

Original Article

The localization and distribution of cells labeled by a somatic stem cell-recognizing antibody (A3) in rat colon development; possible presence of a new cell type forming the intestinal stem cell niche

Hironobu Nishina¹, Chisa Katou-Ichikawa¹, Mizuki Kuramochi¹, Takeshi Izawa¹, Mitsuru Kuwamura¹, and Jyoji Yamate^{1*}

¹Laboratory of Veterinary Pathology, Osaka Prefecture University, 1-58 Rinku-Ourai Kita, Izumisano, Osaka 598-8531, Japan

Abstract: A3, generated as a monoclonal antibody against rat malignant fibrous histiocytoma (MFH)-derived cloned cells, recognizes somatic stem cells (bone-marrow/hair follicle stem cells). We investigated the distribution of cells immunoreactive to A3 in the developing rat intestine (particularly, the colon), focusing on the ontogenic kinetics of A3-positive cells. In the rat intestine, A3 labeled spindle-shaped stromal cells localized in the submucosa and labeled endothelial cells of capillaries in the lamina propria forming villi in the early development stage. With development progression, A3-positive cells were exclusively localized around the crypts of the colon. Double immunofluorescence revealed that A3-positive cells around the crypts reacted to vimentin (for mesenchymal cells) and Thy-1 (for mesenchymal stromal cells) but not to α -SMA (for mesenchymal myofibroblastic cells) or CD34 (for hematopoietic stem cells), indicating that A3-positive cells around the crypts may have characteristics of immature mesenchymal cells. In addition, A3 labeled a few epithelial cells at the base of colon crypts. Furthermore, immunoelectron microscopy revealed that A3-positive cells lay inside myofibroblasts adjacent to the epithelium of the crypts. A3-positive cells were regarded as a new type of immature mesenchymal cells around the crypts. Collectively, A3-positive cells might take part in the stem cell niche in the colon, which is formed through epithelial-mesenchymal interaction. (DOI: 10.1293/tox.2018-0037; J Toxicol Pathol 2019; 32: 37–48)

Key words: stem cell, stem cell niche, malignant fibrous histiocytoma, monoclonal antibody, colon development, F344 rat

Introduction

Antibodies specific to certain cell types are very useful to pursue the kinetics and participation in genesis and lesion development of cells. To investigate the histogenesis of rat malignant fibrous histiocytoma (MFH), A3 was generated as a monoclonal antibody against MT-8 cells as the antigen^{1–4}. MT-8 cells were established from a spontaneous rat MFH and regarded as primitive pluripotential mesenchymal cells that may be capable of differentiating into well-differentiated mesenchymal cells, such as adipogenic, osteogenic, and myofibroblastic cells^{4, 5}. In addition to rat MFH cells, interestingly, A3 labeled somatic stem cells such as bone marrow stem cells, hair follicle stem cells, and pericytes (considered a mesenchymal stem cell) in normal rat

tissues^{3, 5}. These somatic stem cells have been considered to show pluripotency^{6, 7}. Based on these findings obtained by A3 immunohistochemistry, MFHs might be derived from somatic stem cells.

Ontogenetically, the intestinal epithelium is generated from the endoderm and forms the crypt-villus unit in the mucosa. On the other hand, the mesenchyme (undifferentiated mesenchymal cells) is generated from the mesoderm. The differentiation of mesenchyme is regulated by epithelial-mesenchymal interaction, thereafter, forming the lamina propria, submucosa, and smooth muscle layers in the intestine^{8, 9}. In adulthood, the intestinal mucosal epithelium is continuously renewed by intestinal stem cells localized in the crypt every 3 to 5 days throughout life; this self-renewal is tightly regulated by tissue-specific microenvironments: stem cell niches. The intestinal subepithelial myofibroblasts, well-known to be localized at the base of intestinal crypts, are considered as supportive cells for the stem cell niche. These myofibroblasts are immunohistochemically characterized by α -smooth muscle actin (α -SMA) expression^{10–12}.

The aim of the present study was to investigate the distribution of cells immunoreactive to A3 in the developing rat intestine (particularly, the colon, because the colon is well known to have clear crypts with the stem cell niche^{8, 9}), focusing on the ontogenic kinetics of A3-positive cells.

Received: 12 June 2018, Accepted: 9 August 2018

Published online in J-STAGE: 9 September 2018

*Corresponding author: J Yamate

(e-mail: yamate@vet.osakafu-u.ac.jp)

©2019 The Japanese Society of Toxicologic Pathology

This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives

(by-nc-nd) License. (CC-BY-NC-ND 4.0: <https://creativecommons.org/licenses/by-nc-nd/4.0/>).



Materials and Methods

Animals

Two pregnant F344/DuCrj rats (15-day gestation) were obtained from Charles River Laboratories Japan (Yokohama, Japan). These animals were housed in an animal room with a controlled temperature of $22 \pm 3^\circ\text{C}$ and a 12-hour light-dark cycle; they were allowed free access to a standard commercial diet (DC-8, CLEA Japan, Tokyo, Japan) and tap water. Intestine samples were obtained from fetal rats on gestation days 18 and 20 ($n=3$ each). Colon samples were obtained from 3 neonatal rats aged 1, 3, 6, 10, 15, and 20 days ($n=3$ each). Additionally, colon tissues were also prepared as adult samples from rats more than 6 weeks old ($n=3$). Pregnant rats were deeply anesthetized with isoflurane for caesarean section and were euthanized by exsanguination with deep isoflurane anesthesia after removing the fetuses. Fetal rats were euthanized by decapitation. Neonatal and adult rats were euthanized by exsanguination with deep isoflurane anesthesia. Animal housing and sampling conformed to the institutional guidelines for animal care of Osaka Prefecture University.

Tissue preparation and histology

Samples for morphological examination were fixed in periodate-lysine-paraformaldehyde (PLP) fixative solution, and others were frozen immediately in Tissu Mount® (Chiba Medical, Saitama, Japan) and stored at -80°C . The samples immersed in the PLP fixative for 6 h at 4°C were then embedded in paraffin by the AMeX (acetone-methyl benzoate-xylene) method¹³. PLP-AMeX-processed tissues were cut at a thickness of $4 \mu\text{m}$ and stained with hematoxylin and eosin (HE) for morphological examination. The fresh frozen samples kept -80°C were cut at a thickness of $10 \mu\text{m}$ for immunohistochemistry and double immunofluorescence.

Immunohistochemistry and double immunofluorescence

The information for primary antibodies used in this study is shown in Table 1; the following antibodies were mainly used: RECA-1 for endothelial cells, laminin for basement membrane, vimentin and α -SMA for mesenchymal/myofibroblastic cells, Thy-1 and CD34 for stem cells, and cytokeratin 19 (CK19) for epithelial cells.

For immunohistochemistry, PLP-AMeX-processed tissue sections were used for all antibodies except for CK19 and RECA-1, for which fresh-frozen sections were used. For antigen retrieval, microwaving for 20 min in citrate buffer (pH 6.0) was performed for A3, Thy-1, and vimentin. These sections were treated with 5% skimmed milk in phosphate buffered saline (PBS) for 15 min. The section for CD34 was treated with 10% normal horse serum in PBS for 15 min. The sections were incubated with each primary antibody for 1 h at room temperature or overnight at 4°C . The sections were then incubated with 3% H_2O_2 in PBS for 15 min to quench endogenous peroxidase, followed by 1 h of incubation with horseradish peroxidase-conjugated secondary antibody (Histofine Simple Stain MAX PO, Nichirei, Tokyo, Japan). Anti-goat IgG secondary antibody (ImPRESS™ REAGENT Anti-Goat Ig, Vector Laboratories, Inc., Burlingame, CA, USA) was used for CD34. Positive reactions were detected with 3,3'-diaminobenzidine (DAB; Substrate Kit, Nichirei). Sections were counterstained lightly with hematoxylin. A3 reactivity was scored as follows: -, negative; +, weakly positive; ++, moderately positive; and +++, strongly positive.

