'Green mice' as a source of ubiquitous green cells

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Abstract The green fluorescent protein (GFP) is responsible for the green bioluminescence of the jellyfish *Aequorea victoria*. Many classes of GFP mutants exist that display modified fluorescence spectra and an increased extinction coefficient. We produced transgenic mouse lines with an 'enhanced' GFP (EGFP) cDNA under the control of a chicken beta-actin promoter and cytomegalovirus enhancer. All of the tissues from these transgenic lines, with the exception of erythrocytes and hair, were green under excitation light. The fluorescent nature of the cells from these transgenic mouse lines would facilitate their use in many kinds of cell transplantation experiments.

Key words: Green fluorescent protein; Transgenic mouse; Transplantation; Stem cell; Nerve cell; Lymphocyte

1. Introduction

The jellyfish *Aequorea victoria* yields a natural green fluorescence which can be viewed in a dark environment. The green fluorescent protein (GFP) is the ultimate source of this green bioluminescence. The GFP chromophore is derived from the primary amino acid sequence through the modification and cyclization of the amino acids Ser–Tyr–Gly at positions 65 to 67 [1]. Although the mechanism for producing the dehydrotyrosine and cyclic polypeptides is unknown, additional substrates from *A. victoria* are not needed for the production of the fluorescence [2].

The expression of GFP in a heterologous system, *Caenorhabditis elegans* [2], attracted much interest for this novel and simple reporter as a potential in vivo marker. Transgenic animals and plants such as *Drosophila melanogaster* [3], zebrafish [4,5], *Dyctyostelium* and *Arabidopsis thaliana* [6,7] have since been produced using wild-type GFP.

Recently, we inserted the wild-type GFP into pCAGGS (containing the chicken beta-actin promoter and cytomegalovirus enhancer, beta-actin intron and bovine globin poly-adenylation signal [8]) and produced transgenic mouse lines [9,10]. Although a bright green light emission was observed in the muscle and pancreas of more than 20 of these transgenic mouse lines, GFP expression was not ubiquitous and light emission was not visible to the naked eye in other tissues. Since the time of our initial work, various mutant GFPs with modified fluorescence spectra and an increased extinction coefficient have been produced [11,12]. We therefore modified our expression construct to include the enhanced green fluorescent protein (EGFP). Here we describe the production of mouse lines that express the EGFP(S65T+F64L) transgene in the entire body, from pre-implantation embryo to adult stages. These mice can be used as a source of marker cells that are fluorescent green.

2. Materials and methods

2.1. Construction of vectors

The transgene used was prepared as we described previously [9,10]. Briefly, the cDNA encoding EGFP was amplified by PCR with primers, 5′-ttgaattcgccaccatggtgagc-3′ and 5′-ttgaattcttacttgtacagctcgtcc-3′, using pEGFPC1 (Clontech Co. Ltd.) as a template. No additional amino acid sequence was added on either side of the EGFP. The *Eco*RI sites included in the PCR primers were used to introduce the amplified EGFP cDNA into a pCAGGS expression vector containing the chicken beta-actin promoter and cytomegalovirus enhancer, beta-actin intron and bovine globin poly-adenylation signal [8]. The entire insert with the promoter and coding sequence was excised with *Bam*HI and *Sal*I and gel-purified.

2.2. Production of transgenic mice

Transgenic mouse lines were produced by injecting the purified *Bam*HI and *Sal*I fragment into BCF1 × BCF1 fertilized eggs. In total, 272 DNA-injected eggs were transplanted to pseudo-pregnant mice, resulting in 52 newborns. The incorporation of the transgene was examined by placing 1-day-old pups under a fluorescent microscope. Twelve of these were found to be transgenic. To date, five of six lines sired offspring when mated with BCF1 mice and transmitted the *EGFP* gene (TgN(beta-act-EGFP)01Obs to 05Obs).

2.3. Observation of the green fluorescence

The expression of EGFP in F1 pups from each transgenic founder mouse was examined by the naked eye or under a fluorescent microscope using excitation light. The photos were taken under UV light without filters using a Fujix DS-505 digital card color camera (F6.7, shutter 1/16 s). No image intensifying procedure was applied on any of the photos.

