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Differential tissue expression of enhanced green fluorescent protein in 'Green mice'

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Summary. In order to clarify tissue expression of enhanced green fluorescent protein (EGFP) in 'green mice' from a transgenic line having an EGFP cDNA under the control of a chicken beta-actin promoter and cytomegalovirus enhancer, we studied the expression of EGFP in various organs and tissues from these 'green mice' by immunohistochemistry with anti- EGFP antibody in conjunction with direct observation for EGFP fluorescence using confocal laser scanning microscopy.

On immunohistochemical examination and on direct observation by confocal laser scanning microscopy, the level of EGFP expression varied among organs and tissues. EGFP expression was diffusely and strongly observed in the skin, pituitary, thyroid gland, parathyroid gland, heart, gall bladder, pancreas, adrenals and urinary bladder. There was only sporadic and weak expression of EGFP in the epithelium of the trachea, bronchus of the lung, stratified squamous epithelium and gastric glands of the stomach, hepatic bile ducts of the liver, glomeruli and renal tubules of the kidney and endometrial glands of the uterus. Furthermore, EGFP was only demonstrated within the goblet and paneth cells in the colon and small intestine, the tall columnar cells in the ductus epididymis, and the leydig cells in the testis.

In conclusion, our results show that EGFP is differentially expressed in organs and tissues of 'green mice', which indicates that 'green mice' may prove useful for research involving transplantation and tissue clonality.

Key words: Green fluorescent protein, Tissue distribution, Immunohistochemistry, Confocal laser scanning microscopy, Beta-actin

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Introduction

Since the green fluorescent protein (GFP) derived from the jellyfish Aequorea victoria was first found to be a useful marker of gene expression without the need for substrate loading or other pretreatment, various forms of GFP, such as enhanced green fluorescent protein (EGFP), have become important reporters of gene transfer and expression for both in vitro and in vivo studies (Chalfie et al., 1994). Okabe (1997) produced 'green mice' that were transgenic lines having an EGFP cDNA under the control of a chicken beta-actin promoter and cytomegalovirus enhancer (Okabe et al., 1997). The transgenic mice were uniformly green with the exception of their hair and red blood cells, which indicated that EGFP could be used as a novel reporter in almost the entire mouse body from pre-implantation stage to adulthood.

Frequently, molecular biological assays (Northern blots, RT-PCR) are applied to detect GFP expression in target organs. However, for these methods, tissue and cell structures have to be destroyed before processing, and examination of the resultant homogenate gives no information about tissue or sub-cellular distribution of the GFP, which would be of great interest, especially where tissue-specific promoters have been used (Walter et al., 2000). To the best of our knowledge, little is known about histological and/or cellular distribution of EGFP expression in 'green mice'.

To clarify tissue distribution and expression levels of EGFP in 'green mice', we performed immunohistochemistry using anti-EGFP antibody in conjunction with direct observation for EGFP fluorescence using confocal laser scanning microscopy in paraformaldehyde-fixed paraffin sections of various organs and tissues from male/female 'green mice' at different ages from 7 days to 8 weeks.

Materials and methods

Histology

The animal experimental procedure and care of laboratory animals followed the Guidelines for Animal Experiments of the Yamanashi University. We used ten 'green mice' of C57BL/6-Tg(CAG-EGFP)C14-Y01-FM131Osb (5 male mice and 5 female mice) that were kindly gifted to us by professor Okabe of Osaka University and ten control mice (C57BL/6, 5 male mice and 5 female mice). Samples (ten samples of one organ/tissue except for the epididymis, testis, uterus, breast, and ovary (5 samples)) from twenty-five organs (skin, cerebrum, cerebellum, pituitary, thyroid gland, parathyroid gland, heart, trachea, lung, stomach, small intestine, colon, liver, gall bladder, thymus, spleen, pancreas, adrenals, kidney, urinary bladder, uterus, epididymis, testis, breast, and ovary) and three tissues (bone tissue, skeletal muscle tissue, and adipose tissue) from 'green mice' and control mice were obtained by dissection, fixed in 8% buffered paraformaldehyde in PBS for 2h at room temperature, and embedded in paraffin. Two μm-thick sections were cut and mounted on glass slides pretreated with poly-L-lysine for immunostaining and confocal laser scanning microscopy.

