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Novel Classifier Orthologs Of Bovine And Human Oocytes Matured In Different Melatonin Environments

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Running title: Novel classifier orthologs for different melatonin environment of oocytes

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Abstract

It has been demonstrated that melatonin influences the developmental competence of both *in vivo* and *in vitro* matured oocytes. It modulates oocyte-specific gene expression patterns among mammalian species. Due to differences among study systems, the identification of the classifier orthologs—the homologous genes related among mammals that could universally categorize oocytes matured in environments with varied melatonin levels is still limitedly studied. To gain insight into such orthologs, cross-species transcription profiling meta-analysis of *in vitro* matured bovine oocytes and *in vivo* matured human oocytes in low and high melatonin environments was demonstrated in the current study. RNA-Seq data of bovine and human oocytes were retrieved from the Sequence Read Archive database and pre-processed. The used datasets of bovine oocytes obtained from culturing in the absence of melatonin and human oocytes from old patients were regarded as oocytes in the low melatonin environment (Low). Datasets from bovine oocytes cultured in 10-9 M melatonin and human oocytes from young patients were considered as oocytes in the high melatonin environment (High). Candidate orthologs differentially expressed between Low and High melatonin environments were selected by a linear model, and were further verified by Zero-inflated regression analysis. Support Vector Machine (SVM) was applied to determine the potentials of the verified orthologs as classifiers of melatonin environments. According to the acquired results, linear model analysis identified 284 candidate orthologs differentially expressed between Low and High melatonin environments. Among them, only 15 candidate orthologs were verified by Zero-inflated regression analysis (FDR $\leq$ 0.05). Utilization of the verified orthologs as classifiers in SVM resulted in the precise classification of oocyte learning datasets according to their melatonin environments (Misclassification rates < 0.18, area under curves > 0.9). In conclusion, the cross-species RNA-Seq meta-analysis to identify novel classifier orthologs of matured oocytes under different melatonin environments was successfully demonstrated in this study- delivering candidate orthologs for future studies at biological levels. Such verified orthologs might provide valuable evidence about melatonin sufficiency in target oocytes- by which, the decision on melatonin supplementation could be implied.

Keywords: Classifier orthologs; Melatonin; Oocytes; Cross-species RNA-Seq meta-analysis

1. Introduction

in vitro embryo production (IVP) is regarded as a well-established assisted reproductive technology (ART) in both humans and animal species [1]. *in vitro* oocyte maturation (IVM) is one of the multiple steps of IVP that the oocytes are recovered directly from the follicles and are cultured in a specific condition. Despite its advancement, incomparable *in vitro* and *in vivo* environment is still a major problem contributing to the success of IVM performance [2,3]. During *in vivo* oocyte development, several delicate substances and hormones are locally secreted in follicular fluid by ovarian and granulosa cells to nourish oocytes. This includes melatonin which was widely studied for its contribution to oocyte quality [2–5]. Melatonin was most recognized for its scavenging function on reactive oxygen species (ROS). Since ROS play a major role in impairing oocyte development [6,7], the lack of melatonin in conventional *in vitro* culture environments has contributed to ROS-induced poor oocyte quality in several mammals [3,8,9].

Several factors also affect *in vivo* oocyte development. This includes reproductive aging—one of the most common causes of female infertility. It has been reported that reproductive aging induces a variety of physiological aberrations withering oocyte quality [9–12]. One of them is melatonin deprivation, caused by reduced melatonin production from aging granulosa and ovarian cells. Melatonin deprivation was hereby blamed as a major contributor to aging oocyte's incompetency [2,9]. Aging also results in other diverse hormonal imbalances and assorted physiological changes [9,13]. All these factors represent complex negative effects during *in vivo* oocyte aging. Therefore, it has been difficult to exclusively determine the melatonin deprivation effects on the oocyte during *in vivo* development in such circumstances. By means of this, it was difficult to exclusively determine the melatonin deprivation effect on *in vivo* oocyte in such circumstances.