For double immunofluorescence, fresh-frozen samples kept at -80°C were cut at a thickness of $10 \mu\text{m}$, and then these sections were fixed for 15 min with the PLP fixative at room temperature. The prepared frozen tissue sections for all antibodies except for CD34 were treated with 10% normal goat serum in PBS for 15 min to reduce nonspecific reactions; for CD34, 10% normal horse serum in PBS

Table 1. Primary Antibodies Used for Immunohistochemistry and Double Immunofluorescence

Antibody	Poly/ Mono	Clone	Format	Dilution	Pretreatment	Source
A3	mouse mono	A3	purified	1:500	Microwave in Citrate buffer, 20 min	TransGenic, Inc., Kobe, Japan
RECA-1	mouse mono	HIS52	Alexa Fluor 488-labeled	1:200	-	Novus Biologicals, Littleton, USA
laminin	mouse mono	D18	purified	1:200	-	NeoMarkers, California, USA
Vimentin	mouse mono	V9	purified	1:1000	Microwave in Citrate buffer, 20 min	abcam, Cambridge, UK
α -SMA	mouse mono	1A4	purified	1:1000	-	Dako, Glostrup, Denmark
Thy-1(CD90)	mouse mono	OX-7	Alexa Fluor 488-labeled	1:500	Microwave in Citrate buffer, 20 min	Bio Rad, California, USA
CD34	goat poly	-	purified	1:1000 or 1:5000	-	R&D systems, Inc., Minneapolis, USA
Cytokeratin 19	mouse mono	BA17	Alexa Fluor 488-labeled	1:200	-	eBioscience, Inc., San Diego, USA

was used. Then, the sections were incubated with each primary antibody for 1 h at room temperature or overnight at 4°C. Visualization of antibody-specific binding was performed with fluorochrome-conjugated secondary antibodies (Table 2). Next, sections were incubated with a conjugated or unconjugated second primary antibody for 1 h and then treated with an appropriate fluorochrome-conjugated secondary antibody. These sections were mounted with mounting medium including 4',6-diamidino-2-phenylindole (DAPI; Fluoro-KEEPER Antifade Reagent, Nacalai Tesque, Inc., Kyoto, Japan) for nuclear staining, and images were captured with a VS120 Virtual Slide System (Olympus Corporation, Tokyo, Japan) or a laser scanning confocal imaging microscope (C1Si, Nikon, Tokyo, Japan) and processed with EZ-C1 Viewer (Nikon).

Immunoelectron microscopy

An adult rat colon was sampled and was then immersion fixed in a mixture of 0.1% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 4 h at 4°C. The colon was then treated sequentially with an increasing gradient of 10%, 20%, and 30% sucrose in PBS at 4°C overnight and embedded in Tissue Mount® (Chiba Medical, Saitama, Japan). Frozen sections of the colon were washed with PBS and blocked in 10% normal goat serum for 15 min. The tissue sections were then incubated with A3 (diluted 1 in 500) overnight at 4°C. Thereafter, sections were washed three times with PBS and incubated with 3% H₂O₂ in PBS for 15 min to quench endogenous peroxidase, followed by 1 h of incubation with horseradish peroxidase-conjugated secondary antibody (Histofine Simple Stain MAX PO, Nichirei, Tokyo, Japan). Positive reactions were detected with DAB (Substrate Kit, Nichirei) before being fixed in 1% glutaraldehyde for 10 min at room temperature. Sections were then postfixed with 0.5% osmium for 2 h at 4°C. Thereafter,

they were dehydrated through a graded series of ethanol and propylene oxide for 20 min and embedded in epoxy resin. Ultrathin sections were examined with an electron microscope (H-7500, Hitachi, Tokyo, Japan) at 80 kV.

Results

Development of the rat intestine

The intestine of the fetal rat was analyzed as the whole intestine, because it was difficult to distinguish between the small intestine and large intestine. As shown in Fig. 1, in the intestine of fetal rats on gestation days 18 and 20, the lengths of the villus and epithelial cells were still short. In the colon of neonatal rats, the villi showed irregular lengths like the small intestine. On neonate day 1, mucosal epithelial cells notably showed vacuoles in the cytoplasm transiently. The muscle layer and goblet cells were more developed from neonate day 3. Invagination of epithelial cells (crypts) began to be seen on neonate day 6, and the development of the colon was almost completed on neonate days 15–20, being close to structures of the mature colon in adulthood.

A3 immunoeexpression in the developing intestine

The scoring for A3 expression is summarized in Table 3. In the intestine of fetal and early neonate rats, A3 strongly labeled spindle-shaped stromal cells, which were mainly localized in the submucosa, and endothelial cells of capillaries in the lamina propria forming villi. The A3 reactivity of stromal cells was weaker with age from neonate days 6 to 10. On neonate day 15, interestingly, A3-positive cells began to be seen around the crypts in the subsurface of the mucosal epithelium (Fig. 2). A3 also labeled occasionally endothelial cells of the vessels in the submucosa.

In adult rats, A3-positive cells were exclusively localized around the crypts, that is, surrounding the base to

Table 2. Secondary Antibodies Used for Double Immunofluorescence

Antibody	Label	Host	Source	Target primary antibody
Anti-mouse IgG	Alexa 488	Goat	Thermo Fisher Scientific, Massachusetts, USA	A3 when using CD34
Anti-mouse IgG1	Alexa 568	Goat	Thermo Fisher Scientific, Massachusetts, USA	A3
Anti-mouse IgG2a	Alexa 488	Goat	Thermo Fisher Scientific, Massachusetts, USA	α -SMA
Anti-goat IgG	Alexa 568	Donkey	Thermo Fisher Scientific, Massachusetts, USA	CD34

Table 3. Expression Grades of A3 in Developing Rat Intestines

		Intestine		
		lamina propria	pericrypt	villus
Intestine	F18	++	NA	++
	F20	+	NA	+
	N1	++	NA	+
	N3	++	NA	+
Colon	N6	+	+	–
	N10	– ~ +	– ~ +	–
	N15	+	+++	++
	N20	+	+++	++
	adult	– ~ +	+++	– ~ +

