Folliculostellate cell interacts with pericyte via TGFβ2 in rat anterior pituitary

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Abstract

The anterior pituitary gland comprises five types of endocrine cells plus non-endocrine cells including folliculostellate cells, endothelial cells, and capillary mural cells (pericytes). In addition to being controlled by the hypothalamic–pituitary–target organ axis, the functions of these cells are likely regulated by local cell and extracellular matrix (ECM) interactions. However, these complex interactions are not fully understood.

We investigated folliculostellate cell-mediated cell-to-cell interaction. Using S100β-GFP transgenic rats, which express GFP in folliculostellate cells, we designed a three-dimensional cell culture to examine the effects of folliculostellate cells. Interestingly, removal of folliculostellate cells reduced collagen synthesis (Col1α1 and Col3α1). Because pericytes are important collagen-producing cells in the gland, we stained for desmin (a pericyte marker). Removal of folliculostellate cells resulted in fewer desmin-positive pericytes and less desmin mRNA. We then attempted to identify the factor mediating folliculostellate cell–pericyte interaction. RT-PCR and in situ hybridization revealed that the important profibrotic factor transforming growth factor beta-2 (TGFβ2) was specifically expressed in folliculostellate cells and that TGFβ receptor II was expressed in pericytes, endothelial cells, and parenchymal cells. Immunocytochemistry showed that TGFβ2 induced SMAD2 nuclear translocation in pericytes. TGFβ2 increased collagen synthesis in a dose-dependent manner. This action was completely blocked by TGFβ receptor I inhibitor (SB431542). Diminished collagen synthesis in folliculostellate cell-deficient cell aggregates was partially recovered by TGFβ2. TGFβ2-mediated folliculostellate cell–pericyte interaction appears to be essential for collagen synthesis in rat anterior pituitary. This finding sheds new light on local cell–ECM interactions in the gland.

Introduction

Each cellular function is controlled by systemic regulators (endocrine, nerve, and immune systems) and local regulators. The latter include paracrine and autocrine factors and extracellular matrix (ECM) and are important in tissue formation, homeostasis, and regeneration (Hwang et al., 2009). The ECM is a composite of fibers,
adhesive glycoproteins, glycosaminoglycans, and other soluble proteins. These molecules are partially responsible for the mechanical properties of tissues and cell functions. The collagens are the most abundant ECM in the body and comprise more than 20 types. Type I and III collagens form insoluble fibers that define tissue stiffness and tensile strength. The fibrillar collagens also affect cellular functions such as cell morphology, migration, and proliferation (see the report by Gelse et al. 2003 for more information). The collagens are also important in providing these biophysical and biological characteristics to tissues and cells in the anterior pituitary gland.

Our group has identified various collagen types (type I, III, IV, and VI collagens) in rat anterior pituitary gland (Kaidzu et al. 2000). These collagens are distributed around endocrine cells and capillaries and support tissue structure. With respect to function, type I and/or type III collagens act on anterior pituitary cells to modulate hormone and ECM production (Kuchenbauer et al. 2001, Syaidah et al. 2013) and alter cellular behaviors (Toral et al. 2007, Horiguchi et al. 2010). It is generally accepted that fibroblasts and myofibroblasts are collagen-producing cells in many tissues; however, these cells have not been identified in the anterior pituitary gland. Recently, we investigated tissue-specific collagen-producing cells in rat anterior pituitary gland and found that desmin-immunopositive cells around capillaries, namely pericytes, produce collagen (Fujwara et al. 2010). Although the importance of collagens as a local regulator has been suggested, the regulatory mechanism underlying collagen synthesis in pericytes has not been studied.

The anterior pituitary gland has five types of endocrine cells plus folliculostellate cells, which do not produce adenohypophysial hormones. Folliculostellate cells are believed to act as stem/progenitor cells or phagocytes or to regulate hormone release (Inoue et al. 1999, Allaerts & Vankelecom 2005). This study designed a three-dimensional (3D) cell culture of rat anterior pituitary cells in the presence and absence of folliculostellate cells and investigated folliculostellate cell-mediated cell-to-cell interaction. Interestingly, we observed a novel cell-to-cell interaction between folliculostellate cells and pericytes in the gland and found that transforming growth factor-beta-2 (TGFβ2) acts as a mediator that promotes collagen synthesis in pericytes.

