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Identification and profiling of microRNAs from ovary of estrous Kazakh sheep induced by nutritional status in the anestrous season

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ABSTRACT

Estrous regulation in sheep has an important role in the sheep industry in improving production of meat and wool. It has been reported that an enhanced nutritional status can induce estrus resulting in an end of the anestrous season earlier than occurs in ewes with a lesser nutritional status. However, the endocrine and physiological mechanisms that induce the increased incidence of estrus remains unclear. In the present study, the differences in amounts and characteristics of miRNAs in ewes at estrus or during the anestrous season were screened by using the Illumina HiSeq sequencing technology. In total, 294 miRNAs, including 174 novel miRNA candidates, were identified in ewes with an enhanced nutritional status (OEN) through assessment of the OEN library for this group and 307 miRNAs including 186 novel miRNA candidates were identified in the ewes with a lesser nutritional status (OAN) through assessing the OAN library, among which there were nine conserved and 104 novel miRNAs in differential amounts between the two libraries. Based on poly (A) q-PCR, six miRNAs were assessed to verify the accuracy of the library database. Furthermore, the family of the known miRNAs, the target genes and related pathways were also analyzed. The results indicated that the nutritional status had important roles in estrous regulation in sheep. The PLA2G4D can directly regulate ovarian follicle development, or indirectly influence leptin secretion involved in the regulation of the reproductive endocrine and physiological systems during the anestrous season. The identification of significantly different miRNAs expanded the repertoire of sheep miRNAs that have been examined and could contribute to further studies on the molecular mechanism of regulation of initiation of estrous cycles in previously anestrous ewes as influenced by different nutritional status.

1. Introduction

Reproductive functions of sheep are strongly influenced by photoperiod with the transition from anestrus to estrus occurring in the late summer or early autumn and the period over which estrous cycles occur ceasing in the late winter or early spring (Greives et al., 2007; Thimonier, 1981; Shinomiya et al., 2014). Reproductive behavior of

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sheep is also modulated by other environmental (e.g., nutrition) or social (e.g., male effects or interactions of ewes and rams) factors, which directly affect the production efficiency and economic costs which bring enormous benefits in the sheep industry (Forcada and Abecia, 2006). Therefore, increasing the incidence of behavioral estrous rate by shortening the anestrous season is very important in the sheep industry.

MicroRNAs (miRNAs) are a new class of single-stranded endogenous non-coding small RNA molecules (~22 nt) first discovered in 1993 and named as lin-4, a gene known to control the timing of *C. elegans* larval development (Rosalind et al., 1993; Bruce et al., 1993). However, miRNAs were not recognized as a class of biological regulators with conserved functions until the second miRNA, let-7, was identified (Reinhart et al., 2000). Over the past decades, tens of thousands of miRNAs have been detected in the *arabidopsis thaliana*, *Caenorhabditis elegans* (Johnston and Hobert, 2003), *Drosophila melanogaster* (Ashraf et al., 2006), *Mus musculus* (Krutzfeldt et al., 2005), *Danio rerio* (Giraldez et al., 2005) and *Homo sapiens* (Lewis et al., 2005) which have diverse biological functions in control of cell proliferation, cell death, fat metabolism, neuronal cell fate, hormone secretion, and other disease processes (Johnston et al., 2005; Naguibneva et al., 2006). A small portion of miRNAs (e.g., let-4 and lin-7) are present in a physiological stage-specific manner, while a large number of miRNAs are present in tissue-specific patterns (Ruvkun, 2001). A majority of miRNAs inhibit target gene expression by binding to complementary sequences in the 3′ untranslated regions (3′ UTR of mRNAs; Ruvkun, 2001). Although the miRNAs that are 22 nt in length are more abundant, there are fewer of the larger transcripts of approximately 70 nt which are implicated in regulation of the Dicer protein which mediates maturation of miRNAs and subsequently cleavage or translation repression of target mRNAs (Bernstein et al., 2001). A single miRNA may have one to several hundred target mRNAs, and a target mRNA may be regulated by one to several hundred miRNAs, and it is estimated that miRNAs may regulate 30% of protein-coding genes (Lewis et al., 2005).

