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Ccdc85C, a causative protein for hydrocephalus and subcortical heterotopia, is expressed in the systemic epithelia with proliferative activity in rats

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Summary. Coiled-coil domain containing 85c (*Ccdc85c*) is a causative gene for spontaneous mutant mouse with non-obstructive hydrocephalus and subcortical heterotopia. Detailed functions of Ccdc85C protein have not been clarified. To reveal roles of Ccdc85C, we examined the distribution and expression pattern of Ccdc85C in the systemic developing organs in rats. Ccdc85C was expressed in various simple epithelia but not stratified epithelia. In the various epithelia, Ccdc85C was localized at cell-cell junctions and its expression was strong at apical junctions. Furthermore, intense expression was seen at developing period and gradually decreased with advancing development. Distribution of Ccdc85C coincides with that of proliferating epithelial cells. These results suggest that Ccdc85C plays an important role in the proliferative property of simple epithelia.

Key words: Apical junction, Ccdc85C, Cell proliferation, Hydrocephalus, Rat

Introduction

The *hhy* (hemorrhagic hydrocephalus) mouse is a spontaneous mutant with non-obstructive hydrocephalus, subcortical heterotopia and frequent brain hemorrhage (Kuwamura et al., 2004). We previously revealed that the *hhy* homozygous mouse has a mutation in a coiled-coil domain containing 85c (*Ccdc85c*) gene and lacks its expression (Mori et al., 2012). In the developing cerebrum of wild-type mice, Ccdc85C protein is strongly expressed in the radial glia adjacent to ventricles. Lack of Ccdc85c expression in *hhy* homozygous mice results in abnormal dispersing of radial glia, formation of subcortical heterotopia and ependymal agenesis, which leads to hydrocephalus. Thus, the function of Ccdc85C is considered to be closely correlated with maintenance of neural progenitor cells and neurogenesis.

Recently, human CCDC85C protein was shown to regulate Yes-associated protein 1 (YAP1) (Wang et al., 2014). The Hippo pathway is known as a tumor suppressor pathway and regulates organ size determination (Zhao et al., 2011a). YAP1 is a major transcription factor of Hippo pathway and up-regulates genes promoting cell proliferation and inhibiting apoptosis. CCDC85C negatively regulates YAP1 by inhibiting YAP1 nuclear translocation *via* direct protein-protein interaction. Therefore, recent studies have demonstrated a part of the function and localization of Ccdc85C to some extent. However, detailed functions of Ccdc85C remain to be clarified; for example, expression pattern of Ccdc85C in the systemic organs except for the cerebrum is undetermined.

In this study, to elucidate the detailed roles of Ccdc85C, we examined distribution of Ccdc85C protein in the whole body at various developing stages in rats. We here revealed that various epithelia express Ccdc85C at cell-cell junctions and Ccdc85C expression are related to the proliferative property in the epithelia.

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Materials and methods

Animals

We purchased the Crl: CD (SD) rats from Charls River Japan (Shiga, Japan) and maintained them under specific pathogen-free conditions in a room with controlled temperature and 12-h light-dark cycle at the Animal Facility of Osaka Prefecture University. Food and water were provided *ad libitum*. All animals were handled according to the Guidelines for Animal Experimentation of Osaka Prefecture University. SD rats at postnatal day 0 (P0), P7, P14 P21, P30 and P60 were euthanized by isoflurane. Subsequently, we removed systemic organs. We also collected organs of fetuses at embryonic day 20 (E20) from a pregnant rat under deep anesthesia. We embedded the samples in TISSU MOUNT® (Chiba medical, Saitama, Japan) at -80°C for frozen sections.

Reverse transcription PCR (RT-PCR) analyses

Liver, spleen, thymus, kidneys, heart, lungs, trachea, stomach, duodenum, ileum, colon, urinary bladder, testes, ovaries, uterus, skin at left thigh area, left thigh skeletal muscle, cerebrum, cerebellum, spinal cord (cervical part), and eyes of the SD rats at P0 were removed. Total RNA of these organs was extracted using SV Total RNA Isolation System (Promega, WI, USA) according to the manufacturer's instructions. cDNA was synthesized from 0.5 μg of total RNA by using SuperScript® VILO™ cDNA Synthesis Kit (Life Technologies, CA, USA). cDNA was amplified by a thermal cycler (GeneAtlas 485, Astec, Fukuoka, Japan) with Go Taq® Green Master Mix (Promega) to examine the gene expression of *Ccdc85c* (forward primer, 5-GTGCATGCCATGAAGGTTCT-3; reverse primer, 5-CCCAGTTTCCTCCAGACCA-3). RT-PCR cycling conditions were an initial denaturation of 95°C for 2 min, followed by 25 cycles of 95°C for 20 sec, 60°C for 20 sec, 72°C for 20 sec, and a final extension of 72°C for 5 min. β-actin (Rat Housekeeping Gene Primer Set, TaKaRa, Shiga, Japan) was used as internal control.

Production of rabbit polyclonal anti-rat Ccdc85C antibody

To produce new antibody against rat Ccdc85C, the C-terminal 174 amino acid peptide of rat Ccdc85C was expressed as a GST-fusion protein in *Esherichia coli* DH5α. Using this protein, a rabbit polyclonal anti-rat Ccdc85C antibody was produced at Eve Bio-Science Co. Ltd. (Wakayama, Japan).