Materials and methods

Animals

Wistar rats were purchased from Japan SLC (Shizuoka, Japan). The S100β-green fluorescent protein (GFP) transgenic Wistar-crlj stain (S100β-GFP) of rat was produced by integrating the reporter gene GFP driven by the rat S100β promoter (Itakura et al. 2007) and was bred in our laboratory. Male rats of 8–10 weeks old weighing 250–300 g were used. Animals were given ad libitum access to food and water and were maintained at 22°C under a 12 h light:12 h darkness cycle. All animal experiments were carried out in a humane manner after receiving approval from the Institutional Animal Experiment Committee of Jichi Medical University and were conducted in accordance with the Institutional Regulations for Animal Experiments and Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions, under the jurisdiction of the Japanese Ministry of Education, Culture, Sports, Science and Technology.

Cell sorting

After deep anesthesia was induced with intraperitoneally injected pentobarbital sodium (Kyoritsu Seiyaku, Tokyo, Japan), 10–20 anterior pituitary glands were excised from S100β-GFP transgenic rats, after which anterior pituitary cells (approximately 1.0–1.5 × 10⁶ cells/gland) were isolated, as described previously (Horiguchi et al. 2008). The isolated cells were separated into GFP-positive cells (folliculostellate cells) and GFP-negative cells using a MoFlo XDP cell sorter (Beckman Coulter, Brea, CA, USA). About 1.0–2.0 × 10⁶ and 1.0–2.0 × 10⁵ cells/gland were collected for the GFP-positive and GFP-negative fractions respectively. The GFP-positive and GFP-negative cells were then processed for hanging drop 3D cell culture and reverse transcription polymerase chain reaction (RT-PCR) analysis (see below).

Hanging drop 3D cell culture

GFP-positive and GFP-negative cells, or isolated anterior pituitary cells from Wistar rats, were resuspended in cell culture media containing Medium 199 with Earle’s salts (Life Technologies) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 0.5 U/mL penicillin, and 0.5 μg/mL streptomycin (Life Technologies). The hanging drop method was used for 3D cell culture, as described previously (Tsukada et al. 2013). Briefly, a 25 μL drop containing 5000 cells was placed on the undersurface of 100 mm Petri dish lids, which were then cultured over sterile phosphate buffer saline (PBS) at 37°C in a humidified incubator with 5% CO₂. Regarding the GFP-positive and

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GFP-negative cells, the cells were mixed at a proportion of 0% GFP-positive cells (without folliculostellate cells) or 5% GFP-positive cells (with folliculostellate cells), a proportion of 5–10% folliculostellate cells is equivalent to that in normal adult rat anterior pituitary (Hosoya & Watanabe 1997, Perez-Castro et al. 2012) and then cultured in hanging drops. The resulting cell aggregates were processed for each experiment (see below). We previously confirmed that an in vivo-like folliculostellate cell meshwork and type I and III collagens had formed 5 days after plating (Tsukada et al. 2013). An IX71 inverted fluorescence microscope (Olympus) was used for observation of cell aggregates.

Transmission electron microscopy

After 5 days of 3D cell culture, 50–100 cell aggregates were collected in a 1.5 mL tube and centrifuged at 200 g for 5 min at room temperature. The pellets were immediately fixed with 2.5% glutaraldehyde (Merck) in 10mM phosphate buffer (PB) for 2 h. After washing in PB, the samples were postfixed in ice-cold 1% OsO4 in PB for 90 min. The samples were then dehydrated in an ethanol series and embedded in Quetol 812 epoxy resin (Nissin EM, Tokyo, Japan). Ultrathin sections were prepared, stained with 2% uranyl acetate and lead citrate, and then observed using an HT7700 transmission electron microscope (Hitachi).

