Matrix metalloproteinase-9 expression in folliculostellate cells of rat anterior pituitary gland

Cimi Ilmiawati, Kotaro Horiguchi, Ken Fujiwara and **Takashi Yashiro**

Division of Histology and Cell Biology, Department of Anatomy, Jichi Medical University School of Medicine, 3311-1 Yakushiji, Shimotsuke, Tochigi 329-0498, Japan

(Correspondence should be addressed to T Yashiro; Email: tyashiro@jichi.ac.jp; C Ilmiawati; Email: d1030@jichi.ac.jp)

Abstract

Folliculostellate (FS) cells of the anterior pituitary gland express a variety of regulatory molecules. Using transgenic rats that express green fluorescent protein specifically in FS cells, we recently demonstrated that FS cells *in vitro* showed marked changes in motility, proliferation, and that formation of cellular interconnections in the presence of laminin, a component of the extracellular matrix, closely resembled those observed *in vivo*. These findings suggested that FS cells express matrix metalloproteinase-9 (MMP-9), which assists their function on laminin. In the present study, we investigate MMP-9 expression in rat anterior pituitary gland and examine its role in motility and proliferation of FS cells on laminin. Immunohistochemistry, RT-PCR, immunoblotting, and gelatin zymography were performed to assess MMP-9 expression in the anterior pituitary gland and cultured FS cells. Real-time RT-PCR was used to quantify MMP-9 expression in cultured FS cells under different conditions and treatments. MMP-9 expression was inhibited by pharmacological inhibitor or downregulated by siRNA and time-lapse images were acquired. A 5-bromo-2′-deoxyuridine assay was performed to analyze the proliferation of FS cells. Our results showed that MMP-9 was expressed in FS cells, that this expression was upregulated by laminin, and that laminin induced MMP-9 secretion by FS cells. MMP-9 inhibition and downregulation did not impair FS motility; however, it did impair the capacity of FS cells to form interconnections and it significantly inhibited proliferation of FS cells on laminin. We conclude that MMP-9 is necessary in FS cell interconnection and proliferation in the presence of laminin.

Journal of Endocrinology (2012) **212,** 363–370

Introduction

The anterior pituitary gland regulates homeostasis by meticulous adjustment of hormonal secretion. Folliculostellate (FS) cells are present in the anterior pituitary gland but do not secrete classical hormones. Although FS cells are agranular, evidence suggests that they are important in coordinating anterior pituitary function via the homotypic cellular network through gap junction communication in the gland (Fauquier *et al.* 2001, Shirasawa *et al.* 2004). Formation of clusters and elongated cytoplasmic processes are the structural hallmarks of FS cells (Soji & Herbert 1989). However, FS cells are known for another unique feature: apposition of their cytoplasmic processes and the extracellular matrix (ECM) of the basement membrane (Inoue *et al.* 1999, Shirasawa *et al.* 2004).

In a series of experiments that investigated the influence on FS cells of laminin – an ECM component of the basement membrane – we found that FS cells exhibited a motile phenotype and enhanced proliferation and that they established numerous interconnections, which closely resembled their cellular arrangement *in vivo* (Horiguchi *et al.* 2010, 2011*a*,*b*). The high motility of FS cells on laminin suggested that, to migrate on the ECM, they must express matrix-degrading enzymes. Among the enzymes capable of ECM degradation is the matrix metalloproteinase (MMP) family. MMP-9 has been extensively studied under numerous physiological and pathological conditions. In addition to its well-known proteolytic action, MMP-9 modulates cell motility and proliferation (Moon *et al.* 2003, Cauwe *et al.* 2009, Sans-Fons *et al.* 2010). Despite many studies of MMP-9 at the molecular and cellular levels in various organs, MMP-9 expression and function in FS cells of the anterior pituitary have not been investigated. Thus, the role of MMP-9 in FS cell motility and proliferation under the influence of laminin needs to be clarified.

We investigated MMP-9 expression and localization in FS cells. In addition, we studied the effect of laminin on MMP-9 expression in primary culture and attempted to confirm the role of MMP-9 in FS cell interconnection and proliferation.

Materials and Methods

Animals

Transgenic S100b-green fluorescent protein (GFP) rats (Itakura *et al.* 2007), which express GFP under the promoter control of the S100b protein gene (a marker of FS cells), were

DOI: 10.1530/JOE-11-0433
0022–0795/12/0212–363
Online version via http://www.endocrinology-journals.org

donated by Prof. K Inoue of Saitama University and bred in our laboratory. Eight- to ten-week-old male rats weighing 250–300 g were given *ad libitum* access to food and water and housed under a 12 h light:12 h darkness cycle. Rats were sacrificed by exsanguination from the right atrium under deep Nembutal anesthesia and then perfused with Ca^{2+}- and Mg^{2+}-free Hanks' solution for primary culture or with 4% paraformaldehyde (PFA) in 0·05 M phosphate buffer (pH 7·4) for immunohistochemistry. All animals were treated in accordance with the Jichi Medical University Guidelines for Animal Experimentation.

