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Rat hair follicle-constituting cells labeled by a newly-developed somatic stem cell-recognizing antibody: a possible marker of hair follicle development

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Summary. A3 was generated as an antibody recognizing somatic stem cells in rat tissues. We investigated the distribution of A3-positive cells in developing rat hair follicles by immunolabeling. A3-positive cells began to be seen in the hair germ and peg in fetuses and neonates; the positive cells were epithelial cells above basal cells. Furthermore, A3-positive cells were seen in the outer root sheath adjacent to the bulge in mature hair follicles. Double immunofluorescence revealed that these A3-positive epithelial cells reacted to E-cadherin (for all epithelial elements) but not to CK15 (for basal cells/epithelial stem cells) or to nestin (for stem cells), indicating that A3-positive epithelial cells are suprabasal cells in the developing epidermic hair follicle. Additionally, spindle-shaped mesenchymal cells surrounding the hair peg and mature hair follicle reacted to A3; in double immunofluorescence, the A3-positive cells were located outside collagen type IV-positive glassy membrane, and reacted to vimentin (for mesenchmal cells), Thy-1 (for immature mesenchymal cells), CD34 (for stem cells) and nestin, but not to α-smooth muscle actin (for myofibroblasts); the positive cells were regarded as immature mesenchymal cells with stem cell nature in the connective tissue sheath of developing hair follicles. A3-positive epithelial and mesenchymal cells did not show proliferating activity. Collectively, it is considered that A3-positive cells seen in developing rat hair follicles may be quiescent post-progenitor cells with the potential to differentiate into either highly-differentiated epithelial or mesenchymal cells. A3 would become a useful antibody to know the kinetics of rat hair follicle-constituting cells.

Key words: Antibody, Immunohistochemistry, Rat hair follicle, Somatic stem cells, Suprabasal cells

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Introduction

A monoclonal antibody capable of labeling a special cell type would be useful to investigate the cell distribution in normal tissues and the appearance in pathological lesions (Yamate et al., 2000). To pursue the histogenesis of malignant fibrous histiocytoma (MFH), of which the origin has been considered to be undifferentiated mesenchymal cells (Fletcher, 2006; Tarkkanen et al., 2006), we previously generated a monoclonal antibody (A3) using a rat MFH-derived cloned cell line (MT-8) as the antigen; A3 specifically labeled rat MFH cells (Kumagai et al., 2000; Yamate et al., 2007). MFH, characterized by an admixture of fibroblastic, histiocytic and undifferentiated mesenchymal cells arranged in a storiform pattern (Maruno et al., 2006; Matushansky et al., 2009; Terada, 2011), is regarded as synonymous with undifferentiated pleomorphic sarcoma in humans (Matushansky et al,. 2009). It has been considered that MFH may contain stem cells with pluripotential differentiations, because cellular components of well-differentiated mesenchymal cells, such as osteogenic, lipogenic and leiomyogenic cells, have been observed in human MFHs (Hashimoto et al., 1990; Fabre-Guillevin et al., 2006). Interestingly, it was found that A3 labeled with perivascular cells (pericytes) and bone marrow-stem cells, as well as immature mesenchymal cells in developing tissues of visceral organs in rats (Yamate et al., 2007). Pericytes

are believed to be stromal stem cells in the connective tissues (Caplan, 2008; Crisan et al., 2008). A3 would be beneficial for the identification of somatic stem cells and immature mesenchymal cells generating from the stem cells, although the functional properties of A3 have not yet been decided (Yamate et al., 2007).

The hair follicle is a highly sensitive mini-organ which undergoes a dynamic cycling for producing hair and self-maintenance. The mature hair follicles have complex tissue structures, consisting of concentric cylinders of epithelial cells of the inner root sheath (IRS) and outer root sheaths (ORS) in the infundibulum, isthmus, and dermal bulb (Fuchs, 2007). The hair shaft is veiled by the epidermic hair follicles, and these epithelial elements are surrounded by the connective tissue sheath (CTS) as the outermost components of hair follicles (dermic hair follicles). Ontogenetically, the hair follicle is generated from fetal epidermis through the hair placode, germ and peg in the order of development, and the interaction between epidermal cells and immature mesenchymal cells beneath the fetal epidermis play important roles in the development of hair follicles (Fuchs, 2007; Ito et al., 2007).

The bulge of hair follicles contains stem cells which can be activated at anagen to generate a new hair follicle (Amoh et al., 2005b; Ito et al., 2005; Fuchs, 2007) and CTS cells may possess the nature of stromal stem cells (Jahoda et al., 2003). The bulge-area stem cells reacting to nestin, the neuronal stem cell marker protein, can differentiate into not only hair follicle-constituting cells but also neurons and other non-follicle cell types (Amoh et al., 2005a,b, 2010; Liu et al., 2011; Uchugonova et al., 2011). Because A3 labels some somatic stem cells in rat tissues (Yamate et al., 2007), in the present study we investigated the possible distribution of A3-positive cells in developing hair follicles of rats by means of single immunohistochemistry with A3 and double immunofluorescence with A3 and marker antibodies for epithelia, mesenchyme, stem cells and proliferating cells. Finally, we found that inner epithelial cells of the hair germ and peg gave a positive reaction to A3. In maturing hair follicles, additionally, epithelial cells in the ORS adjacent to the bulge and mesenchymal cells in the CTS reacted to A3. These positive cells were regarded as possible post-progenitor cells for either epidermic or dermic hair follicle. Because hair follicles are a unique mini-organ with dynamic cycling and play important roles in cutaneous wound healing (Amoh et al., 2005a, 2010; Ansell et al., 2011; Hoogduijn et al., 2006; Jahoda et al., 2003; Liu et al., 2008), A3 could be used as an antibody for detection of some hair follicle-constituting cells.

Materials and methods

Animals

Pregnant F344/DuCrj rats (15-day gestation; Charls River Japan, Hino, Shiga, Japan) were obtained. These animals were housed in an animal room with a controlled temperature of 22±3°C and with a 12-hour light-dark cycle; they were provided a standard commercial diet (DC-8; Crea, Tokyo, Japan) and tap water. Skin samples were obtained from fetal rats on gestation days 18 and 20, as well as neonatal rats aged 1, 4, 8, 10, 15, 20 and 21 days. Skin tissues were also prepared as adult tissues from rats more than 6 weeks old. These animals were euthanized by exsanguination with deep isoflurane anesthesia. Animal housing and sampling conformed to the institutional guidelines of animal care of Osaka Prefecture University.

Tissue preparation and histopathology

Skin tissues harvested from the trunk were fixed in 10% neutral buffered formalin and periodate-lysine-paraformaldehyde (PLP) fixative solutions; some parts of the skin were frozen immediately in the Tissue Mount (Chiba Medical, Saitama, Japan) and stored at -80°C. Skin samples immersed in the PLP fixative for 6 hours at 4°C were then embedded in paraffin by AMeX (acetone-methyl benzonate-xylen) methods (Suzuki et al., 2002). The formalin-fixed, paraffin-embedded and PLP-AMeX-processed tissues were cut at 4 μm in thickness and stained with hematoxylin and eosin (HE) for morphological observations.

Immunohistochemistry and double immunofluorescence

The information of primary antibodies used in this study is shown in Table 1. E-cadherin and cytokeratin 15 (CK15) were used for epithelial cell markers; vimentin, Thy-1, and α-SMA for mesenchymal markers; nestin and CD34 for stem cell markers; Ki-67 for proliferating cell marker.

