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BRIEF COMMUNICATION

Establishment and characterization of CAG/EGFP transgenic rabbit line

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Abstract Cell marking is a very important procedure for identifying donor cells after cell and/or organ transplantation in vivo. Transgenic animals expressing marker proteins such as enhanced green fluorescent protein (EGFP) in their tissues are a powerful tool for research in fields of tissue engineering and regenerative medicine. The purpose of this study was to establish transgenic rabbit lines that ubiquitously express EGFP under the control of the cytomegalovirus immediate early enhancer/beta-actin promoter (CAG) to provide a fluorescent transgenic animal as a bioresource. We microinjected the EGFP expression vector into 945 rabbit eggs and 4 independent transgenic candidate pups were obtained. Two of them died before sexual maturation and one was infertile. One transgenic male candidate founder rabbit was obtained and could be bred by artificial insemination. The rabbit transmitted the transgene in a Mendelian manner. Using fluorescence in situ hybridization analysis, we detected the transgene at 7q11 on chromosome 7 as a large centromeric region in two F1 offspring (one female and one male). Eventually, one transgenic line was established. Ubiquitous EGFP florescence was confirmed in all examined organs. There were no gender-related differences in fluorescence. The established CAG/EGFP transgenic rabbit will be an important bioresource and a useful tool for various studies in tissue engineering and regenerative medicine.

Keywords CAG · EGFP · Transgenic rabbit · Bioresource

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Introduction

During the last two decades, transgenic animal technology has gained importance in biomedical science. In particular, marking cells by introducing biomarker genes such as the green fluorescent protein (GFP) gene is a popular technique in many fields of research. Transgenic animals expressing the GFP gene have been reported for mouse (Okabe et al. 1997; Chiocchetti et al. 1997; Zhuo et al. 1997), rat (Hakamata et al. 2001; Inoue et al. 2005), and pig (Kurome et al. 2006). These animals are very useful tools for identifying donor cell migration after cell/tissue/organ transplantation without requiring cell staining (Inoue at al. 2004; Tahara et al. 2005). Because GFP expression in tissues or cells in the recipient animals is easily detected by fluorescent microscopy and flow cytometry, these animals are very valuable for studies in various fields, such as cell therapy, tissue engineering, and regenerative medicine.

The intermediate body size of rabbits between laboratory rodents and domestic animals is favorable for surgical and transplantation studies, for example, lung (Yoshida et al. 2005), heart (Furukawa et al. 2005), bone (Li and Li 2005; Judas et al. 2005; Ohya et al. 2005), and hepatocytes (Attaran et al. 2004). Enhanced GFP (EGFP) transgenic rabbits were described previously (Wang et al. 2001; Chesne et al. 2002; Chrenek et al. 2005). Recently, Al-Gubory and Houdebine reported that the fibered confocal fluorescence microscopy with optical mini-probe could detected fluorescent signals and visualize detailed tissue architecture and cell morphology of living EGFP transgenic rabbit, and GFP is a powerful imaging marker (Al-Gubory and Houdebine 2006). However there are no published data of ubiquitous GFP-expressing transgenic rabbit organs with detailed profiling of the fluorescence intensity.

In this study, we developed a biofluorescent transgenic rabbit that ubiquitously expresses the EGFP gene in the body by introducing the CAG expression vector of EGFP, composed of a cytomegalovirus enhancer, chicken beta-actin promoter, beta-globin intron, and a beta-globin polyA sequence fragment (CAG/EGFP). We examined the expression profiles of the fluorescence intensity in various organs of a CAG/EGFP transgenic rabbit. This transgenic rabbit is expected to be advantageous for studies in tissue engineering and regenerative medicine in which the transplanted cells/tissues/organs are identified with a biofluorescence marker, and will be a useful bioresource.

Materials and methods

Animals and preparation of the pronuclear ova

Experiments were performed in accordance with the guidelines at the experimental facilities of Kitayama Labes Co. Ltd (Nagano, Japan), for recombinant DNA experiments and for the care and use of laboratory animals. Seven-week-old out-bred Japanese White (Kbs:JW) rabbits (specific-pathogen-free/virus, antibody-free, 3.0–4.0 kg) were housed in an environmentally controlled room with a 12-h dark:12-h light cycle, at a temperature of 20 ± 3°C, and humidity of 60 ± 15%. They were fed pelleted rabbit chow (Oriental Yeast Co., Shizuoka, Japan) and filtered water ad libitum. Multiple ovulation treatments were performed as described previously (Hashimoto et al. 2004). In brief, super-ovulation of female rabbits was induced by a single subcutaneous injection of 3-AU refined porcine follicle stimulating hormone (FSH; Antrin R, Denka Pharmaceuticals Inc., Kanagawa, Japan) dissolved in 10% polyvinylpyrrolidone (168-03115, K-90, Wako Chemical Industries, Osaka, Japan). The females were then intravenously injected with 75 or 150 IU human chorionic gonadotropin (hCG; Teikoku Zouki, Tokyo, Japan) 72 h after the injection of FSH and were mated with male Kbs:JW rabbits. The oviduct ampullae were flushed with TCM 199 supplemented with 10% fetal bovine serum (FBS, Equitech Bio, Kerrville, TX, USA) at 16–18 h after hCG injection and recovered eggs were maintained in TCM 199 supplemented with 10% FBS at 38.5°C in 5% CO_2 in air.

