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Immunolocalization of a Novel Collectin CL-K1 in Murine Tissues.

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CL-K1 IN MURINE TISSUES

IMMUNOLOCALIZATION OF A NOVEL COLLECTIN CL-K1 IN MURINE TISSUES

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ABSTRACT

We have recently identified a novel collectin, CL-K1, that may play a role in innate immunity as a member of the collectin family. In this study using mice, we investigated the tissue distribution of CL-K1 for better understanding of its pathophysiological relevance. Real-time PCR analyses demonstrated that CL-K1 mRNA was expressed in all tissues tested. Immunohistochemical analyses demonstrated that CL-K1 was expressed in proximal tubules of kidney, in mucosa of the gastrointestinal tract, and in bronchial glands of bronchioles similar to the localization of SP-A and SP-D in these pulmonary structures. Immunohistochemistry also showed that CL-K1 was highly expressed in hepatocytes around the central veins in liver, which suggests that murine CL-K1 might be mainly produced in the liver and secreted into the blood stream as is human CL-K1. CL-K1 was especially detected in vascular smooth muscle in several types of tissue. In addition, it was also expressed in intestinal Paneth cells, in mesangial cells of kidney, in pancreatic islet D cells, and in neurons of the brain. It is of interest that this profile of CL-K1 expression is unique among the collectins. Taken together, these histological findings may be useful for the understanding biological function of this novel collectin.

Key words: CL-K1, colec11, collectin, MBL, mouse, somatostatin, Paneth cells

INTRODUCTION

Collectins are a family of proteins that contain two characteristic structures, a collagen-like region and a carbohydrate recognition domain (CRD) (Drickamer et al. 1998). There are three classical collectins in humans: mannan-binding lectin (MBL) (Kawasaki et al.1983, Sastry et al.1991, Laursen et al.1998, 2000), and surfactant proteins A and D (SP-A and SP-D) (Benson et al.1985, Haagsman et al. 1987, Andersen et al. 1992). MBL, a plasma collectin synthesized in the liver (Sastry et al.1991, Andersen et al.1992, Hansen et al.2002), can kill bacteria through activation of the complement pathway or by opsonization via collectin receptors (Kawasaki et al. 1989, Schweinle et al. 1989). SP-A and SP-D are mainly produced by alveolar type II cells and Clara cells in the lung (White et al. 1985, Lu J et al. 1992, Madsen et al. 2000, 2003, Paananen et al. 2001) and can mediate opsonization of bacteria and neutralization of viral growth. In addition, SP-A and SP-D associate directly with macrophages and stimulate phagocytosis or oxidant-dependent microbial clearance (Sano et al. 2005). Thus, collectins play an important role in innate immunity.

Recently, cDNAs encoding three novel collectins, collectin liver 1 (CL-L1) (Ohtani et al. 1999), collectin placenta 1 (CL-P1) (Nakamura et al. 2001, Ohtani et al. 2001), and collectin kidney 1 (CL-K1) (Keshi et al. 2006) were isolated and characterized by our group as well as by other investigators. CL-L1 is mainly expressed in liver as a cytoplasmic protein. CL-P1 is a membrane type collectin expressed in vascular endothelial cells which binds to oxidized low density lipoprotein (OxLDL) as a scavenger receptor. We have very recently demonstrated a novel human and murine collectin, CL-K1 (Keshi et al. 2006). According to the Mouse Genome Informatics database (http://www.informatics.jax.org/), CL-K1 was first cloned and deposited as RIKEN cDNA 1010001H16 in 2001. It was later assigned the name Colec11 (collectin sub-family member 11) in 2003. The Colec11 gene name is that used in

62 the major databases, including the Genome Expression Omnibus
63 (http://www.ncbi.nlm.nih.gov/projects/geo/) that has 675 entries for expression data of this
64 gene as determined by cDNA microarray. CL-K1 harbors a 25-amino-acid signal sequence
65 and is a secreted type of collectin present in human plasma. CL-K1 can also bind to microbial
66 lipopolysaccharide (LPS) and lipoteichoic acid (LTA), suggesting that it might play an
67 important role in innate immunity. However, little is known about the tissue distribution of
68 CL-K1. In the present study, we generated specific antibody against this collectin for use in
69 immunohistochemistry and determined the tissue distribution of CL-K1 in mice.

MATERIALS and METHODS

Animals and Tissues

Nine-week-old male C57Bl/6Ncrj mice (Charles River Tokyo, Japan) were housed at 22 ºC under 12 hr light/dark cycle (lights on at 7 a.m.) conditions, and were allowed access to food and water ad libitum. For histology and immunohistochemistry, mice were anesthetized with 2.5% avertin and perfused through the left ventricle with 20 ml of ice-cold PBS and then with 4% paraformaldehyde in PBS at 4 ºC for 20 min. Various tissues were then collected, and specimens were dehydrated and embedded in paraffin. For double staining of CD31 and CL-K1, mouse various tissues were fixed by IHV Zinc Fixative (BD Biosciences Pharmingen, San Diego, CA, USA) at room temperature for 24 hour and embedded in paraffin. Five μm-thick sections were stained for immunohistochemistry (IHC) and with Mayer's hematoxylin. All experiments were carried out in accordance with the rules and guidelines of the Animal Experiment Committee of Asahikawa Medical College.

RNA isolation and first strand cDNA synthesis

Total RNA was isolated from small pieces of mouse tissue (80-100 μg) using Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA was reverse-transcribed using RETROscript (Ambion, Austin, Texas, USA). One μg of total RNA was mixed with 2 μl of Random decamers and nuclease-free water in a total volume of 12 μl and heated at 80 ºC for 3 min. The mixture was then chilled on ice and incubated with 2 μl of 10 x RT buffer, 4μl dNTP mix, 1 μl RNase inhibitor, and 1 μl reverse transcriptase, at 44 ºC for 60 min. The reaction mixtures were further incubated for 10 min at 92 ºC. The cDNA was stored at –30 ºC until used for real-time PCR.

Analysis of mRNA expression by real-time PCR

Real-time PCR was performed with 7500 Real Time PCR system (Applied Biosystems,

Foster City, CA, USA) according to the manufacturer's instructions. A Taqman probe primer set for CL-K1 (Mm01289834-m1) was purchased from Applied Biosystems.

Antibodies

Recombinant human CL-K1 including the neck and CRD domains (amino acids 107-271) together with six histidines was expressed in Escherichia coli GI724 using pPLH3 expression vector as described previously (Keshi et al. 2006). CL-K1-CRD-his protein was extracted and purified with Ni-NTA Agarose (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The N-terminal amino acid sequence of the purified recombinant protein was confirmed to be CL-K1-CRD-his. The purified recombinant protein was further characterized as CL-K1-CRD-his by SDS-PAGE and immunoblotting. New Zealand White rabbits were injected three times at 2-week intervals with 200 μg of the above fusion protein in incomplete Freund's adjuvant. After immunization, whole sera from rabbits were applied to HiTrap Protein G HP (Amersham Biosciences Piscataway, NJ, USA) and anti-CL-K1 rabbit IgG fractions were eluted with 0.1 M glycine-HCL buffer at pH 2.5. Furthermore, the anti-CL-K1 IgG was affinity-purified using a CL-K1-CRD-his conjugated antigen column, HiTrap NHS-activated HP (Amersham Biosciences Piscataway, NJ, USA), as described previously (Takeuchi et al. 1997). The IgG fraction which passed through the CL-K1 antigen column was used as the control IgG. The extent of purification was determined by ELISA as described.

