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Original article

Knockdown of melatonin receptor 1 and induction of follicle-stimulating hormone on the regulation of mouse granulosa cell function

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ABSTRACT

Melatonin receptor 1 (MT1) performs a critical role in the regulation of the animal reproductive system, particularly in follicular growth, and has a considerable effect on reproductive performance. However, the role that MT1 plays in regulating hormones associated with reproduction remains unclear. This study was designed to examine the physiological role of constitutive MT1 silencing and follicle stimulating hormone (FSH) treatment in reproduction, making use of mouse granulosa cells (mGCs) as a model. To understand the constitutive role of MT1 in ovarian physiology, the RNAi-Ready pSIREN-RETROQ-ZsGreen Vector mediated recombinant pshRNA was used to silence *MT1* gene expression. Furthermore, we observed that the expression of MT1 was successfully inhibited both at the protein and mRNA levels ($P < 0.001$). We demonstrated that RNAi-B-mediated MT1 down-regulation significantly promoted apoptosis ($P < 0.001$), inhibited proliferation, and regulated the cell cycle at the S-phase; conversely, FSH treatment partially aided the apoptotic effect and improved proliferation but showed a significant effect at the S-phase of the cell cycle. Transitory knockdown of MT1 proved essential in the function of mGCs, as it significantly decreased cyclic adenosine monophospahte (cAMP) level and increased cell apoptosis. Following knockdown of MT1, the expression of Bax was significantly up-regulated ($P < 0.001$), but Bcl-2 was slightly down-regulated, both at the transcriptional and at translational levels. Moreover, the silencing of MT1 and its constitutive effect on FSH significantly promoted an increase in estradiol ($P < 0.001$) and slightly decreased the concentration of progesterone. Together, our data indicates that MT1 suppression leads to interference in the normal physiological function of the ovary by enhancing follicular apoptosis, inhibiting proliferation, and influencing hormonal signaling, whereas constitutive FSH treatment counteracted the negative down-regulatory effects of MT1 on mGCs.

1. Introduction

Folliculogenesis is a complex and core reproductive process that is governed by both paracrine and endocrine factors. This process involves functional and morphological changes in theca, cumulus, and granulosa cells (GCs), which are the main components of ovarian follicles. Along the processes of follicular development, the vast majority of follicles and oocytes undergo atresia before ovulation, and this phenomenon remains mysterious. Melatonin (MLT; *N*-acetyl-5-methoxytryptamine) is a neurohormone that is secreted from the vertebrate pineal gland [2]. It is involved in various mammalian reproductive functions and also acts as an antioxidant [17]. Several previous studies have reported on the robust expression of *MLT* and its two membrane receptors (*MT1* and *MT2*) in the ovaries of various mammalian species including humans [18], rats [26,36], cattle [32], and mice [16]. It has also been reported that *MT1* and *MT2* are either individually or co-expressed in reproductive and cardiac tissues [7,23]. Apart from normal cells and tissues, the expression of MLT has been reported in malignant or cancerous cells [8].

Several lines of evidence have shown the involvement of MLT in a wide range of pathological processes such as reproductive and cardiac disorders [28] and cancer [31]. In addition to MLT, both of its receptors have been shown to play a role in several diseases, including cancer, Alzheimer's, and Parkinson's [20]. The stimulation of these receptors depends on multiple cellular signaling pathways as well as the inhibition of adenyl cyclase (cAMP) activity, which is the *MT1* signaling pathway [7,8,32].

MLT has been shown to have a prominent role in follicular growth,

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oocyte maturation, luteinization, and early development [4,5,19,25,30]. MLT appears to govern most of its cellular action through its ability to bind to its G-protein-coupled receptors, MT1 and MT2, and thereby enhances G-protein activity [15]. Many previous studies have documented the crucial role of both MT1 and MT2 in the normal physiological processes of mammalian reproduction. As such, a recent study conducted by He et al. showed that MLT/MT1 regulates luteinizing hormone (LH) and subsequent luteinization in both bovine and mice ovaries [12]. Another group reported that the anti-apoptotic property of MLT functioned as a consequence of binding to MT1 and 2 in cattle and pigs [13,32]. Moreover, the combined effect of follicle stimulating hormone (FSH) and MLT has been explored on the preantral follicle growth of the caprine *in vitro,* and the results revealed that the interaction of FSH and MLT was vital for the integrity and growth of follicles [24].

Although the role of MLT and its receptors has been extensively investigated using various model animals, the vast majority of studies have been focused on the study of MLT and both of its receptors. Furthermore, most of these studies have been carried out by modulation of MLT using exogenous supplementation of agonists or antagonists. Due to the robust expression of ML1, its unique physiological function should be elucidated.

The interaction of MLT and FSH influences the growth of follicles and their maturation, and MT1 is a downstream effector of MLT. Here, we investigated whether the silencing of *MT1* by RNAi followed by FSH treatment affected the mouse GC (mGC) cell cycle, apoptosis, proliferation, and genes related to these processes.

