

## Maintenance of the Extracellular Matrix in Rat Anterior Pituitary Gland: Identification of Cells Expressing Tissue Inhibitors of Metalloproteinases

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The extracellular matrix (ECM) is important in creating cellular environments in tissues. Recent studies have demonstrated that ECM components are localized in anterior pituitary cells and affect cell activity. Thus, clarifying the mechanism responsible for ECM maintenance would improve understanding of gland function. Tissue inhibitors of metalloproteinases (TIMPs) are endogenous inhibitors of matrix metalloproteinases and participate in ECM degradation. In this study, we investigated whether cells expressing TIMPs are present in rat anterior pituitary gland. Reverse transcription polymerase chain reaction was used to analyze expression of the TIMP family (TIMP1-4), and cells producing TIMPs in the gland were identified by using *in situ* hybridization. Expression of TIMP1, TIMP2, and TIMP3 mRNAs was detected, and the TIMP-expressing cells were located in the gland. The TIMP-expressing cells were also investigated by means of double-staining with *in situ* hybridization and immunohistochemical techniques. Double-staining revealed that TIMP1 mRNA was expressed in folliculostellate cells. TIMP2 mRNA was detected in folliculostellate cells, prolactin cells, and thyroid-stimulating hormone cells. TIMP3 mRNA was identified in endothelial cells, pericytes, novel desmin-immunopositive perivascular cells, and folliculostellate cells. These findings indicate that TIMP1-, TIMP2-, and TIMP3-expressing cells are present in rat anterior pituitary gland and that they are involved in maintaining ECM components.

**Key words:** extracellular matrix, tissue inhibitor of metalloproteinases, anterior pituitary, *in situ* hybridization, immunohistochemistry

### I. Introduction

Extracellular matrix (ECM) components provide mechanical support for tissues and are important in cell growth, proliferation, differentiation, and migration. In the creation of the cellular environment, turnover of ECM components is highly regulated by (1) the production of ECM components, (2) the cleavage of ECM components by

enzymes, and (3) the inhibition of degradation. Matrix metalloproteinases (MMPs) are a major group of enzymes involved in ECM degradation (for a review, see [3]). Tissue inhibitors of metalloproteinases (TIMPs) are secretory proteins that function as endogenous inhibitors of MMPs. Four such molecules have been identified in vertebrates: TIMP1, TIMP2, TIMP3, and TIMP4 [8, 25, 31, 34]. The balance between MMPs and TIMPs is critical for ECM deposition and remodeling in tissues.

The anterior pituitary gland is composed of hormone-secreting cells, folliculostellate cells which do not produce

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**Table 1.** Sequences of the specific primers for the studied genes

Genes	Primer sequence (5'→3')	Product size	Genbank accession number
<i>Timp1</i>	Forward: TAGAGACACGCTAGAGCAGATACC Reverse: GCTGAACAGGAAAACACTG	497 bp	NM_053819
<i>Timp2</i>	Forward: AAGGACCTGACAAGGACATC Reverse: ACCCACTTGGATGATTGATG	542 bp	NM_021989
<i>Timp3</i>	Forward: GGCAACTTTGAGGAAAAGAG Reverse: CGAAATTGGAGAGCATGTC	570 bp	NM_012886
<i>Timp4</i>	Forward: GATTCTCAGTGATGGAAAAGTC Reverse: CAGCCCACTCAGGATATAGTC	736 bp	NM_001109393
<i>Gapdh</i>	Forward: CCATCACCATCTTCCAGGAG Reverse: TTCAGCTCTGGGATGACCTT	457 bp	NM_017008

classical anterior pituitary hormones, and capillary endothelial cells and pericytes. These cells are surrounded by various ECMs such as laminin, collagen, and fibronectin [9, 18, 20, 33]. Anterior pituitary cells are responsive to ECMs by adhesion. Horacek *et al.* showed that adult rat anterior pituitary cells express integrin, a receptor of ECM, and adhere to ECMs [10]. ECM components and signal transduction help control anterior pituitary hormone production and cell proliferation (for a review, see [24]). We previously reported that particular cells actively respond to ECM in the anterior pituitary gland, which suggests that the ECM and cell-ECM interaction are important in regulating gland function [11, 12].