Immunofluorescence microscopy

Around 20–30 cell aggregates were mounted on an MAS-coated glass slide (Matsunami Glass, Osaka, Japan) and immediately fixed with ice-cold 4% paraformaldehyde (PFA) in 50mM PB (pH 7.4) for 3 h. The cells were permeabilized in PBS containing 0.2% Triton X-100 (Sigma-Aldrich) for 20 min at room temperature and then incubated in blocking solution (2% normal goat serum in PBS) for 30 min at room temperature, after which they were incubated with primary antibodies for 90 min at 30°C. The primary antibodies included rabbit polyclonal anti-type I and anti-type III collagen and anti-desmin antibodies (see Table 1 for information on primary antibodies). The cells were then incubated with Alexa Fluor 568-conjugated goat anti-rabbit IgG (1:200; Life Technologies) in PBS for 30 min at 30°C. Cover slips were mounted onto the cells using Vectashield mounting medium containing 4’,6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Stained cells or GFP-expressing folliculostellate cells were subsequently analyzed with an FV1000 confocal laser microscope (Olympus). Images were processed for presentation using Photoshop CS5 (Adobe Systems). The absence of an observable nonspecific reaction was confirmed using normal rabbit serum.

In situ hybridization

After anesthesia, Wistar rats were perfused through the left ventricle with ice-cold 4% PFA in 50mM PB (pH 7.4) for 5 min. Pituitary glands were then excised and immersed in the same fixative for 24 h at 4°C, after which the tissues were immersed for 2 days in 50mM PB (pH 7.2) containing 30% sucrose at 4°C. The tissues were then embedded in Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo, Japan), and frozen sections (8 µm) were obtained using a cryostat (CM3000, Leica Microsystems, Wetzlar, Germany). In situ hybridization was performed with digoxigenin (DIG)-labeled cRNA probes, as described in our previous report (Fujiwara et al. 2007a). The following DNA fragments of rat TGFβ2 (NM_031131) and TGFβ receptor II (NM_031132) were amplified from rat pituitary cDNA by PCR (see Table 2 for primer information). Amplified cDNA fragments were ligated into the pGEM-T vector (Promega) and cloned. Gene-specific antisense or

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**Table 1** Information on primary antibodies used.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Type</th>
<th>Dilution</th>
<th>Source; Catalog number</th>
<th>References for validation of antibodies used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I collagen</td>
<td>Rabbit polyclonal</td>
<td>1:1000</td>
<td>LSL; LS-1102</td>
<td>Hagiwara et al. (2010)</td>
</tr>
<tr>
<td>Type II collagen</td>
<td>Rabbit polyclonal</td>
<td>1:1500</td>
<td>LSL; LS-1393</td>
<td>Hagiwara et al. (2010)</td>
</tr>
<tr>
<td>Desmin</td>
<td>Rabbit polyclonal</td>
<td>1:1000</td>
<td>Abcam; ab15200</td>
<td>Azuma et al. (2015)</td>
</tr>
<tr>
<td>Desmin</td>
<td>Mouse monoclonal</td>
<td>1:50</td>
<td>DAKO; Clone D33</td>
<td>Hamzah et al. (2008)</td>
</tr>
<tr>
<td>S100</td>
<td>Rabbit monoclonal</td>
<td>1:1000</td>
<td>DAKO; S100</td>
<td>Azuma et al. (2015)</td>
</tr>
<tr>
<td>SMAD2</td>
<td>Rabbit monoclonal</td>
<td>1:200</td>
<td>Cell Signaling Technology; S3395</td>
<td>Narimatsu et al. (2015)</td>
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</tbody>
</table>
sense DIG-labeled cRNA probes were generated using the Roche DIG RNA labeling kit (Roche Diagnostics). DIG-labeled cRNA probe hybridization was performed at 55°C for 16 h. Detection of each type of mRNA was performed with alkaline phosphatase-conjugated anti-DIG antibody (Roche Diagnostics) using 4-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Roche Diagnostics). 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. 2007b). The sections were incubated with rabbit polyclonal anti-S100 protein or desmin antibody (Table 1) for 90 min at 30°C and then with biotinylated anti-rabbit IgG (Vector Laboratories) and 3,3′-diaminobenzidine (Dojindo Laboratories, Kumamoto, Japan). The absence of an observable nonspecific reaction was confirmed using normal rabbit serum.