Immunohistochemistry

For immunofluorescence, 10 μm frozen sections were cut by a cryostat and air dried at room temperature for 1 h. The tissue sections were fixed in Zamboni's solution (0.21% picric acid, 2% paraformaldehyde) for 15 min at room temperature. After that, the sections were washed with 0.3% Triton X-100 in phosphate buffered saline (PBS) for 15 min and treated with 10% normal goat serum (Sigma, MO, USA) for 30 min. Then, the sections were incubated with rabbit polyclonal antibodies against Ccdc85C (1:100,000) and ZO-1 (1:500, Life Technologies) or mouse monoclonal antibodies against E-cadherin (1:500, BD Transduction Laboratories, CA, USA), N-cadherin (1:500, Life Technologies), β-catenin (1:500, Life Technologies) and cytokeratin 19 (Leica Biosystems, Newcastle, UK) at 4°C overnight. The sections were incubated with Alexa488 labelled anti-rabbit IgG (1:1,000; Life Technologies) or Alexa568 labelled anti-mouse IgG secondary antibodies (1:1,000; Life Technologies) for 45 min at room temperature, and then covered with mounting medium with DAPI (Vector Laboratories, USA). Signals of the stained section were detected with a confocal imaging system (C1Si; Nikon, Tokyo, Japan).

EdU labeling

To label proliferating cells, SD rats were intraperitoneally injected with 50 mg/kg thymidine analogue 5-ethynyl-2-deoxyuridine (EdU) (Carbosynth, Berkshire, UK) in normal saline 1 h before the sacrifice. EdU staining was performed after the secondary antibody reaction of immunofluorescence. Sections were washed with 3% bovine serum albumin (BSA) in PBS twice and incubated with Click-iT® reaction cocktail (Click-iT® EdU Alexa Fluor® 488 Imaging Kit, Life Technologies) for 30 min at room temperature under light shielded condition. Then sections were washed with 3% BSA in PBS once and normal PBS once, followed by coverslipping with mounting medium with DAPI.

Results

Systemic distribution of Ccdc85C protein in rats

We first examined *Ccdc85c* mRNA expression in the systemic organs of SD rats at P0 by semiquantitative RT-PCR analysis. We found that most rat organs express *Ccdc85c* at P0 (Fig. 1). *Ccdc85c* expression was especially strong at the small intestine, the cerebrum, the cerebellum and the eye. On the other hand, the liver, the skin and the skeletal muscle only faintly expressed *Ccdc85c*.

Next, we investigated the distribution of Ccdc85C protein in the systemic organs by immunofluorescence. In rat organs, many epithelial cells expressed Ccdc85C. Ccdc85C was expressed at hepatocytes (Fig. 2A, arrows) and cholangiocytes (Fig. 2A, arrowhead in inset) of the liver; renal tubule and collecting tubule epithelia of the kidney; epithelia of the nasal cavity, the trachea, the bronchi, the bronchioli (Fig. 2B) and the adnexal glands; epithelia of the glandular stomach (Fig. 2C), the intestine, the salivary gland and exocrine part of the

pancreas (Fig. 2D); and seminiferous tubules (Fig. 2E), epithelia of the epididymal ducts and the uterus (Fig. 2F). In these organs, Ccdc85C expression was also detected at the serosae (Fig. 2G). In the central nervous system, Ccdc85C expression was observed at choroid plexus epithelia, the surface of the ventricles of the brain (Fig. 2H) and the central canal of the spinal cord. In the sensory organs, Ccdc85C expression was observed at the outermost layer of the retina (Fig. 1I) and the cochlear ducts. In these epithelia, the expression pattern of Ccdc85C was meshwork-like (Fig. 2C,H) and linear (Fig. 2G,I).

We then characterized the epithelia expressing Ccdc85C in detail. Ccdc85C expression was observed at simple cuboidal epithelia (Fig. 3A, renal tubules of the kidney), simple columnar epithelia (Fig. 3B, the intestinal mucosa), pseudostratified columnar epithelia (Fig. 3C, the nasal cavity) and simple squamous epithelia (Fig. 3G, arrowheads, cytokeratin 19-positive mesothelia at the liver and the stomach). On the other hand, stratified squamous epithelia (Fig. 3D, the nasal cavity), stratified squamous keratinizing epithelia (Fig. 3E, the skin) and transitional epithelia (Fig. 3F, the urinary duct) did not express Ccdc85C. Therefore, we concluded that simple epithelia but not stratified epithelia express Ccdc85C.

Ccdc85C localization in the epithelia

To examine the subcellular localization of Ccdc85C in epithelia, we performed double immunofluorescence for Ccdc85C and cell adhesion molecules. Ccdc85C was colocalized with E-cadherin (Fig. 4A) or N-cadherin (Fig. 4B and C). Ccdc85C also was colocalized with β-catenin (Fig. 4D). These cell adhesion molecules were expressed throughout the cell surface. In contrast, Ccdc85C expression was strong at apical region of cell surface. By immunofluorescence using serially sliced sections of epididymis, we revealed that expression pattern of Ccdc85C coincided with that of ZO-1, a marker for tight junction protein (Fig. 4E,F). We also obtained similar results in other epithelia expressing Ccdc85C.

