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Study on stiffness of culture substrate for astrocytic  
differentiation of mouse embryonic neural stem cells  
(胎生期マウス神経幹細胞のアストロサイト分化に作  
用する培養基盤の硬さに関する研究)

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# Abbreviations

ALS: amyotrophic lateral sclerosis  
BMP: bone morphogenetic protein  
BSA: bovine serum albumin  
DMSO: dimethyl sulfoxide  
GAPDH: glyceraldehyde 3-phosphate dehydrogenase  
GFAP: glial fibrillary acidic protein  
HRP: horseradish peroxidase  
iPS: induced pluripotent stem  
MRLC: myosin regulatory light chain  
NSCs: neural stem cells  
PBS: phosphate-buffered saline  
PP-MRLC: di-phosphorylated myosin regulatory light chain  
qPCR: quantitative polymerase chain reaction  
Rho A: Ras homolog family member A  
RNA: ribonucleic acid  
ROCK: Rho-associated kinase  
SCI: spinal cord injury  
SDS: sodium dodecyl sulfate  
STAT3: signal transducer and activator of transcription 3  
S100B: S100 calcium binding protein  
Tuj1: neuron-specific class III beta-tubulin

# Chapter 1

## General Introduction

### **1-1 Differentiation to astrocyte from neural stem cells**

Neurons and glia are differentiated from NSCs. Astrocyte, a type of glia is expected to be applicable in regenerative medicine for neurodegenerative diseases, such as spinal cord injury and amyotrophic lateral sclerosis [1-4]. Astrocytes can be obtained from NSCs by passaging NSCs repeatedly or by adding serum and/or recombinant proteins in culture medium [5-8]. On the other hand, these previously known methods are not ideal for regenerative medicine applications. For example, passaging NSCs or the repeated use of recombinant proteins is time-consuming and costly. Moreover, many undefined proteins, including unknown pathogens are contained in serum [9]. Therefore, fast and low-cost methods for differentiating astrocytes from NSCs under pathogen-free conditions are in great demand.

### **1-2 Two types of NSCs**

We can obtain two types of NSCs from the mammalian brain. One is embryonic NSCs isolated from the ganglionic eminence of the embryonic brain [10], and the other is adult NSCs, isolated from the subventricular zone of the lateral ventricles and the subgranular layer of the hippocampus of the adult brain [11].

### **1-3 Astrocytic differentiation from human NSCs**

Human NSCs are also existed in the brain. On the other hand, these human NSCs cannot be isolated from the brain by surgery for regenerative medicine. Alternatively, human NSCs can be obtained by differentiating human iPS cells into NSCs. However, NSCs induced from the human iPS cells have embryonic characteristics and astrocytic

differentiation from human iPS cells is difficult. Over 10 passages/80 days of human NSCs are required to prepare for differentiation and 14 days of prepared NSCs to differentiate into human astrocytes in the serum contained culture medium [12]. Therefore, faster and easier methods for differentiating embryonic and neurogenic NSCs into astrocytes are strongly required for regenerative medicine for neurodegenerative diseases, such as ALS and SCI.

#### **1-4 Influence of culture substrate stiffness to differentiation of NSCs**

Culture substrate stiffness influence differentiation of various types of cells strongly [13-17]. It has been shown that the differentiation of adult NSCs is affected by stiffness of culture substrates [18-20]. Adult NSCs tend to differentiate into astrocytes on stiff culture substrates, whereas they easily differentiate into neurons on soft culture substrates regardless of the presence or absence of serum. On stiff substrates, embryonic NSCs preferably differentiate into astrocytes in a culture medium containing serum, whereas in serum-free media, they easily differentiate to neurons [21]. However, there are no reports that the differentiation of embryonic NSCs is regulated by soft substrate.

#### **1-5 MRLC regulated by substrate stiffness**

Actomyosin is a cytoskeletal complex that generates mechanical contractile force in cells. It has been reported that actomyosin is involved in the regulation of cell fate and differentiation [22-23]. PP-MRLC upregulate actomyosin contractility and is increased in the cells on stiff substrates, and as a result, the cellular contractile force is enhanced [24-25]. Meanwhile, MRLC dephosphorylation loosen cellular contractile force in cells on soft substrates. It is known

that the activation of the RhoA signaling cascade enhance Di-phosphorylation of MRLC [26]. ROCK is a key protein in the RhoA signaling cascade. It was shown that the ROCK inhibitor prevents the phosphorylation of MRLC in various cells and alters the morphology of the cells on stiff substrates [24,27-30]. On the other hand, whether PP-MRLC in NSCs is regulated by substrate stiffness, and whether it directs the differentiation of NSCs is not well understood.

#### **1-6 Aim of this study**

In this report, we examined whether astrocytic differentiation of embryonic NSCs was influenced by substrate stiffness in the absence of serum.

# **Chapter 2**

## **Materials and Methods**

## **2-1 Preparing culture plates**

In this report, we used the commonly used polystyrene culture plates (3815-012; Iwaki, Tokyo, Japan, or 3513; Corning, NY, USA) and Softwell culture plates (Matrigen, CA, USA) with stiffness of 1 kPa (SW12-EC-1 EA) and 12 kPa (SW12-EC-12 EA) for culturing cells on substrates with various stiffnesses. We measured the stiffness of the polystyrene culture plate and Softwell at 1 kPa and 12 kPa under the conditions used in this report using atomic force microscopy (Nano Wizard 4; JPK, Berlin, Germany). The stiffness of the polystyrene culture plate was 2.8 GPa (SD: 0.85 GPa), and that of plates with 1 kPa and 12 kPa was 0.560 kPa (SD: 0.146 kPa) and 15.697 kPa (SD: 2.390 kPa), respectively. We coated culture plates with poly-D-lysine, laminin, and fibronectin before seeding the cells. To coat these molecules, we placed aliquots of 0.5 mL of 20  $\mu\text{g}/\text{mL}$  poly-D-lysine (Corning) in sterile PBS in the wells of the culture plates. Then, we incubated the plate overnight at 37°C. The next day, we rinsed the plates twice with sterile PBS and incubated with 0.5 mL of 10  $\mu\text{g}/\text{mL}$  mouse laminin (Thermo Fisher Scientific, MA, USA) and 0.5  $\mu\text{g}/\text{mL}$  human fibronectin (Thermo Fisher Scientific) in sterile PBS for at least 3 h at 37°C. We rinsed the plates twice with sterile PBS prior to use.