For single immunohistochemistry, the PLP-AMeX-processed tissue sections were used. Epitope retrieval was performed at 80°C for 6 hours in 10 mM citrate buffer at pH 6.0. Sections were then incubated for 30 minutes with 5% skimmed milk in phosphate-buffered saline (PBS) to prevent non-specific reaction. These sections were incubated with both of the unconjugated primary antibodies for 12-14 hours at 4°C, followed by endogenous peroxidase blocking and application of horseradish peroxidase-conjugated secondary antibody (Histofine simplestain MAX PO®; Nichirei, Tokyo, Japan). Positive reaction was visualized with 3, 3'-diaminobenzidine (DAB; Vector Laboratories Inc., Burlingame, CA, USA). Tissue sections were lightly counterstained with hematoxylin.

For double immunofluorescence, skin samples kept at -80°C were cut at 10 μm in thickness as fresh frozen sections, and then these sections were fixed for 5 minutes by a cold mixture of equal parts of acetone and methanol or fixed for 15 minutes in 4% paraformaldehyde (PFA) solution at room temperature. The prepared frozen tissue sections were incubated with 10% normal goat serum in PBS for 30 minutes to reduce

non-specific reactions. Bovine serum albumin (3% in PBS) was used for blocking when using the goat antibody. Subsequently, sections were reacted with both of the unconjugated first primary antibody (Table 1) for 12-14 hours at 4°C. The visualization of antibody-specific binding was performed with fluorochrome-conjugated secondary antibodies (Table 2). Next, sections were incubated with conjugated or unconjugated second primary antibody for 1 hour and then treated with appropriate fluorochrome-conjugated secondary antibody. These sections were mounted with mounting medium including 4', 6-diamidino-2-phenylindole (DAPI; VECTASHIELD®; Vector Laboratories, Burlingame, CA, USA) for nuclear staining, and captured by a laser scanning confocal imaging microscope (Nikon C1Si, Tokyo, Japan) and processed with EZ-C1 Viewer (Nikon).

Results

Development of rat hair follicles

The hair placode, which appears as small epidermal invaginations into the underlying dermis at the basal cell layer of the stratified epidermis of fetal skin, began to be seen in rat fetuses on gestation day 18 (Fig. 1a). Thereafter, the placode elongated towards the dermis and developed into the hair germ consisting of aggregates of increased epithelial cells; the formation of hair germ is directed by the underlying dermal spindle-shaped mesenchymal cells (Fig. 1b) (Paus et al., 1999; Fuchs, 2007). The hair germ-constituting epithelial cells gradually proliferated and more grew down into the dermis, forming the hair peg (Fig. 1c). The hair peg engulfed the dermal condensate (a small cap-like condensation of immature mesenchymal cells beneath the hair peg) to form a dermal papilla (Fig. 1c); the hair peg-constituting cells gradually differentiated into matrix epithelial cells. In mature hair follicles, the matrix epithelial cells move up to form three distinct cylindrical layers of hair follicles: IRS, ORS and hair shaft (Fig. 1d) (Paus et al., 1999). In the hair peg and mature hair follicle, the epidermic hair follicle was surrounded by a few layers of spindle-shaped mesenchymal cells (Fig. 1c) generated from the dermal condensate and papilla (Fuchs, 2007); these mesenchymal cells finally constitute the CTS in the lower part of hair follicles (Fig. 1d).

On gestation days 18 and 20, as well as neonate days 1-10, developing hair follicles at various stages (such as hair placode, germ and peg) were observed; hair placode and germ were more common on gestation days 18 and 20 (Fig. 1a,b), whereas hair pegs were more frequent on neonate days 1-10 (Fig. 1c). On neonate days 15-21, maturing hair follicles were mainly observed (Fig. 1d), in addition to a small number of hair pegs. Mature hair follicles on neonate day 21 and at 6 weeks old were composed of the epithelial components, such as IRS,

Table 1. Primary antibodies used for immunohistochemistry and double immunofluorescence.

Antibody	Poly/Mono	Clone	Dilution	Source	Specificity
A3	Mouse mono	A3	1:1,000	TransGenic Inc., Kumamoto, Japan	Somatic stem cells and immature mesenchymal cells
E-cadherin	Mouse mono	36	1:100	BD Biosciences, Franklin Lakes, NJ, USA	All epithelial cells
CK15	Mouse mono	LHK15	1:200	Thermo Fisher Scientific, Waltham, MA, USA	Epithelial basal cells and epithelial stem cells
Vimentin	Mouse mono	V9	1:400	Dako, Denmark	Mesenchymal cells
Thy-1 (CD90)	Mouse mono	OX-7	1:500	Cedarlane Laboratories, Burlington, Canada	Immature mesenchymal cells (Stem cells)
α-SMA	Mouse mono	1A4	1:500	Dako, Denmark	Myofibroblasts
CD34	Goat poly	-	1:500	R&D Systems, Minneapolis, MN, USA	Stem cells
Nestin	Mouse mono	Rat-401	1:500	Millipore, Billerica, MA, USA	Stem cells (Neural stem cells)
Ki-67	Rabbit poly	-	1:500	Abcam, Cambridge, UK	Proliferating cells

CK15: cytokeratin 15. α-SMA: α-smooth muscle actin.

Table 2. Secondary antibodies used for double immunofluorescence.

Antibody	Label	Host	Source	Target primary antibody
Anti-mouse IgG	Cy3	Donkey	Jackson Immunoresearch, West Grove, PA, USA	Mouse primary antibodies
Anti-mouse IgG1	Alexa 568	Goat	Invitrogen, Carlsbad, CA, USA	A3
Anti-mouse IgG2a	Alexa 488	Goat	Invitrogen, Carlsbad, CA, USA	α-SMA, cytokeratin 15 and E-cadherin
Anti-goat IgG	FITC	Rabbit	Jackson Immunoresearch, West Grove, PA, USA	CD34
Anti-rabbit IgG	Alexa 488	Goat	Invitrogen, Carlsbad, CA, USA	Ki-67

FITC: fluorescein isothiocyanate.

ORS and hair shaft, surrounded by the CTS (Fig. 1d).

A3 immunoexpression in the developing hair follicle

A3 expression began to be seen in epithelial cells of the hair germ (Fig. 2a); the A3-positive epithelial cells were located in the inside of the hair germ and hair peg (Fig. 2a). The A3-positive inner epithelial cells gradually extended, along with development of hair pegs (Fig. 2b, c). In the mature hair follicle, A3-positive epithelial cells were seen adjacent to the bulge (Fig. 2d). The stratified epidermis, including the placode and basal cells of the hair germ and peg, as well as epithelial elements in the bulge and dermal bulb in mature hair follicle, did not react to A3 (Fig. 2a-d).

Additionally, spindle-shaped mesenchymal cells surrounding the hair peg and mature hair follicle reacted to A3 in varying degrees depending on hair follicle development (Fig. 2c,d). In mature hair follicle, A3-positive mesenchymal cells existed at the lower third to half part, surrounding the epithelial elements of the ORS and dermal bulb (Fig. 2d). The dermal condensate-constituting immature mesenchymal cells beneath the hair placode and spindle-shaped cells surrounding the hair germ at the early stage did not react to A3 (Fig. 2a,b).