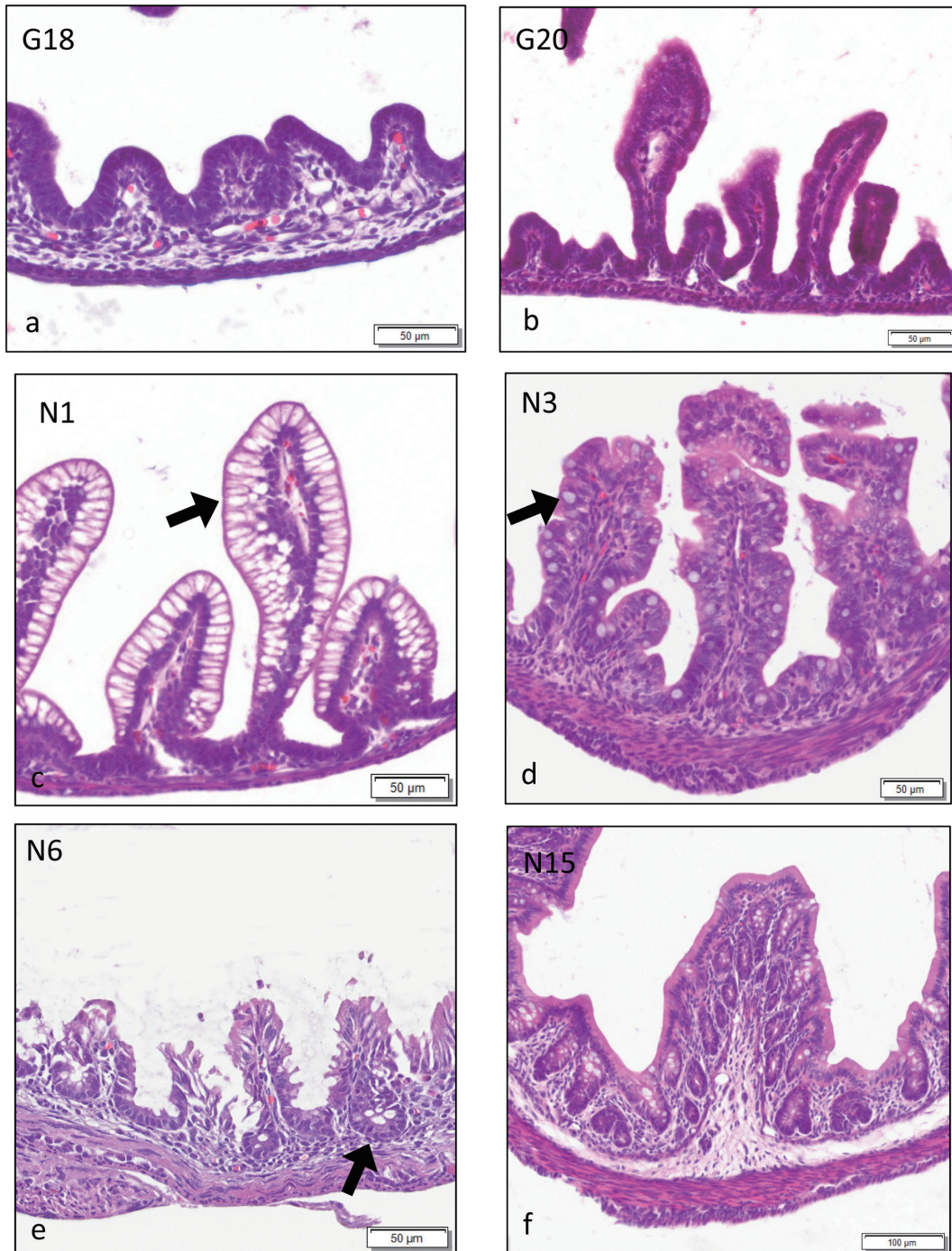


Fig. 1. Normal developing colon of rats. The lengths of the villus and epithelial cells are short on gestation days 18 and 20 (a, b). The length of the villus is irregular during development; epithelial cells are vacuolated notably on neonate day 1 (arrow) (c). The muscle layer and goblet cells (arrow) begin to be seen on neonate day 3 (d). Engraftment of epithelial cells begins to be seen on neonate day 6 (arrow) (e). The development of the colon is almost complete on neonate days 15-20 (f). H&E staining. Bars = 50 μm (a-e), 100 μm (f). G, gestation day; N, neonate day.

middle of crypts with decreased expression at the apex of the villus (Fig. 3a, b). A3 reactivity, which may correspond to spindle-shaped stromal cells in the submucosa, became much weaker than in the developing colons. A3 labeled capillary endothelial cells in the submucosa (Fig. 3a); interestingly, a few epithelial cells constituting the crypts reacted

variously to A3 (Fig. 3c).

Characterization of A3-positive cells in the developing intestine

To further investigate the characteristics of A3-positive cells, double immunofluorescence labeling was performed

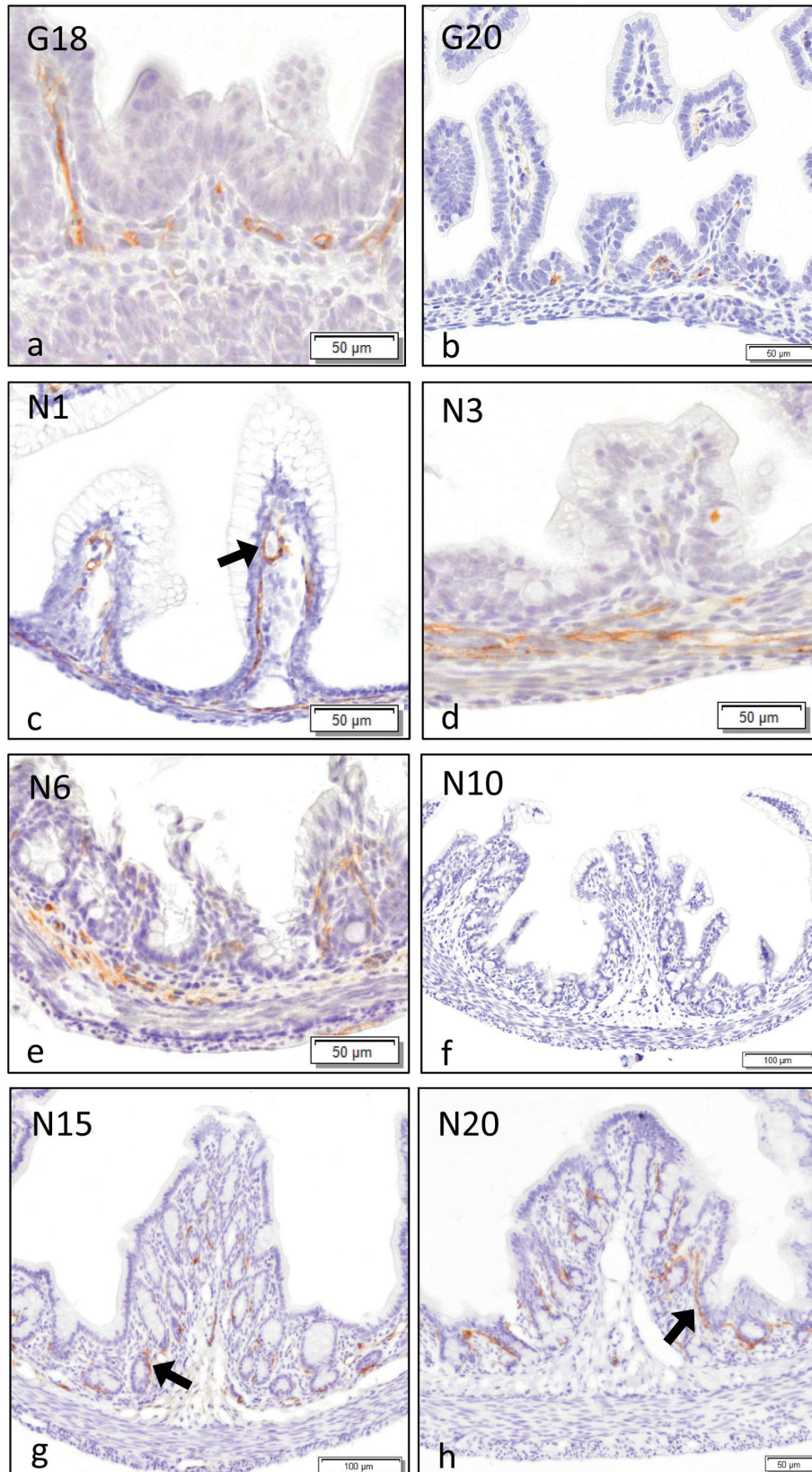


Fig. 2. A3-immunopositive cells in the developing colon. In fetal and neonatal rats, A3 strongly labels spindle-shaped cells and endothelial cells of capillaries (arrow) in the submucosa (a–d). The A3 reactivity of stromal cells is weaker with age from neonate days 6 (e) to 10 (f). A3-immunopositive cells begin to be seen around the crypts in the subsurface of the mucosal epithelium (arrow) on neonate days 15 (g) and 20 (h). Immunohistochemical staining for A3. Bars = 50 μm (a–e, h), 100 μm (f, g). G, gestation day; N, neonate day.

