表　　題　ラット下垂体前葉におけるフィブロモジュリン発現機構の解明

Fibromodulin expression in rat anterior pituitary gland

論文の区分　論文博士

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２０１７年０４月１５日申請の学位論文

紹介教員　人間生物学系　専攻　機能細胞学

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Contents

1. Introduction

Extracellular matrix (ECM) is a complex of collagens, laminins, proteoglycans, and other soluble proteins. They function as mechanical scaffold and physical barrier and also interact with neighboring cells to exert biological actions on cell proliferation, differentiation, and migration (for comprehensive reviews see Paez-Pereda *et al.* (2005)).

The anterior pituitary gland is composed of hormone-secreting cells and non-hormone-secreting cells, including folliculostellate cells and the cells of capillaries (endothelial cells and pericytes). ECM components in the anterior pituitary communicate with these cells and influence the behavior and biological processes of cells within the gland. At a minimum, they participate in controlling hormone synthesis and secretion (Perez *et al.* 1995). A series of studies by our group demonstrated that folliculostellate cell functions are influenced by the interaction between folliculostellate cells and ECM (Horiguchi *et al.* 2010, 2011). In addition, Tsukada *et al.* (2016) showed that transforming growth factor-beta 2 (TGFβ2) secreted from folliculostellate cells strongly induced collagen synthesis in pericytes.

Our group has also identified that folliculostellate cells and pericytes of anterior pituitary gland produce small leucine-rich proteoglycans (SLRPs), a major group of proteoglycans (Horiguchi *et al.* 2013). Fibromodulin is a member of SLRPs that has been found to regulate collagen fibrillogenesis, to contribute ECM assembly (Kalamajski and Oldberg 2010) and to act as a potent endogenous antagonist by binding directly to TGFβ (Hildebrand *et al.* 1994). Other studies suggest that fibromodulin also has key roles as a potent effector in cell signaling pathways and is important in modulating cellular

function and modifying the ECM environment (Hildebrand *et al.* 1994; Dellet *et al.* 2012). However, little is known of the function and regulatory mechanism underlying fibromodulin synthesis in pituitary gland.

This study examined the effects of ECM and growth factor in order to clarify the systems that regulate fibromodulin expression within the gland. Experiment I investigated whether the major components of the anterior pituitary ECM, namely laminin and type I collagen, modulate fibromodulin expression. Experiment II assessed whether TGFβ signaling is associated with fibromodulin expression in folliculostellate cells and pericytes.

2. Experiment I: Laminin and collagen modulate expression of the small leucine-rich proteoglycan fibromodulin in rat anterior pituitary gland

2.1. Purpose

The purpose of this experiment was to evaluate the possible effects of laminin and type I collagen—the major components of anterior pituitary ECM—on the expression of fibromodulin in rat anterior pituitary cells. Immunostaining was performed to see the localization and distribution of fibromodulin in folliculostellate cells and pericytes. Western blot analysis, real-time PCR and immunostaining were used to investigate primary monolayer cultures of anterior pituitary cells in the absence and presence of laminin or type I collagen.

2.2. Materials and Methods

2.2.1. Animals

S100β-green fluorescent protein (GFP) rats (Itakura *et al.* 2007), which express GFP under control of the promoter of the S100 β protein gene (a marker of folliculostellate cells) were donated by Professor K. Inoue of Saitama University and were bred in our laboratory. Male rats aged 8–10 weeks and weighing 250–300 g were given ad libitum access to food and water and were housed under conditions of 12 h light and 12 h darkness. Rats were killed by exsanguination from the right atrium under deep pentobarbital anesthesia and then perfused with Ca^{2+}-free and Mg^{2+}-free Hanks' solution for primary culture or with 4% paraformaldehyde in 50 mM phosphate buffer (pH 7.4) for immunohistochemistry. All animal experiments were performed after approval had been received from the Institutional Animal Experiment Committee of Jichi Medical University, based on NIH guidelines for the Care and Use of Laboratory Animals.

2.2.2. Immunohistochemistry

Pituitary glands were removed from male transgenic GFP rats and fixed in 4% paraformaldehyde at 4°C overnight. Next, samples were immersed at 4°C for 2 days in phosphate buffer (pH 7.2) containing 30% sucrose and embedded in Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo, Japan) before rapid freezing. Sections (thickness: 8 μ m) were obtained by using a cryostat (CM3000; Leica Microsystem, Wetzlar, Germany), mounted on glass slides and stained according to the protocol described previously (Horiguchi *et al.* 2010). Rabbit polyclonal anti-rat fibromodulin (1:100; Santa Cruz

Biotechnology, Santa Cruz, CA, USA) and biotinylated anti-rabbit IgG (1:150; Vector Laboratories, Burlingame, CA, USA) were used. The ABC method (Vector Laboratories) was performed with 3,3'-diaminobenzidine (Dojindo Laboratories, Kumamoto, Japan) as the substrate. For double-immunostaining, the following primary antibodies were used: anti-fibromodulin and mouse monoclonal anti-desmin antibody (1:100; DAKO Glostrup, Denmark). Secondary antibodies were: Alexa-Fluor-568-conjugated goat anti-rabbit IgG and Alexa-Flour-633-conjugated goat anti-mouse IgG (Invitrogen, Carlsbad, CA, USA) diluted to 1:200 in phosphate-buffered saline (PBS). Absence of an observable nonspecific reaction was confirmed by using normal mouse or rabbit serum. A confocal laser microscope (FV1000; Olympus, Tokyo, Japan) was used to observe the reaction.

2.2.3. Cell culture

Primary cell culture with anterior pituitary cells of male S100 β -GFP rats was performed according to the protocol described previously (Horiguchi *et al.* 2010). A cell sorter (MoFlo XDP; Beckman Coulter, Fullerton, CA, USA) was used to separate GFP-positive (GFP+) cells from GFP- negative (GFP−) cells. Next, separated cells were plated onto 8-well glass chamber slides (1 cm^2/well; Nalge Nunc International, Rochester, NY, USA), with or without a coating of 10 μ g/cm^2 laminin (Merck Millipore, Bedford, MA, USA) or 3 μ g/cm^2 type I collagen (Nitta Gelatin, Osaka, Japan), at a density of 1×10^5 cells/cm^2 in 400 μ l Medium 199 with Earle's salts (Invitrogen) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 0.5 U/ml penicillin and 0.5 μ g/ml streptomycin (Invitrogen). Cells were then cultured for 72 h at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air.

2.2.4. Immunocytochemistry

Cultured cells fixed with 4% paraformaldehyde in 25 mM phosphate buffer (pH 7.4) for 20 min at room temperature were first immersed in PBS containing 2% normal goat serum (NGS) for 20 min at 30°C, followed by incubation of antibody against fibromodulin (Santa Cruz Biotechnology) overnight at 4°C. After being washed with PBS, cells were incubated in PBS with Alexa-Fluor-568-conjugated goat anti-rabbit IgG (Invitrogen) diluted to 1:200. Sections were mounted with Vectashield Mounting Medium containing 4,6-diamidino-2-phenylindole (Vector Laboratories). Cells were scanned at a thickness of 1 μm in a confocal laser microscope.

2.2.5. Immunoblot analysis

Whole anterior pituitary gland, cultured anterior pituitary cells and 8×10^5 GFP+ and GFP− cell fractions were washed in PBS and lysed in 2× sample buffer (125 mM Tris-HCl, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, pH 6.8). Total protein was estimated by using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions and 20 μg protein from each sample was separated by 12% SDS-polyacrylamide gel electrophoresis and transferred electrophoretically to Immobilon-P transfer membrane (Merck Millipore). The blots were blocked with 5% skim milk in T-TBS (50 mM Tris-HCl, 100 mM NaCl, 0.1% Tween 20, pH 7.4) for 1 h and probed overnight with a 1:20,000 dilution of antibody against fibromodulin (Santa Cruz Biotechnology) diluted in Can Get Signal Solution (Toyobo, Osaka, Japan), followed by incubation for 1 h with horseradish peroxidase (HRP)-labeled secondary antibodies (1:100; Envision+System- HRP,

anti-rabbit; DAKO). Anti–β-actin antibody (0.1 μg/ml; BioVision; Mountain View, CA, USA) was employed as a control. Immunoreactive bands were visualized by enhanced chemiluminescence plus western Blotting Detection Reagents (GE Healthcare, Mississauga, ON, Canada) with Lumi-shot film (Fujifilm, Tokyo, Japan). The film was scanned and densitometry was analyzed by using ImageJ software (NIH, Bethesda, MD, USA). The data were normalized against β-actin. Each analysis was performed in triplicate.