Blood vessel-surrounding cells (pericytes) seen in the dermis of developing skins showed a positive

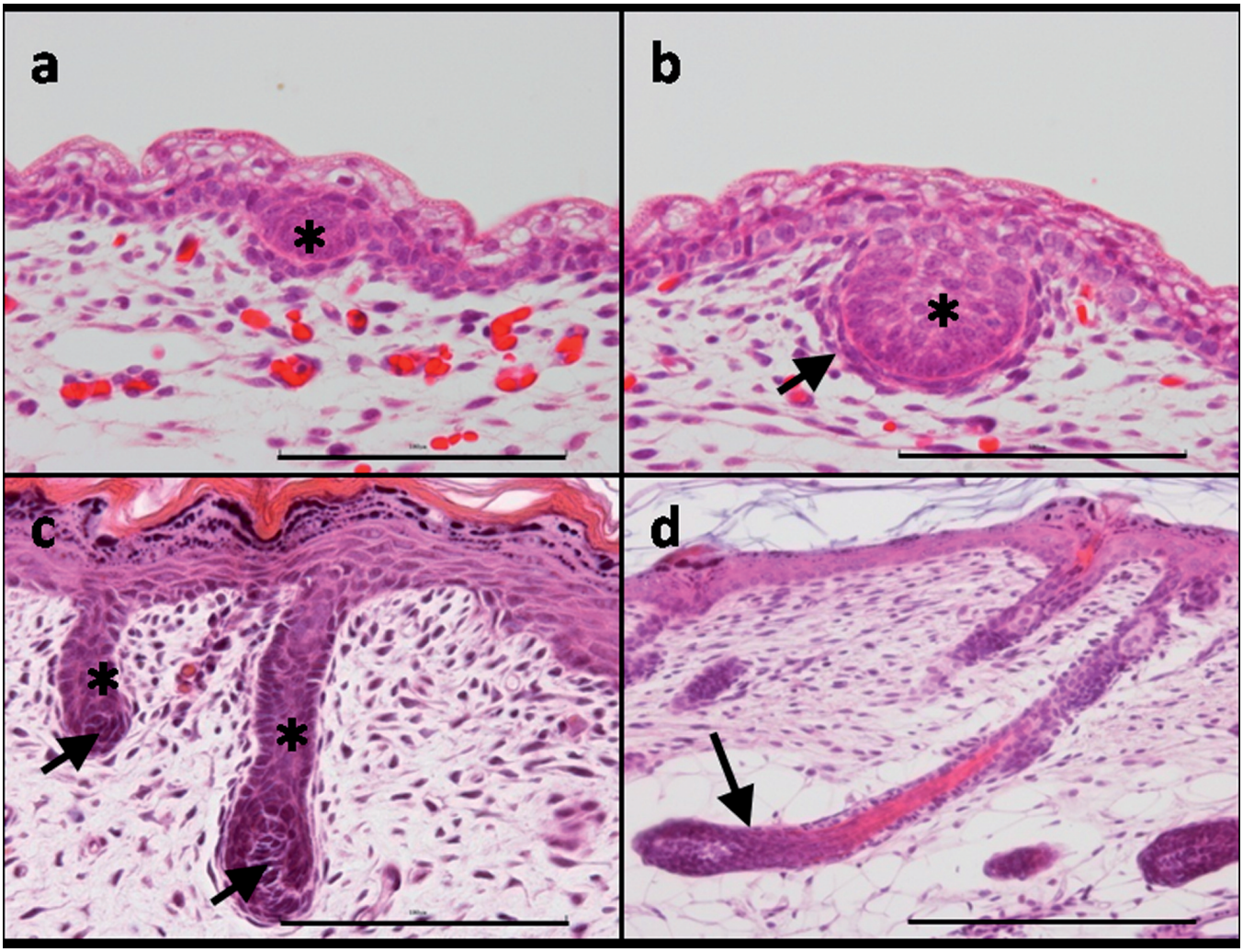

Fig. 1. Developing hair follicles in fetal and neonatal rats **(a-d). a.** A placode (asterisk), characterized by small epidermal invaginations into the underlying dermis, is seen at the basal cell layer of stratified epidermis of fetal skin on gestation day 18. **b.** A hair germ (asterisk) developed from placode consists of aggregates of increased epithelial cells and is surrounded by a few spindle-shaped mesenchymal cells (arrow). **c.** Two hair pegs (asterisks) elongated from hair germs into the dermis engulf dermal condensate (a small cap-like condensation consisting of immature mesenchymal cells) to form dermal papillae (arrows). **d.** A mature hair follicle (arrow), consisting of the epidermic hair follicle and connective tissue sheath (CTS), is seen in the dermis on neonate day 21. HE stain. Bars: a, c, d, 100 μm; b, 200 μm.

reaction to A3 (Fig. 2d).

Localization of A3-positive epithelial cells in the developing hair follicle

To identify the A3-positive epithelial cells of the developing hair follicle (Fig. 2a-d), double immunofluorescence labeling for A3 was carried out with E-cadherin or CK15 antibody. E-cadherin is expressed in all epithelial cell types of the stratified epidermis and epidermic hair follicle (Tinkle et al., 2004). A3-positive cells seen in the hair germ and peg, as well as the mature hair follicle, showed co-expression with E-cadherin (Fig. 3a,b). CK15 is expressed exclusively in the basal cells of the stratified epidermis and epithelial stem cells of the bulge (Kaur, 2006; Hoang et al., 2009). A3-positive cells in the hair germ and peg did not correspond to CK15-positive cells (Fig. 3c); in the mature hair follicle, CK15 was expressed in the basement cells in the ORS and bulge cells, which was different from A3-positive cells (Fig. 3c,d); in immunofluorescence labeling, the A3-positive cells were present above the CK15-positive cells of the bulge (Fig. 3c), and appeared to be located in the outermost layer of the ORS adjacent to the bulge (Fig. 3d). These findings indicated that A3 immunoreaction was seen in the inner epithelial cells above the basal cells of the hair germ and peg, as well as in epithelial cells above the basal cells in the ORS adjacent to the bulge.

Localization of A3-positive mesenchymal cells in the developing hair follicle

The glassy membrane is between the epidermic hair follicle and the CTS (Paus et al., 1999). A3-positive cells seen in the CTS were outside collagen type IV-positive glassy membrane in double immunofluorescence (Fig. 4a). The CTS consists of collagens, fibroblasts and fibroblastic cells expressing α-SMA; the α-SMA-expressing fibroblastic cells may be regarded as myofibroblasts possessing both the nature of fibroblasts and smooth muscle cells; the myofibroblasts are located in the innermost layer of the CTS (Urabe et al., 1992; Hinz, 2010). A3-positive cells in the CTS were present outside the α-SMA-positive myofibroblastic cells, and there were no cells co-expressing A3 and α-SMA in the CTS (Fig. 4b). All cells in the CTS (Fig. 4c) and dermal cells reacted strongly to vimentin, indicating the mesenchymal nature of these cells. Some vimentin-positive cells in the CTS also reacted to A3 in double immunofluorescence (Fig. 4c-e). These findings indicate that A3-positive cells seen in the CTS are mesenchymal cells without myofibroblastic features.

