In Vitro Differentiation of Chicken Astrocytes: Growth, Morphology, and Protein Expression of Astrocytes in Primary Cultures

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In Vitro Differentiation of Chicken Astrocytes: Growth, Morphology, and Protein Expression of Astrocytes in Primary Cultures

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Astrocytes regulate synaptic transmission in the central nervous system. Astrocytes in vivo have "stems" that express glial fibrillary acidic protein (GFAP), intermediate filaments, and peripheral astrocyte processes (PAPs), which contain actin-rich cytoskeletal structures. At the PAPs, the perisynaptic glia contacts and enwraps synapses, and modulates glia-neuronal communication. Cultured astrocytes have been an invaluable tool for studying roles of astrocytes; however, the morphology of mammalian primary astrocytes cultured in conventional medium containing fetal bovine serum (FBS) was similar to that of fibroblasts, and many culture conditions have been developed to generate stellate astrocytes observed in vivo. Avian astrocytes have been prepared from embryonic chick forebrain and maintained at a high cell density in conventional FBS-containing medium as mammalian astrocytes, thus the morphological analysis of chicken astrocytes has not yet been performed. In the present study, we report that the morphology of astrocytes freshly harvested from the forebrain of a chicken embryo in serum-free Neurobasal medium with B-27 supplement and basic fibroblast growth factor (bFGF) is similar to that of the astrocyte morphology in vivo. We also find that astrocytes in this medium express similar levels of GFAP and two actin-binding proteins as astrocytes in conventional FBS-containing medium, although they have different morphologies. Furthermore, we confirmed that cryopreserved astrocytes differentiate faster than freshly harvested astrocytes.

Key words: astrocyte, cell culture, differentiation, peripheral astrocyte process, actin-binding protein

INTRODUCTION

Astrocytes, which have stellate morphology, are the most abundant glial cells in the central nervous system. Astrocytes regulate neuronal excitability and synaptic transmission, releasing neurotransmitters and other signaling molecules (Ben Haim and Rowitch, 2016). They also have key roles in maintaining the blood-brain barrier (Abbott et al., 2006). In vivo, astrocytes possess "stems" expressing intermediate filaments of glial fibrillary acidic protein (GFAP) and peripheral astrocyte processes (PAPs), which contain a rich actin-based cytoskeleton. At the PAPs, the perisynaptic glia contact and enwrap the synapses and modulate glial-neuronal communication (Reichenbach et al., 2010). Preferential localization of actin-binding proteins, such as ezrin, provides structural support of the actin cytoskeleton in PAPs (Lavialle et al., 2011).

Most information of astrocytes has been derived from studies on cultured astrocytes prepared from the brain of mammalian species (reviewed by Saura, 2007). Most protocols for preparing astroglial-enriched cultures from whole cerebrum or dissected tissues (cortex, hippocampus, spinal cord, etc.) of rat/mouse late embryos/neonates. The most frequently used medium to culture rodent astrocytes is DMEM (Dulbecco's Modified Eagle Medium) supplemented with fetal bovine serum (FBS), which is widely used for primary cultures and established cell lines. FBS is thought to contain growth factors, and fulfills numerous metabolic requirements of cultured cells. Such cultures have served as invaluable tools to investigate the role of astrocytes in physiological and pathological states. Many astrocytic functions in metabolism, neurotransmission, and calcium signaling were first ascertained using cultured astrocytes, and most of these results were subsequently obtained in vivo (reviewed by Lange et al., 2012).

Nevertheless, morphologically intact astrocytes can be observed immediately after dissociation (Haseleu et al., 2013). The morphology of primary astrocytes cultured in conventional DMEM supplemented with FBS changed to

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fibroblast-like cells, showing reduced stems and PAPs, and did not completely mimic the astrocytes in vivo; however, these cultured cells expressed GFAP, a selective astrocyte marker (Aumann et al., 2017). Moreover, standard culture protocols using conventional medium recommend using a high density of cells, which is not suitable for the observation of cellular morphology. To analyze the functions of PAPs, the culturing conditions facilitating differentiation (forming stems and PAPs) of isolated astrocytes in vitro have been discussed previously (Wolfes et al., 2017).

Avian astrocytes have been prepared from embryonic chick forebrain; conventional FBS-containing medium was used in these studies. In addition, the cells were maintained at a high cell density for metabolic analysis, and the cells showed fibroblast-like morphology similar to mammalian astrocytes cultured in FBS-containing medium (Adachi et al., 2002; Sorbel et al., 2002; Peters et al., 2005). Thus, morphological analysis of chicken astrocytes has not yet been performed.

We found that, when maintained in serum-free Neurobasal medium containing B-27 supplement and basic fibroblast growth factor (bFGF) developed for chicken tectal neurons, astrocytes freshly prepared from the forebrain of a late-stage chicken embryo formed stems and PAPs similar to those of astrocytes observed in vivo. In the present study, we compared the growth, morphology, and protein expression of astrocytes grown in serum-free medium and conventional medium supplemented with serum. We found that chicken astrocytes cultured in Neurobasal medium containing B-27 supplement bFGF proliferated steadily at various cell densities and showed higher GFAP expression rates than astrocytes in DMEM. We also observed similar expression levels of GFAP and two actin-binding proteins, although their morphologies were markedly different, suggesting that regulation of cell shape is determined by complex molecular mechanisms. Additionally, we confirmed that cryopreserved astrocytes differentiated in the same manner as freshly harvested astrocytes, and their differentiation rate was greater than that of freshly harvested astrocytes.

MATERIALS AND METHODS

Antibodies and reagents

Cy3-labeled anti-GFAP monoclonal antibody (clone G-A-5, 1:4000, Sigma-Aldrich, St. Louis MO, USA), anti-neurofilament (clone 3H11, 1:5000, Merck Millipore, Darmstadt, Germany), and Alexa Fluor 488-labeled anti-mouse IgG antibody (Thermo Fisher, Waltham, MA, USA) were used for immunostaining. Alexa Fluor 488-labeled phalloidin (0.066 μM, Thermo Fisher) and Hoechst 33342 (0.1 μg/mL, Sigma-Aldrich) were used for F-actin staining and nuclear staining, respectively. Anti-actin (pan) antibody (clone C4, 1:5000, Cederlane, Burlington, Ontario, Canada), anti-tubulin (N356, 1:5000, GE Healthcare, Chicago, IL, USA), anti-GFAP antibody (clone G-A-5, 1:5000, Sigma-Aldrich) were used for immunoblotting. Cross-absorbed polyclonal antibodies against chicken lasp-1 (1:1000) and lasp-2 (1:200) for immunoblotting were prepared as described by Terasaki et al. (2004).

