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BASIC RESEARCH

Rescue of the albino phenotype by introducing a functional tyrosinase minigene into Kunming albino mice

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Abstract

AIM: To use the tyrosinase minigene as a visual marker to perform microinjection training and improve the techniques related with transgene to greatly elevate the efficiency of gene transfer.

METHODS: A mouse tyrosinase minigene, i.e., TyBS, in which the 2.25-kb authentic genomic 5′ non-coding flanking sequence of mouse tyrosinase was fused to a mouse tyrosinase cDNA, was introduced into the fertilized eggs of outbred Kunming albino mice.

RESULTS: Of the 11 animals that developed from the injected eggs, two mice (P1 and #8) exhibited pigmented hair (P1) and eyes (P1 and #8), as confirmed by PCR analysis for the tyrosinase minigene integrated into the genome. When founder P1 was bred to Kunming male mouse, six progeny out of 11 offspring inherited the transgene and the pigmented-eye phenotype.

CONCLUSION: Taken together, these results suggest that this minigene encodes the active tyrosinase protein and that its 5′ flanking region contains the sequences regulating the expression of mouse tyrosinase gene as expected. We have rescued the albino phenotype by introduction and expression of a functional tyrosinase minigene in the Kunming albino mouse and the transgene can be passed to subsequent generation. These findings also indicate that TyBS can be a useful visual marker gene in the co-transgenic experiments.

Key words: Kunming mouse; Albino; Tyrosinase minigene; Transgenic mice; Melanization; Phenotypic rescue

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INTRODUCTION

Visible pigmentation in the mammals results from the synthesis and distribution of melanin in skin, hair bulbs and eyes[1-3]. Tyrosinase is the first and rate-limiting enzyme in the pathway for melanin production in melanocytes of the skin and eyes[1-3]. Mutation of the tyrosinase gene is a common cause of a similar phenotype in all vertebrates, known as albinism, due to a lack of melanin pigment[1,3]. In mouse, the albino phenotype is characterized by a total absence of pigmentation due to a mutation in the tyrosinase gene; several point mutations within the tyrosinase gene have been found, which can inactivate its function to result in oculocutaneous albinism (OCA)[1,3]. In mouse, the classical albino (*c*) mutation corresponds to a single-point mutation in the first exon of the tyrosinase gene, which brings about an amino acid mutation Cys103Ser, leading to the accumulation of a non-functional protein[4,5]. When mice are homozygous (*c*/*c*) for mutations that inactivate the tyrosinase gene, mice are albino regardless of the genotype at the other loci[1,3]. The entire common albino inbred strains of laboratory mice, such as FVB/N, BALB/c, *etc*, belonging to OCA, have the same point mutation in the tyrosinase gene, indicating that these strains are derived from a common ancestor[5]. The albino phenotype has been successfully corrected through the tyrosinase transgene, which can express the active tyrosinase in transgenic mice[5-18], rabbits[19], fish[20-22] and other vertebrates

expressing tyrosinase functional transgenes[23].

The Human and Model Organism Genome Projects have revealed the sequence information of many genes. A significant challenge for scientists over the next few decades is to annotate the human and model organism genomes with functional information. Genetically engineered mice will play a vital role in the study of the functional genome.

The production of transgenic mice, involving an intensive sequence of procedures in genetics, molecular biology, embryology and animal science, is usually time-consuming and labor-consuming. One problem with learning to do microinjections is that it can be a long wait between the time the microinjections are done and the time that the results are known, particularly if one waits until the microinjected embryos have developed into weaning age mice before screening. How to easily and rapidly assay for a successful pronuclear? There are a number of constructs that are particularly useful when learning to do microinjections. Among them, tyrosinase can be used to allow the visual identification of transgenic mice at birth in the first and all subsequent generations. Microinjection of a tyrosinase minigene into embryos from an albino mouse strain can result in gene cure of the albino defect and the pigment synthesis[5,6,18]. Pigmented mice with dark eyes can be easily identified by simply visible inspection at birth. In fact, the pigment epithelial cells of the retina begin to synthesize melanin by P10.5 of embryonic development[8,26] so that transgenic mice can be typically identified by visual inspection of the fetuses 2 wk after microinjection. The microinjection can be done using albino inbred strains (such as FVB/N and BALB/c) and inexpensive outbred albino strains (such as ICR and Kunming mice). Another advantage of the tyrosinase minigene is the fact that it is not detrimental to the health of the transgenic animals.