**RT-PCR**

After cell sorting, total RNA was extracted from GFP-positive cells (approximately 3.0–5.0 × 10^5 cells/extraction) and GFP-negative cells (approximately 1.0–2.0 × 10^6 cells/extraction) using an RNeasy mini kit and an RNase-free DNase set according to the manufacturer’s instructions (Qiagen). cDNA was synthesized using the PrimeScript RT reagent kit (Takara Bio) with oligo-(dT) 20 primer (Life Technologies). cDNAs were mixed with gene-specific primers (Table 2), Blend Taq DNA polymerase, buffer, and dNTPs according to the manufacturer’s instructions (TOYOBO, Osaka, Japan) and then subjected to 2 min at 94°C, 26–30 cycles of 30 s at 94°C, 30 s at 58°C, 30 s at 72°C, and an additional 7 min at 72°C in a GeneAmp PCR System 9700 (Applied Biosystems). The products were run on 1.5% agarose gels and visualized with ethidium bromide.

**Treatment of TGFβ2 and TGFβ receptor inhibitor (SB431542)**

Synthetic human TGFβ2 (PeproTech, Rocky Hill, NJ, USA) and selective TGFβ receptor I inhibitor (SB431542: Merck Millipore) were diluted in Hanks’ balanced salt solution (Life Technologies) containing 0.1% bovine serum albumin (BSA) and dimethyl sulfoxide (DMSO), respectively, and stored at −20°C until use. For 24-h treatment, 120–150 anterior pituitary 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 medium containing 100 nM synthetic TGFβ2 and 10 µM of SB431542, and the pituitary cell aggregates were cultured for another 24 h.

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

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Symbol</th>
<th>GenBank Acc. number</th>
<th>Forward sequence (5′–3′)</th>
<th>Reverse sequence (5′–3′)</th>
<th>Size (bp)</th>
<th>Use</th>
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<tr>
<td>Type I collagen</td>
<td>Col1a1</td>
<td>NM_053304</td>
<td>ACCCCAAGGAAGAAGACAT</td>
<td>GGCTGCAAAGATGATGCTCAT</td>
<td>110</td>
<td>Real-time PCR</td>
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<tr>
<td>Type III collagen</td>
<td>Col3a1</td>
<td>NM_023085</td>
<td>GGGTGGATGAGAAGGCAT</td>
<td>ACTGTGCAAGATGATGCTCAT</td>
<td>105</td>
<td>Real-time PCR</td>
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<tr>
<td>Desmin</td>
<td>Des</td>
<td>NM_022531</td>
<td>CAGGCTCTAGAGAGAGAGA</td>
<td>GCCTCTTGACGTGTCATC</td>
<td>113</td>
<td>Real-time PCR</td>
</tr>
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<td>TGFβ1</td>
<td>Tgf1</td>
<td>NM_021578</td>
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<td>GGTGGGAGCTGATCCCCAT</td>
<td>642</td>
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<td>TGFβ2</td>
<td>Tgf2</td>
<td>NM_031131</td>
<td>ACTCTACCTGACCCCTGCTG</td>
<td>GCGGACGATTTCTGAAGACAT</td>
<td>570</td>
<td>RT-PCR</td>
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<tr>
<td>TGFβ3</td>
<td>Tgf3</td>
<td>NM_013174</td>
<td>GAAAGCTGACATCAAGAGGAT</td>
<td>GCAAGCTCTCCCAAGTGC</td>
<td>682</td>
<td>cRNA probe</td>
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<tr>
<td>TGFβ receptor-I</td>
<td>Tgfr1</td>
<td>NM_012775</td>
<td>ACCTCTGATCATCCAGGTTG</td>
<td>AACGCCAGTGTTGAGAGAG</td>
<td>615</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>TGFβ receptor-II</td>
<td>Tgfr2</td>
<td>NM_031132</td>
<td>TCACGTGACCTGTCATGCG</td>
<td>ATCTGCGGTGTCCTGCTC</td>
<td>626</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>TGFβ receptor-III</td>
<td>Tgfr3</td>
<td>NM_017256</td>
<td>GCCGAGACCTGGCTCAGTG</td>
<td>TGTCCTCTCGGCTCGCTC</td>
<td>490</td>
<td>cRNA probe</td>
</tr>
<tr>
<td>S100β</td>
<td>S100b</td>
<td>NM_013191</td>
<td>TACTCCACACAGGGAGAGC</td>
<td>ATAGCACTCCCTGAGGAGC</td>
<td>681</td>
<td>RT-PCR</td>
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<tr>
<td>β-actin</td>
<td>Actb</td>
<td>NM_031144</td>
<td>TGCCACCCACACTTACATG</td>
<td>GGCTCACTTTTACCGGTTG</td>
<td>106</td>
<td>RT-PCR, real-time PCR</td>
</tr>
</tbody>
</table>