2.2.6. Quantification of mRNA levels by real-time reverse transcription-PCR

Total RNA fractions were prepared using RNeasy mini-kit and an RNase-free DNase set according to the manufacturer's instructions (Qiagen, Hilden, Germany). cDNA was synthesized using the PrimeScript RT reagent kit (Takara Bio, Shiga, Japan) with oligo-(dT)20 primer (Thermo Fisher Scientific, Waltham, MA, USA). Quantitative real-time RT-PCR (ABI PRISM 7900HT; Applied Biosystems, Carlsbad, CA, USA) was performed by using gene-specific primers (*Fmod*: NM_080698, Table 1) and SYBR Premix Ex Taq (Takara Bio) containing SYBR Green I. For normalization, glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*, M_17701, Table I) was also quantified: Relative quantification was conducted using the standard curve method and was performed at least three times.

2.2.7. Statistical analysis

All data are presented as means ± SEM for four samples in each group. The significance of differences between groups was determined by the two-tailed multiple *t*-test with Bonferroni correction after one-way analysis of variance.

Table 1 Primers used for real-time polymerase chain reaction

Genes	Primer sequences (5'-3')	Product size	Gen Bank acc #
Fmod	Forward : AGGCACTTGGAGAGGCTGTA	108 bp	NM_080698
	Reverse : CCGAGAGATCTGGTTGTGGT		
Gapdh	Forward : AAGGGCTCATGACCACAGTC	116 bp	M_17701
	Reverse : GGATGCAGGGATGATGTTCT		

2.3. Results and Discussion

Fibromodulin belongs to the SLRP family of ECM constituents and is abundant in dense connective tissue (Iozzo 1998). A previous study using in situ hybridization showed that fibromodulin is expressed and produced specifically by folliculostellate cells and pericytes of the anterior pituitary gland at the transcription level (Horiguchi *et al.* 2013). To gain a deeper understanding, immunohistochemistry and western blot analysis were used in this experiment to examine the protein distribution and expression of fibromodulin in rat anterior pituitary gland. Sections of S100 β -GFP rat pituitary were stained with hematoxylin-eosin (Fig. 1a). GFP (Fig. 1b) and fibromodulin (Fig. 1c, d) were detected in the anterior and posterior lobes. The anterior pituitary showed GFP specifically in folliculostellate cells (Fig. 1e). Next, double-immunostaining was performed to detect desmin (Fig. 1f), a marker of pericytes, and fibromodulin (Fig. 1g). In the anterior lobe, fibromodulin was colocalized with S100 protein folliculostellate cells, primarily in the cytoplasm. It was also detected near capillaries, colocalized with pericytes (Fig. 1h). The presence of fibromodulin in the anterior pituitary was confirmed by western blot analysis and appeared as an immunoreactive band at approximately 44 kDa, which corresponds to the size of fibromodulin (Fig. 1i). SLRPs are composed of a protein core with leucine-rich repeat linked to one or more glycosaminoglycan chains and can be divided into five distinct classes based on their similar structure and genomic units (Schaefer and Schaefer 2010). SLRPs, including fibromodulin, have important roles in modulating collagen fibrillogenesis (Iozzo 1998). The presence of fibromodulin expression in folliculostellate cells and pericytes in the anterior pituitary suggests that both these cells have roles in collagen fibrillogenesis in the pituitary gland.

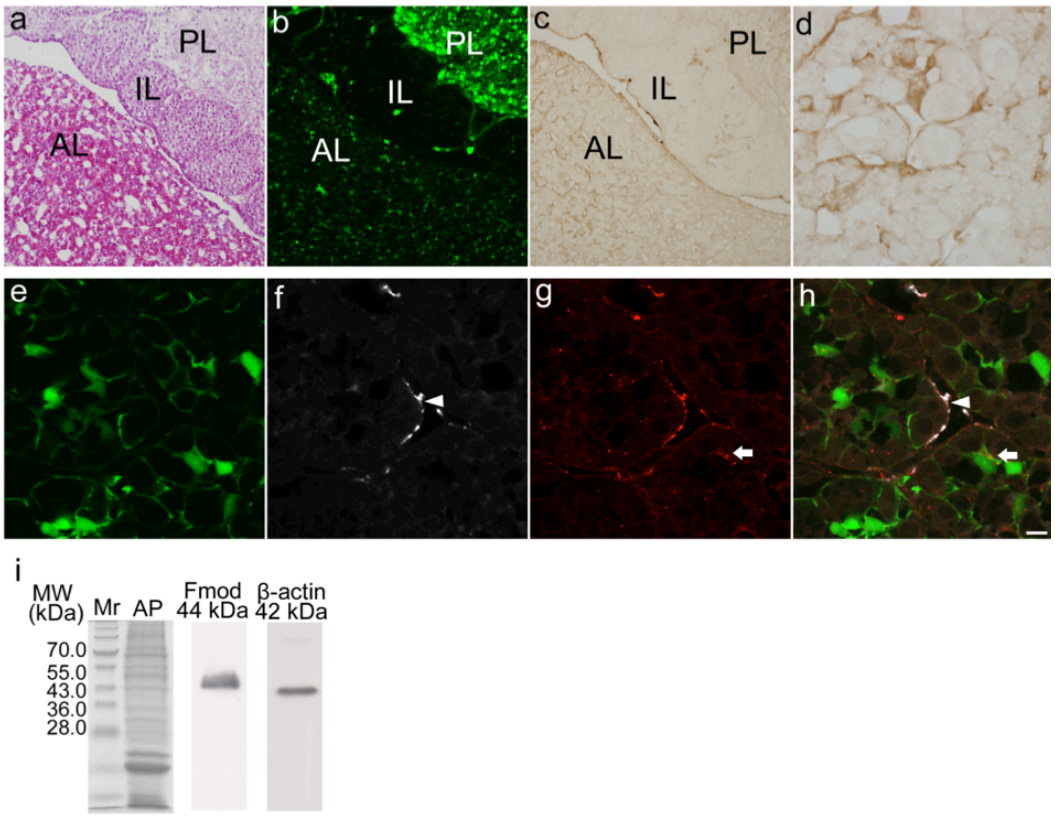

Fig 1. Fibromodulin expression in the anterior pituitary gland. **a** Pituitary cryosection taken from S100b-GFP rat and stained with hematoxylin-eosin: anterior lobe (*AL*), intermediate lobe (*IL*) and posterior lobe (*PL*). **b** Pituitary cryosection from S100b-GFP rat showing GFP-positive cells in anterior lobe (*AL*), intermediate lobe (*IL*) and posterior lobe (*PL*). In the anterior lobe, S100b expression (*green*) is limited to folliculostellate cells. **c** Fibromodulin immunoreactivity (*brown*) in the pituitary. d Higher magnification of **c**. **e–h** Double-immunostaining of fibromodulin and desmin in S100b-GFP rat anterior pituitary section showing a cluster of GFP+ cells (*green*, **e**), desmin immunoreactivity (*white*, **f**), fibromodulin immunoreactivity (*red*, **g**) and an overlay image (**h**) of **e–g** (arrow fibromodulin immunopositivity on folliculostellate cells, arrowhead fibromodulin immunopositivity on pericytes). Bar 10 μm (**d–h**). **i** Western blotting showing immunoreactivity of fibromodulin and β-actin in anterior pituitary gland. Left Protein marker (*Mr*) and CBB staining of anterior pituitary (*AP*) proteins (*MW (kDa)* molecular weight in kiloDaltons, with 70.0, 55.0, 43.0, 36.0 and 28.0 being molecular weights). Center fibromodulin (*Fmod*). Right Loading control (*β-actin*)