Immunohistochemistry staining for EGFP

The procedure for immunohistochemistry staining for EGFP has been described elsewhere (Walter et al., 2000). Briefly, after removing paraffin wax in three changes of xylene and a series of ethanols, endogenous peroxidase was blocked by immersing the sections in 3% H_2O_2 for 10 min. The sections were incubated for 1 h at room temperature with primary antibody (rabbit anti-Aequorea victoria Green Fluorescent Protein, Molecular Probes, Eugene, OR, USA, 1:1000). Tissue sections were then reacted with anti-rabbit EnVision+™ (anti-rabbit immunoglobulins conjugated to peroxidase-labeled dextran polymer, DAKO corporation, Carpinteria, CA, USA) for 40 min at room temperature. Visualization of EGFP was performed with diaminobenzidine for 6 min. After counterstaining with haematoxylin for 10 s, sections were dehydrated in a series of graded ethanols, cleared in 3 changes of xylene and coverslipped for light microscopy examination.

Confocal laser scanning microscopy

The sections were deparaffinized in xylene and rehydrated in a descending series of ethanols. Deparaffinized sections were counterstained with TOPRO-3 (Invitrogen, Carlsbad, CA) at a concentration of 1:500 in PBS for 1h at room temperature. After counterstaining, sections were coverslipped for light microscopy examination. Light micrographs were obtained by using a confocal laser scanning microscopy (FV1000; Olympus, Tokyo, Japan).

Results

The characteristic green fluorescence was directly observed in the newborn green mice under excitation light (Fig. 1).

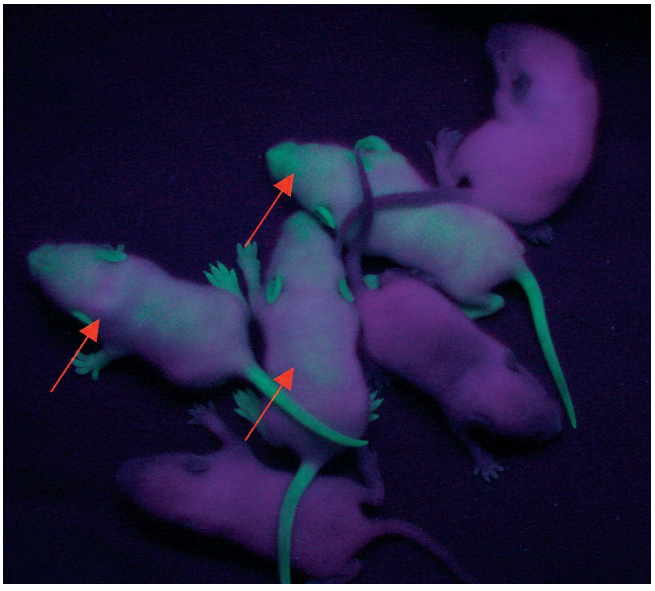

Fig 1. Newborn 'green mice' (red arrows) and control mice (C57BL/6). Please note the green fluorescence of EGFP in the green mice under excitation light.

Table 1. Summary of immunohistochemistry for EGFP in organs/tissues of 'green mice'.

Organs/tissues	Positive pattern	Main positive parts or cells
Skin	Diffuse	Epidermis
Pituitary	Diffuse	Adenohypophysis
Thyroid	Diffuse	Follicles
Parathyroid	Diffuse	Chief cells
Heart	Diffuse	Myocardium
Trachea	Sporadic	Epithelium
Lung	Sporadic	Bronchus; Bronchioles
Stomach	Sporadic	Stratified squamous epithelium; Gastric glands
Small intestine	Sporadic	Goblet cells; Paneth cells
Colon	Sporadic	Goblet cells; Paneth cells
Liver	Sporadic	Bile ducts
Gall bladder	Diffuse	Mucosa
Pancreas	Diffuse	Islets; Acini
Adrenals	Diffuse	Cortex; Medulla
Kidney	Sporadic	Glomeruli; Renal tubules
Urinary bladder	Diffuse	Mucosa
Uterus	Sporadic	Endometrial glands
Epididymis	Sporadic	Tall columnar cells
Testis	Sporadic	Leydig cells
Skeletal muscle	Diffuse	Striate muscle cells
Bone marrow	Sporadic	Hematopoietic cells

