Transgenic rats with green, red and blue fluorescence: powerful tools for bioimaging, cell trafficking, and differentiation

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ABSTRACT

The rat represents a perfect animal for broadening medical experiments, because its physiology has been well understood in the history of experimental animals. In addition, its larger body size takes enough advantage for surgical manipulation, compared to the mouse. Many rat models mimicking human diseases, therefore, have been used in a variety of biomedical studies including physiology, pharmacology, transplantation, and immunology. In an effort to create the specifically designed rats for biomedical research and regenerative medicine, we have developed the engineered rat system on the basis of transgenic technology and succeeded in establishing various transgenic rat strains. The transgenic rats with green fluorescent protein (GFP) were generated in the two different strains (Wistar and Lewis), in which GFP is driven under the chicken beta-actin promoter and cytomegalovirus enhancer (CAG promoter). Their GFP expression levels were different in each organ, but the Lewis line expressed GFP strongly and ubiquitously in most of the organs compared with that of Wistar. For red fluorescence, DsRed2 was transduced to the Wistar rats: one line specifically expresses DsRed2 in the liver under the mouse albumin promoter, another is designed for the Cre/LoxP system as the double reporter rat (the initial DsRed2 expression turns on GFP in the presence of Cre recombinase). LacZ-transgenic rats represent blue color, and LacZ is driven the CAG (DA) or ROSA26 promoter (Lewis). Our unique transgenic rats' system highlights the powerful performance for the elucidation of many cellular processes in regenerative medicine, leading to innovative medical treatments.

Keywords: GFP, DsRed2, LacZ, Cre/LoxP, organ-specific gene expression, tissue regeneration

1. INTRODUCTION

The rat has offered important animal models for biology and medical research, as it has been a historical wealth of physiological and pharmacological data. More than 200 inbred models of human complex disease have been developed for over a century [1, 2], and the larger body size than the mouse have allowed us to make various physiological techniques that may prove to have biological significance. In addition, the Rat Genome Project is also providing biomedical researchers [2, 3] with a powerful set of tools with which to develop much better models of human disease at the phenotypic and genomic level. Synergized with recent advances in genetic engineering technology, the transgenic rat system is thus providing innovative animal tools and a platform for better and deep understanding in new biomedical research fields, such as regeneration medicine.

To enhance our understanding of biological processes as they occur in living animals, imaging strategies have been developed and refined that reveal cellular and molecular events of biology in real time. In particular, fluorescence imaging, which utilizes fluorescent proteins (e.g. GFP and DsRed) as internal biological light sources, offers important opportunities for investigating a wide variety of physiological or disease processes [4, 5]. Cell differentiation or fusion, for example, is the end result of a complex series of biologic events that potentially leads to the functional recovery in damaged tissues [6-9]. Visualization of their fate needs appropriate cellular marking procedure. Indeed it is easy to use fluorescent dye, but there is a drawback in that fluorescent intensity decreases during *in vivo* cellular proliferation. Thus, genetically encoded biological probes provide high performance to examine the cellular fate in living animals.

In this proceeding, we introduce our transgenic rat system and demonstrate potential applications to particular biologic events including cellular trafficking, transdifferentiation, and tissue regeneration: 1) use of GFP-transgenic rats in cellular trafficking, 2) role of bone marrow-derived cells in liver regeneration using the liver-specific DsRed2 transgenic rat, 3) spatiotemporal gene control by the Cre/LoxP system and cellular fusion, 4) role of bone marrow-

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derived cells in skin wound healing using lacZ transgenic rats. Furthermore, we also refer to immunological antigenicity against these marker proteins using conventional skin grafting.

2. METHODOLOGY

2.1 Animals

The Lewis (LEW) (MHC haplotype: $RT1^l$), Wistar ($RT1^k$), DA ($RT1^a$) rats were purchased from Charles River Japan, Inc. (Yokohama, Japan). All animals had free access to standard chow and drinking water, and were maintained on a 12-hr light/dark cycle. All experiments in this study were performed in accordance with the Jichi Medical School Guideline for Laboratory Animals.