Therefore, we decided to use the tyrosinase minigene as a visual marker to perform microinjection training and improve the techniques related with transgene to greatly elevate the efficiency of gene transfer in our center.

MATERIALS AND METHODS

Production of the tyrosinase minigene transgenic mice

The tyrosinase minigene TyBS[5] used for microinjection was generously provided by Dr. P.A. Overbeek (Howard Hughes Medical Institute, Department of Cell Biology, Baylor College of Medicine, Houston, TX, USA) and Dr. F Beermann (Swiss Institute for Experimental Cancer Research, Switzerland).

Transgenic mice were generated by microinjection of single cell embryos using standard techniques[27]. The Kunming mouse strain, supplied by Center of Experimental Animals, Sun Yat-Sen University, was used as the source of embryos for the micromanipulation and for the subsequent breeding trials. For microinjection, the 4.1-kb fragment of tyrosinase minigene (Figure 1) was released free from the vector backbone of pTyBS[5] via digestion with *Eco*R I and *Kpn* I, thereafter isolated and purified using the QIA quick gel extraction kit (Qiagen, Hilden, Germany), diluted to a final concentration of 2

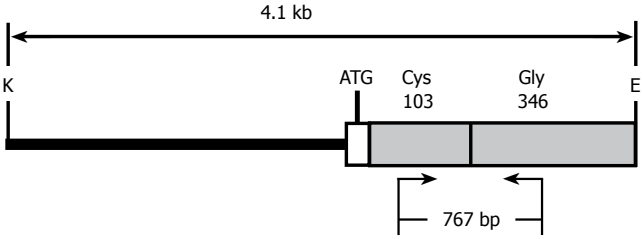

Figure 1 The structure of tyrosinase minigene construct TyBS used for microinjection. The construct contains a 2.25-kb tyrosinase promoter, i.e., 5' non-coding flanking sequence of mouse tyrosinase, as a single thick line plus 65 bp of tyrosinase exon I (to the *Xho* I site) and 1.785-kb *Xho* I-*Eco*R I fragment (shaded) (derived from Tyrs-J) containing tyrosinase cDNA and 3' non-coding flanking sequence. TyBS encodes cysteine at amino acid 103 and glycine at amino acid 346. The 4.1-kb injected fragment was obtained by pTyBS digestion with *Kpn* I and *Eco*R I. The restriction sites are: E, *Eco*R I; K, *Kpn* I. The primers specific for TyBS used in PCR reaction (small arrows) and the expected size of PCR products are indicated.

μg/mL DNA in injection buffer (10 mmol/L Tris/0.1 mmol/L EDTA, pH 7.4), and then microinjected into the pronuclear of one cell-stage fertilized embryos [Kunming mouse (♀) × Kunming mouse (♂)]. About 20-25 DNA-injected fertilized eggs that survived microinjection were implanted into the oviducts of one recipient pseudopregnant Kunming mouse 2-3 h after injection or the next day as previously described[27]. Potential transgenic founders were weaned at 3 wk of age. The offsprings were firstly screened for the presence of the transgene via pigmentation phenotypes derived from the existence of the functional tyrosinase minigene, followed by PCR analysis performed on the tail genomic DNA prepared with standard protocols[28]. All animal care and experimentation were performed according to the Study and Ethical Guidelines for Animal Care, handling and termination established by the Subcommittee of Sun Yat-Sen University on laboratory animal care. The presented work was approved by the ethical committee of Sun Yat-sen University and is covered by Chinese animal husbandary legislation.

Genotype analysis by PCR

PCR was performed on tail genomic DNA to further identify which mice have tyrosinase minigene integrated into their genome. The sequences of the forward primer (FP) within exon 1 and reverse primer (RP) within exon 4 used to amplify a 767-bp fragment of the tyrosinase minigene were: 5'-GGTTTCAACTGCGGAAACTG-3' (forward) and 5'-TGTGAGTGGACTGGCAAATC-3' (reverse) (Figure 1). PCR conditions were as follows: pre-denaturation at 94℃ for 7 min, followed by 30 amplification cycles of denaturation at 94℃ for 1 min, primer annealing at 58℃ for 1 min, and extension at 72℃ for 1 min 30 s, and finally an additional extension at 72℃ for 10 min. TyBS construct DNA was used as the positive control for each PCR reaction, and genomic DNA from normal Kunming mice was employed as a negative control for each PCR test. DNA samples were considered positive for a particular transgene if a band of the predicted size in the test samples was present with no amplification occurring in the control sample. Endogenous genomic

tyrosinase sequence was not amplified under this PCR conditions chosen here.

