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

2

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10

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12

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15 **Title of the manuscript:** Novel classifier orthologs of bovine and human oocytes matured in different melatonin
16 environments

17

18 **Abstract**

19 It has been demonstrated that melatonin influences the developmental competence of both *in vivo* and *in*
20 *vitro* matured oocytes. It modulates oocyte-specific gene expression patterns among mammalian species. Due to
21 differences among study systems, the identification of the classifier orthologs—the homologous genes related
22 among mammals that could universally categorize oocytes matured in environments with varied melatonin levels
23 is still limitedly studied. To gain insight into such orthologs, cross-species transcription profiling meta-analysis
24 of *in vitro* matured bovine oocytes and *in vivo* matured human oocytes in low and high melatonin environments
25 was demonstrated in the current study. RNA-Seq data of bovine and human oocytes were retrieved from the
26 Sequence Read Archive database and pre-processed. The used datasets of bovine oocytes obtained from culturing
27 in the absence of melatonin and human oocytes from old patients were regarded as oocytes in the low melatonin
28 environment (Low). Datasets from bovine oocytes cultured in 10⁻⁹ M melatonin and human oocytes from young
29 patients were considered as oocytes in the high melatonin environment (High). Candidate orthologs differentially
30 expressed between Low and High melatonin environments were selected by a linear model, and were further
31 verified by Zero-inflated regression analysis. Support Vector Machine (SVM) was applied to determine the
32 potentials of the verified orthologs as classifiers of melatonin environments. According to the acquired results,
33 linear model analysis identified 284 candidate orthologs differentially expressed between Low and High
34 melatonin environments. Among them, only 15 candidate orthologs were verified by Zero-inflated regression
35 analysis (FDR ≤ 0.05). Utilization of the verified orthologs as classifiers in SVM resulted in the precise
36 classification of oocyte learning datasets according to their melatonin environments (Misclassification rates <
37 0.18, area under curves > 0.9). In conclusion, the cross-species RNA-Seq meta-analysis to identify novel
38 classifier orthologs of matured oocytes under different melatonin environments was successfully demonstrated in
39 this study- delivering candidate orthologs for future studies at biological levels. Such verified orthologs might
40 provide valuable evidence about melatonin sufficiency in target oocytes- by which, the decision on melatonin
41 supplementation could be implied.

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

43 1. Introduction

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

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

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

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

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

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

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

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

102

103 **2. Materials and methods**

104

105 2.1 Sample datasets

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

120

121 2.2 Data pre-processing

122 Data pre-processing was performed with the process similar to that described in our previous study
123 [27]□. In brief, all sequences were trimmed to remove contaminated adapter sequences and unqualified
124 sequences (length < 25 nucleotides and mean Phred score < 25). Duplicated sequences removal, sequence
125 alignments (>85%), and GC base-balance bias correction were accomplished prior to gene-level counting. In this
126 study, the read counts were normalized by the trimmed mean of M-values (TMM). bovine-human orthologs were
127 assigned with the previously reported procedure [19]□. Data quality control was carried out [28]□ along with
128 cell-cycle effect removal to minimize the bias from within-cell-type heterogeneity [29]□. Count read numbers of
129 all samples were $> 14 \times 10^6$, and none of them was prone to apoptosis (Fraction of mitochondrial reads < 1.5%).
130 The mutual nearest neighbors (MNN) in ortholog expression space was identified for cross-species batch
131 correction [30]□ of pooled cDNA library created from bovine and human oocyte count data using ‘scater’
132 package [31]□.

133

134 2.3 Candidate ortholog selection for oocyte sample categorization based on melatonin environments

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

143

144 *2.4 Verification of the candidate orthologs*

145 Concerning about unrealistic mean-variance relationship produced by the batch-corrected data, the
146 candidate orthologs were verified by Zero-inflated regression analysis with proper model matrix using raw count
147 data to avoid the bias from the batch correction process with ‘MAST’ package [32]. The same criteria of
148 candidate ortholog selection was applied—by which the verified candidate orthologs must be differentially
149 expressed between low and high melatonin environments both by intra-species and cross-species analyses (FDR
150 ≤ 0.05).

151

152 *2.5 Oocyte sample classification by Support Vector Machine*

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

157

158 *2.6 Data visualization*

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

165

166 3. Results

167

168 *3.1 Oocyte samples could be clustered by their melatonin environments using the candidate orthologs as*
169 *features.*

170 Analysis of bias-corrected data by linear model revealed 284 candidate orthologs differentially
171 expressed between oocytes in low and high melatonin environments (importance value ≥ 2.5). According to PAM
172 clustering results based on expressions of these candidate orthologs (Table 2 and Fig 1), oocyte samples were
173 correctly categorized into 2 clusters corresponding to their melatonin environments regardless of the species (Fig
174 2).

175

176 *3.2 All verified candidate orthologs were significantly expressed in oocytes in the high melatonin environment.*

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

185

186 *3.3 Verified candidate orthologs were potential classifiers for the melatonin environments of oocytes.*

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

190

191 4. Discussion

192 A cross-species transcription profiling meta-analysis of *in vitro* matured bovine oocyte data and *in vivo*
193 matured human oocyte data to identify classifier orthologs for different melatonin environments was performed
194 in this study. Despite the discreet procedures manifested, some limitations in result interpretation should be
195 greatly concerned to avoid over-assumption. As described in the methodology, the *in vitro* and *in vivo* matured
196 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.

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

240

241 **5. Conclusion**

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

248

249 **Conflict of interest**

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

252

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258

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389

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

392
393 **Figure 2.** Heatmap of PAM clustering results. Rows of heatmap represented scaled expression values of the
394 candidate orthologs clustered by k-means clustering, while the columns represented oocyte samples clustered by
395 PAM.

396
397 **Figure 3.** Bubble plot of the verified candidate orthologs. The blue and red colors indicated scaled expression
398 levels of target orthologs expressed by oocytes in the low melatonin environment comparing to those obtained
399 from the high melatonin environment. The color intensities were corresponded to the $-\log_{10}\text{FDR}$ values, while
400 the dot sizes were proportional to the absolute $\log_2\text{fold-change}$ values of differentially expressed orthologs.

401
402 **Figure 4.** Predicted probability plots and ROC curves. Predicted probabilities plots were displayed by colored
403 dots, where red dots corresponded to class 0—low melatonin environment, and green dots for class 1—high
404 melatonin environment. The misclassification rate acquired from each learning dataset was shown under the plot
405 (5-fold cross-validation, Bootstrap, and Monte-Carlo-cross-validation). Adjacent to each predicted probability
406 plot, the ROC curve generated from its same learning dataset was drawn with AUC value demonstrated.