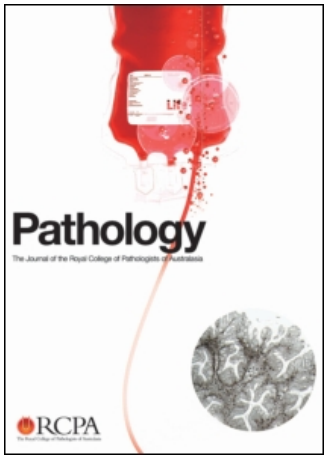

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Sandra A. Biankin [a]; Michael I. Collector [b]; Andrew V. Biankin [a]; Lindsey J. Brown [a]; Wolfram Kleeberger [b]; Wendy L. Devereux [b]; Cynthia A. Zahnow [b]; Stephen B. Baylin [b]; D. Neil Watkins [b]; Saul J. Sharkis [b]; Steven D. Leach [ab]
[a] Surgery,
[b] Oncology, Baltimore

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EXPERIMENTAL PATHOLOGY

A histological survey of green fluorescent protein expression in 'green' mice: implications for stem cell research

SANDRA A. BIANKIN*, MICHAEL I. COLLECTOR†, ANDREW V. BIANKIN*, LINDSEY J. BROWN*, WOLFRAM KLEEBERGER†, WENDY L. DEVEREUX†, CYNTHIA A. ZAHNOW†, STEPHEN B. BAYLIN†, D. NEIL WATKINS†, SAUL J. SHARKIS† AND STEVEN D. LEACH*†

*From the Epithelial Stem Cell Working Group, Departments of *Surgery and †Oncology, Sidney Kimmel Cancer Center, The Johns Hopkins University School of Medicine, Baltimore, USA*

Summary

Aims: The transgenic enhanced green fluorescent protein (EGFP) expressing 'green' mouse (C57BL/6-TgN(ACTbEGFP)1Osb) is a widely used tool in stem cell research, where the ubiquitous nature of EGFP expression is critical to track the fate of single or small groups of transplanted haematopoietic stem cells (HSC). Our aim was to investigate this assumed ubiquitous expression by performing a detailed histological survey of EGFP expression in these mice.

Methods: Fluorescent microscopy of frozen tissue sections was used to perform a detailed histological survey of the pattern of EGFP expression in these mice. Flow cytometry was also used to determine the expression pattern in blood and bone marrow.

Results: Three patterns of EGFP expression were noted. In most tissues there was an apparently stochastic variegation of the transgene, with individual cell types demonstrating highly variable rates of EGFP expression. Certain specific cell types such as pancreatic ductal epithelium, cerebral cortical neurones and glial cells and glomerular mesangial cells consistently lacked EGFP expression, while others, including pancreatic islet cells, expressed EGFP only at extremely low levels, barely distinguishable from background. Lastly, in the colon and stomach the pattern of EGFP expression was suggestive of clonal inactivation. Only cardiac and skeletal muscle showed near ubiquitous expression.

Conclusions: These findings raise questions regarding the 'ubiquitous' expression of EGFP in these transgenic mice and suggest caution in relying overly on EGFP alone as an infallible marker of donor cell origin.

Key words: GFP Mice, GFP expression, stem cell transplantation.

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INTRODUCTION

The transgenic β-actin-enhanced green fluorescent protein mouse, C57BL/6-TgN(ACTbEGFP)1Osb, (hereafter referred to as the β-actin-EGFP mouse) was developed by Okabe and colleagues[1] and is widely utilised as a source of labelled cells for use in cell mixing and transplantation studies.[2] It has been more recently used in the arena of stem cell research,[3–7] where the ubiquitous nature of EGFP expression is critical to track the fate of single or small groups of transplanted haematopoietic stem cells (HSC) in wild type recipients.