Temporal expression of Ccdc85C during organ development

Our previous studies revealed that Ccdc85C was strongly expressed at the gestation to perinatal periods, and decreased rapidly with development in the murine cerebrum (Mori et al., 2012). We temporally examined Ccdc85C expression in the epithelia by immunofluorescence (Table. 1). In the kidney at E20 (Fig. 5A) and P0 (Fig. 5B), many tubular epithelia strongly expressed Ccdc85C. However, Ccdc85C expression became weak with development of the kidney (Fig. 5C, P21) and mostly disappeared at P30 (Fig. 5D). In the ileum, Ccdc85C expression was strong immediately after birth (Fig. 5E, P0). Unlike the kidney, Ccdc85C expression was relatively preserved in the ileum at P60 (Fig. 5F). On the other hand, Ccdc85C expression was weakly detected at the seminiferous tubules at P0 (Fig. 5G). From P21, Ccdc85C expression became strong at the outermost layer of the seminiferous tubules (Fig. 5H). Ccdc85C expression at the seminiferous tubules was the strongest at P30 (Fig. 5I). At P60, Ccdc85C expression at the seminiferous tubules decreased compared to that at P30 (Fig. 5J). In the other epithelia, except for the testes, Ccdc85C expression reached a peak around the period from E20 to P0.

Table 1. Summary of Ccdc85C expression patterns in systemic organs of rat.

	E20	P0	P7	P14	P21	P30	P60
Kidney	+++	+++	+++	++	+	+	-
Stomach	+++	+++	+++	++	+	+	+
Small intestine	+++	+++	+++	+++	+++	++	++
Testis	*	+	+	+	++	+++	++
Cerebrum	+++	+++	+++	++	+	+	-

+++, strong expression; ++, moderate expression; +, weak expression;

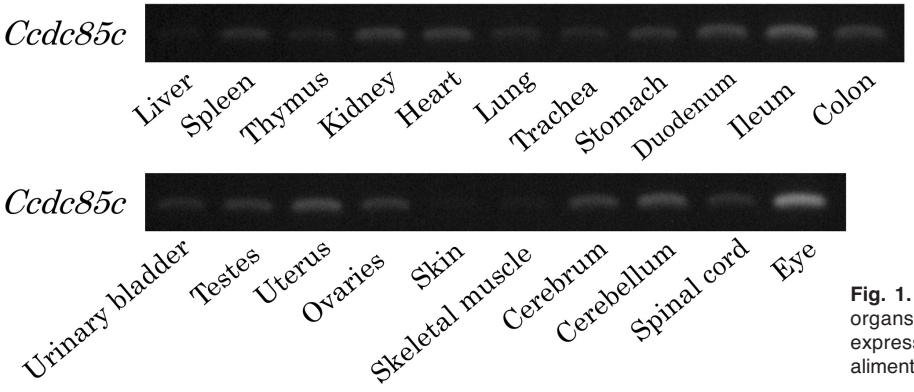

Fig. 1. RT-PCR analysis for Ccdc85c in the systemic organs of SD rats at postnatal day 0 (P0). Ccdc85c expression is detected in many organs, especially in alimentary tract, brain and eye.

Ccdc85C expression and proliferative activity

To investigate the relationship between Ccdc85C expression and proliferative activity in the epithelia, we compared the distribution of Ccdc85C expressing cells and proliferating cells in the kidney and the ileum by imunofluorescence for Ccdc85C and EdU labeling. We used EdU, which is incorporated into DNA at S-phase, as a marker of proliferating cells. In the kidney at P0 and P7, many EdU-labeled cells were located in subcapsular