**Note:** Bolded sequences denote the antisense strand.
media, and all cell aggregates were replated 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 incubated for an additional 24 h at 37°C in a humidified incubator with 5% CO2. BSA (0.1%) and DMSO were diluted in the same manner and used as vehicle controls for TGFβ2 and SB431542 respectively.

For the 5-day treatment, anterior pituitary cells from Wistar rats and GFP-negative cells from S100β-GFP transgenic rats were resuspended in media containing TGFβ2 and SB431542 at the indicated concentrations respectively. The cells were cultured in hanging drops (120–150 drops/treatment) for 5 days at 37°C in a humidified incubator with 5% CO2.

SMAD2 nuclear translocalization analysis
Isolated anterior pituitary cells from Wistar rats were plated onto eight-well glass chamber slides (1 cm²/well; Nalge Nunc Int., Rochester, NY, USA) at a density of 4 × 10⁵ cells/cm² in 400 µL cell culture media and incubated for 3 days at 37°C in a humidified incubator with 5% CO2. After a 30-min treatment with TGFβ2 (50 ng/mL) or BSA, the cells were fixed in 4% PFA in 25 mM of PB (pH 7.4) for 20 min at room temperature. After permeabilization and blocking (see above), the cells were incubated with rabbit monoclonal anti-SMAD2 and mouse monoclonal anti-desmin antibodies (Table 1) for 90 min at 30°C, followed by Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:200, Life Technologies) and Alexa Fluor 568-conjugated goat anti-mouse IgG (1:200; Life Technologies) for 30 min at 30°C. Stained cells were mounted with DAPI and analyzed as described above. The absence of an observable nonspecific reaction was confirmed using normal rabbit and mouse sera.

Real-time PCR quantification of mRNA levels
After treatment with synthetic TGFβ and/or SB431542, total RNA from cell aggregates was extracted, and cDNA was synthesized as described above. Quantitative real-time PCR (ABI PRISM 7900HT; Applied Biosystems) was performed using gene-specific primers (Table 2) and SYBR Premix Ex Taq (Takara Bio) containing SYBR Green I. For normalization, β-actin (Actb: NM_031144) was quantified. All measurements were made in duplicate, and relative quantification was conducted using the standard curve method.

Statistical analysis
All results are presented as mean ± s.e.m. The unpaired Student’s t-test for two-group comparison or one-way analysis of variance (ANOVA) followed by Dunnett’s test for multiple comparisons was performed using Prism version 6 (GraphPad Software). A P value <0.05 was considered to indicate statistical significance.

Results
Folliculostellate cell–pericyte interaction in collagen synthesis
Effects of the absence of folliculostellate cells on cell aggregate formation Dispersed anterior pituitary cells from S100β-GFP rats were cultured with and without folliculostellate cells in hanging drops for 5 days, after which the resulting cell aggregates were examined by light microscopy (Fig. 1A). The aggregates containing folliculostellate cells had a round/oval shape with a smooth outer layer (Fig. 1Aa). In the absence of folliculostellate cells, the aggregates had an irregular shape, and the fringe of aggregates was not smooth (Fig. 1Ab). These morphological differences in cell aggregate formation were also observed in our previous study (Tsukada et al. 2014). Transmission electron microscopy was then performed to compare the morphological characteristics of aggregates with and without folliculostellate cells (Fig. 1B). In the aggregate with folliculostellate cells, fibrillar collagen with a periodic striation pattern was observed in the extracellular space (Fig. 1Ba and inset). In contrast, no extracellular structure was observed in the folliculostellate cell-deficient cell aggregates (Fig. 1Bb).

