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The first whole transcriptomic exploration of pre-oviposited early chicken embryos using single and bulked embryonic RNA-sequencing

--Manuscript Draft--

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Abstract:	Background The chicken is a valuable model organism, especially in evolutionary and embryology research because its embryonic development occurs in the egg. However, despite its scientific importance, no transcriptome data have been generated for deciphering the early developmental stages of the chicken because of practical and technical constraints accessing pre-oviposited embryos. Findings Here, we determine the entire transcriptome of pre-oviposited avian embryos, including oocyte, zygote, and intrauterine embryos from Eyal-giladi and Kochav stage I (EGK.I) to EGK.X collected using a non-invasive approach for the first time. We also compare RNA-sequencing data obtained using bulked embryo sequencing and single embryo/cell sequencing technique. The raw sequencing data were pre-processed with two different genome builds, Galgal4 and Galgal5, and the expression of 17,108 and 26,102 genes was quantified in the respective builds. There were some differences between the two techniques, as well as between the two genome builds, and these were affected by the emergence of long intergenic non-coding RNA annotations. Conclusion The first transcriptome datasets of pre-oviposited early chicken embryos based on bulked and single embryo sequencing techniques will serve as a valuable resource for investigating early avian embryogenesis, for comparative studies among vertebrates, and for novel gene annotation in the chicken genome.
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Response to Reviewers:	Dear Editor,

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Herewith we are submitting our revised manuscript entitled “The first whole transcriptomic exploration of pre-oviposited early chicken embryos using single and bulked embryonic RNA-sequencing” (Manuscript ID: GIGA-D-17-00277).

We would like to express our sincere gratitude to the editor and reviewers who handled this manuscript. We also appreciate the positive and thoughtful comments of the both reviewers who have acknowledged the value of the data submitted. We believe that their comments have greatly improved the reproducibility and readability of our work, especially about the quality control and methodologies of our data. This manuscript was substantially revised according to all of the reviewers’ comments. We also prepared a point-by-point response to the reviewer’s comments and submitting herewith.

We are looking forward to meet editorial and reviewer suggestion regarding this revised manuscript.

Sincerely yours,

Jae Yong Han, Ph.D.

The authors are very grateful for the response on the originally submitted manuscript. In accordance to the comments by the reviewers, we have provided point-to-point responses to all of your comments. We believe that this revision and our responses will satisfy your point of view on our manuscript.

<Response to reviewer 1 comments>

The authors present a new dataset they generated with first analytical steps. Emphasizing that chicken embryos are a very useful model to study because, in part of the accessibility of the embryo in the egg and thus to key steps of the development. However studies so far are focusing their efforts on the accessible stages of the development, i.e. those occurring in the egg and we are missing information on the development stages happening before oviposition.

The authors produced RNA seq data of those pre-oviposition stages thus giving access to the first expression map of chicken embryos at those early stages in development. Aware of technical biases, the authors sequenced the data from 2 different sources, single embryo vs bulked embryos. This gives an interesting overview of potential differences and strength of both approaches in the context of sequencing those very early stages. Also aware that researchers in the chicken community are still using Galgal4 reference build for comparison purposes, the authors aligned their sequenced data on the 2 most recent builds Galgal4 and Galgal5. This allows to appreciate intrinsic differences between both builds.

This dataset is of interest for any researcher in embryology, in chicken or not, that would like to access expression data of early stages of development.

The authors made a good job at controlling the quality their data for the most part. I would definitely recommend to publish this work provided some minor additions/modifications.

1. L55: precise this first set is bulked

Response: We are thankful of the comments for improving our manuscript by the reviewer. As the reviewer’s suggestion, we added “bulked” in that sentence (Line 54).

2. L79: stages could be put in chronological order

Response: As the reviewer pointed out, we re-ordered to “the oocyte, the zygote, EGK.I, EGK.III, EGK.VI, EGK.VIII and EGK.X” (Line 78-79).

3. L81: to what extend this technique could have an impact on the embryo integrity, and consequently RNA quality? More generally, for the bulked sequences, how many hens were used? What genetic background are they? Could the authors justify the choice of choosing different animals for the bulk sequencing and the same ones for different stages for the single embryo sequencing?

Response: The embryo integrity and RNA quality are not affected by the abdominal massage technique for harvesting early embryos in chicken. Using these embryos, we can perform the downstream experiments such as in situ hybridization, qRT-PCR, and

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RNA sequencing. Also, we used total 137 hens for bulked embryonic sequencing after egg-laying time checked.
On the other hands, White leghorn flock we used is registered in Domestic Animal Diversity Information System (DAD-IS; http://www.fao.org/dad-is/) as “White Leghorn SNU” and they have been systematized since they were brought from National Institute of Animal Science in 1992. Thus, the chickens for bulked embryo and single embryo are considered to be the closed population with a genetically similar background. Nevertheless, the expression profiles were shown to be different between bulked and single oocyte and zygote based on Galgal5. This seems to be caused by individual variation and maternal effects in terms of gene expressions during very early stages such as oocyte and zygote, but not in EGK.X. In addition, we changed the words, “genetic information” and “genetic backgrounds” into “the individual gene expression diversity” (Line 170, 172), “its own gene expression” (Line 174), and “various individuals” (Line 176) for not making the readers confused the variation of gene expression with the variation of genetic background.

4. L90: what is the rationale behind the number of embryos pooled together at each stage? Why did the authors chose a higher number for the latest stage?
Response: We thank the reviewer for pointing out this important issue. When we did sampling oocyte, zygote, and intrauterine stages, firstly we checked the stage of embryo morphologically. Immediately after identifying the stage of the embryos, we pooled at least three embryos per one replicate in each stage. In this procedure, it is difficult to use the exact number of samples in all stages, owing to the limited acquisition of intrauterine embryos. In the case of EGK.X which is at oviposition, we could easily obtain a relatively larger number of embryos as one replication.

5. L123: It would be extremely interesting to have on idea of the RNA quality. What is the RIN for each sequenced sample? This information might be useful to interpret some of the further results.
Response: As the reviewer’s question, we prepared rRNA ratio during pre-ovipositional development and RIN of all samples in revised Table S1: Additional file 1. RIN number below 7 were observed from zygote to EGK.VIII stage in few samples although same RNA isolation procedure was applied in oocyte and EGK.X. This is because of common phenomenon that rRNA ratio (28s: 18s) is lower than 1.8 from zygote to EGK.VIII stages, not caused by RNA quality. The low levels of 28s rRNA prior to maternal-to-zygotic transition (MZT) were generally found during early embryonic development. In chicken, the relative amount of 28s rRNA was reduced markedly after the zygote and recovered gradually after EGK.VIII at MZT occurring (Hwang et al., FASEB J 2017), like as bovine until morula stage (Gilbert et al., Mol Reprod Dev 2009). We also added the related sentences to the revised manuscript (Line 100-103 and Line 123-124).

6. L129: replace with 150bp paired-end reads
Response: According to reviewer’s suggestion, we replaced it (Line 134-135).

7. L135: What are the criteria used for filtering after FastQC? We would need some more information to help explain the differences in quality between single embryo and bulked.
Response: Thanks for good suggestion. Here, after Trimmomatic, minimum read length > 75 bp and Phred score > 30 were checked using FastQC (Line 142-144). In addition, we have uploaded all FastQC results in GigaDB including figures and data derived from FastQC.

Location of directory in the GigaDB:
(1) /0.FastQC/1.Bulked_embryo_fastqc.zip: FastQC results for fastq files from bulked RNA-seq data
(2) /0.FastQC/2.Single_Embryo_fastqc.zip: FastQC results for fastq files from single embyonic RNA-seq data

8. L161: This statement depends on what is the genetic diversity of hens used in the study and how close the hens are from the reference genome. The authors could give other clues to explain those differences. What is the duplication rate? What is the proportion of reads that are uniquely mapped?
Response: Thank you very much for providing us with a clue to our hypothesis. In

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revised Table 1, deduplicated percentage derived from FastQC and uniquely mapped reads derived from HISAT2 log were added based on the reviewer's comment. First, we observed significant differences between bulk and single RNA-seq data in terms of duplication rate. In the bulked samples, 31.3% and 42.96% were observed for read 1 and read 2, respectively (In case of, single embryonic sample, 44.78% and 49.33% were observed for read 1 and read 2, respectively). In other words, this result showed a higher read duplication rate in RNA-seq performed with a single embryo sample, demonstrating that there is lower expressional diversity in single embryonic samples. This result has been added to the result body in revised manuscript (Line 170-173).

