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Transcriptome Comparisons of Multi-Species Identify Differential Genome Activation of Mammals Embryogenesis

CHUNSHEN LONG[1], WEI LI[1], PENGFEI LIANG[1], SHUAI LIU[1,2], AND YONGCHUN ZUO[1]

[1]The State Key Laboratory of Reproductive Regulation and Breeding of Grassland Livestock, College of Life Sciences, Inner Mongolia University, Hohhot 010070, China

[2]College of Computer Science, Inner Mongolia University, Hohhot 010010, China

Corresponding author: Yongchun Zuo (yczuo@imu.edu.cn)

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ABSTRACT The identification of underlying differences is crucial for understanding the embryogenesis of species specificity. In this paper, a comparative transcriptome analysis of multi-species (CTAMS) was first developed to detect the genome activation differences of embryogenesis among human, mouse, and bovine. The annotation of intra-species and the detection of inter-species difference were separately to avoid potential batch effects. The profile clusters and Pearson correlation showed the human and bovine embryos displayed a high similarity in genome activation with a dramatic maternal to zygotic transition between 4–8 cell stages. But the mouse embryos maintained the high activity during the whole embryogenesis. Moreover, the stage-specific differentially expressed genes (DEGs) exhibited the specification of trophectoderm and inner cell mass may occur at blastocyst stage in human and bovine embryos, at morula stage in the mouse. The transcription and translation associated pathways were widely activated at morula stage in human embryos, 8-cell stage in bovine embryos and 2-cell stage in mouse embryos. The signal transduction pathways also displayed species-specific expression patterns. Finally, a probable pathway activated landscape of embryonic genome was given with the development axis.

INDEX TERMS Embryogenesis, comparative transcriptome of multi-species, genome activation, underlying differences.

I. INTRODUCTION

The preimplantation embryonic development (PED) of mammalian encompasses the period from fertilization to implantation into the endometrium. After fertilization, the zygote undergoes a series of cell divisions within the maternal deposited gradually decreased and the transcriptional expression of the embryonic genome started, which is also called zygotic genome activation (ZGA) [1]. The ZGA ranges from 1- to 2-cell stage in mouse embryos [2] to 4- to 8-cell stage in human and bovine [3], [4]. Before ZGA, all substances that are required for the initial of embryonic growth and development are provided by mature oocytes, including a variety of RNA proteins and organelles which constitute the maternal deposited of early embryonic development [5], [6]. Thus, the zygotic genes are actively transcribed and translated before they fully grown. Afterward the transcription of the zygotic genome replaces the maternal transcription directly acting on the early development, allowing the embryo to be compacted into the morula. Then the embryo develop to blastocyst through the formation of an internal cavity and further cellular differentiation [7], [8].

Although PED extends over only a few days in mammals, successive critical events are encompassed, including paternal and maternal genome reprogramming, ZGA, and the differentiation of trophectoderm (TE) and inner cell mass (ICM) [9]–[12]. So far, many studies have proved all of the molecular events critically depend on a tightly controlled and well-orchestrated program of gene expression [13]–[15]. About the destruction of maternal transcripts, Giraldez *et al.* and Ramachandra *et al.* found specific microRNAs