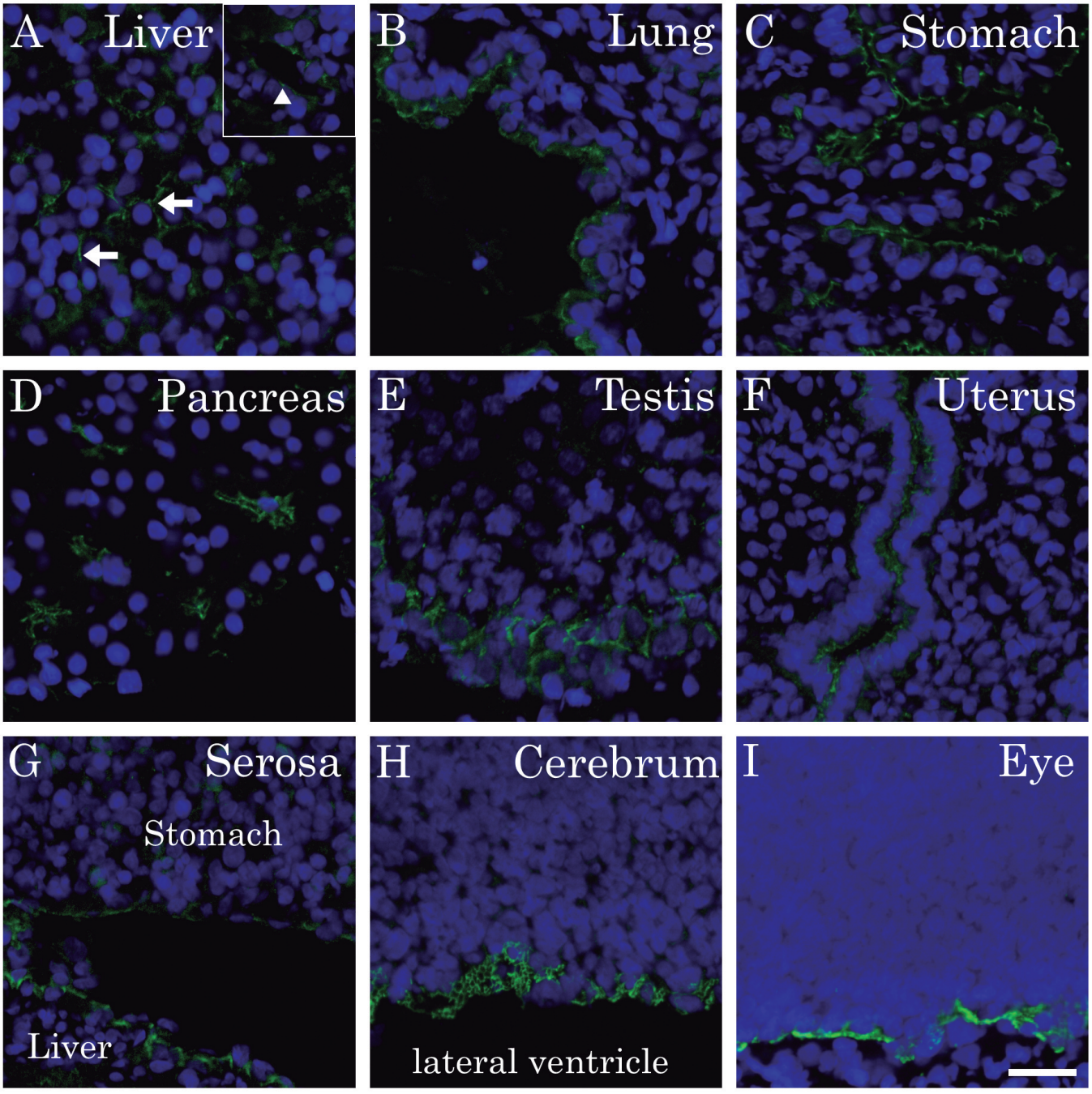

Fig. 2. Ccdc85C protein expression in various rat organs. Many kinds of epithelial cells express Ccdc85C. **A.** Liver, P0. Ccdc85C expression was observed at hepatocytes (arrows) and cholangiocytes (inset, arrowhead). **B.** Lung, bronchiole, P0. **C.** Glandular stomach, P0. **D.** Exocrine pancreas, P0. **E.** Testis, seminiferous tubule, P30. Dashed line indicates basement membranes of the seminiferous tubule. **F.** Uterus, P30. **G.** Serosa of stomach and liver, P0. **H.** Cerebrum, lateral ventricle, E20. **I.** Eye, retina, P0. Arrowheads indicate the outermost layer of the retina. Scale bars: 25 μm.

cortex, known as proliferative region at development period (Nagata et al., 1993; Combs et al., 1998) (Fig. 6A,B, between arrowheads). EdU-labeled cells in other regions were relatively many at P0; however, those cells decreased at P7. After P14, EdU-labeled cells decreased and were diffusely distributed (Fig. 6C). At all time points, Ccdc85C expression was stronger in the subcapsular cortex than that in other region. In other

