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Development of new inbred transgenic strains of rats with LacZ or GFP

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Abstract

The ideal goal of regeneration medicine is to restore form and function to damaged tissues. While stem cell transplantation is considered a promising therapeutic approach, knowing the fate of transplanted cells using appropriate markers is essential. We developed new inbred transgenic rat strains with lacZ and GFP based on the transgenic (Tg) animal technique in rats. These Tg animals expressed most of their marker genes ubiquitously, compared to previous Tg rats. Immunological antigenicity against marker proteins was evaluated using conventional skin grafting, and results suggested lacZ-Tg-derived skin was much less immunogenic than that of GFP-Tg. However, GFP-positive cells from parental transgenic rats were still potential candidates for the study of cellular fate in immune privilege sites, such as the brain. Taking advantage of less immunogenic lacZ, we also examined the role of bone marrow-derived cells (BMDCs) in skin wound healing using an in vivo biological imaging system. Although transplantation of BMDCs enhanced wound healing at the injection site, BMDCs were detected only for a short time, suggesting a transient contribution of autologous BMDC-transplantation in wound healing. Our Tg-rat system may provide great benefits for the elucidation of the cellular process of regenerative medicine, including cell and tissue transplantation.

Keywords: Transgenic rat; LacZ; GFP; Immunogenicity; Regenerative medicine; Cellular trafficking

To restore form and function to damaged tissues, a cell and/or tissue transplantation strategy has emerged as a potential therapeutic approach involving the use of autologous cells [1–3]. To examine the fate of the transplanted cells, however, appropriate and stable marking is required for visualization. While it is easy to use fluorescent dye, there is a drawback in that fluorescent intensity decreases during in vivo cellular proliferation. Based on the genetic engineering technique used in rodents [4–7], it is possible to express stable reporter proteins such as β-galactosidase (LacZ) and green fluorescent protein (GFP) in rats and mice. Cells from transgenic animals that express marker genes provide potential benefits for the stable visualization of cellular fate.

We previously developed lacZ- and GFP-Tg rats on a different genetic background, and demonstrated that these Tg rats were suitable for the monitoring of cellular fate in certain experiments [4,5]. However, these Tg animals have a few weak points: all of the tissues did not express enough marker genes in the established animal lines, although both reporter genes were driven under a ubiquitous CMV enhancer/chicken β-actin promoter (CAG promoter) [8]. Furthermore, transplanted cells

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or tissues were occasionally rejected by immune responses due to a mismatch of the minor histocompatibility complex (mHC) derived from an outbred strain of Wistar rats [4]. Herein we redeveloped both lacZ- and GFP-expressing Lewis rats harboring the same genetic background (MHC haplotype: $RT1^l$). These Tg animals express most of their marker genes ubiquitously, compared to previous Tg rats. We also evaluated immunological antigenicity against these marker proteins using conventional skin grafting, and discovered that the skin from lacZ-Tg rats was much less immunogenic than that of GFP-Tg. Nonetheless, GFP-positive cells from parental transgenic rats were available for the observation of cellular fate in an immune privilege site, such as the brain. We also examined the role of bone marrow-derived cells (BMDCs) in skin wound healing using the less immunogenic lacZ-Tg rat and autologous cell transplantation. Although transplantation of BMDCs enhanced wound healing at the injection site, BMDCs were not detected for a long times despite using a sensitive biological imaging system, suggesting the transient and limited contribution of autologous BMDC transplantation in wound healing. An animal model using our Tg-rat system may provide great benefits for the study of regenerative medicine, including cellular and tissue transplantation.

Materials and methods

Animals. An inbred rat strain, Lewis (LEW) (MHC haplotype: $RT1^l$), was purchased from Charles River Japan, (Yokohama, Japan). LacZ-expressing DA (CAG/LacZ-DA) transgenic rats (MHC haplotype: $RT1^a$) and GFP-expressing Wistar (CAG/GFP-Wistar) transgenic rats (MHC haplotype: $RT1^k$) have been described previously [4,5]. Both of the transgenes were driven under the CAG promoter [8]. All animals had free access to standard chow and drinking water, and were maintained on a 12-h light/dark cycle. All experiments in this study were performed in accordance with the Jichi Medical School Guide for Laboratory Animals.

Generation of transgenic rats. To generate ROSA/LacZ- and CAG/GFP-LEW Tg rats, the authentic microinjection technique was used as described previously [4]. Briefly, the *Nco*I and *Nhe*I fragment (LacZ cDNA) from a pMOD-LacZ plasmid (InvivoGen, San Diego, CA) was inserted into the *Nco*I and *Xba*I sites of a pBROAD2 expression plasmid (InvivoGen) containing ROSA26 promoter [9], and the *Pac*I fragment containing the promoter and coding sequence was injected into the fertilized Lewis rat egg. Transgene expression was examined by β-galactosidase staining (detailed below).