F1 animals were killed at the age of 4–6 weeks and various organs were removed. Specimens were placed under the excitation light at a distance of about 15 cm from the objective lens (×10). To expose the sample to the light, the sample stage of the microscope was removed. Photographs were taken with a Fujix DS-505 digital card color camera (Fuji Film Co. Ltd.: F6.7, shutter speed 1/8 s) with an Olympus IF550 filter.

Eggs were collected from the oviducts of females mated with males and placed into microdrops of kSOM medium [13]. Sperm suspensions were prepared by releasing the contents of each caudal epididymis from mature males into TYH medium [14]. Photographs of eggs were taken with a PXL KAF1400-G2 digital camera (Photometrics Co. Ltd.: shutter speed 1 s) with a Leica K3 blue-filter and were then pseudo-colored.

2.4. FACS analysis

The thymus, spleen and testicular cells were prepared by squeezing the organ between a pair of slide glasses with phosphate-buffered saline (PBS). Leaving large pieces of tissue behind, cells were filtered through a nylon mesh and washed with PBS. The cell suspensions prepared as above were subjected to analysis with a cell sorter (FACS-Calibur, Becton Dickinson Co. Ltd.).

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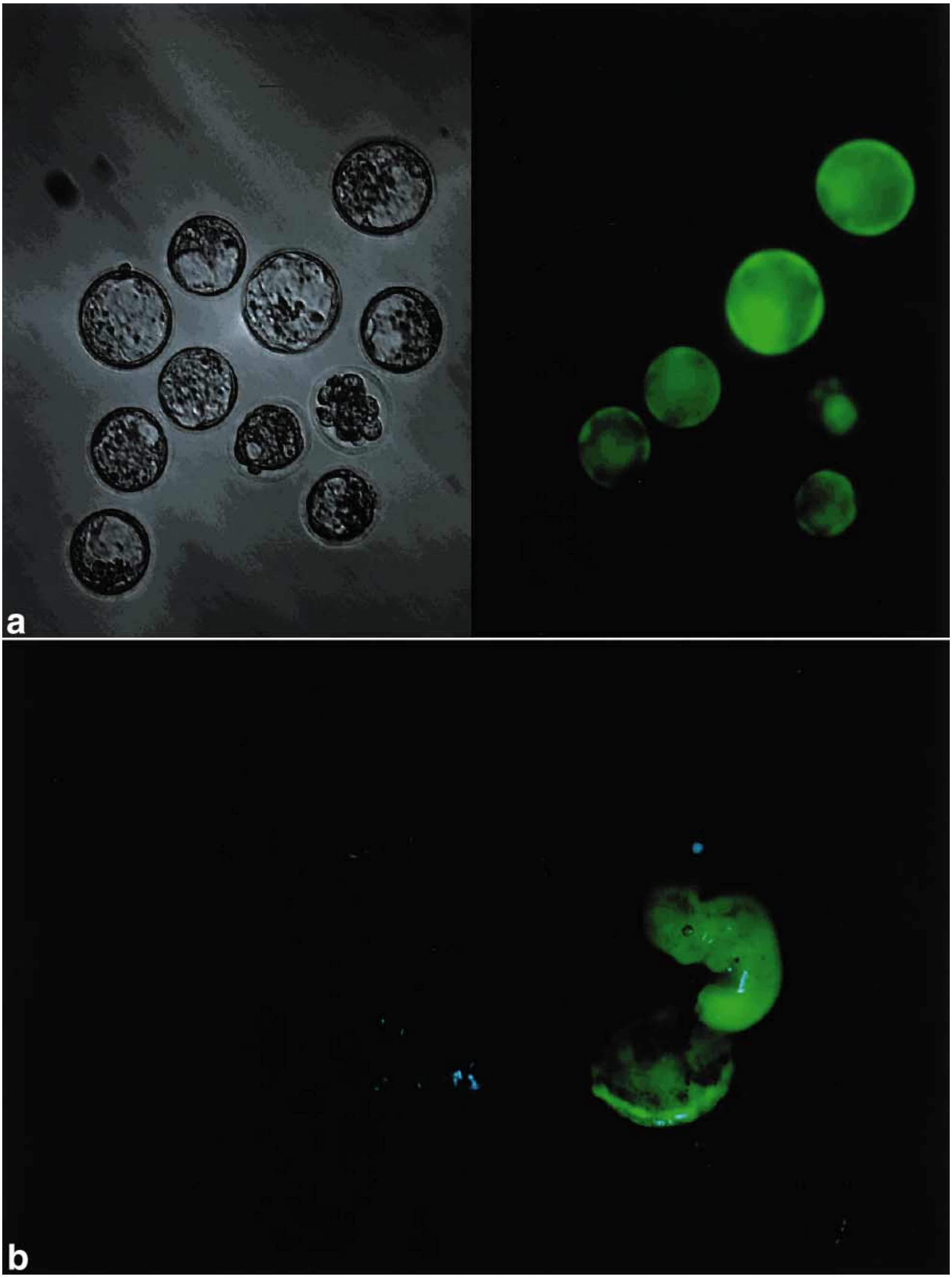


