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SHORT COMMUNICATION

Expression of small leucine-rich proteoglycans in rat anterior pituitary gland

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Abstract Proteoglycans are components of the extracellular matrix and comprise a specific core protein substituted with covalently linked glycosaminoglycan chains. Small leucine-rich proteoglycans (SLRPs) are a major family of proteoglycans and have key roles as potent effectors in cellular signaling pathways. Research during the last two decades has shown that SLRPs regulate biological functions in many tissues such as skin, tendon, kidney, liver, and heart. However, little is known of the expression of SLRPs, or the characteristics of the cells that produce them, in the anterior pituitary gland. Therefore, we have determined whether SLRPs are present in rat anterior pituitary gland. We have used real-time reverse transcription with the polymerase chain reaction to analyze the expression of SLRP genes and have identified the cells that produce SLRPs by using in situ hybridization with a digoxigenin-labeled cRNA probe. We have clearly detected the mRNA expression of SLRP genes, and cells expressing decorin, biglycan, fibromodulin, lumican, proline/arginine-rich end leucine-rich repeat protein (PRELP), and osteoglycin are located in the anterior pituitary gland. We have also investigated the possible double-staining of SLRP mRNA and pituitary hormones, S100 protein (a marker of folliculostellate cells), desmin (a marker of capillary pericytes), and isolectin B4 (a marker of endothelial cells). Decorin, biglycan, fibromodulin, lumican, PRELP, and osteoglycin mRNA have been identified in S100-protein-positive and desmin-positive cells. Thus, we conclude that folliculostellate cells and pericytes produce SLRPs in rat anterior pituitary gland.

Keywords Anterior pituitary gland · Small leucine-rich proteoglycans · Folliculostellate cell · Pericytes · In situ hybridization · Rat (Wistar, male)

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Introduction

The extracellular matrix (ECM) provides mechanical integrity, rigidity, and elasticity and regulates cell growth, development, and differentiation. ECMs are complex composite materials composed of insoluble fibers, microfibrils, a wide range of soluble proteins, and glycosaminoglycans. Proteoglycans comprise a specific core protein substituted with covalently linked glycosaminoglycan chains. They are classified into four categories: small leucine-rich proteoglycans (SLRPs), modular proteoglycans, basement membrane proteoglycans, and cell-surface proteoglycans (Schaefer and

Schaefer 2010). As endogenous ligands, SLRPs form a network for signal regulation. They are upstream of multiple signaling cascades, such as the transforming growth factor-β superfamily, bone morphogenetic protein, insulin-like growth factor-I receptor, and toll-like receptors (Schaefer and Schaefer 2010). In the last two decades, SLRP expression has been analyzed in many tissues and has been identified in skin, tendon, bone, cartilage, kidney, retina, liver, and heart, among other tissues (Kalamajski and Oldberg 2010). However, little is known of SLRP expression, or of the characteristics of the cells that produce them, in the anterior pituitary gland.

The anterior pituitary gland is composed of five types of hormone-producing cells, folliculostellate cells (which do not produce classical anterior pituitary hormones), and fenestrated sinusoids (i.e., endothelial cells and pericytes). These cells are surrounded by various types of ECMs, which are essential for the cells to perform their respective roles (Kaidzu et al. 2000; Paez-Pereda et al. 2005). In this study, we have determined whether proteoglycans are present in rat anterior pituitary gland. First, we have focused on SLRPs in the proteoglycans family and analyzed the expression of SLRP genes by using real-time reverse transcription with the polymerase chain reaction (RT-PCR). Second, we have used in situ hybridization to identify the cells that produce SLRPs.

Materials and methods

Animals

Male Wistar rats (aged 8–10 weeks) were purchased from Japan SLC (Shizuoka, Japan), maintained on a 12-h light/dark cycle, and given conventional food and water *ad libitum*. Room temperature was maintained at approximately 22°C. All animal experiments were performed 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 Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Japanese Ministry of Education, Culture, Sports, Science and Technology.

