Caveolin 3-mediated integrin β 1 signaling is required for the proliferation of folliculostellate cells in rat anterior pituitary gland under the influence of extracellular matrix

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Abstract

Folliculostellate (FS) cells in the anterior pituitary gland are believed to have multifunctional properties. Using transgenic rats that express green fluorescent protein (GFP) specifically in FS cells in the anterior pituitary gland (S100b-GFP rats), we recently revealed that FS cells in primary culture exhibited marked proliferation in the presence of laminin, an extracellular matrix (ECM) component of the basement membrane. In a process referred to as matricrine action, FS cells receive ECM as a signal through their receptors, which results in morphological and functional changes. In this study, we investigated matricrine signaling in FS cells and observed that the proliferation of FS cells is mediated by integrin β 1, which is involved in various signaling pathways for cell migration and proliferation in response to ECM. Then, we

analyzed downstream events of the integrin β 1 signaling pathway in the proliferation of FS cells and identified caveolin 3 as a potential candidate molecule. Caveolin 3 is a membrane protein that binds cholesterol and a number of signaling molecules that interact with integrin β 1. Using specific small interfering RNA of caveolin 3, the proliferation of FS cells was inhibited. Furthermore, caveolin 3 drove activation of the mitogen-activated protein kinase (MAPK) signaling cascades, which resulted in upregulation of cyclin D1 in FS cells. These findings suggest that matricrine signaling in the proliferation of FS cells was transduced by a caveolin 3-mediated integrin β 1 signaling pathway and subsequent activation of the MAPK pathway.

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Introduction

The anterior pituitary gland is composed of five types of hormone-producing cells and folliculostellate (FS) cells that do not produce classical anterior pituitary hormones. These cell aggregations form cell cords or clusters surrounded by different types of extracellular matrices (ECM) that provide the mechanical integrity, rigidity, and elasticity that are essential for these cells to perform their respective roles (Soji & Herbert 1989, Kaidzu *et al.* 2000, Paez-Pereda *et al.* 2005). FS cells have a star-like appearance and form a pseudolumen at the central portion of FS cell clusters (Soji & Herbert 1989). Physiologically, it has been speculated that FS cells may be stem cells, phagocytes, or regulating cells for hormone release (Allaerts & Vankelecom 2005) and that they accomplish cell-to-cell communication via the gap junction in the anterior pituitary gland (Fauquier *et al.* 2001, Sato *et al.* 2005).

Generally, cells can receive ECM as a signal that causes changes such as migration, proliferation, and differentiation – a

process referred to as ‘matricrine’ action (Miyamoto *et al.* 2007). In our recent attempt to investigate matricrine action in anterior pituitary cells, FS cells markedly extended their cytoplasmic processes and promoted gap junction formation between them under the influence of laminin that is an ECM component of the basement membrane (Horiguchi *et al.* 2010, 2011). In addition, we showed that FS cells exhibited marked proliferation in the presence of ECM (Horiguchi *et al.* 2010). However, it is unclear at present how FS cells receive laminin as a signal and exhibit these phenomena, which is crucial for understanding matricrine action in FS cells. In this study, we, therefore, attempted to determine the matricrine signaling pathway in the proliferation of FS cells. First, we examined whether proliferation of FS cells in the presence of laminin is mediated by integrin β 1 as a laminin receptor. Then, we analyzed the downstream events of the integrin β 1 signaling pathway with mediation by caveolin (Echarri *et al.* 2007), which is a membrane protein that binds cholesterol and a number of signaling molecules potentially linked with integrin function.

Materials and Methods

Animals

Transgenic S100b-green fluorescent protein (GFP) rats express GFP under control of the promoter of the S100b protein gene, a marker of FS cells. The rats were donated by Prof. K Inoue of Saitama University and were bred in our laboratory (Itakura *et al.* 2007). Male rats aged 8–10 weeks weighing 250–300 g were given *ad libitum* access to food and water and housed under conditions of 12 h light:12 h darkness. The rats were killed by exsanguination from the right atrium under deep Nembutal anesthesia and were then perfused with Ca^{2+} - and Mg^{2+} -free Hank's solution for primary culture. All animals were treated in accordance with the Guidelines for Animal Experimentation of Jichi Medical University, which are based on the NIH Guidelines for the Care and Use of Laboratory Animals.

