A Novel Methodology for Analysis of Cell Distribution in Chimeric Mouse Organs Using A Strain Specific Antibody

Moriaki Kusakabe,* Minesuke Yokoyama,‡ Teruyo Sakakura,§ Tatsuji Nomura,‡ Howard L. Hosick‖ and Yasuaki Nishizuka¶

* Institute for Laboratory Animal Research, Nagoya University School of Medicine, Showa-ku, Nagoya, Japan; ‡ Central Institute for Experimental Animals, Nogawa, Kawasaki, Japan; § Laboratory of Cell Biology, Tsukuba Life Science Center, Riken, Tsukuba, Ibaragi, Japan; ‖ Department of Zoology, Washington State University, Pullman, Washington; and ¶ Aichi Cancer Center Research Institute, Laboratory of Experimental Pathology, Chikusa-ku, Nagoya, Japan

Abstract. Chimeric animals are very useful for analysis of cell lineage, homeostasis in tissue architecture, and cell–cell interactions during both organogenesis and carcinogenesis. However, there is not a generally effective means for marking cells of chimeric mice. We have therefore developed a polyclonal antibody that is useful for this purpose. This antibody specifically recognizes those cells derived from C3H strain mice. The specificity of this antibody was checked by both immunoblotting and immunoadsorption methods. The antigens were immunohistochemically detected in cytoplasm of both epithelial and mesenchymal cells of C3H/HeN strain mouse in many different organs, but not the corresponding cell types from BALB/c or C57BL/10 or several other mouse strains. The validity of these antibodies as markers for C3H cells was further checked by tissue recombination experiments and in mixed cultures of mouse and rat cells. In each case the antibody recognized only the C3H mouse cells. Next, chimeric mice were prepared between strains C3H/HeN and BALB/c, and C3H/HeN and C57BL/10 mice. Chimeras 2-mo old were examined for antigen distribution using the indirect immunofluorescence method. Many tissues in chimeric mice were com-

posed of cells that were both stained and unstained by the anti–C3H specific antigen. The chimeric patterns were classified into four types, A–D. In well-defined structural units such as intestinal crypts, small intestinal villi, kidney convoluted tubules, exocrine gland acini, ovarian follicles, thyroid gland follicles, stomach glands, adrenal cortex, lingual papillae, etc., (A) each unit was composed entirely of either positive or negative cells, or else (B) in some organs each unit was composed of both types of cells. In the uniform tissues without such distinguishable units, such as stratified squamous epithelium, mesenchymal tissue, corpora lutea, pituitary gland, Islets of Langerhans, adrenal medulla etc., (C) the tissue was composed of definite small cell groups made entirely of either positive or negative cells, or else (D) the tissue was composed of both types of cells which were intermingled with one another.

These findings strongly suggest that the chimeric patterns demonstrated here reflect the cell proliferative unit in each tissue. This cell marker system has proven useful for analysis of cell lineage and cell renewal systems in many organs of chimeric mice.

S INCE the first experimental production of mammalian chimeras was reported (33), many developmental biologists have recognized the potential value of such animals for assessing the cell lineages of components of organs and tissues (21, 24, 29). For such studies, however, the problem has been the lack of suitable autonomous cell markers for different mouse strains which are detectable in histological sections of various organs. Without this capability, an identification of the strain origin of the cells in chimeric organs is impractical. As discussed previously (15, 21), the

ideal histological marker molecule should fulfill six criteria: (a) Cell-localized, i.e., not secreted extracellularly; (b) cell autonomous, not transferred between cells or affecting other cells; (c) stable both within the first marked cells and all of their mitotic progeny; (d) ubiquitous throughout development among both the internal and external tissues of the body; (e) easy to detect, both grossly and/or in histological sections without elaborate processing; and (f) developmentally neutral, not causing cell selection or influencing developmental processes such as cell mixing. Recently, numerous histological markers have been used for analysis of mosaicism in chimeric mice. Albino/pigmented chimeras have been used to investigate pigmentation in the coat (17) and in the retinal pigment epithelium of the eye (34). Nuclear morphology of Ichtyosis mutant mice was used to analyze cell distribution in chimeric brain (7). The Gus-s^h variant

was used to demonstrate chimeric patterns histochemically in situ (2, 36). Anti-H-2 antibody has been used (22) and anti-glucose phosphate isomerase-1B (GPI)[1] antibody has been developed to detect allozymes immunohistochemically in Purkinje cells (6, 12). Recently, peroxidase-conjugated *Dolichos biflorus* agglutinin was used as a biochemical marker to demonstrate two populations of cells in fixed sections of vascular and intestinal epithelium (23, 24, 31). In a very different approach, interspecific chimeras between *Mus caroli* and *Mus musculus* were made and mosaic patterns demonstrated using differences in satellite DNA sequences (29, 30). Other specialized labeling methods have been used to identify one cell population: for example differential tritiated thymidine incorporation (5, 8, 32). In spite of advantages of several of these methods in specialized situations, the use of mouse chimeras has been restricted to analyses of a small number of organs by the lack of ubiquitous and autonomous markers. No ideal markers exist that fulfill all six criteria. We have now developed an anti-C3H strain specific antigens (CSAs) antibody that is useful as a strain-specific cell marker. The characteristics of this antibody, and the results of its use to assess cell distribution patterns in numerous organs of chimeric mice, are described here.

Materials and Methods

Animals

Wistar rats were purchased from Charles River Japan, Astugi, Japan. C3H/HeN, BALB/c, C57BL/10, SJL/J, AKR, and ICR mouse strains were purchased from the Central Institute for Experimental Animals (Nogawa, Kawasaki, Japan). BALB/c, AKR, (BALB/c × AKR) Fl, SJL/J and (BALB/c × SJL) Fl female mice were used for immunization. The following mouse tissues were immunohistochemically examined: digestive system (tongue, esophagus, forestomach, glandular stomach, small and large intestine, salivary glands, pancreas, liver), muscle tissues (striated skeletal muscle of hind legs, striated cardiac muscle, smooth muscle of digestive tract, and bladder), nervous system (cerebellum, cerebrum, spinal cord, ganglion), urinary system (kidney, bladder), endocrine system (pituitary gland, thyroid gland, adrenal gland), reproductive system (ovary, oviduct, uterus, vagina, testis, epididymis, prostate gland), immune system (thymus, spleen), hair and skin.