## **2-2 Isolating and culturing embryonic mouse NSCs**

We obtained embryonic mouse NSCs via the modification of a previously described method [46]. Briefly, we purchased pregnant female C57BL/6J mice from CLEA Japan and dissected after cervical spine dislocation with isoflurane anesthesia (871129; Pfizer, NY, USA). We took out E11.5 mice from these mice and dissected the forebrains of the E11.5 mice and dispersed mechanically. We cultured aliquots of 50,000 cells/mL mouse NSCs in

KBM neural stem cell medium (KOHJIN BIO, Saitama, Japan) in ultra-low attachment culture flasks (3814; Corning). We added the medium twice daily. We passaged the cells into new flasks 3 days after seeding. Four to six days after seeding, we rinsed the neurospheres that had formed in sterile PBS and dispersed with Accutase (Innovative Cell Technologies, CA, USA) at 37°C for 5 min. We used the dispersed NSCs for subsequent experiments. We used 36 of pregnant female C57BL/6J mice in this report for obtaining adequate number of NSCs needed for our experiments. Shionogi's Institutional Animal Care and Use Committee approved animal care and experimental procedures. We performed animal care and experimental procedures in accordance with guidelines provided by Shionogi's Institutional Animal Care and Use Committee. We carried out the study in accordance with the ARRIVE guidelines (<https://arriveguidelines.org>).

### **2-3 Differentiation of NSCs**

After washing with sterile PBS, we dissociated the NSCs into single cells with Accutase. Before seeding the cells on poly-D-lysine, laminin, and fibronectin-coated 12-well plates, we filled the wells with 500  $\mu$ L of Neurobasal plus B27 medium (Thermo Fisher Scientific) with 0.5% DMSO, 500  $\mu$ L of 20  $\mu$ M Y27632 (Wako, Osaka, Japan) in Neurobasal plus B27 medium with 0.5% DMSO, or 50  $\mu$ M blebbistatin (Sigma Aldrich, MO, USA) in Neurobasal plus B27 medium with 0.5% DMSO, or 0.2 nM Calyculin A (Wako) in Neurobasal plus B27 medium with 0.5% DMSO. Then, we added 500,000 cells in 500  $\mu$ L of Neurobasal plus B27 medium to each well. We changed the medium to 500  $\mu$ L of Neurobasal plus B27 medium with 0.25% DMSO, 500  $\mu$ L of 10  $\mu$ M Y27632 in Neurobasal plus B27 medium with 0.25% DMSO, or 500  $\mu$ L of 25  $\mu$ M blebbistatin in Neurobasal plus B27 medium with 0.25%

DMSO, or 500  $\mu$ L of 0.1 nM Calyculin A in Neurobasal plus B27 medium with 0.5% DMSO of daily. After 3 days of culture, we analyzed the cells.

#### **2-4 Immunocytochemical staining**

We fixed Cells with 4% paraformaldehyde in PBS for 15 min at room temperature. We permeabilized the cells with 0.2% Triton-X100 in PBS with 1% normal goat serum (Vector Laboratories, CA, USA) for 60 min at room temperature and incubated with primary antibodies in PBS containing 1% normal goat serum at 4°C overnight. After washing the primary antibodies with PBS, we incubated the cells with secondary antibodies conjugated with Alexa Fluor® 488 anti-mouse IgG (1:500; Invitrogen, CA, USA) in PBS or Alexa Fluor® 555 anti-rabbit IgG (1:500; Invitrogen) in PBS for 60 min at room temperature. We stained the nuclei with Hoechst 33423 (1:10,000; Invitrogen) for 15 min at room temperature. We used the commercial antibodies used for immunostaining as follows: Dako for GFAP (rabbit polyclonal/M0761/1:2,000) and Covance for Tuj1 (mouse monoclonal/MMS-435P/1:500). We observed stained cells and imaged using a fluorescent microscope with a 20x objective (BZ-9000; Keyence, Osaka, Japan). For the quantification of immunostaining, we analyzed four fields per well, and calculated the average number of cells for three wells in three independent experiments (animal preparation). To calculate the population of astrocytes or neurons, we excluded strong Hoechst 33423-positive cells with smaller nuclei (dead cells) from the analysis. Then, the number of cells with Hoechst 33423 positive nuclei inside the GFAP-positive area or Tuj1 positive area were counted.

## **2-5 Western blotting**

For the detection of GFAP, Tuj1, GAPDH and beta actin, we lysed the cells in radioimmunoprecipitation assay buffer (RIPA buffer, Sigma Aldrich) with protease inhibitors (Roche Life Science, Penzberg, Germany) and incubated for 3 min at 95°C with the addition of sample buffer solution (Nacalai Tesque, Kyoto, Japan). We separated proteins in the cell lysates by SDS polyacrylamide gel electrophoresis with 5–20% polyacrylamide gels (e-PAGEL; ATTO, Tokyo, Japan) using Tris-glycine-SDS running buffer (Thermo Fisher Scientific) and transferred onto polyvinylidene fluoride membranes (iBlot gel transfer PVDF; Invitrogen). We blocked the membranes with 5% skim milk (Nacalai Tesque) in Tris-buffered saline-Tween 20 (TBS-T, Cell Signaling, MA, USA) for 60 min at room temperature and incubated with 1:1,000 anti-GAPDH (14C10) rabbit monoclonal antibody (Cell Signaling) in 1% bovine serum albumin (BSA)/TBS-T or 1:1,000 anti-beta actin (13E5) rabbit monoclonal antibody (Cell Signaling) in 1% BSA/TBS-T at 4°C overnight. After washing with TBS-T, we incubated the membranes with 1:2,000 anti-rabbit IgG HRP linked antibody (Cell Signaling) in 1% BSA/TBS-T for 60 minutes at room temperature. We induced signal emission using ECL Prime Western Blotting Detection Reagent (GE Healthcare, IL, USA). We performed imaging of ECL blots and densitometric analysis using LAS 3000 luminoimage analyzer (FUJIFILM, Tokyo, Japan). After detection, we detached antibodies on the membrane by incubation with stripping solution (Wako) for 120 min at 37°C and incubated with 1:2,000 anti-GFAP (D1F4Q) rabbit monoclonal antibody (Cell Signaling) in 1% BSA/TBS-T or anti-Tuj1 (D71G9) rabbit monoclonal antibody (Cell Signaling) in 1% BSA/TBS-T at 4°C overnight. After washing with TBS-T, we incubated the membranes with 1:2,000 anti-rabbit IgG HRP-linked antibody in 1% BSA/TBS-T for 60 min at room temperature. We detected signal emission in the same manner as described above.