Cell culture

Anterior pituitary cells of S100b-GFP male rats were dispersed as described in a previous report (Horiguchi *et al.* 2008). The dispersed cells were then sorted by a MoFlo XDP (Beckman Coulter, Inc., Fullerton, CA, USA) into GFP-positive (GFP+) and GFP-negative (GFP−) cell fractions. GFP+ cells were plated onto eight-well glass chamber slides (1 cm^2/well; Nalge Nunc International, Rochester, NY, USA), with or without a coating of 10 μg/cm^2 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% fetal bovine serum (Sigma–Aldrich Corp.), 0·5 U/ml penicillin, and 0·5 μg/ml streptomycin (Invitrogen). Cells were then incubated at 37 °C in an atmosphere of 5% CO_2 and 95% air. For MMP-9 inhibition, cells were treated with 100 μM synthetic MMP-9/MMP-13 Inhibitor I (444252; Merck), and control groups were treated with an equal volume of dimethyl sulfoxide 9 (DMSO); (Wako, Osaka, Japan) in the medium from 0 h. For MMP-9 knockdown, a siRNA sequence against rat MMP-9 (SI102004247; Qiagen), with a nontargeting siRNA (1027283; Qiagen) as control, was transfected into cells by using an INTERFERin transfection reagent (PolyPlus Transfections, Inc., New York, NY, USA) 24 h after seeding. Cells were time-lapse recorded using a digital camera (ORCA-ER; Hamamatsu Photonics, Shizuoka, Japan) and MetaMorph Software (Molecular Devices Corp., Downingtown, PA, USA) from 2 to 72 h after seeding in a CO_2 gas culture chamber (Sankei Corp., Tokyo, Japan) with a thermostat (Kokensha Engineering Corp., Tokyo, Japan) on a fluorescence-inverted microscope (IX71; Olympus Corp., Tokyo, Japan). Each observation was performed in triplicate.

RT-PCR and real-time RT-PCR

For RT-PCR, total RNA fractions were prepared with TRIzol reagent (Invitrogen) from anterior pituitary gland, primary culture of anterior pituitary cells, and the GFP+ and GFP− cell fractions of S100b-GFP male rats. Expression of MMP-9 and cyclin D1 mRNA in primary cultured FS cells was measured by real-time RT-PCR. Total RNA fractions were prepared with the RNeasy Mini kit (Qiagen) from cultured FS cells, and all RNA samples were incubated with RNase-free DNase I (1 U/tube; Promega Corp.) and heated to inactivate DNase I. cDNA was synthesized using a Superscript III RT kit with oligo-$(dT)_{20}$ primer (Invitrogen). The PCR mix consisted of the RT reaction product, PCR buffer containing dNTPs, KOD Dash DNA Polymerase (2·5 U/μl; Toyobo, Osaka, Japan), and each oligonucleotide primer. The primer pairs used and putative product lengths were as follows: MMP-9 (GenBank accession no. NM_031055), forward: 5′-AGG GTC GGT TCT GAC CTT TT-3′, reverse: 5′-TGA GGG ATC ATCc TCG GCT AC-3′ (522 bp); S100b (BC_087026), forward: 5′-ATA GCA CCT CCG TTG GAC AG-3′, reverse: 5′-CAT CTC AGT GGC CCT TCA TT-3′ (527 bp); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; M_17701), forward: 5′-CCA TCA CCA TCT TCC AGG AG-3′, reverse: 5′-TTC AGC TCT GGG ATG ACC TT-3′ (457 bp).

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, Tokyo, Japan) containing SYBR Green I. To amplify cDNA fragments, the following primers were used: MMP-9, forward: 5′-CCT GAA AAC CTC CAA CCT CA-3′, reverse: 5′-GGA CTG CTT CTC TCC CAT CA-3′ (100 bp); cyclin D1 (NM_171992.4), forward: 5′-TGC AAA TGG AAC TGC TTC TG-3′, reverse: 5′-GCG GAT GAT CTG CTT GTT CT-3′ (125 bp). As reference, we also quantified GAPDH, forward: 5′-AAG GGC TCA TGA CCA CAG TC-3′, reverse: 5′-GGA TGC AGG GAT GAT GTT CT-3′ (116 bp). Relative quantification was conducted using the standard curve method and was performed in triplicate.

Immunoblot analysis

Anterior pituitary gland, GFP+ and GFP− cell fractions separated by cell sorter and primary cultured GFP+ cells were lysed in RIPA buffer (20 mM Tris, 150 mM NaCl, 2 mM EDTA, 0·1% w/v SDS, 1% v/v Triton X-100; pH 7·5), and total protein was estimated by Bradford assay (Sigma). Twenty micrograms of protein from each sample were applied to 10% SDS–PAGE. Proteins were then transferred electrophoretically onto Immobilon-P transfer membranes (Millipore). Membranes were blocked with 5% skim milk in TBST (50 mM Tris, 100 mM NaCl, 0·1% v/v Tween 20; pH 7·4), probed overnight at room temperature with rabbit monoclonal anti-MMP-9 antibody diluted 1:15 000 (ab76003; Abcam, Inc.) or mouse anti-β-actin antibody (0·1 μg/ml; BioVision, Mountain View, CA, USA), diluted in Can Get Signal solution (Toyobo) followed by TBST washes, and incubated for 1 h with HRP-labeled secondary antibodies (Envision+System–HRP, anti-rabbit; Dako, Glostrup, Denmark). Immunoreactive bands were visualized by ECL Plus Western Blotting Detection Reagents (GE Healthcare, Mississauga, ON, Canada). Each blot was performed in triplicate.

Gelatin zymography

Gelatin zymography to detect MMP-9 activity was performed according to the method described in a previous report (Zhang & Gottschall 1997). In brief, protein samples from anterior pituitary gland and GFP+ and GFP− cell fractions were prepared using nonreducing sample buffer containing 0·5 M Tris–HCl (pH 6·8), 10% SDS, and glycerol. GFP+ cells were cultured as described above for 48 h. Then, medium was replaced with serum-free medium supplemented with 0·1% BSA (Roche Diagnostics), and cell culture was continued for 24 h. Conditioned medium was collected from each well of three experimental replicates and centrifuged at 15 000 ***g*** for 3 min at 4 °C to remove cellular debris. Samples were electrophoretically run in copolymerized 10% SDS–acrylamide (Bio-Rad Laboratories, Inc.) and 0·1% gelatin (Difco Laboratories, Detroit, MI, USA) gel. Gel was incubated in regeneration buffer containing 2·5% Triton X-100 for 2 h at room temperature followed by incubation in reaction buffer containing 10 mM $CaCl_2$ for 24 h at 37 °C. To visualize gelatinolytic bands, the gel was stained with Coomassie Brilliant Blue (ATTO Corp., Tokyo, Japan).