To examine whether ECM had differential effects on fibromodulin expression in folliculostellate cells and pericytes, GFP+ and GFP− cells were separated, and protein levels of fibromodulin were analyzed after 72-h incubation on uncoated, laminin-coated and type I collagen-coated surfaces. GFP+ cells represent folliculostellate cells, as they express S100 protein (Fig. 2a, b, e, f, i, j), whereas GFP− cells represent pericytes, hormone-producing cells and endothelial cells (Fig. 2c, d, g, h, k, l). Folliculostellate cells exhibited morphological changes, i.e., flattened elongated cytoplasmic processes, on ECM-coated surfaces (Fig. 2e, f, i, j; Horiguchi *et al.* 2010). In contrast, no morphological changes were detected in GFP− cells in the presence of ECM components (Fig. 2c, d, g, h, k, l). Fibromodulin in folliculostellate cells was detected on cell bodies and cytoplasmic processes on the uncoated surface (Fig. 2b) and ECM-coated surface (Fig. 2f, j) and expression was greater and more widely distributed along the processes when folliculostellate cells were cultured on laminin and type I collagen (Fig. 2f and j). However, no difference was seen in the fibromodulin expression of pericytes (Fig. 2d, h, l). An immunoreactive band of fibromodulin was detected at 44 kDa in mixed anterior pituitary cells (GFP+ and GFP− cells; Fig. 3a), in GFP+ cells (Folliculostellate cells; Fig. 3b) and in GFP− cells including pericytes (Fig. 3c). When normalized against β-actin, the relative concentration of fibromodulin protein was higher on the ECM-coated surfaces than on the uncoated surface in anterior pituitary cells (Fig. 3d) and folliculostellate cells (Fig. 3e) but not in GFP− cells, at 72 h (Fig. 3f).

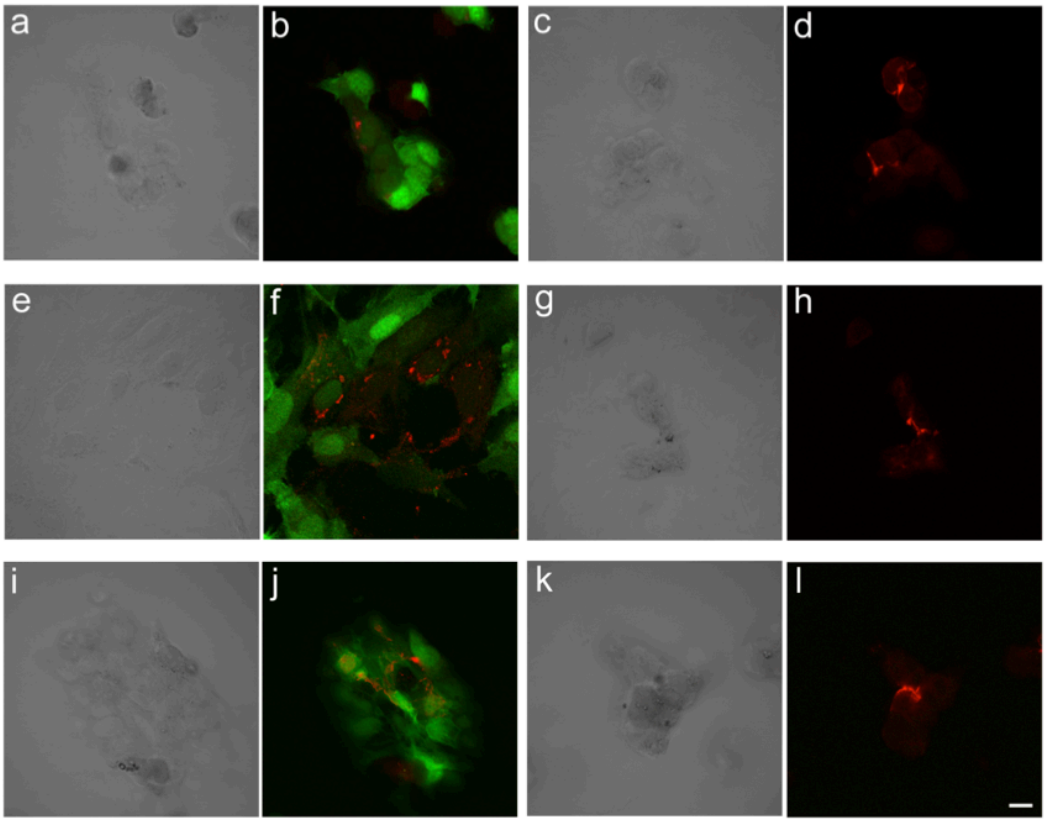

Fig 2. Localization of fibromodulin in primary culture of S100b-GFP rat anterior pituitary cells. **a, c, e, g, i, k** Phase-contrast images. **b, f, j** Confocal image of fibromodulin immunoreactivity (*red*) in FS cells (*green*) after 72 h incubation on uncoated, laminin-coated and type I collagen-coated surfaces, respectively. **d, h, l** Fibromodulin immunoreactivity (*red*) in GFP− cells cultured on uncoated, laminin-coated and type I collagen-coated surfaces, respectively. Bar 10 μm

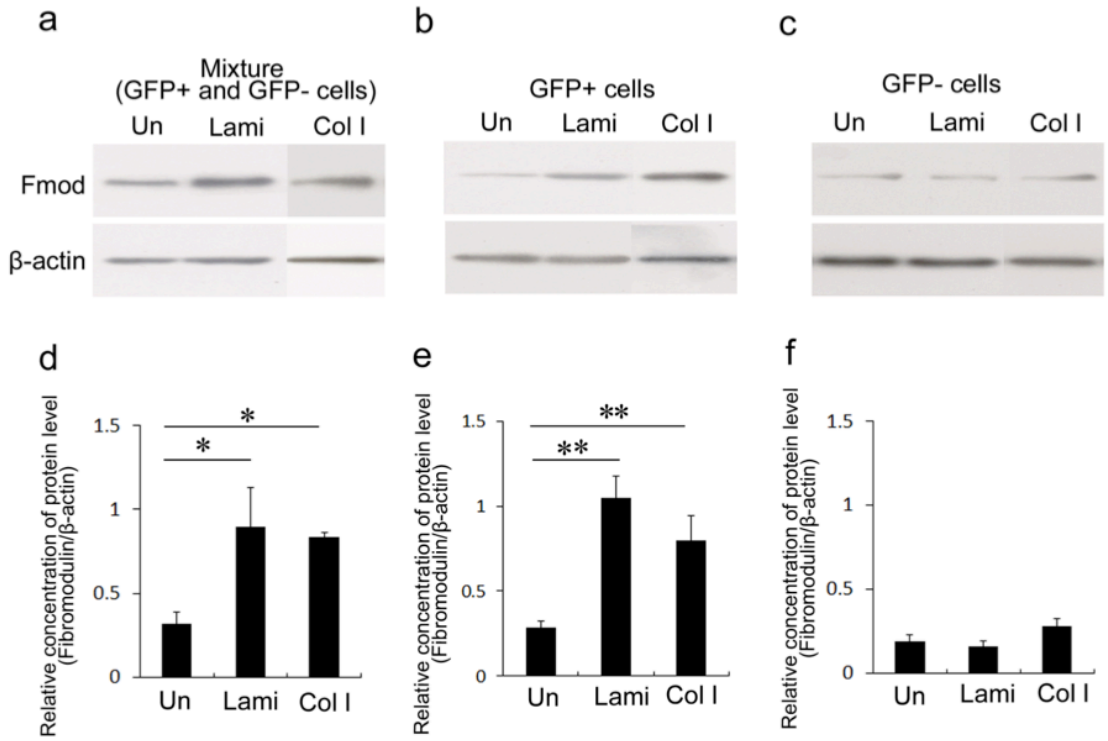

Fig 3. Effect of laminin and type I collagen on fibromodulin expression in FS cells. **a–c** Western blotting showing immunoreactivity of fibromodulin in primary culture of anterior pituitary cells in a mixture of GFP+ and GFP− cells (**a**), in GFP+ cell (**b**) and GFP− cell (**c**) fractions after 72 h incubation on uncoated (*Un*), laminin-coated (*Lami*) and type I collagen-coated (Col I) surfaces. Top, fibromodulin (*Fmod*). Bottom, loading control (β *-actin*). **d–f** Immunoblot analysis of **a–c** presented as fold induction normalized against β-actin, respectively. **d** Mixture of GFP+ and GFP− cells. **e** GFP+ cells. **f** GFP− cells. Data represent means ± SEM; n = 4. **P* < 0.01