Relationship of Thy-1-positive immature mesenchymal cells with A3 expression in the developing hair follicle

Because Thy-1 has been considered to be expressed in immature mesenchymal cells (mesenchymal stem

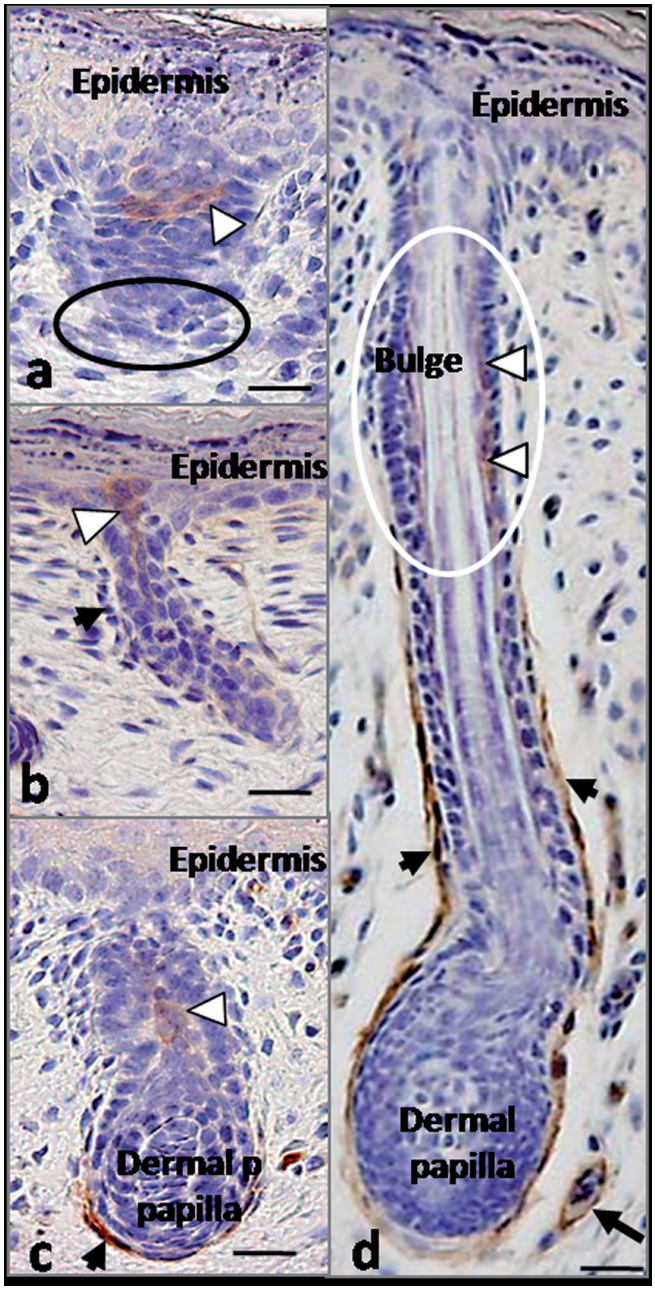

Fig. 2. A3-immnopositive epithelial and mesenchymal cells in developing hair follicles. **a.** A3-positive epithelial cells are seen in the inner site of hair germ (white arrowhead); dermal condensate (circle) consisting of immature mesenchymal cells do not react to A3. **b.** There are A3-positive epithelial cells in the inner site of hair peg at the early stage (white arrowhead); surrounding mesenchymal cells do not react to A3 (arrow). **c.** A3-positive epithelial cells (white arrowhead) are seen in the inside of hair germ with dermal papilla, and some immature mesenchymal cells surrounding the dermal bulb also react to A3 (black arrow). **d.** In the almost mature hair follicle, A3 immunoexpression (white arrowheads) seems to be present in epithelial cells adjacent to the bulge (circle), and immature mesenchymal cells in the CTS show a positive reaction to A3 (small black arrows); blood vessel-surrounding cells (pericytes) reacting to A3 are also seen (longer black arrow). A3 immunohistochemistry, counterstained with hematoxylin. Bars: a, d, 30 μm; b, c, 50 μm.

cells) (Mercati et al., 2009; Patel et al., 2010), we investigated the expression pattern of Thy-1 in developing rat skins. Dermal mesenchymal cells beneath the stratified epidermis of fetal and neonatal rats reacted to Thy-1 in varying degrees (Fig. 5a,b). Particularly, mesenchymal cells in the dermal condensate beneath the hair placode, germ and peg strongly expressed Thy-1 (Fig. 5a). Additionally, Thy-1-positive cells surrounded