ELISA

Microtiter plates were coated overnight at 4 ^{o} C with 10 μg/ml of various collectins, namely, CL-L1-CRD-his, CL-P1-CRD-his, CL-K1-CRD-his, mouse CL-K1-CRD-his, and MBL-CRD-his, in the coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.05% NaN_3, pH 9.6). The plates were washed with TBS (Tris-buffer saline containing 20 mM Tris-HCl and

140 mM NaCl, pH7.4) / TC (0.05% Tween 20 and 5 mM $CaCl_2$), and incubated at 37 ºC for 1 hr with various preparations of anti- CL-K1 antibodies containing the IgG fraction of the anti-CL-K1 serum, the affinity-purified anti-CL-K1 IgG, or the control IgG fraction. After washing, they were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Chemicon International, Temecula, CA, USA) followed by color development using a TMB-Peroxidase Substrate System (Kierkegaard & Perry Laboratories. Gaithersburg, MD, USA). The reaction was stopped with 1 M phosphoric acid and absorbance at 450 nm was measured.

Immunocytochemistry

CHO-K1 cells (ATCC, Rockville, MD, USA) were stably transfected with human CL-K1 expression vectors as described previously (Keshi et al. 2006). The transfected cells (CHO/CL-K1) were plated in 14-mm wells of 35-mm plastic culture dishes (Matsunami Glass Industries, Tokyo, Japan) and cultured in Ham's F-12 medium containing 5% fetal bovine serum. CHO/CL-K1 cells were fixed with 4% paraformaldehyde in PBS at 4 ºC, and permeabilized and blocked in BlockAce (Dainippon Seiyaku, Osaka, Japan) for 1 hr at room temperature. The cells were then incubated with affinity-purified CL-K1 IgG or control IgG (1μg/ml) overnight at 4 ºC, followed by treatment with anti-rabbit IgG-conjugated Alexa 488 and TO-PRO-3 (Molecular Probes, Eugene, OR, USA). Fluorescent images were observed with a confocal laser-scanning microscope FV1000 (Olympus Optical, Tokyo, Japan). All immunofluorescence images show fluorescence overlaid on phase contrast images.

Immunohistochemistry and immunofluorescence analyses

Immunohistochemical staining was carried out by using the avidin-biotin complex method and for immunofluorescence, the indirect fluorescence staining method was employed. Five

142 μm-thick tissue sections were cut and placed onto slides and almost all sets of slides were
143 processed together in the following steps. Slides were deparaffinized through a series of
144 xylene and ethanol baths. Sections were blocked in BlockAce for 1 hr at room temperature,
145 and then incubated in affinity-purified anti-CL-K1 IgG or control IgG (5 μg/ml) overnight at
146 4 ^{o} C. Each section was incubated with biotinylated guinea pig anti-rabbit IgG for 1 hr
147 followed by incubation with avidin-biotin-alkaline phosphatase complex for 1 hr. Finally, the
148 sections were treated with Alkaline Phosphatase Substrate Kit II (Vector Laboratories,
149 Burlingame, CA, USA). Endogenous alkaline phosphatase activity was blocked with
150 Levamisol solution (Vector Laboratories, Burlingame, CA, USA). Sections were briefly
151 counterstained with hematoxylin. In the case of immunofluorescence staining, secondary
152 antibodies were incubated with Alexa Fluor 488 anti-rabbit IgG and TO-PRO-3 (Molecular
153 Probes, Eugene, Oregon, USA) for 1 hr. For double staining with somatostatin, glucagon, or
154 insulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) of stomach and pancreas,
155 secondary antibody was used together with Alexa Fluor 594 anti-goat IgG.
156 Immunohistochemistry and fluorescent images were examined with a confocal
157 laser-scanning microscope. For double staining with mouse CD31 (BD Biosciences
158 Pharmingen San Diego, CA, USA), secondary antibody was used together with Alexa Flour
159 594 anti rat IgG.

RESULTS

CL-K1 mRNA expression in murine tissues.

To investigate the tissue distribution of CL-K1 mRNA, real-time PCR analyses were performed with RNAs purified from a number of mouse tissues. We used specific primer pairs and a Taqman probe to detect the CL-K1 neck-CRD fragment. The data in figure 1 shows CL-K1 mRNA per 18S RNA and were further normalized to kidney defined as 1.0. The data of CL-K1 mRNA expression were normalized to kidney because we identified CL-K1 from kidney. Using real-time PCR analysis, CL-K1 mRNA was detectable in almost all organs tested (Figure 1). CL-K1 mRNA was found at the highest level in heart, and a relatively high expression of CL-K1 mRNA was detected in liver, testis, white adipose tissue, brain, and kidney.

Characterization of the CL-K1 affinity antibody

We first generated a specific antibody against CL-K1 for use in immunohistochemistry. This antibody was raised against the CL-K1neck-CRD region which is highly conserved in humans, mice, and rats (Keshi et al. 2006). To increase the titer of the antibody, we employed a CL-K1 affinity column after IgG purification. As shown in Figure 2A and 2B, an ELISA for measuring titers of several antibodies against CL-K1 recombinant proteins revealed that CL-K1 antibodies were strongly reactive with the mouse CL-K1 protein. Since the affinity-purified antibody had an approximately 10 times higher titer than the unpurified preparation, we used the former in the following experiments. Figure 2B indicates that the CL-K1 IgG reacted specifically with CL-K1 rather than with CL-L1, CL-P1, or MBL. Figure 2C shows that the affinity-purified antibody was capable of detecting mouse CL-K1 as well as human CL-K1. CL-K1 overepressed CHO cells (CHO/CL-K1) or empty vector transfected CHO cells (mock) were stained with the affinity-purified CL-K1 or control

antibody, respectively. As shown in Figure 2D (left and middle panels), the CL-K1 affinity-purified antibody could detect human CL-K1 protein in the cytoplasm of CHO/CL-K1 cells while the control IgG could not. In addition, the CL-K1 antibody failed to react with anything in CHO/mock cells, as shown in the right panel of Figure 2D, clearly demonstrating the high specificity of the affinity-purified antibody.

CL-K1 expression in murine tissues.