3. Results and discussion

The green female mice produced 'green' eggs; therefore the maternal EGFP transgene was already active in the unfertilized egg. The sperm of green males showed very faint fluorescence in the midpiece, where a small amount of cytoplasm

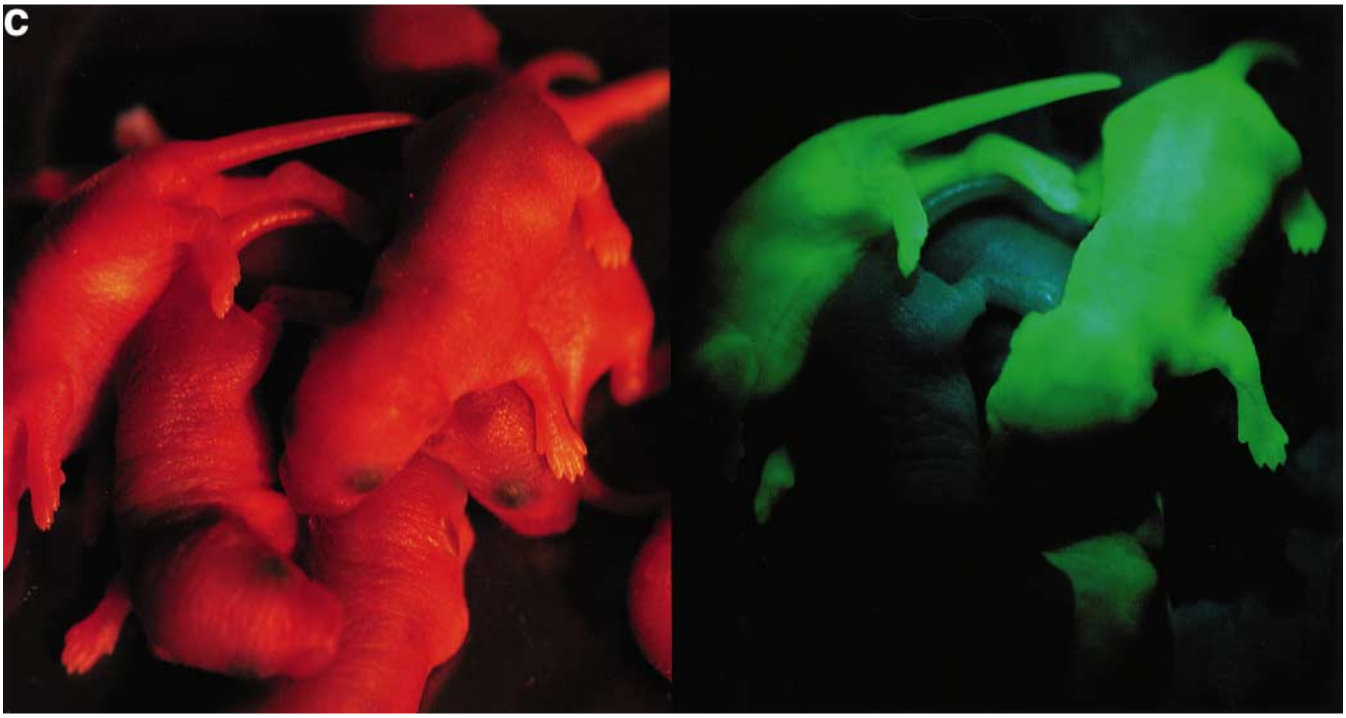


Fig. 1. a: Approximately half of the fertilized eggs derived from wild female and hemizygous males were fluorescent green, starting from 4-cell stage (the figure shows blastocyst-stage eggs). The implanted embryos and placentas (the figure shows day-12 embryo and placenta; note that the wild-type embryo and placenta were not visible in this condition) in panel (b) and the newborn mice (c) were also green under excitation light. (d–g) Expression of green fluorescence in an EGFP transgenic mouse (TgN(beta-act-EGFP)04Obs). Transgene expression was apparent in brain (d), liver (e), adrenal gland and kidney (f), and testes (g). h: Lung, heart, muscle, intestine, colon and adipose tissues. The pancreas and muscle were brighter than other tissues. Note that places lacking hair, such as paws and around the mouth, glowed green. Therefore