A rapid and non-invasive selection of transgenic embryos before implantation using green fluorescent protein (GFP)

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Abstract Non-invasive selection of transgenic mice was performed at the stage of preimplantation embryos. The morulae collected from wild female mated with hemizygous transgenic male expressing *Aequorea victoria* green fluorescent protein (GFP) under chicken β-actin promoter could be classified as green or non-green under a fluorescent microscope. All the green embryos were shown to carry the transgene by PCR analysis. Taking advantage of the detection of GFP expression can be done non-invasively, the selected embryos were demonstrated to be able to developed to term with 100% of accuracy of the selection.

Key words: Green fluorescent protein; Transgenic mouse; Preimplantation embryo; β-Actin promoter; PCR; Marker

1. Introduction

The discernment of transgenic pups from non-transgenic siblings is an important step in producing homozygous transgenic mice and establishing transgenic lines. The methods mainly used in detection of transgenes are PCR analysis or Southern blotting following extraction of DNA from the tail of 3–4-week-old pups [1,2]. However, both techniques require skill and consume time if applied to a large number of transgenic animals.

A quick separation of transgenic mice after birth has been reported to be possible by co-injecting a marker transgene [3–5]. For example, the mice lacking tyrosinase become albino and the injection of tyrosinase minigene into albino eggs will render the coat color of the transgenic mice agouti or black [3]. In our previous paper, we reported that a separation of transgenic pups expressing the green fluorescent protein was possible non-invasively by observing their green color under 390 nm excitation light on the day of parturition [6]. One of the advantages of this method was its application to mice of any coat color.

A procedure simplifying detection of transgenic pups would be useful in establishing transgenic mouse lines. Moreover, for production of large transgenic animals such as bovine or porcine, detection of the transgene at the preimplantation stage would be desirable considering the long gestation period and limited number of offspring.

Blastocyst biopsy and subsequent PCR analysis enable us to separate X- and Y-chromosome-bearing embryos [7–9]. The same technique might prove effective in detection of transgene-bearing embryos [10]. However, non-invasive detection methods would be desirable and the development of such technique would be awaited.

The reporter genes so far used for this purpose have some detrimental effects on the embryos. Recently, use of a secreting type of luciferase was reported as an effective non-invasive way to separate the transgenic eggs [11] but was not extend to the birth of the separated embryos.

Green fluorescent protein (GFP) found in *Aequorea victoria* absorbs blue light and emits green fluorescence without exogenous substrates or cofactors. Therefore the detection of the GFP requires neither the extraction, substrate loading, steps as the commonly used CAT, β-galactosidase, and firefly luciferase markers do.

In the present paper, we demonstrated that GFP could be expressed in preimplantation embryos and that it worked well as a reporter gene by showing selective birth of transgenic mice following a color separation of preimplantation embryos.

2. Materials and methods

2.1. Construction of vectors

The plasmid containing the GFP coding sequence (pGFP10.1) was donated by Drs. Prasher and Chalfie [12]. We introduced the Kozak sequence by PCR as described in our previous paper [6]. The PCR product including GFP structural gene was digested with *Eco*RI and inserted into the pCAGGS expression vector [13] to make pCX-GFP. The *Sal*I/*Bam*HI DNA fragment of pCX-GFP was separated from the vector by electrophoresis and recovered from agarose gel.

2.2. Production of transgenic mice

Fertilized eggs were collected from the oviducts of B6C3F1 females mated with B6C3F1 males. The DNA fragments purified with QIAEX (Qiagen, CA) were injected into male pronuclei. The injected eggs were transferred to female mice of ICR strain made pseudopregnant by mating with vasectomized males. This resulted in 3 transgenic lines (#2, #10 and #14), as previously reported [6]. Hemizygous transgenic F1 mice were identified by observing the GFP expression in their tails.

2.3. Preparation of embryos

Six-week-old wild females were superovulated by injecting 5 IU of PMS and hCG (Teikoku Zouki, Japan), respectively, at 48-h intervals. The 2-cell stage embryos were collected from oviducts of pregnant females (1.5 day pc) mated with hemizygous transgenic males or B6C3F1 males. Recovered embryos were placed into 30 μl of microdrops of kSOM medium [14] under paraffin oil and cultured for 2 days.