Cell culture

Mixed neuronal/glial cultures were prepared from 14- to 15-day-old embryonic chick forebrain, following the method of Adachi et al. (2002) with a slight modification (Adachi et al., 2002). The cerebral meninges and superficial blood vessels were carefully removed in phosphate-buffered saline (PBS), and tissues were minced and treated with 0.1% trypsin-EDTA (WAKO, Tokyo, Japan) for 15 min. DMEM containing 10% FBS was then added to inactivate trypsin and the cells were dissociated by pipetting. The cells were then filtered through Kimwipes (Kimberly-Clark, Irving, TX, USA) in a Swinnex filter holder (Merck Millipore) and plated on 35-mm culture dishes (IWAKI, Tokyo, Japan) with circular coverslips of 15 mm diameter (Matsunami, Tokyo, Japan) coated with 0.08 mg/mL Poly-L-Lysine (Sigma-Aldrich) at various cell densities. Cultures were incubated in DMEM containing 10% FBS or serum-free Neurobasal medium used for chicken tectal neurons (Goldsbury et al., 2008) with modification by LONZA (Basel, Switzerland, method VPG-1002); Neurobasal (Thermo Fisher) supplemented with 2% B-27 supplement (Thermo Fisher), 0.5 mM L-glutamine, 25 μM Glutamax (Thermo Fisher), and 5 ng/mL bFGF (Thermo Fisher). For cells freshly prepared from the forebrain, the medium was replaced completely by fresh medium on the next day of culture, and half of the medium were changed approximately twice a week. For cryopreserved astrocytes, half of the medium was changed approximately twice a week after three days in culture. Chicken forebrain neurons were prepared from 8-day-old embryos in accordance with the method of Pettmann et al. (1979) and cultured in the same serum-free Neurobasal medium as chicken astrocytes.

For subculture, astrocytes were dissociated from dishes with 0.25% Trypsin (WAKO), washed with DMEM containing 10% FBS and plated on coverslips coated with Poly-L-Lysine.

Cryopreservation

Astrocytes cultured in serum-free Neurobasal medium for 2–3 days were dissociated with 0.25% Trypsin were dissolved in Cell Banker 1 (ZENOAQ, Tokyo, Japan) and stored at –80°C until use. Cells in cryotubes were thawed using water at 37°C and washed with serum-free Neurobasal medium. Trypan Blue Solution (Sigma-Aldrich) was used as a cell stain to assess cell viability.

Proliferation assay

Cells were imaged with an inverted microscope (CK40, Olympus, Tokyo, Japan) at 20× magnification, equipped with a digital camera (CAMEDIA, Olympus). The percentage of area covered by cells at various proliferation stages were manually determined and estimated from phase-contrast images using ImageJ software; coverage indices were determined for 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100% area coverages. Coverage indices for astrocytes cultured in DMEM containing FBS and those cultured in serum-free Neurobasal medium were determined separately (see Supplementary Figure S1). Cell area fractions of randomly selected images were determined using these indices. If a cell area fraction was between 5% and 10%, we rounded up the value to 10%.

We also analyzed the relationship between the cell area and numbers of nuclei from fluorescence images by staining F-actin to visualize the cell outline and Hoechst to visualize the nuclei in low magnification (10 x). We counted each nucleus manually using the multi-point tool in the ImageJ software.

Immunocytochemistry

Astrocytes cultured on coverslips were fixed with 4% formaldehyde in phosphate-buffered saline (PBS) for 5 min at 37°C, and permeabilized with 0.1% Triton-X100 and 10% goat serum in PBS, for 30 min at room temperature. Nonspecific background staining was reduced with signal enhancer (Thermo Fisher). The cells were stained with Cy3-labeled anti-GFAP antibody in the dilution buffer (1% BSA, 0.5% casein, 0.1% gelatin in PBS) for 2 h, rinsed with PBS, and stained with mixture of Alexa Fluor 488-labeled-phalloidin and Hoechst 33342 for 10 min. Finally, the cells were mounted on slide glasses using SlowFade Gold antifade reagent (Thermo Fisher).

Analysis of GFAP expression

GFAP expression rates were determined from the number of cells identified with nuclear staining with Hoechst 33342 and number of GFAP-expressing cells identified with anti-GFAP antibody staining. Cells with stained dots clearly distinguishable from the background and fibrillar staining were identified as GFAP-positive cells. Three randomly selected images from each experiment were analyzed.

Fluorescence microscopy

Fixed cells were observed using an inverted fluorescence microscope (Axiovert135, Carl Zeiss) equipped with a ×40 phase-contrast objective lens, fluorescence filters (Zeiss 39, 38HE, and 43HE), and a Coolsnap HQ charge-coupled device (CCD) camera (Photometrics) operated by the IPLab software (Scanalytics, Billerica, MA, USA).

cDNA preparation and semi-quantitative PCR

Total RNA and total protein were extracted simultaneously from the cultured astrocytes, using the RNeasy Plus Mini Kit (Qiagen, Venlo, Netherlands). cDNAs were prepared from 1 μg of total RNA using Superscript III reverse transcriptase (Thermo Fisher) and amplified using primers for GAPDH and lasp-2 (GAPDH: forward: 5′-GCACGCCATCACTATCTTCC-3′ reverse: 5′-GCAGGTCAGGTCAACAACAGAG-3′, lasp-2: forward: 5′-CGCTGTGGGAAAGTGGTCTATC-3′ reverse: 5′-CTTGGGACTGGCTTTGCTG-3′). For quantification, the PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide.

Western blotting

Protein samples prepared with RNeasy Plus Mini Kit were precipitated and lysed in SDS sample solution buffer (4% SDS, 0.125 M Tris-HCl pH 6.8, 0.004% BPB, 10% 2-ME) and total protein concentration was determined measured using PlusOne 2D Quant Kit (GE Healthcare). SDS-PAGE was performed following the method of Laemmli (1970), using 10% polyacrylamide resolving gels. Immunoblotting was performed in accordance with the method developed by Towbin et al. (1979) with modifications. The electrophoresed proteins were electroblotted onto polyvinylidene difluoride membranes, and the membranes were blocked with 3% skimmed milk in TBS (20 mM Tris-HCl, 0.5 M NaCl, pH 7.6). The membranes were then incubated with primary antibodies in Can Get Signal solution (TOYOBO, Tokyo, Japan) for 2 h, then incubated with alkaline phosphatase-labeled secondary antibodies (MP Biomedicals, Aurora, OH, USA) for 1 h, and developed with BCIP (Bromo-4-chloro-3-indolyl phosphate) and nitro blue tetrazolium (NBT). Gels and membranes were stained with Coomassie Brilliant Blue (BIO-RAD) and low molecular weight marker (GE Healthcare) was used to confirm the mobility of reacted bands.

Statistical analyses

All data are expressed as mean ± standard error of mean (SEM) obtained from at least three independent experiments. “N” refers to the numbers of independent experiments.

RESULTS

Most cells freshly harvested from the 14- to 15-day-old chick embryo forebrains were isolated longitudinal cells, which were suspected to be astrocytes; immunostaining for GFAP and neurofilament confirmed that a large number of the cells differentiated as astrocytes and a small number of neurons remained in the culture (described later).