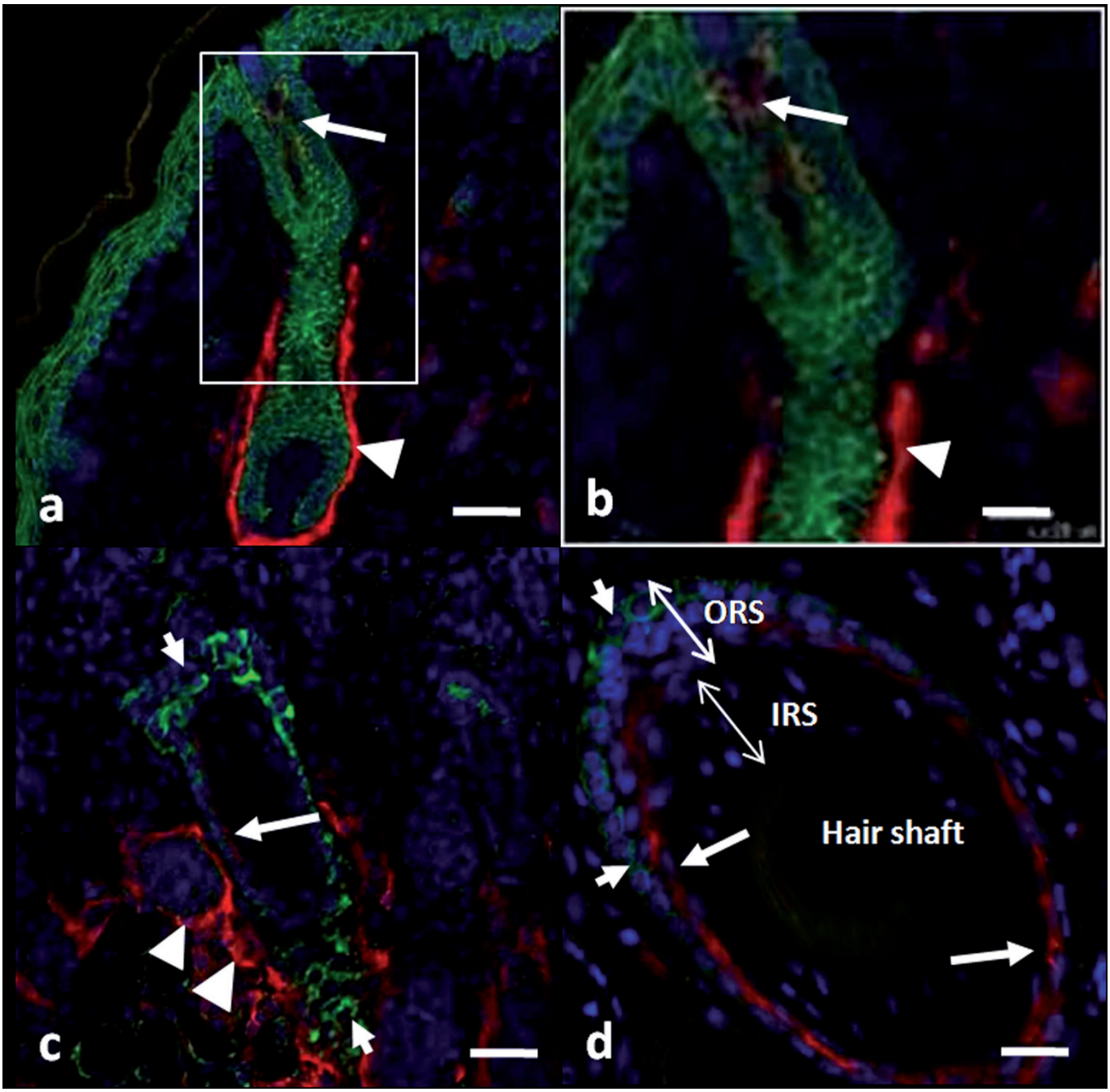

Fig. 3. Double immunofluorescence for A3 and E-cadherin **(a, b)** or CK15 **(c, d);** the confocal images were merged from red (A3 for **a-d)**, green (E-cadherin for **a** and **b**; CK15 for **c** and **d**), and blue (nuclei for **a-d**) fluorescences. **a.** A3-positive epithelial cells in the inner site of hair peg co-express with E-cadherin (arrow). **b.** A higher magnification of **a** (white rectangle), showing co-expression of A3 and E-cadherin. **c.** CK15-positive epithelial cells are located in the basement layer of the bulge (small arrows) in the mature hair follicle, but the positive cells do not show co-expression of A3 (larger arrow). **d.** In cross cut section of the bulge portion A3-positive epithelial cells (large arrows) do not correspond to CK15-positive basal cells in the basement layer (small arrow); the A3-positive epithelial cells appear to be in the outermost layer of the ORS, being located above the CK15-positive basal cells. **a-d**, immature mesenchymal cells in the CTS react to A3 (white arrowheads). Immunofluorescence, counterstained with DAPI. Bars: a, c, d, 40 μm; b, 60 μm.

the hair germ and peg as a thin layer (Fig. 5b), and dermal papilla-forming mesenchymal cells showed a strong reaction to Thy-1 (Fig. 5b). In the mature hair follicle, Thy-1-positive cells were seen mainly in the dermal papilla and mesenchymal cells in the CTS. Pericytes in the dermis reacted to Thy-1 (Fig. 5b). In double immunofluorescence, CTS cells and pericytes also gave a positive reaction to Thy-1 (Fig. 6a-c).

Relationship between A3-positive cells and cells expressing stem cell markers (CD34 and nestin) in the developing hair follicle

CD34 is expressed both in epithelial and mesenchymal stem cells (Trempus et al., 2003; Tumbar et al., 2004). A3-positive epithelial cells seen in the hair germ and peg, as well as the mature hair follicle did not react to CD34 (Fig. 7a-c). On the other hand, A3-positive mesenchymal cells seen in the CTS reacted partly to CD34 (Fig. 7a-c).

Nestin is a type VI intermediate filament protein expressed in neuronal and stromal stem cells (Toma et al., 2001; Wiese et al., 2004; Amoh et al., 2010; Liu et al., 2011; Uchugonove et al., 2011). It was difficult to conduct double immunofluorescence labeling for A3 and nestin, because there was no appropriate fluorochrome-conjugated secondary antibody available for recognizing different expressions. Therefore, positive cells for A3

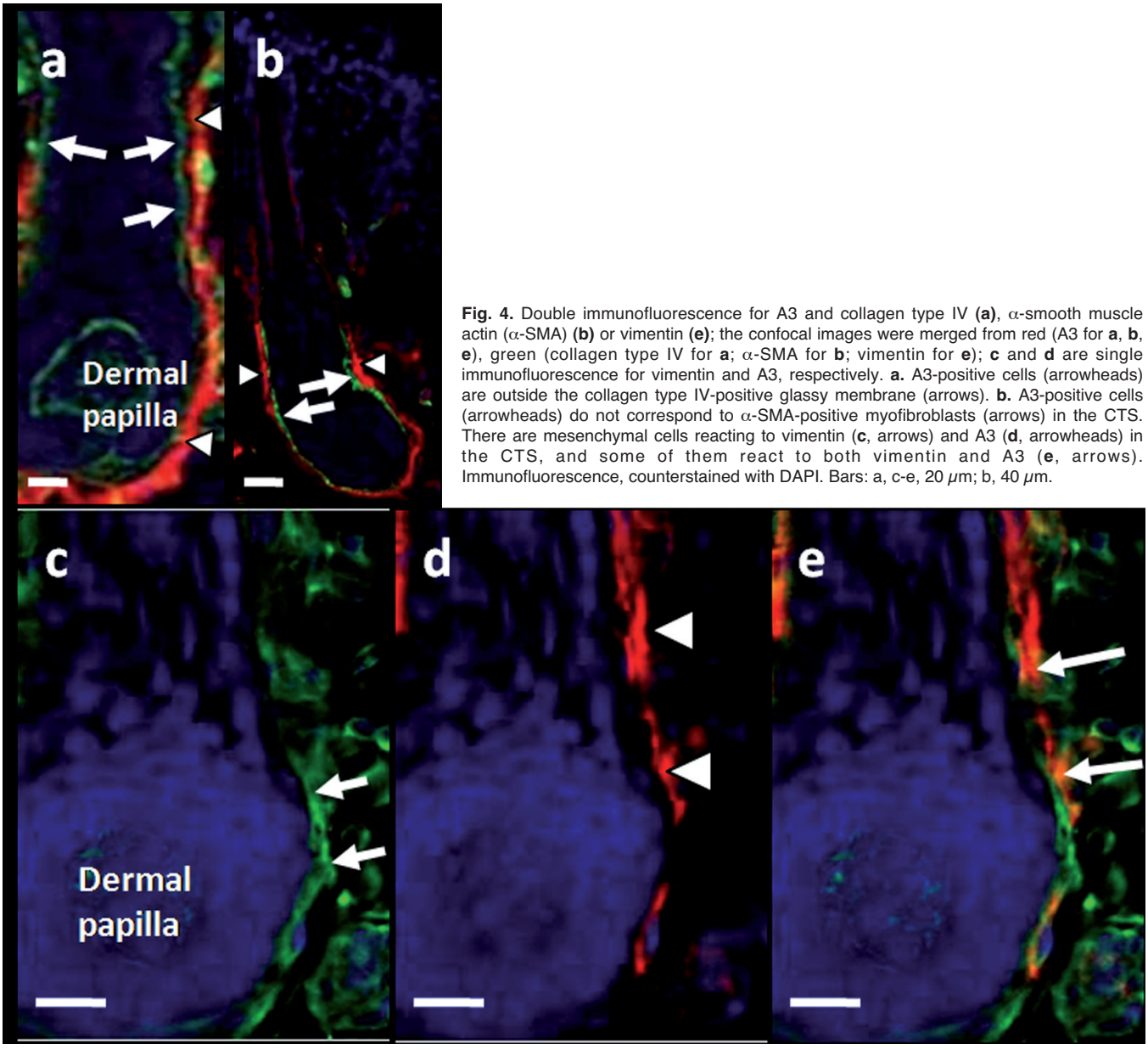

Fig. 4. Double immunofluorescence for A3 and collagen type IV **(a)**, α-smooth muscle actin (α-SMA) **(b)** or vimentin **(e)**; the confocal images were merged from red (A3 for **a**, **b**, **e**), green (collagen type IV for **a**; α-SMA for **b**; vimentin for **e**); **c** and **d** are single immunofluorescence for vimentin and A3, respectively. **a.** A3-positive cells (arrowheads) are outside the collagen type IV-positive glassy membrane (arrows). **b.** A3-positive cells (arrowheads) do not correspond to α-SMA-positive myofibroblasts (arrows) in the CTS. There are mesenchymal cells reacting to vimentin (**c**, arrows) and A3 (**d**, arrowheads) in the CTS, and some of them react to both vimentin and A3 (**e**, arrows). Immunofluorescence, counterstained with DAPI. Bars: a, c-e, 20 μm; b, 40 μm.

and nestin were compared to each other in individual immunofluorescence labeling (Fig. 8a,b). Nestin expression was seen exclusively in CTS-constituting cells and mesenchymal cells beneath the epidermis (Fig. 8a); additionally, a small number of cells reacting to nestin were present in the bulge area (Fig. 8a). Apparently, nestin-positive cells in the CTS corresponded to A3-positive cells; however, in A3 immunofluorescence (Fig. 8b) there were no A3-positive cells coinciding with the bulge-area nestin-positive cells (Fig. 8a).