it was possible to distinguish the transgenic mouse just by observing the paws in adulthood. i: The green color of the spleen was dim, probably because of a high proportion of red blood cells, which do not express the transgene. h,i: The organs were taken as a group under hand-held 360 nm UV light. Although EGFP requires a blue excitation light (488 nm), emission of the green light was visible to the naked eye.

is stored. Wild-type eggs fertilized with green male sperm were not green at the 2-cell stage, but became green afterwards during stages of prenatal development (Fig. 1a,b) and newborns were fluorescent green (Fig. 1c). The green fluorescence was visible in the live animals until hair emerged to cover the body.

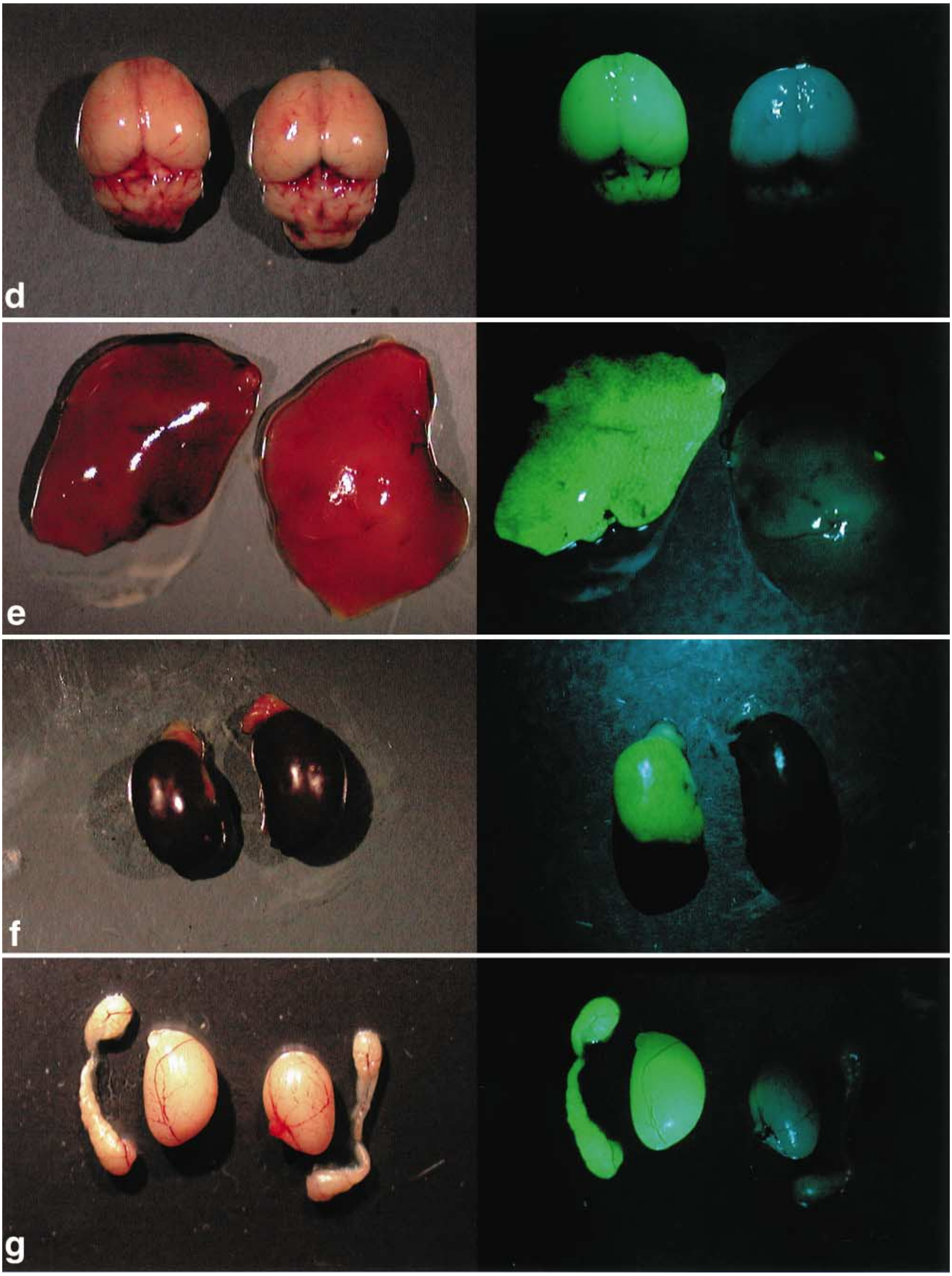
Transgenic mice were uniformly green with the exception of hair and red blood cells. The brain, liver, kidney, adrenal gland and testis (Fig. 1d–g), lung, muscle, heart, intestine, and adipose tissue (as a group in Fig. 1h,i), thymus, spleen and testicular cells (Fig. 2) of all transgenic mouse lines were naturally green when irradiated with excitation light.