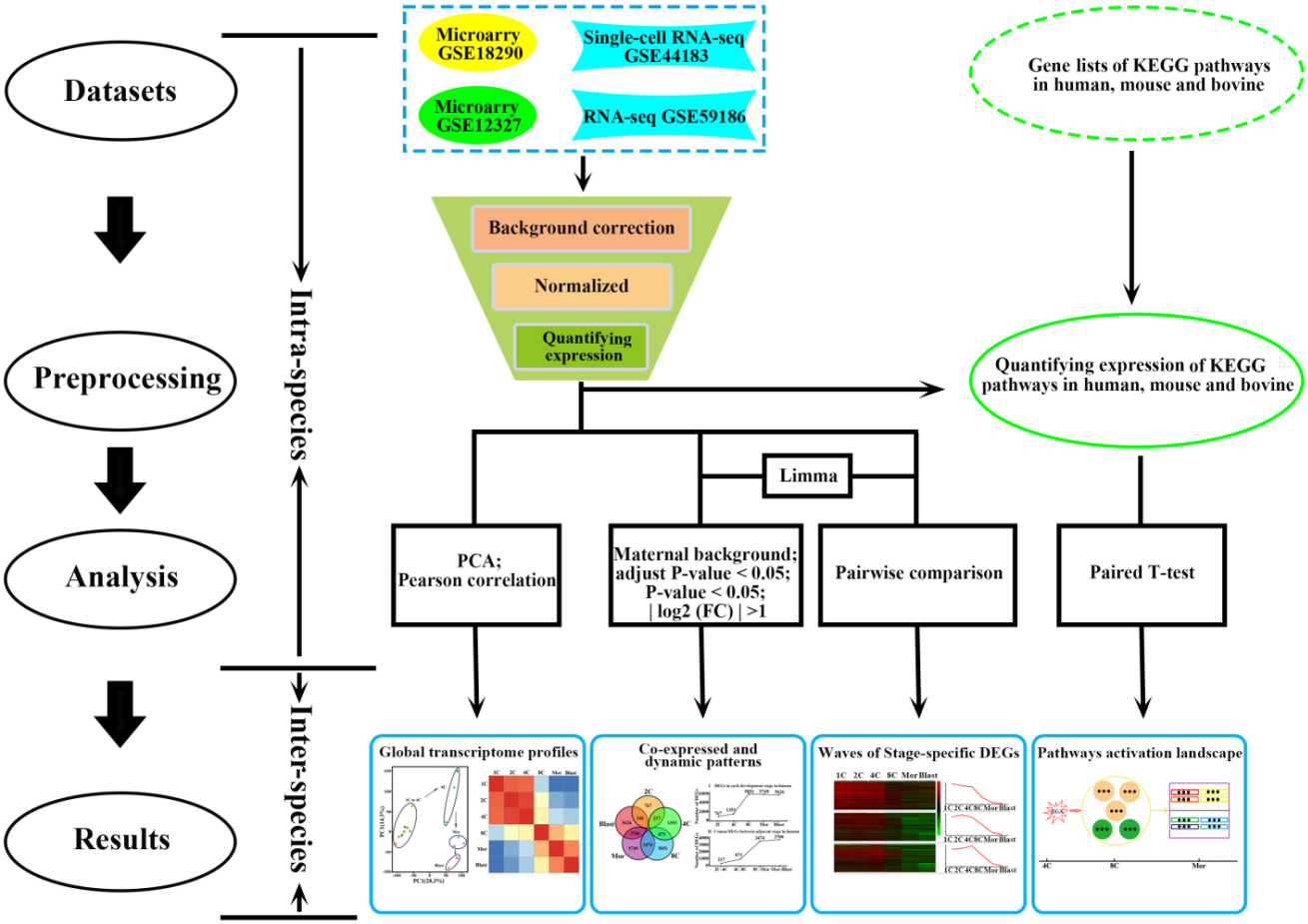

FIGURE 1. Flowchart of comparative transcriptome analysis of multi-species (CTAMS) used in this study.

(miRNAs) produced by the embryo have been shown to be involved in the destruction of maternal transcripts [16], [17]. In terms of maternal-to-zygotic transition (MZT), Mondou *et al*. observed an increase in the abundance of the mature forms of miR-130a and miR-21 and of the precursor form of miR-130a, which was at least in part of embryonic origin, from the 1-cell to the 8-cell stage, correlated with MZT. Bultman *et al*. [18] found Brg1 is the first gene required for ZGA in mammals. For genome reprogramming, Nakamura *et al*. [19] revealed PGC7/Stella is indispensable for the maintenance of methylation involved in epigenetic reprogramming after fertilization. Shen *et al*. [20] defined the respective functions of Tet3 and DNA replication in paternal DNA demethylation and revealed an unexpected contribution of Tet3 to demethylation of the maternal genome. Also, several key transcription factors (TFs) and signaling pathways have been identified in initiating and or maintaining the first and second cell-fate segregations [21]–[23].

The progress of PED is the basis of notable inter-species disparities appears after blastocyst, and the success of ART and cloning is mostly dependent on the culture stage of preimplantation embryos in vitro [24], [25]. Transcriptomic comparisons and studies among human, mouse and bovine are valuable for two reasons: First, due to ethical constraints, the derivation, acquisition, and driving differentiation of human pluripotent stem cells are mainly based on the research conclusion of pathways underlying mouse embryonic development [9]. However, because of fundamental developmental differences between species, direct application of mouse embryology to the human system has not always been successful [26]. The complex effects about the functional pathways and underlying mechanisms of these molecular events among mammals remain unclear. Second, bovine is an economically important species for which advanced reproduction technology has been developed [27]. Jiang *et al*. found that there were more similarities between the embryonic transcriptomes of bovine and human than those between human and mouse, making bovine an alternative to the mouse for the analysis of mammalian preimplantation development and understanding the causes of aberrations in embryonic and fetal development in human [28]–[35].

Here, in order to reveal the underlying differences of inter-species in the high conserved PED stages, we proposed a method of the comparative analysis of multi-species (CTAMS). According to the CTAMS flowchart in Fig. 1, the transcriptome datasets were firstly annotated and

analyzed separately within intra-species to avoid potential batch effects and inter-species differences. Then, for the inter-species comparisons, we mainly performed the overlap analysis by DEGs and pathways derived from three species [9]. A series of bioinformatics analysis were combined into the CTAMS method, including the global transcriptome profiles, co-expressed and dynamic patterns, waves of stage-specific DEGs. At last, a probable pathway activated landscape was given with development axis. The genome activation differences of our study may provide new insight into the embryogenesis and assisted reproductive techniques (ARTs).