Immunohistochemistry

After perfusion with 4% PFA in 0·05 M PB (pH 7·4), the pituitary glands were carefully dissected and fixed overnight in the same fixative solution at 4 °C. Next, tissues were immersed in 0·05 M PB (pH 7·2) containing 30% sucrose for 2 days at 4 °C, embedded in Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo, Japan), and snap-frozen. Frontal sections (8 μm) were incubated in PBS containing 2% normal goat serum for 20 min at 30 °C to block nonspecific antigen binding, then incubated with anti-rat MMP-9 rabbit monoclonal antibody diluted 1:25 in PBS for two nights at 4 °C, followed by incubation with Alexa Fluor 568-conjugated goat anti-rabbit IgG diluted 1:200 (Invitrogen) for 30 min at 30 °C. For immunostaining of cultured GFP+ cells, cells were fixed with 4% PFA in 0·025 M PB (pH 7·4) for 20 min at room temperature, immersed in PBS containing 2% normal goat serum for 20 min at 30 °C, incubated with anti-rat MMP-9 rabbit monoclonal antibody diluted 1:100 overnight at room temperature, then probed with Alexa Fluor 568-conjugated goat anti-rabbit IgG. Absence of an observable nonspecific reaction was confirmed using normal rabbit serum instead of primary antibody.

Proliferation assay

To observe FS cell proliferation, the nucleotide analog 5-bromo-2′-deoxyuridine (BrdU; Sigma) was incorporated into primary culture and detected in fixed cells according to previously described procedures (Horiguchi *et al.* 2010). Thirty random fields were imaged per well using a confocal laser microscope with a 60-fold objective lens. Observations were done in triplicate for each experimental group.

Statistical analysis

Results are presented as mean±S.E.M. Student's *t*-test was used to compare differences between groups, which were considered to be statistically significant at a *P* value of $<0{\cdot}05$.

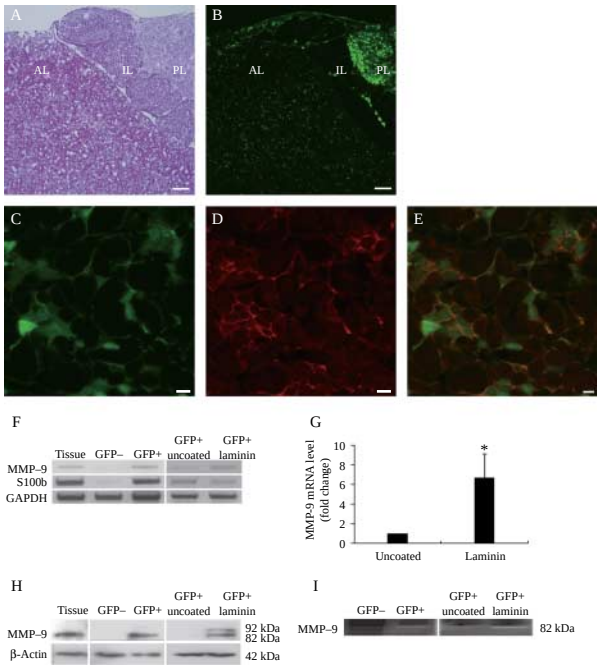

Figure 1 MMP-9 expression in FS cells. (A) Pituitary cryosection from S100b-GFP rat stained with hematoxylin–eosin showing the anterior lobe (AL), intermediate lobe (IL), and posterior lobe (PL). Scale bar, 100 μm. (B) Pituitary cryosection from S100b-GFP rat showing GFP+ cells in anterior lobe (AL), intermediate lobe (IL), and posterior lobe (PL). In anterior lobe, S100b expression (green) is limited to FS cells. Scale bar, 100 μm. (C) S100b-GFP rat anterior pituitary showing clusters of GFP+ FS cells. (D) MMP-9 immunoreactivity (red) in anterior pituitary section. (E) Overlay image of (C) and (D), MMP-9 immunoreactivity (red) is observed in most GFP+ cells (green). Scale bars for C-E, 10 μm. (F) Expression of MMP-9 gene in anterior pituitary. Total RNA fractions extracted from anterior pituitary tissue (tissue), GFP− and GFP+ cell fractions (by cell sorter), and primary culture of GFP+ cells on uncoated (GFP+ uncoated) and laminin-coated (GFP+ laminin) surfaces were analyzed by RT-PCR of MMP-9, S100b, and GAPDH. (G) Expression of MMP-9 mRNA as determined by real-time RT-PCR after 72 h incubation on uncoated (uncoated) and laminin-coated (laminin) surfaces, normalized to internal control (GAPDH; mean±S.E.M., $n=3$, $^*P<0{\cdot}05$). (H) Immunoblotting showing MMP-9 expression in anterior pituitary tissue (tissue), GFP− and GFP+ cell fractions, and primary cultured GFP+ cells on uncoated (GFP+ uncoated) and laminin-coated (GFP+ laminin) surfaces. (I) Gelatin zymography showing MMP-9 activity in anterior pituitary cell fractions (GFP−, GFP+) and conditioned medium of FS cells (GFP+) cultured on uncoated (GFP+ uncoated) and laminin-coated (GFP+ laminin) surfaces.

Results

MMP-9 expression in anterior pituitary gland

Localization of MMP-9 in anterior pituitary gland was investigated immunohistochemically. Figure 1A shows hematoxylin–eosin staining of S100b-GFP rat pituitary gland. Transgenic S100b-GFP rats expressed GFP specifically in FS cells of the anterior pituitary (Fig. 1B). Frozen frontal sections of S100b-GFP rat pituitary tissue were probed with anti-rat MMP-9 rabbit monoclonal antibody (Fig. 1C and D). MMP-9 immunoreactivity was observed throughout the anterior pituitary, specifically in clusters of GFP+ cells and in their long cytoplasmic processes (Fig. 1E). Most FS cells showed MMP-9 immunoreactivity on their cell bodies and cytoplasmic extensions. Next, expression of MMP-9 gene in FS cells was examined by RT-PCR. MMP-9 was detected in anterior pituitary cells both *in vivo* and *in vitro* (Fig. 1F). Analysis of GFP+ and GFP− cell fractions showed identical results, with the exception of the S100b, which was not detected in the GFP− cell fraction (Fig. 1F). Real-time RT-PCR was performed to quantify the relative expression of MMP-9 in FS cells cultured on uncoated and laminin-coated surfaces (Fig. 1G). Expression of MMP-9 was markedly higher in GFP+ cells cultured on laminin than in those cultured on an uncoated surface ($P<0{\cdot}05$).

