Theriogenology 101 (2017) 99–108

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

iTRAQ-based proteomic profiling of granulosa cells from lamb and ewe after superstimulation

Jiapeng Lin [a, b, 1], Yangsheng Wu [b, 1], Bing Han [b, 2], Ying Chen [b, 2], Liqin Wang [b, 2], Xiaolin Li [b, 2], Mingjun Liu [b, *], Juncheng Huang [b, **]

[a] College of Animal Science and Technology, Shihezi University, Shihezi, 832003, China
[b] Biotechnology Research Institute, Xinjiang Academy of Animal Science, Urumqi, 830000, China

ARTICLE INFO

Article history:
Received 8 December 2016
Received in revised form
9 June 2017
Accepted 10 June 2017
Available online 10 June 2017

Keywords:
Lamb
Granulosa cells
Proteomics
iTRAQ

ABSTRACT

The number of oocytes obtained from lambs after FSH treatment is far greater than those acquired from adult ewes. However, these oocytes typically have reduced viability in comparison with adult ewe oocytes. However, the molecular mechanisms of differences in viability between lamb and ewe oocytes remain unknown. In the present research, we applied iTRAQ coupled with LC-MS/MS proteomic analysis in order to investigate the proteomic expression profile of granulosa cells from lambs and ewes following stimulation with FSH. We detected 5649 proteins; 574 were differentially expressed between adults and juveniles. Based on Gene Ontology enrichment and KEGG pathway analysis, the majority of DEPs are participated in metabolic processes, ribosome and MAPK signaling pathways. Expression levels in ewes turned out to be lower than lambs. Protein interaction network analysis generated by STRING identified MAPK1, SMAD2, SMAD4, CDK1, FOS and ATM as the major findings among 54 significant differentially expressed of proteins. Quantitative real-time PCR analysis was applied to verify the proteomic analysis. These proteins which were identified in lambs may contribute to the reduction of oocyte quality compared to adults. The present research provides understanding of the molecular mechanism for follicle development in lambs.

1. Introduction

Sheep (Ovis aries) are important agricultural animals, raised for the meat, milk, skin and wool. In order to improve reproduction rates in breeds of sheep and other livestock, assisted reproductive technologies have been widely applied all over the world. For instance, in sheep superovulation and embryo transfer technology, when a donor ewe is stimulated with follicle stimulating hormone (FSH), 5–10 suitable embryos are typically retrieved for transplantation to rapidly expand herds. In order to reduce generation intervals, lambs can serve as donors to produce embryos *in vitro* using oocytes harvested from prepubertal lambs. This process is known as juvenile *in vitro* embryo transfer (JIVET). In this research, 4–8 weeks old lambs were injected with follicle stimulating hormone (FSH) which stimulates oocyte production and recovery, and were used for *in vitro* embryo production and embryo transfer. This procedure has been successfully applied in lamb [1], calf [2] and goat [3]. In comparison to traditional sheep embryo transfer, this technology can shorten 6–7 months of the generation interval. Donor lambs can offer 60–80 or even more oocytes [1,4], which is far more than adult ewes. The physiological explanation for this is because fetal ovarian antral follicles were first observed at around 135 days of gestation, and they maintained relatively high numbers after birth [1]. The maximum number of follicles is observed 4–8 weeks after birth. Until the first ovulation, this number is gradually reduced and remains stable [5].

However, the maturation process of nuclear and cytoplasmic does not occur completely synchronise during *in vitro* maturation of prepubertal oocytes, which results in low fertilization efficiency [6–8] and increases fetal loss. What's more, the chance of mummified fetuses was also increased [4]. Meanwhile, in prepubertal oocytes, the raise of ATP is delayed and peaked at levels lower than what is normally observed in adult ones; what else, it can also be observed that the active mitochondria are evenly

* Corresponding author. Tel.: +86 991 3075280.
** Corresponding author. Tel.: +86 991 3075299.
E-mail addresses: mingjun_l@sina.com (M. Liu), h_jc@sina.com (J. Huang).
[1] Tel.: +86 991 3075297.
[2] Tel.: +86 991 3075298.

http://dx.doi.org/10.1016/j.theriogenology.2017.06.014
0093-691X/

homogenously distributed throughout the cytoplasm, while in adults they are aggregated in big clusters. These data deduce that prepubertal oocytes with low developmental competence have slower energetic metabolism which delays further development [9]. On the other hand, previous studies have shown that differences in the follicular environment differences may also contribute to development of juveniles oocytes at lower rates compared with what is seen in adult animals [10].

In mammals, one of the most significant roles of the ovary is to undergo continuous folliculogenesis, which serves to ensure that the viable and competent oocytes can be produced and are available [11]. Folliculogenesis is a complex process which involves a series of morphological and functional changes depending on the type of cells and developmental stage [12]. However, the pattern of follicle distribution in the lamb ovary differed from adult sheep. The follicles and corresponding oocyte growth were discordant, which seems to be contributing factors to additional intrinsic causes of immaturity of prepubertal gametes [6]. Hence, understanding the molecular mechanism of follicular development is essential to unravel the complex synergies orchestrated during the process of fertilization *in vitro* or *in vivo*, and also during JIVET or multiple ovulation and embryo transfer (MOET). Although, we have previously described [13–15] methods which can promote lamb or ewe developmental competence *in vitro*, embryos produced, the presence of altered gene expression patterns in granulosa cells as determined by RNAseq, and the proteomic expression (iTRAQ) regulation mechanisms that lead to follicular development, especially after FSH superstimulation remain elusive.