Interestingly, real-time reverse transcription plus the polymerase chain reaction revealed that fibromodulin expression at the transcription level was much higher on type I collagen-coated surfaces than on laminin-coated surfaces (Fig. 4); these data suggest that type I collagen is more important in modulating fibromodulin expression. The mechanism responsible for this is not fully understood. Fibromodulin can interact with type I collagen, thereby influencing collagen scaffold formation (Kalamajski and Oldberg 2010). This interaction might give feedback to mRNA synthesis, thereby explaining the poor correlation between mRNA levels (Fig. 4) and protein (Fig. 3d) when mixed cells were cultured on type I collagen. In addition, the possible effects of other factors, such as post-transcriptional mechanisms and experimental conditions, cannot be excluded. Further study is necessary to explain the relevant mechanism.

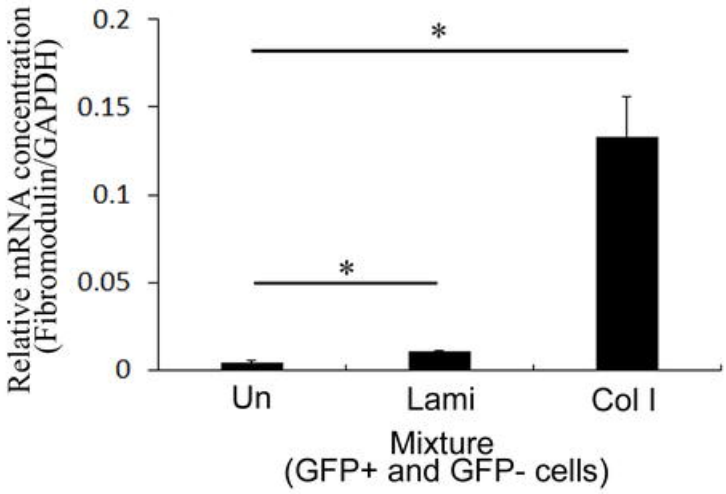

Fig 4. Comparison of the mRNA fibromodulin expression levels in mixture anterior pituitary cells after 72 h incubation on uncoated (*un*), laminin-coated (*Lami*), and type I collagen-coated (*Col I*) surfaces. The relative mRNA expression levels were quantified by PCR and normalized with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. means ± SEM. n = 3. **P*<0.05.

In anterior pituitary gland, type I collagen is produced by pericytes (Fujiwara *et al.* 2010) and altered fibromodulin expression in folliculostellate cells should shed light on the interaction between pericytes and folliculostellate cells. In addition, considerable experimental evidence suggests that type I collagen regulates the expression of other ECM components, such as fibronectin, thrombospondin-1 and type IV collagen, in epithelial cells of mammary gland (Streuli and Bissell 1990) and trachea (Davenport and Nettesheim 1996). However, the effect of ECM on fibromodulin expression has not been studied. Our results are the first to show that fibromodulin expression is influenced by other ECM components. Previously, Horiguchi *et al.* (2010) have demonstrated that the presence of laminin and collagen can enhance the proliferation of folliculostellate cells, a possible reason for the up-regulation of fibromodulin. In the present experiments, however, the increase in fibromodulin expression was much greater than the increase in the number of folliculostellate cells observed in the previous report. Thus, the present increase is likely directly induced by laminin and type I collagen, as these ECM components affect folliculostellate cells via integrin $\beta 1$ and the activated mitogen-activated protein kinase (MAPK) pathway (Horiguchi *et al.* 2011) and that SLRP synthesis itself is mediated via the MAPK pathway (Kobayashi *et al.* 2003).

In contrast with fibromodulin expression in folliculostellate cells, fibromodulin protein expression in pericytes does not differ in relation to the presence of ECM components. Although integrin $\beta 1$ is expressed in pericytes and folliculostellate cells (Silva *et al.* 2008; Horiguchi *et al.* 2010) in the mediation of ECM signaling, Horiguchi *et al.* (2011) have shown that ECM signaling via integrin $\beta 1$ is mediated by caveolin-3. Because this mechanism is specific to folliculostellate cells, it is likely that pericytes cannot be further stimulated by collagen. This might explain the difference in regulation of fibromodulin

expression between folliculostellate cells and pericytes under the influence of ECM components.

In conclusion, the present results show that the presence of ECM constituents (i.e., laminin and type I collagen) increases fibromodulin expression in folliculostellate cells of the anterior pituitary gland. Laminin and type I collagen appear to promote folliculostellate cells function in the anterior pituitary gland by increasing fibromodulin expression.

3. Experiment II: Fibromodulin expression in folliculostellate cells and pericytes is promoted by TGFβ signaling in rat anterior pituitary gland

3.1. Purpose

The aim of this experiment was to determine whether TGFβ signaling is associated with fibromodulin expression in folliculostellate cells and pericytes of anterior pituitary gland. In situ hybridization followed by immunohistochemistry was performed to identify TGFβ receptor gene expression in the anterior pituitary gland. Real-time PCR and western blotting were performed to determine the effects of TGFβ2 on fibromodulin gene and protein expressions in cultured anterior pituitary cells. Primary monolayer culture and hanging drop 3D culture methods were used.

3.2. Materials and Methods

3.2.1. Animals

Male Wistar rats were purchased from Japan SLC (Shizuoka, Japan) and S100β-GFP transgenic rats were supplied by the National BioResource Project for the Rat in Japan, Kyoto University (Kyoto, Japan) and bred in our laboratory. All the animals were maintained and handled under the same condition in the experiment I.

3.2.2. *In situ* hybridization

Frozen sections (8 μm) of pituitary gland were obtained using the same protocol as experiment I. *In situ* hybridization was performed with digoxigenin (DIG)-labeled cRNA probes, as described previously (Fujiwara *et al.* 2007). The DNA fragments of rat TGFβ receptor II (*Tgfbr2*; NM_031132) were amplified from rat pituitary cDNA by PCR with gene-specific primers (Table 2). Amplified DNA fragments were ligated into the pGEM-T vector (Promega, Fitchburg, WI, USA) and cloned. A Roche DIG RNA labeling kit (Roche Diagnostics, Penzberg, Germany) was used to generate gene-specific antisense or sense DIG-labeled cRNA probes. The hybridization was performed at 55°C overnight, and alkaline phosphatase-conjugated anti-DIG antibody (Roche Diagnostics) using 4-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) was used for the detection of mRNA. In a control experiment, no specific signal was detected in sections processed with the DIG-labeled sense RNA probes.

For double staining, subsequent immunohistochemistry was performed as described previously (Fujiwara *et al.* 2007). The sections were incubated with blocking solution containing 2% normal NGS in PBS for 20 min at room temperature and then with primary antibody for 90 min at 30°C. Primary antibodies included rabbit polyclonal anti-S100 protein

(1:1000, DAKO) and anti-desmin (1:1000, Abcam, Cambridge, UK). The sections were then incubated with biotinylated anti-rabbit IgG (Vector Laboratories) for 30 min at 30°C, and the immunoreactive signal was detected with a Vectastain ABC kit (Vector Laboratories) and 3,3'-diaminobenzidine (Dojindo Laboratories).

3.2.3. Cell culture

Monolayer primary cell culture using anterior pituitary cells from Wistar or S100β-GFP transgenic rats was performed as described in the experiment I. Hanging drop 3D cell culture using anterior pituitary cells from Wistar rats was performed according to the protocol described previously (Tsukada *et al.* 2013). A 25-µl drop containing 5,000 anterior pituitary cells was placed on the undersurface of 100-mm Petri dish lids, which were then cultured over sterile PBS at 37°C in a humidified incubator with 5% CO_2.