Immunohistochemistry and immunofluorescent analyses were performed in several murine tissues to investigate expression of the CL-K1 protein. Figure 2E shows that CL-K1 antibody could react with the testis, but the pass-through IgG used as a control could not detect any antigen in the testis, suggesting a specificity of the CL-K1 antibody. Using the CL-K1 affinity-purified IgG, immunohistochemical and immunofluorescent analyses were performed with tissues of murine kidney, lung, heart, testis, liver, pancreas, digestive organs including the esophagus, stomach, small intestine, and large intestine, and brain. Figure 3A of showing results immunofluorescence analysis of renal cortex demonstrates that CL-K1 was expressed in mesangial cells, podocyte or microvascular endothelial cells of glomerulus (A: red arrow), and in the brush border of proximal tubules (A: yellow arrow). To further characterize the CL-K1 immunoreactive cells in the renal cortex, immunofluorescent analysis using both CL-K1 and antibody against CD31, a marker for endothelial cells, was performed. As demonstrated in Figure 3C, the merge image showed that endothelial cells do not express CL-K1, supporting that CL-K1 may be expressed in the mesangial cells. Figure 3B shows that CL-K1 was also expressed in the vascular portion of the kidney. As shown in Figure 4A and 4B, CL-K1 was observed in vascular portion of the heart and small intestine as well as in those of kidney. Furthermore, the double immunofluorescence analyses presented in Figure 4C and 4D indicated that CL-K1 was expressed specifically in smooth muscle cells

but not in endothelial cells. This indicates that vascular smooth muscle cells in all tissues are made up of primary cells expressing CL-K1.

Immunohistochemical localization of CL-K1 in lung, heart, testis, and brain is shown in Figure 5. CL-K1 expression was strong in bronchial glands of bronchium (Figure 5A-C). CL-K1 was also expressed in bronchial glands of bronchioles (Figure 5A and 5B, red arrow) and respiratory bronchioles (Figure 5C black arrow). Figure 5D indicates that CL-K1 was expressed in whole myocardium as well as in the vascular portion of this tissue, but not in endocardium. Figure 5E shows that CL-K1 was expressed in the cytoplasm of spermatocytes. In brain, CL-K1 was abundantly and ubiquitously expressed in neurons of the central nervous system (data not shown). Figure 5F indicates that representative neurons were stained in the medulla oblongata. Immunohistochemical localization of CL-K1 in liver and pancreas is shown in Figure 6. CL-K1 was expressed in hepatocytes, especially around the central veins (black arrow in Figure 6A). Figure 6B and 6C shows that CL-K1 was expressed in pancreatic acinar cells and islet cells. In the case of the islets, CL-K1 was especially expressed in the marginal cells. The double immunofluorescence analyses presented in Figure 6D indicate that CL-K1 was expressed specifically in D cells that produce somatostatin but not in alpha and beta-cells which produce glucagon and insulin, respectively. Figure 7 shows immunohistochemical localization of CL-K1 in murine digestive tract. CL-K1 was expressed in epithelial cells of all mucosa of the digestive tract including the esophagus (Figure 7A), stomach (Figure 7B and 7E), small intestine (Figure 7C) and large intestine (Figure 7D). CL-K1 was strongly stained on the surface of esophageal mucosa. In stomach, CL-K1 was expressed in whole mucosa of gastric glands. Double immunofluorescence analyses revealed that CL-K1 in stomach was also specifically localized in D cells containing somatostatin. In small intestinal mucosa, CL-K1 was expressed in Paneth cells as well as in intestinal crypt

232 (yellow arrow in Figure 7C). In the large intestine, CL-K1 was expressed in epithelial

233 mucosa (Figure 7D).

DISCUSSION

Collectins interact with glyco-conjugated and lipid moieties present on the surface of microorganisms and allergens, as well as with receptors on host cells. Through these interactions, they play a crucial role in innate immunity. However, a single type of collectin cannot meet the requirements for all of the functions of innate immunity and several collectins are required for host defense (van de Wetering et al. 2004). In our previous report, we demonstrated that CL-K1 could bind to bacterial LPS and LTA. Thus, this novel collectin might be involved in host defense against microorganisms. With regard to the tissue distribution of human CL-K1, we have shown by RT-PCR that CL-K1 mRNA is expressed in most human tissues (Keshi et al. 2006). The present study using mice was carried out to determine the precise tissue distribution of CL-K1 protein expression in order to reach a better understanding of the biological functions of this novel collectin. For this purpose, we generated a new affinity-purified anti-CL-K1 antibody. This polyclonal antibody raised against the CL-K1 neck-CRD domain recognized full-length CL-K1 over-expressed in CHO cells. We have previously demonstrated by RT-PCR that CL-K1 mRNA expression is ubiquitous in human tissues (Keshi et al. 2006). In this study, we quantitatively evaluated the tissue expression of CL-K1 mRNA in mice using real-time PCR. The real-time PCR study demonstrated that CL-K1 mRNA was distributed in all organs. Among the murine tissues expressing CL-K1 mRNA (see Figure 1), a relatively high level of expression was observed in heart, liver, testis, kidney, and white adipose tissue. Results of immunostaining of these tissues clearly demonstrated that heart, liver, testis, and kidney express CL-K1 protein, in strong agreement with the observations of mRNA expression by real-time PCR. The major finding in the present study was that CL-K1 was expressed in proximal tubules in kidney, bronchial glands of bronchioles, and mucosa of gastrointestinal tract. CL-K1 is a secreted

type of collectin and would be expected to be secreted into lumen of these various tissues. This expression pattern is similar to those of SP-A and SP-D in the bronchial glands of bronchioles (Madsen et al. 2000, 2003). Sites of CL-K1 expression in kidney, lung, and gastrointestinal tract coincide with areas subject to microbial growth, suggesting that CL-K1 has an important role in defense against microorganisms invading the urinary tract, respiratory tract, and lumen of the digestive tract. In kidney, CL-K1 was identified in mesangial cells of glomeruli in addition to the proximal tubules. We have reported in our recent publication that CL-K1 is made in the liver and might secret into the blood stream (Keshi et al. 2006). In addition, molecular weight of CL-K1 is around 37kDa. One may speculate that collectin could be passively deposited in the mesangium. It is therefore speculated that CL-K1 immunoreactivity found in the mesangial cells may be passively deposited from systemic circulation. We could not rule out the possibility at this moment. However, the possibility might be low because native CL-K1 exists as oligomer structure in the blood and its molecular weight is more than 100kDa as described in our recent publication (Keshi et al). These evidences indicate that CL-K1 immunoreactive products in the mesangial cells could not be passively deposited. Further studies such as in situ hybridization should be needed to clarify whether CL-K1 is indeed produced by mesangial cells or other cells stained with the CL-K1 antibody. Recent studies on IgA glomerulonephritis have demonstrated that IgA2 harboring polysaccharide chains tend to be agglutinated with each other so that deposits of IgA2 accumulate in mesangial cells and activate the lectin pathway in glomeruli (Hisano et al. 2001, 2005, Oortwijn et al. 2006). These experiments indicate that IgA2 with sugar chains are important in agglutination and adhesion in glomeruli. However, characterization of the ligands involved has not been carried out. Our findings suggest that CL-K1 might be involved in the triggering of