For the GFP-expression plasmid, GFP cDNA from a pEGFP vector (Clontech, Palo Alto, CA) was inserted into a pCAGGS expression plasmid [4,8], and the *Hin*dIII–*Sal*I fragment was injected into the fertilized Lewis rat egg. Transgene expression was confirmed under an excitation light (489 nm). In this study, hemizygous Tg rats (LEW-Tg(Rosa-LacZ)15Jmsk and LEW-Tg(CAG-GFP)ys) were used.

Detection of lacZ expression. Samples were embedded in OCT compound (Miles laboratory, IN), frozen in liquid nitrogen, and cut into thin (8–10 μm) sections under freezing conditions. Sections were fixed with a fixative solution (0.2% glutaraldehyde, 2 mM $MgCl_2$, and 5 mM EGTA) in phosphate-buffered saline (PBS) for 5 min at room temperature (RT), and washed three times in a washing solution (2 mM $MgCl_2$, 0.01% sodium deoxycholate, and 0.02% Nonidet-P40) in PBS. Specimens were treated with a β-gal staining solution (1 mg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 2 mM $MgCl_2$, and 5 mM potassium hexacyanoferrate [III], 5 mM potassium hexacyanoferrate [II] trihydrate) at 37 °C for 1–4 h [10]. Eosin was then used for counter-staining.

To visualize lacZ expression in vivo and in vitro, an in vivo bioimaging system (IVIS) (Xenogen, Alameda, CA) was used. Animals were anesthetized using isoflurane (Abbot, Chicago, IL), and Beta-glo (Promega, Madison, WI) was administered locally to the artificial dermis or skin graft (50 μl of the reagent/animal). LacZ expression photo-images were taken by IVIS and quantified using Living Image software (Xenogen), which measured photon/s/cm^2/steradian. Longitudinal changes in lacZ expression of artificial dermis in the same animal were followed using IVIS.

Skin grafting. Skin transplantation was performed using 6- to 8-week-old female rats. Full-thickness donor skin grafts were transplanted onto a dorsal area of recipients under diethyl ether anesthesia using our coupled skin grafting method [11]. Grafted skins were fixed physically using 5–0 nylon tie-over sutures and bandages. Grafts were monitored regularly by visual and tactile inspection after the removal of the bandage on day 6, as described by Billingham and Medawar [12]. Rejection was defined as the start of contraction of the skin graft according to previously defined criteria [11].

Neurosphere preparation and transplantation into the injured brain. Neurospheres were prepared by culturing cells from the fetus of CAG/GFP-LEW Tg rats as described by Reynolds et al. [13]. GFP-positive fetuses of 14.5 day gestation were obtained from pregnant rats. After decapitation, brains were mechanically excised and collected in cold PBS. Each sample was mined with a razor blade following centrifugation for 500*g*, 10 min. The precipitate was resuspended in PBS containing 0.1% trypsin and 0.04% DNase, and then incubated at 37 °C for 30 min to facilitate dissociation into single cells. The dissociated cells were cultured in serum-free Dulbecco's modified Eagle's medium/Ham's F12 (DEMEM/F12: Gibco, Grand, Island, NY) with basic fibroblast growth factor (bFGF, 10 mg/ml, Sigma) and epidermal growth factor (EGF, 20 mg/ml, Sigma). GFP expression in the cultured cells was analyzed using a flow cytometer.

The left-middle cerebral artery (MCA) in adult male LEW rats was occluded for 60 min using the intraluminal filament method [14]. Five days after MCA occlusion, the rats were anesthetized with ketamine (60 mg/kg, ip) and xylazine (6 mg/kg, ip) and then placed in a stereotactic frame (Type SR-50 Narishige, Tokyo, Japan). GFP^+ neural stem cells (1.1×10^4 cells) were transplanted into the left side of the lateral ventricle through a glass micropipette.

After transplantation of the neurosphere, the host rats were sacrificed at 28 days. Each rat was perfused transcardially with heparinized saline followed by a phosphate-buffered solution containing 4% paraformaldehyde and 7.5% sucrose. The brains were removed and serially sectioned in the coronal plane at a thickness of 2-mm. GFP expression in each section was observed using a fluorescence stereoscopic microscope.

BMDC preparation and artificial dermis grafting. BMDCs were harvested by flushing femurs, tibiae and humeri of rats with ice cold PBS. Red blood cells were lysed with ACK buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA, pH 7.2) at 4 °C for 30 min. Cells were then washed with PBS three times and re-suspended in PBS just before injection.

The head hair of rats was clipped under anesthetic conditions. Full-thickness skin defects (2-cm × 2-cm) were made on the head. The artificial dermis (Terudermis, Terumo, Tokyo, Japan) containing 10^7 BMDC cells (100 μl) was grafted onto the skin defects. As a control group, artificial dermis containing PBS (100 μl) was grafted. For histological evaluation, animals were killed 1, 2, 3, 4, and 8 weeks after transplantation, and specimens were stained using a β-gal solution.