9. L166: rephrase this sentence
Response: We have rephrased it and corrected the error (0.028 and 0.001 to 2.79 and 0.14) in this sentence (Line 176-180).

10. L190: replace "which RNAs" with "which RNA category" or "which RNA type"
Response: According to reviewer's suggestion, we replaced it (Line 203).

11. L194: what is the threshold of expression used to call a gene expressed?
Response: Here, we just used mapped reads on the reference genome across all samples. Based on the read count of each genes, we designated them as expressed genes (Line 208).

12. L205: this might be linked to the quality of those samples. Is it harder to extract quality RNA from very early stages?
Response: As shown in revised Table S1: Additional file 1, the extracted RNA from these embryos have good quality. Thus, RNA quality does not seem to be related to the different correlation between Galgal4 and Galgal5.

13. L242: The author might want to moderate this sentence. Here bulked embryos were from different genetic background and extracted with a very specific technic. We would like to know to what extend those choices can impact the quality of the data as well. It might not be solely due to the pooling process.
Response: As the above answers, the sample quality does not seem to be relevant to these effects. In terms of the evidences we have found, the pooling effect from the individual gene expression diversity and the difference of gene annotations could impact on such results between oocyte and zygote (Line 257-258).

14. Table1: "surviving": would suggest to rephrase
Response: We have corrected "Surviving reads" to "QC passed reads" and "Surviving rates" to "QC passing rate" in revised Table 1.

15. Figure 2b is it a pairwise comparison? What difference are you testing here?
Response: Here, the null hypothesis assumes that the RNA concentration values of all developmental stages are the same, and the alternative hypothesis hypothesized that RNA concentration values differ in at least one stage. In other words, F-test was performed on a statistical model of one-way Analysis of variance (ANOVA) format. Consequently, The RNA concentration, amount of RNA, and total RNA per embryo did not differ significantly among the groups (Line 398-400).

16. Figure3a-b: what is the correspondence between those lists and the annotation? What is the percentage of overlap? In other words, how many of those genes that are expressed differentially between galgal4 et galgal5 are actually the ones that are not annotated in one of the builds?
Response: Thank you for suggesting the issues from different references. It is quite difficult to the answer to the reviewer's comment. First, we can't perform analysis identifying differentially expressed genes (DE analysis) between Galgal4 and Galgal5 with the genes in a particular build. Please understand that in order to perform DE analysis between both builds, we can only perform on approximately 11,000 genes that were commonly annotated in both builds. Second, if we perform DE analysis on genes that were commonly annotated in both builds, there is a statistical issue. Currently, RNA-seq statistical analysis is performed on a generalized linear model (GLM). Hypothesis testing for differences between two builds violates the independence assumption in this model. To resolve this issue, generalized linear mixed-effect model (GLMM) should be employed, but this model has not yet been proven as standard

method. This question is very interesting, but adding it to a discussion in the main text does not seem to fit the article type of the Data Note, so it only answers the letter.

17. Figure 3c is redundant with Table 2. Y axis: anntoated —> annotated
Response: First, typo that the reviewer pointed out has been fixed in Fig. 3c. But, Figure 3c and Table 2 are different. In case of revised Table 2, the contents include only annotation comparison between Galgal4 and Galgal5. On the other hand, Figure 3c demonstrates that how many newly annotated genes were actually expressed in terms of mapped reads across all samples. In order to improve the readability for the potential readers, Table 2 and Fig. 3c citation of the main text were modified in Line 204-206.

18. Figure 4: would be interesting to correlate those coordinates with known covariates to show what are in the main axis of variation.
Response: Thanks for reviewer pointing out an important issue. As part of the reviewer, we proceeded with factor analysis through various environmental variables (Batch effects and etc.) with vectors obtained through dimensional reduction. As a result, the variation of the projected vector values of dimension 1 and dimension 2, as mentioned in the text, describes the sub-cluster structure of developmental stages best. First dimension showed that developmental stages progressed in a negative direction during intrauterine development. In case of second dimension, variation of values seem to explain the difference between oocyte and fertilized embryos from zygote to EGK.X (Line 417-420).

<Response to reviewer 2 comments>
Hwang and coauthors report their RNA sequencing data of pre-oviposited early chicken embryos. The authors used single cell as well as standard whole tissue RNA-seq analysis and also assess differences between gene annotation in the two most recent chicken genome builds. Given the wide usage of the chicken embryo as a model system to study vertebrate development, this contribution is very important to the research community. I have several issues for the authors to consider revising.

1. Quality of total RNA was assessed using several methods including an Agilent Bioanalyzer (lines 97- 100). The RNA integrity number (RIN) should be reported for all samples. Typically, RNA samples with a RIN ≤ 7 are not suitable for accurate RNA-seq analysis.
Response: We are thankful to the considerate comments by the reviewer. As the reviewer's suggestion, we prepared rRNA ratio during pre-ovipositional development and RIN of all samples in revised Table S1: Additional file 1. RIN number below 7 were observed from zygote to EGK.VIII stage in few samples although same RNA isolation procedure in oocyte and EGK.X. This is because of common phenomenon that rRNA ratio (28s: 18s) is lower than 1.8 from zygote to EGK.VIII stages, not caused by RNA quality. The low levels of 28s rRNA prior to maternal-to-zygotic transition (MZT) were generally found during early embryonic development. In chicken, the relative amount of 28s rRNA was reduced markedly after the zygote and recovered gradually after EGK.VIII at MZT occurring (Hwang et al., FASEB J 2017), like as bovine until morula stage (Gilbert et al., Mol Reprod Dev 2009). We also added the related sentences (Line 100-103 and Line 123-124).

2. The methodology for Illumina sequencing library preparation is insufficiently reported (lines 125- 129). More detail should be added here including the method of transcript enrichment and average size of library fragments.
Response: As the reviewer's suggestion, we added detailed methods including specific library prep kit product used in this study and the average size of cDNA libraries (Line 131-133)

3. Table 1 demonstrates the number of reads that passed Trimmomatic filtering, however, representative FastQC plots such as per bas and/or per sequence quality plots should be shown in the main text or supplement to demonstrating the quality of the data.
Response: Thanks for good suggestion. Based on suggestion from reviewers, we have uploaded all FastQC results in GigaDB including figures and data derived from FastQC.

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	Location of directory in the GigaDB: (1) /0.FastQC/1.Bulked_embryo_fastqc.zip: FastQC results for fastq files from bulked RNA-seq data (2) /0.FastQC/2.Single_Embryo_fastqc.zip: FastQC results for fastq files from single embyonic RNA-seq data 4. All settings for Trimmomatic filtering software (line 135) HISAT2 alignment software (line 155), and HTSeq-count transcript quantification software (line 171) should be reported. Response: Thanks for reviewer's suggestion to improve the reproducibility of our paper. Based on the reviewer's comment, we add specific option of used tools as follows: (1) Trimmomatic ver. 0.33 with "-phred33 and ILLUMINACLIP:/home/Program/Trimmomatic-0.32/adapters/TruSeq3-PE-2.fa:2:30:10 MINLEN:75 option" (Line 141-142). (2) HISAT2 ver. 2.0.0 [15] was used with the "--rna-strandness RF –x [File name of Galgal4 or Galgal5 reference] -1 [File name of left lead] -2 [File name of right read] 2> [Sample name].log" (Line 163-165). (3) HTSeq-count [17] with following option, "python -m HTSeq.scripts.count -f bam --stranded=reverse [File name of bam file] [File name of annotation (.GTF file)] > [Output file name] (Line 183-185). 5. Figure 3 alludes to interesting differences in gene annotation between Galgal4 and Galgal5 genome builds but does not report what these newly annotated transcripts are in the updated annotation. A table of genes/transcripts represented in Fig3a-c should be included. Response: In this study, we just used Galgal4 and Galgal5 reference genome and gene annotations rather than do-novo based assembly. Thus, those annotations have been already provided in Ensembl DB; (Galgal4, jul2016.archive.ensembl.org/Gallus_gallus/Info/Annotation and Galgal5, www.ensembl.org/Gallus_gallus/Info/Annotation). The major difference between two gene builds is shown to be the presence of long non-coding genes and the increased number of gene transcripts. 6. Figure 4 alludes to interesting differences in gene expression between developmental stages but there are no reports on what these genes are in the main body of the paper. An additional figure or table should be added to report several aspects of differential gene expression between embryo stages. Response: Since this article type is a Data Note and according to the Criteria of Data Note in GigaScience, we have focused on the results obtained from the pre-processing step as much as possible. As shown in Figure 4, the expression pattern of these genes will vary according to the developmental stages, we expect potential users to unveil their-own downstream analyses based on the raw-count and TMM normalized matrix we provided. Moreover, because of the possibility of duplication of processed data regarding differential gene expression in our further research article, please excuse that we could not provide it completely.
Additional Information:	
Question	**Response**
Are you submitting this manuscript to a special series or article collection?	No
Experimental design and statistics Full details of the experimental design and statistical methods used should be given in the Methods section, as detailed in our Minimum Standards Reporting Checklist. Information essential to interpreting the data presented should be made available	Yes

