Theriogenology 85 (2016) 1439–1445

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Global transcriptional expression in ovarian follicles from Tsaiya ducks (*Anas platyrhynchos*) with a high-fertilization rate

Shyh-Jong Wu [a], Yu-Shin Cheng [b], Hsiao-Lung Liu [b], Hsing-He Wang [c], Hsiu-Lin Huang [c,*]

[a] Department of Medical Laboratory Science and Biotechnology, Kaohsiung Medical University, Kaohsiung, Taiwan
[b] Livestock Research Institute, Council of Agriculture, Tainan, Taiwan
[c] Department of Post-Modern Agriculture, MingDao University, Changhua, Taiwan

ARTICLE INFO

Article history:
Received 12 March 2015
Received in revised form 6 November 2015
Accepted 4 January 2016

Keywords:
Fertilization rate
Differentially expressed transcript
Ovarian follicle
Duck

ABSTRACT

Novel candidates for biomarkers of a high-fertilization rate were identified here through global transcriptional profiling of ovarian follicles. Some other differentially expressed candidate genes were first noted to influence animal reproduction in our previous cDNA microarray analysis and are now recognized as markers for marker-assisted selection. In the present study, we compared gene expression in ovarian follicles from animals with high- and low-fertilization rates using an oligonucleotide array. On the basis of a fold change of greater than 1.2 and less than −1.2, a difference of >100 Affymetrix arbitrary units between the two groups, and a P value of less than 0.05, 47 genes were found to be associated with fertilization rate. GOEAST and MetaCore software were further used to identify the functional categories of genes that were differentially expressed. Then, we focused on three interesting genes associated with a high-fertilization rate: one of these genes was discovered to participate in signaling pathways of fertilization, and two genes take roles in lipid metabolism. An oligonucleotide array showed that the levels of orthodenticle homeobox 2 (*OTX2*) and lecithin:cholesterol acyltransferase *(LCAT*) gene expression were 1.62-fold and 1.95-fold higher in the high-fertilization rate group than in the low-fertilization rate group, respectively ($P < 0.05$). The level of apolipoprotein A-I (*APOA1*) gene expression was also higher in the high-fertilization rate group, with a difference of 2.31-fold ($P < 0.05$). The data were validated through quantitative polymerase chain reaction analysis. These results confirm the usefulness of the array technique and data mining methods in the discovery of new biomarkers and add knowledge to our understanding of the factors affecting fertilization rates in ovarian follicles.

1. Introduction

Transcriptomes comprise the RNA content of cells, and transcriptomics is the name given to the investigation of transcriptomes and their function [1,2]. Microarrays that produce genome-wide expression profiles permit quantitative and repeated measurements of transcripts and allow comparisons of transcriptomes [3–5]. The exploration of different biological events through bioinformatics provides a comprehensive view of transcriptomics [6]. A targeted approach to gene expression profiling, such as quantitative polymerase chain reaction (qPCR), is critical for the validation of expression profiles identified [7,8]. Those data from microarray, qPCR, and data mining can lead to unprecedented advances in our understanding of the functional and regulatory architecture of the genome.

* Corresponding author. Tel.: +886 4 8876660x8316; fax: +886 4 8871774.
E-mail address: hlhuang@mdu.edu.tw (H.-L. Huang).

0093-691X/$ – see front matter © 2016 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2016.01.005

Ovarian follicles are the basic units of ovaries, and each follicle contains an oocyte that is surrounded by somatic (granulosa) cells [9,10]. The follicle has a significant effect on oocyte quality [11,12]. However, most studies have focused on hormonal treatment of ovaries [13–16], and the explicit role of early follicular cell gene expression in the competence of enclosed duck oocytes is unclear. Successful oocyte signaling cascades involving many genes are essential for the regulation of ovulation and subsequent fertilization [17]. Ovarian follicles provide a microenvironment to foster oocytes also through signaling pathways and interact with oocytes via gap junction [18]. There are currently no precise morphologic criteria that can be used to predict the fertilization rate of oocytes [19,20]. Therefore, much remains to be investigated to discover markers of high-fertilization rates in follicular cells.