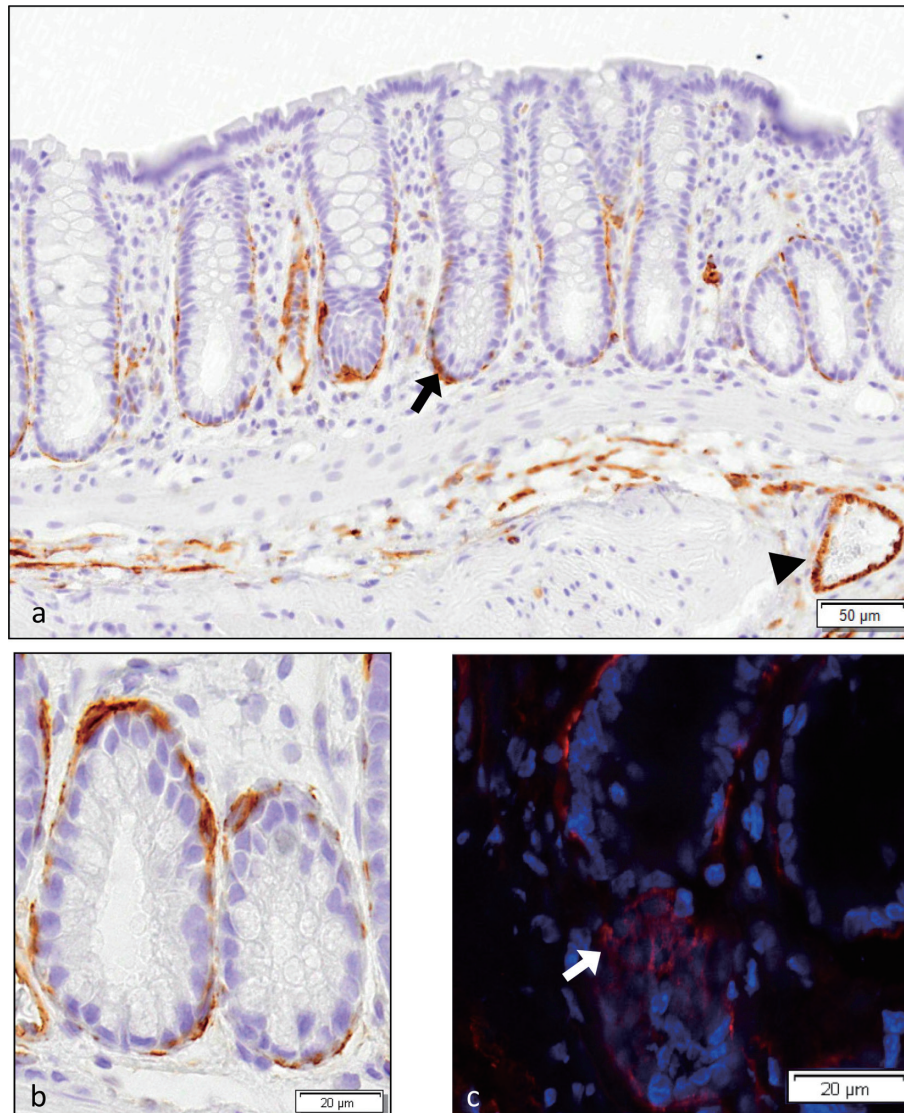


Fig. 3. A3-immunopositive cells in the adult colons. A3 labels spindle-shaped cells at the periphery of the crypts (arrow) and endothelial cells of vessels (arrowhead) and capillaries in the submucosa (a): higher magnification of the crypts (b). Some epithelial cells express the epitopes recognized by A3 (arrow) (c). Immunohistochemical staining (a, b) and immunofluorescence (c) for A3. Bars = 50 µm (a), 20 µm (b, c).

with other marker antibodies. In fetal and early neonate rats, the majority of A3-positive cells seen in the submucosa and villus cluster co-expressed RECA-1 (for rat endothelial cells)¹⁴ (Fig. 4). In adult rats, RECA-1 reactivity was not seen in A3-positive cells around the crypts, and A3-expressing endothelial cells were drastically decreased (Fig. 5).

Characterization of A3-positive cells around the crypts of the colon in adult rats

In adult rats, A3-positive cells were exclusively localized around the crypts of the colon. To further investigate the properties, these cells were analyzed by focusing on the relationship with myofibroblasts and immature mesenchymal cells, which have been considered to participate in the intestinal stem cell niche^{15, 16}. In the double immunofluores-

cence method, A3-positive cells were located outside the basement membrane reacting to laminin (Fig. 6c). Cells reacting to vimentin and Thy-1 simultaneously co-expressed A3-recognizing antigen (Fig. 6f, 6i). α -SMA-expressing myofibroblasts seen outside the basement membrane did not react to A3 (Fig. 7c). Although CD34-expressing endothelial cells co-expressed A3 in the lamina propria of the intestine, A3-positive cells around the crypts did not react to CD34 (Fig. 7f). More interestingly, co-expression of CK19 was seen in a few A3-positive cells (Fig. 7i).

Based on these findings, it was considered that A3-positive cells around the crypts might have features of immature mesenchymal cells (vimentin and Thy-1 reactivity) but not show a myofibroblastic (α -SMA reactivity) or endothelial (CD34 reactivity) nature. Interestingly, A3-positive cells