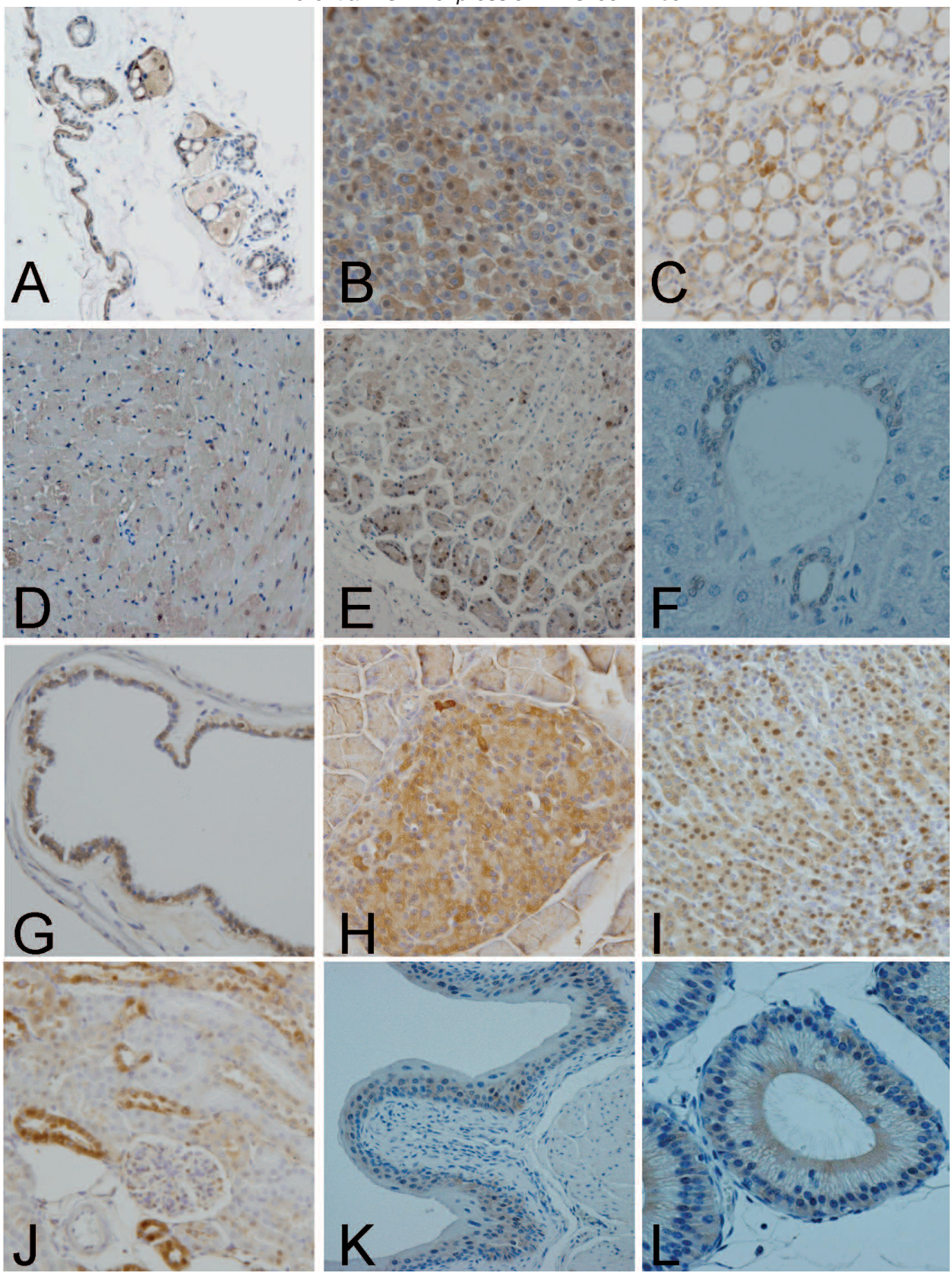

Fig. 2. Immunohistochemical results showing EGFP in various organs from 8-week-old 'green mice'. Diffuse EGFP expression was observed in skin **(A)**, pituitary **(B)**, thyroid gland **(C)**, heart **(D)**, gall bladder **(G)**, pancreas **(H)**, adrenals **(I)** and urinary bladder **(K)**. Sporadic positive expression was observed in the gastric gland of the stomach **(E)**, hepatic bile ducts of the liver **(F)**, and glomeruli and renal tubules of the kidney **(J)**. Only tall columnar cells in the epididymis **(L)** were distinctively positive for EGFP. A, D, E, K, x 200; B, C, F-J, L, x 400