3.2.4. Treatment of TGFβ2 and TGFβ receptor I inhibitor (SB431542)

Recombinant human TGFβ2 (PeproTech, Rocky Hill, NJ, USA) and selective TGFβ receptor I inhibitor (SB431542, Merck Millipore) were diluted in Hanks' balanced salt solution (Thermo Fisher Scientific) containing 0.1% bovine serum albumin (BSA) and dimethyl sulfoxide (DMSO), respectively, and stored at −20°C until use. To examine Smad2 nuclear translocation, the cells on a glass chamber slide (2D culture) were treated with TGFβ2 (50 ng/ml) or BSA for 30 min at 37°C in a humidified incubator with 5% CO_2. For real-time PCR analysis, 120–150 cell aggregates from Wistar rats were collected in a 15-ml tube 4 days after 3D cell culture and then centrifuged at 200×g for 2 min. Cell culture media were replaced with 470 µl of fresh cell culture media, and all cell aggregates were re-plated onto a 24-well plastic plate. To obtain the indicated concentrations, 30 µl of TGFβ2 and/or

SB431542 diluted by cell culture media was added to the well (final volume, 500 μl). The cell aggregates were then incubated for an additional 24 h at 37°C in a humidified incubator with 5% CO_2. BSA and DMSO were diluted in the same manner and used as vehicle controls for TGFβ2 and SB431542, respectively. For western blot analysis, dispersed anterior pituitary cells from Wistar rats were re-suspended in media containing TGFβ2 (50 ng/ml) or BSA and then cultured in hanging drops (120–150 drops/treatment) for 5 days at 37°C in a humidified incubator with 5% CO_2.

3.2.5. Immunofluorescence microscopy

To analyze the nuclear translocation of Smad2, cells treated with TGFβ2 and BSA were fixed in 4% PFA in 25 mM of PB (pH 7.4) for 20 min at room temperature. The cells were permeabilized in PBS containing 0.2% Triton X-100 (Sigma-Aldrich) for 20 min and then incubated in PBS containing 2% NGS for 30 min at room temperature. The cells from S100β-GFP transgenic rats were incubated with rabbit monoclonal anti-Smad2 antibody (1:200, Cell Signaling Technology, Danvers, MA, USA), and the cells from Wistar rats were incubated with rabbit monoclonal anti-Smad2 and mouse monoclonal anti-desmin (1:50, DAKO) antibodies, for 90 min at 30°C, followed by a secondary antibody containing DAPI (0.5 μg/ml) for 30 min at 30°C. The secondary antibodies were Alexa Fluor 568-conjugated goat anti-rabbit IgG, Alexa Fluor 488-conjugated goat anti-rabbit IgG, and Alexa Fluor 568-conjugated goat anti-mouse IgG antibodies (all 1:200, Thermo Fisher Scientific). Stained cells were subsequently analyzed with an FV1000 confocal laser microscope (Olympus). Images were processed for presentation using Photoshop CS5 (Adobe Systems, San Jose, CA, USA).

3.2.6. Real-time PCR quantification of mRNA levels

After treatment with TGFβ2 and/or SB431542 for 24 h, real-time PCR was used to quantify mRNA level. The method used was identical to that described for experiment I. Fibromodulin primers (*Fmod*: NM_080698; Table 2) were used in this experiment, and β-actin (*Actb*: NM_031144; Table 2) was used for normalization. All measurements were made in duplicate, and cycle threshold values were converted to relative gene expression levels by using the $2^{-(\Delta Ct\ sample-\Delta Ct\ control)}$ method.

3.2.7. Immunoblot analysis

TGFβ2- and BSA-treated cell aggregates were treated using the same method, as Experiment I. Eight µl protein sample were used per-lane in this experiment.

3.2.8. Statistical analysis

All results are presented as mean±SEM. The unpaired Student's *t*-test for 2-group comparison or 1-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons was performed using Prism Version 6 (GraphPad Software, San Diego, CA, USA). A P value of <0.05 was considered to indicate statistical significance.

Table 2. Primers for real-time PCR and cRNA probes

Genes	Primer sequence (5'-3')	Prod size	GenBank acc.#	Use
Tgfbr2	Forward: GGCGAGACCTTCTTCATGTG Reverse: TGTCCTTCTCCGTTTTCCAC	490	NM_031132	cRNA probe
Fmod	Forward: CCACAATAAGATGGGGAAGA Reverse: GCACAGATCATGTTGGTCAG	92	NM_080698	real-time PCR
Actb	Forward: TGGCACCACACTTTCTACAATGAGC Reverse: GGGTCATCTTTTCACGGTTGG	106	NM_031144	real-time PCR

3.3. Results

TGFβ receptor II expression in fibromodulin-expressing cells

We previously reported that folliculostellate cells and pericytes express fibromodulin in rat anterior pituitary gland (Horiguchi *et al.* 2013). The present study attempted to determine whether these cell types possess TGFβ receptor; thus, *in situ* hybridization for TGFβ receptor II (*Tgfbr2*) was performed. *Tgfbr2* is associated with canonical TGF β signal transduction: it forms a heteromeric complex with TGFβ receptor I. Figure 5a shows hematoxylin-eosin staining of a rat pituitary section. *Tgfbr2*-expressing cells were localized in the posterior, intermediate, and anterior lobes (Fig. 5b). *Tgfbr2*-expressing cells were found in parenchymal cells, perivascular cells, and endothelial cells of the anterior lobe (Fig. 5c). No specific signal was detected in a section processed with the DIG-labeled sense cRNA probe for *Tgfbr2* (Fig. 5d). The *Tgfbr2*-expressing cells were further stained for S100 protein (a marker of folliculostellate cells) and desmin (a marker of pericytes). The results of double staining showed that *Tgfbr2*-expressing cells were co-stained with S100 protein (Fig. 5e) and desmin (Fig. 5f).