II. MATERIALS AND METHODS

A. PREPARING DATASETS FROM MULTIPLE PLATFORMS

The microarray datasets are derived from NCBI Gene Expression Omnibus under accession no. GSE18290 and GSE12327, the RNA-seq datasets are under no. GSE59186 and the single-cell RNA-seq datasets are under no. GSE44183 in this study, including the 18 microarray samples and 30 single-cell RNA-seq samples of human, 18 microarray samples and 17 single-cell RNA-seq samples of mouse, 39 microarray samples of bovine and 16 RNA-seq samples of bovine in vivo preimplantation embryos [4], [36]–[38]. The representative stages of PED include oocyte, pronuclear, zygote, 1-cell, 2-cell, 4-cell, 8-cell, morula, and blastocyst in human, mouse and bovine. All of the datasets in this study have been approved and consented by ethics committee, which were listed in their original reference.

B. ANNOTATING INDIVIDUAL DATASETS

For the microarray datasets, we first downloaded the ''.cel'' format files. The ''rma'' method of affy R packages was used to for background correction. The ''quantile'' method was used to normalize the datasets. At last, the ''exprs'' function was used to extract the expression levels. The annotation files of microarray datasets were downloaded on the Affymetrix web site, and the genome version is hg19 in human, mm10 in mouse and bosTauMd3 in bovine. Each probe set ID of the whole profile was mapped to its corresponding the gene symbol according to the annotation file by R, and that has no corresponding gene annotation were discarded to reduce the potential noise. The multiple gene names for the same probe and multiple probes for the same gene name have been reserved. For the RNA-seq datasets and the single-cell RNA-seq datasets, we downloaded the processed data provided by original papers.

C. COMPARATIVE TRANSCRIPTOME ANALYSIS OF MULTI-SPECIES (CTAMS)

In the comparative analysis of multi-species (CTAMS) method, all of the transcriptome datasets were annotated and analyzed separately within intra-species to avoid potential batch effects, including the global transcriptome profiles, co-expressed and dynamic patterns, waves of stage-specific DEGs, pathways activation landscape mentioned in the article. Finally, when it comes to inter-species comparisons, we mainly performed the overlap analysis by DEGs and pathways derived from three species [9]. The detail description of CTAMS method was displayed in flowchart of Fig. 1.

D. PREPARING GENE SETS OF PATHWAYS AND CORRESPONDING TO EXPRESSION LEVELS

The gene sets in pathways of the three species were downloaded from the KEGG website, respectively, including Genetic Information Processing, Environmental Information Processing, and Cellular Processes. The gene symbols were matched with expression levels by transcriptome list. In this study, statistical analysis and data visualization were carried out mainly by the R, including the R/Bioconductor.

E. HIERARCHICAL CLUSTERING AND PRINCIPAL COMPONENT ANALYSIS (PCA)

For hierarchical clustering, we converted the gene expression levels into ''Euclidean'' and performed a hierarchical cluster analysis based on ''ward.D'' by hclust function [39]. For PCA, we performed a principal components analysis on the gene expression levels matrix and returned the results as an object of class prcomp. The calculation was done by a singular value decomposition of the (centered and possibly scaled) data matrix. And then, we used the first two most important principal component features to quantitatively describe the clustering relationship between embryo samples in two-dimensional space. Both Hierarchical clustering and PCA were calculated by R.

F. IDENTIFYING DEGs OF ADJACENT STAGES AND COMMON GENES

The comparison between every two adjacent stages was performed by differential expression functions in limma packages of R [40]. Genes with adjust P-value and P-value less than 0.05, $|\log2\ (\text{Fold Change})| > 1$ were selected as differently expressed genes. These genes with the higher expression levels than the previous stage were characterized up-regulated genes ($\log2\ (\text{Fold Change}) > 1$). Otherwise, they were regarded as down-regulated genes ($\log2\ (\text{Fold Change}) < -1$). Then we used 1- cell stage (human and mouse) as background compared with other developmental stages, respectively. Genes with adjust P-value and P-value less than 0.05, and the higher expression levels comparing to the 1-cell stage ($\log2\ (\text{Fold Change}) > 1$) were identified as DEGs at corresponding stage, and the intersection of DEGs between every two adjacent stages was identified as common DEGs.

G. IDENTIFYING MATERNAL GENES AND ZYGOTIC GENES

The zygotic genes were defined as the DEGs with adjust P-value and P-value less than 0.05, $\log2\ (\text{Fold Change}) < -1$ in 1-cell stage vs. blastocyst stage. The maternal genes were defined as the DEGs with adjust P-value and P-value less than 0.05, $\log2\ (\text{Fold Change}) > 1$ in 1-cell stage vs. blastocyst stage.