Relationship between A3-positive cells and proliferating cells in the developing hair follicle

The basement epithelial cells from the infundibulm to dermal bulb showed a proliferating activity demonstrable by Ki-67 immunofluorescence; however,

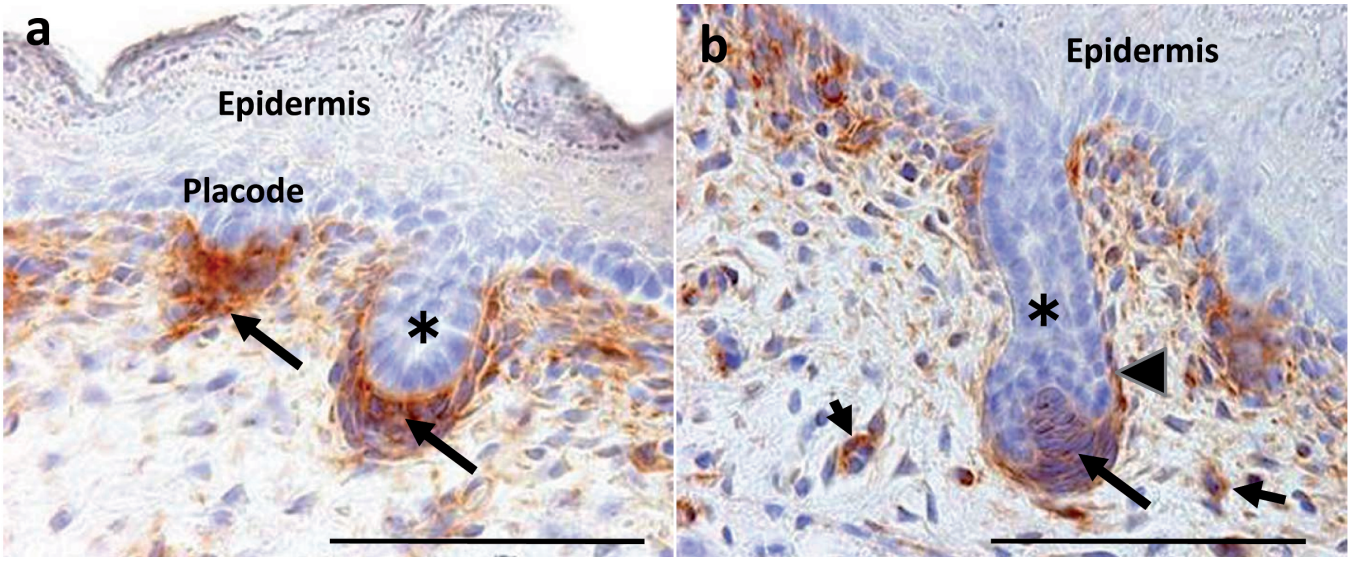

Fig. 5. Thy-1 immunohistochemistry in the hair placode, germ and peg. **a.** Mesenchymal cells in the dermal condensate beneath the hair placode and germ (asterisk) strongly react to Thy-1 (arrows). **b.** Thy-1-positive cells surround the hair peg as a thin layer (arrowhead), and dermal papilla-forming mesenchymal cells show a strong reaction to Thy-1 (large arrow); blood vessel-surrounding cells (pericytes) also react to Thy-1 (small arrow). Dermal mesenchymal cells beneath the stratified epidermis of fetal and neonatal rats are positive to Thy-1 in varying degrees (**a**, **b**). Immunohistochemistry, counterstained with hematoxyline. Bar: 100 μm.

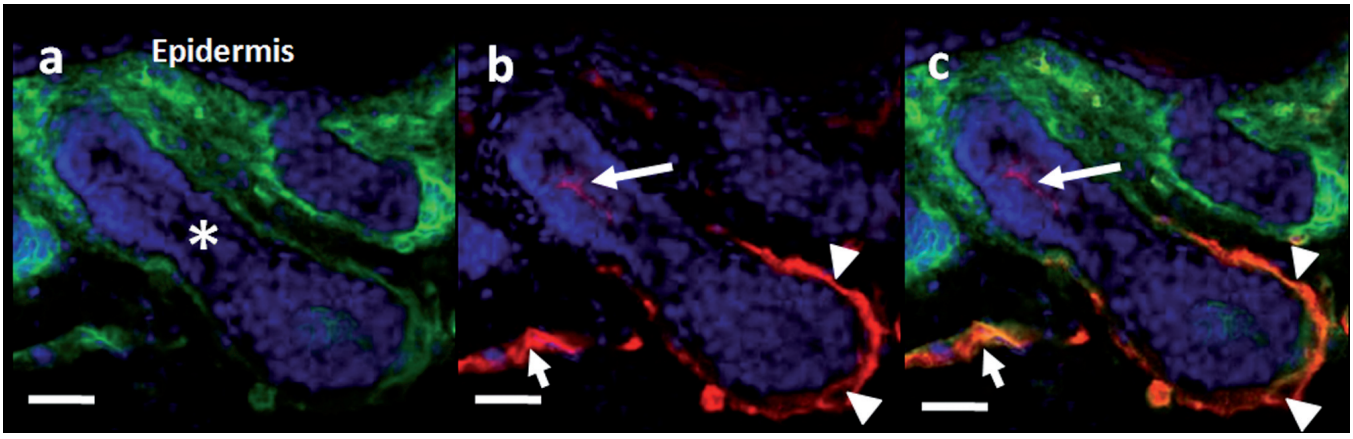

Fig. 6. Single immunofluorescence for Thy-1 **(a)** and A3 **(b)**, and double immunofluorescence for A3 and Thy-1 **(c)** in the hair peg; the confocal image was merged from green (Thy-1 for **a**), red (A3 for **b**) and blue (nuclei for **a-c**). **a.** Thy-1-positive mesenchymal cells are seen beneath the epidermis and around the hair peg (asterisk). **b.** A3-positive cells are present in the CTS (arrowheads) and pericytes (small arrow), as well as epithelial cells in the inner site of hair follicle (large arrow). **c.** Mesenchymal cells in the CTS (arrowheads) and pericytes (small arrow) react to both Thy-1 and vimentin, although A3-positive epithelial cells do not react to Thy-1(large arrow). Immunofluorescence, counterstained with DAPI. Bar: 20 μm.