Recently, our group identified various types of ECM-producing cells for type I and III collagens [6], laminin chains [28], and small leucine-rich proteoglycans [13] in rat anterior pituitary gland. Our study of the catabolic enzyme for ECM components in the gland revealed that folliculostellate cells express MMP9 [14]. These studies helped to show that the mechanism of ECM remodeling in the gland involved production and degradation of ECM components. However, information on inhibition of degradation is essential for a complete understanding of this mechanism. Although TIMP1, TIMP2, and TIMP3 were previously detected in normal human pituitary by Western blotting [1], there are no data on expression of TIMPs in rat anterior pituitary gland. Therefore, using *in situ* hybridization, we investigated the possibility that TIMPs and TIMP-expressing cells are present in the rat anterior pituitary gland.

## II. Materials and Methods

### Animals

Four male Wistar rats (age 8–10 weeks) were purchased from Japan SLC (Shizuoka, Japan), maintained on a 12-hr 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.

### Analysis by reverse transcription polymerase chain reaction

Reverse transcription polymerase chain reaction (RT-PCR) was performed as described previously [11]. Total RNA was extracted by using an RNeasy mini-kit and an RNase-free DNase set (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA was synthesized by a Superscript III reverse transcription kit with oligo-(dT)<sub>20</sub> primer (Life Technologies, Carlsbad, CA, USA). Sequences of the gene-specific primers are described in Table 1. Samples were subjected to 2 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 58°C, 30 s at 72°C, and then an additional 7 min at 72°C in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). Amplified products were analyzed on 1.5% agarose gels and visualized by ethidium bromide staining. Negative controls were subjected to RT-PCR without reverse transcriptase, cDNA, or Blend Taq DNA polymerase (Toyobo, Osaka, Japan) and showed no reaction bands.

### In situ hybridization and immunohistochemistry

Rats were perfused through the left ventricle with 4% paraformaldehyde 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 hr 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 [5]. PCR was used to amplify DNA fragments from rat anterior pituitary cDNA. Primer sequences are shown in Table 1. Amplified cDNA fragments were ligated into the pGEM-T vector (Promega, Madison, WI, USA) and cloned. Gene-specific antisense or sense DIG-labeled cRNA probes were made using 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 hr. Each type of mRNA was visualized with an alkaline-phosphatase-conjugated anti-DIG antibody 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 TIMP mRNAs had been detected by *in situ* hybridization, the section was immunostained as described in our previous report [4]. Sections were then incubated overnight at room temperature in phosphate-buffered saline (PBS) with primary antibodies. Primary antibodies used in the experiments were rabbit anti-porcine adrenocorticotrophic hormone 1–39 (ACTH; Dr. F. Nakamura, Hokkaido University, Hokkaido, Japan; dilution, 1:3,200), anti-rat growth hormone (GH; Dr. K. Wakabayashi, Gunma University, Gunma, Japan; dilution, 1:12,800), anti-rat prolactin (National Institutes of Health (NIH), Bethesda, MD, USA; dilution, 1:12,800), anti-rat thyroid-stimulating hormone (TSH)  $\beta$  subunit (NIH; dilution, 1:3,200), anti-ovine luteinizing hormone (LH)  $\beta$  subunit (Dr. K. Wakabayashi; dilution, 1:12,800), and anti-S100B protein (DAKO, Glostrup, Denmark; dilution, 1:500), as reported previously [4], and desmin (Abcam, Tokyo, Japan; dilution, 1:200), as reported previously [16]. Biotinylated isolectin B4 (1:25; Vector Laboratories, Burlingame, CA, USA) was used for lectin histochemistry. Absence of an observable nonspecific reaction was confirmed by using normal rabbit serum. The ABC method (Vector Laboratories) was performed with 3,3'-diaminobenzidine (Dojindo Laboratories, Kumamoto, Japan) as the substrate.