Interestingly, similar aging aberrance also occurred with *in vitro* cultured oocytes in a time-dependent manner. *in vitro*-aging phenomenon also included ROS-induced quality deterioration due to the absence of melatonin in the conventional culture environment [2–5,9,11]. In agreement with the protective effects of *in vivo* melatonin produced by ovarian follicles, *in vitro* melatonin supplement was evidenced to restore the oocyte developmental competency in several species [3–5,9]. Unlike *in vivo*-aging condition divided effects of *in vitro* melatonin deprivation should be conveniently observed due to the simplicity of the culture environment. Through this, analysis of crossed effects between *in vitro* and *in vivo* melatonin deprivation due to aging should hereby contribute a novel insight into the universal melatonin effects independent from other factors.

With comparable reproductive physiology and embryogenesis, cattle are regarded as excellent study models for human IVP [10,14]. Employing this, novel transcriptomic technologies including RNA sequencing (RNA-Seq) have been continuously applied with oocyte samples obtained from both species for studying their

universal transcriptome's regulation. However, the application of standard RNA-Seq or bulk RNA-Seq with mammalian oocytes was not practical due to low oocyte numbers for cDNA library preparation [15,16]. Moreover, oocytes were heterogeneous populations, and should thus not represented with average results. Since cell-to-cell variations are common among isolated oocytes, loss of real features among them in bulk analysis could bring about considerable bias in the analysis [15].

Recently, low-input and single-cell RNA sequencing (RNA-Seq) methods have been implemented with oocytes acquired from various mammals [3,15,17]. Since such technologies allow robust transcript profiling analysis down to a single cell, they enable researchers to explore biological variations among oocytes with reduced bias [3,16,18]. Interestingly, an increase of such data among a variety of mammalian species even allowed cross-species meta-analysis of oocytes. Since cross-species transcription profiling meta-analysis of oocytes could imply universal orthologs regulated among the species in the environment of interest [19], implement of such analysis with RNA-Seq data of oocytes obtained from different *in vitro* and *in vivo* melatonin environments should imply us insight into such orthologs, as well.

In several transcription profiling studies, genomic classification or subtyping of unknown samples can be archived by various machine learning algorithms. Support vector machine (SVM) is a robust learning model widely implemented on a genome-wide scale to prioritize orthologs for sample classification [20,21]. Novel classifier orthologs were identified by SVM in several topics including aging, cancers, infections, and metabolic disorders [22–25]. Regardless of its flexibility, SVM application in oocyte study was still limitedly demonstrated. It was noteworthy that the integration of SVM with cross-species meta-analysis of different melatonin environments could thus help us determining novel classifier orthologs for evaluating the melatonin sufficiency of oocytes. In other words, such cognition would preliminarily assess the beneficial effects of melatonin supplement on target oocytes presented in environments of interest.

To gain insight into universal classifier orthologs for melatonin environments, cross-species transcription profiling meta-analysis of *in vitro* matured bovine oocytes and *in vivo* matured human oocytes in low and high melatonin environments was performed in the current study. The pooled cDNA library based on human-bovine orthologs was constructed with bias correction. With restricted verification procedures and SVM classification, the universal classifier orthologs for melatonin environments of oocytes were successfully implied for the first time in this study.