glomerulonephritis since it would act as a ligand against polysaccharides with IgA. This concept will be further explored in a future study. On the other hand, results of the real-time PCR and immunohistochemistry clearly demonstrated that CL-K1 mRNA was highly expressed in liver and that CL-K1 protein expression was homogenously localized in hepatocytes where it was especially high around the central veins. We have already shown that CL-K1 protein is secreted into human blood (Keshi et al. 2006). These results suggest that murine CL-K1 is mainly produced in hepatocytes in the liver and secreted into the blood stream, as is human CL-K1. In pancreas, CL-K1 was expressed in acinar cells and islet cells. According to the results of immunostaining, it is of interest that CL-K1 was strongly associated with somatostatin in the islets, but not with insulin or glucagons. Moreover, in gastric mucosa, the cells producing CL-K1 corresponded to those producing somatostatin. Somatostatin is a peptide hormone that is known to regulate the endocrine system, affect neurotransmission and inhibit the release of a variety of secondary hormones. Recently, several reports have implicated somatostatin in innate immunity (Zavros et al. 2004, Seboek et al. 2004). These results also suggest that somatostatin might have a special relationship with CL-K1 in host defense mechanisms. In small intestine, CL-K1 was highly expressed in Paneth cells which contain epithelial granulocytes in the basement area of crypts. Defensins are secreted from Paneth cells and contribute to mucosal barrier function through their potent antimicrobial activities (Ouellette et al, 1990,1992, Ayabe et al. 2000). The fact that CL-K1 was localized in Paneth cells indicates that this molecule would be advantageous in host defense because it would likely be secreted into the lumen together with defensins with which they would play a cooperative role as anti-microbial molecules. In the central nervous system, CL-K1 was mainly expressed in neurons of the brain. Since CL-K1 expression was localized in the cytoplasm and not in dendritic portion of the cell, it would not contribute to

306 any specific neuronal network formation. The relatively high expression of CL-K1 mRNA
307 observed in the central nervous system was in agreement with immunohistochemical
308 observations. In lung, gastrointestinal tract and testis, CL-K1 was expressed in the region
309 exposed outer environment, indicating that CL-K1 play an important role in innate immunity
310 systems as other collectins. On the other hand, CL-K1 expressed in heart, liver and brain
311 may play unexpected roles because the sites of CL-K1 expression are unlikely involved in
312 host defense. We do not know the physiological relevance of CL-K1 expressed in heart, liver
313 and neurons in brain. Further studies should be needed to clarify whether CL-K1 possesses
314 what kind of biological actions in addition to its expected action as a collectin.
315 In conclusion, we determined the tissue distribution of CL-K1 protein in mice. These
316 findings may be useful for understanding the biological significance of this novel collectin in
317 future studies.

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LEGENDS

Figure 1

Estimation of the amount of CL-K1 mRNA in different tissues. Relative mRNA levels were measured by TaqMan RT-PCR. Data were normalized based on the value of 18S ribosomal RNA.

Figure 2

The specificity of our CL-K1 polyclonal antibody was analyzed by ELISA, immunocytochemistry and immunohistochemistry. The anti-CL-K1 IgG fraction (IgG) was purified from rabbit serum. After IgG purification, the affinity antibody (post-affinity) was purified on an antigen column, and the pass-through IgG was used as the control IgG (pass-through IgG). Figure A shows results of ELISA analysis using anti-CL-K1 IgG, post-affinity antibody or pass-through IgG. ELISA analyses of anti-CL-K1 antibodies against human CL-K1. Figure B shows the results of ELISA analyses of anti-CL-K1 affinity antibody reactivity with other collectins, namely, CL-L1, CL-P1, and MBL. Figure C shows cross reactivity between human and murine CL-K1 recombinant protein. Figure D shows immunofluorescence in CHO cells overexpressing CL-K1 (left and middle panel) as well as in empty vector expressed CHO cells (mock cells) (right panel). Figure E shows immunohistochemistry staining and immunofluorescence staining with affinity antibody or control IgG in murine testis.

Figure 3

Immunohistochemistry of murine renal cortex (A) and vascular smooth muscle cells in kidney (B). CL-K1 protein was expressed in mesangial cells in glomerulus (A: red arrow)

and in brush border of proximal tubules (A: yellow arrow). Double immunofluorescence staining (C) demonstrates that CL-K1 was not co-localized in microvascular endothelial cell.

Figure 4

Immunohistochemistry of vascular cells in heart (A) and small intestine (B). CL-K1 expression was detected in vascular portion in heart (A), and small intestine (B). Double immunofluorescence staining (C and D) demonstrates that CL-K1 was co-localized in vascular smooth muscle cells but not in endotherial cells.

Figure 5

Immunohistochemical localization of CL-K1 in murine lung, heart, testis, and brain. CL-K1 expression was especially strong in bronchial glands of bronchium (A and B: red arrow). In peripheral lung (C), CL-K1 was also expressed in bronchial glands of bronchium (red arrow) and respiratory bronchioles (black arrow). In heart and testis, CL-K1 was expressed in lamina elastica of coronary artery in myocardium (D) and in cytoplasm of spermatocytes (E). Figure F shows the representative neurons stained with CL-K1 antibody in the reticular formation of the medulla oblongata.

Figure 6

Immunohistochemical localization of CL-K1 in liver and pancreas. In liver (A), CL-K1 was expressed in hepatocytes. A relatively high expression of CL-K1 was seen in hepatocytes around the central vein (black arrow). In pancreas (B), CL-K1 was expressed not only in acinar cells but also in islet cells (C). Double immunofluorescence staining (D) demonstrates that CL-K1 was co-localized in somatostatin-containing D cells but not in

496 glucagon-containing alpha- or insulin-containing beta- cells.

497

498 Figure 7

499 Immunohistochemical localization of CL-K1 in gastrointestinal tract. In esophagus (A),

500 stomach (B), small intestine (C), and large intestine (D), CL-K1 was expressed in epithelium.