Green fluorescent protein (GFP) is an autofluorescent protein isolated from the bioluminescent jellyfish *Aequorea victoria*. More stable mutant variants of this GFP were created, of which the most widely used is enhanced GFP (EGFP). EGFP exposed to blue light has an emission spectrum of approximately 490 nm, which is well suited to standard detection systems used in fluorescence activated cell sorter machines (FACS) and confocal microscopes.[2] In the β-actin-EGFP mouse, EGFP is driven by a pCAGGS promoter, consisting of the chicken β-actin promoter coupled with a cytomegalovirus (CMV) enhancer, β-actin intron and β-globin polyadenylation signal.[2]

These β-actin-EGFP 'green' mice were reported to demonstrate uniform tissue fluorescence under excitation light, with the exception of hair and red blood cells.[1,8] However, while using control tissues from the β-actin-EGFP mouse in our own stem cell transplantation studies, we noted there was far from ubiquitous expression of EGFP in most tissues. EGFP expression varied considerably from mouse to mouse, and also between microscopic fields within the same organ. We were prompted by these initial findings to complete a detailed histological survey to quantify the expression of EGFP in these mice, with the results suggesting the need for considerable caution in assuming that all cells derived from β-actin-EGFP mice are reliably identified by detectable EGFP fluorescence.

MATERIALS AND METHODS

Animals

C57BL/6-TgN (ACTbEGFP)1Osb mice (β-actin-EGFP mice) were obtained from Jackson Laboratories (USA), and bred to syngeneic non-transgenic littermates. For the tissue survey described here, five male and five female mice were used, ranging in age from 2 to 10 months of age. Mammary tissue only from an additional five female virgin mice was also obtained. Bone marrow and peripheral blood was collected from one additional wild type C57/B6 and four 8-week-old β-actin-EGFP male mice.

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All animal study protocols were approved by The Johns Hopkins Medical Institutions Animal Care and Use Committee.

Tissue preparation

Mice were sacrificed and portions of the brain, lungs, mammary glands, heart, liver, pancreas, kidneys, stomach, small intestine and colon were fixed overnight in 4% paraformaldehyde/PBS at 4°C. The tissues were then transferred to a solution of 30% sucrose for 48 h, and subsequently embedded and quick frozen in OCT compound (Sakura Finetek, USA).

Flow cytometry preparation

One normal C57/B6 and four 8-week-old β-actin-EGFP male mice were used for analysis. The mice were anesthetised with Avertin (Aldrich Chemical Company, USA) and bled from the retro-orbital venous plexus. The animals were sacrificed and bone marrow harvested by flushing the femurs and tibias with Minimum Essential Medium, alpha modification (α-MEM; Gibco, Invitrogen Corporation, USA) using a 25 gauge needle.

Histology and immunofluorescence analysis

Cryosections of 5 µm were cut (CM 1850 Cryostat; Leica, Germany) and mounted on Superfrost slides (Fisher Scientific, USA). The sections were rehydrated in phosphate buffered saline (PBS), coverslipped and counter-stained with Vectashield HardSet Mounting Medium with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, USA), and immediately examined using an inverted motorised fluorescent microscope outfitted with DAPI, fluorescein isothiocyanate (FITC), tetramethyl rhodamine isothiocyanate (TRITC) and Texas Red (TR) filters (Axiovert 200M; Carl Zeiss, Germany). Multicolor images were captured using a Zeiss Axiocam and pseudocolors assigned using Zeiss Axiovision software. Excitation wavelengths were 359 nm for DAPI, 494 nm for FITC, 555 nm for TRITC and 595 nm for TR. Fluorescent emission was collected at 461, 525, 576 and 620 nm respectively.

Specific confirmatory immunofluorescent double labelling was performed on pancreatic sections, with the following antibodies used at the indicated dilutions: goat polyclonal anti-amylase (1:1000; Santa Cruz Biotechnology, USA); guinea pig polyclonal anti-insulin (1:500; LINCO Research, USA); rabbit polyclonal antiglucagon (1:500; LINCO Research); rhodamine labelled Dolichos Biflorus Agglutinin (DBA; 1:200; Vector Laboratories); and rhodamine labelled Peanut Agglutinin (PNA; 1:200; Vector Laboratories). The following reagents for detection were purchased from Jackson ImmunoResearch Laboratories: Cy3-conjugated anti-goat, Cy3-conjugated anti-rabbit, Cy3-conjugated anti-guinea pig (all 1:500 dilution).

Assessment of EGFP expression

The overall expression pattern of EGFP in a particular tissue was first assessed at low magnification (×25). Subsequently, 50 representative high power fields (×400) were selected and detailed counts of the different cell types were performed by a single pathologist (SAB). For any given cell type, a minimum of 1000 cells over 10 different sections were assessed in each mouse. Cells were scored positive for EGFP expression based on a level of fluorescence visually distinguishable from background. A mean fraction of EGFP-positive cells was then calculated for each tissue, and where appropriate for individual cell types.