Mouse propagation and transmission

At 6-8 wk of age, founders shown to be transgenic for the tyrosinase minigene were mated with normal Kunming mice to generate F1. Pigmented F1 animals derived from founder, as well as albino non-transgenic littermates were further analyzed for the inheritance of the tyrosinase transgene by PCR using tyrosinase-FP/RP primers. PCR protocols for TyBS were noted above.

RESULTS

Rescue of the albino phenotype by tyrosinase transgene

Within the coding sequences of the tyrosinase gene, a G to C transversion at nucleotide 308, leading to a cysteine (Cys) to serine (Ser) mutation at amino acid 103, is sufficient to abrogate pigment production in mice[5]. This same base pair change is fully conserved in the classical albino strains of laboratory mice, such as FVB/N and BALB/c[5]. Albino Kunming mice are an outbred mouse strain that is homozygous mutant at the albino (*c*) locus. An albino mutation carried in the Kunming mouse strain should be also the result of a base substitution from G to C in exon I. It is, therefore, reasonable to expect that the albino phenotype can be rescued by introducing a functional tyrosinase minigene, such as TyBS, into albino embryos.

The tyrosinase minigene TyBS construct[5] used for microinjection is illustrated in Figure 1. As the expression of the tyrosinase minigene is easily detected by the pigmented phenotype, this gene can be used as a visual marker for the production of transgenic animals.

Of the 45 embryos transferred to the recipient females, 11 embryos developed to term. Two individuals of 11 siblings were transgenic, as demonstrated by pigmentation phenotype in the eyes (Figure 2A-D, F and G) and coat (Figure 2C, D and F), and PCR analyses (Figures 3A and B).

Furthermore, two TyBS transgenic mice, i.e. P1 (Figure 2A and B) and #8 (Figure 2G) which died 48 h after birth, had dark eyes at birth, and were immediately identifiable as transgenic mice. Although the extent of the coat pigmentation was non-standard like the wild-type phenotype, founder P1 exhibited the partially pigmented phenotype (Figure 2C, D and F). Over time, the coat of P1 with nearly black eyes (Figure 2A-D, F) became more heavily pigmented (light grey to dark grey) (Figure 2C, D and F), while the eye and fur phenotypes of non-transgenic littermate controls remained pink and albino throughout life (Figure 2C-E), respectively.

Transmissibility of the foreign transgene

To determine whether the TyBS transgene was transmitted to the next generation, at 6 wk of age female P1 was back-crossed to the parental mouse strain to give F1 generation. The progeny of P1 was analyzed for the inheritance of the transgene by eye phenotype, coat pigmentation and PCR.

From the cross between P1 and normal Kunming mouse, 11 offspring were obtained. Although all of littermates from P1 died immediately at birth, it was found that six out of the 11 siblings exhibited the pigmented eyes at birth (Figure 2H), as verified by PCR (Figure 3C).

Non-mosaic transgenic mice with one site of integration should transmit the transgenic DNA in a Mendelian fashion to about 50% of their offspring, whereas mosaic mice generally show a frequency of transmission of 25% or less. Note that founder mice that have more than one site of integration can produce litters where 75% or more of the offspring are transgenic, although the percent transmission for any one site of integration is expected to be average 50% or less[29,30]. It was concluded that founder P1, successfully transmitting the transgene in a Mendelian fashion to about 55% (6/11) of its progeny, is non-mosaic transgenic mouse.

Taken together, these data demonstrate that founder P1 can transmit the transgene to subsequent generation and its progeny show an inherited characteristic phenotype of pigmented eyes.

DISCUSSION

Coat color of the tyrosinase transgenic mice

Pigmentary genes are the first mammalian genes to be studied, mostly because of the obvious phenotypes associated with their mutations[23]. In this study, founder P1, harboring the tyrosinase minigene TyBS, exhibited light pigmentation, but non-standard wild-type coat color in the skin, although over time, P1 coat became more heavily pigmented. Similarly, the transgenic mice carrying TyBS construct showed considerable variation in the intensity of pigmentation, the coat colors were found to range from grayish to brownish, and none of the mice were black[5].