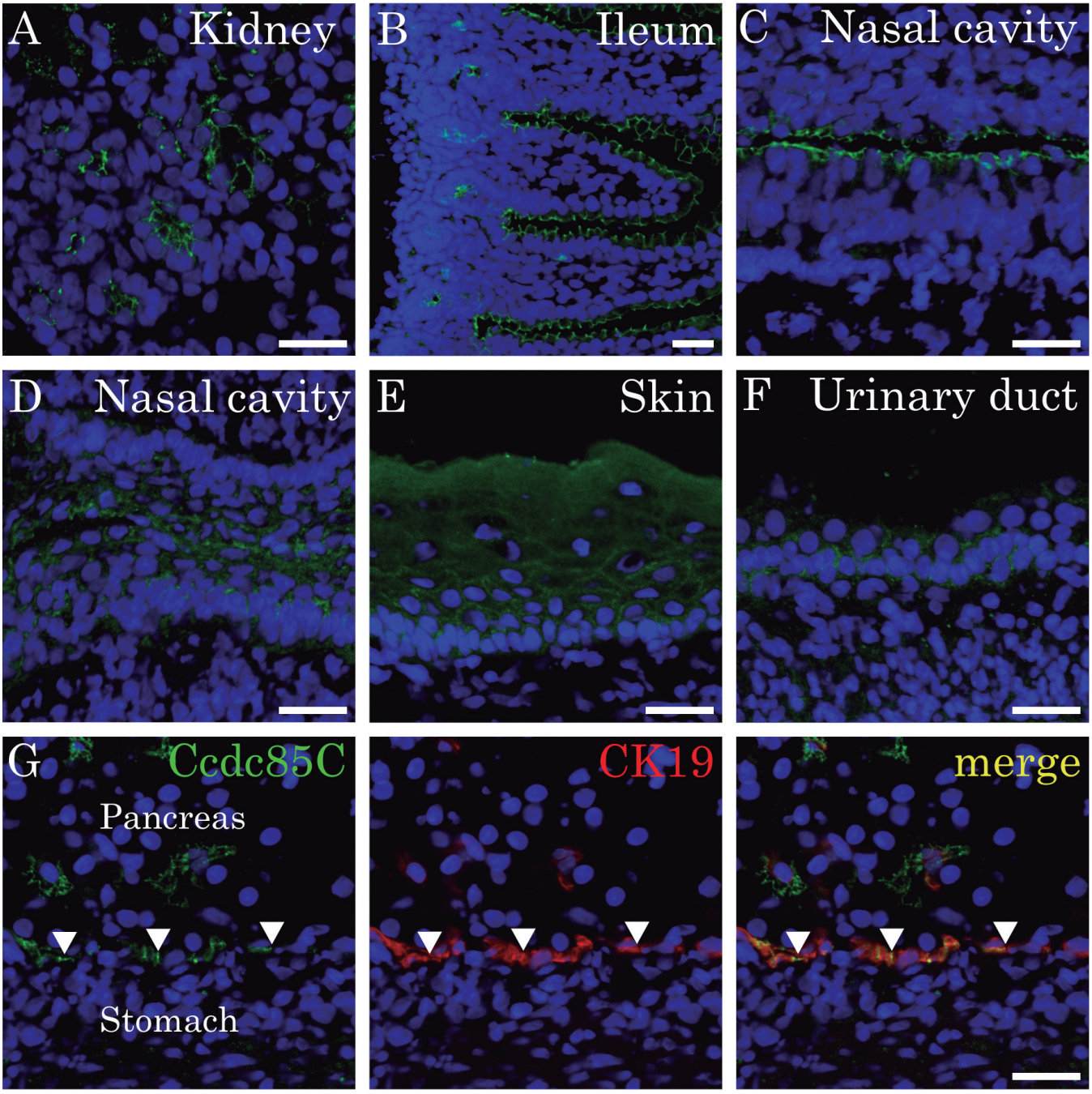

Fig. 3. Types of epithelia expressing Ccdc85C. Various types of epithelia except for stratified epithelia express Ccdc85C. **A.** Simple cuboidal epithelia, renal tubules of kidney, P0. **B.** Simple columnar epithelia, ileal mucosa, P14. **C.** Pseudostratified columnar epithelia, mucosa of nasal cavity, E20. **D.** Stratified squamous epithelia, mucosa of nasal cavity, E20. **E.** Stratified squamous keratinizing epithelia, head skin, P0. **F.** Transitional epithelia, urinary duct, P7. **G.** Simple squamous epithelia, double immunofluorescence for Ccdc85C (green) and CK19 (red), mesothelia (arrowheads) between stomach and pancreas, P0. Scale bars: 25 μm.