501 In stomach, CL-K1 was co-localized with somatostatin in somatostatin-containing cells (E).

502 In small intestine, CL-K1 was expressed in Paneth cells (C: yellow arrow). In large intestine,

503 CL-K1 was expressed in epithelial mucosa (D).

Figure 1

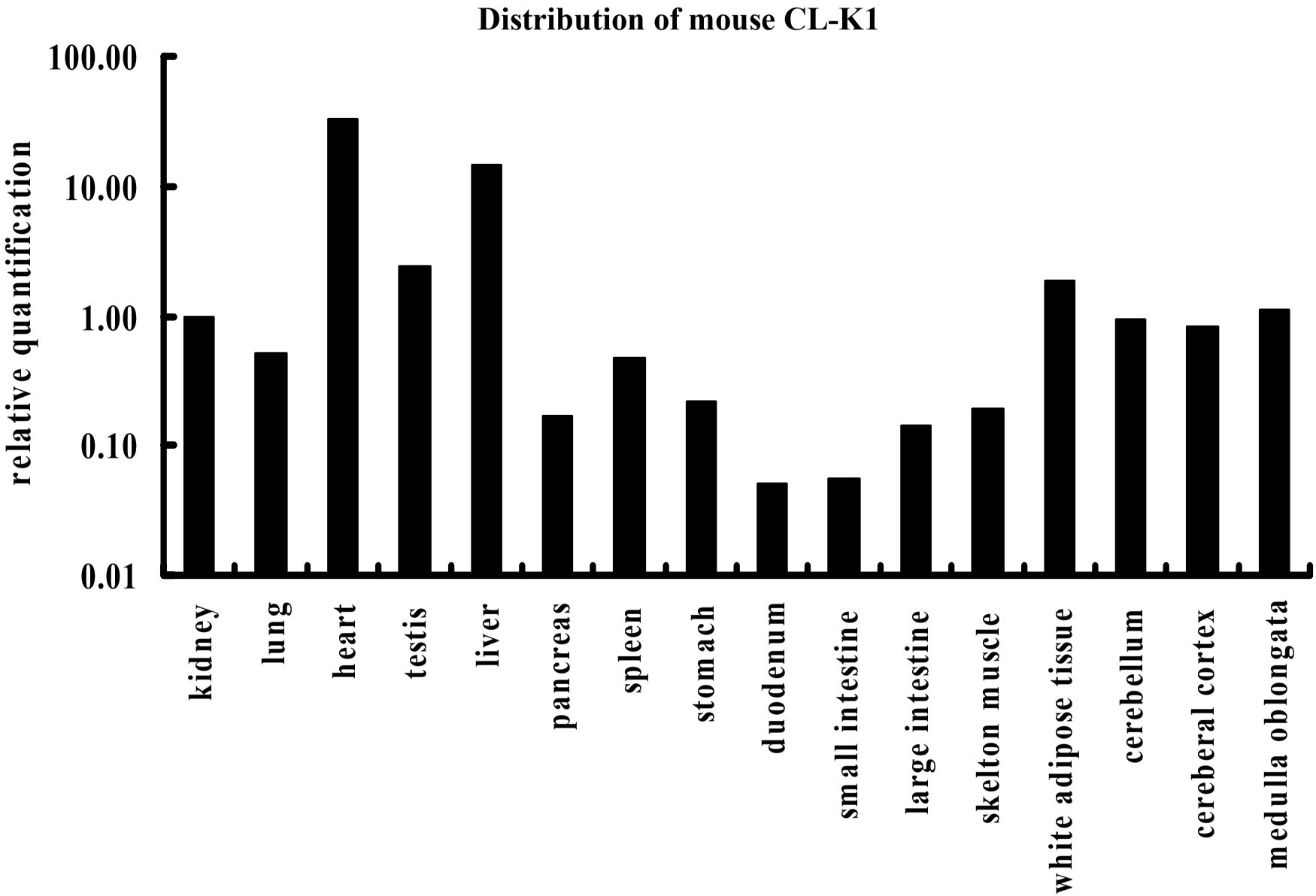

Distribution of mouse CL-K1
relative quantification
100.00
10.00
1.00
0.10
0.01
kidney
lung
heart
testis
liver
pancreas
spleen
stomach
duodenum
small intestine
large intestine
skelton muscle
white adipose tissue
cerebellum
cereberal cortex
medulla oblongata

Figure 2

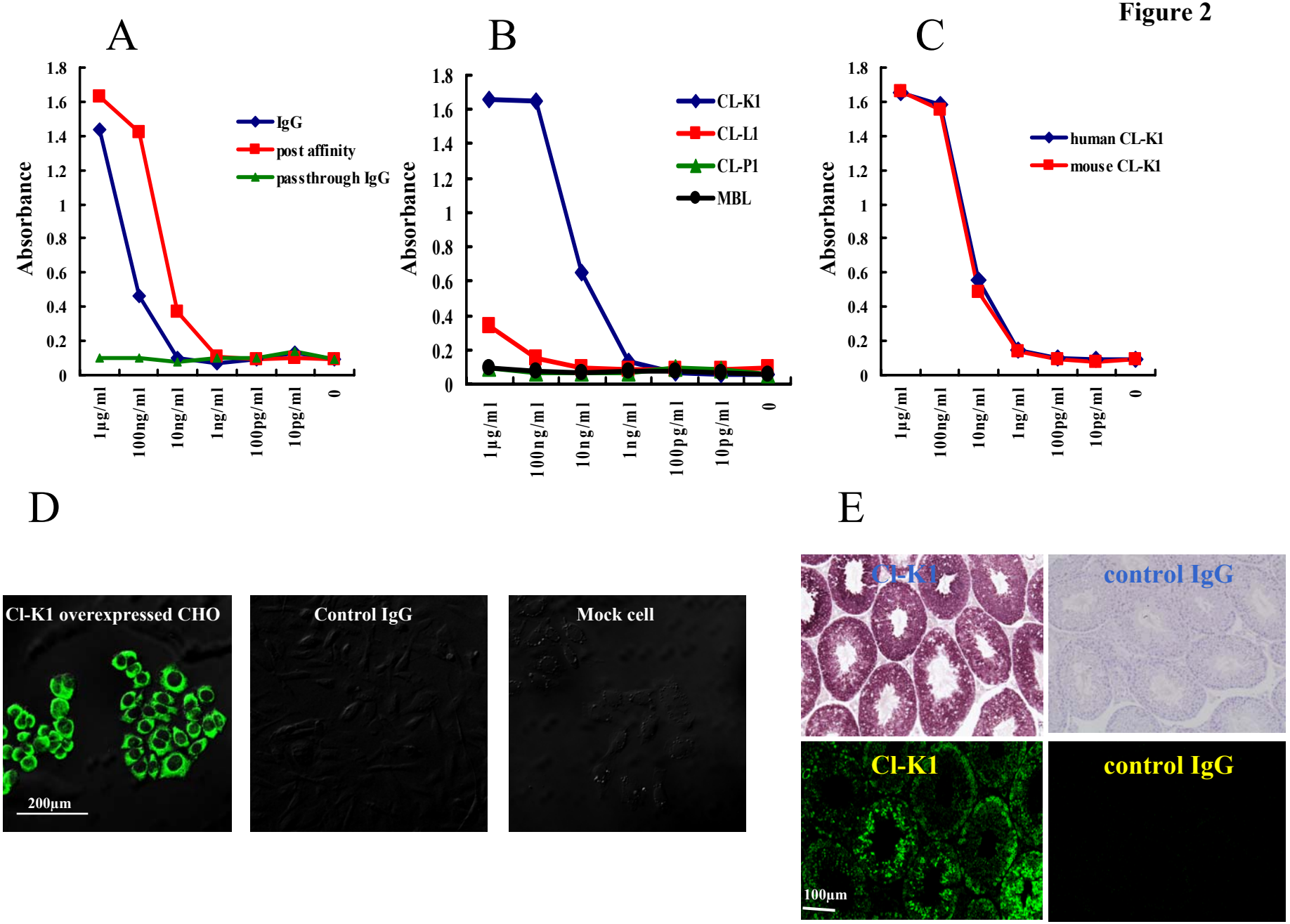

A
B
C
Absorbance
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
IgG
post affinity
passthrough IgG
CL-K1
CL-L1
CL-P1
MBL
human CL-K1
mouse CL-K1
1μg/ml
100ng/ml
10ng/ml
1ng/ml
100pg/ml
10pg/ml
0
D
Cl-K1 overexpressed CHO
200μm
Control IgG
Mock cell
E
Cl-K1
control IgG
Cl-K1
control IgG
100μm