Cell culture

Anterior pituitary cells of male S100b-GFP rats were dispersed as described previously (Horiguchi *et al.* 2008). Dispersed cells were separated into GFP-positive and -negative cells by a cell sorter (MoFlo XDP: Beckman Coulter, Inc., Fullerton, CA, USA). GFP-positive 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 $\mu\text{g}/\text{cm}^2$ of laminin (Millipore, Bedford, MA, USA), at a density of 1×10^5 cells/ cm^2 in 400 μl of Medium 199 with Earle's salts (Invitrogen) supplemented with 10% FBS (Sigma–Aldrich Corp.), 0.5 U/ml penicillin, and 0.5 $\mu\text{g}/\text{ml}$ streptomycin (Invitrogen). In the other experimental group, laminin-coated slides were treated in medium containing 5 $\mu\text{g}/\text{ml}$ monoclonal anti-integrin $\beta 1$ antibody (clone Ha2/5, BD Biosciences, San Jose, CA, USA), which was shown to block signaling mediated by integrin $\beta 1$ (Schultz & Armant 1995); hamster IgM served as control. Cells were then cultured for 72 h at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

Proliferation assay

To visualize the proliferative activities of FS cells, the nucleotide analog 5-bromo-2'-deoxyuridine (BrdU; Sigma–Aldrich Corp.) was added to primary culture for 24 h at a concentration of 3 $\mu\text{g}/\text{ml}$. Cells were fixed in 4% paraformaldehyde in 25 mM phosphate buffer (pH 7.4) for 20 min at room temperature and were then treated with 4 M HCl in PBS for 10 min. Cells were incubated in PBS containing 2% normal goat serum for 1 h at 30 °C and were then incubated with anti-rat BrdU mouse monoclonal antibody (1.5 $\mu\text{g}/\text{ml}$, Sigma–Aldrich Corp.) for 90 min at 30 °C. After washing with PBS, cells were incubated in PBS with Alexa Fluor 568-conjugated goat anti-mouse IgG (Invitrogen) diluted to 1:200. The absence of an observable nonspecific reaction was

confirmed using normal mouse serum. In total, ten random fields were imaged per well using a confocal laser microscope with a 60-fold objective lens. The percentage of immunoreactive cells was calculated by counting at least 100 cells/well. The observations were done three times for each experimental group.

Immunoblot analysis

After primary culture, cells were washed in PBS and lysed in RIPA buffer (20 mM Tris, 150 mM NaCl, 2 mM EDTA, 0.1% w/v SDS, and 1% v/v Triton X-100, pH 7.5), and total protein was estimated by using the Bradford assay reagent (Sigma). From each sample, 20 μg protein was applied to 12% SDS-PAGE. Proteins were then electrophoretically transferred to Immobilon-P transfer membrane (Millipore). The membrane was blocked with 5% (w/v) nonfat dried milk in TBST (50 mM Tris, 100 mM NaCl, 0.1% v/v Tween 20, pH 7.4) for 1 h. After washing with TBST, the membrane was incubated overnight with mouse monoclonal caveolin 1 (12.5 ng/ml; BD Biosciences) or caveolin 3 (5 ng/ml; BD Biosciences), or rabbit polyclonal cyclin D1 (1:2000; NeoMarkers, Inc., Fremont, CA, USA), ERK1/2 (1:3000; Assay Designs, Ann Arbor, MI, USA), or phospho-ERK1/2 (1:5000; R&D Systems, Inc., Minneapolis, MN, USA) antibody, diluted in Can Get Signal solution (Toyobo, Osaka, Japan), followed by TBST washes, and incubated for 1 h with HRP-labeled secondary antibodies (Envision+ System-HRP, anti-rabbit, Dako, Glostrup, Denmark). After washing with TBST, specific immunoreactivity was visualized using Chemiluminescence ECL Plus Systems (GE Healthcare, Mississauga, ON, Canada) with lumi-shot film (Fujifilm, Tokyo, Japan). The film was scanned and densitometric analysis was performed with ImageJ software (NIH, Bethesda, MD, USA). The results were normalized with β -actin. Each analysis was performed in triplicate.