For the detection of PP-MRLC and total MRLC, we fixed the cells in cold 10% trichloroacetic acid (Sigma Aldrich) in PBS for 5 min on ice. We washed the cells three times with cold PBS for 3 min on ice and then lysed in SDS buffer (0.125 M Tris-HCl, 0.2 M dithiothreitol, 4% SDS, 20% glycerol, and 0.01% bromophenol blue, pH 6.8). We subjected the cell lysates to ultrasonic fragmentation and incubated at 95°C for 5 min. We separated proteins in the cell lysates using 10% SDS-polyacrylamide gels and then transferred onto polyvinylidene fluoride membranes. We prepared the membranes separately for total MRLC and PP-MRLC detection because of the same molecular weight (18 kDa) between them. We incubated the membranes with the appropriate primary antibody (rabbit monoclonal/3674/1:1,000 for PP-MRLC, or rabbit monoclonal/3672/1:1,000 for total MRLC; Cell Signaling) in Can Get Signal Solution A at 4°C overnight. Then, we washed membranes three times with TBS-T (10 mM Tris-HCl containing 150 mM NaCl and 0.05% Tween 20, pH 7.5) and incubated with the appropriate secondary antibody (HRP anti-rabbit IgG: 1:10,000 for PP-MRLC, HRP anti-rabbit IgG: 1:5,000 for total MRLC; Cell Signaling) in Can Get Signal Solution B for 1 h at room temperature. We detected protein signals using an Immobilon Western Chemiluminescent HRP substrate (EMD Millipore, MA, USA). MRLC with GAPDH or PP-MRLC with GAPDH on the same membrane were measured, and we used GAPDH-normalized data in the study.

For the quantification of western blots, 500,000 cells/well in 12 well plates (131,579 cells/cm<sup>2</sup>) were seeded and we extracted protein from three wells and collected them into one after 3 days of culture to analyze the expression level of protein in an experiment and calculated the average expression level with GAPDH normalized in three independent experiments (animal preparation).

## 2-6 Real-time quantitative polymerase chain reaction

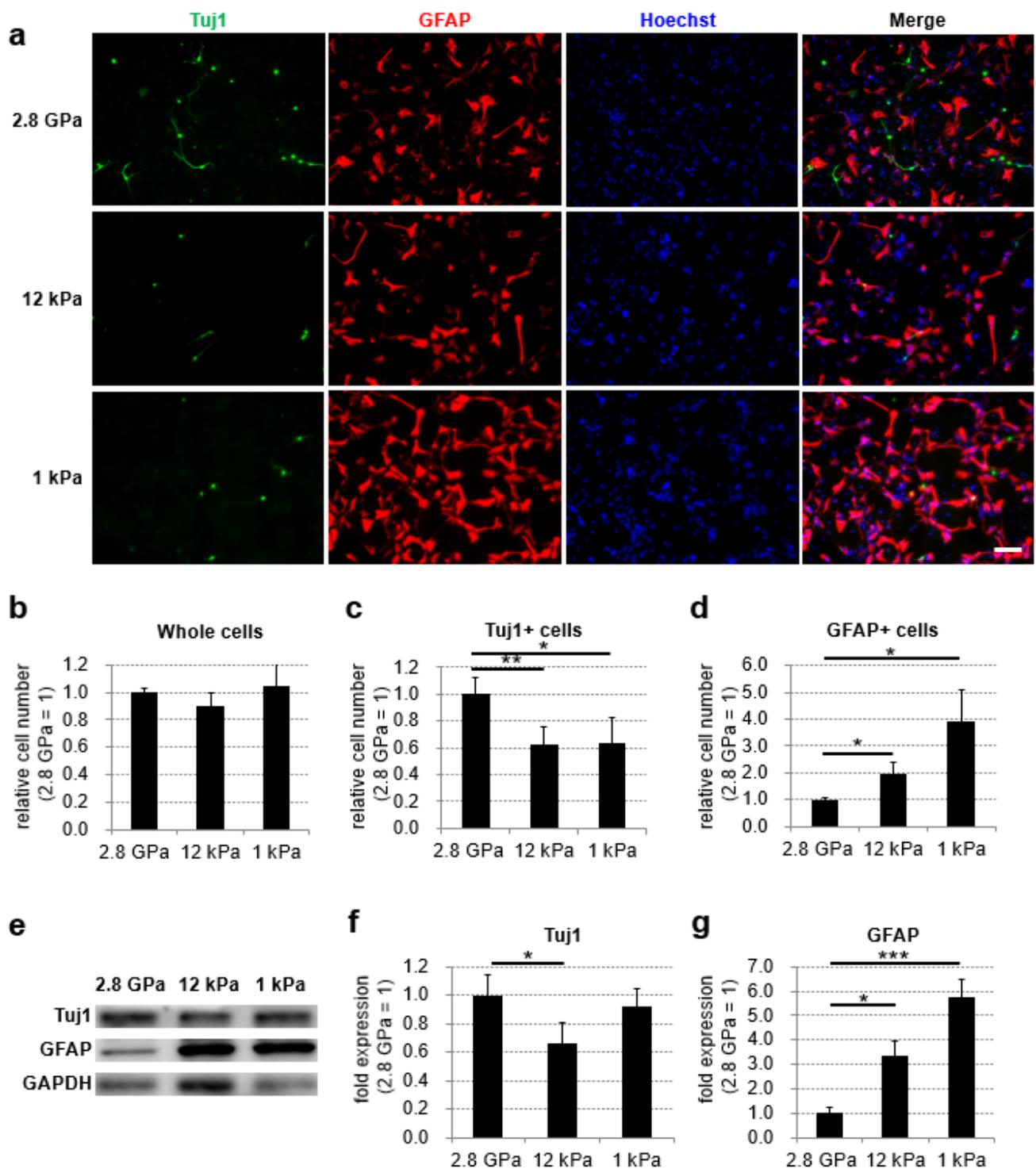
We prepared total RNA from cells cultured under various conditions using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. We carried reverse transcription out using 1 µg of total RNA in a final volume of 1000 µL using SuperScript III First-Strand Synthesis SuperMix (Life Technologies) according to the manufacturer's instructions. We performed real-time qPCR with the 7500 Real-Time PCR System using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). We used the following primers and purchased from Takara Bio Inc. (Shiga, Japan): GFAP (forward: 5'-GACCAGCTTACGGCCAACAG-3', reverse: 5'-TCTATACGCAGCCAGGTTGTTCTC-3'), S100B (forward: 5'-ATCAACAACGAGCTCTCTCACTTCC-3', reverse: 5'-TCGTCCAGCGTCTCCATCAC-3'), Nestin (forward: 5'-GAGGTGTCAAGGTCCAGGATGTC-3', reverse: 5'-GACACCGTCTCTAGGGCAGTTACAA-3'), and GAPDH (forward: 5'-TGTGTCCGTCGTGGATCTGA-3', reverse: 5'-TTGCTGTTGAAGTCGCAGGAG-3').