Expression of MMP-9 was confirmed by immunoblot analysis of anterior pituitary cell lysate *in vivo* and *in vitro*. We detected an immunoreactive band at 82 kDa, which corresponds to the size of activated MMP-9 in anterior pituitary tissue (Fig. 1H). Blotting of the GFP+ cell fraction protein showed a strong immunoreactive band, corresponding to the 82 kDa protein, and a weaker band of 92 kDa that corresponds to the molecular weight of latent MMP-9 (Fig. 1H). No immunoreactive band appeared in the sample of the GFP− cell fraction (Fig. 1H). GFP+ cells of primary culture on uncoated surface showed very weak immunoreactive bands. However, two bands of MMP-9 appeared for GFP+ cells on laminin-coated primary culture (Fig. 1H). MMP-9, also known as 92 kDa gelatinase B, can digest gelatin, i.e. denatured collagen. Gelatin zymography was performed to detect this specific property of MMP-9. Protein samples were collected with nonreducing sample buffer from GFP− and GFP+ cell fractions. The only clear gelatinolytic band for the GFP+ cell fraction corresponded to 82 kDa (Fig. 1I). We then investigated whether FS cells secreted MMP-9 into the media. Serum-free media of FS cells cultured in the absence or presence of laminin were collected. The clear gelatinolytic band corresponding to 82 kDa was stronger for medium from FS cells conditioned in the presence of laminin (Fig. 1I).

MMP-9 localization on FS cells in primary culture

To observe MMP-9 localization on isolated FS cells in primary culture, a cell sorter was used to separate GFP+ FS cells from the anterior pituitary of S100b-GFP rats. These cells were then cultured on uncoated and laminin-coated chamber slides for 72 h. The shape of FS cells differed when grown on uncoated and laminin-coated surfaces (Fig. 2A–D; Horiguchi *et al.* 2010). On the uncoated surface, FS cells acquired a round shape and aggregated (Fig. 2A and B). Under the influence of laminin, FS cells appeared to flatten and form interconnections (Fig. 2C and D). Immunocytochemistry to detect MMP-9 distribution demonstrated that FS cells showed punctate MMP-9 immunoreactivity on the uncoated surface (Fig. 2B). However, under the influence of laminin, MMP-9 had a filament-like distribution on FS cells (Fig. 2D).

Effect of MMP-9 inhibitor on FS cells in primary culture

To determine the role of MMP-9 on FS cells in the presence of laminin, we observed the behavior of living FS cells in primary culture with MMP-9 inhibitor (Fig. 3A and B). Cells were treated with 100 μM MMP-9 synthetic inhibitor or an equal volume of vehicle (DMSO; control group) from 0 to 72 h. In the control group, the behavior of FS cells was similar to that reported previously (Horiguchi *et al.* 2010, 2011*a*), i.e. by 72 h of culture, they attached to laminin by becoming flatter, showed remarkable locomotion, extended their cytoplasm toward other cells in their vicinity, and formed interconnections with other FS cells (Fig. 3A; Supplementary data Movie 1, see section on supplementary data given at the end of this article). In the group treated with MMP-9

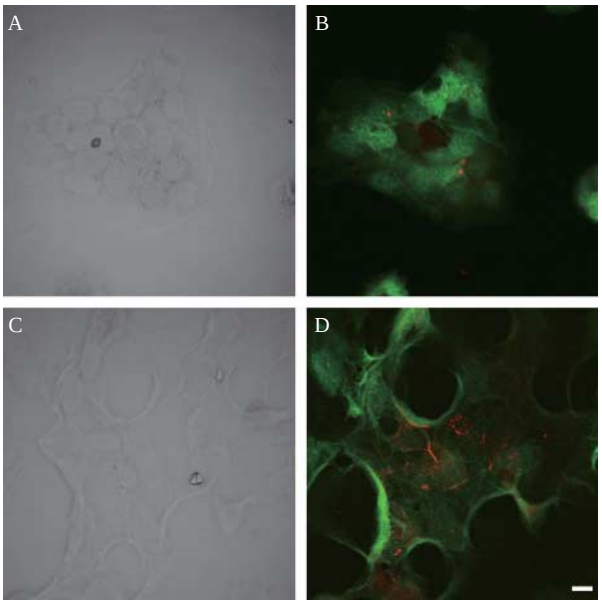

Figure 2 Immunocytochemistry of MMP-9 in primary cultured FS cells. (A and C) Phase contrast images. (B) Confocal image of FS cells cultured on uncoated surface. (D) Confocal image of FS cells cultured on laminin-coated surface. Note that MMP-9 immunoreactivity (red) is higher in FS cells (green) cultured on laminin-coated surface. Scale bar, 10 μm.