Quantification of mRNA levels by real-time RT-PCR

Real-time RT-PCR was performed as described previously (Horiguchi et al. 2011). By using Trizol (Invitrogen, Carlsbad, Calif., USA), total RNA fractions were prepared from anterior pituitary tissue and cultured cells and then incubated with RNase-free DNase I (1 U/tube; Promega, Madison, Wis., USA). After inactivation of DNase I by heating for 10 min at 65°C, cDNA was synthesized by using the PrimeScript RT reagent kit (Takara, Otsu, Japan) with oligo-$(dT)_{20}$ primer (Invitrogen). Quantitative real-time RT-PCR (ABI PRISM 7900HT; Applied Biosystems, Carlsbad, Calif., USA) was performed by using gene-specific primers and SYBR Premix Ex Taq (Takara) containing SYBR Green I. The sequences of the gene-specific primers are described in Table S1 of the Electronic Supplementary Material. For normalization, we also quantified glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Relative quantification was conducted by using the standard curve method and was performed at least three times.

In situ hybridization and immunohistochemistry

Rats were perfused through the left ventricle with 4% formaldehyde in 0.05 M phosphate buffer (pH 7.4) for 5 min under deep sodium pentobarbital anesthesia. Pituitary glands were then excised and immersed in the same fixative for 24 h at 4°C, after which the tissues were immersed for at least 2 days in phosphate buffer containing 30% sucrose at 4°C. In situ hybridization was performed with digoxigenin (DIG)-labeled cRNA probes, as described in our previous report (Fujiwara et al. 2007a). The DNA fragments were amplified from rat pituitary cDNA by using PCR. Primer sequences are detailed in Table S2 of the Electronic Supplementary Material. Amplified cDNA fragments were ligated into the pGEM-T vector (Promega, Madison, Wis., USA) and cloned. Gene-specific antisense or sense DIG-labeled cRNA probes were made by means of the Roche DIG RNA labeling kit (Roche Diagnostics, Penzberg, Germany). A cryostat was used to obtain frozen sections (8 μm), which were then mounted on glass slides. DIG-labeled cRNA probe hybridization was performed at 55°C for 16 h. Visualization of each type of mRNA was performed with alkaline-phosphatase-conjugated anti-DIG antibody (Roche Diagnostics) by using 4-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Roche Diagnostics). Control experiments were performed, and no specific signal was detected in sections processed with the DIG-labeled sense RNA probe.

For double-staining, after SRLP mRNAs had been detected by in situ hybridization, the section was immunostained, as described in our previous report (Fujiwara et al. 2007b). The sections were incubated overnight at room temperature in phosphate-buffered saline (PBS) with primary antibodies. Primary antibodies against the following proteins were used for immunostaining: adrenocorticotropic hormone (ACTH), growth hormone (GH), prolactin, thyroid-stimulating hormone β-subunit (TSHβ), luteinizing hormone β-subunit (LHβ), and S-100 protein, as reported previously (Fujiwara et al. 2007b), plus desmin (Nehls et al.

1992; 1:1,200; Abcam, Tokyo, Japan) and biotinylated isolectin B4 (1:25; Vector Laboratories, Burlingame, Calif., USA). Absence of an observable nonspecific reaction was confirmed by using normal rabbit serum. After being washed with PBS, sections were incubated in PBS with biotinylated anti-rabbit IgG (Vector Laboratories) for 30 min at 30°C. The ABC method (Vector Laboratories) was performed with 3,3′-diaminobenzidine (Dojindo Laboratories, Kumamoto, Japan) as the substrate.

Results and discussion

Quantitative real-time RT-PCR was used to determine whether anterior pituitary cells expressed SLRPs. We detected the expression of decorin, biglycan, asporin, fibromodulin, lumican, PRELP, osteomodulin, osteoglycin, and tsukushi in anterior pituitary cells, whereas keratocan, epiphycan, opticin, chondroadherin, nyctalopin, and podocan-like protein 1 were not detected (Fig. 1). The relative mRNA concentrations of decorin, biglycan, asporin, fibromodulin, lumican, PRELP, osteomodulin, osteoglycin, and tsukushi were 0.138±0.041, 0.015±0.003, 0.003±0.001, 0.003±0.001, 0.018±0.002, 0.024±0.003, 0.024±0.009, 0.002±0.001, and 0.002±0.001, respectively (Fig. 1). Decorin expression predominated, and modest amounts of biglycan, lumican, PRELP, and osteomodulin were also detected. Expression of asporin, fibromodulin, osteoglycin, and tsukushi was barely but consistently detectable in all samples.