We compared the proliferation of the astrocytes in different culture media every day, based on the cell coverage area index from low magnification (20 ×) phase-contrast images of living cells (Supplementary Figure S1). The proliferation of astrocytes cultured in DMEM containing FBS depended on the cell plating densities. Astrocytes cultured in DMEM at a cell density of 1.6×10^5 cells/cm^2 (1.6D in Fig. 1A) which formed cell-cell contacts from the initial day (~25% coverage area) steadily proliferated and reached confluence at approximately one week; however, the astrocytes at a cell density of 0.8×10^5 cells/cm^2 (0.8D) proliferated much slower. The cells dispersed on coverslips formed colonies within a few days and the colonies grew and fused gradually. The cell coverage area at a cell density of 0.4×10^5 cells/cm^2 (0.4D) barely increased, even after seventeen days in culture, as more days were needed for forming colonies. All of GFAP-positive astrocytes were detected in the colonies (details were described in Fig. 2A). In contrast, astrocytes cultured in serum-free Neurobasal medium containing B-27 supplements and bFGF continued to proliferate at various cell densities (0.8N, 0.4N, 0.2N, and 0.1N in Fig. 1A).

Cultured astrocytes in various conditions were fixed after three days, five days, and eight days of culturing and stained with Alexa Fluor 488-labeled-phalloidin to analyze cell coverage area and Hoechst 33342 to detect cell numbers in low magnification (10 ×). Correlation between area and numbers was confirmed in cells cultured in DMEM at a cell density of 0.8×10^5 cells/cm^2 for three days and five days, at a cell density of 0.4×10^5 cells/cm^2 for five days. However, cells cultured in DMEM at a cell density of 1.6×10^5 cells/cm^2 for five days showed a lower correlation, and cells cultured in DMEM at a cell density of 0.8×10^5 cells/cm^2 after eight days showed different cell numbers in similar coverage (~700 and ~1600 cells in the same ~70% coverage). Correlation between area and numbers of cells cultured in Neurobasal medium showed the same tendency in all conditions. Images of astrocytes stained with phalloidin and Hoechst 33342 in low magnification (10 ×) also showed that many cells in DMEM were packed in the colonies and cells in Neurobasal medium uniformly dispersed and formed stems from each cell (Fig. 1C). Cultured astrocytes were also stained with an anti-GFAP antibody to detect differentiation rate of astrocytes at lower magnification (10 ×), and to visualize stems at higher magnification (40 ×) (Supplementary Figure S2, GFAP). Phalloidin was also used to observe formation of PAP in higher magnification (Supplementary Figure S2, F-actin).

A small number of neurons with long axons and rounded cell bodies were observed from the initiating of culture of both media and were detected with anti-neurofilament antibody in lower magnification (Supplementary Figure S3). Obvious difference in the proportion of the contaminated neurons was not observed between the media and cell densities (< 1% of total cells) and number of neurons decreased during long-term culture. In cells cultured in DMEM at a lower density (below 0.8×10^5 cells/cm^2), which needed several days to form colonies, GFAP-negative polygonal cells with tight attachments sometimes appeared (Supplementary Figure S4, a). Observation in higher magnification confirmed the polygonal cells had rich actin filaments and grew on the layers of astrocytes (Supplementary Figure S4, b and c). Proportion of the polygonal cells were varied in cultures (up to ~5%) in lower cell densities, but astrocyte cultured in DMEM at a high cell density (1.6×10^5 cells/cm^2) which reached confluence faster, and astrocytes

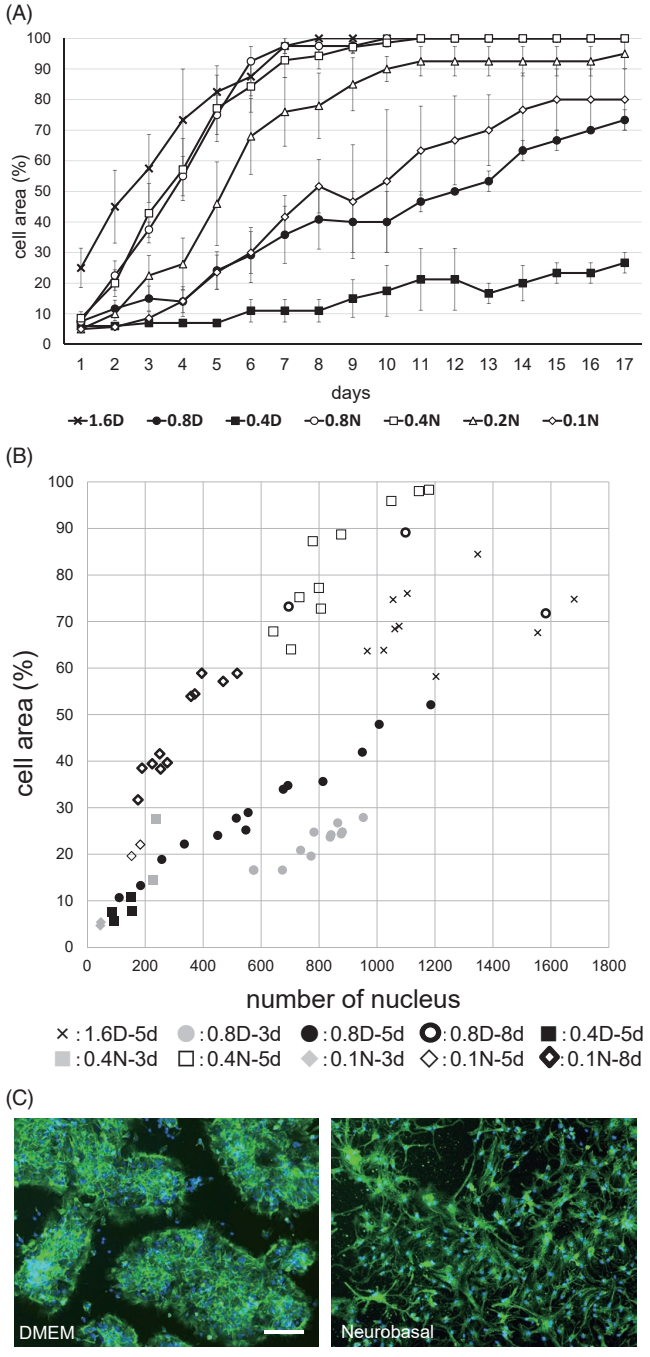

Fig. 1. Proliferation of astrocytes cultured in different media and at different cell densities. **(A)** Cell coverage area of living cells in terms of the percentage of astrocytes cultured in DMEM supplemented with fetal bovine serum and serum-free Neurobasal medium containing B-27 supplement and bFGF was estimated from the coverage index of astrocytes, using image analysis. As shown, 1.6D, 0.8D, and 0.4D indicate astrocytes cultured in DMEM at cell densities of 1.6, 0.8, and 0.4×10^5 cells/cm^2, respectively and 0.8N, 0.4N, 0.2N, and 0.1N indicate astrocytes cultured in Neurobasal medium at cell densities of 0.8, 0.4, 0.2, and 0.1×10^5 cells/cm^2, respectively. The bars represent the mean of independent experiments (DMEM: n = 3–6) (Neurobasal medium: n = 3–7) ± SEM. **(B)** Relationship between area and numbers of fixed cells counted by nuclear staining. **(C)** Fluorescence images of F-actin and nuclei of astrocytes cultured in DMEM (1.6×10^5 cells/cm^2, five days) and Neurobasal medium (0.1×10^5 cells/cm^2, eight days) in similar cell coverage area (~60%). Scale bar = 100 μm.

cultured in Neurobasal medium at various cell densities scarcely contained polygonal cells (data not shown).