2.2 Generation of transgenic (Tg) rats

To generate Tg rats, the authentic microinjection technique was used as described previously [10]. For example, GFP cDNA from a pEGFP vector (Clontech, Palo Alto, CA) was inserted into a pCAGGS expression plasmid (containing CAG promoter) [10, 11], and the *Hind* III–*Sal* I fragment was injected into the fertilized Lewis rat egg. Transgene expression of GFP was confirmed under an excitation light (489nm). Precise generation processes of other Tg-rat lines have been described previously: liver-specific DsRed2-expressing Tg (Alb/DsRed2-Wistar: $RT1^k$) [12], DsRed2/GFP double reporter Tg ($RT1^k$) [13], Cre-expressing Tg ($RT1^k$) [13], LacZ-expressing DA (CAG/LacZ-DA: $RT1^a$) [14], LacZ-expressing LEW (Rosa/LacZ-LEW: $RT1^l$) [15], and GFP-expressing LEW (CAG/GFP-LEW: $RT1^l$) [15]. In a series of the study, hemizygous Tg-rats were used.

2.3 Recombinant adenoviral vectors and their transfer to the target organ

AdV-Cre was propagated and purified as described previously [16]. Recombinant adenoviruses were titered by determining the median tissue culture infection dose (TCID50). Viral stocks were stored in 10% glycerol and kept at –80°C prior to use. For administration of the vectors, AdV-Cre ($1x10^8$ pfu) was injected intramuscularly into the lower limb.

2.4 Chemically-induced liver injury models

Carbon tetrachloride (CCl_4) and 2-actylaminofluorene (2-AAF) were purchased from Sigma-Aldrich (Tokyo, Japan). For the acute liver injury model, animals were exposed with 2-AAF for suppressing hepatocyte proliferation and CCl_4 was administrated for hepatic injury [17]: 2-AAF (2.5 g/l in earthnut oil) was daily administrated to the stomach of rats by a gastric tube for 14 days. On the 7th day of 2-AAF administration, CCl_4 was given into the peritoneal space of rats. A single LD_{50} dose of CCl_4 (1.9 ml (1500 mg)/kg of body weight), which was determined by another experiment using Lewis rats, was administrated in a 1:1 dilution (vol: vol) of corn oil. For chronic liver injury model, CCl_4 was repeatedly administrated as described previously [18, 19]: rats were injected subcutaneously every 4 days with CCl_4 (2.0 ml/kg body weight).

2.5 Preparation of bone marrow-derived cells (BMDCs) and neural progenitor cells (NPCs)

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 [12, 14, 15].

NPCs were prepared by culturing cells from the fetus of CAG/GFP-LEW Tg rats as described by Reyonds et al [20]. 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 500xg, 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.

2.6 Cerebral infarction model

The left-middle cerebral artery (MCA) in adult male LEW rats was occluded for 60 min using the intraluminal filament method [21]. Five days after MCA occlusion, the rats were anesthetized with ketamine (60 mg/kg, i.p.) and xylasine

(6mg/kg, ip), and then placed in a stereotactic frame (Type SR-50 NARISHIGE, Tokyo, Japan). GFP^+ neural stem cells ($1.1x10^4$ 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.

2.7 Portal injection

BMDCs ($5 X10^8$ cells/animal) of Alb-DsRed2 Tg rats [12] were injected into the right liver lobe through the portal vein either 48 hr after CCl_4 administration (for the acute liver injury model) or 4 wks after repeated administration (for chronic liver injury model). Briefly, the middle abdomen of rats was incised to expose the viscera. Left hepatic artery and portal vein were clamped by a vascular microclip. BMDCs were selectively injected into the distal site of the portal vein using 23-gauge needle. At the end of injection, the needle was rapidly withdrawn and hemostasis was secured without hematoma formation by gentle pressure using a cotton swab. The left hepatic vessels were released and the abdominal wall was closed.

2.8 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 diethylether anesthesia using our coupled skin grafting method [22]. 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 [23]. Rejection was defined as the start of contraction of the skin graft according to previously defined criteria [22].

2.9 Limb transplantation model

The hind limb of the DsRed2/GFP double reporter Tg rat was orthotopically transplanted into NCre Tg rats using a microsurgical technique, as described previously [24]. To prevent rejection, 1 mg/kg of FK506 (kindly provided by Fujisawa Pharmaceutical Company, Osaka, Japan) was intramuscularly injected for 14 days after transplantation. DsRed2/GFP expression in the graft limb was observed, and specimens were biopsied at 1, 2, and 4 weeks after transplantation.