Effect of the absence of folliculostellate cells on collagen deposition and synthesis The major fibrillar collagens are the type I and type III collagens. We therefore stained the aggregates with isoform-specific anti-collagen antibodies (Fig. 2A). Although both type I and III collagens were observed in the presence of folliculostellate cells, type III collagen was predominant (Fig. 2Aa and c). Type III collagen was deposited throughout almost the entire extracellular space and exhibited a fibrillar structure. In the absence of folliculostellate cells, neither type I nor III collagen was detected (Fig. 2Ab and d). Consistent with these results, the gene expressions of type I and III collagens (gene symbols Col1a1 and Col3a1 respectively) were significantly lower in the absence of folliculostellate cells (Fig. 2B).
Effect of the absence of folliculostellate cells on collagen-producing cells

Our previous reports showed that pericytes, but not folliculostellate cells, produce type I and type III collagens in rat anterior pituitary gland (Fujii et al. 2010, Jindatip et al. 2013). To examine whether removal of folliculostellate cells affects collagen-producing cells, cell aggregates were

Figure 1
(A) Anterior pituitary cell aggregates with and without folliculostellate (FS) cells in hanging drop 3D cell culture. Anterior pituitary cells of S100β-GFP transgenic rats were used. The top panels are phase–contrast images of cell aggregates superimposed on fluorescence images of GFP-expressing folliculostellate cells (a, b). The bottom panels show folliculostellate cells (c, d). As in our previous study (Tsukada et al. 2014), cells formed round/oval aggregates with a smooth outer layer in the presence of folliculostellate cells (a); however, the fringe of folliculostellate cell-deficient cell aggregates was not smooth (b). Scale bar: 100 µm. (B) Electron micrographs of cell aggregates with and without folliculostellate cells. In the presence of folliculostellate cells (a), insoluble fibrillar architectures were observed in the extracellular space. The inset shows a magnified view of boxed area. The fibers had periodic striations, indicating deposition of fibrillar collagens (arrowheads). However, no fiber was observed in the aggregates without folliculostellate cells (b). Scale bars: 10 µm.

Figure 2
(A) Immunofluorescence of type I and III collagens. Anterior pituitary cells of S100β-GFP transgenic rats were used. Cell aggregates with folliculostellate (FS) cells (a, c) and without folliculostellate cells (b, d) were stained with anti-type I (a, b) and anti-type III collagen antibodies (c, d). Collagens, GFP-expressing folliculostellate cells, and nuclei are shown in red, green, and blue respectively. In the presence of folliculostellate cells, type I collagen deposition was scarce (a, arrowheads), whereas type III collagen deposition was clearly observed (c). However, no collagen deposition was detected in the absence of folliculostellate cells (b, d). Scale bar: 10 µm. (B) Relative mRNA concentration of type I and III collagens (Col1a1 and Col3a1 respectively) was evaluated by quantitative real-time PCR. Gray and white bars represent cell aggregates with and without folliculostellate cells respectively (n = 7, mean ± s.e.m.). Concentrations were normalized with β-actin (Actb) mRNA concentration. Col1a1 and Col3a1 expressions were lower in the absence of folliculostellate cells. *P < 0.05 (Student’s t-test).
stained for desmin, a pericyte marker. Pericytes were detectable in cell aggregates with and without folliculostellate cells (Fig. 3A); however, the number of pericytes was less in the absence of folliculostellate cells. To confirm the staining results, we performed real-time PCR (Fig. 3B), which showed that desmin expression was lower in the absence of folliculostellate cells.