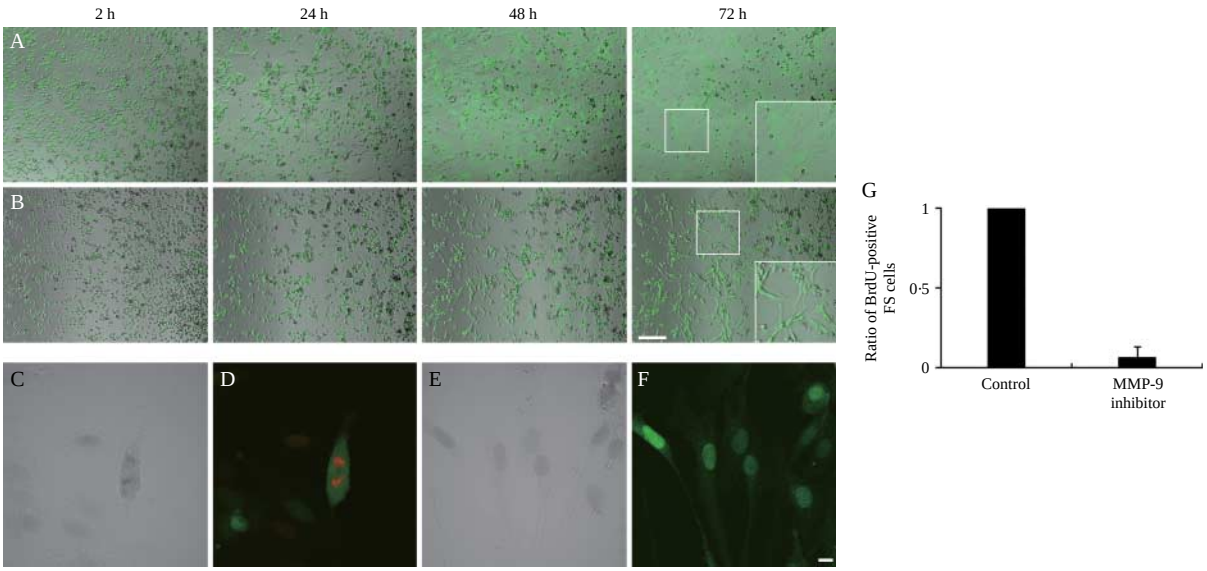

Figure 3 Time-lapse images of FS cells isolated from S100b-GFP rat anterior pituitary cells in primary culture on laminin-coated surface, with or without MMP-9 inhibitor. Cells are time-lapse recorded at 15 min intervals from 2 to 72 h after plating. (A and B) GFP images superimposed on phase contrast images, using an inverted fluorescence microscope, on laminin-coated surface without (A) and with (B) MMP-9 inhibitor. 2, 24, 48, and 72 h: elapsed time from plating of cells. Higher magnification views are shown in the inset images. Scale bar, 100 μm. (C–F) BrdU incorporation into FS cells. (C and D) Control group treated with DMSO. BrdU immunoreactivity (red) is observed in FS cells. (E and F) Representative FS cells under the influence of MMP-9 inhibitor show very little proliferative activity. Scale bar, 10 μm. (G) Ratio of BrdU-positive FS cells cultured on laminin-coated surfaces in the absence (control) or presence of MMP-9 inhibitor (MMP-9 inhibitor; mean±S.E.M., $n=3$, $^{**}P<0{\cdot}01$).

inhibitor, FS cells did not fully flatten but did extend their cytoplasmic processes. In addition, although the motility of these cells was comparable to that of the control group, they only established sparse cellular interconnections by 72 h of culture (Fig. 3B; Supplementary data Movie 2, see section on supplementary data given at the end of this article). To investigate whether MMP-9 affects the proliferative capacity of FS cells, we performed BrdU incorporation in FS cells cultured without or with MMP-9 inhibitor. Figure 3C and D shows BrdU incorporation in FS cells cultured on the laminin-coated surface in the absence of MMP-9 inhibitor. In the presence of MMP-9 inhibitor (Fig. 3E and F), a marked decrease in BrdU-positive cells was observed. As compared with the control group, the ratio of BrdU-positive cells was lower on the laminin-coated surface with MMP-9 inhibitor ($P<0{\cdot}01$; Fig. 3G).

Knockdown of MMP-9 gene by siRNA

The use of siRNA to silence the expression of MMP-9 resulted in a marked change in FS cell shape. By 24 h of culture, plasma membrane projections were already established in most FS cells (Fig. 4A and B, 24 h). Transfection with MMP-9 siRNA from 24 to 72 h after plating led to retraction of these projections; i.e. FS cells adopted a round shape and detached from laminin (Fig. 4B, 48, 72 h; Supplementary data Movie 4, see section on supplementary data given at the end of this article). The number of cellular interconnections was also diminished, resulting in small, scattered clusters by 72 h of culture (Fig. 4B, 72 h). FS cells transfected for an identical period with nontargeting siRNA reconstructed interconnections (Fig. 4A, 72 h; Supplementary data Movie 3, see section on supplementary data given at the end of this article). Quantitative real-time RT-PCR analysis of MMP-9 expression showed that siRNA transfection decreased MMP-9 gene expression as compared with control ($P<0{\cdot}01$; Fig. 4C). We also examined the effect of MMP-9 silencing on FS cell proliferation and found that MMP-9 downregulation decreased the ratio of BrdU-positive FS cells as compared with control ($P<0{\cdot}01$; Fig. 4D). However, MMP-9 silencing did not significantly change the expression of cyclin D1 (Fig. 4E).

Discussion

This study shows that MMP-9 is specifically expressed in FS cells of normal adult rat pituitary, that its expression is upregulated by laminin, and that it is involved in laminin-mediated morphological and proliferative changes in FS cells *in vitro*.