Using the same promoter construct, our former transgenic lines carrying the wild-type GFP(w-GFP) or RS-GFP [11] did not provide ubiquitous expression [9,10], whereas EGFP did in this study. The EGFP sequence was modified from the original jellyfish GFP in two ways: by amino acid changes to GFP, yielding GFPmut1, and by optimized codon usage for human cells, yielding EGFP. A comparison of the fluorescence intensity of GFPmut1 with jellyfish codons to the GFPmut1 with human codons (which equals EGFP) using flow cytometry (FACS) revealed that the mean fluorescence intensity obtained from cells transfected with EGFP was approximately 4-fold greater than GFPmut1 (S. Kain, personal communication).

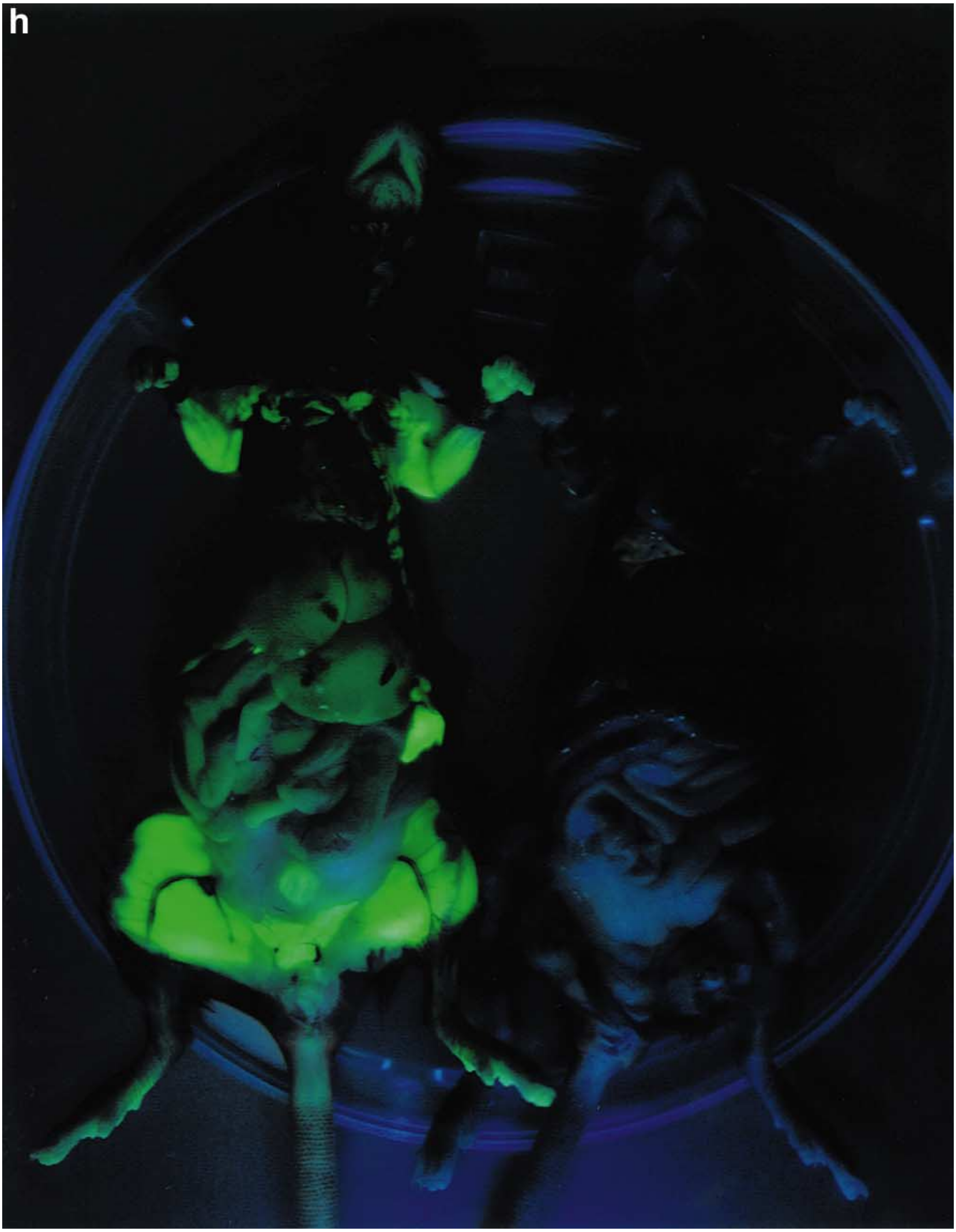
The difference was not due simply to greater sensitivity of EGFP, allowing detection in tissues with lower expression. For example, the green fluorescence observed in the morulae stage grew faint and became invisible in the blastocyst stage through implanted embryos as a rule in the former two GFP-bearing transgenic mouse lines. However, in the EGFP transgenic mice, none of the lines lost their fluorescence, but became increasingly brighter as they developed from the blastocyst stage to become embedded embryo. Moreover, the blood vessels were the least 'green' part of the body in the former two kinds of GFP-bearing lines while the tissue was classified as 'bright' in the EGFP-bearing lines. We assumed that the 'humanized' modification of the codon usage might be responsible for the ubiquitous expression of EGFP.

In jellyfish, GFP is normally sequestered in microbody-like lumisomes. In the transgenic experiment described here, the EGFP was designed to be expressed in the cytosol and the soluble nature of the protein allowed its distribution throughout the cytosol. This situation could have a potentially toxic effect if EGFP were expressed at high levels. However, all of the transgenic mouse lines looked normal and healthy, despite a significant amount of EGFP expression throughout the body, including brain and hormonal glands. Muscle, heart, pancreas, lung and kidney from 12-week-old transgenic males were subjected to histochemical analysis following fixation with Bouin's solution and no abnormality was found in any of the sections examined (data not shown). The expression of EGFP therefore seemed to be non-toxic. These results indicate that EGFP could be used as a novel reporter in almost all of the mouse body from pre-implantation stage to adulthood. A more precise histological description of the GFP expression will be presented elsewhere.