Among ECMs in the anterior pituitary, laminin is produced by LH cells (Tougard et al. 1985), and type I and III collagens are produced by pericytes, which are localized in the perivascular spaces (Fujiwara et al. 2010). However, little is known of the expression of SLRP in the anterior pituitary gland. SLRPs are characterized by a core protein with a leucine-rich repeat domain and an N-terminal variable domain and are classified into five classes on the basis of their structural properties (Schaefer and Schaefer 2010). Class I SLRPs (decorin, biglycan, and asporin) have a typical cluster of Cys residues at N termini and form two disulfide bonds. Class II SLRPs (fibromodulin, lumican, PRELP, keratocan, and osteomodulin) contain a cluster of Tyr sulfate residues at N termini. Class III members (epiphycan, opticin, and osteoglycin) have relatively few leucine-rich repeats. Classes IV (chondroadherin, nyctalopin, and tsukushi) and V (podocan and podocan-like protein 1) SLRPs are new classes. In the present study, we demonstrate the expression of class I, II, III, and IV SLRP genes in the anterior pituitary gland of adult rats (Fig. 1).

Decorin, biglycan, fibromodulin, lumican, PRELP, and osteoglycin mRNAs were detected in the adult pituitary gland by in situ hybridization with a DIG-labeled antisense cRNA probe (Figs. 2b, d, 3a, d, g, j, m). Cells expressing decorin mRNA were located in the posterior, intermediate, and anterior lobes and were more prominent in the posterior pituitary (Fig. 2b). Decorin-gene-expressing cells in the posterior and intermediate lobes were located near capillaries (data not shown). No specific signal was detected in sections processed with the DIG-labeled sense RNA probe of decorin (Fig. 2c). However, asporin, osteomodulin, and tsukushi mRNA were not detected in sections of rat anterior pituitary gland (data not shown). We repeated in situ hybridization using DIG-labeled cRNA probes of several sequences for these mRNA, but no signals were detected. This suggests that the sensitivity of in situ hybridization is too low to detect these mRNAs. We intend to confirm the presence of such cells in the anterior pituitary gland in future studies.

To identify the cells that express SLRP mRNAs, we performed double-staining by using in situ hybridization to

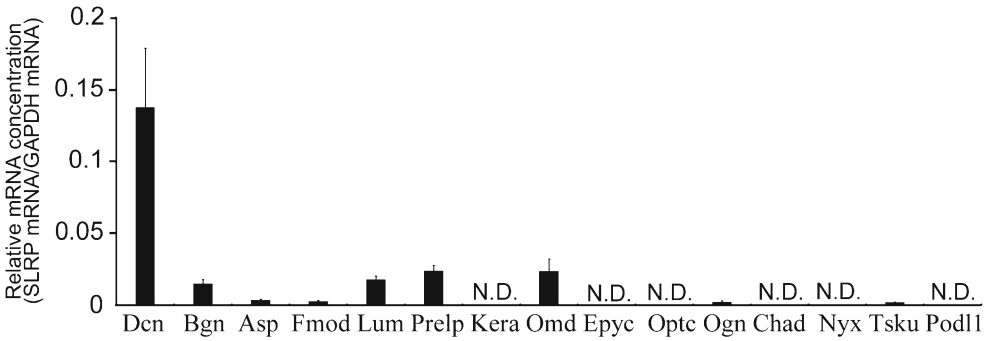

Fig. 1 Small leucine-rich proteoglycan (SLRP) gene expression in rat anterior pituitary gland. Expression of SLRP mRNA as determined by real-time reverse transcription with the polymerase chain reaction was normalized with an internal control (glyceraldehyde-3-phosphate dehydrogenase; *GAPDH*); means ± SEM, *n*=5. SLRPs examined: decorin (*Dcn*), biglycan (*Bgn*), asporin (*Asp*), fibromodulin (*Fmod*), lumican (*Lum*), proline arginine-rich end leucine-rich repeat protein (*Prelp*), keratocan (*Kera*), osteomodulin (*Omd*), epiphycan (*Epyc*), opticin (*Optc*), osteoglycin (*Ogn*), chondroadherin (*Chad*), nyctalopin (*Nyx*), tsukushi (*Tsku*), and podocan-like protein 1 (*Podl1*). Dcn, Bgn, Asp, Fmod, Lum, PRELP, Omd, and Ogn were expressed in the rat anterior pituitary gland; however, Kera, Epyc, Optc, Chad, Nyx, and Podl1 mRNA were not detected (*N.D.*)