We observed differentiation of astrocytes in DMEM and Neurobasal medium at various cell densities with expression of GFAP in stems and PAP formed in the tip of stems in high magnification (40 ×). Primary astrocytes in DMEM and Neurobasal medium were observed as longitudinal cells immediately after plating; however, the astrocytes in DMEM

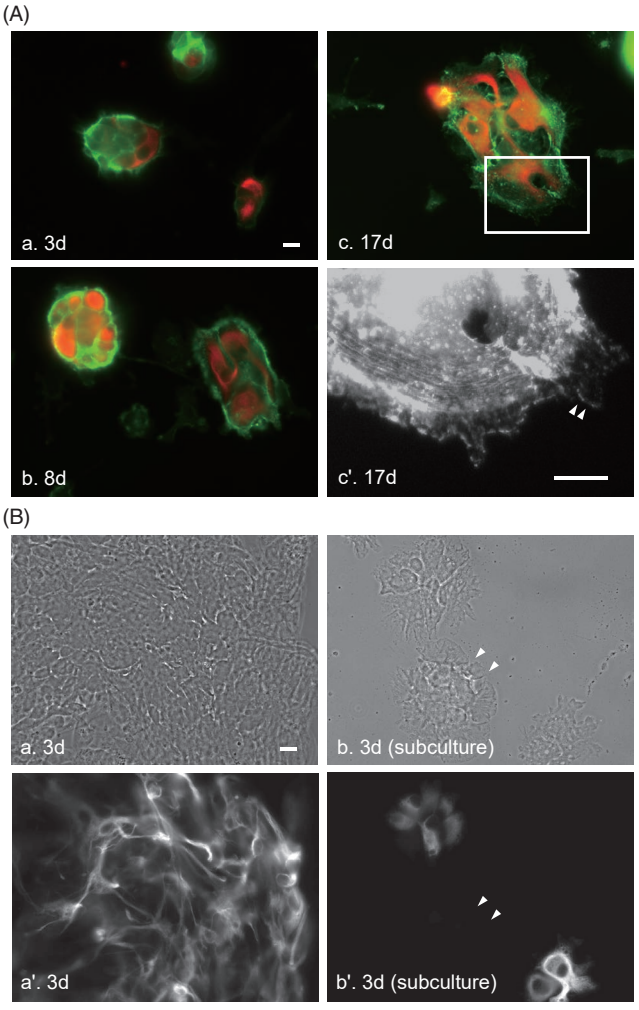

Fig. 2. Differentiation of astrocytes cultured in DMEM. **(A)** Astrocytes cultured in DMEM containing with fetal bovine serum at a cell density of 0.4×10^5 cells/cm^2 for three days (a), eight days (b), and seventeen days (c). Cells were stained with an antibody against the astrocyte marker glial fibrillary acidic protein (GFAP: red) and the F-actin probe (Alexa Fluor 488-labeled phalloidin: green). Nearly all astrocytes formed colonies cultured for three days (a) and the cells proliferated in the colonies (b and c). Lamellipodia were observed as thin sheet-like processes (a, b, c). The magnified image shows short filopodia extending in the peripheral region of the lamellipodia (c', arrowheads). **(B)** Phase-contrast image (a) and fluorescence image of anti-GFAP antibody (a') of astrocytes cultured in DMEM at a cell density of 1.6×10^5 cells/cm^2 for three days. Phase-contrast image (b) and fluorescence image of anti-GFAP antibody (b') of subcultured astrocytes prepared from primary astrocytes cultured in DMEM at a cell density of 0.8×10^5 cells/cm^2 for six days and seeded at a cell density of 0.2×10^5 cells/cm^2 for three days. A part of colonies lost GFAP expression (b', arrowheads). Scale bar = 10 μm.

at a low density (0.4 × 10^5 cells/cm^2) formed colonies of packed cells within a few days. After three days of culture, the majority of these cells were GFAP-negative (Fig. 2A, a). Most cells in these colonies were stained with the anti-GFAP antibody after eight days; however, they had no stems (Fig. 2A, b). GFAP-positive astrocytes proliferated in the colonies and isolated cells expressing GFAP were scarce even after seventeen days of culturing (Fig. 2A, c). Lamellipodia stained with phalloidin were observed as thin, sheet-like processes at the periphery of the astrocyte colonies, and short filopodia extended from the lamellipodia (Fig. 2A, c', arrowheads).

Astrocytes cultured in DMEM at a high cell density (1.6 × 10^5 cells/cm^2) generated cells with multiple stems stained with the anti-GFAP antibody in a thick cell layer for a few days (Fig. 2B, a and a'). PAPs were not identifiable in cells cultured in DMEM at any density, because stems were entangled in the colonies. However, subcultured astrocytes prepared from well-differentiated primary cells and seeded at a low density changed to flat cells and formed colonies, similar to primary astrocytes at low density (Fig. 2B, b). A part of colonies of the subcultured astrocytes lost GFAP expression (Fig. 2B, b', arrowheads), and additional days were necessary for redifferentiation.

Most of the astrocytes in serum-free Neurobasal medium at a low density (0.1 × 10^5 cells/cm^2) were displayed an elongated morphology for the first two to three days (Fig. 3A, a). Multiple branched stems, which stained positive with anti-GFAP antibody, extended approximately five days after plat-