in the figure legends. Have you included all the information requested in your manuscript?	
Resources A description of all resources used, including antibodies, cell lines, animals and software tools, with enough information to allow them to be uniquely identified, should be included in the Methods section. Authors are strongly encouraged to cite Research Resource Identifiers (RRIDs) for antibodies, model organisms and tools, where possible. Have you included the information requested as detailed in our Minimum Standards Reporting Checklist?	Yes
Availability of data and materials All datasets and code on which the conclusions of the paper rely must be either included in your submission or deposited in publicly available repositories (where available and ethically appropriate), referencing such data using a unique identifier in the references and in the "Availability of Data and Materials" section of your manuscript. Have you have met the above requirement as detailed in our Minimum Standards Reporting Checklist?	Yes

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The first whole transcriptomic exploration of pre-oviposited early chicken embryos using single and bulked embryonic RNA-sequencing

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Abstract

Background

The chicken is a valuable model organism, especially in evolutionary and embryology research because its embryonic development occurs in the egg. However, despite its scientific importance, no transcriptome data have been generated for deciphering the early developmental stages of the chicken because of practical and technical constraints accessing pre-oviposited embryos.

Findings

Here, we determine the entire transcriptome of pre-oviposited avian embryos, including oocyte, zygote, and intrauterine embryos from Eyal-giladi and Kochav stage I (EGK.I) to EGK.X collected using a non-invasive approach for the first time. We also compare RNA-sequencing data obtained using bulked embryo sequencing and single embryo/cell sequencing technique. The raw sequencing data were pre-processed with two different genome builds, Galgal4 and Galgal5, and the expression of 17,108 and 26,102 genes was quantified in the respective builds. There were some differences between the two techniques, as well as between the two genome builds, and these were affected by the emergence of long intergenic non-coding RNA annotations.

Conclusion

The first transcriptome datasets of pre-oviposited early chicken embryos based on bulked and single embryo sequencing techniques will serve as a valuable resource for investigating early avian embryogenesis, for comparative studies among vertebrates, and for novel gene annotation in the chicken genome.

Keywords

RNA-seq - Single embryonic sequencing - Single cell sequencing - Early embryo - Chicken

Background

Avian species are valuable animal models in many research areas, especially in embryology, because the avian embryo develops in an egg before hatching. This is an excellent *in vitro*-like *in vivo* system that has allowed extensive research of the developmental events during embryogenesis. Previous studies have examined primitive streak formation and gastrulation after oviposition in avian species [1-4]. Nevertheless, despite the importance of the initial events in avian embryogenesis before oviposition, only a few morphological studies have examined pre-oviposited embryos because of practical difficulties accessing the embryos [5-7]. The temporal regulation of gene expression during the pre-oviposited stages is important for understanding early embryonic development.

Recently, the Bird10K project was initiated because of the intermediate position of birds in the comparative biology of vertebrates and their broad utility for diverse research. This project used the genome sequences of 48 species of birds to construct a phylogenetic hierarchy of avian species and examine the comparative genomics of flight and functional adaptations [8-10]. However, no transcriptomic approach to early bird embryos has been performed. Here, we present whole transcriptome sequencing of bulked pre-oviposited chicken embryos, including oocyte, zygote, and intrauterine embryos from Eyal-giladi and Kochav stage I (EGK.I) to EGK.X (Fig. 1a). Furthermore, a single oocyte, zygote, and EGK.X blastoderm from one hen were sequenced (Fig. 1b) and compared with the results for bulked embryos. Based on the whole transcriptome of early chicken embryos, we mapped our sequencing reads on the two most recent chicken (*Gallus gallus*) genome references, Galgal4 and Galgal5, and examined the differences in gene expression between the two builds with or without long intergenic non-coding RNA (lincRNA) annotations.

Data description

Collection of bulked early chicken embryos

In the chicken, the initial 25 h of embryonic development from fertilization to oviposition

progresses through the oviduct. The mature oocyte on top of the yellow yolk is ovulated into the infundibulum 30 min after oviposition. Then, fertilization occurs and the zygote passes through the magnum without any morphological changes in the embryo. According to the well-defined criteria of Eyal-Giladi and Kochav [5, 6], the first cleavage is observed 5 h after fertilization in the shell gland and has been designated EGK.I. Beginning with this event, the pre-ovipositional development of birds is divided into 10 stages, including the cleavage (EGK.I to EGK.VI) and area pellucida formation (EGK.VII to EGK.X) periods. During the cleavage stages, rapid cellularization and an increase in layers lead to formation of a multi-layered blastula by EGK.VI. In the second half of intrauterine development, the first morphological segregation, including the area pellucida and area opaca regions, occurs with anterior–posterior axis formation and layer reduction. Finally, a thinner, longer, bi-layered blastoderm is established at EGK.X. Based on the morphological dynamics that occur during intrauterine development, we chose critical representative stages to analyze: the oocyte, the zygote, EGK.I, EGK.III, EGK.VI, EGK.VIII and EGK.X (Fig. 1a).

The egg-laying times of white leghorn (WL) hens were recorded, and intrauterine eggs from EGK.I−VIII were harvested using an abdominal massage technique [11]. Briefly, the abdomen was pushed gently until the shell gland was exposed; the surface of the shell gland expands when an egg is present for egg shell formation. After expansion of the shell gland surface, massaging was used to move the egg gently towards the cloaca until the intrauterine egg was released. EGK.X blastoderms were collected from WL hens after oviposition. To collect oocytes and zygotes, WL hens were sacrificed and the follicles were collected. Zygote embryos located in the magnum and showing no cleavage were collected within 1 h post-fertilization according to the recorded egg-laying times. All embryos were classified according to morphological criteria (Fig. 1c). All stages were prepared in triplicate and each replicate contained three to seven embryos, while there were ten embryos per replicate of the post-oviposited EGK.X blastoderm (Fig. 2a). Shortly after collection, the embryos were separated from the egg using sterilized paper, and the shell membrane and

albumen were detached from the yolk. A piece of square filter paper (Whatman, Maidstone, UK) with a hole in the center was placed over the germinal disc. After cutting around the paper containing the embryo, it was gently turned over and transferred to saline to remove the yolk and vitelline membrane and allow embryo collection. Total RNA was isolated from early embryos using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The quality and quantity of the extracted total RNA were determined using the Trinean DropSense96 system (Trinean, Gentbrugge, Belgium), a RiboGreen kit (Invitrogen), and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). We assessed rRNA ratio (28s: 18s) and RNA integrity number (RIN) of bulked embryos (Table S1: Additional file 1). We observed lower rRNA ratio from zygote to EGK.VIII stage, because of the low levels of 28s rRNA before maternal-to-zygotic transition (MZT) during early development [12, 13]. The average concentration and amount of total RNA in the early stages was 157.7 ng/μL and 7,026.2 ng, respectively, with the exception of EGK.X, which contained 368.9 ng/μL and 18,495.8 ng due to the larger number of embryos pooled (Fig. 2b, c). Based on the amount of total RNA and the number of embryos in each sample, we estimated the total amount of RNA per embryo in each stage. On average, the early chicken embryos contained 1,457 ng of total RNA (Fig. 2d).