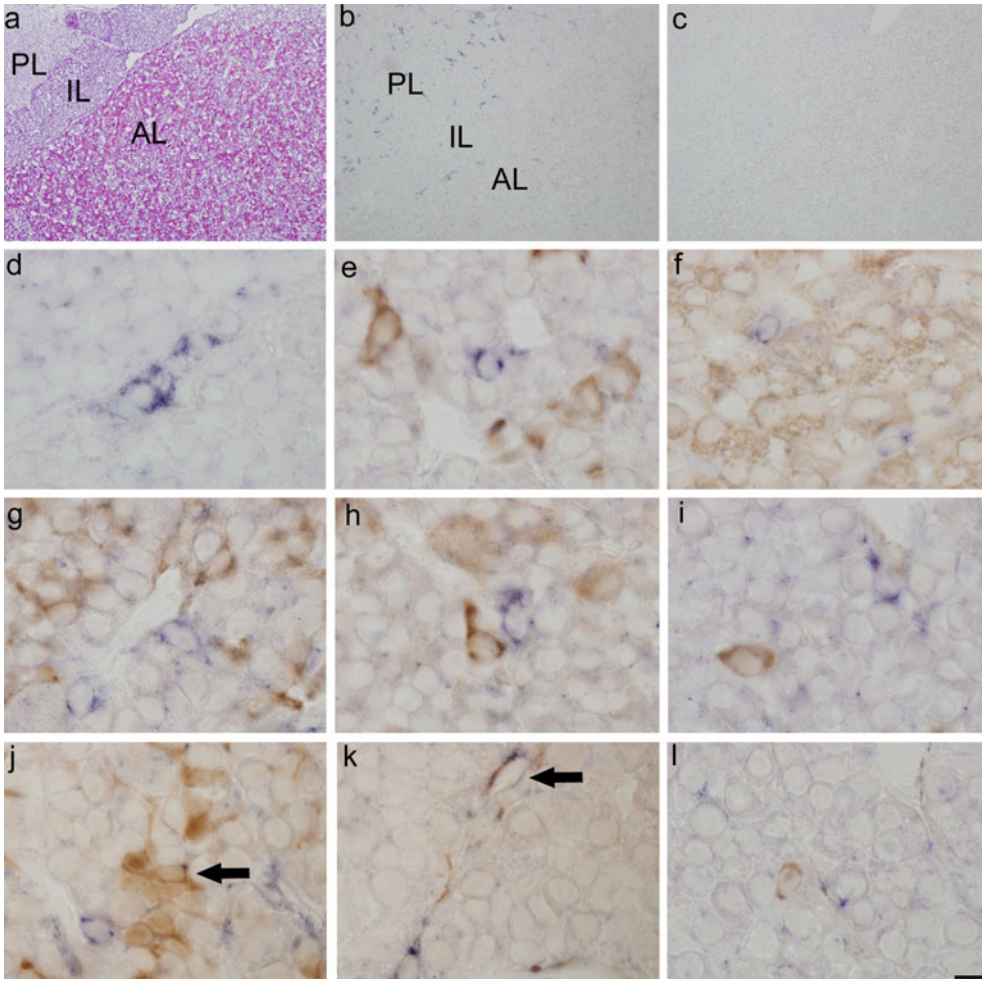

Fig. 2 Double-staining of SLRP mRNAs detected by in situ hybridization and of hormones, S100 protein, desmin, and isolectin B4 detected by immunohistochemistry in rat anterior pituitary gland. **a** Hematoxylin and eosin staining of a cryosection of anterior pituitary gland (*PL* posterior lobe, *IL* intermediate lobe, *AL* anterior lobe). **b** In situ hybridization for decorin. Decorin mRNA was detected in the posterior lobe (*PL*), intermediate lobe (*IL*), and anterior lobe (*AL*). **c** In situ hybridization for the decorin sense probe. No signal was detected in the pituitary gland. **d** High-magnification image of AL (**b**). **e-l** Double-staining of decorin mRNA detected by in situ hybridization and of pituitary hormones, S100 protein, desmin, and isolectin B4 identified by immunostaining. Adrenocorticotropic hormone (ACTH cells; **e**), growth hormone (GH cells; **f**), prolactin (prolactin cells; **g**), thyroid-stimulating hormone β-subunit (TSHβ cells; **h**), luteinizing hormone β-subunit (LHβ cells; **i**), S100 protein (folliculostellate cells; **j**), desmin (pericytes; **k**), and isolectin B4 (endothelial cells; **l**); in situ hybridization with NBT/BCIP (*blue*) and immunoreactivity with 3,3′-diaminobenzidine (*brown*). Decorin mRNA was colocalized with S100 protein and desmin immunoreactivity (*arrows*). *Bar* 10 μm