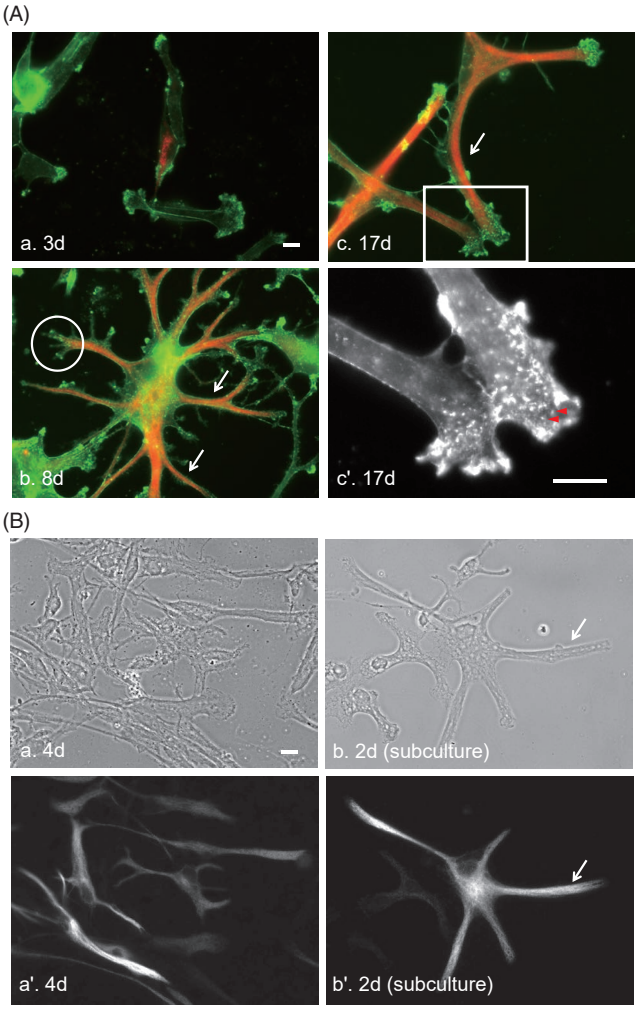

Fig. 3. Differentiation of astrocytes in serum-free Neurobasal medium. **(A)** Astrocytes cultured in Neurobasal medium containing B-27 supplements and bFGF at a cell density of 0.1 × 10^5 cells/cm^2 for three days (a), eight days (b), and seventeen days (c). Many isolated astrocytes were observed in culture for three days (a). Astrocytes cultured for eight days had multiple GFAP-positive stems (arrows) and peripheral astrocyte processes (PAPs, circle), observed as ruffled lamellate processes stained with phalloidin, were observed at the stem periphery (b) and astrocytes cultured for seventeen days still retained the PAPs (c). Many filopodia were observed in the membrane ruffles in the magnified image of PAPs (c', red arrowheads). **(B)** Astrocytes cultured in serum-free Neurobasal medium at a cell density of 0.4 × 10^5 cells/cm^2 for four days (a). The astrocytes were cultured for six days for differentiation and subcultured at a cell density of 0.1 × 10^5 cells/cm^2 for two days (b). In (a), many astrocytes with multiple stems were observed stained with anti-GFAP antibody (a') and the cells still have branched stems (arrow) after subculture at low density (b'). Scale bar = 10 μm.

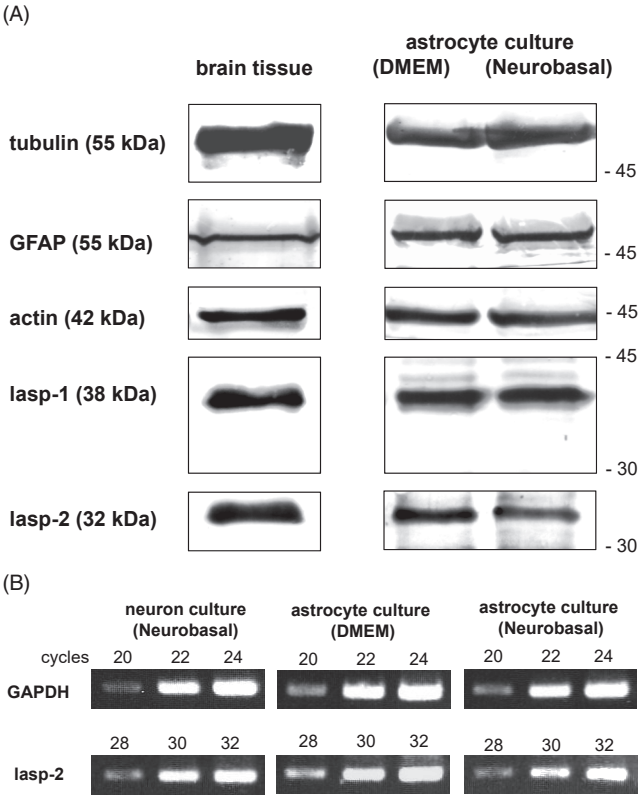

Fig. 4. Expression of cytoskeletal proteins in astrocytes. **(A)** Immunoblotting of tubulin, GFAP, lasp-1 and lasp-2 in adult chicken forebrain tissue and chicken astrocyte culture. Astrocytes were cultured in DMEM containing FBS at a cell density of 0.8 × 10^5 cells/cm^2 or serum-free Neurobasal medium at a cell density of 0.4 × 10^5 cells/cm^2. The tissue sample contained 5 μg of protein and astrocyte culture samples were adjusted with reactivity of the anti-actin antibody. **(B)** Semi-quantitative PCR of lasp-2 and GAPDH mRNA of neurons from 8-day old embryo cultured in serum-free Neurobasal medium, astrocytes from a 15-day-old embryo cultured in DMEM at a cell density of 0.8 × 10^5 cells/cm^2, and astrocytes cultured in Neurobasal medium at a cell density of 0.4 × 10^5 cells/cm^2. All cells were cultured for five days.

ing (data not shown); nearly all astrocytes had stems after eight days (Fig. 3A, b) and retained their stems after seventeen days (Fig. 3A, c). PAPs were observed as ruffled lamellate processes stained with phalloidin at the stem periphery, and numerous filopodia were observed in the membrane ruffles, as shown in the magnified image of PAPs (Fig. 3A, c'). The number of stems varied among cells and tended to increase during culturing. All cells displaying more than four stems were GFAP-positive and all stems of the well-differentiated astrocytes had PAPs. Astrocytes cultured in serum-free Neurobasal medium at a high cell density (0.4×10^5 cells/cm^2) also generated cells with multiple stems stained with anti-GFAP antibody (Fig. 3B, a and a'). Cells subcultured at a low cell density to enable observation of isolated cells had GFAP-positive stems and PAPs as primary astrocytes (Fig. 3B, b and b').

We analyzed the protein expression of an astrocyte marker (GFAP), microtubule component (tubulin), actin-binding protein ubiquitously expressed in various tissues (lasp-1) and highly expressed in neuronal tissues (lasp-2) by immunoblotting (Fig. 4A). Equal amounts of the protein from astrocytes cultured in DMEM and Neurobasal medium were loaded and reacted with the aforementioned antibodies at the same concentrations to compare the expression levels of the proteins in vitro with that of the forebrain samples in vivo. Reactivities of the immunoabsorbed antibodies against lasp-1 and lasp-2 were specific, although they had similar domain structure (see lasp-1 in Fig. 4A) and anti-lasp-2 antibody, which has been reported to show weak immunoreactivity in non-neuronal tissues and non-neuronal/non-glial cells, detected clear bands in astrocyte samples. Reactivities of the antibodies against GFAP, tubulin, lasp-1, and lasp-2 were almost same between the astrocyte samples. Transcript levels of lasp-2 mRNA in neurons and astrocytes cultured in different media were also compared using semi-quantitative PCR and normalized to those of the housekeeping gene GAPDH as an internal control (Fig. 4B). Almost all cells from the 8-day old embryo chicken forebrain were neurons that had elongated axons with rounded cell bodies; astrocytes were hardly observed after five days of