Collection of a single oocyte, zygote, and EGK.X blastoderm from one hen

In accordance with the estimated amount of total RNA per embryo, a single RNA-rich embryo could be used to perform RNA-sequencing (RNA-Seq) without an amplification technique. In this way, probable sequencing errors due to library amplification from low-input RNA can be avoided. Furthermore, the deviation of transcriptomes among early embryos at the same stage can be examined. Chicken physiology allows a single oocyte, zygote, and EGK.X blastoderm to be collected from one hen at the same time, which minimizes any individual variation and maternal effects (Fig. 1b). On the day when single embryos were acquired, a single EGK.X blastoderm was collected and the time was recorded. Within 1 h

post-fertilization according to the recorded egg-laying times, a WL hen was sacrificed and a single oocyte and zygote were simultaneously collected. All stages were prepared in triplicate (Fig. 2a). The subsequent steps, including embryo separation and total RNA isolation and quantification, were the same as for the pooled embryos. RIN of single embryos were comparable to bulked embryos (Table S1: Additional file 1). With the single-embryo approach, the RNA concentration was 105.3 ng/μL and the amount of total RNA averaged 2123.5 ng (Fig. 2b, c). The total amount of RNA for a single embryo was higher and more constant among the different stages than with the bulked embryo collection (Fig. 2d).

Library preparation and whole transcriptome sequencing

Total RNA was used to construct cDNA libraries using the TruSeq Stranded Total RNA Sample Preparation kit with Ribo-Zero Gold (Illumina, San Diego, CA, USA). The resulting average size of the cDNA libraries was approximately 530 bp. The resulting libraries were subjected to transcriptome analysis using the Illumina NextSeq 500 platform to produce 150 bp paired-end reads.

Summary statistics of pre-processing for RNA-seq data

Thirty RNA-seq samples were used in the pre-processing step for the quantification of gene expression in the early developmental stages in the chicken. First, adapter sequences and poor-quality reads were removed from the raw paired-end sequenced files using Trimmomatic ver. 0.33 with “-phred33 and ILLUMINACLIP:/home/Program/Trimmomatic-0.32/adapters/TruSeq3-PE-2.fa:2:30:10 MINLEN:75 option” [14]. The quality of the clean reads, including minimum read length > 75 bp, and Phred score > 30, was verified using FastQC ver. 0.11.2 [15]. On average, 58,930,612 (96.75%) and 39,969,608 (86.16%) paired-end reads remained after the quality-control step for bulked and single-embryo sequencing, respectively (Table 1).

The clean reads were mapped into the two different builds of the Galgal4 and Galgal5 reference genomes, which were obtained from the Ensembl database. The Galgal4 build was the so-called "golden standard" reference chicken genome at the end of 2015, and many studies have employed this build. In December 2016, a new genome build, Galgal5 (Ref Seq assembly accession: GCA_000002315.3) and an improved gene model were established using advanced sequencing techniques. One of the features of Galgal5 compared with Galgal4 is the different read length used when the gene model was established. This change improved inaccurate gene annotations, especially the structure of isoforms, in existing short-read based gene models through an isoform sequencing technique using the Pacific Biosciences (PacBio) long reads. Furthermore, PacBio long-read sequencing technology makes it possible to establish lincRNAs, which is important in developmental biology [16]. Given that our data were not only an early developmental sample of a chicken but also a sample of all types of RNAs, this must be considered when quantifying gene expression levels in the RNA-seq pipeline. Therefore, we decided to quantify the expression level of the entire transcriptome using the two different versions of the genome builds, and then compared the results to examine the differences. In the alignment step, HISAT2 ver. 2.0.0 [17] was used with the "--rna-strandness RF –x [File name of Galgal4 or Galgal5 reference] -1 [File name of left lead] -2 [File name of right read] 2> [Sample name].log". As a result, an average of 76.07 and 73.27% mapping rates were observed in Galgal4 and Galgal5, respectively, in the 21 bulked embryo samples and 84.41 and 84.28% were observed in the nine single embryo or cell samples (Table 1). For Galgal4 and Galgal5, the average observed difference in the mapping rate between the bulked and single embryo samples was 8.35 and 11%, respectively. We suspected that this difference in mapping rates was caused by the individual gene expression diversity. Upon examining the duplication rate of the generated read, higher duplication rate was observed in single cell and/or embryonic RNA-seq, which is evidence that the individual gene expression diversity is lower in single embryonic samples (Table 1). Since transcriptome data generated using

single embryo sequencing technology contains only its own gene expression for a single entity, it is assumed that the mapping rate is increased by alleviating the heterogeneity problem derived from various individuals. We also observed small differences in the average mapping rates in two genome builds; 2.79 and 0.14% decrease respectively for the bulked and single embryo samples in Galgal5 compared to Galgal4, which implies that there are little differences between the two genome builds at the DNA level, but more impact on bulked embryos. Following the alignment step using the two different versions of the genome builds, alignment files (.SAM files) were converted into binary alignment files (.BAM) using SAMtools ver. 1.4.1 [18]. Based on the alignment files, the gene expression levels (number of mapped reads) were quantified using HTSeq-count [19] with following option, “python -m HTSeq.scripts.count -f bam --stranded=reverse [File name of bam file] [File name of annotation (.GTF file)] > [Output file name]” with the Ensembl gene annotation files corresponding to the genome builds (Ensembl release 85 for Galgal4 and 86 for Galgal5). As a result, the number of mapped reads was quantified in each pipeline and 17,108 and 26,102 genes were annotated in the Galgal4 and Galgal5 genome builds, respectively.

Comparison of the gene expression patterns between Galgal4 and Galgal5 in chicken early embryo samples

Based on the mapped-count matrix of the genome builds and the Ensembl annotation, we systematically investigated how many and which types of genes differed between the two genome builds. First, we found that many genes were differentially annotated in each build in terms of their Ensembl IDs (Fig. 3a). Of the 17,108 and 26,102 annotated genes in Galgal4 and Galgal5, respectively, only 11,451 Ensembl IDs were shared by both annotations, while 5,657 and 14,651 Ensembl IDs were annotated only in the respective builds. Next, we compared the two genome builds based on the genes actually expressed in the early embryo samples of chickens. For this comparison, we filtered out genes with no mapped counts across all 30 RNA-seq samples. As a result, 901 and 3,849 genes were filtered out in

the raw gene annotations of Galgal4 and Galgal5, respectively (*i.e.*, 16,207 and 22,253 genes remained). Because the same pattern of results was observed when validated with the filtered Ensembl IDs (Fig. 3b), we then examined which RNA type produced the difference between Galgal4 and Galgal5. As a result, many lincRNAs and protein-coding genes were newly identified in Galgal5 (Table 2) and confirmed to be expressed in early chicken embryos (Fig. 3c). With the development of sequencing technology, lincRNA has been added to over 5,166 new genes, and it was confirmed that it is actually expressed in our data based on the mapped reads. Unlike lincRNA, which was unilaterally added to Galgal5, there were many changes in protein-coding genes (Table 2). A total of 4,892 protein-coding genes were discarded, while 5,613 were added in the new version of the gene annotation (based on the Ensembl ID matching). Since there is still a lack of empirical evidence and practical discussion of the validity of both gene models, it is impossible to determine which genome build is correct for quantifying gene expression in our study. However, we expect to contribute to further studies by providing the entire transcript expression metrics for early embryos of chickens in both builds. Finally, correlations between the 30 samples were examined based on the quantified expression of 11,001 genes common to the gene annotations of these two builds (Fig. 3d). Based on bulked embryo sequencing, high correlations (≥ 0.9) were observed between Galgal4 and Galgal5, except for the oocyte and zygote. In comparison, single embryo and/or cell sequencing showed the high correlation between Galgal4 and Galgal5 including the oocyte and zygote. This demonstrates the excellent reproducibility of the data produced based on the single experimental subject. Most of the embryonic transcriptome data generated to date have involved pooling problems and we expect to be able to perform more sophisticated downstream analysis using single embryo and/or cell sequencing, which is now possible due to technological developments.