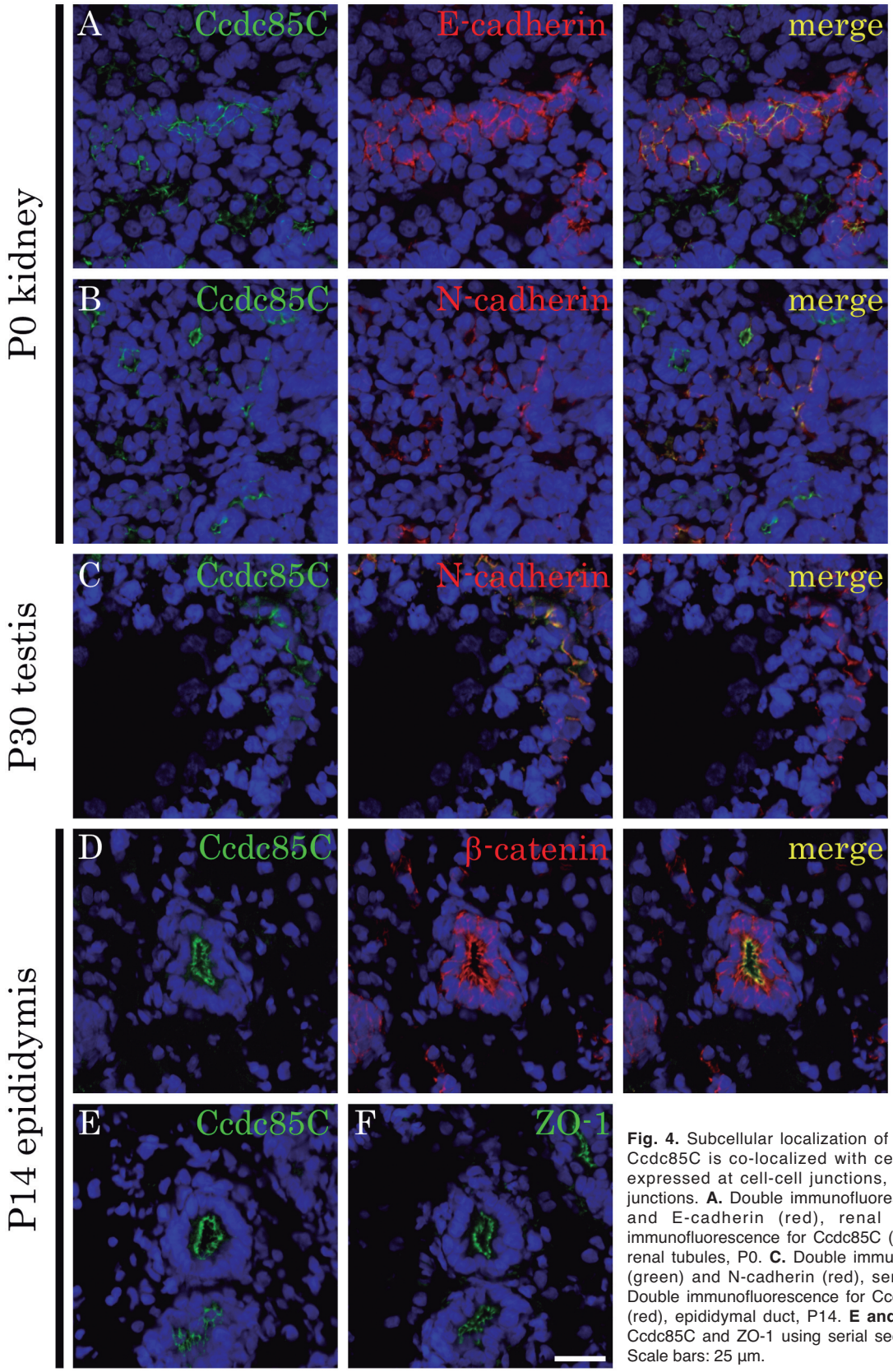

Fig. 4. Subcellular localization of Ccdc85C in epithelial cells. Ccdc85C is co-localized with cell adhesion molecules and expressed at cell-cell junctions, especially strong at apical junctions. **A.** Double immunofluorescence for Ccdc85C (green) and E-cadherin (red), renal tubules, P0. **B.** Double immunofluorescence for Ccdc85C (green) and N-cadherin (red), renal tubules, P0. **C.** Double immunofluorescence for Ccdc85C (green) and N-cadherin (red), seminiferous tubules, P30. **D.** Double immunofluorescence for Ccdc85C (green) and β-catenin (red), epididymal duct, P14. **E and F.** Immunofluorescence for Ccdc85C and ZO-1 using serial sections, epididymal duct, P14. Scale bars: 25 μm.

regions, Ccdc85C expression was relatively strong at P0, and then weakened and finally almost disappeared at P60. In the ileum, Ccdc85C was expressed throughout the mucosa, whereas proliferating cells were localized only at crypts. This homogeneous Ccdc85C expression at the villi was observed at all time points (Fig. 6E,F). Taken together, Ccdc85C was expressed in the region or organ where EdU-positive proliferating cells were abundant, although Ccdc85C expression and EdU signals were not always overlapped in the same cells.

Discussion

Ccdc85C of rats and mice

We demonstrate that Ccdc85C is expressed on the surface of the cerebral ventricles in rats. The characteristics of rat Ccdc85C coincide with those of murine Ccdc85C (Mori et al., 2012). Alignment search for the rat Ccdc85C protein by Basic Local Alignment Search Tool (BLAST) in National Center for Biotechnology Information (NCBI) indicates that Ccdc85C protein of rats has high homology to murine Ccdc85C (identities in protein: 92%). Therefore, the present results suggest that rat Ccdc85C protein has almost the same characteristics and functions as Ccdc85C of mice.

Ccdc85C expression in epithelia

In the present study, we demonstrate that many types of simple epithelia express Ccdc85C in rat tissues and Ccdc85C expression is localized at epithelial junctions, especially strong at the apical region. In the simple epithelia, tight junctions are localized at apical membranes and adherens junctions are localized at basolateral membranes, which form polarization of cell-cell junctions. On the other hand, cell-cell junctions of