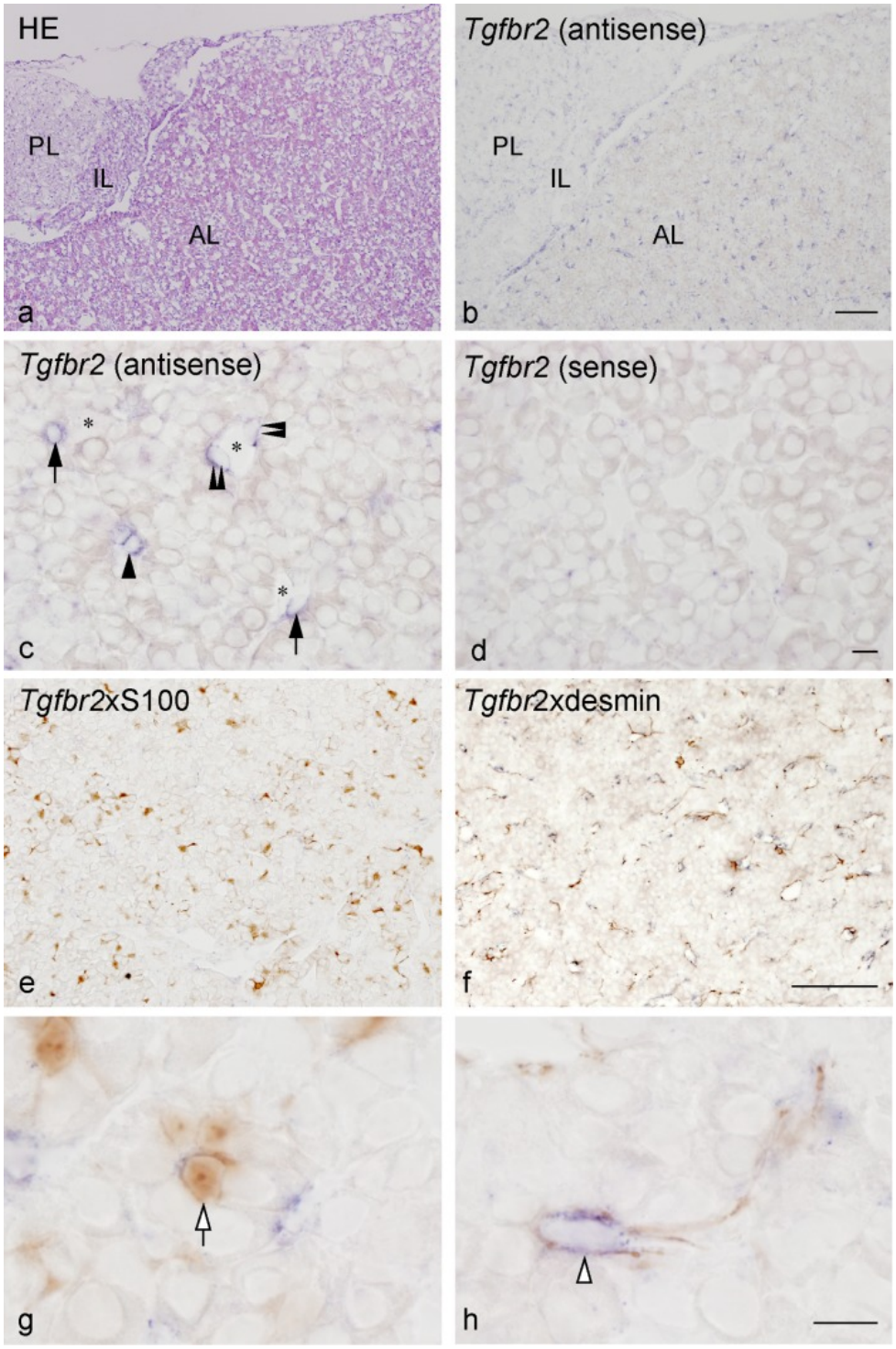
HE
PL
IL
AL
a
Tgfbr2 (antisense)
PL
IL
AL
b
Tgfbr2 (antisense)
c
Tgfbr2 (sense)
d
Tgfbr2xS100
e
Tgfbr2xdesmin
f
g
h

Fig 5. Expression of TGFβ receptor II mRNA in folliculostellate cells and pericytes of rat anterior pituitary gland. The first row panels show hematoxylin and eosin staining of a cryosection of rat anterior pituitary gland (**a**) and in situ hybridization of the TGFβ receptor II antisense probe (**b**). The second row panels show images of *in situ* hybridization of TGFβ receptor II (**c**: antisense, **d**: sense) in the anterior lobe. TGFβ receptor II-expressing cells were observed in parenchymal cells (arrowhead), perivascular cells (arrows), and endothelial cells (double arrowheads). The third row panels show immunohistochemistry of S100 protein (**e**) and desmin (**f**), combined with TGFβ receptor II in situ hybridization. The magnified images of (**e**) and (**f**) are shown in (**g**) and (**h**), respectively. S100 protein was used as a marker for folliculostellate cells, and desmin was used as a marker for pericytes. Immunohistochemical and *in situ* hybridization signals are shown in brown and purple, respectively. S100 protein-cells (**g**: open arrow) and desmin-positive cells (**h**: open arrowhead) expressed TGFβ receptor II. *AL*: anterior lobe, *IL*: intermediate lobe, *PL*: posterior lobe. Bars=100 μm (**a**, **b**, **e**, and **f**) and 10 μm (**c**, **d**, **g**, and **h**). Asterisks: capillary lumen.

TGFβ signal transduction in fibromodulin-expressing cells

To confirm the histochemical findings (Fig. 5), nuclear translocation of Smad2 in these cell types was investigated. Smad2 is a signaling molecule of TGFβ receptor and is transferred into the nucleus in response to TGFβ. Isolated rat anterior pituitary cells were treated with TGFβ2 (50 ng/ml) for 30 min and then stained for Smad2. In the vehicle-treated cells, Smad2 was diffusely stained in both S100β-GFP-positive and desmin-positive cells (Fig. 6Aa, 6Ab, and Fig. 6Ba, 6Bb, respectively); however, cells treated with TGFβ2 had intense Smad2 signals in the nucleus (Fig. 6Ad, 6Ae, and Fig. 6Bd, Be, respectively). There was zero Smad2 nuclear translocalization in vehicle-treated cells but more than 90% nuclear localization in TGFβ2-treated cells.

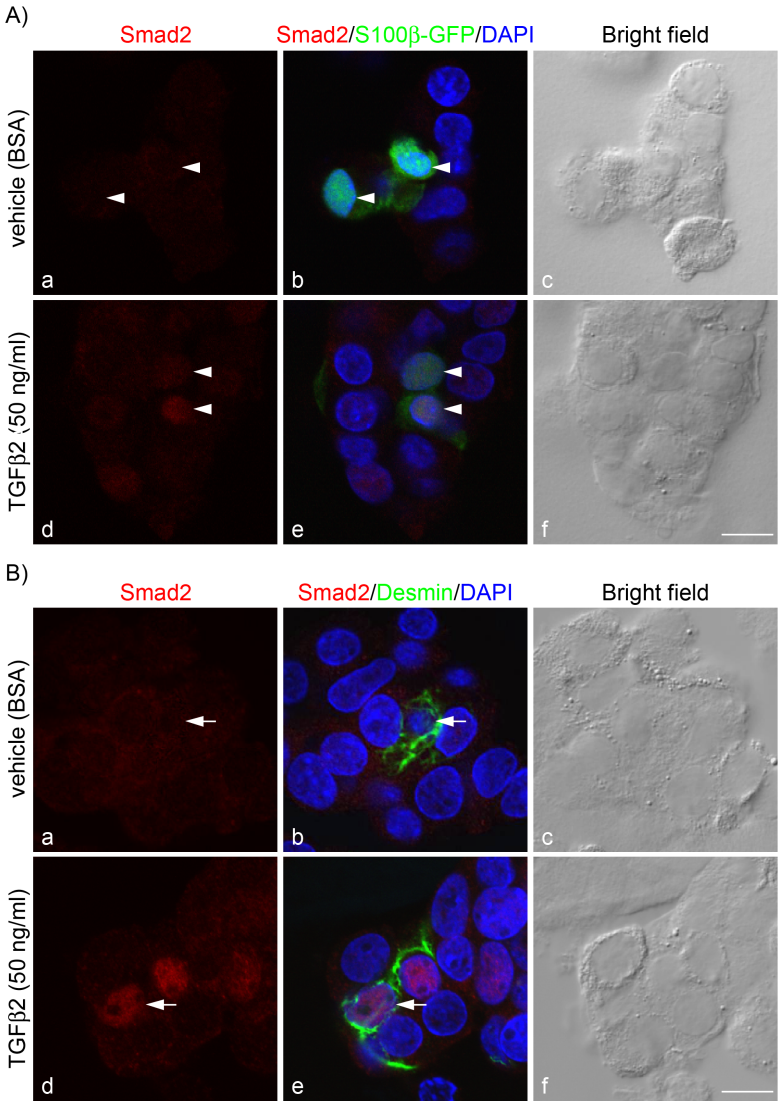

Fig 6. Detection of Smad2 signaling in folliculostellate cells and pericytes. Anterior pituitary cells from S100β-GFP transgenic rats (**A**) or Wistar rats (**B**) were treated with TGFβ2 (50 ng/ml) or 0.1% BSA (vehicle) for 30 min and then stained for Smad2. The left panels show Smad2 staining, the middle panels show a merged image (Smad2: *red*; S100β-GFP/desmin: *green*; DAPI: *blue*), and the right panels show bright-field images. Diffuse cytoplasmic staining of Smad2 was seen in vehicle-treated GFP-positive cells (folliculostellate cells: **Aa**, **Ab**) and desmin-positive cells (pericytes: **Ba**, **Bb**); however, nuclear staining was seen in TGFβ2-treated GFP-positive cells (**Ad**, **Ae**) and desmin-positive cells (**Bd**, **Be**). The cells, Smad2 positive in the nucleus and desmin-negative, in Fig. 6B are considered to be folliculostellate cell or endothelial cell, because they express TGFβ receptor II (Tsukada *et al*. 2016). Arrowheads: nucleus of GFP-positive cells. Arrows: nucleus of desmin-positive cells. Bars=10 μm.

