Molecular Imaging Using Labeled Donor Tissues Reveals Patterns of Engraftment, Rejection, and Survival in Transplantation

Yu-An Cao,[1] Michael H. Bachmann,[1] Andreas Beilhack,[2] Yang Yang,[3] Masashi Tanaka,[5] Rutger-Jan Swijnenburg,[5] Robert Reeves,[1] Cariel Taylor-Edwards,[2] Stephan Schulz,[2] Timothy C. Doyle,[1] C. Garrison Fathman,[2] Robert C. Robbins,[5] Leonore A. Herzenberg,[3,4] Robert S. Negrin,[2,4] and Christopher H. Contag[1,4,6]

Tissue regeneration and transplantation of solid organs involve complex processes that can only be studied in the context of the living organism, and methods of analyzing these processes in vivo are essential for development of effective transplantation and regeneration procedures. We utilized in vivo bioluminescence imaging (BLI) to noninvasively visualize engraftment, survival, and rejection of transplanted tissues from a transgenic donor mouse that constitutively expresses luciferase. Dynamic early events of hematopoietic reconstitution were accessible and engraftment from as few as 200 transplanted whole bone marrow (BM) cells resulted in bioluminescent foci in lethally irradiated, syngeneic recipients. The transplantation of autologous pancreatic Langerhans islets and of allogeneic heart revealed the tempo of transplant degeneration or immune rejection over time. This imaging approach is sensitive and reproducible, permits study of the dynamic range of the entire process of transplantation, and will greatly enhance studies across various disciplines involving transplantation.

Keywords: Molecular imaging, Transplantation, Tissue regeneration, Stem cells.

(*Transplantation* 2005;80: 134–139)

Advances in immunology and stem cell biology have given impetus to systematic investigation aimed at the restoration of functional organs by cell and tissue transplantation. Imaging strategies that noninvasively reveal cell viability and functional information in the context of the living body can accelerate effective development of new methods that would prolong survival of grafts and enhance regeneration of tissues. One of the cornerstone technologies in the field of molecular imaging, in vivo BLI, is based on the observations that light passes through mammalian tissues and that the expression of luciferase is detectable as an internal biological source of light in the living body (*1–3*). A variety of gene transfer tools have been developed to deliver the luciferase reporters to cells (*4–7*) and employed with BLI to elucidate the spatiotemporal trafficking patterns of lymphocytes within the body (*4*, *5*), to follow hematopoietic engraftment by transplanted luciferase-expressing stem cells over time (*6*), and to examine stem cell engraftment in cardiac tissue (*7*).

To assess survival and regeneration using BLI, it is essential that the transplant constitutively and uniformly expresses luciferase throughout the study period. To date, gene transfer efficiency remains as an obstacle, especially for primary cells, tissues and intact organs, and stable reporter expression in particular cell types over time can be variable. Thus, current methods of introducing reporter genes are not satisfactory for transplantation studies. In contrast, cells from a transgenic mouse with a stably integrated reporter gene cassette controlled by a promoter that is largely constitutively expressed meet these criteria and would provide a source of uniformly labeled biological materials for transplantation studies. Here, we report the generation of a "universal donor" transgenic mouse line that incorporates a luciferase reporter driven by a constitutive promoter, and demonstrate in a series of transplantation experiments the ease and success of monitoring transplanted cells, tissue and organs in unlabeled recipient mice.

The transgene in this line is comprised of a CMV-β-actin promoter (*8*) and a biscistronic gene consisting of two reporter genes, firefly luciferase and enhanced green fluorescence protein (eGFP), separated by a 2A ribosome slippage site from foot and mouth disease virus (FMDV), to allow equimolar expression of two open reading frames in cultured cells (*9*) (Fig. 1A). Comparison of homozygous and heterozygous mice in this FVB.luc+ line, designated as L2G85, indicated that both the bioluminescent and fluorescent signals in the heterozygote were lower than those in homozygote (Fig. 1B and C). While GFP is expressed mainly in skin (Fig. 1D),

This work was funded in part through a Small Animal Imaging Resource Program (SAIRP) grant from the National Cancer Institute (NCI, grant number R24 CA 92862), and other grants from the National Institutes of Health (RO1 DK58664, P20 CA86312–01, RO1 CA80006–01, PO1 CA49605–14) and the Juvenile Diabetes Foundation (4–2001–9).