To clarify the mechanisms underlying the marked motility and proliferation of FS cells on laminin, we investigated MMP-9 expression in rat anterior pituitary and isolated FS cells, based on the techniques of our earlier study (Horiguchi *et al.* 2010). We found that FS cells express

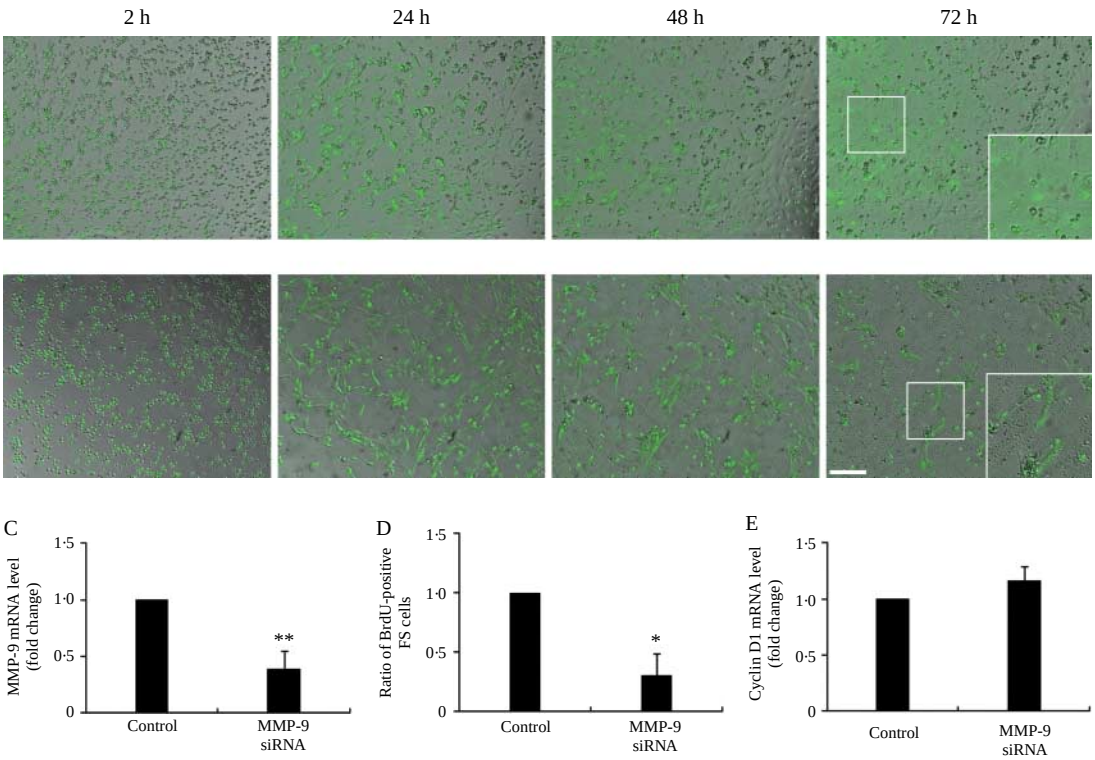

Figure 4 Time-lapse images of FS cells (in primary culture on laminin-coated surface) transfected with nontargeting siRNA or MMP-9 siRNA. (A and B) GFP images superimposed on phase contrast images on laminin-coated surface with nontargeting siRNA (A) and MMP-9 siRNA (B). 2, 24, 48, and 72 h: elapsed time from plating of cells. Higher magnification views are shown in the inset images. Scale bar, 100 μm. (C) Quantitative real-time RT-PCR shows that MMP-9 gene expression level is significantly lower in FS cells transfected with MMP-9 siRNA (MMP-9 siRNA) than in those transfected with nontargeting siRNA (control); results are normalized to internal control (GAPDH; mean±S.E.M., $n=3$, $^{**}P<0{\cdot}01$). (D) Ratio of BrdU-positive FS cells, cultured on laminin-coated surfaces, transfected with nontargeting siRNA (control) or MMP-9 siRNA (MMP-9 siRNA; $^{*}P<0{\cdot}05$). (E) Quantitative real-time RT-PCR shows that cyclin D1 expression is not affected by silencing MMP-9 expression; results are normalized to internal control (GAPDH; mean±S.E.M., $n=3$).

MMP-9 mRNA and protein (Fig. 1D and F). Other studies have used immunohistochemistry (Knappe *et al.* 2003) and zymography (Paez Pereda *et al.* 2000) to investigate MMP-9 expression in normal and tumorous human anterior pituitary glands. However, those earlier studies did not identify MMP-9-expressing cells in the gland.

We also observed that FS cells differentially expressed MMP-9 in the absence and presence of laminin and that this expression was increased by laminin (Fig. 1D–G). In several cell lines, laminin-derived peptide (Freitas *et al.* 2007) and whole laminin (Maity *et al.* 2011) induced MMP-9 secretion and activity upon interaction with integrin by utilizing the ERK pathway. The integrins comprise an α-subunit and β-subunit, which 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, which differ in ligand specificity (Hynes 2002). With respect to these ligand specificities, we recently reported that FS cells bind laminin through integrin-α3β1 and/or integrin-α6β1 (Horiguchi *et al.* 2010). Since laminin induces integrin β1 signaling in FS cells (Horiguchi *et al.* 2011*b*), we hypothesize that the increase in MMP-9 expression in the presence of laminin occurs via a similar pathway. Furthermore, the increases in MMP-9 mRNA expression and protein (in cell lysate and secreted into medium) in the presence of laminin parallel the phenotypic change in FS cell shape from round to stellate (Figs 1D–G and 2). Punctate MMP-9 immunoreactivity was observed in round FS cells on the uncoated surface. In contrast, filament-like signals were seen in clusters of stellate-shaped FS cells on laminin (Fig. 2). MMP-9 was spatially distributed along microfibers (Schumacher *et al.* 2005), and elevated MMP-9 expression was found to be associated with the invasive phenotype of cells (Peters *et al.* 1999). Sbai *et al.* (2010) showed that MMP-9 has a vesicular distribution and that the location of vesicles is associated with the cytoskeleton. Previously, we found that motile FS cells on laminin formed F-actin in their cytoplasmic processes during cellular interconnection (Horiguchi *et al.* 2010). It is thus

possible that the differential spatial distribution of MMP-9 under the influence of laminin is related to variation in the pattern of cytoskeletal arrangement in FS cells.