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

Total RNA fractions were prepared with Trizol (Invitrogen) from cultured cells and incubated with RNase-free DNase I (1 U/tube; Promega Corp.). After inactivation of DNase I by heating for 10 min at 65 °C, cDNA was synthesized using the PrimeScript RT reagent kit (Takara, Otsu, Japan) with oligo-(dT)₂₀ primer (Invitrogen). Quantitative real-time PCR (ABI PRISM 7900HT; Applied Biosystems, Carlsbad, CA, USA) was performed by using gene-specific primers and SYBR Premix Ex Taq (Takara) containing SYBR Green I. The following primers were used to amplify cDNA fragments of cyclin D1 (GenBank accession no. NM_171992): forward 5'-TGCAAATGGAACGTGCTTCTG-3' and reverse 5'-GCGGATGATCTGCTTGTTCT-3' (125 bp); caveolin 1 (NM_031556): forward 5'-GGGAACAGGGCAACATCTAC-3' and reverse 5'-GCGGTTGACCAGATCAATTT-3' (107 bp); caveolin 2 (NM_131914): forward

5'-GGATCTGATCGCAGAACCCTC-3' and reverse 5'-CA-AGGGGATGGCTAGAAACA-3' (127 bp); caveolin 3 (NM_019155): forward 5'-GGTGAACAGAGACCCCAA-GA-3' and reverse 5'-ACGCCATCGAAGCTGTAAGT-3' (99 bp). For normalization, we also quantified glyceraldehyde 3-phosphate dehydrogenase (GAPDH, M_17701): forward 5'-AAGGGCTCATGACCACAGTC-3' and reverse 5'-GG-ATGCAGGGATGATGTTCT-3' (116 bp). Relative quantification was conducted using the standard curve method and was performed at least three times.

Induction of caveolae depletion

The GFP-positive cells were cultured for 72 h on the laminin-coated surface as described above. The culture medium was changed to 400 µl Medium 199 with Earle's salts (Invitrogen) supplemented with 10% FBS (Sigma-Aldrich Corp.), 0.5 U/ml penicillin, 0.5 µg/ml streptomycin (Invitrogen), and 10 mM methyl-β-cyclodextrin (MβCD; WAKO, Osaka, Japan; Bellott *et al.* 2005, Chung *et al.* 2009) and cultured for 8 h. MβCD was used at a concentration of 10 mM because this concentration has been shown to markedly disrupt caveolae structure in cultured cells, without affecting cell viability, by removing cholesterol from the plasma membrane (Ushio-Fukai *et al.* 2001). After washing with Medium 199, the culture medium was changed without MβCD for 40 h. The culture medium without MβCD was used as a negative control. We time-lapse recorded the cells as described previously (Horiguchi *et al.* 2010, 2011) using a digital camera (ORCA-ER; Hamamatsu Photonics, Shizuoka, Japan) and MetaMorph software (Molecular Devices Corp., Downingtown, PA, USA). Each observation was performed in triplicate.

Small interfering RNA for caveolin 1 and 3

For small interfering RNA (siRNA) transfection, the culture medium was replaced by 400 µl Medium 199 with Earle's salts (Invitrogen) supplemented with 10% FBS (Sigma-Aldrich Corp.), 0.5 U/ml penicillin, 0.5 µg/ml streptomycin (Invitrogen), siRNA against caveolin 1 or 3 mRNA (0.2 µM, Qiagen), and INTERFERin (1:100 v/v, PolyPlus Transfections, Inc., New York, NY, USA) transfection reagent at 24 h. Nonsilencing siRNA (Qiagen) with no homology to any known mammalian gene was used as a negative control. After that, the GFP-positive cells were cultured for 48 h. Each analysis was performed at least three times.

Immunocytochemistry

Cultured cells fixed with 4% paraformaldehyde in 50 mM phosphate buffer for 20 min at room temperature were first immersed in PBS containing 2% normal goat serum for 20 min at 30 °C and then incubated overnight with anti-rat caveolin 3 mouse monoclonal antibody (12.5 ng/ml, BD Biosciences) at room temperature. After washing with PBS, cells were incubated in PBS with Alexa Fluor 568-conjugated

goat anti-mouse IgG diluted to 1:200. Absence of an observable nonspecific reaction was confirmed using normal mouse serum. Cells were scanned using a confocal laser microscope (FV1000, Olympus Corp., Tokyo, Japan).