C.H.C. is scientific founder and consultant for Xenogen Corporation, Alameda, CA. Other authors have no competing interests.

[1] Departments of Pediatrics, Radiology, and Microbiology & Immunology, Stanford School of Medicine, Stanford, California.

[2] Department of Medicine, Stanford School of Medicine, Stanford, California.

[3] Department of Genetics, Stanford School of Medicine, Stanford, California.

[4] Program in Immunology, Stanford School of Medicine, Stanford, California.

[5] Department of Cardiothoracic Surgery, Stanford School of Medicine, Stanford, California.

[6] Address correspondence to: Dr. Christopher H. Contag, E-150B Clark Center, 318 Campus Drive, Stanford, CA 94305–5427.

E-mail: ccontag@cmgm.stanford.edu.

Received 31 August 2005. Revision requested 28 October 2004. Accepted 28 January 2005.

ISSN 0041-1337/05/8001-134

DOI: 10.1097/01.TP.0000164347.50559.A3

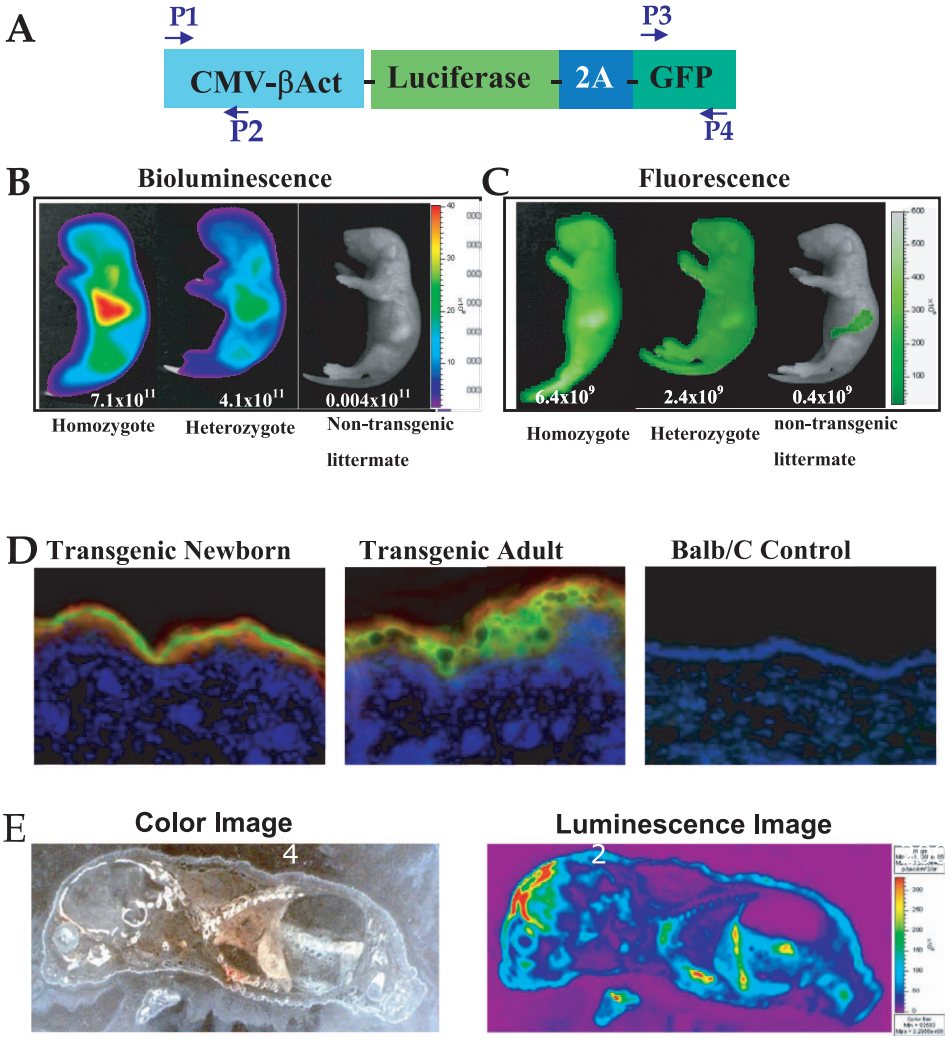

FIGURE 1. Generation of luciferase-expressing transgenic mouse. (**A**) Transgene composition and PCR primer location. The transgene is comprised of a hybrid CMV-chicken-β-actin promoter, a firefly luciferase gene, a FMDV 2A ribosomal slippage site and GFP gene. The luciferase, 2A and GFP are in one open reading frame. Primers P1 & P2 are for amplifying the first 750 bp of CMV-β-actin promoter and P3 & P4 are for GFP. Positive results from both PCR suggest the presence of an intact transgene. (**B**), (**C**) In vivo BLIB and fluorescence imaging (**C**) of newborn luciferase-expressing transgenic mice, line L2G85. (**D**) Microscopic localization of GFP expression. Fluorescence microscopic evaluation of skin samples from newborn (3 days) and adult (8 weeks) L2G85 mice showed a green fluorescent signal in the upper epidermal layers, that is, stratum granulosum and stratum corneum. This signal could be confirmed