Comparison of bulked embryo sequencing and single embryo and/or cell sequencing

with chicken early embryos

To investigate the differences between the two technologies more systematically, multidimensional scaling analysis was performed using information from 30 RNA-seq samples in two gene expression matrixes: Galgal4 and Galgal5. All of the samples in both gene expression matrixes clearly clustered according to their developmental stage, except for the zygote, EGK.I, and EGK.III (Fig. 4). This means that although there are morphological differences, there is no transcriptome change during the early embryonic development of the chicken for a specific time after zygotic gene activation. In fact, the time from the zygote to EGK.III is also very short. While most of the patterns seem to be concordant between Galgal4 and Galgal5, distinct differences were observed between the bulked and single embryo RNA-seq techniques for the oocyte and zygote samples based on the Galgal5 gene expression matrix. However, no difference was detected between the two techniques for the EGK.X samples, which is presumably due to the difference between the bulked and single cells because we performed single embryo RNA-seq for the oocyte, zygote, and EGK.X stages. The RNA samples from the oocyte and zygote were derived from a single cell, whereas those from EGK.X were derived from bulked cells. As we have already examined the difference in gene annotation between Galgal4 and Galgal5, more than 10,000 genes have been changed, which includes both protein-coding genes and lincRNAs. Of these changes, 5,166 newly added lincRNAs may be a major factor causing this difference because lincRNA plays an important role in the zygote as an epigenetic marker in both humans and mice, which have been subjected to lincRNA annotation and early embryonic transcription studies. Furthermore, epigenetic markers are very sensitive, exhibiting subject- or cell-specific characteristics. Therefore, our RNA-Seq data based on the single embryo and cell technique for oocytes and zygotes is more accurate than ordinary RNA-Seq data because it eliminates epigenetic and genetic pooling effects. For example, bulked zygote samples were separated from the cluster of EGK.I and EGK.III samples in a multidimensional scaling (MDS) analysis based on the Galgal5 gene matrix, whereas there

was no difference in the Galga4 gene expression matrix (Fig. 4, right panel). This shows that quantifying gene expression using the standard RNA-Seq pooled embryo sequencing technique could be affected by the individual gene expression diversity and the difference of gene annotations.

In summary, we produced the first whole transcriptome sequences of pre-oviposited early chicken embryos based on standard RNA-Seq and single embryo sequencing techniques. We then quantified and compared gene expression using the standard gene annotation used for the chicken and a new chicken gene annotation based on the advanced long-read sequencing technique. As a result, we not only demonstrated the accuracy of RNA-Seq data based on single embryo or cell sequencing but also successfully quantified 5,166 lincRNAs in the new chicken gene model, for the pre-oviposited early chicken embryo. We expect that the transcriptome sequences of pre-oviposited early chicken embryos will fill the gap in comparative developmental and evolutionary studies of vertebrates as a valuable resources and provide comprehensive knowledge of early avian embryogenesis. Furthermore, the oocyte and early chicken embryos express numerous types of RNA, including mRNA and lincRNA, so our dataset should help to establish novel transcript and gene annotations for the chicken reference genome. Our large dataset should also be useful for future studies of avian and comparative genomics because the data were generated using the latest sequencing platform and whole transcriptome sequencing enabling the characterization of all RNA transcripts, including primary transcripts, regardless of polyadenylation.

Availability of supporting data

The bulked and single embryo RNA-Seq data have been deposited in the NCBI GEO database (GSE86592 and GSE100798, respectively). Supporting data including pre-processed gene expression levels are also available in the GigaScience database, GigaDB [20].

Abbreviations

EGK: Eyal-giladi and Kochav; lincRNA: long intergenic non-coding RNA; MDS: multidimensional scaling; PacBio: Pacific Biosciences; RNA-Seq: RNA-sequencing; WL: white leghorn.

Experimental animals and animal care

The care and experimental use of chickens were approved by the Institute of Laboratory Animal Resources, Seoul National University (SNU-150827-1). Chickens were maintained according to a standard management program at the University Animal Farm, Seoul National University, Korea. The procedures for animal management, reproduction and embryo manipulation adhered to the standard operating protocols of our laboratory.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

YSH and JYH conceived and designed the experiments. YSH, HJC, and SKK collected embryos. YSH prepared RNA samples and generated whole-transcriptome RNA-seq reads. YSH, MS, HK, and JYH analyzed and wrote the manuscript. All authors read and edited the manuscript.

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Bulked embryonic sequencing								
Samples	**QC passed reads**	**QC passing rate**	**Deduplicated Percentage (R1)**	**Deduplicated Percentage (R2)**	**Uniquely mapped ratio (Galgal4)**	**Uniquely mapped ratio (Galgal5)**	**Mapping rates (Galgal4)**	**Mapping rates (Galgal5)**
Oocyte_S1_Bulked	56024575	94.81%	37.83%	44.47%	83.82%	99.56%	82.73%	84.32%
Oocyte_S2_Bulked	56043780	94.14%	35.35%	42.25%	81.27%	99.34%	82.77%	79.54%
Oocyte_S3_Bulked	59498675	95.54%	35.84%	43.31%	84.03%	99.58%	82.16%	82.39%
Zygote_S1_Bulked	53378148	96.74%	30.17%	37.65%	84.77%	99.74%	82.43%	85.89%
Zygote_S2_Bulked	53999584	96.77%	26.44%	35.21%	84.20%	99.73%	82.19%	79.86%
Zygote_S3_Bulked	50027929	98.02%	25.13%	39.78%	86.17%	99.76%	80.90%	87.58%
EGK.I_S1_Bulked	56909314	97.36%	27.88%	39.30%	86.28%	86.01%	74.55%	70.70%
EGK.I_S2_Bulked	61447014	97.94%	21.97%	36.34%	87.13%	86.86%	73.24%	68.64%
EGK.I_S3_Bulked	50188847	96.80%	28.24%	37.31%	85.33%	84.67%	81.34%	77.01%
EGK.III_S1_Bulked	60876681	97.30%	25.31%	36.29%	85.09%	86.05%	76.06%	69.37%
EGK.III_S2_Bulked	56357690	97.90%	25.78%	38.44%	86.55%	86.28%	75.20%	70.47%
EGK.III_S3_Bulked	45715485	98.02%	28.17%	41.24%	86.72%	86.32%	75.30%	70.38%
EGK.VI_S1_Bulked	62075038	97.53%	27.00%	42.86%	86.62%	86.77%	71.14%	63.68%
EGK.VI_S2_Bulked	65223164	97.77%	23.36%	34.43%	85.85%	85.61%	80.95%	72.89%
EGK.VI_S3_Bulked	49604292	98.16%	27.31%	41.37%	86.86%	86.44%	75.12%	69.22%

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EGK.VIII_S1_Bulked	67401388	97.35%	36.30%	50.09%	87.21%	86.30%	70.10%	67.32%
EGK.VIII_S2_Bulked	56396268	96.82%	35.34%	51.04%	87.61%	87.25%	66.53%	60.37%
EGK.VIII_S3_Bulked	71309063	97.44%	33.44%	49.01%	86.81%	85.62%	70.68%	70.70%
EGK.X_S1_Bulked	67730502	95.70%	41.09%	52.23%	86.37%	85.54%	72.24%	69.29%
EGK.X_S2_Bulked	74109500	95.02%	42.83%	54.66%	86.60%	85.48%	70.64%	69.62%
EGK.X_S3_Bulked	63225919	94.65%	42.42%	54.85%	86.86%	85.82%	71.13%	69.51%
Average	58930612.19	0.967514286	31.30%	42.96%	85.82%	89.94%	76.06%	73.27%
Single embryonic or cell sequencing								
Oocyte_S1_SingleCell	23558381	86.61%	42.82%	45.68%	79.90%	78.06%	86.28%	86.67%
Oocyte_S2_SingleCell	53963445	84.75%	54.54%	58.84%	81.28%	79.71%	85.95%	85.86%
Oocyte_S3_SingleCell	24660386	84.95%	52.01%	56.95%	81.79%	79.99%	84.95%	84.33%
Zygote_S1_SingleEmbryo	31742857	87.17%	27.95%	32.85%	81.75%	80.66%	84.32%	84.40%
Zygote_S2_SingleEmbryo	91033778	85.72%	37.12%	42.18%	81.62%	80.14%	76.59%	76.15%
Zygote_S3_SingleEmbryo	27687195	87.60%	36.35%	41.48%	81.57%	80.07%	86.02%	85.96%
EGK.X_S1_SingleEmbryo	30914824	86.41%	47.58%	51.56%	85.27%	82.92%	83.67%	83.16%
EGK.X_S2_SingleEmbryo	47159061	86.29%	53.42%	58.46%	82.40%	80.75%	88.38%	89.10%
EGK.X_S3_SingleEmbryo	29006546	85.94%	51.19%	55.97%	82.20%	80.59%	83.57%	82.86%
Average	39969608.11	0.8616	44.78%	49.33%	81.98%	80.32%	84.41%	84.27%