To determine the role of MMP-9 in FS cells, we used MMP-9 inhibitor to observe the behavior of living FS cells in primary culture. Inhibition of MMP-9 abrogated FS cell interconnection on laminin (Fig. 3; Supplementary data Movie 2, see section on supplementary data given at the end of this article). Because MMP-13 is a secondary target for the inhibitor used in this study, we next ruled out the effect of MMP-13 inhibition on our observations by silencing MMP-9 expression. The results were similar to those observed with MMP-9 inhibitor, i.e. FS cells failed to reconstruct dense interconnections, due to loss of attachment to laminin (Fig. 4; Supplementary data Movie 4, see section on supplementary data given at the end of this article).

The interconnections between FS cells are believed to serve as a network for conveying cellular messages through gap junctions in the anterior pituitary gland (Soji & Herbert 1989, Fauquier *et al.* 2001, Shirasawa *et al.* 2004). Previously, we discovered that FS cells formed networks in the presence of laminin, a process mediated through integrin β1 signaling (Horiguchi *et al.* 2011*a*). Veeravalli *et al.* (2010) showed that MMP-9 silencing downregulates the α-subunit and β-subunit of integrin *in vitro*. Knocking down MMP-9 expression might inhibit integrin signaling in FS cells and therefore influence adhesion of FS cells to laminin. Our present results confirm that FS cells do at least partly depend on MMP-9 expression to interconnect and assemble their characteristic network on laminin.

MMP-9 promotes cell proliferation in several cell types (Dwivedi *et al.* 2009, Sans-Fons *et al.* 2010, Ingraham *et al.* 2011). The present study showed that MMP-9 inhibition suppressed proliferative activity of FS cells on laminin, which indicates that MMP-9 is necessary in FS cell division (Figs 3G and 4D). Proliferation of FS cells on laminin involves integrin β1, which is associated with caveolin-3, and uses MAPK signal transduction, which upregulates the cyclin D1 expression that drives the cell cycle (Horiguchi *et al.* 2011*b*). Despite the marked decrease in BrdU incorporation after MMP-9 silencing, cyclin D1 expression was unchanged in this study (Fig. 4). This unexpected finding suggests that MMP-9 does not affect the capacity of cells to progress from G1 to S phase via the caveolin-3-mediated signaling pathway, but rather that MMP-9 is necessary for FS cells to synthesize DNA. We are continuing to investigate the role of MMP-9 in FS cell proliferation.

In conclusion, the present study provides evidence that laminin promotes MMP-9 expression in FS cells of rat anterior pituitary gland and that MMP-9 is required for the interconnection and proliferation of FS cells. In the presence of laminin, increased MMP-9 expression appears to promote the characteristic features of FS cells in anterior pituitary gland. During the early postnatal period, a small number of FS cells appear sparsely in the anterior pituitary at day 10; during maturation, they gradually form a more interconnected cell network (Soji *et al.* 1997). It is tempting to speculate that MMP-9 contributes to the expansion of this FS cell network *in vivo*. Future study is required to identify the mechanisms involved in the network arrangement of FS cells.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-11-0433.

Declaration of interest

The authors hereby declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported in the manuscript entitled 'Matrix metalloproteinase-9 expression in folliculostellate cells of rat anterior pituitary gland'.

Funding

This work was partially supported by a Grant-in-Aid for Scientific Research (C) (22590192), a Grant-in-Aid for Young Scientists (B) (22790190) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by promotional funds from the Keirin Race of the Japan Keirin Association.

Acknowledgements

We thank Prof. Kinji Inoue (Saitama University, Japan) for supplying the transgenic rats. We are grateful to Miss Megumi Yatabe for her excellent technical assistance and to Prof. Yutaka Hanazono and Mr Yutaka Furukawa for their support in fluorescence-activated cell sorting. We also thank David Kipler, ELS of Supernatant Communications for revising the language of the manuscript.

References

Cauwe B, Martens E, Proost P & Opdenakker G 2009 Multidimensional degradomics identifies systemic autoantigens and intracellular matrix proteins as novel gelatinase B/MMP-9 substrates. *Integrative Biology* **1** 404–426. (doi:10.1039/b904701h)

Dwivedi A, Slater SC & George SJ 2009 MMP-9, and -12 cause *N*-cadherin shedding and thereby beta-catenin signalling and vascular smooth muscle cell proliferation. *Cardiovascular Research* **81** 178–186. (doi:10.1093/cvr/cvn278)

Fauquier T, Guerineau NC, McKinney RA, Bauer K & Mollard P 2001 Folliculostellate cell network: a route for long-distance communication in the anterior pituitary. *PNAS* **98** 8891–8896. (doi:10.1073/pnas.151339598)

Freitas VM, Vilas-Boas VF, Pimenta DC, Loureiro V, Juliano MA, Carvalho MR, Pinheiro JJ, Camargo AC, Moriscot AS, Hoffman MP *et al.* 2007 SIKVAV, a laminin alpha1-derived peptide, interacts with integrins and increases protease activity of a human salivary gland adenoid cystic carcinoma cell line through the ERK 1/2 signaling pathway. *American Journal of Pathology* **171** 124–138. (doi:10.2353/ajpath.2007.051264)

Horiguchi K, Fujiwara K, Kouki T, Kikuchi M & Yashiro T 2008 Immunohistochemistry of connexin 43 throughout anterior pituitary gland in a transgenic rat with green fluorescent protein-expressing folliculo-stellate cells. *Anatomical Science International* **83** 256–260. (doi:10.1111/j.1447-073X.2008.00239.x)

Horiguchi K, Kikuchi M, Kusumoto K, Fujiwara K, Kouki T, Kawanishi K & Yashiro T 2010 Living-cell imaging of transgenic rat anterior pituitary cells in primary culture reveals novel characteristics of folliculo-stellate cells. *Journal of Endocrinology* **204** 115–123. (doi:10.1677/JOE-09-0333)

Horiguchi K, Fujiwara K, Ilmiawati C, Kikuchi M, Tsukada T, Kouki T & Yashiro T 2011*a* Caveolin 3-mediated integrin beta 1 signaling is required for the proliferation of folliculostellate cells in rat anterior pituitary gland under the influence of extracellular matrix. *Journal of Endocrinology* **210** 29–36. (doi:10.1530/JOE-11-0103)