Actually, all these standard tyrosinase constructs, including TyBS, driven by the limited amount of 5' tyrosinase upstream regulatory sequences (ranging from 270- to 5500-bp promoter sequences) displayed a high degree of variability in coat pigmentation between independent lines[14,30-34], and the coat pigmentation did not reach the normal levels observed in the wild-type phenotype[6,18,30-32,35,36]. For example, in an evaluation of 39 transgenic founder animals and 44 transgenic lines, 5 phenotypic patterns of pigmentation were consistently observed, including albino, dark, light, mottled and Himalayan[32]. In fact, the tyrosinase minigene which is sufficient to produce normal levels of both eumelanin and phaeomelanin can give normal black or brown pigmentation on the appropriate non-agouti genetic backgrounds[5,32]. These abnormally expressional patterns might have been explained by position effects. In summary, these findings demonstrate that other regulatory regions within the tyrosinase gene are required to sustain the faithful expression of tyrosinase transgene, independent of integration site.

By flanking a tyrosinase minigene with tandem copies of the chicken β-globin 5' HS4 insulator, there is a significant reduction in variability among transgenic lines, with the resulting mice exhibiting the similar levels of coat pigmentation, which, in turn, improves the yield of phenotypically expected transgenic founders resulting from each microinjection session, and consequently reduces