Production of Chimeric Mice

Chimeric mice were produced from inbred strain mice by an aggregation procedure (18). Eight-cell stage embryos of two strains (C3H/HeN and C57BL/10, or C3H/HeN and BALB/c) were collected by oviduct flushing and zona pellucidae removed by enzymatic digestion with pronase (16). A pair of embryos of each genotype were then aggregated in Whittingham medium (37). After 1 d in culture, the aggregated embryos that had reached the blastocyst stage were surgically transferred into the uterus of pseudopregnant ICR mouse hosts, brought to term and reared by ICR foster mothers. 35 chimeric mice, 2 mo of age, were used in the analyses reported here.

Preparation of Cultured Cells

10-d embryos from C3H/HeN, C57BL/10 and BALB/c strain mice and 14-d embryos from Wistar rats were used. The pregnant mothers were killed by an overdose of inhaled ether. 10 embryos were taken out and minced with scissors. Chopped tissues were incubated in PBS containing 0.25% trypsin and 0.25% EDTA for 10 min at 37°C. Then, tissues were pipetted to dissociate the cells. After allowing nondissociated tissues to settle the supernatant

1. *Abbreviations used in this paper*: GPI, glucose phosphate isomerase; CSA, C3H specific antigens.

was collected and centrifuged at 1,500 rpm for 10 min. Supernatant was discarded and cells were resuspended into 10 ml of Ham's F-12 medium (Gibco, Grand Island, NY) containing 10% FCS, transferred to Tissue Tek chambers (Miles Laboratories, Malvern, IL) and cultured overnight in an incubator regulated at 5% CO_2 and 95% air and at 37°C. The following morning, most cells had adhered to the bottom of chambers. These cells were fixed with ice-cold 95% ethanol containing 1% acetic acid for 1 h, dehydrated in 100% ethanol for 30 min, dried in air, and stored at −20°C until use. The fixed cells were stained according to the immunofluorescence procedure described below.

Preparation of CSAs and Immunization Procedures

Muscles and livers were collected from C3H/HeN strain mice and stored at −80°C until use. 100 g of each tissue were homogenized in 500 ml extract buffer. Extract buffer (Buf. A) was 0.05 M triethanolamine buffer (pH 8.2) containing 0.1% 2-mercaptoethanol and 1 mM EDTA. The homogenate was centrifuged at 40,000 rpm for 90 min. The supernatant was collected and concentrated by precipitation with 0.8 saturation $(NH_4)_2SO_4$ overnight at 4°C. Precipitates were collected by centrifugation (30 min at 10,000 rpm), and resuspended in 100 ml of Buf. A containing 0.3 M KCl. Nonsoluble components were again removed in the same way. Supernatant was collected and filtered through a Sephacryl S-300 gel column (1 × 100 cm). The fractions containing GPI activity were collected, because the antigen of interest was included in the same fractions as GPI. These fractions were pooled, concentrated to 30 ml with an Amicon concentrator, dialyzed against PBS and used as antigen. Final concentration of antigen was adjusted to 1 mg total protein/ml. BALB/c, AKR, SJL/J, (AKR × BALB/c) Fl and (BALB/c × SJL/J) Fl female mice 6-8-wk old were injected in inguinal lymph nodules with 0.5 ml of an emulsion composed of 5 vol of antigen and 1 vol of Freund's incomplete adjuvant. 30 mice of each strain were used. After 1 mo, second injections were performed in the same way. Usually, we immunized each mouse five times. Existence of an antibody was screened by using immunohistochemical staining of primary cultured embryonic cells derived from C3H/HeN embryos and of tissue sections of C3H/HeN, C57BL/10 and BALB/c strains. Specificity of the antibody was also examined by immunoblotting and an adsorption test using liver cell powder from both strains.

Immunoadsorption and neutralization were performed to check whether this antibody inhibits GPI enzyme activity. It did not (data not shown). The antigen molecules therefore seem not to be GPI or GPI related molecules. Characterization of the distinctive strain specific molecules is in progress and will be reported separately.

Immunoblotting Technique

SDS-PAGE was performed on 10% polyacrylamide gels (7 cm × 8 cm), according to Laemmli's procedure (14). Separated proteins were then transferred onto nitrocellulose membranes in Tris-glycine buffer containing 10% methanol and 0.1% SDS, by constant voltage (8 V/cm) for 2-4 h at 4°C. The membrane was preincubated in PBS containing 5% normal goat serum (NGS) and 1% BSA for 1 h and subsequently incubated with first antibody at room temperature for 1 h or at 4°C overnight. The membrane was then washed with cold Tris-buffered saline (TBS, 50 mM Tris-HCl buffer containing 0.9% NaCl, pH 8.0) three times for 30 min each and then incubated with goat anti-mouse IgG (Zymet) for 1 h at room temperature. After washing with cold TBS, the membrane was incubated with mouse Peroxidase-anti-Peroxidase complex (Zymet) for 1 h at room temperature. The enzymatic reaction of peroxidase was performed with TBS containing 0.05% 3,3'-diaminobenzidine tetrahydrochloride as substrate and 0.05% H_2O_2.

Immunohistochemical Procedures

Mice were fixed by cardiac perfusion with ice-cold 95% ethanol containing 1% acetic acid. After dehydration with 100% ethanol, each tissue was embedded in polyester wax containing 10% ethanol according to Kusakabe's method (12), and cut into 4-μm serial sections. Sections were rinsed with 100% ethanol two times to remove the wax and subsequently ethanol was washed out with PBS. These sections were preincubated with PBS containing 5% NGS and 1% BSA to block nonspecific antibody reactions and then incubated with anti-CSAs antibody for 1 h at room temperature. After washing, sections were incubated with FITC-labeled goat anti-mouse IgG for 1 h at room temperature, washed, and examined by fluorescent microscopy.

Results

Incidence of Antibody

We could recover the C3H specific antibody only from immunized (BALB/c × SJL/J) F1 mice. Other mice failed to develop the C3H specific antibodies. 5 of 30 immunized (BALB/c × SJL/J) F1 mice produced antibodies which recognized only cells of C3H strain.