2.4. Observation and selection of embryos

After various incubation intervals, embryos were placed in FHM microdrops prepared on 5 × 5 cm coverglass covered with paraffin oil. A Nikon Diaphot inverted fluorescent microscope with filters DM430 (EX380-425 and BA510) was used for GFP monitoring. Green and non-green morulae derived from wild females mated with hemizygous transgenic males were separated under a fluorescent microscope. During the short period of excitation (< 10 s), the locations of green eggs were memorized. The separation was performed under dissecting

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microscope using a finely drawn pasteur pipette. The separated embryos were washed with kSOM and cultured further in kSOM overnight to form blastocysts [14]. They were then subjected to PCR analysis or to uterine transfers to 2.5-day pseudopregnant mice.

2.5. PCR amplification of embryo samples

One blastocyst was transferred into an Eppendorf PCR tube with 10 μl of dH_2O, overlaid with 30 μl of light mineral oil (Nacalai Tesque, Japan). The samples were heated for 10 min at 97°C. After cooling down, 1 μl of Proteinase K stock solution (10 mg/ml) was added to each tube, then treated for 1 h at 56°C. Proteinase inactivation was done for 10 min at 97°C. Then 3.5 μl of dH_2O, 2 μl of 10 × PCR buffer (100 mM Tris-HCl pH 9.0, 500 mM KCl, 1% Triton X-100), 1.5 μl of $MgCl_2$ stock solution (25 mM), 1 μl of dNTP mixture (each 5 mM of dATP, dCTP, dGTP and dTTP) and 0.5 μl of each primer (50 μM) were added to individual tubes. The primers used for the detection of the GFP gene were: *5′* primer TGG AGA GGG TGA AGG TGA TGC; and *3′* primer TGT GTG GAC AGG TAA TGG TTG. Finally, 5 μl of Polymerase solution (2.5 units of Taq Polymerase in 1 × PCR buffer) (Toyobo, Japan) was added at 94°C. The PCR conditions were as follows: 40 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s and extension at 72°C for 40 s. After the completion of 40 cycles, 5 μl of each sample was analyzed immediately by 1% agarose gel electrophoresis.

3. Results

3.1. Production of GFP transgenic mice

The construct of the vector and the production of GFP transgenic mice was described in our previous paper [6]. In brief, as shown in Fig. 1, the GFP cDNA introduced the Kozak sequence was ligated to chicken β-actin promoter and hCMV enhancer (pCX-GFP). Three lines of transgenic mice (#2, #10, #14) were obtained by injecting the *SalI/Bam*HI DNA fragment of pCX-GFP into 166 fertilized eggs. Lines #2 and #14 carried about 5 copies and #10 carried about 50 copies of the transgene. In the present experiment, #2 and #10 lines were used.

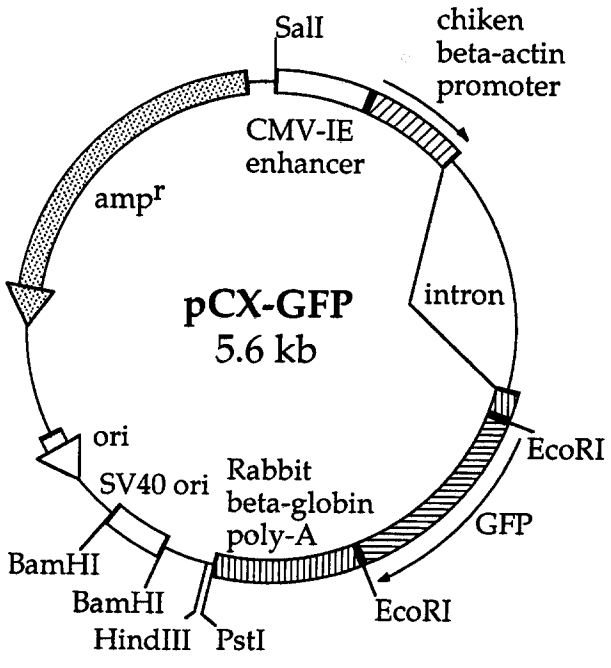

Fig. 1. Construction of pCX-GFP. We introduced the Kozak sequence into the cDNA of GFP and inserted it into the pCAGGS expression vector to make pCX-GFP. The pCAGGS expression vector possesses chicken β-actin promoter and cytomegalovirus enhancer (CMV-IE enhancer).