Effect of TGFβ2 and TGFβ receptor I inhibitor (SB431542) on fibromodulin mRNA synthesis

To determine whether TGFβ2 affects fibromodulin mRNA synthesis, anterior pituitary cell clusters from Wistar rats were treated with TGFβ2 for 24 h, and fibromodulin expression was examined by quantitative real-time PCR (Fig. 7). TGFβ2 significantly increased fibromodulin expressions in a dose-dependent manner (Fig. 7A; by approximately 2-, 4-, 8-, and 22-fold for 1, 5, 10, and 50 ng/ml of TGFβ2, respectively). Furthermore, TGFβ2-induced fibromodulin expression was completely abolished by co-administration of TGFβ receptor I inhibitor (Fig. 7B).

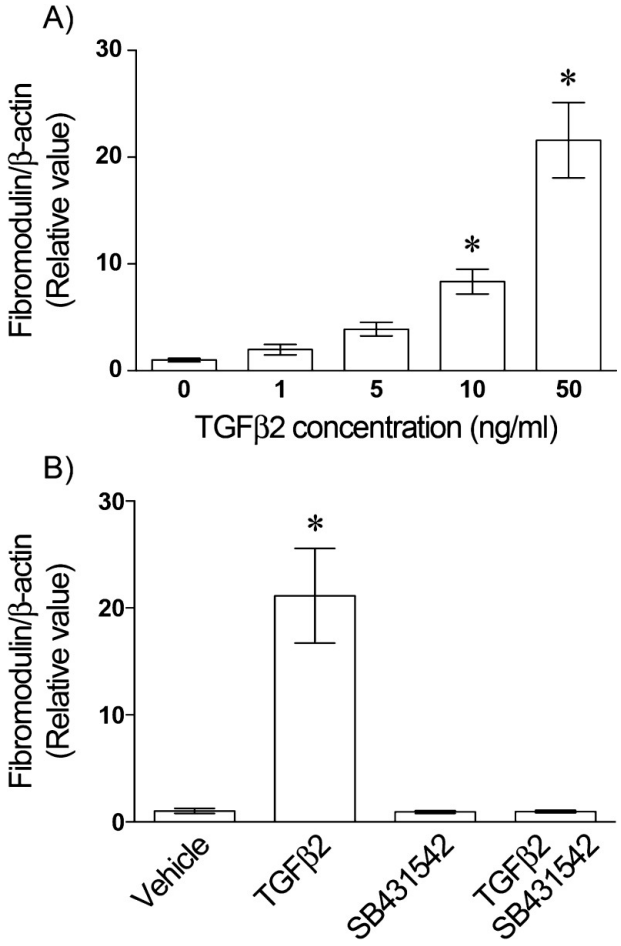

Fig 7. Relative mRNA expression level of fibromodulin in cell aggregates treated with TGFβ2 and/or selective TGFβ receptor I inhibitor (SB431542) evaluated by quantitative real-time PCR. Anterior pituitary cells of Wistar rats were used. mRNA expression levels were normalized with β-actin. **A**) Cell aggregates were treated with different concentrations of TGFβ2 (1–50 ng/ml). TGFβ2 induced fibromodulin synthesis in a dose-dependent manner (n=4, mean±SEM). **B**) Cell aggregates were treated with TGFβ2 (50 ng/ml) and SB431542 (10 μM). BSA and DMSO were the vehicle control for TGFβ2 and SB431542, respectively. SB431542 attenuated TGFβ2-induced fibromodulin synthesis (n=4, mean±SEM). **P*<0.05 (Dunnett's test).

Effect of TGFβ2 on fibromodulin protein expression

Because increased fibromodulin mRNA expression was found in cells treated with TGFβ2, western blot analysis was used to examine the effect of TGFβ2 on fibromodulin protein expression. In this experiment, anterior pituitary cell clusters from Wistar rats were treated with TGFβ2 for 5 days. Major immunoreactive bands for fibromodulin were detected at approximately 67 kDa, the expected size of fibromodulin with *N*-linked oligosaccharides. TGFβ2 at a dose of 50 ng/ml significantly increased fibromodulin protein expression, by approximately 3-fold (Fig. 8).

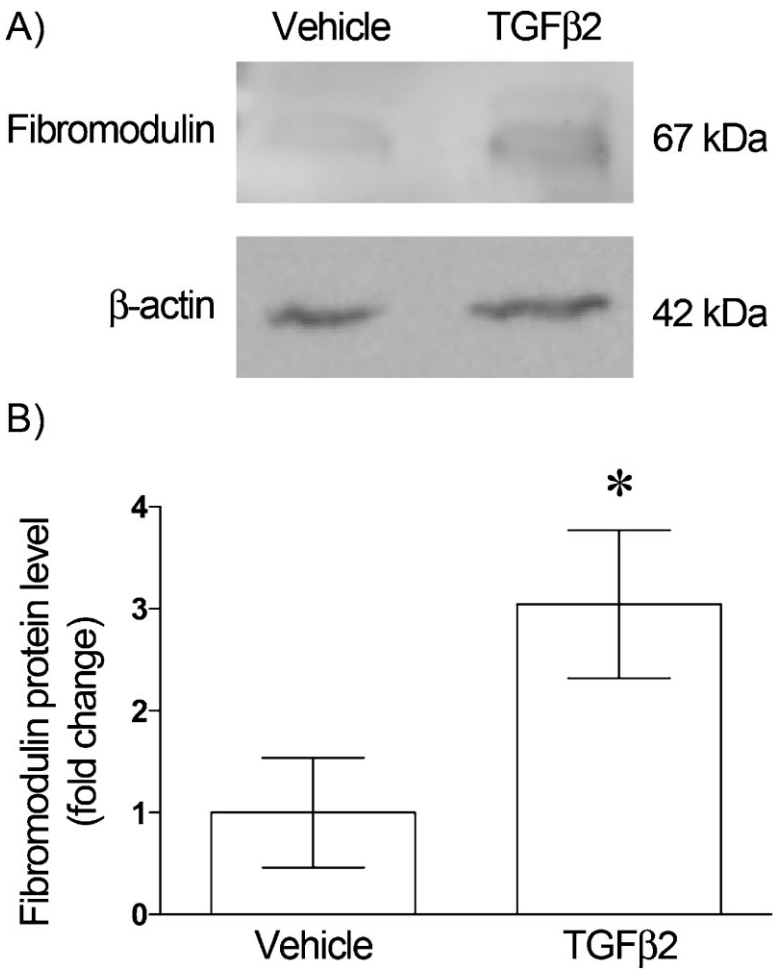

Fig 8. Relative protein expression level of fibromodulin in cell aggregates treated with TGFβ2 evaluated by western blotting. Anterior pituitary cells of Wistar rats were treated with TGFβ2 (50 ng/ml) for 5 days in hanging-drop culture. **A**) Top: Fibromodulin; bottom: β-actin. **B**) The graph shows the results of immunoblot analysis as fold induction normalized against β-actin (n=3, mean±SEM). **P*<0.05 (Student's *t*-test).

3.4. Discussion

The present findings suggest that fibromodulin-expressing cells (folliculostellate cells and pericytes) in rat anterior pituitary gland possess TGFβ receptor II and that both cell types respond to TGFβ2 and increased fibromodulin mRNA and protein expressions. This is first to show that fibromodulin known as an endogenous TGFβ antagonist is regulated by TGFβ signaling in anterior pituitary gland.

In situ hybridization revealed the localization of TGFβ receptor II mRNA in rat anterior pituitary gland (Fig. 5c). As was the case in our previous report (Tsukada *et al.* 2016), TGFβ receptor II mRNA was detected in parenchymal cells, perivascular cells, and endothelial cells. Double staining showed that parenchymal cells were co-stained with S100 protein and that perivascular cells were co-stained with desmin (Fig. 5e, f). Because fibromodulin is produced by folliculostellate cells and pericytes in the anterior pituitary gland (Horiguchi *et al.* 2013), our results suggest that a certain population of fibromodulin-expressing cells possess TGFβ receptor II.