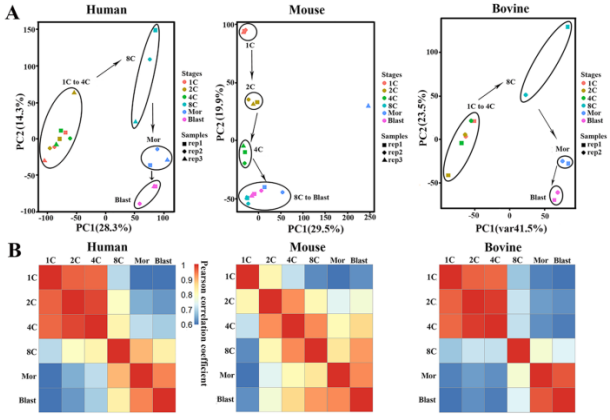

FIGURE 2. **Global transcriptome profiles of PED in three species. A. Principal component analysis (PCA) of gene expression in human, mouse, bovine embryos during representative stages of PED. B. Pearson correlation coefficient (PCC) between stages of pairwise PCCs within each stage in human, mouse and bovine.**

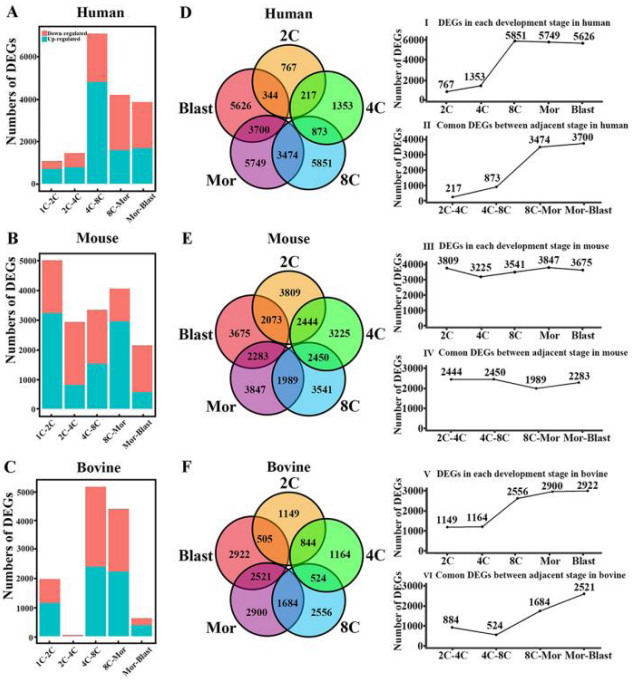

FIGURE 3. **Dynamic changes of gene expression profiles between two adjacent developmental stages. A-C. Histogram showing up- and down-regulated genes between each two adjacent stages in human, mouse and bovine identified by adjust P-value and P-value less than 0.05, |log FC|>1. D-F. The distribution of DEGs in human, mouse and bovine embryos between neighboring datasets take 1-cell stage as background. The numbers of DEGs exclusively regulated at each development stage are shown in the circle. The numbers of the common DEGs are shown in the overlapping regions. I, III, and V represented change of the DEGs at each stage; II, IV and VI represented the change of the common DEGs.**

H. IDENTIFYING STAGE-SPECIFIC DEGs AND GENE ONTOLOGY ANALYSIS

We performed a pairwise comparison across all time points, that is, each time point is compared to every other stage, or all versus all $n * (n-1)/2$ comparisons, where n is the number of developmental stages [9]. The DEGs from the pairwise comparison (adjust P-value < 0.05 and P-value < 0.01,) was selected and divided into each developmental stage based on the highest expression levels as stage-specific DEGs at each developmental stage. Functional annotation of stage-specific DEGs was performed with the Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resource [41]. Gene ontology terms were shown with the P-values.

I. IDENTIFYING DIFFERENTIALLY REGULATED PATHWAYS

The boxplot was used to character comprehensive of gene expression, including median, range, outliers and distribution of one dataset. The line graph of the median was integrated to reflect the activation trend of functional pathways in preimplantation development. The statistical significance of each pathway was calculated by paired T-test validation.

III. RESULTS

A. GLOBAL TRANSCRIPTOME PROFILES OF PED

According to the CTAMS flowchart in Fig. 1, the comparative transcriptome analysis was firstly annotated and analyzed separately within intra-species (Wang *et al.*, 2017). The hierarchical clustering results were shown in **Fig. S1**. For the bovine embryos, three expression datasets containing the microarray and RNA-seq datasets were used to identify the profile of genome activation. The global gene expression profiles derived from the two latest datasets (**Fig. S1D and S1G**) performed highly unified among the biological replicates of the same developmental stages together. Both of them supported that maternal to zygotic transition (MZT) occurs at the 4-8 cell stages in bovine embryos, with the early development process was divided into two clusters. However, the bovine microarray data GSE18290 showed low uniformity in hierarchical clustering and didn't consistent with the other microarray and RNA-seq datasets.