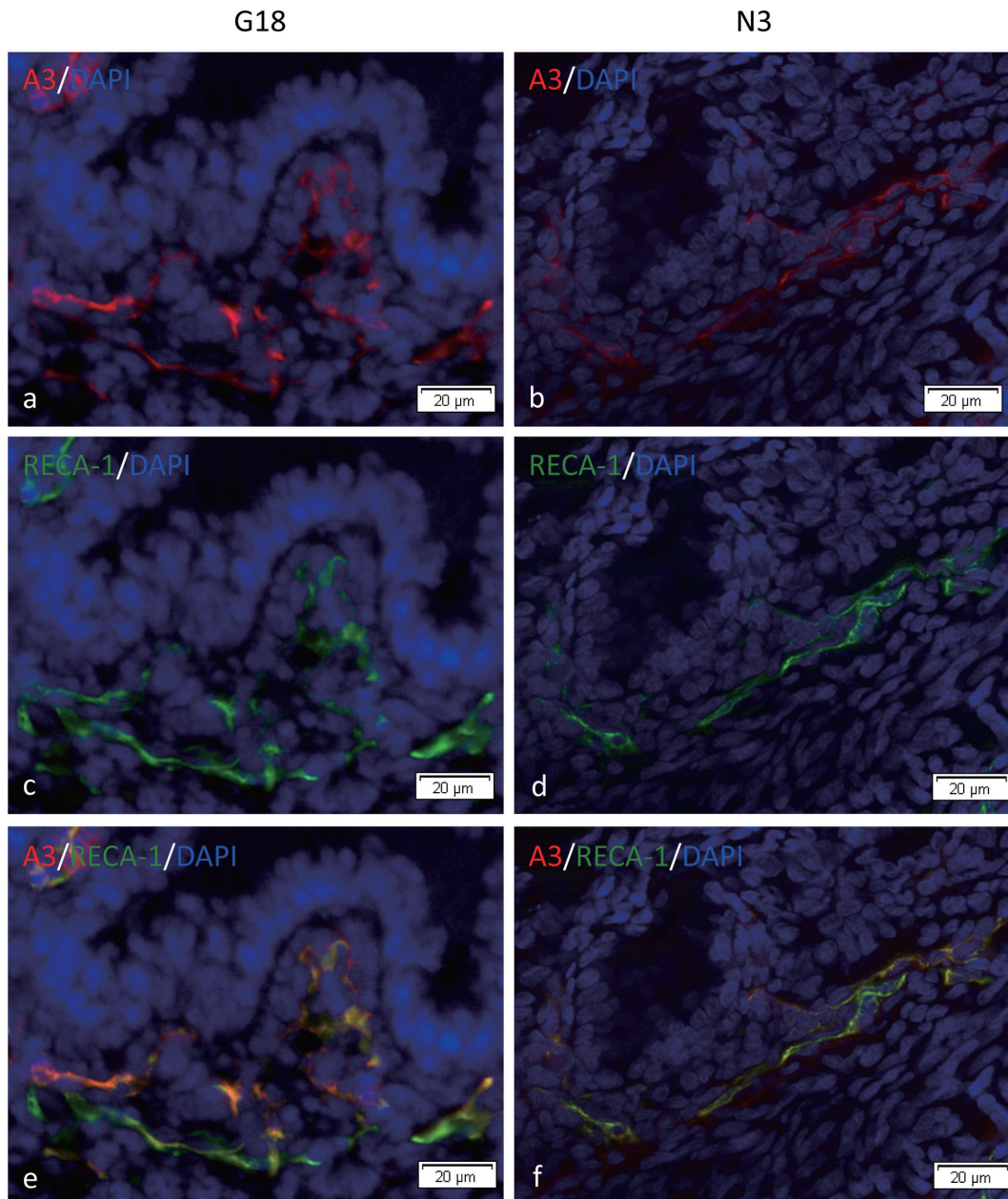


Fig. 4. Immunofluorescence for A3 (a, b) and RECA-1 (c, d), as well as merged findings from double immunofluorescence for A3/RECA-1 (e, f) in the adult colons. DAPI was used for nuclear staining. Bar = 20 µm.

might have the feature of epithelial (CK19 reactivity) partly.

Immunoelectron microscopy of A3 reaction

It was confirmed that A3-positive cells around the crypts were located outside the basement membrane and very adjacent to epithelial cells (Fig. 8a). Reaction products of A3 were seen exclusively on the cytoplasmic membrane (Fig. 8b). These findings indicated that A3-positive cells were located more closely to epithelial cells than the intestinal subepithelial myofibroblasts.

Discussion

A3-positive cells in the colon of the fetus and neonates

It has been reported that A3 labeled spindle-shaped cells in the kidneys and lungs of rat fetuses^{4, 17}. Because of co-expression of vimentin and Thy-1, these cells are considered to be immature mesenchymal cells consisting of loose connective tissue which may participate in visceral organogenesis^{4, 17}. Additionally, perivascular cells (pericytes) around blood vessels in the subcutis, kidneys, and lungs also showed strong reactivity to A3 with Thy-1 expression⁵; pericytes have been regarded as mesenchymal stem cells (im-

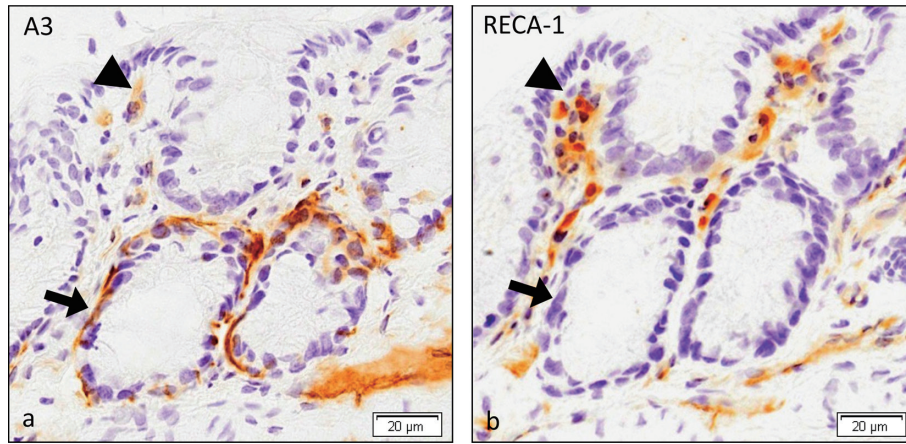


Fig. 5.

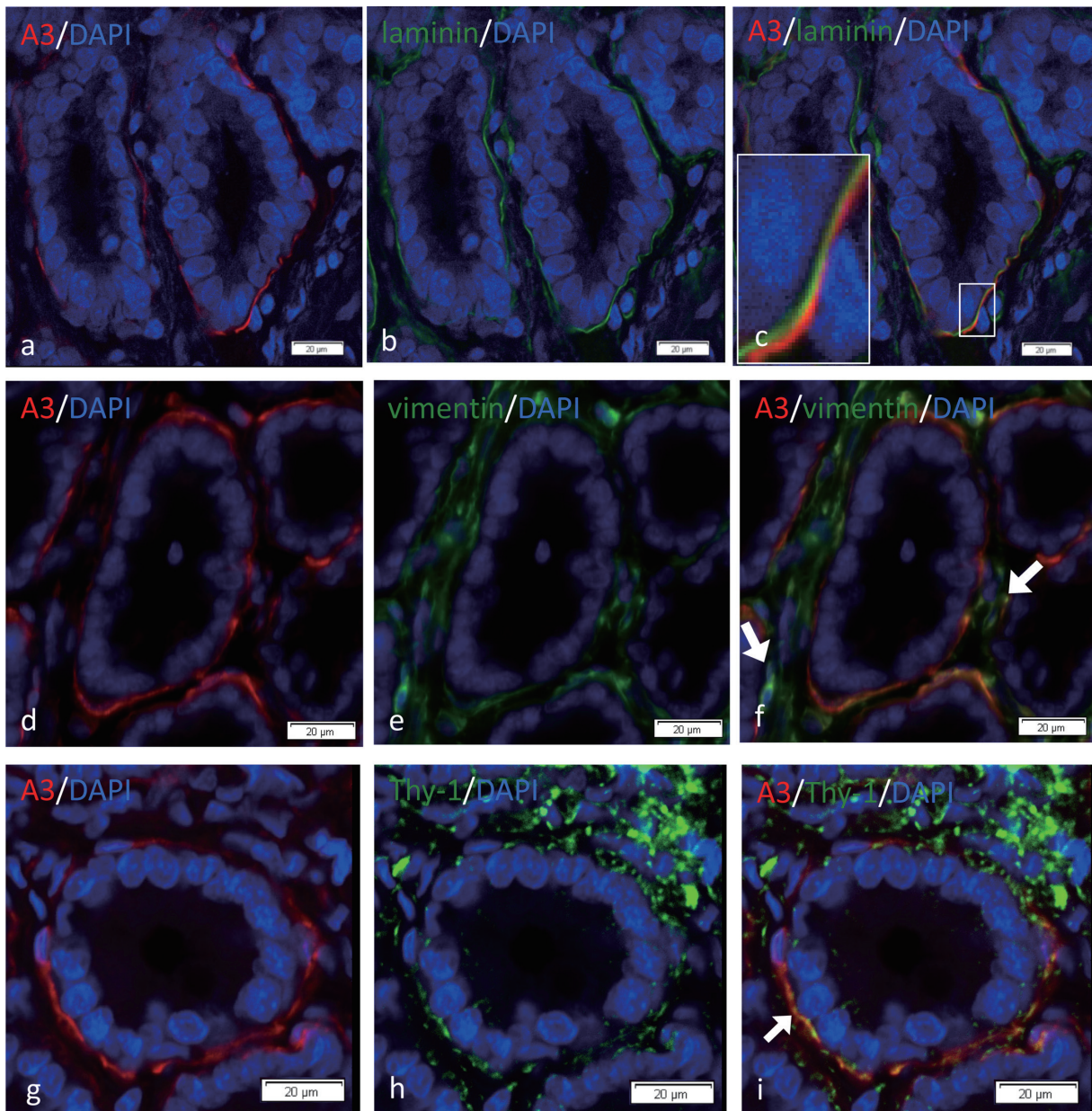


Fig. 6.