Flow cytometry of peripheral blood and bone marrow

Blood and bone marrow were depleted of red blood cells by 30 min 1000 × g centrifugation over a Ficol cushion (Sigma Diagnostics, USA) at specific gravity of 1.091. The interface, containing mononuclear cells, was collected and washed twice in α-MEM. The cells were analysed on a Caliber FACS machine (Becton Dickinson, USA) with excitation at 520 nm for GFP expression. Normal mouse blood and bone marrow were used to set the negative control gate. Positive cells were those cells with a fluorescent intensity exceeding the 99th percentile of the negative control cells. Dead cells, debris and non-nucleated red blood cells were gated out in the analysis.

RESULTS

Patterns of EGFP expression in tissue sections

Three main patterns of EGFP expression were noted. Firstly, the majority of the tissues examined (liver, lung, pancreatic acinar tissue, small intestine and breast ducts) showed an apparently stochastic pattern of EGFP expression. EGFP showed a highly variable expression pattern in these tissues. For example, the frequency of EGFP expression in hepatocytes in individual mice ranged from 2.5 to 62%, with a mean of 40%. However, the arrangement of EGFP positive hepatocytes varied from scattered single cells, to a more mosaic appearance with larger patches composed of EGFP expression hepatocytes, adjacent to similarly large patches lacking EGFP expression (Fig. 1A). In the pancreas, individual acinar cells showed an alternating pattern of single EGFP positive and negative cells in some areas, with whole lobules of contiguously EGFP positive or negative cells also noted in the same pancreas (Fig. 1B).

Secondly, certain specific cell types and structures consistently lacked EGFP expression. Pancreatic ductal epithelium, confirmed by positive immunohistochemical staining for DBA, was consistently EGFP negative. While the luminal contents of some larger pancreatic ducts showed faint fluorescence, the cytoplasmic and nuclear compartments of all examined ductal epithelial cells were EGFP negative. Pancreatic islets were either EGFP negative (Fig. 1C) or showed only very faint expression, barely distinguishable from background levels. Similarly, glomerular mesangial cells (Fig. 1D) and cerebral cortical neurons and glial cells did not express EGFP.

A third pattern was noted in other tissues, where the arrangement of positive cells occurred within presumed clonal units; for example, adjacent columns of EGFP-positive and EGFP-negative cells were frequently observed in colonic crypts (Fig. 1E) and gastric glands (Fig. 1F).

Only cardiac (Fig. 1G) and skeletal muscle (Fig. 1H) showed near ubiquitous expression of EGFP.

There was no concordance in levels of EGFP expression between organs and tissues in individual mice; for example, a mouse that expressed EGFP in only 2.5% of hepatocytes, expressed EGFP in over 98% of pancreatic acinar cells. The results for all tissue are summarised in Table 1.

EGFP expression in peripheral blood and bone marrow

FACS-based examination of mononuclear bone marrow cells from β-actin-EGFP mice revealed a mean of 70% EGFP positive cells (range 65–76%), compared to 45.5% in peripheral blood (range 38–45%). The peripheral blood showed a marked bimodal distribution of EGFP expression levels, with cells mostly divided into either 'high' or 'low' EGFP expression. A bimodal pattern of EGFP expression was also noted in the bone marrow, although it was not as marked as that seen in the peripheral blood (Fig. 2A,B).

DISCUSSION

The original reports of the β-actin-EGFP transgenic mouse line suggested uniform tissue fluorescence under excitation light, with the exception of hair and red blood cells.[1,8] These descriptions of the EGFP expression pattern were based on examination of whole organs under excitation