Immunohistochemistry

Pituitary glands were carefully excised and fixed overnight in 4% paraformaldehyde in 50 mM phosphate buffer (pH 7.4) at 4 °C. Tissues were then immersed in phosphate buffer (pH 7.2) containing 30% sucrose for 2 days at 4 °C, embedded in Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo, Japan), and frozen rapidly. Frozen frontal sections (8 µm thick) were obtained using a cryostat (CM3000; Leica Microsystems, Wetzlar, Germany) and were mounted on slide glasses. Sections were incubated in PBS containing 2% normal goat serum for 20 min at 30 °C and were then incubated overnight with mouse anti-rat caveolin 3 monoclonal antibody (12.5 ng/ml; BD Biosciences) at room temperature. After washing with PBS, sections were incubated in PBS with Alexa Fluor 568-conjugated goat anti-mouse IgG (Invitrogen) diluted to 1:200 and were washed with PBS again. Sections were scanned using a confocal laser microscope.

Statistical analysis

All results were presented as mean ± S.E.M. Statistical analysis was performed using ANOVA with Fisher's protected least significant difference test (StatView 5.0 software, SAS Institute, Inc., Cary, NC, USA) and Dunnett's test. Differences between groups were considered to be statistically significant at a *P* value of <0.05.

Results

Role of integrin β1 in laminin-induced FS cell proliferation

A cell sorter was used to isolate FS cells from male S100b-GFP rat anterior pituitary for primary culture. The histological features of FS cells in primary culture were the same as in our previous study (Horiguchi *et al.* 2011). At 72 h, FS cells on the uncoated surface and laminin-coated surface with integrin β1 antibody formed clusters of ~10–20 cells (Fig. 1A and C). However, almost all FS cells on laminin with or without hamster IgM were flattened and had interconnected cell bodies (Fig. 1B and D). The percentage of BrdU-positive cells among all FS cells was 14.9 ± 0.7 and 13.9 ± 0.5% on the laminin-coated surface with and without hamster IgM respectively (Figs 1J, L and 2A). In contrast, BrdU-positive FS cells were not observed on laminin-coated surfaces with integrin β1 antibody or on uncoated surface (Figs 1I, K, and 2A). Then, we examined whether ERK activation and cyclin D1 expression level were regulated by integrin β1. Using western blot analysis, we detected pERK, ERK, and cyclin D1 immunoreactivity,

Figure 1 Proliferation of FS cells in primary culture. (A–D) GFP images superimposed on phase-contrast images on the uncoated surface, laminin-coated surface, laminin-coated surface with integrin $\beta 1$ antibody, and laminin-coated surface with hamster IgM respectively. Scale bars, 100 μm . (E–H) Phase-contrast image of the same field shown in I–L on the uncoated surface, laminin-coated surface, laminin-coated surface with integrin $\beta 1$ antibody, and laminin-coated surface with hamster IgM respectively. (I–L) Confocal image of BrdU incorporation (green, FS cells and red, BrdU).

at their expected masses, in FS cells (Fig. 2B). When normalized with β -actin, the phosphorylation of ERK and the production of cyclin D1 were greater in FS cells on laminin-coated surfaces with or without hamster IgM than on the uncoated and laminin-coated surfaces with integrin $\beta 1$ antibody (Fig. 2C and D).

Change in caveolin mRNA and protein expression level in FS cells on laminin

To determine whether laminin affects gene expression of caveolins in FS cells, we performed quantitative real-time RT-PCR. As compared with the uncoated surface, caveolin 1 and 3 expression was higher in FS cells on the laminin-coated surface (Fig. 3A and C). However, their expression levels on the laminin-coated surface with anti-integrin $\beta 1$ antibody were lower than those on the laminin-coated surface with hamster IgM. Caveolin 2 expression was not higher in cells on the laminin-coated surface compared with the uncoated surface (Fig. 3B). On western blot analysis, caveolin 1 and 3 immunoreactivity was greater in FS cells on the laminin-coated surface (Fig. 3D–F). In contrast, treatment with anti-integrin $\beta 1$ antibody completely blocked the increases in caveolin 1 and 3 immunoreactivity in FS cells on the laminin-coated surface with hamster IgM (Fig. 3D–F).