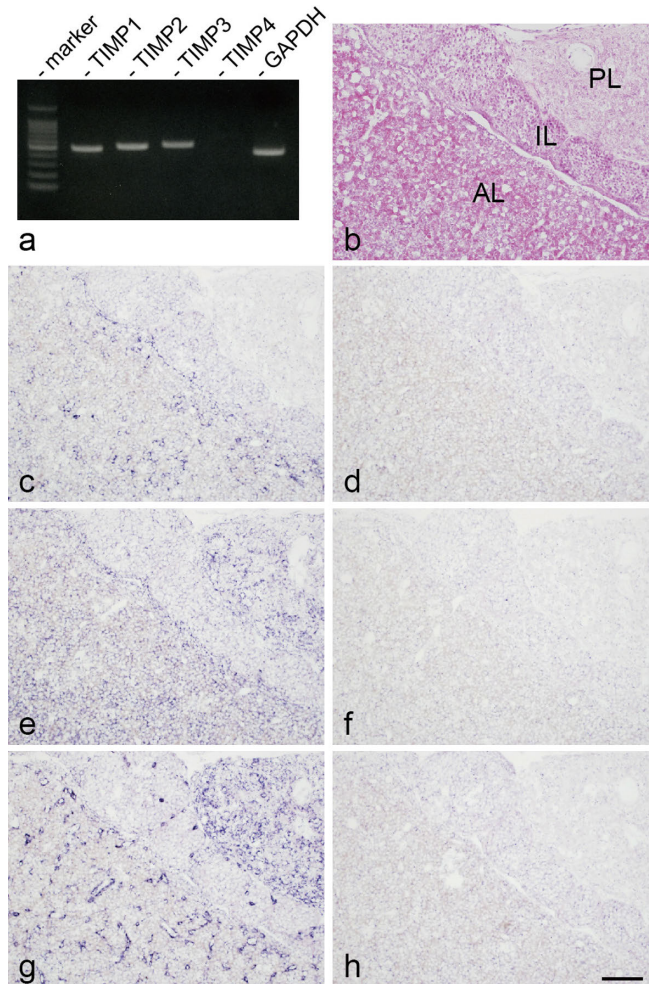
### III. Results

#### *Expression of TIMP family genes in rat anterior pituitary gland*

RT-PCR was used to examine expression of TIMP family genes in rat anterior pituitary gland. TIMP1, TIMP2, and TIMP3 mRNAs were detected (Fig. 1a). Next, localization of TIMP1-, TIMP2-, and TIMP3-expressing cells was examined by *in situ* hybridization with a DIG-labeled antisense cRNA probe. Figure 1b shows hematoxylin-eosin staining of a rat pituitary section. TIMP1-expressing cells were located in the intermediate and anterior lobes (Fig. 1c). TIMP2 and TIMP3 mRNAs were expressed in the posterior, intermediate, and anterior lobes (Fig. 1e, g). No specific signal was detected in adjacent sections processed with the DIG-labeled sense cRNA probes for TIMP1, TIMP2, and TIMP3 (Fig. 1d, f, h).