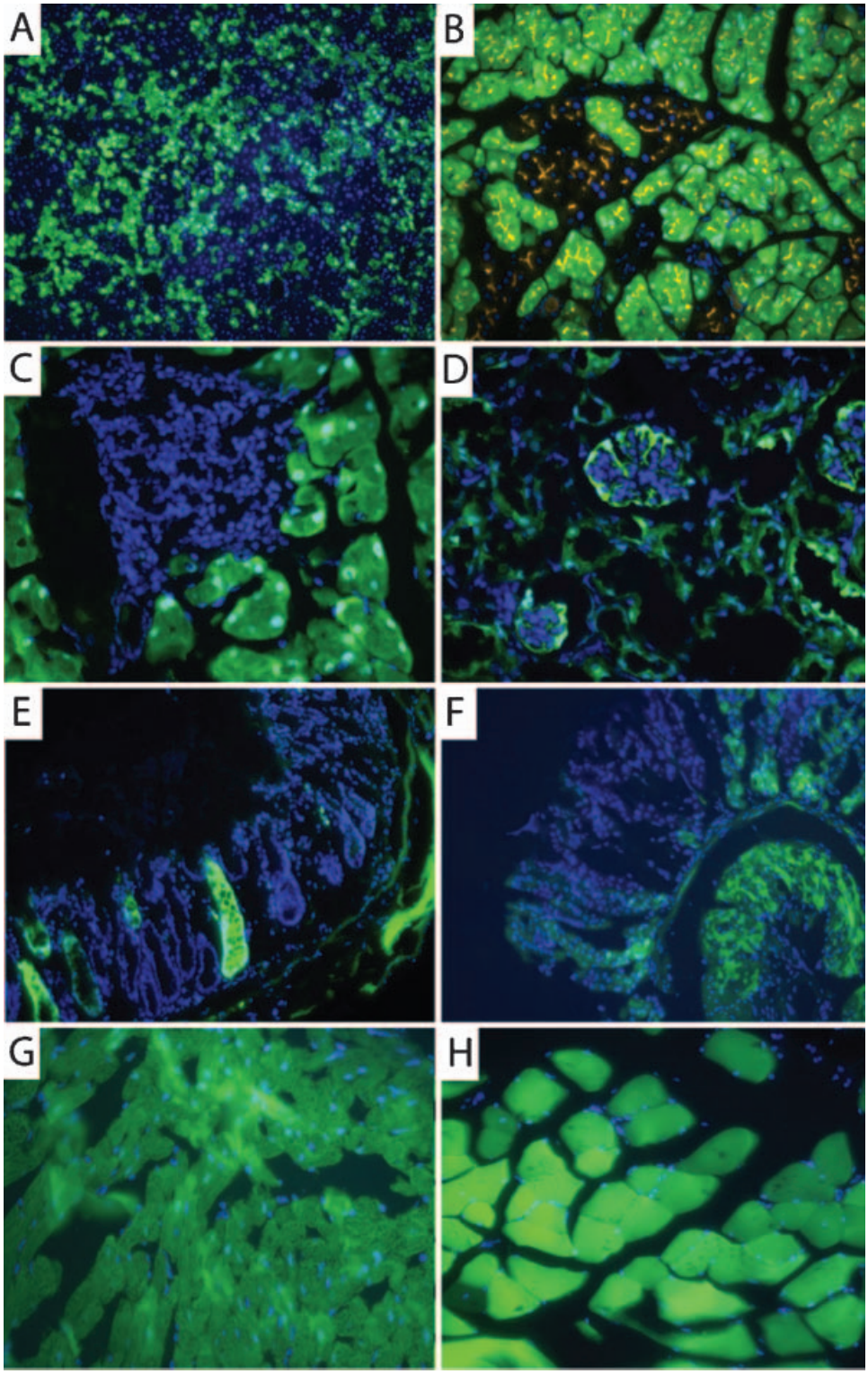

Fig. 1 Survey of EGFP expression in β-actin-EGFP mouse tissues (DAPI counterstained). (A) Liver showing mosaic pattern of EGFP expression (×100). (B) Pancreatic acinar cells, with apical membranes labelled with PNA (orange), show a variegated pattern of EGFP expression (×200). (C) Pancreatic islet showing no detectable EGFP expression, with surrounding acinar cells strongly positive for EGFP (×200). (D) Kidney showing glomeruli with EGFP positive podocytes and non-expressing mesangium with surrounding renal tubules showing variable EGFP expression (×200). (E) In the colon the muscularis propria shows EGFP expression, but only scattered colonic crypts show EGFP positivity (×100). (F) In the stomach the muscularis propria shows strong expression of EGFP, while the gastric glands show only patchy EGFP expression (×100). (G) Cardiac muscle shows strong uniform expression of EGFP in cardiomyocytes (×100). (H) Skeletal muscle with myocytes showing strong, uniform expression of EGFP (×200).