Production of transgenic rabbits

CAG/EGFP expression vector was constructed as follows; a 3.0-kb *Eco*RI coding fragment of the EGFP coding region prepared from the pEGFP vector (#6077-1, Clontech Laboratories Inc., Palo Alto, CA, USA) was introduced into the *Eco*RI site of the pCXN2 expression vector (Niwa et al. 1991) with an *Eco*RI linker. The DNA of the CAG/EGFP expression vector was dissolved in 10 mM Tris–HCl (pH 7.6) containing 0.1 mM EDTA at a concentration of 5 ng/μL. The DNA solution was injected into the male pronucleus in the egg. The microinjected eggs were transferred to the oviduct ampullae of pseudopregnant female recipients (3.4–4.6 kg) that had received 75 IU hCG on the preceding day.

Screening of transgenic rabbits

Rabbits harboring the CAG/EGFP gene were screened by genotyping with Southern hybridization methods. Genomic DNA was prepared from the tail of a weanling or the tissue of dead pups. The rabbit genomic DNA was digested with *Eco*RI, and an *Nco*I–*Not*I fragment of CAG/EGFP expression vector labeled with ^{32}P was used as the hybridization probe.

Expression profiling of the transgenic rabbit line

EGFP expression profiling was performed with F1 male and female progeny. Various organs were examined using a CCD camera under a 488-nm excitation light (fluorescent microscope, MZFL III, Leica).

Breeding of transgenic rabbits

Breeding of transgenic male founder rabbits was performed according to the artificial insemination protocol (Kaneda et al. 1993). In brief, ejaculated sperm was collected in a water-warmed artificial vagina (handmade; 45–50°C). The collected sperm was diluted ten times with phosphate buffered saline, and 0.5 mL of sperm suspension was injected into the vaginal vestibule with a 30-cm glass pipette (handmade; bent 4 cm at the tip).

Fluorescence in situ hybridization analysis of transgene

Determination of the chromosomal location of the CAG/EGFP transgene was performed using fluorescence in situ hybridization (FISH) analysis (Watanabe et al. 1996). Chromosome number was identified as previously reported (Hayes et al. 2002).

Results and discussion

The widespread use of transgenic experimental animals in life science research has led to an increase in the importance of establishing transgenic animals as bioresources. Animals with biomarker genes such as EGFP are very useful for identifying donor cell migration after cell/tissue/organ transplantation and we attempted to establish a transgenic rabbit that ubiquitously expresses biofluorescence using pronuclear microinjection of the CAG/EGFP expression vector mini-gene. Of the 945 microinjected eggs, 867 were transferred to 34 recipient females. Twenty-one females become pregnant and 81 pups were born, of which 20 survived after weaning. Out of the 81 pups, 7 were transgenic but 3 were stillborn. Of the four live born transgenic pups, two were light yellow–green colored and strongly EGFP-fluorescent upon exposure to excitation light, but died before sexual maturation. The third had a faint yellow–green color, but was sterile. Only one transgenic founder rabbit (male), which was also faint yellow–green colored, transmitted the transgene to its progeny. Thus, only one of seven candidate transgenic founders could be bred to a transgenic line. The integration efficiency (0.74%; 7/945) was consistent with our previous report (Hirabayashi et al. 2001), but six of seven candidate founders were stillborn, died prior to reaching sexual maturity, or were infertile. We successfully established a transgenic rabbit line that ubiquitously expressed an EGFP biomarker gene. The overall

production efficiency of the transgenic line of rabbits (0.1%; 1/945) was lower than that reported for mouse (2.1%; 4/192) and rat (3.2%; 3/95) using the same DNA preparation (data not shown). As rabbits produced by the manipulated embryos have a high risk of mortality (Fan et al. 1999; Fan and Watanabe 2003), we carefully raised the pups, including providing a foster mother to nurse early weaning. It is noteworthy that approximately 75% (61/81) of the pups died before sexual maturation. Nevertheless, we successfully established a transgenic rabbit line that ubiquitously expressed EGFP. Forty-two pups were obtained from the fertile male founder rabbit (#GFP-1) by artificial insemination. Eight F1 generation pups were florescent upon exposure to excitation light and had stable transgene copy number as determined by Southern blot hybridization analysis. The number of pups with the transgene was lower than expected based on Mendelian genetics. We had often experienced these results on the F1 generation from founder mouse and rat. In the F2 generation, however, 39 pups were obtained from F1 transgenic rabbit (#GFP-1-4) and 19 of them were transgenic. Thus, the transgene was transmitted according to Mendelian manner. The florescent profile of F1 progeny was examined. Green florescence was detected in all organs examined, but the strength and weakness of the fluorescence differed among organs (Fig. 1). The expression profile of the rabbit line was comparable to those of mouse (Okabe et al. 1997) and rat (Hakamata et al. 2001). There were no gender differences in the EGFP expression profiles in all examined organs (Fig. 1).