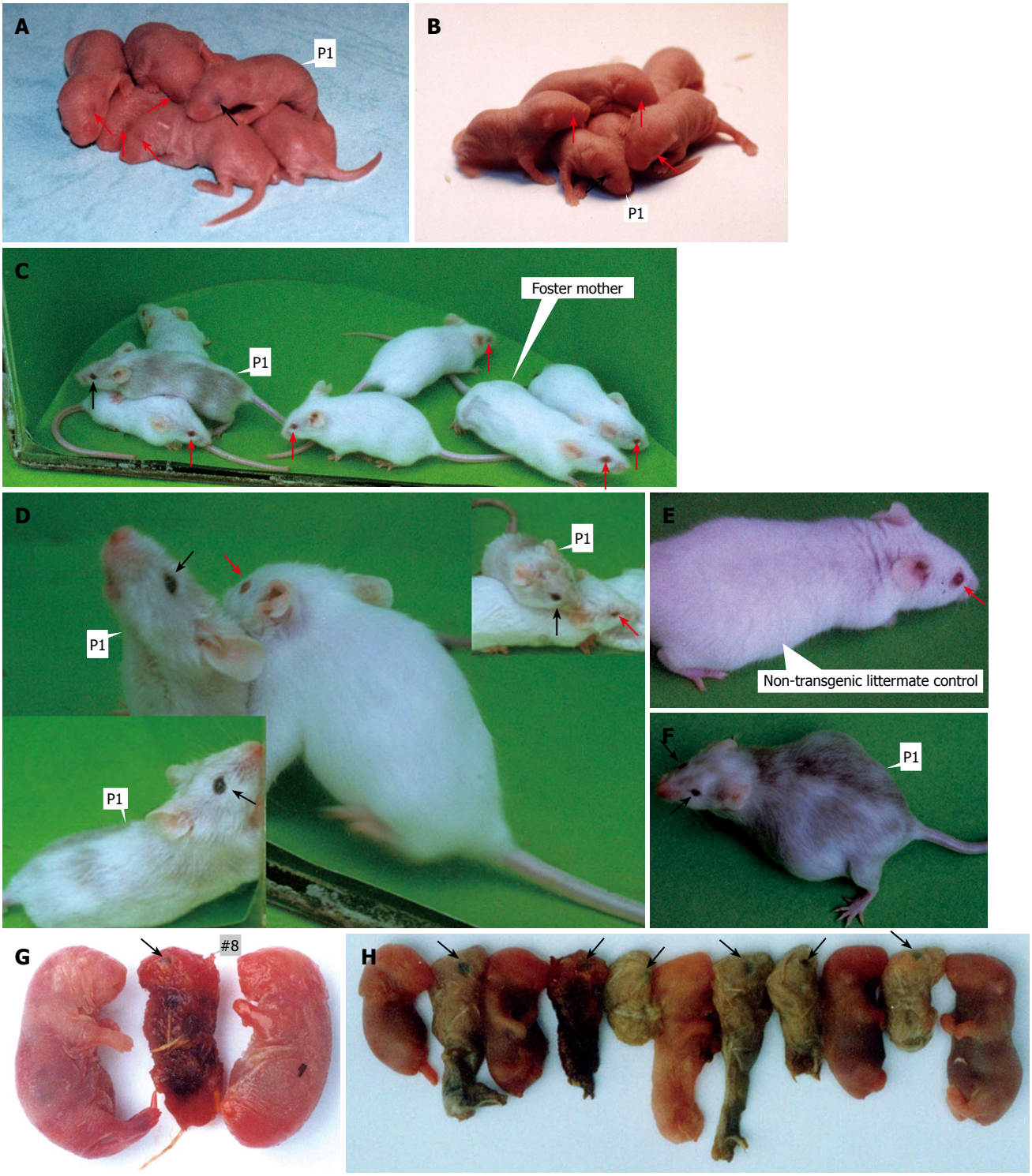

Figure 2 Eye and coat colors of tyrosinase minigene transgenic Kunming albino mice. One foster mother gave birth to six F0 pups (**A**-**F**); among the littermates, only one mouse (referred to as P1) had the pigmented eyes at birth. On July 15, another foster mother produced three F0 pups (**G**); of three siblings, only one mouse (referred to as #8) indicated the pigmented eyes at birth. P1 (♀) was crossed with normal Kunming albino mouse to give birth to 11 F1 offspring (**H**). All of the common albino strains of laboratory mice, such as FVB/N, BALB/c, and Kunming mouse (in China), have pink eyes and albino skin. (**A** and **B**) The **2**-d-old littermates. At birth, one pigmented mouse (P1) with dark eyes could be easily and immediately identified as a transgenic mouse by simple visual inspection. (**C**) The 4-wk-old littermates and Kunming albino foster mother. Founder P1 exhibited black eyes and light grey fur when compared to the non-transgenic littermate controls and Kunming albino foster mother with pink eyes and albino skin. No differences in phenotypes between transgenic mouse and the controls and foster mother except for melanization in eyes and hairs. Actually, the Kunming albino mouse was also used as a recipient strain for TyBS transgene in this project. (**D**) Eye color of the 4-wk-old P1 mouse compared with one of the non-transgenic littermates. One of the non-transgenic littermates (right of the middle map) had pink eyes, while at this age the heterozygote P1 (left of the middle map, upper and lower) had nearly black eyes. (**E** and **F**) The 8-wk-old non-transgenic littermate control and the adult P1 mouse (8-wk old), respectively. The non-transgenic littermate control (**E**, left) had pink eyes and albino coat, while at this age the heterozygote P1 mouse (**F**, right) had nearly black eyes and dark grey coat. Over time, the coat of P1 mouse became more heavily pigmented, while the eye and fur phenotypes of non-transgenic littermate control remained pink and albino throughout the life, respectively. (**G**) Eye color of the 1.5-d old #8 compared with its littermates. At birth, #8 with dark eyes could be easily and immediately identified as transgenic mice by simple visual inspection. Unfortunately, #8 as well as non-transgenic littermates without dark eyes were killed by foster mother 1.5 d after birth. **(H)** Eye color of F1 offspring (11) developed from mating of P1 and normal Kunming albino mouse. Founder P1 (♀) was back-crossed to normal Kunming albino mouse to produce eleven F1 generation. Unluckily, all of F1 offspring (11), born on September 8, died immediately at birth. P1 also deceased one month after delivery as it did not recover from giving birth to pubs. Of the 11 animals that developed from the mating aforementioned, six mice exhibited pigmented eyes. → and → indicate the pigmented eyes and non-pigmented eyes (pink), respectively.