Horiguchi K, Kouki T, Fujiwara K, Kikuchi M & Yashiro T 2011*b* The extracellular matrix component laminin promotes gap junction formation in the rat anterior pituitary gland. *Journal of Endocrinology* **208** 225–232. (doi:10.1677/JOE-10-0297)

Hynes RO 2002 Integrins: bidirectional, allosteric signaling machines. *Cell* **110** 673–687. (doi:10.1016/S0092-8674(02)00971-6)

Ingraham CA, Park GC, Makarenkova HP & Crossin KL 2011 Matrix metalloproteinase (MMP)-9 induced by Wnt signaling increases the proliferation and migration of embryonic neural stem cells at low O_2 levels. *Journal of Biological Chemistry* **286** 17649–17657. (doi:10.1074/jbc.M111.229427)

Inoue K, Couch EF, Takano K & Ogawa S 1999 The structure and function of folliculo-stellate cells in the anterior pituitary gland. *Archives of Histology and Cytology* **62** 205–218. (doi:10.1679/aohc.62.205)

Itakura E, Odaira K, Yokoyama K, Osuna M, Hara T & Inoue K 2007 Generation of transgenic rats expressing green fluorescent protein in S-100 beta-producing pituitary folliculo-stellate cells and brain astrocytes. *Endocrinology* **148** 1518–1523. (doi:10.1210/en.2006-1390)

Knappe UJ, Hagel C, Lisboa BW, Wilczak W, Lüdecke DK & Saeger W 2003 Expression of serine proteases and metalloproteinases in human pituitary adenomas and anterior pituitary lobe tissues. *Acta Neuropathologica* **106** 471–478. (doi:10.1007/s00401-003-0747-5)

Maity G, Sen T & Chatterjee A 2011 Laminin induces matrix metalloproteinase-9 expression and activation in human cervical cancer cell line (SiHa). *Journal of Cancer Research and Clinical Oncology* **137** 347–357. (doi:10.1007/s00432-010-0892-x)

Moon SK, Cha BY & Kim CH 2003 *In vitro* cellular aging is associated with enhanced proliferative capacity, G1 cell cycle modulation, and matrix metalloproteinase-9 regulation in mouse aortic smooth muscle cells. *Archives of Biochemistry and Biophysics* **418** 39–48. (doi:10.1016/S0003-9861(03)00402-8)

Paez Pereda M, Ledda MF, Goldberg V, Chervin A, Carrizo G, Molina H, Muller A, Renner U, Podhajcer O, Arzt E *et al.* 2000 High levels of matrix metalloproteinases regulate proliferation and hormone secretion in pituitary cells. *Journal of Clinical Endocrinology and Metabolism* **85** 263–269. (doi:10.1210/jc.85.1.263)

Peters TJ, Albieri A, Bevilacqua E, Chapman BM, Crane LH, Hamlin GP, Seiki M & Soares MJ 1999 Differentiation-dependent expression of gelatinase B/matrix metalloproteinase-9 in trophoblast cells. *Cell and Tissue Research* **295** 287–296. (doi:10.1007/s004410051235)

Sans-Fons MG, Sole S, Sanfeliu C & Planas AM 2010 Matrix metalloproteinase-9 and cell division in neuroblastoma cells and bone marrow macrophages. *American Journal of Pathology* **177** 2870–2885. (doi:10.2353/ajpath.2010.090050)

Sbai O, Ould-Yahoui A, Ferhat L, Gueye Y, Bernard A, Charrat E, Mehanna A, Risso JJ, Chauvin JP, Fenouillet E *et al.* 2010 Differential vesicular distribution and trafficking of MMP-2, MMP-9, and their inhibitors in astrocytes. *Glia* **58** 344–366.

Schumacher K, Klar J, Wagner C & Minuth WW 2005 Temporal–spatial co-localisation of tissue transglutaminase (Tgase2) and matrix metalloproteinase-9 (MMP-9) with SBA-positive micro-fibres in the embryonic kidney cortex. *Cell and Tissue Research* **319** 491–500. (doi:10.1007/s00441-004-1028-x)

Shirasawa N, Mabuchi Y, Sakuma E, Horiuchi O, Yashiro T, Kikuchi M, Hashimoto Y, Tsuruo Y, Herbert DC & Soji T 2004 Intercellular communication within the rat anterior pituitary gland: X. Immunohistocytochemistry of S-100 and connexin 43 of folliculo-stellate cells in the rat anterior pituitary gland. *Anatomical Record* **278** 462–473. (doi:10.1002/ar.a.20040)

Soji T & Herbert DC 1989 Intercellular communication between rat anterior pituitary cells. *Anatomical Record* **224** 523–533. (doi:10.1002/ar.1092240410)

Soji T, Mabuchi Y, Kurono C & Herbert DC 1997 Folliculo-stellate cells and intercellular communication within the rat anterior pituitary gland. *Microscopy Research and Technique* **39** 138–149. (doi:10.1002/(SICI)1097-0029(19971015)39:2<138::AID-JEMT5>3.0.CO;2-H)

Veeravalli KK, Chetty C, Ponnala S, Gondi CS, Lakka SS, Fassett D, Klopfenstein JD, Dinh DH, Gujrati M & Rao JS 2010 MMP-9, uPAR and cathepsin B silencing downregulate integrins in human glioma xenograft cells *in vitro* and *in vivo* in nude mice. *PLoS ONE* **15** e11583. (doi:10.1371/journal.pone.0011583)

Zhang JW & Gottschall PE 1997 Zymographic measurement of gelatinase activity in brain tissue after detergent extraction and affinity-support purification. *Journal of Neuroscience Methods* **76** 15–20. (doi:10.1016/S0165-0270(97)00065-4)

Received in final form 14 December 2011
Accepted 19 December 2011
Made available online as an Accepted Preprint
19 December 2011