2.10 Detection of lacZ expression

Samples were embedded in OCT compound (Miles laboratory, IN), frozen in liquid nitrogen, and cut into thin (8–10 mm) sections under freezing conditions. Sections were fixed with a fixative solution (0.2% glutaraldehyde, 2 mM $MgCl_2$, 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, 0.02% Nonidet-P40) in PBS. Specimens were treated with a beta-gal staining solution (1 mg/ml of 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside, 2 mM $MgCl_2$, 5 mM potassium hexacyanoferrate [III], 5 mM potassium hexacyanoferrate [II] trihydrate) at 37°C for 1-4 h [25]. Eosin was then used for counter-staining.

To visualize lacZ expression *in vivo* and *in vitro*, an *in vivo* bio-imaging system (IVIS) (Xenogen, Alameda, CA) was used [13, 15, 24]. 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 ml of the reagent /animal). LacZ expression photo-images were taken by IVIS and quantified using Living Image software (Xenogen), which measured photon/sec/cm^2/steradian. Longitudinal changes in lacZ expression of artificial dermis in the same animal were followed using IVIS.

2.11 Skin wound healing model and artificial dermis grafting

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 Corp., Tokyo, Japan) containing 10^7 BMDC cells (100 ml) was grafted onto the skin defects. As a control group, artificial dermis containing PBS (100 ml) was grafted. For histological evaluation, animals were killed 1, 2, 3, 4 and 8 wks after transplantation, and specimens were stained using a beta-gal solution [15].

3. RESULTS

3.1 GFP-transgenic rats

3.1.1 GFP expression profile

Our first generation of GFP-Tg rat was on Wistar background. Use of the Wistar back was indeed useful and effective on the monitoring of cellular fate in certain experiments [10, 26-29]. However, Tg animals derived fromWistar 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 beta-actin promoter (CAG promoter) [11]. Furthermore, transplanted cells 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 [10]. Thus, we redeveloped GFP-expressing Lewis rats harboring the same genetic background (MHC haplotype: $RT1^{l}$). The new CAG/GFP-LEW line expressed GFP strongly and ubiquitously in most of the organs, compared with the former GFP-Tg line of Wistar [10] (Table 1). As shown in Figure 1, representative organs such as the brain and liver demonstrated higher levels of GFP-expression in the new GFP-LEW Tg line.

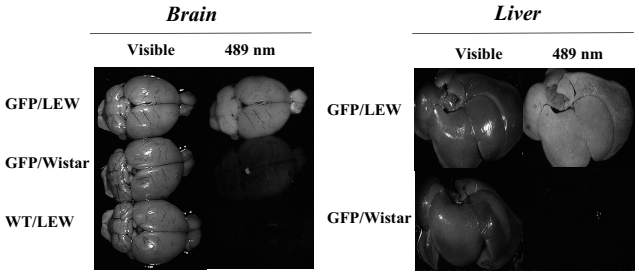

Figure 1:Expression profile of GFP between the adult GFP-Wistar and GFP-LEW Tg rats

Table 1. Differential expression profile of GFP between the adult CAG/GFP-Wistar and CAG/GFP-LEW Tg rats

Organ	GFP-Wistar	GFP-LEW
Brain	+/-	+++
Eye	++	+++
Lung	+/-	+++
Heart	++	+++
Thymus	+	+++
Liver	+	+++
Pancreas	++	+++
Small intestine	+	+++
Colon	+	+++
Kidney	++	+++
Muscle	++	+++
Skin	+	+++

3.1.2 Cellular trafficking

The use of cells from GFP-Tg is much attractive for studies of cellular monitoring due to the stable marker expression and easy visualization under excitation light. Since the brain is well known as an immunologically privileged site, we examined the cellular fate of neural progenitor cells (NPCs) from CAG/GFP-LEW Tg rats without any immunosuppressive drugs in a rat cerebral infarction model. Of note, we described later whether some marker proteins may occasionally exert as immunogens.