by immunofluorescence microscopy, using an antiGFP antibody, detected by a conjugated secondary antibody (Alexa 546, *red*). No specific green fluorescent signal and no anti GFP reaction was observed on corresponding skin samples of adult Balb/c control mice. Nuclei were stained with DAPI (*blue*). (**E**) A representative 30 μm cryosection of a 3-day-old L2G85 Tg mouse is shown. Panel A: color image; Panel B: luminescence image after application of a luciferin/ATP solution. Light emission was recorded as previously described.

BLI of dissected tissues showed that luciferase is expressed in heart, spleen, muscle, pancreas, skin, thymus, and BM (Fig. 1E and data not shown).

To enable the assessment of multilineage hematopoietic reconstitution originating from a small number of BM cells, hematopoietic lineages need to express a detectable level of luciferase to permit monitoring by BLI. We therefore quantified the bioluminescence of purified hematopoietic subsets. Splenocytes were sorted by flow cytometry and then analyzed for luciferase expression in live cell assays and as cell lysates in a standard luciferase assay. Although they usually produced light less intensively immediately after sorting, sorted cells recovered after incubation at 37°C for 4–12 hr and exhibited varying levels of luciferase activity in all leukocyte subsets tested, including $CD4^+$ and $CD8^+$ T cells, $B220^+$ B cells, $NK1.1^+$ NK cells, and $Gr\text{-}1^+Mac\text{-}1^+$ granulocytes (Fig. 2A). Light production of different subsets ranged from 0.02 to 0.14 photons/second/cell in the live cell assay after 12 hr in the incubator and 0.15 to 1.30 photons/second/cell in the lysate assay (Fig. 2A). Luciferase activity is not detected at significant levels in mature erythrocytes ($Ter119^+CD45^-$), but low levels of activity are detected in erythrocyte precur-