#### *Characterization of TIMP1-expressing cells*

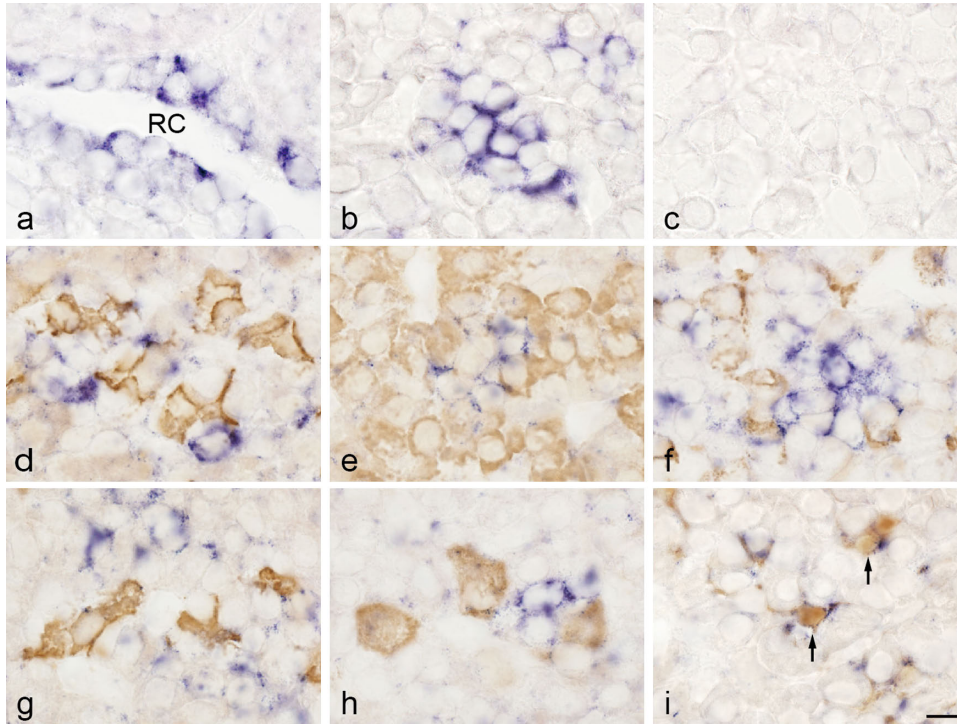
TIMP1 mRNA was detected in cells of the marginal cell layer (in both the anterior and intermediate lobes) (Fig. 2a) and in cells of the anterior lobe (Fig. 2b). A positive signal was observed only in cell cytoplasm. To identify



**Fig. 1.** Expression of tissue inhibitor of metalloproteinase (TIMP) family genes in rat anterior pituitary gland. **a:** Expression of TIMP family (TIMP1-4) mRNAs in rat anterior pituitary gland, as determined by reverse transcription polymerase chain reaction. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal control. **b:** Hematoxylin and eosin staining of a cryosection of rat anterior pituitary gland (AL: anterior lobe, IL: intermediate lobe, PL: posterior lobe). **c, d:** *In situ* hybridization for the TIMP1 antisense probe (**c**) and sense probe (**d**). **e, f:** *In situ* hybridization for TIMP2 antisense probe (**e**) and sense probe (**f**). **g, h:** *In situ* hybridization for TIMP3 antisense probe (**g**) and sense probe (**h**). Images **b-h** are consecutive sections. *In situ* hybridization with NBT/BCIP (blue). Bar=100  $\mu$ m (**b-h**).

cells that express TIMP1 mRNA, we performed double-staining using *in situ* hybridization to detect TIMP1 mRNA and immunohistochemistry to detect pituitary hormone and S100 protein. TIMP1 mRNA was detected in folliculostellate cells, which were identified on the basis of their immunoreaction to S100 protein (Fig. 2i). In this experiment, all TIMP1-expressing cells were detected by immunoreaction for S100 protein. TIMP1 mRNA was not expressed in cells that produced ACTH, GH, prolactin, TSH, or LH (Fig. 2d, e, f, g, h).