NPCs were established from E14.5 of CAG/GFP-LEW Tg rats and maintained *in vitro* for 20 days under appropriate culture conditions (see Methodology section). Neurosphere cells strongly expressed GFP (Figure 2) and Nestin, and kept the phenotype as the NPCs (data not shown). The sphere cells were then transplanted stereotactically into the cerebral ventricle space of wild-type LEW rats at 5 days post-infarction. As shown in Figure 2 (lower panels), GFP-positive cells accumulated in the cerebral infarction area and were able to survive. Thus, even if GFP is highly immunogenic, GFP-positive cells are still useful and attractive materials for the study of cellular fate in immune privilege sites. A similar phenomenon was observed in the case of spinal cord injury (data not shown).

Regarding this migration, tt has recently been reported that this biological event is strongly associated with the chemokine receptor CXCR4 and the ligand CXCL12/SDF-1 [30, 31]. Rat neural progenitors from our transgenic line expressed significant levels of CXCR4 and the cerebral infarction area revealed enhanced mRNA expression of CXCL12 (data not shown). We therefore speculated that those cells were activated and targeted by the interaction of CXCR4 with CXCL12. Furthermore, since there is emerging data suggesting that the axis of CXCR4 and CXCL12 enhances survival of various cells [32, 33], it is likely that cell survival, as well as chemotactic migration, may contribute to the accumulation of GFP-positive neural progenitor-derived cells in the infarction area.

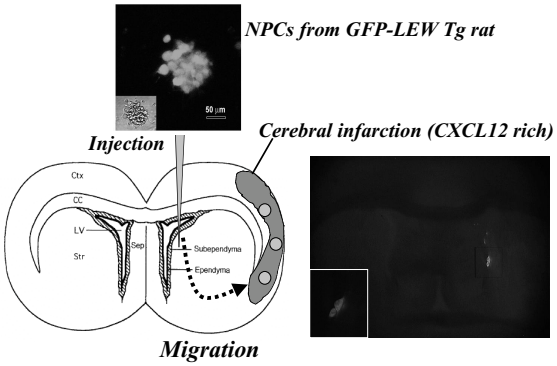

Figure 2: Neural progenitor cells migrate cerebral infarction area

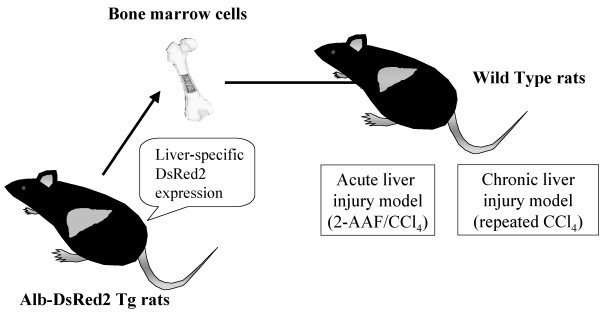

Figure 3: Monitoring transdifferentiation events using bone marrow derived cells of Alb-DsRed2 Tg rats

3.2 DsRed2-Tg rats

3.2.1 Development of the liver-specific reporter Tg-rat

Recent studies have shown that bone marrow-derived cells (BMDCs) are potentially the source for liver regeneration [34-37]. While spontaneous cell fusion is one hypothesis for adaptation of BMDCs into mature hepatocytes [38, 39], it remains still unclear how BMDCs could generate or differentiate into mature cells. Because serum albumin is a characteristic protein much produced from the mature hepatocytes, the albumin gene promoter is to provide an attractive machinery to report cellular events during liver specification and the terminal differentiation. Therefore, we developed a liver-specific reporter Tg-rat (Alb-DsRed2), in which DsRed2 derived from a red coral (*Discosoma*)[40] is expressed as a reporter protein under the control of the mouse albumin enhancer and promoter [41].

In accordance with albumin production from hepatoblasts (early progenitor cell), liver-specific expression of DsRed2 was observed from 14.5 embryonic days in the developmental stage of the Tg rats. DsRed2 expression was observed in the adult liver, but not in BMDCs, lymphocytes, and granulocytes (data not shown).

3.2.2 Differentiation of BMDCs to albumin-producing cells in the damaged liver

In an effort to examine the ability of the Alb-DsRed2 Tg-rat for liver regeneration studies, we used a chemically induced liver damage model with the carbon tetrachloride (CCl_4) (Figure 3). In the acute liver injury model with CCl_4 and 2-AAF, no DsRed2-expressing cells were observed at 19 days after portal injection of BMDCs. However, a few $DsRed2^+$ small cells were recognized in each lower-power view section around 60 days after BMDCs injection (1.5±1.6 cells). Immunohistochemical studies also revealed albumin expression in the $DsRed2^+$ cells, indicating that donor-derived BMDCs migrated and differentiated into the albumin-producing cells in the host liver.