To further compare the similarities and distinctions in the activation of the whole genome-wide profiles, the principal component analysis (PCA) at each developmental stage of three species was further evaluated. As shown in **Fig. 2A**, the development roadmaps for human and bovine were similar. Both of the embryo samples fell into four clusters, one cluster containing 1-cell, 2-cell and 4-cell stage samples; and the other three clusters were 8-cell, morula and blastocyst stage samples, respectively. In mouse embryos, the samples of 8-cell stage to blastocyst were clustered into one group.

The 1-cell, 2-cell, 4-cell stage samples in human and bovine showed a high Pearson correlation coefficient (PCC) (PCC>0.90), and the same result was obtained between morula and blastocyst stages (**Fig. 2B**). The 8-cell stage has lower similarity (PCC<0.9) with other stages, indicating that it is the major phase of MZT in human and bovine embryos. Meanwhile, there was higher heterogeneity among the whole developmental stages in mouse embryos, most of the PCC values were less than 0.88 between any two stages.

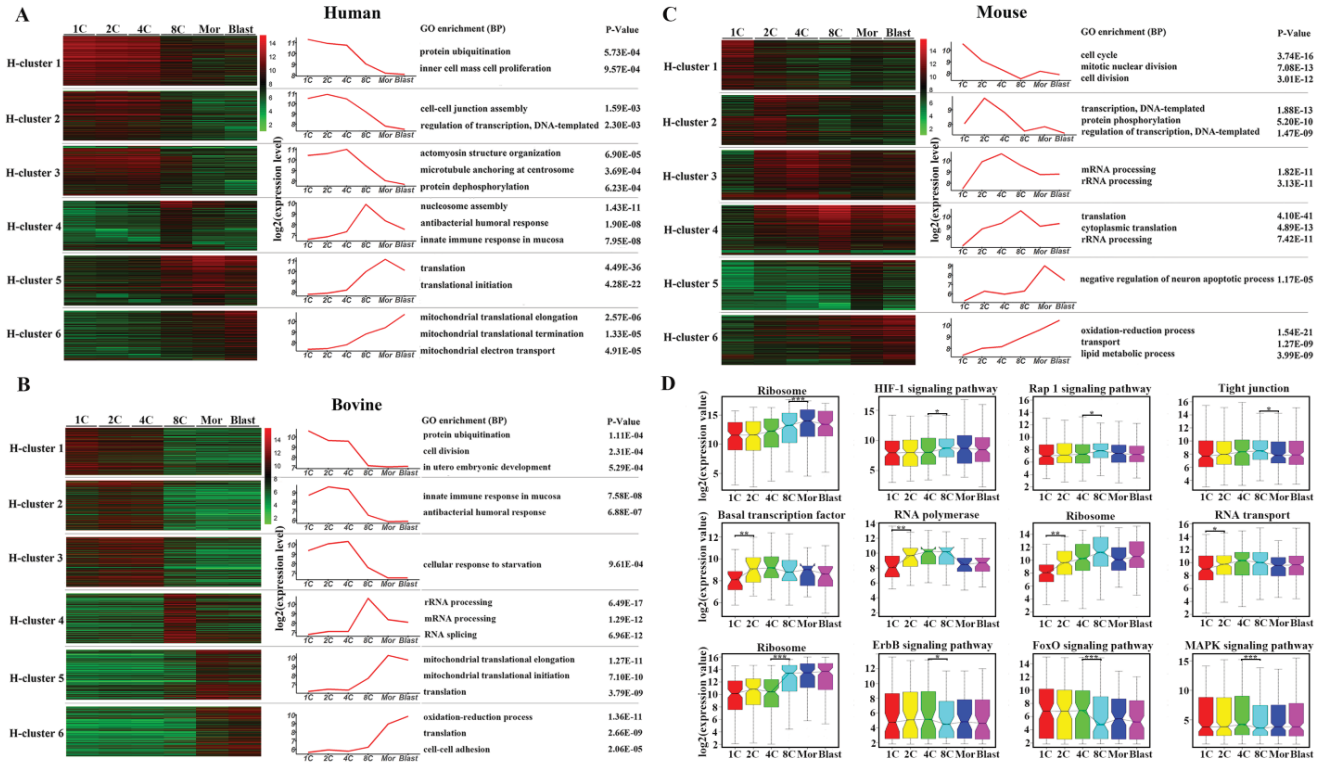

FIGURE 4. Gene expression profiles of stage-specific DEG clusters in human, mouse and bovine embryos. A-C. Stage-specific DEG clusters during representative stages of PED in human, mouse and bovine, and representative GO terms are listed behind. Human-cluster (H-cluster) 1-6, Mouse-cluster (M-cluster) 1-6 and Bovine-cluster (B-cluster) were the stage-specific DEGs of 1-cell, 2-cell, 4-cell, 8-cell, morula, blastocyst in human, mouse and bovine embryos, respectively. *D*. The representative differentially regulated pathways (DRPs).