**Fig. 2.** Double-staining of TIMP1 mRNA detected by *in situ* hybridization and hormones and S100 protein detected by immunohistochemistry in rat anterior pituitary gland. **a, b:** *In situ* hybridization for TIMP1. TIMP1-expressing cells were observed in the marginal layer (**a**) surrounding Rathke's cleft (RC) and in the anterior lobe (**b**). **c:** Negative control with sense probe. **d–i:** *In situ* hybridization of TIMP1 and immunohistochemistry of adrenocorticotropic hormone (ACTH; **d**), growth hormone (GH; **e**), prolactin (**f**), thyroid-stimulating hormone  $\beta$ -subunit (TSH $\beta$ ; **g**), luteinizing hormone  $\beta$ -subunit (LH $\beta$ ; **h**), and S100 protein (folliculostellate cells; **i**). *In situ* hybridization with NBT/BCIP (blue) and immunostaining with 3,3'-diaminobenzidine (brown). TIMP1 mRNA was colocalized with the S100 protein immunoreaction (arrows). Bar=10  $\mu$ m (**a–i**).

#### Characterization of TIMP2-expressing cells

TIMP2 mRNA was detected in the marginal layer cells (Fig. 3a) and in the anterior lobe (Fig. 3b). A positive signal was observed only in cell cytoplasm. To identify cells that express TIMP2 mRNA, we performed double-staining using *in situ* hybridization to detect TIMP2 mRNA and immunohistochemistry to detect pituitary hormone and S100 protein. TIMP2 mRNA was detected in prolactin cells, TSH cells, and folliculostellate cells, which were identified on the basis of immunoreaction to prolactin, TSH $\beta$ , and S100 protein, respectively (Fig. 3f, g, i). TIMP2 mRNA was not expressed in cells that produced ACTH, GH, or LH (Fig. 3d, e, h). TIMP2 mRNA was also expressed in pituicytes, which are glial cells in the posterior lobe (Supplementary Fig. 1).

#### Characterization of TIMP3-expressing cells

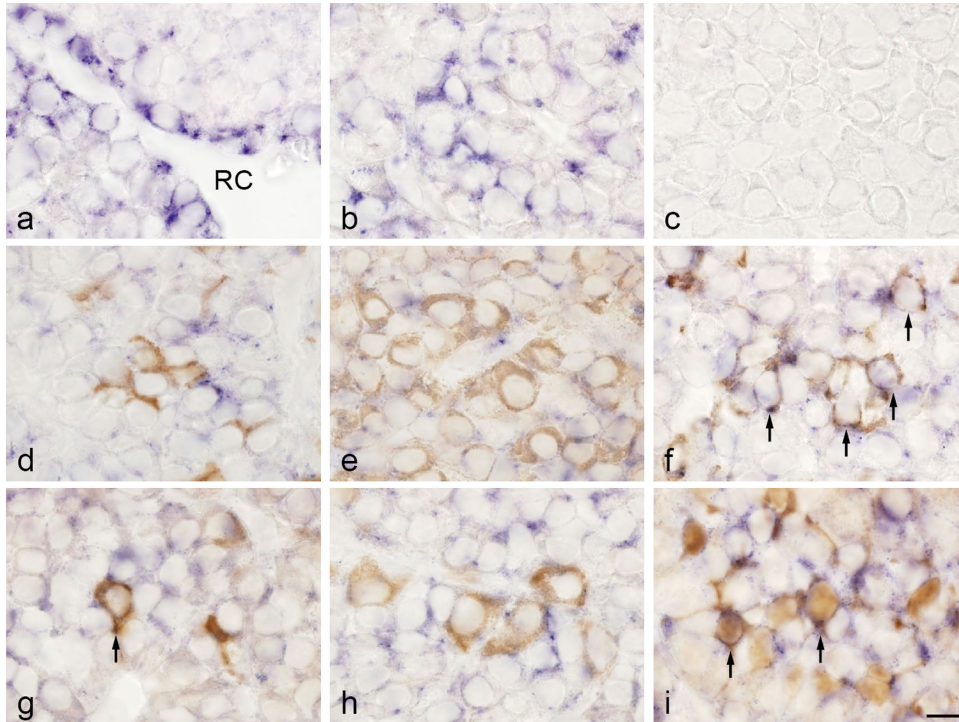
TIMP3 mRNA was detected in cells of capillaries and cells in perivascular spaces (Fig. 4a) and the anterior lobe (Fig. 4b) and was more prominent in perivascular spaces. A positive signal was observed only in cell cytoplasm. To identify cells that express TIMP3 mRNA, we performed double-staining using *in situ* hybridization to detect TIMP3 mRNA and histochemistry to detect isolectin B4, desmin, pituitary hormone, and S100 protein. TIMP3 mRNA was