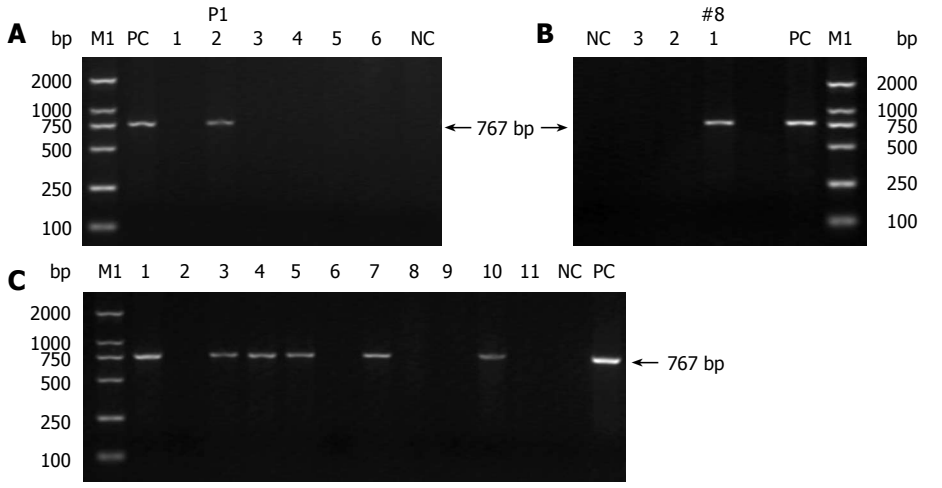

Figure 3 PCR detection of TyBS gene from genomic DNA of the potential transgenic founders (**A** and **B**) and subsequent generation(s) (**C**). Lane M1: DL 2000 DNA Marker (TaKaRa); lane PC: positive control (TyBS as template); lane NC: negative control using genomic DNA from normal Kunming mice as template. The arrows indicate the positions of PCR products amplified by the primers shown in Figure 1. (**A**) Littermates (F0, six mice) were verified for the transgene presence by PCR analysis. Lanes 1-6: genomic DNA from the potential founder(s) of 6 littermates; Lane 2: 767-bp band amplified from genomic DNA of P1 with pigmentation in the eyes. (**B**) Littermates (F0, three mice) were confirmed for the transgene presence by PCR analysis. Lanes 1-3: genomic DNA from the potential founder(s) of three siblings. Lane 1: 767-bp band amplified from genomic DNA of #8 with pigmentation in the eyes. Other details are as in Figure 2G. (**C**) Littermates (F1, 11 mice) were examined for the transgene presence by PCR analysis. The founder P1 (♀) was crossed with normal Kunming mouse to produce 11 littermates (F1) with six mice with pigmented eyes. Lanes 1-11: genomic DNA from F1 offspring derived from P1; Lanes 1, 3-5, 7, 10: 767-bp specific band amplified from genomic DNA of F1 offspring exhibiting pigmented eyes.

animal requirements for transgenic production[37].

Co-injection strategy for visually identifying the transgenic mice

Screening transgenic animals is usually time-consuming and labor-consuming. It would be very helpful if the transgenic animals could be identified by the visible inspection at birth. The functional tyrosinase gene introduced into an albino mouse strain leads to pigmentation in eyes and skin with high penetrance, and pigmented mice with dark eyes can be immediately identified by simply visible inspection at birth[23], as further confirmed by this study.

When two or several transgenic constructs are co-injected into single-cell fertilized embryos, the co-injected constructs typically co-integrate into the genome, where the transgene can independently express[38]. Theoretically, co-injection of tyrosinase transgenic construct with any other construct(s) should result in a certain percentage of transgenic mice carrying both transgenes at a single chromosomal site[23]. Additionally, co-injection experiments with the agouti transgenes and other transgenes demonstrated co-integration of the two constructs at the same chromosomal site in approximately 95% of F1 progeny, allowing transgene inheritance to be visibly detected[39]. The direct and visual detection of pigmentation in tyrosinase transgenic animals generated in the albino genetic backgrounds was repeatedly proposed by independent teams as a visual marker in co-injection strategies for the rapid detection of the successful tran sgenesis[12,30-33,35] and by our practices (data not shown). The utility of tyrosinase minigene co-injection with other construct(s) of interest is a useful adjunct to allow rapidly visual identification of transgenic mice at birth.

Moreover, another advantage of co-injection strategy is the fact that homozygous mice in most families can be identified by simply visual inspection, since the homozygous mice have darker coat colors, reflecting the increased gene dosage[32].

The co-injection strategy improves the yield of phenotypically desirable transgenic founder mice resulting from each microinjection session, and consequently reduces animal requirements for the transgenic production and routine genetic validation of transgenic lines.

In summary, we have successfully rescued the albino phenotype by introducing a functional tyrosinase gene into Kunming albino mouse. It should be pointed out here that TyBS and other tyrosinase transgenic constructs can be fused with any of the other genes and microinjected into fertilized eggs from albino murine strains in order to produce melanin pigments as an excellently visible marker for the generation and breeding of transgenic mice.

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