Time-lapse imaging of FS cells in primary culture with M β CD

To examine the importance of caveolin protein in the proliferation of FS cells under the influence of laminin, FS

cells were exposed to M β CD, a cholesterol-depleting agent that disassembles caveolae (Bellott *et al.* 2005, Chung *et al.* 2009), from 72 to 80 h in primary culture. M β CD treatment inhibited proliferation of FS cells (Fig. 4A, B and Supplementary Movie 1, see section on supplementary data given at the end of this article). After M β CD was removed from medium in primary culture at 80 h, FS cells resumed proliferation (Fig. 4C and Supplementary Movie 2, see

Figure 2 Analysis of proliferation of FS cells in primary culture. (A) Percentage of BrdU-positive cells among FS cells on the uncoated surface (uncoated), laminin-coated surface (laminin), laminin-coated surface with integrin $\beta 1$ antibody (laminin Itgb-1 AB), and laminin-coated surface with hamster IgM (laminin Ham IgM). ND, not determined. (B) The amount of protein was determined by western blotting. The four panels show pERK, ERK, cyclin D1, and β -actin. (C and D) pERK and cyclin D1 protein levels in FS cells on the uncoated surface (uncoated), laminin-coated surface (laminin), laminin-coated surface with integrin $\beta 1$ antibody (laminin Itgb-1 AB), and laminin-coated surface with hamster IgM (laminin Ham IgM) respectively. Western blotting data from three experiments (mean $\pm$ S.E.M., $n=3$) were quantified by densitometry and normalized with β -actin. pERK and cyclin D1 protein levels were calculated as ratios of uncoated values respectively. * $P<0.05$.

Figure 3 Caveolin expression levels in primary culture of FS cells after 72-h incubation. (A–C) The expression of caveolin 1, 2, and 3 mRNA determined by real-time PCR was normalized with an internal control (GAPDH) respectively (mean $\pm$ S.E.M., $n=5$). Caveolin 1, 2, and 3 mRNA levels were calculated as ratios of uncoated values respectively. Caveolin 1 and 3 mRNA expression on the laminin-coated surface (laminin) and laminin-coated surface with hamster IgM (laminin Ham IgM) was higher than that on the uncoated surface (uncoated). (D) The amount of protein was determined by western blotting. The three panels show caveolin 1 (Cav 1), caveolin 3 (Cav 3), and β -actin. (E and F) Caveolin 1 and 3 protein levels in FS cells on the uncoated surface (uncoated), laminin-coated surface (laminin), laminin-coated surface with integrin $\beta 1$ antibody (laminin IgG-1 AB), and laminin-coated surface with hamster IgM (laminin Ham IgM) respectively. Western blotting data from three experiments (mean $\pm$ S.E.M., $n=3$) were quantified by densitometry and normalized with β -actin. Caveolin 1 and 3 protein levels were calculated as ratios of uncoated values respectively. * $P<0.05$.

section on [supplementary data](#) given at the end of this article). However, FS cells on a laminin-coated surface without M β CD from 72 to 120 h continued proliferating (Fig. 4D–F).

Knockdown of caveolin 1 and 3 by siRNA

To examine the functional role of caveolin 1 and 3 in the proliferation of FS cells, we downregulated caveolin 1 and 3 gene expression using siRNA in FS cells of primary culture on laminin-coated surfaces. When caveolin 1 and 3 were downregulated with a specific siRNA, their respective expression levels were also downregulated (Fig. 5A–D). Quantitative analysis revealed that cyclin D1 mRNA level in caveolin 3 siRNA-treated FS cells was lower than that in caveolin 1 siRNA-treated and control FS cells (Fig. 5E). The percentage of BrdU-positive cells among caveolin 3 siRNA-treated FS cells was also lower than that among caveolin 1 siRNA-treated and control FS cells (Fig. 5F).

Localization of caveolin 3 on FS cells

We examined the localization of caveolin 3 by immunocytochemistry at 72 h in primary culture on both the uncoated and the laminin-coated surfaces. Immunoreactive caveolin 3 was located on cell bodies and cytoplasmic processes at FS cells on both the uncoated and the laminin-coated surfaces (Fig. 6A–D). Caveolin 3 immunoreactivity of FS cells was clearly greater on the laminin-coated surface than on the uncoated surface (Fig. 6B and D). We also detected the localization of caveolin 3 by immunohistochemistry in rat anterior pituitary gland. Immunoreactive caveolin 3 was located on FS cells in the anterior pituitary gland (Fig. 6E).