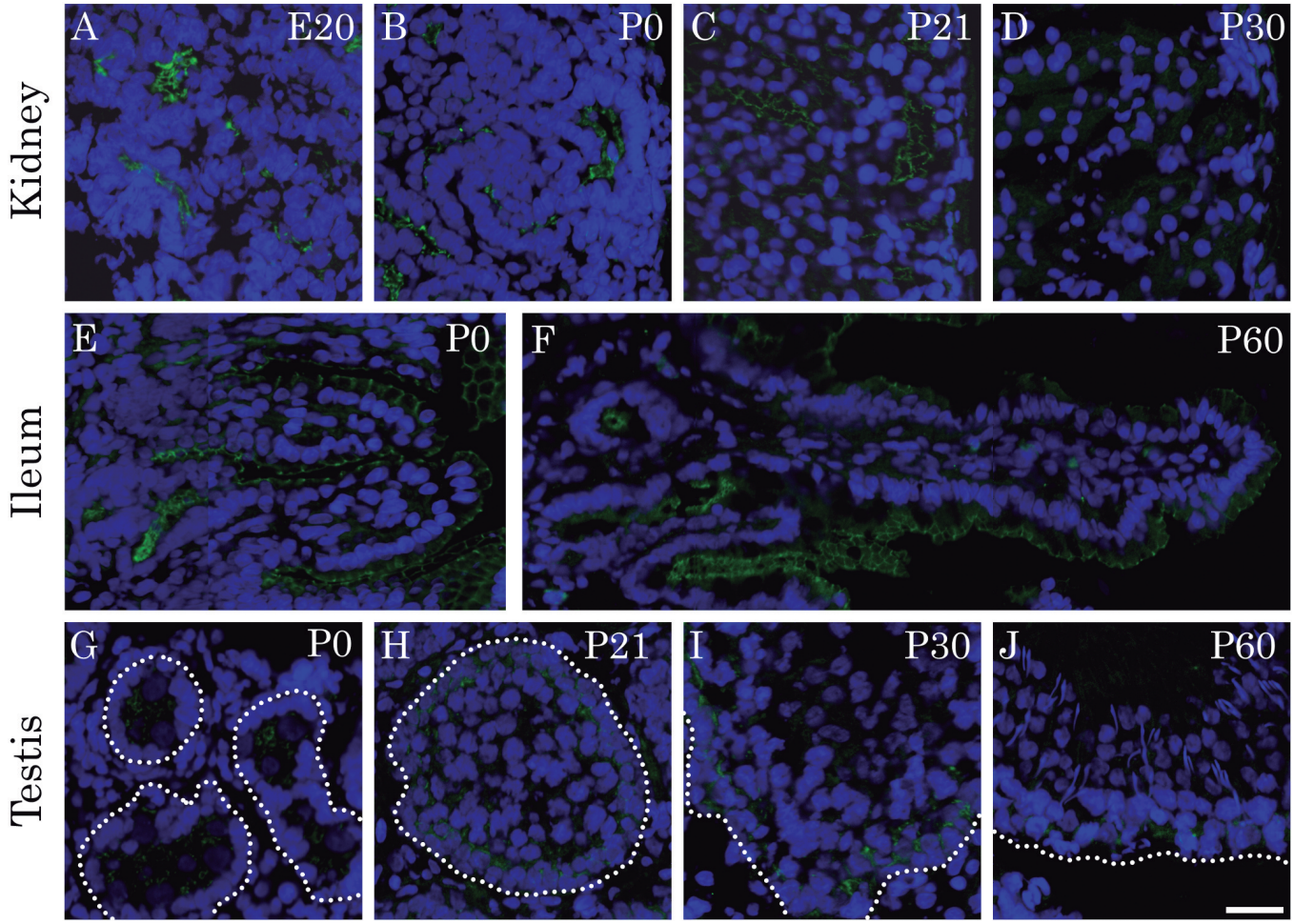

Fig. 5. Temporal change of Ccdc85C expression in organ development. **A-D.** Kidney at E20 **(A)**, P0 **(B)**, P21 **(C)** and P30 **(D)**; Ccdc85c expression is strong at E20 and P0. **E and F.** Ileum at P0 **(E)** and P60 **(F)**; Ccdc85C expression is still detected at P60. **G-J.** Testis at P0 **(G)**, P21 **(H)**, P30 **(I)** and P60 **(J)**; Ccdc85C expression is strong at P30. Dashed lines indicate basement membranes of the seminiferous tubules. Scale bar: 25 μm.