light at low magnification. FACS analysis was also performed for EGFP expression in bone marrow, spleen and testis. Since this initial description we have been unable to locate any more detailed surveys of these mice, and the 'ubiquitous' nature of their EGFP expression has been widely accepted in the literature.[5]

TABLE 1 Quantification of EGFP expression in selected β-actin-EGFP mouse tissues

Organ (no. of mice surveyed)	Mean percentage of cells positive for EGFP (range of cells positive for EGFP)
Pancreas (n=10)	Acinar cells: 79% (65.4–100%) Islet cells: 70% (0–100% faint expression only) Pancreatic ductal epithelium: 0%
Liver (n=7)	Hepatocytes: 40.2% (2–62%) Biliary ductal epithelium: 75.5% (62–90%)
Mammary gland (n=5)	Ductal epithelial cells: 53.1% (29.7–66%)
Heart (n=7)	Cardiac myocytes: 99.7% (98–100%)
Skeletal muscle (n=6)	Myocytes: 100%
Lung (n=7)	Bronchial epithelium: 62% (45–80%) Alveolar epithelium: 26.1% (5–50%)
Kidney (n=7)	Podocytes: 100% Mesangium: 0% Proximal tubules: 74.3% (55–90%) Distal tubules/Collecting ducts: 35% (35–65%)
Stomach (n=7)	Gastric glands containing positive epithelial cells: 21.8% (5–44%)
Small intestine (n=7)	Intestinal villi containing positive epithelial cells: 7.6% (0.5–35%)
Large intestine (n=6)	Colonic crypts containing positive epithelial cells: 10.3% (2–35.4%)
Cerebral cortex (n=7)	Glial cells: 0% Neurons: 0% Blood vessels: Strong, uniform expression
Bone marrow (n=4)	70% (65–76%)
Peripheral blood (n=4)	45.5% (38–45%)

We have demonstrated that, in most tissues, ubiquitous expression of EGFP is not apparent. Only the heart and skeletal muscle showed near ubiquitous expression. EGFP expression patterns in all other organs were widely variable between individuals, between organs and within each individual organ or tissue. Our findings have implications in the field of stem cell transplantation.

Transdifferentiation is the conversion of a cell of one tissue lineage into a cell of an entirely different lineage, demonstrating loss of the specific markers and function of the original cell type, gaining markers and function of the new cell type.[7] Proving this event requires a permanent and reliable method of labelling the initial cell, so that this marker can be detected in the presumed transdifferentiated cell. EGFP has been a widely used marker in such studies of adult stem cell plasticity,[3–5,9,10] because of the its ease of detection and assumed 'ubiquitous' expression. Given our observations regarding highly variable levels of EGFP expression among different cell types, including peripheral blood and bone marrow, and a virtual absence of EGFP expression in select cell types, it is clear that this assumption is not valid and may explain some of the conflicting findings from studies examining adult stem cell plasticity.[7,11] Although it is not clear that transdifferentiated bone marrow derived stem cells will adopt the same variegated EGFP tissue expression noted in our survey, it does seem possible that studies relying only on EGFP as a marker of donor derived cells are likely to underestimate the number of actual transdifferentiation events. This may prove to be a particular problem where the cells of interest, for example pancreatic islet cells[3] or cerebral neurons,[10] show minimal or no expression of EGFP in the β-actin-EGFP mouse. Further complicating this issue is our finding that expression of EGFP in mononuclear cells in bone marrow and peripheral blood, is also far from ubiquitous.