On the other hand, we also evaluated the contribution of BMDCs to the chronic liver injury model with CCl_4. CCl_4 was repeatedly administrated into the subcutaneous space of rats. Several $DsRed2^+$ cells were easily identified around 30 days after portal injection of BMDCs (lower magnification). Different sizes of $DsRed2^+$ cells were observed at 60 days after portal injection, and their number was also significantly increased in comparison with the case of the acute liver injury model (89.3±17.6 cells vs. 1.5±1.6 cells). Of note, it is unlikely that differentiated BMDCs were simply adhered to the damaged liver since significant DsRed2 expression was not observed in BMDCs and peripheral blood in the Tg rats. Thus, we concluded that BMDCs were differentiated into $DsRed2^+$ albumin-producing cells in the damaged liver conditions.

3.3 Double reporter Tg rat: from red to green

3.3.1 DsRed2/GFP double reporter Tg rat

Advanced genetic manipulation has progressed less far in the rat than in the mouse. Generation of cloned rats and the mutagenesis-based gene knockout rat promises much [42, 43]. Herein we introduce the rat Cre/LoxP system, which allows examination of a particular gene function in a temporal and tissue-specific manner by means of conditional gene recombination.

The double reporter Tg rats possess DsRed2 cDNA flanking LoxP sites at both ends as a stuffer and GFP downstream of the DsRed2. Their expression is controlled under a ubiquitous CMV enhancer/chicken beta-actin

promoter (CAG promoter), and DsRed2 is expressed in the rat before Cre/LoxP site-specific excision (Figure 4A). The skeletal muscle, pancreas, heart myocardium, and bronchus showed relatively strong DsRed2 expression in adult Tg rats (data not shown). During the developmental stage, DsRed2 expression was detected in the embryos at the 2-cell stage (1.5 embryonic days) under 560-nm wavelength excitation light, indicating that the CAG promoter functions in rats in many tissues and from early embryo development.

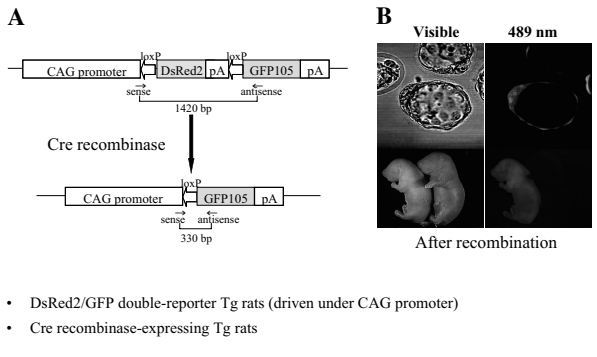

Figure 4: Cre/LoxP recombination system for monitoring cell fusion

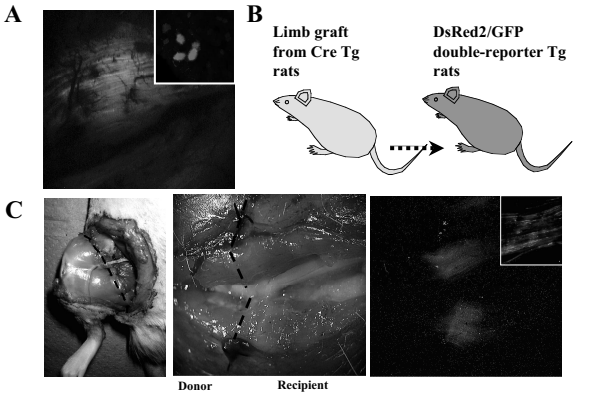

Figure 5: Spatiotemporal control of gene expression and cell fusion

3.3.2 Spatio-temporal control of gene expression by the rat Cre/LoxP system

In order to functional excision of the DsRed2 stuffer gene by Cre recombinase, we first mated DsRed2/GFP double reporter Tg rats with the other NCre Tg rats expressing Cre recombinase (with nuclear localizing signal from SV40 virus large T antigen). In mating of the heterozygous male DsRed2/GFP double reporter Tg rat with the homozygous female of NCre Tg rat, blastocysts at 5.5 embryonic days do not express red fluorescence (DsRed2), but show green fluorescence (GFP) especially in the inner cell mass (Figure 4B, upper right panels). Newborns of double Tg (DsRed2/GFP x NCre) rat also displayed ubiquitous green fluorescence (Figure 4B, lower panels: left newborn rat). These results show that this rat Cre/LoxP system is already functioning in the developmental stage. The polymerase chain reaction (PCR) analysis also showed that the DsRed2 stuffer gene in the double reporter Tg rat was completely excluded by Cre recombinase (data not shown).