The MiRNAs have important roles in the development of primordial germ cells. Otsuka et al. (2007) reported that the down-regulation of the Dicer enzyme in ovaries of mice led to a failure of pregnancy in the wild type mice. Recent studies provided evidence that ovulation and fertilization maybe normal in these mice, however, the related hormones (prolactin, progesterone and luteinizing hormone) may be down-regulated in the mice as a result of Dicer knockdown (Otsuka et al., 2008). Another study showed that Dicer knockdown could inhibit the transformation of the primordial germ cells to oogonia (Hong et al., 2008). The miR-129-2 and miR-290-295 were up-regulated in the development of primordial germ cells and the latter was closely associated with the primordial germ cell migration and survival, while the miR-141, miR-200a, miR-200c and miR-323 were down-regulated in the differentiation process of primordial germ cells (Hayashi et al., 2008). Another report showed that there were 13 down-regulated miRNAs after TGFβ treatment in ovary granular cells, among which miR-224 regulated the proliferation of ovary granular cells and the expression of the aromatase gene (Cyp 19a1) by targeting the SMADS in the TGFβ1/SMADS pathway (Yao et al., 2010). There was, however, another report that provided evidence that the miR-383 was down-regulated in TGFβ treated mice, and its target, RBMS1, positively regulated the aromatase gene (Cyp19a1) expression and E_2 secretion (Yin et al., 2012).

To explore the influence of nutritional status on regulation of the initiation of sheep estrous cycles in anestrous ewes, the present study was designed to observe the profiles of microRNA in Kazakh sheep ovaries during estrus as a result of enhanced nutritional (OEN) and in the anestrous condition in a group of ewes that were fed the typical diet (OAN) during the anestrous season. The Illumina HiSeq sequencing technology was used to assess amounts of miRNA in the two groups. Subsequently, target gene-dependent KEGG pathways were analyzed by incorporating information about known reproduction pathways to find the regulatory network of enhanced nutritional status involving in regulating the initiation of estrous cycles in seasonally anestrous ewes.

Table 1
Nutritional content of alfalfa and concentrate supplemented feed (%).

Components	Alfalfa (%)	Concentrate supplement (%)
Crude protein	16.05	18.0
Crude fiber	58.3	11.0
Crude ash	9.11	11.0
Crude fat	2.73	2
Calcium	1.43	0.8–1.5
Phosphorus	0.15	0.4

Nutrient composition of the alfalfa and concentrate supplement were determined, respectively.

2. Materials and methods

2.1. Animal feeding and tissue collection

Healthy Kazakh ewes ($n = 36$) were selected and randomly divided into two groups (18 per group). Ewes were maintained at an Animal Experimental Station (College of Animal Science and Technology, Shihezi University, Xinjiang, China). The control group was managed by feeding a typical diet of alfalfa hay (1.5 kg/per sheep) and had *ad libitum* access to water. The group with the enhanced nutritional status was fed a concentrate supplement (0.3 kg/per sheep) with greater nutrient content than the control diet (Table 1). In the anestrous season (February to June, Xinjiang, China), the rate at which estrus occurred was monitored twice a day for each ewe by introducing a ram into the pen with the ewe. The date when a ewe first accepted mounting by the "teaser" ram along with detection of vaginal bleeding and pro-genital swelling were considered characteristics of estrous onset and these details were recorded as the determining indicators of the first day of the estrous cycle at the end of anestrus (Chen et al., 2012). Three ewes in the standing estrous state (day of onset of estrous cycle at the end of the anestrous period) were selected and humanely killed for ovary collection. Subsequently, another three ewes that had a sustained ane-