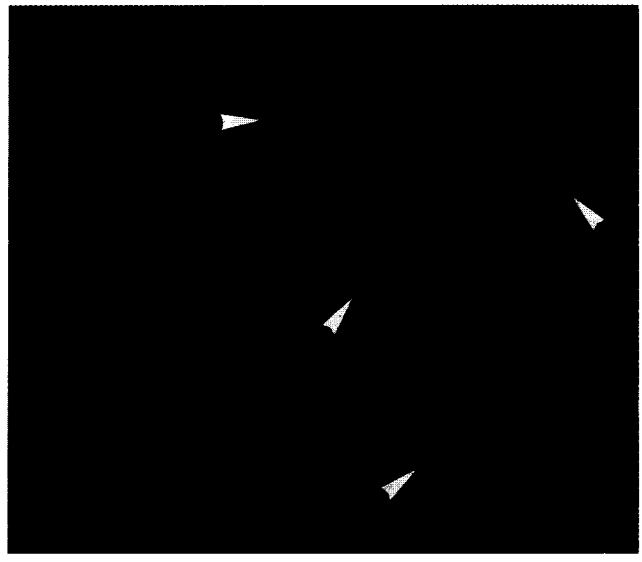

Fig. 2. GFP expression in pre-implantation embryos. The morulae collected from B6C3F1 females mated with hemizygous transgenic males. Arrowheads indicate green embryos.

3.2. GFP expression in preimplantataion embryo

Green fluorescence was observed in half of the eggs obtained from wild female mated with hemizygous transgenic male. The paternally transmitted GFP expression became detectable at the 4-cell stage and the fluorescence reached its peak at the morula stage. The green eggs were considered to possess the transgene (Fig. 2).

3.3. Selection of transgenic embryos before implantation

Morulae obtained from wild female mated with hemizygous transgenic male (line #2 and #10) were separated into 'green' or 'non-green' groups under a fluorescent microscope. All the embryos survived the selection procedure and formed blastocysts within the following 18 h. The individual embryos were subjected to PCR analysis to check the transgene. A total of 49 embryos was separated and subjected to PCR analysis. Positive bands of GFP gene at 512 bp position were observed in all the PCR products from 26 green morulae. There were no positive signals in the remaining 23 non-green samples (Fig. 3).

3.4. Transfer of separated embryos

In the next experiment, separated embryos (line #10) were transferred into the uterus of pseudopregnant females (day 2.5). As shown in Table 1, a total of 154 morulae was separated into two groups depending on the green fluorescence. Eighty-two of

Table 1
Exclusive production of GFP-transgenic mice by selecting preimplantation embryos

Embryos recovered	Embryos transferred*	Pregnant/ recipients	Transgenic/pups
154	82 (green)	5/6	32/32
154	72 (non-green)	4/6	0/20

*Embryos were separated under a fluorescent microscope. Shedding the excitation light on the embryos was within a few seconds.

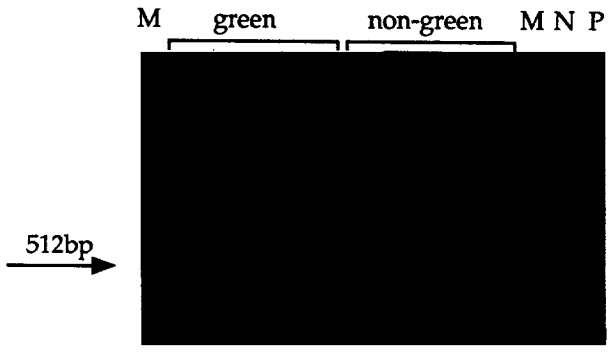

Fig. 3. PCR analysis of separated embryos. M = ϕX174 *Hae*III digest, N = negative control, P = positive control. The 512 bp fragments showed that all the selected green-embryos inherited GFP transgene.

the green, and 72 of the non-green, embryos were transferred; 32 (39%) and 20 (28%) of them developed to term. The tails from pups were cut at 3–4 weeks of age and extracted DNA was subjected to PCR analysis. All the mice born from green eggs proved to be carrying the GFP transgene while the non-green eggs did not carry the transgene at all.