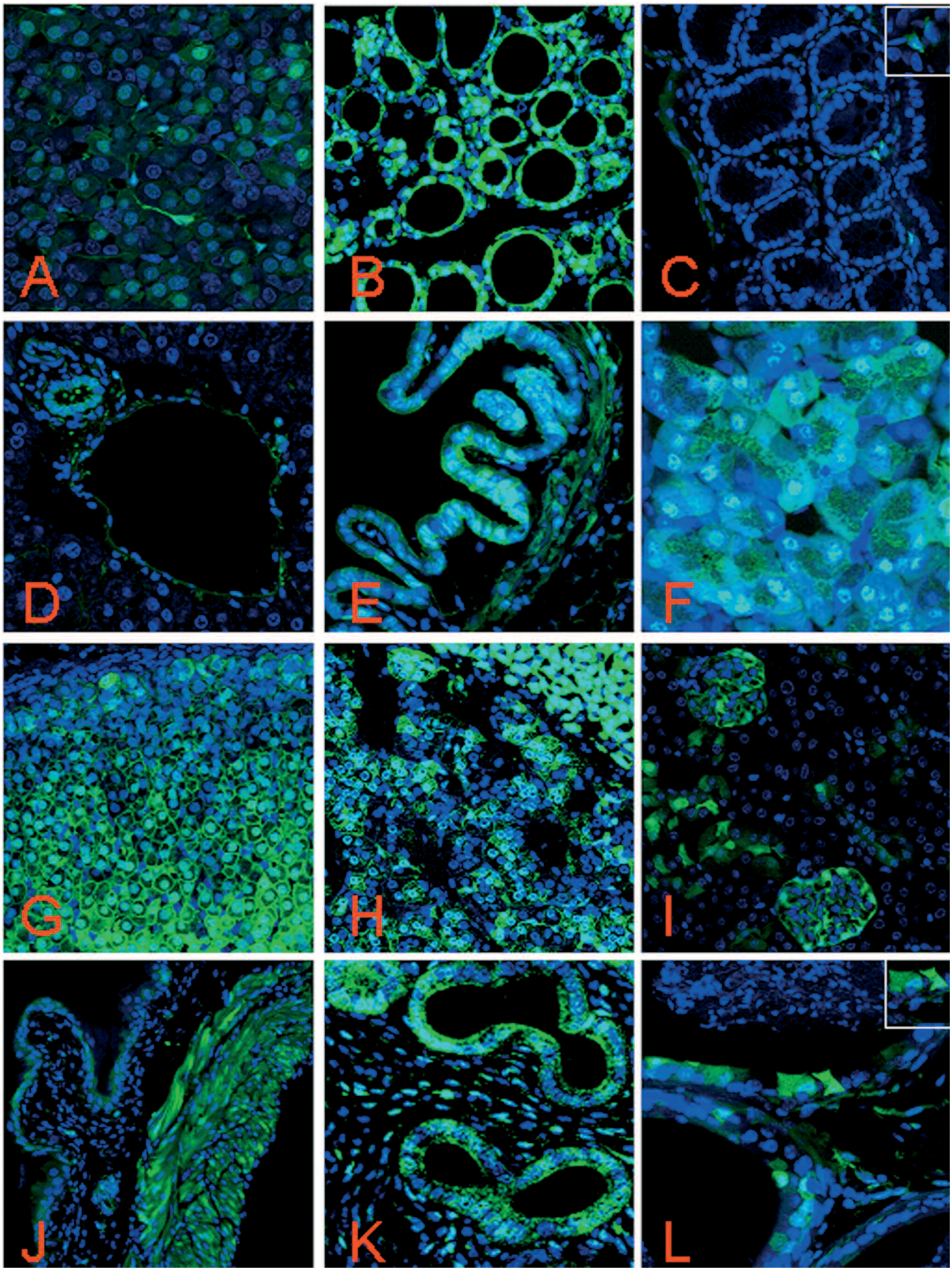

Fig. 3. Direct observation by confocal laser scanning microscopy in various organs from 8-week-old 'green mice'. Expression patterns of EGFP in pituitary **(A)**, thyroid **(B)**, colon **(C),** liver **(D)**, gall bladder **(E)**, pancreas **(F)**, cortex of adrenals **(G)**, medulla of adrenals **(H)**, kidney **(I)**, urinary bladder **(J)**, uterus **(K)**, and epididymis **(L)** were almost identical to expression patterns found with immunohistochemistry. Insets: magnified images for goblet cell in the colon and tall columnar cells in the epididymis. A-I, K, L, x 400; J, x 200; insets, x 800