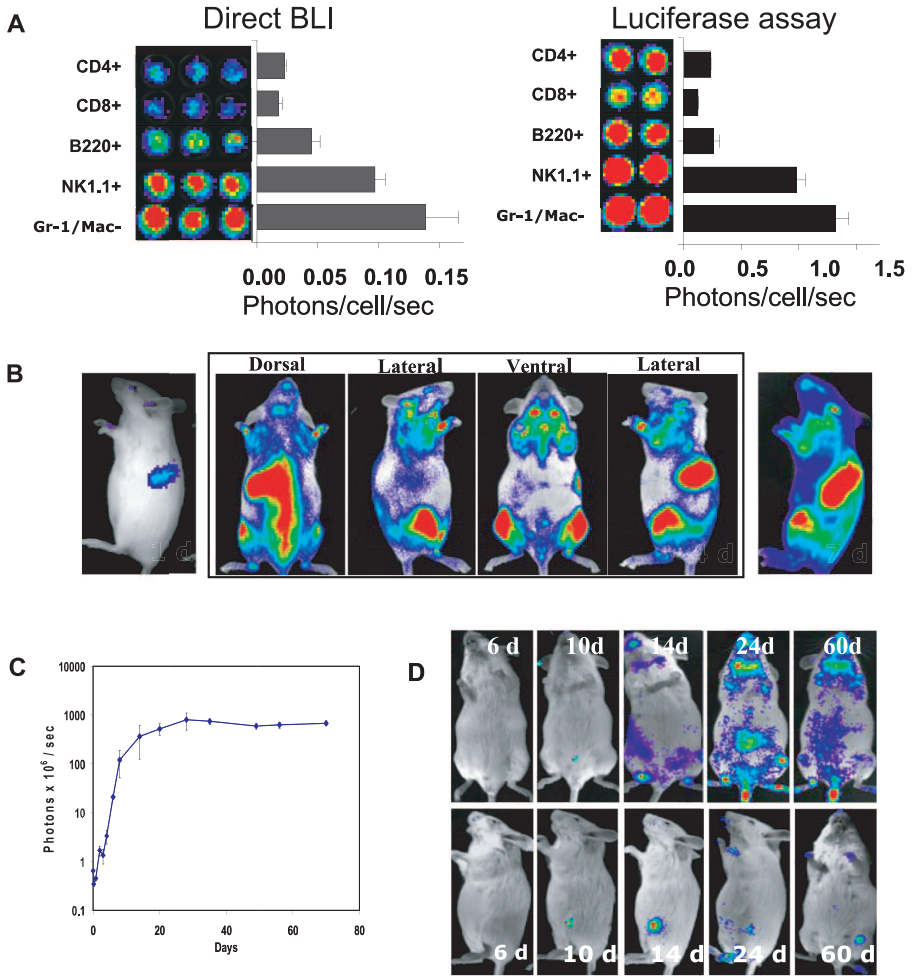

FIGURE 2. Dynamics of hematopoietic reconstitution. **(A)** Hematopoietic Lineage analyses and light output. 2×10^5 FACS sorted cells from L2G85 were either imaged directly (left panel) or luciferase assay was performed (right panel), and signals in multiple cell lineages were detected. **(B), (C)** Dynamics of hematopoietic reconstitution after BM transplantation. 5×10^6 whole BM cells from L2G85 were transferred via tail vein into lethally irradiated syngeneic recipients and bioluminescent imaging was performed at different time points (day 0 through 70). (**B**) representive images during hematopoietic engraftment. (**C**) Kinetics of hematopoietic reconstitution. **(D)** Small numbers of transgenic BM cells can be detected as individual foci of engraftment. Two hundred whole BM cells were transferred into lethally irradiated recipients along with 3×10^5 helper non-transgenic BM cells. Bioluminescent foci were detectable 10 day after transplantation. Some of the foci expanded and engrafted hematopoietic compartment at a detectable level (upper panel) and others contributed little to hematopoietic reconstitution (lower panel).

sors. Isolated c-Kit$^+$Thy-1.1^{lo}Lineage$^-$Sca-1$^+$ (KTLS) hematopoietic stem cells (HSCs) express luciferase at the highest level among the different hematopoietic cell types, with 3 photons/second/cell in live cell assays (*10*). The differences in light emission between the live cell and the lysate assays of the same number of isolated cells may be due to insufficient ATP levels in the sorted live cells since there are excess amounts of ATP and a CoA enzyme in the lysate assay. The consistent pattern of activity between the two assays indicates that the live cell assay reflects relative expression levels in each cell type.

To test if we could visualize the dynamics of hematopoietic engraftment from whole BM cells of this transgenic line and how the kinetics of reconstitution from whole BM differ from that of highly purified KTLS HSCs (*10*), 5 million whole BM cells were transplanted into lethally irradiated syngeneic recipients. Bioluminescent signal was detected from the splenic area 15 min after cell transfer (data not shown). Images obtained at 1 day post-transplant indicated signals primarily from the spleen (Fig. 2B). Three days later, four views of the animals revealed intense signals from the bones, most likely from the marrow, and secondary lymphoid organs, spleen and lymph nodes. Hematopoietic reconstitution, measured by integrated whole body photon emission, rapidly progressed (Fig. 2B). Engraftment from luc$^+$ BM peaked at 5 weeks