Figure 3

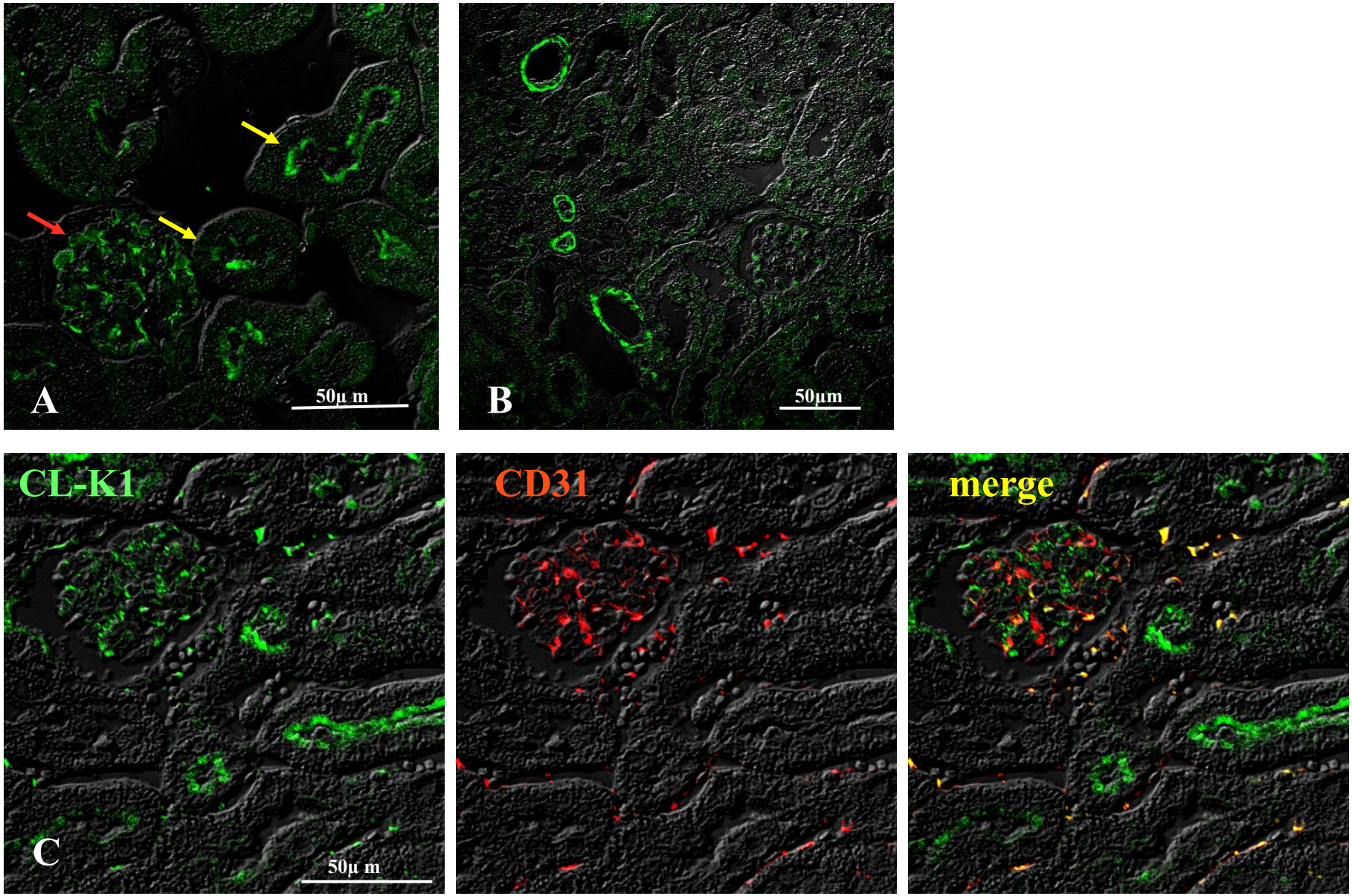


Figure 4

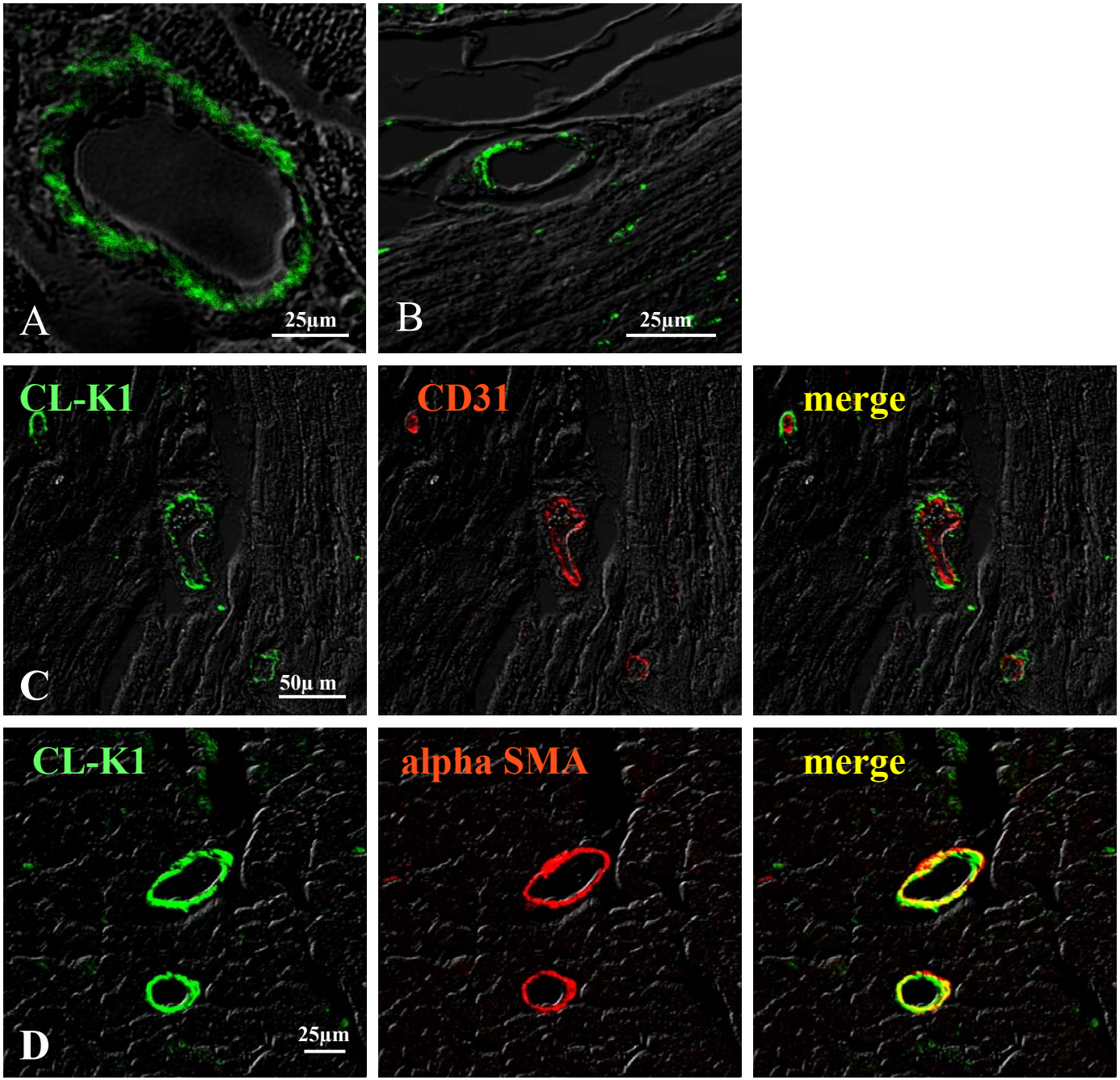

A
25µm
B
25µm
CL-K1
C
50µ m
CD31
merge
CL-K1
D
25µm
alpha SMA
merge

Figure 5