Fig. 5. Immunohistochemical staining for A3 and RECA-1 in the adult colons. A3 labels spindle-shaped cells at the periphery of the crypts (arrow) and endothelial cells of a few capillaries (arrowhead) (a). RECA-1 labels endothelial cells of capillaries but not spindle-shaped cells at the periphery of the crypts (b). Bar = 20 μ m.

Fig. 6. Immunofluorescence for A3 and respective markers (laminin, vimentin, and Thy-1), as well as merged findings from double immunofluorescence in the adult rat colon. A3-immunopositive cells (a) are located outside the basement membrane reacting to laminin (b). Cells reacting to vimentin (e) and Thy-1 (h) simultaneously co-express A3-recognizing antigen (d, g). DAPI was used for nuclear staining. Bar = 20 μ m.

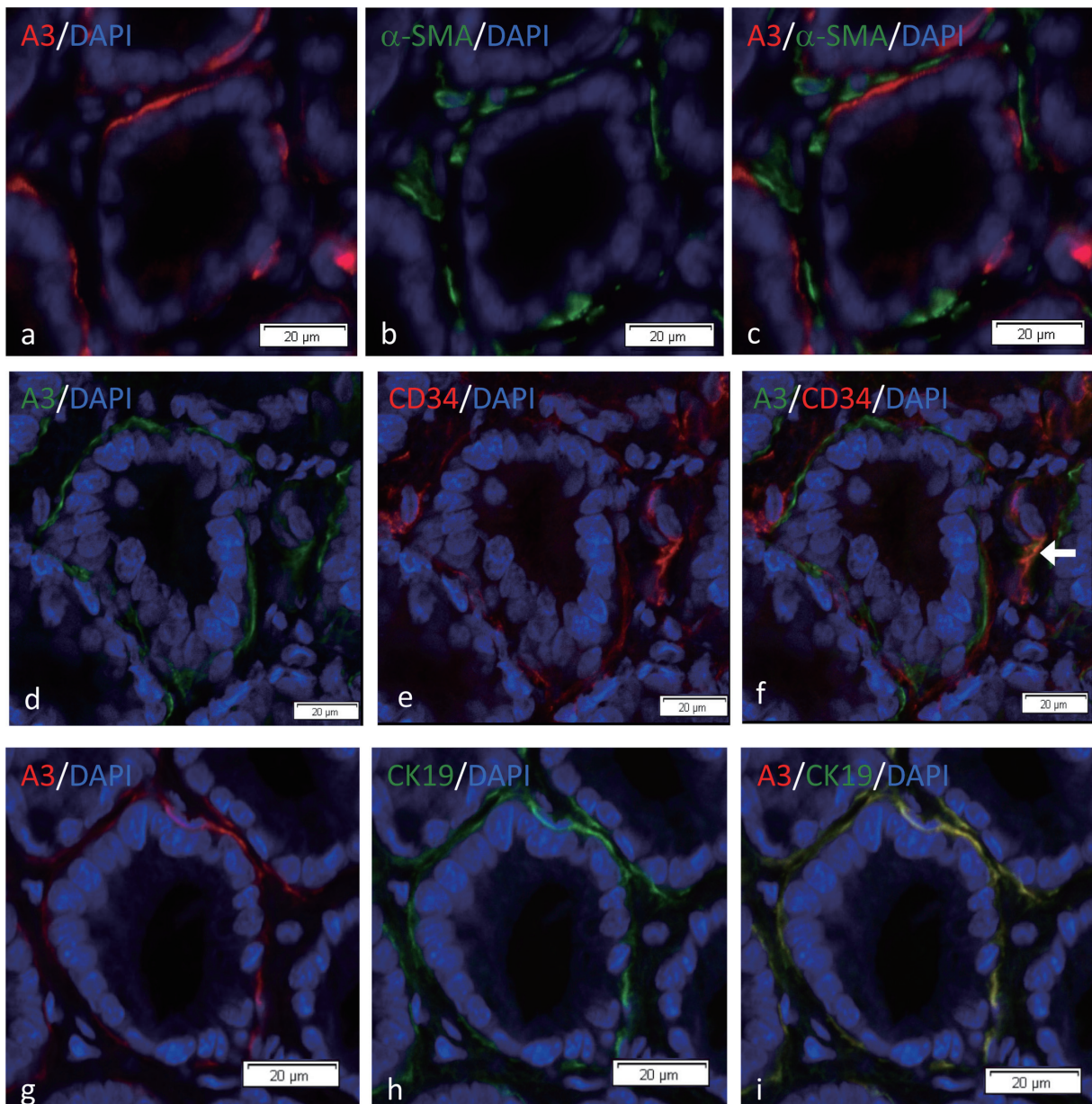


Fig. 7. Immunofluorescence for A3 and respective markers (α -SMA, CD34, and CK19), as well as merged findings from double immunofluorescence in the adult rat colon. α -SMA-expressing myofibroblasts (b) seen outside the basement membrane do not react to A3 (a). A3-immunopositive cells (d) around the crypts do not react to CD34 (e), although CD34-expressing endothelial cells co-express A3-recognizing antigen in the lamina propria of the colon (arrow). Cells reacting to CK19 (h) co-express A3-recognizing antigen (g). DAPI was used for nuclear staining. Bar = 20 μ m.

