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Molecular characterization and hormonal regulation of tissue inhibitor of metalloproteinase 1 in goat ovarian granulosa cells

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Tissue inhibitor of metalloproteinase 1 (TIMP1) belongs to a group of endogenous inhibitors that control the activity of matrix metalloproteinases and other metalloproteinases. TIMP1 is ubiquitously expressed and implicated in many physiological and pathologic processes. In this study, the full-length complementary DNA of goat (*Capra hircus*) *Timp1* was cloned from adult goat ovary for the first time to better understand the regulatory role of TIMP1. The putative TIMP1 protein shared a high amino acid sequence identity with other species. Real-time polymerase chain reaction results showed that *Timp1* was widely expressed in adult goat tissues, and messenger RNA expression was higher in the ovary than in other tissues; meanwhile, increasing expression of *Timp1* was also discovered during the process of follicle growth and corpus luteum. We then investigated *Timp1* expression patterns in different types of ovarian follicular cells from goats. In small or large antral follicles, *Timp1* expression was higher ($P < 0.05$) in theca cells than in granulosa cells, cumulus cells, and oocytes. Increasing expression of *Timp1* in theca and granulosa cells was observed as the variation of the follicle size. Immunohistochemical analyses further revealed the presence of the TIMP1 proteins in follicles at all antral stages of development. The most intense staining for TIMP1 was observed in the theca cells and granulosa cells of large antral follicles and corpus luteum. *Timp1* was highly ($P < 0.05$) induced in granulosa cells in vitro after treatment with the luteinizing hormone agonist, human chorionic gonadotropin. Treatments with forskolin, phorbol 12-myristate 13-acetate, or phorbol 12-myristate 13-acetate + forskolin could also stimulate *Timp1* messenger RNA expression. The effects of human chorionic gonadotropin were reduced ($P < 0.05$) by the inhibitors of protein kinase A, protein kinase C, MAPK kinase, or p38 kinase, indicating that *Timp1* expression could be adjusted by luteinizing hormone–initiated activation of these signaling mediators. Our results suggested that TIMP1 may be involved in regulating ovarian follicle development and ovulation.

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1. Introduction

Tissue inhibitors of metalloproteinases (TIMPs) belong to endogenous protein regulators of the matrix metalloproteinases and other metalloproteinases [1,2]. In mammals, 4 TIMPs (TIMP1–4) that share substantial sequence homology and structural identity at the protein level have been identified [2,3]. TIMP proteins possess a similar domain structure, which is composed of an N-terminal domain and a C-terminal domain [4]. Previous studies have shown that the members of TIMPs have key functions in various physiological processes, including

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morphogenesis, reproduction, cancer, arthritis, and angiogenesis [5–10]. TIMP1 is the first member of the TIMPs family with multiple functions in numerous human and rodent cells [4,11]. It is expressed in a wide range of mammalian tissues, notably in the reproductive organs [12,13]. Earlier studies have reported that *Timp1*-null mice could cause reproductive cycle disorder and fertility reduction [14–16]. In the ovary, TIMP1 has been postulated to have a critical role in extracellular matrix remodeling associated with ovulation and luteal formation and regression [17,18].

The gonadotropin, luteinizing hormone (LH) is an essential regulator of ovarian endocrine function and female fertility [19]. The LH-induced preovulatory changes are mediated by inducing a complex pattern of gene expression in the granulosa cells (GCs) that is regulated by different signaling cascades such as cAMP/protein kinase A (PKA), ERK1/2, and phosphatidylinositol-3 kinase (PI3K) cascades [20]. Evidence has pointed out the key role of TIMP1 during the ovulatory process. Studies on rodents have identified a gonadotropin-dependent upregulation of TIMP1 in GCs after LH and/or human chorionic gonadotropin (hCG) stimulation [21,22]. The hCG-stimulated expression of *Timp1* messenger RNA (mRNA) can be inhibited by inhibitors of PKA (H89), protein kinase C (PKC and GF109203X), and MAPK (SB2035850) pathways [22].