**Folliculostellate cell–pericyte interaction via TGFβ2**

**Expression and localization of TGFβ isoforms and TGFβ receptors**  To determine whether folliculostellate cells secrete a factor that acts on pericytes and promotes collagen production, we examined expression of the major profibrotic cytokine TGFβ in folliculostellate cells. Among the three TGFβ isoforms (gene symbols Tgfb1–3), Tgfb2 was exclusively expressed in GFP-positive cells (folliculostellate cells), whereas Tgfb1 and Tgfb3 were expressed in GFP-positive and GFP-negative cells (Fig. 4). Tgfb1 expression was predominant in GFP-negative cells, and Tgfb3 expression was similar in GFP-positive and GFP-negative cells. Expressions of TGFβ receptor I and II (gene symbols Tgfbr1 and Tgfbr2), which form a heteromeric complex, were higher in GFP-negative cells than in GFP-positive cells, whereas TGFβ receptor III (gene symbol Tgfbr3) was undetectable in GFP-positive and GFP-negative cells (Fig. 4). In situ hybridization showed that Tgfbr2- and Tgfbr2-expressing cells were present in anterior pituitary gland (Fig. 5b and d). Tgfbr2-expressing cells formed a cell cluster in parenchymal cells and were costained with S100 protein (folliculostellate cell marker; Fig. 5c). In contrast, Tgfbr2-expressing cells were detected in parenchymal cells, endothelial cells, and perivascular cells (Fig. 5d and e). Tgfbr2-expressing perivascular cells were stained for desmin (pericytes, Fig. 5e). Next, we investigated the nuclear localization of SMAD2 protein in pericytes to determine whether TGFβ receptors in pericytes are functional. After 30-min treatment of synthetic TGFβ2 in anterior pituitary cells, SMAD2 was intensely stained in the nuclei of desmin-immunopositive cells (Fig. 6e and f) but diffusely stained in cytoplasm treated with vehicle (Fig. 6B and C).

**Effect of TGFβ2 and TGFβ receptor I inhibitor (SB431542) on collagen synthesis**  Anterior pituitary cell clusters from Wistar rats were treated with TGFβ2 for 24 h, and Col1a1 and Col3a1 expressions were examined by real-time PCR (Fig. 7). TGFβ2 significantly increased Col1a1 and Col3a1 expressions in a dose-dependent manner.
(Fig. 7A). In contrast, a 5-day treatment of TGFβ receptor I inhibitor significantly decreased Col3a1 expression in a dose-dependent manner (Fig. 7B). Although TGFβ receptor I inhibitor tended to decrease Col1a1 expression, the difference was not significant (Fig. 7B). TGFβ2-induced Col1a1 and Col3a1 expressions were completely abolished by co-administration with TGFβ receptor I inhibitor (Fig. 7C). When the folliculostellate cell-deficient cell aggregates were treated with TGFβ2 for 5 days, Col1a1 and Col3a1 expressions (which were reduced by removal of folliculostellate cells) were partially rescued (Fig. 8).

**Discussion**

This study using a 3D culture system highlights the importance of folliculostellate cells in collagen synthesis. Our previous study showed that 3D cell aggregate exhibited (1) highly elongated folliculostellate cells, (2) complex folliculostellate cell meshwork formation, and (3) dense collagen fiber deposition, all of which were observed in anterior pituitary in vivo (Tsukada et al. 2013). Because such in vivo-like cell and ECM architectures are not observed in conventional two-dimensional (2D) culture, the 3D culture system has the potential to elucidate cellular and molecular functions that cannot be adequately investigated by conventional 2D culture.

Type I and III collagens are important fibrillar collagens in rat anterior pituitary gland (Kaidzu et al. 2000). Several effects of collagen have been reported. Type I collagen decreases ACTH biosynthesis and inhibits proliferation of AtT-20 corticotroph tumor cells (Kuchenbauer et al. 2001). This study showed that cell aggregates without folliculostellate cells had less mRNA for type I and III collagens and no collagen deposition (Figs 1B and 2). This is the first report that folliculostellate cells are indispensable in collagen synthesis and deposition.
As shown in Fig. 3, the removal of folliculostellate cells reduced desmin-positive cells concomitantly with a reduction in desmin mRNA level. Desmin is expressed in pericytes in rat anterior pituitary gland (Fujiwara et al. 2010, Jindatip et al. 2013) and in cell aggregates (Supplementary Fig. 1, see section on supplementary data given at the end of this article). Thus, the present findings reveal a novel interaction between folliculostellate cells and pericytes in the gland. Pericytes are mural cells of capillaries and share a basement membrane with vascular endothelial cells (Shepro & Morel 1993). Because pericytes are important collagen-producing cells in the gland (Fujiwara et al. 2010), removal of folliculostellate cells might influence collagen production in pericytes, resulting in the absence of collagen deposition. However, because pericytes exhibit cellular plasticity (Armulk et al. 2005, Birbrair et al. 2013), it is not clear whether the reductions in desmin mRNA and protein in folliculostellate cell-deficient cell aggregates are attributable to trans differentiation into another cell type that does not express desmin, to loss of pericytes, or to reduced proliferative activity in pericytes.