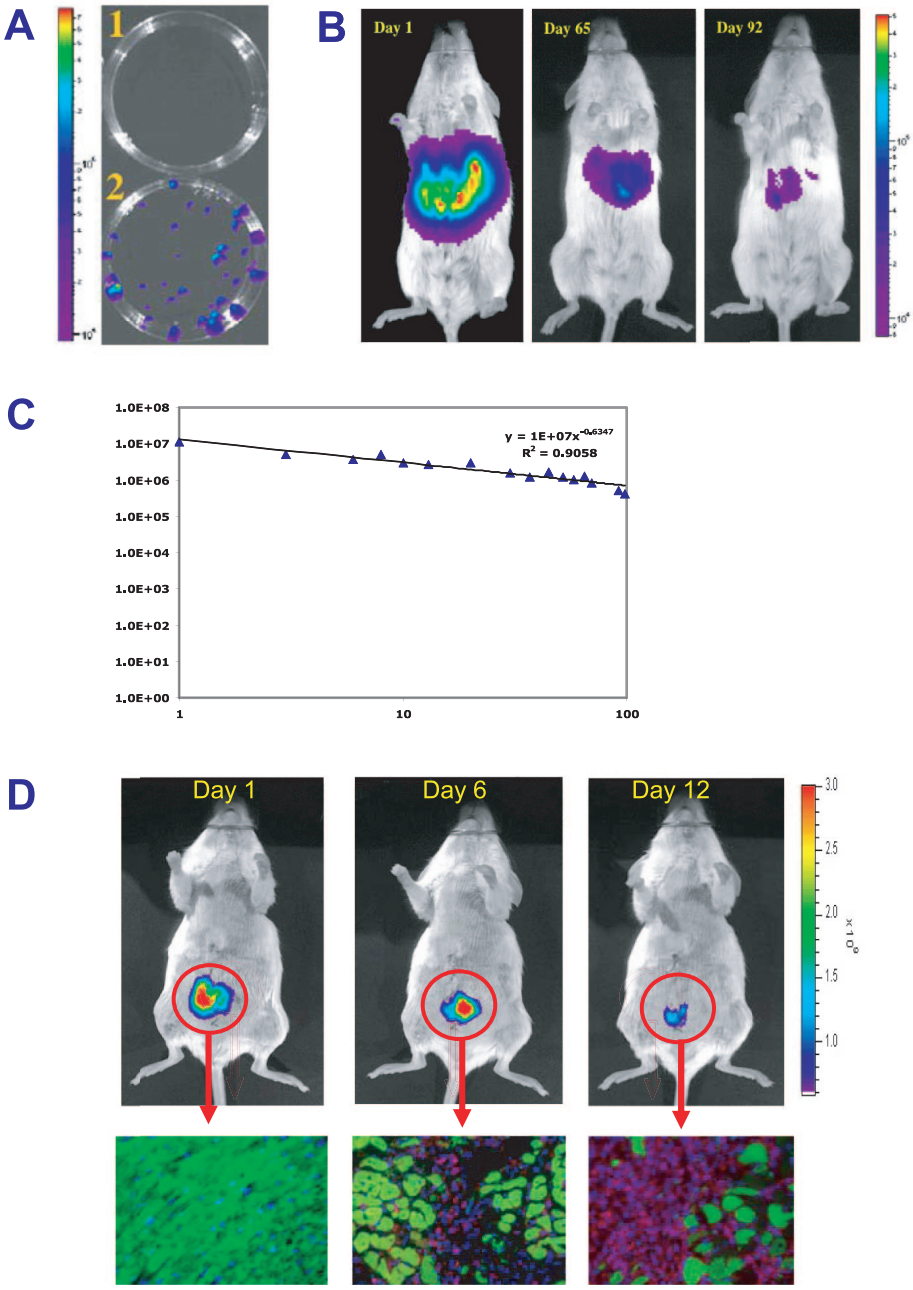

FIGURE 3. Pancreatic islet and cardiac transplantations. **(A–C)** Transplanted bioluminescent pancreatic islets expire at an exponential rate. (**A**) BLI of isolated pancreatic islets from two transgenic mouse lines: 1. CMV-GFP-yLuc, 2. L2G85. Light emission [photons/sec/cm^2] is represented according to the adjacent scale in a false color image overlay. **(B)** BLI of a FVB non-transgenic mouse that has received 500 pancreatic islets isolated from a L2G85 mouse via injection into the portal vein. **(C)** Logarithmic plot of time [log (days)] vs. light emission [log (photons/sec)] obtained of the FVB mouse shown in **(B)** by BLI of the abdominal region of interest. The formula best fitting to the data is a power law equation, describing a linear curve in a log-log plot, with an R^2 value of 0.906. **(D)** Transplanted bioluminescent cardiac allograft showed a decrease in light production over time by 12 days after transplantation. L2G85 mice were bred with a GFP transgenic line (GFPU). Cardiac allografts from the offsprings of this breeding (FVB) were transplanted into Balb/c recipients and all acutely rejected by 12 days. Correlating with decrease in light production, immunohistochemistry shows a decline in viable cardiomyocyte structure and an increase in inflammatory cells. In the lower panel of histology pictures, *red*, inflammatory cells (CD45 stain); *green*, cardiomyocyte (GFP); *blue*, nuclei (DAPI stain) (magnification=400×).