Discussion

In this study, we revealed that matricrine signaling of the proliferation in FS cells was transduced by the association of caveolin 3 with integrin $\beta 1$, which promotes mitogen-activated protein kinase (MAPK).

By using transgenic rats (S100b-GFP rats) that express GFP specifically in FS cells in the anterior pituitary gland (Itakura *et al.* 2007), we were able to use a cell sorter to obtain pure GFP-positive FS cells, which allowed us to examine the direct involvement of ECM on FS cells. As shown in Fig. 1, isolated FS cells directly receive laminin as a signal and thus proliferate in the absence of GFP-negative cells, which are primarily hormone-producing cells.

ECM generally transduces signals to cells through a family of transmembrane receptors called integrins (Hynes 2002). Many reports suggest that integrin signaling induces proliferation of cells, including mesangial cells, mammary

Figure 4 Time-lapse images of FS cells isolated from male S100b-GFP rat anterior pituitary cells in primary culture on laminin-coated surface with or without 10 μ M M β CD. Cells were time-lapse recorded at 15 min intervals from 72 to 120 h after cells were plated. (A–F) GFP images superimposed on phase-contrast images on the laminin-coated surface. (A) 72 h, (B) 80 h of the same field shown in 72 h with M β CD for 8 h incubation. (C) 120 h of the same field shown in 80 h, after M β CD was removed for 40 h incubation. (D–F) 72, 80, and 120 h without M β CD. The elapsed time from plating of cells was 72, 80, and 120 h respectively. Scale bar, 100 μ m.

Figure 5 Downregulation of caveolin 1 and 3 by siRNA. (A and B) The expression of caveolin 1 and 3 mRNA determined by real-time PCR was normalized with an internal control (GAPDH) for 48 h incubation after FS cells were transfected by siRNA respectively (mean $\pm$ S.E.M., $n=4$). Caveolin 1 and 3 mRNA levels (Cav 1 siRNA and Cav 3 siRNA) were calculated as ratios of control values (control). Caveolin 1 and 3 mRNA expression of experimental group was lower than that of control. (C and D) The protein levels of caveolin 1 and 3 were determined by western blotting. The upper panel shows caveolin 1 (Cav 1) or 3 (Cav 3) and the lower panel shows β -actin as the loading control. Western blotting data from three experiments (mean $\pm$ S.E.M., $n=3$) were quantified by densitometry and normalized with β -actin. The protein level of caveolin 1 and 3 in FS cells of siRNA treatment (Cav 1 siRNA and Cav 3 siRNA) was significantly lower than that of control (control). (E) Expression of cyclin D1 mRNA determined by real-time PCR was normalized with an internal control (GAPDH) for 48 h incubation after FS cells were transfected by siRNA. Cyclin D1 mRNA levels in FS cells of caveolin 1 and 3 siRNA treatment (Cav 1 siRNA and Cav 3 siRNA) were calculated as ratios of control values (control) respectively (mean $\pm$ S.E.M., $n=4$). (F) The percentage of BrdU-positive cells among FS cells on the laminin-coated surface with siRNA of caveolin 1 and 3 (Cav 1 siRNA and Cav 3 siRNA) respectively. The number of BrdU-positive cells with siRNA of caveolin 3 was significantly lower than that of control (control). * $P<0.05$.

epithelial cell, and mesenchymal cells, in the presence of ECM (Schöcklmann *et al.* 2000, Li *et al.* 2005, Fernandes *et al.* 2006). Integrins comprise an α -subunit and a β -subunit that form a heterodimer. In mammals, 18 types of α -subunits and eight types of β -subunits are known; their various combinations give rise to 24 integrin heterodimers that differ in ligand specificity (Hynes 2002). With respect to these ligand specificities, we recently reported that FS cells bind laminin through integrin- $\alpha 3\beta 1$ and/or integrin- $\alpha 6\beta 1$ in the