TGFβ receptor II forms a heteromeric complex with TGFβ receptor I, which induces intracellular signal transduction, including phosphorylation and nuclear translocation of SMADs (for a review see Macias *et al.* 2015). To determine whether expressed TGFβ receptor II in fibromodulin-expressing cells is functional, Smad2 immunocytochemistry was performed using rat anterior pituitary cells (Fig. 6); Intense Smad2 immunoreactivity in the nuclei of S100β-GFP-positive cells and desmin-positive cells after TGFβ2 treatment were noted (Fig. 6Ad and Bd, respectively). These data strongly suggest that both folliculostellate cells and pericytes respond to TGFβ2 and have canonical TGFβ signals.

The previous study using a 3D cell culture system of rat anterior pituitary cells showed

that TGFβ2 acts on pericytes and induces type I and III collagen synthesis (Tsukada *et al.* 2013). In the present experiment, the same technique was used to evaluate the effect of TGFβ2 on fibromodulin expression. Fibromodulin mRNA expression was significantly higher after TGFβ2 treatment, and the effect was dose-dependent (Fig. 7A). Consistent with this increased mRNA expression, fibromodulin protein levels, as evaluated by western blot analysis, were also enhanced by TGFβ2 (Fig. 8). That TGFβ2 increases SLRP fibromodulin expression in anterior pituitary is a novel finding, although some reports noted that TGFβs induce small proteoglycans in other tissues (Burton-Wurster *et al.* 2003, Kahari *et al.* 1991, Schonherr *et al.* 1993, Yang *et al.* 2010). Furthermore, TGFβ2-induced fibromodulin expression was completely abolished by co-administration of SB431542, an inhibitor of TGFβ receptor I (Fig. 7B). These results suggest that canonical TGFβ signals in some of fibromodulin producing cells are associated with fibromodulin gene regulation. Further studies are needed in order to clarify the mechanism by which the fibromodulin gene is regulated by TGFβ-induced SMAD signaling in these cells.

TGFβ is a secreted growth factor and has various effects on the biological activities of cells (Roberts *et al.* 1993). It is a potent regulator of ECM synthesis, in which signaling stimulates synthesis of ECM components (Roberts *et al.* 1986, Tsukada *et al.* 2016, Verecia *et al.* 2002). Indeed, our past and present findings show that TGFβ2 induced fibromodulin and collagen synthesis in pericytes (Tsukada *et al.* 2016). It is highly likely that fibromodulin and collagen synthesis in pericytes utilizes the same pathway. Because TGFβ2 is exclusively expressed in folliculostellate cells in anterior pituitary gland (Tsukada *et al.* 2016), the action of TGFβ2 on pericytes is probably a paracrine mechanism. Fibromodulin binds different types of collagen, stimulates early protofibril assembly, and facilitates maturation of fibrils (Ezura *et al.* 2000). Thus, the simultaneous increase in fibromodulin and collagen expressions in pericytes may be important for proper fibrillogenesis in anterior pituitary gland.

In contrast, the action of TGFβ2 on folliculostellate cells is considered to be an autocrine mechanism. In addition to collagen, fibromodulin binds TGFβ and potently sequesters its activity (Hilderbrand *et al.* 1994). The possibility of increased fibromodulin expression in folliculostellate cells probably modulates the action of active TGFβ by its antagonistic action and functions as negative feedback regulator. Fibromodulin expression in folliculostellate cells is also increased when cells are cultured with collagen and laminin (Syaidah *et al.* 2013); thus, there is another pathway to regulate fibromodulin expression in folliculostellate cells.

Evidence indicates that local interactions are mediated by ECM within the gland (Paez-Pereda *et al.* 2005). Studies of the functions of collagen in anterior pituitary gland show that it induces folliculostellate cell proliferation (Horiguchi *et al.* 2013) and SLRP synthesis (Syaidah *et al.* 2013), alters cell morphology (Toral *et al.* 2007), and promotes hormone production (Kuchenbauer *et al.* 2001). Because of its variety of actions on anterior pituitary cells, it is probable that collagen is regulated in anterior pituitary. The present study may highlight the importance of interaction between TGFβ signaling and fibromodulin in collagen regulation in anterior pituitary gland.

4. Conclusion

This series of studies revealed a number of novel findings, as follows.

a. The major constituents of the anterior pituitary ECM, namely laminin and type I collagen, increased fibromodulin expression in folliculostellate cells but not in pericytes.

   - Folliculostellate cells exhibited morphological changes and the expression of fibromodulin was shown higher with wider distribution on ECM-coated surfaces cell culture while no morphological changes nor expression differences were detected in pericytes

   - Protein concentration of fibromodulin was higher on ECM-coated surfaces than on uncoated surfaces in anterior pituitary cells and folliculostellate cells, but not in pericytes.

b. Fibromodulin expression at the transcription level was much higher on type I collagen-coated surfaces than on laminin-coated surfaces

c. Folliculostellate cells and pericytes exhibited canonical TGFβ2 signaling, which probably is associated with fibromodulin production.

   - Fibromodulin-expressing cells (folliculostellate cells and pericytes) in rat anterior pituitary gland partly possessed TGFβ receptor II and this receptor responded to TGFβ2 by showing Smad2 nuclear translocalization
   - TGFβ2 affected fibromodulin gene and protein expression in rat anterior pituitary cells in a dose-dependent manner
   - TGFβ2-induced fibromodulin expression was completely abolished by co-administration of TGFβ receptor I inhibitor

In sum, these studies showed that both ECMs and growth factor are important in regulating fibromodulin synthesis in anterior pituitary. Fibromodulin is important in modulating collagen fibrillogenesis, thus, these studies may highlight the importance of interaction between TGFβ signaling and fibromodulin in collagen regulation and may give a novel view about ECM-to-cell communication and cell-to-cell interaction within pituitary gland.

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This dissertation includes two articles published in peer reviewed journal, as follows:

Dissertation	Published article	Publication title/full citation
Experiment I: Fig. 1 Page 10 Fig. 2 Page 12 Fig. 3 Page 13 Fig. 4 Page 15	Published as: Fig. 1 Page 635 Fig. 2 Page 636 Fig. 3 Page 636 Supplementary Fig. S1	Laminin and collagen modulate expression of the small leucine-rich proteoglycan fibromodulin in rat anterior pituitary gland Rahimi Syaidah, Kotaro Horiguchi, Ken Fujiwara, Takehiro Tsukada, Motoshi Kikuchi, Takashi Yashiro Cell and Tissue Research 354; 633–638, 2013
Experiment II : Table 2 Page 23 Fig. 5 Page 25 Fig. 6 Page 28 Fig. 7 Page 30 Fig. 8 Page 32	Published as: Table 1 Page 172 Fig. 1 Page 175 Fig. 2 Page 176 Fig. 3 Page 177 Fig. 4 Page 177	Fibromodulin Expression in Folliculostellate Cells and Pericytes Is Promoted by TGFβ Signaling in Rat Anterior Pituitary Gland Rahimi Syaidah, Takehiro Tsukada, Morio Azuma, Kotaro Horiguchi, Ken Fujiwara, , Motoshi Kikuchi, Takashi Yashiro Acta Histochem Cytochem 49 (6): 171–179, 2016

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8. Declaration of Previous Publication

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Title
Syaidah R, Horiguchi K, Fujiwara K, Tsukada T, Kikuchi M, Yashiro T Laminin and collagen modulate expression of the small leucine-rich proteoglycan fibromodulin in rat anterior pituitary gland *Cell and Tissue Research* 354; 633–638, 2013
Syaidah R, Tsukada T, Azuma M, Horiguchi K, Fujiwara K, Kikuchi M, Yashiro T. Fibromodulin Expression in Folliculostellate Cells and Pericytes Is Promoted by TGFβ Signaling in Rat Anterior Pituitary Gland *Acta Histochem Cytochem* 49 (6): 171–179, 2016

April 15, 2017

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