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Research paper

Analysis of miRNA expression profiles in melatonin-exposed GC-1 spg cell line

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ABSTRACT

Melatonin is an endocrine neurohormone secreted by pinealocytes in the pineal gland. It exerts diverse physiological effects, such as circadian rhythm regulator and antioxidant. However, the functional importance of melatonin in spermatogenesis regulation remains unclear. The objectives of this study are to: (1) detect melatonin affection on miRNA expression profiles in GC-1 spg cells by miRNA deep sequencing (DeepSeq) and (2) define melatonin affected miRNA–mRNA interactions and associated biological processes using bioinformatics analysis. GC-1 spg cells were cultured with melatonin (10^{-7} M) for 24 h. DeepSeq data were validated using quantitative real-time reverse transcription polymerase chain reaction analysis (qRT-PCR). A total of 176 miRNA expressions were found to be significantly different between two groups (fold change of > 2 or < 0.5 and FDR < 0.05). Among these expressions, 171 were up-regulated, and 5 were down-regulated. Ontology analysis of biological processes of these targets indicated a variety of biological functions. Pathway analysis indicated that the predicted targets were involved in cancers, apoptosis and signaling pathways, such as VEGF, TNF, Ras and Notch. Results implicated that melatonin could regulate the expression of miRNA to perform its physiological effects in GC-1 spg cells. These results should be useful to investigate the biological function of miRNAs regulated by melatonin in spermatogenesis and testicular germ cell tumor.

1. Introduction

Melatonin is mainly secreted by the pineal gland and regulated by light and darkness (Al-Hussain, 2006). This hormone mediates its pivotal actions through either receptor-mediated or receptor-independent mechanism (Reiter et al., 2007). The most significant role of melatonin is the circadian rhythm regulation in mammalians (Cajochen et al., 2003). Melatonin also plays as a scavenger of reactive oxygen species (ROS) to possess anti-oxidative properties (Vishwas et al., 2013). The role of melatonin in reproduction has been supported by growing studies. In female reproduction, follicle growth, oocyte maturation, ovulation, and luteinization produce excessive ROS. Melatonin and its metabolites play central roles in these processes as a powerful scavenger to attenuate oxidative stress. A study by Li et al. showed that melatonin application protected porcine oocytes from thermo stress in vitro (Li et al., 2015). In male reproduction, melatonin was involved in testicular development by affecting testosterone secretion (Redins et al., 2002). Accumulated studies revealed that melatonin processed a beneficial function on human sperm motility (Ortiz et al., 2011), ram and pig sperm quality (Casao et al., 2010; Jang et al., 2010), and testicular damage due to testicular torsion in rats (Kanter, 2010) by its anti-oxidative properties.

MicroRNAs (miRNAs) are small noncoding RNAs. The mature miRNA performs its biological functions by binding to a seed region in the 3′ untranslated region (UTR) but may also bind to the 5′ UTR of target mRNA (Lytle et al., 2007) to enhance mRNA degradation or inhibit translation and result in the decrease of protein expression. Growing studies have demonstrated that miRNAs are considered as key regulators in reproduction. A series of conditional knockout (cKO) mice experiments of *Drosha*, *Dgcr8* and *Dicer* used a variety of Cre-mediated promoters at approximate time points, revealing the diverse and central roles of miRNAs in ovarian function and spermatogenesis (Wang et al., 2007; Nagaraja et al., 2008; Papaioannou et al., 2009; Lei et al., 2010; Wu et al., 2012). The research by Fiedler et al. demonstrated that

Abbreviations: miRNAs, microRNAs; DeepSeq, deep sequencing; ROS, reactive oxygen species; UTR, untranslated region; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; qRT–PCR, quantitative real-time reverse transcription polymerase chain reaction; SSC, spermatogonial stem cell

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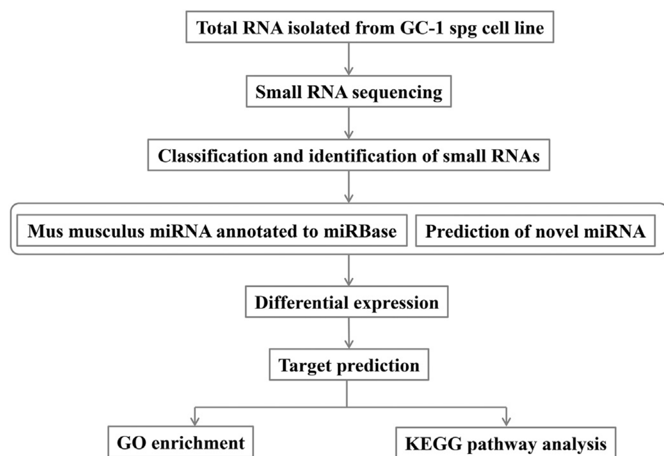

Fig. 1. Workflow of small RNA DeepSeq. Total RNA was isolated from GC-1 spg cell line treated with/without melatonin for 24 h. Rfam was used for miRNA annotation. Target prediction was performed using miRanda and RNAhybrid. Gene enrichment and functional annotation analyses were conducted using GO and KEGG.

miRNA could be regulated by LH/hCG at post-transcriptional level to affect gene expression in mouse granulosa cells (Fiedler et al., 2008). Another study showed that miRNAs were involved in FSH-mediated progesterone production in rat granulosa cells in vitro (Yao et al., 2010).