High expression of the EGFP gene is desirable for cell marking, but it is undesirable for EGFP toxicity against the transgene host. The established transgenic line resulted from a compromise between those contradictions. As reported previously (Okabe et al. 1997; Hakamata et al. 2001), the EGFP expression profile in various organs is very valuable for cell therapy, tissue engineering, and regenerative medicine. Although an EGFP transgenic rabbit was reported previously (Wang et al. 2001; Chesne et al. 2002; Chrenek et al. 2005), the EGFP expression profile was not described. Further, the chromosome location of the transgene was examined by using FISH analysis. The CAG/EGFP transgene was detected as a single site of chromosome 7 on the long arm region consisting of the 1.1 domain (Fig. 2).

In the present study, we reported basic data about the profile of EGFP expression in a transgenic rabbit, and evaluated the established

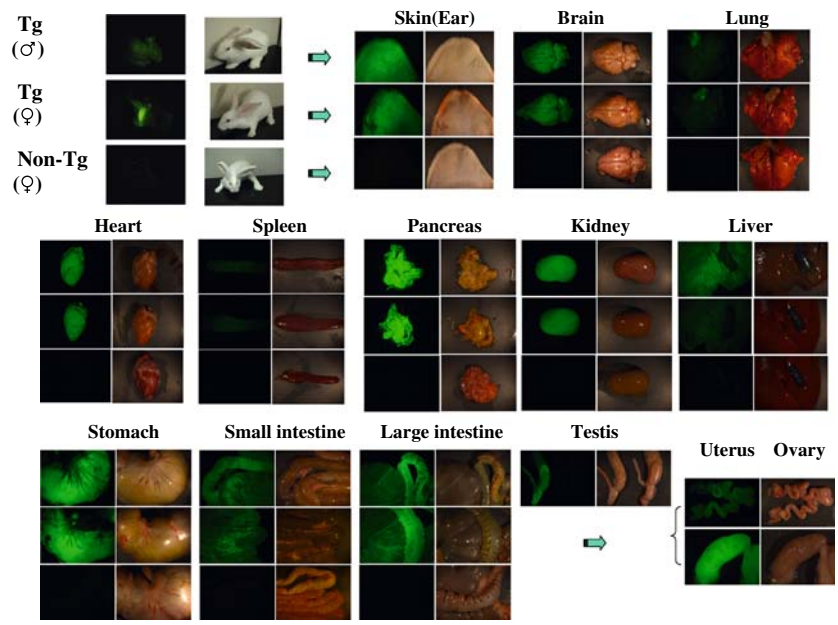

Fig. 1 Profile of EGFP expression in various tissues of transgenic male and female F1 rabbits. All photos were taken under the same intensity of 489 nm excitation light (*left side*) and visible light (*right side*)

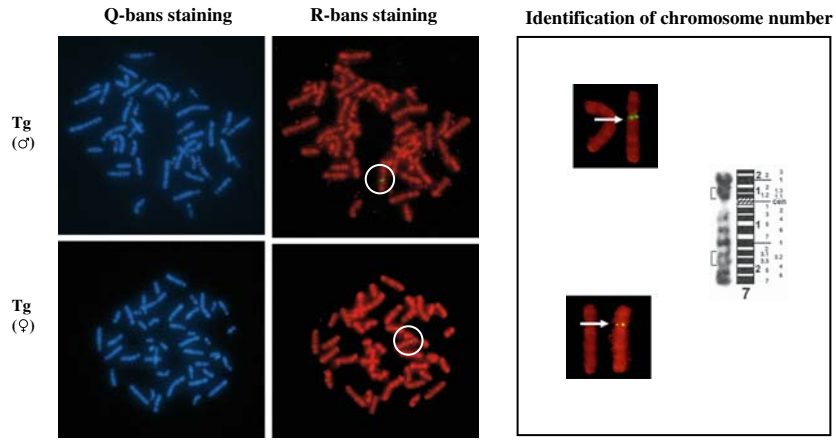

Fig. 2 FISH analysis of the transgene. The entire CAG/EGFP DNA fragment was labeled with FITC. The hybridization signal (indicated by *arrow*) was detected at 7q11 on chromosome 7 as a large centromeric chromosome. The signal existed hemi-zygous on its chromosome

transgenic rabbit for its use as a bioresource. The established CAG/EGFP transgenic rabbit will be a useful tool for various studies (Ajiki et al. 2005; Sato et al. 2005; Inamatsu et al. 2006) such as tissue engineering, regenerative medicine, and developmental and differentiational biology.

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