# Chapter 3

## Results

### **3-1 Soft culture substrates promote the astrocytic differentiation of embryonic NSCs in the absence of serum**

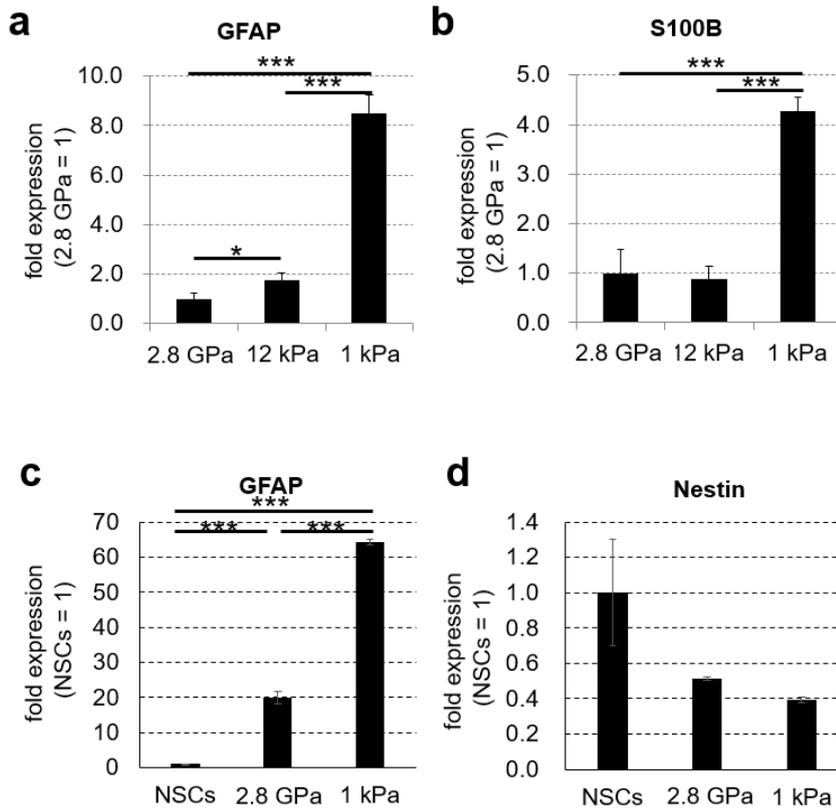
To examine the influence of culture substrate stiffness on the differentiation of embryonic NSCs into astrocytes in the absence of serum, we used neurogenic NSCs from embryonic day (E) 11.5 mouse embryonic forebrain for this report. We cultured these NSCs on three types of plate with different stiffness (1 kPa plates, 12 kPa plates, and commonly used stiff plastic plates [2.8 GPa]) for 3 days with a serum-free medium supplemented with B27. Immunostaining revealed that the number of whole cells was not significantly different among the cells on these plates, whereas the number of neuronal marker Tuj1 positive cells was increased significantly in the cells on the stiff substrates compared with the cells on the soft substrates (Figs. 1a, 1b, and 1c). Moreover, the number of GFAP positive cells was significantly decreased in the cells on the stiff substrate plates compared with the soft substrates (Figs. 1a and 1d). Western blotting also showed that the protein expression of Tuj1 was lower in the cells on substrates at 12 kPa than in the cells on a plastic plate (Figs. 1e and 1f). On the other hand, GFAP protein expression in the cells on stiff substrates was lower than that on soft substrates (Figs. 1e, 1g and Appendix 1). Furthermore, qPCR analysis showed that the mRNA expression of GFAP and mature astrocyte marker S100B was lower in the cells on stiff substrates than those on soft substrates (Figs. 2a, 2b, and 2c). In addition, the mRNA expression of a marker of undifferentiated NSCs decreased after seeding on each plate (Fig. 2d). These results indicate that soft culture substrates inhibit the neuronal differentiation of embryonic NSCs and promote the astrocytic differentiation of NSCs in the absence of serum.



**Fig. 1. Soft substrates increase astrocytic differentiation from mouse embryonic NSCs in the absence of serum**

(a) We cultured mouse embryonic NSCs on three types of plate (1 kPa plates, 12 kPa plates, and commonly used plastic plates [2.8 GPa]) for 3 days in serum-free condition and assessed by immunofluorescence staining. We

visualized neurons by Tuj1 (green), astrocytes by GFAP (red), and counterstained cell nuclei with Hoechst (blue). Scale bar, 100  $\mu\text{m}$ . **(b)** We counted whole cell number, **(c)** the number of Tuj1 positive cells, and **(d)** the number of GFAP positive cells on each plate and normalized relative to the control. **(e)** We detected protein expression of Tuj1, GFAP, and GAPDH (for loading control) on each plate by western blotting analysis. We normalized the expression levels of **(f)** Tuj1 and **(g)** GFAP proteins relative to GAPDH protein. Error bars represent the standard deviation; \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.005$  (Student's  $t$  test with Bonferroni correction).

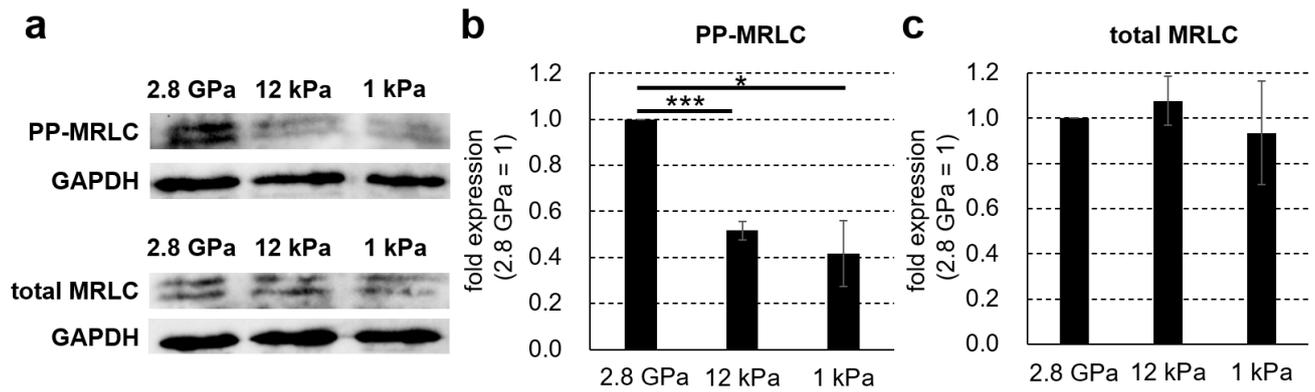


**Fig. 2. Soft substrates decrease mRNA expression of NSC marker and increase mRNA expression of astrocytes marker after 3 days of differentiation of mouse embryonic NSCs in the absence of serum**

We cultured mouse embryonic NSCs on three types of plates (1 kPa plates, 12 kPa plates, and commonly used plastic plates [2.8 GPa]) for 3 days in serum-free conditions. We detected the mRNA expression of GFAP, S100B, and GAPDH on each plate type by qPCR analysis. We normalized the expression levels of (a) GFAP and (b) S100B relative to that of GAPDH. We cultured mouse embryonic NSCs on two types of plates (1 kPa plates and commonly used plastic plates [2.8 GPa]) for 24 hours in serum-free conditions. We detected the mRNA expression of GFAP, Nestin, and GAPDH on each plate type and pre-seeding NSCs by qPCR analysis. We normalized the expression levels of (c) GFAP and (d) Nestin relative to that of GAPDH. Error bars represent the standard deviation; \* $p < 0.05$ , \*\*\* $p < 0.005$  (Student's  $t$  test with Bonferroni correction).