Previous studies revealed that melatonin could be regulated by and could regulate miRNAs to perform its pivotal functions. A study from Clokie et al. showed that melatonin synthesis in the pineal gland could be regulated by miR-483 (Clokie et al., 2012). Other studies reported that melatonin could regulate miRNA expressions to perform its anticancer function in different cancers (Han et al., 2011; Lee et al., 2011; Mori et al., 2016). Our previous study showed that melatonin could alter the expression profiles of miRNAs in mouse testis, and further investigation suggested that melatonin could promote the proliferation of GC-1 spg cells by reducing miRNA-16 expression by targeting *Ccnd1* (Li et al., 2016). However, a miRNA expression pattern has not yet been reported in melatonin exposed GC-1 spg cell line. Therefore, the objectives of this study are to (1) determine the effects of melatonin on the miRNA expression profiles in GC-1 spg cell line using DeepSeq and (2) define the affected miRNA–mRNA interactions and associated biological processes using integrated target prediction analyses.

2. Materials and methods

2.1. Cell culture and RNA extraction

GC-1 spg cell line (ATCC, CRL-2053) was cultured in DMEM with high glucose (Hyclone, USA) containing 10% FBS (Gibco, USA) and incubated at 37 °C with 5% CO_2. When cells reached the 70%–80% confluence of the six-well plate, medium was changed to serum-free medium with/without melatonin of 10^{-7} M for another 24 h. Three replicates were prepared for each group. Total RNA was extracted from each sample using Trizol (NEB, UK) reagent following the manufacturer's protocol. RNA quantity was determined with a Nanodrop 2000 spectrophotometer (Thermo, USA), and RNA quality was determined using 1% agarose gel electrophoresis and analyzed with Alpha Imager EP (Alpha Innotech, USA).

2.2. DeepSeq and data analysis

The NEBNext Multiplex Small RNA Library Prep Set for Illumina (NEB, USA) was used for RNA library preparation according to the manufacturer's protocol. Briefly, 3′ and 5′ SR Adaptor for Illumina were ligated to total RNA through ligation reactions separately, and reverse transcription was accomplished using ProtoScript II Reverse Transcriptase (NEB, USA) for 1 h at 50 °C. PCR amplification was performed using ProtoScript® II First Strand cDNA Synthesis Kit (NEB, USA), and purification was done using 15% non-denaturing PAGE gel. Bio-Qseq-100 was used to analyze size distribution, Qubit 2.0 Fluorometer was used to assess miRNA library concentration, and library quantification was performed using KAPA Biosystem Library Quantification kit (Kapa Biosystems, USA).

DeepSeq was performed using an Illumina HiSeq 2500 instrument (Illumina Inc., USA) at 50 bp single-end condition. FastQC (Babraham Bioinformatics, UK) was used to do quality control checks on raw sequence data. Ligated adapters from the 3′ end of the sequenced reads were removed. Reads with poly A/poly T shorter than 15 nucleotides in length and low qualities were eliminated from the total reads. Filtered reads were annotated with miRBase (ftp://mirbase.org/pub/mirbase/CURRENT/). Fold change ≥ 2.0 or ≤ 0.5 at FDR < 0.05 was identified as difference.

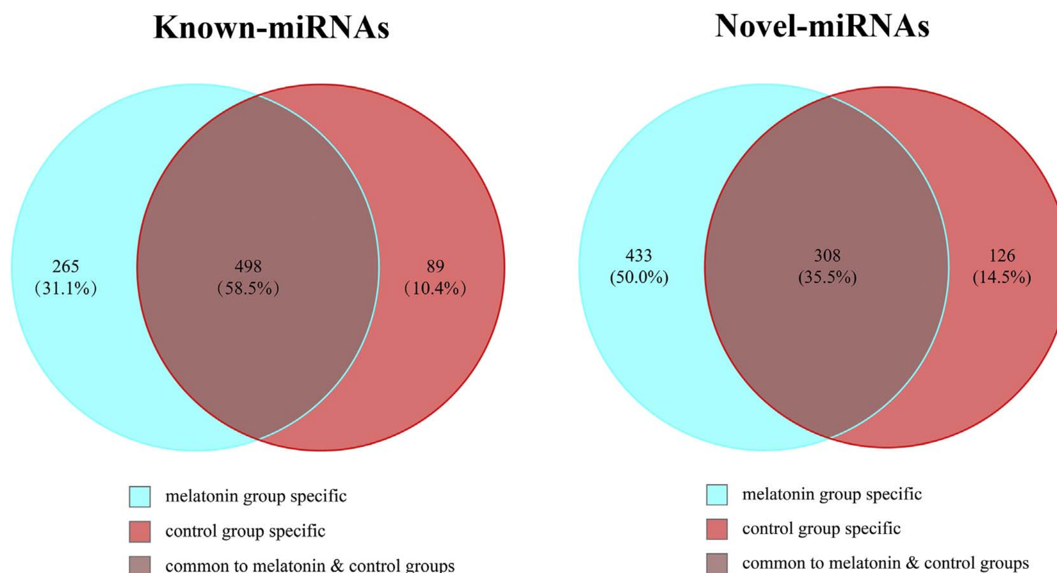

Fig. 2. Venn diagrams illustrating unique and overlapping differential miRNA expression domains for melatonin-exposed and control groups. DeepSeq results showed that 763 known and 741 novel miRNAs were expressed in the melatonin-exposed group. Both in known and novel miRNA groups, more miRNAs were detected in melatonin-exposed group.

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