detected in endothelial cells, desmin-immunopositive cells, and folliculostellate cells, which were identified on the basis of their histochemical reaction to isolectin B4, desmin, and S100 protein, respectively (Fig. 4d, e, k). Recently, our group found two types of desmin-immunopositive cells in rat anterior pituitary gland: pericytes and novel desmin-immunopositive perivascular cells [17]. TIMP3 mRNA was not expressed in cells that produced ACTH, GH, prolactin, TSH, or LH (Fig. 4f–j). TIMP3 mRNA was also detected in cells located around the endocrine cell cluster in the intermediate lobe (Supplementary Fig. 2a). In addition, TIMP3 mRNA was expressed in pituicytes, endothelial cells, and pericytes in the posterior lobe (Supplementary Fig. 2b–e).

## IV. Discussion

This is the first report to describe TIMP expression in rat anterior pituitary gland. Double-staining with *in situ* hybridization and immunohistochemistry revealed that folliculostellate cells express TIMP1, TIMP2, and TIMP3; that endothelial cells, pericytes, and novel desmin-immunopositive perivascular cells express TIMP3; and that prolactin cells and TSH cells express TIMP2.

A central mechanism in ECM remodeling is the cleav-



**Fig. 3.** Double-staining of TIMP2 mRNA detected by *in situ* hybridization and hormones and S100 protein detected by immunohistochemistry in rat anterior pituitary gland. **a, b:** *In situ* hybridization for TIMP2. TIMP2-expressing cells were observed in the marginal layer surrounding Rathke's cleft (RC) (**a**) and in the anterior lobe (**b**). **c:** Negative control with sense probe. **d–i:** *In situ* hybridization of TIMP2 and immunohistochemistry of ACTH (**d**), GH (**e**), prolactin (**f**), TSH $\beta$  (**g**), LH $\beta$  (**h**), and S100 protein (folliculostellate cells; **i**). *In situ* hybridization with NBT/BCIP (blue) and immunostaining with 3,3'-diaminobenzidine (brown). TIMP2 mRNA was colocalized with the prolactin, TSH $\beta$ , and S100 protein immunoreactions (arrows). Bar=10  $\mu$ m (**a–i**).