### **3-2 PP-MRLC is decreased in NSCs on soft substrates**

It has been shown that mechanical stress in the cells is generated by actomyosin contractility, and as a result, cell fate decision and differentiation are regulated [22-23]. PP-MRLC enhance mechanical stress generated by actomyosin contractility. We showed that stiff substrates increased PP-MRLC, which enhanced cellular contractile force [24-25]. Therefore, we examined whether substrate stiffness regulates the expression of PP-MRLC in NSCs. We cultured mouse embryonic NSCs for 3 days on plates with different stiffness (1 kPa plates, 12 kPa plates, and commonly used stiff plastic plates [2.8 GPa]) in serum-free conditions and examined the expression of total MRLC and PP-MRLC. Western blotting showed that PP-MRLC was significantly increased in cells on the stiff substrates compared with the cells on the soft substrates, although there was no significant difference in the expression of total MRLC among the cells on these plates (Figs. 3a, 3b, and 3c). These results indicate that the expression of PP-MRLC was reduced in NSCs on soft substrate.

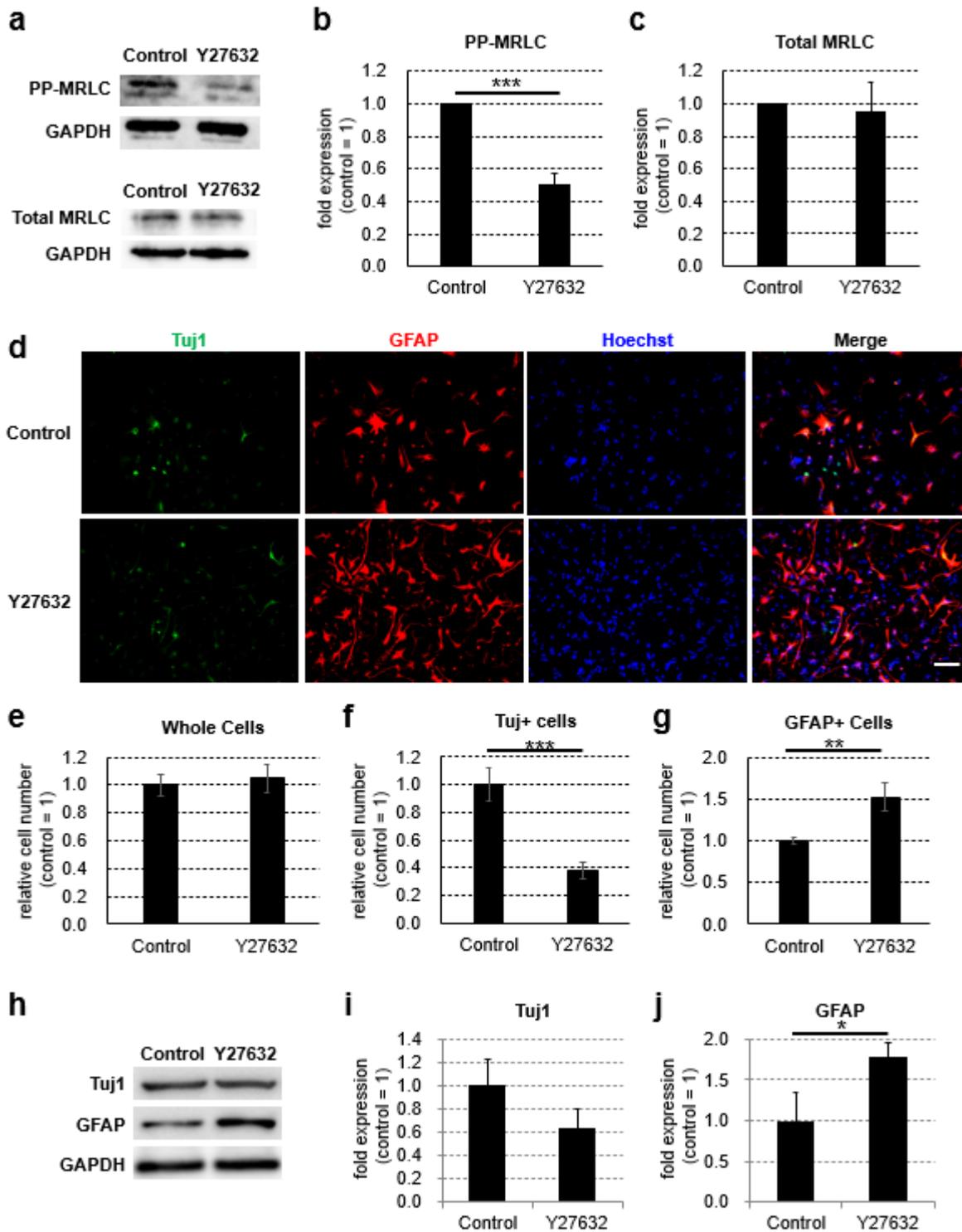


**Fig. 3. PP-MRLC is decreased in mouse embryonic NSCs on soft substrates in the absence of serum**

(a) We cultured mouse embryonic NSCs on three types of plate (1 kPa plates, 12 kPa plates, and commonly used plastic plates [2.8 GPa]) for 3 days in serum-free condition. We detected protein expression of PP-MRLC, total MRLC, and GAPDH (for loading control) on each plate type by western blotting analysis. We normalized the expression levels of (b) PP-MRLC and (c) total MRLC proteins relative to that of GAPDH protein. Error bars represent the standard deviation; \* $p < 0.05$ , \*\*\* $p < 0.005$  (Student's  $t$  test with Bonferroni correction).