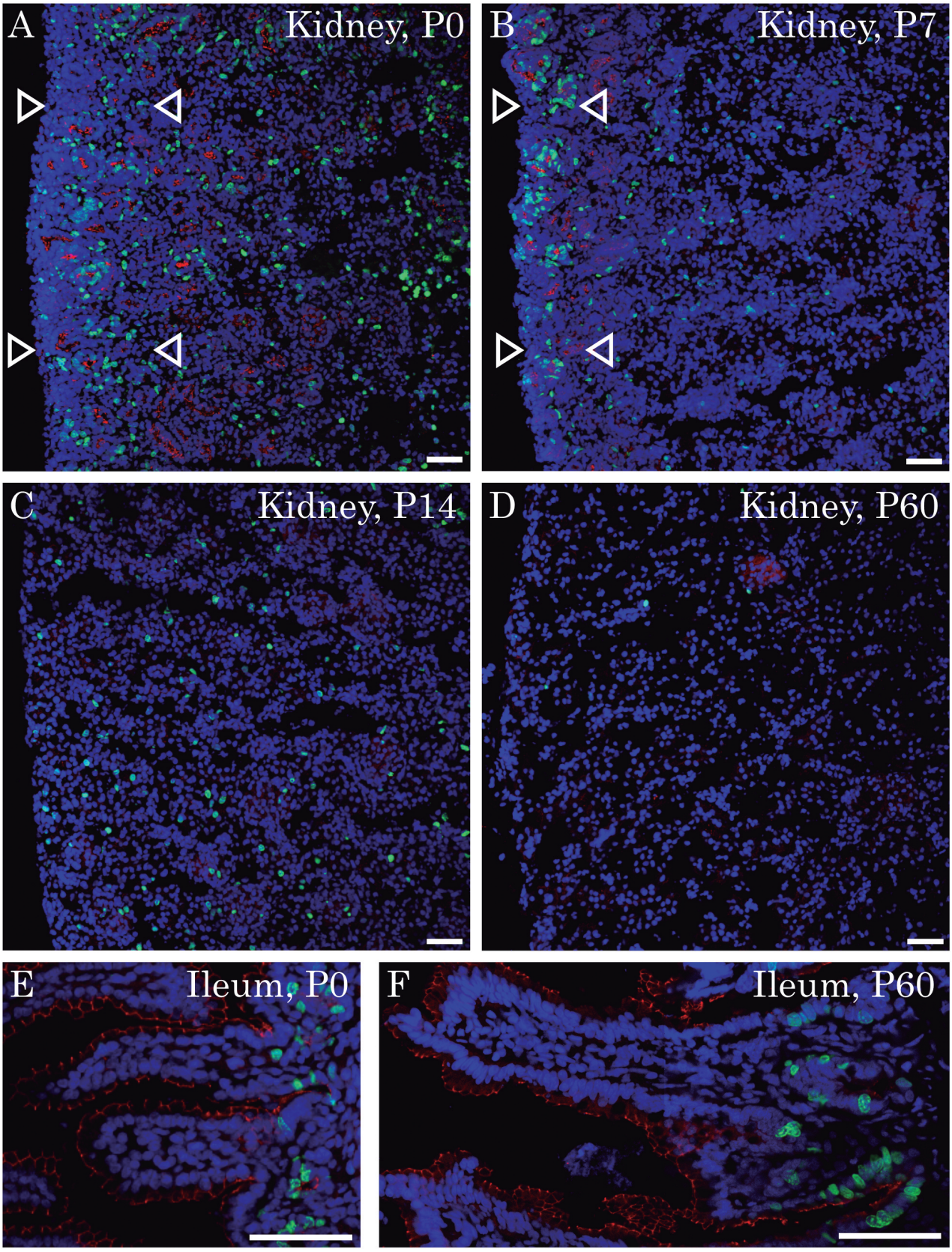

Fig. 6. Distributions of Ccdc85C and 5-ethynyl-2-deoxyuridine (EdU)-labeled proliferating cells. **A-D.** Kidney at P0 **(A)**, P7 **(B)**, P14 **(C)** and P60 **(D)**; Distribution of Ccdc85C is consistent with that of EdU-labeled cells at all time points. Arrowheads indicate subcapsular cortical regions. **E and F.** Ileum at P7 (E) and P60 **(F)**; Ccdc85C distributes from the crypts to the tip of villi, whereas EdU-labeled proliferating cells are located only at crypts. Scale bars: 50 μm.

stratified epithelia differ from those of simple epithelia. For example, in the skin, basal keratinocytes lack tight junctions and zonula adherens, and E-cadherin expression is found all around the cell cortex (Muroyama and Lechler, 2012). Therefore, we speculate that Ccdc85C plays some roles in the tight junction in the simple epithelia. In the epithelial cell junctions, Ccdc85C colocalized with adhesion molecules. A recent study has demonstrated that p120-catenin, a cell adhesion molecule stabilizing cadherin expression, bound to exogenous Ccdc85C in Madin-Darby canine kidney (MDCK) cells (Markham et al., 2014). These facts indicate that Ccdc85C may interact and function with these cell adhesion molecules for the development of epithelial cells.

Ccdc85C and organ development

Strong expression of Ccdc85C was observed at E20 and P0 in many organs. Subsequently, Ccdc85C expression decreased with the advancing organ development. In contrast, Ccdc85C expression becomes strong from P21 and was the strongest at P30 in the testes. Pubertal development starts from approximately P40 in SD rats (Korenbrot et al., 1977). Therefore, our data indicates that Ccdc85C expression in the seminiferous tubules is considered to correlate with testicular development during the sexual maturation period.

Ccdc85C and cell proliferation

In the present study, temporal expression of Ccdc85C in the kidney of rats shows similar pattern to cerebrum of mice (Mori et al., 2012), whereas epithelia of the alimentary tract, especially the intestine, continue to express Ccdc85C even at P60. In the adult alimentary tract, self-renewing stem cells and transit-amplifying cells reside at the isthmus of the stomach and crypts of the intestine and constantly undergo cell division to produce massive epithelia (Barker et al., 2007; Mills and Shivdasani, 2011). These results suggest that Ccdc85C expression may be associated with cell proliferation for not only tissue development but also postnatal maintenance of mucosal epithelia. Ccdc85C was expressed in the apex of intestinal villi, whereas EdU-positive proliferating cells did not reside. This indicated that Ccdc85C expression is not restricted in self-renewal cells. Further studies are needed to reveal detailed relationship between Ccdc85C expression and proliferative activity.

Ccdc85C and Hippo pathway

A recent study has revealed that human CCDC85C directly binds to YAP1 and promotes YAP1 translocation from nuclei to cytoplasm (Wang et al., 2014). Our present study shows the relationship between Ccdc85C expression, cell proliferation and organ development. These results may support the theory that Ccdc85C / CCDC85C regulates organ development through interaction with YAP1 in the Hippo pathway. In mammalian polarized epithelia, some upstream components of Hippo pathway are localized at apical membrane and tight junction, such as KIBRA, Merlin or Crumbs (Varelas et al., 2010; Zhang et al., 2010; Xiao et al., 2011). Already known apical proteins associated with tight junction or cellular polarity, like ZO-2 or aPKC, are suggested to be related with the Hippo pathway (Grzeschik et al., 2010; Oka et al., 2010). Furthermore, AMOT is reported to be localized at tight junction and induces YAP to localize at tight junction by binding to YAP, like CCDC85C (Zhao et al., 2011b). Thus, the cell-cell junction of polarized epithelia is an important location for the Hippo pathway. In the present study, Ccdc85C is localized at tight junctions in the various epithelia. Another recent report has indicated that CCDC85C is involved in part of a mechanism integrating different signal inputs from cell junctions (Moya and Halder, 2014).

Ccdc85C and hhy mice

We found Ccdc85C expression at radial glia of the spinal cord and retinal progenitor cells. Our previous study showed that lack of Ccdc85C expression causes subcortical heterotopia in cerebrum of *hhy* mice by early migration of neural progenitor cells (Mori et al., 2012). In the study using chicken, YAP regulates neural progenitor cell number by affecting cell proliferation, differentiation and survival (Cao et al., 2008). Therefore, these facts suggest that lack of Ccdc85C in *hhy* mice causes disruptions of neuronal lamination by YAP1 dysregulation of neural progenitors. However, further studies are needed to demonstrate this hypothesis.

In conclusion, we found Ccdc85C expression in the systemic simple epithelia with proliferative activity in rats. Our present study sheds light on the properties of Ccdc85C. Our antibody against Ccdc85C would be helpful to study the biological property of Ccdc85C. Further studies will be required to reveal Ccdc85C function in the proliferative epithelia and the interaction between Ccdc85C and Hippo pathway.

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