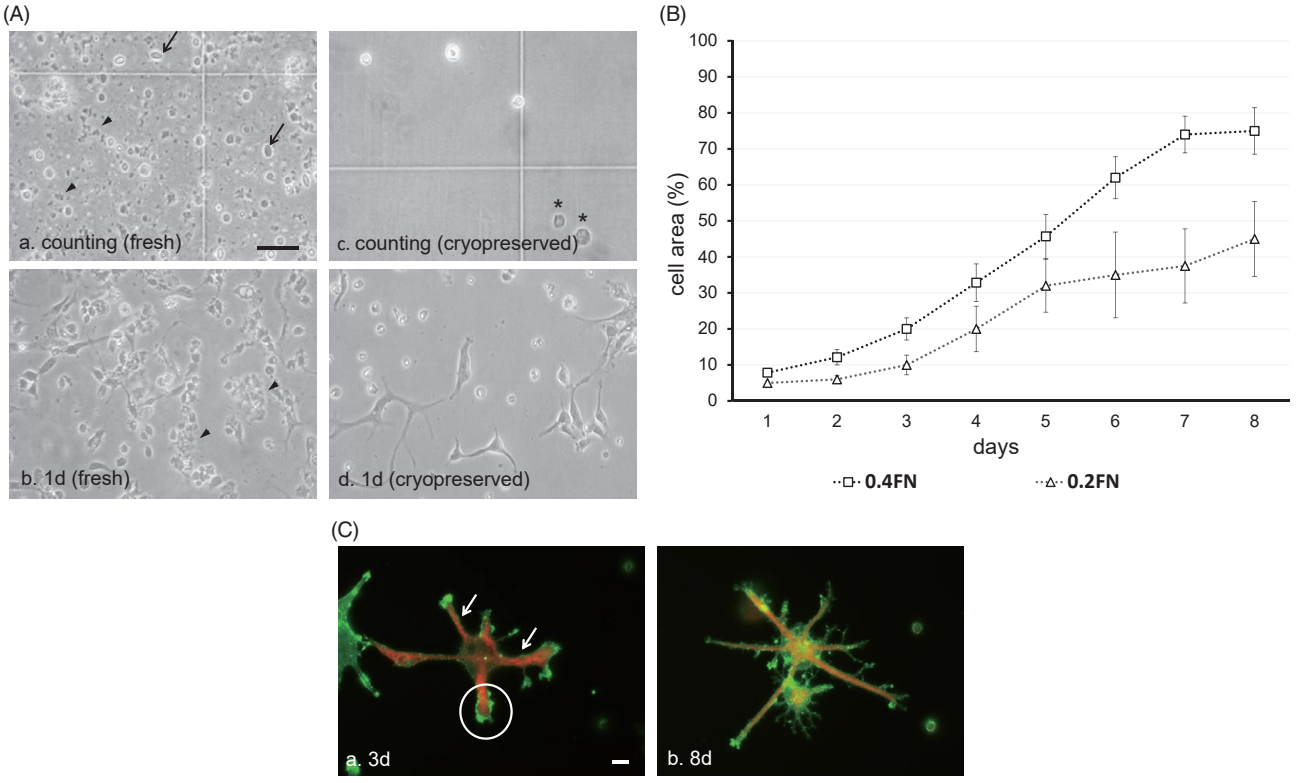

Fig. 5. Proliferation and differentiation of cryopreserved astrocytes. **(A)** A lower magnification view of freshly prepared astrocytes and cryopreserved astrocytes. Contamination of oval-shaped erythrocytes (arrows) and debris of dead cells and extracellular matrix (arrowheads) were observed in astrocytes freshly prepared from 15-day-old chicken forebrain, enumerated using a hematocytometer (a). The debris (arrowheads) was still observed in astrocytes freshly harvested from the forebrain, after one day of culturing in Neurobasal medium containing supplements and on changing the medium (b). Contamination with erythrocytes and debris was rarely observed in astrocytes revived from freeze-stocks before plating, and dead cells (asterisk) were easily identified with Trypan Blue staining (c). Multiple stems were observed in cryopreserved astrocytes and cultured for one day (d). Scale bar = 50 μm. **(B)** Cell coverage area in terms of the percentage of astrocytes revived from freeze-stocks and cultured in Neurobasal medium containing supplements at different cell densities. As shown, 0.4 FN and 0.2 FN indicate the cryopreserved astrocytes cultured in Neurobasal medium at cell densities of 0.4 and 0.2×10^5 cells/cm^2, respectively. The bars represent the mean of independent experiments (n = 4–7) ± SEM. **(C)** Astrocytes cultured in Neurobasal medium at a cell density of 0.2×10^5 cells/cm^2 after three days (a) and eight days (b). After culturing for three days, many astrocytes had multiple glial fibrillary acidic protein-positive stems (arrows) and peripheral astrocyte processes (PAPs, circle), similar to those observed in freshly prepared astrocytes (a), and they retained their morphology after eight days (b). Scale bar = 10 μm.

culturing (data not shown). Lasp-2 expression levels in neurons, astrocytes cultured in DMEM, and astrocytes cultured in Neurobasal medium were almost the same.

We also prepared cryopreserved astrocytes from cells cultured in serum-free Neurobasal medium for two or three days and compared their differentiation with freshly harvested astrocytes from the forebrain. In freshly harvested astrocytes from 15-day-old chicken forebrains, contamination with oval-shaped erythrocytes (Fig. 5A, a, arrows) and debris of dead cells and extracellular matrix (arrowheads) was observed using a hematocytometer, which was used to measure the cell density before plating. The debris (arrowheads) was still observed in astrocytes after the first medium change on the next day (Fig. 5A, b). In cryopreserved cells, contamination of erythrocytes and debris were rarely observed, and dead cells (asterisk) were easily identified with Trypan Blue staining before plating (Fig. 5A, c). Viability of the cryopreserved astrocytes was approximately 60–80%. Cell morphology was observed on the next day of plating (Fig. 5A, d). Proliferation of the cryopreserved astrocytes was rather slower than that of the freshly harvested astrocytes; however, they continued proliferating as freshly harvested astrocytes (Fig. 5B). Multiple branched stems stained with anti-GFAP antibody extended faster in case of the cryopreserved astrocytes than in freshly harvested astrocytes (Fig. 5C, a), and they retained their stems after eight days of culturing (Fig. 5C, b). Morphology of the stems and PAPs were the same as that for the freshly prepared astrocytes. Transcript levels of lasp-2 mRNA for cryopreserved astrocytes was the same as those for freshly prepared astrocytes (data not shown).

GFAP expression rates of freshly prepared astrocytes and cryopreserved astrocytes were compared on the basis of the number of cells identified with nuclear staining with Hoechst 33342 and the number of GFAP-expressing cells (Fig. 6). Intermediate filaments are expressed in monomeric forms and observed as puncta in various cells; hence, we identified cells with stained dots and fibrillar staining as GFAP-positive. Immunostaining of freshly prepared astrocytes cultured for one or two days could not be performed due to contamination by debris. After the second medium change in the 3-day cultured samples, GFAP expression rates of freshly prepared astrocytes cultured in DMEM and Neurobasal medium were compared. GFAP expression rate of astrocytes cultured in DMEM at a cell density of 0.8×10^5 cells/cm^2 and 0.4×10^5 cells/cm^2 varied in experiments (0.8D and 0.4D in Fig. 6) because astrocytes slowly proliferated at this density and the proportion of contaminated cells between the astrocyte samples fluctuated (0.8D and 0.4D in Fig. 1). The GFAP expression rate of astrocytes cultured in DMEM at a cell density of 1.6×10^5 cells/cm^2 seemed higher after three days of culture; however, it was difficult to count the nuclei as some astrocytes were overlaid in culture dishes (Fig. 2B). GFAP expression rate of astrocytes cultured in Neurobasal medium at a cell density of 0.4×10^5 cells/cm^2 (0.4N in Fig. 6) was the same as that for the astrocytes cultured in DMEM at a cell density of 0.8×10^5 cells/cm^2 and 0.4×10^5 cells/cm^2 after three days of culture; however, the expression rate of GFAP approached 70% after five days, and approximately 90% after eight days. Astrocytes cultured in Neurobasal medium at a cell density of 0.2×10^5 cells/cm^2 and 0.1×10^5 cells/cm^2 (0.2N and 0.1N in Fig. 6) showed similar rates to 0.4×10^5 cells/cm^2. GFAP expression rates of cryopreserved astrocytes could be analyzed on the next day, because they did not contain debris; the rates were the same as those of freshly harvested astrocytes after three days of culturing. GFAP expression rates of cryopreserved astrocytes after three days of culturing were the same as those of freshly harvested astrocytes after five days of culturing (0.4FN and 0.2FN in Fig. 6). We stained cryopreserved cells with anti-neurofilament to detect contaminated neurons as primary cultures, but a small number of cells with rounded cell bodies and short projections was occasionally stained, and cells with long neurites as primary culture were very rarely observed (data not shown).