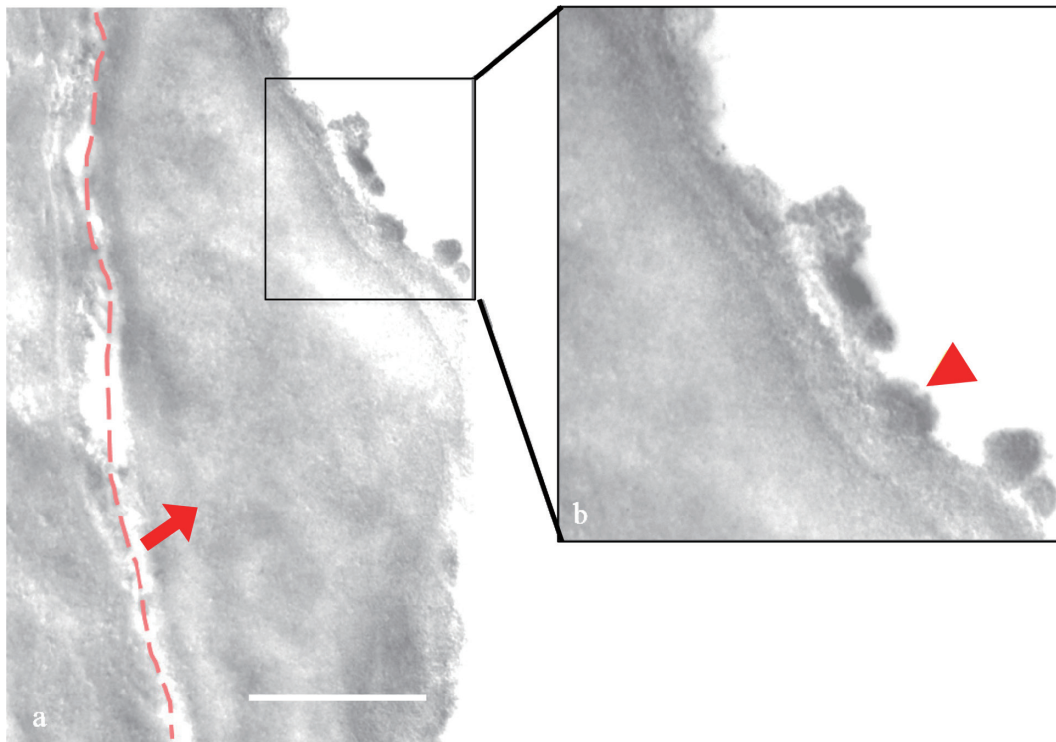


Fig. 8. Immunoelectron microscopy for A3 in an adult rat colon. A3-immunopositive cells around the crypts (arrow) are located outside the basement membrane (dotted line) (a). Reaction products of A3 (arrowhead) are seen exclusively on the cytoplasmic membrane (b). Bar = 800 nm.

mature cells with pluripotency)^{7, 18}. Furthermore, because MFH-forming cells have been considered to be immature mesenchymal cells capable of differentiating towards well-differentiated mesenchymal cells⁵, the spindle-shaped interstitial cells recognized by A3 may be regarded as mesenchymal stem cells of the developing colon.

In this study, we investigated the distribution and localization of A3-positive cells in the developing rat intestine, focusing on the ontogenic kinetics. In the rat fetus, the spindle-shaped cells recognized by A3 simultaneously expressed endothelial marker RECA-1. Ontogenetically, endothelial cells and pericytes form a complex plexus to supply nutrients systemically from epithelial cells¹⁹. Recently, endothelial cells in fetuses and injured lesions have been considered to have the potential to be multipotent stem-like cells²⁰. In adulthood, myofibroblasts, which participate in maintaining the intestinal stem cell niche around the crypts, are considered to be evoked partly by dedifferentiation via the endothelial-to-mesenchymal transition²⁰. Additionally, the mesenchyme adjacent to the intestinal epithelium contributes to formation of the villus²¹. This study showed that A3-positive cells aggregated as a villus cluster and co-expressed vimentin, RECA-1, and CD34. CD34 is used as a marker for bone marrow stem cells, as well as some endothelial cells^{22–24}. Based on this information, A3-positive cells may be regarded as immature mesenchymal cells which participate in formation of the villus via epithelial-mesenchymal interaction. The endothelial cells reacting to

A3 decreased drastically with the progress of development. Because A3 conventionally reacted to immature cells^{5, 25}, endothelial cells reacting to A3 in fetuses may be more immature than endothelial cells which do not react to A3 in adulthood. Likewise, A3-immunoreactive spindle-shaped cells present in the apex of the villus, which were seen in the developing intestine but not in adulthood, may be more immature.

A3-positive cells in the adult colon

In the adult rat colon, A3-positive cells were exclusively localized around the crypts, where the stem cell niche regulated by mesenchymal cells (such as α -SMA-expressing myofibroblasts)^{15, 16} is present. In double immunofluorescence, A3-positive cells reacted simultaneously to vimentin and Thy-1. The vimentin antibody has been widely used to identify mesenchymal cells, and Thy-1 is known to be expressed by immature mesenchymal cells including fibroblasts, pericytes, and myofibroblasts¹⁵. In fact, a previous study observed Thy-1 expression in immature mesenchymal cells beneath developing hair follicles and pericytes in the dermis²⁶. Therefore, A3-positive cells seen around crypts may have characteristics of immature mesenchymal cells. It is worth mentioning that the A3-positive cells around the crypts did not react to α -SMA. The intestinal subepithelial myofibroblasts (reacting to α -SMA) are considered supportive cells within the intestinal stem cell niche, although their nature is still under investigation^{10–12}. In this present

study, although α -SMA-expressing myofibroblasts were confirmed around the crypts by immunohistochemical and electron microscopy, double immunofluorescence did not show co-expression of A3 and α -SMA. Further, immunoelectron microscopy revealed that A3-positive cells were adjacent more closely to the epithelium of the crypts than intestinal myofibroblasts. These findings showed that A3-positive cells around the crypts were different from myofibroblasts forming the intestinal stem cell niche. A3-positive cells were regarded as a new type of immature mesenchymal cells around the crypts. On the basis of the localization of A3-positive cells, the A3-positive cells may participate in the intestinal stem cell niche with myofibroblasts via epithelial-mesenchymal interaction. Further investigations of the relationship between A3-positive cells and myofibroblasts, including the factor/cell signals (such as BMP and Wnt signal) that maintain the stem cell niche, by gene expression analysis are needed.

Interestingly, the present study showed that a few cells forming the crypt epithelium reacted variously to A3. It has been reported that A3-positive cells seen in the hair germ and peg, as well as mature hair follicles, reacted to E-cadherin, indicating that A3 labels epithelial elements²⁶; these epithelial cells in the hair follicle were considered to be suprabasal cells, which are post-progenitor cells capable of developing into prickle cells^{27, 28}. Therefore, A3-positive cells seen in the crypt epithelium are regarded as immature epithelial cells forming the crypts. The localization of A3-positive cells around the crypts may be similar to the compartments forming microenvironments within the stem cell niche^{1, 16, 29}. In a future study, it may be interesting to investigate the relation of Lgr5-expressing intestinal epithelial stem cells with A3-reacting epithelial cells.

In conclusion, it was considered that A3-positive cells might take part in the stem cell niche in colon which may be formed by the interaction between of immature mesenchymal and epithelial cells, because A3 labelled both immature mesenchymal cells and epithelial cells with greater reactivity in the mesenchymal cells. Recently, the epitope of A3 has been regarded to be the sugar chain (data not shown). The N-linked sugar chain is related to differentiation of embryonic stem cells and inducible pluripotential stem (iPS) cells³⁰. A3-recognizing antigen may be important in stem cell functions and formation of the stem cell niche in the colon, although the significance of the A3-immunopositive cells in the colon should be investigated further.

Disclosure of Potential Conflicts of Interest: The authors indicate that they have no potential conflicts of interest.

Acknowledgments: This work was supported in part by JSPS KAKENHI grant numbers 26292152 and 23658265 (to Yamate) and a JSPS Research Fellowship for Young Scientists (DC1: 25-9632 to Katou-Ichikawa).