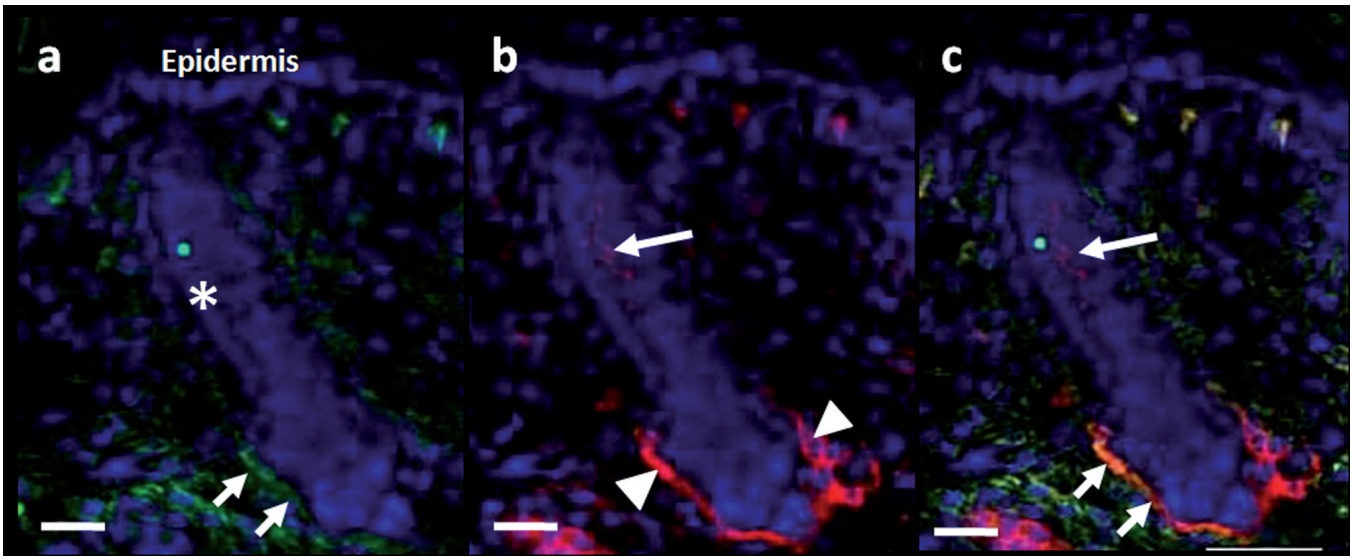

Fig. 7. Single immunofluorescence for CD34 **(a)** and A3 **(b)**, and double immunofluorescence for A3 and CD34 **(c)** in the hair peg (asterisk); the confocal image was merged from green (CD34 for **a**), red (A3 for **b**) and blue (nuclei for **a-c**). **a.** CD34-positive cells are seen sporadically in the CTS (arrows) and dermis. **b.** A3 expression is seen in epithelial cells in the inner site (large arrow) and mesenchymal cells in the CTS (arrowheads) of hair peg. **c.** Some mesenchymal cells in the CTS react to both CD34 and A3 (small arrows), but epithelial cells (large arrow) do not react to CD34. Immunofluorescence, counterstained with DAPI. Bars: 40 μm.

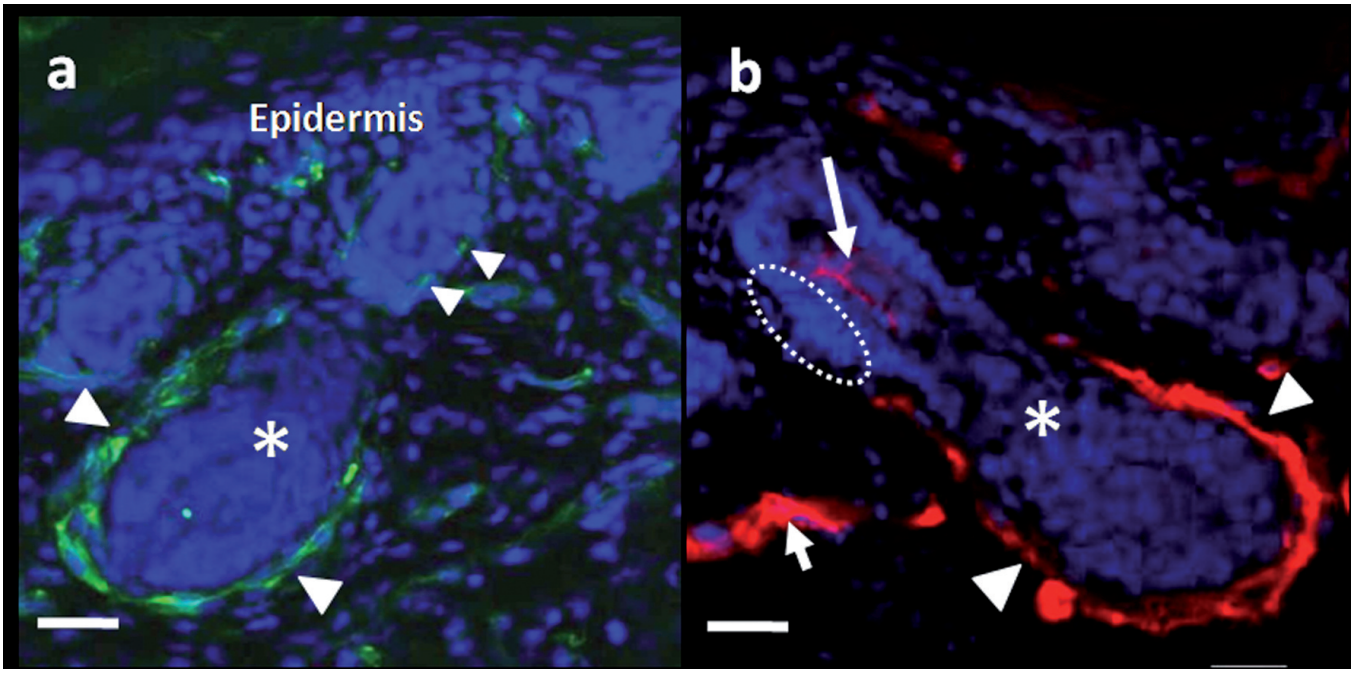

Fig. 8. Single immunofluorescence for nestin (**a**) and A3 (**b**) in the maturing hair peg (asterisks). **a.** In the CTS nestin-positive mesenchymal cells (**a**, arrowheads) correspond to cells reacting to A3 (**b**, arrowheads), indicating that mesenchymal cells in the CTS express both nestin and A3; some cells in the bulge area react faintly to nestin (small arrowheads), but there are no A3-reacting cells coinciding with the nestin-positive cells in A3 immunofluorescence (**b**, dotted circle). **b.** Epithelial cells in the inner site of hair peg (large arrow) and pericytes (small arrow) show a positive reaction to A3. Immunofluorescence, counterstained with DAPI. Bars: a, 40 μm; b, 20 μm.

A3-positive epithelial cells and mesencymal cells in developing hair follicles did not react to Ki-67 in double immunofluorescence (Fig. 9), indicating the absence of proliferating activity of the A3-positive cells.

Discussion

It has been reported that A3 labels immature mesenchymal cells in visceral organs, pericytes and bone marrow-stem cells in rats by DAB immunohistochemistry (Kumagai et al., 2000; Yamate et al., 2007). In this study, in agreement with findings reported previously (Yamate et al., 2007), we confirmed that pericytes in the subcutis of fetuses and neonates reacted to A3. Pericytes have been considered to be stromal stem cells with mesenchymal differentiation potential (Caplan, 2008; Crisan et al., 2008). Although molecular characteristics of antigen recognized by A3 are under investigation, A3 may become a useful tool to know the localization of cells with potential stem cell nature in developing organs of fetal and neonatal rats. In this study, we found that there were epithelial and mesenchymal cells reacting to A3 in the developing rat hair follicle. To identify these cell types, double immunofluorescence was carried out with a panel of antibodies for epithelial cell, mesenchymal cell, stem cell and proliferating cell markers.