The similar transcription profiles between human and bovine were shown in the hierarchical clustering, both PCA and Pearson correlation analysis, supporting that bovine is more suitable than mouse in studying human embryos during PED.

B. DYNAMIC CHANGES IN EXPRESSION PROFILES BETWEEN ADJACENT DEVELOPMENTAL STAGES

In order to detect the quantitative changes in expression differences between adjacent developmental stages in three species, the limma R package was introduced to select DEGs between two adjacent stages [40]. From the **Fig. 3**, we can find that the largest wave of DEGs appeared between 4-8 cell stages in human and bovine embryos (**Fig. 3A-3C**). According to the adjust P-value and P-value < 0.05, there were 4795 up-regulated DEGs and 2279 down-regulated DEGs in human, 2399 up-regulated DEGs and 2752 down-regulated DEGs in bovine. In mouse embryos, the overall level of DEGs maintained at high until blastocyst stage, consistent with an overall accelerated pace of mouse embryonic development [36]. In addition, the human and bovine shared more DEGs after maternal to zygotic transition (MZT), while mouse showed little overlap with human and bovine (**Fig. S2A**). Moreover, we roughly distinguished maternal genes and zygotic genes by DEGs (adjust P-value and P-value < 0.05,$|$ log2 (Fold Change) $|$ >1) in 1-cell stage vs. blastocyst stage [42] **(Fig. S2B)**. During MZT, the ratio of zygotic genes in up-regulated DEGs was 43% in human embryos, 55% in mouse embryos and 60% in bovine embryos, respectively. And the ratio of maternal genes in down-regulated DEGs was 74% in human embryos, 66% in mouse embryos and 65% in bovine embryos, respectively. It confirms that MZT was characterized by drastic maternal mRNA decay and zygotic mRNA biosynthesis [4], [43].

The oocyte is the crucial driver for successful fertilization and the outcome of consequent embryo development [44], [45]. To observe the dynamic changes of expression profiles at subsequent developmental stages, the 1-cell stage was taken as maternal background (adjust P-value and P-value < 0.05, $|$log2 (Fold Change) $|$ >1) **(Fig. 3D-3F)**. In human and bovine embryos, the DEGs increased significantly after 4-cell stage and the common DEGs went up dramatically after 8-cell to morula stages. Otherwise, the DEGs and common DEGs kept high levels throughout the PED in mouse embryos.

C. IDENTIFICATION OF REPRESENTATIVE STAGE-SPECIFIC PATTERNS

To study the functional time frames of genes and reveal the diversity of gene expression patterns for different mammals, we further performed a pairwise comparison across

all developmental stage. Each stage was compared to each other stage, or all versus all $n*(n-1)/2$ comparisons, where n is the number of developmental stages [9]. The DEGs (P-value $<$ 0.001 and adjust P-value $<$0.05) derived from the pairwise comparison were clustered into each developmental stage based on the highest expression levels as stage-specific DEGs. The representative Gene Ontology (GO) terms for each cluster were listed behind (**Fig. 4**) and the complete stage-specific DEGs were listed in **Table S1.** Human-cluster (H-cluster) 1-6, Mouse-cluster (M-cluster) 1-6 and Bovine-cluster (B-cluster) were the stage-specific DEGs of 1-cell, 2-cell, 4-cell, 8-cell, morula, blastocyst in human, mouse and bovine, respectively.

Beyond doubt, each cluster was highly expressed at their represent developmental stages in the three species (**Fig. 4**). In human and bovine samples, H-cluster 1-3 and B-cluster 1-3 gradually down-regulated expressed along with embryogenesis, while H-cluster 4-6 and B-cluster 4-6 gradually up-regulated expressed. These data indicated the distinct stage-specific function after the mainly ZGA in human and bovine embryos. For H-cluster 1-6, the stage-specific DEGs for protein ubiquitination, cell-cell junction assembly, actomyosin structure organization, nucleosome assembly, translation, mitochondrial translational were enriched **(Fig. 4A)**. B-cluster 1-6 mainly included protein ubiquitination, innate immune response in mucosa, cellular response to starvation, rRNA processing, mitochondrial translational elongation, oxidation-reduction process, respectively **(Fig. 4B)**. In mouse samples, M-cluster 1-6 displayed an apparent stage-specific expression patterns. And the most representative biological processes of M-cluster 1-6 were cell cycle, transcription and DNA-templated, mRNA and rRNA processing, translation, negative regulation of neuron apoptotic process, oxidation-reduction process, respectively (**Fig. 4C**).