Specificity of the Antibody Against C3H/HeN and BALB/c Strain Mice

As shown in Fig. 1, this antibody specifically reacted with two proteins with molecular weights of ∼67,000 and 66,000 D, respectively, from C3H/HeN strain mouse. These molecular weights were calculated by using pre-stained SDS-PAGE standards (Bio-Rad Laboratories, Richmond, CA) and immunoblotting methods. The antibody did not react with these standard proteins or with any proteins from BALB/c strain mouse, using immunoblotting methods. When this antigen was preadsorbed with acetone-fixed liver cell powder (LCP) from both strains, it was completely adsorbed by LCP from C3H/HeN, but not by BALB/c LCP (Fig. 2).

General Observations of CSAs Localization in Cultured Cells and Normal Tissues

The specificity of antibody was further checked by immunofluorescent techniques. To establish suitable procedures for making histological sections and localizing CSA, both primary cultured embryonic cells and tissues of adult mice fixed by cardiac perfusion were used. Many tissues were collected, embedded in polyester wax and sectioned according to the procedure described in Materials and Methods. These cells and sections were immunohistochemically stained with anti-CSAs antibody. We also tested various fixative solutions, including buffered neutral 10% formalin and 4% paraformaldehyde, but ice-cold 95% ethanol containing 1% acetic acid solution was better than any of these for maintaining the antigenicity of the CSAs. Cardiac perfusion was also better than immersion to retain both antigenicity and fine structure of tissues.

CSAs were recognized as small fluorescent particles uniformly dispersed through the cytoplasm of cells from C3H/HeN strain, but not in cells from BALB/c strain (Fig. 3 a). No CSAs were found in the nucleus or plasma membrane. The tissues examined are listed in Table I. CSAs were detected in all types of cell including both mesenchyme and epithelium. However, the particles in epithelium were much
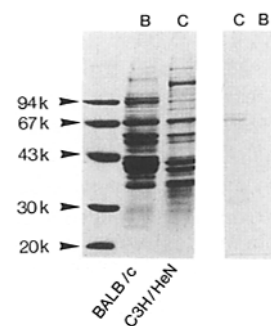

Figure 1. Test for the strain specificity of the anti-CSAs antibody by immunoblotting technique. (Lane *B*) Soluble fraction of homogenized liver from BALB/c. (Lane *C*) Soluble fraction of homogenized liver from C3H/HeN. Only two bands in lane *C*, molecular weight ∼67,000 and 66,000, were recognized by the antiserum.
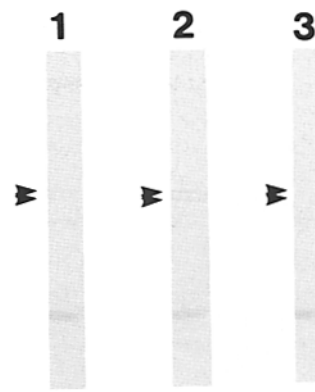

Figure 2. Immunoadsorption test for the strain specificity of the anti-CSAs antibody by immunoblotting technique. Soluble fraction of homogenized liver from C3H/HeN were applied to all lanes. Lane *1* was stained by the nonadsorbed antibody. Two positive bands are shown (*arrowheads*). Other two bands are light and heavy chains of immunoglobulin. Lane 2 was stained by the antibody preadsorbed by aceton fixed liver cell powder (LCP) of BALB/c. Same bands (*arrowheads*) were recognized. Lane *3* was stained by the antibody preadsorbed by LCP of C3H/HeN. These bands were not detected.

more numerous than those in mesenchyme. The density of these particles seemed to depend on the status of the cells. When cells were filled with products, such as mucus, hormones and zymogen granules etc., occupying a large portion of the cytoplasm, the fluorescent particles were reduced in number in histological sections. Fluorescence was observed in axons and dendrites of neural cells. Only enucleated keratinocytes (which exist in the superficial layer of squamous epithelium) entirely lacked CSAs. Endothelial cells in section barely stained enough to visualize. CSAs were consistently absent from the BALB/c cells (Fig. 3, *b–d*).