The great advantage of the GFP as a reporter is that the

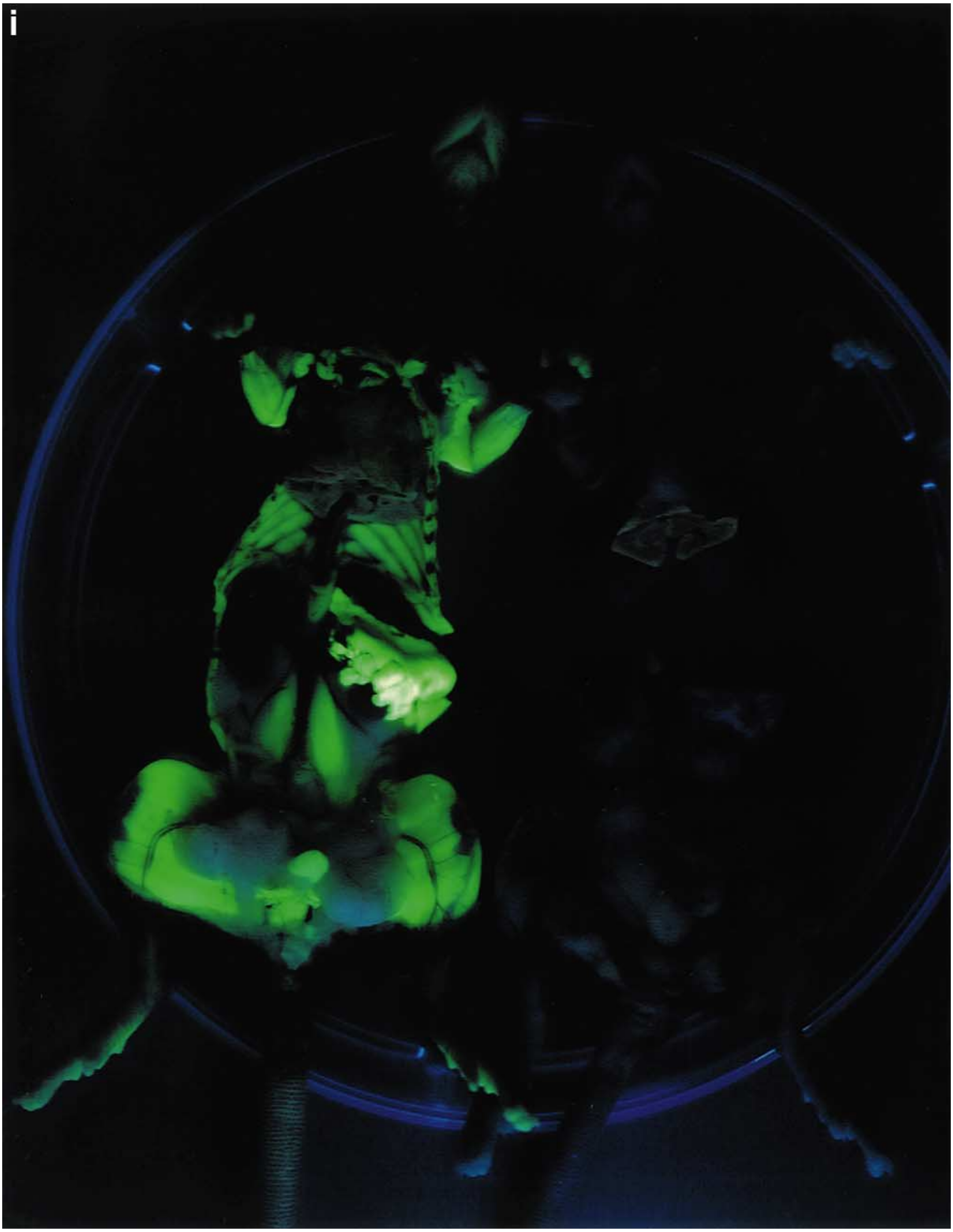


introduction of a substrate is not required, unlike other commonly used reporter genes such as beta-galactosidase, firefly luciferase, alkaline phosphatase, chloramphenicol acetyltransferase and beta-glucuronidase. This enabled us to observe the



fluorescence from live cells and in intact form on a real-time basis.

Because the excitation optimum for EGFP is close to 488 nm, cells from the 'green mice' were also suitable for analysis by flow cytometry. The green fluorescence from thymus, spleen and testicular cells was measured by a cell sorter (Fig. 2). The difference of the peak between transgenic and wild-type cells was significant. It should be noted that all of



the cells (except red blood) derived from thymus, spleen and testis were green. The B-cell population appeared as one bright peak, whereas the T-cells were split into bright and less bright populations with one exception (TgN(beta-act-EGFP)05Obs). The cause of the two peaks and their relationship to the T-cell population has not been determined.