This binary double reporter system allows us to control spatiotemporal gene expression. For example, targeted tissue delivery of the Cre-expressing vector is a promising strategy for regulating gene expression. We then introduced Cre-expressing adenovirus (AdV-Cre) into the muscle of the adult double reporter Tg rats by intramuscular injection. Local infection of the Adv-Cre into the muscle changed red-fluorescent muscle fibers expressing DsRed2 to green-fluorescent fibers expressing GFP (Figure 5A), implying excision of the DsRed2 stuffer gene.

To enhance gene control to the specific-target organ, we have also developed a catheter-based gene transfer system. For example, to target the pancreas in the living animals, we successfully performed retrograde intra-pancreatic duct injection of the adenovirus vector using a fine catheter. As a control delivery, *in vivo* luciferase activity was evaluated following AdV-luciferase infection, indicating pancreas-selective gene expression after AdV administration (described in the proceeding number 5704-26 by Murakami et al) [13]. This procedure is an excellent methodology for specific gene expression in the pancreas of living animals, leading to easy control of spatiotemporal gene expression.

3.3.3 Visualization of cell fusion using DsRed2/GFP double reporter Tg rat

We applied our own method based on Cre/LoxP recombination to detect cell fusion events in the muscles. We transplanted a hind limb of the DsRed2/GFP double reporter Tg-rats orthotopically to the NCre Tg rats (Figure 5B). Four weeks after limb transplantation (Figure 5C, left panel), GFP positive muscle fibers were detected in the proximal side of the recipient limb (Figure 5C, right panel). Chromosomal translocation in the anastomosis site was also confirmed by PCR (data not shown). These results demonstrate that GFP expression resulted from fusion event between donor and recipient myofibers.

3.4 Blue Tg rats

3.4.1 LacZ expression profiling

We have developed two types of LacZ-Tg rats: one is driven under a beta-actin promoter (CAG promoter) [11, 14], and another is driven under the ROSA26 promoter [44]. Both promoters provide potentially ubiquitous expression. However, their characters were a little bite different each other. Various organs were removed from transgenic animals and their expression pattern and intensity were determined. Regarding LacZ-LEW transgenic rats, we compared lacZ expression of the previously established LacZ-DA Tg (CAG/LacZ-DA) line [14] with that of the LacZ-LEW (ROSA/LacZ-LEW) line. While skeletal muscle and myocardium revealed strong lacZ expression in CAG/LacZ-DA rats, ROSA/LacZ-LEW rats showed weak and heterogeneous expression in these tissues. In contrast, ROSA/LacZ-LEW rats showed superior expression in the liver (hepatocytes), skin (epidermis and hair follicles), small intestine and cartilage, compared to CAG/LacZ-DA rats. Expression patterns of lacZ and intensity are summarized in Table 2. It is notable that bone marrow cells were not stained by this histological staining procedure, but their expression was visualized by the Beta-glo™ bioluminescent system (described later).

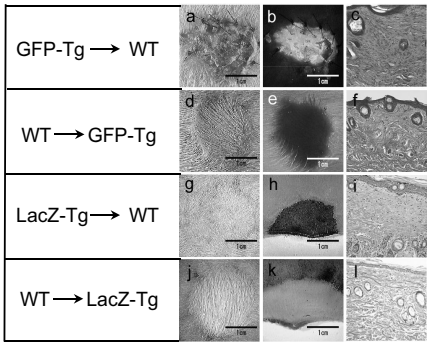

Figure 6: LacZ is less immunogenic than GFP

Table 2: *Differential expression profile between the adult CAG/LacZ and ROSA/LacZ-Tg rats*

Organ	CAG/LacZ	ROSA/LacZ
Brain	-	+
Heart	+++	+
Skeletal muscle	+++	+
Vessels	-	++
Liver	+	+++
Pancreas	++	++
Small intestine	+	+++
Kidney	+	+
Cartilage	++	+++
Nerve	-	-
Skin	++	+++
Bone*	-	-
Bone marrow	-	-

*, Born matrix was LacZ-negative, but osteocytes were LacZ-positive.