Tests of the Six Criteria for a Strain-Specific Marker

Primary cultured cells from embryos of C3H strain mice and conditioned medium from such cultures were screened with antibody. CSAs were detected in the cells, but not in the medium. Next, C3H cells and rat cells were co-cultured using the Millicell-CM (Millipore) system to test the possibility that CSAs are transferable between cells. In this system, there is no direct contact between the two different cell populations, which are cultured on two sides of a permeable membrane. Cells and medium were screened by immunoblotting methods. CSAs were detected only in the C3H cells, and not in the rat cells or in the common culture medium. These outcomes indicated that CSAs are cell-localized and not secreted extracellularly. Primary cultured C3H cells were also passaged three times and stained when both confluent and nonconfluent. All cells in passaged cultures stained with the antibody. This indicates that CSAs are expressed in the mitotic progeny of cells containing the antigen. Next, C3H cells were mixed with cultured rat fibroblastic cells to test whether CSAs can transfer between adjacent cells in vitro or not. Identification of cells of each species was based on differences in the Hoechst dye staining patterns of nuclei of mice and rats (3, 19). All antigen positive cells had the mouse pattern, and negatives were of the rat pattern. We also tested for exchange of antigen between tissues in vivo, using a tissue-recombination chimeric system. Salivary glands were dissected from 14-d embryos of C3H and BALB/c mice, and each organ was separated into epithelial and mesenchymal components by collagenase digestion (13). Mammary fat pad precursor tissue (FP) was also dissected
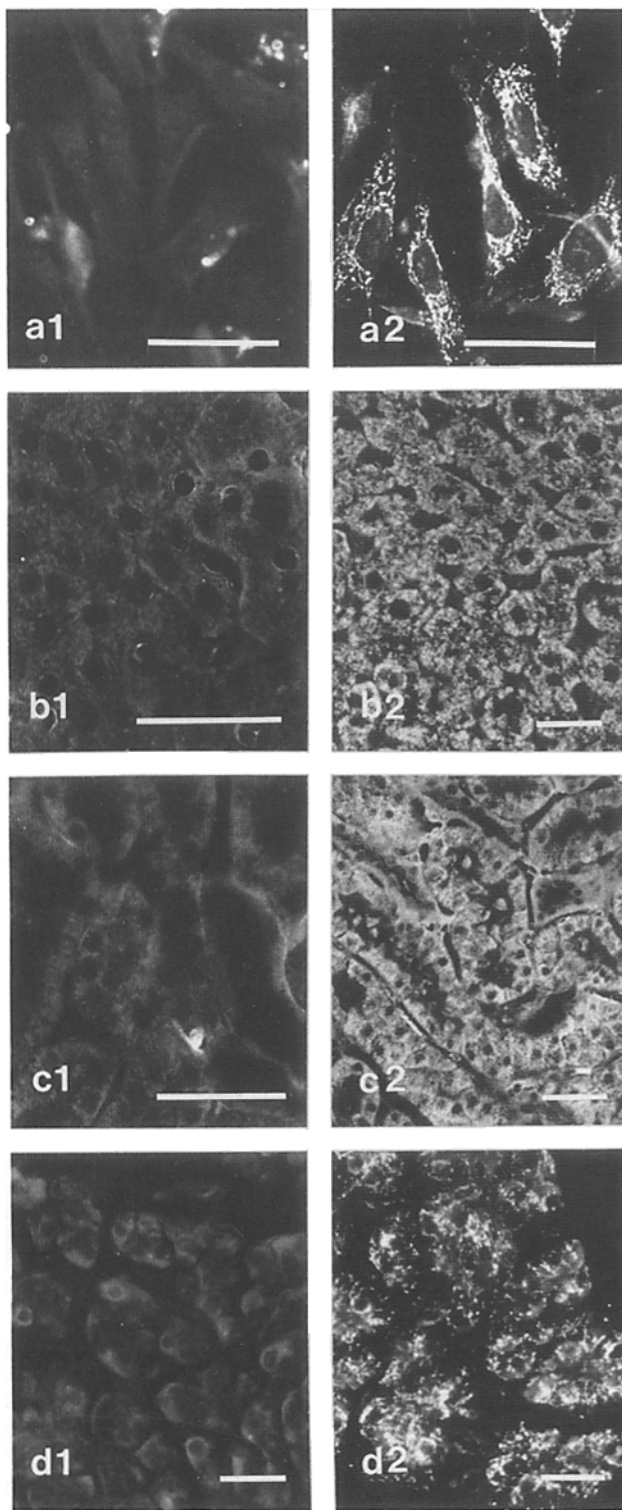
Figure 3. Indirect immunofluorescent staining of cultured embryonic cells derived from BALB/c (*a1*) and from C3H/HeN (*a2*). CSAs were exhibited as a particle only in the cytoplasm of the cells derived from C3H/HeN, but not in those from BALB/c. FITC particles in *a1* are nonspecific binding to the dust or cell debris. Indirect immunofluorescent staining of normal tissues from BALB/c (*left*) and from C3H/HeN (*right*). Tissues from C3H/HeN were positively stained by anti–CSAs antibody, but not those from BALB/c. Liver (*b*, *1* and *2*), kidney (*c*, *1* and *2*), and pancreas (*d*, *1* and *2*). Bars, 50 μm.

from these embryos. Then, salivary epithelium of each strain was recombined with FP of the other strain and transplanted under kidney capsules of BALB/c nude mice. Resulting tissues were recovered after 2 wk, fixed and sectioned, and examined immunohistochemically. As shown in Fig. 4, in each recombinant CSAs were demonstrated only in the tissue type supplied by the C3H donor. These findings demonstrated that CSAs are cell autonomous, not transferred between cells either in vitro or in vivo. Furthermore, expression of CSAs is not affected by the surrounding tissue environment. Finally, embryonic salivary epithelium from BALB/c strain was recombined with salivary mesenchyme from either the BALB/c or C3H strain, and recombinants were maintained under kidney capsules as described above. Both kinds of recombinant developed into histologically normal salivary gland structures. This demonstrated that tissues that express CSAs do not influence the developmental process in neighboring tissues, and that CSA-expressing cells are capable of entirely normal development themselves.

CSAs-staining Patterns in Chimeric Organs

35 aggregation chimeras between C3H/HeN and BALB/c, and C3H/HeN and C57BL/10, were examined. In sections of the tissues listed in Table I, both positive cells and negative cells were observed in the same tissues in most organs. The features of immunostaining in the positive cells were basically similar to those in tissue and cultured cells from C3H mice (described above). The positive cells and negative cells were generally observed in distinctive groups or clusters. The border between positive and negative populations was often very definite. We could classify the chimeric tissues into four definite categories, based on staining patterns (Fig. 5). In the tissues made up of definite structural units, such as intestinal crypt and villus, liver, glandular stomach, acinus of exocrine gland, islet of Langerhans, prostate gland, seminiferous tubes, thyroid follicles, ovarian follicles, the three layers of adrenal cortex, hair follicles, convoluted tubules etc., two different mosaicisms were observed: Pattern A: Each unit was composed entirely of either positive or negative cells. For example, the small intestinal crypts were composed of either positive or negative cells, and positive crypts were often located close to each other (Fig. 6, *a* and *c*). However, each villus was composed of both types of cell (Fig. 6 *b*), because the villus maintains its morphology by the migration of cells from several adjacent crypts. Mesenchymal cells and muscle layers of the small intestine however were composed of both types of cell intermingled with one another. Features of immunostaining of crypts, mesenchymal cells and muscle layers in the large intestine were the same as those of small intestine. Pattern B: Each unit such as pancreatic acinus, thyroid follicle, villus of small intestine etc., was composed of both positive and negative cells. In the chimeric pancreas, parenchymal cells exhibited FITC-labeled particles in the cytoplasm, but the particles were sparse. The intercalated duct and pancreatic duct were composed of both positive and negative cells. Some acini consisted of both types of parenchyma (Fig. 7). In islets of Langerhans, there was no correspondence between positive or negative cells and each type of endocrine cell. Mesenchymal cells were derived from both types of cells. In kidney, the ducts of uriniferous tubules were composed of both types

Table I. List of Chimeric Patterns in Tissue Sections Stained by Anti–CSAs Antibody