Cell-to-cell communication is generally mediated by paracrine factors and/or juxtacrine factors and by gap junction-mediated intercellular interaction. In rat anterior pituitary gland, folliculostellate cells and pericytes do not have direct contact because of the basement membrane between them (Inoue et al. 1999). We hypothesized that a paracrine factor mediates the interaction and thus focused on the TGFβ family. The TGFβ family is known as profibrotic growth factor and...
The present RT-PCR analysis detected transcripts of TGF\(\beta\) receptor II in GFP-positive fractions (Fig. 4). Therefore, TGF\(\beta\) receptor II-expressing parenchymal cells may include lactotrophs and folliculostellate cells. To confirm whether pericytes can transduce TGF\(\beta\) signals, double immunocytochemistry for SMAD2 and desmin was performed. As shown in Fig. 6, immunoreactive SMAD2 was localized in the nuclei of pericytes when cultured anterior pituitary cells were in the presence of TGF\(\beta\)2, indicating that TGF\(\beta\)2 directly acts on pericytes through TGF\(\beta\) receptors I and II.

TGF\(\beta\) induces collagen synthesis in fibroblasts (Roberts et al. 1986). Thus, we examined whether TGF\(\beta\)2 has a similar action on pericytes and found that TGF\(\beta\)2 increased collagen synthesis in a dose-dependent manner and that TGF\(\beta\)2-induced collagen synthesis was completely blocked by SB431542, a TGF\(\beta\) receptor I inhibitor (Fig. 7A and C). These results suggest that canonical TGF\(\beta\) signals in pericytes are associated with collagen gene regulation. We also showed that SB431542 significantly reduced type III collagen synthesis (Fig. 7B). These results suggest that endogenous TGF\(\beta\)2 from folliculostellate cells affects collagen synthesis of pericytes in a 3D cell culture. Furthermore, reduction of collagen synthesis in folliculostellate cell-deficient cell aggregates was partially rescued by TGF\(\beta\)2 treatment, which suggests that loss of TGF\(\beta\)2 in folliculostellate cell-deficient cell aggregates reduced collagen synthesis. However, the collagen mRNA level could not be recovered to the level in aggregates containing folliculostellate cells; thus, an effect by a factor other than TGF\(\beta\)2 cannot be excluded.

In sum, we demonstrated that TGF\(\beta\)2-mediated interaction between folliculostellate cells and pericytes is important in collagen synthesis and deposition in rat anterior pituitary gland. In general, the functions of anterior pituitary cells are controlled by hormones from the hypothalamus and target organs. However, existing evidence indicates that surrounding cells and ECM also influence pituitary cell functions (Paez-Perepa et al. 2005, Le Tissier et al. 2012). Indeed, our recent study found that type I and III collagens act on folliculostellate cells to induce cell proliferation (Horiguchi et al. 2010) and small leucine-rich proteoglycan production (Syaidah et al. 2013). Past and present evidence, taken together, suggests the following explanation for folliculostellate cell-mediated cell and ECM interaction: 1) folliculostellate cells act on pericytes via TGF\(\beta\)2, 2) pericytes synthesize collagen in response to TGF\(\beta\)2, and 3) deposited collagens affect folliculostellate cell functions. Thus, folliculostellate cell, pericyte, and collagen interactions are mutually interdependent rather than independent.
Furthermore, our recent study identified another cell-to-cell interaction between folliculostellate cells and gonadotrophs in rat anterior pituitary gland (Tsukada et al. 2014). Gonadotrophs produce the major basement membrane component laminin (Holck et al. 1987, Ramadhani et al. 2012). We showed that humoral factor from folliculostellate cells is required for the release of laminin from gonadotrophs (Tsukada et al. 2014). We also observed various laminin actions on folliculostellate cells, such as proliferation, migration, and gap junction formation, as well as production of ECM and matrix metalloproteinase (Horiguchi et al. 2010, 2011, 2012, Ilmiawati et al. 2012, Syaidah et al. 2013). Thus, folliculostellate cells, gonadotrophs, and laminin are also interdependent. These past and present findings reveal much about complex cell-to-cell and/or cell-to-ECM interactions in the anterior pituitary gland. Nevertheless, additional studies are needed if we are to understand fully the local interactions in the anterior pituitary.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-16-0033.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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