3.4.2 LacZ is less immunogenic than GFP

The transplantation of cells and tissues is a well-established strategy used in an effort to evaluate the nature of cellular processes. Transplanted cells expressing a marker protein, however, have occasionally disappeared during the observation period, even with syngeneic transplantation, and this has sometimes been explained in terms of immunogenicity [45-47]. Therefore, we evaluated how tissues from inbred Tg animals possess immunogenic potential. The skin grafting model was used to clarify the antigenic relationship between Tg and wild-type LEW rats because the skin is the most immunogenic organ and the skin grafting technique provides an easy experimental procedure to look at cellular immune responses. The skin grafts of either CAG/GFP-LEW or ROSA/LacZ-LEW rats were transplanted onto a dorsal area of parental LEW rats. The skin of CAG/GFP-LEW Tg rats resulted in graft rejection (Figure 6, a-c); this rejection reaction occurred within 6-9 days after skin transplantation, suggesting acute graft rejection (by cellular immune responses). In contrast, Rosa/LacZ-LEW skin graft remained intact for more than 90 days (Figure 6, g-i). Microscopic studies showed that CAG/GFP-LEW Tg skin grafting introduced more intense cellular infiltrate than ROSA/LacZ skin grafting (Figure 6, c and i). These cellular infiltrates represented $CD8^+$ T cells, while $CD4^+$ T cells were scarcely observed using immunostaining (data not shown). Rejection patterns after grafting are summarized in our recent paper [15]. Skin grafting of wild-type rats to both Tg animals did not show any rejection reaction more than 30 days after transplantation (Figure 6, d-f, and j-l). These results therefore demonstrate that LacZ is less immunogenic than GFP, and suggest that cells from ROSA/LacZ-LEW Tg rats may be more useful for the evaluation of cellular process through a transplantation technique than those of GFP-LEW Tg rats.

GFP is a fluorescent product of the jellyfish (*Aequorea victria*) and is used for a variety of biological experiments as a reporter molecule [4]. While GFP possesses advantages for the non-invasive imaging of viable cells, GFP-positive cells still need to be considered potential xeno-antigens [45, 47]. In fact, Gambotto et al [47] synthesized a number of candidate H2-Kd-binding peptides derived from the EGFP protein using an epitope prediction program, and identified the peptide, HYLSTQSAL (corresponding to EGFP200-208), as a naturally occurring epitope of EGFP, which strongly bound to H2-Kd molecules (in BALB/c mice). Our authentic skin grafting experiments also showed that GFP induced striking immune responses, but lacZ was less immunogenic than GFP (Figure 6). Similar immunogenecity was

observed in luciferase-transduced cells [48], though substantial epitopes against immune responses remain to be determined as their xenogeneic antigen. Thus, long-term studies of GFP^+ cell transplantation may have to be limited to immune privilege sites (e.g. brain and testis), and it is much safer to use LacZ-positive cells in other sites.

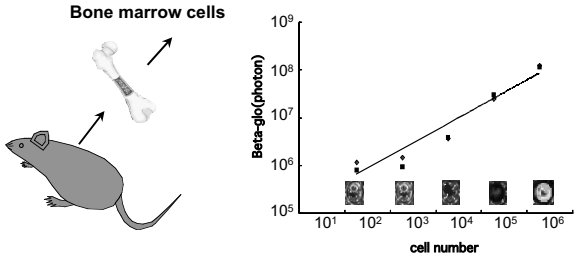

Figure 7: LacZ-expression of BMDCs is visualized in the presence of a luminescent substrate (Beta-glo™, Promega)