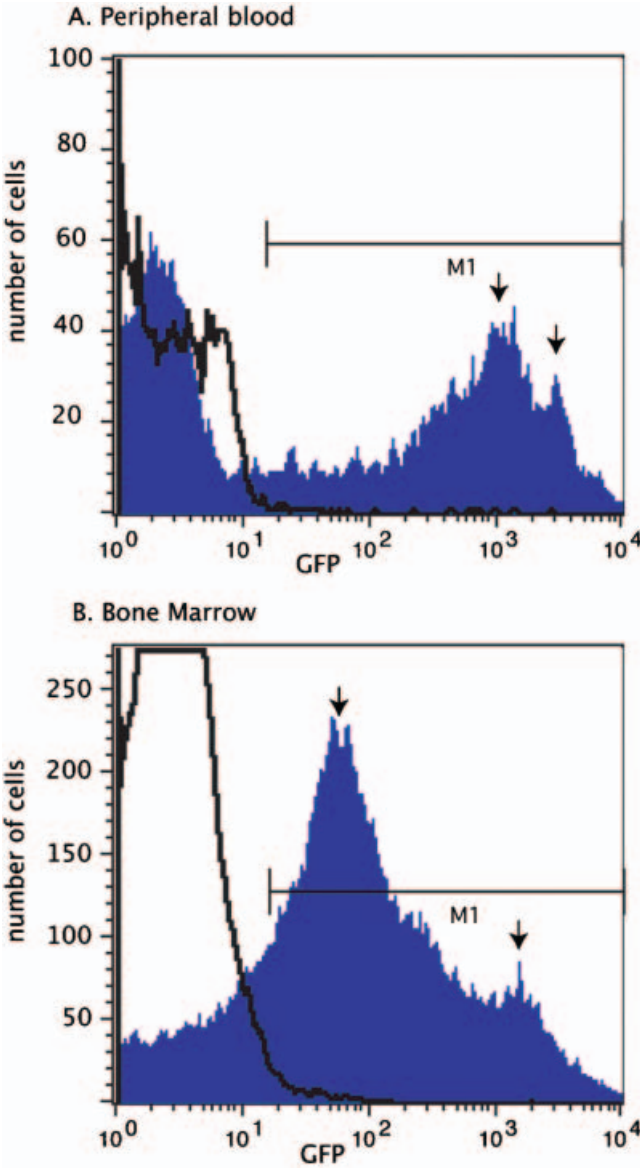

Fig. 2 Representative FACS analyses of mononuclear cells isolated from β-actin-EGFP mouse (A) peripheral blood and (B) bone marrow. In each graph the solid black line represents the results from a wild type control mouse and the solid blue area shows the results for the β-actin-EGFP mouse. M1 indicates cells with EGFP fluorescence exceeding 99th percentile of control cells. Dead cells, debris and non-nucleated red blood cells were gated out in the analysis. (A) Note the marked bimodal distribution of cells from peripheral blood, such that most cells demonstrate either high or low levels EGFP expression (arrows indicate peaks). (B) A bimodal distribution of EGFP fluorescence (arrows indicate peaks) is also noted in analysis of bone marrow but is not as marked as in peripheral blood.

It is not clear what ultimate post-transdifferentiation fate 'high' and 'low' EGFP expressing peripheral blood and bone marrow β-actin-EGFP derived donor cells may adopt but it is possible that they may demonstrate different potentials for transdifferentiation.

Our study does not address mechanisms underlying the specific patterns of variegated EGFP expression in these transgenic mice. However, it is well known that epigenetic modification can affect transgene expression,[12] and it is possible that the nature of epigenetic modification may vary between specific cells and tissues. Transgene expression has also been noted to decrease with increasing mouse

age;[13] this has significance in stem cell studies as mice are often not sacrificed until many months after haematopoietic stem cell transplantation, with a corresponding decrease in EGFP expression of the donor mouse tissue.

In summary, we have found that there is widely variable expression of EGFP in the β-actin-EGFP mouse, with only cardiac and skeletal muscle showing near ubiquitous expression of EGFP. The pancreas, liver, kidneys, lung and gastrointestinal tract show much lower rates of expression, and specific cell types such as pancreatic duct cells, glomerular mesangium and cerebral neurones are consistently negative for EGFP. We recommend that EGFP expression be surveyed in multiple tissues and mice, even if the reporter is driven by a specific tissue promoter, before EGFP transgenic mice are used in transplantation studies. We suggest that alternative methods of identifying donor cell origin, such as detection of the Y chromosome in sex mismatched transplants,[14] also be employed. While β-actin-EGFP 'green' mice are still a useful tool, these results suggest caution in relying overly on EGFP as an infallible marker of donor cell origin.

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Address for correspondence: Dr S. A. Biankin, Garvan Institute of Medical Research, 384 Victoria St, Darlinghurst, NSW 2010, Australia. E-mail: s.biankin@garvan.org.au

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