detect SLRP mRNAs and immunohistochemistry to detect pituitary hormone, S100 protein, desmin, and isolectin B4. Decorin mRNA was detected in folliculostellate cells and pericytes, which were identified on the basis of their immunoreactivity to S100 protein and desmin, respectively (Fig. 2j, k). Interestingly, the decorin gene was expressed in a subset of folliculostellate cells (apparently about 5%) and in most pericytes in this study (Fig. 2j, k). However, it was not expressed in ACTH cells, GH cells, prolactin cells, TSH cells, LH cells, or endothelial cells (Fig. 2e–i, l). In addition, biglycan, fibromodulin, lumican, PRELP, and osteoglycin mRNA were also expressed in some folliculostellate cells and most pericytes (Fig. 3b, c, e, f, h, i, k, l, n, o) but were not detected in hormone-producing cells or endothelial cells (data not shown). We also detected decorin-producing cells in the posterior lobes (Fig. S1). The decorin gene was not expressed in S100-protein-positive cells (pituicytes) or endothelial cells (Fig. S1c, e) but was detected in pericytes (Fig. S1d).

Pericytes are multifunctional cells associated with vascular development, stabilization, maturation, and remodeling (Allt and Lawrenson 2001). Some reports have shown that retinal and gingival pericytes produce SLRPs (Kaji et al. 2004; Alimohamad et al. 2005). Recently, SLRPs have been recognized as regulators of collagen fibril assembly (Kalamajski and Oldberg 2010). Several SLRPs bind