2. Materials and methods

2.1 Sample datasets

RNA-Seq data of bovine and human oocytes were retrieved from the sequence read archive (SRA) database (https://www.ncbi.nlm.nih.gov/sra) (Table 1). Both bovine and human RNA-Seq data were presented in NCBI with accession numbers GSE122738 [3] and GSE125300 [26] (https://www.ncbi.nlm.nih.gov/geo/). The sources of bovine [3] and human oocytes [26] utilized for data preparation were summarized in Table 1. In brief, bovine cumulus- oocyte complexes (COCs) were collected from slaughterhouses and cultured in the presence (In_vitro_MII+Mela) or absence (In_vitro_MII) of 10^{-9} M melatonin for 24 hours, respectively. Metaphase II (MII) oocytes were isolated from COCs and divided into 6 samples—each containing 7 oocytes of their groups. On the contrary, each human MII oocyte sample was retrieved directly from single follicles of young (In_vivo_young_MII), or old (In_vivo_old_MII) patients by ultrasound-guided technique. In this study, the term—melatonin environment was used to infer the amount of melatonin in the environment surrounding the target oocyte samples. Through this, the bovine oocytes matured in the absence of melatonin supplement, and human oocytes acquired from old patients were regarded as oocytes in the low melatonin environment. On the other hand, bovine oocytes matured in the presence of melatonin supplement, and human oocytes acquired from young patients were considered as oocytes in the high melatonin environment.

2.2 Data pre-processing

Data pre-processing was performed with the process similar to that described in our previous study [27]. In brief, all sequences were trimmed to remove contaminated adapter sequences and unqualified sequences (length < 25 nucleotides and mean Phred score < 25). Duplicated sequences removal, sequence alignments (>85%), and GC base-balance bias correction were accomplished prior to gene-level counting. In this study, the read counts were normalized by the trimmed mean of M-values (TMM). bovine-human orthologs were assigned with the previously reported procedure [19]. Data quality control was carried out [28] along with cell-cycle effect removal to minimize the bias from within-cell-type heterogeneity [29]. Count read numbers of all samples were > 14 x10^{-6}, and none of them was prone to apoptosis (Fraction of mitochondrial reads < 1.5%). The mutual nearest neighbors (MNN) in ortholog expression space was identified for cross-species batch correction [30] of pooled cDNA library created from bovine and human oocyte count data using 'scater' package [31].

2.3 Candidate ortholog selection for oocyte sample categorization based on melatonin environments

Candidate orthologs for oocyte sample categorization by melatonin environment were selected using procedures as described in our previous study [19]. In brief, candidate bovine-human orthologs differentially expressed between oocytes in low and high melatonin environments were selected from 1,000 learning datasets using the linear model and empirical Bayes methods. In this study, we consider differentially expressed orthologs with importance value ≥ 2.5. Only orthologs declared significant both by intra-species and cross-species analyses would be selected as candidate orthologs. With expression values of the candidate orthologs, Partitioning around medoids (PAM) clustering (2-4 clusters) was performed, and clustering validation was determined by Connectivity, Dunn, and Silhouette scores.

2.4 Verification of the candidate orthologs

Concerning about unrealistic mean-variance relationship produced by the batch-corrected data, the candidate orthologs were verified by Zero-inflated regression analysis with proper model matrix using raw count data to avoid the bias from the batch correction process with 'MAST' package [32]. The same criteria of candidate ortholog selection was applied—by which the verified candidate orthologs must be differentially expressed between low and high melatonin environments both by intra-species and cross-species analyses (FDR ≤ 0.05).

2.5 Oocyte sample classification by Support Vector Machine

To determine the potential of the verified candidate orthologs as classifiers for melatonin environments, Support Vector Machine (SVM) with linear kernel classifier was performed. Different training data were generated by 5-fold cross-validation, Bootstrap, and Monte-Carlo-cross-validation strategies—1,000 iterations for each strategy. All procedures could be archived by class prediction functions using the 'CMA' package [20].

2.6 Data visualization

Heatmap illustrating candidate orthologs was drawn using the "ComplexHeatmap" package [33]. The silhouette width values acquired from PAM clustering were illustrated by the bar plot drawn by the 'ggplot2' packages. A bubble plot manifesting $-\log_{10}$FDR values and $\log_2$fold-change values of the verified candidate orthologs was drawn using the 'ggplot2' package. Probability plots and Receiver operating characteristic (ROC) curves were drawn to illustrate the classification accuracy of the verified candidate orthologs by the 'ggplot2' package.