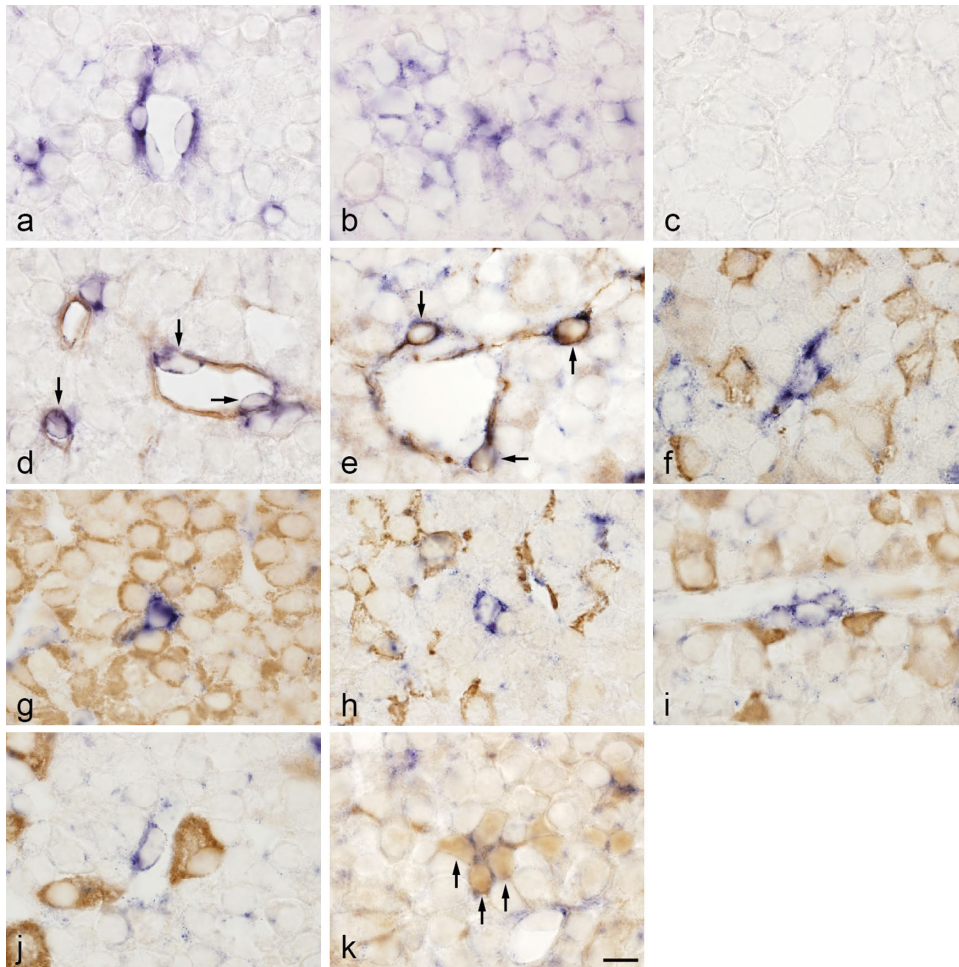
age of ECM components by MMP. TIMPs inhibit MMP activity and are thus important in the maintenance of ECM components. Homeostasis of ECM is controlled by the combination of MMP activation and inhibition by TIMPs (for a review, see [2]). Although all TIMPs inhibit the proteolytic activity of MMPs, they differ somewhat in expression pattern and interaction with MMP zymogens [7, 23]. It has been reported that TIMP1, TIMP2, and TIMP3 are widely expressed. In contrast, TIMP4 was definitely detected in heart, brain, ovary, and skeletal muscle [19, 26]. In the present study, RT-PCR showed expression of TIMP1, TIMP2, and TIMP3 mRNA, but not TIMP4 mRNA, in rat anterior pituitary gland (Fig. 1). To further understanding of the mechanism responsible for maintaining local ECM in the gland, we attempted to identify the cells that express TIMPs. Histological observations using *in situ* hybridization revealed that TIMP1, TIMP2, and TIMP3 mRNA were distributed in different cell populations in rat anterior pituitary gland (Fig. 1c–h).

Folliculostellate cells express S100 protein and regulate endocrine cells by secreting several humoral factors, such as basic fibroblast growth factor, vascular endothelial growth factor, and interleukin-6 (for a review, see [15]). In this study, we found that folliculostellate cells expressed TIMP1, TIMP2, and TIMP3 in rat anterior pituitary gland