Gel-free Proteomics methods have been widely used in the examination of physiologic mechanisms of animal husbandry [16,17]. The isobaric Tag for Relative and Absolute Quantification (iTRAQ)-based Proteomics through isolation and high performance liquid chromatography-tandem Mass Spectrometry (MS/MS) is also widely used in the identification, characterization and expression analysis of the proteins throughout embryo development [18]. Here, we attempted to measure levels of FSH and other differentially expressed proteins in granulosa cells of lambs and adult sheep. Additionally, we sought to reveal the age based differences in mechanisms of pathway activation following FSH treatment of oocytes.

2. Materials and methods

All chemicals were obtained from Sigma Chemical Company (St. Louis, MO), except where indicated otherwise. All experiments were performed in accordance with relevant guidelines and regulations set by the Ministry of Agriculture of the People's Republic of China.

2.1. Animal preparation

Four weeks old lambs and 2 years old ewes were used in the experiments which were generated conventionally produced from natural mating of the mature Merino ewes, located in the Sheep Research Centre, Urmuqi, The Xinjiang Uygur Autonomous Region as previously reported [13]. Nine lambs and nine adult ewes were randomly placed into three pools for three biological replicates. Each lamb was then injected 160 IU of FSH (SanSheng Inc., NingBo, China) in 4 × 2 mL treatments at 12 h intervals to induce superstimulation. Twelve hours after the last FSH injection, the lambs underwent ovariectomy under general anaesthesia. Each ewe was injected with 4 injections of FSH (SanSheng Inc., NingBo, China) after carrying aprogesterone impregnated intravaginal CIDR for 12 days. 12 h after the last FSH injection, the ewes underwent ovariectomy and CIDRs were removed.

2.2. Follicle categorization and granulosa cell collection

Ovaries were obtained surgically after 12 h stimulation with FSH in lambs and ewes. Tissues surrounding the ovaries were removed and ovaries were rinsed in 4 ml PBS. Follicles were dissected from the ovarian stroma and their cross-sectional diameters were measured. The sizes of follicles dissected from lambs were relatively uniform, they varied from 1 to 3 mm. However, this was not the case for follicles dissected from ewes, the sizes of their follicles varied from 1 to 5 mm (Fig. 1). For the purposes of the present study, all follicles with diameters of approximately 3–4 mm were obtained from lambs and ewes. Healthy follicles with a well-vascularised follicular wall and transparent, amber-colored follicular fluid lacking debris were included in the present study. Granulosa cells were obtained by dissecting follicles into hemispheres and gently scraping follicle walls with blunt-ended forceps as described previously [19]. All samples were immediately frozen in liquid nitrogen for total protein extraction.

2.3. Protein preparation and iTRAQ labeling

The frozen granulosa cell samples were disrupted and centrifuged at 1000×*g* for 5 min, and washed three times with ice-cold PBS. Cell pellets were re-suspended in 300 μL RIPA lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM sodium chloride, 2 mM EDTA, 1 mM phenylmethanesulfonyl fluoride (PMSF), 1% Triton X-100) supplemented with protease inhibitors (Thermo Scientific, Shanghai,PRC). Afterwards the samples were sonicated and centrifuged at 12000g for 20 min at 4 °C. Supernatants were analyzed as total lysates. Total protein concentration was determined using the BCA Assay Kit (Thermo Scientific, Shanghai,PRC) according to the manufacturer's instructions. Bovine serum albumin (BSA) was used as a reference standard for all assays.

iTRAQ tagging and analysis was performed as previously described [20]. Briefly, 200 μg of protein from each sample was reduced, alkylated, and digested with trypsin (Promega). The digested peptides were then dried and reconstituted in 50 μl 1 M TEAB. The dried peptides were labeled following the manufacturer's recommendations (iTRAQ 8-plex kits, AB Sciex, USA). Peptides were labeled with iTRAQ tags as follows: 118, 119 and 121 for three biological replicates of ewe samples; 114, 116 and 117 for three biological replicates of lamb samples.

2.4. High pH reverse phase fractionation

High pH Reverse Phase Fractionation (hpRP) chromatography was performed using a LC-20AD Nano HPLC (Shimadzu, Kyoto, Japan) as described previously [21]. The iTRAQ-tagged tryptic peptides were reconstituted in buffer A (20 mM NH_4HCO_2, pH 10) and loaded onto Gemini-NX C18 columns (3 μm, 2 × 150 mm, 110 Å, Phenomenex) with 20 mM NH4HCO2 as buffer A and 80% ACN/20% 20 mM NH_4HCO_2 as buffer B. The peptides were eluted at a flow rate of 200 μL/min and 24 fractions were collected at 1-min intervals, based on UV absorbance at 214 nm/280 nm utilizing a multiple fraction concatenation strategy.

2.5. Nano RPLC-MS/MS analysis

Peptides were subjected to nanoelectrospray ionization followed by tandem mass spectrometry (MS/MS) in the Q Exactive system (Thermo Fisher Scientific, Bremen, Germany) which was interfaced with a Dionex UltiMate 3000 RSLC nano system (Thermo Fisher Scientific, MA, USA). The peptide mixture was loaded onto a PepMap C18 RP column (2 μm, 75 μm × 150 mm, 100 A) at a flow rate of 300 nL/min. Peptides were eluted from the HPLC column