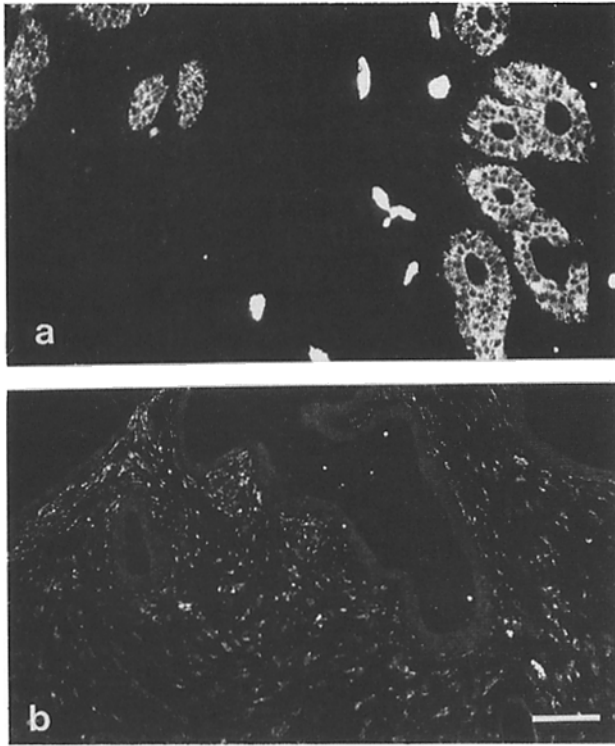| Name of tissue	Pattern Epi/Mes	Fig. No.	Name of tissue	Pattern Epi/Mes	Fig. No.
Cerebral cortex	D/No		Pancreas	-/D	7
Cerebellum	D/No		*Acinus	B/-	
Choroid plexus	D/D		*Islet of Langerhans	B/-	
Peripheral neuron	D/D				
Spinal ganglion	D/No		*Ducts	B/-	
Ganglionic plexus	D/No		Skin	C/D	
Striated skeletal muscle	D/D	10 a	Hair		
			*follicle	B/D	
Smooth M.	D/No	10 b	Kidney		8
Cardiac M.	D/No	10 c	*Bowman's capsule		
Thymus			Visceral	D/-	
*Reticular cell	D/No		Parietal	B/-	
*Lymphocyte and			*Uriniferous Tube	B/-	
Macrophage	D/No				
Tongue	C/D	9	Bladder	-/D	
Esophagus	C/D		*Transitional		
Forestomach	C/D		Epithelium	D/D	
Glandular stomach			Pituitary gland	-/D	
*Gastric pit	D/D		*Pars distaris	D/-	
*Gastric gland	B/D		*Pars intermedia	D/-	
Small intestine	-/D	6 a	Thyroid gland	-/D	
*Villus	B/-	6 b	*Follicle	B/-	
*Crypt	A/D	6 c	*Parafollicular cell	D/-	
Large intestine	A/D				
Liver	B/No		Adrenal gland	-/D	
Ovary	-/D		*Cortex (3 layers)	B/-	
*Oocyte	A/-		*Medulla	D/-	
*Granulosa cell	D/-		Testis	-/D	
*Theca follicule	D/-		*Seminiferous tube	B/-	
*Corpora Lutea	D/-				
Oviduct	C/D		Sertoli	D/-	
Uterus	D/D		Germ cell	D/-	
Vagina	C/D		*Interstitial cells	D/D	
Salivary glands	-/D				
*Acinus	B/-		Epididymis	B/D	
*Ducts	B/-		Prostate gland	B/D	

No: Not identified.
* Fine structure in each tissue. A, B, C, and D definitions; refer to Fig. 5.

of cell (Fig. 8). These cells were well intermingled with one another. The loop of Henle in the medulla and both parietal and visceral layers of Bowman's capsule were also composed of both types of cell. Interstitial cells and kidney capsule cells were composed of stained and unstained cells intermingled with one another.

Different staining patterns were observed in tissues lacking such distinctive functional units, such as pars distalis and pars intermedia of pituitary gland, stratified squamous epithelium of skin, tongue, esophagus, forestomach, and vagina, epithelium of gastric pits, muscle tissues, central and peripheral nervous system, thymus, spleen, bladder, lung mesenchymal tissues, etc. Staining patterns of these tissues were: Pattern C: Tissues constituted of definite small cell groups composed of either positive or negative cells. As shown in Fig. 9, epithelial cells in the tongue surface were arranged in distinct cell groups. The positive cell group was located on one side of each filiform papilla. Superficial enucleated keratinocytes were not stained by the anti–CSAs antibody. Connective tissues in the lamina propria were
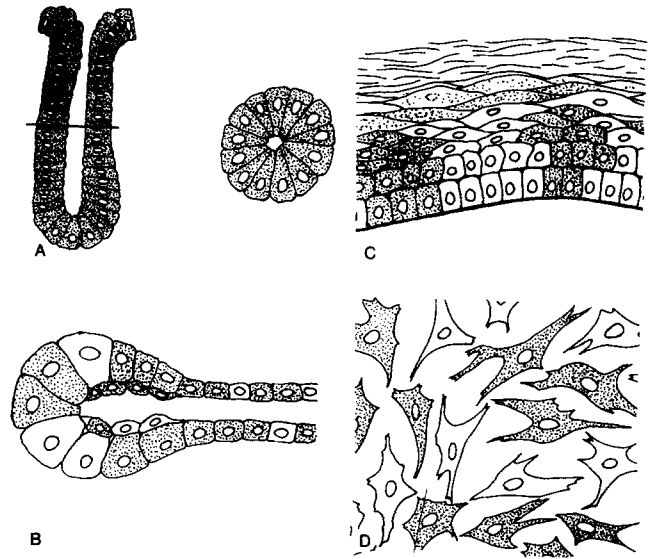
composed of both types of cell. These cells intermingled with one another. The mosaic pattern in tongue muscle was similar to that in striated skeletal muscle described later. Pattern D: Tissues composed of both cell types randomly intermingled with one another. However, in pattern D, positive cells appeared to be in groups close to each other. The mesenchymal cells in many chimeric organs were organized in this way. In transverse sections of striated muscle fibers, diffuse immunofluorescent particles were detected (Fig. 10 a). In some fibers, FITC-labeled particles were dispersed throughout all of the cytoplasm; in other fibers, particles were not detectable; in some fibers, only a few particles were observed. The variability seemed to depend on where along their length the fibers cut. As shown in Fig. 10 b, cytoplasm of smooth muscle stained very well. Positive cells were in groups and the edge of each group was not well defined. FITC-stained particles were uniformly detected in cytoplasm of cardiac muscle cells. The positive cells intermingled with negative cells, but tended to group close to each other (Fig. 10 c).