after transplantation, and remained high thereafter (Fig. 2C). To confirm the presence of significant numbers of donor-derived hematopoietic cells in the recipients, we sorted myeloid and lymphoid cells from peripheral blood. In vitro luciferase assays of these sorted cells showed that there were significant numbers of donor-derived peripheral blood cells and that hematopoietic reconstitution was multilineage (data not shown). We did not observe transplant rejection in these syngeneic models, as based on the reporter activity.

To assess whether engraftment of small numbers of cells in BM could be detected, we transferred 200 whole BM cells into lethally irradiated syngeneic recipients along with 3×10^5 nontransgenic helper BM cells. Bioluminescent foci were detected at 12 to 17 days posttransplant most frequently at anatomic sites corresponding to the location of the spleen, skull, vertebrae and femurs (Fig. 2D and data not shown). Confirmation of tissue origin was obtained by imaging from different views and in some instances, immediately postmortem. Surprisingly, we detected bioluminescent foci arising from engrafting cells in 12% of recipients who had received 200 BM cells each. This suggests that the foci observed may be derived from both HSCs and hematopoietic progenitors since the HSC frequency in BM is usually about 0.01% (*11*).

Transplantation of pancreatic islets into patients suffering from type 1 diabetes mellitus has become increasingly applied but ascertaining the presence and quantity of beta cells has proven difficult (*12*). BLI has been used to track lentiviral vector transduced human or mouse islets after transplantation (*13–15*). Since a transgenic donor with uniform integration sites could potentially offer greater signal stability, we purified Langerhans islets from L2G85 pancreas (*16*) for transplantation. These islets emitted significant amounts of bioluminescence (Fig. 3A). When five hundred of these islets were transferred into a non-transgenic syngeneic recipient via portal vein injection, high levels of bioluminescence over the abdominal region of the liver were detected within 24 hr (Fig. 3B). Monitoring abdominal bioluminescence of the recipient for more than 90 days revealed a decline over time that was best described by a power law equation (Fig. 3C). Islet transplantation under the kidney capsule of syngeneic mice produced comparable results whereas allogeneic recipients rejected the grafts within 3 weeks (data not shown). The decline of bioluminescent signal over time in the syngeneic recipient could be due to islet primary nonfunction occuring with high frequency in islet transplant models (*17*) or to a gradual loss of luciferase gene expression in the transplanted islets since the bioluminescent signal remains stable in NOD-scid mice (*14*). Further research needs to analyze the reason(s) for the decline. Nevertheless, BLI offers the possibility, in conjunction with conventional assays, to rapidly evaluate the efficacy of therapeutic strategies to improve islet transplantation.

Hearts from the L2G85 mice also exhibit bioluminescence that can serve as a marker for survival and rejection of transplants. When transplanted in an acute rejection model (*18*) into the abdominal cavity (*19*) of Balb/c mice, i.e. across a major MHC mismatch barrier, all L2G85 cardiac allografts were acutely rejected by 12 days. Light production from the cardiac allografts correlated with their viability, as it gradually declined over time after transplantation (Fig. 3D). L2G85 mice were bred with a GFP transgenic line (FVB.Cg-Tg(GFPU)5Nagy) (*20*) to generate luciferase-GFP double transgenic mice L2G85XGFPU. Hearts of these double transgenic mice were rejected after transplantation with kinetics identical to those of L2G85 transplants. The course of rejection was corroborated by immunohistochemical analysis of transplanted hearts of L2G85XGFPU demonstrating an increase of inflammatory cell infiltration and a decrease in the number of viable cardiomyocytes with time during the acute rejection phase (Fig. 3D).