### **3-3 PP-MRLC in NSCs is reduced by a Rock inhibitor**

It has been shown that Inhibition of ROCK activity reduce the amount of PP-MRLC in various cells [24, 27-30]. On the other hand, there are no reports that a ROCK inhibitor inhibits the PP-MRLC in NSCs. Therefore, we examined whether PP-MRLC expression in NSCs was decreased by treatment with a ROCK inhibitor. We cultured mouse embryonic NSCs for 3 days with 10  $\mu$ M Y27632, a common ROCK inhibitor, on plastic plates in serum-free conditions and examined the expressions of PP-MRLC and total MRLC. The results of western blotting revealed that total MRLC was not significantly different between untreated and treated cells with Y27632, whereas the expression of PP-MRLC in NSCs was significantly reduced by treatment with Y27632 (Figs. 4a, 4b, and 4c). These results indicated that PP-MRLC in NSCs is reduced by treatment with a ROCK inhibitor.



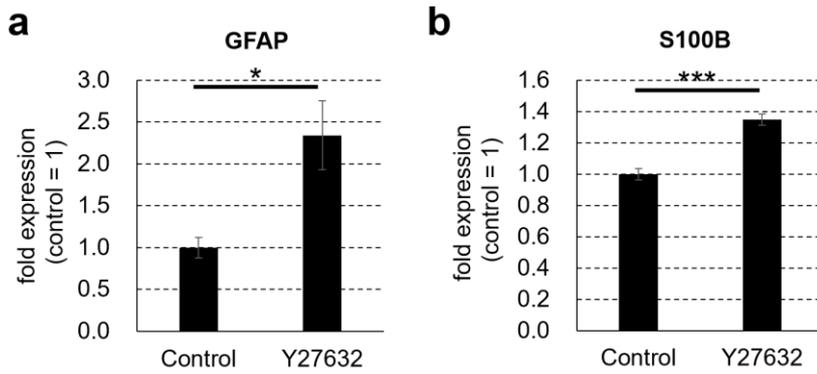
**Fig. 4. Treatment of ROCK inhibitor decreases PP-MRLC and increases astrocytic differentiation of mouse embryonic NSCs in the absence of serum**

(a) We cultured mouse embryonic NSCs on commonly used plastic plates for 3 days without (control) or with 10  $\mu$ M

Y27632 (Y27632) in serum-free condition. We detected protein expression of PP-MRLC, total MRLC, and GAPDH (for loading control) by western blotting analysis. We normalized the expression levels of **(b)** PP-MRLC and **(c)** total MRLC proteins relative to GAPDH protein. **(d)** We assessed differentiated cells by immunofluorescence staining. We visualized neurons by Tuj1 (green), astrocytes by GFAP (red), and counterstained cell nuclei with Hoechst (blue). Scale bar, 100  $\mu\text{m}$ . We counted **(e)** whole cell number, **(f)** the number of Tuj1 positive cells, and **(g)** the number of GFAP positive cells on each plate and normalized relative to the control. **(h)** We detected protein expression of Tuj1, GFAP, and GAPDH (for loading control) by western blotting analysis. We normalized the expression levels of **(i)** Tuj1 and **(j)** GFAP proteins relative to GAPDH protein. Error bars represent the standard deviation;  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.005$  (Student's *t* test).

### **3-4 Treatment with a ROCK inhibitor increases astrocytic differentiation of embryonic NSCs on stiff substrates in the absence of serum.**

To confirm whether the differentiation of embryonic NSCs is regulated by PP-MRLC, we added the ROCK inhibitor Y27632 in the culture media and analyzed the differentiation of the NSCs into neurons or astrocytes. Mouse embryonic NSCs were cultured for 3 days with or without 10  $\mu$ M Y27632 on plastic plates in serum-free conditions and compared the expression of Tuj1 and GFAP. Immunostaining revealed that the whole cell number was not significantly different between the cells. However, the number of Tuj1-positive cells were decreased and the number of GFAP-positive cells were increased significantly by the treatment with Y27632 (Figs. 4d, 4e, 4f, and 4g). In addition, western blotting revealed that the protein expression of Tuj1 was not significantly different between Y27632-treated NSCs and that in untreated cells (Figs. 4h and 4i). In contrast, GFAP protein expression was significantly higher in Y27632-treated NSCs than that in untreated cells (Figs. 4h, 4j and Appendix 2-3). In addition, qPCR analysis showed that the mRNA expression of GFAP and S100B was increased in Y27632-treated NSCs than in untreated cells (Figs. 5a and 5b). A myosin II inhibitor blebbistatin also significantly increased the expression of GFAP in NSCs (Appendix 4). Furthermore, calyculin A, which has been shown to increase PP-MRLC [31], decreased the GFAP expression in NSCs on soft substrates (Appendix 5). These results suggest that dephosphorylation of MRLC by ROCK inhibition increases the astrocytic differentiation of embryonic NSCs.



**Fig. 5. Treatment with ROCK inhibitor increases mRNA expression of astrocyte markers after 3 days of differentiation of mouse embryonic neural stem cells in the absence of serum**

(a) We cultured mouse embryonic NSCs on commonly used plastic plates for 3 days without (control) or with 10  $\mu$ M Y27632 (Y27632) in serum-free condition. We detected mRNA expression of GFAP, S100B and GAPDH on each plate type by qPCR analysis. We normalized the expression levels of (a) GFAP and (b) S100B relative to that of GAPDH. Error bars represent the standard deviation; \* $p < 0.05$ , \*\*\* $p < 0.005$  (Student's  $t$  test).

# Chapter 4

## Summary and Remaining Questions

In this report, the results showed that soft culture substrates promoted the astrocytic differentiation of embryonic NSCs in serum-free conditions. On the other hand, embryonic NSCs preferably differentiate into neurons on stiff culture substrates in serum-free media. This is the first report to show that differentiation from embryonic NSCs into astrocyte is mediated by substrate stiffness. It is shown that adult NSCs tend to differentiate into neurons on soft culture substrates, but they easily differentiate to astrocytes on stiff culture substrates regardless of the presence or absence of serum [18-20]. Moreover, embryonic NSCs preferably differentiate to neurons in serum-free media on stiff culture substrates, but they easily differentiate to astrocytes in a culture medium containing serum [21]. Therefore, it is thought that the inconsistency of the differentiation tendency of NSCs induced by stiffness of culture substrates is due to the difference between adult NSCs and embryonic NSCs, not due to the absence or presence of serum. It is suggested that in soft culture substrates, embryonic neurogenic NSCs generate more astrocytes., whereas adult gliogenic NSCs generate more neurons.

In this study, we used GAPDH as a housekeeping gene in order to normalize expression. On the other hand, alterations in the elastic modulus of the substrate have been recently proposed to modulate GAPDH activity [32]. Therefore, it was also revealed that soft culture substrates or the ROCK inhibitor promoted the astrocytic differentiation of embryonic NSCs in the absence of serum by using beta-actin as a housekeeping protein (Appendix 1-2). Substrate stiffness could affect any housekeeping genes/proteins; therefore, we recommend the use of more than one housekeeping gene for relative quantification.

