with neuritis. Scale bar, 100 μm.

cence instead of red fluorescence suggesting that loxP-site-specific recombination results in the expression of EGFP instead of DsRed. We also confirmed *EGFP* expression using RT-PCR (Supplementary Fig. 3B). Taken together, these results indicated that infection of the medaka larvae with AdexCAG-NL-Cre induced loxP-site-specific recombination and permanent expression of EGFP driven by the β-actin promoter.

4. Discussion

Our results demonstrate that the medaka brain can be infected with human Ad vectors and induced to express foreign genes. Injection of AdexCAG-mCherry and AdexCAG-NL-LacZ into the mesencephalic ventricle in the larval head demonstrated infection with Ad vectors in several cells at the rostral end of the telencephalon, the boundary area between the telencephalon and the optic tectum, the midline of the tectum, and the boundary area between the tectum and cerebellum. This is the first demonstration of in vivo infection of small teleost fish with human Ad vectors. Confocal microscopy of the torus longitudinalis revealed that some infected cells had a neuron-like morphology (Fig. 3G white and yellow arrows). Some of these cells projected toward the optic tectum (Fig. 3G white arrow). In the carp torus longitudinalis, some neurons were reported to have efferent projection to the optic tectum [13], suggesting that the infected cells included some neurons, but we could not confirm the cell type of all infected cells. In the present study, strong signals were not detected in all the Ad-vector-injected larvae (19/25), which may be due to a difference in the extent of the diffusion of the Ad vector solution.

Selective infection with Ad vectors in those brain regions raises following possibilities. One possibility is that the Ad vector solution might not have diffused throughout the entire brain. The Ad vector could not penetrate into distant cells from the ventricles and might tend to accumulate at the brain edge, sulcus, ventricle, and boundaries between brain structures, resulting in a higher probability of Ad infection in those brain regions. The other possibility is that infection efficiency might be different among cell types. The coxsackievirus and Ad receptor (CAR) is involved in Ad infection [14]. In zebrafish, the gene homologous to human CAR (zCAR) interacts with human adenovirus type 5 [15]. The medaka gene homologous to CAR (medaka CAR) may also be involved in Ad infection in the larval brain and differential expression of CAR among cell types might result in a difference in infection efficiency.

Next, Cre-loxP recombination in vivo in medaka brain mediated by Ad infection was demonstrated for the first time. We confirmed that some cells had green fluorescence instead of red fluorescent (Fig. 3G). Detection of strong red fluorescence in a few cells with green fluorescence might be due to insufficient degradation of DsRed. As Cre-loxP systems have been used extensively in mammals [1], the ability to use the Cre-loxP system mediated by Ad vectors in the central nervous system of small teleost fish will promote analyses of molecular and neural functions in specific neurons.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.06.047.

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