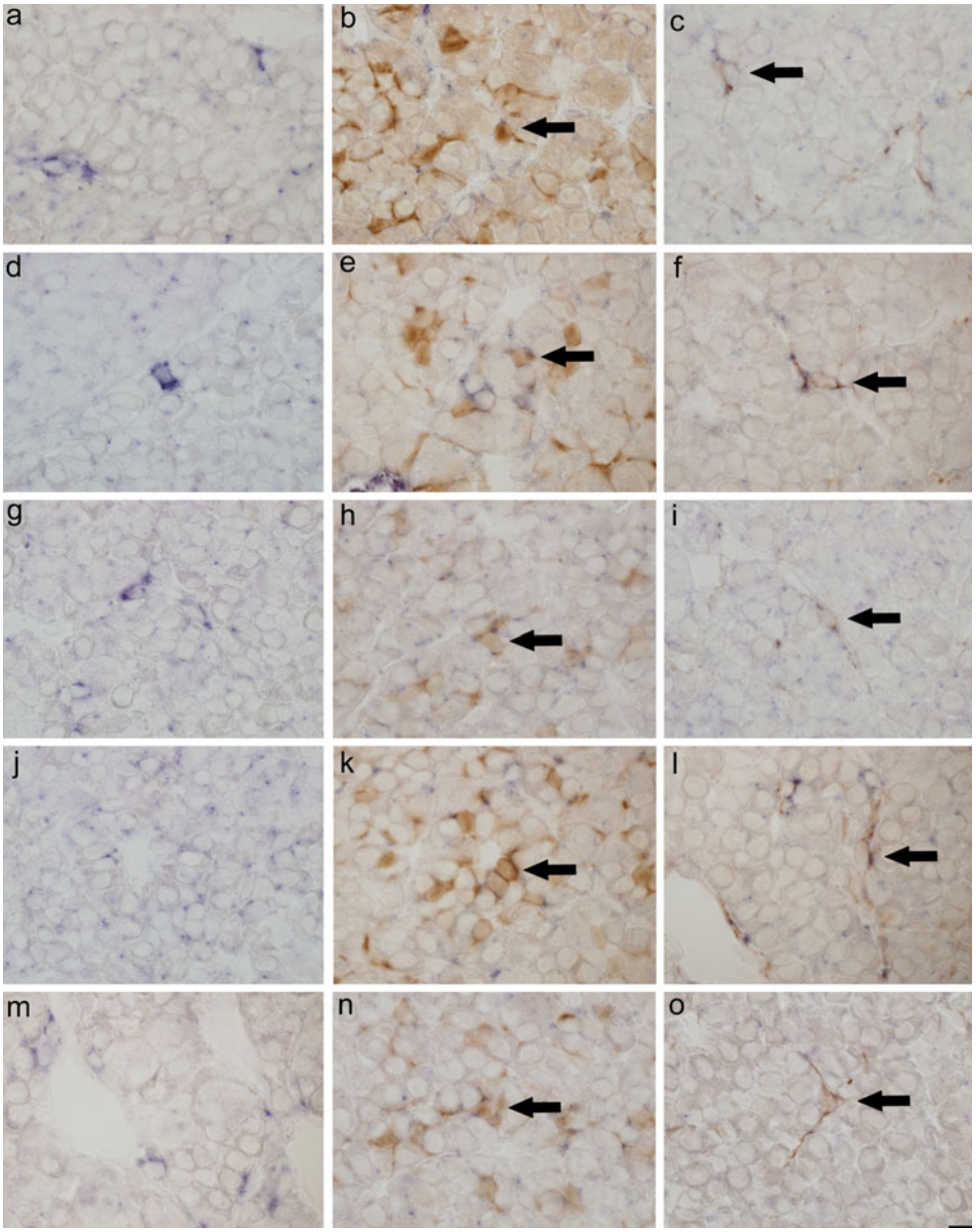

Fig. 3 Double-staining of SLRP mRNAs detected by in situ hybridization and of S100 protein and desmin detected by immunohistochemistry in rat anterior pituitary gland. **a**, **d**, **j**, **g**, **m** In situ hybridization of biglycan, fibromodulin, lumican, PRELP, and osteoglycin. **b**, **e**, **h**, **k**, **n** In situ hybridization of biglycan (**b**), fibromodulin (**e**), lumican (**h**), PRELP (**k**), and osteoglycin (**n**), and immunohistochemistry of S100 protein, respectively. **c**, **f**, **i**, **l**, **o** In situ hybridization of biglycan (**c**), fibromodulin (**f**), lumican (**i**), PRELP (**l**), and osteoglycin (**o**), and immunohistochemistry of desmin. In situ hybridization (*blue*) and immunoreactivity (*brown*). Biglycan, fibromodulin, lumican, PRELP, and osteoglycin mRNA were colocalized with S100 protein and desmin immunoreactivity (*arrows*). *Bar*

fibrillar collagens, including type I and III collagens, and inhibit fibril formation (Vogel et al. 1984; Rada et al. 1993). Fujiwara et al. (2010) have reported that, in the rat anterior pituitary, only pericytes produce type I and III collagens. These findings suggest that SLRPs produced by pericytes interact with collagen to remodel ECMs.

In the present study, we have found that folliculostellate cells also express SLRPs in the anterior pituitary (Figs. 2, 3). Accumulating evidence indicates that folliculostellate cells have numerous possible functions. In the postnatal development of the anterior pituitary gland, folliculostellate cells have been reported to act as stem cells and proliferative progenitor cells (Kikuchi et al. 2011). They also possess phagocytic activity (Ogawa et al. 1997). Furthermore, folliculostellate cells regulate hormone-producing cells by secreting paracrine factors such as basic fibroblast growth factor (Ferrara et al. 1987), vascular endothelial growth factor (Gospodarowicz and Lau 1989), interleukin-6 (Vankelecom et al. 1989), and annexin 1 (Buckingham et al. 2006). Decorin, biglycan, and fibromodulin bind to transforming growth factor β, thereby modulating its cascade/downstream (Cabello-Verrugio and Brandan 2007). Furthermore, SLRPs are involved in the initial triggering of multiple responses through epidermal growth factor receptor, insulin-like growth factor receptor, toll-like receptor, and the caveolin-mediated pathway (Schaefer and Schaefer 2010). Because hormone-producing cells and folliculostellate cells express these receptors (Ocrant et al. 1989;

Tichomirowa et al. 2005; Horiguchi et al. 2011), SLRPs might regulate the function of anterior pituitary cells as new paracrine factors produced by folliculostellate cells.

In conclusion, we have found that pericytes and folliculostellate cells produce SLRPs in the rat anterior pituitary gland. Further studies are needed to clarify the mechanism that regulates SLRP expression and the function of SLRPs in the gland.

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