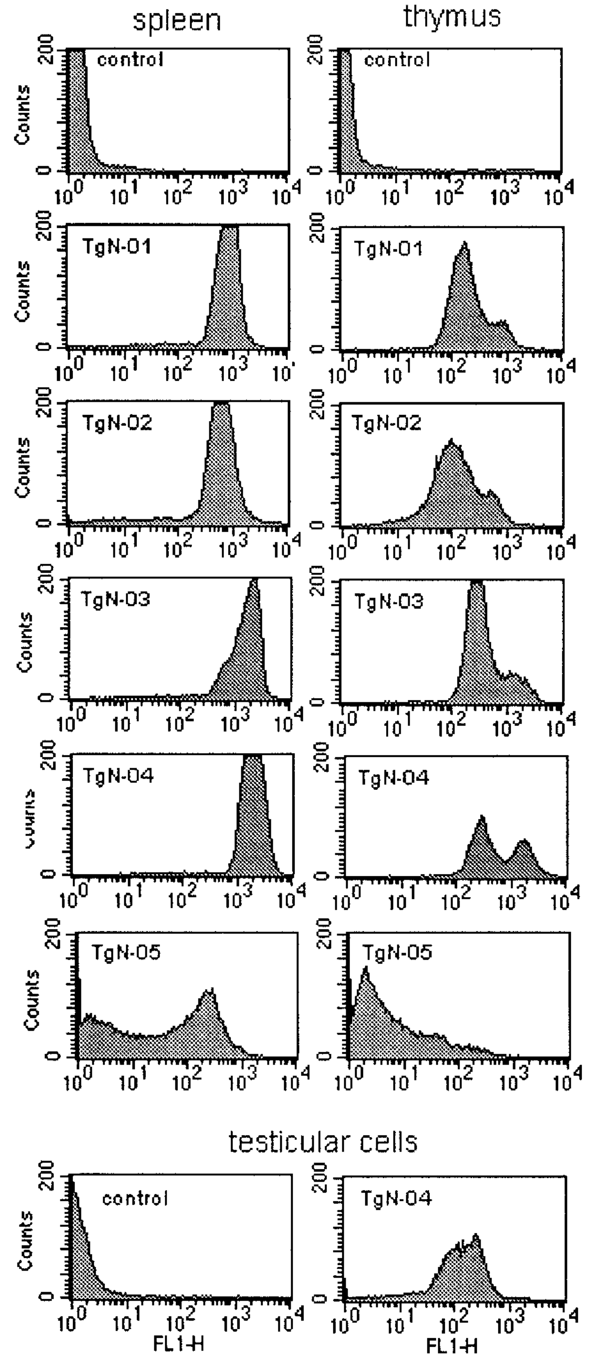


Fig. 2. Thymus (a), spleen (b) and testicular (c) cells from TgN(beta-act-EGFP)04Obs were subjected to FACS analysis. The green fluorescence of the cells from green mice was apparently different from that of wild-type cells.

Since the newborns from all the transgenic lines were green fluorescent, the construct was shown to be useful as a transgenic marker when co-injected with a desired transgene. Moreover, it was very simple to identify the few transgenic cells mixed with many wild-type cells. The detection and recovery of the transgenic cells in their intact form would be possible since no fixation or substrate addition is necessary. Taken together, these facts suggest that the EGFP transgenic mice would provide a powerful and useful cell source, such as in bone marrow, nerve and spermatogonia stem cell transplantation. They will also provide a source of green pre-implantation stage embryo, which can be used for production of chimeric mice by the injection or aggregation of non-green embryonic stem cells. This approach is frequently taken when the ES cells have been genetically manipulated, and is particularly useful when the genetic changes are embryonic lethal [15]. By generating chimeras comprised of green blastocysts derived from the EGFP transgenic mice and non-green ES cells, the ES cell-derived tissues can be easily identified. Another use would be in the study of tumorigenesis, wherein 'green mice' could be implanted with non-green tumor cells. Indeed, the usage would extend to virtually all types of experiments in which a chimeric analysis is needed.

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