Recently, we used microarrays to screen for novel DNA markers for use in marker-assisted selection for genetic breeding [21–24]. These genes were later confirmed to play a role in animal reproduction. The Tsaiya duck is the major egg-laying duck in Taiwan [25,26]. In this study, unknown target sequences were prepared from Tsaiya ducks classified as having high- or low-fertilization rates and were hybridized with commercially available chicken oligonucleotide chips containing known sequences. Chicken sequences were used due to the lack of available arrays containing duck probes; however, duck sequences are sufficiently close to those of chicken [24,27]. Genome-wide transcriptional profiles of ovarian follicles were analyzed with targets from the two duck groups, and the expression levels of selected genes were verified using qPCR. The results provide valuable insights into molecular pathways that are altered in ovarian follicles with high- and low-fertilization rates. We also focused on three candidate markers associated with high-fertilization rates and discuss the possible implications of these markers on increasing female ability in reproduction.

2. Materials and methods

2.1. Preparation of samples

After sexual maturation, fertility data were obtained from 12 laying brown Tsaiya ducks, and the animals were slaughtered for sample collection at the age of 42 weeks, which is still in the high-laying-performance phase of development. To investigate the significantly differentially expressed transcripts in ovarian follicles from ducks with high fertility, we divided all samples in pairs in terms of fertilization rates. The ducks were classified as having total averages of high- (72.32 ± 7.02 days, $n = 6$) or a low-fertilization rate (59.12 ± 6.05 days, $n = 6$). Total RNA samples were isolated from small ovarian follicles (around 5 mm in diameter) from each of the animals using RareRNA reagent (GenePure, Kaysville, UT, USA). Equal amounts of RNA samples extracted from two ducks in the same group were pooled as an independent RNA pool, resulting in a total of three independent RNA pool pairs for use in microarray hybridization or qPCR analysis. The means (%) of fertilization rate for ducks of each RNA pool pair are as follows: 75.98 versus 54.13 (high- vs. low-fertilization rate groups), 70.45 versus 65.91, and 70.49 versus 57.33. All samples were collected from birds raised in the breeding stock of the Taiwan Livestock Research Institute, Council of Agriculture, Executive Yuan. The care and use of research animals were within standard ethical guidelines, and the protocols were approved by the Livestock Research Institute.

2.2. Affymetrix GeneChip analysis

The cRNA used as the microarray hybridization target was prepared with GeneChip 3′ IVT reagents (Affymetrix Inc., Santa Clara, CA, USA), according to the method described previously [24]. Briefly, the total RNA sample was reverse-transcribed and then converted into a double-stranded DNA template for further *in vitro* transcription. The cRNA amplification was processed for 16 hours with biotin, and the labeled product was subsequently purified and fragmented to 35 to 200 nt cRNA. The biotin-labeled fragments were then hybridized onto an Affymetrix GeneChip Chicken Genome Array (Affymetrix Inc.), and the expression profiles of 32,773 transcripts were monitored on each of six chips: three biological repeat pairs hybridized with three pairs of the high-versus the low-fertility-group targets from the six independent RNA pools. Finally, GeneChip washing and image acquisition were performed according to the manufacturer's recommendations.

2.3. Functional mining of microarray data

The results of the Affymetrix GeneChip arrays were acquired from GeneChip Operating Software (GCOS; Affymetrix Inc.) in the form of CIMFast Event Language files. The raw data were then processed and normalized using a dChip Analyzer. Briefly, we used the invariant set normalization method to normalize the intensities of the arrays according to the median overall brightness of the probe signal in a baseline array. The background signal of each array was subtracted using a model-based method of perfect match/mismatch difference. Significantly differentially expressed transcripts were filtered on the basis of a signal log ratio ($\log_2$ fold change) less than -1.2 and greater than 1.2 between the high- and low-fertilization rate groups; a difference of at least 100 Affymetrix arbitrary units between the aforementioned groups; a signal detection P value less than 0.05. This is important to note that a small fold change of 1.2 in microarray experiments can be biologically relevant [28–31], and we wanted important changes to be detected. Therefore, a signal log ratio less than -1.2 and greater than 1.2 between the high- and low-fertilization rate groups was set as described previously. We then used the Expression Console analysis package (Affymetrix Inc.) to measure Pearson correlations, and a cluster analysis graph was plotted using a dChip Analyzer (Fig. 1). We mapped differentially expressed transcripts to biological pathways and networks using the Web-based tool, Gene Ontology Enrichment Analysis Software Toolkit (GOEAST; http://omicslab.genetics.ac.cn/GOEAST/index.php) and MetaCore analytical software.

To analyze the data in GOEAST, the option of the Affymetrix platform was chosen. Pathways and networks were