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Table 2. Comparison of Galgal4 and Galgal5 gene annotations

RNAs	Annotated in Galgal4 only	Commonly annotated	Annotated in Galgal5 only
lincRNA	0	0	5,166
miRNA	204	487	253
misc_RNA	15	71	43
Mt_rRNA	2	0	2
Mt_tRNA	10	0	14
protein_coding	4,892	10,213	5,613
pseudogene	29	10	25
rRNA	6	8	58
scaRNA	0	0	4
snoRNA	41	172	44
snRNA	7	40	30
Total	5,206	11,001	11,252

Figure legends

Fig. 1. The bulked and single embryonic RNA-Sequencing (RNA-Seq) in early chicken development. a) The diagram of bulked embryonic RNA-seq. Total 137 pre-oviposited embryos were collected. Each replicate contains from three to ten embryos pooled. The bulked embryo RNA-Seq was performed in triplicate. b) The diagram of single embryonic RNA-Seq. The single oocyte, zygote, and Eyal-giladi and Kochav stage X (EGK.X) blastoderm were obtained from one hen simultaneously. Samples was collected from three hen. Single embryo was sequenced as one replicate and each stage consists of triplicated embryos from three hen, respectively. c) The representative stages of chicken early embryos used for RNA-Seq. Dorsal views of whole embryos from the oocyte to EGK.X are shown. A germinal vesicle oocyte in the ovary and fertilized zygote in the magnum without cleavage were obtained. The intrauterine embryos were obtained 5.5 (EGK.I), 8.5 (EGK.III), 15.5 (EGK.VI), and 20.5 (EGK.VIII) h after fertilization. The EGK.X embryo was obtained after oviposition. Scale bar, 1000 μm.

Fig. 2. Collection of bulked and single embryos during early chicken development. a) The number of embryos in each sample. b) The RNA concentration and c) total amount of RNA for each stage used in RNA-Seq. d) The estimated total RNA per embryo in the bulked samples and the total amount of RNA in a single embryo. The RNA concentration, amount of RNA, and total RNA per embryo did not differ significantly among the groups (Kruskal–Wallis test, $P > 0.05$).

Fig. 3. Comparison of two different builds of gene annotation for the early chicken embryo samples. a) Using the Ensembl annotation with the two different genome builds, annotated genes were compared based on the Ensembl ID. As a result, 5,657 and 14,651 Ensembl IDs were identified in Galgal4 and Galgal5, respectively, while 11,451 Ensembl IDs are common to the two different annotations. b) Based on the expressed genes at any stage of the

chicken early embryos, the gene lists were compared between Galgal4 and Galgal5. c) Investigation of the change in annotated genes in Galgal5 among genes expressed in early chicken embryos. As a result, a large number of lincRNAs was added as new features in Galgal5. d) A correlation analysis of the total gene expression based on 11,001 common annotated genes shared between Galgal4 and Galgal5.

Fig. 4. Multidimensional scaling plots based on all annotated genes in Galgal4 and Galgal5. The gene expression patterns of early chicken embryos quantified based on Galgal4 were clearly differentiated by developmental stage regardless of the sequencing technique used. In comparison, there was a difference between the bulked and single embryo sequencing techniques in the oocyte and zygote in Galgal5. The first dimension (Coordinate 1) is the progression of developmental stages in a negative direction during intrauterine development and the second dimension (Coordinate 2) is the difference between oocyte and fertilized embryos from zygote to EGK.X.

Figure 1

Click here to download Figure Fig1.pdf

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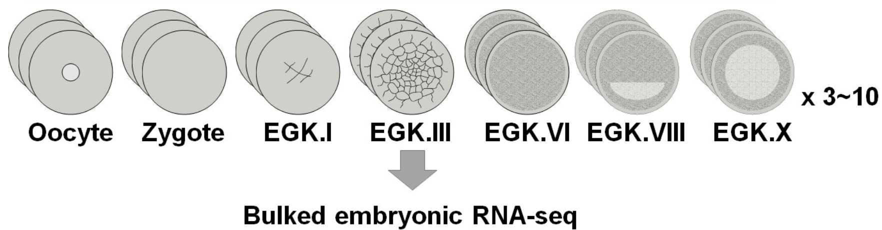

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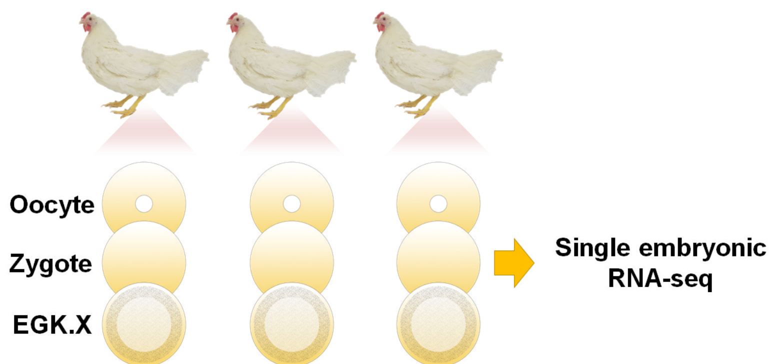