Figure 5. Classification of chimeric patterns stained by anti–CSAs antibody. (*A*) Tissue consists of definite structural units composed of either positive or negative cells. (*B*) Tissue consists of definite structural units composed of both types of cell. (*C*) Uniform tissue consists of many definite small cell groups composed of either positive or negative cells. (*D*) Uniform tissue consists of both types of cell intermingled with one another.

Figure 4. Indirect immunofluorescent staining of the sections of tissue recombinants between embryonic salivary epithelium and mammary fat pad precursor tissue transplanted under the kidney capsule for 2 wk. (*a*) Recombinant between salivary epithelia from C3H/HeN and fat pad precursor tissue from BALB/c, (*b*) recombinant between salivary epithelia from BALB/c and fat pad precursor tissue from C3H/HeN. Both salivary epithelia (*a*) and fat pad precursor tissue (*b*) from C3H/HeN were positively stained by anti–CSAs. In *b*, negative cells in mesenchyme were derived from the host (BALB/c-nude mouse). Bar, 50 μm.

Discussion

Recently, many attempts have been made to demonstrate mosaicisms in chimeric tissues for analysis of cell lineage. However, there has been no ideal cell-marking method which effectively satisfied the six criteria proposed previously (15, 21). Many markers have restricted utility because they are not persistently expressed, or recognizable, in all kinds of tissue, especially in mesenchyme. Our goal therefore was to develop a more generally useful marker that could be applied to a broader spectrum of organs and tissues. We therefore made polyclonal antibody by immunizing (BALB/c × SJL/J) F1 with widely-expressed C3H proteins and used this antibody to stain chimeric tissues. This antibody could stain all primary cultured cells from C3H strain embryos but not cells from BALB/c embryos. FITC-tagged particles were widely distributed in the cytoplasm of cells, including such narrow structures as axons and dendrites. The specificity of this antibody was demonstrated using immunoblotting and immunoadsorption tests. The proteins (molecular weight 67,000 and 66,000 D) recognized by this antibody were detected only in the C3H strain mouse. We have checked the specificity of the antibody against other strains including A/HeN, DBA/2N, 129/VJ, LT/Sv, C58/J, C57BL/6J, NFS/N, SJL/J, CBA/J, AKR/N, and GR (data not shown). The antiserum recog-

nized only C3H strain, but not any others. We have used this antibody to stain histological sections. CSAs were demonstrated in such sections as well as in cultured cells. The antigenicity of CSAs seemed to be labile to aldehyde fixatives, but is preserved by 95% ethanol containing 1% acetic acid. Antigenicity was not effectively preserved in cryosections. The optimal procedure is to embed tissues in polyester wax (12) after fixation by cardiac perfusion. This method is simple and effectively maintains both fine structure and antigenicity.

It is of the utmost importance to demonstrate that CSAs remain in the cells that produce them, and are not passed between cells. Beta-glucuronidase, for example, is a protein that is transferred between cells in vitro (20) and in mouse chimeras (4, 11). If CSAs are transferable, it is possible that they will mark adjacent nonproducing cells in chimeras. Several experimental tests were therefore carried out to check for antigen transfer between cells, and all of the rest of the six criteria for an ideal cell marker. As described in Results, CSAs seem to be autonomous proteins located only inside the cells and not secreted extracellularly. Even when C3H cells were immediately adjacent to nonCSAs-producing cells, both in vitro and in vivo, CSAs were not transferred. CSAs seemed not to affect development processes during the histogenesis observed in embryonic tissue recombinants. This marker system therefore effectively fulfills the six criteria of a usable cell-strain marker.

As shown in many chimeric organs, the chimeric patterns were made conspicuous in histological sections by this antibody. As summarized in Table I, most organs of chimeras consist of both types of cell. The chimerisms observed in some organs such as intestine (22–24, 31), brain (7, 21), and liver (36), were revealed by our marker system as effectively as by other markers used previously. However, our marker
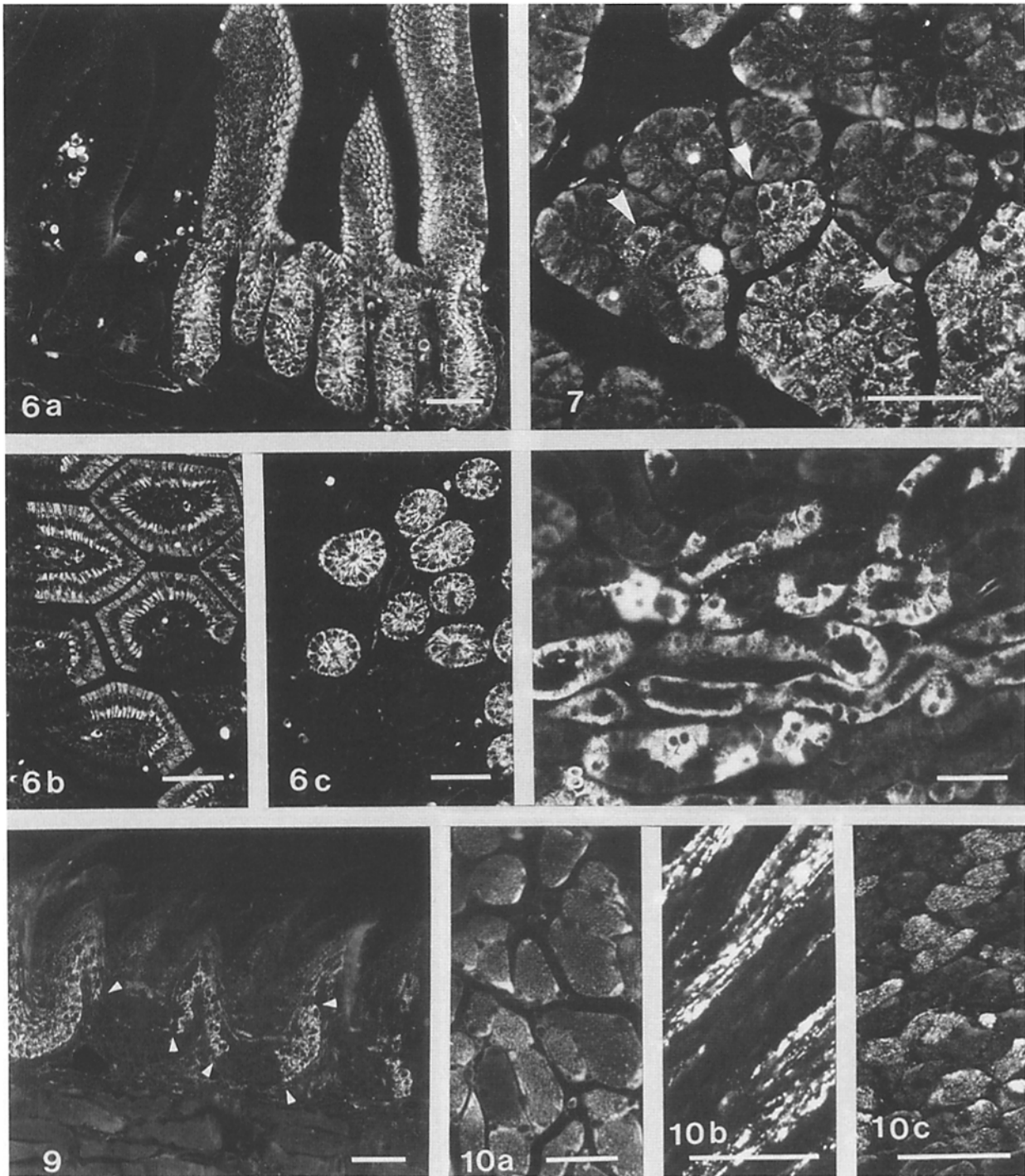
Figures 6–10. (Fig. 6) Small intestine: Sagittal section of small intestine (*a*) and transverse section of villus (*b*) and of crypt (*c*). Chimeric villi were observed (*b*), but there are no chimeric crypts (*c*) shown. (Fig. 7) Pancreas: Single acinus consisted of two types of cell (*arrowheads*). (Fig. 8) Kidney: Uriniferous tubules were composed of both types of cell intermingled well with one another. (Fig. 9) Tongue: Positive cells in filiform papilla were observed as definite cell groups (*arrowheads*). (Fig. 10) Muscular tissues: Transverse section of striated skeletal muscle (*a*), sagittal section of the smooth muscle (*b*) and transverse section of striated cardiac muscle (*c*). Bars, 50 µm.