3. Results

3.1 Oocyte samples could be clustered by their melatonin environments using the candidate orthologs as features.

Analysis of bias-corrected data by linear model revealed 284 candidate orthologs differentially expressed between oocytes in low and high melatonin environments (importance value ≥ 2.5). According to PAM clustering results based on expressions of these candidate orthologs (Table 2 and Fig 1), oocyte samples were correctly categorized into 2 clusters corresponding to their melatonin environments regardless of the species (Fig 2).

3.2 All verified candidate orthologs were significantly expressed in oocytes in the high melatonin environment.

Zero-inflated regression analysis revealed 158 orthologs differentially expressed between oocytes in low and high melatonin environments (FDR≤0.05). However, only 15 orthologs were found intersected with the candidate orthologs acquired from the previous selections—AAMP, ADM, ATP6V0A2, BCL7B, CYB561D2, ENDOV, F2RL1, KIAA0586, LPCAT4, NR1H3, PLEKHM1, SLC47A1, SYT11, RFT1, and MRPS18C (Fig 3). While all oocytes in the high melatonin environment expressed these orthologs higher than those in the low environment, only ADM, CYB561D2, ENDOV, KIAA0586, NR1H3, PLEKHM1, SLC47A1, SYT11, RFT1, and MRPS18C rendered substantial fold-change values (≥ 1.5). According to our review of their biological functions, such orthologs were associated with a variety of cell metabolisms (Table 3).

3.3 Verified candidate orthologs were potential classifiers for the melatonin environments of oocytes.

The verified candidate orthologs were used as classifier orthologs in SVM performance. Consistent results were acquired among learning datasets—by which all of them rendered Misclassification rates < 0.18, and their ROCs had Area Under Curves (AUCs) > 0.9 (Fig 4).

4. Discussion

A cross-species transcription profiling meta-analysis of *in vitro* matured bovine oocyte data and *in vivo* matured human oocyte data to identify classifier orthologs for different melatonin environments was performed in this study. Despite the discreet procedures manifested, some limitations in result interpretation should be greatly concerned to avoid over-assumption. As described in the methodology, the *in vitro* and *in vivo* matured oocyte samples used in this study were procured from different studies with limited information about them. For

197 instance, melatonin concentrations in follicles containing *in vivo* matured human oocytes were not indicated,
198 while the concentrations were clearly stated in bovine oocytes' culture systems. Undetermined differences in
199 melatonin concentrations between such environments should reduce the sensitivity of differential analysis in this
200 study. With more available *in vivo* matured oocyte profiling data with well-defined melatonin environments in
201 the future, the list of classifier orthologs could be improved for better generalization.

202 As expected, our previously reported procedures [19] were also applicable to the selection of
203 candidate orthologs differentially expressed between low and high melatonin environments (Fig 2 and Table 2).
204 While these orthologs could be used to annotate essential melatonin functions on *in vivo* and *in vitro* matured
205 oocytes, this study aimed to identify the most reliable classifier orthologs among them. Due to such delegation,
206 we further verified the candidate orthologs with the zero-inflated regression test to reduce the mean-variance bias
207 (Fig 3).

208 Encoded products of the verified candidate orthologs were associated with in various oocyte
209 metabolisms (Table 3). In detail, ADM encoded an angiogenic factor, adrenomedullin—an important
210 vasorelaxant required during folliculogenesis and oocyte maturation processes [34,35]. CYB561D2 encoded a
211 member of trans-membrane (TM) protein family involving stress defense and iron metabolism [36]. ENDOV
212 encoded Endoribonuclease V—an enzyme involving with the DNA repair process of oocyte [37]. KIAA0586
213 encoded a conserved centrosomal protein required for sonic hedgehog/SHH signaling during the oocyte
214 developmental process [38]. NR1H3-activating ligands helped with oocyte meiosis resumption [39].
215 PLEKHM1 could regulate the autophagy process which was crucial in the regulation of oocyte metabolism [40–
216 42]. SLC47A1 encoded an extrusion protein for drug and toxic metabolites [43]. SYT11 encoded
217 synaptotagmin-11 for exocytosis and endocytosis regulation [44]. RFT1 encoded a major folate transporter
218 [45], which helped to accumulate folate for normal oogenesis and early embryogenesis [46]. Lastly, MRPS18C
219 encoded a mitochondrial ribosomal protein and thus was required for other mitochondrial protein syntheses
220 [47].