Notably, some vital factors for pluripotency and differential displayed more diverse patterns (**Table S2**). The pluripotent TF, POU5F1, preferred to express at morula stage for human and bovine embryos, however, in mouse, it appeared at 8-cell stage. And SOX2 appeared at morula stage in human and mouse embryos. Moreover, several histone variants (e.g., HIST2H2AA3, HIST1H2AC and HIST1H3J) which play critical roles in modulating chromosome accessibility fell into the H-cluster 4 (8-cell stage-specific DEGs) in human embryos. Other key TFs that regulate the TE specification (e.g., SOX17) [46] and ICM (e.g., TBX3) [47] were clustered into H-cluster 6 (blastocyst stage-specific DEGs). It indicated that the specification of TE and ICM may start at the blastocyst stage in human, which was consistent with the previous report [9], [48]. In mouse embryos, the genes that are important for maternal RNA decay (e.g., YAP1) [49] fell into M-cluster 2 (2-cell stage-specific DEGs) and the TFs of TE specification (e.g., Gata2 and Gata3) belonged to M-cluster 5 (morula stage-specific DEGs) and M-cluster 6 (blastocyst stage-specific DEGs), respectively. In bovine embryos, the TET2 which regulate the specification of ICM fell into B-cluster 6 (blastocyst stage-specific DEGs).

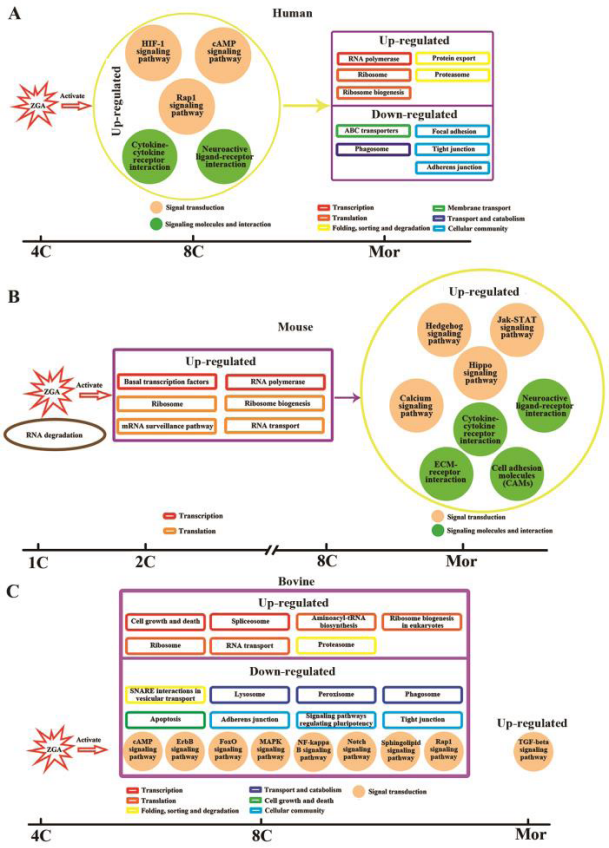

FIGURE 5. **The probable timing activation of functional pathways after ZGA in human, mouse and bovine embryos.**

D. THE PROBABLE UNDERLYING DIFFERENCES OF TIMING ACTIVATION FOR DECISIVE PATHWAYS

In order to put insight into what and when the functional pathways playing the most important roles in regulation of early embryogenesis, the dynamic roadmap of genome activation should be comprehensive discussed for the decisive pathways [50]. We downloaded all of the genes in each pathway in the three species from the KEGG website, including Genetic Information Processing, Environmental Information Processing, and Cellular Processes. The statistical significance of each pathway was calculated by paired T-test validation. The representative differentially regulated pathways (DRPs) were shown in **Fig. 4D**, and the other DRPs were put into**Fig. S3, Fig. S4 and Fig. S5**, which showed a series of key function pathways were activated after ZGA in the three species.

According to the expression patterns of DRPs, we presented a probable dynamic landscape of some key functional pathways in the three species (**Fig. 5**). In human embryos, several signal transduction, signaling molecules and interaction pathways up-regulated expressed obviously in major ZGA (4- to 8-cell stage). And some other pathways preferred to the morula stage (**Fig. 5A**). In mouse embryos, RNA degradation pathway was highly expressed between 1- and

2-cell stages, indicating that there was considerable degradation of maternal deposited RNA (**Fig. 5B**). Afterwards, the transcription and translation related pathways were activated along with ZGA, and kept high expression levels until 8-cell stage. From 8-cell to morula stage, the mouse embryos undergone a series of activation of functional pathways which are critical for stem cell proliferation and differentiation, including Jak-STAT signaling pathway, Hedgehog signaling pathway etc. In bovine embryos, transcription and translation related pathways were up-regulated during the ZGA (4-8 cell stages), whereas partial transport and metabolism, cell growth and death, cellular communication, and signal transduction pathways were up-regulated at the same time. In addition, TGF-beta signaling pathway was up-regulated at morula stage (**Fig. 5C**).