system also revealed chimerisms in other organs which have not been amenable to examination before. Basically, chimeric staining patterns seem to be classifiable into four groups (Fig. 5). Group A includes units such as the intestinal crypt and Group C includes the stratified squamous epithelium; multicellular chimeric units in these structures are likely to correspond with cell proliferative units, or clones (9, 10, 25–28). It is likely that patterns B and D also reflect patterns of cell replication and migration, but the underlying basis of pattern development has not yet been determined. Although chimerisms demonstrated here were all observed in 2-mo old mice, it is reasonable to expect that the same pat-

terns will be maintained throughout life as each tissue grows and lost cells are replaced. However, the pattern observed in the adult may differ from that in the younger mouse. An example of this is the intestinal crypt. Previous papers (24, 31) on the chimeric crypt indicated that each crypt in the adult mouse is derived from a single progenitor cell. However, our preliminary observations suggest that chimeric crypts are common in young mice 40 d-old (Kusakabe, M., M. Yoko-yama, and T. Sakakura, manuscript in preparation). Crypt formation is occurring actively before weaning. From 4 w onward a steady-state number of crypts appears to be reached (1). At this point, it appears that there is a transition from the chimeric crypt of young mice into the adult pattern. The way in which this occurs is unknown. We are now studying chimeric mice of different ages to explore this issue.

A previous report on the adrenal gland (35) indicated that the adrenal cortex consists of clonal proliferating units in the chimeric rat. However, our observations on chimeric adrenal cortex of the mouse showed that each glomerulosa consisted of two types of cell. Perhaps the nonconcordance between our findings and previous work reflects the sensitivity of our marker system. It is difficult to detect a single negative cell or positive cell in a positive or negative cell mass by using radioactive cell constituents. It is also to be noted that certain other well defined functional units such as the acini of exocrine glands consisted of both types of cell (group B). These findings suggest that each acinus of an exocrine gland develops from a few originator cells derived from both strains. However, the pattern in the liver lobules was too complex to explain the cell proliferative unit in any obvious or simple way, but proliferative units clearly must exist in every organ like liver which has a complicated chimeric pattern. Group D includes the brain, thymus epithelium, pituitary gland, mesenchymal cells in many organs, etc. These tissues and organs are established in early embryonic stages and their chimeric patterns seem to reflect active cell movement during morphogenesis.

These four different chimeric patterns should be helpful for understanding the cell renewal system in a variety of organs. To consider cell lineage as a dynamic morphogenetic process, our techniques can be used to analyze embryonic morphogenesis. The technique should also prove useful for understanding various other normal and pathological processes such as regeneration, carcinogenesis, infiltration of cancer, and metastasis in chimeric mice. Such analyses are now underway.

One disadvantage of this system is that the amount of antisera is very small, because the polyclonal antibody was developed in mice. It is also difficult to maintain uniform specificity, because each immunized mouse develops a slightly different quality and spectrum of antibody. We have now developed a monoclonal antibody to the same CSAs (Kusakabe, M., and T. Sakakura, manuscript in preparation). This antibody has the advantages of the polyclonal antibody for analyzing chimeric patterns, and overcomes the shortcomings listed above. Using the monoclonal antibody we have also initiated analyses of the structure and function of the distinctive cell-specific antigens.