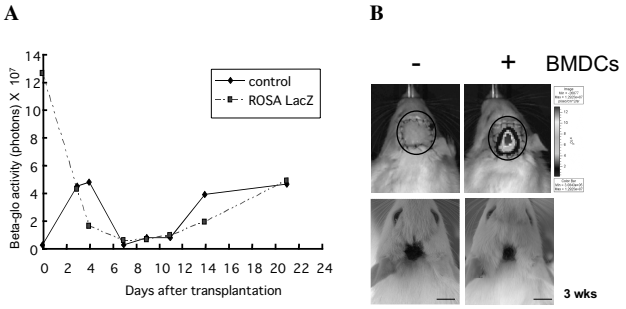

Figure 8: BMDCs can enhance skin wound healing, but their contribution may be low and perhaps transitory

3.4.3 Evaluation of the BMDCs' contribution to skin wound healing

Considering the less immunogenic lacZ and sensitive luminescence assay for the expression of lacZ, we evaluated the contribution of BMDCs to skin wound healing. LacZ-expression of BMDCs was visualized in the presence of a luminescent substrate (Beta-glo™, Promega), and at least 2,000 cells were able to give rise to successful imaging *in vitro* (5 $X10^6$ photons) (Figure 7). Full-thickness skin defects (2-cm X 2-cm) were made on the head of wild-type LEW rats, and BMDCs (10^7 cells/ 100 ml) from ROSA/LacZ-LEW rats with the artificial dermis (Terudermis®) were transplanted into the skin defects of these rats (artificial dermis plus PBS 100 ml was grafted as the experimental control) (Figure 8B, upper panels). Substantial luminescent images were only obtained for a few days (Figure 8A), but BMDCs contributed to skin wound healing, as also recently demonstrated by Yamaguchi et al [49] (Figure 8B, lower panels). The wound area reduction rate was 9.6±2.7% in the BMDC-administered group and 12.9±6.9% in the control group. BMDCs administered to a wound area shortened the healing period. Nonetheless, their cellular signals were equivalent to the background level after 4 days post-grafting, and cell fate was not monitored throughout the healing period (Figure 8A). These results therefore suggest that BMDCs can indeed enhance skin wound healing, but their contribution may be low and perhaps transitory.

We also evaluated the BMDCs' contribution to a myocardial injury (by a cryo-injury) in the BMDC (from LacZ Tg rats)-transplanted (BMT) rats. Very few numbers of $LacZ^+$ cells were detected at 28 days after the cryo-myocardial injury (less than 0.05% of myocardial cells) [14]. These $LacZ^+$ cells in myocardium were cTnI-positive (the cardiac specific marker), and we denied that possibility that these $LacZ^+$ cells were adherent macrophages or lymphocytes because other immune cell markers were negative and BMDCs and peripheral blood cells were LacZ-negative. Here is also demonstrating that the BMDCs' contribution is still low. Thus, the contribution of administered BMDCs to damaged tissues may perhaps involve an appropriate supply of certain kinds of beneficial growth factors.

4. CONCLUSION

Herein we demonstrated remarkable features in our transgenic rat system: (1) CAG/GFP-LEW Tg expressed GFP ubiquitously and strongly in all of the tissues we examined and the cellular source from GFP-LEW Tg rats provides a high-performance tool for looking at cellular fate especially in immune privilege sites (e.g. brain and testis) (the GFP-Wistar line is also useful in leukocyte-trafficking studies (shortly described by Sato A et al [50])); (2) the Alb-DsRed2 Tg-rat was able to show role of BMDCs for liver regeneration studies; (3) the DsRed2/GFP double reporter Tg rats allowed gene expression control under the Cre/LoxP site-specific recognition, providing the effective materials to elucidate the cellular fusion process; (4) ROSA/LacZ-Tg was strongly expressed in the liver, small intestine, cartilage and skin (CAG/LacZ-DA Tg line expressed much LacZ in the heart and skeletal muscle), and they represent still attractive animal tools because cells from LacZ-Tg were less immunogenic than those of GFP-Tg and the sensitive luminescent technology is now available for visualizing *in vivo* cellular fate in living animals.

Since the rat is larger than the mouse, studies using the former animals make available various physiological techniques. Coupled with recent advances in genetic engineering of the rat such as rat cloning and mutagenesis [42, 43], the transgenic rats presented in this proceeding should provide innovative animal tools and help to broaden understanding in new biomedical research fields, including regeneration medicine. The spatiotemporal information using the rat Tg system can accelerate the development of experimental therapeutic strategies. These high-demand animals (Tg rat embryos) are now available from the Health Science Research Resources Bank at hsrrb@osa.jhsf.or.jp.

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