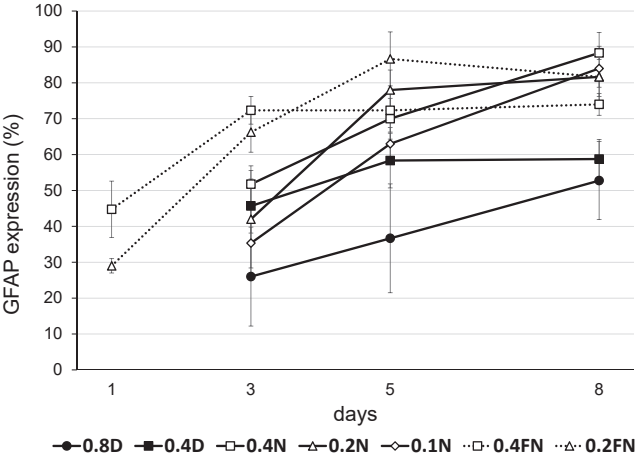

Fig. 6. Glial fibrillary acidic protein (GFAP) expression rates of freshly harvested astrocytes and cryopreserved astrocytes. GFAP expression rates were determined from the number of cells and the number of GFAP-expressing cells. As shown, 0.8D and 0.4D indicate astrocytes cultured in DMEM at densities of 0.8 and 0.4×10^5 cells/cm^2. Furthermore, 0.4N, 0.2N, and 0.1N indicate astrocytes cultured in Neurobasal medium at cell densities of 0.4, 0.2, and 0.1×10^5 cells/cm^2, respectively. Moreover, 0.4FN and 0.2FN indicate cryopreserved astrocytes cultured in Neurobasal medium at cell densities of 0.4 and 0.2×10^5 cells/cm^2, respectively. The bars represent the mean of independent experiments (n = 3–4) ± SEM.

DISCUSSION

In addition to GFAP-positive stems, astrocytes display extremely fine processes, termed PAPs. The close structural relationship between synaptic structures and PAPs makes astrocytes an important partner of neurons in the organization and functioning of synaptic connections (Bernardinelli et al., 2014). Astrocytes also respond to CNS damage and disease through a process called astrogliosis, which involves morphological changes, including cell body hypertrophy and thickening of the major processes (Pekny and Pekna, 2014). The detailed mechanisms underlying these cell-cell interactions via PAPs and these morphological changes are unknown; however, actin-binding proteins are expected to play numerous roles in these processes, because PAPs are actin-rich structures with ruffled lamellate processes and filopodia. Moreover, immunocytochemistry of rat brain sections and cultured astrocytes demonstrated that the actin-binding protein ezrin is localized at the PAPs

(Lavialle et al., 2011). Ezrin siRNA or dominant-negative ezrin in primary astrocytes indicates that filopodia formation and motility require ezrin in the membrane/cytoskeleton-bound form. Knockdown of the Arp2/3 subunit and a chemical compound that inhibit the activity of Arp2/3 complex blocked driving rat cortical astrocytes into a stellate morphology with actin bundle disassembly, cytoplasmic shrinkage and process outgrowth by Forskolin stimulation (Murk et al., 2013). The rate of Ca^{2+} -induced PAPs outgrowth of rat astrocyte was reduced by overexpressing of profilin mutant and the knockdown of two isoforms of profilins of murine astrocytes also reduced the morphological complexity of astrocytes (Molotkov et al., 2013; Schweinhuber et al., 2015). Localization of actin-binding proteins mentioned above was observed in mammalian astrocytes cultured in "standard protocol" using DMEM supplemented with FBS and had poor stems and PAPs.

"Standard protocols" for rodent astrocytes recommend that cells be maintained at a high density in medium supplemented with FBS (Mccarthy and de Vellis, 1980; de Velis and Cole, 2012). For example, the recommended cell density of the initial mixed glial cell cultures of newborn rat brain is 2×10^5 cells/cm^2. They maintained the culture in high density for almost a week for stratification of astrocytes and oligodendrocytes and subcloned astrocytes at lower density (0.5×10^5 cells/cm^2) in the same medium.

The composition of astrocyte culture medium is critical for primary cultured astrocytes. Various growth factors expressed in the nervous system serve as important signals during developmental of mammalian central nervous system (reviewed by Reuss and Von Bohlen Und Halbach, 2003) and it has been reported that cell culture supplements such as growth factors, affected proliferation and morphology of mammalian astrocytes. Morrison and de Vellis, (1981) observed that combination of FGF, putrescine, insulin, prostaglandin, and hydrocortisone had a synergic effect on growth of astrocytes subcloned from rat cerebral cortex at a rather lower density (0.6×10^5 cells/cm^2) than primary culture and the cells had many long branching process. Morita et al. (2003) revealed that rat astrocytes subcultured in astrocyte-defined medium (ADM) containing basic FGF (bFGF), epidermal growth factor (EGF), apotransferrin, D-biotin, sodium selenite, fibronectin, heparin sulfate, and insulin developed by Miller et al. (1995) promoted calcium oscillation. Further, they showed hypertrophic morphology with fibers strongly stained by the anti-GFAP antibody. They also observed that astrocytes cultured in ADM without EGF and FGF were flat and showed mesh-like GFAP staining in the perinuclear region, and the morphological effects of growth factors were suppressed by pro-inflammatory cytokines.

Recently, Neurobasal medium with growth factors and B-27 supplement containing insulin, transferrin, progesterone, and other supplements (concentrations are confidential) was reported to promote differentiation of mammalian astrocytes. Kang et al. (2014) reported that Neurobasal medium containing B-27 supplement, 30 ng/mL bFGF, or 25 ng/mL FGF-8 promoted the branching of GFAP-positive stems of mouse cortical astrocytes. Stellate astrocyte monoculture of rat cortices was also achieved by transferring of astrocyte maintained in DMEM to Neurobasal medium supplemented with B-27 supplement and heparin-binding EGF (Wolfes et al., 2017).