221 The high melatonin environment was shown to enhance expressions of all verified candidate orthologs
222 (Fig 3 and Table 3). Supporting this notice, enhanced expression of KIAA0586 by melatonin was already
223 notified in the other study [8]. Since functions of these orthologs were strongly associated with qualified oocyte
224 development as previously mentioned, their diminished expressions in the low melatonin environment should
225 thus adversely affect the oocyte development (Table 3). As far as we know, shreds of evidence of enhanced
226 functions of most verified orthologs by melatonin were still not warranted in mammalian oocytes—by which
227 future studies to confirm such effects were encouraged.

In the current study, the classifying potential of the verified orthologs was determined by SVM to assess their reliability and reproducibility. According to the result, low misclassification scores and high AUCs manifested among tested learning datasets (Fig 4) hereby indicated such orthologs as potential classifiers for melatonin environments. Though utilization of cross-species data in this study partially suggested such classifier orthologs to be universally applicable among mammalian oocytes, further confirmation in other mammals with known melatonin environments were still required. Since overall expression levels of acquired classifier orthologs could imply sufficiency of melatonin, we, on a certain level proposed them as novel parameters to provide some clues about beneficial effects of melatonin supplement on target oocytes in any culture environments of interest. Such knowledge greatly relieved burdens for researchers to screen for universal melatonin effects on all ortholog expressions which were not possible by the conventional approach. It should however be noted that these classifier orthologs might not only be specific to the melatonin environment and thus required further classification tests with other possible oocyte environments.

5. Conclusion

Novel classifier orthologs of oocytes matured under low and high melatonin environments were identified by cross-species transcription profiling meta-analysis in this study. Such an outcome not only offered us future melatonin research topics to evaluate classifying potentials of the verified orthologs at biological levels but also the complete process to acquire them from available RNA-Seq data. We would like to note that the analytical procedures demonstrated in the current study could be applied with other oocyte environments of interest—by which identification of other novel parameters of other oocyte culture environments was possible.

Conflict of interest

We certify that there is no conflict of interest with any financial organization regarding the material and methods discussed in the manuscript.

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Figure 1. Silhouette plot of PAM clustering. The average silhouette width was 0.395 when dividing oocyte samples into 2 clusters.

Figure 2. Heatmap of PAM clustering results. Rows of heatmap represented scaled expression values of the candidate orthologs clustered by k-means clustering, while the columns represented oocyte samples clustered by PAM.

Figure 3. Bubble plot of the verified candidate orthologs. The blue and red colors indicated scaled expression levels of target orthologs expressed by oocytes in the low melatonin environment comparing to those obtained from the high melatonin environment. The color intensities were corresponded to the $-\log_{10}$FDR values, while the dot sizes were proportional to the absolute $\log_2$fold-change values of differentially expressed orthologs.

Figure 4. Predicted probability plots and ROC curves. Predicted probabilities plots were displayed by colored dots, where red dots corresponded to class 0—low melatonin environment, and green dots for class 1—high melatonin environment. The misclassification rate acquired from each learning dataset was shown under the plot (5-fold cross-validation, Bootstrap, and Monte-Carlo-cross-validation). Adjacent to each predicted probability plot, the ROC curve generated from its same learning dataset was drawn with AUC value demonstrated.