Future applications of BLI with luciferase transgenic mice are distributed widely across biomedical fields. While the studies presented here demonstrate the use of this luciferase transgenic mice as a highly useful donor for tracking transplanted cells, tissues and organs, realization of the full potential of this system may greatly benefit the study of the physiological behavior and function of transplanted cells, tissue and organs, and the evaluation of new transplantation protocols in an rapid and reliable fashion.

ACKNOWLEDGMENTS

We thank Peggy Kemper for excellent technical assistance.

REFERENCES

1. Contag PR, Olomu IN, Stevenson DK, et al. Bioluminescent indicators in living mammals. *Nature Medicine* 1998; 4: 245.
2. Contag CH, Contag PR, Mullins JI, et al. Photonic detection of bacterial pathogens in living hosts. *Molecular Microbiology* 1995; 18: 593.
3. Contag CH, Spilman SD, Contag PR, et al. Visualizing gene expression in living mammals using a bioluminescent reporter. *Photochemistry and Photobiology* 1997; 66: 523.
4. Edinger M, Cao YA, Verneris MR, et al. Revealing lymphoma growth and the efficacy of immune cell therapies using in vivo bioluminescence imaging. *Blood* 2003; 101: 640.
5. Nakajima A, Seroogy CM, Sandora MR, et al. Antigen-specific T cell-mediated gene therapy in collagen-induced arthritis. *Journal of Clinical Investigation* 2001; 107: 1293.
6. Wang X, Rosol M, Ge S, et al. Dynamic tracking of human hematopoietic stem cell engraftment using in vivo bioluminescence imaging. *Blood* 2003; 102: 3478.
7. Wu JC, Chen IY, Sundaresan G, et al. Molecular imaging of cardiac cell transplantation in living animals using optical bioluminescence and positron emission tomography. *Circulation* 2003; 108: 1302.
8. Niwa H, Yamamura K, Miyazaki J. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 1991; 108: 193.
9. Donnelly ML, Luke G, Mehrotra A, et al. Analysis of the aphthovirus 2A/2B polyprotein 'cleavage' mechanism indicates not a proteolytic reaction, but a novel translational effect: a putative ribosomal 'skip'. *J Gen Virol* 2001; 82: 1013.
10. Cao YA, Wagers AJ, Beilhack A, et al. Shifting foci of hematopoiesis during reconstitution from single stem cells. *Proc Natl Acad Sci U S A* 2004; 101: 221.
11. Morrison SJ, Qian D, Jerabek L, et al. A genetic determinant that specifically regulates the frequency of hematopoietic stem cells. *J Immunol* 2002; 168: 635.
12. Sweet IR, Cook DL, Lernmark A, et al. Systematic screening of potential beta-cell imaging agents. *Biochem Biophys Res Commun* 2004; 314: 976.
13. Lu Y, Dang H, Middleton B, et al. Bioluminescent monitoring of islet graft survival after transplantation. *Mol Ther* 2004; 9: 428.
14. Chen J, Kaufman DB. Bioluminescent Imaging of Pancreatic Islet Transplants. *Current Medicinal Chemistry – Immunology, Endocrine and Metabolic Agents.* 2004; 4: 301.

15. Powers AC, Jansen ED. Current Medicinal Chemistry – Immunology. *Endocrine and Metabolic Agents* 2004; 4: 339.
16. Lejon K, Fathman CG. Isolation of self antigen-reactive cells from inflamed islets of nonobese diabetic mice using CD4high expression as a marker. *J Immunol* 1999; 163: 5708.
17. Ricordi C, Strom TB. Clinical islet transplantation: advances and immunological challenges. *Nat Rev Immunol* 2004; 4: 259.
18. Miura M, Morita K, Kobayashi H, et al. Monokine induced by IFN-gamma is a dominant factor directing T cells into murine cardiac allografts during acute rejection. *J Immunol* 2001; 167: 3494.
19. Corry RJ, Winn HJ, Russell PS. Primarily vascularized allografts of hearts in mice. The role of H-2D, H-2K, and non-H-2 antigens in rejection. *Transplantation* 1973; 16: 343.
20. Hadjantonakis AK, Gertsenstein M, Ikawa M, et al. Generating green fluorescent mice by germline transmission of green fluorescent ES cells. *Mech Dev* 1998; 76: 79.