anterior pituitary gland (Horiguchi *et al.* 2010). As shown in Fig. 2, an antibody that inhibits the integrin $\beta 1$ cascade blocked the increase of ERK phosphorylation and cyclin D1 production, which suggests that integrin $\beta 1$ receives laminin as a signal on FS cells and that its signaling activates MAPK signaling cascades, leading to cyclin D1 transcription and contributing to cell cycle progression. However, there are several signal transduction molecules between integrin $\beta 1$ and MAPK, including focal adhesion kinase (Li *et al.* 2001, Ding *et al.* 2009), protein kinase C (Han *et al.* 2004, Kudirka *et al.* 2007), and caveolin (Echarri *et al.* 2007). Caveolin is a cholesterol-binding membrane protein component of caveolae that are small (50–100 nm) cell invaginations that play a role in numerous cell functions (Parton 2001). Caveolin isoforms 1 and 3 are involved in the formation of

Figure 6 Caveolin 3 expression in anterior pituitary gland. (A–D) Immunocytochemistry for caveolin 3 in primary culture of FS cells; (A and B) 72 h culture of FS cells on the uncoated surface; (C and D) 72 h culture of FS cells on the laminin-coated surface; (A and C) phase-contrast images; (B and D) fluorescent images. (E) Immunohistochemistry of caveolin 3 in the anterior pituitary gland. Caveolin 3 (red) immunoreactivity was observed on FS cells (green). Scale bar, 10 μ m.

caveolae and interact with integrin $\beta 1$ signaling molecules via a specific domain (Echarri *et al.* 2007). Caveolin 3 is expressed in smooth and skeletal muscle cells, cardiac myocytes, and astrocytes (Song *et al.* 1996, Ikezu *et al.* 1998). As shown in Figs 3 and 6, FS cells in anterior pituitary also expressed caveolin 3, and its expression level increased in the presence of laminin. Furthermore, the proliferation of FS cells was inhibited by M β CD, which abolishes the distribution of caveolin protein, and by siRNA specific for caveolin 3 (Figs 4 and 5). These results suggest that integrin $\beta 1$ signaling in the proliferation of FS cells was mediated by caveolin 3. We recently reported that hormone-producing cells also expressed integrin $\beta 1$ and showed almost no proliferation in the presence of laminin (Horiguchi *et al.* 2010). Thus, the difference in response to laminin must be due to the expression of caveolin 3. Our findings in this study showed that the caveolin 3-mediated integrin $\beta 1$ signaling pathway was specific to FS cells in anterior pituitary. Integrin $\beta 1$ signaling in the presence of laminin also triggered an increase in gap junction formation between FS cells in our previous report (Horiguchi *et al.* 2011). There are some reports that caveolin 3 upregulated the expression of connexin 43 in gap junction channel protein (Chung *et al.* 2009, Liao *et al.* 2010). This leads us to speculate that caveolin 3 plays a role in gap junction formation between FS cells. In anterior pituitary gland, it has been shown that the lobular structures surrounded by basement membrane are responsible for organizing the functional unit and that the cytoplasmic processes of FS cells attach to the basement membrane (Soji & Herbert 1989, Shirasawa *et al.* 2004). In addition to these histological features, we hypothesize that FS cells exert matricrine action via caveolin 3-mediated signaling for their functional roles.

In this study, we observed increased expression of not only caveolin 3 but also caveolin 1, under the influence of laminin in FS cells (Fig. 3). However, caveolin 1 has no effect on the proliferation of FS cells (Fig. 5). Kurzchalia *et al.* (1992) showed that caveolin 1 is localized to plasma membrane caveolae, the Golgi apparatus, and trans-Golgi-derived transport vesicles. In a previous study, we confirmed by electron microscopy that, in the presence but not in the absence of laminin, FS cells displayed well-developed Golgi apparatus that consisted of several layers of Golgi lamellae and many vesicles and vacuoles in the Golgi area (Horiguchi *et al.* 2011). These results suggest that the increase in caveolin 1 is due to the hyperfunctional status of FS cells under the influence of laminin.

In a series of studies investigating matricrine action in FS cells (Horiguchi *et al.* 2010, 2011), we have shown that FS cells in the presence of laminin may play important functional roles in the anterior pituitary gland. In this study, we have succeeded in elucidating the specific mechanism of matricrine action in FS cells. Our findings suggest that proliferation of FS cells is induced by a caveolin 3-mediated integrin $\beta 1$ signaling pathway, leading to activation of the MAPK pathway in the anterior pituitary gland.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-11-0103>.

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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