References

1. Yamate J, Tajima M, Shibuya K, Ihara M, and Kudow S. Morphologic characteristics of a transplantable tumor derived from a spontaneous malignant fibrous histiocytoma in the rat. *Nippon Juigaku Zasshi*. **51**: 587–596. 1989.
2. Yamate J, Tajima M, Togo M, Shibuya N, Shibuya K, and Kudow S. Characteristics of in vitro passaged cells derived from a rat transplantable malignant fibrous histiocytoma. *Nippon Juigaku Zasshi*. **51**: 861–869. 1989.
3. Yamate J, Tajima M, Togo M, Shibuya K, Ihara M, and Kudow S. Heterogeneity of cloned cell lines established from a transplantable rat malignant fibrous histiocytoma. *Jpn J Cancer Res*. **82**: 298–307. 1991.
4. Kumagai D, Yamate J, Tajima T, Tsukamoto Y, Yasui H, Kuwamura M, Kotani T, and Sakuma S. Distribution of cells labelled by a monoclonal antibody (A3) against a cloned cell line derived from a rat malignant fibrous histiocytoma. *J Comp Pathol*. **123**: 77–87. 2000.
5. Yamate J, Ogata K, Yuasa T, Kuwamura M, Takenaka S, Kumagai D, Itoh K, and LaMarre J. Adipogenic, osteogenic and myofibroblastic differentiations of a rat malignant fibrous histiocytoma (MFH)-derived cell line, and a relationship of MFH cells with embryonal mesenchymal, perivascular and bone marrow stem cells. *Eur J Cancer*. **43**: 2747–2756. 2007.
6. Hoffman RM. The pluripotency of hair follicle stem cells. *Cell Cycle*. **5**: 232–233. 2006.
7. Gökçinar-Yagci B, Uçkan-Çetinkaya D, and Çelebi-Saltık B. Pericytes: Properties, functions and applications in tissue engineering. *Stem Cell Rev*. **11**: 549–559. 2015.
8. Spence JR, Lauf R, and Shroyer NF. Vertebrate intestinal endoderm development. *Dev Dyn*. **240**: 501–520. 2011.
9. Slack JMW. Early development. In: *Essential developmental biology*, 2nd ed. JMW Slack (ed). Blackwell Publishing Ltd., Oxford. 11–13. 2006.
10. Powell DW, Mifflin RC, Valentich JD, Crowe SE, Saada JI, and West AB. Myofibroblasts. II. Intestinal subepithelial myofibroblasts. *Am J Physiol*. **277**: C183–C201. 1999.
11. Kosinski C, Li VS, Chan AS, Zhang J, Ho C, Tsui WY, Chan TL, Mifflin RC, Powell DW, Yuen ST, Leung SY, and Chen X. Gene expression patterns of human colon tops and basal crypts and BMP antagonists as intestinal stem cell niche factors. *Proc Natl Acad Sci USA*. **104**: 15418–15423. 2007.
12. Lei NY, Jabaji Z, Wang J, Joshi VS, Brinkley GJ, Khalil H, Wang F, Jaroszewicz A, Pellegrini M, Li L, Lewis M, Stelzner M, Dunn JC, and Martín MG. Intestinal subepithelial myofibroblasts support the growth of intestinal epithelial stem cells. *PLoS One*. **9**: e84651. 2014.
13. Suzuki M, Katsuyama K, Adachi K, Ogawa Y, Yorozu K, Fujii E, Misawa Y, and Sugimoto T. Combination of fixation using PLP fixative and embedding in paraffin by the AMeX method is useful for histochemical studies in assessment of immunotoxicity. *J Toxicol Sci*. **27**: 165–172. 2002.
14. Ulger H, Karabulut AK, and Pratten MK. Labelling of rat endothelial cells with antibodies to vWF, RECA-1, PECAM-1, ICAM-1, OX-43 and ZO-1. *Anat Histol Embryol*. **31**: 31–35. 2002.
15. Powell DW, Pinchuk IV, Saada JI, Chen X, and Mifflin RC. Mesenchymal cells of the intestinal lamina propria. *Annu Rev Physiol*. **73**: 213–237. 2011.
16. Walker MR, Patel KK, and Stappenbeck TS. The stem cell

- niche. *J Pathol.* **217**: 169–180. 2009.
17. Iwasaki H, Isayama T, Johzaki H, and Kikuchi M. Malignant fibrous histiocytoma. Evidence of perivascular mesenchymal cell origin immunocytochemical studies with monoclonal anti-MFH antibodies. *Am J Pathol.* **128**: 528–537. 1987.
 18. Crisan M, Yap S, Casteilla L, Chen CW, Corselli M, Park TS, Andriolo G, Sun B, Zheng B, Zhang L, Norotte C, Teng PN, Traas J, Schugar R, Deasy BM, Badylak S, Buhring HJ, Giacobino JP, Lazzari L, Huard J, and Péault B. A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell Stem Cell.* **3**: 301–313. 2008.
 19. Wells JM, and Spence JR. How to make an intestine. *Development.* **141**: 752–760. 2014.
 20. Medici D, Shore EM, Lounev VY, Kaplan FS, Kalluri R, and Olsen BR. Conversion of vascular endothelial cells into multipotent stem-like cells. *Nat Med.* **16**: 1400–1406. 2010.
 21. Karlsson L, Lindahl P, Heath JK, and Betsholtz C. Abnormal gastrointestinal development in PDGF-A and PDGFR-(alpha) deficient mice implicates a novel mesenchymal structure with putative instructive properties in villus morphogenesis. *Development.* **127**: 3457–3466. 2000.
 22. Fina L, Molgaard HV, Robertson D, Bradley NJ, Monaghan P, Delia D, Sutherland DR, Baker MA, and Greaves MF. Expression of the CD34 gene in vascular endothelial cells. *Blood.* **75**: 2417–2426. 1990.
 23. Pusztaszeri MP, Seelentag W, and Bosman FT. Immunohistochemical expression of endothelial markers CD31, CD34, von Willebrand factor, and Fli-1 in normal human tissues. *J Histochem Cytochem.* **54**: 385–395. 2006.
 24. Testa JE, Chrastina A, Oh P, Li Y, Witkiewicz H, Czarny M, Buss T, and Schnitzer JE. Immunotargeting and cloning of two CD34 variants exhibiting restricted expression in adult rat endothelia in vivo. *Am J Physiol Lung Cell Mol Physiol.* **297**: L251–L262. 2009.
 25. Hori M, Juniantito V, Izawa T, Ichikawa C, Tanaka M, Tanaka K, Takenaka S, Kuwamura M, and Yamate J. Distribution of cells labelled by a novel somatic stem cell-recognizing antibody (A3) in pulmonary genesis and bleomycin induced pulmonary fibrosis in rats. *J Comp Pathol.* **148**: 385–395. 2013.
 26. Ichikawa C, Izawa T, Juniantito V, Tanaka M, Hori M, Tanaka K, Takenaka S, Kuwamura M, and Yamate J. Rat hair follicle-constituting cells labeled by a newly-developed somatic stem cell-recognizing antibody: a possible marker of hair follicle development. *Histol Histopathol.* **28**: 257–268. 2013.
 27. Blanpain C. Stem cells: Skin regeneration and repair. *Nature.* **464**: 686–687. 2010.
 28. Snippert HJ, Haegebarth A, Kasper M, Jaks V, van Es JH, Barker N, van de Wetering M, van den Born M, Begthel H, Vries RG, Stange DE, Toftgård R, and Clevers H. Lgr6 marks stem cells in the hair follicle that generate all cell lineages of the skin. *Science.* **327**: 1385–1389. 2010.
 29. Umar S. Intestinal stem cells. *Curr Gastroenterol Rep.* **12**: 340–348. 2010.
 30. Sasaki N, Okishio K, Ui-Tei K, Saigo K, Kinoshita-Toyoda A, Toyoda H, Nishimura T, Suda Y, Hayasaka M, Hanaoka K, Hitoshi S, Ikenaka K, and Nishihara S. Heparan sulfate regulates self-renewal and pluripotency of embryonic stem cells. *J Biol Chem.* **283**: 3594–3606. 2008.