References

1. Cheng, H., and M. Bjerknes. 1985. Whole population cell kinetics and post natal development of the mouse intestine epithelium. *Anat. Rec.* 211: 420–426.
2. Condamine, H., R. P. Custer, and B. Mintz. 1971. Pure-strain and genetically mosaic liver tumors histochemically identified with the beta-glucuronidase marker in allophenic mice. *Proc. Natl. Acad. Sci. USA.* 68: 2032–2076.
3. Cunha, G. R., and K. D. Vanderslice. 1984. Identification in histological sections of species origin of cell from mouse, rat, and human. *Stain Technol.* 59:7–12.
4. Feder, N. 1976. Solitary cells and enzyme exchange in tetraparental mice. *Nature (Lond.).* 263:67–69.
5. Garner, W., and A. McLaren. 1974. Cell distribution in chimeric mouse embryos before implantation. *J. Embryol. Exp. Morph.* 32:495–503.
6. Gearhart, J., and M. L. Oster-Granite. 1980. An immunofluorescence procedure for the tissue localization of glucose phosphate isomerase. *J. Histochem. Cytochem.* 28:245–249.
7. Goldowitz, D., and R. J. Mullen. 1982. Nuclear morphology of Ichthyosis mutant mice as a cell marker in chimeric brain. *Dev. Biol.* 89:261–267.
8. Hillman, N., I. Sherman, and C. Graham. 1972. The effect of spatial arrangement on cell determination during mouse development. *J. Embryol. Exp. Morph.* 28:263–278.
9. Hume, W. J., and C. S. Potten. 1976. The ordered columnar structure of mouse filiform papillae. *J. Cell Sci.* 22:149–160.
10. Hume, W. J., and C. S. Potten. 1983. Proliferative units in stratified squamous epithelium. *Clin. Exp. Dermatol.* 8:95–106.
11. Herrup, K., and R. J. Mullen. 1979. Intracellular transfer of beta-glucuronidase in chimeric mice. *J. Cell Sci.* 40:21–31.
12. Kusakabe, M., T. Sakakura, Y. Nishizuka, M. Sano, and A. Matsukage. 1984. Polyester wax embedding and sectioning technique for immunohistochemistry. *Stain Technol.* 59:127–132.
13. Kusakabe, M., T. Sakakura, M. Sano, and Y. Nishizuka. 1985. A pituitary-salivary mixed gland induced by tissue recombination of embryonic pituitary epithelium and embryonic submandibular gland mesenchyme in mice. *Dev. Biol.* 110:382–391.
14. Laemmli, V. K. 1970. Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680.
15. McLaren, A. 1976. Mammalian Chimareras. Cambridge University Press, London, New York.
16. Mintz, B. 1962. Experimental study of the developing mammalian egg: removal of the zona pellucida. *Science (Wash. DC).* 138:594–595.
17. Mintz, B. 1967. Gene control of mammalian pigmentary differentiation. I. Clonal origin of melanocytes. *Proc. Natl. Acad. Sci. USA.* 58:344–351.
18. Mintz, B. 1971. Allophenic mice of multi-embryo origin. *In* Methods in Mammalian Embryology. J. C. Daniel Jr., editor. Freeman, San Francisco. 186.
19. Moser, P. G., B. P. Dorman, and F. H. Ruddle. 1975. Mouse-human heterokaryon analysis with A 33258 hoechst-giemsa technique. *J. Cell Biol.* 66:676–680.
20. Olsen, I., Dean, M. F., Harris, G., and Muir, H. 1981. Direct transfer of a lysosomal enzyme from lymphoid cells to deficient fibroblasts. *Nature (Lond.).* 291:244–247.
21. Oster-Granite, M. L., and J. Gearhart. 1981. Cell lineage analysis of cerebellar purkinje cell in mouse chimeras. *Dev. Biol.* 85:199–208.
22. Ponder, B. A. J., M. M. Wilkinson, and M. Wood. 1983a. H-2 antigens as markers of cellular genotype in chimeric mice. *J. Embryol. Exp. Morph.* 76:83–93.
23. Ponder, B. A. J., and M. M. Wilkinson. 1983b. Organ-related differences in binding of Dolichos biflorus agglutinin to vascular endothelium. *Dev. Biol.* 96:535–541.
24. Ponder, B. A. J., G. H. Schmidt, M. M. Wilkinson, M. J. Wood, M. Monk, and A. Reid. 1985. Derivation of mouse intestinal crypts from single progenitor cells. *Nature (Lond.).* 313:689–691.
25. Potten, C. S. 1974. The epidermal proliferative unit: The possible role of the central basal cell. *Cell Tissue Kinet.* 7:77–88.
26. Potten, C. S., and J. H. Hendry. 1975a. Differential regeneration of intestinal proliferative cells and cryptogenic cells after radiation. *Int. J. Radiat. Biol.* 27:413–424.
27. Potten, C. S. 1975b. Small intestinal crypt stem cells. *In* Stem Cells of Renewing Cell Population. A. B. Cairnie, P. K. Lala, and D. G. Osmond, editors. Academic Press. New York. 78–84.
28. Potten, C. S. 1981. Cell replacement in epidermis (Keratopoiesis) via discrete units of proliferation. *Int. Rev. Cytol.* 69:271–318.

29. Rossant, J., M. Vijh, L. D. Siracusa, and V. M. Chapman. 1983. Identification of embryonic cell lineage in histological sections of M. musculus in-equilibrium M. caroli chimeras. *J. Embryol. Exp. Morph.* 73: 179–191.

30. Rossant, J., and V. M. Chapman. 1983. Somatic and germline mosaicism in interspecific chimeras between Mus musculus and Mus caroli. *J. Embryol. Exp. Morph.* 73:193–205.

31. Schmidt, G. H., M. M. Wilkinson, and B. A. J. Ponder. 1985. Cell migration pathway in the intestinal epithelium: An in situ marker system using mouse aggregation chimeras. *Cell.* 40:425–429.

32. Spindle, A. 1982. Cell allocation in preimplantation mouse chimeras. *J. Exp. Zool.* 219:361–367.

33. Tarkowski, A. K. 1961. Mouse chimeras developed from fused eggs. *Nature (Lond.).* 190:857–860.

34. Tarkowski, A. K. 1964. Patterns of pigmentation in experimentally produced mouse chimera. *J. Embryol. Exp. Morph.* 12:575–585.

35. Weinberg, W. C., J. C. Howard, and P. M. Iannaccone. 1985. Histological demonstration mosaicism in a series of chimeric rats produced between congenic strains. *Science (Wash. DC).* 227:524–527.

36. West, J. D. 1976. Patches in the livers of chimeric mice. *J. Embryol. Exp. Morph.* 36:151–161.

37. Whittingham, D. G. 1971. Culture of mouse ova. *J. Reprod. Fert.* 14 (Suppl.):7–21.