It has been demonstrated that Hippo signaling pathway can activate the Sox2 [51] transcription for the inside cells of mouse embryos and Cdx2 [52] for the outside cells to drives ICM and TE lineage formation. In human and bovine embryos, the Hippo signaling pathway didn't exhibit significant activation, but the Sox2 was also activated at morula developmental stage in human embryos (**Fig. S6**), which revealed the differential molecular mechanism of ICM and TE lineage formation for different mammals.

IV. CONCLUSION

In this study, the CTAMS method was introduced to reveal the underlying differences of genome activation in preimplantation embryogenesis for the three species of mammals. Based on the time series transcriptome datasets from embryos, the global gene expression profiles and dynamic patterns demonstrated that human and bovine displayed high similarity in zygotic genome activation (ZGA). The quantitative analysis of the gene expressed dynamic changes revealed a dramatic MZT at 4-8 cell stages in human and bovine embryos with the significant increase of the DEGs. The high degree of heterogeneity among the developmental stages and the high levels of DEGs throughout the PED in mouse embryos indicated an accelerated pace of mouse embryonic development at the molecular level. Moreover, the human and bovine shared more DEGs after maternal to zygotic transition (MZT), while mouse showed little overlap with human and bovine throughout the process of PED.

The clusters of stage-specific DEGs exhibited the similar expression patterns in human and bovine embryos, however, some vital factors for pluripotency and differential displayed disparate expression patterns. For example, the pluripotent TF POU5F1 was specifically expressed at morula stage in human and bovine embryos, and 8-cell stage in mouse embryos. We also confirmed that the specification of TE took place at blastocyst stage in human embryos. But in mouse embryos, the gene markers occurred simultaneously at morula stage and blastocyst stage. In bovine embryos, the ICM was restricted to blastocyst stage. At last, by putting the biological time axis on the same scale, a more clear activation landscape was displayed among the three species, including transcription, translation and signal transduction associated pathways.

The success of ART and cloning rigorously depend on the quality of *in vitro* embryos [6]–[8], so the genome activation differences among the three species during PED were meaningful. The finding of this study will provide new insight into the understanding of embryogenesis and improvement of assisted reproductive techniques (ARTs) [4].

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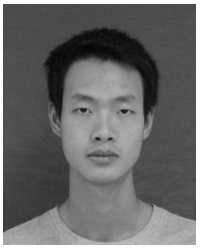
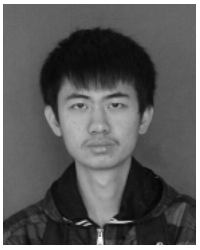
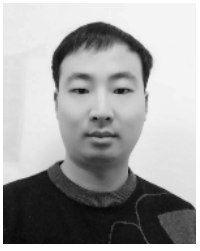


CHUNSHEN LONG is currently pursuing the master's degree with The State Key Laboratory of Reproductive Regulation and Breeding of Grassland Livestock, College of Life Sciences, Inner Mongolia University, Hohhot, China. His research interests include bioinformatics, systems biology, and developmental biology.



WEI LI is currently pursuing the bachelor's degree with The State Key Laboratory of Reproductive Regulation and Breeding of Grassland Livestock, College of Life Sciences, Inner Mongolia University, Hohhot, China. His research interests include bioinformatics, systems biology, and developmental biology.



PENGFEI LIANG is currently pursuing the master's degree with The State Key Laboratory of Reproductive Regulation and Breeding of Grassland Livestock, College of Life Sciences, Inner Mongolia University, Hohhot, China. His research interests include bioinformatics, systems biology, and developmental biology.

SHUAI LIU received the Ph.D. degree. He is currently a Professor with the College of Computer Science, Inner Mongolia University, Hohhot, China. His current research interests include complex computing and big data analysis. He is a member of the Chinese Computer Society. He is a long-term Reviewer of several TOP journals, including the IEEE TRANSACTIONS ON FUZZY SYSTEMS, *Information Sciences*, the IEEE TRANSACTIONS ON IMAGE PROCESSING, and so on.

YONGCHUN ZUO is currently a Professor with the State Key Laboratory of Reproductive Regulation and Breeding of Grassland Livestock, College of Life Sciences, Inner Mongolia University. His research interests include bioinformatics and integration analysis of multi-omics in cell reprogramming.