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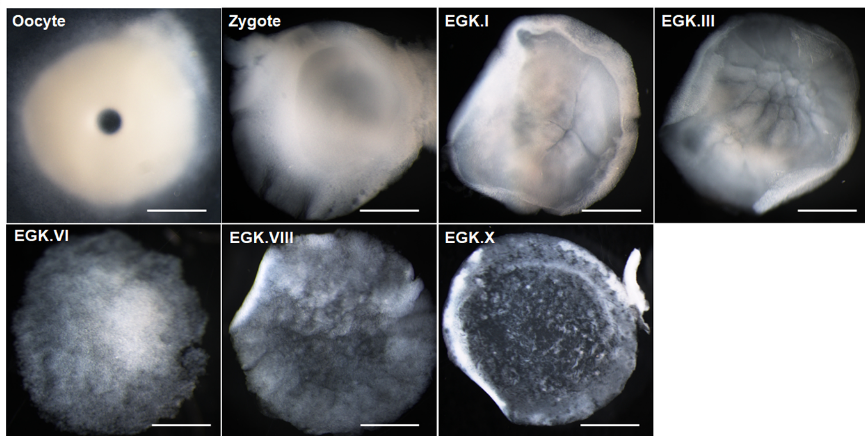
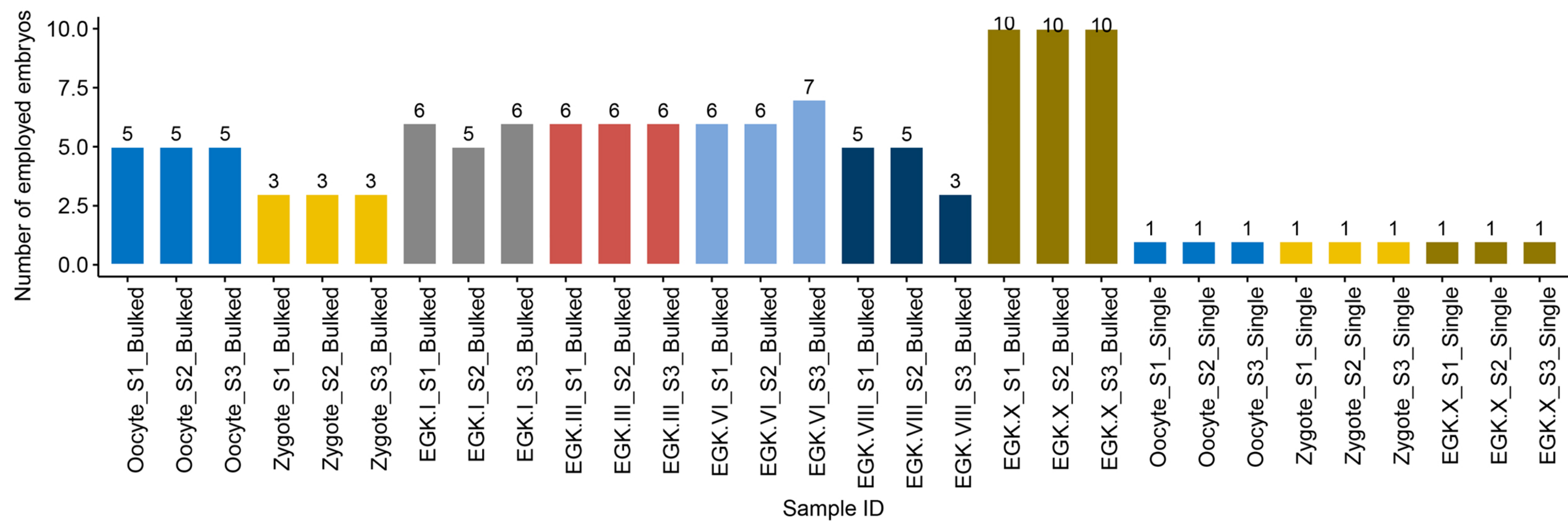
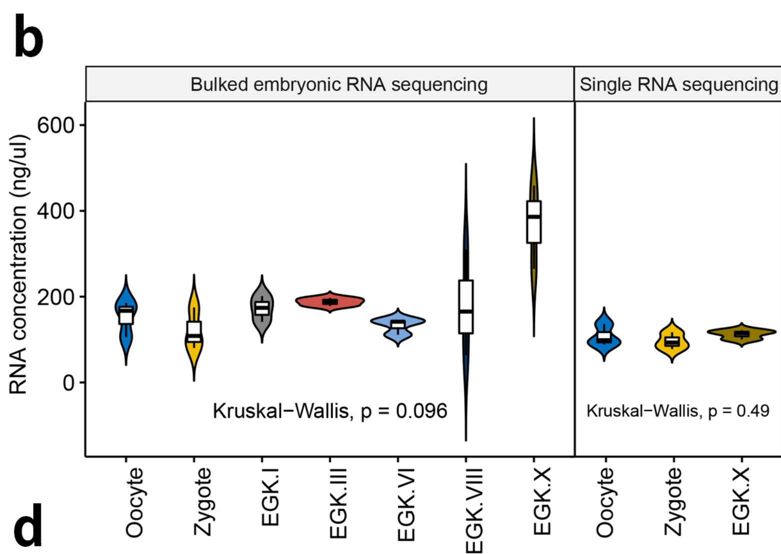
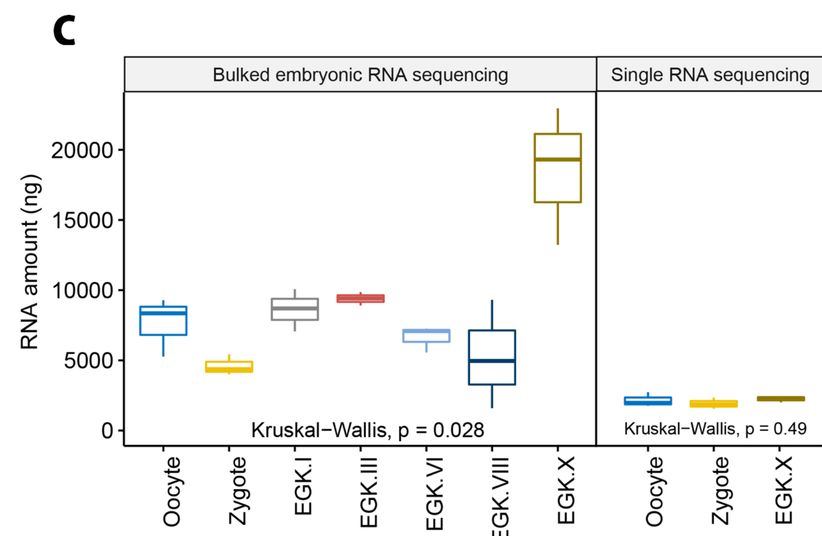
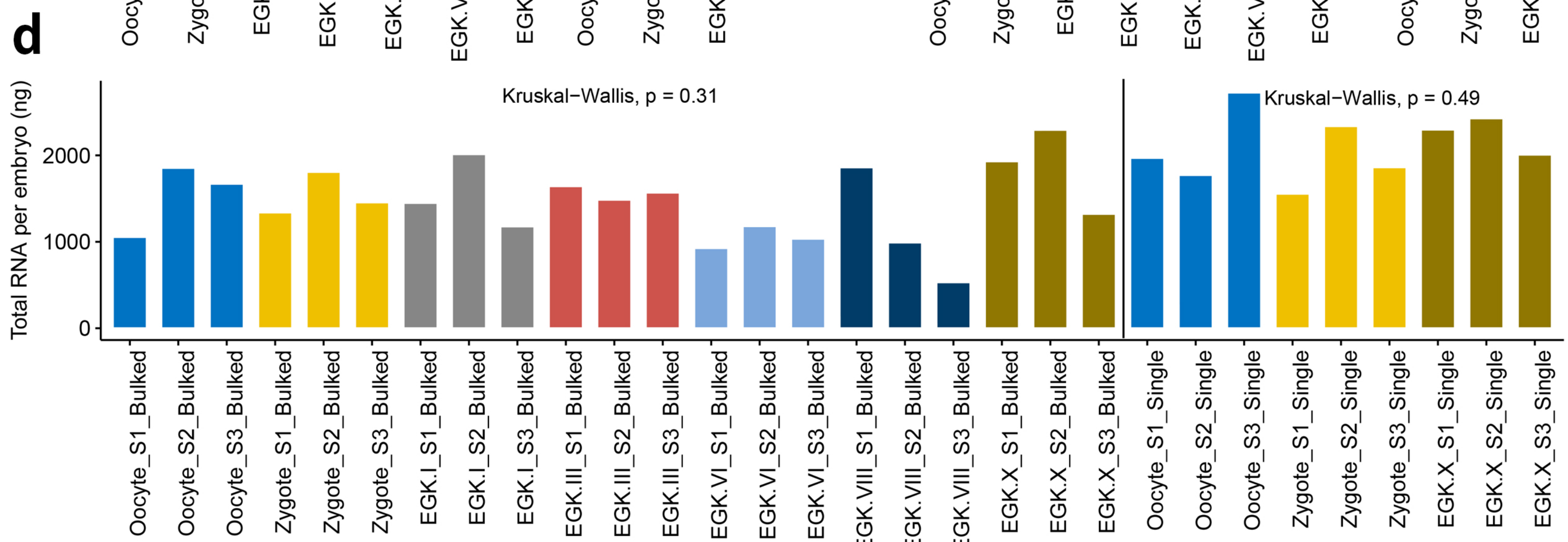


Figure 2 Click here to download Figure Fig2.pdf









Revised Figure 3

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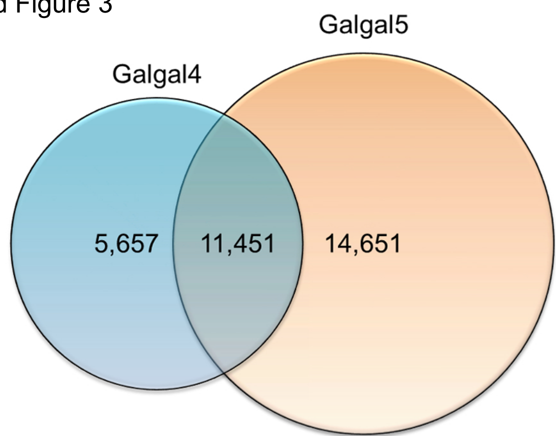