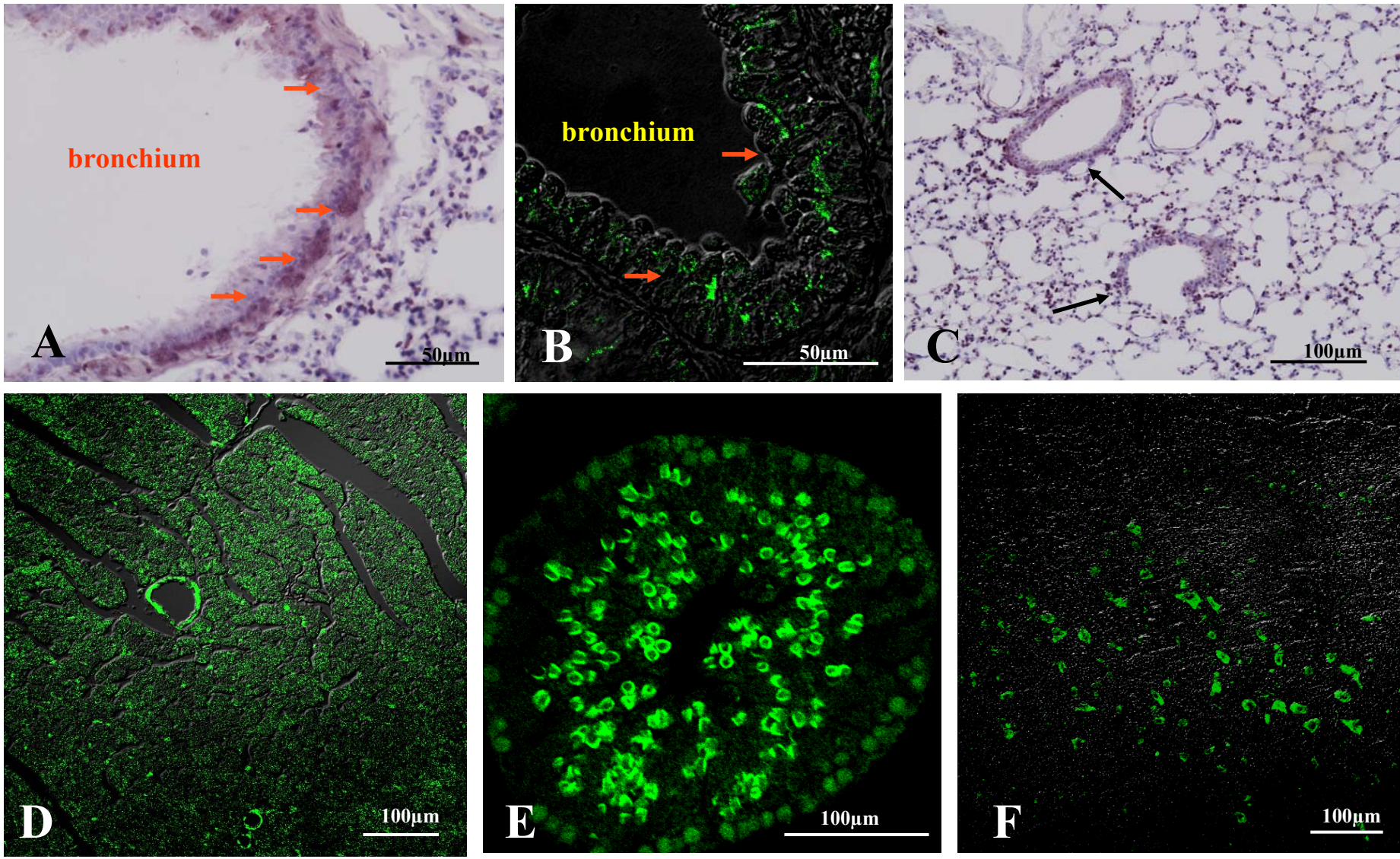

bronchium
A
50μm
bronchium
B
50μm
C
100μm
D
100μm
E
100μm
F
100μm

Figure 6

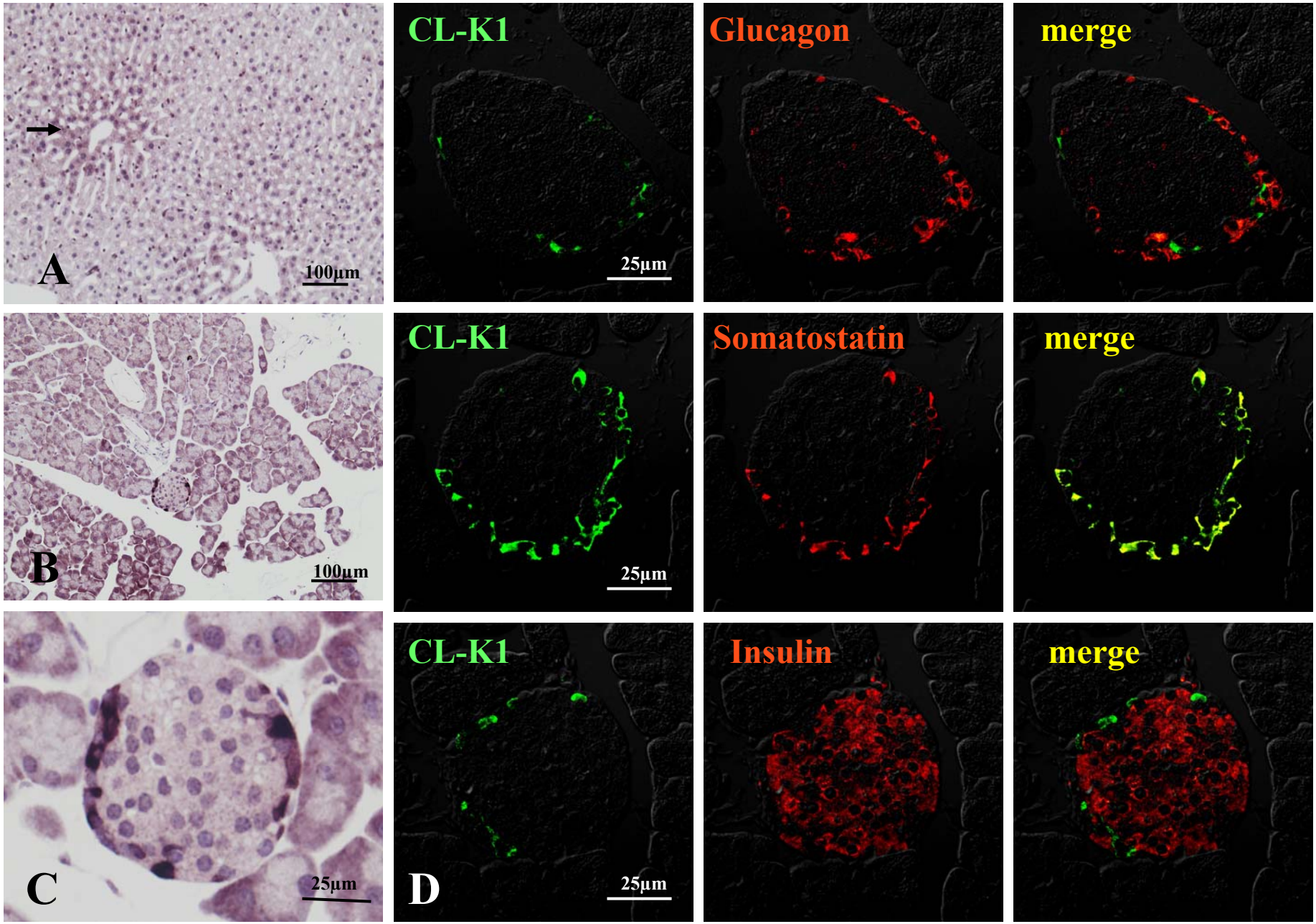
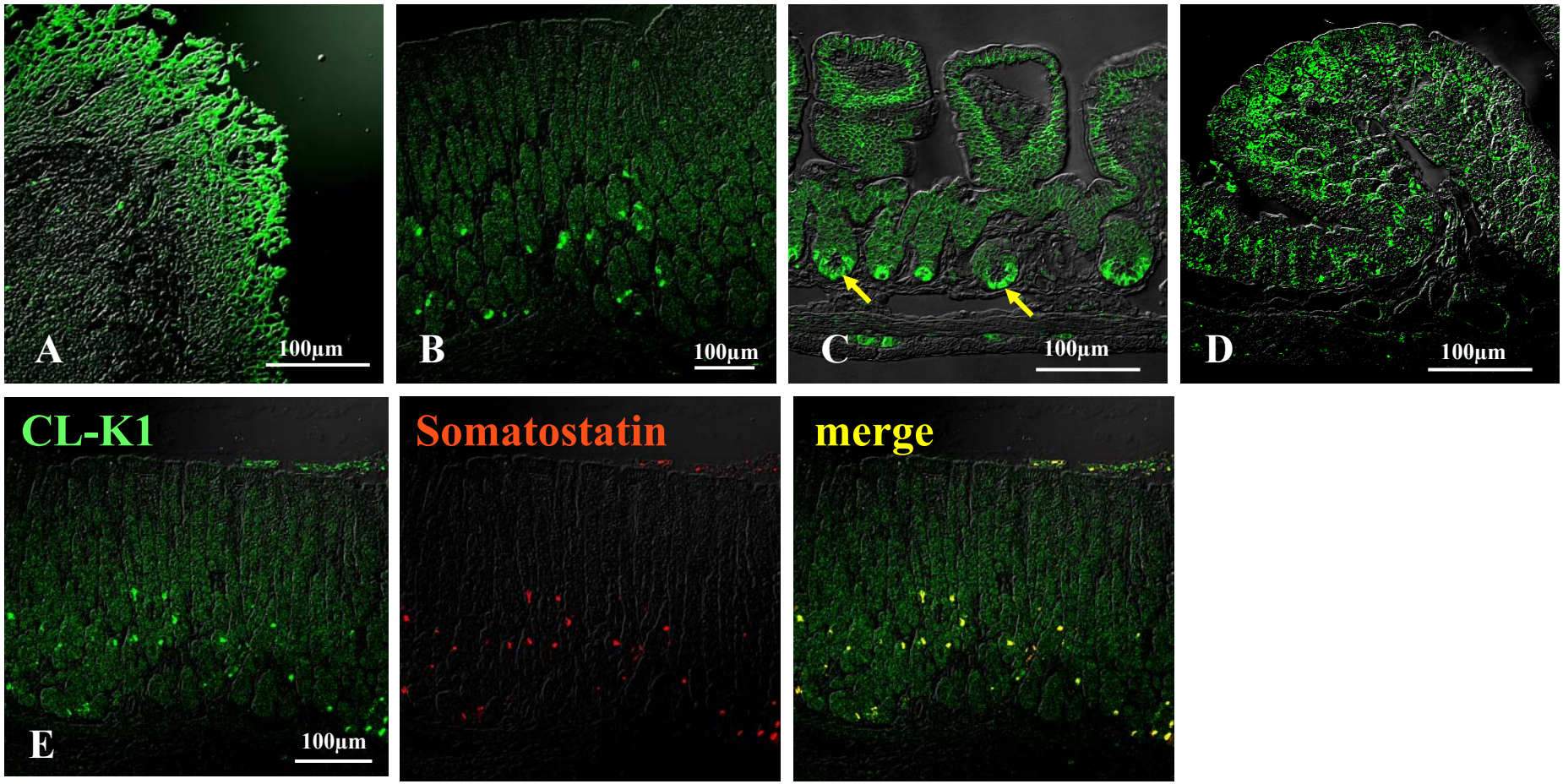

A
100μm
CL-K1
25μm
Glucagon
merge
B
100μm
CL-K1
25μm
Somatostatin
merge
C
25μm
D
CL-K1
25μm
Insulin
merge

Figure 7


A
100μm
B
100μm
C
100μm
D
100μm
CL-K1
E
100μm
Somatostatin
merge