On immunohistochemistry examination and on direct observation by confocal laser scanning microscopy, the expression levels of EGFP varied among organs and tissues (Table 1). All male/female mice examined from the newborn to the 8-week-old 'green mice' showed the same pattern of EGFP expression in all the tested organs and tissues. The EGFP expression was diffusely and strongly observed in the skin (Fig. 2A), pituitary (Figs. 2B, 3A), thyroid gland (Figs. 2C, 3B), parathyroid gland, heart (Fig. 2D), gall bladder (Figs. 2G, 3E), pancreas (Figs. 2H, 3F), adrenals (Figs. 2I, 3G, 3H) and urinary bladder (Figs. 2K, 3J), while trachea, lung, stomach, small intestine, colon, liver, kidney, uterus, epididymis, and testis exhibited sporadic and weak expression. Focal positive expression was observed in the epithelium of the trachea, bronchus of the lung, stratified squamous epithelium and gastric gland of the stomach (Fig. 2E), hepatic bile ducts of the liver (Figs. 2F, 3D), glomeruli and renal tubules of the kidney (Figs. 2J, 3I), and endometrial glands of the uterus (Fig. 3K). Remarkably, only goblet and paneth cells in the colon and small intestine (Fig. 3C), tall columnar cells in the ductus epididymis (Figs. 2L, 3L), and leydig cells in the testis were distinctly positive for EGFP. Organs such as cerebrum, cerebellum, thymus, spleen, breast, and ovary were negative for EGFP. Skeletal muscle and bone marrow showed strong EGFP reactivity and adipose tissue was negative for EGFP. All organs and tissues from the control mice examined were negative for EGFP.

Discussion

High quality structural tissue preservation is essential when evaluating EGFP expression at the single cell level and for intracellular localization of fluorescence. Since EGFP is very soluble, care must be taken regarding fixation. To fix highly soluble protein EGFP, perfusion fixation before dissection the organs is often used. After perfused fixation with 4% paraformaldehyde solution and being placed in 4% paraformaldehyde solution for two hours and subsequently in 30% sucrose solution overnight, cryoscetions mounted on glass slides can be used for histochemical detection of EGFP and direct EGFP fluorescence observation (Akagi et al., 1997). However, Jockusch (2003) has proved that performing enzyme and immunohistochemistry on cryostat sections from prefixed tissues is unproductive because of the previous fixation (Jockusch et al., 2003). In the current research, we found that fixation in 8% buffered paraformaldehyde for 2 h did not impede EGFP-fluorescence in tissue. Solvents (xylene, ethanol) and a high temperature (56°C) while embedding the tissue in paraffin wax had no extinguishing effect on EGFP-fluorescence.

Immunohistochemical detection of EGFP expression in the transgenic mice is usually more sensitive than that of direct EGFP fluorescent observation. Walter reported that the cells of nerves, ganglia, and secretory ducts of submandibular gland were slightly positive after immunohistochemical staining with anti-EGFP, but fluorescence was under the detection threshold for confocal microscopy (Walter et al., 2000). These distinctions are explained by the very low amount of fluorescent protein in these cells but enough to be detectable by the antibody (Walter et al., 2000). In the present research, our results demonstrated that the sensitivity achieved through direct observation by confocal laser scanning microscopy is equal to the sensitivity of immunohistochemistry in the organs/tissues examined. Histological preparation and evaluation with confocal laser scanning microscopy may be suitable for determining tissue-, cell-, and even subcellular- specific distribution patterns of EGFP expression in tissues. The sensitivity and ease of the histological procedure using confocal laser scanning microscopy may facilitate EGFP application for determining of the activity of tissue-specific promoters in transplantation and clonality research.

In the current study, we analyzed an array of sections from organs of male/female 'green mice' at different ages from 7 days to 8 weeks and found significant differences in EGFP expression among organs and tissues using immunohistochemistry and direct observation. Interpreting tissue-specific patterns of EGFP expression was difficult, although significant differences in EGFP expression detected between various organs, and frequently single cells in organs, show promoter activity. Such histological information is of particular interest, especially when tissue-specific promoters are evaluated. EGFP was distinctly expressed in the secretarial cells, especially endocrine cells such as pituitary hormone producing cells, adrenal cells, thyroid follicular cells, parathyroid cells, and islet cells of the pancreas. These results indicate that it may be possible for 'green mice' to be used when determining tissue clonality of endocrine organs in chimeric mice and for determining the activity of tissue-specific promoters in transplantation research. Cells and tissues with increased hemoglobin content in 'green mice' exhibit reduced fluorescence as development progresses (Hadjantonakis et al., 1998). We failed to demonstrate a strong expression of EGFP in lung and liver, possibly due to the higher hemoglobin content.

In conclusion, our results suggest that EGFP is differentially expressed in organs and tissues of 'green mice', which could facilitate research in areas such as transplantation, and clonality. Evaluation with confocal laser scanning microscopy and immunohistochemistry in paraffin sections was suitable for determining a pattern of EGFP expression within organs and tissues at the single cell level.

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