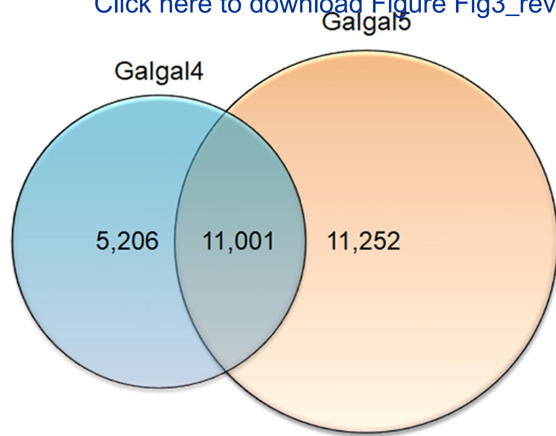

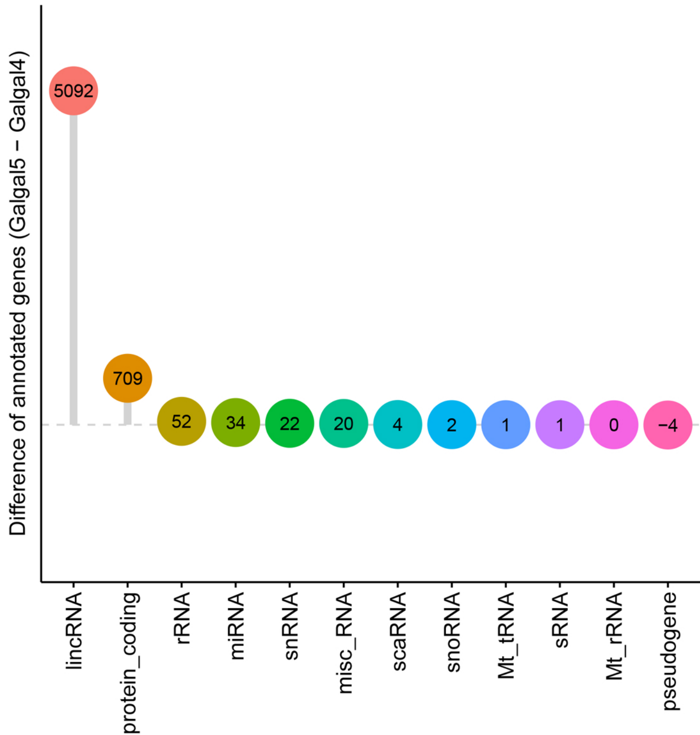

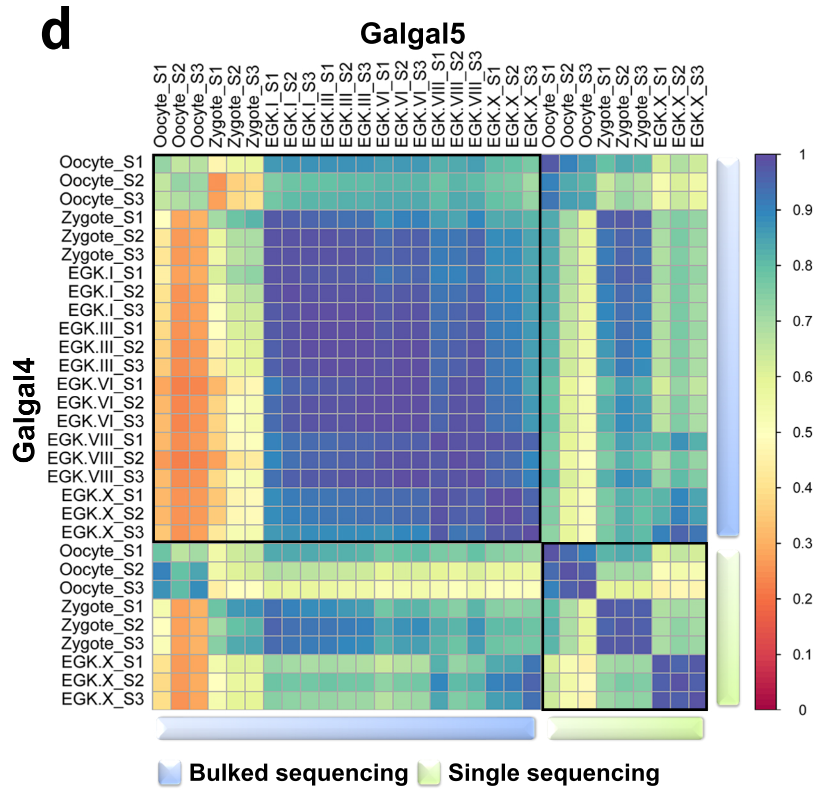

Figure 4

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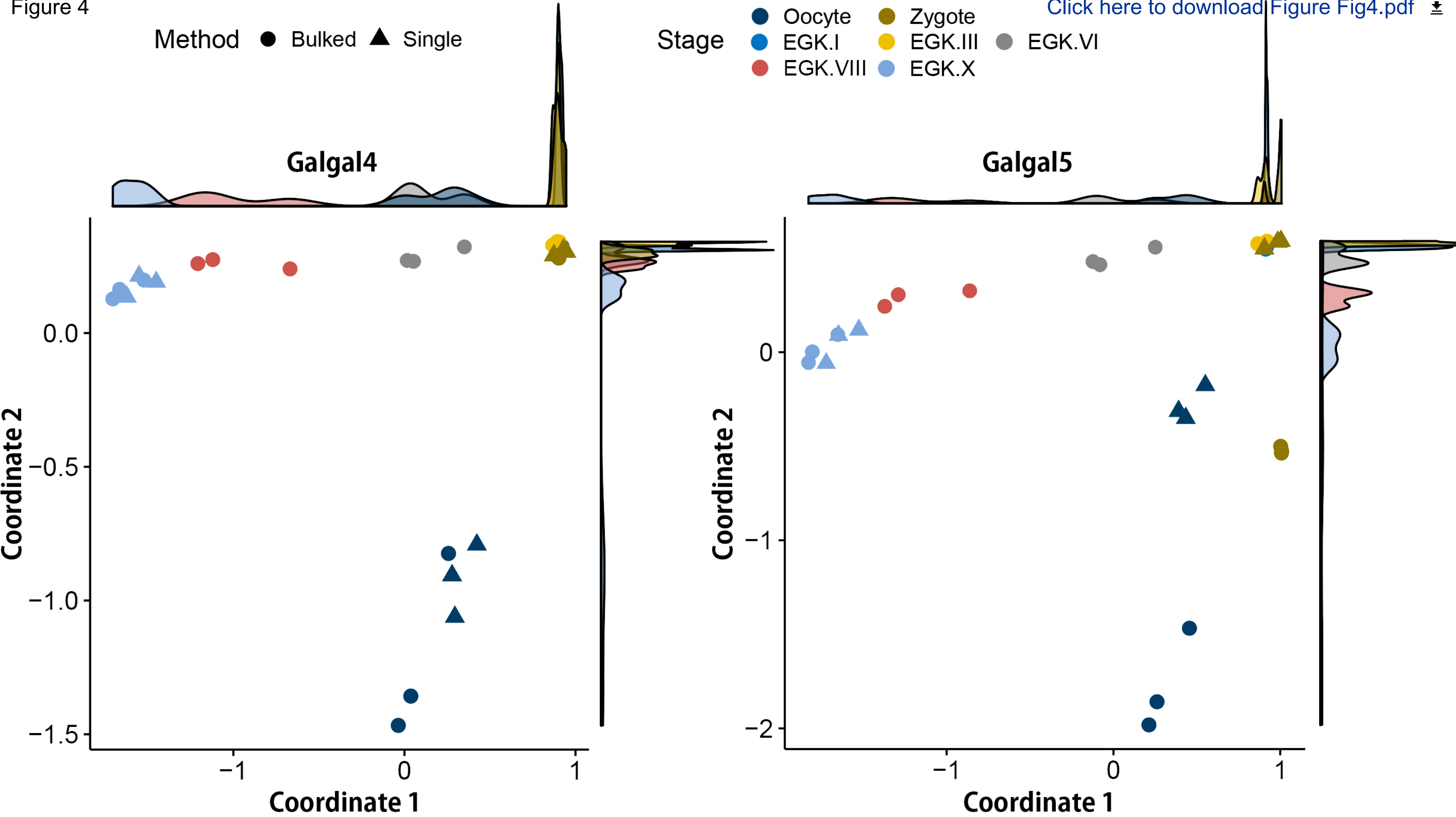

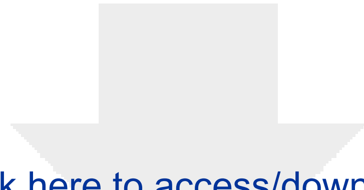

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