In a previous study, we have demonstrated that *Timp1*, a differentially expressed gene, was screened from ovarian tissues between polytocous and monotocous Guanzhong dairy goats by suppressive subtractive hybridization [23]. The results confirmed that the different levels of *Timp1* mRNA expression in the ovarian tissues of polytocous and monotocous goats could lead to different ovulation rates, which causes a difference in litter size. To identify the role of TIMP1 in the development of GCs during folliculogenesis, in the present study, we cloned and characterized the goat *Timp1* gene. The sequence of the deduced form of TIMP1 protein was analyzed, and its phylogenetic relationship was compared with TIMP1 from various species. The expression level in various tissues was analyzed by real-time polymerase chain reaction (PCR). Furthermore, we explored the expression abundance of the *Timp1* gene transcript at different ovarian follicle stages and the modulatory effects of gonadotropin on the expression of *Timp1* mRNA in goat ovarian GCs in vitro. The results of this study provided information on the *Timp1* gene with regard to its sequence, tissue expression profile, and influence of gonadotropin on its expression.

2. Materials and methods

2.1. Reagents

hCG (human chorionic gonadotropin) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Chemicals and reagents including H89, LY294002, PD98059, GF109203X, and forskolin (FSK) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phorbol 12-myristate 13-acetate (PMA) and SB2035850 were purchased from Beyotime Biotechnology (Jiangsu, China). The DMEM/F12, FBS (fetal bovine serum), and Platinum SYBR Green qPCR SuperMix-UDG were the products of Life Technologies Inc (Carlsbad, CA, USA).

2.2. Collection of tissues and cells

To evaluate the tissue-specific pattern of *Timp1* expression, different tissues (uterus, spleen, kidney, heart, liver, lung, ovary, muscle, fat, mammary gland, and oviduct) were isolated from 4 adult Guanzhong dairy goats. Healthy adult Guanzhong dairy goats at the estrous cycle stage were stunned with a captive bolt and slaughtered to collect the different tissues. In 0.1% w/v diethylpyrocarbonate water, the different tissues of the goats were cut into small pieces of approximately 1 g and immediately placed in liquid nitrogen until RNA isolation.

Ovaries of cyclic Guanzhong dairy goats were collected and fixed in paraformaldehyde for immunohistochemical localization of TIMP1 protein or used to collect follicles and luteal tissue to study the mRNA expression of *Timp1* using real-time PCR. During the breeding season, ovaries were recovered from slaughtered cyclic adult Guanzhong dairy goats and transported to the laboratory in phosphate-buffered saline (PBS, 4°C) supplemented with penicillin (100 IU/mL) and streptomycin (50 mg/mL). The ovaries were washed 3 times in PBS, and the different cell types were recovered according to their follicular diameter. The small (1 mm–3 mm) and large (>3 mm) healthy antral follicles were isolated from 30 ovaries and dissected free from stromal tissue using 25-gauge needles as describe by Lucci et al [24]. After 3 times of brief washing with PBS, the small (60 follicles per pool; $n = 4$ independent sample pools) and large (48 follicles per pool; $n = 4$ independent sample pools) healthy antral follicles were cut into pieces, and then, an aseptic needle was used to release the GCs. The cumulus–oocyte complexes (COCs) and layers of theca cells (TCs) were collected under a stereomicroscope (Nikon, Tokyo, Japan). The COCs that were surrounded by at least 3 layers of cumulus cells (CCs) and with evenly granulated cytoplasm were selected. After collecting the COCs, the CCs were mechanically separated by careful and repeated pipetting until no adherent CCs could be observed under the stereomicroscope. Oocytes were denuded, and the CCs from the COCs were collected. GCs were harvested by centrifuging (800*g*) for 10 min and washed twice in PBS. To collect TCs, layers of TCs were washed 3 times in PBS then vortex mixed for 1 min in 1 mL of PBS, transferred to 1 mL of fresh buffer, and centrifuged for 1 min. The cell pellets were homogenized in 0.5 mL of RNAiso Plus (TaKaRa, Dalian, China) and stored at −80°C until RNA extraction.

2.3. Isolation and culture of GCs

Goat ovaries were obtained from a local slaughterhouse, irrespective of stage of the estrous cycle, and then, placed into PBS supplemented with penicillin (100 IU/mL) and streptomycin (50 mg/mL) at 4°C. The samples were then transported to the laboratory within 2 h. The ovaries were washed 3 times with prewarmed PBS. The method of collecting and culturing GCs has been described previously [25]. Briefly, the tissue was first washed with 75% alcohol for 1 min and then washed 3 times with PBS to eliminate

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