and that TIMP1 is expressed specifically in folliculostellate cells (Fig. 2i, Fig. 3i, Fig. 4k). Matsumoto *et al.* reported that TIMP2 is secreted from TtT/GF, a mouse folliculostellate cell line, as a cell survival factor in the pituitary gland [22]. The present study is the first to show that three types of TIMP are produced in folliculostellate cells of the anterior pituitary gland *in vivo*. Interestingly, our series of studies demonstrated that folliculostellate cell functions are influenced by the interaction between folliculostellate cell and ECMs [11, 12]. In addition, folliculostellate cells produce MMP9, which is required for interconnection and proliferation of folliculostellate cells on laminin [14]. Our present and previous observations indicate that folliculostellate cells not only secrete growth factors for regulating endocrine cells, but also actively respond to ECMs and modulate the amount of ECMs involved in creating the cellular environment of the gland by producing the required catabolic enzymes and inhibitors. In immunohistochemical studies, Tomita showed that normal human anterior pituitary cells contain MMP2, MMP9, and lesser amounts of TIMP1 and TIMP2 [32]. To further elucidate the mechanism for ECM remodeling in rat anterior pituitary gland, we need to assess the correlations between the enzymes and their inhibitors.

Anterior pituitary cells form lobules surrounded by





**Fig. 4.** Double-staining of TIMP3 mRNA detected by *in situ* hybridization and isolectin B4, desmin, hormones, and S100 protein detected by immunohistochemistry in rat anterior pituitary gland. **a, b:** *In situ* hybridization for TIMP3. TIMP3-expressing cells were observed in blood capillaries, perivascular spaces (**a**), and the anterior lobe (**b**). **c:** Negative control with sense probe. **d–k:** *In situ* hybridization of TIMP3 and immunohistochemistry of isolectin B4 (endothelial cells; **d**), desmin (**e**), ACTH (**f**), GH (**g**), prolactin (**h**), TSH $\beta$  (**i**), LH $\beta$  (**j**), and S100 protein (folliculostellate cells; **k**). *In situ* hybridization with NBT/BCIP (blue) and immunostaining with 3,3'-diaminobenzidine (brown). TIMP3 mRNA was colocalized with the isolectin B4, desmin, and S100 protein immunoreactions (arrows). Bar=10  $\mu$ m (**a–k**).

ECMs, and the blood capillaries run around the lobules in the rat gland [30]. Basement membrane proteins and collagen fibers were observed in the perivascular space of the gland [18, 29]. Our previous studies revealed that the two types of desmin-immunopositive cells (pericytes and novel desmin-immunopositive perivascular cells) in the gland produce type I and III collagens [6, 17]. In addition, the endothelial cells in the gland express the basement membrane component laminin [27, 28]. The present study revealed that the endothelial cells, pericytes, and novel desmin-immunopositive perivascular cells express TIMP3 in rat anterior pituitary gland (Fig. 4d, e). A previous study showed that, unlike other TIMPs, TIMP3 is entrapped in the ECM by binding to sulfated glycosaminoglycans [35]. Our findings suggest that ECM-producing cells, endothelial cells, pericytes, and novel desmin-immunopositive perivascular cells also secrete TIMP3, which remains with the ECM in the perivascular spaces of rat anterior pituitary

gland and contributes to the deposition of ECM components in those spaces.

We found that TIMP2 mRNA is expressed in endocrine cells, prolactin cells, and TSH cells (Fig. 3f, g). This suggests that the active state of endocrine cells affects TIMP2 expression and can alter the ECM state in rat anterior pituitary gland. This result is important in clarifying the interaction between hormone-secreting cells and ECM in the gland.

Mander and Morris showed that macrophages are present in rat anterior pituitary gland [21]. We examined expression of TIMPs in macrophages of the gland by double-staining with *in situ* hybridization for TIMPs and immunohistochemistry for markers of macrophages (data not shown). We found that the number of macrophages was extremely low in the gland. Furthermore, only some macrophages were positive in *in situ* hybridization for TIMPs in the gland. The low number of macrophages suggests that

TIMPs of macrophages are not important in the maintenance of ECM in the gland.

The present results strongly suggest that appropriate utilization of TIMPs and TIMP-expressing cells is crucial for local ECM remodeling in the rat anterior pituitary gland.

## V. Declaration of Interest

The authors have no conflict of interest that might prejudice the impartiality of this research.

## VI. Acknowledgments

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