The motor activity of SCI mice model and the lifespan of ALS mice model were increased by transplantation of astrocytes to these animals [1-4]. Human iPS cells will be good sources of astrocytes for the treatment of ALS and

SCI. On the other hand, preparing an adequate amount of astrocytes from human iPS cells is time-consuming because over 94 days are required to differentiate human astrocytes from human NSCs owing to their embryonic neurogenic NSC features [12, 33-34]. To differentiate human astrocytes from human iPS cells, we needed to differentiate human NSCs from human iPS cells for 12 days and passage these NSCs for over 10 times, then differentiate human astrocytes from these passaged NSCs for 14 days in the serum contained culture medium [12]. In this study, we revealed that the astrocytic differentiation of mouse embryonic NSCs was induced only 3 days after seeding on soft substrates with serum-free media. These results suggest that this rapid method of preparing astrocytes by culturing embryonic NSCs on soft substrates without serum is better than the conventional methods and may be applicable to human iPS cells derived NSCs.

Cytokines belonging to the BMP family play important roles in the astrocytic differentiation of NSCs. It has been shown that activated STAT3/Smad1 under the signaling cascade of these cytokines forms a complex with the transcription coactivator p300, and consequently, induces transcriptional activation of astrocyte-specific genes [35-38]. In addition, BMP cascade signaling has been shown to be regulated by the stiffness of culture substrates [39]. In addition, previous studies have shown that BMP signaling reduces Nestin expression in NSCs and increases GFAP and S100B expression [8, 39-40]. In this report, we found that the expression of the mature astrocyte marker S100B mRNA was higher in the cells on soft substrates than on stiff substrates (Fig. 2a and 2b). However, qPCR analysis revealed that the mRNA expression of BMP2, BMP4, and BMP8b was lower in the cells on soft substrates than in the cells on stiff substrates (Appendix 6). Therefore, further studies are required to determine whether BMP cascade signaling is involved in soft-substrate-induced astrocyte differentiation.

Triple coating (poly-D-lysine, laminin, and fibronectin) was used in this study because this triple coating had the best cell adhesion on culture plates as a result of various examinations on coating. On the other hand, it has been shown that coating materials affect characteristics of neural stem precursor cells [41]. Moreover, it was reported that the presence or absence of differentiation-inducing factors, differences in the origin of NSCs, and the stiffness of the culture substrate affect astrocytic differentiation [18-20, 42-43]. In addition, in previous studies examining the effect of stiffness on differentiation of NSCs, a relatively soft range (1-30 kPa) was used, whereas a hard range (10-10000 kPa) was used in some other studies [18-19]. In this report, the effect of stiffness on differentiation of NSCs within a relatively wide range (1 kPa - 2.8 GPa) was examined. Since differences in the effect of stiffness on differentiation of NSCs have been reported even in a relatively narrow range, different results may be obtained if a narrower range of stiffness in our experimental system is examined. Therefore, further studies are required to determine the optimal method for astrocytic differentiation.

The results of this study revealed that PP-MRLC of NSCs was decreased by culturing on soft substrates or by treating them with the ROCK inhibitor or the myosin II inhibitor. A previous study showed that treatment with blebbistatin inhibited substrate stiffness-dependent differentiation of brain, muscle, and bone cells from mesenchymal stem cells [13]. Moreover, stiff substrates induced osteogenic differentiation of mesenchymal stem cells dependent on RhoA activity, which is critical for the contraction of actomyosin [45]. Therefore, it is possible that substrate stiffness regulates the differentiation of various stem cells, including NSCs by modulating cellular contractile forces generated by actomyosin.

The results of this study revealed that differentiation from embryonic NSCs into astrocytes was induced by

culturing them on soft substrates in the absence of serum. In addition, it was also found that astrocytic differentiation of NSCs on soft substrates was dependent on the dephosphorylation of MRLC. These results suggest that culturing embryonic NSCs on soft substrates or treating them with Y27632 or blebbistatin to reduce MRLC activity might be a suitable method to prepare astrocytes in regenerative medicine.

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## **Acknowledgments**

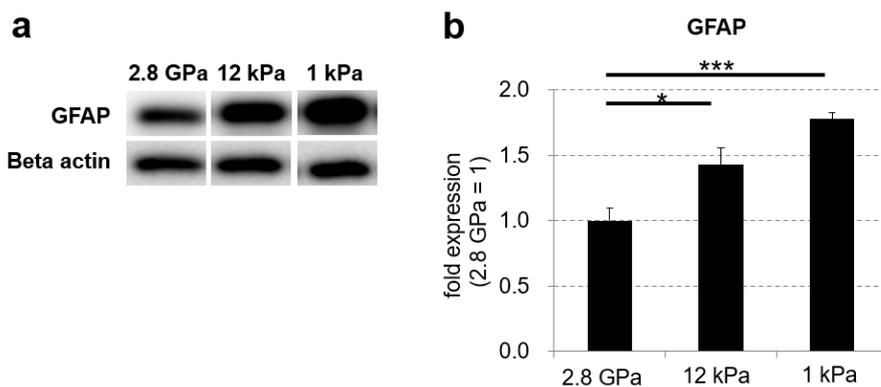
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## Appendix

### Appendix 1. Soft substrates increase GFAP expression after 3 days of differentiation of mouse embryonic

#### NSCs in the absence of serum

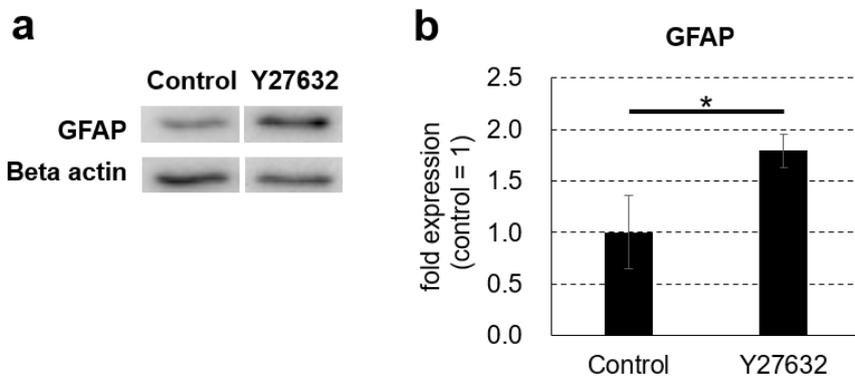
(a) We cultured Mouse embryonic NSCs on three types of plates (1 kPa plates, 12 kPa plates and commonly used plastic plates [2.8 GPa]) for 3 days in serum-free condition. We detected protein expression of GFAP, and beta actin (for loading control) on each plate by western blotting analysis. We normalized the expression levels of (b) GFAP proteins relative to beta actin protein. Error bars represent the standard deviation; \* $p < 0.05$ , \*\*\* $p < 0.005$  (Student's  $t$  test with Bonferroni correction).