A3-positive epithelial cells in the developing hair follicle

A3-positive epithelial cells seen in the hair germ and peg, and mature hair follicles reacted to E-cadherin but not to CK15. E-cadherin is expressed in all types of epithelial cells of the epidermis and hair follicle (Tinkle et al, 2004). CK15 expression is specific for basal cells of epidermis and hair follicles, and CK15-positive cells may be regarded as stem cells with potential epithelial differentiation (Kaur, 2006; Poblet et al., 2006). Ki-67-positive reaction was not seen in A3-positive epithelial cells. These findings indicate that A3-positive epithelial cells in the hair germ and peg may not be epithelial stem cells with proliferating activity. The suprabasal cells in the epidermis have been considered to be a post-progenitor which can develop into prickle cells (Blanpain, 2010; Snippert et al., 2010). Since the A3-positive epithelial cells were located above basal cells in the hair germ and peg, they may be regarded as suprabasal cells with potential differentiation towards well-differentiated stratified epithelial cells. Epithelial stem cells in the hair bulge did not react to A3, and in A3 immunofluorescence there were no A3-positive cells that correspond to nestin-positive cells seen occasionally in the bulge-area. In hair follicles at anagen, bulge stem cells differentiate into epithelial elements to form a new hair follicle by migrating downwards and upwards along the ORS (Paus et al., 1999; Fuchs, 2007; Hsu et al., 2011; Uchugonova et al., 2011). In the maturing hair follicle, the A3-positive epithelial cells were seen adjacent to the bulge, and were located above CK15-positive basal cells; therefore, these A3-positive epithelial cells may be epithelial cells with features similar to those of the suprabasal cells. The companion layer between the ORS and IRS may play important roles in migration and maturation of epidermic hair follicles (Fuchs, 2007). Because the epidermic hair follicle is complicated in tissue structure, the cellular nature of the A3-positive epithelial cells should be investigated further.

A3-positive mesenchymal cells in the developing hair follicle

There were A3-positive cells surrounding the hair peg and mature hair follicle, which were different in location from epithelial cells reacting to A3. The CTS consisting of three components, such as collagens, fibroblasts and myofibroblastic cells, was divided from the epidermic hair follicle by the glassy membrane (Urabe et al., 1992). On the basis of findings in double immnofluorescence with A3 and vimentin (for mesenchymal cells), collagen type IV (for glassy membrane) or α-SMA (for myofibroblasts), A3-positive cells were regarded as mesenchymal cells without

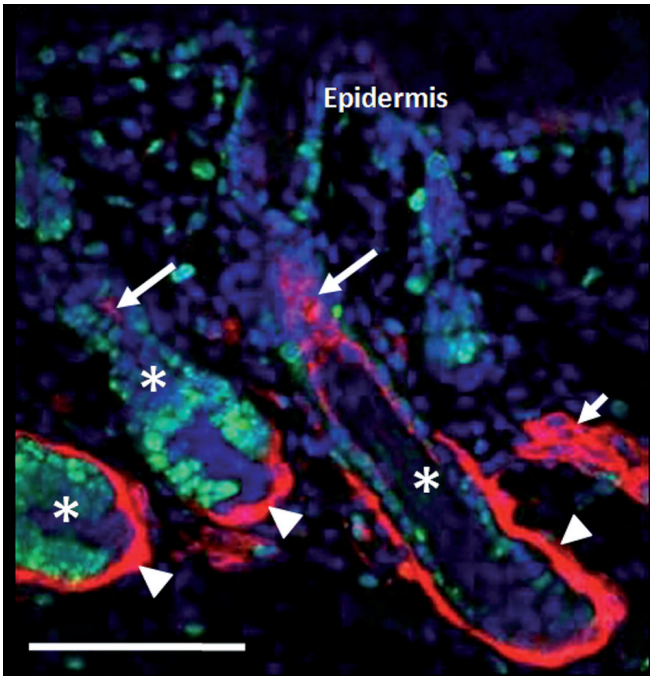

Fig. 9. Double immunofluorescence for Ki-67 and A3 in the hair peg and maturing hair follicle (asterisks); the confocal image was merged from green (Ki-67), red (A3) and blue (nuclei). Ki-67-positive cells (green) are seen in the basement layer of hair peg and maturing hair follicle; although epithelial cells in the inner site of hair peg (large arrows), mesenchymal cells in the CTS (arrowheads) and pericytes (small arrow) react to A3, there are no cells reacting to both Ki-67 and A3. Immunofluorescence, counterstained with DAPI. Bar: 150 μm.

myofibroblastic nature located in the CTS outside the glassy membrane.

Recently, mesenchymal cells in the CTS around the dermal bulb have been considered to possess potential mesenchymal differentiation like a stromal stem cell (Hoogduijn et al., 2006; Sellheyer and Krahl, 2010). Nestin-positive cells in the dermal papilla are regarded as pluripotential stem cells of the hair follicle and the skin itself (Uchugonova et al., 2011), and have been believed to differentiate into neuronal and glial cells under appropriate conditions (Amoh et al., 2005a; Liu et al., 2011). The A3-positive mesenchymal cells in the CTS also reacted to Thy-1. Thy-1 is known to be expressed by immature mesenchymal cells and pericytes (Sellheyer and Krahl, 2010). Besides the pericytes, in fact, the present study showed that Thy-1 was expressed in immature mesenchymal cells in the dermal condense of placode and beneath the epidermis of fetuses and neonates. Furthermore, A3-positive cells in the CTS also gave a positive reaction to CD34, and the distribution of A3-positive cells was similar to that of nestin-positive cells in the CTS. CD34 and nestin are used as markers for stem cells and post-progenitors (somewhat differentiated cells from stem cells) (Toma et al., 2001; Amoh et al., 2005a; Uchugonova et al., 2011). Taking these findings together, it is considered that A3 can label immature mesenchymal cells with stem cell nature in the CTS (Yamate et al., 2007). It is interesting to note that A3-positive mesenchymal cells in the CTS did not show proliferating activity demonstrable with Ki-67 immunohistochemistry. A3-positive mesenchymal cells may be regarded as quiescent post-progenitor cells which may be activated under some stimuli.

In conclusion, the present study showed that there were two types of cells that can react to A3 in developing rat hair follicles; one is epithelial cells present in the inner site of the hair germ and peg, and in the ORS adjacent to the bulge of the maturing hair follicles; the other is immature mesenchymal cells in the CTS of hair peg and mature hair follicle. Based on anatomical localization and double immunofluorescence findings, the A3-positive epithelial and mesenchymal cells may be regarded as post-progenitors with potential to differentiate into epithelial and mesenchymal cells, respectively. A3 was generated as an antibody specific for rat MFH cells by using rat MFH cells as the antigen (Kumagai et al., 2000; Yamate et al., 2007). Human MFHs are considered to be pleomorphic undifferentiated sarcomas derived from pluripotential stem cells. Recently, epitheliod variant has been reported in a part of human MFHs (Maruno et al., 2011; Terada, 2011). A3 labeled both epithelial and mesenchymal cells at the immature stage in developing rat hair follicles. Although the cellular nature of these A3-positive cells in developing rat hair follicles and molecular properties of A3-recogninizing antigen should be investigated further, A3 could become a useful antibody to discover the kinetics of hair follicle-forming cells in hair cycle and regeneration after injury.

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