2. Materials and methods

2.1. Experimental animals

In this experiment, we collected ovaries from untreated sexually immature mice mimic natural conditions. The ovaries were collected from 21-day-old mice, when growth, proliferation, and other physiological functions of GCs are most active. At this stage the ovarian are predominately composed of primary follicles. Three-week-old Kunming strain female mice were obtained from the Hubei Animal Research Experimental Center (Wuhan, China) and housed in a solitary room with a controlled temperature (20 °C–25 °C), humidity (5% CO_2) and a 12 h light/dark cycle. The mice were provided free access to water and feed. All protocols were approved by the Ethical Committee of the Huazhong Agricultural University (Approval ID: SCXK (Hubei) 2008-0005).

2.2. Isolation and culture of mGCs

Mice were dissected and ovaries were collected in 60-mm cell culture plates containing fresh phosphate buffered saline (PBS). Then, the mGCs were punctured and centrifuged at 1500 rpm for 5 min as previously described [35]. The supernatants were discarded and the pellets were cultured in a medium composed of 89% Dulbecco's modified Eagle's medium (DMEM), 10% FBS and 1% of streptomycin/penicillin. The cells were maintained under conditions of 37 °C with 5% CO_2 for 48 h and were supplemented with fresh medium every 24 h.

2.3. RNA extraction and real-time PCR

Total RNA was extracted from cultured mGCs using TRIzol (Invitrogen, Pacific Grove, CA, USA) and reverse transcription was done using the first strand cDNA kit (Toyobo, Osaka, Japan) following the manufacturer's instructions. The expression of *MT1* and other related genes was detected with Real-time PCR (Table 1). Reactions were conducted in a total of 20 µl composed of 0.3 µl of both forward and reverse primers (10 µM), 5 µl SYBR green, 0.4 µl template, and 10 µl ddH_2O was used. Beta-actin (*β-actin*) was used as a reference gene for the normalization of the expression of mRNA levels of related genes. For transparency, melting curve analysis was performed to monitor PCR product purity.

Table 1
List of primers used for Real-time PCR.

Gene	Sequence of Primer (5′–3′)	Annealing Temperature (°C)	Product Length (bp)
MT1	F: CCATTTCATCGTGCCTATG R: GTAACTAGCCACGAACAGC	53	259
Bcl-2	F: GCTACCGTCGTGACTTCGC R: ACCCAGCCTCCGTTATCC	57	173
Bax	F: GCCTTTTTGCTACAGGGTTTCAT R: TATTGCTGTCCAGTTCATCTCCA	58	320
PCNA	F: GTCGGGTGAATTTGCACGTA R: GACGGTCCACCTTTATGTT	59	178
P21	F: AACTGACTGCTCCCCTGTCTA R: CTCTATGGTTACCGCCTCCTC	57	108
BETA-ACTIN	F: AGGTCATCACTATTGGCAAC R: ACTCATCGTACTCCTGCTTG	54	400

2.4. Construction of MT1-recombinant plasmid and transfection

Using the short hairpin RNA (shRNA) structure, two shRNA target sequences were identified from cds of MT1 (Accession number: NM_008639) at cds positions 434 and 1245, and were chosen for plasmid construction (Table 2). To obtain (shRNA) for silencing the target *MT1* gene, oligonucleotides 65 bp in length were constructed, consisting of 19 bp of the sense strands, 19 bp of the antisense strand, 6 bp of the BamHI restriction sites, 9 bp of a short hairpin loop, 6 bp of a terminator, and 7 bp of an EcoRI restriction site. Following construction of the plasmid, two complementary strands, bottom and top oligonucleotides, were annealed and inserted into the BamHI and EcoRI plasmid RNAi-Ready pSIREN-RetroQ-ZsGreen (BD Biosciences, Clontech, Mountain View, CA, USA). The recombinant plasmids were named RNAi-A and RNAi-B and RNAi-negative control, which encoded a nonsense sequence that did not match the mouse or human genome. The plasmids were purified using the Endo-free plasmid kit from Omega Bio-Tek, (Beijing, China). 48 h prior to transfection, the mGCs were cultured in six-well cell culture plates and supplemented with medium until 70%-80% confluence was attained. Three plates of cultured mGCs were transfected to RNAi-A, RNAi-B, and RNAi-negative control using Lipofectamine LTX with Plus (Invitrogen) based on the manufacturer's instructions. Six hours after transfection, fresh medium without antibiotics was added, and after 48 h the cells were subjected to total RNA and protein extraction for subsequent experiments. Part of the culture medium was preserved for hormonal assays.

2.5. Measurement of cAMP

mGC cells (1×10^5 viable cells in 200 µl medium) were transfected in 96-well plates, with or without FSH stimulation, with three wells for each sample. Then, the cells were washed with pre-warmed PBS and treated with 1 µM 3-methylxanthine (IBMX, Sigma-Aldrich, St. Louis, USA) for 30 min followed by the addition of forskolin (10 µM, Sigma–Aldrich) for 10 min. cAMP was measured using the cAMP-Glo measuring kit (10 µM, Sigma-Aldrich) for 10 min.

Table 2
shRNA description.

shRNA	Target sequences (5′–3′)	Position on cds
RNAi-A	GAACTCGCTGCTACGTG	434
RNAi-B	GCAGGCACCATATGTTA	1245
Negative control	TGGACATGGCGACGTG	