4. Discussion

Recently, embryo sexing by PCR analysis from a part of the embryo has been performed in a variety of species such as human [9,15], bovine [8] and mouse [7]. This method is also effective in identifying transgenic animals [10]. However, biopsy procedures include embryo holding, partial dissection of zona pellucida, removal of a single blastomere from the embryo and transfer of the blastomere into PCR tube. Moreover, the PCR procedures include DNA extraction, amplification of the objective gene, and electrophoresis. Since much skill and time are required, it is difficult to handle many embryos at a time [16].

To avoid such complicated procedures, a simple phenotypical separation technique can be used in separating transgenic embryos or cells. Reddy et al. introduced the LacZ gene into embryonic stem cells and separated the transgenic cells by fluorescence-activated cell sorting [17]. Matsumoto et al. used a firefly luciferase and detected enzyme activity at the 2-cell stage of mouse embryo without killing cells [18]. However, these methods need the step of loading the substrate inside the cells and the toxicity of the substrates is well known. Recently, as an improved method, non-invasive detection of the marker protein was demonstrated by Thompson et al. [11]. According to their report, transgenically expressed *vargula* luciferase was secreted from living embryos and aliquots of culture medium were subjected to photon counting using a high sensitive chilled type charge-coupled device (CCD) camera. As indicated in their paper, the transgenic embryos may be discernible by the phenotype. However, to ascertain the accuracy of the separation, the separated embryos had to be re-examined genotypically. Furthermore, to prove that the non-invasive separation was not detrimental to the further development of the embryo, we had to demonstrate that it is possible to obtain pups from the separated embryos.

In the present study, we used the GFP as a reporter and tried to distinguish the transgenic embryos from non-transgenic ones. One characteristic of the GFP is that no substrates or co-factors are needed for the emission of green fluorescence [12]. This eliminates the possibility of any toxic effects from the substrates, a potential problem with luciferase or β-galactosidase. Moreover, the observations can be done while the cells are alive and intact. As shown in section 3, the GFP worked well to separate the embryos in 2 out of 3 transgenic lines with fluorescent microscope in a non-invasive manner. The real usage of the separation of the transgenic embryo would emerge in in vitro fertilization using one transgenic male for many non-transgenic females, since numerous gametes could be obtained from male but not from female. Therefore, a more successful detection method of paternally inherited transgene is required.

However, the eggs are known to be sensitive to UV light [19]. If the eggs were observed under fluorescent microscope for longer than a few minutes, many eggs failed to form blastocysts. However, as reported earlier [20], a brief irradiation was not toxic to the eggs. Under our conditions, it was also shown that the short exposure of eggs to excitation light did not affect their viability. All the eggs selected at morula stage developed to blastocysts within the following 18 h. And the eggs transferred to the uterus of pseudopregnant foster mothers developed to term effectively.

Recently, Youvan et al. and Tsien et al. have reported a mutated type of GFPs in which the excitation wave length was shifted to a longer side (around 490 nm) [21–23]. Combining these mutated GFP with the high sensitive CCD camera as Thompson did [11], we should be able to make the separation procedure even safer for the embryos.

No detrimental effects of GFP per se in cells have been found. The transmission rate of GFP from hemizygous male or female was about 50% following the Menderian rule [6], even though the fertilized eggs expressed GFP as shown in this paper. The mice from all three GFP transgenic lines we obtained expressed GFP strongly in muscle, pancreas and kidney. However, we could not find any sign of abnormality in all of these organs examined [6].

In the present paper, we demonstrated the separation of GFP transgenic embryos and subsequent transfer generating transgenic pups. GFP would be useful as a marker of gene expression, particularly in embryogenesis, since the observation of live embryo is a direct monitoring of gene expression.

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