## Appendix 2. ROCK inhibitor increases GFAP expression of differentiated cells from mouse embryonic NSCs

### in the absence of serum

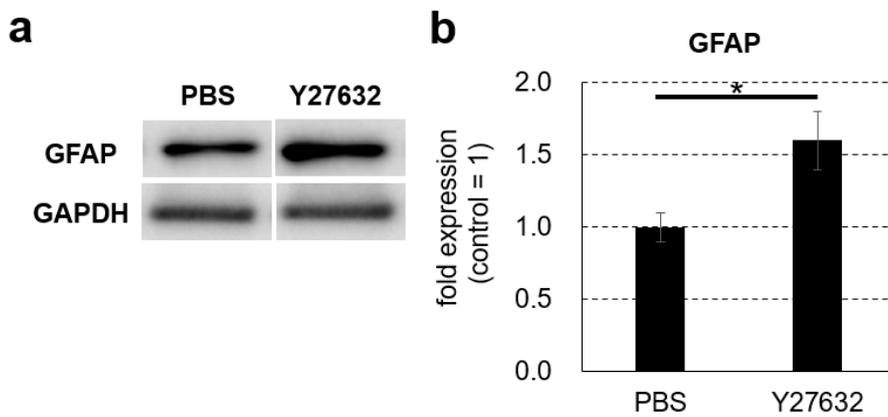
(a) We cultured mouse embryonic NSCs on commonly used plastic plates for 3 days without (control) or with 10 mM Y27632 (Y27632) in serum-free condition. We detected protein expression of GFAP, and beta actin (for loading control) by western blotting analysis. We normalized the expression levels of (b) GFAP proteins relative to beta actin protein. Error bars represent the standard deviation;  $*p < 0.05$  (Student's *t* test).



### Appendix 3. ROCK inhibitor increases GFAP expression of differentiated cells from mouse embryonic NSCs

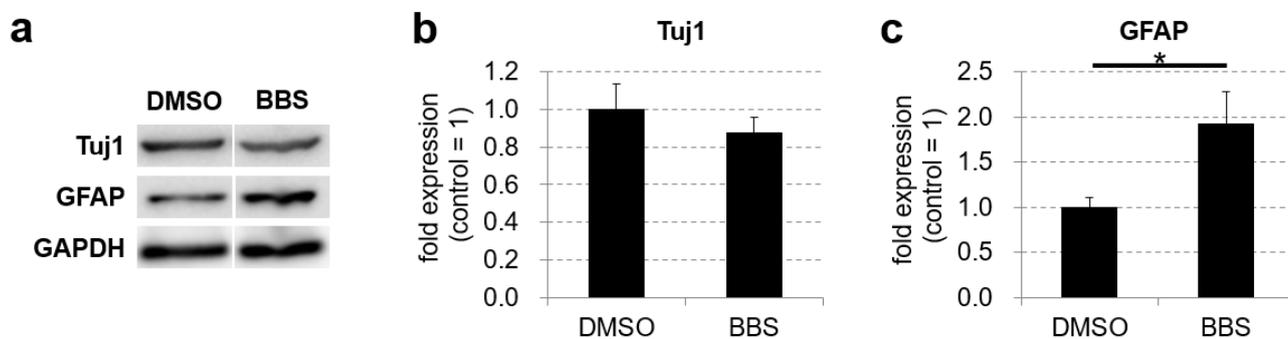
#### in the absence of serum

(a) We cultured mouse embryonic NSCs on commonly used plastic plates for 3 days without (PBS) or with 10 mM Y27632 (Y27632) in serum-free condition. We detected protein expression of GFAP, and GAPDH (for loading control) by western blotting analysis. We normalized the expression levels of (b) GFAP proteins relative to GAPDH protein. Error bars represent the standard deviation;  $*p < 0.05$  (Student's *t* test).



**Appendix 4. Blebbistatin increases the GFAP expression of differentiated cells from mouse embryonic NSCs in the absence of serum**

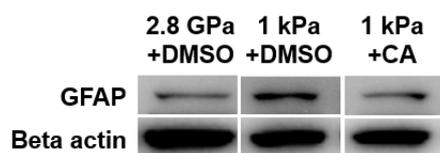
(a) We cultured mouse embryonic NSCs on commonly used plastic plates for 3 days without (DMSO) or with 25 mM blebbistatin (BBS) in serum-free condition. We detected protein expression levels of Tuj1, GFAP, and GAPDH (for loading control) by western blotting analysis. We normalized the expression levels of (b) Tuj1 and (c) GFAP proteins relative to GAPDH protein. Error bars represent the standard deviation;  $*p < 0.05$  (Student's *t* test).



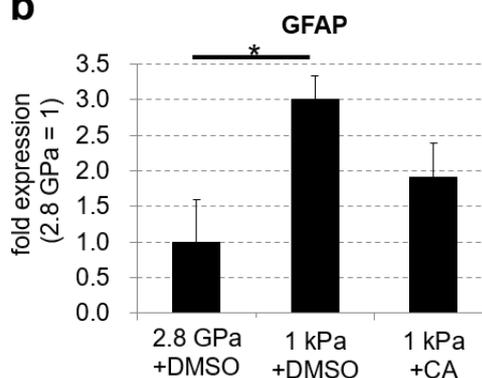
**Appendix 5. Calyculin A inhibits the increase of GFAP expression of differentiated cells from mouse embryonic NSCs on a soft substrate in the absence of serum**

(a) We cultured mouse embryonic NSCs on two types of plates (1 kPa plates and commonly used plastic plates [2.8 GPa]) for 3 days without (DMSO) or with 0.1 nM Calyculin A (CA) in serum-free condition. We detected protein expression of GFAP, and beta actin (for loading control) by western blotting analysis. We normalized the expression levels of (b) GFAP proteins relative to beta actin protein. Error bars represent the standard deviation;  $*p < 0.05$  (Student's *t* test with Bonferroni correction).

**a**



**b**



## Appendix 6. Soft substrates decrease expression of BMPs after 3 days of differentiation of mouse embryonic

### NSCs in the absence of serum

(a) We cultured mouse embryonic NSCs on three types of plates (1 kPa plates, 12 kPa plates, and the commonly used plastic plates [2.8 GPa]) for 3 days under serum-free condition. We detected the mRNA expression of BMP2, BMP4, BMP8b, and GAPDH on each plate type using qPCR analysis. We normalized the expression levels of (a) BMP2, (b) BMP4, and (c) BMP8b relative to GAPDH. Error bars represent the standard deviation;  $*p < 0.05$  (Student's *t* test with Bonferroni correction).