We found astrocytes from the chicken forebrain were observed as isolated cells with stems and PAPs in serum-free Neurobasal medium containing 2% B-27 supplement and 5 ng/mL bFGF in long-term culture of forebrain neuron. We confirmed that almost cells from forebrain of a 15- to 16-day-old chicken embryo were astrocytes and the cells proliferated steadily in various cell densities in Neurobasal medium (Fig. 1A). The cells formed stems and PAPs both in primary culture and in subculture (Fig. 3A and 3B) and showed higher GFAP expression rates than astrocytes in DMEM in 8-day culture (Fig. 6). The medium composition was developed by manufacture (LONZA) from the medium for chicken tectal neurons, which was originally reported by Goldsbury et al. (2008) without growth factors, and in which astrocyte generation had not occurred; however, its composition is similar to the medium for rat astrocytes reported by Kang et al. (2014).

We demonstrated that chicken astrocytes cultured in DMEM at a high density (1.6×10^5 cells/cm^2) which had cell-cell contact from the initial day of culture reached confluent, but astrocytes in DMEM at a low density (0.4×10^5 cells/cm^2) did not proliferate (Fig. 1A). Astrocytes in DMEM at a low density proliferated in colonies (Fig. 1C) and astrocytes with stems were observed in the colonies (Fig. 2A), thereby suggesting that proliferation and differentiation of chicken astrocytes in serum-containing medium requires self-secretion of growth factors. Subcultured astrocytes from well-differentiated cells caused dedifferentiation supported the idea (Fig. 2B). We also observed chicken astrocytes cultured in DMEM contained more cells than Neurobasal medium in the same coverage area because packed colonies with many nuclei were often observed (Fig. 1B and C) and small numbers of polygonal cells sometimes contaminated in cultures (Supplementary Figure S4). Lower correlation between area and numbers of cells in DMEM at a high density may affected by packed colonies or contaminated cells. Small proportions of oligodendrocytes, neurons, ependymal cells, fibroblasts, endothelial cells or microglial cells may be present in astrocyte cultures (reviewed by Saura, 2007), and DMEM supplemented with FBS was suitable not only for astrocytes but for other types of cells. The main types of contaminating cells of chicken astrocytes in Neurobasal medium were neurons and polygonal cells were scarcely observed, thus, the medium condition would be suitable for proliferation and differentiation of astrocytes.

It has been proposed that bFGF derived from glia may promote neuronal survival and neurite extension and astrocyte morphology is controlled by neuron-derived FGF (Agarwal and Bergles, 2014). In fact, mouse cortical astrocytes formed well-branched stems in glia-neuron mix culture at a high density and morphology of astrocytes in cortical organotypic slice cultures is similar to their appearance in vivo, but isolated astrocytes were fibroblast-like structure (Schweinhuber et al., 2015). We analyzed the proportion of neurons to evaluate interactions between astrocytes and remaining neurons (Supplementary Figure S3). Only a small number of contaminated neurons were detected in primary culture (Supplementary Figure S3) and cryopreserved astrocytes that hardly contained any neurons differentiated as

primary astrocytes (Fig. 5), therefore, effects of serum-free Neurobasal medium on proliferation and differentiation of astrocytes are thought to be affected by medium composition.

We also confirmed that the cryopreserved astrocytes differentiated in the same manner as the freshly harvested astrocytes, and their differentiation was faster than that of the freshly harvested astrocytes (Figs. 5 and 6). Neural extracellular matrix (ECM) was rich in the brain at the late developing stage (Heikkinen et al., 2014), and it deters immunostaining. Cryopreserved astrocytes resolved this issue and differentiated faster than freshly prepared astrocytes (Figs. 5C and 6), which is thought to be attributable to their having differentiated partially before freezing. Cryopreserved rat astrocytes are commonly used as feeder layer for preparing low-density dissociated-cell cultures of hippocampal neurons (Kaech and Banker, 2006), but the cells are maintained at a high density and support neurons not with attaching but with secreting growth factors.

This is the first report of morphology of chicken astrocytes, so we analyzed expression levels of GFAP, tubulin, and two actin-binding proteins (lasp-1 and lasp-2) in astrocytes cultured in DMEM and Neurobasal medium to determine any cytoskeletal protein would affect their morphology (Fig. 4). Lasp-1 is ubiquitously expressed in non-muscle tissues and lasp-2 is highly expressed in brain (Terasaki et al., 2004), so we predicted that these proteins would be co-expressed in astrocytes. As we expected, anti-lasp-1 antibody and anti-lasp-2 antibody reacted with astrocyte samples, indicating high reactivity of anti-lasp-2 antibody against brain tissue is derived from both neurons and astrocytes. However, protein expression levels of these actin-binding proteins and other two cytoskeletal proteins analyzed in this study showed similar expression patterns in DMEM and Neurobasal medium though they had different morphologies (Fig. 4A). Semi-quantitative PCR also showed that mRNA expression levels of lasp-2 were also similar (Fig. 4B). It was reported that mammalian astrocytes adopt a stellate, process-bearing morphology with treatments of lysophosphatidic acid, a mitogen that signals through its cognate G protein-coupled receptor, or forskolin that is commonly used as a tool to raise levels of cyclic AMP in cells (Hotulainen and Lappalainen, 2006). These data suggested that activity of cytoskeletal proteins affected by complex signal transduction mechanism altered cell shape. Comparison of phosphorylation status of cytoskeletal proteins of chicken astrocytes in serum-containing medium and serum-free medium would yield much information on the molecular mechanisms determining astrocyte shape. The idea that mammalian astrocytes are not uniform cells and perform different functions in vivo was reported from morphological differences and reactivity of the antibody (Ben Haim and Rowitch, 2016; Matyash and Kettenmann, 2010). Further analysis of chicken astrocytes is needed to reveal whether they should be classified similarly to mammalian astrocytes.

In this study, we reported culture method of isolated avian astrocytes, which have similar structure to astrocytes in vivo. Researchers will be able to obtain astrocytes easily with incubation of eggs (without keeping and mating mouse or rats) and cryopreserved astrocytes are also suitable for experiments. In mammalian astrocytes, many protocols (immunostaining, transfections of fluorescent protein vectors, siRNA, visualization of membrane traffic, analysis of Ca^{2+} signaling, morphology / motility assay) have been established and should be readily applicable to avian astrocytes. We have been investigating function of lasp-2, an actin-binding protein identified from chicken brain (Terasaki et al., 2004), and confirmed lasp-2 is highly expressing in avian astrocytes. Thus, avian astrocytes can serve as a useful tool for studying how lasp-2 regulates astrocyte morphology and motility.

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COMPETING INTERESTS

The authors have no competing interests to declare.

AUTHOR CONTRIBUTIONS

TS, TA, KM, FY, NS and TK performed the culture experiments. NS and AN performed the immunoblotting and the semi-quantitative PCR experiments. HN assisted with microscopic observation. AT conceived and designed the study, performed the culture experiments, and wrote the manuscript.

SUPPLEMENTARY MATERIALS

Supplementary materials for this article are available online. (URL: https://bioone.org/journals/supplementalcontent/10.2108/zs180102/10.2108.zsj.36.458.s1.pdf).

Supplementary Figure S1. Coverage indices in the present study.

Supplementary Figure S2. Staining of F-actin, glial fibrillary acidic protein (GFAP), and the nuclei in chick cultured astrocytes.

Supplementary Figure S3. Contaminated Neurons in chick primary cultured astrocytes.

Supplementary Figure S4. Non-glial cells in primary cultured astrocytes.

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